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Molecular diversity of the microsporidium *Kneallhazia solenopsae* reveals an expanded host range among fire ants in North America

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ABSTRACT

Kneallhazia solenopsae is a pathogenic microsporidium that infects the fire ants *Solenopsis invicta* and *Solenopsis richteri* in South America and the USA. In this study, we analyzed the prevalence and molecular diversity of *K. solenopsae* in fire ants from North and South America. We report the first empirical evidence of *K. solenopsae* infections in the tropical fire ant, *Solenopsis geminata*, and *S. geminata* × *Solenopsis xyloni* hybrids, revealing an expanded host range for this microsporidium. We also analyzed the molecular diversity at the 16S ribosomal RNA gene in *K. solenopsae* from the ant hosts *S. invicta, S. richteri, S. geminata* and *S. geminata* × *S. xyloni* hybrids from North America, Argentina and Brazil. We found 22 16S haplotypes. One of these haplotypes (WD_1) appears to be widely distributed, and is found in *S. invicta* from the USA and *S. geminata* from southern Mexico. Phylogenetic analyses of 16S sequences revealed that *K. solenopsae* haplotypes fall into one of two major clades that are differentiated by 2–3%. In some cases, multiple *K. solenopsae* haplotypes per colony were found, suggesting either an incomplete homogenization among gene copies within the 16S gene cluster or multiple *K. solenopsae* variants simultaneously infecting host colonies.

1. Introduction

The red imported fire ant, *Solenopsis invicta*, is an invasive ant that has plagued the southern United States since its introduction in the 1930s from South America. It has spread throughout the southern USA and more recently has been introduced into California. It is also currently found in Australia, Taiwan, mainland China, northern Mexico, and the Caribbean (Buren, 1982; Buckley, 1999; MacKay and Fagerlund, 1997; Davis et al., 2001; McCubbin and Weiner, 2002; Huang et al., 2004; Chen et al., 2006; Sánchez-Peña et al., 2005; Wetterer and Snelling, 2006). *S. invicta* causes an estimated $6.3 billion in damage annually in urban and agricultural sectors in the USA alone (Lard et al., 2006). In addition, within areas infested by fire ants, nearly 1% of the human population is at risk for anaphylaxis from fire ant stings (Rhoades et al., 1989; Prahlow and Barnard, 1998). Although insecticides are highly effective at controlling *S. invicta* populations, they must be used on a regular basis to maintain areas free of fire ant populations. Thus, discovery and development of biological control agents remains a high research priority to sustainably control *S. invicta*.

Kneallhazia solenopsae was originally described as *Thelohania solenopsae* by Knell et al. (1977) from *S. invicta* collected in Brazil, but has since been reclassified into the genus *Kneallhazia* (Sokolova and Fuxa, 2008). *K. solenopsae* infects all castes and life stages of *S. invicta* and is transmitted both horizontally and vertically (transovarial transmission) (Briano et al., 1996; Valles et al., 2002; Sokolova and Fuxa, 2008). While the mechanisms of transmission are poorly understood, brood raiding (Tschinkel, 2006) seems to be one pathway for horizontal transmission of *K. solenopsae* in fire ants (Oi and Williams, 2003). Numerous studies have demonstrated its impact on *S. invicta* by the debilitation of fire ant queens, resulting in lower weight, reduced fecundity, and premature death (Knell et al., 1977; Williams et al., 1999; Oi and Williams, 2002, 2003). Thus, *K. solenopsae* was identified as a natural enemy with potential for use in the USA against the red imported fire ant.

Few species of microsporidia infect multiple distantly related hosts, and specificity to one host or a related group of hosts is very common (Baker et al., 1995; Vossbrinck et al., 2004; McClymont et al., 2005). *K. solenopsae* spores have been detected microscopically in the black fire ant, *Solenopsis richteri*, in South America

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(Allen and Silveira-Guido, 1974; Briano et al., 1995), in other *Solenopsis* species from Brazil (Allen and Buren, 1974), and in *Solenopsis daguerrei*, a social parasite of fire ants (Briano et al., 1996). *K. solenopsae* spores were not found in other ant genera in South America (Briano et al., 2002), suggesting that the host range of *K. solenopsae* is restricted to the genus *Solenopsis*. An extensive survey for fire ant pathogens was conducted in the mid 1970s in the USA (Jouvenaz et al., 1977). Among 1007 colonies of *S. invicta* examined, a microsporidian infection was present in only one colony. The same microsporidium was found in four of 307 colonies of the tropical fire ant *Solenopsis geminata*, leading the authors to conclude that *S. geminata* was the natural host (Jouvenaz et al., 1977). No further description of this microsporidium was provided in the study (Jouvenaz et al., 1977). A more recent survey in 1996 indicated that *K. solenopsae* was widely distributed in *S. invicta* populations throughout the southern USA (Williams et al., 1998), and subsequent examination of archived *S. invicta* samples from Texas revealed *K. solenopsae* infections in ants dating as far back as 1984 (Snowden and Vinson, 2006). Surveys of other non-*S. invicta*, field-collected ants, including *S. geminata*, *Dorymyrmex bureni*, *Pheidole metallescens*, *Pheidole moerens*, *Camponotus floridanus*, *Trachymyrmex septentrionalis*, and *Brachymyrmex depilis*, were all negative for *K. solenopsae* (Williams et al., 1998).

Recently, in an unpublished report, Snowden and Vinson (2007) described *K. solenopsae* in *S. geminata* from Texas. These authors sequenced a portion of the 16S ribosomal RNA gene of *K. solenopsae* collected from 64 *S. invicta* and 29 *S. geminata* colonies, and found a total of 12 16S sequences that differed by 0–5%. Further, some of the sequences were shared between sympatric *S. invicta* and *S. geminata* colonies. Another molecular study of *K. solenopsae* spores isolated from *S. invicta* from Florida and Brazil, and *S. richteri* from Argentina showed that the *K. solenopsae* variant from Florida was genetically distinct but closely related (~1% base pairs differences) to the microsporidium found in *S. invicta* from Brazil and *S. richteri* from Argentina (Mosser et al., 2000). These results suggest an expanded host range for *K. solenopsae*, as well as high genetic diversity within nominal *K. solenopsae*. The primary objective of our study was to confirm whether *K. solenopsae* infects *S. geminata*. In addition, we conducted comparative phylogenetic analyses of *K. solenopsae* sequences that were isolated from different *Solenopsis* hosts in North America, from *S. invicta* from Brazil and *S. richteri* from Argentina (Mosser et al., 2000), as well as the microsporidium *Kneallhazia* sp. from a thief ant, *Solenopsis carolinensis*.

