



## *Myrmecomorba nylanderiae* gen. et sp. nov., a microsporidian parasite of the tawny crazy ant *Nylanderia fulva*



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### ABSTRACT

A new microsporidian genus and species, *Myrmecomorba nylanderiae*, is described from North American populations of the tawny crazy ant, *Nylanderia fulva*. This new species was found to be heterosporous producing several types of binucleate spores in both larval and adult stages and an abortive octosporoblastic sporogony in adult ants. While microsporidia are widespread arthropod parasites, this description represents only the fifth species described from an ant host. Molecular analysis indicated that this new taxon is phylogenetically closely allied to the microsporidian family Caudosporidae, a group known to parasitize aquatic black fly larvae. We report the presence of 3 spore types (Type 1 DK, Type 2 DK, and octosporos) with infections found in all stages of host development and reproductive castes. This report documents the first pathogen infecting *N. fulva*, an invasive ant of considerable economic and ecological consequence.

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### 1. Introduction

While microsporidian parasites are extremely diverse and pervasive within the animal kingdom, only four species have been described from ants to date, all from the host genus *Solenopsis*. The evolutionary adaptation of microsporidia as intracellular parasites has resulted in the evolution of highly reduced genomes in some species and loss of mitochondria and associated metabolic functionality (Keeling and Fast, 2002), yet these remarkable parasites exhibit intricate life cycles with multiple spore types (Andreadis, 2007; Becnel and Andreadis, 2014). A clear phylogeny has only recently been developing which has included their placement within the fungi (Capella-Gutiérrez et al., 2012; Keeling, 2014) and comparative relationships between the taxa (Smith, 2009; Vossbrinck and Debrunner-Vossbrinck, 2005; Vossbrinck et al., 2014). Of note are the apparent aquatic origins of most microsporidian clades within which some recent acquisitions of terrestrial hosts have occurred and this appears to be the case with the four previously described species of microsporidian parasites of ants (*Kneallhazia solenopsae*, *K. carolinensae*, *Vairimorpha invictae* and *Burenella dimorpha*).

The discoveries of the four microsporidian ant pathogens have been associated with studies of several *Solenopsis* species where there is an interest in the potential use of microsporidia as host-specific biological control agents for the invasive red imported fire ant, *Solenopsis invicta* (Oi and Valles, 2009). Another invasive ant species in North America is *Nylanderia fulva*, the tawny crazy ant, which is native to central South America (Gotzek et al., 2012), but has recently become an important invasive species in the southeastern U.S. and several Caribbean islands where it can reach extremely high local densities (Wetterer et al., 2014) and displace native fauna (LeBrun et al., 2013). Efforts are underway in a number of U.S. laboratories to understand the biology (LeBrun et al., 2013, 2014) and discover natural enemies (Valles et al., 2012a) of this important developing pest ant. During laboratory studies, some *N. fulva* workers were observed exhibiting distended gasters, a characteristic observed in some *Kneallhazia* microsporidian infections of *Solenopsis invicta* (R. Plowes, N. Jones pers. obs.). Indeed, microscopic examination of the abdomens of *N. fulva* worker ants exhibiting distended gasters revealed spores consistent with microsporidia. Morphological, developmental and molecular analyses have confirmed that the spores found in *N. fulva* represent a new microsporidian taxon, here designated *Myrmecomorba nylanderiae* gen. et sp. nov.

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## 2. Materials and methods

### 2.1. Host ant collections

*N. fulva* ant samples were collected from sites in Texas, Florida, and St. Croix. Samples were preserved in 90% ethanol and maintained at  $-20^{\circ}\text{C}$ . Live ants with queens and brood were maintained in plastic trays with ventilated lids and side-walls coated with Fluon™ to contain the ants. The live ants were provided water, 10% sugar solution and fed with crickets, house fly larvae, or adult wingless *Drosophila* flies. All life stages were examined for symptoms of disease such as enlarged or reduced body parts and discoloration.

### 2.2. Microscopy

Colonies of *N. fulva* were collected from the environs of Gainesville, Florida, and established in the laboratory as above. Microsporidian-infected colonies were identified by examining slides of wet mounted extracts of macerated ants for the presence of spores under phase contrast microscopy. All life stages of *N. fulva* were examined using both light and transmission electron microscopy (TEM). Air dried smears were prepared from queens, eggs, larvae, pupae, adults and alates, fixed with methanol for 5 min and stained for 10 min with 10% Giemsa stain buffered at pH 7.4. Fresh preparations of the same stages were also examined with phase contrast microscopy. Spore measurements of the longest axis and a broadest width perpendicular to that axis were obtained from both Giemsa and fresh-mounted preparations using SPOT Imaging Software (ver. 4.7). For TEM, pupae and adults were dissected in 2.5% glutaraldehyde and held for a minimum of 2 h, then fixed 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. Sections were then stained with 2.5% uranyl acetate and lead citrate and viewed and photographed on a Hitachi H-600 electron microscope.

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

Genomic DNA for sequencing was extracted from approximately 10 ant workers from sites in Florida and Texas using Qiagen DNeasy™ Blood and Tissue kits after the specimens were homogenized for 20 s in a Mini-Bead Beater™ in 200  $\mu\text{l}$  molecular grade water. DNA yields were improved if the ant venom (formic acid) was first removed by dissection of venom glands or by squeezing the tip of the ant gasters to discharge the venom into distilled water that was then discarded. This step may have reduced the effect of an unidentified endogenous component of *N. fulva* abdomens that can severely inhibit downstream molecular assays (Valles et al., 2012b). PCR amplification was conducted in a Labnet MultiGene Gradient PCR thermal cycler in a 25  $\mu\text{l}$  volume: 3  $\mu\text{l}$  of template DNA 20–60 ng/ $\mu\text{l}$ , 2.5  $\mu\text{l}$  of primer mix 10  $\mu\text{M}$ , 12.5  $\mu\text{l}$  Qiagen Multiplex mix and 7  $\mu\text{l}$  ddH<sub>2</sub>O.

Molecular confirmation that the spores observed were of microsporidian origin was achieved using oligonucleotide primers V1 (5'-CACCAGGTTGATTCTGCCTGAC) and PMP2 (5'-CCTCTCCGGAACCAAACCTG) to amplify a 270-bp amplicon from the 16S rDNA gene using established PCR conditions (Fedorko et al., 1995; Müller et al., 1999). The consensus sequence yielded strong matches (>70%) for microsporidians of the Caudosporidae family in NCBI using a Blastn search (Altschul et al., 1997).

An alternate antisense oligonucleotide primer 1492 (5'-GGTACCTTGTACGACTT) (Zhu et al., 1993) was utilized in combination with the V1 primer in an effort to extend the consensus sequence. Thermocycling conditions were as follows: initial

denaturing at  $94^{\circ}\text{C}$  (3 min), then 35 cycles of denaturing at  $94^{\circ}\text{C}$  (1 min), annealing at  $51.8^{\circ}\text{C}$  (2 min), and extension at  $72^{\circ}\text{C}$  (3 min), followed by a final polishing step at  $72^{\circ}\text{C}$  for 10 min. The V1/1492 forward reads provided a consensus sequence of 996 nucleotides while the reverse reads often contained both microsporidian and host ant products.

To overcome ant host contamination we designed new oligonucleotide primers NF1201 (5'-TGACGGAAGGACACCACAAG) and NR1701 (5'-GTACAAAGGACAGGGACGCA) from conserved regions of 16s rDNA genes from several Caudosporidae species in GenBank (accession numbers: *Weiseria palustris* AF132544, *Caudospora simulii* AY973624, *Polydispyrenia simuli* AY090069, *Flabelliforma montana* AJ252962). The thermal cycler conditions used for the primer set NF1201/NR1701 were initial denaturing at  $95^{\circ}\text{C}$  (15 min), then 35 cycles of denaturing at  $94^{\circ}\text{C}$  (30 s), annealing at  $51.8^{\circ}\text{C}$  (90 s), and extending at  $72^{\circ}\text{C}$  (90 s), followed by final extension at  $72^{\circ}\text{C}$  for 10 min. PCR amplification yielded two amplicons of 420-bp (microsporidian) and 500-bp (host ant). The products from the combined sequencing efforts (V1/PMP, V1/1492 and NF1201/NR1701) generated a 1408-bp consensus sequence.

