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Ultrastructural Description of *Microsporidium brevirostris* sp. n., Parasite of the Teleostean *Brachyhypopomus brevirostris* (Hypopomidae) from the Amazon River

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Summary. *Brachyhypopomus brevirostris* sp. n. (family Hypopomidae), a fish from the estuarine region of the Amazon River, collected near the city of Belém, Brazil, is parasitized by numerous microsporidian spores that form xenomas. These xenomas are found in the skeletal muscle adjacent to the abdominal cavity. The xenoma wall, with an irregular surface, consists of concentric laminated layers of compressed cells, possibly fibroblasts. Developing cells are in direct contact with host cell cytoplasm. Among the mature spores, small groups of juxtaposed immature spores are observed. The spores are ellipsoidal and uninucleate, measuring ~ 2.95 × 1.68 µm (n = 50). The isofilar filament (~ 55 nm diameter), consists of a regular coil in a single layer with 9-10 (or rarely 8) turns surrounding the posterior vacuole of the spore. The vacuole occupies about half of the total volume of the spore. The angle of tilt of the turns is ~ 37°. The spore wall is in direct contact with the cytoplasm of the host cells. A few larger grouped spores, measuring ~ 6.9 × 2.5 µm (n = 20), were observed mainly at the periphery of the most xenomas. The filament consists of two or three irregular layers of coils with 27-28 turns surrounding the posterior vacuole. The ultrastructural morphology of the spores and host specificity suggest that they may be included in the collective group of new *Microsporidium* species and named *Microsporidium brevirostris.* The taxonomic affinities and morphological comparisons with other similar species of some genera were discussed.

Key words: fish parasite, microsporidian, *Microsporidium brevirostris* sp. n., spore, ultrastructure.

INTRODUCTION

Microsporidia are common parasites of fish from different geographical areas (Canning and Lom 1986, Lom and Dyková 1992, Sprague *et al.* 1992, Dyková 1995, Larsson 1999, Shaw and Kent 1999, Lom and Nilsen 2003). Several microsporidian species parasitizing fish have been assigned: *Glugea, Heterosporis, Ichthyosporidium, Kabatana, Loma, Microfilum, Microgemma, Neonosemoides, Nosemoides, Nucleospora, Ovipleistophora, Pleistophora, Spraguea, Tetramicra and Amazonspora* (Vinckier 1975, Matthews and Matthews 1980, Ralphs and Matthews 1986, Lom

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and Dyková 1992, Sprague *et al.* 1992, Faye *et al.* 1996, Larsson 1999, Shaw and Kent 1999, Sprague and Becnel 1999, Lom 2002, Azevedo and Matos 2003, Lom and Nilsen 2003). Among them, however, only few are xenoma-forming genera of Microsporidia.

Although there is considerable information on the species of Microsporidia (Lom and Dyková 1992, Sprague *et al*. 1992, Lom 2002, Lom and Nilsen 2003), little is known about those from South America, and particularly those from the Amazon River, where a diverse assemblage of several hundred species of fish live. Light microscopicy and ultrastructural data are available for only two Amazonian species, *Loma myrophis* (Azevedo and Matos 2001) and *Amazonspora hassar* (Azevedo and Matos 2003). Here, a detailed ultrastructural study is presented of the xenoma, the spores and the host cell interaction of a parasite of *Brachyhypopomus brevirostris* (family Hypopomidae), a fish from the estuarine region of the Amazon River. Based on the ultrastructural features and host specificity observed, we propose the creation of a new microsporidian species.

MATERIALS AND METHODS

Forty specimens of the teleost *Brachyhypopomus brevirostris* (Steindachner, 1868) (family Hypopomidae) (common Brazilian name, "itui rajado"), were collected in the estuarine region of the Amazon River (01° 11´ 30´´ S / 47° 18´ 54´´ W) near the city of Belém (Pará), Brazil. Infection was determined by the presence of xenomas located in the skeletal muscle of the abdominal cavity, recognizable by the naked eye. Measurements of xenomas and fresh spores were made in wet mount preparations with an eye-piece micrometer at ×1,000. After crushing the xenoma, the spores were identified by differential interference contrast microscopy (DIC). For transmission electron microscopy (TEM), the xenoma and surrounding tissues were fixed in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at 4°C for 20-24 h, rinsed overnight in the same buffer at 4°C and post-fixed in 2% OsO_4 in the same buffer at 4°C for 4 h. After dehydration in an ascending ethanol series (70, 80, 90, 95 and 100% (2 h in each change) and in propylene oxide (two changes of 3 h each), the infected tissues were embedded in Epon (10-12 h in each change). Semithin sections were stained with 1% methylene blue, 1% Azur II (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and observed with a JEOL 100CXII TEM operated at 60 kV.

