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ORIGINAL PAPER

Morphological and genetical description of *Loma psittaca* sp. n. isolated from the Amazonian fish species *Colomesus psittacus*

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Abstract A previously unrecognised fish-infecting microsporidia (*Loma psittaca* n. sp.), found adherent to the intestinal mucosa of the freshwater puffer fish *Colomesus psittacus* (Teleostei, Tetraodontidae) from lower Amazon River, was described based on light and transmission electron microscope and phylogenetic analysis. The whitish xenoma was completely filled by numerous spores, including several developmental stages of the parasite. In all of these stages, the nuclei were monokaryotic. The merogonial plasmodium divided by binary fission and the sporont gave rise to disporoblastic ovoid spores measuring 4.2 ± 0.4 × 2.8 ± 0.4 μm. In mature spores, the polar filament was arranged in 10–11 (rarely 12) coils in one row in turn of posterior vacuole. The polaroplast had two distinct regions around the manubrium. The polyribosomes were organised in coiled tapes. The small subunit rRNA gene was sequenced and maximum parsimony analysis placed the microsporidian described here in the clade that includes the genera *Ichthyosporidium*, *Loma* and *Pseudoloma.* Based on differences from previously described microsporidians, such as ultrastructural characteristics of the xenoma, developmental stages including the spore and phylogenetic analysis supported the recognition of a new species, herein named *L. psittaca* n. sp.

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Introduction

The members of the phylum Microsporidia Balbiani, 1882 are widespread, minute, obligatory intracellular parasites found in most invertebrate phyla and in vertebrates, with the majority of species in insects and fish (Lom and Dyková 1992; Sprague et al. 1992; Larsson 1999; Lom 2002). Presently, there are at least 144 available genera (Larsson 1999), 18 of them occurring in teleost fishes from the different geographic areas and habitat (Azevedo and Matos 2003; Lom and Nilsen 2003; Baquero et al. 2005; Casal et al. 2008), and some of them are recognised as serious pathogens for their hosts. Fishes are hosts to 156 recorded species of microsporidia, 11 species belonging to the genus *Loma* Morrison and Sprague, 1981 and the other eight parasitoses were classified as *Loma* spp. (Lom 2002). One of them, *Loma myrophis*, was found in the subepithelial tissues of the fish gut *Myrophis platyrhynchus* from Amazonian fauna (Azevedo and Matos 2002; Matos et al. 2003). About those from South America, particularly from the Amazon River where lives a diverse assemblage of

several hundred species of fishes, little is known. Recently, some other microsporidiosis were described in Amazonian fishes: *Amazonspora hassar* was found in the gill of the teleost *Hassar orestis* (Azevedo and Matos 2003), *Microsporidium brevirostris* in the skeletal muscle of the abdominal cavity of the fish *Brachyhypopomus brevirostris* (Matos and Azevedo 2004), and *Potaspora morhaphis* adherent to the wall of coelomic cavity of the freshwater fish, *Potamorhaphis guianensis* (Casal et al. 2008).

Ultrastructurally, the genus *Loma* is characterised to form xenoma, the nuclei to be unpaired during all stages of development and the sporogony to be polysporoblastic within parasitophorous vacuole bound with host cell-derived membrane (Morrison and Sprague 1981; Lom and Pekkarinen 1999; Lom 2002). Presently, there is little information about the origin of vacuole formed during the sporogony of *Loma* species (Matthews et al. 2001). Small subunit (SSU) ribosomal DNA (rDNA) sequence comparison is a well-recognised technique for providing valuable information about phylogenetic relationships (Hillis and Dixon 1991). Only for three *Loma* species was the SSU rDNA gene sequenced: *Loma embiotocia* in shiner perch *Cymatogaster aggregata* (Shaw et al. 1997), *Loma salmonae* found in *Oncorhynchus mykiss* (Docker et al. 1997) and *Loma acerinae* (Cheney et al. 2000). Phylogenetic analysis using SSU rDNA gene show evidences that *Loma* spp. do not comprise a monophyletic group, being placed in the same clade with the genera *Ichthyosporidium* and *Pseudoloma* (Lom and Nilsen 2003). Sometimes, the phylogenetic trees do not support traditional taxonomic schemes (Sprague et al. 1992). Important morphological characters presented by those genera, such as the number of nuclei per spores and the presence of a parasitophorous vacuole or sporophorous vesicle, are not in concordance with molecular data.

In this paper, we describe a new species of a microsporidian based on morphological and ultrastructural observations. Phylogenetic relationships comparing the *Loma psittaca* SSU rRNA gene with those of other fish-infecting microsporidian species were also done. The morphological characteristics and taxonomic position are discussed.

Materials and methods

Fish, location of infection and prevalence

Thirty specimens of freshwater teleost puffer fish *Colomesus psittacus* Bloch and Schneider, 1801 (Teleostei, Tetraodontidae) (Brazilian common name "baiacú") were collected from the estuarine region of the Amazon River (02°14′ S, 48°57′ W) near the city of Cametá (Pará State), Brazil. The specimens were anaesthetised by MS 222 (Sandoz Lab.) and later measured (8–12 cm in length). Infection was determined by the presence of xenomas located in the intestinal mucosa. The prevalence of infection was 30% (nine fishes in 30 examined) in both sexes.

