

Title Page

Kinetoplastid flagellates overlooked by universal primers dominate in the oxygenated hypolimnion of Lake Biwa, Japan

Indranil Mukherjee¹, Yoshikuni Hodoki², Shin-ichi Nakano¹

¹*Center for Ecological Research, Kyoto University, Otsu, Shiga, Japan;* ²*Department of Biology, Keio University, Yokohama, Japan*

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Running Title: Dominance of kinetoplastid flagellates in a Lake

Correspondence: S Nakano, Center for Ecological Research, Kyoto University, Hirano 2-509-3, Otsu 520-2113, Shiga, Japan.

Tel: +81-77-549-8239

Fax: +81-77-549-8201

E-mail: nakano@ecology.kyoto-u.ac.jp

Abstract

1
2 Kinetoplastid flagellates, microscopically often detected from various aquatic environments
3 and considered ubiquitous are seldom reported in molecular diversity studies with universal
4 eukaryote DNA primers. To investigate this inconsistency, we examined nanoflagellate
5 diversity in Lake Biwa, Japan by 18S rRNA gene clone libraries using universal eukaryote
6 and kinetoplastid-specific primers. We also examined the abundance of kinetoplastids by
7 catalyzed reporter deposition–fluorescence *in situ* hybridization. No kinetoplastid sequences
8 were detected in the universal eukaryote primers library from epilimnion and hypolimnion in
9 different seasons. However, kinetoplastid flagellates were detected with kinetoplastid-specific
10 probe from all of the seasons and contributed up to 11.9% and 36.0% of total eukaryotes in
11 the epilimnion and hypolimnion, respectively. Thus kinetoplastids probably are a significant,
12 sometimes dominant, group in the hypolimnion, contributing up to 43.7% of the total
13 flagellates. Using group-specific primers, kinetoplastid sequences were also obtained from
14 both epilimnion and hypolimnion library. Therefore, we attributed the inconsistency to the
15 divergent nature of 18S rRNA gene of kinetoplastids, which lead to their un-detection in the
16 universal eukaryote primer libraries. The present study revealed that kinetoplastids have
17 significant ecological importance in the hypolimnion of Lake Biwa, suggesting that
18 kinetoplastids have been overlooked in other studies using universal eukaryote primers.

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Introduction

23
24 The ecological importance of nanoflagellates in the planktonic food web is well known
25 (Azam *et al.* 1983; Caron and Goldman 1990; Sherr and Sherr 1994; Nakano *et al.* 1998,
26 2001), and studies using molecular techniques have revealed an unexpectedly high diversity
27 of these organisms in various aquatic environments (Lovejoy *et al.* 2006; Massana *et al.*
28 2006; Stoeck *et al.* 2010; Edgcomb *et al.* 2011a). However, the population ecology (Sherr
29 and Sherr 1992), diversity and dynamics of individual nanoflagellate groups have received
30 relatively less attention (Logares *et al.* 2012). The scarcity of information about individual
31 groups prohibits our understanding of the ecological role of different flagellate groups in the
32 microbial food web (Lim *et al.* 1999; Beardsley *et al.* 2005; Piwosz *et al.* 2013).

33 Kinetoplastids are a ubiquitous group of protists (Simpson *et al.* 2006) with high
34 diversity (von der Heyden and Cavalier-Smith 2005; Edgcomb *et al.* 2011b) that have a
35 characteristic structure named kinetoplast, which is a highly modified mitochondrion with
36 densely packed DNA (Vickerman 1976). Kinetoplastids diverged from other eukaryotes due
37 to the massive evolutionary change of their 18S ribosomal (r) RNA gene and thus occupies a
38 basal position in the eukaryotic 18S rRNA gene phylogenetic trees (Simpson *et al.* 2006).
39 Studies using conventional microscopic observations have detected kinetoplastid flagellates
40 from various aquatic environments, though not as a dominant member of the nanoflagellate
41 community (Brandt and Sleigh 2000; Weitere and Arndt 2003; Lukes *et al.* 2014). However,
42 in contrast to morphological studies, kinetoplastids are rarely reported in eukaryote diversity
43 studies that use molecular techniques with the universal eukaryote 18S rRNA gene primers
44 (Lopez-Garcia *et al.* 2002; von der Heyden and Cavalier-Smith 2005; Scheckenbach *et al.*
45 2010; Risse-Buhl *et al.* 2013). These techniques target a large group of organisms based on
46 their conserved region of 18S rRNA gene.

47 These contrasting observations could be attributed to the various biases associated
48 with molecular techniques which underestimate the true diversity of microorganisms (Valster
49 *et al.* 2009; Lepère *et al.* 2010). Biases in molecular studies might arise due to number of
50 reasons, some of which are, artifacts arising during DNA extraction and plasmid ligation,
51 selectivity of gene amplification of specific groups and high copy number of 18S rRNA gene
52 of some organisms (Caron *et al.* 2009; Stoeck *et al.* 2010). The Catalyzed Reporter
53 Deposition–Fluorescence *in Situ* Hybridization (CARD-FISH) is an efficient tool to
54 circumvent the biases due to culture and molecular study and is widely used to enumerate
55 microorganisms from natural habitat (Pernthaler *et al.* 2002; Acinas *et al.* 2005). However,
56 limited numbers of probes are available for eukaryotic microorganisms relative to that of
57 bacteria (Lim *et al.* 1999; Loy *et al.* 2007), with only a few group-specific probes for
58 flagellates (Massana *et al.* 2006; Lepère *et al.* 2010; Piwosz and Pernthaler 2010). A
59 kinetoplastid group-specific probe has been constructed by correcting a mismatch in the
60 universal eukaryote probe (EUK516) sequence due to which kinetoplastids were not targeted
61 and thus underestimated in total eukaryote counts (Bochdansky and Huang 2010).
62 Information about the abundance and diversity of kinetoplastids is limited, with only a few
63 studies concerning the abundance of kinetoplastids using FISH probes in oceans (Edgcomb *et*
64 *al.* 2011b; Morgan-Smith *et al.* 2011, 2013). A surprisingly high abundance of kinetoplastids
65 was reported in all of these studies, with their increased abundance in deeper waters. In
66 addition, kinetoplastid sequences were detected from various environments including some
67 extreme environments e.g. hypersaline anoxic basins, abyssal sea floor, etc. using
68 kinetoplastid-specific primers (Scheckenbach *et al.* 2010; Edgcomb *et al.* 2011b; Salani *et al.*
69 2012).

