U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Ultrastructure and phylogeny of a microsporidian parasite infecting the big-scale sand smelt, *Atherina boyeri* Risso, 1810 in the Minho River, Portugal

Marília Catarina dos Santos Margato

Dissertation for Master in Marine Sciences and Marine Resources – Marine Biology and Ecology

2014

MARÍLIA CATARINA DOS SANTOS MARGATO

Ultrastructure and phylogeny of a microsporidian parasite infecting the big-scale sand smelt, *Atherina boyeri* Risso, 1810 in the Minho River, Portugal

Dissertation for Master's degree in Marine Sciences and Marine Resources – Marine Biology and Ecology submitted to the Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal

Supervisor – Doctor Carlos Azevedo
Category – "Professor Catedrático Jubilado"
Affiliation – Institute of Biomedical Sciences Abel Salazar, University of Porto
Co-supervisor – Sónia Raquel Oliveira Rocha
Category – Doctoral Student
Affiliation – Institute of Biomedical Sciences Abel Salazar, University of Porto

Agradecimentos

A execução e entrega desta tese só foi possível com a ajuda e colaboração prestada por várias pessoas. A quem quero expressar os meus sinceros agradecimentos pelo apoio e motivação, nomeadamente:

aos meus orientadores, Professor Doutor Carlos Azevedo e Sónia Rocha, pelos conselhos, dicas e enorme colaboração e orientação neste trabalho, sem os quais não seria possível a sua execução,

à Doutora Graça Casal que, pelo trabalho desenvolvido neste filo, deu uma assistência imprescindível para o desenvolvimento desta tese,

às técnicas Ângela Alves e Elsa Oliveira por toda a assistência técnica prestada e auxílio na execução dos procedimentos laboratoriais,

aos meus colegas de mestrado pelos encontros de desabafo sobre teses e laboratórios que fomentaram a troca de ideias e informação, e me motivaram para finalizar este trabalho e ter esperança para o futuro,

a todos os meus amigos e às meninas da tuna que de alguma forma contribuíram, motivando-me a continuar em frente nos momentos de maior desespero e aflição, e por toda a preocupação e apoio que demonstraram,

ao Raúl por ter sido o meu maior apoio psicológico e o meu melhor amigo, e por todas as horas que passou a ouvir os meus desabafos e a aconselhar-me da melhor forma que pôde, por me ter ajudado a manter a minha sanidade mental e a combater os maus momentos de bloqueio de escrita, de desespero e também de preguiça,

e, por fim, aos meus pais, que ao longo da minha vida sempre apoiaram as minhas decisões, sem questionar mas aconselhando, e que sem eles nada disto seria possível, obrigada pelo incondicional apoio que sempre me deram, permitindo o meu desenvolvimento como pessoa, mesmo que para isso ficasse longe de casa e não conseguisse ir tantas vezes como gostariam.

Preface

The present thesis was developed for my master's degree in Marine Sciences and Marine Resources, specialization on Marine Biology and Ecology in the Institute of Biomedical Sciences Abel Salazar of the University of Porto. The work developed focused in parasitology, more specifically in the phylum Microsporidia, as parasites on freshwater fishes of the Minho River, Portugal.

The first chapter is composed by an introduction to parasitism, a description of the site of sampling and infected species and then a summary of the phylum Microsporidia, including its taxonomic, morphological and biological features, as well as diagnosis, prevention and control measures. The main features of the genus *Glugea* are also summarized. The second chapter consists of an article that results from the work developed and is organized following the outline of the indexed journal chosen for publication. The third chapter gives general discussion and conclusion to this thesis, being followed by a References section corresponding to the citations made in the first and third chapters. Lastly, an Appendage composed by supplementary information is presented.

Abstract

The phylum Microsporidia Balbiani, 1882 is composed by unicellular organisms, obligatory intracellular parasites of all five classes of vertebrates, as well as some invertebrates such as mollusks, cnidarians, nematodes, rotifers, annelids, bryozoans and arthropods and even other microparasites such as Myxozoa, Ciliata and Apicomplexa. Microsporidians present mostly simple life cycles, being capable of horizontal and vertical transmission. These parasites form resistant spores that measure 1-40 µm in length and display a trilaminate cell wall. Within the spores, the polar filament coils forming single or double rows around the posterior vacuole. This structure is part of a complex mechanism of transmission that allows the extrusion and injection of the infectious agent into a neighboring host cell. Being able to pierce cell membranes, the polar filament forms a hollow tube that can reach 50-500 µm in length.

There are 156 species belonging to 18 microsporidian genera described infecting fish. The present study aimed to identify and describe parasitic species infecting fish in the Minho River, by sampling and dissecting fish for the macroscopic and microscopic detection of parasitic infection, with infected tissues later being prepared for ultrastructural and molecular analysis. Of the 23 specimens of the big-scale sand smelt *Atherina boyeri* Risso, 1810 analyzed, 9 presented cyst-like structures resulting from a microsporidian infection (prevalence of infection was estimated at ~39%).Cysts were macroscopically visible in the abdominal cavity and, upon microscopic analysis, contained many mature spores and small groups of developmental stages. Spores were ellipsoidal and measured 5.9 ± 0.4 µm in length and 2.6 ± 0.5 µm in width. The rRNA genes were sequenced and analyzed using maximum parsimony, maximum likelihood and neighbor-joining methodologies in MEGA 5.05. The use of combined microscopic and molecular data resulted in the identification of the Microsporidia as *Glugea atherinae* Berrebi, 1979, a species that was first identified as parasite of the fish species *Atherina boyeri* in French coastal lagoons of the Mediterranean Sea. This study constitutes the first report of a microsporidian species parasitizing a fish in the Minho River, as well as the first report of a *Glugea* in Portugal.

Key words: Fish · Parasite · *Glugea atherinae* · Ultrastruture · Phylogeny · Minho River

Resumo

O filo Microsporidia Balbiani, 1882 é composto por organismos unicelulares, parasitas intracelulares obrigatórios de todas as cinco classes de vertebrados, alguns invertebrados como moluscos, cnidários, nemátodes, rotíferos, anelídios, briozoários e artrópodes e até mesmo de outros microparasitas como Myxozoa, Ciliata e Apicomplexa. O ciclo de vida dos microsporídios pode ser complexo ou, na maioria dos casos, simples com apenas um hospedeiro, pondendo ocorrer transmissão horizontal ou vertical. Estes parasitas formam esporos resistentes com comprimentos entre 1 e 40 µm, que exibem uma parede celular trilaminar e contêm um filamento polar que se enrola formando uma fiada simples ou dupla em torno do agente infecioso. O filamento polar faz parte de um mecanismo de transmissão complexo, o qual permite a extrusão e entrada do agente infecioso numa célula hospedeira vizinha. Sendo capaz de perfurar a membrana celular, o filamente polar constitui um tubo ôco que mede 50-500 µm em comprimento.

Existem 156 espécies distribuídas por 18 géneros que parasitam peixes. O presente estudo teve por objetivo identificar e descrever espécies de parasitas que infetam peixes do Rio Minho, através da amostragem e dissecção de peixes para a deteção macro e microscópica de infeções parasíticas, sendo os tecidos infetados preparados para análises microscópicas e moleculares. Dos 23 espécimes de peixe-rei *Atherina boyeri* Risso, 1810 analizados, 9 apresentavam cistos resultantes de infeção por um Microsporidia (a prevalência de infeção foi determinada em ~39 %). Os cistos eram visíveis a olho nu na cavidade abdominal, mediante observação microscópica, verificou-se que continham numerosos esporos maduros e pequenos grupos de estadios em desenvolvimento. Os esporos apresentavam forma elipsoidal e mediam 5,9 ± 0,4 µm em comprimento e 2,6 ± 0,5 µm em largura. Os genes ribossomais foram sequenciados e analisados no software MEGA 5.05 utilizando os métodos de “maximum parsimony”, “maximum likelihood” e “neighbor-joining”. A utilização combinada de dados microscópicos e moleculares resultaram na identificação do microsporídio como *Glugea atherinae* Berrebi, 1979, uma espécie que foi primeiro identificada como parasita da espécie de peixe *Atherina boyeri* em lagoas da costa Francesa do Mediterrâneo. Este estudo constitui o primeiro relato de um microsporídio parasitante numa espécie de peixe do Rio Minho, bem como o primeiro *Glugea* descrito em Portugal.

Palavras-chave: **:** Peixe · Parasita · *Glugea atherinae* · Ultrastrutura · Filogenia · Rio Minho

Table of Contents

Chapter 1 - General Introduction

1.1 Introduction

Symbiosis defines all interactions between two or more different biological species. The term symbiosis derives from the Ancient Greek, meaning “living together” and applies to different types of interactions, being mutualism, commensalism and parasitism the three most known. Parasitism occurs when one of two organisms involved, the parasite, benefits, gains shelter, nutrition and a way to reproduce on the expense of the other, the host, that may suffer from a wide range of biological disorders, namely disease and even death. Parasites are organisms that evolved and adapted themselves to live on or within the hosts organisms; some have suffered dramatic reduction, as well as physical, genomic and functional adaptations. The parasitic way of life is generally so successful that it has evolved in almost every phylum of animals and plant groups, as well as in a diversity of species of bacteria, fungi, protozoa, helminthes, arthropods and myxozoans (Roberts and Janovy 2009).

Relationships between parasites and hosts can be very strict and intimate, becoming biochemically challenging; this boosted the science of parasitology to develop and evolve in order to understand and combat parasitic diseases of humans, as well as animals and plants of economic interest. Presently, molecular, cellular and microscopic techniques are widely used to describe the morphology, ultrastructure and phylogeny of the many species of parasites, as well as the study of immunology, transmission, life cycle and control techniques. The present thesis considers only the parasitic phylum Microsporidia Balbiani, 1882.

The Minho River or Miño (in Spanish), has an extension of about 300 km, the source being in Spain, in Meira’s hills, at an height of 700 meters, 230 km of its extension lie in Spain and in the last 70 km, the river defines the border between the extreme northwest of Portugal and Spain and flows into the Atlantic Ocean in the Portuguese coast. The international hydrographic region of the Minho River has a total area of 17080 km^2, from which 1934 km^2 are in the international sub-basin. From the total basin area, 16250 km^2 (95 %) lies in Spain and only 799 km^2 (5 %) are located in Portugal. The Minho River is an ecosystem with slightly polluted waters and, in Portuguese territory there are no significant permanent sources of pollution, only a few sporadic episodes of contamination due to forestry and agriculture. It constitutes a highly biodiverse ecosystem, being home to at least 51 identified species of fish (Antunes and Rodrigues 2004, Nunes 2012).

One of the fish families represented in the Minho River is the family Atherinidae. This family is composed only by the genus *Atherina* in the eastern Atlantic (Quignard and Pras 1986, Creech 1992), whose members are commonly referred to as sand

smelt. The big-scale sand smelt *Atherina boyeri* Risso, 1810 occurs along the east Atlantic Ocean coast, from the Kattegat Sea and Scotland to Mauritania, penetrating into Mediterranean waters near the Strait of Gibraltar. This small pelagic species displays a maximum length of 20 cm and inhabits coastal and brackish waters, feeding on small crustaceans and fish larvae and reproducing during the spring and summer (Quignard and Pras 1986, Billard 1997).

1.2 Microsporidia

The phylum Microsporidia is formed by obligate intracellular parasites. These unicellular parasites present small dimensions (1 to 40 µm) and are best recognized by their spore form, which is the only stage of their life cycle that can survive outside the host, mainly because of its resistant, trilaminar wall. Microsporidians possess worldwide range, having been reported from all principal regions of the world (Azevedo and Matos 2002, Freeman et al. 2004, Forest et al. 2009, Abdel-Baki et al. 2012, Mansour et al. 2013). They are highly reduced eukaryotes, at every level, from morphology and ultrastructure, to biochemistry and metabolism, and even at the molecular level in genes and genomes (Keeling and Fast 2002). The great divergence and phenotypic variability of Microsporidia is mirrored in the size and structure of its genome. *Encephalitozoon cuniculi* was the first microsporidian to be sequenced, and was found to have the smallest genome of any eukaryote, with only 11 chromosomes and a total genome size of 2.9 Mb (Katinka et al. 2001); to date, the larger microsporidian genome known is from *Brachiola algerae* with approximately 23 Mb (Belkorchia et al. 2008). This shows high genome variability and extreme genome compaction for some species.

Since the first microsporidia was described and named *Nosema bombycis* by Nageli in 1857, over 1300-1500 species and over 197 genera have been identified and described (Vávra and Lukeš 2013), with fish being hosts to 156 species from 18 genera (Lom and Nilsen 2003, Casal et al. 2012). Microsporidians were originally classified by Nageli as members of the class Schizomycetes (Fungi) and later, at the end of the nineteenth century, reclassified as protozoans, a classification that was accepted for over 100 years until the development of molecular phylogenetic techniques reintroduced them as members of the kingdom Fungi (Germot et al. 1997, Hirt et al. 1997, Cavalier-Smith 1998, Keeling and McFadden 1998, Keeling et al. 2000, Van de Peer et al. 2000, Keeling and Fast 2002, Tanabe et al. 2002, Lom and Nilsen 2003).

Microsporidia are known to infect many invertebrate groups, as well as all vertebrate orders; their hosts include protists, bryozoan, nematodes, annelids, insects, fish and mammals, including humans (Becnel and Andreadis 2001, Canning et al. 2002, Lom and Nilsen 2003, Didier 2005, Morris et al. 2005, Fokin et al. 2008, Troemel et al. 2008). The first microsporidia ever identified in mammals was *Encephalitozoon cuniculi*, which was reported from rabbits in 1922 (Wright and Craighead 1922), and about a dozen times from humans between 1924 and 1985 (Wittner and Weiss 1999). Since 1985, many human microsporidiosis caused by several different species of microsporidia have been reported worldwide; nowadays, these parasites are frequently recognized as etiologic agents of opportunistic infections in immunosuppressed and and immunocompetent patients (Schwartz et al. 1996, Keeling and McFadden 1998, Coyle et al. 2004, Lanternier et al. 2009, Talabani at al. 2010, Sak et al. 2011). Nevertheless, only seven genera (*Enterocytozoon, Encephalitozoon, Nosema, Vittaforma, Pleistophora, Trachipleistophora* and *Brachiola*) and a few unclassified microsporidia are known to infect humans (Wittner and Weiss 1999, Franzen and Muller 2001).

In the aquatic environment, microsporidiosis extensively affects the profitability of aquaculture and commercial fish. The large expansion of the world aquaculture production in the last decades is trying to anticipate future pressures of supply demand of commercial fish. Disease in wild and farmed fish increases these pressures on aquaculture production, causing reductions in fitness and thus corresponding to a reduction in catch value. Microsporidia belonging to *Glugea, Loma, Nucleospora* and *Heterosporis* genera are responsible for many diseases in economically important fish. The symptoms can include leukemia-like conditions, emaciation, disfigurement from xenoparasitic growths or tissue necrosis, and growth inhibition (Lom and Dyková 2005).

In Portugal, few Microsporidia have been identified from humans, mammals, trematodes, crustaceans and insects (Azevedo 1987, Azevedo and Canning 1987, Maddox et al. 1999, Matos et al. 2002, Lobo et al. 2003, 2006), but none from fish. Considering the importance of these microparasites, which are known to infect many species of wild and farmed fish in several geographic locations, causing death and reduction in catch value and, therefore, significant economic losses, it's mandatory that the species and genera present in our rivers and seas are recognized. Only through the establishment of this knowledge basis is it possible to prevent transmission and to control infection, specifically in farmed species. Although the study of Microsporidia has been growing in the last 100 years, many new species are yet to be identified and described. The development of new techniques, mostly molecular, brought new

perspectives and new phylogenetic developments, allowing more accurate classifications and phylogenetic trees. However, the classification characters are not unanimous, and phylogenetic advances cause controversy.

1.2.1 Background and phylogeny

It was in 1857 that Carl Wilhelm von Nageli identified the first Microsporidia. The silk industry in the Southern Europe was being shattered by the "prébine" (pepper disease), affecting the silkworm and causing massive economic losses. The agent responsible for the disease was named *Nosema bombycis,* after the silkworm *Bombyx mori* (Franzen 2008) and it was the first Microsporidia ever identified, despite being initially considered as an yeast by Nageli and classified as a Schizomycete fungi. Because of the economic interest in the disease, many researchers started investigating it, but Louis Pasteur was the most outstanding and the only one to actually prove that the disease was caused by the parasite later determined as *Nosema bombycis*. He was assigned to lead a commission of the Department of Agriculture, in 1865, to learn all possible information about this "pepper disease". Pasteur found that the parasite infected not only the silkworm but also their moths and ova, and showed how the microscopic examination of the ova and silkworms and selection of only the non-infected would be very successful overcoming the disease. His results were published in 1870 as "*Études sur la maladie des vers à soie"* (Studies on the disease of the silkworm) (Franzen 2008). In fact, the earliest known report of a Microsporidia was given in 1838 by Gluge, who observed a fish parasite later identified as the microsporidian *Glugea anomala*. Similar parasites were observed by Creplin in 1842 and Muller in 1841 (Franzen 2008); nevertheless, Nageli's talk on *N. bombycis,* more than 150 years ago, is considered the beginning of Microsporidiology.

The taxonomic designation of the Microsporidia was controversial for several years, and researchers regarded them variously as unicellular alga, nuclei of degraded erythrocytes, tumor cells, or yeast spores (Franzen 2008). It was only in 1882 that Balbiani first suggested the separate taxon Microsporidia for *N. bombycis,* recognizing that this organism lacked several Schizomycetes characteristics but shared similarities with the Sporozoa Leuckart, 1879 (Balbiani 1882), the spore-forming parasites composed by the now known Apicomplexa, Myxozoa, Actinomyxidia, Haplosporidia and a handful of individual genera. However, in 1979, Sprague created the phylum Microspora (Sprague et al. 1992), only to, in 1998 (Sprague and Becnel 1998) acknowledge that the phylum name Microsporidia Balbiani, 1882, was the correct author and date.

