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Use of ribosomal DNA sequence data to characterize and detect a neogregarine pathogen of *Solenopsis invicta* (Hymenoptera: Formicidae)

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Abstract

A neogregarine parasite of the red imported fire ant, *Solenopsis invicta*, was discovered recently in Florida and tentatively placed in the *Mattesia* genus based on morphological characterization. *S. invicta* infected with this *Mattesia* species exhibited a characteristic yellowing of the cuticle which was designated *Mattesia* "yellow-head disease" (YHD). The 18S rRNA gene sequence from *Mattesia* YHD was elucidated and compared with the neogregarine pathogens, *Mattesia geminata* and *Ophriocystis elektroscirrha*. The sequence data support the previous conclusion that *Mattesia* YHD is a new species that infects *S. invicta*. Furthermore, high sequence identity between *Mattesia* YHD, *M. geminata* (95.7%), and *O. elektroscirrha* (86.2%) correctly place the YHD organism in the *Mattesia* genus and Neogregarinorida order. Oligonucleotide primer pairs were designed to unique areas of the 18S rRNA genes of *Mattesia* YHD and *S. invicta*. Multiplex PCR resulted in sensitive and specific detection of *Mattesia* YHD infection of *S. invicta*. Published by Elsevier Inc.

Keywords: *Mattesia* sp.; *Solenopsis invicta*; Multiplex PCR; Neogregarinorida; 18S rRNA

1. Introduction

The red imported fire ant, *Solenopsis invicta* Buren, introduced into the southeastern United States in the 1930s, has become a major pest causing billions of dollars of damage annually (Williams et al., 2001). In areas where *S. invicta* is indigenous, the infestation rate is a fraction of that observed in the United States, a fact that has been attributed to the lack of natural enemies in introduced areas (Porter et al., 1992). Thus, sustainable control of *S. invicta* populations across its US range will depend on the use of biological control agents.

In the last several years, a number of parasites and parasitoids of *S. invicta* have been released or discovered in the US (Graham et al., 2003; Pereira et al., 2002; Porter et al., 2003; Williams et al., 1998). One parasite, a neogregarine, was discovered recently in Florida (Pereira et al., 2002) and tentatively placed in the *Mattesia* genus based on morphological characterization. *S. invicta* infected with this *Mattesia* species exhibited a characteristic yellowing of the cuticle which was aptly designated "yellow-head disease" (Pereira et al., 2002).

To further substantiate the designation of the yellow-head disease (YHD) *Mattesia* organism (hereafter, *Mattesia* YHD) as a new species, we have cloned and sequenced the nuclear 18S rRNA genes of *Mattesia* YHD and *Mattesia geminata*, a neogregarine species infecting the North American native fire ant, *Solenopsis geminata*. In addition, to facilitate epidemiologic studies, we developed a PCR technique capable of detecting *Mattesia* YHD in *S. invicta*.

2. Materials and methods

2.1. Amplification and sequencing of 18S rRNA genes

Genomic DNA was isolated as described by Valles et al. (2002). Briefly, 20 ants were added to a 1.5 ml microcentrifuge tube containing 150 μl of lysis buffer

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(50 mM Tris–HCl, pH 8, 4% sodium dodecyl sulfate, and 5% of 2-mercaptoethanol). The insects were homogenized with a disposable plastic pestle for 15 s and the mixture was incubated at 100 °C for 15 min. The mixture was allowed to cool on ice for 1 min, followed by the addition of 200 μl of phenol:chloroform:isoamyl alcohol (Tris–HCl saturated, pH 8). The mixture was inverted five times and centrifuged at room temperature for 5 min at 16,000*g*. The supernatant was removed, nucleic acids precipitated with ice-cold isopropanol, and the pellets were washed twice with 70% ethanol. Pellets were dried at 37 °C, then dissolved in 30 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8).

PCRs were conducted in an MJ Research PTC-200 thermal cycler (Waltham, MA) in 50 μl volumes containing 2 mM $MgCl_2$, 200 μM dNTP mix, 1 U of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.4 μM of each primer, and 1 μl of the genomic DNA preparation (50–500 ng). For all experiments, negative controls were run alongside treatments. Amplicons selected for sequencing were purified by separation on a 1.2% agarose gel, ligated into pGEM-T easy (Promega, Madison, WI), and used to transform Solopack Gold supercompetent *Escherichia coli* DH5α cells (Statagene, La Jolla, CA). Insert-positive clones were sequenced by the Interdisciplinary Center for Biotechnology Research, University of Florida.

