Phylogenetic diversity of the picocyanobacterial community from a novel winter bloom in Lake Biwa

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Abstract

In Lake Biwa, picocyanobacteria blooms usually occur during the summer–autumn thermal stratification period. Intriguingly, a novel bloom was detected in winter 2015–2016, in which picocyanobacterial cell density increased by one order of magnitude despite lower water temperature, suggesting the possibility that "cold-water-preference" species dominate in the picocyanobacterial community. In the present study, we investigated the phylogenetic diversity of picocyanobacteria in Lake Biwa by analyzing the 16S rRNA gene. We found that the picocyanobacterial community were highly diverse in Lake Biwa, with eight *Synechococcus*-related operational taxonomic units (OTUs) detected in different seasons. These OTUs fell into distinct phylogenetic groups, and the majority were closely related to clusters reported previously. Notably, OTU04, detected during the winter bloom, was highly affiliated with sequences found in a variety of lakes, such as Tibetan lakes and Lake Superior, where the water bodies generally have a low trophic state and temperature, and different concentrations of total dissolved solids. Thus, we inferred that the group containing OTU04 may be a psychrotolerant lineage that is widely distributed in oligotrophic water systems with low–intermediate salinity.

Keywords: 16S rRNA; picocyanobacteria; phylogenetic diversity; psychrotolerant

Introduction

Picocyanobacteria, which are generally unicellular cyanobacteria smaller than 2 µm, are ubiquitous photosynthetic microorganisms in aquatic ecosystems (Stockner and Antia

1986; Stockner 1988). Despite their small size, picocyanobacteria contribute significantly to primary production and form the basis of food webs in various types of lakes and oceans (Stockner 1991; Weisse 1993; Callieri et al. 2013). In freshwaters, they are mainly represented by the genera *Synechococcus* and *Cyanobium*, which often cannot be distinguished clearly (Callieri 2008).

Seasonal variation in picocyanobacterial abundance in lake ecosystems has received considerable attention during the last few decades. It has been widely acknowledged that temperature is an important driver of picocyanobacterial growth and abundance (Beardall and Raven 2004; Vörös et al. 2009; Jodłowska and Śliwińska 2014; Śliwińska-Wilczewska et al. 2018), and picocyanobacteria generally reach maximum cell densities (10^5–10^6 cells mL^{-1}) during summer–autumn thermal stratification in temperate lakes (Stockner et al. 2000; Callieri et al. 2013). Previous studies in Lake Biwa, the largest freshwater lake in Japan, have also reported a similar pattern, i.e., that picocyanobacteria form significant blooms in the epilimnion during summer and early autumn and decline in other seasons (Nagata 1986; Maeda et al. 1992; Nakano et al. 1996; Wakabayashi and Ichise 2004).

Intriguingly, we found an increase in picocyanobacterial cell densities in Lake Biwa during winter 2015–2016 despite decreased water temperature (Cai et al. 2020). It is likely that psychrotolerant or psychrophilic species dominated the community during that period. Psychrotolerant *Synechococcus* have been frequently found in winter oceans (Choi et al. 2013) and polar seas (Tang and Vincent 1999), and some can maintain slow but sufficient growth even at very low temperatures (−1.8°C to 4°C in Cottrell and Kirchman 2009; nearly 4°C in Xu et al. 2015). In addition, psychrophilic eukaryotic

picophytoplankton that achieve optimal growth rates at 6–8°C have also been reported in polar oceans (Lovejoy et al. 2007). However, unlike those in marine systems, freshwater picocyanobacterial lineages that adapt to low temperature conditions remain largely unexplored.

In the present study, we investigated the phylogenetic diversity of the picocyanobacteria community in Lake Biwa by analyzing the 16S rRNA gene. We collected water samples in different seasons and determined the 16S rRNA sequences by clone-library analyses. By comparing the community structure between summer and winter, we inferred whether the picocyanobacteria that proliferated during the winter were “cold-water-preference” species.

