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# Diversity, infection patterns and host-parasite associations of apicomplexan parasites in reptiles

João Pedro Moura da Costa Maia

Tese de Doutoramento apresentada à
Faculdade de Ciências da Universidade do Porto
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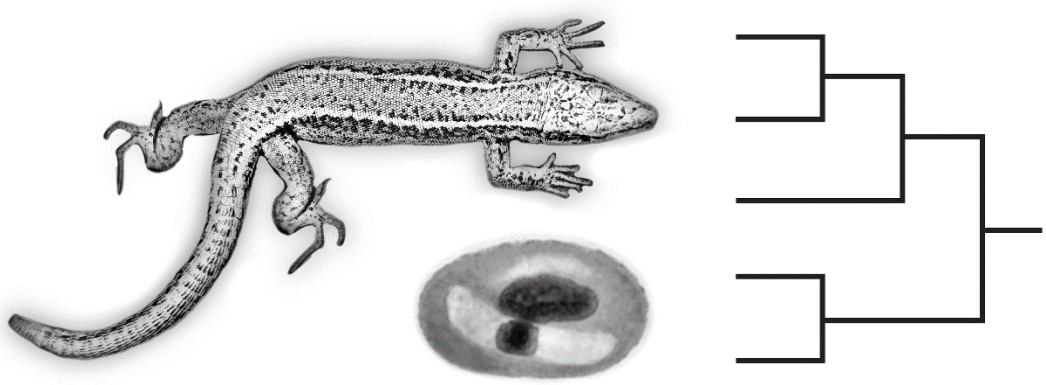

# Diversity, infection patterns and host-parasite associations of apicomplexan parasites in reptiles

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Programa Doutoral em Biodiversidade, Genética e Evolução

Departamento de Biologia

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Versão definitiva

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# Nota Prévia

Na elaboração desta tese, e nos termos do número 2 do Artigo 4º do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31º do Decreto-Lei 74/2006, de 24 de Março, com a nova redação introduzida pelo Decreto-Lei 230/2009, de 14 de Setembro, foi efetuado o aproveitamento total de um conjunto coerente de trabalhos de investigação já publicados ou submetidos para publicação em revistas internacionais indexadas e com arbitragem científica, os quais integram alguns dos capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados com a colaboração de outros autores, o candidato esclarece que, em todos eles, participou ativamente na sua conceção, na obtenção, análise e discussão de resultados, bem como na elaboração da sua forma publicada. A Faculdade de Ciências da Universidade do Porto foi a instituição de origem do candidato, tendo o trabalho sido realizado sob orientação do Doutor David James Harris, Professor Associado Convidado no Departamento de Biologia da Faculdade de Ciências da Universidade do Porto e Investigador Principal do Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO-InBio). A instituição de acolhimento foi o Instituto de Biología Evolutiva do Consejo Superior de Investigaciones Científicas – Universitat Pompeu Fabra (IBE-CSIC-UPF), sob a coorientação da Investigadora Doutora Elena Goméz-Díaz e do Professor Doutor Salvador Carranza. O trabalho laboratorial foi realizado no CIBIO-InBio e no IBE-CSIC-UPF.

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What lies in our power to do, it lies in our power not to do.

Aristotle (philosopher)

**Diversity, infection patterns and host-parasite associations of apicomplexan parasites in reptiles**

João Pedro Moura da Costa Maia
Faculdade de Ciências, Universidade do Porto – CIBIO-InBio / IBE-CSIC-UPF

# Abstract

Parasites may account for half of the total number of species in the world but only a fraction of the diversity within these organisms has been detected and fewer described. Parasites have obligate relationships with their hosts and for this reason are good models to study the ecological and evolutionary processes that drive diversification and speciation. In addition, parasites have implications for conservation as these may cause adverse effects and affect host fitness, playing a crucial role at the individual, population and ecosystem levels.

Apicomplexa is one of the most important parasite phylum composed almost excusably of obligate intracellular parasites, yet potentially one of the least understood. Within Apicomplexa, three main groups of parasites concern this thesis, the hemogregarines (Adeleorina), the haemosporidians (Haemosporida) and the eimeriorinids (Eimeriorina). These parasites differ in their lifecycle, life-history traits and ecology of transmission, which makes them an ideal group to study host-parasite evolutionary phenomena. What these have in common is the lack of understanding of the real diversity and phylogenetic relationships within these groups. In particular, the study of these groups in reptiles has been scarce but their importance is increasing in recent years with the growing use of molecular tools to estimate parasite distribution, diversity and phylogenetic relationships.

Genetic information can be obtained using molecular tools and this information can be assessed through phylogenetic analyses. These analyses are thus of great importance in parasitology as these allow the study of diversity, coevolutionary and ecological processes of these organisms in relation to their hosts. Nonetheless, different methodologies can have different sensitivities and performances at estimating parasite infection parameters and may result in erroneous biological inferences of these estimates, which are a first step towards understanding host-parasite associations and specificity.

Therefore, a chapter of this thesis aimed to compare the performance of different biological samples and extraction protocols on the estimation of biologically relevant parasite infection parameters. The findings of this chapter showed that different protocols yield significantly different estimations of parasite infection. Therefore, the estimation of parasite infection parameters should be conducted using complementary approaches to validate the significance of the results obtained. For this reason, the limitations of and recommendations for the various methodologies, as well as proposed lines of work, are discussed.

One of the main objectives of this thesis was to contribute to the knowledge on the diversity, phylogenetic relationships and host-specificity of apicomplexan parasites in reptile hosts.

Throughout various chapters, this objective has been achieved at different levels. First, the species *Hepatozoon domerguei* (Adeleorina), a known hemogregarine of reptiles from Madagascar, has been genetically characterized and included in a phylogenetic framework for the first time. This allowed to corroborate prey-predator transmission that had been previously reported in experimental studies for this parasite species. Second, a new *Hepatozoon* species exclusively found in reptiles from Oman has been described based on morphological and genetic distinctive characters in comparison with available information (proposed name *Hepatozoon omanensis* n. sp.). The principal host species is endemic to Oman, which could be an indication that this parasite is also endemic to this region. This parasite species might be also of interest for studying parasite adaptation in the future since it is found in a wide range of other reptile species in this region. Third, unexpectedly high diversity has been observed in a molecular screen of various reptile species with different geographical ranges across Oman. In this study, a previously unreported lineage that diverged by more than 5% for the 18S rRNA gene from the most closely published sequences has been reported. Fourth, genetically distinct lankesterellids (Eimeriorina) have been detected in reptiles and amphibians from Oman. These two latter points could represent new taxonomic entities and warrant further investigation of the developmental stages and vectors of these parasites. Fifth, a possible new *Haemocystidium* (Haemosporida) parasite species has been detected in two gecko species from Oman, also highlighting the importance of screening wild hosts from remote regions. And sixth, unique hemogregarine lineages have been found in two lacertid lizard species living in sympatry in Oukaimeden (Morocco). This could be an indication of host-specificity and specialization, as well as a possible explanation for the variation of hemogregarine intensity levels observed in these hosts.

Furthermore, in an effort to compile the recent effort that has been done to determine the diversity of hemogregarines (using the 18S rRNA gene) and haemosporidians (using the cytochrome *b* gene), a chapter of this thesis was dedicated to an overview of the genetic information available on public databases for these parasite groups. In accordance with recent studies, the taxonomic inconsistencies of present taxonomy have been evidenced and discussed in the light of the diversity and relationships obtained. In this chapter, common problems such as parasite taxon sampling and host sampling bias have also been addressed and recommendations for future studies are provided.

An additional objective of this thesis was to study host-parasite associations and elucidate transmission dynamics across different host taxa. By analysing parasite distributions and the host range of particular hemogregarine lineages in wild hosts (i.e. lizards and rodents as potential prey, and snakes and canids as potential predators) occasional occurrence of prey-predator transmission has been corroborated. These findings could either be an indication of low host-specificity of some hemogregarine lineages and/or the occasional accidental transmission to “dead-end” hosts that do not transmit the infection further. In either case, these events may be of importance for increasing parasite host range and leading to parasite diversification because could increase the probability of occurrence of host shifts and thus should be further investigated.

Finally, this thesis also aimed to study hemogregarine infection patterns in different scenarios in order to disentangle the factors that may influence these patterns. These scenarios were: i) several related and unrelated reptile host species; ii) two closely related lizard species living in sympatry across different time points; and iii) two related and one unrelated lizard species living in sympatry across different time points. Infection patterns were generally maintained over time between species and sexes in both systems. This shows that that host unique biological characteristics and ecological preferences may be major drivers of the distribution of these parasites.

All in all, the studies presented in this thesis have shown the usefulness of conducting parasite molecular surveys in wild hosts from remote regions, as well as the need for adopting integrative and complementary approaches in the study of these parasite groups. The study of asexual and developmental stages on the different hosts of these heteroxenous parasites in combination with molecular characterization using multiple genes and accurate estimation of infection patterns, could revolutionize this field and provide answers to the taxonomic incongruences evidenced.

**Key-words:** phylogeny; taxonomy; incongruence; systematic revision; diversity; new species; host-specificity; host-parasite associations; host-parasite interactions; temporal; spatial; seasonality; evolutionary history; differentiation; specialization; lizard; lacertid; gecko; hemoparasite; blood parasite; cyt *b* gene; 18S rRNA gene; microscopy; morphology; molecular screen; detection; infection; quantification; absolute quantification; PCR; qPCR; prevalence; intensity; infection status; infection patterns; abundance; prey predator transmission; host factors; ecological factors; sympatry; host relatedness; phylogeography; Oman; Morocco; Madagascar; North Africa; Western Mediterranean; Portugal; Spain; Adeleorina; Haemosporida; Eimeriorina; hemogregarine; haemosporidian; eimeriorinid; *Proteromonas*; *Hepatozoon*; *Bartazoon*; *Karyolysus*; *Haemoproteus*; *Haemocystidium*; *Lankesterella*; *Schellackia*; *Sarcocystis*; *Hepatozoon domerguei*; filarial nematode*; Foleyella furcata*; *Hepatozoon omanensis*; vector; transmission dynamics; genbank.

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# Resumo

Estima-se que os parasitas representem cerca de metade do número total de espécies no mundo, no entanto apenas uma pequena parte da sua diversidade tem sido documentada. Os parasitas têm uma relação obrigatória para com os seus hospedeiros e por esta razão representam modelos ideias para o estudo de processos evolutivos e ecológicos que despoletam a diversidade e especiação dos parasitas e hospedeiros. Além disso, os parasitas têm implicações a nível da conservação de espécies podendo provocar efeitos adversos nos hospedeiros e afetar a sua aptidão, desempenhando desta forma um papel crucial a nível individual, populacional e do ecossistema.

Apicomplexa representa um dos mais importantes filos de parasitas e é composto quase exclusivamente por parasitas intracelulares obrigatórios, todavia é um dos menos compreendidos. Dentro do Apicomplexa, três grupos principais de parasitas são relevantes para esta tese, as hemogregarinas (Adeleorina), Haemosporida e Eimeriorina. Estes parasitas diferem no seu ciclo de vida, características evolutivas e ecologia de transmissão, o que os torna num grupo ideal para o estudo de processos de evolução entre parasita-hospedeiro. O que eles têm em comum é um défice na compreensão da sua verdadeira diversidade e relações filogenéticas dentro destes grupos. Em particular, o estudo destes grupos em répteis é escasso, mas a sua importância tem vindo a crescer recentemente com o aumento no uso de métodos moleculares para estimar a distribuição, diversidade e relações filogenéticas destes parasitas.

Informação genética pode ser obtida através de métodos moleculares e esta informação pode ser comparada através de análises filogenéticas. Desta forma, estas análises são de grande interesse no campo da parasitologia porque permitem o estudo da diversidade, coevolução e processos ecológicos destes organismos em relação aos seus hospedeiros. No entanto, diferentes metodologias podem ter diferente sensibilidade e exatidão na estimativa de parâmetros de infeção de parasitas, podendo resultar em inferências biológicas erróneas destas estimativas, as quais representam um primeiro passo para a compreensão da especificidade dos parasitas e interações parasita-hospedeiro.

Deste modo, um capítulo desta tese teve como objetivo a comparação do desempenho entre vários protocolos e tipos de amostras biológicas na estimativa de parâmetros de infeção dos parasitas. Os resultados deste capítulo mostraram que diferentes protocolos produzem estimativas de infeção significativamente diferentes. Assim, a estimativa destes parâmetros deve ser baseada no uso de abordagens complementares para que seja possível validar a significância dos resultados obtidos. Por esta razão, as limitações e recomendações para os vários tipos de metodologias, bem como possíveis linhas de trabalho, são discutidas.

Um dos principais objetivos desta tese foi contribuir para o conhecimento da diversidade, relações filogenéticas e da especificidade dos parasitas apicomplexos em répteis. Ao longo de

vários capítulos, este objetivo foi alcançado em diferentes níveis. Primeiro, a espécie *Hepatozoon domerguei*, a qual é conhecida de répteis em Madagáscar, foi pela primeira vez caracterizada geneticamente e colocada num contexto filogenético. Isto permitiu corroborar a ocorrência de transmissão presa-predador que havia sido reportado para esta espécie através de estudos experimentais. Segundo, uma nova espécie de *Hepatozoon*, detetada exclusivamente em répteis de Oman foi descrita com base em carateres morfológicos e genéticos distintivos em comparação com informação publicada (nome proposto *Hepatozoon omanensis* n. sp.). O hospedeiro principal é endémico de Oman, o que pode ser uma indicação que esta espécie seja também endémica desta região. Esta espécie pode também ser de interesse para estudos futuros relacionados com adaptação de parasitas, uma vez que foi encontrada em várias espécies de répteis desta região. Terceiro, durante um rastreio molecular de várias espécies de répteis de várias áreas geográficas de Oman foi inesperadamente encontrada uma elevada diversidade. Neste estudo, foi reportada uma linhagem que nunca tinha sido reportada, a qual diverge das sequências mais relacionadas publicadas em mais de 5% para o gene 18S rRNA. Quarto, membros da família Lankesterellidae distintos geneticamente de dados publicados foram também detetados em répteis e anfíbios desta região. Estes últimos dois pontos podem representar novas entidades taxonómicas e merecem mais investigação, nomeadamente o estudo dos seus vetores e estágios de desenvolvimento. Quinto, uma possível nova espécie de *Haemocystidium* foi detetada em duas espécies de osgas de Oman, evidenciando também a importância do rastreio de animais selvagens de regiões remotas. E sexto, linhagens únicas de hemogregarines foram encontradas em duas especies de lacertídeos que coexistem em Oukaimeden (Marrocos). Isto pode ser uma indicação de especificidade e especialização do parasita, bem como uma possível explicação para a variação dos níveis de intensidade de hemogregarinas encontradas entre estas duas espécies.

Além disto, de forma a compilar o esforço conduzido recentemente na investigação da diversidade das hemogregarinas (para o gene 18S rRNA) e Haemosporida (para o gene citocromo *b*), um capítulo desta tese foi dedicado a uma sinopse da informação genética disponível em bases de dados públicas para estes grupos de parasitas. Em concordância com estudos anteriores, os resultados deste capítulo evidenciam as incongruências da atual taxonomia, que são discutidas face à diversidade e relações encontradas. Neste capítulo, problemas comuns tais como obtenção de amostragem de parasitas e desequilíbrios na amostragem de hospedeiros foram assinaladas e recomendações para estudos futuros foram nomeadas.

Um objetivo adicional desta tese foi o estudo das associações parasita-hospedeiro e elucidar a dinâmica de transmissão de parasitas através de diferentes grupos de hospedeiros. Ao analisar a distribuição de parasitas e a gama de espécies hospedeiras de certas linhagens de hemogregarinas em hospedeiros selvagens (isto é, lagartos e roedores como possíveis presas, e cobras e canídeos como possíveis predadores) a ocorrência ocasional de transmissão presa-predador foi corroborada. Estes resultados podem indicar por um lado que algumas destas linhagens de hemogregarinas

possuem uma baixa especificidade e/ou por outro lado a transmissão ocasional para hospedeiros "sem-saída" que não transmitem o parasita para outros hospedeiros. Em qualquer destes casos, estas ocorrências podem ter um papel importante em aumentar a gama de hospedeiros de um parasita e podem desencadear a diferenciação do parasita, por aumentarem a probabilidade da ocorrência de trocas de hospedeiros, devendo assim ser estudados em mais detalhe.

Por fim, esta tese teve também como objetivo o estudo dos padrões de infeção das hemogregarinas em diferentes cenários de modo a discernir os fatores que possam influenciar estes padrões. Estes cenários foram: i) várias espécies de répteis relacionadas e não relacionadas; ii) vários intervalos temporais em duas espécies de lagartos relacionadas que coexistem numa região; e iii) vários intervalos temporais em duas espécies de lagartos relacionadas e uma não relacionada que coexistem numa região. Os padrões de infeção entre as espécies e sexos dos hospedeiros mantiveram-se constantes de uma forma geral nos intervalos estudados em ambos os sistemas. Isto demonstra que características biológicas únicas dos hospedeiros e as suas preferências ecológicas podem ser impulsionadores importantes da distribuição destes parasitas.

Em suma, os estudos apresentados nesta tese demonstraram a utilidade de realizar rastreios moleculares de parasitas em hospedeiros selvagens de regiões remotas, bem como a necessidade de adotar abordagens complementares e integrativas no estudo destes grupos de parasitas. O estudo de estágios assexuados e de desenvolvimento em diferentes hospedeiros destes parasitas conjugado com a caracterização molecular usando vários genes e uma estimativa exata dos padrões de infeção, podem revolucionar o campo e providenciar as respostas para as incongruências taxonómicas evidenciadas.

**Palavras-chave:** filogenia; taxonomia; incongruência; revisão sistemática; diversidade; nova espécie; especificidade parasita-hospedeiro; associações parasita-hospedeiro; interações parasita-hospedeiro; temporal; espacial; sazonalidade; história evolutiva; diferenciação; especialização; lagarto; lacertídeo; gecko; hemoparasita; parasita sanguíneo; gene cyt *b*; gene 18S rRNA; microscopia; morfologia; rastreio molecular; deteção; infeção; quantificação; quantificação absoluta; PCR; qPCR; prevalência; intensidade; estatuto de infeção; padrão de infeção; abundância; transmissão presa-predador; fatores dos hospedeiros; fatores ecológicos; simpatria; semelhança entre hospedeiros; filogeografia; Oman; Marrocos; Madagáscar; Norte de África; Mediterrâneo; Portugal; Espanha; Adeleorina; Haemosporida; Eimeriorina; hemogregarina; hematozoa; *Proteromonas*; *Hepatozoon*; *Bartazoon*; *Karyolysus*; *Haemoproteus*; *Haemocystidium*; *Lankesterella*; *Schellackia*; *Sarcocystis*; *Hepatozoon domerguei*; filária; nematode*; Foleyella furcata*; *Hepatozoon omanensis*; vetor; transmissão; genbank.

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# Appendices

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# Abbreviations

AIC – Akaike information criterion

ANOVA – Analysis Of Variance (also referred to as AOV)

BI – Bayesian Inference

BIC – Bayesian information criterion

BLAST – Basic Local Alignment Search Tool

bp – base pairs

COX1 – Cytochrome c oxidase subunit I (also referred to as COI)

Ct – threshold cycle in quantitative PCR (also known as Cp, Crossing Point)

Cyt *b* – Cytochrome *b*

DNA – deoxyribonucleic acid

ERDF – European Regional Development Fund

ESF – European Social Fund

FCT – Fundação para a Ciência e Tecnologia

FID – Flight Initiation Distance

GB – GenBank

GLM – General Linear Model

GZLM – Generalized Linear Model

GZLMM – Generalized Linear Mixed Model

LM – Linear Models

MCA – Melting Curve Analysis

MIQE – Minimum Information for Publication of Quantitative Real-Time PCR Experiments

ML – Maximum Likelihood

NSRF – National Strategic Reference Framework

ON.2 – North Portugal Regional Operational Program

PCR – Polymerase Chain reaction

POPH-QREN – Programa Operacional Potencial Humano – Quadro de Referência Estratégico Nacional

qPCR – Quantitative Polymerase Chain reaction (also known as Real-Time PCR)

RNA – Ribonucleic acid

rRNA – ribosomal ribonucleic acid

SD – Standard Deviation

SS – Sums of Squares

SVL – Snout-vent length

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# 1 GENERAL INTRODUCTION

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Chapter 1 provides a general introduction into the field of parasitology, with a particular focus on apicomplexan parasites. In this chapter, the following topics are introduced:

i. the importance of parasites in ecosystems and their contribution to biodiversity (section 1.1);
ii. the influence of host relatedness and specificity on our understanding of host-parasite associations (section 1.2);
iii. the ecology and transmission dynamics of host-parasite interactions (section 1.3);
iv. the concept of parasite phylogeography and how this information can be used to improve our understanding of host and parasite coevolutionary and ecological history (section 1.4);
v. the effects of parasites on their hosts at individual and population level (section 1.5);
vi. diversity, phylogeny and lifecycle of the apicomplexan parasites relevant for this thesis (section 1.6);
vii. a brief overview of the most common methodologies used to detect and identify blood parasites (section 1.7).

## 1.1 Importance of parasites to wildlife and ecosystems

In recent years, there has been an emergence of infectious diseases, including vector-borne diseases, for which the primary factor has been anthropogenic change, due to actions such as deforestation, wildlife trade and pollution (Aguirre, 2009). The study of these parasite infections in wildlife is important because these hosts provide a "zoonotic pool" from which unknown pathogens may emerge (Daszak, 2000), which may be of concern for wildlife-domestic animal interactions and public health. Parasites are increasingly recognized as "ecosystem-engineers" (Thomas *et al.*, 2000), and the diversity of parasite communities can be a sign of ecosystem health (Hudson *et al.*, 2006; Hatcher *et al.*, 2012). These organisms play a role in the biodiversity of free living species by interfering in processes as diverse as competition, migration, speciation and ecosystem stability (Combes, 1996), and can play keystone roles in determining species coexistence and biodiversity (Hudson, 1998; Tompkins *et al.*, 2011). They may for instance mediate competition between host species by apparent competition, parasite-mediated coexistence and parasite-mediated trophic cascade (Hatcher *et al.*, 2012), or manipulate host behaviour in order to facilitate transmission (Berdoy *et al.*, 1995; Poulin, 2010). These effects are important because reductions in the abundance of a particular host due to parasite infections may not only have important consequences for the competitors, predators or prey of that host species, but also cause profound ecosystem changes when combined with other factors (Tompkins *et al.*, 2011). Many studies have shown that the removal or introduction of parasites can have profound implications to the structure of a community (Lefèvre *et al.*, 2009) and that parasites may even be as powerful as predators in regulating abundance and distribution of species (Dobson, 2005). For example, parasites may influence ecosystem stability by doubling the food web connections in a network framework (Lafferty *et al.*, 2006). This results in increased dynamic stability, meaning that an ecosystem is able to return

to a normal state when perturbed (i.e. community resilience), which is fundamental for the health of an ecosystem (Hatcher *et al.*, 2012). Parasites can also contribute to energy budgets of ecosystems by concomitant (when preyed upon accidently when an infected prey is eaten) or intentional predation (Johnson *et al.*, 2010).

Furthermore, the loss of parasite biodiversity as a result of the loss of host biodiversity may have unpredictable threats to ecosystem health (Carlson *et al.*, 2013) [although due to climate change, although some vector-borne parasites may increase in certain areas of the world (Møller *et al.*, 2013)]. In particular, specialist parasites are most vulnerable to secondary extinction following their host extinction, which should be considered for conservation policies (Dobson *et al.*, 2008). For this reason, a great deal of debate has been going on for decades on the need to include parasites in endangered and threatened species lists (e.g. IUCN) (Whiteman and Parker, 2005). This is a call for parasite conservation, emphasizing the importance of these organisms not only in terms of the biodiversity index itself but also in terms of the information they may bare about their hosts and ecosystems.

### 1.1.1 Biodiversity of parasites

Understanding why certain organisms are common and abundant while others are rare has been one of the central aims of ecology (Poulin, 2006). Parasites represent a substantial portion of global biodiversity because most free-living metazoan species host one or more unique parasite species (Nadler and De León, 2011). Although it is estimated that parasites may account for more than half of all species on Earth (Price, 1977; Poulin, 2011), only a small fraction of their diversity has been collected and fewer described (Morrison, 2009). The composition of parasite populations may be influenced by many variables including phylogenetic history, host-specificity, parasite competition, and parameters of host biology such as population size, habitat, diet, migration, range, microhabitat use and anti-parasite defenses (Clayton and Walther, 2001; Poulin, 2007).

#### *1.1.1.1 Parasite differentiation and speciation*

Parasites are ideal models for the study of diversification, ecological specialization and speciation mechanisms (Poulin and Morand, 2000). Parasite differentiation and speciation may occur in different ways across time and space through processes that induce reproductive isolation. Hence, when studying these processes it is important to consider host and parasite mobility, host and parasite biological traits, host-specificity and abiotic factors, as all of these factors may influence reproductive isolation (Huyse *et al.*, 2005). Examples of processes that induce parasite differentiation and speciation may occur:

i. as an adaptation to different sympatric hosts (McCoy, 2003; Criscione *et al.*, 2005);
ii. due to non-overlapping ecologies between sympatric host species that may lead to physical separation between groups of parasites (McCoy, 2003).

iii. by accidental host switch to a host on which this parasite did not occur, which may trigger an adaptation to this new host (Clayton *et al.*, 2003; Martinsen *et al.*, 2008) (Figure 1-1);

iv. when parasites of the same species adapt to different tissues in a single host species (Perkins, 2001);

v. when parasites add host species to their host spectrum that can induce additional adaptations (Poulin, 1997), or when reproductive barriers affect the parasite lineage but not the host (Clayton *et al.*, 2003) (without the host speciating, i.e. duplication in Figure 1-1);

However, parasites may fail to adapt to a new host or host lineage and thus are lost (miss the boat, Figure 1-1) or may fail to diverge when their hosts do (failure to diverge, Figure 1-1) (Charleston and Perkins, 2006). Moreover, generalist parasites have a high potential of undergoing specialization on different host species because they are subjected to different selection pressures (which may increase with host genetic distance) (Mccoy *et al.*, 2001).

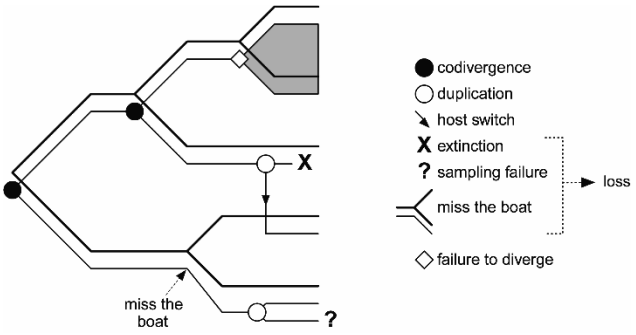


Figure 1-1 Macroevolutionary events that explain parasite differentiation and speciation.
[Adapted from Charleston and Perkins (2006)]

### *1.1.1.2 Cryptic parasite diversity*

The study and documentation of parasite diversity has been traditionally done using morphological characters (Valkiūnas, 2005; Telford, 2009). Assessment of these characters allows the identification of groups of lineages that are morphologically similar, which could be an indication of similar parasite ecology (Valkiūnas, 2005; Cosgrove *et al.*, 2008). However, the limited morphological characters of many parasitic helminths and protozoans have resulted in an underestimation of the real diversity within these groups (Bensch *et al.*, 2004; Dobson *et al.*, 2008; Perkins *et al.*, 2011). For this reason, the term “cryptic species” has been coined to refer to organisms that are morphologically indistinguishable but genetically distinct (de León and Nadler, 2010; Nadler and De León, 2011), and it has been shown that cryptic species can co-occur in the same individual host (Bensch *et al.*, 2004; Pérez-Tris *et al.*, 2007). By recognizing new host records and identifying parasite species and cryptic lineages, we increase our understanding of parasite ecology, evolution and diversity. This is useful information that can be used for the management of vertebrate host species, especially for endangered species (Whiteman and Parker, 2005). Diversity of parasites is often not considered in ecological studies, but this can be an important source of variation in host-parasite interactions (Cosgrove *et al.*, 2008). Molecular methods allow a more precise identification

of parasite species and the distinction between species that have identical or indistinguishable morphologies. The importance of correct species identification can be illustrated, for example, by a study of malarial parasites in humans from Malaysian Borneo. These parasites were morphologically identified as *Plasmodium malariae*, a parasite of humans, but later genetically characterized identified as *Plasmodium knowlesi*, a parasite of macaque monkeys (Singh *et al.*, 2004). This resolution contributed to the investigation of the transmission dynamics between humans and macaque monkeys.

Species delimitation recommendations and procedures have long been a subject of discussion, especially in parasitology, because it is often difficult to decide when a particular taxon is “different enough” based on morphological and/or molecular data from other described species (Nadler and De León, 2011). A common approach that is generally accepted for the proposition of new species has been the formation of a monophyletic group by the proposed new species and considerable genetic divergence relative to other closely related species. De León and Nadler (2010) proposed a set of steps for describing new parasite species, taking into account the possibility of existing cryptic species. If the parasite of study is not distinct enough, both morphologically and genetically, relative to a closely related described species then it should be considered as intraspecific variation of that species (Figure 1-2). On the other hand, if it is distinct enough and it forms a well-supported monophyletic group, it should be described and named as a new parasite species (Figure 1-2). The threshold for this genetic divergence depends on the molecular marker and its variability, for instance the accepted cut-off for the cyt *b* gene in haemosporidians is 5% genetic divergence (Outlaw and Ricklefs, 2014), while for the 18S rRNA gene new species may be described based on one or two base pair differences due to its less variability (O’Dwyer *et al.*, 2013). Finally, parasites that occur in multiple host species (i.e. low host-specificity), in different habitats and/or in extensive geographical areas are likely to undergo differentiation and speciation (Nadler and De León, 2011). Therefore it is important to investigate the diversity and host spectrum of parasites using molecular tools to identify the occurrence of cryptic diversity.

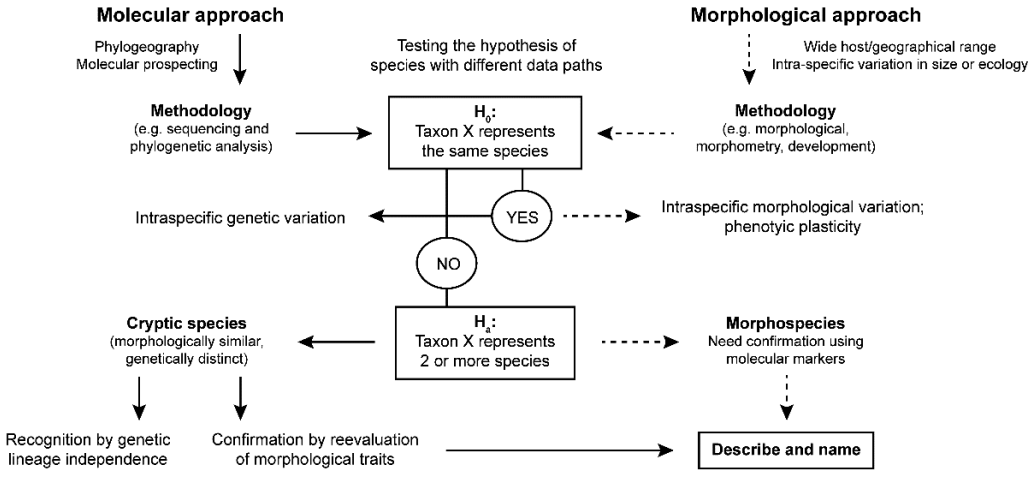


Figure 1-2 Hypothesis formulation in description of new species, with a focus on cryptic diversity.
[Adapted from de León and Nadler (2010)]

## 1.2 Host-spectrum and host-specificity in parasites

The host-spectrum (or host range) of a parasite can be defined as the number of host species that it is able to infect. In this perspective, one can consider two main filters that constrain the host spectrum of parasites, the encounter filter and the compatibility filter (Figure 1-3). The encounter filter is associated with the probability of contact between the parasite and potential host species, while the compatibility filter is associated with the probability of these organisms having a durable interaction after the encounter filter has been surpassed (Combes, 2001). The probability of a parasite to encounter host species is dependent on the distribution of biodiversity (associated with environmental and historical filters) and on host behaviour (associated with dispersal filters), in the sense that these organisms may never be in contact because they do not exist in the same ecosystem or due to behavioural reasons (Figure 1-3) (Combes, 2001; Guégan *et al.*, 2005). In addition, hosts and parasites may only be compatible if a host can provide the necessary resources and conditions for parasite development and if host defenses, such as immune response, do not eradicate infection (Figure 1-3).

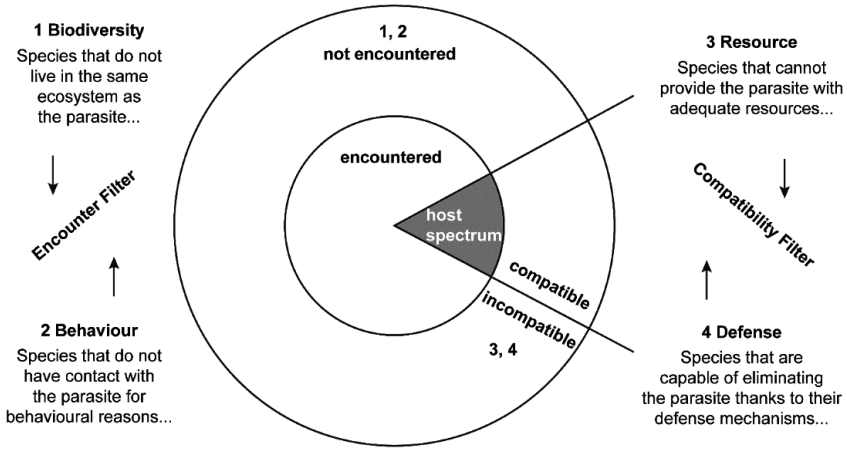


Figure 1-3 Factors involved in the formation of a host spectrum.
[Adapted from Combes (2001)]

Host-specificity in parasites is linked with their host-spectrum and so may be determined by ecological, behavioural, physiological and biochemical traits of its host species that the parasite has adapted to (Krasnov and Poulin, 2010). The characterization of parasite diversity and determination of host-specificity requires an assessment of the prevalence and intensity of parasite infections, rather than simply investigating the range of hosts parasitized (Tripet and Richner, 1997). Determining host-specificity is also important because it can provide information on parasite specialization and efficiency of host exploitation. A parasite with high host-specificity, i.e. one that can infect a narrow range of taxonomically related hosts, may be more efficient at exploiting its host because they are specifically adapted to them (Poulin, 2005). On the other hand, a parasite species that is able to infect a wide range of unrelated hosts may be less efficient at exploiting them, however

it may have a wider plasticity and resilience to co-extinction (Combes, 2001; Dunn *et al.*, 2009). If a parasite is specific to few closely related host species (Figure 1-4 D), then it displays a high basic and phylogenetic specificity towards its hosts. On the other hand, if a parasite is specific to only a few host species that are distantly related (Figure 1-4 B), then it displays a high basic specificity but a low phylogenetic specificity. In cases in which the parasite can be found in several closely related host species (Figure 1-4 C), it shows that it has a high phylogenetic specificity but low basic specificity. Finally, if a parasite infects several host species, of which some are closely related while others are distantly related, (Figure 1-4 A), then it displays both low phylogenetic and basic specificity (Poulin *et al.*, 2011).

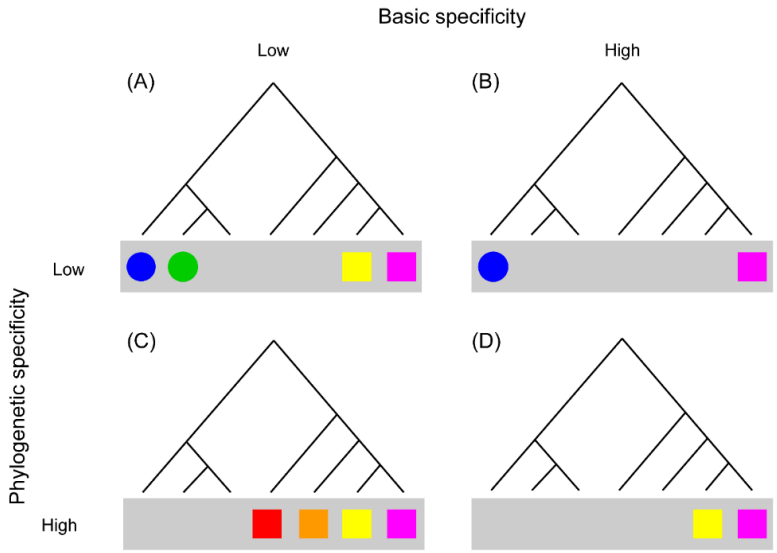


Figure 1-4 Contribution of host phylogeny to the measurement of host-specificity. Parasite populations are indicated by grey boxes and host species by coloured symbols. For any level of basic host-specificity, a parasite could show high phylogenetic specificity if it only exploits host species closely related to each other, or low phylogenetic specificity if its host species are not closely related. [Adapted from Poulin *et al.* (2011)]

### 1.2.1 Host relatedness and parasite distributions

As noted by Combes (2001), “to live in two or more host species, a parasite must be capable of encountering organisms that have different behaviours, exploiting different resources, successfully confronting different competitors, and avoiding different immune systems. […] These costs probably increase very quickly when the genetic distance between hosts increases” (p. 87). Hence, a first step when investigating host-specificity is to determine its correlation with host relatedness. Host relatedness can be calculated based on taxonomic distances between hosts in a Linnean taxonomic tree path length linking the two host species (Figure 1-5), or based on the genetic distance between the two host species. It is also important to consider the distribution of the parasite among the different host species, by determining its prevalence and intensity of infection. The principal host species can be considered as that in which the parasite reaches its highest levels of prevalence, intensity and abundance, while the other host species are auxiliary hosts in which these parameters

are lower (Poulin and Mouillot, 2004, 2005). Auxiliary hosts that are more closely related with the principal host, are more likely to have a similar exposure to parasites (e.g. due to similar diet and behaviour) and to offer similar living conditions to the parasites (e.g. immune defences, nutrient quality and availability) (Poulin, 2005). In contrast, other auxiliary hosts will differ greatly from the principal hosts with respect to these characteristics.

Poulin and Mouillot (2005) developed a host-specificity index that accounts for the phylogenetic and ecological information of the hosts. This index includes a $S_{TD}$ measure, which considers the average taxonomic/genetic distances, and a $VarS_{TD}$, which considers the asymmetries in these distances between the host species exploited by the parasite. High taxonomic/genetic distances between hosts exploited by a parasite results in an increase in $S_{TD}$, and if a parasite infects one or a few hosts that are distinctly related to the other hosts results in an increase in $VarS_{TD}$ (Figure 1-5 B) (Poulin, 2005). In other words, the lower the $S_{TD}$, the more specific a parasite is, and the lower the $VarS_{TD}$ the more symmetrical parasite distribution is regarding host relatedness (Figure 1-5 A). Intuitively, the greater the taxonomic distance between principal and auxiliary hosts for parasites with higher specificity, the lower parasite infection parameters are in more distantly related host species (Figure 1-5 A).

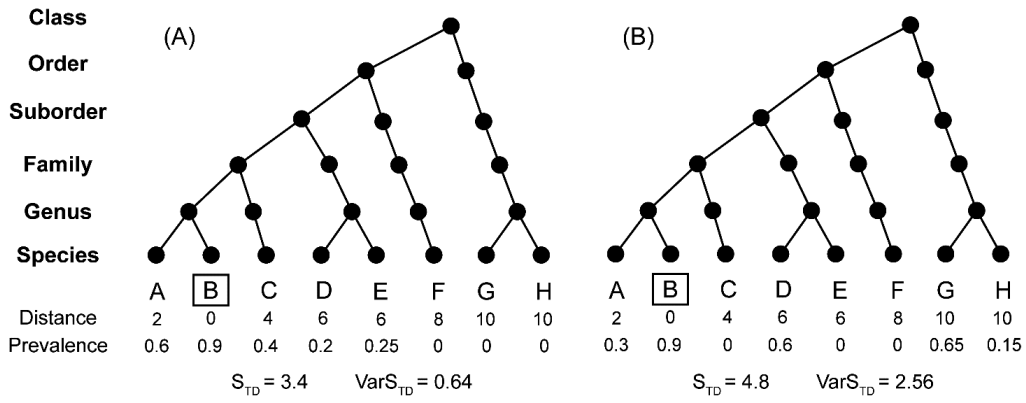


Figure 1-5 Hypothetical example on the influence of parasite distribution among host species for host-specificity indexes. The principal host is shown by a rectangle and numbers refer to the taxonomic distance (i.e. the number of branches) between the principal host and the other hosts. Calculation of the indexes $S_{TD}$ and $VarS_{TD}$ were conducted using TaxoBioDiv2 (http://www.otago.ac.nz/parasitegroup/Downloads/TaxoBiodiv2.zip) (Poulin and Mouillot, 2005). [Adapted from Poulin (2005), Poulin and Mouillot (2005)]

However, numerous other factors that may be directly or indirectly associated with host relatedness can influence infection patterns and other host-parasite interactions, such as differences in the immune system, behaviour and microhabitat preferences between the different hosts (Poulin, 2005). Vector-borne parasites are especially constrained by host dispersal abilities, which could drive patterns of geographical specificity. Geographical specificity can be defined as the consistency in host use across communities of potential host species that change in composition from one locality to the next (Poulin *et al.*, 2011). In addition, a parasite species can show high specificity at the local scale but a low specificity at the global scale, thus it may be important to distinguish between local species diversity and the difference in species composition among localities (Poulin *et al.*, 2011).

However, this may require an extensive knowledge of the number and distribution of hosts at both local and global scale, which is often not available in less studied parasites.

### 1.2.2 Coevolutionary patterns in host-parasite associations

Apart from host phylogenetic factors, ecological factors may greatly influence the coevolutionary patterns in host-parasite associations. Variation in microhabitat sharing between hosts and parasites may influence these interactions at different levels, from individuals to populations and species. Next, four examples illustrating this variation are given. First, within a single host individual, a parasite with a patchy microhabitat distribution (particularly important in macroparasites) may have increased probability of duplication (see Figure 1-1) (Clayton *et al.*, 2003). Second, within a host population, a parasite with low prevalence and/or intensity may have increased probability of missing the boat (i.e. to miss translocation with their hosts to a new geographical area) since dispersal founder individuals are more likely to be parasite-free (Clayton *et al.*, 2003). Aggregations of parasites among individuals within a population may also increase the probability of a parasite to miss the boat or become extinct (Clayton *et al.*, 2003). This again may be because parasites may be absent from hosts involved in founder events, assuming that parasitized individuals are not able to disperse as easily due to poorer condition. Third, parasites can have different abundances among host populations, for which low host densities or gaps in parasite distributions can create a dispersal barrier for parasites or limit gene flow between parasite populations in different parts of the host's geographic range (Clayton *et al.*, 2003). And fourth, the distribution of parasites among host species is directly associated with host-specificity. A parasite that fails to complete host switching because it could not disperse and/or establish in the new host results in a parasite merely incorporating a new host species, which reduces its host-specificity (Clayton *et al.*, 2003). Thus, limitations of dispersal are important in maintaining specificity of parasites among related hosts, while limitations to establishment (e.g. host defence) are important in maintaining the specificity of parasites in unrelated hosts (Clayton *et al.*, 2003).

#### *1.2.2.1 Sympatric host species*

Sympatric and closely related host species present a great opportunity for studying host-parasite associations and host-specificity, and the influence of host-relatedness and ecological factors on the evolutionary patterns of the interacting species. Coexisting species would be selected to tolerate each other's parasites or to avoid them by using different microhabitats (Price *et al.*, 1986) and related host species are likely to be susceptible to the same or similar parasite species (Thomas *et al.*, 1995). When sympatric, closely related species are compared, divergences in infection patterns and phylogeographic patterns can be attributed to derived life-history traits or ecological traits (Nieberding *et al.*, 2004). In some cases, ecology may be more important than host phylogeny or taxonomy (Figure 1-6), which suggests that some parasites can be more opportunistic because they

can exploit host species with similar ecological requirements (“resource tracking”) rather than being limited to their evolutionary history (“phyletic tracking”) (Clayton *et al.*, 2003; Poulin, 2005). As an example, Poulin (2005) found that the level of infection attained by Trematodes, Cestodes, Nematodes, Acanthocephalans and Copepods infecting fish species in auxiliary host species was not determined or constrained by the taxonomic distinctiveness of the auxiliary host to the principal host. For this reason, the study of closely related sympatric species is important because phylogenetic confounding factors are accounted for, which allows for a better discrimination of the possible ecological factors influencing parasite distributions.

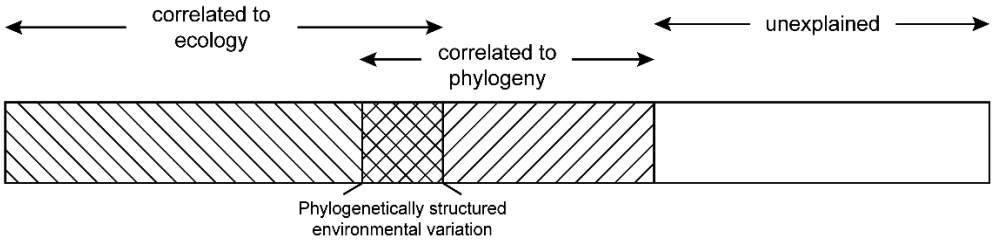


Figure 1-6 Estimated influence of ecology and phylogeny on parasite distribution. [Adapted from Morand and Poulin (2003)]

## 1.3 Disease ecology and transmission dynamics

Disease ecology is a highly interdisciplinary subject that studies the underlying principles that influence the spatio-temporal patterns of diseases (Kilpatrick and Altizer, 2012). This encompasses a wide range of factors that may influence parasite transmission and reproduction such as mode of transmission, lifecycle, epidemiology and ecology of the environment. The environment includes abiotic features such as climate and weather, soil moisture and composition, water and humidity, solar irradiation and topography; as well as biotic factors including other individuals from the parasite and host own species, a range of other organisms including competitors, predators, vectors, alternate hosts, other parasites and humans (Wolinska and King, 2009). The environment can influence the expression of host life-history traits and fitness (Agnew *et al.*, 2000), and spatial structure, dispersal patterns, and landscape-level heterogeneity can influence the spatial distribution of parasites (Simberloff, 2010; Kilpatrick and Altizer, 2012). Thus, it is important to consider the relationship between each organism with the environment within which it occurs.

The interactions between biotic and abiotic factors dictate the success of parasite transmission and fitness. For instance, genetically identical parasites may have different fitness in two genetically distinct hosts (Figure 1-7 A), two genetically distinct parasites can have different fitness in the same host (Figure 1-7 B), or two genetically distinct hosts can have opposite fitness patterns in two genetically distinct hosts in the same environment (Figure 1-7 C). In addition, genetically identical parasites that infect genetically identical hosts can have different fitness depending on their environment (Figure 1-7 D and E), or genetically distinct parasites can have opposite fitness patterns

on genetically identical hosts that inhabit different environments (Figure 1-7 F and G). Finally, different environmental traits in different environments may have opposite effects on parasite fitness (Figure 1-7 H).

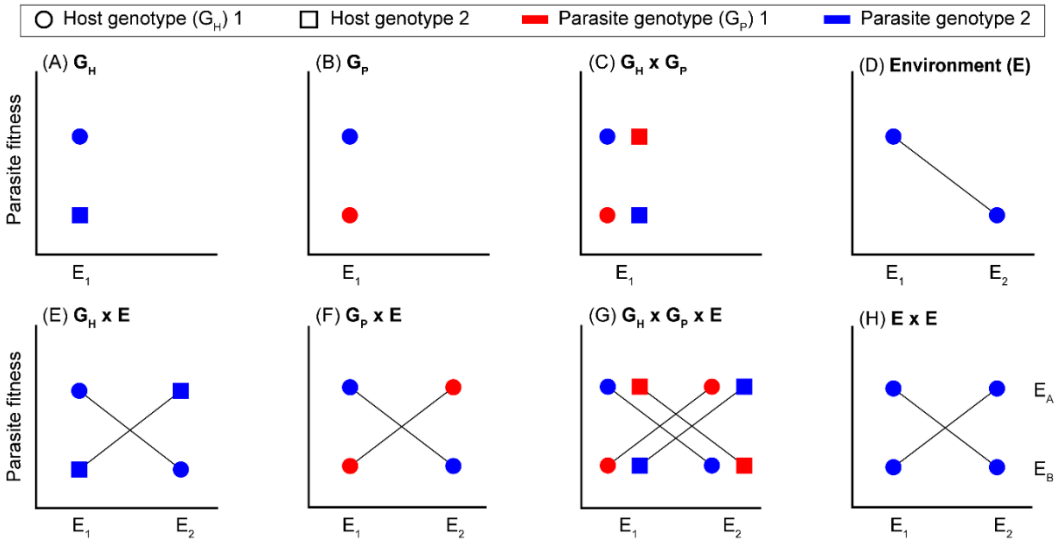


Figure 1-7 Influence of host genotype, parasite genotype, environment and their interactions on parasite fitness. Shapes indicates host genotype, colours indicate parasite genotype, Y-axis indicates parasite fitness (which is associated with host susceptibility and vector competence) and X-axis two different environments in which this parasite system exists. [Adapted from Wolinska and King (2009), Lefèvre *et al.* (2013)]

Depending on the transmission mode and lifecycle of a parasite, it can be exposed to several different factors that may influence host-parasite interactions. Parasites can have direct lifecycles, in which parasites infect single host species, or indirect lifecycles, in which parasites infect more than one host species in order to complete their lifecycle. Vector-borne parasites usually have indirect lifecycles and are transmitted by a vector, usually an arthropod host. Therefore, these parasites may be subjected to very distinct pressures during different stages of their lifecycle and often include a wide range of hosts, which complicates our understanding of interactions between their hosts and their environment. Next, a brief overview of some of the main interactions between parasites and hosts is given, regarding parasite characteristics that can manipulate hosts and influence parasite transmission dynamics, as well as other environmental components that can interfere with parasite fitness.

### 1.3.1 Transmission dynamics

Disease transmission within and among individuals within a population can reveal interactions among hosts (Whiteman and Parker, 2005). Transmission success is an important component of parasite's reproduction and can be influenced by several traits, such as parasite virulence, host susceptibility/resistance, host condition, host vagility, competition for resources within the host, ecological opportunities for transmission, environmental heterogeneity and the dynamics of transmission (Poulin, 1997; Agnew and Koella, 1999; Beldomenico and Begon, 2010). To

understand the ecology of a multi-host disease it is critical to identify all the potential hosts, and to quantify the transmission rates within and between the species involved. Transmission in vector-borne diseases may be facilitated by host habitat sharing and distribution of competent vectors; host grooming with ingestion of infected invertebrates and trophic transmission by ingestion of infected prey or by concomitant predation of infected invertebrate with prey (Ewing and Panciera, 2003; Johnson *et al.*, 2009). In arthropod-borne diseases, not all hosts contribute equally to the spread of the disease and some of these parasites are likely to end-up in non-competent hosts (Poulin, 2010). Some hosts may present adverse conditions for the multiplication of parasites (e.g. strong immune response), serving as “sinks” or “dead-ends” for parasite transmission (Hellgren *et al.*, 2009) (Figure 1-8). Some of these “dead-end” hosts may reduce, or even eliminate, infection within the vector itself (Lane *et al.*, 2006), reducing the percentage of infected vectors in the community (Johnson *et al.*, 2008c). However, these “dead-end” hosts still provide a blood meal for the vector and thus may help maintain the vector population (Figure 1-8), ultimately helping the pathogen to persist (Hudson, 1998). These hosts may occur at two stages in vector-borne parasites with heteroxenous lifecycles: when a vector takes a blood meal from an unsuitable host, or when an unsuitable host feeds on an infected intermediate host by predation or scavenging (Figure 1-8).

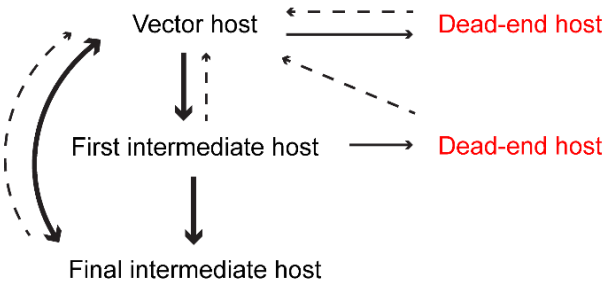


Figure 1-8 Role of "dead-end" hosts in transmission dynamics of vector-borne diseases.
Thin arrows indicate transmission to dead-end hosts and dashed arrows indicate
blood meal source for vectors, which is important for the maintenance and survival of these hosts.
Red colour indicates end of parasite cycle due to unsuccessful further transmission.

Moreover, one of the main questions regarding transmission dynamics and host-parasite interactions in the past decade, has been the concept of biodiversity-disease relationships. Parasite transmission may be host density dependent, in which both infectious and susceptible hosts are important, or may be host frequency dependent, in which only infectious hosts are important for successful transmission (Johnson *et al.*, 2013a). In density dependent cases (Figure 1-9 A), a reduction in host biodiversity leads to a reduction in overall parasite prevalence, which in return may lead to the proliferation of susceptible host populations. If parasite prevalence has been lowered in excess, then parasites will not be able to colonize the new populations, thus closing the cycle. In frequency dependent cases (Figure 1-9 B), a reduction in overall host diversity may or not lead to an increase in infectious hosts, that is, hosts that are competent and can transmit the disease. This may lead to an increase in parasite prevalence, which may reduce overall host population density

but support a greater proportion of highly competent host species (Hatcher *et al.*, 2012; Johnson *et al.*, 2013a). This scenario is of special importance for more vulnerable host populations that in this case are more prone to extinction, which in return results in a decrease in host diversity and the cycle may continue (Figure 1-9 B).

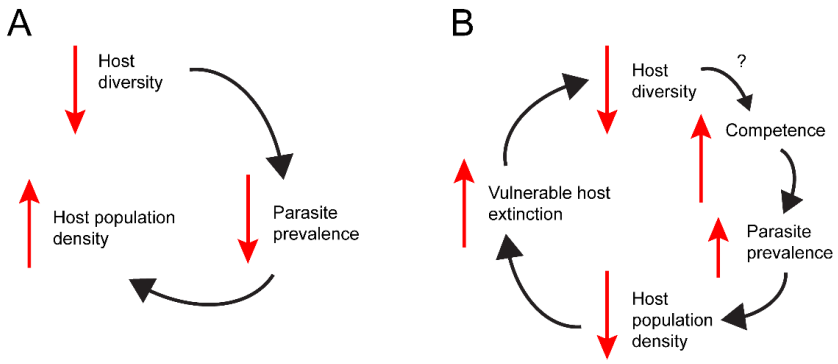


Figure 1-9 A conceptual model of biodiversity-disease relationship.
[Adapted from Hatcher *et al.* (2012)]

Furthermore, an increase in parasite and/or host richness can contribute to a reduction in transmission success. For example, recent studies on amphibian decline and the virulent trematode *Ribeiroia ondatrae*, which leads to limb malformation in amphibians, have shown that increased species parasite richness reduced transmission of this trematode by more than half (Carlson *et al.*, 2013). This may be a result of increased parasite competition in the intermediate host (Johnson *et al.*, 2013b) or due to increased immune genetic diversity in hosts as a result of multiple parasite exposures (Froeschke and Sommer, 2012). Moreover, higher host richness may contribute to a reduction in infection success due to the dilution effect and encounter reduction (Dobson, 2004; Keesing *et al.*, 2006; Johnson *et al.*, 2013a). One example of this would be the possible reduction of human transmission by zooprophylaxis, that is to have non-human animals that serve as alternative hosts and redistribute the blood meals for the mosquito vectors (encounter reduction) (Saul, 2003). Another example would be the reduction of the bacteria *Bartonella* infection in wood mice *Apodemus sylvaticus* due to the presence of bank voles *Clethrionomys glareolus* (Telfer *et al.*, 2005). However, there are cases in which increased species diversity can increase disease risk if the added species can act as an alternative source of infection (spillover) or lead to an increase in the number of vectors (vector augmentation) (Morand and Poulin, 1998; Daszak, 2000; Dobson, 2004; Keesing *et al.*, 2006). Many factors can influence broad-scale disease dynamics, such as sickness behaviour that may result in loss of aggression, which results in increase encounters between healthy and infected individuals, facilitating transmission (Hawley and Altizer, 2011). As a matter of fact, transmission dynamics in multi-host and vector-borne infections are subjected to more complex patterns and associations imposed by environmental factors that are still not well understood, which may result in spatial and temporal variation of the density of vectors, intermediate stages and susceptible hosts (Johnson *et al.*, 2013a).

### 1.3.2 Host factors

Our understanding of the relationship between host traits and parasite infection parameters is still far from clear. As Poulin (2007) noted, “For every positive relationship between a host trait and parasite species richness currently published, one can find a negative relationship and some non-significant relationships between the same trait and parasite richness in other studies”. This variation is probably associated with the combination of several factors that are intrinsic to the characteristics of each host and environment (see Figure 1-10 and Figure 1-12).

The evolutionary ecology of host-parasite relationships can be studied by integrating fitness-related parameters in a life-history theory context (Agnew and Koella, 1999). Life-history traits of organisms are those associated with fitness, such as growth, reproduction, survival, behaviour and nutrition. The term “ecological immunity” has been coined to the notion that there is an energetic trade-off for the host when mounting an immune defence and that, for this reason, individuals have less energy to devote to other life-history activities (Sheldon and Verhulst, 1996; Tripet *et al.*, 2008). The costs of parasitism can be reduced mainly by behavioural avoidance, such as avoiding contact with parasites and infected conspecifics, or by having immune defensive mechanisms that prevent the establishment and/or clear an infection (Agnew *et al.*, 2000). Parasites can indirectly affect host behaviour by consuming host resources and having an impact on the host’s nutritional state, which in return affects host feeding behaviour and diet (Figure 1-10) (Ponton *et al.*, 2011). Host diet is directly associated with immune function and microbial community in the gut (Cirimotich *et al.*, 2011), which interfere with the host’s nutritional status and parasite infections, ultimately dictating overall host fitness (Figure 1-10).

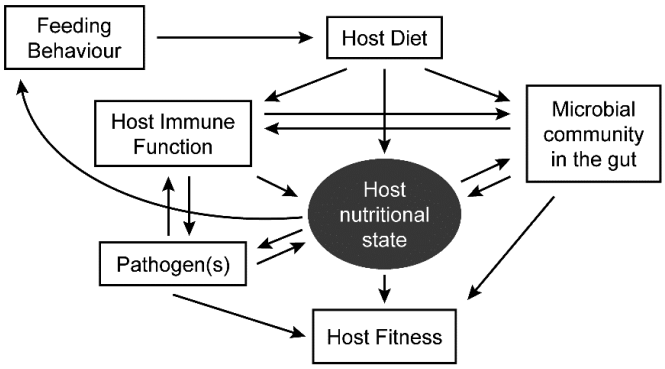


Figure 1-10 Interaction between host nutrition, behaviour, immunity and fitness.
[Adapted from Ponton *et al.* (2011)]

#### *1.3.2.1 Vertebrate host immunity*

Host immunocompetence plays a major role in clearing parasite infections and it is known to vary greatly between and within species if they are subjected to different ecological pressures (Brunham *et al.*, 1993; Schmid-Hempel, 2003; Telfer *et al.*, 2010). In vertebrates, males usually have lower immune response and resistance to infection than females (Schmid-Hempel, 2003). This trend has

been coined as the “immunocompetence handicap” hypothesis, which suggests there is a trade-off between increased level of testosterone and male mating success, and the ability to build an immune response against parasitic infections (Folstad and Karter, 1992). Besides its immunosuppressive effects, testosterone has been shown to have important effects on transmission-relevant behaviours, such as contact rate, aggressiveness, and territory density (Grear *et al.*, 2009; Hawley and Altizer, 2011). For this reason, males that are sexually mature are often regarded as superspreaders of wildlife diseases by contributing disproportionately to parasite transmission (Salkeld and Schwarzkopf, 2005; Hawley and Altizer, 2011; Molnár *et al.*, 2013). During mating period, testosterone levels increase greatly in males (Cox and John-Alder, 2005; Gowan *et al.*, 2010; Restif and Amos, 2010), which is important for secondary sexual development that potentially increases mating success (Combes, 2001) (Figure 1-11 A). However, there is a trade-off with this potential increase in mating success because testosterone is a known immunosuppressive hormone [(Folstad and Karter, 1992; Salvador *et al.*, 1996; Klein, 2004) but see (Hasselquist *et al.*, 1999)]. Thus, with the exception of males that have other “good genes” that allow them to overcome these immunosuppressive effects of testosterone, this leads to a decrease in male immune defenses and consequently an increase in parasite load, which has a negative impact on host fitness (Figure 1-11B) (Hamilton and Zuk, 1982; Combes, 2001). Restif and Amos (2010) suggest that, assuming no acquired immunity in males, the constant re-infection events in males selects for lower resistance, which leads to the counterintuitive situation where males evolve lower immunocompetence despite being more exposed to infection than females.

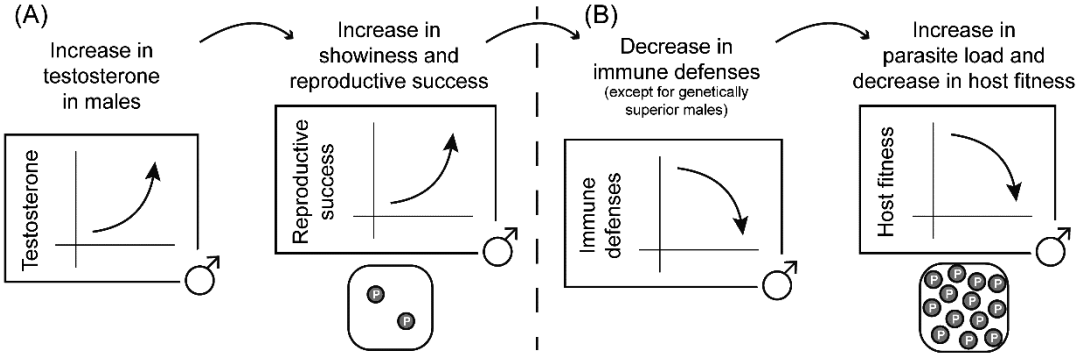


Figure 1-11 The immunocompetence handicap hypothesis in males.
(A) individuals with other attributes (e.g. “good genes”) that allow them to have maintain low levels of parasites and achieve greater reproductive success.
(B) individuals that cannot cope with immunosuppressive effects of testosterone, resulting in an increase in parasite load and consequent decrease in reproductive success.
[Adapted from Combes (2001)]

Moreover, other widely studied vertebrate host factors that may influence parasite distributions include body size and age. Host body size can be a proxy of the resources and the number of niches for parasites that an individual host can offer (Clayton and Walther, 2001). In endo-macroparasite systems, such as nematodes, host body size is often positively associated with parasite richness and intensity because larger hosts are expected to harbour higher parasite fauna (Morand and

Poulin, 1998), although these relationships are diminished after controlling for sampling effort and host phylogeny (Poulin, 1997). These associations in endo-microparasite systems are still unclear, with studies showing contrasting results depending on the parasite, host and geographical location. Nevertheless, for hemogregarine parasites of reptiles, there seems to be a trend towards a positive correlation with body size in short-lived reptile species (Amo *et al.*, 2004; Garrido and Pérez-Mellado, 2013; Molnár *et al.*, 2013), but a negative correlation in long-lived reptile species (Madsen *et al.*, 2005; Brown *et al.*, 2006; Madsen and Ujvari, 2006; Godfrey *et al.*, 2011).

Furthermore, vertebrate host microhabitat preferences, behaviour and consequent exposure to possible arthropod vectors (e.g. ticks, mites and mosquitoes) may result in differential hemoparasite transmission (Boyer *et al.*, 2010). It is possible that repeated use of rock crevice refugia may facilitate accumulation and transmission of ectoparasites and thus result in higher hemoparasite infection levels (Reardon and Norbury, 2004). Alternatively, individuals may use many different refuges, which has been shown to decrease the number of tick load in the sleepy lizard *Tiliqua rugosa* (Leu *et al.*, 2010).

#### *1.3.2.2 Vector competence*

In the case of vector-borne diseases, a part of host (vertebrate) immunity, numerous factors influence the transmission potential by a given vector individual. Vector competence is a combination of parasite infectivity and vector susceptibility, and thus includes both vector host resistance and parasite infective mechanisms (Lefèvre *et al.*, 2013). Vector genetics and vector-parasite genetic interactions, play an important role in determining vector competence (Mitri *et al.*, 2009; Harris *et al.*, 2010). Like vertebrate host populations, vector populations consist of many individuals that besides having different genetic backgrounds, also differ in life-history traits that may influence their competence, such as age, reproductive status, immune status and body size (Lefèvre *et al.*, 2013). In addition to this, vectors may also be subjected to other non-genetic factors that may result in differential vector competence, such as different environmental conditions and other parasite infections or microbiota composition, which interact with their physiological state, immune function and behaviour, as well as with parasite development (Figure 1-12) (see (Wolinska and King, 2009; Lefèvre *et al.*, 2013) for an overview of environment determinants on host-parasite interactions).

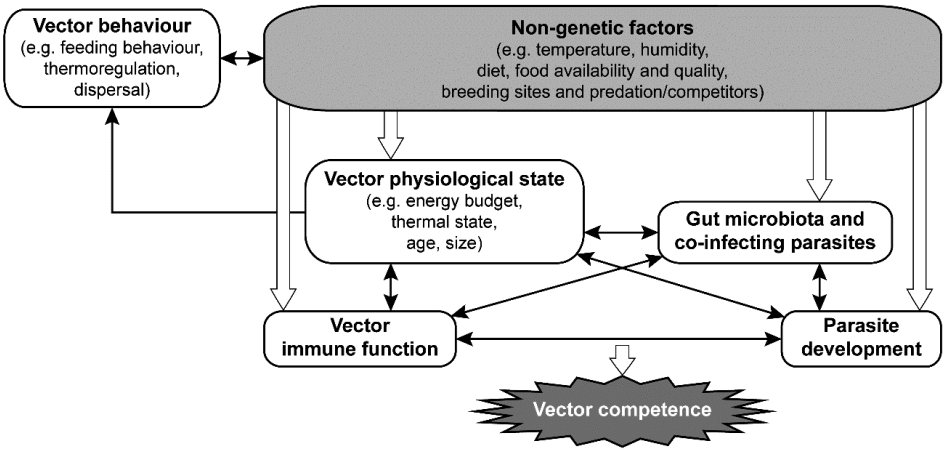


Figure 1-12 Non-genetic factors that influence competence of arthropod vectors in parasite transmission. [Adapted from Lefèvre *et al.* (2013)]

## 1.4 Parasite phylogeography

The geographic component of species diversity is of great interest (Poulin and Morand, 2000). Phylogeographical analyses aim to reconstruct the historical biogeography of populations (e.g. vicariance events) and identify major genetic subdivisions within species (Avise, 2000). The study of host and parasite phylogeographic patterns is necessary to understand the coevolutionary and historical biogeographical processes in shaping the diversity of parasite assemblages (Poulin and Mouillot, 2003; Nieberding *et al.*, 2004). Parasites are convenient models for studying geographic patterns and variation in the ecological parameters of a species. First, replicated samples of parasites (e.g. host individuals, populations or species) are easy to obtain, and second, individual hosts represent well-defined ecological niches of these organisms (Krasnov and Poulin, 2010). Parasite genetic diversity and population structure may be influenced by several factors such as host range, vagility and sociality (Whiteman and Parker, 2005; Criscione *et al.*, 2005), as well as parasite breeding system, selection, and gene flow, the current and historical effective size, and the mutation rate (Nieberding *et al.*, 2004).

Hence, studying parasite distribution is the first step towards understanding host-parasite interactions and opens up the possibility of using parasite information beyond the scope of the parasite, for example, to understand host biogeography and behavioural history at a finer scale (Whiteman and Parker, 2005). The study of these patterns is more complex in heteroxenous parasites because these require more than one host to complete their lifecycle and so are also subjected to vector dispersal (Perkins, 2001). A potential pitfall in biogeographic studies is the risk that diversity among geographic regions merely reflects the variability in research effort, especially in relatively poorly known organisms (Poulin and Morand, 2000). To minimize these issues, studies tend to focus on direct and specific parasites (Nieberding *et al.*, 2004; Nieberding and Olivieri, 2007).

In this perspective, parasites are closely linked to their hosts and thus often share similar coevolutionary history and phylogeographical patterns (Criscione *et al.*, 2005). For this reason,

parasites have been used to infer host population history (Nieberding *et al.*, 2004, 2008), which increases their overall importance for conservationist biologists and ecologists (Whiteman and Parker, 2005). Parasite DNA usually evolves more rapidly than host DNA, thus parasite population structure can be used to infer recent host structure that is not possible using host markers. An example of this is a study by Nieberding and colleagues (2004), in which host population structure and phylogeographic history are unveiled as a results of a higher differentiation of the parasite (Figure 1-13). In this study, while host phylogeny only produced three well-supported main clades, parasite phylogeny allowed to uncover additional well-supported subdivision within those main clades, as well as a new clade (Nieberding *et al.*, 2004) (Figure 1-13).

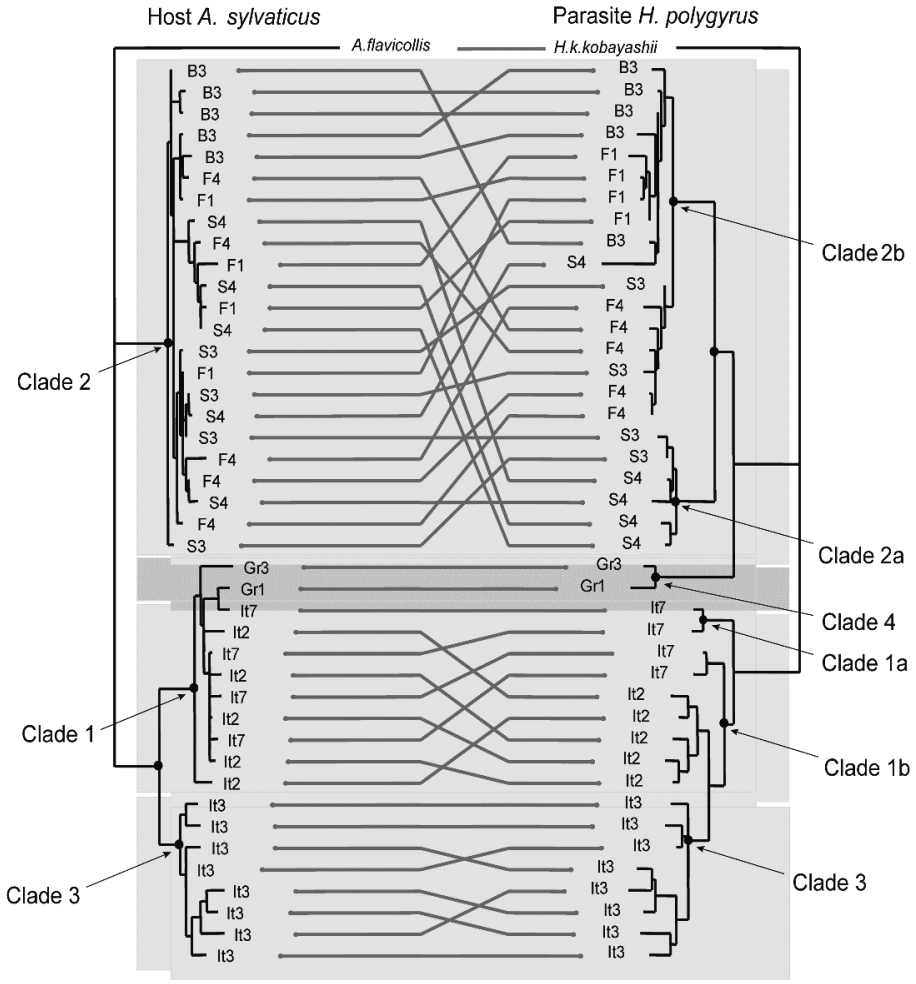


Figure 1-13 Host-parasite association patterns, showing how parasites can be used to infer their hosts phylogeographic history. Greater differentiation of the parasite unveils phylogeographic patterns of their hosts that were not possible using only molecular markers on their hosts. Darker grey indicates a clade only obtained through parasite phylogeny [Adapted from Nieberding *et al.* (2004)]

Furthermore, the study of parasite biogeography also provides information on parasite evolutionary history, for example, to disentangle between parasite allopatric differentiation and specialization (Figure 1-14) either due to geographical separation or to host differentiation (Nieberding *et al.*, 2008). If a parasite population genetic structure reflects a geographical pattern independent of the sampled host species, it suggests that parasite and host co-differentiation did not

occur (Nieberding *et al.*, 2008). On the other hand, if co-differentiation has occurred this can be observed intra-specifically by having genetic lineages that cover similar allopatric areas (Nieberding *et al.*, 2004). For instance, the analyses of cyt *b* gene differentiation within the host *Apodemus* and its parasite *Heligmosomoides*, have shown that the parasite genetic lineages segregated according to the individuals' geographic origin rather than according to the host species, while displaying some regional differentiation in some of the geographical locations (Nieberding *et al.*, 2008).

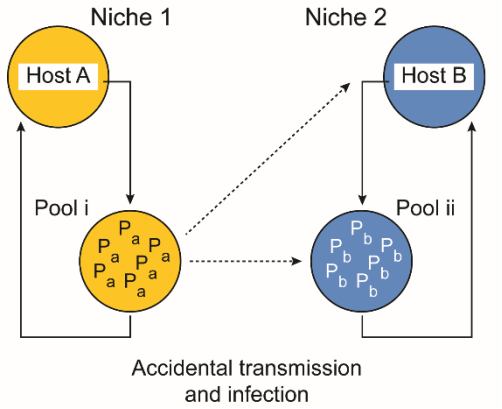


Figure 1-14 Allopatric parasite speciation by accidental host switch between hosts using different niches.
Parasite infective stages are present in pools within niches of an environment and occasional accidental transmission can occur.
[Adapted from McCoy (2003)]

## 1.5 Effects of parasites on their hosts

Parasites can have negative effects and reduce their host fitness (Marzal *et al.*, 2005), and they can also alter various host traits such as: host behaviour [e.g. manipulate behaviour of infected hosts or lead to defensive behaviour in uninfected hosts (Hawley and Altizer, 2011)], reproductive strategies [e.g. inducing an early or later investment in reproduction (Fredensborg and Poulin, 2006)], and immune status [e.g. allocating resources to immune defence (Schmid-Hempel, 2003)]. Pathology is the ability of an organism to cause a disease, while virulence is the degree of pathology caused by that organism that causes the loss of fitness in the host. There is always a minimum cost to parasitism, even if a parasite does not apparently harm its host, although it is easy to confuse reduced pathology with the absence of pathology (Combes, 2001). In this perspective, even apparently non-pathogenic parasites can reduce host fitness and survival because parasitic infections require shifts in energy use, which may render hosts more vulnerable to predation, reduce their ability to compete reproductively, or be more prone to infections by other parasites (Combes, 2001). The cost of pathogenic parasites is easier to evaluate because they can seriously impair hosts and even cause death. In a host-parasite system, selective pressures may act on hosts to resist or tolerate infections, while on parasites these pressures act to reduce virulence in order to maximize its reproductive success (Thomas *et al.*, 2009). This is because parasites depend on their hosts for survival, thus there is usually no advantage on over-exploiting the host as this may lead to a significant decrease in host fitness and consequently may decrease parasite fitness (Figure 1-15).

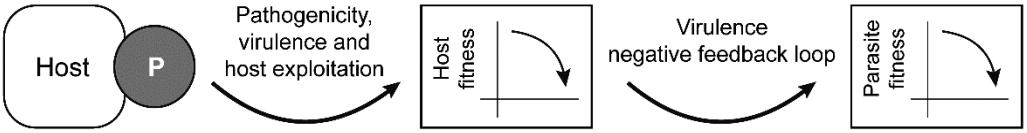


Figure 1-15 Parasite virulence limitations regarding host fitness.
Other factors may simultaneously influence both host and parasite fitness.
[Adapted from Combes (2001)]

Over time parasites tend to reach an optimal level of virulence and hosts an optimal level of tolerance, which allows for a durable interaction between these organisms, often with no visible trade-offs (Combes, 2001). When optimal virulence and tolerance levels are achieved, positive relationships are often found between host traits and parasite infection parameters (Poulin, 1999a). For instance, in the case of mammalian nematodes of the order Strongylida body weight and host population density were significantly related with parasite abundance, after controlling for the effects of each variable (Arneberg *et al.*, 1998). In addition, infection by less virulent parasites may actually protect a host against more pathogenic forms by “spatial preemption” (occupying the space in the host) and "temporal preemption” (by contributing to the host immune system defences) (Price *et al.*, 1986). Nonetheless, with more virulent parasites, co-infections (or mixed infections) are likely to occur because hosts may allocate resources to combat one parasite, which may render them more susceptible to infection by other parasite species (Tompkins *et al.*, 2011). The outcomes of co-infections and interactions among parasite heterospecifics in a single host are often asymmetrical (Poulin, 1999b), which may result in the effects of the coexistence of two parasites being more detrimental than the additive effects of single infections (Petney and Andrews, 1998; Tompkins *et al.*, 2011).

### 1.5.1 Effects at individual and population level

Understanding how parasites are distributed among species in an ecosystem and how they affect host species and host communities (e.g. if they have differential infection patterns and the outcomes of this on competition) is extremely important as parasites play a role in structuring host populations within communities (Thomas *et al.*, 2000).

Host susceptibility and host condition are important factors that influence the effects, success of establishment and transmission of disease (Beldomenico and Begon, 2010). These factors may vary as, for example, a result of resource shortage, competition and/or environmental stress. At the individual level, high stressing conditions may result in impoverished host defenses that make these individuals enter a vicious circle of poor condition that predisposes individuals to higher levels of infection. This in return causes poorer body condition, not only as a result of specific pathogenic effects but also because parasites extract host resources and induce a nutritionally demanding immune response (Figure 1-16) (Beldomenico *et al.*, 2008; Beldomenico and Begon, 2010). This ultimately results in reproductive failure and death of the host. On the other hand, low stressing

conditions allow hosts to have high resilience to parasite infection, which results in high probability of survival and reproduction (Figure 1-16). A similar pattern is observed at the population level, in which infection intensity is both a cause and consequence of the host body condition. The occurrence of many hosts in poor condition that enter in a vicious circle of higher intensities and poorer condition, leads to an increased mortality and poor overall reproductive success as a result of greater pathogen density, which causes a decline in that host population (Figure 1-16).

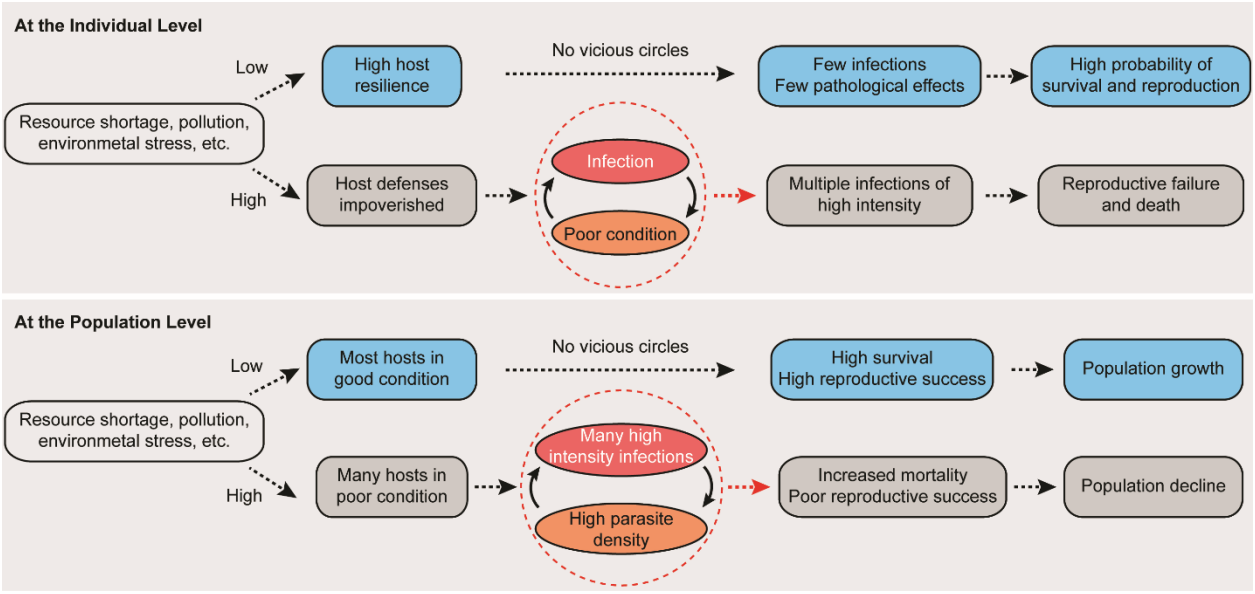


Figure 1-16 Effects and vicious cycles of parasite infections at the individual and population level. [Adapted from Beldomenico and Begon (2010)]

## 1.6 The Phylum Apicomplexa

The phylum Apicomplexa is the largest group of unicellular organisms, composed of more than 5000 species. However, its diversity is still one of the poorest known with only an estimated 0.1% of the total number of species named (Morrison, 2009). Apicomplexans are obligate intracellular parasites that possess a unique feature that originated their name, the apical complex, which consists of three structures present in the infectious stage of these organisms. Almost all are parasites, although a recent study reported a beneficial apicomplexan symbiont in marine animals (Saffo *et al.*, 2010). This phylum includes some of the most important disease agents of both economical and medical/veterinary impact, such as the genera *Plasmodium*, *Cryptosporidium* and *Toxoplasma*, and common but often poorly studied groups of blood parasites, such as hemogregarines and lankesterellids.

Apicomplexa can be divided into four main hypothetical groups: gregarines, cryptosporidia, hematozoa (which includes haemosporidians and piroplasms) and coccidia (which includes hemogregarines, enteric coccidia (monoxenic coccidia), cyst forming coccidia, and hemococcidia) (Figure 1-17). Coccidia is a subclass of apicomplexan parasites that undergo merogony,

gametogony, sporogony and syzygy for gamete production, which leads to marked anisogamy. In addition, the apical complex of all or most asexual motile stages of members of this subclass contain a conoid structure made of tubulin fibres (Hu *et al.*, 2002; Adl *et al.*, 2012). Hematozoa, a synonym of Aconoidasida (Figure 1-18) (Šlapeta and Morin-Adeline, 2011), differs from coccidians because members of this group lack a conoid in their apical complex (with some exceptions) (Valkiūnas, 2005).

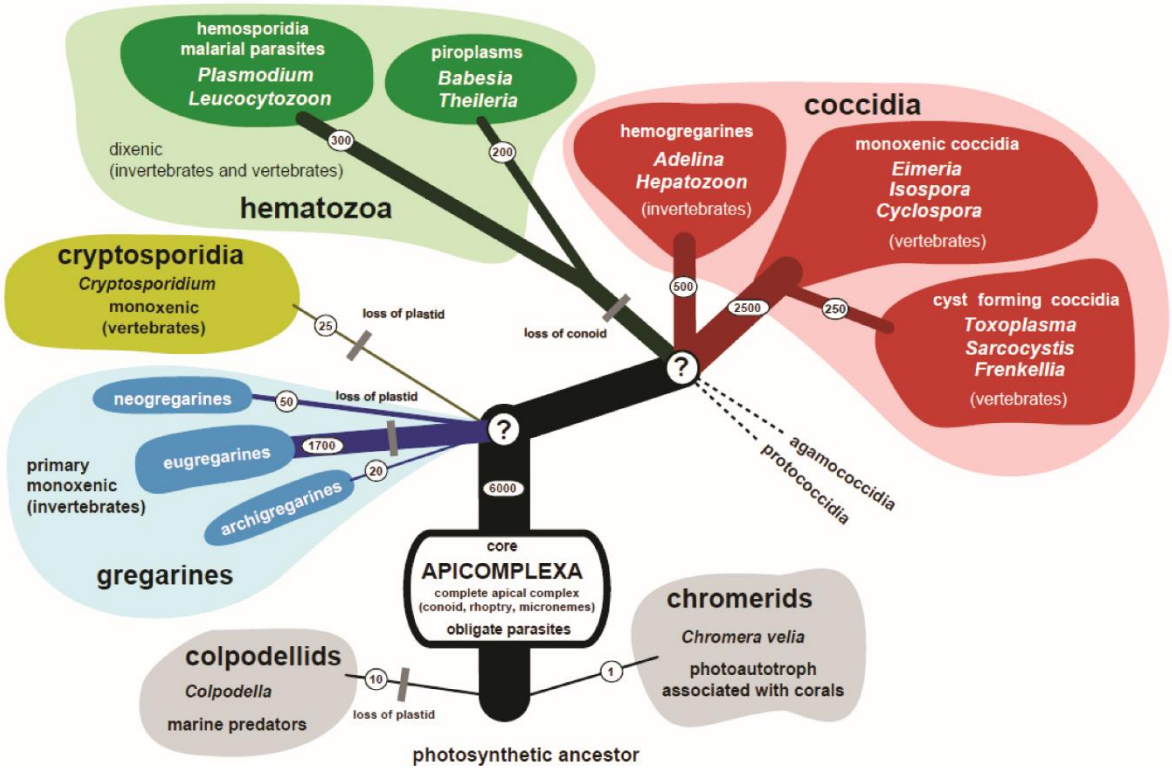


Figure 1-17 Hypothetical tree of the main groups of apicomplexan parasites.
[Adapted from Šlapeta and Morin-Adeline (2011)]

The taxonomy and phylogeny of the Apicomplexa phylum has been controversial and frequently revised due to the wide variety of parasite species that may exhibit very distinct or, in contrast, very similar characters (Escalante and Ayala, 1995; Adl *et al.*, 2005, 2012; Beck *et al.*, 2009). Parasite classification has classically been based on lifecycle and host associations (Valkiūnas, 2005; Telford, 2009). However, some studies suggest that these characteristics may not reflect the evolutionary history within Apicomplexa (Barta *et al.*, 2001), thus a growing number of molecular studies are being conducted in this group. Next, the state of the art on the diversity and phylogenetic relationships of the three apicomplexan groups that concern this thesis is provided, that is, the hemogregarines, the eimeriorinids and the haemosporidians (Figure 1-18).

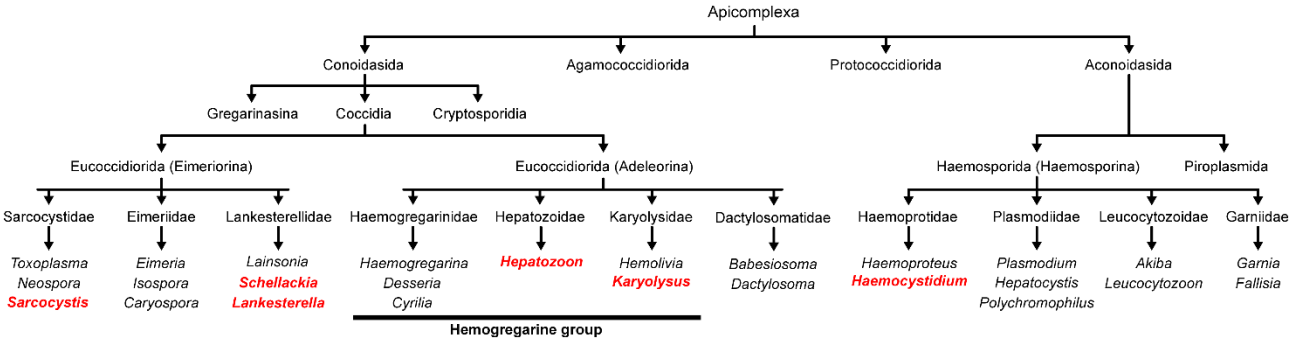


Figure 1-18 Classification of the apicomplexan parasites relevant for this work.
Taxonomic levels are: phylum, class, subclass, suborder, family and genera.
In red are the genera that were detected in the works of this thesis.
[Based on Adl *et al.* (2012)]

### 1.6.1 Hemogregarines (Adeleorina)

Hemogregarine is the term used to collectively describe the genera *Hepatozoon* (Hepatozoidae), *Haemogregarina*, *Desseria* and *Cyrilia* (Haemogregarinidae), *Karyolysus* and *Hemolivia* (Karyolysidae) from the suborder Adeleorina of the Apicomplexa phylum (Figure 1-18). Adeleorina is a suborder of apicomplexan parasites for which all species produce gametes by syzygy, and is divided into two groups: the Adelines that are monoxenous parasites of invertebrates, and the Hemogregarines that are heteroxenous parasites of various invertebrates and vertebrates. Adelines are composed of two families, Adeleidae and Legerellidae, while hemogregarines are composed of three families (Barta *et al.*, 2012). Hemogregarines can infect all vertebrate groups and are the most common and widely distributed hemoparasites of reptiles (Telford, 2009). Within hemogregarines, the most predominant genus is *Hepatozoon*, for which research using morphological and molecular tools increased over the last decade.

#### *1.6.1.1 Diversity and phylogeny*

Many studies have shown that hemogregarines present great taxonomic diversity, variation in life cycles and hosts, and have a cosmopolitan distribution, suggesting they are an ancient and successful group (Perkins and Keller, 2001; Maia *et al.*, 2011; Tomé *et al.*, 2014). Recent estimates of the phylogeny of hemogregarines based on the 18S rRNA gene have evidenced paraphyly of the Hepatozoidae family in relation to the Karyolysidae family. As shown in Figure 1-19, *Hemolivia* may be sister taxa to one of the clades of *Hepatozoon* that is found in a wide range of hosts (e.g. amphibians, rodents and reptiles) (Kvičerová *et al.*, 2014), and *Karyolysus* sequences are identical to previous identified *Hepatozoon* parasites from reptile hosts based on this marker (Haklová-Kočíková *et al.*, 2014). In addition, a recent study proposed an alternative systematic revision of the adeleorinid hemogregarines based on biological life cycles and phylogenetic reconstructions (Karadjian *et al.*, 2015). These authors proposed the separation into four hemogregarine types: *Hepatozoon* (type I), *Karyolysus* (type II), *Hemolivia* (type III) and a new genus *Bartazoon* (type IV) (Figure 1-19). However, the studies in this thesis were prepared before the publication of Karadjian

*et al.* (2015), therefore the taxonomy used throughout this thesis is prior to Karadjian *et al.* (2015). The genus *Hepatozoon* has extraordinary diversity (Harris *et al.*, 2015) and the need for a taxonomic revision of this genus, the only of the Hepatozoidae family, has long been noted (Smith and Desser, 1997; Smith *et al.*, 1999). The 18S rRNA gene has been the most widely used molecular marker for assessing the phylogenetic relationships within adeleorinids (Barta *et al.*, 2012), but the first *Hepatozoon* mitochondrial genome has been recently released [*Hepatozoon catesbianae* from frogs (Leveille *et al.*, 2014)]. Therefore it should be possible in a near future to conduct multigene phylogenies of adeleorinids in order to clarify the current taxonomic inconsistencies.


Figure 1-19 Hemogregarine topology based on the 18S rRNA gene.
**Karyolysus* sequences are placed together with *Hepatozoon* sequences in a recent study (Haklová-Kočíková *et al.*, 2014). Colours indicate hemogregarine clades. New classifications proposed in a recent systematic revision based on lifecycle and phylogenetic reconstruction are given (Karadjian *et al.* 2015). However, this thesis uses the taxonomy prior to that study.
[Adapted from Kvičerová *et al.* (2014)]

A comprehensive review by Smith (1996) documented *Hepatozoon* species according to vertebrate host group and included: 1 in amphibians and salamanders, 6 in crocodilians, 19 in birds, 42 in anurans, 74 in lizards, 46 in mammals, and 121 in snakes. However, this was conducted about 20 years ago, when identification and classification of blood parasites was mainly based on morphological characters. The development of molecular tools to obtain genetic information for phylogenetic analyses overcame some of the limitations of morphological characters, especially in

cases of parasite species with similar morphological characters (see section 1.7). Thus, it is possible that a much higher number of species are found in these host groups (Bensch *et al.*, 2004) and that many reassignments and reclassifications are needed, as previously proposed (Smith and Desser, 1997; Smith *et al.*, 1999). *Hepatozoon* may be transmitted by a wide range of invertebrate hosts and can be found in a wide range of vertebrate hosts, which complicates our understanding of host-specificity that is often important for taxonomic purposes. On the other hand, few vector groups are recognized as vectors of the other hemogregarine genera that make *Hepatozoon* paraphyletic, such as mites for the genus *Karyolysus* (Haklová-Kočíková *et al.*, 2014) and ticks for the genus *Hemolivia* (Kvičerová *et al.*, 2014). It is likely that hemogregarines have a stronger coevolution history with their invertebrate hosts, as shown in a recent molecular assessment of the phylogenetic position of adeleorinid coccidia (Barta *et al.*, 2012), which is plausible given that sexual reproduction occurs in these hosts and this is the most important phase of their lifecycle.

The genus *Karyolysus* has been reported in lacertid lizards, including *Lacerta* and *Podarcis*, and also skinks and some amphibians, and are transmitted by gamasid mites (Mesostigmata) (Svahn, 1974; Telford, 2009; Haklová-Kočíková *et al.*, 2014). Some gamont stages can be morphologically similar to other hemogregarine parasites, such as *Hepatozoon* (see Figure 1-30), however members of *Karyolysus* typically cause lysis of the nucleus of infected cells (Figure 1-20) (Telford, 2009), hence the name derived from “karyolysis” that means dissolution of a cell nucleus. *Hepatozoon* and *Karyolysus* differ in the known invertebrate host range, by the site where oocysts are located on the definitive hosts and by transovarial transmission that is present in *Karyolysus* but not reported in *Hepatozoon* (Haklová-Kočíková *et al.*, 2014). The molecular characterization of these parasites was only conducted recently for the first time and indicated genetic similarity with some *Hepatozoon* sequences obtained from reptiles (Figure 1-19) (Haklová-Kočíková *et al.*, 2014). This evidenced the taxonomic uncertainty of this genus and of some of the previously identified *Hepatozoon* parasites (Figure 1-19) (Maia *et al.*, 2011; Harris *et al.*, 2012; Tomé *et al.*, 2014) and the need for further research within this group of parasites to better understand their diversity and phylogeny.

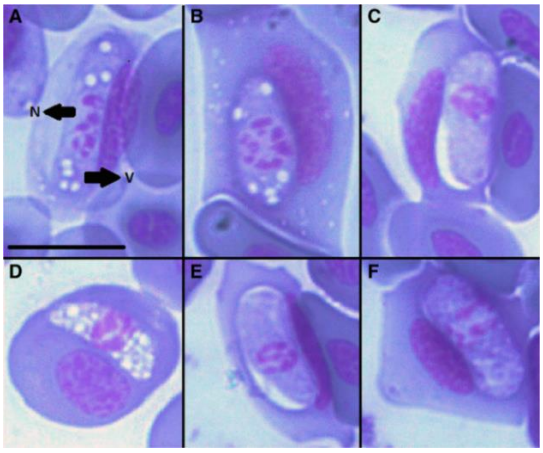


Figure 1-20 Morphological characteristics of *Karyolysus latus* infecting *Podarcis muralis*.
A, B and D represet trophozoites, and C, E and F gamonts. Scale bar = 10 µm. [Adapted from Haklová-Kočíková *et al.* (2014)]

### *1.6.1.2 Lifecycle*

The typical hemogregarine lifecycle involves sexual reproduction in an invertebrate host and asexual reproduction in a vertebrate host. However, each genus of hemogregarines is transmitted by particular invertebrate hosts and contain distinct life stages. Thus, for simplicity, this section is dedicated to the lifecycle of the genus *Hepatozoon*, which is the most common intracellular protozoan genus found in reptiles (Telford, 2009). These are unicellular, intraerythrocytic, heteroxenous, apicomplexan parasites, that infect all major tetrapod groups, such as lizards, snakes, turtles, crocodilians, mammals and birds, and are transmitted by invertebrate hosts, mainly arthropods such as mites, ticks, mosquitoes and fleas, but also leeches (Smith, 1996; Davies and Johnston, 2000). This means in order to complete their lifecycle, *Hepatozoon* parasites must alternate between invertebrate hosts (definitive hosts), where sexual reproduction and sporogony occur, and vertebrate hosts (intermediate hosts), where asexual reproduction occurs with the formation of gametocytes.

Transmission of *Hepatozoon* typically occurs when an infected hematophagous invertebrate host is ingested by a vertebrate host, which is the intermediate host for the parasite. There may be two kinds of intermediate hosts: a first intermediate host that acts as a paratenic host in which the formation of cysts occurs, such as lizards and rodents (Figure 1-21 H-I); and a final intermediate host that becomes infected when it preys on the first infected intermediate vertebrate host, such as snakes, canids and felids (Figure 1-21). The latter is an alternative mode of transmission that has been demonstrated experimentally (Landau *et al.*, 1972; Sloboda *et al.*, 2008; Johnson *et al.*, 2009) and is likely to occur in nature across different prey-predator systems (Allen *et al.*, 2011; Tomé *et al.*, 2012). In addition, there may be a second intermediate host, when a saurophagous lizard preys on an infected lizard, become infected and undergoes cyst formation. In fact, lizard cannibalism has been shown to be a successful mode of transmission in other apicomplexan parasites, such as *Sarcocystis* (Matuschka and Bannert, 1987). These lizards may then be preyed upon the final intermediate host, such as snakes, where the asexual reproduction occurs (Smith, 1996). In some hosts, such as canids, grooming ticks off their own coats and social grooming of group members may be an important direct mode of transmission to the final vertebrate host (Johnson *et al.*, 2010), but grooming behavior is not necessarily the only route of *Hepatozoon* transmission. It is assumed that infection only occurs through ingestion of an infected invertebrate and not by salivary transmission for *Hepatozoon* between hosts in nature (Telford, 2009). However, experimental transmissions have demonstrated that it is possible (Desser *et al.*, 1995; Sloboda *et al.*, 2007; Telford *et al.*, 2008). This is also likely because salivary transmission has been widely described for other vector-borne pathogens, such as *Plasmodium* (Paul *et al.*, 2003) and *Haemogregarina*, a closely related genus (Davies *et al.*, 2004). In addition, vertical transmission may also occur by placental passage of merozoites, as suggested in a recent redescription of *Hepatozoon felis*, in which PCR from fetal tissue was positive for this parasite (Baneth *et al.*, 2013).

The cycle starts when a hematophagous invertebrate host takes a blood meal containing intraerythrocytic gamonts (female macrogamonts and male microgamonts) from an infected vertebrate host (Figure 1-21 A). These gamonts penetrate the gut wall of the invertebrate, where syzygy (gamete alignment) followed by gametogenesis and fertilization occur, resulting in the formation of a zygote (Figure 1-21 B-D). The zygote undergoes fission in a process called sporogony and produces an early oocyst (Figure 1-21 E) that matures and becomes a multisporocystic oocyst (Figure 1-21 F), containing infective sporozoites (Figure 1-21 G). The formation of sporocysts during sporogony, which may occur in the haemocoel, malphighian tubules or gut wall of the invertebrate host, is one of the distinctive characteristics of *Hepatozoon* species, which, for example, is absent in the *Haemogregarina* genus. Sporozoites are the infective stages of *Hepatozoon* and when a vertebrate host ingests an infected invertebrate host, sporocysts release sporozoites that penetrate the gut wall, enter the circulatory system and migrate to the liver or lungs of the vertebrate (Figure 1-21 H-J). Here, they form schizonts (Figure 1-21 J-K) that undergo merogony (multiple fission, Figure 1-21 L), resulting in meront cysts that form merozoites in a series of divisions (Figure 1-21 M). Some meront cysts and merozoites probably encyst in new tissues, providing recurring events of infection (Telford, 2009). Finally, merozoites infect erythrocytes and develop trophozoites (young gamonts) that then become mature gamonts, in a process called gamontogony (Figure 1-21 A). The cycle is restored when a hematophagous invertebrate host has a blood meal containing mature gamonts from the infected vertebrate host.

Lifecycle characteristics are important for species recognition or description. When describing the lifecycle of a new parasite species based on morphology, it is important to describe in detail all the morphological and developmental stages, both in the vertebrate and the invertebrate hosts. For instance, the confirmation of the tick *Amblyomma sphenodonti* as the vector of *Hepatozoon tuatarae* in the New Zealand tuatara *Sphenodon punctatus* was possible by showing the development of a sporoblast and mature oocysts containing sporozoites in the mid-gut epithelial cells of the tick. The mid-gut contents of the tick contained tuatara erythrocytes that were ingested as a blood meal, with some infected with gamonts structurally similar to the ones found in the tuatara host (Herbert *et al.*, 2010).

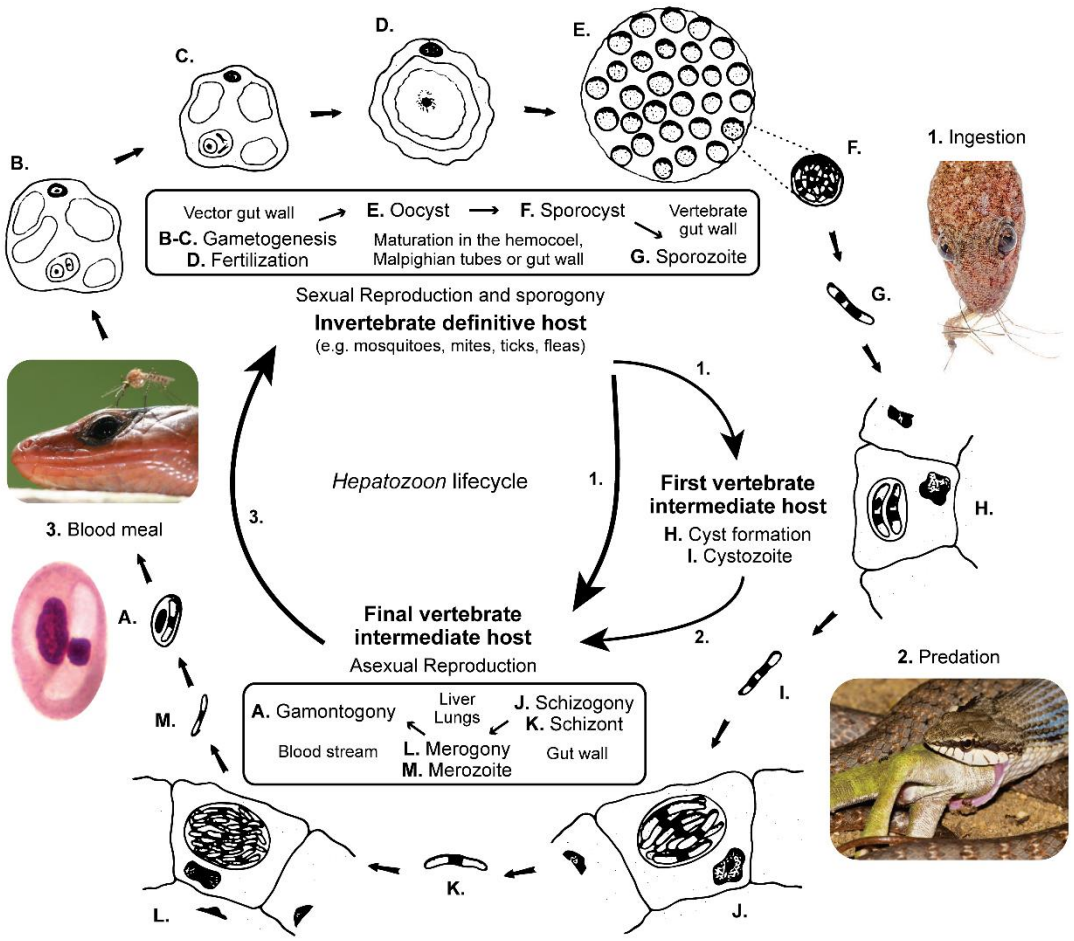


Figure 1-21 Typical lifecycle of *Hepatozoon* parasites.
[Based on Smith (1996), Telford (2009). Image 1 from http://thumbs.dreamstime.com/x/geckoeatmosquito23549562.jpg, image 2 from Crottini *et al.* (2010), image 3 from http://www.hrrna.com/RNA/images/Reptiles%20and%20Amphibs/BroadHeadSkink%20mosq.jpg; gamont figure from *Hepatozoon confusus* of *Coluber constrictor priapus* Telford (2009) page 218]

### *1.6.1.3 Ecology and transmission*

Hemogregarines are vector borne parasites and thus transmission is associated with vector abundance, dispersal and competence. These vector characteristics are dependent on the environment and are susceptible to seasonal and spatial fluctuations such as temperature and moisture (Bajer *et al.*, 2006). Exposure to vectors may be influenced by host intra- and inter-specific heterogenic behaviour, which could be associated with variations of infection patterns within exposed host populations with, for example, host sex, body size and reproductive status (Godfrey *et al.*, 2011). In fact, there may be a males-bias in infection patterns due to testosterone immunosuppressive effects (see section 1.3.2.1), as well as the possibility that exposure to competent vectors of hemogregarine parasites increases with host age (Amo *et al.*, 2004; Salkeld and Schwarzkopf, 2005). However, these relationships are often contradictory in the literature (Madsen *et al.*, 2005; Brown *et al.*, 2006; Godfrey *et al.*, 2011), which could indicate that these differences may be a combination of several factors that are difficult to measure in wild hosts, such as susceptibility to infection, or other factors not yet understood. Hemogregarines may be transmitted by a wide range of invertebrate hosts, such as mosquitoes, mites, ticks and leeches, which inhabit

distinct microhabitats in a same geographical area. Therefore, host behaviour and microhabitat preferences are additional factors that may influence the ecology and transmission of these parasites.