Oligonucleotide primers were designed from conserved areas of known 18S and 16S rRNA sequences of species in the phyla Apicomplexa and Microspora retrieved from GenBank, respectively. After a number of empirical evaluations for amplification with a number of primers, PCR was successfully conducted with primers p10 and p37 (Table 1) using genomic DNA prepared from *Mattesia* YHD-infected and -uninfected *S. invicta* workers under the following conditions: 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 53 °C for 15 s, and 68 °C for 1 min, followed by an elongation step of 5 min at 68 °C. Two amplicons approximately 900 base pairs (bp) in size were generated from genomic DNA prepared from *Mattesia* YHD-infected *S. invicta* and -uninfected *S. invicta*. The overlapping 900 bp amplicons from *Mattesia* YHD-infected *S. invicta* and the single 900 bp amplicon from -uninfected *S. invicta* were digested in separate reactions with *Ava*I, *Sau*3AI, and *Ban*I for 20 h at 37 °C. *Ava*I successfully digested only the ant amplicon (that is, only the amplicon generated from ants) but did not digest the amplicon from *Mattesia* YHD DNA. Therefore, the uncut 900 bp amplicon unique to *Mattesia* YHD was cloned and sequenced. This sequence was used to develop *Mattesia* YHD specific 18S rRNA primers for use in inverse PCR.

Table 1
List of oligonucleotide primers used in PCR

Name	Sequence
p10	5′GAAAACGGCCATGCACCAC
p37	5′GGAGARGRAGCCTKAGARAYSG
p41	5′CCTCCAATTGATACTCGCATGGG
p42	5′AGGAATTGACGGAAGGGCACC
p52	5′GATCGTCGCAAGACGGACAGAAG
p53	5′CTAACCATAAACGATGCCAGCTAGC
p71	5′CATGCTGGAGTATTCAGGGCGTAAC
p72	5′TTGGTGGGTGGTTGTGTAGTGTGAC
p73	5′TATCCAGGCGGCTGGACACATAGG
p75	5′GGTTCACCTACAGATACCGTGTTACGAC
p77	5′GGTGGATCCTGCCAGTAGTCATATG
p78	5′GATCCTTCTGCAGGTTCACCTACG
p79	5′AAGTGGCGTATTTTTGACAGCTTC
p80	5′TAGTTTTGCAATTGGAATGAGTTTGA
p81	5′GCCTTTCGGCCAGGGAAC
p86	5′TTCCTTTAAGTTTCAGCTTTGCAACC

Genomic DNA (100 ng) prepared from *Mattesia* YHD-infected *S. invicta* was cut with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, and *Spe*I in separate reactions at 37 °C for 7 h. Restriction enzyme-digested DNA was phenol–chloroform purified and ligated at 16 °C overnight. Ligated DNA served as template for inverse PCR with primers p41 and p42 using the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) under the following conditions: 94 °C for 2 min, 10 cycles at 94 °C for 10 s, 59 °C for 30 s, 68 °C for 5 min, then 25 cycles at 94 °C for 10 s, 59 °C for 30 s, 68 °C for 5 min (increasing 20 s per cycle), and a final elongation step of 68 °C for 5 min. Successful amplifications were cloned and sequenced.

BLAST analysis of the partial sequences of the ~2400 bp amplicon produced from *Hin*dIII-digested DNA by inverse PCR revealed strong identity to 18S rRNA sequences from species in the phylum Apicomplexa (Cavalier-Smith, 1993). Using this sequence, additional oligonucleotide primers (p73, p79, p80, and p75, Table 1) were synthesized that allowed amplification of the entire 18S rRNA gene of *Mattesia* YHD. Primer pairs p73 and p79 and p75 and p80 produced overlapping amplicons approximately 1300 bp in size which were cloned and sequenced in triplicate (Fig. 1).

Mattesia geminata-infected *S. geminata* were collected from areas in Gainesville, FL. Genomic DNA was prepared from pupae exhibiting characteristics of *M. geminata* infection (Jouvenaz and Anthony, 1979).

