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

G. CASAL[1,2,3], E. MATOS[4], M. L. TELES-GRILO[5] *and* C. AZEVEDO[1,3]*

[1] *Department of Cell Biology, Institute of Biomedical Sciences, University of Porto (ICBAS/UP), Lg. A. Salazar no. 2, P-4099-003 Porto, Portugal*
[2] *Department of Sciences, High Institute of Health Sciences, P-4585-116 Gandra, Portugal*
[3] *Laboratory of Pathology, Centre for Marine Environmental Research (CIIMAR/UP), 4050-123 Porto, Portugal*
[4] *Carlos Azevedo Research Laboratory, Federal Rural University of Amazonia, 66.077-530 Belém (Pará), Brazil*
[5] *Genetics Molecular Laboratory, Institute of Biomedical Sciences, University of Porto (ICBAS/UP), Lg. A. Salazar no. 2, P-4099-003 Porto, Portugal*

(*Received 3 March 2008; revised 9 May 2008; accepted 10 May 2008*)

SUMMARY

A fish-infecting Microsporidia *Potaspora morhaphis* n. gen., n. sp. found adherent to the wall of the coelomic cavity of the freshwater fish, *Potamorhaphis guianensis*, from lower Amazon River is described, based on light microscope and ultrastructural characteristics. This microsporidian forms whitish xenomas distinguished by the numerous filiform and anastomosed microvilli. The xenoma was completely filled by several developmental stages. In all of these stages, the nuclei are monokaryotic and develop in direct contact with host cell cytoplasm. The merogonial plasmodium divides by binary fission and the disporoblastic pyriform spores of sporont origin measure $2{\cdot}8 \pm 0{\cdot}3 \times 1{\cdot}5 \pm 0{\cdot}2\,\mu$m. In mature spores the polar filament was arranged into 9–10 coils in 2 layers. The polaroplast had 2 distinct regions around the manubrium and an electron-dense globule was observed. The small subunit, intergenic space and partial large subunit rRNA gene were sequenced and maximum parsimony analysis placed the microsporidian described here in the clade that includes the genera *Kabatana*, *Microgemma*, *Spraguea* and *Tetramicra*. The ultrastructural morphology of the xenoma, and the developmental stages including the spores of this microsporidian parasite, as well as the phylogenetic analysis, suggest the erection of a new genus and species.

Key words: Amazonian fish, parasite, Microsporidia, ultrastructure, developmental stages, phylogeny, *Potaspora morhaphis* n. gen, n. sp.

INTRODUCTION

The phylum Microsporidia Balbiani, 1882 is represented by at least 144 available genera. It is characterized by unicellular eukaryotic microorganisms living as obligate intracellular parasites, commonly infecting fishes, insects, crustaceans, and other invertebrate and vertebrate groups from different geographical areas (Lom and Dyková, 1992; Sprague *et al*. 1992; Larsson, 1999; Lom, 2002). In a recent paper, Lom and Nilsen (2003) described the following 15 microsporidian genera as infecting fish: *Glugea* Thélohan, 1891; *Pleistophora* Gurley, 1893; *Ichthyosporidium* Caullery and Mesnil, 1905; *Heterosporis* Schubert, 1969; *Nosemoides* Vinckier, 1975; *Spraguea* Weissenberg, 1976; *Loma* Morrison and Sprague, 1981; *Tetramicra* Matthews and Matthews, 1980; *Microgemma* Ralphs and Matthews, 1986; *Microfilum* Faye, Toguebaye and Bouix, 1991; *Nucleospora* Hedrick, Graff and Baxa, 1991; *Neonosemoides* Faye, Toguebaye and Bouix, 1996; *Kabatana* Lom, Dyková and Tonguthai, 1999; *Pseudoloma* Matthews, Brown, Larison, Bishop-Stewart, Rogers and Kent, 2001; *Ovipleistophora* Pekkarinen, Lom and Nilsen, 2002. Recently 2 new genera were identified as infecting fish: *Amazonspora* in the gills of an Amazonian fish (Azevedo and Matos, 2003) and *Myosporidium* in muscle of commercial hake (*Merluccius* sp.) from fisheries near Namibia (Baquero *et al*. 2005).

There is very little knowledge about microsporidiosis in the ichthyological fauna of South

* Corresponding author: Department of Cell Biology, Institute of Biomedical Sciences, University of Porto, Lg. A. Salazar no. 2, P-4099-003 Porto, Portugal. Tel: +351 22 206 22 00. Fax: +351 22 206 22 32/33. E-mail: azevedoc@icbas.up.pt, gcasal@icbas.up.pt

doi:10.1017/S0031182008004654 Printed in the United Kingdom

America. Two Microsporidia were found in Amazonian fishes *Loma myrophis* (Azevedo and Matos, 2002; Matos *et al*. 2003) and *Microsporidium brevirostris* (Matos and Azevedo, 2004) in *Myrophis platyrhynchus* and *Brachyhypopomus brevirostris* host species respectively.

In this paper, we described a new genus and new species of a microsporidian through morphological and ultrastructural observations, with special reference to the ultrastructural aspects of the xenoma wall and the spore differentiation. Phylogenetic relationships comparing the *Potaspora morhaphis* SSU rRNA gene with that of other fish infecting microsporidian species was also done. The morphological characteristics and taxonomic position are discussed.

MATERIALS AND METHODS

Fish, location of infection and prevalence

Thirty specimens of freshwater teleost fish *Potamorhaphis guianensis* Schomburgk, 1843 (Teleostei, Belonidae) (Brazilian common name 'Peixe-Agulha'), were collected from the estuarine region of the Amazon River (01°11′ S/47°18′ W) near the city of Belém (Pará State), Brazil. The specimens were anaesthetized by MS 222 (Merck) and later measured (19–25 cm in length). Infection was determined by the presence of several xenomas located in the coelomatic cavity near the anal region. The prevalence of infection was 40% (12 fishes in 30 examined), in both sexes.

Light (LM) and transmission electron microscopy (TEM)

For LM smears of xenoma and free spores were observed directly without any fixation or stain by a light microscope equipped with Nomarski interference-contrast (DIC) optics.

For ultrastructural studies, the xenomas were excised and fixed in 3% glutaraldehyde in 0·2 M sodium cacodylate buffer (pH 7·2) at 4 °C for 24 h. After washing overnight in the same buffer at 4 °C and post-fixation in 2% osmium tetroxide in the same buffer and temperature for 3 h, the fragments were dehydrated through a graded ethanol ascending series, followed by propylene oxide (3 changes of 2 h each) and embedded in Epon (12 h in each change). Semi-thin sections were stained with methylene blue-Azur II and observed by DIC optics. Ultrathin sections were contrasted with aqueous uranyl acetate and lead citrate and observed with a JEOL 100CXII TEM, operated at 60 kV.

