



Tetra disseminated microsporidiosis: a novel disease in ornamental fish caused by *Fusasporis stethaprioni* n. gen. n. sp.

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Abstract

A novel microsporidial disease was documented in two ornamental fish species, black tetra *Gymnocorymbus ternetzi* Boulenger 1895 and cardinal tetra *Paracheirodon axelrodi* Schultz 1956. The non-xenoma-forming microsporidium occurred diffusely in most internal organs and the gill, thus referring to the condition as tetra disseminated microsporidiosis (TDM). The occurrence of TDM in black tetra was associated with chronic mortality in a domestic farmed population, while the case in cardinal tetra occurred in moribund fish while in quarantine at a public aquarium. Histology showed that coelomic visceral organs were frequently necrotic and severely disrupted by extensive infiltrates of macrophages. Infected macrophages were presumed responsible for the dissemination of spores throughout the body. Ultrastructural characteristics of the parasite developmental cycle included uninucleate meronts directly in the host cell cytoplasm. Sporonts were bi-nucleated as a result of karyokinesis and a parasite-produced sporophorous vesicle (SPV) became apparent at this stage. Cytokinesis resulted in two spores forming within each SPV. Spores were uniform in size, measuring about 3.9 ± 0.33 long by 2.0 ± 0.2 μm wide. Ultrastructure demonstrated two spore types, one with 9–12 polar filament coils and a double-layered exospore and a second type with 4–7 polar filament coils and a homogenously electron-dense exospore, with differences perhaps related to parasite transmission mechanisms. The 16S rDNA sequences showed closest identity to the genus *Glugea* ($\approx 92\%$), though the developmental cycle, specifically being a non-xenoma-forming species and having two spores forming within a SPV, did not fit within the genus. Based on combined phylogenetic and ultrastructural characteristics, a new genus (*Fusasporis*) is proposed, with *F. stethaprioni* n. gen. n. sp. as the type species.

Keywords Microsporidia · *Fusasporis* · Disease · Fish · Phylogeny · Pathology

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Introduction

The world ornamental fish industry, annually valued at \$15–30 billion US dollars, is dominated by freshwater species, 90% of which are farm-raised, while the remaining are wild-collected fish (Raghavan et al. 2013). Of these, the tetras are a popular group of aquarium fish, which are diverse characid fishes spanning several subfamilies. The black tetra *Gymnocorymbus ternetzi* Boulenger 1895 and cardinal tetra *Paracheirodon axelrodi* Schultz 1956 in the subfamily Stethaprioninae are both native to South America and are important species in the ornamental fish trade. The cardinal tetra, a close relative to the neon tetra *Paracheirodon innesi* (Myers, 1936), is native to the upper Orinoco and Rio Negro rivers in South America, and sustainable wild-harvest of this species is vital to the local economy in Barcelos Municipality in Brazil (Zehev et al. 2015). Though historically depending on sustainable wild-harvest, captive rearing of cardinal tetra

has become more popular in countries including the Czech Republic, Vietnam, and Indonesia (Evers et al. 2019). The black tetra is a subtropical fish native to the Guaporé drainage and Paraguay River basins in southern Brazil, Argentina, and Bolivia (Géry 1977). This is a popular aquarium species that is farmed domestically in the USA.

Infectious disease contributes to losses in ornamental fish, and biosecurity is a challenge in wild-captured fishes. Furthermore, the international trade of these species may lead to inadvertent spread of infectious agents to other facilities, geographic locales, and fish populations. Several microsporidial diseases are known in ornamental fish, most notably “neon tetra disease” caused by *Pleistophora hyphessobryconis*. Though originally found in neon tetra, this microsporidium causes muscular degeneration in a broad range of ornamental fish hosts including characids, cichlids, cyprinids, and cyprinodontids (Steffens 1962; Sanders et al. 2010; Li et al. 2012), and in the non-ornamental siluriform hybrid known as jundiara, *Leiarius marmoratus* (Gill 1870) × *Pseudoplatystoma reticulatum* Eigenmann & Eigenmann, 1889 (Winters et al. 2016). Additionally, a microsporidium consistent with *Glugea anomala* (Moniez 1887), which forms xenomas throughout the intestinal wall and multiple organs, has been shown to infect and be highly lethal to a variety of cyprinodontid ornamental fish species (Lom et al. 1995). Herein, we describe another unique microsporidial disease that we refer to as tetra disseminated microsporidiosis (TDM), characterized from two ornamental fish species, the cardinal tetra and black tetra.

Microsporidia are obligate intracellular parasites considered to be either a basal branch or a sister group to fungi (Lee et al. 2008; Han and Weiss 2017; Bass et al. 2018). Microsporidia in finfish are highly diverse, with species reported in over 20 genera (Lom and Nilsen 2003; Kent et al. 2014). Historically, taxonomic descriptions of microsporidia were based on host species, tissue tropism, spore morphology, and the developmental cycle, though genetics of the small subunit rRNA gene have considerably improved taxonomic classifications (Nilsen et al. 1998; Lom and Nilsen 2003; Vossbrinck and Debrunner-Vossbrinck 2005). In fact, it has been suggested that ultrastructural features and morphology have been less useful in higher level taxonomic rankings, as these characters may show higher variability at the genus, species, and population levels (Vossbrinck and Debrunner-Vossbrinck 2005). Nonetheless, ultrastructural features of the developmental cycle provide further comparative traits and insights into the evolution and life cycle of microsporidial species (Vávra and Larsson 2014). Herein, we genetically and morphologically characterize the microsporidial species responsible for TDM to better understand its life cycle and taxonomic position.

Materials and methods

Case material and sampling

Black tetras were raised year-round in ponds at two different locations in the Southeastern United States. Temperature was maintained between 22 and 28 °C during cooler seasons by covering the ponds with clear plastic liners to induce a greenhouse effect and allow for year-round grow-out. Black tetras were transferred to multi-tank recirculating aquaculture systems (RAS) within indoor greenhouses throughout the year. The RAS consisted of mechanical and biological bead filters, UV filtration, heaters for temperature control during cooler seasons, and multiple individual 757-L concrete burial vault-style tanks. Standard water quality parameters (temperature, pH, ammonia, nitrite, hardness, and alkalinity) were kept within normal limits for the species and checked at minimum once weekly. During cooler periods, heaters maintained RAS at 25–28 °C, and during warmer periods, ambient air temperature maintained RAS temperature at 27–30 °C. Breeder fish were handled at minimum once a month and transferred to a breeding room where they were pair spawned in static tanks for 24–48 h before being returned to greenhouses. Chronic mortality of black tetras, with low daily mortality in single to low double digits (exact numbers are unavailable), was first noted in the summer of 2017 and subsequently occurred thereafter.

During the mortality period, moribund fish were euthanized humanely using buffered tricaine methanesulfonate (MS-222). No obvious gross lesions were observed. Microscope squash preparations of the gastrointestinal tract and liver were viewed using differential interference contrast (DIC) microscopy which revealed microsporidial spores. A squash sample containing spores was preserved in 95% ethanol for further molecular processing. Euthanized or freshly dead fish were incised along the abdomen to facilitate fixation and fixed whole in 10% neutral buffered formalin for histology. The liver and gut were dissected from two fish selected arbitrarily and fixed in Karnovsky's fixative and maintained at 4 °C for transmission electron microscopy (TEM). A microscope squash preparation was prepared from a portion of fixed liver, examined with a Zeiss Axioplan 2 research microscope using DIC microscopy and photographed with a microscope-mounted ProgRes Gryphax Arktur CMOS digital camera (Jenoptik AG). Spore measurements were taken directly from digital images taken using the × 100 objective under oil immersion. Each spore was measured along its longest axis to obtain length, and a perpendicular measurement was obtained at the widest point for width. A total of 70 spores were measured to obtain the mean and a range of spore lengths and widths. The remaining tissue in Karnovsky's fixative was stored at 4 °C until further processing for TEM.

Approximately 500 cardinal tetras were acquired from a commercial distributor and held in a quarantine system at a public aquarium. While the exact origin of this group is unknown, many of these cardinal tetras had co-infections with myxozoan parasites within the central nervous system, which has been reported in fish collected from Brazil (Camus et al. 2017), suggesting these cardinal tetras likely originated from South America. In March 2018, three cardinal tetras were found moribund and euthanized (as described above for black tetras) a day after ending prophylactic treatment for suspected bacterial infection with a sulfamethoxazole/trimethoprim combination drug. An additional fish died during transfer from quarantine to an exhibit 5 weeks later. All four fish were preserved whole in 10% neutral buffered formalin and submitted for histopathological examination.

Fixed tissue samples from both black and cardinal tetras were routinely processed for histology, including dehydration through an ascending series of ethanols, clearance in xylene, and infiltration and embedding in paraffin wax. Sections of 5- μm thickness were cut from each fish, mounted on glass slides, and stained with either hematoxylin and eosin (H&E), modified Brown-Hopps (Gram) stain, or Luna stain (Luna et al. 1968).