70 The abundance and diversity of kinetoplastids in freshwater lakes are poorly known
71 due to fewer quantitative studies regarding nanoflagellates in freshwater lakes (del Campo

72 and Massana 2011). Moreover, studies have mainly focused on surface waters (Lefranc *et al.*
73 2005; Richards *et al.* 2005; Mangot *et al.* 2009; Nolte *et al.* 2010), and information about
74 deeper waters is scarce (Salbrechter and Arndt 1994; Lepère *et al.* 2010). Only one study
75 reported a high abundance of kinetoplastid-like flagellates in the hypolimnion of deep lakes
76 (Steinberg *et al.* 1983). However, the study by Steinberg *et al.* (1983) was conducted using
77 inverted microscope, and therefore no sequence data is available to compare or confirm the
78 organism observed. Information about the diversity and abundance of nanoflagellates in
79 different layers of deep lakes is important to understand the contribution and roles that
80 different flagellates play in the freshwater food web.

81 To investigate the dynamics of kinetoplastids in freshwater lakes, the present study
82 was conducted in Lake Biwa as it is deep (maximum depth 104 m) and largest freshwater
83 lake in Japan. We constructed 18S rRNA gene clone libraries using universal eukaryotic
84 primers from different depths and seasons to examine whether the method underestimates the
85 relative abundance of kinetoplastid flagellates. CARD-FISH analysis was conducted using
86 kinetoplastid-specific probes to study their seasonal and vertical abundance. Additionally,
87 clone libraries were constructed from epilimnion and hypolimnion sample using
88 kinetoplastid-specific DNA primers. The molecular analysis (clone library) and microscopic
89 analysis (CARD-FISH) were combined to understand the potential bias that is responsible for
90 the contradictory results given by the two methods to detect kinetoplastids.

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Materials and Methods

93 *Study site and Sampling*

94 The study was conducted in the monomictic and mesotrophic Lake Biwa, the largest
95 freshwater lake in Japan (surface area 674 km², maximum depth 104 m). The lake has an
96 oxygenated hypolimnion with minimum dissolved oxygen concentrations generally above
97 100 μM (Kim *et al.* 2006). During winter (January-March), the water column of the lake is
98 vertically mixed due to cooling and a strong seasonal wind, whereas during the rest of the
99 year, the water column is thermally stratified (Thottathil *et al.* 2013).

100 The samples were collected from January 2012 to January 2013 on a monthly basis
101 and once on August 2013 from station Ie-1 (35° 12' 58" N, 135° 59' 55" E, depth 73 m), a
102 long term limnological survey station of Center for Ecological Research, Kyoto University,
103 Japan. The hydrographic structure was determined with a conductivity-temperature depth
104 profiler (SBE-911 plus; Sea Bird Electronics, Sea-logger, WA, USA) equipped with an
105 oxygen sensor (13 E, SBE). Water samples from six depths (5, 10, 15, 20, 50 and 70 m)
106 representing the epilimnion, metalimnion and hypolimnion were collected with a 5 litre
107 Niskin sampler (General Oceanics, Miami, USA). The samples were drawn into clean plastic
108 bottles that were rinsed three times with sample water before collection; they were kept cool
109 and dark in an icebox and transported to the laboratory within three hours of collection.

110 The chlorophyll *a* concentration was determined by filtering 150 ml of the water
111 sample through a GF/F filter (diameter 25 mm, Whatman) and analysing the sample
112 following the *N,N*-dimethylformamide fluorometric method (Moran & Porath 1980).

113 *Total count of bacteria and nanoflagellates*

114 Samples were fixed with 1% final concentration of glutaraldehyde immediately after
115 collection and were stored at 4°C until filtration. A 1 to 2 ml water sample was filtered
116 through a polycarbonate membrane filter (pore size 0.2 μm, diameter 25 mm, Advantec), and
117 stained with 4, 6-diamidino-2-phenylindole (DAPI) to enumerate the total bacteria (Porter

118 and Feig 1980). The bacterial cells were visualized under UV light with an epi-fluorescent
119 microscope (Olympus BX- 50, Japan). Duplicate samples from each depth were counted at
120 1000× magnification from 20 randomly chosen fields (on an average, a minimum of 300 cells
121 were counted). For the enumeration of the total heterotrophic and autotrophic nanoflagellates
122 (HNF and ANF), 30 ml of epilimnion and metalimnion (5-20 m) and 50 ml of hypolimnion
123 (50 and 70 m) waters were filtered through polycarbonate membrane filters (pore size 0.8
124 μm, diameter 25 mm, Advantec) and stained with primulin (Caron 1983). HNF and ANF
125 cells were observed with an epi-fluorescent microscope under UV and green excitation,
126 respectively. For each sample, 100 fields were counted at 1000× magnification (on average, a
127 minimum of 100 cells were counted for HNF and simultaneously ANF cells were also
128 counted in each field).

129 *DNA extraction and Clone library analysis*

130 The samples for the clone libraries were collected in each season from the epilimnion (5 m or
131 10 m) to obtain the maximum abundance of nanoflagellates (Nagata 1988) and once from the
132 hypolimnion in August, 2013 (Table 1). Water samples were collected after pre-filtering with
133 a 20 μm plankton net, and 1-2 litre portions of the filtrate were filtered through polycarbonate
134 membrane filters (pore size 0.8 μm, diameter 47 mm, Costar) at low vacuum (5 cmHg) and
135 stored at -80°C. The DNA extraction was conducted using the Power Soil DNA Isolation Kit
136 (MO BIO Laboratories, Carlsbad, CA, USA). The eukaryotic 18S rRNA genes were
137 polymerase chain reaction (PCR) amplified by universal eukaryote primers EukA and EukB
138 (Medlin *et al.* 1988). PCRs were performed in 20 μl of reaction volume with Blend Taq PCR
139 kit (Toyobo, Osaka, Japan). The amplification was performed under the following conditions:
140 initial denaturation at 95°C for 2 min; 35 cycles (95°C for 30 s, 59.5 °C for 30 s, 72°C for 2
141 min); and final extension at 72°C for 7 min. Two separate clone libraries were constructed
142 from epilimnion and hypolimnion using kinetoplastid-specific primers kineto14F and

143 kineto2026R following the PCR condition mentioned by von der Heyden and Cavalier-Smith
144 (2005) to isolate kinetoplastid sequences (Table 1). The PCR products were purified with the
145 Exo-I and TSAP enzymes and cloned using the pT7 Blue Perfectly Blunt Cloning Kit
146 (Novagen, Madison, WI, USA) according to the manufacturer's instructions.

