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ORIGINAL PAPER

Potaspora aequidens n. sp. (Microsporidia, Tetramicridae), a parasite infecting the freshwater fish *Aequidens plagiozonatus* (Teleostei, Cichlidae) from Brazil

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Abstract Morphological and molecular procedures were used to describe a new species of microsporidian that infects the muscles of the sub-opercular region and the caudal fins of the freshwater *Aequidens plagiozonatus* in Brazil. This microsporidian forms whitish xenomas containing variable number of spores, reaching up to ~0.4 mm in diameter. The mature spores, pyriformin shape, with slightly round ends, measured 3.4±0.5 μm long and 1.9±0.3 μm wide (n=50) and showed characteristics typical of Microsporidia. The average thickness of the spore wall was 100 (96–108) nm (n=50), and the spore wall was composed of two layers, a thin, electron-dense exospore and a thick electron-transparent endospore. The exospore was surrounded by a thin, irregular layer of granular material. The anchoring disc was mushroom-like, located in the apical region of the spore in an eccentric position relative to the spore axis, rendering bilateral asymmetry to the spore. The anterior part of the polar filament (PF) (manubrium) measured approximately 125 (122–128) nm thick (n=30), and the angle of tilt between the anterior PF and the spore axis was ~45°; the posterior part was packed in 8–9 coils. Phylogenetic analysis showed a strongly supported clade containing family Sprague idae Weissenberg, 1976, family Tetramicridae Matthews and Matthews, 1980, *Microsporidium* sp. RBS1, and *Kabatana* spp. In conclusion, the available morphological, ultrastructural, and molecular data shows that this microsporidian is a new species belonging to group 4, classified as *Potaspora aequidens* n. sp. This is the second species described in the genus *Potaspora.*

Keywords Amazonian fish · Parasite · Microsporidia · Ultrastructure · Phylogeny

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Introduction

The phylum Microsporidia Balbiani, 1882, is comprised of obligatory intracellular parasites (Franzen 2008). The life cycle can be divided into two phases: extracellular and intracellular. The extracellular phase consists of a spore with a solid wall, one sporoplasm, either uni or binucleate, and an extrusion apparatus that consists of a tube containing a polar filament, which is characteristic of the phylum (Wittner and Weiss 1999). This polar filament can be ejected and break the spore, transferring the sporoplasm to the host cell (Xu and Weiss 2005).

This phylum contains a very diverse group of organisms that have a host range spanning over most of the invertebrate phyla (especially insects) and all classes of vertebrates.

Studies on this phylum gained attention when microsporidia were identified as important opportunistic pathogens for HIV-infected individuals (Mathis 2000; Mathis et al. 2005).

Of the 187 genera of microsporidians described to date, 20 genera can infect fish belonging to the class Osteichthyes (bony fish). They may occur in hosts inhabiting freshwater, brackish, and marine environments, including temporary water bodies, lakes, rivers, estuaries, the open ocean, and the deep ocean (Stentiford et al. 2013; Vávra and Lukes 2013).

Of the microsporidia that infect fish, the genus *Potaspora* is comprised of only one species, *Potaspora morhaphis*, which parasitizes the coelomic cavity and anal region of the teleost *Potamorhaphis guianensis*, in the Amazon region (Casal et al. 2008).

In Amazonia, six species of microsporidia that parasitize fish have been described, *Kabatana rondoni* (Casal et al. 2010), *Loma myrophis* (Azevedo and Matos 2002), *Loma psittaca* (Casal et al. 2009), *Microsporidium brevirostris* (Matos and Azevedo 2004), *Amazonspora hassar* (Azevedo and Matos 2003), and *P. morhaphis* (Casal et al. 2008); the latter two were also described as new genera.

In this paper, we describe a new species of microsporidian, *Potaspora aequidens* n. sp., using morphological, ultrastructural, and molecular characteristics.

Materials and methods

Fish sampling and examination

Forty-five specimens of the freshwater fish *Aequidens plagiozonatus* Kullander, 1984 (Teleostei, Cichlidae) (Brazilian common name Acará Pixuna), were collected between January and October 2013 near the city of Peixe Boi, State of Pará, Brazil. The live specimens were taken to the Carlos Azevedo Research Laboratory at Federal Rural University of Amazonia (UFRA). The animals were anesthetized with tricaine methanesulfonate (MS222: Sigma, Saint Louis, MO, USA) and dissected using a stereomicroscope that is appropriate for the analysis of parasites.