In 1922, Weissenberg introduced for the first time the term “xenon” (meaning the guest-house), for the formations induced by some Microsporidia in fish hosts, that later developed to “xenoma” (Weissenberg 1968). Richard Roksabro Kudo was the most renowned protozoologist of his time. In 1924, he published a monograph revising the state of knowledge on Microsporidia, “*A Biologic and Taxonomic Study of the Microsporidia”,* in which he listed 4 families, 14 genera and 178 species (Kudo 1924). Kudo was the first researcher to engage in microsporidian research throughout his academic career.

Since its discovery, the phylum Microsporidia has always posed a difficult evolutionary problem, because of their lack of several features that are considered to be universal to eukaryotes, including mitochondria, peroxisomes, classical stacked Golgi membranes, 80S ribosomes (Microsporidia have 70S ribosomes) and 9 + 2 microtubule structures such as cilia or flagella. They were originally considered to be ancient organisms, branching from prokaryotes, but later, more evidences suggested a more recent origin (Edlind et al. 1996, Keeling and Doolittle 1996, Hirt et al. 1999, Keeling et al. 2000) as eukaryotes that underwent gene compaction and lost several genes, as a result of their growing adaptation to intercellular parasitism (Keeling and Fast 2002).

The Archezoa theory

Though Microsporidia were first classified as fungi by Nageli, in 1983, Cavalier-Smith brought attention to a possible new evolutionary significance for the Microsporidia and a new classification. Proposing that the origin of the eukaryotes might have preceded the endosymbiotic origin of the mitochondrion, he implied they might be primitively amitochondriate eukaryotes, since they lack mitochondria (Cavalier-Smith 1987). Four lineages of amitochondriate eukaryotes were identified, and collectively named Archezoa Haeckel, 1894: Archamoebae (e.g., *Entamoeba*), Metamonada (e.g., *Giardia*), Parabasalia (e.g., *Trichomonas*), and Microsporidia (Cavalier- Smith 1983). With the development of molecular techniques more evidences were found that supported this theory, Charles Vossbrinck found that ribosomes of *Vairimorpha necatrix* lack the 5.8S RNA subunit, thought to be a universal eukaryotic characteristic (Vossbrinck and Woese 1986), and using molecular sequencing methods for the construction of a life tree, showed that Microsporidia diverged from other eukaryotes before the evolution of mitochondria, supporting the theory of ancient amitochondriate eukaryotes. These four phyla differ from the standard Eukaryotes by having 70S ribosomes, like bacteria, instead of 80S ribosomes as in most eukaryotes

and, in never having mitochondria, peroxisomes, hydrogenosomes or well-developed Golgi dictyosomes.

Phylogenetic analysis of many proteins of the translational apparatus, as translation elongation factors EF-1a and EF-2, glutamyl tRNA synthase, all supported this theory (Kamaishi et al. 1996). But the fact that Microsporidia are obligate intracellular parasites of eukaryotes with mitochondria, unlike the other Archezoa phyla, which have free-living members, produced some doubts as to their primitively amitochondrial character, and researchers started questioning this theory and the possibility that Microsporidia might have suffered extreme parasitic reduction, including the loss of mitochondria, peroxisomes and also lysosomes, cilia and centrioles.

The Fungi Theory

The fungal origin of microsporidia was first suggested based on the analysis of gene sequences for beta and alpha tubulin between microsporidia and fungi (Edlind et al. 1996, Keeling and Doolittle 1996, Keeling et al. 2000) and later supported by the phylogenetic analyses of gene sequences for the large subunit of RNA polymerase II (Hirt et al. 1999), the TATA-box binding protein and mitochondrial HSP70 (70 kDa heat shock protein) (Germot et al. 1997, Hirt et al. 1997, Fast et al. 1999). Other features supporting this theory are the chitinous spore wall and intranuclear division. Additionally, many of the molecular data that placed microsporidia at the base of the eukaryotic tree have recently been reanalyzed with more sophisticated methods and the resulting trees didn't support an early and primitive origin for microsporidia (Hirt et al. 1999). Contrary to the Archezoa theory, a gene encoding HSP70, a protein involved in folding other proteins during import into the mitochondrion, was characterized from three microsporidian genera: *Encephalitozoon*, *Vairimorpha* and *Nosema* (Germot et al. 1997, Hirt et al. 1997, Peyretaillade et al. 1998). In this HSP70 phylogeny, the Microsporidia, a phylum that previously was assumed to emerge close to the base of the eukaryotic tree, appears as the sister-group of the fungi.

More mitochondrion-derived genes encoding metabolic proteins were sequenced, Fast and Keeling (2001) characterized genes encoding the alpha and beta subunits of pyruvate dehydrogenase complex E1 (PDH E1) from *Nosema locustae*. This complex is found in nearly all mitochondriate eukaryotes and is a strong evidence for mitochondrion-derived metabolic activity in Microsporidia, whereas in amitochondriate protists, such as *Trichomonas*, *Giardia* and *Entamoeba*, PDH appears to be absent and was replace by pyruvate:ferrodoxin oxidoreductase (Muller 1998). However it is yet unclear whether microsporidia have retained a mitochondrion in some altered form or have completely lost it (Fast and Keeling 2001).

More evidence for the mitochondriate origin of the microsporidia, a tiny mitochondrion-derived organelle, the mitosome, was detected. The first evidences of this organelle came from genes of mitochondrion-derived proteins in the nuclear genomes of several Microsporidia and, later, the complete genome of *Encephalitozoon cuniculi* revealed many more mitochondrion-derived protein-encoding genes. The molecular function of this organelle remains poorly understood. The mitosome has no genome, so it must import all its proteins from the cytosol. In other fungi, the mitochondrial protein import machinery consists of a network series of heterooligomeric translocases and peptidases, but in Microsporidia, only a few subunits of some of these complexes have been identified to date (Burri et al 2006, Waller et al. 2009)

In the present, molecular studies and the complete sequence of the *Encephalitozoon cuniculi* genome identified Microsporidia as members of the Kingdom Fungi. Despite them being true eukaryotes with a nuclear envelope and an intracytoplasmic membrane system, some doubts concerning the correct phylogenetic relationships of Microsporidia still persist due to the lack of several typical eukaryotic characteristics and inconclusive analysis data (Franzen 2008). The dissimilar phylogenetic molecular data for tree construction may be explained by the long-branch attraction artefact of many phylogenetic methods, leading to erroneously grouping fast-evolving lineages at the base of the tree when they are analyzed together with other slowly evolving lineages (Forterre and Philippe 1999).

1.2.2 Taxonomy and Systematics

Microsporidian taxonomy has also proven to be very challenging. They are classified as a separate phylum within the Protista kingdom. The first classification systems developed were based exclusively on morphological characters (Issi 1986, Sprague et al. 1992, Cavalier-Smith 1993), which are distinguishable using light microscopy, but were prone to the subjectivity of the researchers. When electron microscopy was introduced for taxonomic purposes in the mid-1970s, other morphological characters started being used to classify Microsporidia. Thélohan, Doflein and Pérez classification was based on the modes of spore formation, particularly the number of spores produced by each sporoblast (Franzen 2008).

The division of Microsporidia into classes was previously based on characters such as whether the sporoblast appeared surrounded by a membrane (Pansporoblastina versus Apansporoblastina) (Tuzet et al. 1971), whether they were uninucleate or binucleate throughout their life cycle, or the type of nuclear division

(Haplophaseate versus Dihaplophasea) (Sprague et al. 1992). The Dihaplophasea are then separated into those in which the diplokaryon is formed through meiosis (Meiodihaplophasida) and those in which the diplokaryon is formed through nuclear dissociation (Dissociodihaplophasida). Morphological data provides information that often is unique to a genus or even species. Presently, the morphological characters that are mostly used for classification are the number of nuclei, one or two, the number of spores or sporonts, the length, the arrangement, structure and number of coils of the polar filament and many other details of the life cycle. The main obstacle is that many morphological features may change very rapidly during the parasites' adaptation to different hosts or tissues, and some characters, apparently, evolve several times simultaneously in distinct lineages of Microsporidia. Therefore, over time, new techniques were developed to assist Microsporidia classifications. The development of molecular techniques brought new perspectives to the taxonomy of Microsporidia, specifically, brought a revolution of new results and classifications.

Through the analyses of 125 microsporidian species, Vossbrinck and Debrunner-Vossbrinck came to the conclusion that groups or clades are formed based on habitat and host (Vossbrinck and Debrunner-Vossbrinck 2005). Based on their SSU rRNA analysis, they stated that structural and ultrastructural characters are erratic for distinguishing among higher-level microsporidia taxa and suggested three classes reflecting the habitat of each group: the Aquasporidia, the Marinosporidia and the Terresporidia, which, respectively, corresponded to a group found mainly in freshwater habitats, another found in marine habitats, and the third from terrestrial habitats. However, this new classification was not completely accepted by the scientific community, and Larsson completely disagreed about the usefulness of cytological characters for microsporidian systematics, stating that none of Vossbrinck and Debrunner-Vossbrinck classes were strictly confined to the particular habitat (Larsson 2005). All this proves that morphology-based methods need to be supplemented with molecular data (Lom and Nilsen 2003) as do need molecular methods with morphological data. Molecular techniques are excellent for identifying species and providing data for proposing evolutionary relatedness through phylogenetic analysis. Knowing that morphology is the visual expression of the genome, molecular and morphological data should be in agreement, providing phylogenies and classifications with greater certainty. Although the microsporidian systematics is in constant reviewing, additional sequence data from new host groups continues to support division of the phylum into five major deep-rooted clades (Terry et al. 2004, Vossbrinck and Debrunner-Vossbrinck 2005). Clade I is composed mainly by species infecting dipteran host species; clade II contains only three species *Weiseria palustris, Polydispyrenia*

simulii and *Flabelliforma montana*; clade III consists mainly of parasites such as *Loma*, *Glugea* and *Pleistophora* which infect fish (Lom and Nilsen 2003); clade IV is composed by the majority of mammal-infective species; and clade V contains parasites found in primitive animals such as bryozoans and oligochaetes (Canning et al. 2002, Morris et al. 2005).

1.2.3 Spore Ultrastructure

Microsporidia display some unique features, namely the lack of mitochondria, peroxisomes or typical Golgi apparatus throughout their life cycle, and the fact that their ribosomes resemble more the ribosomes of prokaryotic organisms than the typical eukaryotic type (Vávra and Larsson 1999). Since Thélohan first published his observations on the structure of the microsporidian spore in 1894, many researchers have published diverse views concerning the internal morphology of these microparasites; in Fig. 1 the general uninucleate mature spore ultrastructure is represented.

The spores are unicellular, presenting ovoid, spheroid or cylindric form. Mature spores dimensions range between 1 to 40 µm in length and 1.5 to 5 µm in width (Franzen and Müller 1999, Vávra and Larsson 1999). Walls are complete, without suture lines, pores or other openings, and trilaminar, composed of an outer electron-dense exospore, an electron-lucent middle layer endospore and a thin membrane

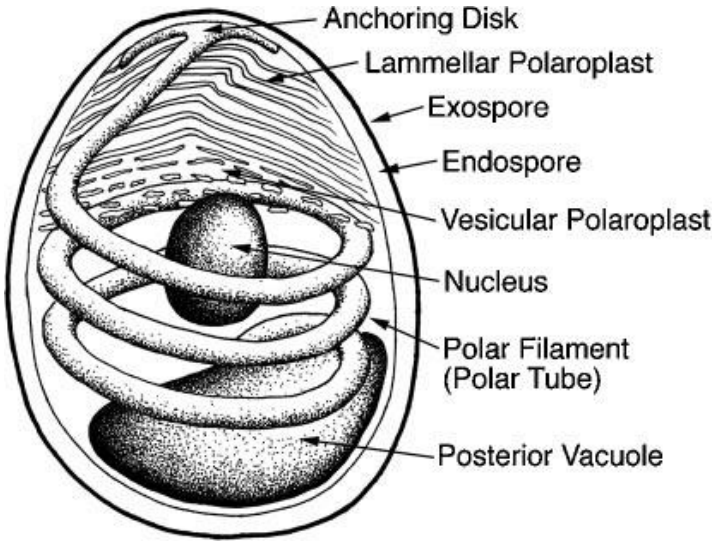

Figure 1 – Diagram of the microsporidian spore ultrastructure, indicating the position of the anchoring disk in the anterior region, the lamellar and globular polaroplast, the exospore and endospore, the nucleus, the polar filament and the posterior vacuole at the posterior region of the spore. From Keeling and Fast 2002.

surrounding the cytoplasmic contents. Ultrastructural studies of the genus *Encephalitozoon* using transmission electron microscopy (TEM), as well as freeze-fracture and deep-etching, showed the complexity of the exospore, which is composed by three layers: an outer spiny layer, an intermediate electron-lucent lamina and an inner fibrous layer (Bigliardi et al. 1996). The endospore was observed as a space crossed by bridges connecting the exospore to the plasma membrane; these bridges were thought to be composed by chitin, as well as part of the fibrilar system of the exospore (Erickson and Blanquet 1969, Vávra 1976, Bigliardi et al. 1996). The spore possesses a hollow coiled polar filament joined with a polar cap and anchoring disk at the anterior pole. Before the extrusion of the polar filament occurs, the filament is composed of a membrane and glycoprotein layers, ranging from 0.1 to 0.2 µm in diameter. The filament is straight from one third to one half of the spore, the remainder is helically coiled around the posterior vacuole. The number of coils, their arrangement and the angle of helical tilt are conserved and describe particular species (Sprague et al. 1992, Vávra and Larsson 1999). Microsporidia display a complex extrusion apparatus, but have no polar capsules as do Myxozoa and neither is the polar filament formed by a separate capsulogenic cell. They develop a single sporoplasm with many free ribosomes and some endoplasmic reticulum and have a large vacuole in the posterior part of the spore. The polaroplast is an organization of membranes in the anterior part of the spore usually divided into two portions: the lamellar polaroplast, which is located anteriorly and consists of numerous highly organized and closely stacked membranes; and the globular polaroplast, which appears located posteriorly and consists of several widened cisternae or inflated vesicle-like cisternae loosely organized. Generally, spores are of a single type, presenting uniform shape and size but, in some species, the formation of macrospores and microspores occur, and spores differ in size, as well as in the number of polar filament coils. Species presenting these two types belong to the genera *Pleistophora, Ovipleistophora* and *Heterosporis* (Dyková 2006).

1.2.4 Life cycle

Microsporidia are obligate intracellular pathogens with no active stages outside their host cell. They are widespread and infect many species of vertebrates and invertebrates; this success lies in the diversity and flexibility of their transmission strategies and life cycles. The general life cycle pattern can be divided into three phases:

- the infective phase, which is the spore;
- the proliferative phase (merogony), responsible for the massive increase of parasitic cells inside the host cell;
- the spore productive phase (sporogony), as is outlined in Fig. 2,

not without fundamental diversity.

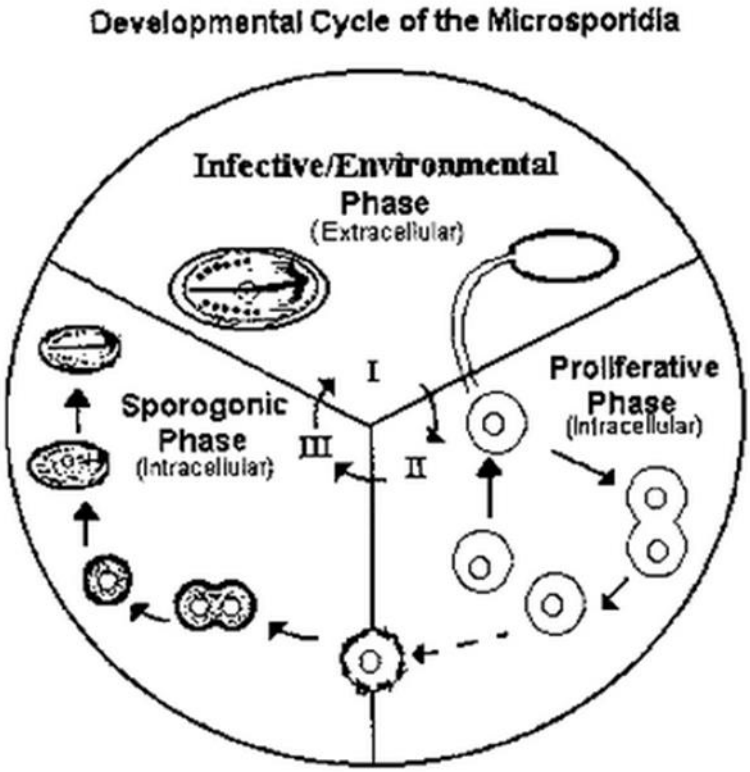

Figure 2 – Diagram of a typical developmental cycle of the Microsporidia. The three regions represent the three phases of the microsporidian life cycle. Phase I is the infective/environmental phase, the extracellular phase of the cycle. It contains the mature spores in the environment. Under appropriate conditions the spore is activated (e.g., if the spore is ingested by an appropriate host, it is activated by the gut environment) and triggered to extrude its polar filament (which becomes a hollow tubule). If the polar tubule pierces a susceptible host cell and injects the sporoplasm into it, phase II begins. Phase II is the proliferative phase, the first phase of intracellular development. During the proliferative part of the microsporidian life cycle, organisms are usually in direct contact with the host cell cytoplasm and increase in number. The transition to phase III, the sporogonic phase, represents the organism's commitment to spore formation. In many life cycles this stage is indicated morphologically by parasite secretions through the plasmalemma producing the thickened membrane. The number of cell divisions that follow varies depending on the genus in question, and the result is spore production. From Cali and Takvorian 1999.

