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Ultrastructure and molecular characterization of a microsporidium, *Tubulinosema hippodamiae*, from the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville

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ABSTRACT

Hippodamia convergens, the convergent lady beetle, is available for aphid control in home gardens and in commercial food production systems throughout the United States and Canada. Beetles received from commercial insectaries for biological control are occasionally infected with a microsporidium. The objective of this study was to describe the pathogen by means of ultrastructure, molecular characterization and tissue pathology. All stages of the microsporidium were in direct contact with the host cell cytoplasm. Early developmental stages were proximal to mature spores and both were observed throughout the tissue sections that were examined. Merogony resulted from binary fission. Early-stage sporoblasts were surrounded by a highly convoluted plasma membrane and contained an electron-dense cytoplasm and diplokaryon. Ovoid to elongated late-stage sporoblasts were surrounded by a relatively complete spore wall. The polar filament, polaroplast, and anchoring disk were readily observed within the cell cytoplasm. Mature spores were typical of terrestrial microsporidia, with a thickened endospore surrounded by a thin exospore. Spores contained well-defined internal structures, including a diplokaryon, lamellar polaroplast and a slightly anisofilar polar filament with 10–14 coils arranged in a single or double row. A prominent indentation was evident at the apical end of the spore wall proximal to the anchoring disk. Aberrant spores were also observed. These had a fully developed endospore and exospore but lacked any discernable internal spore structures, and were, instead, filled with lamellar or vesicular structures. Typical and aberrant spores measured 3.58 ± 0.2 × 2.06 ± 0.2 μm (n = 10) and 3.38 ± 0.8 × 2.13 ± 0.2 μm (n = 10), respectively. Spores were observed in longitudinal muscle surrounding the midgut and within the fat body, Malpighian tubules, pyloric valve epithelium, ventral nerve cord ganglia, muscles and ovaries. The hindgut epithelium was often infected but the connective tissues were rarely invaded. The life cycle and pathology of the microsporidium bears some resemblance to *Nosema hippodamiae*, the only microsporidium reported from *H. convergens* by Lipa and Steinhaus in 1959. Molecular characterization of the pathogen genomic DNA revealed that it is 99% similar to *Tubulinosema acridophagus* and *T. ratisbonensis*, two pathogens that infect *Drosophila melanogaster* and 98% similar to *T. kingi* from *D. willistoni*. Based on similarities in pathogen ultrastructure and the molecular information gained during this study, we propose that the microsporidium in *H. convergens* be given the name *Tubulinosema hippodamiae*.

1. Introduction

During the late 19th century, the Australian lady beetle, *Rhodolia cardinalis* Mulsant was imported and released for control of cottony cushion scale, *Icerya purchasi* Maskell, on California citrus (DeBach, 1964). The resounding success of this effort resulted in further exploitation of some predaceous lady beetle species for pest control in agroecosystems. One such example is the importation and release of convergent lady beetles, *Hippodamia convergens* Guérin-Méneville, for aphid control on field and greenhouse crops.

One hundred years ago, *H. convergens* were first collected from their winter aggregation sites in the Sierra Nevada Mountains of California for redistribution among orchard fruit and vegetable crops that were infested with aphids (Carnes, 1912), including thousands of acres of cantaloupes that were susceptible to the melon aphid, *Aphis gossypii* Glover. Control of this pest was so impressive that it prompted an ongoing tradition of collecting large aggregations of beetles during the winter months, subjecting them to conditions that prolong hibernation and shipping them for use as aphid control agents during the subsequent growing season.

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H. convergens are currently available for aphid control in home gardens and in commercial food production systems throughout the United States and Canada.

A problem associated with the mass collection and release of field specimens for augmentative biological control is the potential to inadvertently transport and release insect pathogens and parasitoids along with the host. In the case of *H. convergens*, some adults are parasitized by *Dinocampus coccinellae* Schrank, a common endoparasitoid of several lady beetle species, whereas others are infected with microsporidia, eugregarines and fungi (Bjørnson, 2008). Host ranges of these parasites and pathogens are unknown.

Nosema coccinellae, *N. tracheophila* and *N. hippodamiae* have been reported from lady beetles (Cali and Briggs, 1967; Lipa, 1968; Lipa et al., 1975) but *N. hippodamiae* is the only microsporidium described from *H. convergens*. The formal description of this pathogen is based on light microscopic observations of the pathogen life cycle and a description of the tissues that are infected (Lipa and Steinhaus, 1959). First recovered from *H. convergens* from California in 1959, *N. hippodamiae* infects primarily the midgut and fatbody of *H. convergens* pupae and adults. The pathogen divides by binary fission to produce ovoid spores that measure 3.3–5.4 × 2.2–2.7 μm. Another undescribed microsporidium that infects *H. convergens* has been the subject of recent studies (Joudrey and Bjørnson, 2007; Saito and Bjørnson, 2006, 2008; Bjørnson, 2008). In this paper, we describe this pathogen by means of ultrastructure, molecular characterization and tissue pathology. Where possible, comparisons will be made between the undescribed microsporidium and what is known about *N. hippodamiae*.

2. Materials and methods

Green peach aphids (*Myzus persicae* Sulzer) reared on nasturtium (*Tropaeolum nanum* L., Jewel Mixed, Stokes Seeds, ON) were used as food for *H. convergens*. Uninfected and microsporidia-infected beetles were reared individually in 120-ml clear, polyethylene cups (Canemco-Marivac Inc, QC). A 2.2-cm hole in the side of each cup was covered with fine mesh screen (80 μm, Bioquip, CA). Beetles were fed aphids and artificial diet (20 mL Lacewing and Ladybug Food, Planet Natural, MT; 20 mL honey; 1 mL water). Distilled water was provided daily on a moistened cotton roll (Crosstex International, NY). Plants, aphids and beetles were reared under controlled conditions (25 ± 1 °C, 16L:8D).

2.1. Pathogen ultrastructure

The infection status of individual larvae was confirmed through the microscopic examination of eggs and cohort larvae from individual parent females. Uninfected and infected larvae were reared independently within polyethylene cups until eclosion. Twelve specimens (6 uninfected and 6 infected adults) were processed simultaneously. The embedment protocol was repeated six times on six different dates (total specimens: 36 uninfected and 36 infected).

