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Ultrastructure and Phylogeny of *Pleistophora beebei* sp. nov. (Microsporidia) Infecting the Amazonian Teleostean *Brachyhypopomus beebei* (fam. Hypopomidae)

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Abstract. A new microsporidian, *Pleistophora beebei* sp. nov., parasitizing the freshwater benthopelagic teleostean fish *Brachyhypopomus beebei* Schultz, 1944 (fam. Hypopomidae) collected from the Amazon River is described based on molecular and morphological studies. The parasite develops in the skeletal muscle of the abdominal cavity, forming a whitish cyst-like containing several groups of two types of spores (macrospores and microspores), which were observed in close contact with the myofibrils. Small groups of macrospores (ovoid elongate, tapering more anteriorly than posteriorly and measuring about $7.8 \pm 0.4 \times 4.7 \pm 0.2$ µm) were observed among the numerous microspores (lightly pyriform to ellipsoidal with rounded ends, measured about $4.7 \pm 0.3 \times 2.8 \pm 0.4$ µm)**.** Both types of spores possessed a single large posterior vacuole containing flocculent material. The ultrastructural aspects observed, together with the formation of a cyst-like, suggest that the parasite belongs to the genus *Pleistophora.* This taxonomic positioning was confirmed by the molecular analysis of the SSU rRNA gene and Maximum-likelihood (ML) inference. Comparison to similar species previously described, recognized this as a new species, herein named *Pleistophora beebei* sp. nov.

Keywords: Dimorphism, SSU rRNA gene, parasite, Brazil

INTRODUCTION

The ichthyofauna of South America is particularly diversified, with an estimated 8,000 species in Brazil alone (Cellere *et al*. 2002). The family Hypopomidae, also known as bluntnose knifefish, comprises small sized teleosts that are confined to the continental waters of South America. Although there is some commercial exploitation of species from the genus *Brachyhypopomus*, its importance is mainly ecological, since they represent a significant percentage of the biomass (Crampton 1996). Few parasitological studies have been conducted in hypopomids. Five new species of

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Urocleidoides (Monogenoidea) have been reported from *Brachyhypopomus occidentalis* (Mendoza-Franco and Reina 2008) and, more recently, the myxozoan parasite *Henneguya torpedo* was described from the nervous system of *Brachyhypopomus pinnicaudatus* in the Amazon region (Azevedo *et al*. 2011). An infection caused by a microsporidian has also been reported in specimens of *Brachyhypopomus brevirostris* captured from the same hydrographic area (Matos and Azevedo 2004).

Microsporidians are minute obligatory intracellular parasites that display a wide habitat distribution, occurring in almost all taxonomic groups, including unicellular organisms (Larsson 1999, Vávra and Larsson 1999). Nonetheless, these parasites are best known for the diseases they cause in commercially important fish hosts (Lom and Dyková 1992, Lom and Nilsen 2003). Fish-infecting microsporidia are distributed among 21 genera (Lom and Dyková 1992; Lom 2002; Lom and Nilsen 2003; Stentiford *et al*. 2013; Diamant *et al*. 2010, 2014). Among these, seven infect teleost fish from South America, with five occurring in freshwater fish from the Amazonian watershed. Two of the latter were originally described from this geographic area: the genus *Amazonspora*, with the type species *Amazonspora hassar* from the gills of *Hassar orestis* (Doradidae) (Azevedo and Matos 2003), and the genus *Potaspora*, with the type species *Potaspora morhaphis* adhering to the wall of the coelomic cavity of *Potamorhaphis guianensis* (Casal *et al*. 2008). *Potaspora aequidens* has very recently been reported parasitizing the muscles of the sub-opercular region and the caudal fins of the freshwater fish *Aequidens plagiozonatus* (Cichlidae) (Videira *et al*. 2015). Two species have also been reported from the genus *Loma*: *L. myrophis* infecting the sub-epithelial gut tissue of *Myrophis platyrhynchus* (fam. Ophichthidae) (Azevedo and Matos 2002) and *L. psittaca* infecting the intestinal mucosa of the freshwater puffer fish *Colomesus psittacus* (Tetraodontidae) (Casal *et al*. 2009). *Kabatana rondoni* was described from the skeletal muscle of the abdominal cavity of *Gymnorhamphichthys rondoni* (Rhamphichthyidae) (Casal *et al*. 2010). The microsporidian *Pleistophora hyphessobryconis* has been described from several families of ornamental fish, mainly from the Amazon River watershed (Lom 2002) and Winters *et al*. (2016) very recently reported an infection in the skeletal muscle of a non-ornamental hybrid fish (*Leiarius marmoratus* × *Pseudoplatystoma reticulatum*) from a Brazilian aquaculture facility. Matos and Azevedo (2004) described a microsporidian infection occurring in the skeletal muscle of *Brachyhypopomus brevirostris*. Not being able to classify the infective agent at the genus level, *Microsporidium brevirostris* remains included in the collective group (Matos and Azevedo 2004).

Aiming to provide new information on microsporidian parasites infecting teleost fish in the Amazon region, the present study describes the morphological, ultrastructural and molecular features of a new species occurring in this geographic area.

MATERIALS AND METHODS

Thirty specimens of the freshwater benthopelagic fish *Brachyhypopomus beebei* Schultz, 1944 (fam. Hypopomidae, order Gymnotiformes) (Brazilian common name: "Itui tuanga") were collected from the Amazon River near the City of Peixe Boi (01º 11´ S / 47º 18´ W), State of Pará, Brazil and transported live to the laboratory in UFRA- Belém. Specimens were kept for 2-5 d, in an aquarium containing water from the collection site and maintained at the same temperature (25-28 ºC). The fish were euthanized with an overdose of the anaesthetic MS 222.

Using light microscopy, several irregular whitish cyst-like were observed in the skeletal muscle of the ventral abdominal cavity, near the gut. These were removed from the infected fish and isolated fresh spores were measured using Nomarski differential interference contrast (DIC) optics.

