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Taxonomic revision of the genus *Zygorhizidium*: *Zygorhizidiales* and *Zygophlyctidales ord. nov.* (*Chytridiomycetes*, *Chytridiomycota*)

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Abstract: During the last decade, the classification system of chytrids has dramatically changed based on zoospore ultrastructure and molecular phylogeny. In contrast to well-studied saprotrophic chytrids, most parasitic chytrids have thus far been only morphologically described by light microscopy, hence they hold great potential for filling some of the existing gaps in the current classification of chytrids. The genus *Zygorhizidium* is characterized by an operculate zoosporangium and a resting spore formed as a result of sexual reproduction in which a male thallus and female thallus fuse via a conjugation tube. All described species of *Zygorhizidium* are parasites of algae and their taxonomic positions remain to be resolved. Here, we examined morphology, zoospore ultrastructure, host specificity, and molecular phylogeny of seven cultures of *Zygorhizidium* spp. Based on thallus morphology and host specificity, one culture was identified as *Z. willei* parasitic on zygnematophycean green algae, whereas the others were identified as parasites of diatoms, *Z. asterionellae* on *Asterionella*, *Z. melosirae* on *Aulacoseira*, and *Z. planktonicum* on *Ulnaria* (formerly *Synedra*). According to phylogenetic analysis, *Zygorhizidium* was separated into two distinct order-level novel lineages; one lineage was composed singly of *Z. willei*, which is the type species of the genus, and the other included the three species of diatom parasites. Zoospore ultrastructural observation revealed that the two lineages can be distinguished from each other and both possess unique characters among the known orders within the *Chytridiomycetes*. Based on these results, we accommodate the three diatom parasites, *Z. asterionellae*, *Z. melosirae*, and *Z. planktonicum* in the distinct genus *Zygophlyctis*, and propose two new orders: *Zygorhizidiales* and *Zygophlyctidales*.

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INTRODUCTION

Chytrids are early diverging lineages of fungi and characteristically reproduce with posteriorly uniflagellate zoospores. Traditionally, chytrids were classified based on thallus morphology (Sparrow 1960, Karling 1977) and once accommodated in the phylum *Chytridiomycota* (Barr 2001). During the last decade, systematics of the *Chytridiomycota* has dramatically changed based on molecular phylogenies and zoospore ultrastructure (Powell & Letcher 2014). *Chytridiomycota sensu* Barr (2001) was divided into three basal phyla, *Chytridiomycota*, *Blastocladiomycota*, and *Neocallimastigomycota* in the kingdom *Fungi* (James *et al.* 2006, Hibbett *et al.* 2007). Currently, chytrids (*s. str.*) are accommodated in the class *Chytridiomycetes* and their classification is based on molecular phylogenies and zoospore ultrastructure (Powell & Letcher 2014). Molecular phylogenetic analysis of chytrids revealed that the orders *Chytridiales* and *Spizellomycetales sensu* Barr (2001) were polyphyletic and separated into several monophyletic lineages (James *et al.* 2006). Subsequently, five of the lineages revealed by James *et al*. (2006) were defined based on zoospore ultrastructure and established as new orders: *Rhizophydiales* (Letcher *et al.* 2006), *Rhizophlyctidales* (Letcher *et al.* 2008), *Cladochytriales* (Mozley-Standridge *et al.* 2009), *Lobulomycetales* (Simmons *et al.* 2009), and *Polychytriales* (Longcore & Simmons 2012).

Recently, metabarcoding surveys of aquatic and terrestrial environments have revealed a number of undescribed lineages in the *Chytridiomycetes* (Lefèvre *et al*. 2008, 2012, Freeman *et al*. 2009, Monchy *et al*. 2011, Jobard *et al*. 2012, Comeau *et al*. 2016, Tedersoo *et al*. 2017). These results indicate that there are large gaps in the current systematics of chytrids. Taxonomic studies on chytrids have been primarily conducted on saprotrophic chytrids, which can grow on culture media (Letcher *et al*. 2006, Mozley-Standridge *et al.* 2009, Simmons *et al*. 2009, Longcore & Simmons 2012). However, many chytrids are obligate parasites (Sparrow 1960, Longcore 1996), which have not been sequenced yet due to difficulty of culturing them. Consequently, their phylogenetic positions remain largely unclarified (Wijayawardene *et al.* 2018). Generally, parasitic chytrids cannot grow on culture media and require living hosts to complete their life cycle, *i.e*. they must

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be maintained as a dual culture of chytrid and its host. Recent efforts in cultivating parasitic chytrids of algae highlighted that parasitic chytrids belong to novel lineages of known orders (Lepelletier *et al*. 2014, Seto *et al*. 2017, Van den Wyngaert *et al.* 2017, Rad-Menéndez *et al.* 2018, Seto & Degawa 2018a, b), as well as order-level novel clades (Karpov *et al*. 2014, Seto *et al*. 2017, Van den Wyngaert *et al*. 2018). These results indicate that parasitic chytrids corresponds with the gaps in the current systematics of chytrids.

The genus *Zygorhizidium* was erected by Löwenthal (1905) to include chytrids characterized by epibiotic, operculate zoosporangia and resting spores formed as a result of sexual reproduction, in which a male thallus and female thallus fuse via a conjugation tube. The genus contains 11 described species, all of which are parasitic on green algae, chrysophycean algae, or diatoms (Karling 1977). Taxonomy of *Zygorhizidium* in the current classification of chytrids based on zoospore ultrastructure and molecular phylogeny remains to be clarified. Beakes *et al.* (1988) examined zoospore ultrastructure of *Zygorhizidium* for the first time and revealed that *Zygorhizidium planktonicum*, parasitic on the diatom *Asterionella formosa*, possessed unique characters among the known orders in the *Chytridiomycetes*. Recently, Seto *et al.* (2017) established dual cultures of two species of diatom parasites, *Z. planktonicum* on *As. formosa* and *Z. melosirae* on *Aulacoseira* spp. and examined their phylogenetic positions. Results showed that these two species belonged to "Novel Clade II (Jobard *et al*. 2012)", an order-level novel clade including only environmental sequences of uncultured chytrids. Although a new order should be proposed for the novel clade including *Zygorhizidium*, taxonomic treatment was hindered because the phylogenetic position of type species *Z. willei* has not been examined.

In the present study, we established three new cultures of *Zygorhizidium* spp., including the type species *Z. willei*, that were studied along with four previous cultures (Seto *et al.* 2017). The purpose of this study was to clarify the taxonomic position of the genus *Zygorhizidium* based on both zoospore ultrastructure and molecular phylogenetics, and to re-examine species identification of diatom parasites, *Z. planktonicum* and *Z. melosirae*, based on their host specificity, zoospore ultrastructure, and molecular phylogenetics. Herein we identified seven cultures as four species: one species is *Z. willei* parasitic on zygnematophycean green algae, and the other three are diatom parasites: *Z. asterionellae* on *Asterionella*, *Z. melosirae* on *Aulacoseira*, and *Z. planktonicum* on *Ulnaria* (formerly *Synedra*, see Discussion). According to their zoospore ultrastructural characters and phylogenetic positions, we describe two new orders in the *Chytridiomycetes*: *Zygorhizidiales* including *Z. willei* and *Zygophlyctidales* including three diatom parasites, which are accommodated in the distinct genus *Zygophlyctis*.

MATERIALS AND METHODS

Isolation and culturing

We newly established three dual cultures of chytrids and their host algae (KS97, KS109, and SVdW-SYN-CHY1). Host algal and chytrid cultures were obtained and maintained as described in Seto *et al*. (2017) and Van den Wyngaert *et al.* (2017). We also used the previously established cultures (C1, KS94, KS98, and KS99; Seto *et al*. 2017) for cross-inoculation experiments, transmission electron microscopic observations, and molecular phylogenetic analyses. Detailed information on cultures can be found in Table 1.

Light microscopy

For morphological observations of the chytrid cultures, 12–60-h-old cultures were used. Living cultures mounted in WC medium (Guillard & Lorenzen 1972) were observed on microscope slides. Thalli on the host alga were imaged using an Olympus BX53 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP73 CCD camera (Olympus) or a ZEISS Axio Imager 2 microscope (Carl Zeiss, Tokyo, Japan) equipped with a ZEISS Axiocam 512 color (Carl Zeiss).

Transmission electron microscopy

For observations of zoospore ultrastructure of chytrid cultures C1, KS97, KS98, KS99, and SVdW-SYN-CHY1, zoospore suspensions were obtained as described below. A 3-d-old culture (100 mL) was concentrated (~8 mL) by centrifugation and incubated at room temperature for 2–3 h. When swimming zoospores were confirmed, the culture was filtered through a 5 µm nylon mesh filter to exclude the host algal cells, and we retained 7.5 mL of zoospore suspension. For fixation, the zoospore suspension was mixed with an equal volume of 2.5 % glutaraldehyde and 2 % osmium tetroxide in WC medium (final concentration of 1.25 % glutaraldehyde and 1 % osmium tetroxide). The mixture was incubated on ice for 90 min. Fixed zoospores were pelleted at 2 000 × *g* and 0 °C for 30 min. After washing in distilled water, the pellet was embedded in 1.5 % agarose type VII-A (Sigma-Aldrich, MO). Agarose blocks containing zoospores were then dehydrated in an ethanol series (10 %, 30 %, 50 %, 70 %, 75 %, and 90 % for 15 min per step, and 95 % once and 100 % twice

Table 1. Dual cultures used in this study.

Culture number	Species	Host (culture number)	Source	Date of sampling
C1	*Zygophlyctis melosirae*	*Aulacoseira ambigua* (C5)	Lake Inbanuma, Chiba, Japan	July 30, 2012
KS94	*Zygophlyctis melosirae*	*Aulacoseira ambigua* (KSA24)	Lake Shirakaba, Nagano, Japan	September 23, 2014
KS97	*Zygorhizidium willei*	*Gonatozygon brebissonii* (KSA15)	Lake Suwa, Nagano, Japan	June 7, 2015
KS98	*Zygophlyctis asterionellae*	*Asterionella formosa* (KSA60)	Pond Biwa, Nagano, Japan	October 31, 2015
KS99	*Zygophlyctis melosirae*	*Aulacoseira granulata* (KSA17)	Lake Suwa, Nagano, Japan	October 24, 2015
KS109	*Zygophlyctis melosirae*	*Aulacoseira granulata* (KSA17)	Lake Teganuma, Chiba, Japan	October 18, 2017
SVdW-SYN-CHY1	*Zygophlyctis planktonica*	*Ulnaria* sp. (HS-SYN2)	Lake Melzersee, Mecklenburg-Vorpommern, Germany	April 15, 2015

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for 20 min per step) and embedded in Agar Low Viscosity Resin (Agar Scientific, Stansted, UK). For ultrastructural observation of the thallus of KS97, a 12–24-d-old culture was prepared as described above. Ultrathin sections were prepared with an RMC MT-X ultramicrotome (RMC Products, AZ). Sections were stained with platinum blue (Inaga *et al*. 2007) and lead citrate (Venable & Coggeshall 1965). Sections were then imaged using a Hitachi HT7700 transmission electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 80 kV.

Cross-inoculation experiments

We performed cross-inoculation experiments using chytrid cultures (C1, KS98, KS99, and SVdW-SYN-CHY1) and 12 host diatom cultures. Diatom cultures included three cultures of each species *Au. ambigua*, *Au. granulata*, *As. formosa*, and *Ulnaria* sp. (Table 2). To obtain zoospore suspensions, 3- or 4-d-old chytrid cultures were filtered through a 5 µm nylon mesh filter and approximately 25 mL of zoospore suspensions were retained for each chytrid culture. In each well of a 24-well plate, 0.5 mL of exponentially growing host diatom culture (1-wk-old culture), 0.5 mL of the zoospore suspension, and 0.5 mL of WC medium were added. Triplicates were prepared for each diatom culture. Infection results of the experiments were evaluated by observation with an inverted light microscope after 3, 5, 7, 9, 11, 13, 15 d.

DNA extraction, amplification, and sequencing

We harvested about 10 zoospores from chytrid culture KS97 by micro-pipetting and transferred them into a 200-µL PCR tube, and they were used as the template for direct PCR. We amplified 18S rDNA, ITS1-5.8S-ITS2, and 28S rDNA (D1/D2 region) loci by PCR using KOD FX (TOYOBO, Osaka, Japan) with the following primer sets: NS1 and NS8 (White *et al.* 1990) for 18S rDNA, ITS5 and ITS4 (White *et al.* 1990) for ITS1-5.8S-ITS2, LR0R (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990) for 28S rDNA. Thermal cycling conditions for PCR amplification were: (1) 95 °C for 5 min, (2) 10 cycles of denaturation at 98 °C for 10 s, annealing at 55–50 °C (0.5 °C decrease per cycle) for 30 s, and extension at 68 °C for 3 min (18S rDNA) or 1 min (ITS1-5.8S-ITS2 and 28S rDNA), and (3) 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 68 °C for 3 min (18S rDNA) or 1 min (ITS1-5.8S-ITS2 and 28S rDNA). PCR products were purified by PEG precipitation. Cycle sequence reactions were conducted using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit and the following primers: NS1, NS4, NS6, NS8, SR2, and SR5 (White *et al.* 1990, Nakayama *et al*. 1996) for 18S rDNA, ITS5 and ITS4 for ITS1-5.8S-ITS2, and LR0R and LR5 for 28S rDNA. DNA sequences were analyzed using an ABI PRISM 3130 Genetic Analyzer.

DNA was extracted from chytrid cultures KS109 and SVdW-SYN-CHY1 using the HotSHOT method (Truett *et al.* 2000). We amplified 18S rDNA, ITS1-5.8S-ITS2, and 28S rDNA (D1/D2 region) loci by PCR using KOD FX Neo (TOYOBO) with the following primer set: NS1 and LR5. We used the thermal cycling conditions as described above but extension time was 4 min. PCR products were purified by ExoSAP-IT (Thermo Fisher Scientific, MA). DNA sequences were analyzed by Fasmac sequencing service (Kanagawa, Japan) using the following primers: NS1, NS4, NS6, NS8z (O'Donnell *et al.* 1998) for 18S rDNA, ITS5 and ITS4 for ITS1-5.8S-ITS2, and LR0R and LR5 for 28S rDNA.