Parasitological survey was conducted on several organs and tissues, including the muscles and fins. The diagnosis was later confirmed by a preliminary microscopic analysis of smear preparations using differential interference contrast (DIC) optics. Parasitized tissue fragments were collected and prepared for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) and for molecular procedures.

This study was approved by the Ethics Committee for Experiments with Animals/UFRA (report number 013/2014).

For LM, smears of xenoma and free spores were observed directly, without fixation or staining, using a light microscope Zeiss Primo Star (Carl Zeiss, Jena, Germany) equipped with DIC.

For TEM, isolated xenomas were fixed in 5 % glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.2) at 4 °C for 24 h, washed overnight in the buffer at the same temperature and post-fixed in 2 % osmium tetroxide in the same buffer for 3 h at 4 °C. Dehydration in an ascending ethanol series was followed by oxide propylene treatment and inclusion in EPON (Fluka, Hauppauge, NY). Semithin sections were stained with methylene blue-Azure II for LM. Ultrathin sections were double contrasted with uranyl acetate and lead citrate for observation and photographed using a JEOL 100CXII TEM (JEOL Ltd., Tokyo, Japan) operated at 60 kV.

For SEM, free spores were fixed in 5 % glutaraldehyde and post-fixed in 2 % osmium tetroxide. The samples were then dehydrated in an ascending ethanol series, dried to the critical point, covered with a thin film of gold, and photographed with a SEM (LEO 1459 VP) (Zeiss, Thornwood, NY).

DNA extraction, amplification, and sequencing

Several xenomas were excised from the infected fish, followed by homogenization to isolate the spores, which were then stored in 80 % ethanol at 4 °C. The genomic DNA from approximately 5×10^6 spores was extracted using GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, Saint Louis, MO, USA) as per the manufacturer's instructions for animal tissue.DNA was stored in 50 μl of TE buffer at −20 °C until further use.

The majority of the gene coding for the small subunit (SSU) rRNA was amplified by PCR using the primers V1f (5′-CACC AGGTTGATTCTGCC-3′) and 1492r (5′-GGTTACCTTGTT ACGACTT-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, the primers HG4F (5′-GCGGCTTAATTTGACTCAAC) and HG4R (5′-TCTCCT TGGTCCGTGTTTCAA) were used (Gatehouse and Malone 1998). The 5′-end of the SSU gene was amplified using a primer designed for this study, HG5F_rev (5′-TCACCCCACTTGTC GTTA-3′). PCR was performed in 50-μl reactions using 10 pmol of each primer, 10 nmol of each dNTP, 2 mM of $MgCl_2$, 5 μl of ×10 *Taq* polymerase buffer, 1.25 units of *Taq* DNA polymerase (Invitrogen Products, Waltham, MA, USA), and 3 μl of genomic DNA. The reactions were run on a Hybaid PxE Thermocycler (Thermo Electron Corporation, Milford, MA, USA).

The amplification program consisted of denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min. A final elongation step was performed at 72 °C for 10 min. Five-microliter aliquots of the PCR products were visualized with ethidium bromide staining after electrophoresis on 1 % agarose gel.

PCR products for the SSU gene and the ITS region are approximately 1,400 and 1,100 bp in length, respectively. PCR products were cleaned using the MinElute PCR purification Kit (Qiagen, Germany), and then three purified PCR

products were sequenced in both directions. Sequencing was performed using BigDye Terminator v1.1 of the Applied Biosytems Kit, and the sequencing reactions were run on an ABI3700 DNA Analyzer (Perkin-Elmer, Applied Biosystems, Stabvida, Co., Oeiras, Portugal).