#### 1.6.1.3.1 Alternative mode of transmission: the prey-predator example

Transmission of some apicomplexans has a strict prey-predator association (e.g. *Sarcocystis*), while other apicomplexans can exploit prey-predator systems as alternative modes to expand their host range and increase their transmission (e.g. *Hepatozoon*). Recently, studies have shown that paratenic hosts of *Hepatozoon* help maintain its lifecycle because the parasite undergoes cystozoite stages that are infective for competent predator hosts (Johnson *et al.*, 2008a; Sloboda *et al.*, 2008). For example, the importance of endogenous cysts located in the tissues of a secondary host in the transmission of *Hepatozoon domerguei* in Malagasy reptiles has been demonstrated experimentally for a long time (Landau *et al.*, 1970, 1972) (Figure 1-22). In this system, arthropod vectors (definitive hosts) infect lizards (first intermediate hosts), which undergo cyst formation and, depending on the host species, may also undergo asexual reproduction that ultimately produces gamonts (Figure 1-22). These lizards are preyed upon by snakes (final intermediate hosts), in which the parasite reproduces asexually to produce gamonts and may also produce infective cystozoites (Figure 1-22). Infective cystozoites may transmit infection to other predators or scavengers, and the cycle is restored when a vector host has a blood meal containing gamonts from these vertebrate hosts. The awareness of this mode of transmission has increased and used to justify low prevalence levels of *Hepatozoon* parasites in hosts that are nearly exclusively insectivorous and predominantly feed on termites, but that can occasionally feed on small rodents, birds and eggs [such as bat-eared foxes (Pinto *et al.*, 2013)]. In addition, the presence of infected competent vectors attached to an unsuitable host for the development of a parasite, may still serve as a vehicle of transmission to suitable predator hosts [concomitant predation (Johnson *et al.*, 2010)].

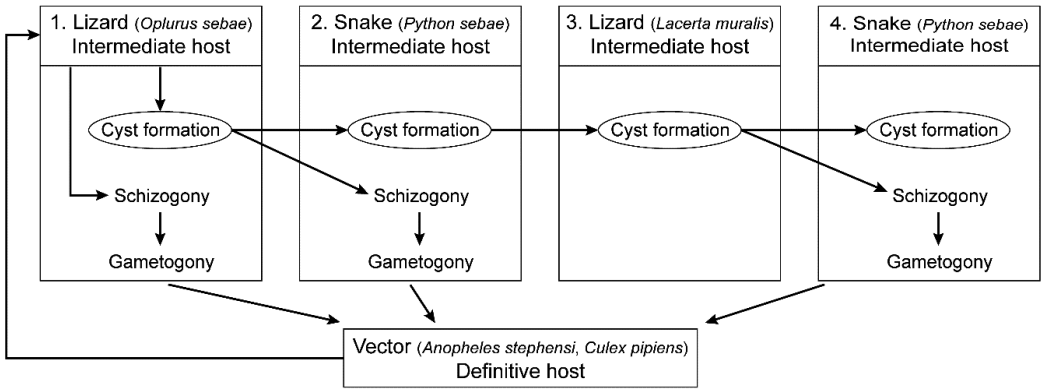


Figure 1-22 Possible routes of infection for different reptiles with *Hepatozoon domerguei*, with a focus on prey-predator transmission.
[Adapted from Landau *et al.* (1972)]

#### *1.6.1.4 Pathogenesis*

Pathology and virulence of hemogregarine parasites in wildlife is still poorly understood. These parasites mainly occupy the erythrocytes of reptile hosts [but are occasionally found in leukocytes (Godfrey *et al.*, 2011)], and therefore may cause anaemia (Oppliger and Clobert, 1997). On the other hand, hemogregarines mainly occupy leukocytes in mammals (Baneth *et al.*, 2003). In dogs, the disease is often asymptomatic but can cause varying degrees of granulomatous inflammation in various organs, mainly in the muscles, which leads to anorexia, weight loss, weakness, diarrhoea and eye discharge (Mundim *et al.*, 2008; Johnson *et al.*, 2008b). However, the impact of these parasites on their hosts is probably intrinsically related with host life-history traits and body condition, because studies on different host species from different geographical locations often show different patterns [e.g. a negative impact on fitness of snake hosts (Wozniak *et al.*, 1996; Madsen *et al.*, 2005), or no apparent impact (Brown *et al.*, 2006)]. It is difficult to assess the fitness and health of wildlife, hence some studies have assessed the relationship between parasite load and the ability of hosts to recognize, avoid and escape predators, which may be an indication of host survival and fitness status. Two recent studies have estimated the distance separating an approaching predator when prey escape begins (Flight Initiation Distance FID) and the total distance covered during flight (distance fled DF) (Damas-Moreira *et al.*, 2014; Garrido *et al.*, 2015). These studies concluded that hemogregarines may not greatly affect the fitness in the studied lizard populations because no direct correlation was observed between parasite load and escape distance. In fact, the latter study even reported a positive correlation between FID and parasite load, but a negative correlation for DF (Garrido *et al.*, 2015). It is possible that infections by different parasite genetic lineages of the same parasite species, and even very closely related parasite lineages, have significantly different outcomes of their hosts, and that the same host is parasitized by multiple parasite lineages of a same parasite species (Reullier *et al.*, 2006; Pérez-Tris *et al.*, 2007). However, the implications of these infections are still unclear. These studies are often based on microscopy identification and estimation of parasite infection parameters, and therefore these relationships may differ when the effect of different parasite lineages can be compared in a same host.

### 1.6.2 Haemosporidians (Haemosporida)

Most of the knowledge available for haemosporidians is regarding the genera with most anthropogenic importance, such as *Plasmodium* (Plasmodiidae). However, a great research effort has been conducted over the last decade on investigating this and two other common genera, *Haemoproteus* (Haemoproteidae) and *Leucocytozoon* (Leucocytozoidae), in wild avian hosts (Hellgren *et al.*, 2004; Valkiūnas, 2005; Bensch *et al.*, 2009; Valkiūnas *et al.*, 2010). Four families are currently recognized in Haemosporida: Plasmodiidae, characteristic to have hemozoin pigment and erythrocytic merogony; Haemoprotidae, characteristic to have hemozoin pigment but no erythrocytic merogony; Garniidae, characteristic to have erythrocytic merogony but no hemozoin

pigment; and Leucocytozoidae, characteristic not to have both of these characteristics (Perkins, 2014). Despite this, this group of parasites is composed of many other genera and subgenera that remain poorly known (Table 1-1).

Table 1-1 Classification of haemosporidians with known vertebrate hosts and vectors. Numbers in brackets indicate the number of described species as in Martinsen *et al.* (2008). [Adapted from Perkins (2014)]

| **Classification** | **Vertebrate hosts** | **Vectors** |
|---|---|---|
| Family Plasmodiidae | | |
| Genus *Plasmodium* (199) | | |
| Subgenus *Plasmodium* | Primates | Anophelines |
| Subgenus *Laverania* | Apes | Anophelines |
| Subgenus *Vinckeia* | Rodents | Anophelines |
| Subgenus *Bennettinia* | Birds | *Culex* |
| Subgenus *Giovannolaia* | Birds | Culicidae |
| Subgenus *Haemamoeba* | Birds | Culicidae |
| Subgenus *Huffia* | Birds | *Culex* |
| Subgenus *Novyella* | Birds | *Culex,* Culiseta |
| Subgenus *Asiamoeba* | Lizards | ? |
| Subgenus *Carinamoeba* | Lizards | ? |
| Subgenus *Lacertamoeba* | Lizards | Culicidae |
| Subgenus *Ophidiella* | Snakes | ? |
| Subgenus *Paraplasmodium* | Lizards | Phlebotomines |
| Subgenus *Sauramoeba* | Lizards | ? |
| Genus *Hepatocystis* (25) | Primates, bats, ungulates, rodents | Culicoides |
| Genus *Polychromophilus* | Bats | Nycterbids |
| Genus *Nycteria* | Bats | ? |
| Genus *Bioccala* | Bats | ? |
| Genus *Biguetiella* | Bats | ? |
| Genus *Dionisia* | Bats | ? |
| Genus *Saurocytozoon* | Lizards | ? |
| Genus *Mesnilium* | Fish | ? |
| Family Haemoproteidae | | |
| Genus *Haemoproteus* (202) | | |
| Subgenus *Haemoproteus* | Birds | Hippoboscids |
| Subgenus *Parahaemoproteus* | Birds | Culicoides |
| Genus *Haemocystidium* | | |
| Subgenus *Haemocystidium* | Squamates | ? |
| Subgenus *Simondia* | Chelonians | Tabanids |
| Family Leucocytozoidae | | |
| Genus *Leucocytozoon* (91) | | |
| Subgenus *Leucocytozoon* | Birds | Simulids |
| Subgenus *Akiba* | Birds | Culicoides |
| Family Garniidae | | |
| Genus *Fallisia* | | |
| Subgenus *Fallisia* | Lizards | ? |
| Subgenus *Plasmodoides* | Birds | ? |
| Genus *Garnia* | Lizards | ? |
| Genus *Progarnia* | Crocodilians | ? |

### *1.6.2.1 Diversity and phylogeny*

The classification of haemosporidians was traditionally based on morphological characters (e.g. number and position of pigments, parasite cell size, shape and orientation in host cell), life-history traits, host taxa and geographical location (Valkiūnas, 2005; Perkins, 2014). However, these

characters vary during different stages of parasite maturity and different host taxa, thus it has become clear the limitations for distinguishing among parasite species and genera based solely on these characters (Telford *et al.*, 1989; Bensch *et al.*, 2004; Perkins, 2014). Molecular analyses of protozoan parasites are bound to change considerably as more information builds up and as phylogenetic tools progress through the years. These tools are especially important for the discrimination of insect-vertebrate host associations (Kim *et al.*, 2009; Ferraguti *et al.*, 2013) and in cases where not all life stages are observed or if blood smear fixation and staining alter cell morphology (Valkiūnas *et al.*, 2008; Perkins, 2014). The taxonomy of this group has been subjected to many rearrangements, especially with the inclusion of molecular analyses that allowed to greatly increase the number of recognized species compared to those originally identified using microscopy (Bensch *et al.*, 2004; Hellgren *et al.*, 2004). Several subgenera have been formulated in order to separate these parasites based on the morphological similarities and genetic distinctiveness of some of the clades obtained using molecular tools (Table 1-1).

Up to recently, three molecular hypotheses existed for explaining the evolutionary history of Haemosporida that mainly differed on the number of parasite taxa included in each analysis and on the outgroup used to root the phylogenies (Figure 1-23) (Perkins, 2014). The first hypothesis, proposed more than a decade ago, was generated based on 21 mammalian species, 11 from lizards and 18 from birds, and rooted with *Theileria* and *Leucocytozoon* (Perkins and Schall, 2002). The main finding of this study was the identification of a mammalian parasite clade and a bird/reptile clade (Figure 1-23 A). The second hypothesis was generated based on 11 mammalian parasite species, 7 from lizards and 39 lineages from avian hosts, and rooted with *Leucocytozoon* (Figure 1-23 B). This resulted in a similar overall topology and the main finding of this study was that the major cladogenic events of Haemosporida seemed to be related to host switches between vectors (Martinsen *et al.*, 2008). This suggests that the parasite might occasionally have been ingested by an unusual vector, which was suitable for parasite development, resulting in continued transmission and subsequent establishment, radiation, differentiation and speciation (Perkins, 2014). Finally, the third hypothesis was generated based on both previous datasets plus additional sequences available on public databases (including *Polychromophilus* genus of bats, although authors do not precise the exact sequences used) (Outlaw and Ricklefs, 2011). The novelty of this study was the use of an outgroup-free approach that provided striking findings, such as the fact that *Leucocytozoon* was a derived and not an ancestral lineage, therefore not being a suitable outgroup for rooting the Haemosporida tree for the cyt *b* gene, and that mammalian *Polychromophilus* was more closely related with bird and reptile haemosporidians than with other mammalian haemosporidians (Figure 1-23 C). However, a recent study using a multi-gene approach found an alternative hypothesis, including *Leucocytozoon* as basal to *Haemoproteus* and *Plasmodium*, therefore making this a suitable outgroup, and that mammalian *Plasmodium* was sister taxa to bird and reptile *Plasmodium* (Borner *et al.* 2015).

Due to the exponential increase on the genetic information from malarial parasites of avian hosts, a dedicated database was recently created with the objective to reconcile all the information and allow researchers for a quick and easy verification of the novelty of a particular lineage, or to better identify and characterize the diversity known lineages (MalAvi (Bensch *et al.*, 2009)).

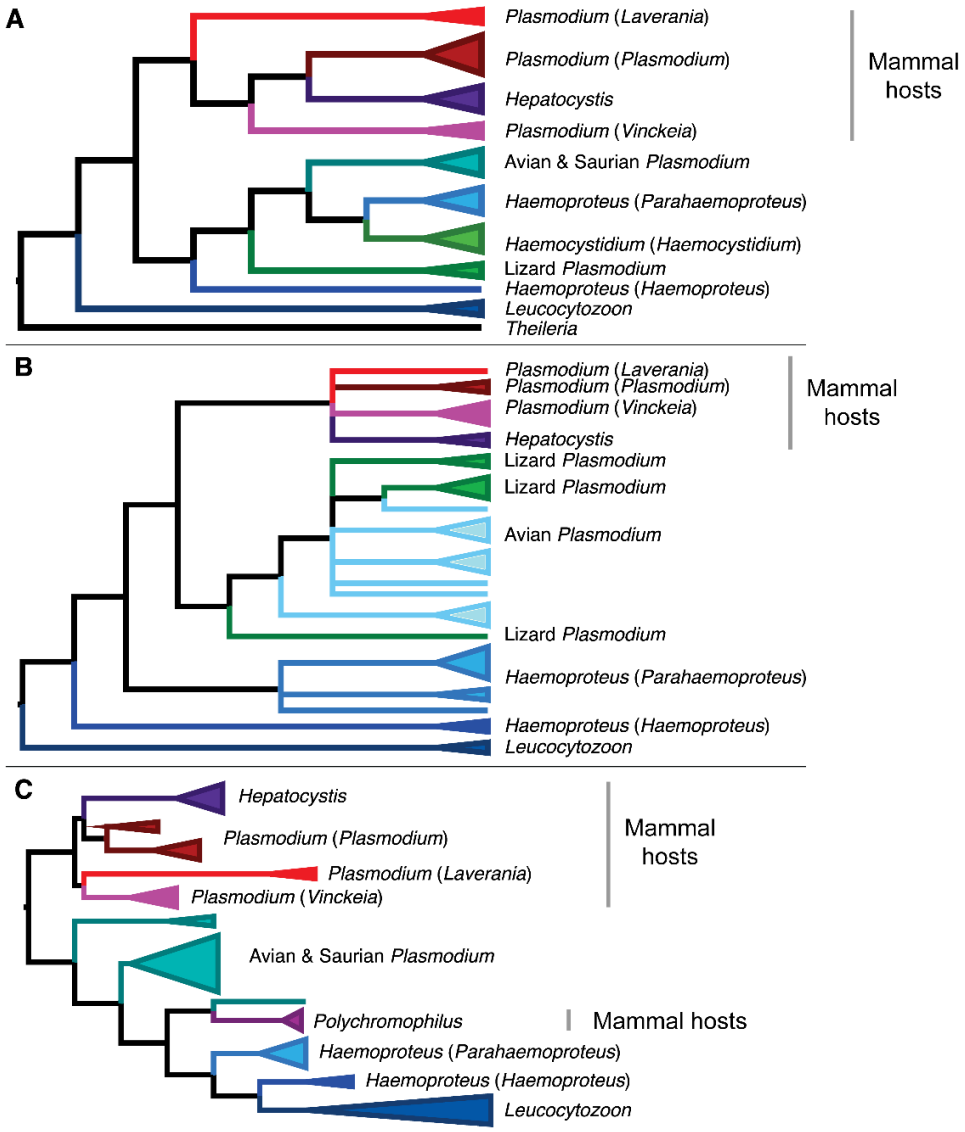


Figure 1-23 Three molecular phylogenetic hypotheses of the Haemosporida.
(A) Rooted topology with *Theileria* and *Leucocytozoon* using cyt *b* gene sequences (Perkins and Schall, 2002). (B) Rooted topology with *Leucocytozoon* with additional sequences from avian parasites using a multilocus approach (cyt *b*, coI, clpc and asl genes) (Martinsen *et al.*, 2008). (C) Unrooted Bayesian topology showing *Leucocytozoon* as a derived lineage rather than ancestral using the cyt *b* gene (Outlaw and Ricklefs, 2011). Blue indicates bird hosts and green indicates reptile hosts. [Adapted from Perkins (2014)]

#### 1.6.2.1.1 The hemoproteid parasites of avian and reptile hosts (Haemoprotidae)

Hemoproteid parasites in birds have been classified as *Haemoproteus*, while in reptiles may be referred to as *Haemocystidium*. Subgenera have been proposed for each of these genera based on the diversity, intermediate and vector hosts in which they occur. Much research has been conducted on avian *Haemoproteus* over the past decade (Valkiūnas *et al.*, 2010; Iezhova *et al.*, 2011; Levin *et al.*, 2012; Matta *et al.*, 2014) and for this reason the taxonomy of these parasites in these hosts is

much better understood in comparison with reptilian hemoproteids. *Haemoproteus* (*Haemoproteus*) are transmitted by hippoboscid flies, while *Haemoproteus* (*Parahaemoproteus*) are transmitted by *Culicoides* midges (Bennett *et al.*, 1965; Valkiūnas, 2005). The genetic information of members of both these subgenera have corroborated this division for a long time (Figure 1-23). Reptilian hemoproteids have been subjected to many reclassifications. For example, *Haemocystidium* was created in 1904 (Castellani and Willey, 1904) but has subsequently been synonymized with *Plasmodium* (Wenyon, 1915) and *Haemoproteus* (Wenyon, 1926; Levine, 1988), or resurrected as a separate genus (Mackerras, 1961; Telford, 1996). *Haemocystidium* (*Simondia*) are transmitted by tabanid flies and found in chelonians, while *Haemocystidium* (*Haemocystidium*) are found in squamates and their vectors are still unknown (Telford, 1996; Perkins, 2014) (Table 1-1). Only recently have these parasites been genetically characterized and this has indicated that reptilian hemoproteids are distinct from avian hemoproteids (Figure 1-24) (Pineda-Catalan *et al.*, 2013). This highlights the importance of including reptilian parasites in estimates of parasite phylogeny and taxonomy.

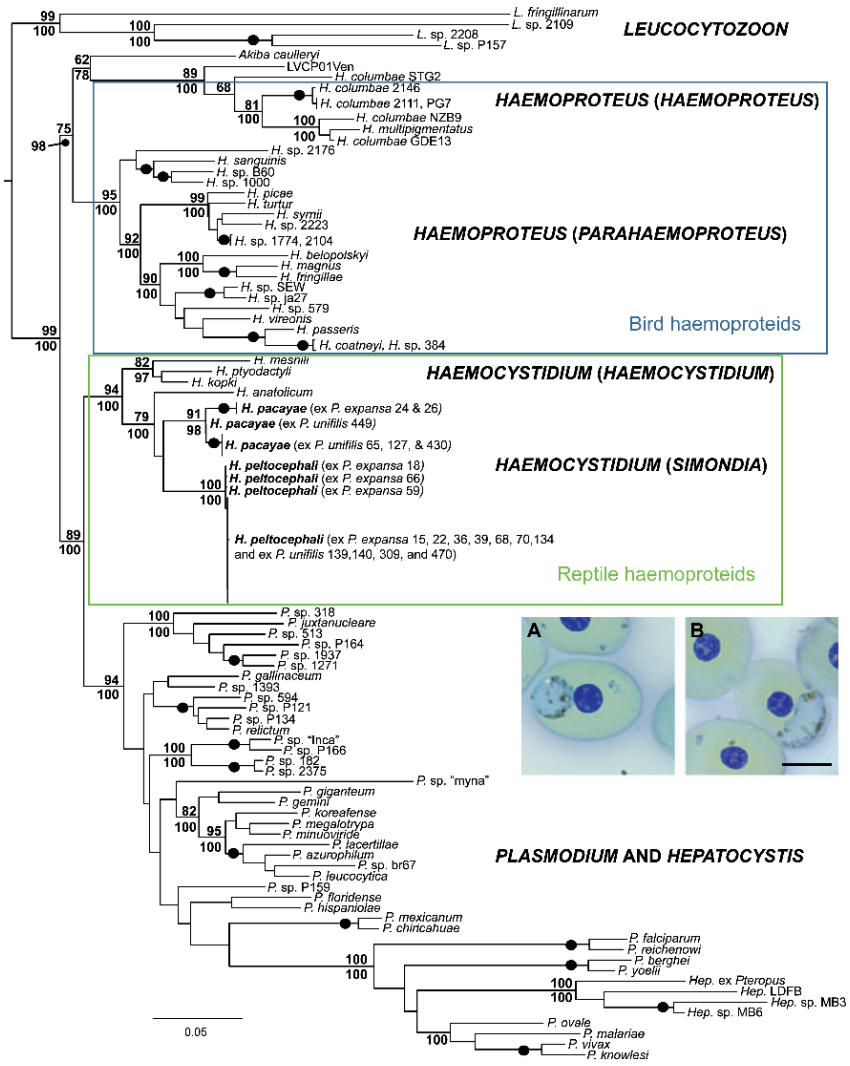


Figure 1-24 Multigene phylogeny (cyt *b*, cox1 and clpC) of haemosporidians, with a focus on avian and reptilian hemoproteids. A and B represent *Haemocystodium* (*Simondia*) *pacayae* gametocytes infecting *Podocnemis unifilis* turtles. [Adapted from Pineda-Catalan *et al.* (2013)]

### *1.6.2.2 Lifecycle*

The typical lifecycle of haemosporidians involves sexual reproduction in invertebrate hosts and asexual reproduction in vertebrate hosts. Vertebrates become infected after inoculation of infective sporozoites from an infected invertebrate host and agamic stages undergo asexual division to produce exoerythrocytic meronts (merogony) or schizonts (schizogony) depending on the parasite species (Figure 1-25 1-2) (Valkiūnas, 2005). Then, unicellular merozoites are formed and these are distributed within the organism of the host to various tissues (Figure 1-25 3-7 and 9-12). These forms induce the formation of gametocytes or gamonts in the blood cells of the vertebrate host and later differentiate into macrogametocytes and microgametocytes (Figure 1-25 8 and 13). The characters of sexual dimorphism may be distinguishing features of haemosporidians (e.g. Haemoproteidae and Plasmodiidae species possess hemozoin pigment granules in microgametocytes but usually not in macrogametocytes) (Valkiūnas, 2005). Gametocytes are infective to vectors when these take a blood meal on infected vertebrate hosts, and exflagellation of microgametes occurs. In invertebrate hosts, gametogenesis (Figure 1-25 14-15) and fertilization (Figure 1-25 16-17) occur, and the zygote develops as an oocyst that undergoes sporogony (Figure 1-25 18-19) and produces thousands of sporozoites (infective forms to vertebrate hosts, Figure 1-25 20) that move to the salivary glands of these hosts (Figure 1-25 21) and the cycle is ready to restart (Lapointe *et al.*, 2012).

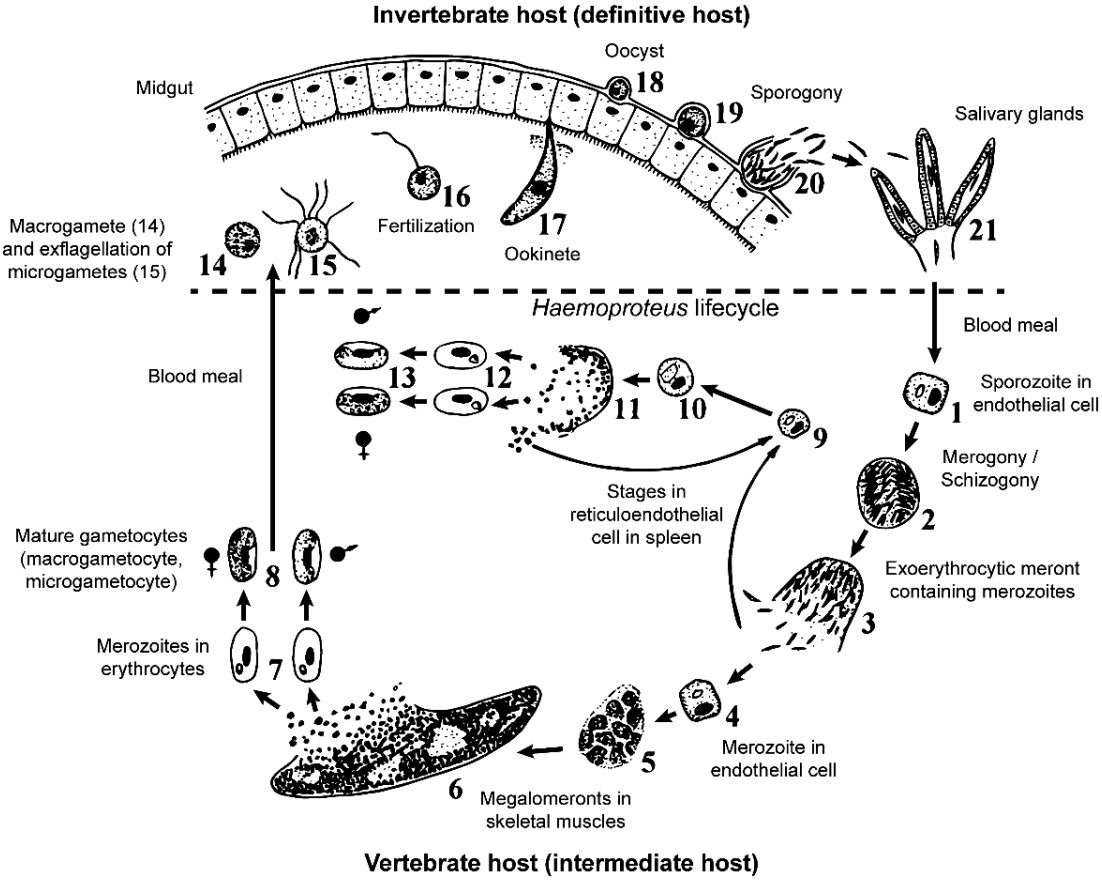


Figure 1-25 Lifecycle of *Haemoproteus mansoni* in avian hosts.
[Adapted from Valkiūnas (2005)]

### *1.6.2.3 Ecology and transmission*

In the same perspective as hemogregarines and other vector borne parasites, the distribution of haemosporidians is intrinsically associated with that of their vectors (Rogers and Bates, 2007; Ishtiaq *et al.*, 2008) and dispersal and competence for successful transmission (Sol *et al.*, 2000). It has been demonstrated that the development of malaria parasite species in *Anopheles* mosquitoes may be strongly influenced by environmental temperature fluctuations (Paaijmans *et al.*, 2010) or rainfall (Lapointe *et al.*, 2012). For this reason, these parasites are emerging in new regions due to climate change that are associated with higher temperatures and frequent rainfalls (Maroli *et al.*, 2008; Møller *et al.*, 2013), which influence vector larval habitat availability and adult survivorship. In addition, the abundance of suitable vectors for parasite transmission varies with altitude and seasonality due to variation in suitable conditions for vector and parasite development (Tanga *et al.*, 2010; Rooyen *et al.*, 2013; Atkinson *et al.*, 2014).

Furthermore, haemosporidian infection in avian hosts includes several phases: prepatent, acute, crisis, chronic and latent (Valkiūnas, 2005; Lapointe *et al.*, 2012). The later two may persist in these hosts from many years, thus infected hosts may serve as a continuous source of infection to vectors and contribute to the transmission of these parasites in a host population. Transmission of these parasites may also be influenced by intensity levels of infection. For example, higher levels may allow the parasite to produce more transmissible forms per unit of time and/or may lead to a slower parasite clearance rate, thus increasing the time of infection from which to transmit from (Mackinnon and Read, 2004) (but see section 1.5 for the trade-offs of increased parasite intensity levels and virulence). Haemosporidians with a broad host range can be abundant in host communities (Fallon *et al.*, 2005) and their vectors are likely to transfer parasite between different host species (Hellgren *et al.*, 2009). Nonetheless, parasites can have different development success rates in different host species due to incompatilibities between hosts and parasites (e.g. heterogeneity in immune response between hosts). This could affect the probability of a particular parasite lineage being found in certain host species (Mackinnon and Read, 2004).

### *1.6.2.4 Pathogenesis*

Haemosporidian parasites may be of major epidemiological concern because some can cause serious health issues including host death, and also exert great economic costs to public health (Talisuna *et al.*, 2004; Hotez *et al.*, 2007). The most widely studied examples are from *Plasmodium* species that cause malaria in a wide range of hosts, such as mammals, birds and reptiles. However, the implications of these parasites have mostly been studied in humans due to their public health importance (Mackinnon and Read, 2004), thus the pathogenenicity of these parasites in birds and reptiles is still poorly understood [(Lapointe *et al.*, 2012) but see (Lachish *et al.*, 2011a; b) for effects of malaria infections in bird populations]. Despite this, it has been shown that the introduction of *Plasmodium reticulum* to the Hawaiian Islands have played a major role in the decline and extinction

of native bird species (Atkinson *et al.*, 2000). *Haemoproteus* parasitize birds and reptiles (referred to as *Haemocystodium* in this thesis, see section 1.6.2.1.1) and an effort has been made to document the importance of these parasites in wild avian hosts (Merino *et al.*, 2000; Marzal *et al.*, 2005; Arriero and Møller, 2008; del Cerro *et al.*, 2010; Synek *et al.*, 2013; Dunn *et al.*, 2014), but less in reptilian hosts (Lainson and Naiff, 1998; Telford, 2007, 2009; Orkun and Güven, 2012). *Haemoproteus* infections in wild hosts may be non-detrimental at low levels, but it has been shown that high levels of infection may be associated with host death (Ferrell *et al.*, 2007; Cannell *et al.*, 2013).

## 1.6.3 Eimeriorinids (Eimeriorina)

Eimeriorina is a suborder within the Apicomplexa phylum that comprises many genera and species (Duszynski *et al.*, 2000; Duszynski and Upton, 2009; Megía-Palma *et al.*, 2015). Of these, the genera that are of importance for the works in this thesis are: *Schellackia* and *Lankesterella* (Lankesterellidae), and *Sarcocystis* (Sarcocystidae) (Figure 1-18). Lankesterellids are often referred to as hemococcidia because they can be found in the blood of vertebrates, while sarcocystids are referred to as cystforming coccidia because they form cysts in the tissues of vertebrates.

### *1.6.3.1 Diversity and phylogeny*

Similarly to haemosporidians, the majority of the knowledge available for Eimeriorina regards some of the genera with anthropogenic importance, such as *Eimeria*, *Toxoplasma* and *Sarcocystis*. However, the increasing genetic information on eimeriorinid parasites of reptiles has clearly indicated taxonomic inconsistencies that have long been suspected (Barta *et al.*, 2001; Jirků *et al.*, 2009). Recent studies have highlighted the need for a taxonomic revision of Eimeriidae by showing paraphyly of this family (Megía-Palma *et al.*, 2013) and of Lankesterellidae (if *Schellackia* is considered as a member of this family) by showing polyphyly of this family (Megía-Palma *et al.*, 2014) (Figure 1-26). Thus, the two main genera of lankesterellids, *Lankesterella* and *Schellackia*, are sometimes considered as belonging to the same family (Lankesterellidae) (Telford, 2009), or to separate families, *Schellackiidae* and *Lankesterellidae* (Megía-Palma *et al.*, 2014). This shows the lack of a clear understanding of the real diversity within this group and the usefulness of molecular tools to aid understanding the relationships within and between these parasites. Therefore, there is a need for assessing the diversity in animals from understudied regions and to verify the validity of species described solely based on morphological characters with molecular techniques. The current plausible solution to the needed taxonomic revision of lankesterellids seems to be the resurrection of the family Schellackiidae (Figure 1-26). However, for this to be possible the two possibly misidentified *Eimeria* species that appear in this monophyletic clade (*Eimeria arnyi* from snakes and *Eimeria ranae* from amphibians, Figure 1-26) need to be confirmed as *Schellackia* species (Megía-Palma *et al.*, 2014). In addition, this would implicate that the lack of exogenous oocysts that is a

characteristic of lankesterellids, arose independently for *Schellackia* and *Lankesterella* (Megía-Palma *et al.*, 2013).

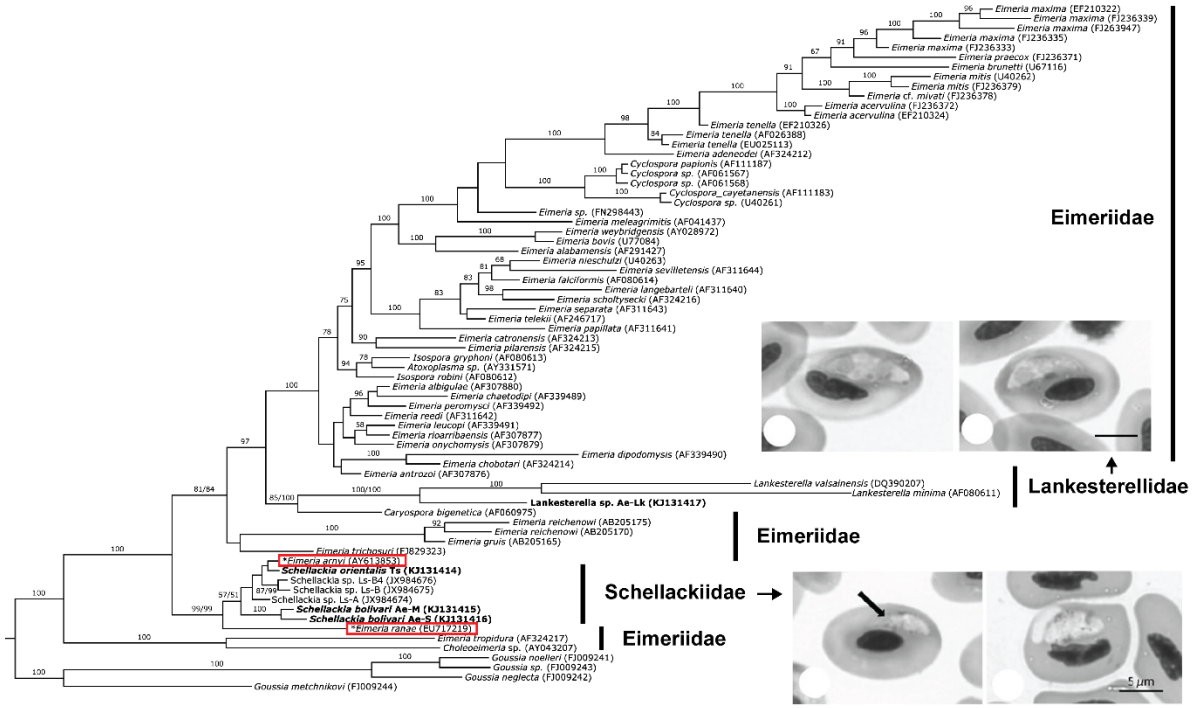


Figure 1-26 Phylogenetic relationships among eimeriorinid parasite families.
Figures represent *Lankesterella* and *Schellackia* sporozoites infecting lizards (Megía-Palma *et al.*, 2013, 2014).
Red squares indicate two *Eimeria* species that are placed inside the Schellackiidae clade.
[Adapted from Megía-Palma *et al.* (2014)]

The family Sarcocystidae comprises some genera of medical importance, such as *Toxoplasma* and *Neospora*, and of veterinary importance, such as *Sarcocystis*. The latter genus was the only sarcocystid detected in the works of this thesis. *Sarcocystis* has a two-host lifecycle and transmission involves prey-predator transmission or cannibalism (Matuschka and Bannert, 1987; Matuschka, 1988; Harris *et al.*, 2012). *Sarcocystis* species have been traditionally identified based on sarcocyst and sporocyst morphology using microscopy. However different species may display similar morphologies, and so the use of molecular tools has become standard for the assignment of these parasites (Dahlgren and Gjerde, 2008; Gjerde, 2013, 2014b). Reptiles are host to a wide range of *Sarcocystis* species (Duszynski and Upton, 2009). Some studies that analyzed the phylogenetic relationships between *Sarcocystis* species, suggest that these parasites are more linked to their final hosts than to their intermediate hosts (Doležel *et al.*, 1999; Lau *et al.*, 2013) (Figure 1-27).

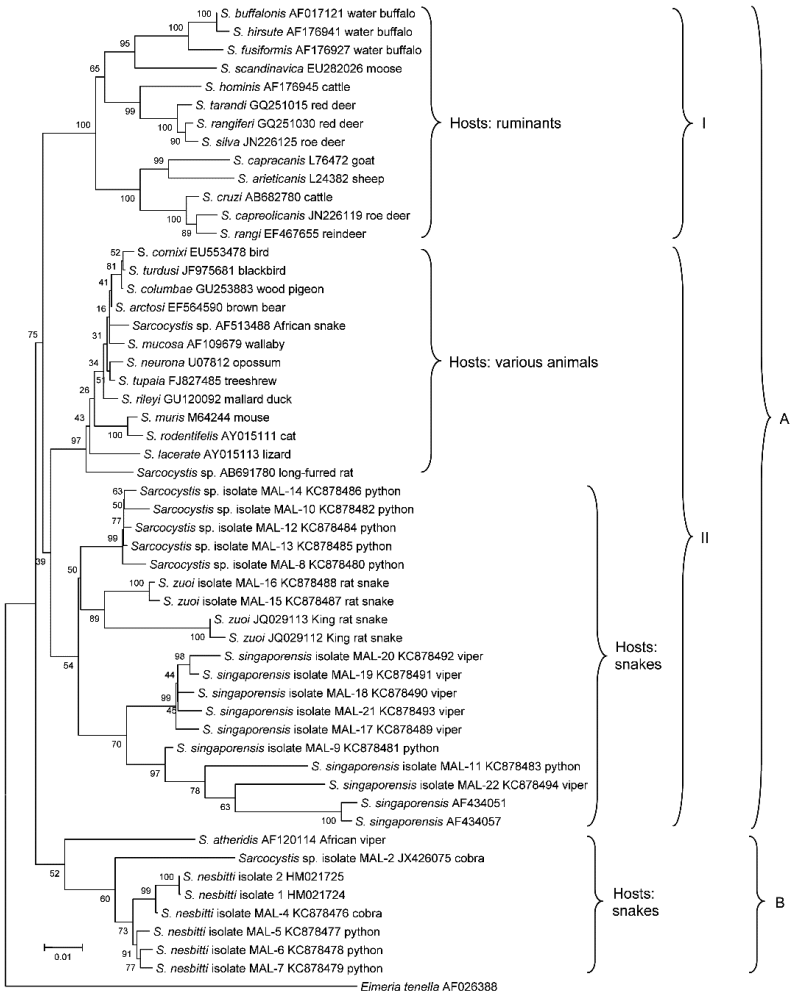


Figure 1-27 Phylogenetic relationships of *Sarcocystis* species based on the 18S rRNA gene. [Adapted from Lau *et al.* (2013)]

### *1.6.3.2 Lifecycle and transmission*

Lankesterellids are heteroxenous parasites but the sexual and replicative stages occur in the gut tissues of the vertebrate hosts, unlike hemogregarines and haemosporidians for which these stages occur in the invertebrate host. Thus, invertebrate vectors play only a mechanical role in the lifecycle of these parasites, with no development or multiplication in these hosts (Telford, 2009). Transmission occurs when a vertebrate host ingests sporozoites (Figure 1-28 A) from an infected invertebrate vector, but it might also occur directly through ingestion of infected blood and liver of infected reptiles (Figure 1-28) (Bristovetzky and Paperna, 1990; Finkelman and Paperna, 1998). Sporozoites enter epithelial cells and form meronts (Figure 1-28 B), which develop merozoites (Figure 1-28 C) that become gamonts and produce macro- and microgametes (Figure 1-28 D) (Telford, 2009). Fertilization occurs in the vertebrate host, usually in the lamina propria of the intestinal wall, forming a zygote (Figure 1-28 E) that develops oocysts (Figure 1-28 F), which produce eight sporozoites without the formation of sporocysts (Figure 1-28 A). Finally, sporozoites infect white or red blood cells and transmission is ready to restart. The number of sporozoites is a distinctive characteristic of

*Schellackia* and *Lainsonia* (both produce 8), in relation to *Lankesterella* (produces 32 or more); while the intestinal site and type of blood cell used as part of these parasites lifecycle is used for species discrimination (Telford, 2009). These parasites can be detected using microscopy by observing sporozoites inside host blood cells (Figure 1-26).

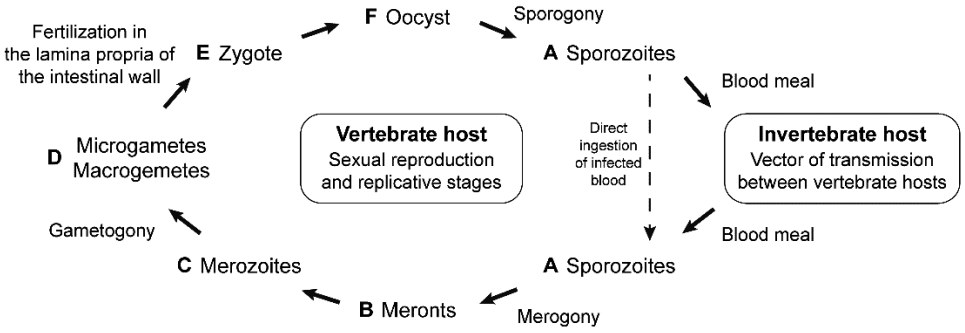


Figure 1-28 Typical lifecycle of lankesterellids.
[Based on Telford (2009)]

On the other hand, sarcocystids have a direct prey to predator lifecycle, with mainly herbivores and omnivores as intermediate hosts and carnivores as definitive hosts (Gjerde, 2014a). Intermediate hosts, such as rodents and lizards, become infected by ingesting oocysts or sporocysts in fecally contaminated feed or water, while definitive hosts become infected by preying or scavenging on intermediate hosts that contain sarcocysts in striated muscle cells (Duszynski and Upton, 2009; Gjerde, 2014b) (Figure 1-29 A). Once sporocysts are ingested by the intermediate host, these release sporozoites that invade the intestinal tissues of the intermediate host, where initial rounds of merogony occur with the formation of meronts (Figure 1-29 B) followed by additional rounds of merogony resulting in the formation of merozoites (Figure 1-29 C) (Duszynski and Upton, 2009). Merozoites then proliferate throughout the host organism and enter striated muscles, where they initiate the formation of cysts, from metrocytes to sarcocysts and finally to bradyzoites, which are infective to the definitive host (Figure 1-29 D-E). The definitive host becomes infected by preying on infected intermediate hosts and consequently ingesting cysts that contain bradyzoites in its striated muscles. Once ingested, cysts release bradyzoites that penetrate intestinal wall cells and undergo sexual reproduction by forming microgametocytes and macrogametocytes (Figure 1-29 F). These fertilize and generate a zygote (Figure 1-29 G) that develops into an oocyst (Figure 1-29 H). This oocyst undergoes sporogony to generate sporocysts containing sporozoites (Figure 1-29 A), the infective form to the intermediate host (Duszynski and Upton, 2009). Sporocysts are released to the environment through the faeces of the definitive host and the cycle restarts when an intermediate host ingests these sporocysts.

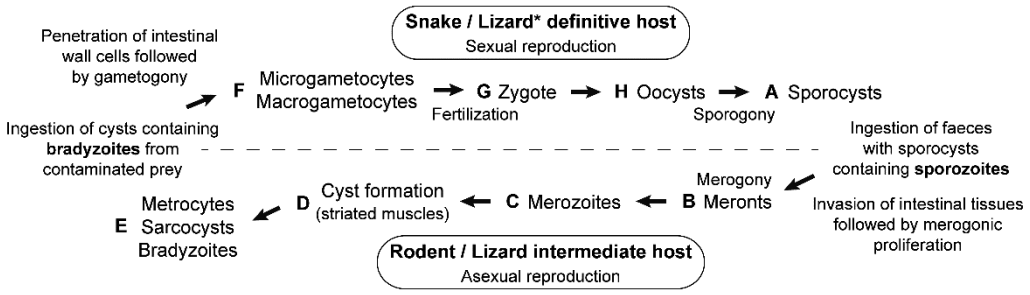


Figure 1-29 Typical lifecycle of *Sarcocystis* species.
* In some host-parasite systems, lizards can be both the intermediate and definitive hosts through cannibalism (Matuschka and Bannert, 1987)
[Based on Duszynski and Upton (2009)]

### *1.6.3.3 Pathogenesis*

Lankesterellids are usually present at low intensity levels in reptiles and no apparent effect rather than occasional displacement of nuclei within host cells is observed (Telford, 2009). However, information about pathogenesis of this group of parasites is pratically inexistent, thus it is possible that some lineages of these parasites may be pathogentic to their hosts. On the other hand, some *Sarcocystis* may be of veterinary and economic importance in livestock, and domestic and wild animals, with some infections causing clinical symptoms such as anemia, reduced general condition, anorexia or even death (Dubey *et al.*, 2006; Olias *et al.*, 2009; Caspari *et al.*, 2011).

## 1.7 Methodologies for parasite detection and identification

Assessing the prevalence and intensity of infection of hemoparasites in vertebrates is a first step towards understanding the ecological interactions in the relationships between parasites and hosts (Poulin and Mouillot, 2005). Microscopy has been the traditional and “gold standard” method for detection and identification of parasites through the examination of Giemsa-stained blood smears (Valkiūnas, 2005; Telford, 2009), but advances in molecular biology have allowed an improvement of both sensitivity and accuracy for detecting parasites.

The observation of parasite morphological characters and developmental stages through microscopy allows an understanding of the course of infection itself, and also to increase the knowledge on the lifecycle of a parasite. With most apicomplexans that infect blood cells of vertebrates (e.g. haemosporidians and hemogregarines), infections are detected by identifying gamonts inside these cells (Figure 1-30 A-F). Nonetheless, correct identification and characterization of new hemoparasite species is often dependent on the characterization of the parasite developmental stages in the definitive host (Figure 1-30 G-I), where sexual reproduction occurs. Hence, microscopy is a valuable tool because it allows to: i) assess prevalence and intensity of infection; ii) observe morphological characters of taxonomic and evolutionary importance (Figure 1-30); iii) identify mixed infections and which cells or tissue the parasites are inhabiting (Figure 1-30);

iv) to perform inexpensive routine screenings; and v) to identify double gametocyte infections in single host cells (Moody, 2002; Jovani *et al.*, 2004; Valkiūnas *et al.*, 2008).

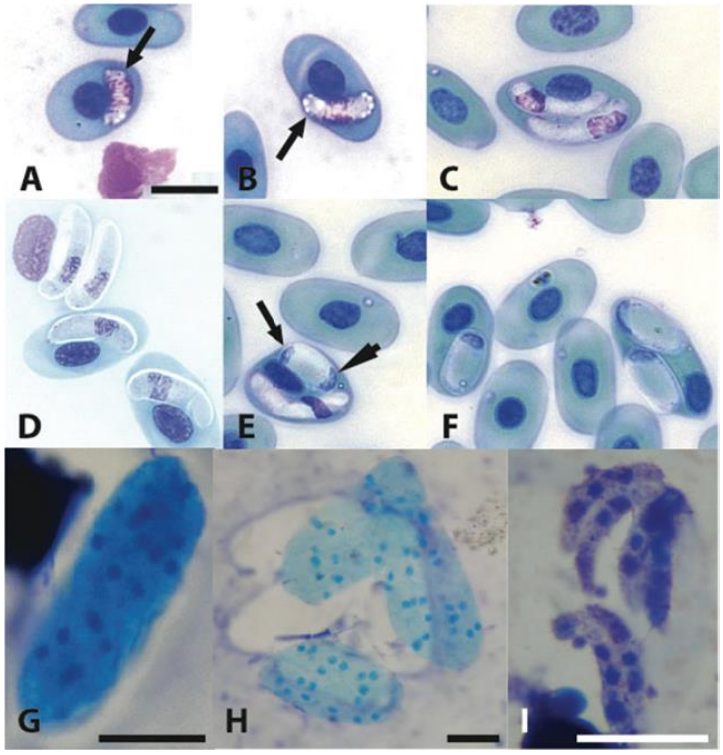


Figure 1-30 Morphological characteristics observed by microscopy of two *Hepatozoon* species in terrestrial chelonians. (A-B) possible trophozoites or merozoites. (C-D) gamonts of *H. fitzsimonsi*, with double gametocyte infection in C. (E) mixed infection with *H. fitzsimonsi* and *H. parvula*. (F) gamonts of *H. parvula*, with double gametocyte infection. (G-H) sporocysts containing sporozoites. (I) sporozoites from ruptured sporocysts. Scale bar = 10 µm. [Adapted from Cook *et al.* (2014)]

However, when compared to molecular methods, microscopy is significantly less sensitive (due to a large proportion of false negatives) and less accurate (due to frequent misdiagnosis, even by skilled parasitologists) in determining parasite prevalence and intensity, especially in subpatent infections in hosts with low levels of parasitemia (Perandin *et al.*, 2004; Mangold *et al.*, 2005) (see Valkiūnas *et al.* (2008) for a review on the shortcomings of microscopy). Fluorescence microscopy may facilitate detection over traditional microscopy, especially by non-skilled technicians, but molecular methods continue to be much more sensitive (Safeukui *et al.*, 2008). Nonetheless, studies comparing different methodologies often report different levels of sensitivity, which could be an indication that examiner/technician experience and quality of the samples and materials or protocols used are key issues. For instance, the rate of drying and/or staining protocol may lead to bad quality blood smears that reduce detection rates (Perkins and Austin, 2009), while unsuitable preservation of biological samples may result in poor genetic extractions and amplifications (Espy *et al.*, 2006). Therefore, it is often important to perform a priori assessments on the performance of different methodologies. A critical step in molecular methods is the design of the primers, which have to take into consideration a large variety of sequences from various related and unrelated organisms in order to certify that they are specific to the organism of interest. Occasional unsuccessful application of

the organism of interest may happen in PCR protocols. For instance, (Perkins and Martin, 1999) showed that a supposedly set of conserved primers for the 18S rRNA region (Wozniak *et al.*, 1994) failed to amplify solely the parasite, also amplifying a wide variety of organisms including the host (see also section 2.1 for a similar result obtained in one of the works of this thesis). Therefore, PCR protocols need optimization and may not work well across laboratories, influencing the success of molecular tools, depending on the DNA extraction method as well as the type of samples used (Karagenc *et al.*, 2006), *Taq* supplier and PCR machine may influence success rates (Freed and Cann, 2003, 2006) or the amount of host DNA may inhibit the PCR reaction (Cogswell *et al.*, 1996). Traditional DNA extraction protocols are generally regarded as being less effective than commercial kits, despite being inexpensive and easy to use, but few studies have addressed this (Espy *et al.*, 2006). In terms of contamination issues, they both require multiple manipulations and appropriate academic training to minimize these risks (Espy *et al.*, 2006).

### 1.7.1 Molecular detection

Molecular markers can be used in parasite species identification to: i) elucidate lifecycles by establishing the range of intermediate, paratenic and definitive hosts; ii) assess the diversity and search for cryptic species, termed “cryptic species prospecting”; and iii) to link morphologically indistinguishable early lifestages with later stages of known parasite species (Jousson *et al.*, 1999; Bartoli *et al.*, 2000; Criscione *et al.*, 2005). Thus, these tools provide an opportunity to uncover parasite biodiversity by characterizing their genetic diversity, differentiation and population structure (Nadler and De León, 2011).

Two main molecular techniques are used in parasitological studies: conventional PCR and quantitative PCR (qPCR). The main advantage of conventional PCR is that it provides genetic information, with much greater length and ease than qPCR protocols, which can be used to assess the phylogenetic relationships between parasites (Allsopp and Allsopp, 2006; Perkins and Austin, 2009). On the other hand, qPCR allows to simultaneously estimate prevalence, intensity of infection [quantification of the number of parasite gene copies by means of a standard curve that is calculated using a set of plasmid dilution series (Figure 1-31)] and distinction between mixed infections based on their melting temperature. Melting temperature differences are dictated by the degree of divergence between sequences, and so as long as parasites species or parasites lineages differ by a few base pairs, they will be distinguishable by a change in melting temperature (Mangold *et al.*, 2005; Alvarez *et al.*, 2013; Kamau *et al.*, 2014). Quantitative PCR protocols depend on the reagents used, which depend on the purpose of the qPCR assay. The two main reagents that are widely used are SYBR green, which binds to all double-stranded DNA and emits a fluorescent signal when bound, and *Taq*Man, which uses probes that bind to the single stranded DNA template but only emit fluorescence if that strand is synthesized by the *Taq* polymerase after successful binding of the primers (Smith and Osborn, 2009). The studies of this thesis use SYBR green because the objective

of the designed qPCR assay (section 2.2) was to maximize the detection and amplification of parasite DNA using highly-specific primers. The validity of the qPCR assay and its reproducibility is evaluated based on the Efficiency values, which are derived from the slope values. Under-amplification is expressed by lower efficiency values, while over-amplification by higher efficiency values.

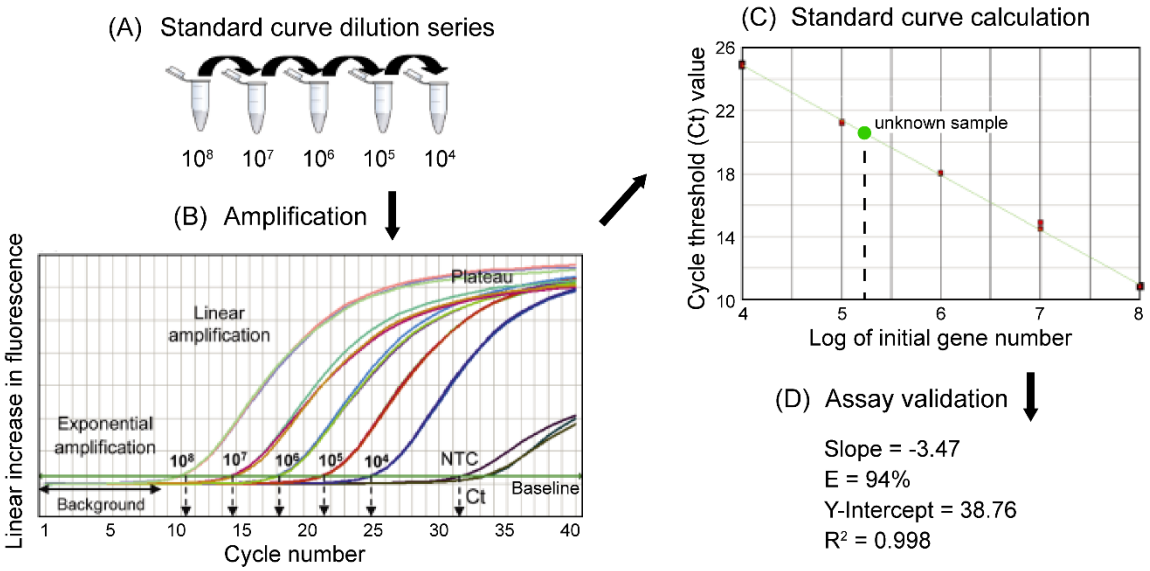


Figure 1-31 qPCR amplification from known concentrations of DNA to construct standard curves for quantification of unknown samples. (A) series of dilutions of a known sample. (B) amplification produces S shaped curves, with a linear and plateau phase, which differ by 3-4 Cts for successful dilutions. The Ct (cycle threshold, also known as Cp, crossing point) corresponds to the number of cycles required for the fluorescence signal to cross the threshold (defined by the baseline). (C) construction of a standard curve, and (D) validation of the assay by analysing the value of efficiency, which is calculated based on the slope of the curve. [Adapted from Smith and Osborn (2009)]

Due to the extreme sensitivity of qPCR assays, general guidelines were developed in order to standardize the methodology and procedures for validating these assays [MIQE guidelines (Bustin *et al.*, 2009, 2010; Bustin, 2010)]. Thus, qPCR protocol design for SYBR green assays should follow strict guidelines, such as, short amplicon amplification (less than 300bp, ideally 80-150bp) to obtain high level of fluorescence without compromising PCR efficiency, and extensive optimization of primer concentrations to avoid amplification of nonspecific products. In addition, due to the limitations of the manufacturer's software programs to properly analyze the qPCR results, researchers developed free programs that allow for better and more precise analyses of the raw data. An example of this is LinReg (http://www.hartfaalcentrum.nl/), a software that determines a baseline fluorescence, does a baseline subtraction and calculates the starting concentration per sample based on the Ct (cycle threshold) or Cp (crossing point) and PCR efficiency per sample (Ruijter *et al.*, 2009, 2012). This program was used to treat the raw data obtained from the manufacturer's program in the works of this thesis (see sections 2.2, 5.2 and 5.3).

### 1.7.2 Phylogenetic analyses

Evolutionary tree inference methods, such as Bayesian Inference and Maximum Likelihood, can be used to test hypothesis for molecular sequence data. These methods analyse character-state data, such as aligned nucleotide sequences, and may employ complex and realistic substitution models (Nadler and De León, 2011). These models can be tested to select the best-fit substitution model and parameters for each dataset (Posada and Crandall, 1998; Posada, 2008; Darriba *et al.*, 2012). Each tree inference method has different implicit and explicit assumptions. The advantage of sequence data is the large number of potential characters available to infer relationships (Nadler, 1995). However, its resolution is limited by several factors, such as outgroup choice, taxon sampling, alignment methodology, gene selection and sequence length, data treatment and parameter optimization (Smith, 1994; Sanderson and Shaffer, 2002; Rich and Xu, 2011).

Different methods or parameters applied to the same dataset often recover different groups or even topologies depending on the sequence alignment and gap penalty criteria, especially for the variables regions of ribosomal genes (Morrison and Ellis, 1997). Computational algorithms to align sequence data have become a common place in phylogenetics and allowed researchers to have more confidence on their tree inferences [e.g. (Thompson *et al.*, 1994; Talavera and Castresana, 2007; Katoh and Standley, 2013)]. In addition to this, two other common limitations associated with inaccurate inference of parasite phylogenies are the outgroup choice and taxon sampling (Rich and Xu, 2011). These factors may greatly affect the topology and inference of relationships between parasite groups due to the lack of knowledge on the diversity and relationships of these organisms. An acceptable outgroup would be one that is closely related to the ingroup but that is confidently excluded from the ingroup [e.g. sister group to the ingroup (Smith, 1994)]. Recently, models that estimate independent rates of molecular evolution for each lineage (i.e. relaxed molecular clock) and outgroup-free approaches to estimate phylogenetic relationships (Drummond *et al.*, 2012) have been developed. These methodologies may open new perspectives to the study of the evolutionary history of some parasite groups, as demonstrated in Haemosporida (Outlaw and Ricklefs, 2011). In addition, increasing taxon sampling may uncover unexpected relationships between parasite taxa (e.g. polyphyly and paraphyly) that challenge traditional taxonomy, especially when including previously unavailable parasite species or genera (Putaporntip *et al.*, 2010; Pineda-Catalan *et al.*, 2013; Haklová-Kočíková *et al.*, 2014).

## 1.8 Objectives of the thesis

The objectives of this PhD thesis were to:

1. Compare the performance, specificity and accuracy of various detection techniques [microscopy, conventional PCR and quantitative PCR (qPCR)] in estimating parasite infection parameters.
2. Determine the diversity and phylogenetic relationships of apicomplexan parasites in reptiles. Use this and available information to conduct an overview of the present knowledge of diversity and relationships of hemogregarine and haemosporidian parasites.
3. Assess host-parasite associations and host-specificity of apicomplexan parasites of reptiles in wild populations.
4. Investigate the influence of host ecology and individual host factors on parasite infection parameters by investigating spatial and temporal patterns of hemogregarine infection in sympatric hosts.

## 1.9 Outline of the thesis

The studies in this thesis form 4 chapters, ending with a general discussion, a glossary and appendices that contain all the supplementary information regarding these studies.

Chapter 2 is composed of two published articles that demonstrate the challenges often faced when studying blood parasites: i) the need to verify the validity of positive amplifications when using molecular tools (section 2.1); and ii) the differences in performance of various molecular protocols on parasite detection and identification to obtain biologically relevant information to investigate variation in infection patterns within and between host species (section 2.2). In the latter section, a qPCR assay for estimating hemogregarine infection was developed and applied for the first time in reptiles to study hemogregarine infection patterns in these hosts.

Chapter 3 is composed of two published articles and one in preparation, in which the genetic diversity and phylogenetic relationships of apicomplexan parasites are explored in several reptile host species from various geographical locations. In this chapter: i) hemogregarine parasites from the Western Mediterranean region detected through a large-scale molecular study are included for the first time in a phylogenetic framework (section 3.1); ii) a previously described hemogregarine species typical from reptiles from Madagascar is for the first time characterized genetically and included in a phylogenetic framework (as well as a filarial nematode species, section 3.2); and iii) a description of a new hemogregarine species is proposed from reptiles from Oman based on both molecular and morphologic data (section 3.3).

Chapter 4 is composed of one published article and one short-note in preparation. This chapter provides an overview of the current available genetic information for two of the most common blood parasite groups of wild animals, the cyt *b* gene for haemosporidians (section 4.1) and the 18S rRNA

gene for hemogregarines (section 4.2), and discusses the implications of the observed diversity and phylogenetic relationships to the present taxonomy of these parasites.

Chapter 5 is composed of one published article and two articles in preparation that explore host-parasite-environment interactions in wild hosts. This chapter provides new insights into: i) the transmission dynamics of hemogregarine parasites across different host groups (section 5.1); ii) the host-specificity, phylogeography and infection patterns of apicomplexan parasites in amphibians and reptiles from Oman (section 5.2); and iii) the temporal dynamics of hemogregarine infection patterns in sympatric lizard species (section 5.3). The latter two sections employed the qPCR protocol developed in section 2.2.

Finally, Chapter 6 provides a general discussion of all the previous chapters, in which the relevance and importance of the works presented is discussed, as well as suggestions for future perspectives that arise from this thesis.

Research from this thesis has been published in international peer-reviewed journals, such as Acta Parasitologica (Maia *et al.*, 2012b), Folia Parasitologica (Maia *et al.*, 2012a), PLoS One (Maia *et al.*, 2014a), Journal of Wildlife Diseases (Maia *et al.*, 2014c), Parasite (Maia *et al.*, 2014b) and Parasitology International (Maia *et al.*, 2015). Also, research from this thesis has also been presented in international congresses, such as the “2nd Mediterranean Congress of Herpetology (CMH2)” in Marrakesh, Morocco, in 2011 termed “Molecular assessment of hemoparasites from European and North African reptiles”; in “Trends in Biodiversity and Evolution: Integrative Approaches In Evolutionary Biology” (TiBE2012) in Porto, Portugal, in 2012 termed “Patterns of apicomplexan parasite diversity in reptiles”; the “Malaria and Related Haemosporidian Parasites of Wildlife” in Vilnius, Lithuania, in 2013 termed “Infection estimates of apicomplexan hemoparasites in reptiles: a comparison of multiple quantification methods”; and the “XIII Iberian Congress of Herpetology” in Aveiro, Portugal, in 2014 termed “Hiding deep in the blood: a survey of hemoparasites of wild endemic reptiles from Madagascar”.

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# 2 CHALLENGES IN PARASITE DETECTION AND IDENTIFICATION

**Article I - Maia, J. P. M. C.**, Gómez-Díaz, E. and Harris, D. J. (2012). Apicomplexa primers amplify *Proteromonas* (Stramenopiles; Slopalinida; Proteromonadidae) in tissue and blood samples from lizards. *Acta Parasitologica*, 57, 337–341.

**Article II - Maia, J. P.**, Harris, D. J., Carranza, S. and Gómez-Díaz, E. (2014). A comparison of multiple methods for estimating parasitemia of Hemogregarine Hemoparasites (Apicomplexa: Adeleorina) and its application for studying infection in natural populations. *PLoS One*, 9, e95010.

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## 2.1 Article I - Apicomplexa primers amplify *Proteromonas* (Stramenopiles; Slopalinida; Proteromonadidae) in tissue and blood samples from lizards

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**Abstract**

Microscopy has traditionally been the most common method in parasitological studies, but in recent years molecular screening has become increasingly frequent to detect protozoan parasites in a wide range of vertebrate hosts and vectors. During routine molecular screening of apicomplexan parasites in reptiles using the 18S rRNA gene, we have amplified and sequenced *Proteromonas* parasites from three lizard hosts (less than 1% prevalence). We conducted phylogenetic analysis to confirm the taxonomic position and infer their relationships with other stramenopiles. Although our phylogeny is limited due to scarcity of molecular data on these protists, our results confirm they are closely related to *Proteromonas lacertae*. Our findings show that unexpected parasites can be amplified from host samples (blood and tissue) using general procedures to detect hemoparasites, and stress that positive PCR amplifications alone should not be considered as definitive proof of infection by particular parasites. Further validation by sequence confirmation and thorough phylogenetic assessment will not only avoid false positives and biased prevalence estimates but also provide valuable information on the biodiversity and phylogenetic relationships of other parasitic organisms. More generally, our results illustrate the perils of general diagnosis protocols in parasitological studies and the need of cross-validation procedures.

**Keywords:** Lizard; hemogregarine; 18S rRNA gene; molecular screening; microscopy; hemoparasites.

## Introduction

Traditionally, microscopy has been the gold-standard in parasitological studies and there has been considerable effort to identify and classify the diversity of protist biota in reptiles, namely intestinal flagellates (e.g., Wood 1935; Wenrich 1947; Janakidevi 1961a, b; Krishnamurthy 1968; Telford 1970; Dollahon and Janovy 1971; Saratchandra and Narasimhamurti 1980a, b; Telford and Bursey 2003) and hemoparasites (e.g., Amo *et al.* 2005, Austin and Perkins 2006, Roca and Galdon 2010). Recently, molecular methods have been increasingly used for the detection of protists in both wild and domestic animals. Compared to more traditional approaches, such as microscopy, molecular methods benefit from good specificity and sensitivity in detecting parasitic infections (e.g., Rubini *et al.* 2005, Merino *et al.* 2009), especially at low parasitemia levels (Moody 2002), they are straightforward and are relatively low cost and not time consuming, thus allowing the screening of large numbers of samples in a reproducible manner. Moreover, molecular data provides additional and valuable information on the diversity of parasitic organisms and their genetic relationships. However, some studies estimate infection prevalence solely based on PCR screening without sequence confirmation (e.g., Ujvari *et al*. 2004, Vardo *et al*. 2005), which could lead to biased estimates since other related or unrelated organisms may be amplified. In recent years, specific primers for the amplification of Apicomplexa in reptiles have been developed, and have been shown to be very useful, considerably increasing the sensitivity of detection (e.g., Ujvari *et al*. 2004, Harris *et al*. 2011, Maia *et al*. 2011, Tomé *et al*. 2012). In this study we demonstrate that these primers can also amplify other protists, namely *Proteromonas* (Stramenopiles, see Cavalier-Smith and Chao 2006). At present, limited molecular data is available for the genus *Proteromonas*; there are currently only data from *P. lacertae* for the mitochondrion genome (Perez-Brocal *et al*. 2010) and from rRNA genes (Leipe *et al*. 1996, Hoevers and Snowden 2005). Phylogenetic studies have shown that *Proteromonas* and other members of the Slopalinida are related with the genus *Blastocystis*, a group of protozoan parasites of medical and veterinary importance (e.g., Tan 2004, Kostka *et al*. 2007).

## Materials and Methods

DNA was extracted from blood or tail tissue using standard High Salt methods (Sambrook *et al*. 1989), DNeasy Blood & Tissue kit (Qiagen), or Speedtools Tissue DNA extraction kit (Biotools). Primers used were HepF300 and HepR900 (Ujvari *et al.* 2004) targeting part of the 18S rRNA gene and PCR cycling consisted of 94°C – 30 sec, 60°C – 30 sec, 72°C – 1 min (35 cycles) (see Harris *et al*. 2011 for more details). Negative and positive controls were run with each reaction. PCR products were electrophoresed on 2% agarose gels, stained using sybr safe or gel red and visualized using a UV-transilluminator. The positive PCR products were purified and sent for direct sequencing (Macrogen Inc). All amplifications were sequenced in both directions. Sequence chromatograms were checked manually and assembled using Geneious v. 5.6.4 (Biomatters Ltd.). We then performed a similarity analysis using the Basic Local Alignment Search Tool (BLAST) to find the best

match (E-value ≤ 10–8) for the sequences against published sequences in GenBank (http://www.ncbi.nlm.nih.gov). All sequences obtained matched the single *P. lacertae* 16S-like rRNA gene sequence available (U37108) with 98% similarity. The new sequences have been submitted to GenBank (accession numbers JX276957-JX276959). To assess phylogenetic relationships, these sequences were aligned with the following sequences retrieved from GenBank: 1) representatives of Proteromonadidae family: *P. lacertae* (U37108), *Karotomorpha* sp. (DQ431242 and DQ431243); 2) representatives of the Opalinidae family: *Protoopalina japonica* (AB175929), *Protoopalina intestinalis* (AY576545), *Opalina ranarum* (AF146089); 3) *Blastocystis* sp. (AY135412) and *Blastocystis pythoni* (AY266472), which were used as outgroups for rooting the phylogenetic trees following Kostka *et al.* (2007). Alignments were performed using the MUSCLE algorithm (Drummond *et al.* 2012) using default parameters implemented in Geneious 5.6.4. The final alignment consisted of 11 sequences of 575 bp. Two different phylogenetic analyses (Maximum Likelihood and Bayesian Inference) were conducted. Maximum Likelihood (ML) analysis included random sequence addition (100 replicate heuristic searches), and support for nodes was estimated using the bootstrap technique (Felsenstein 1985) with 1000 replicates, using PhyML 3.0 (Guindon *et al*. 2010). The AIC criteria carried out in jModeltest 0.1.1 (Posada 2008) were used to choose the model of evolution employed (GTR+I+G). Bayesian analysis was implemented using Mr. Bayes 3.1 (Huelsenbeck and Ronquist 2001) with parameters estimated as part of the analysis. The analysis was run for $5 \times 10^6$ generations, saving one tree each 1000 generations. The log-likelihood values of the sample point were plotted against the generation time and all the trees prior to reaching stationary were discarded. Remaining trees were combined in a 50% majority consensus tree (Figure 2-1). Upon collection, slides were air-dried and later fixed with methanol and stained with Giemsa following Telford (2009). Microscopy was conducted at 400x and 1000x magnification using an Olympus CX41 microscope with an in-built digital camera (SC30).

**Results**

During routine molecular screening of Apicomplexa parasites in reptiles, we have amplified and sequenced a portion of the *Proteromonas* 18S rRNA gene in three reptile samples out of around 600 reptile samples analysed, less than 1% prevalence. These samples belong to three different lizard genera: two from the family Lacertidae (tail tissue from *Acanthodactylus erythrurus* from Spain, sample ALC5, and blood drop from *Darevskia armeniaca* from Armenia, sample K18910), and one from the family Gekkonidae (blood drop from *Pristurus carteri* from Oman, sample IBES7122). Of these samples, a blood smear was only available for IBES7122, in which flagellate stages of *Proteromonas* could be identified (Figure 2-2). A comparison between U37108 and the Apicomplexa specific primers used in this study (HEP300F and HEP900R) showed only two and four mismatches, respectively (Table 2-1). Our phylogenetic analysis using sequences obtained in this study together with previously published sequences of other related Stramenopiles, confirms these *Proteromonas*

18S rRNA gene sequences to be closely related to *Proteromonas lacertae* (Figure 2-1). In the conserved regions of the alignment, the three sequences obtained in this study differ from the *P. lacertae* retrieved from GenBank (Leipe *et al*. 1996) by one (K18910), two (ALC5) and four positions (IBES7122). Additionally, there is a short (20 nucleotides) hyper-variable region in which all samples differ considerably in sequence and length, and where there is variability within the single host sample for K18910 and IBES7122. The sequence from GenBank also has an ambiguity (A or G) in the same region.

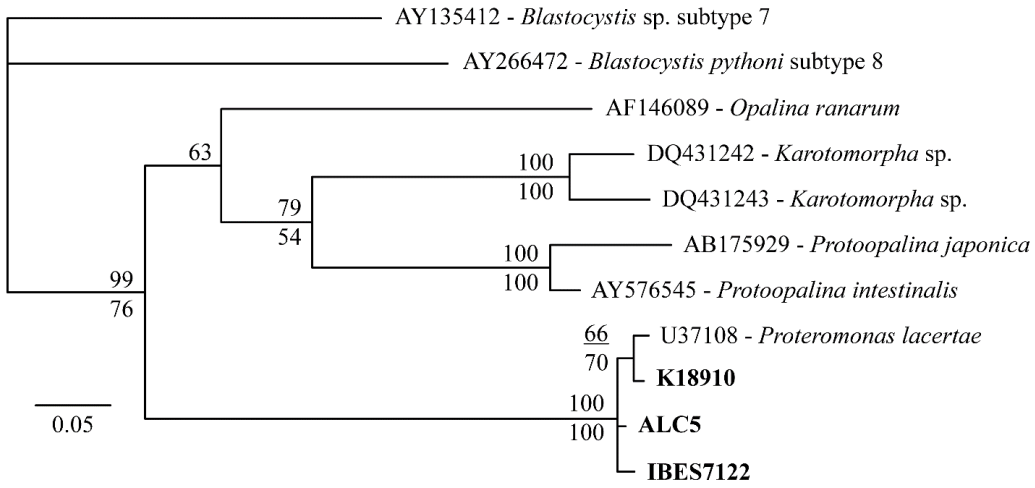


Figure 2-1 Bayesian Inference tree of the new *Proteromonas* sequences together with sequences retrieved from GenBank. Support for the Bayesian and for ML analysis are given above and below the nodes, respectively.

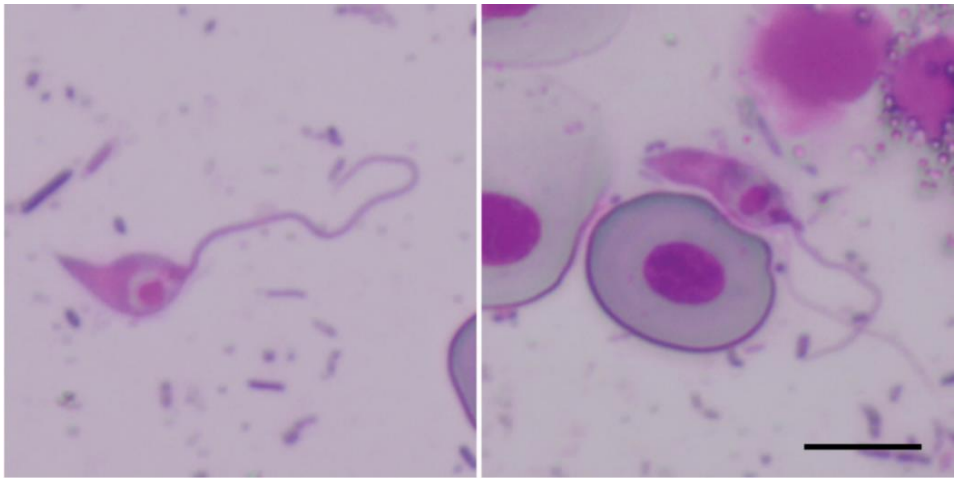

Figure 2-2 Microscopy picture of the blood smear of sample IBES7122 from *P. carteri* showing flagellate stages of *Proteromonas*. Scale bar = 0.01 mm.

Table 2-1 Comparison between *P. lacertae* sequence retrieved from GenBank (U37108) together with the Apicomplexa specific primers used in this study.
Mismatches are indicated in bold.

| | HEP300F | HEP900R |
|---|---|---|
| Primer | GTTTCTG**A**CCTATCAGCTTTCGA**C**G | GTCAGAGGTGAAATTC**TTA**GATTT**G** |
| U37108 | GTTTCTG**C**CCTATCAGCTTTCGA**T**G | GTCAGAGGTGAAATTC**AAG**GATTT**A** |

**Discussion**

In this study, we show that sequences of unexpected parasites can be amplified from host samples (blood and tissue) using general procedures to detect hemoparasites. We have amplified and sequenced a segment of the *Proteromonas* 18S rRNA gene from three lizard host genera using specific primers for the amplification of Apicomplexa. The taxonomic position of these sequences was further confirmed by our phylogenetic analyses. Although our phylogeny is limited due to scarcity of molecular data on these protists, the relationships are congruent with those obtained in previous published works (Kostka *et al.* 2004, 2007; Nishi *et al.* 2005; Hoevers and Snowden 2005). The position of *Karotomorpha* and of opalinids, agrees indeed with the topology obtained by Kostka *et al.* (2007) that showed the family Proteromonadidae as paraphyletic, with the genus *Karotomorpha* being more closely related to opalinids than to the genus *Proteromonas*. Interestingly, *Proteromonas* is a genus of obligate anaerobe protists that live as commensals in the colon of lizards. However, in this study we detected *Proteromonas*-like organisms in blood and tail-tissue samples. One hypothesis is that faecal runoff (containing cysts) could be present on the skin of the reptile tissue sampled. Indeed, lizards often defecate when being processed, so there is the possibility that the parasites present in the faeces may be transferred to the blood drops or remain in the skin of the tail tip. Alternatively, cysts or flagellate stages of these organisms may be naturally present in the blood stream (the latter shown in Figure 2-2), or occasionally the trophic stages may be able to invade tissues. Other unexpected parasites have also recently been reported from blood samples, including *Eimeria*, which is also typically detected in faecal samples (Harris *et al*. 2012). This clearly warrants further investigation. Regardless of the source, our study demonstrates the perils of molecular diagnosis protocols in parasitological studies and the need of cross-validation procedures between and within methodologies. First, the primers used were originally designed in a study where 100 pythons were found to have 100% prevalence of presumably *Hepatozoon* spp. based solely on PCR (Ujvari *et al.* 2004). However, these primers are actually less specific and can amplify other distantly related protists (e.g., *Proteromonas*) or even fungi (Tome *et al*. 2012), as well as various apicomplexans such as *Eimeria* and *Sarcocystis* (Harris *et al*. 2012), which could pose an important bias for infection estimates. Nonetheless, the fact that primers are less specific may also open up new possibilities in providing relevant information on other parasitic organisms, as long as these are confirmed through sequencing and phylogenetic analyses. It therefore should be stressed that it is important not to rely solely on PCR amplifications when screening for infections; PCR products should be sequenced to confirm identification of the parasite detected. This will not only avoid false positives, if other unrelated parasites are amplified, but also has the potential to provide valuable information on the diversity and evolutionary relationships of poorly-known or less common parasites.

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## 2.2 Article II - A comparison of multiple methods for estimating parasitemia of Hemogregarine Hemoparasites (Apicomplexa: Adeleorina) and its application for studying infection in natural populations

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**ABSTRACT**

Identifying factors influencing infection patterns among hosts is critical for our understanding of the evolution and impact of parasitism in natural populations. However, the correct estimation of infection parameters depends on the performance of detection and quantification methods. In this study, we designed a quantitative PCR (qPCR) assay targeting the 18S rRNA gene to estimate prevalence and intensity of *Hepatozoon* infection and compared its performance with microscopy and PCR. Using qPCR, we also compared various protocols that differ in the biological source and the extraction methods. Our results show that the qPCR approach on DNA extracted from blood samples, regardless of the extraction protocol, provided the most sensitive estimates of *Hepatozoon* infection parameters; while allowed us to differentiate between mixed infections of Adeleorinid (*Hepatozoon*) and Eimeriorinid (*Schellackia* and *Lankesterella*), based on the analysis of melting curves. We also show that tissue and saline methods can be used as low-cost alternatives in parasitological studies. The next step was to test our qPCR assay in a biological context, and for this purpose we investigated infection patterns between two sympatric lacertid species, which are naturally infected with apicomplexan hemoparasites, such as the genera *Schellackia* (Eimeriorina) and *Hepatozoon* (Adeleorina). From a biological standpoint, we found a positive correlation between *Hepatozoon* intensity of infection and host body size within each host species, being significantly higher in males, and higher in the smaller sized host species. These variations can be associated with a number of host intrinsic factors, like hormonal and immunological traits, that require further investigation. Our findings are relevant as they pinpoint the importance of accounting for methodological issues to better estimate infection in parasitological studies, and illustrate how between-host factors can influence parasite distributions in sympatric natural populations.

**Keywords:** Eimeriorina; microscopy; PCR; qPCR; intensity; extraction method; biological source.

## Introduction

Parasites can be major drivers of host ecology and evolution and play key roles in ecosystem functioning and structure. Parasitism can for example alter host life-history traits and fitness [1,2], as well as influence predator-prey or competitive interactions between and within species [3–5]. However, the ecological niche in which both host and parasite occur is complex and multifaceted, and there is considerable variation in patterns of parasitism in natural populations at multiple spatial and temporal scales [6], from individual to community levels [7,8]. Natural variation in infection parameters can be due to differences in the environmental exposure to parasites [9], or to variation in the susceptibility or resistance to infection [10]. Investigating the underlying patterns can contribute to a better understanding of the impact and evolution of parasitism [11–13], while also providing valuable epidemiological and conservation information.

Importantly, the estimation of biologically relevant and realistic infection patterns is constrained by differences in the accuracy, specificity and sensitivity of the detection and quantification protocols used [14,15]. The use of less accurate protocols may lead to erroneous ecological and epidemiological inferences, which is particularly critical in host-parasite systems with low intensity levels or with mixed parasite infections. In recent years, quantitative PCR (qPCR) has been used in parasitological studies as it allows the simultaneous detection and quantification of parasite DNA from various biological sources, such as host and vector blood and tissues [16–20]. Compared to more traditional approaches, such as microscopy or conventional PCR (PCR), qPCR has increased accuracy and sensitivity of detection [21,22]. Despite the advantages, the use of this quantitative method in parasitology has been primarily used for clinical samples and/or pathogens of human-health and veterinary importance [17]. Although this technique is now routinely applied for clinical studies in domestic animals [21], its applications in wild animal populations, is just beginning to emerge [6,19,23–25]. More importantly, studies comparing the performance of the different methods under various experimental conditions are generally lacking (but see [15]).

Sympatric and closely related species represent natural model systems and an ideal opportunity where to investigate between-host differences in infection parameters [26]. Closely related species are likely to be susceptible to the same or similar parasite species due to common ancestry, and the same can happen with sympatric host species due to common ecological determinants [27]. In these systems, differences in infection may arise due to inter- and intra-host variation in susceptibility and/or tolerance to infection, while controlling for general environmental differences in exposure. Reptiles have become model organisms for parasitological studies, in part due to their low dispersal potential, and the occurrence of sympatric speciation [28]. Despite the growing number of studies addressing infection variation in animal populations [29–32], these remain largely enigmatic in the case of wild reptiles. In reptiles, body size and weight have been commonly used as a proxy measure to explain individual as well as population and species differences in susceptibility and/or tolerance to parasite infection [33–37], because it is often positively correlated with host life-history traits such

as longevity, survival and fecundity, which relate to fitness [38]. Other factors that might drive variation in parasite infection include host behaviour and morphology [39,40], immunity [10,41] and ecology [27,42]. However, previous studies have shown contrasting patterns of association with those factors [34–36,43], which may be in part due to the effects of sampling and methodological bias, and/or confounding phylogenetic or environmental factors [42,44,45].

*Hepatozoon* (Apicomplexa: Adeleorina), is a highly diverse genus of intracellular hemogregarine parasites with more than 300 species described [46], and the most common and widely distributed hemoparasites found in reptiles [47]. Yet few studies to date have examined prevalence and intensity of infection in wild reptile populations (but see [35,37,48–51]). Arthropod vectors, such as mites, ticks and mosquitoes, are the definitive hosts in a complex heteroxenous lifecycle that includes a wide range of vertebrates as intermediate hosts [46]. Pathogenesis caused by *Hepatozoon* infections in reptiles is unclear, with studies reporting from apparently non-detrimental infections in natural hosts [36,52], to severe and life-threatening illness in unnatural hosts [53]. This has been mostly studied in domestic animals, for which the most common symptoms include anaemia, lethargy, weight loss, weakness and cachexia [54,55], while the pathogenic effects on wildlife are mostly unknown. In addition, other apicomplexans, such as *Schellackia* sp. (Apicomplexa: Eimeriorina), are often found at lower prevalence and intensities in wild reptile hosts, and mixed infections with *Hepatozoon* can also occur [56,57]. In this study, we investigate *Hepatozoon* infection parameters in two sympatric closely related lacertid species, *Podarcis bocagei* and *P. hispanica*. These two host species present an attractive system because: first, they live in sympatry and, despite having similar ecological requirements, there are apparent preferences for microhabitat: *P. hispanica* is frequently found on rocks (saxicolous), while *P. bocagei* is mainly ground-dwelling [58,59]; second, despite being genetically closely related [60], they present considerable morphological differences, with *P. bocagei* being generally larger [61,62]; third, size sexual dimorphism has been reported for both species, with adult males being larger than females (Kaliontzopoulou *et al.*, 2008, 2012); and fourth, recent studies report high prevalence of *Hepatozoon* and low prevalence of *Schellackia* infections for these species in the Iberian Peninsula [50,56]. Therefore, this model system provides an ideal scenario for investigating between host-species, sex and inter-individual differences in patterns of *Hepatozoon* infection, while controlling for confounding factors such as ecology and host and parasite phylogeny.

The objectives of this study are: i) to evaluate the accuracy and sensitivity of different detection methods (microscopy, PCR and qPCR) and protocols (biological source and DNA extraction protocol) for detecting and quantifying hemogregarine parasites in reptile samples, as well as the occurrence of mixed infections, and ii) apply the most sensitive approach, i.e. qPCR, to determine prevalence and intensity of *Hepatozoon* infection in two sympatric closely related lacertid species, *P. bocagei* and *P. hispanica*, and assess the relative role of inter-individual (intra-species) and between-species factors on these parameters.

## Materials and Methods

### *Study species and study site*

Samples were collected from two lacertid lizard species, *P. bocagei* and *P. hispanica*, from a single location in Gerês, Northern Portugal (41.782340, -8.145140), in July 2011. Capture permits were issued by the ICNB (Instituto da Conservação da Natureza e da Biodiversidade, I.P.), license numbers 67-75/2011/CAPT. These hosts are small, diurnal, insectivorous lizards, with adult snout-vent length (SVL) of 45-65 mm and 37–70, respectively. A total of 87 adult individuals were sampled (51 *P. bocagei* and 36 *P. hispanica*, see Table 2-2). Each individual was handled by experienced herpetologists, a small piece of the tail-tip was collected and, when enough blood was obtained, a blood drop was stored in Whatman filter paper and the rest was used to make a blood smear. No animals were sacrificed. SVL was measured using a vernier caliper, animals were photographed and sex was determined based on the existence of enlarged femoral pores. After processing, animals were released at the site of capture. This protocol has been approved by the ethical committee of the University of Porto. Tissue samples were preserved in 96% ethanol and stored at room temperature and blood drops stored at -20ºC. Blood smears were air-dried, fixed with methanol on the day of collection and stained with diluted Giemsa (1:9 of distilled water) for 55 minutes within a week of collection.

### *Microscopic examination*

Blood smears were examined using an Olympus CX41 microscope with an in-built digital camera (SC30). Several photographs per slide were taken under the 40X magnification lenses and stitched using cell^B software (basic image-acquisition and archiving software). Prevalence was estimated as the proportion of infected hosts and intensity of infection was estimated as the number of parasites per 4,000 erythrocytes from a total of 72 blood smears [64,65]. Counts were done using the manual cell counter plug-in available in the image processing software ImageJ ver. 1.44p [66]. Figure 2-3 shows mature intraerythrocytic gamonts of *Hepatozoon* sp. and sporozoites of *Schellackia* sp. infecting the two lizard species.

### *DNA extraction and sample preparation*

For the total number of samples (*n*=87), DNA was extracted from blood using the Speedtools tissue DNA extraction kit (Biotools, Madrid). In a subset of 47 individuals, in which we compared various methodological approaches, DNA was extracted from two biological sources: blood drops stored in Whatman filter paper (approximately 3 mm by 3 mm) and tail-tip muscle tissue (approximately 2 mm by 2 mm with skin removed). On these samples we used two extraction protocols: the Speedtools commercial kit following manufacturer's instructions, and the standard saline protocol [67]. Briefly, the saline method used consisted of adding 600 µl of lysis buffer (0.5M tris; 0.1M EDTA; 2% SDS; pH 8.0) and 10 µl of proteinase K (25 mg/ml) to the cut material, which was incubated at 56ºC overnight. After incubation, 300 µl of ammonium acetate (5M; pH 8.0) was

added and centrifuged for 15 min at 14000 rpm at 4ºC. The supernatant was carefully transferred to new eppendorf tubes, 600 µl of ice-cold isopropanol was added and samples were frozen between 3h to overnight. After incubation, samples were centrifuged for 25 min at 14000 rpm at 4ºC, and the supernatant was carefully discarded. Then, 1000 µl of ice-cold ethanol (70%) was added and centrifuged for 15 min at 14000 rpm at 4ºC. The supernatant was carefully discarded and samples were left to evaporate at room temperature. When completely evaporated, 50 µl of ultra-pure water was added and samples were left to hydrate at ambient temperature in an agitator for 2h. For qPCR analyses, samples were diluted to 10ng/µl with nuclease-free water (QIAGEN) using an ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). Quality of DNA for all samples was verified with the nm wavelength within the excepted range for pure DNA according to the manufacturer's manual (260nm/280nm ratio mean was 1.98, and the 260nm/230nm ratio mean was 2.1). This standardization procedure allows to control for the amount of host DNA, which can interfere with amplification of parasite DNA [16,68].

*Conventional PCR, sequencing and phylogenetic analysis*

PCR amplifications were performed on all 87 samples using primers HepF300 and HepR900 [69] that target part of the *Hepatozoon* 18S rRNA gene. The PCR reactions using the Hep primers were run in a 20 µl reaction mixture containing 1 U of GoTaq® DNA Polymerase (5u/µl), 1.5 mM $MgCl_2$ (25 mM), 0.125 mM of each nucleotide, 1 X GoTaq® Flexi Buffer, 0.6 mM of each primer, and 2 µl of DNA. The reaction mix was heated to 94ºC for 3 min, and amplification was performed through at 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 1 min, in 35 cycles, with a final 10 min extension at 72ºC. Two negatives (one blank and one known *Hepatozoon* negative) and one positive control (one known *Hepatozoon* positive) were run with each reaction. Sequencing of the positive PCR products was performed in both directions outsource (Macrogen Europe, The Netherlands). Geneious v6.0.3 was used for contig assembly and visualization of sequences. The "heterozygotes plugin" with 30% peak similarity was used to search for possible double peak positions. Consensus sequences for each individual were deposited in GenBank under the accession numbers KJ189387-KJ189433 (*Hepatozoon*), KJ189382-KJ189385 (*Schellackia*) and KJ189386 (*Lankesterella*). One representative of each of the three *Hepatozoon* haplotypes (KJ189418, KJ189426 and KJ189390), of the two *Schellackia* haplotypes (KJ189382 and KJ189384), and the single *Lankesterella* haplotype (KJ189386), were aligned with published data on GenBank using the MUSCLE alignment [70] with default parameters and "Refine Existing Alignment" option implemented in Geneious. The alignment consisted of 75 taxa and was 641 bp long. Two different phylogenetic analyses (Maximum Likelihood and Bayesian Inference) were conducted. Maximum Likelihood (ML) analysis with random sequence addition (100 replicate heuristic searches) was used to assess evolutionary relationships, using the software PhyML 3.0 [71]. Support for nodes was estimated using the bootstrap technique [72] with 1000 replicates. The AIC criterion conducted in jModeltest 0.1.1 [73] was used to choose the model

of evolution (TIM1+G). Bayesian analysis was implemented using Mr. Bayes v.3.1 [74] with parameters estimated as part of the analysis. The analysis was run for $10 \times 10^6$ generations, saving one tree with random tree sampling each 1000 generations. All trees prior to reaching stationarity (25% of the run) were discarded as burn-in samples, and the remaining trees were combined in a 50% majority consensus tree.

*qPCR design and protocol*

qPCR assays for detecting *Hepatozoon* species in canids have been previously designed [18,20]. We first tested the former protocol in reptile-infected samples, and we obtained a double dissociation peak in the Melting Curve Analysis (MCA). Therefore we designed a qPCR assay targeting the 18S rRNA gene of hemogregarine species in reptiles taking into account the variation previously reported in *Hepatozoon* and other apicomplexans (Figure S1). For this purpose we designed Hemogregarine specific primers JM4_F (5'-ACTCACCAGGTCCAGACATAGA-3') and JM5_R (5'-CTCAAACTTCCTTGCGTTAGAC-3') using Primer3Plus (Rozen and Skaletsky, 2000). We constructed a reference plasmid containing the target *Hepatozoon* 18S rDNA fragment (171 bp) amplified from a positive *P. hispanica* individual (JX531917) that we used as amplification standard for the qPCR assay. The PCR product was cloned to a plasmid using the TOPO TA Cloning® kit (Invitrogen, California) following the manufacturer's instructions. One Shot® TOP10 Competent Cells were used for transformation and we used the Purelink™ Quick Plasmid Miniprep Kit (Invitrogen, California) to isolate the plasmid (aliquots are available upon request). Successful plasmid integration of the *Hepatozoon* gene fragment was assessed using M13 primers following manufacturer's instructions. Serial 10-fold dilutions containing from 500,000 to 5 copies of plasmid were performed to produce a standard curve for *Hepatozoon* quantification that was included in each qPCR plate. A MyiQ qPCR machine (Applied Biosystems) was used with the following protocol: 95ºC 10 min, 95ºC 10 s, 63ºC 20 s, 72ºC 25 s (with melting), 95ºC 1 min, 63ºC 30 s, melting from 63-95ºC by increments of 0.2ºC every 10 s. Reactions contained iQ™ SYBR® Green supermix at 0.5x, each primer at 0.5mM, 2µl of 10ng/µl genomic DNA, in a total volume mix of 20 µl. To estimate the number of copies in unknown samples, raw qPCR results were exported using the program iQ5 R&D version 2.1 (Biorad) and the baseline threshold was determined individually for each plate using the algorithm implemented in LinRegPCR [76]. Validation was performed by direct sequencing on a total of 33 qPCR positive individuals. These included qPCR positives displaying a single peak (negatives and positives for *Hepatozoon* from microscopy and PCR) and all those displaying mixed infection peaks. These sequences have been deposited in GenBank under accession numbers KJ189434-KJ189463. For this experiment, samples with all threshold cycles (CTs) higher than 35 were considered negative because repeatability decreased significantly after cycle 35, with most replicates differing by more than one Ct. Mixed infection intensity estimates ($n$=6) were discarded from subsequent analyses.

*Statistical analyses*

Normality tests were performed using Shapiro-Wilk and homogeneity of variances tested using Levene's non-parametric test. To approach normality, infection estimates obtained from qPCR (*Hepatozoon* copy number) were log-transformed using the formula *log (x+1)*. Fitness of the models was tested by Normal Q-Q plots of the Pearson residuals from the model analysis.

*Between methods' comparisons* - We first investigated the correlation between *Hepatozoon* intensity levels detected by microscopy and qPCR using the Spearman correlation coefficient (*n*=66, out of 72 blood smears after excluding 6 mixed infections). We used McNemar's test to compare sensitivity of the three detection methods (*n*=87) and the four extraction protocols (*n*=47) in estimating *Hepatozoon* prevalence. Then, to compare *Hepatozoon* intensity among biological sources and extraction protocols (*n*=41, out of the subset of 47 samples after excluding 6 mixed infections) we fitted a Generalized Linear Mixed Model (GZLMM), as recommended for unbalanced data with repeated measures (random effects) [77], with normal distribution and log link function. The full model included copy number as the target variable, the biological source, extraction protocol and their interaction as fixed factors, and subject individuals as random effect. Pairwise LSD mean contrasts for each factor were included in the model.

*Between hosts' comparisons* - Previous studies have found various associations between infection parameters and body size [43,51], and therefore prior to building the models we first examined variation in body size between host species and sexes using ANOVA with Type I sums of squares (SS). For this analysis, body size was the response variable and host species and sexes (and their interaction) were treated as fixed factors. We then tested for inter- and intra-specific associations between body size and intensity of infection using the Spearman correlation coefficient. A positive correlation was found and body size was used as a covariate in all subsequent analyses. Then, using qPCR estimates as a proxy, we investigated differences in infection parameters between the two *Podarcis* host species and between sexes within each species. Prevalence of *Hepatozoon* infection between host species, between sexes within each species and between the same sexes of both species (*n*=87) was compared using a Chi-square test (http://www.quantpsy.org/chisq/chisq.htm). For intensity comparisons between host species and sexes (*n*=81, out of 87 samples after excluding 6 mixed infections), while accounting for the effects of body size, we used a Generalized Linear Model (GZLM) with normal distribution and log link function using Type I SS. The full model included body size, host species and sexes (and their interactions) as fixed factors. All statistical analyses were conducted in IBM® SPSS® Statistics 21, except for McNemar tests that were conducted in R (R version 3.0.2, R Development Core Team).

Table 2-2 Prevalence and mean intensity of *Hepatozoon* and Eimeriorina parasites for the two lizard species analysed in this study, estimated using three different methods.

| | | *Hepatozoon* | | | | | Eimeriorina | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | qPCR | | PCR | Microscopy | | qPCR (Blood Kit) | PCR | Microscopy[1] | |
| **Host species** | **Sex** | **Prevalence** | **Mean Intensity [log(copy number)]** | **Prevalence** | **Prevalence** | **Mean Intensity (% ± std)** | **Prevalence** | **Prevalence** | **Prevalence** | **Mean Intensity (% ± std)** |
| *Podarcis* | Female | 13/22 (59%) | 1.23 ± 0.26 | 10/22 (45%) | 5/16 (31%) | 0.12 ± 0.10 | 0/22 (0%) | 1/22 (5%) | 0/16 (0%) | - |
| *bocagei* | Male | 24/29 (83% | 2.20 ± 0.28 | 17/29 (59%) | 14/25 (56%) | 0.22 ± 0.08 | 3/29 (10%) | 2/29 (7%) | 3/25 (12%) | 0.16 ± 0.12 |
| | | 37/51 (73%) | 1.74 ± 0.20 | 27/51 (53%) | 19/41 (46%) | 0.18 ± 0.06 | 3/51 (6%) | 3/51 (6%) | 3/41 (7%) | - |
| *Podarcis* | Female | 16/18 (89%) | 1.80 ± 0.26 | 11/18 (61%) | 7/14 (50%) | 0.11 ± 0.05 | 0/18 (0%) | 0/18 (0%) | 1/14 (7%) | 0.02 |
| *hispanica* | Male | 16/18 (89%) | 2.94 ± 0.33 | 13/18 (72%) | 12/17 (71%) | 0.73 ± 0.24 | 1/18 (6%) | 2/18 (11%) | 1/17 (6%) | 0.02 |
| | | 32/36 (89%) | 2.37 ± 0.23 | 24/36 (67%) | 19/31 (61%) | 0.45 ± 0.15 | 1/36 (3%) | 2/36 (6%) | 2/31 (6%) | 0.02 ± 0.00 |
| | | *n* = 87 (79%) | *n* = 81 | *n* = 87 (59%) | *n* = 72 (53%) | *n* = 72 | *n* = 87 (5%) | *n* = 87 (6%) | *n* = 72 (7%) | *n* = 72 |

[1] These were found inside erythrocytes, except for one that was found in a leukocyte (Figure 2-3).

## Results

### *qPCR assay validation*

Intensity of *Hepatozoon* infection estimated by microscopy and qPCR were significantly correlated (ρ=0.893, *P*<0.001), with qPCR being much more sensitive and detecting as few as 5 copies of parasite DNA. Mean threshold cycle (Ct) standard deviation (SD) was 0.16, mean efficiency 81.1% (SD ± 2.6) and mean $R^2$ = 0.991 (SD ± 0.008) (Figure S2). Mean SD values for intra-assay repeatability for sample replicates was 0.198 (SD ± 0.147) and 0.192 for plasmid dilution replicates (SD ± 0.166). Melting Curve Analysis of plasmid dilutions displayed a single peak at 81.4ºC and amplification charts were S-shaped. Sequences retrieved from qPCR amplifications were BLASTed on GenBank. Those samples displaying a single melting peak curve all matched *Hepatozoon* spp. sequences, while the ones displaying two distinct peaks (range from 82.6-83.8ºC) presented a mixed electropherogram in the sequencing analyses (Figure S3). These mixed infections detected by qPCR matched those detected by conventional PCR sequencing.

### *Parasite diversity and identification*

A total of 56 sequences were obtained from PCR sequencing with the Hep primers. Of these, 51 BLASTed with previously published GenBank sequences of *Hepatozoon* spp. (Apicomplexa: Adeleorina), 4 with *Schellackia* spp. and 1 with *Lankesterella* spp. (Apicomplexa: Eimeriorina). These were considered positive for PCR prevalence (Table 2-2). In mixed infections, these conventional PCR primers preferentially amplified Eimeriorina parasites so they resulted in single electropherograms by sequencing analysis. The 47 *Hepatozoon* sequences (4 were discarded from further analyses due to poor quality) resulted in 3 unique haplotypes (and individuals with double peak positions corresponding to these haplotypes, see Table S1) that differed in 15 polymorphic segregating sites. These haplotypes clustered within a previously identified *Hepatozoon* lineage obtained from Western Mediterranean and North African reptiles (Figure 2-4). The melting curve analysis (MCA) was able to differentiate between different Apicomplexa genera in mixed infections (Figure S3). But we did not find differences in melting temperatures between the three closely related *Hepatozoon* haplotypes, which are identical for the targeted fragment in the qPCR assay (Figure S3). Two haplotypes of *Schellackia* sp. were obtained, one haplotype from a single individual of *P. bocagei* was identical to *Schellackia* sp. from *P. hispanica* (JQ762306 and JX984676), and the other haplotype (two sequences from *P. hispanica* and one from *P. bocagei*) was closely related with two haplotypes from *Lacerta schreiberi* (JX984674 and JX984675) (Figure 2-4). Finally, the single *Lankesterella* sp. sequence obtained in this study is related with *Lankesterella minima* from the American bullfrog *Lithobates* (formerly *Rana*) *catesbeianus* (AF080611) and *Lankesterella valsainensis* from the blue tit *Parus caeruleus* (DQ390207) (Figure 2-4).

