# Phylogeographic patterns in two North Saharan reptiles from the Western Maghreb


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Todas as correções determinadas
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O Presidente do Júri,

Porto, _____/_____/_____

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# ABSTRACT

The Western Maghreb region is considered a hotspot of biodiversity and an important reservoir of endemism due to the heterogeneous climate and topography of this region and its complex geological history which have shaped the availability and distribution of numerous and varied habitats. Molecular assessments have successfully revealed a complexity of genetic lineages within many species, especially reptiles. However, the actual existing biodiversity is clearly underestimated due to large area cover by this region, the difficult access to remote areas and the political instability of many of these regions. In the Western Maghreb, the few studies available have focused on the northern regions of the Atlas Mountains. Therefore to contribute towards a better knowledge about diversity patterns of North Saharan species, this study involved a phylogeographic assessment, using a multilocus approach on two case studies.

*Ptyodactylus oudrii*, the fan-footed gecko, inhabits the Eastern foothills of the Atlas Mountains and proximal arid regions from Morocco to Algeria. Previous preliminary studies detected considerable levels of genetic variability within this species, potentially indicating the presence of a cryptic species complex. In this work, we carried out a comprehensive phylogeographic screening, particularly in the possible contact zones between lineages, in order to determine the level of genetic variability and gene-flow within *P.oudrii* and to assess whether these lineages represent cryptic species. Maximum likelihood and Bayesian phylogenetic analyses based on two mitochondrial (12S and Cyt-b) and two nuclear (MC1R and RAG-*2*) markers showed four divergent genetic lineages with a strict association with geography. None of these lineages were found in sympatry and the level of haplotype sharing between them was minimal at all loci investigated. The orogeny of the Atlas Mountains took place at about the same time of the cladogenetic events between these lineages, thus suggesting a possible scenario of allopatric divergence mediated by the onset of a geographical barrier. An integrative approach will be necessary in order to assess the taxonomy of these lineages which should be treated distinct “species”, as according to molecular data.

*Uromastyx nigriventris*, the spiny-tailed lizard, inhabits semi-deserts of sand, gravel and small rocks, in Morocco and Algeria. Previous works, focused on the *Uromastyx* genus, suggested only recently that *U. nigriventris* is a distinct species with low levels of intraspecific variation. We performed Maximum likelihood and Bayesian Inference phylogenetic analysis based on one mitochondrial gene (ND2*)* and two nuclear (RAG-1 and MC1R) markers to assess the genetic diversity and phylogeographic patterns of *U.*

*nigriventris*. We found two divergent mitochondrial lineages with a clear association with geography: one in the northeastern and the other in the southwestern portions of the species' range. The recent divergence of the lineages can be associated with the low variation of nuclear loci and the few instances of nuclear haplotype sharing between the two lineages suggesting a pattern of incomplete lineage sorting. Further research is needed to identify whether the lineages occur in sympatry or not. We speculated on a possible role of the Erg Chebbi formation as a long-term barrier between the two lineages in addition of the expansion of arid climate, during the Pleistocene and Pliocene climatic oscillations. Further studies are needed to confirm this hypothesis and to assess whether species co-distributed with *U. nigriventris* show similar phylogeographic patterns.

Distinct processes associated with past climate and geologic events have been responsible for shaping the pattern of diversity, in the two studied Saharan species. Further research is required to understand the phylogeographic patterns and evolutionary processes shaping the distribution and genetic variation of species from the Western Maghreb, and in order to identify similar phylogeographic patterns and ecologic responses. Those findings will contribute to identify cryptic diversity, as well as priority target for conservation in terms of species and areas.

## KEYWORDS

Phylogeography; Western Maghreb; Biodiversity; Atlas Mountains; Pleistocene/Pliocene; climatic oscillations; *Ptyodactylus oudrii*; *Uromastyx nigriventris*;

# RESUMO

A região Ocidental do Magrebe é considerada um *hotspot* de biodiversidade e um reservatório importante de endemismos, devido à heterogeneidade climática e topográfica desta região e à sua complexa história geológica, que tem moldado a disponibilidade e a distribuição de inúmeros e variados habitats. Análises moleculares têm revelado com sucesso uma complexidade de linhagens genéticas em diversas espécies, especialmente em répteis. No entanto, a biodiversidade que existe atualmente está claramente subestimada devido à extensa área desta região, à dificuldade de acessos a áreas remotas e à instabilidade política de muitas dessas zonas. No Magrebe Ocidental, os poucos estudos disponíveis têm-se focado nas regiões a norte das Montanhas do Atlas. Portanto, a fim de contribuir para um melhor conhecimento sobre os padrões de diversidade nas espécies norte Sarianas, este estudo envolveu uma análise filogeográfica, usando uma abordagem multilócus, em dois casos de estudo.

*Ptyodactylus oudrii* habita nas regiões este das Montanhas do Atlas e próximo a zonas áridas, desde Marrocos a Argélia. Estudos preliminares detetaram níveis consideráveis de variabilidade genética dentro desta espécie, indicando a presença de um potencial complexo de espécies crípticas. Neste estudo, realizámos uma análise filogeográfica abrangente, com especial atenção em possíveis zonas de contacto entre as linhagens, de forma a determinar o grau de variação genética e de fluxo genético em *P. oudrii* e avaliar se essas linhagens representam espécies crípticas. Análises a partir dos métodos de máxima verosimilhança e inferências bayesianas, com base em dois marcadores mitocondriais (12S e Cyt-b) e dois nucleares (MC1R e RAG-2), demonstraram quatro linhagens genéticas distintas com uma associação estrita a nível geográfico. Nenhuma dessas linhagens foi encontrada em simpatria e o nível de partilha de haplótipos, em todos os marcadores analisados, foi mínimo. A orogenia das Montanhas do Atlas ocorreu aproximadamente ao mesmo tempo que os eventos cladogenéticos entre as linhagens, sugerindo assim um possível cenário de divergência alopátrica mediado pelo aparecimento de barreiras geográficas. Uma abordagem integrativa será necessária de modo a avaliar a taxonomia destas linhagens, as quais devem ser tratadas como “espécies” distintas, tendo em conta os dados moleculares.

*Uromastyx nigriventris*, o lagarto de cauda espinhosa, habita em zonas semi-desérticas de areia, gravilha e pequenas rochas, em Marrocos e Argélia. Estudos anteriores, focados no género *Uromastyx*, sugeriram apenas recentemente que *Uromastyx nigriventris* é uma espécie distinta com baixos níveis de variação intraespecífica. Nós

realizámos análises filogenéticas de máxima verosimilhança e inferência bayesiana com base em um marcador genético mitocondrial (ND2) e dois nucleares (MC1R e RAG-1), de forma a avaliar a diversidade genética e os padrões filogeográficos de *U. nigriventris.* Nós descobrimos duas linhagens mitocondriais divergentes com clara associação geográfica: uma na região nordeste e outra na porção sudoeste da área de distribuição da espécie. A recente divergência das linhagens pode estar associada com a pequena variação nos marcadores nucleares e os poucos casos de partilha de haplótipos nucleares entre as duas linhagens sugerem um padrão de retenção de polimorfismos ancestrais. São necessárias mais pesquisas para identificar se as linhagens ocorrem em simpatria ou não. Nós especulámos sobre o possível papel da formação do Erg Chebbi como uma barreira a longo prazo entre as duas linhagens, em conjunto com a expansão de clima árido, durante as oscilações climáticas do Pleistoceno e Plioceno. No futuro, é preciso mais estudos a fim de confirmar esta hipótese e de avaliar se espécies que coabitam na mesma área de distribuição apresentam padrões similares aos de *U. nigriventris*.

Processos distintos e associados a eventos climáticos e geológicos do passado têm sido responsáveis por moldar os padrões de biodiversidade, nas duas espécies sarianas estudadas. São necessárias mais investigações para compreender que padrões filogeográficos e processos evolutivos estão na base da distribuição e variação genética das espécies do Magrebe Ocidental, e de forma a identificar padrões filogeográficos e respostas ecológicas idênticas. Essas conclusões irão contribuir para identificar a diversidade críptica, sendo esta um alvo prioritário para a conservação em termos de espécie e áreas.

# PALAVRAS CHAVE

Filogeografia; Magrebe Ocidental; Biodiversidade; Montanhas do Atlas; oscilações climáticas; Pleistoceno/Plioceno; *Ptyodactylus oudrii*; *Uromastyx nigriventris;*

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| | |
|---|---|
| BI | Bayesian Inference |
| BSA | Bovine Serum Albumin |
| Cyt-b | Cytochrome b |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide Triphosphate |
| GBIF | Global Biodiversity Information Facility |
| GPS | Global Position System |
| HPD | Highest Posterior Density |
| MCMC | Markov Chain Monte Carlo |
| MC1R | Melanocortin 1 receptor |
| $MgCl_2$ | Magnesium chloride |
| ML | Maximum Likelihood |
| MP | Maximum Parsimony |
| MRCA | Most Recent Common Ancestor |
| MtDNA | Mitochondrial Deoxynucleotide Triphosphate |
| My(a) | Million years (ago) |
| NCBI | National Center for Biotechnology Information |
| ND2 | NADH Dehydrogenase Subunit 2 |
| NJ | Neighbor-Joining |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| RAG-1 | Recombination Activating Gene 1 |
| RAG-2 | Recombination Activating Gene 2 |
| RNA | Ribonucleic Acid |
| Stdev | Standard Deviation |
| UK | United Kingdom |
| 12S | 12S Ribossomal RNA gene |
| 16S | 16S Ribossomal RNA gene |

# GENERAL INTRODUCTION

## 1.1. Background

### 1.1.1. Biodiversity and its conservation

Biodiversity embraces the overall variety of life, embodied in all the organisms' forms, from microorganisms to big mammals, and the interactions among them, which forms communities (DeLong 1996). The biodiversity concept encompasses different levels: the species, the genetic, and the ecosystem levels of diversity. The species level is the number of living forms, in all shape and size; while, the genetic level includes the genetic inheritance, which passes through generations; and, finally, the ecosystem level comprises species' populations and non-living environmental components, which interact dynamically to form an ecosystem and to define species' responses. The three levels cannot be separated and they interact and influence one each other.

Currently, the Global Biodiversity Information Facility (GBIF) database registers 1 643 948 species worldwide (*www.gbif.org;* Roskov *et al.* 2016). These values are positively biased by the amount of sampling efforts and studies in regions with high economic resources and access facilities, such as Europe, North America, South Africa and Australia (GBIF). It is also biased by the level of attractiveness for researchers and on the availability of institutions specialized in certain taxa or specific geographic areas (Ficetola *et al.* 2012). The number of species listed in the GBIF is likely underestimated as we are still far from an accurate estimate of biodiversity, due to limited information on species distribution and taxonomy, with the existence of species that are yet to be described (Ficetola *et al.* 2012), especially in regions such as the Maghreb (Ficetola *et al.* 2012) or the tropical rainforests of Madagascar (Vieites *et al.* 2009) or Amazonia (Fouquet *et al.* 2007). These regions provide an obstacle to biodiversity assessment because of their large size, political instability, and remoteness of habitats. These three main obstacles differently affect many understudied areas and, in some cases, such as, for the Sahara Desert, all these three type of impediments contribute to the scares biodiversity knowledge on this region (Brito *et al.* 2014). Additionally, a methodological bias in species identification can also be anticipated. Traditionally species have been identified and described based on morphological characters. However, in the last decades the use of molecular tools has revealed and increasing number of cryptic species, which shared a similar morphology, as well as of species which have been

erroneously classified, due to high similarity in appearance and often unclear boundaries between them (Bohm *et al.* 2013). Unfortunately, the real biodiversity values will be difficult to achieve, even for the best studied groups, and, consequently, some species could be extinct before to be described (Butchart *et al.* 2010; Ficetola *et al.* 2012).

The decline of biodiversity, reported in the last decades (Butchart *et al.* 2010), is highly related to the emergence of multiple threats, associated to humans activities (Vitt & Caldwell 2014). Increasingly, some local impacts, such as agricultural activities, pollution, biological resources use, introduction of invasive species and urban development, are adding up to global impacts, promoting drastic change in ecosystems. For example, global warming, described as an increase of global temperature and of ultraviolet radiation, is recognized as one major threat for biodiversity together with habitat destruction and fragmentation (Araújo *et al.* 2006; Vitt & Caldwell 2014).

The global impacts effect species in three general ways, at the demographic, genetic and environmental level (Vitt & Caldwell 2014). The demographic level comprises changes in both population size and sex ratio, which can effect reproductive behaviour and success rates (Vitt & Caldwell 2014). The genetic level includes the loss and reduction of both genetic diversity and species' adaptive plasticity, that is, the ability to change phenotype in response to environmental changes (Vitt & Caldwell 2014). The genetic effect is closely associated to the demographic effects, as small population sizes increase the strength of stochastic processes such as genetic drift due to bottlenecks or inbreeding depression, characterized by the inbreeding of close relatives, which increases homozygosity (Vitt & Caldwell 2014). Finally, the environmental level involves unpredictable changes in abiotic and biotic factors that disturbs the availability of resources and the equability of environmental conditions (Vitt & Caldwell 2014). These changes include weather and climate changes, catastrophes, predators, invasive species and diseases and parasites. All of these issues function as threats to populations and species persistence, which may or may not be able to respond to such environmental changes.

The ability of species to cope with global impacts is not equal across groups, and as a consequence some groups are generally more threatened than others. For example, herpetofauna, comprising reptiles and amphibians species, are, in general, particularly sensitive to habitat degradation and loss. In this case, the low dispersal ability, the morphological specialization on substrate type, the moderately small home ranges and the thermoregulatory constraints are the main causes of their sensitivity (Huey 1976;

Kearney *et al.* 2009). Therefore, the need to implement conservation strategies for this group is particularly high.

Conservation and management plans should be designed in order to maintain the evolutionary processes within species and ecosystems. The challenges to set conservation priorities are, in most part, the required knowledge about species diversity and distribution, and the identification of the ecologic responses and evolutionary processes behind the observed pattern of diversity (Whittaker *et al.* 2005). Hence, the study of populations and ecosystems are the first step towards conservation of biodiversity. However, it is impossible to protect the overall biodiversity and it is crucial to identify regions where a high number of species are concentrated (Myers *et al.* 2000; Brooks *et al.* 2006). This has led to the recognition of the importance of "hotspots" of biodiversity, where it could be possible to protect a larger proportion of species with the same investment (Myers *et al.* 2000). The identification of "hotspots" are only possible with multi-approach studies focused on multiple groups of organisms, and studies on these groups further require a multi-disciplinary approach integrating ecological genetic and morphological data in a biogeographical and evolutionary framework.

### 1.1.2. Phylogeographic insights into the structure of biodiversity

More than just quantifying the level of genetic variability, it is also important to understand the role of past climatic and geologic events and associated demographic and evolutionary processes in shaping the current degree of genetic diversity found, between and within species. In this regard, the application of phylogenetic methods and of models from the coalescent theory within the main field of phylogeography has provided crucial insights.

Phylogeography is the study of how genetic lineages are distributed across geographic regions, especially within and among closely related species (Avise 1998, 2009; Hickerson *et al.* 2010). The phylogeographic concept emerged in the 1990's decade, following Avise *et al*.'s (1987) work and rapidly expanded. The principal aims of this discipline is to relate historical evolutionary processes to spatial, temporal and environmental factors, in order to understand the contemporaneous spatial distribution of species and of their genetic variation (Avise *et al.* 1987; Avise 1998; Hickerson *et al.* 2010). Moreover, microevolutionary processes operating within species can be extrapolated to explain macroevolutionary differences among species and higher taxa (Avise 1998, 2009). Therefore, phylogeography is considered a multidisciplinary discipline, since the analysis and interpretation of lineages distribution requires input

from phylogenetic, population genetics, biogeography, molecular ecology, demography, geography and geology (Avise 1998, 2009; Nielsen & Beaumont 2009).

Additionally, phylogeography can be used to recognize different patterns between populations and species, for example, to identify separated lineages among geographic regions; to identify contact zones (i.e. two lineages inhabiting the same space) with genetic admixture, which leads to absence of spatial separation; to recognize isolated populations, or to identify an intermediate state (hybrids), where the levels of gene flow are considerable, which induce a partial spatial separation (Avise *et al.* 1987). These patterns reflect the way a species has been interacting with environmental and geographic space, across time, and it is influenced by the ecological requirements, which determines species responses, to the surrounding environment. Given the above, phylogeographic studies have provided an important contribution to identify ecological and evolutionary processes as conservation targets and to biodiversity assessment, detecting lineages and delimiting species.

### 1.1.3. Molecular data and species delimitation

The delimitation of species is a complex and much debated subject in science, and it has been confused by the lack of consensual species concepts (de Queiroz 2007). Nowadays, it is generally accepted that the species term is not fixed, and that biological diversity has discontinuities among morphological, genetic and ecological levels (Hausdorf 2011; Gunnarsson *et al.* 2012). The transition from a traditional species delimitation, typically based on morphological data, to a more complex and multi-scale approach, comprising different biological disciplines - the integrative taxonomy - was a key step towards a more robust recognition of species and lineages (Dayrat 2005; Padial *et al.* 2010).

Phylogeographic studies are based on molecular data, which can provide important insights into species delimitation. Molecular data and new informatics tools have changed the way biodiversity is perceived. In some cases, molecular tools are the only methodology available (Patwardhan *et al.* 2014). For example, in cryptic species complex - species that are morphological similar but with high genetic differentiation - sometimes only molecular assessments are able to recognize and to define them (Avise *et al.* 1987). Among reptiles groups such as amphisbaenians, lizards and geckos have been demonstrated numerous examples of species complex with distinct genetic lineages sharing an overall similar morphology (Perera & Harris 2010; Kaliontzopoulou *et al.* 2011; Barata *et al.* 2012b; Sampaio *et al.* 2015). In all these cases, it is crucial to

understand when genetic differences are associated to reproductive isolation and sufficient large to describe (or not) genetic groups as distinct species.

Phylogeography was developed using mitochondrial DNA (mtDNA) (Avise *et al.* 1987; Grechko 2002) but rapidly studies started to incorporate nuclear data (Saint *et al.* 1998; Hare 2001; Godinho *et al.* 2008; Avise 2009; Hoshino *et al.* 2012). The mtDNA was the first choice for phylogeographic studies for numerous reasons. Firstly, the number of varied sites, in mitochondrial DNA, is higher because of the higher evolutionary rate (Hewitt & Zhang 2003; Avise 2009), which becomes useful for studies focus on population and closely related species. However, in ancient population events, mtDNA may not be able to reconstruct the phylogenetic history, since more slow evolving genes are required (Hewitt & Zhang 2003; Patwardhan *et al.* 2014). The high evolutionary rate is a consequence of relatively inefficient mechanisms of DNA repair in the mitochondria (Hurst & Jiggins 2005). Also, the mitochondrial genome is haploid, meaning that each individual carries only a unique sequence (Avise 2009), and is maternally inherited. This simplifies the genetic history of a taxa, and part of the information will be lost (Hewitt & Zhang 2003). On the other hand, nuclear markers allow us to have multiple copies of the genetic signature, from both parents. Furthermore, the mutation rate is typically lower, which means a slower genetic evolution. Nevertheless, this type of data also comes with some problems and challenges, such as the presence of recombination, heterozygosity, insertion/deletion polymorphism, and low divergence but also, the gene specific variation in rate and history, and the practical difficulties of working with one single copy within the nuclear genome (Avise & Wollenberg 1997; Hewitt & Zhang 2003; Avise 2009; Hoshino *et al.* 2012). In sum, both molecular markers contribute to reveal different aspects of a complex evolutionary history, at different depths of perception, and thus are complementary (Hewitt & Zhang 2003). Moreover, phylogenetic and phylogeographic studies should follow a multi-locus approach, since a single gene tree only provides a single realization of a stochastic process. In order to reconstruct species or population evolutionary history, we need to sampling many gene trees and combining their genealogical information.

## 1.1.4. Phylogenetic tools

Inferring phylogenies is not an easy procedure and becomes more difficult with large molecular datasets (Blair & Murphy 2011). Fortunately, several approaches are now available with different advantages and disadvantages, although none of them is the perfect method. Moreover, the phylogenetic revolution has allowed phylogenetic tools to

be used at the intraspecific level to reconstruct genealogies from the sampled alleles, within populations. The most common analysis methods include Neighbor-joining (NJ) (Saitou & Nei 1987), Maximum parsimony (MP) (Fitch 1971), Maximum likelihood (ML) (Felsenstein 1981) and Bayesian inference (Huelsenbeck & Ronquist 2001). The use of probabilistic methods, such as ML and BI, has gained popularity due to their ability to incorporate various models of sequence's evolution to best explain the data (Blair & Murphy 2011).

The Maximum likelihood method tests a hypothesis, estimating an evolutionary tree that better predicts the observed data, assigning quantitative probabilities to mutational events in order to compare possible trees and find the final tree with the highest probability value (this probability is the likelihood score of the tree). There are various software available to perform this method and to get the statistical support for the branch nodes, such as PAUP (Swofford 2002) and PHYML (Guindon & Gascuel 2003). Recently, more sophisticated ML algorithms, such as, GARLI (Zwickl 2006) and RaxML (Stamatakis 2014) have been developed for a rapid analysis, and the last one is able to deal with large datasets incorporating mixed models (Blair & Murphy 2011).

The Bayesian Inference method uses similar models to ML, but it produces a posterior probability that fits to the data and with a confidence value estimated, evaluating features in common among the sampled trees. The Markov chain Monte Carlo (MCMC) tool is then used to infer the posterior probabilities values for each tree. The posterior probability of a tree can be interpreted as a summation over all trees and, for each tree, integration over all possible combinations of the branch lengths and the substitution models. Additionally, some packages, such as BEAST (Drummond *et al.* 2012), are now able to implement relaxed molecular clocks and coalescent models, in a Bayesian framework, which is particularly adequate for phylogeographic studies.

The coalescent theory is a retrospective stochastic mathematical model that predicts the time of the common ancestor (MCRA) between two lineages, which is the time when two lineages coalesce into a single lineage (Kingman 1982). The theory also predicts that the rate at which lineage coalesce depends on the size of a population, since genetic drift is a factor, so that smaller populations will have a faster rate of drift and, consequently, of coalescent lineages (Lemey *et al.* 2009). This theory has been applied to genes trees, within a population, in order to understand the phylogenetic history of a species (Hey *et al.* 2005; Nielsen & Beaumont 2009). Each gene has an associated mutation rate, which is defined by the number of mutations that are expected in each generation and influenced by stochastic sorting (and in some cases by selection), which

results in different patterns across different genes (Lemey *et al.* 2009). Thus, the phylogenetic trees result in a combination of all the mutation processes that were absorbed, through time, within populations.

Lastly, for more recent divergences such as at the intraspecific level, the interactions between sequences sampled can be represented by networks, since it offers more resolution in terms of haplotype relationships (Posada & Crandall 2001). This method is able to represent alternative evolutionary paths as recombination or homoplasies by inserting loops into the network (Nielsen & Beaumont 2009). The most used methods are the statistical parsimony approach, implemented in TCS (Clement *et al.* 2000) and the median joining approach, implemented in NETWORK (Polzin, 2014-2016), for example. The statistical parsimony estimates the maximum number of differences among the haplotypes and connects them by the number of different sites (Posada & Crandall 2001). On the other hand, median joining network is a simple model that defines simple distances between haplotypes, based on the number of mutations in each nucleotide position.

## 1.2. Study region and study species

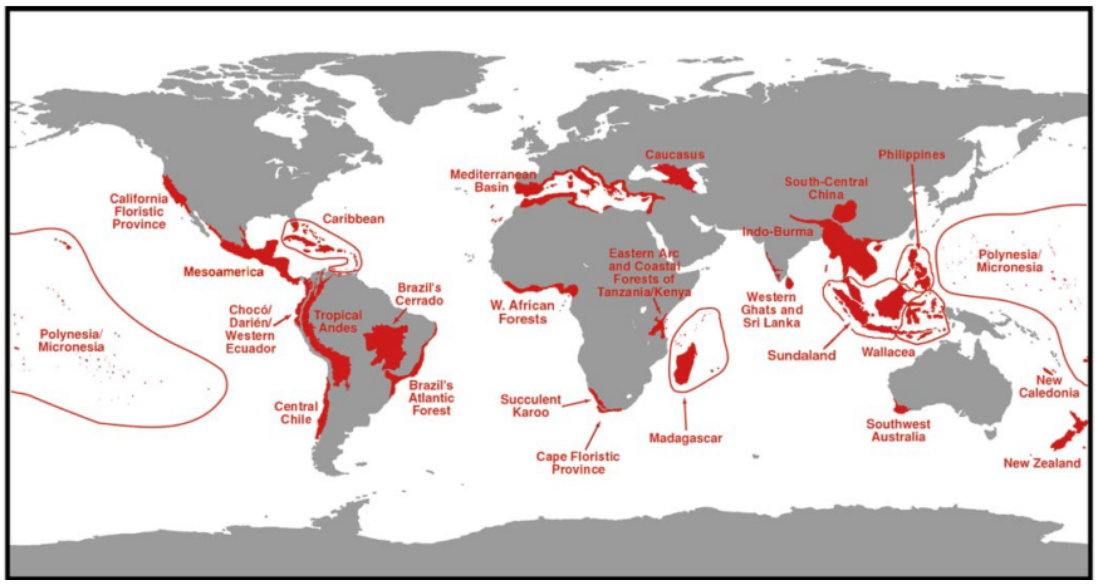


**Fig. 1**- The 25 hotspots of biodiversity in the world. (Source: Myers *et al.* 2000)

This study aims to uncover the patterns of genetic diversity of two species inhabiting the North Africa region, and in particular within the Maghreb. This region is included in the Mediterranean global biodiversity hotspot (Myers *et al.* 2000; Fig. 1) and has been identified as one of the understudied region for which a high portion of biodiversity is yet to be described (Ficetola *et al*. 2012). The general aim of this study is therefore to

contribute our understanding on the evolutionary processes responsible for the observed patterns of biodiversity in this understudied region.

## 1.2.1.The Maghreb region, a hotspot of diversity

The Maghreb region includes Morocco, Algeria, and Tunisia. The heterogeneity of climatic conditions and the topographical complexity of this region coupled with past climatic changes and geologic events have determined the richness and the diversification of habitats from Mediterranean environment to very arid deserts, including also mountainous areas (Le Houérou 1997). A great level of biodiversity is hosted in the Maghreb, especially within reptiles (Bons & Geniez 1996; Padial 2006; Pleguezuelos *et al.* 2010).

