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NOTE

Morphological and Molecular Characterization of the *Thelohania solenopsae* Complex (Microsporidia: Thelohaniidae)

More than 1000 microsporidian species in 144 genera have been described from a broad range of host organisms but most species are known from insects (Didier *et al.*, 1998; Sprague and Becnel, 1999). Only about 25 species are recorded from Hymenoptera, most of which cause diseases in beneficial Hymenoptera like honey bees (Fries, 1993) and parasitic wasps (Brooks, 1993). Certain microsporidia, however, are pathogenic to Hymenoptera that are considered pests, including sawflies (Darwish *et al.*, 1992; Smirnov, 1974) and fire ants.

Microsporidia of the genera *Burenella*, *Vairimorpha*, and *Thelohania* are found in fire ants. *Burenella dimorpha* infects the tropical fire ant *Solenopsis geminata*, a fire ant species native to the United States (Jouvenaz and Hazard, 1978). *Thelohania solenopsae* and *Vairimorpha invictae* have been described from the red imported fire ant *Solenopsis invicta* (type host) in Brazil (Allen and Buren, 1974; Knell and Allen, 1977; Jouvenaz and Ellis, 1986). Unnamed species of *Thelohania* and *Vairimorpha* have been reported from the black imported fire ant *Solenopsis richteri* in Argentina and Uruguay (Briano *et al.*, 1995; Allen and Silveira-Guido, 1974). These unnamed species cannot be distinguished from *T. solenopsae* and *V. invictae*, respectively, by light- and electron-microscopic observations (Moser *et al.*, 1998).

Recently, a microsporidium has been discovered in *S. invicta* in Florida that is indistinguishable from *T. solenopsae* (Brazil) and *Thelohania* sp. (Argentina) by comparison of stages with the light microscope (Williams *et al.*, 1998). Because these three *Thelohania* isolates are difficult to distinguish, we suggest grouping them into a *T. solenopsae* complex. By definition, "complex" is "a neutral term for a number of related taxonomic units, most commonly involving units in which the taxonomy is difficult or confusing" (Mayr and Ashlock, 1991).

In this publication we present further comparative morphological (spore ultrastructure) and molecular studies (sequence comparisons of the 16S rRNA genes) designed to clarify the relationship of *Thelohania* sp. (United States) to *Thelohania solenopsae* (Brazil) and *Thelohania* sp. (Argentina).

Workers and male and female alates of *S. invicta* infected with *Thelohania* sp. (United States) were

collected near Gainesville, Florida. *S. invicta* infected with *T. solenopsae* (Brazil) and *S. richteri* infected with *Thelohania* sp. (Argentina) have been obtained and processed previously from the area of Cuiaba, Brazil, and the area of Saladillo, Buenos Aires province, Argentina, respectively (Moser, 1995).

Thelohania sp. (United States) spores were prepared for transmission electron microscopy and examined as outlined by Becnel *et al.* (1986). For spore harvest and purification, *S. invicta* (United States) adults were ground in homogenizing buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% SDS) in a Tekmar tissuemizer and filtered through cotton to remove large body parts. The crude spore suspension was further purified by differential centrifugation on a continuous Ludox (DuPont) gradient (Undeen and Alger, 1971). The spores were stored at 4°C in Tris-EDTA (TE), pH 8.0, or distilled water. Microsporidian spores isolated from three colonies (approximately 1×10^7 spores/colony) were broken mechanically in a mini beadbeater, and a crude DNA extract was prepared from the resulting homogenate by the methods of Baker *et al.* (1995). The DNA preparation was used for PCR immediately or stored at -20°C for later PCR analysis.

PCR amplification was optimized for each new DNA template. Based on the optimizations, standard conditions for PCR were as follows: Each 50- μ l reaction contained 1 μ l of DNA (<10 ng), 1.6 μ M of each primer, 0.2 mM of each dNTP (Qiagen), and 0.6 U of *Taq* DNA polymerase (Qiagen). The reactions were overlaid with a drop of mineral oil (Sigma) and carried out in an MJ Research thermocycler using the following temperature profile: An initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C–72°C (one minute each). A final extension step of 72°C for 15 min was done after 35 cycles. PCR products from three reactions were pooled, purified with the QIAquick PCR Purification Kit (QIAGEN) by following the manufacturer's instructions, and stored.