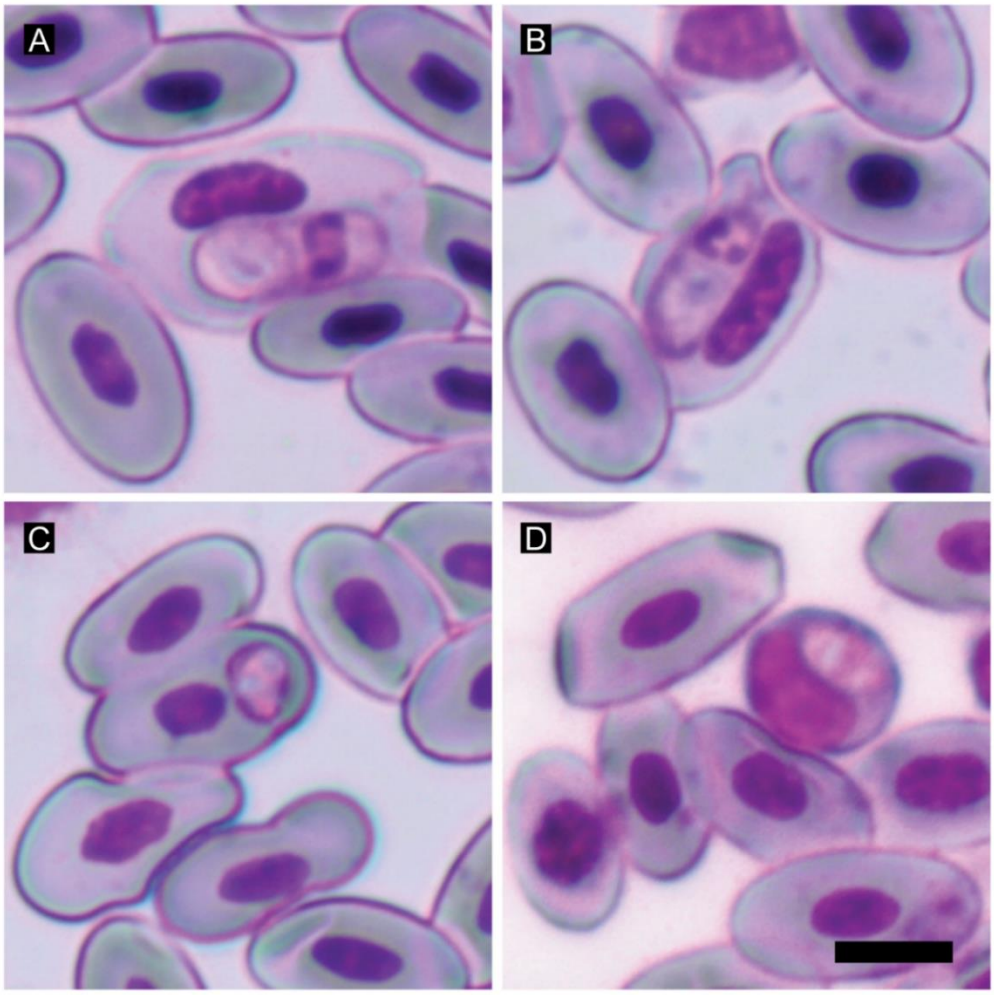


Figure 2-3 Parasites found in common wall lizards analysed from Gerês, Portugal.
Hemogregarines infecting erythrocytes from *P. bocagei* (A), and *P. hispanica* (B).
*Schellackia* sp. infecting an erythrocyte from *P. bocagei* (C), and a leukocyte from *P. hispanica* (D).
Scale bar = 5 µm.

### *Methodological comparison*

We first compared the accuracy and sensitivity of different detection methods for estimating *Hepatozoon* prevalence, including microscopy, PCR and qPCR (Table 2-2). The qPCR assay gave the highest prevalence estimates and differed significantly from microscopy ($n$=72, McNemar $Chi^2$=17.053, df=1, $P$<0.001) and PCR ($n$=87, McNemar $Chi^2$=11.529, df=1, $P$<0.001). Microscopy and PCR also differed significantly ($n$=72, McNemar $Chi^2$=4.167, df=1, $P$=0.041). Among molecular methods: 23% (16/69) of qPCR *Hepatozoon* positives and 75% (3/4) of qPCR Eimeriorina positives were not detected by PCR, while the contrary was found only for 2% (1/51) *Hepatozoon* and 20% (1/5) Eimeriorinid positives (Table 2-2 and Table 2-3). *Hepatozoon* negatives for PCR and qPCR (considering only blood and the kit extraction protocol) were also negative by microscopy, but two Eimeriorina positives by microscopy were not detected by PCR (Table 2-3).

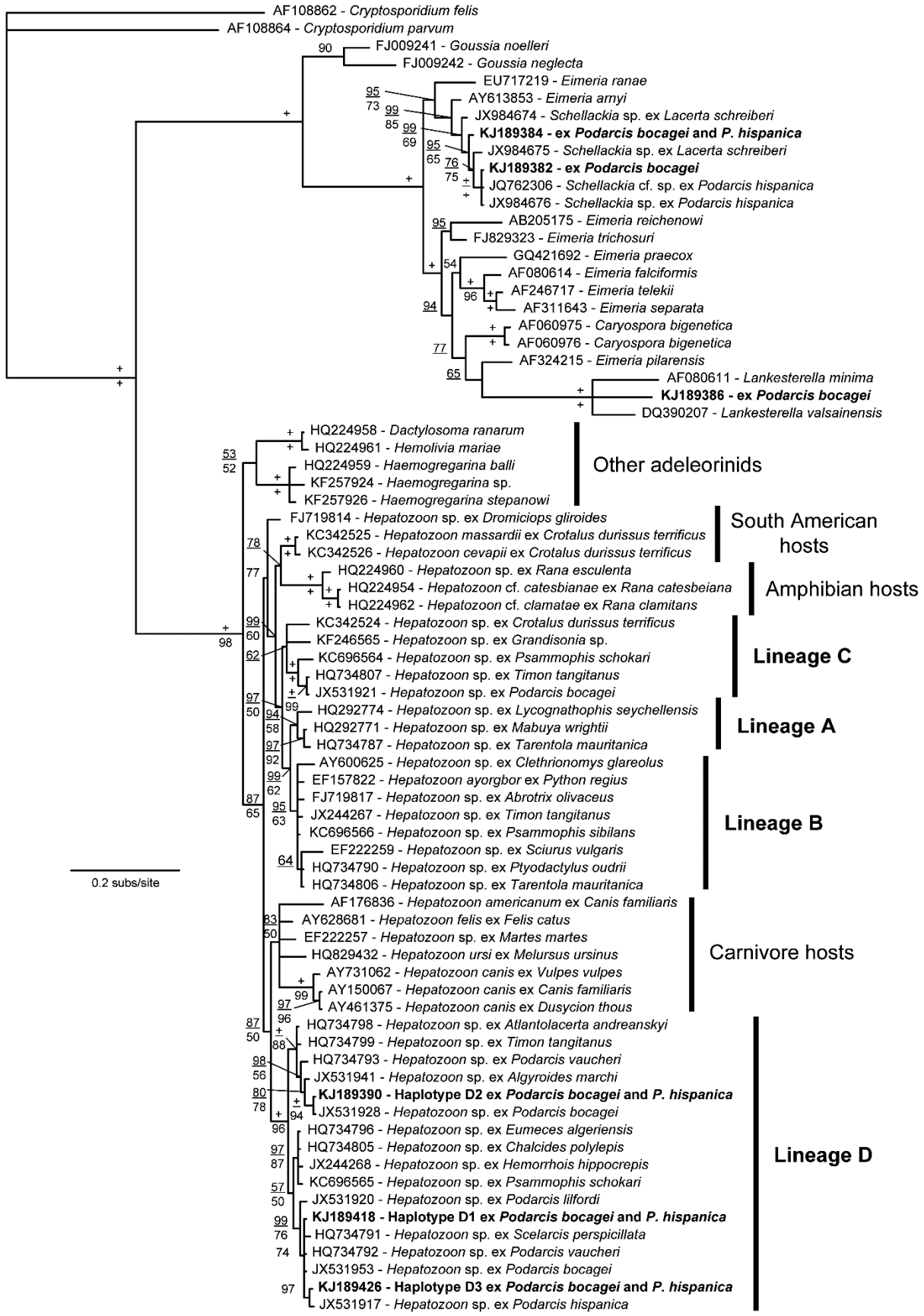


Figure 2-4 Phylogenetic relationships for the 18S rRNA gene of the hemoparasites analyzed in this study. Haplotypes retrieved from this study are in bold. Letters refer to lineages found previously in North African lizards [122].

Table 2-3 False negatives for each of the three detection methods compared in this study.

| | **False negatives (*Hepatozoon*/Eimeriorina)** | | |
|---|---|---|---|
| | Microscopy | PCR | qPCR (BK) |
| Microscopy (*n*=72) | - | 5 /2 | 19 / 1 |
| PCR (*n*=87) | 0 / 2 | - | 16 / 3 |
| qPCR (BK) (*n*=87) | 0 / 0 | 1[2] / 1 | - |

Then, based on the qPCR estimates, we compared the performance of two biological sources (blood and tissue) and two extraction protocols (kit and saline) in estimating *Hepatozoon* infection for a subset of 47 individuals (Table 2-4). Prevalence differed significantly for the type of source (McNemar $Chi^2$=10.083, df=1, *P*<0.001 for kit, and McNemar $Chi^2$=7.692, df=1, *P*=0.003 for saline), but not for the type of extraction protocol (McNemar $Chi^2$=0, df=1, *P*=1.000 for blood and for tissue). In terms of *Hepatozoon* intensity of infection, the GZLMM analysis showed that *Hepatozoon* intensity differed significantly between sources (F=155.096, df1=1, df2=160, *P*<0.001) (Figure 2-5 C-D) and between extraction protocols (F=4.526, df1=1, df2=160, *P*=0.035), but their interaction was marginally significant (F=3.736, df1=1, df2=160, P=0.055). Pairwise comparisons further show that the two tissue extractions differ significantly (F=4,980, df1=1, df2=160, *P*=0.027) (Figure 2-5 B), but not for the two blood extractions (F=0.046, df1=1, df2=160, *P*=0.830) (Figure 2-5 A), which shows that the previous differences between extraction protocols for the GZLM analysis are due to differences observed between tissue extractions. That is, for tissue the saline method provides slightly lower *Hepatozoon* intensity estimates than the kit (Table 2-4). Overall, however, both protocol extractions result in proportional estimates of *Hepatozoon* intensity (Figure 2-5 A-B) and provide similar number of false negatives (Table 2-4). In addition, for the same extraction protocol, tissue provided proportionally lower intensity estimates compared to blood (Figure 2-5 C-D). Finally, blood extractions performed similarly at detecting overall prevalence but differed for samples with extremely low parasitemia levels (Figure 2-5 A and Table 2-4).

### *Between-host differences in infection patterns*

ANOVA analyses showed significant differences in body size between host species (F=11.991, df=1, *P*=0.001), *P. bocagei* being bigger, and sexes (F=5.023, df=1, *P*=0.028), males being bigger in size. Given that qPCR and blood-kit DNA extraction provided good estimates of infection parameters for the methodological subset comparison, we used this protocol for investigating the relationship between host-factors and infection patterns. Prevalence was higher in *P. hispanica* females (89%) than in *P. bocagei* females (59%) ($X^2$=4.409, df=1, *P*=0.036), but there were no significant differences between species ($X^2$=3.434, df=1, *P*=0.064), or sexes (*P. bocagei*: $X^2$=3.519, df=1, *P*=0.061; *P. hispanica*: $X^2$=0, df=1, *P*=1) (see Table 2-2).

[2] Ct was higher than 35 and differed by more than 1 Ct in two trials so was considered negative (see Materials and Methods)

Table 2-4 Prevalence and mean intensity levels of *Hepatozoon* and prevalence of Eimeriorina parasites using different biological sources (blood and tissue) and extraction protocols (kit and saline) on a subset of samples tested with qPCR.

| | | *Hepatozoon* | | Eimeriorina | **False negatives (*Hepatozoon*/Eimeriorina)** | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **Extraction** | **Prevalence** | **Mean Intensity [log(copy number)]** | **Eimeriorina Prevalence** | Blood Kit | Blood Saline | Tissue Kit | Tissue Saline |
| Blood | Kit | 38 (81%) | 2.17 ± 0.23 | 4 (9%) | - | 5 / 1 | 0 / 2 | 0 / 1 |
| | Saline | 37 (79%) | 2.17 ± 0.23 | 3 (6%) | 6 / 2 | - | 0 / 1 | 1 / 0 |
| Tissue | Kit | 26 (55%) | 1.30 ± 0.19 | 2 (4%) | 12 / 4 | 10 / 2 | - | 2 / 0 |
| | Saline | 26 (55%) | 1.09 ± 0.18 | 1 (2%) | 12 / 4 | 11 / 2 | 2 / 1 | - |
| | | *n* = 47 | *n* = 41 | *n* = 47 | | | | |

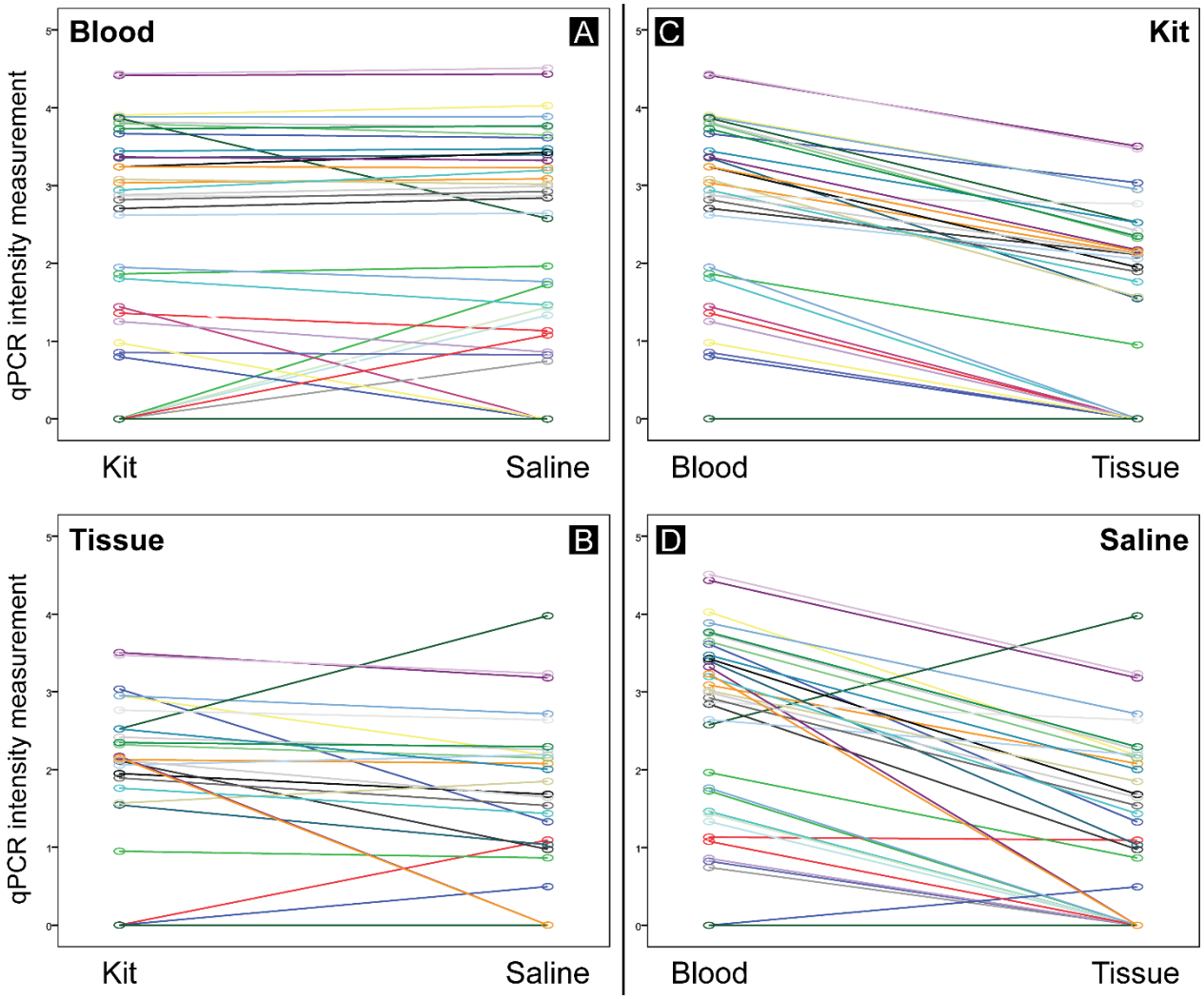


Figure 2-5 Comparison of the performance of various methods in estimating Hemogregarine infection intensity. The two biological sources: blood (A) and tissue (B), and DNA extraction protocols: kit (C) and saline (D). Each line represents the logged *Hepatozoon* intensity measurement from qPCR for an individual extraction (*n*=41, out of the subset of 47 samples after excluding 6 mixed infections).

In terms of intensity of infection, intensity was significantly related with body size for both host species (*P. bocagei*, ρ=0.521, *P*<0.001, and *P. hispanica*, ρ=0.417, *P*=0.014) (Figure 2-6). The GZLM analysis showed a significant effect of host species (Wald $X^2$=8.407, df=1, *P*=0.004) and sexes (Wald $X^2$=7.208, df=1, *P*=0.007), on *Hepatozoon* intensity levels, and confirmed a significant effect of the covariate factor body size (Wald $X^2$=4.254, df=1, *P*=0.039). The interaction between body size and host species was also significant (Wald $X^2$=4.571, df=1, *P*=0.033), which indicates that the effect of host species on the intensity of infection is body-size dependent (Figure 2-6).

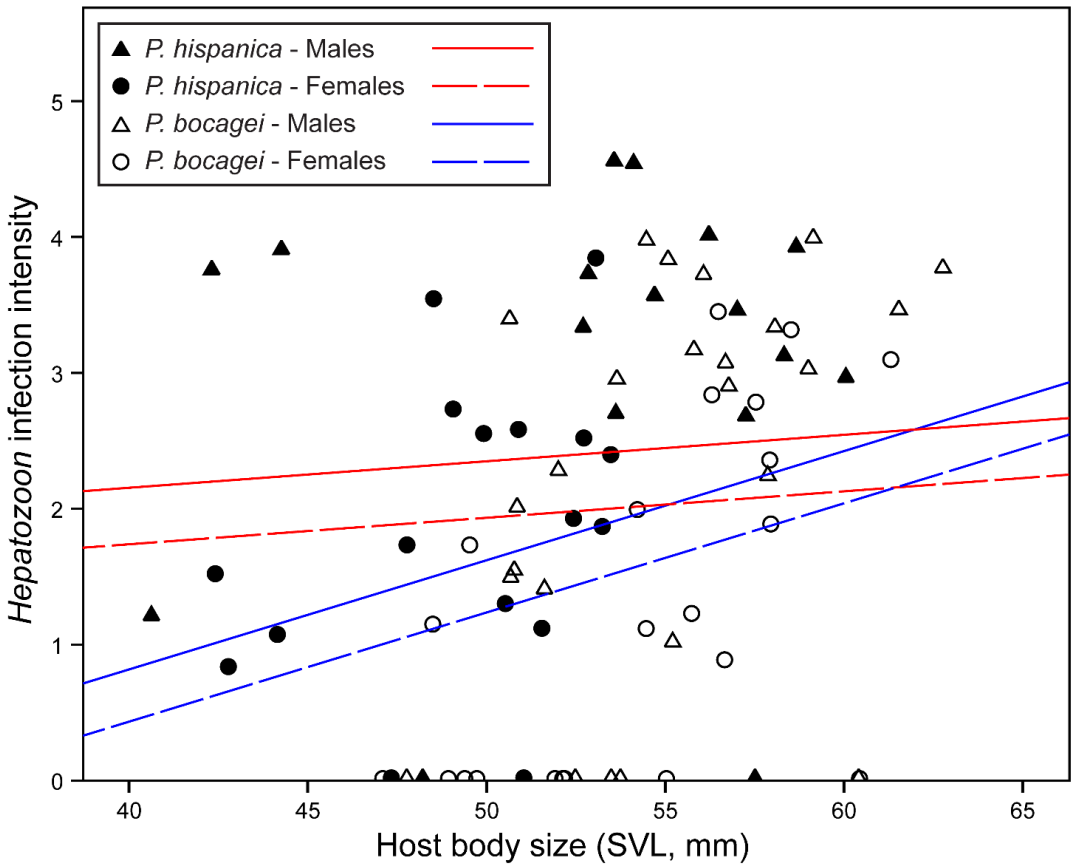


Figure 2-6 Relationship between *Hepatozoon* intensity and host body size (SVL).
Different host species are represented by colours and sexes by shapes. Fit lines were calculated from the parameter estimates of a regression model only considering the significant factors (intensity~body size+host species+sex+host species*body size).

## Discussion

### *Methodological recommendations*

In this study we compared the performance of various detection methods and DNA extraction protocols, and evaluated how the detection method can constrain the estimation of biological relevant infection parameters. First, by comparing various methodological approaches, we found that qPCR was the most accurate and sensitive method for estimating *Hepatozoon* prevalence and intensity, especially in cases of low intensity levels, which is concordant with previous studies conducted on *Plasmodium* [21,22]. To our knowledge, this is the first quantitative PCR assay of

*Hepatozoon* parasites of reptiles, with the few qPCR studies on *Hepatozoon* focusing on carnivore hepatozoonosis [17,18,20,54]. Second, by comparing various extraction protocols on standardized DNA samples, we show that blood samples provide the best estimates of *Hepatozoon* prevalence and intensity levels, regardless of the extraction protocol, and should therefore be the preferred collection method during fieldwork when possible. Our results also show that the traditional saline DNA extraction protocol can be used as an effective low-cost alternative to the tested commercial DNA extraction kit, since it provides similar estimations of hemoparasite infections. This result is in agreement with previous parasitological studies using alternative protocols to commercial kits [50,78–80]. At low intensity levels, protocols had different sensitivities, which pinpoints that performing a preliminary methodological comparison prior to the commencement of the study can be a cost/time effective strategy. Finally, the performance of tissue samples was poorer than blood, with higher number of false negatives for an equivalent DNA concentration (i.e. 10ng/µl of total DNA). However, tissues still represent a valuable biological source in parasitological studies because voucher specimens and tail-tip tissue have been traditionally collected in reptile studies, and can provide information about past infections and the evolution of specific pathogens [81]. For each DNA extraction protocol, *Hepatozoon* infection intensity from tissue was proportionally lower than blood, probably because a piece of tail-tip muscle contains less DNA, than a blood drop stored in Whatman filter paper. It is possible that increasing the DNA concentration of tissue extractions used in qPCR would yield similar results to blood, but this was not tested in this study. The same applies to tissue extracted with kit and tissue extracted with the saline protocol, the latter providing lower overall estimates of *Hepatozoon* infection. Thus, we recommend the use of higher DNA concentrations when using tissue samples. Our results highlight the need of comparing multiple methods in a case-base manner as a preliminary step, in order to minimize imperfect detection that can ultimately lead to erroneous ecological and epidemiological inferences [14,16,82].

In this study we found mixed infection of *Hepatozoon* (Adeleorina) with two genera of Eimeriorinid parasites (*Schellackia* and *Lankesterella*). These parasites were detected by the three detection methods (microscopy, PCR and qPCR), but with different sensitivities. Conventional PCR using the Hep primers only allow the amplification of either hemogregarines or other apicomplexans, and preferentially amplify non-hemogregarine parasites when present in mixed infections [57]. So this method requires at least one additional PCR run using hemogregarine-specific primers (e.g. HEMO [83]). If mixed infections are suspected, we recommend complementing the qPCR approach with conventional PCR that allows newly discovered parasite lineages to be set in a phylogenetic framework. On the other hand, microscopy and qPCR allow the simultaneous detection and differentiation of mixed infections, but failed to detect a few of the *Schellackia* positives detected by PCR. The false Eimeriorina negative of the qPCR approach could be due to handling errors (e.g. pipetting) or, most likely, because of the preferential binding of the qPCR primers towards *Hepatozoon*. Despite the qPCR melting curve analysis allowed us to differentiate between parasite

genera, specificity was not high enough to discriminate at lower levels, i.e. between closely related *Hepatozoon* haplotypes. This result is not unexpected, as the aim of this study was to simultaneously detect and quantify various hemogregarine parasites in reptile samples with high accuracy and sensitivity, so the primer set used herein targets an invariable region of the 18S rRNA gene (see Figure S1). Thus, future studies aimed at discriminating between and within closely related hemogregarine species may need to refine the proposed qPCR approach by targeting a more variable region or a faster-evolving gene [84]. But in order to do so, a more comprehensive characterization on hemogregarine diversity and taxonomy in multiple hosts and across populations is needed. Eimeriorina infections estimates agree with previous studies that also report low prevalence of these parasites in *P. hispanica* [56,57]. This finding is relevant since these coccidian parasites are poorly studied and taxonomy is controversial in some taxa [85,86]. The sequences obtained here resemble *Schellackia* and *Lankesterella* parasites (Apicomplexa: Lankesterellidae). The fact that they are present in both *Podarcis* species at low prevalence and low intensities may be an indication of the opportunistic nature of the interaction, and/or the fact that this parasite may have detrimental effects on hosts. Co-infection implications in wildlife hosts remain poorly investigated but interaction among parasite heterospecifics in mixed infections is often asymmetrical [3] and the effects of their coexistence may be more detrimental than the additive effects of single infections, resulting in stronger selective pressures on hosts [8,9]. More sensitive and accurate approaches such as the qPCR assay developed in this study, which simultaneously detects hemogregarine and eimeriorinid parasites, can allow the investigation of the occurrence of mixed infections in natural populations in a systematic and more standardized way.

*Between-host differences in infection patterns*

Based on qPCR estimates (from blood-kit DNA extraction) we investigated *Hepatozoon* infection parameters in two *Podarcis* sympatric species. Our results show high levels of prevalence with no significant differences between host species and sexes within each species. Prevalence can be determined by the rate at which parasites encounter suitable hosts [9], environmental factors [87] and by the distribution of suitable vectors [88]. Thus, the observed pattern may arise because these hosts' species share the same habitat and are exposed to the same parasites and vectors, both being suitable hosts for hemogregarine parasites. This finding differs however with results from a previous study showing differences in prevalence in these two host species from different locations in the Iberian Peninsula [50]. These contrasting patterns may be the result of the differences between method detection accuracy, as shown in this study, or due to the influence of temporal, ecological, evolutionary and behavioural factors in hemoparasite infection [6,44]. Interestingly, we found three haplotypes and individuals with double peak positions, consistent with what has been previously reported by other studies [50]. The importance of this variation should be further studied as the co-occurrence of various parasite species and lineages within a host may contribute increasing parasite

and/or host diversity and speciation [89] and parasite clonal diversity can maintain high parasite diversity in host populations [90]. Clearly, integrative studies that involve all these variables are needed in wild reptiles.

Regarding *Hepatozoon* intensity of infection, we found that it was positively associated with body size within each host species, which seems to be a common pattern among short-lived reptile species (e.g. *Iberolacerta monticola* [43], *P. lilfordi* [51] and *Lacerta viridis* [91]). Body size has been used as an estimator of age in reptiles [92,93], with older individuals of the same species usually being larger in size. It is assumed that intensity of infection may increase with longevity due to more encounters with parasites, more time to develop infections and less immunocompetence in older animals [37,42,94]. However, this pattern is not linear among hemogregarine infections, especially when considering long-lived reptile species. Studies conducted on snakes [35,36,95] and tuataras [96] have found an inverse association to that reported here, with larger individuals having lower intensities. These contrasting infestation patterns may be related to differences in the pathogenesis of hemogregarine infections between short- and long-lived reptiles. In short-lived species, such as lizards, hemogregarine infections are often regarded as non-detrimental to host condition [97], while in long-lived species hemogregarine infection appears linked to detrimental effects on life-history traits, including growth rate, juvenile survival and female reproductive output [35]. These contrasting effects may be due to the continued parasite acquisition through time in larger, and older, individuals, in the case of non-detrimental infections [26], while in detrimental infections only those individuals that manage to reduce or clear infection can have longer lifespans and reach larger sizes [9,35]. Given the strong relationship of host longevity and body size on parasite infection in reptiles in nature, it would be interesting to investigate hemogregarine infection patterns in species with intermediate size and longevity to determine if such an intermediate parasite infection pattern is found.

When comparing intensity levels between the two species, the smaller sized species (*P. hispanica*) harboured significantly higher intensity levels of *Hepatozoon* parasites. The association between body size and intensity of infection differed for the two species, being more pronounced in *P. bocagei*, for which a lower proportion of smaller animals and a higher proportion of bigger animals, are infected. Also, males had higher intensities than females. Several factors may explain these patterns including: host immunocompetence and susceptibility to infection [41,98,99], parasite specialization to both the intermediate [44] and definitive hosts [100–102], host microhabitat preference [88], abundance of suitable vectors [101,103,104], and behaviour heterogeneity of host species and sexes [9,40,105]. In our system, the smaller-sized species, *P. hispanica*, and males within each species, can be more susceptible to infection and/or can differ immunologically/hormonally, and thus harbour higher hemogregarine intensity levels. Differences in leukocyte counts have been reported between *Podarcis* species [106], which can result in differential immune responses. Associations between individual- and species- specific immune responses and parasitemia levels for various blood parasite groups have been widely reported in birds [107–110],

and, to a lesser extent, in reptiles [95,111]. Likewise, a male-biased pattern is commonly observed in hemoparasite infections, which is often attributed to hormonal and immunocompetence differences between sexes [112–114]. Future studies should now look for an association between immunological variables and hemogregarine infection in sympatric species.

Hemoparasite infection parameters can vary temporally and spatially, which is especially important in complex parasite systems that have heteroxenous lifecycles and are vector-borne, such as *Hepatozoon*. For instance, a cross-sectional study of two malaria parasite species has shown parasite-specific variation in the spatial patterns of disease risk in two closely related sympatric bird species, with consistency between years [115]. Seasonal variations (or environmental variations due to climate change [116]) can not only affect vector abundance [117] and influence rates of parasite development in these vectors [118], but also influence vertebrate host behaviour [119] and host immune condition [120,121]. So studies on various populations and/or across different seasons and years should be conducted in these sympatric species, provided that this system presents a good host-parasite interaction assessment for comparing differences between species and sexes. Finally, a fundamental question on the biology of these parasites that remains, is to identify and compare host-specificity and competence of the definitive invertebrate host(s) for reptile hemogregarines which is fundamental to better understand the evolutionary ecology and transmission dynamics of these parasites.

**Conclusion**

Here, we present a qPCR assay to detect and quantify hemogregarine hemoparasites in reptiles, which can also differentiate mixed infections by different apicomplexan genera. We then tested the performance of the qPCR approach in natural settings and compared infection patterns in a pair of lizard species. Our study illustrates that closely related sympatric hosts, for which factors like behaviour, morphology and habitat preferences are well-known, represent a good model for studying among-host variation in infection. Future studies are needed to assess the influence of other intrinsic factors, such as physiological and immunological traits, as well as to investigate the evolutionary and ecological significance of mixed infections. Overall, this study illustrates the importance of using accurate detection and quantification methods for estimating infection parameters, and the need of considering methodological aspects when comparing parasitological data within and between studies. We also advocate that, due to differences in performance between detection methodologies and extraction protocols, preliminary studies should be carried out prior to choosing the most appropriate approach in a case-study basis to avoid erroneous inferences.

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# 3 UNCOVERING THE UNKNOWN: HEMOGREGARINE DIVERSITY AND HOST-ASSOCIATIONS IN REPTILES

**Article III - Maia, J. P. M. C.**, Perera, A. and Harris, D. J. (2012). Molecular survey and microscopic examination of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) in lacertid lizards from the western Mediterranean. *Folia Parasitologica*, 59, 241–248.

**Article IV - Maia, J. P.**, Crottini, A. and Harris, D. J. (2014). Microscopic and molecular characterization of *Hepatozoon domerguei* and *Foleyella furcata* in wild endemic reptiles from Madagascar. *Parasite*, 21, 47.

**Article V - Maia, J. P.**, Harris, D. J. and Carranza, S. In preparation. Description of a new hemogregarine species *Hepatozoon omanensis* n. sp. (Apicomplexa, Haemogregarinidae) found in reptiles from Oman.

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## 3.1 Article III - Molecular survey and microscopic examination of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) in lacertid lizards from the western Mediterranean

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**Abstract**

The genus *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) is composed of intracellular haemogregarine parasites that are widely distributed among all tetrapod groups. The present study combines microscopic and molecular data on haemogregarine parasites from lizards in the western Mediterranean. We screened tissue samples and examined blood smears for the presence of species of *Hepatozoon* from four lizards, namely *Algyroides marchi* Valverde, endemic to Southeast Spain, *Podarcis bocagei* Seoane from Spain and Portugal, *P. hispanica* Steindachner from Spain, and *P. lilfordi* Günther from Cabrera, Balearic Islands (Spain). Our results show that prevalence and intensity of *Hepatozoon* parasites vary between and within lizard species from different regions. *Algyroides marchi* and *P. bocagei* from Spain had the lowest values, whereas *P. hispanica* had the highest. Phylogeny based on 18S rRNA gene sequences indicates that most of the new *Hepatozoon* sequences are part of a clade exclusive from North African and Iberian lizards, except for a single *P. bocagei* isolate that is found related to another clade including isolates from other reptile host species and rodents. Interestingly, isolates from *Algyroides* form a distinct monophyletic subgroup, which could be a signal of strict host-specificity within this host genus.

**Keywords:** Apicomplexa; haemogregarine; 18S rRNA; phylogeny; tissue; blood smears.

**Introduction**

The study of parasites is important not only in terms of understanding biodiversity as a whole but also for seeking answers to more complex questions related to host-specificity and coevolution (Poulin and Mouillot 2005, Paterson and Piertney 2011). However, there is a bias within the study of parasites, with most research focusing on parasites that are considered of great veterinary, medical and public health importance. Hence, most information available is dedicated to parasites affecting domestic animals rather than wild species, with parasites infecting groups such as reptiles being still poorly studied. Haemogregarines represent an important parasite group with veterinary importance for some groups of their hosts, such as dogs (Baneth et al. 2003), and they are one of the most common parasites found in reptiles. Haemogregarines are a group of apicomplexan (Apicomplexa, Adeleorina) intracellular parasites and four genera within this group are known to infect reptiles: *Hepatozoon* Miller, 1908, *Haemogregarina* Danilewsky, 1885, *Karyolysus* Labbé, 1894 and *Hemolivia* Petit, Landau, Baccam et Lainson, 1990 (Smith 1996, Smith and Desser 1997, Telford 2009). The genus *Hepatozoon* is the most widely distributed among reptiles and also has been reported in all other tetrapod groups (Telford 2009). Lizards of several genera, e.g., *Iberolacerta* Arribas, *Podarcis* Wagler, *Psammodromus* Fitzinger and *Timon* Tschudi, from the Iberian Peninsula have been shown to have high levels of haemogregarine infection using traditional blood smear inspection methods (Álvarez-Calvo 1975, Amo et al. 2004, 2005a, b, Jovani et al. 2004, Martín et al. 2007, Roca and Galdón 2010, Sacchi et al. 2011). This makes lizards from this region potentially good models for studying host-parasite interactions. In contrast, to our knowledge there are no studies on the prevalence and intensity of *Hepatozoon* in species of *Algyroides* Bibron et Bory de Saint-Vicent from the Iberian Peninsula. Molecular data of *Hepatozoon* parasites in public databases, such as GenBank, are also mainly biased towards socio-economically important species, especially domestic and wild mammals. This is also the case of the Iberian Peninsula, for which multiple *Hepatozoon* parasites have been found in mammals and their definitive hosts, i.e. arthropods (Garcia et al. 1990, Criado-Fornelio et al. 2003, 2006, 2007, 2009, Ortuno et al. 2008, Tabar et al. 2008, Yabsley et al. 2008, Lledó et al. 2010). Although *Hepatozoon* sequences from reptiles are scarce in GenBank, there are a few from snakes (Ujvari et al. 2004, Harris et al. 2011, Moço et al. 2012, Tomé et al. 2012) and lizards, with records from the Seychelles (Harris et al. 2011), North Africa (Maia et al. 2011) and the Iberian Peninsula and Balearic Islands (Harris et al. 2012). These studies have shown that 18S rRNA gene primers successfully amplify *Hepatozoon* from tail tips collected from lizards, highlighting the utility of this method for screening and providing new insights into parasite phylogeny. *Hepatozoon* currently includes more than 300 species (Smith 1996) with variable morphological characteristics, diverse life histories (Smith and Desser 1997) and a wide range of host species. Nevertheless, many of these species have been described solely on morphological characteristics of the parasites found in different host species, which may be problematic if host-specificity is low (Mathew et al. 2000). Based on diverse morphological and

developmental characteristics, Smith and Desser (1997) suggested that the genus should be partitioned into two genera of adeleorin parasites, which was supported by a recent study on the phylogeny of the adeleorinid coccidia, which has indicated paraphyly of *Hepatozoon* (Barta et al. 2012). In addition, Barta et al. (2012) showed that these parasites seem to have a relatively high level of host-parasite specificity with their definitive hosts (invertebrates) rather than intermediate hosts (vertebrates), in particular with the separation of parasites from leeches as a distinct clade from those from arthropods. Although four main lineages based on 18S rRNA gene sequences have been described in lizards so far (Maia et al. 2011), the phylogenetic relationships within *Hepatozoon* seem to be complex and remain largely unresolved. The aim of this study is to increase the knowledge of haemogregarines in lizards from the Iberian Peninsula and Cabrera Island by combining both molecular and morphological methods, and to relate this information with the current literature on *Hepatozoon*. When possible, parasite load was quantified from positive samples.

## Materials and Methods

### *Sample collection*

Blood smears and tissue samples were collected from lizards from different localities from the Iberian Peninsula: Gião and Viana do Castelo (Portugal), Alba de Tormes, Tanes, Palacios de Compludo, Albacete, Jaén, Rambla los Vaquerizos and Pedro Andrés (all from Spain) and Cabrera (Balearic Islands, Spain). Species were identified by experienced herpetologists in the field, body size (snout-vent length, SVL, to the nearest millimetre) and sex (based on colour pattern and the existence of developed femoral pores) of the host were also registered. After sample collection, animals were released at the capture site. Tissue samples were preserved for molecular analysis (tail tips containing blood stored in 96% ethanol) and used to screen the presence of *Hepatozoon* parasites. When enough blood was available from the autotomized tail, blood smears were made, then air-dried, fixed with methanol and stained with Giemsa (Telford 2009). A total of 104 tissue samples from two species were collected (Table 3-1). Additionally, 19 *Podarcis hispanica* Steindachner type 1b samples from Spain (June 2009) and ten *P. lilfordi* Günther samples from Cabrera Island (September 2010) included in Harris et al. (2012) were also used in this study for microscopic examination and further molecular and phylogenetic analyses.

### *DNA extraction, amplification and sequencing*

DNA was extracted from tissue using standard High Salt methods (Sambrook et al. 1989). Detection of parasites was made using PCR reactions with the primers HEMO1 and HEMO2 (Perkins and Keller 2001), targeting part of the 18S rRNA gene region, and using the primers HepF300 and HepR900 (Ujvari et al. 2004), targeting another partially overlapping part of the 18S rRNA gene region. Conditions of the PCR are detailed in Harris et al. (2011). Briefly, PCR cycling for the HEMO primers consisted of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min (35 cycles), whereas

for the Hep primers annealing temperature was 60 °C. A total of 133 samples were analysed using PCR, with negative and positive controls run with each reaction. The positive PCR products obtained were purified and sequenced by a commercial sequencing facility (Macrogen Inc., Seoul, Korea). All fragments were sequenced in both directions. Sequences were deposited in GenBank under the accession numbers JX531906 to JX531973. Samples from *P. hispanica* from Spain and *P. lilfordi* from Cabrera Island were used in a previous study using a shorter fragment of the *Hepatozoon* 18S rRNA gene (Harris et al. 2012). In this study we amplified and sequenced the missing region using the Hep primers to be able to compare the full 1401 bp length (see Table 3-1).

*Phylogenetic analysis*

Consensus sequences for each individual were created by combining the sequences of the two partially overlapping 18S rRNA gene regions and analysed using Geneious 5.6.2 (Drummond et al. 2012). Some sequences had double peak positions, which could indicate the existence of different *Hepatozoon* isolates in the same individual, or variation within the multiple copies of the 18S rRNA gene within the same parasite. These were given the corresponding IUPAC code letter. We conducted two separate analyses, one containing a longer 18S rRNA gene segment (1401 bp, combining sequences obtained with Hep and HEMO primers) (Harris et al. 2011, Maia et al. 2011) and the other containing a shorter 18S rRNA gene segment (562 bp, obtained with Hep primers) in order to compare their phylogenetic resolution and because many previously published sequences were available only for the shorter fragment. Since not all samples worked for both fragments, a total of 49 new parasite sequences were obtained for both fragments, whereas 68 parasite sequences were obtained only for the shorter segment. Sequences were blasted in GenBank and all matched known *Hepatozoon* sequences. *Hepatozoon* sequences available were downloaded from GenBank and those representing different *Hepatozoon* haplotypes for the major clades were included in the phylogenetic analysis. In addition, the newly available sequences from other adeleorinid parasites were also included (HQ224961 *Babesiosoma stableri* Schmittner et McGhee, 1961, HQ224957 and HQ224958 *Dactylosoma ranarum* Labbé, 1894, and HQ224959 *Haemogregarina balli* Paterson et Desser, 1976). Although there is a sequence from *Hemolivia mariae* Smallridge et Paperna, 1997 available in GenBank (JN211118), it was not included in the analysis because it only partially overlapped with the short fragment used here. The final alignments contained 113 *Hepatozoon* sequences that were 1401 bp long, whereas the second analysis contained 151 *Hepatozoon* sequences that were 562 bp long. Sequences were aligned with the ClustalW algorithm using default parameters implemented in Geneious 5.6.2 and checked by eye. Two different phylogenetic analyses (Maximum Likelihood and Bayesian Inference) were conducted. Maximum Likelihood (ML) analysis with random sequence addition (100 replicate heuristic searches) was used to assess evolutionary relationships, using the software PhyML 3.0 (Guindon et al. 2010). Support for nodes was estimated using the bootstrap technique (Felsenstein 1985) with 500 replicates. The AIC

criterion conducted in Modeltest 3.06 (Posada and Crandall 1998) was used to choose the model of evolution and the parameters employed (TVM+G for the shorter fragment, TVM+I+G for the longer fragment). Bayesian analysis was implemented using Mr. Bayes v.3.1 (Huelsenbeck and Ronquist 2001) with parameters estimated as part of the analysis. The analysis was run for 10 × 106 generations, saving one tree each 1000 generations. The log-likelihood values of the sample point were plotted against the generation time and all the trees prior to reaching stationary were discarded, ensuring that burn-in samples were not retained. Remaining trees were combined in a 50% majority consensus tree, in which frequency of any particular clade represents the posterior probability (Huelsenbeck and Ronquist 2001). *Adelina* Hesse, 1911 was used as an outgroup for rooting the phylogenetic tree (Morrison 2009). Pairwise uncorrelated differences (p-distance) were estimated using MEGA 5.05 (Tamura et al. 2011). In order to facilitate the analysis of the phylogenetic relationships within lineage 2 isolates, a network was made using only the longer sequences from this lineage. The network was produced using a Median-Joining analysis with default parameters in the software Network 4.6.1.0 (Bandelt et al. 1999).

*Microscopic examination*

Blood smears were examined using an Olympus CX41 microscope with an in-built digital camera (SC30). Several photomicrographs per slide were taken at 400x magnification and stitched using cell^B software (basic image-acquisition and archiving software, Olympus, Germany). Intensity of infection, i.e. the percentage of infected cells within a host, was estimated as percentage based on counts of haemogregarines per 2000 cells for a total of 44 blood smears (Table 3-1). Counts were done using the manual cell counter plug-in available in the image processing software ImageJ ver. 1.44p (Abramoff et al. 2004). Figure 3-1 shows intracellular gamonts of *Hepatozoon* infecting the four lizard species.

*Statistics*

Comparison in prevalence between sexes per locality was assessed using Fisher's Exact Test, which is more appropriate than a chi-square test in cases of 2 x 2 tables with low frequencies (Zar 2009). Correlation between host body size (snout-vent length) and parasitemia levels was investigated using a Spearman correlation. All analyses were performed using R (R Development Core Team 2009).

**Results**

Of the 133 samples analysed using molecular markers, 77 were positive, which gives an overall prevalence of 58%. Primer performance differed between the two sets used in this study. Overall, the Hep primers performed better at detecting, amplifying and sequencing *Hepatozoon* than the HEMO primers, except in double infection cases (Table 3-1). Prevalence of *Hepatozoon* sp. was highest in *Podarcis hispanica* (100%), followed by *P. lilfordi* (70%), *P. bocagei* Seoane from Portugal

Table 3-1 Summary of the samples analysed in this study.
Prevalence is based on positive PCR results.

| Host | Country | Sample size (males/females) | Molecular screening | | | | Microscopic examination | |
|---|---|---|---|---|---|---|---|---|
| | | | N positives/ total (males/females) | Prevalence (%) | HEMO | Hep | Positives analysed (males/females) | Mean intensity % (min–max) |
| *Algyroides marchi* | Spain | 66 | 29 | 44 | 19 | 29 | 7 (3/4) | 0.57 (0.17–2.05) |
| *Podarcis bocagei* | Portugal | 26 (14/12) | 17 (10/7) | 65 | 13 | 17 | 6 (3/3) | 2.95 (0.10–6.88) |
| *Podarcis bocagei* | Spain | 12 (7/5) | 5 (2/3) | 42 | 4 | 5 | 5 (2/3) | 0.49 (0.25–1.14) |
| *Podarcis hispanica* type1b[3] | Spain | 19 (10/9) | 19 (10/9) | 100 | 17 | 19 | 19 (10/9) | 2.52 (0.30–11.55) |
| *Podarcis lilfordi*[3] | Cabrera | 10 (3/7) | 7 (3/4) | 70 | 7 | 2[4] | 7 (2/5) | 0.97 (0.42–2.77) |
| | | 133 | 77 | 58 | 60 | 72 | 44 (20/24) | 1.79 (0.10–11.55) |

[3] Samples from Harris *et al.* (2012).
[4] As described by Harris *et al.* (2012); HEP primers preferentially amplified *Sarcocystis* in double infections.

(65%) and *Algyroides marchi* Valverde (44%) with *P. bocagei* from Spain having the lowest prevalence (42%) (Table 3-1). There was no significant variation of prevalence between sexes (Fisher's Exact Test, in all cases $p > 0.05$). Intensity of infection, i.e. the percentage of infected cells, varied across and within host species and geographical locations. *Algyroides marchi* and *P. bocagei* from Spain showed the lowest haemogregarine intensities, whereas the highest intensities were found in *P. bocagei* from Portugal and *P. hispanica* type 1b. There was no relation between snout-vent length and intensity of infection in any of the populations analysed (Spearman Correlation, in all cases $p > 0.5$).

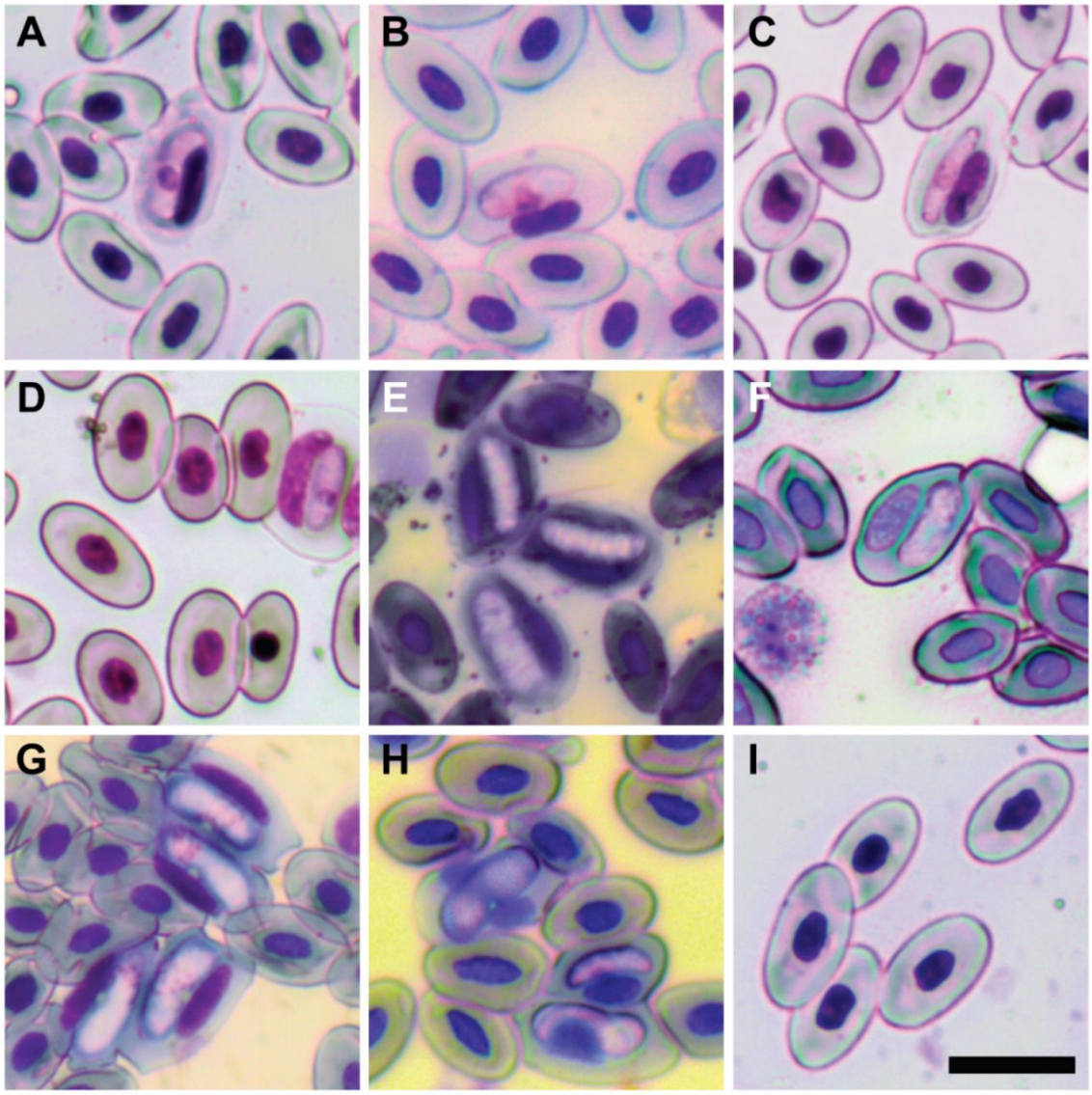


Figure 3-1 *Hepatozoon* parasites infecting erythrocytes from lacertid lizards. *Algyroides marchi* from Spain (DB9365AmSP and DB9379AmSP) (**A**, **B**), *Podarcis lilfordi* from Cabrera Island (DB10319PlCA and DB10553PlCA) (**C**, **D**), *P. bocagei* from Portugal (3251PbPO and 3284PbPO) (**E**, **F**), and *P. hispanica* from Spain (5204PhSP, 5212PhSP) (**G**, **H**). Normal, uninfected, lizard erythrocytes (from DB9171AmSP) (**I**). Scale bar = 10 µm.

The two phylogenetic methodologies used to estimate phylogeny produced similar tree topologies and thus only one is presented (Figure 3-2). Two main lineages were found, one composed of a single *Hepatozoon* isolate from the lacertid *P. bocagei* from Portugal (lineage 1) and other lineage of the remaining Iberian and Cabrera *Hepatozoon* isolates (lineage 2) (see Figure 3-2). Lineage 1 is closely related to one of the lineages found in North Africa that also infects a lacertid host (*Timon tangitanus* Boulenger). In contrast, all other *Hepatozoon* isolates (from *A. marchi*, *P. hispanica*, *P. bocagei* and *P. lilfordi*) are closely related to a generally unresolved *Hepatozoon* lineage found in skinks (*Eumeces algeriensis* Peters and *Chalcides polylepis* Boulenger) and several lacertids from North Africa (including another *Podarcis* species, *P. vaucheri* Boulenger), and lacertids from the Iberian Peninsula and Balearic Islands (Figure 3-2). Sequence divergence between the isolate found in the lineages 1 and 2 is 1.3–1.6% (for the longer segment) and 3.1–3.3% (for the shorter segment). Within the lineage 2, all *A. marchi* isolates form a monophyletic subgroup, whereas relationships between isolates from *P. bocagei* and *P. hispanica* are not well resolved. The network of the unresolved lineage 2 shows that *Hepatozoon* parasites from *P. bocagei* and *P. hispanica* can be found in two groups, whereas those from *A. marchi* form a unique subgroup (Figure 3-3).

**Discussion**

Our results show that both parasite prevalence and intensity vary between and within host species and their geographical locations. Overall, prevalence was high in our study, which is in accordance with the morphological studies on lizards from the same region. A microscopic survey of *Podarcis carbonelli* Pérez Mellado and *P. bocagei* from northwest Portugal showed similar prevalence of haemogregarines, 69.7% and 74.7%, respectively, although intensities were not reported (Roca and Galdón 2010). Another microscopic survey on *Podarcis muralis* Laurenti from Spain found 58.1% prevalence and intensity levels of less than 0.5% (Amo et al. 2005a). In this study we found varying prevalence values for parasites infecting the genus *Podarcis* Wagler, with all individuals infected from one population of *P. hispanica*, whereas for *P. bocagei* from Spain less than half of the individuals analysed were infected. In fact, within *P. bocagei* from Portugal we found high prevalence (65%, n = 26) and high mean intensity levels (2.95%, n = 6), whereas in Spain prevalence and intensity values were much lower (42%, n = 12, and 0.49%, n = 5, respectively).

Nevertheless, since only 12 individuals were screened for *P. bocagei* in Spain, this difference could be biased by non-representative sampling. Interestingly, mean intensity values for *P. bocagei* are similar to those of *P. muralis* reported by Amo et al. (2005a). Differences in prevalence and intensity of haemoparasites (either between or within host species) could be associated with factors such as microhabitat characteristics (Davis et al. 2012), feeding habits and seasonality. Indeed, seasonality has been shown to be associated with differences in parasite load (Salkeld and Schwarzkopf 2005, Santos et al. 2005). However, since our samples were collected during different seasons and in different years, we cannot draw conclusions regarding this. This could be

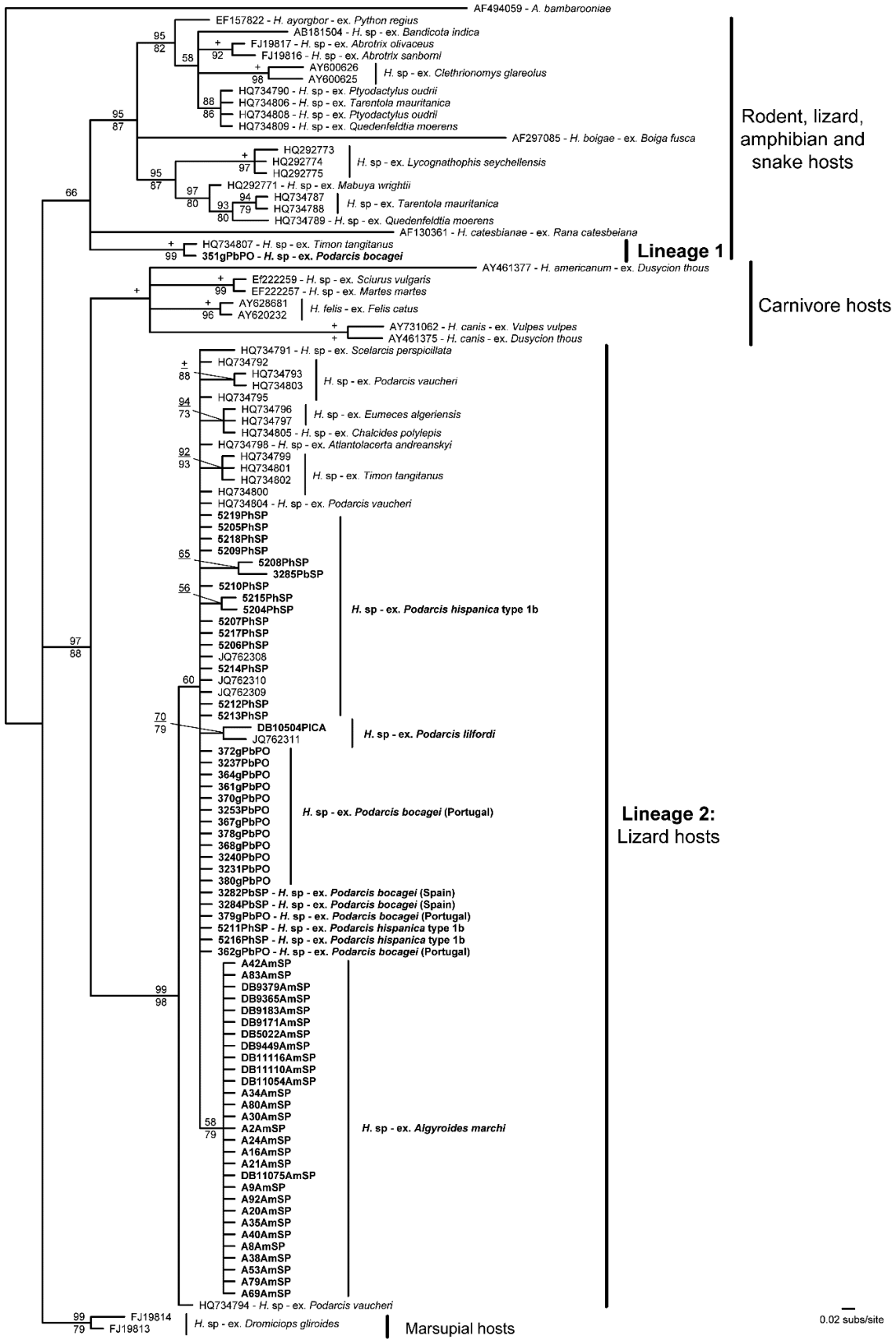


Figure 3-2 Bayesian estimate of relationships of *Hepatozoon* species based on 562 bp 18S rRNA gene sequences. Bayesian posterior probabilities are given above the nodes and ML bootstrap values below them. When both values were 100%, this is indicated with a +. New haplotypes from this study are in bold. The branches of JN181157, AF130361 and AF297085 were shortened by 50%.

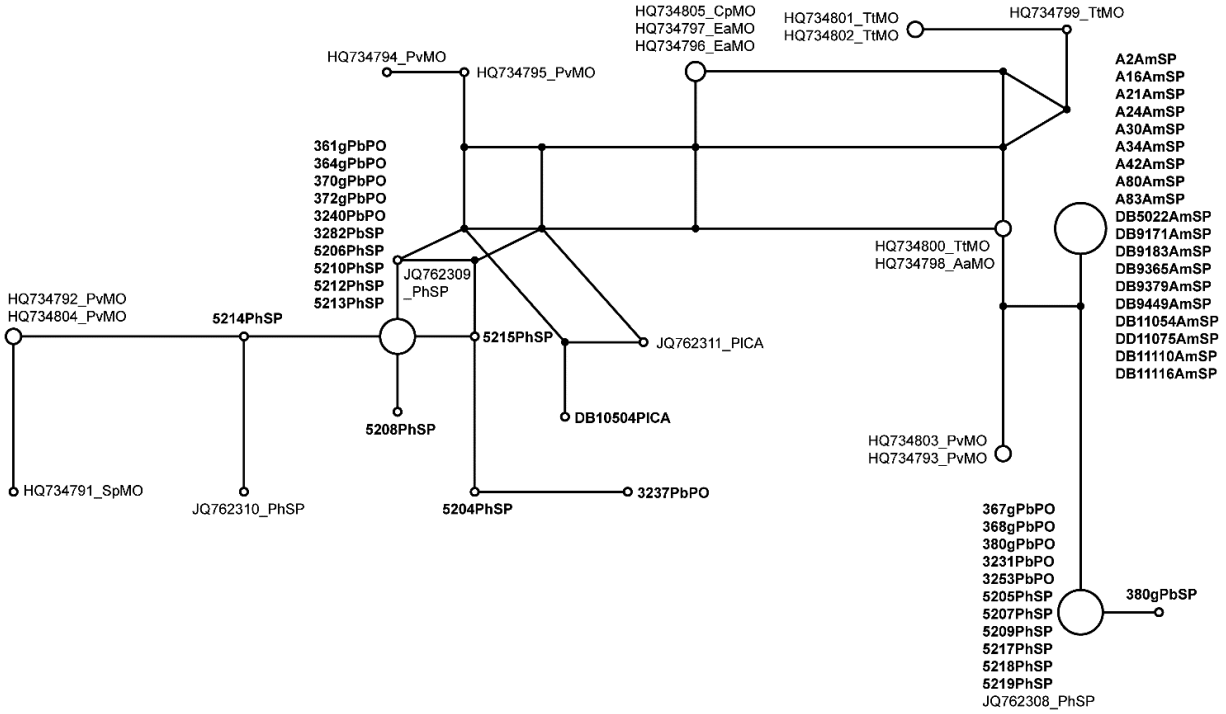


Figure 3-3 Median-Joining Network analysis of lineage 2, using 1401 bp 18S rRNA gene sequences. Last four letters indicate host species initials (first two letters) and country (last two letters). New haplotypes from this study are in bold.

investigated by conducting large-scale sampling of individuals from several species from a single location and from the same species from different locations at different seasons. Also, we still know little about the influence that these parasites may have on host health and fitness, and this could also be investigated using immunological and performance tests (Sacchi et al. 2007). To our knowledge, this is the first assessment of the occurrence of haemogregarines in the species *Algyroides marchi*. There is only a record of haemogregarine infection in *Algyroides nigropunctatus* Duméril et Bibron (from Italy), with a mean intensity of about 0.2% (no prevalence records are provided), in a survey performed with the purpose of comparing counts and morphology of leukocytes in 12 lizards species (Sacchi et al. 2011). In our study, intensity levels in *A. marchi* were very similar to those found in its Italian relative (mean = 0.57, min.–max.: 0.17–2.05). Our study highlights the utility of the short 18S rRNA fragment amplified using the Hep set of primers for largescale molecular surveys of *Hepatozoon* parasites, since the estimate of phylogenetic relationships is similar to those retrieved using the longer fragment. In addition, Hep primers usually perform better at detecting infections and normally yield better quality sequences. Although a larger fragment provides more genetic information and acts as a safety net against contamination, because it requires two separate PCRs, the sole use of Hep primers provides similar estimates of phylogenetic relationships at lower cost. Nonetheless, as realized recently, Hep primers have the potential of amplifying DNA of other apicomplexan parasites and in double infection some primers may preferentially amplify other apicomplexans, such as *Eimeria* Schneider, 1875 and *Sarcocystis* Lankester, 1882 coccidia (Harris et al. 2012). Hence, in these situations samples should also be

tested with the HEMO primers to check for the presence of haemogregarines, since these are more specific primers.

The phylogenetic relationships estimated for *Hepatozoon* parasites remain similar to those previously described (Maia et al. 2011, Harris et al. 2012), despite the addition of new sequences for the 18S rRNA gene in this study. However, the present phylogenetic analysis increases the known geographical distribution of this group and the number of host species infected by these parasites. One lineage from North Africa, previously known only from the lacertid host *Timon tangitanus*, appears to be related with one of the new isolates from *P. bocagei*, whereas all the other new isolates fall in a group with another lineage from North Africa found in lacertids and skinks. The occurrence of two very distinct genetic lineages of *Hepatozoon* isolates from the same host species demonstrates that a single host species can harbour genetically distinct isolates. Since the 18S rRNA is a slow evolving gene, the high sequence divergence found between the lineages 1 and 2 could indicate that these represent different, unrelated species of *Hepatozoon*. Unfortunately, no blood smear was made at the time of collection for the unique sample from lineage 1 from *P. bocagei* and thus it is not possible to couple microscopy with phylogeny for this lineage. Still, this result emphasizes that *Hepatozoon* may have low host-specificity (Telford et al. 2001), at least concerning some intermediate host species such as *P. bocagei*. Thus, the transmission cycle should be fully studied in the future, by collecting from a single location (with known parasitic infections) both the vertebrate host and the potential vectors that are the definitive hosts (arthropods which are often found attached to the intermediate host, such as ticks and mites, but also mosquitoes and reduviids bugs – Telford 2009). This is especially important since relatively high degree of host-parasite association of various species of Apicomplexa with their definitive hosts have been observed recently (Barta et al. 2012). In contrast, the fact that *Hepatozoon* isolates from *Algyroides* form a monophyletic subgroup within the lineage 2 demonstrates some degree of strict intermediate host-specificity. Finally, the phylogenetic relationships between the isolates found in *P. bocagei* and *P. hispanica* remain largely unresolved and given that the hosts are closely related species (together forming a species complex – see Pinho et al. 2008) it is possible that these hosts share the same parasite communities. Nonetheless, further research should be conducted using faster evolving genes, to obtain a better resolution of this lineage.

By including other adeleorinid sequences in our phylogeny, we demonstrate in accordance with Barta et al. (2012) that these are clearly distinct from our *Hepatozoon* sequences, thus discarding the possibility of the lineage 1 being from other adeleorinids, a possibility that could not previously be rejected (Maia et al. 2011). Support levels for the major groups within *Hepatozoon* are also low, further indicating that more data are necessary prior to any taxonomic changes being appropriate. Finally, our phylogeny also presents some incongruences in sequences deposited in GenBank. For example, the sequence identified as the snake *Cerastes cerastes* Linnaeus in GenBank (EF125058) is actually a *Hepatozoon* sp., presumably accidentally amplified instead of the host. This kind of

erroneous amplification of parasites instead of hosts, and subsequent mislabelling in databases, has also been recorded for flatworms (Heneberg 2012), and further highlights the need for care when comparisons are made with sequences from GenBank.

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## 3.2 Article IV - Microscopic and molecular characterization of *Hepatozoon domerguei* and *Foleyella furcata* in wild endemic reptiles from Madagascar.

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**Abstract**

Madagascar is one of the world's top twelve ''megadiversity'' hot spots hosting unique and threatened flora and fauna. Parasites are a major component of biodiversity but remain largely uncharacterized in wildlife. In this study we combine microscopic and molecular assessment of hemoparasites in endemic reptile species from Madagascar. We detected three distinct parasites: the apicomplexans *Hepatozoon* and *Sarcocystis*, and filarial nematodes. The prevalence and intensity of these apicomplexans were low overall, while microfilarial infections in chameleons were relatively high. We detected mixed infections of two *Hepatozoon* haplotypes in *Madagascarophis colubrinus*, and of *Hepatozoon* and microfilariae in a *Furcifer* sp. Phylogenetic analyses of *Hepatozoon* showed evidence of prey-predator transmission, with identical sequences found in the snakes *M. colubrinus* and *Ithycyphus oursi*, and their prey *Furcifer* sp. Based on previous studies regarding the life cycle of *Hepatozoon domerguei* Landau, Chabaud, Michel, and Brygoo, 1970 in these hosts and due to their morphological similarity, we propose that this *Hepatozoon* haplotype is *Hepatozoon domerguei*. Future studies, including the examination of invertebrate hosts, are needed to verify this preliminary taxonomic identification. A distinct hemogregarine haplotype was found in *Oplurus* sp., which displayed morphologically different gametocytes, some of which were apparently inside leukocytes. The *Sarcocystis* identified from *Tracheloptychus petersi* was identical to that reported in a North African snake, indicating that the same lineage is found in geographically distinct regions. By combining morphological and genetic information, *Foleyella furcata* (Linstow, 1899) filarial nematodes were identified in several *Furcifer* chameleons. This study provides insights into the distribution, diversity and host-parasite interactions of hemoparasites in wild reptile populations from Madagascar.

**Keywords:** Hemogregarine; Sarcocystis; Apicomplexa; Nematode; Filaria; Arthropod-borne diseases.

## Introduction

Madagascar is the fourth largest island in the world, and as one of the world's top 12 ''megadiversity'' hot spots [54] hosts an almost unparalleled concentration of endemic, diverse and threatened fauna and flora [54, 88]. The native reptile fauna has a high level of endemism at the species level (about 92%), and is composed of at least 25 extant independent lineages [17, 24]. Currently, more than 400 reptile species are known in Madagascar [56], and this list will increase in the future as a result of intense research activities and widespread application of integrative taxonomic approaches [24, 56], as shown by the numerous recent species descriptions [18, 25, 69]. Parasites have been increasingly recognized as a main component of biodiversity; however, their study clearly lags behind that of their hosts [53]. Documenting the diversity of parasites is important for several reasons, since they (1) coevolve and interact with their hosts [59, 75], (2) play an important role in structuring animal communities [31, 65], and (3) are important in ecosystems and conservation [61, 72]. Hemoparasites typically have complex life cycles, requiring more than one host to complete it. The life cycle of several filarial and coccidian parasites has been described in endemic hosts from Madagascar: *Foleyella furcata* (Linstow, 1899) [44], an onchocercid described from the chameleon *Furcifer verrucosus* (Cuvier, 1829) [7], and *Hepatozoon domerguei* (Landau, Chabaud, Michel and Brygoo, 1970) [37], a hemogregarine described from the lamprophiid snake *Madagascarophis colubrinus* (Schlegel, 1837) [38]. Both are arthropod-borne parasites, and the mosquito *Culex quinquefasciatus* (*Culex pipiens fatigans*) Say, 1823 has been used experimentally as a vector [7, 38]. *Foleyella* species have a limited geographic distribution and have been found only in the lizard families Agamidae and Chameleonidae [10]. Four species compose the genus *Foleyella*, of which *F. furcata* and *Foleyella brevicauda* (Chabaud and Brygoo, 1962) [14] are generally common in Malagasy chameleons [12]. Morphological identification to the species level is possible through analysis of adult forms [10]; however, the advent of molecular tools and the use of fast-evolving genetic markers now allow the placement of parasites in a phylogenetic framework, allowing assignment to the species level more easily [40, 55]. The genus *Hepatozoon* is part of the hemogregarine group and is one of the most common hemoparasites in reptiles [73]. *Hepatozoon* can be transmitted by direct ingestion of infected invertebrate hosts by vertebrate hosts or by prey-predator transmission through infective cysts in prey that can cause infection in receptive hosts [38]. Molecular parasitological studies in mammals and reptiles corroborate the latter mode of transmission by reporting identical parasite lineages in predator and prey hosts [49, 81], thus providing new insights into parasite-host interactions. Hemogregarines from continental African reptiles have shown high genetic diversity comprising various unrelated lineages [47, 82], compared with rather limited genetic diversity from the Seychelles islands [27]. Occurrence of these hemoparasites can be easily detected through microscopy, by observing hemogregarine gamonts inside erythrocytes and leukocytes [79], by observing onchocercid microfilarial stages in blood smears stained with Giemsa [33], and through molecular screening of host samples using parasite-

specific primers [47, 62]. Parasite species can be better identified by combining genetic and morphological data [1, 29, 57]. Although this practice is currently easy to apply, and despite the wide range of parasites that can be found in reptile blood samples [28], studies using this approach to assess parasite prevalence and diversity are still generally lacking. Parasite research in Madagascan amphibian and reptile hosts has mainly focused on a few groups, such as the malarial parasite *Plasmodium* [71], monogenean polystomatids [11, 66], nematodes [35, 42], or it has been focused on well-known conservation threats such as the amphibian chytrid fungus *Batrachochytrium dendrobatidis* Longcore et al., 1999 [16, 19, 86, 87], while other groups remain less studied.

The aims of this study are to: (i) provide preliminary information on the prevalence and intensity of hemogregarines and filarial nematodes in endemic reptile species from Madagascar, (ii) place these parasites in a phylogenetic framework to determine the specificity of the detected parasite lineages by comparing them with known parasite species from different hosts and geographical locations, and (iii) to detect prey-predator transmission of *Hepatozoon* lineages by analyzing predators and their prey from this region.

## Materials and methods

### *Sample collection*

Samples were collected from 73 reptile specimens (Table 3-2) from several localities in the center and south-west of Madagascar, mostly Ranomafana, Ambalavao, Isalo, Ifaty, Toliara, and Lavenombato. For each individual a small tail tip was collected for molecular identification, and when enough blood was naturally available this was used to prepare a blood smear. Tissue was preserved in 96% ethanol. Individuals were released at the site of capture. Blood smears were air-dried, fixed with methanol and stained with diluted Giemsa (1:9 of distilled water) for 55 min.

### *Microscopic examination*

Blood smears were screened initially at 400x magnification to search for extracellular hemoparasites, such as microfilariae, and at 1000x for intracellular parasites, using an Olympus CX41 microscope with an in-built digital camera (SC30) (Olympus, Hamburg, Germany). Prevalence was estimated as the proportion of infected hosts, and intensity of infection was estimated as the number of parasites per 5000 erythrocytes [13, 51] (Table 3-2). To reduce errors in manual counts, intensity counts for blood smears of infected individuals were counted three times and averaged. Mature hemogregarine gamonts and sheathed microfilariae in reptiles from Madagascar were observed (Figure 3-4 and Figure 3-5, respectively). Hemogregarine gametocytes and infected host erythrocytes were measured at 1000x magnification (see Table 3-4) and microfilariae at 400x magnification (see Table 3-5) using cell ^B software (basic image acquisition and archiving software; Olympus, Münster, Germany). For sheathed microfilariae, measurements include the sheathed part of the parasite. Length and width were taken using the horizontal and vertical distance tool for

hemogregarines and polygon length tool for microfilariae, while the area and perimeter were taken using the area/perimeter tool in the Measure menu of the cell ^B software.

Table 3-2 Reptile samples collected in different localities of Madagascar in 2009.
Prevalence and intensity estimates for hemogregarines and filarial nematodes for each reptile species.

| | | | **Hemogregarines** | | ***Foleyella furcata*** | |
|---|---|---|---|---|---|---|
| **Host group** | **Host species** | *n* | Prevalence | Intensity (%) ± SD (Min-Max) | Prevalence | Mean Intensity (%) ± SD (Min-Max) |
| Chameleon | *Calumma crypticum* | 3 | | | | |
| | *Calumma gastrotenia* | 1 | | | | |
| | *Calumma nasutum* | 1 | | | | |
| | *Calumma oshaughnessyi* | 3 | | | | |
| | *Furcifer* sp. | 18 | 1 (6%) | 0.35 | 7 (39%) | 0.26 ± 0.24 (0.02 - 0.81) |
| | *Furcifer antimena* | 1 | | | | |
| | *Furcifer lateralis* | 9 | | | 1 (11%) | 1.34 |
| | *Furcifer oustaleti* | 12 | | | 2 (17%) | 0.31 ± 0.17 (0.14 - 0.47) |
| | *Furcifer verrucosus* | 3 | | | 3 (100%) | 0.10 ± 0.04 (0.05 - 0.14) |
| | | 51 | 1 (2%) | | 13 (25%) | |
| Lizard | *Blaesodactylus sakalava* | 1 | | | | |
| | *Chalarodon madagascariensis* | 1 | | | | |
| | *Lygodactylus pictus* | 1 | | | | |
| | *Oplurus* sp. | 1 | 1 (100%) | 0.07 | | |
| | *Tracheloptychus petersi* | 1 | | | | |
| | | 5 | 1 (20%) | | | |
| Snake | *Compsophis laphystius* | 1 | | | | |
| | *Dromicodryas bernieri* | 2 | | | | |
| | *Ithycyphus oursi* | 1 | 1 (100%) | 0.04 | | |
| | *Leioheterodon modestus* | 1 | | | | |
| | *Madagascarophis colubrinus* | 7 | 2 (29%) | 0.2 ± 0.03 (0.17 - 0.23) | | |
| | *Mimophis mahfalensis* | 1 | | | | |
| | *Thamnosophis lateralis* | 3 | | | | |
| | *Typhlops arenarius* | 1 | | | | |
| | | 17 | 3 (18%) | | | |
| | | 73 | 5 (7%) | | 13 (18%) | |

### *DNA extraction, amplification, and sequencing*

DNA was extracted from tissue using standard high-salt methods [50, 70]. Presence of hemogregarines was determined using PCR reactions with the apicomplexan primers HepF300 and HepR900 [83] targeting the overlapping part of the hemogregarine 18S rRNA region. For positive samples we used the primers HEMO1 and HEMO2 [63] to amplify a partially overlapping fragment of the 18S to obtain a longer portion of this gene. PCR cycling for the Hep primers consisted of

94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min (35 cycles), while for HEMO primers the annealing temperature was 48 °C [27]. Microfilariae were detected in blood smears, and three pairs of primers were used to taxonomically identify these parasites: the 18S rRNA gene [62], COX1 and 12S rRNA gene [40]. Negative and positive controls were run with each reaction. The positive PCR products were purified and sequenced by two commercial sequencing facilities (Macrogen Inc., Seoul, Korea; and CTM, Porto, Portugal). All sequences were performed in both directions. Sequences were deposited in GenBank under the accession numbers KM234619–KM234629 (*Foleyella furcata* COX1 sequences), KM234630–KM234637 (*F. furcata* 12S rRNA gene sequences), KM234638–KM234645 (*F. furcata* 18S rRNA gene sequences), KM234646–KM234650 (hemogregarine 18S rRNA gene sequences), and KM234651 (*Sarcocystis* sp. 18S rRNA gene sequence).

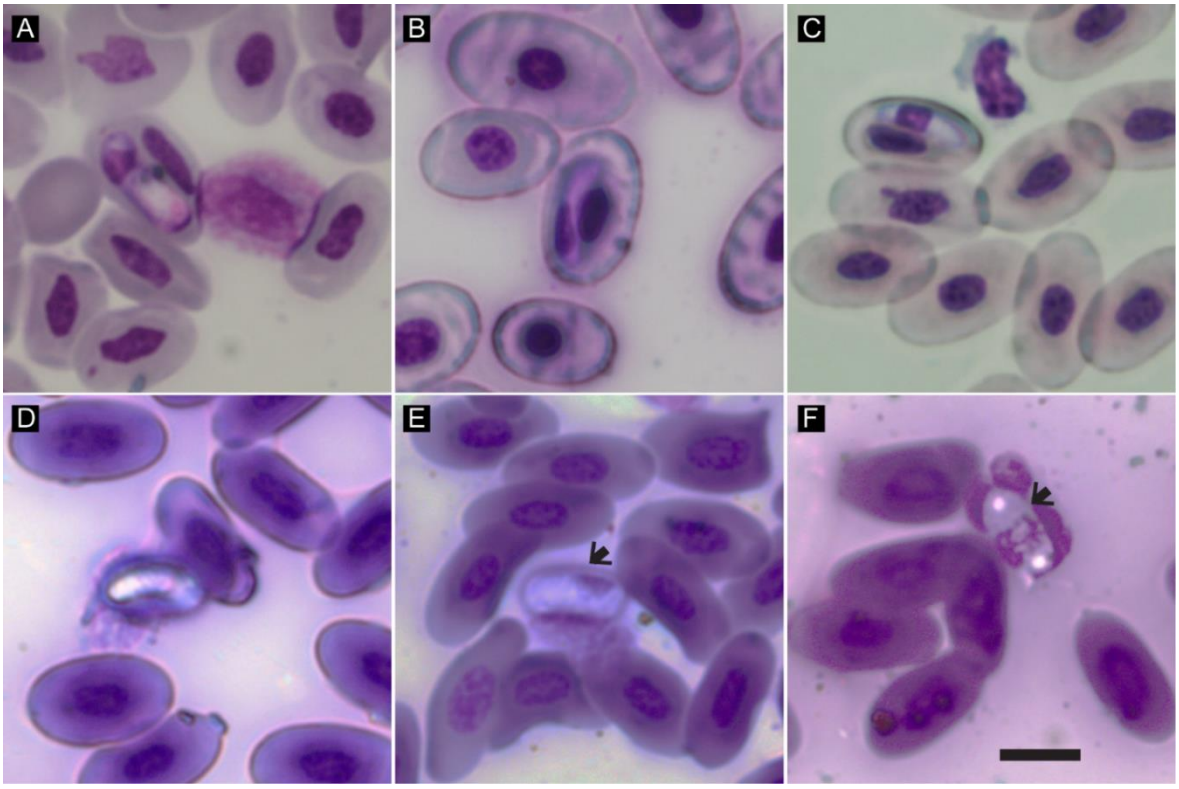


Figure 3-4 Hemogregarine mature gamonts in two snake species and one lizard species endemic to Madagascar. (A) *Hepatozoon* in *Ithycyphus oursi* (ACZC1932); (B) *Hepatozoon* in *Madagascarophis colubrinus* (ACZC1827); (C) *Hepatozoon* in *M. colubrinus* (ACZC1963); (D, E, F) hemogregarine infections in *Oplurus* sp. (x49). (F) Could represent a young stage based on the characteristics of the nucleus. *Hepatozoon* infecting *Furcifer* sp. is presented in Figure 3-5 A. Arrows indicate hemogregarine parasites apparently inside leukocytes. Scale bar = 10 µm.

### *Molecular identification*

The apicomplexan Hep primers amplified 4 of the 5 hemogregarine infections observed under the microscope. These were compared with data in GenBank using BLAST [4]. One sequence (sample ACZC1827 from *Madagascarophis colubrinus*, which could correspond to the gamonts observed under the microscope) displayed 4 heterozygous positions for the 18S rRNA gene. Two haplotypes (KM234646 and KM234647) were derived from this sequence and included in the phylogenetic analysis. The HEMO set of primers only amplified 3 of the 5 hemogregarine infections and no mixed

infections. For this reason, and since this produces similar tree topologies to those estimated using the longer fragment, we conducted phylogenetic analysis using the shorter fragment to include all sequences [48], although the longer fragments were deposited in GenBank when available. The sample infected with *Sarcocystis* sp. (KM234651 (sample ACZC1899) from *Oplurus* sp.) was identical to a published sequence (KC696571), thus no phylogenetic analysis was conducted for this parasite. Three genes were amplified for filarial nematodes, the 18S rRNA gene, the COX1 and 12S rRNA gene. All 18S and 12S sequences were identical for the 8 samples analyzed, while for COX1 four closely similar haplotypes were obtained. The BLAST results for the 12S rRNA gene sequences (418 bp) indicated 99% similarity with the sequence AJ544841 from *F. furcata* and 93% identity with FR827906 from *Foleyella candezei* (Fraipont, 1882) [23] in GenBank. For this reason, we only present the results of the phylogenetic analyses of COX1. Sequences were analyzed using Geneious 6.0.3 [20], each electropherogram was carefully checked and aligned with MUSCLE algorithm implemented in this software. The new sequences were aligned with sequences retrieved from GenBank from various host species and the final datasets contained: 66 sequences of 590 bp in length for the 18S rRNA gene fragment of hemogregarines; and 105 sequences of 590 bp for the COX1 gene of filarial nematodes.

Two different phylogenetic analyses (Maximum Likelihood, ML, and Bayesian Inference, BI) were conducted for each group. ML analysis with random sequence addition (100 replicate heuristic searches) was used to assess evolutionary relationships, using the software PhyML 3.0 [26]. Support for nodes was estimated using the bootstrap technique [22] with 1000 replicates. The AIC criterion conducted in jModeltest 0.1.1 [64] was used to choose the best model of evolution and the parameters employed (TVM+G for hemogregarines and TIM3+I+G for filariae). BI was implemented using Mr. Bayes v.3.1 [32] with parameters estimated as part of the analysis. The analysis was run for $10x10^6$ generations, saving one tree each 1000 generations. The log-likelihood values of the sample points were plotted against the generation time and all the trees prior to reaching stationarity were discarded, ensuring that burn-in samples were not retained. Remaining trees were combined in a 50% majority consensus tree [32]. For hemogregarines, *Dactylosoma ranarum* (Lankester, 1882) [39] (HQ224958) and *Haemogregarina balli* Paterson and Desser, 1976 [60] (HQ224959) were used as outgroups, while for spirurid nematodes, *Ascaris lumbricoides* Linnaeus, 1758 [43] (JN801161), *Contracaecum rudolphii* Hartwich, 1964 [30] (NC014870), and *Heterakis isolonche* Linstow, 1906 [45] (FJ009626) were used as outgroups [40].

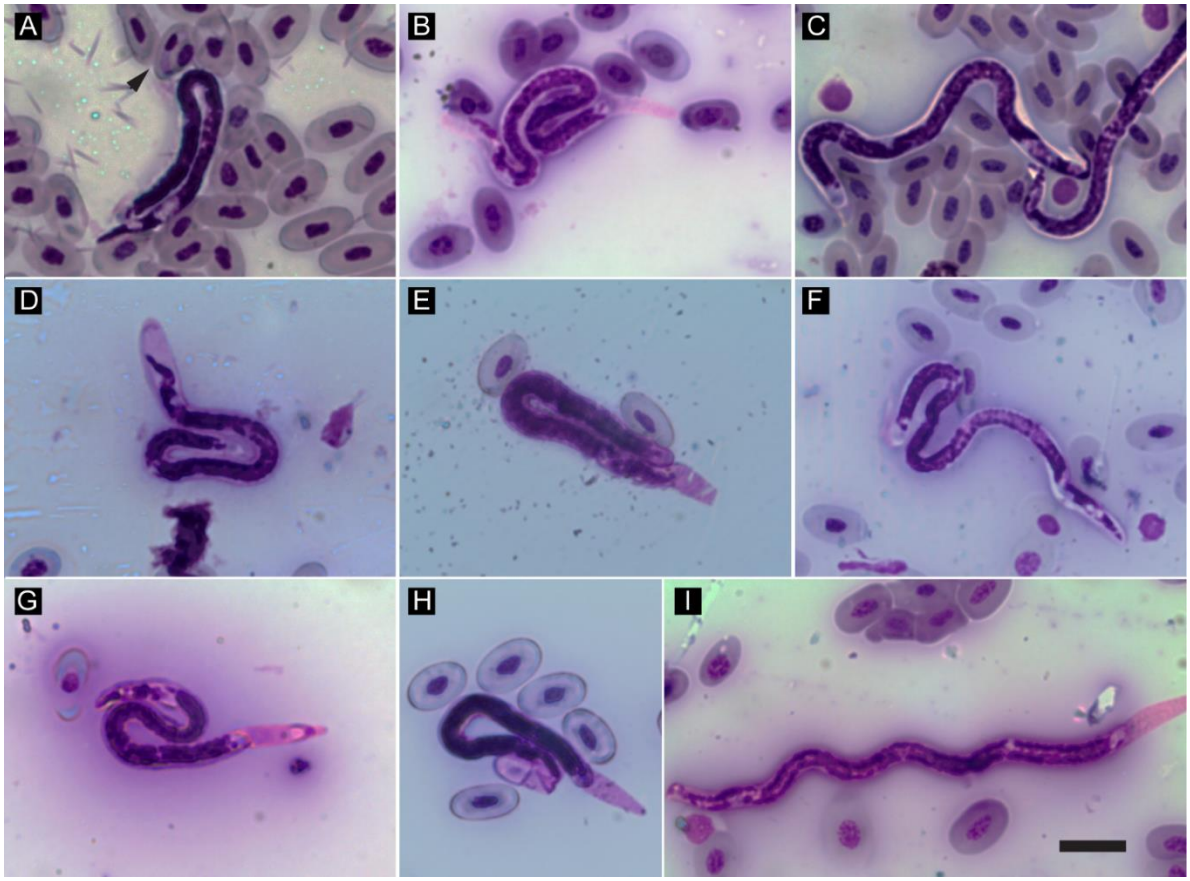


Figure 3-5 *Foleyella furcata* nematode infections in Malagasy chameleons of the genus *Furcifer*.
(A) Mixed infection of *Hepatozoon* (arrowhead) and microfilariae in *Furcifer* sp.;
(B) infection in a *F. lateralis* individual; (C, D) infections in two *F. oustaleti* individuals;
(E, F, G) infections in three *F. verrucosus* individuals;
(H, I) infections in two *Furcifer* sp. individuals.
Scale bar = 20 µm.

## Results

A total of 5 animals from different host species were infected with hemogregarines based on microscopy, resulting in an overall prevalence of only 7% (5/73) (Table 3-2). One chameleon was observed with both hemogregarine and filarial infections (Figure 3-5 A). Intensity levels were low overall, with *Ithycyphus oursi* Domergue, 1986 and Oplurus sp. having the lowest estimates, while the genus *Furcifer* had the highest (Table 3-2). Of the 5 hemogregarines identified by microscopy, 4 were sequenced for the 18S rRNA gene and resulted in 3 haplotypes with some genetic divergence (Table 3-3 and Figure 3-6). One haplotype was found in the predator-prey system composed of the chameleon *Furcifer* sp. (KM234649), and the snakes *I. oursi* (KM234648) and *Madagascarophis colubrinus* (KM234646). The mean measurements of gamonts from these genetically identical haplotypes (Table 3-4) match the descriptions of *Hepatozoon domerguei* from *M. colubrinus* (mean of 14 µm in length and 3 µm in width [38]). The second haplotype was found in the same infected individual of *M. colubrinus* (KM234647) and was more similar to other *Hepatozoon* sp. from continental African snakes (e.g. KJ508511) and lizards (e.g. HQ734806) (Figure 3-6). Finally, the third haplotype was found in the iguanid lizard *Oplurus* sp. and clusters in a group with parasites

identified from Chilean rodents (e.g. FJ719817). In fact, this parasite displayed distinct morphological characteristics (Figure 3-4 D and E, and Table 3-4), with some gamonts apparently inside leukocytes (Figure 3-4 E and F). The *Sarcocystis* sequence detected in the lizard *Tracheloptychus petersi* Grandidier, 1869 was identical (100%, 543 bp) to that reported in the snake *Psammophis schokari* Forsskål, 1775 (KC696571) from Algeria, and less similar to *Sarcocystis* spp. from lizards (AY015112 *Sarcocystis gallotiae* Matuschka and Mehlhorn, 1984 [52] from *Gallotia galloti eisentrauti* Bischoff, 1982 from Canary Islands, AY015113 *Sarcocystis lacertae* (Babudieri, 1932) [5] from *Podarcis muralis* (Laurenti, 1768) from Slovakia, and JQ762307 *Sarcocystis* sp. from *Podarcis lilfordi* (Günther, 1874) from the Balearic Islands).

Table 3-3 Estimates of evolutionary divergence between the three haplotypes obtained in this study. The number of base substitutions per site between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model [76]. There were a total of 565 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [77]. Haplotype 1 (Hap 1) is composed of sequences KM234646, KM234648 and KM234649; haplotype 2 (Hap 2) of sequence KM234647; and haplotype 3 (Hap 3) of sequence KM234650 (Figure 3-6).

| | Haplotype 1 | Haplotype 2 | Haplotype 3 |
|---|---|---|---|
| Haplotype 1 | - | - | - |
| Haplotype 2 | 0.013 | - | - |
| Haplotype 3 | 0.035 | 0.025 | - |

A relatively high number of chameleons from the genus *Furcifer* were infected with sheathed microfilariae (13/45, 37%) (Figure 3-5) with varying infection intensities (Table 3-2). Mean microfilaria measurements per host species (Table 3-5) match the descriptions of *Foleyella furcata* in *Furcifer verrucosus* (range 125–157 µm and 6–7 µm) and are different from *Foleyella candezei* (83–96 µm and 6.5–7 µm [8]) and *Foleyella brevicauda* (225 µm and 7 µm [6]). *Foleyella* species are characterized by having a loose prominent sheath that completely encloses the body [10], as was observed in this study (Figure 3-5). We sequenced 8 samples for three filarial genes (18S rRNA gene, COX1 and 12S rRNA gene). Sequences were identical for the 18S and 12S rRNA genes and the latter was similar to the previously published *F. furcata* (AJ544879; 99% identity). This is the first 18S rRNA gene sequence for *F. furcata*, and the closest matches were *Loa loa* (Guyot, 1778) (DQ094173), *Onchocerca cervicalis* (Railliet and Henry, 1910) [67] (DQ094174), *Breinlia mundayi* (Spratt and Varughese, 1975) [76] (JF934735), *Dipetalonema* sp. (DQ531723), and *Setaria digitata* (Linstow, 1906) [45] (DQ094175) with 99% identity (895 bp). Phylogenetic analysis of the COX1 gene confirms that these sequences belong to the species *F. furcata* (Figure 3-7 and Figure 3-8) and the tree topology resembles that of previous studies [40].

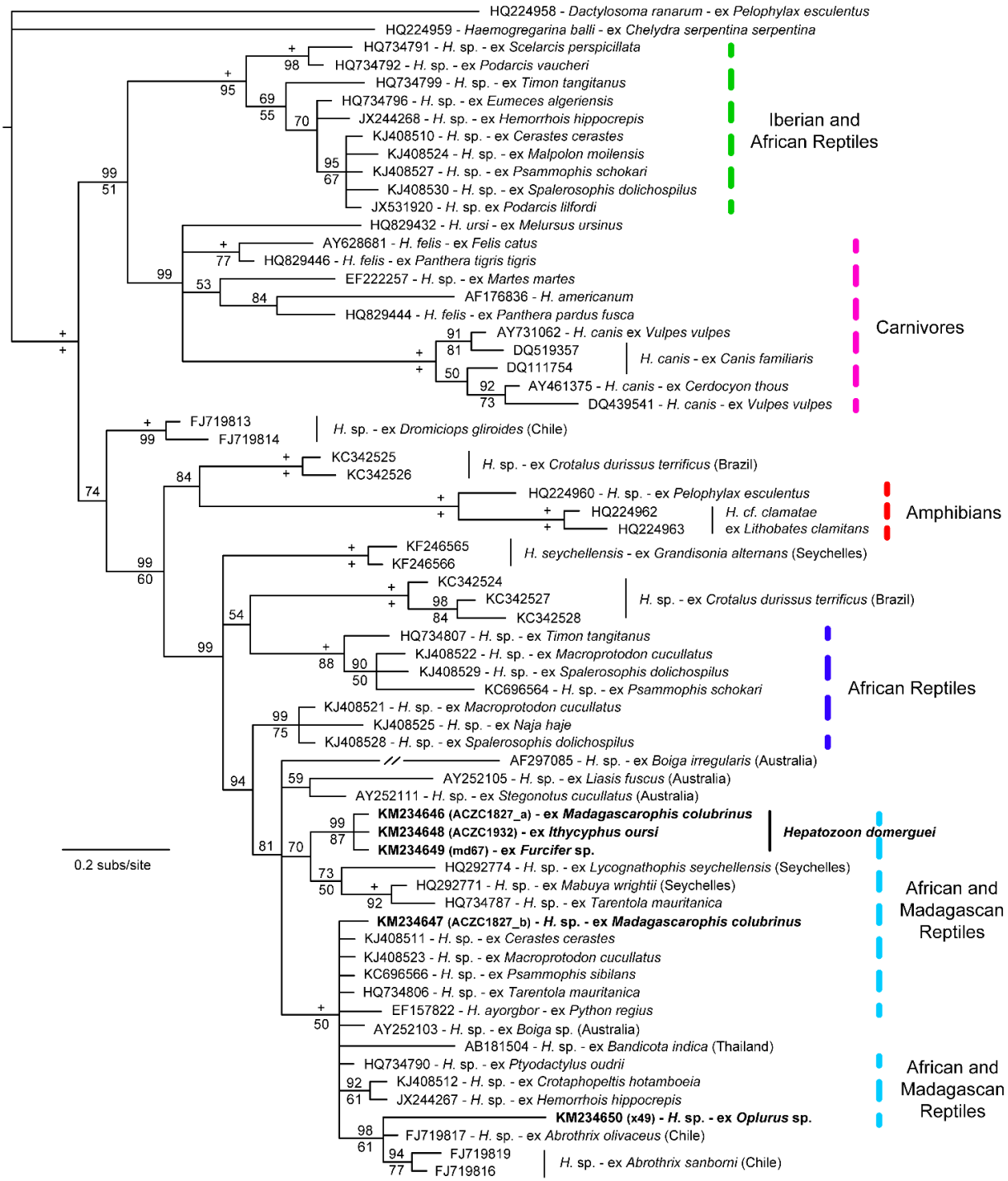


Figure 3-6 Tree derived from a Bayesian Inference analysis of the hemogregarine 18S rRNA gene sequences. Bayesian Posterior Probability values are given above relevant nodes, and Bootstrap values for ML analyses below them. + indicates when support is 100. The branch for sequence AF297085 was shortened by 50%. The new sequences are in bold.

Table 3-4 Microscopy measurements of hemogregarine intracellular parasites and infected host erythrocytes under 1000x magnification. An extracellular gametocyte was also detected for the sample ACZC1827 but was not included in these measurements (19.22 µm in length and 2.26 µm in width, similar to mean measurements for free gamonts in previous studies [38]). n refers to the number of hemogregarine gamonts or infected host cells measured per sample. Samples md67, ACZC1932 and ACZC1827 yielded identical 18S rRNA gene sequences (Figure 3-6), so a mean is presented for these as they may correspond to the same *Hepatozoon* species (*Hepatozoon domerguei*, see Discussion and Figure 3-6). GenBank accession numbers are given in Figure 3-6.

| | | Hemogregarines - Mean ± Sd (min-max) | | | | | Host cell - Mean ± Sd (min-max) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Host species | code | *n* | Vertical | Horizontal | Area | Perimeter | *n* | Vertical | Horizontal | Area | Perimeter |
| *Furcifer* sp. | md67 | 10 | 12.89 ± 0.94 (11.09-14.14) | 4.31 ± 0.71 (2.95-5.10) | 49.67 ± 5.36 (39.19-56.40) | 34.78 ± 2.00 (31.23-38.36) | 10 | 17.84 ± 1.45 (15.19-19.89) | 11.02 ± 0.87 (9.97-13.01) | 157.67 ± 11.48 (137.15-173.51) | 51.74 ± 2.00 (48.77-54.79) |
| *Ithycyphus oursi* | ACZC1932 | 5 | 14.49 ± 0.46 (13.95-15.28) | 3.77 ± 0.86 (2.56-5.21) | 58.64 ± 9.21 (45.28-72.23) | 39.12 ± 1.07 (37.59-40.66) | 5 | 18.29 ± 1.35 (16.32-19.58) | 10.82 ± 1.53 (8.13-12.76) | 163.41 ± 31.87 (112.91-208.07) | 52.84 ± 4.98 (45.46-58.57) |
| *Madagascarophis colubrinus* | ACZC1827 | 10 | 13.82 ± 0.56 (12.55-14.61) | 2.97 ± 0.55 (1.83-3.59) | 42.77 ± 8.73 (24.78-57.26) | 36.43 ± 2.13 (32.60-40.81) | 8 | 19.93 ± 1.59 (16.32-22.08) | 11.20 ± 1.01 (9.97-13.20) | 180.91 ± 10.63 (164.88-194.65) | 56.80 ± 1.23 (55.10-58.87) |
| | | | 13.73 ± 0.66 (11.09-15.28) | 3.68 ± 0.71 (1.83-5.21) | 50.36 ± 7.77 (24-78-72.23) | 36.78 ± 1.73 (31.23-40.81) | | | | | |
| *Madagascarophis colubrinus* | ACZC1963[5] | 10 | 11.53 ± 1.16 (10.12-14.40) | 3.42 ± 0.35 (2.94-4.02) | 35.93 ± 4.07 (30.85-42.80) | 30.76 ± 2.33 (27.95-36.59) | 9 | 15.99 ± 2.20 (12.54-19.30) | 9.72 ± 1.41 (8.24-12.76) | 123.34 ± 13.97 (92.00-142.14) | 46.35 ± 3.42 (40.00-52.88) |
| *Oplurus* sp. | x49[6] | 10 | 12.13 ± 0.51 (11.25-13.19) | 5.88 ± 0.67 (4.72-7.01) | 59.71 ± 5.90 (48.30-66.92) | 34.26 ± 1.24 (32.38-36.08) | 6 | 14.61 ± 1.21 (12.33-15.80) | 10.54 ± 1.19 (8.24-11.84) | 124.23 ± 15.90 (96.91-141.63) | 45.15 ± 2.78 (41.68-49.10) |

[5] PCR was negative for sample ACZC 1963.
[6] Sample·x49 presented morphologically distinct parasites (Figure 3-4), some of which apparently found inside leukocytes and yielded a genetically distinct 18S rRNA gene haplotype (Figure 3-6).

Table 3-5 Microscopy measurements of *Foleyella furcata* microfilaria in Giemsa-stained blood smears under 400x magnification. As confirmed by PCR sequencing, see Figure 3-7 and Figure 3-8. n refers to the number of microfilaria measured per sample. GenBank accession numbers are given in Figure 3-7 and Figure 3-8.

| | | | *Foleyella furcata* - Mean ± Sd (min-max) | | | |
|---|---|---|---|---|---|---|
| Host species | code | *n* | Length | Width | Area | Perimeter |
| *Furcifer lateralis* | x43[7] | 10 | 148.46 ± 10.83 (131.06-168.77) | 6.93 ± 0.43 (5.92-7.59) | 969.32 ± 101.36 (766.98-1106.76) | 332.43 ± 23.37 (293.12-376.73) |
| *Furcifer oustaleti* | md57[7] | 10 | 155.88 ± 6.05 (144.31-168.24) | 7.40 ± 0.47 (6.56-8.16) | 1058.15 ± 52.58 (988.72-1156.45) | 345.32 ± 15.85 (313.75-373.17) |
| | md59 | 10 | 125.84 ± 11.92 (106.80-145.62) | 5.95 ± 0.37 (5.12-6.70) | 682.61 ± 98.77 (543.62-819.30) | 288.45 ± 27.16 (248.42-329.20) |
| | | | 140.86 ± 8.99 (106.80-168.24) | 6.68 ± 0.42 (5.12-8.16) | 870.00 ± 75.68 (543.62-1156.45) | 316.89 ± 21.52 (248.42-373.17) |
| *Furcifer verrucosus* | ACZC1898 | 10 | 118.75 ± 9.06 (105.31-135.48) | 5.51 ± 0.46 (4.64-6.40) | 608.21 ± 76.41 (478.54-714.50) | 264.17 ± 20.84 (235.38-299.71) |
| | md56[7] | 10 | 136.50 ± 17.88 (106.96-172.86) | 6.34 ± 0.49 (5.76-7.24) | 753.88 ± 154.45 (509.39-1076.02) | 316.10 ± 49.67 (240.59-383.25) |
| | x67 | 10 | 122.02 ± 3.92 (116.16-128.87) | 6.00 ± 0.51 (5.12-7.04) | 663.08 ± 58.64 (587.52-805.07) | 276.46 ± 9.06 (258.61-286.48) |
| | | | 125.75 ± 10.29 (105.31-172.86) | 5.95 ± 0.49 (4.64-7.24) | 675.06 ± 96.50 (478.54-1076.02) | 282.06 ± 22.84 (235.38-383.25) |
| *Furcifer* sp. | md67 | 10 | 115.84 ± 13.01 (97.73-137.74) | 5.40 ± 0.76 (4.00-6.56) | 524.46 ± 83.49 (408.76-639.08) | 267.21 ± 32.22 (219.58-319.37) |
| | md68[7] | 5 | 136.38 ± 26.72 (102.02-181.86) | 6.18 ± 1.04 (4.16-7.04) | 749.68 ± 195.57 (421.09-972.21) | 303.14 ± 59.61 (231.40-407.32) |
| | md70[8] | 10 | 108.51 ± 6.15 (100.14-117.74) | 4.34 ± 0.29 (4.00-4.80) | 396.60 ± 36.37 (350.00-480.38) | 245.40 ± 13.00 (225.58-263.99) |
| | md71[7] | 4 | 128.03 ± 6.83 (117.83-137.06) | 6.24 ± 0.30 (5.76-6.58) | 762.91 ± 73.76 (689.18-878.59) | 281.34 ± 16.62 (256.14-302.82) |
| | md87[8] | 10 | 117.17 ± 7.93 (100.81-130.29) | 5.70 ± 0.64 (4.22-6.40) | 624.74 ± 59.61 (525.00-713.83) | 264.65 ± 17.43 (229.24-294.14) |
| | x46[7] | 10 | 126.20 ± 7.80 (113.58-139.09) | 6.23 ± 0.46 (5.44-6.88) | 713.36 ± 55.59 (610.00-795.57) | 283.11 ± 17.66 (257.93-311.08) |
| | x47[7] | 10 | 142.37 ± 12.93 (124.13-163.79) | 5.96 ± 0.39 (5.50-6.56) | 790.25 ± 102.58 (638.49-940.39) | 317.31 ± 26.90 (278.56-360.18) |
| | | | 124.93 ± 11.62 (100.14-181-86) | 5.72 ± 0.55 (4.00-7.04) | 651.71 ± 86.71 (350.00-972.21) | 280.31 ± 26.21 (219.58-407.32) |

## Discussion

This study shows that multiple parasites can be found in endemic reptile species from Madagascar. We detected hemogregarines at an overall low prevalence and intensity of infection in two snake species (*Ithycyphus oursi* and *Madagascarophis colubrinus*), a chameleon (*Furcifer* sp.) and an iguanid lizard (*Oplurus* sp.), while filarial infections were relatively high in chameleons (*Furcifer* genus). We found an identical *Hepatozoon* 18S rRNA gene haplotype in the prey-predator system composed of the snakes *I. oursi* and *M. colubrinus*, and their prey *Furcifer* sp. [15]. This mode of transmission has been increasingly detected by molecular tools in reptiles from continental Africa [80, 81] and in mammals [2, 3, 50], and it has already been described for *Hepatozoon*

[7] Some microfilaria displayed larger sheaths that were included in the measurements.
[8] Microfilaria were found in a coiled position, which complicates measurements and may explain lower values compared to the others.