Methods

Sampling and DNA isolation

Sample collection was conducted biweekly at a pelagic site (Ie-1) in the north basin of Lake Biwa from July 2015 to June 2017 (Cai et al. 2020). Unfiltered water samples were collected from the epilimnion (5 m). Cell densities of picocyanobacteria were determined by visualizing the autofluorescence under green excitation (530–550 nm) with an epifluorescence microscope (Cai et al. 2020). For DNA extraction, four water samples were collected in August 2015 (2015sum), March 2016 (2015win), July 2016 (2016sum), and March 2017 (2016win). For each sample, 0.5 to 1 L of water was filtered through a 0.2-µm-pore-size polycarbonate membrane filter (Advantec K020A047A; Toyo Roshi Kaisha, Japan). DNA was extracted from seston on the filter using the PowerSoil DNA Isolation kit (MOBIO, USA) according to the manufacturer's

instructions. Extracted DNA was eluted in TE buffer and stored at −20°C until downstream application.

PCR amplification, cloning, and sequencing

Partial 16S rRNA gene of *Synechococcus* was amplified using the primer set CYA359F (5'-GGG GAA TYT TCC GCA ATG GG, Nübel et al. 1997) and 1499R (5'-CAC CTT CCG GTA CGG CTA C). PCRs were conducted in a 150-µL reaction mixture with the following final reactant concentrations: 1 × PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgSO_4$, 0.3 µM of each primer, ca. 600 ng of template DNA, and 1 U of KOD-Plus-Neo (Toyobo, Japan) polymerase. The PCR conditions were as follows: initial activation of the KOD-Plus-Neo polymerase for 2 min at 94°C, followed by 35 cycles of 10 s denaturation at 98°C, annealing for 30 s at 60°C, and extension for 30 s at 68°C, and a final extension at 68°C for 7 min. The PCR products were purified using a NucleoSpin Tissue kit (Macherey-Nagel, Germany), and cloned using a TArget Clone kit (Toyobo) and Competent High DH5α (Toyobo) following the manufacturer's instructions.

Approximately 40 positive colonies containing PCR products were randomly selected from each sample. The inserted DNA was re-amplified with the T7 and U19 primers, and the length of the PCR products was verified by agarose gel electrophoresis. Only PCR products containing the target sequence were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and a 3130 genetic analyzer (Applied Biosystems).

Phylogenetic analysis

DNA sequences (V3–V8 region) amplified with the primer set for cyanobacteria were checked against the GenBank database using BLAST (Altschul et al. 1997). Chimeras were identified using vsearch (v.2.6.2; Rognes et al. 2016) and excluded from further analyses. Only sequences related to *Synechococcus* were grouped together into an operational taxonomic unit (OTU) if their similarity was greater than 99%, considering the high conservation of the 16S rRNA gene (Edgar 2018). Reference sequences of currently known picocyanobacterial clusters and the closest relative of each OTU were downloaded from the GenBank database. In addition, sequences of the V3–V4 region that were highly similar to the OTUs detected in winter were obtained to evaluate the geographical distribution of winter picocyanobacteria. The sequence data were aligned using the MUSCLE algorithm, as implemented in MEGA X (Kumar et al. 2018). Maximum-likelihood trees were constructed using FastTree (Price et al. 2010) and then edited with the "ggtree" package (Yu et al. 2017) in R software (v.3.4.3; R Development Core Team, 2018). The 16S rRNA gene sequences obtained in the present study were deposited in GenBank with accession numbers MT772216 to MT772235.

Results

In Lake Biwa, picocyanobacteria formed significant blooms (up to 4.5×10^5 cells mL^{-1}) in the epilimnion during June and October (i.e., the thermal stratification period) in both 2015 and 2016 (Fig. 1). Notably, an increase in picocyanobacterial abundance was also observed during December 2015 and March 2016. Cell densities of picocyanobacteria increased from 3.9×10^3 to 3.4×10^4 cells mL^{-1}, while the water temperature decreased

from 17 to 8°C. Afterwards, the density decreased to 7.5×10^3 cells mL^{-1} when the temperature rose to 11°C in April 2016, thus forming a winter bloom. During this period, a negative Spearman's rank correlation ($r_s = -0.915$, $p < 0.001$) was found between picocyanobacterial abundance and water temperature. However, no bloom was observed in winter 2016–2017. After the summer bloom in 2016, picocyanobacterial abundance decreased gradually to 1.5×10^2 cells mL^{-1} along with temperature, and did not increase until the temperature increased in March 2017.