DNA isolation and PCR amplification

Several cysts were dissected from fishes, following homogenization to isolate the spores, and were then stored in 80% ethanol at 4 °C. The genomic DNA of about 5×10^6 spores was extracted using a GenElute™ 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 until used. The DNA concentration was estimated with the Qubit™ Fluorometer (Invitrogen). The majority of the region coding for the small subunit (SSU) rRNA gene was amplified by PCR using the primers V1f (5′CACCAGGTTGATTCTGCC3′) and 1492r (5′GGTTACCTTGTTACGACTT3′) (Vossbrinck *et al*. 1993; Nilsen, 2000). To amplify the 3′-end of the SSU, internal transcribed spacer (ITS) and 5′-end of the large subunit (LSU) rRNA gene, HG4F (5′GCGGCTTAATTTGACTCAAC) and HG4R (5′TCTCCTTGGTCCGTGTTTCAA) primers were used (Gatehouse and Malone, 1998). To obtain the 5′-end of the SSU gene region a primer was designed (454r – 5′AATTAAGCCGCACACTCCAC). PCR was carried out in 50 μl reactions using 10 pmol of each primer, 10 nmol of each dNTP, 2 mM of $MgCl_2$, 5 μl of 10X *Taq* polymerase buffer, 1·25 units *Taq* DNA polymerase (Invitrogen products), and 3 μl of the genomic DNA. The reactions were run on Hybaid PxE Thermocycler (Thermo Electron Corporation, Milford, MA). The amplification program consisted of 94 °C denaturation for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min. A final elongation step was performed at 72 °C for 10 min. Five μl aliquots of PCR products were visualized with ethidium bromide staining after running on a 1% agarose gel.

DNA sequencing

PCR products for the SSU gene and ITS region have approximate sizes of 1400 bp and 1100 bp respectively. They were cleaned using the MinElute PCR purification kit (QIAGEN) and then 3 purified PCR products were sequenced in both directions. Sequencing was done using BigDye Terminator v1.1 of Applied Biosytems Kit and the sequence reactions were run on an ABI3700 DNA analyser Perkin-Elmer, Applied Biosystems, Stabvida, Co., Oeiras, Portugal).

Distance and phylogenetic analysis

To evaluate the relationship of *Potaspora morhaphis* to other Microsporidia, we have used the 42 rDNA sequences, listed with their hosts in Table 1, obtained from GenBank data. The corresponding sequences and GenBank/NCBI Accession number of *Endoreticulatus schubergi* (L39109), *Enterocytozoon bieneusi* (L07123), *Vairimorpha necatrix* (Y00266) and *Vittaforma corneae* (L39112) were used as the

Table 1. Hosts and GenBank Accession numbers for the SSU rRNA sequences of 42 microsporidian that parasite fishes species used in the phylogenetic analyses

Microsporidian	Host	Accession number
Glugea anomala	*Gasterosteus aculeatus*	AF044391
Glugea atherinae	*Atherina prebyster*	U15987
Glugea plecoglossi	*Plecoglossus altivelis*	AJ295326
Glugea stephani	*Platichthys flesus*	AF056015
Glugea sp. GS1	*Gasterosteus aculeatus*	AJ295325
Glugea sp.	*Epinephelus awoara*	AY090038
Heterosporis anguillarum	*Anguilla japonica*	AF387331
Heterosporis sp. PF	*Perca flavescens*	AF356225
Ichthyosporidium sp.	*Leiostomus xanthurus*	L39110
Kabatana takedai	*Oncorhyncus masu*	AF356222
Kabatana newberryi	*Eucyclogobius newberryi*	EF202572
Kabatana seriolae	*Seriola quinqueradiata*	AJ295322
Loma acerinae	*Gymnocephalus cernuus*	AJ252951
Loma embiotocia	*Cymatogaster aggregate*	AF320310
Loma salmonae	*Oncorhynchus tshawytscha*	U78736
Loma sp.	*Encelyopus cimbrius*	AF104081
Microgemma caulleryi	*Hyperoplus lanceolatus*	AY033054
Microgemma tincae	*Symphodus tinca*	AY651319
Microgemma vivaresi	*Taurulus bubalis*	AJ252952
Microsporidium cypselurus	*Cypselurus pinnatibarbatus japonicus*	AJ300706
Microsporidium prosopium	*Prosopium williamsoni*	AF151529
Microsporidium sp. GHB1	*Sparus aurata*	AJ295324
Microsporidium sp. RSB1	*Pagrus major*	AJ295323
Microsporidium sp. STF	*Salmo trutta fario*	AY140647
Microsporidium MYX1	*Takifugu ruripes*	AJ295329
Myosporidium merluccius	*Merluccius* sp.	AY530532
Nucleospora salmonis	*Oncorhynchus tshawytscha*	U78176
Ovipleistophora mirandellae	*Gymnocephalus cernuus*	AF356223
Ovipleistophora ovariae	*Notemigonus crysoleucas*	AJ252955
Pleistophora ehrenbaumi	*Anarhichas lupus*	AF044392
Pleistophora finisterrensis	*Micromesistius poutassou*	AF044393
Pleistophora hippoglossoideos	*Hippoglossoides platessoides*	AJ252953
Pleistophora typicalis	*Myoxocephalus scorpius*	AF044387
Pleistophora sp. 1	*Glyptocephalus cynoglossus*	AF044394
Pleistophora sp. 2	*Zeugopterus punctatus*	AF044389
Pleistophora sp. 3	*Taurulus bubalis*	AF044390
Pseudoloma neurophilia	*Danio rerio*	AF322654
Spraguea americana	*Lophius americanus*	AF056014
Spraguea lophii (1)	*Lophius piscatorius*	AF104086
Spraguea lophii (2)	*Lophius piscatorius*	AF033197
Spraguea sp.	*Lophius litulon*	AY465876
Tetramicra brevifilum	*Scophthalmus maximus*	AF364303

outgroup. Sequences were aligned as described by Azevedo *et al*. (2006). Alignment using Clustal W (Thompson *et al*. 1994), in MEGA 4 software (Tamura *et al*. 2007), with an opening gap penalty of 10 and a gap extension penalty of 4 was done for both pairwise and multiple alignments. Subsequent phylogenetic and molecular evolutionary analyses were conducted using MEGA 4, with the 42 rDNA sequences for microsporidian species and the outgroup species selected. Distance estimation was carried out using the Kimura-2 parameters model distance matrix for transitions and transversions. For the phylogentic tree reconstructions, maximum parsimony analysis was conducted using the close neighbour interchange (CNI) heuristic option with a search factor of 2 and random initial trees addition of 2000 replicates. Bootstrap values were calculated over 100 replicates.

RESULTS

Macroscopical and light microscopical observations

Some spherical to elipsoidal whitish cysts (xenomas) were macroscopically observed adherent to the internal wall of the coelomatic cavity of the teleost fish near the anal region. These xenomas with a variable number (up to 7) could reach dimensions of up to $\sim$0·8 mm (Fig. 1A). In semi-thin section, the thick xenoma wall showed a lucent area surrounded by a