For transmission electron microscopy (TEM), a small fragment of the infected tissues was fixed in 3% glutaraldehyde with a 0.2 M sodium cacodylate buffer (pH 7.2) for 20 - 24 h at 4 ºC, washed overnight in the same buffer at 4 ºC and post-fixed in 2% OsO_4, buffered in the same solution for 3 h at the same temperature. After dehydration in an ascending ethanol series and propylene oxide, the fragments were embedded in Epon. Semi-thin sections were stained with blue methylene for light microscopy. Ultrathin sections were contrasted with both aqueous uranyl acetate and lead citrate and observed with a JEOL 100CXII TEM operated at 60 kV.

For molecular analysis, several whitish cyst-like were dissected and homogenized. Isolated spores were then stored in 80% ethanol at 4 °C. The genomic DNA of about 3×10^6 spores was extracted using a GenEluteTM Mammalian Genomic DNA Miniprep Kit (Sigma) following the manufacturer's instructions for animal tissue, except for the incubation time. The DNA was stored in 50 µl of TE buffer at –20 ºC. The majority of the region coding for the SSU rRNA gene was amplified by PCR using the primers V1f (5′-CACCAGGTTGATTCTGCC-3′) (Nilsen 2000) and HG5F_rev (5′-TCACCCCACTTGTCGTTA-3′) (Abdel-Baki *et al*. 2015). The primers HG4F (5′-CGGCTTAATTTGACTCAAC-3′) and HG4R (5′-TCTCCTTGGTCCGTGTTTCAA-3′) (Gatehouse and Malone 1998) were used to amplify the 3′ end of the SSU rRNA gene, the internal transcribed spacer (ITS) and the 5′ end of the LSU rRNA gene. PCR reactions were carried out in 50 µl reactions using 10 pmol of each primer, 10 nmol of dNTP, 2.5 mM of $MgCl_2$, 5 µl 10 × Taq polymerase buffer, 1.5 units Taq DNA polymerase (Nzytech), and 3 μl of the genomic DNA. Reactions were run on a Hybaid PxE Thermocycler (Thermo Electron Corporation, Milford, MA), 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. Five-μl aliquots of the PCR products were electrophoresed through a 1% agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide. The PCR products obtained were purified using a single-step enzymatic clean-up that eliminates unincorporated primers and dNTPs. 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, Stabvida, Co., Oeiras, Portugal).

The various forward and reverse sequence segments were aligned manually with ClustalW (Thompson *et al*. 1994) in MEGA 5 software, and ambiguous bases were clarified using corresponding ABI chromatograms. To evaluate the relationship of *Pleistophora beebei* sp. nov. to other microsporidians, we first selected all rDNA sequences that have a fish as their host (results not shown) and then used 34 rDNA sequences belonging to groups 2 and 3 according to the classification provided by Lom and Nilsen (2003). The rDNA sequence of *Potaspora morhaphis* (EU534408) was used as outgroup. The alignment was performed using MUSCLE (Edgar 2004) in MEGA 5 software (Tamura *et al*. 2011), following the default parameters. After trimming the LSU rRNA 3'-end, the resulting alignment comprised 2013 informative characters in the final dataset. Subsequent phylogenetic and molecular evolutionary analyses were conducted in MEGA 5 using the maximum likelihood methodology. The general time reversible substitution model with 4 gamma distributed rate variation among sites was performed. All positions with less than 75% site coverage were eliminated from the trees and the bootstrap consensus tree was inferred from 500 replicates.

Distance estimation was carried out in MEGA 5 using the p-distance model distance matrix for transitions and transversions, with all positions with less than 75% site coverage eliminated. The number of base differences per sequence from between sequences were also shown.

RESULTS

Pleistophora beebei sp. nov.

Systematic position

Phylum Microsporidia Balbiani, 1882

Class Marinosporidia Vossbrinck and Debrunner-Vossbrinck, 2005

Family Pleistophoridae Doflein, 1901

Genus *Pleistophora* Gurley, 1893

Species *Pleistophora beebei* sp. nov.

Sporogonic stages

Few advanced sporogonic stages were observed. Inside the whitish cyst-like a lot of mature spores of two sizes were observed (Fig. 1 A-G).

Mature spores

The main morphological dissimilarities found between the two spore types were the dimensions of the spores and the polar filament arrangements. Microspores measured 4.7 ± 0.3 (4.4 – 5.0) × 2.8 ± 0.4 (2.3 – 3.0) μm (n = 25) (Figs 1-3) and contained a polar filament coiled in 9-10 turns, organized in a single row that surrounded the posterior vacuole (Fig. 1 D-F). Macrospores measured 7.8 ± 0.4 (7.4 – 8.1) × 4.7 ± 0.2 (4.3 – 4.9) μm (n = 25) and contained a polar filament irregularly coiled in 28-30 turns organized in 2-3 rows (Figs 1 E-G).

Description of the species

Eighteen of the 30 specimens of *Brachyhypopomus beebei* Schultz, 1944 (family Hypopomidae, order Gymnotiformes) analysed contained several irregular whitish cyst-like, located in the skeletal muscle of the ventral abdominal cavity. Semi-thin and ultrathin sections from each cyst-like were examined and found to contain two types of spores: a smaller number of macrospores surrounded by numerous microspores (Fig. 1 B and C). At the periphery, several fibroblasts surrounding the cyst-like were observed (Fig. 1 D). Both types of spores displayed similar ultrastructural characteristics, both having pyriform shape, tapering more anteriorly than posteriorly, and presenting the wall composed by two distinct layers: an external thin electron dense layer (~ 60 nm thick), and an internal thick electron-lucent layer (~ 125 nm thick) (Figs 1 F-G and 2). Moreover, the anchoring disc and lateral extensions at the apical end of the spore were found to be in continuity with the anterior part of the polar filament (manubrium), which was posteriorly projected obliquely in relationship to the spore axis. The membranous system constituting the polaroplast was folded around the manubrium (Fig. 1 F-G). The nucleus contained little chromatin, and was located between the polaroplast and the posterior vacuole. The latter contained flocculent material (Fig. 1 F-G).

