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Establishment of a new microsporidian genus and species, *Pseudoberwaldia daphniae* (Microsporidia, Opisthosporidia), a common parasite of the *Daphnia longispina* complex in Europe

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ABSTRACT

Microsporidia are among the most common microparasites of cladocerans and have potentially significant impact on host populations. However, many of these pathogens are known only from molecular-based studies. We provide ultrastructural data supported by molecular phylogeny for a common microsporidium infecting the *Daphnia longispina* complex, important planktonic filter-feeders in reservoirs and ponds in the temperate Holarctic region. This parasite, previously characterized only by molecular means, infects adipose cells around the *Daphnia* midgut and eventually fills the centre of the host body with ovoid-shaped spores. A new microsporidian genus and species belonging to the Agglomeratidae superclade is described as *Pseudoberwaldia daphniae* gen. et sp. nov. Molecular data indicate its widespread presence in Central European reservoirs (reported as isolate "MIC1") but also in Swedish coastal rockpools ("Ängskärs-klubben"). The most closely related lineage was reported from a caddisfly larva; we thus speculate that this taxon may have an insect secondary host in its life cycle. Morphological characterization and differential diagnosis of most commonly encountered microsporidian taxa infecting hosts in the *D. longispina* complex in Europe opens new possibilities for studies of their ecological and evolutionary interactions.

1. Introduction

Microsporidian species are among the most common parasites of animals (Vávra and Lukeš, 2013; Wittner and Weiss, 1999; Sprague, 1977). Microsporidian hosts of special interest are aquatic invertebrates, which are constantly exposed to spores of microsporidia that originate from different host species and are distributed by water currents. These microsporidia have the potential to infect and colonize new species of hosts.

Water fleas of the genus *Daphnia* (Crustacea, Branchiopoda) are ecologically important planktonic crustaceans of temperate inland waters (Lampert, 2006, 2011). These effective filter-feeders are frequently infected by microsporidia (Ebert, 2005). While a few of these microparasites became important models for evolutionary research (e.g., Ebert, 2008; Altermatt and Ebert, 2008), the identity of many other *Daphnia*-infecting microsporidia is known only by ssu rDNA sequence data (e.g., Refardt et al., 2002, 2008; Weigl et al., 2012).

Several microsporidian species infecting the *Daphnia longispina* species complex in European lakes and reservoirs were recently identified by molecular methods (Refardt et al., 2008; Wolinska et al., 2009; Weigl et al., 2012). The *Daphnia* complex comprises several evolutionary lineages (Petrusek et al., 2008, 2012) that often are involved in interspecific hybridization (e.g., Taylor and Hebert 1992; Schwenk and Spaak 1995; Seda et al., 2007). *Daphnia* are particularly important as they often are prevalent in the zooplankton of large water bodies across the Holarctic region (Benzie, 2005). Altogether eight microsporidian lineages causing infections of body cavity of *Daphnia* were detected by Wolinska et al. (2009) and Weigl et al. (2012) in Central European waters. However, two closely related microsporidian taxa were the most frequently collected and are likely the ecologically most important of this group of *Daphnia* parasites in the studied habitats. One of these microsporidia was erroneously reported as "*Berwaldia schaeffernai*" due to a sequence match to the GenBank Acc. No. AY090042 labelled by that name. In our previous contributions, we documented that the genus *Berwaldia* actually comprises other cladoceran microsporidian parasites (Vávra et al., 2017), and we formally described this commonly

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encountered clade, misidentified under the name *B. schaeffernai,* as *Conglomerata obtusa* (Vávra et al., 2018).

The present paper provides morpholgical data and a formal description of another widespread microsporidian infecting the *D. longispina* complex that was previously reported from Central Europe under the label MIC1 (Wolinska et al., 2009; Weigl et al., 2012; González-Tortuero et al., 2016). Based on the unique characteristics of this taxon, we also erect a new microsporidian genus. Detailed ultrastructural characterization as well as characteristics allowing its discrimination from other *Daphnia*-infecting species using light microscopy opens the possibility of future investigations of this pathogen's biology and ecology, which has been so far studied in detail only by molecular approaches (Weigl et al., 2012; González-Tortuero et al., 2016).

2. Methods

2.1. Hosts and sampling

Specific hosts of the MIC1 microsporidium are cladocerans of the *Daphnia longispina* complex (for nomeclature of these *Daphnia*, see Petrusek et al., 2008). When the infection is well developed, the hosts look conspicuously white when observed in incident light. The infected daphnids were sampled by diagonal hauls of plankton nets during summer months (August 2014 and 2017) in two localities in the Czech Republic: drinking water reservoir Žlutice (50.087N, 13.127E) and the carp pond Hořejší near Nový Jáchymov (49.978N, 13.913E).

2.2. Microscopy

Microscopic examination included common methods of light (LM) and transmission electron microscopy (TEM) as described in Vávra et al. (2017). In brief, spores were recorded and measured on agar monolayers (Vávra, 1964), permanent slide preparations were made with smears stained by routine Giemsa, or negative staining by bacteriological Burri Ink (Vávra and Maddox, 1976). TEM investigation included fixation in cacodylate-buffered glutaraldehyde, osmium post-fixation, plastic embedding and ultrathin section examination (Becnel, 2012).

2.3. Molecular phylogeny

2.3.1. DNA isolation, primers, PCR

Microsporidian DNA was obtained from infected daphnids that were individually evaluated for characteristic infection patterns by light microscopy. After proteinase K digestion (Schwenk et al., 1998), extract of total DNA was used for PCR with microsporidia-specific primers. Primer pairs 16SmicGen 361F/16SmicGen 869R (Weigl et al., 2012), 530F/580R and 18F/1492R (Weiss and Vossbrinck, 1999) were used to amplify the SSU rDNA. PCR protocols followed Weigl et al. (2012). PCR products were separated, purified and sequenced using methods described in Vávra et al. (2016b).

2.3.2. Alignments and phylogenetic analysis

The alignment was constructed from three newly obtained microsporidian SSU rDNA sequences, 17 sequences of MIC1 available in GenBank, 26 representatives of microsporidia from the superclade Agglomeratidae, and 23 other microsporidian isolates and species including three sequences of the genus *Amblyospora* set as outgroup. The dataset was aligned using MAFFT v7.308 (Katoh et al., 2002) with the E-INS-i multiple alignment method, gap opening penalty 1.0 and gap extension penalty 0.0. The length of the alignment was 1656 nt positions.

