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 \( \mu m \) long by 1.19 ± 0.05 \( \mu m \) wide. Each spore contained eight coils of iso-\( f \)ilar 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-ösine, 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 \( \mu m \) de long et 1,19 ± 0,05 \( \mu m \) de large. Chaque spore contenait huit spirales 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 appartiennent 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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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 (Dictyocoea 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 microsporian. 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 1. Sampling sites in Lake Superior where Diporeia sp. (Amphipoda, Gammaridae) were collected.
(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
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'-CACCAGGTGATTCTGCCTGAC-3' [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 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.
manufacturer’s protocol, and sequenced using the M13f (5’-GTT TTC CCA GTG 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:72ECFC50-E46FE-9562-13E86B043A5

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 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.
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 $1.49 \pm 0.11 \mu m$ in diameter. No developing sporoblasts were observed. Mature spores measured $1.99 \pm 0.09 \mu m$ long by $1.19 \pm 0.05 \mu m$ wide ($n = 14$). Eight coils of isofilar polar filaments were arranged in single ranks. Polar filaments measured $71.27 \pm 3.33 \text{nm}$ 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 wall was $0.99 \pm 0.07 \text{nm}$.

**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 berillonii* | AJ438957               |
| *D. berillonum* | *Echinogammarus marinus* | JQ673481                 |
| *D. mueleri*    | *Gammarus duebeni celticus* | AJ438955              |
| *D. duebenum*   | *Gammarus duebeni duebeni* | FN434091              |
| *D. mueleri*    | *Gammarus roeseli*     | AJ438956                 |
| *D. mueleri*    | *Gammarus duebeni duebeni* | FN434090              |
| *D. duebenum*   | *Gammarus duebeni duebeni* | AF397404              |
| *Dictyocoela sp.* | *Gammarus pseudolimnaeus* | HM991451                |
| *D. duebenum*   | *Echinogammarus marinus* | JQ673482                 |
| *D. cavinana*   | *Talitrus sp.*         | AJ438959                 |
| *D. deshayesum* | *Talorchestia deshayesi* | AJ438961                |
| *D. cavinana*   | *Orchestia cavinana*   | AJ438960                 |
| *D. gammarellum* | *Orchestia gammarellus* | AJ438958                 |
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 sporophorous 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. duabenum 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.

**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 duabenum (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 duabenum (FN434091) | 0.936 | 0.975 | 0.960 | 0.946 | 0.961 | 0.985 | 0.945 |
| Dictyocoela muelleri (FN434090) | 0.949 | 0.971 | 0.990 | 0.955 | 0.992 | 0.972 |
| Dictyocoela cavimatum (AJ438959) | 0.922 | 0.924 | 0.927 | 0.937 | 0.930 | 0.922 | 0.938 | 0.911 | 0.924 | 0.929 |
| Dictyocoela cavimatum (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 |
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