147 *Sequencing of clones and phylogenetic analysis*

148 The sequencing of clones was performed using the Euk 528F (Elwood *et al.* 1985) primer for
149 the V4 region (Lovejoy *et al.* 2006) and the BigDye Terminator v3.1 Cycle Sequencing Kit
150 (Applied Biosystems, Foster City, CA, USA) to obtain an 800-900 bp segment of the gene.
151 The sequencing was performed with an ABI Genetic Analyzer 3130xl Avant Capillary
152 automated sequencer. The quality of the sequences was assessed using the Sequencing
153 Analysis Software v5.3.1 (Applied Biosystem) and the sequences were manually corrected
154 and trimmed using the MEGA6 software (Tamura *et al.* 2013). The closest match to each
155 sequence was obtained from BLAST search (Altschul *et al.* 1997), and sequences whose
156 closest match was under 97% were checked for chimeras with additional BLASTs of several
157 sections of the sequence. Sequences were also checked for chimeras using KeyDNATools
158 (<http://KeyDNAtools.com>). Chimeras, low-quality sequences and sequences less than 500 bp
159 were excluded from further analysis. Sequences affiliated with eukaryotes other than
160 flagellates (e.g. Ciliophora, Fungi and Diatoms) were not included. The sequences were
161 aligned using the CLUSTAL W package (Thompson *et al.* 1994). The operational taxonomic
162 units (OTUs) were separated at 97% similarity, and the similarity matrix was calculated using
163 Bioedit (Hall 1999). The selected clones which represented one member of each flagellate
164 OTU were sequenced using the internal forward and reverse primers, 1055F (Holman *et al.*
165 2003) and D978 (Zimmermann *et al.* 2011) and EukB primer.

166 Kinetoplastid sequences obtained using kinetoplastid-specific primers from the
167 epilimnion and hypolimnion were closely related to *Rhynchomonas nasuta* and *Neobodo*
168 *designis* in the BLAST search, respectively. The phylogenetic analysis therefore was
169 conducted with closely related *Rhynchomonas* and *Neobodo* sequences available in the
170 GeneBank (<http://www.ncbi.nlm.nih.gov/>) and also with other members belonging to
171 Neobodonida (e.g. *Cruzella marina*, *Dimastigella trypaniformis*, and *Rhynchobodo* sp.) and
172 closely related bodonids. Six 18S rRNA gene sequences were retrieved from GeneBank
173 affiliated to slow-evolving kinetoplastids (*Ichthyobodo* and *Perkinsiella*-like sp.) and used as
174 outgroup (Moreira *et al.* 2004). Total 60 partial and full length sequences were aligned by
175 PRANK algorithm with Trust insertions (+F) (Löytynoja and Goldman 2008) from 100
176 alternative bootstrap alignments. Ambiguously aligned positions were masked from our
177 analysis using GUIDANCE (Penn *et al.* 2010) with specific residue cutoff value of 0.93.
178 Maximum likelihood (ML) tree was constructed using MEGA6 software based on Kimura 2-
179 Parameter model of nucleotide substitution with a six category discrete approximation of a Γ
180 distribution plus invariable sites (K2+ Γ + I model) (Kimura 1980). Model test confirmed that
181 K2+ Γ + I model was the most appropriate model for the given data. Initial trees for the
182 heuristic search were obtained by applying the Neighbor-Joining method to a matrix of
183 pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach.
184 ML trees were constructed from 1000 bootstrap replicates. To further confirm the results
185 obtained from the ML tree, Neighbor joining (NJ) analysis was performed using Maximum
186 Composite Likelihood model with a six category Γ distribution of rate variation among sites.
187 NJ trees were constructed from 2000 bootstrap replicates. The result of only ML tree was
188 discussed as the topology of consensus NJ tree was the same as ML tree.

189 *CARD-FISH*

190 Water samples were pre-filtered through a 20 μm mesh plankton net and fixed in a 2% final
191 concentration of formaldehyde (freshly prepared by filtering through 0.2 μm syringe filter)
192 for at least 3-4 hours before filtration. The nanoflagellate cells were harvested on
193 polycarbonate filters (pore size 0.8 μm , diameter 25 mm, Advantec); they were rinsed twice
194 with 1X PBS and twice with MilliQ water, air dried and frozen at -20°C until further
195 processing.

196 CARD- FISH was performed according to the protocol of Okazaki *et al.* (2013),
197 based on Pernthaler *et al.* (2004). Permeabilization with lysozyme or proteinase K was not
198 performed (Bochdansky and Huang 2010). The filters were embedded in 0.1% low-gelling-
199 point agarose and cut into eight sections, which were hybridized at 35°C for 12 hours with
200 $0.5 \mu\text{g ml}^{-1}$ concentration of probe and 30% concentration of formamide. A formamide
201 stringency test was performed with a gradient of formamide (0-55%) with 35°C and 45°C
202 temperatures, where optimal stringency was achieved at 35°C with 30% formamide. Optimal
203 stringency test was conducted using a cultured kinetoplastid sp. isolated from Lake Biwa and
204 also with hypolimnion samples from Lake Biwa. The probes, (Table 2) were purchased from
205 the Thermo Electron Co. (Ulm, Germany). Counting was performed using an Olympus BX50
206 epifluorescence microscope under $1000\times$ magnification at blue/UV excitation. For the
207 kinetoplastids, either 100 microscopic fields were counted, or when the densities were low,
208 the complete filter piece was screened per sample. To confirm the kinetoplastid cells, the
209 hybridized cells were counter checked under DAPI staining for the nucleus and kinetoplast
210 (Fig. 1). The total eukaryotes were counted simultaneously with the kinetoplastid cells by
211 DAPI staining under UV excitation.

212 *Statistics*

213 Spearman's rank correlation coefficient was calculated for testing the relationship between
214 abundance of kinetoplastids with environmental and biological parameters. Bonferroni's
215 correction was also conducted to correct the familywise error rate. The statistics were
216 computed in the R environment (www.r-project.org).

217

218 Results

219 *Physico-chemical and biological characteristics*

220 The water column was completely mixed from January to March with a mean temperature of
221 $7.6 \pm 0.5^\circ\text{C}$ (Fig. 2a). Stratification commenced in April, and the epilimnion water (5 m)
222 temperature varied widely from 11.6°C in April to a maximum of 29.1°C in August and
223 decreasing to 10.2°C in December. However, the hypolimnion water temperature remained
224 stable with a mean temperature of $7.6 \pm 0.2^\circ\text{C}$ throughout the study period (Fig. 2a).

225 The maximum chlorophyll *a* concentration ($31.1 \mu\text{g l}^{-1}$) was detected in May (5 m)
226 with an average concentration of $4.5 \pm 5.7 \mu\text{g l}^{-1}$ during the study period (Fig. 2b). High
227 values remained until July, decreased drastically in August and September and increased
228 slightly in October and November. However, the chlorophyll *a* concentration reduced from
229 December and remained low and uniform throughout the water column during winter. The
230 concentration of chlorophyll *a* always remained low in the hypolimnion, with the lowest
231 concentration ($0.3 \mu\text{g l}^{-1}$) in October (50 m). The bacterial abundance was high in the
232 epilimnion, with two peaks (maximum at 5 m in May and July with $4.6 \times 10^6 \text{ cells ml}^{-1}$ and
233 $4.8 \times 10^6 \text{ cells ml}^{-1}$, respectively) with an average concentration of $2.5 \pm 0.9 \times 10^6 \text{ cells ml}^{-1}$
234 during the study period (Fig. 2c). The abundance of bacteria was relatively low in the

235 hypolimnion, and the lowest densities were detected from 50 m. Low and uniform bacterial
236 abundance was found throughout the water column during the winter mixing period.