Transmission electron microscopy

Karnovsky's fixative was washed from the tissues with two changes of 0.1 M phosphate buffer each for 10 min, followed by fixation for 1 h in 1% osmium tetroxide in 0.1 M phosphate buffer. Following fixation, tissues were washed twice in distilled water for 10 min each and dehydrated in an ascending series of ethanols to anhydrous ethanol. Tissues were cleared in propylene oxide for 10 min and gradually infiltrated with EMBED 812 resin (Electron Microscopy Sciences), first in a 1:1 concentration of resin:propylene oxide for 2 h, followed by 3:1 concentration of resin:propylene oxide overnight, and finally in pure resin overnight in a vacuum desiccator. Tissues were embedded in flat capsules and polymerized for 48 h at 60 °C. Semi-thin sections (0.5 μm) were cut from 10 samples, mounted on slides, stained with Toluidine Blue O stain (Electron Microscopy Sciences), and screened with a light microscope. Four samples that contained high concentrations of microsporidia in various stages of development were selected and re-trimmed and ultrathin sections (90 nm) were cut and mounted onto 100-mesh copper grids. Sections were stained with 1% uranyl acetate in 50% ethanol for 30 min, followed by washing in distilled water, staining with a modified Sato lead stain (Hanaichi et al. 1986) for 2 min, and washing again in distilled water. Samples were viewed using a Philips CM12 transmission electron microscope operated at 80 kV and images taken with a mounted AMT-XR11 digital camera located at the

Department of Pathology, Robert Wood Johnson Medical School, Rutgers University.

DNA extraction, PCR, sequencing, and phylogenetic analysis

A pooled liver and intestine sample from a black tetra was freshly frozen at –20 °C and submitted to the Wildlife and Aquatic Veterinary Disease Laboratory, College of Veterinary Medicine, University of Florida for molecular identification. DNA was extracted from the sample using a QIAcube (Qiagen) and a DNeasy Blood and Tissue Kit (Qiagen) using the manufacturer's protocol for animal tissues. The extracted DNA was resuspended in 100 μl AE buffer and stored at –80 °C. The sample was amplified by PCR with the V1F-1492R primer pair that amplifies approximately 1300 bp of the 16S rDNA sequence (Vossbrinck et al. 1993, 2004). The reaction volume was 50 μl and consisted of 0.25 μl of Platinum Taq DNA Polymerase (Invitrogen), 5.0 μl of 10× PCR buffer, 2.0 μl of 50 mM MgCl₂, 1.0 μl of 10 mM dNTPs, 2.5 μl of 20 μM forward and reverse primers, 32.25 μl of molecular grade water, and 4.5 μl of DNA template. An initial denaturation step of 94 °C for 5 min was followed by 36 cycles of a 94 °C denaturation step, a 55 °C annealing step, and a 72 °C extension step, each step runs for 1 min, and a final extension step at 72 °C for 5 min. PCR products were subjected to electrophoresis in 1% agarose gel stained with ethidium bromide. Amplicons were purified from 10 μl of the PCR products by excision from an agarose gel using a QIAquick PCR Purification Kit (Qiagen) and cloned using a TOPA TA Cloning Kit (Invitrogen). Transformed bacterial colonies were screened using PCR, and plasmids containing relevant inserts were purified using a QIAprep Spin Miniprep Kit (QIAGEN). Two purified plasmid clones were sequenced using M13 forward and reverse primers with an ABI 377 automated sequencer (Applied Biosystems). The sequence data were assembled and edited and primer sequences removed using CLC Genomics Workbench 7.5 software (Qiagen). BLASTn analysis was performed with generated sequence data compared against known sequences maintained in GenBank.

To identify microsporidia within formalin-fixed, paraffin-embedded (FFPE) cardinal tetras, DNA was extracted from tissue scrolls using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for FFPE tissues. According to the type sequence, forward (5'-CGCGGYAAGGCTCAGTAAC-3') and reverse (5'-CCTACTTACTAACTGATGGGATGGC-3') primers, spanning nucleotide positions 61–79 and 189–213, respectively (supplemental figure), were designed to target a variable region of the 16S rRNA gene that is unique to the type species from black tetras. This

region was determined by aligning sequence data from the type species and other closely related microsporidia to find a less conserved region within the 16S sequence. Conventional PCR using extracted DNA was performed using a T100™ thermal cycler (Bio-Rad, Hercules, CA) with the following protocol: 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 51 °C for 30 s, and 68 °C for 30 s, with a final extension step of 68 °C for 5 min. A 200 µl reaction solution, divided into eight, 25 µl reactions, consisted of 5 units of NEB One Taq Polymerase (New England Biolabs, Ipswich, MA), 10 pmol of forward and reverse primers, 60 µl of DNA template, and nuclease-free water to volume. PCR products from each reaction were combined, visualized by gel electrophoresis and UV light, extracted using a QIAquick Gel Extraction Kit (Qiagen), and directly submitted for Sanger sequencing (Genewiz, South Plainfield, NJ). Sequences were assembled and manually edited using Geneious® 11.1.5 and identified in GenBank with a BLASTn search for somewhat similar sequences.

The nucleotide sequence of the type species from black tetra was queried using BLASTn to determine other sequences within the National Center for Biotechnology Information's GenBank database with closest nucleotide identity. Only sequences with > 90% sequence coverage were used for phylogenetic analysis. Sequence alignment and phylogeny was conducted in MEGA X (Kumar et al. 2018). A total of 40 related nucleotide sequences within clade III representing the class Marinospordia were included, with *Spraguea lophii* Sprague and Vavra, 1976 used as an outgroup, based on a previous phylogenetic analysis conducted by Vossbrinck and Debrunner-Vossbrinck (2005). The most suitable DNA model was determined using MEGA X; the phylogenetic analysis was conducted by using the maximum likelihood method and general time reversible model (Nei and Kumar 2000). There were a total of 1465 positions in the final dataset. The tree with the highest log likelihood (-9340.93) was shown. A total of 1000 bootstrap replicates were run and the percentage bootstrap support is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3934)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 26.50% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Host species of microsporidia, habitat, and whether the microsporidium is xenoma-forming or not are depicted in the tree.

Results

Clinical observations and pathology of tetra disseminated microsporidiosis

The primary clinical observations of affected black tetras were first noted in the summer of 2017 and consisted of a history of poor breeding performance coupled with low-level chronic mortalities. Occasional external protozoan parasites, including *Trichodina* spp. and *Ichthyophthirius multifiliis*, were noted. Therapeutic attempts, including 3–5 treatments with 2 ppm copper applied every other day for *Ichthyophthirius* and 3 treatments of 25 ppm with formalin applied every other day for *Trichodonella*, were unsuccessful at resolving the underlying clinical observations. No evidence of internal parasites or mycobacterial granulomas was observed on initial microscopic squash preparations of internal organs. Bacterial cultures of the brain and posterior kidney on tryptic soy agar with 5% sheep's blood were consistently negative. A few of the initial cohorts of affected black tetras had individual fish that exhibited tight spinning behaviors or swam in large circles, which in both instances were consistently in a counterclockwise direction. It was these initial behavioral observations in combination with the ongoing low, chronic mortality, and poor breeding performance that led to the initial histopathologic investigation in an attempt to determine the etiology.

Squash preparations of the internal organs viewed with DIC microscopy showed disseminated infection with microsporidial spores, particularly in the liver and the gastrointestinal tract (Fig. 1a–d). Spores most frequently occurred in pairs within infected cells (Fig. 1b–e) and cell lysis freed spores from cells (Fig. 1d). In histologic specimens, Luna stain consistently stained the spores red (Fig. 1f), as expected for microsporidia.

Light microscopic examination revealed that lesions in black tetras (Fig. 2a–f) and cardinal tetras (Fig. 3a–f) had similar distribution and severity. In most fish, coelomic visceral organs were frequently necrotic and severely disrupted by extensive infiltrates of macrophages and scattered lymphocytes (Figs. 2a–d and 3a). The cytoplasm of most macrophages was markedly distended by numerous, approximately $2 \times 4 \mu\text{m}$, ovoid, microsporidian spores with distinct polar vacuoles and basophilic polaroplasts (Figs. 2e, f and 3e, f). Infected macrophages frequently contained an enlarged, prominent, basophilic nucleolus within the nucleus (i.e., epithelioid conformation). Macrophages containing spores transmurally infiltrated the proximal intestinal wall, extending into the coelomic cavity, hepatic sinusoids, splenic sinuses, and interstitial stroma of the pancreas, ovary, testis, and kidney (Figs. 2a–f and 3b–f). Free microsporidia and macrophage infiltrates severely disrupted the intestinal submucosa and muscularis, the hepatic parenchyma, and testicular and ovarian interstitial stroma, occasionally causing parenchymal

