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Multilamina teevani gen. et sp. nov., a microsporidian pathogen of the neotropical termite *Uncitermes teevani*

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ABSTRACT

A new genus and species of microsporidia is described from adults of the termite *Uncitermes teevani* (Emerson) (n. comb., formerly *Armitermes teevani*), collected in Ecuador. Masses of elongate, ovoid, uninucleate spores were localized to the coelomic cavity of adult workers and measured 6.29 × 3.33 μm (fresh) and 5.83 × 3.00 μm (fixed). These spores were individually contained within a multi-layered sporophorous vesicle and contained an isofilar polar filament with 24–28 coils. Blast-n analysis revealed that the small subunit ribosomal DNA (ssrDNA) sequence of this new species exhibited 85% identity with that of a *Varimorpha* species from the fire ant, *Solenopsis richteri*, and slightly less (78–85% identity) to a large clade of microsporidian parasites from mosquitoes and microcrustacea. The morphological and sequence data support the conclusion that *Multilamina teevani* gen. et sp. nov. is a novel microsporidium and distinct from any previously described genera or species.

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1. Introduction

Termites (Isoptera) are known from all tropical, subtropical, and some temperate regions of the world (Jones and Eggleton, 2011) with more than 3000 described species (Engel and Krishna, 2004). While most often associated with economic damage, less than 3% of all species are known to cause damage to manmade structures, forestry and agriculture products (Edwards and Mill, 1986; Logan et al., 1990). The use of pathogens for microbial control of economically important termites has been considered a popular subject of research but efforts have been largely ineffective (Chouvenc et al., 2011). Most evaluations have been conducted with generalist pathogens (mainly bacteria, fungi and nematodes) isolated from non-termite hosts as few naturally occurring pathogens have been identified. While it appears microbial control holds limited promise, it is evident that the diversity and role of natural pathogens in termites are poorly known. In this report a new genus and species of microsporidia is described from termites collected in Ecuador based on host, morphology and 16S rDNA sequence data.

2. Materials and methods

2.1. Termite collections

Foraging *Uncitermes teevani* workers and soldiers were collected (RHS) on 1 June 2011 on terra firma in a dense rainforest on the shore of the Tiputini River, Orellana Province, Ecuador (0.67530 S, −76.36864 W, 247 m elevation; accession No. EC1163, University of Florida Termite Collection, Davie, FL).

Taxonomic note: Emerson (1925) described *Armitermes teevani* from Kartobo, Guyana. At that time, most mandibulate nasute species were combined in the genus *Armitermes*, *Cornitermes*, or *Syntermes*. Currently there are eighteen genera of mandibulate nasutes (Constantino, 1998; Cancello and Myles, 2000; Constantino and Carvalho, 2011; Rocha et al., 2012) in the newly erected subfamily Syntermitinae (Engel and Krishna, 2004). Rocha et al. (2012) acknowledged a very close affinity of *U. teevani* with the genus *Rhynchotermes* but still opted to erect *Uncitermes* to prevent increased heterogeneity in *Rhynchotermes*. Although *U. teevani* soldiers and workers have a procoxal keel instead of a spine, the mandible shape and dentition, the nasus length and shape, and the gut structure fall well within the *Rhynchotermes* as a whole (Mathews, 1977; Scheffrahn, 2010). A genetic molecular analysis is needed to confirm the correct generic assignment for *U. teevani*.

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2.2. Microscopy

Adults of *U. teevani* with symptoms of microsporidian infection were received preserved in 85% ethanol (ETOH). Specimens were rehydrated in a descending alcohol series of 50%, 25% and 10% ETOH (2 h at each concentration) into deionized H_2O. The large, porcelain white cysts were dissected from adult workers of *U. teevani* (Fig. 1) and examined using both light and transmission electron microscopy (TEM). Smears prepared from cysts were stained with Giemsa for observation under light microscopy. Fresh preparations were viewed with phase contrast microscopy. Measurements of the longest axis and a broadest width perpendicular to that axis of spores were obtained from both Giemsa and fresh-mounted preparations using SPOT Imaging Software (ver. 4.7). For TEM observations, cysts were placed into 2.5% glutaraldehyde for 2 h, postfixed in 2% osmium tetroxide for 1.5 h, dehydrated in an ethanol series to absolute acetone and embedded in epon-araldite (Becnel, 2012) and thin-sectioned. Sections were post-stained with 2.5% uranyl acetate and lead citrate and viewed and photographed on a Hitachi H-600 electron microscope.

