A new species of Haplosporidium *Caullery & Mesnil, 1899 in the marine false limpet* Siphonaria lessonii *(Gastropoda: Siphonariidae) from Patagonia*

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A new species of *Haplosporidium* Caullery & Mesnil, 1899 in the marine false limpet *Siphonaria lessonii* (Gastropoda: Siphonariidae) from Patagonia

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Abstract A new species of *Haplosporidium* Caullery & Mesnil, 1899 parasitising the pulmonate gastropod *Siphonaria lessonii* Blainville in Patagonia, Argentina, is described based on morphological (scanning and transmission electron microscopy) and sequence (small subunit ribosomal RNA gene) data. Different stages of sporulation were observed as infections disseminated in the digestive gland. *Haplosporidium patagon* n. sp. is characterised by oval or slightly subquadrate spores with an operculum that is ornamented with numerous short digitiform projections of regular height, perpendicular to and covering its outer surface. The operculum diameter is slightly larger than the apical diameter of the spore. Neither the immature nor mature spores showed any kind of projections of the exosporoplasm or of the spore wall. Regarding phylogenetic affinities, the new species was recovered as sister to an undescribed species of *Haplosporidium* Caullery & Mesnil, 1899 from the polychaete family Syllidae Grube from Japanese waters. The morphological characters (ornamentation of the operculum, spore wall structure, shape and size of spores, and the lack of spore wall projections) corroborate it as an as yet undescribed species of *Haplosporidium* and the first for the phylum in marine gastropods of South America. *Siphonaria lessonii* is the only known host to date.

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Introduction

Haplosporidians are endoparasitic protists that are characterised by the production of spores and parasitism in a variety of marine invertebrates (Perkins, 2000) and a few freshwater hosts (Burreson, 2001; Messick, 2009; Molloy et al., 2012). There are approximately 40 named species and at least 20 unnamed putative species in the phylum (Hine et al., 2009). Molecular phylogenetic studies have clarified some aspects of the systematics of the Haplosporida Caullery & Mesnil, 1899, confirming the monophyly of the phylum (Siddall et al., 1995; Reece et al., 2004; Burreson & Reece, 2006) and contributing to knowledge of its diversity at the generic level and including species of *Bonamia* Pichot, Comps, Tige, Grizel & Rabouin, 1980 for which spores have been characterised in only one species (Azevedo et al., 2006; Carnegie et al., 2006). Differentiating the remaining three traditionally recognised genera, *Minchinia* (Labbé, 1896), *Haplosporidium* Caullery & Mesnil, 1899

and *Urosporidium* Caullery & Mesnil, 1905, is based on the morphology and ultrastructure of the spore (particularly the presence or absence of a hinged operculum covering spore orifice) and by the presence, characteristics and origin of spore ornamentation (Burreson & Reece, 2006; Hine et al., 2009). Species of *Urosporidium* lack an operculum, the spore projections in species of *Minchinia* originate in the epispore cytoplasm (from which they disappear at the end of sporulation), whereas in species of *Haplosporidium* spore ornamentation is derived from spore wall and is permanent (Reece et al., 2004; Azevedo et al., 1999, 2006).

Haplosporidians infecting marine molluscs have been widely reported around the world (Burreson & Ford, 2004). Most species parasitising molluscs have been described from bivalves and only four species are known from gastropods: *Haplosporidium pickfordi* Barrow, 1961 from the freshwater physid snail *Physella parkeri* (Currier), *Haplosporidium lusitanicum* Azevedo, 1984 from the limpet *Helcion pellucidus* Linnaeus, *Haplosporidium montforti* Azevedo et al., 2006 from the abalone *Haliotis tuberculata* Linnaeus, and *Haplosporidium tuxtlensis* Vea & Siddall, 2011, recently described from the striped false limpet *Siphonaria pectinata* (Linnaeus) in the Gulf of Mexico (Vea & Siddall, 2011).

In the Southwestern Atlantic Ocean, the knowledge of haplosporidians parasitising marine invertebrates is poor. Kroeck & Montes (2005) reported a case of bonamiasis in *Ostrea puelchana* d′Orbigny from San Matías Gulf (Argentina) and a *Bonamia exitiosa*-like species was the causative agent of mass mortality among *O. puelchana* cultured at San Matías Gulf (Kroeck, 2010).

Siphonaria lessonii Blainville is a common pulmonate gastropod occurring in high and medium zones of intertidal rocky shores along Patagonia in Argentina. Its distribution ranges from Santa Catarina (Brazil) in the Atlantic coast southern to Tierra del Fuego and Malvinas (Falkland) Islands, and into the Pacific where it has been reported as far North as Paita, Peru (Rios, 1994).

Here, a new species of the genus *Haplosporidium* is described from *Siphonaria lessonii* based on spore morphology, including ultrastructure, as well as molecular information that permitted determination of the phylogenetic position of the new species among other haplosporidians from various hosts.

