Taxonomic Studies on Parasitic Chytrids on Algae

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Abstract

Chytrids are basal lineages of true fungi that reproduce with posteriorly uniflagellate zoospores. During the last decade, the classification system of chytrids has been dramatically changed from the traditional system based on thallus morphology to the current system based on molecular phylogeny and zoospore ultrastructure. However, a large number of gaps in the current classification system were revealed by the recent environmental DNA surveys of chytrids. Although more than 400 species of parasitic chytrids have been morphologically described under the traditional classification system, less than 10 species of parasitic chytrids were sequenced and their zoospore ultrastructure were examined. To establish the complete classification system of chytrids, taxonomic examinations of parasitic chytrids which are missing in the current system are indispensable. The object of this study is determination of taxonomic position of parasitic chytrids on algae in the current classification system. To do this I tried to establish dual cultures of parasitic chytrids and their host algae. Using these cultures, I identified them based on thallus morphology and discussed their taxonomy based on molecular phylogeny and zoospore ultrastructure.

I isolated chytrids and algae from the water samples collected at several lakes in Japan and established six dual cultures (KS93, KS94, KS97, KS98, KS99 and KS100). Culture C1 was provided by Dr. Maiko Kagami (Toho University).

Five cultures were assigned to the genus *Zygorhizidium* based on operculate zoosporangium and resting spore formed as a result of sexual reproduction, in which a male thallus and female thallus fuse via a conjugation tube. They were identified as three known species: *Zygorhizidium willei* (KS97) parasitic on zygnematophycean green algae, *Zygorhizidium planktonicum* (KS98) and *Zygorhizidium melosirae* (C1, KS94 and KS99) parasitic on diatoms. The molecular phylogenetic analysis revealed that the *Zygorhizidium* was polyphyletic and separated into two distinct clades. KS97 (*Z. willei*) was sister to an environmental sequence of uncultured chytrid and distinguished from any known orders in Chytridiomycetes. The four cultures (*Z. planktonicum* and *Z. melosirae*) were placed in a novel clade that was previously reported as an undescribed clade composed of only

the environmental sequences. The transmission electron microscopic observations on KS97 (*Z. willei*) and KS98 (*Z. planktonicum*) revealed that the zoospore ultrastructure of these chytrids were remarkably different and also unique among the known orders in Chytridiomycetes. Based on these results, I proposed two new orders: the Zygorhizidiales including *Z. willei* (type species of *Zygorhizidium*) and the Zygophlyctidales including *Z. planktonicum* and *Z. melosirae* which were segregated from the *Zygorhizidium* and accommodated in the genus *Zygophlyctis*.

Culture KS93 was parasitic on diatom *Aulacoseira granulata*, and its morphological characters were distinguished from those of any known species. In the phylogenetic tree, KS93 was sister to environmental sequences of uncultured chytrids and placed in the basal position of the family Chytriomycetaceae in the order Chytridiales. The zoospore of KS93 possessed the characters of Group I type zoospore which are specific to the Chytriomycetaceae. Based on these results, I described a new species in a new genus of Chytriomycetaceae, *Pendulichytrium sphaericum*.

Culture KS100 was parasitic on green algae *Microglena coccifera* and its morphological characters were distinguished from those of any known species. In the phylogenetic tree, KS100 belonged to the order Rhizophydiales and were distinguished from any known family in the order. The zoospore of KS100 was revealed to have the kinetosome-associated structure which was distinguished from that of any other family in the Rhizophydiales concerning its morphology and position. Based on these results, I described a new species in a new genus, *Collimyces mutans*, and erected a new family Collimycetaeae to accommodate it.

As a result, I clarified the taxonomic position of seven cultures of parasitic chytrids in the current classification system of chytrids. They were identified as five species. Three of them were the known species but accommodated in the two new orders in the Chytridiomycetes. The other two were novel taxa in the known orders: the Chytridiales and the Rhizophydiales, respectively. Finally, I discussed 1) environmental sequences, 2) host specificity, 3) nutritional traits, 4) sexual reproduction and 5) zoospore ultrastructure, with the new knowledges obtained in this study, from the point of view of their taxonomic implications. I provided the future perspective for taxonomic study of chytrids.

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Chapter 1
General introduction

1.1. General description of chytrids

Chytrids are true fungi and characteristically reproduce with posteriorly uniflagellate zoospores. During asexual reproduction, they form a sac-like structure called the zoosporangium, in which zoospores are produced. They also produce thick-walled resting spores sexually or asexually. As chytrids occupy a basal position in the kingdom fungi (James et al. 2006a), they must hold the key to understand the early evolution of fungi such as origin of fungi and terrestrialization of fungi (Stajich et al. 2009).

Chytids are essentially ubiquitous, inhabiting freshwater environments such as streams, ponds and lakes as well as terrestrial environments such as forest, agricultural and desert soils (Barr 2001; Powell 1993). Some are found from brackish water, marine environment and coastal, saline soil (Barr 2001). Recent molecular environmental surveys revealed that chytrids dominate the fungal community in marine and freshwater habitats and high-elevation soils (Comeau et al. 2016; Freeman et al. 2009; Hassett and Gradinger 2016). The sequences of chytrids are also found from unique habitats such as anoxic environment, cold-seep sediments and hydrothermal vent in deep-sea (Bass et al. 2007; Le Calvez et al. 2009; Nagahama et al. 2011; Stoeck and Epstein 2003).

Nutritional traits are varied among the chytrid taxa (Barr 2001; Powell 1993). Some are saprotrophs, decomposing refractory substrates such as pollen, organic materials containing chitin, keratin and cellulose. Others are parasites of microorganisms such as algae, protists, other fungi and small invertebrates. Plant pathogenic chytrids such as *Synchytrium* spp. and *Olpidium* spp. are also known. *Synchytrium endobioticum* (Schilberszky) Percival is the causal agent of potato wart disease and has the potential to cause significant economic damage (Obidiegwu et al. 2014). Only two amphibian parasitic chytrids, *Batrachochytrium dendrobatidis* Longcore and *Batrachochytrium salamandrivorans* Martel are known to be parasitic on vertebrates. They are considered to cause the population declines of amphibians (Berger et al. 2016; James et al. 2015; Martel et al. 2013).

In aquatic habitats, parasitic chytrids influence the population dynamics of host phytoplankton

species (Canter and Lund 1948, 1951, 1953; Kudoh and Takahashi 1990; Van Donk and Ringelberg 1983). Chytrids have been shown to play an important role in aquatic food webs (Gleason et al. 2008; Kagami et al. 2014; Sime-Ngando 2012). Parasitic chytrids were previously found to transfer nutrients and energy from inedible phytoplankton to zooplankton such as *Daphnia* via zoospores (Kagami et al. 2007b, 2011). This new pathway from the phytoplankton to the zooplankton via the zoospores of parasitic chytrids named as "Mycoloop (Kagami et al. 2007a)" is considered to be an important factor to shape the food-web dynamics in aquatic ecosystems (Kagami et al. 2007a, 2014).

1.2. A history of chytrids classification

The first described chytrid is *Chytridium olla* Braun which is an obligate parasite of oogonia of green algae, *Oedogonium* (Braun 1851, 1856). Subsequently, approximately 1000 species have been described to date (Longcore 1996; Sparrow 1960). In the traditional classification, which is based on the morphological features of thallus (Karling 1977; Sparrow 1960), chytrids were included in the single class Chytridiomycetes (Phycomycetes in Sparrow 1960) and divided into three orders: Chytridiales, Blastocladiales and Monoblepharidales (Table 1). Chytridiales was the largest order in the Chytridiomycetes including more than 75 genera, and the classification of the order was based on thallus morphology. Sparrow (1960) divided the Chytridiales into two groups based on presence or absence of the operculum, a lid-like structure on zoosporangium, which opens during zoospore discharge. In contrast, other researchers such as Barr (1978) and Karling (1977) used development of thallus to divide the Chytridiales to the families. Under the traditional classification system, many species of chytrids had been described based on the direct observations on the collected samples or the observations on the crude culture using the substrates such as pollen. However, the morphological observations on pure culture of chytrids revealed the plasticity of important taxonomic characteristic in the traditional classification (Powell and Koch 1977). Therefore, revision of classification system of chytrids has been demanded.