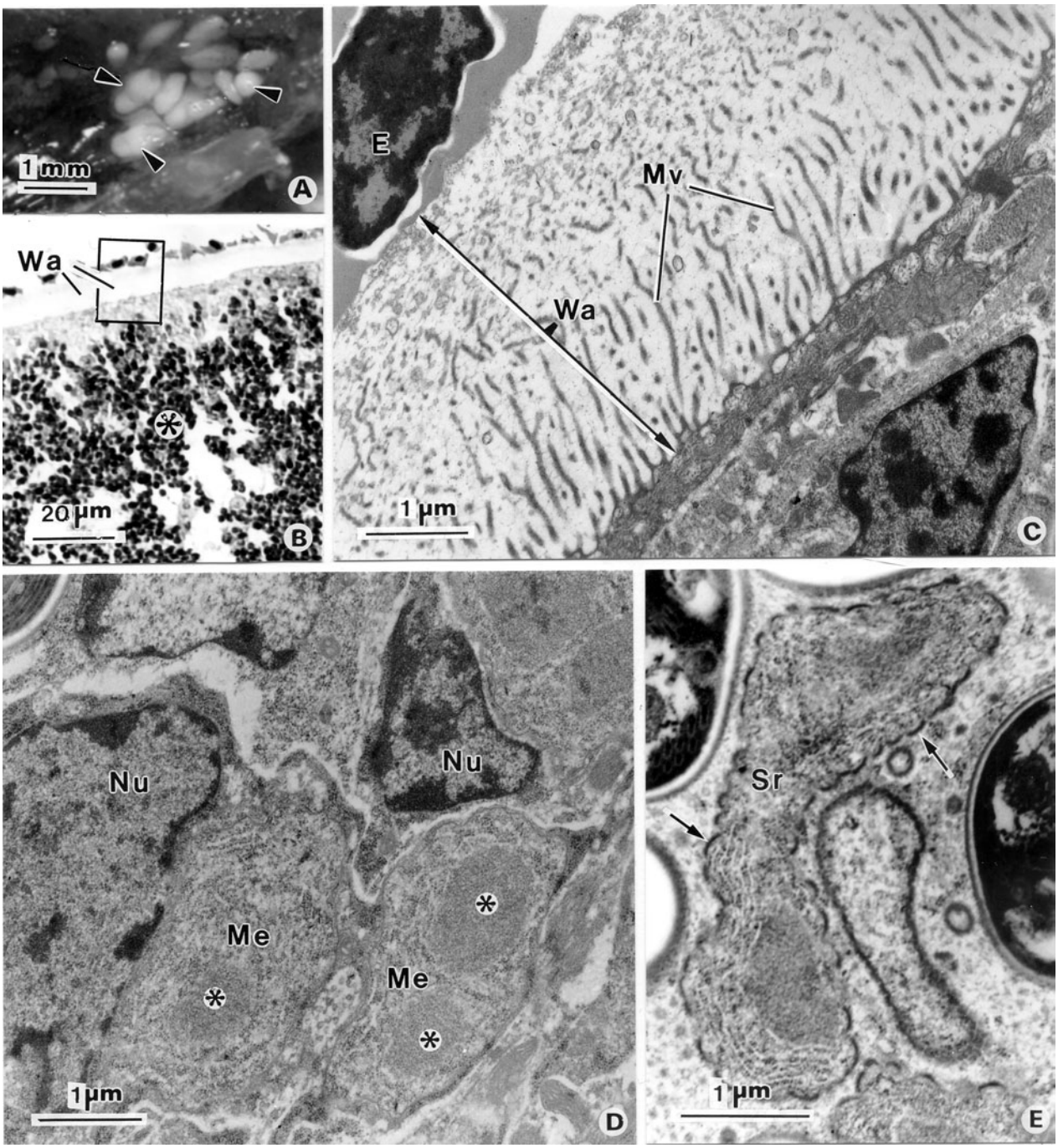

Fig. 1. (A–E) Light and transmission electron micrographs of the microsporidian *Potaspora morhaphis* n. gen., n. sp. (A) Some xenomas (arrowheads) on the abdominal cavity. (B) Semi-thin section of the xenoma periphery, showing the xenoma wall (Wa) and the matrix of the xenoma containing developmental stages including spores (*). The boxed area is enlarged in the figure C. (C) Ultrathin section of the xenoma wall (Wa) showing numerous filiform and anastomosed microvilli-like structures (Mv) projected toward the periphery and, externally, an erythrocyte nucleus (E) in contact with the wall. (D) Ultrathin section of the internal periphery of the xenoma, showing several host cell nuclei (Nu) and a dividing meront (Me), showing some nuclei (*), in direct contact with the host-cell cytoplasm. (E) Ultrathin section of a sporogonial plasmodium in division (Sr) showing the wall formation by a gradual deposition of the dense material on the membrane (arrows).

layer of cells and inside was filled with numerous spores and other developmental stages (Fig. 1B). After rupture of the xenoma wall, the free spores were easily identified as belonging to the phylum Microsporidia (Fig. 4).

Ultrastructural observations

Xenoma. The xenoma wall was formed by numerous filiform and anastomosed microvilli-like structures, with a regular diameter, projected from the