Materials and methods

Host sampling and examination

Specimens of *S. lessonii* (n = 119) were collected during the austral autumn (April, 2011) from the mid-intertidal rocky littoral zone at Puerto Deseado (47°45′S, 65°55′W), Santa Cruz, Argentina. The gastropods were dissected and the digestive gland was inspected with a stereomicroscope in order to search for infections. Pieces of the digestive glands suspected of being parasitised were squashed and fresh preparations were made to determine the presence of spores with a compound microscope. From infected tissues, live, fixed free spores and small fragments of digestive gland were observed under phase contrast and dark field light microscopy.

Transmission and scanning electron microscopy

Small pieces of infected tissues (5 × 5 mm) allocated for transmission electron microscopy (TEM) were fixed in cold 2.5% glutaraldehyde with 4% formalin (prepared from paraformaldehyde) in filtered seawater, for 20 h at 4 °C. After rinsing in seawater, samples were post-fixed in 1% osmium tetroxide, rinsed in 0.2 M cacodylate buffer, dehydrated in an ascending ethanol series (70 to 100%), and transferred to Spurr's resin via propylene oxide. Semithin sections were stained with 1% water solution of Tolouidine blue. Ultrathin sections were double stained with uranyl acetate and lead citrate, and examined in a Jeol 1200 EX II transmission electron microscope. Fresh and fixed in glutaraldehyde spores from each of the isolates from infected specimens were mounted for scanning electron microscopy (SEM), viewed in a Philips EM301. Spore measurements (length and width in micrometres) were taken from SEM images and semithin sections for TEM (n = 30) and are given as the mean ± standard deviation.

Molecular phylogenetic analysis

The DNeasyH Tissue Kit (Qiagen Valencia, California) was used for extraction. Amplification reactions (PCR) were conducted in an Eppendorf MastercyclerH (Eppendorf, Hamburg, Germany) and employed Taq Gold and 50 cycles of 94 °C (45 s), 47 °C (30 s), and 68 °C (90 s) following a 10 min pre-melt at 94 °C with primers A and B, as described in Phillips et al. (2010). PCR amplification products were purified with AM-Pure (Agencourt Bioscience Corporation, Beverly,

Massachusetts). Cycle sequencing reactions were performed with an Eppendorf Mastercycler using 1 ml Big Dye, 1 ml of 1 mM [A, L, C, Y, O, and B described in Phillips et al. (2010)], and 3 ml of cleaned PCR template (13 ml total volume) and analysed with an ABI PRISM 3730 sequencer (Applied Biosystems). CodonCode Aligner (CodonCode Corporation, Dedham, Massachusetts) was used to edit and reconcile sequences. The new sequence was deposited in GenBank (Accession no. KJ534587).

Molecular phylogenetic analysis included all available sequences (small subunit rRNA gene) for the phylum Haplosporida and related parasites used to root the tree (AY542903, GQ385242, EU016528, DQ356000, GQ366703, AF262995, AF492442, AY449715, AY435093, AF387122, DQ458793, AY781176, U447851, AY449713, DQ219484, AB080597, AY452724, DQ444238, JN368430, AY449711, FJ518816, EF165631, AY449712, AY449710, U20319, U47852, AY449714, JX185413, HQ285783). Sequences were aligned with MUSCLE version 3.7 (Edgar, 2004) through the European Bioinformatics Institute server (http://www.ebi.ac.uk/Tools/msa/muscle/). Parsimony analyses were accomplished with TNT (Goloboff et al., 2008) in which gapped regions were treated as missing and with 100 bootstrap replicates; all searches employed three rounds of fusing, five rounds of the ratchet and sectorial searches in which minimum length trees were found ten times. Maximum likelihood analyses were conducted with PhyML 3.0 (Guindon et al., 2010). The best fitting nucleotide model was assessed with FindModel (http://www.hiv.lanl.gov/cgi-bin/findmodel/findmodel.cgi) yielding the general time-reversible model including gamma distributed among-site variation (GTR+Γ; Akaike Information Criterion score = 30,571.03).

Voucher specimens are deposited in the Collection of Parasitology, Museo Argentino de Ciencias Naturales, Buenos Aires (MACN-Pa).

Results

The digestive glands of seven out of 119 (5.88%) studied specimens of *S. lessonii* collected during the austral autumn in 2011 were infected. Infections were disseminated throughout the whole digestive gland, which appeared swollen, with a characteristic discoloration of tissues, being brownish instead of dark green as in healthy specimens. The intensity of each infection was so high that the structure of the digestive tubule epithelium appeared deeply altered. In heavily infected gastropods the acini of the digestive gland were nearly completely replaced by the developing parasites; however the ducts of the gland were not affected (Fig. 1A). Neither the intestinal epithelium surrounded by the acini of the digestive gland nor other organs of the visceral mass appeared to be affected by haplosporidians (Fig. 1B).

Haplosporidium patagon n. sp.

Type-host: *Siphonaria lessonii* Blainville (Pulmonata, Bassomatophora, Siphonariidae).
Site of infection: Digestive gland.
Type-locality: Puerto Deseado (47°45′S, 65°55′W), Santa Cruz Province, Argentina.
Type-material: One hapantotype, SEM stub MACN-Pa 566-1, with numerous spores.
Other material examined: 2 semithin sections, MACN-Pa 566-2 and 566-3.
Etymology: The species name refers to the Patagones, the name the Spanish conquerors gave to the aboriginal inhabitants of vast coastal areas along the southern tip of South America.

