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Microsporidian genus *Berwaldia* (Opisthosporidia, Microsporidia), infecting daphnids (Crustacea, Branchiopoda): Biology, structure, molecular phylogeny and description of two new species

Jiří Vávra^{a,b,*}, Miroslav Hyliš^c, Ivan Fiala^{a,b}, Veronika Sacherová^d, Charles R. Vossbrinck^e

^aInstitute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic

^bDepartment of Parasitology, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

^cLaboratory of Electron Microscopy, Faculty of Science, Charles University in Prague, Czech Republic

^dDepartment of Ecology, Faculty of Science, Charles University in Prague, Czech Republic

^eThe Connecticut Agricultural Experiment Station, New Haven, CT, USA

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Abstract

Structural, molecular and life cycle data are presented for two microsporidian species of the genus *Berwaldia*: *B. singularis* Larsson, 1981 (type species of the genus) and *B. schaefernai* Vávra and Larsson, 1994, parasites of *Daphnia pulex* Leydig, 1860 and *Daphnia galeata* Sars, 1863, respectively. Analysis of the SSU rDNA gene confirmed the species status of both species and showed that the GenBank sequence data submitted previously in GenBank for the genus *Berwaldia*, are from microsporidia that are not *Berwaldia*. Correct SSU rDNA gene sequences for *B. schaefernai* and *B. singularis* are now deposited in GenBank. The life cycle of these two species appears incomplete as the spores collected from their respective infected hosts will not infect the same host when fed per os. *B. schaefernai* appears as a frequent parasite of *Daphnia longispina/galeata* complex daphnids, influencing the behaviour of the infected host. In addition, two new species, of *Berwaldia*, one infecting fat body tissues of *Daphnia longispina/galeata* complex, and the other, infecting hypodermis and fat cells of *Simocephalus vetulus* (O. F. Müller, 1776) are described.

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Keywords: *Daphnia*; Fungi; Microsporidia; Parasite; SSU rDNA phylogeny; Transmission

Introduction

Microsporidia are parasitic protists (unicellular eukaryotic organisms), living in cells of animals and, rarely, in cells of other protists (Vávra and Lukeš 2013). The origin

of microsporidia is not understood but, currently, they are believed to be either members of the early branching Fungi or a sister group to Fungi called Opisthosporidia (James et al. 2013; Karpov et al. 2014; Keeling 2014). Microsporidia are a monophyletic taxon characterized by a preformed injection tube within the mature spore, an apomorphic character. The injection tube (“polar filament”) everts explosively when spores germinate inside the body or digestive tract of the host and spore contents, in the form of a small cell, are

*Corresponding author at: Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic.

E-mail address: vavrajir@natur.cuni.cz (J. Vávra).

injected through the tube into a host cell. Microsporidia are frequent parasites with about 200 genera and more than 1500 species described (Becnel et al. 2014; Vávra and Lukeš 2013). Although found in most animal phyla, microsporidia most frequently parasitize arthropods among invertebrates and fish among vertebrates (Becnel and Andreadis 2014; Stentiford and Dunn 2014). Several microsporidian species infect mammals, including humans (Snowden 2014). Small planktonic crustacea (cladocerans and copepodes) are commonly hosts of microsporidia (Refardt et al. 2002; Vávra et al. 2016b) and research on this host group is of interest from the perspective of natural diversity, ecology and host-parasite associations. Microsporidian ancestors probably originated in water as their phylogenetic relatives (Rozellomycota and aphelids) are parasites of algae, water molds and some amoebozoia (Corsaro et al. 2014; Karpov et al. 2014). Research on microsporidia of planktonic crustacea is thus motivated by the hypothesis that ancestral forms will eventually be found in these hosts, which are principal grazers (daphnids) and predators (copepods) in the freshwater environment and have many opportunities to acquire different parasites. In fact, one phylogenetically basal microsporidian was recently described as a parasite of *Daphnia magna* (Haag et al. 2014).

Approximately 40 species in twelve microsporidian genera have been described from daphnids (Crustacea, Branchiopoda) (Becnel et al. 2014; Larsson and Voronin 2000), among them two species of the genus *Berwaldia*: *B. singularis* Larsson, 1981, a parasite of *Daphnia pulex* Leydig, 1860, and *B. schaefernai* Vávra and Larsson, 1994, a parasite of *Daphnia galeata* Sars, 1863. The first sequence of the SSU rRNA gene of *Berwaldia* sp. (*B. schaefernai*) was deposited in NCBI GenBank by Vossbrinck et al. (2004) (Accession # AY090042). This sequence enabled researchers to publish data on the occurrence of microsporidia of similar molecular sequence in various water reservoirs, study the population genetic structure and transmission mode of *Berwaldia* parasites in *Daphnia* spp., and deposit a number of *B. schaefernai* and *Berwaldia* sp. SSU rRNA partial sequences in GenBank (Weigl et al. 2012; Wolinska et al. 2009). In July 2017, 119 sequences of *Berwaldia* isolates were accessioned in GenBank.

We have recovered a microsporidium identical to *B. singularis* (the type species of the genus) in a population of *D. pulex* (the type host). Surprisingly, the SSU rRNA gene sequence we obtained from this material was unrelated to *B. schaefernai* sequences deposited in the GenBank. This prompted us to reinvestigate in full the two *Berwaldia* species in their original hosts, using morphological, life cycle and molecular (SSU rRNA) characters, to reaffirm their species status and to deposit the correct sequences of SSU rRNA for both *B. singularis* and *B. schaefernai* in GenBank. We have also recovered two additional isolates of *Berwaldia* like microsporidia with distinctive structural characters, one in *Daphnia longispina/galeata* complex specimens, and another infecting fat and hypodermal cells of *Simocephalus vetu-*

lus. Both isolates are described below as new *Berwaldia* species.

Material and Methods

Hosts and sampling

Information concerning sampling of infected daphnids, handling of the sampled material, acronyms used for individual microsporidia and tissue infected are presented in Table 1. Infected animals were selected due to their milky appearance in incident light and were identified using the key of Benzie (2005). In addition to microscopy, the *D. galeata* specimens were identified using the mitochondrial gene 12S rRNA as molecular tag (Taylor et al. 1996; Tokishita et al. 2017) (see Supplementary material 1.1. for details). Spore numbers in individual infected *D. pulex* were determined by using Bürker's haemacytometer (Jírovec 1947).

Microscopy

Methods described in Refardt et al. (2008), Vávra (1964a) and Vávra et al. (2016a,b); Vávra et al. (2016a,b), were used for light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (see Supplementary material 1.2. for details).

DNA isolation, sequencing and phylogeny

The protocols published in Vávra et al. (2016a), were followed, except that only the primers ss530f: ls580r (Weiss and Vossbrinck 1999) served for rDNA amplification. Details of phylogeny analysis and tree construction are presented in Supplementary material 1.3.

Infection experiments

Juvenile *D. pulex* individuals were fed spores of *B. singularis* isolated from their infected adult congeners (fresh or stored), or spores present in the sediment of water from the habitat where the infection was flourishing (see Supplementary material 2 for details).