2. Materials and methods

2.1. Collection of ants

Ants were collected from nests, pitfalls, and baits using aspirators or forceps and immediately preserved in 95% ethanol. A total of 450 samples was analyzed; 117 collections were from *S. invicta*, and 273 samples included *S. geminata* and *S. geminata* × *Solenopsis xyloni* hybrids (Table 1). Collections were obtained in the USA (Florida, South Carolina, Texas), and Mexico (Tamaulipas, Nuevo León and Veracruz) (Table 1, Fig. 1). Taxonomic identification of ants was made by the collectors with confirmation when necessary by one of the authors (David H. Oi) based on Trager (1991). Ants from Tamaulipas and Nuevo León were collected at baits and had insufficient major workers for a definitive identification of *S. geminata*, and were thus specified as *S. cf. geminata*.

2.2. DNA extraction and microsporidia screening

Total genomic DNA was extracted from groups of 10–15 individual worker ants (bulk extractions) using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota). Microsporidia were detected using a polymerase chain reaction (PCR) assay developed by Valles et al. (2004) that amplifies a portion of the 16S ribosomal RNA (16S rRNA) gene of the microsporidian genome. This assay includes specific primers from two microsporidia known to infect fire ants, *K. solenopsae* and *Vairimorpha invictae*. *K. solenopsae* DNA produces a 318-base pairs (bp) PCR amplicon, whereas *V. invictae* DNA produces a 791-bp PCR amplicon. The assay is very sensitive and PCR amplicons are produced even when using bulk DNA extractions where as few as one in 10 individuals are infected. This assay is also more effective in detecting low numbers of spores and pre-sporal stages of *K. solenopsae* infections in fire ants relative to microscopic methods (Milks et al., 2004). The screening assays were conducted employing the primers 1TsS, 2TsAs, Vinp90 and Vinp93 as described in Valles et al. (2004). PCRs were carried out in 25-μL reactions containing 12.5 μL of 2X Taq-Pro COMPLETE (2.0 mM $MgCl_2$; Denville Scientific, Metuchen, New Jersey), 1.6 μM of each primer, 2–4 μL of total genomic DNA (40–200 ng) and water. The thermal cycling profile began with initial denaturation at 94 °C (4 min) and ending with a final extension of 68 °C (5 min). The screening protocol consisted of 35–45 cycles of 94 °C (20 s), 55 °C (30 s), and 68 °C (45 s). All sets of PCR amplifications were performed in parallel with one blank reaction (PCR negative-control: no DNA) and one positive control (DNA from known infected nest). Five microliters of the PCR product were loaded onto an agarose gel and subjected to electrophoresis. The gel was stained with ethidium bromide and amplicons were visualized with UV light.

2.3. PCR amplification and sequencing

PCR amplification to obtain a larger portion of the 16S gene was conducted on *K. solenopsae*-positive samples. Two sets of primers were used. The first set included primers: 18f (5′-CACCAGGTTGA TTCTGCC-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Baker et al., 1995) that are extensively used for microsporidia, and amplify a fragment of about 1200 bp. The second set was specifically designed using *K. solenopsae* sequences and amplifies a region of about 1484 bp. Primers for this PCR were: P933 (5′-TAGTATGTTT TGTAAGGGAGAACATAGACTATGACG-3′) and P935 (5′-ATACGGGA CTATAACCCTGTA TCGTGTCTGT-3′).

Amplification reactions conducted using primers 18f and 1492r included 5.6 μL of 2X Taq-Pro COMPLETE (2.0 mM $MgCl_2$; Denville Scientific, Metuchen, New Jersey), 0.4 μM of each primer, 2–4 μL of total genomic DNA (40–200 ng) and water to a final volume of 15 μL. The thermal cycling profile began with initial denaturation at 94 °C (4 min), followed by 35 cycles of 94 °C (45 s), 58 °C (30 s), and 68 °C (45 s) and ending with a final extension of 68 °C (5 min). PCR amplicons were purified using magnetic beads (Agencourt AMPure, Beverly, Massachusetts) and used in standard fluorescent cycle-sequencing PCR reactions (ABI Prism BigDye™ Terminator chemistry, Applied Biosystems, Foster City, California). Sequencing reactions were purified using Agencourt CleanSEQ (Beverly, Massachusetts) magnetic beads and run on an automatic sequencer at the sequencing core facility (ICBR) at the University of Florida.

For PCRs conducted using primers P933 and P935, thermal cycling conditions were as follows: 1 cycle at 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 59 °C for 15 s, 68 °C for 1.5 min, and a final extension cycle at 68 °C for 5 min. In this case, PCRs were carried out in 25-μL reactions containing Platinum *Taq* (hot start) (Invitrogen, Carlsbad, California), 0.4 μM of each primer, 0.5–1 μL of total genomic DNA (25–50 ng) and water. Gel-purified amplicons were ligated into the pCR4-TOPO vector and transformed into TOP10 competent cells (Invitrogen, Carlsbad, California). Three clones from each sample were sequenced from both directions at the sequencing core facility (ICBR) at the University of Florida.

Table 1
Prevalence of *Kneallhazia solenopsae* infections in fire ant colonies from North America.

Country State	Locality	Host species	No. colonies surveyed	Percentage of colonies infected
USA				
Florida	Ocala	*S. invicta*	67	64
	Gainesville	*S. geminata*	100	0
South Carolina	Aiken	*S. invicta*	92	6.5
Texas	Austin	*S. invicta*	18	0
		S. geminata	5	20
	Riviera	*S. geminata*	4	25
		Hybrid	4	100
Mexico				
Tamaulipas	Tampico	*S. cf. geminata*	4	50
	El Longoreño		1	0
Nuevo León	Yerbaniz	*S. cf. geminata*	5	60
	La Boca		6	17
	Linares		13	8
	Santa Maria		1	0
Veracruz	Catemaco	*S. geminata*	20	20
	Tlacotalpan		10	20
	Tejada		10	20
	Cabada		10	0
	Tuxtla		10	0
	Comoapan		10	0
	Eyipantla		10	0
	Acayucan		10	10
	San Juan Evangelista		10	10
	Rodriguez Clara		10	10
	Ciudad Isla		10	10
	Sayula		10	20

Note: Hybrids occur between *Solenopsis geminata* and *S. xyloni*.

