Journal of Invertebrate Pathology 182 (2021) 107578

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Morphological and molecular characterization of a new microsporidium, *Janacekia tainanus* n. sp. from the adipose tissue of *Kiefferulus tainanus* (Diptera: Chironomidae) in China

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ARTICLE INFO

Keywords:
Microsporidia
Chironomid
Janacekia-Jirovecia-Bacillidium lineage

ABSTRACT

We reported a new microsporidium *Janacekia tainanus* n. sp. from the adipose tissue of the midge *Kiefferulus tainanus* Kieffer, 1912 collected from a eutrophic pond in Daye city, Hubei Province, China. Infected chironomid larvae with hypertrophied adipose tissue exhibited porcelain-white. All developmental stages possessed large nuclei. The earliest stages observed were diplokaryotic meronts which were in direct contact with the host adipocyte cytoplasm. Diplokaryotic meronts developed into sporonts with the deposition of electron-dense coagulum on their surface. Multinucleate sporogonial plasmodia developed into uninucleate sporoblasts by the rosette-like division. Mature spores were oval and monokaryotic, measuring 6.14 ± 0.27 (5.65–6.67) μm long and 3.71 ± 0.12 (3.43–3.98) μm wide. Bipartite polaroplast consisted of a narrow anterior lamella and a wide posterior lamella. Isofilar polar filaments coiled 13–17 turns and arranged in one row. The exospore was thin and of no stratification, but remarkably covered with tubular secretions. The electron-lucent endospore was thick and measured 145–352 nm wide. Phylogenetic analysis based on the obtained SSU rDNA sequence indicated that the present species clustered closely with *Jirovecia sinensis*, a species with rod-shaped mature spores isolated from the coelomocytes of *Branchiura sowerbyi*. Consistent with the previous result, the monophyletic clade of *Jirovecia-Bacillidium-Janacekia* was sister to *Pseudonosema* clade and then collectively nested within Clade V of Class Aquasporidia sensu Vossbrinck and Debrunner-Vossbrinck (2005). The novel species did not form an independent monophyletic lineage with the congener, *Janacekia debaisieuxi*. Based on the morphological characters and ultrastructural features, as well as SSU rDNA-inferred phylogenetic relationships, a new species in the genus *Janacekia*, *Janacekia tainanus* n. sp. was designated. This is the first report of aquatic arthropod-infecting microsporidia in China.

1. Introduction

Microsporidia are ubiquitous obligate intracellular eukaryotic parasites that can infect nearly all vertebrates and invertebrates, including humans (Cali and Takvorian, 2014). Microsporidia possess a unique extrusive structure, the evaginable polar tube, by which the infective sporoplasm can be directly injected into the host cytoplasm to initiate the infection during the spore germination (Vávra and Lukeš, 2013). More than 1600 microsporidia belonging to about 200 genera have been described worldwide, among which more than 57 genera are known to infect dipteran insects (Becnel and Andreadis, 2014; Vávra et al., 2017). Most of them, however, were described in the 20th century by the traditional taxonomic approach which was mainly based upon spore morphological features and ultrastructural data of the life developmental stages. Due to the unreliability of ultrastructural features of life cycle developmental stages as taxonomic criteria, the high plasticity of

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https://doi.org/10.1016/j.jip.2021.107578
Received 11 November 2020; Received in revised form 19 February 2021; Accepted 24 February 2021
Available online 19 March 2021

spore morphology and the frequent occurrence of spore dimorphism or polymorphism of some species, the traditional taxonomy of Microsporidia is increasingly challenged by the SSU rDNA sequence-based molecular phylogeny, no matter at the high taxonomic level or at the genus and species level (Sokolova et al., 2007; Stentiford et al., 2013a; Vossbrinck and Debrunner-Vossbrinck, 2005). Therefore, a comprehensive taxonomic approach of integrating morphological features, life cycle, host and habitat information and molecular phylogeny of based predominantly upon the SSU rDNA sequence data has been reached a consensus of being a necessity for the novel microsporidian species description and the validation of previously poorly described species (Bekircan, 2020; Stentiford et al., 2013b; Tokarev et al., 2012). Insufficiently available sequence data due to the undersampling, however, prevents severely from figuring out the more reasonable classification and phylogeny of the phylum Microsporidia.

Chironomid larvae (Diptera: Chironomidae) are a group of insects of high ecological importance in the freshwater ecosystem (Armitage et al., 1995) and the common host of aquatic Diptera-infecting microsporidia (Tokarev et al., 2010a). To date, more than 51 microsporidia belonging to 24 genera have been described from chironomids (Tokarev et al., 2010b; Voronin, 1999). One possible explanation for the high diversity of chironomid larvae-infecting microsporidia is the long developmental process and mass propagation of chironomid hosts in the silt layer of water bottom of ponds and lakes which provides a favorable condition for the prolonged acquisition of infective microsporidian spores from various sources (Tokarev et al., 2010a). As such, most of the chironomids-infecting microsporidia were described and identified on the basis of light and electron microscopic studies and sequence data was available for only five species among them (Issi et al., 2012; Tokarev et al., 2010a, 2010b, 2012).