Results

The *Berwaldia* parasites (*B. singularis*, *B. schaefernai*) in *D. pulex* and *D. galeata*, their respective hosts

Microsporidia identity

Multiple specimens of *D. pulex* infected by *B. schaefernai* (BSING) and collected at a single habitat were examined (Table 1). As documented below, the *Berwaldia*

Table 1. Habitats: **1.** semipermanent forest marsh, near Běleč, Central Bohemia region, Czech Republic ($50^{\circ}3'13.842''N$; $14^{\circ}1'7.955''E$). **2.** Large carp pond “Velký Pálenec”, near the town of Blatná, South Bohemia region, Czech Republic ($49^{\circ}24'48.01''N$; $13^{\circ}48'54.37''E$), this pond belongs to a system of mutually related carp-breeding ponds from which *Berwaldia schaefernai* was originally described, see Vávra and Larsson 1994. **3.** Small carp pond “LUKES”, in the vicinity of the village Nižbor, Central Bohemia region, Czech Republic ($49^{\circ}59'10.851''N$; $14^{\circ}0'5.461''E$). **4.** Abandoned carp pond turning into marsh, near pond LUKES of above ($49^{\circ}59'13.136''N$; $13^{\circ}59'52.638''E$). **5.** Carp pond “Hřešíš”, near Karlov, Central Bohemia, Czech Republic ($49^{\circ}58'40.958''N$; $13^{\circ}54'43.136''E$).

Microsporidian organism	Host	Habitat	Tissue	Acronym	Host determination.no. of inspected	No. of DNA isolations
<i>Berwaldia singularis</i> Larsson, 1981	<i>Daphnia pulex</i> Leydig, 1860	1	Fat cells	BSING	LM (multiple)	4#
<i>Berwaldia schaefernai</i> Vávra and Larsson, 1994	<i>Daphnia galeata</i> Sars, 1863	2	Fat cells	BSCH-1	LM + MB (multiple)	3#
<i>Berwaldia schaefernai</i> Vávra and Larsson, 1994	<i>Daphnia galeata</i> Sars, 1863	3	Fat cells	BSCH-2	LM + MB (multiple)	2#
<i>Berwaldia hypodermica</i> sp. n. Berwaldia nana sp. n.	<i>Simocephalus venulus</i> (O.F. Müller, 1776) <i>Daphnia longispina/galeata</i> complex	4 3,5	Fat cells, hypoderm Fat cells	— —	LM (2) LM	— —

Host determination: LM, light microscopy; MB, molecular biology.
#multiple infected hosts examined in order to reveal intraspecies variability.

parasites infecting *D. pulex* specimens were found to be mutually identical. Mutually identical were also the *Berwaldia* parasites (labeled BSCH-1 and BSCH-2), which infected *D. galeata* specimens sampled in two, geographically distant habitats (Table 1). This allows us to treat below the daphnid microsporidia in question as *Berwaldia singularis* Larsson, 1981, (BSING) and *Berwaldia schaefernai* Vávra and Larsson, 1994, (BSCH), respectively.

Occurrence

Microsporidia BSING and BSCH infected fat cells of their hosts and the overall aspect of infection looked similar in both hosts. The infected fat cells had a globular character in early stages of infection (Fig. 1 a) and turned progressively into a confluent mass of spore filled tissue as infection progressed. Infected daphnids carried none or very few eggs, appeared milky yellow with a slight pinkish (BSING) or greenish (BSCH) tinge. In BSING the infection repeatedly occurred in a dense daphnia population each spring over 4 years of observation (2013–2016), with the peak prevalence reaching in one year 26% of adult females in May. Infection prevalence gradually declined, but infected individuals were occasionally found during the summer months and early autumn. Each large infected *D. pulex* female produced spore loads numbering from 1.3×10^5 (minimum), to 2.3×10^5 (average), to 3.6×10^5 (maximum). No quantitative data are available for the seasonal dynamics and spore loads of the BSCH as both samples (BSCH-1, BSCH-2) were obtained at a single plankton sampling at their respective habitats.

Structural evidence

Light microscope and TEM observations confirmed the identity of both BSING and BSCH, conforming to data reported by Larsson (1981) and Vávra and Larsson (1994) (Figs. 1a–k, 2 a–c). Characteristic for both microsporidia were similar, medium-long oval spores, slightly more acute at one pole. There was some variability in size and shape of spores in both BSING and BSCH. In individual daphnid hosts the spores appeared to be more plump, in others, they appeared to be thinner and longer (Figs. 1b, c, 2a, b). Spores of both species were of similar size: $4.4 \times 2.3 \mu\text{m}$ for BSING ($3.8\text{--}5.0 \times 2.0\text{--}2.7$, $n=80$) and $4.4 \times 2.2 \mu\text{m}$ for BSCH ($3.7\text{--}5.0 \times 1.9\text{--}2.6$, $n=100$) (fresh spores). The posterior vacuole was easily observable and spores in India Ink wet preparations in both species were without the exospore mucous layer. The presence of large, single nucleus in the spore (Figs. 1d, 2c) and in developmental stages (Fig. 1e), was characteristic for both BSING and BSCH. Ultrastructurally the BSING and BSCH were almost identical. Most characteristic for these microsporidia, was the occurrence of blister-like dense patches on plasma membrane of developmental stages at the onset of sporogony. This material was deposited between the plasma membrane and another outside membrane produced during sporogony and was the precursor material of the future sporophorous vesicle (Fig. 1f). Mature spores were enclosed in individual sporophorous vesicles

