Journal of Invertebrate Pathology 107 (2011) 1–10

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Morphology, molecular characteristics and prevalence of a *Cystosporogenes* species (Microsporidia) isolated from *Agrilus anxius* (Coleoptera: Buprestidae)

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ARTICLE INFO

Article history:
Received 9 November 2009
Accepted 9 December 2010
Available online 15 December 2010

Keywords:
Agrilus anxius
Cystosporogenes species (Microsporidia)
Morphological and molecular characterization
Phylogenetic analysis
Field prevalence

ABSTRACT

A microsporidium was isolated from the bronze birch borer, *Agrilus anxius* Gory (Coleoptera: Buprestidae), collected near Sudbury and Sault Ste Marie, Canada. Light and electron microscopic investigations showed that gross pathology and ultrastructure of the investigated *Cystosporogenes* species was similar to those characterized and described for other *Cystosporogenes* species. Small subunit rRNA gene sequence data and comparative phylogenetic analysis confirmed that the microsporidian species from *A. anxius* is most closely related to the genus *Cystosporogenes* clade of microsporidia. Infection average in the Sudbury and Sault Ste Marie beetle populations was >80% and relatively stable in 2006–2007 but declined in 2008. Field prevalence of the *A. anxius* isolate, mechanisms that may potentially be involved in its horizontal (autoinfection) and vertical (transovarial) transmission, and disease dynamics are discussed. The congeneric relationship between *Agrilus planipennis* and *A. anxius* makes it imperative to study the virulence of this *Cystosporogenes* species in *A. planipennis*.

1. Introduction

The bronze birch borer, *Agrilus anxius* Gory (Coleoptera: Buprestidae), is one of the most destructive native insect pests of indigenous and exotic birch species in North America. Since the late 1800's, *A. anxius* has been recorded to cause widespread damage to forests, woodlands and ornamental birches in the north-eastern United States and Canada (Katovich et al., 2000).

Adult bronze birch borers primarily attack birches that are weakened or stressed by drought, old age, insect defoliation, soil compaction, or physical injury to the stem or root (Barter, 1957; Williamson and Pellitteri, 2003). Adults feed on birch foliage throughout the entire stage (June to early August). At the end of the maturation period the adults oviposit dozens of eggs under loose flakes, in cracks and crevices of the bark. Neonates drill into the bark until reaching the phloem and sapwood where they feed until pupation. Tree mortality is caused largely by meandering feeding galleries that girdle the branches and trunk. Continual attacks lead to interruption of nutrient translocation and eventual death of the tree.

Agrilus anxius is a congener to emerald ash borer, *Agrilus planipennis* an invasive pest that is spreading and devastating ash trees in North America at an alarming rate (McCullough et al., 2008). The life cycle and developmental stages of these agrilid species are quite similar (Barter, 1957, 1965; Cappaert et al., 2005). *Agrilus* species typically kill trees within 2–4 years after the beetle population in an area reaches a lethal density threshold. The cryptic habits of these buprestids have hindered efforts to develop appropriate management methods. Adverse impacts on forest biodiversity and resources as well as on riparian and urban areas have fueled intensive suppression efforts by Canadian and American authorities. Although chemical substances have been used, consistent and effective control has not yet been achieved (Katovich et al., 2000; McCullough et al., 2004, 2005; Smitley et al., 2005a,b).

Despite the massive outbreaks and wide distribution of these *Agrilus* species, little is known about natural enemies of these wood boring beetles. Suppression of pest organisms by natural enemies is recognized as one of the most suitable long-term pest management strategies for introduced pests and includes the use of entomopathogenic microorganisms (Hajek et al., 2007). Microbial agents may have the potential to suppress populations of *A. planipennis* (Liu and Bauer, 2006, 2008a,b). In an effort to identify microbial agents for control of *A. planipennis* in Canada, we are investigating entomopathogens that infect closely related species (phylogenetically and behaviorally). We therefore collected agrilid species to look for pathogens that have potential as biological control agents against *A. plannipenis*. We hope that self-sustaining biological control agents, such as microsporidia, could become a major factor in long-term suppression of established *A. plannipenis* populations by causing direct mortality or by serving as stress factors predisposing this beetle to mortality caused by other factors.

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doi:10.1016/j.jip.2010.12.010

Microsporidia are obligate intracellular parasites and the phylum consists of approximately 143 genera and over 1200 species (Wittner and Weiss, 1999). They are among the most widespread pathogens of insects, causing disease in both beneficial and pest species (Sprague and Becnel, 1999), and have the potential to act as natural regulators of pest insect populations (Onstad and Carruthers, 1990; Tanada and Kaya, 1993). Microsporidia typically produce chronic infections in host populations, causing suppressed pupal weight and significant reductions in size, fecundity and adult longevity (Thomson, 1958; Wilson, 1983; Fries et al., 1984; Bauer and Nordin, 1988; Anderson and Giacon, 1992). Microsporidia may be transmitted vertically, horizontally, or by both means, depending on species-specific microsporidium–host interactions (Becnel and Andreadis, 1999; Goertz et al., 2007). Given these attributes, microsporidia can rapidly establish and spread within host populations relatively unnoticed until a high prevalence reduces productivity and performance.

There is lack of information on the discovery, morphological identification and molecular characterization of microsporidia parasitizing wood boring beetles. In this paper, we characterize a microsporidian isolate detected in adult *A. anxius*. Detailed studies of the morphological and ultrastructural features, development and molecular analyses of the SSU-rDNA sequence were conducted in order to determine its generic identity and to confirm its taxonomic position. Furthermore, we investigated the prevalence of this previously unreported microsporidium in two Ontario populations of *A. anxius*.

2. Material and methods

2.1. Collection of specimens

Adult *A. anxius* were obtained from infested white birch, *Betula papyrifera* Marsh logs cut from unmanaged forests in two localities near Sudbury and Thunder Bay, and from landscape trees in Sault Ste Marie, Ontario, Canada (Table 1). *Agrilus anxius*-infested log sections were stored in the laboratory in breeding chambers at 24 °C (±1 °C) under long day conditions (*L*:*D* = 16:8). Breeding chambers were emptied daily between 13:00 h and 17:00 h (peak flight activity) until no beetles emerged for several consecutive weeks. To determine microsporidian development post beetle emergence, a subsample of the early emerged beetles was stored frozen until processing, and another cohort was reared on birch twigs for approximately two weeks under the same environmental conditions as above. For the Sault Ste Marie samples, beetles were collected by hand from infested trees during the peak flight activity (mid-July) and reared on birch twigs. In addition, alcohol-preserved adult emerald ash borers, *A. planipennis*, *A. anxius* and the two-lined chestnut borers, *A. bilineatus* (Weber) were kindly provided by Dr. Barry Lyons, Great Lakes Forestry Center, Sault Ste Marie, Canada, and tested for microsporidian infections.

