A freshwater radiation of diplonemids

Indranil Mukherjee^{1#}, Michaela M Salcher¹, Adrian-Ştefan Andrei^{1*}, Vinicius Silva Kavagutti^{1,2}, Tanja Shabarova¹, Vesna Grujčić³, Markus Haber¹, Paul Layoun^{1,2}, Yoshikuni Hodoki⁴, Shin-ichi Nakano⁴, Karel Šimek^{1,2}, Rohit Ghai^{1#}

¹*Department of Aquatic Microbial Ecology, Institute of Hydrobiology, Biology Centre of the Czech Academy of Sciences, , Na Sadkach 7, 37005, České Budějovice, Czech Republic*

²*Department of Ecosystem Biology, Faculty of Science, University of South Bohemia, Branišovská 1760, 37005, České Budějovice, Czech Republic*

³*KTH Royal Institute of Technology, Science for Life Laboratory, Department of Gene Technology, School of Engineering Sciences in Chemistry, Biotechnology and Health, Stockholm, Sweden*

⁴*Center for Ecological Research, Kyoto University, Otsu, Shiga, Japan*

^{*}*Current address: Limnological Station, Institute of Plant and Microbial Biology, University of Zurich, Seestrasse 187, 8802, Kilchberg, Switzerland*

Keywords: 18S amplicon sequencing/ CARD-FISH/ diplonemids/ freshwater lakes/ protists/ metagenomic 18S rRNA assembly

Running Title: Diplonemids in freshwater lakes

[#]Authors to whom correspondence should be addressed

Indranil Mukherjee (indranilmukherjee04@yahoo.com)

Rohit Ghai (ghai.rohit@gmail.com)

Abstract

Diplonemids are considered marine protists and have been reported among the most abundant and diverse eukaryotes in the world oceans. Recently we detected the presence of freshwater diplonemids in Lake Biwa, Japan. However, their distribution and abundances in freshwater ecosystems remain unknown. We assessed abundance and diversity of diplonemids from several geographically distant deep freshwater lakes of the world by amplicon-sequencing, shotgun metagenomics and CARD-FISH. We found diplonemids in all the studied lakes, albeit with low abundances and diversity. We assembled long 18S rRNA sequences from freshwater diplonemids and showed that they form a new lineage distinct from the diverse marine clades. Freshwater diplonemids are a sister-group to marine isolates from coastal and bay areas, suggesting a recent habitat transition from marine to freshwater habitats. Images of CARD-FISH targeted freshwater diplonemids suggest they feed on bacteria. Our analyses of 18S rRNA sequences retrieved from single cell genomes of marine diplonemids shows they encode multiple rRNA copies that may be very divergent from each other, suggesting that marine diplonemid abundance and diversity both have been overestimated. These results have wider implications on assessing eukaryotic abundances in natural habitats by using amplicon-sequencing alone.

Introduction

Diplonemids appear as one of the more intriguing findings in amplicon-sequencing surveys of the global oceans microbiota [1]. While the abundance and distribution of these protists were nearly unknown till the last decade, they are now considered (based mainly on metabarcoding approaches) as one of the most abundant and diverse microbial eukaryotic group in the world oceans [2,3]. Marine diplonemid 18S rRNA sequences exhibit high diversity and are the sixth most frequently recovered eukaryotic signatures in

the world's oceans in amplicon-based surveys [3]. They appear to be more abundant in the mesopelagic zone of the oceans [1,4]. These discoveries have ignited interest in understanding the importance and distribution of these protists in other aquatic non-marine ecosystems. Diplonemids are considered marine protists [5] and were till recently never reported from freshwater ecosystems. Our interest in studying the distribution of free-living kinetoplastids (a sister group of diplomonads) from freshwater lakes using kinetoplastid-specific primers accidentally led us to the discovery of freshwater diplomonads in deep freshwater lakes of Japan [6]. Similar to kinetoplastids, diplomonads are generally not targeted by commonly used eukaryotic V4 primers due to their divergent 18S rRNA genes [7,8] and this explains their exclusion from most diversity studies in freshwater environments. This finding suggested that diplomonads are not limited to marine ecosystems and are likely present in other freshwater lakes of the world. To examine the distribution of diplomonads in freshwater habitats, we sampled seven deep freshwater lakes (different depths, both monthly time series and isolated samples) of various trophic statuses from Japan, Czech Republic, and Switzerland. The diversity and abundance of diplomonads were analyzed using PCR based amplicon sequencing, shotgun metagenomic sequencing and CARD-FISH analyses. Our results suggest a broad distribution (albeit with low abundance) of diplomonads in freshwater ecosystems, and the presence of one widespread freshwater clade that appears to be a radiation of known marine isolates.

Results and Discussions

Freshwater diplomonad 18S rRNA gene sequences

A single diplomonad sequence (OTU_4) was described recently from the lakes Biwa and Ikeda using 18S rRNA amplicon sequencing [6]. This sequence provided the first

intriguing evidence for the existence of freshwater diplomonads, which have been until now considered as marine eukaryotes [5]. However, more conclusive evidence for the presence of these eukaryotes and their distribution in other freshwater habitats is lacking. As the first diplomonad sequence was described from Lake Biwa, in an effort to more clearly examine the depth- or seasonal-related abundance of freshwater diplomonads, we carried out multiple sampling campaigns with different sampling frequencies at this site. The first campaign in Lake Biwa lasted for three months in spring/summer 2016 and samples were collected from three different depths (Table 1, Supplementary Figure S3). The second campaign lasted throughout the year 2017 with a monthly sampling frequency (Table 1, Supplementary Figure S4). A single depth profile was also collected from Lake Ikeda to examine the vertical distribution of diplomonads (Table 1, Supplementary Figure S5). To expand the known distribution of diplomonads outside Japan, we also collected samples from five European lakes (Řimov, Medard, Zurich, Constance and Lugano). A complete list of sampling sites and their locations, depths and analyses performed with the samples from each site is given in Table 1.

