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Phylum Haplosporidia Caullery & Mesnil, 1899: ultrastructural morphology of spores of microparasites of aquatic fauna

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Abstract

The Kingdom "Protista" or "Protoctista" encompasses a diverse group of microparasites, which includes the phylum Haplosporidia Caullery & Mesnil, 1899, where around 50 species, most of them reported as pathogenic, are described. This phylum divided into four genera: *Minchinia*, *Haplosporidium, Urosporidium* and *Bonamia*. Most species of haplosporidia infect bivalves and gastropods, and may infect other taxonomic groups. The taxonomic nomenclature was very controversial at the time the name of the phylum was created, with a nomenclatural variation, with the unanimously accepted designation of Haplosporidia prevailing, defended by Caullery & Mesnil in 1899. The first haplosporidian species described were the genera *Minchinia* Labbé, 1896 and *Haplosporidium* Caullery & Mesnil, 1899. However, the taxonomic characteristics described for each of the genera were not based on the same morphological aspects of the same organelles. Most haplosporidian species in this taxonomic group were initially described based on comparative morphology by LM observations; subsequently, ultrastructural morphology and, more recently, molecular technology of SSU rDNA sequences have allowed us to clarify the taxonomic position of some species. In few species the life cycle has been reported, although there are isolated states without continuity of development and cellular differentiation.

Keywords: Haplosporidian species, ultrastructural morphology, spores, microparasites, aquatic fauna.

Resumo - Filo Haplosporidia Caullery & MesniL, 1899: morfologia ultrastructural de esporos de microparasitas da fauna aquática

O Reino "Protista" ou "Protoctista" engloba um grupo diversificado de microparasitas, que inclui o filo Haplosporidia Caullery & Mesnil, 1899, onde estão descritas cerca de 50 espécies, a maioria das quais reportadas como patogénicas. Este filo divide-se em quatro géneros: *Minchinia, Haplosporidium, Urosporidium e Bonamia.* A maioria das espécies de haplosporídeos infeta bivalves e gastrópodes, podendo também infetar outros grupos taxonómicos. A nomenclatura taxonómica era à data da criação do nome do filo, muito controversa, existindo variadas nomenclaturas, prevalecendo a designação unanimamente aceite Haplosporidia, defendida por Caullery & Mesnil em 1899. As primeiras espécies de haplosporidios descritas foram do género *Minchinia* Labbé, 1896 e o género *Haplosporidium* Caullery & Mesnil, 1899. No entanto, as características taxonómicas descritas para cada um dos géneros não se basearam nos mesmos aspetos morfológicos dos mesmos organelos. A maioria das espécies deste grupo taxonómico foi descrita com base na morfologia comparativa das observações em LM e na morfologia ultrastrutural e, recentemente, com base na análise molecular das sequências da SSU rDNA. Em algumas espécies o ciclo de vida foi reportado, embora existam estados isolados sem continuidade de desenvolvimento e diferenciação celular.

Palavras-chave: espécies de haplosporídios, morfologia ultrastrutural, esporos, microparasitas, fauna aquática.

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Resumen - Phylum Haplosporidia Caullery & Mesnil, 1899: morfología ultraestructural de las esporas de microparásitos de la fauna acuática

El Reino "Protista" o "Protoctista" engloba un grupo diverso de microparásitos, que incluye el filo Haplosporidia Caullery & Mesnil, 1899, donde se describen alrededor 50 especies, la mayoría de ellas reportadas como patógenas. Este filo se divide en cuatro géneros: *Minchinia, Haplosporidium, Urosporidium* y *Bonamia.* La mayoría de las especies de haplosporidios infectan bivalvos y gasterópodos y también pueden infectar otros grupos taxonómicos. La nomenclatura taxonómica fue muy controvertida en la época en que se creó el nombre del filo, existiendo una variación en la nomenclatura, prevaleciendo la designación unánimemente aceptada de Haplosporidia, defendida por Caullery & Mesnil en 1899. Las primeras especies de haplosporidios descritas fueron el género *Minchinia* Labbé, 1896 y el género *Haplosporidium* Caullery & Mesnil, 1899. Sin embargo, las características taxonómicas descritas para cada uno de los géneros no se basaron en los mismos aspectos morfológicos de los mismos organelos. La mayoría de las especies de este grupo taxonómico se han descrito basándose en la morfología comparativa de observaciones de LM y la morfología ultraestructural y, recientemente, basándose en el análisis molecular de secuencias de DNAr de SSU. En algunas especies el ciclo de vida ha sido reportado, aunque existen estados aislados sin continuidad del desarrollo y diferenciación celular.

Palabras clave: especies de haplosporidios, morfología ultraestructural, esporas, microparásitos, fauna acuática.