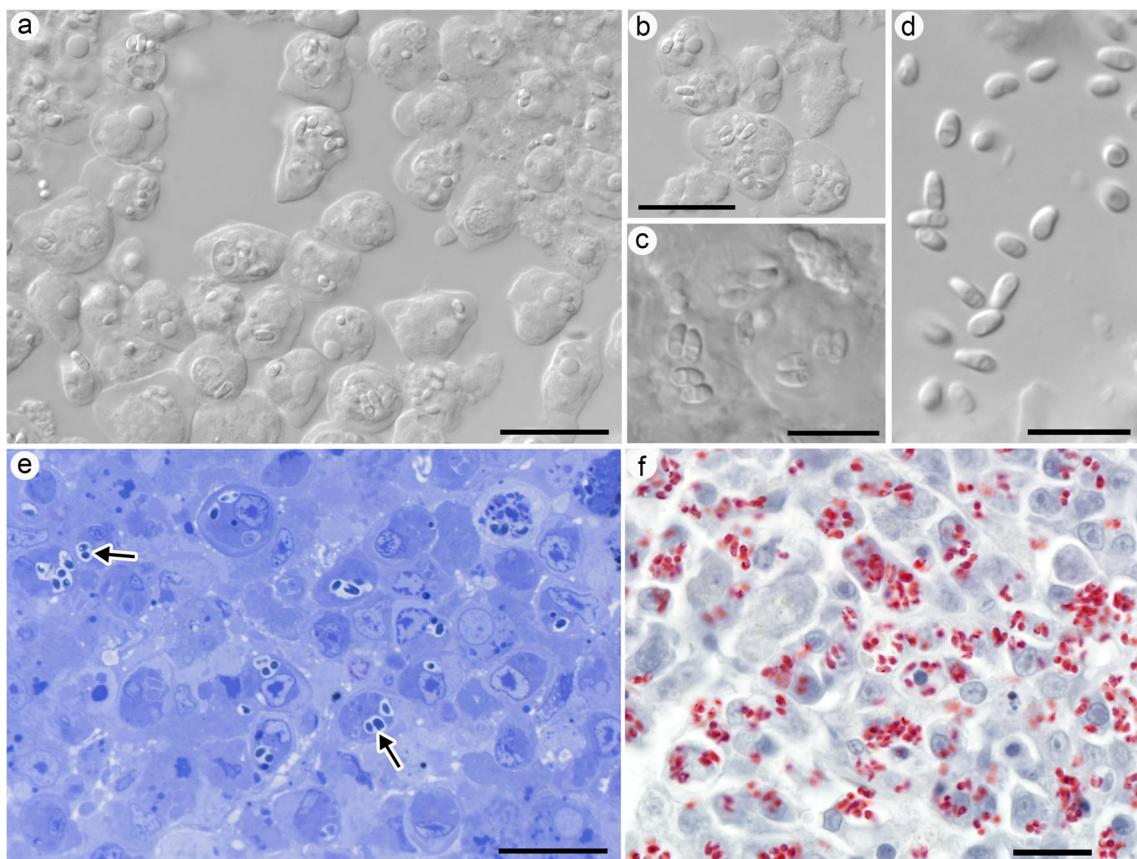


Fig. 1 *Fusasporis stethaprioni* n. gen. n. sp. spores in black tetra in various microscopic preparations. **a–d** Spores in a microscope squash preparation of affected liver showing paired or single spores within host cells (**a–c**) and free outside of cells (**d**); notice the typical posterior

vacuole of the spores. **e** Semi-thin section stained with Toluidine Blue O stain demonstrating paired spores within sporophorous vesicles (arrows). **f** Luna stain, showing red-stained microsporidial spores in histology of liver. Magnification bars in **a, b, e, f** = 20 μ m and **c, d** = 10 μ m

necrosis (Figs. 2a–f and 3b–f). Within the liver, it was unclear whether microsporidia were additionally present within the cytoplasm of hepatocytes or were limited entirely to macrophages. Low to moderate numbers of free and intrahistiocytic spores were widely disseminated throughout the body (often associated with the vasculature) in various organs including the spleen, adipose tissue, common bile duct, skeletal muscle, skin, choroid rete of the eye, gill filaments, pseudobranch, meninges, spinal cord, and peripheral ganglia. Spores were bright red and nearly black with Luna (Fig. 2f) and Gram (Fig. 3f) stains, respectively.

Developmental cycle, host-parasite interface, and spore morphology

Infected cells were mildly hypertrophic and contained numerous parasite developmental stages (Fig. 4a, b). Infected cells were devoid of lysosomes or residual bodies and no cellular response was noted to developing spores. Host cell cytoplasm contained abundant ribosomes and mitochondria. Infection did not induce formation of xenomas, and only limited spore replication occurred within each cell.

Earliest observed stages were uninucleate meronts that occurred directly within the cell cytoplasm surrounding the cell nucleus (Fig. 4c). No more than 12 uninucleate meronts were observed within a single host cell. The cytoplasm of meronts predominantly contained ribosomes bound by a plasma membrane. Occasionally, there were inclusions of electron-dense material in the cytoplasm of meronts and sporonts. Sporonts had increased ribosomes which caused the cytoplasm to appear more electron-dense, and the plasma membrane became increasingly electron-dense prior to formation of the sporophorous vesicle (SPV). Karyokinesis occurred around the sporont stage, producing two nuclei, at which point the sporonts become more elongated (Fig. 4d). No more than two nuclei were observed in any of the sporonts. In many instances, after development of the SPV, the plasma membrane of sporonts was not noticeably more electron-dense than meront stages, making this distinction difficult. Host cell mitochondria were often in close association with sporonts. The SPV was first evident in the bi-nucleated sporonts, with the SPV membrane blistering from the sporont plasma membrane (Fig. 4d, e). Cytokinesis occurred simultaneously with the generation

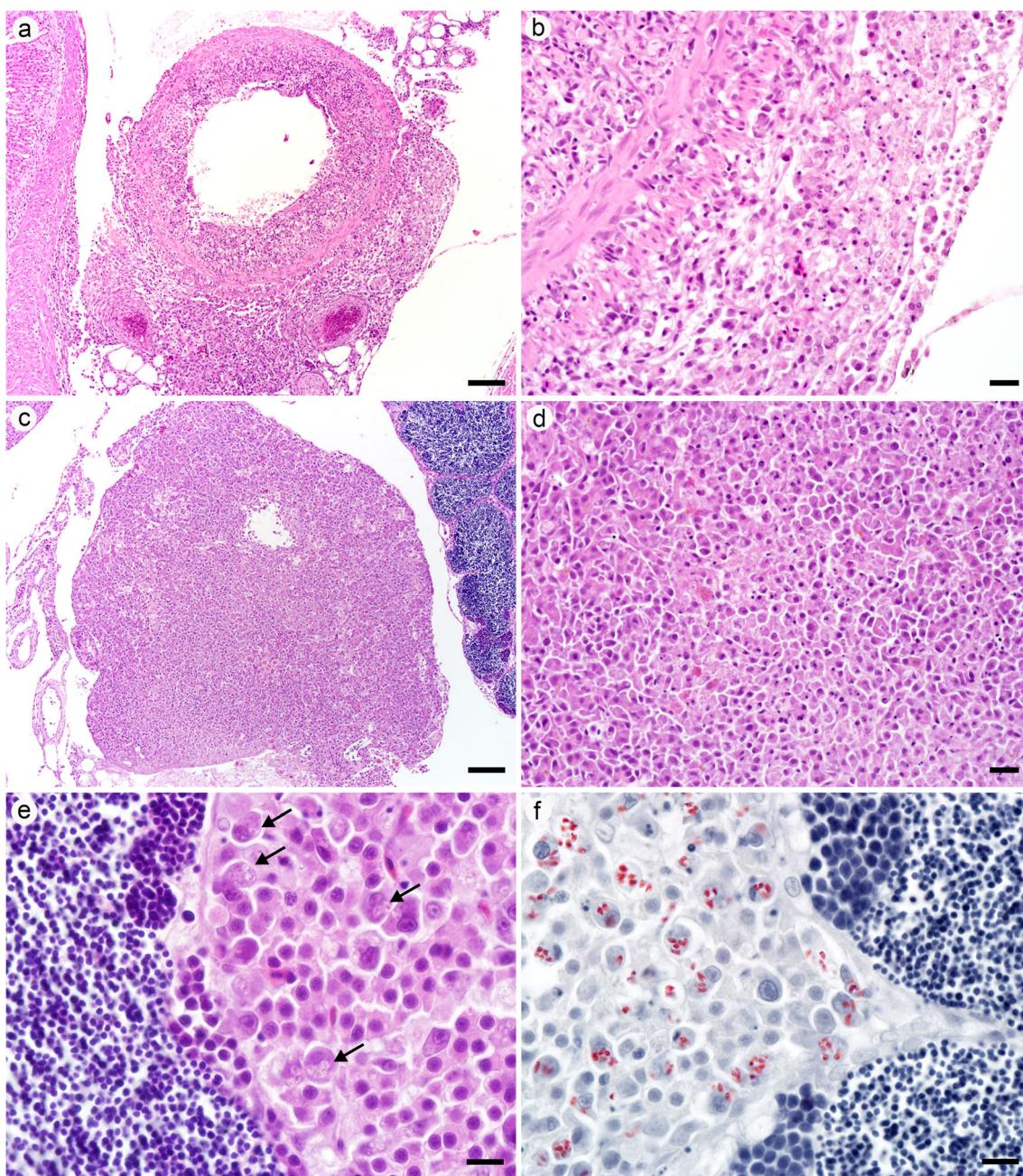


Fig. 2 Histopathology associated with *Fusasporis stethaprioni* n. gen. n. sp. in black tetras. **a** Focally extensive transmural inflammation of the intestine and adjacent coelomic cavity, mesentery, and pancreas. H&E. **b** Necrotizing enteritis within the submucosa, muscularis, and serosa along with extensive coelomitis. H&E. **c** Expansion and disruption of the hepatic parenchyma due to necrosis and inflammatory infiltrates. H&E. **d** Hepatocellular necrosis and disruption of cords and sinusoids due to

infiltrating macrophages. H&E. **e** Expansion of the testicular interstitial space due to infiltrating macrophages which frequently contain intracytoplasmic microsporidia (arrows). H&E. **f** Free and intracytoplasmic microsporidia throughout the interstitial infiltrates in the testis. Luna. Magnification bars in **a, c** = 100 μ m; **b, d** = 20 μ m; and **e, f** = 10 μ m

of the SPV, resulting in the formation of two sporonts within each SPV (Fig. 4f).