In China, aquatic microsporidia have been sporadically recorded from teleosts, crustaceans and benthic oligochaetes; however, to the best of our knowledge, no microsporidium has been found from chironomids (Ding et al., 2016a; 2016b; Liu et al., 2019; 2020; Wang et al., 2017). To further uncover the species and genetic diversity of aquatic microsporidia in China, we conducted an investigation of the diversity of chironomid-infecting microsporidia in the middle and lower reaches of the Yangtze River. In the present work, a novel chironomid-infecting species, *Janacekia tainanus* n. sp. was characterized with morphological, ultrastructural and molecular data.

2. Materials and methods

2.1. Collection of specimens and light microscopical observation

Chironomid larvae were collected from a eutrophic pond in Daye city, Hubei province, China (30°17′46.49″ N, 114°44′8.53″ E) in June of 2019. Samples were transported live immediately to the laboratory for further parasitological examination. Sampled chironomid larvae were morphologically identified (Tang, 2006) and screened for possible microsporidian infection using the porcelain white appearance of their teguments. Infected adipose tissues were used to make wet mount preparations that were then observed under light microscopy. Spore images were captured using an Olympus BX 53 microscope equipped with an Olympus DP72 digital camera (Olympus, Japan). The remaining infected adipose tissues 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 infected adipose tissues were trimmed and washed in sodium cacodylate buffer twice (10 min) and placed into 1% osmium tetroxide (OsO4) in the same buffer for 1 h. After dehydration through a gradually ascending series of ethanol and propylene oxide, samples were embedded in Spurr resin. Ultrathin sections (70–90 nm) were mounted on an uncoated copper grid and stained with uranyl acetate and lead citrate (Liu et al., 2019). Sections were examined under a Hitachi HT-7700 TEM. Two infected chironomid specimens were examined by TEM.

2.3. DNA extraction, PCR, and sequencing

Ethanol-fixed infected adipose tissues were washed with distilled water 3 times to remove ethanol remnants. The isolated adipose tissues were homogenized using Lysing Matrix B FastPrep® tubes and a FastPerp cell disrupter (2 min at 8 m s^{-1}). The genomic DNA was extracted from the prepared homogenate using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. The general microsporidian primer pair V1f (5′-CACCAGGTTGATTCTGCC-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) was used to amplify the partial SSU rRNA gene (Weiss and Vossbrinck, 1999). PCR was carried out in a 50 μl reaction system, containing PCR buffer, 200 mM dNTP, 2 mM $MgCl_2$, 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, purified using a PCR purification kit (CWBiotech, Beijing, China) and cloned into a PMD-18 T vector system (Takara, Tokyo, Japan). Two positive clones were randomly selected to be sequenced with the ABI BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3100 Genetic Analyzer.

2.4. Molecular characterization

The obtained sequences of fragments were assembled by BioEdit (Hall, 1999). The obtained consensus sequences were verified as a microsporidium by a BLAST search. Sequences with high similarity and those of our interest were retrieved from the GenBank database. A total of 27 sequences were aligned with Clustal X by default setting (Thompson et al., 1997). This alignment was corrected manually using the alignment editor function within MEGA 6.0 (Tamura et al., 2013). *Vittaforma corneae* (U11046), *Crispospora chironomi* (GU130407) and *Anisofilariata chironomi* (GU126383) 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 determined to be GTR + I + G by ModelTest 3.7 using Akaike information criteria. Two independent runs were conducted with four chains for one 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. Tree was 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).

3. Results

3.1. Light microscopy

In total, 295 chironomid larvae were collected, examined, and morphologically identified to be *Kiefferulus tainanus*, *Einfeldia insolita* and *Paratanytarsus* sp. The suspected microsporidian infection, however, was only found in the adipose tissue of *K. tainanus*. The infection caused significant hypertrophy of host adipocytes and the porcelain white coloration of the host tegument. The prevalence of infection was 28.3% (30/106). Porcelain white hypertrophied adipose tissues were fully filled with xenoma-like formations after rupturing. Fresh spores were oval and measured 6.14 ± 0.27 (5.65–6.67) μm in length and 3.71 ± 0.12

(3.43–3.98) μm in width (N = 40) (Fig. 1a).