Introduction

Phylum Haplosporidia Caullery & Mesnil, 1899 is a small group consisting of about 50 species apportioned by 4 genera (*Haplosporidium, Minchinia, Urosporidium* and *Bonamia*) (Ball & Neville, 1979; Azevedo & Hine, 2016; Urrutia et al., 2019; Cho, 2020; Hine et al., 2020) which infect a varied number of host species, more often, bivalves, gastropods and crustaceans different degrees of pathogenicity and prevalence, most of the infections occurring mortality of host species (Perkins, 1969; Azevedo, 1984; Utary et al., 2012; Stentiford et al., 2013; Catanese et al., 2018; Panarese et al., 2019). The specific characters that identify the spores of the different species of the reported phylum correspond to spore morphology and ornaments of their wall and the presence, among others, of specific cytoplasmic structures called "haplosporosomes" and spherulosomes (Ball, 1980; Azevedo, 1984; Burreson & Reece 2006; Bearham et al., 2008a, b; Ford et al., 2009; Azevedo & Hine, 2016; Hine et al., 2020).

Most of the species included in this phylum were described based on the morphology observed in light microscopy (LM) (Caullery & Mesnil, 1899; Debaisieux, 1920; Vilela, 1951; van Banning, 1977; Ormières et al., 1973; Ormières, 1980) based on old rudimentary techniques that did not reveal the morphological characteristics, which current methodologies reveal with the use of observation in transmission electron microscopy (TEM) (Perkins, 1969, 1975; Marchand & Sprague, 1979; Ball, 1980; Ormières, 1980; Azevedo et al., 2003, 2006; Azevedo & Hine, 2016), scanning electron microscopy (SEM) (Azevedo, 1984; Azevedo et al., 1999, 2003, 2006, and others (De Turk, 1940; Perkins, 1975; Ball, 1980; Anderson et al., 1993; Carballal et al., 2005; Cho, 2020; Davies et al., 2020). This situation has allowed the observation of morphological characters, hitherto never reported, advising nominal transfers of some genera (Labbé, 1896; Vilela, 1951; Sprague, 1963; Azevedo, 2001), as reported in this manuscript.

Historically, the phylum Haplosporidia for many years comprised three genera *Minchinia* (Lankester, 1895) Labbé, 1896; *Haplosporidium* (Caullery & Mesnil, 1899) Lüke, 1900; and *Urosporidium* Caullery & Mesnil, 1905). Recent molecular phylogenetic analyses support the inclusion of the genus *Bonamia* Pichot et al., 1979 within the phylum Haplosporidia (Carnegie et al., 2006). The genera were characterized based on the morphological peculiarities of the spore and the presence and origin of spore wall ornamentation as the main morphological characters.

Some of the described haplosporidian species based on microscopic specific characters of the spores were confirmed by molecular analysis of SSU rDNA gene sequences (Azevedo et al., 2006; Burreson & Reece, 2006; Vea & Siddall, 2011; Winters & Faisal, 2014; Urrutia et al., 2019; Cho et al., 2020).

The theme of this manuscript aims to review the ultrastructural morphology of spores from different haplosporidian species.

Material and Methods

The haplosporidian species belonging to phylum Haplosporidia described here were collected in various aquatic environments in different host species and processed for studies in LM, TEM and SEM, using frequently a double fixation of the microparasites with 2-3% glutaraldehyde and 2% osmium tetroxide, both buffered with 0.2 M sodium cacodylate, according to the standardized technologies followed in the Laboratory of Cell Biology of ICBAS/UP, as reported in detail in the manuscripts that supported the present results (Azevedo 1984; Azevedo & Corral, 1985; Azevedo et al., 2006). Most haplosporidian species were described based on light and ultrastructural morphology of the spores. Phylogeny based on the analysis of SSU rDNA gene sequences has been little explored in this group of microparasites, having been done in only a few species.

Results

Taxonomy of the Phylum Haplosporidia Caullery & Mesnel, 1899

The taxonomic classification of this phylum has been reported by some authors, with different nomenclatures, currently identified as synonyms of the phylum Haplosporidia. Among them, we cite Aplosporidia Caullery & Mesnel, 1899; Ascetospora Sprague, 1979, (La Haye et al., 1984) and Balanosporidia Sprague, 1979), which are currently without taxonomic acceptance. The following taxonomic classification is currently widely accepted:

Kingdom Protista Haeckel, 1866

Phylum Haplosporidia Caullery & Mesnil, 1899

Class Haplospora Caullery, 1953

Order Haplosporida Caullery & Mesnil, 1899

Family Haplosporiidae Caullery & Mesnil, 2005

Genus *Minchinia* Labbé, 1896

Genus *Haplosporidium* Caullery & Mesnil, 1899

Genus *Bonamia* Pichot et al., 1979

Family Urosporiidae Sprague, 1979

Genus *Urosporidium* Caullery & Mesnil, 1905

Phylum Haplosporidia Caullery & Mesnil, 1899

The phylum Haplosporidia is characterized by having spores consisting of a uninucleate cell whose morphology is oval to ellipsoidal, consisting of a wall where covers an operculum is differentiated in the apical portion that have small orifice called a micropyle covers by an operculum, or having an open micropyle without an operculum. Having various types of external ornaments, named tails, filaments, slender projections, folds adhered to its wall with varied shapes and varied lengths or presenting the spore wall phases that are approximately 3-6 µm long and 2-3 µm wide (Figure 1).
The spore wall surrounds an endosporoplasm where a nucleus is located in its cytoplasm, generally in a basal position; - a spherulosome constituted complex membranous structure located in apical position next to the micropyle; and several dense vesicles called haplosporosomes and mitochondria and other cytoplasmic organelles (Figure 1a).