Amplikon-sequencing was performed for the sampling campaigns from Lake Biwa and Lake Ikeda (Figure 1A, B, C and Supplementary Figure S3, S4, S5). Relative abundance of diplomonad sequences in these samples was always below 1%. A total of 18 sequences were recovered, somewhat more frequently from the hypolimnion. The highest relative abundance was observed in the hypolimnion of Lake Ikeda (100 m), contributing up to 0.96 % of total sequence reads (Figure 1C). Although rare, they were present in nearly all the seasons in Lake Biwa (Figure 1) in both epilimnion (5 m) and metalimnion (10-20 m) but only occasionally in the hypolimnion (50-60 m). The highest amplikon read abundance in Lake Biwa was observed in the hypolimnion of May (0.5% of total sequences, Figure 1A). Diplomonad sequences were more abundant in the epilimnion and

114 metalimnion during the early part of the spring and as the stratification developed their
 115 numbers increased in the hypolimnion (Figure 1A). However, data from the entire year
 116 showed the absence of diplonemid sequences in the hypolimnion during summer and
 117 autumn (Figure 1B). This discrepancy could be due to the sample collection in different
 118 years (spring sampling in 2016 and monthly sampling in 2017) and depths (50 vs. 60 m).
 119 However, the low numbers of recovered sequences preclude definitive conclusions
 120 regarding seasonal or depth related preferences for diplonemids. Similar results were
 121 obtained upon examining the abundance of freshwater diplonemids using an existing
 122 shotgun metagenomic timeline from the Řimov reservoir (Figure 1D). Here, diplonemid
 123 sequences in the epilimnion were found only during spring or early summer with low
 124 abundance (maximum 0.04% of total eukaryotic 18S rRNA reads) (Figure 1D). In contrast
 125 to Lake Biwa, diplonemid sequences were found at all seasons in the hypolimnion of the
 126 Řimov reservoir with relatively higher proportions (up to 0.6% of total metagenomic
 127 reads) (Supplementary Table S2) although these samples were pre-filtered with 5 µm
 128 filters, which may have reduced the number of diplonemids. Moreover, the differences in
 129 trophic status and sample collection from different depths (Table 1) might be responsible
 130 for the observed variation. It is also relevant to mention that dynamics of diplonemids
 131 inferred from raw sequence reads is not fully quantitative, as we observed multiple copies
 132 of 18S rRNA in single cell genomes from marine diplonemids (Supplementary Table S1).

133 To further examine the distribution of diplonemids at other locations, we sampled
 134 several European lakes for shotgun metagenomics (Table 1). As the sequences obtained
 135 by amplicon-sequencing were short (300 bp), we wondered if it was possible to identify
 136 longer assembled 18S rRNA sequences from freshwater diplonemids in assembled
 137 metagenomic contigs. Additionally, we also used previously available metagenomic
 138 samples from the Řimov reservoir [17] and other published metagenomic datasets from

the deep Lake Baikal [18,19]. Multiple long sequences were retrieved from all samples except for Lake Baikal and all of them were closely related to the amplicon-derived sequences (See Figure 2). The longest assembled 18S rRNA sequence was 2029 bp suggesting the less examined potential for assembled metagenomic sequence data for recovery of long eukaryotic 18S rRNA sequences.

Long sequences (>300 bp) of diplomonids from the metagenomic datasets allowed us to construct a robust maximum-likelihood phylogenetic tree including all closely related sequences of diplomonids from public databases (Figure 2). We observed that the sequences recovered from geographically distant freshwater lakes were highly similar and appeared to be a sister clade of several marine isolates. High similarity of diplomonid sequences from freshwater lakes of the world reiterates what has been suggested already [32] that geographic distance does not restrict the distribution of protists in freshwater lakes around the world. The freshwater sequences formed a separate freshwater cluster that was separated from the marine cultured and environmental sequences with high bootstrap values, suggesting that freshwater diplomonids diverged from the marine ones and are phylogenetically different. The highly congruent freshwater clade provides conclusive evidence for an evolutionary radiation of diplomonids in freshwater ecosystems. Intriguingly, a single diplomonid isolate (accession MF422200) that was obtained from Tokyo Bay also appeared to be part of this freshwater clade. This might suggest that even though this isolate was obtained in the bay of Tokyo (Supplementary Table S3), it is possible that it is actually derived from the freshwater inflow into the bay. Moreover, the freshwater cluster is closely related to the marine isolates (Figure 2) and majority of those isolates were from bay, estuarine and near-shore regions (Supplementary Table S3) further suggesting a habitat transition of diplomonids from marine to freshwaters.

Occurrence, morphology, and potential feeding mode of diplonemids

We conducted CARD-FISH with the diplonemid-specific probe ‘Diplo1590R’ [31] targeting all freshwater and marine sequences, to enumerate their abundance in freshwater lakes. However, the number of CARD-FISH stained cells was too low to confidently assess their abundance and dynamics in all the studied lakes. Their abundance was low even during spring, when 18S rRNA read numbers were relatively high. In total, we detected 22 CARD-FISH stained cells from all lakes, again pointing at their rarity in freshwater habitats. However, images of these cells provide some insights about cell size, morphology and potential trophic strategies. All detected diplonemids were ovoid cells in the range of 5-10 μm in length (Figure 3, Supplementary Figure S6). Thus, the freshwater diplonemids observed in the present study are smaller when compared to the cultured marine diplonemids (which are around 20 μm in size), and also the ones observed from marine waters by single cell sorting, which are much diverse in size (7-20 μm) and shape [20].