As a rapid and inexpensive diagnostic tool, we developed a Chelex™ extraction protocol and sequence-specific oligonucleotide primers for this *Nylanderia* microsporidian. Oligonucleotide primer V1 (above) and NR614 (5'-CCATGCTAGACCAGCCATT) yielded a 614-bp amplicon. The DNA extraction used 10–20 ants homogenized as above, centrifuged at 10,000 RCF for 2 min, followed by addition of 8% Chelex (300  $\mu\text{l}$ ) and protease-K (2  $\mu\text{l}$ ). The solution was incubated at  $65^{\circ}\text{C}$  for 2 h,  $100^{\circ}\text{C}$  for 10 min, centrifuged for 15 min at 14,000 RCF and the supernatant retained as template. To serve as extraction controls for *N. fulva* host DNA we developed oligonucleotide primers for the elongation factor alpha one (EFA) gene of the ant host (EFA3f Forward (5'-GACAATATGCTCGAGCCGTC), EFA3r Reverse (5'-CGAACCTTTGAGACGGTGAA)). Diagnostic PCR assays included both the V1/NR614 and EFA3f/EFA3r primer sets in a multiplexed reaction. PCR was run with Qiagen™ Multiplex in a 10  $\mu\text{l}$  reaction volume, with 1  $\mu\text{M}$  primer and 1  $\mu\text{l}$  Chelex extraction supernatant. The thermal cycling conditions were as for NF1201/NR1701 with an annealing temperature of  $55^{\circ}\text{C}$ .

Phylogenetic analysis was conducted to establish the relationship of *M. nylanderiae* with known microsporidia using 16S rDNA partial sequences. Representative species (Table 1) were included from each of the major microsporidian clades (Vossbrinck and Debrunner-Vossbrinck, 2005) with *Heterococcus pleurococcoides* (phylum Ochrophyta) included as an outgroup. We included sequences for all known ant-hosted microsporidia although no sequences or specimens were available for *B. dimorpha*, a parasite of *S. geminata*. Phylogenetic analyses were performed in Geneious software using Neighbor Joining with 1000 bootstrap replications (Saitou and Nei, 1987).

## 3. Results and discussion

### 3.1. *Myrmecomorba* n. g. Plowes, Becnel, LeBrun, Oi, Valles, Jones, Gilbert

#### 3.1.1. Definition

Heterosporous microsporidian with sporulation sequences in immatures and adult life stages. Multiple binucleate spore types produced by disporoblastic sporogony during which secretions can be produced but binucleate spores are not enclosed in a sporophorous vesicle (SV). Octosporous sequence present with spore-onts developing within an SV but the sequence may be abortive in some species. The 16S rDNA sequence assembly of the type

**Table 1**

Sequences used for 16S rDNA phylogenetic analysis. From NCBI GenBank. Outgroup was AJ579335 *Heterococcus pleurococcoides* from the phylum Ochrophyta. The new species *M. nylanderiae* was represented by the 1408 bp consensus assembly GenBank Acc. No. KR704917.

GenBank accession	Organism
KR704917	<i>Myrmecomorba nylanderiae</i>
ACU68473	<i>Amblyospora californica</i>
AF024655	<i>Antonospora scoticae</i>
AF027682	<i>Parathelohania anophelis</i>
AF027683	<i>Culicosporella lunata</i>
AF031538	<i>Kneallhazia solenopsae</i>
AF031539	<i>Vairimorpha</i> sp. ( <i>S. richteri</i> )
AF056014	<i>Glugea americanus</i>
AF069063	<i>Anncaliia algerae</i>
AF132544	<i>Weiseria palustris</i>
AF439320	<i>Gurleya daphniae</i>
AF484695	<i>Trichnosema pectinatellae</i>
AJ252962	<i>Flabelliforma montana</i>
AJ438956	<i>Dictyocoela muelleri</i>
AJ579335	<i>Heterococcus pleurococcoides</i>
AJ581995	<i>Bacillidium vesiculoformis</i>
AY008373	<i>Nosema bombi</i>
AY090041	<i>Marssoniella elegans</i>
AY090042	<i>Berwaldia schaefermai</i>
AY090067	<i>Hazardia milleri</i>
AY090069	<i>Polydispyrenia simili</i>
AY233131	<i>Cystosporogenes legeri</i>
AY305324	<i>Paranosema locustae</i>
AY973624	<i>Caudospora similii</i>
EZORGOA	<i>Encephalitozoon cuniculi</i>
GU173849	<i>Kneallhazia carolinensae</i>
KC990122	<i>Multilamina teevani</i>
NSU78176	<i>Nucleospora salmonis</i>

species *M. nylanderiae* (GenBank Accession Number KR704917) serves as the reference sequence for the genus.

### 3.2. *M. nylanderiae* n. sp. Plowes, Becnel, LeBrun, Oi, Valles, Jones, Gilbert

#### 3.2.1. Type host

*Nylanderia fulva* (Mayr, 1862) (Hymenoptera: Formicidae: Formicinae). This species was recently transferred from the genus *Paratrechina* (LaPolla et al., 2010).

#### 3.2.2. Sites of infection

Infected cells (possibly haemocytes) in larvae and pupae were filled with meronts (Fig. 1A). Sporocytosacs (cysts) containing spores (Sokolova et al., 2005) were composed of an outer region of unidentified host cells bound by a basement membrane (Fig. 2C) and localized to the abdominal cavity in adult workers and alates (not shown). Host eggs contained binucleate sporoplasms (not shown). Molecular assays using the specific primers V1/NR614 detected the microsporidian in eggs and 1st–4th instar larvae.

#### 3.2.3. Transmission

Vertical (transovarial) transmission appears likely based on observed binucleate sporoplasms in eggs. Horizontal transmission mechanisms are unknown but it is assumed to occur within the extensive super-colonial populations of host *Nylanderia* ants given the widespread infections within such populations.

#### 3.2.4. Development

All developmental stages observed were diplokaryotic. The main proliferative phase occurs in larvae and pupae with reproduction of diplokaryotic meronts and sporonts (Fig. 1A and B). Division occurs primarily by binary fission but occasionally quadrinucleate stages were observed (not shown). These stages led to the

production of two morphologically distinct binucleate spores and rarely uninucleate spores (termed octospores here but equivalent to meiospores). The main sporulation sequence began with the production of meronts in pupae (Fig. 1C and D). The cytoplasm of meronts was ribosome rich with few organelles and typical diplokaryotic arrangement of the nuclei (Fig. 1C). Changes in the region of the closely appressed membranes of the diplokaryon first appeared in late meronts with vacuoles formed to begin separation of the nuclear envelopes (Fig. 1D). This separation of the region between the two nuclei continued in early sporonts until the separation was complete in late sporonts (Fig. 1E). Sporonts were additionally characterized by becoming elongate with a denser cytoplasm and prominent regions of endoplasmic reticulum (Fig. 1E). Sporonts divided once (disporous sporulation) to produce two sporoblasts that undergo spore morphogenesis (Fig. 1F) and transform into elongate-narrow binucleate spores (of the Type 2 DK of Sokolova and Fuxa, 2008) (Fig. 2A–F). A second, infrequently observed sporulation sequence occurred in pupae and adults to produce elongate-oblong binucleate spores (of the Type 1 DK of Sokolova and Fuxa, 2008) (Fig. 3A–D). A third sporulation sequence began with diplokaryotic meronts in adults (Fig. 4A). At some point, diplokaryotic sporonts (Fig. 4B) were produced that undergo octosporoblastic sporogony within a sporophorous vesicle (Fig. 4C) to produce uninucleate sporoblasts (Fig. 4D). Development after this stage in sporulation appears to abort and only rarely produced groups of normal octospores within a sporophorous vesicle (Fig. 5A–E).