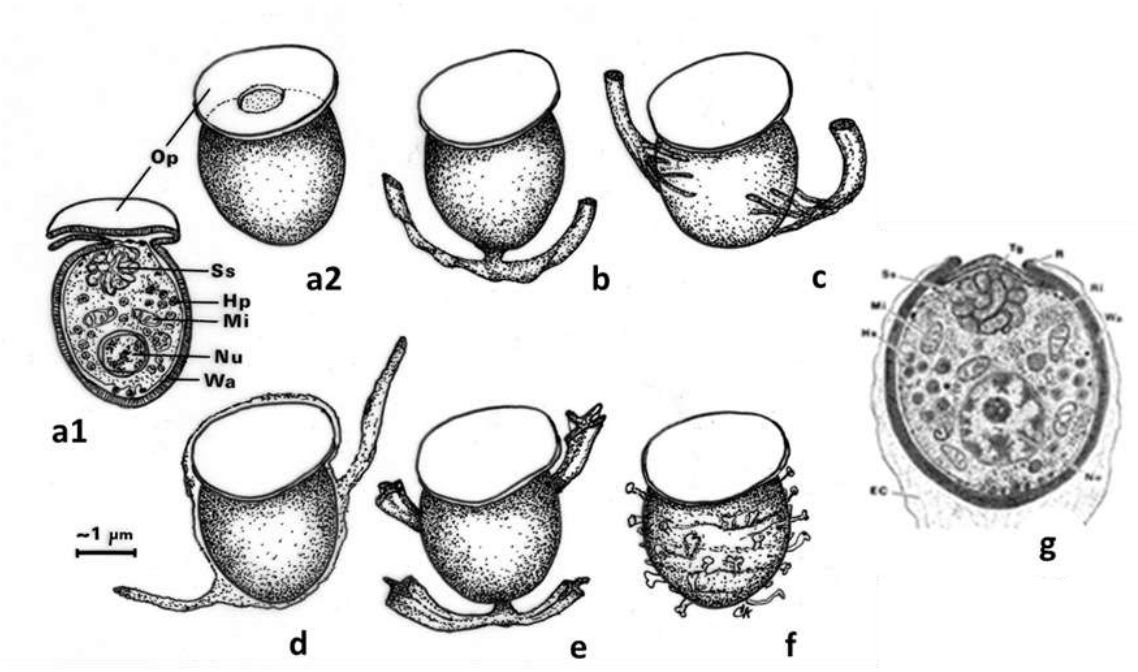

Figure 1. Schematic drawings the morphology of spores of different haplosporidian species: **a1), a2)** *Minchinia tapetis* (Op - Operculum; Ss - Spherulosome; Hp - Haplosporosome; Mi- Mitochondria; Nu - Nucleus; Wa- Spore wall); **b)** *Haplosporidium lusitanicum*; **c)** *H. armoricanum*; **d)** *H. edule*; **e)** *H. montforti*; **g)** *Urosporidium* sp.

Thirty-four species of the genus *Haplosporidium* are described, found mostly, in molluscs (bivalves and gastropods) of different species collected in various geographic areas (Perkins, 1969; Pichot et al., 1979; Ball, 1980; Azevedo, 1984; Burreson 2001; Hine et al., 2009; Catanese et al., 2018; Urrutia et al., 2019). Among the different species described in this genus, we report the ultrastructural morphology of the spores and the complex life cycle of the species *Haplosporidium lusitanicum* Azevedo, 1984, which was found in cysts located near the testis of the gastropod mollusk *Helcion pellucidus* living on the brown algae of the genus *Laminaria* (Phaeophyta), collected on the Atlantic coast of northern Portugal (Azevedo, 1984).

Ultrastructural Morphology of Haplosporidian Spores

The species of the genera *Haplosporidium*, *Minchinia, Urosporidium* and *Bonamia* reported here are characterized based of different specificities on the morphology of their spores, mainly observed in TEM and SEM (Marchand & Sprague 1979; Azevedo, 1984; La Haye et al., 1984) with, however, some descriptions obtained in molecular analyses of the SSU rDNA nucleotide sequences (Azevedo et al., 2006; Burreson & Reece, 2006; Vea & Siddall, 2011; Winters & Faisal, 2014; Urrutia et al., 2019).

