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Short Communication

# *Kneallhazia carolinensae* sp. nov., a microsporidian pathogen of the thief ant, *Solenopsis carolinensis*

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ABSTRACT

A new species of microsporidia is described from adults of the thief ant, *Solenopsis carolinensis*, collected in Florida, USA. Morphological and genetic characterization of this new species showed that it is most closely related to the genus *Kneallhazia* and is therefore formally designated, *Kneallhazia carolinensae* sp. nov. Masses of ovoid, binucleate spores were localized to fat body of adult workers and measured 6.2 ± 0.1 × 3.1 ± 0.1 μm (fresh) and 6.0 ± 0.1 × 3.4 ± 0.1 μm (fixed). These spores were in direct contact with the cell cytoplasm and contained an isofilar polar filament with 12–15 coils. Blastn analysis revealed that the *K. carolinensae* 16S rDNA sequence exhibited 91% identity with the 16S rDNA gene of *K. solenopsae*. The morphological and sequence data support the conclusion that *K. carolinensae* is a novel microsporidian species distinct from *K. solenopsae*.

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## 1. Introduction

Microsporidia are specialized obligate intracellular pathogens of fungal origin that infect a variety of eukaryotes. These organisms are often exploited as biological control agents against invasive insect species. *Kneallhazia solenopsae*, for example, was first described from red imported fire ant, *Solenopsis invicta*, colonies in South America (the native range of *S. invicta*) and was recognized as a potential biological control agent (Knell and Allen, 1977). It has since been spread intentionally throughout the southeastern United States in an attempt to provide widespread control of this invasive ant species (Williams et al., 2003). Studies have been conducted to examine the life cycle (Sokolova and Fuxa, 2008), the nature of the host-pathogen interaction (Cook et al., 2003; Shapiro et al., 2003; Valles et al., 2009), spore morphologies (Chen et al., 2004; Sokolova and Fuxa, 2001; Sokolova et al., 2005), effects against field populations of *S. invicta* (Fuxa et al., 2005; Oi et al., 2004), and interactions with traditional insecticides (Oi and Valles, 2009; Valles and Pereira, 2003).

*K. solenopsae* was originally classified in the genus *Thelohania* and was recently re-classified into the newly created monotypic genus *Kneallhazia* based on unique morphological and genetic characteristics (Sokolova and Fuxa, 2008). While examining specimens of the ant, *Solenopsis carolinensis*, we discovered microsporidian spores with characteristics similar to but obviously distinct from *K. solenopsae*. We provide morphological and genetic characterization that distinguish it from *K. solenopsae* and designate this new ant pathogen infecting the thief ant, *S. carolinensis*, as *Kneallhazia carolinensae* sp. nov.

## 2. Materials and methods

### 2.1. Ant collections

*S. carolinensis* is in the subgenus *Diplorhoptrum* whose members are called thief ants. Ants were collected in north Union Co., FL (N 30.126; W 82.204), and in Alachua, Co., FL, at the USDA-ARS, Center for Medical, Agricultural and Veterinary Entomology compound, in Gainesville, Florida (N 29.636; W 82.360). The worker caste was collected by using baited traps or pitfall traps. Baited traps consisted of capped 74-ml plastic vials containing a piece (~1 g) of a commercially-available pecan shortbread cookie. The vial cap was punctured with small (0.5–1 mm) holes to permit entry by the ants. The traps were buried in soil in a horizontal position at a depth of 10–15 cm and retrieved after 1 to 3 days. Live-collected ants were maintained on pecan shortbread cookies and provided access to water and nesting harborage in the laboratory in plastic trays (15 × 15 × 3 cm) with the sides coated with Fluon® (Asahi Glass Fluoropolymers, Inc., Chadds Ford, PA) to prevent escape.

Pitfall traps consisted of 25 ml plastic tubes (4 cm diameter) filled with approximately 8 ml of propylene glycol, which were buried upright into the soil until the tube opening was level with the soil surface. The traps were left in the field for 1–4 days before

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being retrieved. Once the traps were returned to the laboratory, the arthropods were separated from the anti-freeze liquid, preserved in 70% ethanol, and classified into taxonomic categories. Thief ants, highly cryptic and distributed widely in Florida (Deyrup, 2003), were identified using the key of Thompson (1989).

*S. carolinensis* worker ants were examined for the presence of pathogens by preparing a macerate of several individuals from a collection sample and observing it under phase contrast microscopy. If overt signs of infection (e.g., the presence spores) were observed, additional ants from the same collection were examined under a dissecting microscope or phase contrast microscope for the presence of spores inside the ant body.