Table 2. Results of cross inoculation experiment with chytrid cultures C1, KS98, KS99, and SVdW-SYN-CHY1 - = no infection, + = weakly infected (< 5 %), ++ = moderately infected (5-50 %), +++ = highly infected (> 50 %), ND = not determined.

Host species	Culture	*Zygophlyctis asterionellae* KS98	*Zygophlyctis melosirae* C1	*Zygophlyctis melosirae* KS99	*Zygophlyctis planktonica* SVdW-SYN-CHY1
Asterionella formosa	AST1	-	ND	ND	-
	KSA59	+	-	-	-
	KSA60	+++	-	-	-
Aulacoseira ambigua	C5	-	+++	+	-
	KSA24	-	+++	+	-
	KSA35	ND	+++	+	ND
Aulacoseira granulata group_1	KSA17	-	+	+++	-
Aulacoseira granulata group_2	KSA47	-	+	+	-
	KSA55	ND	+	++	ND
Ulnaria sp.	HS-SYN2	-	-	-	+++
	KSA32	-	ND	ND	+
	KSA56	-	-	-	+

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DNA was extracted from 12 diatom cultures using the HotSHOT method. We amplified the 18S rDNA locus of *Aulacoseira* spp. cultures (*Au. ambigua*: C1, KSA24, KSA35; *Au. granulata*: KSA17, KSA47, KSA55) and large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) gene of *Asterionella* (AST1, KSA59, KSA60) and *Ulnaria* (KSA32, KSA56, HS-SYN2) by PCR using KOD FX Neo (TOYOBO) with the following primer sets: SR1 and SR12 (Nakayama *et al*. 1996) for 18S rDNA, and Diat-rbcL-F and Diat-rbcL-R (Abarca *et al.* 2014) for *rbcL*. The thermal cycling conditions were as described above but extension time was 2 min (18S rDNA) or 1 min (*rbcL*). DNA sequences were analyzed by Fasmac sequencing service (Kanagawa, Japan) using the following primers: SR1, SR7, SR9, SR12 (Nakayama *et al*. 1996) for 18S rDNA, Diat-rbcL-F and Diat-rbcL-R for *rbcL*.

The newly obtained sequences were deposited as LC482213–LC482227 and LC483759– LC483764 in GenBank.

Phylogenetic analysis

For phylogenetic analysis of chytrids, we created datasets of 18S, ITS1-5.8S-ITS2, and 28S rDNA sequences (Table 3). Some environmental sequences of uncultured chytrids that are related to our cultures of *Zygorhizidium* spp. were added to the 18S rDNA and 28S rDNA dataset. *Rozella allomycis* and *Rozella* sp. (JEL374) were selected as outgroup taxa. Sequences were automatically aligned with MAFFT v. 7.409 (Katoh & Standley 2013) independently for each region. Ambiguously aligned regions were excluded using trimAl v. 1.2 (Capella-Gutiérrez *et al*. 2009) with a gappyout model. The ITS1 and ITS2 regions were excluded from the alignment because it was not possible to align them unambiguously. A concatenated alignment was generated and partitioned by genes for an analysis with maximum likelihood (ML) and Bayesian methods. The ML tree was inferred using RAxML v. 8.2.7 (Stamatakis 2014). We ran an analysis under the GTR + GAMMA + I model and used the "-fa" option to conduct a rapid bootstrap analysis with 1 000 replicates combining 200 searches for the optimal tree. A Bayesian analysis was run using MrBayes v. 3.2.6 (Ronquist *et al*. 2012) under the GTR + GAMMA + I model and 5 million generations, with sampling every 100 generations. The first 25 % of trees were discarded as "burn-in". Bayesian posterior probability and branch lengths were calculated from remaining 75 % of trees.

For phylogenetic analysis of *Aulacoseira* spp., we created a dataset of 18S rDNA sequences. *Melosira varians* was selected as an outgroup taxon. Sequences were aligned and ambiguously aligned regions in the alignment were excluded as described above. The phylogenetic tree was inferred by the neighbor-joining method using MEGA v. 7 (Kumar *et al.* 2016), with the Maximum Composition Likelihood model and 1 000 bootstrap replicates.

Table 3. List of rRNA genes used in phylogenetic analysis of chytrids. New GenBank numbers are in bold.

Species	Culture Number	GenBank accession no.		
		18S	5.8S	28S
Outgroup (Cryptomycota)				
Rozella allomycis	BK47-054	AY635838	AY997087	DQ273803
Rozella sp.	JEL347	AY601707	AY997086	DQ273766
Blastocladiomycota				
Allomyces arbusculus	Brazil2	AY552524	AY997028	DQ273806
Blastocladiella emersonii	BK49-1	AY635842	AY997032	DQ273808
Catenophlyctis variabilis	JEL298	AY635822	AY997034	DQ273780
Neocallimastigomycota				
Cyllamyces aberensis	EO14	DQ536481	AY997042	DQ273829
Neocallimastix sp.	GE13	DQ322625	AY997064	DQ273822
Orpinomyces sp.	OUS1	AJ864616	AJ864475	AJ864475
Chytridiomycota				
Monoblepharidomycetes				
Gonapodya sp.	JEL183	AH009066	AY349112	KJ668088
Hyaloraphidium curvatum	SAG235-1	Y17504	AY997055	DQ273771
Monoblepharella mexicana	BK78-1	AF164337	AY997061	DQ273777
Oedogoniomyces sp.	CR84	AY635839	AY997066	DQ273804
Chytridiomycetes				
Chytridiales				
Chytriomyces hyalinus	MP4	DQ536487	DQ536499	DQ273836
Dendrochytridium crassum	JEL354	AY635827	AY997083	DQ273785
Phlyctochytrium planicorne	JEL47	DQ536473	AY997070	DQ273813
Rhizoclosmatium sp.	JEL347-h	AY601709	AY997076	DQ273769
Cladochytriales				
Endochytrium sp.	JEL324	AY635844	AY997044	DQ273816

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Table 3. (Continued).

Species	Culture Number	GenBank accession no.		
		18S	5.8S	28S
Cladochytrium replicatum	JEL180	AY546683	AY997037	AY546688
Cladochytriales sp.	JEL72	AH009044	AY349109	AY349083
Nowakowskiella sp.	JEL127	AY635835	AY997065	DQ273798
Gromochytriales				
Gromochytrium mamkaevae	CALU_X-51	KF586842	KF586842	KF586842
kor_110904_17	Uncultured	FJ157331	—	—
Lobulomycetales				
Clydaea vesicula	PL70	EF443138	EU352774	EF443143
Lobulomyces angularis	JEL45	AF164253	AY997036	DQ273815
Lobulomyces poculatus	JEL343	EF443134	EU352770	EF443139
Maunachytrium keaense	AF021	EF432822	EF432822	EF432822
Mesochytriales				
Mesochytrium penetrans	CALU_X-10	FJ804149	—	FJ804153
PFF5SP2005	Uncultured	EU162641	—	—
T2P1AeB05	Uncultured	GQ995415	—	—
Polychytriales				
Arkaya lepida	JEL93	AF164278	AY997056	DQ273814
Karlingiomyces asterocystis	JEL572	HQ901769	—	HQ901708
Lacustromyces hiemalis	JEL31	AH009039	—	HQ901700
Polychytrium aggregatum	JEL109	AY601711	AY997074	AY546686
Rhizophlyctidales				
Rhizophlyctis rosea	JEL318	AY635829	AY997078	DQ273787
	BK47-07	AH009028	—	—
	BK57-5	AH009027	—	—
Rhizophydiales				
Boothiomyces macroporosus	PLAUS21	DQ322622	AY997084	DQ273823
Kappamyces laurelensis	PL98	DQ536478	DQ536494	DQ273824
Rhizophydium brooksianum	JEL136	AY601710	AY997079	DQ273770
Uebelmesseromyces harderi	JEL171	AF164272	AY997077	DQ273775
Spizellomycetales				
Brevicalcar kilaueaense	JEL355	DQ536477	AY997093	DQ273821
Gaertneriomyces semiglobiferus	BK91-10	AF164247	AY997051	DQ273778
Spizellomyces punctatus	ATCC48900	AY546684	AY997092	AY546692
Thoreauomyces humboldtii	JEL95	AF164245	AY997075	DQ273776
Synchytriales				
Synchytrium decipiens	DUH0009362	DQ536475	AY997094	DQ273819
Synchytrium endobioticum	P-58	AJ784274	—	—
Synchytrium macrosporum	DUH0009363	DQ322623	AY997095	DQ273820
Zygophlyctidales ord. nov.				
Zygophlyctis asterionellae	KS98	LC176289	LC176299	LC176294
Zygophlyctis melosirae	C1	LC176287	LC176297	LC176292
	KS94	LC176288	LC176298	LC176293
	KS99	LC176290	LC176300	LC176295
	KS109	**LC482216**	**LC482217**	**LC482218**
Zygophlyctis planktonica	SVdW-SYN-CHY1	**LC482219**	**LC482220**	**LC482221**
AY2009A5	Uncultured	HQ219392	HQ219392	—
B86-172	Uncultured	EF196796	—	—

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Table 3. (Continued).

Species	Culture Number	GenBank accession no.		
		18S	5.8S	28S
BiwaFcA1	Uncultured	AB971229	—	—
CH1_2B_29	Uncultured	AY821989	—	—
KRL02E73	Uncultured	JN090912	—	—
InbaSyA	Uncultured	AB971237	—	—
PA2009D11	Uncultured	HQ191415	HQ191415	—
PFH1AU2004	Uncultured	DQ244009	—	—
PG5.12	Uncultured	AY642734	—	—
Zygorhizidiales ord. nov.				
Zygorhizidium willei	KS97	**LC482213**	**LC482214**	**LC482215**
Novel Clade II *sensu* Lefèvre *et al.* (2008)				
AY2009D3	Uncultured	HQ219449	HQ219449	—
PA2009B8	Uncultured	HQ191387	HQ191387	—
PA2009E8	Uncultured	HQ191289	HQ191289	—
PFH9SP2005	Uncultured	EU162642	—	—
***incertae sedis*_1**				
Dangeardia mamillata	SVdW-EUD2	MG605054	—	MG605051
E4e4731	Uncultured	—	—	KF750554
LLMB2_1	Uncultured	—	—	JN049538
P34.43	Uncultured	AY642701	—	—
***incertae sedis*_2**				
Rhizophydium scenedesmi	EPG01	MF163176	—	—
Elev_18S_563	Uncultured	EF024210	—	—
L73_ML_156	Uncultured	FJ354068	—	—
PFE7AU2004	Uncultured	DQ244008	—	—

RESULTS

Morphology of chytrids

Zygorhizidium willei culture KS97 (Fig. 1) is parasitic on the zygnematophycean green alga *Gonatozygon brebissonii* culture KSA15. Zoospores were spherical, 2.5–3 µm diam, containing a single lipid globule, with a posteriorly-directed, eccentrically-inserted, ~12 µm long flagellum (Fig. 1A). The zoospore encysted and germinated on the cell surface of *G. brebissonii* (Fig. 1B, C). The developing thallus was spherical (Fig. 1D). The mature zoosporangium was broadly obpyriform, nearly spherical, 8.7–13.5(–21.2) µm in height, 7.1–14.7(–20.9) µm in width (Fig. 1E). Zoospores were discharged from an apical or subapical, operculate discharge pore (Fig. 1F). The operculum was convex, approximately 4 µm wide, and separated from the discharge pore (Fig. 1G). Rhizoid was delicate and poorly visible under light microscopy. Transmission electron microscopic observation revealed that rhizoid penetrated the host cell wall and formed an apophysis (Fig. 1L, M). Branched rhizoids extended from the apophysis (Fig. 1M). Resting spore was produced as a result of sexual reproduction. The content of a small male cell was transferred to a large female cell via a conjugation tube, and the female cell became a resting spore (Fig. 1H–J). The resting spore was subspherical, 9.1–10.6 µm in height, 7.4–10.4 µm in width, with a smooth thick wall containing numerous lipid globules (Fig. 1J). After 3 mo incubation at 5 °C and under dark condition followed by incubation at 20 °C and under light condition, germination of resting spores was observed. As a result of germination of the resting spore, a zoosporangium was produced (Fig. 1K). Zoospore discharge was not observed but zoosporangium was operculate (data is not shown).

Zygophlyctis planktonica culture SVdW-SYN-CHY1 (Fig. 2) is parasitic on the diatom *Ulnaria* sp. culture HS-SYN2. Morphology of SVdW-SYN-CHY1 was similar to that of KS97. Zoospore was spherical, 3.0–3.5 µm diam, containing a single lipid globule, with a ~16.0 µm long flagellum (Fig. 2A). The zoosporangium was obpyriform, 6.1–12.5 µm in height, 4.6–11.0 µm in width (Fig. 2E). The operculum was convex, approximately 3 µm wide (Fig. 2G). The resting spore was ellipsoidal, 7.3–9.6 µm in height, 6.2–8.1 µm in width or spherical 6.2–9.1 µm diam, with a smooth, thick wall, containing several lipid globules (Fig. 2I, J).

Morphology of *Zygophlyctis asterionellae sp. nov.* culture KS98 parasitic on *As. formosa*, *Zygophlyctis melosirae comb. nov*. culture C1, KS94 (on *Au. ambigua*), and KS99 (on *Au. granulata*) were described previously (Seto *et al.* 2017). *Zygop. melosirae* culture KS109 (data not shown) was morphologically similar to three other cultures but shape and size of zoosporangia of these four cultures (C1, KS94, KS99, and KS109) slightly differed from each other.