Fig. 1 Light and transmission electron micrographs of the microsporidian *L. psittaca* n. sp. parasite of *Colomessus psittacus*. *1* A group of fresh spores observed in DIC. *Scale bar*, 10 μm. *2* An isolated fresh mature spore observed in DIC. *Scale bar*, 10 μm. *3* Semi-thin section of the xenoma showing the wall (*W*) and the matrix of the xenoma containing numerous spores. *Scale bar*, 50 μm. *4* Semi-thin section of the xenoma periphery showing the wall (*W*) and the matrix containing developmental stages (*asterisk*) and numerous spores. *Scale bar*, 10 μm. *5* Ultrathin section of a xenoma showing the wall formed by several fibroblast layers (*Fb*). The matrix shows some spores (*Sp*). *Scale bar*, 5 μm. *6* Ultrathin section of some spores (*Sp*) sectioned at different levels showing the internal organisation. *Scale bar*, 1 μm. *7* Ultrastructural details of the spore apical zone showing the spore wall (*Wa*), the anchoring disc (*AD*) and the polar filament sections (*PF*) of which the anterior part was surrounded by two types of polaroplast lamellae (*Pp*). Several polyribosomes organised in long tapes (*arrows*) are observed. *Scale bar*, 0.5 μm. *8* Ultrastructural details of the polyribosomes arranged in long coiled tapes (*arrows*). The wall (*Wa*) and some transverse section of the polar filament (*PF*) are also observed. *Scale bar*, 0.2 μm. *9* Ultrastructural details of some transverse sections of the polar filaments (*PF*) containing some internal concentric layers. The spore wall (*Wa*) was composed by two layers of different densities (*arrowheads*). *Scale bar*, 0.2 μm

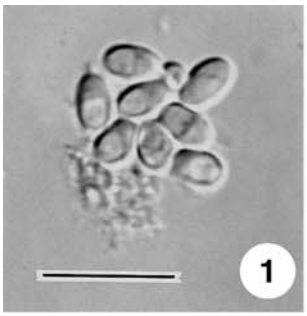
Light and transmission electron microscopy

For light microscopy, smears of xenoma and free spores were observed directly without fixation or stain by a light microscope equipped with Nomarski interference contrast [differential 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-fixed 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 (three 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 xenomas were dissected from fishes following homogenisation to isolate the spores and then were stored in 80% ethanol at 4°C. The genomic DNA of about 5×10^6 spores was extracted using a GenElute™ Mammalian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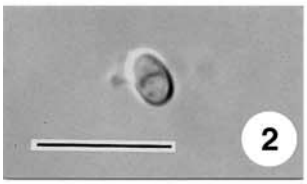
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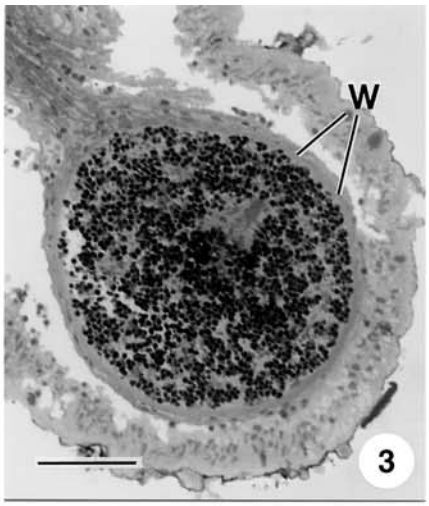
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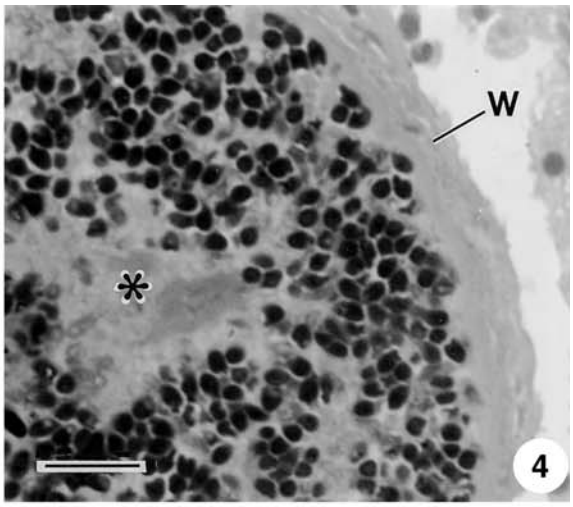
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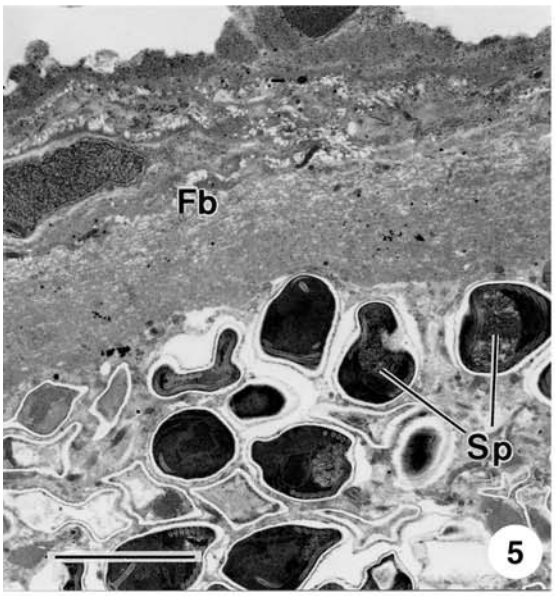
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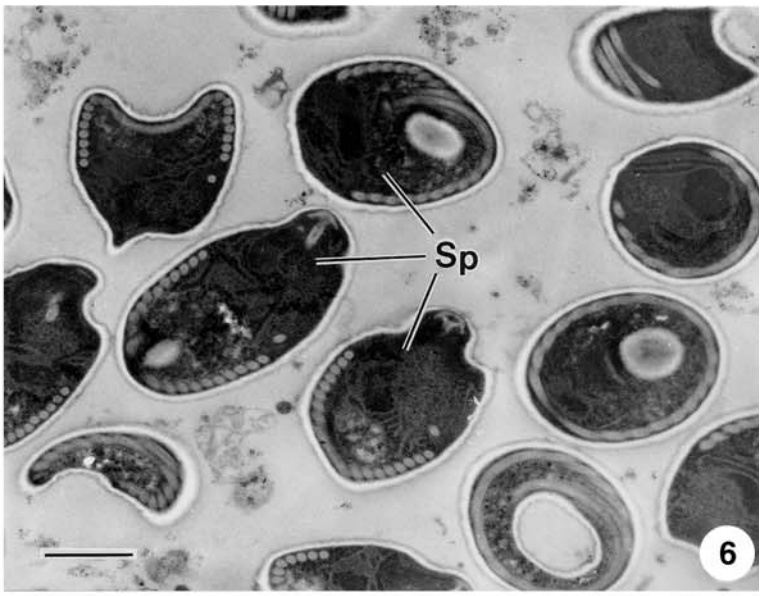
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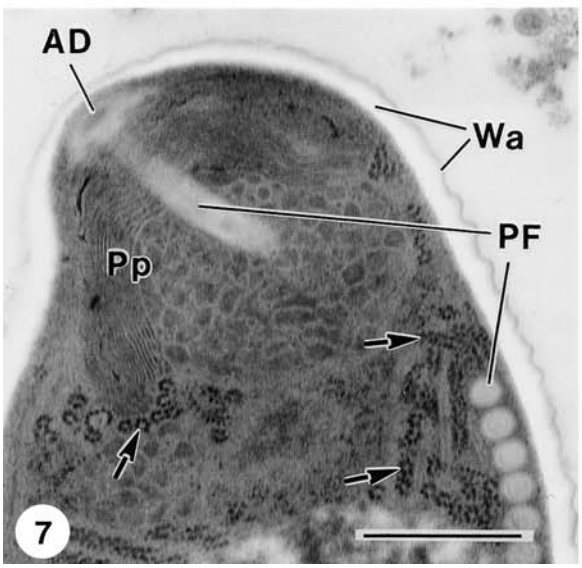
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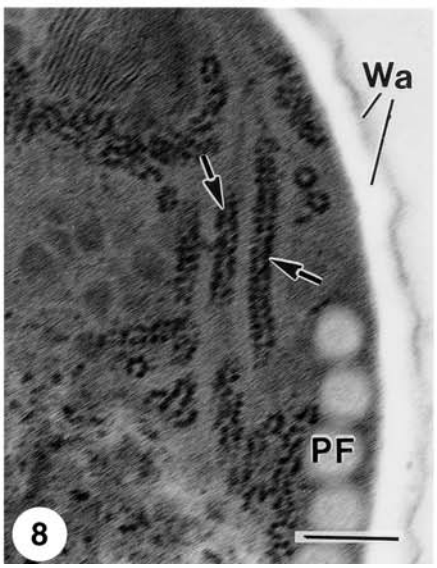
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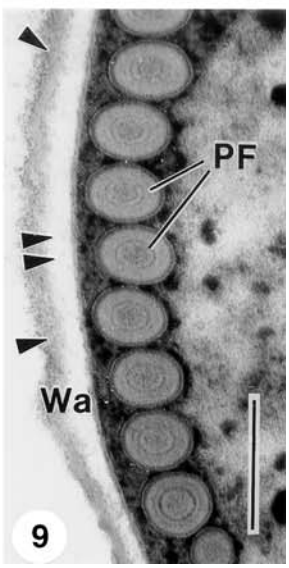
PF
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9