237 *HNF and ANF abundance*

238 HNF were abundant in the epilimnion and metalimnion during the stratified period, and a
239 high abundance of HNF fluctuated vertically, depending on the seasons. The maximum
240 abundance was found at 5 m in May with 36.9×10^3 cells ml⁻¹ with an average concentration
241 of $7.1 \pm 5.2 \times 10^3$ cells ml⁻¹ throughout the study period (Fig. 2d). The high abundance of HNF
242 fluctuated at 5 m in May and June, which shifted to 10 m and 15 m during July to September
243 and shifted back to 5 m in October and November. Similarly, the peak of ANF abundance
244 was found fluctuating between 5 m to 15 m during May to November. Maximum abundance
245 of ANF was found at 5 m in September and 20 m in December, with 2.8×10^3 cells ml⁻¹ and
246 an average concentration of $1.0 \pm 0.8 \times 10^3$ cells ml⁻¹ (Fig. 2e). Similar to the bacteria, the
247 abundance of both ANF and HNF were low in the hypolimnion. During the winter, the HNF
248 density was relatively low, whereas high densities of ANF were found throughout the water
249 column.

250 *Clone library*

251 The five clone libraries from the epilimnion representing four seasons, and the hypolimnion
252 yielded 403 clones (Table 1) where flagellate groups contributed to 84 unique OTUs.
253 Cryptophyta had the highest diversity in the epilimnion nanoflagellate communities, with
254 their highest contribution in May which accounted for 57% of the total OTUs (Fig. 3). Other
255 groups obtained from the epilimnion libraries with significant contribution were Perkinsozoa
256 and Cercozoa. Dinophyta were the most diverse group in the hypolimnion library and
257 contributed to 34% of the total OTUs followed by Cercozoa, Bicosoecida and Chrysophyta.

258 The OTUs of various phylogenetic groups of nanoflagellates were detected including
259 singletons. These singletons most likely represented rare taxa, whereas no sequence affiliated
260 with kinetoplastid flagellates was obtained from the libraries. However, both epilimnion and
261 hypolimnion libraries with kinetoplastid-specific primers yielded kinetoplastid sequences.
262 The epilimnion library with kinetoplastid specific primers yielded four kinetoplastid
263 sequences (Table 1) which belonged to one OTU and having the closest match with
264 *Rhynchomonas nasuta* in the BLAST search. Whereas, hypolimnion library with
265 kinetoplastid-specific primers yielded six kinetoplastid sequences (Table 1) which belonged
266 to two OTUs and had the closest match with *Neobodo designis*. The phylogenetic analysis
267 revealed that kinetoplastid from the epilimnion of Lake Biwa is related to *R. nasuta* (Clade 2,
268 Fig. 4), having closest similarity with a *R. nasuta* strain from deep sea sediment with a low
269 bootstrap support. The phylogenetic analysis of kinetoplastids from the hypolimnion of Lake
270 Biwa revealed that both the OTUs were closely related to each other with 79% bootstrap
271 support and were included in a clade (Clade 1) with *N. designis* sequences only from
272 freshwater environment (Fig. 4). Nevertheless, kinetoplastids from the hypolimnion of Lake
273 Biwa were separated from other closely related sequences in the clade with a high bootstrap
274 support.

275 *Dynamics of kinetoplastids*

276 The CARD-FISH positive kinetoplastid cells were detected throughout the year (Fig. 5a),
277 although, at some depths they were not detected during winter and spring. Kinetoplastid
278 abundance was low in the epilimnion in April and May, despite the high chlorophyll *a*
279 concentrations and high abundance of bacteria and HNF (Fig. 2). Statistical tests indicated
280 that kinetoplastid abundance was significantly correlated ($p < 0.001$) only with chlorophyll *a*
281 concentration (Table 3). The abundance of kinetoplastids increased in the epilimnion during
282 the summer, with relatively high values from June to December (Fig. 5a). Two peaks of high

283 abundance were found, one during July and August and the other during November and
284 December. The highest abundance of kinetoplastids in the epilimnion was recorded in
285 December (10 m) with 9.3×10^2 cells ml^{-1} , which accounted for 11.9% of the total eukaryotes
286 (Fig. 5b) and 12.1% of the total flagellates (HNF+ANF) (Fig. 5c). The kinetoplastid cell
287 density was low during winter mixing throughout the water column. Interestingly, in the
288 hypolimnion, we found that kinetoplastids were the dominant nanoplankton during summer
289 and autumn (Fig. 5a). The abundance of kinetoplastids in the hypolimnion increased from
290 June, with peak abundances observed in August and September. Their abundance remained
291 high until December in both 50 m and 70 m with the maximum cell numbers at 70 m in
292 August (1.2×10^3 cells ml^{-1}). The percentage contribution of the kinetoplastids in the
293 hypolimnion in August (2012) accounted for 31.6% and 36.0% of the total eukaryotes (Fig.
294 5b) and 41.8% and 43.7% of the total flagellates (Fig. 5c) at 50 m and 70 m, respectively.
295 The hypolimnion library in August (2013) by the universal eukaryote primers did not detect
296 any kinetoplastid sequences, however, the percentage contribution of CARD-FISH positive
297 kinetoplastids was 47.8% and 53.6% of the total eukaryotes at 50 m and 70 m respectively
298 (data not shown), which was even higher than their contribution in the previous year (2012).

299

300

Discussion

301 We found a high abundance of HNF in the upper layers of Lake Biwa during the study period
302 with two peaks in early summer and autumn with the highest abundance in early summer (Fig.
303 2d) which corroborates with the other studies in HNF (Nagata 1988; Auer and Arndt 2001;
304 Auer *et al.* 2004; Mangot *et al.* 2009). Abundance of HNF during early summer in the
305 present study was higher than the HNF abundance in German mesotrophic lakes (Auer and
306 Arndt 2001), but was in the similar range of that in French oligomesotrophic and mesotrophic

307 lakes (Lepère *et al.* 2010). Abundance of kinetoplastids, on the other hand increased in the
308 epilimnion during late summer and autumn (Fig. 5), and this corroborates with the study in
309 German lakes (Auer and Arndt 2001).