Sample fixation and embedding were performed according to the method outlined by Becnel (1997). Each specimen was dissected while submerged in 2.5% gluataraldehyde. The elytra, legs and thorax of each specimen were removed to maximize penetration of the fixative. One longitudinal and two lateral incisions were made, dividing the abdomen into six sections. Adults were examined because their tissues are more substantial than larval tissues. Tissues were embedded in Epon 812/Spurr resin. Specimens were placed under vacuum (15 PSI) while in 100% resin.

A Leica UCT ultramicrotome was used to cut ultra-thin sections. These were placed onto Formvar®-coated grids and were then stained with uranyl acetate and lead citrate. Digital micrographs were generated with a GATAN ES500W Erlangshen CCD camera that was side mounted to an Hitachi H7500 transmission electron microscope (operating at 80 kV). Imaging software (Zeiss Axiovision) was used for measuring spores.

2.2. Tissue pathology

Ten *H. convergens* virgin females were randomly selected from uninfected and microsporidia-infected laboratory colonies. Females were mated with males from the uninfected colony and mated pairs were provided aphids, artificial diet and distilled water for a minimum of 7 days. Females were confirmed as either uninfected or infected by examining the eggs produced by each female for microsporidian spores.

Twenty live specimens (10 uninfected and 10 infected) were processed for light microscopic examination in accordance to the protocol outlined by Becnel (1997). Uninfected *H. convergens* females (controls) were examined to detect tissue abnormalities in microsporidia-infected conspecifics. Whole specimens were immersed in Carnoy's fixative. While immersed, the head, pronotum, elytra, hindwings and legs of each specimen were removed to maximize infiltration of the fixative. Additional openings were made in the body by inserting a fine dissecting needle under the metasternum and making small incisions on both sides of the abdomen between ventrite 4 and 5. Two minor modifications were made to Becnel's procedure: (1) tissues were left in 100% ethanol:100% butanol (1:1) overnight (rather than for 2 h), and (2) tissues were later immersed in 100% butanol: paraffin (3:1) for 20 min, then 100% butanol:paraffin (1:1) overnight (both in a 60 °C oven) prior to being immersed in 100% paraffin. Whole abdomens of female beetles were embedded in Paraplast® Plus (Sigma–Aldrich Inc., melting point 56 °C).

Eight specimens (4 uninfected and 4 infected) were sectioned with a rotary microtome (5 μm): 4 specimens were sectioned longitudinally (2 uninfected and 2 infected) and the other 4 were sectioned parasagittally. Eight slides were prepared for each specimen: 12 sections (2 rows of 6 consecutive sections) were mounted on each microscope slide that had been coated with a thin film of protein solution (1 g gelatin, 2 g solid phenol, 15 ml glycerin, 100 ml distilled water) to improve specimen adherence. Before the sections were mounted, a few drops of 1% formalin were placed on each slide so that the sections would float to the surface of the protein mixture. Microscope slides with sections were placed on a slide warmer (40 °C, Fisher Scientific) overnight to dry completely. Sections were stained with Harris Hematoxylin (Fisher Scientific, SH30–500D) and Alcoholic Eosin Y (Fisher Scientific, SE22-500D).

2.3. Molecular characterization

Microsporidia genomic DNA was extracted from whole *H. convergens* eggs that were less than 24 h old. DNA was isolated and purified with a DNeasy® Blood & Tissue Kit (50; Qiagen) following the manufacturer's suggested protocol. Design of the primers (18f (CACCAGGTTGATTCTGCC)/1492 (GGTTACCTTGTTACGACTT); Eurofins Laboratories) were based on those described by Vossbrinck et al. (2004). DNA was amplified by Polymerase Chain Reaction (PCR) with the following the protocol: (1) 4 min at 95 °C (1 cycle); (2) 45 s at 95 °C followed by 45 s at 45 °C and 90 s at 72 °C (35 cycles); and (3) 7 min at 72 °C (1 cycle). Molecular cloning with pGEM®-T and pGEM®-T Easy Vector Systems (Promega Corp.) was performed to check for the possibility of multiple microsporidian DNA sequences. Then plasmid DNA with the successful insert was extracted with PureYield™ Plasmid Miniprep System (Promega Corp.), following the recommended protocol and sent to Macrogen (Korea) for sequencing.

Sequences of *Tubulinosema acridophagus*, *T. kingi*, *T. ratisbonensis* and *Thelohania solonopsae* were obtained from NCBI GeneBank. *T. solonopsae* was used for comparison as an outgroup (see Franzen et al., 2005). Automated sequence outputs were examined using chromatographs. Multiple sequences were automatically aligned using ClustalX, version 1.81 with default parameters (Thompson et al., 1997). Phylogenetic analyses were based on the comparison of 1371 base pairs.

Phylogenetic analysis was carried out using the maximum-parsimony (MP) method using PAUP*, version 4 beta 10 Win (Swofford, 2003). All characters were treated equally and were specified as unweighted and unordered. Indels longer than 3 bp were excluded from the analysis. Most-parsimonious trees were obtained through a heuristic search using the Tree Bisection-Reconnection (TBR) option. Overall character congruence was estimated by the consistency index (CI) and retention index (RI). Bootstrap values with 1000 replications were calculated using the PHYLIP package, version 3.6b (Felsenstein, 1993) by performing a heuristic search using the TBR option.

2.4. Host specificity

To determine whether the *H. convergens* microsporidian isolate is one of the two *Tubulinosema* species described from *Drosophila*, an attempt was made to infect *Drosophila* with this pathogen. Wild-type *Drosophila melanogaster* Meigen adults (Wards Natural Science, ON) were anesthetized with ether. Flies were sexed and each female was isolated in a rearing container ($n = 10$). Meridic diet consisting of Instant *Drosophila* Medium (Wards Natural Science, ON) hydrated in sterile water with five grains of yeast (*Saccharomyces cerevisiae* Meyen ex E.C. Hansen). Three drops of antibiotic solution (0.5% Penicillin G., Fisher Scientific) was added to prevent bacterial growth within the medium. Vials were stoppered with sponge plugs and maintained under controlled conditions (25 ± 0.5 °C, dark).