Genomic DNA Miniprep kit (Sigma) according to the manufacturer's instructions for animal tissue protocol, except for the incubation time. The DNA was stored in 50 µl of TE buffer at −20°C until use. The DNA concentration was estimated with the Qubit™ Fluorometer (Invitrogen). The majority of the region coding for the SSU rRNA gene was amplified by polymerase chain reaction (PCR) using the primers V1f (5′ CACCAGGTT GATTCTGCC 3′) and 1492r (5′ GGTTACCTTGTTAC GACTT 3′) (Vossbrinck et al. 1993; Nilsen 2000). 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 10X *Taq* polymerase buffer, 1.25 U *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, USA). The amplification programme 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. Aliquots (5 µl) of PCR products were visualised with ethidium bromide staining after running on a 1% agarose gel.

DNA sequencing

PCR product for the SSU rRNA gene has an approximate size of 1,400 bp. It was cleaned using the MinElute PCR purification kit (Qiagen) and then three 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 *L. psittaca* to other microsporidians, a homology search was performed using BLAST programme (Altschul et al. 1990). We used 44 rDNA sequences belonging to the microsporidians parasitising fish species. The sequence and NCBI accession number data obtained from GenBank are the following: *Aspalatospora milevae* (EF990668); *Glugea anomala* (AF044391); *Glugea atherinae* (U15987); *Glugea plecoglossi* (AJ295326); *Glugea stephani* (AF056015); *Glugea*

Table 1 Comparative measurements (in µm) from *Loma* spp.

Loma sp.	Host and local infection	Habitat countries	Spore shape	Spore		Polar filament		References
				Length	Width	Coils	Row	
L. branchialis (=*L. morhua*)	*Melanogrammus aeglefinus* Gill filaments	Marine Boreo-Artic	Ellispoidal / ovoid	4.2 6	2.0 4	16–17 16–19	isofilar	(Morrison and Sprague 1981)
L. salmonae	*Oncorhynchus mykiss* Gill filaments	Freshwater Several countries	Pyriform/ ellipsoidal	3.7 4.4	2.2 2.3	12–14 14–17		(Putz et al. 1965)
L. fontinalis	*Salvelinus fontinalis* Gill lamellae	Freshwater Canada	–			12–14		(Morrison and Sprague 1983)
L. dimorpha	*Gobius niger* (and others species) Connective tissue of digestive tract	Marine France and Spain	Ovoid/ ellipsoidal	4.5	1.8–2.0	13–15	Isofilar	(Loubès et al. 1984)
L. diplodae	*Diplodus sargus* Vessels of the gill filaments	Marine France	Ovoid	4.17	2.22	17–18		Bekhti and Bouix 1985)
L. trichiuri	*Trichurus savala* Gill filaments	Marine India	Pyriform	3.0	2.0	–		(Sandeep and Kalavati 1985)
L. camerounensis	*Oerochromis niloticus* Oesophagus to intestine	Freshwater Cameroon	Ovoid	3.96	2.16	11–12		(Fomena et al. 1992)
L. boopsi	*Boops boops* Liver and digestive tract	Marine Senegal	Ovoid	3.7	2.4	12–14 16–18	Isofilar	(Faye et al. 1995)
L. embiotocia	*Cymatogaster aggregate* Gills	Marine Canada	Ovoid	4.8	2.6	14–18		(Shaw et al. 1997)
L. acerinae	*Gymnocaphalus cernuus* Intestine wall	Freshwater Czech Republic	Ellipsoidal	4.64	2.19	11–23	Isofilar	(Lom and Pekkarinen 1999)
L. myrophis	*Myrophis platyrhynchus* Subepithelial gut tissue	Freshwater Brazil	Ellipsoidal	4.06	1.61	13–14	Isofilar	(Azevedo and Matos 2002)
L. psittaca n. sp.	*Colomesus psittacus* Intestinal wall	Freshwater Brazil	Ovoid	4.2	2.8	11–12	Isofilar	This study

