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Analysis of the Ribosomal DNA Sequences of the Microsporidia *Thelohania* and *Vairimorpha* of Fire Ants

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Sequences of the 16SrRNA gene of three microsporidia pathogenic to imported fire ants, *Solenopsis invicta* and *Solenopsis richteri*, were determined and compared to each other and 15 other species of microsporidia. The sequences of 2 *Thelohania* species are nearly identical (99.2% identity), supporting light-microscopic and ultrastructural evidence that *Thelohania solenopsae* and *Thelohania* sp. are closely related but probably not conspecific. Sequence comparisons further revealed that *Vairimorpha* sp. has a sequence identity of about 73% with the two *Thelohania* species and *Vairimorpha necatrix*, the type species of the genus *Vairimorpha*. This, together with information on spore morphology, suggests that *Vairimorpha* sp. represents a genus distinct from that of the fire ant *Thelohania*. Its placement in the genus *Vairimorpha* must also be reevaluated. Two new sister taxa, one containing *T. solenopsae* and *Thelohania* sp. and one containing *Vairimorpha* sp., were found to have diverged early in the microsporidian lineage. © 1998 Academic Press

Key Words:* microsporidia; *Thelohania solenopsae; Vairimorpha* sp.; ribosomal gene phylogeny; *Solenopsis invicta; Solenopsis richteri.

INTRODUCTION

The red imported fire ant *Solenopsis invicta* is a major agricultural and urban pest in the southeastern United States despite extensive chemical control efforts (Stimac and Alves, 1994). Alternative control strategies have focused on natural enemies of *S. invicta,* which are extremely rare in the United States (Buren *et al.,* 1978; Buren, 1983) but abundant in South America (Jouvenaz, 1983; Stimac and Alves, 1994). The microsporidium *Thelohania solenopsae* is the first specific pathogen described from *S. invicta* in Brazil (Allen and Buren, 1974). Subsequently, another microsporidium, *Vairimorpha invictae,* was detected in *S. invicta* in Brazil (Jouvenaz and Ellis, 1986). Infections with *T. solenopsae*-like and *V. invictae*-like microsporidia, hereforth called *Thelohania* sp. and *Vairimorpha* sp., also occur in the black imported fire ant *Solenopsis richteri* from Argentina (Briano *et al.,* 1995a) and may appear as dual infections in the same host. It is still debated whether the red and black imported fire ants are color variants of one species or separate species (Vander Meer and Lofgren, 1988). Briano *et al.* (1995b) showed that infection with *Thelohania* sp. substantially reduces *S. richteri* population densities in Argentina, making it a possible biological control candidate for imported fire ants in the United States.

Light microscopic and ultrastructural studies on the developmental sequences and spores could not differentiate *T. solenopsae* from *Thelohania* sp. or *V. invictae* from *Vairimorpha* sp. (Moser, 1995). As discussed elsewhere (Baker *et al.,* 1995), morphological markers may not be sufficient for systematic analysis of microsporidia and should be supported by molecular characterization. Results on molecular studies of the fire ant microsporidia involving restriction fragment length polymorphism (RFLP) analysis and sequence comparisons of the 16S rRNA genes for these species are presented in this investigation.

MATERIALS AND METHODS

Specimen Collection

R. S. Patterson and J. Briano collected *S. richteri* infected with *Thelohania* sp. and *Vairimorpha* sp. in the area of Saladillo, Buenos Aires province, Argentina, and *S. invicta* infected with *T. solenopsae* and *V. invictae* in the area of Cuiaba, Brazil.

The identity of the ants was confirmed by determination of cuticular hydrocarbon components with gas chromatography (GC). Both *S. invicta* and *S. richteri*

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have signature cuticular hydrocarbon profiles (Vander Meer and Lofgren, 1988).

Spore Harvest and Purification

Spore-filled fat body cysts of *Thelohania* sp. from *S. richteri* and *T. solenopsae* from *S. invicta* were dissected from the abdomens of 25–30 adult workers and collected on ice in 0.1% sodium dodecyl sulfate (SDS). The spores were washed twice by centrifugation in deionized water, counted, and stored in deionized water at 4°C until the DNA was extracted.