### 1.2.1.1. Climate evolution of the Maghreb

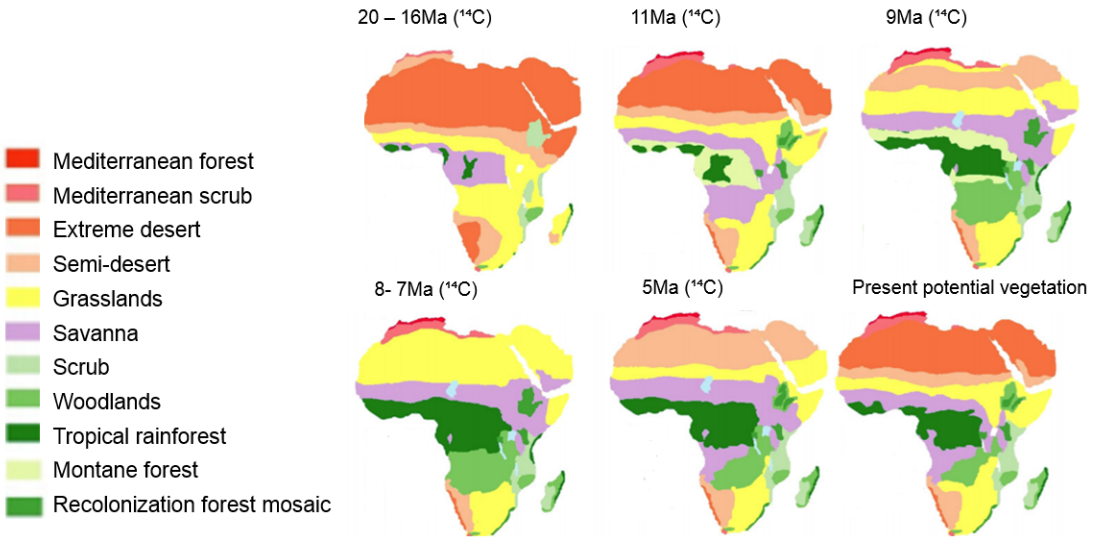


**Fig. 2 -** Evolution of the African land cover during the last 20Mya. Modified from www.esd.ornl.gov/projects/qen/nercAFRICA.html.

The African continent has experienced different climatic conditions during the Cenozoic Era with associated changes in ground cover and habitat distribution (Fig.2) (Gasse 2006; Kröpelin *et al.* 2008). The thermal maximum with a more humid climate was registered during the middle Miocene (between 17-15My ago) (Feakins & Demenocal 2010). These periods were characterized by high levels of precipitation, feeding rivers and lakes and to cover regions with rain forest vegetation (Demenocal 1995; Kohler *et al.* 2010). In the late Miocene the climate changed towards aridity, leading to the emergency of dry and open woodlands, fragmenting the previously continuous forest

habitat (Pound *et al.* 2012). These climatic changes promoted the expansion of ranges in several species adapted to arid environments such as agamids and geckos of the genera *Uromastyx* sp. (Amer & Kumazawa 2005), *Agama* sp. (Gonçalves *et al.* 2012), *Ptyodactylus* sp. (Metallinou *et al.* 2015) and *Saurodactylus* sp. (Rato & Harris 2008) .

The Pliocene and Pleistocene periods were characterized by more intense climatic changes with oscillation between cold and dry periods and hot and humid periods. In high latitudes of the Northern Hemisphere alternate cycles of glacial and inter-glacial stages were associated to contraction and expansion (respectively) of temperate biomes, (Hewitt 1996, 2000). In subtropical and tropical regions, the glaciation events between 3.2Mya and 2.6Mya and the associated southern spread of polar ice sheets promoted the movement of warmer and vegetation zones towards the Equator (Demenocal 1995; Hewitt 2000). During such stages the clime became drier and arid, the desert and savannah areas increased so that the Sahara desert considerably increased in size (Demenocal 1995; DeMenocal 2004; Kohler *et al.* 2010). Between 2.8Mya and 1Mya, during the inter-glacial phase, the north African climate was moderate and the aridity decreases (Demenocal 1995), allowing the evolution of temperate xerophytic woods and warm mixed forest (Prentice *et al.* 2001). A new glacial maximum at 1.0Mya was characterized by intense drier and arid climate conditions in subtropical and tropical African regions (Sarnthein 1978; Demenocal 1995).

These rapid climatic fluctuations had a profound effect on the distribution of fauna and flora, with notable consequences in the genetic backgrounds of species (Hewitt 1996; Schmitt 2007; Kröpelin *et al.* 2008). The repeated expansion and contraction of species ranges caused several events of isolation followed by secondary contact and admixture. The short time frame in which climate condition changed forced rapid species' responses (Demenocal 1995). Mountainous regions had a greater range of life-supporting environment, through the presence of a continuous climatic altitudinal shifts (Hewitt 2001). For example, in Europe mountains provided more suitable opportunities for cold-adapted species, even at low altitudes, during the glacial times; and also, for temperate species, which during the interglacial times extended their ranges to higher altitudes. In contrast arid adapted species, from North Africa region, were favoured during glacial times, since they had suitable habitats in higher altitudinal ranges. Several studies have shown how in many group of organisms such as mammals (Cossons *et al.* 2005) and reptiles (Harris *et al.* 2004a; b; Fritz *et al.* 2005) the main genetic subdivision in genetic lineages or separation of sister taxa observed in North African species are associated to Miocene climatic events suggesting a common response, of these species, to past

climate related environmental changes (Taberlet *et al.* 1998). However, in other cases species showed an individual response to similar events that affected in a different way their pattern of distribution and of genetic variation (Stewart & Lister 2001).

### 1.2.1.2. Geologic evolution of the Maghreb

Concerning to geological aspects, the Maghreb is situated in a triple junction between the Africa continent, the Atlantic Ocean and the active plate collision zone of the Alpine bel system (Michard *et al.* 2008). As a consequence of diverse tectonic systems, several geological episodes have shaped the topography and influenced the weather in the Maghreb region (Michard *et al.* 2008; de Lamotte *et al.* 2009). The distribution of flora and fauna were remarkable affected by these geological events, and also at the intraspecific level, with major changes in past population connectivity and isolation, which had consequences in the evolution of genetic lineages. Phylogeographic literature on species occurring in western Maghreb have shown two major biogeographic barriers involved in shaping the current pattern of species distribution and of their genetic variation: the Strait of Gibraltar and the Atlas mountains.

#### 1.2.1.2.1. The Strait of Gibraltar

The Western Mediterranean Basin has suffered intensive geological changes during the Cenozoic, since it is located in a convergent plate's margin, between Euroasia and African plates (Fig.3) and which forms a subduction zone in the western part of the basin. The slow convergence between Euroasia and Africa led to a drifting and rotation of several continental terrains, such as Corsica, Sardinia, the Baleartic Islands, the Kabylies blocks, Calabria and the Betic-Rif (Rosenbaum *et al.* 2002). The dispersion, collision and deposition of land fragments allowed the formation of diverse marine zones, as the Alborán Sea, the Algerian-Provençal Basin, the Valencie Through, the Ligurian Sea and the Thyrrhenian Sea (Rosenbaum *et al.* 2002). This region was not only influenced by glacio-eustatic and tectonic processes, but also by the orogenic systems, from North Africa and Western Europe (Krijgsman *et al.* 1999). The orogenic uplift of the Alborán basin (16-14Mya) created the betic-rif corridor, linking the southern part of the Betic region to the African continent, and formed the present Rif Mountains (Rosenbaum *et al.* 2002). Later (8-10Mya), the fragmentation of the betic-rif corridor started to occur

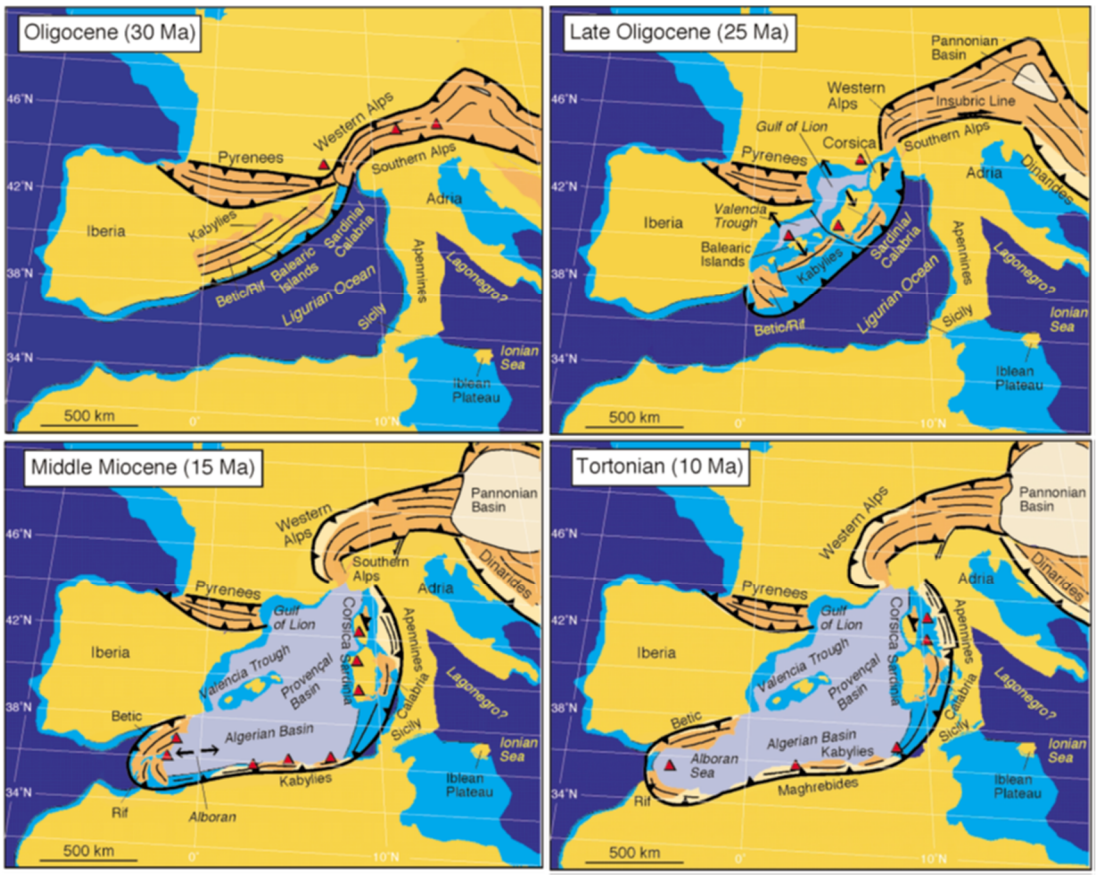


**Fig. 3 –** Geological events retracting the opening and re-opening of the Strait of Gibraltar and the consequences for the Mediterranean Sea. (Source: Rosenbaum *et al*., 2002)

(Rosenbaum *et al.* 2002). Additionally, at about 5.96My ago, the southern Iberia land masses and the Strait of Gibraltar were connected, but the causes remain unclear (Krijgsman *et al.* 1999; Rosenbaum *et al.* 2002). Consequently, the Mediterranean became isolated from the Atlantic Ocean, successive evaporitic events and a subsequent desiccation of the Mediterranean Sea, resulting in the Messinian Salinity Crisis (Krijgsman *et al.* 1999). Around, 5.33My ago the re-opening of the Strait of Gibraltar allowed the Mediterranean to refill, remaining until the present day (Krijgsman *et al.* 1999). These palaeogeographic changes had repercussion on the distribution of fauna and flora, across the Western Mediterranean basin, between Europe and Africa. The presence of land corridors, connecting these two continents, allowed migration and expansion, from Europe to North Africa and vice versa, of many territorial species, such as terrapins, *Testudo graeca* (Alvarez *et al.* 2000); lizards, including *Podarcis vaucheri* (Pinho *et al.* 2006; Kaliontzopoulou *et al.* 2011), *Acanthodactylus erythrurus* (Harris *et al.* 2004a; Fonseca *et al.* 2009) and *Psammodromus algirus* (Carranza *et al.* 2006b); geckos, such as *Tarentola* forms (Harris *et al.* 2004b), and snakes, including *Natrix maura* (Guicking *et al.* 2008), *Hemorrhois hippocrepis*, and *Malpolon monspessulanus* (Carranza *et al.* 2006a). Consequently, vicariance and speciation events were promoted,

since both continents were disconnected, which modified the genetic structure of species and populations on both sides of the Western Mediterranean Sea.

#### 1.2.1.2.2. The Atlas Mountains

The Atlas comprises different Mountains belts: the High, the Middle and Anti-Atlas in Morocco, the Saharan Atlas and Aurès Mountains in Algeria, and the Tunisian Atlas in Tunisia (Fig.4). The Atlas Mountains are not geologically homogenous across their range, with major differences due to distinct and exclusive deformations (faults and compressions) separating the Moroccan Atlas, mainly the High Atlas region, from the Algerian and Tunisian complex (Piqué *et al.* 2002; Michard *et al.* 2008; de Lamotte *et al.* 2009). Also in terms of elevation the Atlas Mountain are not homogeneous with altitude decreasing from west to east. The highest peaks are found in Morocco in the High Atlas (Jebel Toubkal, 4167 m above sea level) and also in the Anti-Atlas range many peaks exceeds 3000m, (Michard *et al.* 2008). Lower altitudes are recorded in the Algerian and Tunisian mountains (Michard *et al.* 2008; de Lamotte *et al.* 2009).

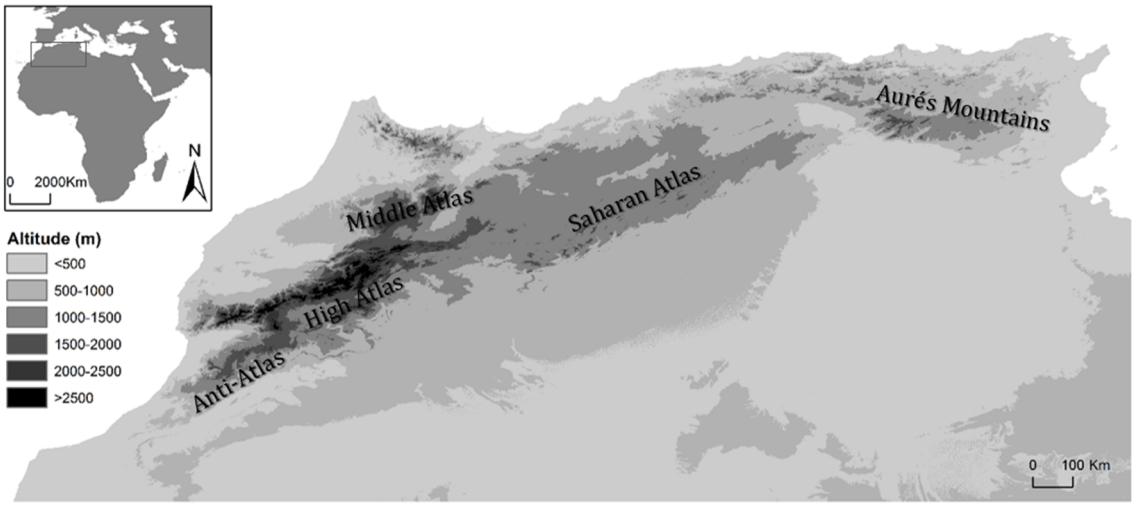


**Fig. 4 –** Representation of the Atlas Mountains complex, from North Africa.

Several works, based on different methods, agree that the formation of the Atlas system was due to tectonic events occurring during the Cenozoic (Missenard *et al.* 2006; Babault *et al.* 2008, 2012; de Lamotte *et al.* 2009) and it was similar in terms of age and intensity, for the entire system (de Lamotte *et al.* 2009). However, those tectonic events are not able to explain the differences found, in altitude, across the Atlas, which seems to be due to the presence of a thermal anomaly component, formed also during the Cenozoic and present in the western region of the Atlas - the “Moroccan Hot Line” (Missenard *et al.* 2006; de Lamotte *et al.* 2009). The formation of the Atlas Mountains included two major events which are distinct in time. The first event is dated to the Late Eocene and

it consisted in a generalized tectonic episode characterized by a depression of the Atlas regions relative to adjacent areas, starting the creation of elevations and by a related inversion of normal faults (Piqué *et al.* 2002; Missenard *et al.* 2006; Babault *et al.* 2008; de Lamotte *et al.* 2009). The second event, occurred in the middle-late Miocene, during the Tortonian stage (11.6 - 7.2My) and is present up to now, consisting of a second crustal shortening and relief building by compressive activity (de Lamotte *et al.* 2009). These time estimations are in agreement with the irregularity of temperature (thermal anomaly), generated by convection currents of the mantle, also underlined by the beginning of volcanism activity in the Morocco region (Missenard *et al.* 2006). However, some dating, based on geomorphological details, have associated this second event with the convergence forces in the subduction zone of Africa-Euroasia plates and with the Messinian Salinity Crisis, revealing a more recent age for the main uplift episode of the Middle and High Atlas (7.1 – 5.3My), in Pliocene to Quaternary, (Gomez *et al.* 2000; Lamotte & Mercier 2000; Babault *et al.* 2008). Moreover, the authors that refer the middle late Miocene, for the main uplift events in the Atlas Mountains, suggest that a more recent (Pliocene to Quaternary) and independent deformation occurred in the Anti-Atlas region (Missenard *et al.* 2006; Babault *et al.* 2008).

#### 1.2.1.3. Species responses to paleoenvironmental changes in the Western Maghreb

The Western Maghreb region was clearly affected by the climatic and geologic events above mentioned, and the species inhabiting this region were not an exception. Species adapted to arid environments have experienced expansion of their ranges, during the increase of arid conditions, in the middle Miocene, and they have been isolated during the humid and temperate periods. Temperate species, in turn, have been isolated, such as in mountains, during the arid periods and they have expanded their ranges during more temperate environmental conditions. However, even within main ecological types, species are able to respond individually, since dispersal ability and habitat type preference vary according different species. For example, species with high dispersal abilities and tolerance of a wide Mediterranean and sub arid condition were less impacted by past climate changes, such as the case of snakes *Hemorrhois hippocrepis* and *Malpolon monspessulanus*, which show a continuous distribution in a large area of North Africa and South Europe, with limited phylogeographic structure and genetic variation (Carranza *et al.* 2006a). On the other hand, species highly specialized in one habitat type with more ecological requirements and limited dispersal ability tend to be severely impacted by past climate and geologic events. In general geckos are small

reptiles, limited to rock or ground-dwelling habitat and they have low dispersal abilities, consequently, they have shown high levels of population fragmentation in distinct lineages, likely as consequence of past prolonged isolation, as in the case of *Ptyodactylus oudrii* (Perera & Harris 2010) and *Saurodactylus* sp (Rato & Harris 2008).

Most phylogeographic studies have focused on the species distributed to north of the main mountain range of the Western Maghreb, and also on mountain species, such as *Quedenfeldtia* sp. (Barata *et al.* 2012b) and *Atlantolacerta andreanskyi* (Barata *et al.* 2012a). Unfortunately, little is known about distribution and genetic structure of species inhabiting in the south of the Atlas Mountains, specifically in arid and semi-arid northern regions of the Sahara desert – usually called the north Saharan species (Geniez *et al.* 2000).

## 1.2.2. North Saharan species

The species inhabiting semi-desert habitats, in the northern margin of the Sahara desert, are included in the north Saharan species category (Geniez *et al.* 2000). North Saharan species includes different reptile species, including following genera: lacertids *Acanthodactylus* and M*esalina*, geckos, *Stenodactylus stenodactylus* and *Tropiocolotes tripulitanus*, snakes *Coluber algirus and Telescopus obtusus*, vipers from the *genus Cerastes* and amphibians, *Bufo brongersmai* (Bons & Geniez 1996; Geniez *et al.* 2000). In this thesis we focused our attention on two north Saharan reptiles, endemic of the western Maghreb: Oudri's fan-footed gecko, *Ptyodactylus oudrii* (Lataste1880)*,* and the Moroccan Spiny-tailed lizard, *Uromastyx nigriventris* (Rothschild & Hartert 1912)*.*

### 1.2.2.1. The Oudri's fan-footed gecko, *Ptyodactylus oudrii*

*Ptyodactylus* is one of the most characteristics genera of geckos of the Phyllodactylidae family (Fig.5). Phyllodactylidae includes 7-10 different genera, characterized by the presence of digits lamellae in the form of a fan (Gamble *et al.* 2008). The studies of Gamble and colleagues (2008, 2011) showed that the "leaf-toed" morphology (defined as digits with broad, divided, terminal scansors) evolved independently several times, with clear evidence of convergent or parallel evolution with gains and losses in distinct lineages (Gamble *et al.* 2012; Fig.5). The evolutionary history of this family was only recently investigated, mainly due to the large area of distribution, to insufficient data, to taxonomic uncertainties and to the poor performance of morphology assessments in identifying species groups (Gamble *et al.* 2008).

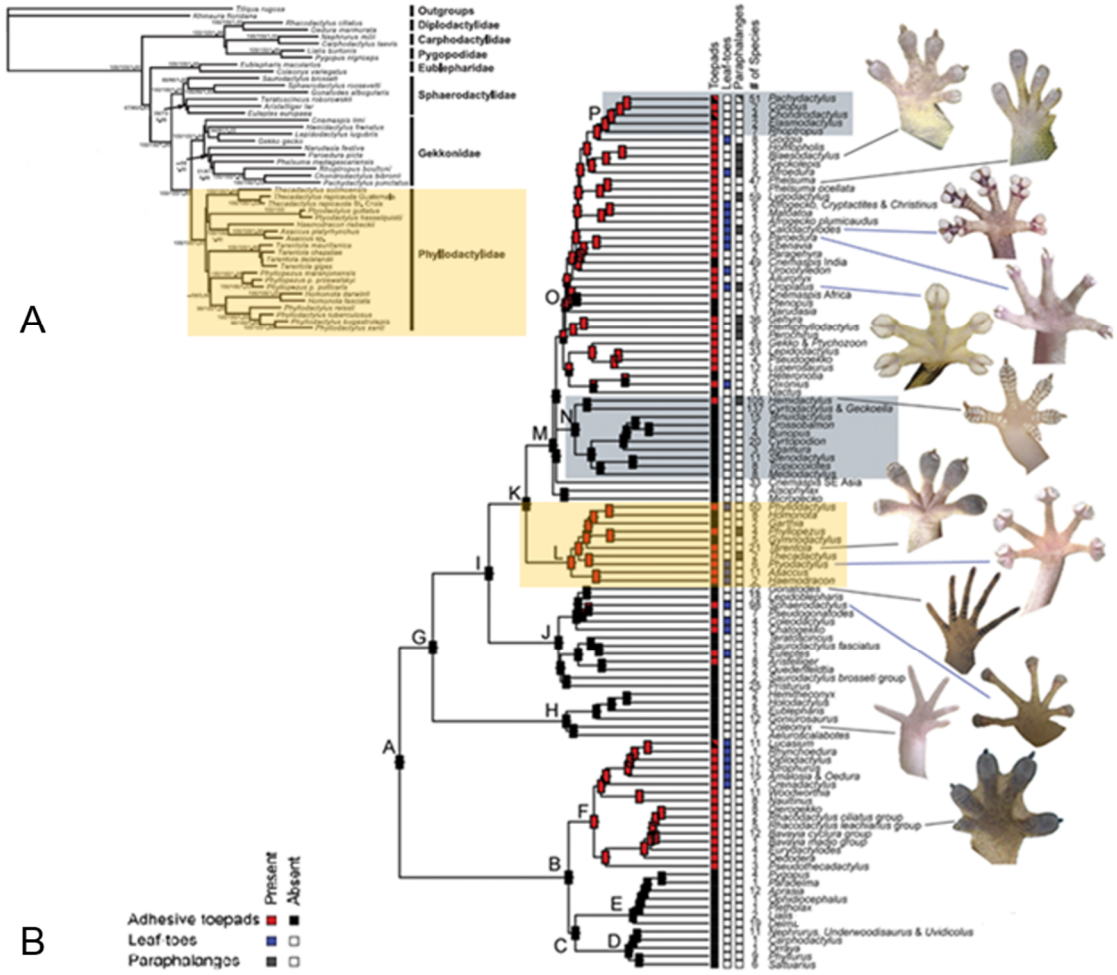


**Fig. 5 – A.** Phylogeny of the Gekkota families, with emphasis to Phyllodactylidae family, based on Maximum Likelihood, Maximum Parsimony and Bayesian Inference methods (Modified from Gamble *et al*. 2008); B. Gecko phylogeny and the repeated evolution of adhesive toepads, based on Maximum Likelihood approach (Modified from Gamble *et al*. 2012).

The origin of the genus *Ptyodactylus* was estimated in the Late Oligocene and the diversification of the group started approximately 27My ago, during the expansion of arid habitats in the middle Miocene (Metallinou *et al.* 2015). A similar timing for the diversification was estimated for the arid-adapted species of the genus *Agama* (Gonçalves *et al.* 2012). It is likely that the range of rock dwelling geckos, such as *Ptyodactylus*, has been restricted by the increase of sand habitat, during the middle Miocene, given the requirement of these species for hard substrate. The extreme specialization of *Ptyodactylus* geckos to rocky substrate and to rock-dwelling life style implies that all species in this genus, even between phylogenetically distant species, shared similar morphological characters and habitat, as a result of a high conservatism of these two aspects (Metallinou *et al.* 2015). The taxonomy of *Ptyodactylus* genus is not completely resolved. Six species are currently described: *P. guttatus* (Heyden 1827), *P. puiseuxi* (Boutan 1893), *P. homolepsis* (Boutan 1893), *P. ragazzii* (Anderson 1898), *P. hasselquistii* (Donndorf 1798) and *P. oudrii* (Lataste 1880). The last two taxa represent

species complexes with unclear species separation between constituent species (Perera & Harris 2010; Metallinou *et al.* 2015). Baha El Din (2006) "tentatively" tried to elevated *P. siphonorhina* (Anderson 1896) as a species, completing seven species in total, and its genetic differentiation was confirmed by Metallinou *et al.* (2015). More, recently, the taxonomic revision by Nazarov *et al*., (2013) described three species within the *Ptyodactylus hasselquistii* complex, *P. ananjevae*, *P. dhofarensis* and *P. orlovi* however, the use of few individuals from single localities and the lack of geographic information of the distribution of these three species led to them being treated as part of the *Ptyodactylus hasselquistii* complex (Metallinou *et al.* 2015).

The Oudri's fan-footed gecko, *Ptyodactylus oudrii* is endemic to Morocco, Algeria and Tunisia and is distributed along rocky habitats in the south slopes of the Atlas Mountains complex (Fig. 6 and 7) (Bons & Geniez 1996; Perera & Harris 2010). Very recently it was found in the oriental foot-hills of the Middle Atlas (François *et al.* 2016).