For a long period of time, the morphological characters of the spores that distinguished the genus *Haplosporidium* from the genus *Minchinia* were controversial. After a detailed taxonomic analysis, some species of these genera were transferred to the other genus (Debaisieux, 1920; Sprague, 1963; van Banning, 1977; Azevedo et al., 1999).

Genus *Haplosporidium* has spores with a thick wall that presents morphological particularities that identify the species, that is: presence of operculum and spore wall with ornaments of various externally types attached to the periphery of the spore (Figures 1-6).

This genus, which encompasses around 34 species, reports in this document ultrastructural details of the following species:

a) *Haplosporidium lusitanicum* Azevedo, 1984

This species was described in the gastropod mollusk *Helion pelucidus* from the Portuguese Atlantic coast. This microparasite was found, incidentally, in gastropod species at a time when we were studying "spermatology of aquatic fauna". Spores contained in cysts of the parasite were found among spermatozoa, with which they were confused due to the similarity of the spores, which had two evident episporal tails (Figure 2), like, to the tails of spermatozoa, when observed in LM. The ultrastructural morphology is shown in images obtained in TEM and SEM, as well as in schematic drawings obtained in ultrathin serial sections and observed in TEM (Figure 2).

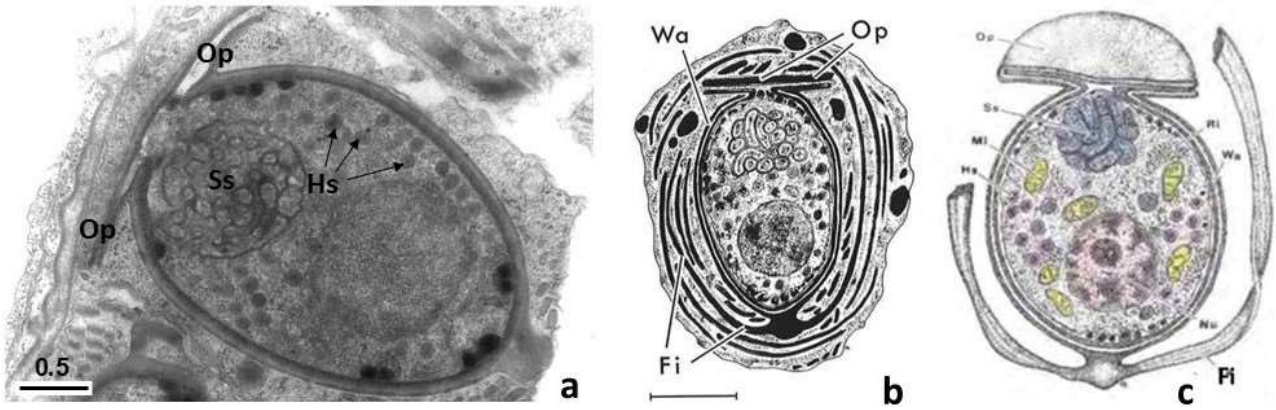

Figure 2. Spores of *Haplosporidium lusitanicum*: **a)** Longitudinal section of ultrastructural image; **b)** Schematic drawings of immature spore and **c)** mature spore. (S - Spore; Wa - Spore wall; Op – operculum; Fi - Filaments or tails; Ss - Spherulosome; Mi - Mitochondria; Hs - Haplosporosome; Ri - Ribosomes; Nu - Nucleus) Scale bars in µm.

b) *Haplosporidium armoricanum* Azevedo et al., 1999

Spores of a microparasite found in the oyster, *Ostrea edulis*, which had previously been observed and described as belonging in the genus *Minchinia, as M. armoricana* (van Banning, 1977), were subsequently reexamined in LM and TEM observations showing the presence of ornaments on the spore wall, as well as the generic morphological characteristics corresponding to the *Haplosporidium* genus. Based on these observations, the genus *Minchinia* was replaced by the genus name *Haplosporidium*, such as *H. armoricanum* (Azevedo et al., 1999) (Figure 3).

The spores contained two long epispore cytoplasm extensions each possessing cytoskeletal structures corresponding to the filaments or tails. However, the 2 long filaments (about 130 µm) persisted that were closely attached, in opposition to the spore wall by a bundle of 9-13 fibres. The ultrastructural morphology of this species is detailed in the images obtained in TEM and SEM as well as, in schematic drawings of the spores (Figure 3).