There is little information on the feeding behaviour of diplonemids apart from the consensus that they are heterotrophic. Some studies have also reported diplonemids as mainly benthic dwellers feeding by phagotrophy on bacteria, osmotrophy or by detritivory [33,34]. Protists of size ranges observed here are also known to be efficient grazers of bacteria in aquatic ecosystems [35–39]. All CARD-FISH positive cells observed here also appeared to contain several bacteria (Figure 3). However, it is impossible to conclude from these images whether or not these microbes are inside the cells (suggesting phagotrophic behavior or symbiosis) or outside (epibionts, commensals, etc.) (Figure 3). Although there is no direct evidence of their feeding behavior from environmental samples, diplonemid sequences were detected from the larger planktonic fractions (180-

2000 μm) in TARA Ocean data and suggested to possess a parasitic or symbiotic lifestyle [40]. Moreover, a recent study reported the presence of endosymbiotic bacteria inside the cells of some cultured diplomonads [41], which might mimic bacterivory. Direct evidence using FLB (fluorescently labelled bacteria) uptake experiments [42,43] are necessary to confirm their phagotrophy on bacteria.

Copy number of 18S rRNA genes in diplomonads

Diplomonads have divergent rRNA genes and due to the large number of mismatches, they are generally not targeted in diversity studies using universal eukaryotic primers (especially V4 primers) [7,8]. This explains why they remained undetected in a majority of eukaryotic diversity studies in the world oceans until the TARA expedition. TARA strategically used primers targeting the V9 region [3], which is a short region (~150 bp) considered best to capture a large diversity [10]. Although sequence abundance of diplomonads was high in the global oceanic waters, direct quantitative evidence of their actual abundances is scarce. Only a single study from the Atlantic Ocean quantified the abundance of diplomonads from various depths using CARD-FISH and found their abundance to be <1% of total planktonic eukaryotes [31], similar to our observations from freshwaters. In the microscopic analysis we could barely observe CARD-FISH positive cells even in those samples, which had relatively higher sequence numbers assigned to diplomonads. These observations suggest that diplomonads (especially marine ones) possess high copy numbers of divergent 18S rRNA genes, that could contribute to their amplified sequence abundance [44]. In order to test this, we analyzed ten previously available single cell genomes from marine diplomonads [20]. These genomes are very incomplete (most complete being 9%), so the number of actual 18S rRNAs detected in these is likely to be severely underestimated. Even so, we detected multiple 18S rRNA

gene sequences in these incomplete genomes (up to 21 sequences >300bp in cell_13, Supplementary Table S4). Moreover, the sequences from the same cell did not cluster with each other in a phylogenetic tree in several cases (e.g. cell-13, cell-27 and cell-4sb), suggesting a high intra-genomic diversity of this frequently used gene for assessing protist diversity [44] (Supplementary Figure S2). As the raw sequence data for the single cell genomes are not available, we used the coverage of the contigs to obtain an estimate of 18S rRNA copy number (Supplementary Table S4). A conservative estimate, using median coverage of an 18S rRNA containing contig vs coverage of the remaining contigs (>3kb) suggests multiple copies of 18S rRNA genes in one single cell (up to 141 copies, Supplementary Table S1 and Supplementary Table S4). Only a single, related isolate genome is available (*Diplonema papillatum* sp.) with an estimated genome completeness of ca. 30% (Supplementary Figure S1). We could detect 6 copies of 18S rRNAs, which appears to be somewhat low [22]. However, we could not estimate the putative number of rRNAs, as coverage information for contigs was not available. Given such wide variations in the number of 18S rRNA copies and sequence divergence in the presently available incomplete genomic data, accurate quantifications of protist diversity or abundance by sequencing data alone are expected to remain a pressing issue until improved genomes are available.

Conclusions

We describe a clade of freshwater diplonemids from multiple lakes around the world. Freshwater diplonemids are related to cultured marine diplonemids and distinct from the as yet uncultured marine diplonemids. Direct observations using CARD-FISH also suggest that freshwater diplonemids are consistently smaller in size in comparison to those from the marine habitat. Although diplonemid 18S rRNA gene sequences were found to

be highly abundant in marine waters [3], a prior quantification of marine diplomonads using CARD-FISH suggests that they are rare components of the marine habitat [31]. It has also been recently suggested that estimates from amplicon sequencing are far from quantitative [44,45]. Taken together with the highly divergent and multiple 18S sequences derived from single diplomonad cells, it appears the actual abundances of marine diplomonads using sequence data might have been overestimated. Direct counts by CARD-FISH using an identical probe that targets both freshwater and marine clades suggest that diplomonad abundances in both marine and freshwater ecosystems are very low. However, amplicon-sequencing results from both habitats are drastically different, while a large diversity of diplomonad sequences have been obtained from the marine habitat [3], very limited diversity was observed in freshwaters. This suggests that freshwater diplomonads are less diverse than their marine counterparts and their close relationship indicates a recent habitat transition from marine to freshwaters. Nevertheless, it is important to note that this contradictory result of diversity and abundance might not be restricted to diplomonads alone and may also extend to other protistan groups as well [7,43,45]. Further studies using single-cells, cultures, genome sequencing and experimental approaches will be necessary for an improved understanding of the ecological role and evolutionary history of these poorly understood groups of protists.

Materials and Methods

Sampling: Samples were collected from seven freshwater lakes, in Japan (Biwa- 35°12' N 135°59' E and Ikeda- 31°14' N 130°33' E), Czech Republic (Řimov- 48°51' N 14°29' E, and Medard- 50°10' N 12°35' E), and Switzerland (Zurich- 47°18' N 8°34' E, Lugano- 45°59' N 8°58' E, and Constance- 47°32' N 9°31' E) (Table 1). All these lakes are >100m deep with the exception of the Řimov reservoir and Medard, which are 43 and 50m deep,

respectively. Samples from Lake Biwa, Japan were collected from three depths representing epilimnion, metalimnion and hypolimnion from station Ie-1, a long-term limnological survey station of Kyoto University, Japan. These samples were collected monthly for one year in 2017 (from depths 5, 20 and 60 m) and also from a high frequency weekly sampling, which was conducted for three months during the spring of 2016 (from depths 5, 10 and 50 m) (Table 1). Samples from the Řimov Reservoir, Czech Republic were collected seasonally for two years (2016 and 2017) from two depths (0.5 and 30 m), representing epilimnion and hypolimnion (Table 1) from a station located at the dam (deepest part of the reservoir). For other lakes, samples were collected once or twice during the thermal stratification period from depths representing the epilimnion and hypolimnion from stations located at the deepest part of the lakes. Samples were collected with a 5-liter Niskin sampler (General Oceanics, Miami, USA) or with a Freidinger sampler. Temperature, concentration of chlorophyll *a* and dissolved oxygen was determined with a multiparameter probe (911 plus, Sea Bird Electronics, Inc., USA; or YSI 6600 multiparameter sonde V2, Yellow Springs Instruments, USA) (Figure 1).