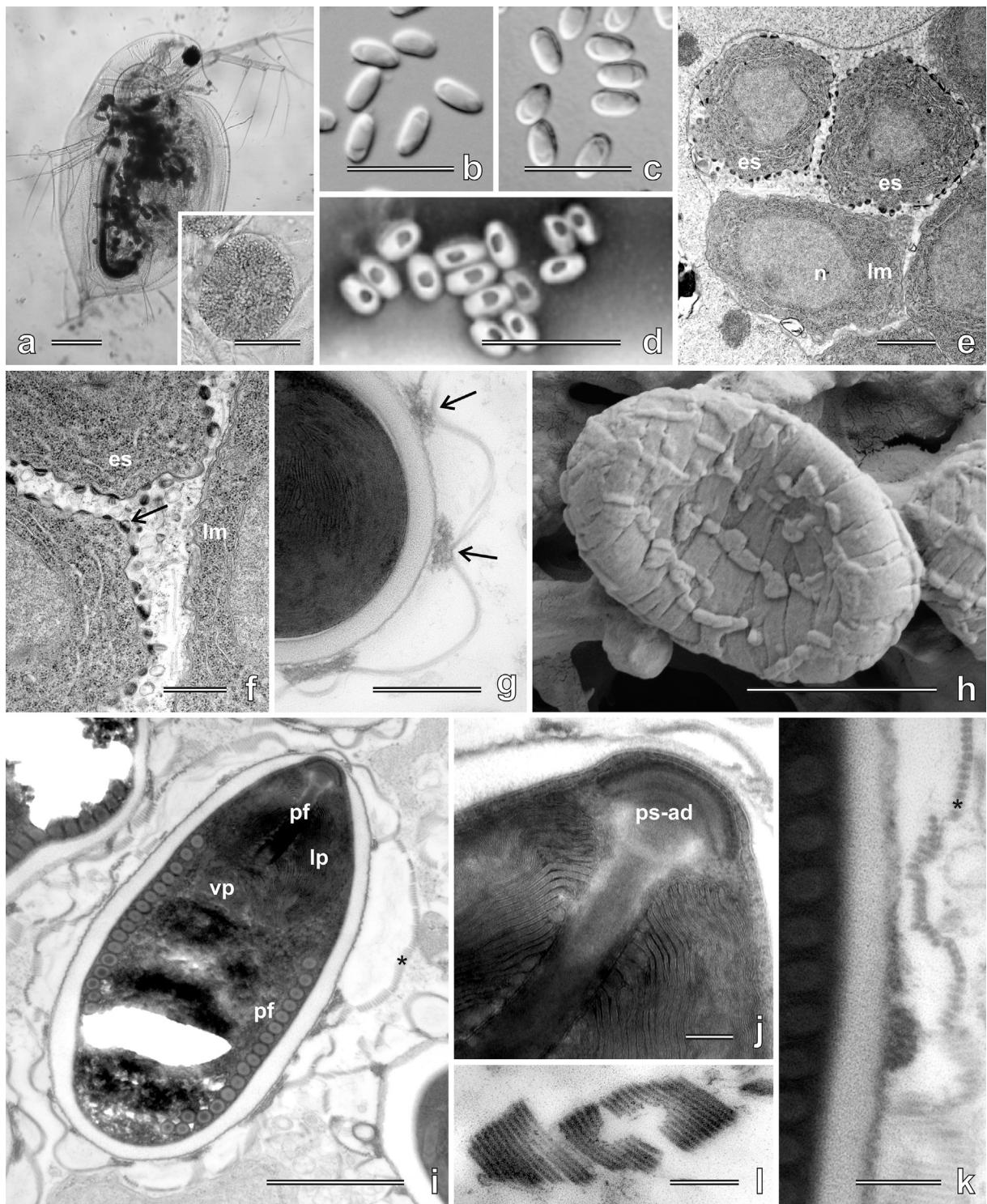


Fig. 1. *Berwaldia singularis* Larsson 1981 in *Daphnia pulex* Leydig, 1860.

(a) Lightly infected host. Note the nodular appearance of infected tissue Bar = 500 µm. Inset shows an enlarged fat cell filled with spores. Bar = 50 µm. (b, c) Variability of fresh spores—two extreme spore morphs are shown (Nomarski contrast). Bar = 10 µm. (d) Spores showing the characteristic large nucleus. Robinow staining-Burri Ink. Bar = 10 µm. (e–g, i–l) TEM, (h) SEM. (e) Late meronts (lm) and early sporonts (es). Note the large nuclei (n), characteristic of developmental stages of *Berwaldia*. Bar = 1 µm. (f) Detail of the construction of the cell membrane of meronts (lm) and of the cell wall material patchily deposited on sporonts (es), characteristic for the genus *Berwaldia*. The outer membrane limiting the deposits is the future sporophorous vesicle (SPOV) membrane (arrow). Bar = 0.5 µm. (g) Partial view of spore covered with sporophorous vesicle in the form of undulatory waves. The bottom of each wave is anchored to the exospore by an accumulation of dark material (arrows). Bar = 0.5 µm. (h) SEM photograph of spore surface shows the collapsed blisters of the SPOV.

(SPOV) that were undulatory in appearance in TEM sections and formed a kind of chamber-like blisters on the spore surface. These blisters were somewhat more voluminous in BSING (Fig. 1i) than in BSCH (Fig. 2c) and were collapsed in SEM preparations showing the spore surface (Fig. 1h). The bottom of each undulation of the SPOV was anchored to the exospore membrane by a clump of dense granular material (Fig. 1g). The wall of the SPOV was formed by sheets of closely adherent fine tubuli, 20 nm thick, interspaced by less dense material of 15 nm (Figs. 1g, i, k, l). The tubuli were difficult to fix and preserve properly and the tubular nature of the SPOVs walls was not always evident. The interior of SPOV blisters contained very fine, irregularly deposited filaments (not shown). The spores of BSING and BSCH had the same structural organization: a thick, uniform, single-layer endospore (100 nm), a thin, dense, single-layer exospore (20 nm) and 16–17 polar filament coils, with the last 4–5 coils revealing a more complex inner structure (Figs. 1i, k, 2c). Polaroplast had compact lamellae in its upper part, followed by a vesicular part (Figs. 1i, j, 2c). The specific character of BSCH spores, reporting that one of the polar filament coils was not aligned with other coils (Vávra and Larsson 1994), was not found in the present material.

Molecular phylogeny

Four PCR products of SSU rRNA of 1400 bp in length from individual daphnias infected by BSING, and three and two respective products of similar length from BSCH-1 and BSCH-2 were obtained. Direct sequencing revealed sequence polymorphism in all sequenced microsporidians. A 594 bp long part of the SSU rDNA without sequence polymorphism was used for phylogeny and tree construction (Fig. 4). Uncorrected p-distances were calculated from alignment containing 653 positions limited to the SSU rDNA region. (Supplementary material 3). The partial SSU rDNA sequence obtained from BSING was different in 13 nt positions from the two, mutually identical sequences of BSCH-1, BSCH-2. The respective sequences were deposited in the GenBank.

Infection experiments

At several occasions we tried to transmit the infection by BSING using direct spore feeding to daphnias (see Supplementary material 2.1 for details). Time lapse investigation of daphnids exposed to spores showed that spores do germinate in the daphnia gut. The proportion of germinated spores increased with time, probably due to repeated passage throughout the digestive system. In TEM micrographs, sporoplasms were found in tissues of daphnids exposed to spores (Supplementary material 2.2); however, infection

could not be transmitted by feeding spores to uninfected daphnids. None of the animals exposed to spores were found infected 4–6 weeks post exposure, nor were infections found in the offspring of experimental animals during the course of the experiments. Transmission experiments using BSCH microsporidia were not attempted as previous experiments with the same microsporidium and the same host failed (Vávra 1964b).

Other *Berwaldia* microsporidia

The search for *Berwaldia*-like microsporidia in various daphnids resulted in finding two microsporidian isolates, incontestably belonging to the genus *Berwaldia* as based on structural characters. Both microsporidia were extremely rare (two infected host specimens for each isolate) and we failed to find additional samples in an extensive search of their respective collection sites. The scarcity of these microsporidia did not allow for characterization by molecular methods.

Isolate 1 infected the hypodermis and fat cells of *Simocephalus vetulus* (O.F. Müller, 1776) (Table 1, Fig. 2d–j). Nearly all hypodermal cells of the host were infected, the fat cells in the body were also heavily parasitized (Fig. 2d). Spores were ovoid in shape (Fig. 2e), 4.1 × 2.4 μm (3.8–4.2 × 2.2–2.8, n = 30) (fresh spores), in size. TEM revealed almost exclusively mature spores (Fig. 2g), the few developmental stages found were sporonts showing large patches of dense material on the plasma membrane, a typical character of *Berwaldia* spp. microsporidia during SPOV formation (Fig. 2f) (see Discussion). Spores had the ultrastructural organization, typical of *Berwaldia* spp. microsporidia (Fig. 2g, h). The spore had a large single nucleus, bipartite polaroplast (lamellar and vesicular), and 13 (12–14) polar filament coils in a single row. The endospore was formed of one uniform layer, 100 nm thick. The exospore was thin (20 nm) and uniform. Each spore was enclosed in an individual SPOV, forming a sachet of irregular, blister-like folds, touching the exospore without being attached to it (Fig. 2g–i). The “membrane” of the SPOV was formed by a thin layer of dense material to which a layer of 20-nm tubuli adhered on the external side (Fig. 2j). The preservation of SPOV subunits was difficult to achieve, and the tubular substructure of the SPOV was only rarely seen. There is no doubt that the isolate is another *Berwaldia* species and the name *B. hypodermica* is proposed for this organism (see Taxonomic summary).