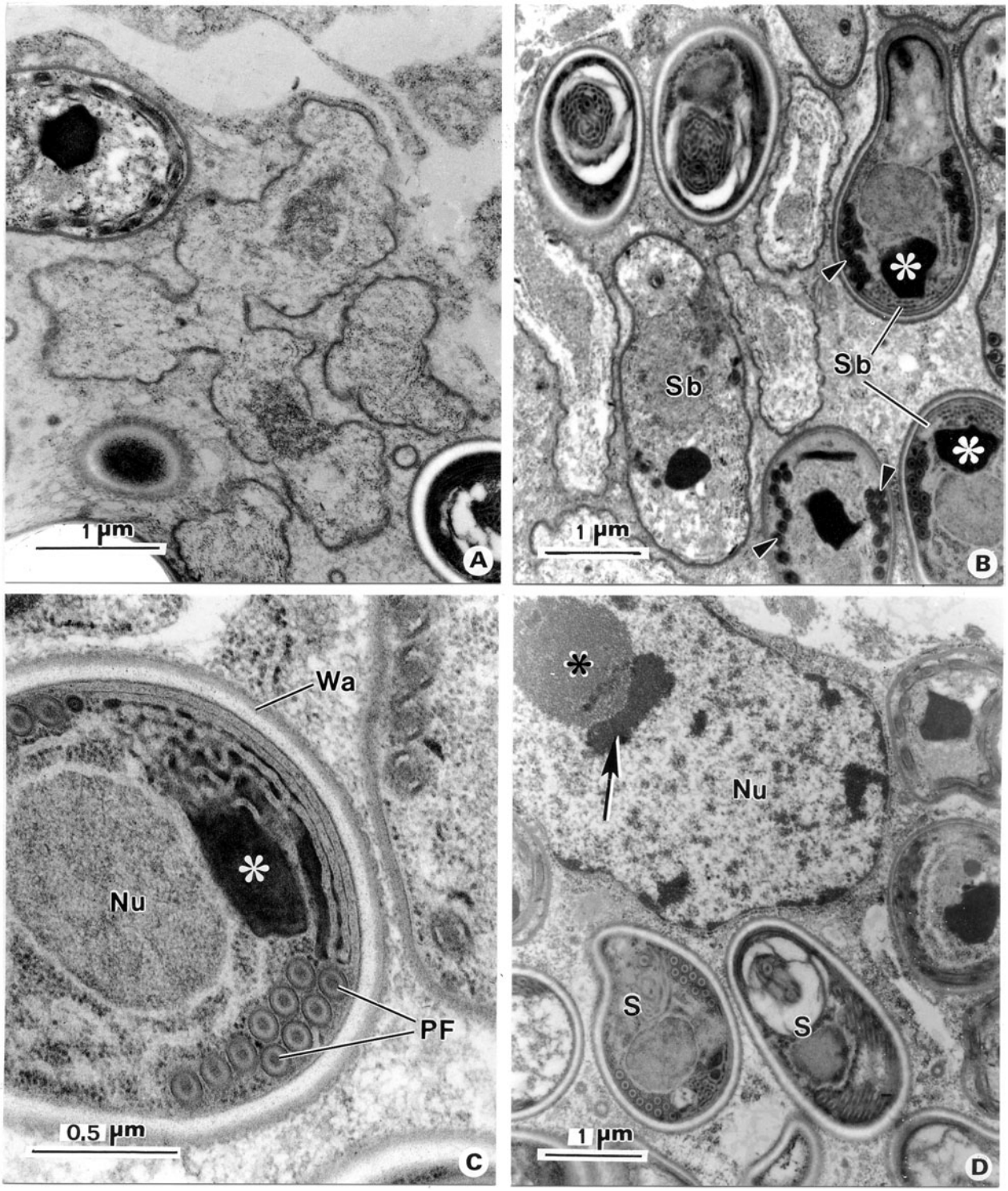

Fig. 2. (A–D) Late sporogonic development of the microsporidian *Potaspora morhaphis* n. gen., n. sp. (A) Sporogonial plasmodium in division giving rise to 4 sporoblasts. (B) Some sporoblasts (Sb) in different developmental stages showing a dense globule (*) that gradually decreases in density and the polar filament in differentiation (arrowheads). (C) Detail of an immature spore showing the dense globule (*) strongly associated to the polar filament formation (PF). Nucleus (Nu). (D) Ultrastructure of a host cell showing the nucleus (Nu) and the nucleolus (*) with peripheral nucleolar heterochromatin (arrow) surrounding the nucleolus. The mature spores (S) are contained in the cytoplasm of the host cell.

surface toward the periphery. The microvilli were intermingled by an amorphous, finely granular material. In favourable sections the microvilli were 4–5 μm long (Fig. 1C). Some zones towards the apical region of the microvilli were in contact with an external layer of erythrocytes (Fig. 1B, C). The asynchronous development was characterized by several merogonic and early sporogonic stages

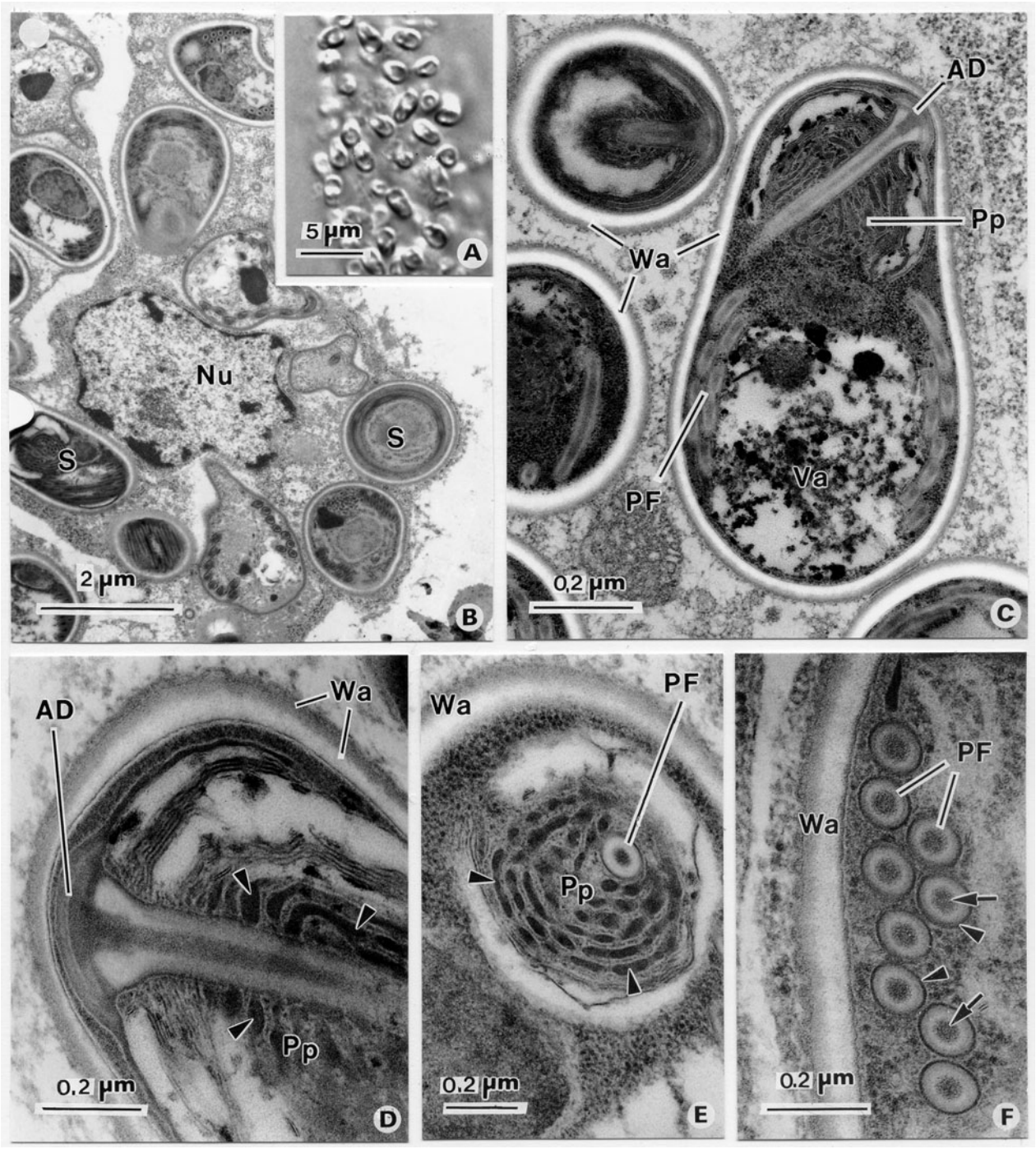

Fig. 3. (A–F) Morphological and ultrastructural details of the microsporidian *Potaspora morhaphis* n. gen., n. sp. (A) Several isolated mature spores observed by DIC microscopy. (B) Some spores (S) in different stages of development in close contact with the cytoplasm of the host cell that shows the (Nu). (C) Ultrathin longitudinal and two transverse sections of a spore showing the typical microsporidian structures and organelles. Wa, wall; AD, anchoring disc; Pp, polaroplast; PF, polar filament; Va, vacuole. (D) Ultrastructural detail of the apical region of a spore showing anchoring disc (AD) in close contact with the wall (Wa) and the lamellar region of the polaroplast (Pp) containing dense material (arrowheads). (E) Ultrastructural detail of a transverse section of a spore showing the lamellar region of the polaroplast (Pp) containing dense material (arrowheads), the polar filament (PF) and the wall (Wa). (F) Ultrastructural detail of the wall (Wa), the polar filament coils (PF) showing the external membrane (arrowheads), as well as a central dense mass (arrows).

predominantly along the xenoma periphery, while immature and mature spores were more internally localized in the centre of the xenoma (Fig. 1B). Internally, the matrix of the xenoma possessed numerous host cells, their nuclei showing a prominent nucleolus, and great mass of peripheral heterochromatin (Figs 1D and 2D). Inside each host cell, the parasite was always in direct contact with the

cytoplasm of host cells, without any surrounding membrane and frequently in the same stage of the developmental life cycle (Fig. 3B).

Description of the development stages

Meronts. These cells grow into multinucleate plasmodia. They appeared in ultrathin sections as round to elliptical uninucleated or binucleated cells always with the nuclei unpaired. In these cells, the chromatin was homogeneous in contrast to the nuclei of the host cells in which the chromatin was organized in dense masses. Their cytoplasm possessed numerous free ribosomes and was uniformly granular and poorly endowed with cytoplasmatic organelles (Fig. 1D). Meronts divided by binary fission and transformed into sporonts (Figs 1E and 2A).