### 2.2. Microscopy

Once microsporidian-infected *S. carolinensis* were identified, they were examined using both light and transmission electron microscopy (TEM). Smears prepared from infected worker ants were stained with Giemsa for observation under light microscopy. Fresh preparations were viewed with phase contrast microscopy. For TEM observations, internal tissues and microsporidian spores were dissected from worker ants in 2.5% glutaraldehyde for 2 h, postfixed in 2% osmium tetroxide for 1.5 h, dehydrated in an ethanol series to absolute acetone and embedded in epon-araldite (Becnel, 1997) and thin-sectioned. Spore measurements were obtained. Measurements of the longest axis and a broadest width perpendicular to that axis were obtained from both Giemsa and fresh-mounted preparations using a microscope-mounted ocular micrometer.

### 2.3. Amplification and sequencing of 16S rDNA gene

Genomic DNA was isolated from microsporidium-infected *S. carolinensis* workers as described by Valles et al. (2002). Briefly, approximately 50 ants were added to a 1.5 ml microcentrifuge tube containing 150 μl of lysis buffer (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 isopropanol, and the pellets were washed twice with 70% ethanol. Pellets were dried at 37 °C, then resuspended in 30 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8).

PCR was conducted in an MJ Research PTC-200 thermal cycler (Waltham, MA) in 50 μl volumes containing 2 mM $MgCl_2$, 200 μM dNTP mix, 1U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), 0.4 μM of each primer, and 1 μl of the genomic DNA preparation (50–100 ng). Negative controls were included. 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* DH5a cells (Statagene, La Jolla, CA). Insert-positive clones were sequenced by the Interdisciplinary Center for Biotechnology Research, University of Florida. The regions amplified were sequenced from at least 3 different clones yielding a consensus sequence.

Oligonucleotide primers were designed from conserved areas of microsporidia 16S rDNA genes retrieved from GenBank. PCR was conducted with primer p414 (5′ACAAATGCCTCTTCACAGCAGCATC) and p415 (5′ACCAGGTTGATTCTGCCTGGTATGTGT) using genomic DNA prepared from microsporidium-infected and uninfected *S. carolinensis* workers under the following conditions: 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 59 °C for 15 s, and 68 °C for 1.5 min, followed by an elongation step of 5 min at 68 °C. Amplicons were cloned and sequenced as described above. DNA prepared from uninfected ants failed to produce an amplicon.

Blastn analysis of the *K. carolinensae* 16S rDNA partial sequence (1273 nucleotides) was conducted to identify closely related organisms based on nucleotide identities. Multiple alignments were carried out for the nucleotide sequences of 16S rDNA genes of microsporidia infecting hymenopteran insects. These sequences were acquired from the Genbank database, aligned and subsequently used to construct a phylogenetic tree using the neighbor-joining method (Saitou and Nei, 1987) in ClustalX (Thompson et al., 1994). The statistical significance of branch order was estimated by performing 1000 replications of bootstrap re-sampling of the original aligned nucleotide sequences. Trees were generated with TreeView (Page, 1996).

## 3. Results and discussion

*Kneallhazia carolinensae* sp. nov.

*Type host Solenopsis carolinensis* Forel, 1901 (Hymenoptera: Formicidae). *S. carolinensis* is a cryptic, subterranean ant species described and known from the adult stages (Thompson, 1989).

*Site of infection* Fat body in adult workers; other stages of *S. carolinensae* not observed.

*Transmission* Unknown.

*Interface* Sporulation stages were in direct contact with the host-cell cytoplasm. No sporophorous vesicles were present at any point in the observed developmental cycle.

*Other parasite host cell relations* False hypertrophy of infected fat body cells.

*Development* Only diplokaryotic stages observed. Sporogony by binary fission with each sporont dividing once to produce two binucleate sporoblasts (based on examination of Giemsa-stained smears). Sporogenesis began with the concurrent formation of the exospore and polar filament followed by cell elongation.

*Spores* Only one binucleate spore type was observed. Spores measured $6.2 \pm 0.1 \times 3.1 \pm 0.1$ μm (fresh, $n = 10$) and $6.0 \pm 0.1 \times 3.4 \pm 0.1$ μm (fixed, $n = 10$). Masses of spores (similar to the 'sporocytosacs' described by Sokolova et al., 2005) represented "false hypertrophy" of fat body cells (Sokolova and Fuxa, 2008) and were localized to the head, thorax and abdomen. They could be observed directly through the ant's cuticle (Fig. 1A). Spores were ovoid in sagittal section, binucleate and in direct contact with the host cell cytoplasm (Fig. 1B and C). The polar filament was isofilar and contained between 12 and 15 coils (average number of coils was 13.6 from a sample size of 20); the coil arrangement was uniform to irregular. Transverse sections of the polar filament revealed several layers with an electron dense outer layer and an inner core separated by an electron lucent layer (Fig. 1D). The polaroplast was poorly preserved in most examples but appeared to occupy only about one fourth of the anterior region of the spore. The spore wall was bound by an inner plasmalemma with an unlayered electron-dense exospore and a slightly thicker electron transparent endospore (Fig. 1D). Binucleate spores were in direct contact with the host cell cytoplasm and no other spore types were observed in adult worker ants.