Isolate 2 was found in fat cells of two specimens of *Daphnia longispina/galeata* complex (Table 1, Fig. 3a–h). Spores were egg-shaped and 4.0 × 2.5 μm (3.8–4.3 × 2.4–2.6, n = 20) in size (fresh). The presence of a small, slit-like pos-

Bar = 2 μm.

(i) Spore, total view in TEM. (lp and vp – lamellar and vesicular polaroplast, pf – polar filament with 17 coils). Bar = 1 μm. (j) Detail of the spore apex with the terminal part of the polar filament (ps-ad, polar sac-anchoring disc) and regularly arranged polaroplast lamellae. Bar = 100 nm. (k) Detailed view of polar filament coils and of the tubular nature of the SPOV membrane. Bar = 200 nm. (l) Tangential section throughout the tubules forming the SPOV. Bar = 200 nm.

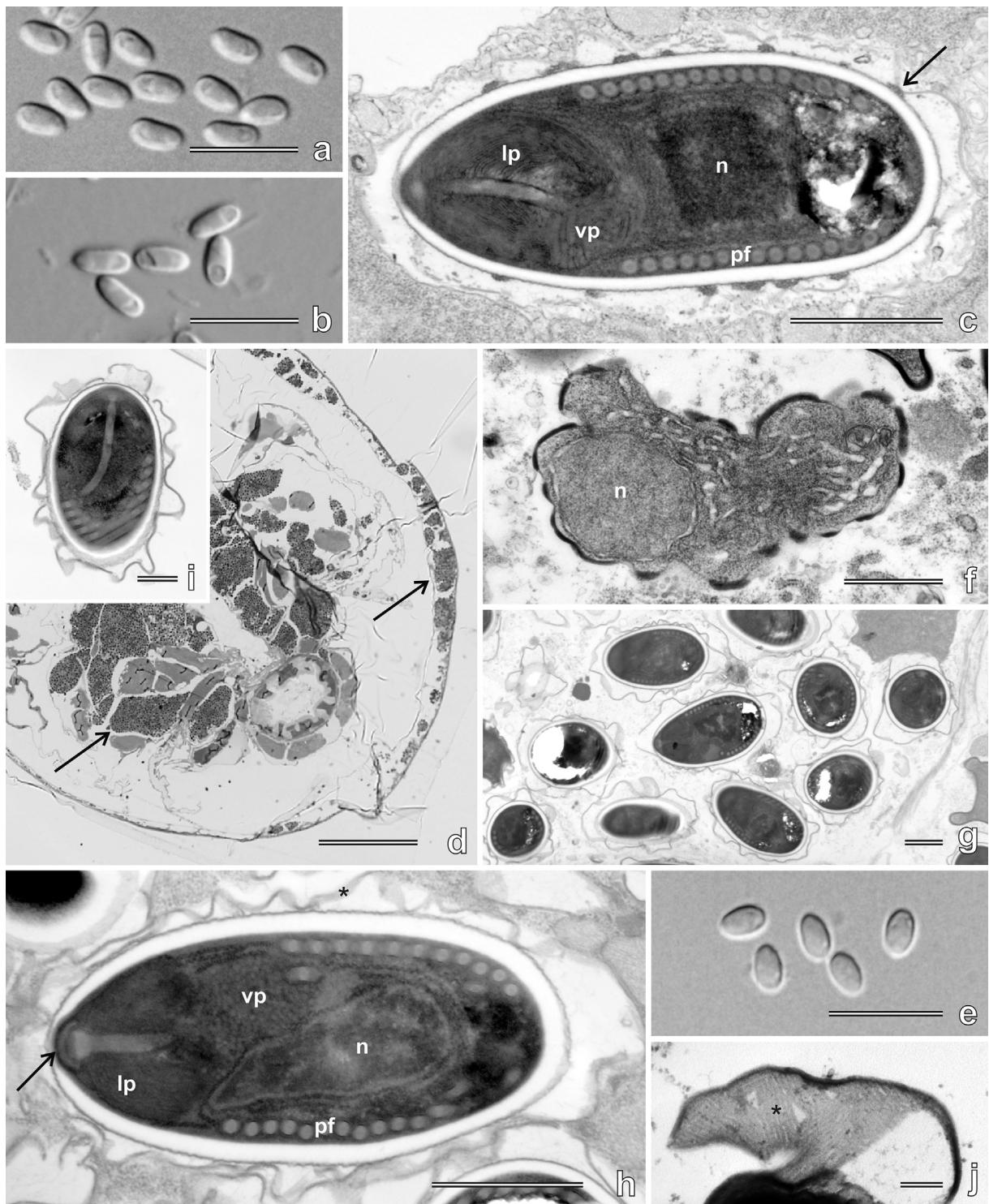


Fig. 2. (a–c) Spores of *Berwaldia schaefernai* Vávra and Larsson 1994 in *Daphnia galeata* Sars, 1863. (a, b) variability of fresh spores—two extreme spore morphs are shown (Nomarski contrast). Bar = 10 µm. (c) spore seen by TEM (lamellar—lp, vesicular—vp, polaroplast, nucleus—n, polar filament—pf). Bar = 1 µm. (d–j) Isolate 1 (=*Berwaldia hypodermica* sp. n.) in *Simocephalus vetulus* (O. F. Müller, 1776). (d) Semithin section throughout the host showing that both the hypodermis and the fat cells are infected (arrows). Bar = 100 µm. (e) Spores in Nomarski contrast. Bar = 10 µm. (f–i) TEM. (f) Sporont during the early phase of the formation of the SPOV (n-nucleus). Bar = 1 µm. (g) Low magnification view of host tissue with spores. Bar = 1 µm. (h) Spore with nucleus (n), lamellar (lp) and vesicular (vp) polaroplast, 12 coils of the polar filament (pf) and the polar sac-polar cap filament terminus (arrow). Note the absence of densifications at sites of contact of the SPOV with the exospore. Bar = 1 µm. (i) free spore with the SPOV. Bar = 0.5 µm. (j) High magnification view of a part of the SPOV wall showing the presence of tubuli (asterisk). Bar = 200 nm.

