



Temporal variation in community structure of zoosporic fungi in Lake Biwa, Japan

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ABSTRACT: Zoosporic fungi play an important role in aquatic environments, but their diversity, especially that of parasitic fungi of phytoplankton, has still not been fully revealed. We conducted monthly analyses of the community structure of zoosporic fungi at a pelagic site in Lake Biwa, Japan, from May to December 2016. Metabarcoding analysis, targeted to a large subunit region of ribosomal DNA in the nano-size fraction of particles (2–20 µm), was carried out on the samples. We also counted large phytoplankton and chytrid sporangia attached to the hosts. We detected 3 zoosporic fungal phyla (Blastocladiomycota, Chytridiomycota and Cryptomycota) within 107 operational taxonomic units (OTUs), in which Chytridiomycota was the most diverse and abundant phylum. Few fungal OTUs overlapped between months, and specific communities were detected in each month. These results showed that diverse zoosporic fungi with high temporal variability inhabited the lake. Five large phytoplankton species were found to be infected by chytrids: *Staurastrum dorsidentiferum*, *S. rotula*, *Closterium aciculare*, *Asterionella formosa* and *Aulacoseira granulata*. Some chytrids were detected by metabarcoding analysis: *Zygophlyctis asterionellae* infecting *A. formosa*, *Staurastromyces oculus* infecting *S. dorsidentiferum* and *Pendulichytrium sphaericum* infecting *A. granulata*. One OTU detected in association with infected *C. aciculare* by microscopic counting might have been an obligate parasitic chytrid of *C. aciculare*. The results indicated that a combination of metabarcoding and microscopic analysis revealed more information on zoosporic fungi, including those that are parasitic.

KEY WORDS: Zoosporic fungi · High-throughput sequencing · LSU rDNA · Chytrid · Community diversity

1. INTRODUCTION

Zoosporic fungi include facultative and obligate parasites and saprotrophs, belonging mainly to the phyla Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Cryptomycota and Aphelidiomycota (James et al. 2006, Hibbett et al. 2007, Karpov et al. 2014, Letcher & Powell 2018), and they play important roles in aquatic environments (Kagami et al. 2007a, Frenken et al. 2017, Grossart et al. 2019,

Yoneya et al. 2021). Among zoosporic fungi, Chytridiomycota and Aphelidiomycota are prominent parasites of phytoplankton not only in freshwater lakes (Kagami et al. 2006, Karpov et al. 2014), but also in marine ecosystems (Garvetto et al. 2019, Kiliass et al. 2020). Zoosporic fungi release motile zoospores that attach to the surface of host phytoplankton cells. These zoospores have often been miscounted as flagellates, owing to their uncharacteristic morphological features (Kagami et al. 2007b, Lefèvre et al. 2007,

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Jobard et al. 2010). Parasitic chytrids potentially influence food web dynamics by transferring nutrients and energy from large phytoplankton cells to zooplankton, in a process known as the ‘mycoloop’ (Kagami et al. 2007a).

Recently, the taxonomy of zoosporic fungi has been greatly expanded with the development of molecular phylogenetics and ultrastructure analysis (Frenken et al. 2017). However, owing to missing DNA reference data, parasitic fungi are often not identifiable, even by deep amplicon sequencing. Phylogenetic analysis could assign taxonomic positions of unidentifiable sequences and shed light on their ecology based on phylogenetic positions (Seto et al. 2020). Counting fungal sporangia attached to host phytoplankton cells is helpful in identifying the parasitic fungi of phytoplankton. Combining microscopic counts and metabarcoding analysis may improve the detection of parasitic fungi and allow us to understand the community structure of zoosporic fungi.

In Lake Biwa, the largest lake in Japan, parasitic chytrids have been observed to infect the dominant green alga, *Staurastrum dorsidentiferum*, and to influence its temporal dynamics and material flows (Kagami & Urabe 2002, Kagami et al. 2004). Diverse fungi including Cryptomycota and Aphelidiomycota, as well as Chytridiomycota, have also been detected from different phytoplankton species in Lake Biwa by analysis of the single sporangium-like cell attached to a phytoplankton cell or colony (Ishida et al. 2015). The spatio-temporal dynamics of these host-parasite relationships in Lake Biwa remain uncertain. High-throughput sequencing of the internal transcribed spacer 2 (ITS2) region of ribosomal DNA (rDNA) revealed fungal communities in Lake Biwa; however, only 1 zoosporic fungal phylum (Chytridiomycota) was detected (Song et al. 2018). The ITS2 region of chytrids is too variable for alignment by phylogenetic analysis, and not many chytrid ITS2 sequences have been deposited in databases yet (Frenken et al. 2017, Heeger et al. 2019). The large subunit (LSU) region of rDNA has less genetic variation than the ITS2 region, but has adequate variation for chytrids, and many sequences of known chytrid species have already been deposited in databases and have been applied to analyze chytrid communities in several lakes (Lefèvre et al. 2012, Wurzbacher et al. 2016). In the present study, we determined the community structure of zoosporic fungi using high-throughput sequencing and phylogenetic analysis of the LSU region of rDNA extracted from nano-sized particles (2–20 µm), to clarify the diversity and tem-

poral variation in the zoosporic fungal community in Lake Biwa. Chytrid sporangia attached to phytoplankton were also counted by staining, using epi-fluorescence microscopy to identify the parasitic chytrids infecting host phytoplankton.

2. MATERIALS AND METHODS

2.1. Sample collection

Water samples were collected monthly from 6 May to 12 December 2016, when phytoplankton were abundant, with a Van Dorn sampler (volume: 10 l) from each depth at 1, 3, 5, 10 and 15 m at Stn 3 (70 m deep, 35° 19.8' N, 136° 08.9' E) in the north basin of Lake Biwa (mean depth, 41.2 m), Japan. For molecular analyses, equal volumes (500 ml) of water collected from each depth were mixed and combined into 1 sample; this was because chytrid distribution and any environmental parameters were vertically homogeneous above 15 m during the study period (Figs. S1 & S2 in the Supplement at www.int-res.com/articles/suppl/a087p017_supp.pdf). All biotic and abiotic parameters were therefore calculated as averages throughout the 0–15 m water column (Table S1). The samples for molecular analysis were transferred to the laboratory within 2 h in an insulated container to keep the samples cold. Aliquots of the samples from each depth for counting phytoplankton and chytrid sporangia cells attached to the host phytoplankton were preserved immediately after collection with buffered formalin at a final concentration of 1%. Vertical profiles of water temperature were determined with a CTD profiler (JFE Advantech, ACL1183-PDK) on each sampling occasion.

