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±0.4 × 4.7±0.2 µm) were observed among the numerous microspores (lightly pyriform to ellipsoidal with rounded ends, measured about 4.7±0.3 × 2.8±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.

**Key words:** 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
Urocleidoides (Monogeneidea) 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 morphapis 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. 2016). Two species have also been reported from the genus Loma: L. myrophis infecting the sub-epithelial gut tissue of Myrophis platyrhynchos (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 hyphesobryconis 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: “Huatuanga”) were collected from the Amazon River near the City of Peixe Boi (01°11′S / 47°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°C). 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°C, washed overnight in the same buffer at 4°C and post-fixed in 2% OsO4, 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 × 106 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°C. The majority of the region coding for the SSU rRNA gene was amplified by PCR 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°C. The majority of the region coding for the SSU rRNA gene was amplified by PCR using the primers V1f (5′-CCCAAGGGTTGATTCTGC-3′) (Nilsen 2000) and HG5F_rev (5′-GCAACCCACTTCGTTTAAC-3′) (Abdel-Baki et al. 2015). The primers HG4F (5′-CCGTTAATTGTACCTC-3′) and HG4R (5′-TCTCCTTGGCTGTGTTC-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 MgCl2, 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 microsporidia, 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 (EU334408) 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 (Fig. 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 1–3) and contained a polar filament coiled in 9–10 turns, organized in a single row that surrounded the posterior vacuole (Fig. 1 D–F). 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.
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 + 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 (*Lettarius marmoratus × Pseudoplattystoma reticulatum*) (KM458272). The next closest matches among the *Pleistophora* spp. sequenced corresponded to entries of *Ovipleistophora* spp., *Heterosporis* spp. and *Dasyatipora* 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 Dolfin, 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...
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. aegyptica* (Abdel-Ghaffar et al. 2012), *P. finisterrensis* (Leiro et al. 1996), *P. pagri* (Morsy et al. 2012), *P. hippoglossoides* (Morrison et al. 1984) and *P. hypphesso-bryconis* (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).

| (1) Pleistophora beebei sp. nov. (KX099692) | 1861 – 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 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 96.1 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 – 98.5 97.3 97.4 96.7 96.5 96.3 96.6 96.2 97.0 95.2 94.4 94.3 94.2 94.2 93.5 94.0 93.4 |
| (6) Ovipleistophora mirandeliae (AF356223) | 1363 45 52 52 53 31 – 98.5 96.1 95.9 96.0 95.8 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 94.6 94.0 94.4 94.4 94.4 94.0 93.8 93.6 |
| (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 94.0 93.8 93.6 |
| (9) Heterosporis anguillare (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 94.1 94.1 93.6 93.2 |
| (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 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 93.9 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 hippocloboideos (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 chironibaui (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 morphas* 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. beebei* 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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