Sporonts. The transition from merogony to sporogony is characterized by the acquisition of a thick and dense cell coat located on the outer surface of the plasmalemma (Figs 1E and 2A). Early on, the discontinuous coat of the sporogony stages appeared to be formed by isolated patches (Fig. 1E). They were rounded and uninucleated cells and in their cytoplasm several well-developed cisternae of rough endoplasmatic reticulum and small vesicles were observed. Before the sporont transformed in uninucleate sporoblasts they divided again by multiple fission giving rise to 4 sporoblasts (Figs 1E and 2A).

Sporoblasts. The sporoblasts do not have the capacity to divide further and gradually differentiate the organelles typical of the spores, composed of an anchorage disc, polaroplast, polar filament and posterior vacuole. In the sporoplasm a very electron-dense irregularly-shaped globule that persists until sporogenesis is concluded, was frequently observed (Fig. 2B). This structure is associated with the reticular body present in the sporoplasm during differentiation of the spores and later appears to be immersed into a posterior vacuole (Fig. 2C).

Systematic position

Phylum Microsporidia Balbiani, 1882; Class Haplophasea Sprague, Becnel and Hazard, 1992; Family Tetramicridae Matthews and Matthews, 1980.

Description of the genus

Name: Potaspora n. gen.
Diagnosis: Xenoma formation has several nuclei and the plasmalemma differentiates numerous filiform and anastomosed microvilli-like structures projected externally. In all developmental stages the nuclei are monokaryotic and develop in direct contact with host cell cytoplasm. The merogony stages are binucleated and divide by binary fission. Each meront differentiates into a sporont by a gradual development of a thick electron-dense coat. The sporont divides by multiple fission into 4 sporoblasts. In this stage a very electron-dense irregular-shaped body differentiates. Monomorphic spores containing polaroplast with 2 distinct kinds of lamellae.

Description of the species

Name: Potaspora morhaphis n. gen., n. sp.
Type host: *Potamorhaphis guianensis* Schomburgk, 1843 (Teleostei, Belonidae).
Type Locality: Estuarine region of the Amazon river (01°11′S and 47°18′W) near the city of Belém (Pará State), Brazil.
Location in the host: Xenoma in the coelomatic cavity near the anal region.
Prevalence of infection: Twelve of 30 (40%).
Type specimens: One slide containing mature free spores and another with semi-thin sections of tissues containing spores and different developmental stages of hapantotype were deposited in the International Protozoan Type Slide Collection at Smithsonian Institution Washington, DC. 20560, USA with acquisition number (USNM 1113817). The histological semi-thin sections containing different developmental stages were deposited at the laboratory of the senior author.
Etymology: The genus name is the prefix from the name of the host genus and the specific name is derived from the suffix of the host genus name.
Description of the spores: Pyriform spores measuring $2{\cdot}8 \pm 0{\cdot}3 \times 1{\cdot}5 \pm 0{\cdot}2\ \mu$m and containing all the typical characteristic structures of the Microsporidia (Figs 3A, C and 4). The spore wall was about 125 (114–131) nm thick ($n = 30$), except for the anterior end where the anchoring disc contacted with the wall, which was ~50–70 nm thick (Fig. 3C, D). The spore wall consisted of an electron-lucent endospore and an electron-dense exospore each with just about the same thickness (Fig. 3C, D, E, F). The exospore was externally surrounded by a thin irregular layer of granular material (Fig. 3D).

The anchoring disc is located in the apical region of the spore in an eccentric position in relation to the spore axis, giving the spore bilateral asymmetry (Fig. 3C, D). The anterior part of the polar filament (FP) (manubrium) measured about 145 (140–149) nm ($n = 25$) and the angle of tilt anterior PF to the spore axis was ~45° (Fig. 3C, D). The PF was isofilar arranged into 9–10 (rarely 11) coils in 2 layers and, when sectioned transversally, the PF exhibited concentric layers (Fig. 3F). The polaroplast (Pp) has 2 distinct lamellae folded around the PF. In the anterior zone the lamellae were without a lumen and were irregularly packed with a lucent space between them, while in the posterior lamellae the

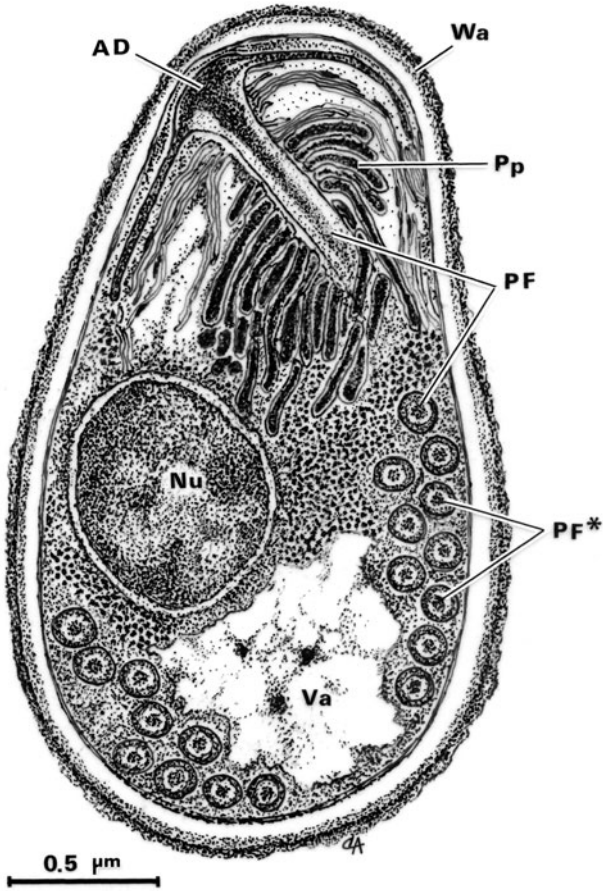

Fig. 4. Semi-schematic drawing of a spore of *Potaspora morhaphis* n. g., n. sp. showing specific characters, such as spore shape and dimensions, spore wall (Wa), polaroplast (Pp), anchoring disc (AD), polar filament (PF) coils (PF*), nucleus (Nu) and vacuole (Va).

lumen was filled with electron-dense material approximately 35–40 nm thick (Fig. 3D, E). The nucleus, containing a moderately uniform nucleoplasm and surrounded by numerous ribosomes, was situated laterally between the polaroplast and the posterior vacuole. The posterior vacuole, situated at the basal part of the spore between the PF coils, was irregular and contained some masses of dense material (Fig. 3C).

Molecular analysis

Two bands of approximately 1·4 kb and 1·1 kb were obtained after amplification of the microsporidian genomic DNA. The primers used were V1f-1492r and HG4F-HG4R, respectively. The sequences were assembled and the resulting consensus DNA sequence of the complete SSU rRNA, ITS, and the 5'-end of the LSU rRNA gene was 1826 bp in length. This sequence with a GC content of 47% was deposited in GenBank (Accession number EU534408). In total, 42 SSU rDNA sequences, including those with the highest BLAST scores, were aligned with the *Potaspora morhaphis* SSU rDNA sequence. Only sequences belonging to species parasitizing fishes were included in the final analyses (Table 1). *Trachipleistophora hominis* found in muscle of humans, some *Pleistophora* spp. found in crustacean species and several *Dictyocoela* spp. parasitizing amphipods were excluded. The length of the aligned sequences used for phylogenetic analysis was 1527 bases after trimming the 3′end. Before phylogenetic analysis, only those sites which could be unambiguously aligned among all Microsporidia and outgroups were used, resulting in an alignment of 1321 bases long.