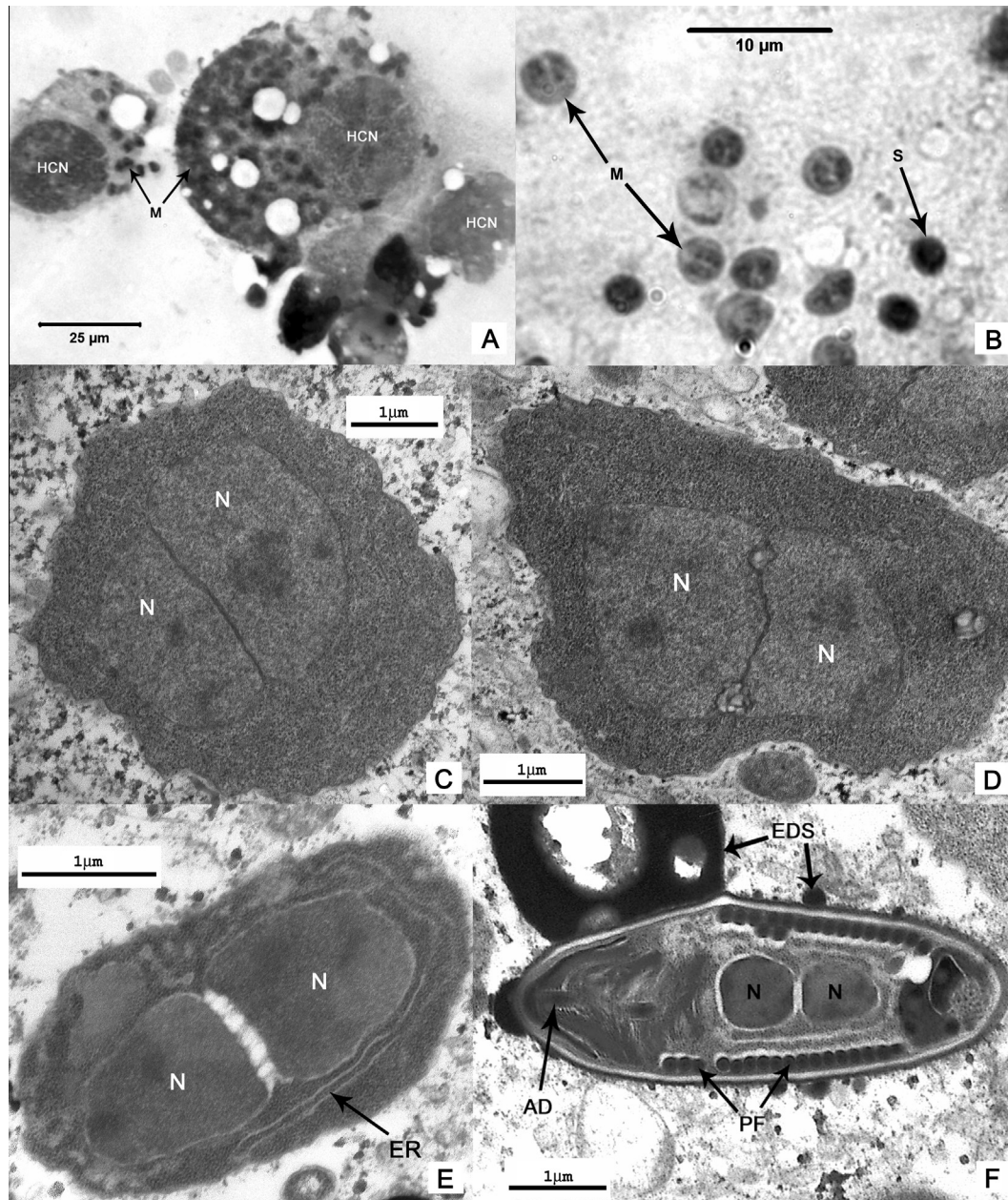
#### 3.2.5. Spores

In adults, the spore produced in large numbers is an elongate-narrow binucleate spore (Type 2 DK) that was slightly tapered toward the posterior end (Fig. 2A and B). This spore measured  $6.57 \pm 0.09 \times 1.59 \pm 0.03 \mu\text{m}$  (mean  $\pm$  SE, fresh,  $n = 30$ ) and  $6.40 \pm 0.07 \times 1.83 \pm 0.02 \mu\text{m}$  (mean  $\pm$  SE, fixed,  $n = 30$ ). The membranous polaroplast was of the uniform lamellar type and occupied the anterior third of the spore (Fig. 2D and E). The long, isofilar polar filament had a prominent anchoring disk and made 19–21 turns arranged in a single row (Fig. 2D and F). The spore wall was composed of a thin, electron transparent endospore and a slightly thicker, unlayered electron-dense exospore (Fig. 2F). A second spore type was observed in pupae and adults (Type 1 DK), which were elongate-oblong binucleate spores found to occur in relatively small numbers (Fig. 3A, B and D) and many had already germinated (Fig. 3C). This spore measured  $5.57 \pm 0.11 \times 2.29 \pm 0.05 \mu\text{m}$  (mean  $\pm$  SE, fixed,  $n = 20$ ). The injection complex contained a typical anchoring disk, a short, isofilar polar filament making 4–5 turns and a membranous polaroplast that occupied about a quarter of the anterior end of the spore (Fig. 3D). The posterior vacuole was large (Fig. 3D) and the unlayered exospore was approximately one-quarter the thickness of the thicker endospore at its widest point (Fig. 3D).

In adults, the octosporoblastic sequence was initiated but rarely produced normal octospores and mostly aberrant spores were produced (Fig. 5A, B and D). Because of this, measurements were made from spores in groups of six to eight and yielded a size of  $2.79 \pm 0.08 \times 1.86 \pm 0.05 \mu\text{m}$  (mean  $\pm$  SE, fresh,  $n = 30$ ). Octospores that appeared to be mature were characterized by incompletely formed spore organelles with a short, isofilar polar filament, with 3–4 posterior-anterior coils and a polaroplast of the lamellar type (Fig. 5C and E). The exospore was crenulated and layered with a thicker, electron lucent endospore (Fig. 5E).

#### 3.2.6. Interface

Sporogenesis of the Type 2 DK elongate-narrow binucleate spores occurred in a specialized host parasite association where host cells limited by a basement membrane formed a cyst wall



**Fig. 1.** Developmental stages of *Myrmecomorba nylanderiae* in larvae and pupae of *Nylanderia fulva*. (A) Numerous meronts (M) in the cytoplasm of host cells (possible haemocytes) in larvae and pupae (host cell nucleus, HCN). (B) Groups of diplokaryotic meronts (M) and sporonts (S) released from infected host cells in pupae. (C) Early diplokaryotic meront (N, nucleus). (D) Late diplokaryotic meronts. The closely appressed membranes between the two nuclei (N) begin separating at the margins. (E) Sporont with prominent regions of endoplasmic reticulum (ER) and complete separation of the region between the two nuclei (N). (F) Immature, elongate-narrow binucleate spore (Type 2 DK) with electron dense secretions (EDS) first indicated as small blebs on the developing exospore that expand into large masses. The developing anchoring disk (AD), coils of the long polar filament (PF) and nuclei (N) are evident. (A and B) giemsa-stained; (C–F) transmission electron micrographs.

(Fig. 2C). During sporogony, electron dense secretions accumulated around the spores (Fig. 2D and E) and eventually resulted in a uniform, electron dense matrix within which spores were embedded (Fig. 2C). This type of cyst is similar to a xenoparasitic complex (xenoma) found in several groups of microsporidia (Sprague et al., 1992) but unusual in the lack of a membranous sporophorous vesicle (SV) to contain the spores as commonly found in xenomas in fish (Vávra and Larsson, 2014) and some insects (Becnel and Andreadis, 2014).