DNA extraction, amplicon sequencing and data processing: Amplicon sequencing was conducted only from samples collected from Biwa and Ikeda lakes. Water from the different sampling depths was pre-filtered just after collection using a 20- μ m mesh plankton net to exclude larger organisms. One to two liters were filtered onto 0.8 μ m polycarbonate filters (47 mm diameter, Costar) at low vacuum (7.5 cm Hg) and immediately frozen at -30°C until analysis. DNA was extracted with the Power Soil DNA isolation and purification kit (MoBio laboratories, Carlsbad, CA, USA) and quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Polymerase chain reactions (PCR) were conducted using

universal eukaryotic primers Euk1391f and EukBr to amplify the V9 region of 18S rRNA genes of all eukaryotes [9,10]. PCRs were performed in 25 µl of reaction volume according to the 18S Illumina amplicon protocol of the Earth Microbiome Project (<http://www.earthmicrobiome.org/protocols-and-standards/18s/>). Amplicons were examined using agarose gel electrophoresis and DNA was purified using magnetic beads. Samples were quantified using Qubit. The samples were pooled to have uniform DNA concentration in each and were sequenced using the Illumina MiSeq platform. The processing and quality control of the sequencing data were conducted using DADA2 [11]. Chimera check was conducted with DADA2 and also with the reference-based chimera searches against the PR2 database [12]. Amplicons with quality score more than Q30 were retained and amplicons were trimmed to have 300 bp using the ‘Filter and Trim’ function of DADA2. A total of 656,427 reads were obtained after processing. Classification of unique sequences was conducted against the SILVA 132 reference database [13]. The unclassified sequences were additionally examined for their closest relatives with BLAST searches against the NCBI NT database [14].

Filtration and DNA extraction for shotgun metagenomic analysis: Shotgun

metagenomic sequencing was conducted for all lakes except for the Japanese ones (Biwa and Ikeda). Water samples (ca. 10 L each) from lakes Řimov (Czech Republic, depths 0.5 m and 30 m), Medard (Czech Republic, depths 5 m and 20 m), Zurich (Switzerland, depths 5 m and 80 m), Constance (Switzerland/Germany, depths 5 m and 200 m) and Lugano (Switzerland/Italy, depths 5 m and 50 m) were progressively filtered through 20 µm, 5 µm, and 0.22 µm polycarbonate or polysulfone membrane filters (Sterlitech, USA). DNA extraction was performed from the 0.22 µm filters (containing the 5–0.22 µm

microbial size fraction) using the ZR Soil Microbe DNA MiniPrep kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions.

Preprocessing of metagenomic datasets: Shotgun metagenome sequencing was performed with Novaseq 6000 (2×151 bp) (Novogene, HongKong, China). Low quality bases/reads and adaptor sequences were removed from the raw Illumina reads using the bbmap package [15]. Briefly, the reads were quality trimmed by bbdduk.sh (using a Phred quality score of 18). Subsequently, bbdduk.sh was used for adapter trimming and identification/removal of possible PhiX and p-Fosil2 contamination. Additional checks (i.e., *de novo* adapter identification with bbmerge.sh) were performed in order to ensure that the datasets meet the quality threshold necessary for assembly. Each metagenomic dataset (i.e. each sample) was assembled independently with MEGAHIT (v1.1.5) [16] using the k-mer sizes: 49, 69, 89, 109, 129, 149, and default settings. Previously available metagenomes and assemblies from Řimov reservoir [17] and Lake Baikal were included in the analysis [18,19].

Genomic data processing: Assembled sequence data for single cell derived genomes from 10 published marine diplomonads were downloaded [20]. Genome completeness estimates performed by us (and similar to those reported earlier) for these were found to be very low with the best assembly missing 91% of conserved single copy orthologs (as reported by BUSCO [21]). The assembled *Diplonema papillatum* genome [22] was downloaded from NCBI (accession LMZG000000000). Only the assembled genome is available and raw data was not available although it was mentioned to be 21.5Gb. The genome assembly size is ca. 107Mb (shortest contig 1000bp, longest contig 100kb). The authors estimated the genome size to be 175Mb, but no details were provided on how this

estimate was obtained [22]. Genome completeness estimates for all available genomes (*Diplonema papillatum* genome and the 10 marine diplonemid SAGs) were obtained using BUSCO [21] with the euglenozoa lineage (euglenozoa_odb10 version 2019-11-21) (busco -m genome -i genome.fna -o output --lineage_dataset euglenozoa_odb10) (Supplementary Figure S1). Eukaryotic small ribosomal subunit sequences were detected in these genomic assemblies using ssu-align [23]. Only those sequences that were >300bp were retained for further analysis. The results are summarized in Supplementary Table S1.

Retrieval of 18S rRNA sequences from assembled shotgun metagenomic datasets: All diplonemid sequences were extracted from the SILVA database version 138 [13]. These sequences along with the freshwater diplonemid sequence (OTU_4) described previously [6], were used as queries for BLASTN against the metagenomic datasets. Hits with e-value <1e-5, alignment length >200 bp and percentage identity >90% to the queries were extracted. These candidate sequences were aligned to the global SILVA alignment for rRNA genes using the SINA web aligner [24] and subsequently inserted into the guide tree of SILVA SSU database 138 RefNR 99 in ARB [25] using maximum parsimony and only those sequences that affiliated to diplonemids and of length >300 bp were retained.

