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Molecular and ultrastructural characterization of *Dictyocoela diporeiae* n. sp. (Microsporidia), a parasite of *Diporeia* spp. (Amphipoda, Gammaridea)

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Abstract – *Dictyocoela diporeiae* n. sp. is described from *Diporeia* spp. (Amphipoda, Gammaridea) collected from Lake Superior (USA), and its morphology and taxonomic affiliation are discussed. In hematoxylin- and eosin-stained sections of infected amphipods, the microsporidian was observed to infect muscle tissue surrounding the ovaries. Melanized hemocytic encapsulations were often observed in or near masses of microsporidians. The microsporidians appeared as spores measuring 1.99 ± 0.09 µm long by 1.19 ± 0.05 µm wide. Each spore contained eight coils of isofilar polar filaments that were arranged in single ranks. Polar filaments measured 71 ± 3 nm in diameter. A prominent lamellar polaroplast composed of ordered concentric membranes was found at the apical end of the spore surrounding the polar filament. A distinct posterior vacuole was observed at the distal end of the spore. Phylogenetic analysis based on 16s RNA sequences showed that the microsporidian belongs to the genus *Dictyocoela*, and is most similar to *D. berillonum*, yet distinctly different. The species is new, based on its morphology, genetic sequence, host, and location within the host.

Key words: *Dictyocoela diporeiae* n. sp., Microsporidia, *Diporeia*, Small subunit ribosomal DNA.

Résumé – Caractérisation moléculaire et ultrastructurale de *Dictyocoela diporeiae* n. sp. (Microsporidia), un parasite de *Diporeia* spp. (Amphipoda, Gammaridea). *Dictyocoela diporeiae* n. sp. est décrit de *Diporeia* spp. (Amphipoda, Gammaridea) prélevé dans le Lac Supérieur (USA), et sa morphologie et affiliation taxonomique sont discutées. Dans les coupes d'amphipodes infectés colorées à l'hématoxyline-éosine, il a été observé que la microsporidie infecte les tissus musculaires entourant les ovaires. Des encapsulations hémocytiques mélanisées ont été souvent observées dans les masses de microsporidies ou à proximité. La microsporidie est apparue sous forme de spores mesurant 1,99 ± 0,09 µm de long et 1,19 ± 0,05 µm de large. Chaque spore contenait huit spires de filaments polaires isofilaires disposés en rangs simples. Les filaments polaires mesuraient 71 ± 3 nm de diamètre. Un polaroplaste lamellaire important, composée de membranes concentriques ordonnées, a été trouvé à l'extrémité apicale de la spore et entoure le filament polaire. Une vacuole postérieure distincte a été observée à l'extrémité distale de la spore. L'analyse phylogénétique basée sur les séquences d'ARN 16S a montré que la microsporidie appartient au genre *Dictyocoela*, et est très semblable à *D. berillonum*, tout en s'en démarquant. L'espèce est nouvelle de par sa morphologie, séquence génétique, hôte, et localisation dans l'hôte.

Introduction

Over the past three decades, a steady decline in amphipods of the genus *Diporeia* has been observed in four of the Laurentian Great Lakes in North America. This is concerning since *Diporeia* spp. constitute an important component of the food web and traditionally have been a major prey item for a number of commercial fisheries (e.g., lake whitefish, *Coregonus*

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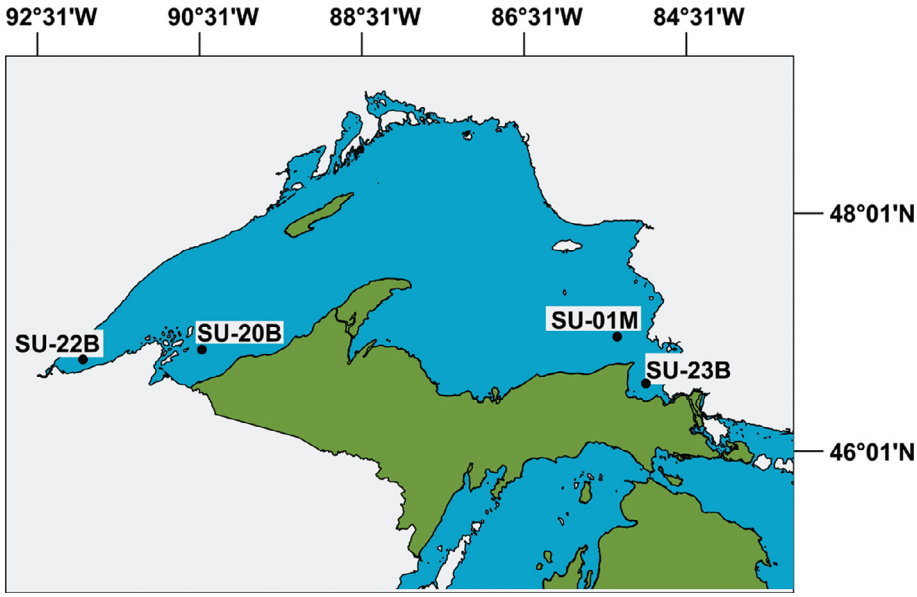