RESULTS

Irregular whitish xenomas, were macroscopically observed only in the internal muscular tissue of the abdominal cavity of the fish, *Brachyhypopomus brevirostris* (family Hypopomidae). Eighteen of 40 specimens were infected (45%). The xenomas, measuring 85 to 465 µm (n=15), were photographed by DIC (Fig. 1). Most of the xenoma was filled with numerous spores (Fig. 2). Among the spores (microspores) contained in the xenoma, small groups of immature spores were also observed (Figs 2, 10). After rupture of the xenomas, numerous ellipsoidal spores were identified as belonging to the phylum Microsporidia. Unfixed spores observed in DIC optics were 2.95 ± 0.32 µm long and 1.68 ± 0.18 µm wide (n=50) (Fig. 3).

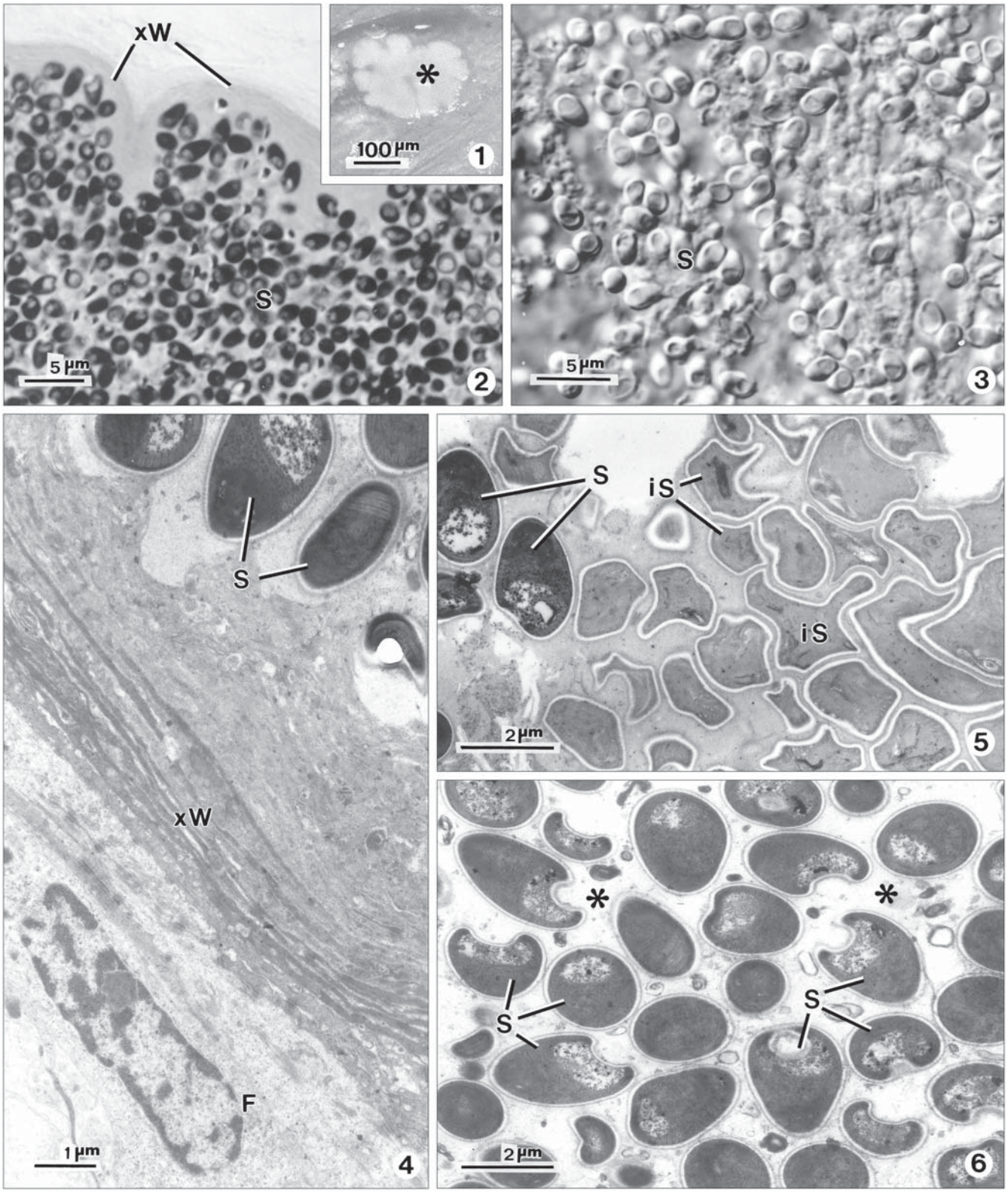
The xenoma wall has an irregular surface and is formed of concentric laminated structures, spaced by electron-lucent layers intermingled with layers of the compressed cell coat (possibly fibroblasts), which forms an electron-dense substance (Fig. 4). Some fibroblasts were observed external to the xenoma (Fig. 4). Small groups of juxtaposed immature spores were observed among the mature spores (Fig. 5). The spores were in direct contact with the host cell cytoplasmic matrix, where no cytoplasmic structure was observed other than some vesicular structures and granular cytoplasmic debris (Figs 4, 6). The walls of mature spores (~ 37 nm thick) are composed of an electron-dense exospore (~ 15 nm thick) and an electronlucent endospore (~ 22 nm thick) (Figs 7-9). The anchoring disk was eccentric and the polar tube had an oblique position in relation to the longitudinal axis of the spore (Figs 6, 7). The manubrium constituted a straight anterior part of the polar filament measuring ~ 125 nm diameter at its midpoint (Fig. 8).