**Fig. 6 –** An exemplar of *Ptyodactylus oudrii* species (on the left) and its habitat type (on the right).

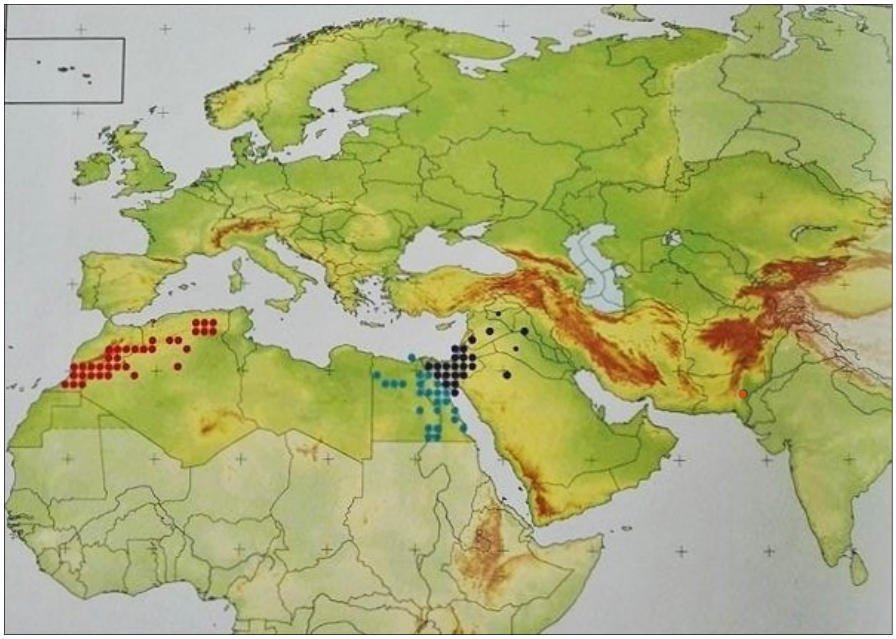

**Fig. 7 –** Distribution map of *Ptyodactylus oudrii* (in red), *P. guttatus* (in blue) and *P. homolepsis* (in brown). (Source: Sindaco & Jeremcenko 2008)

In the past, this species was included as a subspecies of *P. halssequistii* and only later, based on electrophoretic and morphological studies and taking into account the differences found in the biogeographic pattern, *P. oudrii* was raised to the species level (Heimes 1987). Previous phylogeographic studies revealed considerable intraspecific genetic variation, and four main genetic lineages have been identified, based on mitochondrial and nuclear markers (Fig. 8 and 9) (Perera & Harris 2010; Metallinou *et al.* 2015). These genetic lineages have a mainly allopatric distribution: one lineage is restricted to Algeria, and the other three lineages are endemic to Morocco: two from the High Atlas (one in the west and another in the east), and one lineage from the Anti-Atlas, in south Morocco. The Algerian lineage is the more divergent (Perera & Harris 2010; Metallinou *et al.* 2015), however the few samples analysed are not enough to recognize possible sublineages divided by barriers. The additional samples, from the western Algeria, analysed in Metallinou *et al*. (2015) allowed to speculate on the presence of a contact zone between this lineage and the one inhabiting the eastern parts of the High Atlas.

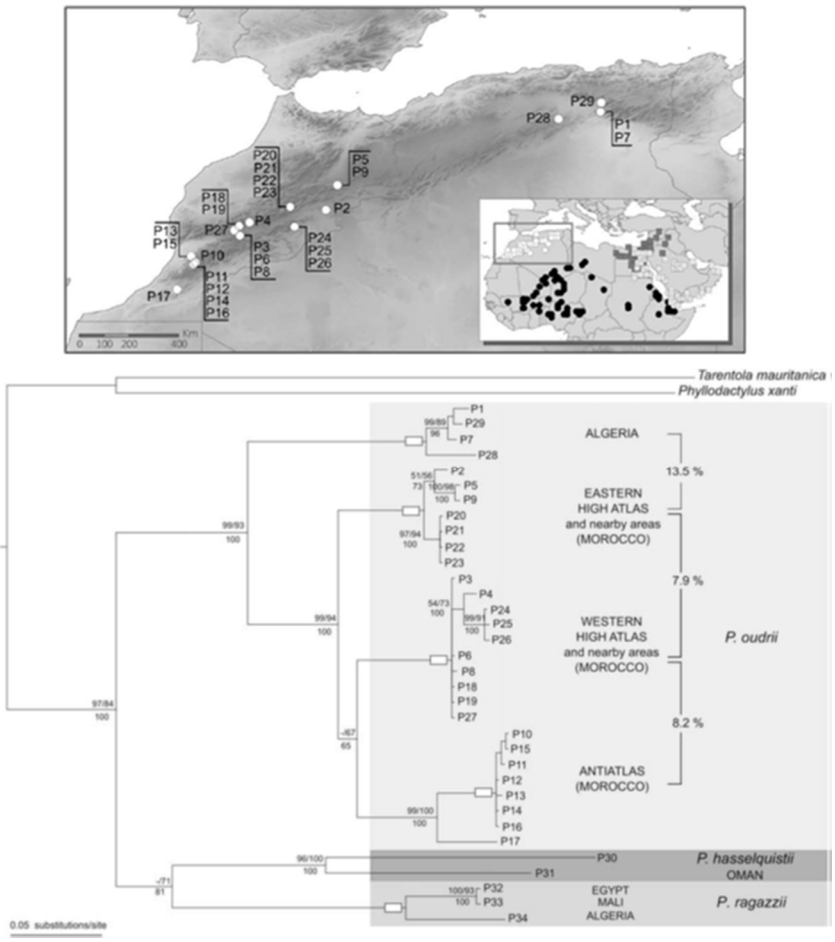


**Fig. 8 -** Samples localities of *Ptyodactylus oudrii* used in Perera & Harris, 2010 study (on the top) and the correspondent phylogenetic tree, of 12S and 16S rDNA genes, based on Maximum Likelihood and Bayesian Inference methods. (Source: Perera & Harris 2010).

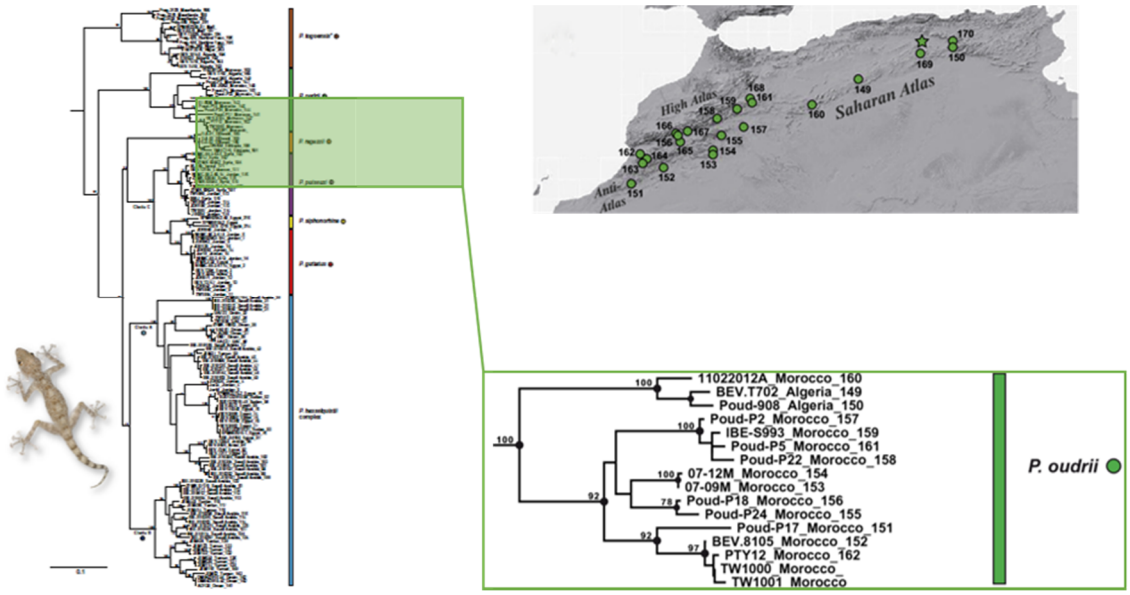


**Fig. 9 -** Samples localities of *Ptyodactylus oudrii* used in Metallinou *et al. 2015 (*on top-right) and Maximum Likelihood phylogenetic tree of the genus *Ptyodactylus*, emphasizing the results obtained for *P. oudrii* using six-locus concatenated dataset (Source: Metallinou *et al.* 2015).

Concerning the three Moroccan lineages, again high genetic variation was found (Perera & Harris 2010; Metallinou *et al.* 2015). Clearly, *Ptyodactylus oudrii* constitutes a cryptic complex with high intraspecific variation, however no information was available regarding the level of genetic admixture in areas where divergent lineages potentially meet. Additionally, it is interesting to understand if the Atlas Mountains could be the more plausible cause for the observed pattern of genetic structure, as suggested by Perera & Harris (2010), or other paleogeologic or paleoenvironmental event that occurred in the Maghreb region.

### 1.2.2.2. The Moroccan Spiny-tailed lizard – *Uromastyx nigriventris*

The first description of *Uromastyx* specimens dates back to the second half of the 18th century, as *Lacerta aegyptia* (Wilms & Böhme 2007; Wilms *et al.* 2007). The genus belongs to the Agamidae family, which together with Chamaeleonids forms the Acrodonta group. The monophyly of Agamidae is not consensual between authors (Joger 1991; Honda *et al.* 2000; Okajima & Kumazawa 2010), but several studies have identified Uromastycinae and Leiolepidinae subfamilies as sister taxa, and this pair being in turn, sister taxon to all remaining Agamidae (Okajima & Kumazawa 2010).

The diversification within the genus, *Uromastyx*, is associated to geologic and climatic episodes during the Miocene and Pliocene (Amer & Kumazawa 2005). Rögl (1998) based on paleogeographic reconstructions, demonstrated that the principal factors

responsible for diversification of African and Arabian groups were the tectonic activities, which have separated Africa from other continents, and, then, the formation of a non-permanent Gomphoterium land bridge connecting the Eurasia and Africa plates, in the Arabian Peninsula region (Rögl 1998). Additionally, the climate changes towards aridity, during the middle/later Miocene, led to an expansion of grasslands and desert environment, interrupting the continuous African forest (Demenocal 1995). Based on genetics, the estimated time of divergence between the main African and Arabian forms of *Uromastyx* (11-15Mya), coincides with the expansion of arid regions during the middle Miocene (McClanahan & Young 1996; Amer & Kumazawa 2005). The last divergent taxa within the genus are the North Africa's species, such as *U. acanthinura* and *U. nigriventris* (Amer & Kumazawa 2005). One possible scenario for the split of North African species is the repeated cold and warm cycles, which led to habitat fragmentation and isolation of local populations, resulting in rapid speciation events. Similar patterns of diversification were also found for other reptile desert species, such as *Acanthodactylus* sp. (Tamar *et al.* 2016), *Pseudotrapelus* sp. (Melnikova *et al.* 2015) and *Agama* sp. (Gonçalves *et al.* 2012).

The spiny-tailed lizards are large generalist herbivores, feeding on the scarce vegetation across the Old World desert belt region, from North Africa to north western India (Wilms & Bohme 2001; Amer & Kumazawa 2005; Harris *et al.* 2007; Wilms *et al.* 2007). The distribution of these animals is primarily limited by the availability of food and appropriate thermal refuges (Wilms *et al.* 2009). *Uromastyx* spp. is included in the Appendix II of CITES, since they are commonly collected both for food and the pet trade (Harris *et al.* 2007; Wilms *et al.* 2007; Ching & Chng 2016). Therefore, it increases the need to understand the conservation status of the species within this genus.

Currently there are 15 recognized species: *U.benti* (Anderson 1894), *U. ornata* (Heyden 1827), *U. ocellata* (Lichtenstein 1823), *U. princeps* (O'Shaughnessy 1880), *U. aegyptia* (Forsskål 1775), *U. alfredschmidti* (Wilms & Böhme 2001), *U. shobraki* (Wilms & Schmitz 2007), *U. thomasi* (Parker 1930), *U. yemenensis* (Wilms & Schmitz 2007), *U. macfadyeni* (Parker 1932), *U. occidentalis* (Mateo, Geniz, López-Jurado & Bons 1998), *U. dispar* (Heyden 1827), *U. geyri* (Müller 1922), *U. acanthinura* (Bell 1825) and *U. nigriventris* (Rothschild & Hartert 1912). The later four species occur in the Northwest Africa region, forming the *Acanthinura* group.

Morphological and molecular studies on the genus *Uromastyx* have shown difficulties regarding delimitation of specific and subspecific taxa leading to an uncertain taxonomy of this group (Wilms & Bohme 2000; Wilms *et al.* 2007). The first studies based solely on

morphological characters (Wilms & Bohme 2000, 2001; Wilms & Böhme 2007) faced difficulties due to the overall, morphological similarity of *Uromastyx* species, likely due to similar ecological adaptations. Therefore most of the morphological characters used have little value for inferring phylogenetic relationships and have resulted in taxonomic instability as in the case of *U. nigriventris* (Wilms & Bohme 2001; Wilms *et al.* 2007). Early molecular assessment based on immunological data (1991), suffered from poor immune-distance estimates, as a result of subjective estimate of the intensity of precipitin-arc, and were based on an insufficient number of samples (Wilms & Bohme 2001). Harris *et al.* (2007), based on mitochondrial markers (rDNA 12S, rDNA 16S and Cyt-b) suggested a revision of the taxonomic status of the North Africa's *Uromastyx* species. They analysed 20 individuals from Mauritania, Morocco, Libya and Niger and found four distinct groups (Fig.10). The specimens assigned as *U. acanthinura* formed two paraphyletic and not sister groups, one from Morocco and the other from Libya (Harris *et al.* 2007). Although, they only used one specimen from Libya, it was suggested that *U. acanthinura* should be split into two different species. In the same year, it was published a work based on 16S and 12S (Wilms *et al.* 2007), where it was demonstrated that *U. acanthinura* and *U. nigriventris* (Lineage C in Figure 10) should be considered as independent species (Fig. 11).

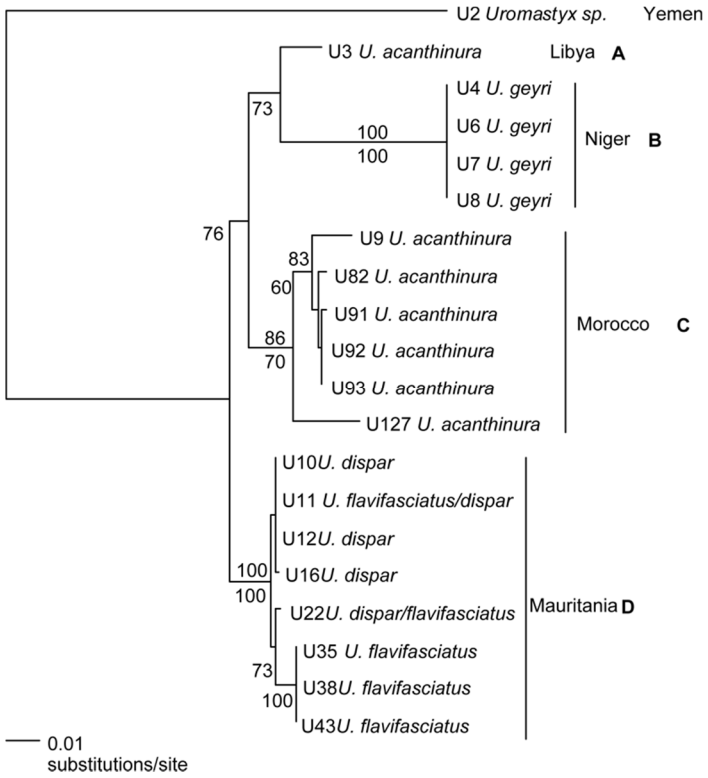


**Fig. 10 –** Phylogenetic tree inferred by Maximum Likelihood methods from North African *Uromastyx* species, based on rDNA 12S, 16S and Cyt-b genes. (Source: Harris *et al.* 2007).

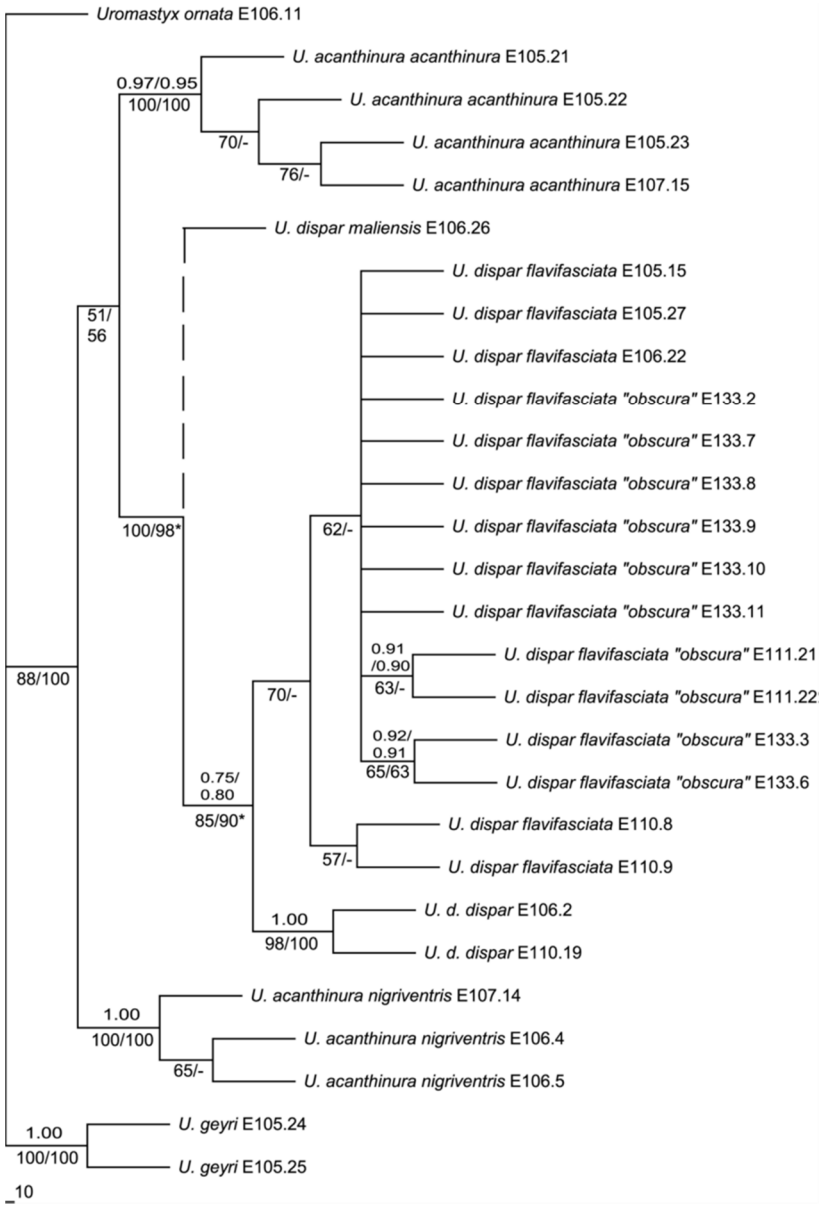


**Fig. 11 -** Cladogram of the tree recovered by the analyses based on the combined 16S and 12S rDNA genes, for the acanthinura-group. Upper values at the nodes are Bayesian posterior probabilities; lower values on the right are maximum-parsimony bootstrap replicates; lower values on the left are neighbor-joining bootstrap. (Source: Wilms *et al.* 2007).

The taxon *nigriventris* is restricted to semi-arid deserts from west, east and south of the Atlas Mountains, in Morocco, and to Sahara Atlas in western Algeria (Fig.12 and 13; Wilms *et al* 2007). Harris *et al*. (2007) identified a relatively high within-group variation, up to 4.1% at the cytochrome b gene between samples from the Morocco region. However, no further work has been done in order to identify genetic groups across the species' distribution.

**Fig. 12 –** Exemplar of *Uromastyx nigriventris* species, one adult (on the top-left; Source: Salvi, D.) and one juvenile (on the top-right) specimen, and the habitat type of the species (down).

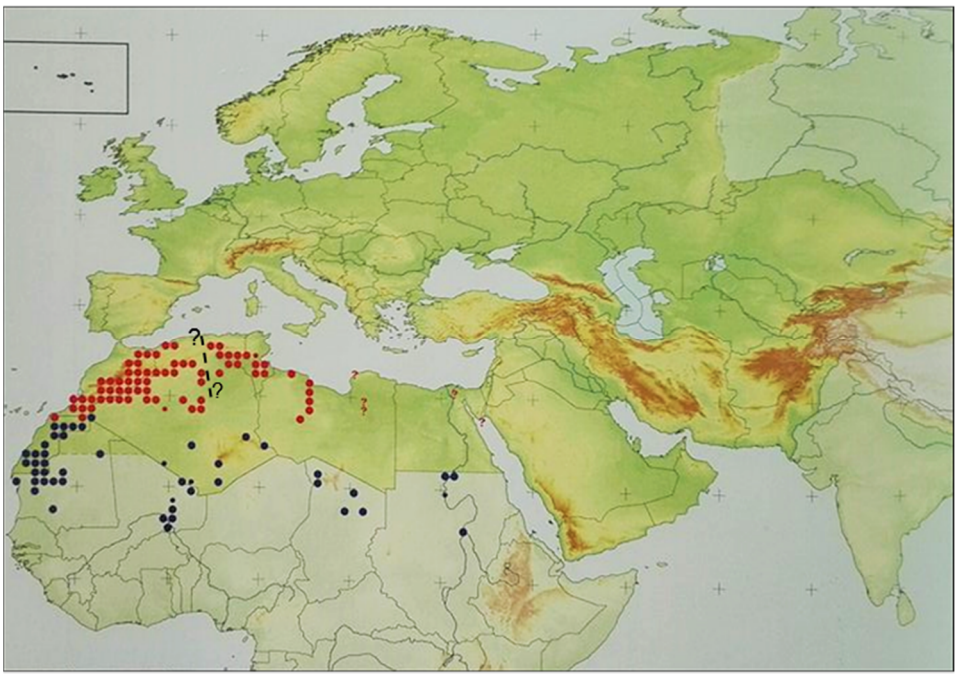

**Fig. 13 –** Distribution map of *Uromastyx dispar* (in dark blue) and *U. acanthinura* (in red, east part) and *U. nigriventris* (in red, western part). The black dashed line with question marks represent the possible unidentified barrier between these two last species. (Source: Sindaco & Jeremcenko 2008).

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# 2. OBJECTIVES

The main aim of this study is to describe the phylogeographic patterns of two reptiles species endemic to the Western Maghreb and inhabiting the north Saharan range, specifically, Oudri's fan-footed gecko, *Ptyodactylus oudrii* (Lataste 1880) and the Moroccan Spiny-tailed lizard, *Uromastyx nigriventris* (Rothschild & Hartert 1912. In general, in this work, we aim:

a) To assess the phylogeographic patterns of both *Ptyodactylus oudrii* and *Uromastyx nigriventris*, in the Maghreb region, based on mitochondrial and nuclear molecular markers;
b) To determine if patterns observed by nuclear DNA sequence, from independent loci, are concordant between them and, with estimated relationships based on mitochondrial DNA;
c) To estimate the divergence times associated to the split of the genetic groups, using evolutionary molecular clocks, applied to mitochondrial data;
d) To explore the possible association between the spatial distribution of the genetic lineages and their estimated time of split, with known palaenvironmental and geological events, which may have acted as long-term barriers to gene-flow;
e) To compare the phylogeographic history of the two North Saharan species, in the context of the evolutionary processes that act in the Maghreb region.

In the following chapters we present each study, separately, and in the last chapter we provide a general discussion of the main results and implication of the two studies.

# 3. CHAPTER I

## Genetic lineages or distinct species? Multilocus genetic variation in the Oudri's fan-footed geckos, *Ptyodactylus oudrii* complex

## 3.1. Introduction

The North Africa region represents a hotspot of biodiversity, with high levels of endemisms within some groups, such as reptiles (Myers *et al.* 2000; Padial 2006; Pleguezuelos *et al.* 2010). The magnitude of the past climatic fluctuations and the complex geologic structures are the primary responsible for such high biodiversity levels (Alimen 1965; Rognon 1987; Le Houérou 1997; DeMenocal 2004; Michard *et al.* 2008). Previous studies have suggested prominent natural barriers to organism's dispersion as the cause of many speciation events (DeMenocal 2004; Joger *et al.* 2007). The uplift of the Atlas Mountains, during the late Miocene (Gomez *et al.* 2000; Missenard *et al.* 2006; Babault *et al.* 2008; de Lamotte *et al.* 2009), promoted isolation, particularly in species with low dispersal abilities, where the connectivity between population groups was lost (Hewitt 2001; DeMenocal 2004). For this reason, the Atlas Mountains are considered a centre of diversification, for numerous species such as *Tarentola* sp. (Rato *et al.* 2012) *Quedenfeldtia* sp. (Barata *et al.* 2012b), *Atlantolacerta andreanskyi* (Barata *et al.* 2012a), scorpions (Habel *et al.* 2012) and in *Mauremys leprosa* (Fritz *et al.* 2005).

Biodiversity in the North Africa region is known to be underestimated (Ficetola *et al.* 2012). The limited information on species distribution and taxonomy is, in most part, a result of the large geographical area of North Africa, the lack of easy access to remote areas and the political constraints associated to some countries (Ficetola *et al.* 2012; Brito *et al.* 2014). Additional, the few studies developed in the region are often limited by the availability of specialized institutions (Ficetola *et al.* 2012). Furthermore, in the last decades, studies have revealed that a considerable component of biodiversity present in this region is hidden in the form of cryptic species. Cryptic species includes organisms that are visually similar but in reality are genetically distinct (Bickford *et al.* 2007). Cryptic species are particularly common in groups inhabiting extreme environmental conditions, resulting in similar environmental adaptations, and in turn species sharing a similar morphology (Hewitt 1996; Vitt & Caldwell 2014). Apart from the need of an accurate estimation of biodiversity, the identification and description of cryptic species are crucial for the efficiency of conservation strategies (Butchart *et al.* 2010; Ficetola *et al.* 2012). The emergence of sophisticated molecular tools has allowed more insights on the structure of biodiversity (Miller 2007; Hickerson *et al.* 2010; Patwardhan *et al.* 2014). In particular, more cryptic species have been identified and higher levels of biodiversity and endemism have been exposed, for different regions of the world (Myers *et al.* 2000; Olson *et al.* 2001; Vieites *et al.* 2009).