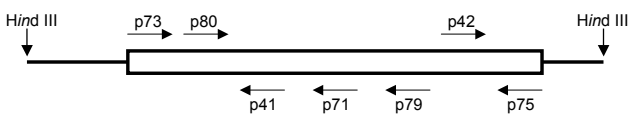

Fig. 1. Diagrammatic representation of the *Mattesia* 18S rRNA gene within the *Hin*dIII restriction digested genomic DNA. Primer pairs p73/p79 and p75/p80 were used to amplify overlapping fragments (~1300 bp) of part of the external transcribed spacer and the complete 18S rRNA gene. Primers p71 and p80 were specific for *Mattesia* YHD and were used to discern infection in *S. invicta*. Primers p41 and p42 were used to conduct inverse PCR.

Oligonucleotide primers designed toward *Mattesia* YHD were used to amplify the *M. geminata* 18S rRNA gene. Primers p75 and p80 and p72 and p79 successfully amplified two overlapping fragments (~1300 and ~1200 bp, respectively) from *M. geminata*-infected *S. geminata* using the thermal cycling protocol described for *Mattesia* YHD 18S. These amplicons were cloned, sequenced, and assembled in triplicate.

The 18S rRNA genes of *S. invicta* and *S. geminata* were amplified from primers developed from the published 18S rRNA sequence of the ant *Leptothorax acervorum* (Accession No. X89492). PCR was conducted with genomic DNA and oligonucleotide primers p77 and p86, and p53 and p78 under the following optimized temperature regime: 1 cycle at 94 °C for 2 min, then 35 cycles at 94 °C for 15 s, 57 °C for 15 s, and 68 °C for 50 s, followed by a final elongation step of 5 min at 68 °C. Amplicons were cloned, sequenced, and assembled in triplicate.

To verify that the 18S rRNA gene sequence was indeed from *Mattesia* YHD, genomic DNA was prepared from oocysts purified by isopycnic centrifugation on a gradient of the colloidal silica, Redigrade (Amersham Biosciences, Piscataway, NJ). Briefly, 50 worker ants exhibiting the characteristic yellow color and confirmed for infection by identification of oocysts by microscopy were homogenized in 250 µl of isotonic KCl (1.15%), applied to a 9:1 solution of Redigrade: NaCl (1.5 M), and centrifuged at 105,000*g*Max for 15 min. Spores were removed, washed with KCl (1.15%), and concentrated by centrifugation. PCR was conducted using this DNA as template and primers specific for 18S rRNA genes of *Mattesia* YHD and *S. invicta*.

2.2. Detection of Mattesia YHD infection in S. invicta by multiplex PCR

Oligonucleotide primers were designed to unique areas of *Mattesia* YHD and *S. invicta* 18S rRNA genes in order to detect infection by multiplex PCR. Primers p71 and p80 were specific for *Mattesia* YHD 18S rRNA and produced a 310 bp amplicon while primers p52 and p81, specific for *S. invicta* 18S rRNA gene, produced a 637 bp amplicon. Experiments were conducted to evaluate the detection limits of the multiplex PCR method. *Mattesia* YHD spores were purified by isopycnic centrifugation on Redigrade colloidal silica. Suspensions of purified *Mattesia* YHD oocysts of known concentrations were added to groups of uninfected worker ants (20) so final preparations had 10–10,000 oocysts (simulating varying infection levels). Genomic DNA was extracted as described above. Each experiment was replicated three times.

We also examined several developmental stages from an infected *S. invicta* colony for the presence of *Mattesia* YHD using PCR. Twenty-five fourth instars, pupae, and adult workers were taken from an infected colony and analyzed by multiplex PCR with primers p52, p71, p80, and p81.

Phylogenetic relationships, based on 18S rRNA gene sequences, were analyzed by the Neighbor-joining method (Saitou and Nei, 1987). Nucleotide sequences were obtained from the GenBank database for other protozoa in the Eugregarinorida, Neogregarinorida, Piroplasmorida, and Eucoccidiorida.

3. Results and discussion

The 18S rRNA nucleotide sequences for *Mattesia* YHD (1985 bp), *M. geminata* (1832 bp), *S. invicta* (1863 bp), and *S. geminata* (1863 bp) obtained during our studies were submitted to GenBank and assigned Accession Nos., AY334569, AY334568, AY334566, and AY334567, respectively. Nucleotide identity comparisons were conducted on the *Mattesia* YHD and a number of species within the Apicomplexa phylum which are believed to be closely related to the Neogregarinorida (Table 2). *Mattesia* YHD exhibited the greatest identity (95.7%) to *M. geminata*. Pereira et al. (2002) placed the yellow head disease-causing organism into the *Mattesia* genus based on morphological and life history characteristics. The sequence data we provide here support the conclusion that *Mattesia* YHD is a new species distinct from *M. geminata*. Moreover, the high sequence identity among *Mattesia* YHD, *Mattesia geminata*, and *Ophriocystis elektroscirrha* correctly place the YHD-causing organism in the Neogregarinorida order and *Mattesia* genus.