Spore cysts of *Vairimorpha* sp., very small in comparison to spore cysts of *Thelohania* sp., cannot be collected by dissection of adults. Hence, *S. richteri* 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 resulting 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 until DNA was extracted. Infection levels with *V. invictae* were so low that large numbers of its spores could not be isolated from the ants.

DNA Extraction from Microsporidia

Microsporidian spores (approximately 1×10^7 spores of each species) 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.

Gene Amplification by PCR

The DNA sequences for the forward and reverse primers to amplify the 16S rRNA gene segment of the microsporidian genomic DNA were kindly provided by C. R. Vossbrinck and M. D. Baker (personal communication). The primers were modified to incorporate restriction sites at the 5′ ends to allow subsequent directional cloning of the PCR products (Table 1).

PCR amplification was optimized for each new DNA template. Based on the optimizations, standard conditions for PCR were as follows: Each 50-μl reaction contained 1 μl of DNA (<10 ng), 1.6 μM each primer, 0.2 mM each dNTP (Boehringer Mannheim), and either 1.6 U of Taq DNA polymerase (Boehringer Mannheim) or 0.6 U of Primezyme DNA polymerase (Biometra). The reactions were overlaid with either 100 μl sterile mineral oil or 50 μl Chill-out 14 Liquid Wax (MJ Research) and carried out in an MJ Research thermocycler using the following temperature profile: 94°C for 5 min, then 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min for 35 cycles. 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 at −20°C.

TABLE 1
List of PCR and Sequencing Primers Used

	Nucleotide sequence of primer (5′–3′)
Forward primer	
JM27/18f	TTT GAA TTC CAC CAG GTT GAT TCT GCC
RP6/18f	AAG GTA CAA GGT TGA TTC TGC CTG ACG
RP7/530f	GTG CCA GC(AC) GCC GCG G
RP9/1061f	GGT GGT GCA TGG CCG
RP11f	GGT CGT TGT AAA CTC
RP12f	GGA GTG GAT TAT ACG G
M13f(−20)	GTA AAA CGA CGG CCA GT
Reverse primer	
RP4/1492r	TTT GGA TCC GGT TAC CTT GTT ACG ACT T
RP8/1047r	AAC GGC CAT GCA CCA C
RP10/530r	CCG CGG C(GT)G CTG GCA C
M13r(−24)	AAC AGC TAT GAC CAT G

Cloning of the 16S rRNA Gene

The PCR products were pretreated by digestion with Proteinase K and 0.5% SDS to remove the Taq DNA polymerase bound to the DNA (Crowe *et al.*, 1991).

A 1:3 molar ratio of vector:PCR product DNA was used for ligation (Bethesda Research Laboratories, 1979). The vector was pTZ19r and the bacterial host for transformation was *Escherichia coli* JM109. The heat shock method was used to transform competent cells. (Bethesda Research Laboratories Life Technologies, Inc. transformation protocol).

Dot blots with nick-translated PCR product probe (USB Nick Translation Protocol) were performed on the clear colonies to confirm the positive clones (Sambrook *et al.*, 1989). The alkaline lysis method combined with DNA precipitation by polyethylene glycol (PEG) was used to purify the plasmid DNA carrying the cloned DNA from *E. coli* transformants (Nicoletti and Condorelli, 1993).

Restriction Fragment Length Polymorphisms of the 16S rDNA

Several enzymes were tested: Sau3A, HhaI, and HaeIII (New England Biolabs). Digests were performed according to manufacturer's instructions in 20-μl volumes using ~700 ng of PCR product. Restricted DNA samples were electrophoresed on a 3% Nusieve GTG/1% Seakem LE agarose gel.

Sequencing of the 16S rDNA

Pooled PCR products of *Thelohania* sp. and *Vairimorpha* sp. were sequenced directly. The sequence was

completed by redundant sequencing of both strands. Cloned *T. solenopsae* rDNA was used for sequencing. The consensus sequence was obtained by redundant sequencing of both strands of three clones.