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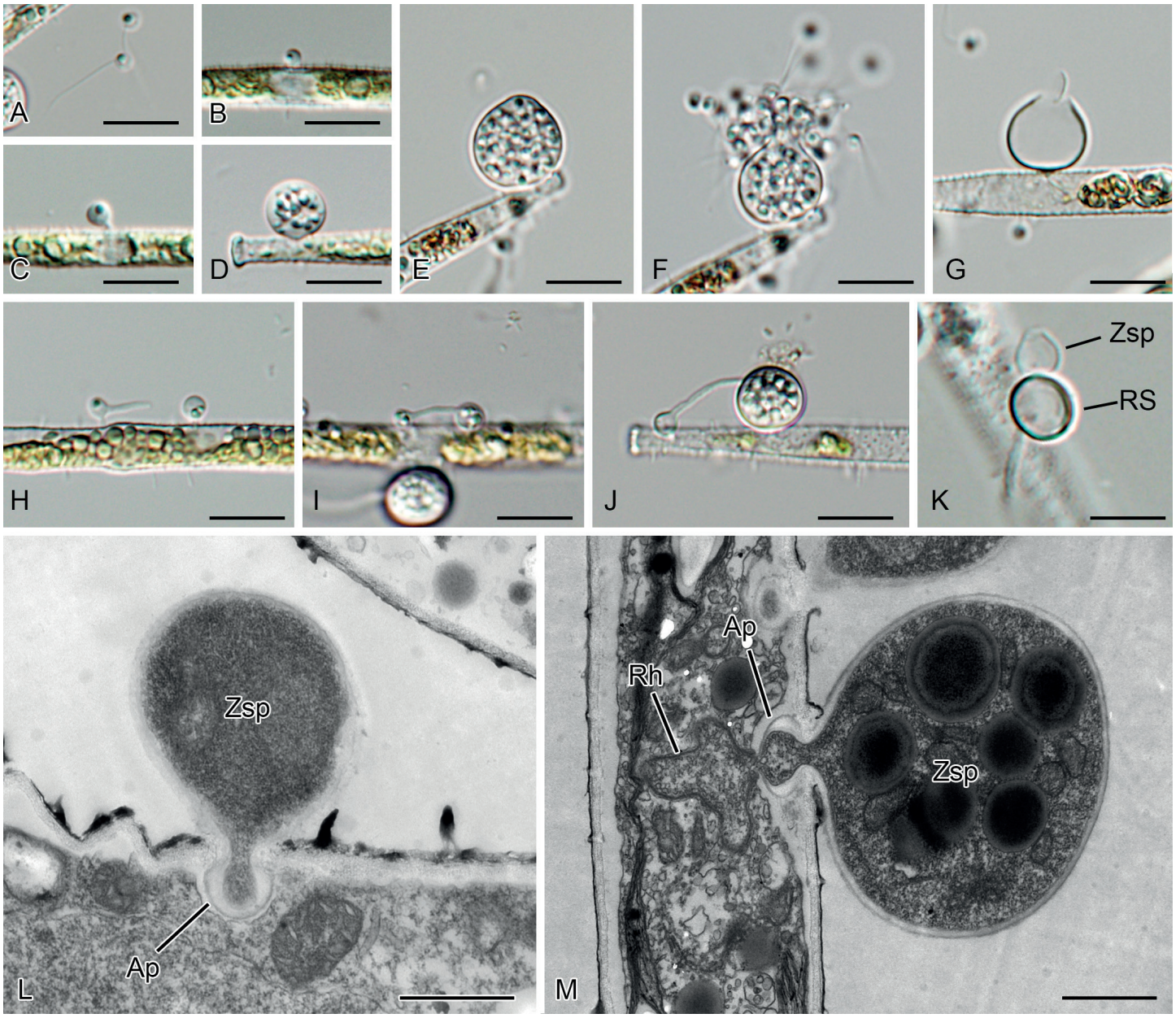

Fig. 1. Thallus morphology of *Zygorhizidium willei* (KS97) on the host *Gonatozygon brebissonii* (KSA15). **A.** Zoospores. **B.** Encysted zoospore. **C.** Germinating spore. **D.** Developing zoosporangium. **E.** Mature zoosporangium. **F.** Zoospore discharge. **G.** Empty zoosporangium with an operculum. **H, I.** Conjugation of two thalli. **J.** Resting spore and empty male thallus connected by a conjugation tube. **K.** Germinated resting spore. **L, M.** Transmission electron microscopic images of zoosporangium and rhizoidal system. Abbreviations: Ap = apophysis; Rh = rhizoid; RS = resting spore; Zsp = zoosporangium. Scale bars: A–K = 10 µm; L, M = 1 µm.

Zoospore ultrastructure of chytrids

The zoospore of *Zygor. willei* culture KS97 has a flagellum which occurred from an eccentric, posterior position of the cell (Fig. 3A, B). A single lipid globule was positioned at the lateral area of the zoospore (Fig. 3A, B). A single mitochondrion was anteriorly associated with the lipid globule (Fig. 3A, B). A microbody was appressed to the lipid globule and nearby the plasma membrane at the lateral side of the zoospore (Fig. 3A). A nucleus was positioned at the lateral side opposite to the lipid globule (Fig. 3A). Ribosomes were not aggregated but dispersed in the cytoplasm. A large vesicle enclosed by an electron dense, thick membrane was at the side of kinetosome and somewhat associated with the lipid globule (Fig. 3B). The membrane of the large vesicle was thicker and partially opened near the plasma membrane (Fig.3F–I, L). A fenestrated cisterna partially covered the lipid globule and was closely associated with the kinetosome (Fig. 3C–E, I). Fenestrations of fenestrated cisterna were 30–35 nm diam, 30–40 nm in height. A nonflagellated centriole (NfC) was composed of nine triplet microtubules and laid at an angle of about 45° to the kinetosome (Fig. 3C–E, J, K). Two types of banded structures laid at the area between the kinetosome and the large vesicle (Fig. 3B, F–I). One was a fan-like structure in the transverse section (BS1 in Fig. 3B, H). The other one was a more conspicuous structure than the former one and associated with the large vesicle (BS2 in Fig. 3B, G). Small dense bodies which were spherical, electron dense structures existed near the large vesicle and kinetosome (Fig. 3J–L). A microtubular root and Golgi apparatus were not observed.

In the zoospore of *Zygop. asterionellae* culture KS98, a single lipid globule was located at the central region of the cell (Fig. 4A). A single mitochondrion and a nucleus were associated

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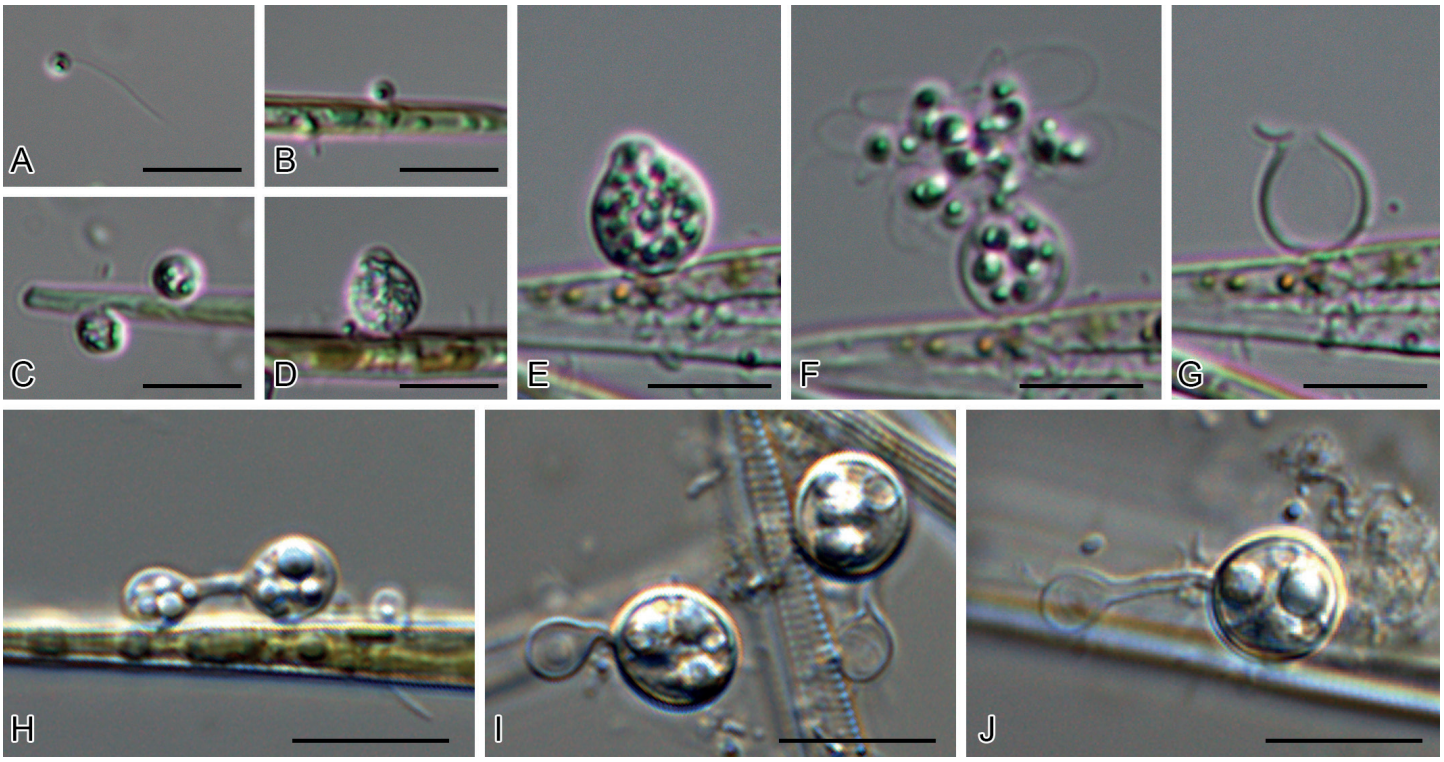

Fig. 2. Thallus morphology of *Zygophlyctis planktonica* (SVdW-SYN-CHY1) on the host *Ulnaria* sp. (HS-SYN2). **A.** Zoospore. **B.** Encysted zoospore. **C, D.** Developing zoosporangia. **E.** Mature zoosporangium. **F.** Zoospore discharge. **G.** Empty zoosporangium with an operculum. **H.** Conjugation of two thalli. **I, J.** Resting spores and empty male thalli connected by a conjugation tube. Scale bars = 10 µm.

with the lipid globule (Fig. 4A). A microbody was appressed to the lipid globule (Fig. 4B). The ribosomes were not aggregated but dispersed in the cytoplasm. A fenestrated cisterna partially covered the lipid globule and was associated with a fibrillar vesicle (Beakes *et al.* 1988) which contained fibrillous materials (Fig. 4A, C). Fenestrations of fenestrated cisterna were 40–45 nm diam, 60–65 nm in height. The fibrillar vesicle contained two components (Fig. 4C): central electron-transparent one with loosely packed fibrils (C_1) and electron denser, peripheral one (C_2). A dense particulate body (dense particulate vesicle in Beakes *et al.* 1988) was associated with the mitochondrion (Fig. 4A, E). The position of the NfC was not stable. When the NfC was positioned near the kinetosome, its angle to the kinetosome varied, parallel (Fig. 4F, I) to orthogonal (Fig. 4G). The NfC was often separated from the kinetosome and positioned near the mitochondrion (Fig. 4H). The NfC was composed of a ring of nine singlet microtubules (Fig. 4I, J) but occasionally included two or three triplet microtubules (Fig. 4K). These irregular NfC had a cartwheel structure similar to the kinetosome (Fig. 4I–K). The kinetosome was average to other chytrids, composed of nine triplet microtubules (Figs. 4I, L). A microtubular root and Golgi apparatus were not observed.

The zoospore of *Zygop. planktonica* culture SVdW-SYN-CHY1 (Fig. 5) and *Zygop. melosirae* culture C1 and KS99 (Fig. 6) had similar characters as *Zygop. asterionellae* culture KS98: a single mitochondrion and a nucleus associated with a central lipid globule (Figs 5A, B, 6A), ribosomes dispersing in the cytoplasm, a fenestrated cisterna associated with a fibrillar vesicle (Figs 5D, E, 6C, D), a dense particulate body associated with the mitochondrion (Figs 5F, 6E), and an unstable position and unique structure of NfC (Figs 5G–L, 6F, G). Fibrillar vesicles of these three species can be distinguished from each other. The fibrillar vesicle of *Zygop. planktonica* culture SVdW-SYN-CHY1 included three components (Fig. 5D): central one containing fibrillar materials (C_1) and peripheral electron dense one (C_2), and more electron dense one (C_3) associated with fenestrated cisterna. The components C_1 and C_2 were similar to those of *Zygop. asterionellae* culture KS98 (Fig. 4C). The fibrillar vesicle of *Zygop. melosirae* cultures C1 and KS99 possessed two components (Fig. 6C): a central electron dense component that is similar to C_3 of *Zygop. planktonica* and a peripheral electron transparent component with fibrillar materials (C_1) which is similar to C_1 of *Zygop. asterionellae* and *Zygop. planktonica*.

A general scheme of the zoospore ultrastructure of *Zygor. willei* culture KS97 and *Zygop. asterionellae* culture KS98 is illustrated in Fig. 7.

Molecular identification of diatom cultures

The *rbcL* sequences of cultures AST1, KSA59, and KSA60 were completely identical to each other and had 100 % similarity with

Fig. 3. Zoospore ultrastructure of *Zygorhizidium willei* (KS97). **A, B.** Longitudinal sections of zoospore. **C–E.** Longitudinal serial sections through the base of flagellum. **F–I.** Transverse serial sections through the base of flagellum. **J, K.** Serial sections including transverse section of nonflagellated centriole. **L.** Section of thickened region of large vesicle and small dense bodies. Abbreviations: BS1 = banded structure 1; BS2 = banded structure 2; FC = fenestrated cisterna; K = kinetosome; L = lipid globule; LV = large vesicle; Mb = microbody; Mt = mitochondrion; N = nucleus; NfC = nonflagellated centriole; SDB = small dense bodies. Scale bars: A, B = 500 nm; C (for C–E), F (for F–I), J (for J, K), L = 200 nm.