2.3. Amplification and sequencing of 16S rDNA gene

Genomic DNA (gDNA) was isolated from $\sim 6 \times 10^6$ microsporidia spores from a single patently infected *U. teevani* following the methods of Valles et al. (2002). The *U. teevani* individual was homogenized thoroughly using a DNAse/RNAse free pestle and microcentrifuge tube in 150 μL of lysis buffer (50 mM Tris–HCl, pH 8; 4% of sodium dodecyl sulfate; and 5% of 2-mercaptoethanol) and incubated at 100 °C for 15 min. The homogenate was placed on ice for 1 min, after which 200 μL of phenol:chloroform:isoamyl alcohol (25:24:1, Tris saturated, pH 8) was added followed by inverting the tube several times to mix thoroughly. The mixture was centrifuged at room temperature for 5 min at 21,000*g*, and the supernatant was removed into a clean microcentrifuge tube. Ethanol precipitation of nucleic acids was performed with the addition of 2 μg of muscle glycogen (G-Biosciences, St. Louis, MO), and pellets were dried at room temperature and resuspended in 30 μL Ambion Elution Solution (Ambion/Life Technologies, Grand Island, NY).

The microsporidian ssrDNA sequence was amplified from infected *U. teevani* gDNA using universal primers 18f (5′-CACCAGGTTGATTCTGCC-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Baker et al., 1997) on a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) with the following PCR conditions: 94° for 3 min; 35 cycles of 94 °C for 0.5 min, 50 °C for 1 min, and 72 °C for 1.5 min; and a final elongation step at 72 °C for 10 min. Reaction volumes were 20 μL containing 0.1 mM dNTP mix, 0.5 U Taq DNA polymerase (Invitrogen/Life Technologies, Grand Island, NY), 0.1 μM of each primer, 4 mM $MgCl_2$, and 1 μL of either a 1:10, 1:20, or 1:50 dilution of gDNA. Amplicons were visualized by loading a small sample of PCR product (PCRp) on a 1% agarose gel, and the remainder of the PCR reaction was purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and sequenced by Macrogen USA (Rockville, MD). A consensus sequence of the microsporidia ssrDNA (1177 bp) was generated using sequences from 3 different PCR reactions.

3. Results and discussion

3.1. Multilamina gen. nov. Becnel, Scheffrahn, Vossbrinck and Bahder

3.1.1. Definition

Ovoid, uninucleate spores with a long, isofilar polar filament. Each spore is individually contained within a persistent, multi-layered sporophorous vesicle. The wall of the sporophorous vesicle contains irregularly spaced vesicles and/or short tubules. Exospore is multi-layered and ornamented with small spheres or short tubules.

3.2. Multilamina teevani sp. nov. Becnel, Scheffrahn, Vossbrinck and Bahder

3.2.1. Type host

Uncitermes teevani (Isoptera: Termitidae: Syntermitinae).

