



A new microsporidium, *Apotaspora heleios* n. g., n. sp., from the Riverine grass shrimp *Palaemonetes paludosus* (Decapoda: Caridea: Palaemonidae)

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ABSTRACT

We report a new microsporidium from a key species of the estuarine communities of the Gulf States, the Riverine grass shrimp, *Palaemonetes paludosus*. A milky-white shrimp was found in the Mobile Bay Delta, a large, oligohaline-freshwater wetland in Alabama, USA. Light microscopy of smears and thick sections of the abdominal tissues demonstrated infection with microsporidian spores enclosed in sporophorous vesicles (SVs) in sets of eight. Broadly oval spores measured $2.9 \pm 0.06 \times 1.7 \pm 0.03 \mu\text{m}$ ($2.5\text{--}3.3 \times 1.6\text{--}1.9 \mu\text{m}$, $n = 11$). SVs with a persistent membrane ranged from 4.4 to $5.6 \mu\text{m}$ in diameter. Subcuticular epithelium and underlying musculature were packed with sporonts, sporoblasts, and spores. Electron microscopy demonstrated diplokaryotic meronts that gave rise to sporont mother cells with a large single nucleus. The meront plasma membrane turned into a SV envelope, and the sporont wall segregated internally. The sporont nucleus underwent meiosis followed by two mitotic divisions accompanied by internal budding to produce four sporonts, each dividing in two uninucleate sporoblasts. Eight-spore SVs were filled with fibrillary-tubular secretions. Spores possessed $90\text{--}110\text{-nm}$ thick envelopes (exospore, $40\text{--}60 \text{ nm}$ + endospore, $30\text{--}50 \text{ nm}$), a triangle-shaped nucleus, isofilar polar filament of $10\text{--}13$ coils arranged in two-three rows, bipartite polaroplast, and a mushroom-shaped polar disk. The SSU rDNA sequence of the novel species was deposited in GenBank under Accession number MG 708238. SSU rDNA-based phylogenetic analysis indicated that the Riverine grass shrimp microsporidium was a new species and placed it in one branch with two species of *Potasporea*, xenoma-forming microsporidia from freshwater perciform fishes. Because morphological and developmental characters of the novel species did not fit the diagnosis of the genus *Potasporea*, and, based on SSU rDNA-inferred phylogenetic analyses, different host specificity, pathogenesis, and ecological considerations, we erect here the new genus *Apotaspora* for the Riverine grass shrimp microsporidium and name the new species *Apotaspora heleios*. Grouping together fish and crustacean parasites on SSU rDNA phylogenetic trees suggests that polyxenous life cycles might be a common feature of extinct and/or extant members of the studied lineage of the Microsporidia.

1. Introduction

The phylum Microsporidia Balbiani 1882 belongs to the Aphelidea-Rozellamycota-Microsporidia (ARM) clade, a basal Fungi or sister-to-Fungi lineage (Karpov et al., 2014; Letcher et al., 2013). Microsporidia are ubiquitous intracellular parasitic protists (= unicellular eukaryotes) reported from nearly every class of living bilaterian animals as well as from all biotopes, including deep sea methane seeps (Sapir et al., 2014). Microsporidia are especially abundant in arthropods, both terrestrial and aquatic, and in fishes. About a half of the known genera infect aquatic hosts, with presumably thousands of taxa yet unknown

(Stentiford et al., 2016). Crustaceans, the major aquatic arthropods, host at least 48 genera of Microsporidia, 21 of which parasitize representatives of the order Decapoda in all five infraorders. Penaeid shrimps (Penaeidea) host four genera, crayfish and lobsters (Astacidea) – three, hermit crabs (Anomura) – two, crabs (Brachyura) – five, and caridean shrimps (Caridea) – seven (Canning et al., 2002; Ding et al., 2016; Ovcharenko, 1984; Sokolova et al., 2015; Sprague, 1977; Stentiford and Dunn, 2014; Stentiford et al., 2013, 2017; Wang et al., 2013). Overall, 12 species of microsporidia were reported worldwide to infect 11 species of caridean shrimps, 8 of which represent members of the closely related genera *Palaemonetes* and *Palaemon/Exopalaemon*

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Table 1
Species and genera of Microsporidia reported from caridean shrimps (Crustacea: Decapoda: Caridea).

Parasite name	Host name (type of habitat)	Locality	Tis-sue	SV [#]	LM/EM/MPH [±]	References
<i>Gurleya pontica</i>	<i>Palaeomon elegans</i> (B)	Black Sea coastal water bodies (Ukraine)	Mu	4	+/-/-	Ovcharenko (1984)
<i>Chapmanium macrocystis</i> (Gurley 1883)	<i>Palaeomonetes varians</i> (F)	Minchir River, Verona, Italy	Mu	8	+/-/-	Hazard and Oldacre (1976)
<i>Inodosporus octosporus</i> (Henneguy 1892)	<i>Palaeomon retrostris</i> (M)	Atlantic (France; England)	Mu	8	+//+/+	Azevedo et al. (2000), Codreanu and Bălcescu-Codreanu (1974), Corsaro et al. (2014), Overstreet and Weidner (1974), Stentiford et al. (2017)
(syn. <i>Thelohania octospora</i>)	<i>Palaeomon serratus</i> (M)	Black Sea (Romania)	Mu	8		
<i>Inodosporus spraguei</i>	<i>Palaeomon elegans</i> (M)		Mu	8	+//+-	
	<i>Palaeomonetes pugio</i> (F/B)	Gulf of Mexico coastal marshes (Mississippi, Texas, USA)	Mu	8		
	<i>Palaeomonetes kadiakensis</i> (F/B)		Mu	8		
<i>Ovipleistophora arto</i>	<i>Palaeomon serratus</i> (B)	The River Fal Estuary, English Channel, GB	Mu	> 16	+//+/+	Stentiford et al., 2017
	<i>Palaeomonetes pugio</i> (M)	Atlantic (Sapelo Island, Georgia, USA)	Mu	> 8	+/-/-	Sprague, 1977; Street and Sprague, 1974
<i>Pleistophora lintoni</i>	<i>Atyphira</i> sp.*(F)	Japan	Gut	> 8	+/-/-	Sprague, 1977
	<i>Macrobrachium nipponense</i> (F)	China	Mu	?	+//+/+	Ding et al., 2016
	<i>Exopalaeomon carnicauda</i> (M)	Pacific: East China Sea coast (China)	?	?	-/-/+	GenBank, Acc# JX853814
	<i>Cardina formosa</i> * (F)	Taiwan	Mu	> 8	+//+/+	Wang et al., 2013
	<i>Palaeomonetes paludosus</i> (F/B)	Gulf of Mexico coastal marshes (Alabama, USA)	Mu	8	+//+/+	Herein

B, brackish; F, freshwater; M, marine; Mu, muscles; * the species belong to the family Atyidae, all other species in the table represent palaemonids; # number of spores in sporophorous vesicles; ± the species has been studied by light microscopy (LM), electron microscopy (EM), and molecular phylogenetic analysis (MPH).

(Table 1). The genus *Palaemonetes* is globally distributed and includes species that tolerate all grades of salinity from fresh to marine (Strenth, 1976). We report here a new microsporidium from one of the key species of the estuarine communities of the Gulf States, the Riverine grass shrimp, *Palaeomonetes paludosus* (Gibbes 1850), not previously reported as hosting microsporidia.

2. Materials and methods

2.1. Sampling and light microscopy

On 12 December 2016, a 24-mm long Riverine grass shrimp with an unusual milky-whitish abdomen was collected in Little Bateau Bay (30°43' 36.81"N Latitude; 87°57'34.32"W Longitude), a small bay in the Mobile Bay Delta of the Gulf of Mexico, Alabama, USA. The smears prepared from the shrimp were first observed and photographed fresh using an Olympus BX53 microscope and attached JENOPTIK ProgRes® SpeedXT core 5 digital camera. Later, some smears were fixed with methanol, stained with Giemsa and Modified Trichrome Blue stains, and examined using a Zeiss Axioplan microscope equipped with an Olympus DP73 digital camera. The shrimp was divided into portions and stored in 0.8% NaCl, Bouin's solution (75 ml picric acid, sat. aq. sol.; 25 ml formaldehyde, 40% sol.; 11.5 ml glacial acetic acid; and 33.5 ml distilled water), in Karnovsky's fixative (kit # 22872, Polysciences, Inc.), and 70% molecular grade ethanol until further examination.

