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Nosema adaliae sp. nov., a new microsporidian pathogen from the two-spotted lady beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae) and its relationship to microsporidia that infect other coccinellids

T. Steele, S. Bjørnson *

Department of Biology, Saint Mary's University, 923 Robie Street, Halifax, NS B3H 3C3, Canada

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ABSTRACT

The two-spotted lady beetle, *Adalia bipunctata* L., is a tree-dwelling lady beetle endemic to parts of Europe, Central Asia and North America that is commercially available for aphid control in Europe and North America. Lady beetles host a wide variety of symbionts including parasitoids, viruses, eugregarines, fungi, bacteria, nematodes and microsporidia. Four species of microsporidia have been described from lady beetles, and an undescribed microsporidium was recently isolated from local populations of *A. bipunctata* in Nova Scotia, Canada. In a previous study, this pathogen prolonged the development of *A. bipunctata* larvae but had no effect on adult fecundity, longevity or sex ratios. The objective of this study was to formally describe the microsporidium by means of its ultrastructure, tissue pathology and molecular characterization. All stages of the microsporidium were diplokaryotic and developed in direct contact with the host cell cytoplasm. Mature spores measured 4.25 ± 0.09 × 1.82 ± 0.03 μm (SE, n = 49, from micrographs) and fresh spores measured 6.10 ± 0.06 × 3.01 ± 0.05 μm (±SE, n = 60; range: 5.0–6.9 × 2.18–3.86 μm). The polar filament was isofilar with 10–18 coils that were frequently arranged in a single row. The lamellar polaroplast was not typically visible and spores contained a relatively small posterior vacuole. Both the flight muscles and fat body were heavily infected and a large number of spores were observed within and between the cells of these tissues. The ovaries, developing oocytes, spermatocytes and accessory glands within the testes, midgut epithelium, Malpighian tubules, ileum, colon, and ventral nerve cord were also infected but not as heavily. Connective tissue near the cuticle and surrounding the trachea were lightly infected. The presence of spores in both the alimentary canal and ovaries (particularly within developing oocytes) suggests that the microsporidium can be transmitted *per os* (horizontally) and transovarially (vertically). Molecular analysis of the genome of the microsporidium described in this study was 97% similar to *Nosema bombi* and 96% similar to *Nosema thomsoni*, *Nosema vespula* and *Nosema oulemae*. Based on information gained during this study, we propose that the microsporidium in *A. bipunctata* be given the name *Nosema adaliae* sp. nov.

1. Introduction

The two-spotted lady beetle, *Adalia bipunctata* L., is a tree-dwelling aphidophagous lady beetle endemic to Europe, Central Asia and North America (Majerus, 1994). *A. bipunctata* consume a wide range of aphids along with coccids and diaspids (scale insects) as alternative prey but they also feed on pollen when aphids are absent or scarce (Hemptinne and Desprets, 1986). The two-spotted lady beetle has been commercialized for aphid control in Europe since 1999 and was made available for biological control in North America shortly thereafter (De Clercq et al., 2005). Early attempts at establishing two-spotted lady beetles for biological pest control were unsuccessful until *A. bipunctata* were used to control the rosy apple aphid, *Dysaphis plantaginea* (Passerini) in Switzerland (Wyss et al., 1999).

An emerging concern with regard to the overall success of biological control is the quality and condition of the biological control agents that are used (van Lenteren, 2003). Lady beetles are known to host a wide variety of symbionts including parasitoids, viruses, eugregarines, fungi, bacteria, nematodes and microsporidia (see Richerson, 1970; Hodek and Honěk, 1996; Riddick et al., 2009). The most studied symbionts in *A. bipunctata* are maternally inherited, male-killing bacteria that are known to reduce egg hatch and result in female-biased sex ratios (Hurst et al., 1996, 1999).

Microsporidia are common pathogens of lady beetles. Four species of microsporidia have been described from field-collected coccinellids, including *Nosema hippodamiae* from the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville (see Lipa and Steinhaus, 1959); *Nosema tracheophila* from the seven-spotted lady beetle, *Coccinella septempunctata* L. (Cali and Briggs, 1967);

* Corresponding author. Fax: +1 902 420 5261.
E-mail address: susan.bjornson@smu.ca (S. Bjørnson).

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Nosema coccinellae from several lady beetle species, including *C. septempunctata* (see Lipa, 1968; Lipa et al., 1975); and *Tubulinosema hippodamiae* from *H. convergens* (Bjørnson et al., 2011). Two of the four species of microsporidia also infect *A. bipunctata*; *N. coccinellae* is reported to infect field-collected beetles in Poland (Lipa, 1968; Lipa et al., 1975) and *T. hippodamiae* has been transmitted from *H. convergens* to *A. bipunctata* under laboratory conditions (Saito and Bjørnson, 2008).

Recently, an undescribed microsporidium was isolated from local populations of *A. bipunctata* that were collected in Nova Scotia, Canada. This pathogen prolongs the development of *A. bipunctata* larvae but has no observable effect on adult fecundity, longevity or sex ratios (Steele and Bjørnson, 2012). The objective of this study was to formally describe this microsporidium by means of its ultrastructure, tissue pathology and molecular characterization.

2. Materials and methods

Microsporidia-infected *A. bipunctata* adults used in this study were isolated from laboratory-reared colonies established from field-collected individuals. Uninfected *A. bipunctata* were isolated from a single shipment of 100 larvae that were obtained from a commercial insectary.