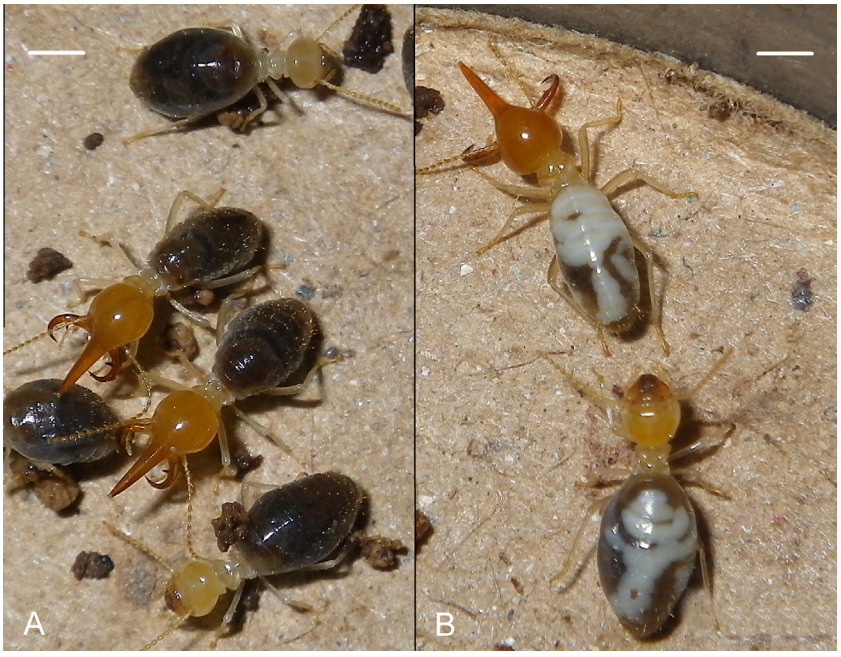

Fig. 1. *Uncitermes teevani* foragers near Tiputini River, Ecuador. Uninfected workers and soldiers (A) and *Multilamina teevani* infected soldier (top) and worker (bottom) demonstrating white encysted areas typical of microsporidian infections (B). Scale bar = 1 mm.

3.2.2. Site of Infection

Masses of spores were localized to the coelomic cavity in adult workers and soldiers (Fig. 1). The originally infected tissues/cells are not known but because soil-feeding termites do not have fat body, we speculate that cells of the midgut or integument are the original sites of infection. Replication and sporulation results in greatly hypertrophied cells that produce the large masses of spores that were observed to fill the coelomic cavity; immature stages of *U. teevani* not examined.

3.2.3. Transmission

Unknown.

3.2.4. Development

Only specimens with advanced, terminal infections were available for examination. All stages observed were uninucleate and in the final phases of sporulation (sporogony plus spore morphogenesis). Sporogenesis began with the concurrent formation of the exospore and polar filament followed by cell elongation.

3.2.5. Spores

One uninucleate spore type was observed that in phase contrast was highly refractive (Fig. 2A). The halo observed around each spore is believed to represent the sporophorous vesicle (SV). In Giemsa-stained smears, a single nucleus was present and what are believed to be remnants of the collapsed SV appeared as a short, thin filament at the ends of the spores (Fig. 2B). Spores measured $6.29 \pm 0.05 \times 3.33 \pm 0.03$ μm (mean ± SE, fresh, $n = 30$) and $5.83 \pm 0.05 \times 3.00 \pm 0.04$ μm (mean ± SE, fixed, $n = 30$). Masses of spores could be observed directly through the cuticle of the adult termite workers (Fig. 1). Spores were ovoid in sagittal section, uninucleate and individually contained within a SV (Figs. 3A and 4A).

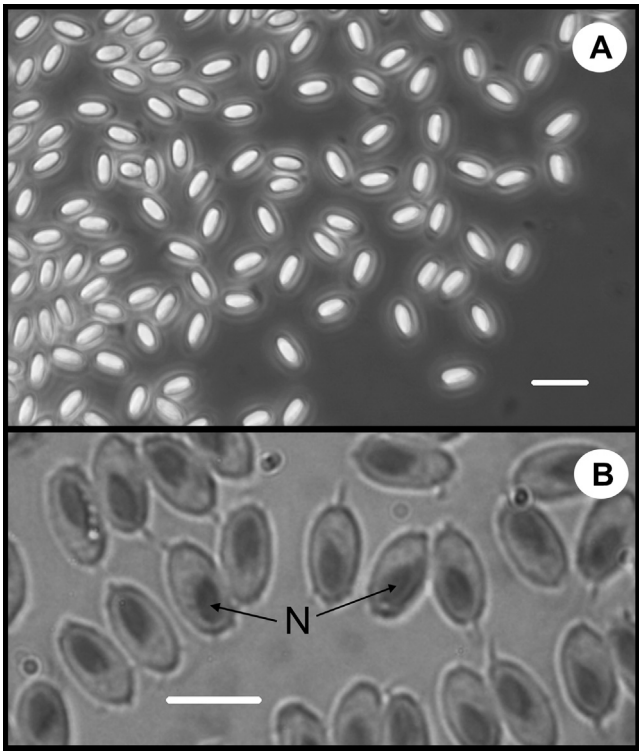

Fig. 2. Light micrographs of spores from adult workers of *Uncitermes teevani* infected with *Multilamina teevani*. (A) Ovoid, elongate spores individually contained within a SV (phase, fresh, Scale bar = 10 μm). The halo observed around each spore is believed to represent the SV. (B) Spores stained with Giemsa demonstrating the single nucleus (N) and what are believed to be remnants of the collapsed SV appearing as short, thin filaments at the ends of the spore. Scale bar = 5 μm.