The isofilar polar filament (~ 55 nm diameter) consisted of a regular coil in a single layer with 9-10 turns (or rarely 8 turns) surrounding the posterior vacuole (Figs 7, 9) that occupied about half of the spore length (Fig. 7). In a favourable series of longitudinal ultrathin sections, it was possible to measure the angle of tilt as being about 37° (35-41°) (n=15) (Fig. 7).

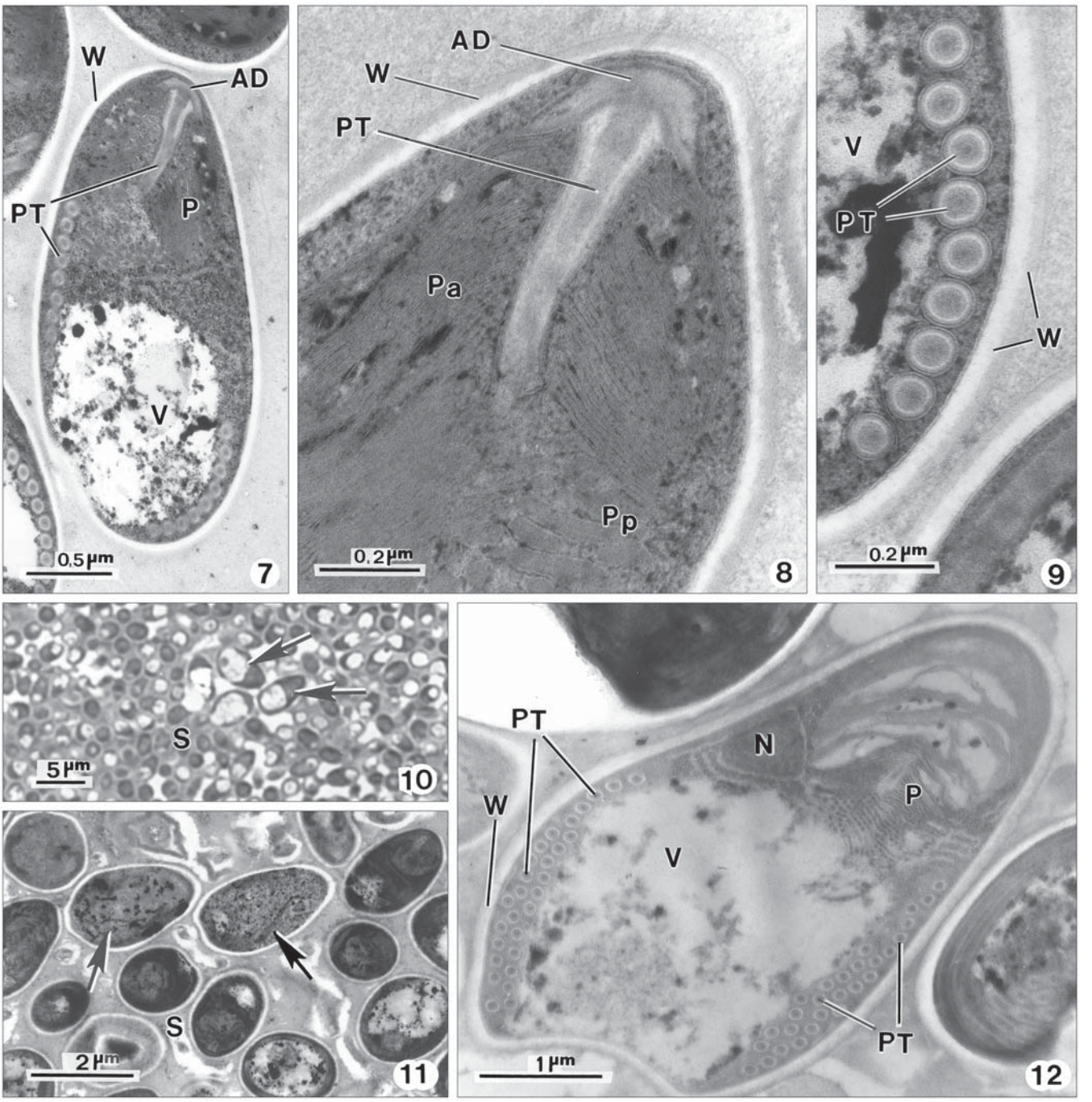
The lamellate polaroplast occupied the apical position around the anterior portion of the polar filament and consisted of two membranous system. The anterior region contained closely packer and arranged lamellae, while the posterior was more widely spaced lamellae (Figs 7, 8).

