



# Molecular and structural assessment of microsporidia infecting daphnids: The “obtusa-like” microsporidia, a branch of the monophyletic Agglomeratidae clade, with the establishment of a new genus *Conglomerata*



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## ABSTRACT

Microsporidia (Opisthosporidia, Microsporidia) are frequent parasites of planktonic cladocerans, including *Daphnia* (Crustacea, Branchiopoda). Analysis of available molecular data (ITS region and partial ssu and lsu rDNA) of these parasites indicates that many microsporidia infecting daphnids have a common ancestor and represent a large clade, which splits during evolution into a number of well supported subclades. These subclades are cytologically different but may be most conveniently characterised by their specific ITS barcode. We have analysed one of these subclades and we describe a new microsporidian genus and species combination, and assemble a large group of structurally indistinguishable microsporidian parasites that infect adipose cells of their hosts and form pyriform spores of a certain type (“obtuse spores”). Obtuse spores are non-infectious by feeding to their crustacean hosts and it is plausible that microsporidia forming them actually are parasites of insects with aquatic larval stages, with an obligate two-host life cycle, analogous to the *Amblyospora* life cycle involving copepods and mosquitoes.

## 1. Introduction

Nearly any naturalist working with planktonic Crustacea has met specimens of the cladoceran genus *Daphnia* (Crustacea, Branchiopoda) that appear white-opaque in incident light. These “white daphnids” are usually infected with microsporidia (Opisthosporidia, Microsporidia). Such infections are common in many *Daphnia* habitats, sometimes reaching epizootic levels, and they involve either the hypodermis or the internal organs (“body cavity”; see Weigl et al., 2012). Only those infecting internal organs (adipose tissue cells) are considered here. When squashed, infected *Daphnia* release clouds of microsporidian spores of characteristic size and shape. Some of these spores are ellipsoid or ovoid in shape. The taxonomy of the microsporidia producing such spores was described earlier (Vávra et al., 2017). However, microsporidia forming spores of other shapes infect cladocerans as well, and they are even more common.

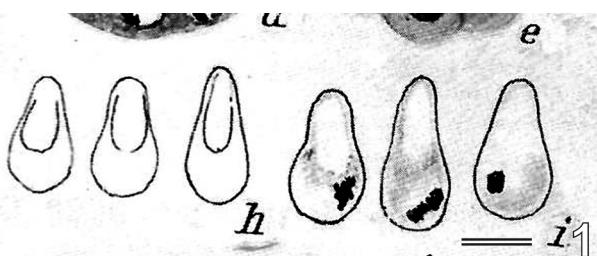
In this paper we strive to define a group of *Daphnia* infecting microsporidians forming spores of “obtuse” shape. Microsporidia

producing obtuse spores were described as early as the 1880s (Moniez, 1887). Jírovec (1936) studied and made drawings of microsporidia producing such spores. Fig. 1 shows his interpretation of the obtuse form of spores as pyriform (see more information in the Material and Methods section). Jírovec claimed that microsporidia producing obtuse spores are the most common parasites of the “body cavity” of daphnids. Using light microscopy he studied their development in three different *Daphnia* species and proposed that they represent a single species, which he identified as *Plistophora obtusa* (Moniez, 1887). In 1942, Jírovec recognized the adipose tissue as the infection site for *P. obtusa* (Jírovec, 1943), and Weiser (1945) depicted various spore shapes of *P. obtusa* spores in *Daphnia pulex* Leydig, 1860 and *Daphnia magna* Straus, 1820, collected in different carp ponds. All these observations indicated that a species, or a group of microsporidian species, exists that are characterized by the host taxon (*Daphnia* spp.), the tissue specificity (adipose cells) and by the shape of spores produced. Surprisingly, very limited effort was made to properly characterize these microsporidia, although they have been the subject of several technically advanced

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**Fig. 1.** Otto Jírovec's drawing of "obtuse spores" of microsporidia infecting daphnids (from Jírovec, 1936). Bar = 2  $\mu$ m.

papers dealing with microsporidian distribution, molecular identification, diversity and infection mode (Wolinska et al., 2009, Weigl et al., 2012, González-Tortuero et al., 2016). In the present paper we use the acronym ACPS (adipose cells, pyriform spores) for such microsporidia. These are characterized using ultrastructural and phylogeny data.

## 2. Material and methods

### 2.1. Hosts and sampling in different habitats

Infected *D. pulex* were collected in a semi-permanent forest marsh near Běleč, Central Bohemia region, Czech Republic (50.054N, 14.019E; habitat H-1). Infected *D. magna* were collected in a pond in Warsaw, Poland ("Park na Książęcem", 52.2304N, 21.0285E; H-2). Infected members of the *Daphnia longispina* complex, either *D. galeata*, *D. longispina* or their interspecific hybrids (see Petrušek et al., 2008a, b), were collected at the following sites: water reservoir Žlutice (50.087N, 13.127E; code ZLU, H-3); carp pond Lukeš (49.9864N, 14.0015E; code LU, H-4); carp pond Hořejší near Nový Jáchymov (49.978N, 13.913E; code JACH, H-5); pond Šeberák in Prague-Šeberov (50.010N; 14.498E; code SE, H-6); ponds Hadí and Roudenský near Blatná (49.414N, 13.828E; code HA, H-7; and 49.426N, 13.863E; RO, H-8). Habitats H-1 and H-3 to H-8 are situated in the Czech Republic and some of them were sampled repeatedly during the years 2012–2017.

### 2.2. Host selection and spore shapes

*Daphnia* appearing white in incident light, infected in their "body cavity" (but not in the hypodermis or the gut) were selected for examination and the spore shape was used for distinguishing ACPS microsporidia from other microsporidia infecting these cladocerans. The ACPS microsporidia spores were short pyriform, their side walls tapering towards the spore apex were straight or slightly concave (Fig. 1). Ellipsoid, oval and pyriform spores with slightly convex side walls belonged to other microsporidian taxa not studied here.

### 2.3. Microscopy

Methods described in Refardt et al. (2008), Vávra (1964) and Vávra et al. (2016a, b), were used for light microscopy (LM) and transmission electron microscopy (TEM).

### 2.4. DNA isolation, sequencing and phylogeny

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

### 2.5. Infection experiments

In three series of experiments, 10–20 adult females of *D. pulex* from the habitat H-1 were fed *ad libitum* twice a week with suspension of

*Monoraphidium* algae mixed with spores obtained from squashed infected daphnids from the same population. After one month, the experimental animals were checked microscopically for potential development of infection.

## 3. Results

Our observations involved ACPS microsporidia infecting respectively *D. pulex*, *D. magna* and the *D. longispina* complex, i.e. the *Daphnia* spp. recorded as hosts of *Plistophora obtusa* by Jírovec (1936).