3.2. TEM

The earliest stages observed were late meronts which were in direct contact with the host cell cytoplasm. The meronts contained a remarkable large diplokaryotic nucleus which occupied the major volume of the cell and were surrounded by an amorphous electron-dense membrane (Fig. 1b). The subsequently observed stages were sporonts which possessed a large elliptical nucleus and measured 2.7 μm in diameter. Electron-dense granular coagulum in a spotty pattern could be found to accumulate on the surface of sporonts (Fig. 1c). Following the further nuclei division, uninucleate sporonts developed into multinucleate sporogonial plasmodia. Subsequently, nuclei moved to the periphery and multinucleate sporogonial plasmodia split into lobes in a rosette-like fashion, with one nucleus in each bud, to produce 4–12 uninucleate sporoblasts (Fig. 1d). Early sporoblasts possessed a round nucleus 2.0–2.3 μm in diameter and sufficient endoplasmic reticulum. The electron-dense coagulum progressively became flat during the development of sporonts to sporoblasts (Fig. 1e). Finally, the electron-dense coagulum gave rise to a number of tubules on the surface of sporoblasts which traversed the sporophorous vesicle envelope (Fig. 1f, fi). Meanwhile, the tubular secretions were visible in the sporophorous vesicle envelope (Fig. 1f). After a series of further development, the cytoplasm of sporoblasts became denser and the polar filament was formed. The electron-dense substance deposited on the surface of sporoblasts formed fleece-like structures (Fig. 2a-b). Development of uninucleate sporoblasts to mature spores involved the differentiation of typical spore organelles, including the trilaminar spore wall, anchoring disk, bipartite polaroplast and polar filaments. An individual sporophorous vesicle covered only a single oval mature spore. Mature spores were covered with tubular secretions on their surface which connected them to the sporophorous vesicle envelope (Fig. 2c, 2d). The spore wall exhibited typical three layers, including a 24–52 nm thick electron-dense exospore, a 145–352 nm thick electron-lucent endospore and an 11 nm thick plasma membrane (Fig. 2d). The polaroplast was bipartite with a narrow anterior lamella and a wide posterior lamella (Fig. 2e). An umbrella-like polar disc locating in the apex of spores was surrounded by the polaroplast (Fig. 2e). The isofilar polar filament was coiled 13–17 turns and arranged in one row (Fig. 2c). The polar filament measured 153–157 nm in diameter and exhibited seven discontinuous density concentric circles which included a 4 nm thick unit membrane, a 5 nm thick moderately electron-lucent layer, a 4 nm thick electron- dense layer, an 8 nm thick moderately dense layer, a 7 nm thick moderately electron- lucent layer, a 15 nm thick electron-dense layer and a wide lucent center (Fig. 2f).

3.3. Molecular characterization

The SSU rDNA sequence similarity of two clones of the described microsporidium was 99.58%, with variations of only six loci along with the 1442 bp fragment. Sequences were deposited in GenBank under the accession number MT622752 and MT622753. A BLAST search indicated that these two SSU rDNA sequences were not identical to any microsporidian sequence available in GenBank, but most similar to *Jirovecia sinensis* (MN752317, 92.95%) which were found from coelomocytes of the freshwater oligochaete *Branchiura sowerbyi*. Other closely related species included *Bacillidium vesiculoformis* (AJ581995, 92.03%) infecting haemocytes of *Nais simplex* (Annelida), *Pseudonosema cristatellae* (AF484694, 88.43%) infecting epithelium of *Cristatella mucedo* (Bryozoa) and *Bacillidium* sp. (AF104087, 83.06%) infecting coelomocytes of *Lumbriculus* sp. (Annelida). The present novel species, however, is comparatively genetically distant from the congener, *Janacekia debaisieuxi* (below 82% sequence similarity). The pairwise distances/