A persistent SV was produced by the sporont (Fig. 4B) during the octosporoblastic sporulation sequence. The SV separates from the sporont plasmalemma followed by divisions to produce sporoblasts (Fig. 4C and D) and spores contained within the SV

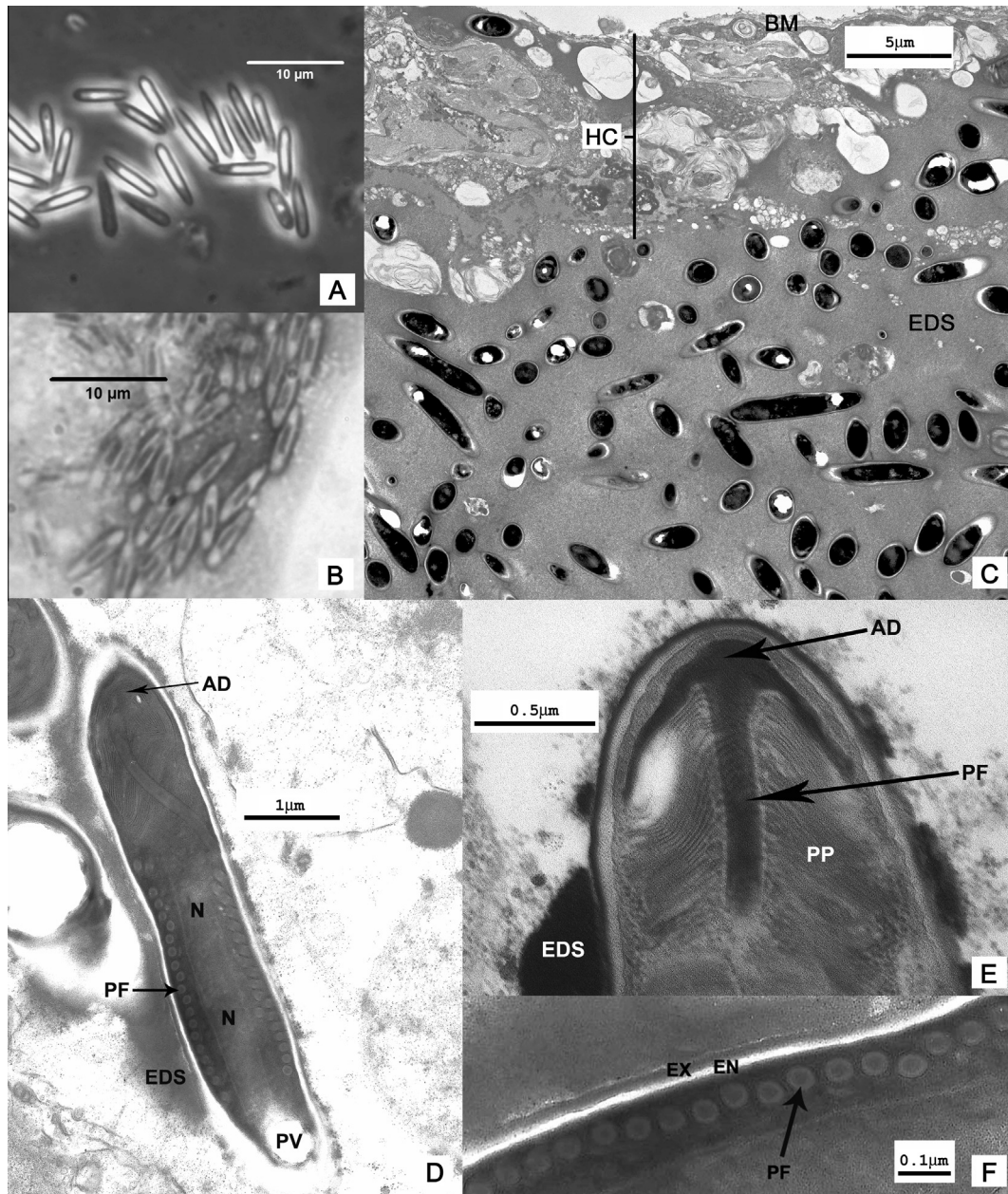
(Fig. 5A and C). During sporoblast formation, tubules were produced and connected the SV with the developing wall of the sporoblast (Fig. 4D). As sporogony progressed, tubules were reduced or disappeared completely (Fig. 5C–E).

### 3.2.7. Type locality

Florida, Alachua Co., Gainesville, SW 24th Ave., 29.630042°N, 82.464258°W, 26 August 2013.

*Additional collections.* Florida, Alachua Co., Gainesville, Petra Design Inc., 29.700115°N, 82.333452°W, 20 February 2014.

Florida, Nassau Co., Callahan, Callahan Waste Treatment Plant, 30.574514°N, 81.827069°W, 11 October 2013.



**Fig. 2.** Elongate-narrow binucleate spores (Type 2 DK) of *Myrmecomorba nylanderiae* in adult workers of *Nylanderia fulva*. (A and B) Groups of elongate-narrow binucleate spores in adult workers characterized by a highly refractive anterior end (A, fresh) and a prominent dark band in the region of the nuclei (B, stained). (C) Sporocytosac (cyst) containing mature spores (cross and longitudinal sections) embedded in the matrix of electron dense secreted material (EDS). The cyst wall is limited by a basement membrane (BM) below which is a region composed of host cells (HC) in various stages of deterioration. (D) Mature diplokaryotic spore (N, nucleus) with accumulations of electron dense secretions (EDS), the anchoring disk (AD), cross sections of the many coils of the polar filament (PF) arranged in a single row and a prominent posterior vacuole (PV). (E) Details of the anchoring disk-polar sack (AD), polar filament (PF), the uniform lamellar polaroplast complex (PP) and electron dense secretions (EDS). (F) The unlayered exospore (EX) is only slightly thicker than the electron lucent endospore (EN) and details of the cross-sections of the isofilar coils of the polar filament (PF) are illustrated. (A) fresh, phase-contrast; (B) giemsa-stained; (C–F) transmission electron micrographs.

Texas, Bastrop Co., Buescher State Park, 30.0414°N, 97.1618°W, 30 October 2014.

Texas, Brazoria Co., East Columbia, 29.1370°N, 95.6202°W, 1 May 2014.

Texas, Brazoria Co., Iowa Colony, 29.4348°N, 95.4443°W, 7 October 2014.

Texas, Comal Co., Guadalupe State Park, 29.7757°N, 98.1593°W, 7 October 2014.

Texas, Harris Co., Armand Bayou, 29.5980°N, 95.0649°W, 9 October 2013.

Texas, Wharton Co., El Campo, 29.2275°N, 96.3166°W, 7 October 2014.