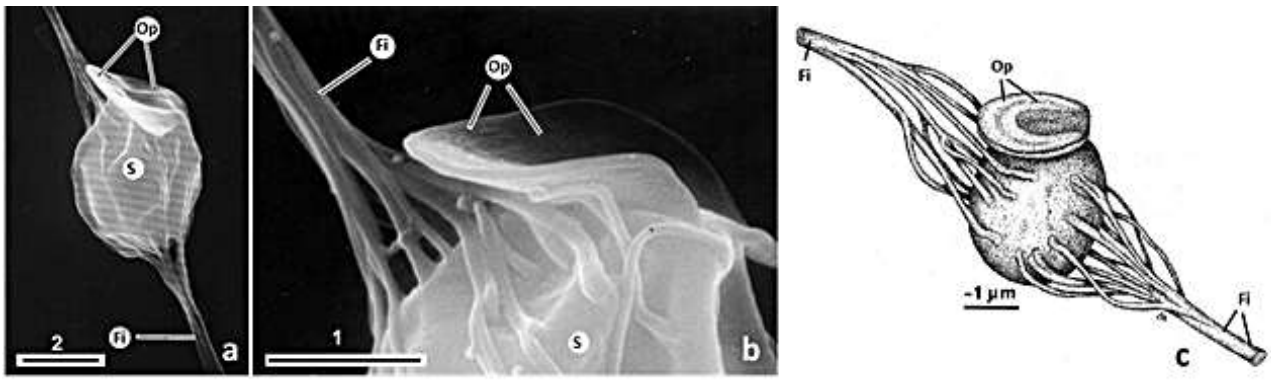

Figure 3. *H. armoricanum* spore (S): **a, b)** Two aspects of the spore (S) observed in SEM with evidence of the operculum (Op) and filaments (Fi); **c)** Schematic drawing of spore morphology. Scale bars in µm.

c) *Haplosporidium edule* Azevedo et al., 2003

The species *H. edule* was described from digestive gland tissue of the cockle *Cerastoderma edule* collected in Galicia (northwest Spain). The fine structure of spores, mainly, their surrounding ornaments attached to the spore wall, have been described from LM and TEM (Figure 4). The plasmodial development and sporoblast formation stages of the life cycle were observed. The spores were ovoid to ellipsoidal (3.20 × 2.20 µm) and the apical zone of the wall modified into a complex opercular system covering the 0.5–0.8 µm diameter micropyle. The spore wall (~0.10 µm thick) was composed of three layers. The electron-dense material forming the outer layer was extended into numerous surrounding folds and thin projections attached to the spore wall. Each projection had a forked tip, with branches about ~0.25 µm long (Figure 4). The taxonomic affinities of this species were discussed in comparisons made, mainly, with the ornamentation of the spores of other species of the same genus, showing large morphological differences (Azevedo et al., 2003).

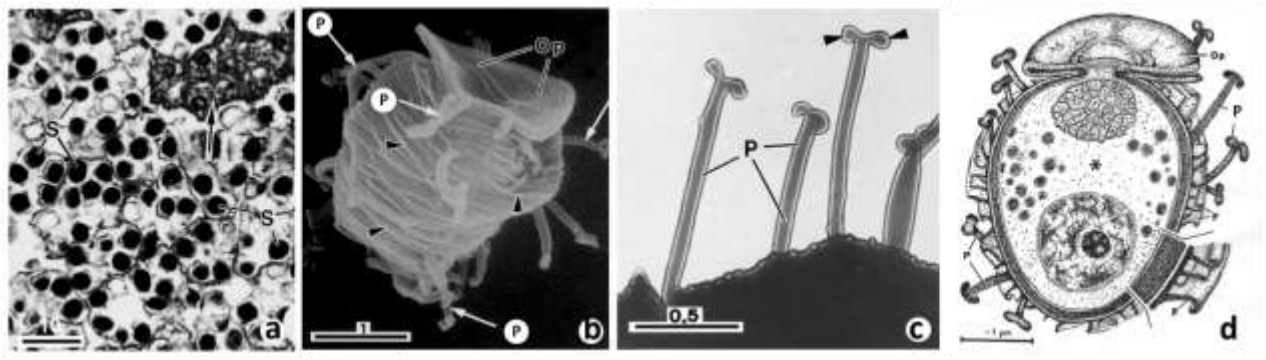

Figure 4. *H. edule*: a) Spores (S) and some life cycle stages (arrows) observed in LM; b) Spore observed in SEM showing several projections (P) and several folds (arrowheads) projected from the spore wall; c) Detail of slender bifurcated projections (P); d) Schematic drawing of a spore showing its ultrastructural morphology. Scale bars in µm.

d) *Haplosporidium montforti* Azevedo et al., 2006

Haplosporidium found infecting connective tissue, gills, digestive gland and foot of gastropod *Haliotis tuberculata* imported from Ireland to Galicia (NW Spain). TEM observation and molecular characterization of the ribosomal SSU rRNA gene sequence confirmed the ultrastructural morphology of the spores and their surrounding ornaments attached to the spore wall. Systemic infection with uninucleate and multinucleate plasmodia containing spherical nuclei were observed among multiple sporocysts containing different stages of spore maturation. Spores were spherical to slightly ellipsoidal (2.4 ± 0.5 / 2.3 ± 0.6 µm). The apical area of the spore wall had a complex opercular system covering a circular hole (micropyle) that measured about 0.5 µm in diameter. The spore wall was about 110 nm thick, with 4 filaments (20–28 µm long) consisting of the same material that formed the wall. Internally, the uninucleate endosporoplasm contained structures such as haplosporosomes, ribosomes and a spherulosome in the apical position (Figure 5).