In the early stages of SPV formation, there was abundant material within the SPV lumen (Fig. 5). Extensions of the sporont cytoplasm led to smaller fragments of sporonts in the SPV lumen (Fig. 5a, b). These sporont extensions became narrower in diameter, became more electron-dense, and

developed an extensive glycocalyx (Fig. 5c). Additionally, the SPV lumen contained narrow diameter electron-dense tubules measuring between 30 and 40 nm and membranous structures composed of two closely abutted membranes with a striated electron-dense pattern (Fig. 5d). As development progressed, the two sporonts became sporoblasts that had highly electron-dense cytoplasm and evidence of polar

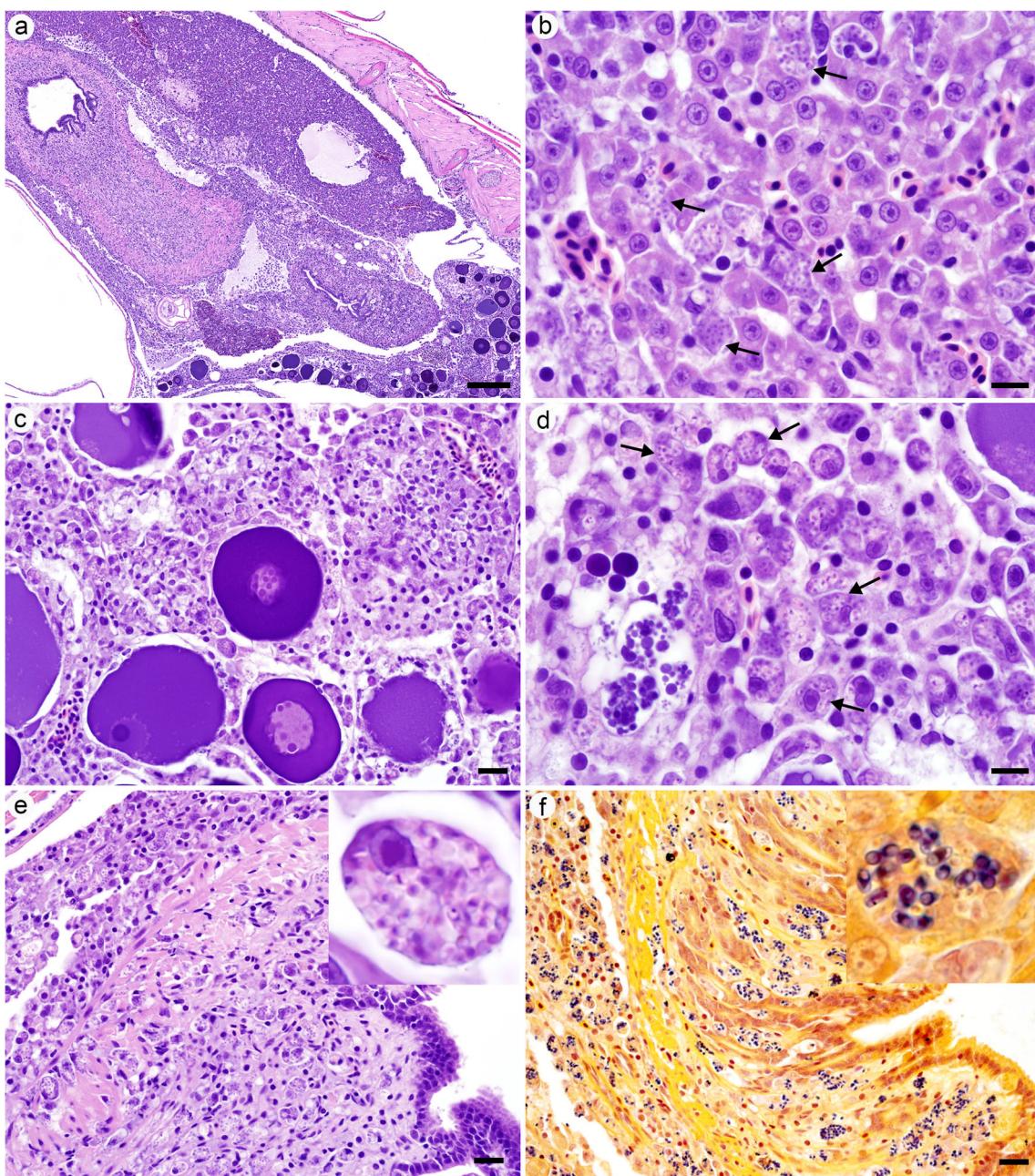


Fig. 3 Histopathology associated with *Fusasporis stethaprioni* n. gen. n. sp. in cardinal tetras. **a** Subgross magnification of the coelomic cavity with dense inflammatory infiltrates that partially obscure the viscera. H&E. **b** Hepatic sinusoids are distended by lymphocytes and macrophages filled with numerous, intracytoplasmic microsporidia (arrows), which disrupt the adjacent parenchyma. H&E. **c** Oocytes are displaced by inflammatory infiltrates, primarily macrophages and lymphocytes. **d** Macrophages within the ovary are frequently distended

by numerous microsporidia (arrows). H&E. **e** Massively distended macrophages containing microsporidia (inset) and free microsporidia transmurally cross the intestinal wall into the coelomic space. H&E. **f** Free and intracytoplasmic (inset) microsporidia throughout all layers of the intestinal wall and adjacent coelom. Gram stain. Magnification bars in **a** = 200 μ m; **b**, **d** = 10 μ m; and **c**, **e**, **f** = 20 μ m. Magnification bars do not apply to insets

filament formation. In nearly all instances, two spores formed within each SPV, with one exception where 4 sporonts occurred within an SPV. As spores matured, less structural material was observed in the SPV lumen and was limited to uniform narrow diameter tubules (Fig. 5a, b).

Spores were uniform in size, measuring 3.9 ± 0.33 μ m (range = 3.2–3.7 μ m) long by 2.0 ± 0.2 μ m (range = 1.6–2.5) wide ($n = 70$). The posterior vacuole was clearly visible by light microscopy (Fig. 1). All spores were monokaryotic. Two types of mature spores occurred based on the

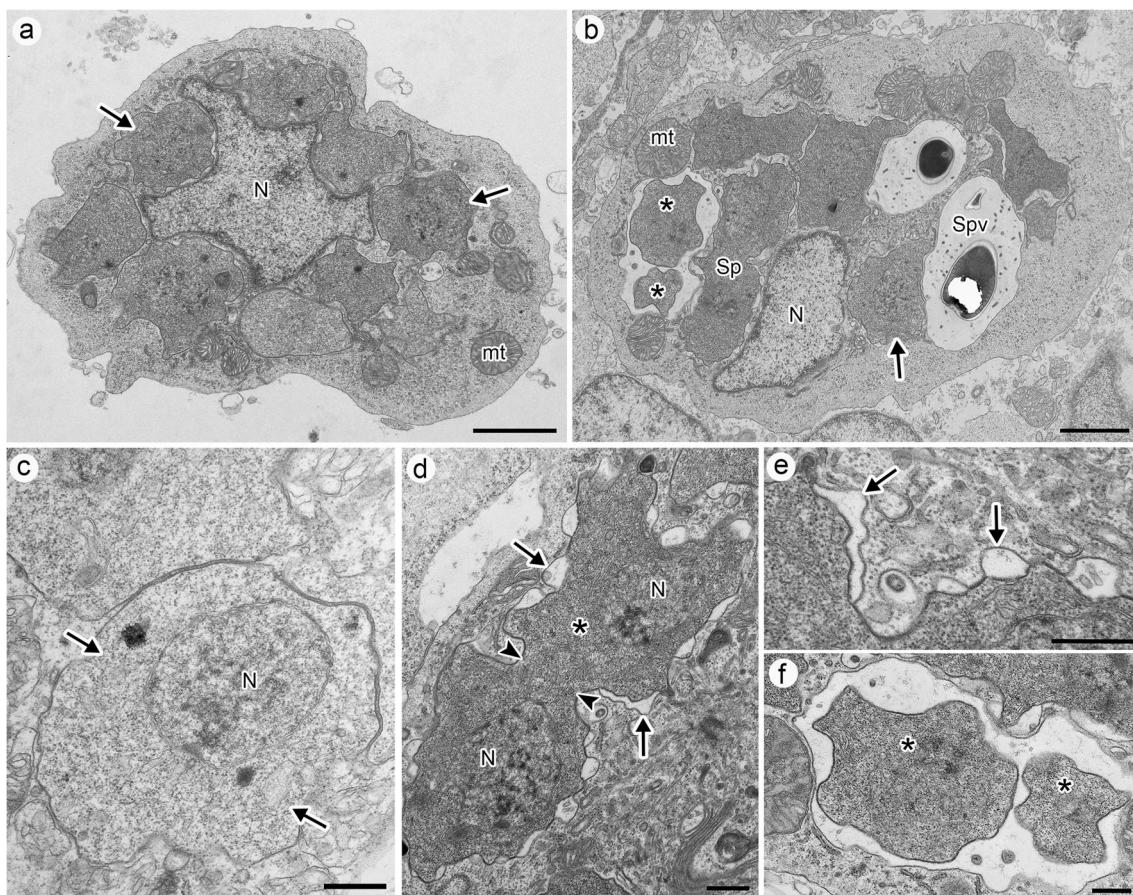


Fig. 4 Ultrastructure of the developmental cycle of *Fusaspis stethaprioni* n. gen. n. sp. in black tetras. **a** Infected host cells containing meronts (arrows) surrounding the host cell nucleus (N); host cell mitochondria (mt). **b** Infected host cell with multiple stages of development, including meronts directly in the host cell cytoplasm (arrow), an elongated sporont with two nuclei (Sp), sporonts (*) within a sporophorous vesicle, and mature spores in sporophorous vesicles (Spv). **c** Higher magnification of meront (arrows) directly in the host cell