A total number of 20 sequences putatively derived from *Synechococcus* were detected among all samples, except for 2016win. These sequences were clustered into eight OTUs (01–08) based on 99% similarity and fell into distinct phylogenetic groups (Fig. 2). OTU01 was detected in summer 2015 and 2016 and was closely affiliated with *Cyanobium* JJ9-A3. OTU02 and OTU03, both of which were detected in summer 2015, were closely related to the Lake Biwa cluster (Ernst et al. 2003) and *Cyanobium* Suigetsu-CG4 (group IV in Ohki et al. 2012), respectively. OTU04 and OTU05 were detected in winter 2015, with the former being highly affiliated with the Tibetan cluster (Xing et al. 2009; Huang et al. 2014). OTU05 was closely related to uncultured bacteria detected in Feicui Reservoir and Green Lake. OTU06–08 were detected in summer 2016. OTU06 was closely related to *Cyanobium* JJM10D5. OTU07 was closely related to the clade containing the Lake Biwa cluster and group E (Robertson et al. 2001), which has been described as "Lake Biwa strains" (Crosbie et al. 2003). OTU08 was highly affiliated with group H (Crosbie et al. 2003).

The additional phylogenetic analysis based on the V3–V4 region (Fig. 3) showed that OTU04 and OTU05 were related to LSI/LSII and PDII, respectively, all of which were previously reported in Lake Superior (Ivanikova et al. 2007).

Discussion

No previous studies conducted in Lake Biwa have investigated seasonal variation of the picocyanobacterial community at the genetic level, and their phylogenetic diversity remains largely unknown. In the present study, by analyzing the partial 16S rRNA gene, we found that picocyanobacteria in Lake Biwa were highly diverse and the community structure varied by season (Fig. S1). The presence of OTU01 in both summers also indicated that the same species may proliferate in the same time period regardless of year. Unfortunately, due to the low abundance (i.e., no *Synechococcus*-related sequence was detected in winter 2016; Fig. 1), it was unclear whether OTU04 or OTU05 was a ubiquitous species in the winter season. One possible reason for the lack of a winter bloom in 2016 was grazing pressure by cladocerans, since the individual density of *Daphnia* spp. during the winter increased from 3.3 individuals L^{-1} in 2015 to 23.1 individuals L^{-1} in 2016 (Cai et al. 2020).

Most OTUs found in summer were closely related to clusters that have been previously reported (Fig. 2). The relationship among OTU02 (Lake Biwa cluster), OTU03 (group IV in Lake Suigetsu), and group E was especially robust, as confirmed by the similar topology of phylogenetic trees in previous studies (Crosbie et al. 2003; Ohki et al. 2012). By contrast, the group of OTU01 or OTU06 appeared to be a distinct cluster that has not yet been described. The two groups may be lineages that adapt to

meso-eutrophic conditions, since relative sequences were found in a variety of meso-eutrophic lakes (Rajaniemi-Wacklin et al. 2008; Komárek et al. 2011; Cai and Kong 2013; Kojima et al. 2014). However, further studies are necessary to clarify their ecological features.

Two OTUs were detected during the winter bloom, and the negative correlation between abundance and temperature suggested that either or both of them may be psychrotolerant species. Notably, OTU04 dominated the picocyanobacterial community in winter (66.6%, Fig. S1) and was highly affiliated with the Tibetan cluster (III–V in Wu et al. 2010 and Huang et al. 2014; Fig. 2), which was ubiquitous and abundant in a variety of cold, oligotrophic lakes on the Tibetan Plateau (Table 1). Most of these lakes had low concentrations of total dissolved solids (TDS), except for Lake Nam Co, which was oligosaline (Huang et al. 2014). The phylogenetic analysis based on the V3–V4 region (Fig .3) showed that OTU04 was also affiliated with the Lake Superior cluster containing LSI and LSII, which have been reported as a distinct group endemic to Lake Superior, an extremely oligotrophic lake with low water temperature and TDS content (Table 1; Ivanikova et al. 2007; Callieri et al. 2013). Moreover, environmental sequences identical to that of OTU04 have been frequently found in other water bodies, such as Lake Baikal, high-altitude Pyrenean lakes, and waters in the Arctic region, where temperature and trophic states are generally low. Therefore, it is likely that the group of OTU04 is a psychrotolerant lineage distributed widely in oligotrophic water systems with low–intermediate salinity.

