

1 **Title: Identification of species and genotypic compositions of *Cryptomonas* (Cryptophyceae)**  
2 **populations in the eutrophic Lake Hira, Japan, using single-cell PCR**

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20

1 **Abstract**

2 Single-cell PCR and gene sequencing were conducted to evaluate species and genotypic compositions of  
3 *Cryptomonas* in the eutrophic Lake Hira, Japan. We determined the sequences of nuclear internal  
4 transcribed spacer 2 region from single *Cryptomonas* cells with a high success rate (83.3-97.9%),  
5 excluding one case (56.3%). A total of 325 sequences were obtained over 8 sampling days from May 28  
6 to October 3 2012, and phylogenetic analysis indicated that all sequences were divided into six groups.  
7 Four groups were clustered together with known sequences of *C. curvata*, *C. marssonii*, *C. pyrenoidifera*  
8 or *C. tetrapyrenoidosa*, although the sequences of the other two groups did not show high similarity to  
9 known *Cryptomonas* species. *Cryptomonas curvata* dominated during the study period (45-98%), and  
10 unidentified *Cryptomonas* species (group 2) became dominant at high water temperatures. The genotypic  
11 composition of *C. curvata* also varied temporarily, suggesting that the genotypic composition of  
12 *Cryptomonas* was susceptible to environmental changes. These results indicated that single-cell PCR can  
13 be used to analyze the species composition and ecology of *Cryptomonas*.

14  
15 **Key words:**

16 *Cryptomonas*, nuclear internal transcribed spacer 2 (ITS2), single-cell PCR, genotypic composition  
17

## Introduction

1  
2 Microorganisms have four types of nutritional modes: heterotrophy, autotrophy, photoheterotrophy and  
3 mixotrophy. Based on specific nutritional strategies and evolutionary interests of mixotrophic algae,  
4 previous studies have explored their population, quantitative ecological role and life history traits in  
5 microbial communities (Porter 1988; Sanders and Porter 1988; Sanders 1991; Holen and Boraas, 1995).  
6 The genus *Cryptomonas* Ehrenberg (Ehrenberg 1831) is one of the most common mixotrophs that is  
7 ubiquitously distributed in lakes and ponds (Rott 1988; Menezes and Novarino 2003; Carino and Zingone  
8 2006). Many ecological studies have been performed on *Cryptomonas* and have demonstrated that  
9 *Cryptomonas* plays an important role in aquatic ecosystems, such as periodical dominance (Graham et al.  
10 2004), and acts as a grazer of bacterioplankton (Urabe et al. 2000) and prey of zooplankton (Porter 1973).  
11 However, the majority of studies have evaluated the dynamics of *Cryptomonas* populations at the genus  
12 level, and less information is available on species composition and ecological features of each species.

13 Classification criteria for *Cryptomonas* had been based on morphological features such as cell  
14 size, cell shape, furrow/gullet system, periplast structure, number of pyrenoids and internal anatomy  
15 (Novarino and Lucas 1993; Clay et al. 1999; Deane et al. 2002). However, previous molecular  
16 phylogenetic studies have clarified complex divergences in *Cryptomonas* and incongruence between  
17 morphological and phylogenetic classifications (Hoef-Emden and Melkonian 2003; Hoef-Emden 2005;  
18 Hoef-Emden 2007). Moreover, some *Cryptomonas* species show dimorphism (cryptomorph and  
19 campylomorph) or morphological plasticity and cannot be distinguished based on light microscopic  
20 observation alone (Hoef-Emden and Melkonian 2003; Hoef-Emden 2007). Therefore, molecular analysis

1 should be conducted to identify *Cryptomonas* species.

2 To evaluate the microorganism species composition, PCR-based molecular analyses, such as  
3 clone library analysis and denaturing gradient gel electrophoresis, have been applied. However, the  
4 quantitative accuracy of this data may be low since these results are biased by inhomogeneous  
5 amplification of PCR (von Wintzingerode et al. 1997). On the other hand, single-cell PCR methods  
6 without any bias for quantification of the microbial community have also been developed (Ki et al. 2005;  
7 Auinger et al. 2008; Bachvaroff et al. 2012). Nuclear internal transcribed spacer 2 region (ITS2) is  
8 variable and used for phylogenetic analysis and the identification of potential biological species for some  
9 lineages (e.g., Coleman 2000), though the plants and fungi have relatively conserved ITS2 regions  
10 (Caisová et al. 2013; Caisová and Melkonian 2014). The nuclear ITS2 region is also used for  
11 phylogenetic analysis of the genus *Cryptomonas* and reported that the high variabilities among the clades  
12 are identical within putative biological species (Hoef-Emden and Melkonian 2003). Although the ITS2  
13 region seems not to be enough resolution for analyzing phylogenetic relationships among the species  
14 correctly (Hoef-Emden and Melkonian 2003; Hoef-Emden 2007), it has sufficient information to identify  
15 *Cryptomonas* at species level. Therefore, single-cell PCR targeting the nuclear ITS2 region seems to be  
16 the one of useful methods for evaluating species composition within the *Cryptomonas* community.