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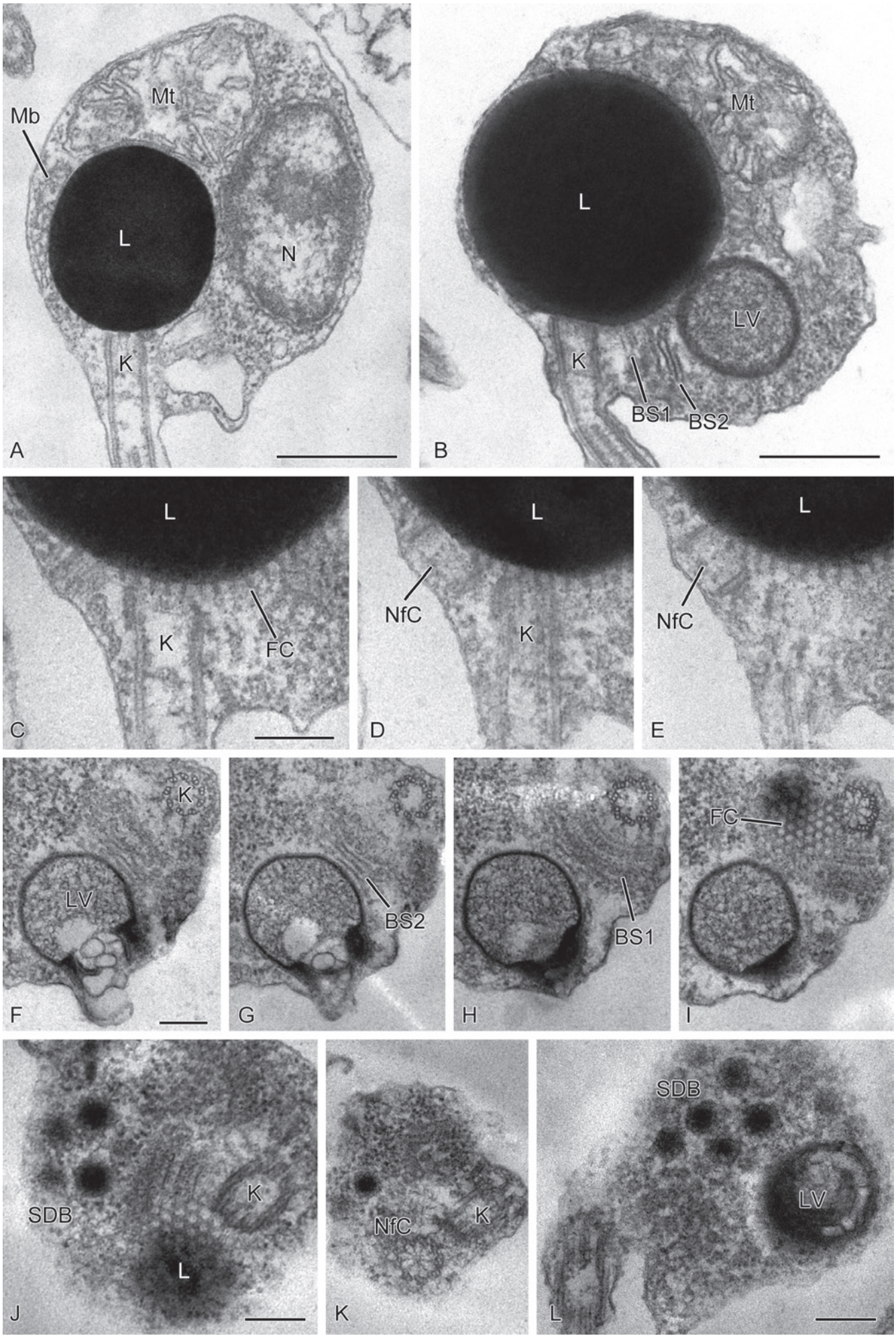

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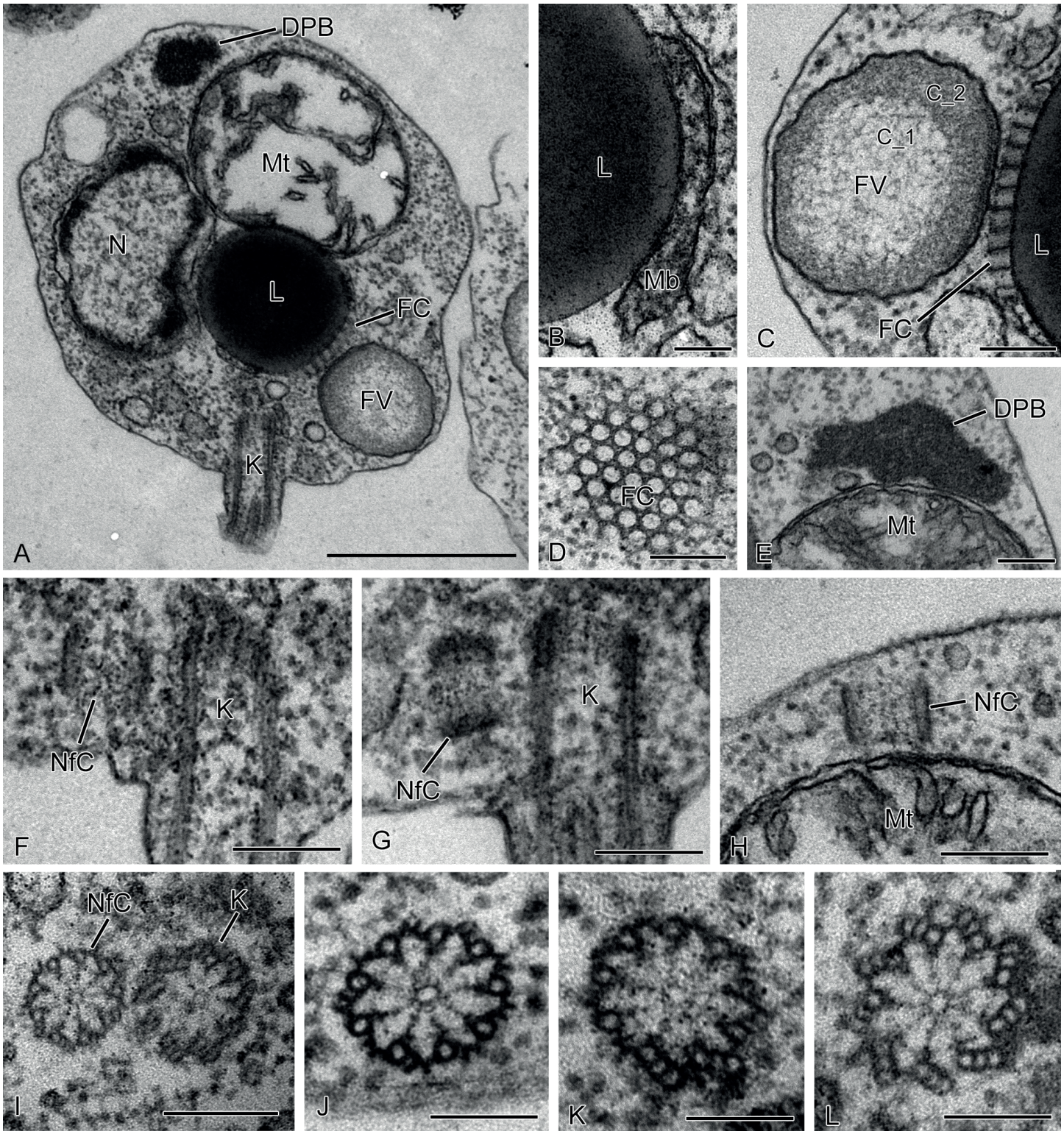

Fig. 4. Zoospore ultrastructure of *Zygophlyctis asterionellae* (KS98). **A.** Longitudinal section of zoospore. **B.** Microbody associated with lipid globule. **C.** Longitudinal section of fenestrated cisterna associated with fibrillar vesicle including two components C_1 and C_2. **D.** Transverse section of fenestrated cisterna. **E.** Dense particulate body associated with mitochondrion. **F, G.** Longitudinal sections of the base of flagellum including kinetosome and nonflagellated centriole. **H.** Nonflagellated centriole associated with mitochondrion. **I.** Transverse section of base of flagellum including kinetosome and nonflagellated centriole. **J, K.** Transverse sections of nonflagellated centriole. **L.** Transverse section of kinetosome. Abbreviations: DPB = dense particulate body; FC = fenestrated cisterna; FV = fibrillar vesicle; K = kinetosome; L = lipid globule; Mb = microbody; Mt = mitochondrion; N = nucleus; NfC = nonflagellated centriole. Scale bars: A = 500 nm; B–I = 200 nm; J–L = 100 nm.

As. formosa cultures s0339 and UTCC605 (GenBank Acc. no. AB430671 and HQ912497 respectively).

The *rbcL* sequences of cultures KSA32 and KSA56 were identical to each other and they were 99.67 % identical (only 2 base differences) to culture HS-SYN2. KSA32 and KSA56 were 99.84 % identical (one base difference) to *Ulnaria ulna* culture UTEX FD404 (GenBank Acc. no. HQ912454). HS-SYN2 was 100 % identical to *Ulnaria acus* culture G9 (GenBank Acc. no.

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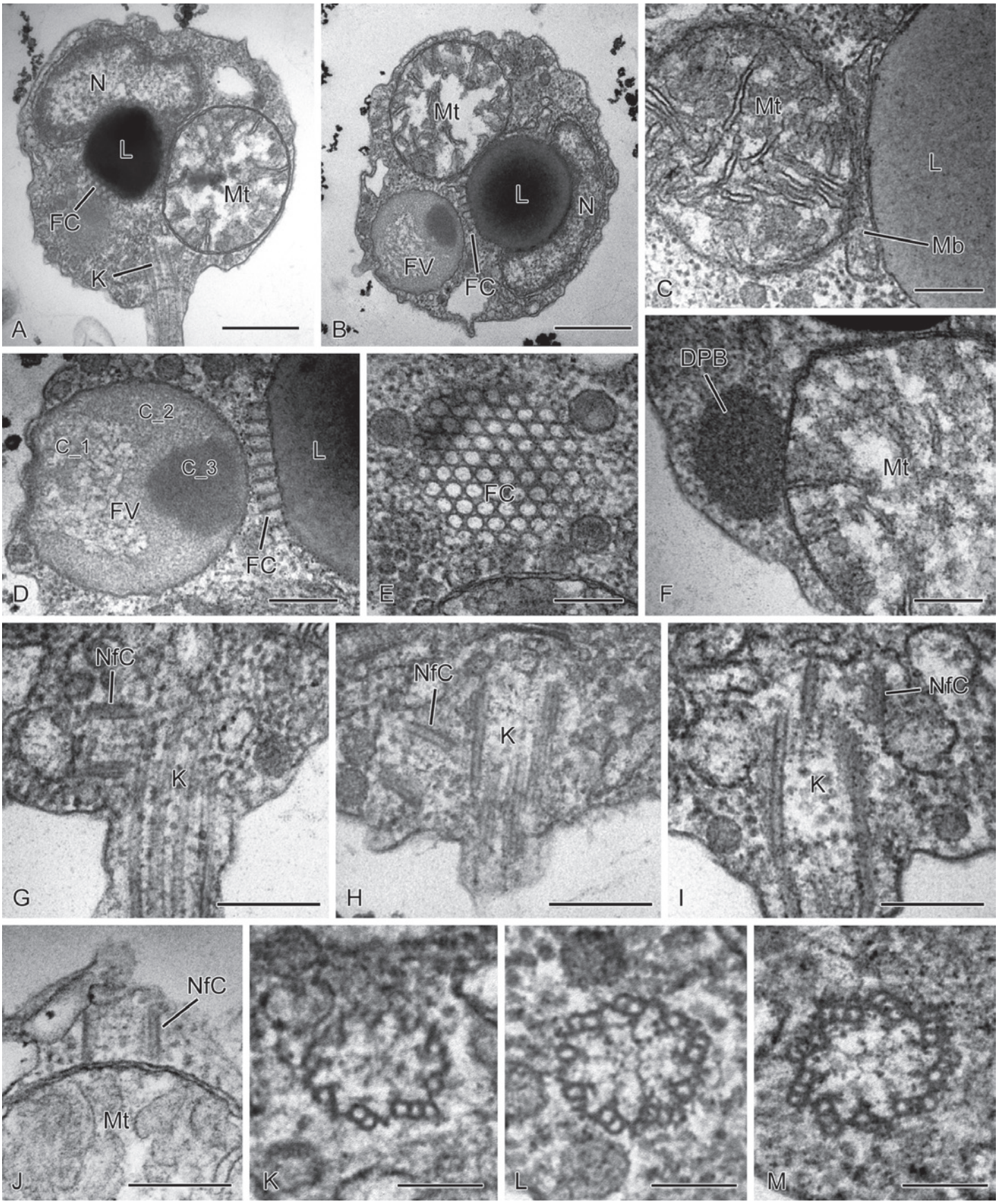

Fig. 5. Zoospore ultrastructure of *Zygophlyctis planktonica* (SVdW-SYN-CHY1). **A, B.** Longitudinal sections of zoospore. **C.** Microbody associated with lipid globule and mitochondrion. **D.** Longitudinal section of fenestrated cisterna associated with fibrillar vesicle including three components C_1, C_2, and C_3. **E.** Transverse section of fenestrated cisterna. **F.** Dense particulate body associated with mitochondrion. **G–I.** Longitudinal sections of the base of flagellum including kinetosome and nonflagellated centriole. **J.** Nonflagellated centriole associated with mitochondrion. **K, L.** Transverse sections of nonflagellated centriole. **M.** Transverse section of kinetosome. Abbreviations as Fig. 4. Scale bars: A, B = 500 nm; C–J = 200 nm; K–M = 100 nm.