Adult beetles were held in 120 ml clear, polyethylene cups (Canemco-Marivac Inc., QC) under controlled conditions (16:8 L:D; 25 °C:20 °C) in environmental growth chambers (Sanyo MLR-350H). Each cup had a 2.2-cm hole cut in the side, which was covered with a fine mesh screen (80 μm) to allow air circulation. Beetles were maintained on green peach aphids (*Myzus persicae* Sulzer) and artificial diet (Lacewing and Ladybug Food, Planet Natural, MT). Distilled water was provided on moistened cotton wicks (Crosstex International, NY). Green peach aphids were reared on nasturtium (*Tropaeolum minus* L.) (Dwarf Jewel Mixed, Stokes Seed Ltd., ON) in separate environmental chambers under controlled conditions (16:8 L:D; 25 °C:20 °C).

To confirm the infection status of the beetles used, sibling eggs from each female parent were randomly selected for microscopic examination. Eggs were smeared on microscope slides, stained with a 5% Giemsa solution (2 h, pH 6.9, Sigma Diagnostics), and examined for presence or absence of microsporidian spores by light microscopy (400× magnification).

2.1. Pathogen ultrastructure

Microsporidia-infected *A. bipunctata* adults were submerged in 2.5% glutaraldehyde fixative (Becnel, 1997) for examination by transmission electron microscopy (TEM). The head, thorax, elytra, wings and legs of each specimen were removed. Additional incisions were made in the abdominal sternites to maximize penetration of the fixative. Samples were stored in fixative at 4 °C until they were processed (48 h) in three different batches on separate days.

Tissues were embedded in Jembed 812/Spurr resin (Canemco-Marivac, QC) according to Becnel (1997) with the following modification: tissues were placed under vacuum (15 PSI) overnight once they were immersed in pure resin. Tissue blocks were chosen at random for examination. Ultra-thin sections (~70 nm thick) were cut with a Leica UCT ultramicrotome. Sections were stained with uranyl acetate and led citrate prior to viewing. Micrographs were digitally generated with a GATAN ES500W Erlangshen CCD camera side mounted to a Hitachi H7500 transmission electron microscope at 80 kV. Imaging software (ImageJ, 1.45s) was used to determine spore measurements.

Microsporidian vegetative stages were observed in *A. bipunctata* eggs that had been smeared on coverslips, stained with Giemsa (5%, 2 h), rinsed in acidic tap water (pH 5.5) and inverted on glass microscope slides (light microscopy, 1000× magnification). Fresh spores were measured from infected egg preparations.

2.2. Tissue pathology

Sixteen uninfected and microsporidia-infected beetles were embedded for histological examination. A total of 12 individuals were examined (4 uninfected and 8 microsporidia-infected). Beetles were submerged in Carnoy's fixative (60 ml ethanol, 30 ml chloroform, 10 ml glacial acetic acid) and the head, thorax, elytra, wings and legs of each specimen were removed. Incisions were made in the abdominal sternites to enhance penetration of the fixative. Samples were held in fixative at 4 °C until they were processed (48–72 h).

Tissues were embedded in Paraplast® Plus (Sigma–Aldrich Inc.) according to the procedure by Becnel (1997) with the following modifications: (1) tissues remained submerged in 1:1 ethanol:butanol overnight in a > 25.5 °C oven (instead of 2 h at room temp); (2) tissues were submerged in 3:1 butanol:paraffin for 20 min in a 60 °C oven (this step was inserted between 100% butanol and 1:1 butanol:paraffin); and (3) tissues remained in 1:1 butanol:paraffin overnight (instead of 2 h). Longitudinal sections (5 μm thick) were stained with Harris hematoxylin and Alcoholic Eosin Y (Fisher Scientific) and examined by light microscopy. Images of spores and infected tissues were obtained using Zeiss Axiovision imaging software.

2.3. Molecular characterization

Microsporidia genomic DNA was extracted from randomly selected *A. bipunctata* eggs (24–48 h old) according to the methods described by Bjørnson et al. (2011). DNA was isolated and purified with a DNeasy® Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Microsporidian primers described by Vossbrinck et al. (2004) were used to design primers for this study (18f (CACCAGGTT GATTCT GCC)/1492 (GGTTACCTTGTTACGACTT); Eurofins Laboratories). DNA was amplified by Polymerase Chain Reaction (PCR), cloned using pGEM®-T and pGEM®-T Easy Vector Systems (Promega Corp.), extracted with PureYield™ Plasmid Miniprep System (Promega Corp.), and sent to Macrogen (Korea) for sequencing.

The top 9 most closely related species were selected for molecular comparison according to their maximum identity score (Table 1). Sequences of *Nosema bombi, Nosema thomsoni, Nosema vespula, Nosema oulemae, Oligosporidium occidentalis, Nosema portugal, Vairimorpha disparis, Nosema carpocapsae* and *Vairimorpha necatrix* were obtained from NCBI GenBank. *Nosema apis* was used for comparison as an out group. Automated sequence outputs were visually inspected with chromatographs. Multiple sequence alignments were made using ClustalX with default parameters and additional manual edits to minimize gaps (Thompson et al., 1997). Phylogenetic analysis using the maximum-parsimony (MP) method was performed with the computer program PAUP* ver. 4 beta 10 (Swofford, 2003). All characters were specified as unweighted and unordered, and gap-only columns were excluded in the analyses. The most parsimonious trees were constructed by performing a heuristic search using the Tree Bisection-Reconnection (TBR) with the following parameters: MulTrees on and ten replications of random addition sequences with the stepwise addition option. Multiple parsimonious trees were combined to form a strict consensus tree. Overall character congruence was estimated by the consistency (CI), and the retention index (RI). To infer the robustness of clades, bootstrap values with 1000 replications (Felsenstein, 1985) were calculated by performing a heuristic search using the TBR option with MulTrees on.