Based on pairwise comparisons among the SSU rDNA sequences, the maximal similarity was observed with *Microgemma tincae*, *Microgemma caulleryi* and *Tetramicra brevifilum* species, 87·3%, 87·2% and 87·2%, respectively (Table 2). Phylogenetic analyses using maximum parsimony placed *Potaspora morhaphis* clustered with the sequences of the *Kabatana* (AF356222, AJ295322, EF202572), *Microgemma* (AJ252952, AY651319, AY033054), *Spraguea* (AF104086, AF033197, AY465876, AF056014), *Tetramicra* (AF364303) genera and *Microsporidium* (AJ295323, AJ295324) collective group. This clade has 72% bootstrap support. Only *Spraguea* (68% bootstrap) clade suggested monophyly (Fig. 5). Neighbour-joining and maximum likehood analyses resulted in identical tree topology.

DISCUSSION

Ultrastructural studies

The ultrastructural organization of the xenoma, as well as aspects of the developmental stages described in the present study, showed that all structures were typically from Phylum Microsporidia, Class Haplophasea and family Tetramicridae (Lom and Dyková, 1992; Larsson, 1999; Lom and Nilsen, 2003).

Of at least 156 fish microsporidian species distributed among 17 genera (Azevedo and Matos, 2003; Lom and Nilsen, 2003; Baquero *et al*. 2005), only 12 develop xenoma. These formations are a characteristic consequence of the host cell defence to the parasite development having features specific to the genus and species (Lom and Nilsen, 2003). Among these, the xenoma wall, of only 4 genera (*Ichthyosporidium*, *Tetramicra*, *Microfilum* and *Amazonspora*), possesses a structure characterized by numerous anastomosed microvilli-like structures, which could partially resemble the xenoma wall of the parasite reported by us. However, some ultrastructural aspects of the developmental stages of those genera are very distinct. In *Ichthyosporidium*, the xenoma wall presents microvilli-like ramified projections irregularly intermingled in the wall, but this parasite has the nuclei organized as a diplokaryon during all sporogonic stages and the polar filament (up to 46 coils) is the largest of the microsporidian

Table 2. Comparison of some SSU rDNA sequences: percentage of identity (top diagonal) and pairwise distance (bottom diagonal) obtained by Kimura-2 parameter analysis

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
(1) *Potaspora morhaphis*	—	86·8	86·8	86·8	86·8	86·4	85·9	87·3	87·2	87·2	85·2	85·2	85·2	86·0
(2) *Spraguea lophii* (1)	0·132	—	100	98·2	100	99·6	95·3	98·2	98·9	98·9	91·0	91·0	91·0	96·4
(3) *Spraguea* sp. *Lophius litulon*	0·132	0·000	—	98·2	100	99·6	95·3	98·2	98·9	98·9	91·0	91·0	91·0	96·4
(4) *Microgemma vivaresi*	0·132	0·018	0·018	—	98·2	97·9	94·1	98·6	98·2	98·2	90·6	90·6	90·6	95·3
(5) *Spraguea lophii* (2)	0·132	0·000	0·000	0·018	—	99·6	95·3	98·2	98·9	98·9	91·0	91·0	91·0	96·4
(6) *Spraguea americana*	0·136	0·004	0·004	0·021	0·004	—	94·9	97·9	97·9	98·6	98·6	91·6	91·6	96·0
(7) *Kabatana takedai*	0·141	0·047	0·047	0·059	0·047	0·051	—	94·1	94·9	94·9	90·2	90·2	90·2	96·4
(8) *Microgemma tincae*	0·127	0·018	0·018	0·014	0·018	0·021	0·059	—	98·2	98·2	90·6	90·6	90·6	95·3
(9) *Tetramicra brevifilum*	0·128	0·011	0·011	0·018	0·011	0·014	0·051	0·018	—	100	91·6	91·6	91·6	96·0
(10) *Microgemma caulleryi*	0·128	0·011	0·011	0·018	0·011	0·014	0·051	0·018	0·000	—	91·6	91·6	91·6	96·0
(11) *Microsporidium* sp. RSB1	0·148	0·090	0·090	0·094	0·090	0·094	0·098	0·094	0·094	0·094	—	100	100	89·8
(12) *Kabatana seriolae*	0·148	0·090	0·090	0·094	0·090	0·094	0·098	0·094	0·094	0·094	0·000	—	100	89·8
(13) *Microsporidium* GHB	0·148	0·090	0·090	0·094	0·090	0·094	0·098	0·094	0·094	0·094	0·000	0·000	—	89·8
(14) *Kabatana newberryi*	0·140	0·036	0·036	0·047	0·036	0·040	0·036	0·047	0·040	0·040	0·102	0·102	0·102	—

group (Sprague and Vernick, 1974; Casal and Azevedo, 1995). In *Microfilum*, the xenoma wall was described as a very dense region covered by numerous apparently disorganized and ramified microvilli. This parasite gives rise to a spore characterized by a manubrium inserted on a laterally offset anchoring disc and extruding into a short non-coiled polar filament, which was very different from those of the present study (Faye *et al*. 1991). A xenoma wall including a microvillous surface layer formed by anastomosed elongated cytoplasmic processes have been described in the genus *Tetramicra* (Matthews and Matthews, 1980). Meronts located within a vacuole in the host cytoplasm and spores with conspicuous posterosomes surrounded by a membrane and located inside the posterior vacuole.

Recently in an Amazonian fish, a new genus and species (*Amazonspora hassar*) having a xenoma, strongly encapsulated, consisting of numerous anastomosed microvilli-like projections penetrating the 1–3 first layers of collagen fibres was described. Up to approximately 22 juxtaposed crossed layers of collagen fibres were observed (Azevedo and Matos, 2003).

The presence of dense globules of unknown nature in the sporoplasm was also seen in other fish-infecting Microsporidia. Several electron-dense inclusion bodies, sometimes very large, measuring up to 1·38 μm in diameter, were described in sporoblasts and spores of *Tetramicra brevifilum* species (Matthews and Matthews, 1980). In *Kabatana arthuri* (Lom *et al*. 1999) and *K. takedai* (Lom *et al*. 2001) a very similar globule was reported, while in *Loma acerinae* (Lom and Pekkarinen, 1999) 1–3 homogeneous dense globules occupying all the space of the posterior vacuole were observed. In our observations a large inclusion consisting of reticular material like that reported in *Ichthyosporidium giganteum*, was also found (Sprague and Vernick, 1974; Casal and Azevedo, 1995).

The polaroplast of *Potaspora morhaphis* has a bipartite structure comprising the anterior region having folds with a lamellar organization and the posterior region with larger lamellae (cisternae) with dense contents. A similar organization was reported by Lom *et al*. (1999) in the species *Kabatana arthuri* which infects the trunk muscles of fishes from the South-East Asia freshwater fish, *Pangasius sutchi*, as well as in *Kabatana takedai* (Lom *et al*. 2001). The polaroplast of the microsporidian, *Spraguea americana*, found in the nervous tissues of the Japanese anglerfish *Lophius litulon* (Freeman *et al*. 2004) has a similar organization.