Conversely, OTU05 was not related to any known clusters. Nevertheless, it has a similarity greater than 97% with strains such as LBP1 (97.91%); therefore, the group of

OTU05 is probably a *Synechococcus* lineage. The closest relatives were found in the epilimnion of Feicui Reservoir in December (Kojima et al. 2014) and near/under the thermocline of Green Lake in September (Meyer et al. 2011; Fig. 2). The former is a subtropical lake where the water temperature can reach as high as 20°C in winter, while the latter is a saline meromictic lake with temperatures lower than 10°C near the thermocline (Brunskill and Ludlam 1969). Unlike OTU04, the key determinant of the distribution of this lineage remains unclear. Although both Feicui Reservoir (Chang and Wen 1997) and Green Lake (Wisconsin Department of Natural Resources) are mesotrophic, sequences of the V3–V4 region related to OTU05 have been detected in both oligotrophic (e.g., Lake Superior) and eutrophic (e.g., Lake Waahi) lakes (Fig. 3). Further studies are still needed to elucidate the characteristics of the group of OTU05; studies on picocyanobacteria have been mostly conducted during warm seasons, when they are abundant. Shedding more light on the winter communities will improve understanding of the phylogeny and ecology of picocyanobacteria.

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Table 1. Comparison of environmental parameters in Lake Biwa (2015win), Tibetan lakes, and Lake Superior.

Lake	Biwa[1]	Tibetan Plateau[2]						Superior[3]
		Zhaling	E'ling	Tuosuhai	Xinxinhai	Nam Co	Puma Yumco	
Sampling date	Mar 2016	Jul 2005	Jul 2005	Jul 2005	Jul 2005	Jul 2004	Oct 2008	Sep 2004
Sampling depth (m)	5	0–0.5	0–0.5	0–0.5	0–0.5	0–0.5	0	5
Temperature (°C)	8.5	9.5	9.5	12.5	13	10.8	7.6	15
pH	7.3	8.42	8.74	8.83	8.47	9.4	9.2	8
TDS (mg L^{-1})	48	616.4	428.2	566.3	673.2	1958	200	56
T-N (mg L^{-1})	0.28	0.352	0.384	0.252	0.723	0.025		0.396
T-P (mg L^{-1})	0.011	0	0	0	0	0.025		0.006
Trophic state	meso	oligo	oligo	oligo	oligo	oligo	oligo	oligo

1. Data were collected from Cai et al. 2020 and Japanese Ministry of Environmental Public Water database.

2. Data were cited from Xing et al. 2009 and Huang et al. 2014.

3. Data were collected from Guildford et al. 2000; Chapra et al. 2012; Dupont et al. 2012; and Dove and Chapra 2015.

T-N: total nitrogen; T-P: total phosphorus; Zero means below the detection limit.

Figure 1. Seasonal dynamics of picocyanobacterial abundance (line with black dots) and water temperature (gray area) in the epilimnion (5 m) of Lake Biwa from July 2015 to June 2017. The data were modified from Cai et al. 2020.