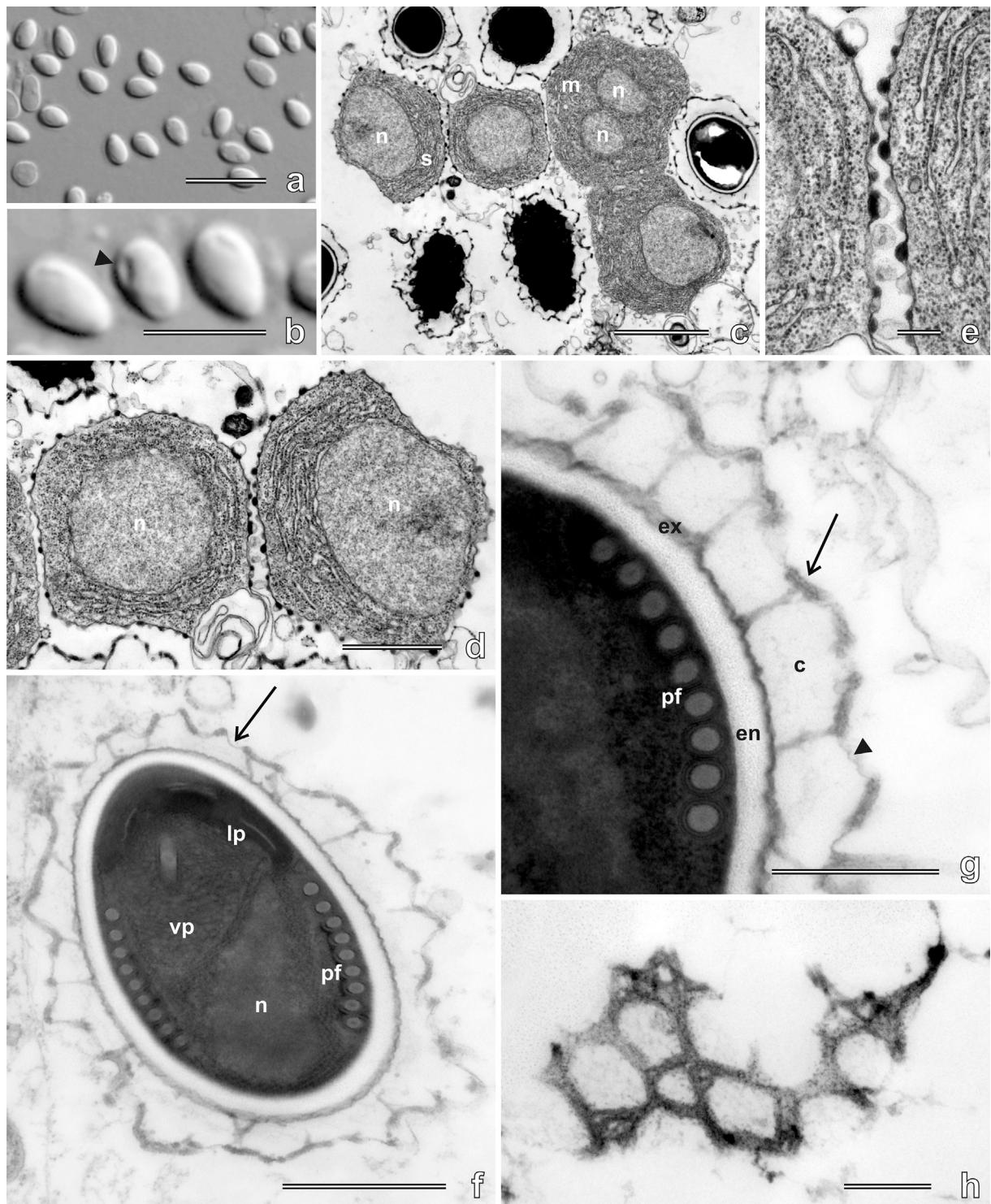


Fig. 3. Isolate 2 (=*Berwaldia nana* sp. n.) in *Daphnia longispina/galeata* complex. (a) Spores in Nomarski contrast. Bar = 10 μm . (b) Detail of the spore showing the typical appearance and position of the posterior vacuole (arrowhead). Bar = 5 μm . (c–h) TEM. (c) Meronts (m) and early sporonts (s) with their large nuclei (n). Bar = 2 μm . (d) Two cells of early sporonts showing large nuclei (n) and dense blebs of the primordium of the SPOV membrane. Bar = 1 μm . (e) Detailed view of the blebbing as origin of the SPOV on sporont cells. This mode of origin is typical for microsporidia of the *Berwaldia* genus. Bar = 200 nm. (f) Spore with its large nucleus (n), bipartite polaroplast (lp, vp), nine coils of the polar filament (pf). The wrinkled SPOV (arrow) ensheats the spore at a distance. Bar = 1 μm . (g) Detail of the spore wall showing the polar filament of nine coils (pf), the thick endospore (en), thin exospore (ex) and the SPOV chambers (c) lined by a thin SPOV membrane (arrowhead) with dense material deposited at the top outline of the chambers (arrow). Bar = 500 nm. (h) tangential view of the SPOV showing the reticulate nature of the surface of the SPOV. Bar = 200 nm.