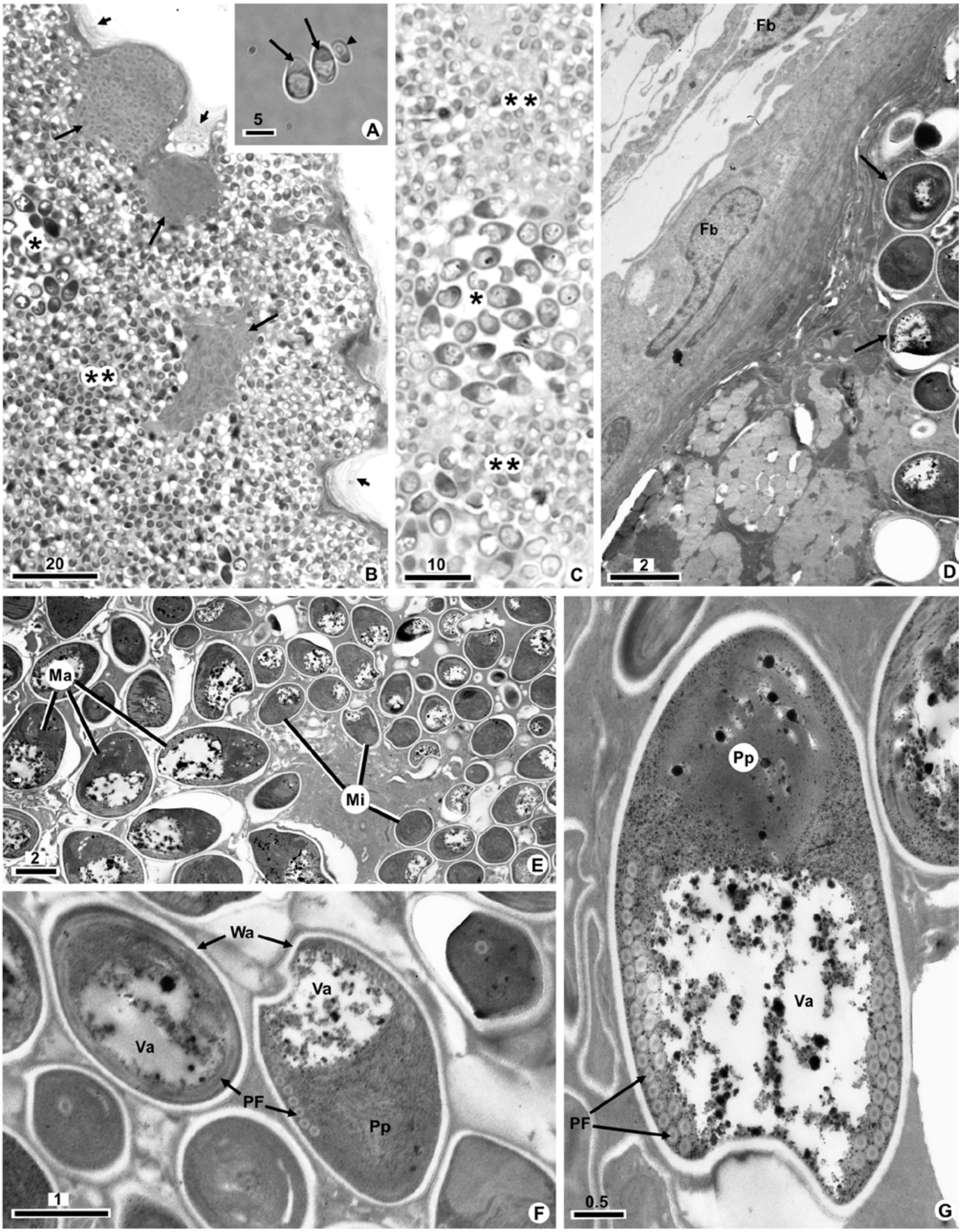
Prevalence and other characters

Type host: *Brachyhypopomus beebei* Schultz, 1944 (fam. Hypopomidae, order Gymnotiformes).

Host size: 15-18 cm in length.

Type locality: Estuarine region of the lower Amazon River, near the city of "Peixe Boi" (01° 11′ S /47° 18′ W), State of Pará, Brazil.

Location in the host: Skeletal muscle of the internal abdominal cavity, near the gut.



5
A
20
B
10
C
2
D
Fb
Fb
Ma
Mi
2
E
Wa
Va
Va
PF
Pp
1
F
Pp
Va
PF
0.5
G

Prevalence and intensity: Eighteen out of 30 fish were infected (60%). No statistical difference between sexes was observed.

Type specimens: One glass slide with semithin sections containing mature spores of the hapantotype was deposited in the Type Slide Collection of the Laboratory of Animal Pathology at the Interdisciplinary Centre of Marine and Environmental Research, Porto, Portugal, reference CIIMAR/UP 2016.12.

Etymology: The specific name "beebei" derives from the species epithet of the host species.

Molecular analysis

The pair of primers V1f / HG5F_rev and HG4F / HG4R amplified fragments with an approximate size of 900 bp and 1100 bp, respectively. Once aligned, the forward and reverse sequences permitted the construction of a consensus sequence with 1863 bp in length, and a GC content of 50.5%, providing an almost complete SSU rRNA gene sequence + ITS region and partial LSU rRNA gene for *Pleistophora beebei* sp. nov. This sequence was deposited in GenBank with the accession number GX099692. BLAST analysis was performed, with the highest score being found to correspond to the SSU rRNA sequences obtained from fish-infecting microsporidians, particularly those positioned in group 3, following the classification provided by Lom and Nilsen (2003). Overall, *P. hyphessobryconis* (KM458272) was the sequence that presented the closest relationship, according to BLAST.

Pairwise distances between the SSU rRNA sequences revealed that *P. beebei* sp. nov. exhibits greatest similarity (98.5%) to *P. hyphessobryconis*, a microsporidian described from the ornamental fish *Paracheirodon innesi* (neon tetra) (GU126672) and *Puntius tetrazona* (JN575482), and one other non-ornamental hybrid fish (*Leiarius marmoratus* ×*Pseudoplatystoma reticulatum*) (KM458272). The next closest matches among the *Pleistophora* spp. sequenced corresponded to entries of *Ovipleistophora* spp., *Heterosporis* spp. and *Dasyatispora* sp., with similarities between 96.4% and 98.0%. Comparing with others *Pleistophora* species the percentage of identity varies between 93.4% and 96.3% (Table 1).