cytoplasm; meront nucleus (N). **d** A sporont with two nuclei (N) with newly forming sporophorous vesicle (arrows) blistering from the sporont membrane. Notice the constriction of the sporont (arrowheads) indicating the region of cytokinesis. **e** Close up showing the new sporophorous vesicle (arrows) blistering from sporont surface. **f** Two sporonts (*) forming within a sporophorous vesicle. Magnification bars in **a**, **b** = 2 μ m and **c–f** = 500 nm

ultrastructure of the exospore and number of coils of the polar filament. The most frequent spore type occurred in pairs within SPVs (Fig. 6a, b). These spores had 9–12 coils in the isofilar polar filament with a mean of 10 coils ($n = 37$). The exospore occurred in two electron-dense layers with an area of reduced electron density in the middle (Fig. 6b). Polyribosomes occurred frequently within the spore cytoplasm. The second spore type (Fig. 6c–e) occurred less frequently and had 4–7 coils in the isofilar polar filament with a mean of 5 coils ($n = 10$). The exospore was uniform in electron density in these spores and they occurred individually within collapsed SPVs (Fig. 6c–e) or in pairs within an SPV.

Small subunit rDNA sequence analysis

PCR of the pooled liver and intestinal sample from black tetra yielded an amplicon of the expected size. After removal of primer sequences, the sample yielded 1286 bp (4 \times coverage);

this sequence was deposited to GenBank under accession number MW077214. PCR from FFPE tissue of the microsporidium from cardinal tetras yielded a 114 bp sequence, which spanned from nucleotide positions 100–213 when aligned with the type sequence from black tetra (supplemental figure). The sequence was identical to the type species and the next closest microsporidium sequence in GenBank shared 87% identity (AY140647), indicating that this was consistent with *F. stethaprioni* n. gen. n. sp. BLASTn analysis of the type species revealed that the highest sequence identity (94.39%) was to an unpublished account of an uncharacterized microsporidium (AY140647) from brown trout *Salmo trutta* Linnaeus, 1758. The next closest identities were to species within the genus *Glugea* with identities ranging from 92.69% in *Glugea* sp. (MK568064) to 91.07% in *Glugea anomala* (AF044391). Phylogenetic analysis showed two main clades (Fig. 7): clade I with *Glugea* spp., *Loma* spp., and *F. stethaprioni* n. gen. n. sp., and clade II that contained

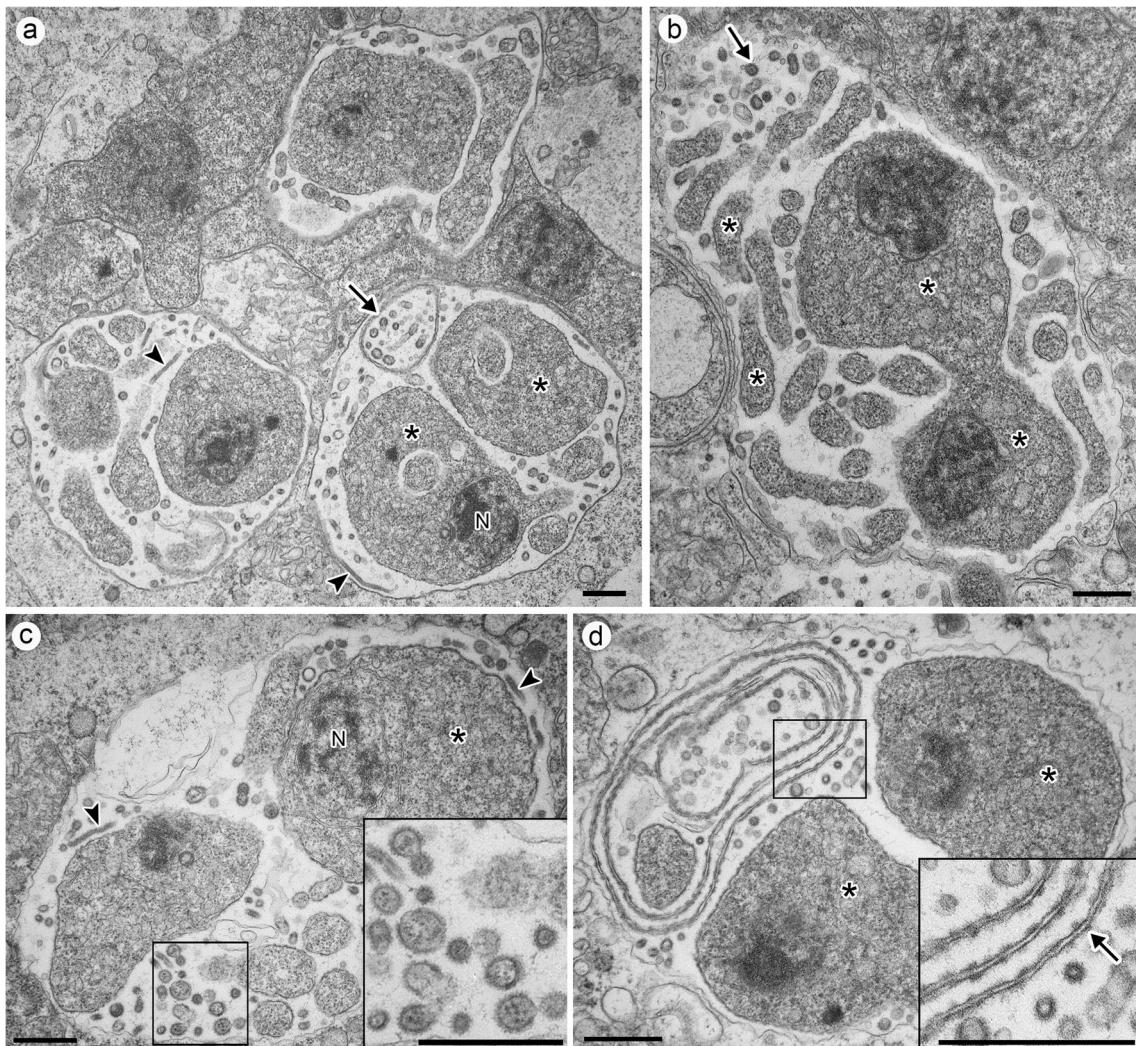


Fig. 5 Ultrastructure of the newly formed sporophorous vesicles of *Fusasporis stethaprioni* n. gen. n. sp. in black tetras. **a** A sporophorous vesicle containing sporonts (*), narrow diameter tubules (arrowheads), and variously sized tubular elements enclosed within double-membraned structures (arrows). **b** A bi-nucleated sporont undergoing cytokinesis within the sporophorous vesicle, which contains fragments or extensions

of the sporonts (*) and electron-dense spherical structures (arrows). **c** The sporophorous vesicle containing sporonts (*), tubules (arrowheads), and spherical structures with an extensive glycocalyx (inset). **d** Sporophorous vesicle containing tubular structures and a network of double-membraned structures with striated electron-densities (inset arrow). Magnification bars = 500 nm

the genera *Heterosporis*, *Dasyatispora*, most *Pleistophora* spp., *Trachipleistophora*, and *Vavraia* spp.

Clade I comprised all aquatic hosts, all being ray-finned fish except for one which occurred in a brown shrimp, *Penaeus aztecus* Ives, 1891. The majority of hosts in clade I were also associated with marine or brackish environments, with the only exceptions being *F. stethaprioni* described herein and a *Glugea* sp. from Great Lakes (USA) slimy sculpin *Cottus cognatus* Richardson, 1836. Clade I contained mainly xenoma-forming species, with the only exceptions being *F. stethaprioni* n. gen. n. sp. and *Pleistophora penaei* Contransitch, 1970 (AJ252958). *Microsporidium* sp. (AY140647) was uncharacterized; therefore, the predilection of this species to form xenomas is unknown. *Pleistophora penaei* made the genus *Pleistophora* polyphyletic, likely a

result of incorrect placement of *P. penaei* into this genus. The *Glugea* spp. formed two sister groups, which was reasonably well supported (69% bootstrap support). *Fusasporis stethaprioni* n. gen. n. sp. grouped with *P. penaei* (AJ252958) and an uncharacterized species, *Microsporidium* sp. (AY140647), as a basal branch to the *Glugea* spp. (Fig. 7). This branching from *Glugea* spp. was very poorly supported, likely due to inadequate taxa sampling in other closely related species. The *Loma* spp. and *Ichthyosporidium weissii* formed basal branches to *Glugea* spp. and *F. stethaprioni* n. gen. n. sp. Clade II included more variable host species, with ray-finned fish being most common, but also including an elasmobranch, amphipod, salamander, insects, and human. Most of the aquatic hosts were also from marine and brackish habitats, with only 3 related to freshwater environments and the