310 *Dominance of kinetoplastid flagellates in deeper waters of Lake Biwa*

311 Kinetoplastids are considered less important in their contribution to total HNF (Arndt
312 *et al.* 2000) and in oligomesotrophic Lake Mondsee, kinetoplastids and other flagellates were
313 found to reduce at greater depths (Salbrechter and Arndt 1994). By contrast, in Lake Biwa,
314 kinetoplastids were found to be the dominant member of HNF community in the hypolimnion
315 and were also present throughout the year in the water column (Fig. 5c). The dominance of
316 kinetoplastids in deeper layers indicated their importance in the hypolimnion HNF
317 community characterized by a low abundance of flagellates. Thus, kinetoplastids play a major
318 role in the matter cycling of Lake Biwa during the summer stratification because the
319 dominant taxa have the major contribution to the ecosystem's function (Scheckenbach *et al.*
320 2010).

321 A flagellate identified as *Phyllomitus apiculatus* (Cryptophyceae) was found to
322 dominate in the hypolimnion of some deep lakes to avoid zooplankton grazing and their
323 maximum densities were observed just after dense diatom populations (Steinberg *et al.* 1983).
324 The dynamics of the kinetoplastids in Lake Biwa were similar to the dynamics of *P.*
325 *apiculatus*, since they were dominant in the hypolimnion in the summer and their abundance
326 in the epilimnion increased in August and December, immediately after the chlorophyll *a*
327 concentration was reduced. The taxonomic affinities of organisms assigned to the genus
328 *Phyllomitus* are complex (Lee 2002), however the *P. apiculatus* reported by Steinberg *et al.*
329 (1983) resembles *Rhynchobodo spp.*, which belongs to the class Kinetoplastidae (Vickerman
330 2000; Lee 2002). Therefore, the study by Steinberg *et al.* (1983) might be the first report of

331 the dominance of kinetoplastid flagellates in the hypolimnion of deep lakes. The kinetoplastid
332 sequences obtained from the hypolimnion of Lake Biwa had the closest match to *Neobodo*
333 *designis* in BLAST search. According to the updated kinetoplastid phylogeny (Moreira *et al.*
334 2004), both *N. designis* and *Rhynchobodo* belongs to the same order Neobodonida. No
335 sequence is available for the reported organism by Steinberg *et al.* (1983), therefore
336 phylogenetic analysis or comparison with Lake Biwa kinetoplastid is not possible, but both
337 belonging to same species or are a close relative cannot be ruled out.

338 In the phylogenetic analysis, kinetoplastids from the hypolimnion of Lake Biwa
339 belonged to a *N. designis* clade (Clade 1) that consisted of sequences from freshwater and
340 mainly from freshwater sediments (Fig. 4). Nevertheless, Lake Biwa kinetoplastids branched
341 from other members of the cluster with high bootstrap support. The results indicate novelty of
342 the Lake Biwa kinetoplastid sub-cluster due to little information known about Neobodonid
343 diversity and low amount of research conducted on free living kinetoplastids in limnetic
344 ecosystems. However, *Neobodo* clade is a newly created group of kinetoplastids from which
345 was earlier considered as members of *Bodo* (Moreira *et al.* 2004). *Neobodo* clade is
346 complicated with many subclades (von der Heyden *et al.* 2004), and clear demarcation of its
347 members is not available. It is therefore not possible to assign a specific taxonomic affinity to
348 the Lake Biwa kinetoplastids.

349 Kinetoplastids had an inverse relationship with temperature during stratification (Fig.
350 2a, 5a), due to the dominance in the hypolimnion with low and stable water temperature
351 (7°C). Thus, low water temperature may be favorable for the growth of kinetoplastids.
352 However, the dominance of kinetoplastids was not observed in the water column with low
353 water temperature during winter mixing period, therefore we could not find any significant
354 relationship with kinetoplastid abundance and temperature (Table 3). Hence, only
355 temperature cannot explain the dominance of kinetoplastids in the hypolimnion. On the other

356 hand, Salani *et al.* (2012) have reported that free-living kinetoplastids especially *Neobodo*
357 and *Rhynchomonas* were found to be abundant in ocean abyssal depths characterized by
358 permanent stratification with constant and homogenous environment. *N. designis* is one of the
359 most ubiquitous freshwater flagellates (Patterson and Lee 2000) and the majority of *N.*
360 *designis* strains are not tolerant to a wide variety of environmental conditions (von der
361 Heyden and Cavalier-Smith 2005). Thus, in the present study, the hypolimnion during the
362 stratification period having constant and homogenous environment might be the reason for
363 the dominance of these flagellates.

364 The bacterial group CL500-11 (phylum *Chloroflexi*) is the dominant bacteria in the
365 hypolimnion of Lake Biwa (Okazaki *et al.* 2013). The dynamics of CL500-11 in the
366 hypolimnion were similar to the dynamics of kinetoplastids which suggested that similar
367 environmental conditions may favor the growth of kinetoplastids and CL500-11 in Lake
368 Biwa. Large size and curved shape of CL500-11 bacteria might protect them from grazing
369 and thus allow this group of bacteria to dominate in the hypolimnion of Lake Biwa. However,
370 some flagellates, especially bodonids are capable of feeding on large bacteria (Gonzalez *et al.*
371 1990; Šimek and Chrzanowski 1992; Posch *et al.* 1999). Therefore kinetoplastids might have
372 an advantage over other flagellates in the hypolimnion due to their ability to feed on large
373 bacteria dominant in the deeper layers of Lake Biwa.

374 Another possible explanation for the dominance of kinetoplastids is their grazing on
375 deposited bacteria. Previous studies demonstrated that *Bodo saliens* (probably *N. designis* or
376 a close relative belonging to the order Neobodonida) grew on deposited rather than
377 suspended bacteria in laboratory systems (Zubkov and Sleigh 2000). Kinetoplastids are
378 known to feed on attached bacteria (Caron 1987), inhabiting on macroaggregates such as
379 *Rhizosolenia* mats and marine snow (Caron *et al.* 1982; Artolozaga *et al.* 2000). In the
380 present study, dominance of kinetoplastids after the decrease in the chlorophyll *a*

381 concentration (Fig. 2b, 5) might be due to their increased abundance in detritus. This point is
382 further clarified by the fact that kinetoplastids had only significant negative correlation with
383 chlorophyll *a* concentration (Table 3). In the present study, the kinetoplastids, *R. nasuta* and
384 *N. designis* identified from the epilimnion and hypolimnion respectively are known to be
385 poor swimmers and glide over surfaces (Boenik and Arndt 2000). Therefore increased
386 abundance of kinetoplastids in the hypolimnion water might be due to their growth in the
387 associated detritus sinking from the epilimnion. Thus, more studies are needed to understand
388 the factors responsible for the dominance of kinetoplastids and whether kinetoplastid
389 flagellates prefer the oxygenated hypolimnion of deep lakes.