2.4. Sequence editing, alignment and haplotype reconstruction

Sequences were edited using Sequencher v4.8 (Gene Codes Corporation) with base calling confirmed by eye. Consensus sequences were generated using both forward and reverse sequences per direct sequencing reaction and for each clone. In some cases clone sequence variation was observed, suggesting polymorphic sites; thus, we considered these clone-sequences as different haplotypes. For the sequences obtained through direct sequencing, heterozygous sites were found. In some cases we re-sequenced the same sample, and consistently found the same heterozygous sites. From the aligned heterozygous sequences, we obtained pseudohaplotypes using the haplotype reconstruction option implemented in the software DnaSP v5 (Librado and Rozas, 2009). This option uses a coalescent-based Bayesian method to infer the haplotypes defined in the program PHASE 2.1 (Stephens et al., 2001). All sequences were aligned using MUSCLE v4 (Edgar, 2004).

2.5. Phylogenetic analysis

A matrix of pairwise differences using uncorrected *p*-distances (proportion of nucleotide sites at which two sequences being compared are different) among 16S haplotypes was calculated with MEGA version 4 (Tamura et al., 2007). To treat alignment gaps and sites with missing information, we used the pairwise-deletion option, which computes a distance for each pair of sequences, ignoring only those gaps that are involved in the pairwise comparison. The genetic relationships of all 16S sequences were estimated by constructing neighbor-joining (NJ) trees (Saitou and Nei, 1987) using the F84 parameter model. The PHYLIP program package (Felsenstein, 2004) was used to construct the trees, with branch support evaluated by bootstrapping across loci (1000 replicates). Maximum parsimony analysis was performed using the heuristic search option of PAUP* 4.0b10 (Swofford, 2003), with 100 random addition replicates. Bootstrapping was performed using 1000 pseudo-replications of the data set. The appropriate model of sequence evolution for maximum-likelihood (ML) analysis was first determined using the program jModelTest (Posada, 2008). The model of sequence evolution that best fit the observed data, selected by the Akaike information criterion (AIC), was the transversional model with a proportion of invariable sites (*p-inv* = 0.558219), with site rate variation following a discrete gamma distribution ($\alpha = 0.764$) (TVM + I + G). This model was used in PAUP* 4b10 (Swofford, 2003) to compute ML bootstrap percentages after 100 pseudo-replications with 10 random additions of the sequences and tree bisection-reconnection (TBR) branch-swapping. Sequences of closely related microsporidia were aligned with our sequences and included *Anncaliia* (formerly *Brachiola*) *algerae* (Aalg_1, GenBank accession number: AY963290 and Aalg_2, GenBank accession number: AY230191), and *Kneallhazia* sp. from a thief ant, *S. carolinensis* (Kcar, GenBank accession number: GU173849). The genealogical relationships among haplotypes within each fire ant *K. solenopsae* clade were analyzed using the program TCS version 1.13 (Clement et al., 2000) with gaps treated as missing data.

3. Results

3.1. Population screening

Our survey data showed that the prevalence of *K. solenopsae* varied among sampling sites (Table 1, Fig. 1). Forty-three of the 67 *S. invicta* colonies from Ocala (Florida) were infected with *K. solenopsae* and only six of 92 *S. invicta* colonies from Aiken (South Carolina) were infected. In contrast, none of the 18 *S. invicta* colonies surveyed from Austin (Texas) were infected. Among all USA *S. geminata* surveyed, none from Alachua County (Florida) were infected and only a single colony was infected in Austin (Texas) and Riviera (Texas). Among the six sampling sites from northern Mexico, at four sites *S. cf. geminata* samples harbored *K. solenopsae*