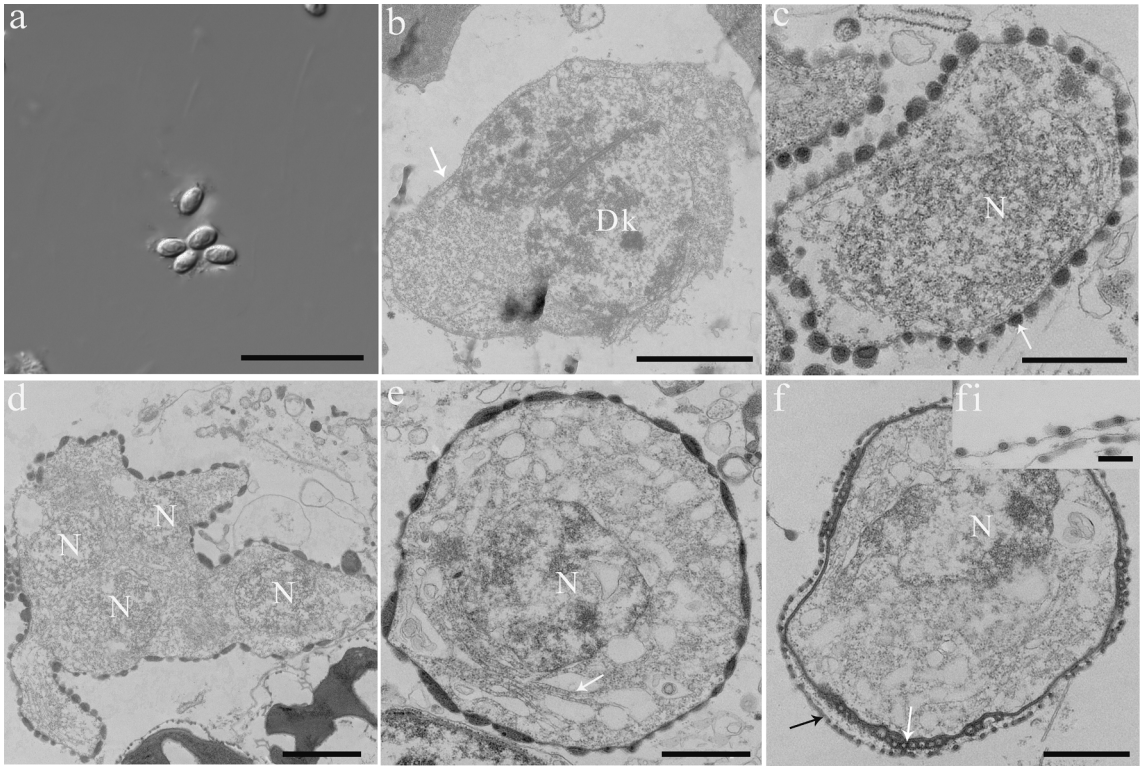

Fig. 1. Light microscopy and transmission electron microscopy of *Janacekia tainanus* n. sp. a. Fresh spores observed under light microscopy. Bar = 20 μm. b. A diplokaryotic meront surrounded by a simple plasma membrane (arrow). Bar = 2 μm. c. Sporonts with an elliptical nucleus covered by electron-dense coagulum (arrow). Bar = 1 μm. d. Multinucleate sporogonial plasmodia developed into sporoblasts by rosette-like division. Bar = 2 μm. e. Early sporoblasts possessed a round nucleus and prominent endoplasmic reticula (arrow). Bar = 1 μm. f. Uninucleate sporoblasts enclosed within sporophorous vesicle (black arrow). Tubular secretions (white arrow) were visible. Bar = 1 μm. fi. The magnification of the sporophorous vesicle. Bar = 200 nm. Dk, diplokaryon; N, nucleus.

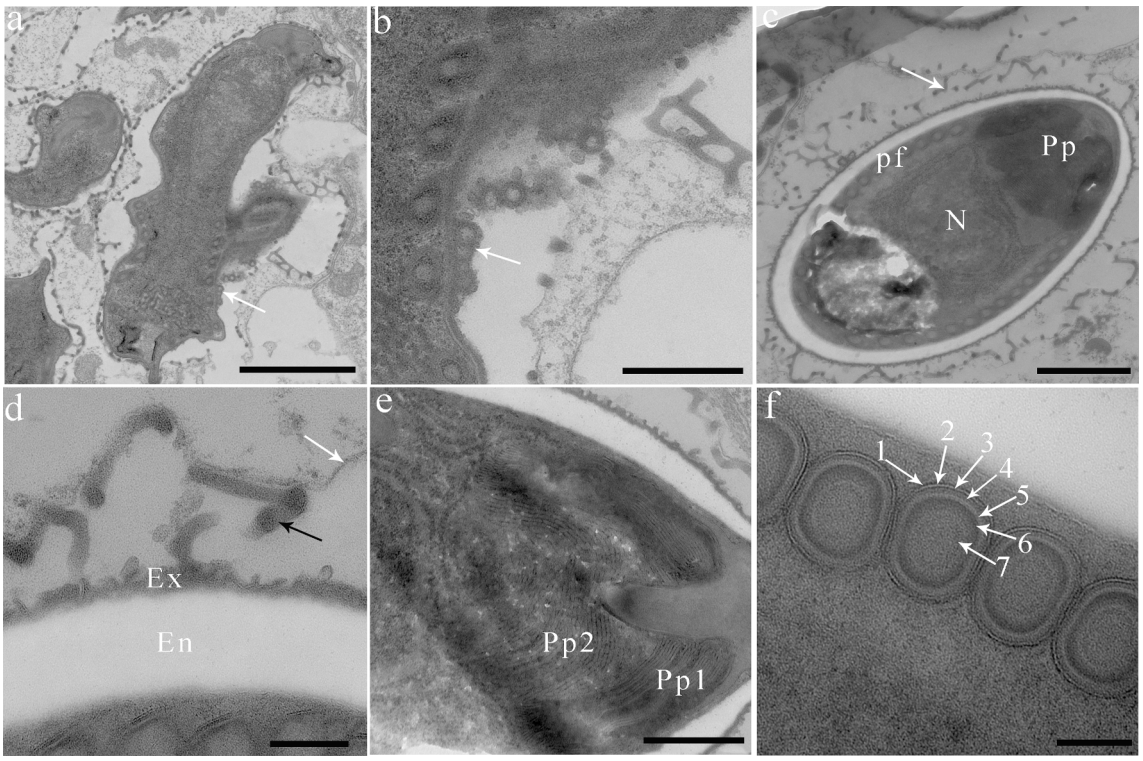

Fig. 2. Electron microscopy of *Janacekia tainanus* n. sp. a. Sporoblasts with denser cytoplasm, electron-dense substance (arrow) deposited on the surface of sporoblasts to form fleece-like structure. Bar = 2 μm. b. The magnification of fleece-like (arrow) structure. Bar = 500 nm. c. A mature spores with typical microsporidian features of internal structure, including the isofilar polar filaments, a bipartite polaroplast, a large nucleus and a trilaminar spore wall consisting of an electron-dense exospore, an electron-translucent endospore and a plasma membrane. An individual sporophorous vesicle (arrow) covered a single oval mature spore. Bar = 1 μm. d. Trilaminar spores wall consisting of an electron-dense exospore, an electron-translucent endospore and a plasma membrane. Tubular secretions (black arrow) on the surface of spores connecting the exospore with the sporophorous vesicle envelope (white arrow). Bar = 200 nm. e. The bipartite polaroplast including narrow lamellae and wide lamellae. Bar = 500 nm. f. Transverse section of polar filament coils exhibiting seven discontinuous density concentric circles. Bar = 100 nm. AD, anchoring disc; En, endospore; Ex, exospore; N, nucleus; Pf, polar filament; Pp, polaroplast.