Sequencing was completed with three primers in each direction (Table 1). Sequences for RP7/530f, RP9/1061f, RP8/1047r, and RP10/530r primers were obtained from C. R. Vossbrinck and M. D. Baker (personal communication). Both manual and automated DNA sequencing methods were employed. Manual cycle sequencing was performed by the dideoxynucleotide chain termination sequencing method (Sanger *et al.,* 1977) using the fmol sequencing kit and following the manufacturer's instructions (Promega fmol sequencing kit technical manual).

Automated sequencing was done by the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research. Sequencing was accomplished by employing the Taq DyeDeoxy Terminator (Part No. 401388) Cycle Sequencing protocol developed by Applied Biosystems (a division of Perkin–Elmer Corp., Foster City, CA) using fluorescent-labeled dideoxynucleotides. The labeled extension products were analyzed on an Applied Biosystems Model 373A DNA Sequencer. Sequence Accession Nos. are AF031537, AF031538, and AF031539, deposited with GenBank, Los Alamos, New Mexico.

Sequence Data Analysis

Ribosomal gene sequences of microsporidia from a variety of host organisms including insects (Hymenoptera, Lepidoptera), fish, humans, and the protozooan *Giardia lamblia,* used as outgroup, were obtained from GenBank (*G. lamblia,* Sogin *et al.,* 1989; *Ameson michaelis,* Zhu *et al.,* 1993; *Endoreticulatus schubergi, Ichthyosporidium* sp., *Nosema bombycis, Encephalitozoon hellem,* Baker *et al.,* 1995; *Pleistophora* sp., *Glugea atherinae, N. corneum,* DaSilva *et al.,* unpublished, direct submission, 1994; *Encephalitozoon cuniculi,* Zhu *et al.,* 1993; *Enterocytozoon bieneusi,* Zhu *et al.,* 1993; *Septata intestinalis,* Visvesvara *et al.,* 1995; *N. apis,* Malone *et al.,* 1994; *N. trichoplusiae,* Pieniazek *et al.,* unpublished, direct submission, 1994; *N. vespula,* Ninham, unpublished, direct submission, 1994; *V. necatrix,* Vossbrinck *et al.,* 1987). *Nosema vespula* will be referred to as *Nosema* sp. because so far no data other than the 16S ribosomal gene sequence are published on this microsporidium.

A multiple sequence alignment of those sequences together with the sequences of *Vairimorpha* sp., *Thelohania* sp., and *T. solenopsae* was generated with the programs PileUp and Clustal (Genetics Computer Group, Inc., Madison, Wisconsin) and edited visually. About 1200 bp of the sequences were used in the alignment. Alignment files were analyzed using distance (MEGA: Molecular Evolutionary Genetics Analysis version 1.02, Kumar *et al.,* 1993) and parsimony (PAUP version 3.1.1, Swofford, 1993) methods. Sequences were converted to distances using Jukes–Cantor and Kimura two-parameter models of base substitution. Trees were constructed from the distance data using neighbor-joining and Fitch–Margoliash algorithms. The heuristic option of PAUP was used to find the most parsimonious tree, and a bootstrap analysis (1000 replicates) was performed to place confidence estimates on the groups contained in the optimal tree.

RESULTS

A size difference was observed between the amplified DNA fragments of *Vairimorpha* sp. (~1300 bp) and those of *Thelohania* sp. and *T. solenopsae* (~1400 bp). *Vairimorpha* sp. was also differentiated from *Thelohania* sp. and *T. solenopsae* by a different restriction profile when cut with Sau3A, HhaI, or HaeIII (Fig. 1). The restriction patterns for each enzyme showed differences among *Vairimorpha* sp. and the two *Thelohania* species, but the two *Thelohania* species had identical restriction profiles. As detected by gel electrophoresis, the two *Thelohania* species had two restriction sites each for Sau3A and HhaI and four restriction sites for HaeIII. *Vairimorpha* sp. had one restriction site for Sau3A, three restriction sites for HhaI, and four restriction sites for HaeIII.