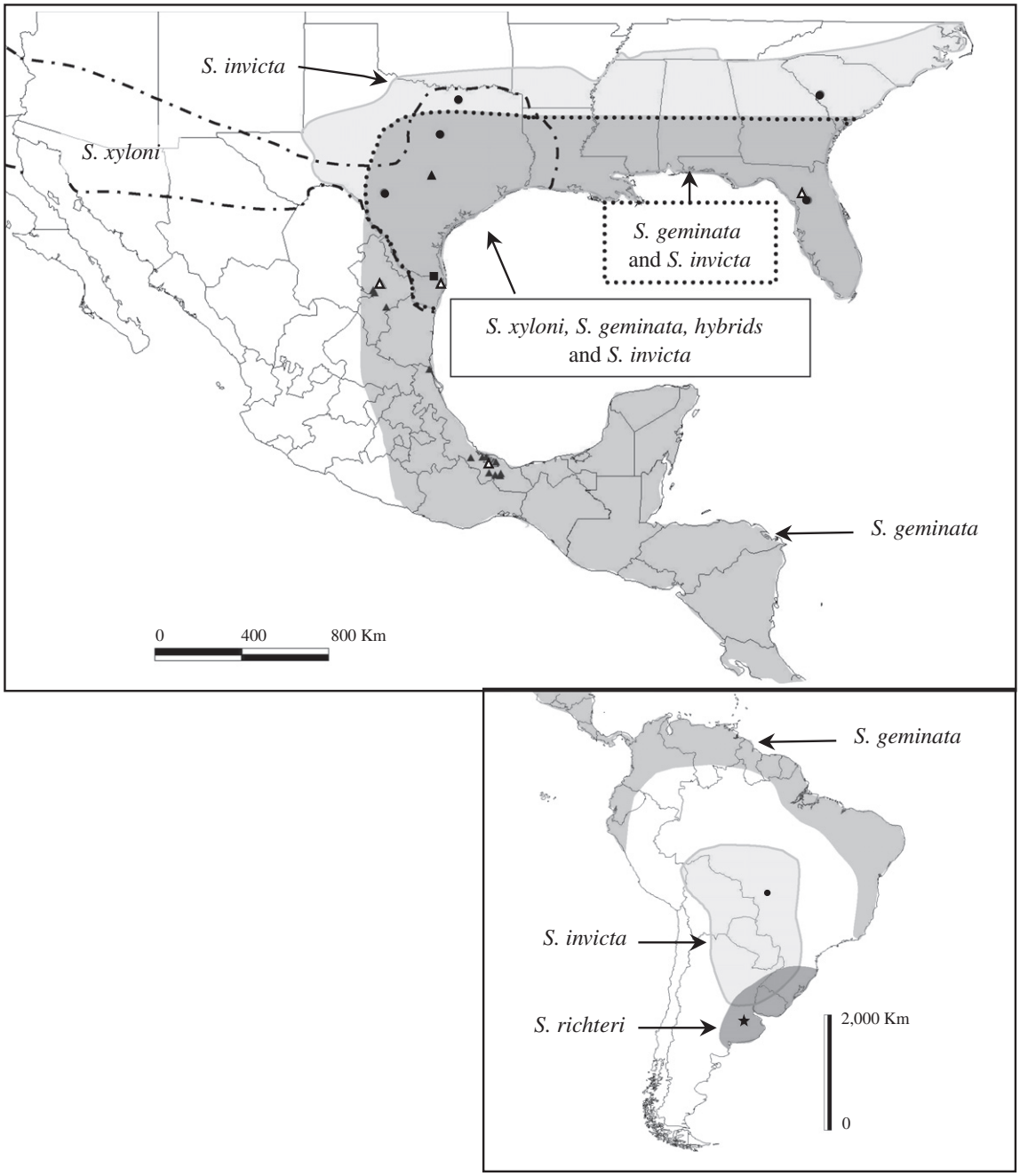

Fig. 1. Collection sites of the *Solenopsis* species analyzed in the current study. The geographic distribution of each species is as follows: top, native distribution of *S. xyloni* (delineated with combined dash and dots line), *S. geminata* (shaded medium gray) and invasive distribution of *S. invicta* (shaded light gray) in North America based on Tschinkel (2006), Helms-Cahan and Vinson (2003) and the red imported fire ant quarantine map from USDA-APHIS-PPQ (2009). Areas of sympatry of each species also are indicated. Bottom, native distribution in South America of *S. geminata* (same color as above, medium gray), *S. invicta* (light gray) and *S. richteri* (dark gray), based on Tschinkel (2006). Filled circles represent sites for *S. invicta* with *K. solenopsae* infection; filled triangles are sites where *S. geminata* ants have the microsporidian infection, whereas empty triangles are *S. geminata* sites without the infection; filled squares show the site where both *S. geminata* and the *S. geminata* × *S. xyloni* hybrids were infected with *K. solenopsae*; and filled star in South America indicates the site from which infected *S. richteri* was collected.

infection. In the state of Veracruz, in southern Mexico, at eight of 12 sites, *S. geminata* colonies were infected. All four *S. geminata* × *S. xyloni* hybrid colonies from Riviera (Texas) yielded positive PCR results for *K. solenopsae.*

3.2. 16S sequence variation and phylogenetic analysis

A total of 22 16S haplotypes of *K. solenopsae* were identified (Table 2). Sequence comparisons for alignment of 877 nucleotides revealed 34 substitutions and two insertion–deletions (indels) of one and four base pairs, respectively (Supplementary Table 1). Phylogenetic trees obtained using maximum likelihood (ML), maximum parsimony (MP) and neighbor-joining (NJ) methods yielded similar topologies with only minor differences in bootstrap support values for some nodes (Fig. 2, Supplementary Fig. 1). All phylogenetic methods supported the presence of two major clades (100% bootstrap support): a North America clade (NA) that included *K. solenopsae* sequences obtained from ants in northern Mexico and Texas, and a Neotropical clade (NeoT) that included *K. solenopsae* sequences obtained from ants in southern Mexico, Florida, South

Carolina, Texas, and South America. Ant species harboring NA-clade *K. solenopsae* included: *S. cf. geminata* in northern Mexico, *S. geminata* × *S. xyloni* hybrids and *S. geminata* in Texas. *K. solenopsae* sequences of the NeoT-clade were observed in *S. geminata* from southern Mexico, *S. invicta* from the USA and Brazil, and *S. richteri* from Argentina. Moreover, one 16S *K. solenopsae* sequence was shared between *S. geminata* from southern Mexico and *S. invicta* from Florida and South Carolina, which was labeled widely distributed (WD_1) (Table 2, Fig. 2).

We constructed haplotype networks using the statistical parsimony method implemented in the program TCS to further understand the relationships among the 16S haplotypes within each major clade. Within the NA clade, haplotypes were connected by 1–11 mutational steps (Fig. 3A). It is interesting to note that haplotype NA_12 (found in *S. geminata* × *S. xyloni* hybrids from Texas) presented a central distribution with respect to the three northern Mexican haplotypes (NA_1, 2, and 3) found at the tips of the network. The high diversity among the USA 16S haplotypes contrasted with the low diversity among the northern Mexico haplotypes. This may suggest that these northern Mexican haplotypes occupies a geographical southern marginal distribution within the NA clade. Within the NeoT-clade, the WD_1 haplotype occupied a central position with respect to the other two southern Mexican haplotypes (Mex_1 and Mex_2) (Fig. 3B). Two of the three USA haplotypes (USA_2 in Florida and 3 in South Carolina) were more closely related to the WD_1 than to the South American haplotypes. Finally, USA_1 (present in Texas and South Carolina) was equally distantly related (three mutational steps) to WD_1 and SA_2.

3.3. Pairwise divergence

We used *p*-distance to estimate the pairwise genetic distances among the 16S sequences (Table 3). We summarized these values by considering the clusters determined by the phylogenetic methods (Table 4). Within the NeoT and the NA clades, *p*-distances ranged from 0.11% to 0.92%, whereas between-clade *p*-distance values ranged from 2% to 3% (Tables 3 and 4).

4. Discussion

We surveyed the fire ants *S. invicta* and *S. geminata*, as well as *S. geminata* × *S. xyloni* hybrids for *K. solenopsae* (or a variant thereof) infections. All species were infected, though the prevalence of infections varied considerably among populations. Although the presence of *K. solenopsae* in *S. geminata* has been reported previously (Snowden and Vinson, 2007), this is the first report of this microsporidium in *S. geminata* × *S. xyloni* hybrids. This is also the first attempt at describing the genetic diversity in this microsporidium among several fire ant hosts and from different geographic sites. Subsequent analyses of these 16S sequence data revealed that (a) numerous sequences of *K. solenopsae* exist that comprise two divergent clades and (b) one sequence is geographically widespread in distribution and is found in both *S. invicta* and *S. geminata*.

4.1. Genetic diversity within and among NeoT and NA clades of *K. solenopsae*

Phylogenetic analyses revealed that all *K. solenopsae* variants fall into one of two major clades (Fig. 2). The significance of this divergence in terms of whether the two major lineages of *K. solenopsae*, or perhaps even lineages within each clade, correspond to different microsporidia species is difficult to interpret. Indeed, assigning species status based solely on sequence data from a single gene is unjustified; however, it is not without precedent. For example, the divergence between lineages we report is above the level of

Table 2
Descriptive information of *Kneallhazia* sp. samples and their hosts. Haplotype composition bases on *Kneallhazia* 16S rRNA gene sequences.