The nucleus occupies a position between the polaroplast and the posterior vacuole, and some helically arranged aggregate polyribosomes could be observed surrounding the nucleus.

In some sections, it was possible to observe the presence of a few larger uninucleate spores, measuring



Figs 1-6. *Microsporidium brevirostris* sp. n. **1 -** single xenoma (*) observed in the internal abdominal wall of the fish; **2 -** semithin section of the xenoma periphery showing the xenoma wall surrounding numerous ellipsoidal spores; **3 -** fresh spores released from a xenoma observed in DIC optics. Electron micrographs showing: **4 -** fibroblast and possibly other compressed fibroblasts located at the periphery of the xenoma wall. Internally some spores are present; **5 -** small groups of immature spores next to mature spores; **6 -** several spores without a surrounding membrane, sectioned in different planes and located in an amorphous matrix (*) of host cytoplasm. F - fibroblast, iS - immature spores, S - spores, xW - xenoma wall.



Figs 7-12. *Microsporidium brevirostris* sp. n. electron micrographs of: **7 -** mature spore sectioned longitudinally showing the different typical structures of a microsporidian spore. The nucleus occupied a central position, was not observed in this section; **8 -** details of the apical region of a spore, showing the spore wall, anchoring disk, the initial portion of the polar tube (manubrium) and the organization of the polaroplast; **9 -** of transverse sections of the polar tube located between the posterior vacuole and spore wall. The vacuole contains dense masses; **10** - semithin section of the xenoma showing some macrospores (arrows) among numerous microspores; **11** - some macrospores (arrows) among microspores; **12 -** macrospores sectioned longitudinally showing the spore wall, the polar tube with its 27-28 turns, the vacuole, the nucleus and the polaroplast. AD - anchoring disk, N - nucleus, P - polaroplast, Pa - anterior region, Pp - posterior region, PT - polar tube, S - microspores, V –vacuole, W - spore wall.

~ 6.9 (6.4-7.2) µm long and × 2.5 (2.0-2.8) µm wide (n=20) (Fig.10), containing polar filaments with two or three irregular coils of 27-28 turns surrounding the posterior vacuole (Figs 11, 12). These spores appeared in grouped, containing from 4-10 in number among the microspores (Fig. 10).