Females that emerged were used for rearing progeny. When larvae began to emerge from the media to pupate, each female was removed from the rearing container, smeared, stained with 5% Giemsa and examined for pathogens. After pupation, 20 female progeny were isolated within rearing vials (one female per vial) that had been previously inoculated with microsporidian spores. The inoculum consisted of four drops of a suspension of crushed microsporidia-infected *H. convergens* eggs and larvae in distilled water that had been transferred by pipette onto the surface of the diet. Once progeny were visible within the diet, the parent female was removed from the rearing container and examined for microsporidian spores by light microscopy. Ten larvae from each vial were also examined for microsporidian spores.

3. Results

3.1. Pathogen ultrastructure

All stages of the microsporidium were in direct contact with the host cell cytoplasm. Early developmental stages were proximal to mature spores and both were observed throughout the tissue sections that were examined. All developmental stages were diplokaryotic but occasionally meronts and sporonts with a single nucleus were observed. When diplokarya were present, their combined shape was round to ovoid with a shared central nuclear membrane that defined the two nuclei (Fig. 1). The shared membrane was not always prominent.

The meront was round to slightly ovoid (Fig. 1). Surrounded by a thin plasma membrane, the cytoplasm possessed many free ribosomes and evidence of a weakly developed endoplasmic reticulum.

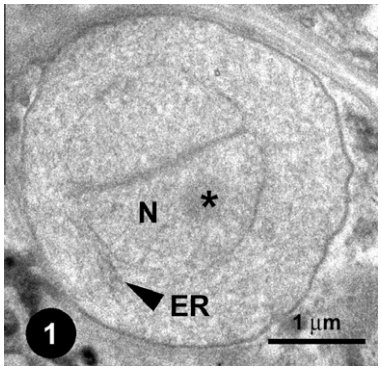

Fig. 1. Diplokaryotic meront in direct contact with the host cell cytoplasm. The sporoplasm is surrounded by a thin plasma membrane and contains many free-floating ribosomes, a developing endoplasmic reticulum (ER) and a distinct diplokaryotic nucleus (N). Condensed chromatin (*) is visible within the lower half of the diplokaryon. Scale bar: 1 μm.

The diplokaryon occupied approximately two-thirds of the cell and condensed chromatin was occasionally observed within it. Meronts proliferated by means of binary fission (Fig. 2). Sporonts were surrounded by an electron dense membrane (Fig. 3) and the sporogonial cytoplasm contained a well-developed and prominent endoplasmic reticulum and few free ribosomes.

Early-stage sporoblasts (Fig. 4) were surrounded by a plasma membrane that often appeared highly convoluted. The cytoplasm of these cells was electron dense. Cell nuclei were observed frequently within but these were not always observed as distinct diplokarya. Other internal structures, including the polaroplast and polar filament, were not observed. Ovoid to elongated, late-stage sporoblasts (Figs. 5 and 6) were surrounded by a relatively complete spore wall. The polar filament, polaroplast, and anchoring disk were readily observed within the cell cytoplasm where these structures appeared to undergo various stages of development and organization. The exospore appeared to develop at the same time as the polar filament, whereas the endospore, along with other internal spore structures such as the polaroplast and polar vacuole, developed afterward. Cell nuclei were not observed within the ovoid sporoblasts.

Both typical and aberrant spores were observed. Mature spores (Figs. 7–9) were slightly pyriform and had a thickened endospore that was surrounded by a thin exospore (spore wall thickness, 0.32 ± 0.04 μm, $n = 10$). These spores contained well-defined internal spore structures, including the diplokaryon, polaroplast and polar filament. The slightly anisofilar polar filament possessed 10–14 coils ($n = 10$) and was arranged in a single or double row.

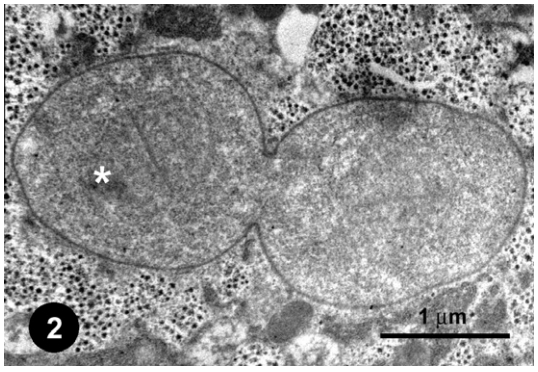

Fig. 2. Diplokaryotic meront undergoing binary fission. Condensed chromatin (*) is evident within the diplokaryon. Scale bar: 1 μm.

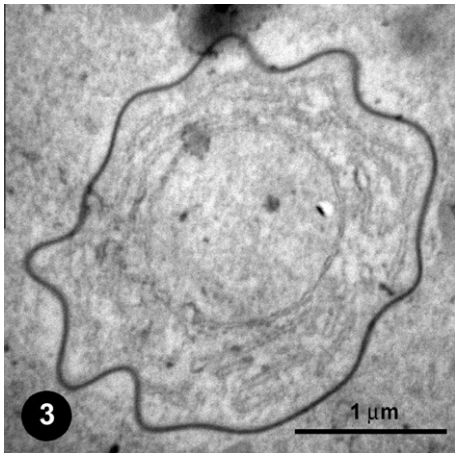

Fig. 3. Sporont characterized by a thick membrane that surrounds the cell and a well-developed rough endoplasmic reticulum that surrounds the diplokaryon. Scale bar: 1 μm.

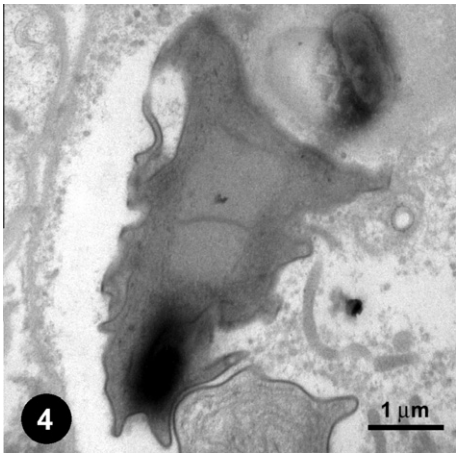

Fig. 4. An irregular-shaped early-stage sporoblast displaying a distinct diplokaryon within an electron-dense cytoplasm. Scale bar: 1 μm.