Quantification of 18S rRNA sequences of freshwater diplonemids in a shotgun metagenomic time-series from Řimov reservoir: All assembled freshwater diplonemid sequences (from previous section) along with OTU_4 from Lake Biwa [6] were added to the existing SILVA 138 database of 18S sequences. At least half the reads from each metagenomic dataset (minimum 94 million reads, maximum 277 million reads) were scanned by ssu-align to extract potential eukaryotic 18S reads (minimum ca. 65,000, maximum 180,000 found) and these were used as queries against the SILVA138 database

supplemented with freshwater diplonemid sequences using mmseqs2 [26]. Metagenomic query sequences matching the freshwater diplonemid sequences with >95% identity, evaluate $<1e-5$ and alignment length >50bp were considered to originate from freshwater diplonemids (Supplementary Table S2).

18S rRNA phylogenetic tree: Assembled 18S rRNA sequences identified to be from freshwater diplonemids were aligned to all known clades of Euglenids using PASTA [27] and a maximum likelihood phylogenetic tree (Figure 2) was made using IQ-TREE 2 using automatic model selection (TIM3e+R10 model), ultrafast bootstraps and SH-aLRT tests (-bb 1000 -alrt 1000) [28–30]. Additionally, all 18S sequences (>300 bp) from the single cell diplonemid genomes and the *Diplonema papillatum* genome were aligned to the same set of sequences and a phylogenetic tree (Supplementary Figure S2) was created as described above.

Catalyzed Reporter Deposition-Fluorescent in situ Hybridization (CARD-FISH):

Samples for CARD-FISH were collected from the same depths as the samples used for DNA extractions (Table 1). Water samples were fixed in a 2% final concentration of formaldehyde (freshly prepared by filtering through 0.2 μm syringe filter) for at least 3-4 hours before filtration. 50 ml of epilimnion/metalimnion and 100 ml of hypolimnion samples were filtered through polycarbonate filters (pore size 0.8 μm , Advantec), rinsed twice with 1X PBS and twice with MilliQ water, air dried and frozen at -20°C until further processing. CARD-FISH was performed according to the method described previously [7]. The filters were hybridized at 35°C for 12 h with a $0.5 \mu\text{g ml}^{-1}$ probe concentration and a 30% concentration of formamide. The probe targeting diplonemids ‘DiploR1792’ [31] was purchased from Biomer, Germany. Counting was performed using an Olympus

BX 53 epifluorescence microscope under 1000 × magnification at blue/UV excitation. Total microbial eukaryotes were counted simultaneously by DAPI staining under UV excitation. Images were taken and processed using a semiautomatic image analysis system (NIS-Elements 3.0, Laboratory Imaging, Prague, Czech Republic).

Data Accessibility

The sequence data of Japanese lakes generated from amplicon sequencing were submitted to European Nucleotide Archive (ENA) and are available under the BioProject: PRJEB37597, BioSamples ERS4413153–226. Sequence data for all metagenomes generated in this work are archived at EBI European Nucleotide Archive and can be accessed under the BioProject PRJEB35770, BioSamples ERS4146214-50 (Řimov reservoir), BioProject PRJEB35640, BioSamples ERS4065084-85 (Lake Medard), ERS4065070-71 (Lake Lugano), BioProject PRJEB35770, BioSamples ERS4409418–21 (Lake Constance), BioSamples ERS4409422–23 (Lake Zurich). All 18S rRNA sequences, alignments and trees are available at figshare (<https://doi.org/10.6084/m9.figshare.12091749.v1>). This study did not generate any code.

Author's Contributions

IM and RG conceived the study. IM performed sampling, amplicon analyses and CARD-FISH. RG performed metagenomic and phylogenetic analyses. MMS, A-ŞA and VSK performed sampling and phylogenetic analyses. A-ŞA, TS, VG, MH, PL, YH, SN, KS performed sampling and DNA extractions. IM and RG drafted the manuscript with the input of all other authors.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the JSPS Bilateral Japanese-Czech Joint Research Project No. JSPS-17-17, funding the collaboration of KS, SN, IM, VG and YH. IM was supported by the research grants CZ.02.1.01/0.0/ 0.0/16_025/0007417 (ERDF/ESF) and 20-12496X (Grant Agency of the Czech Republic). RG was supported by the research grants 17-04828S, 20-12496X (Grant Agency of the Czech Republic) and CZ.02.1.01/0.0/ 0.0/16_025/0007417 (ERDF/ESF). MMS was supported by the research grants 20-12496X (Grant Agency of the Czech Republic) and 310030_185108 (Swiss National Science Foundation). A-ŠA was supported by the research grants 17-04828S (Grant Agency of the Czech Republic) and MSM200961801 (Academy of Sciences of the Czech Republic). VSK was supported by the research grants 17-04828S, 20-12496X (Grant Agency of the Czech Republic) and 116/2019/P (Grant Agency of the University of South Bohemia in České Budějovice 2019-2021). MH was supported by the research grant 310030_185108 (Swiss National Science Foundation). PL was supported by the research grant 19-23469S (Grant Agency of the Czech Republic) and 022/2019/P (Grant Agency of the University of South Bohemia in České Budějovice 2019-2021).

Acknowledgements

We are thankful to the captains of the research vessel ‘Hasu’, Y. Goda and T. Akatsuka for their help in sample collection in Lake Biwa. We are also thankful to Shohei Fujinaga for his help in sample collection and data analysis. P. Znachor, P. Rychtecky and J. Nedoma are acknowledged for help in sampling of Řimov Reservoir and Lake Medard. T.

Posch, E. Loher and V. Lanta are thanked for help in the sample collection of Lake Zurich. S. Dirren and the crew of the research vessel ‘Kormoran’ are thanked for help in sampling of Lake Constance, and F. Leporelli and V. Lanta for help in sampling of Lake Lugano.