Table 1
Species name, primary host, GenBank accession number and maximum identity score (MI) of nine microsporidia that are closely related to the undescribed microsporidium from *Adalia bipuncata*.

Microsporidia	Host	GenBank accession #	MI (%)
Nosema bombi	*Bombus agrorum* (Hymenoptera: Apidae)	AY008373.1	97
Nosema thomsoni	*Choristoneura conflictana* (Lepidoptera: Tortricidae)	EU219086.1	96
Nosema vespula	*Vespula germanica* (Hymenoptera: Vespidae)	U11047.1	96
Nosema oulemae	*Oulema melanopus* (Coleoptera: Chrysomelidae)	U27359.1	96
Oligosporidium occidentalis	*Metaseiulus occidentalis* (Mesostigmata: Phytoseiidae)	AF495379.1	96
Nosema portugal	*Lymantria dispar* (Lepidoptera: Erebidae)	AF033316.1	96
Vairimorpha disparis	*Lymantria dispar* (Lepidoptera: Erebidae)	AF033315.1	96
Nosema carpocapsae	*Cydia pomonella* (Lepidoptera: Tortricidae)	AF426104.1	96
Vairimorpha necatrix	*Pseudaletia unipuncta* (Lepidoptera: Noctuidae)	DQ996241.1	95

In addition to MP analysis, maximum-likelihood (ML) and Bayesian analyses were also performed. For ML analysis, eight nested models of sequence evolution were tested for each data set using PhyML 3.0 (Guindon and Gascuel, 2003). For each data set, the general time-reversible (GTR) (Lanave et al., 1984) substitution model led to a largest ML score compared to the other 7 substitution models: JC69 (Jukes and Cantor, 1969), K80 (Kimura, 1980), F81 (Felsenstein, 1981), F84 (Felsenstein, 1993), HKY85 (Hasegawa et al., 1985), TN93 (Tamura and Nei, 1993) and custom (data not shown). As a result, the GTR model was used in the Bayesian analysis using MrBayes 3.1 (Ronquist and Huelsenbeck, 2005). MrBayes 3.1 was run with the program's standard setting of two analyses in parallel, each with four chains, and an estimated convergence of results by calculating the standard deviation of split frequencies between analyses. In order to make the standard deviation of split frequencies fall below 0.01 so that the occurrence of convergence could be certain, 20,000 generations of data were run for the small subunit rRNA gene. Samples were taken every 1000 generations under the GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites. For all analyses, the first 25% of samples from each run were discarded as burn-in to ensure the stationarity of the chains. Bayesian posterior probability (PP) values were obtained from a majority rule consensus tree generated from the remaining sampled trees.

A BLAST analysis was also conducted to determine if any of the microsporidia described from lady beetles were genetically similar to the microsporidium examined in this study; however, only *T. hippodamiae* from *H. convergens* had a genome submitted to GenBank.

3. Results

3.1. Pathogen ultrastructure

All stages of the microsporidium developed in direct contact with the host cell cytoplasm. Early developmental stages were observed adjacent to mature spores. Meronts were round (53/88 observations; 60.2%) to slightly irregular (35/88; 39.7%) and were surrounded by a thin plasma membrane (Fig. 1). Diplokaryotic nuclei were primarily observed (52/88 observations; 59.1%). Each nucleus was round and occupied roughly one-third of the cell. Numerous ribosomes were within the cytoplasm but there was no evidence of an early developing endoplasmic reticulum. The examination of vegetative stages by light microscopy revealed diplokaryotic meronts that divide by binary fission. Round to oval sporonts (Fig. 2) were surrounded by a thickened, convoluted plasma membrane (17/27 observations; 63%). Ribosomes were more abundant within the cytoplasm when compared to the meront. The central area of the sporonts was occupied by a diplokaryon that was surrounded by the endoplasmic reticulum.

Sporoblasts (Fig. 3) were highly irregular in shape and were surrounded by a thickened and convoluted plasma membrane. The diplokaryon was less apparent than it was in other vegetative stages. Sporoblasts contained a developing polar filament, a lamellar polaroplast and posterior vacuole. Mature spores (Fig. 4) were diplokaryotic and measured $4.25 \pm 0.09 \times 1.82 \pm 0.03$ μm (±SE, $n = 49$, from micrographs). Fresh spores measured $6.10 \pm 0.06 \times 3.01 \pm 0.05$ μm (±SE, $n = 60$; range: 5.0–6.9 × 2.18–3.86 μm). The polar filament was arranged in 10–18 coils ($n = 42$); however, several spores contained polar filaments that were arranged in 9 ($n = 2$), 20 ($n = 1$), 21 ($n = 1$) or 22 ($n = 3$) coils. The isofilar polar filament was frequently arranged in a single layer (37/49 observations; 75.5%) but double layers were also observed (12/49; 24.5%). The lamellar polaroplast (Fig. 5) was often not visible (14/49 observations) and the majority of spores contained a round membranous structure where the polaroplast is normally located (not shown). A prominent posterior vacuole was frequently observed (31/49 observations; 63.3%) and electron-dense material was often observed along its internal perimeter (21/31; 67.7%). Spores were surrounded by a well-developed endospore and exospore (0.12 ± 0.004 μm and 0.03 ± 0.003 μm respectively, $n = 49$, from micrographs). Evacuated (germinated) spores were frequently observed (Fig. 6).