Figure 1. Sampling sites in Lake Superior where *Diporeia* sp. (Amphipoda, Gammaridae) were collected.

clupeaformis) [3, 6, 18, 21, 22]. In a previous study [20], the authors reported on the presence of multiple parasites and fungi infecting *Diporeia* spp. collected from Lake Michigan (USA). Among these, microsporidia were found in 0.68% (21/3, 082) of *Diporeia* collected from nine sites in Lake Michigan between 1980 and 2007. Microsporidian spores were observed in high densities where they filled and replaced muscle tissue. Melanized encapsulating host hemocytes were often observed in or near masses of microsporidians, suggesting that the parasite is pathogenic to *Diporeia*.

Microsporidia are a diverse and ubiquitous group of obligate intracellular single-cell fungi with an extraordinary host range; from protists to humans. In shrimp and crayfish species, microsporidia infect multiple tissues and organs, including the heart, connective tissues, hepatopancreas, hemocyte-forming organs, and other tissues [9, 15, 17], causing pathologies ranging from inflammation to tissue destruction. For this reason, microsporidiosis has been called one of the most globally significant diseases of freshwater crayfish globally [1]. In amphipod crustaceans of the family Gammaridae, vertically transmitted microsporidia have commonly been reported to occur at high prevalences and have been shown to have a range of effects on host behavior, fitness, population size, stability, and sex ratio [7, 8, 10, 12, 16, 26].

While a wide genetic diversity of microsporidia has been reported to infect gammarids in France, Scotland [26], and Iceland [13], little is known about microsporidia infecting gammarids in the Great Lakes basin. In one study, Ryan and Kohler et al. [24] used PCR and DNA sequence analyses to reveal the presence of two microsporidia (*Dictyocoela* sp. and *Microsporidium* sp.) infecting *Gammarus pseudolimnaeus* populations from four cool-water streams in Southwestern Michigan, USA, providing evidence that a range of genetically diverse microsporidia are impacting amphipod populations in the Great Lakes. While multiple studies have employed light microscopy techniques to investigate microsporidia infections in *Diporeia*, due to the lack of phylogenetic and detailed ultrastructural studies, the taxonomic affiliation of microsporidia infecting *Diporeia* is currently unknown. Herein, we report the phylogenetic relationship of a microsporidian infecting Lake Superior *Diporeia* to other microsporidia reported to infect amphipods. We also shed light on morphological criteria of importance in classifying the novel microsporidian. The potential ecological impact of the observed microsporidian infection is discussed.

Materials and methods

Sample collection and morphological analysis

A total of 338 *Diporeia* were collected from four sites in Lake Superior for determining the presence of microsporidian infection (Fig. 1). Samples were collected by taking Ponar grabs (sampling area 0.251 × 0.251 m/8.2 L) at depths between 18 and 136 m. Benthic samples were sieved (mesh = 0.25 mm) and *Diporeia* were identified according to Bousfield [4] and placed in either 10% neutral buffered formalin for histopathological analysis or filter-sterile (0.2 μm) 80% ethanol for molecular analysis. An average of 80 amphipods was sampled from each site. The taxonomic system for microsporidia infecting *Diporeia* was based on the morphological criteria used for taxonomy detailed in Wittner and Weiss [29].

For histopathological analysis, amphipods preserved in formalin were dehydrated in a graded series of alcohols, embedded in paraffin, cut into 3–4-μm-thick serial sections, and stained with Mayer's hematoxylin and eosin [19]. Ultrastructural studies were performed on a representative, heavily infected *Diporeia* sample collected from site SU-01M in Lake Superior that was embedded in a paraffin block. The sample was deparaffinized, post-fixed, and processed for transmission electron microscopy (TEM). For TEM, ultra-thin sections

