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ULTRASTRUCTURAL AND MOLECULAR CHARACTERIZATION OF A NEW MICROSPORIDIUM PARASITE FROM THE AMAZONIAN FISH, *GYMNORHAMPHICHTHYS RONDONI* (RHAMPHICHTHYIDAE)

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ABSTRACT: A new species of a microsporidium found in the freshwater teleost *Gymnorhamphichthys rondoni*, collected on the lower Amazon River, is described based on light, ultrastructural, and phylogenetic studies. This parasite develops in the skeletal muscle of the abdominal cavity, forming whitish cyst-like structures containing numerous spores. Mature spores, lightly pyriform to ellipsoidal with rounded ends and measuring 4.25 ± 0.38 × 2.37 ± 0.42 μm (n = 30), were observed. The spore wall, which measured about 102 nm, was composed of 2 layers with approximately the same thickness. The isofilar polar filament was coiled, with 9–10 (rarely 8) turns. The posterior vacuole appeared as a pale area, occupying about 1/3 of the spore length, and contained a spherical posterosome composed of granular material that was denser at the periphery. The myofibrils located near the spores appeared to be in advanced degradation. Molecular analysis of the rRNA genes, including the ITS region, and phylogenetic analyses using maximum parsimony, maximum likelihood, and Baysesian inference were performed. The ultrastructural characteristics of the spores and the phylogenetic data strongly suggested that it is a new species related to *Kabatana*, *Microgemma*, *Potaspora*, *Spraguea*, and *Tetramicra*. We named this new microsporidian from Amazonian fauna as *Kabatana rondoni* n. sp.

Microsporidia are intracellular parasites that occur in almost all taxonomic groups (Canning and Lom, 1986; Sprague et al., 1992; Larsson, 1999; Lom, 2002) and are best known to cause diseases in commercially important fish hosts (Lom and Dyková, 1992; Lom, 2002; Lom and Nilsen, 2003). Microsporidian species, simultaneously parasitizing freshwater and marine fishes from different geographic areas, are included among 18 genera assigned to approximately 150 genera of Microsporidia (Lom, 2002; Lom and Nilsen, 2003; Azevedo and Matos, 2003; Baquero et al., 2005; Casal et al., 2008).

Presently, there are 156 Microsporidia species; 2 of them were identified as new genera and new species in freshwater fishes from the Amazon fauna, i.e., *Amazonspora hassar*, which occurs in the gills of *Hassar orestis* (Azevedo and Matos, 2003) and *Potaspora morhaphis* in the coelomic cavity of *Potamorhaphis guianensis* (Casal et al., 2008). Another 2 Microsporidia from the same region have also been described, i.e., *Loma myrophis*, parasitizing the sub-epithelial gut tissues of *Myrophis platyrhynchus* (Azevedo and Matos, 2002) and *Microsporidium brevirostris* in the skeletal muscle adjacent to the abdominal cavity of the teleost fish *Brachyhypopomus brevirostris* (Hypopomidae) (Matos and Azevedo, 2004). The last species, and the microsporidian described in the present report from *Gymnorhamphichthys rondoni* (fam. Rhamphichthyidae), represent the first reference to microsporidiosis in teleost knifefishes (Gymnotiformes). Phylogenetic studies based on the molecular analysis of the rRNA genes have been a powerful tool in the identification of new genera and species, as well as in grouping in family taxa (Weiss and Vossbrinck, 1999; Vossbrinck and Debrunner-Vossbrinck, 2005). Presently, there are several SSU rRNA sequences available in GenBank corresponding to 44 microsporidian species in fish. According to Lom and Nilsen (2003), fish Microsporidia are clustered in 5 groups and only some of the genera are monophyletic.

Using light and electron microscopy, we describe several morphological features of a new microsporidian species found in a fish from the Amazon River. Molecular characterization and phylogenetic relationships for the SSU rRNA gene were also performed, as well as an analysis of the pathology induced by spores in the muscle.

MATERIALS AND METHODS

Fish, location of infection, and prevalence

Several irregular, whitish aggregations of spores (cyst-like structures), located in the skeletal muscles of the internal wall of the ventral abdominal cavity, were removed from the freshwater fish *Gymnorhamphichthys rondoni* (fam. Rhamphichthyidae) (Brazilian common name: Itui transparente). The fish were collected in the lower Amazonian region (01°46′S, 47°26′W) near Irituia City, Pará State, Brazil. The fish (12–25 cm long) were taken alive to the laboratory, where they were anesthetized with MS 222 and necropsied. For measurements, fresh isolated spores were observed in the Nomarski differential interference–contrast (DIC) optics. The prevalence of infection was 36% (18 fishes in 50 examined).

Electron microscopy

For transmission electron microscopy (TEM), small fragments of the infected tissues were fixed in 3% glutaraldehyde with 0.2 M sodium cacodylate buffer (pH 7.2) for 12 hr at 4 C, washed overnight in the same buffer at 4 C, and post-fixed in 2% OsO_4 buffered in the same solution for 3 hr at same temperature. After dehydration in an ascending ethanol series and propylene oxide, the fragments were embedded in Epon. The semi-thin sections were stained with blue methylene-Azure II for light microscopy. The ultra-thin sections were contrasted with both aqueous uranyl acetate and lead citrate and observed with a JEOL 100CXII TEM (JEOL, Ltd., Tokyo, Japan) operated at 60 kV.