2.2. Electron microscopy

For transmission electron microscopy (TEM), at about 2 weeks after fixation, the samples were cut into small pieces, transferred to a fresh portion of the same fixative for 2 h, washed in 0.1 M cacodylate buffer supplemented with 5% sucrose, post-fixed in 2% osmium tetroxide, dehydrated in ascending ethanol series, transferred to propylene-oxide, and embedded in Epon-Araldite. Thin (70–80 nm) sections were stained with uranyl acetate and Reynold's lead citrate and then examined in a JEOL JEM 1011 transmission electron microscope equipped with HAMAMATSU ORCA-HR digital camera (Tokyo, Japan). For scanning electron microscopy (SEM), specimens were dehydrated through the ethanol series and followed by exchanging ethanol with CO₂ in a Polaron E3000 Standard Critical Point Drier. Dried samples were mounted on 13-mm aluminum mount specimen stubs, covered with carbon adhesive tabs, sealed with colloidal silver paste, coated with Gold/Palladium in an EMS 550X Sputter Coater for 4 min to achieve a coating thickness of 20–25 nm, and examined in a FEI Quanta 200 ESEM at a high vacuum mode and 20 kV. All reagents for light microscopy (LM) were from Sigma-Aldrich (St. Louis, MO) and for EM from EMS Chemicals (Fort Washington, PA), except those indicated above.

2.3. DNA isolation

The shrimp portion in saline was stored at –80 °C for about 2 weeks before being used for DNA isolation. The DNA was isolated by Zymo Research Quick-DNA™ Universal Kit (Zymo Research, Irvine, CA) using the "Solid Tissue Protocol" with modifications. Briefly, about a 10-mg piece of frozen shrimp tissue was thawed in a water bath at 37 °C and homogenized with a Teflon pestle. Microscopic examination of the obtained suspension revealed presence of numerous spores alongside with remnants of host tissues. The sample was then digested in 100 µl of "Solid Tissue Buffer" containing 20 mg/ml Proteinase K for 4 h at 55 °C. To release the DNA content from spores, 100 mg of 0.1-mm diameter glass beads were added to the Eppendorf tube with the samples. The tube was shaken with a Bullet Blender™24 bead-beater (Next Advance, Inc., Averill Park, NY) for 90 sec at maximal speed. The tube was placed on a hot plate at 95 °C for 3–5 min and then transferred to ice. Beads and debris were spun down by centrifugation at 12,000g. The

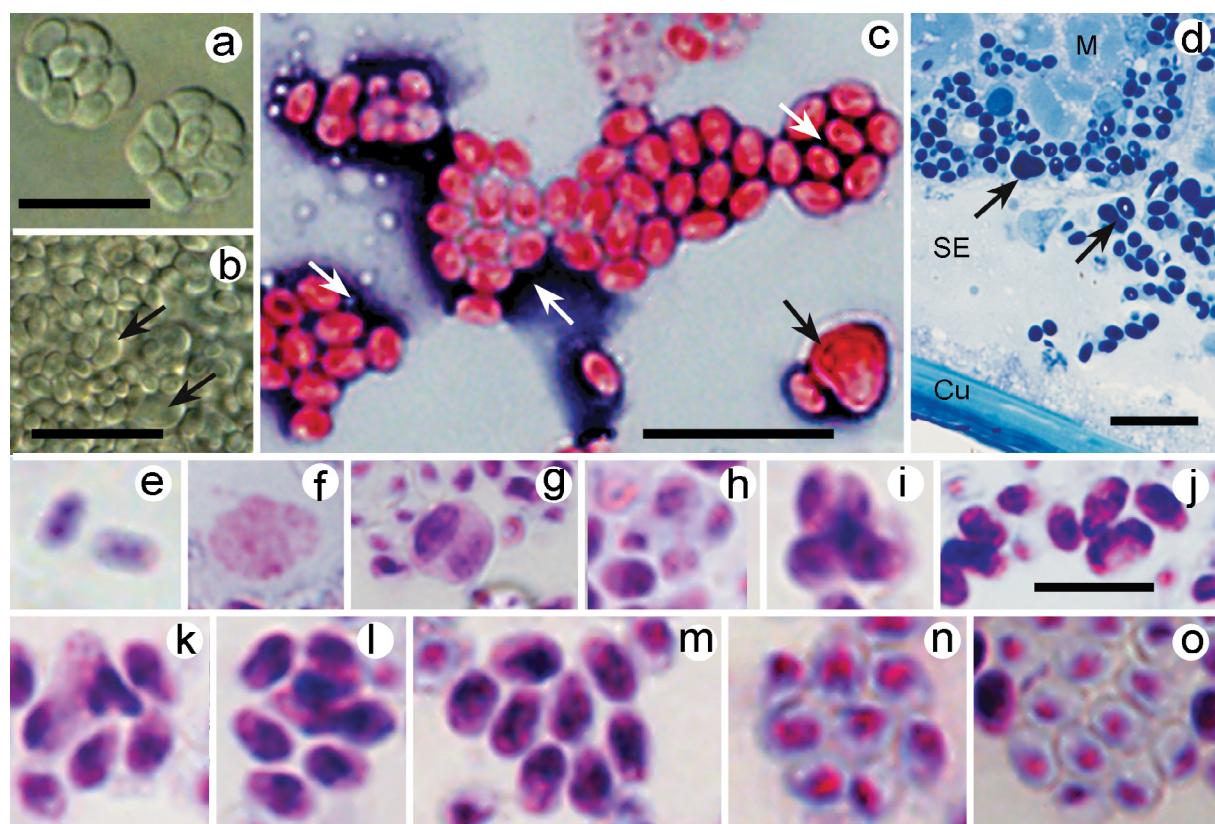


Fig. 1. *Apotaspaspora heleios* n. sp. from *Palaemonetes paludosus*: Light Microscopy. (a) Roundish sporophorous vesicles with 8 spores each, DIC. (b) Mass of spores released from the infected tissue: black arrows point to teratoid spores. (c) A methanol-fixed smear from infected tissue stained with Trichrome Blue: black arrows point to teratoid spores; white arrows indicate depositions of organic non-chitinous material around spores, and on the external surface of SV envelopes intensively counterstained by Evans Blue. (d) A 500-nm thick sections stained with Methylene blue demonstrates that infection localized in muscles, but a few spores are visible within subcuticular epithelium. Arrows point to teratoid spores. (e–n) Life cycle stages of the microsporidium that can be revealed on methanol-fixed Giemsa-stained smears from infected crustacean: (e) oval small diplokaryotic meronts; (f) roundish weakly stained cells with voluminous nucleus occupying nearly the whole cell volume, presumably sporont mother cells prior the meiosis; (g) cells of similar shape and size with 2–4 large nuclei likely resulted from the first and second meiotic divisions; (h–i) cells with 4-lobed nucleus in rosette-like arrangement at different steps of sporont segregation; (j) SVs with 4 sporoblasts or spores; (k–o) SVs with 8 sporoblasts and spores at different stages of maturation. Cu, cuticle; M, muscle fibers; SE, subcuticular epithelium. Bars: a, c, 10 µm; b, d, 20 µm; e–o, 5 µm.

supernatant (about 75 µl) was subjected to DNA isolation in accordance with the manufacturer's protocol. The DNA was eluted in 20 µl of DNA elution buffer and used as a template in downstream polymerase chain reactions (PCRs).