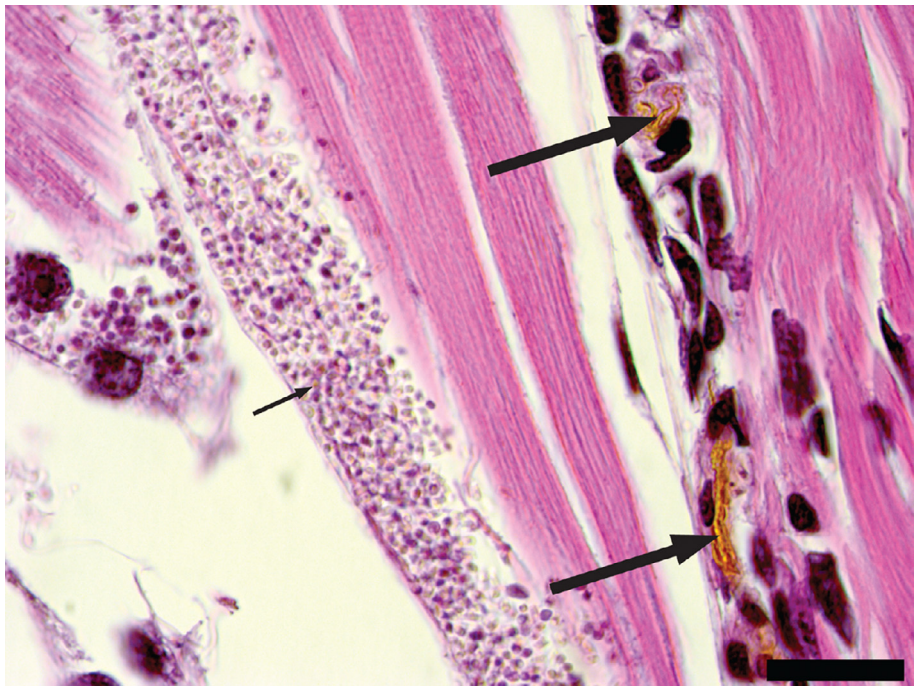

Figure 2. Histological sections (hematoxylin and eosin) of *Dictyocoela diporeiae* n. sp. developmental stages in an infected *Diporeia* sp. collected from Lake Superior. Notice the individual spores (small arrow) replacing skeletal muscle and melanized hemocytic infiltration in adjacent muscle tissue (large arrows). Scale bar = 25 μm.

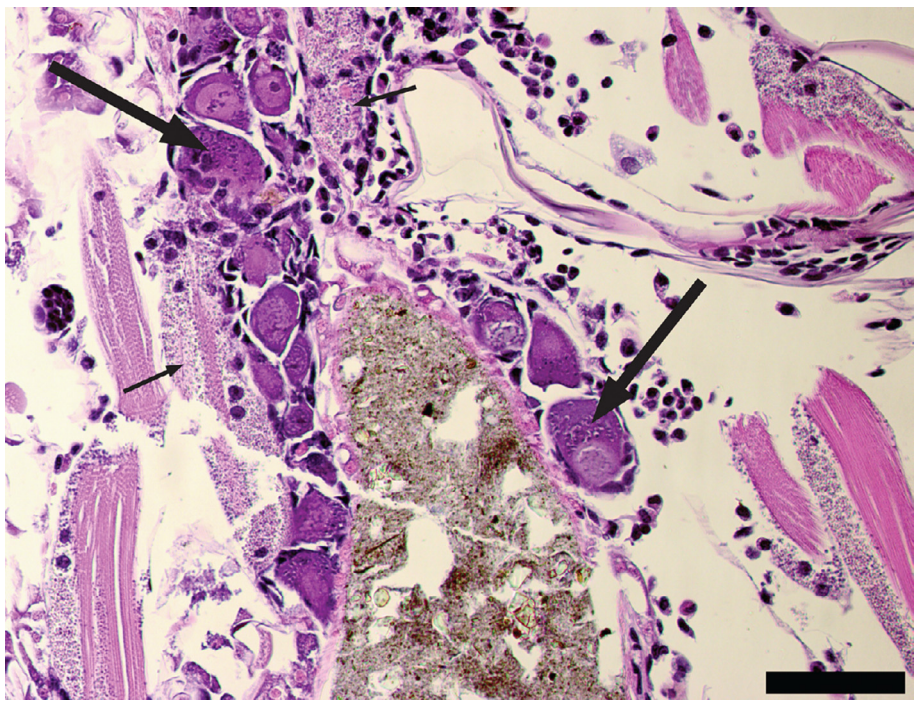

Figure 3. Histological sections (hematoxylin and eosin) of a *Diporeia* sp. sample collected from Lake Superior. Notice the microsporidians (*Dictyocoela diporeiae* n. sp.) filling and replacing muscle tissues (small arrows) surrounding the ovaries (large arrows). Scale bar = 100 μm.

(60–100 nm) were stained with 2% (w/v) uranyl acetate in 50% ethanol followed by Reynold's lead citrate and examined in a JEM-100 CX II electron microscope at an accelerating voltage of 100 kV.