Molecular phylogenetic analyses

To evaluate the relationships of the present species with other described microsporidian species, a homology search was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). The SSU rDNA sequences for 56 microsporidian species available in the GenBank database were aligned with the newly generated sequence (Table 1). *Nucleospora salmoni* (AF186003), *Nucleospora bieneusi* (L07123), *Microsporidium* sp. (FN610845), *Trachipleistophora hominis* (AJ002605), *Endoreticulatus schubergi* (L39109), *Enterocytozoon bieneusi* (L07123), *Vairimorpha necatrix* (Y00266), and *Vittaforma corneae* (L39112) were used as outgroups. The sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalo/). The resulting alignment was verified and manually corrected using BioEdit (Hall 1999).

Calculation of the estimated frequency of each nucleotide bases and replacement rates were performed for each marker separately, and the uncorrected genetic distances, *p*, were calculated using PAUP 4.0 (Swofford 1998).

Phylogenetic relationships were determined through Bayesian inference using Markov chain Monte Carlo (MCMC) tree searches with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The most appropriate model for evolution was determined using jModelTest 2.0.2 (Posada 2008), under the Akaike Information Criterion (AIC). We performed two parallel runs of four simultaneous MCMC searches for 5 million generations each, sampling one tree every 500 generations and discarding the results of the first 1,000 trees as "burn-in." MrBayes was used for the remaining trees to estimate the posterior probability of each node in our phylogenetic reconstruction.

Results

Morphological description of the parasite

Macroscopic observation of the skeletal musculature located in the sub-opercular region and the fins revealed the presence of whitish rounded cysts (xenomas) (Fig. 1); this diagnosis was later confirmed by preliminary microscopic analysis of smear preparations using LM. Of the 45 fish examined, 30 were infected with microsporidian parasites (66.6 %).

These xenomas contained variable number of spores and could reach up to ~0.4 mm in diameter. A large number of mature spores were usually observed after the cysts ruptured.

Ultrastructurally, the isolated parasite cysts were bordered by a single-layered cyst wall enclosing different developmental stages of the parasite, which showed asynchronous growth. During the final stages of sporogenesis, the xenomic structure was occupied only by mature spores (Fig. 2a) and externally, several layers of fibroblasts associated with tissue were also observed (Fig. 2d).

The mature spores were pyriform ellipsoids (Fig. 2b, c) with lightly rounded ends, measuring 3.4±0.5 μm long and 1.9±0.3 μm wide (n=50), showing all the characteristics typical of Microsporidia (Fig. 2e). The spore wall measured 100 (96–108) nm (n=50) and was composed of two layers, with a thin electron-dense exospore and a thick electron-transparent endospore (Fig. 2g). The exospore was surrounded by a thin, irregular layer of granular material.

The anchoring disc was mushroom like and located in the apical region of the spore in an eccentric position relative to the spore axis, giving the spore bilateral asymmetry (Fig. 2f). The anterior part of the polar filament (PF) (manubrium) measured approximately 125 (122–128) nm (n=30); the angle of tilt between the anterior PF and the spore axis was ~45°, and the posterior part was packed in 8–9 coils. The polaroplast (Pp) contained two distinct lamellae folded around the PF. The posterior vacuole, one third of the total spore length, generally contained 1–2 posterosomes.

The spore morphology is shown in a schematic drawing, to provide a better overview of the morphological and ultrastructural characteristics (Fig. 3).

Taxonomic summary

Name: *Potaspora aequidens* n. sp. (Microsporidia, Tetramicridae)

Type host: *Aequidens plagiozonatus* Kullander, 1984 (Teleostei, Peciforme, Cichlidae), (Brazilian common name: "Acará-pixuna").

Site of infection: Xenomas containing numerous spores were located in the fins and the skeletal musculature of the sub-opercular region.

Type locality: Region of Peixe Boi river (01°11′S/47°18′W), near the city of Peixe Boi, State of Pará, Amazonia, Brazil.

Prevalence: Occurs in 30 out of 45 sampled fish (66.6 %). No difference in prevalence was observed between sexes.

Type material: A glass slide with a semithin section displaying a cyst containing numerous mature spores of the hapantotype was deposited in the International Protozoan Type Slide Collection at the Instituto Nacional da Pesquisa da Amazônia (INPA), Manaus, State of Amazonas, Brazil, under acquisition 004/2015.

Etymology:The specific epithet "aequidens" refers to the genus name of the host species.