17 In the present study, we evaluated species/genotype compositions of *Cryptomonas* community  
18 in eutrophic Lake Hira, Japan, using a single-cell PCR method. We seasonally monitored nuclear ITS2  
19 sequences from each *Cryptomonas* cell directly isolated from lake water and evaluated temporal  
20 variations in species compositions of *Cryptomonas*, as well as the genotypic compositions of each species

1 during the study period. These results, together with the biological and chemical factors that are thought  
2 to affect *Cryptomonas* populations, were used to explore the environmental factors that affect species and  
3 genotype compositions.

4

5

## Materials and methods

### 6 Study site and sampling procedure

7 Lake Hira (35°2' 57.12"N, 135°55'22.73"E) is a eutrophic satellite lake located on the eastern shore of  
8 Lake Biwa in Shiga prefecture, western Japan (Supplement a). The surface area and average depth are  
9 0.134 km<sup>2</sup> and 1.5 m, respectively. Lake Hira connects to Lake Yanagihira via a small canal (Canal 1 in  
10 Supplement b) approximately 100 m long. The water from Lake Yanagihira flows into Lake Hira and  
11 finally into Lake Biwa through canal 2 (Supplement b). The water from Lake Biwa rarely flows into Lake  
12 Hira, even when the water level of Lake Biwa is higher than that of Lake Hira.

13 Samples from Lake Hira were collected semimonthly from 28 May 2012 to 19 November 2012.

14 Because of the small area and the shallow depth, we supposed that Lake Hira is a typical continuous  
15 warm polymictic lake (Lewis 1983) and therefore collected the sample from the surface water. Surface  
16 water temperature was measured using a bar thermometer on each sampling day. Two liters of surface  
17 water were collected with a bucket and stored in a 4°C cooler box until the sample treatment.

18

### 19 Environmental factors

20 All treatments for chemical and biological analyses of the water samples were processed within 3 hours

1 after sample collection. Aliquots of water samples were filtered through pre-combusted Whatman GF/F  
2 filters (3 h at 420°C), and the filtrates were used to analyze dissolved inorganic nitrogen (DIN) and  
3 phosphorus (DIP). Nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ) and phosphate ( $\text{PO}_4^{3-}$ ). These concentrations were  
4 measured using colorimetric analysis with an AACS II Auto Analyser (Bran+Luebbe, Norderstedt,  
5 Germany). The ammonium concentration was determined fluorometrically (Holms et al. 1999). For  
6 chlorophyll *a* (Chl. *a*) concentrations, seston in 10 ml of water were collected onto glass microfiber filters  
7 (Whatman® GF/F, GE Healthcare UK Ltd.) and stored at -30°C. Chlorophyll *a* concentrations were  
8 determined using the method of Welschmeyer (1994) with a spectrofluorophotometer (RF-5300,  
9 Shimadzu Corporation, Kyoto, Japan) after extraction with 10 mL of N,N dimethylformamide (Suzuki  
10 and Ishimaru 1990). We also measured daily precipitation at the Otsu station (Fig. 1) based on weather  
11 static information from the Japan Meteorological Agency (Japan Meteorological Agency 2013) and  
12 evaluated the effect of precipitation on the *Cryptomonas* community during the study period.

13

#### 14 **Estimation of *Cryptomonas* cell density**

15 To estimate *Cryptomonas* cell density, 100 ml water samples were preserved in 1% glutaraldehyde. All  
16 *Cryptomonas*-like cells with orange fluorescence derived from phycobiliproteins under green excitation  
17 light were enumerated using a fluorescent microscope (BX50F4, OLYMPUS, Japan). At least 200 fields  
18 were counted on each slide at 400× magnification for three replications, and the density was calculated  
19 from the average cell number.

20

## 1 **Single cell PCR and sequence analysis**

2 We analyzed pigment-containing *Cryptomonas*-like (cryptomorph and campylomorph) cells, and did not  
3 evaluate colorless cells, including *C. paramaecium* (synonym: *Chilomonas paramaecium*). A total of 36  
4 to 48 cells were isolated for each sample using a narrow-width glass capillary under a microscope, and  
5 cells were washed three to five times in sterilized water and put into 0.2-mL PCR tubes containing 20  $\mu$ L  
6 of 10% Chelex® (Bio-Rad Laboratories, Hercules, California, USA) solution. The tubes were incubated  
7 at 95°C for 20 min. Since Chelex® solution inhibits PCR amplification, 10  $\mu$ L of each supernatant was  
8 collected from extracted samples and used for template DNA of PCR amplification.

9         The ITS2 region of *Cryptomonas* is variable enough for species identification (Hoef-Emden  
10 and Melkonian 2003), and we designed a new primer set for obtaining the PCR product of the ITS2  
11 region efficiently. To design a new primer set, a total of 31 *Cryptomonas* sequences were selected from  
12 Hoef-Emden and Melkonian (2003) and Hoef-Emden (2005) (Accession No., AJ566140, AJ  
13 566142-566153, AJ566155-566157, AJ566159-566170, AJ715444 and AJ715454-715455, Fig. 2), and  
14 aligned using MEGA 5 (Tamura et al. 2011). The end region of 5.8S ribosomal DNA and the beginning  
15 region of 28S ribosomal DNA of all reference sequences were examined and the new primer set (Forward  
16 primer : 5'-GCACGCCTGTTTGAGGRA-3') and (Reverse primer :  
17 5'-TGCTTAAGTTCAGCGGGTAG-3') was designed using Primer 3 Plus (Untergasser et al. 2007).