The polar filament was isofilar and contained between 24 and 28 coils; the coil arrangement was irregular (Fig. 4A). Transverse sections of the polar filament revealed several layers with an electron dense outer layer and an inner core separated by an electron lucent layer (Fig. 4B). The polaroplast was poorly preserved in most examples but appeared to occupy about one fourth of the anterior region of the spore. The spore wall was bound by an inner plasmalemma with a thick electron transparent endospore of approximately 170 nm and a four layered electron-dense exospore of approximately 75 nm (Fig. 4B). The spore wall was ornamented with arrays of small (~30 nm) membrane bound spheres with an electron dense core (Fig. 4A and B).

3.2.6. Interface

Each spore (Fig. 4A) is contained within a persistent, multi-layered sporophorous vesicle (SV). Few pre-sporulation stages were observed but there is an indication that precursors of the SV began to accumulate on the surface of sporonts as electron dense granules (Fig. 3A, inset). Multiple electron dense layers that are formed on the surface of sporoblasts separate and transform into the future SV (Fig. 3B). The mature SV is composed of seven layers that in cross section appears to have intermittent breaks (Fig. 4A and B). Grazing cuts of the SV revealed that the breaks represent double membrane vesicles or short tubules irregularly spaced in the wall of the SV (Fig. 4C, inset).

3.2.7. Type locality

Amazonian Ecuador.

3.2.8. Etymology

Genus name refers to the multiple layers of the sporophorous vesicle. The specific name refers to the host, *Uncitermes teevani* (Emerson, 1925).

3.2.9. Molecular characterization

The ssrDNA nucleotide sequence for *Multilamina teevani* (1177 bp) was submitted to GenBank and assigned accession number KC990122. Blast-n analysis of this sequence revealed significant expectation scores from organisms in the phylum Microsporidia showing it to be most closely related (85% maximum identity) to a "*Vairimorpha*" species isolated from the fire ant, *Solenopsis richteri*. Phylogenetic analysis reveals that these two parasites of ground dwelling insects are members of Clade I (Vossbrinck and Debrunner-Vossbrinck, 2005) which also contains a number of aquatic species from mosquitoes and microcrustaceans. Fig. 5.

The sister clade, Clade II, contains *Ovavesicula popillae* described from the grub of the Japanese beetle *Popillia japonica* (Andreadis and Hanula, 1987; Vossbrinck and Andreadis, 2007). Both *O. papillae* and the fire ant parasite have been shown to directly infect their host, requiring survival of spores in the terrestrial environment (Oi et al., 2005; Petty et al., 2012).

3.2.10. Deposition of type specimens

Two type slides have been deposited with the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, DC (USNM 1208004 and 1208005). Additional slides and specimens embedded in plastic resin are archived at the Center for Medical, Agricultural and Veterinary Entomology, USDA, Gainesville, Florida.

3.2.11. Bionomics

Termites were collected under a decomposed log. Gut contents of *U. teevani* suggest that this species, like most Syntermitinae, are soil feeders. Based on host symptomology, two similar microsporidian infections were observed in workers of the termite *Rotundit-*

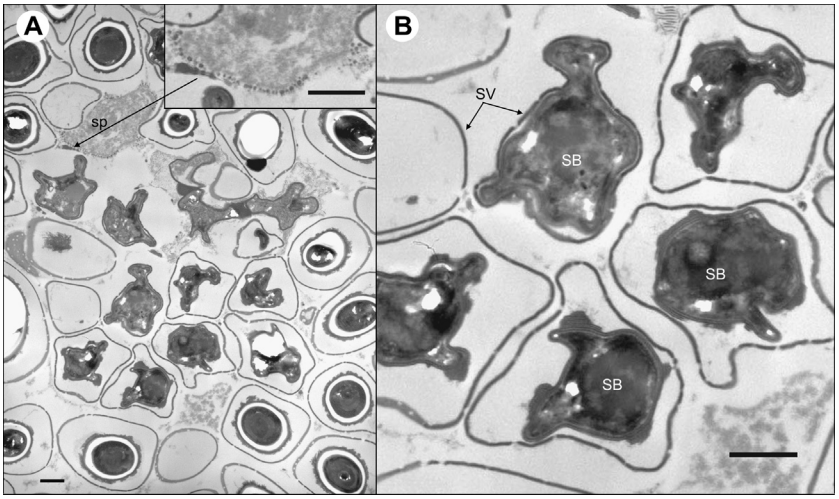

Fig. 3. Electron micrographs of *Multilamina teevani* stages in the final phases of sporulation. (A) Section through the cyst demonstrating a sporont with small granules accumulation on the surface (inset) and sporoblasts and spores each contained within a sporophorous vesicle. (B) Sporoblasts (SB) of *M. teevani*. One early sporoblast is in transition with the future sporophorous vesicle (SV) separating from the surface of the wall. Scale bar = 1 μm.

ermes bragantinus (Termitidae, Nasutitermitinae) and in workers and soldiers of *Cyrillotermes* sp. (Termitidae, Syntermitinae) both about 1 km from the *Uncitermes* site.