Sequences of the SSU rDNA gene sequence, corroborating morphological data, suggested that it was an undescribed species. Based on in the ultrastructural characteristics of the spore and the sequence of the SSU rDNA gene, a new microparasite called *Haplosporidium montforti* was described (Azevedo et al., 2006) (Figure 5).

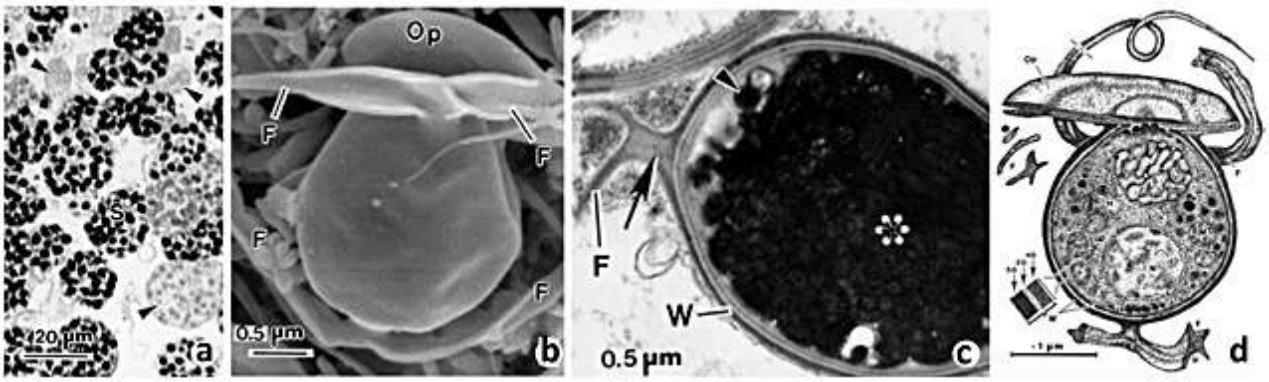

Figure 5. *H. montforti*: a) Group of spores (S) and developmental stages (arrowheads) observed in LM; b) Spore observed in SEM; c) Ultrastructural aspect showing the filaments (F) adherent to the spore wall (W); d) Schematic drawing of a spore showing its ultrastructural morphology. (F- Filament; Op – Operculum; W – spore wall; *- endosporoplasm). Scale bars in µm.

e) *Minchinia tapetis* (Vilela, 1951) Azevedo, 2001

This species was formerly described as belonging to the genus *Haplosporidium,* under the designation *Haplosporidium tapetis* Vilela, 1951. The morphology of the spore is similar to that of the spore of the genus *Haplosporidium*, having an operculum, but the spore wall did not have any type of ornamentation, morphological character absent in the genus *Minchinia* (Figure 6). This parasite was the first species of the phylum Haplosporidia described in Portugal (Vilela, 1951). It was found in the clam *Tapes decussatus*, in which "apparent projections" of the spore wall were observed. This observation led the researcher to describe in 1951, the parasite included in the genus *Haplosporidium,* as *H. tapetis* Vilela, 1951.

Subsequently, an ultrastructural morphological reanalysis of the spores showed the absence of any external ornaments, namely filaments or tails, contrary to what was reported by Vilela (1951) through observations in LM. Thus, this species was transferred from the genus *Haplosporidium* to the genus *Minchinia* (Vilela, 1951) Azevedo, 2001.

The failure in the microscopic observation, regarding the presence or absence of ornaments, was possibly due to rudimentary characteristics of the microscope in which the observation was made in 1951 by Professor H. Vilela.

Based on the similar morphological characters of the uninucleated endoplasm and absence of ornaments, two species formerly described as belonging to genus *Haplosporidium* was transferred to genus *Minchinia*, with the same specific epithed, as *Minchini*a *chitonis* (Lankester, 1885) Labbè, 1896 and *Minchinia tapetis* (Vilela, 1951) Azevedo, 2001 (Figure 6).