DNA isolation and PCR amplification

To isolate the spores, several cysts dissected from fishes were homogenized and subsequently stored in 80% ethanol at 4 C. The genomic DNA of approximately 5×10^6 spores was extracted using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, Missouri) following the manufacturer's instructions for animal tissues, except for our incubation time (12 hr). The DNA was stored in 50 μl of TE buffer at −20 C until further use. The DNA concentration was estimated

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with the Qubit™ Fluorometer (Invitrogen, Carlsbad, California). The majority of the region coding the small subunit (SSU) rRNA gene was amplified by PCR using the primers V1f (5′-CACCAGGTTGATT CTGCC-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Vossbrinck et al., 1993; Nilsen, 2000). To amplify the 3′-end of the SSU internal transcribed spacer (ITS), and the 5′-end of the large subunit (LSU) rRNA gene, HG4F (5′-GCGGCTTAATTTGACTCAAC) and HG4R (5′-TCTCCTTGGTCCGTGTTTCAA) primers were used (Gatehouse and Malone, 1998). 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 10× *Taq* polymerase buffer, 1.25 units *Taq* DNA polymerase (Invitrogen), and 3 µl of the genomic DNA. The reactions were run on a Hybaid PxE Thermocycler (Thermo Electron Corporation, Milford, Massachusetts). 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-microliter aliquots of the PCR products were electrophoresed through a 1% agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide.

DNA cloning and sequencing

The PCR product for the SSU gene, with an approximate size of 1,400 bp, was excised from the agarose gel and purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). The DNA was cloned into a pGEM-T Easy Vector System II (Promega, Madison, Wisconsin) following the manufacturer's instructions. JM109 competent cells, high efficiency (Promega), were transformed and 2 positive clones selected. The plasmid DNA isolations were carried out with a NucleoSpin Plasmid (Macherey-Nagel) according to the manufacturer's manual. Cloning was confirmed by digestion with the restriction enzyme *EcoRI* (Promega) and through sequencing with the universal sequencing primers T7 forward/SP6. For the ITS region, a PCR product of about 1,100 bp was sequenced directly, after cleaning. The sequencing reactions were done using a BigDye Terminator v1.1 kit (Applied Biosystems, Carlsbad, California) and were run on an ABI3700 DNA analyzer (Perkin-Elmer, Applied Biosystems, Stabvida, Co., Oeiras, Portugal).

Distance and phylogenetic analysis

Previously, the various forward and reverse sequence segments were aligned manually with ClustalW (Thompson et al., 1994) in MEGA 4 software, and ambiguous bases were clarified using corresponding ABI chromatograms. To evaluate the relationship of the new species to other Microsporidia, a homology search was performed using BLAST software (NCBI, Bethesda, Maryland). We used 45 rDNA sequences belonging to the Microsporidia that had fish as hosts. The sequence and NCBI accession number data obtained from GenBank are as follows: *Aspalatospora milevae* (EF990668); *Glugea anomala* (AF044391); *Glugea atherinae* (U15987); *Glugea plecoglossi* (AJ295326); *Glugea stephani* (AF056015); *Glugea* 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); *Kabatana* sp. (EU682928); *Loma acerinae* (AJ252951); *Loma embiotocia* (AF320310); *Loma 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); *Spraguea lophii* (2) (AF033197); *Spraguea* sp. (AY465876); and *Tetramicra brevifilum* (AF364303). *Endoreticulatus schubergi* (L39109), *Enterocytozoon bieneusi* (L07123), *Vairimorpha necatrix* (Y00266), and *Vittaforma corneae* (L39112) were used as outgroup.

The alignment was performed with ClustalW 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 45 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, the maximum parsimony analysis was performed using the close neighbor interchange heuristic option with a search factor of 2 and a random initial trees addition of 2,000 replicates. Clade support was assessed with bootstrapping of 100 replicates.

For maximum likelihood (ML) and Bayesian inferences (BI) analysis, the sequences were aligned with ClustalW and the ambiguous regions, i.e., those containing gaps, poorly aligned, or both, were subsequently removed with Gblocks using the default parameters through the Phylogeny.fr platform (Dereeper et al., 2008). The ML method was implemented in the PhyML program (v3.0 aLRT) (Guindon et al., 2005). The GTR substitution model was selected assuming an estimated proportion of invariant sites (of 0.282) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 1.386). Reliability for the internal branch was assessed using the bootstrapping method (100 bootstrap replicates). BI was performed with the MrBayes program (v3.1.2) (Ronquist and Huelsenbeck, 2003) with the following parameters: The standard (4 by 4) model of nucleotide substitution was used, the number of substitution types = 6, and rates variation across sites was fixed to "invgamma." Probability distributions were generated using Markov Chain Monte Carlo methods. Four chains were run for 2×10^6 generations, sampling every 100 generations, with the first 10,000 sampled trees discarded as "burn-in." Finally, a 50% majority rule consensus tree was constructed. Both the trees were built with the TreeDyn program (Chevenet et al., 2006).

DESCRIPTION

Kabatana rondoni n. sp.

(Figs. 1–3)

General diagnosis: Isolated and grouped, whitish cyst-like structures in skeletal muscle of abdominal cavity (Figs. 1a, b). This parasite does not develop xenomas and spores in direct contact with myofibrils (Fig. 1c).

Description of the spores: Monomorphic, uninucleated mature spores, lightly pyriform to ellipsoidal with rounded ends; 4.25 ± 0.38 µm long and 2.37 ± 0.42 µm wide (n = 30) (Figs. 1a, b). Nucleus in central position between apical polaroplast and posterior vacuole (Figs. 1d, 3). Polaroplast lamellate, bipartite, with elements of distal position somewhat expanded (Fig. 1e). Isofilar polar filament formed by 3 concentric layers of membranes (Fig. 1f), 115 (110–121) nm in diameter (n = 50), an angle of tilt of about 45° (42–47) (n = 10) (Fig. 1e), and posteriorly arranged in packed double-layered coils with 9–10 (rarely 8) turns (Figs. 1d, f). Posterior vacuole with 1/3 of spore length generally contained 1–2 conspicuous inclusions, i.e., posterosome, consisting of central granular mass surrounded by amorphous and irregular material, denser at periphery (Figs. 1d, g). Spore wall about 102 (95–110) nm thick (n = 50) composed of 2 layers, an electron-dense exospore of ~ 50 nm width and an electron lucent endospore, both with approximately the same thickness (Figs. 1d–h). Light incisions distributed regularly on exospore (Fig. 1h). Spores inside sphorophorous vesicles never observed.