Colony name	Haplotype composition	Host	Geographical origin	Major lineage	GenBank accession number
NW59	WD_1/USA_2	*S. invicta*	Introduced: Florida, USA	NeoT	HM026467 HM026465
NW11	WD_1/USA_2	*S. invicta*	Introduced: Florida, USA	NeoT	HM026467 HM026465
FL_AF	WD_1	*S. invicta*	Introduced: Florida, USA	NeoT	AF134205
57C2	USA_1/USA_3	*S. invicta*	Introduced: South Carolina, USA	NeoT	HM026464 HM026466
10B4	WD_1/USA_3	*S. invicta*	Introduced: South Carolina, USA	NeoT	HM026467 HM026466
TX_AY	USA_1	*S. invicta*	Introduced: Texas, USA	NeoT	AY312502
Mex_s2	WD_1	*S. geminata*	Native: Nayarit, Mexico	NeoT	HM026467
Mex_s7	WD_1	*S. geminata*	Native: Nayarit, Mexico	NeoT	HM026467
Mex_s12	WD_1	*S. geminata*	Native: Nayarit, Mexico	NeoT	HM026467
Mex_s10	Mex_1	*S. geminata*	Native: Nayarit, Mexico	NeoT	HM026468
Mex_s13	Mex_2	*S. geminata*	Native: Nayarit, Mexico	NeoT	HM026469
BA_1	SA_1	*S. richteri*	Native: Buenos Aires, Argentina	NeoT	AF031537
Cuiaba_1	SA_2	*S. invicta*	Native: Mato Grosso, Brazil	NeoT	AF031538
NMex_s9	NA_1/NA_3	*S. cf. geminata*	Native: Nuevo León, Mexico	NA	HM026470 HM026472
NMex_s8	NA_2/NA_3	*S. cf. geminata*	Native: Nuevo León, Mexico	NA	HM026471 HM026472
Gem651	NA_4/NA_5 NA_6	*S. geminata*	Native: Texas, USA	NA	HM026473 HM026474 HM026475
Hyb664	NA_7/NA_8 NA_12	Hybrid	Native: Texas, USA	NA	HM026476 HM026477 HM026481
Hyb661	NA_9/NA_11 NA_13	Hybrid	Native: Texas, USA	NA	HM026478 HM026480 HM026482
Hyb665	NA_10/NA_14	Hybrid	Native: Texas, USA	NA	HM026479 HM026483
S. carolinensis	Kcar	*S. carolinensis*	Native: Florida, USA	–	GU173849

Note: Hybrids occur between *Solenopsis geminata* and *S. xyloni*.

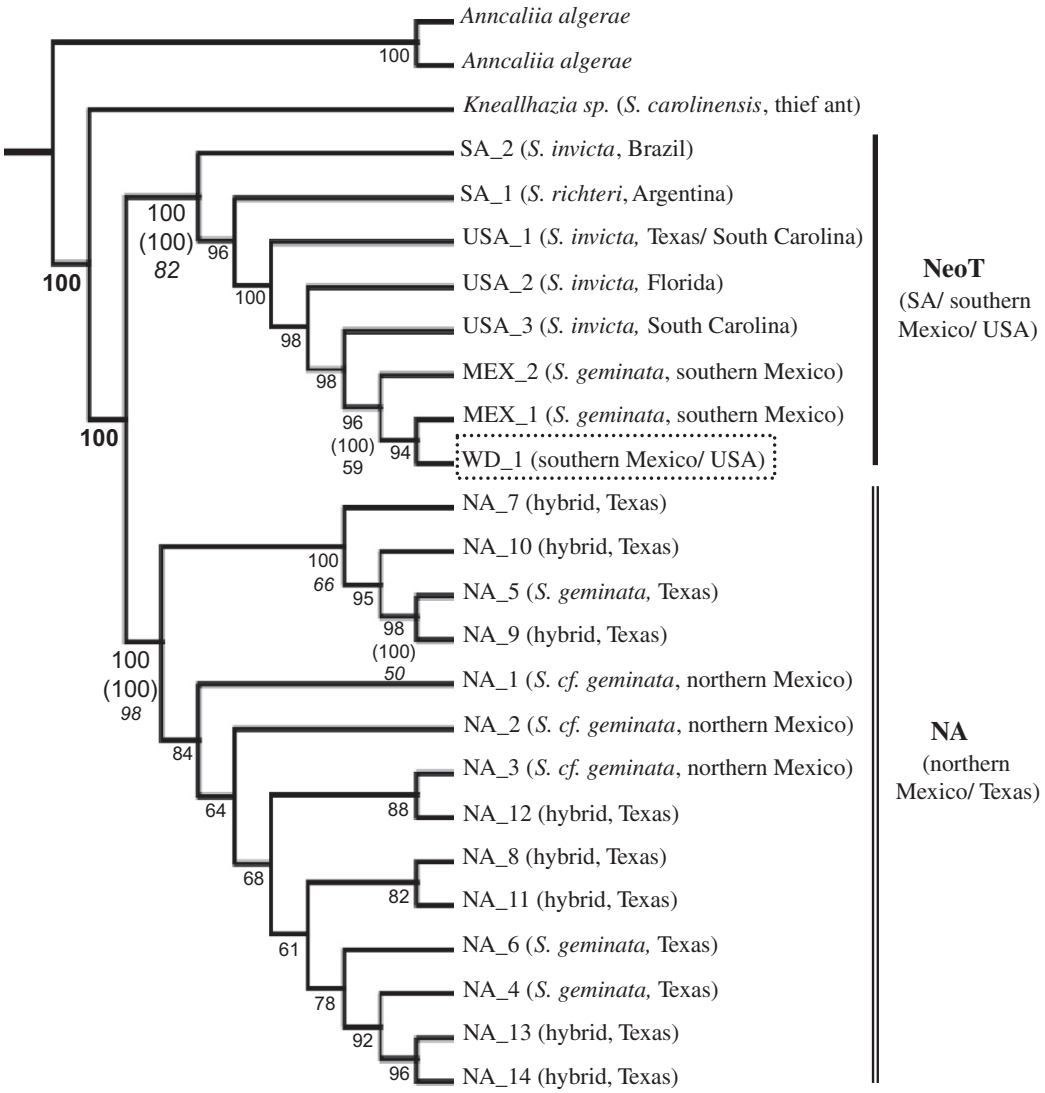

Fig. 2. Neighbor-joining (NJ) tree constructed from 16S rRNA sequence data of microsporidia included in the study. Numbers on branches represent bootstrap support values (only values greater than 50% are shown) for each tree reconstruction method. Bold numbers represent instances where bootstrap support values were the same for all tree phylogenetic methods employed (NJ, MP, ML), whereas numbers in parentheses are those obtained using parsimony and numbers in italics are the values obtained using ML. Trees were rooted using both *Anncaliia algerae* sequences as outgroups. The two major clades, NeoT (Neotropical) and NA (North America), are indicated by vertical bars. SA: South America. The single haplotype (WD_1) found in both *S. invicta* (USA: Texas, Florida, South Carolina) and *S. geminata* (southern Mexico: Veracruz, Mexico) is enclosed by dotted rectangle.