18         PCR amplification was performed with a 20- $\mu$ L PCR mixture containing 1.0- $\mu$ L template DNA  
19 from supernatant liquids of Chelex® extractions, 2  $\mu$ L of  $\times 10$  Buffer for Blend Taq with 20 mM of Mg<sup>2+</sup>  
20 (TOYOBO Co. Ltd., Osaka, Japan), 2 mM of each dNTP, 2.5 U Blend Taq-Plus- DNA polymerase

1 (TOYOBO Co. Ltd., Osaka, Japan) and 0.2  $\mu$ L of each primer (0.2 mM). After pre-incubation at 94°C for  
2 3 min, 35 cycles were performed at 94°C for 30 s, 56.6°C for 30 s and 72°C for 2 min, followed by a final  
3 extension at 72°C for 5 min. PCR products were purified using the QIAquick PCR Purification Kit  
4 (Qiagen, Valencia, California, USA) and sequenced using a Big Dye Terminator ver. 3.1 Cycle  
5 Sequencing Kit (Applied Biosystems (Life Technologies Japan Ltd., Tokyo, Japan)) using an ABI3130xl  
6 Genetic Analyzer (Applied Biosystems (Life Technologies Japan Ltd., Tokyo, Japan)).

7

#### 8 **Alignment of ITS2 region and phylogenetic analysis**

9 The obtained nuclear ITS2 alignments were folded using the mfold web server (Zuker 2003) for RNA  
10 secondary structure. The partial ITS2 region, mainly consisting helix domain III, was used in the unrooted  
11 phylogenetic analysis (cf, Hoef-Emden 2007). To infer helix domain III region in our obtained ITS2  
12 sequences, we firstly made secondary structure prediction of the ITS2 region in all our sequences. We  
13 then compared our graphs with that of *C. obovoidea*, *C. erosa*, *C. commutata*, *C. loricata* and *C.*  
14 *phaseolus* (Hoef-Emden 2007) and determined each helix III regions. Other known *Cryptomonas* species  
15 were referenced from Hoef-Emden (2007), DDBJ (DNA database of Japan) and BLAST search. The  
16 alignments were refined by eye and non-alignable regions were excluded prior to analysis. The 157  
17 positions were used for the analysis.

18 Because ITS2 region is hyper variable and cannot be properly aligned among Cryptophyto  
19 sequences (Hoef-Emden 2007), the unrooted phylogenetic analysis was performed. For the unrooted  
20 phylogenetic analysis, neighbor-joining (NJ) and maximum likelihood (ML) analysis were performed



1 using MEGA 6.06 (Tamura et al 2013). In ML analysis, the selected optimum model was a Kimura 2  
2 parameter (Kimura 1980) with gamma-distributed rates using a model selection option (a maximum  
3 likelihood value,  $\ln L = -2402.4983$ , Tamura et al 2011). Thus we used this model for the unrooted  
4 phylogenetic analysis and set the bootstrap value at 2000. In NJ model, a bootstrap value was set at 2000.  
5 The topology of the consensus tree was the same as ML (data not shown), thus we only showed the result  
6 of the ML analysis.

7

## 8 **Results**

### 9 **Variations in environmental factors in Lake Hira**

10 Water temperature increased gradually from mid-June and reached a maximum of 34°C on August 16,  
11 and then decreased to 23°C by 3 October (Fig. 1a). Because the rainy season ranges from Jun to mid-July  
12 in Japan, the amount of daily precipitation varied during the study period, and high amounts of daily  
13 precipitation was observed more frequently during this period than other periods (Fig. 1a).

14 Nutrients were supplied mainly from inflow and increased during the rainy season (Fig. 1b).  
15 The DIN concentration peaked (180.8  $\mu\text{g L}^{-1}$ ) on 4 July and gradually decreased to less than 20  $\mu\text{g L}^{-1}$   
16 until 7 August (Fig. 1b). The concentration of DIP fluctuated from 28 May to 4 July and peaked (17.9  $\mu\text{g}$   
17  $\text{L}^{-1}$ ) on 19 July (Fig. 1b). The concentration gradually decreased and reached 0  $\mu\text{g L}^{-1}$  on 25 September.

18

### 19 **Variations in *Cryptomonas* density and species composition**

20 The abundance of phytoplankton was affected by the high turnover rate of the lake water due to high

1 precipitation. The chlorophyll *a* concentration remained low (less than 10  $\mu\text{g L}^{-1}$ ) from mid-June to  
2 mid-July (Fig. 1c), although DIN and DIP were replete (Fig. 1b). Chlorophyll *a* concentration increased  
3 gradually from mid-July and reached 212.1  $\mu\text{g L}^{-1}$  on 25 September.