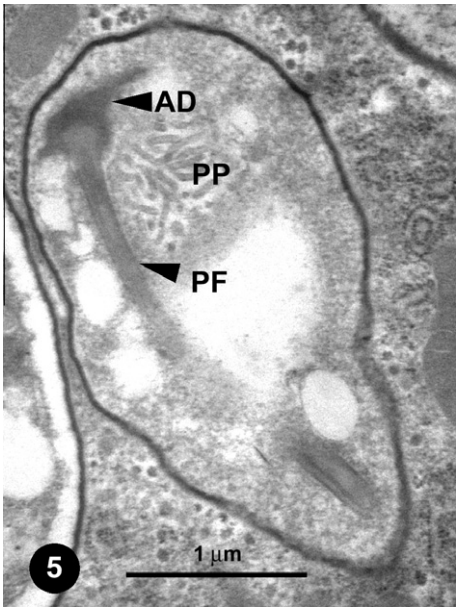

Fig. 5. Late-stage sporoblast showing early development of polar filament (PF), anchoring disk (AD) and polaroplast (PP). Scale bar: 1 μm.

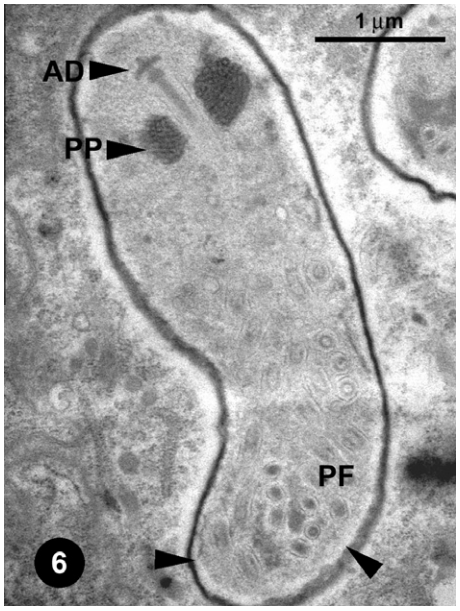

Fig. 6. Late-stage sporoblast displaying early development of the spore wall (arrow). The polar filament (PF), lamellar polaroplast (PP) and anchoring disk (AD) are well developed. Scale bar: 1 μm.

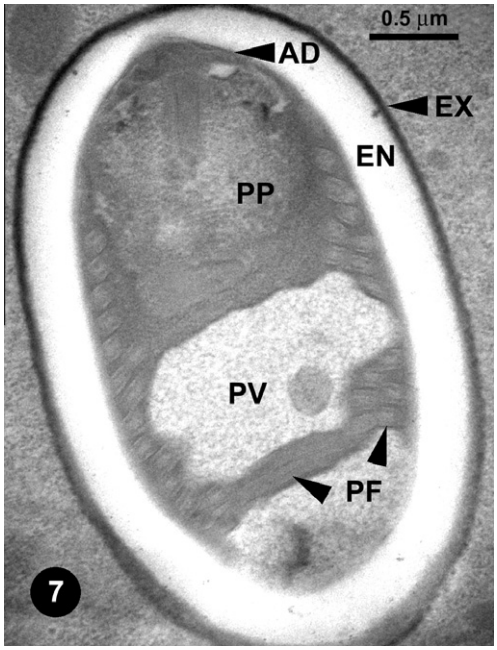

Fig. 7. Mature spore with a fully developed cell wall composed of the exospore (EX) and thickened endospore (EN). An isofilar polar filament (PF) is arranged in two layers. A lamellar polaroplast (PP) and polar filament anchoring disk (AD) are located within the apical end of the spore. A polar vacuole (PV) occupies the posterior half of the spore. Scale bar: 0.5 μm.

Coils were perpendicular to the longitudinal axis of the spore. The diplokaryon occupied approximately two-thirds of the spore and was surrounded by rough endoplasmic reticulum. A lamellar polaroplast occupied about one-third of the anterior region (Fig. 7) and a polar vacuole occupied approximately one-third of the posterior region. However, the polar vacuole was not consistently observed. A prominent indentation was evident at the apical end of the spore wall proximal to the anchoring disk (Fig. 8). Aberrant spores (Figs. 10–13) had a fully developed endospore and exospore but

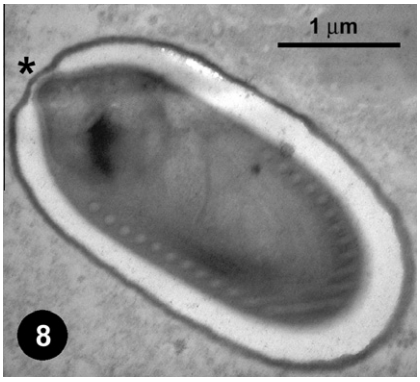

Fig. 8. Mature spore. An isofilar polar filament is arranged in a single layer. A thinning of the spore wall is apparent at the apical end of the spore (*). Scale bar: 1 μm.

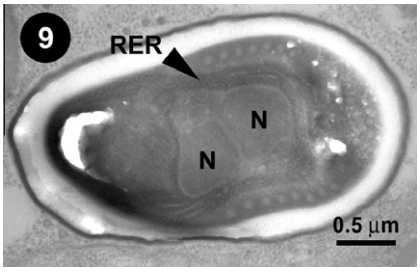

Fig. 9. Mature spore displaying a well-defined diplokaryon (N), rough endoplasmic reticulum (RER) surrounding the nucleus, and isofilar polar filament arranged in a single layer. Scale bar: 0.5 μm.