### 3.1. *Daphnia pulex* Leydig, 1860 ACPS microsporidium "DHM"

#### 3.1.1. Occurrence, pathology and light microscopy

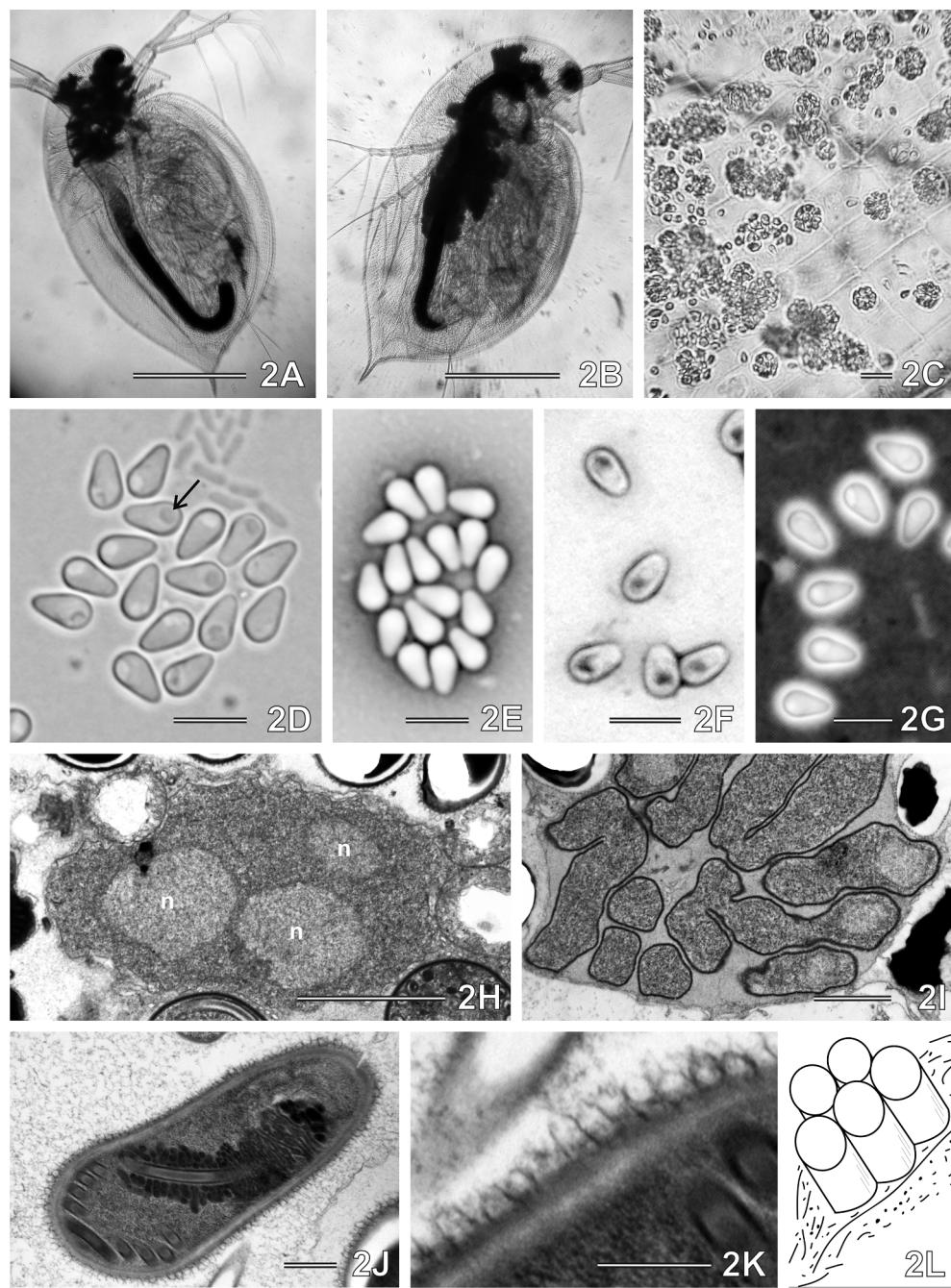
The parasite was found in a semi-permanent forest marsh (habitat H-1), which hosted a dense population of *D. pulex* in spring months (beginning of May to end of June) of several consecutive years (2012–2017). The prevalence of infection fluctuated widely in different years reaching up to 18% in one year. The parasite first invaded the adipose cells around the hepatopancreas and the anterior midgut of the host (Fig. 2A), later spreading to adipose cells around the gut (Fig. 2B). The acronym DHM ("Daphnia Head Microsporidium"), used for the organism in this publication, is derived from the initial site of infection in the head region of *Daphnia*. In heavily infected hosts the spores were released into the body cavity and some spores were seen circulating in the haemolymph and occurred inside haemocytes. This explains the older observations of microsporidia infecting the body cavity ("Körperhöhle") of daphnids (Jírovec, 1936). Squashing of infected animals released round packets of spores, around 10  $\mu$ m in size (Fig. 2C). Each packet contained approximately 10–26 spores (average 15 spores/packet, packets n = 20). Spores were short pyriform (wedge shaped), with the length/width ratio of approximately 1.6–1.8, measuring 4.3 (4.2–4.5)  $\times$  2.4  $\mu$ m (n = 30). A relatively large (approximately 1.5  $\mu$ m), eccentrically located vacuole was situated at the posterior pole of the spore (Fig. 2D). The spore shape was relatively well preserved in dry smears (Fig. 2E). Each spore contained a single nucleus (Fig. 2F). A thick layer of mucous material surrounded fresh spores suspended in water (Fig. 2G). The spore yield in a massively infected *Daphnia* could approximate 425–500  $\times$  10<sup>3</sup> spores.

The spores were non-infectious for the original host. None of the experimental animals fed spores developed patent infections, although the spores germinated in the digestive tract of *D. pulex* and sporoplasms were found by TEM in different tissues of the host. Such sporoplasms, however, quickly degenerated (not shown).

#### 3.1.2. Ultrastructure

The earliest stages encountered were late meronts with several single nuclei (Fig. 2H). Sporonts were observed, dividing by finger-like budding into uninucleate sporoblasts inside a thin-walled sporophorous vesicle (SPOV; Fig. 2I). Immature spores were elongated cells in which the polar tube and the nascent polaroplast were visible (Fig. 2J). In well-fixed immature spores, the ornamentation of the future exospore layer appeared as densely packed tubular outgrowths with electron dense wall material. The tubules were 60–90 nm long and 60–70 nm wide (Fig. 2K). These tubules (in fact hollow cylinders as drawn in Fig. 2L) formed the characteristic ornamentation of the spore surface. Spore formation was accompanied by the occurrence, inside the SPOV, of several dense formations with periodic structure, in which lines of dense material of 11 nm were interposed with electronlucent lines of the same thickness. Large bodies of the material with such structure were found in SPOVs containing mature spores (Fig. 3A, B).