2.4. PCR and sequencing

The SSU rRNA sequence was amplified by PCR with 1–3 µl of DNA eluate using either OneTaq® Quick-load® master mix (New England Biolabs, Inc., Ipswich, MA) or PureTaq™ Ready-To-GO™ PCR beads (GE Healthcare, Buckinghamshire, UK). For amplification of SSU rDNA V1 (5' – CAC CAG GTT GAT TCT GCC TGA C – 3') and 1492r (5' – GGT TAC CTT GTT ACG ACT T-3') primers were used (Vossbrinck et al., 2004). The PCR cycle included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles with a denaturation at 95 °C for 30 sec, annealing at 45 °C for 60 sec and elongation at 72 °C for 120 sec, and a final extension at 72 °C for 10 min. The expected amplicon sizes were approximately 1200 base pairs. Amplicons were loaded onto a 2% agarose gel, and bands of the expected size were excised and purified using a QIAquick Gel Extraction Kit (QIAGEN, Germantown, MD). The purified PCR bands were sequenced with the Applied BioSystems BigDye Terminator technology (version 3.1), and the resulting chromatogram was obtained by a Beckman Coulter Seq 8000 DNA. The primers for sequencing were V1, 530r (5' – CCG CGG C(T/G)G CTG GCA C – 3'), 530f (5' – GTG CCA GC (G/A) GCC GCG G), 1061f (5' – GGT GGT GCA TGG CCG – 3'), and 1492r (Vossbrinck et al., 2004; Weiss and Vossbrinck,

1999). These primers produced overlapping sequences that were assembled with ChromasPro 1.34 software (<http://www.technelysium.com.au/ChromasPro.html>).

2.5. Phylogenetic analysis

For the SSU rRNA-based phylogenetic analyses, two subsets of SSU rDNA sequences were retrieved from GenBank. The first subset included 30 sequences of about 1200 base pairs. These sequences included 21 closest matches in BLAST search, 7 selected sequences from microsporidia infecting freshwater and marine crustaceans, and 2 outgroup sequences, one from *Paranosema grylli*, an insect microsporidium and one from an uncultured metchnikovellid to represent the most basal microsporidian lineage (Mikhailov et al., 2017). The second dataset contained 12 shorter sequences belonging to the *Potaspaspora-Spraguea-Microgemma-Kabatana* lineage including the sequence of *Potaspaspora macrobrachium*. All sequences in this database were trimmed to match a short 819 bp sequence of *P. macrobrachium*. For species names and GenBank accession numbers, see Fig. 4 and Fig. 5. The sequences were aligned by Muscle (MEGA 5.05), with default parameters (Edgar, 2004). The first dataset resulted in 889 informative positions, the second – in 728. Pairwise genetic distances were calculated by the Kimura-2 parameter method with a gamma distribution (Tamura et al., 2011). Both alignments were subjected to phylogenetic reconstructions by maximum likelihood (ML) using MEGA 5.05 (Tamura et al., 2011). As suggested by Modeltest, the ML phylogenetic analyses were based on

the General Time Reversible model for the first alignment and on the Tamura-Nei model for the second alignment. A discrete gamma distribution was used to model the evolutionary differences among sites (GTR + G and TN + G) (Hasegawa et al., 1985). Bootstrap tests were limited to 1000 replications in both analyses.

3. Results

3.1. Prevalence and light microscopy

Prevalence of the infection in December 2016 was as low as one infected shrimp among approximately one hundred or more. In March 2018, we followed up with another collection at the same site with no obvious infection present among several hundred shrimp. Light microscopic examination of smears prepared from the shrimp revealed numerous spores, most of which were enclosed in persistent sporophorous vesicles (SVs) in sets of 8. These octospores measured $2.9 \pm 0.06 \times 1.7 \pm 0.03 \mu\text{m}$ ($2.5\text{--}3.3 \times 1.6\text{--}1.9 \mu\text{m}$, $n = 11$). Polar filaments artificially extruded after adding distilled water to dried fresh spores $5.9\text{--}22.7 \mu\text{m}$ long, $n = 15$. The SVs ranged $4.4\text{--}5.6 \mu\text{m}$ in diameter and averaged $5.1 \pm 0.12 \mu\text{m}$ (Fig. 1a). Besides SVs, a few free spores were occasionally seen on smears. In addition to uniform spores of the mentioned above size, a relatively large proportion of SVs with 1–4 teratoid of larger size and irregular shape were seen (Fig. 1b–d). Teratoid sporogony in heavily infected tissues had been previously observed in other octosporogenous microsporidia, like *Knealhazia sole-nopsae* and *Agmasoma penaei* (see Sokolova et al., 2015; Sokolova and Fuxa, 2008). On the Trichrome Blue-treated sections, chitin spore envelopes were stained bright red with Trichrome, whereas depositions of organic non-chitinous material around spores and on external surfaces of SV envelopes were intensively counterstained by Evans Blue (Fig. 1c). On Giemsa-stained smears, the following developmental stages could be distinguished: oval small diplokaryotic meronts (Fig. 1e); spherical, weakly stained cells with a voluminous nucleus occupying nearly the whole cell volume, presumably sporont mother cells prior to meiosis (Fig. 1f); cells of similar shape and size with 2–4 large nuclei likely resulting from the first and second meiotic divisions (Fig. 1g); cells with 4-lobed nucleus in rosette-like arrangement at different steps of segregation of sporonts (Fig. 1h, i); SVs with 4 sporoblasts or spores (Fig. 1j); SVs with 8 sporoblasts; and spores at different stages of maturation (Fig. 1k–o). The latter greatly prevailed over all other stages on smears and sections. Thick 500-nm sections stained with methylene blue demonstrated that infection was restricted primarily to muscles, even though subcuticular epithelium was occasionally infected (Fig. 1d).

3.2. Electron microscopy

The earliest stages observed were diplokaryotic meronts of two types. The first type, “early meronts,” demonstrated homogenous cytoplasm and relatively small diplokaryon with tightly associated nuclei and a barely distinguishable envelope (Fig. 2a). Meronts of the second type, “late meronts,” exhibited prominent endoplasmic reticulum and large diplokaryon delineated by a double-membrane nuclear envelope (Fig. 2b). A similar stage was regularly observed prior to karyogony and meiosis at the onset of octosporogenous sporogony in *A. penaei* and other microsporidia (Andreadis, 2007; Sokolova et al., 2015; Sokolova and Fuxa, 2008). Meronts developed in direct contact with the cell cytoplasm and contained double-membrane structures, seemingly mitochondria, in vicinity of a nucleus (Fig. 2a, b). The next observed stage was a unicellular cell with a slightly thickened plasma membrane and a large single nucleus of irregular shape. Both nuclear and plasma membranes displayed numerous invaginations, and the cytoplasm was filled with membrane structures (Fig. 2c). Within the otherwise homogenous nucleoplasm, membrane- or thread-like structures were occasional seen representing the presumed remnants of membranes