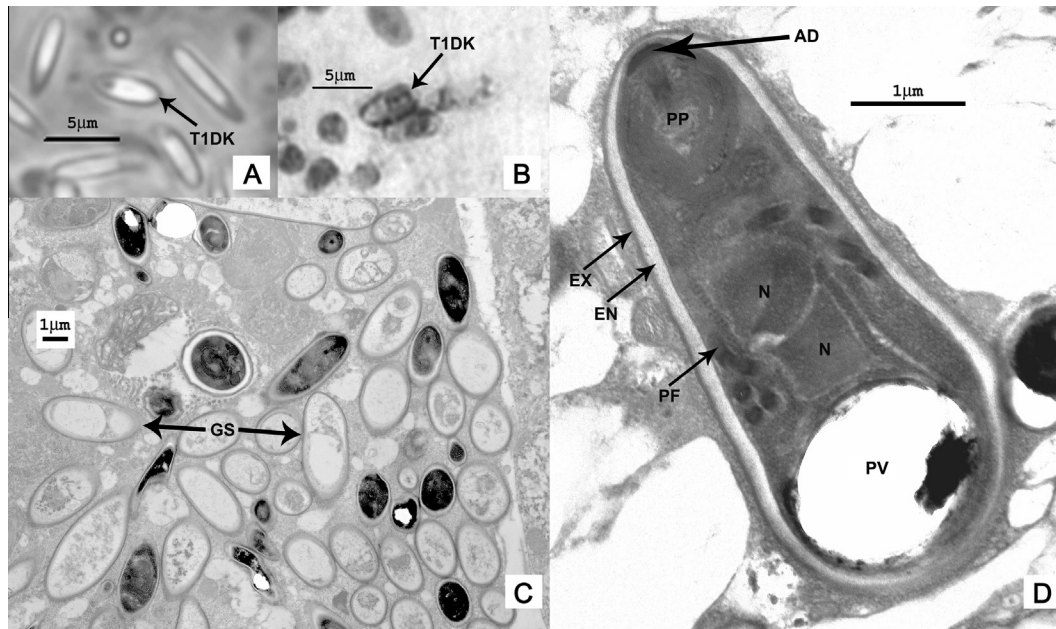
USVI, St. Croix, Mt Victory orchard, 17.747°N, 64.868°W, 15 December 2013.

### 3.2.8. Etymology

The genus name refers to this microsporidian as a disease of ants, a combination of *myrmeco-* (pertaining to ants) and *morbus* (disease). The specific name refers to the host ant genus of the type species.

### 3.2.9. Molecular characterization

The 16s rDNA nucleotide sequence making up the 1408-bp consensus assembly for *M. nylanderiae* was submitted to GenBank and



**Fig. 3.** Elongate-oblong binucleate spores (Type 1 DK) of *Myrmecomorba nylanderiae* in pupae and adult workers of *Nylanderia fulva*. (A and B) Mature spores Type 1 DK (T1DK) characterized by a highly refractive anterior end and large posterior vacuole (A, fresh) and a prominent dark band in the region of the nuclei (B, stained). (C) Region with numerous germinated spores (GS) and apparent lack of electron dense secretions. (D) Mature diplokaryotic spore (N, nucleus) demonstrating the anchoring disk (AD), a lamellar polaroplast (PP) and a large posterior vacuole (PV). Cross-sections of the short polar filament (PF) making 4–5 turns in the mid-section of the spore bound by an unlayered exospore (EX) approximately one-quarter the thickness of the thicker endospore (EN). (A) fresh, phase-contrast; (B) giemsa-stained; (C and D) transmission electron micrographs.

assigned Accession Number KR704917. NCBI Blastn scores for this sequence showed highest similarities with 3 microsporidia species of the Caudosporidae group (*Caudospora simulii*: Max score 1097, E-value 0.0, Identity 79%; *Polydispyrenia simuli*: Max score 1095, E-value 0.0, Identity 79%; *Weiseria palustris*: Max score 1077, E-value 0.0, Identity 79%).

Neighbor Joining phylogenetic analysis of the 16S rDNA sequences (Fig. 6) produced the same major clades reported by Vossbrinck and Debrunner-Vossbrinck (2005). The placement of *M. nylanderiae* was found to occur within Clade II and sister to the caudosporid parasites of simuliid black flies (Adler et al., 2000).

### 3.2.10. Deposition of type specimens

Two holotype slides have been deposited with the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, DC (USNM Nos. 1281267, 1281268). Additional paratype material including slides, specimens embedded in plastic resin, and host specimens in ethanol, are archived at the Center for Medical, Agricultural and Veterinary Entomology, USDA, Gainesville, Florida, and in the University of Texas Insect Collection, Austin, Texas.

### 3.2.11. Bionomics

*N. fulva* ant colonies infected with *M. nylanderiae* have been encountered at several sites across Texas, Florida and USVI St. Croix. In Texas this invasive ant species forms polydomous super-colonies of many interconnected nests that contain multiple queens (Horn, 2009). The spatial distribution of infected nests within populations was sometimes observed to be patchy potentially implying some barriers to horizontal transmission. The infection rates of individual workers from three infected nests were 6/8, 6/10 and 14/20 (infected/total). Workers with advanced infections show a characteristic level of reduced melanization with distended gasters and expanded intersegmental membranes (Fig. 7).

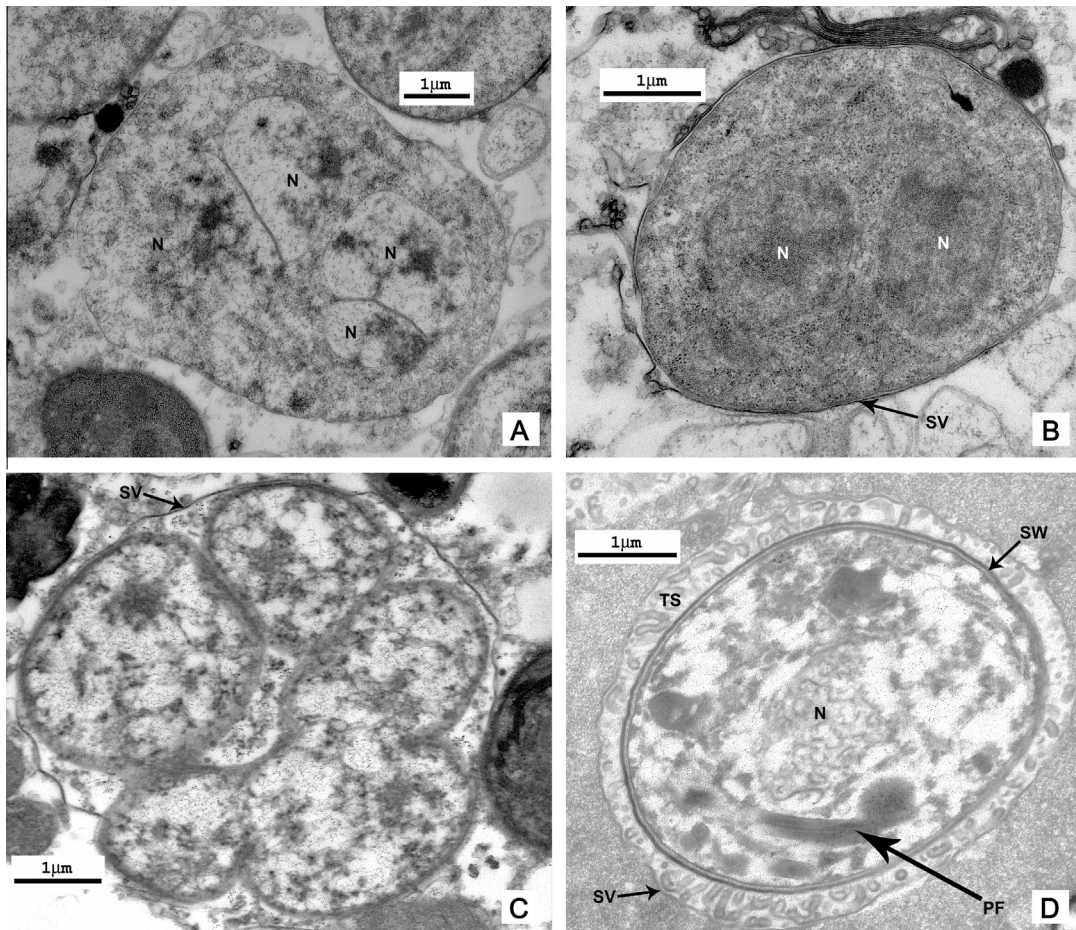
No populations have yet been tracked across sufficient time to determine the impacts of *M. nylanderiae* on ant mortality at a

colony level. The reported declines of some *N. fulva* populations in Florida (D. Oi pers. comm.) and St. Croix (Wetterer et al., 2014) have not been tied to infections by *M. nylanderiae* and should be examined further.