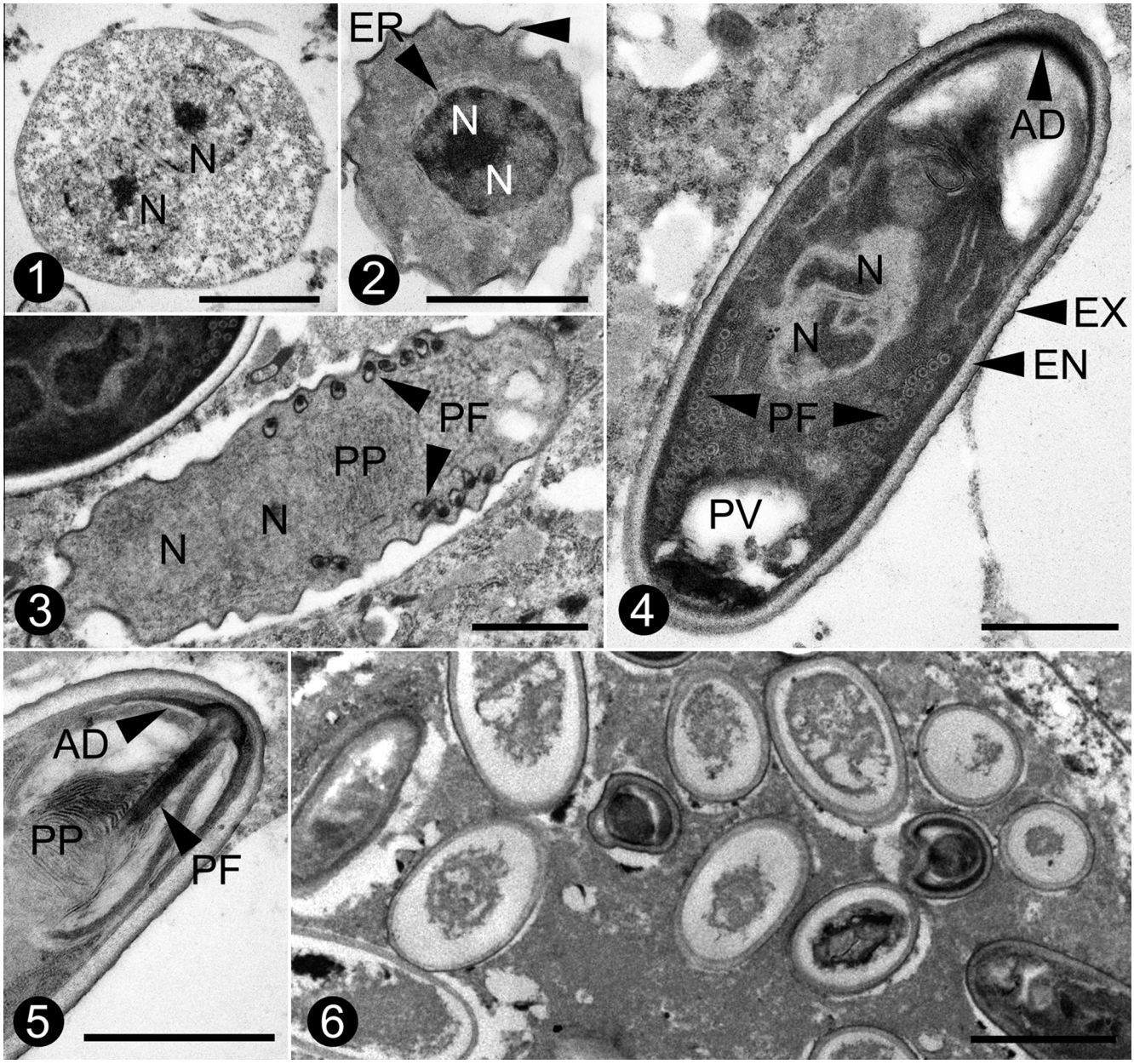
Numerous tubular-like structures surrounded by a delimiting membrane were observed within a single meront (Fig. 7) as well as in the central region of mature spores (4 observations, Fig. 8). Spherites (Fig. 9) were occasionally observed in proximity to microsporidian spores and vegetative stages.

3.2. Tissue pathology

Spores were observed in the ovaries and developing oocytes in all four of the microsporidia-infected females examined. The spermatocytes and accessory glands within the testes of the four males examined were infected but the sperm bundles remained uninfected. In both sexes, the flight muscles and fat body were heavily infected and large numbers of spores were observed within and between the cells of these tissues. In the fat body, spores were frequently found within the cell nuclei. Spores were also observed within the cells of the Malpighian tubules and in neurons that surround the ventral nerve cord. The midgut, ileum and colon epithelia were lightly infected and few microsporidian spores were observed in these tissues. Connective tissue beneath the cuticle and surrounding the trachea were also lightly infected.

3.3. Molecular characterization

Only one sequence was produced from microsporidia-infected *A. bipunctata* eggs, suggesting that only one species of microsporidia was present. Molecular analysis of the genome showed the pathogen described in this study was 97% similar to *Nosema bombi* (Accession no: AY008373.1) and 96% similar to *N. thomsoni* (Accession no: EU219086.1), *N. vespula* (Accession no: U11047.1), *N. oulemae* (Accession no: U27359.1), *Oligosporidium occidentalis*