separating diplokaryon counterparts. This infrequently observed stage probably displayed an intermittent stage that followed a momentary event of the diplokaryon nuclei fusion before segregation of the sporont mother cell from the periplasmic space, i.e., the future lumen of the sporophorous vesicle (SV). All further development of the microsporidium occurred within the 8–14-nm thick SV membrane. Uninucleate sporogonial plasmodia were surrounded by electron-dense envelopes composed of two layers separated by a narrow electron lucid space (Fig. 2d, 2d insert). The nuclear membrane was hardly seen at this stage; nucleoplasm was homogenous and contained synaptonemal complexes (Fig. 2d, 2d insert), suggesting meiosis. Sporophorous vesicles contained either dividing sporogonial plasmodia (Fig. 1e) or 2–4 sporonts resulting from plasmodial division (Fig. 2f). Lumens of SVs with sporonts were filled with finely granulated material and membranous inclusions. Regularly, SVs with 4 sporoblasts in rosette-like arrangement were observed indicating abortive tetra-spore sporogony, i.e., failure of the last mitotic division. Lumens of SVs with those teratoid sporoblasts contained fibrillary material (Fig. 2g). In accordance with LM observations, TEM repeatedly revealed SVs with defective irregularly shaped spores with an increased number of organelles (Fig. 2h, 3a). However, most SVs contained normal sporoblasts and spores at different steps of maturation (Fig. 3b–g). The lumens of SVs with mature spores were filled with granular and fine fibrillary material. Some spores exhibited a tubular-like episporal secretion (Fig. 3c). In the scanning electron microscope (Fig. 3h), the fibrillary content of SVs was obvious, particularly when SV envelopes had been accidentally damaged (Fig. 3h). The spores on thin sections measured $2.5\text{--}3.1 \times 1.3\text{--}1.7 \mu\text{m}$. Their envelopes were 90–110 nm thick and contained a relatively thick (40–60 nm) electron dense exospore and an endospore about 30–50 nm thick. The nucleus displayed a triangular shape at certain planes of sectioning (Fig. 3d, f). The polar filament was isofilar with 10–13 coils about 90–100 nm in diameter and arranged in two or three rows. The posterior vacuole was not prominent, but some spores contained posterosome-like structures (not shown). Polaroplast was bipartite with an apical electron-dense region of “tight lamellas” that embraced a region with an unresolved structure (Fig. 3f, g). The polar disk was mushroom-shaped with a relatively short manubrial portion.

3.3. Phylogenetic relationship with other microsporidia based on SSU rRNA

The sequence recovered from the novel microsporidium was 1329 base pairs long and contained 52.1% GCs (GenBank MG 708238). Twenty one of the total 30 sequences from the analysis demonstrated the highest scores (1200–1766) when aligned with sequence of the novel microsporidium from *P. paludosus* (MPP) by Basic Local Alignment Search Tool (BLAST). These 21 sequences belong to predominantly fish microsporidia of the genera *Potaspora*, *Spraguea*, *Kabatana*, *Microgemma*, *Tetramicra*, and *Glugea* (Table S1). The sequences of *Potaspora aequidens* and *P. morhaphis*, xenoma-forming microsporidia that infect neotropical teleostean fishes, displayed maximal identity (91%) with the MPP sequence. The next closest match (88% identity) was the sequence (JX853814) designated in GenBank as “*Potaspora* sp. YW-2013.” This sequence was the only one in this group of “the close matches” that had been recovered not from a fish microsporidium but from a species that infects a caridean shrimp in China. To assess the relation of the novel species with other microsporidian taxa, the alignment composed of 30 SSU rDNA sequences was subjected to phylogenetic analysis by a Maximum Likelihood algorithm. The resultant phylogram exhibited a topology basically concurrent with previously published microsporidian phylogenies (Stentiford and Dunn, 2014; Vossbrinck and Debrunner-Vossbrinck, 2005; Vossbrinck et al., 2014). The studied taxa were distributed among all five major clades defined by Vossbrinck and Debrunner-Vossbrinck (2005) as “Clade I” and “Clade III,” microsporidia of freshwater origin, “Clade II and IV,” microsporidia of terrestrial origin, and “Clade V,” microsporidia of

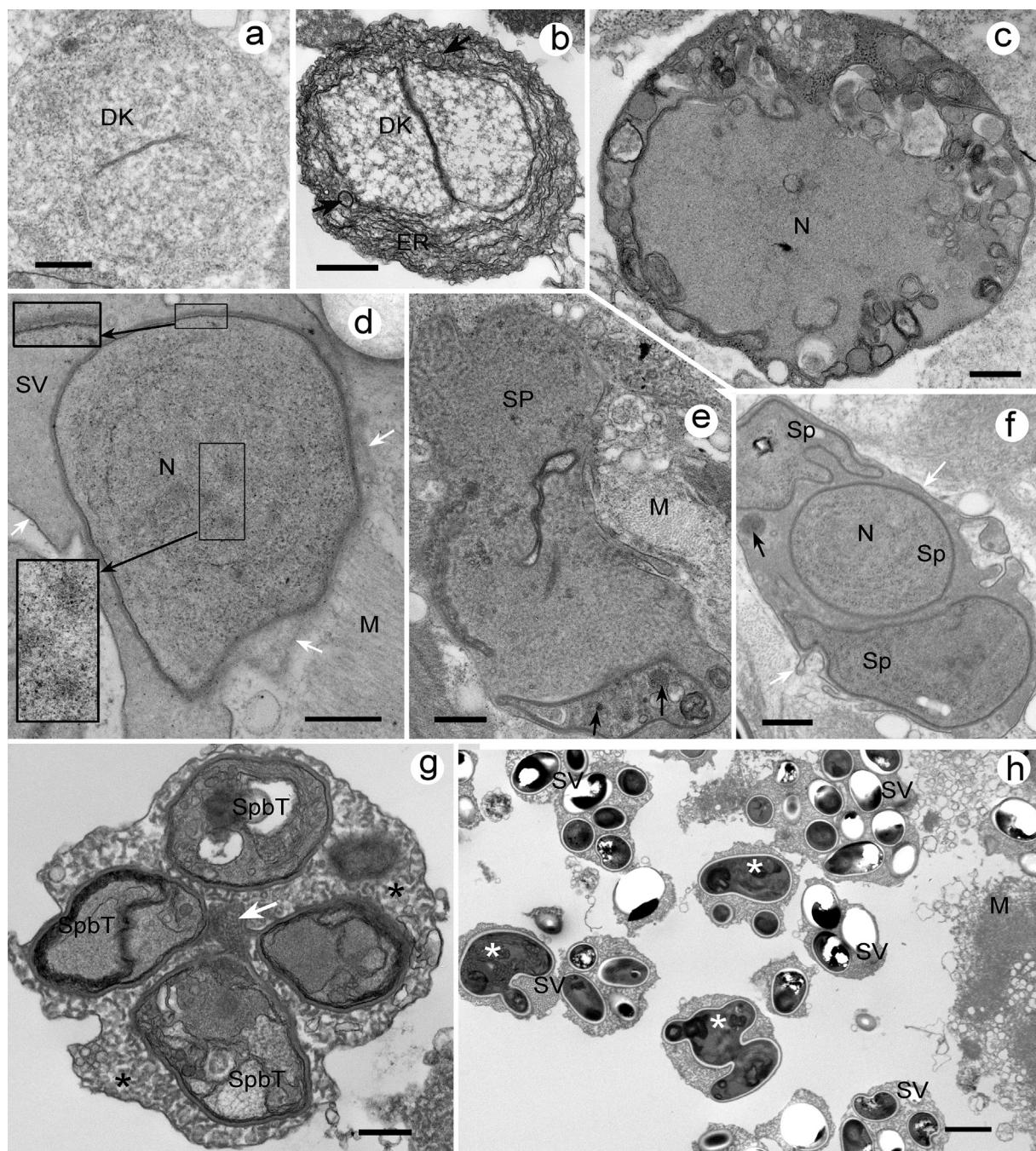


Fig. 2. *Apotaspora heleios* n. sp. from *Palaemonetes paludosus*: Electron Microscopy I. (a) An early meront with homogenous cytoplasm and small diplokaryon. (b) A late meront exhibiting prominent endoplasmic reticulum and large diplokaryon. Arrows indicate double-membrane structures (mitosomes?) in vicinity of the nucleus. (c) A pre-sporogonial transitional stage that followed a momentary event of the DK counterparts fusion, just before segregating a sporont mother cell within a sporophorous vesicle. (d) Section through a sporont with a single nucleus containing synaptonemal complexes (lower insert indicated by black arrow). The sporophorous vesicle (SV) is filled with homogenous fine granular material and is limited by a three-layered membrane indicated by white arrows, enlarged on the insert (upper box indicated by black arrow). (e) SV with a dividing sporontal plasmodium. Black arrows indicate electron dense membranous inclusions within SV. (f) Section through a SV with 3 sporonts in the view. Black arrow indicate electron dense inclusions in otherwise homogenous SV content. White arrows point to SV membrane. (g) SV with 4 sporoblasts in rosette-like arrangement demonstrating g abortive tetra-spore sporogony, i.e., the failure of the last mitotic division. Lumens of SVs with such teratoid sporoblasts contained unusual fibrillary material (asterisks). White arrow points to the residual body (a remnant of mother sporont cytoplasm that connected sporonts before separation). (h) SVs at lower magnification. Some SVs contain irregularly shaped spores (asterisk). DK, diplokaryon; M, muscle fibers; N, single nucleus; Sp, sporont; Spb, sporoblast; SV, sporophorous vesicle. Bars: a-g, 0.5 μ m; h, 2 μ m.