Phylogenetic trees were constructed using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) method. ML analysis was done in RAxML v8.2.11 (Stamatakis, 2006) under a GTR + Γ model. MP was done in PAUP* v4.0b10 (Swofford, 2002) with a heuristic search, Ts:Tv = 1:2 and a random addition of taxa. Bootstrap supports were calculated from 500 replicates in ML and 1000 replicates in MP analysis. BI was done using MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001) with the GTR + Γ model of evolution (6 rates of substitution; gamma rate variation across sites; 4 categories were used to approximate gamma distribution). MrBayes was run to estimate posterior probabilities over 1 million generations via 2 independent runs of 4 simultaneous Markov Chain Monte Carlo (MCMC) algorithms with every 100th tree saved. Tracer v1.4.1 (Rambaut and Drummond, 2007) was used to ascertain a sufficient length of the burn-in period.

Genetic distances (converted to similarities in %) among and within selected sequences of Agglomeratidae subclades and among related microsporidia (Supplementary material, Table 2) were computed in PAUP* v4.0b10 with default P parameter.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jip.2019.02.004.

3. Results

3.1. MIC1 microsporidium characterization

3.1.1. Tissue infected

Adipose cells situated around the midgut of *Daphnia* individuals were infected. Hypertrofied infected tissue characteristically formed a dense clump in the centre of the host body (Fig. 1A). This usually allowed distinction of the MIC1-infected *Daphnia* hosts from those infected by other microsporidia (see Section 4.6).

3.2. Spore LM

When squashed, infected *Daphnia* released a mass of free spores (Fig. 1B); no defined spore packets occurred, but occasionally irregular rosettes of adhering spores were observed (Fig. 1B inset). Individual spores were wedge-pyriform in shape (with straight-sided walls tapering to the spore apex, and lacking any distinct concavity). The widest part of the spore was very close to the posterior end (with the posterior vacuole). Spores measured 4.4 (4.1–4.6) × 2.6 (2.4–2.8) μm in size (n = 10). The typical spore shape was preserved in dry smears that were negativelly stained by bacteriology Burri-Ink (Fig. 1C), as well as within the infected tissue prepared for transmission electron microscopy (Fig. 1D). The spore shape allowed distinction of MIC1 spores from spores of other microsporidia infecting adipose tissues of the same host taxa in the sampled habitats (see discussion).

3.3. Development

The earliest developmental stages encountered were binucleate plasmodia (single nuclei), with homogenous cytoplasm filled by ribosomes and encircled by a simple plasma membrane (Fig. 1E). Patches of dark cell wall material developed inside these plasmodia under the cell surface (Fig. 1F and G). These progressively merged to form a circle situated under the plasma membrane of the plasmodium. Part of the plasma membrane of the plasmodium thus remained exterior to the cell wall material and its outline delimited a large sporophorous vesicle (SPOV) in which sporogony took place (Fig. 1G). Nuclear divisions and finger-like division of the plasmodium (Fig. 1H), usually appearing distorted, led to the formation of uninucleate segments, each segment surrounded by a dense wall of electron-dense material, complex in structure (Fig. 1I), representing the future complex spore envelope. These uninucleate segments developed into uninucleate sporoblasts (Fig. 1J) and later spores.