Table 1 Hosts and GenBank accession numbers for the SSU rRNA sequences of microsporidian that parasite fishes species used in the phylogenetic analyses (except outgroup)

Species	Host	Accession no.
Glugea anomala	*Gasterosteus aculeatus*	AF044391
Glugea atherinae	*Atherina prebyster*	U15987
Glugea hertwigi	*Osmerus mordax*	GQ203287
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 weissi	*Clevelandia ios*	JQ062988
Ichthyosporidium sp.	*Leiostomus xanthurus*	L39110
Kabatana takedai	*Oncorhyncus masu*	AF356222
Kabatana newberryi	*Eucyclogobius newberryi*	EF202572
Kabatana seriolae	*Seriola quinqueradiata*	AJ295322
Kabatana rondoni	*Gymnorhamphichthys rondoni*	FJ843105
Kabatana sp.	*Gobiusculus flavescens*	EU682928
Loma acerinae	*Gymnocephalus cernuus*	AJ252951
Loma embiotocia	*Cymatogaster aggregate*	AF320310
Loma salmonae	*Oncorhynchus tshawytscha*	U78736
Loma psitacca	*Colomesus psittacus*	FJ843104
Loma sp.	*Encelyopus cimbrius*	AF104081
Microgemma carolinus	*Trachinotus carolinus*	JQ085991
Microgemma caulleryi	*Hyperoplus lanceolatus*	AY033054
Microgemma tincae	*Symphodus tinca*	AY651319
Microgemma vivaresi	*Taurulus bubalis*	AJ252952
Microsporidium cerebralis	*Salmo salar*	JQ316511
Microsporidium cypselurus	*Cypselurus pinnatibarbatus japonicus*	AJ300706
Microsporidium prosopium	*Prosopium williamsoni*	AF151529
Microsporidium sp. RSB1	*Pagrus major*	AJ295323
Microsporidium sp. STF	*Salmo trutta fario*	AY140647
Microsporidium MYX1	*Takifugu ruripes*	AJ295329
Microsporidium seriolae	*Seriola quinqueradiata*	AJ295322
Microsporidium prosopium	*Prosopium williamsoni*	AF151529
Myosporidium merluccius	*Merluccius sp.*	AY530532
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
Potaspora moraphis	*Potamorrhaphis guianensis*	EU534408
Spraguea americana	*Lophius americanus*	AF056014
Spraguea gastrophysus	*Lophius gastrophysus*	GQ868443
Spraguea lophii	*Lophius piscatorius*	AF033197
Spraguea sp. 1	*Lophius litulon*	AY465876
Spraguea sp. 2	*Seriola quinqueradiata*	JQ820239

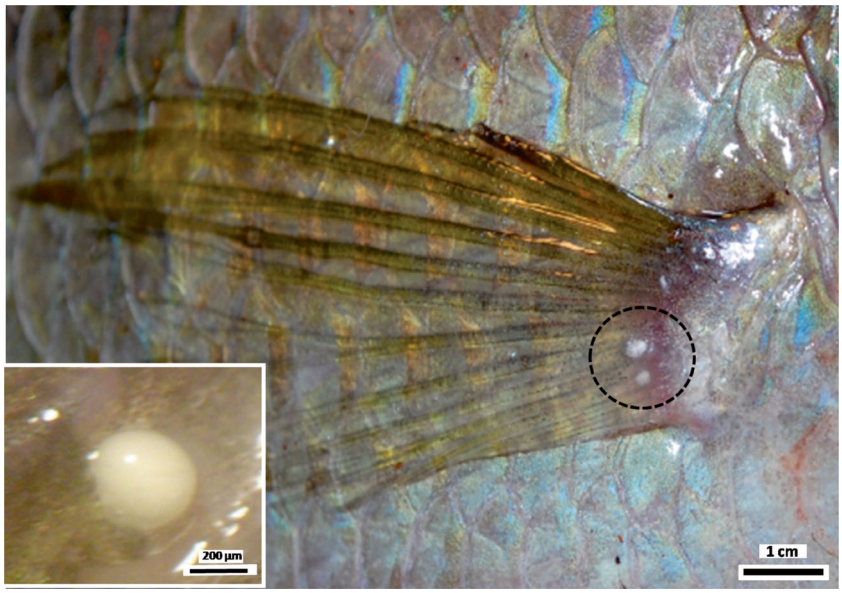

Fig. 1 Whitish cysts (xenomas) located in the fins of *Aequidens plagiozonatus* (*circle* and detail)