Pooled gene product from three separate PCR reactions for each of the three colonies was sequenced directly. Automated sequencing was done by the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research. The sequences were completed by redundant

sequencing of both strands and compared to those of *T. solenopsae* (Brazil) and *Thelohania* sp. (Argentina). PCR and sequencing primer sequences have been published (Moser *et al.*, 1998). The sequence accession number of *Thelohania* sp. (United States) is AF134205, deposited with Genbank (Los Alamos, NM). Sequence accession numbers of *Thelohania* sp. (Argentina) and *T. solenopsae* (Brazil) are AF031537 and AF031538, respectively. A best fit analysis (Genetics Computer Group, Inc., Madison, WI, Version 9) was chosen for pairwise sequence comparisons. This program creates an optimal alignment between two sequences.

Meiospores of *T. solenopsae* (Brazil), *Thelohania* sp. (Argentina), and *Thelohania* sp. (United States) and free spores of *Thelohania* sp. (Argentina) and *Thelohania* sp. (United States) had similar features and could not be differentiated by ultrastructural comparison (Figs. 1–5). Diplokaryotic free spores were extremely rare (<1%), and it was not possible to obtain a good photo of a free spore of *T. solenopsae* (Brazil).

The meiospores were pyriform in sagittal section with the posterior end more broadly rounded than the

anterior end. They were uninucleate with a lamellar polaroplast anteriorly and vacuolated posteriorly. Polyribosomes bordered the nucleus. The polar filament was isofilar with 10–12 coils with the coil arrangement variable from uniform to irregular. Cross sections of the polar filament revealed several layers with an electron-dense outer layer and an inner core separated by an electron-transparent zone. The spore wall, composed of exospore and endospore, was relatively thin and rugose. The exospore consisted of two to three layers and was about one-fifth the thickness of the electron-transparent endospore.

Free spores of *Thelohania* sp. (United States) and *Thelohania* sp. (Argentina) were ovoid in sagittal section with polyribosomes bordering the nuclei and vacuolated posteriorly. The polar filament, composed of several layers, was isofilar with ~14 coils. The spore wall was relatively thin and smooth, with an unlayered electron-dense exospore and electron-transparent endospore.

The sequences of *Thelohania* sp. (United States) from the three colonies were identical, and the com-

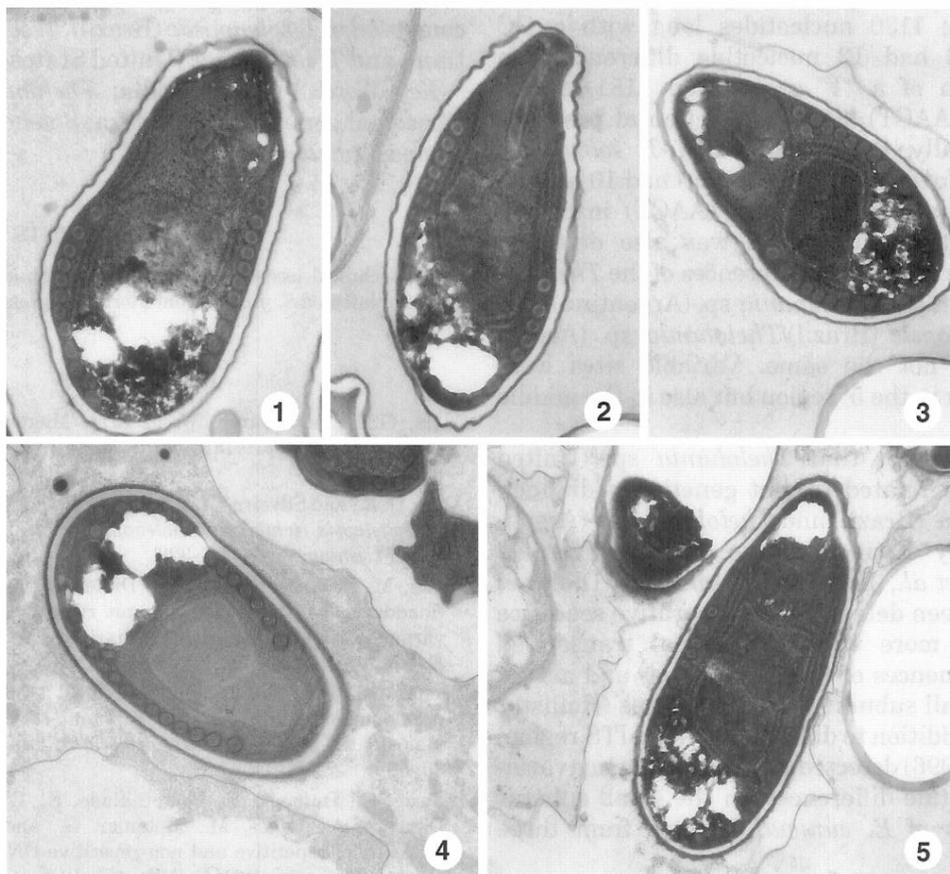


FIG. 1–5. Electron micrographs of meiospores and free spores of the *Thelohania solenopsae* complex isolated from different populations of imported fire ants. **FIG. 1.** Meiospore of *Thelohania* sp. (Argentina), 25,000 \times . **FIG. 2.** Meiospore of *T. solenopsae* (Brazil), 25,000 \times . **FIG. 3.** Meiospore of *Thelohania* sp. (United States), 20,000 \times . **FIG. 4.** Free spore of *Thelohania* sp. (Argentina), 20,000 \times . **FIG. 5.** Free spore of *Thelohania* sp. (United States), 20,000 \times .