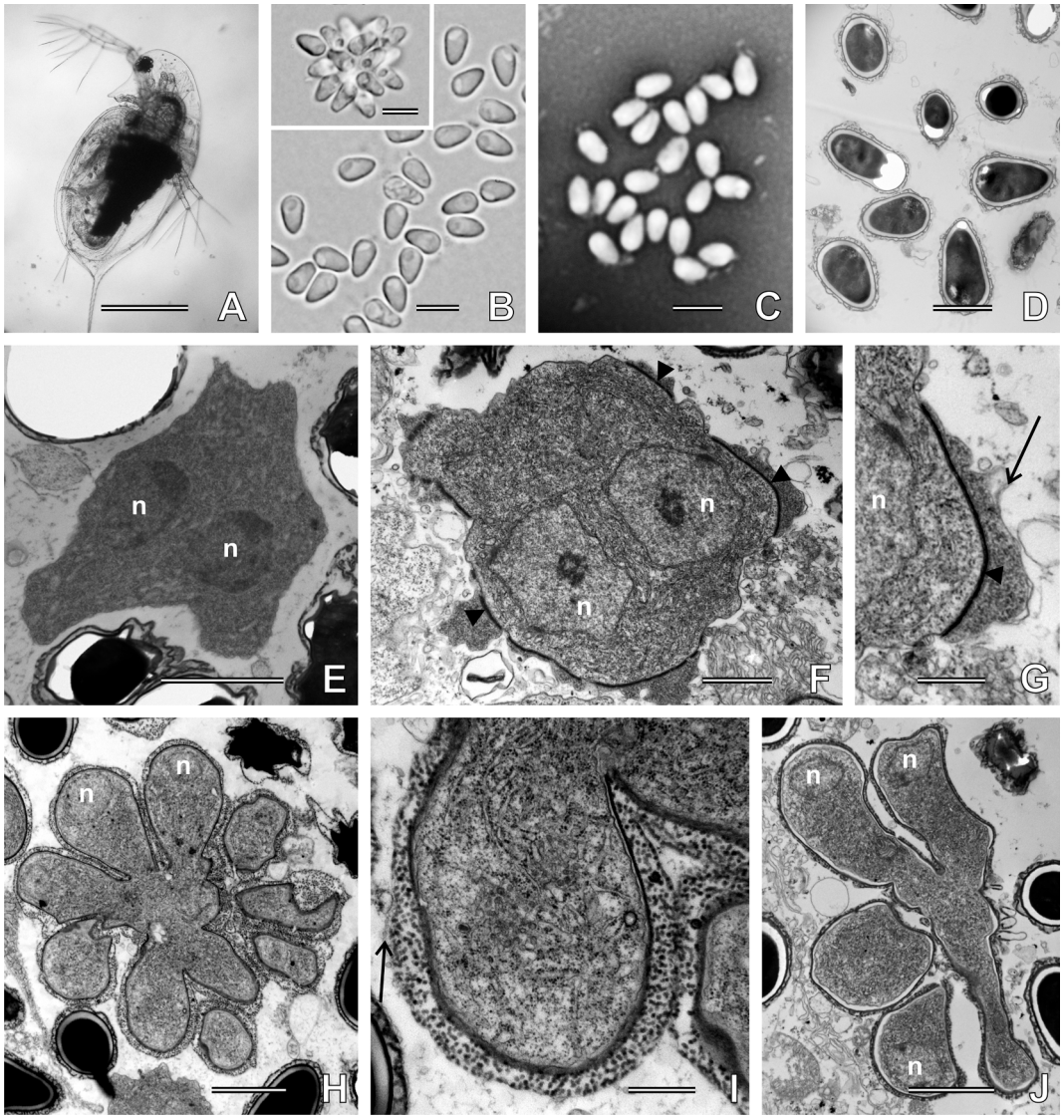

Fig. 1. *Pseudoberwaldia daphniae*, gen. et sp. nov. as seen in light microscopy (A–C) and transmission electron microscopy (D–J). A. *Dapnia longispina* infected by MIC 1 microsporidium. Note the accumulation of infected tissue in the middle of body length. Bar 500 µm. B. Fresh spores on an agar monolayer. Bar 5 µm. B inset. Group of spores formed from a single sporont. Bar 5 µm. C. Spores on a dry smear negatively stained by bacteriology Burri-Ink. Bar 5 µm. D. Total view of spores as seen on a TEM section. Bar 2 µm. Note that all these techniques well preserve the typical spore shape. E. Late, binucleate (n: single nuclei) meront. Bar 2 µm. F. Early binucleate (n) sporont with patches of dark cell wall material (arrowheads). Bar 1 µm. G. Detail of F. The sporophorous vesicle (SPOV, arrow) is located externally to the future spore exospore (arrowhead), (n; nucleus). Bar 500 nm. H. Multiple, finger-like division of sporont into individual sporoblasts, each with a single nucleus (n). Bar 2 µm. I. Detail of the cell organization of the sporoblast formation. The nascent SPOV is at arrow. Bar 500 nm. J. More typical and more frequent mode of sporogony (n: nuclei). Bar 2 µm.

3.4. Spore TEM

The organization of the MIC1 spore corresponded to the general pattern seen in spores of other aquatic microsporidia of the Agglomeratidae superclade (Vávra et al., 2017; Vávra et al., 2018). However, it was the construction of the spore case and its surrounding sporophophorous vesicle which made the MIC1 spores structurally unique.

3.4.1. Sporophorous vesicle

Spores occurred in individual monosporic SPOVs (Fig. 2A), and the spore exospore and the SPOV formed a structurally integrated whole (Fig. 2B). The spore was surrounded (from the interior outwards) by a plasma membrane, 150–155 nm thick electronlucent chitinous endospore (thinning to minimum at the apex of the spore), and an approximately 50 nm thick electron-dense exospore consisting of a double layer of very dense material (difficult to resolve), extending into a complex maze of anastomosing outgrowths 30–35 nm thick (Fig. 2B). These outgrowths were slightly less opaque than the exospore. The SPOV was externally delimited by a fine membrane 80–190 nm (typically 150 nm) following the outline of the spore (Fig. 2A). In addition to the maze of exospore outgrowth, the SPOV was filled with fine granular material (Fig. 2B). Rarely, two spores were included in the same SPOV.

3.4.2. Polar tube

The anchoring disc was bulbous in shape (360 nm); a thin layer of electron-dense matter, but no endospore material, was observed at the apex. Some of the polar filament coils (typically 7, more rarely 8–11) appeared structurally different when seen in cross-section: several anterior coils were shown as circular cross-sections of the tube (usually 4 coils, approximately 120 nm in diameter each, with a dark center), followed by 1 or 2 coils of the same diameter, but without the dark

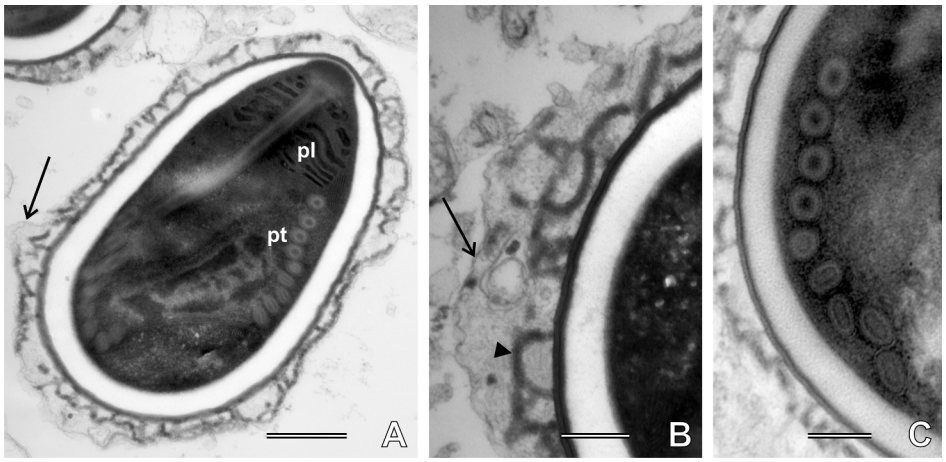

Fig. 2. *Pseudoberwaldia daphniae*, gen. et sp. nov. – ultrastructure of spore and of sporophorous vesicle. A. Total view of spore organization inside of its monosporic SPOV (arrow), (pl: polaroplast, pt: polar tube). Bar 500 nm. B. Detail of the SPOV-exospore interface (SPOV is at arrow, exospore outgrowths – thick threads – are at arrowhead). Bar 200 nm. C. Polar filament (tube) organization. Bar 200 nm.

center. The remainder (usually 3 coils) were thinner coils (80 nm in diameter) with internal structure sectioned at an angle (Fig. 2A and C). The filament appeared, therefore, to be anisofilar, but the appearance might have been due to the geometry of coils, with the last few coils lying at a steep angle to the section plane. Some photographs suggested that the last (most posterior) coil extended to or communicated with a thin, very long (700 nm), whip-like fibre of several layers, delimiting the anterior border of the posterior vacuole. The vacuole material was coarsely granular, distinct from the cytoplasm of the spore (Fig. 2A).

3.4.3. Polaroplast

The polaroplast was finely lamellar. In most spores we observed, the top lamellae appeared as flat vesicular lacunae with dense, structureless material between less dense membranes (Fig. 2A). The lacunae extended posteriorly into an area of very fine, tightly compressed membranes.

3.5. Phylogeny

Newly obtained sequences (deposited in GenBank under accession numbers MK053814–MK053816) clearly clustered within the large group of Agglomeratidae, along with other MIC1 microsporidian sequences from Czech localities (Wolinska et al., 2009; Weigl et al., 2012; González-Tortuero et al., 2016) and, apparently, the same microsporidium from Sweden ("Ängskärs-klubben"; Refardt et al., 2008) (Fig. 3). Bootstrap analysis supported this group by high nodal values in ML (91%) and BI (0.93) and rather low support in MP (61%). Very short branch lengths for the MIC1 isolates suggested high sequence similarities. MIC1 microsporidia were most closely related to a microsporidium from a caddisfly *Hydropsyche* sp. (Acc. No. KX137919) and they all a sister group to Agglomeratidae Clade III microsporidia (three sequences; two assigned to the genus *Gurleya* and one unidentified). *Conglomerata obtusa* forming Clade I of Agglomeratidae according to Vávra et al. (2018) is another taxon closely related to MIC1. The specific barcode motif in the ITS region reported by Vávra et al. (2018) was identified and the sequence for MIC1 is TTTTTATAATGTGGGTAA.