sp. GS1 (AJ295325); *Glugea* sp. (AY090038); *Heterosporis anguillarum* (AF387331); *Heterosporis* sp. PF (AF356225); *Ichthyosporidium* sp. (L39110); *Kabatana takedai* (AF356222); *Kabatana newberryi* (EF202572); *Kabatana seriolae* (AJ295322); *Loma acerinae* (AJ252951); *Loma embiotocia* (AF320310); *L. salmonae* (U78736); *Loma* sp. (AF104081); *Microgemma caulleryi* (AY033054); *Microgemma tincae* (AY651319); *Microgemma vivaresi* (AJ252952); *Microsporidium cypselurus* (AJ300706); *Microsporidium prosopium* (AF151529); *Microsporidium* GHB1 (AJ295324); *Microsporidium* sp. RSB1 (AJ295323); *Microsporidium* sp. STF (AY140647); *Microsporidium* MYX1 (AJ295329); *Myosporidium merluccius* (AY530532); *Nucleospora salmonis* (U78176); *Ovipleistophora mirandellae* (AF356223); *Ovipleistophora ovariae* (AJ252955); *Pleistophora ehrenbaumi* (AF044392); *Pleistophora finisterrensis* (AF044393); *Pleistophora hippoglossoideos* (AJ252953); *Pleistophora typicalis* (AF044387); *Pleistophora* sp. 1 (AF044394); *Pleistophora* sp. 2 (AF044389); *Pleistophora* sp. 3 (AF044390); *Potaspora morhaphis* (EU534408); *Pseudoloma neurophilia* (AF322654); *Spraguea americana* (AF056014); *Spraguea lophii* (1) (AF104086); *S. lophii* (2) (AF033197); *Spraguea* sp. (AY465876); *Tetramicra brevifilum* (AF364303). *Endoreticulatus schubergi* (L39109); *Enterocytozoon bieneusi* (L07123); *Vairimorpha necatrix* (Y00266) and *Vittaforma corneae* (L39112) were used as outgroup. Sequences were aligned as described by Casal et al. (2008). Alignment was done through 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 for both pairwise and multiple alignments. Subsequent phylogenetic and molecular evolutionary analyses were conducted using MEGA 4, with the 44 rDNA sequences for microsporidian species and the outgroup species selected. Distance estimation was carried out using the Kimura-2 parameter model distance matrix for transitions and transversions. For the phylogenetic tree reconstructions, maximum parsimony analysis was conducted using the close neighbour interchange heuristic option with a search factor 2 and random initial trees addition of 2,000 replicates. Bootstrap values were calculated over 100 replicates.

Results

Some spherical whitish xenomas were macroscopically observed adherent to the intestinal mucosa of the fish. After rupture of the xenoma wall, free spores (1 and 2 in Fig. 1) were easily microscopically observed and identified as belonging to the phylum Microsporidia. These xenomas with up to ~310 μm diameter, filled with numerous spores and different developmental stages, contained a thick xenoma wall formed by several juxtaposed fibroblast layers (3 and 4 in Fig. 1).

Description of *L. psittaca* n. sp.

Systematic position (Figs. 1 and 2)
Phylum Microsporidia Balbiani, 1882
Class Haplophasea Sprague, Becnel and Hazard, 1992
Order Glugeida Issi, 1986
Family Glugeidae Thélohan, 1892
Genus *Loma* Morrison and Sprague, 1981
Species: *L. psittaca* n. sp.

Description of the species

Type host: *C. psittacus* Bloch and Schneider, 1801 (Teleostei, Tetraodontidae) (Brazilian common name “baiacú”).

Type locality: Estuarine region of the Amazon River (02° 14′ S, 48°57′ W) near the city of Cametá (Pará State), Brazil.

Pathogenecity: The whitish cysts (xenoma) wall was formed by several juxtaposed collagen layers intermingled with some fibroblasts, but no other tissue reactions were observed and no clinical signs were detected.

Location in the host: Xenoma in the intestinal mucosa.

Prevalence of infection: Nine of 30 (30%).

Type specimens: One glass slide containing mature free spores and others 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 1123998. The histological semi-thin sections containing different developmental stages were deposited at the laboratory of the senior author.

Etymology: The specific name is derived from the generic name of the host species.

Description of the spores

Ovoid spores measuring 4.2 ± 0.4 × 2.8 ± 0.4 μm (n = 30) contained all typical characteristic structures of the Microsporidia (1, 2, 6 and 7 in Fig. 1). The spore wall was about 87 nm thick, except for the anterior end where the central zone of the anchoring disc contacted with the wall, which was about 20–35 nm thick consisting of an electron-lucent

endospore and a thin electron-dense exospore (7–9 in Fig. 1). The anchoring disc is located in the apical region of the spore in an eccentric position in relation to the spore axis, giving a bilateral asymmetry (6 and 7 in Fig. 1). The anterior part of the polar filament (PF) (manubrium) measured about 125 (118–131) nm in diameter and the angle of tilt anterior PF to the spore axis was ~48° (7 in Fig. 1). The PF was isofilar arranged into 10–11 (rarely 12) coils in one row, and when sectioned transversally, it had 80–90 nm in diameter and exhibited three concentric layers (9 in Fig. 1). The last coil measured ~60 nm in diameter (9 in Fig. 1). The polaroplast had two distinct lamellar structures folded around the PF. In the anterior zone, the compacted lamellae was without lumen, whilst in the posterior lamellae, the lumen was filled with electron-dense material (7 in Fig. 1). The nucleus, containing a moderately uniform nucleoplasm, was surrounded by numerous polyribosomes forming coiled tapes (7 and 8 in Fig. 1). The posterior vacuole, situated at the basal part of the spore between the PF coils, was irregular and contained light material (7 in Fig. 1).