divergence differentiating *Nosema* species infecting lepidopterans (0.1–1.5%; Kyei Poku et al., 2008), but below the level of divergence used to differentiate *Nosema* species infecting bees (6.4% between *Nosema ceranae* and *Nosema apis*; Williams et al., 2008). A previous study by Mosser et al. (2000), which analyzed only three *K. solenopsae* isolates from *S. invicta* in Florida and Brazil, and *S. richteri* from Argentina, questioned the conspecificity of the different *K. solenopsae* isolates and suggested grouping them into a single species complex (*K. solenopsae* complex). Cross-infection assays between species have shown that inoculations with *K. solenopsae* isolates from *S. invicta* from Florida and *S. richteri* collected in Argentina resulted in 56% fewer infections when inoculations were not made to conspecific colonies (Oi, unpublished data). While these reductions in infection could be attributed at least partly to poor cross-fostering of brood inocula (Oi et al., 2010), the data support the possibility of different *K. solenopsae* species, or, at the very least, different variants that exhibit host preferences. Additional studies are needed to compare the biology, structure, and distribution of *K. solenopsae* from each clade to determine whether these are different species.

4.2. The distribution of the WD_1 16S sequence among S. geminata and S. invicta

One 16S sequence (WD_1) was widely distributed geographically and found both in *S. geminata* from southern Mexico and *S. invicta* from the USA (Florida and South Carolina). In addition, as part of an ongoing project, additional *S. invicta*-infected nests from Texas have been analyzed to characterize *Kneallhazia* haplotypes. All three sequences obtained from *S. invicta-infected* nests belonged to the haplotype WD_1 (University of Texas, unpublished data). Locations of *S. invicta-infected* nests in Texas are indicated in Fig. 1. Phylogenetic analyses suggested that this WD_1 sequence was more closely related to other *K. solenopsae* 16S sequences found in *S. invicta* in the USA than to the 16S sequence in *S. invicta* from South America. We also found a second sequence present in

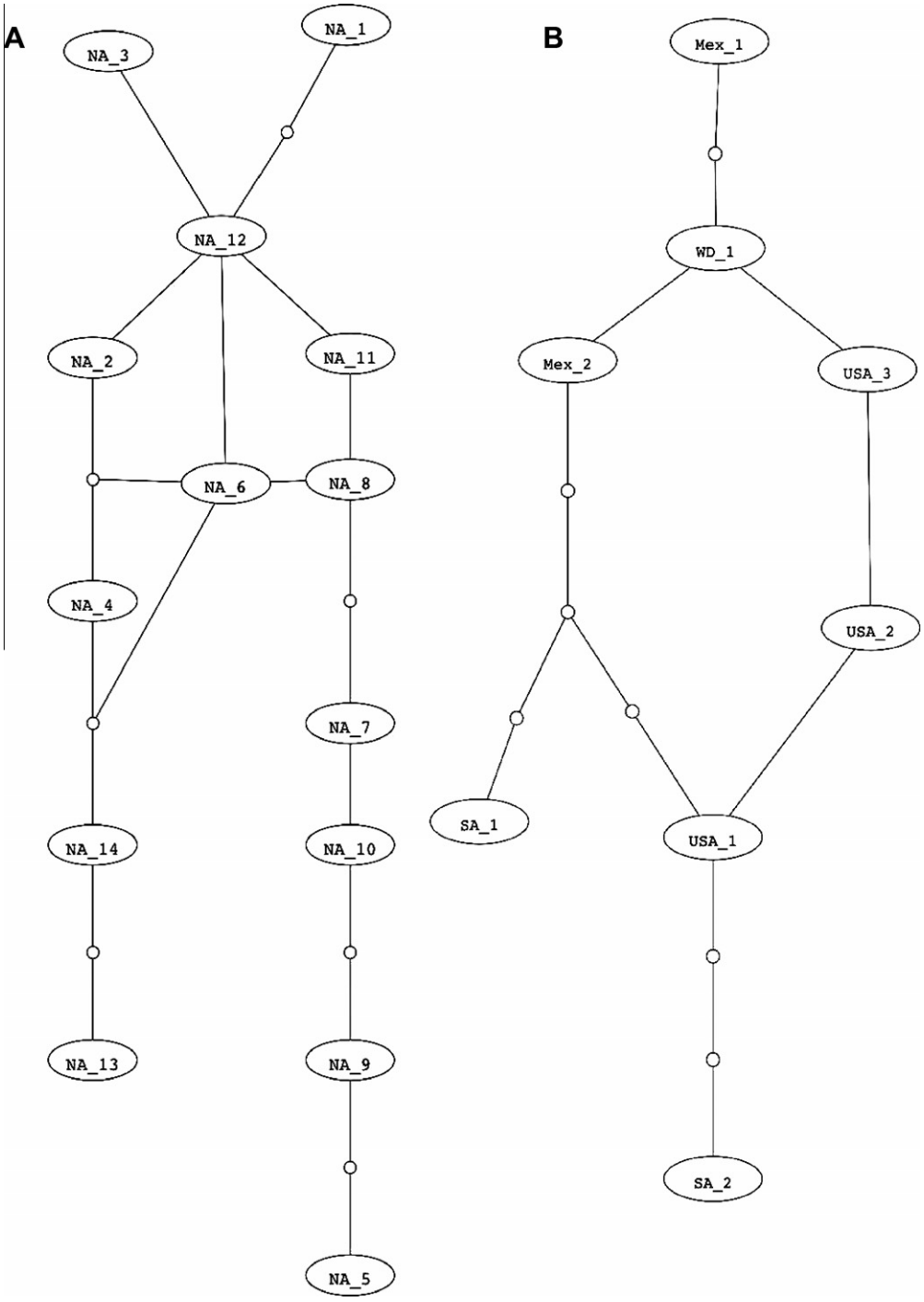

Fig. 3. Statistical parsimony networks for the 16S haplotypes found in the NA clade (A) and the NeoT-clade (B). Each connecting branch represents a single mutational step and inferred missing intermediate haplotypes are represented by open circles.