marine origin, or “Marinosporidia.” The MPp belonged to the latter and clustered together with *Potasporea* spp. from fishes forming a sister group to the sequence “*Potasporea* sp. YW-2013” from a Chinese shrimp. The “*Potasporea* spp. – YW-2013 – MPp” lineage grouped with the *Spraguea* – *Microgemma* branch. All node supports were solid, varying

from 98 to 100 (Fig. 4). Sequences of the fish microsporidia *P. morhapis* and *P. aequidens* demonstrated the lowest pairwise divergence values (“evolutionary distances”) against the MPp sequence, 0.050 and 0.060, respectively, in the K-2-parameter analysis. The evolutionary distance separating MPp and *Potasporea* YW-2013 was 0.095, and MPp

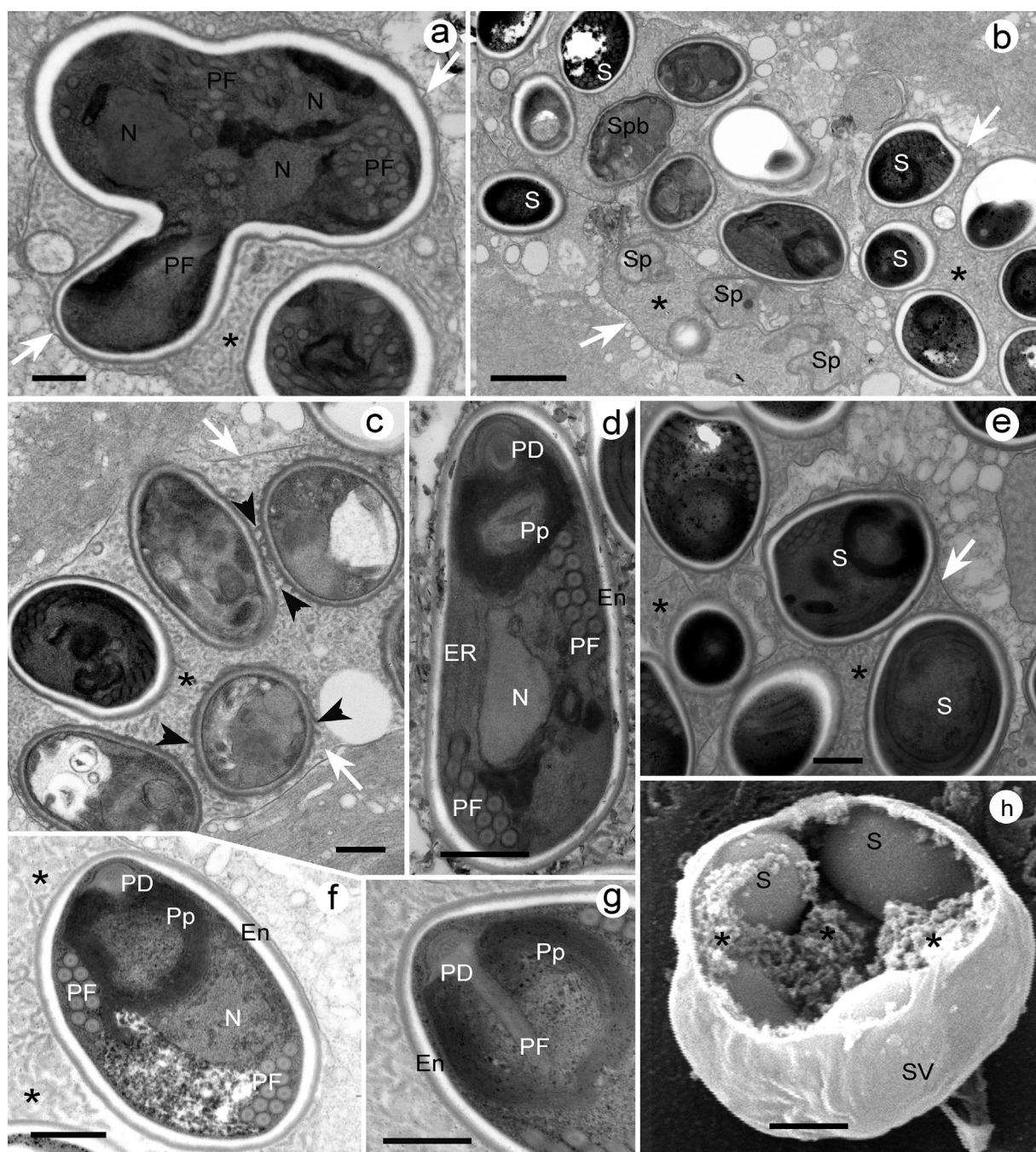


Fig. 3. *Apotaspula heleios* n. sp. from *Palaemonetes paludosus*: Electron Microscopy II. (a) A teratoid spore displaying irregular shape and increased number of organelles. (b) SVs with sporonts, sporoblasts and spores at different steps of maturation. (c) A section through a CV, in which some spores exhibit tubular-like episporal secretion (arrowheads). (d–g) Sections through mature spores demonstrating typical microsporidian features of internal structure. Note characteristic bipartite polaroplast, isofilar polar filament arranged in 2–3 layers, and triangle shaped nucleus. (h) Scanning electron microscopy image of SV with accidentally damaged membrane and clearly seen internal matter, the spores and abundant fibrillary material. En, endospore; ER, endoplasmic reticulum; N, nucleus; PD, polar disk; PF, polar filament; PP, polaroplast; S, spore; Sp, sporont; Spb, sporoblast; SV, sporophorous vesicle. Bars: a, c–g, 0.5 μ m; b, 2 μ m; h, 1 μ m.

and *Spraguea lophii* was 0.099 (Table S1). To better resolve the position of MPP, we focused on the relationships within the *Spraguea* – *Microgemma* – *Potaspula* clade. We added more taxa of this lineage in the analysis, including the sequence of the microsporidium recently described from a caridean shrimp in China as *Potaspula macrobrachium* (Ding et al., 2016). The SSU rDNA sequence of *P. macrobrachium* (KU307278) did not produce a significant score in the initial BLAST analysis because of a small coverage (61%). The sequences of the first dataset and *P. macrobrachium* overlapped by about only 800 bp. However, the identity in these SSU orthologues between *P. macrobrachium*

and MPP was relatively high, 88% in the BLAST search (Table S2). The alignment of 12 sequences belonging to the *Spraguea*-*Microgemma*-*Potaspula* lineage was subjected to the distance analysis, which demonstrated that the lowest evolutionary distances (0.091–0.093), as in the previous analysis, separated MPP and fish *Potaspula* spp. The value of sequence divergence between MPP and *P. macrobrachium* was greater (0.113). For pairs MPP – *Spraguea* spp., MPP – *Microgemma* spp., MPP – *Kabatana* sp., and MPP – *Tetramicra brevifilum*, these values ranged between 0.139 and 0.145 (Table 2). The topology of the resultant phylogenetic tree (Fig. 5) was identical to the framed part of the