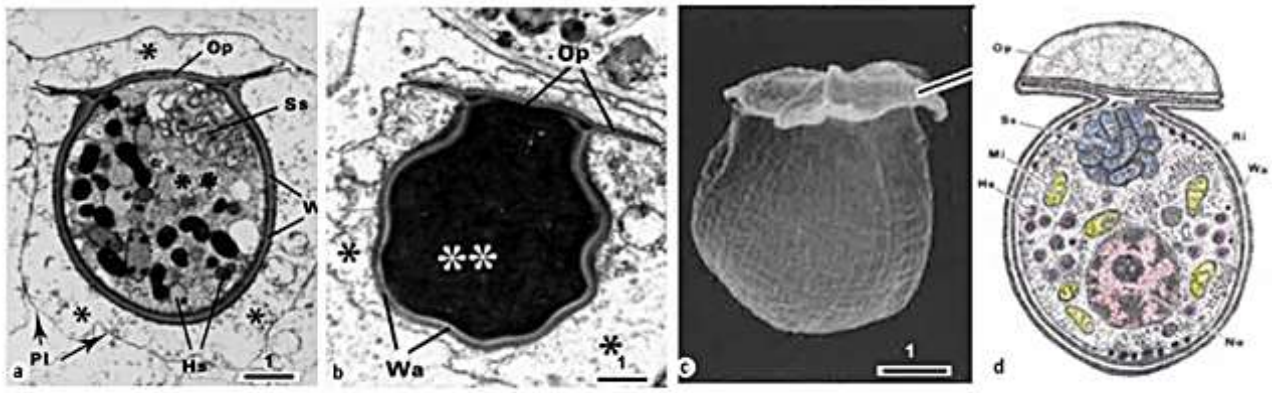

Figure 6. *Minchinia tapetis*: **a)** Ultrastructural aspect of an immature spore (S) observed in longitudinal section; **b)** Ultrastructure of a mature spore; **c)** Scanning image; **d)** Schematic drawing of a spore, showing its internal organization. (Op – Operculum; Wa– Wall; Hs – Haplosporosome; Ss – Spherulosome; * - exosporoplasm; ** - endosporoplasm). Scale bars in µm.

f) Genus *Urosporidium* Caullery & Mesnil, 1905

The genus *Urosporidium* is characterized by the peculiarity of the spore having an opening in its wall in the apical region of the spores, obturated with an internal disc of wall material that covers the spore orifice and not having an operculum. The external surface of the spore wall of different species may be smooth or have ornaments, that is, tails or episporal extensions associated with their wall. The internal organization of the uninucleated endosplasm contains the similar typical organelles as described in *Minchinia* and *Haplosporidium* endoplasm, as haplosporosomes and spherulosome (Figure 7).

Eight species of this genus have been described, discovered in various geographic regions, infecting several organs, most of them are hyperparasites located in various turbellarian tissues, generally located in the form of cysts with spores (De Turk, 1940; Zaika & Dolgikh, 1963; Perkins, 1971; Anderson et al., 1993; Carballal et al., 2005; Le et al., 2015) (Figure 7).

Some descriptions of these species were supported based on molecular analyses (Cho et al., 2020), while others were described based on microscopic observations by LM and TEM and host specificity (Anderson et al., 1993; Carballal et al., 2005; Le et al., 2015).

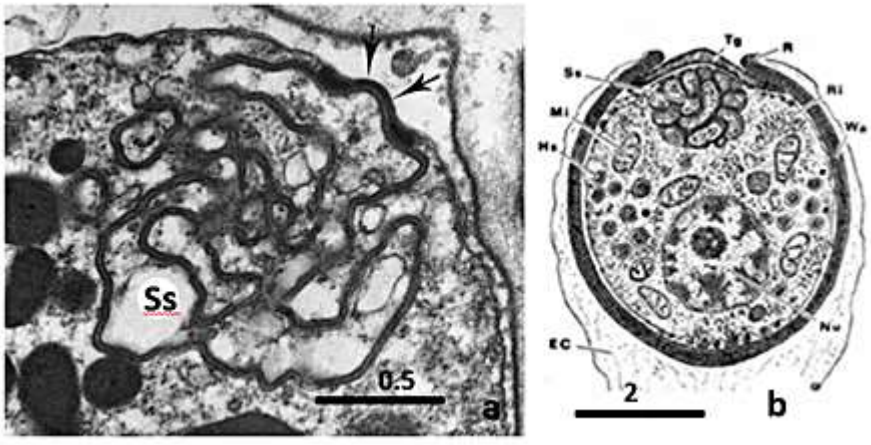

Figure 7. Genus *Urosporidium*: **a)** Ultrastructural morphology of the spherulosome (Ss) membrane system, located in the apical region of the spore; **b)** Schematic drawing of a mature spore, observed in longitudinal section, showing (Scale bars in µm):
Tg - língula;
R - Rim;
Ss - Spherulosome;
Mi - Mitochondria;
Hs - Haplosporosome;
Ri - Ribosomes.