Developmental stages

Developmental stages with asynchronous distribution and a hypertrophic nucleus centrally positioned were observed (5 in Fig. 1). In the cytoplasm xenoma, it was possible to see many mitochondria surrounding the parasites (10–12 in Fig. 2).

Meronts

They appeared in ultrathin sections as round to elliptical uninucleated or binucleated cells with the unpaired nuclei. These nuclei presented homogeneous chromatin without apparent nucleolus. The cytoplasm possessing numerous free ribosomes was uniformly granular and poorly endowed with cytoplasmatic organelles (10 in Fig. 2). Meronts divided by multiple fissions and transformed into sporonts (10 and 12 in Fig. 2).

Sporonts

These cells were characterised by a gradual acquisition of a thick and dense discontinuous cell coat formed by isolated patches located on the outer surface of the plasmalemma (12 in Fig. 2). The multinucleated sporogonial plasmodia had several cisternae of RER surrounding the nucleus. Between the sporont and host cytoplasm, a small space appeared, growing up until transforming into parasitophorous vacuole (PV) (membrane lining the vacuole originated by host cell). The cytoplasm of the host cell in close contact with the sporogony vacuole gradually accumulated a great quantity of electrodense material (11 and 12 in Fig. 2). Later, this material appears to be transferred to PV space, and simultaneously, the sporont divided into sporoblast cells.

Fig. 2 Ultrastructural aspects of some developmental stages of *L. psittaca* n. sp. parasite of *C. psittacus*. *10* A dividing meront (*Mr*) located amongst mature spores (*Sp*). *Scale bar*, 2 μm. *11* A sporogonial plasmodium in division showing the wall formation by deposition of dense material around the plasmalemma (*arrowheads*). *Scale bar*, 2 μm. *12* Some early sporoblasts (*Sb*) located amongst dividing meront (*Mr*) and mature spores (*Sp*). A dense granular substance (*arrowheads*) was interposed between early sporoblasts. *Scale bar*, 2 μm. *13* Some free mid sporoblasts (*Sb*) located in the parasitophorous vacuole space (*asterisk*) containing some dense granular substances (*arrowheads*) and tubular appendages (*arrows*). *Scale bar*, 2 μm. *14* Some late sporoblasts (*Sb*) in the parasitophorous vacuole (*asterisk*) containing some dense granular substances (*arrowheads*). *Scale bar*, 1 μm

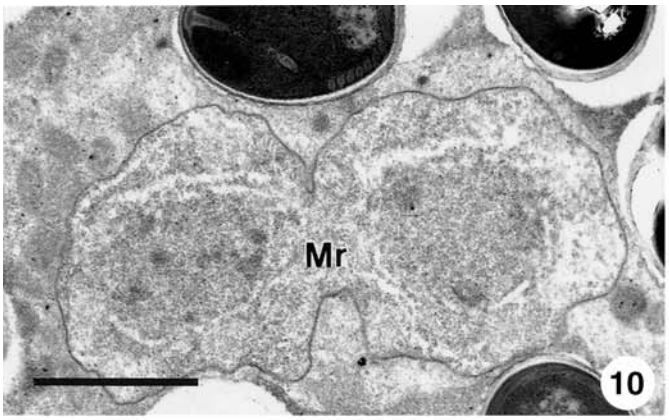
Sporoblasts

The sporoblasts gradually differentiate the typical organelles of the spores and became with irregular contours (13 and 14 in Fig. 2). Sporoplasm became dense and the endospore (internal portion of the wall) became more evident. Simultaneously, inside the PV space, the mass of electrodense material dispersed between the sporoblasts seemed to dissipate into tubular structures (13 in Fig. 2).

Molecular analysis

Conserved SSU rDNA primers V1f /1492r permitted to amplify a fragment with approximately 1.4 kb. After sequencing both strains, a sequence 1,260 bp in length corresponding to the almost SSU rRNA gene was obtained. This sequence with a GC content of 55.5% was deposited in GenBank (accession number FJ843104). Blast search confirmed that it belongs to 16S rDNA and bears the closest similarity to other microsporidians that have fish species as a host. Forty-four SSU rDNA sequences were aligned with the *L. psittaca* SSU rDNA sequence. The length of the aligned sequences used for phylogenetic analysis was 1,459 bases after trimming the 3′ end. Before phylogenetic analysis, only those sites which could be unambiguously aligned amongst all microsporidians and outgroups were used, resulting in an alignment of 1,339 bases long.