Fish Microsporidia generally have a simple life cycle. On very few genera and species has a dimorphic life cycle been identified. For instance, species of *Spraguea* Sprague and Vávra, 1976, were described with dimorphic life cycle (Lom and Nilsen 2003). In insects, crustaceans and other host groups, intermediate hosts have been

identified and experiments conducted by Sweeney et al. (1985) proved the existence of an intermediate host involved in the life cycle of *Amblyospora* sp. These proofs of intermediate hosts in some Microsporidia life cycles made them potential species to control pests and much research has been taking place about that matter.

Microsporidia have many strategies for parasite maintenance in the host population such as direct and indirect life cycles and horizontal or vertical transmission and, in some cases, even both (Dunn and Smith 2001). It is believed that fish-infecting microsporidia possess a direct way of transmission, vertical transmission, but it had only been shown for *Nosema salmonis*, *Loma salmonis*, *Glugea anomala*, *Glugea stephani*, *Ovipleistophora ovariae, Glugea atherinae, Pleistophora typicalis, Trachypleistophora horminis, Pleistophora mirandellae, Spraguea lophii* (Dunn and Smith 2001), until Terry et al. (2004) showed that vertical transmission occurs in all major lineages of the phylum. However, the most common way of transmission is horizontal transmission, which occurs when the fish either ingests spores that are free in the water column or when they prey on infected aquatic invertebrates or fish (Weissenberg 1968). It can occur between related or unrelated hosts of the same or different generations, and between hosts from the same or different species (Dunn and Smith 2001).

The way of transmission is determinant in the evolution of virulent host-parasite relationships. It has been verified that parasites that are transmitted horizontally produce higher numbers of transmission stages of the parasite and have higher virulence, often leading to the death of the host (Kellen et al. 1965), while parasites that are transmitted vertically are dependent on the host survival and reproduction for their own transmission and survival, thus being less virulent (Smith and Dunn 1991, Ebert and Herre 1996). Nonetheless, vertical transmission is associated with manipulation of host reproduction; infections are more common in female than males and five out of nine parasite species cause host sex-ratio distortion (Terry et al. 2004), with male killing and feminization.

Some species belonging to xenome-forming genera can stimulate hypertrophy of the infected host cell, which develops hypertrophic nuclei and surface modifications, such as microvilli, invaginations or thick walls (Sprague and Vernick 1968, Weissenberg, 1968), forming a separate entity until maturation of the spores and disintegration of the xenome. This type of Microsporidia are generally transmitted orally, mainly by cohabitation with diseased fishes that defecate and urinate spores into the water, thus facilitating dispersion. Spreading within the host body may occur by rupture of the xenome or by spore discharging through the xenome wall and infection of the surrounding cells. Hereupon, secondary xenomas may develop (Lom and

Dyková 2005), whether originating in connective tissue cells or macrophages, it is yet to be resolved, but massive infections and multiple xenomas can be observed on some species of *Glugea* (Figs. 3 and 4), proving that autoinfection may occur and discarding the possible ingestion of a whole xenome, which is very unlikely.

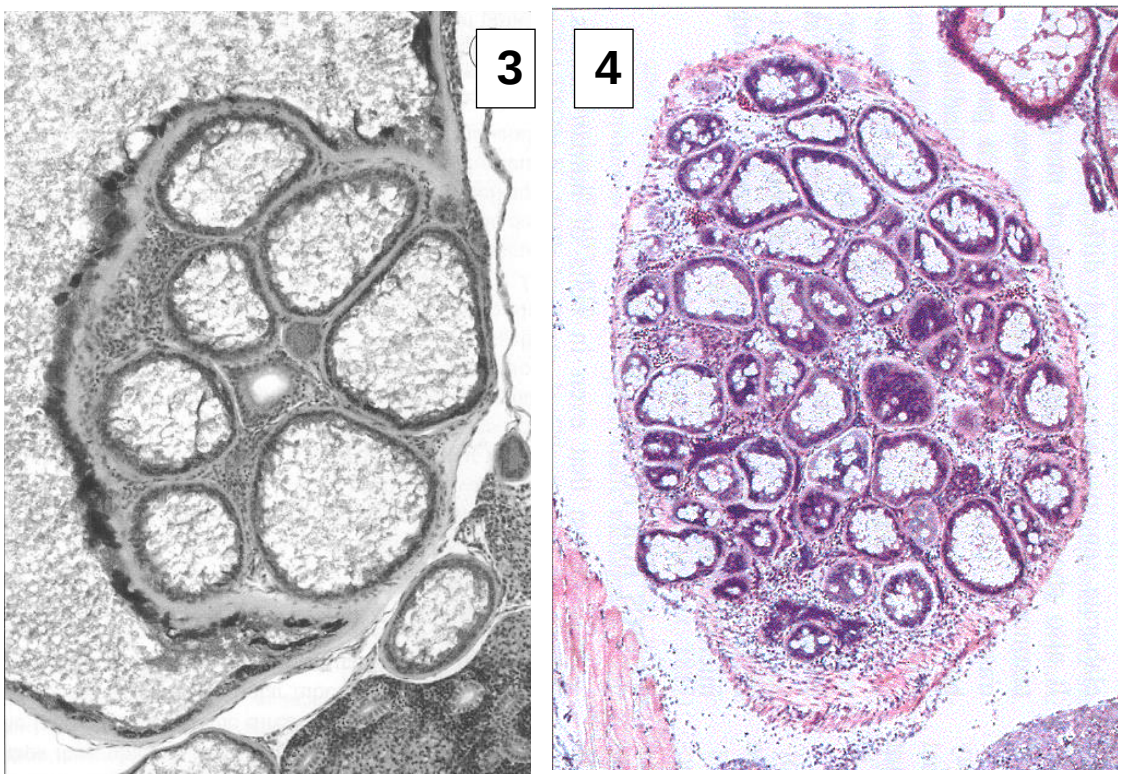

Figure 3 and 4 – Developing of *Glugea anomala* xenomas in fish, from Lom and Dyková 2005. **Fig. 3** - A group of secondary *Glugea anomala* xenomas developing within the old one. H&E, × 280. **Fig. 4 -** Overview of a massive spontaneous infection of *G. anomala* as seen in the intestine of *Gasterosteus aculeatus.* H&E, × 70.

Experimentally, tests of intraperitoneal, intramuscular and intravascular transmission, as well as by anal gavage (Shaw and Kent 1999) have been successful. Shaw et al. (1998) conducted experiments with *Loma salmonae*, and was successful in the transmission of infection by exposing *Oncorhynchus* spp. to spores through cohabitation, intraperitoneal, intramuscular or intravascular injection or administration *per os*, but showed no success in infection by placing spores on the gills.

1.2.4.1 The infective phase

The spore is the most recognizable phase of Microsporidia, representing the extracellular resistant infective phase, displays a thick wall, which provides resistance

to environmental conditions and, at the same time, allows the increase on hydrostatic pressure that causes the spore discharge (Frixione et al.1997).

The polar tube extrusion

The spore discharge is believed to occur following several steps:

- Activation by appropriate stimuli
- Increase in the intrasporal osmotic pressure
- Eversion of the polar tube
- Passage of sporoplasm through the polar tube

The extrusion apparatus occurs between four major structures of the spore as it is represented in Fig. 5: the posterior vacuole, the polar tube, the polaroplast and the anchoring disc (Bigliardi and Sacchi 2001). The posterior vacuole occupies the last third of the spore and is formed by a series of membrane-bound vesicles that may be considered as part of the Golgi apparatus; starting the spore germination, the posterior vacuole swells increasing the spore internal pressure. The polar tube, or polar filament, which is divided into two regions: the anterior straight portion surrounded by a lamellar polaroplast and attached to the inside of the anterior end of the spore by an anchoring disk; and the posterior coiled region that forms from 4 to approximately 30 coils around the sporoplasm, depending on the species (Huger 1960, Vávra et al. 1966, Cali and Owen 1988). The eversion of the polar filament can be compared to the movement of a tube sliding within another tube (Keohane and Weiss 1999) following a screw-like movement, which is thought to accelerate expulsion of the sporoplasm. Once extruded, the polar filament assumes a tube-like aspect, therefore being designated polar tube (Vávra et al. 1966, Weidner 1976). Following the swelling of the posterior vacuole, the polaroplast also swells in response to sudden osmotic changes in its matrix, and both are responsible for the increasing of the spore internal pressure and impelling of the sporoplasm from the spore through the polar tube into its host cell (Oshima 1937, Lom and Vávra 1963).

Inside the spore, the polar filament is composed of electron dense and electron lucent concentric layers that can range from three to as many as 20 different layers in cross-section (Lom 1972, Sinden and Canning 1974, Vávra 1976). Once the polar tube extrusion begins, a protrusion is visible at the anterior end of the spore at the polar cap (Lom and Vávra 1963, Frixone et al. 1992), where the discharging polar tube breaks through the thinnest region of the spore wall, followed by the rapid emergence of the polar tube in a helicoidal fashion along nearly a straight line. Full discharge of the polar tube occurs in less than 2 seconds, with a maximum velocity of about 105 µm/s

(Frixone et al. 1992). Once extruded the polar tubes range from 50 to 150 µm in length and 0.1 to 0.2 µm in diameter (Kudo and Daniels 1963, Weidner 1976, Frixone et al. 1992).

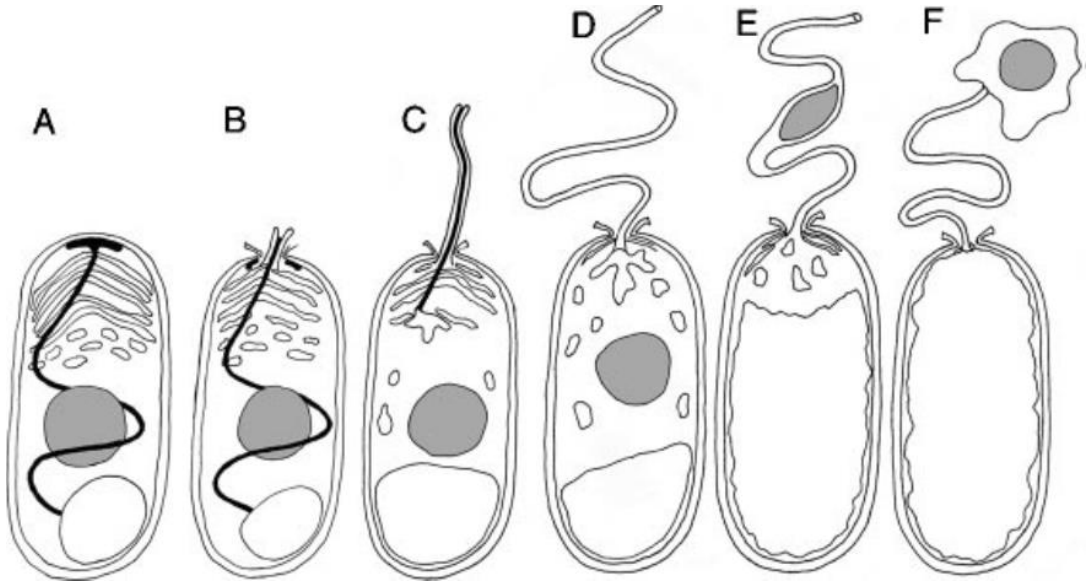

Figure 5 - Polar tube eversion during spore germination. **(A)** Dormant spore, showing polar filament (black), nucleus (gray), polaroplast and posterior vacuole. **(B)** Polaroplast and posterior vacuole swelling, anchoring disk ruptures, and polar filament begins to emerge, everting as it does so. **(C)** Polar filament continues to evert. **(D)** Once the polar tube is fully everted, the sporoplasm is forced into and **(E)** through the polar tube. **(F)** Sporoplasm emerges from the polar tube bound by new membrane. From Keeling and Fast 2002.

Even though the mechanisms mediating the polar tube extrusion and passage of the sporoplasm have been studied, how exactly the polar tube penetrates the host cell membrane is yet unclear. It was initially thought that the polar tube pierces the plasma membrane of the host cell, like a hypodermic needle, to inject the sporoplasm into the host cell (Franzen 2004), but recently some studies suggested that the polar tube enters the host cell by a phagocytic process (Magaud et al. 1997, Foucault and Drancourt 2000, Bigliardi and Sacchi 2001). In different studies with *Encephalitozoon* spp. polar tubes were observed discharging their contents into invaginations of the host cell membrane (Magaud et al. 1997, Bigliardi and Sacchi 2001), suggesting that this phagocytic process is triggered upon contact of the spore apex with the host cell membrane (Magaud et al. 1997), however, there is no evidence for a specific recognition pattern.

Body fluids as blood and lymph, or phagocytic cells and undifferentiated mesenchymal cells are suggested to contribute in the parasite dissemination inside the

host. For most microsporidians, the development of the parasite within the host cell cytoplasm starts immediately after infection; once the polar tube extrusion occurs into a suitable host cell, the sporoplasm initiates the proliferative phase of development, called merogony.

1.2.4.2 Merogony

Development of microsporidian infections in fishes may be dependent of many factors. Among environmental factors, the ambient temperature is known to influence development. Some experiments showed that the growth and development of Microsporidia doesn't occur for temperatures below 15º C, which was verified for *Glugea plecoglossi*, *G. stephani* and *Microsporidium takedai* (Olson 1976, Takahashi and Egusa 1977a). However, under proper conditions, merogony occurs, with meronts generally proliferating and dividing through binary fission (*Encephalitozoon*, *Nosema*, *Vittaforma*), or through karyokinesis without cytokinesis, producing multinucleate cells that form a structure called merogonial plasmodia (*Enterocytozoon*, *Pleistophora*, *Trachipleistophora*) (Bigliardi and Sacchi 2001). Meronts are roundish cells delimited by a typical unit membrane; they have a substantial nuclear region with one or two nuclei, depending on the species. These proliferative cells display a homogeneous granular cytoplasm containing poorly developed endoplasmic reticulum (ER) and many ribosomes that appear free or attached to vesicles of the ER or nuclear envelope, they also have thin filaments of about 5 nm in diameter that are visible in close association with the parasite plasma membrane.

Merogony begins immediately after the infectious sporoplasm enters a suitable host cell with the formation of meronts occurring in direct contact with the host cytoplasm (*Nosema, Enterocytozoon*), within a parasitophorous vacuole lined by a host-produced single membrane (*Encephalitozoon*), within a parasite-secreted amorphous coat (*Pleistophora, Trachipleistophora, Thelohania*), surrounded by the host endoplasmic reticulum (*Endoreticulatus, Vittaforma*) (Frixione et al. 1997, Bigliardi and Sacchi 2001) or within a xenome. The latter is presently understood as the host cell with a completely altered structure and the parasites proliferating inside it, both components being morphologically and physiologically integrated to form a separate entity with its own development within the host at the expense of which it grows (Lom and Dyková 2005). The xenome protects the parasite from the host immune response and confines it to one site, preventing its dissemination throughout the host body (Sprague and Hussey 1980). Xenomas have been found in oligochaetes (e.g., genera

Bacillidium, *Burkea*, *Hrabyeia*, *Jirovecia*, species of the collective group *Microsporidium*), crustaceans (e.g., *Abelspora*, *Mrazekia*), insects (e.g., *Polydispyrenia*, *Thelohania*) and poikilothermic vertebrates, mostly fish (*Alloglugea*, *Amazonspora*, *Glugea*, *Ichthyosporidium*, *Loma*, *Microfilum*, *Microgemma*, *Neonosemoides*, *Pseudoloma*, *Spraguea*, *Tetramicra*) (Lom and Dyková 2005).

At the end of merogony, patches of electron-dense material are deposited externally to the plasma membrane, this event characterizing the beginning of the sporogony.

1.2.4.3 Sporogony

Sporogony is, basically, the development of a meront into a sporont. The developing spore is designated as a sporoblast at its last sporont division and later, through metamorphosis, condenses to become a mature spore smaller than its developmental predecessors. During sporogony, a number of morphological changes occur, the most identifiable of which is the thickening of the electron dense plasmalemma surrounding the parasites (Cali and Takvorian 1999, Lom et al. 2000), but also a progressive increase in cytoplasmic density as more ribosomes, ER and larger Golgi complexes are formed, as well as development of the extrusion apparatus. Sporogony may or not occur within a membranous envelope that originates from the host, the parasite, or from both, and is designated as a sporophorous vesicle (Cali and Takvorian 1999). The sporont may divide in distinct ways but the mechanism of the mitotic process is the same as for meronts. If the karyokinesis is linked to the cytokinesis, sporonts divide by binary fission and two sporoblasts are formed, that's how it happens for the genera *Nosema, Encephalitozoon, Vittaforma*; as for *Enterocytozoon* and *Pleistophora*, cytokinesis is not linked to karyokinesis and multinucleate sporonts develop within a sporogonial plasmodium (Bigliardi and Sacchi 2001). Maturation of the sporoblasts occurs by membrane thickening to form a continuous dense surface, that later becomes the exospore, the sporoblast wrinkles and its cytoplasm becomes more electron-dense due to the development of smooth and rough ER and enlargement of the golgi apparatus (Bigliardi and Sacchi 2001). Both Golgi apparatus and ER are involved in the formation of spore organelles, and once these organelles polarize, the sporoblast can be designated as spore. Liberation of mature spores occurs when the host cell disintegrates.

1.2.5 Diagnosis, Prevention and Control

Heavily infected fish can be easily diagnosed by observation at the naked eye of lesions and clinical signs. Xenome-forming species may display several lesions on the body surface as well as in the body cavity, digestive tract (Fig. 4) and parenchymatous organs, depending on the parasite species and site of development. Nonxenome-forming species cause less defined lesions that appear as whitish foci in the affected tissues. Fresh spores can be easily recognized using light microscope, with optimal visibility through Nomarski differential interference contrast, since the posterior vacuole is easily observed occupying half the spore, particularly on fish-infecting Microsporidia.