similarities calculated by Kimura 2-parameter model between the present species and microsporidian species of high sequence similarity ranged from 0.071/92.95% (*Jirovecia sinensis* MN752317) to 0.212/78.82% (*Schroedera plumatellae* AY135024) (Table 1). Surprisingly, two available sequences of *Janacekia debaisieuxi* from 2 different hosts (AJ252950 and AY090070) are only 91.88% similar. 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 phylogenetic results robustly showed that the present species clustered firstly with *Jirovecia sinensis* with a high support value and then formed a sister group with a late evolutionary branch consisting of *Bacillidium* sp. and *Janacekia debaisieuxi,* which collectively clustered with *Bacillidium vesiculoformis* to form an independent *Bacillidium-Janacekia-Jirovecia* clade. Consistent with our previous analysis, bryozoan-infecting *Trichonosema* spp. was the basal branch to the dichotomy of the *Bacillidium-Janacekia-Jirovecia-Pseudonosema* lineage with representatives infecting oligochaetes and dipterans and the *Bryonosema-Schroedera-Neoperezia* clade with representatives of bryozoan and chironomid-infecting microsporidia (Fig. 3).

Collectively, based on the morphological, ultrastructural, ecological and molecular information, this novel species was provisionally positioned in the genus *Janacekia* and designated as *Janacekia tainanus* n. sp.

4. Taxonomic summary

Name: Janacekia tainanus n. sp.
Phylum: Microsporidia Balbiani, 1882
Family: Neopereziidae Issi et al., 2012
Genus: Janacekia Larsson, 1983

Type host: Kiefferulus tainanus Kieffer, 1912 (Insecta, Diptera, Chironomidae)

Type locality: Eutrophic ponds of Daye city, Hubei province, China (30°17′46.49″ N, 114°44′8.53″ E).

Site of infection: Adipose tissue.

Meronts: Diplokaryotic meronts are surrounded by an amorphous electron-dense membrane.

Sporonts: Diplokaryotic sporonts are not observed. The uninucleate sporonts possess a large elliptical nucleus and electron-dense granular coagulum accumulate on the surface of sporonts. Multinucleate sporogonial plasmodia with isolated nuclei divide in a rosette-like manner.

Sporoblasts: Uninucleate sporoblasts enclosed within individual sporophorous vesicles.

Spores: Oval spores are uninucleate, 6.14 ± 0.27 (5.65–6.67) μm long and 3.71 ± 0.12 (3.43–3.98) μm wide. Isofilar polar filaments coil 13–17 turns and arrange in one row. The anterior part of the bipartite polaroplast is of narrow lamellae and the posterior is of wide lamellae. The trilaminar spore wall consists of a 24–52 nm thick electron-dense exospore, a 145–352 nm thick electron-lucent endospore and an 11 nm thick plasma membrane.

Type material: Syntype specimens of TEM resin blocks deposits in the Museum of Hydrobiological Sciences, Institute of Hydrobiology, Chinese Academy of Sciences with accession number of MTR20190618.

Etymology: The species name relates to host species name.

Gene sequences: Depositing in GenBank under accession numbers of MT622752, and MT622753.

Table 1
Pairwise nucleotide sequence identity (upper right) values and evolutionary distances (left bottom) among *Janacekia tainanus* n. sp. and 13 other microsporidium 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	13	14	15
1 *Janacekia tainanus* n. sp. MT622752	–	99.53	92.95	92.03	88.43	83.06	81.70	81.60	81.55	81.54	81.33	81.06	80.55	78.82	78.82
2 *Janacekia tainanus* n. sp. MT622753	0.0047	–	92.73	91.92	88.42	82.93	81.43	81.46	81.28	81.27	80.93	80.93	80.02	78.82	78.82
3 *Jirovecia sinensis* MN752317	0.0705	0.0727	–	89.19	87.23	82.43	81.43	81.84	81.60	82.40	82.25	81.64	81.73	80.96	79.74
4 *Bacillidium vesiculoformis* AJ581995	0.0797	0.0808	0.1081	–	89.13	82.58	81.61	81.26	81.83	82.07	82.99	82.37	81.48	81.35	79.12
5 *Pseudonosema cristatellae* AF484694	0.1157	0.1158	0.1277	0.1087	–	82.47	81.97	82.50	82.31	81.03	82.12	81.76	81.96	79.81	78.54
6 *Bacillidium* sp. AF104087	0.1694	0.1707	0.1757	0.1742	0.1753	–	77.98	79.09	78.64	89.25	78.00	77.30	79.04	89.66	78.89
7 *Bryonosema plumatellae* AF484692	0.1830	0.1857	0.1816	0.1839	0.1803	0.2202	–	93.94	96.05	78.42	78.84	77.75	96.68	76.48	90.90
8 *Schroedera airthreyi* AJ749819	0.1840	0.1854	0.1836	0.1874	0.1750	0.2091	0.0606	–	95.11	78.72	78.76	77.33	94.26	76.92	90.55
9 *Neoperezia semenovaiae* HQ396520	0.1845	0.1872	0.1840	0.1817	0.1769	0.2136	0.0395	0.0489	–	79.47	78.68	77.07	96.26	76.47	91.34
10 *Janacekia debaisieuxi* AJ252970	0.1846	0.1873	0.1760	0.1793	0.1897	0.1075	0.2158	0.2128	0.2053	–	77.73	76.36	79.07	91.88	78.39
11 *Trichonosema pectinatellae* AF484695	0.1867	0.1906	0.1775	0.1701	0.1788	0.2200	0.2116	0.2124	0.2132	0.2227	–	94.29	78.67	78.24	77.06
12 *Trichonosema algonquinensis* AY582742	0.1867	0.1907	0.1836	0.1763	0.1824	0.2270	0.2225	0.2267	0.2293	0.2364	0.0571	–	77.73	76.75	76.26
13 *Neoperezia chironomi* HQ396519	0.1894	0.1907	0.1827	0.1852	0.1804	0.2096	0.0332	0.0574	0.0374	0.2093	0.2133	0.2227	–	77.29	91.01
14 *Janacekia debaisieuxi* AJ252950	0.1945	0.1998	0.1904	0.1865	0.2019	0.1034	0.2352	0.2308	0.2353	0.0812	0.2176	0.2325	0.2271	–	77.41
15 *Schroedera plumatellae* AY135024	0.2118	0.2118	0.2026	0.2088	0.2146	0.2111	0.0910	0.0945	0.0866	0.2161	0.2294	0.2374	0.0899	0.2259	–