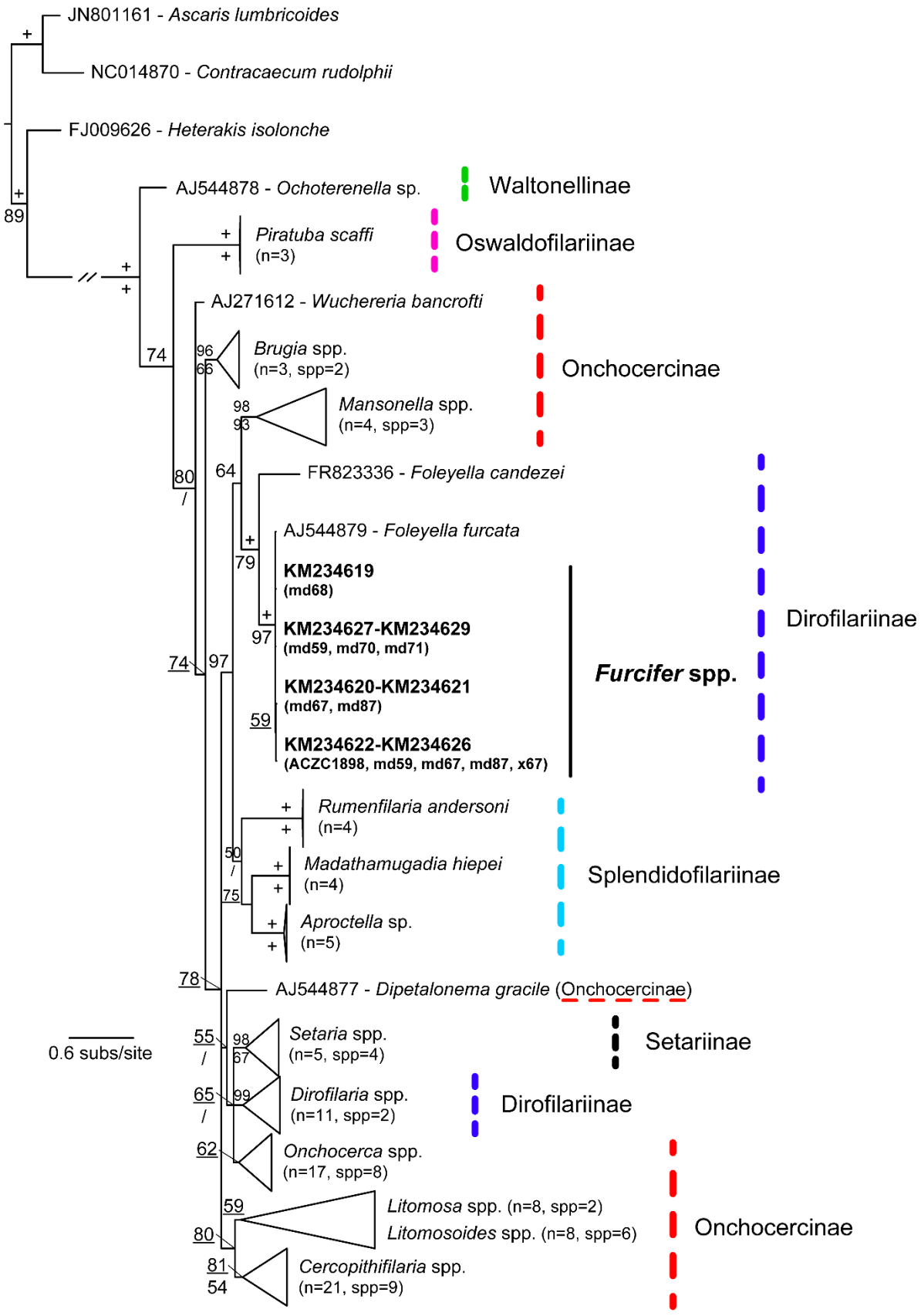


Figure 3-7 Tree derived from a Bayesian Inference analysis of the nematode COX1 gene sequences. Bayesian Posterior Probability values are given above relevant nodes, and Bootstrap supports for ML analyses below them. The symbol + indicates when support is 100 and / when Maximum Likelihood topology differs. n refers to the number of sequences and spp. refers to the number of species that form the collapsed clade. The new sequences are in bold.

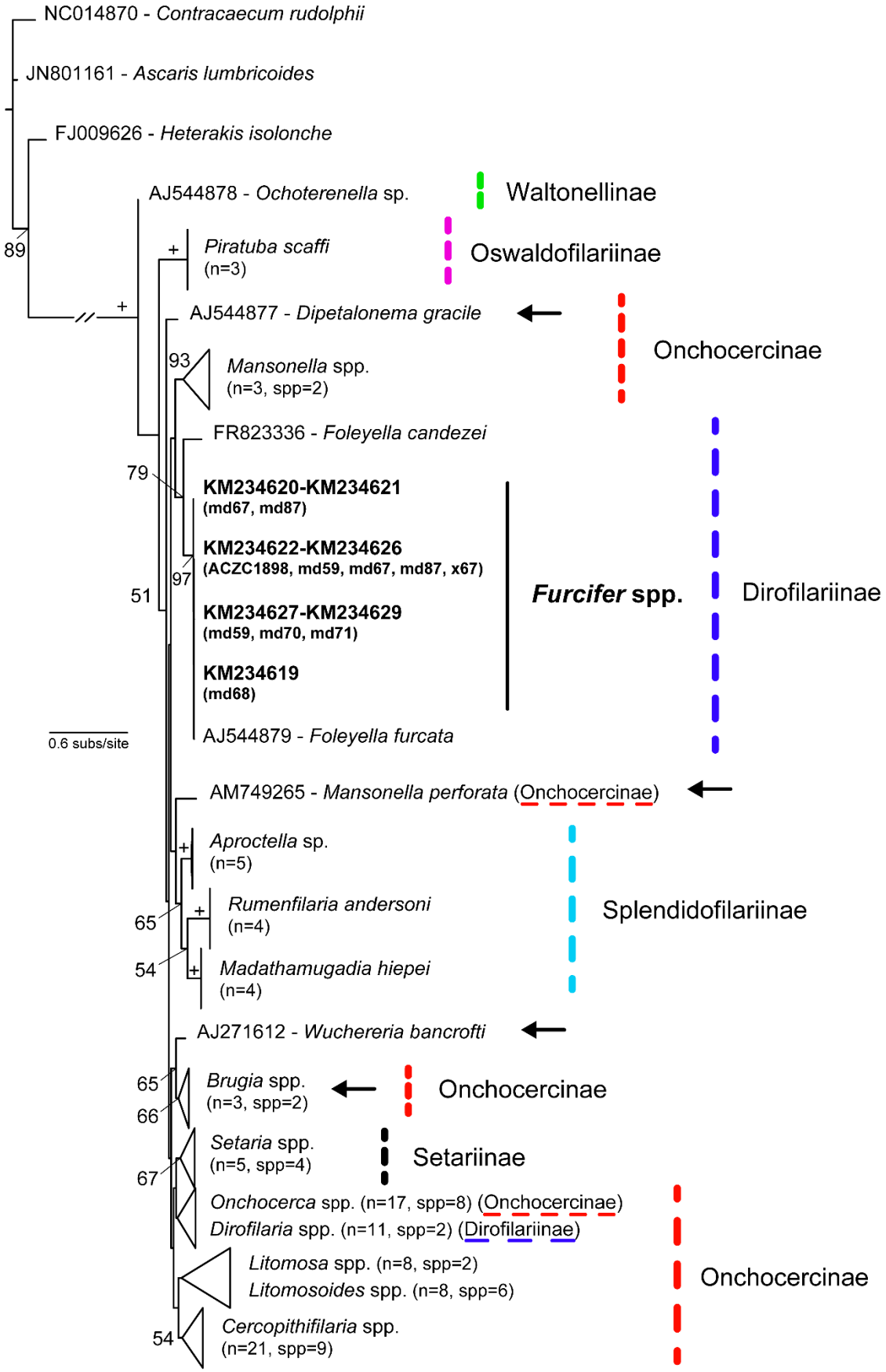


Figure 3-8 Tree derived from a Maximum Likelihood (ML) analysis of the nematode COX1 gene sequences.
The symbol + indicates when support is 100. n refers to the number of sequences and spp. refers to the number of species that form the collapsed clade. The new sequences are in bold and arrows indicate differences between the BI and ML phylogenetic analyses.

*domerguei*, a hemogregarine species whose type host is *M. colubrinus* and type locality is Madagascar [38, 79]. Transmission from prey to predator is possible by ingestion of infective cysts in prey hosts that become infective to a predator and this is a plausible explanation for why the same haplotype was found in these host species. Based on this and the fact that *H. domerguei* gamonts are similar to those found in our study, we propose that this *Hepatozoon* haplotype is from *Hepatozoon domerguei*. However, this needs to be verified by identifying the developmental stages in arthropod vectors. *Hepatozoon* parasites can be transmitted by a wide range of arthropod vectors, such as mites, ticks, and mosquitoes, but the diversity and distribution of competent vectors of these parasites in Madagascar is limited, although sporogony was obtained in the arthropods *Culex quinquefasciatus* and *Anopheles stephensi* (Liston, 1901) [38]. The fact that an individual of *M. colubrinus* was infected with two haplotypes may indicate this host species may have been infected with different hemogregarine species [12, 79], although we did not find major morphological differences in the gamonts from this single individual that could indicate the presence of distinct *Hepatozoon* species in the blood. This may indicate that the second haplotype is a latent infection in the form of tissue cysts that is not visible (or present) in the blood, which may also be a case of dead-end infections, meaning that the parasite does not develop in this host species and is not transmitted further [82]. In addition, the other *M. colubrinus* individual infected with hemogregarine parasites in blood smears (ACZC1963) could not be amplified using the primers employed in this study, but given the distinct morphological characteristics (Table 3-4) this may indicate the presence of another hemogregarine species in this host. Hemogregarine taxonomy is problematic, with evidence that the genus *Hepatozoon* may be paraphyletic [9, 36, 74]. Thus, it is possible that some of these haplotypes belong to different hemogregarine genera. Future studies need to assess the developmental stages of these parasites and the use of faster-evolving genes [41] might help in taxonomic identification of these parasites. It is also worth mentioning that the Hep primers performed better than the HEMO primers by amplifying a broader range of parasites, as observed in other studies [28, 58], allowing for a better assessment of the distribution and diversity of these parasites.

To our knowledge, this is the first report of *Sarcocystis* parasites in the Peters' keeled cordylid lizard *Tracheloptychus petersi*; however, sporozoites of *Sarcocystis* species have been previously reported from reptiles in Madagascar [84]. This lizard species is listed as Vulnerable under the IUCN Red List criteria and is a species with a decreasing population trend [68], thus it is important to assess the real prevalence of this parasite and investigate its implications for the host because Sarcocystis species are known to have adverse effects in some hosts [34]. This parasite is identical to that found in a snake from continental Africa, which provides further evidence that phylogenetic analysis of the 18S rRNA gene of *Sarcocystis* does not reflect the relationships of their final hosts [81]. *Sarcocystis* parasites have a direct life cycle and are transmitted from infected prey to their predator [21], as it has been observed in recent molecular assessments [28, 81]. Given that the

haplotype is identical between a lizard and a snake, it is reasonable to assume that this indicates a lizard-snake life cycle, which is not uncommon [85]. However, the fact that the same lineage of parasite is found in North African snakes and lizards endemic to Madagascar is indicative of low host specificity.

In this work we report a relatively high incidence of microfilarial infections in the chameleon genus *Furcifer*. Although morphological identification of nematodes to the species level requires the use of adult forms, by combining morphological characters and genetic information we were able to identity these microfilariae to the species level. Within the genus *Foleyella*, 4 species are known to infect reptiles, of which *F. furcata* and *F. brevicauda* have been previously reported in chameleons from Madagascar [6, 12]. Since both *F. furcata* and *H. domerguei* are transmitted by the southern house mosquito *Culex quinquefasciatus*, it is possible that the mixed infected chameleons observed in this study were infected by this vector. Future studies should determine the distribution of this vector in natural populations in Madagascar. Both hemogregarines and filarial nematodes are often asymptomatic but, when present at high intensities and/or in the presence of other hemoparasites, they may be associated with health implications and thus their impact in these wild endemic hosts should be further investigated. Sampling sizes across host species were not uniform and may not represent prevalence estimates. Thus, for a more realistic distribution of these parasites larger sampling studies are needed, as well as future studies that consider the ecological characteristics of the different geographical locations analyzed, host susceptibility and abundance of competent vectors. Altogether this and future epidemiological data should be considered when designing and employing conservation measures.

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## 3.3 Article V - Description of a new hemogregarine species *Hepatozoon omanensis* n. sp. (Apicomplexa, Haemogregarinidae) found in reptiles from Oman

In preparation

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**Abstract**

Hemogregarines are the most common apicomplexan parasites of reptiles but their diversity is still underestimated. This is particularly true for host groups from less studied geographical locations, as indicated by recent studies in wild reptiles from Brazil and Oman. In this study, we propose the description of a new hemogregarine species, *Hepatozoon omanensis* n. sp., based both on mature gamont morphology and unique 18S rRNA gene sequences. These sequences form a well-supported monophyletic lineage that is currently known only from reptiles of Oman. The proposed new species is exclusively found in reptiles from Oman, and encompasses two variants. One variant was found in 6 gecko species (*Asaccus platyrhynchus*, *Hemidactylus hajarensis*, *Hemidactylus luqueorum*, *Hemidactylus festivus*, *Ptyodactylus hasselquistii* and *Pristurus rupestris*) and also in the snake species *Echis omanensis*, and the second variant was found in two other gecko species (*Hemidactylus festivus* and *Hemidactylus lemurinus*). These variants differed by four mutations for a fragment of the 18S rRNA gene and the morphology of the mature gamonts was slightly longer and wider in the latter variant. This study highlights the importance of identifying and describing distinct parasites found in wild hosts from remote geographical areas for biodiversity and conservation purposes.

**Keywords:** Herpetofauna; snake; lizard; Arabia; Adeleorina; host-specificity.

## Introduction

Hemogregarines are heteroxenous parasites that are found in a wide range of vertebrate hosts (Smith, 1996; Telford, 2009) and are the most common and widely distributed apicomplexan parasites of reptiles. Although knowledge of hemogregarine diversity is increasing, recent studies have uncovered unexpectedly high diversity in remote geographical locations (O'Dwyer et al., 2013; Tomé et al., 2014). In addition, recent studies have shown that geckos may harbor diverse hemogregarine parasites (Harris et al., 2014a) in comparison to other lizard hosts (Maia et al., 2011). In particular, a recent molecular survey in wild reptiles from Oman has shown that geckos from this region can harbor very distinct hemogregarine lineages (section 5.2). Several different hemogregarine lineages were identified in section 5.2: an exclusive lineage from geckos that is related with the *Hepatozoon*/*Karyolysus* clade, a lineage from snakes that cluster inside the *Hepatozoon*/*Karyolysus* clade, and several haplotypes that cluster in a clade known from *Hepatozoon* species infecting various reptile and rodent hosts. Of the 42 hemogregarine sequences retrieved from geckos in section 5.2, 34 (81%) belonged to a single haplotype found exclusively in reptiles from Oman and 2 (5%) were from a closely related haplotype from 2 gecko species from Oman. Given that these haplotypes were not found anywhere else and that the principal hosts were endemic gecko species in Oman, this may be an indication of a typical lineage occurring in this region. In this study, we propose investigate the morphological characters of the parasites these two haplotypes and propose the description of a new hemogregarine species.

Although developmental stages in the invertebrate hosts are important for species description and identification (Telford, 2009), the combination of mature gamont morphology and genetic information has been increasingly used as a convenient approach to identify and describe new species (Harris et al., 2014b; Netherlands et al., 2014). Gamont morphology for the same parasite species may vary between host species (Telford et al., 2001, 2004; Telford, 2010), and that has led to many descriptions based on the identity of the host, which may not the best practice (Mathew et al., 2000). A good representation of parasite diversity in a wide range of hosts should help to avoid this problem. Furthermore, molecular tools are useful because they allow large scale screenings (section 5.2), offer a more accurate detection (Maia et al., 2014b) and provide genetic data for assessing the relationships between parasites (Hypša, 2006).

The Arabian Peninsula is an attractive region for studying biodiversity due to its high levels of endemism (Carranza and Arnold, 2012; Metallinou et al., 2014; Badiane et al., 2014). The study of parasites in regions with high levels of host endemism and diversity is important not only for implementing conservation measures and reducing disease risk but also for documenting biodiversity due to the potential for uncovering new parasite species. Traditional parasitological studies in hosts from the Arabian Peninsula were based on microscopy (Al-Ghamdy, 2011; Al-Ghamdi et al., 2011; Morsy et al., 2013) but the use of molecular tools is increasing (Abdel-Baki et al., 2014). The aim of this study is to propose the description of a new species with two variants

based on mature gamont morphology and genetic information from 18S rRNA sequences, from a variety of reptile hosts from Oman.

**Materials and Methods**

Samples used in this study were obtained from a field expedition to Oman in May 2011 (section 5.2). Blood smears were available only for a subset of these samples (Table S2). Blood smears were screened using an Olympus CX41 microscope with an in-built digital camera (SC30) (Olympus, Hamburg, Germany). Intensity of infection was estimated as the number of parasites per 4000 erythrocytes (Table S2). Intraerythrocytic parasites and infected host erythrocytes were measured at 1000x magnification (Table S2) using cell ^B software (basic image acquisition and archiving software; Olympus, Münster, Germany). Length and width were taken using horizontal and vertical distance tool, while the area and perimeter were taken using the area/perimeter tool in the Measure menu of cell ^B software. Mature gamonts of hemogregarines are represented in Figure 3-9. For representation purposes, we added gamont figures of hemogregarine species from the literature belonging to the two main clades found in reptile hosts in the phylogenetic tree presented in section 5.2 (Figure 3-10).

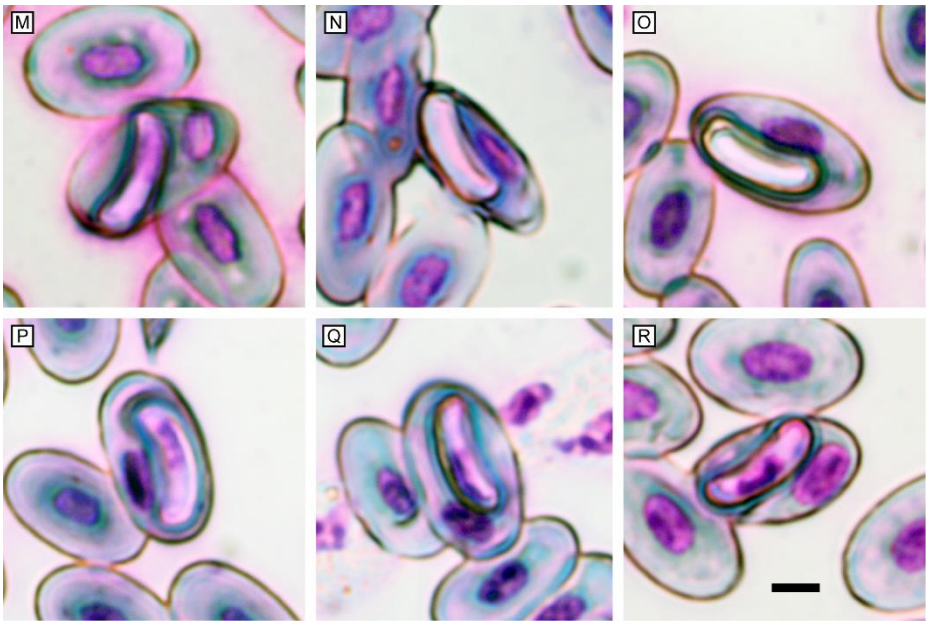


Figure 3-9 Mature gamonts from *Hepatozoon omanensis* n. sp. infections in geckos from Oman.
Letter designation is linked with Table S2 and Figure 3-10. (M) *Hemidactylus lemurinus* (S7134); (N) *Hemidactylus festivus* (S7605); (O) *Asaccus platyrhynchus* (S7429); (P) *Hemidactylus hajarensis* (S7587); (Q) *Hemidactylus luqueorum* (S6080); (R) *Ptyodactylus hasselquistii* (S7123). Scale bar is 5µm.

**Results**

Morphometrics of mature *Hepatozoon* gamonts from each gecko host species are provided in Table S2, and a comparison with known *Hepatozoon* species from lizards and snakes is provided in Table 3-6. Two haplotypes of a fragment of the 18S rRNA gene that differed by 4 mutations were obtained for the new species described herein (for simplicity we keep the original haplotype

designation [haplotype 3 and 4, see section 5.2)]. These haplotypes formed a well-supported monophyletic lineage within a clade known from *Hepatozoon* spp. from reptile and rodent hosts from various geographical locations (e.g. AY252106 from the lizard *Varanus panoptes* from Australia, JQ670908 from invertebrates attached to the snake *Ophiophagus hannah* from Thailand, HQ734787 from the gecko *Tarentola mauritanica* from Algeria, and AY600625 from the rodent *Clethrionomys glareolus* from Spain) (Figure 3-10). Mean measurements for mature gamonts from gecko hosts infected with haplotype 4 were slightly larger and had slightly greater width than gamonts from gecko hosts infected with haplotype 3 (Table 3-6 and Table S2). In addition, mature gamonts were similar within all host species for each haplotype (Table 3-6 and Table S2). In comparison with hemogregarine species of reptile hosts, mature gamonts of the new species described herein are much smaller than *Hepatozoon* species from snake hosts in geographical locations in close proximity to Oman (e.g. Saudi Arabia) (Table 3-6). Our measurements are similar to *Hepatozoon domerguei* from chameleon and snake hosts from Madagascar, putative *Hepatozoon* sp. from *Podarcis* species from Spain, from *Hepatozoon eurytopis* from *Lampropeltis getula floridana* from Florida and *Hepatozoon pictiventris* from *Nerodia fasciata pictiventris* from Florida (Table 3-6).

**Description**

In section 5.2, haplotypes 3 and 4 formed a distinct lineage in comparison to published *Hepatozoon* parasites (Figure 3-10). Haplotype 3 is composed of sequences from 6 gecko species [and one snake species, but for one gecko species and this snake species this was confirmed only based on molecular methods (section 5.2)], and haplotype 4 from 2 *Hemidactylus* species (Table 3-6). Blood smear examination shows morphological similarity between mature gamonts among geckos within each haplotype and only slight differences between haplotypes (Table 3-6 and Table S2). Based on the distinct genetic differences, along with the morphological characteristics we propose the description of a new *Hepatozoon* species typical from reptiles of Oman.

***Hepatozoon omanensis* n. sp.**

(Figure 3-9 M,N,O,P,Q,R)

Gamonts: Mature gamonts are bean-shaped (Figure 3-9) with different forms of staining. Some forms appear to have the nuclei dark stained and situated near the central portion of the gamont (R) or closer to the extremity (P,Q), while other gamont forms were white (N,O). Mean measurements for mature gamonts from all gecko hosts (n=246) were: length 12.94 ± 0.56 (8.28-13.60), width 4.08 ± 0.36 (2.79-4.42), area 50.84 ± 3.81 (30.10-56.18) and perimeter 34.58 ± 1.56 (23.14-36.44) (see Table S2 for gamont measurements for each sample and species). Infected host cell nuclei was found displaced laterally and vertically (M,Q,R) or only laterally (N,O,P). Mean measurements for infected erythrocytes from all gecko hosts (n=246) were length 19.31 ± 1.32 (13.42-21.42), width 9.89 ± 0.71 (7.15-9.60), area 155.58 ± 14.98 (107.25-165.00) and perimeter 52.00 ± 3.45 (36.22-55.06), (see Table S2 for cell measurements for each sample and species).

Table 3-6 Mature gamont morphological comparison between *Hepatozoon omanensis* n. sp. and hemogregarine species infecting lizards and snakes. Tree refers to the letter codes used in the phylogenetic tree. *n* refers to the number of hemogregarine gamonts measured per sample.

| | | | | | Hemogregarine - Mean ± Sd (min-max) | | | |
|---|---|---|---|---|---|---|---|---|
| | **Parasite species** | **Host species** | **Locality** | **tree** | ***n*** | **Length** | **Width** | **Reference** |
| *Haemogregarina* | *Haemogregarina boskiani* | *Acanthodactylus boskianus* | Tunisia | | | (16-16.4) | (4-8.5) | (Abdel-Baki et al., 2012) |
| species of lizards | *Haemogregarina damiettae* | *Acanthodactylus boskianus* | Egypt | | | 18 (16-22 | 5 (4-7) | (Abdel-Haleem et al., 2013) |
| | *Haemogregarina ramadani* | *Acanthodactylus boskianus* | Egypt | | | 12 ± 0.4 (11–13) | 5 ± 0.3 (4–6) | (Abdel-Baki et al., 2012) |
| | *Haemogregarina* sp. | *Acanthodactylus schmidti* | Saudi Arabia | | | 19.8 ± 1.7 | 1.7 ± 0.3 | (Al-Ghamdy, 2011) |
| | *Haemogregarina* sp. | *Gehyra variegata* | Australia | | | 11.0-14.0 | 4.0-6.0 | (Stenhbens and Johnston, 1968) |
| | *Haemogregarina helmymohammedi* | *Hemidactylus flaviviridis* | Egypt | | | (17.5-27) | (3-4.5) | (Saoud et al., 1995) |
| | *Haemogregarina* sp. | *Tarentola annularis* | Sudan | | | 12.4-15.9 | 3.2-5.5 | (Saoud and Younis, 1969) |
| | *Haemogregarina* sp. | *Tarentola annularis* | Sudan | | | 12.5 | 3.6 | (Elwasila, 1989) |
| | *Haemogregarina tarentannulari* | *Tarentola annularis* | Egypt | | | (13-17) | (2.5-3.5) | (Saoud et al., 1995) |
| | *Haemogregarina platydactyli* | *Tarentola mauritanica* | Algeria | | | (14-16) | (6-7) | (Saoud et al., 1995) |
| | *Haemogregarina rawashi* | *Ptyodactylus hasselquistii* | Egypt | | | 17.5 (14-20) | 4.5 (3.5-5) | (Saoud et al., 1995) |
| | *Haemogregarina* sp. | *Ptyodactylus hasselquistii* | Egypt | | | 14.13 ± 2.5 (12.2-19.4) | 10.03 ± 1.9 (6.12-12.2) | (Hussein, 2006) |
| | *Haemogregarina* sp. | *Ptyodactylus hasselquistii* | Egypt | | | 24.3 | 8.5 | (Abdel–Ghaffar et al., 1994) |
| *Haemogregarina* | *Haemogregarina aswanensis* | *Naja haje haje* | Egypt | | | (12.5-17.5) | (2.5-6) | (Saoud et al., 1995) |
| species of snakes | *Haemogregarina floridana* | *Nerodia floridana* | Florida | | 75 | 15.4 ± 0.9 (13-17) | 6.3 ± 0.5 (5-7) | (Telford et al., 2001) |
| | *Haemogregarina roshdyi* | *Varanus griseus* | Egypt | | | (13-20) | (1.5-2.5) | (Ramadan et al., 1996) |
| | *Haemogregarina* sp. | *Cerastes cerastes gasperitti* | Saudi Arabia | | | 17.5 | 3 | (Al-Farraj, 2008) |
| *Hepatozoon* | *Hepatozoon kisrae* | *Agama stellio* | Palestine | | 9 | (12-15) | (2-5.5) | (Telford, 2009) |
| species of lizards | ***Hepatozoon omaniensis* n. sp.** | *Asaccus platyrhynchus* | Oman | O | 151 | 12.77 ± 0.59 (10.97-14.60) | 3.98 ± 0.34 (3.12-4.83) | this study |
| | ***Hepatozoon omaniensis* n. sp.** | *Hemidactylus festivus* | Oman | N | 11 | 13.25 ± 0.53 (12.62-14.37) | 4.44 ± 0.30 (3.92-4.85) | this study |
| | ***Hepatozoon omaniensis* n. sp.** | *Hemidactylus hajarensis* | Oman | P | 20 | 12.82 ± 0.59 (11.64-13.60) | 4.14 ± 0.55 (3.19-5.48) | this study |
| | ***Hepatozoon omaniensis* n. sp.** | *Hemidactylus lemurinus* | Oman | M | 12 | 13.25 ± 0.38 (12.19-13.60) | 3.90 ± 0.24 (3.48-4.42) | this study |
| | *Hepatozoon gracilis* | *Mabuya quinquetaeniata* | Sudan | | | 21.3 ± 1.5 (18-22.5) | 1.14 ± 0.20 (0.9-1.4) | (Telford, 2009) |
| | *Hepatozoon* sp. | *Podarcis bocagei, Podarcis carbonelli* | Portugal | | | 12 ± 1 (10-13) | 4 ± 1 (3-6) | (Roca and Galdón, 2010) |
| | ***Hepatozoon omaniensis* n. sp.** | *Ptyodactylus hasselquistii* | Oman | R | 53 | 12.60 ± 0.69 (8.28-13.91) | 3.94 ± 0.37 (2.79-5.40) | this study |
| | *Hepatozoon hemprichii* | *Scincus hemprichii* | Saudi Arabia | | | 18 ± 1.8 (16–19) | 4 ± 0.8 (3–6) | (Al-Ghamdi et al., 2011) |
| | *Hepatozoon burneti* | *Tarentola mauritanica* | Tunisia | | | 35 | 6 | (Lavier and Callot, 1938) |
| | *Hepatozoon* sp. 1 | *Tarentola mauritanica* | Egypt | | | 14.6-16.5 | 4.8-6.0 | (Abdel–Aziz et al., 2010) |
| | *Hepatozoon* sp. | *Tropiocolotes steudneri* | Egypt | | | 12.4-17.3 | 5.1-6.2 | (El-Nour and El-Toykhy, 2014) |
| *Hepatozoon* species of tuatara | *Hepatozoon tuatarae* | *Sphenodon punctatus* | New Zealand | | | 15.1 (12.9-15.6) | 4 (3.6-4.6) | (Herbert et al., 2010) |
| *Hepatozoon* | *Hepatozoon bitis* | *Bitis arietans* | South Africa | | | (12.5-14) | (3-4) | (Sloboda et al., 2007) |
| species of snakes | *Hepatozoon dogieli* | *Bitis gabonica* | Uganda | | | 14 | 6 | (Sloboda et al., 2007) |
| | *Hepatozoon boodoni* | *Boaedon fuliginosus* | Sudan | | | (14-15) | (2-3; 7) | (Sloboda et al., 2007) |
| | *Hepatozoon musotae* | *Boaedon lineatus* | Uganda | | | 17 | (3.8-4.7) | (Sloboda et al., 2007) |
| | *Hepatozoon boiga* | *Boiga irregularis* | Australia | Z | | (16-17) | (4-5) | (Jakes et al., 2003) |
| | Hepatozoon seurati | *Cerastes cerastes* | Egypt | | | 16.5 ± 1.5 | 3.5 ± 0.4 | (Morsy et al., 2013) |
| | *Hepatozoon seurati* | *Cerastes cornutus* | Algeria | | | (12-16) | (3-5) | (Telford, 2009) |

| | Parasite species | Host species | Locality | tree | Hemogregarine - Mean ± Sd (min-max) *n* | Length | Width | Reference |
|---|---|---|---|---|---|---|---|---|
| | *Hepatozoon confusus* | *Coluber constrictor priapus* | Florida | | 25 | 15.6 ± 0.7 (14.0-17.0) | 4.1 ± 0.4 (3.5-5.0) | (Telford et al., 2005) |
| | *Hepatozoon priapus* | *Coluber constrictor priapus* | Florida | | 25 | 18.0 ± 0.9 (17-20) | 4.2 ± 0.6 (3.5-6) | (Telford et al., 2005) |
| | *Hepatozoon pettiti* | *Crocodylus niloticus* | Senegal | | | (18-25) | (2.5-5.0) | (Telford, 2009) |
| | *Hepatozoon cevapii* | *Crotalus durissus terrificus* | Brazil | G | 100 | 17.05 ± 1.10 (14.46-19.20) | 3.12 ± 0.49 (2.12-4.64) | (O'Dwyer et al., 2013) |
| | *Hepatozoon cuestensis* | *Crotalus durissus terrificus* | Brazil | I | 300 | 17.07 ± 1.44 (12.95-20.54) | 3.6 ± 0.55 (1.85-5.2) | (O'Dwyer et al., 2013) |
| | *Hepatozoon massardii* | *Crotalus durissus terrificus* | Brazil | F | 100 | 17.31 ± 1.00 (15.00-19.94) | 1.95 ± 0.38 (1.77-3.93) | (O'Dwyer et al., 2013) |
| | *Hepatozoon horridus* | *Crotalus horridus* | Florida | | 25 | 15.7 ± 0.9 (13.0-17.0) | 5.1 ± 0.6 (4.0-6.0) | (Telford et al., 2008) |
| | *Hepatozoon minchini* | *Crotaphopeltis degeni* | Kenya | | | (13-14) | (3-4) | (Sloboda et al., 2007) |
| | *Hepatozoon crotaphopeltis* | *Crotaphopeltis hotamboeia* | Uganda | | | 20 | 2 | (Sloboda et al., 2007) |
| | *Hepatozoon punctatus* | *Diadophis p. punctatus* | Florida | | 25 | 13.4 ± 1.0 (12-16) | 5.1 ± 0.5 (4-6) | (Telford et al., 2001) |
| | *Hepatozoon zambiensis* | *Dispholidus typus* | Zambia | | | (14.94-17.79) | (2.35–5.7) | (Sloboda et al., 2007) |
| | *Hepatozoon mehlhorni* | *Echis carinatus* | Egypt | | | 17.2 ± 1.6 | 5.4 ± 0.5 | (Bashtar et al., 1991) |
| | *Hepatozoon vubirizi* | *Gonionotophis capensis* | Uganda | | | (15-17) | (3.8-4.7) | (Sloboda et al., 2007) |
| | *Hepatozoon eurytopis* | *Lampropeltis getula floridana* | Florida | | 25 | 12.6 ± 0.9 (11-14.5) | 4.5 ± 0.5 (4-6) | (Telford, 2010) |
| | *Hepatozoon karyolysi* | *Lampropeltis getula floridana* | Florida | | 25 | 17.6 ± 0.9 (16-20) | 4.9 ± 0.8 (4-7.5) | (Telford, 2010) |
| | *Hepatozoon rexi* | *Lampropeltis getula floridana* | Florida | | 9 | 16.1 ± 1.2 (14-18.5) | 5.0 ± 0.4 (4.5-6) | (Telford, 2010) |
| | *Hepatozoon domerguei* | *Madagascarophis colubrina* | Madagascar | | | 14 | 3 | (Telford, 2009) |
| | *Hepatozoon enswerae* | *Naja melanoleuca* | Uganda | | | 19; 15 | 3; 3.8 | |
| | *Hepatozoon najae* | *Naja tripudians* | India | | | 14 | 3 | (Telford, 2009) |
| | *Hepatozoon fasciatae* | *Nerodia fasciata pictiventris* | Florida | | 25 | 16.5 ± 0.6 (16-18) | 3.7 ± 0.4 (3-5) | (Telford et al., 2001) |
| | *Hepatozoon pictiventris* | *Nerodia fasciata pictiventris* | Florida | | 75 | 13.7 ± 1.0 (11-16) | 4.7 ± 0.4 (4-6) | (Telford et al., 2001) |
| | *Hepatozoon brendae* | *Psammophis schokari* | - | | | (16-17) | (3-4) | (Sloboda et al., 2007) |
| | *Hepatozoon garnhami* | *Psammophis schokari* | Saudi Arabia | * | 50 | 16 ± 1.4 | 2 ± 0.3 | (Abdel-Baki et al., 2014) |
| | *Hepatozoon garnhami* | *Psammophis schokari* | Egypt | | | (15-20) | (1.5-2.5) | (Saoud et al., 1996) |
| | *Hepatozoon matruhensis* | *Psammophis schokari* | Egypt | | | (18-28) | (2.5-6) | (Shazly, 1994) |
| | *Hepatozoon langii* | *Pseudocordylus langi* | South Africa | | | 19.1 ± 1.0 (15.4–28.1) | 6.2 ± 1.1 (3.5–7.9) | (Van As et al., 2013) |
| | *Hepatozoon vacuolatus* | *Pseudocordylus langi* | South Africa | | | 16.5 ± 1.0 (14.7 - 17.6) | 5.9 ± 1.2 (4.0 - 7.7) | (Van As et al., 2013) |
| | *Hepatozoon ayorgbor* | *Python regius* | Ghana | L | | 12.2 (11-13) | 2.9 (2-3.5) | (Sloboda et al., 2007) |
| | *Hepatozoon robertsonae* | *Python regius, Python sebae* | Gambia | | | (12-16) | | (Sloboda et al., 2007) |
| | *Hepatozoon seminatrici* | *Seminatrix p. pygaea* | Florida | | 50 | 16.2 ± 1.1 (14-20) | 4.0 ± 0.5 (3-5) | (Telford et al., 2001) |
| | *Hepatozoon aegypti* | Spalerosophis diadema | Egypt | | | (13-16) | (2.5-3) | (Bashtar et al., 1984) |
| | *Hepatozoon sirtalis* | *Thamnophis s. sirtalis* | Florida | | 75 | 20.0 ± 0.8 (17-22) | 4.1 ± 0.5 (3-6) | (Telford et al., 2001) |
| | *Hepatozoon sauritus* | *Thamnophis sauritus sackenii* | Florida | | 50 | 15.8 ± 0.9 (14-18) | 3.7 ± 0.5 (3-5.5) | (Telford et al., 2001) |
| *Karyolysus* species of lizards | *Karyolysus lacazei* | *Lacerta a. agilis, Lacerta viridis, Lacerta trilineata* ssp. | Hungary, Poland, Romania | E | | 20.69 | 2.8 | (Haklová-Kočíková et al., 2014) |
| | *Karyolysus lacazei* | *Lacerta a. agilis* | France | | 75 | 22.0 (17.7–27.7) | 3.6 (2.7–4.6) | (Telford, 2009) |
| | *Karyolysus lacertae* | *Lacerta a. agilis* | Russia | | 53 | 13.6 (12.3-14.6) | 4.3 (3.9-4.6) | (Telford, 2009) |
| | *Karyolysus latus* | *Podarcis muralis, Lacerta viridis* | Slovakia | F | | 11.53 | 4.83 | (Haklová-Kočíková et al., 2014) |
| | *Karyolysus latus* | *Lacerta a. agilis* | Sweden | | 475 | 14.9 (13.1-17.7) | 7.2 (5.0-10.0) | (Telford, 2009) |
| | *Karyolysus minor* | *Lacerta a. agilis* | Sweden | | 76 | 6.0 (4.6-7.7) | 3.9 (3.1-5.4) | (Telford, 2009) |

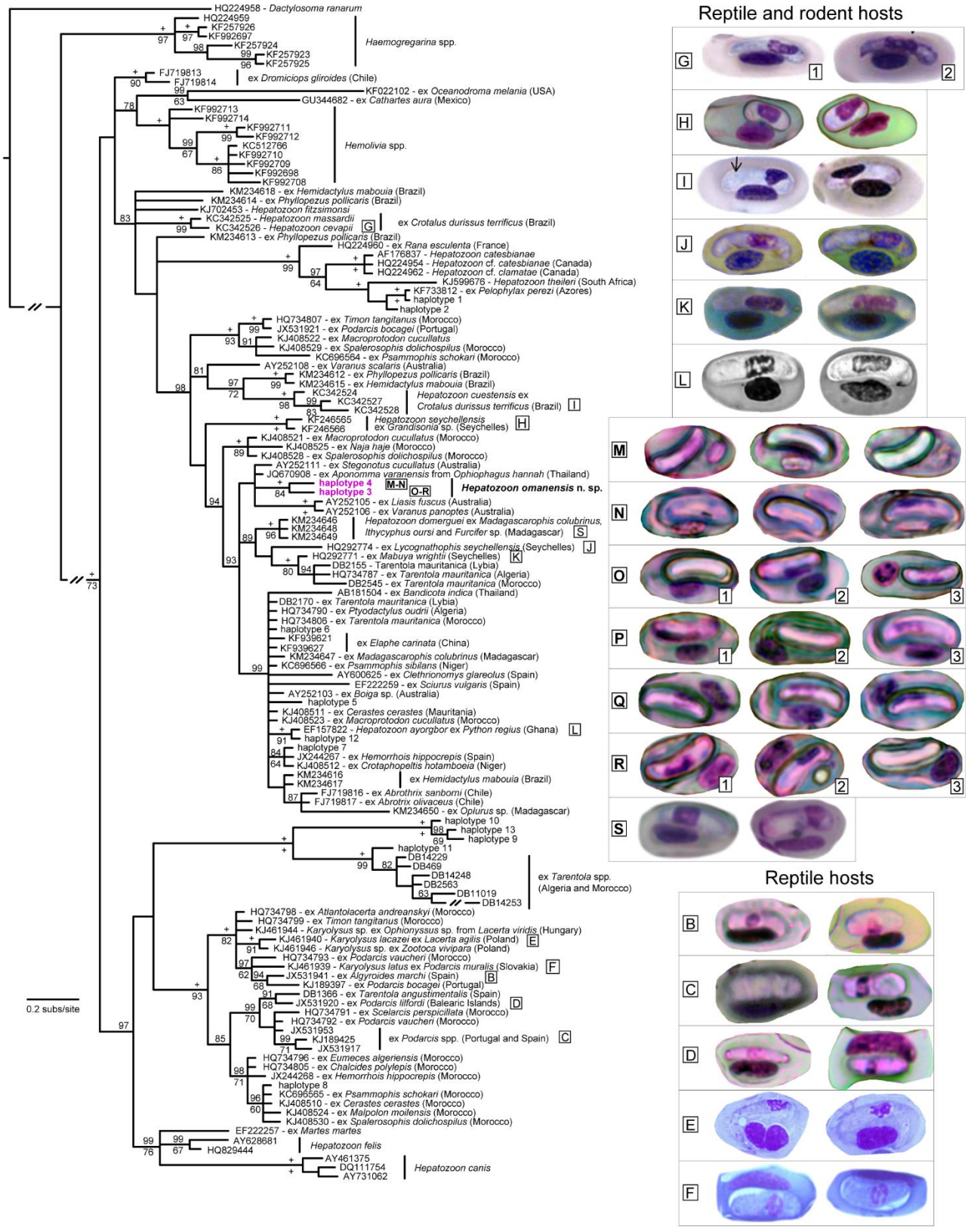


Figure 3-10 Hemogregarine 18S rRNA gene tree adapted from section 5.2.
Letter designation inside squares is linked to Table S2 and Figure 3-9.
Images were adapted from original references: B and D (Maia *et al*., 2012); C (Maia *et al*., 2012, 2014a); E and F (Haklová-Kočíková *et al*., 2014); F, G and I (O'Dwyer *et al*., 2013); H (Harris *et al*., 2014); J and K (Harris *et al*., 2011); L (Sloboda *et al*., 2007); S (Maia *et al*., 2014b). All other figures are from *H. omanensis* n. sp. proposed herein.
Figures are not at scale.

**Taxonomic summary**

*Type host*: *Asaccus platyrhynchus* (haplotype 3)

*Other hosts*: *Echis omanensis*, *Hemidactylus hajarensis*, *Hemidactylus luqueorum, Hemidactylus festivus Pristurus rupestris* and *Ptyodactylus hasselquistii* for haplotype 3; *Hemidactylus festivus* and *Hemidactylus lemurinus* for haplotype 4.

*Type locality*: Jebel Akhdar, Oman.

*Site of infection*: Peripheral blood.

*Other sites of infection*: unknown.

*Vector*: unknown.

*Deposition of voucher specimen*: XXX

*Sequence accession numbers*: XXX

*Etymology*: The specific epithet reflects the country where all the specimens infected by this parasite have been found so far. The type host was chosen based on the host species with the highest abundance levels (section 5.2) and the type locality based on the restricted geographical distribution of this host species.

*Remarks*

*Hepatozoon omanensis* n. sp. could be genetically differentiated from published sequences on GenBank by differences in a fragment of the 18S rRNA gene used in the phylogenetic analyses. The two variants of *Hepatozoon omanensis* n. sp. differ by 4 mutations: sequences of haplotype 4 from the gecko species *H. lemurinus* (M in Figure 3-10) and *H. festivus* (N in Figure 3-10) had unique A-G and G-A mutations at positions 279 and 432, respectively, while haplotype 3 from the gecko species *Asaccus platyrhynchus*, *Hemidactylus hajarensis*, *Hemidactylus luqueorum*, *Hemidactylus festivus Pristurus rupestris* and *Ptyodactylus hasselquistii* (O, P, Q, and R in Figure 3-10) and the snake species *Echis omanensis*, had an additional unique G-A mutation in position 358. In addition, mature gamonts from *Hemidactylus* species with haplotype 4 appear to be slightly larger and wider (length of 13.25 ± 0.45 µm, area of 54.05 ± 2.89 µm, and width of 4.17 ± 0.27 µm) than mature gamonts from hosts infected with haplotype 3, such as: other *Hemidactylus* species (length of 12.78 ± 0.56 µm, area of 49.78 ± 4.29 µm, and width of 4.05 ± 0.52 µm), *A. platyrhynchus* (length of 12.77 ± 0.59 µm, area of 49.22 ± 4.80 µm, and width of 3.98 ± 0.34 µm), and *P. hasselquistii* (length of 12.60 ± 0.69 µm, area of 47.11 ± 4.18 µm, and width of 3.94 ± 0.37 µm) (Table S2). The 18S rRNA gene is a slow evolving gene and this may limit the differentiation of closely related species, and it is therefore possible that this is a cryptic *Hepatozoon* species complex. However, we prefer to be conservative and consider them as a single species, pending further research to identify the invertebrate hosts, to evaluate the developmental stages in invertebrate hosts and to use faster evolving genes for a finer molecular distinction (Leveille et al., 2014).

**Discussion**

Although hundreds of species have been already described for *Hepatozoon* (Smith, 1996; Telford, 2009), recent molecular data has shown an unprecedented genetic diversity within this genus (section 5.2). In particular as new geographical regions are sampled, unique parasite biodiversity is often revealed (O'Dwyer et al., 2013; Tomé et al., 2014; Harris et al., 2014a). In the present study, by combining morphological and genetic data we propose the description of *Hepatozoon omanensis* n. sp. that is found exclusively in reptiles from Oman.

Of the gecko species found infected with *Hepatozoon omanensis* n. sp., there is only a previous record of *Haemogregarina rawashi* in *P. hasselquistii* and *Haemogregarina helmymohammedi* from *Hemidactylus flaviviridis* from Egypt (Saoud et al., 1995). Although there is no genetic information from these hemogregarine species, mature gamont morphologies are very distinct. Additionally, hemogregarine species have been described and identified in four snake species that occur in Oman: *Hepatozoon cantliei* in *Eryx* sp. from North Africa (Smith, 1996); *Hepatozoon echisi* [28% prevalence in West Pakistan (Mohiuddin et al., 1967)], *Hepatozoon mehlhorni* (Bashtar et al., 1991) and *Hepatozoon perfilievi* in *Echis carinatus* from Egypt (Smith, 1996); *Hepatozoon garnhami* in *Psammophis schokari* from Saudi Arabia (Abdel-Baki et al., 2014), and *Haemogregarina* sp. in *Cerastes cerastes gasperettii* from Saudi Arabia (Al-Ghamdy, 2011). However, different host species can have different gamont morphologies for the same *Hepatozoon* species, as shown in a study that described *Hepatozoon ayorgbor* in naturally infected *Python regius* and in 2 other snake species experimentally infected (Sloboda et al., 2007). Also, a snake individual can be infected by multiple different *Hepatozoon* species [sections 3.2, 5.2 and (Tomé et al., 2013)]. Thus, future research should investigate and compare the morphology of mature gamonts in blood smears of snakes from this region and compare them with *H. omanensis* n. sp.

Finally, other hemogregarine lineages recently recovered from Oman need to be further studied for a complete assessment of hemogregarine morphological and genetic diversity, since they may also represent new taxonomic entities. In particular, the unique hemogregarine lineage found in gecko hosts from Oman and the Canary Islands, which is apparently related with hemogregarines from carnivores and with the *Hepatozoon*/*Karyolysus* complex clade, is highly genetically distinct and clearly warrants a complete morphological assessment also.

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# 4 OVERVIEW OF THE PARASITE GENETIC INFORMATION IN PUBLIC DATABASES WITH TAXONOMIC IMPLICATIONS

**Article VI - Maia, J. P.**, Harris, D. J. and Carranza, S. (2015). Reconstruction of the evolutionary history of Haemosporida (Apicomplexa) based on the cyt *b* gene with characterization of *Haemocystidium* in geckos (Squamata: Gekkota) from Oman. *Parasitology International*, 65, 5-11.

**Article VII - Maia, J. P.**, Carranza, S. and Harris, D. J. In preparation. A note on using 18S rRNA gene sequences for estimating relationships of hemogregarines (Apicomplexa, Adeleorina): current limitations and future prospects.

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## 4.1 Article VI - Reconstruction of the evolutionary history of Haemosporida (Apicomplexa) based on the cyt *b* gene with characterization of *Haemocystidium* in geckos (Squamata: Gekkota) from Oman

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**Abstract** (see Figure S4 for graphical abstract)

The order Haemosporida (Apicomplexa) includes many medically important parasites. Knowledge on the diversity and distribution of Haemosporida has increased in recent years, but remains less known in reptiles and their taxonomy is still uncertain. Further, estimates of evolutionary relationships of this order tend to change when new genes, taxa, outgroups or alternative methodologies are used. We inferred an updated phylogeny for the Cytochrome *b* gene (cyt *b*) of Haemosporida and screened a total of 80 blood smears from 17 lizard species from Oman belonging to 11 genera. The inclusion of previously underrepresented genera resulted in an alternative estimate of phylogeny for Haemosporida based on the cyt *b* gene. *Leucocytozoon* and *Haemoproteus* appear as sister taxa to avian and reptilian *Plasmodium* based on Bayesian Inference (BI) analysis, in contrast with the most recent phylogenetic assessment of the evolutionary history of Haemosporida. Paraphyly of mammalian *Plasmodium* and polyphyly of avian *Haemoproteus* support the division into subgenera as suggested in other studies. Avian and Reptilian *Plasmodium* are also polyphyletic, and therefore a similar division may be needed. Reptilian *Haemocystidium* form a monophyletic lineage, sister taxa to avian and reptilian *Plasmodium* and mammalian *Polychromophilus* in the BI analysis but its position in the Maximum Likelihood (ML) analysis is not well supported. This supports its recent reclassification as a separate genus. Our results further corroborate the hypothesis that the phylogeny based on the cyt *b* gene is associated with the invertebrate host families that transmit each genus rather than parasite life-history traits. *Haemocystidium* sp. was detected in two geckos from Oman, which were related with *Haemocystidium* sp. from Malagasy *Oplurus* sp. (<2% genetic divergence) but diverged from other reptilian *Haemocystidium* species by more than 5%. It is likely that these represent a new *Haemocystidium* species and further research on the distribution of vectors and host-vector associations is needed.

**Keywords:** *Haemoproteus*; *Plasmodium*; *Haemocystidium*; *Leucocytozoon*; vector association; life-history traits; Bayesian Inference; Maximum Likelihood.

## 1. Introduction

The apicomplexan order Haemosporida includes many medically important parasites, including *Plasmodium* spp. These parasites may be of major epidemiological concern and exert great economic costs to animal and public health, causing serious health issues including host death [1,2]. They are vector-borne, with sexual stages occurring in invertebrate hosts [3,4]. Apart from the threats posed to human health, these parasites are of concern in conservation because they can lead to extinction of naïve host species [5]. Despite the recent increase in the study of these parasites in some groups of wild hosts, particularly avian hosts [6,7], the distribution and diversity of these parasites in some hosts remain poorly investigated, especially in less studied host groups, such as reptiles from remote geographical regions.

Haemosporida includes four families: Garniidae, Haemoproteidae, Leucocytozoidae and Plasmodiidae that can be distinguished by life-history traits, such as the presence or absence of hemozoin pigmentation and the gain or loss of merogony [8,9]. However, most species within this order belong to three genera, *Plasmodium* (Plasmodiidae), *Leucocytozoon* (Leucocytozoidae) and *Haemoproteus* (Haemoproteidae) [10]. The study of Haemosporida has been primarily focused on the genus *Plasmodium* from a wide range of host groups, including primates [11,12], rodents [13,14], avian [15,16] and reptilian hosts [10,17], and more recently on avian *Haemoproteus* [18,19].

The evolutionary history of haemosporidians has long puzzled parasitologists with many hypotheses arising over time, often limited by the methodologies and analytical tools available [9]. Traditionally, the main characters for describing species and inferring their evolutionary history were based on the vertebrate host taxon and parasite traits observed through microscopy. However, these traits may have evolved convergently to respond to ecological changes, such as host switches [10], and the vertebrate host taxon may not be an adequate taxonomic character due to frequent vertebrate host switches among haemosporidians [20]. For this reason, the implementation of molecular tools to estimate the phylogenetic relationships between haemosporidians has shed new light into their evolutionary history. Initial molecular studies were based on few sequences and thus were prone to taxon sampling biases [21], inappropriate rooting [22] and gene limitations [23]. In fact, tree topologies vary greatly depending on the outgroup and rooting approach [9,24], and on the number of taxa and representatives of each genus [25–28]. Hence, it is important that large-scale phylogenetic reconstructions are conducted from time to time to re-assess the relationships between genera. Studies comparing the resolution of morphological characters, mitochondrial and nuclear genes in *Haemoproteus* species indicate that the diversity observed in the mitochondrial Cytochrome *b* (cyt *b*) gene is representative of the level of differentiation genome-wide [29,30]. For this reason, this gene is the most widely used for taxonomic purposes and to assess the evolutionary history of Haemosporida. The cut-off for identifying new species is considered above 5% in cyt *b*, although there are instances of species that differ by only 1% or less [18,31,32]. In addition, multi-gene

phylogenies have provided important information, for example that the major cladogenic events of Haemosporida seemed to be related to switches between vector hosts [10].

Considerable effort has been put into investigating the diversity of haemosporidians in avian hosts, with many studies analyzing the prevalence, intensity and phylogenetic relationships of the genera *Haemoproteus*, *Leucocytozoon* and *Plasmodium* [33]. These studies have shown that the number of species initially estimated based on microscopy is an underestimation of the real diversity of this group of parasites [8,33]. On the other hand, studies on Haemosporida of reptiles are still scarce, with only a few descriptions of *Haemoproteus* species, such as *Haemoproteus mesnili* and *Haemoproteus balli* in snakes [34], and *Haemoproteus kopki* and *Haemoproteus ptyodactylii* in lizards [28]. A recent molecular study showed that the reptilian *Haemoproteus* clade is clearly distinct from avian *Haemoproteus* and thus should be reclassified as the genera *Haemocystidium* [35].

In this study, we examined the phylogenetic relationships of Haemosporida based on the mitochondrial cyt *b* gene, with a focus on parasites of reptiles, and conducted a morphological and molecular characterization of *Haemocystidium* from geckos from Oman. Information regarding distribution of parasites in these hosts is currently scarce, even though this region is known to have a high degree of reptile endemism and diversity (e.g. [36–38]).

## 2. Materials and Methods

### *2.1. Microscopic examination*

A total of 80 blood smears from geckos from Oman (collected in May 2011) (see Table S6 for exact GPS coordinates) were screened for the presence of haemosporidian parasites (Table 4-1). Blood smears were air-dried, fixed with methanol and stained with diluted Giemsa (1:9 of distilled water) for 55 minutes. Blood smears were screened using an Olympus CX41 microscope with an in-built digital camera (SC30) (Olympus, Hamburg, Germany). Intensity of infection was estimated as the number of parasites per 4,000 erythrocytes (Table 4-1). Intracellular parasites and infected host erythrocytes were measured at 1000x magnification (Table 4-2) using cell ^B software (basic image acquisition and archiving software; Olympus, Münster, Germany). Length, width, vertical and horizontal distance were taken using polygon and arbitrary distance tools, while the area and perimeter were taken using the area/perimeter tool in the Measure menu of cell ^B software.

Table 4-1 Blood smear samples analysed for Haemosporida parasites in reptiles from Oman.
GPS refers to the exact location where the animal was collected given in Table S6.
Numbers in bold indicate locations that were positive for Haemosporida.

| | | **Haemosporida** | | |
|---|---|---|---|---|
| Host species | *n* | Prevalence | Intensity % | GPS |
| *Asaccus platyrhynchus* | 17 | | | 263,350 |
| *Bunopus spatalurus hajarensis* | 2 | | | 289 |
| *Calotes versicolor* | 1 | | | 274 |
| *Hemidactylus luqueorum* | 2 | 1 (50%) | 0.18 | **340**,350 |
| *Hemidactylus alkiyumii* | 1 | | | 277 |
| *Hemidactylus festivus* | 6 | | | 208,279 |
| *Hemidactylus hajarensis* | 3 | | | 289,319 |
| *Hemidactylus lemurinus* | 2 | | | 279 |
| *Messalina adramitana* | 2 | | | 284 |
| *Omanosaura jaykari* | 1 | | | 340 |
| *Pristurus carteri* | 11 | | | 205,268,278,284,286,287 |
| *Pristurus* sp.1 | 2 | | | 278 |
| *Pseudotrapelus dhofarensis* | 1 | | | 205 |
| *Ptyodactylus hasselquistii* | 19 | 1 (5%) | 0.34 | 208,263,289,292, **308**,326,339,340 |
| *Stenodactylus doriae* | 7 | | | 270,301 |
| *Stenodacylus leptocosimbotes* | 2 | | | 284 |
| *Trachylepis tesselata* | 1 | | | 274 |
| | 80 | 2 (2.5%) | | |

### *2.2. Molecular methods*

DNA from the two samples that were identified as infected using microscopy was extracted from blood drops stored in Whatman filter paper stored at −20 °C using the Speedtools tissue DNA extraction kit (Biotools, Madrid), following manufacturer's instructions. PCR amplifications for a fragment of the mitochondrial cyt *b* gene were performed using the nested protocol with the outer primers HaemNFI [5′-CATATATTAAGAGAAITATGGAG-3′] and HaemNR3 [5′ ATAGAAAGATAAGAAATACCATTC-3′] and the inner primers HAEMF (5′-ATGGTGCTTTCGATATATGCATG-3′) and HAEMR2 (5′-GCATTATCTGGATGTGATAATGGT-3′) [33]. The PCR reactions using these primers were run in 20 µl reaction mixture containing 1 U of GoTaq® DNA Polymerase (5u/µl), 1.2 mM MgCl2 (25 mM), 0.125 mM of each nucleotide, 1 X GoTaq® Flexi Buffer, 0.6 mM of each primer, and 2 µl of DNA. The reaction mix was heated to 94ºC for 3 min, and amplification was performed at 94ºC for 30 sec, 50ºC and 54ºC, respectively, for 30 sec, and 72ºC for 1 min, in 35 cycles, with a final 10 min extension at 72ºC. Negative and positive controls were run with each reaction. The positive PCR products were purified and sequenced by a commercial sequencing facility (Macrogen Europe, Netherlands). The two positive sequences, were deposited in GenBank under accession numbers KT364883 (from *Hemidactylus luqueorum*) and KT364884 (from *Ptyodactylus hasselquistii*).

### *2.3. Phylogenetic analysis*

Geneious v. R6.1.6 (Biomatters Ltd.) was used for assembling and editing the chromatographs. We performed a similarity analysis using the Basic Local Alignment Search Tool (BLAST) [39] to find the best match for the sequences against published sequences in GenBank. To produce an updated phylogeny of the cyt *b* gene of Haemosporida [25], we combined the information from published studies [10,28,35,40–43] (see Table S3). Whole cyt *b* gene sequences were included when available, although the alignment consisted of different length sequences due to the discrepancies in sequencing between studies (total alignment length 1131 bp). A rooting approach that does not require an outgroup to be defined a priori was used, as this has been shown to be the most appropriate method to estimate the phylogenetic relationships between Haemosporida [25]. It has also been reported that the cyt *b* gene of these parasites often displays saturation in substitutions and biased frequencies at third-codon positions [44]. Phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. We used PartitionFinder v1.1.0 [45] to infer the partition scheme and the model of sequence evolution for each partition with the following searching criteria: branchlengths = linked; models = raxml or models = mrbayes, depending on whether the output was used for ML or BI analyses; model_selection = BIC; three datablocks (one for each codon position of the cyt b gene); and search = all. The optimal gene partitioning scheme was all three codon positions together (single partition for the cyt *b* gene) and the selected model was the General Time Reversible, taking into account the shape of the gamma distribution and the number of invariant sites (GTR + G + I). Maximum Likelihood analysis was performed with RAxML v.7.0.3 [46], and reliability of the ML tree was assessed by bootstrap analysis [47] with 1000 replications. Bayesian Inference (BI) was performed using BEAST v1.7.5 [48]. Three independent runs of $5 \times 10^7$ generations were carried out, sampling at intervals of 10,000 generations, producing 5000 trees each. Models and prior specifications applied were as follows (otherwise by default): model of sequence evolution for the single cyt *b* partition GTR+I+G; Relaxed Uncorrelated Lognormal Clock; Yule birth death tree prior for the phylogenetic reconstruction; random starting tree; base substitution prior Uniform (0,100); and alpha prior Uniform (0,10). Posterior trace plots and effective sample sizes (ESS N 200) of the runs were monitored in Tracer v1.6 [49] to ensure convergence. The results of the individual runs were combined in LogCombiner discarding 10% of the samples and the consensus tree was produced with TreeAnnotator (both provided with the BEAST package). All trees were displayed in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). The number of variable and parsimony-informative sites, and uncorrected p-distances were calculated in MEGA v6.06 [50].

## 3. Results and Discussion

### *3.1. Phylogenetic relationships of Haemosporida*

The final cyt *b* dataset contained 309 sequences, composed of sequences of different lengths. The number of variable sites was 640 and of parsimony-informative sites 561. The BI and the ML approaches used in this study are similar regarding the topology and composition of most of the lineages and clades (Table S3), although the ML analysis lacks support for most important nodes (Figure 4-1). These poorly-supported nodes might be the result of using sequences of different lengths, which have been included because excluding missing data would require excluding many key taxa from the analyses. However, it has been shown that in general it is preferable to include some missing data rather than not including key taxa when estimating phylogenetic relationships [51].

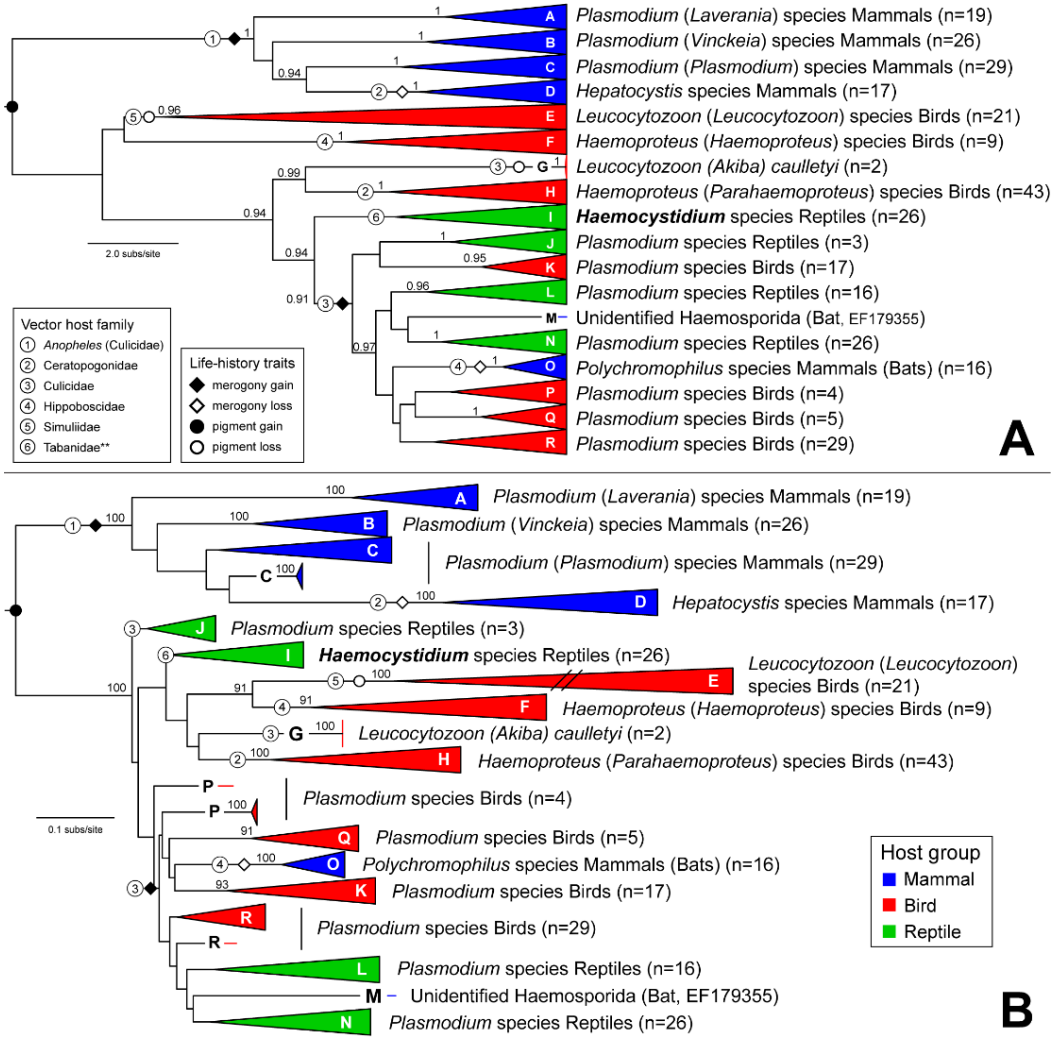


Figure 4-1 Trees derived from Bayesian Inference (BI) and Maximum Likelihood (ML) analyses of the cyt *b* gene of Haemosporida. A) BI using a Relaxed Uncorrelated Lognormal Clock prior that places the root between mammalian *Plasmodium*/*Hepatocystis* and all other parasite lineages. Bayesian Posterior Probability values above 0.90 are shown by the nodes. B) ML rooted between mammalian *Plasmodium*/*Hepatocystis* and all other parasite lineages. Maximum Likelihood bootstrap values above 70 are given above relevant nodes. In the ML analysis (B) the *Leucocytozoon* clade was shortened by 50%. Diamonds indicate merogony gain or loss and circles indicate hemozoin pigment gain or loss [25]. Numbers inside circles indicate the host vector family [9,10]. ** Tabanidae are known vectors of *Haemocystidium* (*Simondia*) that infect North American chelonians, but the vectors of squamate *Haemocystidium* (*Haemocystidium*) are still unknown [9]. Colors indicate the vertebrate host. The new sequences from this study are placed in the highlighted *Haemocystidium* clade (see Table S3, Figure S5 and Figure S6 for more details).

Both estimates of phylogeny differ from the previous most complete analysis for cyt *b* gene [25] mainly in the position of the genera *Haemoproteus* (*Haemoproteus*), *Haemoproteus* (*Parahaemoproteus*) and *Leucocytozoon*. Based on our BI analysis, *Leucocytozoon* and *Haemoproteus* appear as sister taxa to avian and reptilian *Plasmodium*, in contrast with the aforementioned study in which these appear as derived lineages (Figure 4-2). Our dataset contains sequences from genera that were underrepresented in that study, such as *Haemocystidium* and *Leucocytozoon* (*Akiba*), which may explain these differences in topology. These results corroborate the suggestion that *Leucocytozoon* is not appropriate for rooting the Haemosporida tree when using only the mitochondrial cyt *b* gene [10,25] because it branches within the Haemosporida group [24]. Therefore, depending on the phylogenetic analyses and taxon sampling used, these relationships are bound to change from study to study [9] and highlight that the current taxonomic status of Haemosporida may need revision. Our updated cyt *b* phylogeny corroborates the studies that show paraphyly of mammalian *Plasmodium* [10] (clades A–C) as a result of mammalian *Hepatocystis* (clade D) branching within it (Fig. 1), cryptic diversity within mammalian *Plasmodium* [52], polyphyly of bird *Plasmodium* [9,25,28], and the distinction between bird and reptilian *Haemoproteus*, with monophyly of reptilian *Haemocystidium* [35]. Therefore, based on the cyt *b* gene both avian and reptilian *Plasmodium* are polyphyletic as lineages occur in different parts of the tree with unresolved relationships with other genera, such as *Polychromophilus* from chiropteran hosts (clade O).

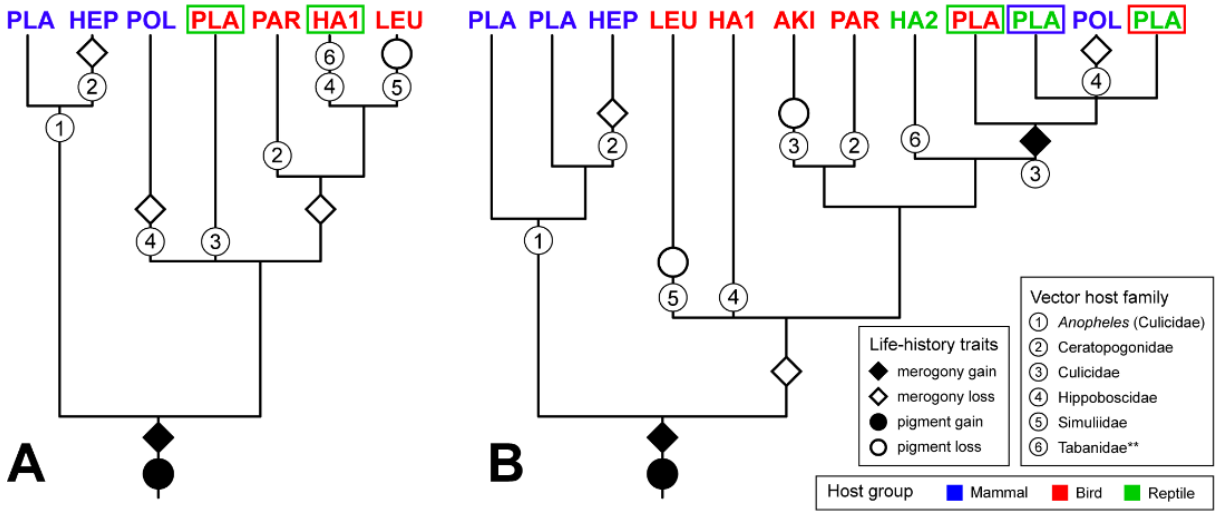


Figure 4-2 Topologies for the evolution of Haemosporida based on cyt *b* under a relaxed molecular clock.
A) Adapted from Outlaw and Ricklefs (2011) topology. B) Most updated enlarged cyt *b* gene dataset. PLA, *Plasmodium*; HEP, *Hepatocystis*; POL, *Polychromophilus*; PAR, *Parahaemoproteus*; HA1, *Haemoproteus*; HA2, *Haemocystidium*; LEU, *Leucocytozoon*; AKI, *Akiba*. Colored rectangles indicate a smaller fraction of the corresponding host group that is also found in this clade (mainly composed of the host colors in the letters). Diamonds indicate merogony gain or loss and circles indicate hemozoin pigment gain or loss [25] and numbers inside circles indicate the host vector family [9,10] (Figure 4-1).

Interestingly, sequence EF179355 identified as an unknown Haemosporida and obtained from the lesser false vampire bat (*Megaderma spasma*) from Cambodia (lineage M) [40] is placed inside reptilian *Plasmodium* clades (clades L and N, Figure 4-1; see Figure S5 and Figure S6). This lineage poses important questions for transmission dynamics of these parasites, such as: is this a new and underrepresented clade of haemosporidians of bats? Is it derived from *Polychromophilus* or has it arisen from horizontal gene transfer from an avian and/or reptilian host?

Regarding the reconstruction of the evolutionary history of Haemosporida based on the cyt *b* gene, our results corroborate the fact that life-history traits do not seem to have played a major role in major cladogenetic events within this order [10]. In our phylogeny, there has been a loss of merogony in the ancestral form that gave rise to avian *Leucocytozoon* and *Haemoproteus* and the ancestral form of avian and reptilian *Plasmodium* regained this trait. In contrast, the major cladogenetic events seem to be associated with vector host shifts as previously proposed [10],with vector host families associated with the main clades (Figure 4-1 and Figure 4-2). Moreover, some studies show that host identity may be an invalid taxonomic character in avian hosts due to frequent host shifts and host-sharing of Haemosporida (e.g. *Haemoproteus* spp. [53–55]). Our results show that this might be particularly frequent for avian and reptilian *Plasmodium* (Figure 4-2). Therefore, when the vector host diversity and specify are better understood, an alternative to the current parasite naming system of haemosporidians would be to take into account the vector host. Regarding reptilian *Haemocystidium*, the chelonian haemosporidians *Haemocystidium* (*Simondia*) may be transmitted by tabanid flies (Tabanidae), however the vectors of *Haemocystidium* (*Haemocystidium*) that infect squamate hosts are still unknown [35]. Our estimate of phylogeny is solely based on a single mitochondrial gene. A recent study that included several nuclear genes found alternative relationships, such as *Leucocytozoon* was basal to *Haemoproteus* and *Plasmodium* or that mammalian *Plasmodium* was sister taxa to bird and reptile *Plasmodium* [59].

Finally, we show some potential misidentifications of sequences deposited in GenBank. This growing problem was already known (e.g. AF069613 that was originally identified as *Haemoproteus columbae*, appears to be a *Plasmodium* species [56] and was corroborated in our study (clade Q, Figure S5 and Figure S6)]. For instance, a sequence previously identified as *Plasmodium* sp. from the lizard *Egernia stokesii* (EU254531) is placed in the *Haemocystidium* clade (see Supplementary Figure S5 and Figure S6). This misidentification may have been due to a lack of taxon sampling of reptile *Haemocystidium* at the time of the study [10,44]. Moreover, sequences identified as *Plasmodium relictum*, the most common *Plasmodium* species in birds, appear in different lineages within clade R of bird malaria (e.g. EU254538 and AY733090). To reduce this kind of misidentifications, the database MalAvi [57] was created in order to better characterize the diversity of avian Haemosporida. Although reptilian Haemosporida are still relatively understudied in comparison to bird Haemosporida, it would be important to create a similar platform for reptilian parasites.

### *3.2. Characterization of Haemocystidium in geckos from Oman*

Of the 80 samples screened with microscopy, only 2 (3%) were infected with Haemosporida. Infected individuals were from northern populations (*P. hasselquistii* from 22.7914 N, 59.22873 E and *H. luqueorum* from 23.18292 N, 57.41627 E, see Table 4-1 and Table S6). Intensity of Haemosporida was low, with less than 14 gametocytes per 4000 erythrocytes (Table 4-1). Microscopic examination of Haemosporida stages in the blood of the two gecko hosts showed ovoid young gametocytes inside erythrocytes (Figure 4-3) [17]. Gametocytes were small, occupying about a quarter of the erythrocyte area (Table 4-2), without considerably changing the cell morphology (Figure 4-3). Most gametocytes stained light pink, with some displaying darker pigments (Figure 4-3 B). These characteristics resemble those of young *Haemocystidium* stages in chelonians [35,43] and lizards [58].

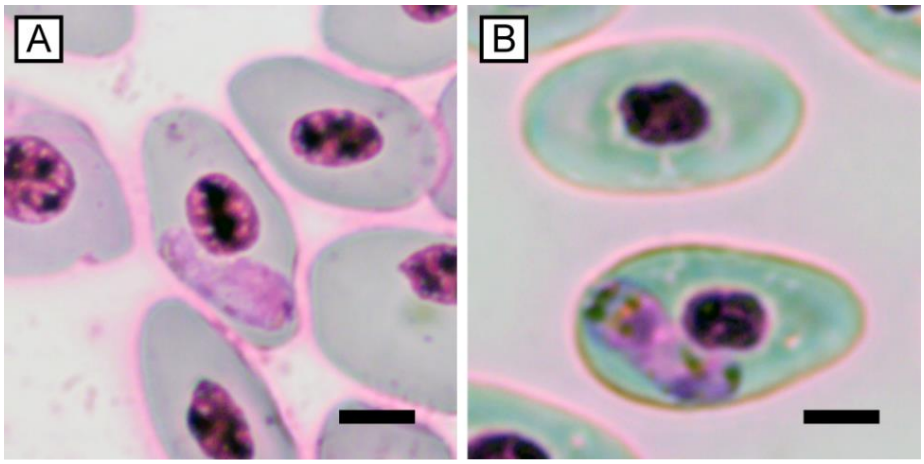


Figure 4-3 Haemosporida parasites found in reptile hosts from Oman.
*Haemocystidium* sp. from *Hemidactylus luqueorum* (sample S7155, Figure 4-3 A);
and from *Ptyodactylus hasselquistii* (sample S7688, Figure 4-3 B). Scale bar is 5 µm.

Table 4-2 Microscopy measurements of Haemosporida intracellular parasites infecting host erythrocytes under 1000x magnification. *n* refers to the number of parasites measured per sample.

| | | | Haemosporida - Mean ± Sd | | | | |
|---|---|---|---|---|---|---|---|
| Host species | sex | code | *n* | Vertical | Horizontal | Area | Perimeter |
| *Hemidactylus luqueorum* | F | S7155 | 13 | 10.80 ± 0.87 (9.50-12.29) | 4.33 ± 0.35 (3.85-5.05) | 41.81 ± 3.54 (36.35-49.00) | 29.69 ± 1.38 (27.78-32.00) |
| *Ptyodactylus hasselquistii* | M | S7668 | 12 | 11.61 ± 0.85 (9.85-12.84) | 4.35 ± 0.53 (3.28-5.25) | 41.65 ± 4.31 (35.12-52.18) | 33.07 ± 1.85 (28.58-35.42) |
| | | | 25 | 11.20 ± 0.86 (9.50-12.84) | 4.34 ± 0.44 (3.28-5.25) | 41.73 ± 3.92 (35.12-52.18) | 31.38 ± 1.62 (27.78-35.42) |

The cyt *b* sequences obtained for these two samples were placed in a clade that is exclusively composed of haemosporidians of reptiles (clade I, Figure 4-1, Figure S5 and Figure S6) and that was recently reclassified as *Haemocystidium* based on morphological and genetic data in comparison to avian *Haemoproteus* (clades F and H, Figure 4-1, Figure S5 and Figure S6) [35]. The aforementioned study was the first to assess the molecular diversity of reptilian *Haemocystidium* and showed the uncertainty of the classification of these parasites. In our BI analysis of the cyt *b* gene,

the *Haemocystidium* clade is sister taxa to reptile and bird *Plasmodium* and mammalian *Polychromophilus* (clades J–R, Figure 4-1) and all these are sister taxa to bird *Haemoproteus* (*Parahaemoproteus*) (clade H, Figure 4-1) and *Leucocytozoon* (*Akiba*) *caulletyi* (clade G, Figure 4-1).

The two new sequences from Oman were genetically identical. The closest match on GenBank to our two new sequences was from *Haemocystidium* sp. from the collared iguana *Oplurus cuvieri* (DQ212191) from Madagascar, with an uncorrected p-distance of 1.9% (Table 4-3). The other estimates of sequence divergence for the cyt *b* gene show that our sequences diverge by 6–7% from *Haemocystidium* species from other reptiles (Table 4-3). The cyt *b* gene has been the most widely used gene for identifying new species, for which the generally accepted cut-off has been above 5% divergence [31]. Finding similar Haemosporida in two gecko species belonging to two different families, *H. luqueorum* (Gekkonidae) and *P. hasselquistii* (Phyllodactylidae) is further evidence that describing new species based on identification in new host species without further information is not a good practice [18]. Indeed, *P. hasselquistii* may have at least two different *Haemoproteus* species, that is, *Haemoproteus ptyodactylii* and that reported in this study. Based on the fact that the new haemosporidian sequences differ from named *Haemoproteus* species by more than 5% in cyt *b*, it is reasonable to assume that these may represent a new species of *Haemoproteus* that is currently found in geckos from Oman and possibly also in an iguanid lizard from Madagascar. However, the determination of the genetic diversity within this putative new species is needed, as well as the identification of the vectors.

Table 4-3 Estimates of evolutionary divergence between selected species of Haemosporida and the haplotype retrieved in this study. The number of base differences per site from between sequences are shown in ascending order for the cyt *b* gene.

| Parasite species | code | p-distance | Host species | Geographic locality | Ref |
|---|---|---|---|---|---|
| *Haemocystidium* sp. | S7155 | - | *Hemidactylus luqueorum* | Oman | this study |
| *Haemocystidium* sp. | S7668 | - | *Ptyodactylus hasselquistii* | Oman | this study |
| *Haemocystidium* sp.[9] | DQ212191[9] | 0.019 | *Oplurus cuvieri* | - | unpub |
| *Haemocystidium pacayae* | KF049495 | 0.059 | *Podocnemis expansa* | Peru | (Pineda-Catalan *et al*., 2013) |
| *Haemocystidium ptyodactylii* | AY099057 | 0.069 | *Ptyodactylus hasselquistii* | Israel | (Perkins and Schall 2002) |
| *Haemocystidium kopki* | AY099062 | 0.073 | *Teratoscincus scincus* | Pakistan | (Perkins and Schall, 2002) |
| *Haemocystidium mesnili* | KF049514 | 0.073 | *Naja annulifera* | South Africa | (Pineda-Catalan *et al*., 2013) |
| *Haemocystidium* sp.[10] | EU254531[10] | 0.074 | *Egernia stokesii* | Australia | (Martinsen *et al*., 2008 |
| *Haemocystidium anatolicum* | JQ039742 | 0.077 | *Testudo graeca* | Turkey | (Orkun and Guven, 2013) |
| *Haemocystidium peltocephali* | KF049491 | 0.085 | *Podocnemis expansa* | Peru | (Pineda-Catalan et al., 2013) |

[9] Identified as *Haemoproteus* sp. on GenBank.
[10] Identified as *Plasmodium* sp. on GenBank.

## 4. Conclusion

Our study presents a reconstruction of the evolutionary history of Haemosporida for the cyt *b* gene and corroborates the need for a taxonomic revision of this order, as well as the need for more taxon sampling, especially regarding reptilian haemosporidians. Phylogenetic relationships may change based on the gene(s) used, therefore future studies should include both mitochondrial and nuclear genes of all genera within Haemosporida. The newly detected *Haemocystidium* sp. in geckos from Oman may represent a different strain or a possible new species that needs to be further sampled and the vectors identified. Our results also exemplify the impact of taxon sampling biases on the phylogenetic relationships of the group based on the cyt *b* gene that are important for understanding their evolutionary history. The observation that major cladogenic events seem to be associated with shifts between vector host families highlights the need for identification of the vectors and the study of the vector stages in underrepresented taxa, such as *Haemocystidium*.

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## 4.2 Article VII - A note on using 18S rRNA gene sequences for estimating relationships of hemogregarines (Apicomplexa, Adeleorina): current limitations and future prospects

In preparation

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**Abstract**

Hemogregarines are a group of apicomplexan parasites composed of three families that infect a wide range of hosts. Many species within these families have been subjected to reclassifications and reassignments, especially since the use of molecular tools to estimate their phylogenetic relationships became more widespread. The 18S rRNA gene has been the only widely used gene for studying the diversity of hemogregarines and recent phylogenetic analyses of this gene have indicated some incongruences with the current taxonomy. To further investigate the current taxonomic situation and potential of using this gene, we conducted an overview of all published 18S rRNA sequence data for hemogregarines. Phylogenetic analysis support the paraphyly of Hepatozoidae with the Karyolysidae family, and shows considerable genetic diversity of the genus *Hepatozoon* with no clear association with intermediate vertebrate host taxonomy or geographical location. These findings may have profound taxonomic implications that are discussed. This overview also shows that there is a bias towards some host groups from particular geographical locations, such as mammals from Asia and Europe. Finally, our knowledge of the genetic lineages found in invertebrate hosts is extremely limited, despite the fact that these hosts are a crucial part of hemogregarine lifecycle. We urge that future studies focus on the development of additional, faster evolving markers and that many host groups and remote geographical locations should be assessed to obtain a more complete estimate of the diversity of this group before a taxonomic revision is conducted.

**Keywords:** *Hepatozoon*, *Karyolysus*, *Haemogregarina*, *Hemolivia*, phylogeny, GenBank, taxonomy, incongruence.

Hemogregarines are cosmopolitan parasites that are known to infect a wide range of vertebrate host groups. Traditionally, these parasites were identified based on microscopy and sexual reproduction stages in the invertebrate host (Telford, 2009). However, diagnostic characters are often difficult to identify with microscopy and the same parasite species may have different morphologies in different host species (Sloboda *et al.*, 2007). This has led to the improper description of many parasite species in the past (Mathew *et al.*, 2000) and for this reason this group has been subjected to many reclassifications and reassignments of species (Morsy *et al.*, 2013; Abdel-Baki *et al.*, 2014; Cook *et al.*, 2014). The 18S rRNA gene has been the only widely used marker for studying genetic variation of hemogregarines, and recent assessments have uncovered unexpectedly high levels of diversity (O'Dwyer *et al.*, 2013; Tomé *et al.*, 2014; Harris *et al.*, 2015) (see also section 5.2). Hemogregarines are composed of the genera *Hepatozoon* (Hepatozoidae), *Haemogregarina*, *Desseria* and *Cyrilia* (Haemogregarinidae), *Hemolivia* and *Karyolysus* (Karyolysidae) (Smith and Desser, 1997; Telford, 2009). These are heteroxenous parasites that require more than one host to complete their lifecycle, at least a vertebrate host in which asexual reproduction occurs and an invertebrate host in which sexual reproduction occurs. The genus *Hepatozoon* is the most commonly reported hemogregarine, which is reflected in the amount of genetic data available for this genus in comparison with the others. This genus was described from rats (Miller, 1908) but has been reported in all tetrapod orders and may be transmitted by a variety of possible vectors, including mosquitoes, ticks, mites and leeches (Smith, 1996). The genus *Haemogregarina* was the first hemogregarine described in reptiles, and includes type species of the family Haemogregarinidae, which are transmitted by leeches (Telford, 2009). A recent phylogenetic study has shown that this genus forms a well-defined cluster outside the *Hepatozoon* phylogeny (Cook *et al.*, 2014). The genus *Hemolivia* is mostly known from anurans and testudines but has also been reported in lizards (Smallridge and Bull, 2000), and is transmitted by ticks (Harris *et al.*, 2013). Phylogenetic estimates indicate that *Hemolivia* may make *Hepatozoon* paraphyletic (Kvičerová *et al.*, 2014). The genus *Karyolysus* has been reported in saurian hosts and is transmitted by mites (Svahn, 1975; Telford, 2009). Another phylogenetic assessment indicates that *Karyolysus* clusters within *Hepatozoon*, in a lineage previously identified from lacertid lizard and snake hosts (Maia *et al.*, 2011; Haklová-Kočíková *et al.*, 2014). Finally, the genera *Desseria* and *Cyrilia* have not yet been genetically characterized. Hemogregarine taxonomy has long been a subject of discussion (Smith and Desser, 1997; Mathew *et al.*, 2000) and these recent studies highlight the need for a taxonomic revision. However, these studies are usually based on a limited number of sequences from selected host species and hemogregarine species. For this reason, we conducted an overview of all 18S rRNA gene data available on public databases for hemogregarines with the objectives to: i) assess the current taxonomy applied to hemogregarines compared to their phylogenetic relationships; and ii) provide prospects for future research by elucidating the less studied host groups and geographical locations.

All hemogregarine sequences available on GenBank were downloaded to produce an overview of the data available for Haemogregarinidae. The following search terms were used: hemogregarine, haemogregarine, hepatozoon, haemogregarina, hemolivia and karyolysus. Sequences from section 5.2 and Tomé et al. (unpublished) were also added to the dataset. A total of 1007 sequences were used and *Dactylosoma ranarum* sequences were designated as outgroup following (Barta *et al.*, 2012) (see Table S5 for more details). These were aligned using MAFFT v7 algorithm (Katoh and Standley, 2013) with the G-INS-1 progressive method and applying parameters by default (Gap opening penalty: 1.53, Offset value: 0.0). RAxML v.7.0.3 (Stamatakis, 2006) using a GTR+GAMMA model to estimate the best tree with 10 ML searches. The resulting tree was used to infer sequences that differed less than 5bp in order to reduce the dataset (see Table S5). The final dataset consisted of 268 sequences of different lengths (ranging from 387bp to 995bp) that were aligned using MAFFT v7 algorithm with the Q-INS-i iterative method and applying parameters by default, which resulted in an alignment of 1083bp in length. RAxML v.7.0.3 was used to assess the phylogenetic relationships with GTR+G model and reliability of the ML tree was assessed by bootstrap analysis (Felsenstein, 1985) with 1000 replications.

Our estimate of 18S rRNA gene phylogeny was similar to previous estimates with the novelty of including all diversity found in each hemogregarine genera. *Haemogregarina* (Haemogregarinidae) formed a distinct group (lineages A and B) from *Hepatozoon* and *Hemolivia*, although not monophyletic if lineage A is confirmed as *Haemogregarina* species (deposited on GenBank as *Hepatozoon* sp., KJ740753 and KJ740754). *Hemolivia* and *Karyolysus* (Karyolysidae, lineages C and K, respectively) made *Hepatozoon* (Hepatozoidae) paraphyletic as in previous studies (Cook *et al.*, 2014; Haklová-Kočíková *et al.*, 2014) (Figure 4-4). *Karyolysus* was placed inside *Hepatozoon* phylogeny and identical to previously identified *Hepatozoon* sequences from reptiles (Table S5) and for this reason we refer to this clade as the *Hepatozoon*/*Karyolysus* complex (lineage K). In addition, the genus *Hepatozoon* displayed a great degree of diversity with no general association with intermediate host taxonomy or geographical location for most lineages present in reptiles (Table 4-4). Reptile hosts may be infected by distinct lineages of *Hepatozoon* parasites (lineages E and G-K in Figure 4-4 and Table 4-4). In lizards there may be a potential separation between hemogregarines from lacertids and geckos, while in snakes most *Hepatozoon* lineages can be found (Table 4-4). For instance, group G was mostly composed of *Hepatozoon* spp. from geckos and snakes (with the exception of two lacertid lizard parasites), while group K was composed of *Hepatozoon*/*Karyolysus* spp. infecting mainly lacertid lizards and snakes, but also skinks, one gecko, one felid and one canid (Table 4-4 and Table S5). In addition, lineages I, J and K from reptile hosts are more closely related with hemogregarines found primarily in carnivores than in other reptiles (Figure 4-4). Regarding other host groups, *Hepatozoon* species from amphibians did not form a monophyletic group. In spite of *Hepatozoon* species from anurans from Asia, Europe and North America forming a monophyletic group (lineage F), a sequence from an anuran from South America

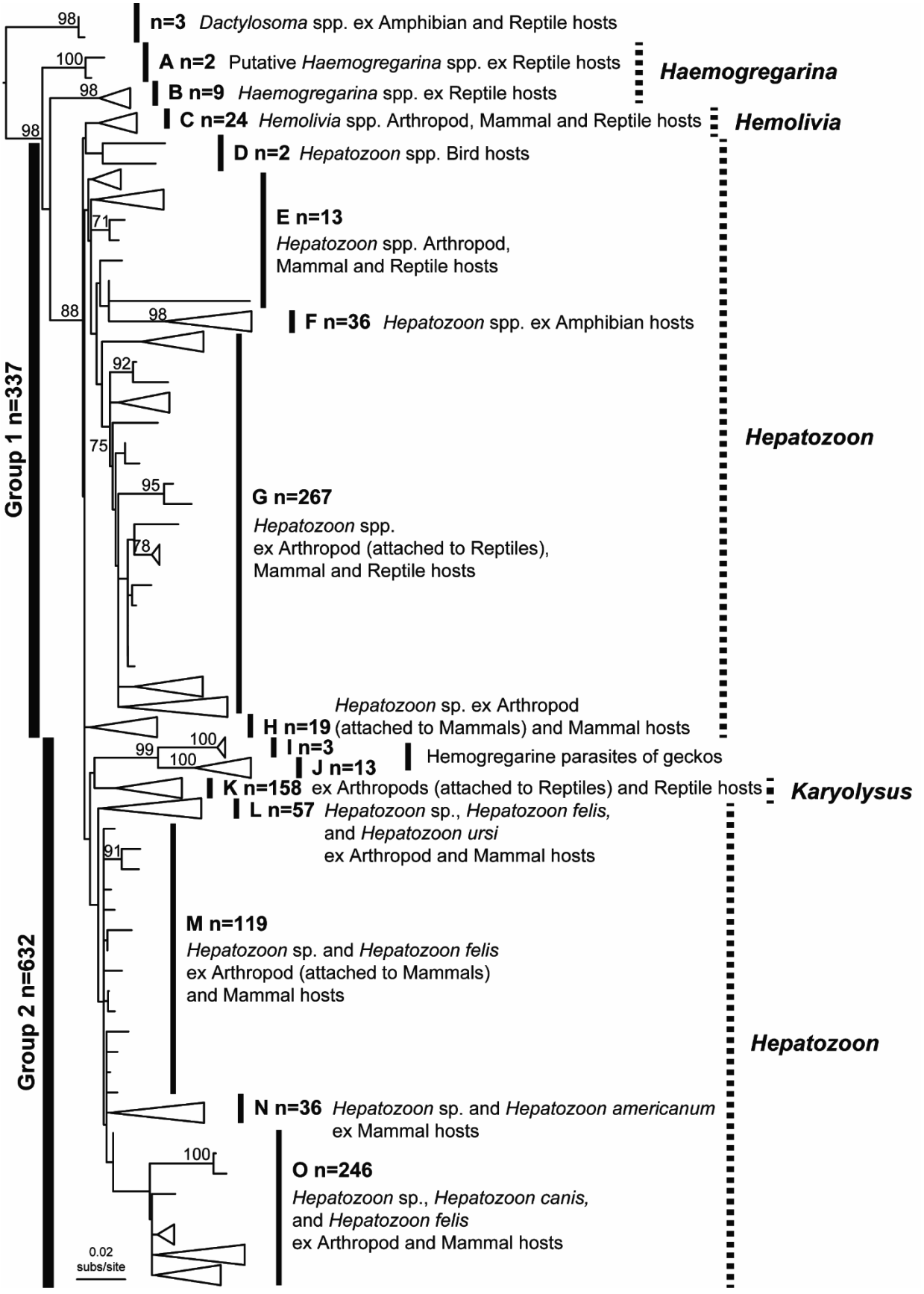


Figure 4-4 Tree derived from a Maximum Likelihood analysis of a representative set of hemogregarine 18S rRNA gene sequences available on GenBank.
See Table S5 for more details. Bootstrap values for Maximum Likelihood above 70 are given.

(JX987775) and *Hepatozoon seychellensis* from caecilians were found in a lineage primarily from reptiles and rodents (lineage G, Figure 4-4 and Table 4-4). For avian hosts, only two *Hepatozoon* sequences were available (GU344682 and KF022102) and these appeared as a distinct lineage with an unresolved position in the hemogregarine phylogeny, sister taxa to *Hemolivia* spp. from testudines (lineages D and C, respectively, Figure 4-4). *Hepatozoon* spp. from marsupial hosts were primarily found in an unresolved position between the two main hemogregarine groups (lineage H, Figure 4-4), with the exception of a sequence from the *Virginia opossum* (JF491225), a host species only found in North America that was placed in lineage E (Table 4-4). Rodent hosts, from which the *Hepatozoon* genus was established (Miller, 1908), may be primarily infected with *Hepatozoon* species from lineage G (Table 4-4). With the exception of a sequence obtained from *Hydrochaeris hydrochaeris* (the largest rodent in the world, EU249993), a sequence from *Marmota monax* (JF491241) and a sequence from cotton rats experimentally infected with *Hepatozoon americanum* (EU249993, see Table S5). Canid hosts may be primarily infected with *Hepatozoon* lineages N and O, with the exception of a five sequences from dogs and foxes that are placed in lineages G (Table S5), a sequence from a wild dog in lineage K (KF270651) and from a dog in lineage L (KF270654). Felid hosts may be infected with several different *Hepatozoon* lineages, with the majority belonging to lineages L and M, with the exception of a sequence from a panther in lineage K, from a cat and a lynx in lineage N, and from cats in lineage O (Table S5). The occasional occurrence of *Hepatozoon* from carnivore hosts in lineages that are primarily found in prey host species (e.g. lineages G and K from reptiles and rodents) is presumably due to predator-prey transmission (Tomé *et al.*, 2012; Maia *et al.*, 2014).

Some potential misidentifications available on GenBank were detected. Two sequences identified as *Hepatozoon felis* on GenBank were placed in lineage O that is typical from canids. In addition, two sequences from *Mauremys leprosa* identified as *Hepatozoon* sp. on GenBank were closely related with *Haemogregarina* species from other testudines, including this host species (Table S5). Therefore it is likely that these sequences belong to this genus and that these were misidentified. And a sequence identified as *Hemolivia mariae* (HQ224961) is instead of *Dactylosoma ranarum*.

Various specific taxonomic issues arise from this overview, such as should *Karyolysus* be considered as a subgenus within *Hepatozoon,* at least until our understanding of hemogregarine diversity has stabilized, or should *Hepatozoon* be split into multiple new genera, as has sometimes been proposed (Smith and Desser, 1997). The fact that *Hepatozoon*/*Karyolysus* lineages (I, J and K) are more closely related to *Hepatozoon* species primarily from carnivores (L-O in main group 2 in Figure 4-4) than from other reptiles in main group 1, indicates that the future clarification of these lineages will undoubtedly have repercussions on some of the most widely known species of domestic animals, such as *Hepatozoon canis*, *Hepatozoon americanum* and *Hepatozoon felis*. The taxonomy is further complicated, in that the type species *H. perniciosum* was described from a rodent but

Table 4-4 Overview of hemogregarine 18S rRNA gene data available on public databases based on host taxonomy and geographical location. This is based in the main groups and lineages found in hemogregarine phylogeny. Capital letters indicate the groups obtained in the overview of hemogregarine phylogeny (Figure 4-4). The sums on the right indicate the total for each geographical location and host group, and the numbers at the bottom indicate the total for each phylogenetic group.

| | | | | Group 1 | | | | | | Group 2 | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Host [Class, Order (Families)]** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** | **M** | **N** | **O** | |
| **Africa** | | 4 | 1 | | 1 | 1 | 87 | | | 12 | 29 | 14 | 2 | | 67 | 218 |
| Amphibia, Anura (Pyxicephalidae) | | | | | | 1 | | | | | | | | | | 1 |
| Amphibia, Gymnophiona (Indotyphlidae) | | | | | | | 2 | | | | | | | | | 2 |
| Arachnida, Ixodida (Ixodidae) | | | 1 | | | | | | | | | | | | 10 | 11 |
| Mammalia, Carnivora (Canidae) | | | | | | | | | | | | | | | 10 | 10 |
| Reptilia, Testudines (Testudinae) | | | 1 | | | | | | | | | | | | | 1 |
| Mammalia, Carnivora (Canidae, Felidae, Hyaenidae) | | | | | | | 1 | | | | 2 | 14 | 2 | | 57 | 76 |
| Mammalia, Rodentia (Dipodidae) | | | | | | | 22 | | | | | | | | | 22 |
| Reptilia, Lacertilia (Chamaeleonidae, Gekkota, Lacertidae, Scincidae) | | | | | | | 34 | | | 12 | 20 | | | | | 66 |
| Reptilia, Serpentes (Colubridae, Elapidae, Lamprophiidae, Pythonidae) | | | | | | | 28 | | | | 7 | | | | | 35 |
| Reptilia, Testudines (Geoemydidae, Pelomedusidae, Testudinidae) | | 4 | | | 1 | | | | | | | | | | | 5 |
| **Asia** | | 2 | 10 | | | 27 | 92 | | 3 | 1 | 2 | 23 | 115 | | 32 | 307 |
| Amphibia, Anura (Bufonidae) | | | | | | 27 | | | | | | | | | | 27 |
| Arachnida, Ixodida (Ixodidae) | | | | | | | 7 | | | | | 1 | 36 | | 19 | 63 |
| Mammalia, Artiodactyla (Suidae) | | | | | | | | | | | | 1 | | | | 1 |
| Mammalia, Carnivora (Felidae) | | | | | | | | | | | | | 36 | | 17 | 53 |
| Reptilia, Serpentes (Elapidae) | | | | | | | 7 | | | | | | | | | 7 |
| Arachnida, Mesostigmata (Macronyssidae) | | | | | | | 1 | | | | | | | | | 1 |
| Mammalia, Carnivora (Canidae, Felidae, Hyaenidae, Mustelidae, Ursidae) | | | | | | | | | | | | 22 | 79 | | 13 | 114 |
| Mammalia, Chiroptera (Hipposideridae) | | | | | | | 3 | | | | | | | | | 3 |
| Mammalia, Rodentia (Muridae) | | | | | | | 1 | | | | | | | | | 1 |
| Reptilia, Lacertilia (Gekkota, Varanidae) | | | | | | | 51 | | 3 | 1 | | | | | | 59 |
| Reptilia, Serpentes (Colubridae, Elapidae, Pythonidae, Viperidae) | | | | | | | 30 | | | | 2 | | | | | 32 |
| Reptilia, Testudines (Geoemydidae, Testudinidae) | | 2 | 10 | | | | | | | | | | | | | 12 |
| **Australia** | | | 6 | | | | 13 | 17 | | | | | | | | 36 |
| Arachnida, Ixodida (Ixodidae) | | | 3 | | | | 3 | 4 | | | | | | | | 10 |
| Mammalia, Marsupialia Dasyuromorphia (Dasyuridae) | | | | | | | | 4 | | | | | | | | 4 |
| Reptilia, Lacertilia (Varanidae) | | | 2 | | | | 2 | | | | | | | | | 4 |
| Reptilia, Serpentes (Colubridae) | | | 1 | | | | 1 | | | | | | | | | 2 |
| Mammalia, Marsupialia Peramelemorphia (Peramelidae) | | | | | | | | 13 | | | | | | | | 13 |
| Reptilia, Lacertilia (Scincidae, Varanidae) | | | 3 | | | | 4 | | | | | | | | | 7 |
| Reptilia, Serpentes (Colubridae) | | | | | | | 6 | | | | | | | | | 6 |

| | | | | Group 1 | | | | | Group 2 | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Host [Class, Order (Families)]** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** | **M** | **N** | **O** | |
| **Europe** | 2 | 2 | 3 | | 3 | 1 | 24 | | | | 127 | 12 | | | 106 | 280 |
| Amphibia, Anura (Ranidae) | | | | | | 1 | | | | | | | | | | 1 |
| Arachnida, Ixodida (Ixodidae) | | | | | | | | | | | 1 | 2 | | | 20 | 23 |
| Mammalia, Carnivora (Canidae) | | | | | | | | | | | | 1 | | | | 1 |
| Mammalia, Primates (Hominidae) | | | | | | | | | | | | | | | 2 | 2 |
| Reptilia, Lacertilia (Lacertidae) | | | | | | | | | | | 1 | | | | | 1 |
| Arachnida, Mesostigmata (Macronyssidae) | | | | | | | | | | | 2 | | | | | 2 |
| Insecta, Siphonaptera (Ctenophthalmidae) | | | | | | | 1 | | | | | | | | | 1 |
| Mammalia, Rodentia (Muridae) | | | | | | | 1 | | | | | | | | | 1 |
| Mammalia, Carnivora (Canidae, Felidae, Mustelidae) | | | | | | | 3 | | | | | 10 | | | 86 | 99 |
| Mammalia, Rodentia (Cricetidae, Sciuridae) | | | | | | | 10 | | | | | | | | | 10 |
| Reptilia, Lacertilia (Gekkota, Lacertidae) | | | | | | | 1 | | | | 124 | | | | | 125 |
| Reptilia, Serpentes (Colubridae) | | | | | 3 | | 9 | | | | | | | | | 12 |
| Reptilia, Testudines (Emydidae, Geoemydidae, Testudinidae) | 2 | 2 | 3 | | | | | | | | | | | | | 7 |
| **North America** | | | 1 | | 1 | 1 | 10 | | | | | 8 | 2 | 31 | 6 | 60 |
| Amphibia, Anura (Ranidae) | | | | | | 1 | | | | | | | | | | 1 |
| Arachnida, Ixodida (Ixodidae) | | | | | | | | | | | | 3 | | | | 3 |
| Mammalia, Primate (Hominidae) | | | | | | | | | | | | 3 | | | | 3 |
| Aves, Cathartiformes (Cathartidae) | | | | 1 | | | | | | | | | | | | 1 |
| Mammalia, Carnivora (Canidae, Felidae, Procyonidae) | | | | | | | | | | | | 5 | | 30 | 6 | 41 |
| Mammalia, Lagomorph (Leporidae) | | | | | | | | | | | | | 2 | | | 2 |
| Mammalia, Marsupalia Didelphimorphia, (Didelphidae) | | | | | 1 | | | | | | | | | | | 1 |
| Mammalia, Rodentia (Cricetidae, Sciuridae) | | | | | | | 8 | | | | | | | 1[11] | | 9 |
| Reptilia, Serpente (Colubridae) | - | - | - | - | - | - | 2 | - | - | - | - | - | - | - | - | 2 |
| **South America** | | | 5 | | 8 | | 41 | 2 | | | | | | 2 | 33 | 91 |
| Amphibia, Anura (Leptodactylidae) | | | | | | | 1 | | | | | | | | | 1 |
| Arachnida, Ixodida (Ixodidae) | | | | | | | | | | | | | | | 5 | 5 |
| Mammalia, Carnivora (Canidae) | | | | | | | | | | | | | | | 3 | 3 |
| Aves, Procellariiformes (Hydrobatidae) | | | | 1 | | | | | | | | | | | | 1 |
| Insecta, Diptera (Culicidae) | | | 1 | | 2 | | | | | | | | | | | 3 |
| Mammalia, Carnivora (Canidae, Felidae, Procyonidae) | | | 1 | | | | 1 | | | | | | | 2 | 28 | 32 |
| Mammalia, Marsupialia Microbiotheria (Microbiotheriidae) | | | | | | | | 2 | | | | | | | | 2 |
| Mammalia, Rodentia (Cricetidae) | | | | | | | 5 | | | | | | | | | 5 |
| Reptilia, (Crocodylidae) | | | | | | | 16 | | | | | | | | | 16 |
| Reptilia, Lacertilia (Gekkota) | | | | | 3 | | 4 | | | | | | | | | 7 |

[11] This rodent sequence was obtained in an experimental study on prey-predator transmission of *Hepatozoon canis* (lineage N) and thus does not represent a natural infection with this parasite.

| | | | | Group 1 | | | | | Group 2 | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Host [Class, Order (Families)]** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** | **M** | **N** | **O** | |
| Reptilia, Serpentes (Colubridae, Viperidae) | | | | | 3 | | 14 | | | | | | | | | 17 |
| Reptilia, Testudines (Geoemydidae) | | | 2 | | | | | | | | | | | | | 2 |
| **not specified** | | 1 | | | | 6 | | | | | | | | 3 | 2 | 12 |
| Amphibia, Anura (Ranidae) | | | | | 1 | 5 | | | | | | | | | | 6 |
| Mammalia, Carnivora (Canidae) | | | | | | | | | | | | | | 2 | 1 | 3 |
| Mammalia, Rodentia (Caviidae, Cricetidae) | | | | | | | | | | | | | | 1 | 1 | 2 |
| Reptilia, Testudines (Chelydridae) | | 1 | | | | | | | | | | | | | | 1 |
| | 2 | 9 | 24 | 2 | 14 | 35 | 267 | 19 | 3 | 13 | 158 | 57 | 119 | 36 | 246 | 1004 |

sequences recovered from natural infections in rodent hosts appear in lineage G, as part of the main group 1 (Figure 4-4). However, it is possible that these hemogregarine sequences from rodent hosts were from hemogregarine parasites, unrelated to *H. perniciosum*, which may have been present as cysts in the muscles of these hosts. Therefore it is currently unclear which of these should retain the name *Hepatozoon* if the taxonomy is changed. The limitations of using a conservative gene, such as the 18S rRNA gene, for estimating cryptic diversity and within parasite genera have been demonstrated in other apicomplexan groups, such as haemosporidians (Escalante and Ayala, 1994; Perkins, 2008). At the same time single trees will not always reflect species phylogeny (Castresana, 2007). Therefore, prior to any taxonomic revision of the group, we urge for the development of routine PCR protocols targeting faster evolving genes (Leveille *et al.*, 2014) and the use of multi-locus approaches to estimate relationships (Ramiro *et al.*, 2012). Furthermore there is still no comparative data from two genera, *Cyrilia* and *Desseria*, which is urgently needed to determine their positions within the phylogeny. Our overview shows that at present the study of invertebrate hosts is clearly lagging behind that of the vertebrate hosts, with only about a tenth of all data available on public databases accounting for invertebrate host records (Table 4-4). Therefore, there is a clear bias towards the screening of some host groups, such as domestic animals from certain geographical locations (e.g. Asia and Europe). This highlights the need for examining host-parasite associations with the invertebrate hosts that are an important part of parasites lifecycle and for sampling additional geographic locations and vertebrate host groups. The recently identified hemogregarine lineages I and J from gecko hosts indicate that entire lineages remain to be placed within the phylogeny and further demonstrate the risk of taxonomic changes prior to more sampling. The ICN code calls for stability of names when possible, and any taxonomic changes made now would, in our opinion, be premature. We suggest to refer to a *Hepatozoon*/*Karyolysus* complex until more data is available, when a new and potentially stable taxonomy can be proposed.