Histopathology: Whitish elongated cyst-like structures containing numerous spores observed in contact with myofibrils of internal wall of abdominal cavity. Infected muscle exhibited degradation characterized by disorganization of myofibrils (Figs. 2a–c). Spores located within cytoplasm of the host cells in close contact with nuclei (Figs. 1d, 2a–c), and its cytoplasm appeared partially destroyed (Figs. 1d, 2a, b). Phagocytic cells, appearing to ingest mature spores, frequently observed near muscle fibers (Figs. 2c, d).

Molecular characterization and phylogeny: Two bands of approximately 1.4 kb and 1.1 kb were obtained after amplification of microsporidian genomic DNA, with primers V1f-1492r and HG4F-HG4R, respectively. All sequences obtained were aligned, and the sequence consensus corresponding to the complete SSU rRNA gene, ITS, and partial LSU rRNA gene was 1,914 bp in length with a GC content of 43.7%. Sequence deposited in GenBank database under accession number FJ843105. BAST analysis was performed and highest alignment excluded all microsporidian SSU rRNA sequences that did not infect fishes. Then, the 3′-end of the SSU rRNA gene was trimmed, resulting in an alignment with 1,536 bp. The alignment of the SSU rRNA gene of *K. rondoni* with the other

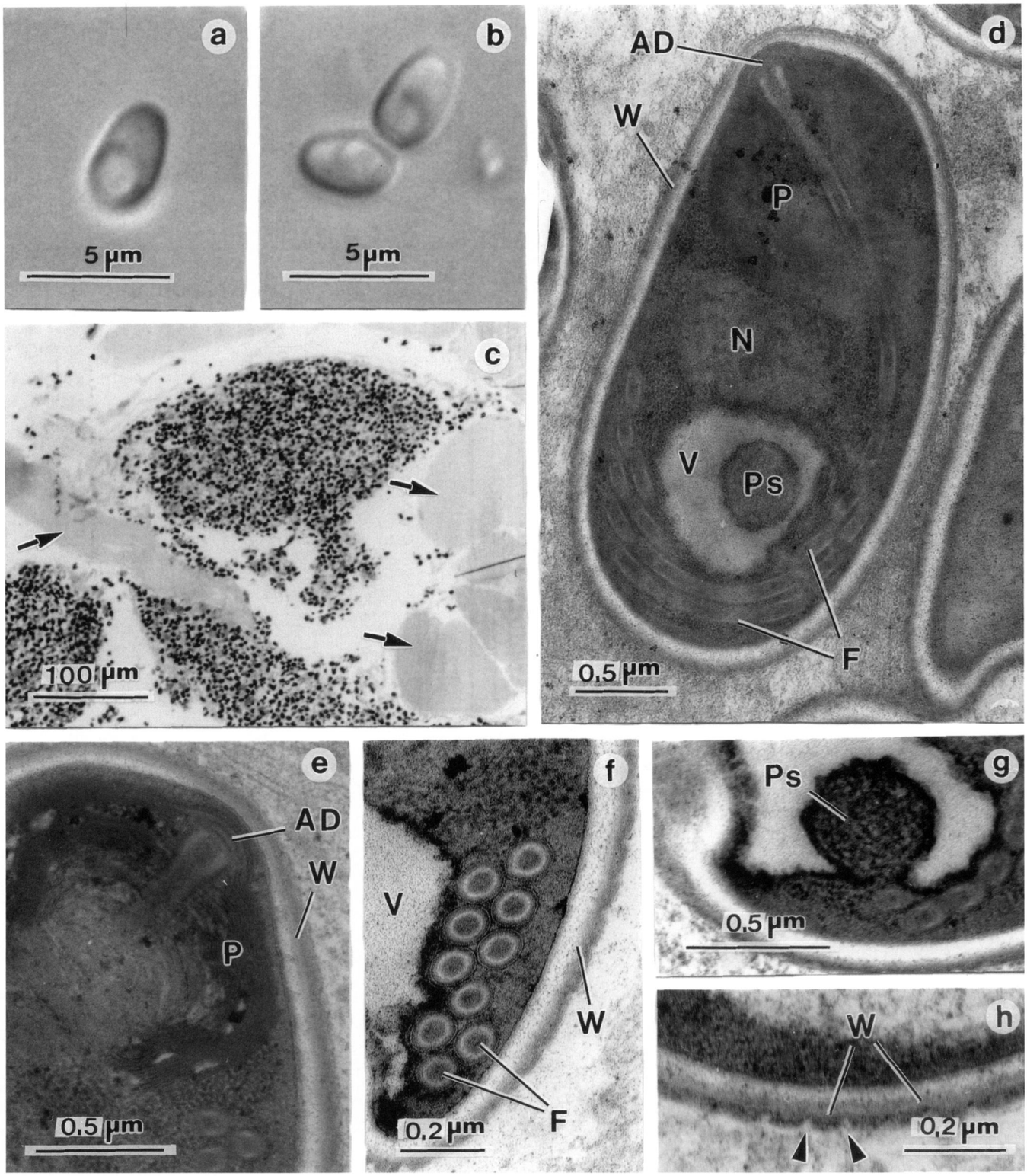

FIGURE 1. Light and transmission electron micrographs of *Kabatana rondoni* n. sp. infecting the muscle fibers of the teleost fish *Gymnorhamphichthys rondoni*. (**a, b**) Fresh spores released from the muscle observed in DIC showing the pyriform to ellipsoidal shape and their prominent posterior vacuole. (**c**) Semi-thin section of whitish patches containing numerous spores, located among muscle