5. Discussion

This study describes and characterizes a novel microsporidium infecting adipocytes of the midge *Kiefferulus tainanus* and is the first report of aquatic arthropod-infecting microsporidia in China. Morphologically, *Janacekia tainanus* n. sp. corresponds almost to the definition of the genus *Janacekia*, including diplokaryotic meronts, uninucleate spores, the division of multinucleate sporogonial plasmodia in the rosette-like fashion, oval or lightly pyriform spores, isofilar polar filament, thin electron-dense and non-stratified exospore and sporophorous vesicle with wide tubules (Larsson, 1983). Three *Janacekia* species have been so far reported, including *J. debaisieuxi* infecting adipocytes of *Odagmia ornata* and *Simulium* sp. (Larsson, 1983; Weiser and Žižka, 1975), *J. undinarum* infecting adipocytes of *Odagmia ornata* (Larsson, 1983) and *J. adipophila* infecting adipocytes of *Ptychoptera paludosa* (Larsson, 1992). The strict morphological comparison between the present species and all congeners is summarized in Table 2. *Janacekia tainanus* n. sp. can be easily differentiated from *J. adipophila* by its smaller size, different polaroplast pattern (narrow lamellae and wide lamellae vs. narrow lamellae and globules) and more coils of the polar filament within mature spores. Moreover, the inclusion of sporophorous vesicle of *J. adipophila* is granular (Larsson, 1992), rather than tubular for the present species. *J. undinarum* also possess a bipartite polaroplast, however, its mature spore is smaller than that of *Janacekia tainanus* n. sp. Also, the number of polar filaments coils of *J. undinarum* (7 vs. 13–17) are also different from that of the novel species. Moreover, the structure of the electron-dense coagulum of *J. undinarum* occurs during the sporoblast stage, rather than during the sporont stage, as observed in *Janacekia tainanus* n. sp. (Larsson, 1983). *J. debaisieuxi*, the type species of the genus, originally identified as *Pleistophora debaisieuxi*, is morphologically similar to the present species in the fleece-like structure on the surface of the sporoblasts and electron-dense coagulum deposition on the surface of sporonts; however, it is distinct from *Janacekia tainanus* n. sp. by its smaller spores and different polar filament pattern (18–24 turns arranged in two or three rows vs. 13–17 turns arranged in one row), as well as the polaroplast pattern (lamellar and globular vs. lamellar and lamellar) (Larsson, 1983; Weiser and Žižka, 1975). So, *Janacekia tainanus* n. sp. is morphologically distinct from all previously reported congeners.

More than 50 microsporidia belonging to 24 genera have so far been recorded from chironomids. The uninucleate sporoblast, spore number per sporophorous vesicle and tubular secretions on the sporophorous