Sequences of *T. solenopsae, Thelohania* sp., and *Vairimorpha* sp. were 1382, 1379, and 1289 bp, respectively, with CG contents of 42, 41, and 54%. DNA sequence comparison revealed that the sequences of

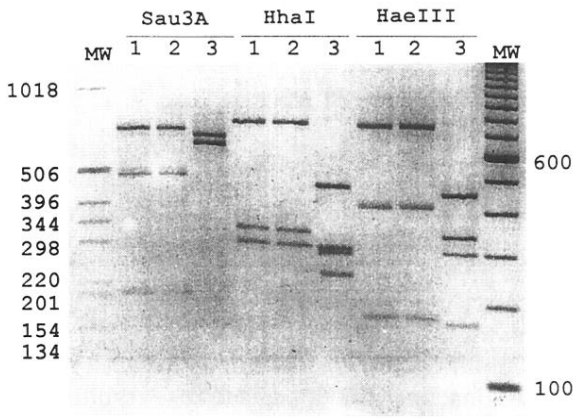

FIG. 1. Restriction profiles of 16S rRNA gene PCR products of three microsporidian species. Photograph of restricted PCR products following gel electrophoresis. About 700 ng of crude PCR product of each species was digested for 2 h at 37°C with *Sau*3A, *Hha*I, or *Hae*III in a 20-μl reaction volume. The samples were electrophoresed with 2 μl of 10× loading dye (50% glycerol, 50 mM EDTA, 0.5% bromphenol blue) on a 3% NuSieve GTG/1% Seakem LE agarose gel in Tris–acetate buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0). Two standards, λ/*Hin*dIII-cut DNA (200 ng) and a 1-kb DNA marker (200 ng), were included as molecular weight markers. Lanes: 1, *Thelohania* sp.; 2, *T. solenopsae;* 3, *Vairimorpha* sp.

TABLE 2

Distance Table Generated the Kimura Two-Parameter Method (Transversions only)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1		0.0037	0.2913	0.2849	0.3063	0.2907	0.2892	0.2720	0.2778	0.2611	0.3093	0.3052	0.3358	0.2417	0.2447	0.2529	0.2633	0.3279	0.3166
2	0.0019		0.2785	0.2873	0.3087	0.3020	0.3000	0.2736	0.2791	0.2742	0.3207	0.3174	0.3726	0.2502	0.2544	0.2593	0.2741	0.3398	0.2873
3	0.0217	0.0224		0.2691	0.3003	0.3256	0.3219	0.2981	0.3061	0.2999	0.2805	0.2728	0.3035	0.2614	0.2605	0.2554	0.2533	0.3397	0.2554
4	0.0204	0.0229	0.0206		0.0543	0.2721	0.2710	0.2686	0.2725	0.2867	0.2739	0.2619	0.2929	0.2429	0.2420	0.2297	0.2274	0.2174	0.3420
5	0.0217	0.0245	0.0226	0.0068		0.2733	0.2722	0.2670	0.2669	0.2811	0.2715	0.2538	0.2949	0.2499	0.2419	0.2351	0.2338	0.2339	0.3523
6	0.0216	0.0252	0.0255	0.0205	0.0204		0.0024	0.1144	0.1112	0.1145	0.1983	0.1925	0.2380	0.2317	0.2307	0.2364	0.2593	0.3166	0.3359
7	0.0215	0.0251	0.0253	0.0204	0.0203	0.0014		0.1112	0.1080	0.1111	0.1969	0.1911	0.2364	0.2314	0.2305	0.2361	0.2590	0.3138	0.3339
8	0.0204	0.0230	0.0235	0.0202	0.0200	0.0109	0.0107		0.0157	0.0277	0.1922	0.1969	0.2422	0.2173	0.2180	0.2235	0.2480	0.3042	0.3677
9	0.0207	0.0234	0.0240	0.0204	0.0199	0.0107	0.0105	0.0036		0.0268	0.1941	0.2007	0.2442	0.2161	0.2167	0.2260	0.2530	0.3080	0.3711
10	0.0198	0.0232	0.0237	0.0215	0.0212	0.0111	0.0109	0.0050	0.0049		0.2032	0.2069	0.2647	0.2194	0.2240	0.2282	0.2597	0.3120	0.3609
11	0.0221	0.0256	0.0217	0.0199	0.0197	0.0159	0.0159	0.0155	0.0156	0.0164		0.0508	0.0903	0.2071	0.2029	0.2036	0.2067	0.3180	0.3470
12	0.0220	0.0255	0.0213	0.0193	0.0187	0.0156	0.0155	0.0159	0.0160	0.0167	0.0066		0.0958	0.1996	0.1968	0.1951	0.1941	0.3143	0.3597
13	0.0239	0.0295	0.0233	0.0212	0.0214	0.0185	0.0184	0.0187	0.0187	0.0202	0.0093	0.0097		0.2313	0.2291	0.2353	0.2374	0.3504	0.3600
14	0.0184	0.0212	0.0209	0.0184	0.0189	0.0181	0.0181	0.0173	0.0171	0.0175	0.0164	0.0159	0.0178		0.0033	0.0414	0.1413	0.3034	0.3323
15	0.0186	0.0215	0.0208	0.0184	0.0183	0.0179	0.0179	0.0171	0.0170	0.0178	0.0160	0.0156	0.0177	0.0016		0.0398	0.1387	0.2947	0.3373
16	0.0191	0.0218	0.0204	0.0176	0.0180	0.0184	0.0183	0.0176	0.0177	0.0180	0.0161	0.0157	0.0180	0.0060	0.0059		0.1526	0.2937	0.3304
17	0.0194	0.0225	0.0201	0.0173	0.0176	0.0194	0.0194	0.0187	0.0189	0.0198	0.0160	0.0153	0.0179	0.0124	0.0122	0.0131		0.2800	0.3288
18	0.0237	0.0275	0.0258	0.0168	0.0177	0.0235	0.0233	0.0227	0.0229	0.0236	0.0231	0.0229	0.0256	0.0227	0.0219	0.0220	0.0208		0.3712
19	0.0220	0.0227	0.0194	0.0237	0.0242	0.0243	0.0241	0.0264	0.0265	0.0263	0.0242	0.0251	0.0254	0.0239	0.0241	0.0238	0.0232	0.0262	