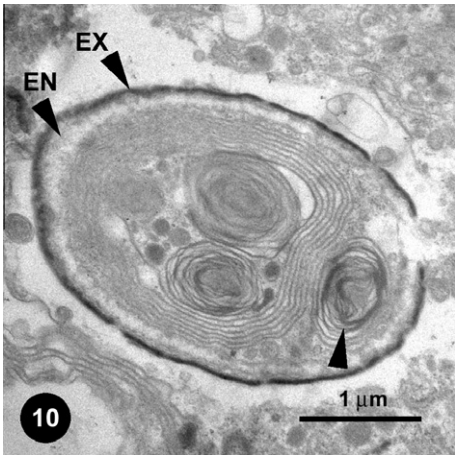

Fig. 10. An early aberrant spore displaying developing exospore (EX) and endospore (EN). Multi-layered concentric rings reminiscent of endoplasmic reticulum are seen surrounding an array of unidentified internal structures including two, centrally-located, large circular masses. A third, large circular mass, located towards the apical portion of the spore surrounds a structure that resembles an underdeveloped anchoring disk (arrow). Scale bar: 1 μm.

lacked any discernable internal spore structures. Instead, they were filled with lamellar or vesicular structures. Aberrant spores were relatively abundant, representing 30–35% of all observed spores. The spore wall was 0.27 ± 0.08 μm (n = 10). This measurement was slightly thinner than for 'normal' spores. Aberrant spores were observed in proximity to typical spores and both spore types were observed throughout all tissues examined. Typical and aberrant spores measured 3.58 ± 0.2 × 2.06 ± 0.2 μm (n = 10) and 3.38 ± 0.8 × 2.13 ± 0.2 μm (n = 10), respectively. Both mature and

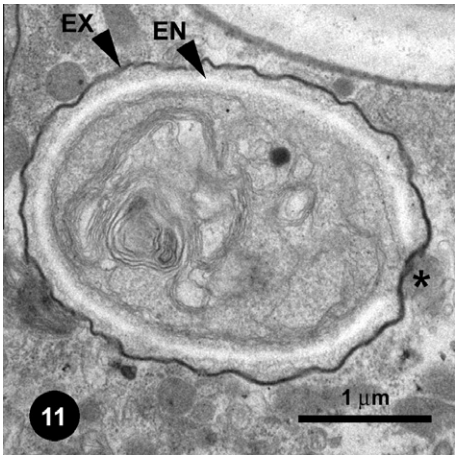

Fig. 11. Aberrant early spore displaying a distinct indentation (*) at the apical end of the developing spore wall. The developing exospore (EX) runs continuously around the early spore; however, the endospore (EN) is discontinuous through the apical indentation. Unidentified vesicular structures occupy the spore interior. Scale bar: 1 μm.

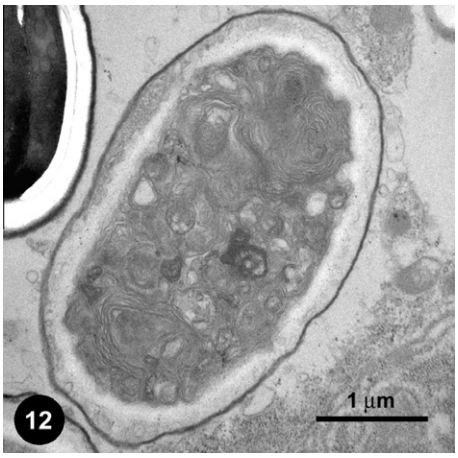

Fig. 12. Aberrant early spore with densely-packed, unidentified vesicular structures that appear unordered within the spore interior. The developing exospore and endospore are relatively uniform in thickness. Scale bar: 1 μm.

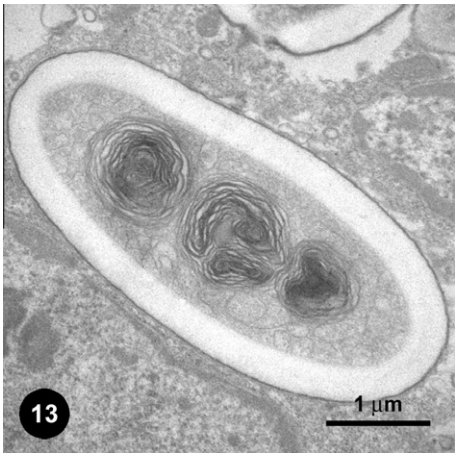

Fig. 13. Aberrant mature spore with fully developed spore wall and three unidentified, centrally-located vesicular masses. These masses are electron dense occupy a significant portion of the spore and appear to be the only structures developing within the spore interior. Scale bar: 1 μm.

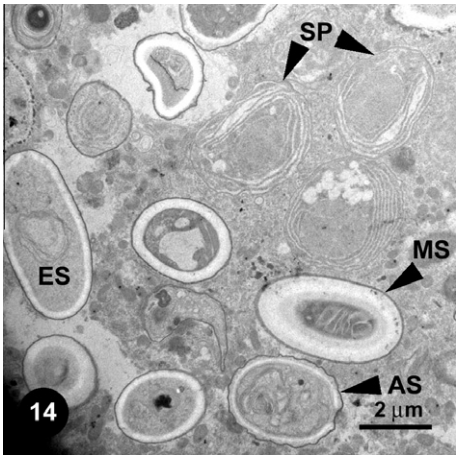

Fig. 14. Various life cycle stages of the microsporidium developing in proximity to one another. Sporonts (SP), mature spore (MS), aberrant early spore (AS, see Fig. 11), evacuated spore (ES). Scale bar: 2 µm.

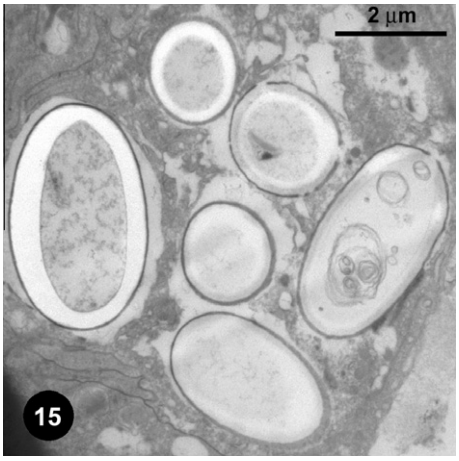

Fig. 15. Evacuated microsporidian spores providing evidence of autoinfection. Scale bar: 2 µm.

aberrant spores were observed together (Fig. 14). Evacuated spores (Fig. 15) provided evidence that autoinfection does occur. Sporophorous vesicles were not observed.