4 *Cryptomonas* cell density increased from  $4.3 \times 10^3$  cells  $\text{mL}^{-1}$  (28 May) to  $4.1 \times 10^4$  cells  $\text{mL}^{-1}$  (4  
5 June) during the first week, and then decreased to  $2.0 \times 10^3$  cells  $\text{mL}^{-1}$  until 13 June (Fig. 1c). The density  
6 remained low and ranged from  $1.4 \times 10^3$  cells  $\text{mL}^{-1}$  to  $7.0 \times 10^3$  cells  $\text{mL}^{-1}$  after 4 June, although a minor  
7 increase and decrease in the density was observed four times (19 to 26 July, 7 August, 12 September and  
8 3 October).

9 Single-cell PCR analysis was conducted eight times during the study period (Fig. 2). We  
10 successfully determined the sequences with high efficiency (83.3% -97.9%), excluding one date (56.3%,  
11 7 August) (Fig. 2). A total of 325 sequences from 8 sampling days were obtained, and all sequences were  
12 clustered into six groups (Fig. 3). Four out of six groups were clustered together with known species and  
13 predicted to be *C. curvata* (group 1, 246 sequences), *C. tetrapyrenoidosa* (group 2, 30 sequences), *C.*  
14 *pyrenoidifera* (group 3, 5 sequences) or *C. marsonii* (group 4, 2 sequences). Groups 5 (23 sequences)  
15 and 6 (19 sequences) were phylogenetically located closest to *C. marsonii*, but did not include any known  
16 sequences (Fig. 3).

17 Group 1, which was closely related to *C. curvata*, dominated during the study period (44.4% to  
18 97.7% of the total abundance), and the relative abundance tended to decrease during high water  
19 temperature periods (Fig. 2). Group 2, which was estimated to be *C. tetrapyrenoidosa*, also appeared  
20 throughout the period and accounted for 2.3% to 17.7% of the total abundance. Unidentified

1 *Cryptomonas* group 5 was detected from 4 June to 12 September, ranging from 4.9% to 15.2% of the total  
2 abundance. On the other hand, Group 3 (*C. tetrapyrenoidosa*) and Group 4 (*C. marsonii*) appeared  
3 sporadically and ranged from 0% to 5.0% and 0% to 6.1%, respectively. Unidentified *Cryptomonas* group  
4 6 appeared and dominated only from 26 June (20.0%) to 7 August (40.7%).

5

### 6 **Genotypic composition in *C. curvata***

7 Although we detected multiple genotypes for each species except for *C. marssonii* (group 4), enough  
8 number of sequence was obtained only for *C. curvata* (group 1) to analyze temporal variation in the  
9 genotypic composition (Fig. 4). A total of 36 genotypes in 246 sequences were presumed to belonging to  
10 *C. curvata* (group 1). Although most of the genotypes were observed with low frequencies during the  
11 study period (less than 8 sequences in total, see Fig. 4), the other 11 genotypes were detected repeatedly  
12 and can therefore be categorized into the following four groups; the first group was present during the  
13 entire study period and seemed to decrease at high water temperatures (C1, C3, C10, C11 and C16), the  
14 second and third groups appeared only in the early or late parts of the study period (C8, C9 and C13), and  
15 the fourth group appeared and dominated from 26 June to 7 August (C15 and C17).

16

17

## **Discussion**

18 Due to the difficulty in the morphological identification of *Cryptomonas*, most of the studies on the  
19 phytoplankton community in natural systems showed *Cryptomonas* by genus level abundance. The result  
20 of this is that information about the species composition and its seasonal variations are quite limited.

1 Moreover, morphological classification does not work for some *Cryptomonas* species (Hoef-Emden and  
2 Melkonian 2003; Hoef-Emden 2007). Using single-cell PCR, we could analyzed the species composition  
3 and its variation even for morphologically indistinguishable species *C. curvata* and *C. pyrenoidifera* (Fig.  
4 3). Therefore, this approach seems to be an effective method for evaluations of the species composition  
5 and diversity in natural phytoplankton communities. On the other hand, single-cell PCR approach largely  
6 lacks the morphological information of target cells, since we extract DNA from living cells without the  
7 fixation and following microscopic observation. This does not affect its availability for the identification  
8 and analysis of the species diversity, but becomes problematic for studies examining the life history of  
9 individual species especially those showing dimorphism. Further change or revision of the method may  
10 be needed to study the evolution of morphological plasticity.

11 At least 14 *Cryptomonas* species have been recognized based on molecular analyses (Hoef-Emden  
12 2007). In Japan, six species (*C. erosa*, *C. ovata*, *C. platyuris*, *C. rostratiformis*, *C. tetrapyrenoidosa* and *C.*  
13 *paramecium*) have been recorded in previous studies (Mizuno 1971; Ishimitsu and Chihara 1984; Erata  
14 and Chihara 1987; Alam et al. 2001), whilst *C. curvata*, *C. pyrenoidifera* and *C. marssonii* were firstly  
15 observed in the present study. On the other hand, these three species do not seem to be rare and have been  
16 frequently reported in Asia such as China and Korea (e.g., Kim et al 2007; Choi et al. 2013; Xia et al.  
17 2013). Because of the difficulty of morphological identification and recent revisions of *Cryptomonas*  
18 species, *Cryptomonas* species observed in Japan have not been fully described, and some species have  
19 been identified as different species. For example, *C. platyuris* NIES-276 isolated in Japan was  
20 morphologically re-identified to *C. borealis* (Hoef-Emden and Melkonian 2003). *Cryptomonas*

1 *rostratiformis* seems to be one of the frequently observed species in Japan (e.g., Ishimitsu and Chihara  
2 1984; Erata and Chihara 1987). However, the species was emended and became a synonym of *C. curvata*,  
3 because the strain M1484 from Germany being re-identified to the campylomorph strain of *C. curvata*  
4 both from morphological and molecular phylogenetic perspective (Hoef-Emden and Melkonian 2003).  
5 Therefore, the *C. rostratiformis* reported and isolated in Japan should be reevaluated in light of the  
6 current classification. Moreover, *C. curvata* and *C. pyrenoidifera* show dimorphism and exhibit similar  
7 cryptomorphs (Hoef-Emden and Melkonian 2003), which may also account for the species disregard in  
8 Japan.