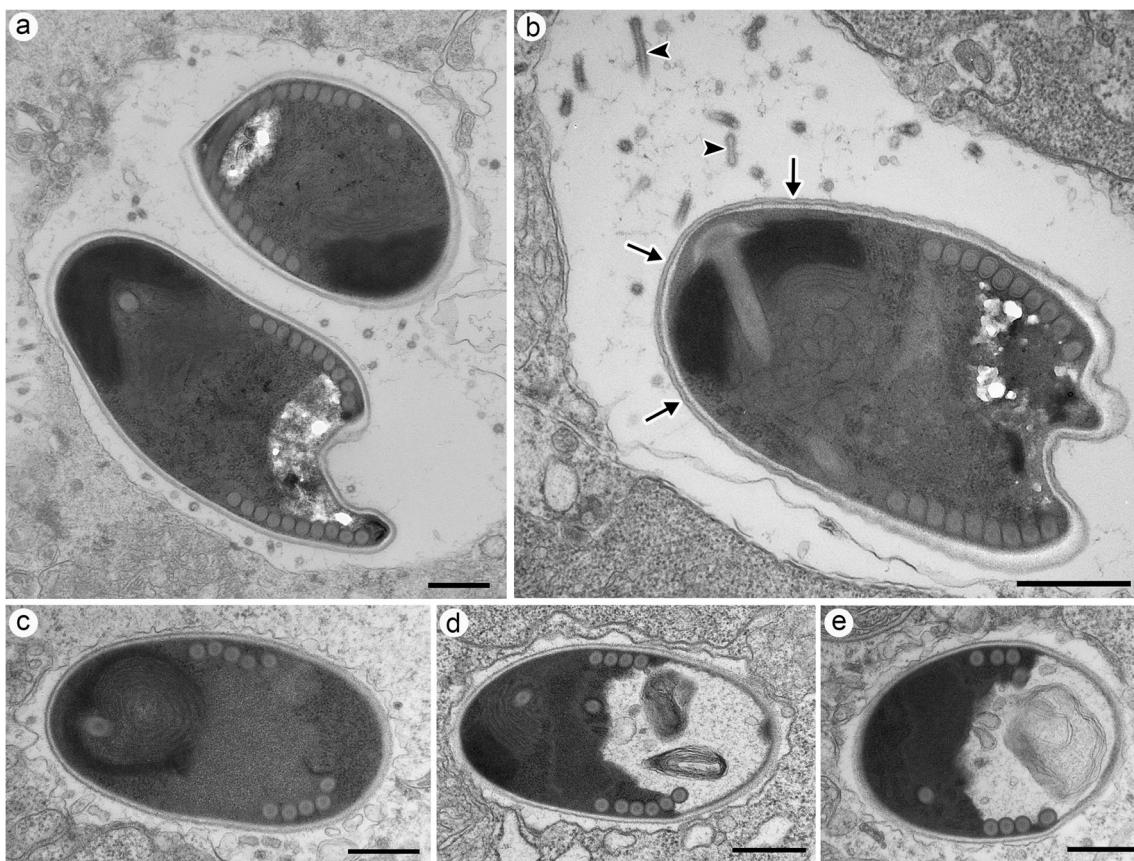


Fig. 6 Ultrastructure showing two types of mature spores of *Fusasporis stethaprioni* n. gen. n. sp. in black tetras. **a, b** One type of spore developing within a sporophorous vesicle containing narrow diameter tubules (arrowheads). These spores have 9–10 polar filament coils. **b**

Notice that the exospore comprised two distinct closely apposed layers (arrows). **c–e** A second spore type with fewer polar filament coils and a homogenously electron-dense exospore. Magnification bars = 500 nm

others being from terrestrial habitats (Fig. 7). All microsporidia within the clade II grouping were non-xenoma-forming species (Fig. 7).

Discussion

Tetra disseminated microsporidiosis (TDM) caused by *F. stethaprioni* n. gen. n. sp. is a novel disease affecting at least two important ornamental fish species. Unlike many microsporidia, a unique characteristic of TDM is the wide range of tissues that are simultaneously infected. Many fish microsporidia have defined tissue tropisms which limit them to certain organ systems. For example, *Pleistophora* most often occurs in skeletal muscle (Sanders et al. 2010; Costa et al. 2016; Winters et al. 2016; El-Garhy et al. 2017), though it is occasionally reported in the gastrointestinal or peritoneal epithelium (Morsy et al. 2012) or in the intestinal wall (Abdel-Baki et al. 2012); *Heterosporis* are limited to the skeletal musculature (Lom et al. 2000; Joh et al. 2007; Al-Quraishy et al. 2012; Phelps et al. 2015); *Ovipleistophora* mainly occur in the ovary (Pekkarinen et al. 2002; Phelps and Goodwin 2008),

though also found associated with the wall of encapsulated trematodes (Lovy and Friend 2017); *Pseudoloma* is generally found in the nervous system (Matthews et al. 2001); *Loma* spp. most frequently occur in the gill, but also in other systemic organs (Speare et al. 1998; Bader et al. 1998; Khan 2005; Brown et al. 2010a) and in the intestinal mucosa (Casal et al. 2009; Ovcharenko et al. 2017); and *Glugea* spp. frequently infect connective tissue in the intestinal wall (Lovy et al. 2009; Abdel-Baki et al. 2015a; Azevedo et al. 2016), subcutaneous tissues (Dezfuli et al. 2004; Vagelli et al. 2005), and the body cavity and viscera (Abdel-Baki et al. 2015b; Casal et al. 2016; Mansour et al. 2020). Some *Glugea* spp. were reported to extend to other internal organs (Su et al. 2014; Ryan and Kohler 2016). The wide range of infected tissues seen with *F. stethaprioni* n. gen. n. sp. herein is likely due to a tropism for macrophages, which traffic the parasite throughout the body. Based on histopathologic analysis, hepatocytes and gonadal interstitial cells may become infected with the microsporidium, though the severe disruption of the hepatic parenchyma made it difficult to know with certainty if hepatocytes were directly infected or displaced by infected macrophages. Similarly, inflammation made it difficult to

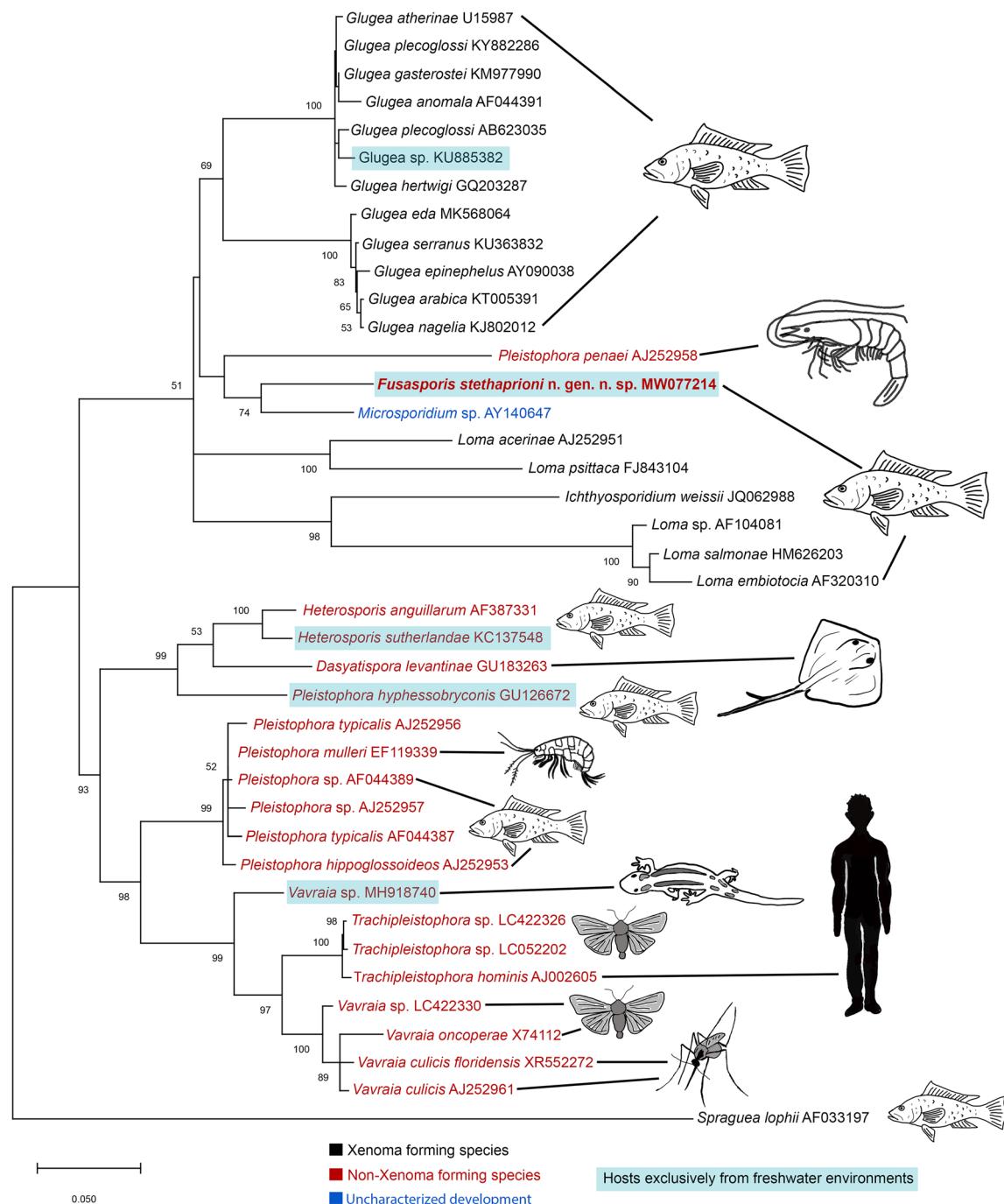


Fig. 7 Maximum likelihood tree including 40 microsporidial species, showing *Fusasporis stethaprioni* n. gen. n. sp. (bold) fitting within a clade made up primarily of xenoma-forming species from marine aquatic hosts. Shading represents aquatic species exclusively from freshwater

environments, whereas all other hosts are either aquatic, involving brackish or marine environments, or terrestrial. A second clade includes non-xenoma-forming microsporidia from various host species, including some from terrestrial habitats. *Spraguea lophii* was used as an outgroup

assess whether there was infection within epithelial cells of the coelomic viscera or if the presence of spores was related to infected macrophages.