### 3.2.12. Remarks

*Myrmecomorba nylanderiae* represents a new genus with molecular affinities to Clade II of Vossbrinck and Debrunner-Vossbrinck (2008), which includes the Caudosporidae associated with aquatic larvae of black flies (Simuliidae) and sandflies (Psychodidae). Phylogenetic analysis revealed that *M. nylanderiae* is well separated from other microsporidian parasites of ants: *Kneallhazia solenopsae*, *K. carolinensae* and *Vairimorpha invictae*. (Specimens of the ant parasite *Burenella dimorpha* were not available for molecular analysis, but *B. dimorpha* is somewhat related to *V. invictae* from which it is readily distinguished by morphology (Table 2) and by its restricted association with host immature stages (Jouvenaz and Hazard, 1978)). Primary differences between *M. nylanderiae* and these other ant parasites are the spore sizes and polar filament coil numbers of Type 2 DK spores and octosporos. Type 2 DK spores in *M. nylanderiae* have 19–21 polar filament coils, while other species have less than 15 or more than 24 coils. Octosporos of *M. nylanderiae* are the smallest of those reported in ant parasites with the least number of polar filament coils (only 3–4 coils vs 9 or more in other species). *M. nylanderiae* is differentiated from other caudosporid microsporidia by the lack of caudae or other ornamentation of the exospores (Adler et al., 2000).

The four microsporidian parasitic genera of ants included in this analysis were placed in separate clades of the microsporidian phylogeny and this may indicate that several independent host-switching events have occurred to ants from ancestral hosts. Furthermore, these clades comprise mainly microsporidian parasites of aquatic organisms (Smith, 2009; Vossbrinck and Debrunner-Vossbrinck, 2005) implying that the ancestral hosts of these ant-parasitic microsporidia may have been aquatic organisms. *Myrmecomorba* is placed within Clade II of Vossbrinck and



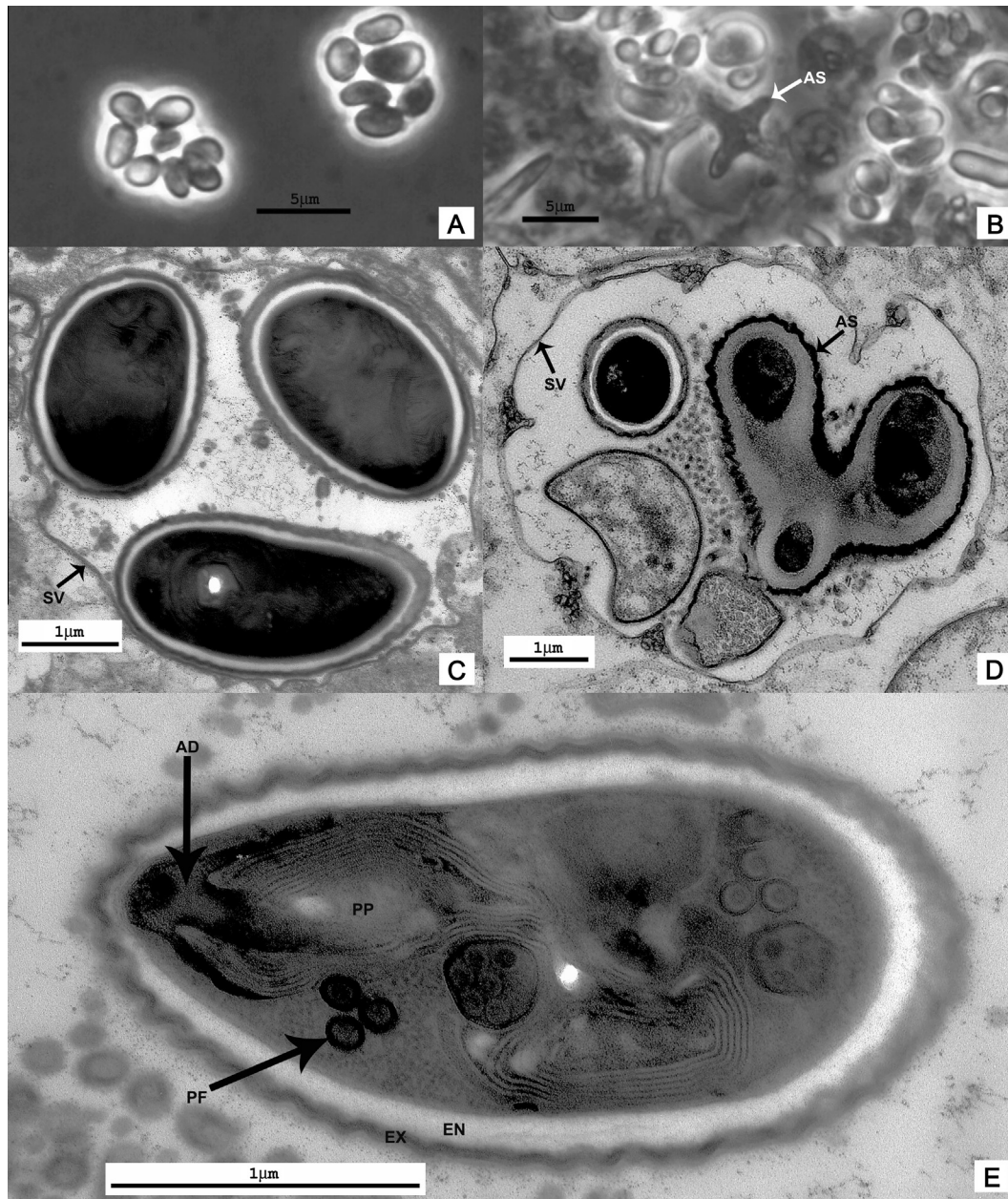
**Fig. 4.** Transmission electron micrographs of developmental stages in the aberrant octosporoblastic sporogony sequence of *Myrmecomorba nylanderiae* in adult workers of *Nylanderia fulva*. (A) Meront with two diplokarya prior to binary fission (N, nucleus). (B) Early diplokaryotic sporont (N, nucleus) with the initial formation of the sporophorous vesicle (SV). (C) Sporogonial plasmodium dividing within the SV to produce sporoblasts. (D) Early sporoblast with the two layers of the spore wall (SW) now evident and the initiation of polar filament formation (PF). Tubular secretions (TS) between the developing SW and the SV.

Debrunner-Vossbrink (2008) and in this analysis *Myrmecomorba* is more closely aligned with the subclade of aquatic caudosporid genera (*Flabelliforma*, *Polydispyrenia*, *Caudospora* and *Weiseria*) and more distant to the subclade of terrestrial genera (*Antonospora* and *Paranosema*). The direction of host switching between aquatic and terrestrial hosts within Clade II remains to be determined. Such host switching would entail a considerable shift in life history selective constraints to cope with the novel ant host environment. The frequency of such events remains to be evaluated requiring additional microsporidian ant parasite discoveries to expand the phylogeny. In the case of *Solenopsis*, several fairly thorough surveys, associated with interests in discovering fire ant pathogens, have revealed three different microsporidian genera (Briano et al., 1995; Jouvenaz et al., 1977, 1980; Jouvenaz and Hazard, 1978; Valles et al., 2011) giving an indication of potential diversity of microsporidia within other ant taxa.

Phylogenetic diversity based on 16S rDNA analysis across a geographic range may reflect the host–parasite relationship. For example, *K. solenopsae* occurs across the entire native ranges of *S. invicta* and *S. geminata* and was shown to comprise multiple strains with genetic distances reaching 3% between clades (Ascunce, 2010). Thus genetic divergence since host acquisition continues to evolve (Ascunce et al., 2010; Moser et al., 2000). In some microsporidia, the 16s rDNA gene exhibits multiple alleles within a spore that may confound measures of evolutionary diversity (Ironside, 2007). In the case of *M. nylanderiae* we found no nucleotide

dissimilarities between the Florida, Texas and St. Croix samples (262-bp) and no evidence that multiple alleles occur. In comparison to the observed diversity within *K. solenopsae*, this lack of genetic diversity in *M. nylanderiae* from multiple sites implies either a recent acquisition by *N. fulva* or a population bottleneck as may have occurred if *M. nylanderiae* had arrived with introductions of its ant host from a shared common origin.