2.2. Phytoplankton and sporangia counts

Large phytoplankton (>20 µm) species were identified and counted under a light microscope (Olympus, BX-50) at a magnification of 400×. Chytrid sporangia attached to the host cells were stained with calcofluor white and counted with blue fluorescence under excitation and emission wavelengths of 385 and 440 nm, respectively (Kagami et al. 2007a), using an epi-fluorescence microscope (Olympus, BX-51) at a magnification of 100–1000×. The prevalence of infection for each phytoplankton species was calculated as the percentage of phytoplankton cells with chytrid sporangia out of the total number of phytoplankton cells.

2.3. DNA extraction

The combined water sample from each depth was pre-filtered through a 20 µm mesh net for separating zoospore fungi (2–5 µm in diameter) from other, larger fungi and those attached to the host phytoplankton and substrates. Next, 500 ml of the filtrate were filtered again through a 2.0 µm pore size membrane filter (Millipore) to collect the zoospores. DNA was extracted directly from the membrane filter with the DNeasy® Plant Mini Kit (Qiagen®). DNA concentration was measured with a Qubit fluorometer (Invitrogen). The extracted DNA was frozen at –20°C until analysis.

2.4. Metabarcoding analysis using Illumina MiSeq

Metabarcoding analysis targeted the fungal-specific LSU regions, which were amplified using the primers LR3R (5'-GTC TTG AAA CAC GGA CC-3') (Rehner & Samuels 1995) and LR5 (5'-TCC TGA GGG AAA CTT CG-3') (Hopple & Vilgalys 1994). All PCR reactions were conducted in 20 µl mixes with Takara Ex TaqHS mix, 0.2 µM of both forward and reverse primers and ~1 ng of extracted DNA. The first PCR cycling consisted of initial denaturation at 94°C for 2 min; followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and elongation at 72°C for 30 s; and then a final extension at 72°C for 5 min. To attach the index sequence to the first PCR products, the second PCR products were processed with the primer set 2nd F: AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-Index2 (8 bp)-ACA CTC TTT CCC TAC ACG ACG C and 2nd R: CAA GCA GAA GAC GGC ATA CGA GAT-Index1 (8 bp)-GTG ACT GGA GTT CAG ACG TGT G (Table S2). The second PCR cycling consisted of initial denaturation at 94°C for 2 min; followed by 8 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s; and then a final extension at 72°C for 5 min. The library quality was assessed using a Fragment Analyzer system. The libraries were sequenced on an Illumina MiSeq platform and 250 bp paired-end reads were generated. We obtained 1 112 173 raw reads from all samples collected, and all raw reads were deposited in the NCBI Sequence Read Archive (SRA) database under accession number SRP119396.

2.5. DNA amplification and sequencing

Barcodes and PCR primers were removed from the raw reads by the `fastq_barcode_splitter` from the

Fastx toolkit software (ver. 0.0.13.2). Low-quality reads (values <20) were trimmed using Sickle software (ver. 1.33). High-quality reads were processed to merge paired-end reads of each sample through FLASH software (ver. 1.2.10) (Magoč & Salzberg 2011). After these processes, the reads obtained were screened by USEARCH software (ver. 8.0.1623-i86linux64) to find chimera sequences (Edgar 2010). Finally, effective reads were generated by removing these chimera sequences, and singletons were removed. UPARSE software (Edgar 2013) was used to cluster effective reads of all samples. Effective reads with 97% similarity were classified into 1 operational taxonomic unit (OTU). Taxonomic assignments were based on `assign_taxonomy.py` scripts of Quantitative Insights Into Microbial Ecology software (QIIME, ver. 1.9.0) (Caporaso et al. 2010). Data that contained the term 'no BLAST hit' in the taxonomic annotation were removed. All OTUs that were not assigned to fungi were discarded from the analysis. OTU sequences belonging to fungi were submitted to the DNA data bank of Japan (DDBJ) Mass Submission System (MSS) under accession numbers LC323648–LC324677. Zoospore fungal sequences (i.e. phyla Blastocladiomycota, Chytridiomycota, Cryptomycota and Neocallimastigomycota) were selected for the downstream analysis.

2.6. Statistical analysis

OTU composition and relative abundances of zoospore fungal assemblages derived from the DNA amplification and sequencing results in each month were calculated to clarify taxonomic diversity in the zoospore fungal community in the lake. In order to visualize the number of unique OTUs detected in each month, we used Gephi software (ver. 0.9.1), which is a tool for discovering the connectivity among the OTUs tested. Prior to the statistical analyses, a rarefied OTU table was generated by QIIME rarefaction analysis script with the rarefied depth in the minimum sequence number. Chao-1 (Chao 1984) and Shannon's *H* (Shannon 2001) indices were calculated as representatives of alpha diversity in fungal communities.

2.7. Phylogenetic analysis

In order to determine which OTUs were closely related to those of cultured chytrids by molecular phylogenetic analysis, we created a data set of LSU

rDNA sequences of the class Chytridiomycetes. Abundant and/or unique Chytridiomycetes OTUs were selected and added into the data set. Three species of the class Monoblepharidomycetes were selected as outgroup taxa. Sequences were automatically aligned with MAFFT (Katoh & Standley 2013). Ambiguously aligned regions were excluded using 'trimAl' (Capella-Gutiérrez et al. 2009) with the 'gappout' option. The maximum likelihood tree was inferred using RAxML 8.2.7 (Stamatakis 2014). We 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.