As the technology of transmission electron microscopy improved, the ultrastructures of cell were observed in every organism. In 1970s to 1980s, the ultrastructural features such as organelles arrangements and flagellar apparatus began to be recognized as the stable taxonomic characteristics for classification of various groups of protists (Hibberd 1976; Hibberd and Leedale 1972; Lynn and Small 1981; O'Kelly and Floyd 1984). The early ultrastructural observations on chytrids (Koch 1961) revealed the significant variation of zoospore ultrastructure and its potential to use for assessing the relationships among the chytrid taxa. Since 1970s, zoospore ultrastructure of many chytrid taxa have been observed and several distinct "zoospore types" were recognized of the basis on a constellation of characters such as the flagellar apparatus and the microbody-lipid globule complex (MLC) which is a unique and intricate assemblage of organelles in the zoospore (Barr 1981; Lange and Olson 1979; Powell 1978). Barr (1980) used the features of zoospore ultrastructure for higher taxonomy of chytrids for the first time. He segregated some taxa from the Chytridiales and placed them in the new order Spizellomycetales based on their unique features of zoospore ultrastructure. Although Heath et al. (1983) added the obligate anaerobic chytrids such as *Neocallimastix frontalis* (Braune) Vavra & Joyon ex Heath in the Spizellomycetales, Li et al. (1993) placed these anaerobic chytrids in the new order Neocallimastigales. In the classification system by Barr (2001), chytrids were included in the phylum Chytridiomycota and divided into five orders: Chytridiales, Monoblepharidales, Blastocladiales, Spizellomycetales and Neocallimastigales (Table 1).

In the early 1990s, molecular phylogenetic analysis began to be used for assessing the classification of each group of the fungi. Based on the molecular phylogeny, the classification of the kingdom Fungi was dramatically changed (Hibbett et al. 2007). As a result of the molecular phylogenetic analysis of Chytridiomycota (James et al. 2000, 2006b), chytrids were divided into three basal phyla in the kingdom Fungi: Chytridiomycota, Blastocladiomycota, and Neocallimastigomycota (Hibbett et al. 2007; James et al. 2006b). In the Chytridiomycota, Monoblepharidales was segregated from Chytridiomycetes including Chytridiales and Spizellomycetales, and placed in the Monoblepharidomycetes (Hibbett et al. 2007).

In the Chytridiomycetes, Chytridiales and Spizellomycetales were revealed to be polyphyletic and separated into several monophyletic clades (James et al. 2000; 2006b). As the clades recognized by molecular phylogeny were also distinguished from each other based on the zoospore ultrastructure (James et al. 2000, 2006b), order-level taxonomy of Chytridiomycetes was rewritten based on the molecular phylogeny and the zoospore ultrastructure (Powell and Letcher 2014). Each monophyletic clade was circumscribed as the new orders. The "*Rhizophlyctis* clade" (James et al. 2006b) was segregated from the Spizellomycetales as the Rhizophlyctidales (Letcher et al. 2008a). The four new orders were established for the clades segregated from the Chytridiales sensu stricto: the Rhizophydiales (Letcher et al. 2006) for the "*Rhizophydium* clade" (James et al. 2000, 2006b), the Cladochytriales (Mozley-Standridge et al. 2009) for the "*Cladochytrium* clade" (James et al. 2006b), the Lobulomycetales (Simmons et al. 2009) for the "*Chytriomyces angularis* clade" (James et al. 2006b) and the Polychytriales (Longcore and Simmons 2012) for the "*Polychytrium* clade" (James et al. 2006b). Currently, the Chytridiomycetes contains nine orders in total (Table 1) with the recently established two new orders Gromochytriales and Mesochytriales (Karpov et al. 2014).

1.3. The problems of taxonomy of chytrids

Molecular environmental studies have revealed a large number of undescribed lineages in Chytridiomycetes (Freeman et al. 2009; Jobard et al. 2012; Lefèvre et al. 2007, 2008, 2012; Monchy et al. 2011). Some of the environmental sequences of uncultured chytrids form the order-level novel clades (Jobard et al. 2012; Lefèvre et al. 2008). These results indicate that there is a large number of gaps in the current classification system of Chytridiomycota.

Recent taxonomic studies on chytrids based on molecular phylogeny and zoospore ultrastructure were mainly conducted using isolates of saprophytic chytrids as their cultures are easily available (Letcher et al. 2006, 2008a, 2008c, 2012; Longcore and Simmons 2012; Mozley-Standridge et al. 2009; Simmons 2011; Simmons and Longcore 2012; Simmons et al. 2009). However, there are also a large number of parasitic chytrids described (Sparrow 1960; Longcore 1996). Although more than 400 species of parasitic chytrids were described, only a few parasitic chytrid species have been

sequenced and their phylogenetic positions were clarified (Karpov et al. 2010, 2014; Küpper et al. 2006; Lepelletier et al. 2014; Sønstebø and Rokrlack 2011; Vélez et al. 2011). Some parasitic chytrids such as *Caulochytrium protostelioides* Olive*, Polyphagus euglenae* (Bail) Nowakowski and *Zygorhizidium planktonicum* Canter were revealed to have unique characters of zoospore ultrastructure (Beakes et al. 1988; Powell 1981a, 1981b), but DNA sequences of them are not available. Some of the environmental sequences of "uncultured" chytirds may be identical or related to the sequences of parasitic chyrids whose culture has not yet been established and whose DNA data have not been obtained yet. Recently, two chytrids parasitic on algae were revealed to be related to the environmental sequences and placed in the order-level novel clades in Chytridiomycetes (Karpov et al. 2014). On the basis of molecular phylogeny and unique zoospore ultrastructural features, Karpov et al. (2014) proposed two new orders: Gromochytriales and Mesochytriales. Lepelletier et al. (2014) described a novel chytrid *Dinomyces arenysensis* Karpov & Guillou which is parasitic on marine dinoflagellates and placed it in the new family in the Rhizophydiales. These results mean that parasitic chytrids corresponds with the gaps in the current systematics of chytrids.

1.4. Purpose of this study

To establish the complete classification system of chytrids, taxonomic examinations of parasitic chytrids which are missing in the current system are indispensable. In this study, I aimed at constructing the taxonomy of parasitic chytrids infecting on the planktonic algae. My object was determination of taxonomic position of parasitic chytrids on algae in the current classification system. To do this I tried to establish dual cultures of parasitic chytrids and their host algae. Using these cultures, I identified them based on thallus morphology and discussed their taxonomy based on molecular phylogeny and zoospore ultrastructure.