fibers (arrows). (**d**) Longitudinal section of a spore showing the wall (W), anchoring disc (AD), different sections of the polar filament (F), polaroplast (P) and the nucleus (N). The posterior vacuole (V) contains a posterosome (Ps). (**e**) Detail of the anterior region of a spore showing the wall (W) composed of 2 evident layers (exospore and endospore), anchoring disc (AD), and polaroplast (P). (**f**) A packed area of double-layer coils of the polar filament (F) with 10 turns between the wall (W) and the vacuole (V). (**g**) Detail of a posterosome (Ps) composed of a granular matrix and surrounded by denser material. (**h**) Transverse section of the spore wall (W) showing the external region of the exospore containing incisions distributed regularly on the spore surface (arrowheads).

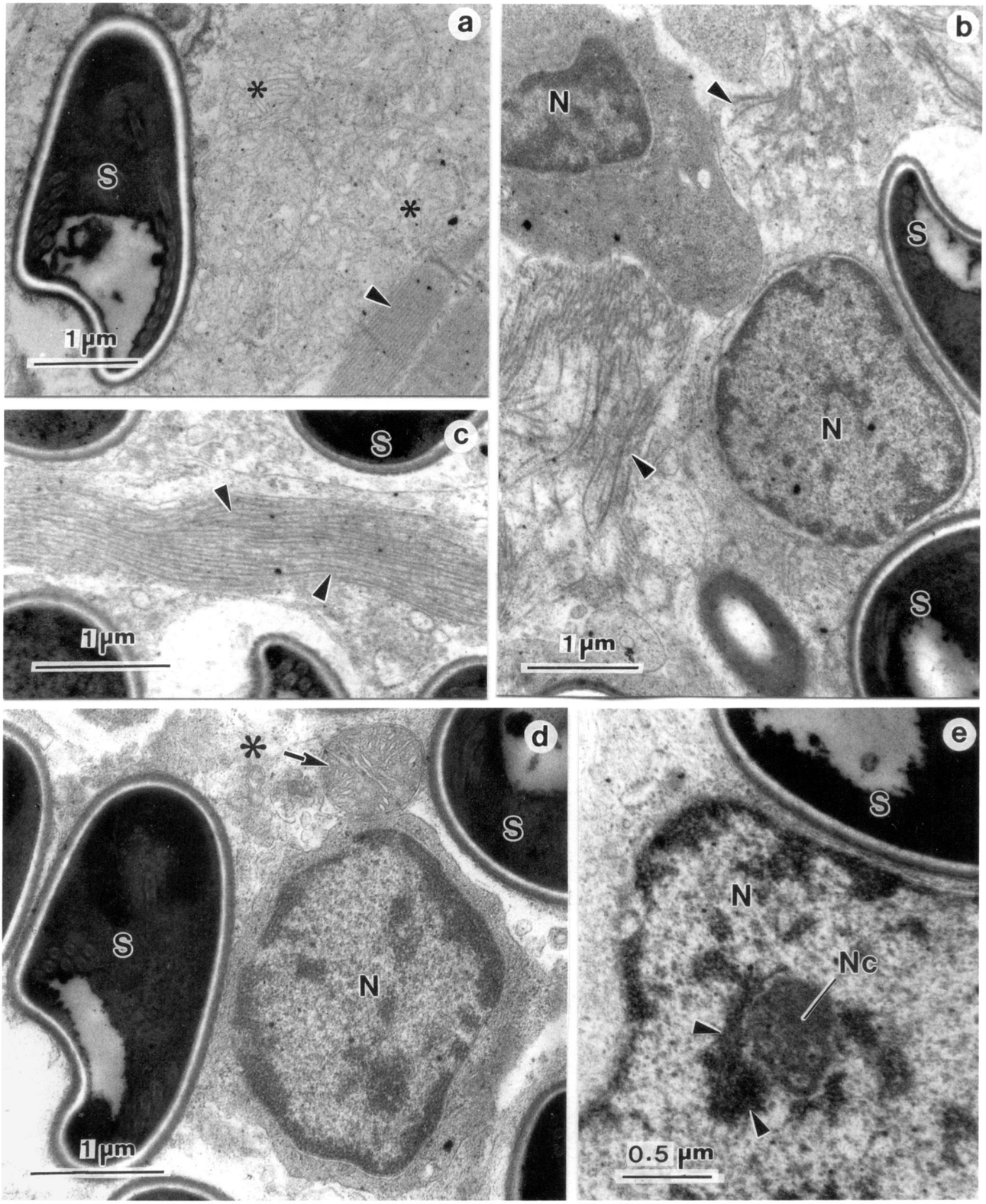

FIGURE 2. Transmission electron micrographs of *Kabatana rondoni* n. sp. infecting the muscle of the teleost fish *G. rondoni*. (**a**) A spore (S), apparently located within the sarcoplasm and containing some mitochondria (*), and evident muscle fibers showing normal myofibrils (arrowhead). (**b**) Some spores (S) in contact with phagocyte cells, each with a nucleus (N), showing among them numerous disorganized myofibrils (arrowheads). (**c**) Numerous disorganized myofibrils (arrowheads) in contact with spores. (**d**) Aspect of a phagocyte with a nucleus (N) located in several spores (S) that seemed to have a disorganized cytoplasm (*) except for the mitochondria (arrow). (**e**) Detail of a spore (S) in close contact with a nucleus (N) of a phagocyte. The nucleus contains a nucleolus (Nc) surrounded by some dense masses of perinucleolar chromatin (arrowheads).