9 In pelagic region of typical dimictic lakes with large water surface area, seasonal variations in  
10 phytoplankton abundance of the surface water are relatively predictable and mainly affected by water  
11 temperature and nutrient supply in the water column by seasonal vertical mixing. On the other hand,  
12 water retention time or water exchange rate become more important factors in water systems with highly  
13 susceptible to variable influent quantities such as small lakes and ponds, upstreams of river-mouth dams,  
14 and riverine and transitional zones of reservoirs (e.g., Thornton et al. 1990; Hodoki and Murakami 2007;  
15 Yamamoto et al. 2013). Lake Hira is small and shallow, and previous study also showed that nutrients  
16 (DIP and DIN) are usually remaining in the water (Mitamura et al. 2014) and seem not to become limiting  
17 factors for phytoplankton growth. Therefore, the frequent lake water exchange due to precipitation  
18 seemed to affect *Cryptomonas* density and total phytoplankton abundance, and kept their levels low from  
19 early June to mid-July (Fig. 1b). On the other hand, biomass in phytoplankton increased after rainy  
20 periods, and nutrient depletion gradually became a limiting factor for phytoplankton growth rate during

1 the study period (Fig. 1c).

2       Because of the limitation of morphological classification, we should pay attention to interpretations  
3 of the ecological features of *Cryptomonas* morphologically analyzed in previous studies. In Lake Hira, *C.*  
4 *curvata* was the dominant species throughout the study period (Fig. 2). This species has been reported  
5 worldwide (e.g., Menezes and Novarino 2003; Melo et al. 2004; Xia et al. 2013). Seasonal changes of the  
6 *Cryptomonas* species composition in a Brazilian reservoir indicated that *C. curvata* tended to dominate  
7 during low temperature periods (15-20°C, between March and September), and then decreased during the  
8 summer (Bicudo et al. 2009). The relative abundance of *C. curvata* in L. Hira also remained low during  
9 the high water temperature period (> 30°C), and our results were consistent with previous reports (Fig. 2).

10       Unidentified *Cryptomonas* groups phylogenetically similar to *C. marssonii* also appeared and  
11 became dominant during high water temperature periods (Fig. 2). *Cryptomonas marssonii* is ubiquitously  
12 distributed under various environmental conditions (Smolander and Arvola 1988; Kim et al. 2007). In  
13 Brazil, *C. marssonii* is one of the most common *Cryptomonas* species (Menezes and Novarino 2003), and  
14 its density increases during high temperature periods (Bicudo et al. 2009). Rott (1988) examined  
15 flagellate assemblages in four mid-altitude lakes (average surface water temperature of 24.3°C) and four  
16 high-mountain lakes (average surface water temperature of 10.8°C) in Austria, and reported that *C.*  
17 *marssonii* was more abundant in mid-altitude lakes. In the present study, the response of the unidentified  
18 groups to water temperature was consistent with previous studies showing higher abundance at high water  
19 temperatures, and environmental characteristics of these groups resembled *C. marssonii*.

20       Genotypic diversity and its role have been investigated for cyanobacteria. For example,

1 high-light-adapted genotypes of *Prochlorococcus* were phylogenetically classified into the same clade  
2 (Moore et al. 1998). *Cuspidothrix issatschenkoi* consists of at least three ecotypes due to the various  
3 combinations of toxin production and nitrogen-fixation abilities, and nitrogen-fixation-lacking and  
4 toxin-producing genotypes prefer more nitrogen-replete environments (Hodoki et al. 2013). Genotypic  
5 composition of *Microcystis aeruginosa* varies temporally and locally depending on the environmental  
6 condition, although all genotypes are distributed ubiquitously (Briand et al. 2009; Sabart et al. 2009;  
7 Ohbayashi et al. 2013). In the present study, the genotypic composition of *C. curvata* also varied  
8 temporary during the study period (Fig. 4). However, we analyzed only ITS 2 regions, and the sequence  
9 information is not enough for speculating the phylogenetic relationships among the genotypes correctly.  
10 Therefore, we could not discuss the response of each genotype to environmental factors, and then the  
11 reason of variations in genotypic composition of *C. curvata* observed in the present study was unclear. To  
12 clarify this problem, further ecophysiological studies are required with increasing the sequence  
13 information analyzed by single-cell PCR.