The host–parasite relationships between microsporidia and ants are likely to reflect the eusocial nature of ant colonies resulting in different selective outcomes compared to those of non-eusocial hosts. There are several life cycle constraints faced by ant-hosted microsporidian species that may result in convergent evolution in this environment. Being parasites of social insects, there are opportunities for horizontal and vertical transmission within a colony, and there is a need for long-lived infections within a colony, and a need to overcome host social-immune responses. The interplay between vertical transmission via queens and horizontal transmission between colony individuals and potential alternate host species provides several routes for sustaining colonial infections and for inter-colonial transmission. Most microsporidia associated with ants have two spore types that could be dispersed between individuals, namely octosporous and Type 2 DK spores. In *B. dimorpha* both spore types are only found in 4th instar larvae and pupae of the host, *S. geminata*, and the infection is lethal at the pupal stage. Transmission of *B. dimorpha* within the colony is likely by ingestion of Type 2 DK spores from cannibalized pupae



**Fig. 5.** Octospores of *Myrmecomorba nylanderiae* in adult workers of *Nylanderia fulva*. (A) Mature spores contained within sporophorous vesicles (SV). (B) Aberrant spores (AS) within the SV demonstrating aborted divisions. (C) Group of 3 spores within the SV. (D) Aberrant spores (AS) with incomplete divisions within the SV. E. Mature spore demonstrating the anchoring disk (AD), lamellar polaroplast (PP) and cross-sections of the coils of the polar filament (PF). The endospore (EN) is approximately 3 times the width of the wavy, two-layered exospore (EX). (A and B) fresh, phase-contrast; (C–E) transmission electron micrographs.

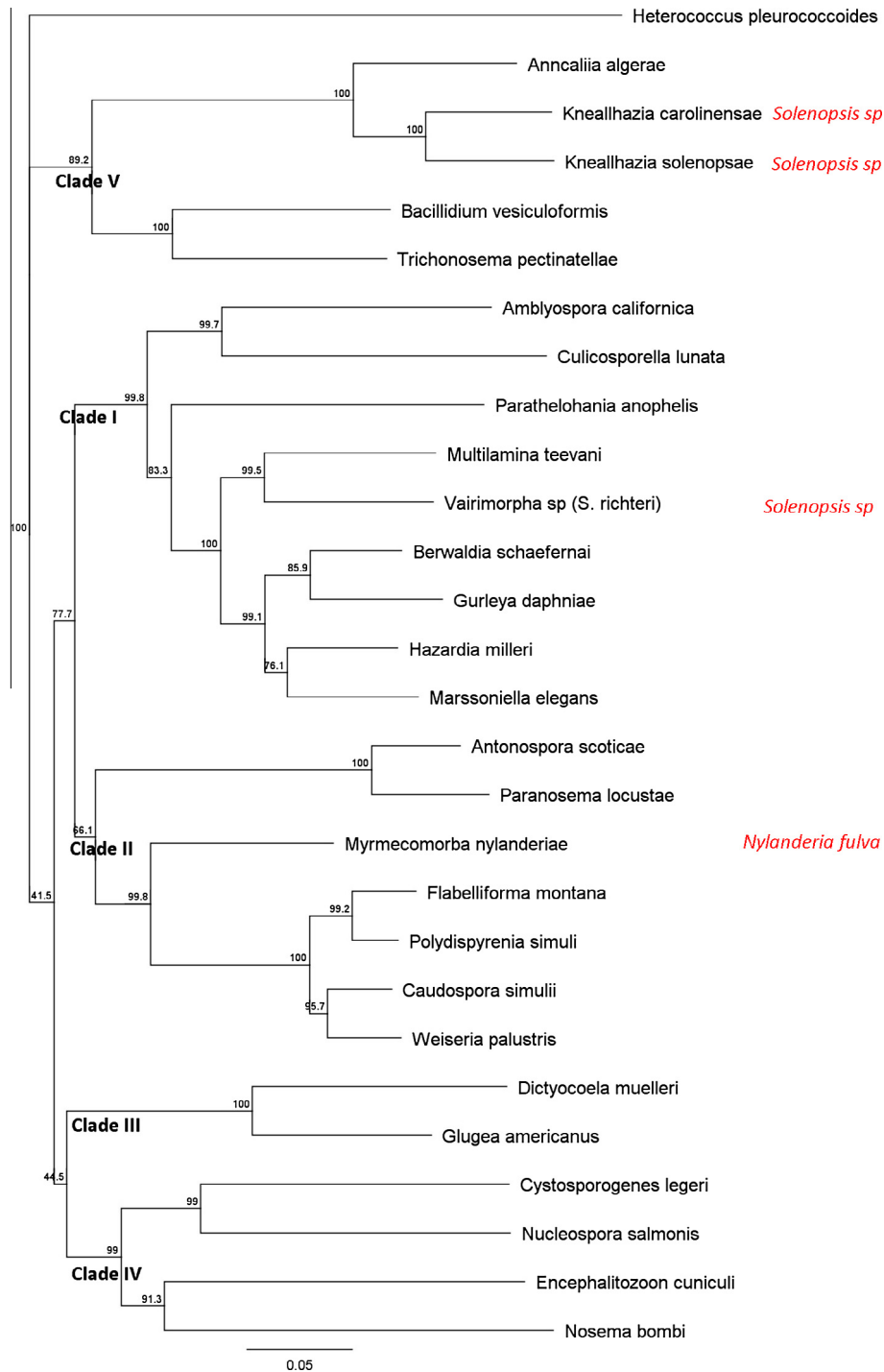
by 4th instar larvae while the octospores are non-infective to larvae and may serve as environmental spores or have an alternate host (Jouvenaz and Hazard, 1978; Jouvenaz et al., 1981).

In contrast, other microsporidian ant parasites, including *M. nylanderiae*, *V. invictae* and *K. solenopsae*, have been found in all ant life stages with a variety of spore types revealing an intricate life history with multiple infection pathways (Sokolova and Fuxa, 2008). Vertical transmission may occur transovarially through infected queens or potentially through mother-offspring social interactions (e.g. trophallaxis) while movement of infected larvae and workers provides a pathway for inter-nest horizontal transmission. It appears that Type 2 DK and octospores are associated with horizontal transmission given the delay in free spore production until the imaginal stage accompanied by high spore densities that occur in worker fat bodies. In *K. solenopsae*, Sokolova and Fuxa

(2008) suggested that octospores may serve primarily as environmental spores. Despite extensive studies of spore sequences, development and transmission in *K. solenopsae* the nature of inter-colony infection pathways is still undetermined. Overall the close similarities between spore types and development phases in *Myrmecomorba*, *Kneallhazia* and *Vairimorpha* species are remarkable given their suggested independent host acquisition and such similarities may be evidence of convergent evolution for overcoming host defenses of social insects and exploiting the unique horizontal and vertical transmission pathways that populations of ant colonies provide.