3.2.12. Remarks

Microsporidia reported from Isoptera are summarized in Table 1. The first described species of microsporidia from a termite was *Duboscqia legeri* from *R. lucifugus* (Rhinotermitidae) collected in France (Pérez, 1908). *D. legeri* is the type species for the genus and a key feature is the production of SVs containing 16 unikaryotic spores. The next report of a microsporidian infection in a termite was made by Georgévitch (1930) when he mentioned the presence of a *Plistophora* sp. in the Malpighian tubules of *Reticulitermes lucifugus* from Yugoslovia but provided no details. Kudo (1943) described *Microsporidium termitis* from *Reticulitermes flavipes* from the United States which was disporous and produced isolated unikaryotic spores not within a SV. Four additional species were described by Kalavati (1976) from several species of termites in India (Table 1). One species was assigned to the genus *Gurleya* Doflein whose type host is from the Cladocera and characterized by SVs with four spores. Another species was assigned to the genus *Stempellia* Leger and Hesse, 1910 whose type host is from the Emphemeroptera and two to the genus *Pleistophora* Gurley, 1893 whose type host is a fish. Kalavati and Narasimhamurti (1976a) described a new species *Duboscqia coptotermi* from the termite *Coptotermes heimi*. In a subsequent report, *M. termitis* was reported from a new termite host *Macrotermes estherae* (Kalavati and Narasimhamurti, 1976b). There have been no additional reports of microsporidia from termites. None of the species described to date have been examined with modern methods to provide ultrastructural or molecular characters for comparative purposes and nothing is known about life cycle characteristics (transmission mechanisms, host range, etc.). This situation precludes a more detailed analysis of the relationships among the previously described species from termites, but based on morphology and host, this microsporidium from *U. teevani* appears to be a new species.

Vairimorpha invictae from the adult workers of the fire ant *S. invicta*, a closely related species to the *Vairimorpha* sp. from *S. richerti* (Moser et al., 1998) that is the most closely related phylogenetically to *M. teevani*, has been examined in detail (Jouvenaz and Ellis, 1986). This species from soil dwelling fire ants is characterized by two morphologically distinct spore types, a large binucleate spore not contained within a SV and uninucleate spores formed in groups