3. RESULTS

3.1. Physicochemical environment and host phytoplankton

According to vertical profiles of water temperature, lake water was vertically stratified from June to September, when the temperature gradually decreased below 5 m (Fig. S1). However, most of the physicochemical parameters other than temperature were vertically homogeneous above 15 m even in the stagnation period (Fig. S1).

We found a total of 12 large phytoplankton species comprising 1 dinoflagellate (*Ceratium hirundinella*), 6 green algae (*Closterium aciculare*, *Micrasterias hardyi*, *Staurastrum arctiscon*, *S. dorsidentiferum*, *S. rotula*, *Xanthidium hastiferum*) and 5 diatom species (*Aulacoseira granulata*, *Asterionella formosa*, *Aulacoseira nipponica*, *Fragilaria crotonensis*, *Synedra* sp.) (Fig. 1). According to the vertical profiles of the dominant phytoplankton species and chytrid sporangia that attached to their hosts, all were almost uniformly distributed throughout the 0–15 m water column (Fig. S2). Therefore, average abundances throughout the 0–15 m water column are shown in the results. Green algae were always the dominant group at >50% of the total counted phytoplankton abundance, while diatoms comprised <38 and <12%, respectively, throughout the study period. *S. dorsidentiferum* was the most dominant species among the phytoplankton (>30% of total abundance) from May until September and had its highest density of 18.0×10^3 cells l^{-1} in August, while *M. hardyi* increased abruptly from October and had its highest density of 78.0×10^3 cells l^{-1} in December.

Chytrid sporangia were found on 5 out of 12 large phytoplankton species (Figs. 2 & 3). The prevalence of infection differed among phytoplankton species: the percentages of infected *S. dorsidentiferum*

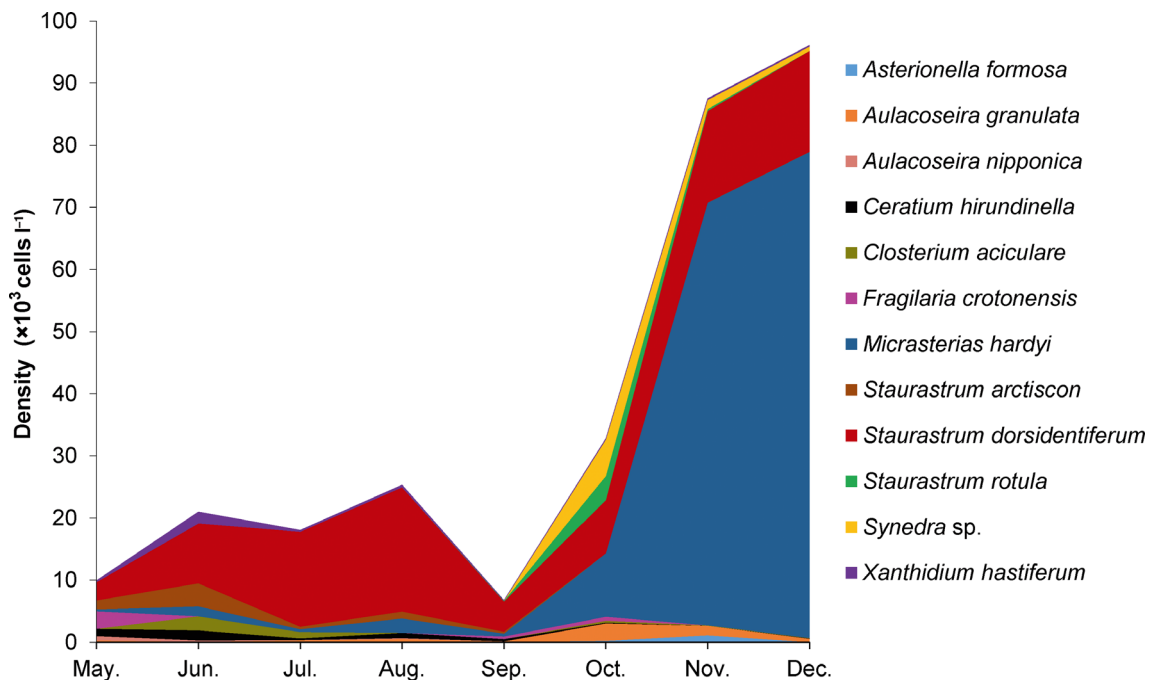
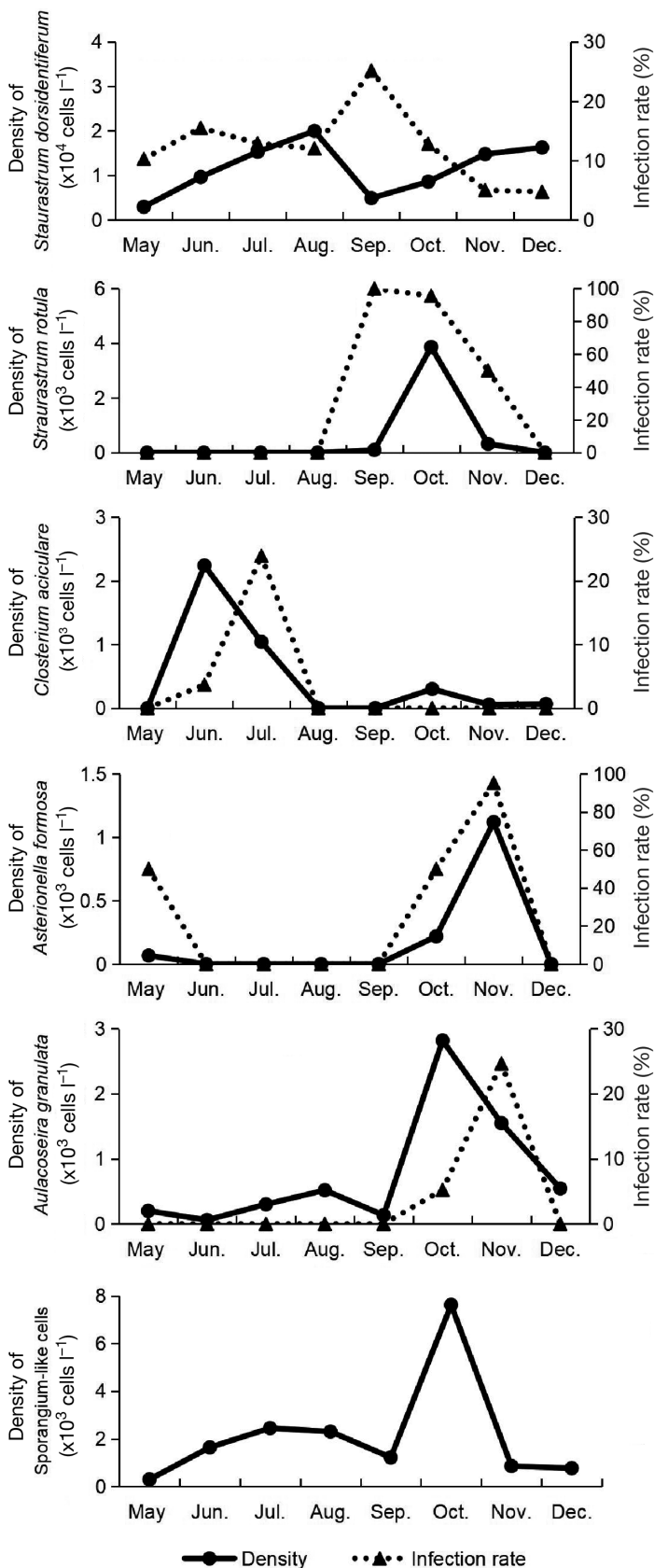