Chapter 2
Isolation and culturing of parasitic chytrid on algae and their phylogenetic position

2.1. Introduction

Although more than 400 species of parasitic chytrids are described (Sparrow 1960; Longcore 1996), a few of them have been cultured. Obligate parasites are generally difficult to culture because they need their host to grow and can't grow as pure culture. Some parasitic chytrids on algae were cultured with their host, and used for the studies of the host-parasite interactions such as the examination of host specificity (Canter and Jaworski 1978, 1982, 1986; Canter et al. 1992; De Bruin el al. 2004; Doggett and Porter 1995; Gromov et al. 1999; Kagami et al. 2004; Müller et al. 1999; Sønstebø and Rokrlack 2011). Transmission electron microscopic observations of some species of parasitic chytrids were also conducted using the dual culture (Barr and Hadland-Hartmann 1978; Beakes et al. 1988, 1993; Powell et al. 1981a, 1981b). However, less than 10 species of obligate parasitic chytrids on algae or other fungi were examined in the molecular phylogenetic studies (Karpov et al. 2010, 2014; Küpper et al. 2006; Lepelletier et al. 2014; Sønstebø and Rokrlack 2011; Vélez et al. 2011), except for the plant pathogenic chytrids such as *Synchytrium* spp. and *Olpidium* spp. (James et al. 2006a, 2006b; Sekimoto et al. 2011; Smith et al. 2014). Therefore, only a few DNA sequence data of parasitic chytrids are available from the databases such as GenBank.

Here, I tried to establish the dual cultures of parasitic chytrids and their host algae for the taxonomic consideration of parasitic chytrids. Using the sequences of rDNA region, I examined the phylogenetic position of established cultures and compared them to the known sequences of saprophytic chytrids and also environmental sequences of uncultured chytrids.

2.2. Materials and methods

2.2.1. Isolation and culturing

I collected water samples from lakes or ponds in Japan and observed them directly to detect parasitic chytrids on algae. To establish algal cultures, I isolated a single algal cell or colony by micro-pipetting and placed it in a well of microplate containing WC medium (Guillard and Lorenzen 1972). To establish dual cultures of parasitic chytrids and their host algae, a single zoosporangium on a host algal cell was isolated by micro-pipetting and transferred into a well of a microplate containing the host algal culture, which was isolated from the same sample. Chytrid-alga dual cultures and host algal cultures were maintained at 14 °C or 20 °C under a photoperiod of 18:6 h (light:dark). I also used the dual culture C1 and host algal culture C5 which were provided by Dr. Maiko Kagami (Toho University). Cultures used in this study were listed in Table 2 and 3.

2.2.2. DNA extraction, amplification and sequencing

Cultures C1, KS93, KS94, KS98, KS99

DNA was extracted from dual cultures using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. 18S rDNA, ITS1-5.8S-ITS2, and 28S rDNA (D1/D2 region) genes were amplified by PCR using KOD FX (TOYOBO, Osaka, Japan) with the following primer set: NS1 (White et al. 1990) and LR5 (Vilgalys and Hester 1990). Thermal cycling conditions for PCR amplification were: i) 95 °C for 5 min, ii) 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 4 min, iii) 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 68 °C for 4 min, and iv) hold at 4 °C. PCR products were purified by PEG precipitation. Cycle sequence reactions were conducted using BigDye® Terminator v3.1 (Applied Biosystems) and the following primers: NS1, NS4, NS6 (White et al. 1990), and NS8z (O'Donnell et al. 1998) for 18S rDNA, ITS5 and ITS4 (White et al. 1990) for the ITS1-5.8S-ITS, and LR0R (Rehner and Samuels 1994) and LR5 for 28S rDNA. DNA sequences were analyzed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Culture KS97

I harvested about 10 zoospores from the dual culture by micro-pipetting, transferred to a 200-ml PCR tube, and used them as template for direct PCR. 18S rDNA, ITS1-5.8S-ITS2, and 28S rDNA (D1/D2 region) genes were amplified using KOD FX with the following primer sets: NS1 and NS8 (White et al. 1990) for 18S rDNA, ITS5 and ITS4 for ITS1-5.8S-ITS2, LR0R and LR5 for 28S rDNA. Thermal cycling conditions for PCR amplification were: i) 95 °C for 5 min, ii) 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 (for 18S rDNA) or 1 min (for ITS1-5.8S-ITS2 and 28S rDNA), iii) 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 68 °C for 3 min or 1 min, and iv) hold at 4 °C. PCR products were purified by PEG precipitation. Cycle sequence reactions were conducted using BigDye® Terminator v3.1 and the following primers: NS1, NS4, NS6 and NS8 for 18S rDNA, ITS5 and ITS4 for the ITS1-5.8S-ITS2, and LR0R and LR5 for 28S rDNA. DNA sequences were analyzed using an ABI PRISM 3130 Genetic Analyzer.

Culture KS100

I harvested about 10 zoospores from the dual culture by micro-pipetting, transferred to a 200-ml PCR tube, and used them as template for direct PCR. 18S rDNA, ITS1-5.8S-ITS2, and 28S rDNA (D1/D2 region) genes were amplified using KOD FX with the following primer sets: NS1 and NS8z for 18S rDNA, ITS1F (Gardes and Bruns 1993) and LR5 for ITS1-5.8S-ITS2 and 28S rDNA. Thermal cycling conditions for PCR amplification were: i) 95 °C for 5 min, ii) 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 2 min, iii) 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 68 °C for 2 min, and iv) hold at 4 °C. PCR products were purified by PEG precipitation. Cycle sequence reactions were conducted using BigDye® Terminator v3.1 and the following primers: NS1, NS4, NS6 and NS8z for 18S rDNA, ITS1F and ITS4 for the ITS1-5.8S-ITS2, and LR0R and LR5 for 28S rDNA. DNA sequences were analyzed using an ABI PRISM 3130 Genetic Analyzer.

Algal cultures

DNA was extracted from each culture using HotSHOT method (Truett et al. 2000). 18S rDNA genes were amplified by PCR using KOD FX with the following primer set: SR1 and SR12 (Nakayama et al. 1996). Thermal cycling conditions for PCR amplification were: i) 95 °C for 5 min, ii) 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 2 min, iii) 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 68 °C for 2 min, and iv) hold at 4 °C. PCR products were purified by PEG precipitation. Cycle sequence reactions were conducted using BigDye® Terminator v3.1 and the following primers: SR1, SR2, SR5, SR7, SR9 (Nakayama et al. 1996) and SR12. DNA sequences were analyzed using an ABI PRISM 3130 Genetic Analyzer.

2.2.3. Phylogenetic analysis

I created a dataset of the 18S rDNA sequences of chytrids. As a result of BLAST searches using the 18S rDNA sequences of my cultures, I found that they were closely related to the sequences of uncultured clones. I selected some of these sequences and added them to the dataset. I also added some environmental sequences which belong to the novel clades in Chytridiomycetes (Jobard et al. 2012; Lefèvre et al. 2008). *Rozella allomycis* Faust and *Rozella* sp. (JEL374) were selected as an outgroup. Sequences were automatically aligned with MAFFT (Katoh and Standley 2013). Ambiguously aligned regions were excluded using trimAl (Capella-Gutiérrez et al. 2009) with a gappyout model. The maximum likelihood (ML) tree was inferred using RAxML 8.2.7 (Stamatakis 2014). I ran an analysis under the GTRGAMMAI model and used the "-fa" option to conduct a rapid bootstrap analysis with 1000 replicates combining 200 searches for the optimal tree. A Bayesian analysis was run using MrBayes 3.2.6 (Ronquist et al. 2012) under the GTRGAMMAI 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 the remaining 75% of trees.

2.3. Results

2.3.1. Isolation and culturing

I newly established six dual cultures of chytrids (KS93, KS94, KS97, KS98, KS99 and KS100). Detailed information on cultures was described in Table 2. Their taxonomy was discussed in Chapter 3, 4 and 5.

