ISSN 1226-9999 (print)
ISSN 2287-7851 (online)

Korean J. Environ. Biol. 36(3) : 424~437 (2018) https://doi.org/10.11626/KJEB.2018.36.3.424

〈Original article〉

Seasonal Changes in Cyanobacterial Diversity of a Temperate Freshwater Paldang Reservoir (Korea) Explored by using Pyrosequencing

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Abstract - The incidence of freshwater algal bloom has been increasing globally in recent years and poses a major threat to environmental health. Cyanobacteria are the major component of the bloom forming community that must be monitored frequently. Their morphological identities, however, have remained elusive, due to their small size in cells and morphological resemblances among species. We have analyzed molecular diversity and seasonal changes of cyanobacteria in Paldang Reservoir, Korea, using morphological and 16S rRNA pyrosequencing methods. Samples were collected at monthly intervals from the reservoir March–December 2012. In total, 40 phylotypes of cyanobacteria were identified after comparing 49,131 pyrosequence reads. Cyanobacterial genera such as *Anabaena*, *Aphanizomenon*, *Microcystis* and *Synechocystis* were predominantly present in samples. However, the majority of cyanobacterial sequences (65.9%) identified in this study were of uncultured origins, not detected morphologically. Relative abundance of cyanobacterial sequences was observed as high in August, with no occurrence in March and December. These results suggested that pyrosequencing approach may reveal cyanobacterial diversity undetected morphologically, and may be used as reference for studying and monitoring cyanobacterial communities in aquatic environments.

Keywords : cyanobacterial diversity, Paldang Reservoir, Han River, pyrosequencing, 16S rRNA

INTRODUCTION

Cyanobacteria are oxygenic photoautotrophic Gram negative bacteria, morphologically diverse and dwelling in almost all environments (Paerl and Paul 2012). They are also known as the oldest oxygen-producing organisms of Earth, with the fossil records of ~3.5 billion years (Schopf 2002). Algal bloom in freshwater lakes and reservoirs is considered as an environmental problem worldwide. Particularly, toxic cyanobacterial blooms in freshwater systems around the world tend to increase in frequency and severity (Paerl and Paul 2012; Steffen *et al*. 2012). However, nutrient over-enrichment of waters by anthropogenic activities has supported the growth of cyanobacteria to form harmful algal blooms (Paerl and Huisman 2008). Cyanobacteria have capabilities to fix atmospheric nitrogen, sequester iron and solubilize phosphate which made them thrive in to various environments with varied nutrient levels from low to enriched (Paerl and Otten 2013). In addition, cyanobacterial blooms create threat to drinking and recreational water by the release of potentially harmful toxins and stinking compounds (Cheung *et al*. 2013). Many bloom forming cyanobacteria known to produce various toxins which can have drastic impact on the ecosystem and surrounding communities. As well, the cyanobacterial blooms can cause hypoxia and leads to disruption of food webs in the system (Cheung *et al*. 2013).

Traditionally, cyanobacteria were identified majorly based on morphological features which require extraordinarily

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skilled personal and chances of misidentification are also high (Komárek and Anagnostidis 1989; Choi *et al*. 2018). In the recent years, advancements in the high-throughput sequencing technology, such as next-generation sequencing (NGS), revolutionized the field of genomics and characterization of diverse microbial communities in the environment. The use of high-throughput parallel pyrosequencing of 16S rRNA genes from environmental DNA allows rapid analysis of microbial communities (Liu *et al*. 2008; İnceoğlu *et al*. 2011). The reads generated by pyrosequencing can resolve the taxonomical information significantly which made possible to analyse the relative abundances of different members of the microbial communities (Boopathi *et al*. 2015; Boopathi and Ki 2016). Over the morphological identification of cyanobacteria, pyrosequencing methods have many advantages including the higher resolving power, speedy analysis and possibilities of automation (İnceoğlu *et al*. 2011). In addition, NGS based approaches facilitate the precise identification of rare and fragile cyanobacterial taxa. Not only the cyanobacteria but combined analysis of co-occurring bacterial community can be useful in assessing their role in cyanobacterial bloom dynamics.

The cyanobacterial diversity of inland waters has been studied all over the world in order to manage and prevent the harmful algal blooms (Paerl and Otten 2013). The current scenario in global climate change can greatly influence the cyanobacterial communities in various environments, particularly in aquatic environment (Paerl and Huisman 2008; Eiler *et al*. 2013). It is also noted that increase in summer water temperature support the incidence and dominance of cyanobacteria over other phytoplanktons (Park *et al*. 2004; Kosten *et al*. 2012). The studies on cyanobacterial communities in the Nakdong River in South Korea using pyrosequencing showed the seasonal dynamics (Hur *et al*. 2013). They reported that the genus *Prochlorococcus* dominated the May month samples and the relative abundance of *Microcystis* and *Anabaena* increased with increase in water temperature of cyanobacteria (Hur *et al*. 2013).