Fig. 1. Species composition and cell densities of phytoplankton from 6 May to 12 December 2016 at Stn 3 in the north basin of Lake Biwa, Japan



ranged from 4.7 to 25.2%, while chytrid sporangia appeared throughout the sampling periods (Fig. 2). The prevalence of infection in *S. rotula* ranged from 50 to 100% from September to November. Chytrids on *C. aciculare* appeared only in June and July, when 3.7 and 23.9% of the total abundance, respectively, were infected. Chytrids were found on *A. formosa* in May, October and November, and the prevalence of infection was relatively high, between 50 and 95.2%. *A. granulata* was mainly infected in October and November, at 5.2–24.6%. The total density of chytrid sporangia was highest in October with 7.6×10^3 cells ml^{-1} , and ranged between 0.3×10^3 and 2.4×10^3 cells ml^{-1} in other months (Fig. 2). No infected cells of *M. hardyi* were found, even when it dominated in November and December.

3.2. Sequence data

After filtering, 956 761 reads included a total of 13 757 OTUs in the final matrix. A total of 22 264 reads and 311 OTUs were assigned as zoosporic fungal sequences. An additional 204 OTUs were removed from the 311 OTUs as singletons, leaving 107 zoosporic fungal OTUs as the final data set, comprising 22 060 reads. OTUs assigned to the phylum Neocallimastigomycota were excluded because phylogenetic analysis indicated that these sequences were not related to Neocallimastigomycota (see Section 4); as a result, 101 OTUs and 15 516 reads were used for the analyses (Table 1).

3.3. Zoosporic fungal diversity and taxonomic characteristics in Lake Biwa

Three zoosporic fungal phyla, namely Blastocladiomycota (1.5% of reads and 2.0% of OTUs), Chytridiomycota (97.4 and 93.0%) and Cryptomycota (1.1 and 5.0%), were detected. Chytridiomycota was the most diverse and abundant phylum among them. The dominant orders were Chytridiales (38.6% of reads and 27.7% of OTUs), Rhizophydiales (32.5 and 28.7%) and Spizellomycetales (10.4 and 14.9%). The

Fig. 2. Temporal changes in cell density of selected phytoplankton species and percentages of the cells with attached chytrid cells (shown as infection rates) from 6 May to 12 December 2016 at Stn 3 in the north basin of Lake Biwa, Japan. Bottom panel shows the total density of chytrid cells attached to host cells during the same time

Table 1. Sequencing information and 2 indices of alpha diversity (Chao-1 and Shannon's H) of the zoosporic fungal community (phyla Blastocladiomycota, Chytridiomycota and Cryptomycota) sampled from 6 May to 12 December 2016 at Stn 3 in the north basin of Lake Biwa, Japan, based on BLAST results. OTU: operational taxonomic unit

Date	Reads	OTUs	Chao-1	Shannon's H
6 May	772	25	25	1.960
22 Jun	3888	23	18	2.062
5 Jul	4010	16	13	1.904
4 Aug	1412	18	18	2.494
12 Sep	1978	28	25	2.152
18 Oct	1841	28	28	2.598
22 Nov	342	18	18	2.296
12 Dec	1273	21	22	2.378
Final matrix	15516	101		

dominant genera were *Chytriomycetes* (23.8 and 10.9%), *Rhizophyidium* (13.0 and 14.9%) and *Phlyctochytrium* (10.6 and 4.0%).

A rarefied OTU table was generated at 342 sequences per sample. Chao-1 and Shannon's H indices in fungal communities varied from 13 to 28 and from 1.904 to 2.598, respectively (Table 1). Both Chao-1 and Shannon's H showed the highest diversity in October, when chytrid cells showed the largest density (Fig. 2). Gephi network analysis is shown in Fig. 3. Results showed that the number of OTUs was very different in all months, and unique OTUs appeared in each month. The highest value of 11 unique OTUs was found in October, while the lowest value of 3 unique OTUs was found in November.

3.4. Temporal variations in zoosporic fungal communities

Temporal variations in zoosporic fungal communities were found during the study period. At the level of order, although the composition of the OTUs did not differ significantly throughout the study period, their sequence compositions showed temporal changes (Fig. 4). The sequence composition of Chytridiales, Rhizophydiales, Spizellomycetales and Monoblepharidales varied from 7.3 to 72.8%, from 13.0 to 56.2%, from 3.3 to 26.7% and from 0 to 19.8%, respectively, during the study period. Chytridiales dominated in May, June and September (>65%) but not in other months (<35%). Spizellomycetales were high contributors (>21%) from October to December but not in other months (<9%). Cladochytriales accounted for >27% in November but

were not detected from June to September. Only 3 known genera (*Chytriomycetes*, *Boothiomycetes* and *Rhizophyidium*) were detected throughout the study period. Six genera (*Allomyces*, *Dendrochytridium*, *Rhizoclostratium*, *Gromochytrium*, *Gonapodya* and *Monoblepharis*) were detected on only 1 occasion.