Detailed information on established cultures of algae used in this study was described in Table 3. Identification of each alga was made based on morphological characters and BLAST searching using the 18S rDNA sequences. Although cultures KSA17, KSA47 and KSA52 were identified as *Aulacoseira granulata* (Ehrenberg) Simonsen based on the morphological characters, the sequence data divided them to two groups. Edgar and Theriot (2004) pointed out that *Au. granulata* is not a single species but a species complex including several intraspecific groups based on the phylogenetic analysis using the morphological (qualitative and quantitative) data. Here, I tentatively called cultures KSA17 and KSA52 as *Au. granulata* group 1, and KSA47 as *Au. granulata* group 2. The 18S rDNA sequences of cultures KSA32 and KSA56 were identical to that of "*Synedra ulna* strain UTEX FD404 (GenBank accession number: HQ912590)". However, morphological characters of two cultures were distinguished from that of *S. ulna*. Thus, I regarded cultures KSA32 and KSA56 as *Synedra* sp.

2.3.2. Phylogenetic analysis

In the ML tree, cultures C1, KS94, KS98 and KS99 were related to the environmental sequences of uncultured chytrids, and they formed a clade which were not assingned to any known orders in the Chytridiomycetes (Fig. 1). This clade was reported as "Novel Clade II" in the phylogenetic survey of fungi at the three lakes in France (Jobard et al. 2012). Lefèvre et al. (2008) also reported a novel clade and named it "Novel Clade II". "Novel Clade II" sensu Jobard et al. (2012) and "Novel Clade II" sensu Lefèvre et al. (2008) were distinct from each other (Fig. 1). Culture KS97 was sister to environmental sequence named P34.43 from Lake Pavin in France (Lefranc et al. 2005) but statistical support of the sister relationship was not high (ML bootstrap value = 46; Bayesian

posteriorly probability = 0.96). KS97-P34.43 clade was distinguished from any other known orders in Chytridiomycetes (Fig. 1). Culture KS93 was placed in the order Chytridiales (Fig. 1) and sister to two environmental sequences 272H08 and 282H08 from Columbia River in USA (Kahn et al. 2014). Culture KS100 was positioned in the order Rhizophydiales (Fig. 1) and there were no related environmental sequences or known species.

2.4. Discussion

A molecular analysis of environmental samples previously indicated the existence of a large number of uncultured chytrids with no affinity for any known species (Freeman et al. 2009; Jobard et al. 2012; Lefèvre et al. 2007, 2008, 2012; Monchy et al. 2011). The phylogenetic analysis of parasitic chytrid cultures established in this study revealed that five cultures are related to the environmental sequences of uncultured chytrids. The 18S rDNA sequences of cultures KS93 and KS98 were almost identical to the environmental sequences: 272H08 and 282H08 from the Columbia River in USA (Kahn et al. 2014) and PFH1AU2004 from Lake Pavin in France (Lefèvre et al. 2007). Some of the other environmental sequences of as yet undiscovered chytrids may be related or identical to parasitic chytrids. On the other hands, cultures KS97 and KS100 were not related to environmental sequences. Especially, no environmental sequences did form the sister clade with culture KS100. That is to say, these two parasitic chytrids have not been found during environmental DNA surveys. Regarding the culture KS97, it had approximately 1000 bp long insertion in the 18S rDNA sequence (data not shown). PCR amplification might be failed for this chytrid. Other possible reason of overlooking the two chytrids is limited occurrence of parasitic chytrids. Parasitic chytrid on algae are known to occur in accordance with the seasonal population dynamics of host algae (Canter and Lund 1948, 1951, 1953; Kudoh and Takahashi 1990). It is possible that the two chytrids did not occur at the time of sampling of environmental DNA surveys in the previous studies.

Chapter 7
Concluding remarks

In this study, I clarified the taxonomic position of seven cultures of parasitic chytrids including six newly established cultures. As a results, seven cultures were identified as five species. Three of them were the known species but accommodated in the two new orders in the Chytridiomycetes. The other two were novel taxa in the known orders: the Chytridiales and the Rhizophydiales respectively. These results reconfirmed that taxonomic studies on parasitic chytrids are indispensable to fill the gaps in the current systematics of chytrids.

Five cultures were identified as three known species of the genus *Zygorhizidium*: *Zygor. willei* (KS97), *Zygor. planktonicum* (KS98) and *Zygor. melosirae* (C1, KS94 and KS99). To date, none of the species in the *Zygorhizidium* have been sequenced. Here, I clarified the taxonomic position of the *Zygorhizidium* in the current classification system of chytrids for the first time. As a results, the *Zygorhizidium* was revealed to be polyphyletic and divided into two order-level novel clades. Based on the uniqueness of zoospore ultrastructure and monophyly indicated by molecular phylogeny, I proposed two new orders: Zygorhizidiales including *Zygo. willei* (type species of the *Zygorhizidium*), and Zygophlyctidales including *Zygop. planktonica* and *Zygop. melosirae*.

Cultures KS93 and KS100 were identified as new species in the known orders. Culture KS93 was assigned as a new species in a new genus *Pendulichytrium sphaericum* of the family Chytriomycetaceae in the order Chytridiales. Culture KS100 was assigned as a new species in a new genus *Collimyces mutans* of the new family Collimycetaceae in the order Rhizophydiales. These results expanded our knowledge of the ecophysiological diversities of chytrids in the known orders which have been considered to contain mainly saprophytic taxa.

As the result of above mentioned discussion on molecular phylogeny, ecology, physiology and morphology of chytrids, the future perspectives of taxonomic studies on chytrids were provided.

The significance of the analysis of environmental DNA sequences on the taxonomic study of chytrid was evaluated. Three of five species identified in this study were identical or related to the

environmental sequences of uncultured chytrids. Indeed, two belonged to the clade that was previously reported as an undescribed clade, “Novel Clade II” composed of only the environmental sequences of uncultured chytrids. The real status of members of the “Novel Clade II” was identified for the first time. The other two of five species did not have any affinity with environmental sequences. That is to say, these taxa were overlooked even in the environmental DNA surveys. To explore the whole diversity of chytrids and fill the gap of the current systematics of chytrids, the more extensive samplings are needed. And also, more culture-based taxonomical studies of parasitic chytrids are indispensable to clarify the identity of the environmental sequences.

The taxonomic implications of host specificity, nutritional traits, and sexual reproduction of chytrids were discussed. Combining with the appropriate molecular marker, the host specificity of chytrids has the potential to be used as taxonomic character for species delineation. Considering the taxonomic distribution of the nutritional traits and the type of sexual reproduction, these characters were revealed to be used as the available taxonomic character for order- to genus-level delineations. For instance, the order Zygophlyctidales proposed in this study is possible to be exclusive for parasitic species. The uniqueness of “*Zygorhizidium* type” sexual reproduction supports the order-level novel taxonomic positions of *Zygorhizidium* spp. Although “*Zygorhizidium* type” sexual reproduction was turned to emerge at least in the two distantly independent lineages, these sexual behaviours of two lineages might be distinguished based on the timing of karyogamy. To evaluate and realize the taxonomic implications of nutritional traits and sexual reproduction, precise observation and analyses of the further additional taxa are needed.

The zoospore ultrastructural characters which have been important as taxonomic characters were discussed in the point of view of their cytological aspects and function. The NfC of *Zygop. planktonica* with the unique morphology and position was interpreted as the immature stage of the centriole during the duplication. The function of the rumposome and the unique membrane bounded vesicle observed in *Zygor. willei* and *Zygop. planktonica* were discussed. Using the culture established in this study, the precise observations and analyses on cytological events like mitosis, zoospore movement and infection process are needed.

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Tables

Table 1. Classification system of chytrids from 1960 to the present.

