



Morphological and molecular characterization of a new species, *Agglomerata daphniae* n. sp. from the hypoderm of *Daphnia magna* (Crustacea: Daphniidae)

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ABSTRACT

A new microsporidian species was described from the hypoderm of *Daphnia magna* sampled from gibel carp (*Carassius auratus gibelio*) ponds located in Wuhan city, China. The infected cladocerans generally appeared opaque due to numerous plasmodia distributed in the host integument. The earliest stages observed were uniciliate meronts that were in direct contact with the host cell cytoplasm. Meronts developed into multinucleate sporogonial plasmodia enclosed in sporophorous vesicles. Sporoblasts were produced by the rosette-like division of sporogonial division. Mature spores were pyriform and monokaryotic, measuring 4.48 ± 0.09 (4.34–4.65) μm long and 2.40 ± 0.08 (2.18–2.54) μm wide. The polaroplast was bipartite with loose anterior lamellae and tight posterior lamellae. Polar filaments, arranged in two rows, were anisofilar with two wider anterior coils, and five narrower posterior coils. The exospore was covered with fibrous secretions and was composed of four layers. Phylogenetic analysis based on the obtained SSU rDNA sequence, indicated that the present species clustered with three unidentified *Daphnia pulicaria*-infecting microsporidia with high support values to form a monophyletic lineage, rather than with the congener, *Agglomerata cladocera*. The barcode motif of the internal transcribed spacer (ITS) region of the novel species was unique among representatives of the “Agglomeratidae” sensu clade (Vávra et al., 2018). Based on the morphological characters and SSU rDNA-inferred phylogenetic analyses, a new species was erected and named as *Agglomerata daphniae* n. sp. This is the first report of zooplankton-infecting microsporidia in China.

1. Introduction

Microsporidia are obligate intracellular eukaryotic parasites that infect protists and invertebrates, as well as all groups of vertebrates, including humans (Cali and Takvorian, 2014). Recent research indicates that microsporidia most likely emerge from the Rozellomycota and is a basal branch or sister group to Fungi (Corsaro et al., 2016; Han and Weiss, 2017). At least 1500 microsporidian species belonging to 200 genera have been described worldwide, among which more than 50

genera are known to infect aquatic arthropods (Stentiford and Dunn, 2014; Vávra and Lukeš, 2013; Vávra et al., 2017). Nearly half of aquatic microsporidia were found infecting planktonic crustaceans, including cladocerans and copepods (Stentiford and Dunn, 2014). Although they represent a significant proportion of the diversity described within the phylum Microsporidia, it is widely accepted that the diversity of aquatic microsporidian is still severely underestimated, especially based on evidence from environmental DNA analysis (Stentiford and Dunn, 2014; Williams et al., 2018). Furthermore, the sampling of aquatic

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microsporidia has been essentially biased, because most species have been recorded from Europe. In China, only several microsporidia have been described from aquatic crustaceans of economic value, such as *Ameson portunus* from the swimming crab *Portunus trituberculatus* (Wang et al., 2017), *Potaspora macrobrachium* from the oriental river prawn *Macrobrachium nipponense* (Ding et al., 2016a), *Hepatospora eriocheir* from the Chinese mitten crab *Eriocheir sinensis* (Ding et al., 2016b; Wang and Chen, 2007) and *Triwangia caridinae* from the Taiwanese shrimp *Caridina formosae* (Wang et al., 2013). However, to the best of our knowledge, no microsporidium has been so far reported from zooplanktons in China. To enrich the knowledge of the diversity of aquatic microsporidia worldwide and explore their potential ecological roles, especially given increasing water pollution and eutrophication, a project was initiated to investigate the diversity of zooplankton-infecting

microsporidia in China.

Numerous microsporidian species have been recorded in Cladocera, a common group of freshwater zooplankton inhabiting various water bodies (Wolinska and Spaak, 2009). In addition, about 40 microsporidian species assigned to 12 genera, have been described to infect daphnid worldwide (Vávra et al., 2017). Interestingly, a recent report found that *Mitosporidium daphniae* isolated from *Daphnia magna*, was phylogenetically positioned at the root of Microsporidia, indicating that ancestral microsporidia probably originated in aquatic habitats (Haag et al., 2014). Elucidating the diversity of aquatic microsporidia will also undoubtedly deepen insights into the evolutionary biology of Microsporidia. As a part of an ongoing project to investigate the diversity of aquatic microsporidia in middle and lower reaches of Yangtze River, we describe a new daphnid-infecting species with morphological and