Maximum Likelihood analyses of the SSU rRNA gene revealed *P. beebei* sp. nov. clustering within the clade composed by species of the genera *Dasyatispora*, *Heterosporis*, *Ovipleistophora* and *Pleistophora* species (group 3, bootstrap 41%). Within this clade, the parasite forms a robust subclade together with some sequences of *Pleistophora* spp. and all *Ovipleistophora* spp. (bootstrap 93%) (Fig. 3).

DISCUSSION

The morphological and ultrastructural aspects here described identify the parasite as a microsporidian species of the family Pleistophoridae Doflein, 1901. This family includes four genera: *Dasyatispora*, *Heterosporis*, *Ovipleistophora* and *Pleistophora*, all of which develop within the cytoplasm, lack the formation of xenomas, and present spore differentiation within sporophorous vesicles, with dimorphism being a common occurrence (Lom and Dyková 1992, Lom 2002). Despite the parasite reported presenting all of the latter, it lacks the differentiation of a secondary thick envelope (sporophorocyst wall), as is typical of the genus *Heterosporis* (Lom *et al*. 2000), it does not infect the oocytes and forms a thick envelope made up of tiny vesicles, as seen in *Ovipleistophora* (Pekkarinen *et al*. 2002), but it presents spore dimorphism, contrarily to what is seen in *Dasyatispora* (Diamant *et al*. 2010). On the other hand, the morphological and ultrastructural data obtained from the spores, combined with the host reaction

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Fig. 1 A-G. Light and TEM micrographs of the microsporidian *Pleistophora beebei* sp. nov. A - two macrospores (arrows) and a microspore (arrowhead) observed with DIC – Nomarski optics. B - semi-thin section of a cyst-like showing some advanced sporogonic stages, in which groups of development stages (arrows), macrospores (*) and microspores (**), as well as the external layer of fibroblasts (double arrows) can be observed. C - semi-thin section of a cyst-like showing some groups of macrospores (*) among numerous mature microspores (**). D - ultrathin section of the periphery of a cyst-like mass showing the external layers of fibroblasts (Fb) and, internally, several mature microspores (arrows). E - ultrathin section showing some groups of macrospores (Ma) and microspores (Mi) located in the same xenoma. F - ultrastructural detail of some microspores showing the wall (Wa), polaroplast (Pp) and sections of the polar filament (PF), which is organized in one row surrounding the posterior vacuole (Va). G - ultrastructural detail of a macrospore showing the polaroplast (Pp) and transverse sections of the polar filament (PF) organized in 2-3 rows around the posterior vacuole (Va). The latter contains flocculent material. (All scale bars in µm).

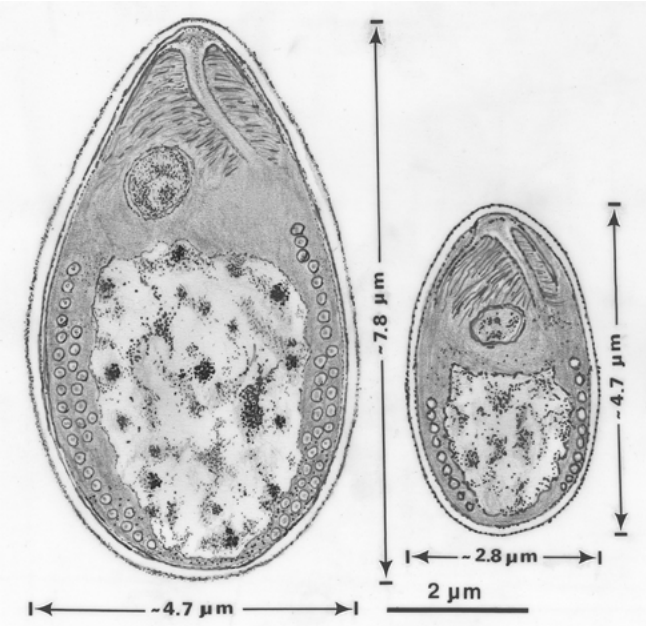

Fig. 2. Semi-schematic drawings of a macrospore (A) and a microspore (B). (The scale bar corresponds to the two schematic drawings).

in terms of the formation of a cyst-like encapsulated by connective tissue, is congruent with the characteristics of the genus *Pleistophora* (Canning and Nicholas 1980; Canning and Hazard 1982; Lom and Dyková 1992, 2005; Larsson 1999). Among the non-xenoma-forming genera, *Pleistophora* are known to cause significant damages in the host tissues, namely in the skeletal muscle of fish (Lom and Dyková 1992, Larsson 1999, Shaw and Kent 1999, Lom 2002). The eight previously described species of the genus *Pleistophora* (Shaw and Kent 1999, Lom 2002) that differentiate macrospores and microspores in fish hosts, differ from the parasite in study in terms of spore morphology, host reaction and host specificity (Table 2). Comparing these data, we may conclude that our results are sufficiently distinct to warrant a new species of the genus *Pleistophora,* which we propose to name *Pleistophora beebei* sp. nov.

Usually the microsporidian classified as *Pleistophora* spp. develops cyst-like granuloma being this complex structure composed of fibroblasts, collagen fibres, phagocytes engulfing spores, free spores, and some destroyed cells among the spores. This structure seems to result from inflammatory cell infiltration into the myosepta, as the presence of the microsporidian provokes an influx of macrophages that phagocytize and digest the spores, as previously reported for other species (Dyková and Lom 1980, Pulsford and Matthews 1991). The engulfment of the spores by the phagocytes in infected skeletal muscle is a physiological process described in several host fish (Morrison 1984). The proliferative granulomatous inflammatory response can cause the destruction of infected skeletal muscle and spores Canning and Lom (1986). Shaw and Kent (1999) further reported the occurrence of myoliquefaction as a result of this process. Although our observations had been at the final phase of sporogenesis, it was not possible to observe the formation of granuloma, structure commonly presents in the genus *Pleistophora*.