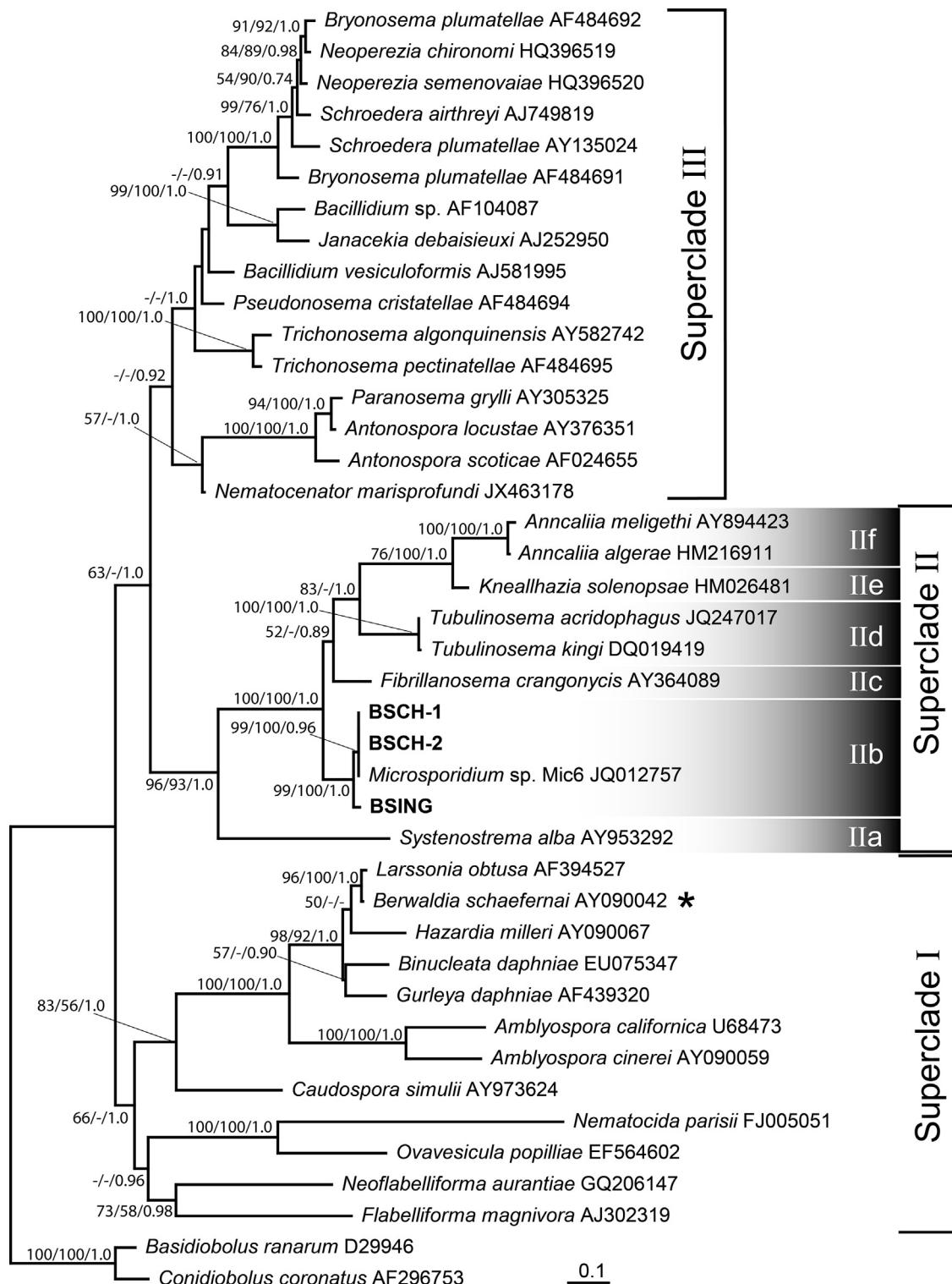


Fig. 4. Maximum likelihood tree based on microsporidian SSU rDNA sequences containing *Berwaldia* spp. Support values are given for each node, the first two numbers are bootstrap values based on the maximum likelihood and maximum parsimony analyses (1000 replicates, less than 50% not shown), the third values representing the posterior probabilities of Bayesian inference. Scale bar is given under the tree. Microsporidia of the genus *Berwaldia* form the cluster IIb within a superclade II, containing microsporidia of terrestrial and aquatic insects, with a single exception of a species infecting a crustacean (clade IIc). The incorrectly named sequence of *Berwaldia schaefernai*, now known to belong to another microsporidian (this paper), is marked by an asterisk.

terior vacuole shifted to the side of spore was characteristic (Fig. 3a, b). Developmental stages, late meronts and early sporonts, were characterized by large nuclei, well developed endoplasmic reticulum and numerous ribosomes (Fig. 3c, d). The SPOV membrane appeared on the plasma membrane of sporonts in the form of small blisters with dense material inside (Fig. 3d, e). This mode of SPOV origin is characteristic for microsporidia of the genus *Berwaldia* (see Discussion). Each spore was enveloped by its own SPOV, loosely ensheathing the spore as a sachet, irregular in outline, bounded by a thin membrane (7 nm), reinforced by electron dense thickenings (30–50 nm) of finely granular material, and joined to the exospore by transverse, irregularly spaced 15–20 nm thick connections (Fig. 3f, g). It appeared that the SPOV formed chambers distributed on the spore surface. This is best shown on a tangential section of the SPOV, where the dense material on the SPOV membrane is revealed in the form of an irregular mesh (Fig. 3h). The spore had a 100-nm thick endospore and a thin, single layer exospore (20 nm) and a large nucleus and bipartite polaroplast (lamellar and vesicular). The polar filament was arranged in 9 coils (Fig. 3f, g). On rare occasions the SPOV membrane formed spikes covered by dense material, around 50 nm thick and 300–400 nm long, stretching to the exterior out of the SPOV (not shown). We believe that the isolate represents another *Berwaldia* species and the name *B. nana* is proposed for this organism (see Taxonomic summary).

Discussion

Tissue, hosts and transmission of *Berwaldia*

Many pathogens infecting daphnids (*Berwaldia* spp. included), thrive in fat cell of their hosts (Ebert 2005). The fat cells are distributed throughout the body and the limbs of daphnids (Martin 1992), and are equivalent to nephrocytes and fat bodies of other arthropods. It is known that microsporidia cause syncytial fusion of infected cells and their hypertrophy (Vávra and Lukeš 2013). Evidently this mechanism of infection-spread operates in daphnids, turning finally the host into a “living sac” filled with spores. In contrast to our earlier observations (Vávra and Larsson 1994), we now believe that ovaries are only secondarily infected in BSCH and that it is the depletion of energetic reserves of the host that causes that infected females carry none or very few eggs. The same is true for BSING infected daphnids.

The seasonal pattern of BSING infection corresponded to seasonal dynamics data on *D. pulex* infections by a different microsporidian: the peak of infections of *D. pulex* by a “*Thelohania* sp.” was situated during five years of observations at May–June, the same period as in BSING infections (Brambilla 1983). The number of spores produced in a daphnia and recorded in the present paper, is the first existing information of its kind. At the same time, the unsuccessful transmission experiments using spore feeding in BSING,

indicated that *Berwaldia* microsporidia should have an indirect life cycle with another host involved. The identity of such host is not known. It is of interest in this respect that the other *Berwaldia* species considered here (BSCH), seems to manipulate its host to aid transmission. BSCH is the most frequent parasite of the *Daphnia longispina/galeata* complex in carp ponds, shows the same spring/early summer prevalence peak as BSING. On sunny days, the infected daphnias assemble in masses in warm water at pond shallows (Vávra and Maddox 1976; Vávra and Pražáková 1983). One can speculate that the alternate host (a chironomid?) is present at that site, and lives in the mat of materials on stones and vegetation exposed to spores released from dead daphnids. Ecology of the presumptive second host might be the reason why Weigl et al. (2012) found the Mic6 parasite (conspecific with BSCH) only on rare occasion and only in two of eight deep water (canyon) reservoirs inspected.

Taxonomy and phylogeny of *Berwaldia*

The present investigation confirmed the existence and taxonomic validity of the two, so far described species of the genus: *B. singularis* in *D. pulex* and *B. schaefernai* in *D. galeata*. These organisms are structurally very similar and their species status was based on minute structural differences and occurrence in different hosts (Vávra and Larsson 1994). The 13 nt difference in the SSU rDNA partial sequence between BSING and BSCH is sufficient to treat them as two different species. In microsporidia, a much smaller difference among SSU rDNA sequences has been judged as a satisfactory, species-determining character (see *Tubulinosema* spp. in the Distance Table in Supplementary material 3).

It is of importance that none of the *Berwaldia* sequences obtained by us were similar to sequences presently deposited in the GenBank for the genus *Berwaldia*. The only sequence in GenBank, that is related to the BSING and BSCH sequences, is the partial SSU rDNA sequence of an unidentified microsporidian Mic6, parasite of the *D. longispina/galeata* complex (JQ012757) (Weigl et al. 2012). The Mic6 microsporidian is evidently conspecific with BSCH (see the Distance Table in Supplementary material 3), but no data on its structure and tissue specificity exist.

Our results show that the sequence for the genus *Berwaldia* deposited under the Acc. No. AY090042, and to which all other GenBank sequences for *Berwaldia* were related, belongs to an unidentified microsporidium that is not related to the genus *Berwaldia*. The infected daphnids from which Vossbrinck et al. (2004) obtained the sequence were sampled by the co-author of the present paper (JV), and were collected in the same pond as the infected daphnids used for the sequence of BSCH-1. Evidently, one animal in the sample was either infected by another, unrecognized microsporidium or there was a dual infection and the “wrong microsporidium” was preferentially amplified during PCR. Indeed, we observed that several *D. pulex* individuals parasitized by

BSING were co-infected by an unidentified microsporidian with small pyriform spores. We believe that the same happened in *Berwaldia*-infected *D. galeata*. Attempts are being made to characterize this microsporidian.

The genus *Berwaldia* represents a well-defined clade (Fig. 4, clade IIb) on the microsporidia phylogenetic tree. This clade is part of the relatively large Superclade II, represented by microsporidia from terrestrial and aquatic insect hosts (Clades IIa, IIId-f), with a single exception, the shrimp parasite *Fibrillanosema crangonycis* (Clade IIc). The close phylogenetic association of the *Berwaldia* clade with insect microsporidia may indicate that *Berwaldia* is an insect microsporidian for which we know only one (aquatic crustacean) phase of its life cycle. The lack of direct oral infectivity to daphnids of spores isolated from daphnids (Vávra 1964b and this paper) seems to support this hypothesis. The *Berwaldia* microsporidia are phylogenetically distant from other microsporidia infecting fat cells and hypodermal tissue of daphnids (Superclade I on Fig. 4 and Clade I of Refardt et al. 2002).