2.2. Light and electron microscopy

Individual beetles were homogenized and screened for microsporidian spores using light microscopy. After wet mount examination of the samples, droplets of microsporidia-infected homogenates were fixed in methanol and stained with Giemsa for further analysis. The size of the Giemsa-stained spores ($n = 50$) was measured with an ocular micrometer (magnification 1000×). In addition, tissue tropism and gross pathology was ascertained by observation of smears prepared from the fat body and midgut tissues excised from beetles ($n = 40$) randomly selected from the infected populations.

For light microscopy, transmission electron microscopy (TEM) and histological investigations, abdominal portions of infected beetles (microsporidia in feces determined by PCR) were fixed overnight in ice cold 2% (v/v) glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) on a rotation device at 4 °C. The tissues were rinsed in cacodylate buffer, postfixed in 2% aqueous osmium tetroxide (w/v), and en bloc stained with 1/2 saturated uranyl acetate, a modification from Karnovsky (1971). Tissue samples were then dehydrated gradually through an ascending ethanol series followed by acetone and ethanol–propylene oxide series. Finally, the samples were embedded in araldite resin. For general histology, semithin (0.5–1 μm) sections were mounted on slides, stained with toluidine blue, and viewed under bright field optics using a Nikon Eclipse E-600 microscope. TEM ultrathin sections were stained with lead citrate and examined with a JEOL1200EX11 electron microscope at 80 kV.

2.3. DNA isolation, amplification, cloning and sequencing

The preserved *A. anxius* homogenates used for the light microscopy examination, and alcohol-preserved *A. anxius*, *A. planipennis* and *A. bilineatus* were used for total genomic DNA extractions. Briefly, beetles were individually macerated in a microcentrifuge tube containing 300 mg glass beads (212–300 μm diameter) (Sigma, St. Louis, MO) and 200 ml of digestion buffer (100 mM NaCl, 10 mM Tris–HCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate, pH 8.0 and 20 mg/ml of proteinase K). The homogenates were then incubated at 55 °C for 3 h and the nucleic acids were extracted twice with an equal volume of chloroform/phenol, then by an equal volume of chloroform followed by ethanol-precipitation. The precipitated DNA was dissolved in 100 μl of ddH_2O and frozen at −20 °C until needed as DNA template in polymerase chain reaction (PCR). Aliquots of DNA from individual beetles were pooled per collection site and used in the molecular analyses.

Table 1
Occurrence and prevalence of *Cystosporogenes* species infecting *Agrilus anxius* populations in Ontario, Canada.

	Year of collection	Total number of beetles screened	No. of beetle infected (total screened)			Total percent infection rate
			Newly emerged[a]	Live post rearing beetle [b]	Dead post rearing beetle	
Sudbury	2006	48	6 (10)	13 (16)	22 (22)	85.4
	2007	38	7(10)	5 (8)	20 (20)	84.2
	2008	31	3(5)	8 (12)	9 (14)	64.5
Sault Ste Marie	2007	30	4 (5)	3 (5)	18 (20)	83.3
	2008	25	3 (5)	4 (6)	12 (14)	76.0
Thunder Bay	2006	40	0 (10)	0 (23)	0 (7)	0.0
	2007	23	0 (5)	0 (12)	0 (6)	0.0

[a] Newly emerged beetles screened for the studied *Cystosporogenes* sp. on day 1 following emergence, no rearing.
[b] Live beetles (survivors) screened for the studied *Cystosporogenes* sp. on day 14 following emergence and rearing on birch twigs.

The universal primer set, 18F/1537R (Baker et al., 1995) was first used to screen the DNA samples for the coding region of microsporidia SSU rRNA gene. New primer sets designed for this work, Ag1F (5′-ATTCTGCCTGACGTAGACGCT-3′) and Ag1R2 (5′-AGATAGCGACGGGCGGTGTGT-3′) was based on SSU rRNA gene sequences from a wide array of microsporidia species and genera retrieved from Genbank (http://www.ncbi.nlm.nih.gov/). Specific internal primers, Ag3F (5′-AGTCGTGGAGGTTGAAATTGGGGCA-3′) and Ag3R4 (5′-AGGGCATCTAAGGGCATCTCT-3′) were designed to screen the *A. anxius* populations for the identified microsporidium infecting *A. anxius*. These new primers were necessary because 18F/1537R failed to amplify products even though microsporidian spores were observed microscopically in the homogenates, while the positive control, *Nosema fumiferanae* DNA did amplify successfully under the same conditions. PCR conditions were as follows: 94 °C for 3 min initial denaturation, followed by 35 cycles of 30 s at 94 °C, 60 s at 56–60 °C and 90 s at 72 °C, and a final extension step of 10 min at 72 °C. Each 25 μl reaction mixture contained l.0 μl of supernatant (template DNA), 2.5 μl of 10× PCR buffer, 4.0 μl of dNTPs, 0.2 μl of TaKaRa LA DNA polymerase Taq (TaKaRa Biotechnology Co. Ltd.), 0.5 μl of each primer set (20 μM) and 16.3 μl of HPLC distilled water. Amplified products were resolved in 1.3% agarose gel with ethidium bromide staining. PCR amplifications were conducted three times on different occasions to establish reproducibility. PCR product of the expected size of ~1100 bp in length, was purified using a QIAquick PCR Purification Kit (Qiagen Company, Valencia, California), and subsequently cloned into the vector pCR2.1 using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA. USA) and the manufacturer's instructions. Three cloned DNA inserts were sequenced in the forward and reverse directions using an ABI 377 XL Automated Sequencer (Perkin-Elmer Applied Biosystems, Foster City, California) using Big Dye 3.1 sequencing chemistry according to manufacturer's protocol.