Sequence identities between *Mattesia* YHD and species in the Eugregarinorida, Neogregarinorida, Piro-

Table 2
Nucleotide sequence identity among partial 18S rRNA gene sequences from GenBank with *Mattesia* YHD 18S

Order/species	Identity (%)
Neogregarinorida	
Mattesia YHD	100
Mattesia geminata	95.7
Ophriocystis elektroscirrha	86.2
Eugregarinorida	
Gregarinia caledia	76.5
Gregarinia chortiocetes	76.9
Gregarinia niphandrodes	70.6
Gregarinia polymorpha	71.4
Leidyana migrator	64.9
Piroplasmorida	
Babesia canis	79.8
Theileria bicornis	81.8
Theileria sp.	81.9
Eucoccidiorida	
Cryptosporidium felis	79.9
Cryptosporidium parvum	82.4

plasmorida, and Eucoccidiorida ranged from 64.9 to 86.2% (Table 2). A phylogenetic tree (Fig. 2) constructed with the Neighbor-joining method places *Mattesia* YHD closest to *M. geminata* and *O. elektroscirrha* which further confirms correct placement of *Mattesia* YHD in the Neogregarinorida order. As anticipated, species within the Eugregarinorida are closely linked and in the same major grouping as the Neogregarinorida.

The construction of a robust taxonomic relationship for organisms in the phylum Apicomplexa has been hindered by the lack of phylogenetically useful morphological characters, their small size, complex life cycles, and a deficient fossil record (Barta, 1997; Levine, 1988). However, molecular examination of small subunit ribosomal RNA (ssrRNA) gene sequences (5.8S, 18S, and internal transcribed spacers [ITS1 and ITS2]) has proven useful in providing phylogenetic affinities among superspecific taxa (Olsen and Woese, 1993) and even for discerning strain variation (Barta, 1997). Our studies represent the first determination of 18S rRNA gene sequences for the *Mattesia* genus. These data may be important in future phylogenetic studies of species in the Apicomplexa.

The 18S rRNA genes from the two ant species, *S. invicta* and *S. geminata*, were also sequenced to provide assurance that the gene sequences corresponded to each respective organism (host and parasite). *S. invicta* and *S. geminata* 18S rRNA gene sequences exhibited 99.2 and 99.4% identity, respectively, with the 18S rRNA gene from the ant, *Leptothorax acervorum*. Sequence identity was 99.8% between *S. invicta* and *S. geminata*. Conversely, *S. invicta* and *S. geminata* 18S rRNA gene sequences exhibited 71.5 and 71.6% identity, respectively, with the *M. geminata* 18S rRNA gene sequence. Similarly, *S. invicta* and *S. geminata* 18S rRNA gene sequences exhibited 71.9 and 71.5% identity, respectively, with the *Mattesia* YHD 18S rRNA gene sequence. Finally, amplification and subsequent sequencing of a portion of the 18S rRNA gene from purified *Mattesia* YHD oocysts was identical to the sequence previously determined from *Mattesia* YHD-infected *S. invicta*.

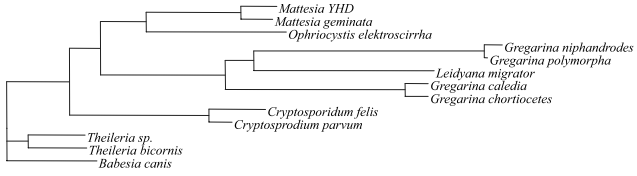

Fig. 2. Unrooted phylogenetic tree inferred from nucleotide sequences of 18S rRNA genes of *M. geminata* (AY334568), *Mattesia* YHD (AY334569), *O. elektroscirrha* (AF129883) (Neogregarinorida); *G. caledia* (L31799), *G. chortiocetes* (L31849), *G. niphandrodes* (AF129882), *G. polymorpha* (AF457129), *Leidyana migrator* (AF457130) (Eugregarinorida); *Cryptosporidium felis* (E493211), *C. parvum* (AY204241) (Eucoccidiorida); *Babesia canis* (AY272047), *Theiloria bicornis* (AF499604), and *Theiloria* sp. (U97048) (Piroplasmorida) using the Neighbor-joining method. GenBank accession numbers are in parentheses.