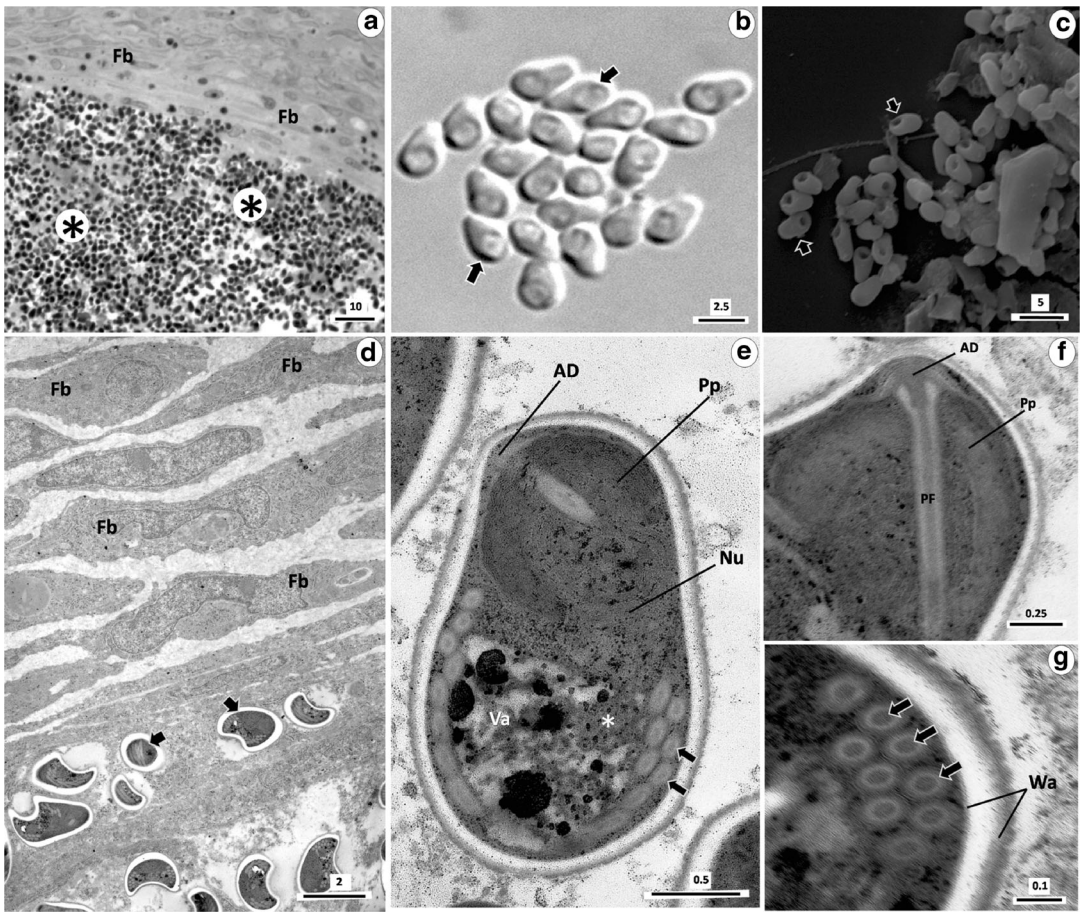

Fig. 2 **a** Semithin section showing an aggregate of the parasites composed of mature spores (*asterisk*). At the periphery, numerous layers of fibroblasts (Fb) can be seen. **b** Some isolated mature spores observed using DIC optics. Inside the vacuole, the posterosome (*arrows*) can be seen. **c** Isolated mature spores observed by SEM (*arrows*). **d** Ultrathin section showing the fibroblasts (Fb) surrounding the aggregate of mature spores (*arrows*). **e** Ultrathin section of a spore showing the typical microsporidian structures and organelles. *AD* anchoring disc, *Pp* polaroplast, *Nu* nucleus, polar filament (*arrows*); posterosome (*asterisk*) inside the vacuole (Va). **f** Ultrastructural detail of the apical region of a spore showing the anchoring disc (AD) in close contact with the wall and the lamellar region of the polaroplast (Pp) surrounding the polar filament (PF). **g** Detail of the transverse section of the polar filament (*arrows*) and double layer and the spore wall (Wa). (All *scale bars* in µm)