2.4. Phylogenetic analyses

Small subunit rRNA sequences obtained from microsporidia infecting *A. anxius* were compared with microsporidia SSU rRNA sequences archived in GenBank to assess putative identifications and likely relationships using BLAST: (http://www.ncbi.nlm.nih.gov/blast/). Twenty-six SSU rRNA sequences belonging to various microsporidia species and genera that produced the highest scores in the BLAST search were introduced into the analyses to determine their relationships with the microsporidian infecting *A. anxius* (see Table 2 and Fig. 2 for Accession numbers). These sequences were aligned using CLUSTAL W (Thompson et al., 1997) and edited manually in the software BioEdit v. 7.0.1 (Hall, 1999). Gaps and ambiguously aligned regions were omitted and the sequences were finally trimmed to a length of 1073 characters before the analyses.

The aligned sequences were used to construct a maximum likelihood (ML) tree as employed in the program PhyML 3.0 (Guindon and Gascuel, 2003)(http://atgc.lirmm.fr/phyml/). The tree was inferred using GTR model for nucleotide substitutions with discrete gamma distribution in 8 + 1 categories and all parameters (gamma shape, proportion of invariants) were estimated from the dataset. The proportion of invariable sites and the g shape parameter were estimated from the data (0.177 and 0.747, respectively). Additional evolutionary history was inferred using the Maximum Parsimony (MP) and Neighbor-Joining (NJ) algorithms as implemented in MEGA 4 program (Tamura et al., 2007). The analyses were based respectively on the two-parameter distance model of substitution (Kimura, 1980) and the max–mini branch and bound option. Confidence estimates and robustness of the branches were assessed by bootstrap analysis based on 500 iterations for MP and ML, and on a 1000 bootstrap replicates for NJ. A pairwise *p*-distance matrix for the microsporidium infecting *A. anxius* and its congeners' based on SSU rRNA sequences was constructed in the

Table 2
Comparison of SSU-rDNA sequences: percent similarity (upper triangle) and pairwise distance (bottom triangle) obtained by Kimura 2-parameter analysis.

	1	2	3	4	5	6	7	8	9	10	11
1. *Cystosporogenes* sp. GKK-2009 GQ379703	–	99.7	99.2	99.2	97.8	89.1	88.7	88.5	88.5	65.2	67.0
2. *Cystosporogenes* sp. GKK-2009 GQ379704	0.8	–	99.5	99.5	98.1	89.3	89.0	88.8	88.8	65.3	67.1
3. *Cystosporogenes* sp. CRV-2004 AY566237	0.8	0.0	–	100	98.1	89.4	89.1	88.9	88.9	64.7	66.2
4. *Cystosporogenes legeri* AY233131	8.1	7.3	7.3	–	98.0	89.6	89.3	89.1	89.1	65.6	67.0
5. *Cystosporogenes operophterae* AJ302320	7.7	6.9	6.9	0.3	–	88.9	88.6	88.4	88.3	65.5	66.9
6. *Endoreticulatus schubergi* L39109	8.0	7.3	7.3	0.2	0.4	–	99.4	99.4	99.3	64.5	65.9
7. *Endoreticulatus* sp. CHW-2004 AY502945	7.9	7.2	7.2	0.4	0.3	0.5	–	99.4	99.3	64.3	66.3
8. *Endoreticulatus* sp. CHW-2004 AY502944	30.7	30.5	30.5	32.9	32.7	32.9	32.7	–	99.6	64.5	65.9
9. *Endoreticulatus bombycis* AY009115	29.3	29.1	29.1	30.7	30.6	30.6	30.7	13.1	–	63.8	65.5
10. *Nosema bombycis* AB093009	1.2	0.4	0.4	.57	7.1	7.4	7.3	30.4	29.1	–	83.3
11. *Vairimorpha necatrix* Y00266	1.0	0.2	0.2	7.3	6.9	7.2	7.1	30.3	28.9	0.2	–

Characterized microsporidian species is in bold lettering.

MEGA 4 program under pairwise deletion of alignment gaps and missing data. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1).

2.5. Microsporidian prevalence in the field

After the discovery of the *Cystosporogenes* isolate in the Sudbury *A. anxius* population in 2006, another survey was conducted to determine its presence and prevalence in *A. anxius* populations from other localities in Ontario, Canada (Table 1). Sites and populations identified as infected with this *Cystosporogenes* species were repeatedly sampled until 2008 to confirm its persistence over time and space. Abdominal tissues of frozen cadavers as well as live beetles were homogenized and DNA was extracted from the samples as previously described. The specific internal primer set Ag3F/Ag3R2, was used to screen DNA from the various *A. anxius* populations. The primer set Ag1F/Ag1R2 was included as the control. We compared prevalence in newly emerged as well as in live and dead adult *A. anxius* beetles ($n = 235$) 2 weeks following rearing on birch foliage. In addition, we monitored the overall infection rate as emergence progressed over a 2-year period in the Sault Ste. Marie population. Level of infection (disease load) of a cohort of these beetles was determined and scored as either light or moderate – heavy based on the intensity of amplified bands. Random subsamples of each sex ♀:♂ (15:15) from the Sudbury population and the same number from the Sault Ste Marie population were screened for microsporidia.

3. Results

3.1. General observations

The *Cystosporogenes* species was recovered from nearly all *A. anxius* adults collected from Sudbury and Sault Ste Marie, but beetles from the Thunder Bay area were not infected with any microsporidia (Table 1). None of the infected beetles exhibited externally visible symptoms or gross signs of infection such as obvious changes in mobility, growth or color compared with uninfected individuals. PCR amplification confirmed that infected beetles shed spores in their feces.