The spore had a single uniform endospore ~100 nm thick, an exospore composed of three conspicuous layers: an inner dense layer of 13 nm, an intermediate less-dense layer of 8 nm and a dense outer layer of 6–9 nm (Fig. 3C, E). Tubules, 60–90 nm long and 70 nm in diameter, densely coated the outer layer, appearing in sections as irregular fibres



**Fig. 2.** A–L. *Conglomerata obtusa*, n. comb., infecting *Daphnia pulex* in the type habitat H-1. A. Early infection. B. Advanced infection of the host. Bars = 500 µm. C. Spore packets released from the host upon squashing. Bar = 10 µm. D. Group of fresh spores released from the packet (as shown in C). The conspicuous posterior vacuole is indicated by the arrow. Bar = 5 µm. E. The obtuse form of spores is well preserved on dry smears (Burri Ink). Bar = 5 µm. F. Spores have a single nucleus (Robinow-Piekarski method, Giemsa stain). Bar = 5 µm. G. Spores are surrounded with a mucous layer, visualised by addition of India Ink. Bar = 5 µm. H. Multinucleate meront with several single nuclei (n). Bar = 2 µm. I. Sporont dividing in a finger-like fashion into individual sporoblasts. Bar = 2 µm. J. Late sporoblast or immature spore with primordia of the extrusion apparatus. Bar = 500 nm. K. The exospore is ornamented by elements of short cylinders (interpretation of this structure shown in L). Bar = 250 nm. L. Schematic drawing of the ornamentation of spores of *Conglomerata*.

stretching out from the exospore. Tangential sections of the spore surface showed that the tubules on the spore surface actually formed a mesh of circular units, wrapping the spore as a mail armour (Fig. 3E, F, G).

The polar tube had a characteristic arrangement. A straight section descended from the polar cap-polar sac complex to the half the spore length and then formed one row of coils situated at an angle of 45° to the long axis of the spore. Thus, in the longitudinally sectioned spore, one row of coils was situated closer to the spore apex, the other row of coils closer to the posterior of the spore (Fig. 3C). In cross sections of individual coils, there was usually one, either complete or incomplete coil, represented by a tube with a dark centre, an electron dense material descending into some length of the coil from the polar cap-polar sac, followed by one or two coils of a simple, structureless tube and 4–5 coils of a tube with a double wall. The total number of coils was 7 or 8. All coils had the same, or nearly the same diameter, although

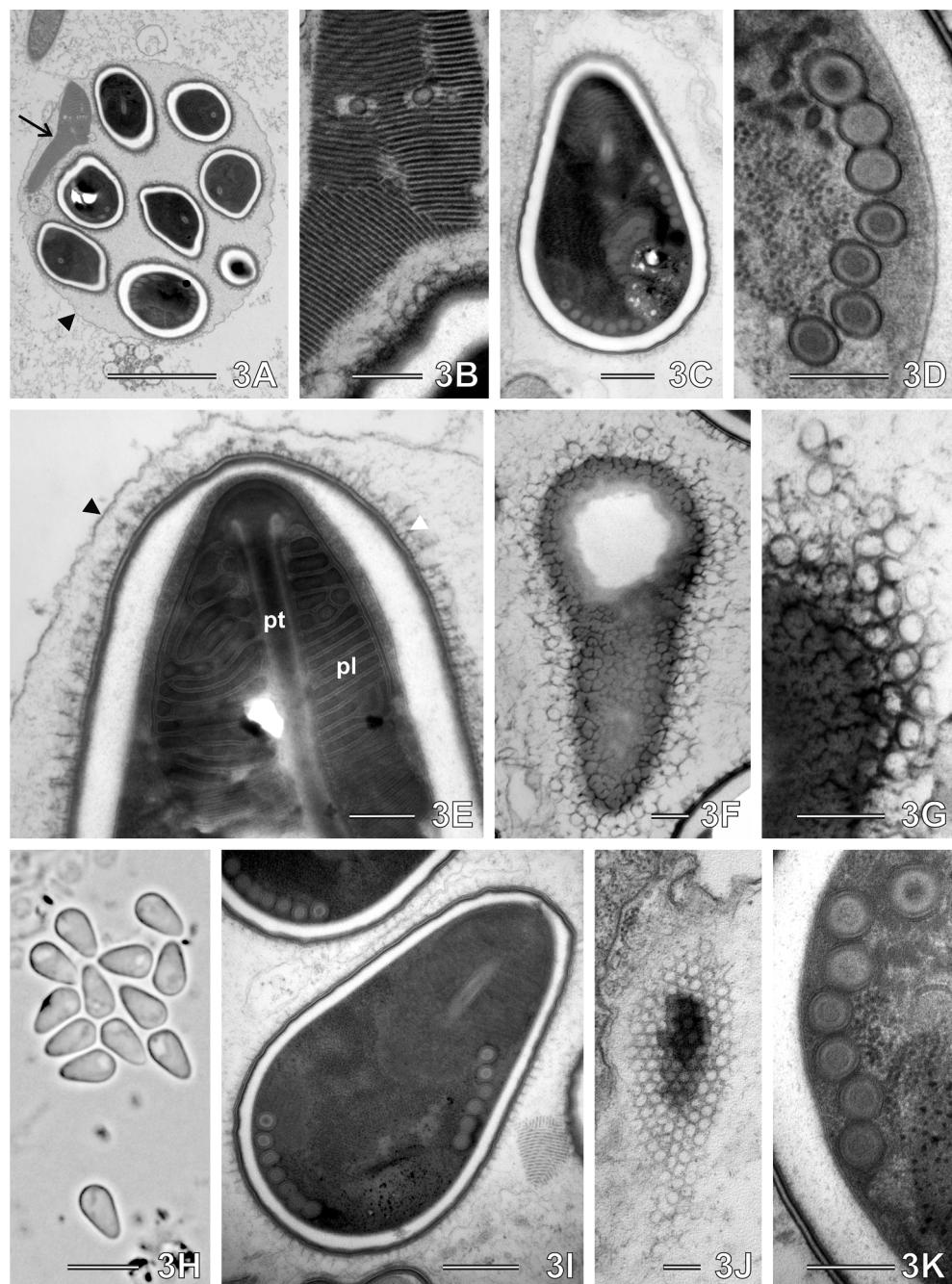
sometimes the anterior coil seemed imperceptibly thicker (Fig. 3D). The polaroplast consisted of three parts, from the posterior portion: large, flat lamellae, very tightly compressed lamellae, and irregular tubules and vesicles of dark material (Fig. 3E).

### 3.1.3. Molecular data

Several identical sequences of the partial ssu rDNA, ITS and partial lsu rDNA, 1453 nucleotides in length, were obtained from *D. pulex* sampled in the habitat H-1, and were deposited in GenBank (Acc. No. MH645034).