Description (Figs. 1–4)

General morphology of spores
Mature spores ellipsoidal to quadrangular, 4.22 ± 0.34 long and 2.48 ± 0.21 wide; width/length ratio 0.59 ± 0.07 (Fig. 1C); developing in sporocysts bearing 11 to 50 spores each (Fig. 1A, B). Spores with an apical ellipsoidal operculum formed by a flange and an eccentrically located lid (Figs. 2C, 3D), both ornamented with minute digitiform projections giving to the outer surfaces a comb-like aspect. Spore wall of mature and immature spores smooth, lacking any kind of wall projections, thickened in mature spores at basal and apical (opercular) portions (Figs. 3A–C, 4B).

Sporulation
The late sporonts or early sporocysts (earliest stages observed) are delimited by an irregular membrane (Fig. 2A). At early stages, spores are nearly spherical; *c*.2.5 in diameter, with a large nucleus and a conspicuous array of Golgi vesicles opposite to the nucleus
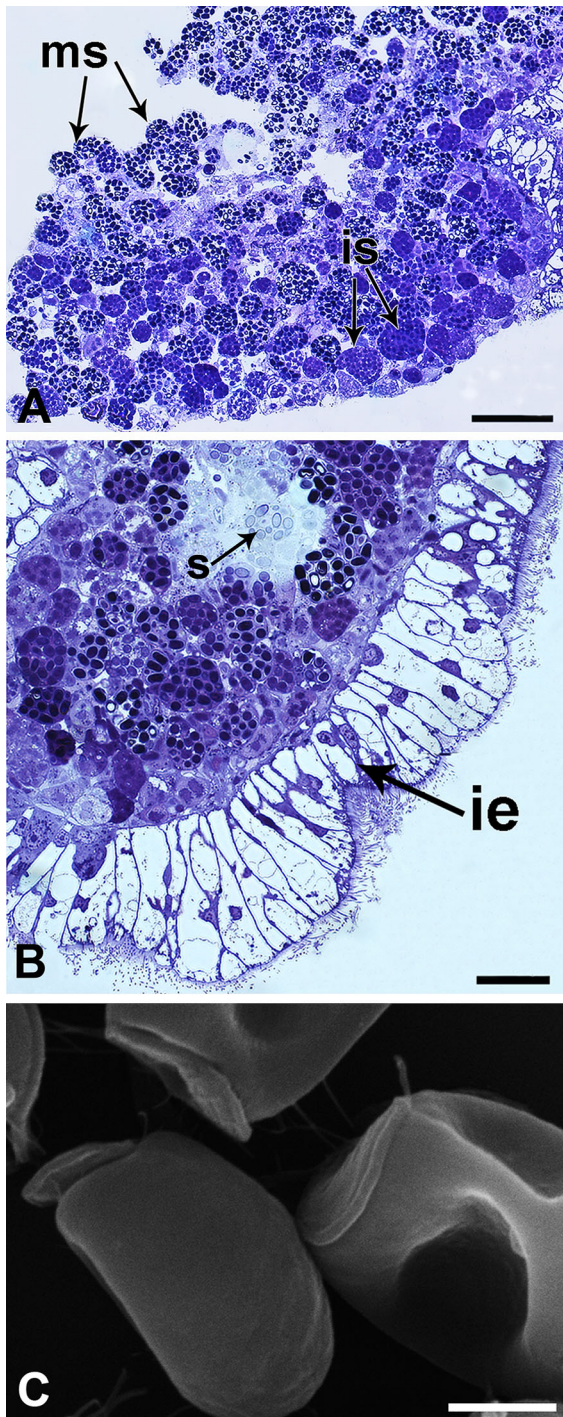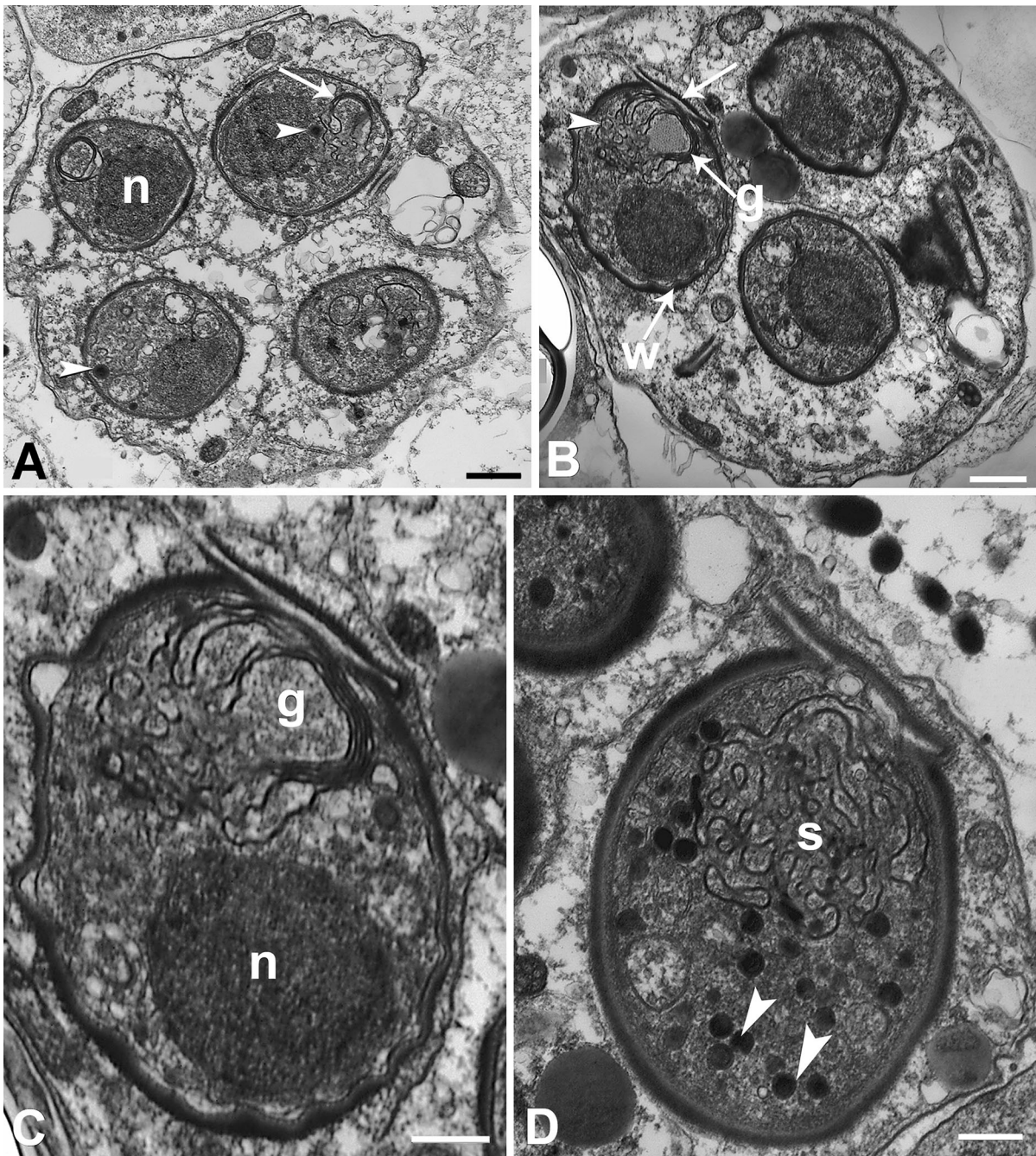
Fig. 1 *Haplosporidium patagon* n. sp. A, B. Semithin sections of the digestive gland of a heavily infected *Siphonaria lessonii*. A, Large number of sporocysts in different stages of sporulation; B, Detail of the infected digestive gland and adjacent healthy intestinal epithelium; C, SEM of mature spores. *Abbreviations*: ie, intestinal epithelium; is, immature sporocyst; ms, mature sporocyst; s, spores. *Scale-bars*: A, 50 μm; B, 20 μm; C, 1 μm