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# 5 HOST-PARASITE INTERACTIONS, SPATIAL AND TEMPORAL DYNAMICS OF HEMOGREGARINE INFECTIONS

**Article VIII - Maia, J. P.,** Álvares, F., Boratyński, Z., Brito, J. C., Leite, J. V. and Harris, D. J. (2014). Molecular assessment of *Hepatozoon* (Apicomplexa: Adeleorina) infections in wild canids and rodents from North Africa, with implications to transmission dynamics across distinct taxonomic groups. *Journal of Wildlife Diseases*, 50, 837-848.

**Article IX - Maia, J. P.**, Harris, D. J., Carranza, S. and Gómez-Díaz, E. In preparation. Assessing the diversity, host-specificity, distribution and infection patterns in apicomplexan parasites of reptiles from Arabia.

**Article X - Maia, J. P.**, Gómez-Díaz, E., Carranza, S. and Harris, D. J. In preparation. Temporal dynamics of hemogregarine infection in two sympatric lizard systems.

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## 5.1 Article VIII - Molecular assessment of *Hepatozoon* (Apicomplexa: Adeleorina) infections in wild canids and rodents from North Africa, with implications to transmission dynamics across distinct taxonomic groups

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**Abstract**

Parasites play a major role in ecosystems, and understanding of host–parasite interactions is important for predicting parasite transmission dynamics and epidemiology. However, there is still a lack of knowledge about the distribution, diversity, and impact of parasites in wildlife, especially from remote areas. *Hepatozoon* is a genus of apicomplexan parasites that is transmitted by ingestion of infected arthropod vectors. However, alternative modes of transmission have been identified such as trophic transmission. Using the 18S rRNA gene as a marker, we provide an assessment of *Hepatozoon* prevalence in six wild canid and two rodent species collected between 2003 and 2012 from remote areas in North Africa. By combining this with other predator–prey systems in a phylogenetic framework, we investigate *Hepatozoon* transmission dynamics in distinct host taxa. Prevalence was high overall among host species (African jerboa *Jaculus jaculus* [17/47, 36%]; greater Egyptian jerboa *Jaculus orientalis* [5/7, 71%]; side-striped jackal *Canis adustus* [1/2, 50%], golden jackal *Canis aureus* [6/32, 18%], pale fox *Vulpes pallida* [14/28, 50%], Rüppell's fox *Vulpes rueppellii* [6/11, 55%]; red fox *Vulpes vulpes* [8/16, 50%], and fennec fox *Vulpes zerda* [7/11, 42%]). Phylogenetic analysis showed further evidence of occasional transmission of Hepatozoon lineages from prey to canid predators, which seems to occur less frequently than in other predator–prey systems such as between snakes and lizards. Due to the complex nature of the *Hepatozoon* lifecycle (heteroxenous and vector-borne), future studies on these wild host species need to clarify the dynamics of alternative modes of *Hepatozoon* transmission and identify reservoir and definitive hosts in natural populations. We also detected putative *Babesia* spp. (Apicomplexa: Piroplasmida) infections in two canid species from this region, *V. pallida* (1/28) and *V. zerda* (1/11).

**Keywords:** *Babesia*; fox; hemogregarine; jackal; jerboa; prevalence; trophic transmission; vector-borne disease.

## Introduction

The role of parasites in ecosystems has been increasingly recognized, and studies have shown that host–parasite interactions can shape the structure of animal communities (Paterson and Piertney 2011) and interfere with food webs and predator–prey systems (Lafferty et al. 2008). Wildlife species are reservoirs to a wide range of important zoonotic parasites and may serve as sentinel species for emerging vector-borne diseases (Aguirre 2009). However, information about the distribution, diversity, and impacts of parasites in wildlife is still scarce, especially from remote areas, despite it being crucial for defining conservation strategies (Daszak 2000). Hepatozoonosis is a vector-borne infectious disease that has been increasingly studied in canids and rodents in the past decade due to its veterinary importance (Criado-Fornelio et al. 2006; Baneth 2011). Hepatozoonosis is caused by species of the genus *Hepatozoon* (Apicomplexa: Adeleorina), intracellular hemogregarine parasites that have been described in all tetrapod vertebrates (Smith 1996). *Hepatozoon* spp. are transmitted by invertebrate hosts such as ticks, mites, lice, fleas, reduviid bugs, sand flies, tsetse flies, mosquitoes, and leeches (Smith 1996), and transmission typically occurs after an infected invertebrate host (i.e., definitive host) has a blood meal or is ingested by a vertebrate host (i.e., intermediate host) (Telford, 2009). In addition, transmission in vector-borne diseases may be facilitated by 1) host habitat sharing and distribution of suitable vectors (Eisen and Wright 2001; Ishtiaq et al. 2008), 2) host grooming that results in ingestion of infected oocysts in the vector (Ewing et al. 2002; East et al. 2008), and 3) trophic transmission by ingestion of infective cystozoites in prey (Sloboda et al. 2008; Johnson et al. 2009) or ingestion of infected vectors attached to prey (Ewing and Panciera 2003; Johnson et al. 2009). Recent molecular studies have supported this trophic mode of transmission by detecting a few distinct *Hepatozoon* haplotypes in canids that were not related to *Hepatozoon canis* (Vojta et al. 2009) or *Hepatozoon americanum* (Almeida et al. 2013), but were rather more related to *Hepatozoon* species infecting typical prey of canids such as rodents and reptiles. These findings, together with molecular assessments of *Hepatozoon* spp. in reptile prey–predator systems (Tomé et al. 2014), emphasize the need for assessing other systems. Paratenic hosts, which are not necessary for the development of a *Hepatozoon* sp., help maintain its lifecycle because the parasite undergoes cystozoite stages that are infective for predator intermediate hosts. However, the ability of certain *Hepatozoon* spp. to infect multiple hosts remains uncertain (Johnson et al. 2008a, b) and, although *Hepatozoon* spp. have a wide range of host-spectrum (Barta et al. 2012), paratenic and reservoir hosts are mostly unknown in wildlife. Thus, there is a need to assess the distribution of *Hepatozoon* spp. in wild animals, and molecular tools present an ideal initial approach, especially for endangered or elusive, free-living host species that are difficult to sample (Wobeser 2007). Furthermore, large-scale parasite screening has the potential to determine if similar lineages occur in various hosts and provide information on transmission dynamics, important for endangered species and human health (Fayer et al. 2004). There are two *Hepatozoon* spp. associated with hepatozoonosis in canids that are geographically disjunct—*H.*

*canis* infects canids in Africa, southern Europe, the Middle East, and Asia; *H. americanum* infects canids in the Americas. In both cases, infections can cause life-threatening illness (Baneth et al. 2003). *Hepatozoon canis* is primarily transmitted by the brown dog tick, *Rhipicephalus sanguineus*, which has a cosmopolitan distribution and can be found in a wide range of host groups, but other vectors have also been associated with *H. canis* (Dantas-Torres 2010). Studies of these *Hepatozoon* spp. have mostly focused on domestic dogs due to their veterinary importance, although the first record of a hemogregarine from an African side-striped jackal (*Canis adustus*) was reported over a century ago (Nuttall 1910a), and recently a series of studies were conducted on wild canid species. *Hepatozoon* spp. have been detected in canid species that occur in Africa such as golden jackals *C. aureus* (Duscher et al. 2013), black-backed jackals *Canis mesomelas* (McCully et al. 1975), African wild dogs *Lycaon pictus* (Williams et al. 2013), and red foxes *Vulpes vulpes* (Gabrielli et al. 2010).

Jackal and fox species are important sentinel species for monitoring emerging infections because they are highly adaptable to different ecosystems and human dominated environments, are reservoirs to several diseases of zoonotic importance, and are long dispersers with wide-ranging movements that have been associated with infections in new locations (Duscher et al. 2013). Additionally, they prey on small mammals and reptiles (Sillero-Zubiri et al. 2004), allowing an assessment of *Hepatozoon* transmission dynamics by investigating infections in different prey of the same geographic region. Rodents are prey of these canids and are often highly infected intermediate hosts for *Hepatozoon* spp. (Smith, 1996), with various parasite species being pathogenic in these hosts, such as *Hepatozoon muris* in rats (*Rattus* sp.; Brumpt 1946) and *Hepatozoon balfouri* in the African jerboa (*J. jaculus*; Hoogstraal 1961). Similarly, studies on the parasite fauna of wild rodents are scarce (Criado-Fornelio et al. 2009), with a bias towards the Muridae family primarily due to their economic and veterinary importance (e.g., mice [*Mus* spp., Karbowiak et al. 2010], voles [*Microtus* spp., Pawelczyk et al. 2004], rats [*Rattus* spp., Webster and Macdonald, 1995], and spiny mice [*Acomys* spp., Bajer et al. 2006]). We used molecular tools to assess *Hepatozoon* infections in six species of wild canids (two jackal and four fox species) and two species of jerboas (*Jaculus* spp.) from remote areas in North Africa. The diversity of *Hepatozoon* parasites in other prey species of canids, such as lizards (Maia et al. 2011), and from other predator species of small mammals such as snakes (Tomé et al. 2014), have already been assessed from this region, thus allowing a detailed comparison between *Hepatozoon* spp. from multiple predators (canids and snakes) and prey (lizards and rodents). Therefore, we pose two questions: 1) How prevalent are *Hepatozoon* spp. in wild canids and rodents from North Africa?, and 2) How specific are *Hepatozoon* lineages to distinct vertebrate host taxonomic levels and what are the implications of this specificity for *Hepatozoon* transmission dynamics?

**Materials and Methods**

Sample collection was conducted between November 2003 and November 2011. We collected tissue samples from two rodent species, *J. jaculus* and *J. orientalis*, for which the phylogeographic structure is already known (Ben Faleh et al. 2012; Boratyński et al. 2012), and from six wild canid species, *C. adustus*, *C. aureus*, *V. pallida*, *V. rueppellii*, *V. vulpes*, and *V. zerda*, in North Africa (Algeria, Egypt, Ethiopia, Libya, Mauritania, Morocco, Niger, Senegal, Tunisia, and Western Sahara; Figure 5-1 and Table 5-1). All samples were preserved in 96% ethanol for molecular analysis and used to screen for hemogregarine parasites. Most samples were road kills that were identified based on morphologic traits; they were digitally photographed and their location registered using a Global Positioning System device. We analyzed 54 rodent samples and 100 canid samples from North Africa (Table 5-1). Chi-square (x2) tests were performed to test significance of differences between frequencies of infected versus uninfected individuals. DNA extraction, amplification, and sequencing DNA was extracted from tissue using a QIAamp DNA Micro Kit (Qiagen, Valencia, California, USA) following manufacturer's instructions. *Hepatozoon* parasites were screened using PCR reactions with the primers HepF300 and HepR900 (Ujvari et al. 2004), targeting part of the 18S rRNA gene. Briefly, PCR cycling for the Hep primers consisted of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 1 min (35 cycles) (see Harris et al. 2011). Negative and positive controls were run with each reaction. The PCR products obtained were purified and sequenced by a commercial sequencing facility (Macrogen Inc., Seoul, Korea).

*Phylogenetic analysis*

Sequences were analyzed using Geneious 6.0.3 (Drummond et al. 2012). The Find Heterozygotes plugin was used to detect double peak positions when peak similarity was above 50%, and the corresponding IUPAC code letter was assigned. Sixty-two parasite sequences (22 from rodent and 40 from canid hosts) were obtained. We then performed a similarity analysis using the Basic Local Alignment Search Tool (BLAST) on GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All matched known *Hepatozoon* sequences, except for two shorter sequences from *V. pallida* (KJ499478) and *V. zerda* (KJ499477) that matched *Babesia* spp. and, thus, were excluded from the phylogenetic analysis. Three *Hepatozoon* sequences (one from *V. rueppellii* from Mauritania and two from *V. vulpes* from Tunisia) were of poor quality and only used to account for prevalence (Table 5-1). DnaSP v5 (Librado and Rozas 2009) was used to estimate the number of haplotypes for individuals with no double peak positions. Representative *Hepatozoon* sequences from the host species analyzed were aligned with sequences retrieved from GenBank of parasites from hosts belonging to distinct taxonomic groups (i.e., carnivores, rodents, lizards, snakes, and amphibians) using the ClustalW algorithm (Thompson et al. 1994) with default parameters implemented in Geneious, and checked by eye. The final dataset contained 77 sequences of 513 base pairs. *Hepatozoon* sequences were deposited in GenBank under the

accessions KJ499479–KJ499515 for canid and KJ499516–KJ499537 for rodent hosts. Two phylogenetic analyses (maximum likelihood, ML, and Bayesian inference, BI) were conducted. The ML analysis with random sequence addition (100 replicate heuristic searches) was used to assess evolutionary relationships using the software PhyML 3.0 (Guindon et al. 2010). Support for nodes was estimated using the bootstrap technique (Felsenstein 1985) with 1,000 replicates. The Akaike Information Criterion (AIC), conducted in jModeltest 0.1.1 (Posada 2008), was used to choose the best model of evolution and the parameters employed (i.e., TVM+I+G). The BI was implemented using Mr. Bayes v.3.1 (Huelsenbeck and Ronquist 2001) with parameters estimated as part of the analysis. The analysis was run for 10 x $10^6$ generations, saving one tree each 1,000 generations. The log-likelihood values of the sample points were plotted against the generation time, and all the trees prior to reaching stationarity were discarded to ensure that burn-in samples were not retained. Remaining trees were combined in a 50% majority consensus tree (Huelsenbeck and Ronquist 2001). Following Barta et al. (2012), *Haemogregarina balli* and *Dactylosoma ranarum* were used as outgroups.

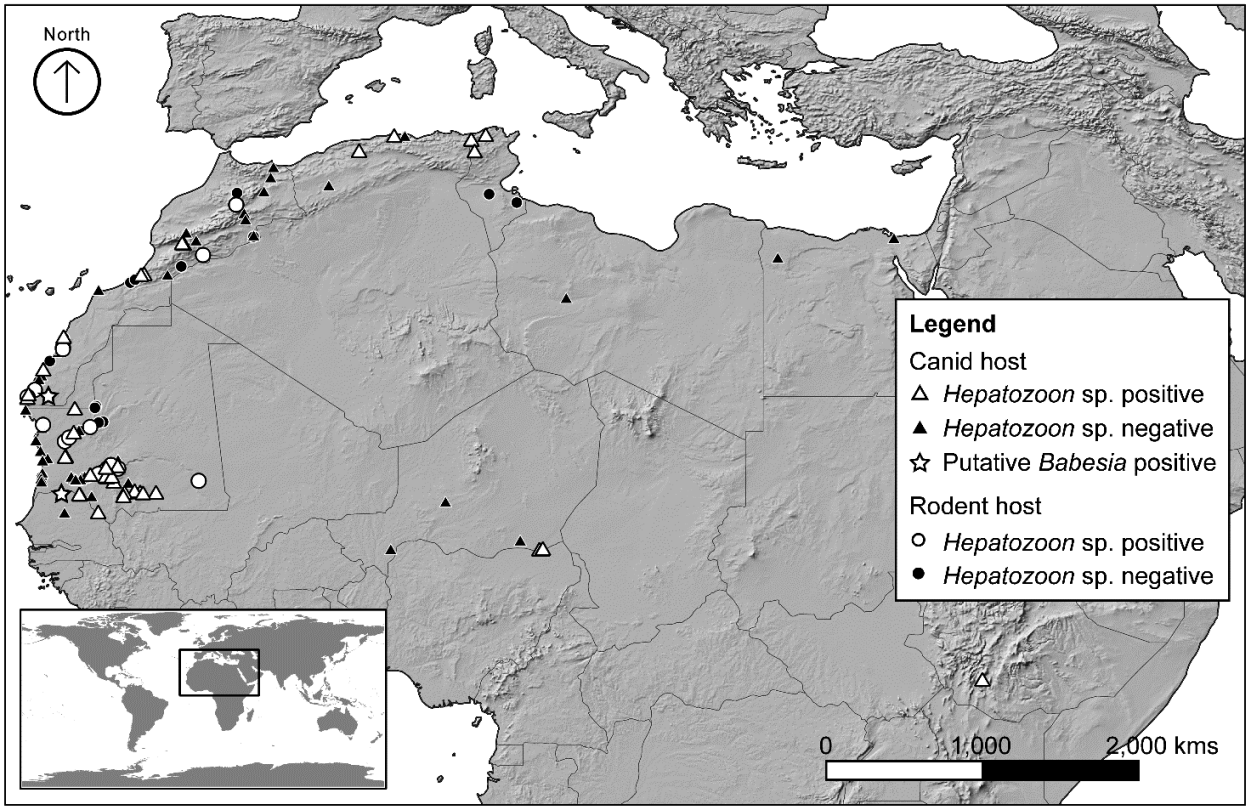


Figure 5-1 Sampling sites of wild canids and rodents examined for *Hepatozoon* parasites and *Babesia* spp. in remote areas of North Africa between 2003 and 2012.

## Results

### *Prevalence*

Overall prevalence of *Hepatozoon* spp. was similar in rodents (41%) and in canids (42%) (Table 5-1), and infection was distributed all across the sampling area (Figure 5-1). Overall prevalence was higher in foxes (35/66) than in jackals (7/34) ($x^2$=59.695, d=51, P<0.001). Additionally, putative *Babesia* spp. were detected in two canid species: *V. pallida* from Mauritania (1/28, 4% prevalence) (KJ499478), for which the most-similar matches were *Babesia conradae* (AF158702) and *Babesia gibsoni* (AF231350) (97% similarity); and *V. zerda* from the Western Sahara (1/ 11, 9% prevalence) (KJ499477), for which the most similar GenBank match was a *Babesia* sp. from the spotted hyena *Crocuta crocuta* from Zambia (KF270672, 96% similarity).

Table 5-1 Prevalence estimates for *Hepatozoon* in samples of wild canid and rodent species from North Africa. Number of positives and total number of samples per country are given in parenthesis.

| Species | Common name | *n* | Positive | Prev (%) | Country |
|---|---|---|---|---|---|
| *Canis adustus* | Side-striped Jackal | 2 | 1 | 50 | Ethiopia (1/1), Senegal (0/1) |
| *Canis aureus* | Golden Jackal | 32 | 6 | 19 | Algeria (2/5), Mauritania (4/16), Morocco (0/9), Libya (0/1), Western Sahara (0/1) |
| *Vulpes pallida* | Pale Fox | 28 | 14 | 50 | Mauritania (10/22), Niger (3/5), Senegal (1/1) |
| *Vulpes rueppellii* | Rüppell's Fox | 11 | 6 | 55 | Egypt (0/1), Mauritania (5[12]/6), Morocco (1/4) |
| *Vulpes vulpes* | Red Fox | 16 | 8 | 50 | Algeria (0/1), Egypt (0/1), Morocco (4/10), Tunisia (4[13]/4) |
| *Vulpes zerda* | Fennec Fox | 11 | 7 | 64 | Mauritania (3/4), Morocco (1/3), Western Sahara (3/4) |
| | Total | 100 | 42 | 42 | |
| *Jaculus jaculus* | Lesser Egyptian Jerboa | 47 | 17 | 36 | Mauritania (12/25), Morocco (1/6), Tunisia (0/2), Western Sahara (4/14) |
| *Jaculus orientalis* | Greater Egyptian Jerboa | 7 | 5 | 71 | Morocco |
| | Total | 54 | 22 | 42 | |

### *Phylogenetic relationships*

Both phylogenetic analyses produced similar estimates of relationships for the 18S rRNA gene of the *Hepatozoon* parasites; thus we present one phylogenetic tree (Figure 5-2). Among the 62 *Hepatozoon* sequences retrieved from rodents and canids from North Africa, 10 haplotypes were found (two from rodent and eight from canid hosts) as well as double peak positions in *C. aureus*, *J. jaculus*, and *V. zerda*. These haplotypes were distributed in two main clades. Parasites from amphibian hosts are sister taxa to clade 1, which is composed of *Hepatozoon* sequences primarily from reptiles and rodents and a few distinct sequences from carnivores (a single sequence from the pale fox *V. pallida*, from Senegal in this study [KJ499479], and published sequences from the

[12] One sequence was of poor quality but matched known *Hepatozoon* sp. using Basic Local Alignment Search Tool.
[13] Two sequences were of poor quality matched known *Hepatozoon* sp. using Basic Local Alignment Search Tool.

domestic dog *Canis lupus familiaris* from Croatia [FJ497023] and the crab-eating fox *Cerdocyon thous* from Brazil [KC127680]). Clade 2 is composed of two weakly supported groups, one of *Hepatozoon* sequences from reptile hosts and another with *Hepatozoon* sequences mainly from mammalian carnivore hosts. Within the mammalian carnivore group, *H. canis* forms a clearly distinct lineage and displays some variation with unresolved relationships.

*Host-specificity and predator–prey systems*

Genetically identical *Hepatozoon* parasites for the 18S rRNA gene fragment analyzed were detected in distantly related host taxa, suggesting that certain *Hepatozoon* spp. may have low host-specificity. For example, a *Hepatozoon* sp. from the pale fox *V. pallida* (KJ499479) is identical to *Hepatozoon ayorgbor* from the royal python *Python regius* (EF157822). Identical *Hepatozoon* sequences were also obtained for more-closely related host taxa such as 1) in the Moroccan eyed lizard *Timon tangitanus* (HQ734807) and the Bocage's wall lizard *Podarcis bocagei* (JX531921); 2) in the hissing sand snake *Psammophis sibilans* (KC696567) and the horseshoe whip snake *Hemorrhois hippocrepis* (JX244267); and 3) in the schokari sand racer *Psammophis schokari* (KC696565) and *He. hippocrepis* (JX244269). In addition, distinct *Hepatozoon* lineages belonging to the two main clades identified in this study were observed in the same host species for several canid hosts (*V. pallida* [KJ499479 and KJ499502, number 5 in Figure 5-2], *C. l. familiaris* [FJ497023 and DQ439540, number 6 in Figure 5-2], and *Ce. thous* [KC127680 and KC127679, number 1 in Figure 5-2]), for snake hosts (*He. hippocrepis* [JX244267 and JX244269, number 4 in Figure 5-2] and *P. schokari* [KC696564 and KC696565, number 2 in Figure 5-2]) and lizard hosts (*T. tangitanus* [HQ734807 and HQ734799, number 3 in Figure 5-2]) and *Podarcis* species [JX531921 and HQ734793]). Furthermore, identical *Hepatozoon* parasite sequences were found in predator and prey host species such as in the domestic dog *C. l. familiaris* (FJ497023), the prey bank vole *Myodes* (*Clethrionomys*) *glareolus* (AY600625), the horned desert viper *Cerastes cerastes* (EF12058), and in the prey common wall gecko *Tarentola mauritanica* (HQ734806) (Figure 5-2).

**Discussion**

We provide the first molecular assessment of *Hepatozoon* infections, an emergent zoonotic disease, in wild (undomesticated) canids and rodents from remote areas of North Africa. Our results provide new insights on the specificity of *Hepatozoon*, and we discuss the implications of these results on the role of paratenic, intermediate, and definitive host species in the transmission of *Hepatozoon* spp. Overall prevalence of *Hepatozoon* infection was high. *Hepatozoon* parasites have heteroxenous lifecycles and are vector-borne; thus, variations in prevalence across host species can be influenced by several factors, such as vector competence and distribution, as shown in other host–parasite systems (Eisen and Wright 2001; Ishtiaq et al. 2008). Also, host immune condition (Schmid-Hempel 2003), habitat characteristics of the geographic regions analyzed (Knowles et al.

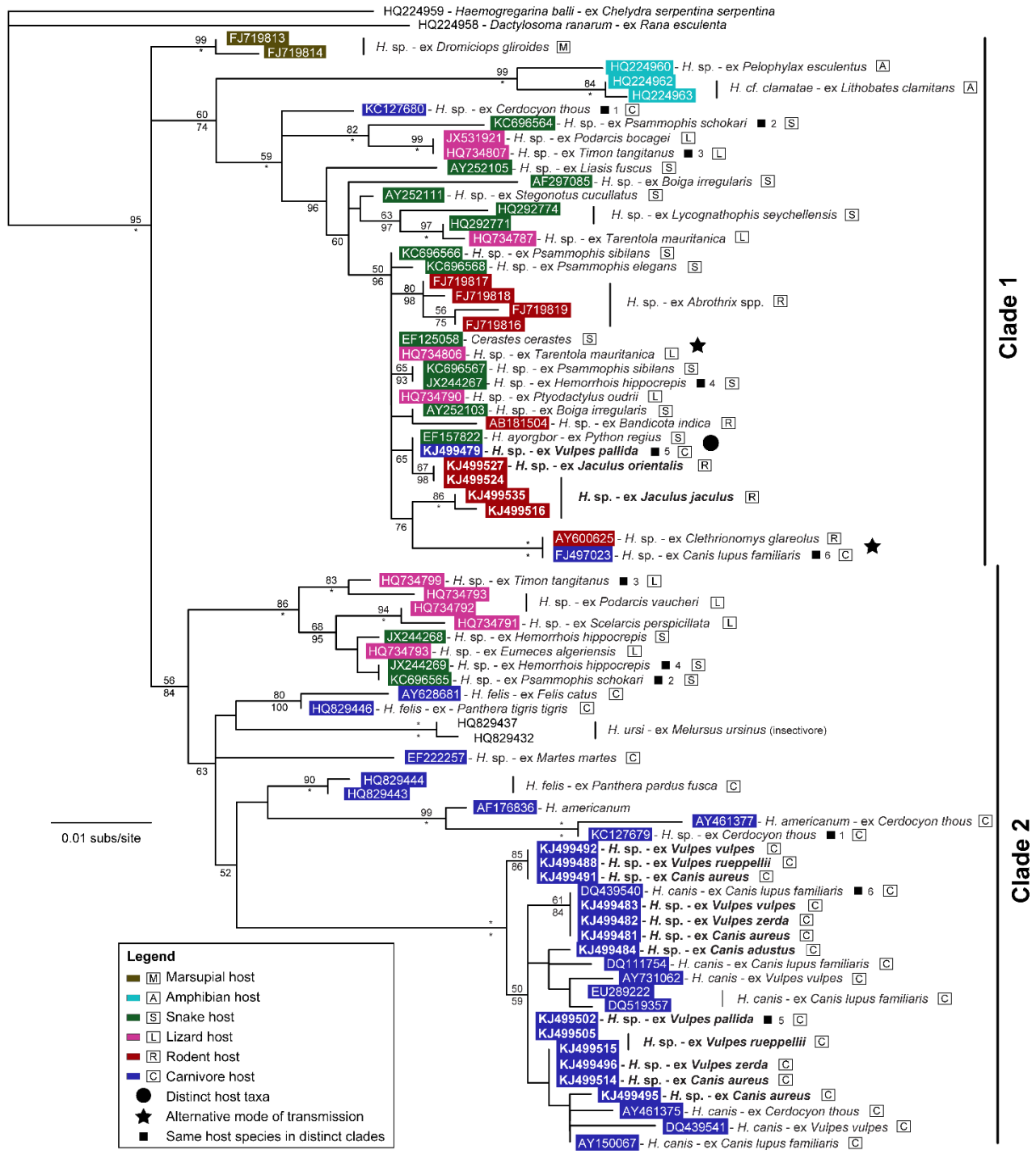


Figure 5-2 Estimate of relationships based on a maximum likelihood (ML) analysis for the 18S rRNA gene of *Hepatozoon*. Bootstrap values for ML are given above relevant nodes and Bayesian posterior probabilities are given below them. When values were 100%, this is indicated with an asterisk (*). The sequences indicated in bold represent those from this study. Letters inside squares and colors indicate the distinct groups of intermediate hosts. Stars indicate examples of alternative modes of *Hepatozoon* transmission, circles indicate examples of the same *Hepatozoon* sequence found in distinct host taxa, and black squares indicate examples when clearly distinct *Hepatozoon* lineages (one in clade 1 and another in clade 2) were found in the same host species.

2011), the detection technique used (O'Dwyer et al. 2013; Maia et al. 2014), and the sample size for each host species (e.g., *C. adustus* in this study) (Jovani and Tella 2006) may interfere with estimates of infection patterns. Previous microscopic surveys on canid (Conceição-Silva et al. 1988) and rodent species (Mbaya et al. 2011) from other regions reported prevalence estimates similar to ours. However, molecular surveys often report that almost all wild canids analyzed are infected with *Hepatozoon* spp. in studies with small sample sizes (Criado-Fornelio et al. 2003; Goller et al. 2010) as well as in studies with large sample sizes (Prager et al. 2012). Thus, it is not clear if variations in prevalence are due to a combination of the above factors. This can only be effectively tested on data that include balanced sample sizes, cover a greater geographic range, and include host-related and ecologic data of these elusive animals. Additionally, we detected putative *Babesia* spp. in two fox species. Prevalence was low, likely because the primers used in this study were designed for *Hepatozoon* spp. (Ujvari et al. 2004) and only amplify other apicomplexans occasionally (Harris et al. 2012; Tomé et al. 2013). *Babesia* spp. have been reported in other African carnivores such as *C. adustus* (Nuttall 1910b), *Cr. crocuta* (Williams et al. 2013), *C. l. familiaris* (Oyamada et al. 2005), *L. pictus* (Matjila et al. 2008), and *V. vulpes* (Maronpot and Guindy 1970). To our knowledge this is the first report of putative *Babesia* spp. in *V. pallida* and *V. zerda*. *Babesia* spp. are piroplasmid, tickborne parasites with an important economic, veterinary, and medical impact worldwide, causing serious health problems (Schnittger et al. 2012); its diagnosis is often difficult due to the relatively low intensity levels in hosts and due to their small size inside erythrocytes, which makes the use of molecular tools a good detection approach. Hence, future studies should analyze these and other wild host species using piroplasmid-specific primers to better assess the prevalence of these parasites.

By combining data from predator–prey systems, we provide new insights into *Hepatozoon* transmission dynamics. We detected a distinct *Hepatozoon* parasite lineage in a pale fox that is closely related with parasites found in rodents, lizards, and snakes. This corroborates previous findings and suggests that finding *Hepatozoon* parasites apparently from prey in canids is a rare event, with an overall ''atypical'' *Hepatozoon* prevalence of 3% (1/37) in this study, 3% (1/30) by Almeida et al. (2013), and 2% (2/108) by Vojta et al. (2009) compared to that found in saurophagous snakes (Tomé et al. 2014). Investigating how predator vertebrate hosts might become infected with parasite lineages found in prey is important because these events can have implications for the transmission dynamics of *Hepatozoon*. These events can have three nonexclusive explanations. 1) Trophic transmission: ingestion of paratenic hosts with infective cystozoites, thus supporting prey–predator transmission (Vojta et al. 2009; Almeida et al. 2013); 2) Concomitant predation: ingestion of infected invertebrate hosts attached to prey (Ewing and Panciera 2003; Johnson et al. 2010); and 3) Host relatedness and ecology: prey and predators may share the same habitat, thus being exposed to the same infected vectors, and host relatedness may be a limiting factor in establishment of infection by competent vectors (Ishtiaq et al. 2008).

First, small mammals and reptiles in clade 1 are often prey of wild canids and, thus, infected prey are likely to occasionally transmit these parasites to their predators; however, direct observation of these parasites by microscopy and experimental studies (e.g., liver analysis) is needed to evaluate the establishment of infections. There is now growing evidence that these methods should be coupled with DNA sequencing and phylogenetic reconstructions and that precaution should be taken when identifying *Hepatozoon* parasites in canids based solely on the presence of *H. canis*-like gamonts (East et al. 2008). Second, invertebrate hosts are of major importance in the transmission of vector-borne diseases, and the current lack of knowledge of these vectors and natural reservoir hosts in the wild limits the interpretation of our results. Attempts to identify the definitive host of *Hepatozoon* spp. infecting rodents are scarce (Hoogstraal 1961), hence it remains unknown. And third, the fact that the habitats of wild hosts, domestic animals, and invertebrate vectors often overlap provides a reasonable scenario for transmission of vector-borne infectious diseases such as hepatozoonosis. However, the fact that these prey–predator transmission events seem to occur more frequently in reptile systems, such as in snakes and lizards, may be an indication that infections can establish more easily in hosts that are more closely related. This can be linked with vector competence (i.e., the ability of a vector to become infected, replicate, and transmit the parasite to a receptive host [Dantas-Torres et al. 2012]), which is known to affect transmission in other systems (Gómez- Díaz et al. 2010; Lefèvre et al. 2013).

In any case, these events might represent dead-end infections (i.e., infections that occur in hosts that are not part of the lifecycle of that parasite species) and thus may not be transmitted further (Tomé et al. 2014). Predators that present dead-end hosts for some parasites, as possibly seen in this study, may contribute to a reduction in parasite transmission (the dilution effect) and to control of disease transmission by an indirect form of parasite predation (Johnson et al. 2010). Thus, the implications of these modes of transmission may be of importance in disease ecology and evolution and need to be further studied. Luong et al. (2013) have shown the usefulness of combining epidemiologic data and molecular analyses of the diet of the host to link predator–prey interactions with exposure to trophically transmitted parasites. Similar approaches could be used in future research regarding the systems analyzed in our study. Finally, *Hepatozoon* diversity in both canids and rodents is rather limited for the 18S rRNA gene when compared to the diversity found in lizards (Maia et al. 2012). All infected jerboas shared genetically similar parasites, distantly related to *H. canis*, suggesting that these jerboas are not likely to be paratenic hosts of *H. canis*, as shown for rodent species and *Hep. americanum* infections in the Americas (Johnson et al. 2008a, b) and as suggested in small mammals in Europe (Criado-Fornelio et al. 2009). Additionally, it would be of interest to assess other endemic and introduced rodent species and samples from other geographic locations (e.g., the Middle East) to verify if the same pattern is observed. Finally, the 18S rRNA gene is slow evolving, and this limited diversity of canid compared with reptilian *Hepatozoon* parasites may be an artefact; thus, faster-evolving genes are needed because protozoa may display the lowest

degree of phylogenetic constraint to carnivore host species in comparison to other pathogens such as viruses and helminths (Huang et al. 2013).

In conclusion, we provide the first molecular screening of *Hepatozoon* infections in wild canids and rodents from North Africa and provide further insights into the transmission dynamics of this parasite. The same *Hepatozoon* parasites can infect distinct taxonomic host taxa, and distinct *Hepatozoon* parasites can infect the same taxonomic host taxa, suggesting low intermediate host-specificity. Potential prey–predator transmission of *Hepatozoon* between jerboas and foxes from North Africa seems to occur occasionally in natural populations, while there is considerable evidence supporting more-widespread trophic transmission in reptiles from the same region. However, these alternative modes of transmission remain to be confirmed, as this can be a result of multiple factors such as host ecology and relatedness and invertebrate competence and distribution; thus, future studies addressing these factors are needed.

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## 5.2 Article IX - Assessing the diversity, host-specificity, distribution and infection patterns in apicomplexan parasites of reptiles from Arabia

In preparation

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**Abstract**

Understanding the processes that shape parasite differentiation and infection patterns provides valuable information on the dynamics and evolution of disease. Parasites are a major part of biodiversity but only a small fraction of their diversity has been identified. In this study, we assessed the diversity, distribution, host-specificity and infection patterns of apicomplexan parasites in amphibians and reptiles from remote areas of the Arabia peninsula, a region known to harbor a high diversity of reptile species and high levels of endemicity. Using molecular techniques we detected three apicomplexan parasites: hemogregarines, lankesterellids and sarcocystids. Thirteen hemogregarine haplotypes were retrieved, which fell into five main clades when placed in a phylogenetic framework. We detected a signal of hemogregarine haplotype specificity at the host family level, suggesting that host relatedness may be an important factor influencing colonization of new hosts. Hemogregarine prevalence and intensity of infection varied significantly between gecko species. We also examined differences between geographical areas and altitude for the gecko species *Pristurus rupestris*, for which we had the widest sampling. There were differences between areas and a negative pattern of correlation between altitude and infection parameters. These results suggest that habitat characteristics associated with altitude may have a role in influencing parasite distributions. Moreover, we found significant differences in intensity between hemogregarine parasite lineages (defined by phylogenetic clustering of haplotypes), suggesting that hosts may respond differently to infection by these lineages. The phylogenetic analysis of the six new lankesterellid haplotypes from this region revealed that these parasites were phylogenetically related, but distinct, from known lankesterellids and thus might represent new taxa. Our results highlight the importance of screening wild hosts from remote regions to estimate parasite diversity, and of host relatedness and parasite distribution patterns in shaping parasite infection patterns and genetic differentiation.

**Keywords:** Hemogregarine; amphibian; eimeriorina; host-parasite associations; altitude; host relatedness.

## Introduction

Parasites play important roles in ecosystems and animal communities (Hudson *et al.*, 2006; Seilacher *et al.*, 2007). Studying parasite diversity is important not only in terms of biodiversity indexes (Dobson *et al.*, 2008) but also for understanding the processes that drive species differentiation and specialization (Poulin and Morand, 2000; Clayton *et al.*, 2003). The study of infection patterns of various parasite species among different hosts and geographic locations can help to a better understanding of the dynamics and evolution of parasite communities as well as the processes that shape their diversity (Whiteman and Parker, 2005; Poulin and Mouillot, 2005; Nieberding *et al.*, 2008). Host-specificity of a parasite reflects its ability to exploit different hosts, which is assumed to be an indication of parasite specialization and coevolution (Poulin and Mouillot, 2004). Using parasite infection parameters to identify the principal and auxiliary hosts of a parasite is a first step towards characterizing host-specificity of parasites with complex lifecycles. The principal host is that in which the higher levels of prevalence, intensity and abundance are achieved by a parasite in a host population. Prevalence can be defined as the number of infected individuals in a population and may be associated with the encounters with the suitable vectors and host behavior. Whereas intensity of infection is the number of parasites in a single host and it may be associated with the ability of the individual immune response to control infection (Poulin, 2006).

There are, however, several factors that should be considered when studying host-specificity and comparing patterns of infection between parasite lineages and host species (Morand and Poulin, 2003; Nieberding *et al.*, 2008). One of these is host relatedness because unrelated host species are more likely to differ in their immune defense mechanisms and to require a higher adaptive effort for successful parasite establishment (Poulin and Mouillot, 2005). Therefore parasite species that display complex lifecycles involving several host species must adapt to different host factors throughout their lifecycle. Another is ecology because host-parasite interactions may differ when subjected to different habitat and environmental conditions (Poulin *et al.*, 2011). In fact, recent studies have shown that host ecology may be a major factor driving parasite differentiation in comparison with host phylogeny (Poulin, 2005; De León and Choudhury, 2005). In this perspective, host species with similar ecological requirements are likely to have similar parasite communities and phylogeographic patterns (Nieberding *et al.*, 2008), especially in closely related host species (Maia *et al.*, 2014). In this study, we analyzed the levels of infection (i.e. prevalence and intensity) in several related and unrelated host species inhabiting the same or different geographical areas, which would allow to explore the influences of both ecology and host factors on the variation of infection patterns.

Apicomplexa is recognized as one of the most diverse parasite groups, however it is estimated that only a small percentage of species have been formally described thus far (Morrison, 2009). In fact, recent studies have shown that there is still a great diversity of these parasites that is potentially still unknown (O'Dwyer *et al.*, 2013; Megía-Palma *et al.*, 2013, 2014; Harris *et al.*, 2015). High levels of endemism have been reported for herpetofauna in the Arabian Peninsula (Mallon, 2011; Cox *et*

*al.*, 2012), with many recent efforts to quantify biodiversity in remote regions from Oman revealing a high level of host diversity and novel species (Carranza and Arnold, 2012; Vasconcelos and Carranza, 2014). Given the tight association of parasites with their hosts and the influence of host ecology and evolution on parasite specialization, it is possible that this extraordinary host diversity is associated with a similar or greater parasite diversity (Lafferty, 2012). But the analysis of host species inhabiting these endemic and remote regions is still lacking.

In this study we estimate apicomplexan genetic diversity, host-specificity, distribution and infection patterns in herpetofauna from the Arabian Peninsula, Oman. For this we used a comprehensive sampling that includes several host species and genera from different geographical areas. The objectives of this study were to: i) determine parasite diversity and define parasite lineages, ii) estimate the prevalence and intensity of infection of apicomplexan parasites between different host species and geographical areas; iii) determine intensity levels of different parasite lineages as well as the importance of host relatedness in shaping their host-specificity.

## Materials and Methods

### *Sample collection*

A total of 234 lizard and amphibian blood samples were collected in Oman in May 2011 and a total of 45 snake tissue samples were collected in Oman between 2009 and 2013 (Table 5-2, Figure S7). For each reptile individual a small tail tip was collected, and for each amphibian individual a toe clip was collected. Tissue samples were preserved in 96% ethanol, while blood was stored in Whatman filter paper and kept at -20ºC prior to molecular analysis. After processing, all individuals were released at the site of capture, which was registered using a GPS device. Based on the limited dispersal of reptile hosts, we grouped sampling locations in an area of 20 by 20 kms radius (see Table S6 for more details).

### *DNA extraction, genetic diversity and phylogenetic analyses*

DNA from geckos and amphibians was extracted from blood drops stored in Whatman filter paper using the Speedtools tissue DNA extraction kit (Biotools, Madrid), following manufacturer's instructions, and DNA from snakes was extracted from tail-tip tissue stored in ethanol using standard saline protocol (Sambrook *et al.*, 1989; Maia *et al.*, 2014).

To estimate genetic diversity and the discovery of new parasite lineages, PCR amplification was performed using the primers HepF300 (5'- GTTTCTGACCTATCAGCTTTCGACG-3') and HepR900 (5'- CAAATCTAAGAATTTCACCTCTGAC-3') (Ujvari *et al.*, 2004), targeting part of the 18S rRNA gene region of apicomplexan parasites, following Maia et al. (2014). Negative and positive controls were run with each reaction. The positive PCR products were purified and sequenced by a commercial sequencing facility (Macrogen Europe, Netherlands). All sequencing reactions were performed in both directions. Sequences were analyzed using Geneious 6.1.6 (Drummond *et al.*,

Table 5-2 Samples analysed for apicomplexan parasites in reptiles and amphibians from Oman.
Prevalence is given for conventional and quantitative PCR (qPCR). Mixed infection with hemogregarines and lankesterellids were detected by qPCR.
GPS refers to the exact location where the animal was collected given in Table S6. Numbers in bold indicate locations that were positive for hemogregarine infections, underlined indicate positive infections of Lankesterellidae, and italics indicate *Sarcocystis* infections. *Sarcocystis* was amplified using primers HepF300 and HepR900, known to amplify various apicomplexan parasites (Harris et al., 2012).

| | | **Hemogregarines** | | | Lankesterellidae | | Sarcocystidae | |
|---|---|---|---|---|---|---|---|---|
| Host species | *n* | PCR | qPCR | Mean Intensity (min-max) | PCR | qPCR (mixed/single) | PCR | GPS |
| Anura: Bufonidae | | | | | | | | |
| *Bufo arabicus* | 20 | 18 (90%) | 20 (100%) | 2.3 ± 0.6 (1.2-3.3) | 1 (5%) | 6 (6/0) (30%) | | **289** |
| | 20 | 11 (55%) | 12 (60%) | 1.7 ± 0.6 (0.7-2.8) | 2 (10%) | 5 (3/2) (25%) | | **350** |
| | 40 | 27 (68%) | 32 (80%) | 2.18 ± 0.7 (0.7-3.3) | 3 (8%) | 11 (9/2) (28%) | | |
| Squamata: Gekkota | | | | | | | | |
| *Asaccus platyrhynchus* | 21 | 19 (90%) | 20 (95%) | 3.9 ± 0.7 (2.8-5.6) | | | | **263**,**350** |
| *Bunopus spatalurus* | 3 | | 2 (67%) | 1.2 ± 0.1 (1.2-1.3) | | | | **289**,319 |
| *Bunopus tuberculatus* | 3 | | 3 (100%) | 1.0 ± 0.3 (0.65-1.3) | | | | **270**,**339** |
| *Hemidactylus atairensis* | 2 | 1 (50%) | 2 (100%) | 2.7 ± 0.3 (2.4-2.9) | | | | **277** |
| *Hemidactylus festivus* | 11 | 1 (9%) | 10 (91%) | 2.1 ± 0.7 (1.2-3.1) | | | | **208**,**279** |
| *Hemidactylus hajarensis* | 9 | 5 (56%) | 7 (78%) | 3.4 ± 1.2 (1.8-4.9) | 1 (11%) | 1 (0/1) (11%) | | **289**,**319**,**349** |
| *Hemidactylus lemurinus* | 4 | 1 (25%) | 3 (75%) | 2.3 ± 1.6 (1.1-4.1) | | | | **279** |
| *Hemidactylus luqueorum* | 3 | 2 (67%) | 3 (100%) | 3.1 ± 1.2 (2.4-4.5) | | | | **340**,**350** |
| *Pristurus carteri* | 14 | | 7 (50%) | 1.7 ± 0.7 (0.9-2.8) | | | | **205**,**268**,**284**,**286**,**287** |
| *Pristurus rupestris* | 93 | 4 (4%) | 59 (63%) | 1.9 ± 0.8 (0.4-5.1) | 2 (2%) | 2 (1/1) (2%) | 1 (1%) | **274**,**276**,**278**,**291-294**,**296**,**297**,**299**,303,304,**308-310**,312,313, 314,**315-320**,322,323,**324**,**325**,327,328,**329**,**330**,**332**,**333**, **336**-***338***,**340**,341,**342**,**343**,**352**-354,358 |
| *Ptyodactylus hasselquistii* | 22 | 9 (41%) | 12 (55%) | 2.9 ± 0.9 (1.9-4.4) | 6 (27%) | 6 (1/5) (27%) | | **208**,**263**,**289**,**292**,**308**,**326**,**339**,**340** |

| Host species | *n* | **Hemogregarines** PCR | qPCR | Mean Intensity (min-max) | Lankesterellidae PCR | qPCR (mixed/single) | Sarcocystidae PCR | GPS |
|---|---|---|---|---|---|---|---|---|
| *Stenodactylus doriae* | 7 | | 3 (43%) | 1.4 ± 0.4 (1.2-1.9) | | | | **270**,301 |
| *Stenodactylus leptocosymbotes* | 2 | | 2 (100%) | 1.1 ± 0.3 (0.8-1.3) | | | | **284** |
| | 194 | 42 (22%) | 133 (69%) | 2.4 ± 1.2 (0.4-5.6) | 9 (5%) | 9 (2/7) (5%) | 1 (1%) | |
| Squamata: Serpentes | | | | | | | | |
| *Atractaspis andersonii* | 1 | | | | | | | 2 |
| *Cerastes gasperettii* | 6 | 6 (100%) | | | | | | **4**,**8**,**9**,**14**,**15**,**18** |
| *Echis carinatus* | 3 | 1 (33%) | | | | | | **5**,6,38 |
| *Echis khosatzkii* | 1 | | | | | | | 1 |
| *Echis omanensis* | 10 | 8 (80%) | | | | | | **21**,24,**27**,**29**,**30**,31,**33**,**34**,**39**,**40** |
| *Echis* sp. | 2 | 1 (50%) | | | | | | 25,**26** |
| *Eryx jayakari* | 3 | | | | | | | 19,20,22 |
| *Lytorhynchus diadema* | 10 | 3 (30%) | | | | | 1 (10%) | 7,8,**10**,11,12,**14**,*16*,17,**23** |
| *Psammophis schokari* | 2 | 1 (50%) | | | | | | 35,**41** |
| *Pseudocerastes persicus* | 3 | 1 (33%) | | | | | | **28**,32,37 |
| *Telescopus dhara* | 4 | 1 (25%) | | | | | | 3,13,23,**36** |
| | 45 | 22 (49%) | | | | | 1 (2%) | |

2012). A total of 105 usable sequences were obtained and we performed a similarity analysis using the Basic Local Alignment Search Tool (BLAST) to find the best match for the sequences against published sequences in GenBank. DnaSP v5 (Librado and Rozas, 2009) was used to estimate the number of haplotypes for individuals with no double peak positions. Double peak positions were identified in some individuals and these double peaks were resolved into two haplotypes by aligning them with the haplotypes found in this study (see Table S8). Sequences for each individual were deposited in GenBank under the accession numbers **** to ****. The number of variable and parsimony-informative sites, and uncorrected *p*-distances were calculated in MEGA v6.06 (Tamura *et al.*, 2013).

Parasites belonging to two distinct apicomplexan suborders were identified. In order to examine phylogenetic relationships in relation to biogeographic patterns, phylogenetic analyses were conducted for each group. No phylogenetic analyses were conducted for *Sarcocystis* because only two sequences were obtained, instead we provide the closest matches on GenBank. For hemogregarines we generated an alignment of 128 sequences with 559 bp in length, and for lankesterellids an alignment of 43 sequences with 605 bp in length. For each of these datasets, two phylogenetic analyses (Maximum Likelihood, ML, and Bayesian Inference, BI) were conducted. Maximum Likelihood analyses were performed with RAxML v.7.0.3 (Stamatakis, 2006) and reliability of the ML tree was assessed by bootstrap analysis (Felsenstein, 1985) with 1000 replications using the GTR+GAMMA substitution model. The AIC criterion conducted in jModeltest 0.1 (Posada, 2008) was used to choose the best model of evolution (i.e. TPM1uf+G for hemogregarines and for TrN+G lankesterellids). BI was implemented using Mr. Bayes v.3.1 (Huelsenbeck and Ronquist, 2001) with parameters estimated as part of the analysis. The analysis was run for $10 \times 10^6$ generations, saving one tree each 1000 generations. The log-likelihood values of the sample points were plotted against the generation time and all the trees prior to reaching stationarity were discarded, ensuring that burn-in samples were not retained. Remaining trees were combined in a 50% majority consensus tree (Huelsenbeck and Ronquist, 2001). *Dactylosoma ranarum* (HQ224958) was used as outgroup for hemogregarines (Barta *et al.*, 2012); *Goussia noelleri* (FJ009241) and *Goussia neglecta* (FJ009242) for lankesterellids (Morrison, 2009; Megía-Palma *et al.*, 2014). All trees were displayed in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

### *Host-specificity index*

To calculate host-specificity index for each hemogregarine haplotype obtained in this study we used the program TaxoBioDiv2 (http://www.otago.ac.nz/parasitegroup/Downloads/TaxoBiodiv2.zip) (Poulin and Mouillot, 2005). We only used samples for which the genetic haplotype was confirmed by conventional PCR. The $S_{TD}$ considers the phylogenetic distance between hosts to infer the specificity of the parasites, for which the lower the value the higher the specificity; while the $VarS_{TD}$ measures the asymmetries in these distances between the host species exploited by the parasite,

for which the higher the value the higher the asymmetry. The taxonomic distance in a Linnean taxonomic tree path length linking two host species (from Class, Order, Suborder, Family, Genus to Species) was used to calculate the specificity index. Haplotypes found in a single host species were assigned a $S_{TD}$ value of zero (Poulin and Mouillot, 2005).

*Real-time PCR detection and quantification*

DNA from 40 amphibian samples and 194 gecko samples (Table 5-2) were diluted to 10ng/µl with nuclease-free water (QIAGEN) using an ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). To estimate hemogregarine prevalence, intensity of infection and mixed infections of hemogregarine and eimeriorinid parasites, qPCR reactions were conducted using the primers JM4_F (5'-ACTCACCAGGTCCAGACATAGA-3') and JM5_R (5'-CTCAAACTTCCTTGCGTTAGAC-3') (Maia *et al.*, 2014). To estimate the number of copies in unknown samples, raw qPCR results were exported using the program iQ5 R&D version 2.1 (Biorad) and the baseline threshold was determined individually for each plate using the algorithm implemented in LinRegPCR (Ruijter *et al.*, 2009). For this experiment, samples with all threshold cycles (CTs) higher than 35 were considered negative because repeatability decreased significantly after cycle 35, with most replicates differing by more than one Ct. To confirm the identity of qPCR positives, 23 were sequenced (5 with double peaks and/or within the Eimeriorina range (83-85ºC), and 18 with melting temperatures in the hemogregarine range (81-82ºC). Eimeriorina peaks (83-85ºC, n=5) matched known eimeriorinid sequences (e.g. *Eimeria tropidura* (AF324217), *Lankesterella* sp. (KJ131417), and *Schellackia bolivari* (KJ131416), with 99% identity). These sequences were deposited in GenBank under GB accession numbers **** to ****. Based on these results, hemogregarine and eimeriorinid estimates of infection for gecko and amphibian hosts refer to qPCR estimates throughout this study.

*Statistical analysis*

For plot representation we log-transformed infection estimates obtained from qPCR (hemogregarine copy number) using the formula *log (x+1)*, and additionally square rooted these estimates to reach normality for statistical models. Homogeneity of variances were tested using Bartlett test and normality was tested using Shapiro-Wilk test. We used the package "ggplots2" implemented in R to create plots. All analyses were conducted in R software version 1.3.0.

To calculate differences in overall prevalence between species we used Chi-square tests. We performed Analysis of Variance (AOV) using Tukey Posthoc tests to calculate differences in overall hemogregarine intensity of infection between gecko species and between main hemogregarine lineages. Main hemogregarine lineages were defined as clusters of haplotypes that had more than 5% genetic divergence (see Table 5-3 and Figure 5-8 for more details). In the gecko species *P. rupestris* for which we had the most widespread sampling, we selected the geographical areas for which we had more than 5 samples and investigated the effects of geographical area (20 by 20 kms radius) and host sex on parasite prevalence and intensity of infection.

For the analysis of prevalence we used Generalized Linear Models (GLM) implemented in the package “MASS” in R with binomial logit distribution and Chi-square test. For parasite intensity of infection analyses we used Linear Models (LM) implemented in R. The best models were selected using a step-wise selection based on AIC and BIC values. Correlation between hemogregarine infection (i.e. infection status) and altitude was tested using Point-biserial correlation implemented in the package “ltm” in R, and correlation between hemogregarine intensity of infection and altitude was tested using Spearman’s rank correlation test.

**Results**

*Diversity and host-parasite associations*

To investigate the diversity of the apicomplexan parasites obtained in this study, these were compared with published data using BLAST algorithm implemented on GenBank and by conducting phylogenetic reconstructions. Of the 105 sequences obtained through conventional PCR, 91 matched hemogregarine (Adeleorina) sequences published on GenBank. Eight haplotypes were obtained for geckos (42 sequences), 4 for snakes (22 sequences), and 2 for *B. arabicus* (27 sequences) (Table S8). Of these, only one haplotype was shared between snakes, the Oman saw-scaled viper *E. omanensis*, and a gecko hosts (haplotype 3). Genetic divergence estimates between the 13 unique haplotypes obtained ranged from 0.4 to 9.3% (Table 5-3).

The 18S rRNA gene hemogregarine haplotypes retrieved from reptiles from Oman clustered in five main clades based on their phylogenetic relationships (Figure 5-3). Haplotypes 1 and 2 were exclusive to *B. arabicus* and grouped together in a single cluster, clade A, which included sequences from various geographical locations belonging to amphibian host species (Figure 5-3). Haplotype 8 was placed in the *Hepatozoon*/*Karyolysus* clade K from lizard and snake hosts (Figure 5-3). The newly found gecko clade C (haplotypes 9, 10, 11 and 13) was sister taxa to this clade. Haplotypes 6 and 7 from snakes and haplotypes 5 and 12 from geckos (the latter also found in a snake host, Table 5-3), were placed in clade B together with other *Hepatozoon* species identified from lizards, snakes and rodents from various geographical locations (Figure 5-3). Only haplotypes 6 and 12 were identical to *Hepatozoon* sp. from other snake hosts (e.g. KJ408511 and EF157822, respectively). Finally, haplotypes 3 and 4 were exclusive to reptiles from Oman and grouped together in a unique well-supported lineage (in clade B), which was related with hemogregarines from reptiles from Australia and Thailand (Figure 5-3).

For Eimeriorina parasites, of the 14 sequences obtained, 12 matched *Lankesterella* (Eimeriorina: Lankesterellidae) and 2 matched *Sarcocystis* (Eimeriorina: Sarcocystidae) (Table S8). For lankesterellids, 6 haplotypes were obtained: 2 from amphibians and 4 from geckos, which formed two separate lineages based on these host groups (Figure 5-4). Although these two lineages were sister taxa, they diverged by more than 7% of genetic distance (Table 5-4). Haplotypes 1 and 6 from

Table 5-3 Estimates of evolutionary divergence between the hemogregarine haplotypes obtained in this study.
The number of base substitutions per site between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). There were a total of 557 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The first two capital letters indicate the genus and the last two letters the species of the host. Amphibian hosts are in italics and snakes hosts in bold. The number of individuals per host species for each haplotype is given in parenthesis (see Table S8).

| Hemogregarine haplotype | Host species (number of individuals) | Hap 1 | Hap 2 | Hap 3 | Hap 4 | Hap 5 | Hap 6 | Hap 7 | Hap 8 | Hap 9 | Hap 10 | Hap 11 | Hap 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hap 1 | *BUar* ($16^{14}$***) | - | - | - | - | - | - | - | - | - | - | - | - |
| Hap 2 | *BUar* ($14^{14}$***) | 0.004 | - | - | - | - | - | - | - | - | - | - | - |
| Hap 3 | ASpl (18), **ECom** ($2^{14}$*), HElu (2), HEha (2), PRru (3), PTha (9) | 0.056 | 0.053 | - | - | - | - | - | - | - | - | - | - |
| Hap 4 | HEfe (1), HEle (1) | 0.054 | 0.051 | 0.007 | - | - | - | - | - | - | - | - | - |
| Hap 5 | PRru (1) | 0.050 | 0.05 | 0.02 | 0.02 | - | - | - | - | - | - | - | - |
| Hap 6 | **CEga** ($4^{14}$*)**, ECom** ($7^{14}$**)**, ECsp** (1)**, LYdi** ($3^{14}$*)**, PSsc** (1)**, PSpp** (1)**, TEdl** (1) | 0.049 | 0.049 | 0.018 | 0.018 | 0.005 | - | - | - | - | - | - | - |
| Hap 7 | **CEga** ($2^{14}$*), **LYdi** ($1^{14}$*) | 0.052 | 0.052 | 0.018 | 0.018 | 0.005 | 0.004 | - | - | - | - | - | - |
| Hap 8 | **CEga** (1)**, ECca** (1) | 0.064 | 0.06 | 0.041 | 0.041 | 0.039 | 0.037 | 0.041 | - | - | - | - | - |
| Hap 9 | HEha (1) | 0.093 | 0.089 | 0.071 | 0.066 | 0.064 | 0.066 | 0.062 | 0.062 | - | - | - | - |
| Hap 10 | HEha (1) | 0.089 | 0.085 | 0.068 | 0.064 | 0.062 | 0.064 | 0.06 | 0.062 | 0.004 | - | - | - |
| Hap 11 | ASpl (1) | 0.076 | 0.072 | 0.054 | 0.051 | 0.052 | 0.058 | 0.054 | 0.052 | 0.039 | 0.035 | - | - |
| Hap 12 | HEat (1), **ECom** ($1^{14}$*) | 0.054 | 0.054 | 0.02 | 0.02 | 0.011 | 0.005 | 0.009 | 0.039 | 0.068 | 0.066 | 0.06 | - |
| Hap 13 | HEha (1) | 0.091 | 0.087 | 0.069 | 0.064 | 0.062 | 0.064 | 0.06 | 0.064 | 0.002 | 0.002 | 0.037 | 0.066 |

Table 5-4 Estimates of evolutionary divergence between the *Lankesterella* haplotypes obtained in this study and published sequences.
The number of base substitutions per site between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). There were a total of 545 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The first two capital letters indicate the genus and the last two letters the species of the host. Amphibian hosts are in italics and snakes hosts in bold. The number of individuals per host species for each haplotype is given in parenthesis (see Table S8).

| *Lankesterella* haplotype | Host species (number of individuals) | *L. minima* (AF080611) | *L. valsainensis* (DQ390207) | Hap 1 | Hap 6 | *L.* sp. (KM234611) | *L.* sp (KJ131417) | Hap 2 | Hap 3 | Hap 4 |
|---|---|---|---|---|---|---|---|---|---|---|
| *L. minima* (AF080611) | - | - | - | - | - | - | - | - | - | - |
| *L. valsainensis* (DQ390207) | *Paus caeruleus* | 0.075 | - | - | - | - | - | - | - | - |
| Hap 1 | *BUar* ($3^{14}$**) | 0.069 | 0.063 | - | - | - | - | - | - | - |
| Hap 6 | *BUar* ($2^{14}$**) | 0.069 | 0.061 | 0.002 | - | - | - | - | - | - |
| *L.* sp. (KM234611) | *Hemidactylus agrius* | 0.079 | 0.081 | 0.071 | 0.073 | - | - | - | - | - |
| *L.* sp (KJ131417) | *Acanthodactylus erythrurus* | 0.107 | 0.109 | 0.096 | 0.097 | 0.043 | - | - | - | - |
| Hap 2 | PRru ($2^{14}$*), PTha (1) | 0.086 | 0.081 | 0.071 | 0.073 | 0.011 | 0.05 | - | - | - |
| Hap 3 | PTha ($3^{14}$*) | 0.088 | 0.084 | 0.075 | 0.077 | 0.011 | 0.05 | 0.007 | - | - |
| Hap 4 | PRru ($1^{14}$*), PTha ($3^{14}$*) | 0.086 | 0.084 | 0.075 | 0.077 | 0.011 | 0.05 | 0.004 | 0.004 | - |
| Hap 5 | HEha (1) | 0.086 | 0.086 | 0.077 | 0.079 | 0.011 | 0.05 | 0.015 | 0.011 | 0.015 |

[14] * the number of asterisks represents the number of sequences derived from infections displaying double peaks (see Materials and Methods).


Figure 5-3 Tree derived from a Bayesian Inference analysis of the 577bp fragment of the hemogregarine 18S rRNA gene. Bayesian Posterior probabilities are given above relevant nodes, and Probability Bootstrap values for Maximum Likelihood below them. + indicate when support is 100. Colors represent the three main linages defined by clusters of haplotypes that had more than 5% genetic divergence between them (see Figure 5-8). New snake haplotypes are shown in bold. New haplotypes include the host species and number of hosts found, as in Table 5-3. The new sequences from this study are colored as in Figure 5-8.

*B. arabicus* hosts were placed in clade 1 with *Lankesterella minima* (AF080611), *Lankesterella valsainensis* from the blue tit *Parus caeruleus* (DQ390207) and *Lankesterella* sp. from the lizard *Podarcis bocagei* from Portugal (KJ189386) (Figure 5-4). In clade 2, *Lankesterella* sp. from the lizard *Acanthodactylus erythrurus* from Spain (KJ131417) and putative *Lankesterella* sp. from the gecko *Hemidactylus mabouia* from Brazil (KM234611) were basal to haplotypes 2, 3, 4 and 5 from gecko hosts (Figure 5-4). Finally, the two *Sarcocystis* haplotypes from the gecko *P. rupestris* and the other from the snake *L. diadema* differed by 4% of uncorrected *p*-distance (588 bp). The closest GenBank matches for these sequences were: *Sarcocystis lacertae* (AY015113) with 99% identity and *Sarcocystis gallotiae* (AY015112) with 97% identity for *P. rupestris* haplotype; and *Sarcocystis* sp. from *Didelphis virginianus* from Mexico (KF278956) and *Sarcocystis neurona* from *Martes pennanti* (HQ709144) with 99% identity for *L. diadema* haplotype.

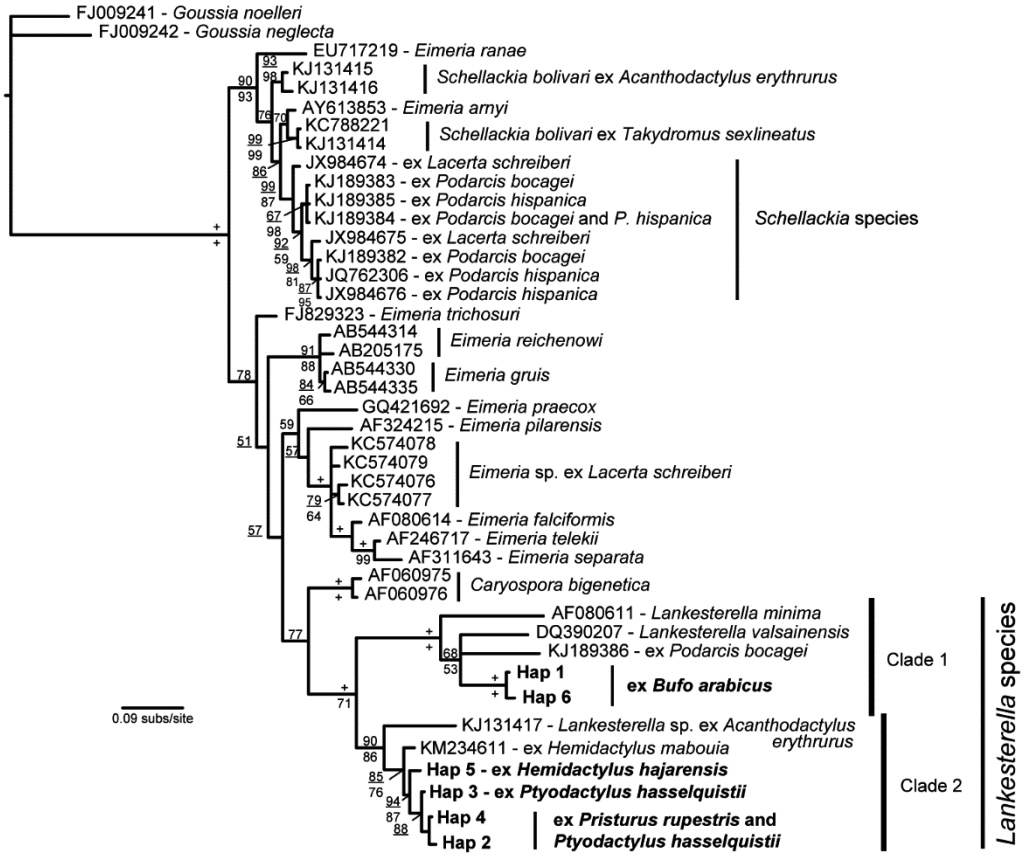


Figure 5-4 Tree derived from a Bayesian Inference analysis of the Eimeriorina 18S rRNA gene sequences of 694 bp in length. Bayesian Posterior probabilities are given above relevant nodes, and Probability Bootstrap values for Maximum Likelihood below them. + indicate when support is 100. The new sequences from this study are in bold.

### *Host-specificity patterns*

To investigate host-specificity of hemogregarine haplotypes, we examined the distribution of these parasites across host species. For this purpose we considered host taxonomic distances (measure of $S_{TD}$, for which lower values indicate higher specificity) and asymmetries in these distances (measure of $VarS_{TD}$, for which higher values indicate higher asymmetries). Haplotype 4 infected two congeneric host species and thus had the lowest $S_{TD}$ value among parasites found in

more than one host species (Table 5-5). On the other hand, haplotype 3 was the least specific haplotype found in our study (i.e. with the highest values of $S_{TD}$ and of $VarS_{TD}$) because it was found in hosts from different families and suborders (Table 5-5). The principal host species for this hemogregarine haplotype was *A. platyrhynchus* (Table 5-5). In addition, host species infected with haplotype 4 may be restricted to a geographical location that was unique for this haplotype (area 30 in Figure 5-5). Haplotype 5 from clade B and haplotypes 9, 10, 11 and 13 from clade C were detected in single host species and thus represent the highest values of host-specificity (Table 5-5).

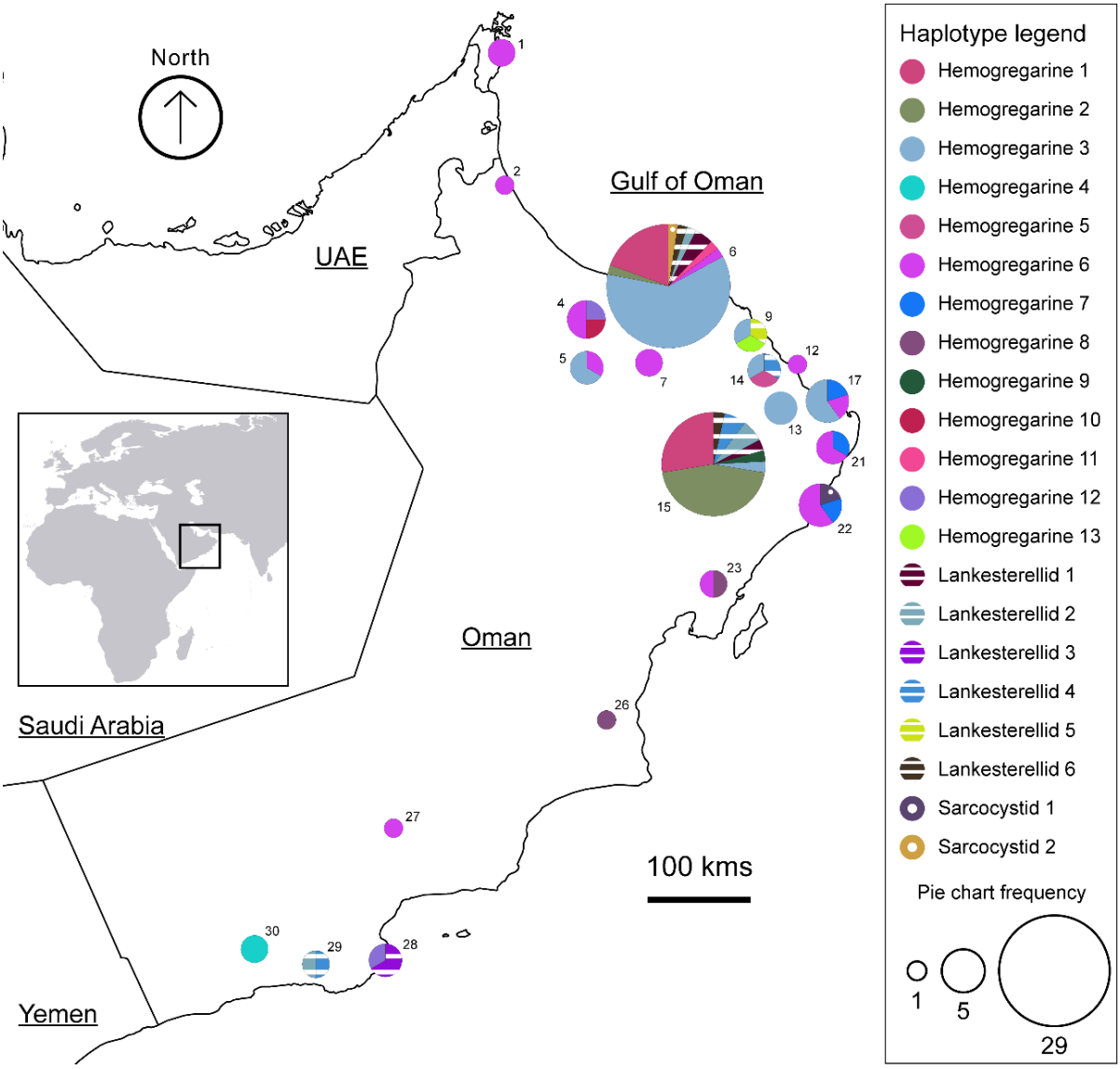


Figure 5-5 Distribution of apicomplexan haplotypes obtained from reptiles in Oman represented in a 20 by 20 kms area radius. See Table S6 for more details. Numbers on map indicate each area code.

At the family level, we detected a pattern of hemogregarine haplotype specificity for the two haplotypes found in a wide range of host species (i.e. haplotypes 3 and 6). However, differences were not significant, possibly due to differences in the sampling available for the different host families (Table 5-5). For haplotype 3, the highest mean prevalence levels was detected in the Phyllodactylidae family (27 positives in 43 individuals, 63%), while lower levels were detected in

Table 5-5 Host-specificity index for each hemogregarine haplotype obtained in this study. This index was calculated using program TaxoBioDiv2 and considers the taxonomic distance between two host species based on the branch lengths in a Linnean taxonomic tree ($S_{TD}$). The first two capital letters indicate the genus and the last two letters the species of the host. Host species are ordered by host family and decreasing levels of prevalence.

| | n | Host family | Prevalence for PCR haplotype | GPS for positives (n) | qPCR Intensity for PCR haplotype [log (x+1)] | Abundance (Prevalence * Intensity | $S_{TD}$ | $VarS_{TD}$ |
|---|---|---|---|---|---|---|---|---|
| **Hap 1** | | | | | | | | |
| *BUar* | 20 | Bufonidae | 40% | 289(8) | 2.4 ± 0.8 (1.4-3.3) | 0.96 | | |
| | 20 | | 40% | 350(8) | 1.8 ± 0.7 (0.7-2.8) | 0.72 | | |
| | | | 40% | | | | 0 | - |
| **Hap 2** | | | | | | | | |
| *BUar* | 20 | Bufonidae | 65% | 289(13) | 2.4 ± 0.5 (1.7-3.2) | 1.56 | | |
| | 20 | | 5% | 350(1) | 2.0 | 0.10 | | |
| | | | 35% | | | | 0 | - |
| **Hap 3** | | | | | | | | |
| *ASpl* | 21 | Phyllodactylidae | 86% | 263(3), 350(15) | 3.9 ± 0.6 (2.9-5.2) | 3.34 | | |
| *PTha* | 22 | Phyllodactylidae | 41% | 263(3), 292(1), 308(3), 339(1), 340(1) | 3.1 ± 0.9 (1.9-4.4) | 1.27 | | |
| *HElu* | 3 | Gekkonidae | 67% | 340(1), 350(1) | 3.4 ± 1.0 (2.4-4.5) | 2.27 | | |
| *HEha* | 9 | Gekkonidae | 22% | 289(1), 319(1) | 3.1 ± 0.3 (2.8-3.3) | 0.69 | | |
| *PRru* | 93 | Sphaerodactylidae | 3% | 292(2), 310(1), | 4.0 ± 0.8 (3.3-5.1) | 0.13 | | |
| *Ecom* | 10 | Viperidae | 20% | 29(1), 34(1) | - | - | | |
| | | | 36% | | | 1.26 | 4.13 | 0.65 |
| **Hap 4** | | | | | | | | |
| *HEfe* | 11 | Gekkonidae | 9% | 279(1) | 2.6 | 0.24 | | |
| *HEle* | 4 | Gekkonidae | 25% | 279(1) | 4.1 | 1.03 | | |
| | | | 17% | | | 0.63 | 2 | - |
| **Hap 5** | | | | | | | | |
| *PRru* | 93 | Sphaerodactylidae | 1% | 310(1) | 1.6 | 0.02 | 0 | - |
| **Hap 6** | | | | | | | | |
| *ECom* | 10 | Viperidae | 70% | 21(1), 27(1), 29(1), 30(1), 33(1), 39(1), 40(1) | - | - | | |
| *CEga* | 6 | Viperidae | 67% | 4(1), 9(1), 14(1), 18(1) | - | - | | |
| *PSpe* | 3 | Viperidae | 33% | 28(1) | - | - | | |
| *PSsc* | 2 | Lamprophiidae | 50% | 41(1) | - | - | | |
| *LYdi* | 10 | Colubridae | 30% | 10(1), 14(1), 23(1) | - | - | | |
| *TEdl* | 4 | Colubridae | 25% | 36(1) | - | - | | |
| | | | 34% | | | | 3.73 | 0.2 |
| **Hap 7** | | | | | | | | |
| *CEga* | 6 | Viperidae | 33% | 15(1), 18(1) | - | - | | |
| *LYdi* | 10 | Colubridae | 10% | 23(1) | - | - | | |
| | | | 22% | | | | 4 | - |
| **Hap 8** | | | | | | | | |
| *ECca* | 3 | Viperidae | 33% | 5(1) | - | - | | |
| *CEga* | 6 | Viperidae | 17% | 8(1) | - | - | | |
| | | | 25% | | | | 3 | - |
| **Hap 9** | | | | | | | | |
| *HEha* | 8 | Gekkonidae | 13% | 289(1) | 4.2 | 0.53 | 0 | - |
| **Hap 10** | | | | | | | | |
| *HEha* | 8 | Gekkonidae | 13% | 349(1) | 4.9 | 0.61 | 0 | - |
| **Hap 11** | | | | | | | | |
| *ASpl* | 21 | Phyllodactylidae | 5% | 263(1) | 5.6 | 0.27 | 0 | - |
| **Hap 12** | | | | | | | | |
| *HEat* | 2 | Gekkonidae | 50% | 277(1) | 2.9 | 1.45 | | |
| *ECom* | 10 | Viperidae | 10% | 33(1) | - | - | | |
| | | | 30% | | | | 4 | - |
| **Hap 13** | | | | | | | | |
| *HEha* | 8 | Gekkonidae | 13% | 319(1) | 4.4 | 0.55 | 0 | - |

other families (Gekkonidae, 4 positives in 12 individuals, 33%, $X^2$=2.221, df=1, P=0.136); Sphaerodactylidae, 3 positives in 93 individuals (3%, $X^2$=57.265, df=1, P<0.001); and other suborders (Serpentes, 2 positives in 10 individuals (20%, $X^2$=4.393, df=1, P=0.036) (see Table 5-5). And for haplotype 6, the highest mean prevalence was observed in snakes of the Viperidae family [12 positives in 19 individuals (63%)], while lower levels were detected in other snake families [(Lamprophiidae, 1 positive in 2 individuals (50%, no Chi-square test available); and Colubridae, 4 positives in 14 individuals (28%, $X^2$=2.600, df=1, P=0.107)].

*Infection patterns across host species*

In order to investigate the influence of host and ecological factors in parasite infection patterns, we examined infection parameters in several related and unrelated host species inhabiting the same or different geographical areas.

*Prevalence*. Overall prevalence of hemogregarine infections was high among for all three host groups examined (80% for *Bufo arabicus*, 69% for geckos, and 49% for snakes) (Table 5-2). Hemogregarines were found in all host species except for *Atractaspis andersonii* (n=1), *Echis khosatzkii* (n=1) and *Eryx jayakari* (n=3), probably due to the low sample size for these host species (Table 5-2). Prevalence of infection differed significantly between the six gecko species with sampling higher than 5 samples ($X^2$=14.916, df=5, P=0.011). Prevalence was higher in the geckos *A. platyrhynchus* (95%) and *Hemidactylus* species (91% in *H. festivus* and 78% in *H. hajarensis*), while was lower in the geckos *P. hasselquistii* (55%) and *Pristurus* species (50% in *P. carteri* and 63% in *P. rupestris*) (Table 5-2). Prevalence was also significantly different between the three snake species with sampling higher than 5 samples ($X^2$=9.652, df=2, P=0.008), with the highest prevalence found in *C. cerastes* (100%), followed by *E. omanensis* (80%) and *L. diadema* (30%) (Table 5-2).

We then examined differences in prevalence between populations in *P. rupestris* for which a more widespread sampling was available. For this gecko species we detected a significant effect of geographical area (GLM, df=7, $X^2$=15.456, P=0.031), but not for host sex ($X^2$=0.554, df=1, P=0.457). In addition, there was a negative correlation between prevalence and altitude in *P. rupestris* (Point-biserial correlation, $\hat{r}_{p.b.}$=-0.34), which showed a tendency for less infected individuals at higher altitudes (Figure 5-6 A). In amphibians, hemogregarine prevalence for *B. arabicus* differed significantly between the two populations examined ($X^2$=7.656, df=1, P=0.006, Table 5-2).

Lankesterellids (Eimeriorina) were found in significantly greater number in *B. arabicus* (11 in 40, 28%) than geckos (9 in 194, 5%) ($X^2$=19.344, df=1, P<0.005, Table 5-2). In addition, prevalence of lankesterellids was identical between the two populations of *B. arabicus* ($X^2$=0, df=1, P=1). In the case of lankesterellids, the low prevalence did not allow for comparisons between populations. Cases of mixed infections with lankesterellids and hemogregarines were also higher in *B. arabicus* (9 cases of mixed infections, in 11 total lankesterellid infected individuals, 82%) than in geckos (2 in 9, 22%) ($X^2$=4.900, df=1, P=0.269, Table 5-2). In contrast, cases of single lankesterellid infections

were higher in geckos (7 in 9, 78%) than in *B. arabicus* (2 in 11, 18%) ($X^2$=4.900, df=1, P=0.269, Table 5-2). Interestingly, *P. hasselquistii* had the highest levels of eimeriorinid infections compared with other gecko species (27%, Table 5-2).

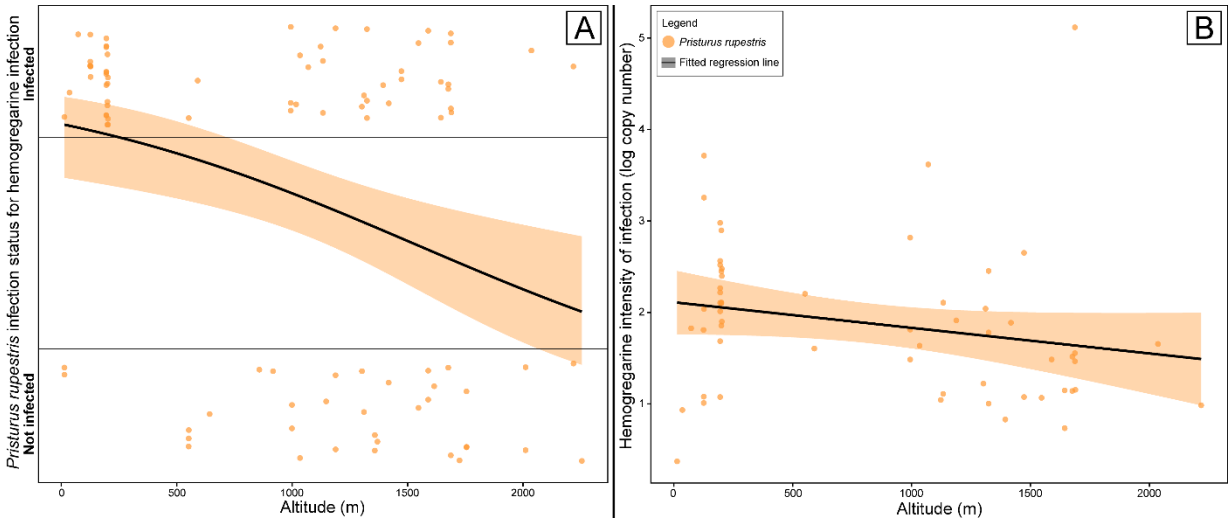


Figure 5-6 Relationship between hemogregarine infection parameters and altitude for *Pristurus rupestris*. This gecko species had the most extensive sampling across Oman. A) Relationship between individual infection status for hemogregarine infection and altitude. Fitted logistic regression line was produced by fitting a GLM with binomial logit distribution for overall prevalence and body size with 95% confidence region. Points representing each individual infection status were spaced and slightly transparentized for easier visualization. B) Relationship between hemogregarine intensity of infection and altitude. Fitted logistic regression line was produced by fitting a LM for overall intensity of infection and body size with 95% confidence region.

*Hemogregarine intensity*. Hemogregarine intensity in *B. arabicus* was significantly higher in the Wadi Bani Khalid population than in the more eastern population (AOV, df=1, sum sq=3.374, F=8.346, P=0.009, Figure 5-7), as observed for prevalence (Table 5-2). Overall intensity of infection differed significantly between gecko species (df=12, sum sq=59.438, F=8.591, P<0.001) (Table S10). In geckos, *A. platyrhynchus*, *H. hajarensis*, and *P. hasselquistii* had the highest intensity levels compared to *Bunopus*, *Pristurus* and *Stenodactylus* species which had the lowest (Table S10 and Figure 5-7). In *P. rupestris* we detected a significant effect of geographical area on hemogregarine intensity of infection (LM, df=6, sum sq=1.315, F=2.991, P=0.018) (Figure 5-7) but not of host sex (df=1, sum sq=0.020, F=0.415, P=0.523). We detected a non-significant negative correlation between intensity of infection and altitude for this host species (Spearman correlation, ρ=0.25, P=0.057), showing a tendency for lower intensity levels at higher altitudes (Figure 5-6 B). In addition, we detected significant differences between 18S rRNA gene main lineages [AOV, df=2, sum sq=35.364, F=27.491, P<0.001 (Figure 5-8)]. Lineage C exclusively from geckos had the highest intensity levels, followed by lineage B also from geckos and lineage A from amphibians (Figure 5-8).

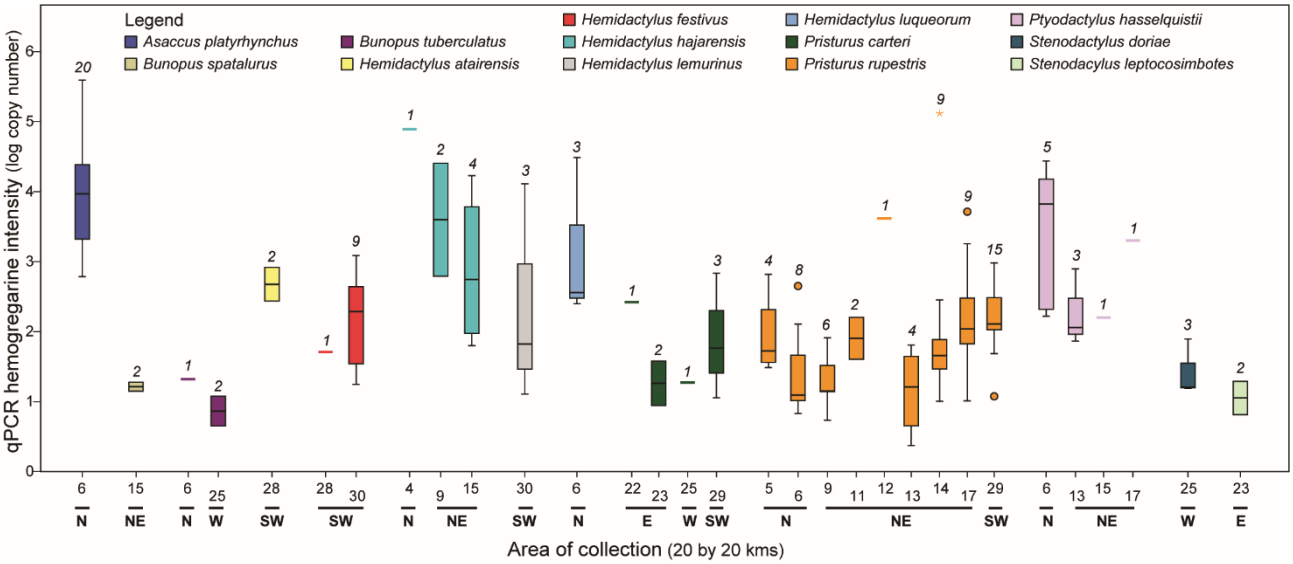


Figure 5-7 Hemogregarine intensity of infection (qPCR) for each host species and area of collection in a 20 by 20 kms radius. Number of positive individuals per area is given above each plot and excludes mixed infections of hemogregarines and eimeriorinids.

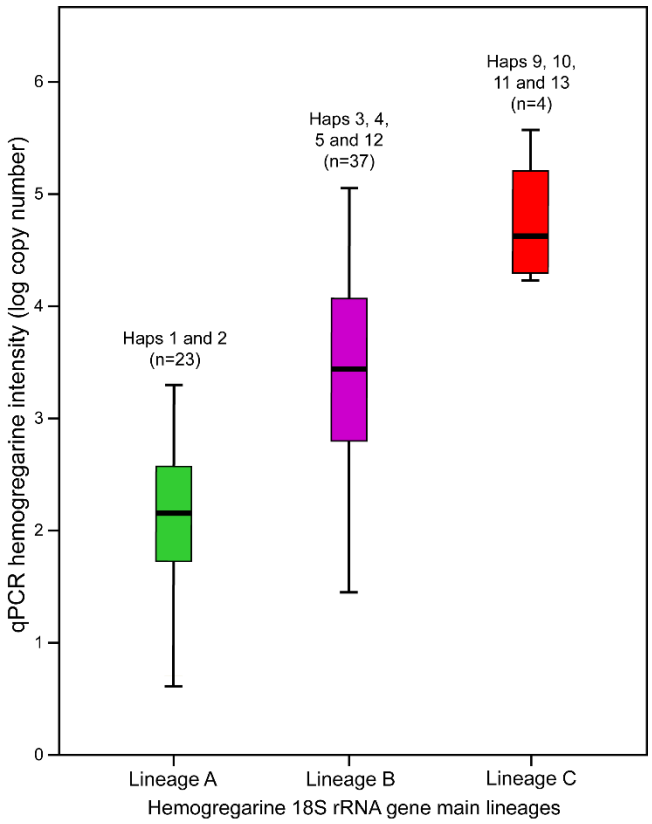


Figure 5-8 Hemogregarine intensity of infection estimated with qPCR for 18S RNA gene main lineages. Number of positive individuals per lineage is provided and excludes mixed infections of hemogregarines and eimeriorinids.

**Discussion**

This study provides the first assessment of apicomplexan diversity, host-specificity and infection patterns in herpetofauna from Oman through a phylogeographic perspective. Previous studies have identified apicomplexan parasites in reptiles from the Arabian Peninsula (Abd-Al-Aal, 1998; Al-Farraj, 2008; Abdel-Ghaffar *et al.*, 2009; Abdel-Baki *et al.*, 2012, 2014) but, to our knowledge, our results represent the first records of apicomplexan parasites in reptiles from Oman. Phylogenetic analyses show that some of these newly discovered parasites could be regarded as new species based on genetic divergence estimates. This highlights the importance of investigating parasite diversity in wild hosts from remote regions.

Identifying processes that shape parasite population structure is often difficult due to a multitude of factors involved (Nieberding *et al.*, 2008), especially in vector borne diseases that may involve multiple hosts throughout their lifecycle. However, we would expect to see a stronger signal of parasite specialization (i.e. host-specificity) between host species that are related to the principal host and a stronger signal of parasite differentiation in unrelated host species (Nieberding *et al.*, 2008). In our study, infection levels were associated with host similarity at the family level, which may indicate that the levels of infection in auxiliary hosts could be constrained by their distinctiveness relative to the principal host (De León and Choudhury, 2005). Since taxonomic distances between hosts are a surrogate measure for similarity in immunology, ecology and physiology (Poulin, 2005), this pattern is expected because related hosts are more likely to be similar in these measures.

In avian malaria studies, it has been suggested that parasite differentiation may be influenced by the acquisition of new hosts followed by divergent selection between host lineages in sympatry (Ricklefs *et al.*, 2004; Fallon *et al.*, 2005). Therefore, the fact that some haplotypes were detected in single host species (i.e. highly specific), could be an indication of divergent selection that is occurring in this host-parasite system. Specialist parasites adapt to the few host species they exploit and this would limit their ability to infect and reach transmissible stages in other host species (Garamszegi, 2006; Hellgren *et al.*, 2009). On the other hand, two haplotypes (3 and 6) were more widely distributed across host species, suggesting the possible occurrence of host-shifts in some parasite lineages (Bensch *et al.*, 2000). This could be an indication of a greater plasticity of these parasite lineages and should be further investigated as this could shed new light into the evolutionary history of these parasites. In any case, some areas from Oman had limited sampling, especially in the West and South of this region, thus further sampling of these and other host groups are needed.

Regarding the hemogregarine haplotype that infected several gecko species (haplotype 3), the host principal host was *A. platyrhynchus*, an endemic gecko species that is restricted to the Jebel Akhdar that is part of the Al Hajar Mountains range in Oman. Two hypotheses can be drawn from the distribution pattern of this haplotype. First, this variant may have adapted and specialized to the host *A. platyrhynchus* and later expanded its host range. This expansion may have occurred through accidental transmission to sympatric gecko species that have wider distribution ranges, such as

*Pristurus* species. Or second, this haplotype already had low host-specificity and could easily colonize unrelated host species, of which *A. platyrhynchus* was the most susceptible to infection of all its host range species. A closely related haplotype (haplotype 4) differed by a few genetic mutations for the 18S rRNA gene and was exclusively found in two additional *Hemidactylus* species. This finding could be an indication that this variant has differentiated and specialized in these two host species and should be further investigated.

Hemogregarine prevalence and intensity differed significantly between gecko species and between amphibian *B. arabicus* populations. In addition, both prevalence and intensity of infection varied significantly between geographical areas for the gecko species *Pristurus rupestris* and we detected a negative association between prevalence and intensity and altitude in *P. rupestris*. These results suggest that both host and ecological factors can influence parasite distributions in this host-parasite system. Differences in prevalence can result from species specific microhabitat preferences, variation in the distribution and abundance of competent vectors, as well as host behavior that could result in differential exposure to parasites and vectors (Eisen and Wright, 2001; Ishtiaq *et al.*, 2008). Hemogregarines are heteroxenous parasites transmitted by arthropod vectors, which are especially sensitive to environmental conditions. At higher altitudes, climatic factors such as temperature, moisture and precipitation are known to be adverse for larval development and survivorship in vectors of *Plasmodium* parasites (Rooyen *et al.*, 2013; Atkinson *et al.*, 2014). Additionally, vector competence may decrease among unrelated hosts due to differences in immune defences of host species, which may result in heterogeneous parasite distributions (Krasnov *et al.*, 2006). It has been reported for example that the competence of arthropods vectors varies with altitude and microheterogeneity of the geographical areas analysed (Bødker *et al.*, 2003; Tanga *et al.*, 2010). Future research is needed to investigate the role of vector distribution and abundance of competent vectors in the reptile host populations studied. Furthermore, differences in intensity of infection are usually associated with host immunology (Klein, 2004; Lindström *et al.*, 2004). Different host species can have different immune defense mechanisms that act to clear or tolerate infections. This may explain why some hosts were more heavily infected than others and also why intensity of infection differed between parasite lineages. The fact that the highest levels of intensity of infection were detected in the newly found lineage of geckos (lineage C found in *A. platyrhynchus* and *H. hajarensis*), increases the relevance of our results and highlights the need to further investigate these host species. Therefore, future research should now focus on investigating parasite pathogenicity and host immune response, as well as host behavior and microhabitat characteristics. It is also important to compare the impact of various parasite lineages in the same and on different host species to better understand heterogeneity in infection and the relationship with host fitness.

## Conclusion

This study provides the first large-scale molecular assessment of apicomplexan parasites from Arabian reptiles. We investigated prevalence and intensity of infection in various host species and geographic locations and show that diversity of hemogregarines and infection patterns may be constrained by host relatedness and ecological factors. Despite a number of studies have started to uncover the diversity of hemogregarines in wild populations, new unexpected lineages that might represent new genera or species continue to be detected. Given the extraordinary diversity of apicomplexan parasites detected on these reptile hosts, it would be important to screen other host groups from remote regions. This would allow to further investigate the host spectrum and transmission dynamics of these and other, potentially unknown, lineages. This has important implications for understanding the diversity of apicomplexan parasites and also for taxonomic purposes. Given the abundance, wide host spectrum and geographic distribution of these parasite lineages, our results can also have implications for conservation as well as for the epidemiology of zoonotic diseases.

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## 5.3 Article X - Temporal dynamics of hemogregarine infection in two sympatric lizard systems

In preparation

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**Abstract**

Sympatric and closely related host species represent natural model systems to investigate between-host differences in infection parameters. Studying these scenarios allows to limit the impact of confounding factors such as host ecology and evolutionary history. Using a sensitive quantitative PCR (qPCR) approach to estimate hemogregarine prevalence and intensity of infection, we investigated the temporal dynamics of hemogregarine parasites across different months and years in two distinct sympatric lizard systems. One composed of two closely related *Podarcis* species (Moledo, Portugal) and the other of two related lacertid species and one distantly related gecko species (Oukaimeden, Morocco). In Moledo, *Podarcis hispanica* had significantly higher hemogregarine intensity levels than *Podarcis bocagei*, which was consistent throughout all time points. Month of collection was not an important variable in our statistical models, showing that infection patterns remained similar across time points. For one year, *P. bocagei* males had significantly higher prevalence levels in Spring (during their mating period) than in Autumn. In Oukaimeden, gecko lizards had significantly lower prevalence and intensity levels compared to the two lacertid species for all three months, also with a consistent pattern between years. Additionally, infection parameters differed significantly between the two lacertid species in Oukaimeden for all time points. These species were infected with unique 18S rRNA gene haplotypes, which could be associated with these heterogeneous patterns. Regarding host characteristics, body size and regenerated tail condition were positively correlated with prevalence. Our findings show that the general lack of temporal variation in hemogregarine infection patterns between host species may be associated with host unique biological features and micro-ecological conditions that need to be further studied.

**Keywords:** *Hepatozoon*; *Karyolysus*; reptile; lacertid; gecko; seasonality; body size SVL; mating period; prevalence; intensity; tail autotomy; month; year; Portugal; Morocco.

**Introduction**

Vector-borne diseases often have complex lifecycles that involve more than one host and thus their dynamics may vary markedly over time as a result of different environmental pressures on these hosts (Cosgrove *et al.*, 2008). In order to understand disease epidemiology in these systems it is crucial to study the environmental and demographic drivers that influence the temporal dynamics in disease transmission (Pascual and Dobson, 2005).

Coupled with the development of sensitive and quantitative molecular techniques to estimate infection, studies on within and between-species variation in prevalence and intensity of infection are increasing (Knowles *et al.*, 2010, 2011; Maia *et al.*, 2014). These studies are important to the study of spatial and temporal patterns of infection, parasite transmission dynamics and epidemiology, as well as to characterize the host-specificity and host range of a parasite (Poulin and Mouillot, 2004). However, only a few studies on hemogregarines thus far have examined seasonal variation in infection parameters in reptile hosts. In birds, several studies have been performed under different experimental conditions and ecological settings but often with conflicting outcomes (Cosgrove *et al.*, 2008; Okanga *et al.*, 2013). These studies suggest that the temporal dynamics of parasite infection is tightly linked to the intrinsic characteristics of the host species and the localities analyzed (Bensch *et al.*, 2007; Godfrey *et al.*, 2011).

In vector-borne parasites, prevalence and intensity of infection can be influenced not only by abiotic factors, such as spatial and temporal differences in habitat characteristics (Lachish *et al.*, 2013), but also by biotic factors, such as host hormonal levels and reproductive effort (Pollock *et al.*, 2012), as well as vector abundance, competence and transmission potential (Eisen and Wright, 2001). The classical example in vertebrate hosts has been the immunosuppressive effect of testosterone (Olsson *et al.*, 2000; Klein, 2004), a hormone often found at higher levels in males during the mating period, in Spring (Gowan *et al.*, 2010). Therefore during the mating season, a higher level of parasite load would be expected, not only as a result of an increase in immunosuppressive hormones but also due to a higher investment in reproductive effort (Hanssen *et al.*, 2005; Knowles *et al.*, 2009; Christe *et al.*, 2012). However, this pattern is not always observed (Cox and John-Alder, 2005; Fuxjager *et al.*, 2011; Ezenwa *et al.*, 2012), which suggests that other factors must be contributing to the dynamics of parasite infection patterns.

Therefore, apart from differences linked to space and time, other confounding factors include host phylogeny and ecology (Poulin *et al.*, 2011b). Phylogeny influences parasite distributions because the more distantly related host species are, the more likely they are to differ in their resistance and tolerance to infection, i.e. immune response, life-history traits and behaviors (Poulin and Mouillot, 2005). Similarly, host species from distinct geographical locations are more likely to be subjected to different habitat conditions and environmental pressures that may result in different probabilities of exposure to parasites or in distinct survival pressures (Hudson *et al.*, 1992; Garrido and Pérez-Mellado, 2015). In this context, sympatric and related host species offer an ideal opportunity to

examine proximate factors influencing patterns of infection and transmission dynamics in wild host populations. That is, by controlling for the confounding effects of host ecology and phylogeny in these systems, other factors can be identified (e.g. host behavior and microhabitat preferences).

Moreover, parasitological studies on wild host populations face important limitations related to the issue of detectability and capture heterogeneity (Jennelle *et al.*, 2007). The accuracy of estimates of prevalence and intensity levels of parasite infection may be affected by sample sizes (Jovani and Tella, 2006). Therefore, detection probabilities and parasite infection patterns may change spatially and temporally that can result in spurious artifacts of variation rather than changes in underlying disease dynamics (Jennelle *et al.*, 2007). For this reason, longitudinal studies are needed to evaluate the occurrence of temporal fluctuations in infection patterns. In the same sense, detectability may also be constrained by the detection method used, with studies reporting differences in performances of various methods (Ricklefs *et al.*, 2005; Valkiūnas *et al.*, 2008; Maia *et al.*, 2014).