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- 4

## Figure captions

- 1
- 2 Supplement. Location of Lake Hira (a) and the study site in Lake Hira (b)
- 3 Fig. 1 Changes in water temperature and the amount of daily precipitation (a), dissolved inorganic  
4 nitrogen (DIN) concentration and dissolved inorganic phosphorus (DIP) concentration (b), and  
5 chlorophyll *a* concentration and *Cryptomonas* density (c)
- 6 Fig. 2 Relative species composition of the *Cryptomonas* community in Lake Hira. Numbers on the bar  
7 indicate numbers of analyzed sequences / isolated cells
- 8 Fig. 3 Unrooted maximum likelihood tree based on ITS2 Helix III regions (157 positions). Bootstrap  
9 values >50% are indicated near the node. Numbers in parentheses indicated near the same sequences  
10 observed during the study period. Our 18 sequences similar to *C. curvata* were omitted from the  
11 phlogenetic analysis, since they were detected only once during study period.
- 12 Fig. 4 Temporal variation in the genotypic composition of *C. curvata* during the study period. Each  
13 number in parentheses represents the number of sequences obtained from each sampling day. Only  
14 genotypes observed more than eight sequences were used for analysis.

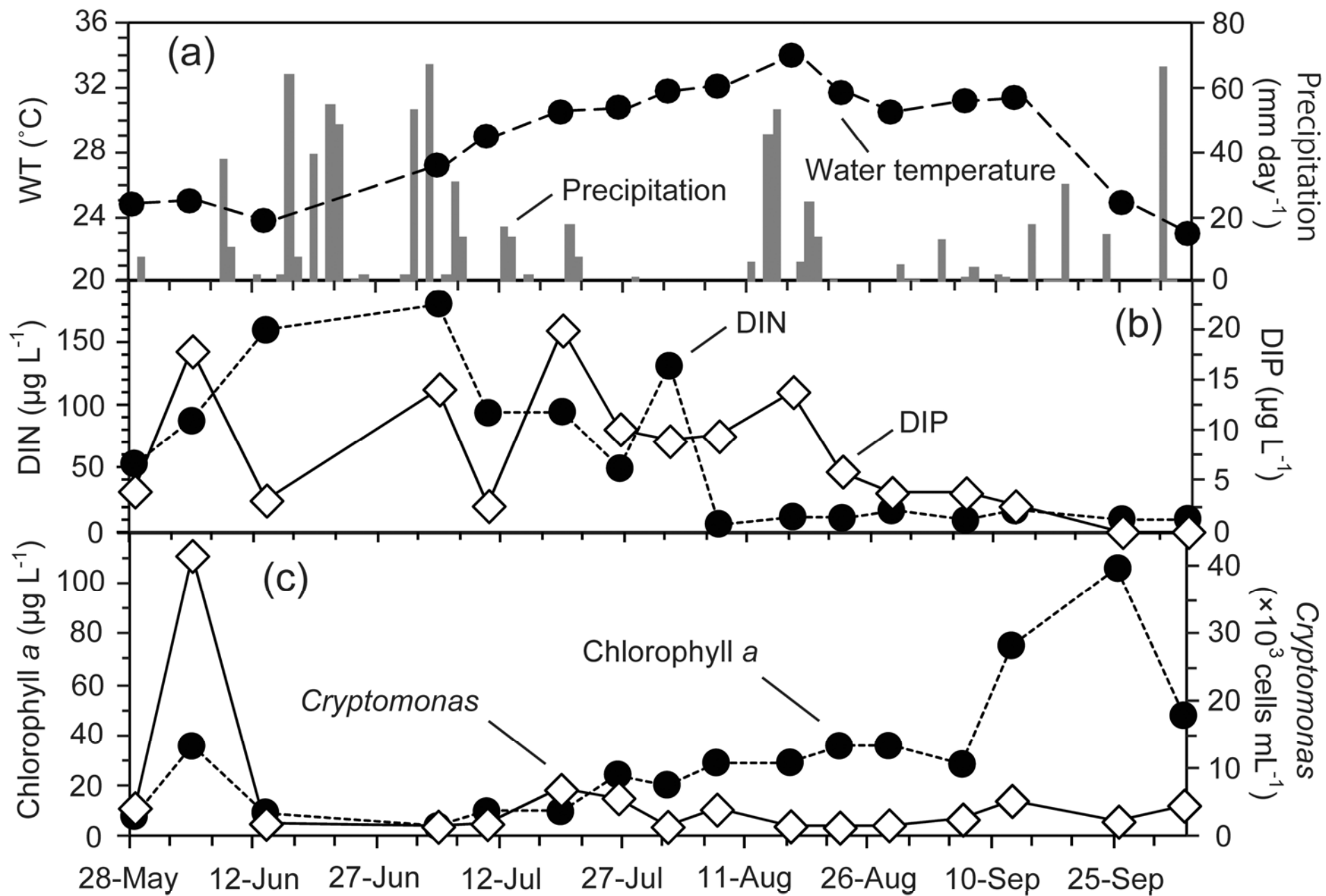


Fig. 1



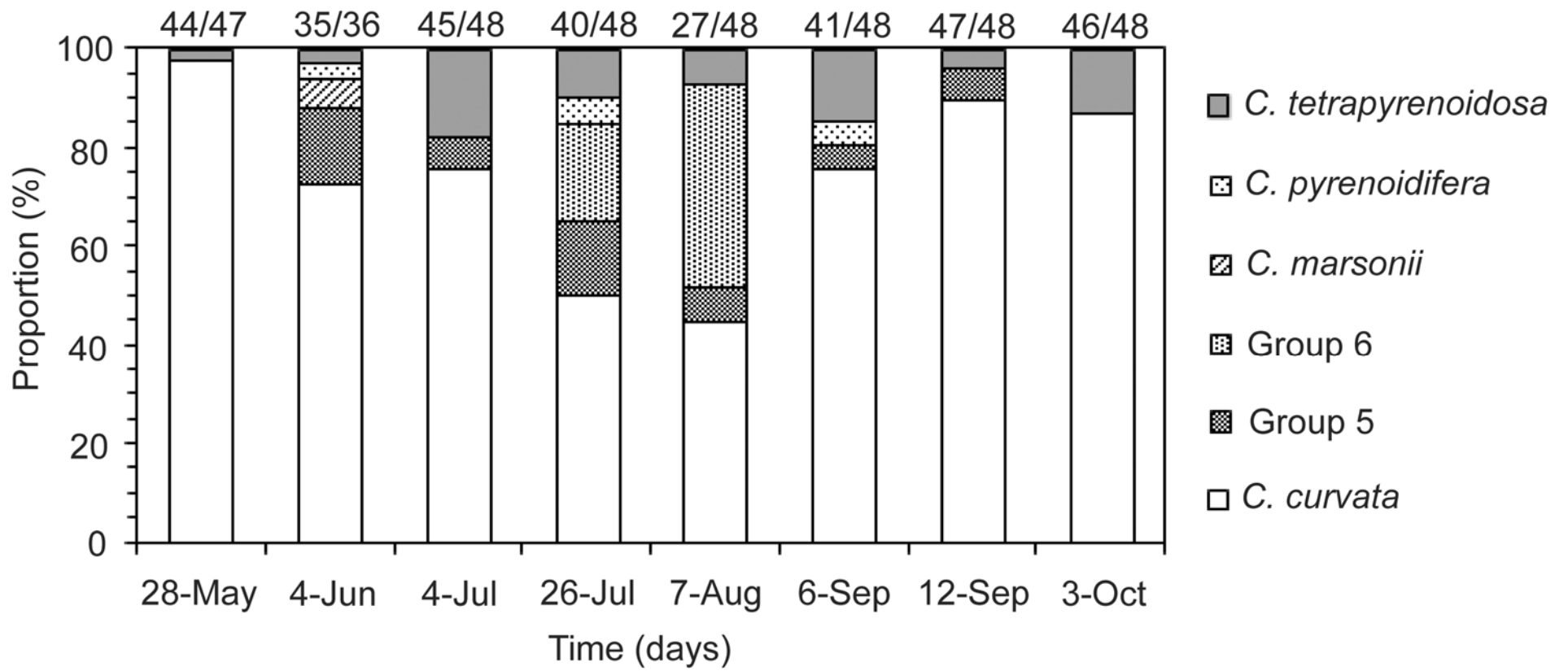


Fig. 2

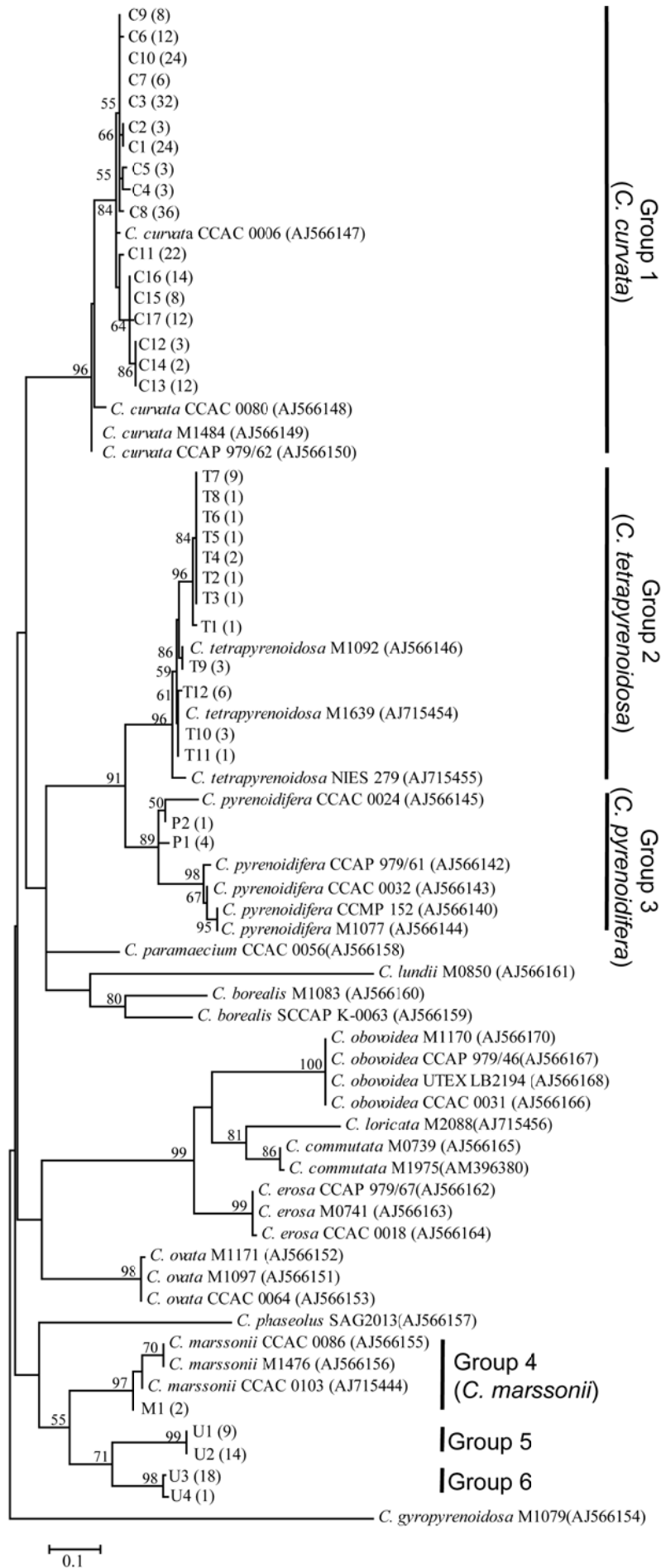


Fig. 3

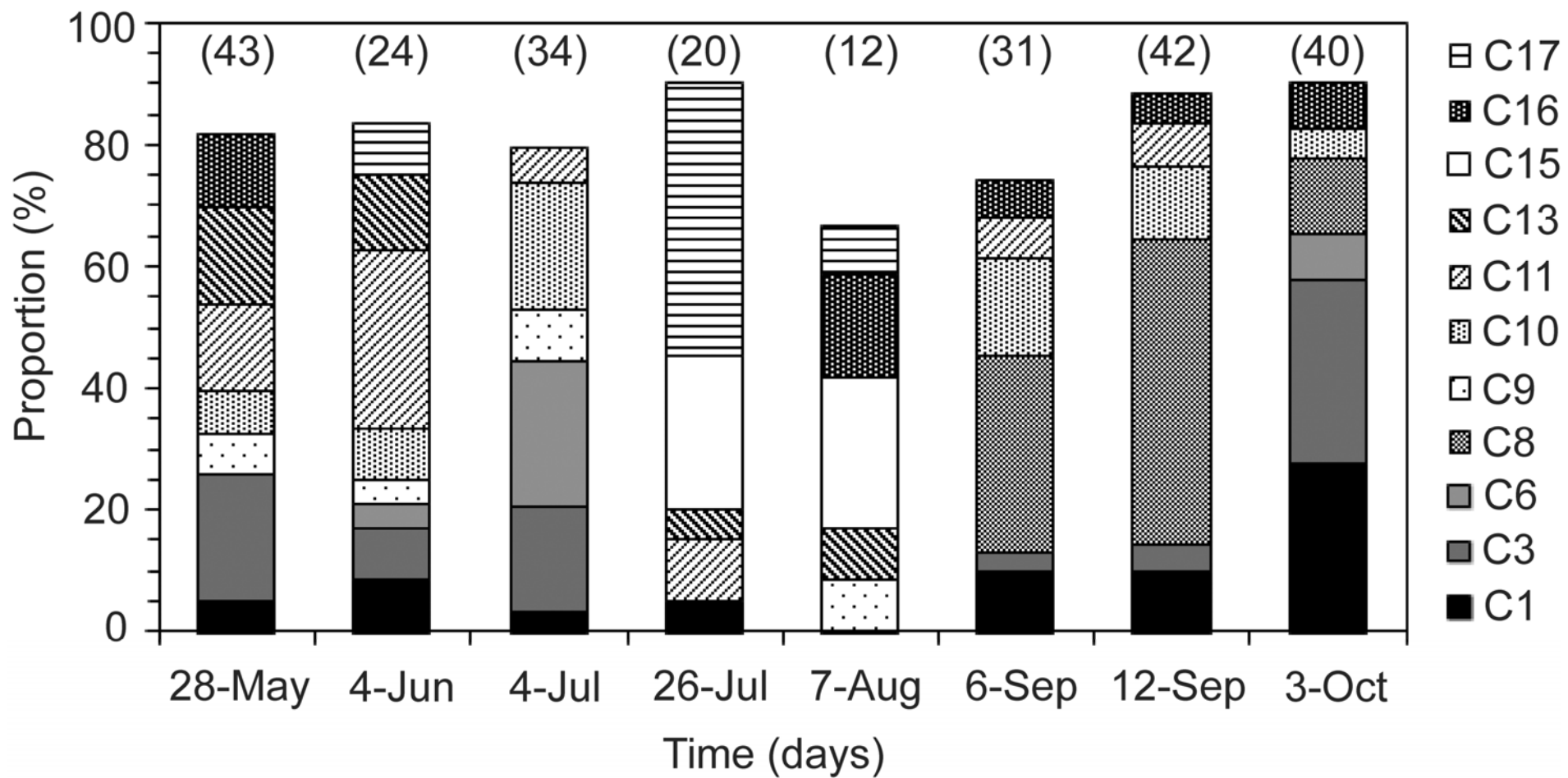


Fig. 4