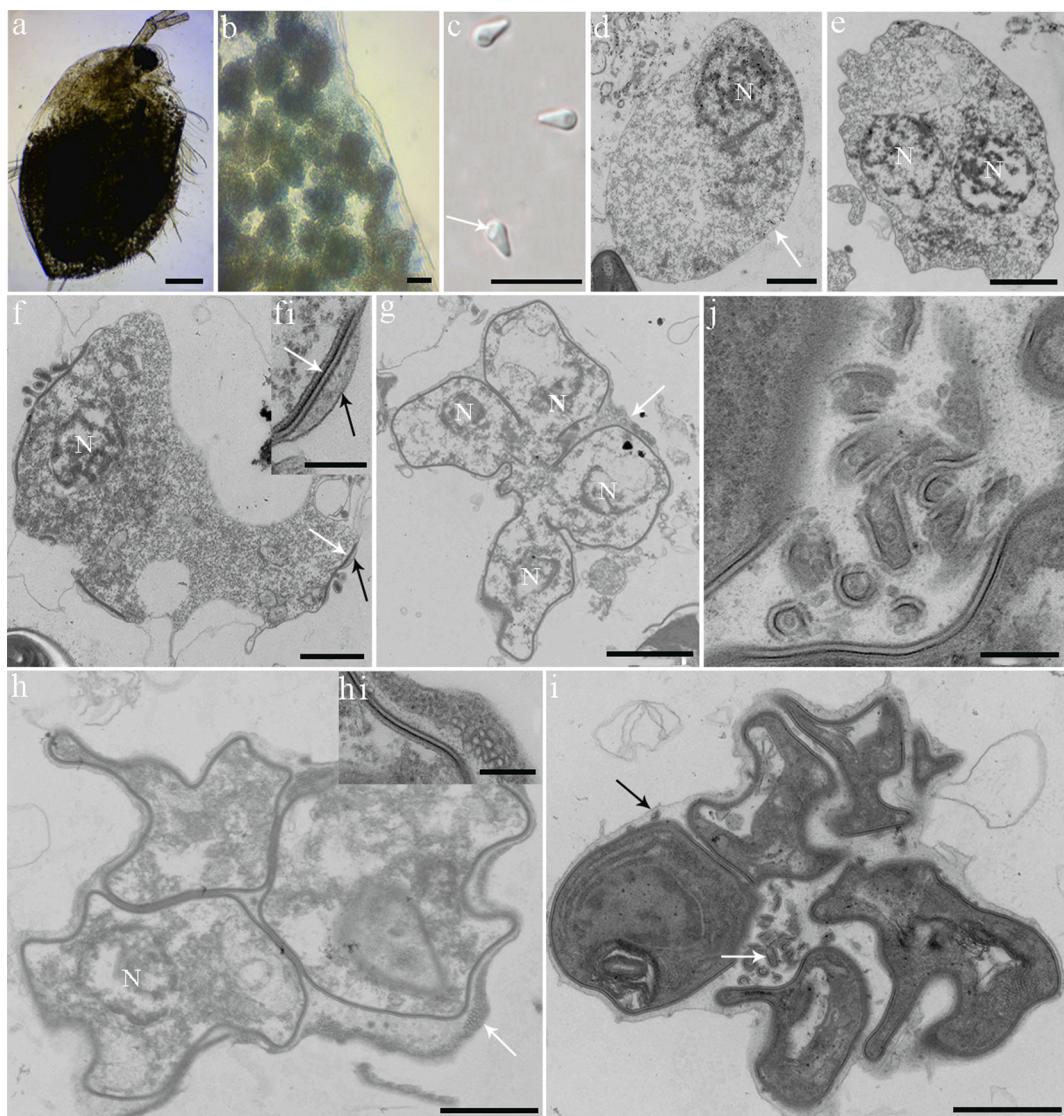


Fig. 1. Microscopic observations of *Agglomerata daphniae* n. sp. **a.** *Daphnia magna* infected by *Agglomerata daphniae* n. sp. appears opaque. Bar = 100 μm. **b.** Numerous spores enclosed within sporophorous vesicle are distributed in the hypoderm of *D. magna*. Bar = 10 μm. **c.** Fresh spores released from infected *D. magna*; the posterior vacuole is easily observed at the posterior end of spores (arrow). Bar = 10 μm. **d.** A uninucleate meront surrounded by a simple plasma membrane (arrow). Bar = 2 μm. **e.** A binucleate meront surrounded by a thickening plasma membrane. Bar = 1 μm. **f.** Meront-sporont transitional stages with electron dense materials deposited on the surface of the cell (white arrow), enclosed within the sporophorous vesicle (black arrow). Bar = 1 μm. **fi.** Magnification of Figure f. showing the structure of sporophorous vesicles membrane (black arrow) and electron dense materials (white arrow). Bar = 500 nm. **g.** Multinucleate sporogonial plasmodia enclosed within sporophorous vesicles undergo division by rosette-like budding (arrow). Bar = 2 μm. **h.** Irregular shaped sporoblasts with vesicular-tubular secretions in the episporontal space (arrow). Bar = 1 μm. **hi.** Magnification of “tubular secretions”. Bar = 200 nm. **i.** Irregular sporoblasts and lamellar secretions (white arrow) enclosed within the sporophorous vesicles (black arrow). Bar = 1 μm. **j.** Magnification of the specific structure of the lamellar secretions. Bar = 200 nm. N, nucleus.

molecular characteristics.

2. Materials and methods

2.1. Collection of specimens and microscopical observation

Using a plankton net (mesh size 112 µm), we collected zooplankton from several eutrophic ponds of the Guanqiao Experimental Station of Institute of Hydrobiology, Chinese Academy of Sciences ($30^{\circ}31'22.78''$ N, $114^{\circ}23'8.1''$ E) in April 2019. Specimens were held on ice and transported immediately to the laboratory for examination. Cladocerans were morphologically identified (Jiang and Du, 1979) and screened for infection or not under the dissecting microscopy (Olympus SZ51) by the opaque coloration of their teguments at 10–50x magnification. The suspected plasmodia were ruptured to prepare glass slide wet mounts and observed at 1000x with an oil immersion objective. Spore images were captured using an Olympus BX 53 microscope equipped with an Olympus DP72 digital camera (Olympus, Japan). Infected cladocerans were preserved in 95% ethanol for further molecular characterization and in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (PH 7.4) for electron microscopic observation.

2.2. Transmission electron microscopy (TEM)

Glutaraldehyde-fixed cladocerans were washed twice for 10 min in sodium cacodylate buffer and then fixed with 1% osmium tetroxide (OsO₄) in the same buffer for 1 h. After dehydration through a gradual ascending series of ethanol and propylene oxide, samples were embedded in Spur resin. Ultrathin sections (70–90 nm) were mounted on an uncoated copper grid and stained with uranyl acetate and lead citrate. Sections of two infected cladocerans were examined using a Hitachi HT-7700 transmission electron microscope (TEM). The images of 40 spores were measured based on TEM images by Adobe photoshop CS6, and measurements were presented as Mean ± SD.