Phylogenetic analysis revealed that most of the OTUs did not have any close affinity with cultured chytrids, but that some were assigned to known species: *Staurastromyces oculus*, *Pateramyces corrientinensis*, *Globomyces pollinis-pini*, *Urceomyces sphaerocarpus*, *Pendulichytrium sphaericum*, *Rhizoclostratium* sp. and *Pseudorhizidium endosporangium* (Fig. 5). Two OTUs were clustered into Zygochlyctidales (Seto et al. 2020).

Three OTUs were matched with 3 chytrid species parasitic on phytoplankton: *S. oculus* as an obligate parasite of the green alga *Staurastrum* sp. (Van den Wyngaert et al. 2017), *P. sphaericum* as an obligate parasite of the diatom *A. granulata* (Seto & Degawa 2018) and *Zygochlyctis asterionellae* as an obligate parasite of the diatom *A. formosa* (Seto et al. 2020). The sequences of these 3 chytrids were always detected when sporangia of chytrids on each host phytoplankton species were found during microscopic examination (Fig. 6). However, their sequences were sometimes not detected even when chytrid sporangia

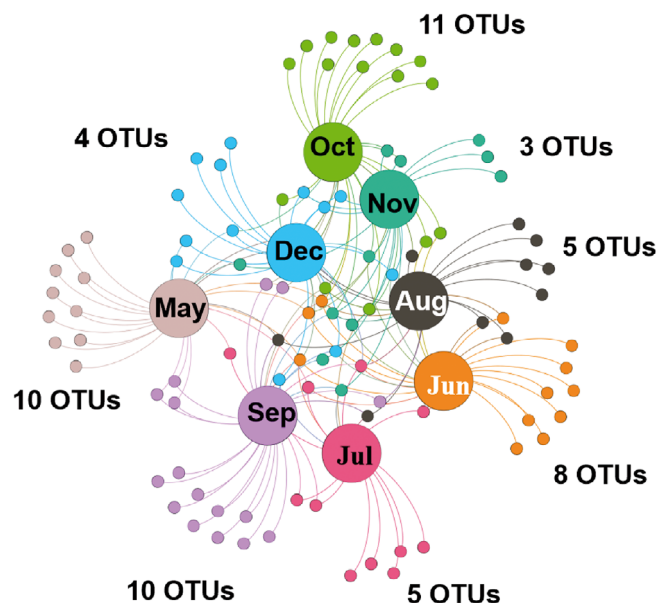


Fig. 3. Gephi network diagram illustrating Blastocladiomycota, Chytridiomycota and Cryptomycota from 6 May to 12 December 2016 at Stn 3 in the north basin of Lake Biwa, Japan, highlighting the number of unique operational taxonomic units (OTUs) based on BLAST results. A small circle (representative OTU) that is only connected to 1 large circle (representative sample) indicates a unique OTU. The number of unique OTUs is shown for each month

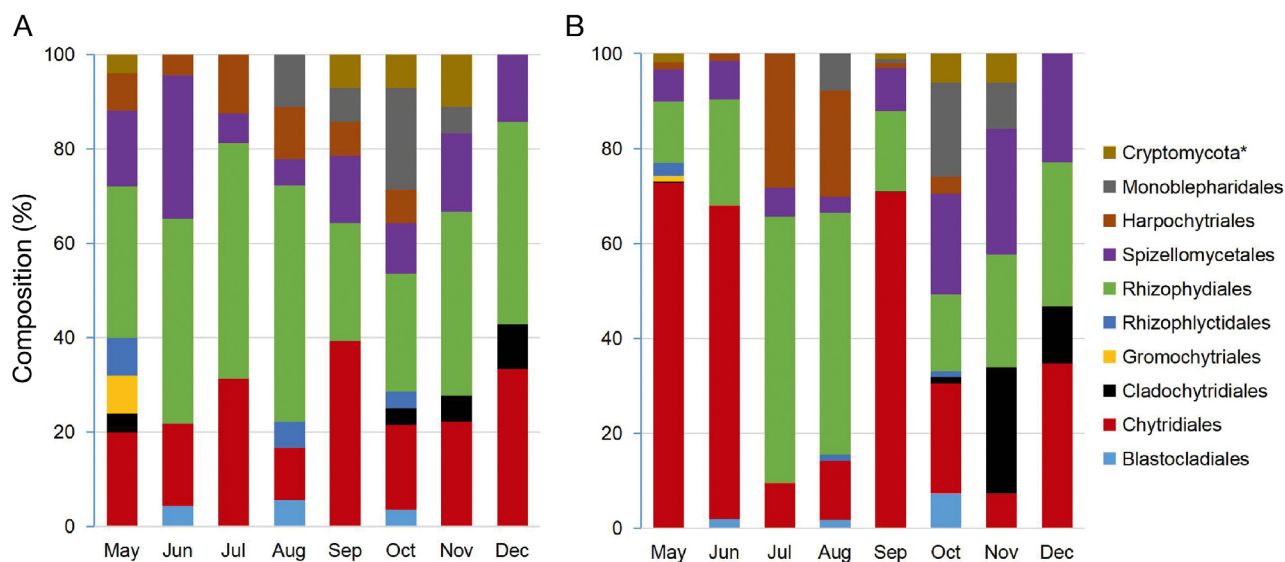


Fig. 4. Composition of zoosporic fungal orders (Blastocladiomycota, Chytridiomycota and Cryptomycota) in (A) operational taxonomic units (OTUs) and (B) number of sequences. Samples were collected from 6 May to 12 December 2016 at Stn 3 in the north basin of Lake Biwa, Japan; fungal composition is based on BLAST results. Asterisk indicates phylum level

were found; thus, dynamics based on sequences and dynamics based on sporangia counts differed from each other.