TABLE 1
Nucleotide Differences in the 16S rRNA Genes of the Members of the *Thelohania solenopsae* Complex

Organism	Nucleotide differences (position ^a)																					
	40	69	153	164	187	193	194	347	407	409	646	661	748	752	765	1030	1031	1032	1033	1151	1155	1177
<i>T. solenopsae</i> (United States)	A	C	A	C	T	G	A	A	A	A	C	G	T	A	A	C	A	A	C	T	A	A
<i>T. solenopsae</i> (Brazil)	G	T	G	A	T	G	A	G	G	T	C	G	C	T	G	C	A	A	C	.	A	G
<i>T. solenopsae</i> (Argentina)	— ^b	T	G	A	A	C	G	G	A	A	A	A	T	T	G	G	G

^a Sequence of *T. solenopsae* (US) used as reference.

^b Missing data.

^c Gap.

plete 16S rRNA gene was 1371 nucleotides long with a GC content of 41%. The identity of one nucleotide at the 3' end at position 1346 could not be resolved satisfactorily. In comparison, the complete 16S rRNA gene of *T. solenopsae* (Brazil) is 1370 nucleotides long with a GC content of 42%. The best fit analysis revealed an insertion/deletion of a "T" at position 1151. The sequences of *Thelohania* sp. (United States) and *T. solenopsae* (Brazil) differed at 11 nucleotide sites (Table 1), with *Thelohania* sp. (United States) used as the reference. The sequences of *Thelohania* sp. (United States) and *Thelohania* sp. (Argentina) (partial sequence of 16S rRNA gene; 1130 nucleotides long with a GC content of 41%) had 12 nucleotide differences, an insertion/deletion of a "T" at position 1151, and a four-nucleotide (AACT) insertion/deletion at positions 1030–1033. Finally, the sequences of *T. solenopsae* (Brazil) and *Thelohania* sp. (Argentina) had 10 nucleotide differences; a four-nucleotide (AACT) insertion/deletion at positions 1030–1033 was also detected. Interestingly, the nucleotide differences of the *Thelohania* sp. (United States)/*Thelohania* sp. (Argentina) pair and the *T. solenopsis* (Brazil)/*Thelohania* sp. (Argentina) pair were not the same. Variable sites were located primarily in the 5' region but also in the middle and 3' regions.

These data suggest that *Thelohania* sp. (United States) is closely related to, but genetically distinct, from *T. solenopsae* (Brazil) and *Thelohania* sp. (Argentina). Genetically distinct strains of *Enterocytozoon bineusi* (Rinder *et al.*, 1997) and *E. cuniculi* (Didier *et al.*, 1995) have been detected by comparative sequence analysis of the more variable internal transcribed spacer (ITS) sequences of the rRNA genes and also of the complete small subunit rDNA sequences (Hollister *et al.*, 1996). In addition to differences in the ITS region, Hollister *et al.* (1996) detected low-level sequence variation (four nucleotide differences) in the small subunit rDNA sequences of *E. cuniculi* isolates from three different hosts.

Molecular and morphological data have provided important information in the characterization of the members of the *T. solenopsae* complex, yet we feel that the question of conspecificity is still not resolved satis-

factorily. The main reason for this is that to date we have not been able to infect fire ants in the laboratory even with their own isolates. Therefore cross-infectivity studies are not possible at this time. Additional molecular studies such as DNA hybridization studies (Stackebrandt and Goebel, 1994) and molecular karyotyping (Biderre *et al.*, 1997) could also be evaluated to resolve uncertainties in the *T. solenopsae* complex. Until the question of conspecificity is resolved unambiguously, we propose to refrain from collectively naming all the different isolates *T. solenopsae*. Instead, we suggest that the different isolates be referred to as a complex composed of *T. solenopsae* (Brazil), *T. solenopsae* (Argentina), and *T. solenopsae* (United States).

Key Words: Microsporidia; *Thelohania solenopsae*; ribosomal genes; Hymenoptera; *Solenopsis invicta*; *Solenopsis richteri*.

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