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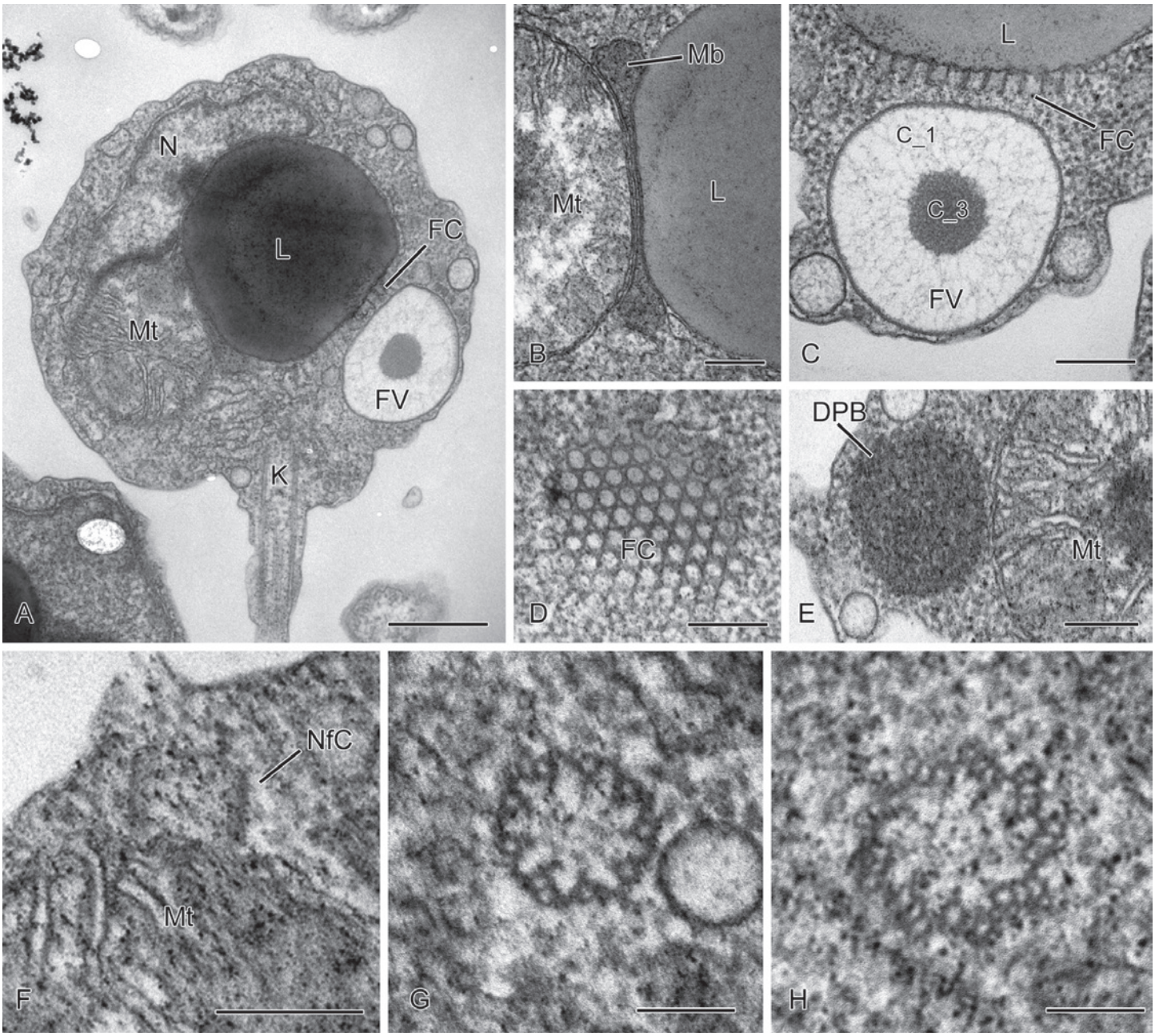

Fig. 6. Zoospore ultrastructure of *Zygophlyctis melosirae* (KS99). **A.** Longitudinal section of zoospore. **B.** Microbody associated with lipid globule and mitochondrion. **C.** Longitudinal section of fenestrated cisterna associated with fibrillar vesicle including two components C_1 and C_3. **D.** Transverse section of fenestrated cisterna. **E.** Dense particulate body associated with mitochondrion. **F.** Nonflagellated centriole associated with mitochondrion. **G.** Transverse section of nonflagellated centriole. **H.** Transverse section of kinetosome. Abbreviations as in Fig. 4. Scale bars: A = 500 nm; B–F = 200 nm; G, H = 100 nm.

JQ088178). We regard these cultures as unidentified species of the genus *Ulnaria* (see Discussion).

The 18S rDNA sequences of cultures C5, KSA24, and KSA35 were 100 % identical to one another and they were identical to some cultures of *Au. ambigua* such as PII7 (GenBank Acc. no. AY569580). Although cultures KSA17, KSA47, and KSA55 were identified as *Au. granulata* based on the morphological characters, phylogenetic analysis using 18S rDNA sequences revealed that they were divided into two groups (Fig. 8). Here, we call culture KSA17 as *Au. granulata* group_1 and cultures KSA47 and KSA55 as *Au. granulata* group_2 (see Discussion).

Host specificity of chytrids

Cross-inoculation experiments revealed that the infection of each chytrid was genus-specific (Table 2). Within the same host genus, each culture showed a different level of susceptibility to infection by a chytrid.

Zygophlyctis asterionellae culture KS98 infected two cultures of *As. formosa* KSA59 and KSA60 but did not infect culture AST1. Between KSA59 and KSA60, susceptibility to infection by KS98 was different. KS98 heavily infected culture KSA60: more than half of algal colonies were infected at 7–9 d after start of the experiment. In culture KSA59, few infections could be observed after 1–3 d but no increase of infected cells occurred afterwards.

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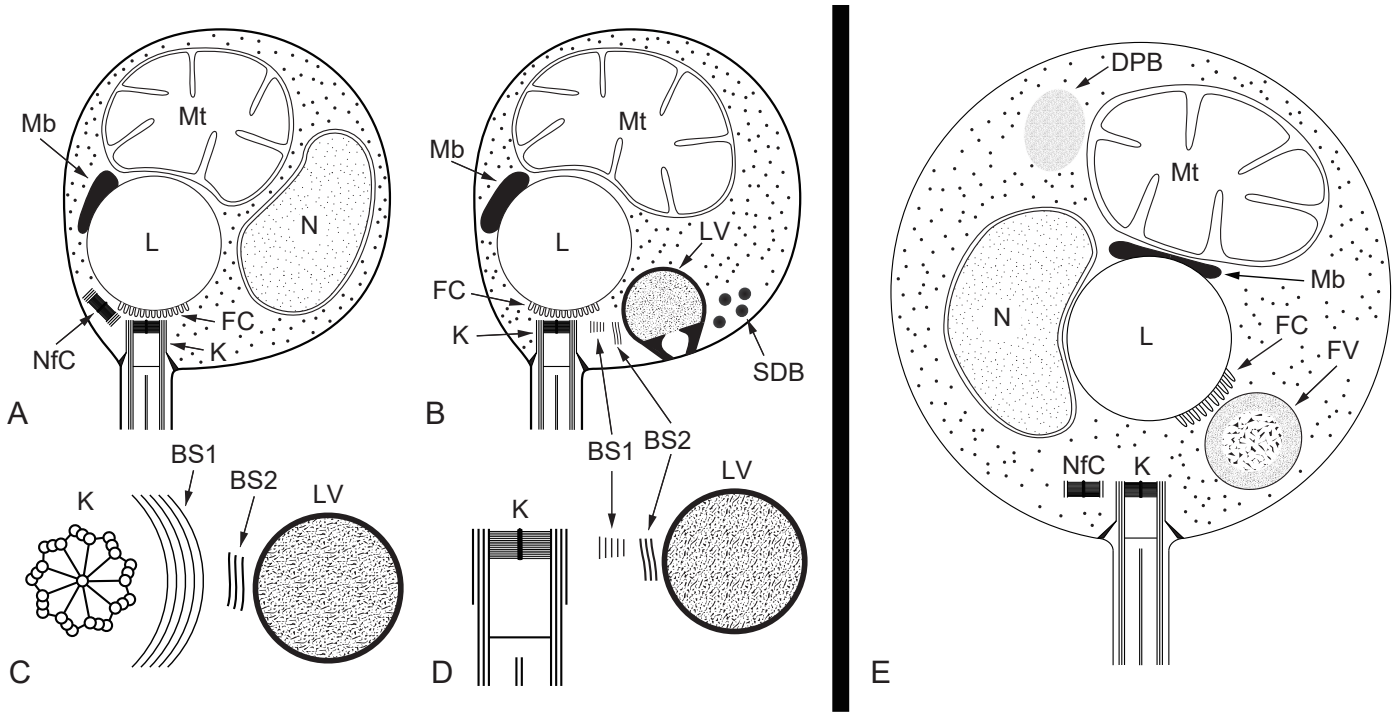

Fig. 7. Schematic drawing of zoospore ultrastructure of *Zygorhizidium willei* (A–D) and *Zygophlyctis asterionellae* (E). **A, B.** Longitudinal sections of zoospore. **C.** Transverse section of the base of flagellum. **D.** Longitudinal section of the base of flagellum. **E.** Longitudinal section of zoospore. Abbreviations as in Figs 3, 4.

Zygophlyctis planktonica culture SVdW-SYN-CHY1 infected only cultures of *Ulnaria* sp. HS-SYN2, KSA32, and KSA56. Among the three cultures of *Ulnaria* sp., susceptibility to infection by SVdW-SYN-CHY1 was different. HS-SYN2 was heavily infected: more than half of algal cells were infected at 7–9 d after start of the experiment. In contrast, only a few infections were realized at day 3–5 in KSA32 and KSA56 but no further spread of infection was observed.

Zygophlyctis melosirae cultures C1 and KS99 infected only the cultures of *Aulacoseira* spp. but both preferred different species of the genus. Culture C1, which is maintained with the culture C5 (*Au. ambigua*), intensively infected all three cultures of *Au ambigua* (C5, KSA24, and KSA35). Three cultures of *Au. granulata* (KSA17, KSA47, and KSA55) were weakly susceptible to infection by C1: infection rate stayed below 5 % during the experiment. By contrast, culture KS99 preferred *Au. granulata* rather than *Au. ambigua* but intensive infection (> 50 %) was observed only in culture KSA17 (*Au. granulata* group_1). KS99 weakly or moderately infected *Au. granulata* group_2 (KSA47 and KSA55): maximum infection rate was less than 5 % in KSA47 and 10 % in KSA55. The infection rate on *Au. ambigua* (C5, KSA24, and KSA35) was similar to that on culture KSA47: less than 5 % infection.

Molecular phylogeny

In the ML tree (Fig. 9), *Zygor. willei* culture KS97 was sister to the clade including *Dangeardia mamillata* culture SVdW-EUD2, which is a parasite of colonial volvocacean algae (Van den Wyngaert *et al.* 2018), and three environmental sequences of uncultured chytrids. Statistical support for this relationship was moderate (ML bootstrap value = 63 %; Bayesian posteriorly probability = 0.91). The environmental sequences in this clade include "E4e4731" from chaparral soil in USA (Lipson *et al.* 2014), "LLMB2_1" from Lake Lurleen in USA (Lefèvre *et al.* 2012), and "P34.43" from Lake Pavin in France (Lefranc *et al.* 2005).

As in the previous study (Seto *et al.* 2017), the parasitic chytrid cultures on diatoms, *Zygop. asterionellae* culture KS98, *Zygop. planktonica* culture SVdW-SYN-CHY1, and *Zygop. melosirae* culture C1, KS94, KS99, and KS109, clustered along with environmental sequences of uncultured chytrids and formed an order-level novel clade (Fig. 9). This clade, which is proposed as a new order in the present study, was previously recognized as "Novel Clade II" in the phylogenetic survey of fungi in lakes in France (Jobard *et al.* 2012). An additional and distinct "Novel Clade II" *sensu* Lefèvre *et al.* (2008) was sister to the order *Rhizophydiales*. *Zygophlyctis asterionellae* was sister to uncultured chytrid clone PFH1AU2004 from lake Pavin in France (Lefèvre *et al*. 2007). *Zygophlyctis planktonica* was sister to the clade including *Zygop. asterionellae* and four uncultured chytrid clones. Four cultures of *Zygop. melosirae* clustered together and were sister to the clade including *Zygop. asterionellae* and *Zygop. planktonica*. Although a previous phylogenetic analysis placed *Rhizophydium scenedesmi* culture EPG01 as sister to *Zygophlyctis* spp. parasitic on diatoms (Ding *et al.* 2018), our phylogeny placed the clade including *R. scenedesmi* and three uncultured chytrid clones as sister to the class Monoblepharidomycetes with moderate statistical support (ML bootstrap value = 66; Bayesian posteriorly probability = 0.94).

Taxonomy

Zygorhizidiales K. Seto, ***ord. nov.*** MycoBank MB831578.

Type family: *Zygorhizidiaceae* Doweld, *Index Fungorum* **102**: 1. 2014.