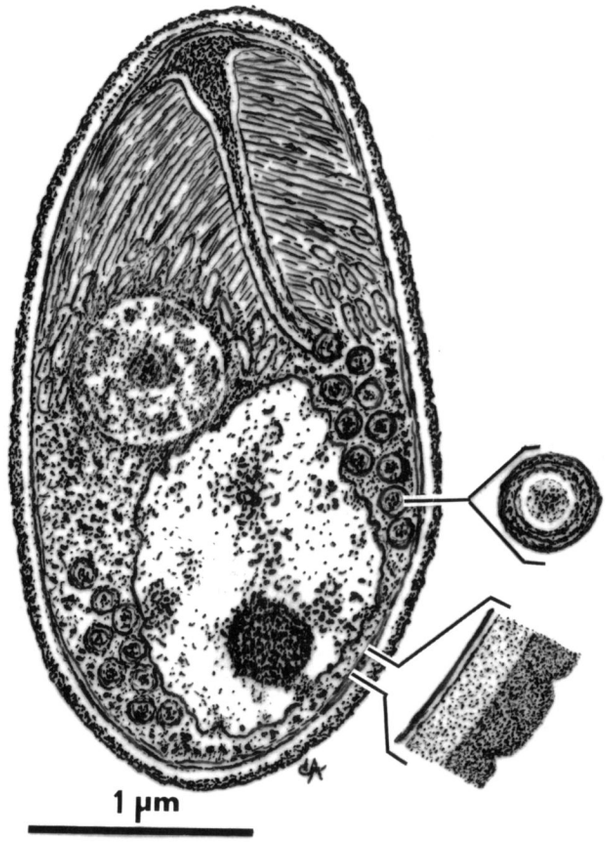

FIGURE 3. Schematic drawing of a longitudinal section of a spore of *Kabatana rondoni* n. sp. showing all typical structures described in the text. Details of transverse sections of the polar filament and spore wall are represented.

microsporidians permitted the recognition of 2 nucleotidic insertions, both with 13 bp, at the 779 and 1057 positions, respectively. Before the phylogenetic analysis, only those sites which could be unambiguously aligned among all Microsporidia and outgroups were used, resulting in an alignment of 1,402 bp. BLAST analysis of the *K. rondoni* sequence showed that *Kabatana takedai* (AF356222) and *Kabatana* sp. (EU682928) had the highest score, followed by 3 *Spraguea* spp. sequences. Based on pairwise comparisons among the SSU rDNA sequences, the maximal similarity (Kimura 2-parameter) of *K. rondoni* with the species of the same clade is for species of *Spraguea* (96.4–96.8%), *Microgemma*, and *Tetramicra* (95.6–96.0%). A longest range of percentage of identity for *Kabatana* species (88.2–95.2%) was also observed (Table I). Maximum parsimony phylogenetic analyses of the SSU rRNA gene strongly supported a clade (bootstrap 91%) with a cluster containing *Kabatana*, *Microgemma*, *Potaspora*, *Spraguea*, *Tetramicra*, and some species of *Microsporidium* (Fig. 4). Within this clade, the new microsporidium forms a sister taxon with *Spraguea* and *Microgemma* species. After BLAST search, we also found a partial SSU rDNA for *Aspalatospora milevae* (EF990668) that showed a 93.9% identify to *K. rondoni*. With an aim to clarify the phylogenetic position of this new species, the BI and ML phylogenetic analyses were also performed, confirming similar topology trees (Fig. 5).

Taxonomic summary

Type host: *Gymnorhamphichthys rondoni* (Miranda-Ribeiro, 1920) (Teleostei: Rhamphichthyidae) with a 12–25 cm length, on average.

Type locality: Lower Amazon River (01°46′S, 47°26′W) near Irituia City, Pará State, Brazil.

Site of infection: Skeletal muscle of the internal abdominal cavity.

Prevalence of infection: Eighteen of 50 (36%), with no statistical difference between sexes.

Type material: One glass slide with semi-thin sections containing mature spores of the hapantotype were deposited in the International Protozoan Type Slide Collection at the Smithsonian Institution, Washington, D.C. 20560 (USNM no. 1123996).

Etymology: The specific epithet "*rondoni*" derives from the species epithet of the host species *G. rondoni*.

Remarks

Of the 18 microsporidian genera found in teleost fishes, only *Heterosporis*, *Kabatana*, *Pleistophora*, and some species of the collective group have affinity to the myocytes of the skeletal muscle, and some induce serious pathological changes (Dyková and Lom, 2000). Species of *Heterosporis*, *Kabatana*, *Ovipleistophora*, and *Pleistophora* are characterized by the inability to develop structures known as xenomas, which confer appropriate conditions for parasite development and simultaneously minimize the proliferation of the parasite to other organs and tissues of the host (Lom, 2002; Lom and Nilsen, 2003).

Based on the spore's morphological data (shape, dimensions), the ultrastructural aspects of the internal organization (with special evidence for the anchoring disc, polaroplast, polar filament coils surrounding the posterior vacuole, and the organization of the posterosome), as well as a lack of sporophorous vesicles differentiation, the site of infection, and absence of xenoma formation), the microsporidium described here seems to be similar, at least in part, to the other species of *Kabatana* (Lom et al., 1999, 2000, 2001; McGourty et al., 2007).