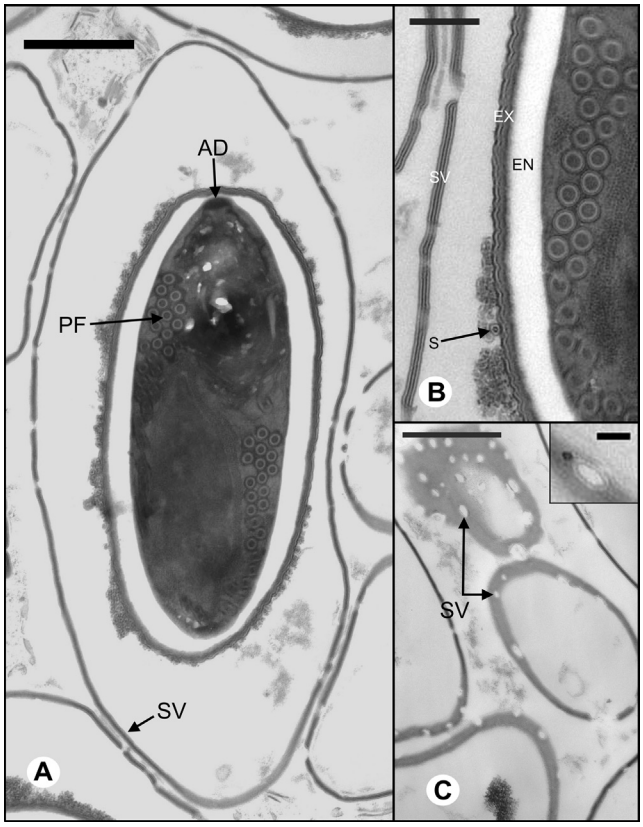

Fig. 4. Electron micrographs of the spore of *Multilamina teevani* from cysts in adult *Uncitermes teevani*. (A) Ultrastructural features of the uninucleate spore, each contained within a sporpophorous vesicle (SV). The spore wall is thinner where the anchoring disc (AD) is attached. The isofilar polar filament (PF) is irregularly arranged in approximately 27 coils around the single nucleus (N). (B) Fine structure of the spore wall composed of a thick (~170 nm) endospore (EN) and a layered exospore (EX) ornamented with arrays of small (~30 nm), membrane bound spheres (S) with an electron dense core. The transversely sectioned polar filament (PF) is composed of several concentrically arranged layers. The sporophorous vesicle (SV) is composed of multiple layers with intermittent breaks. (C) Grazing cuts of the SV demonstrating the small double membrane vesicles/tubules (inset) embedded in the wall. Scale bar = 1 μm for A, C; 0.33 μm for B and 0.1 μm for inset.

of 8 within a persistent SV. *Ovavesicula papillae*, also from a soil dwelling host, has been well characterized with diplokaryotic mer-