The Han River is the largest river system in South Korea which is composed of three major tributaries: North Han River, South Han River and Kyungan Stream. The Paldang Reservoir is situated at the junction of these three rivers. The Paldang Reservoir serves as an important water resource for the people living in Seoul metropolitan area and nearby cities. More than 20 million people are depending on the Paldang Reservoir for drinking water. In addition, more than 95 percent of the inflow to the reservoir comes from the South and North Han Rivers, which have relatively clean water quality. In the recent years, increased occurrence of cyanobacterial bloom has been reported in the Paldang Reservoir (Kim *et al*. 2001). However, the available knowledge on cyanobacterial diversity in the Paldang Reservoir is not up to date (Chang *et al*. 1996; Park *et al*. 2000). Chang and Jeon (1996) recorded the diversity of phytoplankton and reported 9 taxa of cyanobacteria, also the eutrophication of the Paldang Reservoir. More recently, a novel and toxic cyanobacterium *Dolichospermum hangangense* was firstly described from Han River, Korea (Choi *et al*. 2018). Hence, considering the current status of the Paldang Reservoir, comprehensive study on cyanobacterial incidence is highly imperative.

In the present study, we determined the diversity and seasonal variations of cyanobacteria in the Paldang Reservoir, using microscopy and a molecular method. Particularly, this study reported the results of cyanobacterial community structure and diversity in the Paldang Reservoir determined by using pyrosequencing. Moreover, this study can be used as a reference for future cyanobacterial community analysis and monitoring programs in the Paldang Reservoir or any other reservoir ecosystem.

MATERIALS AND METHODS

1. Environmental factors and water sample collections

Water temperature, pH, dissolved oxygen (DO) and conductivity from a monitoring site of the Paldang Reservoir (GPS code: 37°39′15.63″N, 127°17′15.89″E; Fig. 1) were measured using YSI 566 Multi Probe System (YSI Inc., Yellow Springs, OH), when environmental sampling was carried out. In addition, water samples from the sampling site were collected at surface by using a 20 L-bucket from March to December 2012. In a brief, three hundred milliliters of water samples were fixed with 1% Lugol's solution (Sigma-Aldrich), and subsequently used for the identification and quantification of cyanobacteria using light microscope (Axioskop, Zeiss, Oberkochen, Germany).

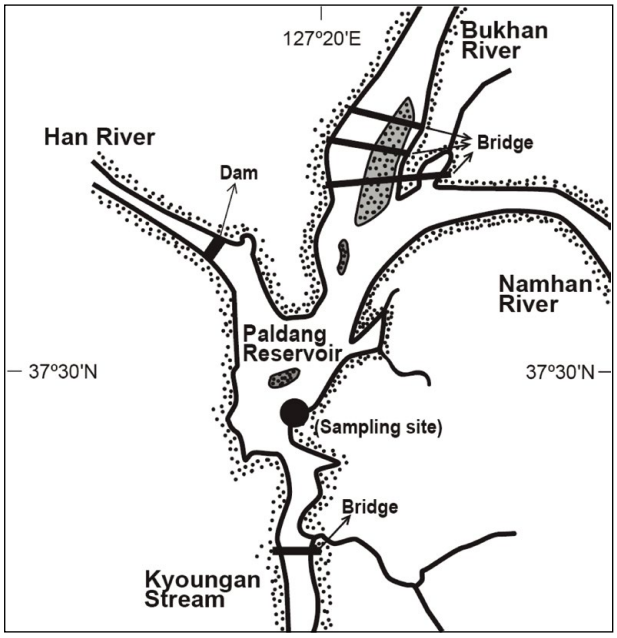

Fig. 1. A map of Paldang Reservoir, Korea. A circle represents a sampling site.

Additionally, samples for environmental DNA extraction were prepared as follows: Firstly, large size organisms such as zooplankton were removed using a 200 μm-pore size mesh. A total of 500 mL of this pre-filtered freshwater was size-fractionated through a 10 μm (Cat. No. TCTP04700, 47 mm diameter, Millipore, Billerica, MA), followed by a 2.0 μm (TTTP04700, 47 mm diameter, Millipore) and 0.22 μm membrane filters (GVWP04700, 47 mm diameter, Millipore), to prevent clogging. The membrane filters were immersed into 0.8 mL extraction buffer (100 mM Tris-HCl, 100 mM Na_2-EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB) and were stored at −80°C until DNA extraction.

2. Nutrient data and chlorophyll *a* measurement

As for nutrient data, total nitrogen (TN) and total phosphorous (TP) were obtained from the Han River Basin Environmental Office (http://www.me.go.kr/hg/web/main.do). The Chlorophyll *a* (Chl-*a*) concentrations was estimated according to Parsons *et al*. (1984). A total of 200 mL of water samples were filtered with GF/F filter (Cat. No. 1825047, 47 mm diameter, Whatman, UK), and those filters were placed in 90% acetone for overnight under the dark in order to extract pigments. The supernatants were used to measure the concentration of Chl-*a* using a DU730 Life Science UV/Vis spectrophotometer (Beckman Coulter, Inc., Fullerton, CA).