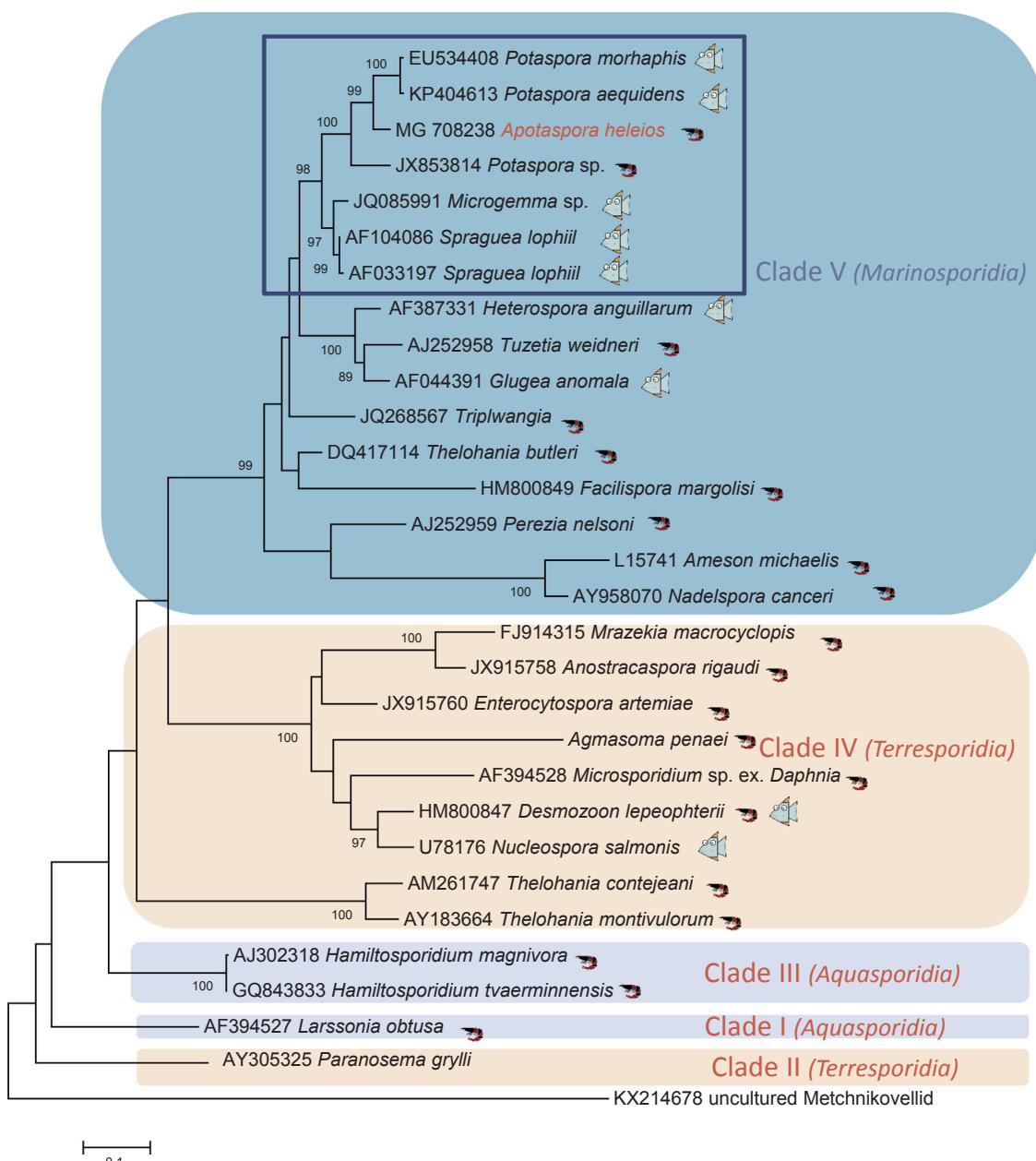


Fig. 4. Phylogenetic position of *Apotaspora heleios* n. sp. from *Palaemonetes paludosus*. SSU rDNA-inferred Maximum Likelihood tree based on 889 informative positions of thirty 1200 bp long microsporidian sequences. Bootstrap values when > 75 are shown at nodes. Fish or crustacean host of the presented species are indicated by corresponding icons. The novel species fell into the Clade V Marinosporidia and clusters within the *Potaspora* – *Microgemma* – *Spraguea* lineage (framed). Scale bar corresponds to 0.1 substitution per site.

phylogram on Fig. 4 and revealed two well-supported lineages, the *Spraguea*-*Microgemma*-*Tetramicra* lineage and the MPp-*Potaspora* lineage. The latter split in two branches, the *Potaspora macrobrachium* – *Potaspora* sp. YW-2-13 dichotomy and the MPp – fish *Potaspora* spp. cluster. The position of *Kabatana* sp. was not resolved (Fig. 5).

4. Discussion

The Riverine grass shrimp, *P. paludosus*, is native to fresh-low salinity bays, ponds, lakes, and streams of the USA coastal plain and spreads from New Jersey to Florida and as far west as eastern Texas. The shrimp was also introduced in the lower Colorado River in Arizona and California. The shrimp has limited value as fish bait, aquarium pets, and food for cultured fish and humans. More importantly, it plays a crucial role in aquatic ecosystems and is considered a keystone forage

species in estuarine communities in which it contributes extensively to the exchange of nutrients among various trophic levels. Not only does it serve as prey for a large number of fishes, including sport and commercial species, birds, and other predators, but, because of its omnivorous eating habits, it is engaged in energy transfer as a primary consumer, decomposer, carnivore, and detritivore (Merritt et al., 1999). Like *Daphnia* spp. or *Cyclops* spp. bearing numerous microsporidia in freshwater inland communities in Europe (Stentiford and Dunn, 2014; Vavra et al., 2016, 2017), *P. paludosus* thrives also at the crossroads of numerous food chains in estuarine communities in the New World, and should be expected to host microsporidia. The absence of records only suggested the scarcity of research. Microsporidia were found in at least five other species of *Palaemonetes/Palaemon* with somewhat similar ecology. Two microsporidian species have been recorded from *P. pugio* and *P. serratos* (Table 1). The novel species can be easily distinguished

Table 2Estimates of evolutionary divergence between SSU rDNA sequences from the taxa within the *Spraguea-Microgemma-Potaspora* clade.*

Accession numbers, species	1	2	3	4	5	6	7	8	9	10	11	12
MG 708,238 <i>Apotaspora heleios</i>	1											
KU307278 <i>Potaspora macrobrachium</i>	2	0.113										
EU534408 <i>Potaspora moraphis</i>	3	0.091	0.126									
KP404613 <i>Potaspora aequidens</i>	4	0.093	0.126	0.013								
JX853814 "Potaspora sp.", YW-2013	5	0.118	0.023	0.128	0.128							
AF104086 <i>Spraguea lophii</i>	6	0.139	0.142	0.163	0.164	0.136						
AF033197 <i>Spraguea lophii</i>	7	0.147	0.152	0.172	0.172	0.146	0.008					
JQ085991 <i>Microgemma</i> sp.	8	0.141	0.134	0.153	0.156	0.130	0.038	0.042				
AJ252952 <i>Microgemma</i> sp.	9	0.145	0.142	0.157	0.160	0.134	0.041	0.046	0.013			
AF364303 <i>Tetramicra brevifilum</i>	10	0.139	0.151	0.163	0.166	0.145	0.047	0.052	0.036	0.038		
EU682928 <i>Kabatana</i> sp.	11	0.143	0.127	0.164	0.168	0.121	0.081	0.088	0.085	0.090	0.087	
KF549987 <i>Agmasoma penaei</i> **	12	0.622	0.610	0.570	0.579	0.600	0.546	0.554	0.553	0.551	0.577	0.551

The novel species and evolutionary distances separating it from other members of the clade are highlighted.

* There was a total of 728 positions in the final dataset. Evolutionary analyses were estimated by Kimura-2-Parameter algorithm; analysis was conducted in MEGA5.