### 3.2. *Daphnia magna* Straus, 1820, ACPS microsporidium, "DM 348"

Adipose cells surrounding the hepatopancreas part of the digestive tube of the host in the habitat H-2 were infected. Spores were pyriform, wedge shaped, 4.0 (3.8–4.2) × 2.4 (2.3–2.4) µm in size (Fig. 3H) and



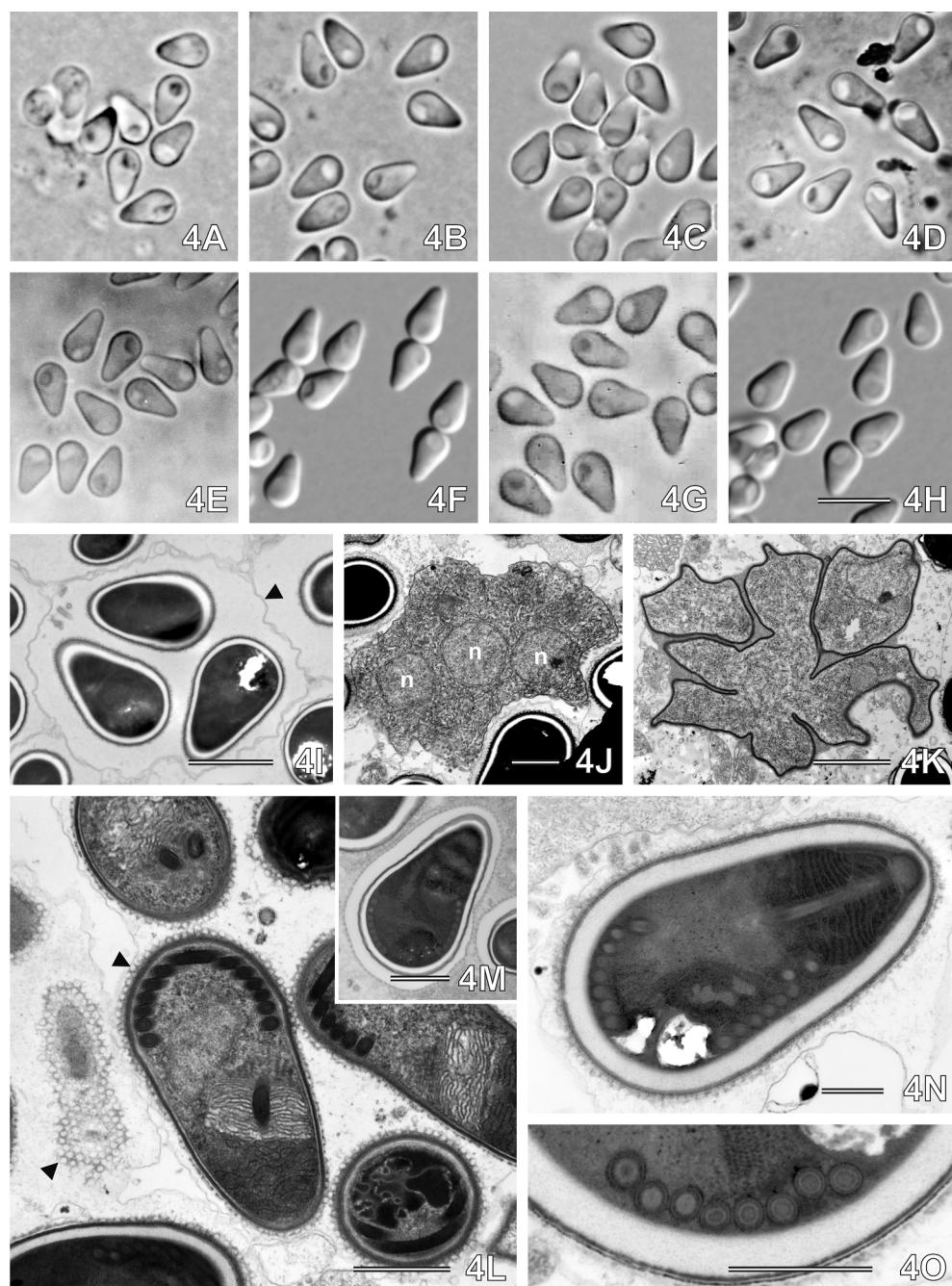
**Fig. 3.** A–G. *Conglomerata obtusa*, n. comb., infecting *Daphnia pulex* in the type habitat H-1. H–K. Fine structure of a *Conglomerata obtusa* isolate from *D. magna* (DM 348, habitat H-2). A. Spore packet showing the presence of the sporophorous vesicle (SPOV) membrane (arrowhead). Metabolic granules appear in the SPOV during sporogenesis (arrow). Bar = 2 µm. B. Detail of the metabolic granule from A, showing its periodic substructure. Bar = 250 nm. C. Spore of *C. obtusa*, total view. Bar = 500 nm. D. The typical organization of the polar filament coils. Bar = 200 nm. E. Section through the anterior part of the *C. obtusa* spore showing the SPOV wall (black arrowhead), material decorating the exospore (white arrowhead), polaroplast lamellae (pl) and the ascending part of the polar tube (pt). Bar = 200 nm. F and G. Tangential sections through the spore of *C. obtusa* shows the circular units decorating the exospore. This is the distinguishing character of microsporidia of the *Conglomerata* genus. Bars = 200 nm. H–K. Fine structure of a *Conglomerata obtusa* isolate from *D. magna* (DM 348, habitat H-2). H. Spores isolated from a spore packet. Bar = 5 µm. I. Total view of the spore, demonstrating its similarity to the *C. obtusa* spore from *D. pulex* (C). Bar = 500 nm. J and K. The exospore decoration (J) and the polar tube construction (K) also conform to those of *C. obtusa* from *D. pulex* (E, F and G). Bars = 200 nm.

their fine structure was indistinguishable from spores of the DHM parasite of *D. pulex* (Fig. 3I). The polar tube had identical construction (Fig. 3K) and the exospore was covered by a similar “mail armour-like” structure (Fig. 3J). The 1329 nucleotides long partial ssu rDNA and ITS sequence from *D. magna* collected at habitat H-2 has been deposited in GenBank (Acc. No. MH645035)

### 3.3. The ACPS microsporidia of the *D. longispina* complex

ACPS microsporidia infecting members of the *D. longispina* complex (*D. longispina*, *D. galeata* and presumably also their interspecific hybrids) were frequently found during summer months in all visited carp ponds and in the reservoir Žlutice (habitats H-3 to H-8). Spores in individual daphnids differed in shape, some being more pointed, some being more obtuse, but their distinguishing character were more pronounced laterally concave side walls. This concavity was more or less

visible in individual hosts, in some hosts the spores were skittle-like, in others these were almost wedge-like (Fig. 4A–H). Spores obtained from fourteen infected *Daphnia* from the reservoir Žlutice (H-3) were examined under LM and were measured, showing large size variability of spores produced in individual hosts. In some hosts the size of spores was  $3.9 \times 2.5 \mu\text{m}$  ( $n = 30$ ), in others it was  $4.2 \times 2.5 \mu\text{m}$  ( $n = 90$ ). Similar variability was found in spores produced in the hosts of the *D. longispina* complex in carp ponds (H-4 to H-8), the largest spores measuring  $4.4 \times 2.6 \mu\text{m}$ . Thirteen ACPS specimens from this host complex (five from Žlutice, eight from three different carp ponds) were examined by TEM. The fine structure of the ACPS microsporidia from *D. gr. longispina* was similar to the DHM microsporidium of *D. pulex* (Fig. 4I–O) and the surface material, characteristic for this group of microsporidia, occasionally detached from the exospore (Fig. 4M). Despite some slight differences in shape and size of the spores in individual animals (observed under LM), their fine structure was similar, and we were not able