Based on GenBank BLAST searches of the SSU rRNA gene, *L. acerinae* (AJ252951) is the most similar species (96.9% of identify), whereas *G. anomala* and *G. atherinae* species had 96% and *P. finisterrensis*, *G. plecoglossi* and *Glugea* sp. GS1 had 95.6%. The distances observed between *L. psittaca* and the other previously described *Loma* species were higher than 10%: *Loma* sp. (12.7%), *L. salmonae* (13.1%) and *L. embiotocia* (14.6%; Table 2). The maximum parsimony phylogenetic analyses of the SSU rRNA showed that *L. acerinae* is a sister species to *L.*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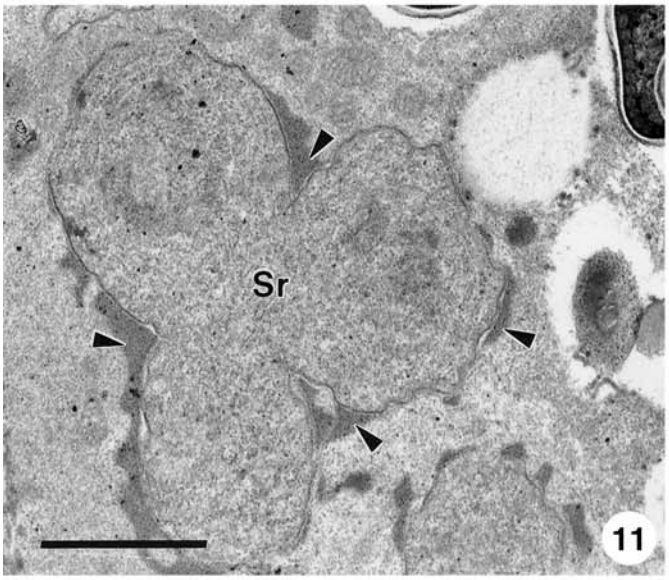
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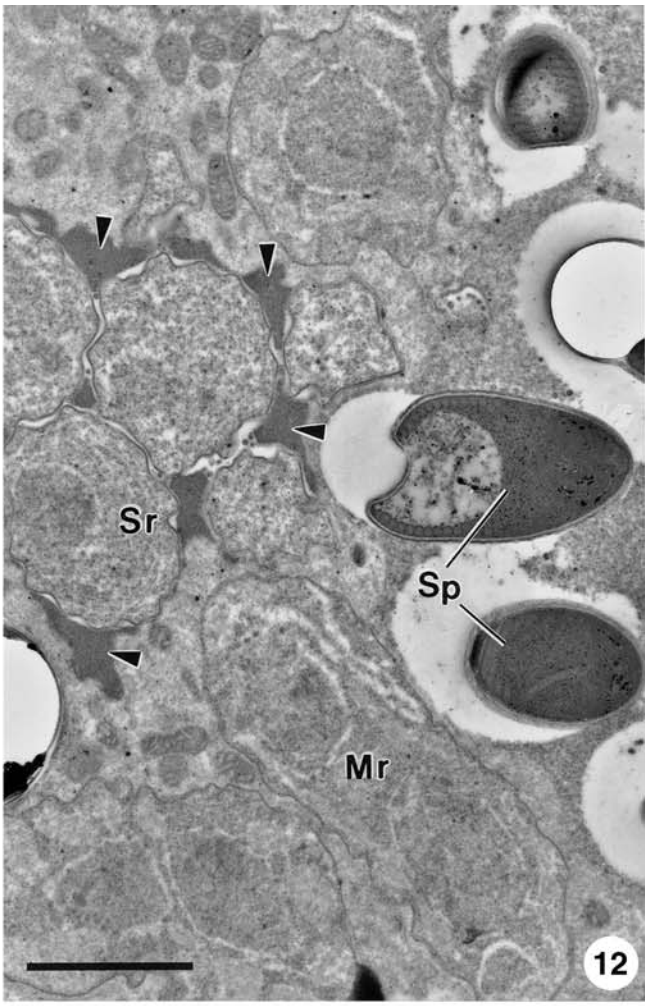
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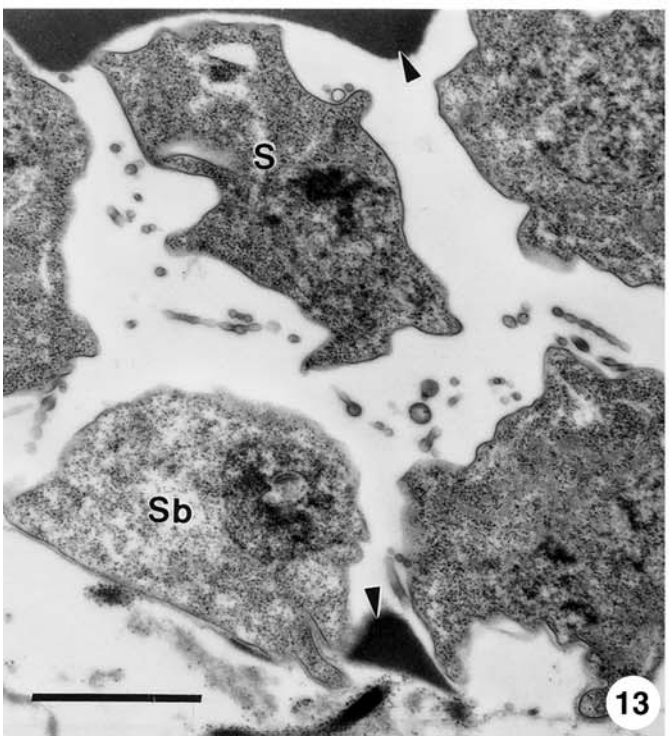
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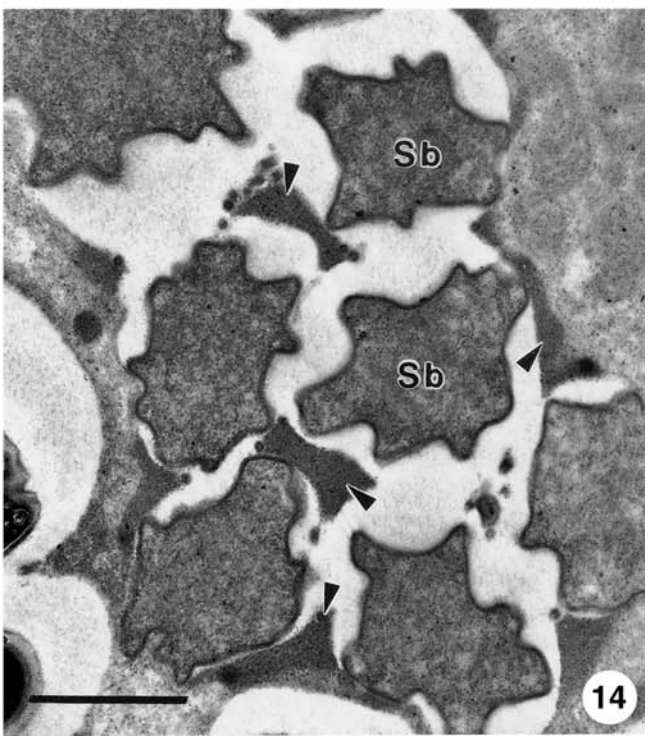
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Table 2 Comparison of some SSU rDNA sequences: percentage of identity (top diagonal) and pairwise distance (bottom diagonal) obtained by Kimura-2 parameter analysis