Other *Berwaldia* species

Although the scarcity of available material did not allow us to produce molecular data for the two proposed species (*B. hypodermica* and *B. nana*), the ultrastructural evidence strongly supports their placement in the genus *Berwaldia*. The unique structural apomorphy of the genus *Berwaldia* is the presence on the spore of chambered, blister-like SPOV. This SPOV type is present in *B. hypodermica* and is constructed similarly to that of BSING, except the lack of dense material gluing each SPOV fold to the exospore (compare Figs. 1g, i, and 2h, i). In *B. nana* n. sp., the chambers of the SPOV are also well developed, but are less regular and their closed nature is evident from tangential sections throughout the distal walls (Fig. 3h). Besides the SPOV apomorphic character, there is a group of plesiomorphic characters (large nuclei in merogony and sporogony, thin 20-nm thick exospore of a single layer, 100-nm endospore, isofilar polar filament, bipartite polaroplast) which together support the placement of both microsporidia into the genus *Berwaldia*. Also of interest is that an unidentified *Berwaldia* type microsporidian (with 13 polar filament coils) was found in *Daphnia cristata* in Western Siberia (Lukyantsev and Simakova 2014).

Taxonomic summary

Phylum Microsporidia Balbiani, 1882

Genus *Berwaldia* Larsson, 1981, emend. (emended diagnosis of Vávra and Larsson 1994)

Merogony and sporogony stages with single, large nuclei.

Disporoblastic. Spores elongate oval or ovoid, monokaryotic, with conspicuously large nuclei and posterior vacuole located terminally in some species or shifted to spore side in some species. Spore covered by a 100 nm endospore and a thin, single layer exospore 20 nm thick. Polar filament isofilar, coils forming a single row (except last 2–3 imperfect coils). Polaroplast bipartite, anterior lamellae closely arranged, posterior part less orderly and vesicular-like. Spores in individual sporophorous vesicles forming a blister-like or chamber-like sachet around the spore. Sporophorous vesicle wall formed by an inconspicuous membranous component overlaid by a single layer of closely packed tubules in some species, or formed by material of medium density to which patches of more dense material are added in other species.

Tissue: fat cells, hypodermal cells, involvement of ovaries unconfirmed.

Species

1. *Berwaldia singularis* Larsson, 1981, type species, parasite of fat cells of *Daphnia pulex* Leydig, 1860. Spores elongate oval, 4.4 (3.8–5.0) × 2.3 (2.0–2.7) µm (fresh). Sporophorous vesicle undulatory, patches of cementing substance occur at sites, where sporophorous vesicle folds attach to the exospore. SSU rDNA sequence: GenBank Acc. No. MF139318.

2. *Berwaldia schaefernai* Vávra and Larsson 1994, parasite of fat cells of *Daphnia galeata* Sars, 1864. Spores elongate oval, 4.4 (3.7–5.0) × 2.2 (1.9–2.6) µm (fresh). Sporophorous vesicle undulatory, patches of cementing substance occur at sites, where sporophorous vesicle folds attach to the exospore. SSU rDNA sequence: GenBank Acc. No. MF139316; MF139317.

3. *Berwaldia hypodermica*, sp. n.

Diagnosis: Spores ovoid, occurring singly, 4.1 (3.8–4.2) × 2.4 (2.2–2.8) µm (fresh), posterior vacuole located terminally. Meronts and sporonts with large nuclei, sporophorous vesicle appearing on sporonts in the form of thick, elongated patches of dense material. Spore: single large nucleus, bipartite polaroplast, 12–13 isofilar polar filament coils in a single row. Endospore thick (100 nm), exospore thin (20 nm) and uniform. Spores in individual sporophorous vesicles, undulatory in appearance, and forming irregular chambers on spore surface. Sporophorous vesicle envelope consists of a thin membrane to which sheets of 20 nm tubules are attached. Sporophorous vesicle folds loosely touch the exospore in some places, but are not cemented to the exospore.

Tissue and host: hypodermis and fat cells of *Simocephalus vetulus* (O. F. Müller, 1776).

Etymology: species name alluding to the location in host's

hypodermal tissues.

Type habitat: former carp pond (a marsh) at 49°59'13.136"N, 13°59'52.638"E, Nižbor, Central Bohemia, Czech Republic.

Type material: Syntypes: Burri-Ink stained slide in J. Weiser's collection of microsporidia slides, in care of the Laboratory of Electron Microscopy, Faculty of Science, Charles University in Prague, Czech Republic. Illustration Fig. 2h, e, this paper.

4. *B. nana*, sp. n.

Diagnosis: Spores egg-shaped with one pole more acute, occurring singly, 4.0 (3.8–4.3) × 2.5 (2.4–2.6) µm in size (fresh), small posterior vacuole forms a laterally shifted slit. Meronts and sporonts with large nuclei, sporophorous vesicle appearing on sporonts in the form of blisters. Ultrastructure of the spore: single large nucleus, bipartite polaroplast and 9 isofilar polar filament coils in a single row. Endospore thick, uniform (100 nm), exospore thin (20 nm) and uniform. Spores occur in individual sporophorous vesicles, loosely ensheathing the spore as a sachet consisting of irregular chambers, consisting of material of medium density, reinforced on the outer face of the sporophorous vesicle by electron dense thickenings connected to the exospore by transverse, irregularly spaced connections.

Tissue and host: fat cells of *Daphnia longispina/galeata* complex.

Etymology: name alluding to the size of spores when compared to the type species of the genus *Berwaldia*.

Type habitat: carp pond LUKES at 49°59'10.851"N, 14°0'5.461"E, Nižbor, Central Bohemia, Czech Republic.

Type material: Syntypes: Burri Ink stained slide in J. Weiser's collection of microsporidia slides, in care of the Laboratory of Electron Microscopy, Faculty of Science, Charles University in Prague, Czech Republic. Illustration Fig. 3a, f, this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejop.2017.07.005>.