Up until the implementation of molecular procedures, several microsporidian species were classified as *Pleistophora*, from both invertebrate and vertebrate hosts. Molecular studies, however, revealed the polyphyly of the genus, consequently leading to the reclassification of some species. The genera *Endoreticulatus*, *Cystosporogenes* and *Vavraia* were erected to encompass those species infecting invertebrates (Pilarska *et al*. 2015). During the last decade, and similarly to the genus *Glugea*, some fish-infecting *Pleistophora* were also reclassified and allocated into new genera, with basis on morphological aspects and molecular data. For example, the parasite found in the eel *Anguilla japonica*, *Pleistophora anguillarum*, was transferred to the genus *Heterosporis* (Lom *et al*. 2000); and the genus *Ovipleistophora* was created for two *Pleistophora* spp. that parasitize oocysts in teleost fish (Lom *et al.* 2002).

A close comparison between *P. beebei* sp. nov. and other *Pleistophora* spp. that form macrospores and microspores, allows it morphological distinction from all of the latter (Table 2). With the exception of *P. macrozoarcidis* (Lom and Dyková 1992), all other species present smaller macrospores and microspores. Furthermore, unlike most *Pleistophora*, *P. dammami* (Abdel-Baki *et al*. 2012) and *P. priacanthusis* (Ding-Ke and Han-Ji 1983) infect the intestinal wall rather than the skeletal muscle. Another differential aspect is the habitat. The species described here parasitizes a freshwater fish. Considering some other *Pleistophora* spp. that have also been molecularly studied, the differentiation of monomorphic spores has been described in *P. aegyptiaca* (Abdel-Ghaffar *et al*. 2012), *P. finisterrensis* (Leiro *et al*. 1996), *P. pagri* (Morsy *et al*. 2012), *P. hippoglossoides* (Morriosn *et al*. 1984) and *P. hyphessobryconis* (Lom and Corliss 1967, Li *et al*. 2012).

Analysing the molecular and phylogenetic data of the microsporidians that have so far been sequenced,

Table 1. Comparison of some rDNA sequences: percentage of identity (top diagonal) obtained by p-distance and nucleotide difference (bottom diagonal).

	pb	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)
(1) *Pleistophora beebei* sp. nov. (KX099692)	1861	-	98.5	98.5	98.5	98.5	98.0	98.0	97.1	96.8	96.8	96.7	96.6	96.4	96.3	94.6	94.4	94.3	94.3	94.2	93.7	93.4
(2) *Pleistophora hyphessobryconis* (GU126672)	1361	40	-	99.9	99.6	99.6	97.7	97.4	96.7	96.5	96.8	95.8	96.5	96.0	96.3	94.8	94.0	93.9	94.0	93.8	93.3	93.0
(3) *Pleistophora hyphessobryconis* (JN575482)	1360	40	6	-	99.6	99.7	97.6	97.4	97.0	96.7	96.9	96.1	96.6	96.0	96.3	95.0	94.0	93.9	94.1	93.8	93.4	93.0
(4) *Pleistophora hyphessobryconis* (KM458272)	1908	81	7	6	-	99.6	97.3	97.4	96.7	96.5	96.5	96.1	96.3	96.0	95.9	94.6	94.0	93.9	94.0	93.8	93.3	93.0
(5) *Pleistophora* sp. KB-2011 (HQ703580)	1467	66	4	3	6	-	97.4	97.5	97.1	96.9	96.9	96.3	96.6	96.2	97.0	95.2	94.4	94.3	94.2	94.2	93.5	93.4
(6) *Ovipleistophora mirandellae* (AF356223)	1363	45	52	52	53	31	-	98.5	96.1	95.9	96.0	96.3	95.9	95.8	94.2	93.3	94.0	93.9	93.7	93.8	92.9	93.0
(7) *Ovipleistophora ovariae* (AJ252955)	1397	56	55	54	57	38	31	-	96.0	95.9	95.8	96.3	95.5	95.9	95.5	93.4	94.0	93.9	93.6	93.8	93.0	93.1
(8) *Heterosporis suntherlandae* (KC137553)	1826	120	53	51	125	104	53	65	-	98.7	98.8	97.8	99.7	97.2	96.7	97.5	94.6	94.5	94.4	94.4	93.8	93.6
(9) *Heterosporis anguillarum* (AF387331)	4250	139	71	70	140	105	82	86	73	-	100	99.6	98.4	97.2	97.9	98.3	94.6	94.5	94.4	94.4	94.0	93.4
(10) *Heterosporis*-like (KT380107)	1210	66	64	65	66	32	76	77	21	1	-	99.5	98.5	97.1	97.9	98.3	94.3	94.2	94.1	94.1	93.6	93.2
(11) *Heterosporis saurida* (JF745533)	950	90	25	24	97	97	23	29	66	20	2	-	96.9	96.9	*	81.6	96.1	95.8	96.1	96.1	95.8	93.2
(12) *Heterosporis* sp. (AF356225)	1311	79	79	79	80	38	81	88	7	35	29	20	-	96.7	96.7	97.5	94.1	94.0	93.9	93.9	93.2	93.0
(13) *Dasyatispora levantinae* (GU183263)	1825	156	87	88	158	117	89	92	107	129	66	85	73	-	96.3	95.5	93.9	93.8	93.6	93.7	93.1	92.6
(14) *Pleistophora pagri* (JF797622)	249	12	12	12	13	6	16	14	8	9	9	*	12	12	-	99.6	90.5	90.5	91.6	90.5	91.1	90.5
(15) *Pleistophora aegyptiaca* (JF514548)	522	28	27	26	28	23	35	34	13	9	9	9	13	23	1	-	92.5	92.5	91.4	92.0	91.1	92.1
(16) *Pleistophora* sp. 2 (AF044389)	1830	174	109	105	186	133	104	115	140	171	91	95	105	192	27	39	-	99.9	99.7	99.8	98.8	96.6
(17) *Pleistophora hippoglossoideos* (AJ252953)	1372	114	112	108	115	60	106	110	76	103	92	24	108	114	27	39	11	-	99.5	99.7	98.4	96.5
(18) *Pleistophora ehrenbaumi* (AF044392)	1397	128	55	54	139	138	60	67	128	130	48	96	55	140	15	39	35	13	-	99.9	98.6	95.9
(19) *Pleistophora typicalis* (AF044387)	1864	175	116	112	191	134	111	122	142	177	93	91	109	194	27	41	18	15	33	-	98.5	96.4
(20) *Pleistophora* sp. 1 (AF044394)	1438	127	65	64	142	140	70	77	123	126	54	93	62	193	18	43	24	26	44	30	-	95.1
(21) *Pleistophora* sp. 3 (AF044390)	1879	236	118	114	241	187	112	121	197	236	101	158	113	244	27	41	157	39	162	168	170	-