Sparrow (1960)	Barr (2001)	Current systematics
	CHYTRIDIOMYCOTA	CHYTRIDIOMYCOTA
PHYCOMYCETES	CHYTRIDIOMYCETES	CHYTRIDIOMYCETES
Chytridiales	Chytridiales	Chytridiales
Monoblepharidales	Spizellomycetales	Cladochytriales
Blastocladiales	Monoblepharidales	Gromochytriales
	Neocallimastigales	Lobulomycetales
	Blastocladiales	Mesochytriales
		Polychytriales
		Rhizophydiales
		Rhizophlyctidales
		Spizellomycetales
		MONOBLEPHARIDOMYCETES
		Monoblepharidales
		NEOCALLIMASTIGOMYCOTA
		NEOCALLIMASTIGOMYCETES
		Neocallimastigales
		BLASTOCLADIOMYCOTA
		BLASTOCLADIOMYCETES
		Blastocladiales

Table 2. Dual cultures used in this study

Culture	Species	Host (culture)	Source	Date of sampling
C1	*Zygophlyctis melosirae* comb. nov.	*Aulacoseira ambigua* (C5)	Lake Inbanuma, Chiba, Japan	30 July 2011
KS93	*Pedulichytrium sphaericum* gen. et sp. nov.	*Aulacoseira granulata* (KSA17)	Lake Teganuma, Chiba, Japan	2 September 2014
KS94	*Zygophlyctis melosirae* comb. nov.	*Aulacoseira ambigua* (KSA24)	Lake Shirakaba, Nagano, Japan	23 September 2014
KS97	*Zygorhizidium willei*	*Gonatozygon brebissonii* (KSA15)	Lake Suwa, Nagano, Japan	7 June 2015
KS98	*Zygophlyctis planktonika*	*Asterionella formosa* (KSA51)	Lake Biwaike, Nagano, Japan	31 October 2015
KS99	*Zygophlyctis melosirae* comb. nov.	*Aulacoseira granulata* (KSA52)	Lake Suwa, Nagano, Japan	24 October 2015
KS100	*Collimyces mutans* gen. et sp. nov.	*Microglena coccifera* (KSA58)	Pond in the park, Chiba, Japan	27 March 2016

Table 3. Algal cultures used in this study

Species	Culture	Source	Date of sampling	Comment
Bacillariophyceae				
Asterionella formosa	KSA51	Lake Biwaike, Nagano, Japan	12 October 2015	Host of KS98
Asterionella formosa	KSA59	Lake Teganuma, Chiba, Japan	27 March 2016	-
Asterionella formosa	KSA60	Lake Chuzenji, Tochigi, Japan	24 May 2016	-
Aulacoseira ambigua	C5	Lake Inbanuma, Nagano, Japan	30 July 2011	Host of C1
Aulacoseira ambigua	KSA24	Lake Shirakaba, Nagano, Japan	23 September 2014	Host of KS94
Aulacoseira ambigua	KSA35	Lake Teganuma, Chiba, Japan	21 April 2015	-
Aulacoseira granulata group 1	KSA17	Lake Teganuma, Chiba, Japan	21 August 2014	Host of KS93
Aulacoseira granulata group 1	KSA52	Lake Suwa, Nagano, Japan	24 October 2015	Host of KS99
Aulacoseira granulata group 2	KSA47	Lake Shirakaba, Nagano, Japan	4 September 2015	-
Aulacoseira subarctica	KSA62	Lake Chuzenji, Tochigi, Japan	24 May 2016	-
Synedra sp.	KSA32	Lake Teganuma, Chiba, Japan	2 March 2015	-
Synedra sp.	KSA56	Lake Inbanuma, Nagano, Japan	19 January 2016	-
Chlorophyceae				
Microglena coccifera	KSA58	Pond in the park, Chiba, Japan	27 March 2016	Host of KS100
Zygnematophyceae				
Gonatozygon brebissonii	KSA15	Lake Suwa, Nagano, Japan	5 July 2014	Host of KS97

Table 5. Comparison of thallus morphology and host diatom of cultures C1, KS94, KS99 and *Zygorhizidium melosirae* Canter emend. (Canter 1967)

Culture/Species	Zoospore	Zoosporangium	Resting spore	Host diatom
C1	spherical, 2.5–3 μm, with a single lipid globule, a ~13 μm long flagellum	narrowly obpyriform, 8–11.5 × 4.4–5.7 μm	ellipsoidal, ovoid, 4.5–6.9 × 4.1–5.4 μm	*Aulacoseira ambigua*
KS94	spherical, 2.5–3 μm, with a single lipid globule, a ~13 μm long flagellum	narrowly obpyriform, 10–16.5 × 5–7.5 μm	ellipsoidal, ovoid, 6–8.5 × 4–6 μm	*Aulacoseira ambigua*
KS99	spherical, 2.5–3 μm, with a single lipid globule, a ~15 μm long flagellum	broadly obpyriform, 7.6–10.8 × 6.3–8.8 μm	ellipsoidal, ovoid, 6.3–8 × 4.8–6.6 μm	*Aulacoseira granulata*
Zygorhizidium melosirae emend.	spherical, 3–3.5 μm, with a single lipid globule, a ~17.5 μm long flagellum	broadly obpyriform, 11.2–15 × 7–9.5 μm	ovoid, 6.6–13 × 3.3–8 μm	*Aulacoseira subarctica*

Figure legends

Fig. 1. Maximum-likelihood tree of chytrids using 18S rDNA sequences. Black circles indicate the environmental sequences of uncultured chytrids. Only bootstrap support ≥ 70% is shown. Nodes supported by Bayesian posterior probabilities ≥ 0.95 are highlighted by bold lines.

Fig. 2. Morphology of *Zygorhizidium willei* KS97 on the host *Gonatozygon brebissonii*. **A.** Two zoospores. **B.** Encysted zoospore. **C.** Germinated thallus. **D.** Developing zoosporangium. **E.** Mature zoosporangium. **F.** Zoospore discharge. **G.** Empty zoosporangium with an operculum. **H, I.** Conjugation of two thalli. **J.** Resting spore. **K.** Germinated resting spore. RS=resting spore, Zsr=zoosporangium. Scale Bars = 10 μm.

Fig. 3. Morphology of *Zygophlyctis planktonica* KS98 on the host *Asterionella formosa*. **A.** Encysted zoospore. **B.** Developing zoosporangium. **C.** Mature zoosporangium. **D, E.** Zoospore discharge. **F.** Conjugation of two thalli. **G, H.** Resting spore. Scale Bars = 10 μm.

Fig. 4. Morphology of *Zygophlyctis melosirae* C1 on the host *Aulacoseira ambigua*. **A.** Encysted zoospore. **B.** Developing zoosporangium. **C.** Mature zoosporangium. **D, E.** Zoospore discharge. **F.** Conjugation of two thalli. **G, H.** Resting spore. Scale Bars = 10 μm.

Fig. 5. Morphology of *Zygophlyctis melosirae* KS94 on the host *Aulacoseira ambigua*. **A.** Encysted zoospore. **B.** Developing zoosporangium. **C.** Mature zoosporangium. **D, E.** Zoospore discharge. **F.** Conjugation of two thalli. **G, H.** Resting spore. Scale Bars = 10 μm.

Fig. 6. Morphology of *Zygophlyctis melosirae* KS99 on the host *Aulacoseira granulata*. **A.** Encysted zoospore. **B.** Developing zoosporangium. **C.** Mature zoosporangium. **D, E.** Zoospore discharge. **F.** Conjugation of two thalli. **G, H.** Resting spore. Scale Bars = 10 μm.

Fig. 7. Ultrastructure of developing zoosporangium and rhizoid of *Zygorhizidium willei* KS97 (A), *Zygophlyctis planktonica* KS98 (B). Ap = apophysis, F = frustule, HCW = host cell wall, Rh = rhizoid, Zsr = zoosporangium. Scale Bars = 1 μm.