3. Environmental DNA extraction

DNA extraction of filtered samples was performed, following a modified protocol by Harder *et al*. (2003). A 2 mL microcentrifuge tube containing each membrane filter (10 μm, 2.0 μm and 0.22 μm) was subjected to freeze-thaw cycles in liquid N_2 and 65°C maintained water bath. Subsequently, 8 μL proteinase K (10 mg/mL in TE buffer) was added and the tube was incubated at 37°C for 30 min. Following incubation, 80 μL 20% sodium dodecyl sulfate (SDS) prepared in double distilled water (ddH_2O) was added and the sample was incubated at 65°C for 2 h. After incubation, the tube were shaken with equal volumes of chloroform-isoamylalcohol (24:1), and centrifuged at 10,000 × g for 5 min. The aqueous phase of mixture was transferred to a new microcentrifuge tube, to which 0.1 volumes of 3 M sodium acetate (pH 5.1, prepared in ddH_2O) and 0.6 volumes of isopropanol (≥99%) were added. The microcentrifuge tube was centrifuged at 14,000 × g for 20 min, the supernatant was discarded, 1 mL cold 70% ethanol was added to the pellet, and the sample was centrifuged again at 14,000 × g for 15 min. The pellet was air dried and reconstituted with 100 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8) (Harder *et al*. 2003).

4. PCR and 454 pyrosequencing

Target rDNA retrieved from the environmental samples was amplified using polymerase chain reaction (PCR). PCR was performed using two universal primers: 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 800R (5′-TAC CAG GGT ATC TAA TCC-3′). Each primer was tagged using multiplex identifier (MID) adaptors according to the manufacturer's instructions (Roche, Mannheim, Germany), which allowed for the automatic sorting of the pyrosequencing derived sequencing reads based on MID adaptors. In addition, MID-linked 27F and 800R1 were linked to pyrosequencing primers, 5′-CGT ATC GCC TCC CTC GCG CCA TCA G-3′ and 5′-CTA TGC GCC TTG CCA GCC CGC TCA G-3′, respectively, according to manufacturer's instructions (Roche, Mannheim, Germany).

Metagenomic PCR reaction was performed with 20 μL reaction mixtures containing 2 μL 10× Ex Taq buffer (TaKaRa, Kyoto, Japan), 2 μL dNTP mixture (4 mM), 1 μL of each primer (10 pM), 0.2 μL Ex Taq polymerase (250 U) and 0.1 μg environmental DNA template. PCR cycling was performed in an iCycler (Bio-Rad, Hercules, CA) at 94°C for 5 min, followed by 35 cycles at 94°C for 20 s, 52°C for 40 s and 72°C for 1 min, and a final extension at 72°C for 10 min. The resulting PCR products were electrophoresed in 1.0% agarose gel, stained with ethidium bromide, and viewed under ultraviolet transillumination.

Prior to pyrosequencing, amplified PCR products were individually purified using a Dual PCR Purification Kit (Bionics, Seoul, Korea), and subsequently, equal volumes of each purified PCR product were mixed together. Pyrosequencing of eight MID-tagged PCR amplicons was performed with a GS FLX Titanium system (Roche, Mannheim, Germany) using a commercial service at Macrogen Inc. (Seoul, Korea).

5. Data cleaning and BLAST (Basic Local Alignment Search Tool) searches

Pyrosequencing data was subjected to systematic checks to remove artifacts and low quality reads using a web-based program (http://microbiomes.msu.edu/replicates/) using default settings. Pyrosequencing artifacts were removed, and the remaining sequence data trimmed using LUCY program (Chou and Holmes 2001). Only high-quality sequence data with long reads *i.e*., those over 150 bp were used for further analyses. Upon comparisons of our pyrosequencing data, perfectly identical sequences were treated as same phylotype, and to reduce data size only single reads with the longest DNA sequence were selected. However, a consensus sequence could also be used as a representative sequence, instead of selecting a longest read. As a result, we constructed a data set that comprised of different genotypic sequence reads. These were subjected to BLAST searches against GenBank database, identified and assigned to their respective taxonomic affiliation. Particularly, we defined species-level phylotypes according to the highest matched species in BLAST search and phylogenetic analysis, with more than 97% sequence identity of the 16S rRNA gene sequence. In addition, those ranging from 92–96.9% similarity were considered to represent same genus, those ranging from 86.0–91.9% similarity were considered to represent same family, and $<$85.9% similarity were considered to represent same order phylotypes. The thresholds stated herein were set based on 16S rRNA sequence comparisons with strains from different species, genera, families and orders.

6. Phylogenetic and statistical analyses

For phylogenetic analysis, we constructed a data set of taxonomic reference sequences; integrating the highest BLAST matched species and well-defined reference sequences for the cyanobacteria. Subsequently, consolidated 16S data set (our unique sequence reads and the selected taxonomic reference sequences) was aligned using Clustal W 1.8 (Thompson *et al*. 1997). The aligned sequences were trimmed at each end to make the same length, and obvious base errors found only in single strands were removed manually. Phylogenetic analyses were performed with the aligned sequences using neighbor-joining (NJ) method with the maximum composite likelihood model in MEGA 5.0 (Tamura *et al*. 2011).