References

- Benzie, J., 2005. The genus *Daphnia* (including *Daphniopsis*) (Anomopoda: Daphniidae). In: Dumont, H. (Ed.), Guides to the Identification of Microinvertebrates of the Continental Waters of the World 21. Backhuys Publ., Kenobi Productions, Ghent & Backhuys Publishers, Leiden, 376 pp.
- Becnel, J.J., Andreadis, T.G., 2014. Microsporidia in insects. In: Weiss, L.M., Becnel, J.J. (Eds.), Microsporidia, Pathogens of Opportunity. Wiley Blackwell, USA, pp. 521–570.
- Becnel, J.J., Takvorian, P.M., Cali, A., 2014. Checklist of available generic names for Microsporidia with type species and type hosts. In: Weiss, L.M., Becnel, J.J. (Eds.), Microsporidia, Pathogens of Opportunity. Wiley Blackwell, USA, pp. 671–686.
- Brambilla, D.J., 1983. Microsporidiosis in a *Daphnia pulex* population. *Hydrobiologia* 99, 175–188.
- Corsaro, D., Walochnik, J., Venditti, D., Steinmann, J., Müller, K.-D., Michel, R., 2014. Microsporidia-like parasites of amoebae belong to the early fungal lineage Rozellomycota. *Parasitol. Res.* 113, 1909–1918, <http://dx.doi.org/10.1007/s00436-014-3838-4>.
- Ebert, D., 2005. Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia*. National Library of Medicine (US), National Center for Biotechnology Information, Bethesda (MD) https://www.ncbi.nlm.nih.gov/corehtml/pmc/homepages/bookshelf/pdf/daph_screenA4.pdf.
- Haag, K.L., James, T.Y., Pombert, J.-F., Larsson, R., Schaer, T.M.M., Refardt, D., Ebert, D., 2014. Evolution of a morphological novelty occurred before genome compaction in a lineage of extreme parasites. *PNAS* 111, 15480–15485, <http://dx.doi.org/10.1073/pnas.1410442111>.
- James, T.Y., Pelin, A., Bonen, L., Ahrendt, S., Sain, D., Corradi, N., Stajich, J.E., 2013. Shared signatures of parasitism and phylogenomics unite cryptomycota and microsporidia. *Curr. Biol.* 23, 1548–1553.
- Jírovec, O., 1947. Zoologická technika. Česká Akademie Věd a Umění, 320 pp.
- Karpov, S.A., Mamkaeva, M.A., Aleoshin, V.V., Nassonova, E., Lilje, O., Gleason, F.H., 2014. Morphology, phylogeny, and ecology of the aphelids (Aphelidea, Opisthokonta) and proposal for the new superphylum Opisthosporidia. *Front. Microbiol.* 5, art. 112.
- Keeling, P.J., 2014. Phylogenetic place of Microsporidia in the tree of Eukaryotes. In: Weiss, L.M., Becnel, J.J. (Eds.), Microsporidia, Pathogens of Opportunity. Wiley Blackwell, USA, pp. 195–202.
- Larsson, R., 1981. A new microsporidium *Berwaldia singularis* gen. et sp. nov. from *Daphnia pulex* and a survey of microsporidia described from Cladocera. *Parasitology* 83, 325–342.

- Larsson, J.I.R., Voronin, V.N., 2000. Light and electron microscopic study of *Agglomerata volgensae* n. sp. (Microsporota: Dubosqiidae), a new microsporidian parasite of *Daphnia magna* (Crustacea: Daphniidae). *Eur. J. Protistol.* 36, 89–99.
- Lukyantsev, V.V., Simakova, A.V., 2014. Infestation of lower crustaceans (Copepoda, Cladocera) with microsporidians (Microsporidia) in Western Siberia (in Russian with English summary). *Parazitologiya* 48, 358–372.
- Martin, J.W., 1992. Branchiopoda. In: Harrison, F.W., Humes, A.G. (Eds.), *Microscopic Anatomy of Invertebrates*, vol. 9. Wiley-Liss, Crustacea, pp. 25–224.
- Refardt, D., Canning, E.U., Mathis, A., Cheney, S.A., Lafranchi-Tristem, N.J., Ebert, D., 2002. Small subunit ribosomal DNA phylogeny of microsporidia that infect *Daphnia* (Crustacea: Cladocera). *Parasitology* 124, 381–389.
- Refardt, D., Decaestecker, E., Johnson, P.T., Vávra, J., 2008. Morphology, molecular phylogeny, and ecology of *Binucleata daphniae*, n. g., n. sp. (Fungi: Microsporidia), a parasite of *Daphnia magna* Straus, 1820 (Crustacea: Branchiopoda). *J. Eukaryot. Microbiol.* 55, 393–408.
- Snowden, K.F., 2014. Microsporidia in higher vertebrates. In: Weiss, L.M., Becnel, J.J. (Eds.), *Microsporidia, Pathogens of Opportunity*. Wiley Blackwell, USA, pp. 469–492.
- Stentiford, G.D., Dunn, A.M., 2014. Microsporidia in aquatic invertebrates. In: Weiss, L.M., Becnel, J.J. (Eds.), *Microsporidia, Pathogens of Opportunity*. Wiley Blackwell, USA, pp. 579–604.
- Taylor, D.J., Hebert, P.D.N., Colbourne, J.K., 1996. Phylogenetics and evolution of the *Daphnia longispina* group (Crustacea) based on 12S rDNA sequence and allozyme variation. *Mol. Phylogenet. Evol.* 5, 495–510.
- Tokishita, S., Shibuya, H., Kobayashi, T., Sakamoto, M., Ha, J.-Y., Yokobori, S., Yamagata, H., Hanazato, T., 2017. Diversification of mitochondrial genome of *Daphnia galeata* (Cladocera, Crustacea): comparison with phylogenetic consideration of the complete sequences of clones isolated from five lakes in Japan. *Gene* 611, 38–46.
- Vávra, J., 1964a. Recording microsporidian spores. *J. Insect Pathol.* 6, 258–260.
- Vávra, J., 1964b. A failure to produce an artificial infection in cladoceran Microsporidia. *J. Protozool.* 11 (Suppl. 110).
- Vávra, J., Hyliš, M., Fiala, I., Nebesářová, J., 2016a. *Globulispora mitoportans* n. g., n. sp. (Opisthosporidia: Microsporidia), a microsporidian parasite of daphnids with unusual spore organization and prominent mitosome-like vesicles. *J. Invertebr. Pathol.* 135, 43–52.
- Vávra, J., Hyliš, M., Fiala, I., Refardt, D., Larsson, J.I.R., 2016b. Microsporidia in a woodland pool I. *Lanatospora costata* sp. n. (Opisthosporidia, Microsporidia), parasite of *Megacyclops viridis* (Crustacea, Copepoda): fine structure and molecular phylogeny. *Acta Protozool.* 55, 269–280.
- Vávra, J., Larsson, J.I.R., 1994. *Berwaldia schaefernai* (Járovec, 1937) comb. n. (Protozoa, Microsporida), fine structure, life cycle and relationship to *Berwaldia singularis* Larsson, 1981. *Eur. J. Protistol.* 30, 45–54.
- Vávra, J., Lukeš, J., 2013. Microsporidia and ‘the art of living together’. In: Rollinson, D. (Ed.), *Advances in Parasitology*, vol. 82. Academic Press, pp. 253–320.
- Vávra, J., Maddox, J.V., 1976. Methods in microsporidiology. In: Bulla Jr., L.A., Cheng, T.C. (Eds.), *Comparative Pathobiology*, vol. 1, *Biology of Microsporidia*. Plenum Press, N.Y., London, pp. 283–319.
- Vávra, J., Pražáková, M., 1983. The influence of a microsporidian infection on the behavior of the infected host. *J. Protozool.* 30, 32A.
- Vossbrinck, C.R., Andreadis, T.G., Vávra, J., Becnel, J.J., 2004. Molecular phylogeny and evolution of mosquito parasitic Microsporidia (Microsporidia: Amblyosporidae). *J. Eukaryot. Microbiol.* 51, 88–95.
- Weigl, S., Körner, H., Petrusk, A., Seda, J., Wolinska, J., 2012. Natural distribution and co-infection patterns of microsporidia parasites in the *Daphnia longispina* complex. *Parasitology* 139, 870–880.
- Weiss, L.M., Vossbrinck, C.R., 1999. Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the Microsporidia. In: Wittner, M., Weiss, L.M. (Eds.), *The Microsporidia and Microsporidiosis*. ASM Press, Washington, D.C, pp. 129–171.
- Wolinska, J., Giessler, S., Körner, H., 2009. Molecular identification and hidden diversity of novel *Daphnia* parasites from European lakes. *Appl. Environ. Microbiol.* 75, 7051–7059.