Note. Distances in the upper right matrix; standard errors in lower left matrix. 1. *Thelohania solenopsae;* 2. *Thelohania* sp.; 3. *Vairimorpha* sp.; 4. *Glugae atherinae;* 5. *Ichthysporidium* sp.; 6. *Nosema bombycis;* 7. *Nosema trichoplusiae;* 8. *Vairimorpha necatrix;* 9. *Nosema* sp.; 10. *Nosema apis;* 11. *Encephalitoxoon hellem;* 12. *Septata intestinalis;* 13. *Encephalitozoon cuniculi;* 14. *Pleistophora* sp.; 15. *Endoreticulates schubergi;* 16. *Nosema corneum;* 17. *Enterocytozoon bieneusi;* 18. *Ameson michaelisr; 19. Giardia lambliae.*

T. solenopsae and *Thelohania* sp. were almost identical to each other but had only ~73% sequence identity to *Vairimorpha* sp. Genetic distance values (Table 2; Kimura two-parameter distance, transversions only) and branching patterns of the phylogenetic tree (Fig. 2) suggested that *Thelohania* sp. and *T. solenopsae* were closely related to each other (distance *Thelohania* sp./*T. solenopsae,* 0.0037). They were not closely related to *Vairimorpha* sp. (distance *Thelohania* sp./*Vairimorpha* sp., 0.2785; *T. solenopsae*/*Vairimorpha* sp., 0.2913) or any other microsporidia including the hymenopteran microsporidia *N. apis* and *Nosema* sp. (distance *Thelohania* sp./*N. apis,* 0.2742; *T. solenopsae*/*N. apis,* 0.2611; *Thelohania* sp./*Nosema* sp., 0.2791; *T. solenopsae*/*Nosema* sp., 0.2778). *Vairimorpha* sp. also was not closely related to any of the other microsporidia including *V. necatrix,* the type species of the genus *Vairimorpha* (distance *Vairimorpha* sp./*V. necatrix,* 0.2981).