Large numbers of aberrant octospores were found arising from the octosporoblast sequence (Fig. 5B and D). While Sokolova and Fuxa (2008) depict several aberrant octospores in *K. solenopsae* (their Figs. 1 and 4) such abnormalities are apparently rare. In early





**Fig. 6.** Molecular phylogeny of microsporidian ant parasites and representatives of clades defined by Vossbrinck and Debrunner Vossbrinck, 2005. Neighbor Joining tree based on 16S rDNA with 1000 bootstrap replicates, showing bootstrap support values. Ant hosts where known are indicated on the right.

classification systems, octosporous sporogony to produce eight octospores within a sporophorous vesicle defined the genus *Thelohania* Henneguy 1892 (Kudo, 1924). Octosporoblastic sporogony is now known to occur in many different genera of microsporidia to include *Amblyospora*, *Burenella*, *Kneallhazia*, *Vairimorpha* and others (Jouvenaz and Ellis, 1986; Jouvenaz and Hazard, 1978; Lukeš and Vávra, 1990; Sokolova and Fuxa, 2008). In species of *Amblyospora*, diplokaryotic sporonts undergo meiosis and octosporoblastic sporogony to produce eight octospores within a sporophorous vesicle. These spores are infectious for a

copepod intermediate host where another spore type is formed that is infectious to mosquito larvae. In many species, such as ant-hosted *K. solenopsae*, *V. invictae* and *B. dimorpha*, the function of the spores produced in octosporous sporogony are unknown. While these species produce normal octospores there are other species that have been shown to exhibit an abortive octosporous sporogony that include *V. imperfecta* from a lepidopteran host and *Culicosporella lunata* and *Edhazardia aedis* from mosquito hosts (Becnel and Fukuda, 1991; Becnel et al., 1989; Canning et al., 1999). In these species there is a concurrent sporulation sequence



**Fig. 7.** *Nylanderia fulva* workers (Left: *M. nylanderiae* uninfected, Right: *M. nylanderiae* infected). Infected workers exhibit a characteristic reduced melanization, abdominal distension, and expansion of intersegment membranes.

that results in the production of large numbers of binucleate spores or uninucleate spores without the involvement of meiosis. It has been speculated for the *Vairimorpha*/*Nosema* clade and the *Amblyospora* clade that complex life-cycles are ancestral and that octosporoblastic sporogony has been completely lost by some, partially lost in others and retained by still others (Becnel et al., 2005; Canning et al., 1999). It has been suggested that some microsporidia in mosquitoes may be evolving away from meiosis and two-host systems to simpler, more efficient single-host systems (Becnel, 1994). The species described here from *N. fulva* with an

abortive octosporoblastic sporogony may represent another example of regressive evolution in microsporidia where this species has partially lost the ability to produce octospores (Becnel, 1994).

Several studies have investigated the influence of temperature on spore production in species with concurrent sporulation sequences. Octospore production was found to be enhanced at lower temperatures and inhibited at higher temperatures for *V. necatrix* and *B. dimorpha* (Jouvenaz and Lofgren, 1984; Maddox and Sprengel, 1978; Maddox, 1966). A subsequent study with *V. necatrix* found that both sporulation sequences occurred at temperatures of 15–30 °C (Mitchell and Cali, 1993) and that the octosporous sequence did not occur in *Vairimorpha* sp. 696 reared at 19 or 32 °C (Sedlacek et al., 1985). The abortive octosporous sporulation sequence for *E. aedis* in *Aedes aegypti* was not altered by rearing temperatures between 20 and 36 °C with few octospores produced regardless of the temperature (Becnel and Undeen, 1992). Whether temperature would influence the octosporous sequence in the species described here is unknown.

Given the ecological and economic importance of the ant host, *N. fulva*, the discovery of this pathogenic microsporidian has interest as a potential biological control agent since both *K. solenopsae* and *V. invictae* have been shown to have considerable impacts on colony health (Briano, 2005; Williams et al., 1999) and *K. solenopsae* has already been distributed as a biological control agent (Oi and Valles, 2009). Further studies are needed to elucidate the ecology of *M. nylanderiae* in both the introduced and native ranges of its ant host, and to evaluate its pathogenicity, transmission mechanisms, host specificity, fitness effects on ant life stages, and potential population level impacts.

**Table 2**

Microsporidian parasites of ants. Only records of microsporidian infections from field collections are included; some other ant species have been forcibly infected by microsporidia in the laboratory. All measurements in  $\mu\text{m}$ , annotated as measured from fresh or fixed material. Terminology: Free Spores are also referred to as NMB (non-membrane bound) and include several types (DK 1, 2, etc.). Type 1 DK binucleate free spores are also referred to as “juvenile”. Type 2 DK binucleate free spores are also referred to as “Nosema-like”. Octospores (uninucleate) are also referred to as “meiospores” or MB (membrane bound), and develop within a sporophorous vesicle (SV). PF = polar filament.

Microsporidia species	<i>Burenella dimorpha</i>	<i>Vairimorpha invictae</i>	<i>Kneallhazia solenopsae</i>	<i>Kneallhazia carolinensae</i>	<i>Myrmecomorpha nylanderiae</i>
Host ant species	<i>Solenopsis geminata</i>	<i>Solenopsis invicta</i> & <i>richteri</i>	<i>Solenopsis invicta</i> & <i>geminata</i>	<i>Solenopsis carolinensae</i>	<i>Nylanderia fulva</i>
Meronts	Two sequences of merogony	1st gen: 4.5–5 $\mu\text{m}$ . 2nd gen: 10–11 $\mu\text{m}$	Two sequences of merogony	Not reported	Two sequences of merogony. Binucleate meronts in queens, larvae & pupae. In eggs: binucleate sporoplasms
Type 1 DK	Not reported	Not reported	4.5 $\pm$ 0.10 $\times$ 2.3 $\pm$ 0.05 (fresh) 3.9 $\pm$ 0.33 $\times$ 2.0 $\pm$ 0.24 (fixed)	Not reported	5.57 $\pm$ 0.11 $\times$ 2.29 $\pm$ 0.05 (fixed)
PF coils			3–4		4–5
Host stage			Only in pupae, larvae L4		Adults & pupae
Type 2 DK	6.4 (6–7) $\times$ 2.9 (2.5–3) (fixed)	11.2 $\pm$ 3.4 $\times$ 3.1 $\pm$ 0.3 (fixed)	4.93 $\pm$ 0.58 $\times$ 1.85 $\pm$ 0.16 (fresh) 4.6 $\pm$ 0.29 $\times$ 2.3 $\pm$ 0.20 (fixed)	6.2 $\pm$ 0.1 $\times$ 3.1 $\pm$ 0.1 (fresh) 6.0 $\pm$ 0.1 $\times$ 3.4 $\pm$ 0.1 (fixed)	6.57 $\pm$ 0.09 $\times$ 1.59 $\pm$ 0.03 (fresh) 6.40 $\pm$ 0.07 $\times$ 1.83 $\pm$ 0.02 (fixed)
PF coils	26	24–26	11–13	12–15	19–21
Host stage	Only in 4th instar larvae and pupae; free spores in hypodermis	In fat bodies of workers	Only in adult castes, fat bodies	In fat bodies of adult workers. Other stages not examined	Worker fat bodies & alates
Megaspores	Not reported	Not reported	6.2 $\pm$ 0.41 $\times$ 3.6 $\pm$ 0.29 (fixed)	Not reported	Not seen
PF coils			19–23		
Host stage			Mainly in alates		
Octospores (meiospores)	6.1 (6–6.5) $\times$ 4.2 (4–4.5) (fixed)	6.3 $\pm$ 0.25 $\times$ 4.2 $\pm$ 0.7 (fixed)	3.32 $\pm$ 0.48 $\times$ 1.95 $\pm$ 0.20 (fresh) 3.1 $\pm$ 0.38 $\times$ 2.1 $\pm$ 0.23 (fixed)	Not reported	2.79 $\pm$ 0.08 $\times$ 1.86 $\pm$ 0.05 (fresh)
PF coils	57	9	9–12		3–4
Host stage	Only in pupal fat bodies	Late pupal stage, maturing in worker fat bodies	Late pupal stage, maturing in worker fat bodies and alates		Late pupal stage, maturing in worker fat cysts
References	Jouvenaz and Hazard (1978)	Jouvenaz and Ellis (1986)	Knell et al. (1977), Sokolova et al. (2004), Sokolova and Fuxa (2008) and Oi et al. (2001)	Valles et al. (2011)	

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