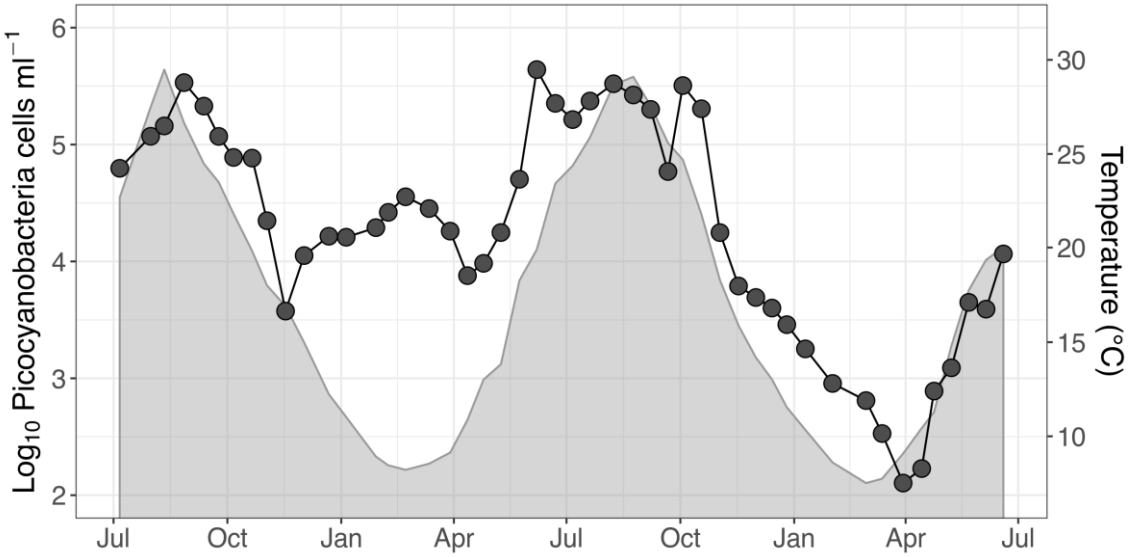

Figure 2. Maximum-likelihood tree inferred from 16S rRNA gene sequences (957 bp, covering the highly variable regions V3–V8) of eight OTUs detected in Lake Biwa. Bootstrap values are shown at nodes. The outgroup was Synechococcus elongatus PCC6301.