References

1. Lukeš J, Flegontova O, Horák A. 2015 Diplonemids. *Curr. Biol.* **25**, R702–704. (doi:<https://doi.org/10.1016/j.cub.2015.04.052>)
2. Lara E, Moreira D, Vereshchaka A, López-García P. 2009 Pan-oceanic distribution of new highly diverse clades of deep-sea diplonemids. *Environ. Microbiol.* **11**, 47–55. (doi:[10.1111/j.1462-2920.2008.01737.x](https://doi.org/10.1111/j.1462-2920.2008.01737.x))
3. de Vargas C, Audic S, Henry N, Decelle J, Mahé F, Logares R, *et al.* 2015 Eukaryotic plankton diversity in the sunlit ocean. *Science* **348**, 1261605. (doi: [10.1126/science.1261605](https://doi.org/10.1126/science.1261605))
4. Flegontova O, Flegontov P, Malviya S, Audic S, Wincker P, de Vargas C, Bowler C, Lukeš J, Horák A. 2016 Extreme Diversity of Diplonemid Eukaryotes in the Ocean. *Curr. Biol.* **26**, 3060–3065. (doi:[10.1016/j.cub.2016.09.031](https://doi.org/10.1016/j.cub.2016.09.031))
5. Tashyreva D, Prokopchuk G, Votýpka J, Yabuki A, Horák A, Lukeš J. 2018 Life cycle, ultrastructure, and phylogeny of new diplonemids and their endosymbiotic bacteria. *MBio* **9**, 1–20. (doi:[10.1128/mBio.02447-17](https://doi.org/10.1128/mBio.02447-17))
6. Mukherjee I, Hodoki Y, Okazaki Y, Fujinaga S, Ohbayashi K, Nakano SI. 2019 Widespread Dominance of Kinetoplastids and Unexpected Presence of Diplonemids in Deep Freshwater Lakes. *Front. Microbiol.* **10**, 1–13. (doi:[10.3389/fmicb.2019.02375](https://doi.org/10.3389/fmicb.2019.02375))

461 7. Mukherjee I, Hodoki Y, Nakano S. 2015 Kinetoplastid flagellates overlooked by
462 universal primers dominate in the oxygenated hypolimnion of Lake Biwa, Japan. *FEMS*
463 *Microbiol. Ecol.* **91**, 1–11. (doi:10.1093/femsec/fiv083)

464 8. Bochdansky AB, Clouse MA, Herndl GJ. 2017 Eukaryotic microbes, principally fungi
465 and labyrinthulomycetes, dominate biomass on bathypelagic marine snow. *ISME J.* **11**,
466 362–373. (doi:10.1038/ismej.2016.113)

467 9. Amaral-Zettler LA, McCliment EA, Ducklow HW, Huse SM. 2009 A method for
468 studying protistan diversity using massively parallel sequencing of V9 hypervariable
469 regions of small-subunit ribosomal RNA Genes. *PLoS One* **4**, 1–9.
470 (doi:10.1371/journal.pone.0006372)

471 10. Stoeck T, Bass D, Nebel M, Christen R, Jones MDM, Breiner HW, Richards TA. 2010
472 Multiple marker parallel tag environmental DNA sequencing reveals a highly complex
473 eukaryotic community in marine anoxic water. *Mol. Ecol.* **19**, 21–31. (doi:10.1111/j.1365-
474 294X.2009.04480.x)

475 11. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016
476 DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods*
477 **13**, 581–583. (doi:10.1038/nmeth.3869)

478 12. Guillou L, Bachar D, Audic S, Bass D, Berney C, Bittner L, *et al.* 2013 The Protist
479 Ribosomal Reference database (PR2): A catalog of unicellular eukaryote Small Sub-Unit
480 rRNA sequences with curated taxonomy. *Nucleic Acids Res.* **41**, 597–604.
481 (doi:10.1093/nar/gks1160)

482 13. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO.
483 2012 The SILVA ribosomal RNA gene database project: improved data processing and
484 web-based tools. *Nucleic Acids Res.* **41**, D590–D596. (doi:10.1093/nar/gks1219)

485 14. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990 Basic local alignment
486 search tool. *J. Mol. Biol.* **215**, 403–410. (doi:10.1016/S0022-2836(05)80360-2)

487 15. Bushnell B. 2016 BBMap Short-read aligner, and other bioinformatics tools.
488 Bioinformatics. (doi:https://sourceforge.net/projects/bbmap/)

489 16. Li D, Luo R, Liu CM, Leung CM, Ting HF, Sadakane K, Yamashita H, Lam TW.
490 2016 MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced
491 methodologies and community practices. *Methods* **102**, 3–11.
492 (doi:10.1016/j.ymeth.2016.02.020)

493 17. Kavagutti VS, Andrei AŞ, Mehrshad M, Salcher MM, Ghai R. 2019 Phage-centric
494 ecological interactions in aquatic ecosystems revealed through ultra-deep metagenomics.
495 *Microbiome* **7**, 0–15. (doi:10.1186/s40168-019-0752-0)

496 18. Cabello-Yeves PJ, Zemskaya TI, Rosselli R, Coutinho FH, Zakharenko AS, Blinov V
497 V, Rodriguez-Valera F, Drake HL. 2018 Genomes of Novel Microbial Lineages
498 Assembled from the Sub-Ice Waters of Lake Baikal. *Appl. Environ. Microbiol.* **84**,
499 e02132–17. (doi: 10.1128/AEM.02132-17)

500 19. Cabello- Yeves PJ, Zemskaya TI, Zakharenko AS, Sakirko M V., Ivanov VG, Ghai R,
501 Rodriguez- Valera F. 2019 Microbiome of the deep Lake Baikal, a unique oxic
502 bathypelagic habitat. *Limnol. Oceanogr.*, lno.11401. (doi:10.1002/lno.11401)

503 20. Gawryluk RMR, del Campo J, Okamoto N, Strassert JFH, Lukeš J, Richards TA,
504 Worden AZ, Santoro AE, Keeling PJ. 2016 Morphological Identification and Single-Cell
505 Genomics of Marine Diplonemids. *Curr. Biol.* **26**, 3053–3059.
506 (doi:10.1016/j.cub.2016.09.013)