2.3. DNA extraction, PCR, and sequencing

Ethanol-fixed cladocerans were washed with distilled water 3 times to remove ethanol remnants. The genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. The general microsporidian primers V1f (5'-CACCAGGTTGATTCTGCC-3') and 580r (5'-GGTCCGTGTTCAA-GACGG-3') were used to amplify the partial 16S ribosomal RNA (rRNA) gene, the complete internal transcribed spacer (ITS), and the partial 23S rRNA gene (Weiss and Vossbrinck, 1999). PCR was carried out in a 50-µl reaction system, containing PCR buffer, 200 mM dNTP, 2 mM MgCl₂, 1.25 units Taq polymerase, 20 pmol each primer, and 2 µl DNA template. Thermocycler parameters were as follows: an initial denaturation step at 95 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 30 s, elongation at 72 °C for 2 min and a final extension at 72 °C for 10 min. The PCR products were excised from an agarose gel and purified using a PCR purification kit (CWBiotech, Beijing, China) and cloned into a PMD-18 T vector system (Takara, Tokyo, Japan). Four positive clones were randomly selected to sequence in both directions with the ABI BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3100 Genetic Analyzer.

2.4. Molecular characterization

The sequence fragments we obtained were assembled by BioEdit (Hall, 1999). The consensus sequence was verified as a microsporidium by a BLAST search. To explore the phylogenetic position of the studied species among a large superclade of aquatic microsporidia named the "Agglomeratidae" clade by Vávra et al. (2018), 35 sequences with high sequence similarity and those of our interest were retrieved from GenBank and aligned using Clustal X by default settings (Thompson et al.,

1997). The obtained alignment was corrected manually using the alignment editor function of MEGA 6.0 (Tamura et al., 2013). *Amblyospora connecticus* (AF025685), *Amblyospora opacita* (AY090052) and *Andreanna caspii* (EU664450) were used as outgroups. Pairwise genetic distances/similarities were calculated using the Kimura-2 parameter model distance matrix for transitions and transversions. Phylogenetic analyses were conducted using the maximum likelihood (ML) method in PhyML 3.0 and Bayesian inference (BI) in MrBayes 3.2.4, respectively. The optimal evolutionary model was GTR + I + G determined by ModelTest 3.7 using the Akaike information criteria. Two independent runs were conducted with four chains for 1 million generations for BI. Phylogenetic trees were sampled every 100 generations. The first 25% of the samples were discarded from the cold chain (burninfrac = 0.25). Bootstrap confidence values were calculated with 100 repetitions for ML. Trees were initially examined in Figtree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>), edited and annotated in Adobe Illustrator (Adobe System, San Jose, CA, USA). Only 16S rRNA gene sequences were used for phylogenetic analysis, because ITS and 23S rRNA gene sequences were only available for a few species in GenBank. Another alignment consisting of full ITS sequences and the tandem short neighbouring portions of SSU and LSU rDNA sequences of 17 species belonging to the "Agglomeratidae" sensu clade (Vávra et al., 2018) was also produced to determine the specific ITS motif of the present species.

3. Results

3.1. Light microscopy

All collected cladocerans were morphologically identified to be *Daphnia magna*, *Daphnia carinata* and *Diaphanosoma leuchtenbergianum*, respectively, however, the microsporidian we studied was only observed in *D. magna*. The prevalence of infection was 1.1% (20/1835). When observed in the light microscope, the infected *D. magna* generally appeared opaque due to numerous plasmodia distributed throughout the whole host integument (Fig. 1a). Microscopic examination showed that plasmodia (34.0 × 23.5 µm) were located in the hypoderm of host (Fig. 1b) and numerous spores were liberated from the ruptured plasmodia. Fresh spores were pyriform, measuring 4.48 ± 0.09 (4.34–4.65) µm long and 2.40 ± 0.08 (2.18–2.54) µm wide (N = 40). A large posterior vacuole could be observed at the posterior end of spores (Fig. 1c).

3.2. Electron microscopy

Various developmental stages were observed within the cytoplasm of host cells under TEM. The earliest stages observed were uninucleate meronts which were in direct contact with the host cell cytoplasm and surrounded by an amorphous cell membrane (Fig. 1d). Uninucleate meronts developed into binucleate pre-sporont stages (Fig. 1e) by division of nuclei, similar to *A. cladocera* and *Binucleata daphniae* (Refardt et al., 2008; Sokolova et al., 2016). Presporonts divided further to produce elongated sporonts with numerous electron-dense materials accumulated on their surface (Fig. 1f). At this stage, an additional envelope appeared that was suspected to be the precursor of the sporophorous vesicle (SV) membrane (Fig. 1f, 1fi). Sporont nuclei underwent division to produce multinucleate sporogonial plasmodia, which further developed into sporoblasts by rosette-like budding within a fragile sporophorous vesicle (Fig. 1g). Each sporophorous vesicle enclosed 2 to 8 sporoblasts, but 4 were most frequently observed. Sporoblasts were of irregular shape. During this stage, vesicular-tubular secretions (Fig. 1h, 1hi) and lamellar-like structures, termed as "lamellar secretion" (Fig. 1i, 1j) occurred in the episporontal space of SVs. Sections through the lamellar secretions showed three layers: two electron-dense layers, and an electron-lucent layer in between. The structure of these secretions resembled the structure of sporoblast envelopes (Fig. 1j). Development of sporoblasts to spores involved differentiation of typical spore organelles, including the trilaminar spore wall, anchoring disk, bipartite