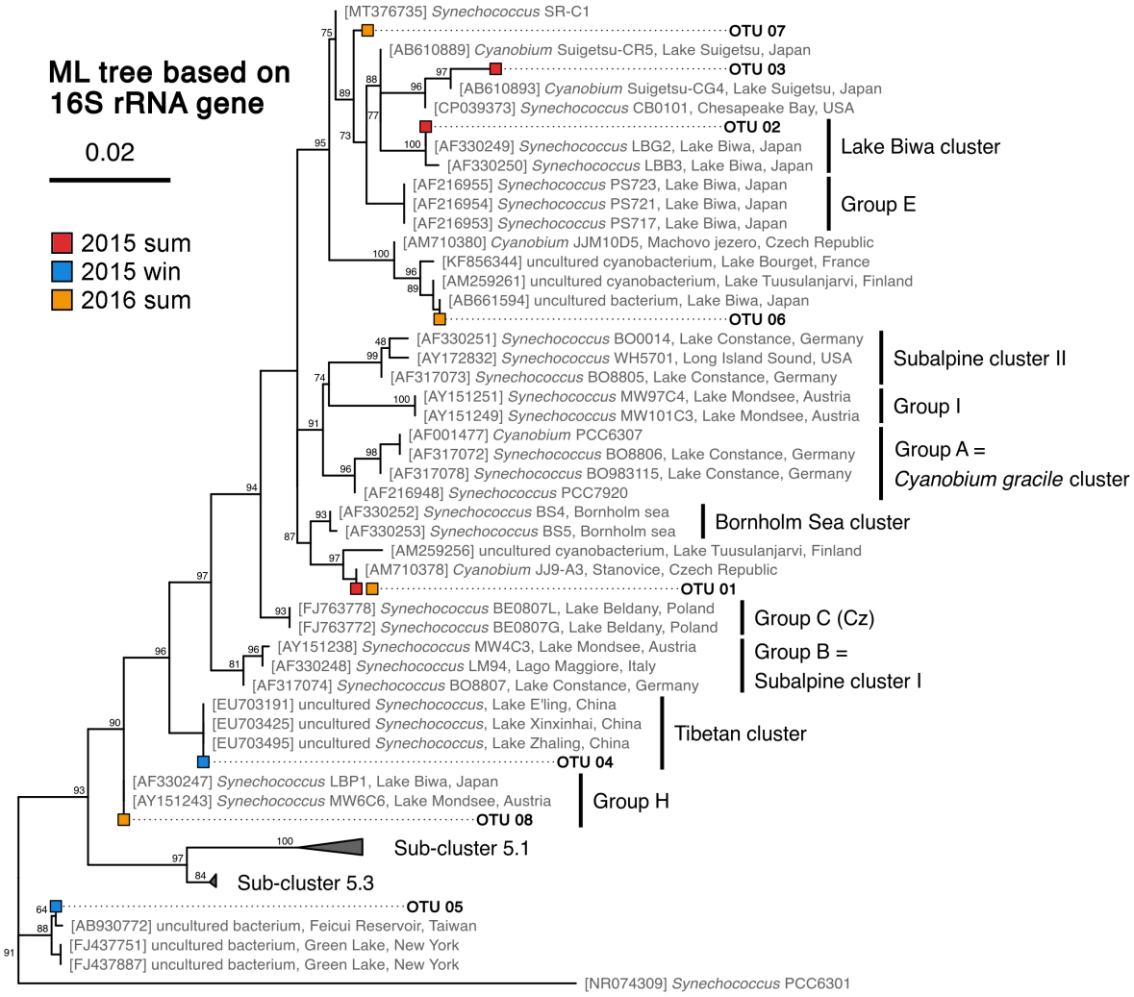

Figure 3. Maximum-likelihood tree based on the alignment of 16S rRNA gene sequences (321 bp, covering the highly variable regions V3–V4) among OTU04, OTU05, and highly similar environmental sequences, including several reference clusters. Bootstrap values are shown at nodes.

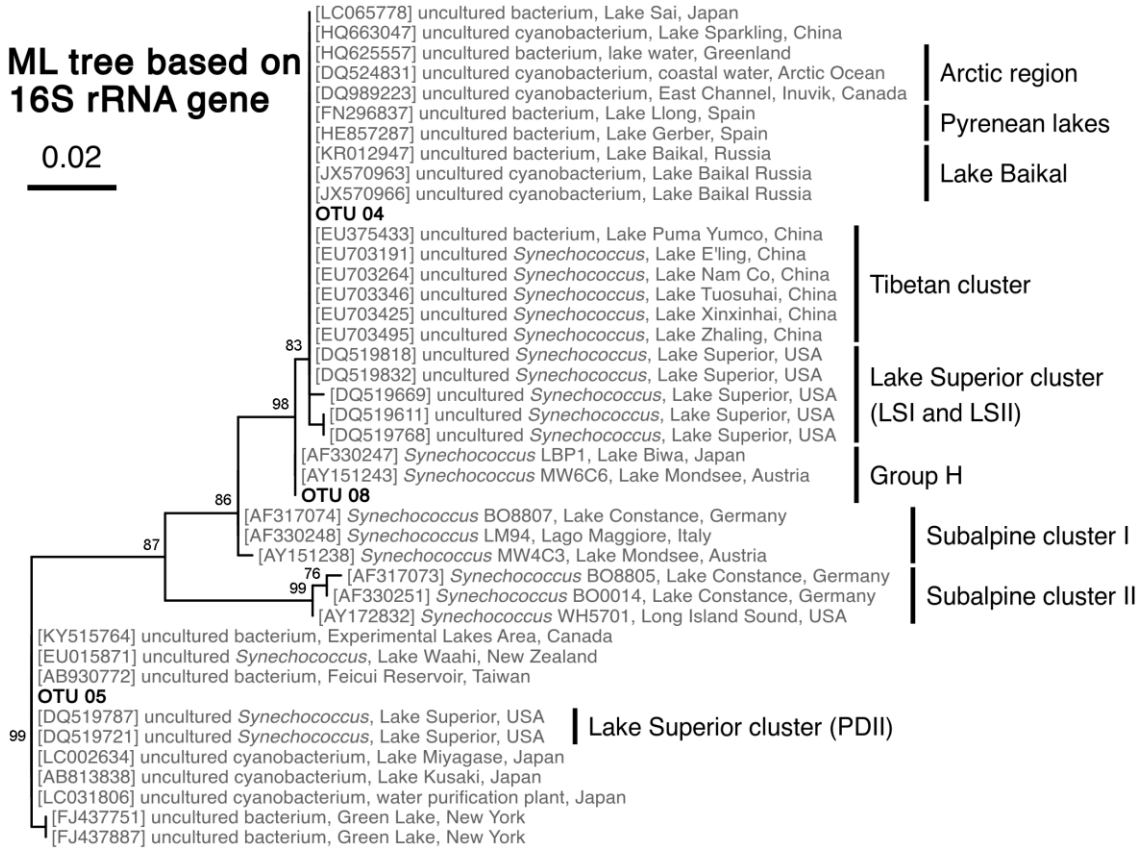

Figure S1. Percentage of read numbers for each OTU detected in Lake Biwa at different seasons.