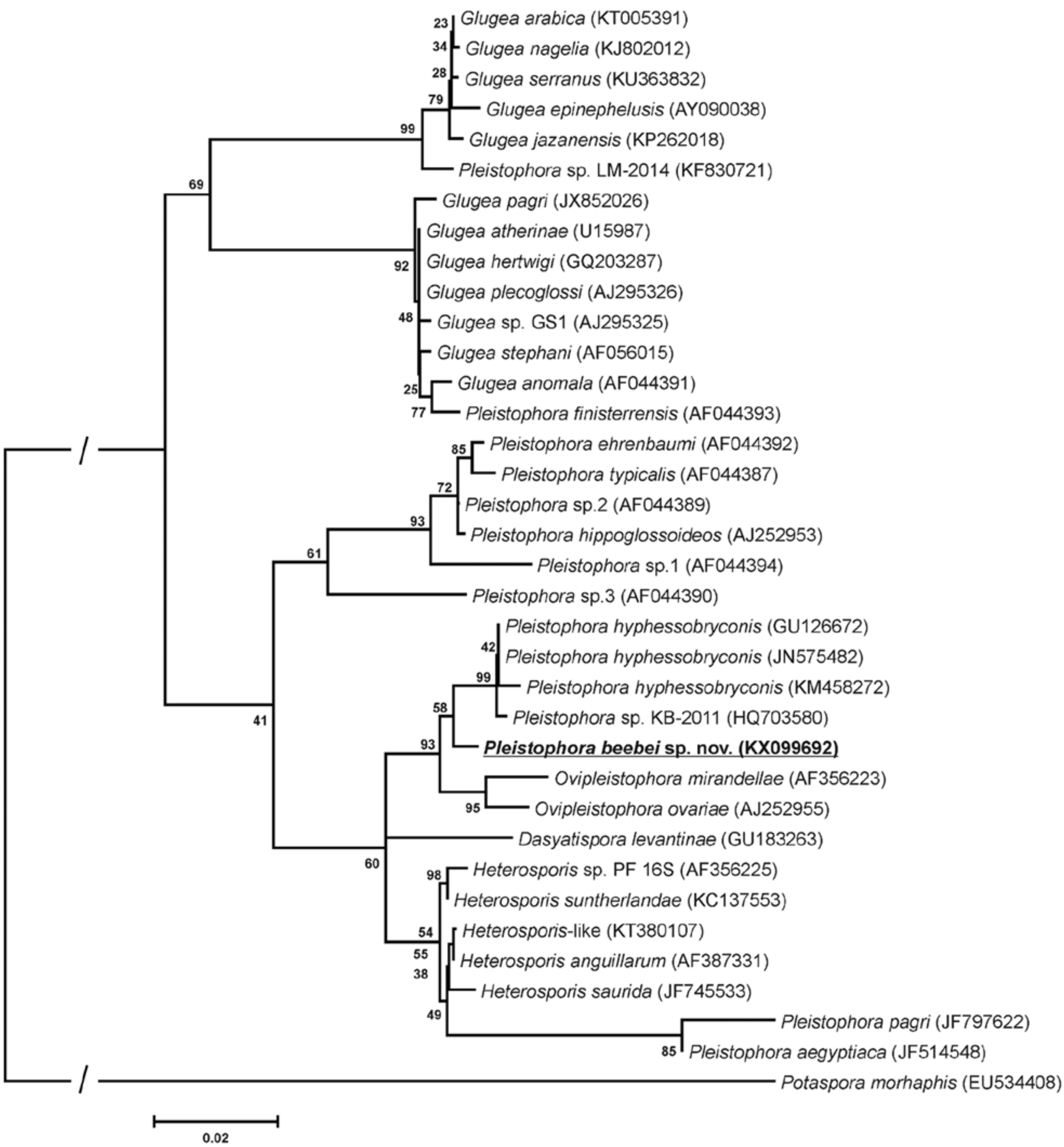

Fig. 3. Maximum Likelihood tree showing the relationship *of Pleistophora beebei* sp. nov. to other microsporidians based on the rDNA sequences. The numbers on the branches are bootstrap confidence levels on 500 replicates for ML trees. The tree was generated using 34 microsporidian selected sequences, with *Potaspora morhaphis* as the outgroup species. The bar indicates the equivalence between the distance and the number of changes. GenBank accession numbers are in parenthesis after the species name. There were a total of 966 positions in the final dataset.

there is also strong evidence that the species described here is indeed a new *Pleistophora* species, residing in a subclade of group III, according to the classification proposed by Lom and Nilsen (2003). This group is composed of two well-supported subclades, one of which divides into two subgroups. *P. beebei* sp. nov. clusters in one of these subgroups, alongside four sequences that, despite corresponding to different hosts, all refer to the same parasitic organism, *P. hyphessobryconis* (GU126672, KM458272, JN575482, and probably HQ703580), as well as *Ovipleistophora ovariae* and *O. mirandellae*, both from the ovaries. The sister subgroup is composed of all *Heterosporis* spp., as well as *P. pagri* and *P. aegyptiaca*. The phylogenetic positioning of these two latter *Pleistophora* species is, however, dubious since their SSU rRNA gene sequences correspond to only 249 and 522 nt, respectively. Also clustering within this subclade is *Dasyatispora levantinae*, which parasitizes the common stingray *Dasyatis pastinaca* in the eastern Mediterranean (Diamant *et al*. 2010). The remaining *Pleistophora* spp. with close molecular identity form a strong subclade within group III of the microsporidians infecting fish. The only exceptions are two microsporidians that cluster in group II, mainly composed by *Glugea* spp. *Pleistophora finnisterrensis*, which clusters in this group, in fact presents morphological aspects that are similar to those of the genus *Glugea*, namely the formation of xenoma and monophormic spores. Consequently, it has been suggested that this species warrants reclassification (Casal *et al*. 2016).