**Fig. 4.** A–O. *C. obtusa* in the *D. longispina* complex hosts, isolated from habitats H-3 to H-8. A–H. A gallery of obtuse spores from various specimens of the *D. longispina* complex, demonstrating spore variability in shape, even in specimens from the same habitat. A: habitat H-3, (ZLU); C: habitat H-4 (LU); D: the habitat H-6 (SE); E: habitat H-7 (HA); F: habitat H-4 (LU); G: habitat H-8 (RO); H: habitat H-4 (LU). Bars = 5 µm. I. The obtuse spores in the *D. longispina* complex develop inside the sporophorous vesicle (arrowhead) as those of *C. obtusa* in *D. pulex*. Bar = 2 µm. J. Multinucleate meront (n indicates single nuclei) of the *C. obtusa* isolate from *D. longispina* in the water reservoir ZLU. Bar = 1 µm. K. Sporont dividing into sporoblasts; *D. longispina* complex, pond JACH (habitat H-5). Bar = 2 µm. L. Several young spores of *C. obtusa* isolate from the *D. longispina* complex, pond JACH (habitat H-5), showing the exospore decoration on spores and on a spore sectioned tangentially (arrowheads). Bar = 1 µm. M. The exospore tubular layer detaches from the spore surface in some cases. *C. obtusa* isolate from the *D. longispina* complex, pond JACH (habitat H-5). Bar = 1 µm. N and O. *C. obtusa* isolate from the *D. longispina* complex, pond JACH (habitat H-5). Both the spore structure (N) and the construction of the polar tube (O) conform to those of *C. obtusa* from the type host, *D. pulex*. Bars = 500 nm.

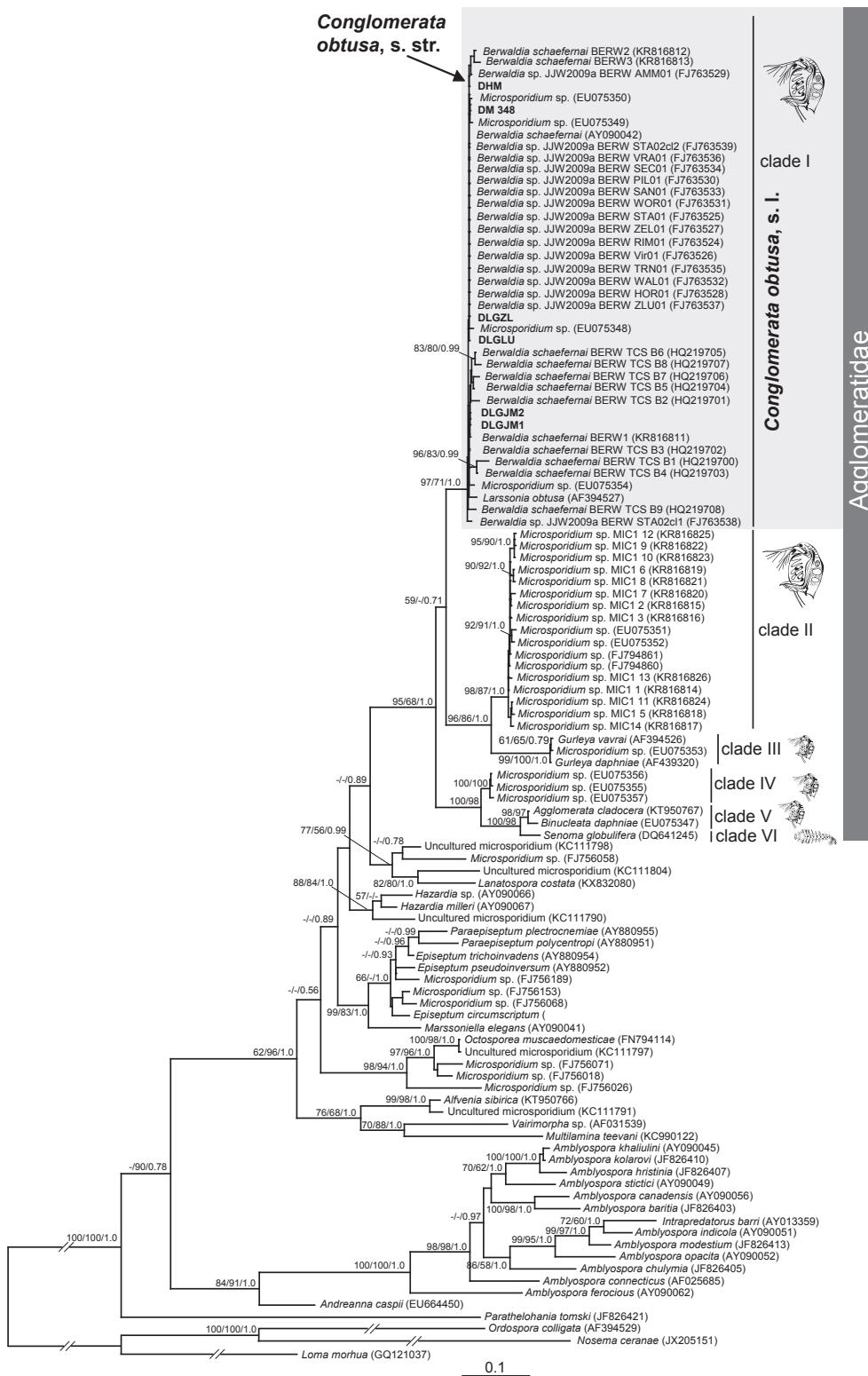
to attribute any specific spore shape to a specific structural character. Several ACPS microsporidia from the reservoir Žlutice, as well as from carp ponds were sequenced and their partial ssu rDNA, ITS and partial lsu rDNA sequences were deposited in GenBank (Acc. Nos. MH645036–MH645039) under the respective acronyms DLGZL (from a *Daphnia* collected at habitat H-3), DLGJM 1 and DLGJM2 (from two host specimens collected at habitat H-5), and DLGLU (from *Daphnia* collected at habitat H-4).

#### 3.4. Molecular phylogeny of the ACPS microsporidia

All studied microsporidians from *D. pulex*, *D. magna* and the *D. longispina* complex clustered with a large group of microsporidia, wrongly labelled in GenBank as *Berwaldia* spp. (Clade I on Fig. 5), e.g. *Berwaldia schaefernai* (GenBank Acc. No. AY090042), yet belonging to

an undescribed microsporidian from *D. galeata* (Vávra et al., 2017). Very short branches indicated high sequence similarity (or identity in many cases) of sequences among representatives of Clade I. The stability of this clade is supported by high ML bootstrap support (97%) and maximum posterior probability of BI. Bootstrap support in MP analysis was moderate (71%). Sequence similarities within sequences of the clade I typically ranged from 98% to 100%, whereas sequence similarities to representatives of the closely related Clades II and III ranged from 85% to 90%.