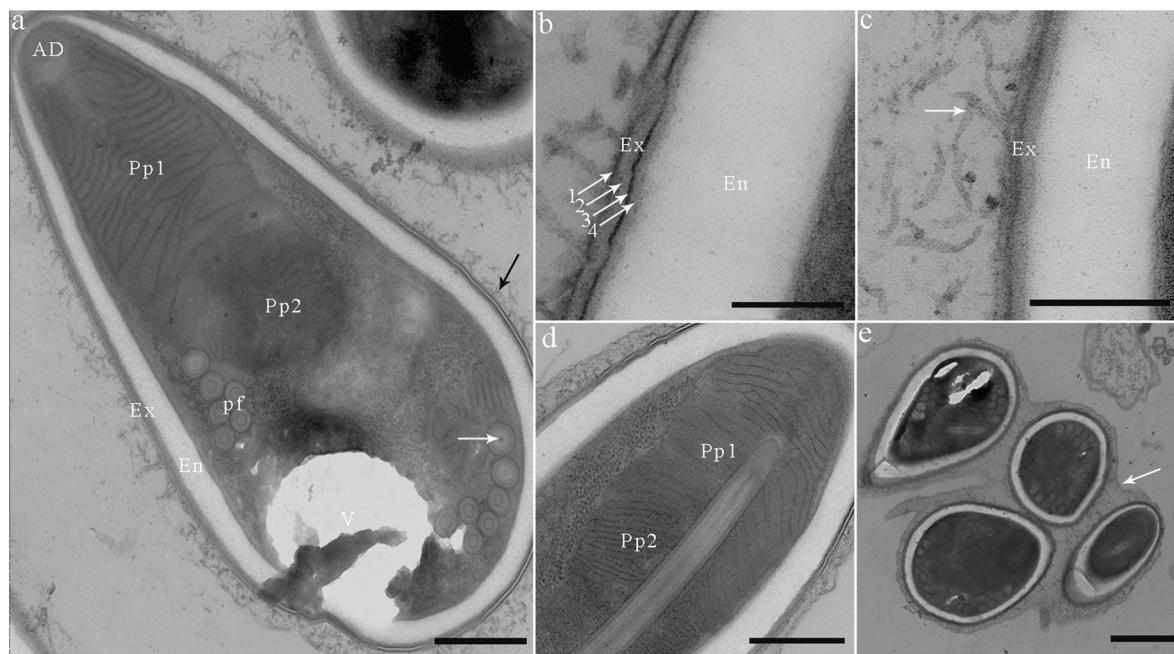


Fig. 2. Microscopic observations of mature spores of *Agglomerata daphniae* n. sp. **a.** Mature spore with typical features of microsporidian internal structure, including the anisofilar polar filaments (pf), a bipartite polaroplast (Pp1, Pp2), a vacuole (V), a mushroom-shaped anchoring disc (AD) and a trilaminar spore wall consisting of an electron-dense exospore (Ex), an electron-translucent endospore (En) and a plasma membrane. The spores were covered with fibrous secretions (black arrow). Anterior coils of polar filaments possess a dark center (white arrow). Bar = 500 nm. **b.** Magnification of the exospore (Ex) showing four distinct layers. Bar = 100 nm. **c.** Fibrous secretions (arrow) scattered on the surface of spores. Bar = 200 nm. **d.** Bipartite polaroplast (Pp1, Pp2). Bar = 500 nm. **e.** Section showing four spores within a sporophorous vesicle (arrow). Bar = 2 μ m.

Table 1

Pairwise nucleotide sequence identity (upper right) values and evolutionary distances (left bottom) among *Agglomerata daphniae* n. sp. and 11 other microsporidian species with high sequence similarity by Kimura-2 Parameter analysis based on SSU rDNA sequences.

| Species (GenBank accession number) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|------|
| 1 <i>Agglomerata daphniae</i> n. sp. (MN892551) | — | 98.4 | 98.3 | 97.9 | 94.4 | 93.8 | 93.7 | 93.5 | 93.4 | 92.8 | 88.6 | 88.4 |
| 2 <i>Microsporidium</i> sp. (EU075356) | 0.0156 | — | 99.9 | 99.3 | 94.1 | 93.6 | 94.0 | 93.1 | 93.7 | 93.2 | 89.1 | 89.0 |
| 3 <i>Microsporidium</i> sp. (EU075355) | 0.0168 | 0.0012 | — | 99.2 | 93.9 | 93.4 | 93.8 | 93.0 | 93.6 | 93.0 | 89.0 | 88.8 |
| 4 <i>Microsporidium</i> sp. (EU075357) | 0.0204 | 0.0072 | 0.0084 | — | 93.5 | 93.0 | 93.6 | 92.6 | 93.3 | 92.8 | 89.2 | 89.1 |
| 5 <i>Agglomerata cladocera</i> (KT950767) | 0.0557 | 0.0595 | 0.0608 | 0.0647 | — | 97.2 | 93.1 | 98.9 | 93.7 | 89.1 | 93.2 | 89.0 |
| 6 <i>Senoma globulifera</i> (DQ241645) | 0.0620 | 0.0645 | 0.0658 | 0.0698 | 0.0277 | — | 90.4 | 96.4 | 90.4 | 89.4 | 86.1 | 86.0 |
| 7 <i>Conglomerata obtusa</i> (MH645035) | 0.0631 | 0.0605 | 0.0618 | 0.0644 | 0.0898 | 0.0964 | — | 90.1 | 99.8 | 98.8 | 90.1 | 90.0 |
| 8 <i>Binucleata daphniae</i> (EU075347) | 0.0648 | 0.0687 | 0.0700 | 0.0740 | 0.0108 | 0.0364 | 0.0995 | — | 89.8 | 89.0 | 85.8 | 85.6 |
| 9 <i>Berwaldia schaeferi</i> (AY090042) | 0.0657 | 0.0631 | 0.0644 | 0.0670 | 0.0925 | 0.0965 | 0.0024 | 0.1022 | — | 98.7 | 90.1 | 90.0 |
| 10 <i>Larssonia obtusa</i> (AF394527) | 0.0724 | 0.0683 | 0.0696 | 0.0723 | 0.1023 | 0.1063 | 0.0120 | 0.1107 | 0.0132 | — | 89.0 | 88.8 |
| 11 <i>Gurleya daphniae</i> (AF439320) | 0.1145 | 0.1090 | 0.1104 | 0.1076 | 0.1333 | 0.1393 | 0.0992 | 0.1421 | 0.0992 | 0.1105 | — | 99.9 |
| 12 <i>Gurleya vavrai</i> (AF395526) | 0.1159 | 0.1104 | 0.1118 | 0.1090 | 0.1348 | 0.1408 | 0.1006 | 0.1436 | 0.1006 | 0.1119 | 0.0012 | — |