In addition, phytoplankton community structure was analyzed with MVSP software (Kovach Computing Service, Anglesey, Wales, UK) for Shannon-Weaver diversity (H') and Evenness index (E). To identify different clustering assemblages from the phylotypes identified during the present study unweighted pair group method with arithmetic mean (UPGMA), with Bray Curtis dissimilarities, was utilized. Cluster analysis was performed with a multivariate statistical package program MVSP3.1 (Kovach Computing Service, Wales, UK).

RESULTS AND DISCUSSION

1. Environmental conditions and phytoplanktons in the Paldang Reservoir

Physicochemical variables, such as water temperature, dissolved oxygen (DO), pH, conductivity, total nitrogen and phosphorous were recorded for all the months from March to December 2012 in the Paldang Reservoir (Fig. 2). The water temperature and DO levels were found to be inverse-

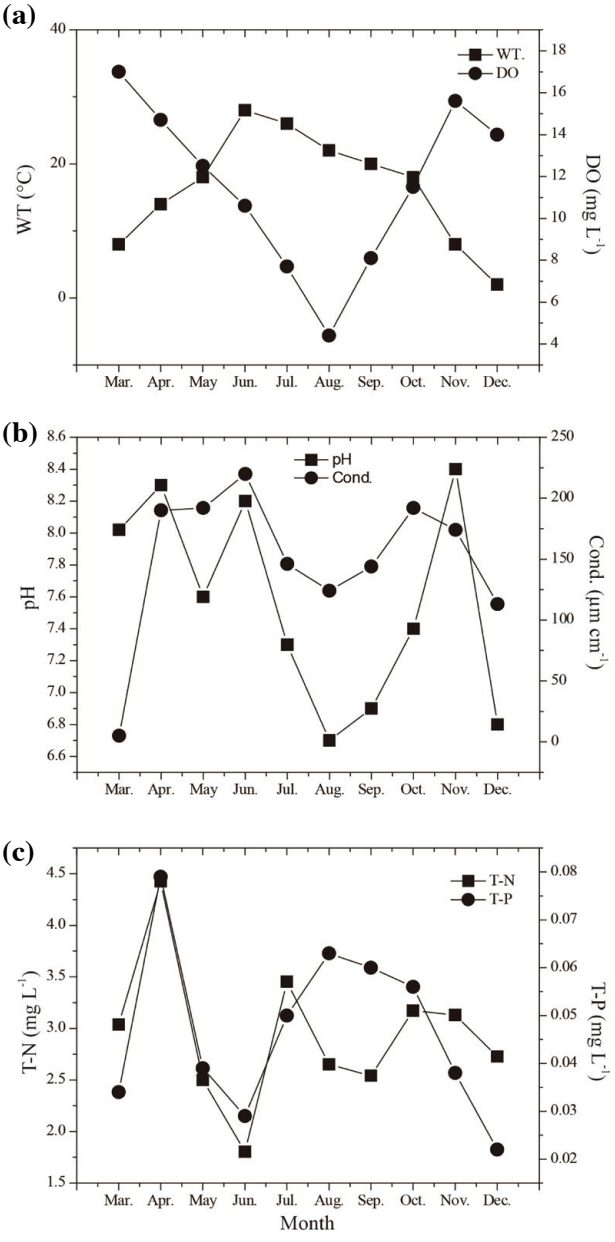

Fig. 2. Monthly variations of (a) Water temperature (WT) and dissolved oxygen (DO); (b) pH and conductivity; (c) Total Nitrogen (TN) and Total Phosphorous (TP) in Paldang Reservoir March-December 2012.

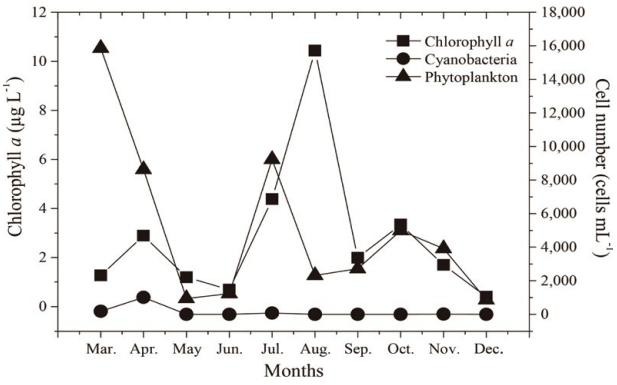

Fig. 3. Monthly variation of Chlorophyll-*a*, phytoplankton and cyanobacteria in Paldang Reservoir March-December 2012.

ly proportional to each other; increased water temperature decreased the DO levels. In addition, total nitrogen and phosphorous levels were found to be synchronous except in August and September month samples. As for biological factors, we analyzed chlorophyll *a* (Chl-*a*), total phytoplanktonic cells and total cyanobacterial cells from the water samples (Fig. 3; Table 1). Chl-*a* concentration was increased in April and subsequently decreased up to July. Then, a sudden increase in Chl-*a* was observed during the month of August. The Chl-*a* concentration was declined in September and followed by a raise during October. The phytoplanktonic cell numbers were found to be high in March, April and July. On the other hand, cyanobacterial cell numbers were high in March, *i.e*., 11.8% of total phytoplankton (Table 1). In addition, we discriminated 18 different cyanobacteria within generic-level identification under microscopic observations (Fig. 4), due to morphological resemblance among species and extremely small in body size. These included *Anabaena*, *Aphanocapsa*, *Chroococcus*, *Dactylococcopsis*, *Gloeotrichia*, *Microcystis*, *Merismopedia*, *Phormidium* and *Pseudoanabaena*. Overall, Chl-*a* concentrations were congruent with fluctuation patterns of both cell numbers and pyrosequence reads detected by microscopy and pyrosequencing, respectively.