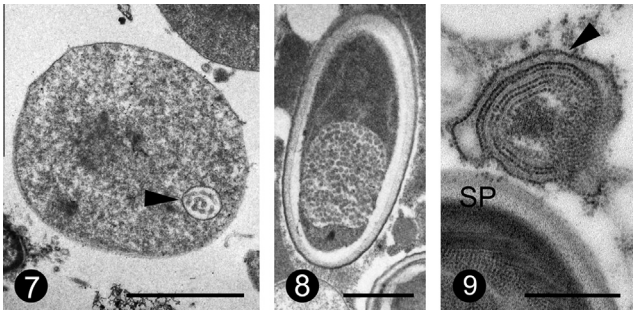
Figs. 1–6. Ultrastructural characteristics of the life cycle of *Nosema adaliae* sp. nov., from the two-spotted lady beetle, *Adalia bipunctata.* 1. Diplokaryotic meront showing two distinct nuclei (N) and ribosomes, surrounded by a thin plasma membrane. 2. Sporont with two nuclei (N) and thickened plasma membrane (arrow). An endoplasmic reticulum (ER) is visible around the perimeter of the nuclei. 3. Early-stage diplokaryotic sporoblast with two nuclei (N), a developing polar filament (PF) and lamellar polaroplast (PP) surrounded by a thickened, convoluted plasma membrane. 4. Mature spore with a fully developed spore wall, composed of an exospore (EX) and endospore (EN), a diplokaryotic nucleus (N) and an isofilar polar filament (PF) arranged in a double layer. A posterior vacuole (PV) is visible within the posterior region of the spore and part of the anchoring disk (AD) at the anterior end. 5. The anterior region of a mature spore showing details of the anchoring disk (AD), polar filament (PF) and lamellar polaroplast (PP). 6. Evacuated (germinated) spores provide evidence that autoinfection occurs. Scale bars: Figs. 1–5, 1 μm; Fig. 6, 2 μm.

(Accession no: AF495379.1), *N. portugal* (Accession no: AF033316.1), *Vairimorpha disparis* (Accession no: AF033315.1) and *N. carpocapsae* (Accession no: AF426104.1; see Table 1). Maximum parsimony analysis using *N. apis* (Accession no: U26534.1) as the out group was conducted (55 parsimony-informative characters, 176 equally most parsimonious trees [CI = 0.801, RI = 0.733]). The separated Bayesian analyses using GTR model resulted in identical trees with mean log-likelihood values −2630.69 and −2641.84 (data not shown). The tree topologies were identical in both ML and Bayesian trees and similar to those generated by MP. Strict consensus trees with bootstrap (1000 replicates) and Bayesian PP values showed three different clades (Fig. 10). The microsporidium from *A. bipunctata* (*Nosema adaliae* n. sp.) formed one clade with *N. bombi* and *O. occidentalis* with 84% bootstrap support (PP = 0.80). Within this clade, *N. bombi* and *O. occidentalis* formed a subclade with 83% bootstrap support (PP = 0.97). A BLAST analysis of the microsporidium from *A. bipunctata* and *T. hippodamiae* (described from *H. convergens*) revealed no significant similarities between the two sequences.

4. Discussion

4.1. Host specificity of microsporidia described from coccinellids

Several microsporidia have been described from lady beetles (Table 2); however, *N. coccinellae* is the only microsporidium



Figs. 7–9. Anomalous structures and germinated spores. 7. Meront with tubular structures (arrow) of unknown origin or significance delimited by a membrane. 8. Numerous tubular structures within the central region of mature spores. 9. Spherite (arrow) adjacent to a mature spore (SP). Scale bars: Figs. 7-8, 1 μm; Fig. 9, 0.5 μm.

reported to infect field-collected *A. bipunctata* (Lipa et al., 1975). The description of *N. coccinellae* was based on light microscopic observations (pathogen life cycle, tissue pathology and spore dimensions) from the hosts *C. septempunctata*, *Hippodamia tredecimpunctata* (L.) and *Myrrha octodecimguttata* (L.). It is impossible to determine if *N. coccinellae* and the microsporidium described herein from *A. bipunctata* are the same species because type specimens and molecular information for *N. coccinellae* are lacking. The original report of *N. coccinellae* infecting *A. bipunctata* (Lipa, 1968), was from a single adult (of 168 examined adults; Lipa et al., 1975). The authors did not include any descriptive information to support their conclusion that the pathogen they observed in *A. bipunctata* was indeed *N. coccinellae*. This raises the question as to whether the pathogen in *A. bipunctata* reported by Lipa (1975) was *N. coccinellae* or another species.

Little information is available regarding *N. coccinellae* ultrastructure. Spores measure 4.4–6.7 × 2.3–3.4 μm (Lipa, 1968; Lipa et al., 1975); however, these measurements also overlap the range of measurements of the three other species of microsporidia reported from lady beetles (Table 2). Although *N. adaliae* and *N. coccinellae* infect similar tissues in their respective hosts, *N. adaliae* was observed in the fat body of *A. bipunctata* whereas *N. coccinellae* was not reported in the fat body of *C. septempunctata*, *H. tredecimpunctata* and *M. octodecimguttata* (there is no information regarding tissue pathology of *N. coccinellae* in *A. bipunctata*).

A particular lady beetle host may be infected by more than one species of microsporidia under laboratory conditions (Saito and

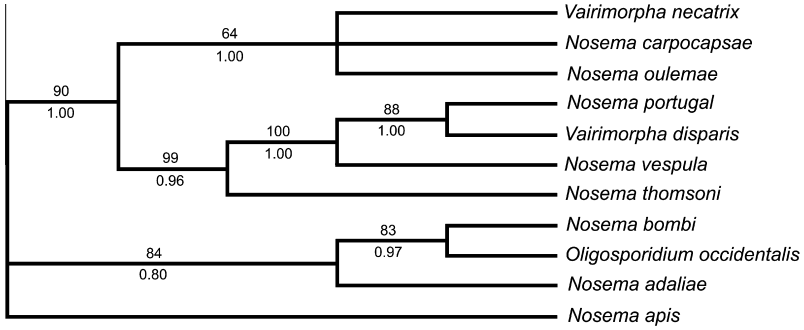


Fig. 10. Strict consensus tree derived from 16S ribosomal RNA gene sequence data conducted using a heuristic search with TBR branch swapping. Numbers above and below branches are bootstrap and Bayesian posterior probability (PP) values, respectively. *Nosema apis* was used as an out group. Consistency index (CI) = 0.801, retention index (RI) = 0.733.