Based on the branching pattern of the most parsimonious tree (Fig. 2), *Vairimorpha* sp. diverged first from the common ancestor, followed by *G. atherinae, Ichthyosporidium* sp., and *A. michaelis* and the two *Thelohania* species. *Vairimorpha* sp. and the two *Thelohania* species did not group closely with any of the other microsporidia. Only minor branching pattern differences were found when generating trees based on the distance data (data not shown). The most parsimonious 50% majority rule consensus tree obtained from the trees generated by 1000 bootstrap replications is shown in Fig. 3. The relationships among the clades composed of *Thelohania* sp. and *T. solenopsae* and *G. atherinae, Ichthyosporidium* sp., and *A. michaelis* as well as the clades composed of *Pleistophora* sp., *E. schubergi, N.*

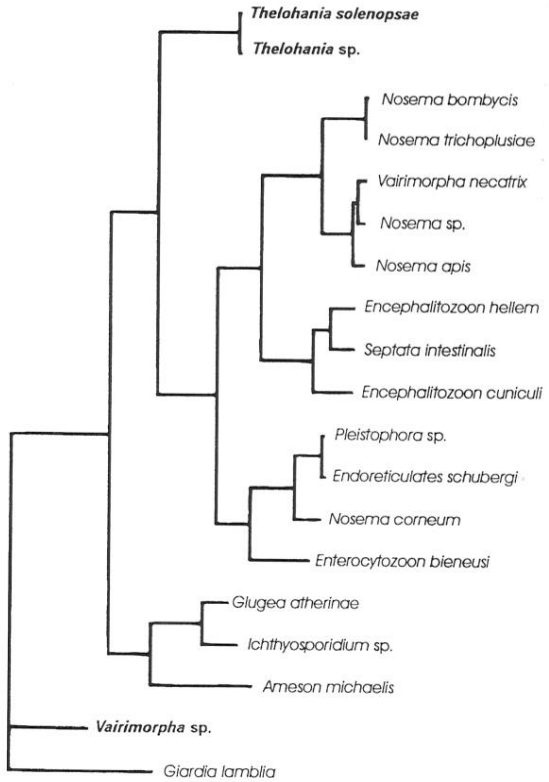

FIG. 2. Most parsimonious tree (3511 steps) based upon the 16S rDNA sequences of 18 species of microsporidia. *Giardia lamblia* was used as the outgroup.

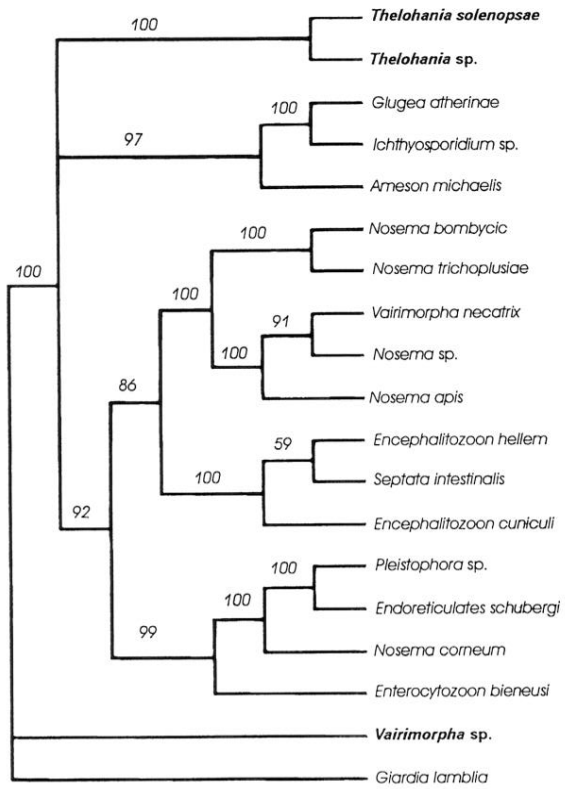

FIG. 3. Majority rule consensus tree obtained in the bootstrap analysis (1000 replicates) of the 16S rRNA sequence data of 18 species of microsporidia. Numbers on the tree indicate the percentage of bootstrap replicates which contained that topology.

corneum, A. penaei and *E. bieneusi* were unresolved in this tree due to a deficiency of informative characters in the data set. A phylogenetic analysis of the other microsporidia to each other has been published (Baker *et al.*, 1995).

DISCUSSION

The RFLP and sequence data analyses support the hypothesis that *Thelohania* sp. and *T. solenopsae* are closely related but probably not conspecific. As detected by agarose gel electrophoresis, *Thelohania* sp. and *T. solenopsae* had identical restriction profiles, and sequence comparison showed 99.2% identity (or a distance of 0.0037) between the two microsporidia.