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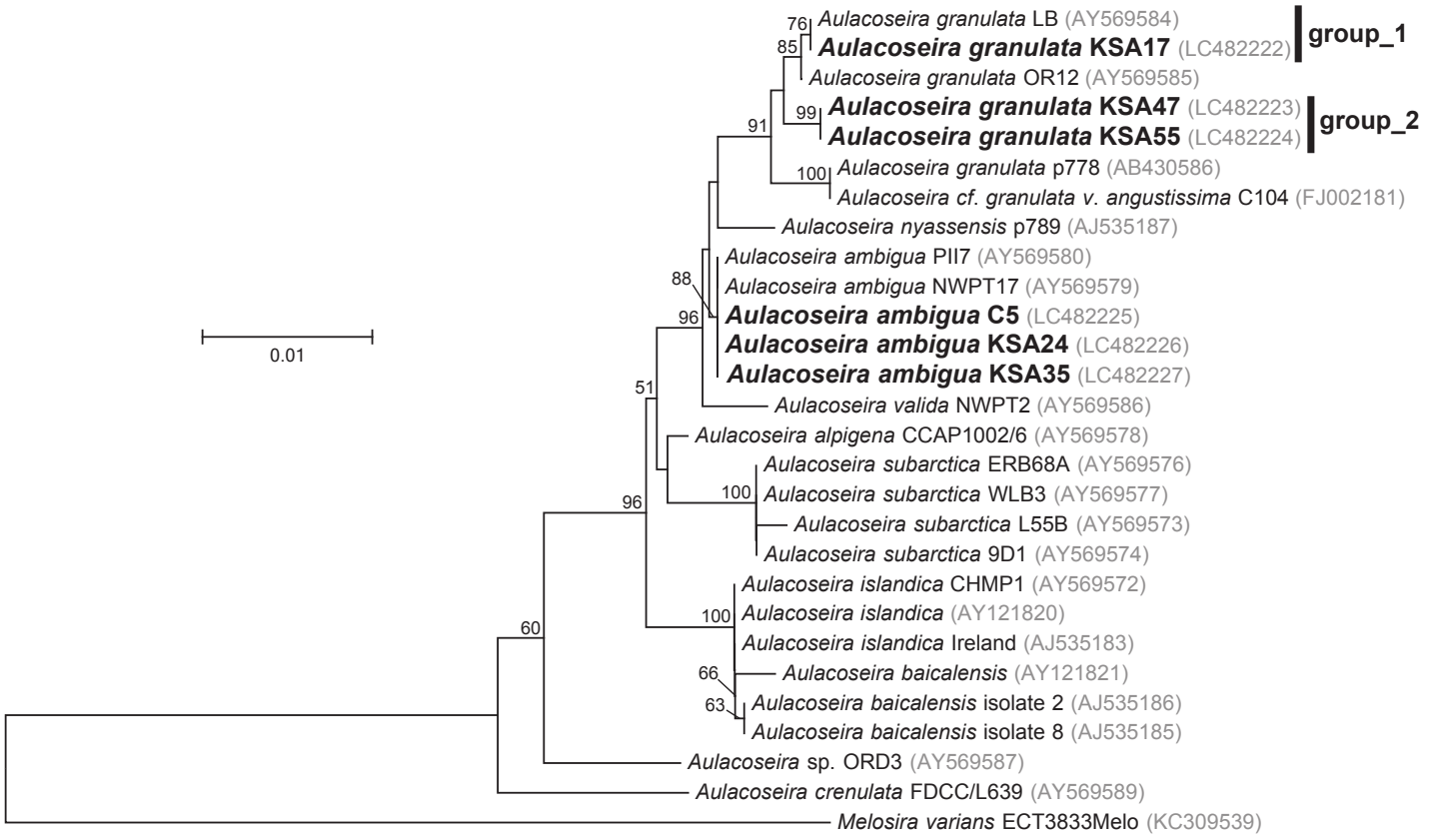

Fig. 8. Phylogenetic tree of *Aulacoseira* spp. inferred by neighbor-joining method using 18S rDNA sequences. GenBank accession number of each OTU is shown in a parenthesis. Only bootstrap support ≥ 50 % is shown.

Zoospore with a single, eccentrically inserted flagellum; a single lipid globule; fenestrated cisterna on lipid globule, closely associated with anterior end of kinetosome; ribosomes dispersed in the cytoplasm; a large vesicle with electron dense, thick membrane, which is thicker and partially opened near plasma membrane; nonflagellated centriole at an angle of *ca*. 45° to kinetosome; two banded structures between kinetosome and large vesicle; banded structure proximal to kinetosome, fan like; banded structure proximal to large vesicle, three or four conspicuous dense lines; small dense bodies spherical, electron dense, near large vesicle.

Zygorhizidiaceae Doweld, *Index Fungorum* **102**: 1. 2014. emend. K. Seto

Type genus: *Zygorhizidium* Löwenthal, *Arch. Protistenk.* **5**: 228. 1905.

Description as for *Zygorhizidiales*; thallus monocentric, eucarpic, endogenous; zoosporangium epibiotic, operculate; resting spore formed after fusion of male and female thallus via conjugation tube.

Notes: *Zygorhizidiaceae* was described by Doweld (2014b) and his description was based on zoospore ultrastructural characters of *Zygophlyctis*. However, *Zygophlyctis* is accommodated in the distinct family and order (see below). Therefore, we emended the description of *Zygorhizidiaceae* based on zoospore ultrastructural characters of *Zygor. willei* observed in the present study.

Zygorhizidium Löwenthal, *Arch. Protistenk.* **5**: 228. 1905.

Type species: *Zygorhizidium willei* Löwenthal, *Arch. Protistenk.* **5**: 228. 1905.

Notes: The genus *Zygorhizidium* includes 11 species (including nomenclaturally invalid names), which are parasites of zygnematophycean green algae (2 spp. including type species), chlorophycean green algae (3 spp.), chrysophycean algae (2 spp.), or diatoms (4 spp.) (Karling 1977). Another species, *Zygor. vaucheriae* has been described as parasite of *Vaucheria* (*Xanthophyceae*) but its affinity to *Zygorhizidium* is questionable because it produces anteriorly uniflagellate zoospores (Karling 1977). At least, four diatom parasites (*Zygor. affluens*, *Zygor. asterionellae*, *Zygor. melosirae*, and *Zygor. planktonicum*) should be excluded from the genus *Zygorhizidium*. The latter three are transferred to a distinct genus in the present study (see below). Recent phylogenetic analysis revealed that *Zygor. affluens* belongs to the *Lobulomycetales* (Rad-Menéndez *et al*. 2018) but taxonomic treatment to transfer *Zygor. affluens* to a distinct genus has not been done yet.

Zygorhizidium willei Löwenthal, *Arch. Protistenk.* **5**: 228. 1905.

Typus: **Norway**, Oslo, from surface of a moist rock, on *Cylindrocystis brebissonii*, May 1904, *Löwenthal* (Arch. Protistenk. 5: pl. 8, figs 8–43, 1905, **lectotype** designated here, MBT388267).

Material examined: **Japan**, Nagano, Suwa, Shimosuwa, from Lake Suwa, on *Gonatozygon brebissonii* (algal culture KSA15), 7 Jun. 2015, *K. Seto*, culture KS97.

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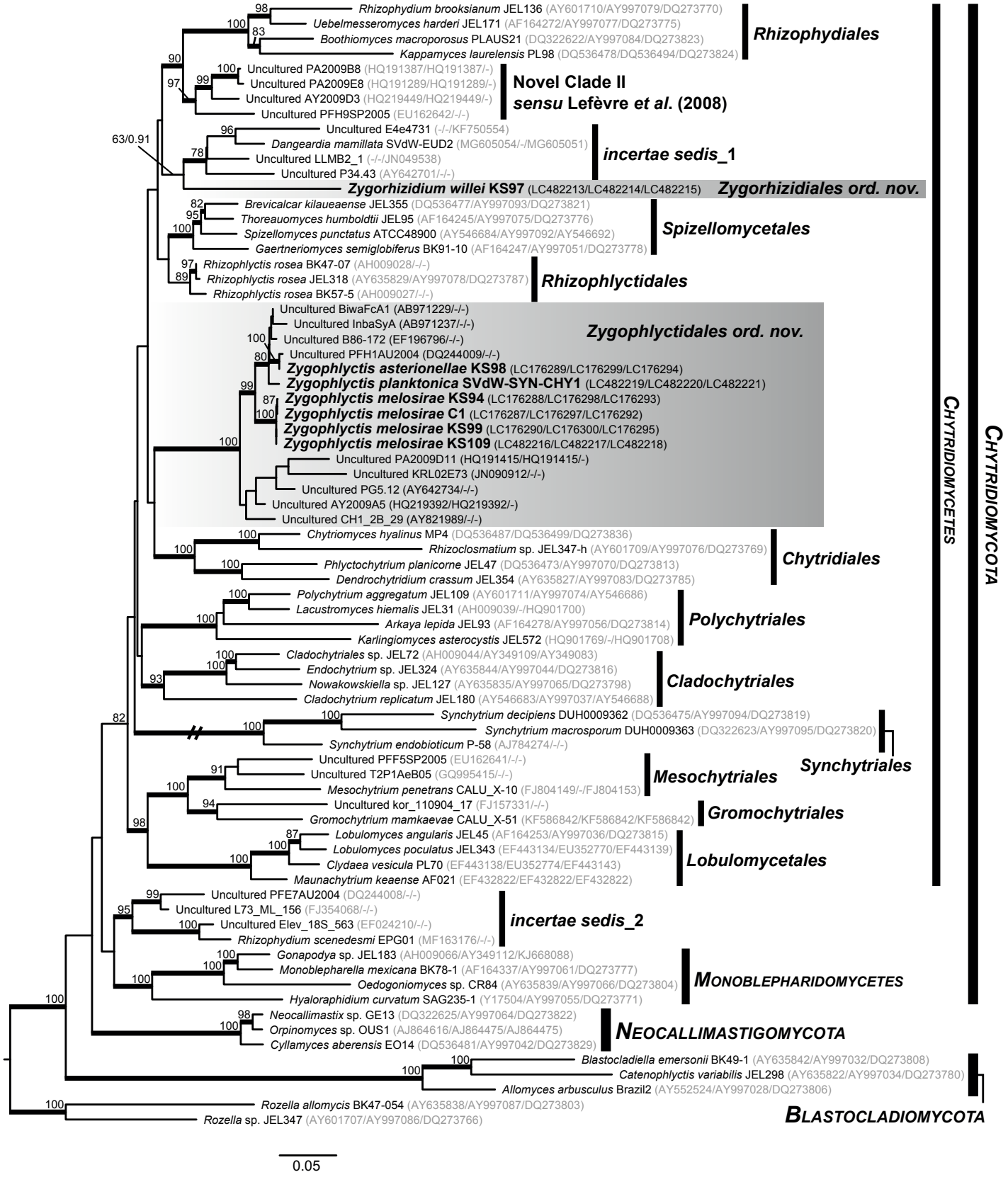

Fig. 9. Maximum-likelihood tree of chytrids using concatenated rDNA sequences (18S, 5.8S, 28S). GenBank accession numbers of each OTU are shown (18S/5.8S/28S). Only ML bootstrap support (MLBP) ≥ 70 % is shown except for the branch described below. Nodes supported by Bayesian posterior probabilities (BPP) ≥ 0.95 are highlighted by bold lines. Branch support for the clade of *Zygorhizidium willei* and *incertae sedis* clade including *Dangeardia mamillata* and three environmental sequences is shown as MLBP/BPP. The branch for *Synchytriales* is shortened by half (indicated by double slash).

Zygophlyctidales K. Seto, ***ord. nov.*** MycoBank MB831579.

Type family: *Zygophlyctidaceae* K. Seto

Zoospore with single lipid globule at central area; fenestrated cisterna on lipid globule orientated to lateral side, associated with fibrillar vesicle; fibrillar vesicle with electron-transparent region containing fibrillous materials and one or two electron

denser regions; dense particulate body near mitochondrion; nonflagellated centriole composed of nine singlet microtubules, or occasionally containing two or three triplet microtubules; nonflagellated centriole positioned near kinetosome and parallel to right angled to kinetosome, or positioned near mitochondrion.

Zygophlyctidaceae K. Seto, ***fam. nov.*** MycoBank MB831580.

Type genus: *Zygophlyctis* Doweld, *Index Fungorum* **114**: 1. 2014.

Description as for *Zygophlyctidales*; Thallus monocentric, eucarpic, endogenous; zoosporangium epibiotic, operculate; resting spore formed after fusion of male thallus and female thallus via conjugation tube.

Zygophlyctis Doweld, *Index Fungorum* **114**: 1. 2014.

Type species: *Zygophlyctis planktonica* Doweld, Index Fungorum **114**: 1. 2014.

Notes: Doweld (2014a) pointed out that the descriptions of *Zygorhizidium planktonicum* by Canter & Lund (1953) and Canter (1967) were invalid because they lacked a Latin description and holotype designation respectively. Even though he did not mention the taxonomic relationship between *Zygorhizidium willei* (type species of the genus) and *Zygorhizidium planktonicum*, he proposed a new genus *Zygophlyctis*, and a new species name *Zygophlyctis planktonica* instead of the invalid name, *Zygorhizidium planktonicum*. Here, we adopt the generic name *Zygophlyctis* by Doweld (2014a) to accommodate three diatom parasites: *Zygop. asterionellae*, *Zygop. melosirae*, and *Zygop. planktonica*.

Zygophlyctis planktonica Doweld, *Index Fungorum* **114**: 1. 2014.

Material examined: **Germany**, Waren (Müritz), from Lake Melzersee in Mecklenburg-Vorpommern, on *Ulnaria* sp. (algal culture HS-SYN2), 15 Apr. 2015, *S. Van den Wyngaert*, culture SVdW-SYN-CHY1.

Notes: *Zygorhizidium planktonicum* was originally described as a parasite of *As. formosa* and *Ulnaria* (formerly *Synedra*) spp. (Canter & Lund 1953, Canter 1967). Later, field observation (Pongratz 1966) as well as cross-inoculation experiments using cultures (Canter & Jaworski 1986, Canter *et al*. 1992, Doggett & Porter 1995) revealed that there are two host specific variants in *Zygor. planktonicum*: one is specific to *As. formosa* and the other is specific to *Ulnaria* spp. Pongratz (1966) separated the parasite of *As. formosa* as a distinct species and proposed a new species *Zygor. asterionellae*, while Canter *et al*. (1992) regarded the two host specific variants as a single species and suggested the *formae speciales* (*Zygor. planktonicum f. sp. asterionellae* and *Zygor. planktonicum f. sp. synedrae*). In the present study, we revealed that the parasite of *As. formosa* (culture KS98) and the one of *Ulnaria* sp. (culture SVdW-SYN-CHY1) were clearly distinguished from each other based on zoospore ultrastructure (character of fibrillar vesicle), host specificity, and molecular phylogeny. In Canter's description (Canter 1967), the specimen collected from Lake Rotsee (Switzerland) in which the chytrid was parasitic on *S. acus var. angustissima* (currently *Ulnaria delicatissima var. angustissima*) was designated as the type collection. Although Doweld (2014a) did not mention the host of *Zygop. planktonica* in his description, he designated Canter's figures of the chytrid on *U. delicatissima var. angustissima* (pl. 2, fig. 3 and pl. 3, fig. 7 in Canter 1967) as the holotype. Therefore, we separate the chytrid on *As. formosa* and the chytrid on *Ulnaria* spp. as distinct species and regard the former as *Zygop. asterionellae* (see below) and the latter as *Zygop. planktonica*.