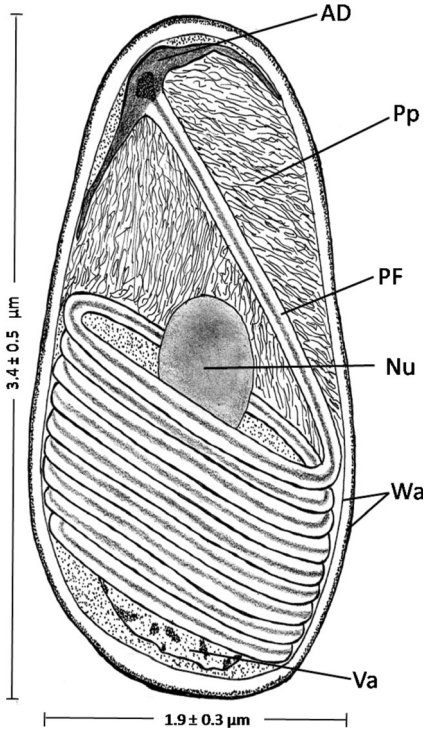

Fig. 3 Schematic drawing of a spore of *Potaspora aequidens* n. sp. showing the typical structures described in the text. Anchoring disc (*AD*), polaroplast (*Pp*), polar filament (*PF*), nucleus (*Nu*), spore wall (*Wa*), vacuole (*Va*)

Phylogenetic analyses

The sequences were assembled, and the resulting consensus DNA sequence of the complete SSU rRNA, ITS, and the incomplete LSU rRNA genes, 1,834 bp in length, was deposited in GenBank (accession number KP404613).

A representative number of sequences from species belonging to the major genera of Microsporidia that parasitize fish and other groups were obtained from GenBank. The resulting alignment consisted of 1,284 bp and was used for phylogenetic analyses.

The Bayesian inference analysis assumed a TIM+I+G model of nucleotide substitution with estimated base frequencies of A=0.2838, C=0.1826, G=0.2974, and T=0.2363, the substitution model (A–C=0.8066, A–G=2.1518, A–T=1, C–G=0.8066, C–T=3.2187, G–T=1), proportion of invariable sites=0.1020, and gamma distribution shape parameter=0.6110.

A BLAST search showed that the parasite infecting *A. plagiozonatus* belongs to the genus *Potaspora*, exhibiting very high sequence similarity (99 %) with *Potaspora moraphis* (the only species described for this genus to date). Other species of Microsporidia showed lower sequence similarity (<88 %).

The value of uncorrected genetic distance, *p*, between *P. moraphis* and *P. aequidens* n. sp. was 1.73 %, whereas among *P. aequidens* n. sp. and other species, *p* was >10 %.

Phylogenetic analysis clearly showed that the new accession belonged to a strongly supported clade containing family Spragueidae Weissenberg, 1976, family Tetramicridae Matthews and Matthews, 1980, *Microsporidium* sp. RBS1, and species of the genus *Kabatana* (Fig. 4).