The wide tissue tropism for *F. stethaprioni* n. gen. n. sp. may lead to various possible transmission routes. Infection of the gill, gut, and kidney would suggest that spores may be

shed directly into the water from live infected fish from damaged gills induced by infection, through fecal material and possibly urine if spores are shed into the urinary system. Additionally, the widespread infection may make it more likely for dead fish left in tank systems to release spores during decomposition. The longevity of microsporidial spores in

decomposing fish has not been well researched, though spore release in decomposing cadavers has been shown to be an important pathway for transmission of invertebrate microsporidia (Goertz and Hoch 2008). This suggests that an important aspect in managing TDM will be in culling moribund fish and quickly removing mortalities to reduce numbers of infectious spores being released into the system. The infected gonads observed herein also suggest that this parasite could be transmitted vertically or at least via contamination of sex fluids or egg surfaces. This is an especially important consideration for ornamental fish farms that depend on captive propagation of these species. These observations suggest that disinfection protocols for eyed eggs are important, despite the known challenges of chlorine disinfection of eggs for microsporidia (Ferguson et al. 2007), and subsequently keeping eggs separated from broodstock should be an important practice when broodstock are suspected to be infected with this microsporidium.

It was historically believed that microsporidia have narrow host ranges, and for some species this holds true. For example, *Loma salmonae* is restricted to salmonids in the genus *Oncorhynchus* (Brown et al. 2010b). Other species are known to have wider host species ranges. Various species of *Glugea* were originally described on the basis of host species, though later genetic data suggested that *G. anomala*, *G. stephani*, *G. atherinæ*, and *G. hertwigi* were in fact conspecific and have a broader host range than originally thought (Pomport-Castillon et al. 2000; Lovy et al. 2009). However, this has not yet been demonstrated through cross-infection experiments. Broad host species ranges occur with *Pleistophora hypessobryconis*, originally described as “neon tetra disease,” though now known to infect at least five different families of fish (Steffens 1962; Sanders et al. 2010; Li et al. 2012; Winters et al. 2016). Similarly, *Pseudoloma neurophilia*, best known from zebrafish, is known to infect at least 7 fish species from 5 different fish families (Sanders et al. 2016). *Fusasporis stethaprioni* n. gen. n. sp. herein is shown to infect two different genera within the Characidae subfamily Stethaprioninae. Characidae is one of the most diverse families of fishes with over a thousand species described including about 145 genera and containing the subfamily Stethaprioninae which represents the South American tetras (Oliveira et al. 2011). Molecular systematics indicates that there are at least 14 other fish genera that have evolved between *Paracheirodon* and *Gymnocorymbus* (Benine et al. 2015), suggesting the possibility of these other fish species as possible hosts for this microsporidium. It would be particularly important to understand if the host range of this microsporidium extends to the popular neon tetra, which is a very close relative of the cardinal tetra (Benine et al. 2015). It is hoped that, with the description of this novel microsporidial disease, awareness will be raised in the aquarium trade, which will help to identify other host fish species and to ensure that precautions are taken to

reduce further spread of this parasite. Based on the broad host ranges of other microsporidial species, the possibility exists of other more distant families of fish in the aquarium trade or in the wild to be impacted by this microsporidium. Both cases described herein were from the USA, one in a domestic farm and the other in quarantine at a public aquarium. With both cardinal and black tetra being native to South America, this parasite may occur in the native range of these species, though it is possible that these fish were infected following their export from that region. In either case, quarantine of these species will be important to avoid introduction of this microsporidium into aquarium systems, farms, or holding facilities.

Phylogenetic analysis of the rRNA gene has aided in understanding the evolutionary divergence of microsporidia and has provided more clarity in their taxonomy. Some of the historically used morphological markers are less reliable than phylogeny based on sequence analysis of the rRNA gene (Nilsen et al. 1998; Vossbrinck and Debrunner-Vossbrinck 2005). Molecular phylogeny of 125 representative microsporidial species indicates five clades, mostly corresponding with host species and habitat, giving rise to three classes of microsporidia (Vossbrinck and Debrunner-Vossbrinck 2005). Based on this phylogeny, the species herein fits within the class Marinosporidia comprising mainly microsporidia of marine origin. The predominant marine habitat of these microsporidia is consistent with the phylogeny conducted in the present study. Microsporidia from multiple freshwater fish are also included in this class and it was proposed that these microsporidia may have originated from marine fish, which later evolved to freshwater environments and brought the parasites with them (Vossbrinck and Debrunner-Vossbrinck 2005). With characiform fishes all being neotropical freshwater fishes, the transition from the marine environment occurred long ago, likely dating back around 115 million years to the Albian period. It is suggested that the most basal characiform fish is the now extinct *Santanichthys diasii* Silva Santos, 1958, which occurred in the marine environment in Brazil (Filleul and Maisey 2004). Due to the long timescale of marine origin of these fishes, perhaps a more likely explanation of *F. stethaprioni* n. gen. n. sp. grouping with marine habitats is that its original host range included marine fish, and it more recently infected these freshwater fish species. Further taxa sampling and better understanding host ranges should better clarify its relation, if any, to marine environments.

The closest microsporidia to *F. stethaprioni* n. gen. n. sp. are the *Glugea* spp., though its relationship to *Glugea* is not yet clear. The branching indicates that *F. stethaprioni* n. gen. n. sp. may form a basal group to the *Glugea* spp. though this branching was only poorly supported, due to inadequate taxa sampling of closely related species. Larger taxa sampling will reveal the relationship of *Fusasporis* spp. to *Loma* and *Glugea*.

spp. The lower sequence identity to species within *Glugea* also supports that it is separated from *Glugea* spp. In GenBank, the sequence identities within named *Glugea* that have sufficient evidence to place them within this genus have 98.6 to > 99% identities to each other, whereas *F. stethaprioni* n. gen. n. sp. had less than 92.7% identity to *Glugea* spp. Equally compelling are the differences in the developmental cycle that distinguishes it from other species of *Glugea* and other microsporidial genera.

To date, all species of *Glugea* and *Loma* induce formation of xenomas in their hosts, whereas *F. stethaprioni* n. gen. n. sp. apparently does not. Xenomas are transformed host cells that become highly hypertrophied, with enlarged and lobulated nuclei, supporting thousands of developing microsporidia (Lom and Dykova 2005). Little is known of the mechanisms by which microsporidia prolong the life of infected host cells and convert them to these spore-forming factories and how this mechanism evolved. This trait is not confined to these genera though, and xenoma-forming species may occur in diverse microsporidia from fish, oligochaetes, crustaceans, and insects spanning at least 20 microsporidial genera (Lom and Dykova 2005). Nonetheless, it is notable that *F. stethaprioni* n. gen. n. sp. seems to lack this characteristic, while phylogenetically fitting within a clade thus far made up mainly of xenoma-inducing microsporidia. Two microsporidial species appear to group with *F. stethaprioni* n. gen. n. sp., and at least one of these, *Pleistophora penaei*, from the brown shrimp, *P. aztecus*, also is a non-xenoma-forming microsporidium, which instead forms sporocysts containing many spores (Couch 1978). Interestingly, this species has also been shown to target numerous organs including the tail muscle, cardiac muscle, hepatopancreas, and walls of the gastrointestinal tract (Couch 1978). Considering the phylogenetic relationship of *P. penaei* within clade I of the analysis herein, this species does not appear to belong to the genus *Pleistophora* and will thus likely need to be reclassified. The species that had the closest identity to *F. stethaprioni* n. gen. n. sp. was *Microsporidium* sp. (AY140647) from brown trout, though unfortunately the developmental stages of this species had not been characterized; thus, it is unknown if it induces xenomas in its hosts.