Upon comparisons of cyanobacterial and total phytoplanktonic cell numbers, we found that there were only very few occurrences of cyanobacteria in March and June; however, phytoplankton was recorded at the highest abundance in the same periods. Microscopic and molecular data clearly showed that it was caused by a huge number of small centric diatoms, *Cyclotella*, *Stephanodiscus* and *Thalassiosira*-like spp. (see Fig. 3; Boopathi and Ki 2016), of which results were well supported by previous works (Kim *et al*. 2001; Jung *et al*. 2009). In considering environmental aspects, the occurrence of cyanobacteria is influenced by many environmental factors, such as light, temperature, pH, nutrients, etc. (Scott and Marcarelli 2012). For examples,

Table 1. Cyanobacteria cell numbers (cells mL^{-1}) of each taxon in Paldang Reservoir

Species	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Anabaena sp.	–	–	–	–	7	4	–	–	–	–
Chroococcus sp.	–	–	–	–	–	4	–	–	–	–
Coelosphaerium sp.	92	–	–	–	–	–	–	–	–	–
Dactylococcopsis sp.	–	1,015	–	–	–	–	–	–	–	–
Merismopedia sp.	–	–	–	1	50	–	–	–	–	–
Oscillatoria sp.	92	–	–	–	–	–	–	–	–	–
Phormidium sp.	–	–	–	–	–	–	–	–	4	–
Pseudanabaena sp.	–	–	–	–	29	–	–	5	9	–
Total cyanobacterial cells	184	1,015	0	1	86	8	0	5	13	0
Total phytoplankton	15,855	8,625	940	1,235	9,235	2,320	2,720	4,990	3,920	860
Percentage of cyanobacteria (%)	1.2	11.8	0.0	0.1	0.9	0.3	0.0	0.1	0.3	0.0