Phylogenetic relationships

The availability, in the public databases, of sequences from different species belonging to the phylum Microsporidia makes the SSU rRNA gene

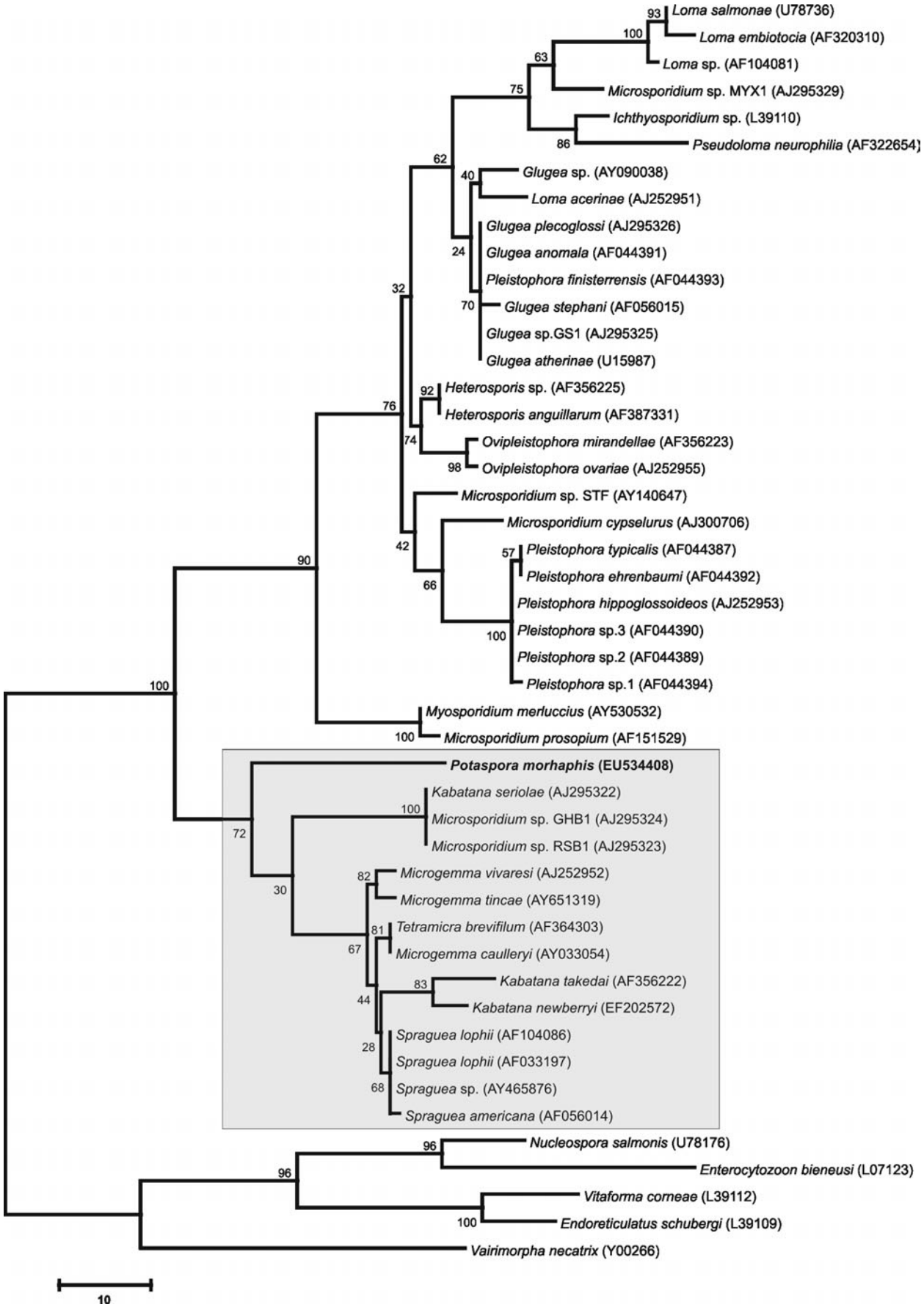

Fig. 5. Parsimony tree of SSU rDNA sequences to compare *Potaspora morhaphis* with selected sequences from other fish-infecting Microsporidia. The analysis was conducted using 1321 aligned nucleotide positions of the highest

the most suitable not only for development of diagnostic tools and species identification, but also for molecular characterization of new parasites through phylogenetic studies (Weiss and Vossbrinck, 1999; Lom and Nilsen, 2003).

In these studies we can see that there is 72% bootstrap support for a clade composed of microsporidian belonging to the *Kabatana* (Lom *et al.* 1999, 2001; McGourty *et al.* 2007), *Microgemma* (Cheney *et al.* 2000; Leiro *et al.* 2000; Mansour *et al.* 2005), *Spraguea* (Freeman *et al.* 2004), *Tetramicra* (Leiro *et al.* 2000) genera, 2 unclassified species of *Microsporidium* group (Bell *et al.* 2001) and the microsporidian reported in this study. This result is in concordance with cladograms previously obtained by Lom and Nilsen (2003) and designated as group IV. Our SSU rDNA sequence analysis also shows that *Potaspora* n. gen. does not have any sister taxa and the lineage is distantly related to the other species examined. Comparing SSU rRNA gene sequences between *P. morhaphis* with species *Microgemma caulleryi* and *Tetramicra brevifilum* (clade with bootstrap 81%) the genetic distances are 12·8% for both species. The smallest genetic distance was observed with the species *Microgemma tincae* (12·7%) but the SSU rRNA was not completely sequenced. The bootstrap support for this species and another *Microgemma vivaresi* is 81%. On the other hand, *K. takedai* and *K. newberryi* group in a clade with 83% bootstrap and all *Spraguea* species are clustered in the clade with 68% bootstrap.

Conclusion

When comparing the xenoma wall of the parasite described here with those fish Microsporidia which form xenoma some structural differences were found, such as the organization of the microvilli-like structures. In addition, the ultrastructural organization of the polaroplast and the presence of a dense globule were the most evident differences found compared with other mature spores of previously described species. Concerning this last aspect, the only exception is the spore of *Kabatana* genus which presents some similarities. However, they were found to parasitize only the muscle fishes and they do not develop inside of xenomas (Lom *et al.* 1999, 2001; McGourty *et al.* 2007). As concerns molecular biology, the most parsimonious cladogram has shown that *Potaspora morhaphis* is placed in the same group as the *Kabatana*, *Microgemma*, *Spraguea* and *Tetramicra* genera, does not have any sister taxa and has the lowest percentage identity within the group.

So, our results suggest that this parasite does not fit into any of the known fish microsporidian genera, and for these reasons we propose a new genus *Potaspora* and a new species, *Potaspora morhaphis*.

This work was partially supported by the Eng°. A. Almeida Foundation (Porto, Portugal), Ph.D. grant from 'CESPU' (G. Casal), 'CNPq' and 'CAPES' – Brazil. We would like to thank the iconographic work of Mr João Carvalheiro. We would like to thank the anonymous reviewers for their helpful suggestions and comments.

REFERENCES

Azevedo, C. and Matos, E. (2002). Fine structure of a new species, *Loma myrophis* (Phylum Microsporidia), parasite of the Amazonian fish *Myrophis platyrhynchus* (Teleostei, Ophichthidae). *European Journal of Protistology* **37**, 445–452.