390 *Kinetoplastids: a rare or an underestimated group of flagellates?*

391 The sequences affiliated with the kinetoplastid flagellate were not detected in clone libraries
392 using universal eukaryote primers, whereas kinetoplastid sequences were detected using
393 kinetoplastid-specific primers from both epilimnion and hypolimnion. The CARD-FISH
394 analysis demonstrated that kinetoplastids were present in all of the seasons and even
395 dominant on some occasions (Fig. 5a). Kinetoplastids were also not detected in the
396 hypolimnion library with universal eukaryote primers (Figure 3) where the abundance of
397 kinetoplastids was high (Figure 5a). These results supported our hypothesis that kinetoplastid
398 flagellates were underestimated when using universal eukaryote primers.

399 A study conducted in the groundwater of a karstified aquifer found several
400 kinetoplastid flagellates by microscopic observation whereas no sequences were detected
401 using universal eukaryote primers (Risse-Buhl *et al.* 2013). Kinetoplastids are also not
402 detected by commonly used universal eukaryote FISH probes (Bochdansky and Huang 2010)
403 due to the divergent nature of their 18S rRNA (Simpson *et al.* 2006). Thus, divergent nature
404 of the 18S rRNA gene might be the possible reason for underestimation of kinetoplastids in
405 the clone libraries when using the universal eukaryotic primers.

406 The high abundance of kinetoplastids was reported from the Atlantic Ocean using
407 kinetoplastid-specific FISH probes (Morgan-Smith *et al.* 2011, 2013). Similarly, a high
408 diversity of kinetoplastid flagellates was reported from various aquatic environments using
409 group-specific primers (von der Heyden and Cavalier-Smith 2005; Rasmussen *et al.* 2001,
410 Edgcomb *et al.* 2011b). However, diversity studies using universal eukaryote primers rarely
411 reported kinetoplastid sequences from marine environments (Stoeck *et al.* 2010; Logares *et*
412 *al.* 2012). Thus, our results suggested that kinetoplastids might have been overlooked in
413 diversity studies in other freshwater or marine environments using universal eukaryote
414 primers, thereby underestimating their importance in the aquatic food web.

415

416 *Conclusion*

417 This study showed that kinetoplastids are an important member of nanoflagellate community
418 in Lake Biwa. We found that these flagellates comprised the major component of microbial
419 eukaryote community in the hypolimnion. Previously, only one study reported dominance of
420 kinetoplastid-like flagellates in some Austrian lakes. A possible reason for the lack of
421 information about the kinetoplastids is the underestimation of their abundance in molecular
422 studies when using the universal eukaryote primers, due to the divergent nature of their 18S
423 rRNA gene. Another reason for limited information is due to lack of studies in oxygenated
424 hypolimnion of deep lakes and this is the first study to reveal the seasonal and vertical
425 dynamics of a particular flagellate group in a lake using CARD-FISH. Kinetoplastids may
426 dominate in oxygenated hypolimnion of other deep lakes in common with previously
427 reported bacterioplankton CL500-11. Studies from other deep lakes and experiments are
428 necessary to identify the factors responsible for their dominance and to understand their role
429 in the ecosystem.

430

431

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438

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445

446

Conflict of Interest Statement

447 The authors declare no conflict of interest.

448

449

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651

651 Table 1 Number of sequences analysed and flagellate OTUs obtained from each library

| Season and year | Depth | Target group | No. of sequences | Flagellate |
|------------------------|-------|----------------|------------------|------------|
| | | | analysed | obtained |
| Spring (May) 2012 | 5m | eukaryotes | 72 | 32 |
| Summer (August) 2012 | 10m | eukaryotes | 151 | 47 |
| Autumn (October) 2012 | 5m | eukaryotes | 79 | 30 |
| Winter (December) 2012 | 10m | eukaryotes | 30 | 11 |
| Summer (August) 2013 | 50m | eukaryotes | 71 | 30 |
| Spring (May) 2012 | 5m | kinetoplastids | 4 | 1 |
| Summer (August) 2013 | 50m | kinetoplastids | 6 | 2 |

652

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653

654 Table 2 CARD-FISH probes used in the present study

| FISH probes | Sequence (5' to 3') | Position* | Target group | References |
|-------------|---------------------|-----------|----------------|------------------------------|
| KIN 516 | ACCAGACTTGTCCTCC | 502-517 | kinetoplastids | Bochdansky and Huang 2010 |
| EUK 516 | ACCAGACTTGCCCTCC | 502-517 | eukaryotes | Bochdansky and Huang 2010 |
| Competitor | | | | Huang 2010 |
| NON 338 | ACTCCTACGGGAGGC | – | – | Wallner <i>et al.</i> 1993 |

655 * positions refer to the 18S rRNA gene of *S. cerevisiae*.

656

656

657 Table 3 Results of Spearman's rank correlation test between abundance of kinetoplastids with
658 environmental and biological parameters. Significant probability is 0.01 after Bonferroni's
659 correction (n=78).

| | Temp. | Chl <i>a</i> | Bacteria | HNF | ANF |
|---------------------------------|-------|--------------|----------|-------|-------|
| ρ (<i>rho</i>) | 0.15 | -0.37 | -0.18 | -0.13 | -0.06 |
| <i>p</i> (<i>probability</i>) | 0.198 | <0.001 | 0.108 | 0.244 | 0.615 |