Overall, the comparisons here performed revealed *P. hyphessobryconis* as the species presenting highest similarity to the parasite in study. Comparing the morphometric and ultrastructural aspects, however, some differences are noticed: *P. hyphessobryconis* forms ovoid spores of equal size, which measurements range between those obtained for the macrospores and microspores in this study, and its polar filament coils in 2−3 layers, forming 36−42 turns that surround the posterior vacuole (Li *et al*. 2012). The small genetic distance found between *P. bebeei* sp. nov. and *P. hyphessobryconis* can be explained by the proximity of these species, which both parasite freshwater fish hosts in the hydrographic basin of the Amazon River.

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REFERENCES

Abdel-Baki A. S., Al-Quraishy S., Al-Qahtani H., Dkill M. A., 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

Abdel-Baki A. S., Al-Quraishy S., Rocha S., Kkhil M. A., Casal G., Azevedo C. (2015) Ultrastructure and phylogeny of *Glugea nagelia* sp. n. (Microsporidia: Glugeidae), infecting the intestinal wall of the yellowfin hind, *Cephalopholis hemistiktos* (Actinopterygii: Serranidae), from the Red Sea. *Folia Parasitol.* **62:** 007

Abdel-Ghaffar F., Bashtar A. R., Morsy K., Mehlhorn H., Al Quraishy S., Al-Rasheid K., Abdel-Gaber R. (2012) Morphological and molecular biological characterization of *Pleistophora aegyptiaca* sp. nov. infecting the Red Sea fish *Saurida tumbil*. *Parasitol. Res.* **110:** 741−752

Azevedo C., Casal G., Matos P., Alves A., Matos E. (2011) *Henneguya torpedo* sp. nov. (Myxozoa), a parasite from the nervous system of the Amazonian teleost *Brachyhypopomus pinnicaudatus* (Hypopomidae). *Dis. Aquat. Org.* **93:** 235−242

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

Azevedo C., Matos E. (2003) *Amazonspora hassar* n. gen. and n. sp. (Phylum Microsporidia, fam. Glugeidae), a parasite of the Amazonian teleost, *Hassar orestis* (fam. Doradidae). *J. Parasitol.* **89:** 336−341

Canning E. U., Hazard E. I. (1982) Genus *Pleistophora* Gurley, 1893: An assemblage of at least three genera. *Protozoology* **29:** 39−49

Canning E. U., Lom J. (1986) The Microsporidia of Vertebrates, Academic Press, London

Canning E. U., Nicholas J. P. (1980) Genus *Pleistophora* (Phylum Microspora): redescription of the type species, *Pleistophora typicalis* Gurley, 1893 and ultrastructural characterization of the genus *J. Fish Dis.* **3:** 317−338

Casal G., Matos E., Teles-Grilo M. L., Azevedo C. (2008) A new microsporidian parasite, *Potaspora morhaphis* n. gen., n. sp. (Microsporidia) infecting the teleostean fish, *Potamorhaphis guianensis* from the River Amazon. Morphological, ultrastructural and molecular characterization. *Parasitology* **135:** 1053−1064

Casal G., Matos E., Teles-Grilo M. L., Azevedo C. (2009) Morphological and genetical description of *Loma psittaca* sp. n. isolated from the Amazonian fish species *Colomesus psittacus*. *Parasitol. Res*. **105:** 1261−1271

Casal G., Matos E., Teles-Grilo L., Azevedo C. (2010) Ultrastructural and molecular characterization of a new microsporidium parasite from the Amazonian fish, *Gymnorhamphichthys rondoni* (Rhamphichthyidae). *J. Parasitol.* **96:** 1155−1163

Casal G., Rocha S., Costa G., Al-Quraishy S., Azevedo C. (2016) Ultrastructural and molecular characterization of *Glugea serranus* n. sp., a microsporidian infecting the blacktail comber, *Serranus atricauda* (Teleostei: Serranidae), in the Madeira Archipelago (Portugal). *Parasitol. Res.* **115**: 3963-3972