(Fig. 2A), the spore wall is thin (*c*.0.3 thick). Later on, the developing spores change the shape to a somewhat elliptical outline, and the spore cytoplasm becomes progressively denser with a well-discernible nucleus; sometimes the spore wall appeared slightly wavy (Fig. 2B). At this stage of sporulation, the initial stages in the formation of the operculum was apparent (Fig. 2C, D), and a defined sporulosome were well discernible (Fig. 2D), a number of dense small spherical haplosporosomes (*c*.0.4 in diameter) were present. In nearly mature spores, one ax-shaped haplosporosome was rarely observed (Fig. 3C).

The lid of the operculum is ellipsoidal, located slightly eccentric at the apical pole of the spore, connected to the spore wall by a curved hinge (Figs. 2C, D, 3D, 4D). In transverse section (minor axis *c*.2 wide) the lid appears as slightly concave with a low dome in the centre (Figs. 3B, C; 4E); in sagittal section (major axis *c*.4 in length) the lid is flat with slightly marked dome in the centre (Fig. 4A, D).

The spore wall lacks of any kind of ornamentation. Throughout the sporulation the spore wall showed changes in its complexity. At early stages, the wall is uniformly thin (*c*.0.14), moderately electron-dense, and formed by two or three weakly discernible layers (Fig. 2C, D). At a more advanced stage, a three-layered structure is clearly visible because of the outermost layer becomes deeply electron-dense (Fig. 3B). In mature or nearly mature spores, the wall thickens markedly, first at the apical (opercular) pole (Fig. 3A, C) and afterwards at the opposite pole (Fig. 3D), remaining thin at level of the equator (Figs. 3A, D, 4A). At this point, the spore wall showed a 6-layer structure, being *c*.0.3 thick (Fig. 4B). The outermost layer of the spore wall does not participate in the structure of the lid or the flange, onto which the operculum rests (Fig. 4D, E). Approaching maturity, the lid showed a character-istic ornamentation at the outer surfaces