3.2. Light and electron microscopy observations

Homogenates of adult *A. anxius* collected from Sudbury and Sault Ste Marie contained large quantities of spores (Fig. 1A). These spores were oval-to kidney-shaped with an average size of $2.60 \pm 0.12 \times 1.66 \pm 0.12$ μm (mean ± standard error of mean) ($n = 50$). Infected fat body tissues were gray or white in color compared to creamy white appearance in healthy beetles. Infected tissues were fragile and contained numerous shiny white nodules. Spores of the microsporidian species infecting *A. anxius* were detected in both fat body and midgut tissues of 80% (32/40) of the dissected beetles. Light and electron micrographs of infected tissues revealed various developmental stages; meronts, sporonts, sporoblasts and mature spores (Fig. 1A–O). Development occurred predominantly in fat bodies (Fig. 1C, D, and H). Mature spores were found in the gut lumen, suggesting that epithelial cells of the midgut may also be infected (Fig. 1B arrow). Hemocyte-like cells and hypodermal cells packed with spores and other developmental stages were observed under the host integument (Fig. 1E and F). The microsporidium was monokaryotic. All developmental stages occurred asynchronously inside interfacial envelopes of a single membrane of unknown origin, which was in direct contact with the host-cell cytoplasm (Fig. 1C, D, and G). We were unable to observe the natural transition from meronts to sporonts on our micrographs. Likewise, the interface between merogony and sporogony was observed as blurred in the life stages of *Cystosporogenes operophterae* parasitizing *Operophtera brumata* (Canning and Curry, 2004). The meront stage could be very short and merogonal divisions may not occur during later stage infections. Meronts transformed directly into spherical uninucleate sporonts which are characterized by a thickening of the plasmalemma and a centrally electron dense cytoplasm (Fig. 1J). Numerous elongated, thick-walled, irregularly shaped structures with dense cytoplasm were considered to be early sporonts or sporoblasts and, with mature spores, lay in tightly fitting vacuoles surrounded by an interfacial envelope (Fig. 1I). A spherical or ovoid uninucleate sporont limited by a simple plasmalemma was seen in the hemocyte-like cells (Fig. 1H). Sporogony was multisporous (Fig. 1C, D, G and I). The vacuolar envelopes were large, filled mostly with spores (intact spores as well as germinated spores) and other life stages (Fig. 1C, D and G). Mature spores have a structure typical of other microsporidia (Vavra and Larsson, 1999). The spores were bean-shaped and showed a distinct electron-opaque and wavy exospore surrounding a thicker electron-lucent endospore (Fig. 1K–O). The internal structures of the spore consisted of; (1) a domed anchoring disk at the anterior end that continued into a polar sac (Fig. 1K); (2) isofilar polar filament arranged around the nucleus in a single row of 8–10 coils extended to posterior half of the spore (Fig. 1L); (3) a single elongate nucleus, occupying more than a third of the length of the cytoplasmic structure and surrounded by several layers of rough endoplasmic reticulum, (Fig. 1M–O); and (4) a small posterior vacuole located in the posterior end of the spore (Fig. 1N and O). Extrusion of polar tubes was not observed, but we observed a mature spore that appeared to be at the point of extrusion with the polar sac extending around the apex of the spore surrounding the dense polaroplast (Fig. 1O).

3.3. SSU rRNA amplification, sequence and phylogenetic analysis

With the exception of the positive control *N. fumiferanae*, the primers 18F/1537R failed to amplify products when tested against the DNA of the agrilid microsporidium. However, we amplified a fragment of the expected size 1121 bp using our designed primer set, Ag1F/Ag1R2 and the microsporidian DNA obtained from the Sault Ste Marie and Sudbury *A. anxius* populations. Neither of the primer sets amplified microsporidian products from *A. anxius* collected from the Thunder Bay area and the other two agrilid species, *A. planipennis* and *A. bilineatus*. These specimens did not harbor any life stages of microsporidia based on microscopic determination, and PCR detection was negative even with serially diluted DNA.

The SSU rRNA sequences of the Sault Ste. Marie and the Sudbury *A. anxius Cystosporogenes* haplotypes span an 1144-bp length with ~49.0% GC content with respective accession numbers GQ379704 and GQ379703. The Sault Ste Marie differs from the Sudbury haplotype by three diagnostic substitutions at positions 178 G/A, 295 C/T and 574 A/G respectively. The BLAST search revealed that the microsporidian species infecting *A. anxius* belongs to Group II of the SSU rRNA phylogeny (Baker, et al. 1994, 1995; Keeling and McFadden, 1998), exhibited maximal similarity with the genus *Cystosporogenes* (~97–99%) and ~89.0% sequence identity to that of the genus *Endoreticulatus* (Table 2). There was a low SSU rRNA sequence similarity (62–65%) to the Group IV microsporidia type species comprising of the genera *Nosema*, (*N. bombycis*) and *Vairimorpha*, (*V. necatrix*).

Close identity of the *A. anxius* isolate with other *Cystosporogenes* species was also shown in the SSU rRNA sequence phylogenetic trees based on parsimony, neighbor joining, and maximum likelihood algorithms. All methods applied revealed the same general tree topology by clustering the *A. anxius* isolate with species belonging to the genus *Cystosporogenes* (Fig. 2, parsimony and neighbor joining data not shown). In all the constructed trees,

