SIMULTANEOUS DETECTION OF *VAIRIMORPHA INVICTAE* (MICROSPORIDIA: BURENELLIDAE) AND *THELOHANIA SOLENOPSAE* (MICROSPORIDIA: THELOHANIIDAE) IN FIRE ANTS BY PCR

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Microsporidia are obligate intracellular protozoan parasites of eukaryotes (Mathis 2000). Two species of microsporidia, *Thelohania solenopsae* (Knell et al. 1977) and *Vairimorpha invictae* (Jouvenaz and Ellis 1986) have been reported to be effective biological control agents against the fire ant, *Solenopsis invicta* (Williams et al. 1999, Briano and Williams 2002). Unfortunately, because the life cycles of these pathogens remain unknown, diagnosis is principally limited to microscopic examination of ant homogenates for the characteristic spore stage. This limitation has hampered epidemiological studies, the elucidation of potential intermediate hosts, and description of the complete life cycle. While a number of PCR-based methods have been developed for detection of *T. solenopsae* (Snowden et al. 2002, Valles et al. 2002) none are available for *V. invictae*. By exploiting nucleotide sequence differences in the 16S rRNA genes of *T. solenopsae* and *V. invictae*, we provide a PCR-based method capable of detecting infection of fire ants by either pathogen.

V. invictae-infected colonies of *S. invicta* were collected in Argentina (near San Javier, Santa Fe Province) in April 2003. Infections were determined by the observation of *V. invictae* spores in wet mount preparations of macerated adult ants under a phase-contrast microscope (400X, Briano and Williams 2002). In addition, 1 *S. invicta* (Santa Fe Province) and 2 *S. richteri* (Entre Rios Province) colonies with dual infections (*V. invictae* and *T. solenopsae*) were collected in Argentina in April 2003. *S. invicta* were keyed to species (Trager 1991) and verified as "*invicta*-like" by chemotaxonomy (Vander Meer and Lofgren 1990). Genomic DNA was extracted from adult ants as described by Valles et al. (2002).

PCR was carried out with primer pairs specific for the 16S rRNA gene of *T. solenopsae* (p1, 5'CGAAGCATGAAAGCGGAGC and p2, 5'CAGCATGTATATGCACTACTGGAGC) and *V. invictae* (p90, 5'CACGAAGGAGGATAACCACGGT and p93, CGCAATCAGTCTGTGAATCTCTTCA). The microsporidian-specific primers were designed by aligning the *T. solenopsae* (accession number AF 134205) and *V. invictae* 16S rRNA gene sequences with the Vector NTI 7.1 program (Informax, Inc., Bethesda, MD) and choosing unique areas from each species. A published nucleotide sequence for the 16S rRNA gene was available in GenBank for a *Vairimorpha* sp. thought to be *V. invictae* (accession number AF031539). To verify that this sequence corresponded to the *V. invictae* 16S rRNA gene, we amplified a fragment of the gene from *V. invictae* with primers p90 and p93. The 791 bp amplicon was purified by separation on a 1.2% agarose gel, ligated into pGEM-T easy (Promega, Madison, WI), and used to transform Solopack Gold supercompetent *E. coli* DH5α cells (Stratagene, La Jolla, CA). Insert-positive clones were sequenced by the Interdisciplinary Center for Biotechnology Research, University of Florida. Three replicates were sequenced.

PCR was conducted by the hot start method in a PTC 100 thermal cycler (MJ Research, Waltham, MA) under the following optimized temperature regime: 1 cycle at 94°C for 2 min, then 35 cycles at 94° C for 15 sec, 55°C for 15 sec, and 68°C for 45 sec, followed by a final elongation step of 5 min at 68°C. The reaction was conducted in a 50-µl volume containing 2 mM $MgCl_2$, 200 µM dNTP mix, 1 unit of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.4 µM of each primer, and 0.5 µl of the genomic DNA preparation (10 to 100 ng). PCR products were separated on a 1.2% agarose gel and visualized by ethidium bromide staining. For all experiments, positive and negative controls were run alongside treatments.

The fragment of the 16S rRNA gene that we amplified from *V. invictae* (host *S. invicta*) was identical to the sequence reported previously by Moser et al. (1998). Despite being found in *S. richteri*, they suspected that the microsporidian with which they were working was *V. invictae*. Indeed, Briano et al. (2002) reported that *T. solenopsae* and *V. invictae* could infect either ant species, *S. invicta* or *S. richteri*. This conclusion was confirmed by successful detection of *V. invictae* from *S. invicta* and *S. richteri* with *V. invictae*-specific primers, p90 and p93.