*Ptyodactylus oudrii* (Lataste 1880), the Oudri's fan-footed gecko, is currently considered a potential cryptic species complex, inhabiting the foothills of the Atlas Mountains range, from Morocco to western Algeria (Perera & Harris 2010a; Metallinou *et al.* 2015). *P. oudrii* was considered a full species only in 1987, based on enzymatic data, morphological and biogeographic patterns (Heimes 1987). Later molecular analyses, which included multiple individuals of *P. oudrii,* recognized the presence of four distinct lineages, one from the Anti-Atlas, two from the High Atlas Mountains (west and east) and other from the Saharan Atlas, in Algeria and Tunisia (Perera & Harris 2010a; Metallinou *et al.* 2015). The level of intraspecific variation within *P. oudri* species, calculated for the mtDNA, was much higher (7.4% for 12S, Perera & Harris 2010a) than that observed in other groups of geckos (Harris *et al.* 2004; Rato & Harris 2008). However, geographic and genetic sampling of these previous works did not allow to determine whether genetic lineages admixed at the boundaries of their ranges or whether those lineages are completely geographically and genetically separated.

In this study, we added more samples to data already available from previous molecular studies (Perera & Harris 2010a; Metallinou *et al.* 2015). We used phylogeographic and phylogenetic analyses to infer gene genealogies and we implement a molecular clock to estimate the time frame of the main cladogenetic events. In addition, we also correlate the distribution of the species and its lineages with variables of habitat, to test for differences in habitat occupancy between lineages. The main aims of this study are to describe the phylogeographic pattern of *Ptyodactylus oudrii* and to infer the processes which may have contributed to its formation. By comparing mitochondrial and nuclear genealogies, we also want to assess whether nuclear markers overall reflect the pattern of the mtDNA and support the hypothesis of cryptic species.

## 3.2. Methods

### 3.2.1. Sampling

In order to assess the degree of admixture between previously described lineages of *Ptyodactylus oudrii* (Perera & Harris 2010a; Metallinou *et al.* 2015), during June 2015, we carried out an intense fieldwork effort within the potential contact zones between Moroccan lineages, resulting in 49 additional samples collected. Moreover, samples from the reptile collection of CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, from the entire species' distribution and data available

in GenBank were included in the analyses, so that in total 128 samples were available (Fig.14).

For each specimen a small tissue of the tail was collected from live animals for genetic analysis and preserved at 96% ethanol solution. Other observations were included, such as GPS coordinate of collection, photographic data and measures for morphological studies, sex, and when possible faecal pellets, a drop of blood for analysis of parasites were collected, for other studies. The animals were then released unharmed at the point of capture. The database (DB) code of each sample, locality and genetic data available are described in Table 1.

### 3.2.2. DNA extraction, amplification and sequence analysis

The DNA extraction was performed using a high-saline method (Sambrook *et al.* 1989) from the tail tip material. Amplification of two partial mitochondrial genes – 12S Ribosomal Gene (12S) and Cytochrome b gene (Cyt-b) – and two partial nuclear genes – Recombination activating gene 2 (RAG-2) and the Melanocortin 1 receptor gene (MC1R) were performed by the Polymerase Chain Reaction (PCR). PCR conditions, primers and source references are listed in Table 2.

These two mitochondrial genes were chosen since the fragments could be compared with the ones used in previous studies. For some older samples the conditions were modified in order to achieve better results, e.g. for the rDNA 12S gene, it was used a new pair of primers specific for this genus targeting the same region of the gene. In the case of the Cyt-b marker, the fragment amplified was longer (768 base pairs) compared with some previous studies (395 base pairs), which resulted in difficult amplifications in some samples. To overcome this problem the Cyt-b genomic fragment was divided into two overlapping parts and a new set of internal primers specific for this species were designed (Fig.15). For the nuclear markers, we sequenced about 30% of the samples sequenced for mitochondrial markers covering all the distribution and representing each genetic lineages.

The PCR's amplifications were performed in a total of 25µl volume, using a GoTaq Polymerase protocol: 13.2µl of purified water, 5µl of GoTaq Buffer, 3-3.2µl of MgCl2, 0.5-1µl of dNTP's and 0.3-0.5µl of Primer Forward and Reverse. 0.4µl of Bovine Serum Albumin (BSA) was used to increase efficiency of difficult samples and, in these cases, volume of purified water was decreased accordingly. In each reaction a negative test was included to control for contaminations, which contains all the reagents except DNA

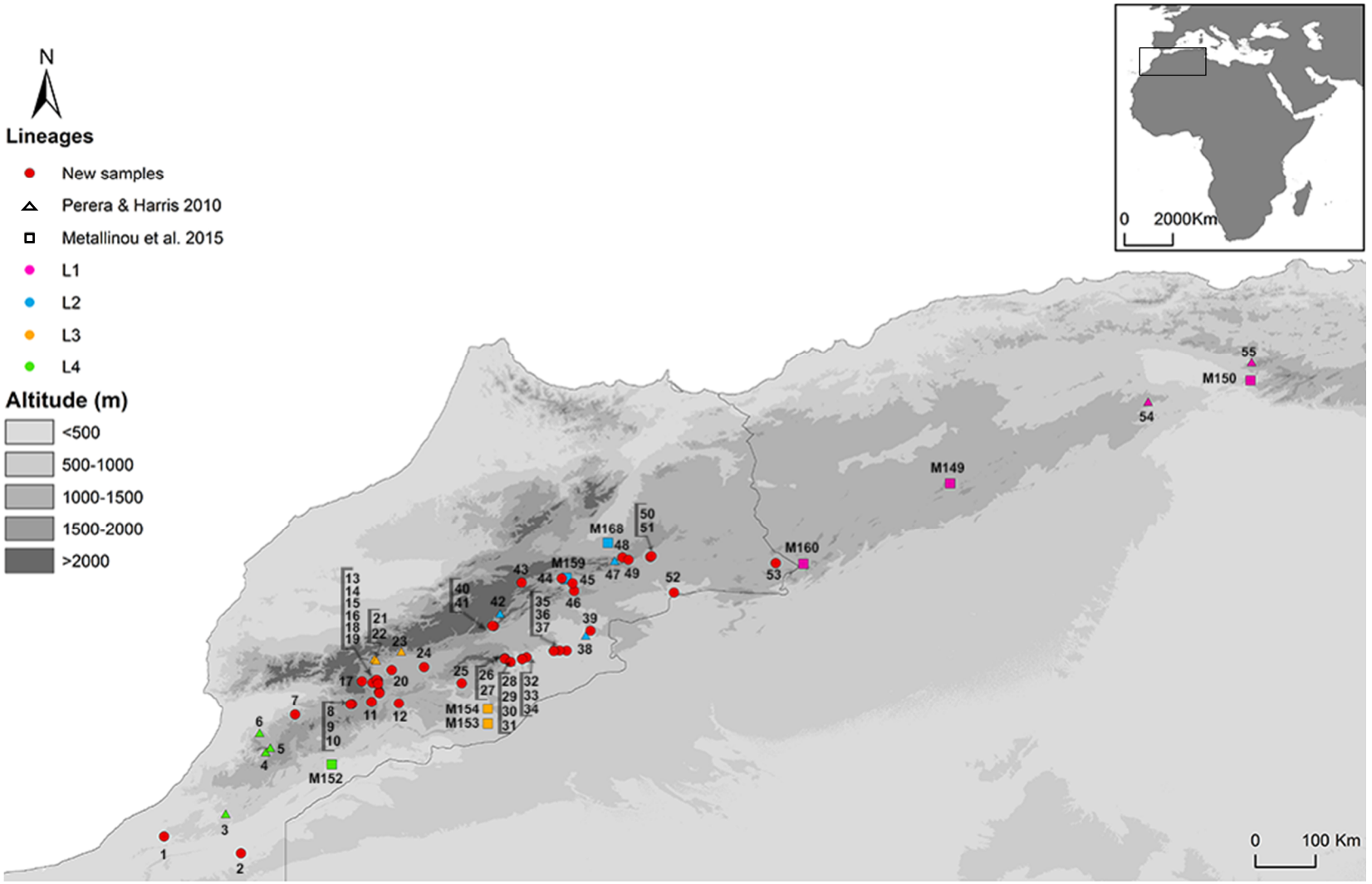


**Fig. 14 -** Samples distribution of *Ptyodactylus oudrii*. Colours represents different genetic lineages achieved based on rDNA 12S marker (L1: Algerian lineage; L2: Eastern High Atlas lineage; L3: Western High Atlas lineage and L4: Anti-Atlas lineage). Symbols represent the sample's source.

**Table 1.** Description about samples used in analyses, database (DB), coordinates of collection and genes amplified and sequenced. X: sequence available; - : sequence not available; GB: sequence from GenBank; a: Perera & Harris 2010a; b: Metallinou *et al.* 2015.

| CODE | LOC | SOURCE | LAT | LONG | 12S | CYT-b | MC1R | RAG-2 |
|---|---|---|---|---|---|---|---|---|
| **DB20044** | 1 | This study | 28.498 | -10.478 | x | x | x | - |
| **DB13422** | 2 | This study | 28.250 | -9.333 | x | x | x | x |
| **DB893** | 3 | a | 28.831 | -9.559 | GU195730 | x | x | - |
| **DB801** | 4 | a | 29.743 | -8.961 | GU195726 | x | - | x |
| **DB805** | 4 | a | 29.743 | -8.961 | GU195728 | x | - | - |
| **DB796** | 5 | a | 29.806 | -8.893 | GU195724 | x | x | x |
| **DB799** | 5 | a | 29.806 | -8.893 | GU195725 | x | - | - |
| **DB802** | 5 | a | 29.806 | -8.893 | GU195727 | x | - | x |
| **DB808** | 5 | a | 29.806 | -8.893 | GU195729 | x | - | - |
| **DB781** | 6 | a | 30.028 | -9.052 | GU195723 | x | x | x |
| **DB14234** | 7 | This study | 30.300 | -8.525 | x | x | x | x |
| **DB25174** | 8 | This study | 30.455 | -7.697 | x | x | x | x |
| **DB25214** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25215** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25230** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25251** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25252** | 8 | This study | 30.455 | -7.697 | x | x | - | x |
| **DB25254** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25255** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25256** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25257** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25258** | 8 | This study | 30.455 | -7.697 | x | x | - | x |
| **DB25259** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25260** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB25262** | 8 | This study | 30.455 | -7.697 | x | x | - | - |
| **DB13196** | 9 | This study | 30.455 | -7.697 | x | x | x | x |
| **DB25209** | 10 | This study | 30.457 | -7.675 | x | x | x | x |
| **DB25185** | 11 | This study | 30.489 | -7.385 | x | x | x | - |
| **DB25194** | 11 | This study | 30.489 | -7.385 | x | x | x | x |
| **DB25196** | 11 | This study | 30.489 | -7.385 | x | x | x | - |
| **DB25198** | 11 | This study | 30.489 | -7.385 | x | x | x | - |
| **DB25223** | 11 | This study | 30.489 | -7.385 | x | x | - | - |
| **DB14218** | 12 | This study | 30.470 | -6.977 | x | x | x | x |
| **DB1382** | 13 | This study | 30.625 | -7.266 | x | x | - | - |
| **DB1415** | 13 | This study | 30.625 | -7.266 | x | x | - | - |
| **DB1632** | 13 | This study | 30.625 | -7.266 | x | x | - | - |
| **DB13361** | 14 | This study | 30.644 | -7.271 | x | x | - | - |
| **DB13362** | 14 | This study | 30.644 | -7.271 | x | x | - | - |
| **DB13363** | 14 | This study | 30.644 | -7.271 | x | x | - | - |
| **DB430** | 15 | a | 30.766 | -7.288 | GU195716 | x | - | - |
| **DB434** | 15 | a | 30.766 | -7.288 | GU195719 | - | - | - |
| **DB435** | 15 | a | 30.766 | -7.288 | GU195721 | x | - | - |
| **DB3363** | 15 | This study | 30.766 | -7.288 | x | x | - | - |
| **DB23818** | 16 | This study | 30.779 | -7.371 | x | x | - | - |
| **DB25188** | 17 | This study | 30.802 | -7.528 | x | x | x | x |
| **DB13364** | 18 | This study | 30.812 | -7.289 | x | x | - | - |
| **DB13365** | 18 | This study | 30.812 | -7.289 | x | x | - | - |
| **DB13366** | 18 | This study | 30.812 | -7.289 | x | x | - | - |
| **DB13367** | 18 | This study | 30.812 | -7.289 | x | x | - | - |
| **DB25173** | 19 | This study | 30.825 | -7.308 | x | x | x | - |
| **DB25202** | 19 | This study | 30.825 | -7.308 | x | x | x | - |
| **DB25208** | 19 | This study | 30.825 | -7.308 | x | x | x | x |
| **DB25219** | 19 | This study | 30.825 | -7.308 | x | x | - | - |
| **DB25220** | 19 | This study | 30.825 | -7.308 | x | x | - | - |
| **DB13371** | 20 | This study | 30.968 | -7.084 | x | x | - | - |
| **DB13372** | 20 | This study | 30.968 | -7.084 | x | x | - | - |

**(CONTINUATION)**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **DB1131** | 21 | a | 31.129 | -7.344 | GU195744 | x | - | - |
| **DB904** | 22 | a | 31.112 | -7.313 | GU195731 | x | - | - |
| **DB907** | 22 | a | 31.112 | -7.313 | GU195732 | x | - | - |
| **DB432** | 23 | a | 31.242 | -6.941 | GU195717 | x | - | x |
| **DB14156** | 24 | This study | 31.013 | -6.600 | x | - | - | - |
| **DB14157** | 24 | This study | 31.013 | -6.600 | x | x | x | x |
| **DB14208** | 25 | This study | 30.771 | -6.039 | x | x | x | - |
| **DB14742** | 26 | This study | 31.135 | -5.399 | x | x | - | - |
| **DB14701** | 27 | This study | 31.142 | -5.396 | x | x | - | - |
| **DB14727** | 27 | This study | 31.142 | -5.396 | x | x | - | - |
| **DB1031** | 28 | a | 31.087 | -5.312 | GU195742 | x | - | - |
| **DB1030** | 29 | a | 31.088 | -5.311 | GU195743 | x | - | - |
| **DB14725** | 30 | This study | 31.088 | -5.311 | x | x | - | - |
| **DB14752** | 30 | This study | 31.088 | -5.311 | x | x | - | - |
| **DB1033** | 31 | a | 31.088 | -5.311 | GU195741 | x | - | - |
| **DB25281** | 32 | This study | 31.130 | -5.140 | x | x | - | - |
| **DB25283** | 32 | This study | 31.130 | -5.140 | x | x | - | - |
| **DB25285** | 32 | This study | 31.130 | -5.140 | x | x | - | - |
| **DB25286** | 32 | This study | 31.130 | -5.140 | x | x | - | - |
| **DB25287** | 32 | This study | 31.130 | -5.140 | x | x | - | - |
| **DB25288** | 32 | This study | 31.130 | -5.140 | x | x | - | - |
| **DB25334** | 32 | This study | 31.130 | -5.140 | x | x | - | - |
| **DB14202** | 33 | This study | 31.133 | -5.141 | x | x | - | - |
| **DB25205** | 34 | This study | 31.155 | -5.073 | x | x | x | - |
| **DB25206** | 34 | This study | 31.155 | -5.073 | x | x | x | x |
| **DB25284** | 34 | This study | 31.155 | -5.073 | x | x | - | - |
| **DB25289** | 34 | This study | 31.155 | -5.073 | x | x | - | - |
| **DB25290** | 34 | This study | 31.155 | -5.073 | x | x | - | - |
| **DB25353** | 35 | This study | 31.250 | -4.665 | x | x | - | - |
| **DB25357** | 35 | This study | 31.250 | -4.665 | x | x | x | x |
| **DB25358** | 35 | This study | 31.250 | -4.665 | x | x | - | - |
| **DB25359** | 35 | This study | 31.250 | -4.665 | x | x | - | - |
| **DB25343** | 36 | This study | 31.257 | -4.580 | x | x | - | - |
| **DB14201** | 37 | This study | 31.255 | -4.474 | x | x | - | x |
| **DB429** | 38 | a | 31.481 | -4.190 | GU195715 | x | - | x |
| **DB25351** | 39 | This study | 31.550 | -4.119 | x | x | x | x |
| **DB25273** | 40 | This study | 31.622 | -5.583 | x | x | - | - |
| **DB24149** | 41 | This study | 31.621 | -5.560 | x | x | - | - |
| **DB24174** | 41 | This study | 31.621 | -5.560 | x | x | x | x |
| **DB1005** | 42 | a | 31.802 | -5.467 | GU195738 | x | - | - |
| **DB1012** | 42 | a | 31.802 | -5.467 | GU195739 | - | - | - |
| **DB1014** | 42 | a | 31.802 | -5.467 | GU195740 | x | - | - |
| **DB1028** | 42 | a | 31.802 | -5.467 | GU195741 | x | - | - |
| **DB1363** | 43 | This study | 32.262 | -5.147 | x | x | x | x |
| **DB25329** | 44 | This study | 32.326 | -4.549 | x | x | - | - |
| **DB25356** | 44 | This study | 32.326 | -4.549 | x | x | - | - |
| **DB3284** | 45 | This study | 32.254 | -4.387 | x | x | x | x |
| **DB25339** | 46 | This study | 32.140 | -4.363 | x | x | - | - |
| **DB25349** | 46 | This study | 32.140 | -4.363 | x | x | - | - |
| **DB433** | 47 | a | 32.586 | -3.761 | GU195718 | - | - | - |
| **DB436** | 47 | a | 32.586 | -3.761 | GU195722 | - | - | - |
| **DB24170** | 48 | This study | 32.636 | -3.641 | x | x | - | - |
| **DB24186** | 49 | This study | 32.602 | -3.553 | x | x | x | x |
| **DB24035** | 50 | This study | 32.645 | -3.227 | x | x | - | - |
| **DB24202** | 51 | This study | 32.656 | -3.211 | x | x | x | x |
| **DB24205** | 51 | This study | 32.656 | -3.211 | x | x | x | x |
| **DB14705** | 52 | This study | 32.116 | -2.875 | x | - | x | x |
| **DB24027** | 53 | This study | 32.552 | -1.358 | x | - | x | x |
| **DB24112** | 53 | This study | 32.552 | -1.358 | - | x | x | x |

**(CONTINUATION)**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **DB24185** | 53 | This study | 32.552 | -1.358 | x | x | x | x |
| **DB491** | 54 | a | 34.966 | 4.197 | GU195745 | - | x | x |
| **DB492** | 55 | a | 35.555 | 5.742 | GU195746 | - | x | x |
| **M149** | M149 | b | 33.749 | 1.244 | KP858152 | KP867983 | - | KP868275 |
| **M150** | M150 | b | 35.281 | 5.725 | KP858344 | KP868054 | KP868485 | - |
| **M152** | M152 | b | 29.561 | -7.977 | KP858192 | KP867985 | KP868405 | KP868297 |
| **M153** | M153 | b | 30.164 | -5.650 | KP858148 | KP867943 | - | - |
| **M154** | M154 | b | 30.388 | -5.650 | KP858149 | KP867944 | - | - |
| **M159** | M159 | b | 32.336 | -4.478 | KP858428 | - | KP868487 | KP868367 |
| **M160** | M160 | b | 32.319 | -4.496 | KP858415 | KP867982 | KP868402 | KP868294 |

and, a positive control of a sample that was successfully amplified previously, to check the success of the amplification. PCR were run in a Biometra TProfessional thermal cycler following the cycles reported in Table 2. In order to test the efficiency and specificity of the reaction, 2µl of PCR product from each sample were run in agarose gel (2%) with GelRed Nucleic Acid Stain and visualized in ultraviolet transiluminator. The images of the results were saved. For the samples with positive results, the PCR product was sent to Genewiz (UK) Company to be purified and sequenced by Sanger methods.

**Table 2**. Description of primers used and PCR conditions.

| Gene | Primer name | Sequence (5'-3') | Source | PCR conditions (ºC/seg) |
|---|---|---|---|---|
| 12S | L-1091 (forward) | AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT | Kocher *et al.*, 1989 | 94º (180), [94º (30), 46º- 48º (45), 72º(60)] x 35, 72º (300), 12º ∞ |
| | H-1478 (reverse) | TGACTGCAGAGGGTGACGGGCGGTGTGT | | |
| | 12Sa Gecko (forward) | CAAACTAGGATTAGATACCCTACATGC | Metallinou *et al.* 2015 | |
| | 12Sb Gecko (reverse) | GAGGGTGACGGCGGTGTGTAC | | |
| Cyt-b | Salvi1 (forward) | TCCAACTACAAAAACCTAATGACCC | Metallinou *et al.*, 2015 | 94º (180), [94 (30), 50-47 (30), 72º (50)] x 35, 72 (300),12∞ |
| | Cb3H (reverse) | GGCAAATAGGAARTATCATTC | Palumbi *et al.*,1991 | |
| | Ptyodr F (forward) | CATCTGYATTTACCTCCACATTGG | This study | 94º (180), [94º (30), 50º (30), 72º (50)] x 35, 72º (300), 72º (300), 12º ∞ |
| | Ptyodr R (reverse) | CACTGGCTGATAGGGCGGAATGTGC | This study | |
| Rag-2 | PY1F (forward) | CCCTGAGTTTGGATGCTGTACTT | Gamble *et al.*, 2008 | 94º (300), [94º (45), 55º (45), 72º (80)] x 40, 72º (300), 72º (300), 12º ∞ |
| | PY1R (reverse) | AACTGCCTRTTGTCCCCTGGTAT | | |
| MC1R | MC1R F (forward) | GGCNGCCATYGTCAAGAACCGGAACC | Pinho *et al.*, 2010 | 92º (240), [92 (30), 62-50 (30), 72º (60)] x 35, 72 (600),12∞ |
| | MC1R R (reverse) | CTCCGRAAGGCRTAGATGATGGGGTCCAC | | |

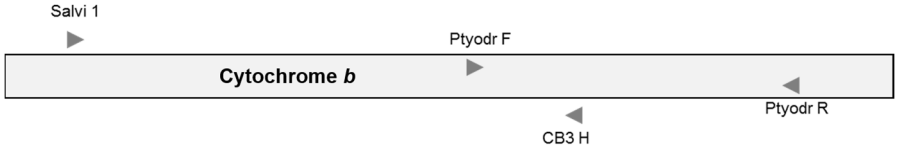


**Fig. 15** – Scheme of primers used for the two small fragments of Cyt-b gene.

### 3.2.3. Sequences data

Firstly, sequences were blasted to the NCBI database of GenBank (Benson *et al.* 2009) to confirm species identity. Chromatographs were checked manually, assembled, aligned and edited using Geneious v.4.8.5 (*www.geneious.com*; Kearse *et al.* 2012). For the nuclear genes, MC1R and RAG-2, polymorphic sites were identified, based on the Geneious tool "find heterozygotes", and coded according to the IUPAC codes. We selected from the GenBank database a *Ptyodactylus ragazzii*'s sequence, in order to use it as outgroup, in the subsequent analysis, since it was demonstrated to be sister taxa of *Ptyodactylus oudrii* (Metallinou *et al.* 2015).

### 3.2.4. Phylogenetic and nuclear network analysis

Phylogenetic analyses were performed for each gene separately and for the complete mitochondrial and nuclear datasets. PartitionFinder v1.1.1 (Lanfear *et al.* 2012) was used, first, to search the best scheme of partitions that fits the coding fragments and, second, according to it, to select the best model that fits the dataset using partition specified for each gene. PartitionFinder analysis was performed with the following parameters: linked branch length; RaxML (Stamatakis 2006) (for the concatenated mitochondrial dataset), BEAST (Drummond *et al.* 2014) and MrBayes models; AIC model selection; user schemes search (the schemes were performed for each coding gene as unique partition, two partition ($1^{st}$ and $2^{nd}$ codon, and $3^{rd}$ codon) and three partition ($1^{st}$ codon, $2^{nd}$ codon and $3^{rd}$ codon), and in the case of rDNA 12S using single partition. Uncorrected *p*-distances with pairwise deletion were calculated for the two mitochondrial gene fragments, using MEGA v. 6 (Tamura *et al.* 2013).

Phylogenetic analyses for each dataset were performed using maximum likelihood (ML) and Bayesian (BI) methods. Maximum likelihood analyses were performed with RaxML v.1.8.2. (Stamatakis 2006), using RAxMLGUI v.1.5. (Silvestro & Michalak 2012) with the specific model and parameters estimated independently for each partition. All ML analyses were carried out with 100 random addition replicates and the support of the nodes was assessed through 1000 bootstrap interactions (Felsenstein 1981). Bayesian analyses were performed in MrBayes (only for the concatenated mitochondrial dataset) and BEAST v. 1.8.1 (Drummond *et al.* 2014). In MrBayes we implemented two partitions, by gene (12S and Cyt-b), and defined the outgroup sequence. A GTR model, with a gamma-distributed rates model across sites with four categories was implemented (GTR+G); the character state frequencies were fixed (0.2633, 0.3680, 0.1340, 0.2348 for rDNA 12S; 0.2731, 0.23645, 0.1269, 0.2355 for Cyt-b); the substitution rates of GTR

model was defined as fixed (8.3404, 34.5324, 8.3404, 1.0000, 34.5324, 1.0000 for rDNA 12S; 1.0000, 8.4870, 1.0000, 4.1995, 1.0000). The analysis was run two times, with a MCMC value of 100 million generations, sampling each 10 000 generations. The consensus tree was calculated after a burnin of 25%. In BEAST v 1.8.1 (Drummond *et al.* 2014), we unlinked both clock and substitution models across partitions. The substitution model were defined specifically for each partition and a Coalescent Model of evolution with constant size was applied (Kingman 1982), since we have mainly intraspecific data. The xml file was manually modified to “Ambiguities = true” for the nuclear partitions to account for variability in the heterozygote positions, instead of treating them as missing data. To estimate the divergence times between lineages we implemented an uncorrelated lognormal clock model with the following mean substitution rates using of mitochondrial gene: 12S with a normal distribution (mean: 0.00755, stdev: 0.00247; substitution per site per million years) and Cyt-b with a normal distribution (mean: 0.0228; stdev: 0.00806). These rates were calculated by Carranza *et al.* (2012) for geckos of the genus *Stenodactylus* and also used by Mettalinou *et al.* (2015). All BEAST analyses were done in CIPRES science gateway (Miller *et al.* 2010). We ran BEAST three times, for a MCMC value of 100 million generations, checked at each 10 000 generations. Tracer v.1.4. (Rambaut *et al.* 2012) was used to assess the posterior trace plots and the effective sample size values (ESS) of the parameters, which should be greater than 200. All runs were then combined with LogCombiner and the Maximum Clade Credibility Tree was calculated with TreeAnnotator, with a 10% of burnin (both available in the BEAST package). FigTree v.1.4.2. (Rambaut 2012) was used to view the tree and export relative graphics. Nodes were considered strongly supported when ML bootstrap values ≥ 80% and posterior probability (pp) support values ≥ 0.95.