4. Taxonomy

4.1. Superphylum Opistoshosporidia, Karpov et al., 2014

Superphylum Opistoshosporidia, Karpov et al., 2014.

4.2. Microsporidia Balbiani, 1882

Microsporidia Balbiani, 1882.

4.3. Microsporidian superclade Agglomeratidae, Clade II (Vávra et al., 2018)

Microsporidian superclade Agglomeratidae, Clade II (Vávra et al., 2018).

4.4. Pseudoberwaldia daphniae

Pseudoberwaldia daphniae, gen. et sp. nov. (the MIC1 parasite of Wolinska et al., 2009; Weigel et al., 2012; González-Tortuero et al., 2016; *Microsporidium* sp. "Ängskärs-klubben" from Refardt et al., 2008). Parasite of the *D. longispina* complex, infecting adipose cells around the midgut. Infected tissue fills characteristically the central body part of the host as a single opaque mass, dark in transmitted light and white in reflected light. Spores seen under light microscopy are pyriform, wedge-like (no concave side walls), the widest part of the spore lies close to spore posterior where the posterior vacuole is situated. Posterior vacuole is shifted to one side from the longitudinal axis. Spores are uninucleate, size 4.4 (4.1–4.6) × 2.6 (2.4–2.8) μm. Early developmental stages are binucleate plasmodia (single nuclei) in which lines of cell wall material appear and merge. Nuclear division followed by separation of uninucleate fragments with characteristic cell wall (electron-dense granular material on the wall material) leads to formation of spores. Each spore is situated in a sporophorous vesicle. Spore wall consists of a transparent, thick endospore and a single layer electron-dense exospore, protruding into numerous outgrowths of electrondense threads and forming an irregular maze in the SPOV volume. Polar tube with 7–11 coils, probably anisofilar, but anisofilarity appearance may be due to geometry of coils. Polaroplast lamellar, with a few anterior lamellar lacunae, followed by posterior part of tightly compressed lamellae.

4.5. Etymology

Etymology: The genus name alludes to the fine structure of *Berwaldia* spp. microsporidia (Vávra et al., 2017) producing spores within individual sporophorous vesicles.

4.6. Differential diagnosis

Four well-defined microsporidian species are presently known to infect adipose tissues of common taxa of the *D. longispina* complex: *Berwaldia schaefernai* and *Berwaldia nana* (Vávra et al., 2017), *Conglomerata obtusa* (Vávra et al., 2018), and the species described herein, *Pseudoberwaldia daphniae* n. gen., n. sp. Under light microscopy, the respective species initially may be difficult to distinguish based on the appearance of fresh spores. However, observation of a monolayer of

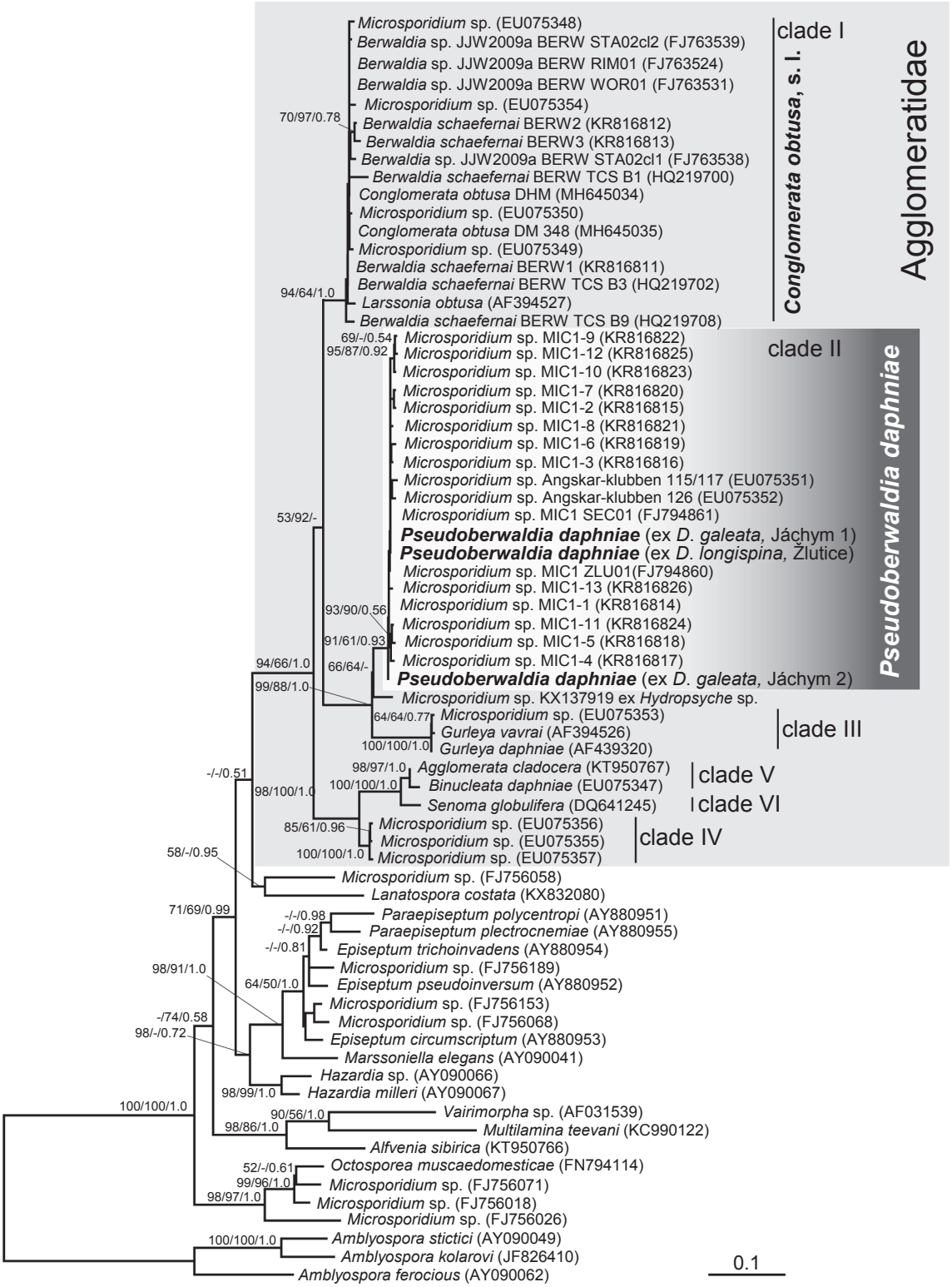

Fig. 3. Phylogenetic relationships of *Pseudoberwaldia daphniae*, gen. et sp. nov., sequences within the family clade Agglomeratidae based on the maximum likelihood analysis of SSU rDNA sequences. Maximum likelihood/maximum parsimony bootstrap support values and Bayesian posterior probabilities are shown at nodes gaining more than 50%/0.5 support. Newly obtained sequences are in bold. NCBI accession numbers are in parentheses. Scale bar is given under the tree.