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Loma psittaca n. sp.	–	96.9	96.0	96.0	95.6	95.6	95.6	95.2	94.4	89.6	89.6	89.2	87.3	86.9	85.4
Loma acerinae	0.031	–	97.3	97.3	96.5	96.5	96.5	96.5	96.5	90.6	89.7	91.0	88.8	88.3	86.9
Glugea atherinae	0.040	0.027	–	100	98.8	98.8	98.8	99.2	97.3	92.7	90.1	91.5	89.6	89.2	87.8
Glugea anomala	0.040	0.027	0.000	–	98.8	98.8	98.8	99.2	97.3	92.7	90.1	91.5	89.6	89.2	87.8
Pleistophora finisterrensis	0.044	0.035	0.012	0.012	–	100	100	98.1	96.5	93.6	89.6	91.0	89.6	90.5	89.2
Glugea plecoglossi	0.044	0.035	0.012	0.012	0.000	–	100	98.1	96.5	93.6	89.6	91.0	89.6	90.5	89.2
Glugea sp. GS1	0.044	0.035	0.012	0.012	0.000	0.000	–	98.1	96.5	93.6	89.6	91.0	89.6	90.5	89.2
Glugea stephani	0.048	0.035	0.008	0.008	0.019	0.019	0.019	–	96.5	91.9	89.6	91.0	88.8	88.3	86.9
Glugea sp.	0.056	0.035	0.027	0.027	0.035	0.035	0.035	0.035	–	90.6	90.1	90.0	87.8	87.4	86.0
Microsporidium sp. MX1	0.104	0.094	0.073	0.073	0.064	0.064	0.064	0.081	0.094	–	91.9	92.8	92.7	93.1	91.8
Pseudoloma neurophilia	0.104	0.103	0.099	0.099	0.104	0.104	0.104	0.104	0.099	0.081	–	93.6	87.8	88.3	86.9
Ichthyosporidium sp.	0.108	0.090	0.085	0.085	0.090	0.090	0.090	0.090	0.090	0.072	0.064	–	90.6	90.2	88.8
Loma sp.	0.127	0.112	0.104	0.104	0.104	0.104	0.104	0.112	0.122	0.073	0.122	0.094	–	98.4	97.3
Loma salmonae	0.131	0.117	0.108	0.108	0.095	0.095	0.095	0.117	0.126	0.069	0.117	0.098	0.016	–	98.8
Loma embiotocia	0.146	0.131	0.122	0.122	0.108	0.108	0.108	0.131	0.140	0.082	0.131	0.112	0.027	0.012	–

psittaca, supported by 78% bootstrap. Both are clustered in a group together with *Ichthyosporidium*, *Loma*, *Pseudoloma* genera and the *Microsporidium* sp. MX1. However, this clade is poorly supported with a bootstrap lower than 50%. The most parsimonious trees suggested paraphyly for *Loma* species (Fig. 3).

Discussion

The light and ultrastructural observation of the xenoma, developmental stages as well as spore morphology described in the present study, showed all structures typical of the parasites belonging to the phylum Microsporidia (Lom and Dyková 1992; Larsson 1999; Lom and Nilsen 2003).

The fishes represented at least 156 species is one of the largest group parasitised by microsporidians. They were found in different geographic area, habitat and local of infection (Lom 2002). The parasite described in the present work is the second occurrence in teleost fish belonging to the family Tetraodontidae. Ogawa and Yokoyama (1998) found in the intestine of the tiger puffer fish, *Takifugu rubripes*, from a mariculture in Japan, another microsporidian, but it was not classified. Comparing the morphology and ultrastructural aspects of the developmental stages of the parasite here described with microsporidian fish previously characterised, it seemed similar to *Loma* spp. (Lom and Dyková 1992; Lom and Nilsen 2003).

Presently, there are 11 *Loma* species and they were reported in the gills and digestive tract of the fresh and marine fishes (Lom 2002; Table 1). The species type *Loma branchialis* was found in the gills of Atlantic cod (Morrison and Sprague 1981), likely as *L. salmonae* in several salmonids species and from different regions (Putz et al. 1965), *Loma fontinalis* (Morrison and Sprague 1983), *Loma dimorpha* found in different hosts (Loubès 1984), *Loma trichiuri* (Sandeep and Kalavati 1985) and *L. embiotocia* (Shaw et al. 1997). Parasitising the intestine, oesophagus or liver, five species were reported: one in Europe, *Loma diplodae* found in *Diplodus sargus* (Bekhti and Bouix 1985); *Loma boopsi* and *Loma camerounensis* identified in African fishes, *Boops boops* from Senegal (Faye et al. 1995) and in the tilapia species *Oreochromis niloticus* from the Cameroon (Fomena et al. 1992), respectively; *L. acerinae* (Lom and Pekkarinen 1999) in the freshwater *Gymnocaphalus cernuus* from Czech Republic. Finally, *L. myrophis* found in the Amazonian fish *M. platyrhynchus* was described by Azevedo and Matos (2002). Concerning the habitat, shape and size of the mature spores and the number of polar filament coils, *L. psittaca* did not seem similar with other previously described species. Comparing with the species from the same geographic area, *L. myrophis* found also in intestinal tissue of an Amazonian freshwater fish pre-