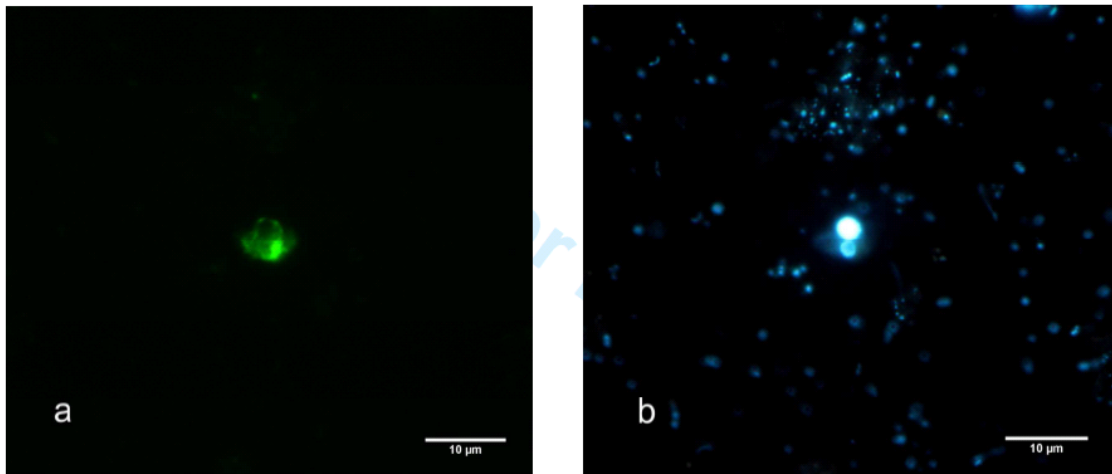
660

661

661

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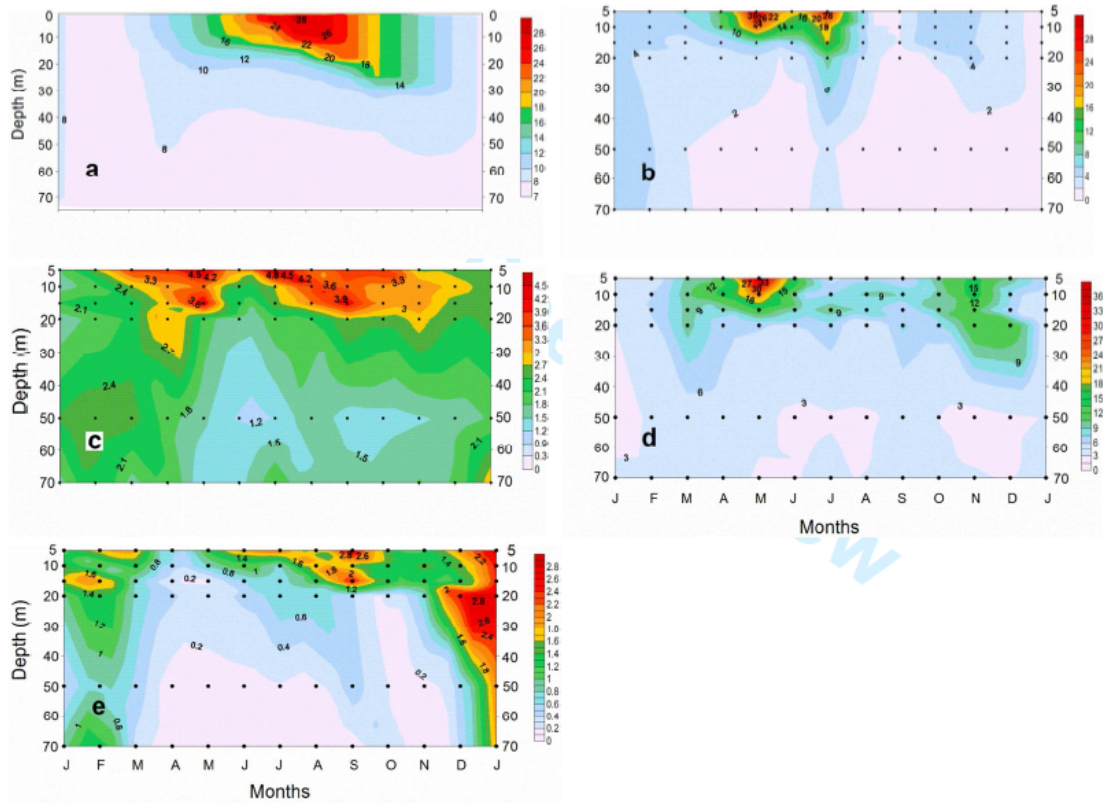
Titles and legend to figures



663

664 **Figure 1:** A kinetoplastid cell of the same microscopic field as seen under (a) blue excitation
665 with CARD-FISH probes (KIN516 and Euk516 competitor) and (b) UV excitation showing
666 nucleus and kinetoplast by DAPI staining. Both the images correspond to the same
667 microscopic field taken with an attached camera at 1000× magnification. Contrast of the
668 photos has been increased from the original microscopic image.

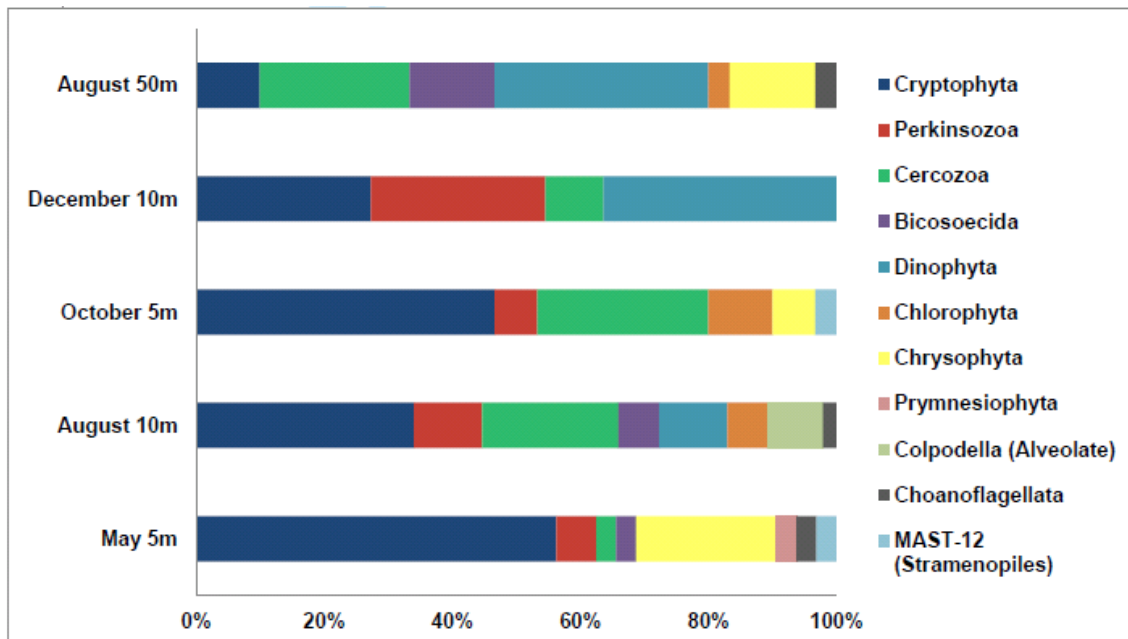
669



669

670 **Figure 2:** Seasonal changes in vertical distribution of (a) temperature ($^{\circ}\text{C}$), (b) chlorophyll *a*
 671 ($\mu\text{g l}^{-1}$), (c) bacterial abundance ($10^6 \text{ cells ml}^{-1}$), (d) total HNF (heterotrophic nanoflagellate)
 672 abundance ($10^3 \text{ cells ml}^{-1}$) and (e) total ANF (autotrophic nanoflagellate) abundance (10^3
 673 cells ml^{-1}). Black dots represent sampling depth.