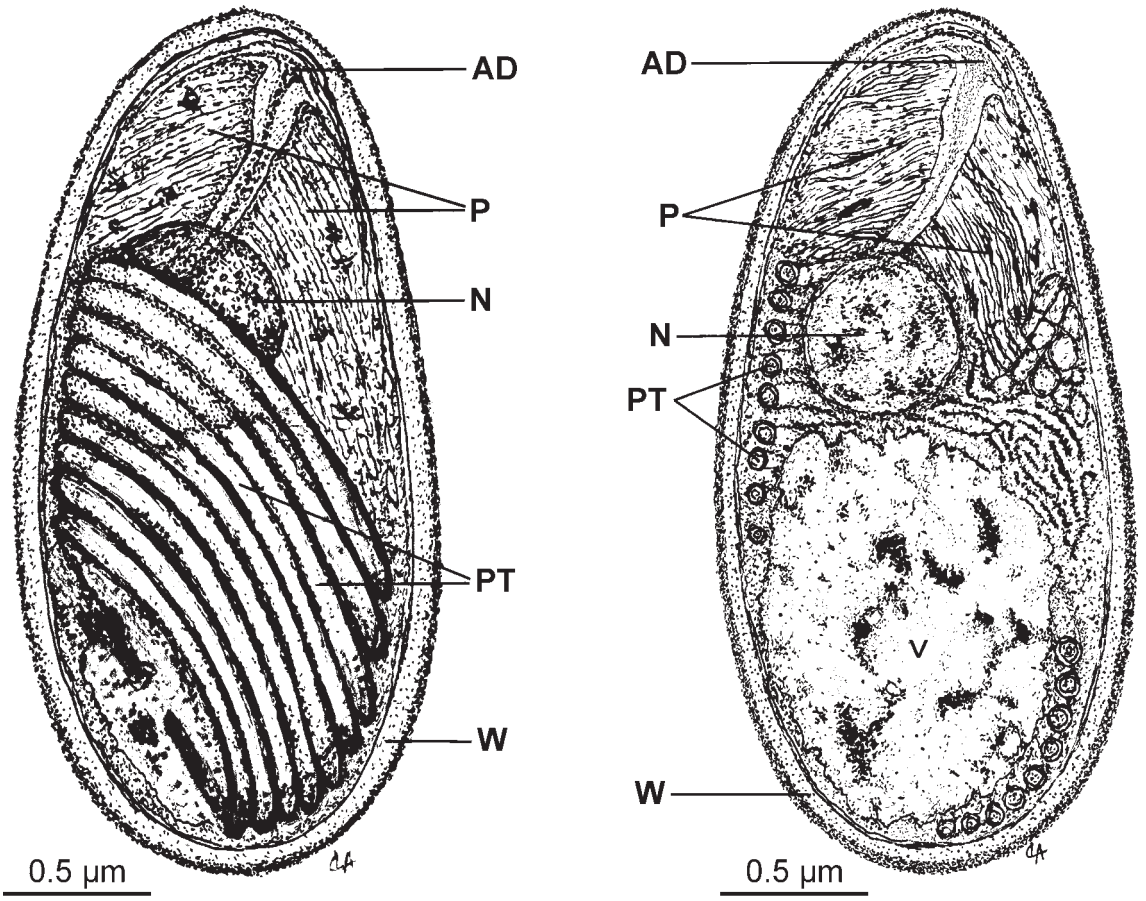


Fig. 13. Two semischematic drawings summarizing the tridimensional morphology (left) of the spore of *Microsporidium brevirostris* sp. n., and the ultrastructural morphology in longitudinal section (right), as described in the text and illustrated in the micrographs.
AD - anchoring disc, N - nucleus, P - polaroplast, PT - polar tube, V - posterior vacuole, W- spore wall.

Schematic drawings of the spore morphology (Fig. 13) were made from serial ultrathin sections.

Taxonomy summary of *Microsporidium brevirostris* sp. n.

Type host: *Brachyhypopomus brevirostris* (Steindachner, 1868) (family Hypopomidae).

Site of infection: Skeletal muscle of the abdominal cavity.

Type locality: Estuarine region of the Amazon River, near city of Belém, Brazil.

(Latitude: 01° 11' 30" S Longitude: 47° 18' 54" W)

Diagnosis: Host cells form macroscopic xenomas filled with spores. Ellipsoidal uninucleate spores in direct contact with the host cell cytoplasm, measured ~ 2.95 × 1.68 µm. Spore wall measured ~ 37 nm thick, were composed of electron-dense exospore (~ 15 nm thick) and electronlucent endospore (~ 22 nm thick). Isofilar polar filament with 9-10 (rarely 8) turns. Angle of tilt was ~ 37°. Polaroplast of the anterior region consisted of closely packer arranged lamellae and the posterior region more spaced lamellae. Larges spores measured ~ 6.9 × 2.5 mm. Polar filament with 27-28 turns in two or three irregular layers.