Zygophlyctis asterionellae K. Seto, ***sp. nov.*** MycoBank MB831581. Fig. 4.

Etymology: Referring to the generic name of the host diatom, *Asterionella*.

Typus: **Japan**, Nagano, Shimotakai, from Lake Biwaike, on *Asterionella formosa* (algal culture KSA60), 31 Oct. 2015, *K. Seto* (*J. Eukaryot. Microbiol*. **64**: 387, fig. 3A–H, 2017, **holotype** designated here), ex-type culture KS98.

Parasitic fungus of diatom *Asterionella formosa*. Thallus monocentric, eucarpic, endogenous. Zoospore spherical, 2.5–3 µm diam, containing a single lipid globule, with a ~15 µm long flagellum. Zoosporangium obpyriform, 6.5–8.8 µm in width, 5.3–8.2 µm in height. Rhizoidal system arising from a single axis which is inserted into the host cell through the girdle region of the frustule, producing short branched rhizoids in the host cell. Zoospore discharge from an operculate discharge pore at the apex of zoosporangium. Operculum convex, 3 µm wide, separating from the discharge pore. Resting spore ellipsoidal, 6.1–8.3 µm × 5.4–7.3 µm, spherical, 6.2–7.4 µm diam, with smooth thick wall, containing several lipid globules, produced after fusion of two thalli via a conjugation tube. Empty male thallus 3–4.1 µm diam.

Notes: *Zygorhizidium asterionellae* was not validly published (Pongratz 1966) because the type was not designated in his description (Turland *et al.* 2018, Art 40.1). Here, we accommodate this species in the genus *Zygophlyctis* and describe it as *Zygop. asterionellae* instead of *Zygor. asterionellae nom. inval*.

Zygophlyctis melosirae (Canter) K. Seto, ***comb. nov.*** MycoBank MB831582.
Basionym: *Zygorhizidium melosirae* Canter, *Ann. Botany* **14**: 283. 1950.

Typus: **UK, England**, Cumbria, Ambleside, from Lake Esthwaite Water, on *Aulacoseira subarctica*, *Canter* (*Ann. Botany* **14**: 282, fig. 13, 1950, **lectotype** designate here, MBT388268).

Materials examined: **Japan**, Chiba, Insai, from Lake Inbanuma, on *Aulacoseira ambigua* (algal culture C5), 30 Jul. 2012, *M.A. Maier*, culture C1; Nagano, Chino, from Lake Shirakaba, on *Aulacoseira ambigua* (algal culture KSA24), 23 Sep. 2014, *K. Seto*, culture KS94; Nagano, Suwa, from Lake Suwa, on *Aulacoseira granulata* (algal culture KSA17), 24 Oct. 2015, *K. Seto*, culture KS99; Chiba, Insai, from Lake Inbanuma, on *Aulacoseira granulata* (algal culture KSA17), 18 Oct. 2017, *K. Seto*, culture KS109.

Notes: In the original description (Canter 1950), *Zygop. melosirae* (*Zygor. melosirae*) was described as a parasite of *Melosira italica* [later identified as *M. italica* subsp. *subarctica* in Canter (1953), currently *Au. subarctica*]. Later, *Zygop. melosirae* was reported as a parasite of *Au. islandica* (Pongratz 1966) and *Au. granulata* (Felix 1977). In the present study, we reported *Au. ambigua* as a

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new host of *Zygop. melosirae*. Although four cultures of chytrids were closely related with each other in our molecular phylogeny, they (at least culture C1 and KS99) were distinguished based on the specificity (preference) to their host. At present, we regard cultures C1 (KS94) and KS99 (KS109) as a single species *Zygop. melosirae* because it is difficult to distinguish them phylogenetically. It is necessary to investigate host specificity and molecular phylogeny of *Zygop. melosirae* infecting other species of *Aulacoseira*, especially *Au. subarctica*, in the future.

DISCUSSION

Chytrid identification: morphology and host specificity

The morphology of culture KS97 is identical to that of *Zygor. willei* in the shape and size of zoosporangium, the rhizoidal characters (delicate and branched rhizoid occurred from apophysis), and the shape and size of the lipid globule rich resting spore. However, there are some differences in the description of zoospores. In the description of Löwenthal (1905), zoospores are asymmetrically ovoid and with a single anterior lipid globule. Although Dómján (1936) and Canter (1947) did not provide an illustration of zoospores in their report of *Zygor. willei*, they noted that the zoospores were "the usual chytridiaceous type" (Canter 1947). In our observation of immotile zoospores of KS97, they were spherical with an eccentrically inserted flagellum and a lateral lipid globule (Fig. 1A). In *Zygor. verrucosum* parasitic on zygnematophycean green alga *Mesotaenium caldariorum*, zoospores are somewhat elongated, with a flagellum laterally inserted near the forward end of the zoospore but directed backwards during motility, and they contain a single anterior globule (Sparrow 1960). We could only photograph the immotile zoospores in the present study. However, when we observed the swimming zoospores of KS97, the lipid globule seemed to be at an anterior position of the zoospore and the flagellum was posteriorly oriented but might occur from nearly anterior or lateral position of the zoospore as with *Zygor. verrucosm*. More precise observation on the movement of zoospore of *Zygor. willei* is necessary. Originally, *Zygor. willei* was described as a parasite of the zygnematophycean green alga *Cylindrocystis brebissonii* (Löwenthal 1905). Later, some researchers reported this species being parasitic on other genera of *Zygnematophyceae* such as *Mougeotia*, *Spirogyra*, and *Zygnema* (Dómján 1936, Canter 1947, Sparrow & Barr 1955). It is not clear whether all reported *Zygor. willei* described above belong to the same species since host specificity of *Zygor. willei* has not been examined. Although we identified our chytrid infecting *G. brebissonii* (culture KSA97) as *Zygor. willei* in the present study, it is necessary to investigate host specificity and molecular phylogeny of *Zygor. willei* parasitic on other zygnematophycean green algae, especially *C. brebissonii*.

Culture KS98 and SVdW-SYN-CHY1 are morphologically identical to *Zygor. planktonicum* (hereafter *Zygop. planktonica*) described by Canter & Lund (1953) and Canter (1967). In their descriptions, *Zygop. planktonica* was described as a parasite of *As. formosa*, *Synedra acus*, and *S. acus var. angustissima* (= *S. delicatissima var. angustissima*). Authors have used the name *Synedra* as the host of *Zygop. planktonica* (Paterson 1958, Pongratz 1966, Canter 1967, Doggett & Porter 1995). However, many of common fresh water *Synedra* spp., such as *S. acus*, *S. delicatissima*, and *S. ulna*, are currently accommodated in the genus *Ulnaria* because the type species of *Synedra* is a distinct marine species (Williams 2011). Therefore, we use the name *Ulnaria* as the host of *Zygop. planktonica* in the present study. As mentioned in "Taxonomy", we regard the chytrid on *Asterionella* and the one on *Ulnaria* as two distinct species, *Zygop. asterionellae* and *Zygop. planktonica* respectively.

Although three host cultures of *As. formosa* examined in the present study (AST1, KSA59, and KSA60) had identical *rbcL* sequences, their susceptibilities to infection by *Zygop. asterionellae* culture KS98 were different. Culture KSA60 was highly susceptible while KSA59 was weakly susceptible and AST1 was not infected. These results are consistent with previous experiments of *Zygop. asterionellae* and *As. formosa* (Canter & Jaworski 1986, De Bruin *et al*. 2004). Canter & Jaworski (1986) performed cross-inoculation experiment using a culture of *Zygop. asterionellae* and five cultures of *As. formosa*. As a result, two of the *As. formosa* cultures were highly susceptible, and the others were infected but infection rate decreased during the experiment. De Bruin *et al*. (2004) established two cultures of *Zygop. asterionellae* and 17 cultures of *As. formosa* from a single lake (Lake Maarsseveen, The Netherlands) and examined genetic variation of *As. formosa* and their susceptibilities to the chytrid. They revealed that different cultures of *As. formosa* differed in their susceptibilities to *Zygop. asterionellae*, and two *Zygop. asterionellae* cultures had different infectivity. They also showed genetic variation of *As. formosa* based on RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) markers. Furthermore, a population genetic study, based on microsatellite markers, strongly suggested the presence of cryptic species within the cosmopolitan *As. formosa* (Van den Wyngaert *et al.* 2015). There might be extensive genetic variation in both *Zygop. asterionellae* and *As. formosa*, indicative of co-evolutionary interaction as discussed in De Bruin *et al*. (2004) and potential co-speciation.

As with *As. formosa*, three cultures of *Ulnaria* sp. (HS-SYN2, KSA32, and KSA56) also showed different susceptibilities to infection by *Zygop. planktonica* culture SVdW-SYN-CHY1. The culture HS-SYN2 was highly susceptible while KSA32 and KSA56 were weakly susceptible. HS-SYN2 and the other two could be distinguished based on a two-base pair difference in the *rbcL* sequence. However, in the present study, we regard our three cultures as an unidentified species of *Ulnaria* because the *rbcL* sequence examined was not variable enough for species identification. Further molecular analysis as well as precise morphological observation of *Ulnaria* are necessary in the future. Canter *et al*. (1992) and Doggett & Porter (1995) examined host specificity of *Zygop. planktonica* independently. Canter *et al*. (1992) performed cross-inoculation experiments using a culture of *Zygop. planktonica* and seven cultures of *Ulnaria* including four species. Two cultures of *U. acus* were highly susceptible while *U. danica*, *U. delicatissima var. angustissima*, and *U. delicatissima* were less aggressively attacked and maintenance of the chytrid was difficult using the cultures of these three species. Doggett & Porter (1995) did a similar experiment as Canter *et al*. (1992). In their experiment, more than 90 % of the cells were infected in five cultures of *U. acus* while *U. ulna* and *Ulnaria* sp. showed minimal infection (5–20 %). *Zygophlyctis planktonica* can infect various species of the genus *Ulnaria* but shows species specific host preference, and there might be several host specific variants as with *Zygop. melosirae* discussed below. It is necessary to find *Zygop. planktonica* parasitic on other *Ulnaria* spp. and examine their molecular phylogeny and host specificity.

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Cultures C1, KS94, KS99, and KS109 are morphologically similar to *Zygor. melosirae* (hereafter *Zygop. melosirae*) described by Canter (1950) but there are a few differences in shape and size of zoosporangium. *Zygophlyctis melosirae* was originally described as a parasite of *Au. subarctica* (Canter 1950). Later, Paterson (1958), Pongratz (1966), and Felix (1977) described *Zygop. melosirae* infecting *Aulacoseira* sp. (reported as *Melosira* sp. but it was probably *Aulacoseira*), *Au. islandica*, and *Au. granulata* respectively. However, host specificity of *Zygop. melosirae* has not been examined. Our cross-inoculation experiment revealed that the two cultures (C1 and KS99) can infect both *Au. ambigua* and *Au. granulata* but they each preferred one of them. C1 preferred three cultures of *Au. ambigua*. KS99 preferred *Au. granulata* but intensive infection was observed only in culture KSA17. Edgar & Theriot (2004) pointed out that *Au. granulata* is not a single species but a species complex including several intraspecific groups. Our phylogenetic analysis revealed that the three cultures of *Au. granulata* examined here were separated into two clades: group_1 including KSA17 and group_2 including KSA47 and KSA55. Chytrid cultures C1 and KS99 are closely related to each other in our phylogenetic tree (only two base differences in the partial 28S rDNA sequences between C1 and KS99). It is possible that *Zygop. melosirae* infecting *Au. subarctica* described by Canter (1950) can parasitize on other *Aulacoseira* spp. but prefers *Au. subarctica*. Furthermore, there might be more variations of *Zygop. melosirae* preferring other species of *Aulacoseira*.

Zoospore ultrastructure and taxonomic position of *Zygorhizidium sensu lato*

The current classification system of chytrids is based on molecular phylogeny and characteristics of zoospore ultrastructure (Powell & Letcher 2014). Our molecular phylogenetic analysis revealed that the genus *Zygorhizidium s. lat.* is polyphyletic and can be divided into two lineages: an order-level *incertae sedis* lineage of *Zygor. willei* (KS97), and "Novel Clade II" *sensu* Jobard *et al.* (2012) including *Zygop. asterionellae* (KS98), *Zygop. melosirae* (C1, KS94, KS99, and KS109), and *Zygop. planktonica* (SVdW-SYN-CHY1). As discussed below, we concluded that the zoospore ultrastructure of *Zygor. willei* and *Zygophlyctis* spp. are remarkably different from each other and both are distinguished from any other known orders in *Chytridiomycetes*. Thus, ultrastructural characters of *Zygor. willei* and *Zygophlyctis* spp. guarantee the independencies of the two novel lineages as the new orders *Zygorhizidiales* and *Zygophlyctidales*, respectively.