** The outgroup.

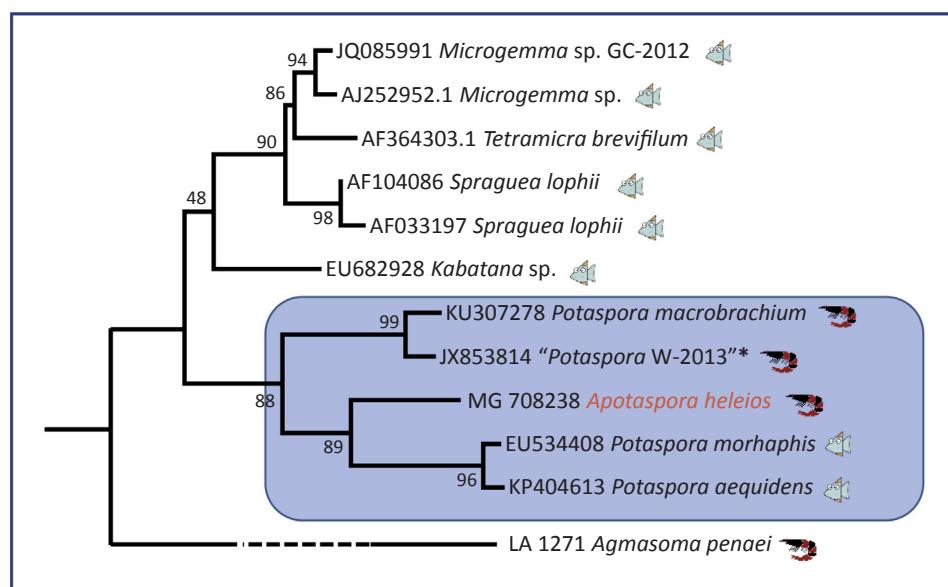


Fig. 5. The clade of *Potaspora*-related microsporidia contains *Apotaspora heleios* n. sp. and polyphyletic genus *Potaspora* comprised of species parasitizing fish and shrimp hosts. SSU rDNA-inferred Maximum Likelihood tree is based on 728 informative positions of 12 sequences, including the sequence of *P. macrobrachium* that warrants re-assigning to a new genus. Bootstrap values are shown at nodes.

from all microsporidia reported to date from *Palaemonetes* spp. and *Palaemon* spp. *Chapmanium macrocystis* that infects *P. varians* produces pyriform sporophorous vesicles (Hazard and Oldacre, 1976), and *Indosporus* spp. parasitizing several shrimp species possess characteristic episporal appendages on the spore surface (Overstreet and Weidner, 1974) and differ significantly by the ultrastructure (Azevedo et al., 2000). *Pleistophora* and *Ovipleistophora* species are characterized by the presence in their life cycles of multinucleate sporogonial plasmodia and numerous spores within pansporoblast membranes (Sprague, 1977). Interestingly, the majority of these species, including MPp, produce *Thelohania*-like octospores inside sporophorous vesicles (Table 1). Unfortunately, no molecular analysis was available at the time many of these species were described, and no way exists to infer their genetic relatedness to the Riverine grass shrimp microsporidium described here. Similarly, it would be informative to know if *Pleistophora myairii* from Japan (Sprague, 1977) is related to the *Pleistophora*-like species parasitizing another atiid shrimp, *Triwangia caridinae*, from Taiwan (Wang et al., 2013) (Table 1). If we concentrate on the species with the known rDNA barcode, the closest relatives of MPp are two *Potaspora* spp. from fish. The latter shared 90% SSU rDNA similarity to MPp, displayed the least evolutionary distance (Table 2, Tables S1, S2.), and fell together with MPp in one well-supported lineage on the phylogenograms (Figs. 4, 5). The type species, *Potaspora moraphis*, was described from

the needlefish *Potamorhaphis guianensis* (Teleostei, Belontidae) native to South America, and it inhabits the estuary of the Amazon River in Brazil (Casal et al., 2008). This microsporidium produces xenomas in the coelomic cavity near the anal region of the fish. These xenomas were distinguished by numerous filiform anastomosing microvilli and were filled with monokaryotic developmental stages residing in direct contact with host cell cytoplasm (Casal et al., 2008). Another species of the genus was described from another freshwater fish, *Aequidens plagiozonatus* (Teleostei, Cichlidae), also from Brazil (Videira et al., 2015). This microsporidium formed xenomas with similar morphological features, although located in muscles of the sub-opercular region and the caudal fins. Regarding spore morphology, both species differ from MPp by having a pyriform shape and fewer polar filament coils. The type-species, unlike MPp, demonstrated characteristic dense granules in the posterior part of the spores, and its polaroplast had electron dense lamellae. Host and tissue tropism, developmental characteristics (i.e., absence of a dikaryotic stage in the life cycle and growth in direct contact with host cytoplasm), and spore morphology of the novel species does not fit the diagnosis of the genus *Potaspora* (Casal et al., 2008, p. 1059). Therefore, MPp does not belong in *Potaspora*. Moreover, the pairwise values of SSU rDNA divergence/similarity of 0.091–0.093/90.9%–90.7% separating *Potaspora* spp. from MPp are within the range of divergence/similarity values typical for the values separating other

genera within the *Spraguea-Micogemma-Potaspora* lineage. For example, only 0.038–0.046 value of sequence divergence (95.4–96.2% similarity) separates *Sprague* from *Micogemma* and 0.085–0.090 (81.5%–90% similarity) – *Kabatana* from *Micogemma* (Table 2). Based on morphological, ecological, developmental, and genetic characters, we erect the new genus *Apotaspora* to accommodate the parasite of *Palaemonetes paludosus* named *Apotaspora heleios* n. sp. Pairwise evolutionary distances separating *Potaspora* spp. from the new species, are less than those between *Potaspora* spp. and other fish microsporidia from the *Spraguea-Micogemma-Potaspora* group (0.139–0.147). This is indicative of a close evolutionary relationship between these fish and shrimp microsporidia different in morphological and ecological features. Given the genetic relatedness and structural dissimilarity, and basing our opinion on the hypothesis about complex polyxenous life cycles, presumably characteristic to ancestral microsporidia (Vossbrinck and Debrunner-Vossbrinck, 2005; Vossbrinck et al., 2014), we speculate that the life cycle of the common ancestor of *Potaspora* and the new species included two phases: asexual sporogony in fishes (the intermediate host) resulting in xenoma formation and a sexual one in shrimp (the definitive host), resulting in mass production of meiospores. Both host species had been tightly interconnected by food chains, like teleostean fishes and caridean or penaeid shrimps in a present day estuarine ecosystem, which would facilitate maintenance of a parasite population spreading among hosts. The decedents, like contemporary *Potaspora* spp. and MPp microsporidium, either converted to monoxygous life cycles or the second host has yet to be discovered. The scenario involving a fish intermediate or primer host has been suggested for *Agmasoma penaei* parasitizing *Litopenaeus setiferus* (Decapoda, Penaeidae) (Overstreet, 1973; Iversen and Kelly, 1976; Pasharawipas and Flegel, 1994; Sokolova et al., 2015). Quite recently, Stentiford et al. (2017) found out that *Inodospora octospora* infecting *Palaemon serratus* in the estuary of the Rivers Fal in England shares as much as 98.4% of SSU rDNA sequence similarity with *Kabatana* sp. from the two-spot goby, *Gobiusculus flavescens*. Surprisingly, the same shrimp was infected with another microsporidium that those authors named *Ovipleistophora arlo*. The latter was genetically (by 99.5%, SSU rDNA sequence) and morphologically extremely similar to *Ovipleistophora* spp. that infect ovaries of freshwater fish (Stentiford et al., 2017). These new data suggest the existence of a complex shrimp-fish life cycle not only among extinct but probably among extant microsporidian species belonging to the Clade V (Vossbrinck and Debrunner-Vossbrinck, 2005) that has yet to be discovered. Direct evidence of a polyxenous fish-crustacean life cycle has been obtained for only *Paranucleospora theridion*, with a salmonid fish (Atlantic salmon, *Salmo salar*, or Rainbow trout, *Oncorhynchus mykiss*) as an intermediate host and a parasitic copepod (*Lepeophtheirus salmonis* or *Caligus elongatus*) as a definite host (Freeman et al., 2003; Freeman and Sommerville, 2011; Nylund et al., 2010).