spores under high-magnification (1000×, oil immersion) offers a relatively easy and reliable distinction. *B. schaefernai* spores are ellipsoid (Fig. 4A), *B. nana* spores are ovoid (Fig. 4B), *C. obtusa* spores in *D. longispina* have side walls slightly concave (Fig. 4C), while *P. daphniae* spores are widest very close to the posterior pole of the spore (Fig. 4D). Further differences can be seen in the location and character of infected tissue in these microsporidia. *Berwaldia* infections usually infect nearly all adipose cells in the *Daphnia* body, including thoracic appendages (Vávra et al., 2017), *C. obtusa* infection is located primarily in the adipose cells in the head of the infected daphnids (Vávra et al., 2018), while *P. daphniae* - infected tissue forms a dense accumulation in the centre of the host's body (Fig. 1A).

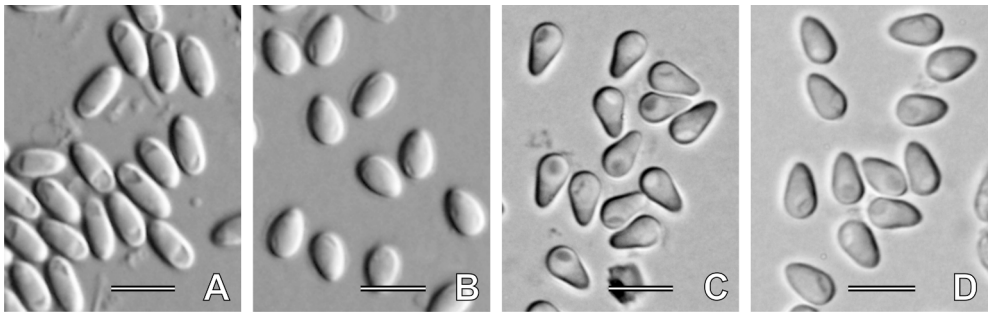

Fig. 4. Gallery of spore types of frequently found microsporidia infecting the adipose tissue of the *Daphnia longispina* complex. A. *Berwaldia schaefernai*, frequent parasite of *D. longispina* in Czech fishponds, but rarely found in reservoirs (from Vávra et al., 2017). Bar 5 μm. B. *Berwaldia nana*, parasite of *D. longispina,* of sporadical occurrence in carp fishponds (from Vávra et al., 2017). Bar 5 μm. C. *Conglomerata obtusa*, Vávra et al., 2018, the most frequent parasite of *D. longispina* in both reservoirs and fishponds. Bar 5 μm. D. *Pseudoberwaldia daphniae*, gen. et sp. nov., this paper, frequent in some fishponds and some reservoirs but rarer than *C. obtusa*. Bar 5 μm.

4.7. Type materials

Burri-Ink stained type slide labelled *Pseudoberwaldia daphniae*, No. 522–50 in J. Weiser's collection of microsporidia slides, in care of the Laboratory of Electron Microscopy, Faculty of Science, Charles University, Prague, Czech Republic. Type ssu rDNA sequence: GenBank Acc. No. MK053815 ("*Pseudoberwaldia daphniae* ex *D. galeata*, Jachym2").

4.8. Ecology and distribution

Little information is available on the ecology and occurrence of *Pseudoberwaldia daphnia* in natural habitats, however, it has been detected in relatively large and deep drinking water reservoirs in Central Europe (Wolinska et al., 2009; Weigl et al., 2012) as well as in small rockpools at the coast of eastern Sweden inhabited by *D. longispina* (Refardt et al., 2008).

5. Discussion

Considering the phylogenetic and ultrastructural differences of the MIC1 microsporidium from others in Agglomeratidae Clade II, we determined it reasonable to describe the isolate as a new species and also to erect a new genus. Available evidence (this paper Fig. 3; González-Tortuero et al., 2016) shows that *P. daphniae* and *C. obtusa* (="*Berwaldia* clade" of Wolinska et al., 2009; Weigl et al., 2012) clearly cluster in the Agglomeratidae superclade and bear the basic structural characters of Agglomeratidae as defined by Larsson and Yan (1988), namely isolated nuclei in development, pyriform spores, complex exospore, multiple-compartment polaroplast, SPOV with many spores. Comparing TEM photographs of *C. obtusa* (Vávra et al., 2018) and those of *P. daphniae* presented in this paper, the microsporidia appear to be structurally very different, particularly in the course of sporogony. *C. obtusa* sporogony takes place inside the SPOV, while *P. daphniae* sporogony is "external" ("finger-like" division of the SPOV) and separates individual spores into 1-cell SPOVs.

P. daphniae is also unique because the SPOV integrates the material from the exospore to form a kind of labyrinth of thick threads on the spore surface (Fig. 2B). No other microsporidian genus with this kind of spore surface has been described, despite the fact that spores of many aquatic microsporidia include various "ornamentations", apparently serving as floating devices or antennae helping ingestion by the host (Vávra et al., 2016a).

There are, however, two microsporidian species with spore structures that, to some extent, resemble those on the spores of *P. daphniae*. In both, the spores are ensheathed by individual SPOVs into which rib-like protrusions extend from the exospore. *B. nana* spores possess wrinkled SPOVs with topical densitifications, some of which connect the spore surface with the SPOV. This species also is a parasite of the *Daphnia longispina* complex and occurs in some habitats similar to those of *P. daphniae*. Although its ssu rDNA is unknown, based on its typical *Berwaldia* ultrastructure (Vávra et al., 2017), *B. nana* should be phylogenetically distant to *P. daphniae*.