Data from nuclear genes were phased using the PHASE algorithm (Stephens *et al.* 2001) implemented in DNAsp v.5 (Librado & Rozas 2009), using 1000 interactions and 100 burn-in. Phased analysis is essential to phylogeographic studies, since it allows haplotype inference avoiding cloning procedure and thus reducing the investment of time an efforts in the lab; this method also show good performance in haplotype reconstruction (Garrick *et al.* 2010). We ran phasing three times, with different random seed numbers in order to test for congruence between phased analyses. For each polymorphic site we selected phase results that were equal for at least two of the runs to build the final phased alignments.

Phylogenetic relationships between nuclear haplotypes were estimated using the statistical parsimony networks (TCS model) and Median joining (MJ) approaches. TCS

v.1.2.1 (Posada et al 2010) was used to assess the statistical parsimony method and the network was checked in TCS beautifier (Santos *et al.* 2015) available on (*www.cibio.up.pt/software/tcsBU/*). NETWORK v5.0 (Polzin *et al*. 2014-2016) was used to run network analysis under MJ model. The missing gaps were treated as 5$^{th}$ state.

### 3.2.5. Habitat pattern among genetic lineages

Input occurrence data were obtained from geographic coordinates for the samples used in this study. In total, 64 different GPS points, from 128 samples, were depicted using ArcMap v10.1 (ESRI 2012) under the WGS 1984 Datum geographic coordinate system. Nineteen categories, from three distinct habitat type of variables, were downloaded from WorldClim – Global Climate Data database (Hijmans *et al.* 2005) (Table 2). We selected all categories that occur across the entire species' distribution. The resolution of the chosen variables varied between 90 and 300 meters so all categories were converted to 300 meters of resolution. The Minimum Bounding Geometry tool was used to design a polygon englobing all the known occurrences of *Ptyodactylus oudrii*, including an additional external buffer of 100km and also a polygon for each genetic lineage. The habitat variables were cut by the polygons generated, using Extract by Mask tool, and then converted to Euclidean Distances. Using Sample tool we obtained one file for each lineage and for all the points, linking each individual to the correspondent distance to each variable used. Moreover, using the polygons designed, it was assessed the distance, of each pixel, to the nineteen variables. In total, ten different files were produced. All the tools described are available in ArcMap v10.1 (ESRI 2012). These files were used as input for two different Principal Component Analyse (PCA) computed in R v.3.2.0 (R Core Team 2015) programme. The sample file comprising the Euclidean distances between variables within all the specie's distribution was reduced to 10% of random entries in R v.3.2.0. programme (R Core Team 2015), in order to be practicable to do the analysis (note that variance will not be lost). The first PCA identify the habitat characteristics selected by the known occurrences in the overall conditions available (using the polygon of species distribution). It also worked as a control to the species' habitat conditions already known. The second PCA identify whether lineages select different ranges of habitat conditions and which are those differences. In this PCA it was used the polygon of each lineage distribution and the environmental conditions associated to each lineage distribution also it was represented in the graph the overall habitat conditions available.

**Table 3**. Categories and correspondent habitat's variables selected for the Principal Component Analysis.

| Type | Variable | Code |
|---|---|---|
| **Lithology** | Metasedimentary | MSED |
| | Non carbonate | NCARB |
| | Carbonate | CARB |
| | Alluvium – others | ALL |
| | Silica | SIL |
| | Alluvium saline | ALLSAL |
| **Land surface** | Smooth plains | SPL |
| | Irregular plains | IRPL |
| | Breaks | BR |
| | Low mountains | LMOU |
| | High mountains | HMOU |
| **Ground cover** | Rainfed croplands | RCROP |
| | Mostly croplands/vegetation | CROP |
| | Mostly vegetation/croplands | VEG |
| | Mostly forest shrublands/ grasslands | FOR |
| | Mostly grasslands/forest shrublands | GRASS |
| | Sparse vegetation | SVEG |
| | Artificial areas | AA |
| | Bare areas | BA |

## 3.3. Results

### 3.3.1. Dataset

We obtained 118 sequences for rDNA 12S (349 bp), 119 sequences for Cyt-b (763 bp), 44 sequences for MC1R (610 bp) and 41 sequences for RAG-2 (388 bp) gene fragments. The concatenated dataset for the mitochondrial DNA includes 113 sequences with a total of 1112 positions. The concatenated file for all genes (12S, Cyt-b, MC1R and RAG-2) comprises 27 sequences and 2110 positions. Table 4 lists information on all markers and datasets used in the phylogenetic and dating analyses, including the correspondent numbers of variable sites, as well as the model of evolution selected for the best partition scheme for each dataset.

### 3.3.2. Phylogenetic and nuclear network analysis

The uncorrected *p*-distances calculated revealed an intraspecific variation within *P.oudrii* of about 6.3% and 10%, for 12S and Cyt-b mitochondrial genes, and within lineages, the genetic variation varies between 1.2% and 3.3% for the rDNA 12S and 2.9% and 6.1% for the Cyt-b. The genetic distances between and within lineages are summarized in Table 5. In addition, the genetic distances between Algerian and Moroccan lineages are 13.3% and 17.5% for the rDNA 12S and Cyt-b genes, respectively.

**Table 4** - Basic information on genetic markers and datasets used in phylogenetic analyses.

| Dataset | Nº ind.[1] | Gene fragment | Nº seq.[2] | Len.[3] | Var.□ | Scheme and model |
|---|---|---|---|---|---|---|
| mtDNA concatenated (ML and BEAST) | 113 | 12S | 113 | 1112 | 98 | 12S: GTR+G |
| | | Cyt-b | | | 382 | Cyt-b ($1^{st}$+$2^{nd}$+$3^{rd}$) : GTR+G+I |
| 4 markers concatenated | 27 | 12S | 27 | 2110 | 106 | 12S + Cyt-b + RAG2:GTR+G |
| | | Cyt-b | | | 326 | MC1R ($1^{st}$): TrN+G |
| | | RAG-2 | | | 47 | MC1R ($2^{nd}$):TrNef+I |
| | | MC1R | | | 24 | MC1R ($3^{rd}$): HKY |

[1] Number of individuals
[2] Number of sequence
[3] Length of the fragment
□ Number of variable positions

**Table 5 –** Uncorrected genetic *p*-distances calculated between lineages for 12S (down-left) and Cyt-b (up-right), and within lineages (bold values) for 12S (down) and Cyt-b (up) values. L1-L4 represent lineage from Algeria, east of High Atlas, west of High Atlas and Anti-Atlas, respectively.

| | L1 | L2 | L3 | L4 |
|---|---|---|---|---|
| **L1** | **0.061 0.033** | 0.184 | 0.163 | 0.195 |
| **L2** | 0.138 | **0.035 0.029** | 0.133 | 0.156 |
| **L3** | 0.137 | 0.091 | **0.029 0.012** | 0.142 |
| **L4** | 0.119 | 0.096 | 0.078 | **0.035 0.013** |

ML and BI analyses based on the mitochondrial dataset recovered congruent results, except for few, not well supported, nodes, which easily allowed identification of the correspondent lineage for the new samples (Fig. 16 and 17). As previously identified *Ptyodactylus oudrii* can be separated into four geographically distinct clades (Fig.16 and 17, Perera & Harris 2010a; Metallinou *et al.* 2015). These lineages are, apparently, not found in sympatry (Fig.17). The Algerian lineage (pink clade) forms the basal clade of the species and it is a sister taxa of the Moroccan lineages (blue, orange and green clades). Within the Moroccan lineages, three independent lineages are found, one for the Anti-Atlas (green clade) and two for the High Atlas, one in the west (orange clade) and another in the east (blue clade). The geographic and genetic separation of Moroccan lineages are evident, however, the phylogenetic relationships between them are not well resolved, as they recovered weak statistical support.

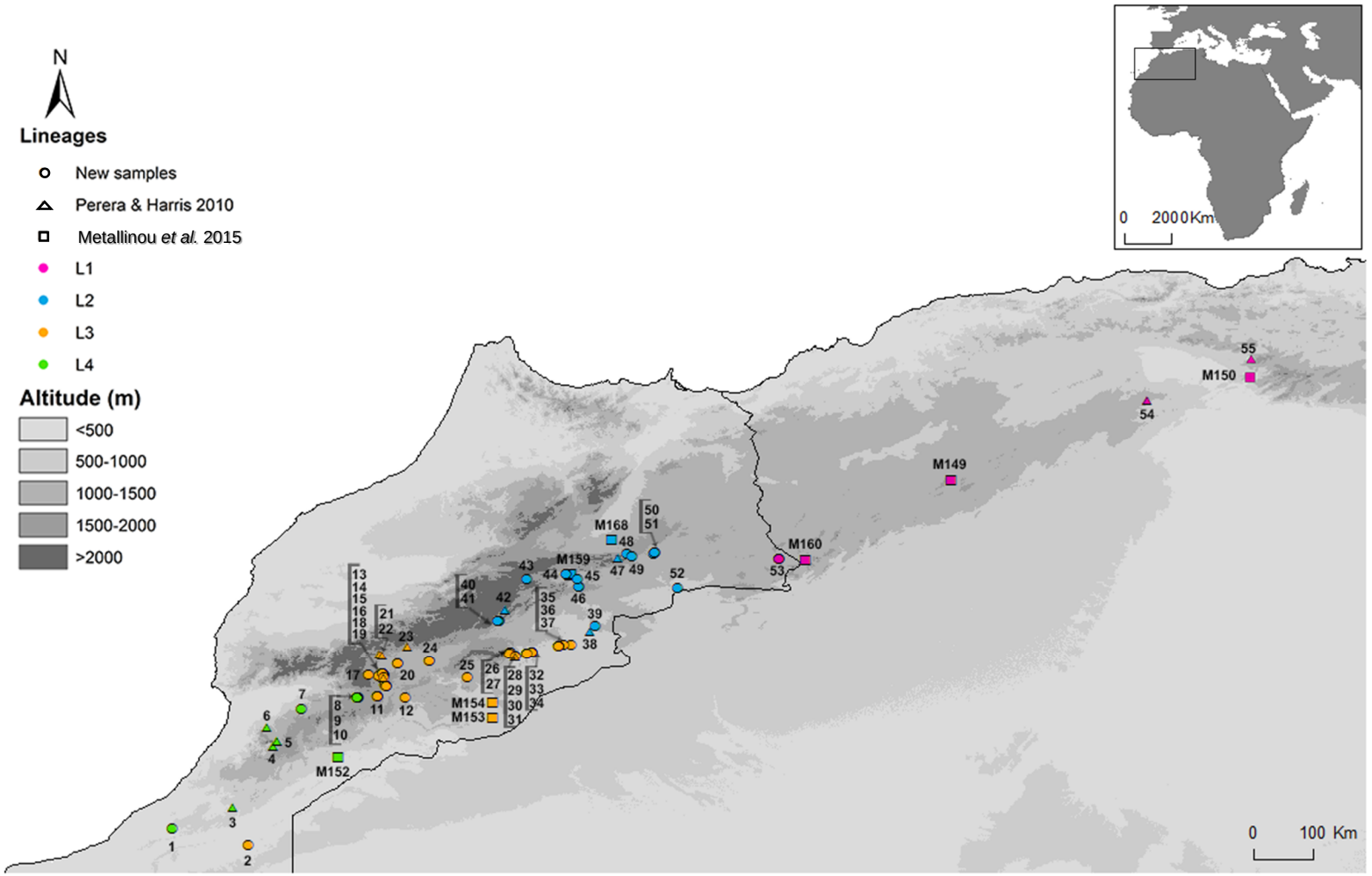


**Fig. 16 -** Distribution of the *Ptyodactylus oudrii* lineages. Colours represents different genetic lineages achieved based on rDNA 12S marker (L1: Algerian lineage; L2: Eastern High Atlas lineage; L3: Western High Atlas lineage and L4: Anti-Atlas lineage). Symbols represent samples' source.

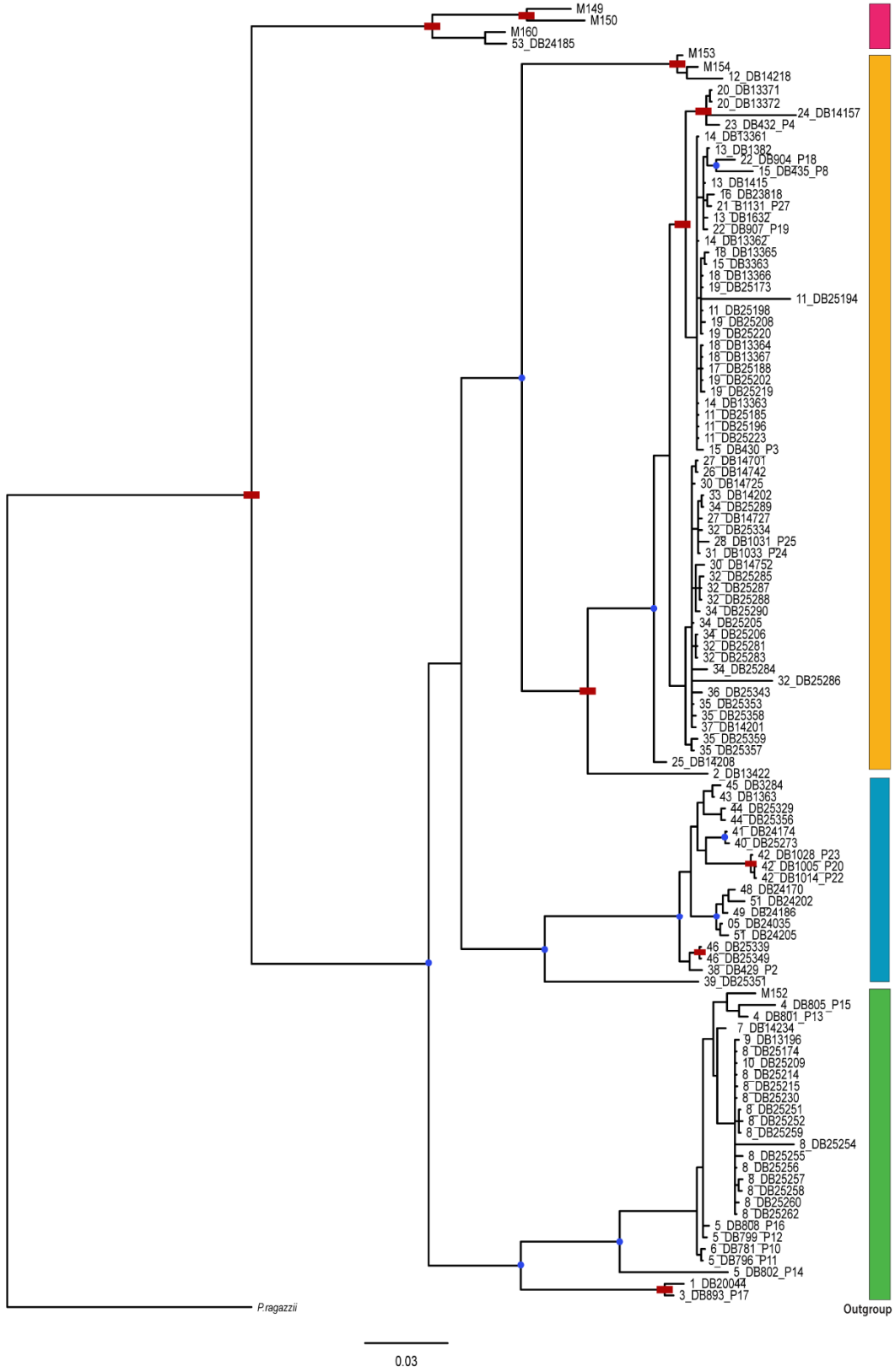


**Fig. 17** - Phylogenetic tree of combined mtDNA (12S and Cyt-b) showing the genetic relationships within *Ptyodactylus oudrii.* Red squares were placed in nodes with 100/100 support. Blue dots were placed in nodes with Bayesian posterior probability>95% and bootstrap values>80%. The coloured bars represents the distinct genetic lineages distributed across different geographic regions - pink: Algeria; blue: Eastern High Atlas; orange: western High Atlas; green: Anti-Atlas.

The haplotype networks were very similar between the two different methods used (TCS and Median-Joining. The haplotype network generated based on the Median-joining, for MC1R and RAG-2 genes are represented in the Figure 18. It suggests a genetic structuring within *Ptyodactylus oudriii* and a low level of haplotype sharing between individuals belonging to distinct mitochondrial lineages. Nuclear haplotype of the Algerian lineage are well separated from nuclear haplotypes of the Moroccan individuals.

### 3.3.3. Divergence time estimations

The age estimation of divergence of *Ptyodactylus oudrii* from its sister taxa, *P. ragazzii*, used as an outgroup, is about 19My ago (Fig.19). Diversification into genetic lineages started around 11Mya, with the separation of the Algerian lineage from the Moroccan ones (Fig.19). The more recent lineages, inhabiting the Atlas Mountains, diverged at similar ages, from 7Mya to 5Mya.

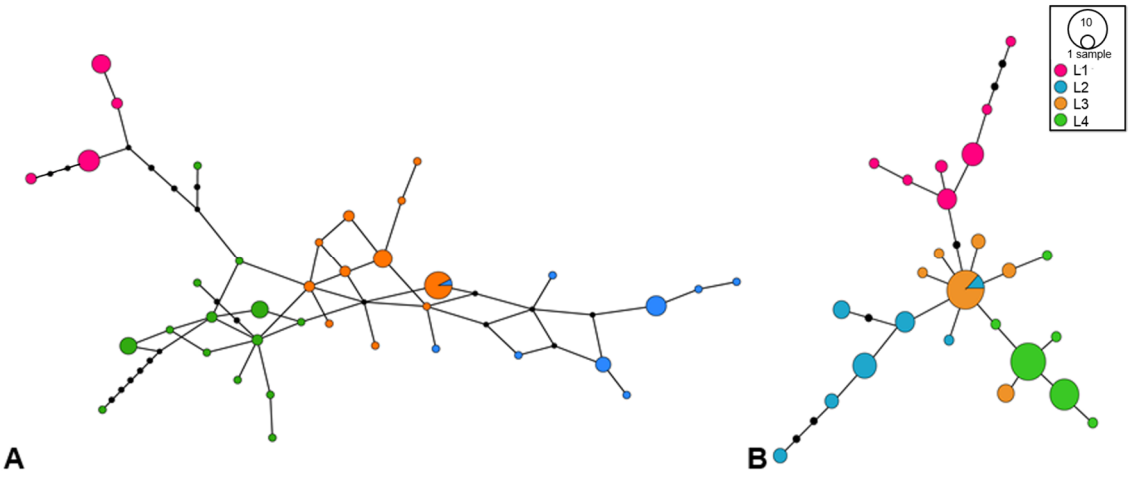


**Fig. 18** – Nuclear haplotype networks based on Median-joining method of representative specimens from the main mitochondrial lineages of *Ptyodactylus oudrii*'s, for MC1R (A) and RAG-2 (B) genes. Different colors represents distinct genetic lineages distributed across different geographic regions - pink: Algeria; blue: Eastern High Atlas; orange: western High Atlas; green: Anti-Atlas.

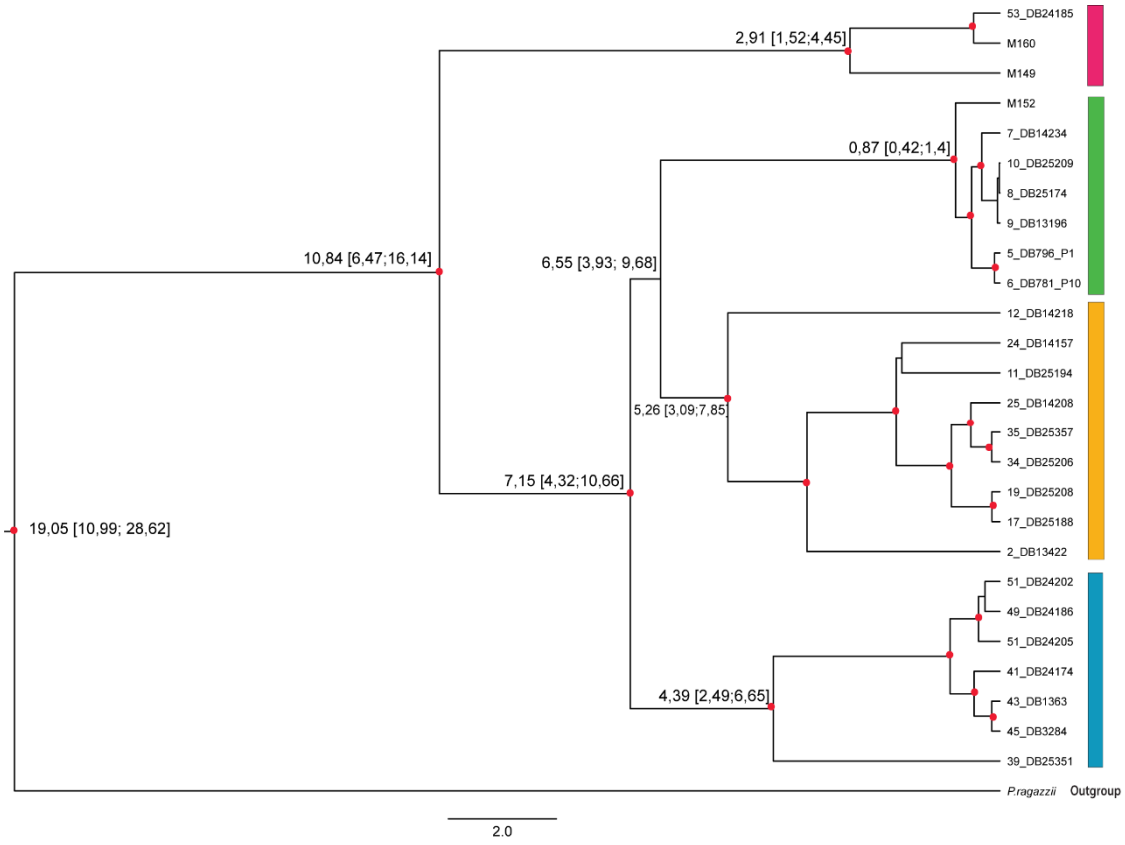


**Fig. 19** – BEAST maximum clade credibility tree for the *Ptyodactylus oudrii* lineages. Divergence times correspond to the mean posterior estimate of their age in million years. The values within parenthesis indicate the 95% HPD (highest posterior density) interval. The red dots represents nodes with posterior probability values above 0.99. The coloured bars represents the distinct genetic lineages distributed across different geographic regions - pink: Algeria; blue: Eastern High Atlas; orange: western High Atlas; green: Anti-Atlas.

### 3.3.4. Habitat pattern among genetic lineages

The first Principal Component Analysis (PCA), relates the known occurrence of *Ptyodactylus oudrii* with the overall habitat conditions available in the defined area of distribution (Fig.9). Nine major axes accounting for most of the variance (92%), however, the first two axis contributed for more than 57% of the variance (Table 6).

The first axis PC1 (38% of variance) defined a gradient of species' proximity to croplands (in all variables related with it), regions with vegetation and sparse vegetation, bare areas and low mountains, following by, high mountains, irregular plains and forests and artificial areas (Fig. 20). In the second axis PC2 (20% of variance) gives more importance to the lithologic level, where the species occurs mostly in areas characterize by carbonates, alluvium (the two variables related to it) and silica, but also, recognize the proximity to high mountains, bare areas and smooth plains (Fig. 20) The following seven axes (PC3

to PC9) give greater importance mostly to the same variable, described above the previous PCA components (PC1-PC2) (see Appendix I.A).

**Table 6.** Loading scores and percentage of total variance explained by the first three component extracted according to the Principal Component Analysis of nineteen habitat variables form the distribution area of *Ptyodactylus oudrii*. Variable codes explained in Table 3, in methods.

| | PCA I | | PCA II | | |
|---|---|---|---|---|---|
| | PC1 | PC2 | PC1 | PC2 | PC3 |
| **% VARIANCE EXPLAINED** | 37.73 | 19.66 | 28.98 | 16.58 | 11.25 |
| **CARB** | -0.17 | 0.24 | 0.25 | 0.25 | 0.17 |
| **NCARB** | -0.15 | 0.18 | 0.12 | 0.09 | 0.04 |
| **MSED** | 0.07 | -0.39 | -0.04 | -0.52 | 0.20 |
| **SIL** | 0.02 | -0.29 | -0.01 | -0.45 | 0.29 |
| **ALLSAL** | -0.08 | 0.44 | 0.23 | 0.33 | 0.35 |
| **ALL** | -0.08 | 0.44 | 0.23 | 0.33 | 0.35 |
| **SPL** | 0.01 | 0.32 | 0.25 | 0.02 | 0.00 |
| **IRPL** | -0.26 | 0.13 | 0.17 | 0.00 | 0.08 |
| **BR** | -0.28 | -0.11 | -0.20 | 0.00 | 0.38 |
| **LMOU** | -0.29 | -0.14 | -0.25 | 0.08 | 0.34 |
| **HMOU** | -0.23 | -0.23 | -0.28 | 0.12 | 0.12 |
| **RCROP** | -0.31 | -0.08 | -0.29 | 0.08 | -0.30 |
| **CROP** | -0.33 | -0.02 | -0.25 | 0.14 | 0.26 |
| **VEG** | -0.33 | -0.04 | -0.37 | 0.12 | 0.01 |
| **FOR** | -0.26 | -0.12 | -0.10 | -0.15 | 0.20 |
| **GRASS** | -0.31 | -0.10 | -0.34 | 0.06 | 0.11 |
| **SVEG** | -0.32 | 0.07 | -0.32 | 0.12 | 0.10 |
| **AA** | -0.26 | 0.03 | -0.10 | 0.07 | -0.27 |
| **BA** | -0.02 | 0.21 | 0.15 | -0.36 | 0.19 |

The second PCA defined the range of habitat conditions, available in the species distribution, selected by the area of distribution of each lineages (Fig. 21). It revealed that nine major axes accounted for most of the variance (91%), in this case, three axes contributed for more than 50% of the variance. In the first axis PC1 (29% of variance), there is no differences between lineages and the results are, as expected, identical to the first PCA analyses (Fig.20). All lineages are preferentially distributed close to land and ground cover types analysed (croplands, grasslands, vegetation, plains and mountains) and in regions of substrate of metasediments, silica and alluvium saline. In the second axis PC2 (17%) there are differences between lineages (Fig. 21). It is possible to recognize three differentiated groups, one comprising the Algerian lineage

(pink), one including the High Mountain lineages (west: blue and east: orange), and the third composing by the Anti-Atlas lineage (green). It defines a pattern of distance, from Algerian lineage (east) to Moroccan lineages from the High and Anti-Atlas (east), regarding to lithologic variables. Carbonated rocks with alluvium are preferentially close to the Algerian lineage and metasediments, silica and alluvium saline are more distant to this lineage and closer to the Anti-Atlas lineage. Besides, the Algerian lineage is near to bare areas. The third axis PC3 (11%) reveals also differences between lineages, however, compared with the previous component (PC2), it is recognized a similarity between the Algeria and Anti-Atlas, suggesting the presence of only two groups (Fig.21). Both Algerian and Anti-Atlas lineages occupy areas with subtracts of silica and saline alluviums. Moreover, they are associated to breaks zones and, preferential occupy low mountains. The Moroccan lineages are associated to rainfed croplands. The remaining six axes (PC4 to PC9) revealed identical results (see Appendix I.B).