consisting of minute digitiform projections (*c*.0.1μm high) ending in a very small rounded tip (Fig. 4C); this ornament gives in transverse section a comb-like appearance. Similar digitiform processes are present in the lower surface of the flange (Fig. 4E). Spore wall thickens along the border of the operculum, appearing in transverse section as rounded electron-dense tips (Fig. 4C–E). The free borders of the operculum (lid and flange) were continuous with the plasma membrane of the sporoblast (Fig. 4D). Ornamentation derived from epispore cyto-plasm was not observed with light microscopy or SEM.
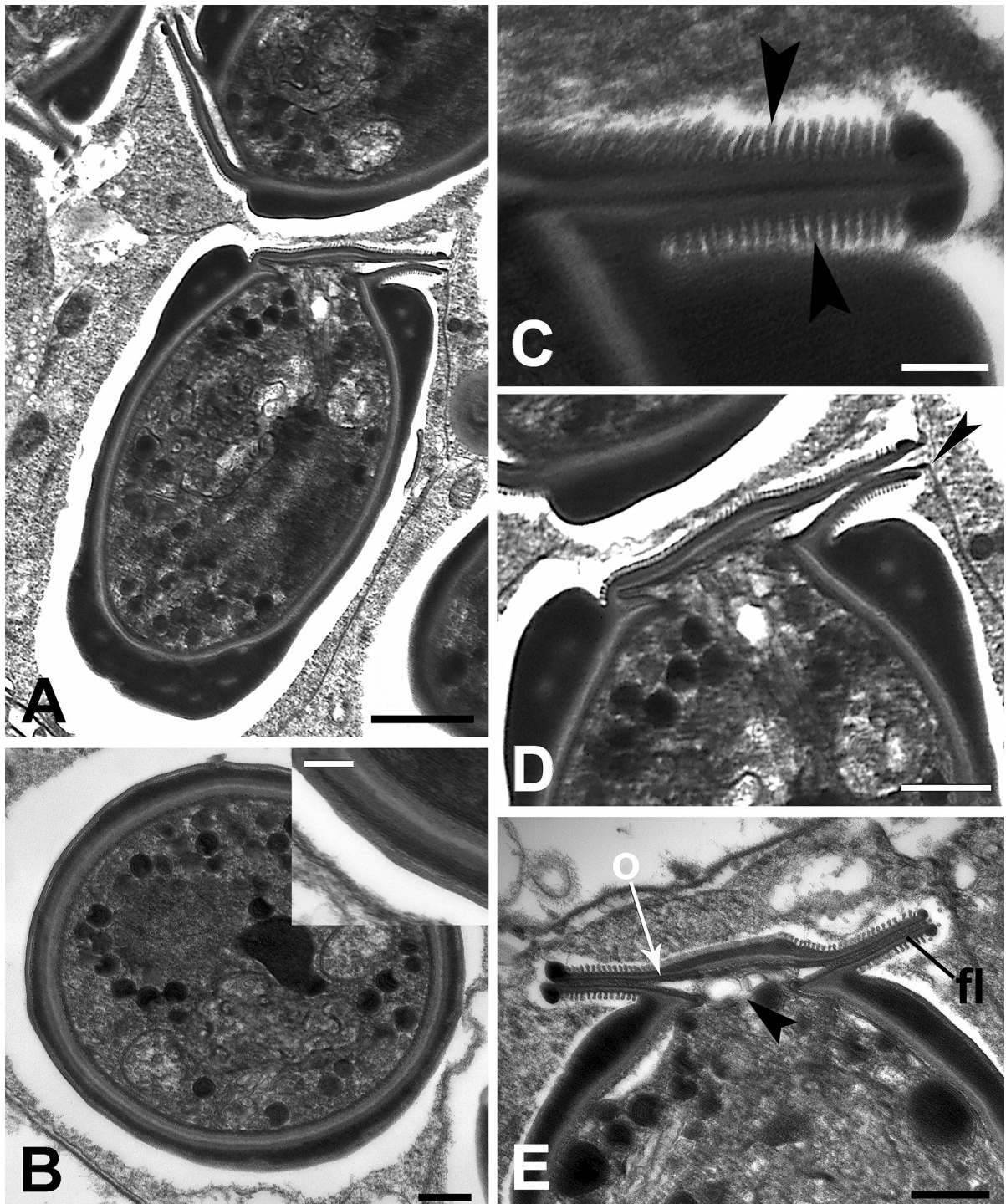
Fig. 2 Ultrastructure of *Haplosporidium patagon* n. sp. A, Sporocyst with four spores at early stages of development showing a large nucleus, arrays of Golgi bodies and a thin monolayer spore wall; B, Sporocyst in a more advanced stage of development, spores show a thicker and denser wall and initial formation of operculum (arrow); C, Detail of a spore with prominent Golgi-like complex just beneath operculum, large nucleus and early development of operculum; D, A further stage in the formation of wall (two layers are clearly visible) and operculum; the spherulosome and multiple haplosporosomes are visible (arrowhead). *Abbreviations*: g, Golgy body; n, nucleus; s, spherulosome. *Scale-bars*: A, B, 1 μm; C, D, 0.5 μm
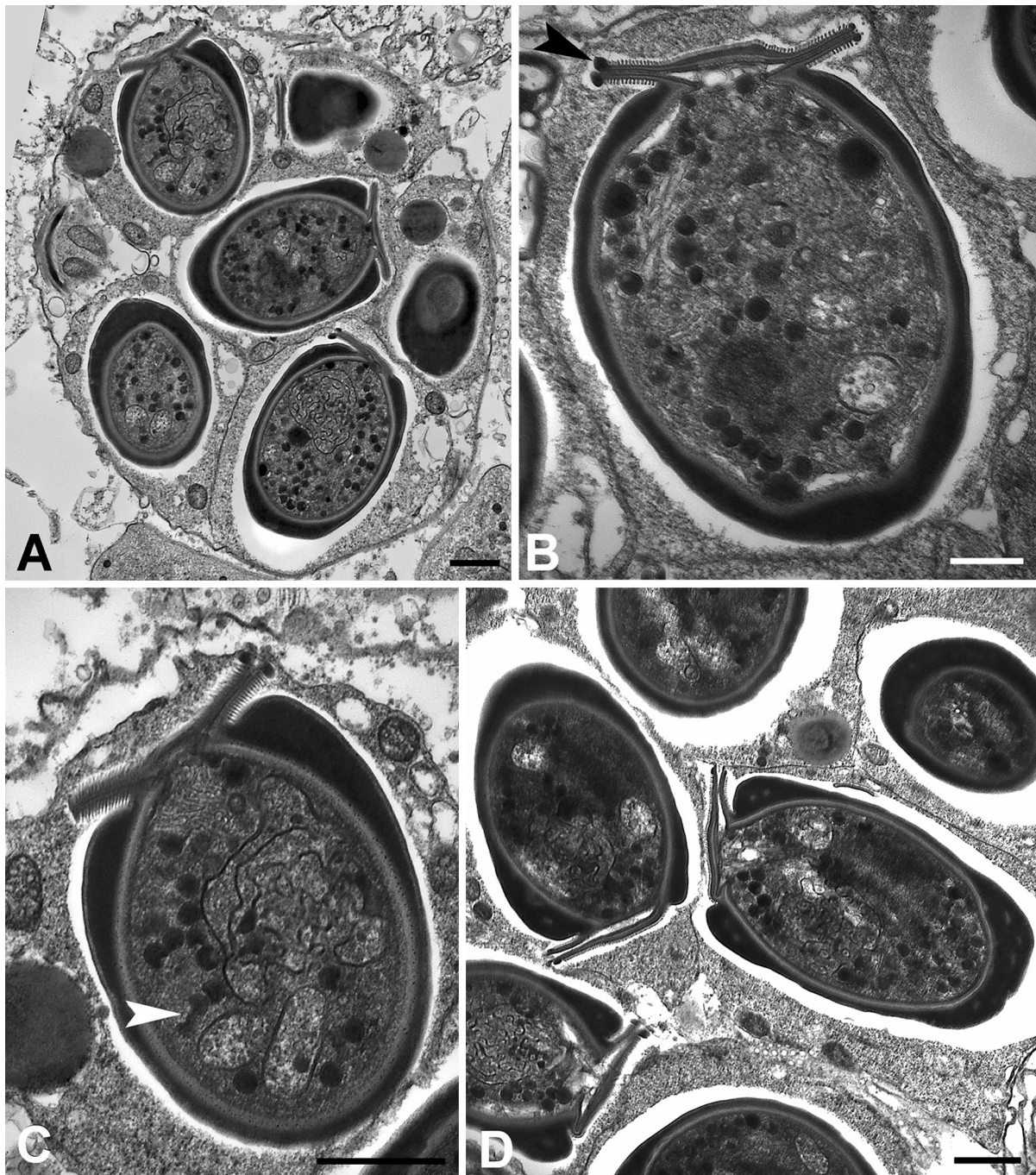
Fig. 3 Ultrastructure of *Haplosporidium patagon* n. sp. A, Sporocyst with several nearly mature spores; B, Transverse section of a spore showing a uniformly thickened spore wall. The border of the operculum and flange show the same thickening of wall (arrow); C, Transverse section of a spore with spore wall thickened at the apical (opercular) pole; D, Saggital sections of two nearly mature spores (centre) with thickening of the spore wall at apical and bottom sides. The cytoplasm is denser than in immature stages and the spore wall is three-layered. *Scale-bars*: A, C, D, 1 μm; B, 0.5 μm