Fig. 8. Zoospore ultrastructure of *Zygorhizidium willei* KS97. **A, B.** Longitudinal section of zoospore. **C–E.** Longitudinal serial sections through the base of flagellaum. BS1 = banded structure 1, BS2 = banded structure 2, K = kinetosome, L = lipid globule, LV = large vesicle, Mt = mitochondrion, N = nucleus, NfC = nonflagellated centriole, Ru = rumposome. Scar Bars = 500 nm (A, B), 200 nm (C–E).

Fig. 9. Zoospore ultrastructure of *Zygorhizidium willei* KS97. **A–D.** Transverse serial sections through the base of flagellum. **E, F.** Serial sections including transverse section of nonflagellated centriole. **G.** Section near the posterior region of the zoospore with small dense vesicles. BS1 = banded structure 1, BS2 = banded structure 2, K = kinetosome, LV = large vesicle, NfC = nonflagellated centriole, Ru = rumposome, SDB = small dense bodies. Scar Bars = 200 nm.

Fig. 10. Zoospore ultrastructure of *Zygophlyctis planktonica* KS98. **A.** Longitudinal section of zoospore. **B.** Microbody associated with lipid globule. **C.** Longitudinal section of rumposome associated with fibrillar vesicle. **D.** Transverse section of rumposome. **E.** Dense particulate body associated with mitochondrion. **F, G.** Longitudinal section of the base of flagellum. **H.** Nonflagellated centriole associated with mitochondrion. **I.** Transverse section of base of flagellum. **J.** Transverse section of kinetosome. **K, L.** Transverse section of nonflagellated centriole. DPB = dense particulate body, FV = fibrillar vesicle, K = kinetosome, L = lipid globule, Mb = microdoby, Mt = mitochondrion, N = nucleus, NfC = nonflagellated centriole, Ru = rumposome. Scale Bars = 500 nm (A), 200 nm (B–H), 100 nm (I–L).

Fig. 11. Maximum-likelihood tree of chytrids using concatenated rDNA sequences (18S, 5.8S, 28S). Black circles indicate the environmental sequences of uncultured chytrids. Only bootstrap support ≥ 70% is shown. Nodes supported by Bayesian posterior probabilities ≥ 0.95 are highlighted by bold lines.

Fig. 12. Morphology of *Pedulichytrium sphaericum* KS93 on the host *Aulacoseira granulata*. **A.** Two zoospores. **B.** Encysted and germinated zoospore. **C.** Developing zoosporangium. **D.** Mature zoosporangium. **E–G.** Zoospore discharge. **H.** Rhizoid inside the host cell (arrowhead). **I.** Resting spore. **K.** Resting spore formed after rhizoidal anastomosis between two thalli. Scale Bars = 10 μm.

Fig. 13. Ultrastructure of *Pedulichytrium sphaericum* KS93. **A.** Longitudinal section of zoospore. **B.** Longitudinal section of rumposome with microtubular root (arrow). **C.** Transverse section of rumposome. **D.** Longitudinal section of base of flagellum with microtubular root (arrow). **E.** Transverse section of microtubular root (arrow). **F.** Longitudinal section of the base of the flagellum with kinetosome associated structure (arrowhead). **G.** Transverse section of the base of the flagellum with kinetosome associated structure (arrowhead). **H.** Developing zoosporangium and rhizoid. CC = cell coat, F = frustule, FP = flagellar plug, G = Golgi apparatus, H = host, K = kinetosome, L = lipid globule, Mt = mitochondrion, N = nucleus, NfC = nonflagellated centriole, R = ribosomes, Rh = rhizoid, Ru = rumposome. Zsr = zoosporangium. Scale Bars = 500 nm (A), 200 nm (B–G), 2 μm (H).

Fig. 14. Maximum-likelihood tree of Chytridiales using concatenated rDNA sequences (18S, 5.8S, 28S). Black circles indicate the environmental sequences of uncultured chytrids. Only bootstrap support ≥ 70% is shown. Nodes supported by Bayesian posterior probabilities ≥ 0.95 are highlighted by bold lines.

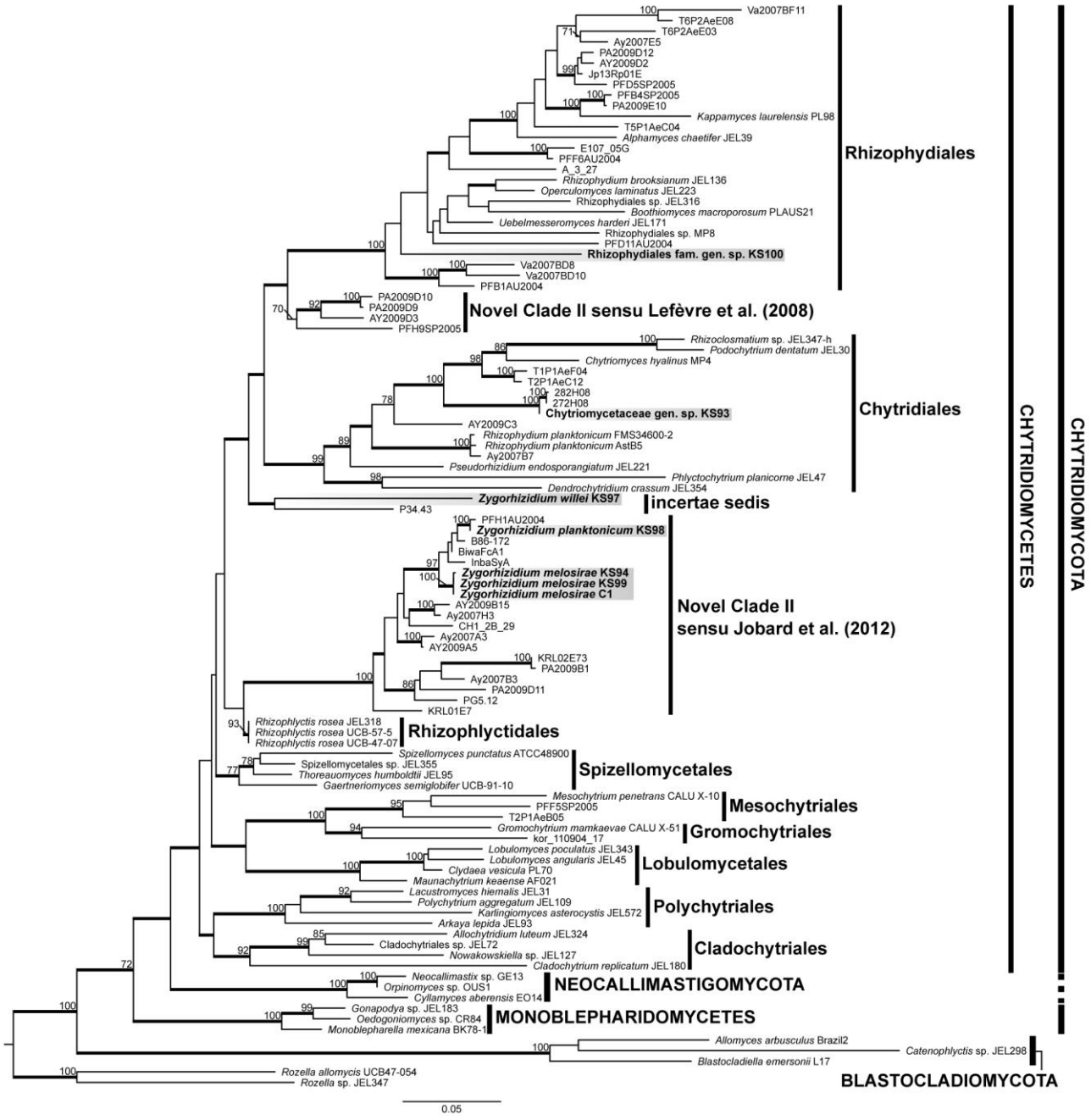
Fig. 15. Morphology of *Collimyces mutans* KS100 on the host *Microglena coccifera*. **A.** Zoospore. **B.** Encysted zoospore. **C.** Developing zoosporangium. **D.** Mature zoosporangium. **E–H.** Zoospore discharge from small pores (arrows) on zoosporangium. **I.** Delicate, thin wall (arrowhead) of empty zoosporangium. Scale Bars = 10 μm.