polaroplast, polar filaments and posterior vacuole (Fig. 2a-d). Mature spores were pyriform (Fig. 2a, e), and covered with the fibrous secretions extending outwards (Fig. 2c). The spore wall consisted of a 31-nm thick electron-dense exospore, a 152-nm thick electron-lucent endospore and an 8-nm thick plasma membrane (Fig. 2a-b). The exospore consisted of four layers, including two electron-dense layers (layer 1 and 3) and two electron-moderate layers (layer 2 and 4) (Fig. 2b). The polaroplast was bipartite; the anterior part occupied approximately two-thirds of the length of the entire polaroplast and was composed of wide lamellae, while the posterior part included more compact lamellae (Fig. 2a, 2d). A mushroom-shaped anchoring disc located at the apex of a spore was surrounded by the anterior portion of polaroplast (Fig. 2a). Polar filaments were anisofilar with two wide anterior coils measuring 162 nm in diameter, and five narrow posterior coils of 132 nm diameter each. Polar filaments were coiled in 7 turns and arranged in 2 rows (Fig. 2a).

3.3. Molecular characterization

Four positive clones produced a consensus sequence 1306 bp long. The sequence was deposited in GenBank under Accession Number MN892551. A BLAST search indicated that the obtained sequence was not identical to any microsporidian sequences available in GenBank, but was most similar to three unidentified microsporidia (Genbank accession EU075356, EU075355, EU075357) isolated from *Daphnia pulicaria* (more than 95% similarity), followed by *A. cladocera* (KT950766) isolated from *D. magna*, with 93.45% similarity. The pairwise distances/similarities calculated by Kimura 2-parameter model between the present species and other microsporidian species of high sequence similarity, ranged from 0.016/98.4% (*Microsporidium* sp. EU075356) to 0.116/88.4% (*Gurleya vavrai* AF395526) (Table 1). Bayesian and maximum likelihood analyses of the aligned SSU rDNA genes generated highly similar topologies, although with different support values at some branch nodes. The novel species clustered with three unidentified *D. pulicaria*-infecting species with high support values. This group is a