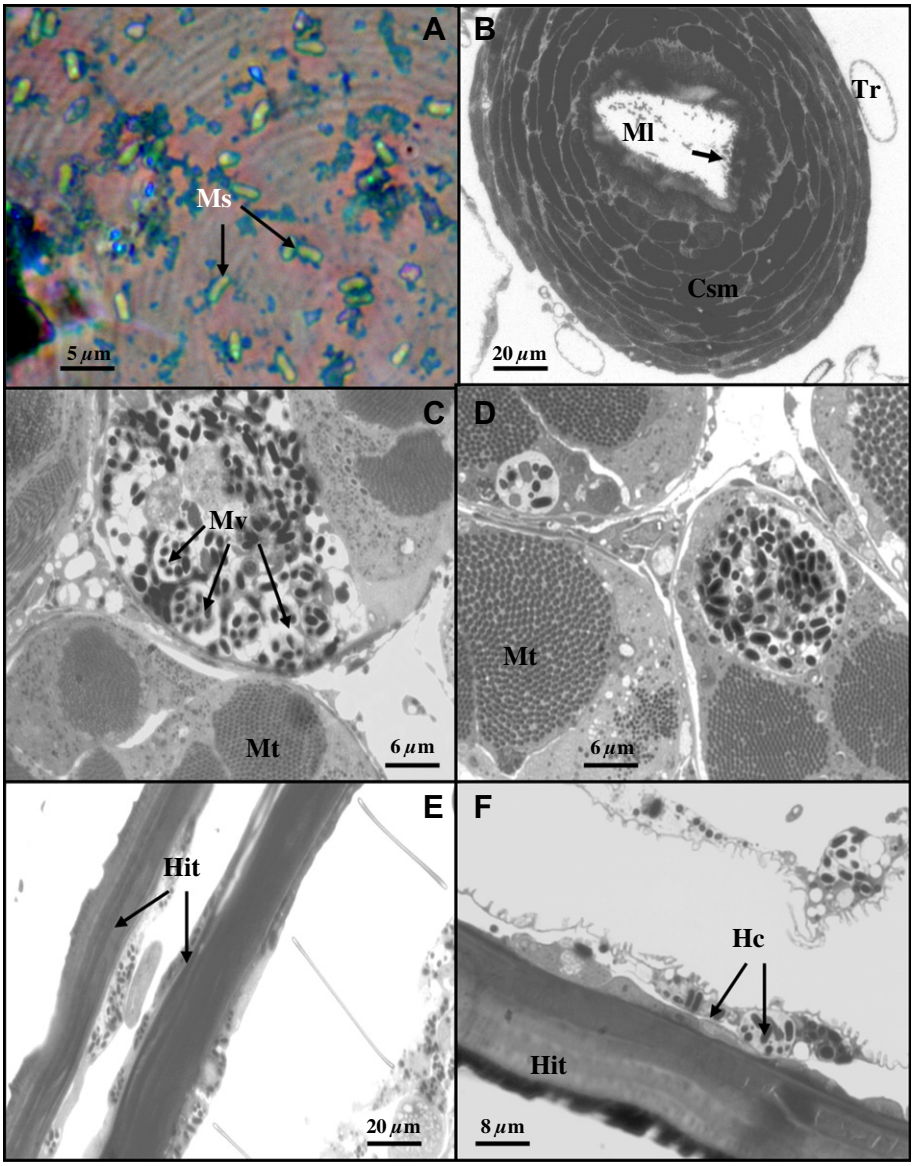

Fig. 1. Light (A–F and H) and electron (G, I–O) microscopy micrographs comprising various developmental stages of the microsporidian species infecting *A. anxius*. (A) Smear from infected tissue containing mainly mature uniform ovoid spores (Ms). (B) Cross section of the gut (Csm) epithelium adjacent to tracheal tubes (Tr) of *A. anxius*; arrow shows mature spores in the midgut lumen (Ml). (C) Multiple vacuoles (Mv) filled with various stages, clustered together in the host-cell cytoplasm, (Mt) muscle tissues. (D) Mature spores within a vacuole surrounded by muscle tissues (Mt). (E and F) Hemocyte-like and hypodermal cells (Hc) filled with spores and other developmental stages under the host integument (Hit). (G) Vacuolar or interfacial envelope containing primary spores and germinated empty spore (Gs). (H) Infected fat body cell under the host integument containing late sporonts (Sp). (I) Interfacial envelope containing early free merogonic or sporogonic stages, sporoblasts (Spb) and primary spores. (J) Late sporont or early sporoblast with a deposition covering the cell membrane and formation of a polar filament; membrane-like envelope is probably the exospore (Ex), single nucleus (N), lacks ribosomes and it is clearly separated from nearby host cells; nascent polar filament coils (arrow). (K–N) Longitudinal sections displaying internal spore structures of the characterized *Cystosporogenes* species. Mature spores were uninucleate (N). At the anterior pole of the spore is the anchoring disk (Ac) and at the posterior is the posterior vacuole (Pv) with vacuoles. Spore showing rough exospore (Ex), the endospore (En), endoplasmic reticulum (Er) running along either side of the nucleus. In addition, the spore shows the polaroplast (Po), polar tube (Pt) and polar filament (Pf) making 8–9 turns. (O) Mature spore at the point of everting its polaroplast.

the bootstrap values supporting the upper branch of genus *Cystosporogenes* clade were high: NJ (99%), MP (99%), and ML (99%) indicating substantial reliability for this taxonomic grouping. Phylogenetic clustering of sequence data was further supported by genetic distances calculated using Kimura's model; average within-group distance for the genus *Cystosporogenes* was between 0.0% and 2.2% (Table 2). The minimum genetic distance observed between the Sault Ste Marie and Sudbury haplotypes was 0.3%.

3.4. Microsporidian prevalence in the field

The newly designed specific-SSU rRNA primers, Ag3F/Ag3R4 amplified PCR products from beetles collected in all locations except those from the Thunder Bay area (Table 1). Of the total 172 beetles screened from the Sudbury and Sault Ste. Marie populations, 137 were infected and the average prevalence was ~79.7% for the study period (Table 1). Prevalence was very high at both localities but differed between sampled years. Taking the combined infections from the Sudbury and Sault Ste. Marie populations, 23 (13.4% of all screened, 65.7% of newly emerged beetles), 33 (19.2% of all screened, 70.2% of live beetles) and 81 (47.1% of all screened, 90.0% of newly emerged beetles) were infected and most of them died during the 2 weeks of laboratory rearing. As shown in the overall infection rate of the Sault Ste. Marie population, early emerging beetles had lower infection rates compared with late emerging individuals (Fig. 3). The 2008 Sault Ste Marie