Fig. 16. Zoospore ultrastructure of *Collimyces mutans* KS100. **A.** Longitudinal section of zoospore. **B.** Longitudinal section of rumposome. **C.** Transverse section of rumposome. **D.** Longitudinal section of kinetosome and nonflagellated centriole with bridge including wide zone of convergence (arrowhead). **E–G.** Transverse serial sections of kinetosome and nonflagellated centriole with bridge (arrowhead), kinetosome associated structure (double arrowhead) and microtubular root (arrow). **H.** Longitudinal section of kinetosome with kinetosome associated structure (double arrowhead). **I.** Longitudinal section of kinetosome with microtubular root (arrow). **J.** Transverse section of microtubular root (arrows) near kinetosome. **K.** Transverse section microtubular root (arrows) near lipid globule**.** K = kinetosome, L = lipid globule, Mt = mitochondrion, N = nucleus, NfC = nonflagellated centriole, R = ribosomes, Ru = rumposome. Scale Bars = 500 nm (A), 200 nm (B–J), 100 nm (K).

Fig. 17. Maximum-likelihood tree of Rhizophydiales using concatenated rDNA sequences (5.8S and 28S). Only bootstrap support ≥ 70% is shown. Nodes supported by Bayesian posterior probabilities ≥ 0.95 are highlighted by bold lines.

Figures

Fig. 1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T6P2AeE03
Ay2007E5
PA2009D12
AY2009D2
Jp13Rp01E
PFD5SP2005
PFB4SP2005
PA2009E10
Kappamyces laurelensis PL98
T5P1AeC04
Alphamyces chaetifer JEL39
E107_05G
PFF6AU2004
A_3_27
Rhizophydium brooksianum JEL136
Operculomyces laminatus JEL223
Rhizophydiales sp. JEL316
Boothiomyces macroporosum PLAUS21
Uebelmesseromyces harderi JEL171
Rhizophydiales sp. MP8
PFD11AU2004
Rhizophydiales fam. gen. sp. KS100
Va2007BD8
Va2007BD10
PFB1AU2004
Rhizophydiales
PA2009D10
PA2009D9
AY2009D3
PFH9SP2005
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Rhizoclosmatium sp. JEL347-h
Podochytrium dentatum JEL30
Chytriomyces hyalinus MP4
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T2P1AeC12
282H08
272H08
Chytriomycetaceae gen. sp. KS93
AY2009C3
Rhizophydium planktonicum FMS34600-2
Rhizophydium planktonicum AstB5
Ay2007B7
Pseudorhizidium endosporangiatum JEL221
Phlyctochytrium planicorne JEL47
Dendrochytridium crassum JEL354
Chytridiales
Zygorhizidium willei KS97
P34.43
incertae sedis
PFH1AU2004
Zygorhizidium planktonicum KS98
B86-172
BiwaFcA1
InbaSyA
Zygorhizidium melosirae KS94
Zygorhizidium melosirae KS99
Zygorhizidium melosirae C1
AY2009B15
Ay2007H3
CH1_2B_29
Ay2007A3
AY2009A5
KRL02E73
PA2009B1
Ay2007B3
PA2009D11
PG5.12
KRL01E7
Novel Clade II
sensu Jobard et al. (2012)
Rhizophlyctis rosea JEL318
Rhizophlyctis rosea UCB-57-5
Rhizophlyctis rosea UCB-47-07
Rhizophlyctidales
Spizellomyces punctatus ATCC48900
Spizellomycetales sp. JEL355
Thoreauomyces humboldtii JEL95
Gaertneriomyces semiglobifer UCB-91-10
Spizellomycetales
Mesochytrium penetrans CALU X-10
PFF5SP2005
T2P1AeB05
Mesochytriales
Gromochytrium mamkaevae CALU X-51
kor_110904_17
Gromochytriales
Lobulomyces poculatus JEL343
Lobulomyces angularis JEL45
Clydaea vesicula PL70
Maunachytrium keaense AF021
Lobulomycetales
Lacustromyces hiemalis JEL31
Polychytrium aggregatum JEL109
Karlingiomyces asterocystis JEL572
Arkaya lepida JEL93
Polychytriales
Allochytridium luteum JEL324
Cladochytriales sp. JEL72
Nowakowskiella sp. JEL127
Cladochytrium replicatum JEL180
Cladochytriales
Neocallimastix sp. GE13
Orpinomyces sp. OUS1
Cyllamyces aberensis EO14
NEOCALLIMASTIGOMYCOTA
Gonapodya sp. JEL183
Oedogoniomyces sp. CR84
Monoblepharella mexicana BK78-1
MONOBLEPHARIDOMYCETES
Allomyces arbusculus Brazil2
Catenophlyctis sp. JEL298
Blastocladiella emersonii L17
BLASTOCLADIOMYCOTA
Rozella allomycis UCB47-054
Rozella sp. JEL347
0.05
CHYTRIDIOMYCETES
CHYTRIDIOMYCOTA