The zoospore ultrastructure of *Zygor. willei* exhibits unique characteristics among the known orders in *Chytridiomycetes*. *Zygorhizidium willei* has a posteriorly directed flagellum that occurred from an eccentric position of the cell. The zoospore of mycoparasitic chytrid *Caulochytrium protostelioides* also exhibits a unique position of the flagellum (Powell 1981). However, the character and arrangement of organelles in the zoospore of *C. protostelioides* is quite different from that of *Zygor. willei*. The fenestrated cisterna of *Zyogor. willei* is adjacent to the kinetosome, in contrast to a typical fenestrated cisterna in chytrids belonging to *Chytridiales* (Letcher & Powell 2014), *Rhizophydiales* (Letcher *et al.* 2006) and some other orders, where it closely associates with the plasma membrane at the lateral side of the zoospore. The plant parasitic chytrid *Synchytrium macrosporum* (*Synchytriales*), green algal parasite *Mesochytrium penetrans* (*Mesochytriales*), and chitinophilic saprotrophic chytrid *Arkaya serpentina* (*Polychytriales*) also have the fenestrated cisterna facing the kinetosome (Montecillo *et al.* 1980, Karpov *et al*. 2010, Longcore & Simmons 2012). However, in all these species, the fenestrated cisterna is near the kinetosome but separated from it. *Zygorhizidium willei* has two types of banded structures between the kinetosome and the large vesicle. These structures are similar to the rhizoplast observed in some chytrids in *Rhizophlyctidales* (Letcher *et al*. 2008) or striated disk observed in some chytrids belonging to *Monoblepharidales* in *Monoblepharidomycetes* (Fuller & Reichle 1968, Reichle 1972, Mollicone & Longcore 1994, 1999). Both structures are distinct from the banded structures of *Zygor. willei*. The rhizoplast in *Rhizophlyctidales* extends from the anterior end of the kinetosome to the posterior end of the nucleus. The striated disk in *Monoblepharidomycetes* surrounds the kinetosome in the transverse section of the flagellar apparatus.

The zoospore of *Zygophlyctis* spp. also has unique characters among the orders of *Chytridiomycetes*. Previously, the zoospore ultrastructures of *Zygop. asterionellae* (Beakes *et al.* 1988) and *Zygop. planktonica* (Canter *et al*. 1992, Doggett & Porter 1995) have been observed. Our results of ultrastructural observations on three species of *Zygophlyctis* were generally consistent with the previous observations. The distinctive features are the fenestrated cisterna adjacent to the fibrillar vesicle (FV) and the dense particulate body near the mitochondrion, both of which are unreported in zoospores of any other chytrids. The FV was originally reported by Beakes *et al.* (1988) as a vesicle containing fibrillar materials in the zoospore of *Zygop. asterionellae*. Later, Canter *et al*. (1992) and Doggett & Porter (1995) showed the morphological difference of the FV between *Zygop. asterionellae* and *Zygop. planktonica*. Our observations also revealed that three species of *Zygophlyctis* exhibit distinct morphology of the FV. *Zygophlyctis planktonica* has the most complex FV including three components (C_1, C_2, and C_3 in Fig. 5D). *Zygophlyctis asterionellae* (C_1 and C_2 in Fig. 4C) and *Zygop. melosirae* (C_1 and C_3 in Fig. 6C) have the FV each with a different combination of two of the three components. *Zygorhizidium willei* also has a distinct vesicle called large vesicle (LV), which is distinguished from the FV of *Zygophlyctis* spp. The LV of *Zygor. willei* has a thicker membrane than the FV of *Zygophlyctis* spp. and it is thickened and partially opened at the region near the plasma membrane. Although the function of both FV and LV is currently unknown, Canter *et al.* (1992) and Doggett & Porter (1995) pointed out that the FV of *Zygophlyctis* spp. is similar to the K-body observed in the zoospore of the taxa of *Saprolegniales* in *Oomycota* (Beakes *et al.* 2014). The K-body is a relatively large vesicle including crystalline matrix and tubular inclusions (Holloway & Heath 1977, Lehnen & Powell 1989) and is considered to include the adhesive materials which are discharged when the zoospore encysts (Lehnen & Powell 1989). Similarly, it is possible that the LV of *Zygor. willei* and the FV of *Zygophlyctis* spp. have the function for attachment of encysted zoospores to host algae.

The non-flagellated centriole (NfC) exhibits irregular morphology and variable position in three species of *Zygophlyctis*. Generally, in chytrid fungi, the NfC is located near the kinetosome and its orientation to the kinetosome is stable in each species. However, in *Zygophlyctis* spp., orientation of the NfC to the kinetosome is unstable; it may be parallel to right angled to the kinetosome. Furthermore, there were several extreme cases, where the NfC was distantly separated from the kinetosome and positioned near the mitochondrion. The NfC of

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Zygophlyctis spp. is composed of mainly singlet microtubules, which is also exceptional for the NfC of chytrids. Although Beakes *et al.* (1988) observed only NfCs composed of nine singlet microtubules, in the present study we observed that some NfCs included some triplets of microtubules. This irregular structure of the NfC is similar to that of the immature centriole during the replication (Cavalier-Smith 1974). During the replication of the centriole, the daughter centriole is reproduced by the following process (Fırat-Karalar & Stearns 2014): 1) cartwheel is produced at first; 2) nine singlet microtubules (A-tubules) are produced around the cartwheel; 3) the other two microtubules of triplet (B- and C-tubules) are produced. It is possible that the NfC of *Zygophlyctis* spp. is at the second stage of replication. As the NfC of *Zygophlyctis* spp. contains a typical cartwheel structure and some of them include triplet microtubules, they may become a typical centriole with nine triplet microtubules during mitosis.

Beakes *et al.* (1988) described the zoospore ultrastructure of *Zygor. affluens* and *Zygop. asterionellae* (as *Zygor. planktonicum*) and compared them. They revealed that characters of zoospore ultrastructure of these two species were remarkably different. Also, the zoospore ultrastructure of *Zygor. affluens* is distinguished from that of *Zygor. willei* observed in the present study. Recently, Rad-Menéndez *et al.* (2018) established the culture of *Zygor. affluens* and clarified its phylogenetic position. In their phylogenetic tree, *Zygor. affluens* was placed in the order *Lobulomycetales* and related to *Algomyces stechlinensis*, which is parasitic on colonial volvocacean green algae (Van den Wyngaert *et al.* 2018). Simmons *et al.* (2009) pointed out that zoospores of *Zygor. affluens* observed by Beakes *et al*. (1988) possess some shared characters of the *Lobulomycetales*. Although Rad-Menéndez *et al.* (2018) did not observe zoospore ultrastructure of their culture of *Zygor. affluens*, this species should be distinct from *Zygor. willei* and *Zygophlyctis* spp. examined in the present study based on molecular phylogeny and zoospore ultrastructure. Therefore, *Zygor. affluens* should be excluded from *Zygorhizidium* and transferred to a distinct genus.

Sexual reproduction of chytrids

In the *Chytridiomycetes*, various types of sexual reproduction have been observed but only a few of the chytrids are well-studied (Powell 2017). The types of sexual reproduction of *Chytridiomycetes* are roughly divided into the following three categories: fusion of two motile gametes, gametangial conjugation, and somatogamy (Sparrow 1960, Powell 2017). The fusion of isogamous planogametes has been observed in the plant pathogenic chytrids such as *Olpidium* and *Synchytrium* (Kusano 1912, Curtis 1921). In the gametangial conjugation, the fusion between male and female thalli occurs. Subsequently, contents of the male thallus are transferred to the female thallus, and the female thallus matures into the thick-walled resting spore. Some distinct types of gametangial conjugation have been observed among the many taxa of chytrids. One of the types in which two thalli fuse via a conspicuous conjugation tube is known mainly in *Zygorhizidium* spp. (Löwenthal 1905, Canter 1967) and also in some species of *Rhizophydium* (Canter 1947, 1954, 1959). In the other type of gametangial conjugation observed in some species of *Rhizophydium* spp. (Scherffel 1925, Canter 1950, 1951), the male gamete directly attaches on the female thallus, which encysts and grows on the host or substrate prior to the fusion. In somatogamy, the resting spore occurs from the anastomosed rhizoids between two thalli. This type of sexual reproduction was well-studied in *Chytriomyces hyalinus* (Moore & Miller 1973, Miller 1977, Miller & Dylewski 1981) and *Polyphagus euglenae* (Wager 1913). Although many types of sexual reproduction are observed in *Chytridiomycetes*, taxonomic implication of sexual reproduction of chytrids has not been argued enough. Most information on the sexual reproduction of chytrids is based on the classical observations of uncultured materials whose phylogenetic positions are currently unknown.

Sexual reproduction of the genus *Zygorhizidium* is unique among the *Chytridiomycetes*, which is adopted as one of the definitive characters of the genus. The uniqueness of the sexual reproduction of *Zygorhizidium* spp. supports the order-level novel phylogenetic and taxonomic position of *Zygorhizidium* spp. However, *Zygorhizidium* spp. were separated into two lineages named as *Zygorhizidiales* and *Zygophlyctidales* in the present study. This means that "*Zygorhizidium* type" sexual reproduction emerged at least twice in the two independent lineages. The "*Zygorhizidium* type" sexual reproduction processes of these independent lineages might be distinguished according to the timing of karyogamy. In the mature resting spore of *Zygor. willei*, two nuclei were observed (Löwenthal 1905). The karyogamy of this species was suspected to occur just before germination of the resting spore (Sparrow 1960). On the other hand, in *Zygop. planktonica*, karyogamy is considered to occur during the development of the resting spore because a single nucleus was observed in the mature resting spore (Doggett & Porter 1996).

Current taxonomy of chytrids rely on zoospore ultrastructure and molecular phylogenetics. The types of sexual reproduction as well as their processes and mechanisms might have the potential to be taxonomically informative characters. It is necessary to accumulate further data on the various types of sexual reproduction of chytrids whose phylogenetic positions are well documented in order to apply them as taxonomic characters for constructing the current classification system of chytrids.

Phylogenetic diversity of parasitic chytrids

Recent molecular phylogenetic studies of parasitic chytrids, especially on algae, have revealed that they often represent novel taxa within *Chytridiomycota*. Some parasitic chytrids belong to order-level novel clades (Karpov *et al.* 2014, Seto *et al.* 2017, Ding *et al*. 2018, Van den Wyngaert *et al.* 2018) while others are positioned in known orders such as *Chytridiales*, *Lobulomycetales*, and *Rhizophydiales* (Lepelletier *et al*. 2014, Seto *et al.* 2017, Van den Wyngaert *et al*. 2017, 2018, Seto & Degawa 2018a, b). In the present study, we revealed that the described parasitic chytrid genus *Zygorhizidium* is separated into two novel lineages which are proposed as new orders *Zygorhizidiales* and *Zygophlyctidales*. Similarly, Karpov *et al.* (2014) established the new order *Mesochytriales* to accommodate the green algal parasite *Mesochytrium penetrans*. Van den Wyngaert *et al.* (2018) revealed that two known parasites of colonial volvocacean algae, *Endocoenobium eudorinae* and *Dangeardia mamillata*, are positioned in independent order-level novel clades. These results indicate that taxonomic reexaminations of described parasitic chytrids whose phylogenetic positions have not been investigated are essential for taxonomic investigations of chytrids. Currently, a number of chytrid genera, many of which are parasitic taxa, still

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remain to be sequenced (Wijayawardene *et al*. 2018). Regarding the other described species of *Zygorhizidium*, *Zygor. verrucosum* is possibly related to *Zygor. willei* because it is parasitic on the zygnematophycean green alga, *Mesotaenium caldariorum* (Sparrow 1960). The other species of *Zygorhizidium* are parasitic on chlorophycean green algae or chrysophycean algae (Karling 1977), and their phylogenetic positions are uncertain. It will be necessary to rediscover them and examine their molecular phylogeny and zoospore ultrastructure.

Metabarcoding analysis of both aquatic and terrestrial environments have unveiled a high number of undescribed lineages of chytrids (Lefèvre *et al.* 2008, Freeman *et al*. 2009, Jobard *et al*. 2012, Comeau *et al*. 2016, Tedersoo *et al.* 2017). Karpov *et al*. (2014) showed that *M. penetrans* belongs to "Novel Clade I", which was reported as an order-level novel clade including only environmental sequences of uncultured chytrids (Lefèvre *et al*. 2008). As with the previous analysis (Seto *et al.* 2017), we showed that *Zygophlyctis* spp. parasitic on diatoms are placed in "Novel Clade II" *sensu* Jobard *et al.* (2012), which is described as *Zygophlyctidales* in the present study. Single-spore PCR techniques (Ishida *et al.* 2015) have revealed that other unidentified chytrids infecting diatoms such as *Fragilaria*, *Cyclotella*, and *Diatoma* also belong to *Zygophlyctidales* (Van den Wyngaert *et al*. unpubl. data). Thus, *Zygophlyctidales* currently represents a lineage of exclusively diatom-specific parasitic chytrids.

Even though metabarcoding is a powerful tool for exploring the diversity of chytrids, not all chytrids can be discovered by this method. In the present study, *Zygop. asterionellae* was closely related to an environmental sequence "PFH1AU2004" from Lake Pavin in France (Lefèvre *et al*. 2007) but *Zygop. melosirae* and *Zygop. planktonica* were distinct from any environmental sequences in this clade. Furthermore, *Zygor. willei* did not have any close affinity with environmental sequences. Similarly, *Pendulichytrium sphaericum* and *Rhizophydium planktonicum*, which are parasitic on *Au. granulata* and *As. formosa* respectively, were closely related to environmental sequences (Seto & Degawa 2018b) while some parasitic chytrids on algae were distinct from any environmental sequences (Karpov *et al.* 2014, Van den Wyngaert *et al*. 2017, 2018, Seto & Degawa 2018a). PCR amplification of some chytrids might fail due to the presence of insertion sequences in the rDNA region or incompatible primers. Actually, the 18S rDNA region of culture KS97 (*Zygor. willei*) includes an insertion sequence of *ca*. 1 000 bases. Other possible reasons for overlooking parasitic chytrids is due to their short and distinct temporal dynamics. Many parasitic chytrids are host specific and occur in accordance with the seasonal population dynamics of their host algae (Canter & Lund 1948, Kudoh & Takahashi 1990). Thus, extensive sampling during various seasons is necessary to explore the whole diversity of chytrids.

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