The presence of 1 or more dense globules, i.e., posterosomes, which lie inside the posterior vacuole, can be observed in the spores of *Kabatana* spp. (Lom et al., 1999, 2001; McGourty et al., 2007) as well as in *Tetramicra brevifilum* (Matthews and Matthews, 1980). Another ultrastructural characteristic common to all *Kabatana* species are small depressions regularly distributed on all surfaces of the external spore's wall (Egusa, 1982; Lom et al., 1999, 2001; McGourty et al., 2007). This differentiation has been reported in microsporidian species of host fishes such as *Spraguea* spp. (Loubès et al., 1979; Freeman et al., 2004). Moreover, for species of *Amazonspora*, although it was not been reported directly, the small fields of the exospore, can be observed in the microphotographs (Azevedo and Matos, 2003).

The location of infection is another characteristic that must be considered. Apparently, the species within a genus often show tissue or organ specificity. All *Microgemma* spp. infect the liver, *Spraguea* spp. the ganglion cells of the nervous tissues, *Kabatana* spp. the skeletal muscular fibers, *Pleistophora* spp. the skeletal and smooth muscles, and almost all *Loma* spp. infect gill filaments. Most of the Microsporidia that infect the muscles could inflict heavy damage on the surrounding muscle cells. Moreover, the enzymatic action induced by the presence of parasites belonging to species of *Kabatana* and *Pleistophora* is clearly present and is similar to the one observed in members of the myxozoan, *Kudoa* spp. (Lom et al., 1999). The presence of the *Kudoa* spp. spores in direct contact with the muscle fibers has been suggested to be the reason for the liquefaction of the muscle tissue (Moran et al., 1999).

Four *Kabatana* species were reported to infect the trunk musculature of freshwater and marine fishes from distinct geographic areas. In Thailand, *K. arthuri* was found in catfish *Pangasius sutchi* (Lom et al., 1990, 1999, 2000). In Japan, yellowtail *Seriola quinqueradiata* is parasitized by *K. seriolae* (Egusa, 1982) whereas *K. takedai* was found in the heart, trunk, and other muscles of freshwater salmonids in Japan and eastern Russia (Lom et al., 2001). Recently, *K. newberryi* was reported in 2 different goby species, i.e., the tidewater goby *Eucyclogobius newberryi* in coastal lagoons in northern California (McGourty et al., 2007) and in two-spotted goby *Gobiusculus flavescens* caught in the Swedish Gullmarsfjord (Barber et al., 2009).

Comparing our results with previously described *Kabatana* spp. (Table II), we found some morphological differences on the spores, mainly in the number and arrangements of the polar filaments coils. In both *K. rondoni* and *K. newberryi*, spores have a similar number of coils (9–10); however, *K. rondoni* typically has the coils organized in 2 rows while *K. newberryi* has 1 or 2 rows. In contrast, the spores of *K. rondoni* are longer than those of *K. newberryi* (McGourty et al., 2007; Barber et al., 2009).

Phylogenetic analysis by MP and ML methods, as well as BIs using SSU rDNA, are in concordance with previous cladograms (Lom and Nilsen, 2003; Casal et al., 2008; Barber et al., 2009). The parasite described here is placed in a clade (MP: 91% bootstrap) composed of Microsporidia