A second species, the copepode microsporidium *Lanatospora costata,* has spore surface organization similar to that of *P. daphniae* (Vávra et al., 2016a). *L. costata* spores are ensheated by individual SPOVs, filled partly by electron-dense ribs from the exospore extending into the volume of SPOV. While similar to *P. daphniae,* the character of *Lanatospora* ribs (dense, rigid structures, perpendicular to exospore) is different from those of *P. daphnia,* which has a labyrinth of soft, thread-like winding structures. *L. costata* is also phylogenetically distant from the *P. daphniae* (Fig. 3). We can only speculate that *L. costata*, being phylogenetically related to other microsporidia found in daphnids, shares some characters of their life cycle.

Differences in morphology, developmental characteristics, ultrastructure, pathogenesis and hosts of the four above-mentioned microsporidia and of other related and similar microsporidia from daphnids and copepods, both within and outside of the Agglomeratidae superclade, are summarized in Table 1. Based on available data, it is apparent that most subclades of the Agglomeratidae superclade (Fig. 3) cannot be currently defined at the morphological level. This is likely because these data represent only the known parts of developmental cycles of the respective microsporidian species/genera. Until the complete life cycles have been discovered, there will be cases where partial developmental sequences of unrelated microsporidia (*Pseudoberwaldia–Berwaldia*, *Pseudoberwaldia–Lanatospora*) are more similar to each other in structural features than the known developmental sequences of more closely related microsporidia (*Pseudoberwaldia–Gurleya*, *Binucleata–Agglomerata*). Additionally, some ultrastructural features are very inconspicuous, so they may be overlooked or misinterpreted by some authors. For example, Voronin (1989) classified *Lanatospora* and Simakova et al. (2018a) classified *Berwaldia* as "apansporoblastic microsporidia" (i.e., lacking SPOV), the latter apparently due to its specific phylogenetic position (Vávra et al., 2017), distant from the "Aquatic outgroup" lineage (sensu Vossbrinck et al., 2004).

At present, the ultrastructural characteristics do not seem to have any clear links to the ecology or known host taxa of the respective microsporidia, although phylogenetic analyses suggest relationships between microsporidia from cladocerans and copepods (and dipterans) (Sokolova et al., 2016).

Thus far, none of the *Daphnia* microsporidia infecting adipose tissue were orally infective to the host from which they were isolated, and it has been speculated they require another, as yet unknown host (Wolinska et al., 2011; González-Tortuero et al., 2016; Vávra et al., 2018). There are no transmission data on *P. daphniae*, but considering its sudden seasonal appearance in the habitats from which it was collected (J. Vávra, unpublished data), an indirect life cycle also might be expected, perhaps with the second host being different from those of *B. schaefernai* and *C. obtusa*. González-Tortuero et al. (2016) also proposed that this might be the case for "MIC1 microsporidium", but based on

Table 1
Comparison of *Pseudoberwaldia daphniae* n. gen., n. sp. with related and structurally similar microsporidia from cladocerans and copepods.

Species, reference/ Main distinctive characters	Host	Site of infection	Spore size, shape	Merontogenetic sac #	Sporogonial plasmodium division	SPOV - no. of spores	SPOV - structure, inclusion	Episporal structures - part of the spore	Polar filament - type, no. of coils	Other specific characteristics
Pseudoberwaldia daphniae n. gen., n. sp. (this paper)	*Daphnia longispina* complex	Adipose cells around the midgut, central body part	4.4 (4.1–4.6) × 2.6 (2.4–2.8) µm (uf), pyriform, wedge-like	No	Finger-like, external (subsequent division of SPOV)	1 (2 rarely)	Persistent, fine granular	Outgrowths of thick electron-dense threads forming an irregular maze	Probably anisofilar (geometry of coils?), 7 (8–11) = 4 + 1–2 + 2–5	Patches of dark cell wall material during transition presporont-sporogonial plasmodium; episporal structures form whole with SPOV
Gurleya tetraspora (Doflein, 1898; Jírovec, 1942)	*Daphnia maxima*(?), *Moina restirostris*	Hypodemal tissue	(–), oval/2.8–3.4 × 1.4–1.6 µm (uf), pyriform	(–)	(–)	4	(–)	Longitudinal grooves (light microscopy)	(–)	Infected host - brownish patches (1st reference), milky white (2nd reference); probably 2 species
Gurleya vavrai (Green, 1974)	*Daphnia longispina*	(–)	5.5–6.0 × 2.8–3.0 µm (uf), oval, slightly tapered to one end	(–)	(–)	4	(–)	(–)	(–)	Preserved specimens do not keep their shape
Gurleya macrocyclopis (Voronin, 1986; Voronin, 1996)	*Macrocyclops albidus*	Fat body	3.8–4.0 × 2.5–2.7 µm (uf), broadly oval	No	(–)	4	Persistent, fibrils and electron-dense secretory granules	Smooth surface (*)	Anisofilar, 12 (8 + 4)	Ribbon-shaped meronts, thick cell wall (patches (*) of material) during transition presporont-sporogonial plasmodium, spherical electron-dense secretory granules (fixation artifacts ? (*))
Gurleya lopukhinae (Voronin 1986; Sokolova et al., 2018)	*Eucyclops serrulatus*	Body cavity	3.2 × 1.9 µm (–), pyriform	No	(–)	4	Subpersistent, thin electron-dense fibrils	(–)	Anisofilar, 8 (2–4) + (1–2) + (2–4)	
Gurleya daphniae (Friedrich et al., 1996)	*Daphnia pulex*	Epidermis	3.8–4.6 × 2.3–2.6 µm (f), pyriform, somewhat angular at the anterior end	No	Finger-like, internal (two lobed followed by four-lobed without subsequent division of SPOV)	4 (1–8) often anomalous concerning size, shape and number of spores	Persistent, fibrous material and tubules	Smooth surface	Anisofilar, 3–4 + 2–5	Thick cell wall (patches (*) of material) during transition presporont-sporogonial plasmodium, later knobs like secretions of granulated electron-dense material (*)
Conglomerata obtusa (Vávra et al., 2018)	*Daphnia pulex, D. magna, D. longispina* complex	Adipose cells around the hepatopancreas and gut (little difference in	4.2–4.5 × 2.4 µm (Dp), 3.8–4.2 × 2.3–2.4 µm (Dm), 3.9–4.4 × 2.5–2.6 µm (Dl) - all (uf), short pyriform, straight or slightly concave	No	Rosette-like, internal (without subsequent	10–30 (irregular)	Non-persistent, metabolic granules with periodic	Short cylindrical projections displayed as fibrillar	Nearly isofilar, 7–8	Characteristic arrangement of polar tube - transitional bend at an angle of 45°,

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Table 1 (*continued*)