Other diagnostic techniques involve adding 2% hydrogen peroxide for polar tube extrusion, staining of the polar cap with periodic acid Schiff reaction to get a red dot and staining the spores dark blue with Giemsa. Lom (2002) presented a practical key for the determination of microsporidian genera based on ultrastructural features obtained through the use of TEM. Nowadays, a combination of morphological, ultrastructural and molecular data is warranted for confirming diagnostics and determining genera and species (Canning and Lom 1986, Larsson 1986, Lom and Dyková 1992, Lom and Nilsen 2003). Several PCR assays have been developed for fish-infecting microsporidia (Barlough et al. 1995, Docker et al. 1997, Brown and Kent 2002), however, the amount of molecular data nowadays available is still insufficient and does not allow the construction of fast and reliable tools for species identification within all genera (Nilsen 2000, Lom and Nilsen 2003). Also, some serological methods as ELISA and western blot assays have proved successful for diagnosing microsporidiosis in animals and humans (Singh et al. 1982, Hollister et al. 1991). Experiments *in vitro* of microsporidia transmission and cell cultures allow the development of pathobiological studies and production of large numbers of spores for *in vitro* and *in vivo* studies and, most important, the generation of monoclonal and polyclonal antibodies, and immunologic, molecular, biochemical, physiologic and animal inoculation studies (Visvesvara 2002, Franzen 2008) that will be used for diagnose.

As transmission in fish-infecting microsporidia is mainly direct, the development of intensive fish cultures, where high fish densities are practiced, increases the scientific interest in the development of more efficient prevention measures. For cultured and ornamental fish is essential to enforce quarantine and examination of fishes collected from natural habitats. Treatment of fish eggs with chlorine at 25 ppm for 10 min is used routinely in zebrafish laboratories and, if it's suspected that parental

specimens are infected and may liberate spores to the water, hatchlings are separated before they begin feeding. Several studies focusing on experimental infections with Microsporidia revealed astonishing results, with xenome formations and nonxenome developmental stages appearing as early as 21 days to 3 weeks (Dyková and Lom 1978, 1980, Rodríguez-Tovar et al. 2002). Short baths of toltrazuril (Bayer AG) have been recommended for treating *G. anomala* infections, using a concentration of 5–20 µg/ml of water for 1 to 4 h applied for 6 days at 2-day intervals and good aerated water (Schmahl and Mehlhorn 1989, Schmahl et al. 1990). On the other hand, experiments using fumagilli (acyclic polyne macrolide) and amprolium to treat microsporidian infections had uncertain results (Awakura and Kurahashi 1967). Fumagillin may cause side effects on high concentrations and prolonged treatments but when 2 or 4 mg of drug/kg per day are mixed with alcohol and sprayed on the feed, there were good results treating infections of *Loma salmonae* (Shaw and Kent 1999). TNP-470 (Takeda Chemical Industries, Ltd), an analogue of fumagillin, showed great results in the treatment of *L*oma *salmonae* and *Nosema salmonis* infections using 0.1–1.0 mg of drug/kg of fish per day for 6 weeks (Shaw and Kent 1999). Nitrofurazone is a bactericidal drug with some antiprotozoan activity and was reported to reduce infections of *Pleistophora ovariae* in *Notemigonus crysoleucas* (Nagel and Summerfelt 1977). Substantial lowering of the intensity of *L. salmonae* infection in rainbow trout juveniles has been achieved in vaccination experiments with a live low-virulence variant of *L. salmonae* (Sanchez et al. 2001) bringing great expectations to a new field of studies focused on protection of fish during the grow-out period through vaccination.

1.2.6 Genus *Glugea* Thélohan, 1891

Trying to group genera according to xenome structure, Lom and Dyková (2005) categorized *Glugea* xenomas as structurally thick walled xenomas, along with the genera *Loma* and *Pseudoloma. Glugea* xenomas form a wall outside the plasma membrane that is constituted by laminar layers of sloughed-off cell coat, the central hypertrophic nucleus is highly branched, and developmental stages are stratified with increasing of maturity to the center of the xenome. Meronts are cylindrical with an electron dense coat on plasmalemma and surrounded by cisterna from the host ER. The sporogonial plasmodium creates a thin membrane-like wall, forming sporophorous vesicles, where sporonts divide by multiple cleavages and generate a variable number of sporoblasts mother cells. The latter divide by binary fission and form sporoblasts that

go through a maturation process to originate monomorphic spores with isofilar polar filament coiled in single layer (Lom 2002).

Lom (2002) made a catalogue of described genera and species of Microsporidia infecting fish, with locations and hosts, wherein he lists, among others, all *Glugea* species described to the date, including 28 species and 8 reports of *Glugea* sp., that all together confirm the worldwide distribution of this genus (Blasiola 1979, Weiser et al. 1981, Mathieu-Daude et al. 1992, Leiro et al. 1999). *Glugea,* alongside *Tetramicra* and *Ichthyosporidium,* presents low host site specificity, being capable of forming xenomas in multiple organs since they infect cells of the connective tissue.

Glugea anomala (Moniez 1887) Gurley, 1893 is the best known and studied species of this genus, commonly infecting *Gasterosteus aculeatus* and *Pungitius pungitius* in Eurasia and North America (Weissenberg 1968, Schmahl et al. 1990). This species forms xenomas that are white, spherical, cyst-like formations that develop up to several mm, causing hypertrophy of the host cells with alterations of their nucleus size and shape. In early xenomas, the meronts are distributed throughout the xenome, while, in more advanced xenomas, meronts are confined to the periphery with spores concentrating in the centre (Dyková 2006). The development of xenomas causes immense pressure atrophy to the surrounding tissues so that when the xenome is filled with mature spores the host cell membrane loses integrity, allowing the host inflammatory reaction to settle in and form granulomas.

Glugea plecoglossi Takahashi and Egusa, 1977 causes one of the most dangerous diseases of cultured *Plecoglossus altivelis* in Japan (Takahashi and Egusa 1977b).

Glugea atherinae Berrebi, 1979 infects the commercially important big-scale sand smelt *Atherina boyeri* in coastal brackish lagoons of the French Mediterranean. The infection is sex dependent, being the females more frequently infected than males and is acquired in the first and second year of life (Berrebi 1978). The visible large xenomas, which measure up to 14 mm in diameter, occur mainly in the body cavity and cause pressure atrophy of affected and surrounding organs that will ultimately contribute for increasing the host mortality rates (Berrebi 1978, Dyková 2006).

Chapter 2

Morphology and phylogeny of *Glugea atherinae* (Microsporidia) infecting the big-scale sand smelt *Atherina boyeri* (Actinopterygii) in the Minho River, Portugal

To be submitted

(To be submitted)

Morphology and phylogeny of *Glugea atherinae* (Microsporidia) infecting the big-scale sand smelt *Atherina boyeri* (Actinopterygii) in the Minho River, Portugal

Sónia Rocha[1,2] · Marília Margato[2] · Graça Casal[1,3] · Pedro Rodrigues[2,4] · Carlos Azevedo[1,2]

[1] *Laboratory of Animal Pathology, Interdisciplinary Centre of Marine and Environmental Research, CIIMAR-CIMAR Associate Laboratory, University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal*

[2] *Laboratory of Cell Biology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal*

[3] *Department of Sciences, High Institute of Health Sciences-North (CESPU), Rua Central da Gandra, 1317, 4585-116 Gandra, Portugal*

[4] *Institute for Molecular and Cell Biology (IBMC), University of Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal*

Address for correspondence: Laboratory of Cell Biology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Rua Jorge Viterbo Ferreira no. 228, 4099-313 Porto, Portugal. Tel.: +351 22 042 8241; E-mail: sonia.oliveira.rocha@gmail.com

Abstract The microsporidian parasite *Glugea atherinae* is for the first time diagnosed from the big-scale sand smelt *Atherina boyeri* Risso, 1810 inhabiting the lower Minho River in Portugal, making this the first report of a microsporidian parasite of the genus *Glugea* from Portuguese waters. Prevalence of infection was detected at approximately 39%. The parasite forms spherical whitish xenomas in the abdominal cavity, which appear adherent to the viscera and contain only mature spores and some developing sporoblasts. Mature spores are ellipsoidal, measure 5.9 ± 0.4 μm in length and 2.6 ± 0.5 μm in width, and present a polar filament coiled in 17-18 (rarely 19) coils. Phylogenetic analysis of the rRNA genes, including the ITS region, using maximum likelihood, neighbor-joining and maximum parsimony methodologies reveal the parasite clustering among a group of closely related *Glugea* species. The high level of rRNA sequence identity between *Glugea atherinae*, *Glugea hertwigi*, *Glugea stephani*, *Glugea* sp. GS1, *Glugea plecoglossi*, *Glugea* sp. LX-2012 and *Glugea anomala* suggests that these may be variants of a single species.

Keywords Microsporidia · Fish-infecting · Parasite · Ultrastructure · rRNA genes

Introduction

Fish species of the family Atherinidae Risso, 1827 occur worldwide in both tropical and temperate waters. Most species inhabit marine and estuarine environments, with few entering freshwater environments (Dyer and Chernoff 1996). Atherinids feed on zooplankton and small bottom-dwelling invertebrates and, in turn, are preyed upon by larger fish, some of which are commercially valuable (Ivantsoff and Crowley 1999). In the eastern Atlantic, the family Atherinidae is represented by the genus *Atherina* Linnaeus, 1758 (Quignard and Pras 1986; Creech 1992), whose members are commonly

referred to as sand smelt. The big-scale sand smelt *Atherina boyeri* Risso, 1810 occurs from the coasts of Portugal and Spain to Nouadhibou in Mauritania and Madeira, and throughout the Mediterranean and Black Sea, with some isolated populations on the coasts of England and the Netherlands (Quignard and Pras 1986). In 1979, Berrebi reported a microsporidian parasite forming xenomas in the digestive tube and abdominal cavity of the big-scale sand smelt in the Mediterranean coastal lagoons of Languedoc at Provence (France), and termed it *Glugea atherinae*. At the time, the classification of this parasite was based on host species, site of infection, geographic location and the morphological and ultrastructural aspects of its life cycle stages within the xenomas it produces, including meronts, sporoblasts and spores. A sequence of the rRNA genes of *Glugea atherinae* is given in GenBank, without information regarding site of infection, host species and geographic location. Therefore, *Glugea atherinae* lacks the comprehensive morphological and molecular description that is necessary for the analysis of its phylogenetic relatedness to other microsporidian species.

The implementation of molecular analyses to the study of Microsporidia demonstrated that morphology alone constitutes an artificial criterion for assessing the taxonomy and phylogeny of microsporidian parasites, which should result from a morphological and molecular combined approach (Weiss and Vossbrinck 1999; Vossbrinck and Debrunner-Vossbrinck 2005). Currently, GenBank provides sequences for the rRNA genes of only 6 species and 3 records of *Glugea*, the molecular analysis of which has raised phylogenetic issues that warrant resolution (Pomport-Castillon et al. 2000; Freeman et al. 2004; Lovy et al. 2009). Following the trend for molecular based studies, the parasite formerly termed *Glugea americanus* was transferred to the genus *Spraguea* (currently *Spraguea americanus*), and the species *Glugea anomala*, *Glugea stephani* and *Glugea hertwigi* were demonstrated as probably representing the same

species (Pomport-Castillon et al. 2000; Freeman et al. 2004; Lovy et al. 2009). The present study provides a combined morphological and molecular approach to the taxonomy and phylogeny of *Glugea atherinae*, subsequently addressing the phylogenetic issues of the genus *Glugea*.

Materials and Methods

Between October and December 2013, a parasitological survey was conducted on several fish collected from the lower River Minho, near Vila Nova de Cerveira (41° 56' N 8° 44' W), Portugal. The fish were kept fresh on ice and immediately transported to the Laboratory of Cell Biology of the Institute of Biomedical Sciences of Abel Salazar (University of Porto). Fish species were identified and the size, weight and gender of the specimens were registered. Necropsy was performed, followed by the macroscopic and microscopic observation of various internal organs and tissues. The eyes, fins and gills were also examined. Among the sampled fish, 23 specimens (8 males and 15 females) of the amphidromous actinopterygian big-scale sand smelt, *Atherina boyeri* Risso, 1810 (Actinopterygii, Atherinidae) (Portuguese common name "peixe-rei"), were analyzed and presented total length and weight range of 7.0-9.8 cm and 2.0-5.0 gr., respectively. Necropsy revealed nine of these specimens displaying whitish parasitic xenomas attached to the viscera in the coelomic cavity. Infected samples were examined and photographed using the Nomarski differential interference contrast optics in a Leitz Dialux 20 light microscope, equipped with a digital camera. Spores' morphometry was determined from fresh material. All measurements include the mean value ± standard deviations (SDs) and number of spores measured (*n*).

For transmission electron microscopy (TEM), xenomas were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.2) at 4 ºC for 24 h, washed

overnight in the same buffer at the same temperature, and post-fixed in 2% osmium tetroxide with the same buffer for 3 h at 4 ºC. After dehydration in an ascending ethanol series ending in propylene oxide, the samples were embedded in EPON. Semi-thin sections were stained with methylene blue-Azure II for light microscopy. Ultrathin sections were contrasted with both aqueous uranyl acetate and lead citrate, observed and photographed using a JEOL 100CXII TEM operated at 60 kV.

For the molecular analysis, DNA was extracted from spores using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma), according to the manufacturer's instructions, and stored in 50 µl of TE buffer at −20 °C. The majority of the region coding for the small subunit (SSU) rRNA gene was amplified using the primer V1f (5′-CACCAGGT TGATTCTGCC-3′) (Nilsen 2000) and the primer HG5F_rev (5′-TCACCCCACTTGTCGTTA-3′), which was designed for this study. The 3′ end of the SSU, the internal transcribed spacer (ITS) and the 5′ end of the large subunit (LSU) rRNA gene were amplified using the primers HG4F (5′-GCGGCTTAATTTGACTCAAC-3′) and HG4R (5′-TCTCCTTGGTCCGTGTTTCAA-3′) (Gatehouse and Malone 1998). PCRs were performed in 50 µl reactions using 10 pmol of each primer, 10 nmol of each dNTP, 2.5 mM of $MgCl_2$, 5 µl 10×*Taq* polymerase buffer, 1.5 units *Taq* DNA polymerase (Nzytech), and 50 to 100 ng of genomic DNA. The reactions were run on a Hybaid PxE Thermocycler (Thermo Electron Corporation), with initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min. The final elongation step was performed at 72 °C for 10 min. Aliquots (5 µl) of the PCR products were electrophoresed through a 1% agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide. PCR products were purified using a single-step enzymatic cleanup that eliminates unincorporated primers and dNTPs by the ExoFast method. The

PCR products were sequenced directly. The sequencing reactions were performed using a BigDye Terminator v1.1 kit from the Applied Biosystems Kit and were run on an ABI3700 DNA analyzer (Perkin-Elmer, Applied Biosystems).

Phylogenetic analysis was performed using the obtained rRNA sequence of the parasite and other microsporidian rRNA sequences retrieved from GenBank, namely, those presenting highest similarity score in Basic Local Alignment Search Tool (BLAST). All rRNA sequences of *Glugea* were considered in the analysis, excepting the former *Glugea americanus* (AF056014) (currently *Spraguea americanus*) (Pomport-Castillon et al. 1997; Freeman et al. 2004). *Nucleospora salmonis* (U78176) was selected as out-group species. To ensure the accuracy of the analysis and maintain a high tree resolution, sequences with less than 1,000 bp were not used, excepting *Glugea* sp. LX-2012 (JX852026) (964 bp); this avoids loss of information due to shortening of the aligned sequences and the appearance of numerous gaps.

Alignments were performed using ClustalW in MEGA 5.05 software (Tamura et al. 2011), with an opening gap penalty of 10 and a gap extension of 4 for both paired and multiple alignments. Subsequent phylogenetic and molecular evolutionary analyses were conducted in MEGA 5.05, using maximum likelihood (ML), neighbor-joining (NJ) and maximum parsimony (MP) methodologies. For ML, the general time reversible substitution model with 4 gamma-distributed rate variation among sites was performed. For NJ, we used Kimura 2-parameter as the substitution model with a gamma distribution (shape parameter = 1.4). For MP, the close neighbor interchange heuristic option with a search factor of 1 and random initial tree addition of 500 replicates was performed. All positions with less than 95% site coverage were eliminated from all trees, resulting in a total of 830 positions in the final dataset. The

bootstrap consensus tree was inferred from 100 replicates for ML and MP, and 500 replicates for NJ.

A second alignment was performed for the rRNA sequence obtained for the parasite and all other *Glugea* rRNA sequences available in GenBank. Distance estimation was carried out in MEGA 5.05, using the Kimura two-parameter model distance matrix for transitions and transversions. All positions with less than 95% site coverage were eliminated, resulting in a total of 889 positions in the final dataset.