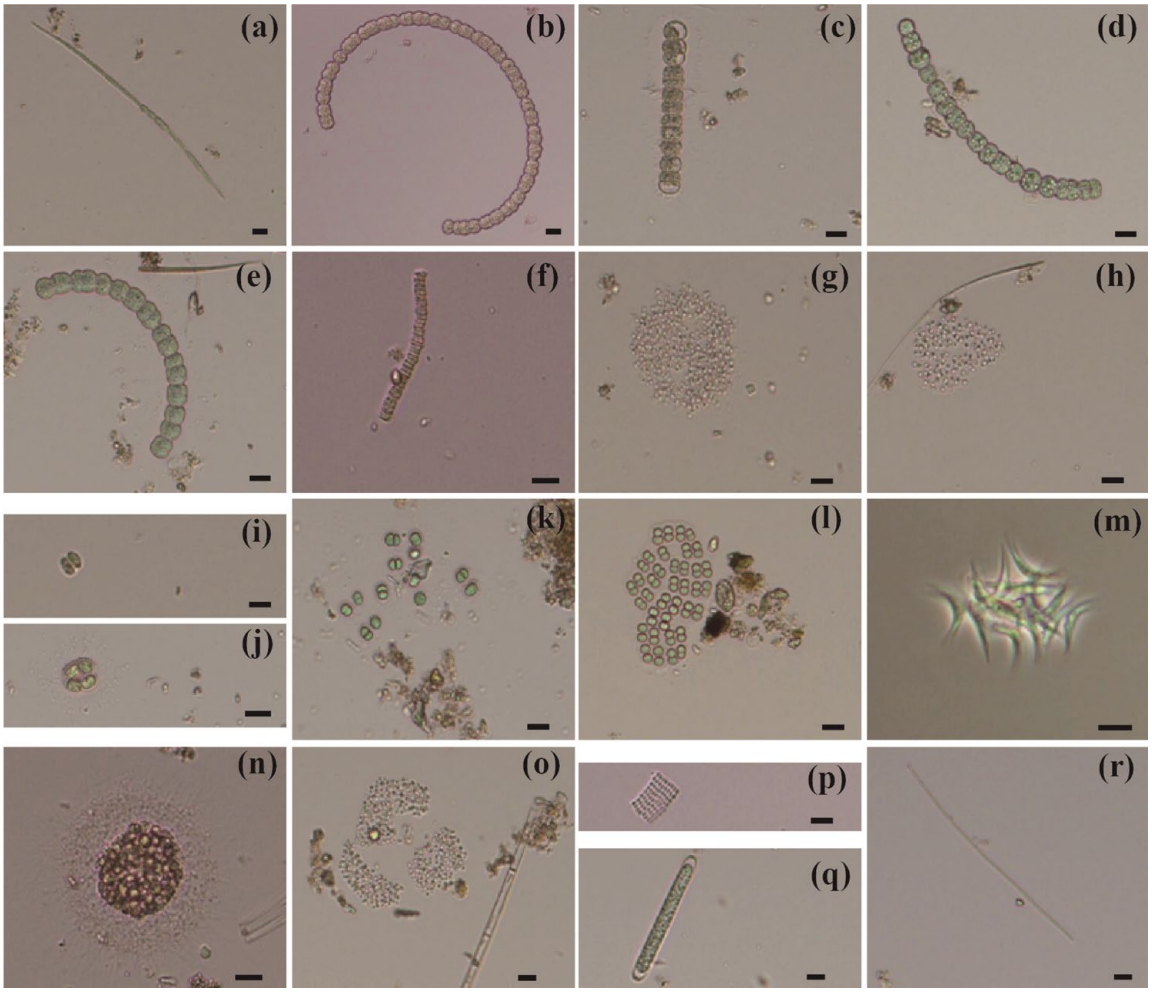

Fig. 4. Cyanobacterial species encountered during microscopic analysis of samples. (a) *Anabaena* sp.1, (b) *Anabaena* sp.2, (c) *Anabaena* sp.3, (d) *Anabaena* sp.4, (e) *Anabaena* sp.5, (f) *Anabaena* sp.6, (g) *Aphanocapsa* sp.1, (h) *Aphanocapsa* sp.2, (i) *Chroococcus* sp.1, (j) *Chroococcus* sp.2, (k) *Chroococcus* sp.3, (l) *Chroococcus* sp.4, (m) *Dactylococcopsis* sp., (n) *Gloeotrichia piscum*, (o) *Microcystis* sp., (p) *Merismopedia* sp., (q) *Phormidium* sp. and (r) *Pseudoanabaena* sp. Scale bars = 10 μm.

Table 2. Pyrosequencing data (a total of 49,131 reads) for bacteria with focus on cyanobacteria

Month	Total reads	Removed reads	Used reads	Cyanobacterial reads	No. of cyanobacterial OTU	%
Mar.	5,364	395	4,969	0	0	0.00
Apr.	6,552	614	5,938	10	5	0.17
May	4,187	308	3,879	2	1	0.05
Jun.	4,592	305	4,287	4	2	0.09
July.	3,483	360	3,123	4	3	0.13
Aug.	8,181	690	7,491	44	23	0.59
Sep.	4,400	471	3,929	0	0	0.00
Oct.	5,251	419	4,832	7	5	0.14
Nov.	3,072	306	2,766	3	3	0.11
Dec.	4,049	481	3,568	0	0	0.00

cyanobacteria dominance was positively influenced by climate-induced changes in the thermal regime rather than direct temperature effects (Wagner and Adrian 2009). In addition, the nutrient concentrations such as total nitrogen or phosphorous not nutrient stoichiometry (*i.e*. N:P ratio) were found to influence the cyanobacterial dominance in the lake environment (Downing *et al*. 2001). Total phosphorus concentration was one of the principal factors influencing cyanobacterial contribution to total algal biomass (Wagner and Adrian 2009). Even, the biomass of cyanobacterial genera such as *Aphanizomenon*, *Anabaena* and *Microcystis* were greatly influenced by various levels of total nitrogen and total phosphorous concentrations (Wagner and Adrian 2009). These well explained phytoplankton and cyanobacterial abundances, suggesting the seasonal succession of phytoplankton in temperate reservoirs (Park *et al*. 2000).

2. Pyrosequence characteristics and taxonomic affiliations

Totally, 44,782 sequences were used for the analysis from 49,131 sequences after removing sequencing artifacts and low quality reads (Table 2). Overall, the phyla Actinobacteria, Proteobacteria and Bacteroidetes were mostly predominated in monthly samples (data not shown). Specifically, the phyla Proteobacteria and Bacteroidetes are found to be dominant (~94–97%) sequences from the samples collected in March. In addition, the sequences belonging to Actinobacteria, Proteobacteria and Bacteroidetes were found to be dominant in June. However, the phyla Actinobacteria, Proteobacteria and Bacteroidetes were constituted a major portion, *i.e*., ~95–98% of sequences belonged to the bacterial phyla. In addition, other groups were also found consistently, but at lower abundance, were belong to phyla Firmicutes, Chloroflexi, Chlorobi, Verrucomicrobia, Acidobacteria, Lentisphaerae and Planctomycetes. This was generally in congruent with Eiler *et al*. (2013), reporting 8.1% of chloroplast and cyanobacterial sequences from a total of 1,116,833 sequences from 259 samples. The chloroplast sequences were found to be high in March, April and August, irrespective of all stations which can be directly attributed to the presence of eukaryotic algal members in the bloom community.