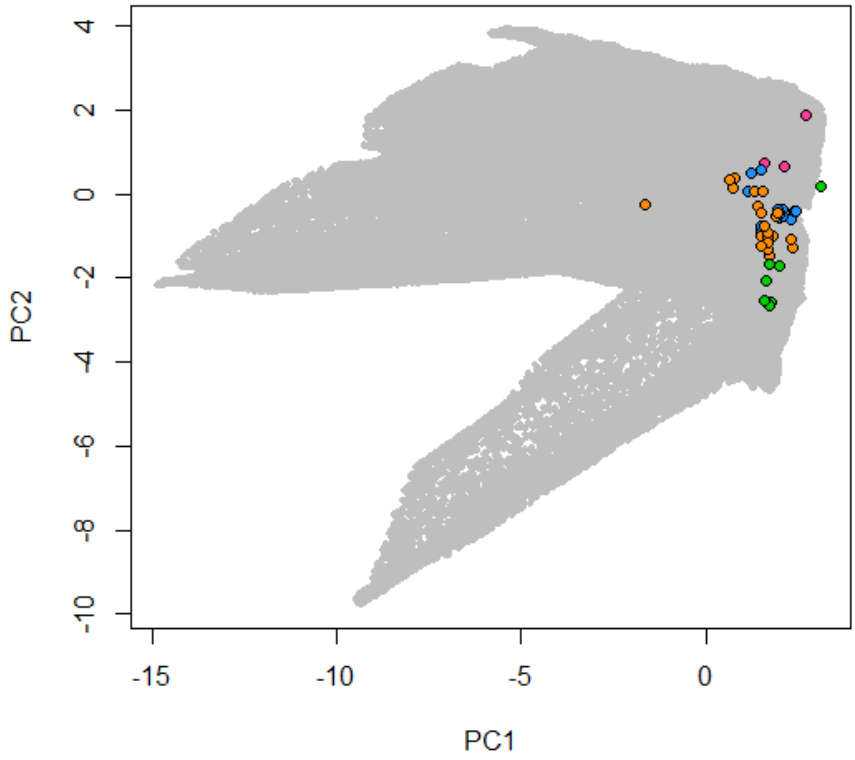


**Fig. 20** – Principal Component Analysis (PCA) of nineteen habitat variables in the three major axis (PC1 and PC2) for the study area (in grey). The coloured points represent the known occurrence for each the four genetic lineages. Different colours represents distinct genetic lineages distributed across different geographic regions - pink: Algeria; blue: Eastern High Atlas; orange: western High Atlas; green: Anti-Atlas.

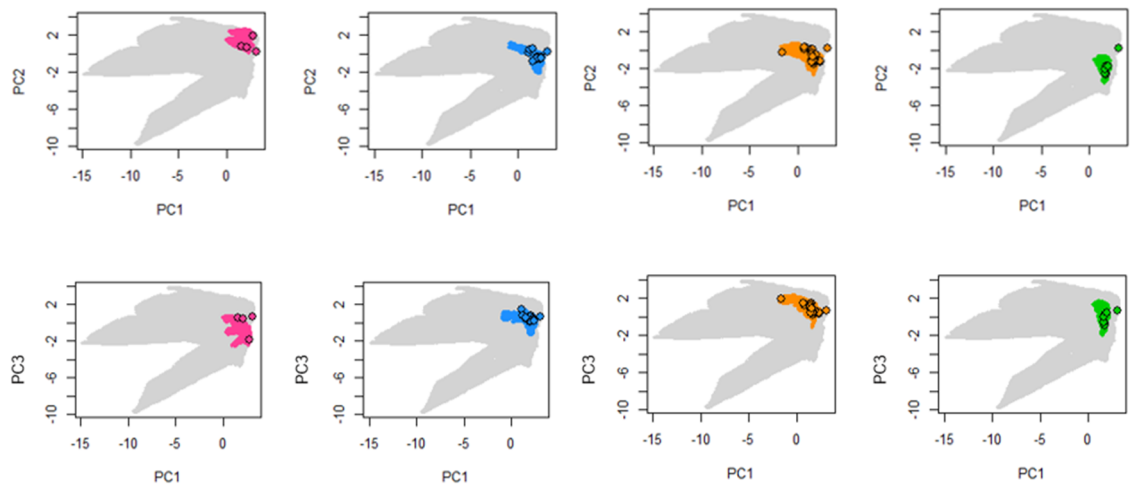


**Fig. 21** – Principal Component Analysis (PCA) of nineteen habitat variables in the three major axis (top: PC1 and PC2; bottom: PC1 and PC3) for the distribution of each genetic lineage (coloured points). The coloured areas represent the habitat conditions available in the area of lineage's distributions. Points represent the known distribution of each lineage. Grey area represents the habitat conditions of the study area in all of the species distribution, specifically, the area not occupied for the species. Different colours represents distinct genetic lineages distributed across different geographic regions - pink: Algeria; blue: Eastern High Atlas; orange: western High Atlas; green: Anti-Atlas.

## 3.4. Discussion

The results of the multi-locus approach implemented in this study are consistent with results from previous studies Perera & Harris (2010a) and Metallinou *et al.* (2015), while providing new insights on the geographic distribution and contact zones between genetic lineages, on their genetic distinctiveness and on the biogeographic process which may have played a role in their formation relationships. A preliminary assessment of habitat associations of distinct lineages provided ecological information which may be useful, together with morphological data, for a future taxonomic assessment of the *P. oudrii* complex.

### 3.4.1. Distribution and genetic variation of *Ptyodactylus oudrii* lineages

Phylogenetic trees based on both Maximum likelihood and Bayesian Inference methods recovered four main genetic lineages within *P. oudrii*, having an allopatric distribution. One lineage occurs in the Algerian Atlas, and the other three in the Atlas Mountains range of Morocco: two in the High Atlas (west and east) and one in the Anti-Atlas. These results are consistent with previous genetic studies (Perera & Harris 2010a; Metallinou *et al.* 2015).

Regarding to these genetic lineages, the specimens from Algeria appear as the basal clade and sister taxa to the remaining lineages. In the genetic variation pattern within the Algerian lineage, a clear structuration and separation between individuals from eastern Algeria, specifically in localities 53 and M160 and the western, in localities 54, 55, M149 and M150, is apparent (Fig 16 and 17). Moreover, genetic distances within the Algerian lineage are relatively high (12S = 3.3%; Cyt-b = 6.1%). Unfortunately, the political unrest within Algeria hinders sampling in the area, and thus additional diversity from unsampled regions can be anticipated. On the other hand, the Algerian specimens are clearly separated from the Moroccan specimens, with well supported nodes for mitochondrial as for nuclear data. The uncorrected genetic *p*-distances calculated between the two groups, Algerian lineage and Moroccan lineage, are higher than in other species in this genus (12S = 13%, Cyt-b = 17%), specifically the species from the Levant, which form the *P.guttatus* complex (*P. guttatus*, *P. puseuxi* and *P. siphonorhina*) (Metallinou *et al.* 2015). Similar value of genetic distance were observed between distinct species of the lizard genus *Timon* from the same region of North Africa. Indeed, Perera & Harris (2010b) found genetic distances of 3.9%, for 12S and 16S rDNA combined, between the two African clades, inhabiting Morocco and Algeria-Tunisia region, *T. tangitanus* and *T. pater*, respectively (Perera & Harris 2010b). Furthermore, significant genetic divergence between the Algerian and Moroccan populations were also found in the widely distributed gecko *Tarentola mauritanica* (Harris *et al.* 2004). The deep genetic divergence between specimens from Morocco and Algeria/Tunisia has been observed not only in reptiles, but also, in other groups such as rodents (Cossons *et al.* 2005) and amphibians (Recuero *et al.* 2007).

Despite the strong genetic divergence between Algerian and Moroccan lineages, the absence of apparent geographic barriers, acting in this region, led to Metallinou *et al.* (2015) to suggest a possible contact zone between the western samples from Algeria and the more eastern clade from the High Atlas. Although, additional samples from this area were not available, we observed no haplotype sharing at the nuclear level between the Algerian lineage and east High Atlas lineage and, also, a high number of mutations separating the two lineages, thus suggesting long-term reproductive isolation between these two groups.

Between the Moroccan lineages, the genetic distances calculated ranged from 7.7% to 9.6% for 12S and 13.2% to 15.6% for Cyt-b. These values are again very high for groups within the same species and similar to some *Ptyodactylus* species, namely between *P. guttatus* and *P. siphornorhina* (12S, 9.4% and Cyt-b, 14.4%) (Metallinou *et al.* 2015). The

nuclear data revealed a pattern of overall lineage sorting, with very low level of haplotype sharing and a sharp geographic separation. Indeed the analyses of individuals from putative contact zones collected in this work, suggested that there are no areas of sympatry between Moroccan lineages. This idea was enforced by field observations, as there was an apparent lack of suitable habitat for the species across the few kilometres that separated the distinct lineages (green-orange and orange-blue).

Moroccan lineages also showed high intra-lineage mitochondrial genetic variation with phylogeographic sub-structuring. First, the individuals from the Anti-Atlas exhibit a clear differentiation between the more southwest localities (Localities 1 and 3) and the remaining ones (Fig.16 and 17). Second, in the western High Atlas lineage a northwest and a southeast group showed genetic differentiation, supported by Bayesian analyses from concatenated mitochondrial data (Fig.17).

Our data confirms the existence of a cryptic species complex within *P oudrii,* given the very high genetic differentiation found between mitochondrial lineages (average distances: 12S = 10.98%; Cyt-b = 16.22%) and the fact that these lineage are well sorted also in nuclear genealogies, with lack of shared haplotypes (Fig. 18). The occurrence of species complex in Morocco has been pointed out by recent studies on mountain species distributed across the Atlas Mountains such as the geckos of the genus *Quedenfeldtia* and the lacertid lizard *Atlantolacerta andreanskyi* (Barata *et al.* 2012a; b). The genus *Quedenfeltia* comprises two species *Q. trachyblepharus* and *Q. moerens* showing 13% of genetic distance, between them, based on the ND4 gene fragment. Within each species, two genetic subclades were found with values of genetic distances between them of 9.7% and 8%, in *Q. trachyblepharus* lineages and *Q. moerens* lineages, respectively, for the same fragment, suggesting that they could actually represent distinct species (Barata *et al.* 2012b). Also for *A. andreaskyi* the average of genetic distances between populations, calculated based on rDNA 12S gene (4.4%) and ND4 (12.6%) exceed values observed between distinct lacertid species (e.g. *Iberolacerta* genus (Crochet *et al.* 2004). High genetic variation was found also in other species, namely between lineages divided by the Atlas range in Morocco, as for example in the gecko of the genera *Saurodactylus* (Rato & Harris 2008) and *Stenodactylus* (Metallinou *et al.* 2012), in amphisbaenians of the genera *Blanus* and *Trogonophis* (Sampaio *et al.* 2015), in the lacertid lizard *Timon tagitanus* (Paulo *et al.* 2008; Perera & Harris 2010b) and in scorpions (Habel *et al.* 2012; Pedroso *et al.* 2013). This suggest a prominent role of the Atlas Mountains in the determination of the phylogeographic patterns found in these multiple species.

### 3.4.2. Scenarios for the divergence within the *P. oudrii* complex

Metallinou *et al.* (2015) estimated a divergence time for the genus *Ptyodactylus* in the Late Oligocene, about 27 Mya, which is considered an old origin. This estimate is similar to those obtained for the diversification of other geckos such as, *Stenodactylus* (Metallinou *et al.* 2012) and *Hemidactylus* (Šmíd *et al.* 2013), and coincides with the geological extreme events which occurs between African and Asian plate, in particular the tectonic activities, which have separated Africa from other continents, and, then, the formation of a non-permanent Gomphoterium land bridge connecting the Eurasia and Africa plates, in the Arabian Peninsula region (Rögl 1998). The inferred scenario of *Ptyodactylus* diversification likely occurred from the eastern to western region of Africa (Metallinou *et al.* 2015). Accordingly, the main clades of *Ptyodactylus* only appeared in North Africa, during the middle Miocene, when the ice-sheets retreated and this region underwent to a progressive aridification (Hewitt 2000; Clark *et al.* 2009; Pound *et al.* 2012). The root age of *P. oudrii* estimated by Metallinou et al (2015) was approximately 19 Mya and the main split within this taxon at 12.7 Mya. We obtain similar estimates for the *P. oudrii* divergence at (10.8 Mya, HPD 6.5 – 16.1, 95%) (Fig. 19). These estimations are much older compared with diversification times inferred for other reptiles, such as *Agama,* in the same area (Brown *et al.* 2002; Gonçalves *et al.* 2012), but similar to the diversification between *Timon tangitanus* (Morocco) and *T. pater* (Algeria/Tunisia) (Paulo *et al.* 2008) and between *Stenodactylus mauritanicus* and *S. sthenodactylus* (Metallinou *et al.* 2012). The split between *P. oudrii* lineages inhabiting the foot-hills of the High Atlas Mountains in Morocco, had a more recent divergence (from 5Ma to 7My ago) likely under a simultaneous event of diversification. Some estimations for the uplift of the Atlas Mountains pointed it during the Tortonian stage (Missenard *et al.* 2006), while others mentioned a more recent age for the main episode, during the Messinian (Gomez *et al.* 2000). The estimated diversification time of lineages within *P. oudrii* fit well with these geologic events, however the intrinsic uncertainty over the molecular clock estimates and the complexity of the uplift process of the High Atlas, which was likely extended for long time, make it difficult to identify a strict relation between cladogenesis within *P. oudrii* and the geologic event of Moroccan mountains (Piqué *et al.* 2002; de Lamotte *et al.* 2009). If we follow the hypothesis of a more recent estimation for the Atlas Mountains' uplift (5Mya-7Mya), the diversification of Algeria and Morocco's specimens would have occurred before such orogenetic event and may have been associated to the extensive aridification occurring in this period in North Africa, which contributed for many expansion and speciation events of several taxa in this region (DeMenocal 2004).

### 3.4.3. Habitat pattern among lineages

Preliminary assessments on the habitat pattern among lineages revealed some differences. The three lineages from the Anti-Atlas, High Atlas and Algeria differ, mostly, in terms of substrate's type and proximity to plains and bare areas, where the Moroccan lineages are associated to metasediments and saline alluvium substrate, the Algerian lineage to bare areas and the Anti-Atlas lineages to plains. However, it is important to note that the observed differences might be the result of the availability of different habitat conditions in distinct portions of the species range, rather than the result of specific lineage requirement. Therefore, in order to assess the statistical significance of the differences found tests of niche overlap should be performed (Warren *et al.* 2008), such as niche similarity and equivalence tests, and also the description of the effective and fundamental niche of each lineage.

### 3.4.4. Taxonomy and conservation implications

There are no doubts regarding the genetic divergence of the described lineages and their distribution limits. The high levels of intraspecific genetic variation, exceeding values of genetic distances found in other reptiles (Rato & Harris 2008; Perera & Harris 2010b; Barata *et al.* 2012a; b), suggest that the genetic lineages have evolved independently, for a long time, because of a long-term barrier prevent their dispersion. Moreover, no apparent sympatric area exists between mitochondrial lineages, which is also well distinct at the two nuclear loci. Even if a possible contact zone exist between lineages, its impact is not strong enough for genetic admixture between individuals from different lineages, which result in a reproductive isolation of the lineages.

The observed genetic pattern is consistent with the hypotheses that the four genetic lineages represent distinct species. However, before a taxonomic revision, an integrative approach is preferred, combined independent data assessments, such as morphological and ecological approaches. Preliminary morphological analyses (unpublished data) suggest that some diagnostic differences exist between Algerian and Moroccan specimens, where, according to genetic assessments, these two groups show the more pronounced differences. Therefore, the description of the genetic forms might be the first step towards a complete taxonomic revision of the *P. oudrii* group.

Finally, regardless of the taxonomic status of the four lineages within *Ptyodactylus oudrii,* they should be considered independently, since they represent distinctive evolutionary

units (Moritz 1994). Consequently, the implementation of management and conservative strategies should be applied to each as separate conservation units.

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# 4. CHAPTER II

## The role of palaeoenvironments in the evolution of genetic diversity within the Moroccan spiny-tailed lizard, *Uromastyx nigriventris*

## 4.1 Introduction

The Western Maghreb region hosts a high level of biodiversity, especially regarding reptiles (Bons & Geniez 1996; Myers *et al.* 2000; Padial 2006; Pleguezuelos *et al.* 2010). The high habitat diversity of this region and its palaeoenvironmental and geologic evolution have had a crucial role in shaping this diversity (Le Houérou 1997). However, the number of studies focused on the region has been restricted by the difficult access and political instability of many remote areas, particularly in Algeria country, which have contributed to the lack of knowledge about species in the Maghreb and their distribution and genetic variation. This results in an expected underestimation of the biodiversity in this region (Ficetola *et al.* 2012).

As a consequence the amount of phylogeographic studies available for Maghreb is not sufficient to draw some general patterns as has been done, for example, for European taxa (Hewitt 1999; Gómez & Lunt 2006). Moreover, comparable to other regions, it is difficult to develop an overall scenario for species, inhabiting similar habitats, as the case of the "southern refugia" hypotheses for European species (Hewitt 1999). In addition, the complexity of past climate and geologic events which have shaped north African habitats during the Miocene, Pliocene and Pleistocene, and thus, the evolutionary patterns of species from the Western Maghreb cannot be explained in one simple history (Demenocal 1995; Michard *et al.* 2008; Brito *et al.* 2014). Assessments of genetic diversity of North Africa, have revealed the role of the uplift of the Atlas Mountains, during the middle/late Miocene (Missenard *et al.* 2006; Michard *et al.* 2008; de Lamotte *et al.* 2009) in the subdivision of several species, for example, in *Quedenfeldtia* (Barata *et al.* 2012b), in *Acanthodactylus erythrurus* (Fonseca *et al.* 2009) and in scorpions of the genus *Buthus* (Habel *et al.* 2012). Also, the Pleistocene climatic oscillations, from expansion of arid regions to increase of tropical vegetation, which promoted shifts of climatic zones, likely played an important role on species diversification and vicariance events due to episodes of contraction and expansion of species ranges (Demenocal 1995; Hewitt 1996; Le Houérou 1997; Kröpelin *et al.* 2008). These scenarios has been inferred for example for the lizards of the genus *Agama* (Gonçalves *et al.* 2012) or in the elephant shrews, *Elephantulus* genus (Douady *et al.* 2003).

Many of the phylogenetic and phylogeographic assessments in the Maghreb were concentrated in the Atlas Mountains regions, such as the case of *Quedenfeltia* species (Barata *et al.* 2012b), *Atlantolacerta andreanskyi* (Barata *et al.* 2012a) *Saurodactylus* species (Rato & Harris 2008) or the *Vipera monticola* complex (Velo-Antón *et al.* 2012)

and in the northern region of the Atlas, for example reptiles of the *Timon* genus (Perera & Harris 2010a), *Podarcis vaucheri* (Pinho *et al.* 2006; Kaliontzopoulou *et al.* 2011), amphisbaenians including *Blanus* genus (Vasconcelos *et al.* 2006; Sampaio *et al.* 2015) and snakes, such as *Psammophis schokari* (Rato *et al.* 2007), *Malpolon monspessulanus* and *Hemorrhois hippocrepis* (Carranza *et al.* 2006). Unfortunately, studies are scarce on species inhabiting the northern margins of the Sahara Desert, also called north Saharan species (Bons & Geniez 1996; Geniez *et al.* 2000), for example *Uromastyx nigriventris, Ptyodactylus oudrii, Mesalina guttulata*, *Tarentola boehmei* and *T. deserti* and some species of the genera *Acanthodactylus* and *Trapelus* (Bons & Geniez 1996). One of the few examples concerns *P. oudrii,* and indicated high levels of intraspecific genetic variation (Perera & Harris 2010b; Metallinou *et al.* 2015). Also phylogenetic and morphological analyses in the *Trapelus mutabilis* species complex revealed a distinct species, *Trapelus boehmei,* occurring in Morocco and Algeria, with complex genetic variation (Wagner *et al.* 2011). In *Acanthodactylus*, *A. boskyanus* and *A. scutellatus* are both considered species complex, comprising diverse species distributed across the Maghreb (Mellado & Olmedo 1990; Tamar *et al.* 2016). Consequently, there emerges the need of a range-wide sampling and a multi-locus approach to be used to determine the complex evolutionary histories that took place in this region.

*Uromastyx* (Merren 1820) is a genus of lizards widely distributed in Arabia and North Africa region, inhabiting sandy areas of semi-desert (Wilms & Bohme 2000; Amer & Kumazawa 2005; Wilms *et al.* 2007). It belongs to the Agamidae family and, currently, fifteen species are recognized within the genus (Wilms *et al.* 2007). The North African acanthinura group comprises the species *U. acanthinura* (Bell 1825), *U. geyri* (Müller 1922), *U. dispar* (Heyden 1827) (and its subspecies) and *U. nigriventris* (Rothschild & Hartert 2012). The Moroccan spiny-tailed lizard, *U. nigriventris* inhabits semi-arid regions across Morocco and Algeria and was only recently considered as a full species (Harris *et al.* 2007; Wilms *et al.* 2007). The main difficulties in studying the taxonomic and diversification within this genus is the overall similarity of some morphological characters (as the arrangement of the annuli of the tail) across the genus, but on the other hand, also the intraspecific variation found in colour patterns (Amer & Kumazawa 2005; Wilms *et al.* 2007, 2010). The use of molecular data from mitochondrial DNA (mtDNA), significantly contributed to understanding the limit between species and their distribution, as well providing a first assessment of their genetic variation. However, the preliminary studies of Amer & Kumazawa (2005) and also the studies of Harris *et al.* (2007) and Wilms *et al.* (2007) were limited to base their studies only in mitochondrial DNA and using

few samples for each species from few localities. In order to understand the evolutionary history of *Uromastyx* species, specifically the level of genetic variation and their diversification, a multi-locus framework approach is clearly required.

In this study, we sampled *Uromastyx nigriventris* individuals across its range in Morocco and sequenced both mitochondrial and nuclear markers. We used phylogeographic and phylogenetic approaches to infer gene genealogies and to estimate the time frame of the main cladogenetic events, implementing evolutionary molecular clocks models. The main aims of this study are to describe the phylogeographic pattern of *Uromastyx nigriventris* and to infer the evolutionary and biogeographic processes which may were responsible for its formation.

## 4.2. Methods

### 4.2.1. Sampling

Several fieldtrips (2005 - 2014) were carried out in different localities across the known distribution of *Uromastyx nigriventris*, in Morocco, and the samples were preserved in the reptile collection of CIBIO (Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto). In total, 57 specimens, from 46 different localities, were used in this study (Fig. 22).

For each specimen a small tissue of the tail was collected from live animals or from road-kills, for genetic analysis, and preserved in 96% ethanol. This process is harmless for living individuals, which were released soon after in the collection point. GPS coordinate of collection was recorded. The database (DB) code of each sample, locality and genetic data available are described in Table 7.

### 4.2.2. DNA extraction, amplification and sequence analysis

The DNA extraction was performed through saline methods (Sambrook *et al.* 1989) from tail tip material. DNA amplification of one partial mitochondrial gene, the NADH dehydrogenase (ND2) and two nuclear genes – the Melanocortin 1 receptor gene (MC1R) and Recombination Activating Gene I (RAG-1) - were performed by Polymerase Chain Reaction (PCR). Furthermore, two more partial mitochondrial genes, 12S ribosomal gene (12S) and 16S ribosomal gene (16S) were amplified for a few individuals, in order to compare genetic distances with sequences from other species

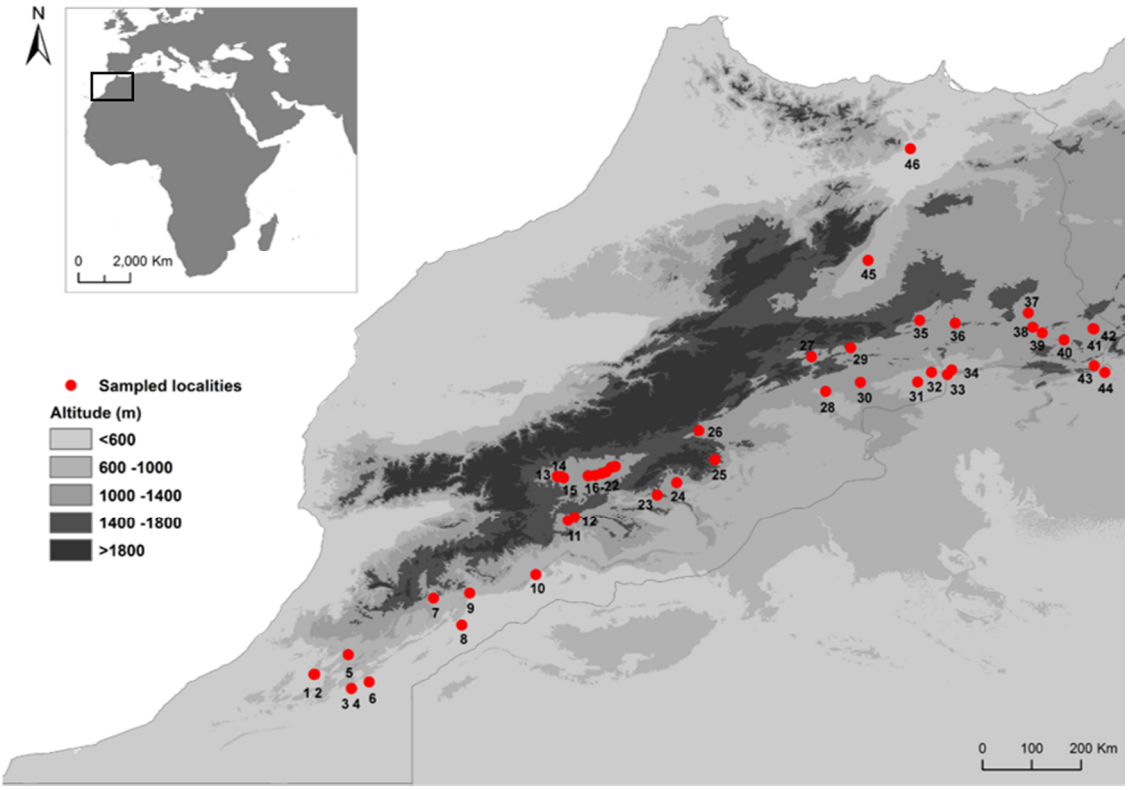


**Fig. 22 –** Distribution map of sampled individuals of *Uromastyx nigriventris* species, in Morocco.

within the genus available in GenBank from previous studies (Wilms *et al.* 2007). All PCR's conditions and primers used were described in Table 8.