Table 2
Histological and ultrastructural comparison of microsporidian pathogens that infect lady beetles.

Microsporidia	Host(s)	Tissues infected	Spore shape	Spore size (μm)	Nuclei (#)	PF coils	PF Type	Reference
Nosema hippodamiae	*Hippodamia convergens*	Mg, FB	Ovoid	3.3–5.4 × 2.2–2.7[a]	1	N/A	N/A	Lipa and Steinhaus (1959)
Nosema tracheophila	*Coccinella septempunctata*	TE, He, CT	Ovoid	4.0–5.3 × 2.2–3.1[b]	N/A	N/A	N/A	Cali and Briggs (1967)
Nosema coccinellae	*Coccinella septempunctata, Hippodamia tredecimpunctata, Myrrha octodecimguttata, Adalia bipunctata*	ME, MT, G, N, M	Elipsoidal	4.4–6.7 × 2.3–3.4[b]	N/A	N/A	N/A	Lipa (1968), Lipa et al. (1975)
Tubulinosema hippodamiae	*Hippodamia convergens* *Adalia bipunctata*[*] *Coccinella septempunctata*[*] *Coccinella trifasciata perplexa*[*] *Harmonia axyridis*[*]	FB, M, MT, PV, Hg, VN, CT, Ov	Pyriform	3.58 ± 0.2 × 2.06 ± 0.2[c]	2	10–14	Anisofilar	Bjørnson et al. (2011)
Nosema thomsoni	*Harmonia axyridis*	He	N/A	N/A	N/A	N/A	N/A	Vilcinskas et al. (2013)
Nosema adaliae	*Adalia bipunctata*	Ov, T, ME, MT, Hg, FB, VN, M	Ovoid	4.25 ± 0.09 × 1.82 ± 0.03[c]	2	10–18	Isofilar	

CT = connective tissues; FB = fat body; G = gonads; He = haemocytes; Hg = hindgut; MT = Malpighian tubules; Mg = midgut; ME = midgut epithelium; M = muscle; N = nerves; Ov = ovaries; PF = polar filament; PV = pyloric valve; T = testes; TE = tracheal epithelium; VN = ventral nerve cord.

[*] Laboratory infection; N/A: Information is lacking from the formal description.

[a] Measurements taken from an alcohol fixed & Giemsa stained smear.

[b] Measurements taken from fresh smear.

[c] Measurements based off of micrographs.

Bjørnson, 2006, 2008; Steele and Bjørnson, 2012); therefore, we cannot assume that *N. adaliae* and *N. coccinellae* are the same pathogen because they share the same host. Furthermore, there is no evidence to confirm that infected *A. bipunctata* from Poland were ever released for biological pest control in Canada.

4.2. Host effects of microsporidia in coccinellids

The majority of microsporidia reported from lady beetles cause chronic, sublethal infections that debilitate their hosts. In a previous study, the microsporidium from *A. bipunctata* prolonged the development of *A. bipunctata* larvae but had no effect on adult fecundity, longevity or sex ratios (Steele and Bjørnson, 2012). Similarly, *T. hippodamiae* (from *H. convergens*) prolongs larval development of several coccinellids, including *A. bipunctata*, *C. septempunctata*, *C. trifasciata perplexa*, and *Harmonia axyridis* while having no effect on larval mortality (Saito and Bjørnson, 2006, 2008). *T. hippodamiae* reduces the fecundity and survival of adult *H. convergens*, its so-called natural host (Joudrey and Bjørnson, 2007; Saito and Bjørnson, 2008) but has no observable effect on these same life history characteristics of *A. bipunctata*, *C. septempunctata* or *H. axyridis* (Saito & Bjornson, 2008).

Vilcinskas et al. (2013) report that a benign microsporidium (related to *N. thomsoni*) infecting *H. axyridis* hemocytes is lethal to *C. septempunctata.* During their study, *C. septempunctata* injected with live microsporidia died within two weeks whereas those injected with heat-activated microsporidia did not. The authors concluded that the microsporidium in *H. axyridis* is lethal if given the appropriate amount of time needed to replicate within *C. septempunctata*. Unfortunately, the study did not include feeding bioassays that would provide confirmation that the microsporidium is transmitted from *H. axyridis* to *C. coccinellae* through a common infection route (*per os*) and that the pathogen is lethal as a result.

4.3. Coccinellids as natural vs. accidental hosts

According to the maximum identity score, the microsporidia that are most closely related to the microsporidium from *A. bipunctata* infect a wide range of different host species (Table 1). *N. bombi*, for example, was first described from the bumblebee *Bombus agrorum*, but it has also been recorded to infect over 40 other *Bombus* species (Tay et al., 2005; Cordes et al., 2012; Li et al., 2012). *N. thomsoni* was described from the large aspen tortrix moth, *Choristoneura conflictana* Walker (Wilson and Burke, 1971). *N. vespula* was isolated from infected larvae of the European wasp, *Vespula germanica* but it is believed to infect a wide range of hosts, including hymenopterans, dipterans, and lepidopterans (Rice, 2001). The description of *N. vespula* was based solely on a genome submission to GenBank. *N. oulemae* infects the cereal leaf beetle, *Ouelma melanopus* but the description of this pathogen was also based solely on a genome submission to GenBank. *V. disparis* was described from the gypsy moth, *Lymantria dispar* (Vavra et al. 2006). *N. carpocapsae* infects the codling moth, *Cydia pomonella* (Malone and Wigley, 1981).