Results

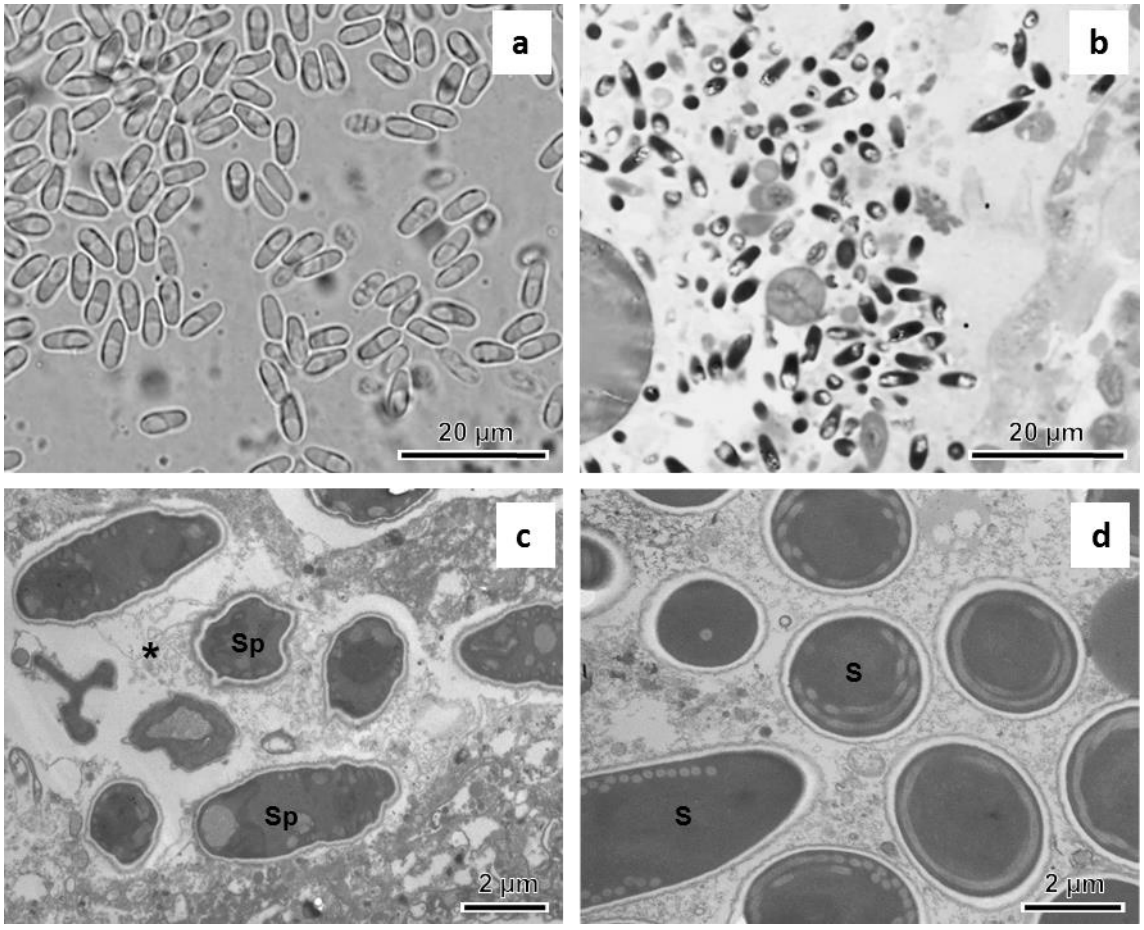

Fig. 1 - Light and transmission electron micrographs of *Glugea atherinae* infecting the big-scale sand smelt *Atherina boyeri* in the Minho River. **a** Fresh mature spores observed using DIC. **b** Semi-thin section of a xenome several mature spores disperse in its matrix. **c** TEM micrograph showing a group of sporoblasts (Sp) developing within a sphorophorous vesicle (*). **d** TEM micrograph showing several mature spores (S) sectioned at different planes.

Prevalence of infection and morphology

Prevalence of infection was determined at ~39%, with 9 infected specimens in 23 examined (4/15 [26.6%] for females and 1/8 [12.5%] for males). Several spherical to ellipsoidal xenomas, measuring up to ~ 3 mm, were observed adhering to the visceral organs in the hosts' coelomic cavity. Each xenome consists of a single hypertrophic

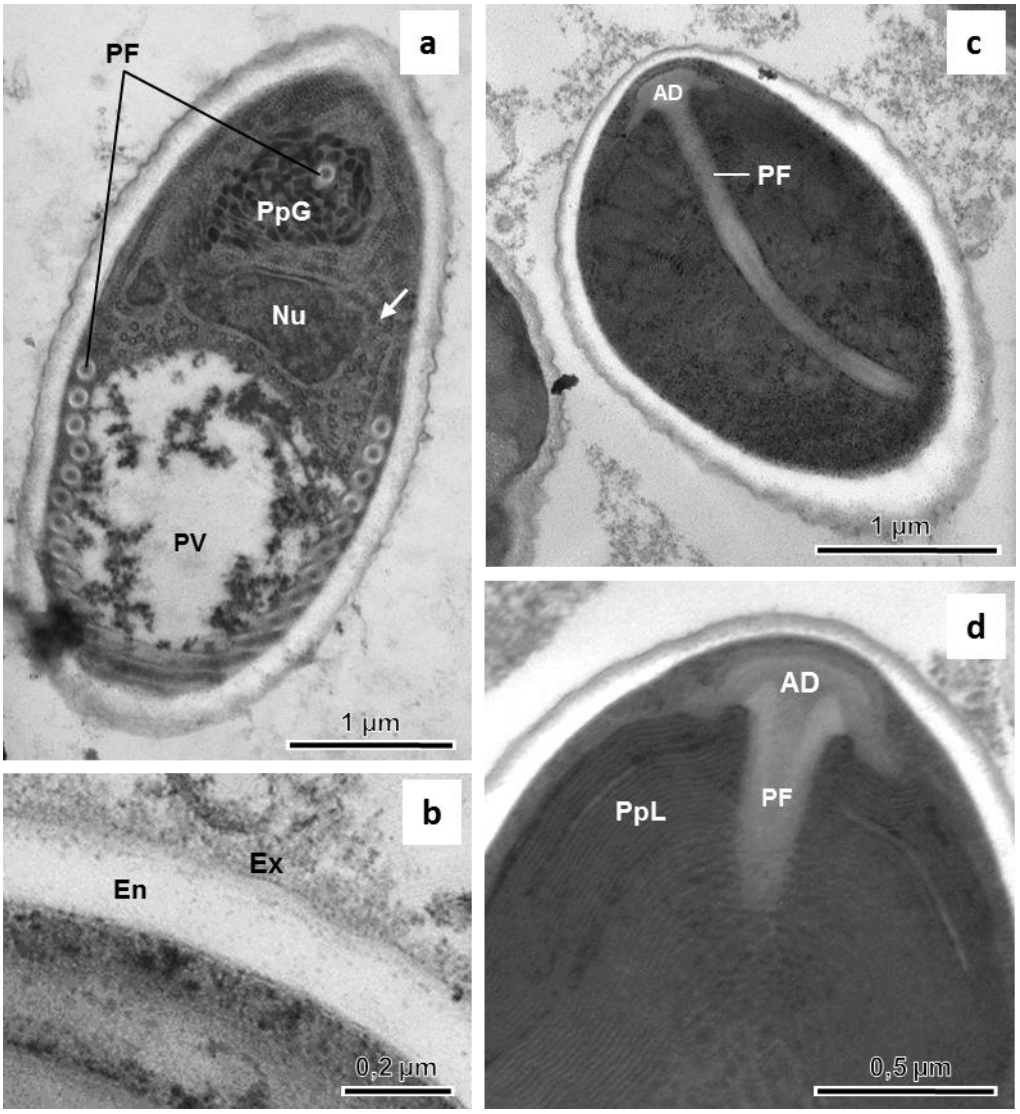

Fig. 2 - Transmission electron micrographs of *Glugea atherinae* infecting the big-scale sand smelt *Atherina boyeri* in the Minho River. a Spore in longitudinal section showing the globular portion of the polaroplast (PpG), the nucleus (Nu) at the centre and the posterior vacuole (PV), around which the polar filament (PF) coils. Notice the polyribosomes (arrow) helicoidally arranged and positioned between the polaroplast and the nucleus. b Ultrastructural detail of the spore's wall evidencing the endospore (En) and the exospore (Ex). c Spore in oblique section displaying the straight portion of the polar filament (PF) attached to the anchoring disk (AD) and extending from the anterior to the posterior pole. d Anterior pole of the spore revealing the anchoring disk (AD) from which the polar filament (PF) extends, and the lamelar portion of the polaroplast (PpL).

cell, in the cytoplasm of which monomorphic mature spores proliferate (Figs. 1a and 1b). Small groups of sporoblasts in the later stages of differentiation were also observed contained within sphorophorous vesicles that appeared close to the xenomas wall (Fig. 1c), but no other earlier life-cycle stages were present.

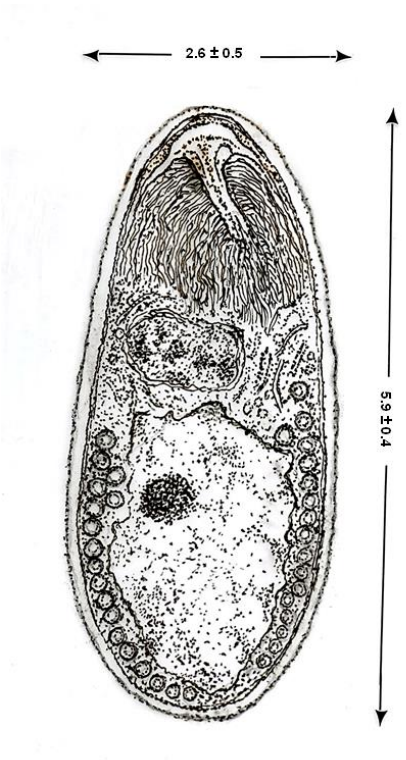

Fig. 3 - Schematic drawing depicting the internal organization of the spores of *Glugea atherinae*. Dimensions are given in µm.

Mature spores are ellipsoidal in shape and measure 5.9 ± 0.4 µm in length and 2.6 ± 0.5 µm (*n* = 50) (Fig. 1a, 1d and 2a). The spores' wall measures 116.6 ± 23.9 nm (*n* = 10) in thickness, except for the apical region, where it contacts the anchoring disc, measuring only 50.3 ± 4.5 nm (*n* = 7). The spores' wall is double-layered, composed by a moderately electron-dense exospore (41.7 ± 12.5 nm (*n* = 10) in width) and an electron-lucent endospore (72.8 ± 19.6 nm (*n* = 10) in width) (Fig. 2b). The anchoring disk appears subterminally, laterally shifted from the anterior pole of the spore; beginning in this structure, the polar filament extends from the anterior to the posterior pole, coiling to form 17-18 (rarely 19) turns in single (rarely double) rows that surround the posterior vacuole at an angle of ~73º (*n* = 15) to the spores' longitudinal axis (Fig. 2a, 2c and 2d). At the manubrium (uncoiled portion near the anchoring disk), the polar filament presents a diameter of 148.8 ± 17.1 nm (*n* = 7), but at its coiled portion is thinner, measuring 101.1± 12.8 nm (*n* = 11) in diameter. The polaroplast surrounds the manubrium and is composed by a lamellar region and a globular region (Figs. 2a and 2d). Several ribosomes arranged helicoidally are located between the polaroplast and the single nucleus, which appears

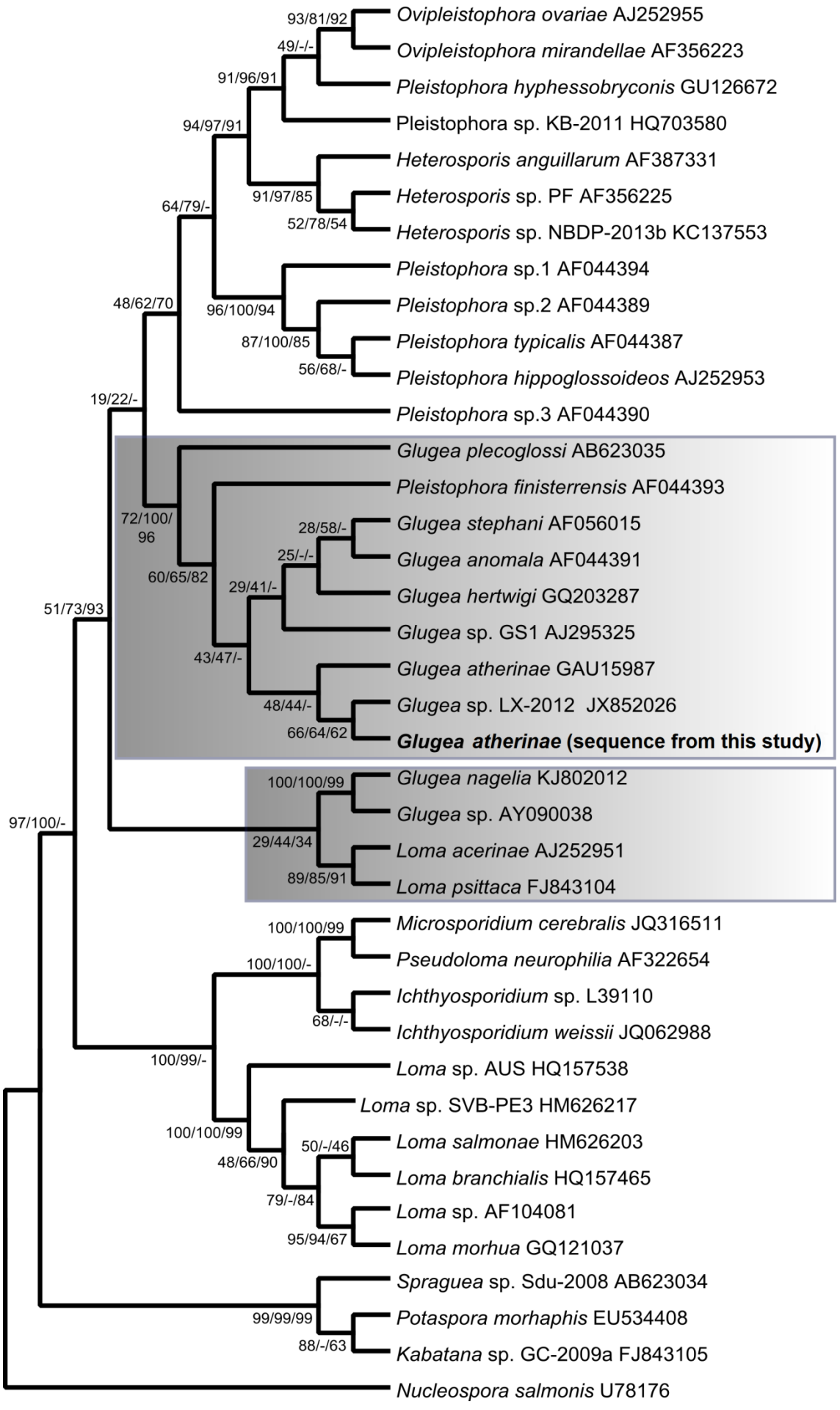
93/81/92
Ovipleistophora ovariae AJ252955
49/-/-
Ovipleistophora mirandellae AF356223
91/96/91
Pleistophora hyphessobryconis GU126672
94/97/91
Pleistophora sp. KB-2011 HQ703580
Heterosporis anguillarum AF387331
91/97/85
Heterosporis sp. PF AF356225
64/79/-
52/78/54
Heterosporis sp. NBDP-2013b KC137553
Pleistophora sp.1 AF044394
48/62/70
96/100/94
Pleistophora sp.2 AF044389
87/100/85
Pleistophora typicalis AF044387
56/68/-
Pleistophora hippoglossoideos AJ252953
19/22/-
Pleistophora sp.3 AF044390
Glugea plecoglossi AB623035
Pleistophora finisterrensis AF044393
72/100/
96
28/58/-
Glugea stephani AF056015
25/-/-
Glugea anomala AF044391
60/65/82
29/41/-
Glugea hertwigi GQ203287
51/73/93
Glugea sp. GS1 AJ295325
43/47/-
Glugea atherinae GAU15987
48/44/-
Glugea sp. LX-2012 JX852026
66/64/62
Glugea atherinae (sequence from this study)
100/100/99
Glugea nagelia KJ802012
Glugea sp. AY090038
97/100/-
29/44/34
Loma acerinae AJ252951
89/85/91
Loma psittaca FJ843104
100/100/99
Microsporidium cerebralis JQ316511
100/100/-
Pseudoloma neurophilia AF322654
Ichthyosporidium sp. L39110
68/-/-
Ichthyosporidium weissii JQ062988
100/99/-
Loma sp. AUS HQ157538
Loma sp. SVB-PE3 HM626217
100/100/99
50/-/46
Loma salmonae HM626203
48/66/90
Loma branchialis HQ157465
79/-/84
Loma sp. AF104081
95/94/67
Loma morhua GQ121037
Spraguea sp. Sdu-2008 AB623034
99/99/99
Potaspora morhaphis EU534408
88/-/63
Kabatana sp. GC-2009a FJ843105
Nucleospora salmonis U78176

Fig. 4 - Maximum likelihood tree for the rRNA sequence obtained and other selected microsporidians. GenBank accession numbers after the species name. The numbers on the branches are bootstrap confidence levels on 500 replicates for ML and 100 replicates for NJ/MP.

at a central position (Fig. 2a). The posterior vacuole is ellipsoidal in shape and measures 2.61 ± 0.58 µm in length and 1.62 ± 0.32 µm in width (n = 11), occupying about half of the spores' total length (Fig. 2a). This structure appears electron-lucent, containing small irregular granular masses randomly disperse in its matrix. The posterosome was never observed. The ultrastructural features here described are represented in a schematic drawing (Fig. 3).

Phylogenetic analysis

The consensus rRNA sequence obtained, composed of 1858 bp, was deposited in GenBank under the accession number XXXXX. The phylogenetic analysis performed revealed the parasite clustering within a clade containing all other sequenced *Glugea*,

Table 1 - Percentage of identity between the rRNA sequences of the *Glugea* species available in GenBank.

ID	Species	GenBank	1	2	3	4	5	6	7	8	9	10
(1)	***Glugea atherinae* (sequence obtained in this study)**	**(xxxxxxxx)**	-									
(2)	*Glugea hertwigi*	(GQ203287)	99.8	-								
(3)	*Glugea atherinae*	(GAU15987)	99.5	99.8	-							
(4)	*Glugea stephani*	(AF056015)	99.5	99.8	99.5	-						
(5)	*Glugea* sp. GS1	(AJ295325)	99.5	99.8	99.5	99.5	-					
(6)	*Glugea* sp. LX-2012	(JX852026)	99.5	99.5	99.5	99.3	99.3	-				
(7)	*Glugea plecoglossi*	(AB623035)	99.4	99.7	99.4	99.4	99.4	99.2	-			
(8)	*Glugea anomala*	(AF044391)	99.3	99.5	99.3	99.3	99.5	99.1	99.2	-		
(9)	*Glugea nagelia*	(KJ802012)	92.4	92.6	92.4	92.4	92.4	92.1	92.6	92.1	-	
(10)	*Glugea* sp.	(AY090038)	92.1	92.3	92.1	92.1	92.1	0.91.9	92.3	91.8	99.3	-

with the exception of *Glugea nagelia* (KJ802012), a species described from the Red Sea in Saudi Arabia, and *Glugea* sp. (AY090038), which origin remains unclear (Fig. 4). Although the resolution within the main *Glugea* clade is median, the bootstrap values supporting the whole clade are high, namely 72% for ML, 100% for NJ and 96% for MP. Pairwise comparisons between all *Glugea* rRNA sequences available revealed that the 7 *Glugea* rRNA sequences clustering together with the rRNA sequence here obtained are also those presenting higher percentage of identity to the parasite, i.e. *Glugea hertwigi* (99.8%), *G. atherinae* (99.5%), *G. stephani* (99.5%), *Glugea* sp. GS1 (99.5%), *Glugea* sp. LX-2012 (99.5%), *G. plecoglossi* (99.4%) and *G. anomala* (99.3%). *Glugea nagelia* and *Glugea* sp. (AY090038) present lower percentage of identity, 92.4 % and 92.1%, respectively (Table 1).