Type specimens: 2 slides containing semithin sections of the xenomas with spores of the holotypes were deposited in the International Protozoan Type Slide Collection at Smithsonian Institution, Washington D. C. 20560, USA with USNM no. 1025353.

Prevalence of infection: 18/40 (45%).

Etymology: The specific epithet, "*brevirostris*", is derived from the specific epithet of the host species.

DISCUSSION

The more conspicuous characteristics of the spores - the shape, wall, polaroplast, polar filament and posterior vacuole - are used to distinguish microsporidia from other taxonomic groups (Sprague *et al.* 1992). The results of our study demonstrate that the ultrastructure of the spore found in xenomas of *Brachyhypopomus brevirostris* (family Hypopomidae) corresponds to that of the phylum Microsporidia (Vávra and Larsson 1999).

In a recent paper it was stated that the 156 fish microsporidian species recorded are distributed among 14 genera (Lom and Nilsen 2003). Some of these produce xenomas: *Glugea* Thélohan, 1891; *Ichthyosporidium* Caullery et Mesnil, 1905; *Loma* Morrison et Sprague, 1981; *Microfilum* Faye, Toguebaye et Bouix, 1991; *Microgemma* Ralphs et Matthews, 1986; *Nosemoides* Vinckier, 1975; *Spraguea* Vávra et Sprague, 1976; and *Tetramicra* Matthews et Matthews, 1980. More recently, a new genus *Amazonspora* was added to these (Azevedo and Matos 2003).

The recently created new genus and species *Pseudoloma neurophilia,* which is found in the central nervous system of the zebrafish (*Danio rerio*), differs from the latter genera because the spores are segregated into clusters of up to 16 spores and appear to develop within a true sporophorous vesicle (Matthews *et al*. 2001). The ultrastructure of the xenoma described in *Pseudoloma* is not typical of must xenomas (Lom 2002). Spores of *Tetramicra* found in xenoma from skeletal muscle have a conspicuous inclusion in the sporoplasm and posterior vacuole, is unique among fish-infecting microsporidian (Lom and Dyková 1992). Such an inclusion was never observed in our study. The xenomas of *Amazonspora,* which consist of a single hypertrophic host cell and a xenoma wall composed of up to 22 juxtaposed crossed layers of collagen fibres (Azevedo and Matos 2003), are very different to the xenomas we describe here. The distinction between the genera *Glugea* and *Loma* is not clear (Cali and Takvorian 1999, Lom and Pekkarinen 1999). However, in the present study using ultrathin sections, the developmental stages, the sporogonial plasmodium dividing into sporoblast mother-cells which gives rise to two sporoblasts (Canning *et al*. 1982), the mature spores and the xenoma wall, all more closely resemble features of the genus *Glugea* (Canning *et al.* 1982).

Compared with these previously described genera, our results show that this parasite has differences in the morphology of the spores and the ultrastructure of the developing cells, xenoma and the xenoma wall. *Ichthyosporidium* sp. and *Kabatana* sp. differ from our results with respect to the developing cells that are in contact with host cells (Lom *et al*. 2000, Lom 2002) and the absence of xenoma formation in *Kabatana* sp. (Lom *et al*. 1999, 2000). In *Microgemma* sp. (Ralphs and Matthews 1986) and *Microfilum* sp. (Faye *et al.* 1991), the life cycles give rise to the formation of xenomas with a microvillous surface, which does not occur in the microsporidia described here. The genus *Ovipleistophora* has both micro- and macrospores, as in *Microsporidium brevirostris*. However these two kind of spores are specific parasite of oocytes (Pekkarinen *et al*. 2002). No microsporidia have been observed or described with comparable spore morphology and picture of infection from freshwater fishes living in the same geographic area. Considering these data and the host specificity, we believe that this microorganism represents a new species that should be included in the collective genus *Microsporidium* Balbiani, 1884, and we propose the name *Microsporidium brevirostris*. However, more detailed studies, particularly, on life cycle stages and host specificity are need to identify the appropriate existing or new genus to include the parasite.

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