4. DISCUSSION

We detected diverse zoosporic fungi with the LSU region as a marker gene: a total of 101 OTUs in 3 zoosporic fungal phyla (Blastocladiomycota, Chytridiomycota and Cryptomycota) and 8 known species (Table 2). In our previous study based on the ITS region, many of the sequences were not assigned or were identified as ‘unclassified fungi’ (Song et al. 2018). Only 1 zoosporic fungal phylum (Chytridiomycota) with 35 OTUs was detected, and 1 parasitic chytrid (*Staurastromyces oculus*) was identified. Compared with the LSU region, there is less information on Chytridiomycota in the reference database of the ITS region for aquatic fungi (Wurzbacher et al. 2016).

Due to lack of chytrid data in public sequence databases, few OTUs were identified as known chytrids in the present study. The remaining OTUs were mostly assigned as unknown taxa, and therefore it is difficult to determine their ecology (Frenken et al. 2017). Eight OTUs, however, were identified as previously known species, and we were able to determine their ecological characteristics (i.e. their nutritional modes, whether they were parasites or saprotrophs, their hosts/substrates and habitats) (Table 3).

Three OTUs were matched with 3 parasitic chytrid species of phytoplankton: *S. oculus* (Rhizophydiales, Staurastromycetaceae) infecting *Staurastrum* sp. (Van den Wyngaert et al. 2017), *Pendulichytrium sphaericum* (Chytridiales, Chytriomycetaceae) infecting *Aulacoseira granulata* (Seto & Degawa 2018) and *Zygothlyctis asterionellae* infecting *Asterionella formosa* (Seto et al. 2020). The morphology of sporangia attached to *Staurastrum dorsidentiferum* was consistent with that of *Staurastromyces oculus*: it had an encysted zoospore in the isthmus region of the host, while sporangia attached on *Staurastrum rotula* were different from any stages of *S. oculus* (Van den Wyngaert et al. 2017). Different patterns in the abundance of sporangia on *S. rotula* and the relative abundance of the sequence reads of *S. oculus* (Fig. 6) also indicated that the chytrids infecting *S. rotula* must have been different species. The chytrid infecting *S. dorsidentiferum* may have been *S. oculus*, although there may have been other species infecting *S. dorsidentiferum* when sequence reads of *S. oculus* were not detected.

According to microscopic analysis, the morphology of sporangia attached to *A. granulata* was consistent with the morphology of *P. sphaericum*: the mature zoosporangium was spherical and stalked (Seto & Degawa 2018) (Fig. S3), and the patterns of read percentage and abundance of sporangia also matched (Fig. 6); thus, it is likely that the chytrid infecting *A. granulata* was *P. sphaericum*. The morphology of sporangia attached to *A. formosa* was consistent with

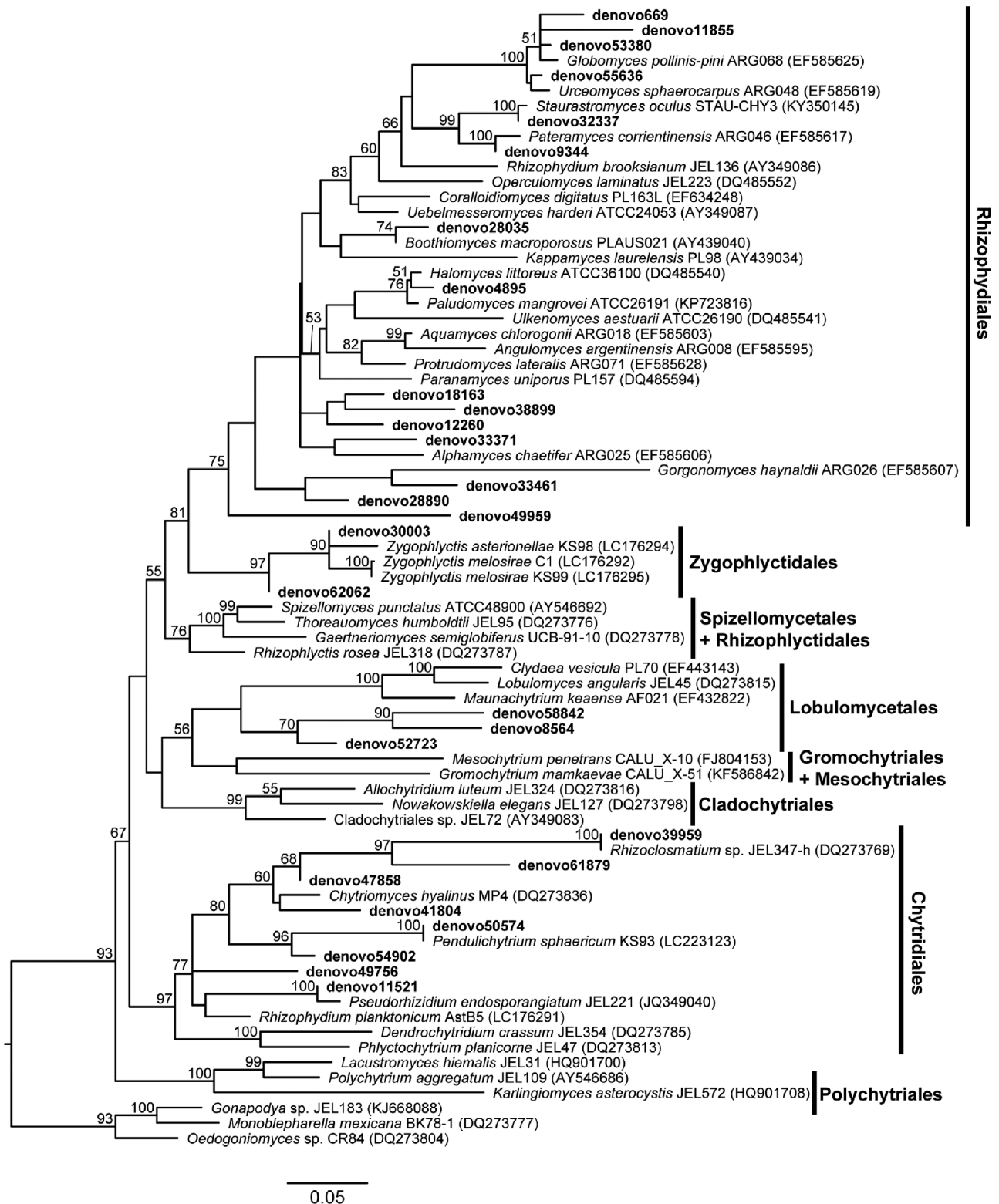


Fig. 5. Phylogenetic tree of the large number of operational taxonomic units (OTUs, designated as 'denovo' in the tree) in chytrids, with large subunit (LSU) sequences of known fungi, based on the maximum likelihood analysis. Samples were collected from 6 May to 12 December 2016 at Stn 3 in the north basin of Lake Biwa, Japan