Figure 1 demonstrates the specificity of the primer pairs for each species 16S rRNA gene. As reported by Valles et al. (2002), the *T. solenopsae*-specific primer pair, p1 and p2, produced a 318 bp amplicon exclusively from *T. solenopsae*-infected *S. invicta* (column 2). Similar specificity was observed for the 16S rRNA gene of *V. invictae* with primers p90 and p93; a 791 bp amplicon was pro-

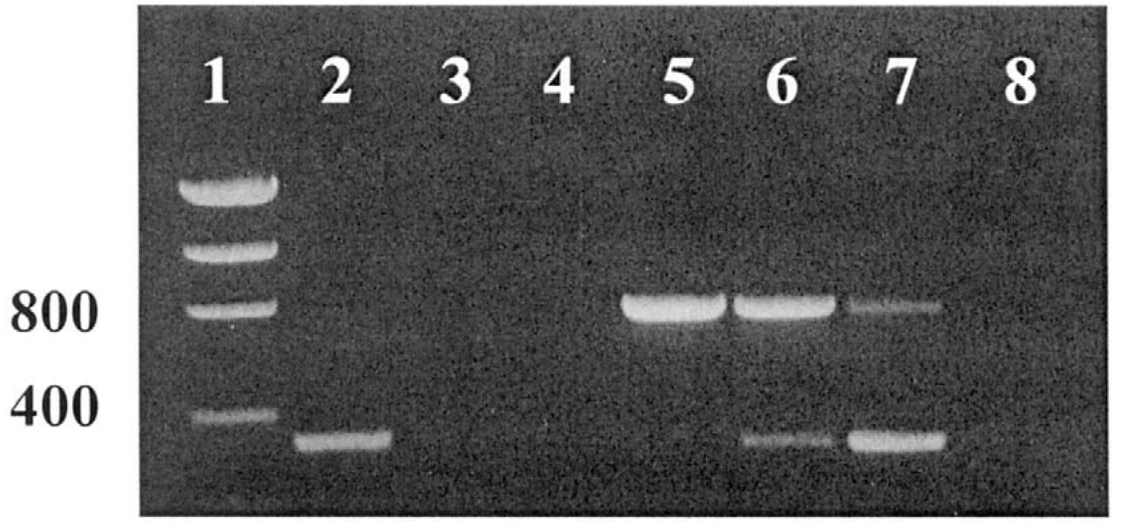

Fig. 1. Banding patterns on a 1.2% agarose gel after multiplex PCR with 16S rRNA-specific oligonucleotide primers. Column 1, molecular weight markers expressed as base pairs (bp);, column 2, DNA prepared from *T. solenopsae*-infected *S. invicta*, oligonucleotide primers p1 and p2 (*T. solenopsae*-specific); column 3, DNA prepared from *T. solenopsae*-infected *S. invicta*, oligonucleotide primers p90 and p93 (*V. invictae*-specific); column 4, DNA prepared from *V. invictae*-infected *S. invicta*, oligonucleotide primers p1 and p2 (*T. solenopsae*-specific); column 5, DNA prepared from *V. invictae*-infected *S. invicta*, oligonucleotide primers p90 and p93 (*V. invictae*-specific); column 6, mixture of DNA prepared from *V. invictae*-infected *S. invicta* and *T. solenopsae*-infected *S. invicta*, oligonucleotide primers p1 and p2 (*T. solenopsae*-specific), and p90 and p93 (*V. invictae*-specific); column 7, DNA prepared from *T. solenopsae-* and *V. invictae*-infected *S. invicta*, oligonucleotide primers p1 and p2 (*T. solenopsae*-specific), and p90 and p93 (*V. invictae*-specific); column 8, DNA prepared from uninfected *S. invicta*, oligonucleotide primers p1 and p2, and p90 and p93.

duced exclusively from *V. invictae*-infected *S. invicta* (column 5). In cases where an ant colony was infected with both organisms, each microsporidian species could be discerned in a single multiplex reaction containing both primers sets (Fig. 1, lanes 6 and 7). Again, *V. invictae* was successfully detected in either *S. invicta* or *S. richteri*.

Microscopic detection of these microsporidia is labor intensive and limited to known stages of development. The multiplex PCR method to detect *T. solenopsae* and *V. invictae* offers a number of advantages over traditional microscopy, including, increased sensitivity, specificity, and the ability to identify all developmental stages. Thus, multiplex PCR decreases the risk of misidentification and will facilitate epizootiological studies concerned with these pathogens.

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Summary

A PCR-based method capable of detecting *Thelohania solenopsae* and/or *Vairimorpha invictae* infection in the red imported fire ant, *Solenopsis invicta*, was developed. Multiplex PCR allows simultaneous detection of both species of microsporidia in a single reaction.

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