Species, reference/ Main distinctive characters	Host	Site of infection	Spore size, shape	Merontogenetic sac #	Sporogonial plasmodium division	SPOV - no. of spores	SPOV - structure, inclusion	Episporal structures - part of the spore	Polar filament - type, no. of coils	Other specific characteristics
		progression among (Dp), (Dm),(Dl)			division of SPOV)		structure (dense and lucent lines)	outgrowths or circular elements		spores with thick layer of mucous material, non-infectious for the original host
Agglomerata simocephali (Voronin 1986; Sokolova et al., 2018)	*Simocephalus vetulus*	Hypoderm	5.0 × 2.8 µm (–), pyriform	(–)	Rosette-like, sporogonial plasmodium with 8 (rarely 12 or 16) nuclei	8 (rarely 12 or 16)	Thin, episporontal space with thin fibrous inclusions	(–)	Slightly anisofilar, 9	Originally *Thelohania simocephali*
Agglomerata sidae (Larsson and Yan, 1988)	*Holopedium gibberum, Sida crystallina, S. brachyura, D. pulex*	Adipose tissue (predominated), hypoderm, haemocytes	1.5–2.0 × 2.5–3.5 µm (f), pyriform	No	Rosette-like, internal (without subsequent division of SPOV)	8–32 (mostly 16)	Persistent, prominent tubular inclusion (sectors of spherical body with concentrical dense and lucent layers)	Fine granular and tubular (*) (continuity between exospore material and SPOV inclusion)	Slightly anisofilar, 1–3 + 3–4	Originally *Duboscqia sidae*
Agglomerata cladocera (Larsson et al., 1996; Sokolova et al., 2016)	*Daphnia magna*	Hypodermal cells (1st reference), hypoderm, fat body, haemocytes (2nd reference)	1.5–2.2 × 3.0–4.5 µm (uf), pyriform (1st reference), 1.9–2.6 × 3.5–4.3 µm (f), pyriform (2nd reference)	No (1st reference), yes (2nd reference)	Rosette-like, internal (without subsequent division of SPOV)	4–16 (usually 8) (1st reference), 8–16 (2nd reference)	Persistent, blister-like protrusion at beginning, granular material is transformed into 2 types - fibrous and wide tubular	Fibril-like exospore coat	Lightly anisofilar, 1–2 + 3–4 (1st reference), slightly anisofilar: 1–2 + 4–5 (2nd reference)	Originally *Glugea cladocera (Thelohania cladocera)*; 2nd reference states additional envelope - a precursor of the SPOV membrane on the surface of presporonts, sporonts and plasmodia and lamellar-like inclusions in SPOV (another species ??)
Agglomerata connexa (Ovcharenko and Wita, 2001)	*Daphnia longispina*	Adipose tissue (primary site), nearly all tissues except muscles and digestive tube	4.08 ± 0.27 × 2.72 ± 0.24 µm (uf), pyriform,	No	Rosette- or finger-like, (with or without subsequent division of SPOV), plasmodium with 8 nuclei (*)	1 (mostly) or up to 4	Fragile, tubular inclusions absent in individual SPOVs, rare in multisporous SPOVs	Fine granular material	Isofilar 5–7	Space outside of SPOVs contains prominent inclusions, which transform into tubules; often, neighbouring SPOVs are connected into short chains
Agglomerata volgensae (Larsson and Voronin, 2000)	*Daphnia magna*	Hypoderm and adipose tissue	3.1–3.7 × 1.7–2.0 (uf), pyriform	No	Rosette-like, external or internal (with or without subsequent division of SPOV), 4–16	4–16 (mostly 8) or 1	Fragile (collective), pad-like primordia are released in a blister-like manner, two	Thin fibrous material	anisofilar, 2–3 + 2–3	Infected hosts have a distinct white color, SPOVs externally connected to each other by dense

(*continued on next page*)

Table 1 (*continued*)

Species, reference/ Main distinctive characters	Host	Site of infection	Spore size, shape	Merontogenetic sac #	Sporogonial plasmodium division	SPOV - no. of spores	SPOV - structure, inclusion	Episporal structures - part of the spore	Polar filament - type, no. of coils	Other specific characteristics
					(usually 8) sporoblasts		type (fibrous and tubular)			fibrillar material (in infected tissue)
Agglomerata lacrima (Bronnvall and Larsson, 2001)	*Acanthocyclops vernalis*	Hypodermis and fat tissue	4.2–4.6 × 2.4–2.8 µm (uf), pyriform,	No	Rosette-like, internal (without subsequent division of SPOV)	4–12 (mostly 8)	Fragile, started to bulge out in a blister-like fashion, loose network of dense material (granular or fibrous)	Different kind of tubuli, formed of excess exospore material (exhibited the same kind of stratification as the exospore)	Anisofilar, 1–2 + 4	Prominent dense white spots visible through cuticle of infected copepods
Binucleata daphniae (Refardt et al., 2008)	*Daphnia magna*	Integumental cells lining the hemocoele cavity of the carapace and of the postabdomen	4.9 × 2.5 µm (uf), elongate pyriform	Yes (halo of dense fibrillo-tubular threads and membranous vesicles inside)	Finger-like, internal	8 or 16	Subpersistent, fine granular secretory material	No (*)	Anisofilar, 4 + 4	Infected host - opaque appearance, all presporogonial stages - dense homogenous coat on plasma membrane, spores autoinfective
Lanatospora bosminae (Voronin 1986; Sokolova et al., 2018)	*Bosmina obtusirostris, B. longirostris, B. coregoni*	Connective tissue of ovaries	2.9–3.6 × 1.7–2.0 µm (f), pyriform	(–)	Rosette-like, (sporonts with 8, 12 or 16 nuclei)	(–)	(–)	Coat composed of threads	Slightly anisofilar, 1 + 1 + 3	Ribbon-like meronts with 1–4 nuclei, spores with mucus capsule, probably *Agglomerata*-like microsporidium (Bronnvall and Larsson, 1995; Vávra et al., 2016a)
Lanatospora macrocyclopis (Voronin, 1989)	*Macrocyclops albidus*	Fat body, possibly ovary	3.2 (3.0–3.3) × 2.0 (1.9–2.2) µm (uf), oviform	No (parasitophorous vacuole)	Rosette-like, external, 6–16 sporoblasts per sporont, drop-like extrusion	6–16 (SPOV not stated)	(–), present (*)	Woolskin-like exosporal coat	Isofilar, 6–8	Polynucleate ribbon-like merogonial plasmodia, whitening of infected hosts, originally *Thelohania macrocyclopis*
Lanatospora tubulifera (Bronnvall and Larsson, 1995)	*Acanthocyclops vernalis*	Connective tissue between muscle strands and beneath the hypodermis	3.5–4 × 2 µm (f), pyriform	No	Rosette-like, external, at least 8 sporoblasts per sporont, electron-dense blisters	1 (individual SPOV)	Thin, connected to the exospore by numerous tubuli	Exospore with a woolskin-like cover	Isofilar, 7–9	Rounded plasmodia with at least four nuclei divide by plasmotomy in second merogony, parasite caused an anomalous yellowish brown