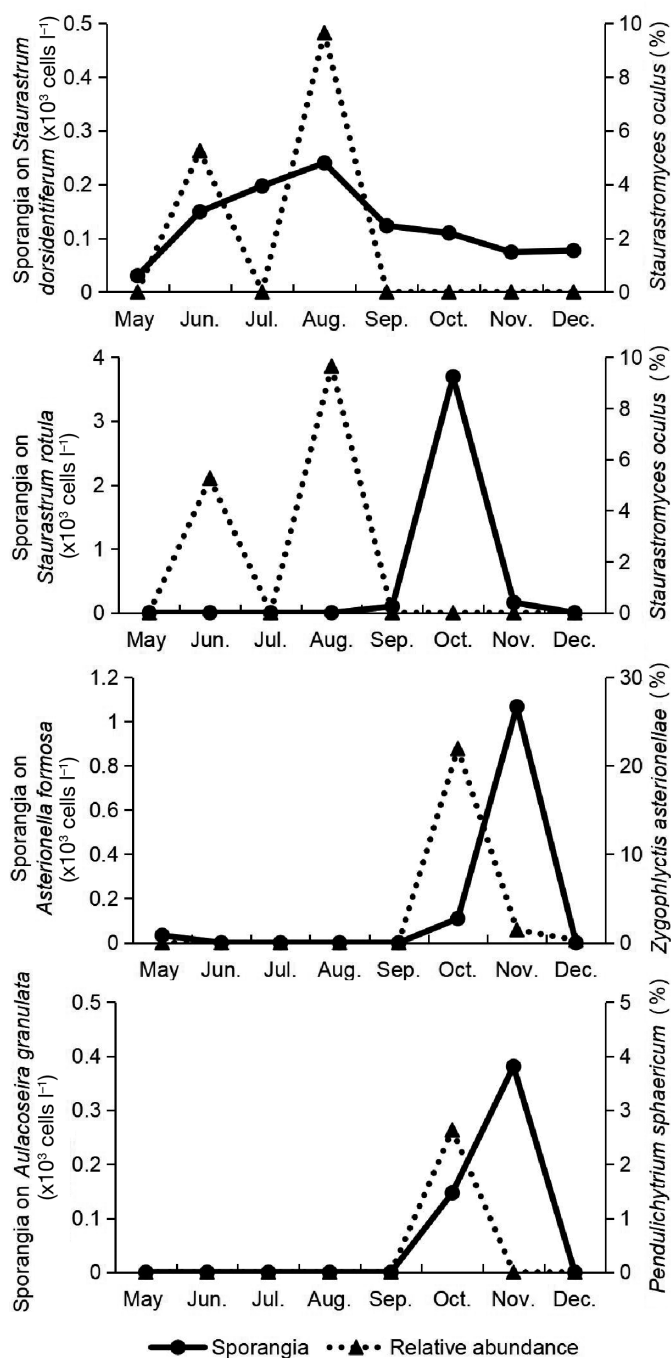


Fig. 6. Comparison of known chytrids between metabarcoding analysis and microscopic counting results from 6 May to 12 December 2016 at Stn 3 in the north basin of Lake Biwa, Japan

that of *Z. asterionellae*, as the encysted zoospore was spherical and the developing thallus was obpyriform (Seto et al. 2017) (Fig. S3). Sequences of *Z. asterionellae* were always detected when chytrid sporangia on *A. formosa* were observed. The peak of *Z.*

asterionellae read percentage was a month earlier than the abundance of sporangia (Fig. 6). Since our DNA analysis targeted zoospores (2–20 μm), instead of sporangia, it is likely that the detected timing recorded a slight gap between zoospores and sporangia. *Rhizophyidium planktonicum* is also a parasite of *A. formosa* and possesses similar morphology to *Z. asterionellae* (Seto et al. 2017), but it was not detected with metabarcoding analysis in this study.

Some other parasitic chytrids might be included in the unknown OTUs. We found high infection rates in *Closterium aciculare* in July (Fig. 2) but could not identify any OTUs corresponding to parasitic chytrids on *C. aciculare*. Although chytrids on *C. aciculare* have been reported previously (Holfeld 1998, Fernández et al. 2011), no DNA sequence data are available for these chytrids. Among all the detected Chytridiomycota sequences in our metabarcoding analysis, 1 unknown chytrid OTU ('denovo 49959') might have been a parasite of *C. aciculare*, which showed relatively high abundance in July when the number of chytrid sporangia on *C. aciculare* was at its highest (Fig. 4). Phylogenetic analysis indicated that denovo 49959 may be a novel clade in the Rhizophydiales (Fig. 5). Further studies are necessary to clarify this, and to identify the parasitic chytrid of *C. aciculare* in Lake Biwa.

In the present study, the chytrid infection rates on 5 large dominant phytoplankton varied along with the abundances of their hosts. The temporal variation in hosts can be strongly influenced by the parasitic fungi, and especially by that of host-specific fungi (Holfeld 2000, Ibelings et al. 2004). In fact, chytrid infection has been shown to affect phytoplankton dynamics on *S. dorsidentiferum* in Lake Biwa (Kagami et al. 2004). Chytrid infection-mediated effects on host phytoplankton dynamics may be plausible in this lake. Compared with the previous study (Kagami et al. 2004), however, the prevalence of infection was low in our study. Since we only took monthly samples, we need more frequent sampling to identify the host-parasite relationships in the lake.