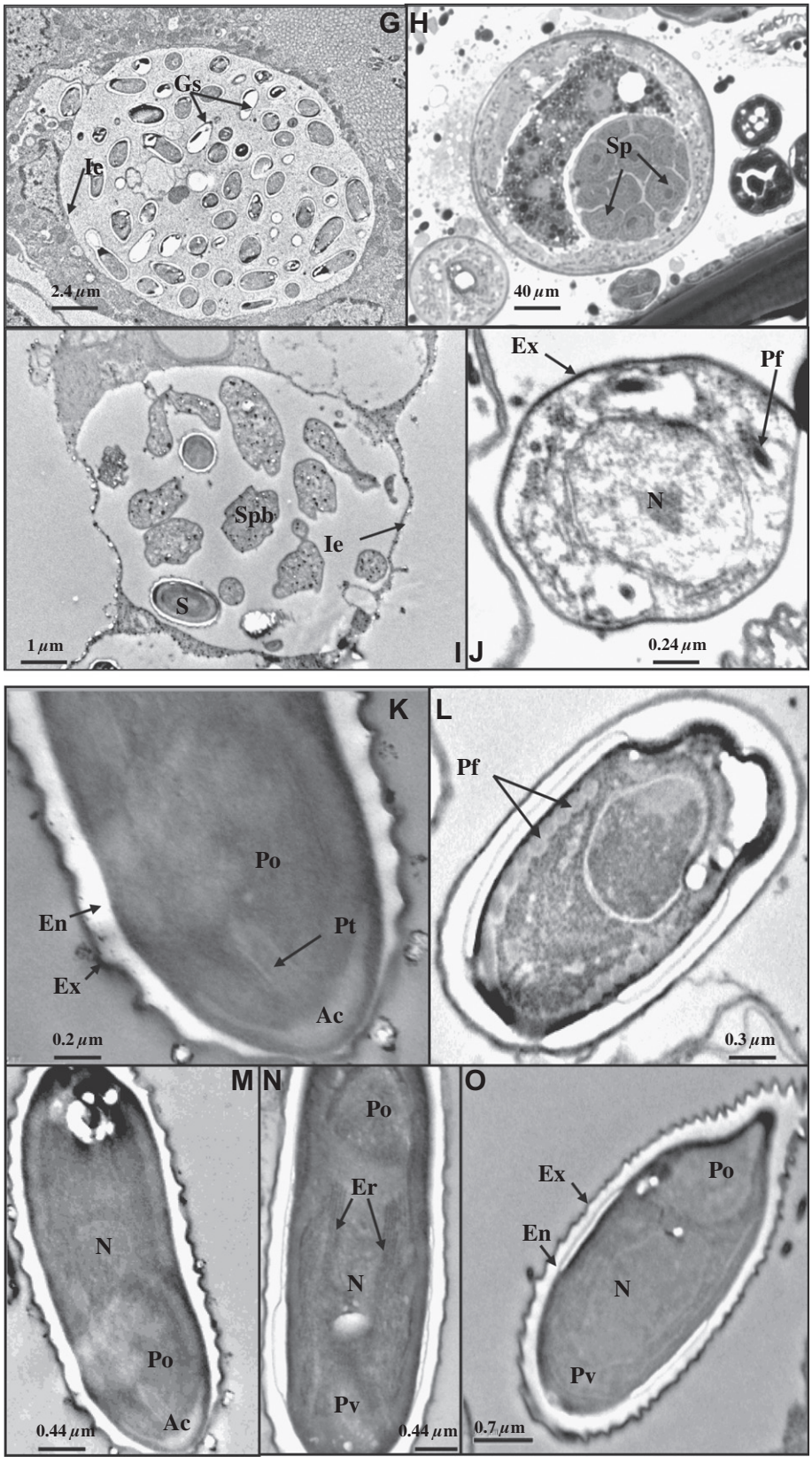

Fig. 1 (*continued*)

collection produced 44.8 % fewer adults than the 2007 cohort. Level of infection (disease load) in early emerged beetles was lighter but increased from moderate to heavy as the emergence period progressed. Overall, dead individuals at any stage in the emergence period were moderate-heavily infected (Fig. 4; lanes 3–6, 7–10 and 11–14). Both genders were infected equally and the sex ratios of infected beetles, ♀:♂ were 1:0.93 and 1:0.87 for the Sault Ste. Marie and Sudbury population respectively.

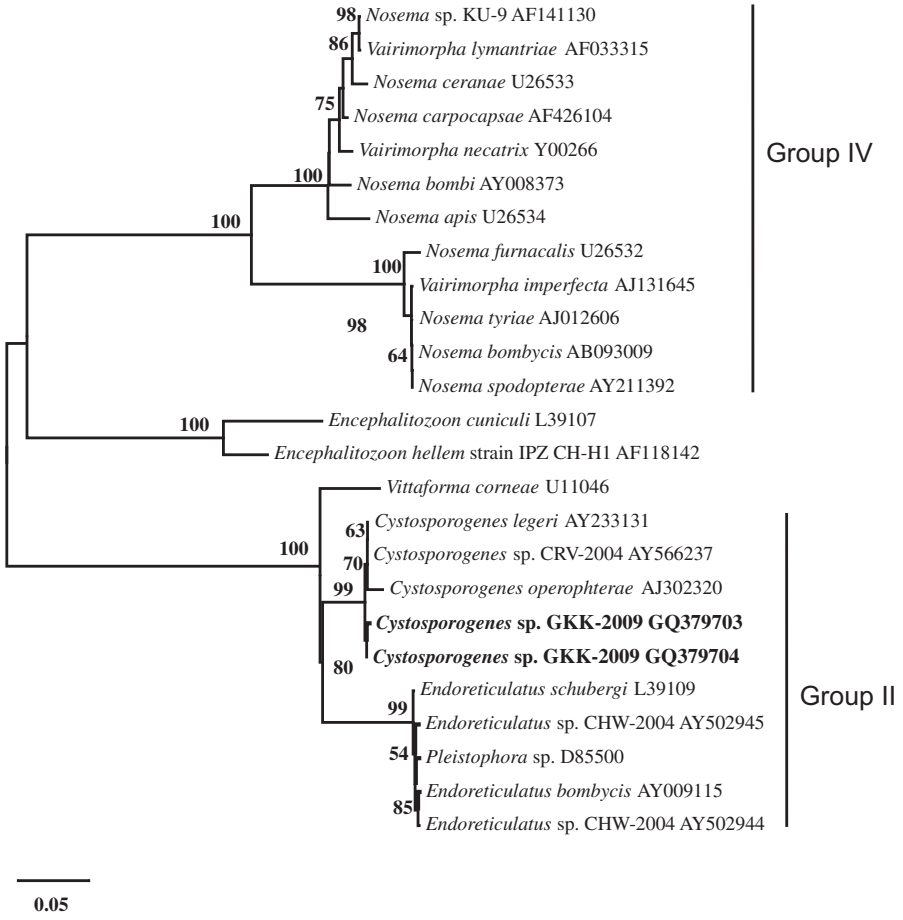

Fig. 2. Phylogenetic relationships among the *Cystosporogenes* species infecting *A. anxius* (bold lettering) and other microsporidia, based on SSUrRNA sequences. The tree was generated using the maximum likelihood (ML) algorithm and Kimura two-parameter distance method. Maximum pasimony (MP) and neighbor joining (NJ) analyses gave identical topologies. Numbers next to the nodes refer to the bootstrap scores (%) in 100 replicates. The groupings are common microsporidial divisions suggested by other authors (Baker, et al. 1994, 1995; Keeling and McFadden, 1998).