*Type locality* Gainesville, Florida, USDA/CMAVE campus.

*Etymology* Named after the host, *Solenopsis carolinensis*.

*Molecular characterization* The 16S rDNA nucleotide sequence for *K. carolinensae* (1273 nucleotides) was submitted to GenBank and assigned Accession number GU173849. Blastn analysis of this sequence revealed significant expectation scores from organisms in the phylum Microsporidia with the highest sequence identity (91%) to the 16S rDNA gene of *K. solenopsae*. The sequence data

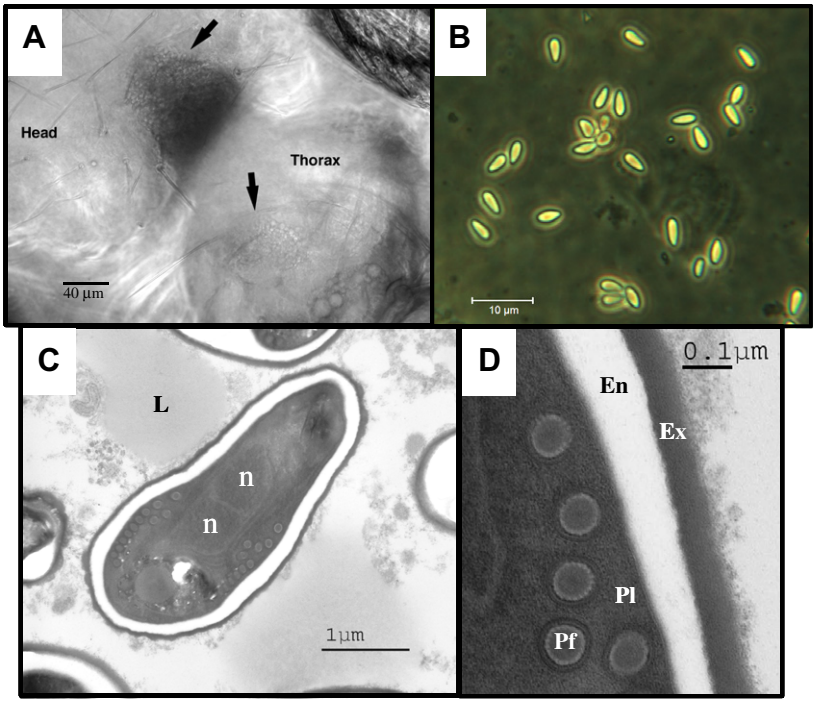


**Fig. 1.** Light and electron micrographs of binucleate spores of *Kneallhazia carolinensae* from adult workers of *Solenopsis carolinensis*. (A) Spores observed through the cuticle of the head and thorax, (B) Spores released from the thorax and abdomen, (C) Binucleate spore in fat body with the polar filament arranged in approximately 12 coils (n = nucleus, L = lipid), (D) Fine structure of the spore wall composed of an unlayered exospore (Ex) and thicker endospore (En) and the inner plasmalemma (Pl). The transversely sectioned polar filament (Pf) was composed of several concentrically arranged layers.

support the morphological data and, consequently, the conclusion that *K. carolinensae* is a novel microsporidian species distinct from *K. solenopsae*. The high sequence identity with *K. solenopsae* correctly places this microsporidium in the *Kneallhazia* genus. Indeed, phylogenetic analysis revealed that *K. solenopsae* and *K. carolinensae* form a monophyletic clade (Fig. 2) providing further support for placement of the new species within the *Kneallhazia* genus. Phylogenetic analysis also revealed distinct separation from other microsporidia of honeybees and other ant species.

*Deposition of type specimens* Two type slides have been deposited with the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, DC (USNM Nos. 1155308 and 1155309). Additional slides and specimens embedded in plastic resin are archived at the Center for Medical, Agricultural and Veterinary Entomology, USDA, Gainesville, Florida.

*Bionomics* Ants with *K. carolinensae* infections did not exhibit any behavioral changes. Two collections were made in Union county (N 30.142; W 82.188) in July 2003 and two in Alachua county (N 29.636; W 82.360) in August 2003 and March 2011. A total of 150 *S. carolinensis* adult worker ants were examined. Total *K. carolinensae* infection prevalence in collected ants was 27.5 ± 7.8%.