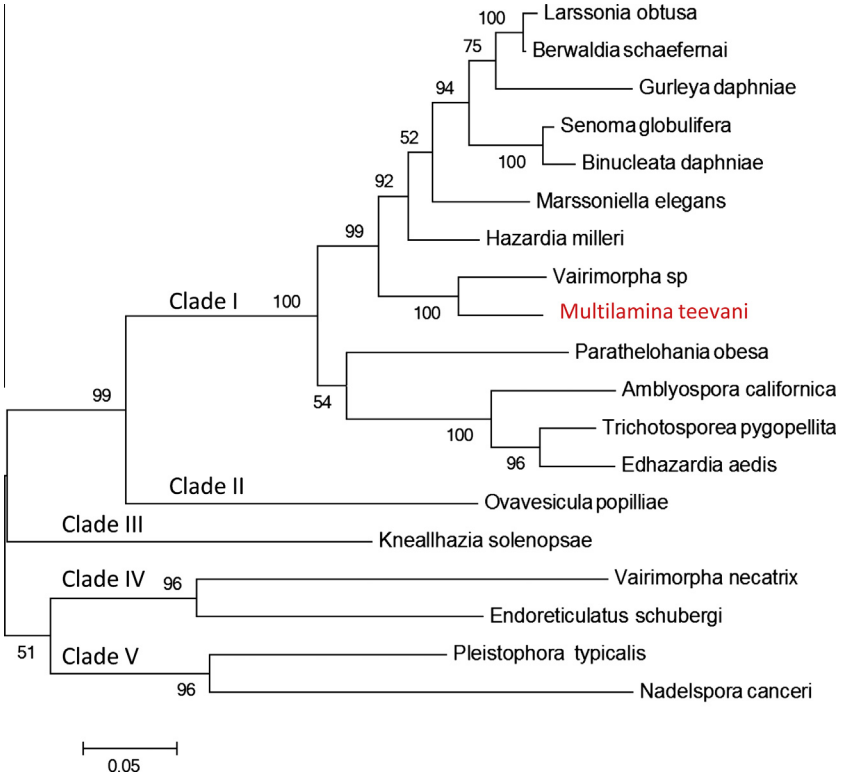

Fig. 5. Maximum likelihood analysis of the ssrDNA nucleotide sequence from 19 species of Microsporidia including *Multilamina teevani* (accession number KC990122) in red. Included are species related to *M. teevani* (Group I) based on BLAST analysis as well as representatives of the other major microsporidian taxa as indicated. Microsporidian species with corresponding Genbank database accession number and the host species are provided: *Kneallhazia* (=*Thelohania*) *solenopsae* (AF134205) host = *Solenopsis invicta*; *Vairimorpha* sp. (AF031539) host = *S. richteri.*; *Hazardia milleri* (AY090067) host = *Culex quinquefasciatus*; *Larssonia obtuse* (AF394527) host = *Daphnia pulex*; *Berwaldia schaefernai* (AY090042) host = *D. galeata*; *Senoma globulifera* (DQ641245) host = *Anopheles messeae*; *Binucleata daphnia* (EU075347) host = *D. magna*; *Gurleya daphnia* (AF439320) host = *D. pulex*; *Trichotosporea pygopellita* (HM594269) host = *Aedes vexans*; *Edhazardia aedis* (AF027684) host = *Aedes aegypti*; *Parathelohania obesa* (AF090065) host = *A. crucians*; *Amblyospora californica* (U68473) host = *Culex tarsalis*; *Pleistophora typicalis* (AF044387) host = *Myoxocephalus scorpius*; *Vairimorpha necatrix* (Y0026) host = *Pseudaletia unipunctata*; *Endoreticulatus schubergi* (L39109) host = *Choristoneura fumiferana*; *Nadelspora canceri* (AY958070) host = *Cancer magister*; *Marssoniella elegans* (AY090041) host = *Cyclops vicinus*; *Ovavesicula popilliae* (EF564602) host = *Popillia japonica*. The statistical significance of branch order is provided at each node from 1000 replications of bootstrap resampling of the original aligned nucleotide sequences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Microsporidia described from Isoptera.

Species	Type hosts	Other hosts	Tissues infected	Spore nuclear arrangement	Spore size	References
Duboscqia legeri Pérez, 1908	*Reticulitermes lucifugus*	Reticulitermes flavipes	Adipose tissue attached to midgut of workers	Unikaryotic	5.0 × 2.0 μm (Perez) and 4.3–5.9 × 2.0–2.8 μm (fixed, Kudo)	Pérez (1908); Kudo (1942)
Duboscqia coptotermi K & N, 1976	*Coptotermes heimi*		Epithelium of worker midgut	Unikaryotic	5.6–6.6 × 2.5–3.5 μm (fresh)	Kalavati and Narasimhamurti (1976a)
Gurleya spraguei Kalavati, 1976	*Macrotermes estherae*		Adipose tissue of workers	Diplokaryotic	4.0–4.5 × 2.0–2.5 μm	Kalavati (1976)
Microsporidium termitis (Kudo, 1943)	*Reticulitermes flavipes*	Macrotermes estherae	Epithelium of worker midgut	Unikaryotic	6.0–8.0 × 4.0–4.7 μm (fresh) and 6–7.5 × 3.6–4.5 μm (fixed, Kudo); 6.0 × 3.6 μm (K & N)	Kudo (1943); Kalavati and Narasimhamurti (1976b)
Multilamina teevani	*Uncitermes teevani*		Unknown, coelomic cavity of adults filled with masses of spores	Unikaryotic	6.29 × 3.33 μm (fresh) and 5.83 × 3.00 μm (fixed)	This study
Pleistophora weiseri Kalavati, 1976	*Coptotermes heimi*		Epithelium of worker foregut	Unikaryotic	5.4–6.0 × 1.8–2.2 μm (fresh)	Kalavati (1976)
Pleistophora ganapatii Kalavati, 1976	*Odontotermes horni*		Epithelium of worker foregut	Unikaryotic	Microspores 8.0–9.0 × 5.0–5.4 μm and macrospores 10.0–12.0 × 6.0–7.0 μm (fresh?)	Kalavati (1976)
Plistophora sp. Georgévitch, 1930	*Reticulitermes lucifugus*		Malpighian tubules	No data	No data	Georgévitch (1930)
Stempellia odontotermi Kalavati, 1976	*Odontotermes* sp.		Epithelium of worker foregut	Usually diplokaryotic, but some unikaryotic	Microspores 4.2 × 3.0 μm and macrospores 12.6 × 4.0 μm (fixed)	Kalavati (1976)

onts and groups of 32 uninucleate spores contained within a persistent SV composed of a thick, two-layered wall (Andreadis and Hanula, 1987). These species have little apparent morphological similarities to *M. teevani* other than the presence of a persistent SV. While each SV is morphologically distinct, its persistent nature and function may be associated with the adaptation to a harsh soil environment where strong selective pressures would favor structures to enhance spore survival.

Most genera of microsporidia that are characterized by spores individually contained within a SV, such as *Tuzetia*, *Alfvenia*, *Berwaldia*, *Janacekia* and *Nelliemba* (Larsson, 1999), are from aquatic crustacean hosts and morphologically and phylogenetically distinct from *M. teevani*. Basic features of the spore (uninucleate and individually contained within a multi-layered SV), tissue infected, molecular phylogeny and host and geographical location precludes this isolate from placement into any previously described genus or species.

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