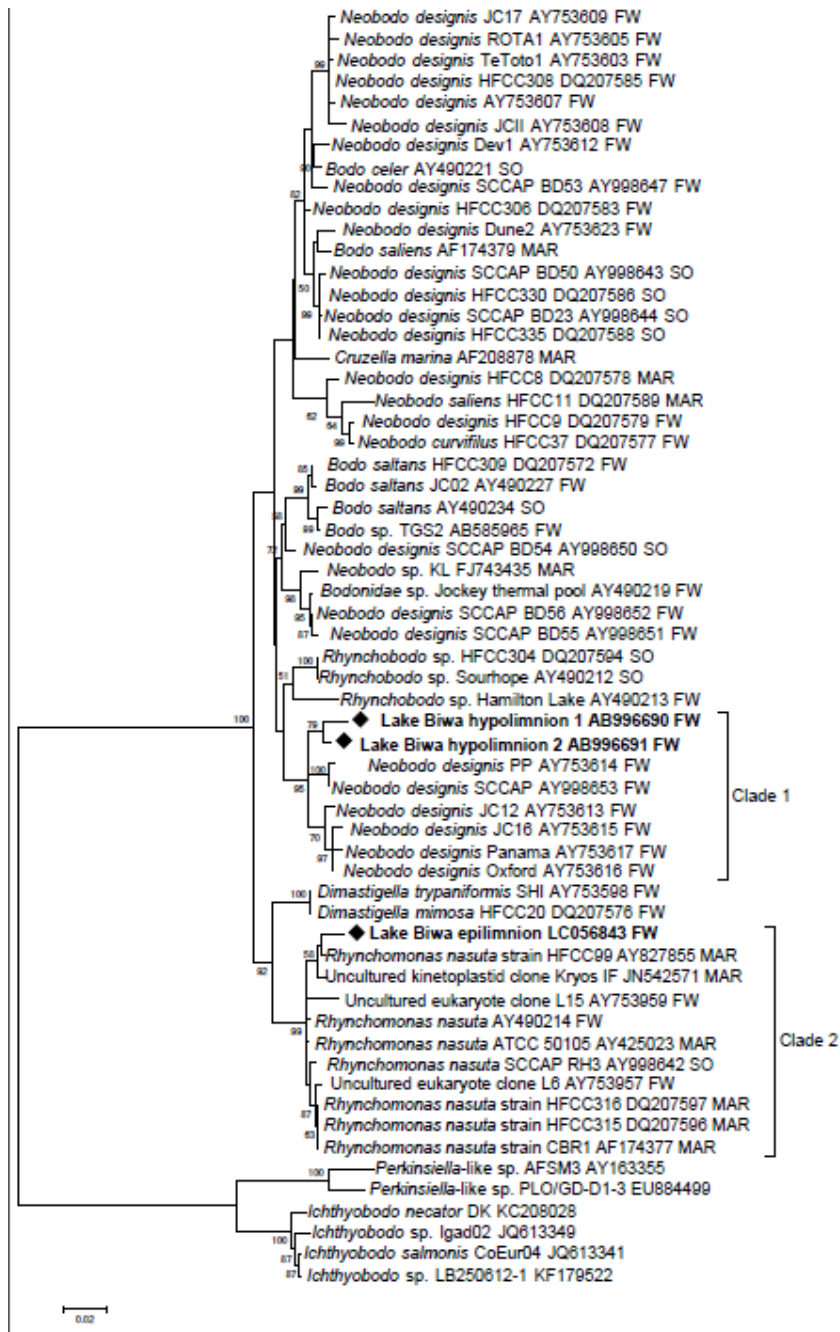
674



674

675 **Figure 3:** Seasonal changes in nanoflagellate communities by 18S rRNA gene clone library
 676 analysis based on the OTU abundance of each group. Sequences affiliated with eukaryotes
 677 other than flagellates (e.g., Ciliophora, Fungi and Diatoms) obtained in clone libraries were
 678 not included in the analysis. x axis represent group percentage of the total OTUs per sample
 679 and y axis represent the month and water depth of sample collection.

680

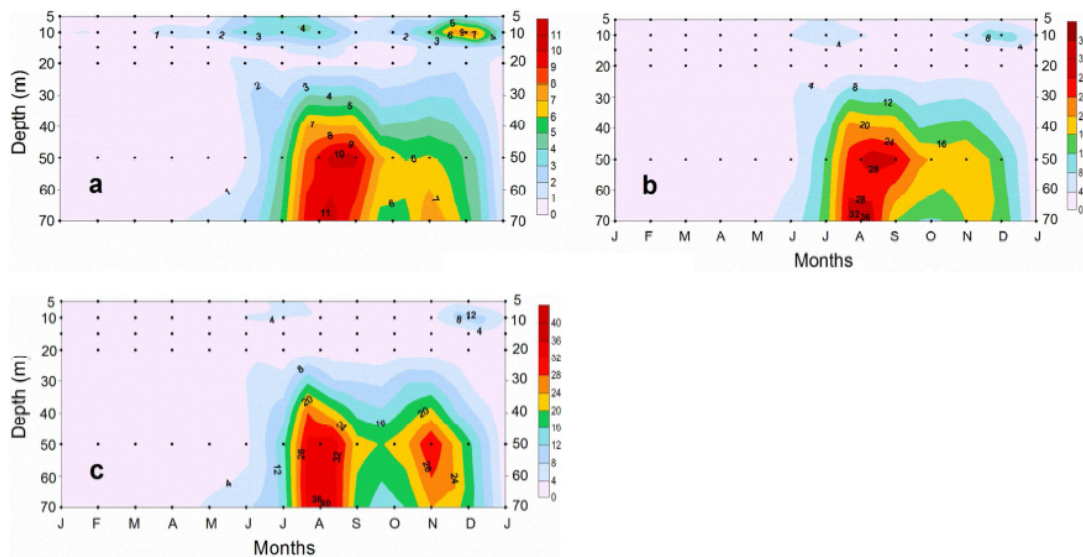


680

681 **Figure 4:** 18S rRNA gene phylogenetic tree of Lake Biwa kinetoplastid flagellates from the
 682 epilimnion and hypolimnion with 54 sequences of neobodonids and closely related bodonids.
 683 6 sequences belonging to slow-evolving kinetoplastids were used as outgroup. Kinetoplastid
 684 sequences obtained from this study are highlighted in bold and marked with black diamond at
 685 the external nodes. Maximum likelihood tree was calculated using K2+ Γ + I model with 1000

686 bootstrap replicates. The bootstrap replicate support percentages > 50% is shown next to the
687 branches. Clades containing kinetoplastids from hypolimnion and epilimnion of Lake Biwa
688 are named as Clade 1 and 2 respectively. Source of each sequence is indicated by labels at the
689 end of their name and accession number (FW- freshwater, MAR- marine and SO- soil). Scale
690 bar = 0.02 substitution per site.

691



691

692 **Figure 5:** (a) Abundance of kinetoplastid cells targeted by KIN516 probe (10^2 cells ml^{-1}), (b)
 693 percentage of kinetoplastids to total eukaryotes, (c) percentage of kinetoplastids to total
 694 flagellates (HNF+ANF). Black dots represent sampling depth.

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