507 21. Seppely M, Manni M, Zdobnov EM. 2019 BUSCO: Assessing genome assembly and
508 annotation completeness. In *Methods in Molecular Biology*, pp. 227–245. Humana Press
509 Inc. (doi:10.1007/978-1-4939-9173-0_14)

510 22. Morales J, Hashimoto M, Williams TA, Hirawake-Mogi H, Makiuchi T, Tsubouchi A,
511 et al. 2016 Differential remodelling of peroxisome function underpins the environmental
512 and metabolic adaptability of diplomonads and kinetoplastids. *Proc. R. Soc. B Biol. Sci.*
513 **283**, 20160520. (doi:10.1098/rspb.2016.0520)

514 23. Nawrocki EP. 2009 Structural alignment and RNA homology search and alignment
515 using covariance models (Ph. D. thesis). Washington University in Saint Louis, School of
516 Medicine. (doi:https://openscholarship.wustl.edu/etd/256/)

517 24. Pruesse E, Peplies J, Glöckner FO. 2012 SINA: Accurate high-throughput multiple
518 sequence alignment of ribosomal RNA genes. *Bioinformatics* **28**, 1823–1829.
519 (doi:10.1093/bioinformatics/bts252)

520 25. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Buchner A, et al. 2004 ARB: a
521 software environment for sequence data. *Nucleic Acids Res.* **32**, 1363–1371.
522 (doi:10.1093/nar/gkh293)

523 26. Steinegger M, Söding J. 2017 MMseqs2 enables sensitive protein sequence searching
524 for the analysis of massive data sets. *Nat. Biotechnol.* **35**, 1026–1028.
525 (doi:10.1038/nbt.3988)

526 27. Mirarab S, Nguyen N, Guo S, Wang LS, Kim J, Warnow T. 2015 PASTA: Ultra-large
527 multiple sequence alignment for nucleotide and amino-acid sequences. *J. Comput. Biol.*
528 **22**, 377–386. (doi:10.1089/cmb.2014.0156)

529 28. Quang B, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, Von Haeseler A,
530 Lanfear R. 2020 IQ-TREE 2: New Models and Efficient Methods for Phylogenetic
531 Inference in the Genomic Era. *Mol. Biol. Evol.* **37**, 1530–1534.
532 (doi:10.1093/molbev/msaa015)

533 29. Kalyaanamoorthy S, Minh BQ, Wong TKF, Von Haeseler A, Jermini LS. 2017
534 ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat. Methods* **14**,
535 587–589. (doi:10.1038/nmeth.4285)

536 30. Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. 2018 UFBoot2:
537 Improving the Ultrafast Bootstrap Approximation. *Molecular biology and evolution. Mol.*
538 *Biol. Evol.* **35**, 518–522. (doi:10.5281/zenodo.854445)

539 31. Morgan-Smith D, Clouse MA, Herndl GJ, Bochkansky AB. 2013 Diversity and
540 distribution of microbial eukaryotes in the deep tropical and subtropical North Atlantic
541 Ocean. *Deep. Res. Part I Oceanogr. Res. Pap.* **78**, 58–69. (doi:10.1016/j.dsr.2013.04.010)

542 32. Boenigk J, Pfandl K, Stadler P, Chatzinotas A. 2005 High diversity of the ‘Spumella-
543 like’ flagellates: An investigation based on the SSU rRNA gene sequences of isolates
544 from habitats located in six different geographic regions. *Environ. Microbiol.* **7**, 685–697.
545 (doi:10.1111/j.1462-2920.2005.00743.x)

546 33. López-García P, Vereshchaka A, Moreira D. 2007 Eukaryotic diversity associated
547 with carbonates and fluid-seawater interface in Lost City hydrothermal field. *Environ.*
548 *Microbiol.* **9**, 546–554. (doi:10.1111/j.1462-2920.2006.01158.x)

549 34. Roy J, Faktorová D, Benada O, Lukeš J, Burger G. 2007 Description of *Rhynchopus*
550 *euleeides* n. sp. (Diplonemea), a free-living marine euglenozoan. *J. Eukaryot. Microbiol.*
551 **54**, 137–145. (doi:10.1111/j.1550-7408.2007.00244.x)

552 35. Massana R, Unrein F, Rodríguez-Martínez R, Forn I, Lefort T, Pinhassi J, Not F. 2009
553 Grazing rates and functional diversity of uncultured heterotrophic flagellates. *ISME J.* **3**,
554 588–595. (doi:10.1038/ismej.2008.130)

555 36. Boenigk J, Arndt H. 2002 Bacterivory by heterotrophic flagellates: community
556 structure and feeding strategies. *Antonie Van Leeuwenhoek* **81**, 465–480. (doi:
557 <https://doi.org/10.1023/A:1020509305868>)