3.2. Tissue pathology

Organs and tissues in *H. convergens* were identified according to Landis (1936), Pradhan (1939), and Rothschild et al. (1986). Microsporidian spores were observed in longitudinal muscle surrounding the midgut and within the fat body, Malpighian tubules, pyloric valve epithelium, ventral nerve cord ganglia, muscles and ovaries. The hindgut epithelium of one of the four *H. convergens* females examined was not infected. Connective tissues were rarely invaded. Significant tissue abnormalities were not observed when uninfected tissues (from *H. convergens* controls) were compared to the same tissues in microsporidia-infected *H. convergens* females. Infected cells of susceptible organs were occasionally hypertrophic due to the large number of spores within them. Encapsulation and melanization were not observed.

3.3. Molecular characterization

Only one sequence was produced from eight successful clones. This suggested that only one species of microsporidia was present in *H. convergens*. According to the National Center for Biotechnology Information (NCBI) BLAST analysis, this genome sequence was 99% similar to *Tubulinosema acridophagus* (AF024658; 1361/1368 identical nucleotides) and *T. ratisbonensis* (AY695845; 1360/1368) from hosts *Schistocerca americana* (Drury), and *Drosophila melanogaster*, respectively, and 98% similar to *T. kingi* (DQ019419; 1348/1368) from *D. willistoni* Sturtevant.

Phylogenetic analysis included 1079 consistent characters, 284 were parsimony-uninformative and eight parsimony-informative. CI and RI were 0.993 and 0.750, respectively. The resulting phylogenetic tree (Fig. 16) shows that the *H. convergens* microsporidium (*T. hippodamiae* n. sp.) forms a sister group to *T. kingi*, *T. acridophagus* and *T. ratisbonensis.*

3.4. Host specificity

Microsporidian spores were not detected in any of the smear preparations that were made from *D. melanogaster* adults or larvae.

4. Discussion

Microsporidia have been described from several lady beetles species; however, *Nosema hippodamiae* Lipa and Steinhaus is the only microsporidium known to infect *Hippodamia convergens* (Lipa and Steinhaus, 1959). Although the description of *N. hippodamiae* is based on light microscopic observations (pathogen life cycle, tissue pathology and spore dimensions), *N. hippodamiae* bears considerable resemblance to the microsporidian pathogen that was the focus of this study.

Molecular characterization of the microsporidium in *H. convergens* revealed that it is 99% similar to *Tubulinosema acridophagus* and *T. ratisbonensis*, and 98% similar to *T. kingi*. According to Franzen et al. (2005), microsporidia belonging to the genus *Tubulinosema* share several distinct characteristics. These include diplokaryotic nuclei, all stages in direct contact with the host cytoplasm and a slightly anisofilar polar filament that is commonly arranged in one, sometimes two, rows on both sides of the diplokaryon. The microsporidium that was examined during this study also shared these ultrastructural characteristics.

4.1. Pathogen ultrastructure

Both *T. ratisbonensis* and *T. kingi* are pathogens of laboratory-reared *Drosophila melanogaster*. There are several similarities in the ultrastructure of *T. ratisbonensis*, *T. kingi* and the undescribed microsporidium from *H. convergens.* Merogony of these microsporidia results from binary fission. In the case of *N. hippodamiae*, Lipa and Steinhaus (1959) note that the pathogen undergoes schizogony, a process that is defined broadly as proliferation by means of binary or multiple fission (Sprague et al., 1992). Schizogony, as described by Lipa and Steinhaus for *N. hippodamiae*, involves the

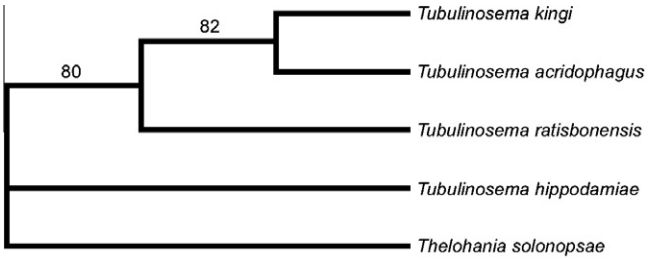

Fig. 16. Maximum-parsimony analysis of small subunit rRNA gene showing the relationship of *Tubulinosema hippodamiae* n. sp. with other microsporidia of the same genus. *Thelohania solonopsae* was included as the outgroup species (CI = 0.993, RI = 0.750).

division of schizonts into two daughter cells (binary fission) and seems to be synonymous with the term merogony.

Microsporidia of the genus *Tubulinosema* are characterized by the appearance of tubular structures on the surface of late-stage meronts. These were not observed on the meronts of the microsporidium from *H. convergens.* It is possible that tubular structures were rather short-lived during pathogen development, appearing with such low frequency that they were not observed. Franzen et al. (2006) observed small tubuli on the surface of *T. ratisbonensis* meronts more frequently than on *T. kingi* meronts. Both early- and late-stage sporoblasts were observed during this study. Early-stage sporoblasts had an electron-dense cytoplasm with visible diplokaryon and was surrounded by a highly convoluted membrane. This type of sporoblast bears close resemblance to the early sporoblast of *T. kingi*, described by the authors as a somewhat shrunken cell having a star-like formation (Franzen et al., 2006).

Spores of the undescribed microsporidium were slightly pyriform, measuring 3.6×2.4 μm (methanol- fixed and Giemsa stained; Joudrey and Bjørnson, 2007). This is consistent with what is reported for *N. hippodamiae* (oval, 3.3–5.4 × 2.2–2.7 μm fixed and stained; Lipa and Steinhaus, 1959). *T. ratisbonensis* and *T. kingi* also have slightly pyriform spores that measure $3.85 \pm 0.05 \times 2.36 \pm 0.05$ μm and $3.6 \pm 0.06 \times 2.4 \pm 0.05$ μm (mean ± SE, methanol-fixed and Giemsa-stained), respectively. As is the case for *T. ratisbonensis* and *T. kingi*, spores of the unidentified microsporidium had a slightly anisofilar polar filament, a thick endospore that was notably thinner near the anchoring disk, and a thin exospore. The polar filament of both *T. kingi* and the microsporidium from *H. convergens* consisted of 10–14 coils arranged in 1–2 rows within the spore, whereas the polar filament of *T. ratisbonensis* was coiled 9–14 times, predominantly in 1 row (Franzen et al., 2005, 2006).