Molecular analysis

Genomic DNA from an infected *Diporeia* collected from a site near SU-01M (SU-23B) was extracted using the DNeasy

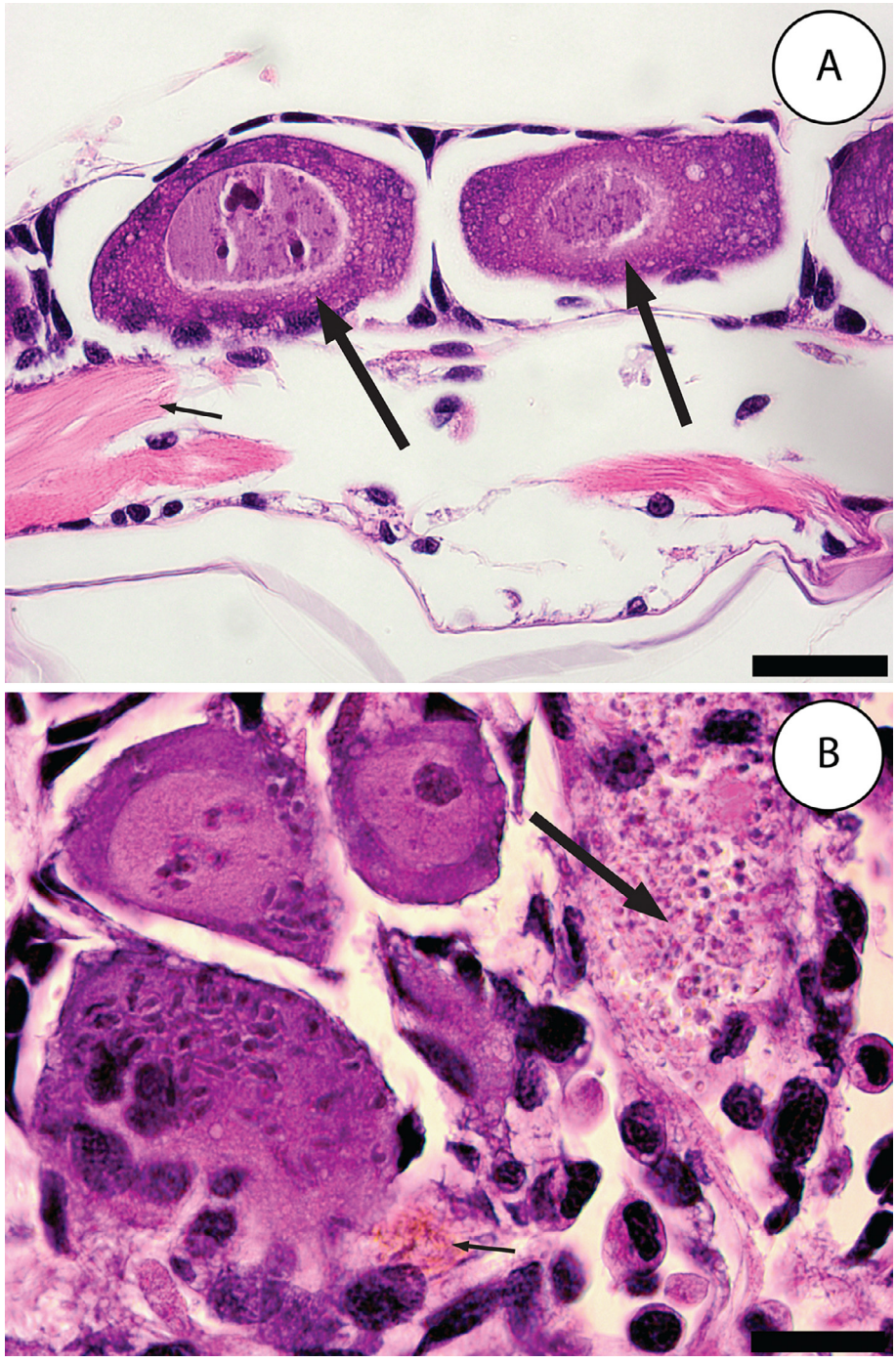

Figure 4. Histological sections (hematoxylin and eosin) of *Diporeia* (Amphipoda) collected from Lake Superior. Notice (A) the histologically normal ovaries (large arrows) of an amphipod not displaying a microsporidian infection in the muscle tissue (small arrow) and (B) melanized hemocytic encapsulation near the ovaries (small arrow) of an amphipod displaying a microsporidian infection (*Dictyocoela diporeiae* n. sp.) in the muscle tissue (large arrow). Scale bar = 25 μm.