To assess the phylogenetic relationships within Clade I in more detail, a detailed sequence comparison was performed on available sequences in which the internal transcribed spacer (ITS) region was represented. ITS is considered by some authors as universal barcode for fungi (Schoch et al., 2012). As microsporidia are related to fungi (James et al., 2013), the same “barcoding specificity” might be applicable to



**Fig. 5.** Maximum likelihood tree based on microsporidian ITS and partial ssu and lsu rDNA sequences. Newly sequenced taxa are in bold. Maximum likelihood/bootstrap support values and Bayesian posterior probabilities are shown at nodes. Dashes indicate bootstrap values < 50 or absence of the respective node in the maximum parsimony or Bayesian tree. NCBI accession numbers are in parentheses. Scale bar is given under the tree. Interrupted branches are shortened three times. Microsporidian sequences from *Conglomerata obtusa*, s. l. are highlighted in grey – names of the sequences designated in the GenBank as *Berwaldia* and *Larssonia* are incorrect (Vávra et al., 2017). Clades I–VI represent well-defined microsporidian phylogenetic groups of the family clade Agglomeratidae. Clades I–V belong to microsporidia infecting *Daphnia* spp., *Senoma globulifera* in clade VI is a parasite of *Anopheles* larvae.

them. Thirty-six ITS containing sequences of microsporidia belonging to the Agglomeratidae clade, including 19 members of Clade I, were assembled and examined (Fig. 6). The alignment of the ITS regions, including a part of the LSU gene, revealed that all microsporidia of Clade I, irrespective of the host involved, shared a high degree of similarity. Many sequences were identical, while several sequences only differed in one or two nucleotides. The analysis also showed that a common

17 bp motif GTTTAATATGTATGTAT is characteristic for microsporidia forming Clade I and could thus be used as their identification tag. At the same time the analysis demonstrated that the respective subclades of the Agglomeratidae clade are distinct in their ITS respective region sequences (see Fig. 6 and the discussion below).

Specific motif in ITS region										
	1	10	20	30	40	50	60	70		
<b>Conglomerata obtusa</b>	CCGCAGGATCAATAAGCAGAGCGT	TTCGGGTTTAAT	ATGTATGT	AT						
ACPS microsporidium (DM 348)	CCGCAGGATCAATAAGCAGAGCGT	TTCGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
ACPS microsporidium (DHM)	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
ACPS microsporidium (DLGMJ1)	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
ACPS microsporidium (DLGMJ2)	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>Microsporidium</i> sp. EU075350	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>Microsporidium</i> sp. EU075349	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW HQ219706	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW2 KR816812	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>Microsporidium</i> sp. EU075348	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW1 KR816811	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW HQ219702	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW3 KR816813	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW HQ219705	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW HQ219707	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW HQ219700	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW HQ219708	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>B. schaefermai</i> BERW HQ219701	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>Larssonia obtusa</i> AF394527	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<i>Microsporidium</i> sp. EU075354	CCGCAGGATCAATAAGCAGAGCGT	TTGGGGTTTAAT	ATGTATGT	ATACATTACGTTCGA						
<b>MIC1</b>	<b>Microsporidium</b> sp. MIC1 KR816814	CCGCAGGATCAATAAAAGAGATCAT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. FJ794861	CCGCAGGATCAATAAAAGAGATCAT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. FJ794860	CCGCAGGATCAATAAAAGAGATCAT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. MIC1 KR816819	CCGCAGGATCAATAAAAGAGATCAT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. MIC1 KR816815	CCGCAGGATCAATAAAAGAGATCAT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. MIC1 KR816818	CCGCAGGATCAATAAAAGAGATCAT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. EU075351	CCGCAGGATCAATAAAAGAGATCAT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. MIC1 KR816820	CCGCAGGATCAATAAAAGAGATCAT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. MIC1 KR816816	CCGCAGGATCAATAAAAGAGATCAT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. EU075353	CAGTAGGATCAATAACAGAGCTT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Gurleya daphniae</i> AF439320	CAGTAGGATCAATAACAGAGCTT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Gurleya vavrai</i> AF394526	CAGTAGGATCAATAACAGAGCTT	TGGGTTTTAT	ATATGIGGGTA	ACACATTGCGTTCGA					
	<i>Microsporidium</i> sp. EU075357	CCGCAGGATCAATAATTAATTA	TTGGGGTTTTAT	ATCTATATAATTATAGTTT	ATACATICATATTTCGA					
	<i>Microsporidium</i> sp. EU075356	CCGCAGGATCAATAATTAATTA	TTGGGGTTTTAT	ATCTATATAATTATAGTTT	ATACATICATATTTCGA					
	<i>Microsporidium</i> sp. EU075355	CCGCAGGATCAATAATTAATTA	TTGGGGTTTTAT	ATCTATATAATTATAGTTT	ATACATICATATTTCGA					
	<i>Binucleate daphniae</i> EU075347	CCGCAGGATCAATAAGCTAACGTT	TTGGGGTTTTAT	ATATTTAATAGTTT	ATACATICATATTTCGA					
	<i>Senoma globulifera</i> DQ641245	CCGCAGGATCAATAAGCTAACGTT	TTGGGGTTTTAT	ATTTATTAATAGTTT	ATACATICATATTTCGA					

Fig. 6. Partial alignment of representatives of all clades of the family clade Agglomeratidae including specific motif in the ITS region.

#### 4. Discussion

##### 4.1. The DHM microsporidium: not Plistophora, not Microsporidium, but an Agglomerata-like microsporidium

Although Jírovec (1936) studied his *P. obtusa* isolate in much detail, he did not recognize that there is a SPOV membrane enveloping the dividing multinucleate sporont or that a packet of spores issued from the sporont. The SPOV membrane is very thin, not persistent, and it is not discernible using a light microscope. The apparent absence of this membrane was the reason that Sprague (1977) renamed the species *Microsporidium obtusum* Moniez, 1887.

Our EM study of microsporidia from three different *Daphnia* host clades (*D. pulex*, *D. magna*, *D. longispina* complex) representing the key widespread evolutionary lineages within the genus (Adamowicz et al., 2009), showed that all these organisms have structural characters of the genus *Agglomerata* Larsson and Yan, 1988. It must be explained why the DHM and related ACPS microsporidia cannot be ranked either into the genus *Agglomerata* or into the genus *Larssonia* that was proposed as replacement for Moniez's *Microsporidium obtusum* (Widtman and Sokolova, 1994), and why it is necessary to create a new genus.