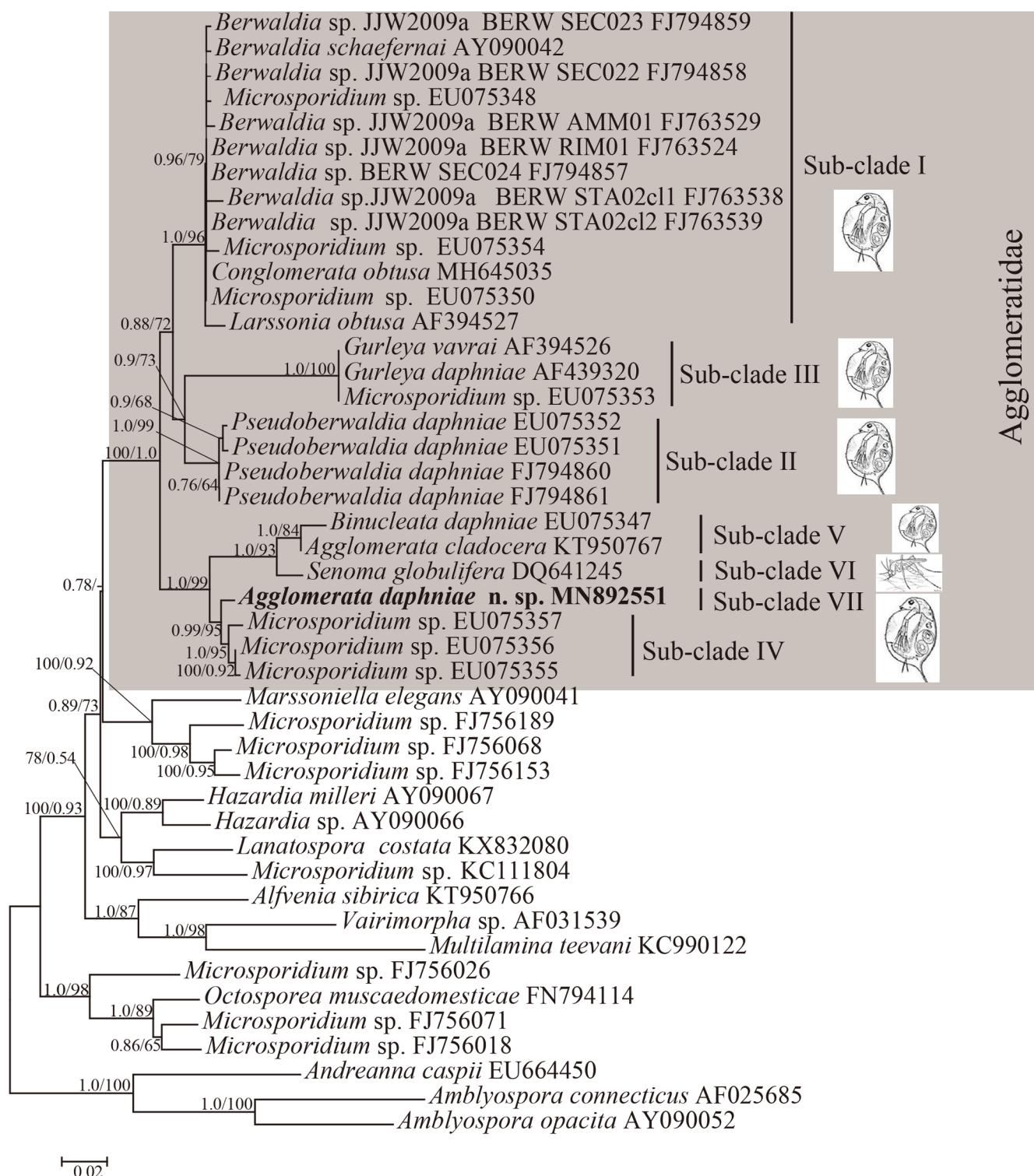


Fig. 3. The SSU rDNA-inferred phylogenetic relationships between *Agglomerata daphniae* n. sp. and other aligned microsporidian species by Bayesian Inference (BI) method. Species names are followed by GenBank accession number. BI posterior probabilities are shown first, followed by ML support values on branch nodes. *A. daphniae* is highlighted in bold.

sister to the *Binucleata-Agglomerata-Senoma* lineage, part of a large monophyletic clade primarily composed of daphnid-infecting genera like *Binucleata*, *Agglomerata*, *Pseudoberwaldia*, *Gurleya*, *Berwaldia*, *Conglomerata*, and *Larssonia*. Interestingly, the present species did not cluster with *A. cladocera*, the only species in the genus *Agglomerata* with available sequence data. *A. cladocera* formed a dichotomy with *A. daphniae* (Fig. 3). Furthermore, the barcode motif in the internal

transcribed spacer (ITS) of the present species (TTTTAATATAATTAA-TAAGTGTAT) was unique among representatives of the “Agglomeratidae” sensu clade (Vávra et al., 2018). Vávra et al. (2018) described six ITS barcode motifs corresponding to subclades I-VI. The novel species represent a new subclade with a characteristic ITS motif (Fig. 4).

Collectively, a new *Agglomerata* species was erected and named *Agglomerata daphniae* n. sp. basing on morphological, ultrastructural and

| Specific motif in ITS region | | | | | | | | | | | |
|---|---|-------------------------------------|-----------------------------|------------------------------|------------------------------|--|--|--|--|--|--|
| <i>Conglomerata obtusa</i> MH645035 | G C CGCAGG A TCAA TA AGCA GAGCGTT TGGTT TAA · · · · · · · · · · · · | TATGT ATGTA · · · · · · · · · · · · | T | · · · · · · · · · · · · | | | | | | | |
| <i>Microsporidium</i> sp.EU075350 | G C CGCAGG A TCAA TA AGCA GAGCGTT TGGTT TAA · · · · · · · · · · · · | TATGT ATGTA · · · · · · · · · · · · | TACATTACGTTCGA | | | | | | | | |
| <i>Larssonia obtusa</i> AF394527 | G C CGCAGG A TCAA TA AGCA GAGCGTT TGGTT TAA · · · · · · · · · · · · | TATGT ATGTA · · · · · · · · · · · · | TACATTACGTTCGA Sub-clade I | | | | | | | | |
| <i>Microsporidium</i> sp.EU075348 | G C CGCAGG A TCAA TA AGCA GAGCGAT TGGTT TAA · · · · · · · · · · · · | TATGT ATGTA · · · · · · · · · · · · | TACATTACGTTCGA | | | | | | | | |
| <i>Microsporidium</i> sp.EU075354 | G C CGCAGG A TCAA TA AGCA GAGCGAT TGGTT TAA · · · · · · · · · · · · | TATGT ATGTA · · · · · · · · · · · · | TACATTACGTTCGA | | | | | | | | |
| <i>Microsporidium</i> sp.EU075351 | G C CGCAGG A TCAA TA · · · · · · · · · · · · | · · · · · · · · · · · · | ATATG GGGTA · · · · · · | CACATTGCGTTCGA | | | | | | | |
| <i>Microsporidium</i> sp.FJ794861 | G C CGCAGG A TCAA TA · · · · · · · · · · · · | · · · · · · · · · · · · | ATATG GGGTA · · · · · · | CACATTGCGTTCGA Sub-clade II | | | | | | | |
| <i>Microsporidium</i> sp.FJ794860 | G C CGCAGG A TCAA TA · · · · · · · · · · · · | · · · · · · · · · · · · | ATATG GGGTA · · · · · · | CACATTGCGTTCGA | | | | | | | |
| <i>Gurleya varvai</i> AF394526 | G C · · · · · · · · · · · · | · · · · · · · · · · · · | A · · · · · · · · · · · · | TGT GGGTA · · · · · · | CACATTGCGTTCGA | | | | | | |
| <i>Gurleya daphniae</i> AF439320 | G C · · · · · · · · · · · · | · · · · · · · · · · · · | A · · · · · · · · · · · · | TGT GGGTA · · · · · · | CACATTGCGTTCGA Sub-clade III | | | | | | |
| <i>Microsporidium</i> sp.EU075353 | G C · · · · · · · · · · · · | · · · · · · · · · · · · | A · · · · · · · · · · · · | A · · · · · · · · · · · · | A · · · · · · · · · · · · | | | | | | |
| <i>Microsporidium</i> sp.EU075356 | G C CGCAGGATCAATAA · · · · · · · · · · · · | · · · · · · · · · · · · | A · · · · · · · · · · · · | TGT GGGTA · · · · · · | CACATTGCGTTCGA Sub-clade IV | | | | | | |
| <i>Microsporidium</i> sp.EU075355 | G C CGCAGGATCAATAA · · · · · · · · · · · · | · · · · · · · · · · · · | A · · · · · · · · · · · · | A · · · · · · · · · · · · | A · · · · · · · · · · · · | | | | | | |
| <i>Microsporidium</i> sp.EU075357 | G C CGCAGGATCAATAA · · · · · · · · · · · · | · · · · · · · · · · · · | A · · · · · · · · · · · · | A · · · · · · · · · · · · | A · · · · · · · · · · · · | | | | | | |
| <i>Binucleata daphniae</i> EU075347 | G C CGCAGGATCAATAACGTTATGGGTTTTAT · · · · · · · · · · · · | · · · · · · · · · · · · | ATT TATAAGTT · · · · · · | ACATCATACTCGA Sub-clade V | | | | | | | |
| <i>Senoma globulifera</i> DO641245 | G C CGCAGGATCAATAAGTTA · · · · · · · · · · · · | · · · · · · · · · · · · | ATT TATAAGTT · · · · · · | ACATCATACTCGA Sub-clade VI | | | | | | | |
| <i>Agglomerata daphniae</i> n. sp. MN892551 | G C CGCAGGATCAATAATTAA · · · · · · · · · · · · | · · · · · · · · · · · · | TATAATTATAAGTGT · · · · · · | ACATCATATTTCGA Sub-clade VII | | | | | | | |