DNA extraction kit (QIAGEN) according to the manufacturer's instructions. PCR amplification of microsporidian 16S rDNA was amplified using the microsporidian 16S primers V1f (forward) 5′-CACCAGGTTGATTCTGCCTGAC-30 [27] and 580r (reverse) 5′-GGTCCGTGTTTCAAGACGG-3′ [2]. A negative control containing no DNA was included in the PCR reaction. The resulting PCR product was visualized by agarose gel electrophoresis to confirm only a single fragment was amplified, cloned using a TOPO TA Cloning Kit® (Invitrogen, CA, USA) following the manufacturer's protocol, cultured on Luria-Bertani agar plates (Fisher Scientific Inc., PA, USA) containing 50 μg/mL Kanamycin as directed by the

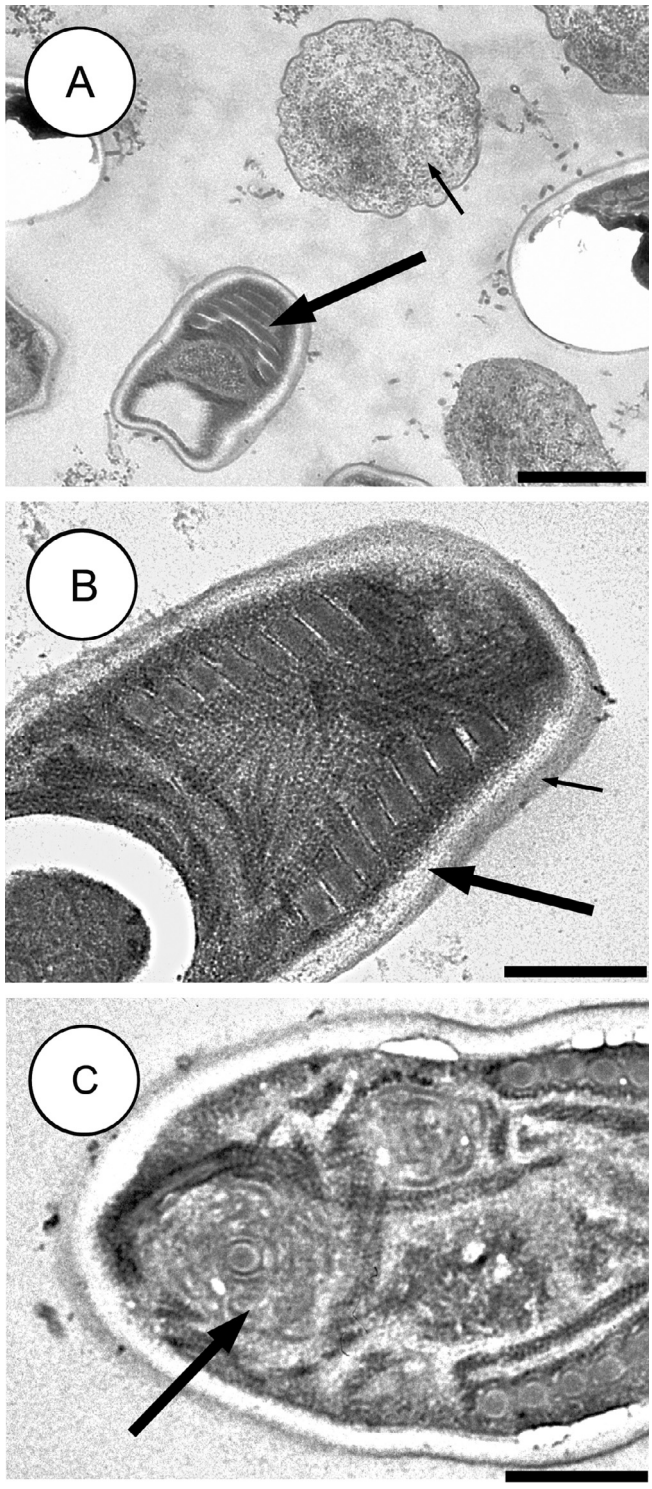

Figure 5. *Dictyocoela diporeiae* n. sp., transmission electron micrograph of the microsporidian infecting *Diporeia* sp. in Lake Superior. Notice (A) the meront (small arrow) and mature spore (large arrow), (B) spore wall composed of a thick electron-lucent endospore (large arrow) overlaid with a thinner electron-dense exospore (small arrow), and (C) lamellar polaroplast composed of ordered concentric membranes surrounding the polar filament (large arrow). Scale bars: A = 1000 nm, B–C = 500 nm.

manufacturer's protocol, and sequenced using the M13f (5′-GTT TTC CCA GTC ACG AC-3′), M13r (5′-CAG GAAACA GCT ATG ACC-3′), and amplification primers. The resulting sequence (1899 bp) was deposited in GenBank (KF537632).