Azevedo, C. and Matos, E. (2003). *Amazonspora hassar* n. gen. and n. sp. (phylum Microsporidia, fam Glugeidae), a parasite of the Amazonian teleost *Hassar orestis* (fam. Doradidae). *Journal of Parasitology* **89**, 336–341.

Azevedo, C., Balseiro, P., Casal, G., Gestal, C., Aranguren, R., Stokes, N. A., Carnegie, R. B., Novoa, N., Burreson, E. M. and Figueras, A. (2006). Ultrastructural and molecular characterization of *Haplosporidium montforti* n. sp., parasite of the European abalone *Haliotis tuberculata*. *Journal of Invertebrate Pathology* **92**, 23–32.

Baquero, E., Rubio, M., Moura, I. N. S., Pieniazek, J. and Jordana, R. (2005). *Myosporidium merluccius* n. g., n. sp. infecting muscle of commercial hake (*Merluccius* sp.) from fisheries near Namibia. *The Journal of Eukaryotic Microbiology* **52**, 476–483.

Bell, A. S., Aoki, T. and Yokoyama, H. (2001). Phylogenetic relationships among Microsporidia based on rDNA sequence data, with particular reference to fish-infecting *Microsporidium* Balbiani 1884 species. *The Journal of Eukaryotic Microbiology* **48**, 258–265.

Casal, G. and Azevedo, C. (1995). New ultrastructural data on the microsporidian *Ichthyosporidium giganteum* infecting the marine teleostean fish *Ctenolabrus rupestris*. *Journal of Fish Diseases* **18**, 191–194.

Cheney, S. A., Lafranchi-Tristem, N. J. and Canning, E. U. (2000). Phylogenetic relationships of *Pleistophora*-like Microsporidia based on small subunit ribosomal DNA sequences and implications for the source of *Trachipleistophora hominis* infections. *The Journal of Eukaryotic Microbiology* **47**, 280–287.

Faye, N., Toguebaye, B. S. and Bouix, G. (1991). *Microfilum lutjani* n. g. n. sp. (Protozoa, Microsporida), a gill parasite of the golden African snapper *Lutjanus fulgens* (Valenciennes, 1830) (Teleost

BLAST score microsporidian sequences and 4 more microsporidian sequences as outgroup. The bar indicates the equivalence between the distance and the number of changes. The numbers on the branches indicate bootstrap support from 100 replicates. *Potaspora morhaphis* is placed within group IV (Lom and Nilsen, 2003) (highlighted box), which includes the sequences of the genera *Kabatana, Microgemma*, *Spraguea*, *Tetramicra*, and *Microsporidium*.

Lutjanidae): Developmental cycle and ultrastructure. *Journal of Protozoology* **38**, 30–40.

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

Gatehouse, H. S. and Malone, L. A. (1998). The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNAgenes and evidence of a large tandem repeat unit size. *Journal of Invertebate Pathology* **71**, 97–105.

Larsson, J. I. R. (1999). Identification of Microsporidia. *Acta Protozoologica* **38**, 161–197.

Leiro, J., Siso, M. I. G., Parama, A., Ubeira, F. M. and Sanmartin, M. L. (2000). RFLP analysis of PCR-amplified small subunit ribosomal DNA of three fish microsporidian species. *Parasitology* **120**, 113–119.

Lom, J. (2002). A catalogue of described genera and species of microsporidians parasitic in fish. *Systematic Parasitology* **53**, 81–99.

Lom, J. and Dyková, I. (1992). Microsporidia (Phylum Microspora Sprague, 1977). In *Protozoan Parasites of Fishes*. *Developments in Aquaculture and Fisheries Sciences* (ed. Lom, J. and Dyková, I.), Vol. 26, pp. 125–157. Elsevier, Amsterdam.

Lom, J., Dyková, I. and Tonguthai, K. (1999). *Kabataia* gen. n., new genus proposed for *Microsporidium* spp. infecting trunk muscles of fishes. *Diseases of Aquatic Organisms* **38**, 39–46.

Lom, J. and Nilsen, F. (2003). Fish Microsporidia: fine structural diversity and phylogeny. *International Journal for Parasitology* **33**, 107–127.

Lom, J., Nilsen, F. and Urawa, S. (2001). Redescription of *Microsporidium takedai* (Awakura, 1974) as *Kabatana takedai* (Awakura, 1974) comb. n. *Diseases of Aquatic Organisms* **44**, 223–230.

Lom, J. and Pekkarinen, M. (1999). Ultrastructural observations on *Loma acerinae* (Jírovec, 1930) comb. nov. (Phylum Microsporidia). *Acta Protozoologica* **38**, 61–74.

Mansour, L., Prensier, G., Jemaa, S. B., Hassine, O. K. B., Méténier, G., Vivarès, C. P. and Cornillot, E. (2005). Description of a xenoma-inducing microsporidian, *Microgemma tincae* n. sp., a parasite of the teleost fish *Symphodus tinca* from Tunisian coasts. *Diseases of Aquatic Organisms* **65**, 217–226.

Matos, E. and Azevedo, C. (2004). Ultrastructural description of *Microsporidium brevirostris* sp. n., parasite of the teleostean *Brachyhypopomus brevirostris* (Hypopomidae) from the Amazon River. *Acta Protozoologica* **43**, 261–267.

Matos, E., Corral, L. and Azevedo, C. (2003). Ultrastructural details of the xenoma of *Loma myrophis* (phylum Microsporidia) and extrusion of the polar tube during autoinfection. *Diseases of Aquatic Organisms* **54**, 203–207.

Matthews, R. A. and Matthews, B. F. (1980). Cell and tissue reactions of turbot *Scophthalmus* maximus (L.) to *Tetramicra brevifilum* gen. n., sp. n. (Microspora). *Journal of Fish Diseases* **3**, 495–515.

Nilsen, F. (2000). Small subunit ribosomal DNA phylogeny of Microsporidia with particular reference to genera that infect fish. *Journal of Parasitology* **86**, 128–133.

McGourty, K. R., Kinzger, A. P., Hendrickson, G. L., Goldsmith, G. L., Casal, G. and Azevedo, C. (2007). A new microsporidian infecting the musculature of the endangered tidewater goby (Gobiidae). *Journal of Parasitology* **93**, 655–660.

Sprague, V., Becnel, J. J. and Hazard, E. I. (1992). Taxonomy of phylum Microspora. *Critical Reviews in Microbiology* **18**, 285–395.

Sprague, V. and Vernick, S. (1974). Fine structure of the cyst and some sporulation stages of *Ichthyosporidium* (Microsporidia). *Journal of Protozoology* **21**, 667–677.

Tamura, K., Dudley, J., Nei, M. and Kumar S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599.

Thompson, J. D., Higgins, D. G. and 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. *Nucleic Acids Research* **22**, 4673–4680.

Vossbrinck, C. R., Baker, M. D., Didier, E. S., Debrunner-Vossbrinck, B. A. and Shadduck, J. A. (1993). Ribosomal DNA sequences of *Encephalitozoon hellem* and *Encephalitozoon cuniculi*: species identification and phylogenetic construction. *The Journal Eukaryotic of Microbiology* **40**, 354–362.

Weiss, L. and Vossbrinck, C. (1999). Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the Microsporidia. In *The Microsporidia and Microsporidiosis* (ed. Wittner, M. and Weiss, L.), pp. 129–171. American Society of Microbiology, Washington, DC.