The limited replication of *F. stethaprioni* n. gen. n. sp. within host cells is also distinct from the formation of sporophorocysts, as seen in *Heterosporis* (Lom et al. 2000; Joh et al. 2007; Phelps et al. 2015), or development of many spores within pansporoblast envelopes as described for *Pleistophora* (Canning and Nicholas 1980). The number of spores forming within either the host-derived parasitophorous vacuole or the parasite-induced sporophorous vesicle (SPV) is a trait that can distinguish microsporidial species (Cali and Takvorian 2014). *Glugea* spp. are polysporoblastic forming 6–32 sporoblasts within the SPV (Voronin 1976; Canning and Nicholas 1980; Cali and Takvorian 2014), *Loma* has been

reported to have up to 12 spores forming within a SPV (Lovy et al. 2004; Ovcharenko et al. 2017), and the genus *Pleistophora* is known to produce many spores within a single SPV (Canning and Nicholas 1980; Li et al. 2012). This distinguishes these genera from *F. stethaprioni* n. gen. n. sp., which have uninucleate meronts and bi-sporous development within a parasite-derived SPV. This difference in development also leads to the disseminated manifestation of TDM. Instead of focal to multifocal cystic aggregates of spores, as seen in species that either induce xenomas or have sporophorocysts with large numbers of spores within a SPV, *F. stethaprioni* n. gen. n. sp. is diffusely spread throughout most internal organs and gill. Though limited replication occurred within each cell, massive numbers of spores still occurred within a host, though this depended on infecting many host cells in various organs.

Another distinguishing feature in the developmental cycle is the types of appendages and tubules found within the SPVs. Five types of appendages or tubules have been described in the parasitophorous vacuole or SPV of developing microsporidial spores (Weidner 1972; Takvorian and Cali 1983; Moore and Brooks 1992). In SPVs with mature spores, narrow diameter tubules like those of *Loma* (Rodríguez-Tovar et al. 2003; Lovy et al. 2004) and *Glugea* spp. (Takvorian and Cali 1983) were present in *F. stethaprioni* n. gen. n. sp., though other structures were present in newly formed SPVs. The aggregations of membranous elements with striated electron density have not been described previously. Also, the apparent fragmentation of the sporonts, which may in fact be extensions of the sporont cytoplasm in the formation of the tubules, has not been previously reported. As these reduced in size, they became more electron-dense and developed an extensive glycocalyx. Type I tubules, as reported from *Glugea stephani*, are extensions of the sporont membrane which terminate with a bulb-like structure and do not extend far from the sporont surface (Takvorian and Cali 1983), which are characteristics unique from those described herein. Though clearly distinguished from previously described tubules or appendages, it is possible that these structures represent a developmental phase in the formation of either type I or III tubules as previously described by Takvorian and Cali (1983).

Spore ultrastructure is useful for taxonomic classification (Vávra and Larsson 2014), although host-related differences and polymorphisms could exist, making phylogenetic-based classification more accurate (Vossbrinck and Debrunner-Vossbrinck 2005). The spore structure may also provide clues on transmission, since spores are the infectious stage. A noteworthy finding with *F. stethaprioni* n. gen. n. sp. was that spores had relative size uniformity, though in TEM two spore types were present. One spore type had more coils (9–12) in the polar filament and had an exospore comprising two distinct layers, whereas the other spore type had fewer coils in the polar filament (4–7) and had a homogenously

electron-dense exospore. The majority of microsporidial species described with bi-morphic spores have had clearly distinct macrospores and microspores, based on size, which also exhibit differences in the number of coils in the polar filament; examples include some species of *Glugea*, *Ichthyosporidium*, *Pleistophora*, and *Ovipleistophora* (Canning and Nicholas 1980; Maurand et al. 1988; Vagelli et al. 2005; Sanders et al. 2012; Abdel-Baki et al. 2012; Lovy and Friend 2017). Using light microscopy, size differences between spores in *F. stethaprioni* n. gen. n. sp. were not apparent. In microsporidial species, having uniform coils in the polar filament is the most common presentation, and this trait has often been used as a taxonomic character. At least one other species has been noted to have high variations in the coils of the polar filament, i.e., *Pleistophora macrozoarcidis*, though spore sizes were not indicated in this description (El-Garhy et al. 2017). Another difference in spores reported herein concerns the exospore structure, with the two spore types having either homogenous or layered exospores. The majority of microsporidial reports describe a single homogenously electron-dense coat, though spores of *Encephalitozoon hellem* were reported to have two-layered exospores (Bigliardi et al. 1996). Exospore composition, particularly if they are layered or homogenous, has been proposed as an important character for understanding taxonomic relationships in microsporidia (Vávra and Larsson 2014). A possible functional reason for these morphological differences in the spores may relate to the strategy of infection. For example, spores may be auto-infective, in that they infect nearby cells or tissues within the same host, or they may be environmental, and leave one host to infect a second host (Solter and Maddox 1998; Rodriguez-Tovar et al. 2003). Considering that the exospore composition may impact polar filament eversion, and the length of polar filament will impact the trajectory of cell infection, it is possible that one type of spore is intended for autoinfection. This strategy would be important for *F. stethaprioni* n. gen. n. sp., which forms a highly disseminated infection throughout the tissues. Perhaps short polar filaments would be better for infecting nearby cells, whereas longer polar filaments would be more suitable for environmental spores that infect new hosts. Though this is possible, autoinfection was not supported by ultrastructural findings of empty spores and/or everted polar filaments within examined tissues in the current study.

With ornamental fish farms and aquariums often utilizing recirculation systems, the avoidance of microsporidia is vital for maintaining healthy stocks. Therapeutics for controlling microsporidia are limited due to their intracellular life cycle, and some microsporidia are resistant to chlorine disinfectants (Ferguson et al. 2007). Albendazole, fumagillin,

and a fumagillin analog (TNP-470) are drugs known to treat microsporidia (Didier 1997). Both fumagillin, originally used to treat *Nosema* infections in honeybees (Higes et al. 2011), and albendazole have shown promise in reducing infections in fish (Speare et al. 1999); however, these are not effective at eliminating the parasite and should be utilized early in the infection cycle for successful reduction in infection levels. Some evidence also suggests that microsporidia overcome the effects of treatment while in fact exacerbating infections, as reported in honeybees treated with fumagillin for *Nosema ceranae* Fries et al., 1996 (Huang et al. 2013). The limited success of therapeutics and the challenges with disinfection emphasize the importance of avoiding microsporidia in an aquaculture and aquarium settings. Once established, eliminating microsporidia from facilities may require difficult actions, such as culling, extensive disinfection, and sanitizing biological filters in recirculation systems, requiring reestablishment of bacteria for biological filtration. Avoidance is best practiced by maintaining strict biosecurity and carefully evaluating newly imported fish during a quarantine period and periodic sacrificial sampling of broodstock and/or newly obtained stock to screen for infection. For example, development and maintenance of zebrafish that are specific pathogen free (SPF) for the microsporidium *Pseudoloma neurophilia* have relied on robust surveillance, testing, and biosecurity (Kent et al. 2011). The characterization of TDM herein should aid in awareness and recognition of these microsporidial pathogens to prevent the spread and establishment of this microsporidium in aquaculture and aquarium settings.

Taxonomic summary

Phylum Microsporidia Balbiani, 1882

Class Marinosporidia (Vossbrinck and Debrunner-Vossbrinck 2005)

Genus *Fusasporis* n. gen.

Diagnosis: Non-xenoma-forming and disseminated throughout internal organs and gills. Monokaryotic throughout all developmental stages. Uninucleate meronts in direct contact with cytoplasm giving rise to bi-nucleated sporonts. Parasite-derived sporophorous vesicle (SPV) appears during sporogony, most often with two spores forming per SPV.

Type species: *Fusasporis stethaprioni* n. gen. n. sp.

Diagnosis: Non-xenoma-forming and limited replication in host cell, forming two spores per SPV. Spores uniform in size measuring $3.9 \pm 0.33 \mu\text{m}$ (range = 3.2–3.7 μm) long by $2.0 \pm 0.2 \mu\text{m}$ (range = 1.6–2.5) wide (Figs. 1 and 2). Two types of spores present based on TEM: one type with 9–12 coils in polar filament and an exospore made up of two concentric layers and a second type with 4–7 coils in polar filament with

a homogenously electron-dense exospore (Fig. 5). Newly formed SPV lumens containing membrane elements with striated electron density and extensions of membrane-bound sporont cytoplasm with pronounced glycocalyx (Fig. 4).

Type host: Black tetra *Gymnocorymbus ternetzi* (Boulenger 1895).

Other hosts: Cardinal tetra *Paracheirodon axelrodi* (Schultz 1956).

Type locality: Identified from captive fish populations from the USA.

Sites of infection: Disseminated in the coelom, liver, wall of the gastrointestinal tract, spleen, kidney, ovary, and gill.

Etymology: Genus name means broadly spread-out spores with the Latin word “fusa” meaning spread out and “sporis” relating to spores of the microsporidium. Specific epithet relates to the Characidae subfamily Stethapronioninae, which includes both host species.

Type material: Histologic slides of infected *G. ternetzi* were submitted to the National Parasite Collection housed at the Smithsonian Institution, National Museum of Natural History, Department of Invertebrate Zoology, under catalog numbers USNM1638543 and USNM1638544. Material from *P. axelrodi* was deposited under catalog numbers USNM1638545 and USNM1638546. Small subunit rDNA sequence was deposited to GenBank under accession number MW077214.

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Data availability Histology slides were submitted to the National Parasite Collection housed at the Smithsonian Institution, National Museum of Natural History, Department of Invertebrate Zoology, under catalog numbers USNM1638543–USNM1638546. Genetic sequence of the small subunit rDNA was deposited to GenBank under accession number MW077214. Original histology blocks and resin-embedded blocks are maintained at the Tropical Aquaculture Laboratory, University of Florida and the Office of Fish and Wildlife Health and Forensics Laboratory, NJ Division of Fish and Wildlife, respectively.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Not applicable.

Consent for participation and publication All authors have provided consent for participation and publication of this study.

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