The 16S rRNA gene sequence was submitted for a BLAST (National Center for Biotechnology Information) search and highly similar matches were included in the dataset for phylogenetic analysis. Selection of sequences included in phylogenetic analyses was based on the findings of Krebes et al. [16]. A total of 22 microsporidian 16S rDNA sequences (the sequence isolated from the *Diporeia* microsporidian, 13 *Dictyocoela* sequences, seven sequences from other microsporidians that parasitize other aquatic animals, and one outgroup sequence from *Enterocytozoon bieneusi*, a microsporidian from a human host) were aligned with ClustalW as implemented in MEGA 5.0 [25] using default settings. The length of final alignment was 1354 nucleotide positions. Estimation of pairwise genetic distances among sequences was also performed in MEGA 5.0 using *p*-distance as a measure of genetic distance.

Bayesian inference phylogenetic construction was performed with MrBayes v 3.1.2 [14] using the transitional model [23] with γ distributed rates (GTR + G) as selected by the program jModelTest [5]. Bayesian analysis included four Monte Carlo Markov chains (MCMC) for 2,000,000 generations with one tree retained every 1000th generation. After discarding the burn-in samples (first 25% of samples), the remaining data were used to generate a 50% majority-consensus tree.

Dictyocoela diporeiae n. sp.

urn:lsid:zoobank.org:act:72ECFCEA-C50E-46FE-9562-13E86B0643A5

Type host: *Diporeia* sp., Amphipoda, Gammaridea.

Type locality: United States: Lake Superior, 46.60° N & 84.81° W, depth = 60 m.

Type material: Reference materials are deposited at the National Museum of Natural History of the Smithsonian Institution, Accession number: 1231538.

Ribosomal DNA sequence: GenBank accession number KF537632.

Etymology: The specific epithet refers to the genus of the host, *Diporeia*.

Description

Spores replace muscle tissue throughout the body of the host. Mature spores measuring 1.99 ± 0.09 µm long by 1.19 ± 0.05 µm wide. Eight coils of isofilar polar filaments arranged in single ranks. Polar filaments measuring 71.27 ± 3.33 nm in diameter. A lamellar polaroplast composed of ordered concentric membranes found at the apical end of the spore surrounding the polar filament. A distinct posterior vacuole at the distal end of the spore.