- 558 37. Šimek K, Kasalický V, Jezbera J, Hornák K, Nedoma J, Hahn MW, Bass D, Jost S,
559 Boenigk J. 2013 Differential freshwater flagellate community response to bacterial food
560 quality with a focus on Limnohabitans bacteria. *ISME J.* **7**, 1519–1530.
561 (doi:10.1038/ismej.2013.57)
- 562 38. Šimek K, Nedoma J, Znachor P, Kasalický V, Jezbera J, Hornák K, Sed'a J. 2014 A
563 finely tuned symphony of factors modulates the microbial food web of a freshwater
564 reservoir in spring. *Limnol. Oceanogr.* **59**, 1477–1492. (doi:10.4319/lo.2014.59.5.1477)
- 565 39. Šimek K, Grujčić V, Hahn MW, Hornák K, Jezberová J, Kasalický V, Nedoma J,
566 Salcher MM, Shabarova T. 2018 Bacterial prey food characteristics modulate community
567 growth response of freshwater bacterivorous flagellates. *Limnol. Oceanogr.* **63**, 484–502.
568 (doi:10.1002/lno.10759)
- 569 40. Lima-Mendez G, Faust K, Henry N, Decelle J, Colin S, Carcillo F, et al. 2015
570 Determinants of community structure in the global plankton interactome. *Science* **348**,
571 1262073. (doi:10.1126/science.1262073)
- 572 41. George EE, Husnik F, Tashyreva D, Prokopchuk G, Horák A, Kwong WK, Lukeš J,
573 Keeling PJ. 2020 Highly Reduced Genomes of Protist Endosymbionts Show Evolutionary
574 Convergence. *Curr. Biol.* **30**, 925–933.e3. (doi:10.1016/j.cub.2019.12.070)
- 575 42. Šimek K, Grujčić V, Nedoma J, Jezberová J, Šorf M, Matoušů A, et al. 2019
576 Microbial food webs in hypertrophic fishponds: Omnivorous ciliate taxa are major
577 protistan bacterivores. *Limnol. Oceanogr.* **64**, 2295–2309. (doi:10.1002/lno.11260)
- 578 43. Grujčić V, Nuy JK, Salcher MM, Jensen M, Šimek K. 2018 Cryptophyta as major
579 bacterivores in freshwater summer plankton. *ISME J.* , 1668–1681. (doi:10.1038/s41396-
580 018-0057-5)
- 581 44. Caron DA, Hu SK. 2018 Are We Overestimating Protistan Diversity in Nature? *Trend*
582 *Microbiol.* **27**, 197–205. (doi:10.1016/j.tim.2018.10.009)

45. Piwosz K, Shabarova T, Pernthaler J, Posch T, Šimek K, Porcal P, Salcher MM. 2020
Bacterial and Eukaryotic Small-Subunit Amplicon Data Do Not Provide a Quantitative
Picture of Microbial Communities, but They Are Reliable in the Context of Ecological
Interpretations. *mSphere* **5**. (doi:10.1128/msphere.00052-20)

608 Table 1. Details of sampling sites

Lake, country	Trophic status	Surface area (km ²)	Z max (m)	Sampling depths (m)	Sampling date	Analyses performed
Biwa, JP	Mesotrophic	674.0	104	5, 10, 50 (2016) 5, 20, 60 (2017)	Mar-May 2016 (weekly) 2017 (monthly)	AS, CF AS, CF
Ikeda, JP	Mesotrophic	11.0	200	5, 50, 100, 200	25.11.2017	AS, CF
Řimov, CZ	Eutrophic	2.1	43	0.5, 30	Apr, Aug, Nov 2016, 2017 (monthly)	MG, CF MG, CF
Medard, CZ	Oligotrophic	4.9	50	5, 20	09.07.2019	MG
Constance, CH/DE/AT	Mesotrophic	536	252	5, 200	03.07.2018	MG, CF
Zurich, CH	Oligo-mesotrophic	88.7	136	5, 80	11.07.2018 13.11.2019	MG, CF CF
Lugano, CH/IT	Eutrophic	48.7	288	5, 50	02.04.2019 05.11.2019	MG CF

609 Abbreviations: JP, Japan; CZ, Czech Republic; CH, Switzerland; DE, Germany; AT, Austria; IT,
610 Italy; AS, 18S amplicon sequencing, MG, shotgun metagenomics; CF, CARD-FISH

611

612

613

Figure 1. Dynamics of freshwater diplomonads (18S rRNA gene sequence abundance) by A) amplicon sequencing in a weekly spring/summer sampling campaign from Lake Biwa, Japan (depths 5, 10 and 50m), B) amplicon sequencing in a one year sampling campaign from Lake Biwa (depths 5, 20 and 60m) C) shotgun metagenomic time series of the Rimov reservoir, Czech Republic (depths 0.5 and 30m) and D) in a single depth profile from Lake Ikeda (depths 0, 50, 100, 150 and 200m).

Figure 2. Maximum-likelihood tree of 18S rRNA of diplomonads. Marine diplomonads, *Hemistasia*, freshwater diplomonads, sequences from isolates and kinteoplastids are shown. Other euglenids (e.g. *EUglena*, *Phacus*) were used as outgroup (not shown and indicated by an arrow). Ultra-fast bootstrap values and SH branch supports are indicated for selected nodes. Scale bar indicates the number of substitutions per site.

Figure 3. Freshwater diplomonids under fluorescent microscope.
A - DAPI, B - FISH, bacteria inside the cells are shown by arrows.

Supplementary Figure S1. Genome completeness estimation for all available diploid genomes using BUSCO (Seppey et al. 2019). Single cell genomes are prefixed with "cell", *Diplonema papillatum* isolate genome: dpap.

Supplementary Figure S2. Maximum-likelihood tree of 18S rRNA gene sequences of diplomonads highlighting the copy numbers in single cells. 18S rRNA sequences from the genome of single cells were obtained from public databases and are highlighted with same color. *D. papillatum* group is highlighted in red in marine isolates. Marine diplomonads, Hemistasia, freshwater diplomonads, sequences from isolates and kinteoplastids are also shown. Other euglenids (e.g. *Euglena*, *Phacus*) were used as outgroup (not shown and indicated by an arrow). Ultra-fast bootstrap values and SH branch supports are indicated for selected nodes. The scale bar indicates the number of substitutions per site.

Supplementary Figure S3. Community composition of microbial eukaryotes in Lake Biwa during weekly spring/summer sampling campaign by 18S amplicon sequencing from A) Epilimnion (5m), B) Metalimnion (10m) and C) Hypolimnion (50m).

Supplementary Figure S4. Community composition of microbial eukaryotes in Lake Biwa in 2017 by 18S amplicon sequencing from (A) epilimnion (5 m), (B) metalimnion (20 m) and (C) hypolimnion (60 m)

Supplementary Figure S5. Community composition of microbial eukaryotes in various depths of Lake Ikeda by 18S amplicon sequencing.

Supplementary Figure S6. Fluorescent microscopic images of freshwater diplomonads from lake Biwa (A-D), Římov (E) and lake Zurich (F). 1 and 2 refers to the same cells visualized by DAPI (under UV excitation) and CARD-FISH (under blue excitation) respectively.