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g) Genus *Bonamia* Pichot et al., 1979

The spore morphology of species of the genus *Bonamia* Pichot et al., 1979, is not similar to that of other genera of haplosporidia, but molecular analyzes demonstrate similarity with species of the phylum Haplosporidia (Carnegie et al., 2006; Audemard et al., 2014) (Figure 8). These microparasites of the genus *Bonamia* are intracellular protistan parasites frequently found of oysters having 4 species have been described *(B. ostreae, B. exitiosa, B. perspora* and *B. roughleyi*), although the status of *B. roughleyi* is controversial. Introduction especially of *B. ostreae* and *B. exitiosa* to host populations has been shown to cause mass mortalities with dramatic impact on oyster production. Histopathology, electron microscopy, and molecular phylogenetic analysis identified characters that permitted a description of the new parasite species, *Bonamia perspora,* which was the first species of the genus *Bonamia* shown to produce typical haplosporidian spore having an orifice and hinged operculum (Carnegie et al., 2006). *Bonamia ostreae* is a flat breath protist parasite that has caused significant losses throughout the world over the last decade (Figure 8).

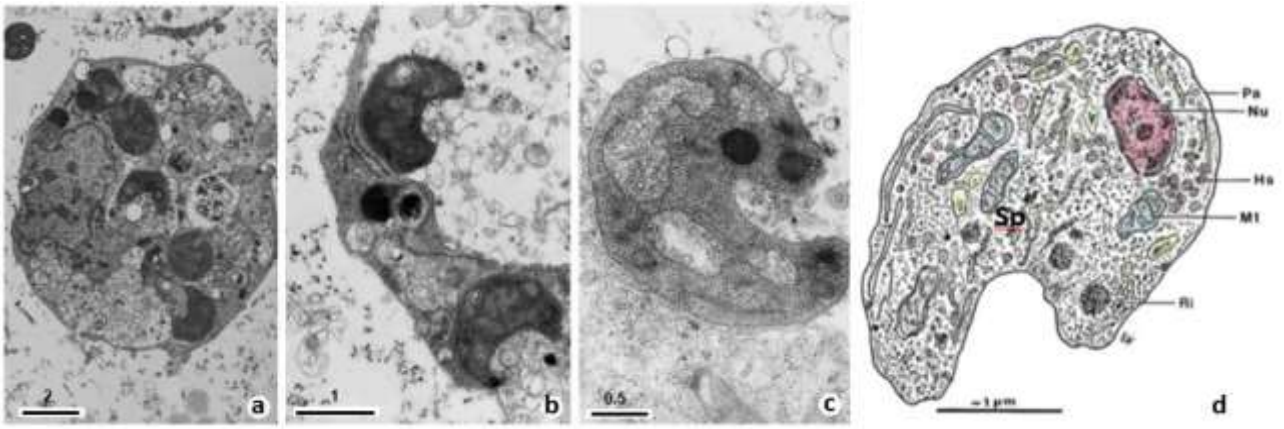

Figure 8. *Bonamia ostreae*: Ultrastructural aspects of different stages of the maturation of its spores; **a)** Host cell infected with several spores; **b)** Two spores (Sp), one of which is in the process of excision from the host (*); **c)** Mature spore; **d)** Schematic drawing of a mature spore, showing the presence of numerous ribosomes. Scale bars in µm.

Discussion

The phylum Haplosporidia Caullery & Mesnil, 1899 corresponds to a small taxonomic group of species of protist microparasites, many of which are reported to be pathogenic, causing mortality in hosts from different aquatic environments (Azevedo, 1984; Anderson et al., 1993; Montes et al., 1994; Azevedo et al., 2006; Carnegie et al., 2006; Bearham et al., 2008a; Le et al., 2015; Catanese et al., 2018). Historically, the phylum Haplosporidia, for many years, engulfed three genera (*Minchinia* Labbé, 1896, *Haplosporidium* (Caullery & Mesnil, 1899) Lüke, 1900, and *Urosporidium* Caullery & Mesnil, 1905). Recent phylogenetic molecular analyzes supported the inclusion of the genus *Bonamia* within the phylum Haplosporidia (Carnegie et al., 2006). The genera were characterized based on the morphological particularities of the spore and the presence and origin of spore wall ornamentation, as majores morphological characters.

Most descriptions of haplosporidian species were described using LM, TEM and SEM data (Debaisieux, 1920; De Turk, 1940; Dyková et al., 1988; Anderson et al., 1993; Carnegie et al., 2006; Bearham et al., 2008a, b), while few species descriptions were made based on molecular analyses (Azevedo et al., 2006; Burreson & Reece, 2006; Winters & Faisal, 2014; Vea & Siddall, 2011; Urrutia et al., 2019; Cho et al., 2020). In addition, many descriptions of these species were complemented with specificity data related to the light and ultrastructural morphologies of the parasites, as well as host specificities. Data on the environmental location in which the parasites were collected were reported to describe some species. A comparative analysis of the presence or absence of the operculum, as well as its morphology and the absence or presence of various types of ornaments (tails or filaments) of the spore wall were characters used to distinguish some species. It is possible that, among some of these described species belonging to the phylum Haplosporidia, a taxonomic reanalysis is needed to confirm the name of the species.

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