PCR amplifications were performed in a total of 25µl volume, using a GoTaq Polymerase protocol: 13.6-14µl of purified water, 5µl of GoTaq Buffer, 1.5-3.2 µl of MgCl2, 0.4-1µl of dNTPs, 0.3 – 0.8µl of Primers (see Table 6) and 0.1 - 0.2µl of GoTaq. 0.3-0.4µl of Bovine Serum Albumin (BSA) was added to increase efficiency of difficult samples and in these cases volume of purified water was decreased accordingly. In each reaction we used a negative test to control for contaminations, which contains all the reagents except DNA, and a positive control, a sample that was successfully amplified previously, to check the success of the amplification. PCR were run in a Biometra TProfessional thermal cycler following the cycles reported in Table 8.

In order to test the efficiency and specificity of the reaction, 2µl of PCR product from each sample were run in agarose gel (2%) with GelRed Nucleic Acid Stain and visualized in an ultraviolet transilluminator. The images of the results were saved. For the samples with positive results, the PCR product was sent to Genewiz (UK) Company to be purified and sequenced by Sanger methods.

**Table 7.** Description about samples used in this study. Database (DB) code, coordinates of collection and genes amplified and sequenced are reported. X: sequence available; - : sequence not available.

| CODE | LOCALITY | LATITUDE | LONGITUDE | ND2 | MC1R | RAG-1 |
|---|---|---|---|---|---|---|
| DB20049 | 1 | 28.84 | -9.72 | x | x | x |
| DB20050 | 2 | 28.84 | -9.71 | x | x | x |
| DB896 | 3 | 28.69 | -9.32 | x | x | x |
| DB20032 | 4 | 28.69 | -9.32 | - | x | x |
| DB1319 | 5 | 29.05 | -9.35 | x | x | x |
| DB1602 | 5 | 29.05 | -9.35 | x | x | x |
| DB12171 | 6 | 28.76 | -9.13 | x | x | x |
| DB13765 | 7 | 29.66 | -8.44 | x | x | x |
| DB3377 | 8 | 29.37 | -8.14 | - | x | x |
| DB3378 | 8 | 29.37 | -8.14 | x | x | x |
| DB14231 | 9 | 29.71 | -8.05 | x | x | x |
| DB14225 | 10 | 29.91 | -7.34 | x | x | x |
| DB1636 | 11 | 30.49 | -7.00 | x | - | x |
| DB1656 | 11 | 30.49 | -7.00 | x | x | x |
| DB23334 | 12 | 30.53 | -6.93 | x | x | x |
| DB24086 | 13 | 30.97 | -7.11 | x | x | x |
| DB24135 | 14 | 30.97 | -7.07 | x | x | x |
| DB14767 | 15 | 30.96 | -7.04 | x | x | x |
| DB14750 | 16 | 30.98 | -6.78 | x | x | x |
| DB24141 | 16 | 30.98 | -6.78 | - | x | x |
| DB13373 | 17 | 30.98 | -6.70 | x | x | x |
| DB14154 | 18 | 31.00 | -6.64 | x | x | x |
| DB13374 | 19 | 31.02 | -6.59 | x | x | x |
| DB3728 | 20 | 31.07 | -6.53 | x | x | x |
| DB3729 | 20 | 31.07 | -6.53 | x | x | x |
| DB3730 | 20 | 31.07 | -6.53 | x | x | x |
| DB14756 | 21 | 31.07 | -6.51 | x | x | x |
| DB23806 | 21 | 31.07 | -6.50 | x | x | x |
| DB14766 | 22 | 31.08 | -6.49 | x | x | x |
| DB14209 | 23 | 30.77 | -6.04 | x | x | x |
| DB1035 | 24 | 30.90 | -5.83 | x | x | x |
| DB14740 | 25 | 31.15 | -5.42 | x | x | x |
| DB14749 | 25 | 31.15 | -5.42 | x | x | x |
| DB3727 | 26 | 31.46 | -5.59 | - | x | x |
| DB3280 | 27 | 32.25 | -4.39 | x | x | x |
| DB14200 | 28 | 31.88 | -4.23 | x | - | x |
| DB4875 | 29 | 32.35 | -3.97 | - | x | x |
| DB14199 | 30 | 31.98 | -3.86 | x | x | x |
| DB14672 | 31 | 31.98 | -3.25 | x | x | x |
| DB3315 | 32 | 32.09 | -3.10 | x | x | x |
| DB14186 | 33 | 32.06 | -2.93 | x | x | x |
| DB3267 | 34 | 32.11 | -2.88 | x | x | x |
| DB24200 | 35 | 32.64 | -3.23 | x | x | x |
| DB24201 | 35 | 32.64 | -3.23 | x | x | - |
| DB24203 | 35 | 32.64 | -3.23 | x | x | x |
| DB24204 | 35 | 32.64 | -3.23 | x | x | x |
| DB24212 | 36 | 32.61 | -2.85 | x | x | x |
| DB14611 | 37 | 32.72 | -2.06 | x | x | x |
| DB3276 | 38 | 32.57 | -2.02 | x | x | x |
| DB24051 | 39 | 32.51 | -1.91 | x | x | x |
| DB24012 | 40 | 32.44 | -1.68 | x | x | x |
| DB3166 | 41 | 32.56 | -1.37 | x | x | x |
| DB24221 | 42 | 32.55 | -1.36 | x | - | x |
| DB24229 | 43 | 32.16 | -1.36 | x | x | x |
| DB3123 | 44 | 32.09 | -1.24 | x | x | x |
| DB3143 | 45 | 33.29 | -3.78 | x | x | x |
| DB3726 | 46 | 34.50 | -3.33 | - | - | x |

**Table 8.** Description of primers and PCR conditions used

| Gene | Primer name | Sequence (5'-3') | Source | PCR conditions (ºC/seg) |
|---|---|---|---|---|
| **ND2** | AlaR2 (forward) | AAAATRTCTGRGTTGCATTCAG | Macey *et al.* 2000 | 94º (90), [ 94º (30), 49º (45), 72º(90)] x 35, 72º (600) |
| | ND2-F17 (reverse) | TGACAAAAAATTGCNCC | Macey *et al.* 1997 | |
| **MC1R** | MC1R-F (forward) | GGCNGCCATYGTCAAGAACCGGAACC | Pinho *et al.* 2010 | 94º (300), [94º (30), 56º (45), 72º (80)] x 35, 72 (300) |
| | MC1R-R (reverse) | CTCCGRAAGGCRTAGATGATGGGGTCCAC | | |
| **RAG-1** | L2408 (forward) | TGCACTGTGACATTGGCAA | Vidal & Hedges 2004 | 94º (120), [94º (20), 52º (50), 72º (3)] x 45, 72º (600) |
| | H2928 (reverse) | GACTGCYTGGCATTCATTTT | | |
| **12S** | 12Sa (forward) | CTGGGATTAGATACCCCACTAT | Kocher *et al.* 1989 | 94º (180), [94º (45), 50º (60), 74º (120)] x 35, 74º (300) |
| | 12Sb (reverse) | GAGGGTGACGGGGCGGTGTGT | | |
| **16S** | 16SL (forward) | CGCCTGTTTATCAAAAACAT | Palumbi *et al.* 1991 | 94º (90), [94º (45), 55º (45), 72º (90)] x 33, 72º (300) |
| | 16SH (reverse) | CCGGTCTGAACTCAGATCACG | | |

### 4.2.3. Sequence data

Initially, sequences were blasted against the NCBI database of GenBank (Benson *et al.* 2009) to confirm species identity. Chromatographs were checked manually, assembled, aligned and edited using Geneious v.4.8.5 (*www.geneious.com;* Kearse *et al.* 2012). For the nuclear genes, MC1R and RAG-2, polymorphic sites were identified, based on Geneious tool "find heterozygotes", and coded according to the IUPAC codes. One ND2 sequence of *Uromastyx dispar* was downloaded from GenBank and used as outgroup in phylogenetic analysis (AB113815 generated in Amer & Kumazawa (2005)), since no *U. acanthinura* (sister taxa to *U. nigriventris*, following Wilms *et al.* 2007) is available.

### 4.2.4. Phylogenetic and nuclear network analysis

To estimate phylogenetic relationships between mitochondrial haplotypes we used TCS v1.2.2. (Clement *et al.* 2000), which implements statistical parsimony network approach. The network were checked in TCS beautifier (Santos *et al.* 2015) available on *(www.cibio.up.pt/software/tcsBU/).* Network approaches are appropriate to represent haplotype relationships at the intraspecific level, mainly within low divergent conspecific datasets, with fewer characters for phylogenetic analysis, which diminish the statistical power of the traditional phylogenetic methods (Posada & Crandall 2001). MEGA v.6 (Tamura *et al.* 2013) was used to calculate mitochondrial (ND2, 12S and 16S)

uncorrected *p*-distances within and between the main genetic groups found using the pairwise deletion option.

PartitionFinder v.1.1.1. (Lanfear *et al.* 2012) was used to select the best model scheme of codon partition and, accordingly, the best substitution model that fits with the protein coding ND2 dataset. PartitionFinder analysis was performed with the following parameters: linked branch length; RaxML (Stamatakis 2006), BEAST (Drummond *et al.* 2014) and MrBayes models; AIC model selection; user schemes search, specifically, one partition, two partitions (1$^{st}$ and 2$^{nd}$ codon + 3$^{rd}$ codon) and three partitions (1$^{st}$ codon + 2$^{nd}$ codon + 3$^{rd}$ codon).

Phylogenetic analyses for each dataset were performed using maximum likelihood (ML) and Bayesian (BI) methods. Maximum likelihood analyses were performed with RaxML v.1.8.2. (Stamatakis 2006), using RAxMLGUI v.1.5. (Silvestro & Michalak 2012) for the mitochondrial dataset, as a single partition as selected by PartitionFinder. The ML analyses were carried out with 100 random addition replicates and the support of the nodes was assessed through 1000 bootstrap interactions (Felsenstein 1981). Bayesian Inference methods were carried out using MrBayes (Huelsenbeck & Ronquist 2001) and BEAST v.1.8.1. (Drummond *et al.* 2014) software. In MrBayes we implemented the model selected by PartitionFinder for the ND2 dataset (GTR+G model); the character state frequencies were fixed (0.2115, 0.1006, 0.3115, 0.3764); the substitution rates of GTR model was defined as fixed (1.0000, 8.0255, 1.0514, 17.4892, 1.0000). We ran the analysis twice, with a MCMC length of 100 million generations, sampling each 10 000 generations. The consensus tree was calculated after a burnin of 25%. The BEAST analysis was run implementing the partition scheme and substitution model estimated by Partition Finder analyses. The Coalescent Model of evolution with constant size (Kingman 1982) was applied, since we have mainly intraspecific data. The monophyly of the intraspecific lineages that were supported by ML and MrBayes analyses genetic group, was enforced by grouping the samples in Beauti v1.8.1. To estimate divergence times between lineages we used an uncorrelated lognormal relaxed clock model with substitution rate estimated in previous studies on Agamidae, which ranged from 0.62% to 0.81% (Macey *et al.* 1998; Schulte *et al.* 2003; Shoo *et al.* 2008; Melville *et al.* 2009; Edwards & Melville 2011; Wagner *et al.* 2011). We implemented these rates using a normal prior distribution with mean=0.00715 and stdev= 0.000578). All BEAST analyses were run in the CIPRES science gateway (Miller *et al.* 2010). The analysis was run three times, with a MCMC length of 30 million sampled every 3000 generations. Tracer v.1.5. (Rambaut *et al.* 2013) was used to assess the posterior trace plots and the effective

sample size values (ESS) of parameters, which should be greater than 200. All runs were then combined with LogCombiner and the Maximum Clade Credibility Tree was calculated with 25% of burnin in TreeAnnotator (both available in the BEAST package). FigTree v.1.4.(Rambaut 2012) was used to view the tree and export relative graphics. Nodes were considered strongly supported when ML bootstrap values (bp) ≥ 80% and posterior probability (pp) support values ≥ 0.95.

Dnasp v.5 (Librado & Rozas 2009) was used to phase the nuclear datasets, using the PHASE algorithm (Stephens *et al.* 2001) with 1000 replications and 100 burnin. We ran phasing three times, with different random seed numbers in order to test for congruence between phased analyses. For each polymorphic site we select phase results which were equal for at least two of the runs to build the final phased alignments.

Phylogenetic relationships between nuclear haplotypes were estimated using the statistical parsimony networks implemented in TCS v.1.2.1 (Clement *et al.* 2000) (TCS model) and the Median joining (MJ) approach implemented in NETWORK v5.0 (Polzin 2014-2016). Gaps were treated as a 5$^{th}$ state. The network produced by TCS was then graphically edited using TCS beautifier (Santos *et al.* 2015) available on *(www.cibio.up.pt/software/tcsBU/*).

## 4.3. Results

### 4.3.1. Dataset

We obtained 51 sequences for ND2 gene (686 bp), 53 sequences for the MC1R gene (620 bp), and 56 sequences for RAG-I (467 bp) gene fragments. Regarding polymorphic sites, the mtDNA (ND2) has 51 sites, while the nuclear data have 13 and 33 sites for MC1R and RAG-I, respectively. The best model of evolution selected for the mitochondrial dataset was GTR+G for the Maximum likelihood analysis and the MrBayes and TrN+G for the BEAST analysis (depending on the set of models available in each software), as a single partition.

### 4.3.2. Mitochondrial phylogenetic analysis

The haplotype network inferred by TCS model, for ND2 gene, revealed two main genetic lineages with geographic coherence: one in the south west and another in the north east of the species range (Fig. 23). The uncorrected *p*-distances average values calculated

between *Uromastyx nigriventris* and outgroup used, *U. dispar maliensis*, was 0.0658. Between the two genetic lineages found within *U.nigriventris*, the uncorrected *p*-distance was 0.038, while within the group L1 and L2 was 0.005 and 0.004, respectively. Concerning to the other two ribossomal mitochondrial genes (12S and 16S) the values of uncorrected *p*-distance between the two genetic lineages of *U.nigriventris* was 0.0225 and 0.007, for 12S and 16S, respectively; while uncorrected *p*-distance within the group L1 and L2, respectively, was 0.0029 and 0.001 for the 12S and 0.001 for the 16S within the L1 group (for L2 insufficient sequences were available).

Both ML and BI tree based on the ND2 data revealed well-supported estimates of relationships with similar branch topology (Fig. 24). The same two main clades obtained in the network analysis were recovered: one from the south west, the other from the north east of the range.

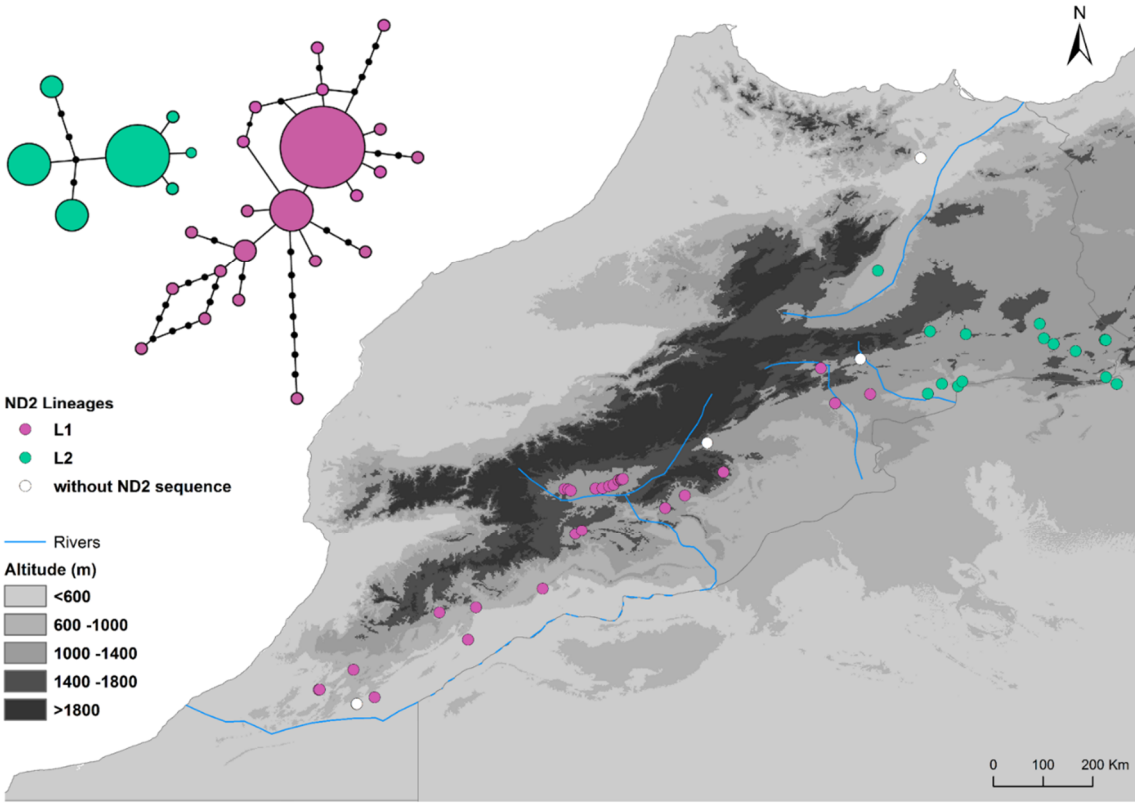


**Fig. 15 -** Distribution of the two genetic lineages of *Uromastyx nigriventris* revealed by phylogenetic parsimony network based on ND2 sequences (top-left). The size of the haplotypes are correspondent to the number of sequences (range from 1 to 8 sequences).

The estimated age of divergence between the two lineages of *Uromastyx nigriventris* is 3.5My ago with a 95% higher posterior density (HPD) interval of 1.57-5.77Mya (Fig. 25).

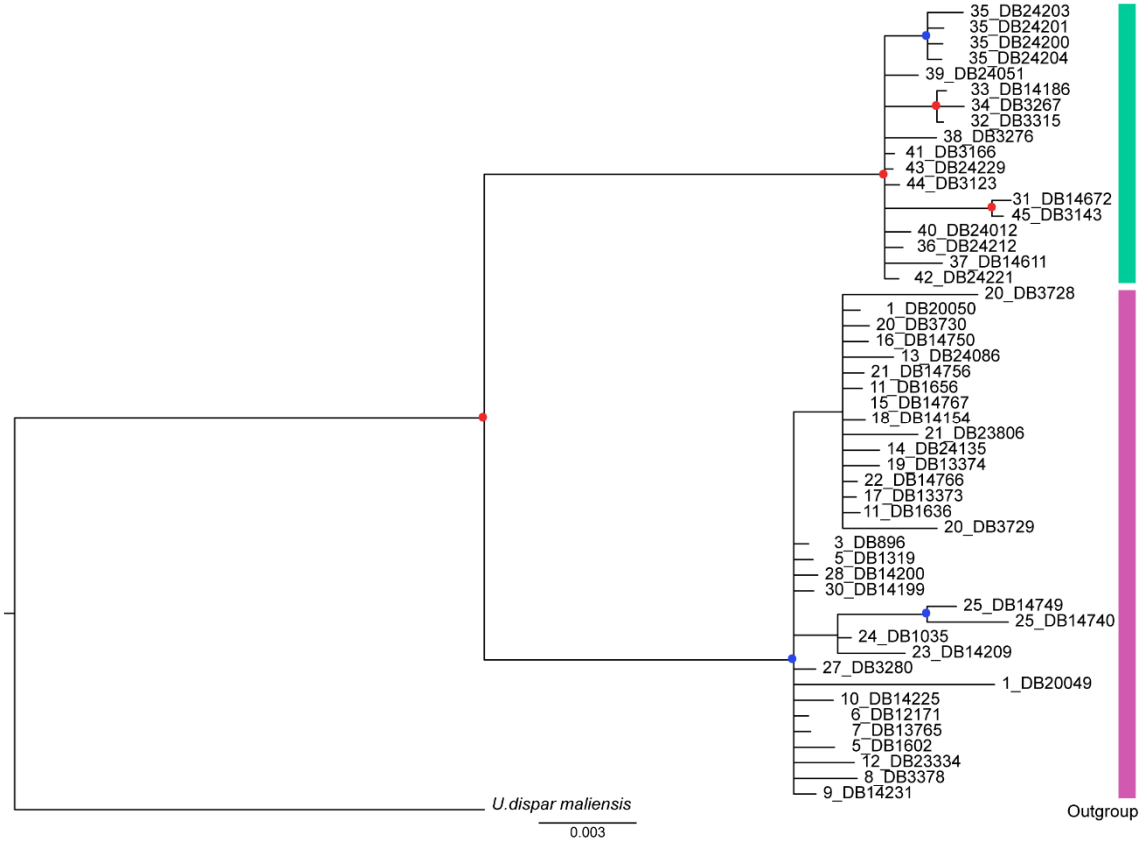


**Fig. 24 –** Phylogenetic tree of mtDNA (ND2) showing the genetic relationships within *Uromastyx nigriventris*, based on Maximum likelihood (RaxML) and Bayesian Inference (MrBayes) methods. The red dots were placed in nodes with both bootstrap support values>80% and Bayesian posterior probabilities >0.95. The blue dots represent supported nodes only for one of the methods used. The coloured bars represents the different genetic lineages: L1 (purple) and L2 (green).

### 4.3.3. Nuclear haplotype network analysis

The haplotype networks of MC1R and RAG-1 markers are shown in Figure 26 and 27. Median joining and TCS model showed identical networks for both genes. The MC1R haplotype network is characterised by sixteen different haplotypes (Fig. 26). Among them only two haplotypes are shared between Individuals from the southwestern and northeastern lineages. Regarding the RAG-1 we found 26 haplotypes with three haplotypes shared by individuals belonging to the two mitochondrial lineages (Fig. 27). Genetic diversity is higher in the northeastern lineage (number of haplotypes in L1 at ND2, MC1R and RAG-1 are equal to 22, 10 and 17, respectively) compared to the southwestern lineage (number of haplotypes in L2 at ND2, MC1R and RAG-1 are equal to 7, 7 and 9 respectively).

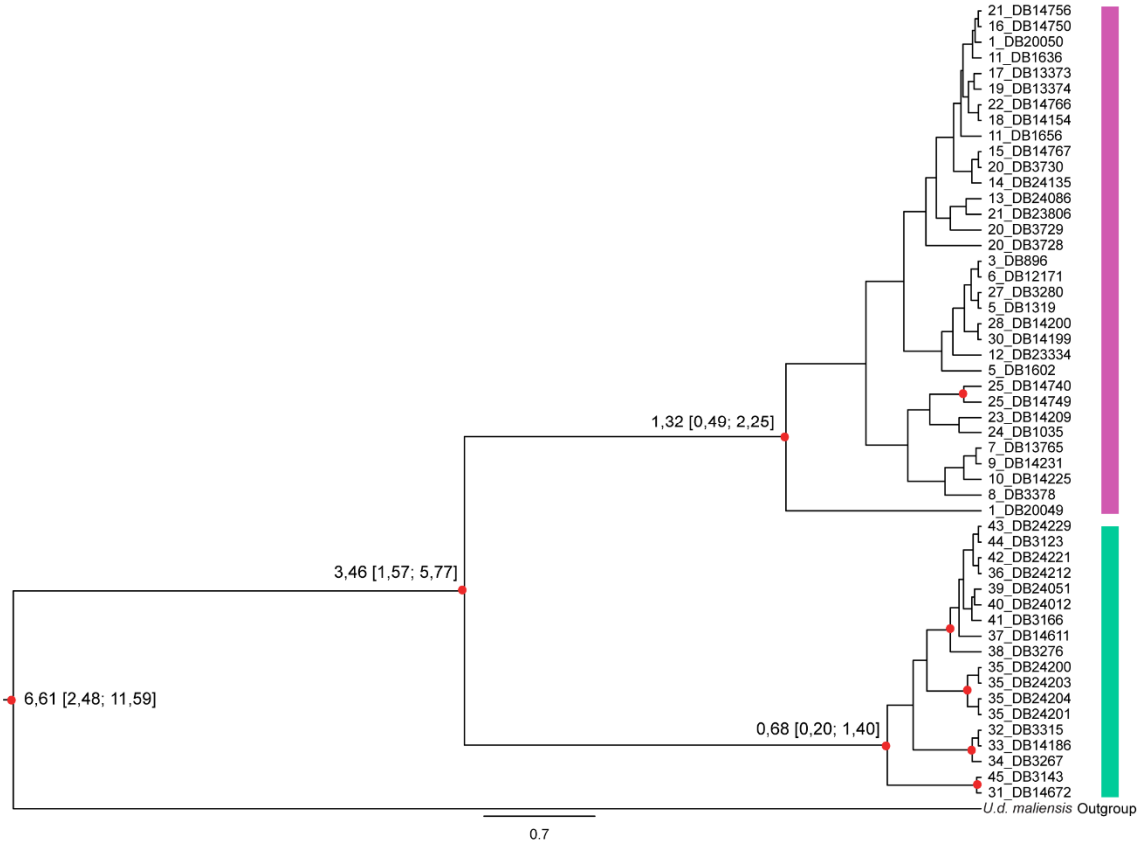


**Fig. 25 -** BEAST maximum clade credibility tree for the *Uromastyx nigriventris* lineages. Divergence times correspond to the mean posterior estimate of their age in million years. The values within parenthesis indicate the height 95% HPD (high posterior density) interval. The red dots represent nodes with posterior probability values above 0.95. The coloured bars represents the different genetic lineages: L1 (purple) and L2 (green).