Discussion

The morphological and ultrastructural organization observed, combined with the molecular analysis of the rRNA genes, recognize the parasite here described as belonging to the genus *Glugea* of the phylum Microsporidia (Lom and Dyková 1992; Larsson 1999; Lom and Nilsen 2003). Although the morphological and molecular data acquired proved insufficient to determine the parasite species, the host species and the site of infection were determinant for its identification as *Glugea atherinae*. The latter was morphologically described forming small xenomas in the digestive tube and large xenomas in the abdominal cavity, adhering to the gall bladder, the liver, the peritoneum and the gonads of the big-scale sand smelt in the Mediterranean coastal lagoons of France (Berrebi 1979). Later, its rRNA sequence was deposited in GenBank, without supplementary information.

Comparison of the parasite here studied to other *Glugea*, including *Glugea atherinae*, reveals an astonishing lack of reliable morphological criteria that can be used for taxonomic and phylogenetic purposes, as it has been observed for most microsporidian taxa (Weiss and Vossbrinck 1999; Vossbrinck and Debrunner-Vossbrinck 2005). At the same time, the few molecular data available for the genus *Glugea* also appears to be highly unreliable for the differentiation of its species (Lovy et al. 2009). In fact, in the case of Microsporidia, the use of molecular tools in general raises taxonomic questions as often as it solves them and it's not only due to the scarcity of molecular data available in certified databases relating to microsporidians in general and to the genus *Glugea* specifically, but also to the artificiality of the criteria used in most original descriptions; thus the importance of combined morphological and molecular approaches for ascertaining the taxonomy and phylogeny of both new and old microsporidian species.

The phylogenetic analysis here performed reveals the rRNA sequence of *Glugea atherinae* obtained showing great similarity percentage not only to the other sequence of this species available in GenBank, but also to all other *Glugea* sharing its clade. In fact, all the rRNA sequences comprising the main *Glugea* clade displayed very high percentages of similarity between them (all above 99%), reinforcing the assumption that they all most likely represent the same species, as suggested by previous molecular and experimental transmission studies (Mathieu-Daude et al. 1992; Leiro et al. 1993; Pomport-Castillon et al. 1997, 2000; Lovy et al. 2009). Mathieu-Daude et al. (1992) showed that *G. atherinae* can cross-infect the flounder *Platichthys flesus*, which is the natural host of *G. stephani*, thus suggesting that these species are cospecific; and furthermore revealed that these species most likely infect yet another fish host, the teleost *Sparus aurata*, forming xenomas in the musculature of the pectoral fins. Leiro et

al. (1993) reported *Glugea atherinae* infecting another member of the family Atherinidae, the sand smelt *Atherina presbyter* Cuvier, 1829 in the Spanish northwest coast, and further showed that it was possible to experimentally infect the turbot *Scophthalmus maximus* Linnaeus, 1758 with this parasite species. Pomport-Castillon et al. (2000) hypothesized that *G. anomala* was also cospecific with the two latter, based on sequence homology. Evidence of *G. hertwigi* possibly being the same species as *G. anomala* was provided by the successful experimental infection of sticklebacks with *G. hertwigi* collected from the European smelt *Osmerus eperlanus* (Weissenberg 1968). Lovy et al. (2009) studied the phylogeny of *G. hertwigi* with similar results to those here obtained; however, at the time, the rRNA sequence of *Glugea nagelia* was not available and, as the origin of the rRNA sequence of *Glugea* sp. (AY090038) is dubious, the study lacked reliable molecular information that could set a term of comparison for evaluating evolutionary relatedness between all *Glugea*. In our phylogenetic study, *Glugea nagelia* and *Glugea* sp. (AY090038) display lower percentage of similarity to all other *Glugea* and form a separate clade, again reinforcing that a common identity should be defined for all the other sequenced *Glugea* species. Also supporting this taxonomic revision are studies that proved that extreme dimorphisms are possible and often result in taxonomic conundrums in the phylum Microsporidia (Canning and Lom 1986; Stentiford et al. 2013); therefore indicating that the reduced dimorphism existent between *G. atherinae*, *G. hertwigi*, *G. stephani*, *Glugea* sp. GS1, *Glugea* sp. LX-2012, *G. plecoglossi* and *G. anomala* probably results from their adaptation to different hosts and sites of infection. In the case of *G. atherinae*, its natural hosts include the big-scale sand smelt *Atherina boyeri*, the sand smelt *Atherina presbyter* and the baitfish *Dorosoma cepedianum*, with few morphometric differences reported between xenomas and spores (Berrebi 1979;

Mathieu-Daude et al. 1992; Leiro et al. 1993; Shaw and Kent 1999). Experimental infection of turbot *Scophthalmus maximus* Linnaeus, 1758 with *G. atherinae* from *Atherina boyeri* has also been demonstrated and, in this host, the parasite forms xenomas in the lamina propria of the digestive tube and in the hepatic parenchyma rather than in the coelomic cavity. In the liver, xenomas appear surrounded by a fibroblastic capsule that was never reported from any of the other sites of infection and hosts (Leiro et al. 1993); therefore acknowledging the occurrence of dimorphisms resulting from this species adaptation to different sites of infection and host species. One of the main criteria used for distinguishing microsporidian species is the number of coils of the polar filament. *Glugea atherinae* was reported having more than 10 coils (Berrebi 1979), with our ultrastructural study establishing a range between 17-18 (rarely 19) coils. *Glugea anomala* has a reported range of 12 to 14 coils, although 11 to 16 coils and 11 to 14 coils were observed in a microsporidian resembling *G. anomala* in the killifish *Nothobranchius korthausae* and *Fundulopanchax filamentosus*, respectively (Lom et al. 1995). This is consistent with the range of 12 to 13 coils of *G. hertwigi* and the 12 coils of the polar filament described for *G. stephani* (Takvorian and Cali 1996). Nevertheless, and despite all of this morphologic, molecular and cross-infection information, further data on intragenomic variability, other genes and experimental transmission should be acquired before any taxonomic revision is suggested, so as not to raise the taxonomic entropy of the phylum Microsporidia.

Turning to consider the sporogonic development, the lack of other developmental stages than the mature spores may be a consequence of the low water temperature that the fish host was exposed in its environment, since all the specimens analyzed were collected during the autumn period. Temperature is a physical factor known to influence the development of microsporidian parasites, with low temperatures

inhibiting their development, namely in the earlier stages of infection (merogony) (Beaman et al. 1999; Speare et al. 1999). The fact that only sporoblasts in the final stage of differentiation and fully mature spores were observed in this study suggests that the parasite developed during the spring/summer months, when the water was warmer, and was no longer replicating. It is possible that the xenomas observed were over-wintering, although it's not known to what extend the spores from over-wintered xenomas can auto-infect the host cells in the following summer. Autoinfection has been proposed for other Microsporidia, such as *Loma* sp. in Atlantic cod *Gadus morhua* (Rodriguez-Tovar et al. 2003), and the Amazonian fish *Myrophis platyrhynchus* (Matos et al. 2003).

The values of prevalence of infection obtained in this study agree that the infection caused by *Glugea atherinae* is sex dependent, with females being more frequently infected than males (Berrebi 1978). However, these values are probably underestimated since the parasite most likely develops during the spring/summer period, when the water temperature is warmer. The size of the xenomas has been used as an indicator of age (Rodriguez-Tovar et al. 2004). *Glugea atherinae* has been described forming large xenomas (up to 14 mm in diameter) (Berrebi 1979), but in this study only small xenomas containing mature spores were observed, suggesting that this criterion does not apply to this parasite species, and to all microsporidian species in general. The lack of larger xenomas may also be the reason why no overall histopathological changes were observed, since these structures are known to cause pressure atrophy of affected and surrounding organs, ultimately contributing to an increase in the host mortality rates (Berrebi 1978).

Acknowledgments This study was partially supported by the Eng. A. Almeida Foundation (Portugal); the Foundation for Science and Technology (FCT), within the

scope of the PhD fellowship grant SFRH/BD/92661/2013 to S. Rocha and the project DIRDAMyx (PTDC/MAR/116838/2010) (Portugal); the project EUCVOA (NORTE-07-0162-FEDER-000116) (Portugal); and the Deanship of Scientific Research of King Saud University, within the scope of the project RGP-VPP-002 (Saudi Arabia). This work complies with the current laws of the countries in which it was performed.

References

Beaman HJ, Speare DJ, Brimacombe M (1999) Regulatory effects of water temperature on *Loma salmonae* (Microspora) development in rainbow trout. J Aquat Anim Health 11:237–245.

Berrebi P (1978) Contribution à l'étude biologique des zones saumaîtres du litoral méditerraneén français. Biologie d'une microsporidie: *Glugea atherinae* n. sp. parasite de l'atérine: *Atherina boyeri* Risso, 1810 (Poisson – Teleostéen) des étangs côtiers. Thesis, Université des Sciences et Techniques du Languedoc, Montpellier, France. pp. 196.

Berrebi P (1979) Etude ultrastructurale de *Glugea atherinae* n. sp., microsporidie parasite de l'athérine *Atherina boyeri* Risso 1810 (poisson téléostéen) dans les lagunes du Languedoc et de Provence. Z Parasitenkd 60:105–122.

Canning EU, Lom J (1986) The Microsporidia of Vertebrates. Academic Press, New York. pp. 289

Creech S (1992) A study of the biology of *Atherina boyeri* Risso, 1810 in Aberthaw Lagoon on the Bristol Channel, in South Wales. J Fish Biol 41:277–286.

Dyer BS, Chernoff B (1996) Phylogenetic relationships among atheriniform fishes (Teleostei, Atherinomorpha). Zool J Linnean Soc, London 117:1–69.

Freeman MA, Yokoyama H, Ogawa K (2004) A microsporidian parasite of the genus *Spraguea* in the nervous tissues of the Japanese anglerfish *Lophius litulon*. Folia Parasitol 51:167–176.

Gatehouse HS, Malone LA (1998) The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNA genes and evidence of a large tandem repeat unit size. J Invert Pathol 71:97–05.

Ivantsoff W, Crowley LELM (1999) Atherinidae. Silversides (or hardyheads), p. 2113-2139. In Carpenter KE, Niem VH (eds.) FAO species identification guide for fishery purposes. The living marine resources of the Western Central Pacific. Volume 4. Bony fishes part 2 (Mugilidae to Carangidae). FAO, Rome

Larsson JIR (1999) Identification of Microsporidia. Acta Protozool 38:161–197.

Leiro J, Bos J, Iglesias R, Estevez J, Fernandez J, Sanmartín ML (1993) Experimental infection of turbot (*Scophthalmus maximus* L.) with a microsporean parasite (*Glugea atherinae* Berrebi 1979) of the sand smelt (*Atherina presbyter* C.). Aquaculture 118:1–7.

Lom J, Dyková I (1992) Microsporidia (Phylum Microspora Sprague, 1977). In Protozoan Parasites of Fishes. Developments in Aquaculture and Fisheries Science, Elsevier, Amsterdam, Netherlands 26:125–157.

Lom J, Nilsen F (2003) Fish Microsporidia: fine structural diversity and phylogeny. Int J Parasitol 33:107–127.

Lom J, Noga EJ, Dyková I (1995) Occurrence of a microsporean with characteristics of *Glugea anomala* in ornamental fish of the family Cyprinodontidae. Dis Aquat Org 21:239–242.

Lovy J, Kostka M, Dyková I, Arsenault G, Pecková H, Wright GM, Speare DJ (2009) Phylogeny and morphology of *Glugea hertwigi* from rainbow smelt *Osmerus mordax* found in Prince Edward Island, Canada. Dis Aquat Org 86: 235–243.

Mathieu-Daude F, Faye N, Coste F, Manier JF, Marques A, Bouix G (1992) Occurrence of a microsporidiosis in marine cultured gilt-head sea bream from the Languedoc coast: a problem of specificity in the genus *Glugea* (Protozoa, Microspora). Bull Eur Assoc Fish Pathol 12:67–70.

Matos E, Corral L, Azevedo C (2003) Ultrastructural details of the xenoma *Loma myrophis* (Phylum Microsporidia) and extrusion of the polar tube during autoinfection. Dis Aquat Org 54:203–207.

Nilsen F (2000) Small subunit ribosomal DNA phylogeny of Microsporidia with particular reference to genera that infect fish. J Parasitol 86:128–133.

Pomport-Castillon C, De Jonckheere JF, Romestand B (2000) Ribosomal DNA sequences of *Glugea anomala*, *G. stephani*, *G. americanus* and *Spraguea lophii* (Microsporidia): phylogenetic reconstruction. Dis Aquat Org 40:125–129.

Pomport-Castillon C, Romestand B, De Jonckheere JF (1997) Identification and phylogenetic relationships of Microsporidia by riboprinting. J Eukaryot Microbiol 44:540–544.

Quignard JP, Pras A (1986) Atherinidae. In: Whitehead P.J.P., Bauchot M.L., Hureau J.C., Nielsen J. and Tortonese E. (eds), Fishes of the North-Eastern Atlantic and the Mediterranean, UNESCO, Paris 1207–1210.

Rodriguez-Tovar LE, Wadowska DW, Wright GM, Groman DB, Speare DJ, Whelan DS (2003) Ultrastructural evidence of autoinfection in the gills of Atlantic cod *Gadus morhua* infected with *Loma* sp. (phylum Microsporidia). Dis Aquat Org 57:227–230.

Rodriguez-Tovar LE, Speare DJ, Markham RJF, Daley J (2004) Predictive modeling of post-onset xenoma growth during microsporidial gill disease (*Loma salmonae*) of salmonids. J Comp Pathol 131:330–333.

Shaw RW, Kent ML (1999) Fish microsporidia. In: Whitner M, Weiss LM (eds) The microsporidia and microsporidiosis. ASM Press, Washington, DC, p. 418–446.

Speare DJ, Beaman HJ, Daley J (1999) Effect of water temperature manipulation on a thermal unit predictive model for *Loma salmonae*. J Fish Dis 22:277–283.

Stentiford GD, Bateman KS, Feist SW, Chambers E, Stone DM (2013) Plastic parasites: Extreme dimorphism creates a taxonomic conundrum in the phylum Microsporidia. Int J Parasitol 43:339–352.

Takvorian PM, Cali A (1996) Polar tube formation and nucleoside diphosphatase activity in the microsporidian, *Glugea stephani*. J Eukaryot Microbiol 43:102S–103S.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739.

Vossbrinck CR, Debrunner-Vossbrinck BA (2005) Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitol 52:131–142.

Weiss LM, Vossbrinck CR (1999) Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the Microsporidia. In: The Microsporidia and microsporidiosis, Wittner M, Weiss L (eds.). American Society of Microbiology, Washington, DC, pp. 129–171.

Weissenberg R (1968) Intracellular development of the microsporidian *Glugea anomala* Moniez in hypertrophying migratory cells of the fish *Gasterosteus aculeatus*, and example of the formation of the xenoma tumors. J Protozool 15:44–57.

Chapter 3 – Discussion and Conclusion

3.1 Discussion

The worldwide consumption of fish and its products has continuously grown in the past few decades, becoming the primary source of protein for many populations. Contrarily to this trend, the fishing industry has been declining significantly, due to over-fishing, loss of fish habitats and environmental pollution, among others. This incapability of the fish industry to respond to the growing commercial demand of this product propelled the aquaculture industry. Several countries developed initiatives and encouragements for private industries to invest in fish cultures, which have come to an exponential growth in the last years, being the fastest-growing food production process in the world. However, the high fish densities practiced in aquaculture facilities are optimal for fish-infecting parasites which are mainly directly transmitted and, therefore, can lead to massive infections and high mortalities. The exponential growth of intensive fish cultures will undoubtedly trigger the emergence of new diseases and the frequent discovery of new species constitutes evidence to the necessity of continuous studies focusing on the diagnosis of potentially harmful microparasites, namely from the phylum Microsporidia. Fish microsporidiosis are known to have caused declines of entire commercial fisheries in the past. Members of the genera *Glugea, Loma, Nucleospora* and *Heterosporis* are known to infect economically important wild, cultured and ornamental fish species (Canning and Lom 1986, Lom and Dyková 2005). In wild fish, *Pleistophora macrozoarcides* was liable for the collapse of the North American ocean pout (*Zoareces anguillaris*) fisheries (Sandholzer et al. 1945), and as for the decline in the rainbow smelt fishery of New Hampshire, it was caused by *Glugea hertwigi* (Haley 1954). In cultured fish, several species of Microsporidia are well known for causing high mortality rates (*Heterosporis anguillarum, Loma salmonae, Nucleospora salmonis*), and in ornamental fish, *Glugea* spp. and *Pleistophora hyphessobryconis* are known to cause mortality and disease (Canning and Lom 1986).