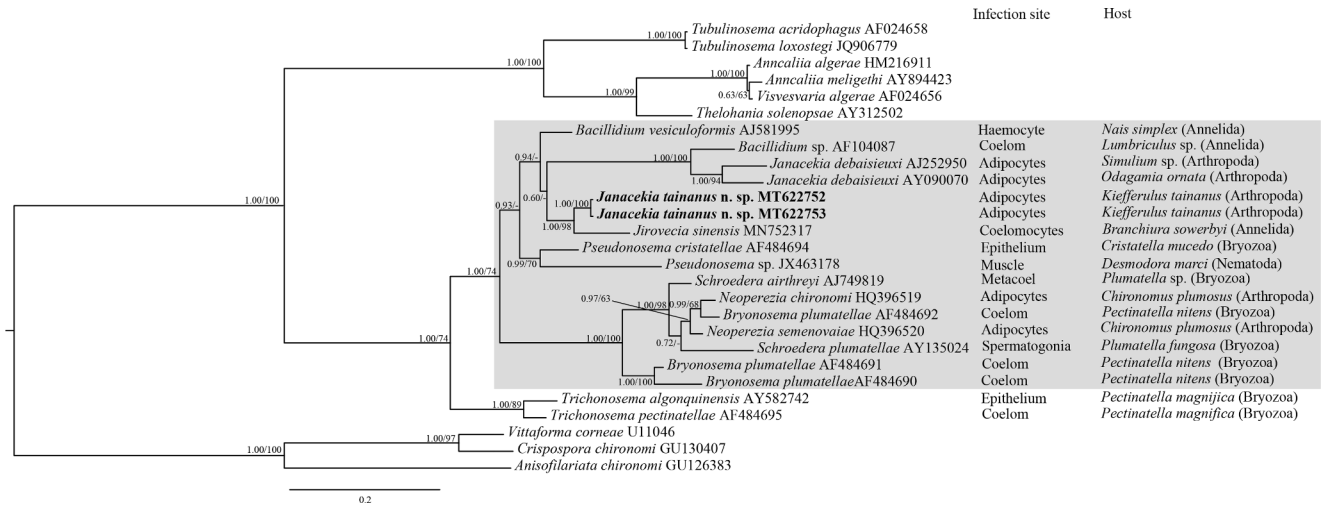

Fig. 3. The SSU rDNA-inferred phylogenetic relationships between *Janacekia tainanus* n. sp. and the other aligned microsporidian species by Bayesian Inference (BI) method. The species names are followed by GenBank accession number. BI posterior probabilities were shown firstly, followed by ML support values on branch nodes. The present species was highlighted in bold. The infection site and host of species belonging to *Bacillidium-Janacekia-Jirovecia-Pseudonosema-Schroedera-Neoperezia-Bryonosema-Trichonosema* cluster were presented.

Table 2
Comparison of *Janacekia tainanus* n. sp. with the most closely morphological related *Janacekia* spp.

Species	Host	Infection sites	Sporogonial plasmodia division, number	Sporophorous vesicle inclusions	Spore shape and size (μm)	Polar filaments number	Polaroplast	References
Janacekia tainanus n. sp.	*Kiefferulus tainanus*	Adipose tissue	Rosette-like, 4–12	Tubular	Oval 5.7–6.7 × 3.4–4.0	13–17 coils arranged one row	Closely packed anterior and wider posterior lamellae	(Herein)
J. debaisieuxi	*Odagmia ornata*, *Simulium* sp.	Adipose tissue	Rosette-like, more than 16	Tubular	Oval 4.5–5.2 × 2.8–3.0	18–24 coils arranged two or three rows	Lamellar anterior and globular posterior parts	(Larsson, 1983; Weiser and Žižka, 1975)
J. undinarum	*Odagmia ornata*	Adipose tissue	Rosette-like, more than 12	(–)	Oval 3.2–3.8 × 2.0–2.2	7 coils arranged one row	Closely packed anterior and wider posterior lamellae	(Larsson, 1983)
J. adipophila	*Ptychoptera paludosa*	Adipose tissue	Rosette-like, 8–16	Granular	Oval 9.1–11.2 × 4.2–6.3	12–13 coils arranged one row	Lamellar anterior and globular posterior parts	(Larsson, 1992)

vesicle envelope can discriminate the present novel species from species of *Bohuslavia*, *Coccospora*, *Cylindrospora*, *Chapmanium*, *Helmichia*, *Napamichum*, *Pernicivesicula*, *Striatospora*, *Thelohania*, *Toxoglngea*, *Toxospora, Gurleya, Scipionospora, Anisofilariata, Crispospora, Evlachovaia, Amblyospora, Jirovecia*, *Bacillidium and Neoperezia,* all of which infect aquatic chironomids (Becnel and Andreadis, 2014; Larsson, 1990, 1994; Tokarev et al., 2010a, 2010b).

Intriguingly, sequence comparison and phylogenetic analysis indicated clearly that *Janacekia tainanus* n. sp. is genetically closer to *Jirovecia sinensis* than the congeneric species, *J. debaisieuxi. Jirovecia sinensis* was recently described from coelomocytes of the freshwater oligochaete *Branchiura sowerbyi* (Oligochaeta: Tubificidae) in China which was characterized by a diplokaryotic nucleus, rod-shaped spores and tail-like posterior prolongation (Liu et al., 2020). Furthermore, like other species with rod-shaped spores belonging to *Bacillidium*, *Hrabyeia*, *Mrazekia* and *Rectispora*, species of *Jirovecia* possessed manubrium-type polar filaments which are significantly different from most Microsporidia (Larsson, 1990; Liu et al., 2020). So, *Janacekia tainanus* n. sp. is significantly distinct from *Jirovecia* species in terms of morphological characteristics and sporogonic process. However, it was shown that Microsporidia of significantly dissimilarities in the life cycle and spore morphology were genetically closely related (Baker et al., 1995; Issi et al., 2012; Refardt et al., 2008; Sokolova et al., 2007). Conversely, Microsporidia with similar spore morphology and ultrastructure are not closely related, as in the case of *Bacillidium vesiculoformis* and *Mrazekia macrocylopis* (Issi et al., 2010; Morris et al., 2005). Therefore, traditional systematics of Microsporidia, no matter at the higher-level taxa, genera and species level, is being severely challenged by the rDNA-based phylogenetic analysis. To find the true synapomorphy of Microsporidian taxon at order, family and genus level by strictly relating the phylogenetic relationships with all morphological, ultrastructural and ecological features is crucial to develop a natural and logical classification of this group of parasites. Here, we suggest deploying molecular data as one of the taxonomical criteria of Microsporidia which has been used for the newly emendation of the genus of the phylum Myxozoa (Jirků et al., 2007).