Hemogregarines are apicomplexan parasites that require at least one invertebrate vector host, in which sexual reproduction occurs, and one vertebrate host, in which gametogenesis occurs (Telford, 2009). Recent studies examined hemogregarine diversity and prevalence among different host species (Maia *et al.*, 2011; Haklová *et al.*, 2014; Tomé *et al.*, 2014), as well as of infection patterns between species (Majláthová *et al*., 2010; Maia *et al.*, 2014). However, few studies have addressed temporal variation in hemogregarine infection patterns across host species and geographical locations (Sorci, 1995). In this study, we analyze the temporal infection dynamics of hemogregarine parasites in two distinct sympatric lizard systems by sampling different host species at different time months and years. The first system consisted of two closely related lacertid species, *Podarcis bocagei* and *Podarcis hispanica*, co-occur in several locations in Portugal, including a coastal area in the north of Portugal. These host species have similar ecological requirements but may have distinct microhabitat preferences (Sá-Sousa *et al.*, 2002). A recent study has shown that the latter host species is more heavily infected than the former and that larger individuals may have higher levels of hemogregarine intensity of infection than smaller individuals (Maia *et al.*, 2014). The second system consisted of two lacertid species, *Atlantolacerta andreanskyi* and *Podarcis vaucheri*, and one gecko species, *Quedenfeldtia trachyblepharus*, which co-occur in the Atlas Mountains in Morocco. These lizard species have distinct habitat requirements and occupy different micro-niches within the same habitat (Carretero *et al.*, 2006). The analysis of these sympatric host species is a good proxy to identify the role of microhabitat preferences on patterns of hemogregarine infection.

Therefore, the objectives of this study were to determine inter- and intra-specific differences in hemogregarine prevalence and intensity of infection between seasons in sympatric species from two geographical locations. In particular, we aimed to investigate whether infection parameters were higher during the mating periods compared with other periods. We also aimed to determine if variation in infection parameters are associated with parasite lineage specificity. Finally, we aimed to investigate the relationships between overall parasite infection parameters and host factors, such

as body size, tail autotomy and habitat preferences (macro and microhabitat). For these purposes, we investigated the temporal dynamics of hemogregarine infections using a recently designed quantitative PCR (qPCR). This assay has been shown to have increased detectability in comparison with traditional methods (Maia *et al.*, 2014), therefore minimizing the limitations related to detectability.

## Materials and Methods

### *Sample and data collection*

Sampling was conducted in two lizard sympatric systems in different countries, Portugal (Moledo N41.8385; W-8.8740) and Morocco (Oukaimeden N31.2035; W-7.8617). A total of 534 blood samples were collected in Moledo from two lizard species living in sympatry: *Podarcis bocagei* (347) and *Podarcis hispanica* (187), with 7 sampling points across 2011-2013 (Table 5-6). A total of 328 blood samples were collected in Oukaimeden from three lizard species living in sympatry: *Atlantolacerta andreanskyi* (64), *Podarcis vaucheri* (93) and *Quedenfeldtia trachyblepharus* (171) with 3 sampling points across 2011-2012 (Table 5-7). The latter species was collected from three proximal areas within approximately 1km of each other. These were defined as microhabitats for this host species and consisted of rocky outcrops separated by unsuitable meadow. For each reptile individual a small tail tip was collected and preserved in 96% ethanol (additionally in Moledo, regeneration of the tail was annotated prior to taking the sample), while blood was stored in Whatman filter paper and kept at -20ºC prior to molecular analysis. The body size of each animal snout-vent-length (SVL) was measured and individuals were searched for the presence of ectoparasites. After processing, all individuals were released at the site of capture. Daily data on mean temperature, minimum temperature, maximum temperature, precipitation sum and averaged sea level pressure were downloaded for a week prior to sampling days (i.e. 2011-04-28, 2011-05-22, 2011-07-28, 2011-09-07, 2011-10-10, 2012-05-16, 2012-07-19, 2012-10-12, 2013-07-04 and 2013-10-30) from E-OBS V11.0 database (Haylock *et al.*, 2008). Exact GPS point information was obtained using the Point Sampling Tool plugin in Quantum GIS v2.8.1 Wien. These measures were averaged and used as an indication of the environmental conditions for that period of the year. This data was only used for Moledo, given the limited between-years sampling for Oukaimeden.

### *DNA extraction and quantitative PCR*

DNA was extracted from blood drops stored in Whatman filter paper using the standard saline protocol (Sambrook *et al.*, 1989; Maia *et al.*, 2014). Samples were diluted to 10ng/µl with nuclease-free water (QIAGEN) using an ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). To estimate prevalence and intensity of hemogregarine infections we used a recently developed qPCR assay using the primers JM4_F (5'-ACTCACCAGGTCCAGACATAGA-3') and JM5_R (5'-CTCAAACTTCCTTGCGTTAGAC-3') (Maia *et al.*, 2014) with triplicates of each sample and

quadruplicates of plasmid dilutions in 384 well plates. A LightCycler® 480 Instrument II machine (Roche) was used following the protocol in (Maia *et al.*, 2014). Reactions were conducted in a total volume mix of 10 µl containing iQ™ SYBR® Green supermix at 1x, each primer at 0.5mM, 1µl of 10ng/µl genomic DNA. Raw qPCR results were exported using the program LightCycler® 480 Software release 1.5.0 (Roche) and the baseline threshold was determined individually for each plate using the algorithm implemented in LinRegPCR (Ruijter *et al.*, 2009). Parasite identity was verified using Melting Curve Analysis (MCA) following (Maia *et al.*, 2014).

*Conventional PCR, sequencing and phylogenetic analyses*

PCR amplification was made for a selection of the qPCR positives in all host species using the primers HepF300 (5'- GTTTCTGACCTATCAGCTTTCGACG-3') and HepR900 (5'- CAAATCTAAGAATTTCACCTCTGAC-3') (Ujvari *et al.*, 2004), targeting part of the 18S rRNA gene region of apicomplexan parasites. PCR reactions and protocols followed (Maia *et al.*, 2014). Negative and positive controls were run with each reaction. The positive PCR products were purified and sequenced by a commercial sequencing facility (Macrogen Europe, Netherlands). Geneious v6.1.6 was used for contig assembly and visualization of sequences. A total of 107 usable sequences were obtained and a similarity analysis using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) was used to find the most similar sequences in GenBank. DnaSP v5 (Librado and Rozas, 2009) was used to estimate the number of haplotypes for individuals with no double peak positions. The "heterozygotes plugin" was used to search for possible double peak positions. Double peaks with more than 30% of peak similarity were resolved based on the unique haplotypes detected in this study (see Table S13 and Table S14). Consensus sequences for each individual were deposited in GenBank under the accession numbers XXXXXX.

For the phylogenetic analysis, one representative of each hemogregarine haplotype for each geographical location (2 for Moledo and 4 for Oukaimeden) was aligned with published data obtained from GenBank. For the alignment we used MUSCLE (Edgar, 2004) with default parameters. The alignment consisted of 91 taxa and was 567 bp long. Two phylogenetic analyses (Maximum Likelihood, ML, and Bayesian Inference, BI) were conducted. Maximum Likelihood (ML) analysis were conducted using RAxML v.7.0.3 with a GTR+GAMMA model for a run of 1000 thorough bootstrap replicates (Stamatakis, 2006). The AIC criterion conducted in jModeltest 0.1 (Posada, 2008) was used to choose the best model of evolution (i.e. TPM1uf+G). BI was implemented using Mr. Bayes v.3.1 (Huelsenbeck and Ronquist, 2001) with parameters estimated as part of the analysis. The analysis was run for $10 \times 10^6$ generations, saving one tree each 1000 generations. The log-likelihood values of the sample points were plotted against the generation time and all the trees prior to reaching stationarity were discarded, ensuring that burn-in samples were not retained. Remaining trees were combined in a 50% majority consensus tree (Huelsenbeck and Ronquist, 2001). *Dactylosoma ranarum* (HQ224958) was used as an outgroup following (Barta *et al.*, 2012)

and the resulting estimate of relationships was displayed in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

*Statistical analysis*

Prevalence is defined as the number of infected individuals in a population. Infection status of an individual refers whether it is infected or not. And intensity of infection is defined as the number of hemogregarine 18S rRNA gene copies in 10 ng/µl of total DNA determined by qPCR.

The continuous variables “intensity of infection” and “host body size” were tested for normality using the Shapiro-Wilk normality test. To reach normality, the parasite copy numbers as estimated by qPCR for hemogregarine intensity of infection were transformed using the formula *log (x+1)*. Homogeneity of variances were tested using Bartlett test for host body size, prevalence and intensity of infection between species, sexes, years and months. Homogeneity of variances was met for all variables (all $P>0.10$, except for prevalence between the three host species in Oukaimeden, df=2, K2=51.046, $P<0.001$). Data from April 2011 for Moledo and September 2011 for Oukaimeden (referred to as odd months in this manuscript) were not available for the year 2012 and 2013 for Moledo and the year 2012 for Oukaimeden and so were excluded when comparing for between-years differences to avoid seasonal biases. Instead, data from these months was used to determine variation within the year 2011 for both datasets (Moledo: April, July and November; and Oukaimeden: May and September).

We used Fisher Exact Test for Count Data to determine differences in prevalence between species and sexes of each species of the same sex across months and years, as well as overall differences for tail condition for Moledo and microhabitats for the gecko species in Oukaimeden. To test for overall correlation between host body size and infection status we used Point-biserial correlation from the package “ltm”, and Spearman’s rank correlation test for host body size and hemogregarine intensity of infection. We created plots using the package “ggplots2”. To determine differences within-sex, intra- and inter-species for intensity of infection between individual time points, as well as overall differences for tail condition for Moledo, we used T-tests (significant at $P<0.05$). Comparison of parasite parameters between time points included the following data subsets: i) overall differences between and within species, ii) between years, iii) within a year; and iv) between the same months of different years to control for seasonal bias between years.

To test for the effect of time of collection (year or month), host species and sex on host body size we conducted Analysis of variance (ANOVA) using a full factorial model with the variables time of collection, host species and host sex. For the Moledo population, a significant effect of host sex on host body size was detected for all data subsets (see results below, all $P<0.05$), while for Oukaimeden a significant effect of host species was detected for all data subsets (all $P<0.05$) and of host sex for the year 2011 (df=1, sum sq=284.3, F=11.151, P=0.001, see Table S11 and Table S12). For these reasons, body size was used as a covariable in model testing when examining

differences between time points. We then modelled the effects of host body size (as a covariate), year or month of collection, host species and host sex on parasite infection parameters (i.e. prevalence and intensity). To model hemogregarine prevalence of infection we used Generalized Linear Models (GZLM) from the package “MASS” with binomial logit distribution and Chi-square significance, and to model hemogregarine intensity of infection we used Linear Models (LM). To test for suitability of the linear models we plotted the distribution of the residuals (all were randomly distributed) and conducted Shapiro-Wilk normality test of the residuals from the linear regression model (all were P>0.05). To obtain the best fitting models we used stepwise simplification under the AIC and BIC criteria starting with a full factorial model that included the dependent variable prevalence or intensity of hemogregarine infection, the covariable host body size, the factors year or month of collection, host species, host sex, and all interactions. All analyses were conducted in R software version 1.3.0.

## Results

### *Parasite identification and genetic structure*

Regarding the hosts from Moledo, a total of 46 hemogregarine sequences for a fragment of the 18S rRNA gene were obtained (28 from *P. bocagei* and 18 from *P. hispanica*, see Table S13). Two closely related haplotypes were retrieved from these sequences. We also detected the presence of mixed infections for these two haplotypes in 14 infected individuals (30%, see Table S13). Regarding the hosts from Oukaimeden, a total of 57 hemogregarine sequences were obtained. Of those 12 were from *A. andreanskyi* and 45 from *P. vaucheri*. (Table S14). No hemogregarine sequences were obtained from *Q. trachyblepharus* by PCR likely due to low infection levels as detected by qPCR (Table 5-7). However, Melting Curve Analysis (MCA) of the few positives obtained by qPCR for this host species matched the reference sequence of *Hepatozoon* used as a standard, confirming the parasite identity of these sequences. This was true for 8 positives obtained except for one of the samples from *Q. trachyblepharus* that appeared distinct based on MCA (single melting peak at 82.3ºC, while hemogregarine peak was at 81.2ºC). This was confirmed by conventional PCR to be an infection by *Sarcocystis* sp. (BLAST results, 99% identity with AY015113 from *Sarcocystis lacertae* infecting a lizard, and KC696570 from *Sarcocystis* sp. infecting the snake *Malpolon monspessulanus*) and thus was excluded from further analysis. The phylogenetic analysis of the hemogregarine parasites shows that all hemogregarine haplotypes cluster in the same main lineage from the *Hepatozoon*/*Karyolysus* complex, known to infect these and other reptile host species from North Africa and the Mediterranean Basin (Figure 5-9). Thus, these are referred to as hemogregarines throughout the manuscript.

Table 5-6 Blood samples collected at different time intervals between 2011-2013 from two lizard species living in sympatry in Moledo (Portugal) screened for hemogregarine parasites.

| | | 2011 | | | | | | | | | 2012 | | | | | | 2013 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | April 2011 | | | July 2011 | | | October 2011 | | | July 2012 | | | October 2012 | | | July 2013 | | | October 2013 | | |
| | | n | inf | intensity (mean±sd) | n | inf | intensity (mean±sd) | n | inf | intensity (mean±sd) | n | inf | intensity (mean±sd) | n | inf | intensity (mean±sd) | n | inf | intensity (mean±sd) | n | inf | intensity (mean±sd) |
| *Podarcis bocagei* | F | 30 | 23 | 2.92±0.14 | 8 | 7 | 2.63±0.13 | 39 | 16 | 2.43±0.19 | 9 | 6 | 2.48±0.51 | 19 | 11 | 2.32±0.27 | 8 | 4 | 2.50±0.40 | 31 | 15 | 2.34±0.29 |
| | M | 41 | 33 | 2.50±0.14 | 10 | 5 | 2.44±0.36 | 41 | 28 | 2.58±0.15 | 18 | 12 | 2.13±0.26 | 26 | 21 | 2.32±0.19 | 34 | 23 | 2.57±0.16 | 33 | 22 | 2.11±0.11 |
| | | 71 | 56 | 2.67±0.11 | 18 | 12 | 2.55±0.16 | 80 | 44 | 2.53±0.11 | 27 | 18 | 2.25±0.24 | 45 | 32 | 2.32±0.15 | 42 | 27 | 2.56±0.15 | 64 | 37 | 2.21±0.13 |
| *Podarcis hispanica* | F | 17 | 12 | 2.90±0.19 | 4 | 2 | 1.97±0.97 | 10 | 5 | 2.73±0.48 | 8 | 7 | 2.59±0.42 | 4 | 4 | 2.68±0.27 | 12 | 8 | 2.55±0.27 | 15 | 12 | 2.57±0.21 |
| | M | 29 | 25 | 2.66±0.15 | 9 | 7 | 2.97±0.22 | 26 | 20 | 2.79±0.12 | 11 | 10 | 2.72±0.26 | 10 | 9 | 2.77±0.19 | 14 | 10 | 2.46±0.35 | 18 | 11 | 2.49±0.22 |
| | | 46 | 37 | 2.74±0.12 | 13 | 9 | 2.75±0.27 | 36 | 25 | 2.78±0.14 | 19 | 17 | 2.67±0.22 | 14 | 13 | 2.74±0.16 | 26 | 18 | 2.50±0.22 | 33 | 23 | 2.53±0.15 |
| Total per month | | 117 | 93 | 2.70±0.08 | 31 | 21 | 2.64±0.15 | 116 | 69 | 2.62±0.09 | 46 | 35 | 2.45±0.16 | 59 | 45 | 2.44±0.12 | 68 | 45 | 2.54±0.12 | 97 | 60 | 2.33±0.10 |

Table 5-7 Blood samples collected at different time intervals between 2011-2012 from three lizard species living in sympatry in Oukaimeden (Morocco) screened for hemogregarine parasites.

| | | 2011 | | | | | | | | 2012 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | May 2011 | | | | September 2011 | | | | May 2012 | | | |
| | | n | inf | % | intensity (mean±sd) | n | inf | % | intensity (mean±sd) | n | inf | % | intensity (mean±sd) |
| *Atlantolacerta andreanskyi* | F | 9 | 8 | 89 | 2.54±0.27 | 11 | 6 | 55 | 1.62±0.25 | 15 | 10 | 67 | 2.4±0.18 |
| | M | 11 | 8 | 73 | 2.00±0.31 | 7 | 3 | 43 | 2.28±0.31 | 11 | 9 | 82 | 2.45±0.31 |
| | | 20 | 16 | 80 | 2.27±0.21 | 18 | 9 | 50 | 1.84±0.21 | 26 | 19 | 73 | 2.43±0.17 |
| *Podarcis vaucheri* | F | 20 | 19 | 95 | 3.32±0.19 | 7 | 4 | 57 | 3.14±0.19 | 6 | 6 | 100 | 3.17±0.10 |
| | M | 21 | 19 | 90 | 2.86±0.18 | 14 | 12 | 86 | 2.78±0.21 | 25 | 20 | 80 | 2.89±0.18 |
| | | 41 | 38 | 93 | 3.09±0.13 | 21 | 16 | 76 | 2.87±0.17 | 31 | 26 | 84 | 2.95±0.14 |
| *Quedenfeldtia trachyblepharus* | F | 21 | 0 | 0 | - | 30 | 2 | 7 | 1.17±0.24 | 35 | 3 | 9 | 0.98±0.23 |
| | M | 18 | 0 | 0 | - | 30 | 2 | 7 | 1.01±0.13 | 37 | 2 | 5 | 0.93±0.08 |
| | | 39 | 0 | 0 | - | 60 | 4 | 7 | 1.09±0.12 | 72 | 5 | 7 | 0.96±0.18 |
| Total per month | | 100 | 54 | 54 | 2.85±0.12 | 99 | 29 | 29 | 2.31±0.17 | 129 | 50 | 39 | 2.11±0.16 |

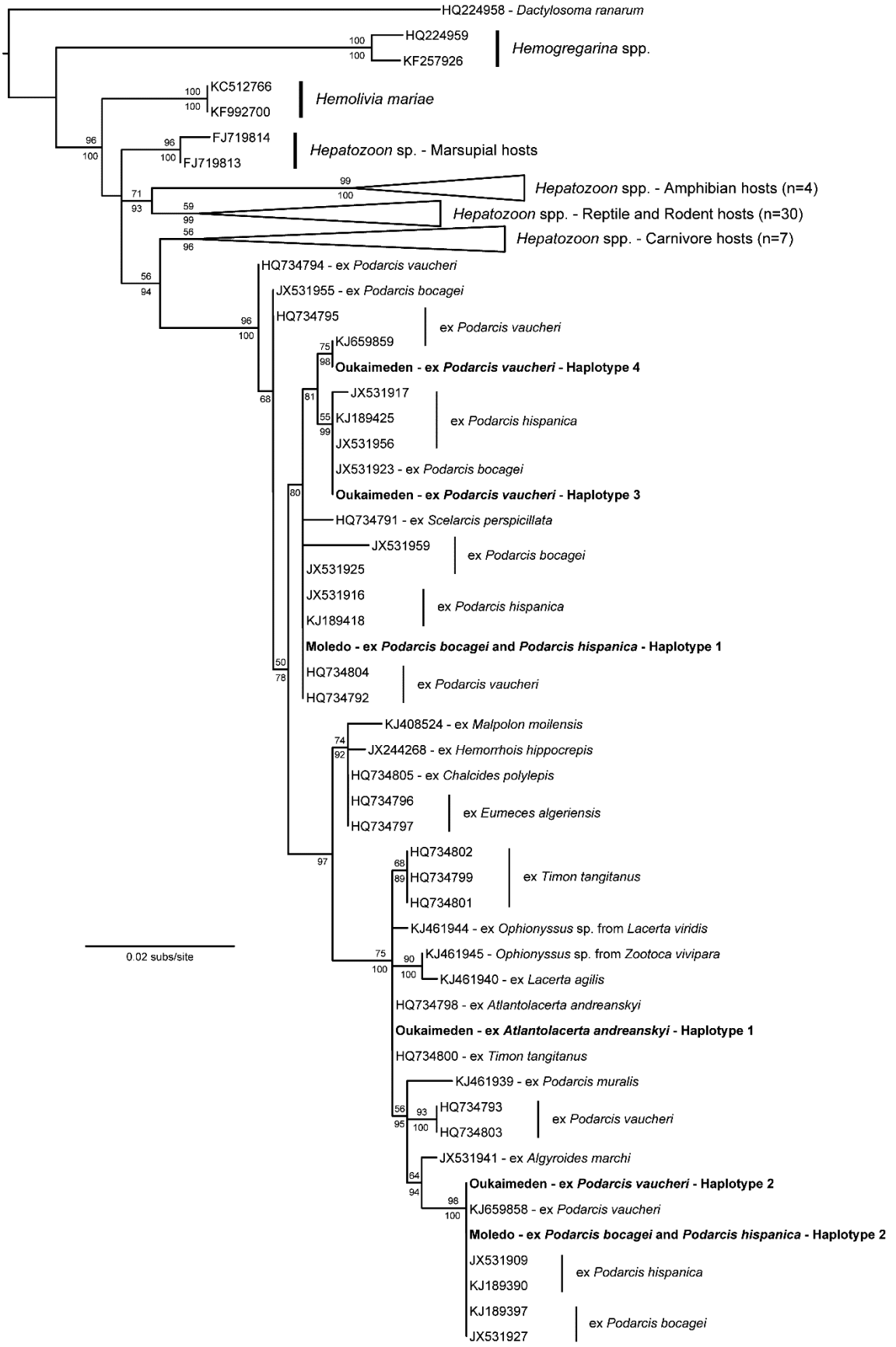


Figure 5-9 Hemogregarine phylogenetic relationships from a Maximum Likelihood analysis (ML) of a fragment of the 18S rRNA gene. Bootstrap support values are given above nodes and Bayesian Posterior probabilities are given below nodes. Haplotypes obtained in this study are in bold (Table S13 and Table S14).

*Case study 1: Moledo, Portugal*

*Between and within species*: we found that prevalence was significantly higher in *P. hispanica* than in *P. bocagei* (Fisher-test, P=0.006). Prevalence was significantly higher in males of *P. bocagei* than in females (P=0.005), while there were no differences between sexes of *P. hispanica* (P=0.881, see Table 5-6). We found significantly higher intensity levels in *P. hispanica* than in *P. bocagei* (t-test, t=2.503, df=316.231, P=0.013) but not between sexes within each species (*P. bocagei*, t=1.339, df=157.865, P=0.183; and *P. hispanica*, t=0.247, df=93.047, P=0.805, see Table 5-6). Regarding host characteristics, we found significant differences in prevalence between individuals with regenerated tails compared to those with intact tails within each species (Fisher test, P<0.005) but not in intensity of infection (t-test, P>0.05). Body size was positively correlated with prevalence for both species (Figure 5-10 A, Point-biserial correlation, $\hat{r}_{p.b.}$=0.42 for *P. bocagei*, and $\hat{r}_{p.b.}$=0.40 for *P. hispanica*) but not significantly correlated with intensity of infection, despite a slight positive slope (Figure 5-10 B, Spearman correlation, *P. bocagei* ρ=0.078, P=0.244, and *P. hispanica* ρ=0.050, P=0.556).

*Within a year variation*: between host species, prevalence was significantly higher in *P. hispanica* than in *P. bocagei* only for the year 2012 (91% and 69%, respectively, Fisher test, P=0.012) but not for 2011 (75% and 66%, respectively, P=0.097) or 2013 (69% and 60%, respectively, P=0.159) (see Table 5-6 and Table 5-8 and Figure 5-11 A). For *P. bocagei* we found significant differences for mean prevalence between the three months of 2011 (April (79%), July (67%) and October (55%), Fisher tests, P=0.002). This was also significant for the same period in females (April (77%), July (88%) and October (41%), P=0.007) but not in males of this species (April (80%), July (50%) and October (68%), P=0.138) (see Table 5-6 and Figure 5-11 A). For *P. hispanica*, no significant differences in prevalence within any of the years were found (all P>0.05). Regarding intensity of infection, for *P. bocagei*, males had significantly higher intensities in July than in October of 2013 (t-test, t=2.255, df=38.826, P=0.030; for all other comparisons P>0.05) (Figure 5-11B). For *P. hispanica*, no significant differences in intensity within any of the years were found (all P>0.05). Therefore, month of collection was not an important variable in our best models for both prevalence and intensity of infection (Table 5-8), showing that hemogregarine infection parameters remained similar between months of each year (see Table 5-6 and Figure 5-11).

*Between-years*: we detected a significant effect of host species on both prevalence and intensity of infection between years when accounting for the variation between years (Table 5-8). This shows that the differences in infection patterns between the two host species are maintained regardless of the year of collection. For both species, prevalence differed significantly between years [Fisher tests, P=0.011 for *P. bocagei* (63% in 2011, 69% in 2012, and 78% in 2013), and P=0.037 for *P. hispanica* (68% in 2011, 91% in 2012, and 69% in 2013)] (see Table 5-6 and Figure 5-11 A). This explains the importance of the variable year of collection in Table 5-8. Between sexes of each species, only

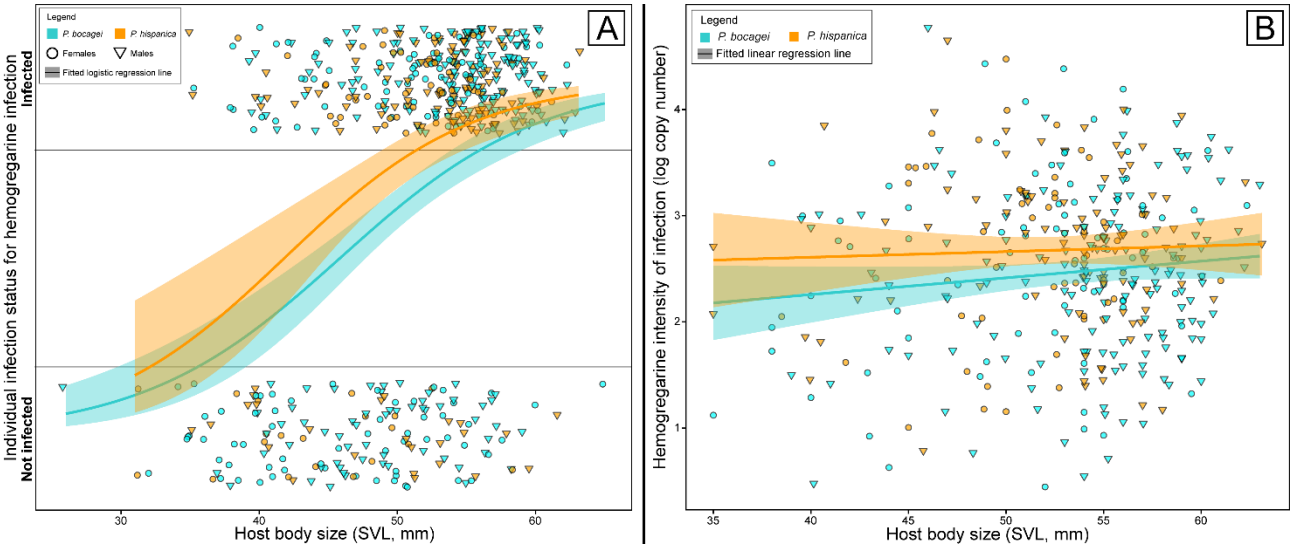


Figure 5-10 Relationship between hemogregarine infection parameters and host body size in two sympatric lizard species in Moledo. Colours represent host species and shapes represent sexes. A) Relationship between individual infection status for hemogregarine infection and host body size. Fitted logistic regression line was produced by fitting a GLM with binomial logit distribution for overall prevalence and body size with 95% confidence region (in grey). Points representing each individual infection status were spaced and slightly transparentized only for representation purposes. B) Relationship between hemogregarine intensity of infection and host body size. Fitted logistic regression line was produced by fitting a LM for overall intensity of infection and body size with 95% confidence region (in grey).

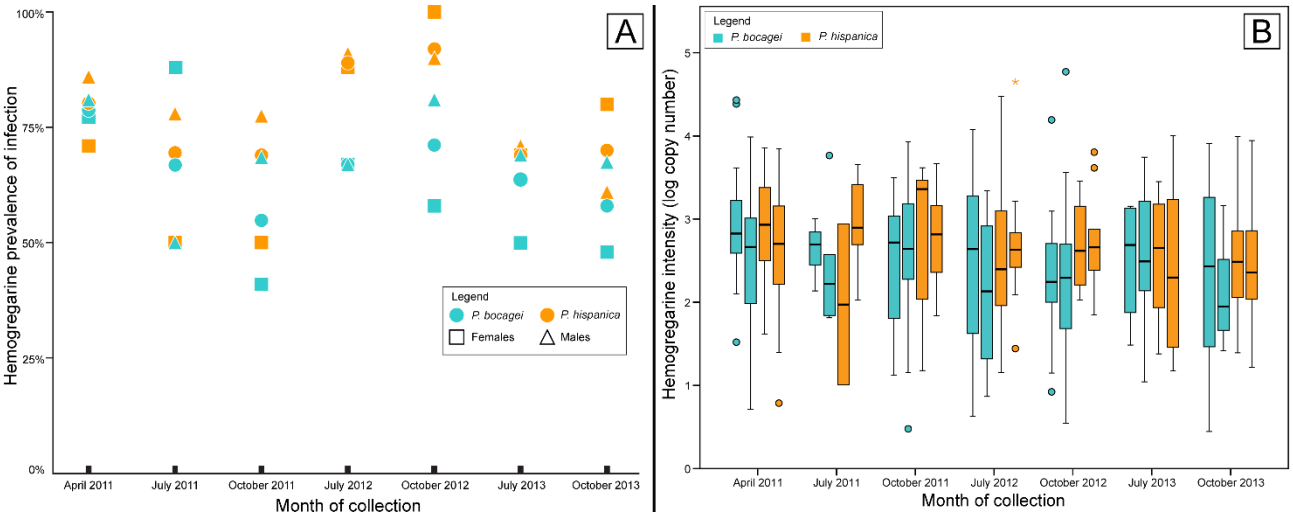


Figure 5-11 Temporal dynamics in hemogregarine infection parameters and host body size in two sympatric lizard species in Moledo. Colours indicate the different host species and dashed line represent the sexes. A) Prevalence of infection variation over time. B) Intensity of infection variation over time. For each host species, the first boxplot represents females and the second boxplot represents males.

prevalence in females of *P. bocagei* differed significantly between years (50% in 2011, 92% in 2012, and 83% in 2013, P=0.040; for all comparisons P>0.05). Regarding intensity of infection, *P. hispanica* had marginal significantly higher intensity levels than *P. bocagei* also for the year 2012 (t-test, t=2.091, df=68.196, P=0.040) but not for 2011 and 2013 (all P>0.05) (see Table 5-6 and Figure 5-11 B). This explains why year of collection is an important variable and host species is a significant variable in our best model (Table 5-8). No differences in intensity of infection were found between years for each species and sexes of each species (t-tests, P.>0.05). Finally, we examined

Table 5-8 Best models for prevalence and intensity of infection regarding temporal dynamics of hemogregarine infection in the two *Podarcis* species in Moledo (Portugal). Models were chosen based on AIC and BIC criteria following a stepwise simplification of a full factorial model. Grey indicates significance and * the level of significance.

| Data subset<br>**Prevalence** | **Body size** | **Time of collection (month/year)** | **Species** | **Sex** | **Species*Sex** | **AIC** | **BIC** |
|---|---|---|---|---|---|---|---|
| 2011 (3 months) | df=1, x2=57.050, P<0.001*** | - | df=1, x2=1.820, P=0.177 | df=1, x2=0.359, P=0.549 | - | 274.299 | 288.603 |
| 2012 (2 months) | df=1, x2=15.029, P<0.001*** | - | df=1, x2=5.754, P=0.016* | df=1, x2=0.086, P=0.770 | - | 102.395 | 113.010 |
| 2013 (2 months) | df=1, x2=21.997, P<0.001*** | - | df=1, x2=2.706, P=0.100 | df=1, x2=0.063, P=0.802 | df=1, x2=2.708, P=0.100 | 198.835 | 211.966 |
| Between-years | df=1, x2=71.388, P<0.001*** | df=2, x2=5.455, P=0.065 | df=1, x2=7.900, P=0.005** | df=1, x2=0.223, P=0.637 | - | 461.949 | 486.147 |
| July across years | df=1, x2=25.916, P<0.001*** | - | df=1, x2=6.396, P=0.011* | df=1, x2=2.355, P=0.125 | - | 151.323 | 163.230 |
| October across years | df=1, x2=46.634, P<0.001*** | - | df=1, x2=3.049, P=0.081 | df=1, x2=2.204, P=0.138 | - | 311.665 | 326.088 |
| **Intensity** | | | | | | | |
| 2011 (3 months) | df=1, sum sq=5.924, F=11.749, P<0.001*** | - | df=1, sum sq=1.051, F=2.085, P=0.150 | df=1, sum sq=1.668, F=3.307, P=0.071 | - | 399.978 | 416.025 |
| 2012 (2 months) | - | - | df=1, sum sq=3.053, F=4.013, P=0.049* | df=1, sum sq=0.021, F=0.028, P=0.867 | | 210.081 | 219.609 |
| 2013 (2 months) | - | - | df=1, sum sq=0.668, F=1.001, P=0.320 | df=1, sum sq=0.067, F=0.100, P=0.753 | | 260.551 | 271.167 |
| Between-years | - | df=2, sum sq=2.242, F=1.752, P=0.174 | df=1, sum sq=4.297, F=6.752, P=0.010* | df=1, sum sq=0.018, F=0.028, P=0.866 | - | 663.087 | 684.787 |
| July across years | - | - | df=1, sum sq=0.601, F=0.819, P=0.368 | df=1, sum sq=0.011, F=0.015, P=0.904 | - | 260.389 | 270.849 |
| October across years | - | - | df=1, sum sq=3.913, F=6.580, P=0.011* | df=1, sum sq=0.009, F=0.015, P=0.902 | - | 408.346 | 420.982 |

differences in prevalence and intensity between years but considering the same month of the year to avoid the effects of seasonal variation of infection. For both July and October, no significant differences in prevalence for each and within each species were found for different years (Fisher tests, all P>0.05, see Table 5-6). For this reason, the variable year of collection was not an important variable in our best models (Table 5-8), which shows that prevalence levels remained similar in the same months of different years. Similar results were obtained regarding intensity of infection. With the exception of males of *P. bocagei* that had significantly higher intensity levels in October 2011 than in 2013 (t-test, t=2.479, df=47.269, P=0.017, Figure 5-11 B), no significant differences were found in July and October between years for each species and within each species (P>0.05).

*Environmental variables*: the year 2012 was associated with significantly higher prevalence and intensity of infection in *P. hispanica* than in *P. bocagei*. This year had the highest minimum temperature and the lowest maximum temperature, while both mean temperature and precipitation sum were between the mean for the other years (Table 5-9). In addition, males of *P. bocagei* had higher intensities in October 2011 than the other months of that same year. This month had intermediate temperatures relative to the other months (Table 5-9).

*Case study 2: Oukaimeden, Morocco*

*Between and within species*: between the two lacertid species, we found that *P. vaucheri* had significantly higher prevalence (Fisher test, P=0.008) and intensity levels (t-test, t=5.198, df=86.369, P<0.005) than *A. andreanskyi*. Compared to the gecko species *Q. trachyblepharus*, both these lacertid species had significantly higher prevalence (all P<0.001) and intensity levels (t=17.904, df=37.348, P<0.005 for *P. vaucheri*, and t=8.994, df=46.021, P<0.005 for *A. andreanskyi*) (Table 5-7). Therefore, hemogregarine infection parameters of the gecko *Q. trachyblepharus* were always significantly different when compared with the two lacertid species (P<0.05, see “all” subsets in Table 5-10). For this reason, only the models using the subsets without this gecko species are discussed further. No significant differences were found in prevalence between sexes of each host species (P=0.595 for *A. andreanskyi*; P=0.752 for *P. vaucheri*; and P=0.746 for *Q. trachyblepharus*) (Table 5-7). Regarding intensity of infection in sexes of each species, we found that females of *P. vaucheri* had significantly higher intensity levels than males (t-test, t=2.480, df=63.063, P=0.016), while no differences were found for *A. andreanskyi* (t=0.032, df=37.838, P=0.975) or *Q. trachyblepharus* (t=0.599, df=5.951, P=0.571) (Table 5-7). Furthermore, we did not find differences in prevalence (Fisher test, P=0.832) or intensity of infection (t-tests, all P>0.05) between the three proximal microhabitats of *Q. trachyblepharus*. Regarding the relationships between body size and infection patterns in lacertid hosts, we found a positive correlation with prevalence for both species [Figure 5-12 A, Point-biserial correlation, weak for *A. andreanskyi* ($\hat{r}_{p.b.}$=0.22), and strong for *P. vaucheri* ($\hat{r}_{p.b.}$=0.46)]. On the other hand, no correlation with intensity of infection for both species [Figure 5-12 B, Spearman correlation, *A. andreanskyi* ρ=0.060, P=0.694, and *P. vaucheri* ρ=-0.159, P=0.160)].

Table 5-9 Environmental variables for the two sampling locations in this study, Moledo (Portugal) and Oukaimeden (Morocco). Variables were downloaded from E-OBS V11.0 database for a week prior to sampling days (see Materials and Methods). In bold are the highest values and in italics the lowest values for each variable

| | **Moledo (Portugal)** | | | | | | | **Oukaimeden (Morocco)** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **2011** | | | **2012** | | **2013** | | | | |
| Environmental variable | **April 2011** | **July 2011** | **October 2011** | **July 2012** | **October 2012** | **July 2013** | **October 2013** | **May 2011** | **September 2011** | **May 2012** |
| **Averaged sea level pressure** | *1011.8 ± 5.0 (1002-1018)* | 1016.8 ± 1.6 (1014-1020) | **1025.4 ± 1.4 (1024-1028)** | 1021.8 ± 2.8 (1018-1025) | 1017.3 ± 2.5 (1014-1020) | 1018.7 ± 2.3 (1015-1022) | 1018.2 ± 7.6 (1005-1027) | - | - | - |
| | | **1021.1 ± 1.5 (1019-1024)** | | 1019.5 ± 2.6 (1016-1023) | | *1018.5 ± 5.0 (1010-1024)* | | | | |
| **Precipitation sum** | 1.9 ± 4.2 (0-12) | *0.0* | *0.0* | 1.5 ± 3.8 (0-11) | 2.6 ± 2.7 (0-8) | *0.0* | **10.6 ± 15.1 (0-46)** | - | - | - |
| | | *0.0* | | 2.1 ± 3.2 (0-10) | | 5.3 ± 7.5 (0-23) | | | | |
| **Mean temperature** | 16.8 ± 1.7 (14-19) | 21.0 ± 1.4 (19-23) | 19.8 ± 2.1 (17-23) | 20.3 ± 3.3 (16-26) | 18.0 ± 2.1 (14-21) | **23.4 ± 2.6 (19-27)** | *13.9 ± 2.1 (11-16)* | *12.5 ± 1.2 (11-15)* | 16.2 ± 3.7 (13-22) | **18.6 ± 0.2 (18-19)** |
| | | **20.4 ± 1. 8 (18-23)** | | 19.2 ± 2.7 (15-24) | | *18.7 ± 2.3 (15-22)* | | | | |
| **Minimum temperature** | 11.9 ± 0.5 (11-13) | 14.2 ± 1.4 (12-16) | 13.7 ± 1.9 (11-16) | 14.1 ± 2.0 (11-18) | 14.9 ± 2.0 (11-17) | **15.2 ± 1.6 (14-18)** | *10.2 ± 3.2 (4-15)* | *8.4 ± 1.4 (6-11)* | 12.3 ± 1.7 (11-15) | **13.3 ± 0.5 (12-14)** |
| | | 13.9 ± 1.6 (11-16) | | **14.5 ± 2.0 (11-17)** | | *12.7 ± 2.4 (10-16)* | | | | |
| **Maximum temperature** | 22.0 ± 3.4 (15-26) | 27.8 ± 2.1 (25-32) | 26.2 ± 2.7 (22-29) | 26.5 ± 5.9 (19-34) | 21.0 ± 2.4 (18-26) | **31.8 ± 3.3 (26-37)** | *18.2 ± 1.4 (17-20)* | *17.0 ± 2.3 (14-21)* | 24.9 ± 3.9 (21-31) | **26.7 ± 1.6 (25-29)** |
| | | **27 ± 2.4 (24-31)** | | *23.8 ± 3.7 (19-30)* | | 25.0 ± 2.4 (21-28) | | | | |

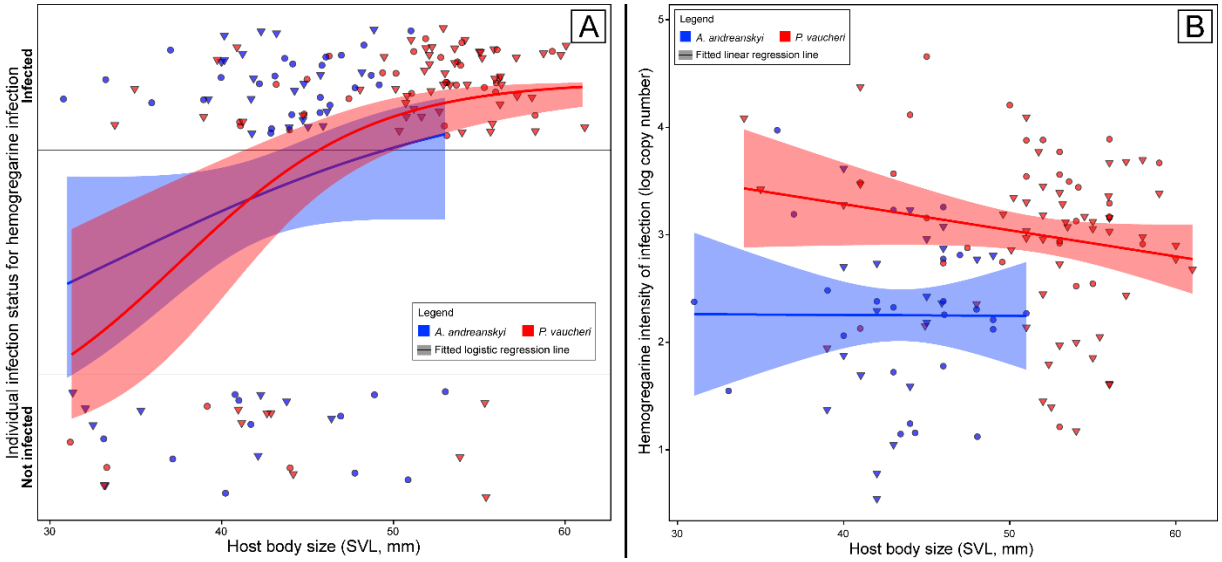


Figure 5-12 Relationship between hemogregarine infection parameters and host body size in the two lacertid lizard species sympatric in Oukaimeden (Morocco)
Colours represent host species and shapes represent sexes. A) Relationship between individual infection status for hemogregarine infection and host body size. Fitted logistic regression line was produced by fitting a GLM with binomial logit distribution for overall prevalence and body size with 95% confidence region (in grey). Points representing each individual infection status were spaced and slightly transparentized only for representation purposes. B) Relationship between hemogregarine intensity of infection and host body size. Fitted logistic regression line was produced by fitting a LM for overall intensity of infection and body size with 95% confidence region (in grey).

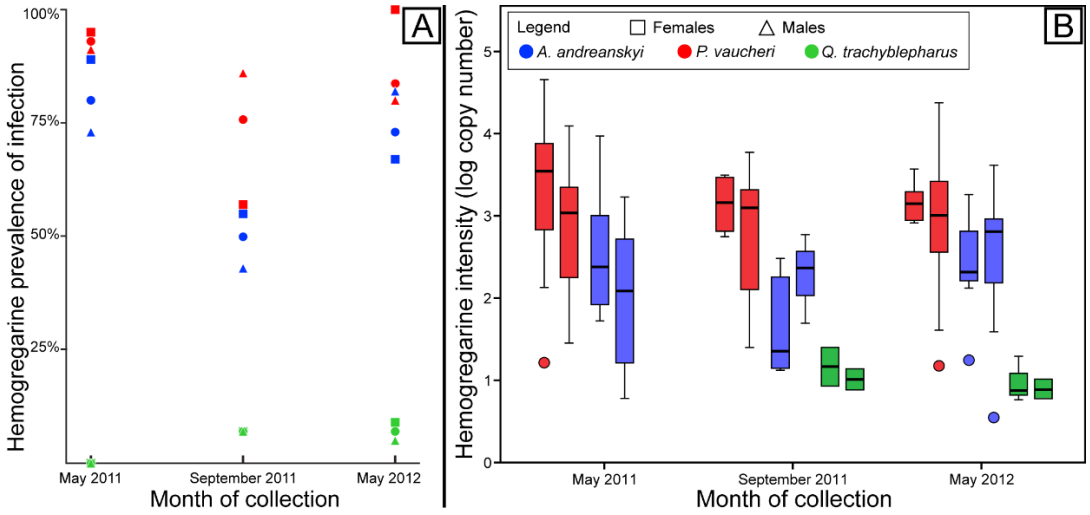


Figure 5-13 Temporal dynamics in hemogregarine infection parameters and host body size in two of the three sympatric lizard species in Oukaimeden (Morocco).
Colours indicate the different host species and dashed line represent the sexes. A) Prevalence of infection variation over time. B) Intensity of infection variation over time. For each host species, the first boxplot represents females and the second boxplot represents males.

*Within 2011 variation*: we did not find significant differences in mean prevalence in each species and sexes of each species for this period (Fisher test, all P>0.05, Figure 5-13 A). However, overall prevalence differed significantly between month of collection (P<0.001) (Table 5-7), which is why the variable month of collection was significant in our best model (Table 5-10). Regarding intensity of infection, we found that *P. vaucheri* had significantly higher levels than *A. andreanskyi* for both months of 2011 (t-test, t=3.272, df=27.562, P=0.003 for May, and t=3.789, df=17.513, P=0.001 for

Table 5-10 Best models for prevalence and intensity of infection regarding temporal dynamics of hemogregarine infection in the three lizard species in Oukaimeden (Morocco). Models were chosen based on AIC and BIC criteria following a stepwise simplification of a full factorial model. For each data subset, "all" refers to the comparison between the three host species *A. andreanskyi*, *P. vaucheri* and *Q. trachyblepharus*, while "no *Qt*" refers to the comparison excluding the latter host species, for which prevalence levels were extremely low compared with the other two host species (Table 5-7). Grey indicates significance and * the level of significance.

| Data subset<br>**Prevalence** | **Body size** | **Time of collection (month/year)** | **Species** | **Sex** | **AIC** | **BIC** |
|---|---|---|---|---|---|---|
| 2011 (all) | df=1, x2=33.017, P<0.001*** | df=1, x2=14.851, P<0.001*** | df=2, x2=110.473, P<0.001*** | df=1, x2=0.379, P=0.538 | 123.654 | 143.414 |
| 2011 (no *Qt*) | df=1, x2=20.988, P<0.001*** | df=1, x2=10.485, P=0.001*** | df=1, x2=0.045, P=0.832 | df=1, x2=0.484, P=0.487 | 80.790 | 93.816 |
| May between-years (all) | df=1, x2=41.262, P<0.001*** | df=1, x2=15.357, P<0.001*** | df=2, x2=127.542, P<0.001*** | df=1, x2=0.175, P=0.678 | 143.548 | 164.150 |
| May between-years (no *Qt*) | df=1, x2=12.528, P<0.001*** | df=1, x2=8.149, P=0.004** | df=1, x2=0.257, P=0.612 | df=1, x2=0.001, P=0.974 | 93.224 | 107.078 |
| **Intensity** | | | | | | |
| 2011 (all) | - | df=1, sum sq=5.491, F=9.666, P=0.003** | df=2, sum sq=10.178, F=17.918, P<0.001*** | df=1, sum sq=1.829, F=3.220, P=0.077 | 195.447 | 209.960 |
| 2011 (no *Qt*) | - | df=1, sum sq=13.476, F=3.448, P=0.067 | df=1, sum sq=13.477, F=22.897, P<0.001*** | df=1, sum sq=1.828, F=3.106, P=0.082 | 188.14 | 200.059 |
| May between-years (all) | df=1, sum sq=1.055, F=1.886, P=0.173 | df=1, sum sq=3.448, F=6.160, P=0.015* | df=2, sum sq=22.440, F=20.046, P<0.001*** | df=1, sum sq=2.492, F=4.452, P=0.037* | 244.867 | 263.445 |
| May between-years (no *Qt*) | df=1, sum sq=0.670, F=1.185, P=0.279 | df=1, sum sq=0.700, F=1.238, P=0.269 | df=1, sum sq=10.915, F=19.309, P<0.001*** | df=1, sum sq=3.036, F=5.370, P=0.023* | 231.35 | 246.916 |

September). This explains why we detected a significant effect of host species on intensity of infection for these two lacertid species (Table 5-10). No significant differences were found in intensity between months of 2011 for *A. andreanskyi* (t-test, t=1.439, df=20.682, P=0.165) or *P. vaucheri* (t=0.993, df=33.97, P=0.323, see Table 5-7 and Figure 5-13 B). We did not detect infections in gecko individuals in May 2011 (in 39 individuals) and only detected 4 infections in September 2011 (in 60, 7%, Table 5-7), so no comparison was made for this species. Within sexes of each species, with the exception of females of *A. andreanskyi* that had significantly higher intensity levels in May 2011 than in September 2011 (t=2.502, df=11.928, P=0.028), no significant differences were detected (all P>0.05, Figure 5-13 B).

*Between-years*: we detected a significant effect of year of collection (Table 5-10). We did not detect differences in prevalence or intensity of infection for each species and sexes of each species between years (Fisher test, all P>0.05, and t-tests, all P>0.05) (Table 5-7 and Figure 5-13 A). This explains why both host species and sex were not significant in our best model (Table 5-10). Despite this, we detected a significant effect of host species and sex on parasite intensity of infection (Table 5-10), which shows that intra-specific differences may exist when accounting for variations due to body size and host species.

**Discussion**

Our study investigates the temporal dynamics of hemogregarine infection in two distinct systems of sympatric lizard populations. For this, we analyzed two closely related host species in one location and two related lacertid species and one unrelated gecko species in another location. Because in each location, host species are exposed to the same macro habitat conditions but may have differential microhabitat usage (Sá-Sousa *et al.*, 2002; Carretero *et al.*, 2006), we can disentangle between host and ecological factors influencing infection patterns. Furthermore, by analyzing a distantly related host species and two related host species, we examine the effect of host relatedness on parasite infection.

Our results show that there were differences in intensity of infection and prevalence between species in both systems and that these differences were consistent between years, seasons and months within a year. In the first host-parasite system, *P. hispanica* had higher intensity levels than *P. bocagei*, and in the second host-parasite system the lacertids *P. vaucheri* and *A. andreanskyi* had the highest levels and the gecko *Q. trachyblepharus* the lowest. For phylogenetically related hosts, differences in parasite infection patterns between host species may be due to several abiotic and biotic factors, the latter including host behavior and immunity (Eisen and Wright, 2001; Hawley and Altizer, 2011). The behavior of the host can for example influence the degree of exposure to vectors, which would explain observed differences in prevalence (Sol *et al.*, 2000). *Podarcis hispanica* is a more generalist species compared to *P. bocagei* (Kaliontzopoulou *et al.*, 2012), which could explain the higher levels of infection due to increased encounters with vectors. Immunity is also often related

to variation in infection (Sol *et al.*, 2003; Huyghe *et al.*, 2010; Westerdahl *et al.*, 2013). Apart from individual variation in immune responses due to body size or nutrition, gender-based differences in immunity are well documented (Klein, 2004; Pollock *et al.*, 2012). In this study, we found that males of *P. bocagei* in Moledo had higher overall prevalence than females, as well as that males had higher intensity levels during Spring (the mating period) than in Autumn for one of the years. These differences might be due to the immunosuppressive effects of testosterone, a hormone that is commonly found in males, especially during the mating season (Hasselquist *et al.*, 1999; Oppliger *et al.*, 2004; Cox and John-Alder, 2005; Grear *et al.*, 2009; Halliday *et al.*, 2014). Apart from the immunosuppressive effects of testosterone, higher levels of this hormone may also increase aggressiveness in males and lead to more frequent encounters with other males. This could increase exposure to parasites, which could explain higher prevalence levels observed in lizard males (Salkeld and Schwarzkopf, 2005; Garrido and Pérez-Mellado, 2013).

Body size and condition have been used as proxies of age and fitness to study the influence of these factors on parasite infection patterns (García-Ramírez *et al.*, 2005; Amo *et al.*, 2006). However, different studies often report diverging associations between these factors and parasite infection. Some studies detect a positive correlation with hemoparasite prevalence and intensity (Amo *et al.*, 2005; Madsen and Ujvari, 2006; Maia *et al.*, 2014), while others detect a negative correlation (Smallridge and Bull, 2000; Brown *et al.*, 2006). For the two cases examined, we found an overall positive correlation between prevalence of infection and host body size. However, we did not find a correlation between intensity of infection and host body size. These patterns were consistent for the various years, seasons and months within a year. This may be an indication that larger, older individuals may have had more time to be exposed to competent vectors (Madsen and Ujvari, 2006). Or that the immune system of lizards is not an effective defense against hemogregarines because these hosts may have little or no acquired immunity (Sorci, 1995).

In addition, tail autotomy is a defense mechanism in lizards that evolved to escape potential predators. It has been also associated with particular lizard behaviors and differences in refuge use (Wilson and Cooper, 2008). After autotomy, lizards have to invest resources to restore the tail (Kuo *et al.*, 2013; McElroy and Bergmann, 2014), which may lead them to be more prone to parasitic infections. We found that *Podarcis* lizards with regenerated tails had higher prevalence than *Podarcis* with intact tails but we did not find differences in intensity of infection. This suggests that the stress of tail loss did not reduce resistance to parasitism once infection is established, a pattern also found in other lacertid species (Oppliger and Clobert, 1997). However, studies often find contrasting patterns regarding the relationship between tail break frequency and prevalence or intensity of infection (Huyghe *et al.*, 2010; Garrido *et al.*, 2015), suggesting that other factors can also influence these patterns.

Environmental factors can also influence parasite infection parameters, particularly in host-parasite systems with complex transmission dynamics, such as vector-borne diseases (Pérez-

Rodríguez *et al.*, 2013, 2014). We found that prevalence and intensity of infection was significantly higher in *P. hispanica* than in *P. bocagei* for the year with the most contrast between minimum and maximum temperature. Studies on *Plasmodium* species have shown that the development of these apicomplexan parasites can be greatly affected by temperature fluctuations (Paaijmans *et al.*, 2010). However, similar studies on influence of temperature fluctuations on hemogregarine and vector development are lacking, thus future research should investigate the optimal conditions for the development of these parasites.

Closely related sympatric host species are more likely to share the same parasites and to be similarly affected by them, while unrelated sympatric host species are more likely to differ in their parasite communities (Poulin *et al.*, 2011a). Both host species in the first system were found to be infected with the same hemogregarine haplotypes, while the two lacertid species in the second system were found to be infected with unique hemogregarine haplotypes, all of which had already been reported for these host species (Maia *et al.*, 2011, 2012; Damas-Moreira *et al.*, 2014). This raises several interesting questions regarding the influence of host relatedness and host-specificity in hemogregarine infection patterns in these systems. First, are the distinct haplotypes in the second system host-specific? Second, are the infection patterns a reflection of how different lineages evolved to affect their hosts? And third, are these infection patterns a result of different susceptibilities of each host species? To develop these hypothesis further, there is a need to use faster evolving gene markers to better understand the diversity and host-specificity of these parasite lineages. The 18S rRNA gene has been widely used for estimating hemogregarine diversity and phylogenetic relationships, however it has limited differentiation of cryptic hemogregarine lineages (Maia *et al.*, 2012; Kvičerová *et al.*, 2014; Haklová-Kočíková *et al.*, 2014). Once these are available, future studies should aim at developing qPCR protocols for simultaneous quantification and differentiation of closely related hemogregarine lineages. This would open new possibilities of study that have been applied to avian malaria host-parasite systems, such as distinguishing cryptic parasite species with minimal genetic differentiation (Falk *et al.*, 2015), investigating seasonal variation of different lineages (Cosgrove *et al.*, 2008), and examining seasonal evolution of lineages among different host species (Pérez-Rodríguez *et al.*, 2015). Although we could not confirm the genetic identity of the hemogregarines infecting the gecko *Q. trachyblepharus*, previous studies have shown that these lizards can harbor different and often unique hemogregarine lineages (Maia *et al.*, 2011; Harris *et al.*, 2015). Therefore, it is possible that also geckos were infected with unique hemogregarine lineages and this should be further studied. Moreover, we detected an unusually high level of an apicomplexan parasite in one gecko individual, which was identified to be a *Sarcocystis* infection. It is possible that the low hemogregarine infection levels found in geckos could be due to the occurrence of other parasites in the same hosts, which could lead to parasite-competition (Bell *et al.*, 2006). Therefore further research should investigate the implications of mixed infections in these hosts.

Finally, our findings show that infection patterns between host species, sexes and individuals reported, are maintained over time. The lack of temporal variation reported indicates that, at least for some host-parasite systems, using samples collected at different time points may produce reliable estimations of parasite infection parameters for inter-specific comparisons. This may be especially important when sampling is reduced and access to more samples is limited or impossible. Thus, these findings may have important applications in parasitological studies because detectability and sampling sizes are some of main limiting factors in these studies (Jovani and Tella, 2006; Jennelle *et al.*, 2007).

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# 6 GENERAL DISCUSSION

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The studies presented in this thesis shed new light on the identification and diversity of apicomplexan parasites in reptiles from various geographical locations, and their relationships with parasites of other vertebrates such as canids, rodents and amphibians. This thesis also contributes to draw attention to some of the methodological limitations in parasitological studies at estimating infection parameters and provides recommendations for a successful line of work. In addition, it draws attention to the current taxonomic uncertainty of various parasite groups and discusses ways to solve them. It also contributes to a better understanding of parasite infection patterns, transmission dynamics, host-specificity and ecology. Therefore, the studies presented in this thesis have focused on:

i. The use of molecular techniques to determine parasite infection parameters across various geographic scales and host species;
ii. The use of genetic information to reconstruct phylogenetic relationships that allow for a better understanding of host-parasite associations and transmission dynamics;
iii. The identification of distinct morphologic and genetic characters of parasite lineages to describe new parasite species and the genetic characterization of previously described parasite species for a placement of these parasites in a phylogenetic framework for the first time;
iv. The investigation of the spatial and temporal variation in hemogregarine infection parameters in different scenarios to assess potential host and ecological factors that may influence these patterns.

## 6.1 Challenges in parasite detection and quantification: the need for integrative analyses

The second chapter of the thesis shows that differences in accuracy, specificity and sensitivity of the detection and quantification protocols used can constrain the estimation of biologically relevant and realistic infection patterns. This is important because the estimation of parasite infection patterns is a first step towards the understanding of disease ecology transmission dynamics and host-specificity (Poulin and Mouillot, 2005; Poulin *et al.*, 2011). Three main detection and quantification techniques are used by parasitologists to estimate infection patterns: microscopy, conventional PCR and qPCR. Microscopy and conventional PCR have been the routine diagnostic tools for many years, but qPCR has been increasingly used because it provides many advantages. Before the studies in this thesis, only one qPCR protocol had been developed and used to study hemogregarine prevalence in canid hosts (Criado-Fornelio *et al.*, 2007). However, it only allowed detection but not quantification (i.e. estimation of intensity levels) of *Hepatozoon* parasites, which is one of the main advantages of this technique. For this reason, a part of this thesis had the aim of developing a quantitative PCR assay and applying it to the study hemogregarine infection patterns in reptiles

(section 2.2). Importantly, it allows for simultaneous detection and quantification of hemogregarines and to detect the occurrence of mixed infections in natural populations in a systematic and standardized way by distinguishing parasite genera based on MCA (see sections 1.7.1 and 2.2).

One of the main limitations in parasitological studies relate to detectability issues and sample sizes (Jovani and Tella, 2006; Jennelle *et al.*, 2007). These limitations may arise during sampling (i.e. depending on the location and host population density that allows for more or less individuals to be collected) or during laboratory work (i.e. depending on the performance and accuracy of the methodologies used). Chapter 2 of this thesis focused on the latter situation because the use of less accurate protocols may lead to erroneous ecological and epidemiological inferences (Espy *et al.*, 2006; Banoo *et al.*, 2008), even if sample numbers were originally high. Critical steps in the protocols of molecular techniques can influence their success such as the protocol for DNA isolation and the optimization of the molecular protocols (Espy *et al.*, 2006). It has been assumed that commercial kits for DNA extraction provide an easier, faster and more reliable way to obtain DNA. However, traditional extraction methods have been successfully applied to obtain parasite DNA from various sample sources and used to assess the distribution and phylogenetic relationships of these parasites (Maia *et al.*, 2011; Tomé *et al.*, 2013, 2014; Perera *et al.*, 2013). Despite this, there were no studies comparing the performance of different biological sources and extraction protocols for estimating parasite infection parameters.

For this reason, in section 2.2 a comparison on the performance of different sources and protocols was carried out in order to determine the most appropriate procedures for each scenario. The new qPCR assay was more accurate than traditional methods for parasite screening and identification, in accordance with previous studies (Perandin *et al.*, 2004; Mangold *et al.*, 2005). For these reasons, this protocol was subsequently used in other studies of this thesis (sections 5.2 and 5.3). When comparing the performance of biological samples for a same amount of DNA concentration, blood samples performed better than tail tissue at estimating hemogregarine prevalence and intensity of infection (section 2.2). However, tail tissue may be the only source available in some situations, especially in museum samples, because these were the only sources originally collected for phylogeographic studies of the hosts (Harris *et al.*, 2007, 2010; Damas-Moreira *et al.*, 2014). Therefore, these could still provide valuable information in these situations and it may be that using higher concentrations of DNA for these samples could increase detectability, but this warrants validation and should be done with precaution since host DNA may inhibit PCR reactions (Cogswell *et al.*, 1996; Gómez-Díaz *et al.*, 2010). In addition, this study showed that traditional extraction protocols provided similar estimates of parasite infection as commercial kits when using blood samples, showing that traditional extraction protocols may be used as an effective low-cost alternative to more expensive commercial kits.

An ideal scenario would be to use the most sensitive and accurate techniques available; however many research projects are often restricted by a particular budget that may not allow for this to

happen, especially in large-scale sampling studies (Jennelle *et al.*, 2007). In these cases and depending on the research objectives, different approaches can be used to study parasites. In this perspective, a proposed line of work is given in Figure 6-1, with a focus on the data that can be obtained using different methodologies that may be adopted based on budget restrictions. Despite the benefits of using molecular tools in parasitological studies, these protocols depend on the specificity of the primers used and on the correct optimization of these protocols on a case-by-case basis. This could result in different sensitivities between studies comparing different methodologies (Richard *et al.*, 2002; Durrant *et al.*, 2006; O'Dwyer *et al.*, 2013). Many factors may influence differences in protocol sensitivities, such as method of collection, time of collection, storage conditions, time of storage, reagent storage, reagent and machine manufacturers (Freed and Cann, 2006). Therefore, the use of cross-validation procedures and integrative approaches should be adopted because they allow to: i) verify the consistency of the results obtained [e.g. molecular protocols may occasionally amplify unexpected organisms, see section 2.1 and (Perkins and Austin, 2009)]; ii) more accurately estimate infection parameters and identify mixed infections [common in natural populations, see sections 2.2 and 5.2 and (Read and Taylor, 2001)]; and iii) obtain both morphological and genetic information that can be used to characterize the diversity within that group and differentiate between parasite species.

For an optimal performance of qPCR protocols, the guidelines recommend the amplification of a small-sized fragment. This may limit the power of discrimination of this technique depending on the molecular marker and on the variability of the amplified fragment. The qPCR assay designed in section 2.2 aimed at amplifying and quantifying various hemogregarine parasites, and for this reason a more conserved region was chosen for the 18S rRNA gene (the only available molecular marker at the time of this study for this group of parasites, see section 1.6.1). Therefore, this assay provided limited intra-specific differentiation of hemogregarine lineages but allowed differentiation of hemogregarines and eimeriorinids. For this reason, a complementary approach was conducted by amplifying a larger fragment of this gene using conventional PCR primers to identify parasite lineages. Nonetheless, without a qPCR protocol that differentiates and quantifies the lineages during amplification, there is always the possibility of amplifying more than one haplotype in cases of mixed infections with closely related parasite lineages. Hence, a finer qPCR assay would allow a better understanding of the real distribution of each cryptic lineage/haplotype and a comparison between intensity levels of these different lineages, which may be associated with host-specificity and host-parasite co-adaptation.

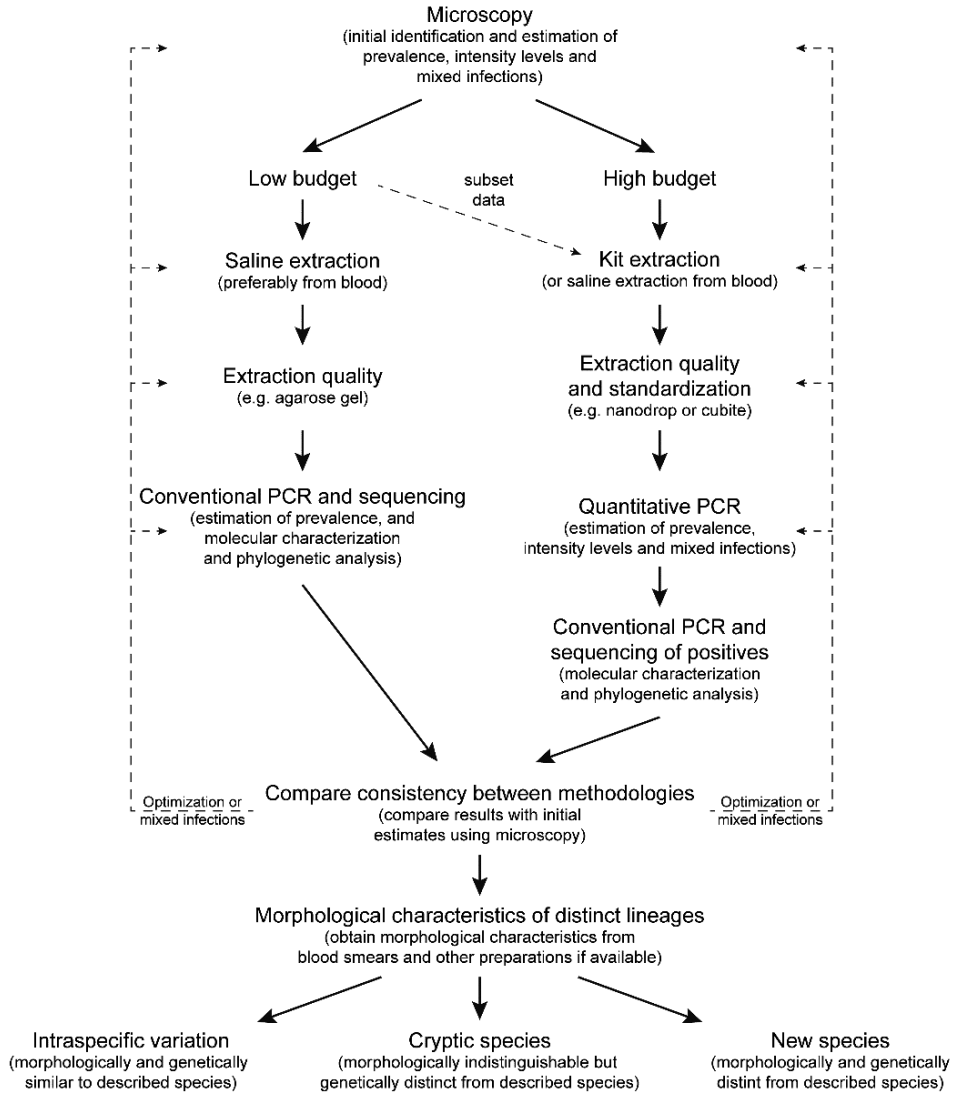


Figure 6-1 Proposed line of work to investigate parasite distribution and diversity, considering the research budget. Research budget can be a limiting factor, especially in large-scale sampling studies.

## 6.2 Improving the knowledge on parasite diversity: the importance of screening wild hosts from remote regions

Before starting this thesis, most information available was dedicated to parasites that are considered of great veterinary, medical and public health importance (e.g. *Toxoplasma*, *Plasmodium* and *Cryptosporidium*). This was highlighted by the lack of studies that focus on parasites infecting wild species, particularly reptiles, despite the frequent attempts to draw attention to the importance of wild hosts as reservoirs for some of these and other emerging infectious diseases (Gondim, 2006; Aguirre, 2009; Tompkins *et al.*, 2011; Baneth, 2014). The studies in this thesis, together with other recent research (O'Dwyer *et al.*, 2013; Tomé *et al.*, 2014; Kvičerová *et al.*, 2014; Haklová-Kočíková *et al.*, 2014; Harris *et al.*, 2015), have highlighted the importance of using molecular tools for screening wild reptiles and other host groups from remote regions, and of placing these parasites in

a phylogenetic framework to assess their diversity. This information can be used to better understand disease ecology and transmission dynamics of these parasites, important for both conservation and epidemiology.

The placement of previously unreported parasite lineages in a phylogenetic framework often challenges the present taxonomy of these parasites (Pineda-Catalan *et al.*, 2013; Kvičerová *et al.*, 2014; Haklová-Kočíková *et al.*, 2014). Remote geographical regions are usually associated with high levels of diversity and endemism, such as the reptile fauna from Morocco (Rato *et al.*, 2007; Barata *et al.*, 2012; Damas-Moreira *et al.*, 2014), Madagascar (Vences *et al.*, 2009; Crottini *et al.*, 2009; Ratsoavina *et al.*, 2011) and Oman (Carranza and Arnold, 2012; Gómez-Díaz *et al.*, 2012; Smíd *et al.*, 2013). The studies of this thesis have assessed the distribution, diversity and host-parasite associations of apicomplexan parasites from these regions in various related and unrelated host species and reported high levels of diversity (e.g. sections 3.2, 3.3, 5.1, 5.2 and 5.3). Given that host vagility is one of the most important factors that contribute to parasite distributions and diversity (Criscione *et al.*, 2005), and the fact that reptile hosts typically have limited dispersal abilities, the high levels of endemism of the host are likely to be linked with high diversity and endemism of their parasite species. In addition, gecko lizards seem to harbour different hemogregarine parasites compared to lacertid hosts. From the findings in section 5.2 and Harris *et al.* (2015), geckos may harbour very distinct hemogregarine lineages, some of which may even be considered as new taxonomic entities. This pattern could be associated with distinct dispersal abilities and microhabitat preferences of the two groups of lizards that may act as a reproductive barrier between parasite populations and promote parasite differentiation.

The studies in sections 3.3 and 5.2 represent the first molecular assessment of the diversity and specificity of apicomplexan parasites from Oman in the Arabian Peninsula. As mentioned before, many distinct apicomplexan lineages were recovered, potentially representing new taxonomic entities. For instance, one of the recovered hemogregarine lineages showed considerable genetic divergence and formed a well-supported monophyletic group composed of two variants. Based on this finding, study 3.3 proposed the description of a new *Hepatozoon* species, *Hepatozoon omanensis* n. sp.. For one variant, the host species in which the highest levels of prevalence and intensity were detected [i.e. principal host (Poulin, 2005)] was *A. platyrhynchus*, an endemic gecko species that is restricted to the Jebel Akhdar that is part of the Al Hajar Mountains range in Oman. This variant was also detected in other gecko species that were sampled from other localities in the northeast of this region (e.g. *P. rupestris* and *P. hasselquistii*), therefore potentially showing low host-specificity. Two hypotheses can be drawn from the distribution pattern of this variant of *H. omanensis*. First, this variant may have adapted and specialized to the host *A. platyrhynchus* and later expanded its host range. This expansion may have occurred through accidental transmission to sympatric gecko species that have wider distribution ranges. Or second, this variant already had low host-specificity and could easily colonize unrelated host species, of which *A. platyrhynchus* was

the most susceptible to infection of all its host range species. The other variant differed by a few genetic mutations for the 18S rRNA gene and was exclusively found in two additional *Hemidactylus* species. This finding could be an indication that this variant has differentiated and specialized in these two host species and should be further investigated.

Finally, the occurrence of mixed infections with different haplotypes of the same parasite species is common in natural host populations [(Read and Taylor, 2001; Hood, 2003) and as shown in sections 2.2, 3.1, 3.2 and chapter 5]. Mixed infections have been detected in the studies of this thesis, by identifying double peak positions that correspond to distinct 18S rRNA gene haplotypes in sequence electropherograms. However, these instances seem to remain largely unreported in the literature regarding hemogregarine parasites. This is of relevance since the co-occurrence of various *Plasmodium* lineages in the same avian host have been shown to contribute to parasite differentiation and speciation (Pérez-Tris *et al.*, 2007; Falk *et al.*, 2015). In addition, the 18S rRNA gene marker is known to occur in multiple paralogous rRNA gene copies in other apicomplexans such as *Cryptosporidium* and *Plasmodium* (Rooney, 2004; Stenger *et al.*, 2015). If variation between copies exists, this could artificially increase parasite diversity estimations in host populations. Furthermore, the occurrence of mixed infections can result in within-host competition, which can have important implications for the epidemiology of the disease. Theoretically, within-host competition is predicted to select for higher parasite virulence, in which the parasite displaying the higher virulence is able to limit the growth of the other parasites that co-exist in that host (Frank, 1996). Host immune system may play a major role in within-host competition because it can change this outcome by interacting with the various parasites (Raberg *et al.*, 2006; Fenton and Perkins, 2010). Therefore, the study of mixed hemogregarine infections in reptile hosts, as well as in other host groups, need to be considered carefully because these are of evolutionary, ecological and epidemiological importance to host-parasite interactions.

### 6.2.1 The importance of studying hemogregarine taxa from reptiles

Hemogregarines are diverse apicomplexans that consist of six genera: *Haemogregarina*, *Desseria* and *Cyrilia* (Haemogregarinidae), *Hepatozoon* (Hepatozoidae), and *Hemolivia* and *Karyolysus* (Karyolysidae). However, up until 2009, only *Hepatozoon* sequences were available on public databases [see (Morrison, 2009) for an overview of the evolution of Apicomplexa]. In 2012, Barta and colleagues conducted a phylogenetic assessment of adeleorinids and published sequences from *Haemogregarina* and *Hemolivia* for the first time (Barta *et al.*, 2012). Since then, many studies have contributed to increase the knowledge on the diversity within these three genera [*Haemogregarina* in turtles (Dvořáková *et al.*, 2014, 2015), *Hemolivia* in turtles (Harris *et al.*, 2013; Kvičerová *et al.*, 2014), and *Hepatozoon* in lizards and snakes (Maia *et al.*, 2011; Tomé *et al.*, 2013, 2014; Harris *et al.*, 2015). However, it was not until 2014 that confirmed *Karyolysus* sequences from lizard hosts became available (Haklová-Kočíková *et al.*, 2014). The phylogenetic placement of these

parasites showed that these were genetically identical to sequences previously identified as *Hepatozoon* [e.g. sections 2.2, 3.1 and (Tomé *et al.*, 2014)], highlighting paraphyly of the *Hepatozoon* genus. The morphologic evidence presented in Haklová-Kočíková *et al.* (2014) was compatible with members of the *Karyolysus* genus, as well as the fact that these parasites were obtained from mite hosts that are the reported vectors of this genus. Nonetheless, *Karyolysus* parasites are expected to lyse the host cell nucleus, which was not reported in the blood smears of the samples previously identified as *Hepatozoon* [(Maia el al. 2012) in section 3.1]. Thus, this key morphological characteristic may not be present in genetically similar parasites for the 18S rRNA gene definition, which, together with the current uncertainty in the classification of this *Hepatozoon*/*Karyolysus* complex, shows the limitations of resolution of both morphological and 18S rRNA gene. In any case, these studies allowed the build-up of important information, such as prevalence and host range of these parasites, which could be important regarding future considerations on the possible solutions to solve the present taxonomic issues. Studies on other equally neglected genera of apicomplexan parasites of reptiles have also evidenced the importance of genetically characterizing previously described genera and placing them in a phylogenetic framework. For instance, the recent morphologic and genetic characterization of reptile hemoproteids justified the resurrection of the genus *Haemocystidium* (Haemosporida) to differentiate between reptile and bird hemoproteids (Pineda-Catalan *et al.*, 2013). Or the fact that morphologic and genetic characterization of reptile eimeriorinids have questioned the validity of the genus *Eimeria* and the need to further characterize these parasites in these hosts (Megía-Palma *et al.*, 2015). Therefore, these and the studies presented in this thesis have important taxonomic implications as this knowledge can partially overcome incongruences related to taxon sampling and outgroup choice as discussed in the next section.

## 6.3 Phylogenetic reconstructions and increase in taxon sampling reveal taxonomic inconsistences in apicomplexan parasite groups

Parasites are complex and diverse organisms and much effort has been done to categorize and organize their classification. This has been traditionally based on morphology and life-history traits (Valkiūnas, 2005; Telford, 2009) but these morphological characters are known to have limited differentiation and resolution power. The build-up of parasite genetic information and the recent effort to assess parasite diversity in wild hosts from remote regions have greatly contributed to a better understanding of parasite diversity and phylogenetic relationships. Public databases contain great amounts of research information (e.g. GenBank, Ensemble, MalAvi), which facilitates the characterization of parasite diversity. Nevertheless, these databases may contain misidentifications and should be used with caution [(Valkiūnas *et al.*, 2008) and sections 3.1, 4.1 and 4.2]. Parasitologists are often faced with missing data problems and/or lack of genetic information for

particular taxa, which complicates the study of parasite evolutionary history (Rich and Xu, 2011). Available information may vary in length for a particular gene due to the use of different primers, leading to missing data. In these situations, researchers usually use a subset of the data available from closely related taxa that matches theirs. Although this is a valid approach that may provide relevant information regarding parasite diversity and host-range, it may generate erroneous relationship inferences and conclusions (Perkins, 2014). For this reason, it is important to conduct overviews of the available data from time to time in order to re-assess the phylogenetic relationships within and between parasite groups [e.g. haemosporidians (Outlaw and Ricklefs, 2011), hemogregarines (Barta *et al.*, 2012) and eimeriorinids (Megía-Palma *et al.*, 2015)]. This was the aim of chapter 4 of this thesis, which included an update on the assessment of the evolutionary history of haemosporidians based on the cyt *b* gene and the first attempt to consider all available genetic information for the 18S rRNA gene for hemogregarine parasites to assess their phylogenetic relationships in the light of the recent studies that have evidenced taxonomic issues.

In hemogregarines, the genus *Hepatozoon* represents some of the most common blood parasites of vertebrates (i.e. intermediate hosts) and this genus is the only member of the family Hepatozoidae. This family includes more than 300 species (Smith, 1996) with variable morphological characteristics, diverse life-histories and a wide range of host species (Smith and Desser, 1997). Many of these species have been described mainly based on morphological characters of parasites found in different intermediate host species (Smith, 1996; Telford, 2009). This approach may be problematic if host-specificity is low (Mathew *et al.*, 2000) and when host-specificity may rather be associated with the invertebrates [i.e. definitive hosts (Barta *et al.*, 2012)]. Previous studies had highlighted the possibility of Hepatozoidae being a complex of different genera due to the diversity found in this family (Smith and Desser, 1997; Smith *et al.*, 1999). Therefore, although the recent studies highlighting paraphyly of this family based on genetic information do not come as a surprise (Kvičerová *et al.*, 2014; Haklová-Kočíková *et al.*, 2014), these have important taxonomic implications. *Hepatozoon* parasites have been primarily studied in domestic animals, namely cats [infected mainly by *Hepatozoon felis* (Baneth *et al.*, 1998)] and dogs [infected mainly by *Hepatozoon americanum* and *Hepatozoon canis* (Baneth *et al.*, 2003)]. In hemogregarine phylogenies, these species and species from most other mammalian carnivores, form a well-supported sister clade to one of the hemogregarine lineages that is found in reptiles (the *Hepatozoon*/*Karyolysus* complex, see sections 1.6.1 and 4.2). Hence, based on 18S rRNA marker phylogenetic reconstructions, *Hepatozoon* parasites from these mammals are more closely related with the *Hepatozoon*/*Karyolysus* complex from reptiles than to other *Hepatozoon* parasites from other mammals such as rodents. However, the genus *Hepatozoon* was originally described from rats [i.e. *Hepatozoon perniciosum* (Miller, 1908)] and *Hepatozoon* parasites occurring naturally in rodent hosts are primarily placed in the lineage that is distinctly related to *Hepatozoon* species from cats and dogs (sections 4.2 and 5.1). Based on sections 4.2 and 5.2, there may be two different ways to attempt to solve this issue. First,

*Hepatozoon* from mammalian carnivores could be renamed as a distinct genus. However this would have complex taxonomic implications. In particular the finding of the new distinct lineage in geckos (Tome et al. submitted and section 5.2) highlights that several other lineages would require formal naming, and the probability of further lineages being found is high. An alternative approach would be that *Karyolysus* could be considered as a subgenus of (or even synonymous with) *Hepatozoon* and the same approach applied to other main *Hepatozoon* clades [similar to what has been done for haemosporidians (Perkins, 2014)]. This would allow a temporary separation of distinct lineages and avoid confusion. However, a recent study proposed an alternative systematic revision of the adeleorinid hemogregarines based on biological life cycles and phylogenetic reconstructions with the separation into four hemogregarine types: *Hepatozoon* (type I), *Karyolysus* (type II), *Hemolivia* (type III) and a new genus *Bartazoon* (type IV) (Karadjian *et al.*, 2015). This would imply that the hemogregarines sequences from rodents (as well as from various reptiles) be classified as *Bartazoon* species. It is possible that these sequences belong to hemogregarine species, unrelated with *H. perniciosum*, which were amplified from cysts in the muscle of these rodents. However, without a genetic reference for the type species *H. perniciosum* from rats, it may be premature to conduct a systematic revision at this point. In any case, the studies in this thesis show that an effort should be made to continue to characterize hemogregarine diversity, which is still potentially unknown, before conducting any major taxonomic revisions. Otherwise this could lead to taxonomic instability in the future. Particularly because these results are based in a single gene tree, which may not reflect the evolutionary history of an organism (Anderson, 2001), as evidenced from the updated cyt *b* gene phylogeny of Haemosporida (section 4.1) and the alternative multi-gene phylogeny by Borner *et al.* (2015). Therefore, multigene analyses for hemogregarine parasites are needed to investigate this further and could bring new insights into possible solutions to this problem. In this sense, the recent publication of the *Hepatozoon catesbianae* mitochondrial genome (Leveille *et al.*, 2014) may be a first step towards generating data from an additional marker across the known diversity, and give new insights regarding hemogregarine phylogeny and evolutionary history.

Haemosporidians have also been primarily studied in mammal hosts, namely humans and primates (Escalante and Ayala, 1994; Escalante *et al.*, 1998) but also in rodents (Perkins *et al.*, 2007), and more recently in bird hosts (Bensch *et al.*, 2004; Hellgren *et al.*, 2007; Zehtindjiev *et al.*, 2012). The study in section 4.1 showed that the inclusion of reptilian parasites can bring new insights into the understanding of these parasites evolutionary history. In addition, the importance of vectors for parasite taxonomy has been overlooked despite the fact that the major cladogenic events in the evolutionary history of Haemosporida may be linked with vector host switches (Martinsen *et al.*, 2008). This may be of importance for a taxonomic revision of this group, in which genera could be assigned based on vector-parasite associations. Apart from taxon sampling and missing data issues, another issue in the study of the evolutionary history of Haemosporida has been the appropriate choice of an outgroup taxa. Earlier phylogenies of this group used *Theileria*, *Babesia* or *Toxoplasma*

species as outgroups (Escalante and Ayala, 1994; Escalante *et al.*, 1998), while following Perkins and Schall (2002) *Leucocytozoon* became the outgroup of choice (Perkins, 2014). Nonetheless, a study using outgroup-free estimations of phylogeny (Outlaw and Ricklefs, 2011) (as the analysis included in section 4.1) has brought a new perspective to the study of the evolutionary history of the group (Rich and Xu, 2011). Our findings, in accordance with that study, have shown that the *Leucocytozoon* genus may not be suitable for rooting this group when using solely the cyt *b* gene because this genus appears as a derived and not ancestral lineage within the group. Nonetheless, a recent study found an alternative pattern for the evolutionary history of Haemosporida based on a multi-gene approach (Borner *et al.* 2015). In contrast with the results obtained in section 4.1, based on an updated cyt *b* gene phylogeny for this parasite order, Borner and colleagues found consistent support for the basal position of *Leucocytozoon* and that sauropsid and mammal-infecting lineages were sister clades. However, their analyses lacked some groups that were included in section 4.1 (e.g. *Haemocystidium* and *Akiba*). Once again, limitations regarding taxon sampling and molecular markers used clearly influence our understanding of the phylogenetic relationships and evolutionary history of these groups of parasites, highlighting the need for multi-gene analyses with increased sampling.

All in all, a good knowledge of parasite diversity and host-parasite associations coupled with accurate and robust estimations of parasite phylogenetic relationships are important for taxonomic purposes and should be the basis for any future taxonomic revision of parasite groups.

## 6.4 Parasite infection patterns and environmental factors

In natural populations, multiple factors can influence infection patterns between host species, including host immunity, microhabitat preferences, parasite lifecycle, transmission dynamics, seasonal variations and vector abundance. To explore the influence of environmental factors on parasite infection patterns, different scenarios were investigated based on the importance of host-relatedness and habitat sharing. These scenarios consisted on the comparison of infection patterns between: i) sympatric and related host species (sections 2.2); ii) several related/unrelated host species from different geographical areas (section 5.2); and iii) between different times of the year and between years in sympatric host species (section 5.3).

The study of parasite distributions in sympatric hosts may allow to control for general ecological confounding factors, while the study of related hosts may allow to control for phylogenetic confounding factors (Poulin and Mouillot, 2004). All things being equal, related hosts that live in sympatry are expected to have similar prevalence and intensity levels (Poulin *et al.*, 2011). However, our findings show that hemogregarine infection levels differ between two closely related *Podarcis* species that live in sympatry, *P. hispanica* and *P. bocagei*. This was verified for two geographical locations where they co-occur in Portugal (Gerês and Moledo) and also across different time points. These findings indicate that, while controlling for generic ecological and phylogenetic confounding

factors, parasite infection levels differed, which shows that unique biological and ecological characteristics associated with each host species may be influencing these patterns. For instance, microhabitat preferences of these species may influence exposure to parasites: *P. hispanica* is frequently found on rocks while *P. bocagei* is mainly ground-dwelling (Sá-Sousa *et al.*, 2002; Sá-Sousa and Harris, 2002).

On the other hand, unrelated hosts living in sympatry are likely to differ in their parasite communities due to exposure to different parasites (Poulin *et al.*, 2011). Our findings from Oukaimeden, Morocco, corroborate this assumption by showing that, of the three widespread lizard species examined, only a few gecko hosts (*Q. trachyblepharus*) were infected with hemogregarines (and those infected had extremely low levels of intensity), while the two lacertid host species (*A. andreanskyi* and *P. vaucheri*) displayed high prevalence and intensity levels. These patterns were similar across different time periods and *P. vaucheri* was always more heavily infected than the other two species. This shows that, at least in this system, the use of samples from different time points may not be a limiting factor in biasing infection estimates for inter-specific comparisons. Interestingly, the two lacertid species were infected by unique hemogregarine lineages: only one haplotype was detected for *A. andreanskyi* (the host species with intermediate levels), while several haplotypes were found for *P. vaucheri* (the host species with highest levels). The qPCR protocol applied does not discriminate between related hemogregarine lineages and only quantifies overall levels of hemogregarine infection. Many *P. vaucheri* individuals had mixed infections with two hemogregarine haplotypes, thus is possible that these higher levels represent cumulative infection levels from various haplotypes. There is also the possibility that these various haplotypes, especially when present as co-infections in the same individual, represent multiple paralogous rRNA gene copies from a single parasite as seen in other apicomplexan parasites such as *Cryptosporidium* and *Plasmodium* (Rooney, 2004; Stenger *et al.*, 2015) (see section 6.2). If this is the case, this could result in an overestimation of hemogregarine diversity in these hosts. On the other hand, the immune system of these host species may react differently to infection, which may result in differential infection levels depending on their effectiveness at controlling or clearing infection. Nonetheless, these heterogeneous patterns of parasite infection are most likely associated with both host and ecological factors that are unique to each host species.

Finally, in section 5.2, the same host species (*P. rupestris*) was found to have heterogeneous hemogregarine infection patterns across different geographical areas in Oman and its prevalence was negatively correlated with altitude. These findings draw attention to the importance of the ecological factors of each area that may influence parasite development and vector dispersal. Higher altitudes are associated with lower temperatures and reduced moisture (Drakeley *et al.*, 2005), which may present unfavourable conditions for some parasites and their vectors. This is a plausible explanation for the pattern observed and an assessment of vector abundance and of infection patterns in other host species is needed.

All in all, various factors can influence disease transmission dynamics in vector-borne parasites. It is increasingly evident that variations in parasite distribution and infection patterns across host species may be the result of the interaction between various factors that may vary from situation to situation. These interactions are summarized in Figure 6-2, which shows the possible interactions at the various levels in an ecosystem (i.e. from the landscape level to the individual level). For instance, disease transmission is dependent on host susceptibility and vector dispersal at the individual level, which may be dependent on seasonal variations at the landscape and population levels that may induce fluctuations in these factors. Or that landscape attributes and habitat connectivity at the community level contribute to host-vector contact rate, which in turn may influence parasite transmission and infection patterns (Figure 6-2).

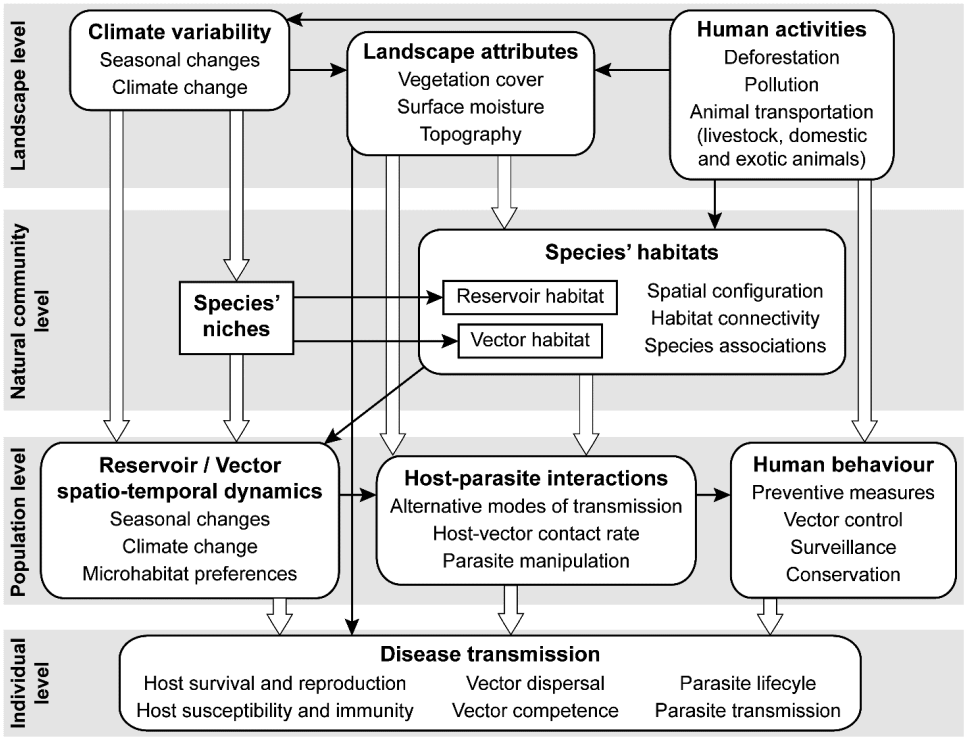


Figure 6-2 A summary of the various factors that may influence disease transmission in vector-borne parasites. [Adapted from Lambin *et al.* (2010), Léger *et al.* (2013)]

### 6.4.1 Parasite infection patterns and Host factors

Body size and weight have been used as proxies to attempt to explain differences in parasite infection patterns (Amo *et al.*, 2005a; b). It is assumed that prevalence and intensity of infection may increase with longevity (estimated through host body size in wild hosts) due to more encounters with parasites, more time to develop infections and less immunocompetence in older individuals (Poulin, 1997; Palacios *et al.*, 2011). This has been observed in studies in this thesis for which there was a positive correlation between lizard body size and hemogregarine prevalence (section 5.3) or a positive correlation between lizard body size and hemogregarine intensity of infection (section 2.2). This tendency has also been observed in studies on hemoparasites conducted on other short-lived

reptile species [e.g. *Lacerta monticola* in Amo *et al.* (2005b); *Podarcis lilfordi* in Garrido and Pérez-Mellado (2013); and *Sceloporus jarrovi* in Halliday *et al.* (2014)]. On the other hand, for studies on long-lived reptile species, such as snakes and tuataras, this correlation is inversed with older individuals having lower intensity levels (Madsen *et al.*, 2005; Brown *et al.*, 2006; Madsen and Ujvari, 2006). In short-lived species, such as lizards, hemogregarine infections are often regarded as non-detrimental to host condition (Amo *et al.*, 2005b), while in long-lived species hemogregarine infection may be linked with detrimental effects on life-history traits, including growth rate, juvenile survival and female reproductive output (Madsen *et al.*, 2005). These contrasting effects may be due to the continued parasite acquisition through time in larger and older individuals in the case of non-detrimental infections (Thomas *et al.*, 1995), while in detrimental infections only those individuals that manage to reduce or clear infection can reach large sizes (Madsen *et al.*, 2005; Simberloff, 2010). It would be interesting to investigate the pattern of infection in intermediate-lived reptile species, as this would allow a better understanding on how host body size and longevity influence parasite infection patterns. These patterns could be intrinsically associated with each host species and/or with other host and microhabitat factors in some cases (see section 1.3). Therefore, although our understanding of why this contrasting pattern occurs is still limited, there may be several hypothetical explanations for these tendencies:

i. increase in pathogenicity and detrimental effects to host condition over time in long-lived species;
ii. survival of individuals that can clear or reduce infection in long-lived species;
iii. behaviour avoidance of older individuals of infected conspecifics in long-lived species;
iv. continued parasite acquisition in short-lived species due to non-detrimental effects;
v. lower immunocompetence in older individuals of short-lived species, but an opposite pattern in long-lived species.

Furthermore, there is a general male-bias in parasite infection parameters in vertebrates (Klein, 2004; Salkeld and Schwarzkopf, 2005). Hormonal levels can contribute to changes in the immune status of an individual, which can result in intra-specific differences in infection patterns (i.e. the immunocompetence handicap hypothesis, see section 1.3.2.1). Testosterone is a hormone with immunosuppressive effects and it has been shown that males with higher testosterone levels can have higher parasite loads (Pollock *et al.*, 2012). Testosterone levels are usually higher during the mating season (Gowan *et al.*, 2010), a period when individuals allocate more resources towards reproductive effort and thus may be more prone to parasitic infections. In addition to its immunosuppressive effects, this hormone may stimulate territorial behaviours and movement of males, which could increase behaviour heterogeneity between sexes, resulting in increased exposure of males to parasites (Perkins, 2001; Reardon and Norbury, 2004). The study in section 2.2 reported significantly higher levels of intensity but not prevalence in males compared to females. However, the study in section 5.3 showed a different scenario, in which males generally had lower

or similar intensity levels than females for most of the times points analyzed. This could be an indication that location, and host and ecological factors must be influencing the distribution of these parasites.

### 6.4.2 Host-range and transmission dynamics in hemogregarines

Hemogregarine parasites have heteroxenous lifecycles, which involve at least one invertebrate and one vertebrate host. The invertebrate hosts act as vectors and for this reason many of these parasites may end-up in unsuitable hosts (Poulin, 2010). This could be the “end of the line” for most parasites, the reason why these unsuitable hosts for parasite proliferation are termed “dead-end” hosts. Nonetheless, accidental transmission may result in occasional host switch or host-range expansion (Clayton *et al.*, 2003). These events are important in an epidemiological perspective because may be associated with possible “spill-over” events from infected populations to new populations and with the increase in reservoir hosts (i.e. hosts that maintain the parasite in a population) (Daniels *et al.*, 2007; Thompson, 2013; Yabsley and Shock, 2013). It has been shown that these events occur in apicomplexan parasites [e.g. piroplasmids (Yabsley and Shock, 2013)], thus the occurrence of occasional atypical hemogregarine lineages in some hosts may be an indication that these events also occur in these parasites. For example, the study in section 3.1 reported a single atypical sequence from a lacertid lizard in a total of 70 positives (1.4%) that was more closely related with previously reported hemogregarine lineages from gecko lizards than with sequences from other lacertid lizards. Another molecular study that screened lacertid, skink and gecko lizards found a similar pattern, with a single atypical sequence from a lacertid host out of a total of 23 positives (5%) (Maia *et al.*, 2011). Another example is the study in section 5.1 that reported a single atypical sequence from a canid host in a total of 37 positives (3%) that is more closely related with sequences found in reptiles and rodents than in other mammalian carnivores. Since these instances occurred at low rates and only large-scale studies were conducted, these may represent “dead-end” hosts that became infected by accidental transmission. However, these “dead-end” hosts still provide a blood meal for the vector and thus may contribute to maintain the vector population, ultimately helping the pathogen to persist (Hudson, 1998). Further research should investigate the establishment of infections in these exceptional events to understand if these do represent dead-end infections or are actually part of the lifecycle of the parasite (Seppälä *et al.*, 2008).

In addition to this, the host-range of a parasite may be influenced by its mode of transmission and its ability to infect and colonize untypical hosts (Poulin, 1997; Beldomenico and Begon, 2010). Some *Hepatozoon* species have been demonstrated experimentally to use prey-predator systems as an alternative mode of transmission by developing cysts in prey that are infective to predators (Landau *et al.*, 1972; Sloboda *et al.*, 2008). The studies 3.2, 5.1 and 5.3 in this thesis highlighted the occasional occurrence of identical parasite lineages in predator and potential prey hosts, which could

be an indication of prey-predator transmission in accordance with recent research (Vojta *et al.*, 2009; Allen *et al.*, 2011; Tomé *et al.*, 2012; Almeida *et al.*, 2013). Theoretically, a parasite has to have low host-specificity (i.e. to be a generalist) to be able to successfully colonize a wide range of vertebrate hosts in prey-predator transmission. Therefore, such findings are important not only for elucidating drivers of parasite diversification and specialization but also in contributing to the knowledge of disease ecology and transmission dynamics and as such deserve to be further investigated (Poulin and Morand, 2000; Clayton *et al.*, 2003).

## 6.5 Concluding remarks

The aim of this thesis was to contribute to the knowledge on parasite diversity, infection patterns and host-parasite interactions in natural host populations. This was achieved at different levels by integrating complementary approaches. The findings in this thesis are relevant for the fields of parasitology, epidemiology, ecology and evolution. In summary, the major conclusions of this work were:

i. The use of different biological sources, extraction protocols and detection methodologies for the study of blood parasites can impact the estimations of biologically relevant infection parameters. The most reliable extraction protocol for estimating parasite infection parameters was blood source extracted with either commercial kit or traditional saline methods.
ii. The qPCR assay designed in this thesis allowed to simultaneously detect and quantify hemogregarines, and this approach was applied to various reptile hosts from various geographical locations. It also allowed to easily detect the occurrence of mixed infections by hemogregarines and eimeriorinids. This assay has higher performance compared with microscopy and conventional PCR and presents a powerful routine method to estimate infection patterns in natural host populations. A line of work including integrative and complementary approaches was proposed in this thesis and could be used a basis for parasitological studies to ensure validation of the results obtained.
iii. Genetic characterization of apicomplexan parasites in wild hosts from remote geographical regions provides new insights into the diversity and phylogenetic relationships of these parasites. Mixed infections of hemogregarine and eimeriorinid parasites were commonly found in natural populations of lizards from various geographical locations. Apicomplexan parasites in reptiles display higher levels of diversity compared to those previously reported using morphology, and illustrate that the biodiversity of these parasites still remains largely underestimated. The consideration of these new and described reptilian parasite taxa is of crucial importance for the study of parasite evolutionary history and taxonomy.
iv. The diversity of apicomplexan parasites in several wild reptile species from Oman was assessed for the first time and showed unprecedented diversity, including possibly new

taxonomic entities within hemogregarines and eimeriorinids. Geckos and snakes harbour genetically distinct lineages and may potentially harbour previously unknown lineages.

v. Hemogregarine haplotype specificity in various related and unrelated host species was assessed. Some haplotypes showed low host-specificity by occurring in distinct taxonomic host groups composed of potential prey and predator hosts. This corroborated the occasional occurrence of prey-predator transmission, which may occur more commonly in snakes then in canids. This highlights the importance of host-specificity on parasite transmission dynamics.

vi. A new *Hepatozoon* species (*Hepatozoon omanensis* n. sp.) found exclusively in reptiles from this region was proposed and the phylogenetic position of a *Hepatozoon* species (*Hepatozoon domerguei*) from reptiles in Madagascar was assessed for the first time, with confirmation of the occurrence of prey-predator transmission in *H. domerguei* as an alternative mode of transmission in natural populations.

vii. Paraphyly of the genus *Hepatozoon* (Hepatozoidae) relative to *Karyolysus* and *Hemolivia* (Karyolysidae) was corroborated. *Hepatozoon* species from mammal carnivores are more closely related to a newly discovered hemogregarine lineage exclusive from gecko hosts and a *Hepatozoon*/*Karyolysus* complex lineage than to other *Hepatozoon* species from mammals. The current taxonomy is clearly inadequate, but more data is needed prior to conducting a systematic revision as proposed by Karadjian *et al.* (2015).

viii. An update to the evolutionary history of Haemosporida based on mitochondrial cyt *b* sequences showed that *Leucocytozoon* and *Haemoproteus* were sister taxa to *Plasmodium* species of birds and reptiles, contrarily to being a derived lineage as suggested in a similar study. This also corroborated the fact that *Leucocytozoon* may not be a suitable outgroup for rooting the Haemosporida phylogeny when using solely the cyt *b* gene. For this reason, further multi-gene phylogenies that include all Haemosporida groups are needed.

ix. In addition, a need for a revision of the present taxonomy is highlighted for the main groups of apicomplexan parasites studied in this thesis (hemogregarines, eimeriorinids and haemosporidians). Despite this, this revision should only be carried out when a more complete estimate of parasite diversity within these groups has been obtained because there may still be potentially unknown lineages, as shown in the studies of this thesis.

x. Our findings regarding temporal dynamics of hemogregarine infection in host species living in sympatry, showed that infection patterns are maintained over time between species and sexes. This shows that that host unique biological characteristics and ecological preferences may be major drivers of the distribution of these parasites. The lack of temporal variation reported for these host systems indicates that using samples collected at different time points may produce reliable estimations of parasite infection parameters. This is

important for parasitological studies because detectability and sampling sizes are common restricting factors in these studies.

xi. Lacertid and gecko lizards living in sympatry displayed drastically different hemogregarine infection patterns. Few geckos were infected and these had extremely low levels of infection, while lacertids had high levels of both prevalence and intensity of hemogregarine infection. These findings reinforce that unique host and ecological characteristics of each species influence parasite distributions. In this perspective, unrelated hosts that have distinct microhabitat preferences vary greatly in their parasite communities and infection levels.

xii. The two lacertid host species also differed in hemogregarine infection levels and were found to be infected with unique hemogregarine lineages. This shows that pathogenicity and virulence of specific lineages may vary between different host species.