##### 4.2. Why not the genus *Agglomerata*?

Two authors previously recognized the structural similarity of some ACPS microsporidia to the genus *Agglomerata*. Lukyansev and Simakova (2014) found an "Agglomerata-like" microsporidium in *D. pulex* in Siberia. Ovcharenko and Wita (2001) described *Agglomerata connexa* in *D. longispina* and their finding is discussed below. However, it is the molecular phylogeny that reveals best the relationship between the ACPS microsporidia and *Agglomerata* spp. The ssu rDNA phylogeny ranks the DHM microsporidium and the related microsporidia from other *Daphnia* hosts we studied into a well-supported clade of microsporidia (Clade I in the tree on Fig. 5), yet distant in phylogeny from *Agglomerata cladocera* in Clade V of the tree. *A. cladocera* is the only member of the genus *Agglomerata* for which the ssu rDNA has been sequenced (Sokolova et al., 2016). *A. cladocera* infects hypodermal tissue of *Daphnia magna*, and its spores are pyriform but longer (length/width ratio of about 2.0, see illustrations in Larsson et al., 1996; Sokolova et al., 2016). In this respect *A. cladocera* is similar to *A. sidae*, the type species of the genus *Agglomerata* (Larsson and Yan, 1988). The spore shape, tissue specificity, molecular phylogeny and exospore ornamentation (in the form of a dense fibrillar brush) make *A. cladocera* distinct from ACPS microsporidia. The DHM organism and the ACPS-related microsporidia cannot be placed into the genus *Agglomerata* if the desirable monophyletic character of genera is to be maintained. Creation of a new genus for the DHM microsporidium and its close relatives is consequently the solution. Concurrently, a new family clade Agglomeratidae is proposed for microsporidia in Clades I to VI (Fig. 5).

The phylogenetic distance of *A. cladocera* (Clade VI) and of the microsporidia considered here (Clade I) in the tree (Fig. 5) is also the reason why the name *Agglomerata connexa* Ovcharenko and Wita, 2001 is not valid for the ACPS microsporidia studied here. *A. connexa* was described as a parasite of *D. longispina* (Ovcharenko and Wita, 2001) and there is no doubt that part of the description fits the characterization of our ACPS parasites of the *D. longispina* complex. However, the description of *A. connexa* has flaws, which, in addition to the phylogeny argument given above, prevent its further taxonomic use. The authors of the description did not fully recognize the structure of the exospore coat and, in some photomicrographs, misinterpreted the artificially separated tubular layer on the spores for a SPOV boundary (Fig. 10 in Ovcharenko and Wita, 2001). Most importantly, the light micrograph of *A. connexa* spores (Fig. 1 in Ovcharenko and Wita, 2001) showed oval (not pyriform), slightly pointed spores with convex side walls, which almost certainly belong to another microsporidian taxon.

##### 4.3. Why not the genus *Larssonia*?

*Larssonia obtusa* was proposed as replacement for Moniez's *Microsporidium obtusa* by Widtman and Sokolova (1994). The distinguishing character of the genus *Larssonia* was the presumed presence of a diplokaryotic nucleus in the cell (late meront or early sporont) initiating the spore formation (Widtman and Sokolova, 1994). There is a single micrograph showing the presumed "diplokaryon" in *Larssonia* (Fig. 3 in Widtman and Sokolova, 1994). However, this photograph is not convincing, the quality of preservation is low and our interpretation

suggests a monokaryotic nucleus with a dividing nucleolus. Jírovec (1936) never mentioned diplokaryotic nuclei in his detailed study of *P. obtusa*, and diplokarya were not found during examination of the three existing slides of *P. obtusa* made by Jírovec (only one of those is well stained). In our study we never encountered diplokaryotic nuclei in the numerous ACPS microsporidia we studied. So, either *Larssonia obtusa* exists and is indeed different from Jírovec's *P. obtusa* (which we find unlikely), or the authors proposing *Larssonia* wrongly interpreted, due to the low quality of their TEM photographs, the dividing nuclei of the sporont (likely option), and the genus *Larssonia* should be treated as a *nomen dubium*. Having no access to the habitat in which *Larssonia* was found, we cannot decide between these two alternatives, but it is probable that *Larssonia* is a wrongly described genus and the organism should be reinvestigated and its name eventually abolished. It must be stressed here that there exists in GenBank the ssu rDNA sequence with the name *Larssonia obtusa* (AF394527). This sequence was not obtained from infected *D. pulex*, from which *Larssonia obtusa* was described (a pond in the Zoological Garden in Kaunas, Lithuania), but from *D. pulex*, collected by Refardt et al. (2002) in a geographically somewhat distant area (Tvärminne, Finland). So, in conclusion, *Larssonia obtusa* is probably a doubtful taxon (*nomen dubium*) in need of reexamination, and it is not supported by proper molecular sequencing. This makes *P. obtusa* (renamed by Sprague in 1977 as *Microsporidium obtusum*) a name available for taxonomy consideration.

## 5. Taxonomy and diagnosis

### 5.1. Microsporidia Balbiani, 1882

#### 5.1.1. Phylogenetic superclade Agglomeratidae (Fig. 5)

5.1.1.1. *Diagnosis*. Large monophyletic superclade assembling, based on ssu rDNA sequences, many (but not all) Microsporidia infecting various daphnidids. The representatives for which structural data are available have pyriform spores and ultrastructural characters as described in Larsson and Yan (1988) for the genus *Agglomerata* (isolated nuclei in development, pyriform spores, complex exospore, three-compartment polaroplast, SPOV with many spores). In many species, but not all, the spores are ornamented by electron-dense convoluted tubules, brush-like fibres or by short electron dense tubules. Available (as yet incomplete) transmission data indicate that many species in the clade may have a complex life cycle requiring another (possibly insect) host.

5.1.1.2. *The Agglomeratidae superclade*. Assembles six clades, all defined by their respective ITS sequences, some of these clades being also supported by some structural and biology data (Fig. 5). The *Conglomerata* Clade I is defined below, MIC-1 Clade II assembles undescribed parasites of the *Daphnia longispina* complex occurring mostly in some deep water reservoirs (González-Tortuero et al., 2016). Clade III assembles two species of the genus *Gurleya* (4 spores inside of a SPOV, infecting epidermis of *D. pulex* and *D. longispina*; Friedrich et al., 1996; Green, 1974) and a single sequence obtained from *D. mendotae* (Refardt et al., 2008). Clade IV assembles three microsporidia known from sequences obtained from parasites of the epidermis of *D. pulicaria* (Refardt et al., 2008). Clade V contains *Binucleata daphniae*, a parasite of the epidermis of *D. magna* (Refardt et al., 2008). *Agglomerata cladocera*, a hypodermal parasite of *D. magna*, belongs to the same clade and is the only member of the genus *Agglomerata* with a known, short partial ssu rDNA sequence (no ITS region sequence available) (Sokolova et al., 2016). Clade VI contains a single sequence of *Senoma globulispora*, parasite of larval *Anopheles messae* (Diptera, Culicidae) (Simakova et al., 2005). It is the only Agglomeratidae sequence from a non-crustacean host and it might be the insect morph of a microsporidium with possible cladoceran-insect developmental cycle, not yet genetically characterized from a cladoceran host.

#### 5.1.1.3. Described genera of Agglomeratidae, their type species and hosts.

*Agglomerata*, *A. sidae* Larsson and Yan, 1988. *Sida crystallina*, Crustacea, Branchiopoda.