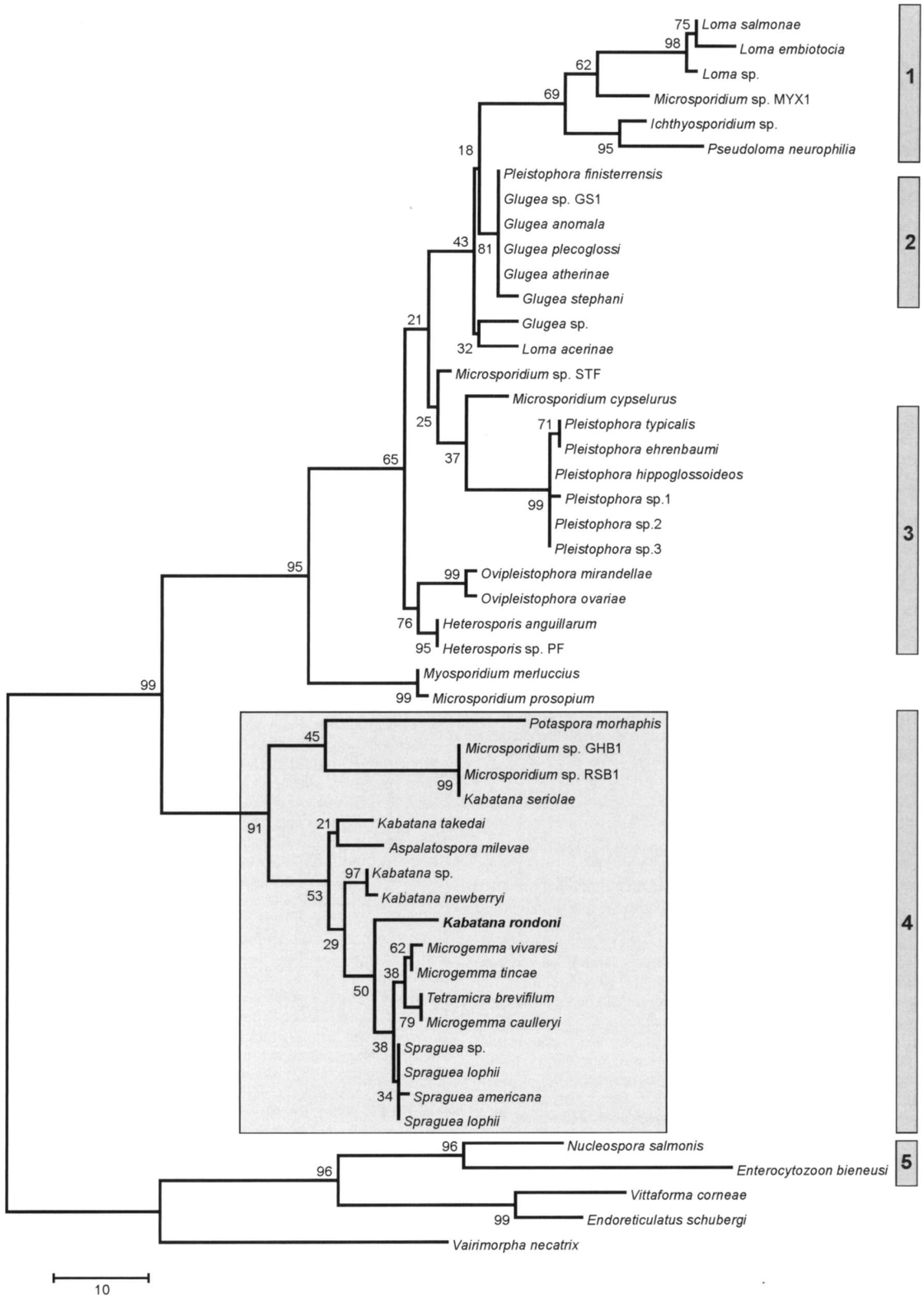

FIGURE 4. The maximum parsimony tree of SSU rDNA sequences of *Kabatana rondoni* n. sp. and other selected Microsporidia. The numbers on the branches are bootstrap confidence levels on 100 replicates. GenBank accession numbers are in parentheses after the species names and the scale is given under the tree. *Kabatana rondoni* places within group 4 (Lom and Nilsen, 2003) (highlighted box), which includes the sequences of *Kabatana*, *Microgemma*, *Potaspora*, *Spraguea*, *Tetramicra*, and *Microsporidium* species.

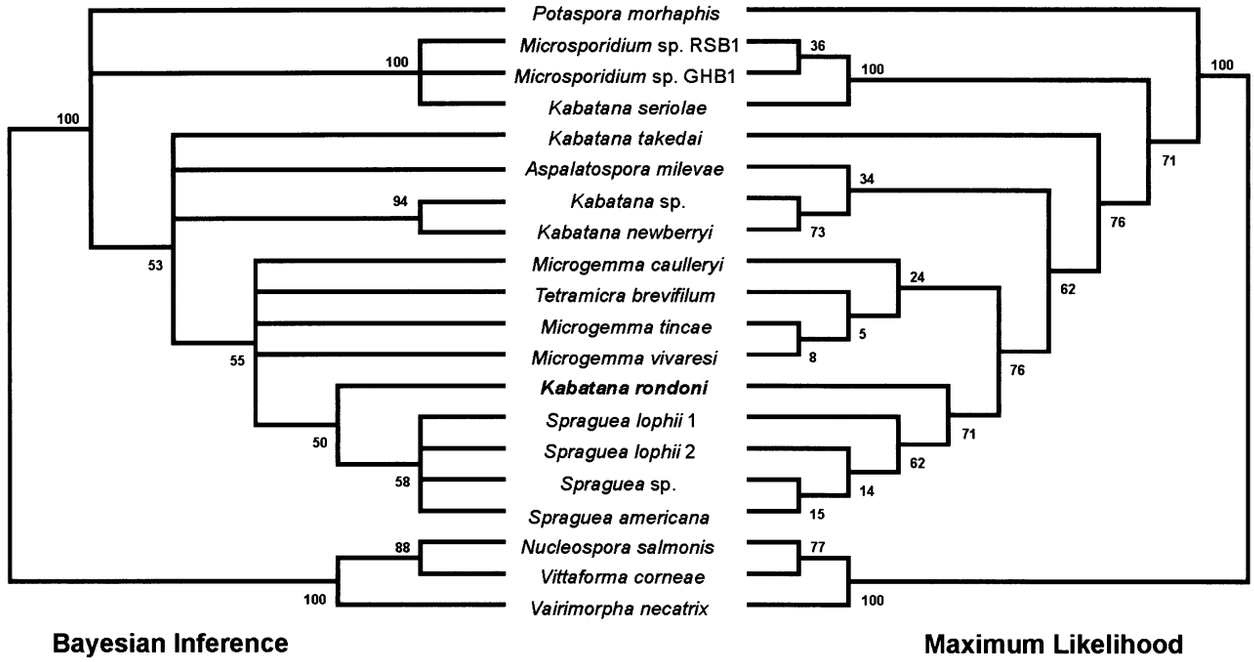

FIGURE 5. Phylogenetic tree based on Bayesian inference and maximum likelihood analysis of SSU rDNA sequences, for both *Kabatana rondoni* n. sp. and Microsporidia positioned in the same clade (Fig. 4, group IV), which provided identical topology.

belonging to *Kabatana*, *Microgemma*, *Potaspora*, *Spraguea*, *Tetramicra*, 2 unclassified species of the *Microsporidium* group, and *Aspalatospora milevae* (species only referred to in the GenBank). Like Matthews et al. (2001), we tried to identify signature sequences. We found 2 regions in the SSU rDNA sequence of *K. rondoni* to be similar to that of *K. takedai*. This kind of analysis has been used to encourage the characterization of the new species (Lom and Nilsen, 2003).