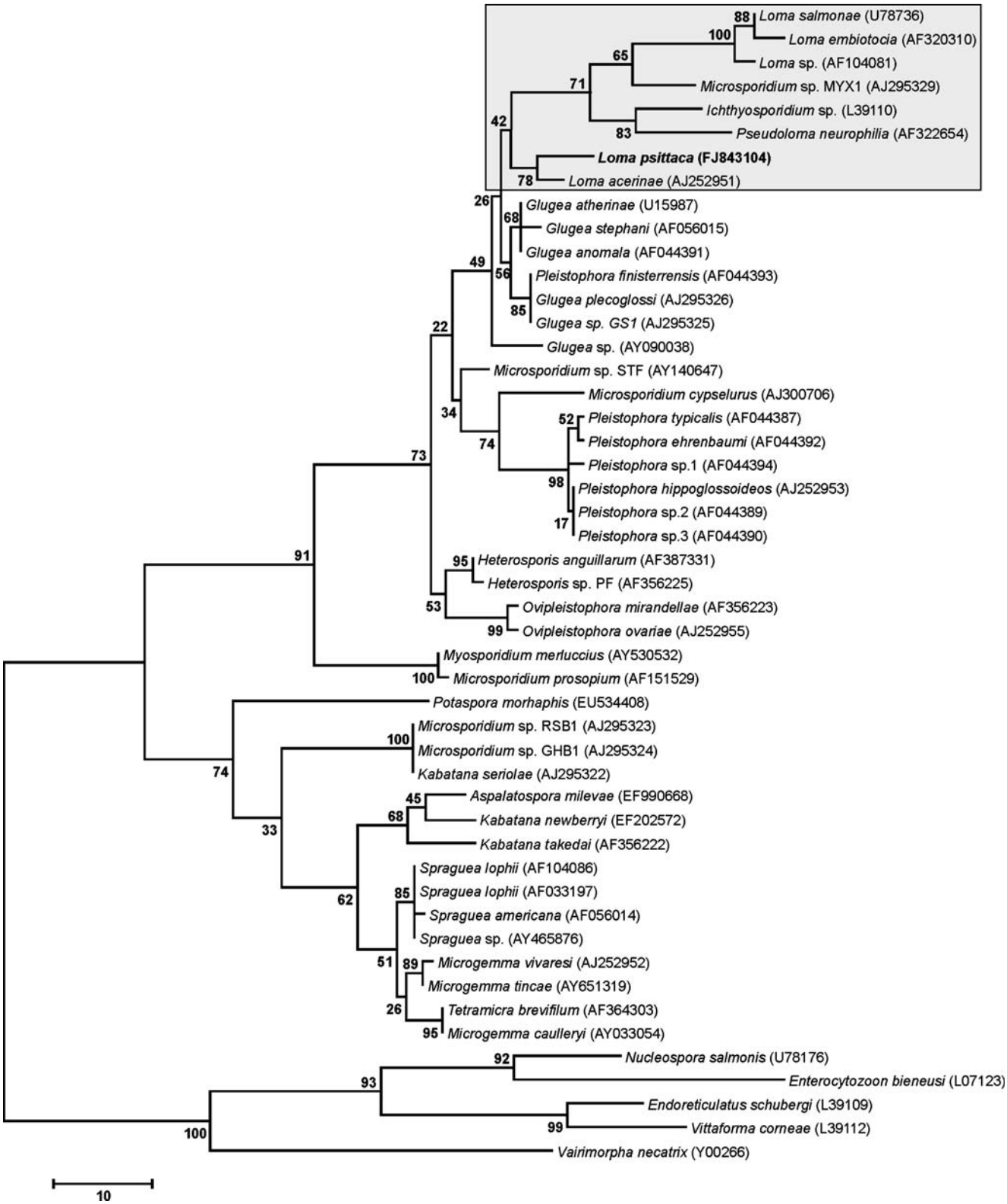

Fig. 3 Parsimony tree of SSU rDNA sequences to compare *L. psittaca* with selected sequences from other fish-infecting Microsporidia. The analysis was conducted using 1,339 aligned nucleotide positions of the highest BLAST score microsporidian sequences and four more microsporidian sequences as outgroup. The *bar* indicates the equivalence between the distance and the number of changes. The *numbers on the branches* indicate bootstrap support from 100 replicates. *L. psittaca* is placed within group I (*highlighted box*), which includes the sequences of the genera *Ichthyosporidium*, *Loma*, *Pseudoloma* and one *Microsporidium* sp.

sented some differences, mainly in the shape and dimensions of spores, being 67% and 39.7% (relationship width/length) in *L. psittaca* and *L. myrophis*, respectively.

Ultrastructural studies

Comparing *L. psittaca* n. sp. with the other *Loma* species, we saw some ultrastructural similarities, namely the developmental stage aspects. Small xenomas with a centrally located hypertrophic host cell nucleus were observed also in *L. branchialis* (Morrison and Sprague 1981), *L. acerinae* (Lom and Pekkarinen 1999) and *L. myrophis* (Azevedo and Matos 2002). In these *Loma* species, like in some *Glugea* species, it was possible to see in the episporontal space electrodense masses that differentiate several tubular appendages. Curiously, in the genus *Loma*, the origin of the episporontal space is not consensus. It has been described for some species by coalescence of host cell vesicles (PV) (Morrison and Sprague 1981, 1983; Lom and Pekkarinen 1999; Azevedo and Matos 2002), whilst in others, apparently episporontal space has been originated from blisters at the surface of the parasite cell (Bekhti and Bouix 1985; Fomena et al. 1992; Faye et al. 1995).

Phylogenetic analysis

Phylogenetic analysis using the SSU rRNA sequences of fish microsporidian suggested that the parasite found in the puffer fish of the Amazonian fauna, *L. psittaca* n. sp., is a sister species of *L. acerinae*. The most parsimonious tree was supported by 78% bootstrap. All previous phylogenetic trees obtained by parsimony and likelihood maximum presented a similar topology (Docker et al. 1997; Lom and Nilsen 2003; Casal et al. 2008), clustering the almost *Loma* species together with *Ichthyosporidium* sp., *P. neurophilia* and *Microsporidium* sp. MX1 in the group I defined by Lom and Nilsen (2003). The same trees also show that the *Loma* species are a paraphyletic group placing *L. acerinae* and *L. psittaca* in a basal position of the group I or alternatively must be considered an outgroup microsporidian of group I, as suggested by Lom and Nilsen (2003). In this study, the genetic distances (Kimura 2-parameter methods) also show that there are some similarity in SSU rRNA sequences with the species belonging to group II, namely with *G. atherinae*, *G. anomala*, *G. plecoglossi*, *G. stephani*, *Glugea* sp. GS1 and *P. finisterrensis* (last one probably needing to change taxonomic group). The diagnosis of *Glugea* and *Loma* genera presents many similarities that have been confirmed by phylogenetic analysis. Definitely, the morphological and ultrastructural aspects of *L. psittaca* do not accommodate within the genera *Glugea* (Canning et al. 1982), characterised by large xenomas with a retractile wall and by the presence of a RER cistern surrounding the meronts during developmental stages.

Based on all these morphological and ultrastructural organisation and host specificity described in the present work and comparing them with those of fish microsporidia, which form xenoma, we have found some ultrastructural differences. On the other hand, the genetic data allowed the diagnosis of other fish-infecting microsporidian, supporting the description of a new species. Lom and Nilsen (2003) have reported that a new genus to accommodate *L. acerinae* and in this case *L. psittaca* also would be created. At the moment, we did not find significant ultrastructural differences that justify the creation of a new genus.

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