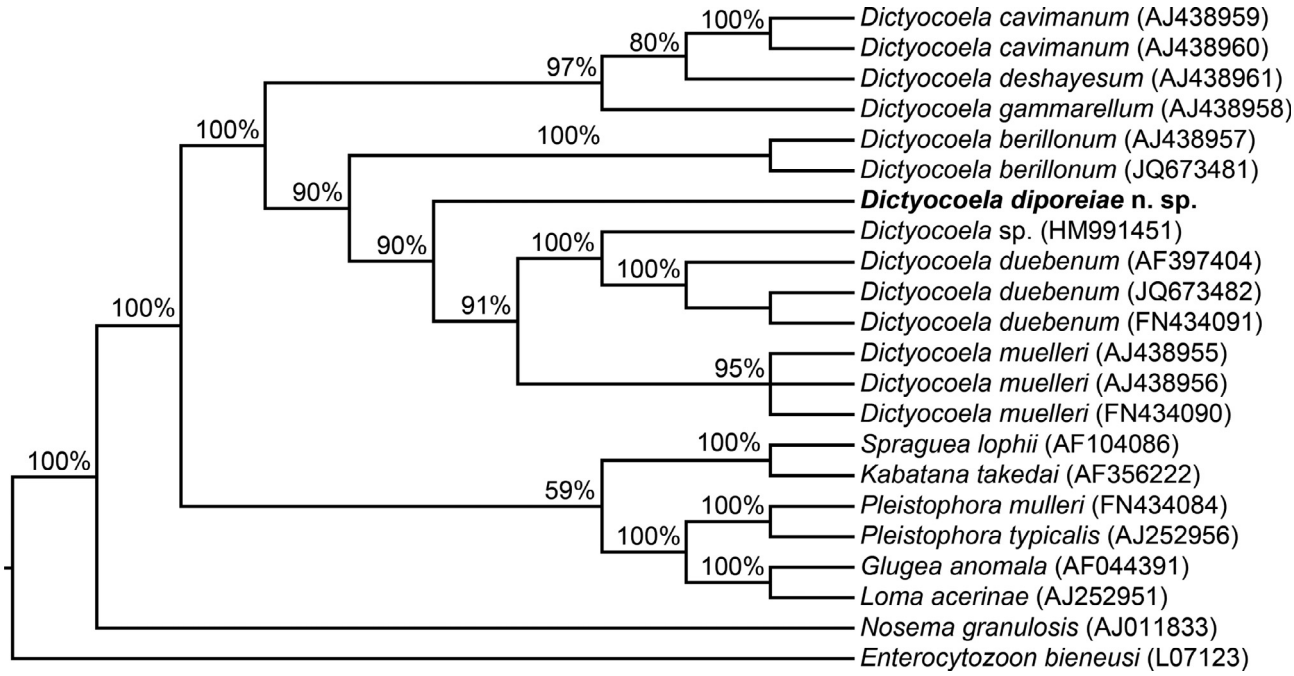

Figure 6. Phylogenetic tree (50% majority-rule consensus) based on Bayesian Inference (MrBayes 3.1.2) of *Dictyocoela* spp. based on the small subunit ribosomal gene. Numbers at the nodes are Bayesian posterior probabilities. *Spaguea lopii*, *Kabatana takedai*, *Nosema granulosis*, *Thelohania parastaci*, *Pleistophra mulleri*, *P. typicalius*, *Glugea anomala*, and *Loma acerinae* were used as an outgroup for *Dictyocoela* spp. based on the results of Krebes et al. (2010).

Table 1. Listing of host record for *Dictyocoela diporeiae* n. sp. and similar *Dictyocoela* strains.

Dictyocoela sp.	Amphipod host	GenBank accession number
D. diporeiae	*Diporeia* sp.	KF537632
D. berillonum	*Echinogammarus berilloni*	AJ438957
D. berillonum	*Echinogammarus marinus*	JQ673481
D. muelleri	*Gammarus duebeni celticus*	AJ438955
D. duebenum	*Gammarus duebeni duebeni*	FN434091
D. muelleri	*Gammarus roeseli*	AJ438956
D. muelleri	*Gammarus duebeni duebeni*	FN434090
D. duebenum	*Gammarus duebeni duebeni*	AF397404
Dictyocoela sp.	*Gammarus pseudolimnaeus*	HM991451
D. duebenum	*Echinogammarus marinus*	JQ673482
D. cavimanum	*Talitrus* sp.	AJ438959
D. deshayesum	*Talorchestia deshayesei*	AJ438961
D. cavimanum	*Orchestia cavimana*	AJ438960
D. gammarellum	*Orchestia gammarellus*	AJ438958

Prevalence, pathology, and morphological characterization

In stained histological sections, microsporidian infections were observed in *Diporeia* collected from three of the four sites sampled. Prevalences for SU-01, SU-20B, SU-22B, and SU-23B were 2.94 (2/68), 1.98 (2/101), 3.23 (3/93), and 0.00% (0/70), respectively, making the overall prevalence for Lake Superior 2.11% (7/332). These infections were always associated with muscle tissues where infected tissues appeared to be replaced with spores. Differentiated, basophilic or melanized encapsulating host hemocytes were often observed in or near masses of microsporidians (Fig. 2). In one amphipod, microsporidians were observed filling and replacing the muscle tissue surrounding the ovaries (Fig. 3) where a melanized hemocytic encapsulation was present near the ovaries (Fig. 4).

By TEM, meronts were roundish cells surrounded by a plasma membrane. Meronts measured 1.49 ± 0.11 µm in diameter. No developing sporoblasts were observed. Mature spores measured 1.99 ± 0.09 µm long by × 1.19 ± 0.05 µm wide (n = 14). Eight coils of isofilar polar filaments were arranged in single ranks. Polar filaments measured 71.27 ± 3.33 nm in diameter. The spore wall was composed of a thick electron-lucent endospore overlaid with a thinner electron-dense exospore. The average thickness of the spore

Table 2. Pairwise genetic distances between *Dictyocoela diporeiae* n. sp. and similar *Dictyocoela* strains based on nearly full-length 16S small subunit rDNA sequences.

Dictyocoela diporeiae n. sp.													
Dictyocoela sp. (HM991451)	0.948												
Dictyocoela muelleri (AJ438955)	0.950	0.971											
Dictyocoela berillonum (AJ438957)	0.950	0.957	0.953										
Dictyocoela muelleri (AJ438956)	0.949	0.972	0.990	0.958									
Dictyocoela duebenum (AF397404)	0.946	0.987	0.973	0.957	0.974								
Dictyocoela berillonum (JQ673481)	0.950	0.958	0.953	0.992	0.958	0.956							
Dictyocoela duebenum (JQ673482)	0.936	0.975	0.960	0.946	0.961	0.985	0.945						
Dictyocoela duebenum (FN434091)	0.948	0.987	0.973	0.959	0.974	0.999	0.957	0.987					
Dictyocoela muelleri (FN434090)	0.949	0.971	0.990	0.955	0.992	0.972	0.955	0.960	0.972				
Dictyocoela cavimanum (AJ438959)	0.922	0.924	0.927	0.937	0.930	0.922	0.938	0.911	0.924	0.929			
Dictyocoela cavimanum (AJ438960)	0.921	0.926	0.926	0.937	0.930	0.924	0.941	0.914	0.925	0.929	0.992		
Dictyocoela deshayesum (AJ438961)	0.922	0.930	0.923	0.937	0.926	0.925	0.941	0.913	0.926	0.926	0.961	0.962	
Dictyocoela gammarellum (AJ438958)	0.905	0.902	0.905	0.905	0.905	0.901	0.905	0.889	0.902	0.905	0.921	0.919	0.914