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Table 1 (*continued*)

Species, reference/ Main distinctive characters	Host	Site of infection	Spore size, shape	Merontogenetic sac #	Sporogonial plasmodium division	SPOV - no. of spores	SPOV - structure, inclusion	Episporal structures - part of the spore	Polar filament - type, no. of coils	Other specific characteristics
										coloration of the host
Lanatospora costata (Vávra et al., 2016a)	*Megacyclops viridis*	Connective and adipose tissue	4,7 × 2,7 µm (uf), egg-shaped	No	Rosette-like, external, 4–8 sporoblasts, dense irregular patches and globules	1 (individual SPOV)	Semipersistent, granular secretory material	Rib-like extension from the exospore, forming system of thick-walled lacunae (basket-like armor at the spore surface)	Isofilar, 8–9	Inner (tubular) substructure of rib-like extensions, infected host - conspicuously white appearance
Berwaldia singularis (Larsson, 1981; Vávra and Larsson 1994; Vávra et al. 2017)	*Daphnia pulex*	Fat body, ovaries, hypoderm	5.5–6.5 × 3.0 µm (uf), broadly oval (1st reference)	No	No, oval binucleate sporont (disporoblastic sporogony)	1 (individual SPOV, two spores glued together)	(–), forming blister-like folds, membranous and tubular components, patches of cementing subst. on exospore	Except the dark anchor material for SPOV waves is surface without appendages	Isofilar, 15–18	2 merogonial sequences, chain or finger-like division of tetranucleate meront in second merogony, infected hosts - milky yellow with a slight pinkish tinge
Berwaldia tschernischovae (Voronin,1986; Sokolova et al., 2018)	*Daphnia cucullata*	Fat body	4,2 × 2.0 µm (–), elongated oval	(–)	(–), sporogony disporoblastic	(–)	With 2-layered envelope, which contacts the exospore in a few places	(–)	Anisofilar, (11–13) + 2 + 3	Originally *Tuzetia tschernischovae*
Berwaldia schaefernai (Vávra and Larsson 1994; Vávra et al. 2017)	*Daphnia galeata*	Fat body, various other tissue	4.14–5.39 × 2.28–2.65 µm (uf), ovoid, slightly assymetrical (1st reference)	No	No, oval binucleate sporont (occurrence of blister-like dense patches on plasma membrane at the onset of sporogony)	1 (individual SPOV, two spores glued together)	(more or less) persistent, forming blister-like (chamber-like) sachets, membranous and tubular components	Except the dark anchor material for SPOV waves is surface without appendages	Isofilar, 15–17	Extreme structural similarity with *B. singularis*, nearly same spore size in later reference, infected hosts - milky yellow with a slight greenish tinge, spores non-infectious for the original host (both species)
Berwaldia hypodermica (Vávra et al. 2017)	*Simocephalus vetulus*	Hypodermis and fat cells	4.1 (3.8–4.2) × 2.4 (2.2–2.8) µm (uf), ovoid	No	No, oval binucleate sporont, dense blister-like patches on plasma membrane at the onset of sporogony)	1 (individual SPOV)	Persistent, thin membrane to which sheets of 20 nm tubules are attached, folds loosely touch but are not cemented to the exospore	No	Isofilar, 12–13	Spores occurring singly (not glued), rare species

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Table 1 (*continued*)

Species, reference/ Main distinctive characters	Host	Site of infection	Spore size, shape	Merontogenetic sac #	Sporogonial plasmodium division	SPOV - no. of spores	SPOV - structure, inclusion	Episporal structures - part of the spore	Polar filament - type, no. of coils	Other specific characteristics
Berwaldia nana (Vávra et al. 2017)	*Daphnia longispina/ galeata* complex	Fat cells	4.0 (3.8–4.3) × 2.5 (2.4–2.6) µm (uf), egg-shaped	No	No, oval binucleate sporont, small blisters on plasma membrane of sporonts)	1 (individual SPOV)	Persistent, loosely ensheathing the spore as a sachet consisting of irregular chambers	Thickenings (30–50 nm) of finely granular material joined to exospore (reinforce of SPOV membrane)	Isofilar, 9	Spores occurring singly (not glued), small posterior vacuole forms a laterally shifted slit, rare species
Berwaldia daphniae (Simakova et al. 2018a; Simakova et al. 2018b)	*Daphnia magna*	Entire host haemocoel	4.8 ± 0.3 × 2.3 ± 0.2 µm (f), elongate, oval	No	No, binucleate sporont	(–)	(–), *Berwaldia*-like SPOV present with an obvious substructure (*)	(–)	Slightly anisofilar, 13–19	Infected host - opaque white or light orange in color

Abbreviations: (–) not given, (*) observation from photodocumentation, (uf) unfixed, (f) fixed, (Dp), (Dm), (Dl), (Hg) - *Daphnia pulex*, *D. magna*, *D. longispina* complex, Holopedium gibberum, SPOV - sporophorous vesicle, # membranous sac - former host cell membrane or abortive sporophorous vesicle membrane formed by the presporont.

patterns of ITS diversity, they speculated that its secondary host is likely less mobile than that of *C. obtusa* infecting *Daphnia* in the same type of habitats. It it intriguing that the most closely related microsporidian sequence (see Fig. 3) was obtained from a stream-dwelling larva of the caddisfly genus *Hydropsyche* (Grabner, 2017). A thorough screening of caddisfly larvae from localities inhabited by *P. daphniae* might reveal whether this insect order is involved in its life cycle.

6. Conclusions

The description of the ultrastructure of the MIC1 microsporidium and its classification as a representative of a new microsporidian genus and species is another step in our effort to clarify the systematics and identification of important microsporidia infecting northern temperate *Daphnia*. The three microsporidia characterized in this and two previous contributions (Vávra et al., 2017, 2018) all infect the *D. longispina* complex, the most common daphnids in both deep and shallow lakes and reservoirs in the temperate Holarctic. They are also found in a wide range of habitats including alpine pools (e.g., Ventura et al., 2014) and coastal rockpools (e.g., Bengtsson and Ebert, 1998). These three microsporidia are now well differentiated and future studies of their ecological interactions or evolutionary biology can be based on solid taxonomic characters.

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