Fig. 4. Partial alignment of representatives of all subclades of the “Agglomeratidae” sensu clade (Vávra et al 2018) including specific motif in the ITS region.

molecular data.

4. Taxonomic summary

Phylum: Microsporidia **Balbiani, 1882****Family:** Agglomeratidae **Vávra et al., 2018****Genus:** *Agglomerata* **Larsson and Yan, 1988***Agglomerata daphniae* n. sp.*Type host:* *Daphnia magna* (Crustacea, Cladocera, Daphniidae)*Type locality:* Eutrophic ponds of Guanqiao Experimental Station of Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan city, Hubei province, China (30°31'22.78" N, 114°23'8.1" E).*Tissue tropism:* Hypoderm.*Interfacial envelopes:* Meronts in direct contact with the host cell cytoplasm. Multinucleate sporogonial plasmodia, sporoblasts and spores develop within sporophorous vesicles.*Meronts:* Uninucleate or binucleate meronts elongated and

surrounded by an amorphous cell membrane.

Sporonts: Uninucleate sporonts not observed, multinucleate sporogonial plasmodia present.**Sporoblasts:** Irregular shape with dense cytoplasm.**Spores:** Spores are uninucleate and pyriform, 4.48 ± 0.09 (4.34–4.65) μm long and 2.40 ± 0.08 (2.18–2.54) μm wide. Seven anisofilar polar filaments arranged in two rows. The two wide anterior coils are 162 nm thick and the five narrow posterior coils are 132 nm thick. The polaroplast is bipartite with loose anterior lamellae and tight posterior lamellae. The spore wall consists of a 31-nm thick electron-dense exospore, 152-nm thick electron-lucent endospore and 8-nm thick plasma membrane. The exospore is composed of four-layers with 2 electron-dense layers and 2 layers of moderate electron density.**Type material:** Syntype specimens of TEM resin blocks were deposited in the Museum of Hydrobiological Sciences, Institute of Hydrobiology, Chinese Academy of Sciences with accession number of MTR20190418.**Etymology:** Referred to host genus name.**Table 2**Morphological comparison of *Agglomerata daphniae* n. sp. with other *Agglomerata* spp.

| Species | Host species | Tissue ^a | Sporogonial plasmodia division | SV ^b , no. of spores | SV, inclusions | Episporal structures | Spore shape size, μm | Polar filament type, number | PP ^c | SV-EX ^d link | EN ^e of layer | Reference |
|------------------------------------|--------------------------------|------------------------------|--------------------------------|---------------------------------|----------------|----------------------|----------------------------------|-------------------------------|-----------------|-------------------------|--------------------------|---|
| <i>Agglomerata daphniae</i> n. sp. | <i>Daphnia magna</i> | Hyp | Rosette-like | 4, 6, 8 | Thread-like | Tubules | Pyriform 2.2–2.5 × 4.3–4.6 | Heterofilar 7 + L | L | Fibrils | 4 | (herein) |
| <i>A. sidae</i> | <i>Holopedium gibberum</i> | Hyp; adip; haem ^e | Rosette-like | 8, 16, 32 | Thread-like | Tubules | Pyriform 1.5–2.0 × 2.5–3.0 | Heterofilar 5–6 + L + T | L | Fibrils | 5 | (Larsson and Yan, 1988) |
| <i>A. cladocera</i> | <i>D. magna</i> | Hyp; adip; haem | Rosette-like | 4, 8, 16 | Thread-like | Fibrils | Pyriform 1.5–2.2 × 3.0–4.5 | Heterofilar 5–6 + L + T | L | Fibrils | 3 | (Larsson et al., 1996; Sokolova et al., 2016) |
| <i>A. volgensae</i> | <i>D. magna</i> | Hyp; adip | Rosette-like | 1, 4, 8, 16 | Thread-like | Fibrils | Pyriform 1.7–2.0 × 2.0–3.1 | Heterofilar 5–6 + L | L | Fibrils | 7 | (Larsson and Voronin, 2000) |
| <i>A. connexa</i> | <i>Daphnia longipinna</i> | Adip | Rosette-like | 1, 4 | Thread-like | Fibrils | Pyriform 2.7 × 4.0 | Isofilar 5–7 + L + T | L | Fibrils | 3 | (Ovcharenko and Wita, 2001) |
| <i>A. lacrima</i> | <i>Acanthocyclops vernalis</i> | Hyp; adip | Rosette-like | 4, 6, 8, 12 | Thread-like | Fibrils | Pyriform 2.6 × 4.4 | Heterofilar 5–6 + L + T | L | Fibrils | 4 | (Bronnwall and Larsson, 2001) |
| <i>A. simocephali</i> | <i>Simocephalus vetulus</i> | Hyp | Rosette-like | 8, 12, 16 | Fibrils | Fibrils | Pyriform 2.8 × 5.0 | Heterofilar 9 + L | (–) | 2 | (Sokolova et al., 2018) | |

^a hyp, hypoderm; adip, adipocytes; haem, hemolymph.^b SV, sporophorous vesicles.^c PP, polaroplast; L, lamellar; T, tubular.^d EX, exospore.^e EN, exospore number.