wall was 97.0 ± 8.3 nm. A lamellar polaroplast composed of ordered concentric membranes was found at the apical end of the spore surrounding the polar filament. A distinct posterior vacuole was observed at the distal end of the spore (Fig. 5).

Phylogenetic analysis

A BLAST search of the 16S rDNA sequence obtained from *Diporeia* showed that the closest matches (95% similarity) were for seven *Dictyocoela* spp. sequences (GenBank Accessions AJ438957, JQ673481, AJ438955, FN434091, AJ438956, FN434090, and AF397404) (Table 1). The resulting phylogeny showed that the sequence obtained from *Diporeia* was positioned deep within a large clade containing *Dictyocoela* spp. but formed a unique clade containing no sister taxa (Fig. 6). Posterior probabilities of branching points based on Bayesian inference indicated that the node support of the Lake Superior *Diporeia* microsporidian taxon was 90%. This result strongly suggested that the Lake Superior *Diporeia* microsporidian is a novel species within the genus *Dictyocoela*.

Phylogenetic analysis of nearly full-length small subunit rDNA sequences demonstrated that the *Diporeia* microsporidian fell deep within the large clade containing the genus *Dictyocoela*. However, electron microscopy revealed that the spores observed in *Diporeia* were not contained in sporophorus vesicles filled with tubules, a defining characteristic for the genus [26]. The genus *Dictyocoela* was proposed based on a group of eight novel sequences that clustered into a discrete clade basal to the major lineage of microsporidia infecting fishes. From these sequences, six species were designated, placing isolates within the same species where sequence dissimilarity was within 1% [26]. Additionally, the study of Wilkinson et al. [28], which investigated the diversity of *Dictyocoela* spp. across Europe and from Lake Baikal in Siberia, supported the designation of *D. berillonum* as a species separate from *D. duebenum* and *D. muelleri* and stated that host species distribution (Table 1) appears to influence structuring of *Dictyocoela* populations. In comparison with the *Diporeia* microsporidian, the results of the current study show that the most similar *Dictyocoela* strains had a 16S rDNA sequence dissimilarity of 5.1% or greater (Table 2), indicating that the observed microsporidian is novel. Based on its morphology, genetic sequence, host, and location in the host, we conclude that this *Dictyocoela* sp. is novel and we propose naming it *Dictyocoela diporeiae* n. sp.

Discussion

All *Dictyocoela* spp. are vertically transmitted parasites that infect both ovarian tissue and adjacent muscle of their amphipod hosts [26]. Observation of microsporidia infecting the muscle surrounding the ovaries of *Diporeia* further suggests its placement in the genus *Dictyocoela*. The impact of this microsporidian on reproduction in *Diporeia* remains to be determined. However, given the extent of infection and involvement of the muscles surrounding ovaries, it is possible that the observed microsporidian can have severe impacts on *Diporeia* populations.

Moreover, it is likely that the observed destruction of muscle tissue caused by microsporidian infection impairs the normal movement, feeding, swimming, and overall functioning and fitness of *Diporeia*. The fact that tissue alteration and host inflammatory immune response were associated with these infections further highlights the negative impacts these infections have on *Diporeia*. Given the fact that *Diporeia* serves as a conduit of nutrients and energy to higher trophic levels and a coupling mechanism between pelagic and benthic zones of the Great Lakes [11], the observed infections could have considerable impacts on the normal functioning of the Great Lakes ecosystem. *Diporeia* was once the most dominant benthic macroinvertebrate throughout the Laurentian Great Lakes. Recently, however, *Diporeia* abundances have effectively been extirpated from many of its habitats in the Great Lakes, as reviewed in Nalepa et al. [21]. Currently, the cause of these declines is unknown. Additional morphological, phylogenetic, and pathological analyses are needed to better understand both the genetic diversity of microsporidia infecting *Diporeia* and the potential impact these infections have on *Diporeia* populations in the Great Lakes. This is the first report of a microsporidian infecting *Diporeia* in Lake Superior.

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