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

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Sequences of the 16S rRNA 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 75% 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.

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, hereafter 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 *The holania* 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 *The holania* 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 tissue homogenizer 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 (Uden 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 × 10⁷ 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).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’–3’</th>
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<tbody>
<tr>
<td>Forward primer</td>
<td>TTT GGA TCC CAT GAG GTT TCT GCC</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>TTT GGA TCC GGT TAT CTT GTT ACG ACT T</td>
</tr>
</tbody>
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PCR amplification was optimized for each new DNA template. Based on the optimization, 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.

**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-translation 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 *The holania* 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 protozoan *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*, Eucephalitozoon hellem, Baker et al., 1995; Pleistophora sp., Glugea atherinae, N. conrneum, DaSilva et al., unpublished, direct submission, 1994; Eucephalitozoon cuniculi, Zhu et al., 1993; *Enterocytozoon bieneusi*, Zhu et al., 1993; *Septata intestinalis*, Visvesvara et al., 1995; *N. apis*, Malone et al., 1994; *N. trichopliusiae*, Pieniazek et al., unpublished, direct submission, 1994; *N. vesupula*, Ninham, unpublished, direct submission, 1994; *V. necatrix*, Vossbrinck et al., 1987). *Nosema vesupula* 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., *Theholania* 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. The sequences were then analyzed using distance (MEGA: Molecular Evolutionary Genetics Analy-

**RESULTS**

A size difference was observed between the amplified DNA fragments of *Vairimorpha* sp. (~1300 bp) and those of *Theholania* sp. and *T. solenopsae* (~1400 bp). *Vairimorpha* sp. was also differentiated from *Theholania* 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 *Theholania* species, but the two *Theholania* species had identical restriction profiles. As detected by gel electrophoresis, the two *Theholania* 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*, *Theholania* 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.](image-url)
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 rRNA sequences of 18 species of microsporidia. Giardia lamblia was used as the outgroup.](image-url)
Corneum, A. penaei and E. bienesi 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 Theolohania sp. and T. solenopsae are closely related but probably not conspecific. As detected by agarose gel electrophoresis, Theolohania 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 mitochondrial protozoa.

To arrive at a biologically meaningful decision as to whether T. solenopsae and Theolohania sp. are separate species, crucial data on the life cycles and host specificities of these microsporidia are still needed. For example, can Theolohania 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 Theolohania sp. to their natural fire ant hosts (Jouveza, 1983).

Knelle and Allen (1977) placed T. solenopsae in the genus Theolohania because of the octosporeoblastic sporogony and the formation of eight spores within a sporophorous vesicle. However, very little information is available on the type species for Theolohania, 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 Theolohania and the genus Theolohania.

As noted by Jouveza 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/Noesma group (composed of N. apis, Noesma sp., N. trichoplusiae, N. bombycis, V. necatrix, and Vairimorpha sp.), and the Endoreticulatus group (E. schubergi, E. bienesi, 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. Gluea atherinae was placed in the Ichthyosporidium group (bootstrap value of 100%). In addition, we found two new taxon groups. one composed of the two Theolohania 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 conjunction with other information such as ultrastructure and tissue specificity of the fire ant microsporidia support the hypotheses that (1) *The holonia* from fire ants is distinct from *Vairimorpha* sp. from *S. richteri*, (2) *The holonia* 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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