Fig. 4 Ultrastructure of *Haplosporidium patagon* n. sp. A, Saggital section of a mature spore still within a sporocyst with scant exosporoplasm; B, Detail of the wall of a mature spore at equatorial level (insert: detail of the six-layer structure); C, Detail of a transverse section of the operculum with surface ornamentation covering the free areas of the operculum and flange (arrows); D, Sagittal section of a spore with a detail of the operculum and hinge: the free tips of the operculum are continuity of the plasma membrane of the sporoblast (arrow), a number of haplosporosomes; E, Detail of a transverse section of a spore at level of operculum, vesicles beneath the operculum (arrow) are visible. *Abbreviations*: fl, flange; o, operculum. *Scale-bars*: A, D, 1 μm; B, E, 0.4 μm; C, B insert, 0.2 μm
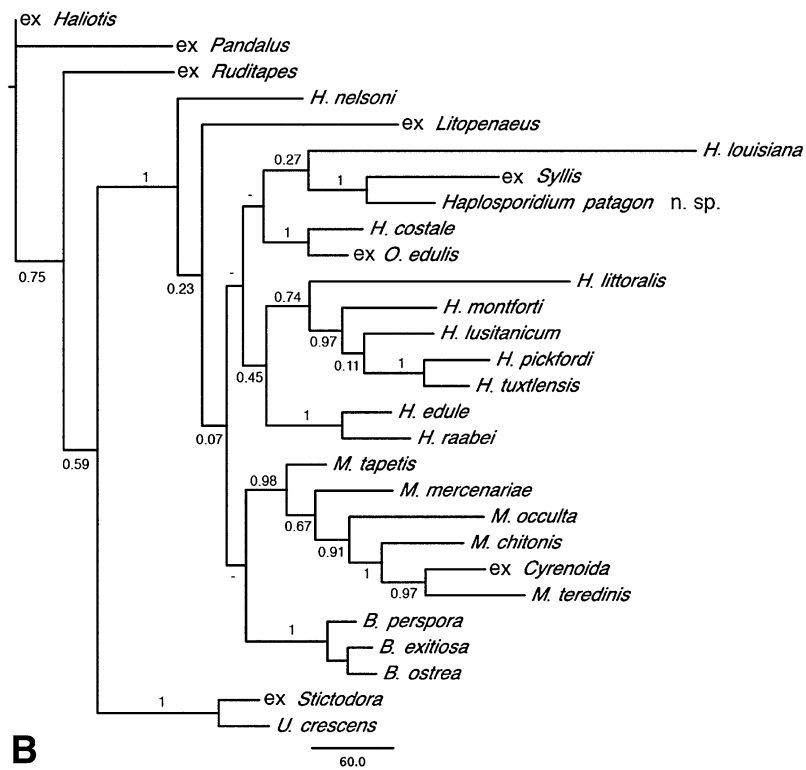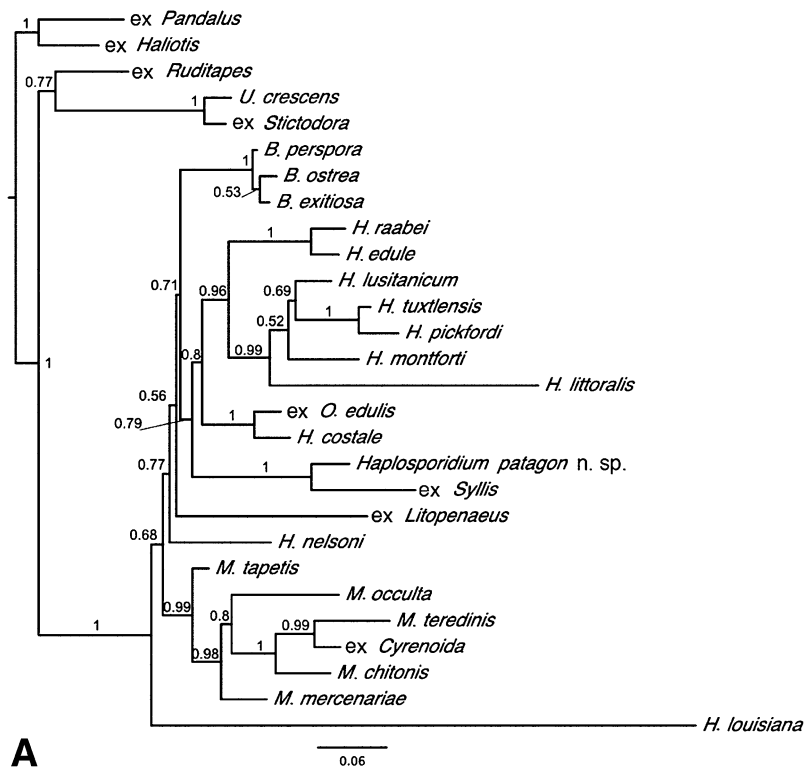
◀ **Fig. 5** Optimal trees found in (A) maximum likelihood (log-likelihood of 10,885.59) and (B) parsimony (with 4,492 steps) analyses for the available species in the haplosporidian genera *Haplosporidium*, *Minchinia* and *Bonamia*. Isolates of undescribed taxa are referred to their hosts. Branches are drawn proportional to the amount of change (substitutions per site in A, steps in B) and bootstrap proportions are assigned to each node