The microsporidium parasitizing muscles of the pond-reared Oriental river prawn *Macrobrachium nipponense* (Caridea, Palaemonidae) in China, was recently described and placed in the genus *Potaspora* based exclusively “on genetic affinity to the genus *Potaspora*” (Ding et al., 2016: p. 61), i.e., exhibiting an 87% identity with *P. morhaphis*. The authors may not have recognized that 85 to 96% pairwise identities between species belonging to different genera within the *Spraguea-Potaspora-Micogemma-Kabatana* lineage are common, and that 87% identity does not necessarily mean belonging to the same genus. They made no attempt to expand the generic diagnosis of *Potaspora* to accommodate the species that they were describing. Hopefully, the confusion caused by a polyphyletic taxon will be corrected by the creation of a new combination for *P. macrobrachium* in future revisions of the genus *Potaspora* and related taxa. However, disregarding taxonomic perplexity, we find it necessary to compare these related species, *P. macrobrachium* and *A. heleios*, which both parasitize palaemonid shrimps, from the Old and New worlds, respectively. Morphologically, these two microsporidia differ significantly by spore size (1.5 × 1.0 µm, *P. macrobrachium* vs. 2.9 × 1.7 µm, *A. heleios*), lack of

persistent SVs in *P. macrobrachium* (although EM photograph Fig. 4, p. 60 by Ding et al., 2016) demonstrated sporogony within SVs, which was not mentioned in the specific description), number of polar filament coils (7–8, *P. macrobrachium* vs. 10–13, *A. heleios*), and some other features of spore ultrastructure. Only late sporonts and spores were demonstrated by Ding et al. (2016), so the pre-sporogony parts of life cycles cannot be compared. Both species parasitize muscle, but histopathological aspects of the infections greatly vary. *Potaspora macrobrachium* infected cardiac muscles and caused formation of melinized granulomas filled with spores and surrounded by macrophages, whereas infection with the novel species was detected in the abdominal subcuticular epithelial layer and underlying muscles and was homogeneously distributed over the infected tissue with no granuloma formation. Finally, these two microsporidia parasitized different palaemonid shrimp genera, each with different ecological preferences and inhabiting distantly isolated geographical areas. Thus, neither morphological nor ecological considerations suggest uniting these two species into one genus. The phylogenetic analysis based on comparison of SSU rDNA orthologues demonstrated that these species are separated by a 0.113 value of evolutionary divergence, which is slightly greater than the value separating fish-infecting *Potaspora* spp. and the new species (0.091–0.093). On the tree, the sequence for *P. macrobrachium* clustered with that belonging to the undescribed microsporidium parasitizing another Chinese palaemonid shrimp, the Ridgetail white prawn, *Exopalaemon carinicauda* (“*Potaspora* sp. YW-2013”, GenBank Acc #JX853814). These two taxa form a sister branch to the fish-infecting-*Potaspora* spp.-MPp microsporidium lineage.

The microsporidium described in this paper brings to attention the existence of a potentially diverse clade of *Potaspora*-related microsporidia with a worldwide distribution currently known from two host groups, teleostean fishes and caridean shrimps (Fig. 5). Despite the discrepancy of structural, ecological, and developmental characters of all members studied to date, this lineage is well supported by all phylogenetic analyses. Grouping together fish and crustacean microsporidia suggests that polyxenous life cycles might be a customary feature for ancestral and likely extant members of this lineage.

5. Taxonomic summary

Following the recent examples (Bojko et al., 2017; Vavra et al., 2016, 2017), we include informal ranking based on SSU-rDNA-inferred phylogenetic analysis in the taxonomic summary presented beneath. This practice seems to be helpful in overcoming current “discomfort” of taxonomy of the Microsporidia connected with “the absence of strict congruity between structural and molecular phylogeny data” (Vavra et al., 2016).

Phylum: Microsporidia Balbiani, 1882

Class-level informal ranking: Clade V Marinosporidia” Vossbrinck and Debrunner-Vossbrinck, 2005

Family-level informal ranking: Clade “the *Potaspora* spp.- related lineage” Sokolova, Overstreet

Apotaspora n. g. Sokolova, Overstreet

Etymology: *Apotaspora* refers to the close relationship of the genus with *Potaspora*, although being different. The Greek prefix “A” means “not,” and the Greek generic name *Apotaspora* should be treated in the feminine gender.

Remarks: At least one developmental sequence results in production of 8 monokaryotic spores within sporophorous vesicles (SVs). Spores of typical ultrastructure with bipartite polaroplast. Because the genus is currently monotypic, other morphological features are yet to be identified. New species placed in this genus, likely, infect muscles and hypodermis of caridean shrimps of the New World. GenBank accession number MG 708,238 serves as the reference sequence for this genus.

Type-species: *Apotaspora heleios* n. sp. Sokolova, Overstreet

Type host: Riverine grass shrimp, *Palaemonetes paludosus* (Gibbes, 1850)

Type locality: Little Bateau Bay ($30^{\circ}43' 36.81''\text{N}$ Latitude; $87^{\circ}57'34.32''\text{W}$ Longitude), Mobile Bay Delta of the Gulf of Mexico, Alabama, USA.

Prevalence: rare, < 1% (one infected shrimp among one hundred or more).

Type-material: Slides with methanol-fixed smears of infected tissues stained with Trichrome and Giemsa stains as well as slides with semi-thin sections, Epon-Araldite blocks, grids with thin sections, and images of thin sections, "Ah ex Mpp" (*Apotaspula heleios* n.g. n.sp. from *Palaemonetes paludosus*) are deposited in the microsporidian collection of the Institute of Plant Protection, St. Petersburg, Russia (Prof. Issi's collection), in the personal collections of YS and RO. Syntype slides with methanol-fixed smears of infected tissues stained with Trichrome and Giemsa stains and slides with semi-thin sections are deposited in the International Protozoan Type Slide Collection, Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC, USA under catalog numbers USNM 1492375–78.

Etymology. The Greek, feminine, adjectival specific name *heleios* refers to the host being marsh-dwelling (Ἐλειος in Ancient Greek = héleios in Latin)

Tissue tropism and pathology: Abdominal musculature and hypodermis; whitish coloration of the abdomen

Merogony is not known, except for the final stage, a diplokaryotic meront

Sporogony: octosporous sporogony results in production of 8 monokaryotic spores within persistent SVs. In this sequence, a diplokaryotic meront gives rise to a sporont mother cell with a large single nucleus after merging of the diplokaryon counterparts. Meront plasma membrane turns into SV envelope, and sporont wall segregates internally. The sporont nucleus undergoes meiosis followed by plasmotomy by internal budding to produce 4 sporonts, each dividing into 2 uninucleated sporoblasts to finally produce 8 spores.

Spores broadly oval measured $2.9 \pm 0.06 \times 1.7 \pm 0.03 \mu\text{m}$ ($2.5\text{--}3.3 \times 1.6\text{--}1.9 \mu\text{m}$, $n = 11$). Sporophorous vesicles with persistent SV membranes ranged from 4.4 to $5.6 \mu\text{m}$ and averaged $5.1 \pm 0.12 \mu\text{m}$. SVs are filled with fibrillary-tubular secretion noticeable as Evans blue-stained regions on methanol-fixed Trichrome blue-stained smears. On thin sections, spores measured $2.5\text{--}3.1 \times 1.3\text{--}1.7 \text{ nm}$, with $90\text{--}110 \text{ nm}$ thick envelopes composed of $40\text{--}60 \text{ nm}$ -thick smooth exospore and $30\text{--}50 \text{ nm}$ -thick endospore, with a single nucleus, with isofilar polar filament $90\text{--}100 \text{ nm}$ in diameter and arranged in 10–13 coils in 2 or 3 rows, and with bipartite polaroplast and mushroom-shaped polar disk. The posterior vacuole is not prominent.

SSUrDNA sequence: GenBank, under Accession number MG 708,238.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jip.2018.05.007>.

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