3. Phylogenetic analysis of cyanobacterial community

In the present study, based on Ribosomal Database Project (RDP) and BLASTn analysis we identified 40 phylotypes of cyanobacteria belonging to 8 genera. The respective positions of these cyanobacteria in the 16S rRNA gene phylogeny were analysed using Neighbor-Joining method (Fig. 5). The sequences from all the months were combined with the 16S rDNA sequences of known cyanobacterial type strain retrieved from NCBI were used for the construction of phylogenetic tree. We used 89 partial 16S rRNA gene sequences for alignment which contained 738 characters among them 281 were conservative and 279 were parsimony informative. The tree was rooted to the *Chlorobium* sp. sequence. However, the cyanobacterial sequences were clearly clustered into four orders, Synechococcales (27.5%), Chroococcales (47.5%), Nostacales (20%) and *Pseudanabaena*les (5%). The major part of the cyanobacterial sequences (65%) were affiliated to uncultured cyanobacteria that were primarily arrived from environmental studies. The sequences of uncultured origins were successfully

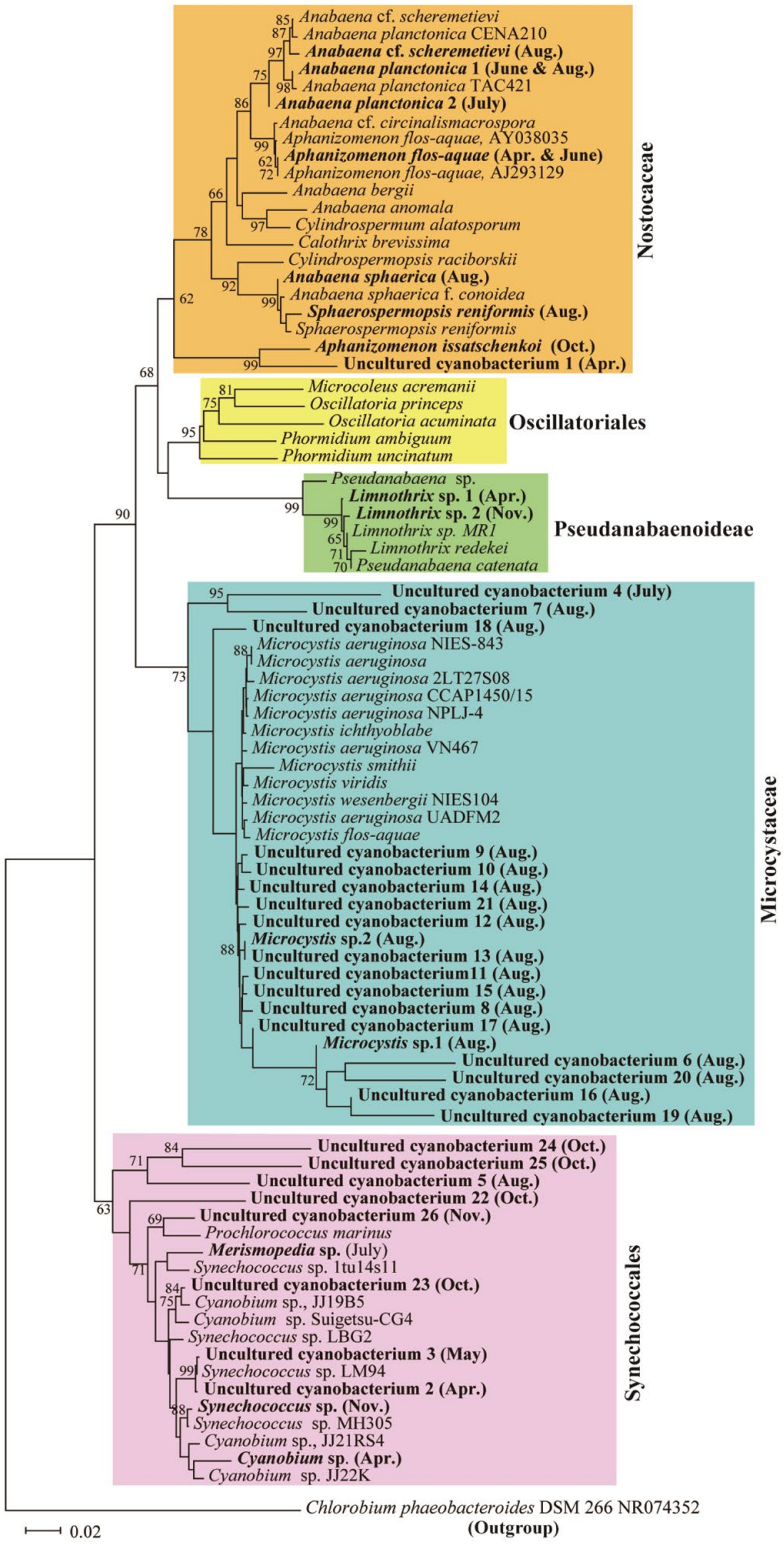

Fig. 5. The phylogenetic tree of cyanobacteria was drawn using Neighbor-Joining method (Acinas *et al*. 2005). The optimal tree with sum of branch length = 2.579 is shown. Percentage of replicate trees in which associated taxa clustered in the bootstrap test (1,000 replicates) are shown next to branches (Chang *et al*. 1996). The tree is drawn to scale, with branch lengths in the same units as those of evolutionary distances used to infer the phylogenetic tree. The names in bold are obtained from this study. Sampling stations followed by month are given in brackets. The phylogenetic tree was rooted to *Chlorobium phaeobacteroides* (NR074352).