Fig. 3

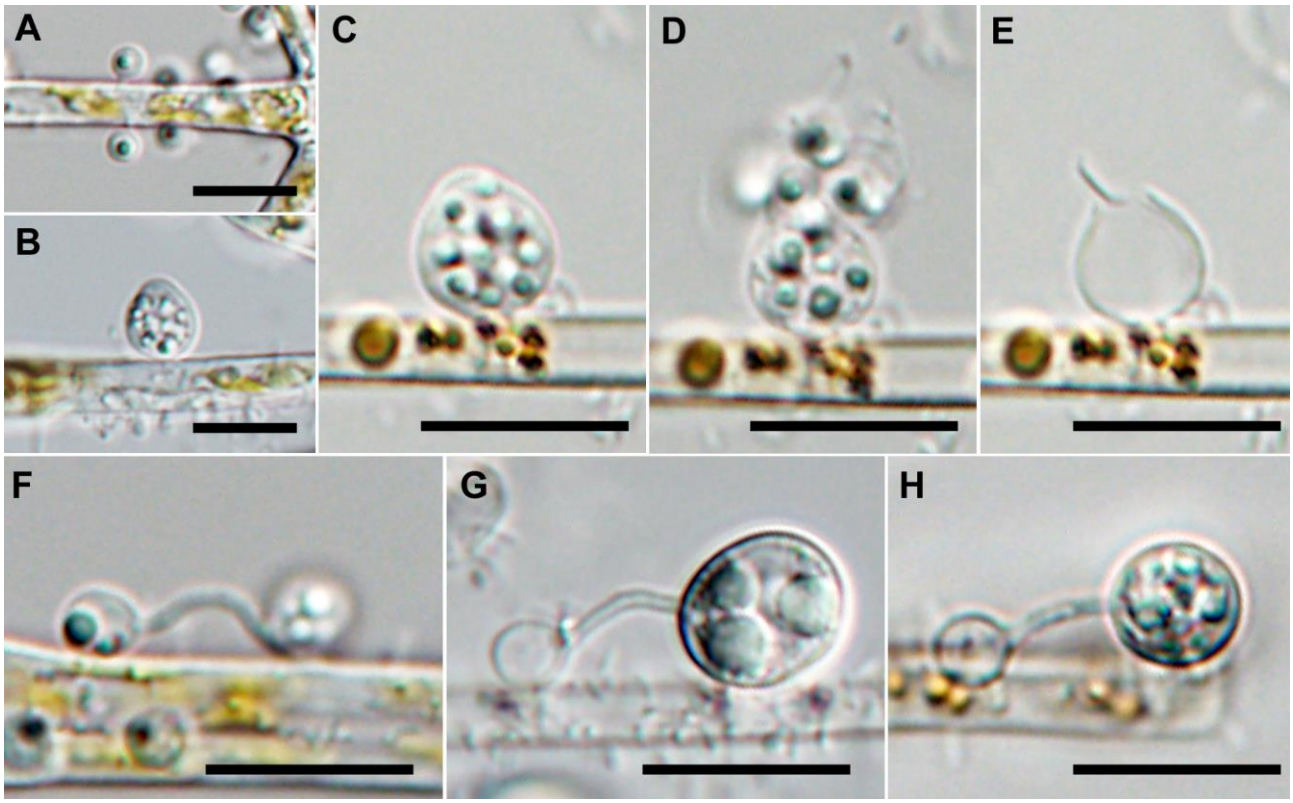

Fig. 4

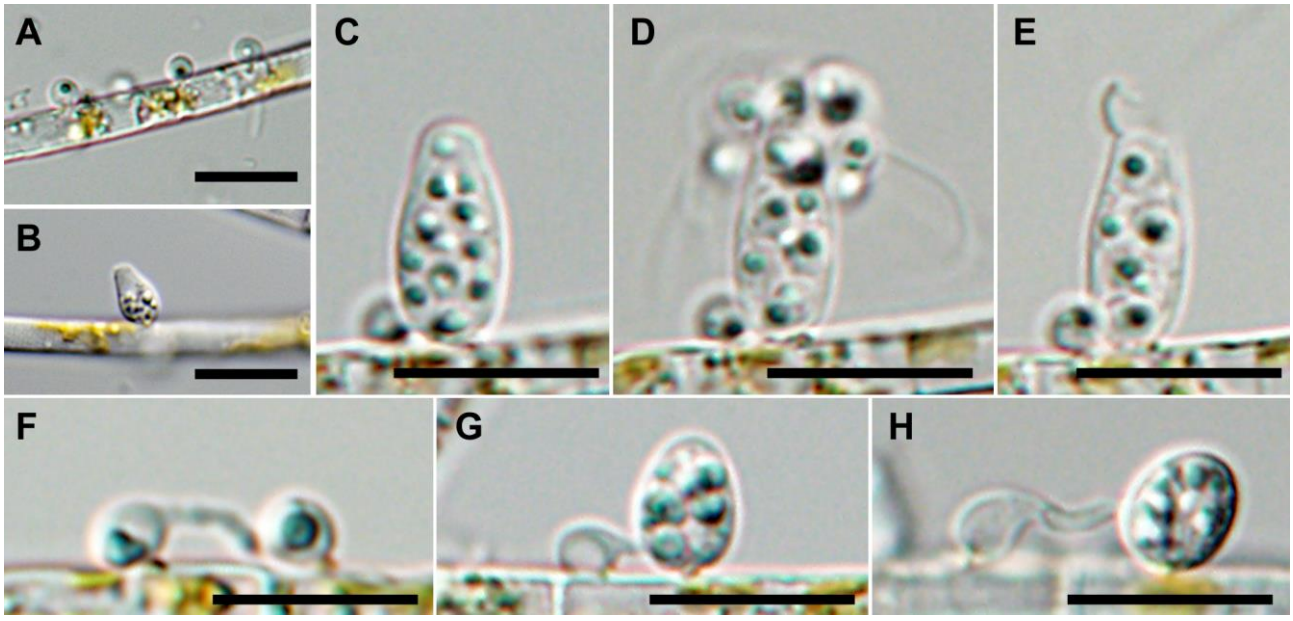

Fig. 5

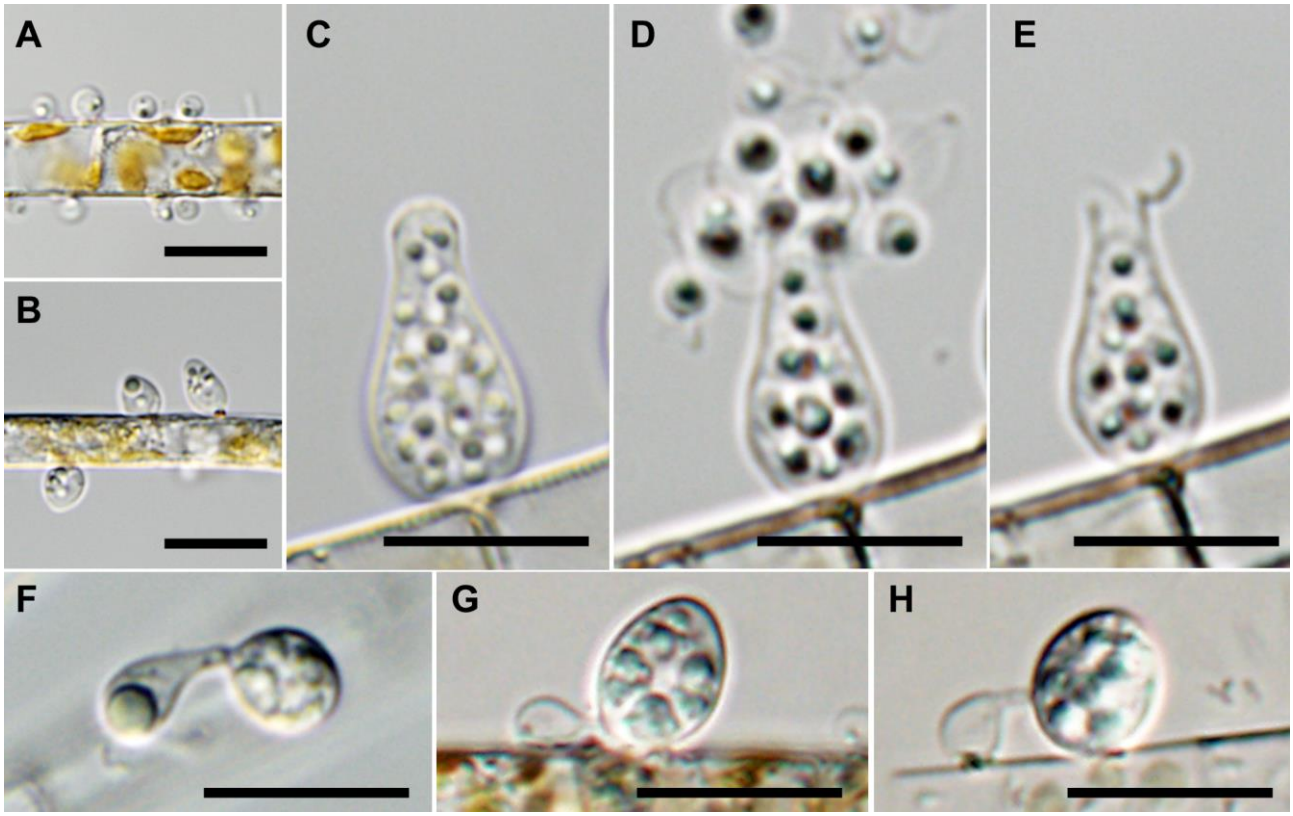

Fig. 6