The undescribed microsporidium from *A. bipunctata* shares some morphological characteristics with *T. hippodamiae* (diplokaryotic nuclei, all stages develop in direct contact with the host cell cytoplasm) but these two pathogens differ at the ultrastructural level. The most noticeable difference is that the polar filament of *T. hippodamiae* is anisofilar with 10–14 coils whereas the polar filament from the microsporidium in *A. bipunctata* is isofiliar with 10–18 coils (Table 2). In addition, *T. hippodamiae* spores are smaller than those of the pathogen from *A. bipunctata* and the results of a BLAST analysis revealed that there was "no significant similarity found" between the genomes of these two microsporidia.

The pathogen in *A. bipunctata* is related to *N. thomsoni* (within the *N. thomsoni* complex). Whether or not this pathogen has a broad or narrow host range is yet to be demonstrated. There is some confusion as to the identity of the microsporidium described by Vilcinskas et al. (2013). The authors indicate that the microsporidium in *H. axyridis* is within the *Nosema/Vairimorpha* clade and is closely related ($\geqslant$99% sequence identity) to *N. thomsoni*. However, the accession number of their isolate indicates that the microsporidium they studied is actually *N. thomsoni* (also spelled *N. thompsoni* by the authors) and is not a close relative of *N. thomsoni*. *Nosema thomsoni* was first described from the large aspen tortrix, *C. conflictana* from Canada (Wilson and Burke, 1971). When compared with other *Nosema* isolates from other lepidopteran defoliators, *N. thomsoni* has an 82% sequence similarity (Kyei-Poku et al. 2008). The authors suggest that the infection of *C. conflictana* by *N. thomsoni* may represent a distinct evolutionary event with respect to the other *Nosema* spp. that infect *C. fumiferana*, *C. pinus*, and C. *occidentalis* and may have resulted from independent transmission events in these lepidopteran hosts. *N. thomsoni* may be relatively host specific but with rDNA that is too conservative to distinguish it from another closely related species.

Although several species of microsporidia are reported from coccinellids, this number may be misrepresented by reports in the literature. In the case of *N. coccinellae*, which has been reported to infect several lady beetle species (Lipa, 1968; Lipa et al., 1975), there is insufficient morphological and molecular information available to confirm whether the infection of these hosts is caused by *N. coccinellae* or more than one microsporidian species. If one microsporidium (*N. coccinellae*) infects all four species of coccinellids (Table 2), the question remains as to which of these lady beetles are natural hosts. These lines are blurred further in the case of *T. hippodamiae*, a microsporidium reported from field-collected *H. convergens* that also infects *A. bipunctata*, *C. septempunctata*, *C. trifasciata perplexa*, and *H. axyridis* under laboratory conditions. Without molecular information, it would be difficult to correctly identify a microsporidian pathogen in these beetles with confidence. In addition to infecting related hosts, microsporidia are known to infect hosts that are distantly related but closely related with respect to host association. For example, *T. hippodamiae* infects the endoparasitic wasp *Dinocampus coccinellae* (Schrank) (Saito and Bjørnson, 2013). Infection with the pathogen has no effect on endoparasitoid development but all major organs and tissues, with exception of the ovaries, are infected. Although vertical transmission from parent to progeny does not occur, a significantly greater proportion of beetles stung by microsporidia-infected wasps do not contain an endoparasitoid larva, suggesting that the pathogen may reduce wasp fecundity or egg viability. *D. coccinellae* is a generalist parasitoid of coccinellids and whether or not it may become infected with the pathogen in *A. bipunctata* (and any associated effects) has not been demonstrated.

4.4.1. Pathogen ultrastructure

Microsporidia belonging to the genus *Nosema* share several characteristics: all stages develop in direct contact with the host cell cytoplasm and diplokaryotic spores have an isofilar polar filament, a thin endospore with a moderately thick exospore and a distinctive, small posterior vacuole (Sprague et al., 1992). The microsporidium from *A. bipunctata* shares these characteristics.

Mature spores of the microsporidium from *A. bipuncata* and those of closely related microsporidia are similar in shape but differ with respect to size and the number of polar filament coils within (Table 3). Fresh spores of the microsporidium from *A. bipunctata* measured $6.10 \pm 0.06 \times 3.01 \pm 0.05$ μm and are distinctly larger than those from the majority of closely related microsporidia.

Ultrastructural studies have been completed for five of the eight species that are most closely related to the pathogen in *A. bipunc-*

Table 3
Ultrastructural comparison of eight microsporidia that are most closely related to the microsporidium from *Adalia bipuncata* with a maximum identity score of 96% or higher.