Given the comparative low sequence similarity of *Janacekia tainanus* n. sp. and *J. debaisieuxi* (81% with AY090070 and 79% with AJ252950), it can be suspected that high diversity of *Janacekia* species waits to be uncovered. Meanwhile, 92% sequence similarity of two isolates of *J. debaisieuxi* from different hosts suggests that it is probably an assemblage of species which is consisted with their heterogeneous morphological and ultrastructural characters documented by Larsson (1983). Furthermore, the genetic relatedness of two *Bacillidium* species, *B. vesiculoformis* and *Bacillidium* sp. is lower than that between *B. vesiculoformis* and the present novel species. So, the taxonomic validity of the genus *Bacillidium* also warrants further work. Consistent with previous reports (Canning et al., 2002; Issi et al., 2012; Morris and Adams, 2002; Morris et al., 2005), the present phylogenetic analysis also supports that Bryozoa-infecting, aquatic Arthropoda-infecting,

Nematode-infecting and Annelida-infecting Microsporidia, including *Bacillidium, Janacekia, Jirovecia, Pseudonosema, Bryonosema, Schroedera* and *Neoperezia* are closely related, although they traditionally belong to the different families, including Pseudonosematidae, Mrazekiidae, Janacekiidae and Neopereziidae, respectively. The sympatric distribution of chironomids, bryozoans, nematodes and oligochaetes and their potential trophic relationships support that these zoobenthos-infecting microsporidia are possible of the different stage of their life cycle (Armitage et al., 1995). Adaptation to different hosts possibly produces variable spore morphology (Stentiford et al., 2013a). Previously, Morris et al. (2005) suggested that another host was involved in the life-cycle of *B. vesiculoformis* for failure to induce direct infection among oligochaetes. Dimorphic or polymorphic development has also been documented in *Amblyospora* spp. and *Hyalinocysta* spp. which have mosquitos and copepods as final host and intermediate hosts, respectively (Andreadis and Vossbrinck, 2002., Andreadis et al., 2018). Thus, it can partially explain why species of morphological dissimilarity of this group of microsporidia are genetically intimated.

In terms of the affiliation of *Bacillidium*, *Janacekia*, *Jirovecia*, *Pseudonosema*, *Bryonosema*, *Schroedera* and *Neoperezia* species to the family, it was previously suggested that members of the Mrazekiidae and Pseudonosematidae formed alternate parts in the life cycles of the same parasites and the Pseudonosematidae maybe a junior synonym of the Mrazekiidae (Morris et al., 2005). However, Mrazekiidae was recently demonstrated to be polyphyletic, rather than monophyletic (Issi et al., 2010). Subsequently, Issi et al. 2012 demonstrated that Pseudonosematidae was a junior synonym of the Neoperezia. The polaroplast of both *Bacillidium* and *Jirovecia* is similar to that of Neoperezia, including bipartite and membrane-bound anterior covering the posterior one in a bell-like manner (Larsson, 1990, 1994). Furthermore, *Janacekia tainanus* n. sp. and *J. debaisieuxi* display maximum structural resemblance with the diagnostic features of the family Neoperezia, including all developmental stages possessing a large nucleus, meronts and meront/sporont transitional stage possessing a diplokaryon, monokaryotic spores, the anterior part of bipartite polaroplast being bounded by a membrane and covering the posterior one in a bell-like manner, isofilar polar filament arranging in one to four rows, and both parasitize the class Insecta (Issi et al., 2012). So, we suggest provisionally place *Bacillidium, Janacekia, Jirovecia, Pseudonosema, Bryonosema, Schroedera* and *Neoperezia* species in the family Neoperezia. Multi-gene loci-based phylogenetic analysis of all members of the family Neoperezia will possibly improve the intra-group's taxonomy and the species identification and delimitation (Tokarev et al., 2020; Tosun, 2020).

In conclusion, comprehensive data from morphological features, ultrastructural characteristics and phylogenetic analysis robustly support that the present species is new to science, which is the first report of aquatic arthropod-infecting microsporidia in China. We suggest to deploy explicitly the molecular data to the taxonomy of the family Neoperezia.

Acknowledgments

This work was supported by grants from the Natural Sciences Foundation of China (31772411), Natural Science Foundation of Hubei Province (2018CFA074), Young experts of Taishan Scholars in Shandong Province and Initiative grant for high-level personnel recruitment in Qingdao Agricultural University awarded to JY Zhang. We appreciate very much the critical comments from two anonymous reviewers which improve the ms much.

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