*Binucleata*, *B. daphniae* Refardt et al., 2008. *Daphnia magna*, Crustacea, Branchiopoda

*Senoma*, *Senoma globulispora* Simakova et al., 2005. *Anopheles messae*, Insecta, Diptera

*Gurleya*, *Gurleya tetraspora* Doflein, 1898. *Daphnia maxima* (= *D. magna*), Crustacea, Branchiopoda

## 5.2. The microsporidian genus *Conglomerata*, gen.n.

### 5.2.1. Diagnosis

Multinucleate meronts with single nuclei acquire in sporogony a thin sporophorous vesicle membrane. The multinucleate sporont with isolated nuclei divides by rosette-like budding inside a thin-walled, non-persistent sporophorous vesicle. Spore packets with irregular number of spores (~10–30) are formed inside the SPOV together with metabolic granules with periodic structure. Spores uninucleate, pyriform, with the length/width ratio of approximately 1.8, fresh spores surrounded by a thick layer of mucous material. Spore wall of complex construction: 100 nm thick endospore, exospore of main three layers (dense, lucent, dense). Outer exospore layer covered by short cylindrical projections, displayed in cross section as fibrillar outgrowths, and in tangential sections as a mesh of 70 nm circular elements. Tripartite polaroplast (wide, flat chambers followed by compact membranous lines and irregular vesicles). Polar tube tilt ~45°. Seven to eight isofilar coils. Molecular tag: presence of the 17-letter motif, GTTTAATATGTA TGTAT, in the ITS region:

### 5.2.2. *Conglomerata obtusa* (Moniez, 1887) n. comb., sp. coll.

(syn. *Plistophora obtusa* f. *typica* Jírovec, 1936; *Plistophora obtusa* Jírovec, 1943; *Microsporidium obtusum* Sprague, 1977; *Larssonia obtusa* Widtman and Sokolova, 1994; *Agglomerata connexa* Ovharenko and Wita, 2001).

5.2.2.1. *Historical remark*. Moniez (1887) described briefly four microsporidian species (*Microsporidium obtusa*, *M. ovata*, *M. elongata*, *M. acuta*) from various daphnidids. Labbé (1899) lumped them together into single species *Plistophora obtusa*. From today's perspective, the information provided by Moniez and Labbé are very unsatisfactory. Jírovec (1936) was the first to study these microsporidia in detail and attributed specific characters to individual parasites and their hosts. He established the taxon *P. obtusa* f. *typica*, treated here as one of junior synonyms of *Conglomerata obtusa*. For formal reasons, however, Jírovec cannot be mentioned as the author of *P. obtusa*; but without that the contribution of Moniez would be treated as *nomen dubium*, a delicate matter needing more detailed analysis.

5.2.2.2. *Type material and neotype*. Because no type material was prepared by former investigators (Moniez, Labbé, Jírovec), and because the only usable, Giemsa-stained slide made by Jírovec does not fulfil requirements for a type slide (no host defined), a neotype is described here as follows. This neotype material ties the specific characters of the *Conglomerata obtusa*, n. comb. to a single host species and habitat (shown as *Conglomerata obtusa*, n. comb., s. stricto on Fig. 5).

5.2.2.3. *Type host and locality*. *Daphnia pulex* Leydig, 1860, adipose cells. Spores pyriform, wedge-like, with the length/width ratio of 1.6–1.8 (fresh), 4.3 × 2.4 µm in size. Forest marsh near Běleč, Central Bohemia, Czech Republic (50.054N, 14.019E). Neotype material deposition: 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, Prague, Czech

Republic. Illustration Figs. 2A and 3G, this paper. Neotype ssu rDNA sequence: GenBank Acc. No. MH645034.

**5.2.2.4. *Conglomerata obtusa* is apparently a collective species.** Available observational and molecular data indicate that there exists an important subset of microsporidia parasitizing *Daphnia* (including *D. magna*, *D. pulex* and the *D. longispina* complex) that are parasites of adipose cells with pyriform spores, “the ACPS microsporidia”, mutually closely related in phylogeny, ultrastructurally indistinguishable from *C. obtusa* n. comb., defined above, yet producing spores of slightly different shapes (Fig. 4A–H). *C. obtusa* n. comb. thus seems to represent an assemblage of closely related morphotypes. We do not know the nature of these morphotypes, yet there is a possibility that they represent different species that cannot, at the current time, be characterized. We speculate that these microsporidia have another developmental phase, possibly in an insect host, and that respective species may differ in specificity to a second host. Thus, we propose *Conglomerata obtusa* (Moniez, 1887) n. comb., as a collective species, assembling phylogenetically close organisms (probably in the ranks of species, subspecies), which cannot be properly distinguished (Zoological Code, Art. 45.1.) It is proposed that individual microsporidia belonging to *Conglomerata obtusa* (Moniez, 1887) n. comb., are listed as respective morphotypes or isolates of *C. obtusa* n. comb. sp. coll. An example of such assemblage is shown in Fig. 5 as *C. obtusa*, n. comb. s. lato.

## 6. Envoi

### 6.1. Relationships

Our paper complements two relatively recently published papers in which microsporidia infecting several populations of the *Daphnia longispina* complex were examined by molecular methods (Wolinska et al., 2009; Weigl et al., 2012). Several microsporidians were identified in these studies as ssu rDNA and ITS-defined isolates, with no structural or taxonomic data provided. Two main lineages of *Daphnia* parasites infecting the “*Daphnia* body cavity” were found, one was identified as the *Berwaldia* clade, the other as the “MIC-1” clade (Fig. 2 in Wolinska et al., 2009; Fig. 3 in Weigl et al., 2012). The “*Berwaldia* clade” is identical with *Conglomerata obtusa* (Moniez, 1887) comb. n., sp. coll., described in this paper, the MIC-1 parasite is another species, the identity of which is presently under investigation.

### 6.2. Are the ACPS microsporidia of daphnids actually insect microsporidia?

The ACPS microsporidia, here ranked (probably provisionally) as a collective species *C. obtusa*, are the commonest parasites of various daphnidids. These microsporidia are not directly infective to their respective hosts (this paper; Vávra, 1964; Refardt et al., 2002, 2008) but other hosts are not known. However, available data indicate there is a substantial gene flow among geographically separated localities (Wolinska et al., 2011; González-Tortero et al., 2016), and it has been suggested this is due to dispersal via a highly motile secondary host. In such situation, and in the analogy to the *Amblyospora*–copepode life cycle pattern (Andreadis et al., 2012; Simakova, 2014), one can speculate that the other hosts in which a sexual cycle takes place are insects with aquatic larval stages, such as chironomid midges. If this assumption is confirmed, the ACPS microsporidia would be, in fact, insect microsporidia using daphnids as secondary hosts in which the multiplicative, haplophasic, non-sexual part of the life cycle takes place.

### 6.3. ITS barcoding

In the present paper, the rDNA-ITS barcoding successfully revealed phylogenetic relationships of a small group of microsporidia parasites. Thus, the ITS barcoding may have potential for revealing phylogeny of

microsporidia, despite the prediction that the existence of intragenomic variability would render the use of rDNA sequences for barcoding microsporidia questionable (Ironside, 2013), and despite within-host parasite nucleotide polymorphisms revealed by cloning in microsporidia infecting the *D. longispina* complex (e.g., Wolinska et al., 2009).

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jip.2018.10.003>.

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