## 6.6 Future perspectives

The estimation of diversity, phylogenetic relationships and evolutionary history of some apicomplexan parasite groups has been primarily based on the use of single locus, as in the case of hemogregarines and eimeriorinids. However, it has been shown that the use of a single locus may not provide an accurate estimation of relationships (Anderson, 2001). For this reason, an obvious topic for future research is the development of additional markers for these and other parasite groups. In this sense, the recent publication of the complete mitochondrial genome of *Hepatozoon catesbianae* may provide a starting point to achieve this (Leveille *et al.*, 2014). A more powerful and promising approach would be the use of Next-Generation Sequencing platforms (NGS) to conduct whole-genome sequencing, the cost of which is decreasingly rapidly. A crucial step for using these platforms is to obtain high-quality, concentrated DNA, which is often difficult for obligate intracellular parasites. Nonetheless, recent techniques that take advantage of parasite and host unique biological features have been developed and successfully applied. One example is a technique developed to isolate apicomplexan DNA from mammals that relies on the filtration of host leukocytes and isolation of both infected and non-infected erythrocytes (Venkatesan *et al.*, 2012). However, this technique is not effective for isolating DNA from apicomplexans of reptiles because these hosts have nucleated erythrocytes, which contain large amounts of host DNA unlike in mammals. Another example is a technique that relies on the selective depletion of host DNA by methylation (Oyola *et al.*, 2013), which seems to be the most promising technique to adapt to reptile apicomplexan parasites. This is because mammals and reptiles seem to have high levels of methylated DNA, while apparently no detectable levels exist in apicomplexans (Varriale and Bernardi, 2006; Gissot *et al.*, 2008). This would allow to obtain high-quality, concentrated DNA, which is a requisite for obtaining good-quality reads from NGS. Attempts to isolate hemogregarine DNA have already been attempted by our research group by adapting these protocols, but further research is needed to optimize them until

high-quality, concentrated DNA suitable for NGS is obtained. Another alternative would be to identify a stage in hemogregarine lifecycle in which forms of these parasites can be isolated with little or no host contamination. This has been achieved for example in the isolation of *Haemoproteus* obtained from avian hosts (also with nucleated erythrocytes) by inducing exflagellation of microgametes *in vitro* [see Figure 1-25 and (Palinauskas *et al.*, 2013)]. In this example, the size and weight of microgametes differ from blood cells, and so these parasite forms can be separated from host cells after centrifugation and DNA can then be extracted using standard extraction protocols (Palinauskas *et al.*, 2013).

Host-parasite coevolution is an increasing and attractive field that needs to be further explored in some apicomplexan groups of parasites, in particular hemogregarines and eimeriorinids. Various tools and methodologies, such as ParaFit (Legendre *et al.*, 2002) and PACo (Balbuena *et al.*, 2013) that are implemented in ape and vegan packages in R, have been applied to study events of co-differentiation and co-speciation in several host-parasite systems [e.g. mammal-bacteria (Lei and Olival, 2014), and fish-monogeneans (Huyse and Volckaert, 2005)]. These packages use a matrix of patristic distances calculated from maximum likelihood phylogenies of host and parasite to quantify the degree of congruence between the two topologies and to identify the individual associations contributing to the cophylogenetic structure (Lei and Olival, 2014). Cophylogenetic studies are based on the assessment of the congruence between a host tree and a parasite tree and the range of host species that the parasite infects. If the parasite occurs in many different host species, the assessment of coevolution is hindered because either the parasite is able to move among hosts easily or they have not diverged with the host species (Charleston, 2002). This is particularly of interest in vector-borne parasites, such as the apicomplexan groups investigated in this thesis, since these parasites may have a wide range of host species. In addition, there it has been suggested that coevolution may be more associated with the vector hosts in haemosporidians (Martinsen *et al.*, 2008) and in hemogregarines (Barta *et al.*, 2012) based on basic phylogenetic inferences. Despite this, the knowledge of hemogregarine distribution and diversity among invertebrate hosts is clearly lagging behind that of the vertebrate hosts (see section 4.2). Therefore, invertebrate hosts may be the crucial missing link in our understanding of the evolutionary history of vector-borne parasites. The apicomplexan parasites included in the studies of this thesis are all vector-borne (i.e. hemogregarines, haemosporidians and eimeriorinids) but have different ecologies and transmission dynamics (see section 1.6). For instance, invertebrates are both the vector and the definitive hosts of hemogregarines and haemosporidians, which may be why they seem to coevolve with these hosts. On the other hand, in the case of lankesterellids, invertebrate hosts are used simply as a mere vehicle of transmission from one vertebrate to another and thus these parasites may be expected to have higher specificity to the vertebrate host. In this perspective, the combination of a better understanding of the vector-parasite associations and the implementation of robust tools to study phylogenetic relationships and coevolution events should allow a clarification of disease

ecology and evolution of these parasites in the near future. Ecology may also be of major importance regarding patterns of diversity and distribution of vector-borne parasites, and therefore future studies should incorporate a measure of habitat condition and characteristics, as well as an assessment of characteristics of microhabitats frequented by different host species.

Finally, despite the fact that hemogregarines are the most common parasites of reptiles, their interactions with and their effects on these hosts are still largely unknown. Nevertheless, this host-parasite system presents an attractive model to study parasite evolution, specialization, transmission dynamics and disease ecology. This is because hemogregarines can have heterogeneous distributions among different host species or the same host species in different habitats and it is of interest for future research from two stand-points, an evolutionary and ecological perspective (already discussed above) and an epidemiological perspective. Regarding the latter, its importance relies on the fact that blood parasites may be associated with negative effects on hosts (Marzal *et al.*, 2005; Arriero and Møller, 2008; Acevedo *et al.*, 2010). This can have implications in terms of host survival and competition for resources (Thomas *et al.*, 2009). For instance, one host species may be dominant over a sympatric host species in the absence of a parasite, but this can be inverted in the presence of a parasite that has a negative impact on the otherwise dominant species (Park, 1948; Thomas *et al.*, 1995). However, it is difficult to measure the implications of parasites in individual wild hosts in natural populations. Therefore, future research should aim at understanding host susceptibility, parasite pathogenicity and virulence in wild populations. Furthermore, mixed infections are common in natural populations and our understanding of their implications is still limited, thus future studies should incorporate a measure of condition and fitness in order to determine the effects of single or mixed infections on these hosts.

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# GLOSSARY

**Allopatry**: nonoverlapping geographical areas in the distribution areas of two species.

**Co-differentiation**: joint genetic differentiation of host and its parasite, which may lead to co-speciation.

**Co-speciation**: the joint speciation between host and its parasite.

**Cryptic species**: species that are morphologically indistinguishable, or practically so, and genetically distinct lineages that are considered to represent separate species.

**Dead-end host**: a host that does not transmit the infection further.

**Definitive host**: host in which sexual reproduction occurs.

**Environment**: elements of a habitat, referring to a wide range of biotic and abiotic conditions.

**Epidemiology**: study of the distribution and determinants of disease in a population.

**Hemoparasite**: parasite living in the blood of a vertebrate host.

**Heteroxenous lifecycle**: parasite that requires more than one host to complete its lifecycle.

**Host spectrum**: the collection of hosts that a parasitic organism can utilize.

**Infectivity**: ability to invade and replicate in a host tissue.

**Intermediate host**: necessary secondary host in which asexual reproduction of a parasite occurs.

**Monoxenous lifecycle**: parasite that requires only one host to complete its lifecycle.

**Parasite intensity**: mean number of parasites per single host individual.

**Parasite prevalence**: number of infected individuals in the total number of a population.

**Paratenic host**: a host that is not necessary for the development of a parasite but that serves to maintain its lifecycle.

**Pathogenicity**: ability of an organism to cause disease.

**Reservoir**: a host in which a parasite is maintained and can multiply.

**Spill-over event**: transmission of infection from a reservoir host population to a new host population.

**Sympatry**: spatial overlap between the distribution areas of two species.

**Trade-off**: a situation which involves the simultaneous gain and loss of qualities.

**Vector**: a carrier that transmits an infective agent from one host to another.

**Virulence**: degree of pathology cause by the organism.

**Zoonosis**: disease agents transmitted from animals to humans.

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# APPENDICES

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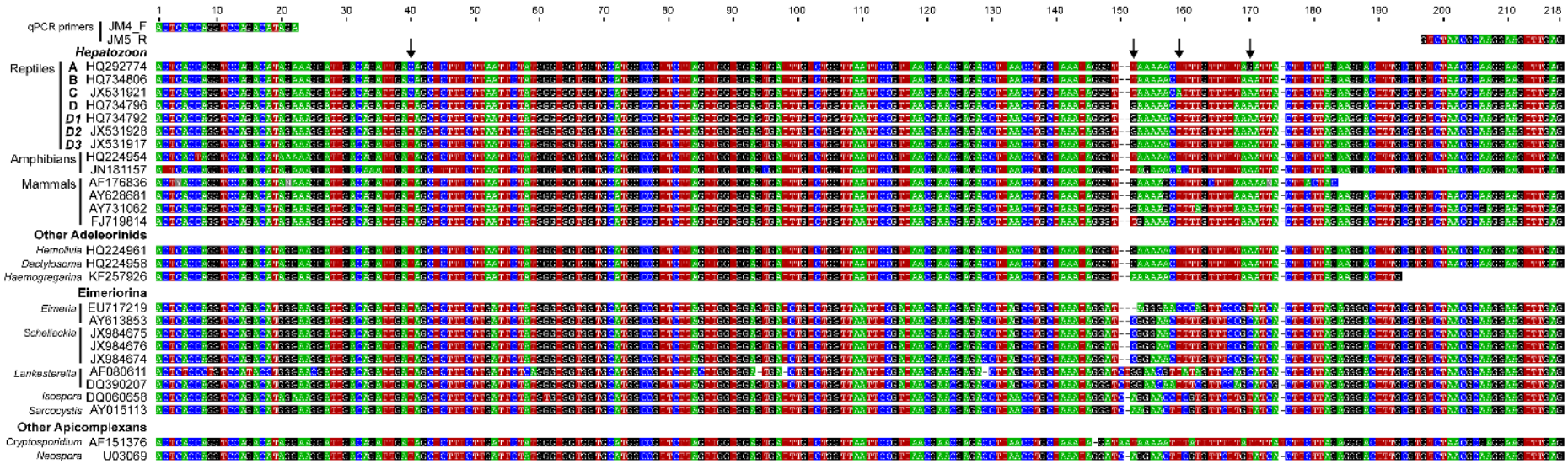


Figure S1 Sequence alignment of the 18S rRNA gene fragment targeted by the qPCR assay.
Arrows indicate nucleotide positions that differ between major *Hepatozoon* lineages (see Figure 2-4).

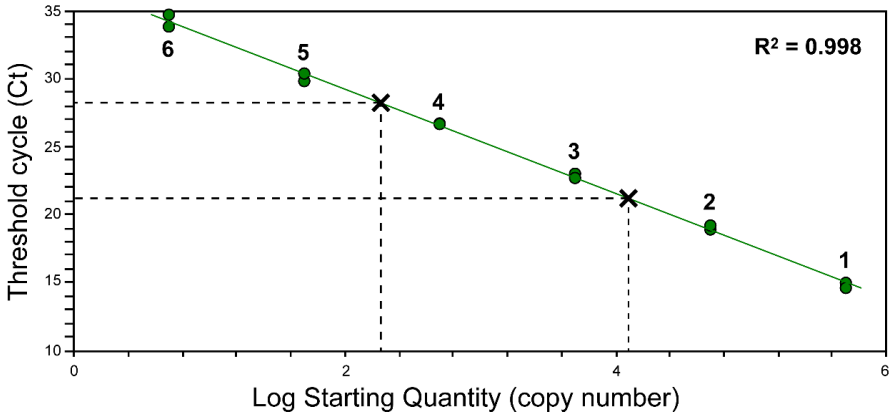


Figure S2 Standard curve obtained from a six 10-fold serial dilutions of the plasmid containing the 18S rRNA gene. Numbers correspond to dilutions from 1 (500,000 copies) to 6 (5 copies). Dashed lines indicate the number of copies for an unknown sample determined based on the starting Threshold cycle (Ct).

Table S1 Number of haplotypes and heterozygous individuals found in this study. + indicates when the first haplotype peaks are higher. & indicates when both haplotype peaks are approximately of the same height.

| | | Haplotypes | | | Heterozygotes | | |
|---|---|---|---|---|---|---|---|
| **Host species** | **Sex** | **D1** | **D2** | **D3** | **D1+D2** | **D2+D1** | **D1&D2** |
| *Podarcis bocagei* | Female | 2 | 2 | 1 | 3 | 2 | 0 |
| | Male | 2 | 6 | 2 | 3 | 0 | 2 |
| | | 4 | 8 | 3 | 6 | 2 | 2 |
| *Podarcis hispanica* | Female | 2 | 5 | 1 | 0 | 0 | 2 |
| | Male | 8 | 1 | 1 | 0 | 1 | 1 |
| | | 10 | 6 | 2 | 0 | 1 | 3 |
| | | 14 | 14 | 5 | 6 | 3 | 5 |

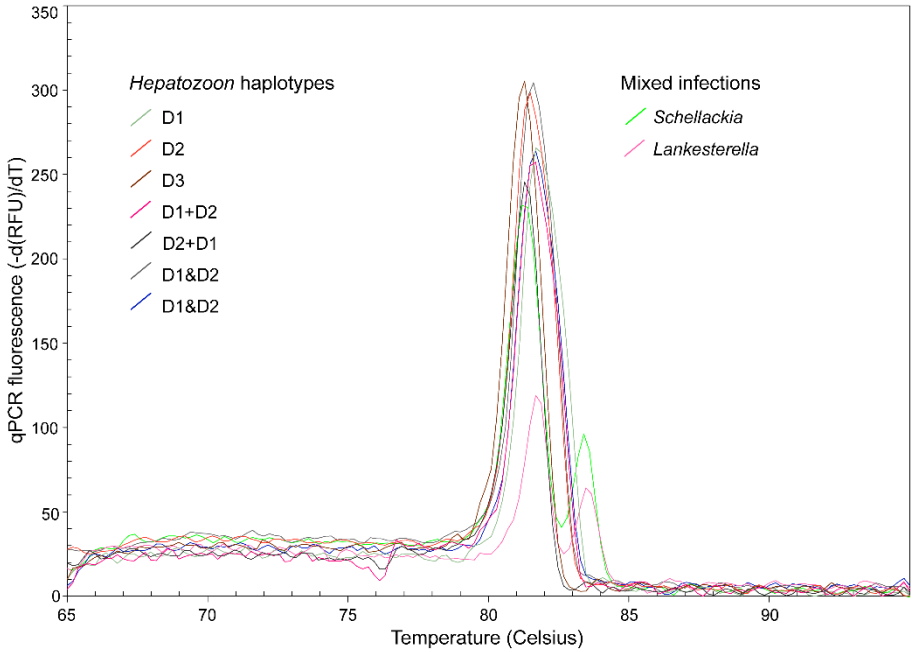


Figure S3 qPCR melting curves that allow distinction between hemogregarine and eimeriorind parasites.

Table S2 Morphological characteristics of *Hepatozoon omanensis* n. sp. mature gamonts and infected host cells, with an estimate of intensity of infection per 4,000 cells. Tree refers to the letter codes used in the phylogenetic tree (Figure 3-10). *n* refers to the number of hemogregarine gamonts or infected host cells measured per sample. GPS coordinates are as follows (Latitude, Longitude): 263 (23.05642, 57.46943), 279 (17.24218, 53.89095), 289 (22.61609, 59.09371), 292 (22.42828, 59.35618), 308 (22.7914, 59.22873), 319 (23.13167, 58.61889), 339 (23.10098, 57.34959), 340 (23.18292, 57.41627), and 350 (23.19809, 57.39045).

| | | | | | | | **Hemogregarine - Mean ± Sd (min-max)** | | | | **Host cell - Mean ± Sd (min-max)** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Host species** | **sex** | **GPS** | **code** | **Hap** | **tree** | **% intensity (4000 cells)** | ***n*** | **Length** | **Width** | **Area** | **Perimeter** | **Length** | **Width** | **Area** | **Perimeter** |
| *Asaccus platyrhynchus* | F | 263 | S7077 | 11 | A1 | 4.170 | 46 | 13.98 ± 1.14 (11.71-16.32) | 3.66 ± 0.46 (2.15-4.72) | 50.77 ± 6.24 (35.43-62.65) | 37.24 ± 3.55 (30.93-48.76) | 18.71 ± 1.43 (16.4-22.36) | 10.22 ± 0.76 (8.13-11.84) | 154.43 ± 17.48 (110.33-196.57) | 51.27 ± 3.03 (45.55-58.59) |
| *Asaccus platyrhynchus* | M | 350 | S6045 | 3 | O | 0.540 | 11 | 13.01 ± 0.47 (12.33-13.88) | 3.86 ± 0.34 (3.44-4.60) | 48.54 ± 5.11 (41.00-58.48) | 34.81 ± 1.86 (31.95-37.47) | 18.94 ± 2.40 (15.12-23.86) | 9.85 ± 0.79 (8.40-11.4) | 149.68 ± 19.63 (111.21-174.69) | 52.69 ± 5.06 (42.55-62.93) |
| | M | 350 | S6050 | 3 | O | 0.022 | 12 | 12.95 ± 0.73 (11.58-13.80) | 4.14 ± 0.36 (3.36-4.69) | 51.73 ± 5.64 (43.53-58.86) | 34.96 ± 2.05 (31.85-37.93) | 19.88 ± 1.29 (17.65-22.78) | 9.92 ± 0.66 (9.01-11.69) | 162.52 ± 13.66 (142.18-188.92) | 54.71 ± 2.57 (51.32-60.38) |
| | F | 350 | S6078 | 3 | O | 0.105 | 12 | 12.36 ± 0.65 (11.70-13.55) | 4.01 ± 0.46 (3.36-4.71) | 47.1 ± 4.95 (39.72-55.07) | 33.27 ± 1.65 (31.21-35.64) | 18.46 ± 1.03 (16.33-20.44) | 9.83 ± 0.75 (8.69-11.36) | 147.66 ± 14.83 (121.57-172.22) | 50.97 ± 2.52 (45.78-55.22) |
| | F | 350 | S6082 | 3 | O | 0.455 | 12 | 13.37 ± 0.74 (12.12-14.23) | 3.80 ± 0.23 (3.40-4.13) | 51.35 ± 5.21 (41.64-59.19) | 35.48 ± 2.01 (31.75-38.01) | 19.17 ± 1.62 (15.35-22.71) | 9.99 ± 0.43 (9.30-10.65) | 159.19 ± 17.54 (120.84-196.64) | 52.80 ± 3.35 (45.74-60.33) |
| | - | 263 | S7168 | 3 | O | 0.024 | 10 | 13.10 ± 0.63 (12.23-14.15) | 4.05 ± 0.35 (3.48-4.56) | 52.88 ± 4.41 (47.00-59.90) | 35.00 ± 1.78 (32.83-38.06) | 19.15 ± 2.46 (13.42-22.11) | 9.64 ± 1.07 (8.01-12.16) | 151.11 ± 17.01 (121.13-172.09) | 52.71 ± 4.00 (45.80-58.86) |
| | F | 350 | S7189 | 3 | O | 0.114 | 12 | 12.59 ± 0.40 (12.05-13.70) | 4.10 ± 0.40 (3.36-4.83) | 49.92 ± 4.03 (43.38-58.00) | 33.60 ± 1.52 (31.30-37.50) | 19.01 ± 0.82 (17.72-20.12) | 10.72 ± 1.11 (8.88-12.49) | 161.31 ± 13.05 (140.19-188.00) | 52.58 ± 1.52 (48.88-55.00) |
| | M | 350 | S7429 | 3 | O1 | 0.045 | 11 | 12.55 ± 0.44 (11.91-13.52) | 4.15 ± 0.36 (3.40-4.64) | 48.46 ± 4.50 (42.10-58.00) | 33.38 ± 1.35 (31.13-36.00) | 19.14 ± 1.40 (15.75-20.94) | 9.65 ± 0.91 (8.03-10.58) | 152.10 ± 13.39 (136.51-175.07) | 52.37 ± 2.37 (48.31-55.83) |
| | F | 350 | S7464 | 3 | O2 | 0.085 | 11 | 12.58 ± 0.49 (11.99-13.62) | 4.09 ± 0.17 (3.75-4.37) | 47.23 ± 3.96 (39.71-53.78) | 33.24 ± 1.32 (31.04-35.96) | 18.82 ± 1.60 (15.75-21.50) | 10.46 ± 0.58 (9.46-11.19) | 160.60 ± 15.02 (133.30-180.00) | 52.68 ± 3.14 (46.12-57.78) |
| | F | 350 | S7474 | 3 | O | 0.070 | 13 | 12.63 ± 0.70 (10.97-13.70) | 4.20 ± 0.39 (3.54-4.74) | 51.38 ± 5.10 (37.54-59.00) | 34.33 ± 1.67 (29.91-36.50) | 17.88 ± 1.13 (16.19-20.21) | 10.99 ± 0.67 (9.73-11.76) | 156.84 ± 12.67 (138.81-185.04) | 50.93 ± 2.09 (47.69-54.95) |
| | F | 350 | S7782 | 3 | O | 0.240 | 11 | 12.79 ± 0.42 (12.22-13.44) | 3.66 ± 0.33 (3.15-4.19) | 47.11 ± 5.44 (37.00-56.69) | 34.20 ± 1.05 (32.46-36.19) | 19.55 ± 1.97 (14.05-21.98) | 9.44 ± 0.41 (8.64-9.96) | 151.38 ± 15.35 (119.09-171.61) | 53.56 ± 2.69 (47.94-59.25) |
| | M | 350 | S7805 | 3 | O | 0.021 | 12 | 12.76 ± 0.63 (12.04-14.60) | 4.15 ± 0.40 (3.58-4.81) | 50.61 ± 3.29 (44.88-58.00) | 33.89 ± 1.58 (31.70-38.00) | 19.32 ± 1.88 (14.31-22.40) | 10.10 ± 0.80 (8.27-11.14) | 156.72 ± 15.46 (125.81-178.00) | 52.76 ± 3.21 (46.44-58.91) |
| | M | 350 | S7835 | 3 | O3 | 0.297 | 13 | 12.03 ± 0.59 (11.07-13.02) | 3.82 ± 0.30 (3.34-4.30) | 44.02 ± 4.58 (36.12-51.60) | 31.85 ± 1.96 (27.41-35.50) | 19.38 ± 1.71 (16.38-22.99) | 9.75 ± 0.82 (8.08-10.96) | 155.61 ± 18.29 (137.24-202.33) | 53.17 ± 3.02 (50.36-59.94) |
| | M | 350 | S7850 | 3 | O | 0.022 | 11 | 13.29 ± 0.79 (11.60-14.22) | 3.76 ± 0.36 (3.12-4.39) | 49.48 ± 6.21 (37.88-59.02) | 34.89 ± 2.17 (29.91-37.61) | 20.70 ± 1.45 (18.29-23.36) | 9.20 ± 1.01 (7.15-10.51) | 159.12 ± 16.66 (139.21-196.17) | 54.48 ± 3.11 (50.37-61.02) |
| | | | | | | | 151 | 12.77 ± 0.59 (10.97-14.60) | 3.98 ± 0.34 (3.12-4.83) | 49.22 ± 4.80 (36.12-59.90) | 34.07 ± 1.69 (27.41-38.06) | 19.19 ± 1.60 (13.42-23.86) | 9.96 ± 0.77 (7.15-12.49) | 155.68 ± 15.58 (111.21-202.33) | 52.80 ± 2.97 (42.55-62.93) |
| *Hemidactylus lemurinus* | F | 279 | S7134 | 4 | M | 0.063 | 12 | 13.25 ± 0.38 (12.19-13.60) | 3.90 ± 0.24 (3.48-4.42) | 52.06 ± 3.28 (46.60-58.5) | 35.62 ± 1.33 (32.30-37.19) | 20.13 ± 1.27 (17.23-22.12) | 10.35 ± 1.02 (8.71-11.92) | 167.64 ± 23.08 (116.24-197.44) | 53.89 ± 3.16 (46.27-57.45) |
| *Hemidactylus festivus* | M | 279 | S7605 | 4 | N | 0.035 | 11 | 13.25 ± 0.53 (12.62-14.37) | 4.44 ± 0.30 (3.92-4.85) | 56.05 ± 2.51 (52.99-60.80) | 35.80 ± 1.45 (32.83-37.98) | 20.02 ± 1.06 (18.07-21.42) | 9.01 ± 0.50 (8.05-9.60) | 150.35 ± 11.32 (129.88-165.00) | 50.81 ± 5.57 (36.22-55.06) |
| | | | | | | | 23 | 13.25 ± 0.45 (12.19-14.37) | 4.17 ± 0.27 (3.48-4.85) | 54.05 ± 2.89 (46.60-60.80) | 35.71 ± 1.39 (32.20-37.98) | 20.08 ± 1.16 (17.23-22.12) | 9.68 ± 0.76 (8.05-11.92) | 158.99 ± 17.2 (116.24-197.44) | 52.35 ± 4.36 (36.22-57.45) |

| Host species | sex | GPS | code | Hap | tree | % intensity (4000 cells) | Hemogregarine - Mean ± Sd (min-max) | | | | | Host cell - Mean ± Sd (min-max) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *n* | Length | Width | Area | Perimeter | Length | Width | Area | Perimeter |
| *Hemidactylus luqueorum* | M | 350 | S6080 | 3 | Q | 0.345 | 13 | 12.70 ± 0.50 (11.85-13.38) | 3.87 ± 0.47 (3.03-4.75) | 47.95 ± 5.90 (35.97-56.92) | 34.08 ± 1.64 (30.89-36.44) | 17.68 ± 1.97 (14.30-21.39) | 10.09 ± 0.51 (8.94-11.04) | 149.70 ± 19.24 (109.75-186.42) | 50.44 ± 4.08 (42.13-58.15) |
| *Hemidactylus hajarensis* | M | 289 | S7170 | 3 | P1/2 | 0.023 | 10 | 12.91 ± 0.58 (11.93-13.60) | 4.19 ± 0.37 (3.69-5.05) | 51.56 ± 4.15 (45.84-57.86) | 33.99 ± 1.43 (31.59-36.09) | 18.33 ± 1.17 (15.89-20.18) | 10.13 ± 0.37 (9.62-10.72) | 152.80 ± 10.04 (131.01-166.16) | 50.60 ± 2.12 (46.10-54.23) |
| | - | 319 | S7587 | 3 | P3 | 0.000 | 10 | 12.73 ± 0.60 (11.64-13.37) | 4.09 ± 0.73 (3.19-5.48) | 49.84 ± 2.83 (45.08-52.65) | 34.17 ± 1.20 (31.73-35.92) | 18.47 ± 1.70 (14.98-21.71) | 9.63 ± 0.68 (8.07-10.78) | 144.66 ± 17.35 (107.25-173.01) | 50.26 ± 4.10 (41.94-57.16) |
| | | | | | | | 33 | 12.78 ± 0.56 (11.64-13.60) | 4.05 ± 0.52 (3.03-5.48) | 49.78 ± 4.29 (35.97-57.86) | 34.08 ± 1.42 (30.89-36.44) | 18.16 ± 1.61 (14.30-21.71) | 9.95 ± 0.52 (8.07-11.04 | 149.05 ± 15.54 (107.25-186.42) | 50.43 ± 3.43 (41.94-58.15) |
| *Ptyodactylus hasselquistii* | M | 292 | S7123 | 3 | R1 | 0.102 | 11 | 11.57 ± 1.39 (8.18-13.41) | 4.31 ± 0.56 (3.21-5.40) | 43.78 ± 6.70 (30.10-53.15) | 30.66 ± 3.54 (23.14-35.69) | 18.38 ± 0.91 (17.29-19.90) | 10.66 ± 0.70 (9.27-11.86) | 156.20 ± 8.65 (139.96-172.29) | 52.57 ± 1.71 (49.53-55.26) |
| | M | 339 | S7357 | 3 | R2 | 0.272 | 14 | 12.67 ± 0.53 (12.07-13.66) | 3.83 ± 0.27 (3.44-4.32) | 48.09 ± 4.18 (44.00-56.18) | 33.75 ± 1.68 (31.49-37.03) | 17.36 ± 1.56 (15.32-19.81) | 10.32 ± 1.11 (7.75-12.14) | 142.49 ± 11.62 (122.58-169.65) | 48.87 ± 2.38 (44.41-52.42) |
| | M | 263 | S7611 | 3 | R | 0.000 | 6 | 12.89 ± 0.29 (12.34-13.28) | 3.83 ± 0.29 (3.33-4.30) | 47.15 ± 2.44 (43.95-50.55) | 33.73 ± 1.01 (32.60-35.84) | 20.19 ± 0.99 (18.90-21.50) | 9.56 ± 0.54 (8.45-10.20) | 160.48 ± 8.55 (148.14-173.82) | 53.33 ± 2.24 (50.38-56.45) |
| | M | 308 | S7668 | 3 | R | 0.042 | 10 | 12.61 ± 0.75 (11.52-13.91) | 3.74 ± 0.50 (2.79-4.29) | 44.91 ± 5.64 (35.55-53.41) | 33.54 ± 2.26 (30.20-37.32) | 18.96 ± 1.72 (16.78-22.15) | 10.26 ± 0.65 (9.16-11.27) | 155.15 ± 14.60 (133.99-180.15) | 53.22 ± 3.93 (48.22-59.26) |
| | F | 340 | S7776 | 3 | R3 | 0.096 | 12 | 13.29 ± 0.47 (12.57-13.90) | 4.02 ± 0.22 (3.66-4.37) | 51.59 ± 1.93 (48.05-55.40) | 35.09 ± 1.10 (32.97-37.00) | 19.17 ± 0.95 (17.22-20.47) | 10.46 ± 0.54 (9.38-11.50) | 163.09 ± 12.80 (144.49-189.82) | 52.30 ± 1.99 (48.31-55.65) |
| | | | | | | | 53 | 12.60 ± 0.69 (8.28-13.91) | 3.94 ± 0.37 (2.79-5.40) | 47.11 ± 4.18 (30.10-56.18) | 33.35 ± 1.92 (23.14-37.32) | 18.81 ± 1.23 (15.32-22.15) | 10.25 ± 0.71 (7.75-12.14) | 155.48 ± 11.24 (122.58-189.82) | 52.06 ± 2.45 (44.41-59.26) |

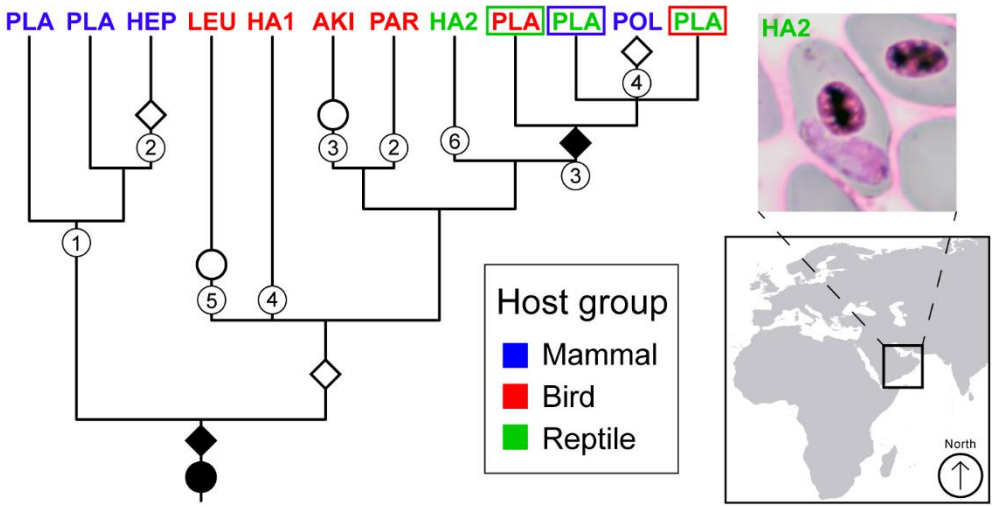


Figure S4 Graphical abstract of section 4.1.

Table S3 GenBank accession numbers for all taxa included in the molecular phylogenetic analysis (a total of 309 sequences). Refer to Table S4 for Reference correspondence.

| Host group | Parasite species | code | Lineage | Subgenus | Host species | Geographic locality | Isolate/Strain | Ref |
|---|---|---|---|---|---|---|---|---|
| Mammal, Primate | *Plasmodium falciparum* | AY282930 | A | *Laverania* | - | - | 3D7 | 21 |
| Mammal, Primate | *Plasmodium falciparum* | AY282961 | A | *Laverania* | - | Haiti | Haiti | 21 |
| Mammal, Primate | *Plasmodium falciparum* | NC_002375 | A | *Laverania* | - | - | NF54 | 9 |
| Mammal, Primate | *Plasmodium falciparum* | AF069605 | A | *Laverania* | *Homo sapiens* | Tropical regions | 3D7 | 13 |
| Mammal, Primate | *Plasmodium falciparum* | AF069607 | A | *Laverania* | *Homo sapiens* | Tropical regions | Santa Lucia | 13 |
| Mammal, Primate | *Plasmodium gaboni* | FJ895307 | A | | Chimpanzee | | K | 33 |
| Mammal, Primate | *Plasmodium gaboni* | GU045318 | A | | Chimpanzee | - | MB753 | 44 |
| Mammal, Primate | *Plasmodium reichenowi* | GU045314 | A | *Laverania* | Chimpanzee | - | MP1309 | 44 |
| Mammal, Primate | *Plasmodium reichenowi* | AF069610 | A | *Laverania* | *Pan troglodytes* | Central Africa | - | 13 |
| Mammal, Primate | *Plasmodium reichenowi* | AJ251941 | A | *Laverania* | *Pan troglodytes* | - | - | 9 |
| Mammal, Primate | *Plasmodium reichenowi* | EU560453 | A | *Laverania* | *Pan troglodytes* | Cameroon | Bana | 46 |
| Mammal, Primate | *Plasmodium reichenowi* | EU560454 | A | *Laverania* | *Pan troglodytes* | Cameroon | Nino | 46 |
| Mammal, Primate | *Plasmodium reichenowi* | EU560455 | A | *Laverania* | *Pan troglodytes* | Cameroon | Max | 46 |
| Mammal, Primate | *Plasmodium reichenowi* | EU560456 | A | *Laverania* | *Pan troglodytes* | Cote d'Ivoire | Rafiki_1 | 46 |
| Mammal, Primate | *Plasmodium reichenowi* | EU560457 | A | *Laverania* | *Pan troglodytes* | Cameroon | Dibamba | 46 |
| Mammal, Primate | *Plasmodium reichenowi* | EU560458 | A | *Laverania* | *Pan troglodytes* | Cote d'Ivoire | Rafiki_2 | 46 |
| Mammal, Primate | *Plasmodium reichenowi* | EU560459 | A | *Laverania* | *Pan troglodytes* | Cote d'Ivoire | Loukoum | 46 |
| Mammal, Primate | *Plasmodium reichenowi* | EU560466 | A | *Laverania* | *Pan troglodytes* | Cameroon | Gabon | 46 |
| Mammal, Primate | *Plasmodium* sp. | HM235115 | A | | Gorilla | Cameroon | - | 24 |
| Mammal, Rodent | *Plasmodium atheruri* | AY099054 | B | *Vinckeia* | *Atherurus africanus* | Congo and Cameroon | - | 39 |
| Mammal, Rodent | *Plasmodium atheruri* | EU254524 | B | *Vinckeia* | *Atherurus africanus* | Congo, Katanga | R20 | 27 |

| Host group | Parasite species | code | Lineage | Subgenus | Host species | Geographic locality | Isolate/Strain | Ref |
|---|---|---|---|---|---|---|---|---|
| Mammal, Rodent | *Plasmodium berghei* | AF014115 | B | *Vinckeia* | - | - | - | unpub |
| Mammal, Rodent | *Plasmodium berghei* | AY099049 | B | *Vinckeia* | *Grammomys surdaster* | Congo, Katanga | NK65 | 39 |
| Mammal, Rodent | *Plasmodium berghei* | DQ414645 | B | *Vinckeia* | *Grammomys surdaster* | Congo | ANKA | 40 |
| Mammal, Rodent | *Plasmodium berghei* | DQ414646 | B | *Vinckeia* | *Grammomys surdaster* | Congo | NK65 | 40 |
| Mammal, Rodent | *Plasmodium berghei* | EF011166 | B | *Vinckeia* | *Grammomys surdaster* | Congo, Katanga | R2 | 26 |
| Mammal, Rodent | *Plasmodium chabaudi* | AF014116 | B | *Vinckeia* | - | - | - | unpub |
| Mammal, Rodent | *Plasmodium chabaudi* | AY099050 | B | *Vinckeia* | *Thamnomys rutilans* | Central Africa | CB | 39 |
| Mammal, Rodent | *Plasmodium chabaudi* | EF011167 | B | *Vinckeia* | *Thamnomys rutilans* | Central African Republic | R16 | 26 |
| Mammal, Rodent | *Plasmodium chabaudi adami* | AB379670 | B | *Vinckeia* | - | - | DS | 8 |
| Mammal, Rodent | *Plasmodium chabaudi adami* | DQ414648 | B | *Vinckeia* | *Thamnomys rutilans* | Congo | 556KA | 40 |
| Mammal, Rodent | *Plasmodium chabaudi chabaudi* | DQ414649 | B | *Vinckeia* | *Thamnomys rutilans* | Central African Republic | AS | 40 |
| Mammal, Rodent | *Plasmodium vinckei* | AY099052 | B | *Vinckeia* | *Grammomys surdaster* | Congo, Katanga | VIBA CyO | 39 |
| Mammal, Rodent | *Plasmodium vinckei* | DQ414650 | B | *Vinckeia* | *Grammomys surdaster* | Cameroon | EL | 40 |
| Mammal, Rodent | *Plasmodium vinckei* | EU254522 | B | *Vinckeia* | *Grammomys surdaster* | Congo, Katanga | R8 | 27 |
| Mammal, Rodent | *Plasmodium vinckei brucechwatti* | DQ414652 | B | *Vinckeia* | *Praomys tullbergi* | Nigeria | - | 40 |
| Mammal, Rodent | *Plasmodium vinckei lentum* | DQ414653 | B | *Vinckeia* | - | Congo | 194ZZ | 40 |
| Mammal, Rodent | *Plasmodium vinckei petteri* | DQ414655 | B | *Vinckeia* | *Thamnomys rutilans* | Central African Republic | - | 40 |
| Mammal, Rodent | *Plasmodium vinckei vinckei* | DQ414651 | B | *Vinckeia* | *Grammomys surdaster* | Congo | VIBA Cy Pl | 40 |
| Mammal, Rodent | *Plasmodium yoelii* | AY099051 | B | *Vinckeia* | *Thamnomys rutilans* | Central African Republic | 17X | 39 |
| Mammal, Rodent | *Plasmodium yoelii* | DQ414657 | B | *Vinckeia* | *Thamnomys rutilans* | Cameroon | EL | 40 |
| Mammal, Rodent | *Plasmodium yoelii* | EU254521 | B | *Vinckeia* | *Thamnomys rutilans* | Central African Republic | R3 | 27 |
| Mammal, Rodent | *Plasmodium yoelii killicki* | DQ414658 | B | *Vinckeia* | *Thamnomys rutilans* | Congo | 193L | 40 |
| Mammal, Rodent | *Plasmodium yoelii nigeriensis* | DQ414659 | B | *Vinckeia* | *Thamnomys rutilans* | Nigeria | N67 | 40 |
| Mammal, Rodent | *Plasmodium yoelii yoelii* | DQ414660 | B | *Vinckeia* | *Thamnomys rutilans* | Central African Republic | 33X | 40 |
| Mammal, Primate | *Plasmodium coatneyi* | AB354575 | C | | - | - | - | 16 |
| Mammal, Primate | *Plasmodium cynomolgi* | AB434919 | C | *Plasmodium* | - | - | - | 16 |
| Mammal, Primate | *Plasmodium cynomolgi* | AF069616 | C | *Plasmodium* | Old world monkeys | Southeast Asia | Ceylonensis | 13 |
| Mammal, Primate | *Plasmodium fieldi* | AF069615 | C | *Plasmodium* | Old world monkeys | Malaysia | - | 13 |
| Mammal, Primate | *Plasmodium fieldi* | AB354574 | C | | - | - | - | 16 |
| Mammal, Primate | *Plasmodium fragile* | AY722799 | C | | - | - | type strain | 20 |
| Mammal, Primate | *Plasmodium gonderi* | AY800111 | C | *Plasmodium* | - | - | - | 30 |
| Mammal, Primate | *Plasmodium gonderi* | JF923750 | C | *Plasmodium* | *Mandrillus sphinx* | Gabon | 17A2/ms | 44 |
| Mammal, Primate | *Plasmodium gonderi* | AF069622 | C | *Plasmodium* | Old world monkeys | Central Africa | - | 13 |
| Mammal, Primate | *Plasmodium hylobati* | AB354573 | C | | - | - | - | 16 |
| Mammal, Primate | *Plasmodium hylobati* | AF069618 | C | | *Hylobates moloch* | Malaysia | - | 13 |
| Mammal, Primate | *Plasmodium inui* | AF069617 | C | *Plasmodium* | Old world monkeys | India and southeast Asia | Mulligan | 13 |
| Mammal, Primate | *Plasmodium inui* | AB354572 | C | | - | - | - | 16 |
| Mammal, Primate | *Plasmodium knowlesi* | AY722797 | C | *Plasmodium* | - | - | Malayan MRA-487 | 20 |
| Mammal, Primate | *Plasmodium knowlesi* | AY598141 | C | *Plasmodium* | *Homo sapiens* | - | - | 20 |
| Mammal, Primate | *Plasmodium knowlesi* | AF069621 | C | *Plasmodium* | Old world monkeys | Malaysia | H | 13 |
| Mammal, Primate | *Plasmodium malariae* | AB354570 | C | *Plasmodium* | - | - | - | 16 |
| Mammal, Primate | *Plasmodium malariae* | AF069624 | C | *Plasmodium* | *Homo sapiens* | Uganda | - | 13 |
| Mammal, Primate | *Plasmodium ovale* | AF069625 | C | *Plasmodium* | *Homo sapiens* | Tropics of Africa and Asia | Harding | 13 |
| Mammal, Primate | *Plasmodium ovale* | AB354571 | C | | - | - | - | 16 |
| Mammal, Primate | *Plasmodium simiovale* | AY800109 | C | *Plasmodium* | - | - | - | 30 |
| Mammal, Primate | *Plasmodium simiovale* | AF069614 | C | *Plasmodium* | Old world monkeys | Sri Lanka | - | 13 |
| Mammal, Primate | *Plasmodium simium* | AY722798 | C | | - | - | Fonseca MRA-353 | 20 |
| Mammal, Primate | *Plasmodium simium* | AF069620 | C | | *Alouatta fuscus* | Brazil | - | 13 |
| Mammal, Primate | *Plasmodium* sp. | AY800112 | C | | - | - | DAJ-2004 | 30 |
| Mammal, Primate | *Plasmodium* sp. | AF069623 | C | | *Mandrillus leucophaeus* | Gabon | - | 13 |

| Host group | Parasite species | code | Lineage | Subgenus | Host species | Geographic locality | Isolate/Strain | Ref |
|---|---|---|---|---|---|---|---|---|
| Mammal, Primate | *Plasmodium vivax* | AY598125 | C | *Plasmodium* | - | Vietnam | VX10 | 20 |
| Mammal, Primate | *Plasmodium vivax* | AF069619 | C | *Plasmodium* | *Homo sapiens* | Brazil | - | 13 |
| Mammal, Primate | *Plasmodium vivax* | NC_007243 | C | | - | El Salvador | SaI-1 | 20 |
| Mammal, Bats (H) | *Hepatocystis* sp | EU254526 | D | | *Cynopterus brachyotis* | Singapore | LDFB | 27 |
| Mammal, Bats (H) | *Hepatocystis* sp | KF159706 | D | | *Epomops buettikoferi* | Guinea | E_buett_G4_2 | 49 |
| Mammal, Bats (H) | *Hepatocystis* sp | KF159689 | D | | *Hypsignathus monstrosus* | Liberia | Hyp_mon_L2_1 | 49 |
| Mammal, Bats (H) | *Hepatocystis* sp | KF159670 | D | | *Micropteropus pusillus* | Guinea | Mic_pus_G2_1 | 49 |
| Mammal, Bats (H) | *Hepatocystis* sp | EU254527 | D | | *Nanonycteris veldkampii* | Guinea | MB6 | 27 |
| Mammal, Bats (H) | *Hepatocystis* sp | EU254528 | D | | *Nanonycteris veldkampii* | Guinea | MB3 | 27 |
| Mammal, Bats (H) | *Hepatocystis* sp | KF159698 | D | | *Nanonycteris veldkampii* | Liberia | Nan_vel_L1_1 | 49 |
| Mammal, Bats (H) | *Hepatocystis* sp | EF587238 | D | | *Pteropus hypomelanus* | Malaysia | - | 32 |
| Mammal, Bats (H) | *Hepatocystis* sp | FJ168565 | D | | *Pteropus hypomelanus* | South-Asia | 2 | 41 |
| Mammal, Bats (H) | Unidentified Haemosporida | EF179356 | D | | *Hipposideros larvatus* | Cambodia | C272 | 11 |
| Mammal, Primate (H) | *Hepatocystis* sp | GU929974 | D | | Macaques | Southeast Asian | HB421 | 45 |
| Mammal, Primate (H) | *Hepatocystis* sp | GU930055 | D | | Macaques | Southeast Asian | M156_b | 45 |
| Mammal, Primate (H) | *Hepatocystis* sp | AF069626 | D | | *Papio nubensis* | Ethiopia | 1 | 13 |
| Mammal, Primate (H) | *Hepatocystis* sp | KC262814 | D | | Primate | Africa | BWC0310 | 53 |
| Mammal, Primate (H) | *Hepatocystis* sp | GU945296 | D | | *Procolobus badius* | Uganda | 56.2 | unpub |
| Mammal, Primate (H) | *Hepatocystis* sp | EU400408 | D | | Wild macaques | Thailand | MFRC11 clone A | 50 |
| Mammal, Rodent (H) | *Hepatocystis* sp | JX090248 | D | | *Callosciurus notatus* | Malaysia: Borneo | K27-G14 | unpub |
| Bird | *Leucocytozoon buteonis* | KF726088 | E | | *Buteo rufinus* | - | Ank-SL1 | unpub |
| Bird | *Leucocytozoon dubreuli* | AY099063 | E | | *Catharus guttatus* | USA: Vermont | - | 39 |
| Bird | *Leucocytozoon fringillinarum* | FJ168564 | E | | *Pipilo chlorurus* | - | - | 41 |
| Bird | *Leucocytozoon fringillinarum* | NC_012451 | E | | *Pipilo chlorurus* | - | - | 41 |
| Bird | *Leucocytozoon majoris* | FJ168563 | E | | *Zonotrichia leucophrys oriantha* | - | - | 41 |
| Bird | *Leucocytozoon majoris* | NC_012450 | E | | *Zonotrichia leucophrys oriantha* | - | - | 41 |
| Bird | *Leucocytozoon sabrazesi* | AB299369 | E | | *Gallus gallus* | Malaysia | - | unpub |
| Bird | *Leucocytozoon schoutedeni* | DQ676823 | E | | *Gallus gallus* | Uganda | 23-379 | 51 |
| Bird | *Leucocytozoon* sp. | EU254519 | E | | *Accipiter brevipes* | Israel | P157 | 27 |
| Bird | *Leucocytozoon* sp. | AB743872 | E | | *Anas crecca* | Japan | 1 | unpub |
| Bird | *Leucocytozoon* sp. | EU254518 | E | | *Buteo jamaicensis* | USA, Massachusetts | 2109 | 27 |
| Bird | *Leucocytozoon* sp. | EU254520 | E | | *Buteo lineatus* | USA, California | 2208 | 27 |
| Bird | *Leucocytozoon* sp. | AY393796 | E | | *Carduelis spinus* | Lithuania | ISISKIN2 | 17 |
| Bird | *Leucocytozoon* sp. | AB741507 | E | | *Columba livia* | Japan | 2 | unpub |
| Bird | *Leucocytozoon* sp. | AB741496 | E | | *Corvus corone* | Japan | 5 | unpub |
| Bird | *Leucocytozoon* sp. | AB741502 | E | | *Corvus macrorhynchos* | Japan | 3 | unpub |
| Bird | *Leucocytozoon* sp. | AB741514 | E | | *Cygnus cygnus* | Japan | 2 | unpub |
| Bird | *Leucocytozoon* sp. | KF479480 | E | | *Heliangelus amethysticollis* | Colombia | 1 IAL-2013 | 28 |
| Bird | *Leucocytozoon* sp. | JN975309 | E | | *Ixos philippinus* | Philippines | L2, AMSI-2011 | 52 |
| Bird | *Leucocytozoon* sp. | AB741510 | E | | *Streptopelia orientalis* | Japan | 4 | unpub |
| Bird | *Leucocytozoon toddi* | AY684973 | E | | *Accipiter francesii* | Madagascar | - | unpub |
| Bird | *Haemoproteus columbae* | EU254548 | F | *Haemoproteus* | *Columba livia* | USA, Massachusetts | 2111 | 27 |
| Bird | *Haemoproteus columbae* | EU254549 | F | *Haemoproteus* | *Columba livia* | Singapore | PG7 | 27 |
| Bird | *Haemoproteus columbae* | EU254553 | F | *Haemoproteus* | *Columba livia* | USA, Massachusetts | 2146 | 27 |
| Bird | *Haemoproteus columbae* | FJ168562 | F | *Haemoproteus* | *Columba livia* | - | - | 41 |
| Bird | *Haemoproteus iwa* | JF833050 | F | | *Fregata minor* | Ecuador | FMINGAL1 | 23 |
| Bird | *Haemoproteus multipigmentatus* | GU296216 | F | | *Zenaida galapagoensis* | Ecuador: Galapagos | JH003W | 56 |
| Bird | *Haemoproteus multipigmentatus* | JN788932 | F | | *Zenaida macroura* | Mexico | SocH1 | 6 |
| Bird | *Haemoproteus multipigmentatus* | JF833051 | F | | *Zenaida galapagoensis* | Ecuador | 1 | 23 |
| Bird | *Haemoproteus* sp. | FJ462674 | F | | *Zenaida galapagoensis* | Ecuador | GDE13 | 48 |
| - | *Akiba caulleryi* | AB302215 | G | | - | Japan | - | 35 |
| - | *Akiba caulleryi* | NC_015304 | G | | - | Japan | - | 35 |

| Host group | Parasite species | code | Lineage | Subgenus | Host species | Geographic locality | Isolate/Strain | Ref |
|---|---|---|---|---|---|---|---|---|
| Bird | *Haemoproteus balmorali* | DQ630007 | H | | *Luscinia luscinia* | Lithuania | L-LULU1 | 18 |
| Bird | *Haemoproteus belopolskyi* | DQ630006 | H | *Parahaemoproteus* | *Hippolais icterina* | Sweden | L-HIICT1 | 18 |
| Bird | *Haemoproteus belopolskyi* | DQ451408 | H | *Parahaemoproteus* | *Sylvia curruca* | Israel | P60 | 25 |
| Bird | *Haemoproteus coatneyi* | EU254550 | H | *Parahaemoproteus* | *Dendroica coronata* | USA, Vermont | 1060 | 27 |
| Bird | *Haemoproteus enucleator* | DQ659592 | H | | *Alcedo leucogaster* | Gabon | - | 3 |
| Bird | *Haemoproteus fringillae* | EU254558 | H | *Parahaemoproteus* | *Zonotrichia albicollis* | USA, Vermont | 169 | 27 |
| Bird | *Haemoproteus ilanpapernai* | DQ451424 | H | *Parahaemoproteus* | *Strix seloputo* | Israel | - | 25 |
| Bird | *Haemoproteus lanii* | DQ630010 | H | | *Lanius collurio* | Europe | L-RB1 | 18 |
| Bird | *Haemoproteus lanii* | DQ630011 | H | | *Lanius collurio* | Sweden | L-RBS2 | 18 |
| Bird | *Haemoproteus magnus* | DQ451426 | H | *Parahaemoproteus* | *Fringilla coelebs* | Israel | - | 25 |
| Bird | *Haemoproteus majoris* | AF254977 | H | | *Parus caeruleus* | - | PARUS1 | 4 |
| Bird | *Haemoproteus majoris* | AY099045 | H | | *Parus caeruleus* | Sweden | - | 39 |
| Bird | *Haemoproteus micronuclearis* | HQ386238 | H | | *Ploceus nigerrimus* | Cameroon | HV39, 26 385 2 | 19 |
| Bird | *Haemoproteus micronuclearis* | EF117230 | H | | *Quelea quelea* | Botswana | H12 | 10 |
| Bird | *Haemoproteus minutus* | DQ630013 | H | | *Turdus merula* | Lithuania | L-TURDUS2 | 18 |
| Bird | *Haemoproteus nucleofascialis* | HQ386243 | H | | *Malimbus rubricollis* | Uganda | HV44, 23 55 2 | 19 |
| Bird | *Haemoproteus pallidus* | DQ630004 | H | | *Ficedula hypoleuca* | Europe | L-PFC1 | 18 |
| Bird | *Haemoproteus parabelopolskyi* | AY831751 | H | | *Sylvia atricapilla* | - | SYAT2 | 37 |
| Bird | *Haemoproteus passeris* | EU254554 | H | *Parahaemoproteus* | *Passer moabiticus* | Israel | P38 | 27 |
| Bird | *Haemoproteus payevskyi* | DQ630009 | H | | *Acrocephalus scipaceus* | Lithuania | L-RW1 | 18 |
| Bird | *Haemoproteus picae* | EU254552 | H | *Parahaemoproteus* | *Picoides pubescens* | USA, Vermont | 1534 | 27 |
| Bird | *Haemoproteus sanguinis* | DQ451410 | H | *Parahaemoproteus* | *Pycnonotus xanthopygos* | Israel | P146 | 25 |
| Bird | *Haemoproteus* sp. | EU254555 | H | *Parahaemoproteus* | *Bonasa umbellus* | USA, Vermont | 2223 | 27 |
| Bird | *Haemoproteus* sp. | EU254561 | H | *Parahaemoproteus* | *Bucephala clangula* | USA, Vermont | 2104 | 27 |
| Bird | *Haemoproteus* sp. | EU254557 | H | *Parahaemoproteus* | *Chamaea fasciata* | USA, California | B60 | 27 |
| Bird | *Haemoproteus* sp. | EU254562 | H | *Parahaemoproteus* | *Dendroica caerulescens* | USA, Vermont | 384 | 27 |
| Bird | *Haemoproteus* sp. | EU254559 | H | *Parahaemoproteus* | *Dumetella carolinensis* | USA, Vermont | 1000 | 27 |
| Bird | *Haemoproteus* sp. | EU254556 | H | *Parahaemoproteus* | *Falco sparverius* | USA, Vermont | 2176 | 27 |
| Bird | *Haemoproteus* sp. | EU254560 | H | *Parahaemoproteus* | *Mergus merganser* | USA, Vermont | 1774 | 27 |
| Bird | *Haemoproteus* sp. | EU254551 | H | *Parahaemoproteus* | *Vireo olivaceus* | USA, Vermont | 579 | 27 |
| Bird | *Haemoproteus* sp. | AY099046 | H | | *Acrocephalus scirpaceus* | India | RW1 | 39 |
| Bird | *Haemoproteus* sp. | KC121053 | H | | *Eudyptula minor* | Australia | 11-626 | 5 |
| Bird | *Haemoproteus* sp. | NC_012425 | H | | *Lichenostomus frenatus* | Australia | jb2.SEW5141 | 2 |
| Bird | *Haemoproteus* sp. | NC_012423 | H | | *Meliphaga lewinii* | Australia | jb1.JA27 | 2 |
| Bird | *Haemoproteus* sp. | AY099042 | H | | *Phylloscopus humei* | India | HLW1 | 39 |
| Bird | *Haemoproteus* sp. | AY099043 | H | | *Phylloscopus occipitalis* | India | LCLW1B19 | 39 |
| Bird | *Haemoproteus* sp. | AY099039 | H | | *Phylloscopus trochilus* | Sweden | WW2 | 39 |
| Bird | *Haemoproteus* sp. | GQ395637 | H | | *Spheniscus mendiculus* | - | DR11DR29 | 22 |
| Bird | *Haemoproteus* sp. | GQ395666 | H | | *Spheniscus mendiculus* | - | NA16K65 | 22 |
| Bird | *Haemoproteus sylvae* | AY099040 | H | | *Acrocephalus arundinaceus* | Sweden | GRW1 | 39 |
| Bird | *Haemoproteus turtur* | DQ451425 | H | *Parahaemoproteus* | *Streptopelia senegalensis* | Israel | - | 25 |
| Bird | *Haemoproteus vacuolatus* | EU770153 | H | | *Andropadus latirostris* | Ghana | H-ANLA1 | 54 |
| Bird | *Parahaemoproteus vireonis* | FJ168561 | H | | *Vireo gilvus* | - | - | 41 |
| Reptile, Lizard | *Haemocystidium kopki* | AY099062 | I | *Haemocystidium* | *Teratoscincus scincus* | Pakistan | - | 39 |
| Reptile, Lizard | *Haemocystidium ptyodactylii* | AY099057 | I | *Haemocystidium* | *Ptyodactylus hasselquistii* | Israel | - | 39 |
| Reptile, Lizard | *Haemocystidium* sp.[15] | EU254531[15] | I | *Haemocystidium* | *Egernia stokesii* | Australia | Circ | 27 |
| **Reptile, Lizard** | ***Haemocystidium* sp.** | **S7155 / S7668** | **I** | ***Haemocystidium*** | ***Hemidactylus* sp. / *Ptyodactylus hasselquistii*** | **Oman** | **S7155 / S7668** | **here** |

[15] Sequence EU254531 identified as *Plasmodium* sp. on GenBank.

| Host group | Parasite species | code | Lineage | Subgenus | Host species | Geographic locality | Isolate/Strain | Ref |
|---|---|---|---|---|---|---|---|---|
| Reptile, Lizard | *Haemocystidium* sp.[16] | DQ212191[16] | I | *Haemocystidium* | *Oplurus cuvieri* | - | 4018 | unpub |
| Reptile, Snake | *Haemocystidium mesnili* | KF049514 | I | *Haemocystidium* | *Naja annulifera* | South Africa | - | 43 |
| Reptile, Testudine | *Haemocystidium anatolicum* | JQ039742 | I | *Simondia* | *Testudo graeca* | Turkey | OMRESN | 36 |
| Reptile, Testudine | *Haemocystidium pacayae* | KF049495 | I | *Simondia* | *Podocnemis expansa* | Peru | E24 | 43 |
| Reptile, Testudine | *Haemocystidium pacayae* | KF049507 | I | *Simondia* | *Podocnemis expansa* | Peru | E26 | 43 |
| Reptile, Testudine | *Haemocystidium pacayae* | KF049506 | I | *Simondia* | *Podocnemis unifilis* | Peru | U65 | 43 |
| Reptile, Testudine | *Haemocystidium pacayae* | KF049509 | I | *Simondia* | *Podocnemis unifilis* | Peru | U127 | 43 |
| Reptile, Testudine | *Haemocystidium pacayae* | KF049513 | I | *Simondia* | *Podocnemis unifilis* | Peru | U449 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049491 | I | *Simondia* | *Podocnemis expansa* | Peru | E134 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049492 | I | *Simondia* | *Podocnemis expansa* | Peru | E15 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049493 | I | *Simondia* | *Podocnemis expansa* | Peru | E18 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049494 | I | *Simondia* | *Podocnemis expansa* | Peru | E22 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049496 | I | *Simondia* | *Podocnemis expansa* | Peru | E36 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049497 | I | *Simondia* | *Podocnemis expansa* | Peru | E39 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049498 | I | *Simondia* | *Podocnemis expansa* | Peru | E59 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049499 | I | *Simondia* | *Podocnemis expansa* | Peru | E66 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049500 | I | *Simondia* | *Podocnemis expansa* | Peru | E68 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049508 | I | *Simondia* | *Podocnemis expansa* | Peru | E70 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049501 | I | *Simondia* | *Podocnemis unifilis* | Peru | U139 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049502 | I | *Simondia* | *Podocnemis unifilis* | Peru | U140 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049503 | I | *Simondia* | *Podocnemis unifilis* | Peru | U309 | 43 |
| Reptile, Testudine | *Haemocystidium peltocephali* | KF049505 | I | *Simondia* | *Podocnemis unifilis* | Peru | U470 | 43 |
| Reptile, Lizard | *Plasmodium agamae* | AY099048 | J | *Sauramoeba* | *Agama agama* | Ghana | - | 39 |
| Reptile, Lizard | *Plasmodium giganteum* | AY099053 | J | *Sauramoeba* | *Agama agama* | Ghana | - | 39 |
| Reptile, Lizard | *Plasmodium giganteum* | EU254534 | J | *Sauramoeba* | *Agama agama* | Ghana | G6 | 27 |
| Bird | *Plasmodium globularis* | EU770151 | K | *Novyella* | *Andropadus latirostris* | Ghana | P-ANLA1 | 54 |
| Bird | *Plasmodium juxtanucleare* | NC_008279 | K | *Bennettinia* | - | - | - | 34 |
| Bird | *Plasmodium juxtanucleare* | AB302893 | K | *Bennettinia* | *Crossoptilon crossoptilon* | Japan: Kanagawa | - | 31 |
| Bird | *Plasmodium juxtanucleare* | AB250415 | K | *Bennettinia* | *Gallus gallus* | Asia | - | 34 |
| Bird | *Plasmodium multivacuolaris* | FJ389157 | K | *Novyella* | *Andropadus latirostris* | | P-ANLA2 | 55 |
| Bird | *Plasmodium parahexamerium* | FJ389155 | K | *Novyella* | *Alethe diademata* | - | P-ALDI1 | 55 |
| Bird | *Plasmodium polare* | DQ659590 | K | *Giovannolaia* | *Parus major* | Sweden | P54 | 3 |
| Bird | *Plasmodium* sp. | EF011198 | K | *Bennettinia* | *Gallus gallus* | Vietnam | PJ4 | 26 |
| Bird | *Plasmodium* sp. | EF011171 | K | *Novyella* | *Agelaius phoeniceus* | USA, Vermont | 318 | 26 |
| Bird | *Plasmodium* sp. | EF011173 | K | *Novyella* | *Seiurus aurocapilla* | USA, Vermont | 513 | 26 |
| Bird | *Plasmodium* sp. | EF011177 | K | *Novyella* | *Turdus migratorius* | USA | 608 | 26 |
| Bird | *Plasmodium* sp. | AF465547 | K | | *Agelaius phoeniceus* | North America | 47 | 47 |
| Bird | *Plasmodium* sp. | AF465550 | K | | *Andropadus latirostris* | Cameroon | 50 | 47 |
| Bird | *Plasmodium* sp. | EU254546 | K | | *Anthus trivialis* | Israel | P164 | 27 |
| Bird | *Plasmodium* sp. | EU254544 | K | | *Hylocichla mustelina* | USA, Vermont | 1271 | 27 |
| Bird | *Plasmodium* sp. | EU254545 | K | | *Turdus migratorius* | USA, Vermont | 1937 | 27 |
| Bird | *Plasmodium* sp. | AF465549 | K | | *Zonotrichia leucophrys* | North America | 49 | 47 |
| Reptile, Lizard | *Plasmodium chiricahuae* | AY099061 | L | *Paraplasmodium* | *Sceloporus jarrovi* | USA: Arizona | - | 39 |
| Reptile, Lizard | *Plasmodium floridense* | AY099059 | L | | *Anolis oculatus* | Dominica | - | 39 |
| Reptile, Lizard | *Plasmodium floridense* | EU254530 | L | | *Anolis oculatus* | Dominica | 5676 | 27 |
| Reptile, Lizard | *Plasmodium floridense* | EF079654 | L | | *Anolis sagrei* | USA | - | unpub |
| Reptile, Lizard | *Plasmodium floridense* | NC_009961 | L | | *Anolis sagrei* | USA | - | unpub |
| Reptile, Lizard | *Plasmodium floridense* | JN187887 | L | | Lizard | Caribbean | DR240 | 14 |
| Reptile, Lizard | *Plasmodium floridense* | JN187902 | L | | Lizard | Caribbean | DR453 | 14 |

[16] Sequence DQ212191 identified as *Haemoproteus* sp. on GenBank.

| Host group | Parasite species | code | Lineage | Subgenus | Host species | Geographic locality | Isolate/Strain | Ref |
|---|---|---|---|---|---|---|---|---|
| Reptile, Lizard | *Plasmodium floridense* | JN187935 | L | | Lizard | Caribbean | M1064 | 14 |
| Reptile, Lizard | *Plasmodium hispaniolae* | JN187890 | L | | Lizard | Caribbean | DR263 | 14 |
| Reptile, Lizard | *Plasmodium hispaniolae* | JN187905 | L | | Lizard | Caribbean | DR490 | 14 |
| Reptile, Lizard | *Plasmodium hispaniolae* | JN187906 | L | | Lizard | Caribbean | DR494 | 14 |
| Reptile, Lizard | *Plasmodium hispaniolae* | JN187914 | L | | Lizard | Caribbean | DR507 | 14 |
| Reptile, Lizard | *Plasmodium mexicanum* | AB375765 | L | *Paraplasmodium* | - | - | - | 16 |
| Reptile, Lizard | *Plasmodium mexicanum* | AY099060 | L | *Paraplasmodium* | *Sceloporus mexicanum* | USA: California | - | 39 |
| Reptile, Lizard | *Plasmodium mexicanum* | EF079653 | L | *Paraplasmodium* | *Sceloporus occidentalis* | USA | - | unpub |
| Reptile, Lizard | *Plasmodium mexicanum* | EU254529 | L | *Paraplasmodium* | *Sceloporus occidentalis* | USA | E1 | 27 |
| Mammal, Bats | Unidentified Haemosporida | EF179355 | M | | *Megaderma spasma* | Cambodia | C289 | 11 |
| Reptile, Lizard | *Plasmodium azurophilum* | AY099055 | N | | *Anolis oculatus* | Dominica | - | 39 |
| Reptile, Lizard | *Plasmodium azurophilum* | AY099058 | N | | *Anolis oculatus* | Dominica | - | 39 |
| Reptile, Lizard | *Plasmodium azurophilum* | EU254532 | N | | *Anolis oculatus* | Dominica | Red5686 | 27 |
| Reptile, Lizard | *Plasmodium azurophilum* | EU254533 | N | | *Anolis oculatus* | Dominica | White5680 | 27 |
| Reptile, Lizard | *Plasmodium azurophilum* | JN187891 | N | | Lizard | Caribbean | DR274 | 14 |
| Reptile, Lizard | *Plasmodium azurophilum* | JN187894 | N | | Lizard | Caribbean | DR304 | 14 |
| Reptile, Lizard | *Plasmodium azurophilum* | JN187915 | N | | Lizard | Caribbean | DR512 | 14 |
| Reptile, Lizard | *Plasmodium azurophilum* | JN187927 | N | | Lizard | Caribbean | DR600 | 14 |
| Reptile, Lizard | *Plasmodium fairchildi* | AY099056 | N | | *Norops cupreus* | Costa Rica | - | 39 |
| Reptile, Lizard | *Plasmodium gemini* | EU834706 | N | | *Hypsilurus modestus* | Papua New Guinea | CCA3595 | 42 |
| Reptile, Lizard | *Plasmodium gemini* | EU834707 | N | | *Hypsilurus modestus* | Papua New Guinea | CCA3596 | 42 |
| Reptile, Lizard | *Plasmodium gemini* | EU834708 | N | | *Hypsilurus modestus* | Papua New Guinea | CCA3597 | 42 |
| Reptile, Lizard | *Plasmodium koreafense* | EU834704 | N | | *Sphenomorphus jobiensis* | Papua New Guinea | CCA2146 | 42 |
| Reptile, Lizard | *Plasmodium lacertiliae* | EU834709 | N | | *Emoia longicauda* | Papua New Guinea | CCA2227 | 42 |
| Reptile, Lizard | *Plasmodium lacertiliae* | EU834710 | N | | *Emoia longicauda* | Papua New Guinea | CCA2228 | 42 |
| Reptile, Lizard | *Plasmodium leucocytica* | JN187892 | N | | Lizard | Caribbean | DR302 | 14 |
| Reptile, Lizard | *Plasmodium leucocytica* | JN187938 | N | | Lizard | Caribbean | M1121 | 14 |
| Reptile, Lizard | *Plasmodium megalotrypa* | EU834705 | N | | *Sphenomorphus simus* | Papua New Guinea | CCA3565 | 42 |
| Reptile, Lizard | *Plasmodium minuoviride* | EU834703 | N | | *Prasinohaema prehensicauda* | Papua New Guinea | CCA0640 | 42 |
| Reptile, Lizard | *Plasmodium* sp. | AY099047 | N | | *Ameiva ameiva* | Brazil | - | 39 |
| Reptile, Lizard | *Plasmodium* sp. | EU254537 | N | | *Ameiva ameiva* | Brazil, Manaus | br67 | 27 |
| Reptile, Lizard | *Plasmodium* sp. | DQ337365 | N | | *Emoia caeruleocauda* | - | LSU CCA 2151 | 1 |
| Reptile, Lizard | *Plasmodium* sp. | DQ337364 | N | | *Emoia kordoana* | Papua New Guinea | LSU CCA 2011 | 1 |
| Reptile, Lizard | *Plasmodium* sp. | DQ337363 | N | | *Emoia longicauda* | Papua New Guinea | LSU CCA 2228 | 1 |
| Reptile, Lizard | *Plasmodium* sp. | DQ337366 | N | | *Emoia longicauda* | Papua New Guinea | LSU CCA 2227 | 1 |
| Reptile, Lizard | *Plasmodium* sp. | DQ337362 | N | | *Sphenomorphus jobiense* | Papua New Guinea | - | 1 |
| Mammal, Bats (P) | *Haemosporida* sp. | AY762070 | O | | *Miniopterus manavi* | Madagascar | SMG12472 | 11 |
| Mammal, Bats (P) | *Haemosporida* sp. | AY762071 | O | | *Miniopterus manavi* | Madagascar | SMG12535 | 11 |
| Mammal, Bats (P) | *Haemosporida* sp. | AY762074 | O | | *Miniopterus manavi* | Madagascar | SMG12918 | 11 |
| Mammal, Bats (P) | *Haemosporida* sp. | AY762075 | O | | *Myotis goudoti* | Madagascar | SMG13064 | 11 |
| Mammal, Bats (P) | *Polychromophilus melanipherus* | JN990708 | O | | *Miniopterus schreibersii* | Switzerland | 3 | 59 |
| Mammal, Bats (P) | *Polychromophilus murinus* | HM055583 | O | | Bat | - | 1 | 29 |
| Mammal, Bats (P) | *Polychromophilus murinus* | HM055584 | O | | Bat | - | 2 | 29 |
| Mammal, Bats (P) | *Polychromophilus murinus* | HM055585 | O | | Bat | - | 3 | 29 |
| Mammal, Bats (P) | *Polychromophilus murinus* | HM055586 | O | | Bat | - | 4 | 29 |
| Mammal, Bats (P) | *Polychromophilus murinus* | HM055587 | O | | Bat | - | 5 | 29 |
| Mammal, Bats (P) | *Polychromophilus murinus* | HM055588 | O | | Bat | - | 6 | 29 |
| Mammal, Bats (P) | *Polychromophilus murinus* | HM055589 | O | | Bat | - | 7 | 29 |
| Mammal, Bats (P) | *Polychromophilus murinus* | JN990713 | O | | *Myotis daubentonii* | Switzerland | 2 | 59 |
| Mammal, Bats (P) | *Polychromophilus* sp. | JQ995284 | O | | *Miniopterus inflatus* | Gabon | 1 | 12 |
| Mammal, Bats (P) | *Polychromophilus* sp. | KF159681 | O | | *Miniopterus villiersi* | Guinea | Min_vil_G3_3 | 49 |
| Mammal, Bats (P) | Unidentified Haemosporida | EF179354 | O | | *Kerivoula hardwickii* | Cambodia | C285 | 11 |

| Host group | Parasite species | code | Lineage | Subgenus | Host species | Geographic locality | Isolate/Strain | Ref |
|---|---|---|---|---|---|---|---|---|
| Bird | *Plasmodium elongatum* | DQ368381 | P | | *Acrocephalus arundinaceus* | - | GRW6 | 38 |
| Bird | *Plasmodium* sp. | EF011168 | P | *Huffia* | *Melospiza melodia* | USA, Vermont | 182 | 26 |
| Bird | *Plasmodium* sp. | EU254543 | P | | *Aegolius acadicus* | USA, Vermont | 2375 | 27 |
| Bird | *Plasmodium* sp. | EU254541 | P | | *Ixobrychus minutus* | Israel | P159 | 27 |
| Bird | *Plasmodium ashfordi* | AF254962 | Q | *Novyella* | *Acrocephalus arundinaceus* | | GRW2 | 4 |
| Bird | *Plasmodium columbae* | AF069613 | Q | | *Columba livia* | Venezuela | - | 13 |
| Bird | *Plasmodium nucleophilum* | JX467689 | Q | *Novyella* | *Alopochen aegyptiacus* | Brazil | 26879 | 7 |
| Bird | *Plasmodium* sp. | EU254542 | Q | | *Acridotheres tristis* | Singapore | myna | 27 |
| Bird | *Plasmodium* sp. | FJ462683 | Q | | *Leptotila verreauxi* | Venezuela | LVCP01Ven | 48 |
| Bird | *Plasmodium cathemerium* | AY377128 | R | *Haemamoeba* | *Wide range Bird* | Wallacean zones | - | 58 |
| Bird | *Plasmodium circumflexum* | JN164734 | R | *Giovannolaia* | *Sylvia atricapilla* | - | TURDUS1 | unpub |
| Bird | *Plasmodium circumflexum* | AF495576 | R | *Giovannolaia* | *Turdus philomelos* | Sweden | TURDUS1 | 57 |
| Bird | *Plasmodium elongatum* | AF069611 | R | *Huffia* | *Passer domesticus* | North America Vietnam | - | 13 |
| Bird | *Plasmodium elongatum* | DQ659549 | R | | *Geothlypis trichas* | USA | P11, PADO M09 | 3 |
| Bird | *Plasmodium gallinaceum* | AB250690 | R | *Haemamoeba* | - | Philippines | - | 34 |
| Bird | *Plasmodium gallinaceum* | DQ212189 | R | *Haemamoeba* | *Gallus gallus* | Vietnam | - | unpub |
| Bird | *Plasmodium gallinaceum* | EU254535 | R | *Haemamoeba* | *Gallus gallus* | Vietnam | RP | 27 |
| Bird | *Plasmodium haemamoeba* | DQ368378 | R | | *Acrocephalus arundinaceus* | - | GRW12 | 38 |
| Bird | *Plasmodium megaglobularis* | EU770152 | R | *Novyella* | *Cyanomitra olivacea* | Ghana | P-CYOL1 | 54 |
| Bird | *Plasmodium relictum* | DQ451404 | R | *Haemamoeba* | *Corvus corone* | Israel | P134 | 25 |
| Bird | *Plasmodium relictum* | EF011193 | R | *Haemamoeba* | *Emberiza hortulana* | Israel | P113 | 26 |
| Bird | *Plasmodium relictum* | AY733090 | R | *Haemamoeba* | *Hemignathus virens* | USA | jb5.NAN015 | 2 |
| Bird | *Plasmodium relictum* | AF495571 | R | *Haemamoeba* | *Passer luteus* | Nigeria | SGS1 | 57 |
| Bird | *Plasmodium relictum* | EU254538 | R | *Haemamoeba* | *Sialia mexicana* | USA, California | B170 | 27 |
| Bird | *Plasmodium relictum* | EU254536 | R | *Haemamoeba* | *Zenaida macroura* | USA, Nebraska | - | 27 |
| Bird | *Plasmodium relictum* | JQ778277 | R | *Haemamoeba* | *Culex pipiens and Parus major* | Switzerland | GRW11 | 15 |
| Bird | *Plasmodium* sp. | EF011187 | R | *Giovannolaia* | *Seiurus noveboracensis* | USA | 1536 | 26 |
| Bird | *Plasmodium* sp. | EF011188 | R | *Giovannolaia* | *Turdus migratorius* | USA | 1542 | 26 |
| Bird | *Plasmodium* sp. | EF011180 | R | *Haemamoeba* | *Dendroica coronata* | USA | 891 | 26 |
| Bird | *Plasmodium* sp. | EF011194 | R | *Haemamoeba* | *Emberiza hortulana* | Israel | P121 | 26 |
| Bird | *Plasmodium* sp. | EF011176 | R | *Haemamoeba* | *Spizella passerina* | USA, Vermont | 594 | 26 |
| Bird | *Plasmodium* sp. | EF011179 | R | *Haemamoeba* | *Zonotrichia albicollis* | USA | 805 | 26 |
| Bird | *Plasmodium* sp. | EU254539 | R | | *Accipiter striatus* | USA, Vermont | 1393 | 27 |
| Bird | *Plasmodium* sp. | AY099041 | R | | *Acrocephalus arundinaceus* | Kenya | GRW4 | 39 |
| Bird | *Plasmodium* sp. | DQ659562 | R | | *Acrocephalus arundinaceus* | Sweden | P22 | 3 |
| Bird | *Plasmodium* sp. | AY099044 | R | | *Acrocephalus orientalis* | Japan | ORW1G278 | 39 |
| Bird | *Plasmodium* sp. | EU254547 | R | | *Larosterna inca* | USA, Washington | Inca | 27 |
| Bird | *Plasmodium* sp. | EU254540 | R | | *Luscinia svecica* | Israel | P166 | 27 |

Table S4 References for studies provided for the GenBank accession numbers in Table S3.

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| Table S3 Ref | Reference |
|---|---|
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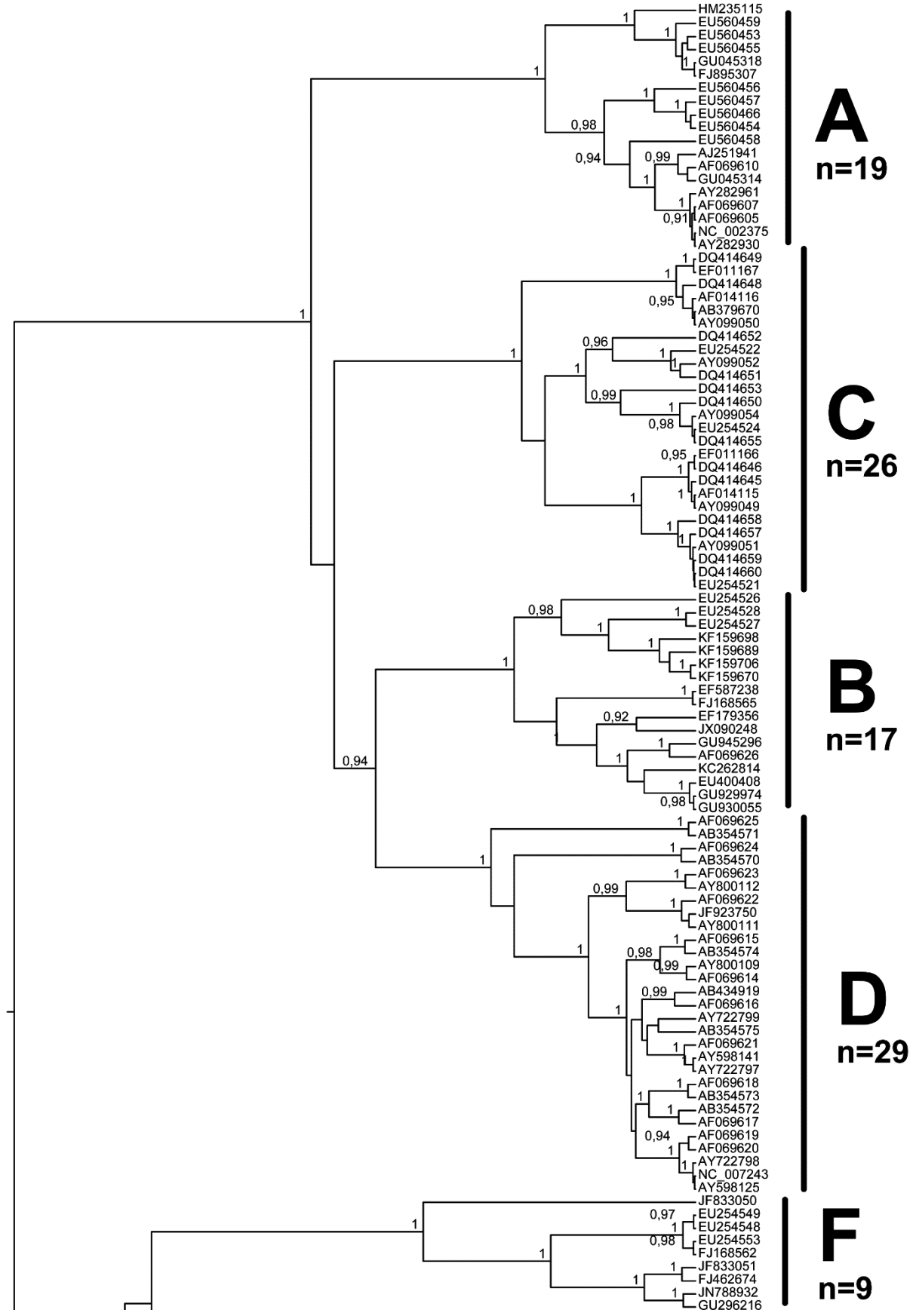
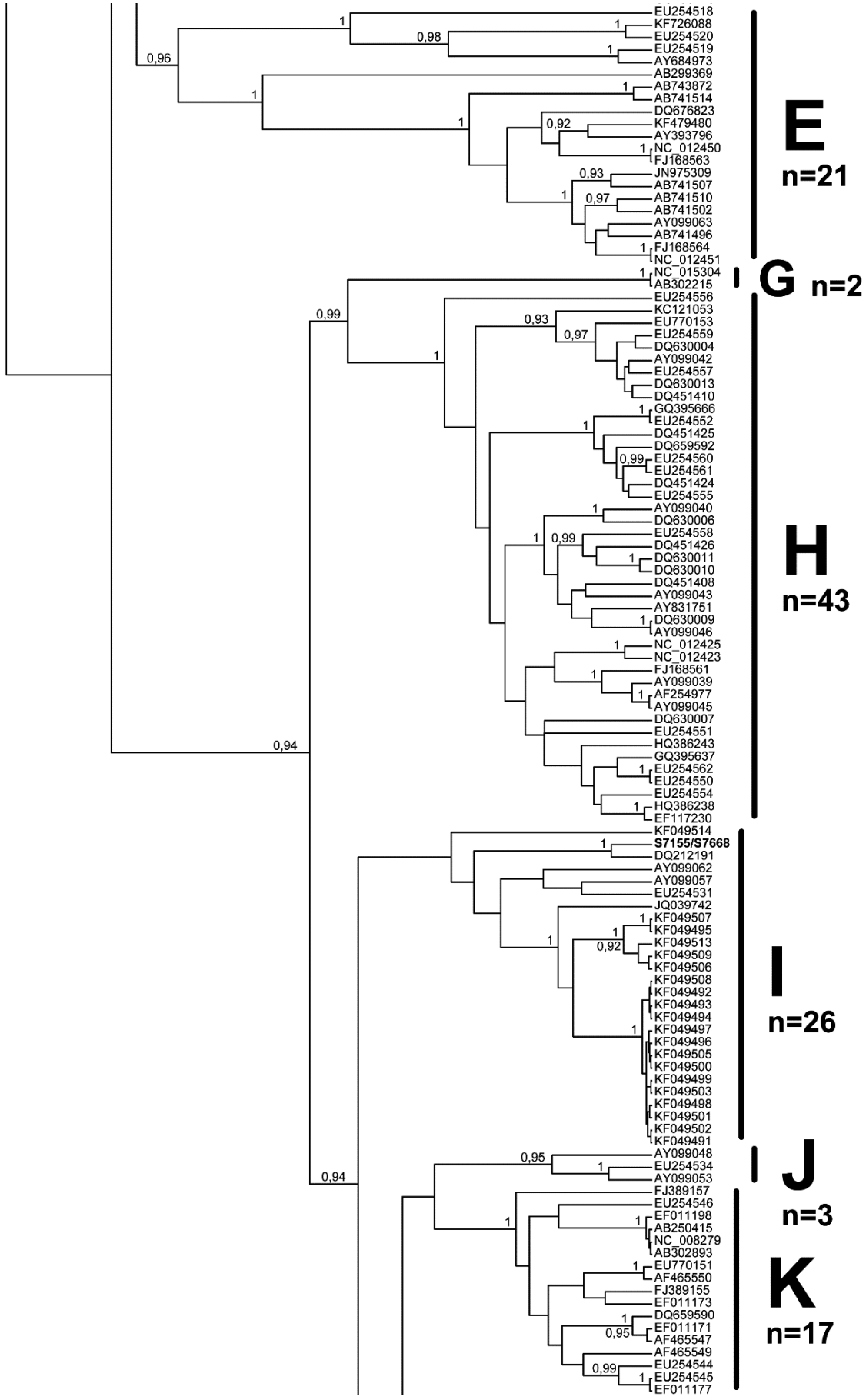
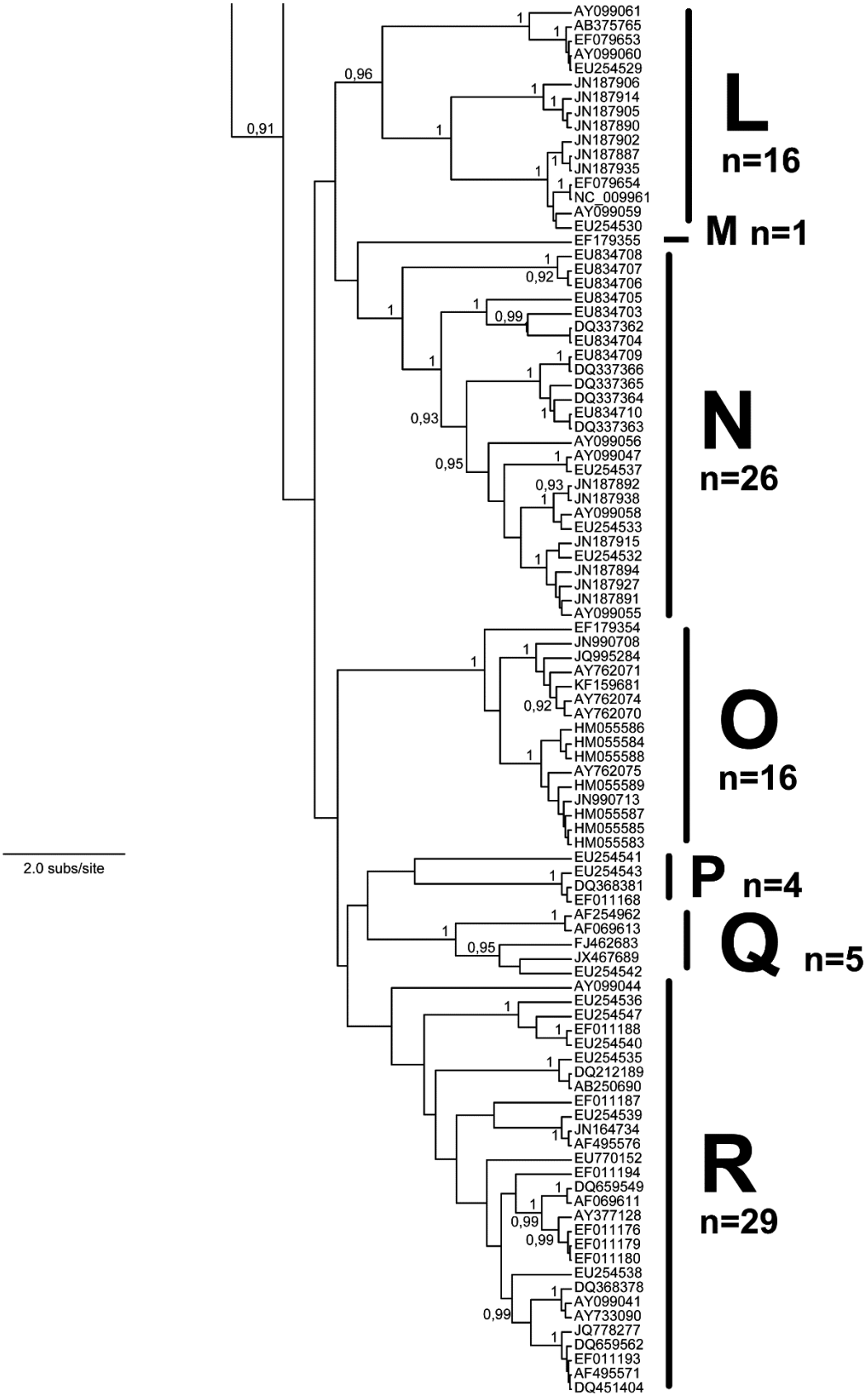



Figure S5 Tree derived from a Bayesian Inference (BI) analysis of the cyt *b* gene of Haemosporida using a Relaxed Uncorrelated Lognormal Clock prior.
Bayesian Posterior Probability values above .0.90 are given above relevant nodes.




(Figure S5 continued)




(Figure S5 continued)

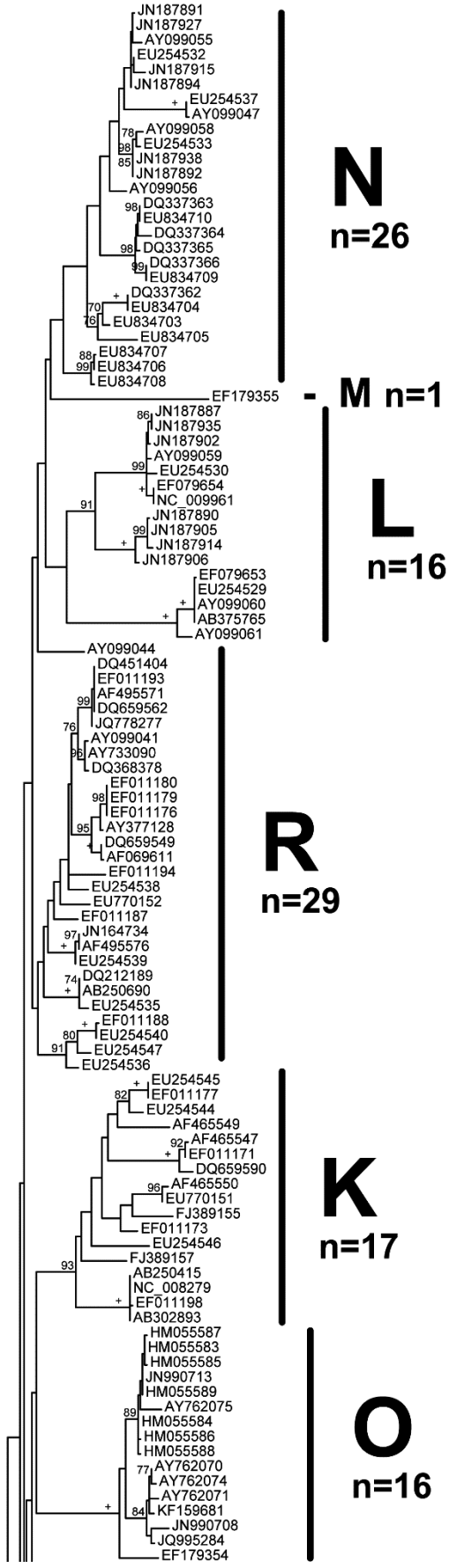
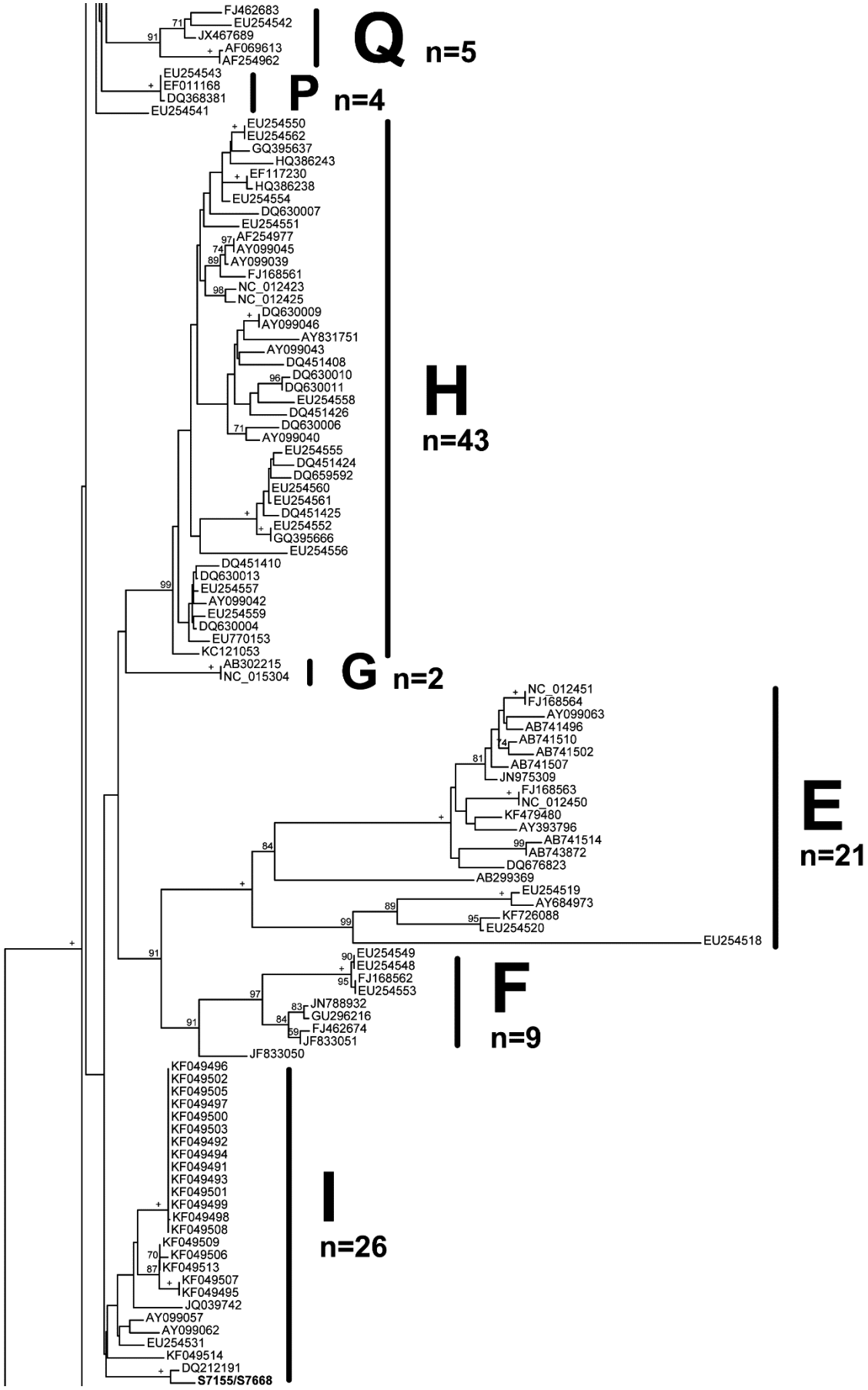
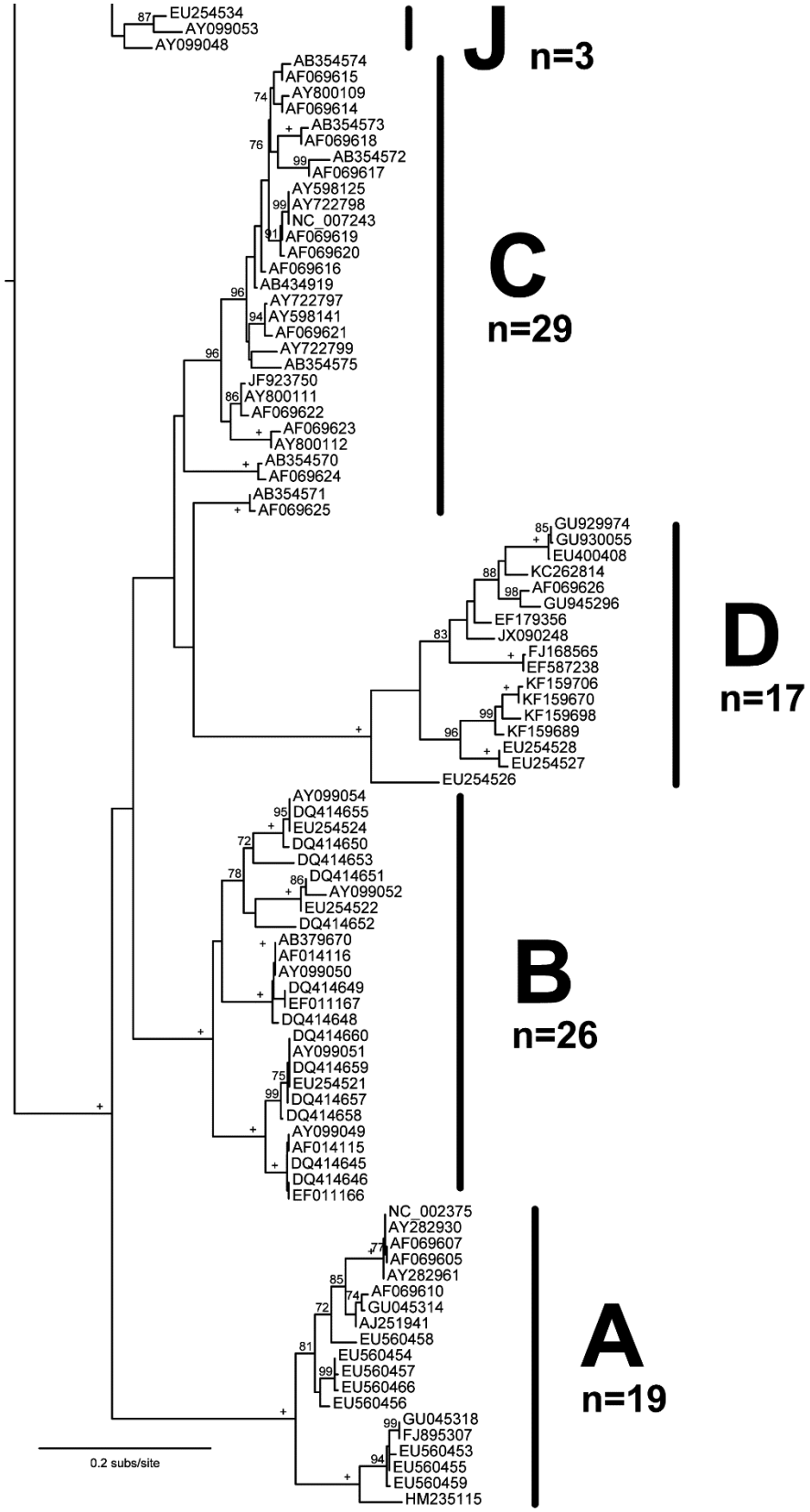



Figure S6 Tree derived from a Maximum Likelihood (ML) analysis of the cyt *b* gene of Haemosporida. Bootstrap values above 70 are given above relevant nodes. + indicates 100 support.




(Figure S6 continued)




(Figure S6 continued)

Table S5 Details for each sequence downloaded from GenBank for an overview of the hemogregarine diversity and phylogenetic relationships.

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| KJ740753 | *Hepatozoon* sp. | A | Reptilia, Testudines, Geoemydidae | *Mauremys leprosa* | Europe | Spain | KJ740753 |
| KJ740754 | *Hepatozoon* sp. | A | Reptilia, Testudines, Geoemydidae | *Mauremys leprosa* | Europe | Spain | KJ740754 |
| HQ224959 | *Haemogregarina balli* | B | Reptilia, Testudines, Chelydridae | *Chelydra serpentina serpentina* | N/A | N/A | HQ224959 |
| KF257928 | *Haemogregarina stepanowi* | B | Reptilia, Testudines, Emydidae | *Emys orbicularis* | Europe | Bulgaria | KF257929 |
| KF257929 | *Haemogregarina stepanowi* | B | Reptilia, Testudines, Geoemydidae | *Mauremys leprosa* | Africa | Algeria | KF257929 |
| KF257926 | *Haemogregarina stepanowi* | B | Reptilia, Testudines, Geoemydidae | *Mauremys caspica* | Asia | Iran | KF257929 |
| KF257927 | *Haemogregarina stepanowi* | B | Reptilia, Testudines, Geoemydidae | *Mauremys rivulata* | Asia | Syria | KF257929 |
| KF992697 | *Haemogregarina stepanowi* | B | Reptilia, Testudines, Geoemydidae | *Mauremys caspica* | Europe | Turkey | KF257929 |
| HQ224959 | *Haemogregarina* sp. | B | Reptilia, Testudines, Pelomedusidae | *Pelusios marani* | Africa | Gabon | HQ224959 |
| HQ224959 | *Haemogregarina* sp. | B | Reptilia, Testudines, Pelomedusidae | *Pelusios subniger* | Africa | Mozambique | HQ224959 |
| KF257923 | *Haemogregarina* sp. | B | Reptilia, Testudines, Pelomedusidae | *Pelusios williamsi* | Africa | Kenya | KF257925 |
| KC512766 | *Hemolivia* sp. | C | Arachnida, Ixodida, Ixodidae | *Hyalomma aegyptium* from *Testudo graeca* | Africa | Algeria | KC512766 |
| EU430236 | *Hepatozoon* sp. | C | Arachnida, Ixodida, Ixodidae | *Amblyomma fimbriatum* from *Liasis fuscus* | Australia | Australia | EU430231 |
| EU430231 | *Hepatozoon* sp. | C | Arachnida, Ixodida, Ixodidae | *Amblyomma fimbriatum* from *Varanus panoptes* | Australia | Australia | EU430231 |
| EU430232 | *Hepatozoon* sp. | C | Arachnida, Ixodida, Ixodidae | *Amblyomma fimbriatum* from *Varanus panoptes* | Australia | Australia | EU430231 |
| JQ080303 | *Hepatozoon* sp. | C | Insecta, Diptera, Culicidae | *Aedes taeniorhynchus* | South America | Galapagos Islands | JQ080303 |
| KF516510 | *Hepatozoon* sp. | C | Mammalia, Carnivora, Procyonidae | *Nasua nasua* | South America | Brazil | KF516510 |
| KF992711 | *Hemolivia mariae* | C | Reptilia, Lacertilia, Scincidae | *Egernia stokesii* | Australia | Australia | KF992711 |
| KF992712 | *Hemolivia mariae* | C | Reptilia, Lacertilia, Scincidae | *Egernia stokesii* | Australia | Australia | KF992711 |
| JN211118 | *Hemolivia mariae* | C | Reptilia, Lacertilia, Scincidae | *Tiliqua rugosa* | Australia | Australia | JN211118 |
| KF992713 | *Hemolivia* sp. | C | Reptilia, Testudines, Geoemydidae | *Rhinoclemmys pulcherrima manni* | South America | Nicaragua | KF992714 |
| KF992714 | *Hemolivia* sp. | C | Reptilia, Testudines, Geoemydidae | *Rhinoclemmys pulcherrima manni* | South America | Nicaragua | KF992714 |
| KF992700 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Iraq | KC512766 |
| KF992701 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Syria | KC512766 |
| KF992702 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Syria | KC512766 |
| KF992703 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Syria | KC512766 |
| KF992704 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Syria | KC512766 |
| KF992705 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Syria | KC512766 |
| KF992706 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Syria | KC512766 |
| KF992707 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Syria | KC512766 |
| KF992708 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Syria | KC512766 |
| KF992709 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Asia | Syria | KC512766 |
| KF992698 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo graeca* | Europe | Turkey | KC512766 |
| KF992699 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo marginata* | Europe | Greece | KC512766 |
| KF992710 | *Hemolivia mauritanica* | C | Reptilia, Testudines, Testudinidae | *Testudo marginata* | Europe | Greece | KC512766 |
| GU344682 | *Hepatozoon* sp. | D | Aves, Cathartiformes, Cathartidae | *Cathartes aura* | North America | USA | GU344682 |
| KF022102 | *Hepatozoon* sp. | D | Aves, Procellariiformes, Hydrobatidae | *Oceanodroma melania* | South America | Mexico | KF022102 |
| JQ080302 | *Hepatozoon* sp. | E | Insecta, Diptera, Culicidae | *Aedes taeniorhynchus* | South America | Galapagos Islands | JQ080302 |
| JQ080304 | *Hepatozoon* sp. | E | Insecta, Diptera, Culicidae | *Aedes taeniorhynchus* | South America | Galapagos Islands | JQ080304 |
| JF491225 | *Hepatozoon* sp. | E | Mammalia, (Marsupalia), Didelphimorphia | *Didelphis virginiana* | North America | USA | JF491225 |
| KM234618 | *Hepatozoon* sp. | E | Reptilia, Lacertilia, Gekkota | *Hemidactylus mabouia* | South America | Brazil | KM234618 |
| KM234614 | *Hepatozoon* sp. | E | Reptilia, Lacertilia, Gekkota | *Phyllopezus periosus* | South America | Brazil | KM234614 |
| KM234613 | *Hepatozoon* sp. | E | Reptilia, Lacertilia, Gekkota | *Phyllopezus pollicaris* | South America | Brazil | KM234613 |
| KJ408513 | *Hepatozoon* sp. | E | Reptilia, Serpentes, Colubridae | *Dolichophis caspius* | Europe | Turkey | KJ408520 |
| KJ408516 | *Hepatozoon* sp. | E | Reptilia, Serpentes, Colubridae | *Hierophis viridiflavus* | Europe | Italy | KJ408520 |
| KJ408520 | *Hepatozoon* sp. | E | Reptilia, Serpentes, Colubridae | *Hierophis viridiflavus* | Europe | Italy | KJ408520 |
| JN181157 | *Hepatozoon sipedon* | E | Reptilia, Serpentes, Colubridae | *Nerodia sipedon sipedon* | N/A | N/A | JN181157 |
| KC342525 | *Hepatozoon cevapii* | E | Reptilia, Serpentes, Viperidae | *Crotalus durissus terrificus* | South America | Brazil | KC342526 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| KC342523 | *Hepatozoon massardii* | E | Reptilia, Serpentes, Viperidae | *Crotalus durissus terrificus* | South America | Brazil | KC342523 |
| KC342526 | *Hepatozoon massardii* | E | Reptilia, Serpentes, Viperidae | *Crotalus durissus terrificus* | South America | Brazil | KC342526 |
| KJ702453 | *Hepatozoon fitzsimonsi* | E | Reptilia, Testudines, Testudinidae | *Chersina angulata* | Africa | South Africa | KJ702453 |
| AF176837 | *Hepatozoon catesbianae* | F | Amphibia, Anura | *Amphibian* | N/A | N/A | AF176837 |
| Bf1[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf10[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf11[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf12[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf13[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf14[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf16[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf17[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf18[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf19[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf2[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf20[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf24[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf25[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf27[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf28[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf3[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf30[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf31[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf36[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf37[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf39[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf4[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf5[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| Bf6[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf7[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_1 |
| Bf8[17] | *Hepatozoon* sp. | F | Amphibia, Anura, Bufonidae | *Bufo arabicus* | Asia | Oman | hap_2 |
| KJ599676 | *Hepatozoon theileri* | F | Amphibia, Anura, Pyxicephalidae | *Amietia quecketti* | Africa | South Africa | KJ599676 |
| KF733812 | *Hepatozoon* sp. | F | Amphibia, Anura, Ranidae | *Pelophylax perezi* | Europe | Portugal | KF733812 |
| HQ224954 | *Hepatozoon cf. catesbianae* | F | Amphibia, Anura, Ranidae | *Rana catesbeiana* | N/A | N/A | HQ224954 |
| HQ224962 | *Hepatozoon cf. clamatae* | F | Amphibia, Anura, Ranidae | *Rana clamitans* | N/A | N/A | AF176837 |
| HQ224963 | *Hepatozoon cf. clamatae* | F | Amphibia, Anura, Ranidae | *Rana clamitans* | N/A | N/A | HQ224963 |
| HQ224960 | *Hepatozoon* sp. | F | Amphibia, Anura, Ranidae | *Rana esculenta* | N/A | N/A | HQ224960 |
| AF130361 | *Hepatozoon catesbianae* | F | Amphibia, Anura, Ranidae | *Rana catesbianae* | North America | USA | AF130361 |
| JX987775 | *Hepatozoon* sp. | G | Amphibia, Anura, Leptodactylidae | *Leptodactylus* sp. | South America | Brazil | JX987775 |
| KF246565 | *Hepatozoon seychellensis* | G | Amphibia, Gymnophiona, Indotyphlidae | *Grandisonia alternans* | Africa | Seychelles | KF246565 |
| KF246566 | *Hepatozoon seychellensis* | G | Amphibia, Gymnophiona, Indotyphlidae | *Grandisonia alternans* | Africa | Seychelles | KF246565 |
| JQ670908 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Aponomma varanense* from *Ophiophagus hannah* | Asia | Thailand | HM585203 |
| KF301647 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Aponomma varanense* from *Ophiophagus hannah* | Asia | Thailand | HM585203 |
| KF301648 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Aponomma varanense* from *Ophiophagus hannah* | Asia | Thailand | HM585203 |
| KF301649 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Aponomma varanense* from *Ophiophagus hannah* | Asia | Thailand | HM585203 |
| KF301650 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Aponomma varanense* from *Ophiophagus hannah* | Asia | Thailand | HM585203 |
| JQ670909 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Aponomma varanensis* from *Ptyas korros* | Asia | Thailand | JQ670910 |
| JQ670910 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Aponomma varanensis* from *Xenochrophis piscator* | Asia | Thailand | JQ670910 |

[17] Sequences added from section 5.2.