Remarks

Haplosporidium patagon n. sp. is similar in spore size to *Haplosporidium tuxtlensis* Vea & Siddall, 2011, *Haplosporidium hinei* Bearham, Spiers, Raidal, Jones, Burreson & Nicholls, 2008, *Haplosporidium costale* Wood & Andrews, 1962 and *Haplosporidium edule* Azevedo, Conchas & Montes, 2003.

The spores of *H. patagon* n. sp. do not exhibit any spore wall-derived ornamentation; in this regard, they are unlike any other described species of *Haplosporidium*. Comps & Pichot (1991) described the mature spores of an unidentified species of *Haplosporidium* as having no prominent ornamentation or filaments, although tangential sections revealed 60 nm tubular ribs on the surface similar to the ribbon-like ornamentation described by Perkins (1969) in *H. costale*.

In addition to the lack of spore ornamentation, *H. patagon* n. sp. is unique in having conspicuous wall thickenings at the poles of the mature spores. Species with small thickenings in the spore wall at the posterior pole include *Haplosporidium pickfordi* Barrow, 1961 and an undescribed species from *Saccostrea cuccullata* (Born) (Burreson, 2001; Hine & Thorne, 2002). Neither resembles the extent of the thickenings observed in *H patagon* n. sp.

The spore operculum in the new species does not surpass spore diameter at the equator, differing from that observed in *H. montforti*. The ornamentation of the surface of the operculum and lid are also features unique among the haplosporidian species yet described. Their digitiform nature is apparent from the uniformity of appearance regardless of sectional plane (figures 3 and 4 in Azevedo et al., 2006).

Regarding ultrastructural aspects of sporulation, the spore spherule has been previously viewed as a modified Golgi complex (Hine et al., 2007). This origin seems clear in *H. patagon* n. sp. in which the Golgi complex is a quite apparent structure close to the operculum, present from the very early stages of spores, that progressively loses its structure by producing a large number of convoluted vesicles that become into the spherule.

Maximum likelihood and parsimony analyses each yielded single resolved trees that differed with respect to internal nodes. The molecular information places *H. patagon* n. sp. as sister to an undescribed species from the polychaete family Syllidae from Japanese waters (Fig. 5). This sister-group relationship received strong support (i.e., 100%) from both parsimony and maximum likelihood analyses. Although *H. tuxtlensis* also infects siphonariids, it was sister to *H. pickfordi* (also with 100% support in both analyses). Maximum likelihood analysis placed *H. louisiana* (Sprague, 1963) outside the genus *Haplosporidium*. Parsimony, in contrast, placed *H. nelsoni* (Haskin, Stauber & Mackin, 1966) outside of the genus while placing the long-branched *H. lousiana* within a monophyletic *Haplosporidium* clade.

Discussion

The phylum Haplosporida contains four genera: *Bonamia*, *Haplosporidium*, *Minchinia* and *Urosporidium* (see Hine et al., 2009). The origin and morphology of spore ornamentations was largely used as a main feature to distinguish among haplosporidian genera (Ormières, 1980; Perkins, 2000). According to these criteria, ornamentation in *Haplosporidium* spp. originates as projections of the spore wall (also present in *Bonamia perspora* Carnegie, Burreson, Hine, Stokes, Audemard, Bishop & Peterson, 2006). In *Minchinia* spp. filaments are derived from the epispore cytoplasm and might eventually breakdown as observed by Azevedo (2001) in *Minchinia tapetis* (Vilela, 1951), leaving a spore without wall-derived ornamentation. The presence of an external operculum and a hinge apparatus in *Haplosporidium*, *Minchinia* and *Bonamia*, was an additional character used to complete the generic identification. External operculum is absent in *Urosporidium*, replaced by an internal flap derived from the spore wall, covering the spore orifice.

The lack of wall projections would place the new haplosporidian species described above within the genus *Minchinia*. However, in spite of the lack of any type of spore ornamentation, the molecular data clearly place *H. patagon* n. sp. within the genus *Haplosporidium*. Regarding the position of *H.*

patagon n. sp in the phylogeny retrieved, it is to note that it groups with an unnamed species from the polychaete *Syllis nipponica* (Imajima) found only as a DNA sequence by Siddall & Aguado (2006) and not (as might have been expected) with *H. tuxtlensis* from the false limpet *Siphonaria pectinata* or other haplosporidians from gastropods. *H. patagon* forms a group that stands as sister to the clades formed by the other known species of *Haplosporidium* from molluscs: *H. montforti*, *H. lusitanicum*, *H. pickfordi*, *H. tuxtlensis*, *H. edule* and *Haplosporidium raabei* Molloy, Giambérini, Stokes, Burreson & Ovcharenko, 2012. Our findings reinforce the evidence for a close relationship between haplosporidians with their most common spirilian hosts with the exception of the sequence obtained from a polychaete, continuing to raise the possibility that annelids serve as intermediate hosts.

The origin and morphology of spore ornamentation has been recognised as one of the most relevant characters to distinguish among genera and species within the phylum Haplosporida. The existence of gaps in the morphological information on many taxa makes difficult specific or generic assignment without the aid of other characters such as those coming from molecular studies. This is the case of *H. patagon* n. sp., in which the lack of any kind of spore ornamentation (derived either from the epispore cytoplasm or from the spore wall) would preclude its generic placement. However, Burreson & Reece (2006) have recognised the incongruence of molecular phylogeny of the Haplosporida and the generic definition based on morphological criteria, namely the origin of spore ornamentation. Final determination of species limits awaits ultrastructural and molecular characterisation of the type-species of the genus *Haplosporidium*, *H. scolopli* Caullery & Mesnil, 1899, and the leveraging of additional loci to resolve the relative monophyly of the genus.

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