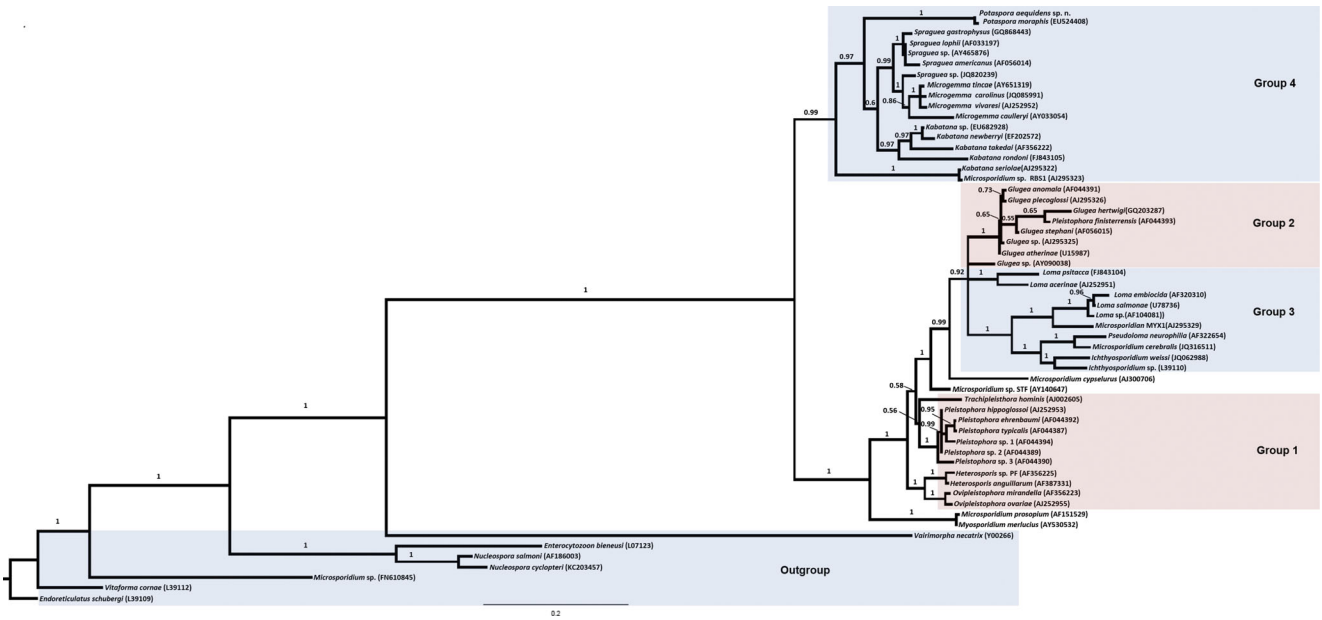

Fig. 4 Phylogram of *Potaspora aequidens* sp. n. inferred from Bayesian analysis using an SSU rRNA data set. The values at each node indicate posterior probabilities. *Highlights* show groups 1 to 4 (from Lom and Nilsen 2003). The species names are followed by the corresponding GenBank accession numbers

Discussion

Analysis of the ultrastructural organization showed that all structures were typical of Phylum Microsporidia (Lom and Dyková 1992).

The new species is similar to *P. moraphis* in terms of spore shape, inclination of the PF, and host habitat, but differs in terms of spore size (3.4×1.9 vs. 2.8×1.5 μm), the size of the front portion of the PF (125 vs. 145 nm), and the number of coils in the PF (9–10 vs. 8–9). In addition, the wall of the xenomas of *P. aequidens* n. sp. did not possess microvilli-like structures, and the xenomas showed numerous layers of fibroblasts.

The current trend for the establishment of microsporidian molecular phylogeny according to family is concordant with the results presented here. The phylogenetic analysis showed a cluster including *P. aequidens* n. sp., which corresponds to group 4, as designated by Lom and Nilsen (2003).

Despite the genetic similarity between *P. moraphis* and *P. aequidens* n. sp., the low values for posterior probability support indicate that they are genetically distinct.

Previous molecular phylogenetic studies have demonstrated a high degree of sequence similarity between members of Microsporidia (Cheney et al. 2000; Nilsen et al. 1998). For example, *Pleistophora aegyptiaca* showed 99.8 % similarity with the type species *P. anguillarum* (Abdel-Ghaffar et al. 2012), and *Microgemma carolinus* showed 99.1 % identity with *Microgemma tincae* (Casal et al. 2012).

According to Vossbrinck and Vossbrinck (2005), the great discrepancies between the evolutionary relationships among Microsporidia proposed based on the analysis of small subunit rRNA sequences and the published taxonomic designations based on the morphological characters must be resolved by proposing a Microsporidia taxonomy based on phylogenetic relatedness, with major taxonomic divisions based on habitat and host.

In conclusion, the available morphological and ultrastructural data and the molecular analyses presented here demonstrate that this Microsporidian is a new species belonging to group 4, classified as *P. aequidens* n. sp. Consistent with other studies (Morsy et al. 2012), these results clearly demonstrate the importance of using classical ultrastructural information in combination with molecular data when describing novel parasite species

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