Despite these stated similarities in pathogen ultrastructure and gene sequences among the microsporidium in *H. convergens* and those of the genus *Tubulinosema* (see Franzen et al., 2005, 2006), the former pathogen did not infect wild-type *D. melanogaster* when adult females were reared on diet to which crushed, microsporidia-infected *H. convergens* eggs and larvae were added. The interpretation of this result should be made with care because positive controls were not used to demonstrate successful transmission in the original host. Spores are likely transmitted from contaminated surfaces or specimens to uninfected individuals. Under laboratory conditions, transmission of the pathogen in *H. convergens* occurs both horizontally and vertically (Joudrey, 2006; Saito and Bjørnson, 2006). This may also be the case for *T. ratisbonensis* infections of laboratory-reared *D. melanogaster* as the origin of this infection is unknown (Franzen et al., 2005).

Based on spore ultrastructure, a characteristic unique to the microsporidium from *H. convergens* was the production of both typical and aberrant spores. Typical spores contained a diplokaryon and internal spore structures were evident; however, aberrant spores were filled with lamellar or vesicular structures. Both typical and aberrant spores were of similar size and spore dimorphism was not observed. The significance of aberrant spores is unclear. In some cases, the development of microsporidian spores is affected by the type of host tissues that are invaded. For example, *Vairimorpha disparis* spores fail to mature in specific tissues of the gypsy moth, *Lymantria dispar* L. (Vavra et al., 2006) but, for the *H. convergens* microsporidium, both typical and aberrant spores were found interspersed in all infected host tissues. Alternatively, the presence of aberrant spores may provide circumstantial evidence that *H. convergens* is not the natural host for this microsporidium. Under laboratory conditions, the pathogen infects several coccinellid species, including *Adalia bipunctata*, *Coccinella septempunctata*, *C. trifasciata perplexa*, and *Harmonia axyridis* (Saito and Bjørnson, 2006, 2008). Whether or not the pathogen produces aberrant spores in these hosts is unknown. Although the microsporidium in *H. convergens* originated from beetles that had been purchased from a commercial insectary, the pathogen may be native to another coccinellid host with tissues that are more conducive to its development. If this is the case, the tissues of *H. convergens* may not provide an adequate environment to support the development of all spores to maturity. It is also possible that the production of aberrant spores is part of the natural life cycle of the pathogen.

The size and shape of microsporidian spores observed during this study ($3.58 \pm 0.2 \times 2.06 \pm 0.2$ μm, $n = 10$, slightly pyriform) are also consistent with what is reported for *N. coccinellae* (3.6–6.2 × 2.0–3.6 μm (fixed and stained); Lipa, 1968; Lipa et al., 1975) and *N. tracheophila* (3.1–4.4 × 1.9–3.2 μm; Cali and Briggs, 1967) from other coccinellid hosts (Table 1). Slight variations in spore dimensions among the microsporidia that infect coccinellids could be attributed to differences in fixation methods and study techniques. Past descriptions of microsporidia from lady beetle hosts have been based on light microscopic study, and because the pathogen from *H. convergens* has a relatively broad host range (Saito and Bjørnson, 2008); questions regarding the true identity of these pathogens remain unanswered.

4.2. Tissue pathology

Nosema hippodamiae, *N. coccinellae*, and *N. tracheophila* infect distinct tissues in their respective hosts (Table 1). Although the host ranges of these pathogens have not been fully investigated, there is some reported host overlap. This suggests that a particular microsporidium may invade different tissues depending on the host species that is infected. Evidence of tissue specificity was observed when *C. septempunctata* and *H. axyridis* were infected with the microsporidium that is the subject of this study (Saito, 2008). In this case, the pathogen did not infect the hindgut epithelium of *C. septempunctata* and neither the hindgut epithelium or connective tissues of *H. axyridis*.

N. hippodamiae, the only microsporidium reported from *H. convergens*, infects the midgut epithelium, fat body, and other unspecified tissues when infection is severe (Lipa and Steinhaus, 1959). The unidentified microsporidium in this study was not observed in the midgut epithelium but spores were present in the fat body and in several other tissues, including the muscles surrounding the midgut, in the Malpighian tubule epithelium, the pyloric valve epithelium, hindgut epithelium, ventral nerve cord ganglia, muscles, connective tissues, and ovaries. The differences in tissue pathology may be attributed to the study techniques that were employed: smear preparations and dissections of *N. hippodamiae*-infected beetles were examined by Lipa and Steinhaus, whereas tissue sections were examined during this study.

The unidentified microsporidium infects both *Coccinella septempunctata* and *Adalia bipunctata* under laboratory conditions (Saito and Bjørnson, 2008), invading the fat body of both hosts but the pathogen was not observed in the midgut epithelium (Table 1). *N. coccinellae* also infects the midgut epithelium, Malpighian tubules, gonads, nerves and muscle tissues of both *C. septempunctata* and *A. bipunctata* (Lipa, 1968). However, the fat body remains uninfected. In the case of *N. tracheophila*, spores are found in the tracheal epithelium, haemocytes and connective tissues of *C. septempunctata* but the pathogen does not infect the fat body (Cali and Briggs, 1967). Spores of the unidentified microsporidium were not observed in the tracheal epithelium of *C. septempunctata* but were present in the connective tissues (Saito, 2008). Haemocytes were not observed but spores were seen throughout the fat body.

Observations with respect to host range, spore size and infected tissue overlap among *N. hippodamiae*, *N. tracheophila*, *N. coccinellae* and the undescribed microsporidium from *H. convergens* have raised questions regarding the true identity of these microsporidia.