All methods provide evidence that *Kabatana* species are a paraphyletic group. The exceptions are *K. newberryi* (a parasite of a goby species from the Pacific coast, United States) and *Kabatana* sp. (parasite of a goby species from the Atlantic coast, Sweden), considered to be of the same species (Barber et al., 2009). MP analysis places *Aspalatospora milevae* in a sister taxon with *K. takedai*. Nevertheless, the bootstrap (21%) for this clade is poorly supported. The species *K. seriolae* is the most genetically

TABLE I. 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	15	16	17
(1) *Kabatana rondoni*	—	96.8	96.8	96.8	96.4	96.0	95.6	95.6	95.6	95.2	94.7	93.9	93.9	88.2	88.2	88.2	83.9
(2) *Spraguea* sp.	0.032	—	100	100	99.6	99.2	98.8	98.8	98.8	96.8	96.4	95.6	95.6	89.5	89.5	89.5	85.3
(3) *S. lophii* (1)	0.032	0.000	—	100	99.6	99.2	98.8	98.8	98.8	96.8	96.4	95.6	95.6	89.5	89.5	89.5	85.3
(4) *S. lophii* (2)	0.032	0.000	0.000	—	99.6	99.2	98.8	98.8	98.8	96.8	96.4	95.6	95.6	89.5	89.5	89.5	85.3
(5) *S. americana*	0.036	0.004	0.004	0.004	—	98.8	98.4	98.4	98.4	96.4	96.0	95.2	95.2	89.1	89.1	89.1	84.9
(6) *Microgemma tincae*	0.040	0.008	0.008	0.008	0.012	—	99.6	99.2	99.2	96.8	96.4	95.6	95.6	89.5	89.5	89.5	85.8
(7) *Microgemma vivaresi*	0.044	0.012	0.012	0.012	0.016	0.004	—	98.8	98.8	96.4	96.0	95.2	95.2	89.1	89.1	89.1	85.3
(8) *M. caulleryi*	0.044	0.012	0.012	0.012	0.016	0.008	0.012	—	100	96.4	96.0	95.2	95.2	89.1	89.1	89.1	85.8
(9) *Tetramicra brevifilum*	0.044	0.012	0.012	0.012	0.016	0.008	0.012	0.000	—	96.4	96.0	95.2	95.2	89.1	89.1	89.1	85.2
(10) *Kabatana* sp.	0.048	0.032	0.032	0.032	0.036	0.032	0.036	0.036	0.036	—	99.6	96.8	97.2	89.5	89.5	89.5	85.3
(11) *K. newberryi*	0.053	0.036	0.036	0.036	0.040	0.036	0.040	0.040	0.040	0.004	—	96.4	96.8	88.0	88.0	88.0	84.8
(12) *Aspalatospora milevae*	0.061	0.044	0.044	0.044	0.048	0.044	0.048	0.048	0.048	0.032	0.036	—	96.8	90.4	90.4	90.4	87.7
(13) *K. takedai*	0.061	0.044	0.044	0.044	0.048	0.044	0.048	0.048	0.048	0.028	0.032	0.032	—	90.4	90.4	90.4	86.1
(14) *K. seriolae*	0.118	0.105	0.105	0.105	0.109	0.105	0.109	0.109	0.109	0.105	0.110	0.096	0.096	—	100	100	85.3
(15) *Microsporidium* sp. GHB1	0.118	0.105	0.105	0.105	0.109	0.105	0.109	0.109	0.109	0.105	0.110	0.096	0.096	0.000	—	100	85.3
(16) *Mi.* sp. RSB1	0.118	0.105	0.105	0.105	0.109	0.105	0.109	0.109	0.109	0.105	0.110	0.096	0.096	0.000	0.000	—	85.3
(17) *Potaspora morhaphis*	0.161	0.147	0.147	0.147	0.151	0.142	0.147	0.142	0.142	0.147	0.152	0.123	0.139	0.147	0.147	0.147	—

TABLE II. Comparative measurements (in µm) from *Kabatana* spp.

Kabatana sp. (Host)	Habitat (Region)	Local infection	Spore			Filament coils (rows)	Exospore	Posterosome	References
			Morphology	L	W				
K. arthuri (*Pangasius sutchi*)	Freshwater (Thailand)	Skeletal muscles	Rounded, pyriform, often curved	3.1 (2.2–4.2)	1.9 (1.5–2.4)	5 to 6 (1)	Divided into small irregular fields	Present	Lom et al. 1999
K. takedai (*Oncorhynchus masou*)	Freshwater (Japan, Russia)	Skeletal muscles	Ovoid	3.3 (4.5–6.2)	1.9 (1.6–2.1)	3 to 4 (1)	Incisions delimiting small fields on the surface	Present	Lom et al. 2001
K. seriolae (*Seriola quinqueradiata*)	Marine (Japan)	Skeletal muscles	Ovoid, pyriform	3.3	2.2	4 to 5	Divided into small irregular fields	—	Egusa 1982, Lom et al. 1999
K. newberryi (*Eucyclogobius newberryi*)	Brackish (California)	Skeletal muscles	Ovoid	2.8 (2.5–3.1)	1.9 (1.5–2.3)	9 to 10 (1 to 2)	Divided into small irregular fields	Present	McGourty et al. 2007
(*Gobiusculus flavescens*)	Marine (Sweden)	Skeletal muscles	Ovoid	3.7 (3.1–4.8)	2.3 (2.1–2.3)	—	—		Barber et al. 2009
K. rondoni (*Gymnorhamphichthys rondoni*)	Freshwater (Brazil)	Skeletal muscles	Pyriform to ellipsoidal	4.25 ± 0.38	2.37 ± 0.42	8 to 10 (2)	Divided into small irregular fields	Present	Present study

distinct (11.8%) and forms a stable clade (bootstrap 100%) together with 2 *Microsporidium* spp. (Bell et al., 2001). Using MP methods, *K. rondoni* occupies a basal position (bootstrap 50%) clustered with all *Microgemma* spp., *Spraguea* spp., and *Tetramicra brevifilum*. Using phylogenetic analyses by ML method (bootstrap 71%) and BI (bootstrap 53%), *K. rondoni* is included with *Spraguea* spp. in the same clade.

In conclusion, the morphological and ultrastructural data available, and the molecular analyses in the present study, demonstrated that this microsporidian is a new species belonging to group 4 and is classified as *Kabatana rondoni* n. sp.

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