In order to establish a reliable database for the efficient diagnosis of potentially harmful microsporidians, strong methods of species classification are warranted. Nowadays, the comprehensive association of morphological and molecular data is mandatory for describing microsporidian species (Nilsen 2000, Lom and Nilsen 2003, Issi et al. 2012). Given the enormous phenotypic variability among Microsporidia, many genera have been wrongly classified (Lom 2002), being that the major criterion for species classification was the spore morphological features (Larsson 1986, Canning and Lom 1986, Lom and Dyková 1992). Old descriptions refer to single spore types infective in isolated hosts, when currently it is known that microsporidians can present

heterospory, as well as morphological variation between host species, evidencing the provable existence of many redundant genera (Dunn and Smith 2001). Hence the importance of applying molecular methodologies, which supplements the morphological descriptions, providing information that limits redundancy and that is reliable for taxonomic classification. Light microscopy, namely the Nomarski differential interference contrast optics, allows the general recognition of the species structure, while TEM details the ultrastructural features of the morphology of spores and developmental stages. Morphological information regarding the developmental stages can be of great value for classification purposes when life cycle details are provided, however, in this case, the observations performed didn't allowed the full description of the parasite life cycle, since several developmental stages were not observed.

Molecular methodologies provide an accurate tool for taxonomic and phylogenetic descriptions (Vossbrinck and Debrunner-Vossbrinck 2005, Stentiford et al. 2013a) that is, however, inefficient if applied alone, as any other data alone does not provide enough information for species description and classification (Larsson 2005).Thus, it is of the utmost importance to create a strong microsporidian database, providing the DNA sequences and morphological data of only well-defined confirmed species (Franzen 2008), seeing that on the existing public databases no one really knows the origin of all the sequences available.

In this thesis, the parasite infecting the big-scale sand smelt in the Minho River is described using morphology-based methods complemented with the sequencing of the rRNA genes, including the ITS region. A phylogenetic analysis is performed using data from GenBank relative to the rRNA genes of fish infecting Microsporidia, using maximum likelihood, neighbor-joining and maximum parsimony methodologies. This comprehensive analysis results in the identification of the parasite as *Glugea atherinae*, but the high level of rRNA sequence identity obtained between *Glugea atherinae*, *Glugea hertwigi, Glugea stephani, Glugea* sp. GS1, *Glugea plecoglossi, Glugea* sp. LX-2012 and *Glugea anomala* suggests that these may be variants of a single species.

In the Appendages section, some transmission electron micrographs of another microorganism also found infecting the big-scale sand smelt are presented. The parasite was identified as belonging to the phylum Apicomplexa Levine, 1970, more specifically to the genus *Goussia* Labbé, 1896, but, considering that the aim of this thesis was to study only microorganisms from the Phylum Microsporidia, no further analysis was performed for the time being. Future studies are necessary to identify this parasite species and describe its morphological and molecular features if it proves to be a new record within its genus.

3.2 Conclusion

Parasitology is a growing science and in order to control and fight parasitic diseases and infections, a quick and reliable diagnosis of the infection agent is necessary. Microsporidia have been studied for over 150 years, but yet many doubts persist regarding their life cycle and biological features, as well as their taxonomy and phylogeny. Careful morphological and molecular studies are indispensable for the correct identification of new and existing species. Sequencing of the rRNA genes of every species described should always be performed, and the sequences obtained listed and freely accessible. Other, more rapidly evolving, non-ribosomal protein-coding genes, such as A-G regions of the largest subunit of RNA polymerase II (Vossbrinck and Debrunner-Vossbrinck 2005, Stentiford et al. 2013b) should also be considered for tracing microsporidian phylogeny and evolution.

The lack of information concerning deeper knowledge of host specificity, cases of indirect transmission and host–parasite relationships at the tissue and cellular levels of fish-infecting Microsporidia is disturbing. New studies of experimental infections focused on the initial stages of life cycles and the spreading within the host, are required, as well as experiments with chemoprophylaxis and chemotherapy. More worrying for the fish industry, there is an immediate need for new methods of appropriate treatment and selection of therapeutic drugs which obey all safety regulations, without leaving residues either in food or residual waters. The establishment of *in vitro* cultures in fish cell lines is of vital importance and may develop results greatly needed for better understanding fish infecting Microsporidia. New species of Microsporidia are still being found and described frequently, and there are much more yet awaiting discovery.

References

Abdel-Baki AS, Al-Quraishy S, Al-Qahtani H, Dkhil MA, Azevedo C, 2012. Morphological and ultrastructural description of *Pleistophora dammami* sp. n. infecting the intestinal wall of *Saurida undosquamis* from the Arabian Gulf, Saudi Arabia. Parasitol. Res., 111: 413-418.

Awakura T, Kurahashi S, 1967. Studies on the *Pleistophora* disease of salmonid fish. II. On prevention and control of the disease. Scient. Rep. Hokkaido Fish Hatchery, 22: 51-68.

Antunes C, Rodrigues H, 2004. Guia Natural do Rio Minho. Os Peixes. Aquamuseu do Rio Minho. Vila Nova de Cerveira. 84p.

Azevedo C, 1987. Fine structure of the microsporidan *Abelspora portucalensis* gen.n., sp.n. (Microsporida) parasite of the hepatopancreas of *Carcinus maenas* (Crustacea, Decapoda). J. Inverteb. Pathol., 49: 83-92.

Azevedo C, Canning EU, 1987. Ultrastructure of a microsporidian hyperparasite, *Unikaryon legeri* (Microsporida), of trematoda larvae. J. Parasitol., 73: 214-223.

Azevedo C, Matos E, 2002. Fine structure of a new species, *Loma myrophis* (Phylum Microsporidia), parasite of the Amazonian fish *Myrophis platyrhynchus* (Teleostei, Ophichthidae). Eur. J. Protistol., 37: 445-452.

Balbiani G, 1882. Sur les microsporidies ou sporogspermies des articules.C. R. Acad. Sci. Paris, 95: 1168-1171.

Barlough JE, McDowell TS, Bigornia L, Slemenda SB, Peniazek NJ, Hedrick RP, 1995. Nested polymerase chain reaction for detection of *Enterocytozoon salmonis* genomic DNA in Chinook salmon *Oncorhynchus tschawytscha*. Dis. Aquat. Org., 29: 17-23.

Becnel JJ, Andreadis TG, 2001. Microsporidia in Insects. In: Microsporidia and Microsporidiosis (ed. Wittner M, Weiss LM). ASM press, Washington DC, 447-501.

Belkorchia A, Biderre C, Militon C, Polonais V, Wincker P, Jubin C, Delbac F, Peyretaillade E, Peyret P, 2008. In vitro propagation of the microsporidian pathogen Brachiola algerae and studies of its chromosome and ribosomal DNA organization in the context of the complete genome sequencing project. Parasitol. Int., 57: 62-71.

Berrebi P, 1978. Contribution à l'étude biologique des zones saumaîtres du litoral méditerraneén français. Biologie d'une microsporidie: *Glugea atherinae* n. sp. parasite de l'atérine: *Atherina boyeri* Risso, 1810 (Poisson – Teleostéen) des étangs côtiers. Thesis, Université des Sciences et Techniques du Languedoc, Montpellier, France, 196p.

Berrebi P, 1979. Etude ultrastructurale de *Glugea atherinae* n. sp., microsporidie parasite de l'athérine *Atherina boyeri* Risso 1810 (poisson téléostéen) dans les lagunes du Languedoc et de Provence. Z. Parasitenkd., 60:105-122.

Bigliardi E, Sacchi L, 2001. Cell biology and invasion of the microsporidia. Microbes Infect., 3: 373-379.

Bigliardi E, Selmi MG, Lupetti P, Corona S, Gatti S, Scaglia M, Sacchi L, 1996. Microsporidian spore wall: ultrastructural findings on *Encephalitozoon hellem* exospore, J. Eukaryot. Microbiol., 43: 181-186.

Billard R, 1997. Les poissons d'eau douce des rivières de France. Identification, inventaire et répartition des 83 espèces. Lausanne, Delachaux & Niestlé, 192p.

Blasiola GC Jr, 1979. *Glugea heraldi* n.sp. (Microsporida, Glugeidae) from seahorse *Hippocampus erectus* Perry. J. Fish Dis., 2: 493-500.

Brown AMV, Kent ML, 2002. Molecular diagnostics for *Loma salmonae* and *Nucleospora salmonis* (Microsporidia). In: Cunningham CO (ed.) Molecular Diagnosis of Salmonid Diseases. Methods and Technologies in Fish Biology and Fisheries, Kluwer, Dordrecht, 3: 267-83.

Burri L, Williams BA, Bursac D, Lithgow T, Keeling PJ, 2006. Microsporidian mitosomes retain elements of the general mitochondrial targeting system. Proc. Natl. Acad. Sci. USA, 103: 15916-15920.

Cali A, Owen RL, 1988. Microsporidiosis. In: Allows AB, Ausler J.R. WJH, Hashi MO, Urano HT, eds. Laboratory Diagnosis of Infectious Diseases: Principles and Practice. New York, Springer-Verlag, 1: 929-950.

Cali A, Takvorian PM, 1999. Developmental morphology and life cycles of the microsporidia. In: Wittner M, Weiss LM, (Eds.), The Microsporidia and Microsporidiosis. ASM Press, Washingtin, D.C., 85-128.

Canning EU, Lom J, 1986. The Microsporidia of Vertebrates. Academic Press, New York and London, 289p.

Canning EU, Refardt D, Vossbrinck CR, Okamura B, Curry A, 2002. New diplokaryotic microsporidia (Phylum Microsporidia) from freshwater bryozoans (Bryozoa, Phylactolaemata). Eur. J. Protistol., 38: 247-265.

Casal G, Matos E, Garcia P, Al-Quraishy S, Azevedo C, 2012. Ultrastructural and molecular studies of *Microgemma carolinus* n. sp. (Microsporidia), a parasite of the fish *Trachinotus carolinus (*Carangidae) in Southern Brazil. Parasitology, 139: 1720-1728.

Cavalier-Smith T, 1983. A 6-kingdom classification and a unified phylogeny. In: Schwemmler W, Schenk HEA, eds. Endocytobiology II. de Gruyter, Berlin, 1027-1034

Cavalier-Smith T, 1987. Eukaryotes with no mitochondria. Nature, 326: 332-333.

Cavalier-Smith T, 1993. Kingdom Protozoa and its 18 phyla. Microbiol. Rev., 57: 953-994.

Cavalier-Smith T, 1998. A revised six-kingdom system of life. Biolog. Rev., 73: 203-266.

Coyle CM, Weiss LM, Rhodes LV, Cali A, Takvorian PM, Brown DF, Visvesvara GS, Xiao L, Naktin J, Young E, Gareca M, Colasante G, Wittner M, 2004. Fatal myostitis due to the microsporidian *Brachiola algerae*, a mosquito pathogen. N. Engl. J. Med., 351: 42-47.

Creech S, 1992. A study of the biology of *Atherina boyeri* Risso, 1810 in Aberthaw Lagoon on the Bristol Channel, in South Wales. J. Fish Biol., 41: 277-286.

Didier ES, 2005. Microsporidiosis: An emerging and opportunistic infection in humans and animals. Acta Tropica, 94: 61-76.

Docker MF, Devlin RH, Richard J, Khattra J,Kent ML, 1997. Sensitive and specific polymerase chain reaction assay for detection of *Loma salmonae* (Microsporea). Dis. Aquat. Org., 29: 41-48.

Dunn AM, Smith JE, 2001. Microsporidian life cycles and diversity : the relationship between virulence and transmission, Microbes Infect., 3: 381-388.

Dyková I, 2006. Phylum Microspora. In: Fish Diseases and Disorders, vol. 1, Protozoan and Metazoan Infections, 2nd edition, ed. Woo PTK, 205-229.

Dyková I, Lom J, 1978. Tissue reaction of the three-spined stickleback *Gasterosteus aculeatus* L. to infection with *Glugea anomala* (Moniez, 1887). J. Fish Dis., 1: 83-90.

Dyková I, Lom J, 1980. Tissue reactions to microsporidian infections in fish. J. Fish Dis., 3: 265-283.

Ebert D, Herre EA, 1996. The evolution of Parasitic Diseases, Parasitol. Today, 12: 96-101.

Edlind TD, Li J, Visvesvara GS, Vodkin MH, McLaughlin GL, Katiyar SK, 1996. Phylogenetic analysis of β-tubulin sequences from amitochondrial protozoa. Mol. Phyl. Evol., 5: 359-67.

Erickson BWJr, Blanquet RS,1969. The occurrence of chitin in the spore wall of *Glugea weissenbergi,* J. Invertebr. Pathol., 14: 358-364.

Fast NM, Keeling PJ, 2001. Alpha and beta subunits of pyruvate dehydrogenase E1 from the microsporidian *Nosema locustae*: mitochondrion-derived carbon metabolism in microsporidia. Mol. Biochem. Parasitol., 117: 201-9.

Fast NM, Logsdon Jr JM, Doolittle WF, 1999. Phylogenetic analysis of the TATA box binding protein (TBP) gene from *Nosema locustae*: evidence for a microsporidia-fungi relationship and sliceosomal intron loss. Mol. Biol. Evol., 16: 1415-1419.

Fokin SI, Di Giuseppe G, Erra F, Dini F, 2008. *Euplotespora binucleata* n. gen., n. sp (Protozoa: Microsporidia), a parasite infecting the hypotrichous ciliate *Euplotes woodruffi*, with observations on microsporidian infections in Ciliophora. J. Eukaryot. Microbiol. 55: 214-228

Forest JJH, King SD, Cone DK, 2009. Occurrence of *Glugea pimephales* in Planktonic Larvae of Fathead Minnow in Algonquin Park, Ontario, J. Aquat. Anim. Health, 21:164-166.

Forterre P, Philippe H, 1999. Where is the root or the universal tree of life? Bioessays, 21: 871-879.

Foucault C, Drancourt M, 2000. Actin mediates *Encephalitozoon intestinalis* entry into the human enterocyte-like cell line, Caco-2. Microb. Pathog., 28: 51-58.

Franzen C, 2004. Microsporidia: how can they invade other cells? Trends Parasitol., 20: 275-279.

Franzen C, 2008. Microsporidia : A Review of 150 Years of Research. Open Parasitol. J., 2: 1-34.

Franzen C, Müller A, 1999. Molecular Techniques for Detection, Species Differentiation, and Phylogenetic Analysis of Microsporidia. Clin. Microbiol. Rev.,12: 243–285.

Franzen C, Müller A, 2001. Microsporidiosis: human diseases and diagnosis. Microbes Infect., 3: 383-400.

Freeman MA, Yokoyama H, Ogawa K, 2004. A microsporidian parasite of the genus *Spraguea* in the nervous tissues of the Japanese anglerfish *Lophius litulon*. Folia Parasitol., 51: 167-176.

Frixione E, Ruiz L, Santillan M, de Vargas LV, Tejero JM, Undeen AH, 1992. Dynamics of polar filament discharge and sporoplasm expusion by microsporidian spores. Cell Motil. Cytoskel., 22: 38-50.

Frixione E, Ruiz L, Cerbon J, Undeen AH, 1997. Germination of *Nosema algerae* (Microspora) spores: conditional inhibition by D2O, ethanol and Hg2+ suggests dependence of water influx upon membrane hydration and specific transmembrane pathways. J. Eukaryot. Microbiol., 44: 109-116.

Germot A, Philippe H, Le Guyader H, 1997. Evidence for loss of mitochondria in Microsporidia from a mitochondrial- type HSP70 in *Nosema locustae*. Mol. Biochem. Parasitol., 87: 159-68.

Haley AJ, 1954. Microsporidian parasite, *Glugea hertwigi*, in American smelt from the Grat Bay region, New Hampshire. Trans. Am. Microsc. Soc., 83: 84-90.

Hirt RP, Healy B, Vossbrinck CR, Canning EU, Embley TM, 1997. A mitochondrial Hsp70 orthologue in *Vairimorpha necatrix*: molecular evidence that microsporidia once contained mitochondria. Curr. Biol., 7: 995-998.

Hirt RP, Logsdon JM Jr, Healy B, Dorey MW, Doolittle WF, Embley TM,1999. Microsporidia are related to Fungi: evidence from the largest subunit of RNA polymerase II and other proteins. Proc. Natl. Acad. Sci. USA, 96: 580-585.

Hollister WS, Canning EU, Wilcox A, 1991. Evidence for widespread occurrence of antibodies to *Encephalitozoon cuniculi* (Microspora) in man provided by ELISA and other serological tests. Parasitology, 102: 33-43.

Huger A, 1960. Electron microscope study on the cytology of a microsporidian spore by means of ultrathin sectioning. J. Insect Pathol.,. 2: 84-105.

Issi IV, 1986. Microsporidia as a phylum of parasitic protozoa. In: Beyer TV, Issi IV, eds., Protozoology, 10: 6-136.

Issi IV, Tokarev YS, Seliverstova EV, Voronin VN, 2012. Taxonomy of *Neoperezia chironomi* and *Neoperezia semenovaiae* comb. nov. (Microsporidia,

Aquasporidia): Lessons from ultrastructure and ribosomal DNA sequence data. Eur. J. Protistol., 48: 17-29.

Kamaishi T, Hashimoto T, Nakamura Y, Nakamura F, Murata S, Okada N, Okamoto K, Shimizu M, Hasegawa M, 1996. Protein phylogeny of translation elongation factor EF-1 alpha suggests microsporidians are extremely ancient eukaryotes. J. Mol. Evo., 42: 257-263.

Katinka MD, Duprat S, Cornillot E, Metenier G, Thomarat F, Prensier G, Barbe VA, Peyretaillade E, Brottier P, Wincker P, Delbac F, El Alaoui H, Peyret P, Saurin W, Gouy M, Weissenbach J, Vivares CP, 2001. Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. Nature, 414: 450-453.

Keeling PJ, Doolittle WF, 1996. Alpha-tubulin from early-diverging eukaryotic lineages and the evolution of the tubulin family. Mol. Biol. Evol., 13: 1297-1305.

Keeling PJ, Fast NM, 2002. MICROSPORIDIA : Biology and Evolution of Highly Reduced Intracellular Parasites. Microbiology, 56: 93-116.