Table 1
Comparison of morphological and ultrastructural features of microsporidia that infect coccinellid hosts.

	Nosema coccinellae	*Nosema hippodamiae*	*Nosema tracheophila*	*Tubulinosema hippodamiae*
Type host (s)	*Coccinella septempunctata*[1] *Hippodamia tredecimpunctata* L.[1] *Myrrha octodecimguttata* L.[1]	*Hippodamia convergens*	*Coccinella septempunctata*	*Hippodamia convergens*
Locality	Poland	California	Ohio (laboratory infection)	
Additional hosts	*Adalia bipunctata* L.[2] *Coccinella quinquepunctata* L.[2] *Exochomus quadripustulatus* L.[2]			*A. bipunctata*[3,a] *C. septempunctata*[3,4,a] *C. trifasciata perplexa*[4,a,b] *Harmonia axyridis*[3,4,a]
Tissue tropism	Midgut epithelium, Malpighian tubules, gonads, nerves, muscle tissues[1,2]	Midgut, fat body and other (unspecified) tissues (in heavy infections)	Hemocytes, tracheal epithelium, connective tissues	Pyloric valve epithelium, hindgut epithelium[c], Malpighian tubules, ovaries, fat bodies, connective tissues[d], muscles, nerves[5]
Spore shape	Ellipsoidal	Ovoid	Ovoid	Slightly pyriform
Size (μm)	4.4–6.7 × 2.3–3.4 (unfixed) 3.6–6.2 × 2.0–3.6 (fixed and stained)	3.3–5.4 × 2.2–2.7	4.0–5.3 × 2.2–3.1 (unfixed) 3.1–4.4 × 1.9 × 3.2 (fixed)	3.9 × 2.5 (fixed and stained)[6] 3.58 × 2.06 (micrographs)
Reference	[1]Lipa (1968) [2]Lipa et al. (1975)	Lipa and Steinhaus (1959)	Cali and Briggs (1967)	[6]Joudrey (2006) [4]Saito and Bjørnson (2006) [3]Saito and Bjørnson (2008) [5]Saito (2008)

1 = reported by Lipa (1968).
2 = reported by Lipa et al (1975).
3 = reported by Saito; Bjornson (2008).
4 = reported by Saito; Bjornson (2006).
5 = reported by Saito (2008).
6 = Joudrey (2006).
[a] laboratory infection resulting from the consumption of microsporidia-infected *H. convergens* eggs.
[b] Not subjected to a histological study.
[c] Not observed in *C. septempunctata* and *H. axyridis*.
[d] Not observed in *H. axyridis*.

Past studies did not employ ultrastructural or molecular studies and it remains possible that some, or all, of these pathogens are a single species. If this is the case, this microsporidium would possess a relatively broad host range and be capable of infecting several lady beetle hosts. Molecular characterization of the pathogens must be compared to confirm if this is the case but this may not be possible if type specimens are unavailable or if sequences cannot be obtained from archival specimens. Molecular characterization of these pathogens is essential to provide clarification regarding the taxonomic classification and organization of microsporidian pathogens that infect coccinellids.

The geographical locality of *N. coccinellae*, *N. hippodamiae* and *N. tracheophila* is distinct: *Nosema coccinellae* is reported from Europe, whereas *Nosema hippodamiae* and *N. tracheophila* are reported from the USA. However, *N. coccinellae* may have been transported inadvertently from Europe to North America because *A. bipunctata* (a known host of *N. coccinellae*) is commercially available from Europe for aphid control in North America. The unidentified microsporidium in *H. convergens* may be the same pathogen as the other three species of microsporidia reported from coccinellids but it may also represent a new species.

The formal description of *Nosema hippodamiae*, the only microsporidium described from *H. convergens*, was based on light microscopic observations of pathogen development and tissue pathology (Lipa and Steinhaus, 1959). Although it is currently unknown as to whether the undescribed microsporidium from *H. convergens* is actually *N. hippodamiae* (or another microsporidium that infects lady beetles), based on the ultrastructural and molecular information gained during this study, we propose that the microsporidium be given the name *Tubulinosema hippodamiae*.

Future molecular characterization of *N. hippodamiae* type specimens may reveal it to be the same pathogen as the one described herein. In this case, the pathogen has been placed in the appropriate genus, *Tubulinosema* and the specific name has not been altered. However, if *N. hippodamiae* is not the same pathogen as the one described in this study, then presumably *N. hippodamiae* would retain the name that was proposed by Lipa and Steinhaus (1959) or the pathogen would be renamed accordingly.

4.2.1. Taxonomic Summary (Tubulinosema hippodamiae)

Tubulinosema hippodamiae n. sp., Bjørnson, Le, Saito and Wang

Type host: *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae).

Other hosts: horizontally transmitted to *Adalia bipunctata*, *Coccinella septempunctata*, *C. trifasciata perplexa*, and *Harmonia axyridis* under laboratory conditions.

Type locality: *H. convergens* purchased from a commercial insectary for biological pest control. The source of origin is likely the Sierra Nevada Mountains of Southern California where *H. convergens* is collected *en masse* for redistribution for aphid control.

Site of infection: Numerous tissues, including the fat body, muscles surrounding the midgut, Malpighian tubule epithelium, pyloric valve epithelium, hindgut epithelium, ventral nerve cord ganglia, muscles, connective tissues and ovaries.

Transmission: Vertical transmission (transovarial) and horizontal transmission (*per os*). Evacuated (germinated) spores in host tissues provide evidence of autoinfection.

Merogony: Diplokaryotic meronts undergo binary fission.

Sporogony: Process unknown.

Interface: All stages develop in direct contact with the host cell cytoplasm.

Spores: Slightly pyriform, diplokaryotic, with a lamellar polaroplast and isofilar polar filament arranged in 10–14 coils (in 1 or 2 layers). Spores were 3.58 ± 0.2 × 2.06 ± 0.2 μm (n = 10, from micrographs). Aberrant spores possessing lamellar or vesicular internal structures were 3.38 ± 0.8 × 2.13 ± 0.2 μm (n = 10).

Etymology: specific name after the host genus.

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