*Remarks* The highly cryptic nature of *S. carolinensis* limited examination to adult ants where very few pre-sporulation stages were observed. Indeed, taxonomic descriptions of these ant species are based on the adult stages only (Thompson, 1989). High concentrations of spores of *K. carolinensae* were observed in the head, thorax and abdomens of worker ants, and often could be observed directly through the cuticle of infected ants (Fig. 1A).

The masses of binucleate spores (sporocytosacs) observed in this study appeared to be localized to fat body cells and could be found in various regions of the adult workers. Fat body of adult *S. invicta* is the main site of infection for *K. solenopsae* where several spore types occur but the predominant type is the octospore (Sokolova and Fuxa, 2008). The binucleate spores of *K. carolinensae*

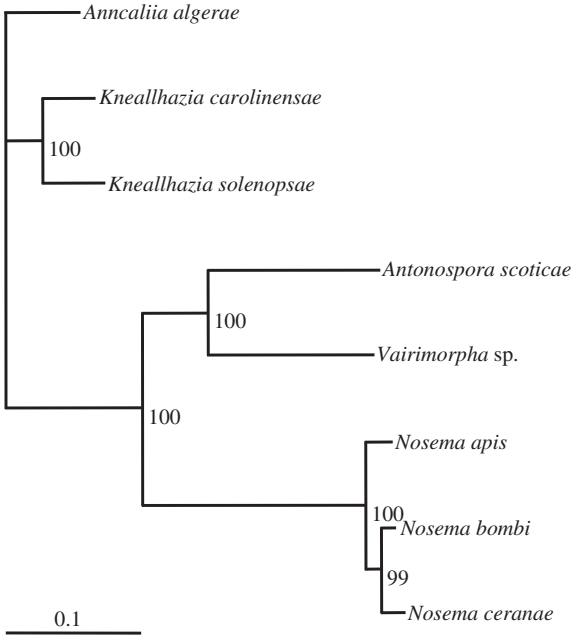


**Fig. 2.** Phylogenetic analysis of the 16S rDNA nucleotide sequences from microsporidia known to infect hymeneopteran insects and the microsporidian *Anncaliia algerae*. Microsporidian species with corresponding Genbank database accession number and the host species are provided: *A. algerae* (AM422905) hosts = *Anopheles stephensi* (original isolate) and *Homo sapiens*; *K. carolinensae* (GU173849) host = *S. carolinensis; K. solenopsae* (AF134205) host = *S. invicta*; *A. scoticae* (AF024655) host = *Andrena scotica*; *Vairimorpha* sp. (AF031539) host = *S. invicta*; *N. apis* (DQ235466) host = *Apis mellifera*; *N. bombi* (AY008373) host = *Bombus terrestris*; and *N. ceranae* (NCU26533) host = *Apis cerana*. The statistical significance of branch order is provided at each node from 1000 replications of bootstrap resampling of the original aligned nucleotide sequences.

were slightly larger than the comparable spore type in *K. solenopsae* referred to as "free spores" (Moser et al., 2000) and Type 2 diplokaryotic spores (Sokolova and Fuxa, 2008) from adult *S. invicta*. Ultrastructurally, the binucleate spores of *K. solenopsae* and *K. carolinensae* were similar, with an isofilar polar filament arranged in a single row with a range of coils 11–13 vs. 12–15, respectively, that overlapped, a thin, smooth exospore and thicker endospore, and a distinct posterior vacuole (Fig. 1C and D). Octospores, the predominant spore type observed in *K. solenopsae* infections of *S. invicta* adults, were not observed. Binucleate *K. solenopsae* spores were rare, usually representing approximately 2% of the spores observed in adult ants (Sokolova et al., 2004). However, it has recently been reported that some individual adult *S. invicta* ants were infected nearly exclusively with binucleate *K. solenopsae* spores (Sokolova and Fuxa, 2008; Sokolova et al., 2004). Factors that control shift from one spore type to another are unknown but may involve adaptations of the pathogen to the life cycle of the host to enhance long term survival. Although no octospores were observed for *K. carolinensae*, the relationship between host and pathogen may be different than that for *K. solenopsae* and its host *S. invicta*.

We fully acknowledge the limited description of this new species of microsporidian. However, because a paucity of microsporidia are known from the Formicidae, we believe it is important to make the information available to the scientific community. The morphological and molecular data provide strong support that this is a unique microsporidian and expands the monotypic *Kneallhazia* genus. Limited descriptions have significant utility, especially when supported by gene sequence data, in aiding elucidation of life cycles; microsporidia often exhibit complex life cycles involving several different host organisms which could be more easily linked with molecular information (Becnel and Andreadis, 1999). Thus, the *K. carolinensae* description expands knowledge of the diversity of microsporidia in ants and other hymenoptera, and provides a genetic basis for future reference.

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