Microsporidia	Host	Shape	Spore size (μm)	Nuclei	PF Coils	PF Type	PP Type	Reference
Nosema bombi	*Bombus agrorum* (Hymenoptera: Apidae)	Ovoid	4.88 ± 0.03 × 2.88 ± 0.03[a]	2	14–18	Isofilar	Lamellar	McIvor and Malone (1995)
Nosema thomsoni	*Choristoneura conflictana* (Lepidoptera: Tortricidae)	Ovoid	1.1–1.8 × 2.1–3.1[a]	1[c]	N/A	N/A	N/A	Wilson and Burke (1971)
Nosema vespula	*Vespula germanica* (Hymenoptera: Vespidae)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nosema oulemae	*Oulema melanopus* (Coleoptera: Chrysomelidae)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Oligosporidium occidentalis	*Metaseiulus occidentalis* (Mesostigmata: Phytoseiidae)	Oblong-ovoid	2.53 ± 0.06 × 1.86 ± 0.06[a]	1	3–5,8–9[d]	Isofilar	Bipartite lamellar	Becnel et al. (2002)
Nosema portugal	*Lymantria dispar* (Lepidoptera: Erebidae)	Ovoid	4.5 ± 0.41 × 1.9 ± 0.17[a]	2	5–8,10–11[d]	Isofilar	Lamellar	Maddox et al. (1999)
Vairimorpha disparis	*Lymantria dispar* (Lepidoptera: Erebidae)	Elongate - ellipsoidal	5.4 ± 0.48 × 2.5 ± 0.37[a,*]	2	4–5,11 – 13[d]	Isofilar	Lamellar	Vavraet al. (2006)
Nosema carpocapsae	*Cydia pomonella* (Lepidoptera: Tortricidae)	Ovoid	2.4–3.9 × 1.3–3.1[b]	2	9–13	N/A	N/A	Malone and Wigley (1981)
Nosema adaliae	*Adalia bipunctata* (Coleoptera: Coccinellidae)	Ovoid	6.10 ± 0.06 × 3.01 ± 0.05[a]	2	10–18	Isofilar	Lamellar	

Shape refers to spore shape; PF = polar filament; PP = polaroplast.
N/A = information is not available; pathogen is either undescribed or information is lacking from description.
[a] Measurements taken from fresh smear preparations.
[b] Measurements taken from fixed & stained (Giemsa) preparations.
[c] Information based on light microscopic observations.
[d] Primary spore and environmental spore; respectively.
[*] Primary spores (secondary spores that measured 5.1 ± 0.34 × 2.6 ± 0.28 and 4.6 ± 0.28 × 2.8 ± 0.26 μm were also reported).

tata (including *N. bombi*, *N. thomsoni*, *O. occidentalis*, *N. portugal* and *N. carpocapsae*). The vegetative stages of the *A. bipunctata* isolate are similar to the majority of these species. Meronts were spherical and primarily diplokaryotic. Sporonts were round to oval with fused diplokaryotic nuclei, which is typical for microsporidia in the genus *Nosema* (Sprague et al., 1992).

Tubular structures observed in spores from *A. bipunctata* were similar to those reported in *Nosema granulosis*. These are described as numerous discrete granules within the polaroplast at the anterior end of the spore (Terry et al., 1999). In the case of the microsporidium in *A. bipunctata*, the tubular structures were located within the central to posterior region of spores but did not occupy the polaroplast. Spherites, identified by their distinct concentric rings, were observed alongside spores and vegetative stages. These spherites were morphologically similar to those observed in the phytophagous lady beetle, *Epilachna cf. nylanderi* that are thought to assist the removal of excess minerals and toxins from the body (Rost-Roszkowska et al., 2010).

4.4.2. Tissue pathology

Microsporidian spores were observed in various tissues including the midgut, hindgut (Malpighian tubules, ileum and colon), reproductive organs, fat body, ventral nerve cord and flight muscles. The presence of spores in both the alimentary canal and ovaries (particularly within developing oocytes) suggests that the microsporidium can be transmitted *per os* (horizontally) and transovarially (vertically). In a previous study, *A. bipunctata* became infected with the isolate when uninfected, first-instar larvae were fed eggs from microsporidia-infected *A. bipunctata* adults (Steele and Bjørnson, 2012). Larvae that later eclosed as adults were used to determine the effects of the pathogen on the life history of adult beetles. All of the first-instar larvae that consumed a single infected egg became infected and horizontal transmission of the pathogen was 100% after 30 days.

Tissue tropism is compared among microsporidia infecting coccinellids in Table 2. Of these, *N. coccinellae* is the only microsporidium reported from field-collected *A. bipunctata*. Lipa (1968) reported that this pathogen infects the midgut epithelium, Malpighian tubules, gonads, nerves and muscle tissues of numerous beetles including *Coccinella septempunctata, Hippodamia tredecimpunctata and Myrrha octodecimguttata*. Information regarding tissue pathology of the *A. bipunctata* isolate was not reported. *T. hippodamiae* infects *A. bipunctata* under laboratory conditions, invading several tissues, including the fat body, muscles, Malpighian tubules, the pyloric valve, hindgut epithelium, ventral nerve cord, connective tissues and ovaries (Saito, 2008).

Based on the pathogen ultrastructure and molecular information gained during this study, we propose that the microsporidium in *A. bipunctata* be considered a new species and given the name *Nosema adaliae* sp. nov.

4.5. Taxonomic summary (*Nosema adaliae*)

Nosema adaliae sp. nov. Steele and Bjørnson

GenBank Accession Number: KC412706
Type host: *Adalia bipunctata* L. (Coleoptera: Coccinellidae)
Other hosts: unknown
Type locality: *A. bipunctata* field-collected in Nova Scotia, Canada
Site of infection: Numerous tissues including the ovaries, developing oocytes, spermatocytes and accessory glands within the testes, midgut epithelium, Malpighian tubules, ileum, colon, fat body, ventral nerve cord and flight muscles.
Transmission: Horizontal transmission (*per os*) and evidence of vertical (transovarial) transmission (spores observed in ovaries and developing oocytes). Evacuated spores in host tissues provide evidence of autoinfection.
Merogony: Binary fission.
Sporogony: Binary fission.
Interface: All stages develop in direct contact with the host cell cytoplasm.
Spores: Oval, diplokaryotic, fresh spores measure 6.10 ± 0.06 × 3.01 ± 0.05 μm (± SE, $n = 60$; range: 5.0–6.9 × 2.18–3.86 μm) with a lamellar polaroplast and relatively small posterior vacuole. The isofilar polar filament is arranged in 10–18 coils in single or double layers.

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