The OTUs identified as *Pateramyces corrientinensis*, *Globomyces pollinis-pini*, *Urceomyces sphaerocarpus*, *Rhizoclostratium* sp. and *Pseudorhizidium endosporangiatum* in the present study may be saprotrophic species, according to the literature (Table 3). They contributed a very small proportion of total chytrid reads (<10%), except in December (21%). *P. corrientinensis*, *G. pollinis-pini* and *U. sphaerocarpus* have been detected from pollen as substrates in aquatic habitats (Letcher et al. 2008). *P. endosporangiatum* has been isolated from pollen in

Table 2. Comparison of the detected zoosporic fungal phyla and orders using internal transcribed spacer (ITS) from our previous study (Song et al. 2018) and large subunit (LSU) regions by metabarcoding analysis. Samples were collected at Stn 3 in Lake Biwa, Japan. Plus (+) represents occurrence. Blank cells therefore indicate that these orders were not found. OTU: operational taxonomic unit

Phylum	Order	ITS (sampling year: 2015)				LSU (sampling year: 2016)							
		Apr	May	Jul	Sep	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Blastocladiomycota	Blastocladales						+		+			+	
Chytridiomycota	Chytridiales		+	+	+	+	+	+	+	+	+	+	+
	Cladochytridiales					+					+	+	+
	Gromochytriales					+							
	Rhizophlyctidiales				+	+			+		+		
	Rhizophydiales	+	+	+	+	+	+	+	+	+	+	+	+
	Spizellomycetales				+	+	+	+	+	+	+	+	+
	Monoblepharidales								+	+	+	+	
	Harpochytriales					+	+	+	+	+	+		
Cryptomycota										+	+	+	
	Number of OTUs	7	15	18	5	25	23	16	18	28	28	18	21

Table 3. List of identified chytrid species detected from 6 May to 12 December 2016 at Stn 3 in the north basin of Lake Biwa, Japan, including their ecology and hosts. –/–: not reported

Species	Ecology	Habitat/host	Reference
<i>Zygothlyctis asterionellae</i>	Obligate parasite	Aquatic/Asterionella formosa	Seto et al. (2020)
<i>Staurastrum oculus</i>	Obligate parasite	Aquatic/ <i>Staurastrum</i> sp.	Van den Wyngaert et al. (2017)
<i>Pendulichytrium sphaericum</i>	Obligate parasite	Aquatic/Aulacoseira granulata	Seto & Degawa (2018)
<i>Urceomyces sphaerocarpus</i>	Saprotroph	Aquatic/pollen	Letcher et al. (2008)
<i>Globomyces pollinis-pini</i>	Saprotroph	Aquatic/pollen	Letcher et al. (2008)
<i>Pateromyces corrientinensis</i>	Saprotroph	Aquatic/pollen	Letcher et al. (2008)
<i>Pseudorhizidium endosporangiatum</i>	Saprotroph	Soil/pollen	Powell et al. (2013)
<i>Rhizoclostridium</i> sp. JEL347-h	Saprotroph	–/–	James et al. (2006)

garden soil (Powell et al. 2013), and therefore in this study, it may have been transferred from terrestrial to aquatic habitats by wind action (Wurzbacher et al. 2010). Saprotrophic zoosporic fungi may depend strongly on substrate quality in the pelagic zone (i.e. carbon sources, sestonic C:N and N:P supply ratios from the watersheds) (Findlay et al. 2002, Güsewell & Gessner 2009), although we were unable to identify the factors influencing the dynamics of saprotrophic fungi in this study.

Some OTUs showed the closest match with Neocallimastigomycota in this study (Table S3). This phylum is regarded as consisting of obligately anaerobic rumen fungi, which are found widely in the guts of ruminants (Gruninger et al. 2014). Both of our sequences matched 2 environmental sequences in GenBank, i.e. LLMB10_1 and LLME10_2 (accession numbers JN049554 and JN049555, respectively), from Lakes Tuscaloosa and Lurleen (Alabama, USA), respectively (Lefèvre et al. 2012), which were affiliated to Neocallimastigomycota. However, a BLAST search and preliminary phylogenetic analysis indicated that these

sequences are not related to Neocallimastigomycota. LLMB10_1 was ca. 96% identical to the green algae *Chlamydomonas* spp. in the BLAST search. In our phylogenetic analysis (Fig. S4), LLME10_2 was not related to Neocallimastigomycota and showed a long branch in Chytridiomycota, indicating that the phylogenetic position of this sequence is uncertain. Wurzbacher et al. (2016) also found an OTU assigned to the Neocallimastigomycota sequence in Lake Stechlin (Germany), but concluded through phylogenetic analysis that the OTU was not related to Neocallimastigomycota but was distributed among other fungal lineages.

In our study, the sequences of the known chytrids were always detected by meta-barcoding analysis when they were observed by microscopic analysis. However, sometimes no sequences were detected even if the chytrid sporangia on phytoplankton were observed. One reason for this may be the nano-sized particle fraction (2–20 µm) that we targeted in metabarcoding analysis, but not in sporangia attached to large phytoplankton species (>20 µm). Metabarcod-

ing analysis was therefore unable to detect chytrid sequences when they mainly presented as sporangia on large phytoplankton. Although calcofluor white stains not only chytrids but also other fungi and protists with cellulose, we observed the morphology of sporangia carefully, and concluded that the detected sporangia were chytrids. The size threshold for chytrid infection has been uncertain, although chytrids tend to infect large hosts (Kagami et al. 2007a). In this study, we never found any chytrids that infected small phytoplankton, although it could be possible that many unexplained OTUs could imply miscounting those attached to small phytoplankton ($\leq 20 \mu\text{m}$). Another reason for this may be the primer bias, since we detected only a few fungi, and most of the sequences belonged to other eukaryotes. Further studies combining microscopic observations and metabarcoding analysis may reveal the existence of more diverse fungi in the water column of Lake Biwa.

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