4. Discussion

This research resulted in the discovery of a *Cystosporogenes* species infecting field populations of *A. anxius* in Ontario, Canada. The isolate was characterized using developmental information as well as morphological and molecular data. The life cycle stages, ultrastructural characteristics and spore dimensions, as well as microsporidian–host relationships, provide characters that traditionally have been used to make taxonomic determinations of microsporidia genera and species (Sprague, 1977; Larsson, 1986). Phase-contrast and electron microscopy of selected tissues from infected *A. anxius* revealed a macro-infection which occurred predominantly in fat bodies, midgut and muscle tissues, while mature spores were observed in the gut lumen. The *Cystosporogenes* species from *A. anxius* is monokaryotic throughout its life cycle. Evidence from gross pathology studies has shown that infection by *Endoreticulatus* is characteristically confined to midgut epithelial cells (Brooks et al., 1988). The genus *Cystosporogenes* initially infects the midgut, and subsequently spreads to gut epithelial cells, fat bodies, Malpighian tubules, muscles, ganglia, gonads, and silk glands to cause a generalized infection (Kleespies et al., 2003; Canning and Curry, 2004; van Frankenhuyzen et al., 2004). In addition, the most consistent taxonomic character used to distinguish between these two genera is the number of membrane-like envelopes that envelop the life cycle stages. All developmental stages including meronts, dividing fusiform sporonts, sporoblasts and mature spores of the microsporidian species infecting *A. anxius* occurred within a vacuole bounded by a single membrane of unknown origin, which is typical for species of the genus *Cystosporogenes* (Kleespies et al., 2003; Canning and Curry, 2004; van Frankenhuyzen et al., 2004). In contrast, species of the genus *Endoreticulatus* develop within a parasitophorous membrane derived from host endoplasmic reticulum (Brooks et al., 1988). Mature spores observed in the present study shared several structural characteristics with previously described *Cystosporogenes* isolates as did gross pathology, life cycle stages and ultrastructural characteristics (Canning et al., 1983; Kleespies et al., 2003; van Frankenhuyzen et al., 2004).

Morphological differences among the *Endoreticulatus/Cystosporogenes* group are supported molecular data. The percentage of sequence similarity or variability and SSU rRNA-based phylogenetic analyses have proven to be a reliable criteria for assessing taxonomic relationships among microsporidian species and are most suitable for molecular characterization of new species (Baker et al., 1994; Franzen et al., 2006; Sokolova et al., 2003; Vossbrinck and Woese, 1986). The high sequence identities and small distance values indicated an extremely close relationship between *Cystosporogenes* sp. CRV-2004, *C. legeri, C. operophterae* and the microsporidian isolate from *A. anxius* and certainly warrant placement of the later in the genus *Cystosporogenes*. We cannot exclude the possibility that the *Cystosporogenes* species infecting the Sudbury and Sault Ste. Marie *A. anxius* populations are haplotypes because the SSU rRNA sequences were approximately 99.7% identical (Table 2). The SSU rRNA sequence-based phylogeny of microsporidia using all analytical methods consistently and stably grouped *A. anxius* isolate with members of the genus

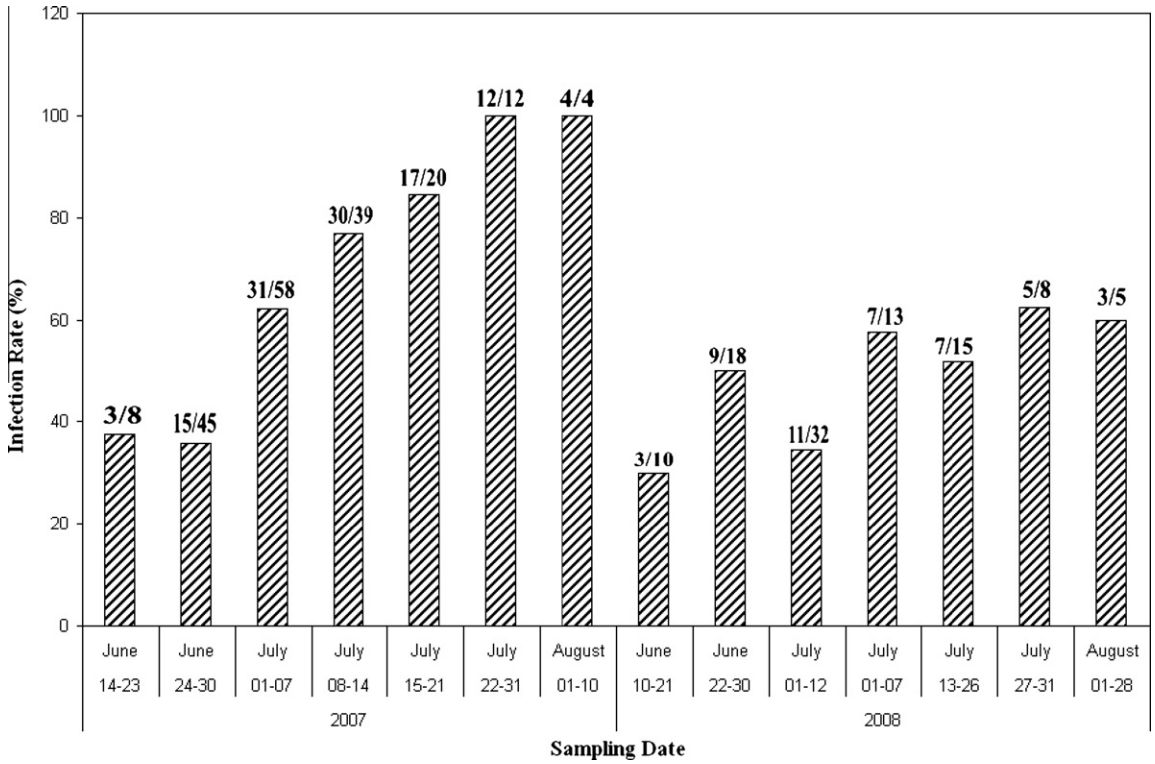

Fig. 3. Infection levels of the studied *Cystosporogenes* species infecting Sault Ste Marie populations of *A. anxius*. The highest infection was observed in the 2007 samples. The bars show number of infected *A. anxius*/total number of emerging beetles during the observation period.