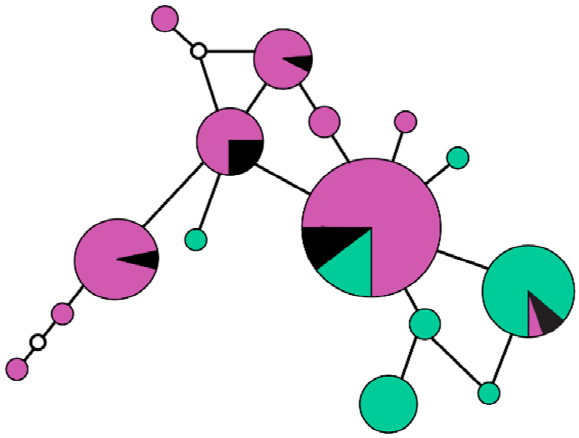

**Fig. 26** - Nuclear haplotype networks based on MC1R sequences of *Uromastyx nigriventris.* The same network was inferred by TCS and Median-joining methods. Haplotype were coloured according to the mitochondrial lineage to which each individual belong (based on phylogenetic analyses of the ND2 dataset), L1 (in purple) and L2 (in green water). MC1R haplotype of individuals without ND2 sequence were colour in black of any lineage. The haplotype size is correspondent to the number of sequences (range from 1 to 37 sequences).

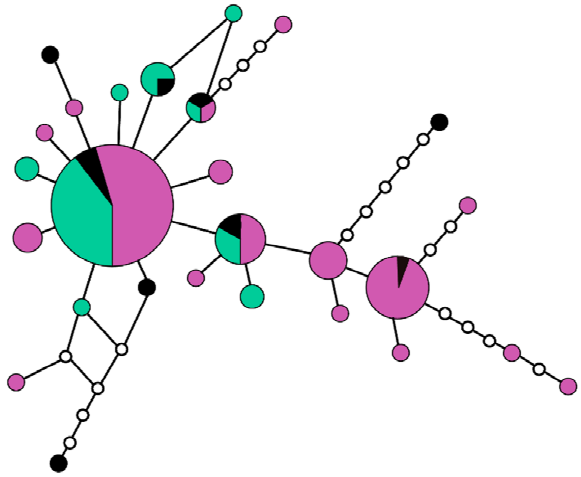

**Fig. 27 -** Nuclear haplotype networks based on RAG-1 sequences of *Uromastyx nigriventris*. The same network was inferred by TCS and Median-joining methods. Haplotype were coloured according to the mitochondrial lineage to which each individual belong (based on phylogenetic analyses of the ND2 dataset), L1 (in purple) and L2 (in green). RAG-1 haplotype of individuals without ND2 sequence were colour in black of any lineage. The haplotype size is correspondent to the number of sequences (range from 1 to 53 sequences).

## 4.4. Discussion

The phylogeographic pattern of *U. nigriventris* shows two main genetic lineages recovered by all phylogenetic analysis based on mitochondrial data. These lineages are geographically well-separated, where one lineage occurs in the south and west, and the other lineage, in the north and east of the range, with a potential contact zone in the southern portion of the Moulouya river valley, around the High Plateau of Debdou (Fig. 23). The genetic divergence between these two lineages is not very high with an average p-distances value of 3.8%, 2.3% and 0.7% for ND2, 12S and 16S mitochondrial genes, respectively. These estimates agree with the minimal genetic diversity reported for the rDNA 16S gene region by Wilms *et al.* (2007). Thus, despite the limited intraspecific sampling in this previous study, there were not sampled divergent genetic lineages across the species distribution range. These values of intraspecific genetic variation are low compared with species inhabiting in mountainous regions (Perera & Harris 2010b; Barata et al. 2012a; b; Sousa et al. 2012), but similar to other arid-adapted species distributed across the North Africa and Arabia region, such as *Agama* sp., *Trapelus* sp. and *Pseudotrapelus* sp. Regarding the nuclear markers there is a degree of haplotype sharing between individuals belonging to different lineages, suggesting an incomplete lineage sorting at the nuclear loci analysed. The differences between phylogeographic patterns observed in mitochondrial and nuclear markers can be explained by the different timing in sorting, of these two types of molecular data. The substitution rates are substantially higher in mitochondrial DNA, since mtDNA is maternally inherit, the

effective population size is low, resulting in general in more polymorphisms accumulated at the same time, when compared with nuclear loci (Brito & Edwards 2009).

The phylogeographic pattern recovered for the *Uromastyx nigriventris* species is consistent with a possible relatively recent separation of the two lineages. According to our estimates, the divergence of *U. nigriventris* started in the Pliocene/Pleistocene (3.46; 95 HPD [1.57 – 5.77]. During this period, in the Maghreb, there was evidence of a rapid expansion of arid regions (in the late Neogene) followed by recurrent dry-humid cycles, caused by the global glaciations and inter-glaciation periods (Demenocal 1995; Dupont & Leroy 1995; DeMenocal 2004). Particularly, marine sediments accumulated off the western and eastern margin of subtropical North Africa provided evidence of recurrent arid-humid climate cycles and progressive increase of African aridity, during the late Neogene (last ca. 5 Mya) (DeMenocal 2004). In addition, a detailed pollen record, also suggested the presence of arid conditions in the region, at 3.5 to 3.2 Mya, in a first step and, also at 2.61-1.74 Mya, as a stronger step of severe dry periods. These events could have played a fundamental role in the diversification of many Saharan species.

However, these climatic changes have affected the entire distribution's range of *Uromastyx nigriventris*, so to explain the historical isolation into two population groups of this species, climatic events must have acted in synergy with other factors. The two genetic lineages are currently separated by the Saoura River but is unlikely that this river has played a role in the diversification of them, since this has been an intermittent river (which was filled only during the humid stages, (Kohler *et al.* 2010), and also, may other similar rivers occurred within the range of each lineage (Fig. 23).

On the other hand, in the eastern margin of the Saoura River, an important geologic formation is present, the Grand Occidental Erg (also called Erg Chebbi) and may have played a role of long term barrier between the two lineages of *Uromastyx nigriventris* (Alimen 1965; Wilson 1973). The Erg Chebbi originated at the Villanfranchian age, overlapping with the end of the Pliocene and beginning of Pleistocene (3.0 – 2.0 Mya), during the expansion of arid zones (Alimen 1965). Thus, the estimated split of *U. nigriventris'* lineages closely match with the time of Erg Chebbi formation (also accounting for the uncertainty associates to the timing of geologic events and to molecular clock estimation). The Erg consists of a desert of dunes, where vegetation is sparse or absent because of the low rainfall and strong wind, which also change constantly dune topography (Wilson 1973). Such instable sandy habitat represent a strong ecological barrier to species such as *Uromastyx nigriventris,* since spiny-tailed lizards are herbivores and live in sandy areas, covered by gravel and small stones

(Wilms *et al.* 2007). The presence of extreme dune-desert, with very hot temperature and without either food or shelter availability, makes for an inhospitable habitat for this species. Also, it is documented that they avoid completely sandy deserts, but they can travel for some kilometres, in order to reach other zones (Wilms & Böhme 2007). However, the Erg Chebbi comprises a high area, with about 103,000km$^2$ of area (Wilson 1973). Moreover, Wilson (1973) suggested that ergs were probably more widespread in the distant geological past, before plants of hardy substrate successfully colonized the dry land. Therefore, a possible scenario for the diversification of the two *U. nigriventris* lineages could be of allopatric divergence triggered by the onset of the ecological barrier represented by sand dunes habitat associated to the Erg Chebbi formation.

The Erg Chebbi may have acted as a long-term barrier to dispersion also in other Saharan species. In *Cerastes vipera* some morphological variations, in the number of ventrals, were verified between populations from the east and west of the Erg Chebbi (Jooris & Fourmy 1996). Also, in *Acanthodactylus scutellatus*, it was suggested morphological differences, in size and number of ventral and dorsal scales, between populations from the Hauts Plateaux (in the west) and the ones from Erfoud and Erg Chebbi (Mellado & Olmedo 1990). Therefore despite the lack of detailed studies on the geographic variation of North Saharan species, the Erg formation appear as a potential phylogeographic barrier which certainly warrants further investigation.

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# 5. FINAL REMARKS

In Europe detailed geographic investigations have uncovered general patterns of phylogeographic historical and demographic trends, in diverse species. Such patterns were not only shaped by glacial cycles, but also by older diversification events mediated by the formation of peninsulas, such as the Iberian, Balkan and Italian peninsulas and mountainous systems, such as the Pyrenees and Alps, associated to the collision of the African and European tectonic plates (Hewitt 1996, 1999, 2000, 2011; Gómez & Lunt 2006). However, for the Maghreb we still have limited number of cases to be compared in order to draw general patterns (Brito *et al.* 2014). Nonetheless, we can identify a major role of the Atlas Mountains complex (Barata *et al.* 2012a; b; Habel *et al.* 2012), or of the Messinian crisis and re-opening of the Strait of Gibraltar (Harris *et al.* 2004a; b; Guicking *et al.* 2008; Kaliontzopoulou *et al.* 2011) and of the Moulouya River basin (Alvarez *et al.* 2000; Vences *et al.* 2014) for northern species. Moreover, almost no studies are available for the Saharan species, occurring to the south and east of the Atlas Mountains.

This work shed new light into the structure and diversification of western Maghrebian species and led to new inferences regarding possible historical events responsible for their formation. The two North Saharan species demonstrated different levels of intraspecific variation, which were originated as a consequence of distinct past events. *Ptyodactylus oudrii* is a semi-desert and rock-adapted gecko, which exhibits a great genetic differentiation, into four main lineages, spatially and genetically well separated (Chapter I). A more plausible explanation for the divergence found could be the allopatric divergence of these group triggered by the uplift of the Atlas Mountains during the middle/late Miocene. The low dispersal abilities of *Ptyodactylus oudrii* and its strong association with rocky-habitat may have contributed to the historical separation between populations. A similar scenario of allopatric divergence, associated to the uplift of the Atlas Mountains was inferred for scorpions of the *Buthus* genus species which also have low dispersal abilities (Habel *et al.* 2012), as well as in many other reptiles. Indeed, the uplift of the Atlas Mountains complex has driven species divergence, acting as barrier for species in the north, such as in *Natrix maura* (Barata *et al.* 2008; Guicking *et al.* 2008), *Timon tangitanus* (Perera & Harris 2010) and *Mauremys leprosa* (Fritz *et al.* 2005), but also in the south of this mountain range, in species like *Ptyodactylus oudrii, Quedenfeltdia* sp. (Barata *et al.* 2012b) and *Atlantolacerta andreanskyi* (Barata *et al.* 2012a). On the other hand, a much more recent divergence was estimated between the two genetic lineages found within *Uromastyx nigriventris* (Chapter II). In this case, range

fragmentation caused by the Pliocene-Pleistocene climatic oscillation coupled with the formation of the Erg Chebbi, during the increase of aridification provided the best explanation for the observed phylogeographic pattern within this species. Moreover, the higher dispersal abilities of *U.nigriventris* favoured the intermixing between populations and thus lower intraspecific genetic variation was found. Also, in other desert species genetic divergence has been associated to the Pliocene/Pleistocene climatic changes, such as in the case of the genera *Agama* (Gonçalves *et al.* 2012) and *Pseudotrapelus* (Melnikova *et al.* 2015), as well as in *Acanthodactylus boskianus* (Tamar *et al.* 2014, 2016) and *A. pardalis* (Fonseca *et al.* 2008) groups and in rodents as *Gerbillus campestris* (Nicolas *et al.* 2014).

In summary, the phylogeographic pattern within the two studied Saharan species are very different on each other, suggesting that it will not be possible to explain their pattern of genetic variation and geographic distribution in one simple history. Clearly more studies are essential to unravel the complexity of biogeographic and evolutionary processes shaping biodiversity patterns of this region.

In addition, by adding nuclear markers to our study we were able to confirm the phylogeographic pattern recovered by the mitochondrial loci and to show different rates of lineage sorting associated to distinct divergent times of lineages. In *Ptyodactylus oudrii* an almost complete lineage sorting as result of ancient lineages and in *Uromastyx nigriventris* an incomplete lineage sorting associated to more recent divergences. Consequently, it emphasises the need of a multilocus approach to have an enlarged view of biogeographical and evolutionary processes.

## 5.1. Future perspectives

Certainly, more studies are necessary to understand the phylogeographic patterns and evolutionary processes shaping the distribution and genetic variation of the north Saharan species and to develop a set of hypotheses about the processes that shaped the biodiversity found in the Maghreb region.

In the case of *Ptyodactylus oudrii* an integrative taxonomic approach, including morphological and ecological data will be carried out in order to reassess the taxonomy of this group, to delimit and to describe the species within this cryptic complex. Also, taking into account the new records for the Middle Atlas (François *et al.* 2016) and a possible larger distribution range. One the other hand, in *Uromastyx nigriventris* more sampling efforts are needed, in order to cover the complete distribution range. Also, it

should be applied different time estimation methods, including alternative molecular clocks and adding fossil calibrations, in order to obtain more precise divergence times of the lineages. Finally, in both cases, demographic analyses will be needed to unravel possible contraction and expansion events of species distribution ranges, associated to past climatic events.

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# 6. APPENDIX

Appendix I. Results of PCA analysis (Chapter 3)

**Table A** – Loading score values and percentage of variance explained of each component of PCA I, relating the occurrence of *Ptyodactylus oudrii* with variables of habitat. Variables codes are explained in Table 3 (Chapter 3)

**Table B** – Loading scores values of PCA II relating the occurrence of *Ptyodactylus oudrii*'s lineages with variables of habitat. Variables codes are explained in Table 3 (Chapter 3)

**Table A.** Loading score values and percentage of variance explained of each component of PCA I, relating the occurrence of Ptyodactylus oudrii with variables of habitat. Variables codes are explained in Table 3 (Chapter 3)

| | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 | PC8 | PC9 | PC10 | PC11 | PC12 | PC13 | PC14 | PC15 | PC16 | PC17 | PC18 | PC19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **% VARIANCE EXPLAINED** | 37.73 | 19.66 | 12.77 | 6.109 | 4.227 | 3.625 | 3.022 | 2.47 | 2.286 | 1.815 | 1.507 | 1.311 | 1.041 | 0.882 | 0.688 | 0.453 | 0.242 | 0.163 | 0 |
| **CARB** | -0.17 | 0.24 | 0.23 | -0.31 | 0.30 | -0.01 | -0.14 | -0.60 | -0.14 | 0.42 | -0.24 | 0.16 | -0.07 | 0.03 | -0.04 | 0.03 | 0.01 | -0.02 | -4.13E-15 |
| **NCARB** | -0.15 | 0.18 | 0.19 | 0.48 | -0.07 | -0.62 | 0.07 | 0.11 | 0.31 | 0.30 | -0.03 | 0.23 | -0.12 | -0.15 | 0.01 | 0.05 | 0.01 | 0.02 | -3.38E-16 |
| **MSED** | 0.07 | -0.39 | 0.33 | -0.19 | -0.20 | -0.14 | -0.04 | 0.01 | 0.01 | -0.01 | 0.04 | 0.15 | -0.01 | 0.10 | -0.10 | 0.18 | -0.09 | -0.74 | 3.12E-14 |
| **SIL** | 0.02 | -0.29 | 0.33 | -0.45 | -0.39 | -0.20 | -0.01 | 0.02 | 0.04 | -0.01 | 0.10 | 0.22 | 0.08 | -0.01 | 0.11 | -0.13 | 0.07 | 0.56 | -2.02E-14 |
| **ALLSAL** | -0.08 | 0.44 | -0.08 | -0.37 | 0.01 | -0.06 | 0.11 | 0.19 | 0.19 | -0.11 | 0.14 | 0.12 | 0.01 | 0.05 | 0.00 | 0.02 | 0.00 | -0.13 | -7.07E-01 |
| **ALL** | -0.08 | 0.44 | -0.08 | -0.37 | 0.01 | -0.06 | 0.11 | 0.19 | 0.19 | -0.11 | 0.14 | 0.12 | 0.01 | 0.05 | 0.00 | 0.02 | 0.00 | -0.13 | 7.07E-01 |
| **SPL** | 0.01 | 0.32 | 0.33 | 0.21 | -0.28 | 0.30 | 0.01 | -0.01 | -0.40 | 0.14 | 0.59 | 0.07 | -0.11 | -0.15 | -0.11 | 0.02 | 0.00 | -0.01 | 4.43E-17 |
| **IRPL** | -0.26 | 0.13 | 0.31 | 0.10 | 0.03 | -0.28 | -0.12 | -0.14 | -0.10 | -0.32 | 0.14 | -0.46 | 0.03 | 0.51 | 0.27 | -0.11 | -0.12 | 0.00 | 3.63E-17 |
| **BR** | -0.28 | -0.11 | 0.24 | 0.00 | 0.39 | 0.01 | 0.09 | 0.35 | -0.27 | -0.10 | -0.04 | 0.09 | 0.01 | 0.02 | 0.02 | 0.17 | 0.67 | 0.01 | -1.39E-16 |
| **LMOU** | -0.29 | -0.14 | 0.19 | -0.01 | 0.37 | 0.09 | 0.14 | 0.32 | -0.17 | -0.03 | -0.04 | 0.23 | 0.00 | -0.09 | -0.02 | -0.22 | -0.66 | 0.07 | 1.12E-16 |
| **HMOU** | -0.23 | -0.23 | 0.07 | 0.04 | 0.16 | 0.19 | 0.57 | -0.34 | 0.45 | 0.03 | 0.36 | -0.10 | 0.13 | 0.02 | -0.11 | 0.11 | 0.04 | 0.05 | -1.38E-17 |
| **RCROP** | -0.31 | -0.08 | -0.18 | -0.03 | -0.22 | 0.02 | -0.01 | 0.18 | -0.03 | 0.29 | -0.06 | -0.11 | -0.18 | 0.47 | -0.62 | -0.15 | 0.04 | 0.08 | 5.80E-17 |
| **CROP** | -0.33 | -0.02 | -0.14 | -0.10 | -0.21 | 0.06 | -0.01 | 0.16 | -0.11 | 0.30 | -0.06 | -0.23 | 0.14 | -0.05 | 0.29 | 0.69 | -0.21 | 0.08 | 1.57E-16 |
| **VEG** | -0.33 | -0.04 | -0.13 | -0.03 | -0.21 | 0.08 | 0.07 | 0.04 | -0.03 | 0.31 | -0.03 | -0.12 | 0.26 | -0.19 | 0.37 | -0.59 | 0.18 | -0.29 | -7.10E-17 |
| **FOR** | -0.26 | -0.12 | -0.02 | 0.09 | 0.10 | 0.31 | -0.67 | 0.03 | 0.44 | -0.01 | 0.25 | 0.23 | -0.12 | 0.07 | 0.15 | 0.01 | 0.04 | 0.02 | -7.19E-17 |
| **GRASS** | -0.31 | -0.10 | -0.16 | -0.11 | -0.15 | -0.06 | 0.12 | -0.17 | -0.08 | -0.28 | -0.03 | -0.06 | -0.77 | -0.30 | 0.11 | -0.01 | 0.02 | -0.04 | -1.53E-16 |
| **SVEG** | -0.32 | 0.07 | 0.10 | -0.03 | -0.10 | -0.09 | -0.26 | -0.11 | 0.03 | -0.33 | -0.10 | -0.20 | 0.37 | -0.50 | -0.48 | 0.03 | -0.01 | 0.00 | -4.55E-17 |
| **AA** | -0.26 | 0.03 | -0.30 | 0.22 | -0.23 | -0.01 | 0.11 | -0.28 | -0.23 | -0.31 | -0.07 | 0.60 | 0.25 | 0.25 | 0.06 | 0.08 | 0.00 | -0.01 | 3.76E-17 |
| **BA** | -0.02 | 0.21 | 0.43 | 0.14 | -0.29 | 0.47 | 0.16 | 0.08 | 0.26 | -0.13 | -0.56 | 0.01 | -0.08 | 0.09 | 0.06 | 0.01 | 0.01 | 0.01 | 1.39E-17 |

**Table B.** Loading score values and percentage of variance explained of each component of PCA II, relating the occurrence of Ptyodactylus oudrii's lineages with variables of habitat. Variables codes are explained in Table 3 (Chapter 3)

| | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 | PC8 | PC9 | PC10 | PC11 | PC12 | PC13 | PC14 | PC15 | PC16 | PC17 | PC18 | PC19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **% VARIANCE EXPLAINED** | 28.98 | 16.58 | 11.25 | 8.595 | 7.813 | 6.122 | 4.288 | 4.138 | 3.073 | 2.328 | 1.749 | 1.194 | 1.181 | 0.87 | 0.656 | 0.598 | 0.49 | 0.09 | 0 |
| **CARB** | 0.25 | 0.25 | 0.17 | 0.26 | 0.05 | -0.18 | -0.14 | 0.21 | -0.40 | -0.16 | 0.08 | -0.62 | 0.07 | -0.02 | 0.02 | 0.05 | 0.33 | 0.00 | 0.00 |
| **NCARB** | 0.12 | 0.09 | 0.04 | -0.39 | -0.42 | 0.02 | -0.05 | -0.49 | -0.52 | 0.21 | 0.04 | 0.12 | 0.02 | 0.14 | 0.11 | 0.01 | 0.19 | 0.03 | 0.00 |
| **MSED** | -0.04 | -0.52 | 0.20 | 0.10 | 0.04 | 0.07 | 0.02 | -0.15 | -0.01 | -0.01 | -0.06 | -0.17 | -0.13 | -0.02 | 0.15 | -0.03 | 0.08 | 0.76 | 0.00 |
| **SIL** | -0.01 | -0.45 | 0.29 | 0.15 | 0.11 | 0.15 | -0.05 | -0.20 | 0.01 | -0.07 | 0.25 | -0.08 | -0.26 | 0.13 | 0.28 | -0.18 | 0.09 | -0.58 | 0.00 |
| **ALLSAL** | 0.23 | 0.33 | 0.35 | 0.14 | 0.10 | 0.00 | -0.09 | -0.15 | 0.14 | 0.03 | 0.14 | 0.16 | -0.15 | 0.12 | -0.02 | -0.07 | -0.16 | 0.15 | -0.71 |
| **ALL** | 0.23 | 0.33 | 0.35 | 0.14 | 0.10 | 0.00 | -0.09 | -0.15 | 0.14 | 0.03 | 0.14 | 0.16 | -0.15 | 0.12 | -0.02 | -0.07 | -0.16 | 0.15 | 0.71 |
| **SPL** | 0.25 | 0.02 | 0.00 | -0.30 | 0.34 | 0.39 | 0.07 | 0.26 | -0.06 | 0.05 | -0.34 | -0.06 | 0.16 | 0.53 | 0.17 | -0.21 | 0.02 | 0.02 | 0.00 |
| **IRPL** | 0.17 | 0.00 | 0.08 | -0.54 | 0.27 | 0.33 | 0.07 | 0.11 | -0.01 | 0.00 | 0.34 | -0.11 | -0.26 | -0.44 | -0.15 | 0.26 | 0.05 | 0.02 | 0.00 |
| **BR** | -0.20 | 0.00 | 0.38 | -0.28 | 0.13 | -0.30 | 0.38 | -0.07 | -0.15 | -0.27 | -0.08 | -0.01 | 0.18 | -0.15 | -0.26 | -0.51 | -0.08 | -0.03 | 0.00 |
| **LMOU** | -0.25 | 0.08 | 0.34 | -0.05 | 0.02 | -0.27 | 0.50 | 0.22 | 0.04 | 0.26 | 0.01 | 0.07 | -0.10 | 0.22 | 0.26 | 0.47 | 0.18 | -0.02 | 0.00 |
| **HMOU** | -0.28 | 0.12 | 0.12 | 0.11 | -0.25 | 0.24 | -0.10 | 0.46 | -0.28 | 0.27 | -0.13 | 0.10 | -0.46 | -0.15 | -0.01 | -0.35 | -0.03 | 0.02 | 0.00 |
| **RCROP** | -0.29 | 0.08 | -0.30 | 0.11 | 0.29 | 0.03 | 0.14 | -0.24 | -0.07 | 0.19 | 0.19 | -0.15 | -0.27 | 0.38 | -0.52 | -0.07 | 0.22 | 0.05 | 0.00 |
| **CROP** | -0.25 | 0.14 | 0.26 | 0.07 | 0.27 | 0.20 | -0.25 | -0.30 | 0.13 | 0.30 | -0.45 | -0.04 | 0.20 | -0.31 | -0.04 | 0.09 | 0.31 | -0.12 | 0.00 |
| **VEG** | -0.37 | 0.12 | 0.01 | 0.00 | 0.12 | 0.14 | -0.04 | -0.16 | -0.25 | 0.06 | 0.05 | -0.37 | 0.09 | 0.04 | 0.19 | 0.18 | -0.71 | -0.01 | 0.00 |
| **FOR** | -0.10 | -0.15 | 0.20 | -0.41 | -0.12 | -0.37 | -0.55 | 0.13 | 0.23 | 0.03 | -0.13 | -0.20 | -0.19 | 0.27 | -0.23 | 0.10 | -0.08 | -0.04 | 0.00 |
| **GRASS** | -0.34 | 0.06 | 0.11 | -0.09 | -0.09 | 0.17 | -0.22 | 0.19 | 0.16 | 0.10 | 0.59 | 0.03 | 0.50 | 0.10 | 0.09 | -0.18 | 0.20 | 0.14 | 0.00 |
| **SVEG** | -0.32 | 0.12 | 0.10 | 0.02 | 0.01 | 0.24 | -0.16 | 0.01 | -0.18 | -0.72 | -0.09 | 0.29 | -0.10 | 0.16 | -0.04 | 0.29 | 0.15 | 0.05 | 0.00 |
| **AA** | -0.10 | 0.07 | -0.27 | -0.12 | 0.52 | -0.42 | -0.27 | 0.01 | -0.21 | 0.02 | 0.09 | 0.27 | -0.14 | -0.11 | 0.43 | -0.14 | 0.08 | 0.09 | 0.00 |
| **BA** | 0.15 | -0.36 | 0.19 | 0.20 | 0.22 | -0.05 | -0.15 | 0.18 | -0.43 | 0.22 | 0.08 | 0.36 | 0.28 | 0.03 | -0.38 | 0.22 | -0.16 | -0.05 | 0.00 |