The interpretation of sequence data from closely related species can be difficult (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994), and it is suggested that more discriminating analysis requires sequence information from more than one gene. Edlin *et al.* (1996), for example, found unexpected differences between β-tubulin gene and 16S rRNA trees in the phylogenetic analysis from amitochondrial protozoa.

To arrive at a biologically meaningful decision as to whether *T. solenopsae* and *Thelohania* sp. are separate species, crucial data on the life cycles and host specificities of these microsporidia are still needed. For example, can *Thelohania* sp. infect *S. invicta* and *T. solenopsae* infect *S. richteri* (cross-infectivity) and can the infection cycle be completed successfully? This cannot be determined, however, because of the inability to horizontally transmit *T. solenopsae* and *Thelohania* sp. to their natural fire ant hosts (Jouvenaz, 1983).

Knell and Allen (1977) placed *T. solenopsae* in the genus *Thelohania* because of the octosporoblastic sporogony and the formation of eight spores within a sporophorous vesicle. However, very little information is available on the type species for *Thelohania, T. giardia* from the marine decapod *Crangon vulgaris.* Therefore, until additional comparative morphological and molecular data are available, it is impossible to determine the relationship between the fire ant *Thelohania* and the genus *Thelohania.*

As noted by Jouvenaz and Ellis (1986), the classification of *V. invictae* may have to be revised, and our data support their suggestion. The tree branching pattern (Fig. 2) and distance of 0.2981 between *Vairimorpha* sp. and *V. necatrix,* the type species of the genus *Vairimorpha,* indicate that *Vairimorpha* sp. may not belong in the genus *Vairimorpha* as currently defined. Other data such as ultrastructure of the spores also support this hypothesis. Both spore types of *Vairimorpha* sp. are ultrastructurally distinct from *V. necatrix.* For example, free spores of *V. necatrix* (Mitchell and Cali, 1993) and *Vairimorpha* sp. differ in the arrangement of the polar filament and polaroplast structure.

The phylogenetic tree (Fig. 2) in this study is similar to the tree published by Baker *et al.* (1995) except that in our analysis we did not include *V. oncoperae* and Baker's *Vairimorpha* sp. Baker's *Vairimorpha* isolate was obtained from the gypsy moth *Lymantria dispar,* while ours was found in the black imported fire ant *S. richteri.* In addition to the species whose sequences were obtained by the authors, we also included *G. atherinae* in the analysis. Baker *et al.* (1995) found four groups: The *Ichthyosporidium* group (composed of *A. michaelis, Vavraia oncoperae,* and *Ichthyosporidium* sp.), the *Encephalitozoon* group (composed of E. *hellem, E. cuniculi,* and *S. intestinalis*), the *Vairimorpha/Nosema* group (composed of *N. apis, Nosema* sp., *N. trichoplusiae, N. bombycis, V. necatrix,* and *Vairimorpha* sp.), and the *Endoreticulates* group (*E. schubergi, E. bieneusi, N. corneum,* and *Pleistophora* sp.). We obtained the same groups in our phylogenetic analysis. Although parsimony and distance methods operate under different assumptions, the trees generated by these methods had only minor branching differences. *Glugea atherinae* was placed in the *Ichthyosporidium* group (bootstrap value of 100%). In addition, we found two new taxon groups, one composed of the two *Thelohania* isolates (bootstrap value of 100%) and one composed of the *Vairimorpha* sp. Additional species need to

be analyzed, however, to support the validity of these two groups.

In conclusion, the sequence analyses data in conjuction with other information such as ultrastructure and tissue specificity of the fire ant microsporidia support the hypotheses that (1) *Thelohania* from fire ants is distinct from *Vairimorpha* sp. from *S. richteri,* (2) *Thelohania* sp. from *S. richteri* and *T. solenopsae* are closely related but probably not conspecific, yet resolution of this issue requires additional biological data such as cross-infectivity testing as well as molecular analysis of other genomic components, and (3) *Vairimorpha* sp. from *S. richteri* should be reclassified.

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