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| EU430234 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Amblyomma fimbriatum* from *Varanus panoptes* | Australia | Australia | EU430234 |
| EU430235 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Amblyomma fimbriatum* from *Varanus panoptes* | Australia | Australia | EU430235 |
| EU430233 | *Hepatozoon* sp. | G | Arachnida, Ixodida, Ixodidae | *Amblyomma moreliae* from *Liasis fuscus* | Australia | Australia | HM585203 |
| KF524358 | *Hepatozoon* sp. | G | Arachnida, Mesostigmata, Macronyssidae | *Ophiophagus hannah* | Asia | Thailand | KJ499535 |
| KJ634066 | *Hepatozoon erhardovae* | G | Insecta, Siphonaptera, Ctenophthalmidae | *Ctenophthalmus agyrtes* from *Apodemus agrarius* | Europe | Hungary | KF418366 |
| KJ499479 | *Hepatozoon* sp. | G | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Senegal | EF157822 |
| FJ497023 | *Hepatozoon* sp. | G | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | HM212627 |
| FJ497024 | *Hepatozoon* sp. | G | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | HM212627 |
| HM212627 | *Hepatozoon* sp. | G | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Croatia | HM212627 |
| KC127680 | *Hepatozoon* sp. | G | Mammalia, Carnivora, Canidae | *Cerdocyon thous* | South America | Brazil | KC127680 |
| KC848055 | *Hepatozoon* sp. | G | Mammalia, Chiroptera | *Hipposideros cervinus* | Asia | Malaysia | KC848055 |
| KC848056 | *Hepatozoon* sp. | G | Mammalia, Chiroptera | *Hipposideros cervinus* | Asia | Malaysia | KC848056 |
| KC848057 | *Hepatozoon* sp. | G | Mammalia, Chiroptera | *Hipposideros cervinus* | Asia | Malaysia | KJ499535 |
| KJ649313 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Clethrionomys glareolus* | Europe | Slovakia | JX644997 |
| AY600625 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Clethrionomys glareolus* | Europe | Spain | HM212627 |
| AY600626 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Clethrionomys glareolus* | Europe | Spain | KF418366 |
| JQ886023 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Myodes glareolus* | Europe | Germany | KF418366 |
| JX644996 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Myodes glareolus* | Europe | Hungary | JX644996 |
| JX644997 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Myodes glareolus* | Europe | Hungary | JX644997 |
| JX644998 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Myodes glareolus* | Europe | Hungary | JX644998 |
| KF418366 | *Hepatozoon erhardovae* | G | Mammalia, Rodentia, Cricetidae | *Myodes glareolus* | Europe | Poland | KF418366 |
| KF418367 | *Hepatozoon erhardovae* | G | Mammalia, Rodentia, Cricetidae | *Myodes glareolus* | Europe | Poland | KF418367 |
| JF491238 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Neotoma fuscipes* | North America | USA | JQ746622 |
| JF491239 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Neotoma fuscipes* | North America | USA | KJ499535 |
| JF491236 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Neotoma micropus* | North America | USA | JF491237 |
| JF491237 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Neotoma micropus* | North America | USA | JF491237 |
| EF620027 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Peromyscus leucopus* | North America | USA | EF620027 |
| JF491235 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Peromyscus leucopus* | North America | USA | JF491235 |
| EF620026 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Sigmodon sp.* | North America | USA | EF620026 |
| FJ719815 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Abrothrix olivaceus* | South America | Chile | FJ719815 |
| FJ719817 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Abrothrix olivaceus* | South America | Chile | FJ719818 |
| FJ719818 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Abrothrix olivaceus* | South America | Chile | FJ719818 |
| FJ719816 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Abrothrix sanborni* | South America | Chile | FJ719815 |
| FJ719819 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Cricetidae | *Abrothrix sanborni* | South America | Chile | FJ719819 |
| KJ499516 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499535 |
| KJ499522 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499522 |
| KJ499523 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499522 |
| KJ499524 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499522 |
| KJ499528 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499522 |
| KJ499530 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499522 |
| KJ499531 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499522 |
| KJ499533 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499522 |
| KJ499534 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499522 |
| KJ499535 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499535 |
| KJ499536 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499535 |
| KJ499537 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Mauritania | KJ499535 |
| KJ499520 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Morocco | KJ499535 |
| KJ499517 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Western Sahara | KJ499535 |
| KJ499518 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Western Sahara | KJ499535 |
| KJ499519 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Western Sahara | KJ499535 |
| KJ499532 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus jaculus* | Africa | Western Sahara | KJ499522 |
| KJ499521 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus orientalis* | Africa | Morocco | KJ499522 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| KJ499525 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus orientalis* | Africa | Morocco | KJ499522 |
| KJ499526 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus orientalis* | Africa | Morocco | KJ499522 |
| KJ499527 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus orientalis* | Africa | Morocco | KJ499522 |
| KJ499529 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Dipodidae | *Jaculus orientalis* | Africa | Morocco | KJ499522 |
| AB181504 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Muridae | *Bandicota indica* | Asia | Thailand | AB181504 |
| EF222259 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Sciuridae | *Sciurus vulgaris* | Europe | Spain | EF222259 |
| JF491240 | *Hepatozoon* sp. | G | Mammalia, Rodentia, Sciuridae | *Sciurus carolinensis* | North America | USA | KC866370 |
| KJ413112 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413115 |
| KJ413114 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413117 |
| KJ413115 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413115 |
| KJ413116 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413115 |
| KJ413117 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413117 |
| KJ413118 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413118 |
| KJ413119 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413119 |
| KJ413120 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413118 |
| KJ413121 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413121 |
| KJ413122 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413118 |
| KJ413123 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413123 |
| KJ413126 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413126 |
| KJ413128 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413128 |
| KJ413129 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413129 |
| KJ413134 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Alligatoridae | *Caiman crocodilus yacare* | South America | Brazil | KJ413134 |
| KJ425235 | *Hepatozoon* sp. | G | Reptilia, Crocodilia, Crocodylidae | *Crocodile* | South America | Brazil | KJ425235 |
| KM234649 | *Hepatozoon domerguei* | G | Reptilia, Lacertilia, Chamaeleonidae | *Furcifer* sp. | Africa | Madagascar | KM234649 |
| HQ292771 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Mabuya wrightii* | Africa | Seychelles | HQ292772 |
| HQ292772 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Mabuya wrightii* | Africa | Seychelles | HQ292772 |
| HQ734790 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus oudrii* | Africa | Algeria | KC696567 |
| HQ734808 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus oudrii* | Africa | Morocco | KC696567 |
| HQ734789 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Quedenfeldtia moerens* | Africa | Morocco | HQ734789 |
| HQ734809 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Quedenfeldtia moerens* | Africa | Morocco | KC696567 |
| DB14212 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola boehmei* | Africa | Morocco | DB2155 |
| DB14214 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola boehmei* | Africa | Morocco | DB2155 |
| DB363 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola deserti* | Africa | Algeria | DB2170 |
| DB14179 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola deserti* | Africa | Morocco | DB2545 |
| DB14185 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola deserti* | Africa | Morocco | DB2170 |
| DB9006 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola deserti* | Africa | Morocco | DB2545 |
| DB9016 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola deserti* | Africa | Morocco | DB2170 |
| DB14219 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola ephippiata* | Africa | Morocco | DB2155 |
| DB14223 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola ephippiata* | Africa | Morocco | DB2170 |
| DB14224 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola ephippiata* | Africa | Morocco | DB2170 |
| HQ734787 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Algeria | HQ734789 |
| HQ734788 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Algeria | HQ734788 |
| DB2155 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Libya | DB2155 |
| DB2170 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Libya | DB2170 |
| DB1411 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB2545 |
| DB160 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB2545 |
| DB201 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB2545 |
| DB2545 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB2545 |
| DB373 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB2545 |
| DB795 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB2545 |
| DB978 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB2545 |
| DB999 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB2545 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| HQ734806 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | KC696567 |
| DB14207 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola* sp. | Africa | Morocco | DB2545 |
| DB3126 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Tarentola* sp. | Africa | Morocco | DB2170 |
| S6045[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S6050[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S6078[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S6082[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7168[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7182[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7189[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7361[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7429[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7464[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7474[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7582[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7750[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7782[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7805[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7835[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7836[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7850[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_3 |
| S7101[17] | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus atairensis* | Asia | Oman | hap_12 |
| S7605[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus festivus* | Asia | Oman | hap_4 |
| S7170[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus hajarensis* | Asia | Oman | hap_3 |
| S7587[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus hajarensis* | Asia | Oman | hap_3 |
| S7134[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus lemurinus* | Asia | Oman | hap_4 |
| S6080[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus luqueorum* | Asia | Oman | hap_3 |
| S7155[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus luqueorum* | Asia | Oman | hap_3 |
| S7509[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Pristurus rupestris* | Asia | Oman | hap_3 |
| S7542[17] | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Pristurus rupestris* | Asia | Oman | hap_5 |
| S7564[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Pristurus rupestris* | Asia | Oman | hap_3 |
| S7590[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Pristurus rupestris* | Asia | Oman | hap_3 |
| S7055[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus hasselquistii* | Asia | Oman | hap_3 |
| S7093[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus hasselquistii* | Asia | Oman | hap_3 |
| S7123[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus hasselquistii* | Asia | Oman | hap_3 |
| S7164[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus hasselquistii* | Asia | Oman | hap_3 |
| S7357[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus hasselquistii* | Asia | Oman | hap_3 |
| S7611[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus hasselquistii* | Asia | Oman | hap_3 |
| S7668[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus hasselquistii* | Asia | Oman | hap_3 |
| S7676[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus hasselquistii* | Asia | Oman | hap_3 |
| S7776[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Lacertilia, Gekkota | *Ptyodactylus hasselquistii* | Asia | Oman | hap_3 |
| KM234615 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus mabouia* | South America | Brazil | KM234612 |
| KM234616 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus mabouia* | South America | Brazil | KJ499535 |
| KM234617 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Hemidactylus mabouia* | South America | Brazil | KM234617 |
| KM234612 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Gekkota | *Phyllopezus pollicaris* | South America | Brazil | KM234612 |
| KM234650 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Lacertidae | *Oplurus* sp. | Africa | Madagascar | KM234650 |
| HQ734807 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Lacertidae | *Timon tangitanus* | Africa | Morocco | HQ734807 |
| JX531921 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | HQ734807 |
| HM585204 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator komaini* | Asia | Thailand | HM585204 |
| HM585205 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator komaini* | Asia | Thailand | HM585205 |
| HQ317910 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator komaini* | Asia | Thailand | HQ317910 |
| HM585203 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | HM585203 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| HM585206 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | HM585203 |
| HM585207 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | HM585203 |
| HM585208 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | HM585203 |
| HM585209 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | HM585203 |
| HM585210 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | EF620027 |
| HM585211 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | HM585203 |
| HM585212 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | HM585212 |
| HQ317909 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | HM585203 |
| HQ317911 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus salvator salvator* | Asia | Thailand | HM585203 |
| AY252106 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus panoptes* | Australia | Australia | AY252105 |
| AY252107 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus panoptes* | Australia | Australia | AY252105 |
| AY252108 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus scalaris* | Australia | Australia | AY252108 |
| AY252109 | *Hepatozoon* sp. | G | Reptilia, Lacertilia, Varanidae | *Varanus scalaris* | Australia | Australia | AY252109 |
| KJ408511 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Cerastes cerastes* | Africa | Mauritania | KC800703 |
| KJ574012 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Cerastes cerastes cerastes* | Africa | Egypt | KJ574012 |
| KJ408512 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Crotaphopeltis hotamboeia* | Africa | Niger | KC696567 |
| HQ292773 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Lycognathophis seychellensis* | Africa | Seychelles | HQ292773 |
| HQ292774 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Lycognathophis seychellensis* | Africa | Seychelles | HQ292773 |
| HQ292775 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Lycognathophis seychellensis* | Africa | Seychelles | HQ292773 |
| KJ408521 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Macroprotodon cucullatus* | Africa | Morocco | KJ408528 |
| KJ408522 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Macroprotodon cucullatus* | Africa | Morocco | KJ408514 |
| KJ408523 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Macroprotodon cucullatus* | Africa | Morocco | KC800703 |
| KM234646 | *Hepatozoon domerguei* | G | Reptilia, Serpentes, Colubridae | *Madagascarophis colubrinus* | Africa | Madagascar | KM234649 |
| KM234647 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Madagascarophis colubrinus* | Africa | Madagascar | KM234647 |
| JX244266 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Malpolon monspessulanus* | Africa | Morocco | KJ499535 |
| KC800704 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Mehelya capensis* | Africa | Swaziland | KC800703 |
| KC800702 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Philothamnus semivariegatus* | Africa | Swaziland | KC800702 |
| KC696568 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Psammophis elegans* | Africa | Mali | KC696568 |
| KC696564 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Psammophis schokari* | Africa | Algeria | KC696564 |
| KC696569 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Psammophis schokari* | Africa | Western Sahara | KJ499535 |
| KC696567 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Psammophis sibilans* | Africa | Burjina Faso | KC696567 |
| KC696566 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Psammophis sibilans* | Africa | Niger | KJ499535 |
| KJ408528 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Spalerosophis dolichospilus* | Africa | Morocco | KJ408528 |
| KJ408529 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Spalerosophis dolichospilus* | Africa | Morocco | KJ408514 |
| KF524356 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Boiga dendrophila melanota* | Asia | Thailand | KJ499535 |
| CN2698[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Cerastes gasparetti* | Asia | Oman | hap_6 |
| CN3768[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Cerastes gasparetti* | Asia | Oman | hap_7 |
| CN3856[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Cerastes gasparetti* | Asia | Oman | hap_6 |
| CN3923[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Cerastes gasparetti* | Asia | Oman | hap_hetero |
| CN7622[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Cerastes gasparetti* | Asia | Oman | hap_6 |
| CN3459[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Lytorhynchus diadema* | Asia | Oman | hap_6 |
| CN3851[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Lytorhynchus diadema* | Asia | Oman | hap_6 |
| CN4093[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Lytorhynchus diadema* | Asia | Oman | hap_hetero |
| CN8365[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Psammophis schokari* | Asia | Oman | hap_6 |
| JQ746622 | *Hepatozoon garnhami* | G | Reptilia, Serpentes, Colubridae | *Psammophis schokari* | Asia | Saudi Arabia | JQ746622 |
| CN205[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Pseudocerastes persicus* | Asia | Oman | hap_6 |
| CN3900[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Telescopus dhara* | Asia | Oman | hap_6 |
| AY252103 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Boiga* | Australia | Australia | AY252103 |
| AF297085 | *Hepatozoon boigae* | G | Reptilia, Serpentes, Colubridae | *Boiga fusca* | Australia | Australia | AF297085 |
| AY252104 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Liasis fuscus* | Australia | Australia | AY252105 |
| AY252105 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Liasis fuscus* | Australia | Australia | AY252105 |
| AY252110 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Stegonotus cucullatus* | Australia | Australia | AY252110 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| AY252111 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Stegonotus cucullatus* | Australia | Australia | AY252111 |
| JX244267 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Hemorrhois hippocrepis* | Europe | Spain | KC696567 |
| KJ408514 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Hemorrhois nummifer* | Europe | Turkey | KJ408514 |
| KJ408515 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Hierophis viridiflavus* | Europe | Italy | HQ734807 |
| KJ408517 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Hierophis viridiflavus* | Europe | Italy | HQ734807 |
| KJ408518 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Hierophis viridiflavus* | Europe | Italy | HQ734807 |
| KJ408519 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Hierophis viridiflavus* | Europe | Italy | HQ734807 |
| KJ408526 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Natrix tessellata* | Europe | Turkey | KJ408514 |
| KJ408531 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Zamenis lineatus* | Europe | Italy | KJ408514 |
| KJ408532 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Zamenis situla* | Europe | Italy | KJ408514 |
| JF491242 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Boa constrictor imperator* | North America | USA | JF491242 |
| KC800705 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Coluber constrictor* | North America | USA | KC800703 |
| KC800706 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Corallus caninus* | South America | South America | KC800703 |
| KF939620 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Elaphe carinata* | South America | China | KJ499535 |
| KF939621 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Elaphe carinata* | South America | China | KJ499535 |
| KF939622 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Elaphe carinata* | South America | China | KF939622 |
| KF939623 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Elaphe carinata* | South America | China | KJ499535 |
| KF939624 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Elaphe carinata* | South America | China | KJ499535 |
| KF939625 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Elaphe carinata* | South America | China | KJ499535 |
| KF939626 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Elaphe carinata* | South America | China | KJ499535 |
| KF939627 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Elaphe carinata* | South America | China | KJ499535 |
| KF939628 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Colubridae | *Elaphe carinata* | South America | China | KJ499535 |
| KC866369 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Elapidae | *Dendroaspis jamesoni camerun* | Africa | Uganda | KC866369 |
| KC866370 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Elapidae | *Dendroaspis jamesoni jamesoni* | Africa | Cameroon | KC866370 |
| KC866368 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Elapidae | *Dendroaspis polylepis* | Africa | Swaziland | HQ292773 |
| KJ408525 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Elapidae | *Naja haje* | Africa | Morocco | KJ408525 |
| KF524357 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Elapidae | *Naja kaouthia* | Asia | Thailand | JQ670910 |
| KM234648 | *Hepatozoon domerguei* | G | Reptilia, Serpentes, Lamprophiidae | *Ithycyphus oursi* | Africa | Madagascar | KM234649 |
| EF157822 | *Hepatozoon ayorgbor* | G | Reptilia, Serpentes, Pythonidae | *Python regius* | Africa | Ghana | EF157822 |
| KC800703 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Pythonidae | *Python sebae natalensis* | Africa | Swaziland | KC800703 |
| KC866367 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Pythonidae | *Morelia viridis* | Asia | Indonesia | KC866367 |
| KF524360 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Pythonidae | *Python molurus* | Asia | Thailand | HM585203 |
| KF524361 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Pythonidae | *Python molurus* | Asia | Thailand | HM585203 |
| KF524362 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Pythonidae | *Python molurus* | Asia | Thailand | HM585203 |
| KF524363 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Pythonidae | *Python molurus bivittatus* | Asia | Thailand | HM585203 |
| KF524359 | *Hepatozoon* sp. | G | Reptilia, Serpentes, Pythonidae | *Python reticulatus* | Asia | Thailand | HM585203 |
| CN2586[17] | *Hepatozoon omanensis* n. sp. | G | Reptilia, Serpentes, Viperidae | *Echis omanensis* | Asia | Oman | hap_3 |
| CN3266[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Viperidae | *Echis omanensis* | Asia | Oman | hap_6 |
| CN3399[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Viperidae | *Echis omanensis* | Asia | Oman | hap_6 |
| CN365[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Viperidae | *Echis omanensis* | Asia | Oman | hap_hetero |
| CN3870[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Viperidae | *Echis omanensis* | Asia | Oman | hap_6 |
| CN729[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Viperidae | *Echis omanensis* | Asia | Oman | hap_hetero |
| CN730[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Viperidae | *Echis omanensis* | Asia | Oman | hap_6 |
| CN8350[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Viperidae | *Echis omanensis* | Asia | Oman | hap_6 |
| CN4375[17] | *Hepatozoon* sp. | G | Reptilia, Serpentes, Viperidae | *Echis* sp. | Asia | Oman | hap_6 |
| KC342522 | *Hepatozoon cuestensis* | G | Reptilia, Serpentes, Viperidae | *Crotalus durissus terrificus* | South America | Brazil | KC342522 |
| KC342524 | *Hepatozoon cuestensis* | G | Reptilia, Serpentes, Viperidae | *Crotalus durissus terrificus* | South America | Brazil | KC342524 |
| KC342527 | *Hepatozoon cuestensis* | G | Reptilia, Serpentes, Viperidae | *Crotalus durissus terrificus* | South America | Brazil | KC342527 |
| KC342528 | *Hepatozoon cuestensis* | G | Reptilia, Serpentes, Viperidae | *Crotalus durissus terrificus* | South America | Brazil | KC342528 |
| EU430237 | *Hepatozoon* sp. | H | Arachnida, Ixodida, Ixodidae | *Ixodes tasmani* from *Sarcophilus harrisii* | Australia | Australia | EU430238 |
| EU430238 | *Hepatozoon* sp. | H | Arachnida, Ixodida, Ixodidae | *Ixodes tasmani* from *Sarcophilus harrisii* | Australia | Australia | EU430238 |
| EU430239 | *Hepatozoon* sp. | H | Arachnida, Ixodida, Ixodidae | *Ixodes tasmani* from *Sarcophilus harrisii* | Australia | Australia | EU430238 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| EU430240 | *Hepatozoon* sp. | H | Arachnida, Ixodida, Ixodidae | *Ixodes tasmani* from *Sarcophilus harrisii* | Australia | Australia | EU430238 |
| EF152218 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| EF152219 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| EF152220 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| EF152221 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| EF152222 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152224 |
| EF152223 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152224 |
| EF152224 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152224 |
| EF152225 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| EF152226 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| EF152227 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| EF152228 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| EF152229 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| EF152230 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Peramelemorphia | *Isoodon obesulus* | Australia | Australia | EF152221 |
| FJ719813 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Microbiotheria | *Dromiciops gliroides* | South America | Chile | FJ719813 |
| FJ719814 | *Hepatozoon* sp. | H | Mammalia, (Marsupialia), Microbiotheria | *Dromiciops gliroides* | South America | Chile | FJ719813 |
| S6061[17] | unidentified Haemogregarinidae | I | Reptilia, Lacertilia, Gekkota | *Hemidactylus hajarensis* | Asia | Oman | hap_10 |
| S7154[17] | unidentified Haemogregarinidae | I | Reptilia, Lacertilia, Gekkota | *Hemidactylus hajarensis* | Asia | Oman | hap_13 |
| S7336[17] | unidentified Haemogregarinidae | I | Reptilia, Lacertilia, Gekkota | *Hemidactylus hajarensis* | Asia | Oman | hap_9 |
| DB11024 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola boehmei* | Africa | Morocco | DB14229 |
| DB14229 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola ephippiata* | Africa | Morocco | DB14229 |
| DB469 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Algeria | DB469 |
| DB11019 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB11019 |
| DB14245 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB14253 |
| DB14248 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB14248 |
| DB14249 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB14248 |
| DB14251 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB14248 |
| DB14253 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB14253 |
| DB2563 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB2563 |
| DB9076 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola mauritanica* | Africa | Morocco | DB14253 |
| DB14204 | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Tarentola* sp. | Africa | Morocco | DB14229 |
| S7077[17] | unidentified Haemogregarinidae | J | Reptilia, Lacertilia, Gekkota | *Asaccus platyrhynchus* | Asia | Oman | hap_11 |
| KJ461941 | *Karyolysus* sp. | K | Arachnida, Ixodida, Ixodidae | *Ixodes ricinus* feeding on *Lacerta viridis* | Europe | Hungary | KJ461941 |
| KJ461944 | *Karyolysus* sp. | K | Arachnida, Mesostigmata, Macronyssidae | *Ophionyssus sp.* from *Lacerta viridis* | Europe | Hungary | KJ461944 |
| KJ461945 | *Karyolysus* sp. | K | Arachnida, Mesostigmata, Macronyssidae | *Ophionyssus sp.* from *Zootoca vivipara* | Europe | Poland | KJ461941 |
| KF270651 | *Hepatozoon* sp. | K | Mammalia, Carnivora, Canidae | *wild Dog* | Africa | Zambia | KF270651 |
| KF270664 | *Hepatozoon* sp. | K | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270664 |
| DB1366 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Gekkota | *Tarentola angustimentalis* | Europe | Spain | DB1366 |
| HQ734798 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Atlantolacerta andreanskyi* | Africa | Morocco | HQ734800 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| HQ734792 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | HQ734791 |
| HQ734793 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | HQ734800 |
| HQ734794 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | HQ734795 |
| HQ734795 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | HQ734795 |
| HQ734803 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | HQ734800 |
| HQ734804 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | HQ734791 |
| KJ659858 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | JX531923 |
| KJ659859 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | JX531923 |
| KJ659860 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | JX531923 |
| KJ659861 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | JX531923 |
| KJ659862 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis vaucheri* | Africa | Morocco | JX531923 |
| HQ734791 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Scelarcis perspicillata* | Africa | Morocco | HQ734791 |
| HQ734799 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Timon tangitanus* | Africa | Morocco | HQ734799 |
| HQ734800 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Timon tangitanus* | Africa | Morocco | HQ734800 |
| HQ734801 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Timon tangitanus* | Africa | Morocco | HQ734799 |
| HQ734802 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Timon tangitanus* | Africa | Morocco | HQ734799 |
| JX531933 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531934 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531935 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531936 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531937 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531938 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531939 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531940 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531941 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531942 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531943 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531944 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531945 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531946 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531947 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531948 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531949 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531950 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531951 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531960 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531961 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531962 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531963 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531964 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531965 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531966 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531967 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531968 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| JX531969 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Algyroides marchi* | Europe | Spain | HQ734800 |
| EU908289 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Lacerta agilis* | Europe | Poland | EU908289 |
| KJ461940 | *Karyolysus lacazei* | K | Reptilia, Lacertilia, Lacertidae | *Lacerta agilis* | Europe | Poland | KJ461941 |
| KJ461942 | *Karyolysus lacazei* | K | Reptilia, Lacertilia, Lacertidae | *Lacerta trilineata* | Europe | Romania | KJ461941 |
| KJ461943 | *Karyolysus lacazei* | K | Reptilia, Lacertilia, Lacertidae | *Lacerta viridis* | Europe | Hungary | KJ461941 |
| JX531922 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | HQ734795 |
| JX531923 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| JX531924 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| JX531925 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| JX531926 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| JX531927 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531927 |
| JX531928 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531927 |
| JX531929 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531927 |
| JX531930 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531927 |
| JX531931 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531910 |
| JX531932 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531927 |
| JX531952 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531927 |
| JX531955 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | HQ734795 |
| JX531958 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189387 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189388 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189389 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189392 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189393 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189395 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189397 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189398 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189400 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189402 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189403 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189405 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189407 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189408 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189410 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189411 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189412 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189414 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189415 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189416 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189421 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189423 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | KJ189404 |
| KJ189424 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189427 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| KJ189428 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Portugal | JX531923 |
| JX531953 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Spain | JX531910 |
| JX531954 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Spain | JX531927 |
| JX531959 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis bocagei* | Europe | Spain | JX531910 |
| KJ189390 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | JX531923 |
| KJ189391 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | JX531923 |
| KJ189394 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | JX531923 |
| KJ189396 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | JX531923 |
| KJ189399 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | JX531923 |
| KJ189401 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189404 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189406 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189409 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189413 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | JX531923 |
| KJ189417 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189418 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189419 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| KJ189420 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189422 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189425 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | JX531923 |
| KJ189426 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | JX531923 |
| KJ189429 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189430 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189431 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189432 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| KJ189433 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Portugal | KJ189404 |
| JQ762308 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531923 |
| JQ762309 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | HQ734795 |
| JX531906 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531927 |
| JX531907 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531927 |
| JX531908 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531927 |
| JX531909 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531927 |
| JX531910 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531910 |
| JX531911 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531910 |
| JX531912 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531927 |
| JX531913 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531927 |
| JX531914 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531927 |
| JX531915 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531923 |
| JX531916 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531923 |
| JX531917 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531927 |
| JX531918 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531923 |
| JX531919 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531923 |
| JX531956 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531923 |
| JX531957 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | KJ189404 |
| JX531970 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531927 |
| JX531971 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531923 |
| JX531972 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis hispanica* | Europe | Spain | JX531923 |
| JQ762311 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis lilfordi* | Europe | Spain | JX531920 |
| JX531920 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis lilfordi* | Europe | Spain | JX531920 |
| JX531973 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Lacertidae | *Podarcis lilfordi* | Europe | Spain | JX531920 |
| KJ461939 | *Karyolysus latus* | K | Reptilia, Lacertilia, Lacertidae | *Podarcis muralis* | Europe | Slovakia | KJ461939 |
| KJ461946 | *Karyolysus* sp. | K | Reptilia, Lacertilia, Lacertidae | *Zootoca vivipara* | Europe | Poland | KJ461941 |
| HQ734805 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Scincidae | *Chalcides polylepis* | Africa | Morocco | HQ734796 |
| HQ734797 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Scincidae | *Eumeces algeriensis* | Africa | Morocco | HQ734796 |
| HQ734796 | *Hepatozoon* sp. | K | Reptilia, Lacertilia, Scincidae | *Eumeces algeriensis* | Africa | Morocco | HQ734796 |
| KJ408510 | *Hepatozoon* sp. | K | Reptilia, Serpentes, Colubridae | *Cerastes cerastes* | Africa | Morocco | KC696565 |
| JX244268 | *Hepatozoon* sp. | K | Reptilia, Serpentes, Colubridae | *Hemorrhois hippocrepis* | Africa | Morocco | HQ734796 |
| JX244269 | *Hepatozoon* sp. | K | Reptilia, Serpentes, Colubridae | *Hemorrhois hippocrepis* | Africa | Morocco | KC696565 |
| KJ408524 | *Hepatozoon* sp. | K | Reptilia, Serpentes, Colubridae | *Malpolon moilensis* | Africa | Morocco | KC696565 |
| KC696565 | *Hepatozoon* sp. | K | Reptilia, Serpentes, Colubridae | *Psammophis schokari* | Africa | Morocco | KC696565 |
| KJ408527 | *Hepatozoon* sp. | K | Reptilia, Serpentes, Colubridae | *Psammophis schokari* | Africa | Morocco | KC696565 |
| KJ408530 | *Hepatozoon* sp. | K | Reptilia, Serpentes, Colubridae | *Spalerosophis dolichospilus* | Africa | Morocco | KC696565 |
| CN2672[17] | *Hepatozoon* sp. | K | Reptilia, Serpentes, Colubridae | *Cerastes gasparetti* | Asia | Oman | hap_8 |
| CN4086[17] | *Hepatozoon* sp. | K | Reptilia, Serpentes, Viperidae | *Echis carinatus* | Asia | Oman | hap_8 |
| JQ751276 | *Hepatozoon* sp. | L | Arachnida, Ixodida, Ixodidae | *Dermacentor atrosignatus* from *Sus scrofa* | Asia | Thailand | JQ751276 |
| KC584779 | *Hepatozoon* sp. | L | Arachnida, Ixodida, Ixodidae | *Ixodes hexagonus* | Europe | Germany | KC584779 |
| JX679178 | *Hepatozoon* sp. | L | Arachnida, Ixodida, Ixodidae | *Ixodes hexagonus* from *Vulpes vulpes* | Europe | Germany | JX679178 |
| KC162910 | *Hepatozoon* sp. | L | Arachnida, Ixodida, Ixodidae | *Amblyomma americanum* from *Homo sapiens* | North America | USA | KC162910 |
| KC162911 | *Hepatozoon* sp. | L | Arachnida, Ixodida, Ixodidae | *Amblyomma americanum* from *Homo sapiens* | North America | USA | KF270644 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| KC162913 | *Hepatozoon* sp. | L | Arachnida, Ixodida, Ixodidae | *Amblyomma americanum* from *Homo sapiens* | North America | USA | KF270644 |
| KF270654 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Zambia | KF270654 |
| KF270642 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270642 |
| KF270643 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270643 |
| KF270644 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270644 |
| KF270646 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270646 |
| KF270658 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270658 |
| KF270663 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270658 |
| KF270665 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270658 |
| KF270667 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270667 |
| KF270668 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270658 |
| KF270669 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zambia | KF270658 |
| GQ926902 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis* | Asia | Thailand | GQ926902 |
| AB872993 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Felis catus* | Europe | Portugal | AB872993 |
| AB872995 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Felis catus* | Europe | Portugal | AB872995 |
| AB896687 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Felis catus* | Europe | Portugal | AB896687 |
| AB896688 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Felis catus* | Europe | Portugal | AB896688 |
| AB896689 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Felis catus* | Europe | Portugal | AB896689 |
| AB896690 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Felis catus* | Europe | Portugal | AB896690 |
| AB896691 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Felis catus* | Europe | Portugal | AB896691 |
| AB896694 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Felis catus* | Europe | Portugal | AB896694 |
| JF491227 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Felidae | *Lynx rufus* | North America | USA | JF491227 |
| KF270659 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Hyaenidae | *Hyena* | Africa | Zambia | KF270659 |
| KF270660 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Hyaenidae | *Hyena* | Africa | Zambia | KF270660 |
| KF270673 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Hyaenidae | *Hyena* | Africa | Zambia | KF270673 |
| EF188809 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Hyaenidae | *Crocuta crocuta* | Asia | Tanzania | KF270644 |
| FJ595127 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes melampus melampus* | Asia | Japan | FJ595127 |
| FJ595128 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes melampus melampus* | Asia | Japan | FJ595127 |
| FJ595129 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes melampus melampus* | Asia | Japan | FJ595129 |
| FJ595130 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes melampus melampus* | Asia | Japan | FJ595130 |
| FJ595131 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes melampus melampus* | Asia | Japan | FJ595131 |
| FJ595132 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes melampus melampus* | Asia | Japan | FJ595132 |
| FJ595133 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes melampus melampus* | Asia | Japan | FJ595132 |
| FJ595134 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes melampus melampus* | Asia | Japan | FJ595132 |
| EF222257 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes martes* | Europe | Spain | EF222257 |
| EU686690 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Mustelidae | *Martes martes* | Europe | United Kingdom | EF222257 |
| JF491243 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Procyonidae | *Procyon lotor* | North America | USA | JF491245 |
| JF491244 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Procyonidae | *Procyon lotor* | North America | USA | JF491245 |
| JF491245 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Procyonidae | *Procyon lotor* | North America | USA | JF491245 |
| JF491246 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Procyonidae | *Procyon lotor* | North America | USA | JF491245 |
| HQ829429 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Melursus ursinus* | Asia | India | EU041717 |
| HQ829430 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Melursus ursinus* | Asia | India | EU041717 |
| HQ829431 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Melursus ursinus* | Asia | India | EU041717 |
| HQ829432 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Melursus ursinus* | Asia | India | EU041717 |
| HQ829433 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Melursus ursinus* | Asia | India | EU041717 |
| HQ829434 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Melursus ursinus* | Asia | India | EU041717 |
| HQ829435 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Melursus ursinus* | Asia | India | EU041717 |
| HQ829436 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Melursus ursinus* | Asia | India | EU041717 |
| HQ829437 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Melursus ursinus* | Asia | India | EU041717 |
| AB586028 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Ursus thibetanus japonicus* | Asia | Japan | EU041717 |
| EU041717 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Ursus thibetanus japonicus* | Asia | Japan | EU041717 |
| EU041718 | *Hepatozoon* sp. | L | Mammalia, Carnivora, Ursidae | *Ursus thibetanus japonicus* | Asia | Japan | EU041717 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| AB983435 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Amblyomma testudinarium* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983392 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Amblyomma testudinarium* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983395 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Amblyomma testudinarium* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983403 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Amblyomma testudinarium* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983411 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis campanulata* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983407 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983386 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983388 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983389 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983390 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983391 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983393 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983394 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB636285 |
| AB983396 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983397 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983398 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983399 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983400 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983401 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983402 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983405 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983406 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis hystricis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983385 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis longicornis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983387 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis longicornis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB983387 |
| AB983404 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis longicornis* from *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771553 |
| AB983410 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis megaspinosa* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| AB983422 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis megaspinosa* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983428 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis megaspinosa* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983433 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis megaspinosa* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983434 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Haemaphysalis megaspinosa* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983408 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983409 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983414 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983420 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983429 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB983430 | *Hepatozoon felis* | M | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| KJ598887 | *Hepatozoon* sp. | M | Mammalia, Carnivora, Felidae | *Acinonyx jubatus* | Africa | Zimbabwe | KJ598887 |
| KJ598886 | *Hepatozoon* sp. | M | Mammalia, Carnivora, Felidae | *Panthera leo* | Africa | Zimbabwe | KJ598887 |
| AB771550 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771550 |
| AB771551 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB771552 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB636285 |
| AB771553 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771554 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771555 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771556 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB771557 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB771558 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB771559 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771560 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771561 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771562 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771563 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB771564 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB771565 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771566 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB771567 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771568 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771569 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771570 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771553 |
| AB771571 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB771573 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB771574 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771574 |
| AB771576 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771576 |
| AB771577 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB771566 |
| AB636285 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB636285 |
| AB636286 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB636285 |
| AB636287 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis iriomotensis* | Asia | Japan | AB771517 |
| AB771501 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| AB771502 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771503 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771505 |
| AB771504 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771505 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771505 |
| AB771506 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771507 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771505 |
| AB771508 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771509 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771510 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771511 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771512 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771513 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771514 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771515 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771505 |
| AB771516 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771505 |
| AB771517 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771518 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771519 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771553 |
| AB771520 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB983387 |
| AB771521 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771522 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771523 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771524 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771525 |
| AB771525 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771525 |
| AB771526 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771527 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771525 |
| AB771528 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771529 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771530 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771531 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771532 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771533 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771533 |
| AB771534 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771535 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771536 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771537 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771538 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771539 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771525 |
| AB771540 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771541 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771542 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771543 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771544 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771545 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771546 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771505 |
| AB771547 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771548 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| AB771549 | *Hepatozoon felis* | M | Mammalia, Carnivora, Felidae | *Prionailurus iriomotensis* | Asia | Japan | AB771517 |
| GQ926901 | *Hepatozoon* sp. | M | Mammalia, Carnivora, Felidae | *Prionailurus planiceps* | Asia | Thailand | AB771517 |
| FJ895407 | *Hepatozoon* sp. | M | Mammalia, Lagomorph, Leporidae | *Sylvilagus aquaticus* | North America | USA | FJ895407 |
| FJ895406 | *Hepatozoon* sp. | M | Mammalia, Lagomorph, Leporidae | *Sylvilagus floridanus* | North America | USA | FJ895406 |
| AF176836 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | N/A | N/A | AF176836 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| EU249992 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | N/A | N/A | JF491230 |
| JX415166 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415166 |
| JX415167 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415167 |
| JX415168 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415166 |
| JX415169 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415169 |
| JX415170 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415169 |
| JX415171 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JF491230 |
| JX415172 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JF491230 |
| JX415173 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JF491230 |
| JX415174 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | AY864676 |
| JX415175 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415175 |
| JX415176 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415176 |
| JF491229 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415183 |
| JF491230 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JF491230 |
| JF491231 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JF491230 |
| JF491232 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415176 |
| JF491233 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JF491230 |
| JX415177 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415183 |
| JX415178 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415183 |
| JX415179 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415183 |
| JX415180 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415183 |
| JX415181 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415183 |
| JX415182 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415183 |
| JX415183 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415183 |
| EU146062 | *Hepatozoon americanum* | N | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | North America | USA | JF491230 |
| AY864676 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | North America | USA | AY864676 |
| EU146065 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | North America | USA | EU146067 |
| EU146066 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | North America | USA | EU146067 |
| EU146067 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | North America | USA | EU146067 |
| AY461377 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Cerdocyon thous* | South America | Brazil | AY461377 |
| KC127679 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Canidae | *Cerdocyon thous* | South America | Brazil | KC127679 |
| JF491228 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Felidae | *Felis catus* | North America | USA | JF491230 |
| JF491226 | *Hepatozoon* sp. | N | Mammalia, Carnivora, Felidae | *Lynx rufus* | North America | USA | JF491226 |
| EU249993 | *Hepatozoon americanum* | N | Mammalia, Rodentia, Cricetidae | *Sigmodon* sp. | N/A | N/A | JF491230 |
| JF491241 | *Hepatozoon* sp. | N | Mammalia, Rodentia, Sciuridae | *Marmota monax* | North America | USA | JX415183 |
| JX027016 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Haemaphysalis adleri* nymph on *Canis lupus familiaris* | Africa | Nigeria | JQ976620 |
| JX027020 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Haemaphysalis adleri* on *Canis lupus familiaris* | Africa | Nigeria | JQ976620 |
| JX027010 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sanguineus* on *dog* | Africa | Nigeria | JX027012 |
| JX027012 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sanguineus* on *dog* | Africa | Nigeria | JX027012 |
| JX027013 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sanguineus* on *dog* | Africa | Nigeria | JQ976620 |
| JX027015 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sanguineus* on *dog* | Africa | Nigeria | JX027015 |
| JX027019 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sanguineus* on *dog* | Africa | Nigeria | JQ976620 |
| JX027011 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus turanicus* on *dog* | Africa | Nigeria | JQ976620 |
| JX027017 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus turanicus* on *dog* | Africa | Nigeria | JQ976620 |
| JX027018 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus turanicus* on *dog* | Africa | Nigeria | JQ976620 |
| AB983415 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Amblyomma testudinarium* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983421 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Haemaphysalis campanulata* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983416 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Haemaphysalis megaspinosa* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| AB983425 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Haemaphysalis megaspinosa* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| JX441117 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Hyalomma anatolicum* | Asia | Pakistan | DQ439540 |
| AB983412 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983413 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983417 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983418 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983419 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983423 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983424 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983426 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983427 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983431 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983432 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983436 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB983437 | *Hepatozoon felis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes tanuki* from *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| KJ605145 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sp.* | Asia | India | DQ060324 |
| KC509529 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Dermacentor marginatus* | Europe | Hungary | AY461375 |
| KF034776 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Dermacentor marginatus* | Europe | Turkey | JX466880 |
| KC584777 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Dermacentor reticulatus* | Europe | Germany | KJ572979 |
| KC509530 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Dermacentor sp.* | Europe | Hungary | AY461375 |
| KC509531 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Haemaphysalis concinna* | Europe | Hungary | AY461375 |
| KC509532 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Haemaphysalis concinna* | Europe | Hungary | KJ572979 |
| KC584776 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes canisuga* | Europe | Germany | KJ572979 |
| KC584780 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes canisuga* | Europe | Germany | AY461375 |
| KC584778 | *Hepatozoon* sp. | O | Arachnida, Ixodida, Ixodidae | *Ixodes canisuga* | Europe | Germany | KJ572979 |
| KC584775 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes hexagonus* | Europe | Germany | KJ572979 |
| KC584774 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes ricinus* | Europe | Germany | KJ572979 |
| GU827130 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes ricinus* | Europe | Luxembourg | GU827130 |
| KF034775 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes ricinus from human* | Europe | Turkey | AY461375 |
| KF034777 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Ixodes ricinus from human* | Europe | Turkey | JX466880 |
| JQ867389 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sanguineus* | Europe | Turkey | JQ867389 |
| KF034778 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sanguineus* | Europe | Turkey | AY461375 |
| KJ605144 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sp.* | Europe | Spain | DQ439540 |
| KJ605146 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus sp.* | Europe | Spain | JQ976624 |
| KJ605147 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus turanicus* | Europe | Italy | AY461375 |
| JF827277 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Tick* | Europe | Italy | DQ439540 |
| FJ743476 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Amblyomma ovale* | South America | Brazil | AY461375 |
| HQ605710 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Rhipicephalus (Boophilus) microplus* | South America | Brazil | AY461375 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| KF972442 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Tick* from *Canis lupus familiaris* | South America | Brazil | KF972444 |
| KF972444 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Tick* from *Canis lupus familiaris* | South America | Brazil | KF972444 |
| KF972445 | *Hepatozoon canis* | O | Arachnida, Ixodida, Ixodidae | *Tick* from *Canis lupus familiaris* | South America | Brazil | KF972444 |
| KJ499484 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis adustus* | Africa | Ethiopia | DQ439540 |
| KJ499481 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Africa | Algeria | DQ439540 |
| KJ499495 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Africa | Algeria | AY461375 |
| KJ499491 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Africa | Mauritania | DQ060324 |
| KJ499498 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Africa | Mauritania | AY461375 |
| KJ499499 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Africa | Mauritania | AY461375 |
| KJ499514 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Africa | Mauritania | KJ499513 |
| GQ395386 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Cape Verde | AB365071 |
| AB365071 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | AB365071 |
| JQ976620 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976620 |
| JQ976621 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976624 |
| JQ976622 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976624 |
| JQ976623 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976624 |
| JQ976624 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976624 |
| JQ976625 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976620 |
| JQ976626 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976620 |
| JQ976627 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976620 |
| JQ976628 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976620 |
| JQ976629 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Nigeria | JQ976620 |
| DQ111751 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Sudan | DQ111754 |
| DQ111752 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Sudan | KJ499513 |
| DQ111753 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Sudan | DQ439540 |
| DQ111754 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Sudan | DQ111754 |
| DQ111755 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Sudan | DQ111754 |
| DQ111756 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Sudan | KJ499513 |
| DQ111757 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Sudan | DQ439540 |
| DQ111758 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Sudan | KJ499513 |
| DQ111759 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Africa | Sudan | DQ439540 |
| KJ499494 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Mauritania | KJ499505 |
| KJ499502 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Mauritania | KJ499505 |
| KJ499503 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Mauritania | KJ499505 |
| KJ499504 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Mauritania | KJ499505 |
| KJ499506 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Mauritania | KJ499505 |
| KJ499507 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Mauritania | KJ499505 |
| KJ499508 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Mauritania | KJ499505 |
| KJ499509 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Mauritania | KJ499505 |
| KJ499512 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Mauritania | KJ499505 |
| KJ499493 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Niger | KJ499505 |
| KJ499510 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Niger | KJ499505 |
| KJ499511 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes pallida* | Africa | Niger | KJ499505 |
| KJ499485 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes rueppellii* | Africa | Mauritania | DQ060324 |
| KJ499488 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes rueppellii* | Africa | Mauritania | DQ060324 |
| KJ499490 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes rueppellii* | Africa | Mauritania | DQ060324 |
| KJ499515 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes rueppellii* | Africa | Mauritania | KJ499513 |
| KJ499505 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes rueppellii* | Africa | Morocco | KJ499505 |
| KJ499486 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Africa | Morocco | DQ060324 |
| KJ499487 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Africa | Morocco | DQ060324 |
| KJ499489 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Africa | Morocco | DQ060324 |
| KJ499492 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Africa | Morocco | DQ060324 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| KJ499480 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Africa | Tunisia | DQ439540 |
| KJ499483 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Africa | Tunisia | DQ439540 |
| KJ499496 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes zerda* | Africa | Mauritania | AY461375 |
| KJ499501 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes zerda* | Africa | Mauritania | AY461375 |
| KJ499513 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes zerda* | Africa | Mauritania | KJ499513 |
| KJ499500 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes zerda* | Africa | Morocco | AY461375 |
| KJ499482 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes zerda* | Africa | Western Sahara | DQ439540 |
| KJ499497 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes zerda* | Africa | Western Sahara | AY461375 |
| JN584477 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Asia | India | DQ439540 |
| JN584478 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Asia | India | DQ439540 |
| AF418558 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Asia | Japan | AY461375 |
| JF827605 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Asia | Jordan | DQ439540 |
| JF827606 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Asia | Jordan | KJ634654 |
| EU289222 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Asia | Taiwan | EU289222 |
| JF459994 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Asia | Taiwan | JF459994 |
| DQ519357 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Asia | Thailand | DQ439544 |
| DQ519358 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Asia | Thailand | DQ439540 |
| HQ829447 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Cuon alpinus* | Asia | India | HQ829448 |
| HQ829448 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Cuon alpinus* | Asia | India | HQ829448 |
| JX466880 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Austria | JX466880 |
| JX466881 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Austria | KJ572979 |
| JX466882 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Austria | KF322143 |
| JX466883 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Austria | KJ572979 |
| JX466884 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Austria | JX466884 |
| JX466885 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Austria | KF322143 |
| JX466886 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Austria | KJ572979 |
| KC886721 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | AY461375 |
| KC886729 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | AY461375 |
| KC886730 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | AY461375 |
| KC886731 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | AY461375 |
| KC886732 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | AY461375 |
| KC886733 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | AY461375 |
| KF322145 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | AY461375 |
| KJ572975 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | KJ572975 |
| KJ572976 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | AY461375 |
| KJ572977 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | FJ497020 |
| KJ634654 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis aureus* | Europe | Hungary | KJ634654 |
| FJ497009 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | KJ572979 |
| FJ497010 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | KJ572979 |
| FJ497011 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | KJ572979 |
| FJ497012 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | FJ497012 |
| FJ497014 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | FJ497014 |
| FJ497015 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | FJ497014 |
| FJ497016 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | FJ497016 |
| FJ497017 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | DQ060324 |
| FJ497018 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | DQ060324 |
| FJ497019 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | FJ497019 |
| FJ497020 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | FJ497020 |
| FJ497021 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | AY461375 |
| FJ497022 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Croatia | FJ497020 |
| KC509526 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Hungary | AY461375 |
| KC509527 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Hungary | AY461375 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| KC509528 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Hungary | KJ572979 |
| JF827276 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Italy | DQ439540 |
| KJ946245 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Malta | DQ439540 |
| KJ946246 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Malta | JF459994 |
| KJ946247 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Malta | DQ439540 |
| AY150067 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Spain | AY461375 |
| AY461378 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Spain | DQ439540 |
| KC138535 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Spain | KC138535 |
| DQ060324 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | DQ060324 |
| DQ060325 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | DQ060325 |
| DQ060326 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | DQ060326 |
| DQ060327 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | DQ060327 |
| DQ060328 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | DQ060324 |
| DQ060329 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | DQ060329 |
| JQ867390 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | DQ439540 |
| KF439866 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | KF439866 |
| KF439867 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | KF439867 |
| KJ513193 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | DQ439540 |
| KJ513198 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | DQ439540 |
| KF439864 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | KF439864 |
| KF439865 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | Europe | Turkey | KF439865 |
| HM212625 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Croatia | KJ572979 |
| HM212626 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Croatia | FJ497020 |
| KC584773 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Germany | KJ572979 |
| KC886722 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | AY461375 |
| KC886723 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | AY461375 |
| KC886724 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | AY461375 |
| KC886725 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | KJ572979 |
| KC886726 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | KJ572979 |
| KC886727 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | KJ572979 |
| KC886728 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | AY461375 |
| KF322142 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | KJ572979 |
| KF322143 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | KF322143 |
| KF322144 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | KJ572979 |
| KJ572978 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | KJ572979 |
| KJ572979 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Hungary | KJ572979 |
| GU371446 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | AY461375 |
| GU371447 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | AY461375 |
| GU371448 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | AY461375 |
| GU371449 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | AY461375 |
| GU371450 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | GU371450 |
| GU371451 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | AY461375 |
| GU371452 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | AY461375 |
| GU376453 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | GU376458 |
| GU376454 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | GU376458 |
| GU376455 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | GU376458 |
| GU376456 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | GU376458 |
| GU376457 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | GU376458 |
| GU376458 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Italy | GU376458 |
| EU165370 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Poland | EU165370 |
| AY731062 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Spain | DQ439540 |
| DQ439541 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Spain | EF622096 |

| Genbank | Parasite species | Lineage | Host taxonomy (Class/Order/Family) | Host species | Geography | Locality | Final alignment |
|---|---|---|---|---|---|---|---|
| DQ439542 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | Europe | Spain | EF622096 |
| AF176835 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canid* | N/A | N/A | AF176835 |
| JX415165 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis latrans* | North America | USA | JX415165 |
| JX112783 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | North America | St Kitts and Nevis | KF972444 |
| EU146063 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | North America | USA | JX415165 |
| EU146064 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | North America | USA | JX415165 |
| JF491234 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Urocyon cinereoargenteus* | North America | USA | JF491234 |
| KF989489 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Vulpes vulpes* | North America | USA | DQ439540 |
| DQ071888 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| DQ198378 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| DQ198379 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | DQ198379 |
| EU571737 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| FJ943578 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| GQ176285 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| JF295088 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| JN835188 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | JN835188 |
| JX118828 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| KF692038 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| KF692039 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | DQ060324 |
| KF692040 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | KF692040 |
| KF972441 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | KF972444 |
| KF972443 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | KF972444 |
| KJ831221 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | KJ831221 |
| AY864677 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| AY864678 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| AY864679 | *Hepatozoon* sp. | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Brazil | AY461375 |
| JN217101 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Colombia | JF459994 |
| JN217102 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Colombia | JN217102 |
| DQ439540 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Venezuela | DQ439540 |
| DQ439543 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Venezuela | DQ439540 |
| DQ439544 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Canis lupus familiaris* | South America | Venezuela | DQ439544 |
| AY461375 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Cerdocyon thous* | South America | Brazil | AY461375 |
| AY461376 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Pseudalopex gymnocercus* | South America | Brazil | EF622096 |
| AY471615 | *Hepatozoon canis* | O | Mammalia, Carnivora, Canidae | *Pseudalopex gymnocercus* | South America | Brazil | AY461375 |
| AB771572 | *Hepatozoon felis* | O | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| AB771575 | *Hepatozoon felis* | O | Mammalia, Carnivora, Felidae | *Prionailurus bengalensis euptilurus* | Asia | Japan | AB983415 |
| DQ315565 | *Hepatozoon canis* | O | Mammalia, Carnivora, Felidae | *Felis catus* | South America | Brazil | DQ315565 |
| DQ315566 | *Hepatozoon canis* | O | Mammalia, Carnivora, Felidae | *Felis catus* | South America | Brazil | DQ315565 |
| EF622096 | *Hepatozoon canis* | O | Mammalia, Rodentia, Caviidae | *Hydrochaeris hydrochaeris* | N/A | N/A | EF622096 |
| HQ224957 | *Dactylosoma ranarum* | outgroup | Amphibia, Anura, Ranidae | *Rana esculenta* | N/A | N/A | HQ224958 |
| HQ224958 | *Dactylosoma ranarum* | outgroup | Amphibia, Anura, Ranidae | *Rana esculenta* | N/A | N/A | HQ224958 |
| HQ224961 | *Hemolivia mariae*[18] | outgroup | Reptilia, Lacertilia, Scincidae | *Tiliqua rugosa* | N/A | N/A | HQ224961 |

[18] Sequence HQ224961 identified as *Hemolivia mariae* was genetically similar to *Dactylosoma ranarum* and was used as an outgroup.

## Article IX

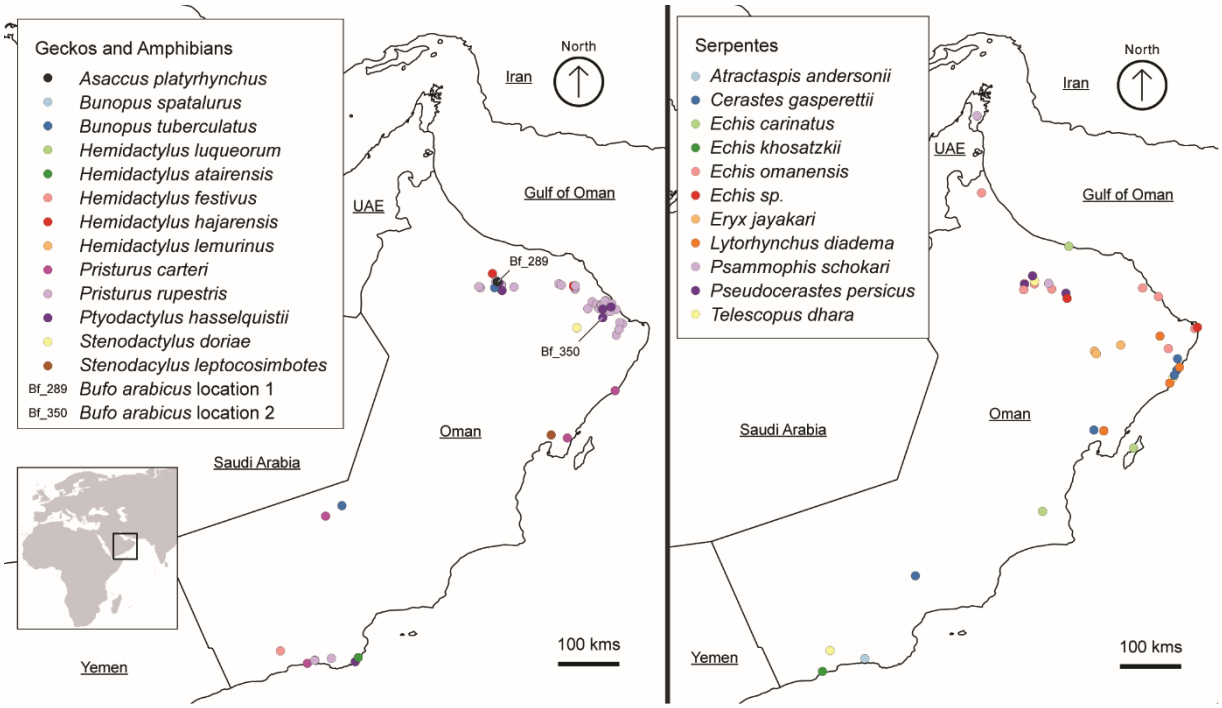



Figure S7 Geographic distribution of collected samples in Oman between 2009 and 2013. Smaller maps indicate sampling for each host group.

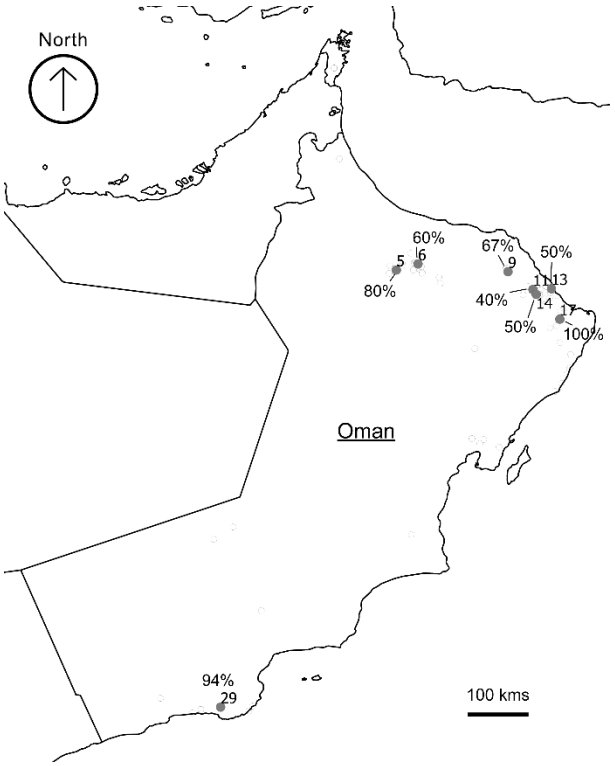



Figure S8 Prevalence estimates in geographical areas for *P. rupestris* from which 5 or more samples were collected.

Table S6 Exact GPS points from Oman used and corresponding geographical areas defined in section 5.2.

| GPS | Latitude | Longitude | Altitude | Zone | Area_20by20 |
|---|---|---|---|---|---|
| 1 | 16.899570 | 53.772640 | 19 | South West | 31 |
| 2 | 17.105310 | 54.454563 | 206 | South West | 29 |
| 3 | 17.235130 | 53.894730 | 694 | South West | 30 |
| 4 | 18.441610 | 55.272190 | 224 | South West | 27 |
| 5 | 19.482000 | 57.324970 | 170 | East | 26 |
| 6 | 20.502590 | 58.795370 | 16 | East | 24 |
| 7 | 20.777920 | 58.312500 | 30 | East | 23 |
| 8 | 20.782010 | 58.314360 | 30 | East | 23 |
| 9 | 20.793600 | 58.154350 | 50 | East | 23 |
| 10 | 21.549710 | 59.379890 | 70 | East | 22 |
| 11 | 21.550450 | 59.380600 | 70 | East | 22 |
| 12 | 21.551210 | 59.379520 | 74 | East | 22 |
| 13 | 21.633860 | 59.428520 | 19 | East | 22 |
| 14 | 21.676630 | 59.451460 | 36 | East | 22 |
| 15 | 21.759110 | 59.492010 | 9 | East | 22 |
| 16 | 21.805860 | 59.534640 | 9 | East | 22 |
| 17 | 21.806760 | 59.534360 | 10 | East | 22 |
| 18 | 21.943530 | 59.504010 | 40 | East | 21 |
| 19 | 22.023190 | 58.192010 | 226 | North East | 19 |
| 20 | 22.066830 | 58.162230 | 233 | North East | 19 |
| 21 | 22.106960 | 59.357030 | 200 | East | 21 |
| 22 | 22.165560 | 58.587950 | 277 | North East | 20 |
| 23 | 22.308730 | 59.221040 | 186 | North East | 17 |
| 24 | 22.431790 | 59.782490 | 22 | North East | 18 |
| 25 | 22.450690 | 59.826190 | 16 | North East | 18 |
| 26 | 22.917040 | 57.721070 | 537 | North | 7 |
| 27 | 22.945980 | 59.195410 | 20 | North East | 12 |
| 28 | 23.000360 | 57.702270 | 1588 | North | 7 |
| 29 | 23.055830 | 57.021110 | 896 | North | 5 |
| 30 | 23.069037 | 57.473240 | 664 | North | 6 |
| 31 | 23.084390 | 58.940670 | 253 | North East | 10 |
| 32 | 23.145610 | 57.033270 | 2254 | North | 5 |
| 33 | 23.152260 | 57.194000 | 762 | North | 4 |
| 34 | 23.156190 | 57.033180 | 1739 | North | 5 |
| 35 | 23.160443 | 57.423298 | 1990 | North | 6 |
| 36 | 23.191890 | 57.198610 | 1834 | North | 4 |
| 37 | 23.281400 | 57.162910 | 2105 | North | 4 |
| 38 | 23.757650 | 57.748030 | 5 | North | 3 |
| 39 | 24.620340 | 56.340020 | 240 | South West | 2 |
| 40 | 25.861550 | 56.269110 | 418 | North | 1 |
| 41 | 25.863970 | 56.267210 | 410 | North | 1 |
| 205 | 17.04136 | 54.32605 | 11 | South West | 29 |
| 208 | 17.0672 | 55.09818 | 7 | South West | 28 |
| 263 | 23.05642 | 57.46943 | 733 | North | 6 |
| 268 | 19.41654 | 54.62015 | 139 | West | 25 |
| 270 | 19.58533 | 54.88407 | 111 | West | 25 |
| 274 | 17.08981 | 54.4428 | 195 | South West | 29 |
| 276 | 17.09183 | 54.4458 | 200 | South West | 29 |
| 277 | 17.13596 | 55.14901 | 9 | South West | 28 |
| 278 | 17.12142 | 54.71404 | 1311 | South West | 29 |
| 279 | 17.24218 | 53.89095 | 678 | South West | 30 |
| 284 | 20.72641 | 58.26222 | 19 | East | 23 |
| 286 | 20.67762 | 58.52362 | 8 | East | 23 |
| 287 | 21.43997 | 59.29431 | 7 | East | 22 |
| 289 | 22.61609 | 59.09371 | 649 | North East | 15 |
| 291 | 22.33769 | 59.31128 | 202 | North East | 17 |
| 292 | 22.42828 | 59.35618 | 127 | North East | 17 |

| GPS | Latitude | Longitude | Altitude | Zone | Area_20by20 |
|---|---|---|---|---|---|
| 293 | 22.53899 | 59.36823 | 126 | North East | 17 |
| 294 | 22.52268 | 59.41361 | 73 | North East | 17 |
| 296 | 22.75099 | 59.30877 | 36 | North East | 13 |
| 297 | 22.8448 | 59.24156 | 14 | North East | 13 |
| 299 | 22.87327 | 59.17228 | 1070 | North East | 12 |
| 301 | 22.45379 | 58.67551 | 360 | North East | 16 |
| 303 | 22.88482 | 59.13114 | 1147 | North East | 12 |
| 304 | 22.89514 | 59.13761 | 858 | North East | 12 |
| 308 | 22.7914 | 59.22873 | 125 | North East | 13 |
| 309 | 22.84148 | 59.09768 | 1547 | North East | 14 |
| 310 | 22.82554 | 59.08568 | 1687 | North East | 14 |
| 312 | 22.82042 | 59.0641 | 1755 | North East | 14 |
| 313 | 22.82374 | 59.00759 | 1369 | North East | 11 |
| 314 | 22.83326 | 58.98821 | 999 | North East | 11 |
| 315 | 22.87384 | 58.92515 | 552 | North East | 11 |
| 316 | 22.76253 | 58.85312 | 591 | North East | 11 |
| 317 | 23.10603 | 58.64444 | 1644 | North East | 9 |
| 318 | 23.13329 | 58.65199 | 1689 | North East | 9 |
| 319 | 23.13167 | 58.61889 | 1676 | North East | 9 |
| 320 | 23.07732 | 58.64769 | 1188 | North East | 9 |
| 322 | 23.14307 | 58.4244 | 552 | North East | 8 |
| 323 | 23.16483 | 58.3853 | 643 | North East | 8 |
| 324 | 22.76619 | 59.03366 | 1324 | North East | 14 |
| 325 | 22.77029 | 59.07579 | 1418 | North East | 14 |
| 326 | 22.75737 | 59.09399 | 1189 | North East | 14 |
| 327 | 22.73958 | 59.10603 | 1358 | North East | 14 |
| 328 | 22.71802 | 59.12097 | 1615 | North East | 14 |
| 329 | 22.70583 | 59.1421 | 2036 | North East | 14 |
| 330 | 22.71755 | 59.21625 | 1589 | North East | 13 |
| 332 | 23.10028 | 57.11915 | 1034 | North | 5 |
| 333 | 23.11864 | 57.09576 | 994 | North | 5 |
| 336 | 23.10397 | 57.35451 | 1017 | North | 6 |
| 337 | 23.12547 | 57.40433 | 1473 | North | 6 |
| 338 | 23.17711 | 57.41018 | 1302 | North | 6 |
| 339 | 23.10098 | 57.34959 | 950 | North | 6 |
| 340 | 23.18292 | 57.41627 | 1133 | North | 6 |
| 341 | 23.19367 | 57.39477 | 917 | North | 6 |
| 342 | 23.21804 | 57.37817 | 1123 | North | 6 |
| 343 | 23.19898 | 57.36444 | 1394 | North | 6 |
| 349 | 23.33012 | 57.31367 | 583 | North | 4 |
| 350 | 23.19809 | 57.39045 | 906 | North | 6 |
| 352 | 23.14927 | 57.46254 | 2218 | North | 6 |
| 353 | 23.13226 | 57.46171 | 2011 | North | 6 |
| 354 | 23.12441 | 57.45664 | 1725 | North | 6 |
| 358 | 23.11336 | 57.65967 | 2254 | North | 7 |

Table S7 Prevalence estimates by host species per area of collection considering a 20 by 20 km radius.
See Table S6 for more details.

| | North | | | | | | North East | | | | | | | | | | | | | East | | | | | South West | | | | | | West |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 26 | 2 | 27 | 28 | 29 | 30 | 31 | 25 |
| **Anura** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *B. arabicus* | | | | | 12/20 | | | | | | | | | 20/20 | | | | | | | | | | | | | | | | | |
| | 12/20 (60%) | | | | | | 20/20 (100%) | | | | | | | | | | | | | - | | | | | - | | | | | | - |
| **Gekkota** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *A. platyrhynchus* | | | | | 20/21 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *B. spatalurus* | | | | | | | | 0/1 | | | | | | 2/2 | | | | | | | | | | | | | | | | | |
| *B. tuberculatus* | | | | | 1/1 | | | | | | | | | | | | | | | | | | | | | | | | | | 2/2 |
| *H. atairensis* | | | | | | | | | | | | | | | | | | | | | | | | | | | 2/2 | | | | |
| *H. festivus* | | | | | | | | | | | | | | | | | | | | | | | | | | | 1/1 | | 9/10 | | |
| *H. hajarensis* | | | 1/1 | | | | | 2/4 | | | | | | 4/4 | | | | | | | | | | | | | | | | | |
| *H. lemurinus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 3/4 | | |
| *H. luqueorum* | | | | | 3/3 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *P. carteri* | | | | | | | | | | | | | | | | | | | | | 1/3 | 2/5 | | | | | | 3/4 | | | 1/2 |
| *P. rupestris* | | | | 4/5 | 9/15 | 0/1 | 0/4 | 6/9 | | 2/5 | 1/3 | 4/8 | 9/18 | | | 9/9 | | | | | | | | | | | | 15/16 | | | |
| *P. hasselquistii* | | | | | 5/7 | | | | | | | 3/4 | 1/1 | 1/6 | | 1/1 | | | | | | | | | | | 1/3 | | | | |
| *S. doriae* | | | | | | | | | | | | | | | 0/3 | | | | | | | | | | | | | | | | 3/4 |
| *S. leptocosimbotes* | | | | | | | | | | | | | | | | | | | | | | 2/2 | | | | | | | | | |
| | 43/54 (79%) | | | | | | 45/82 (55%) | | | | | | | | | | | | | 5/10 (50%) | | | | | 34/40 (85%) | | | | | | 6/8 (75%) |
| **Serpentes** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *A. andersonii* | | | | | | | | | | | | | | | | | | | | | | | | | | | | 0/1 | | | |
| *C. gasperettii* | | | | | | | | | | | | | | | | | | | | 1/1 | 2/2 | 2/2 | | | | 1/1 | | | | | |
| *E. carinatus* | | 0/1 | | | | | | | | | | | | | | | | | | | | | 0/1 | 1/1 | | | | | | | |
| *E. khosatzkii* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 0/1 | |
| *E. omanensis* | 1/1 | | 1/1 | 2/2 | 1/1 | | | | 0/1 | | 1/1 | | | | | | 0/1 | | | 1/1 | | | | | 1/1 | | | | | | |
| *Echis* sp. | | | | | | 1/1 | | | | | | | | | | | 0/1 | | | | | | | | | | | | | | |
| *E. jayakari* | | | | | | | | | | | | | | | | | | 1/2 | 0/1 | | | | | | | | | | | | |
| *L. diadema* | | | | | | | | | | | | | | | | 1/1 | | | | | 2/7 | 0/2 | | | | | | | | | |
| *P. schokari* | 1/1 | | | | 0/1 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *P. persicus* | | | 0/1 | 0/1 | | 1/1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| *T. dhara* | | | 1/1 | | | | | | | | | | | | | 0/1 | | | | | 0/1 | | | | | | | | 0/1 | | |
| | 9/13 (69%) | | | | | | 3/9 (%) | | | | | | | | | | | | | 9/18 (%) | | | | | 2/5 (40%) | | | | | | - |

Table S8 Details for each sequence obtained in section 5.2 for the three apicomplexan parasites amplified. Samples with more than one haplotype represent mixed infections.

| Parasite | Hap | Host species | Code | Latitude | Longitude | Altitude | GPS | Zone | Area |
|---|---|---|---|---|---|---|---|---|---|
| Hemogregarine | 1 | *Bufo arabicus* | Bf1 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf14 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf19 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf24 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf25 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf27 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf30 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf31 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf36 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf37 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf39 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf6 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 1 | *Bufo arabicus* | Bf7 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 1, 2 | *Bufo arabicus* | Bf11 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 1, 2 | *Bufo arabicus* | Bf17 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 1, 2 | *Bufo arabicus* | Bf18 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf10 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf12 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf13 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf16 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf2 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf20 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf28 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf3 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf4 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf5 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 2 | *Bufo arabicus* | Bf8 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S6045 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S6050 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S6078 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S6082 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7168 | 23.05642 | 57.46943 | 733 | 263 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7182 | 23.05642 | 57.46943 | 733 | 263 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7189 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7361 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7429 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7464 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7474 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7582 | 23.05642 | 57.46943 | 733 | 263 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7750 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7782 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7805 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7835 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7836 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Asaccus platyrhynchus* | S7850 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Echis omanensis* | CN2586 | 23.15619 | 57.03318 | 1739 | 34 | North | 5 |
| Hemogregarine | 3, 6 | *Echis omanensis* | CN729 | 23.05583 | 57.02111 | 896 | 29 | North | 5 |
| Hemogregarine | 3 | *Hemidactylus hajarensis* | S7170 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 3 | *Hemidactylus hajarensis* | S7587 | 23.13167 | 58.61889 | 1676 | 319 | North East | 9 |
| Hemogregarine | 3 | *Hemidactylus luqueorum* | S6080 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Hemogregarine | 3 | *Hemidactylus luqueorum* | S7155 | 23.18292 | 57.41627 | 1133 | 340 | North | 6 |
| Hemogregarine | 3 | *Pristurus rupestris* | S7509 | 22.82554 | 59.08568 | 1687 | 310 | North East | 14 |
| Hemogregarine | 3 | *Pristurus rupestris* | S7564 | 22.42828 | 59.35618 | 127 | 292 | North East | 17 |
| Hemogregarine | 3 | *Pristurus rupestris* | S7590 | 22.42828 | 59.35618 | 127 | 292 | North East | 17 |
| Hemogregarine | 3 | *Ptyodactylus hasselquistii* | S7055 | 23.05642 | 57.46943 | 733 | 263 | North | 6 |
| Hemogregarine | 3 | *Ptyodactylus hasselquistii* | S7093 | 22.7914 | 59.22873 | 125 | 308 | North East | 13 |
| Hemogregarine | 3 | *Ptyodactylus hasselquistii* | S7123 | 22.42828 | 59.35618 | 127 | 292 | North East | 17 |
| Hemogregarine | 3 | *Ptyodactylus hasselquistii* | S7164 | 23.05642 | 57.46943 | 733 | 263 | North | 6 |
| Hemogregarine | 3 | *Ptyodactylus hasselquistii* | S7357 | 23.10098 | 57.34959 | 950 | 339 | North | 6 |
| Hemogregarine | 3 | *Ptyodactylus hasselquistii* | S7611 | 23.05642 | 57.46943 | 733 | 263 | North | 6 |

| Parasite | Hap | Host species | Code | Latitude | Longitude | Altitude | GPS | Zone | Area |
|---|---|---|---|---|---|---|---|---|---|
| Hemogregarine | 3 | *Ptyodactylus hasselquistii* | S7668 | 22.7914 | 59.22873 | 125 | 308 | North East | 13 |
| Hemogregarine | 3 | *Ptyodactylus hasselquistii* | S7676 | 22.7914 | 59.22873 | 125 | 308 | North East | 13 |
| Hemogregarine | 3 | *Ptyodactylus hasselquistii* | S7776 | 23.18292 | 57.41627 | 1133 | 340 | North | 6 |
| Hemogregarine | 4 | *Hemidactylus festivus* | S7605 | 17.24218 | 53.89095 | 678 | 279 | South West | 30 |
| Hemogregarine | 4 | *Hemidactylus lemurinus* | S7134 | 17.24218 | 53.89095 | 678 | 279 | South West | 30 |
| Hemogregarine | 5 | *Pristurus rupestris* | S7542 | 22.82554 | 59.08568 | 1687 | 310 | North East | 14 |
| Hemogregarine | 6 | *Cerastes gasperettii* | CN2698 | 20.7936 | 58.15435 | 50 | 9 | East | 23 |
| Hemogregarine | 6 | *Cerastes gasperettii* | CN3856 | 21.67663 | 59.45146 | 36 | 14 | East | 22 |
| Hemogregarine | 6 | *Cerastes gasperettii* | CN7622 | 18.44161 | 55.27219 | 224 | 4 | South West | 27 |
| Hemogregarine | 6, 7 | *Cerastes gasperettii* | CN3923 | 21.94353 | 59.50401 | 40 | 18 | East | 21 |
| Hemogregarine | 6 | *Echis omanensis* | CN3266 | 24.62034 | 56.34002 | 240 | 39 | South West | 2 |
| Hemogregarine | 6 | *Echis omanensis* | CN3399 | 23.069037 | 57.47324 | 664 | 30 | North | 6 |
| Hemogregarine | 6 | *Echis omanensis* | CN3870 | 22.10696 | 59.35703 | 200 | 21 | East | 21 |
| Hemogregarine | 6 | *Echis omanensis* | CN730 | 22.94598 | 59.19541 | 20 | 27 | North East | 12 |
| Hemogregarine | 6 | *Echis omanensis* | CN8350 | 25.86155 | 56.26911 | 418 | 40 | North | 1 |
| Hemogregarine | 6, 12 | *Echis omanensis* | CN365 | 23.15226 | 57.194 | 762 | 33 | North | 4 |
| Hemogregarine | 6 | *Echis* sp. | CN4375 | 22.91704 | 57.72107 | 537 | 26 | North | 7 |
| Hemogregarine | 6 | *Lytorhynchus diadema* | CN3459 | 21.54971 | 59.37989 | 70 | 10 | East | 22 |
| Hemogregarine | 6 | *Lytorhynchus diadema* | CN3851 | 21.67663 | 59.45146 | 36 | 14 | East | 22 |
| Hemogregarine | 6, 7 | *Lytorhynchus diadema* | CN4093 | 22.30873 | 59.22104 | 186 | 23 | North East | 17 |
| Hemogregarine | 6 | *Psammophis schokari* | CN8365 | 25.86397 | 56.26721 | 410 | 41 | North | 1 |
| Hemogregarine | 6 | *Pseudocerastes persicus* | CN205 | 23.00036 | 57.70227 | 1588 | 28 | North | 7 |
| Hemogregarine | 6 | *Telescopus dhara* | CN3900 | 23.19189 | 57.19861 | 1834 | 36 | North | 4 |
| Hemogregarine | 7 | *Cerastes gasperettii* | CN3768 | 21.75911 | 59.49201 | 9 | 15 | East | 22 |
| Hemogregarine | 8 | *Cerastes gasperettii* | CN2672 | 20.78201 | 58.31436 | 30 | 8 | East | 23 |
| Hemogregarine | 8 | *Echis carinatus* | CN4086 | 19.482 | 57.32497 | 170 | 5 | East | 26 |
| Hemogregarine | 9 | *Hemidactylus hajarensis* | S7336 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Hemogregarine | 10 | *Hemidactylus hajarensis* | S6061 | 23.33012 | 57.31367 | 583 | 349 | North | 4 |
| Hemogregarine | 11 | *Asaccus platyrhynchus* | S7077 | 23.05642 | 57.46943 | 733 | 263 | North | 6 |
| Hemogregarine | 12 | *Hemidactylus atairensis* | S7101 | 17.13596 | 55.14901 | 9 | 277 | South West | 28 |
| Hemogregarine | 13 | *Hemidactylus hajarensis* | S7154 | 23.13167 | 58.61889 | 1676 | 319 | North East | 9 |
| Lankesterellidae | 1 | *Bufo arabicus* | Bf32 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Lankesterellidae | 1, 6 | *Bufo arabicus* | Bf22 | 23.19809 | 57.39045 | 906 | 350 | North | 6 |
| Lankesterellidae | 1, 6 | *Bufo arabicus* | Bf9 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Lankesterellidae | 2 | *Pristurus rupestris* | S7267 | 23.10397 | 57.35451 | 1017 | 336 | North | 6 |
| Lankesterellidae | 2, 4 | *Pristurus rupestris* | S7160 | 17.12142 | 54.71404 | 1311 | 278 | South West | 29 |
| Lankesterellidae | 2 | *Ptyodactylus hasselquistii* | S7063 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Lankesterellidae | 3 | *Ptyodactylus hasselquistii* | S7086 | 17.0672 | 55.09818 | 7 | 208 | South West | 28 |
| Lankesterellidae | 3 | *Ptyodactylus hasselquistii* | S7167 | 17.0672 | 55.09818 | 7 | 208 | South West | 28 |
| Lankesterellidae | 3, 4 | *Ptyodactylus hasselquistii* | S7670 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Lankesterellidae | 4 | *Ptyodactylus hasselquistii* | S7198 | 22.61609 | 59.09371 | 649 | 289 | North East | 15 |
| Lankesterellidae | 4 | *Ptyodactylus hasselquistii* | S7652 | 22.75737 | 59.09399 | 1189 | 326 | North East | 14 |
| Lankesterellidae | 5 | *Hemidactylus hajarensis* | S7321 | 23.13167 | 58.61889 | 1676 | 319 | North East | 9 |
| *Sarcocystis* | 1 | *Lytorhynchus diadema* | CN3764 | 21.80586 | 59.53464 | 9 | 16 | East | 22 |
| *Sarcocystis* | 2 | *Pristurus rupestris* | S7251 | 23.17711 | 57.41018 | 1302 | 338 | North | 6 |

Table S9 Haplotype frequency by area in a 20 by 20 kms radius.
To visualize this distribution refer to Figure 5-5.

| Area code (20 by 20 kms radius) | H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 | H10 | H11 | H12 | H13 | L1 | L2 | L3 | L4 | L5 | L6 | S1 | S2 | Total positives per area | Total sampled per area |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | 2 | | | | | | | | | | | | | | | | 2 | 2 |
| 2 | | | | | | 1 | | | | | | | | | | | | | | | | 1 | 1 |
| 4 | | | | | | 2 | | | | 1 | | 1 | | | | | | | | | | 4 | 4 |
| 5 | | | 2 | | | 1 | | | | | | | | | | | | | | | | 3 | 8 |
| 6 | 8 | 1 | 25 | | | 1 | | | | | 1 | | | 2 | 1 | | | | 1 | | 1 | 41 | 69 |
| 7 | | | | | | 2 | | | | | | | | | | | | | | | | 2 | 3 |
| 9 | | | 1 | | | | | | | | | | 1 | | | | | 1 | | | | 3 | 14 |
| 12 | | | | | | 1 | | | | | | | | | | | | | | | | 1 | 4 |
| 13 | | | 3 | | | | | | | | | | | | | | | | | | | 3 | 12 |
| 14 | | | 1 | | 1 | | | | | | | | | | | | 1 | | | | | 3 | 19 |
| 15 | 8 | 13 | 1 | | | | | | 1 | | | | | 1 | 2 | | 2 | | 1 | | | 29 | 32 |
| 17 | | | 3 | | | 1 | 1 | | | | | | | | | | | | | | | 5 | 12 |
| 21 | | | | | | 2 | 1 | | | | | | | | | | | | | | | 3 | 2 |
| 22 | | | | | | 3 | 1 | | | | | | | | | | | | | 1 | | 5 | 13 |
| 23 | | | | | | 1 | | 1 | | | | | | | | | | | | | | 2 | 11 |
| 26 | | | | | | | | 1 | | | | | | | | | | | | | | 1 | 1 |
| 27 | | | | | | 1 | | | | | | | | | | | | | | | | 1 | 1 |
| 28 | | | | | | | | | | | | 1 | | | | 2 | | | | | | 3 | 6 |
| 29 | | | | | | | | | | | | | | | 1 | | 1 | | | | | 2 | 21 |
| 30 | | | | 2 | | | | | | | | | | | | | | | | | | 2 | 15 |
| Grand Total | 16 | 14 | 36 | 2 | 1 | 18 | 3 | 2 | 1 | 1 | 1 | 2 | 1 | 3 | 4 | 2 | 4 | 1 | 2 | 1 | 1 | 116 | 250 |

Table S10 Tukey Posthoc results from an ANOVA comparing intensity of infection (log copy number) between host species. *indicates significance at P<0.05.

| Host-species comparison | diff | lwr | upr | p-adj | sig |
|---|---|---|---|---|---|
| *Bufo arabicus- Asaccus platyrhynchus* | -1.749 | -2.607 | -0.891 | 0.000 | *** |
| *Bunopus spatalurus- Asaccus platyrhynchus* | -2.711 | -4.792 | -0.630 | 0.001 | ** |
| *Bunopus tuberculatus- Asaccus platyrhynchus* | -2.907 | -4.645 | -1.170 | 0.000 | *** |
| *Hemidactylus atairensis- Asaccus platyrhynchus* | -1.248 | -3.329 | 0.834 | 0.735 | |
| *Hemidactylus festivus- Asaccus platyrhynchus* | -1.845 | -2.932 | -0.758 | 0.000 | *** |
| *Hemidactylus hajarensis- Asaccus platyrhynchus* | -0.551 | -1.784 | 0.681 | 0.960 | |
| *Hemidactylus lemurinus- Asaccus platyrhynchus* | -1.576 | -3.314 | 0.161 | 0.119 | |
| *Hemidactylus luqueorum- Asaccus platyrhynchus* | -0.776 | -2.513 | 0.962 | 0.961 | |
| *Pristurus carteri- Asaccus platyrhynchus* | -2.229 | -3.461 | -0.996 | 0.000 | *** |
| *Pristurus rupestris- Asaccus platyrhynchus* | -2.045 | -2.773 | -1.317 | 0.000 | *** |
| *Ptyodactylus hasselquistii- Asaccus platyrhynchus* | -0.994 | -2.081 | 0.093 | 0.112 | |
| *Stenodactylus doriae- Asaccus platyrhynchus* | -2.495 | -4.232 | -0.757 | 0.000 | *** |
| *Stenodacylus leptocosimbotes- Asaccus platyrhynchus* | -2.872 | -4.953 | -0.791 | 0.000 | *** |
| *Bunopus spatalurus-Bufo arabicus* | -0.962 | -3.031 | 1.107 | 0.946 | |
| *Bunopus tuberculatus-Bufo arabicus* | -1.158 | -2.881 | 0.564 | 0.561 | |
| *Hemidactylus atairensis-Bufo arabicus* | 0.502 | -1.567 | 2.570 | 1.000 | |
| *Hemidactylus festivus-Bufo arabicus* | -0.096 | -1.159 | 0.967 | 1.000 | |
| *Hemidactylus hajarensis-Bufo arabicus* | 1.198 | -0.014 | 2.409 | 0.056 | |
| *Hemidactylus lemurinus-Bufo arabicus* | 0.173 | -1.550 | 1.895 | 1.000 | |
| *Hemidactylus luqueorum-Bufo arabicus* | 0.973 | -0.749 | 2.696 | 0.807 | |
| *Pristurus carteri-Bufo arabicus* | -0.480 | -1.691 | 0.732 | 0.986 | |
| *Pristurus rupestris-Bufo arabicus* | -0.296 | -0.987 | 0.396 | 0.972 | |
| *Ptyodactylus hasselquistii-Bufo arabicus* | 0.755 | -0.308 | 1.818 | 0.467 | |
| *Stenodactylus doriae-Bufo arabicus* | -0.746 | -2.468 | 0.977 | 0.969 | |
| *Stenodacylus leptocosimbotes-Bufo arabicus* | -1.123 | -3.192 | 0.946 | 0.847 | |
| *Bunopus tuberculatus-Bunopus spatalurus* | -0.196 | -2.758 | 2.366 | 1.000 | |
| *Hemidactylus atairensis-Bunopus spatalurus* | 1.464 | -1.342 | 4.270 | 0.881 | |
| *Hemidactylus festivus-Bunopus spatalurus* | 0.866 | -1.308 | 3.040 | 0.985 | |
| *Hemidactylus hajarensis-Bunopus spatalurus* | 2.160 | -0.090 | 4.410 | 0.074 | |
| *Hemidactylus lemurinus-Bunopus spatalurus* | 1.135 | -1.427 | 3.696 | 0.963 | |
| *Hemidactylus luqueorum-Bunopus spatalurus* | 1.935 | -0.626 | 4.497 | 0.363 | |
| *Pristurus carteri-Bunopus spatalurus* | 0.483 | -1.767 | 2.732 | 1.000 | |
| *Pristurus rupestris-Bunopus spatalurus* | 0.666 | -1.352 | 2.684 | 0.997 | |
| *Ptyodactylus hasselquistii-Bunopus spatalurus* | 1.717 | -0.456 | 3.891 | 0.292 | |
| *Stenodactylus doriae-Bunopus spatalurus* | 0.216 | -2.345 | 2.778 | 1.000 | |
| *Stenodacylus leptocosimbotes-Bunopus spatalurus* | -0.161 | -2.967 | 2.645 | 1.000 | |
| *Hemidactylus atairensis-Bunopus tuberculatus* | 1.660 | -0.902 | 4.221 | 0.622 | |
| *Hemidactylus festivus-Bunopus tuberculatus* | 1.062 | -0.785 | 2.909 | 0.788 | |
| *Hemidactylus hajarensis-Bunopus tuberculatus* | 2.356 | 0.420 | 4.292 | 0.004 | ** |
| *Hemidactylus lemurinus-Bunopus tuberculatus* | 1.331 | -0.960 | 3.622 | 0.775 | |
| *Hemidactylus luqueorum-Bunopus tuberculatus* | 2.131 | -0.160 | 4.423 | 0.097 | |
| *Pristurus carteri-Bunopus tuberculatus* | 0.679 | -1.258 | 2.615 | 0.995 | |
| *Pristurus rupestris-Bunopus tuberculatus* | 0.862 | -0.799 | 2.524 | 0.885 | |
| *Ptyodactylus hasselquistii-Bunopus tuberculatus* | 1.913 | 0.066 | 3.761 | 0.035 | * |
| *Stenodactylus doriae-Bunopus tuberculatus* | 0.412 | -1.879 | 2.704 | 1.000 | |
| *Stenodacylus leptocosimbotes-Bunopus tuberculatus* | 0.035 | -2.526 | 2.597 | 1.000 | |
| *Hemidactylus festivus-Hemidactylus atairensis* | -0.598 | -2.771 | 1.576 | 1.000 | |
| *Hemidactylus hajarensis-Hemidactylus atairensis* | 0.696 | -1.554 | 2.946 | 0.999 | |
| *Hemidactylus lemurinus-Hemidactylus atairensis* | -0.329 | -2.890 | 2.233 | 1.000 | |
| *Hemidactylus luqueorum-Hemidactylus atairensis* | 0.472 | -2.090 | 3.033 | 1.000 | |
| *Pristurus carteri-Hemidactylus atairensis* | -0.981 | -3.231 | 1.269 | 0.968 | |
| *Pristurus rupestris-Hemidactylus atairensis* | -0.797 | -2.816 | 1.221 | 0.986 | |
| *Ptyodactylus hasselquistii-Hemidactylus atairensis* | 0.254 | -1.920 | 2.427 | 1.000 | |
| *Stenodactylus doriae-Hemidactylus atairensis* | -1.247 | -3.809 | 1.314 | 0.925 | |
| *Stenodacylus leptocosimbotes-Hemidactylus atairensis* | -1.624 | -4.430 | 1.182 | 0.780 | |
| *Hemidactylus hajarensis-Hemidactylus festivus* | 1.294 | -0.089 | 2.677 | 0.092 | |
| *Hemidactylus lemurinus-Hemidactylus festivus* | 0.269 | -1.578 | 2.116 | 1.000 | |
| *Hemidactylus luqueorum-Hemidactylus festivus* | 1.069 | -0.778 | 2.917 | 0.779 | |
| *Pristurus carteri-Hemidactylus festivus* | -0.383 | -1.766 | 0.999 | 1.000 | |

| Host-species comparison | diff | lwr | upr | p-adj | sig |
|---|---|---|---|---|---|
| *Pristurus rupestris-Hemidactylus festivus* | -0.200 | -1.160 | 0.761 | 1.000 | |
| *Ptyodactylus hasselquistii-Hemidactylus festivus* | 0.851 | -0.404 | 2.106 | 0.546 | |
| *Stenodactylus doriae-Hemidactylus festivus* | -0.649 | -2.497 | 1.198 | 0.995 | |
| *Stenodacylus leptocosimbotes-Hemidactylus festivus* | -1.027 | -3.200 | 1.147 | 0.940 | |
| *Hemidactylus lemurinus-Hemidactylus hajarensis* | -1.025 | -2.961 | 0.911 | 0.869 | |
| *Hemidactylus luqueorum-Hemidactylus hajarensis* | -0.225 | -2.161 | 1.712 | 1.000 | |
| *Pristurus carteri-Hemidactylus hajarensis* | -1.677 | -3.177 | -0.177 | 0.014 | * |
| *Pristurus rupestris-Hemidactylus hajarensis* | -1.494 | -2.616 | -0.371 | 0.001 | ** |
| *Ptyodactylus hasselquistii-Hemidactylus hajarensis* | -0.443 | -1.825 | 0.940 | 0.998 | |
| *Stenodactylus doriae-Hemidactylus hajarensis* | -1.943 | -3.880 | -0.007 | 0.048 | * |
| *Stenodacylus leptocosimbotes-Hemidactylus hajarensis* | -2.321 | -4.570 | -0.071 | 0.036 | * |
| *Hemidactylus luqueorum-Hemidactylus lemurinus* | 0.801 | -1.491 | 3.092 | 0.995 | |
| *Pristurus carteri-Hemidactylus lemurinus* | -0.652 | -2.589 | 1.284 | 0.997 | |
| *Pristurus rupestris-Hemidactylus lemurinus* | -0.469 | -2.130 | 1.193 | 0.999 | |
| *Ptyodactylus hasselquistii-Hemidactylus lemurinus* | 0.583 | -1.265 | 2.430 | 0.998 | |
| *Stenodactylus doriae-Hemidactylus lemurinus* | -0.918 | -3.210 | 1.373 | 0.984 | |
| *Stenodacylus leptocosimbotes-Hemidactylus lemurinus* | -1.295 | -3.857 | 1.266 | 0.903 | |
| *Pristurus carteri-Hemidactylus luqueorum* | -1.453 | -3.389 | 0.484 | 0.375 | |
| *Pristurus rupestris-Hemidactylus luqueorum* | -1.269 | -2.931 | 0.392 | 0.345 | |
| *Ptyodactylus hasselquistii-Hemidactylus luqueorum* | -0.218 | -2.065 | 1.629 | 1.000 | |
| *Stenodactylus doriae-Hemidactylus luqueorum* | -1.719 | -4.010 | 0.572 | 0.375 | |
| *Stenodacylus leptocosimbotes-Hemidactylus luqueorum* | -2.096 | -4.658 | 0.466 | 0.240 | |
| *Pristurus rupestris-Pristurus carteri* | 0.184 | -0.939 | 1.307 | 1.000 | |
| *Ptyodactylus hasselquistii-Pristurus carteri* | 1.235 | -0.148 | 2.618 | 0.134 | |
| *Stenodactylus doriae-Pristurus carteri* | -0.266 | -2.202 | 1.670 | 1.000 | |
| *Stenodacylus leptocosimbotes-Pristurus carteri* | -0.643 | -2.893 | 1.607 | 0.999 | |
| *Ptyodactylus hasselquistii-Pristurus rupestris* | 1.051 | 0.090 | 2.012 | 0.018 | * |
| *Stenodactylus doriae-Pristurus rupestris* | -0.450 | -2.111 | 1.212 | 1.000 | |
| *Stenodacylus leptocosimbotes-Pristurus rupestris* | -0.827 | -2.845 | 1.191 | 0.981 | |
| *Stenodactylus doriae-Ptyodactylus hasselquistii* | -1.501 | -3.348 | 0.346 | 0.250 | |
| *Stenodacylus leptocosimbotes-Ptyodactylus hasselquistii* | -1.878 | -4.052 | 0.296 | 0.170 | |
| *Stenodacylus leptocosimbotes-Stenodactylus doriae* | -0.377 | -2.939 | 2.185 | 1.000 | |

Table S11 Effects and significant interactions from full factorial models analysing the effects of time of collection, host species and sex on host body size for *P. bocagei* and *P. hispanica* from Moledo (Portugal).

| **Host body size**<br>Data subset | **Time of collection (month/year)** | **Species** | **Sex** | **Time*Sex** | **Time*Species*Sex** |
|---|---|---|---|---|---|
| 2011 (prevalence) | df=2, sum sq=735.2, F=8.370, P<0.001*** | df=1, sum sq=0.1, F=0.001, P=0.973 | df=1, sum sq=1004.4, F=22.871, P<0.001*** | df=1, sum sq=359.8, F=4.096, P=0.018* | df=1, sum sq=378.0, F=4.304, P=0.015* |
| 2011 (intensity) | df=2, sum sq=122.8, F=1.861, P=0.159 | df=1, sum sq=2.5, F=0.075, P=0.784 | df=1, sum sq=277.3, F=8.407, P=0.004** | - | - |
| 2012 (prevalence) | df=1, sum sq=0.14, F=0.005, P=0.947 | df=1, sum sq=1.98, F=0.062, P=0.804 | df=1, sum sq=171.47, F=5.362, P=0.023* | - | - |
| 2012 (intensity) | df=1, sum sq=16.69, F=0.784, P=0.379 | df=1, sum sq=1.80, F=0.084, P=0.772 | df=1, sum sq=243.44, F=11.439, P=0.001** | - | - |
| 2013 (prevalence) | df=1, sum sq=11.0, F=0.280, P=0.598 | df=1, sum sq=47.8, F=1.214, P=0.272 | df=1, sum sq=248.4, F=6.306, P=0.013* | - | - |
| 2013 (intensity) | df=1, sum sq=3.84, F=0.129, P=0.720 | df=1, sum sq=145.68, F=4.914, P=0.029* | df=1, sum sq=61.96, F=2.090, P=0.152 | - | - |
| Between-years (prevalence) | df=2, sum sq=880.8, F=10.463, P<0.001*** | df=1, sum sq=0.8, F=0.020, P=0.888 | df=1, sum sq=1458.1, F=34.642, P<0.001*** | df=2, sum sq=243.9, F=2.897, P=0.056 | - |
| Between-years (intensity) | df=2, sum sq=181.6, F=3.164, P=0.044* | df=1, sum sq=46.6, F=1.626, P=0.203 | df=1, sum sq=597.8, F=20.832, P<0.001*** | - | - |
| July across years (prevalence) | df=2, sum sq=95.5, F=1.836, P=0.163 | df=1, sum sq=162.7, F=6.083, P=0.014* | df=1, sum sq=288.1, F=11.083, P=0.001** | - | df=1, sum sq=238.0, F=4.576, P=0.020* |
| July across years (intensity) | df=2, sum sq=41.98, F=1.201, P=0.306 | df=1, sum sq=137.60, F=7.871, P=0.006** | df=1, sum sq=151.20, F=8.648, P=0.004** | - | - |
| October across years (prevalence) | df=2, sum sq=708.7, F=7.202, P<0.001*** | df=1, sum sq=109.5, F=2.225, P=0.137 | df=1, sum sq=1046.4, F=21.267, P<0.001*** | df=2, sum sq=333.5, F=3.389, P=0.035* | - |
| October across years (intensity) | df=2, sum sq=166.1, F=2.384, P=0.095 | df=1, sum sq=0.8, F=0.022, P=0.882 | df=1, sum sq=438.8, F=12.592, P<0.001*** | - | - |

Table S12 Effects and significant interactions from full factorial models analysing the effects of time of collection, host species and sex on host body size for species *A. andreanskyi* and *P. vaucheri*, in which prevalence and intensity were higher, from Oukaimeden. Prevalence refers to the subset including all observations, while intensity refers to the subset including only positive observations. Grey indicates significance and * the level of significance.

| **Host body size** Data subset (*A. Andreanskyi* and *P. vaucheri*) | **Time of collection (month/year)** | **Species** | **Sex** | **Time*Sex** |
|---|---|---|---|---|
| May between-years (prevalence) | df=1, sum sq=477, F=15.524, P<0.001*** | df=1, sum sq=1836.5, F=59.765, P<0.001*** | df=1, sum sq=104.8, F=3.411, P=0.674 | - |
| May between-years (intensity) | df=1, sum sq=397.10, F=16.288, P<0.001*** | df=1, sum sq=1771.52, F=72.663, P<0.001*** | df=1, sum sq=5.18, F=0.212, P=0.646 | - |
| 2011 (prevalence) | df=1, sum sq=0.2, F=0.007, P=0.935 | df=1, sum sq=1710.4, F=49.689, P<0.001*** | df=1, sum sq=0.2, F=0.005, P=0.946 | df=1, sum sq=242.8, F=7.054, P=0.009** |
| 2011 (intensity) | df=1, sum sq=4.68, F=1.930, P=0.169 | df=1, sum sq=1169.95, F=45.078, P<0.001*** | df=1, sum sq=4.68, F=0.181, P=0.672 | - |

Table S13 Number of hemogregarine haplotypes found in a total of 46 sequences retrieved from host species from Moledo (Portugal). & indicates when both haplotypes were detected in double peak infections.

| | | **Haplotypes** | | |
|---|---|---|---|---|
| **Host species** | **sex** | **1** | **2** | **1&2** |
| *Podarcis bocagei* | Female | 3 | 3 | 4 |
| | Male | 9 | 4 | 5 |
| | | 12 | 7 | 9 |
| *Podarcis hispanica* | Female | 4 | 1 | |
| | Male | 4 | 4 | 5 |
| | | 8 | 5 | 5 |

Table S14 Number of hemogregarine haplotypes found in a total of 56 sequences retrieved from host species from Oukaimeden. & indicates when both haplotypes were detected in double peak infections.

| | | **Haplotypes** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Host species** | **sex** | **1** | **2** | **3** | **4** | **2&3** | **2&4** | **3&4** |
| *Atlantolacerta andreanskyi* | Female | 6 | | | | | | |
| | Male | 6 | | | | | | |
| | | 12 | | | | | | |
| *Podarcis vaucheri* | Female | | 5 | 3 | 1 | 3 | 5 | 2 |
| | Male | | 4 | 5 | 3 | 2 | 6 | 5 |
| | | | 9 | 8 | 4 | 5 | 11 | 7 |

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