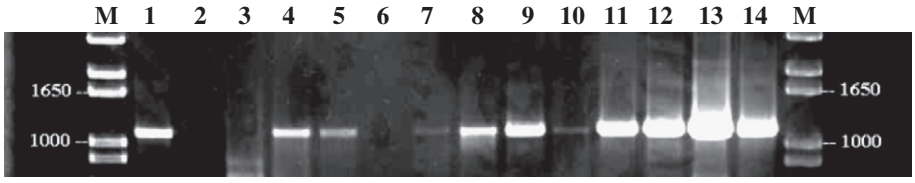

Fig. 4. Detection of the studied *Cystosporogenes* species in early emerging, surviving and dead beetles. DNA was amplified with the primer set, Ag1F/Ag1R2 and generated a fragment size of 1100 bp. DNA from *Nosema fumiferanae* was used as the positive control (lane 1). Water was used as the negative control (Lane 2). Lanes 3–6 represent early emerged adults; lanes 7–10 represent surviving adults 2 weeks post emergence and rearing and lanes 11–14 represents beetles that died during rearing. M = molecular weight marker.

Cystosporogenes and with a higher bootstrap support of 99%. Consequently, considering the observed morphological characters and the comparative sequence analysis that provides robust resolving power for clade identification, we concluded that the appropriate placement of the microsporidian species infecting *A. anxius* is within the *Cystosporogenes* group.

Distribution of *Cystosporogenes* infection in *A. anxius* populations was geographically localized. The beetles sampled from the two localities, Sudbury and Sault Ste. Marie, were in outbreak status and had similar infection rates. Although we did not detect the same *Cystosporogenes* strain or any other microsporidia in the Thunder Bay population, we cannot rule out its presence. It may likely be present but perhaps at a very low prevalence. Beetles collected from two localities, Sudbury and Sault Ste Marie, were infected at high prevalence (>80%) similar to prevalence of other microsporidia in very different hosts (Canning et al., 1999; Sokolova et al., 2006; Fokin et al., 2008). The variation in infection levels (spore load) from light to moderately-heavy commensurate with adult beetle emergence time (early and late emergence) is an indication that the *Cystosporogenes* species infecting *A. anxius* may have killed a high percentage of transovarially infected larvae (Siegel et al., 1986) and retarded larval/pupal development (Solter et al., 1990). The suggested latency in *A. anxius* development may provide ample time for the *Cystosporogenes* to multiply in the host and build up infective spores, a cost to beetle health and fitness.

Some microsporidian species are known to be transmitted both vertically and horizontally via infected females and males, and may result in field prevalence of relatively high levels (Lipa and Madziara-Borusiewicz, 1976; Bruck and Lewis, 1999; Lewis et al., 2006; Goertz and Hoch, 2008). The characterized microsporidian species developed in beetles of both sexes and may play a significant role in vertical (transovarial) transmission as has been observed in other insects (Dunn and Smith, 2001; Solter, 2006). The less intense infection of this *Cystosporogenes* during the early emergence stages of adult *A. anxius* (Fig 4) and especially the late death may imply weak effects on the host and, thus, increased survival of adults and vertical transmission to the next generation. Dunn et al. (2001) suggested that reduced growth of vertically transmitted parasites is an adaptation to increase host survival, and the likelihood of host and parasite reproduction. The presence of spores in the gut lumen (Fig. 1) suggests a steady continual release of spores into the environment in fecal material, thus becoming a source of inoculum to infect newly emerged adults. We were able to amplify products in infected beetle feces prior to selecting individuals for

the electron microscopy work. This observation suggests likelihood of horizontal transmission in field populations of *A. anxius*.

The search for potential and effective entomopathogens for the management of the invasive *A. planipennis* has been the subject of several recent studies (Liu and Bauer, 2006, 2008a, 2008b). It is widely believed that pathogens of closely related insect species will be infectious to congeners (Briano et al., 1995; Chen et al., 2009; Higes et al., 2006; Klee et al., 2007; Knell et al., 1977; Sakamoto et al., 2007). However, there are cases where pathogens isolated from congeners did not result in infection of a target host under laboratory inoculations (Oi et al., 2010). Our goal of this research is to use pathogens of *A. anxius* for the management of its congener, *A. planipennis*. Chronic pathogens such as microsporidia have largely sublethal effects when parasitizing a host. Microsporidia infection can be debilitating to the host especially when combined with specific stress factors, other pathogens and natural enemies. As part of an IPM strategy, and given the various attributes as summarize above on the studied *Cystosporogenes* species, it is apparent that they may have potential as natural control agents and may play a significant role as augmentative control agents for population suppression of *A. planipennis*. There are reports of microsporidian species cross-infecting congeneric hosts (van Frankenhuyzen et al., 2004), but this is not always the case (Solter and Maddox, 1998). It is therefore imperative to conduct infectivity, pathogenicity and transmission studies using spores of the studied *Cystosporogenes* species against *A. planipennis* to determine its potential as a control agent and/or mortality enhancer.

We report the first microsporidian isolate of the genus *Cystosporogenes* parasitizing a coleopteran species, *A. anxius* of the family Buprestidae. Our findings show that the identified *Cystosporogenes* species was widespread in outbreak populations of *A. anxius* in Ontario, Canada. Morphological characterization, in addition to molecular data and phylogenetic analysis of the SSU rRNA gene sequence, was fundamental to understanding phylogenetic relationship of the *Cystosporogenes* isolate from *A. anxius* with other *Cystosporogenes* species. In order to avoid taxonomic inflation, the *Cystosporogenes* isolate from *A. anxius* should be considered as a member of the well-characterized *Cystosporogenes* species until more data are obtained. The high prevalence of infection, progressive mortality observed in infected beetles with mortality occurring over a short period of rearing time in the laboratory, indicates that the identified *Cystosporogenes* is moderately or highly pathogenic to *A. anxius*. These attributes underscore the potential of this microsporidium for use in the management of *Agrilus* species if exploited appropriately. We have selected sites within the epizootic zone for long-term ecological studies which could lead to the development of timely and appropriate control strategies for future outbreaks of *A. anxius*.

Acknowledgments

This study was supported by the Canadian Biotechnology Strategy Genomics initiative through the Canadian Forestry Service, Natural Resources Canada, and the Provinces of Ontario and Manitoba arranged by SERG-International.

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