The species-specific oligonucleotide primers to the *Mattesia* YHD and *S. invicta* 18S rRNA genes (Table 1 and Fig. 1), which we developed for use in multiplex PCR, can be used as aids in epidemiological studies. Oligonucleotide primers p71 and p80, specific for the *Mattesia* YHD 18S rRNA gene, produced a 310 bp amplicon; primers p52 and p81 were specific for the *S. invicta* 18S rRNA gene and produced a 637 bp amplicon (Fig. 3). Using this technique, we evaluated fourth instar larvae, pupae, and adult workers from YHD-infected colonies for the presence of the *Mattesia* YHD 18S rRNA gene. Multiplex PCR indicated clearly that *Mattesia* YHD was present in pupae, and adult workers. Amplification of the *Mattesia* YHD 18S rRNA gene did occur in larvae. However, the band was faint, probably indicating incipient infection on the threshold of the detection limits of the method. Using phase-contrast microscopy, Pereira et al. (2002) were unable to find any signs of the disease in larvae or pupae. Recently, vegetative stages of *Mattesia* YHD have been observed in fire ant pupae from the same colonies used in multiplex PCR studies. The PCR studies were instrumental in selection of colonies with high levels of pupal *Mattesia* YHD infection. Vegetative stages of the neogregarine were subsequently confirmed in these pupae by microscopy.

Our experiments established the level of detection of *Mattesia* YHD in a sample of *S. invicta* worker ants using PCR. When 20 uninfected workers were amended with varying numbers of oocysts, amplification of the 18S rRNA gene occurred consistently to a lower limit of 100 oocysts. A typical infected fire ant worker contains between 2×10^4 and 1.3×10^6 oocysts (Pereira, unpublished

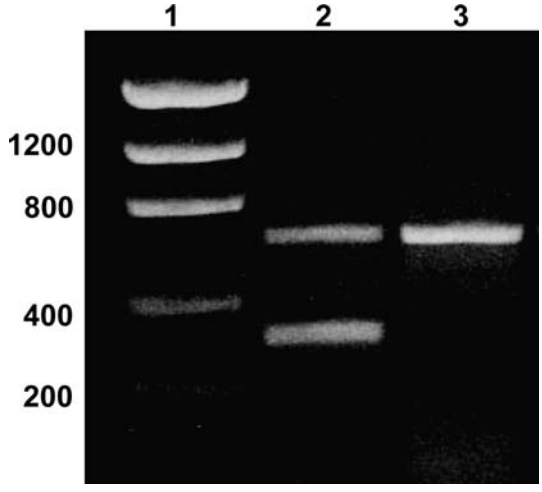

Fig. 3. Banding patterns on a 1.2% agarose gel after multiplex PCR with *Mattesia* YHD 18S-specific primers (p71, p80) and *S. invicta* 18S-specific primers (p52, p81). Lane 1, DNA ladder; lane 2, DNA prepared from *Mattesia* YHD-infected *S. invicta* workers; lane 3, DNA prepared from uninfected *S. invicta* workers.

data) which is 200- to 13,000-fold higher than the multiplex PCR limit of detection. The limit of detection (100 oocysts) was similar to PCR-based detection methods reported previously for microsporidia (Leiro et al., 2002). This detection level allows, with a high probability of success, the detection of the disease in fire ant populations where the YHD prevalence is very low.

The multiplex PCR method to detect *Mattesia* YHD infection in *S. invicta* offers significant advantages over traditional microscopic techniques, including, an increase in the level of detection sensitivity and specificity, and the ability to detect the infection in all developmental stages of the host. The PCR detection method will facilitate epidemiological studies by providing a means of more easily screening potentially infected *S. invicta* colonies as well as intermediate host organisms for the presence of *Mattesia* YHD.

We provide the 18S rRNA gene sequences from *Mattesia* YHD and *M. geminata* and their hosts, *S. invicta* and *S. geminata*. These sequences support the conclusion reached previously (Pereira et al., 2002) of a new Neogregarinorida species infecting *S. invicta*. Using species-specific primers, *Mattesia* YHD was found in larvae and pupae of *S. invicta* for the first time.

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