USA *S. invicta* with a wide geographic distribution (USA_1) occurring in South Carolina and Texas (Fig. 2). One likely explanation for of the broad distribution of these two sequences is that *S. invicta-infected* nests have been inadvertently transported long distances within the USA, which could have concomitantly occurred with the rather rapid expansion of the multiple queen colony social organization (polygyny) in this invasive ant (King et al., 2009). Indeed, because *K. solenopsae* infections in the USA occur predominantly in polygyne colonies (Oi and Valles, 2009), such a hypothesis gains credence.

We propose four potential scenarios for the distribution and diversity of *K. solenopsae* variants in USA *S. invicta*. First, it is possible that all three USA variants plus WD_1 found in *S. invicta* were present among the original founders that invaded the USA, and have since persisted despite the strong bottleneck this species experienced associated with its invasion (Ross and Shoemaker, 2008). Second, only one variant was present in the original invaders from South America and additional variants have arisen via mutation since the initial invasion. Third, it is feasible that none of the original founders were infected and that *Kneallhazia* occurs in USA *S. invicta* as a result of secondary invasions from South America. Finally, considering the absence of *K. solenopsae* in early surveys of *S. invicta* (Jouvenaz et al., 1977) and the fact that both *S. invicta* and *S. geminata share* a single variant (WD_1), *K. solenopsae*

Table 3
Pairwise *p*-distances among microsporidia 16S haplotypes found in *Solenopsis invicta* (SA_2, WD_1, USA_1, USA_2, USA_3), *S. richteri* (SA_1), *S. geminata* (NA_1, 2, 3, 4, 5, 6), *S. geminata* × *S. xyloni* hybrids (NA_7, 8, 9, 10, 11, 12, 13, 14), *S. carolinensis* (Kcar), and the microsporidium *Anncaliia algerae* (Aalg_1 and Aalg_2).

	Aalg_1	Aalg_2	Kcar	SA_1	SA_2	Mex_1	Mex_2	WD_1	USA_1	USA_2	USA_3
Aalg_1											
Aalg_2	0.0012										
Kcar	0.1114	0.1123									
SA_1	0.1193	0.1202	0.0766								
SA_2	0.118	0.1189	0.0775	*0.0058*							
Mex_1	0.1203	0.1212	0.0775	*0.0081*	*0.0092*						
Mex_2	0.1215	0.1224	0.0764	*0.0046*	*0.008*	*0.0034*					
WD_1	0.1203	0.1212	0.0752	*0.0058*	*0.0069*	*0.0023*	*0.0011*				
USA_1	0.1203	0.1212	0.0775	*0.0046*	*0.0034*	*0.0057*	*0.0046*	*0.0034*			
USA_2	0.1203	0.1212	0.0775	*0.0058*	*0.0046*	*0.0046*	*0.0034*	*0.0023*	*0.0011*		
USA_3	0.1192	0.12	0.0764	*0.0046*	*0.0057*	*0.0034*	*0.0023*	*0.0011*	*0.0023*	*0.0011*	
NA_1	0.1251	0.126	0.0766	**0.0266**	**0.0254**	**0.0312**	**0.0289**	**0.0289**	**0.0254**	**0.0266**	**0.0277**
NA_2	0.123	0.1238	0.0755	**0.0243**	**0.0231**	**0.0289**	**0.0266**	**0.0266**	**0.0231**	**0.0243**	**0.0254**
NA_3	0.1253	0.1262	0.0778	**0.0266**	**0.0254**	**0.0312**	**0.0289**	**0.0289**	**0.0254**	**0.0266**	**0.0277**
NA_4	0.1231	0.124	0.0767	**0.0266**	**0.0255**	**0.0313**	**0.0289**	**0.0289**	**0.0255**	**0.0266**	**0.0278**
NA_5	0.1253	0.1262	0.0756	**0.0278**	**0.0266**	**0.0313**	**0.0289**	**0.0289**	**0.0255**	**0.0266**	**0.0278**
NA_6	0.124	0.1249	0.0777	**0.0266**	**0.0254**	**0.0312**	**0.0289**	**0.0289**	**0.0254**	**0.0266**	**0.0277**
NA_7	0.123	0.1238	0.0767	**0.0266**	**0.0254**	**0.0312**	**0.0289**	**0.0289**	**0.0254**	**0.0266**	**0.0277**
NA_8	0.1222	0.1231	0.0781	**0.0278**	**0.0267**	**0.0325**	**0.0302**	**0.0302**	**0.0267**	**0.0278**	**0.0290**
NA_9	0.1251	0.126	0.0789	**0.0289**	**0.0277**	**0.0323**	**0.0300**	**0.0300**	**0.0266**	**0.0277**	**0.0289**
NA_10	0.124	0.1249	0.0777	**0.0277**	**0.0266**	**0.0323**	**0.0300**	**0.0300**	**0.0266**	**0.0277**	**0.0289**
NA_11	0.124	0.1249	0.0777	**0.0266**	**0.0254**	**0.0312**	**0.0289**	**0.0289**	**0.0254**	**0.0266**	**0.0277**
NA_12	0.124	0.1249	0.0766	**0.0254**	**0.0242**	**0.0300**	**0.0277**	**0.0277**	**0.0242**	**0.0254**	**0.0266**
NA_13	0.1275	0.1284	0.0789	**0.0289**	**0.0277**	**0.0335**	**0.0312**	**0.0312**	**0.0277**	**0.0289**	**0.0300**
NA_14	0.1263	0.1272	0.0789	**0.0289**	**0.0277**	**0.0335**	**0.0312**	**0.0312**	**0.0277**	**0.0289**	**0.0300**