Keeling PJ, McFadden GI, 1998. Origins of microsporidia. Trends Microbiol., 6: 19-23.

Keeling PJ, Luker MA, Palmer JD, 2000. Evidence from beta-tubulin phylogeny that microsporidia evolved from within the fungi. Mol. Biol. Evol., 17: 23-31.

Kellen WR, Chapman HC, Clark TB, Lindegren JE, 1965. Host–parasite relationships of some *Thelohania* from mosquitoes (Nosematidae: Microsporidia), J. Invertebr. Pathol., 7: 161-166.

Keohane EM, Weiss LM, 1999. The structure, function, and composition of the microsporidian polar tube. In: Wittner M, Weiss LM, (Eds.), The Microsporidia and Microsporidiosis. ASM Press, Washingtin, D.C., 196-224.

Kudo R, 1924. A biologic and taxonomic study of the Microsporidia. Ill Biol. Monogr., 9: 1-268.

Kudo RR, Daniels EW, 1963. An electron microscope study of the spore of a microsporidian, *Thelohania californica*. J. Protozool., 10: 112-120.

Lanternier F, Boutboul D, Menotti J, Chandesris MO, Sarfati C, Mamzer Bruneel MF, Calmus Y, Mechaï F, Viard JP, Lecuit M, Bougnoux ME, Lortholary O, 2009. Microsporidiosis in solid organ transplant recipients: two *Enterocytozoon bieneusi* cases and review. Transpl. Infect. Dis., 11: 83–88.

Larsson JIR, 1986. Ultrastructure, function and classification of microsporidia. In: Corliss JO, Patterson DJ, (Eds.), Prog. Protistol., Biopress, Bristol, UK, 1: 325-390.

Larsson JIR, 2005. Molecular versus morphological approach to microsporidian classification. Folia Parasitol., 52: 143-44.

Leiro J, Paramá A, Ortega M, Santamarina MT, Sanmartin ML, 1999. Redescription of *Glugea caulleryi*, a microsporidian parasite of the greater sand-eel, *Hyperoplus lanceolatus* (Le Sauvage), (Teleostei: Ammodytidae), as *Microgemma caulleryi* comb. nov. J. Fish Dis., 22: 101-110.

Lobo ML, Teles A, Da Cunha MB, Henriques J, Lourenco AM, Antunes F, Matos O, 2003. Microsporidia detection in stools from pets and animals from the zoo in Portugal: a preliminary study. J. Eukaryot. Microbiol., 50:581-582.

Lobo ML, Xiao L, Cama V, Stevens T, Antunes F, Matos O, 2006. Genotypes of *Enterocytozoon bieneusi* in mammals in Portugal. J. Eukaryot. Microbiol., 53: 61-64.

Lom J, 1972. On the structure of the extruded microsporidian polar filament. Z. Parasitenkd., 38: 200-213.

Lom J, 2002. A catalogue of described genera and species of microsporidians parasitic in fish. Syst. Parasitol., 53: 81-99.

Lom J, Dyková I, 1992. Protozoan parasites of fish. In: Developments in Aquaculture and Fisheries Science, Elsevier Science Publishers, Amsterdam, 26: 125-157.

Lom J, Dyková I, 2005. Microsporidian xenomas in fish seen in wider prospective. Folia Parasitol., 52: 69-81.

Lom J, Dyková I, Wang CH, Lo CF, Kou GH, 2000. Ultrastructural justification of the transfer of *Pleistophora angillarum* Hoshina, 1959 to the genus *Heterosporis* Schubert, 1969. Dis. Aquat. Org., 43: 225-231.

Lom J, Nilsen F, 2003. Fish microsporidia: fine structural diversity and phylogeny. Intern. J. Parasitol., 33: 107-127.

Lom J, Vávra J, 1963. The mode of sporoplasm extrusion in microsporidian spores. Acta Protozool., 1: 81-92.

Maddox JV, Baker MD, Jeffords MR, Kuras M, Linde A, Solter LF, McManus ML, Vávra J, Vossbrinck CR, 1999. *Nosema portugal*, N. SP., Isolated from Gypsy Moths (*Lymantria dispar*L.) Collected in Portugal. J. Invertebr. Pathol., 73: 1-14.

Magaud A, Achbarou A, Desportes-Livage I, 1997. Cell invasion by the microsporidium *Encephalitozoon intestinalis*. J. Eukaryot. Microbiol., 44: 81.

Mansour L, Hassine OKB, Vivares CP, Cornillot E, 2013. *Spraguea lophii* (Microsporidia) parasite of the teleost fish, *Lophius piscatorius* from Tunisian coasts: Evidence for an extensive chromosome length polymorphism. Parasitol. Internat., 62: 66-74.

Mathieu-Daude F, Faye A, Coste F, Manier J-F, Marques A, Bouix G, 1992. Occurrence of a microsporidiosis in marine cultured gilt-head sea bream from the Languedoc coast: a problem of specificity in the genus *Glugea.* Bull. Eur. Ass. Fish Pathol., 12: 67-70.

Matos O, Lobo ML, Goncalves L, Antunes F, 2002. Diagnostic use of 3 techniques for identification of microsporidian spores among AIDS patients in Portugal. Scand. J. Infect. Dis., 34: 591-593.

Morris DJ, Terry RS, Ferguson KD, Smith J, Adams A, 2005. Ultrastructural and molecular characterization of *Bacillidium vesiculoformis* n. sp. (Microspora:

Mrazekiidae) in the freshwater oligochaete *Nais simplex* (Oligochaeta: Naididae). Parasitology, 30: 31-45.

Muller M, 1998. Enzymes and compartmentation of core energy metabolism of anaerobic protists - a special case in eukaryotic evolution? In: Coombs GH, Vickerman K, Sleigh MA, Warren A, (Eds.), Evolutionary relationships among protozoa. London: Chapman and Hall, 1998: 109-132.

Nagel ML, Summerfelt RC, 1977. Nitrofurazone for control of the microsporidian parasite *Pleistophora ovariae* in golden shiners. Progr. Fish Culturist, 39: 18-23.

Nilsen F, 2000. Small subunit ribosomal DNA phylogeny of microsporidia with particular reference to genera that infect fish. J. Parasitol., 86: 128-133.

Nunes TP, 2012. ATLAS de Otólitos de Peixes do Rio Minho. Dissertação de Mestrado, Faculdade de Ciências da Universidade do Porto. Porto, 131p.

Olson RE, 1976. Laboratory and field studies on *Glugea stephani* (Hagenmüller), a microsporidian parasite of pleuronectid flatfishes. J. Protozool., 23: 158-164.

Oshima K, 1937. On the function of the polar filament of *Nosema bombycis*. Parasitology, 29: 220-224.

Peyretaillade E, Broussolle V, Peyret P, Metenier G, Gouy M, Vivares CP, 1998. Microsporidia, amitochondrial protists, possess a 70-kDa heat shock protein gene of mitochondrial evolutionary origin. Mol. Biol. Evol., 15: 683-689.

Quignard JP, Pras A, 1986. Atherinidae. In: Whitehead PJP, Bauchot ML, Hureau JC, Nielsen J, Tortonese E, (eds), Fishes of the North-Eastern Atlantic and the Mediterranean, UNESCO, Paris 1207-1210.

Roberts LS, Janovy J, 2009. Gerald D. Schmidt and Larry S. Roberts' Foundations of Parasitology. 8th ed Boston. McGraw-Hill, 701p.

Rodríguez-Tovar LE, Wright GM, Wadowska DW, Speare DJ, Markham JF, 2002. Ultrastructural study of the early development and localization of *Loma salmonae* in the gills of experimentally infected rainbow trout. J. Parasitol., 88: 244-253.

Sak B, Brady D, Pelikanova M, Květoňová D, Rost M, Kostka M, Tolarová V, Hůzová Z, Kváč M, 2011. Unapparent microsporidial infection among immunocompetent humans in the Czech Republic. J. Clin. Microbiol., 49: 1064-1070.

Sanchez JG, Speare DJ, Markham RJF, Jones SRM, 2001. Experimental vaccination of rainbow trout against *Loma salmonae* using a live low-virulence variant of *L. salmonae*. J. Fish Biol., 59: 442-448.

Sandholzer LA, Nostrand T, Young L, 1945. Studies on an ichthyosporidian-like parasite of ocean pout (*Zoareces anguillaris*). US Fish Wild Serv. Spec. Sci. Rep., 31: 1-12.

Schmahl G, Mehlhorn H, 1989. Treatment of fish parasites. 6. Effects of sym-triazinone (toltrazuril) on developmental stages of *Glugea anomala* Moniez, 1887 (Microsporidia): a light and electron microscopic study. Eur. J. Protistol., 24: 252-259.

Schmahl G, El Toukhy A, Ghaffar FA, 1990. Transmission electron microscopic studies on the effects of toltrazuril on *Glugea anomala* Moniez, 1887 (Microsporidia) infecting the three-spined stickleback *Gasterosteus aculeatus*. Parasitol. Res., 76: 700-706.

Schwartz DA, Sobottka I, Leitch GJ, Cali A, Visvesvara GS, 1996. Pathology of microsporidiosis: emerging parasitic infections in patients with acquired immunodeficiency syndrome. Arch. Pathol. Lab. Med., 120: 173-188.

Shaw RW, Kent ML, 1999. Fish Microsporidia. In: Wittner M, Weiss LM, (Eds.), The Microsporidia and Microsporidiosis. ASM Press, Washingtin, D.C., 418-446.

Shaw RW, Kent ML, Adamson ML, 1998. Modes of transmission of *Loma salmonae* (Microsporidia), Dis. Aquat. Org., 33: 151-156.

Sinden RE, Canning EU, 1974. The ultrastructure of the spore of *Nosema algerae* (Protozoa, Microsporida) in relation to the hatching mechanism of microsporidian spores. J. Gen. Microbiol., 85: 350-357.

Singh M, Kane GJ, Mackinlay L, Quaki I, Yap EH, Ho BC, Ho LC, Lim KC, 1982. Detection of antibodies to *Nosema cuniculi* (Protozoa: Microscoporidia) in human and animal sera by the indirect fluorescent antibody technique. Southeast Asian J. Trop. Med. Public Health, 13: 110-113.

Smith JE, Dunn AM, 1991. Transovarial transmission. Parasitol. Today, 7: 146-148.

Sprague V, Becnel JJ, 1998. Note on the name-author-date combination for the taxon Microsporidies Balbiani, 1882, when ranked as a phylum. J. Invertebr. Pathol., 71: 91-94.

Sprague V, Becnel JJ, Hazard EI, 1992. Taxonomy of the phylum Microspora. Crit. Rev. Microbiol., 18: 285-395.

Sprague V, Hussey KL, 1980. Observations on *Ichthyosporidium giganteum* (Microsporida) with particular reference to the host-parasite relations during merogony. J. Protozool., 2: 169-175.

Sprague V, Vernick SH, 1968. Light and electron microscope study of a new species of *Glugea* (Microsporidia, Nosematidae) in the 4-spined stickleback *Apeltes quadracus*. J. Protozool., 15: 547-571.

Stentiford GD, Bateman KS, Feist SW, Chambers E, Stone DM, 2013a. Plastic parasites: extreme dimorphism creates a taxonomic conundrum in the phylum Microsporidia. Int. J. Parasitol., 43: 339-352.

Stentiford GD, Feist SW, Stone DM, Bateman KS, Dunn AM, 2013b. Microsporidia: diverse, dynamic, and emergent pathogens in aquatic systems. Trends Parasitol., 29: 567-578.

Sweeney AW, Hazard EI, Graham MF, 1985. Intermediate host for an *Amblyospora sp.* (Microspora) infecting the mosquito *Culex annulirostris.* J. Invertebr. Pathol., 46: 98-102.

Takahashi S, Egusa S, 1977a. Studies on *Glugea* infection of the ayu, *Plecoglossus altivelis* III. Effect of water temperature on the development of xenoma of *Glugea plecoglossi*. Jap. J. Fish., 11: 195-200.

Takahashi S, Egusa S, 1977b. Studies on *Glugea* infection of the ayu, *Plecoglossus altivelis* I. Description of the *Glugea* and proposal of a new species, *Glugea plecoglossi*. Jap. J. Fish., 11: 175-182.

Talabani H, Sarfati C, Pillebout E, van Gool T, Derouin F, Menotti J, 2010. Disseminated infection with a new genovar of *Encephalitozoon cuniculi* in a renal transplant recipient. J. Clin. Microbiol., 48: 2651-2653.

Tanabe Z, Watanabe MM, Sugiyama J, 2002. Are Microsporidia really related to Fungi? A reappraisal based on additional gene sequences from basal fungi. Mycolog. Res., 106: 1380-1391.

Terry RS, Smith JE, Sharpe RG, Rigaud T, Littlewood DTJ, Ironside JE, Rollinson D, Bouchon D, MacNeil C, Dick JTA, Dunn AM, 2004. Widespread vertical transmission and associated host sex-ratio distortion within the eukaryotic phylum Microspora. Proc. R. Soc. Lond., 271: 1783-1789.

Troemel ER, Felix MA, Whiteman NK, Barriere A. Asubel FM, 2008. Microsporidia are natural intracellular parasites of the nematode *Caenorhabditis elegans*. PLoS Biology, 6: 2736-52.

Tuzet O, Maurand J, Fize JA, Michel R, Fenwick B, 1971. Proposition d'un nouveau cadre systematique pour les genres de Microsporidies. C. R. Acad. Sci. (Paris), 272: 1268-1271.

Van de Peer Y, Ben Ali A, Meyer A, 2000. Microsporidia: accumulating molecular evidence that a group of amitochondriate and suspectedly primitive eukryotes are just curious fungi. Gene, 246: 1-8.

Vávra J, 1976. Structure of microsporidia. In: Bulla LA, Cheng TC, (Eds.), Comparative Pathobiology, Plenum Press, New York, 1: 1-86.

Vávra J, Joyon L, De Puytorac P, 1966. Observation sur l'ultrastructure de filament polaire des microsporidies. Protistologica, 2: 109-112.

Vávra J, Larsson JIR, 1999. Structure of the microsporidia. In: Wittner M, Weiss LM, (Eds.), The Microsporidia and Microsporidiosis. ASM Press, Washingtin, D.C., 7-84.

Vávra J, Lukeš J, 2013. Microsporidia and 'the art of living together'. Adv Parasitol., 82: 253-319.

Visvesvara GS, 2002. In vitro cultivation of microsporidia of clinical importance. Clin. Microbiol. Rev., 15: 401-413.

Vossbrinck CR, Debrunner-Vossbrinck BA, 2005. Molecular phylogeny of the microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitol., 52: 131-142.

Vossbrinck CR, Woese CR, 1986. Eukaryotic ribosomes that lack a 5.8S RNA. Nature, 320: 287-288.

Waller RF, Jabbour C, Chan NC, Celik N, Likié VA, Mulhern TD, Lithgow T, 2009. Evidence of a Reduced and Modified Mitochondrial Protein Import Apparatus in Microsporidian Mitosomes. Eukaryot. Cell, 8: 19-26.

Weidner E, 1976. The microsporidian spore invasion tube. The ultrastructure, isolation, and characterization of the protein comprising the tube. J. Cell Biol., 71: 23-34.

Weiser J, Kalavati C, Sandeep BV, 1981. *Glugea nemipteri* sp. n. and *Nosema bengalis* sp. n., two new microsporidia of *Nemipterus japonicus* in India. Acta Protozool., 20: 201-208.

Weissenberg R, 1968. Intracellular development of the microsporidian *Glugea anomala* Moniez in hypertrophying migratory cells of the fish *Gasterosteus aculeatus* L., an example of the formation of “xenoma tumors”. J. Protozool., 15: 44-57.

Wittner M, Weiss LM, 1999. The Microsporidia and Microsporidosis. ASM Press, Washington, D.C.

Wright JH, Craighead EM, 1922. Infectious motor paralysis in young rabbits. J. Exp. Med., 36: 135-149.

Appendages

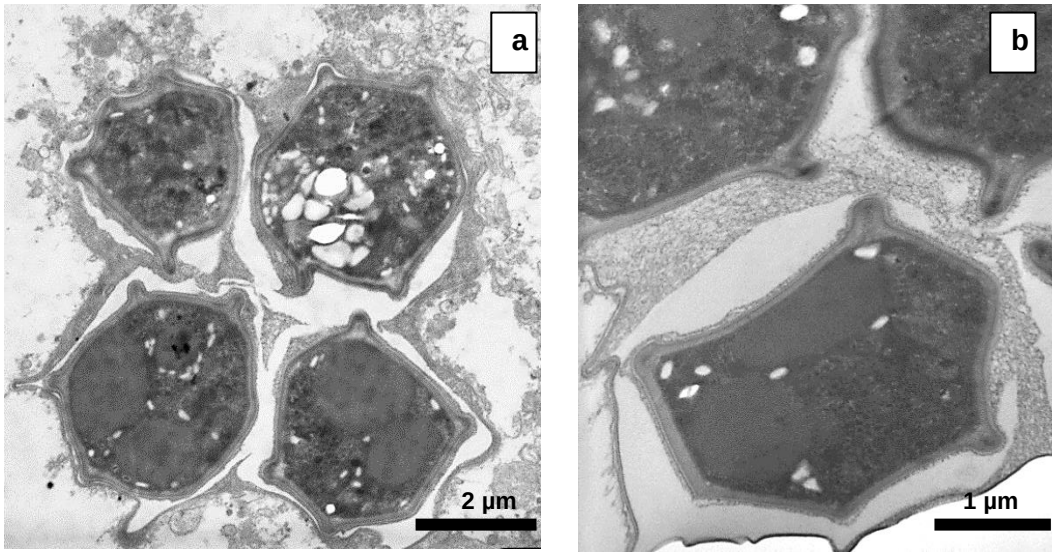

Figure 1a and 1b - Transmission electron micrographs of an Apicomplexan parasite belonging to the genus *Goussia* infecting the big-scale sand smelt *Atherina boyeri* in the Minho River.