Crampton W. G. R. (1996) Gymnotiform fish: An important component of Amazonian floodplain fish communities. *J. Fish Biol.* **48:** 298–301
Cellere E. F., Cordeiro N. S., Adriano E. A. (2002) *Myxobolus obsonus* sp. n. (Myxozoa, Myxosporea) parasitizing *Pimelodus maculatus* (Siluriformes: Pimelodidae), a South American freshwater fish. *Mem. Inst. Oswaldo Cruz* **97:** 79–80
Diamant A., Goren M., Yokes M. B., Galil B. S., Klopman Y., Huchon D., Szitenberg A., Karhan S. U. (2010) *Dasyatispora levantinae* gen. et sp. nov., a new microsporidian parasite from the common stingray *Dasyatis pastinaca* in the eastern Mediterranean. *Dis. Aquat. Org.* **91:** 137–150
Diamant A., Rothman S. B. S., Goren M., Galil B. S., Yokes M. B., Szitenberg A., Huchon D. (2014) Biology of a new xenoma-forming gonadotropic microsporidium in the invasive blotchfin dragonet *Callionymus filamentosus*. *Dis. Aquat. Org.* **109:** 35–54
Ding-Ke H., Han-Ji D. (1983) A new species of *Pleistophora* from big-eyes, *Priacanthus tayenus* Richardson, and *Priacanthus macracanthus* Cuvier and Valenciennes, from the South China Sea. *J. Fish Dis.* **6:** 293-301
Dyková I., Lom J. (1980) Tissue reactions to microsporidian infections in fish. *J. Fish Dis.* **3:** 265–283
Edgar R. C. (2004) "MUSCLE: multiple sequence alignment with high accuracy and high throughput". *Nucl. Acids Res.* **32:** 1792–1797
Gatehouse H. S., Malone L. A. (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. Invertebr. Pathol.* **71:** 97–105
Larsson J. I. R. (1999) Identification of Microsporidia. *Acta Protozool.* **38:** 161–197
Leiro J., Ortega M., Iglesias R., Estevez J., Sanmartin M. L. (1996) *Pleistophora finisterrensis* n. sp., a microsporidian parasite of blue whiting *Micromesistius poutassou*. *Syst. Parasitol.* **34:** 163–170
Li K., Chang O., Wang F., Liu C., Liang H., Wu S. (2012) Ultrastructure, development, and molecular phylogeny of *Pleistophora hyphessobryconis*, a broad host microsporidian parasite of *Puntius tetrazona*. *Parasitol. Res.* **111:** 1715–1724
Lom J. (2002) A catalogue of described genera and species of microsporidians parasitic in fish. *Syst. Parasitol.* **53:** 81–99
Lom J., Corliss J. O. (1967) Ultrastructural observations on the development of the microsporidian protozoon *Plistophora hyphessobryconis* Schaperclaus. *J. Protozool.* **14:** 141–152
Lom J., Dyková I. (1992) Microsporidia (Phylum Microspora Sprague, 1977). In: Protozoan Parasites of Fishes. Developments in Aquaculture and Fisheries Sciences, vol. 26 (Eds. J. Lom, I. Dyková). Elsevier, Amsterdam, 125–157
Lom J., Dyková I. (2005) Microsporidian xenomas in fish seen in wider perspective. *Folia Parasitol.* **52:** 69–81
Lom J., Dyková I., Wang C. H., Lo C. F., Kou G. H. (2000) Ultrastructural justification for the transfer of *Pleistophora anguillarum* 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. *Int. J. Parasitol.* **33:** 107–127
Matos E., Azevedo C. (2004) Ultrastructural description of *Microsporidium brevirostris* sp. n., parasite of the teleostean *Brachyhypopomus brevirostris* (Hypopomidae) from the Amazon River. *Acta Protozool.* **43:** 261–267
Mendoza-Franco E. F., Reina R. G. (2008) Five new species of *Urocleidoides* (Monogenoidea) (Mizelle and Price 1964) Kritsky, Thatcher, and Boeger, 1986, parasitizing the gills of Panamanian freshwater fishes. *J. Parasitol.* **94:** 793–802
Morrison C. M., Marryatt V., Gray B. (1984) Structure of *Pleistophora hippoglossoideos* Bosanquet in the American Plaice *Hippoglossoides platessoides* (Fabricius). *J. Parasitol.* **70:** 412–421
Morsy K., Abdel-Ghaffar F., Mehlhorn H., Bashtar A. R., Abdel-Gaber R. (2012) Ultrastructure and molecular phylogenetics of a new isolate of *Pleistophora pagri* sp. nov. (Microsporidia, Pleistophoridae) from *Pagrus pagrus* in Egypt. *Parasitol. Res.* **111:** 1587–1597
Nilsen F. (2000) Small subunit ribosomal DNA phylogeny of microsporidia with particular reference to genera that infect fish. *J. Parasitol.* **86:** 128–133
Pekkarinen M., Lom J., Nilsen F. (2002) *Ovipleistophora* gen. n., a new genus for *Pleistophora* mirandellae-like microsporidia. *Dis. Aquat. Org.* **48:** 133–142
Pilarska D. K., Radek R., Huang W-F., Takov D. I., Linde A., Solter L. F. (2015) Review of the genus *Endoreticulatus* (Microsporidia, Encephalitozoonidae) with description of a new species isolated from the grasshopper *Poecilimon thoracicus* (Orthoptera: Tettigoniidae) and transfer of *Microsporidium itiiti* Malone to the genus. *J. Invertebr. Pathol.* **124:** 23–30
Pulsford A., Matthews R. A. (1991) Macrophages and giant-cells associated with a microsporidian parasite causing liquefaction of the skeletal-muscle of the Norway pout, *Trisopterus esmarkii* (Nilsson). *J. Fish Dis.* **14:** 67–78
Shaw R. W., Kent M. L. (1999) Fish Microsporidia. In: The Microsporidia and Microsporidiosis, (Eds. M. Wittner, L. M. Weiss). AMS Press, Washington, D.C., 418–446
Stentiford G. D., Feist S. W., Stone D. M., Bateman K. S., Dunn A. M. (2013) Microsporidia: Diverse, dynamic and emergent pathogens in aquatic systems. *Trends in Parasitol.* **29:** 567–578
Tamura K., Dudley J., Nei M., Kuma S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28:** 2731–2739
Thompson J. D., Higgins D. G., Gilson T. J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **22:** 4673–4680
Vávra J., Larsson J. I. R. (1999) Structure of the Microsporidia. In: The Microsporidia and Microsporidiosis, (Eds. M. Wittner, L. M. Weiss). ASM Press, 7–48
Videira M., Casal G., Rocha S., Gonçalves E., Azevedo C., Velasco M., Matos E. (2015) *Potaspora aequidens* n. sp. (Microsporidia, Tetramicridae), a parasite infecting the freshwater fish *Aequidens plagiozonatus* (Teleostei, Cichlidae) from Brazil. *Parasitol. Res.* **114:** 2435–2442
Winters A. D., Langohr I. M., Souza M. A., Colodel E. M., Soares M. P., Faisal M. (2016) Ultrastructure and molecular phylogeny of *Pleistophora hyphessobryconis* (Microsporidia) infecting hybrid jundiara (*Leiarius marmoratus* × *Pseudoplatystoma reticulatum*) in a Brazilian aquaculture facility. *Parasitology*, **143:** 41–49

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