	NA_1	NA_2	NA_3	NA_4	NA_5	NA_6	NA_7	NA_8	NA_9	NA_10	NA_11	NA_12	NA_13
NA_1													
NA_2	0.0035												
NA_3	0.0035	0.0023											
NA_4	0.0058	0.0023	0.0046										
NA_5	0.0069	0.0058	0.0081	0.0058									
NA_6	0.0035	0.0023	0.0023	0.0023	0.0069								
NA_7	0.0046	0.0035	0.0058	0.0035	0.0035	0.0035							
NA_8	0.0046	0.0035	0.0035	0.0035	0.0058	0.0012	0.0023						
NA_9	0.0058	0.0069	0.0092	0.0069	0.0023	0.0069	0.0035	0.0058					
NA_10	0.0035	0.0046	0.0069	0.0046	0.0046	0.0046	0.0012	0.0035	0.0023				
NA_11	0.0035	0.0023	0.0023	0.0046	0.0058	0.0023	0.0035	0.0012	0.0069	0.0046			
NA_12	0.0023	0.0012	0.0012	0.0035	0.0069	0.0012	0.0046	0.0023	0.0081	0.0058	0.0012		
NA_13	0.0035	0.0046	0.0046	0.0046	0.0093	0.0046	0.0081	0.0058	0.0092	0.0069	0.0046	0.0035	
NA_14	0.0058	0.0046	0.0046	0.0023	0.0081	0.0023	0.0058	0.0035	0.0092	0.0069	0.0046	0.0035	0.0023

Note: Underlined values represent the *p*-distances between the *Kneallhazia* 16S haplotypes obtained from *Solenopsis invicta*, *S. geminata* and *S. geminata* × *S. xyloni* hybrids, and *Anncaliia algerae* and *Kneallhazia* sp. Kcar; italicized values are *p*-distances among the *Kneallhazia* 16S haplotypes of *S. invicta* from South America and USA and *S. geminata* from southern Mexico (Neotropical group), bold values indicate *p*-distances between the Neotropical (NeoT) and the North American (NA) group composed of the *Kneallhazia* 16S haplotypes found in northern Mexico and Texas. Values in normal text represent *p*-distances within the NA group.

Table 4
Percent range of pairwise *p*-distances among *Kneallhazia solenopsae* clades of 16S haplotypes, *A. algerae* and *Kneallhazia* sp. Kcar. Values on the diagonal show the range of pairwise *p*-distances within each group: *A. algerae*, NeoT and NA.

	A. algerae	Kcar	NeoT	NA
A. algerae	(0.12)			
Kcar	11	–		
NeoT (SA–Mex–USA)	12	8	(0.11–0.92)	
NA	12	8	2–3	(0.12–0.92)

Note: No value is given for *Kneallhazia* sp. Kcar since only a single variant was found.

infection in *S. invicta* in North America could be the result of horizontal transmission events from the tropical fire ant, *S. geminata*, possibly through brood raiding or colony extirpation by *S. invicta*. Host switching of microsporidia among insect congeners is not without precedent. For example, *Nosema bombi* infects multiple bumblebee (*Bombus*) species (Tay et al., 2005). *N. ceranae*, whose host is the Asian honeybee (*Apis cerana*) has been transmitted to the western honeybee (*Apis mellifera*) (Higes et al., 2006; Huang et al., 2007; Klee et al., 2007; Shafer et al., 2009). These studies have shown that *Nosema* has the ability to switch hosts when suitable conditions occur, e.g., sympatry of Asian and western honeybee created by globalization. *K. solenopsae* may well fit this model where allopatric host species come into contact due to human activities, in this case the accidental introduction of red imported fire ants.

Because none of the *S. geminata* nests in Florida appear to harbor *K. solenopsae* (Table 1), it is possible that horizontal transmission may be unidirectional (e.g. from *S. geminata* to *S. invicta*). In addition, based on the absence of descriptions of *S. geminata* in earlier surveys through the southeast USA (including Louisiana, Mississipi and Alabama) (Wojcik et al., 1976), some authors suggested that the *S. geminata* populations from the southeast USA were introduced (Trager, 1991). If Florida is a new area for *S. geminata*, the ant may lack natural enemies, explaining why no nests were infected in our survey in Florida. Thus, the most feasible scenario for the jump of *K. solenopsae* from *S. geminata* to *S. invicta*

is that it would have occurred in some region of "true native" *S. geminata* distribution (i.e., Texas), and subsequently infected red imported fire ants were transported by humans elsewhere. On the other hand, the lack of WD_1 (and of other Neotropical haplotypes) in native fire ants in Texas and northern Mexico makes this scenario less clear, unless this absence of NeoT haplotypes is due to limited sampling.

4.3. Multiple related microsporidia variants within hosts

Different *K. solenopsae* sequences occasionally were found within single colonies. Two potential explanations for this pattern are (1) incomplete homogenization (lack of concerted evolution) of the rRNA gene clusters, or (2) infection of the colony with multiple microsporidian variants. Among microsporidia there is precedence for both, but distinguishing these in our case will require additional future studies. The multiple copies of the ribosomal gene cluster were thought to be homogenized in sequence through the process of concerted evolution (e.g., Dover and Coen, 1981). However, several studies have shown a lack of, or incomplete, homogenization in the rRNA family in various plants and animals, including microsporidia (O'Mahony et al., 2007). An alternative explanation for the detection of multiple genomic rRNA variants in *K. solenopsae* from individual fire ant hosts is multiple infections by different genetic variants. Double infection of the same host is not uncommon among microsporidia (Weitzel et al., 2001; Haine et al., 2004; Chen et al., 2009). Among fire ants in South America, dual infections with *K. solenopsae* and *Vairimorpha invictae* (Valles and Briano, 2004) have been reported. Although there is little evidence of clonality among microsporidia, a combined pattern of clonality plus multiple infections could explain the presence of multiple variants in some of the fire ant hosts. Further studies using a set of single individuals per nest, single spores and single copy genes could help to clarify these questions.

K. solenopsae infects the native tropical fire ant, *S. geminata*, and *S. geminata* × *S. xyloni* hybrids. Although we provide several possible explanations for the observed pattern of identical sequences of *K. solenopsae* within both *S. invicta* and *S. geminata*, many unresolved questions remain. Does each 16S sequence account for different *K. solenopsae* variants? Do variants differ biologically? What is the pathogenicity of each variant in its original host and in the expanded host? Which ant species is the original host? Further use of *K. solenopsae* as a biological control agent against imported fire ants: *S. invicta*, *S. richteri*, and their hybrids in the USA should be evaluated in light of their genetic makeup and pathogenicity. Genetic variation associated with differences in infectivity and virulence among hosts and/or social form could potentially be exploited to improve biological control. The *Kneallhazia*-fire ant system also provides an ideal model for studying the epidemiological dynamics among host species and patterns of host switching in host hybrid zones and areas of local contact. Such basic knowledge can help understand the origin, reservoirs and transmission of this and possibly other microsporidia.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jip.2010.07.008.

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