Gene sequences: Deposited in GenBank under accession numbers of MN892551.

5. Discussion

The morphological characters of the new microsporidian species we described, *Agglomerata daphniae* n. sp., match the diagnostic features of genus *Agglomerata*: uninucleate or binucleate meronts, sporogonial plasmodia dividing in a rosette-like fashion, pyriform spores, multilayered exospore, anisofilar polar filaments, variable spore number within the sporophorous vesicles, and tubular inclusions in episporal space (Larsson and Yan, 1988). Seven *Agglomerata* species were reported previously worldwide, including *Agglomerata simocephali* infecting the hypoderm of *Simocephalus vetulus* (Sokolova et al., 2018; Voronin, 1986), *A. cladocera* infecting the hypoderm of *D. magna* (Larsson et al., 1996; Sokolova et al., 2016), *Agglomerata lacrima* infecting the hypoderm and fat tissue of *Acanthocyclops vernalis* (Bronnvall and Larsson, 2001), *Agglomerata connexa* infecting adipocytes of *Daphnia longispina* (Ovcharenko and Wita, 2001), *Agglomerata volgensae* infecting the hypoderm and adipocytes of *D. magna* (Larsson and Voronin, 2000), *Agglomerata sidae* infecting the hypoderm of *Holopedium gibberum* (Larsson and Yan, 1988) and *Agglomerata bosminiae* infecting the connective tissue of ovary of *Bosmina obtusirostris*, *Bosmina longirostris* and *Bosmina coregoni*, respectively (Voronin, 1986, 1990). The strict morphological comparison between the present species with all congeners is summarized in Table 2 and shows that *A. daphniae* is distinct from those species. *A. cladocera*, *A. sidae*, *A. connexa* and *A. lacrima* possess tripartite polaroplast (wide lamellae, narrow lamellae and tubules), rather than the bipartite polaroplast (wide lamellae and narrow lamellae), as in the case of the present species, *A. volgensae* and *A. simocephali*. The episporal space of *A. volgensae* and *A. simocephali* contains fibrous inclusions rather than tubular inclusions, as observed in *A. daphniae*. Moreover, the number of polar filament coils in *A. daphniae* (seven) is different from *A. simocephali* (nine) and *A. volgensae* (six) (Larsson and Voronin, 2000; Sokolova et al., 2018; Voronin, 1986). In addition, the exospore of *A. volgensae* consists of seven layers, the most exospore layers observed so far in *Agglomerata* species (Larsson and Voronin, 2000). *A. cladocera* can be easily differentiated from *A. daphniae* by its three-layered exospore and fibrillar structures in the episporal space (Larsson et al., 1996; Sokolova et al., 2016). *A. sidae*, originally identified as *Lanatospora macrocyclospis*, has tubular episporal structures and thread-like inclusions in sporophorous vesicles that is morphologically similar to *A. daphniae*, however, it can be easily distinguished from the novel species by its smaller spores (1.5–2.0 × 2.5–3.0 vs. 2.2–2.5 × 4.3–4.6), the different polaroplast structure (tripartite vs. bipartite) and five layers in the exospore compared to four layers in *A. daphniae* (Larsson and Yan, 1988). The polar filaments of *A. connexa* are coiled in 5–7 turns, similar to *A. daphniae*. However, the polar filament of *A. connexa* is isofilar, unlike that of *A. daphniae*. In addition, the number of mature spores within sporophorous vesicles of *A. connexa* is different from that of *A. daphniae* (1 vs. 4) (Ovcharenko and Wita, 2001). Among *Agglomerata* spp., *A. lacrima* is the only species that infects cyclops (*Ac. vernalis*) but not cladocerans. Furthermore, *A. lacrima* is distinguished from the present species by a different polar filament pattern (5 turns arranged in one row vs. 7 turns arranged in two rows) and the structure of the polaroplast (tripartite vs. bipartite) (Bronnvall and Larsson, 2001). Prior to this study, sequence data was available only for *A. cladocera* among *Agglomerata* spp.

Phylogenetic analysis shows that *Agglomerata daphniae* n. sp. is closely related to three unidentified microsporidia from the epidermis of *D. pulicaria* sampled in the USA. These species share more than 98% sequence similarity with *A. daphniae* and likely belong to the genus *Agglomerata* (Table 1). Further detailed morphological characterization will finally assign their taxonomic affiliation. Our analysis also supports previous reports that most of daphnid-infecting microsporidia, including *Berwaldia* spp., *Larssonia* spp., *Agglomerata* spp., *Binucleata* spp., *Gurleya*

spp. and *Microsporidium* spp. form a monophyletic clade treated by Vávra et al. (2018) as “*Agglomeratidae sensu clade*”. Validation of the taxonomy of *Agglomerata*, as well as phylogenetic relationships with *Senoma globulifera*, a mosquito-infecting species (Simakova et al., 2005), and *Binucleata* spp. (Refardt et al., 2008) warrants further work. Our results clearly show that the ITS barcode motif of *A. daphniae* is different from that of other species of the “*Agglomeratidae sensu clade*” using available ITS sequences. This further supports that ITS motif is a potential candidate barcode to differentiate morphologically similar daphnid-infecting microsporidia.

The integrative evidence from ecological, morphological, ultrastructural and phylogenetic data supports the erection of a new species, *Agglomerata daphniae* n. sp. This is the first report of zooplankton-infecting microsporidia in China.

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