assigned to respective genera using this phylogenetic tree. In the phylogenetic tree 65.3% of the uncultured cyanobacterial sequences were clustered into the Microcystaceae clade. Among the Nostocales, high numbers of sequences with high similarity (99−100%) to *Anabaena* sp. were observed. The order Nostacales resolved genera including *Aphanizimenon*, *Dolichospermum* and *Sphaerospermopsis*. The cluster Pseudanabaenoideae comprised of *Limnothrix* sp. However, 30.7% of the uncultured cyanobacterial sequences were clustered into the clade Synechococcales. The cyanobacterial genera including *Merismopedia*, *Cyanobium* and *Synechococcus* were clearly resolved in this clade. Of the obtained cyanobacterial sequences, majority of the sequences were belongs to the uncultured cyanobacteria and many other closely related to the marine cyanobacterial species which shows the lack of comparable sequences from freshwater origins in the available database. Therefore, it is necessary to culture and characterized the freshwater taxa which would be helpful in comparing the results from environmental surveys. We observed that the cyanobacterial genera *Anabaena*, *Microcystis*, *Synechococcus*, *Aphanizomenon*, *Cyanobium* and *Limnothrix* were present in the Paldang Reservoir. It was also found that many of observed cyanobacterial species were previously reported as potential toxin producers (Bittencourt-Oliveira *et al*. 2012; Humpage *et al*. 2012). Thus, the continuous monitoring of cyanobacteria in the Paldang Reservoir is imperative to control the noxious bloom formation at the earlier stage.

In addition to this, the UPGMA cluster analysis of bacterial diversity at the phylum level using Bray Curtis showed dissimilarities among samples from different months. The Bray Curtis dissimilarities varied between zero (complete similarity) to 1 (total dissimilarity). It showed three clusters, in which the samples from consecutive months were found more similar (Fig. 6). For example, the samples from April and March; October and November were found to exhibit the similar pattern of diversity. However, the August month sample shown to be much different from the other samples.

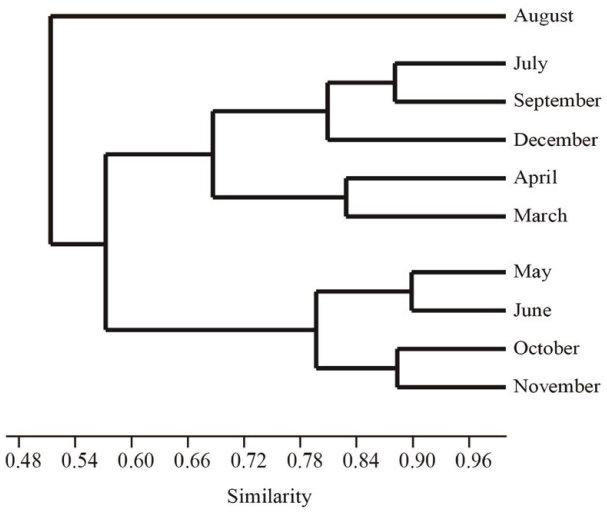

Fig. 6. Cluster analysis (UPGMA) based on bacterial diversity at phylum level using Bray Curtis dissimilarities among sampling sites.

4. Cyanobacterial distribution pattern – pyrosequencing and microscopic data

As described previously, totally eight cyanobacterial genera (not species level) were identified by morphological observations. Different cyanobacterial species were identified from the different month samples; for examples, *Anabaena* sp. and *Chroococcus* sp. were detected from the samples from August; *Coelosphaerium* sp. and *Oscillatoria* sp. in March; *Dactylococcopsis* sp. in April; *Merismopedia* sp. in June and July; *Phormidium* sp. in November and *Pseudanabaena* sp. in July, October and November. On the other hands, pyrosequencing analysis revealed quite different compositions of cyanobacterial species, and only one genus (*Merismopedia* sp) was detected in both methods. In the present study, we identified a total of 74 pyrosequence reads belonging to 40 phylotypes of cyanobacteria, which accounted for 0.16% of the total reads (Table 2). Seasonal comparisons revealed that the cyanobacteria were found to be absent in samples collected from March, September and December. Among the bacterial phyla, relative abundance of cyanobacteria exhibited a seasonal pattern. The cyanobacteria were initially absent in March and then a notable increase in April followed by an increasing pattern from May to August. Interestingly, the highest number of cyanobacterial reads was observed in August (*i.e*. 0.59%), which well matched with chlorophyll data rather than cyanobacterial cell counts.

In addition, relative abundance of individual species was calculated with pyrosequence reads (Fig. 7). For examples, *Anabaena planctonica* was present in June, July and

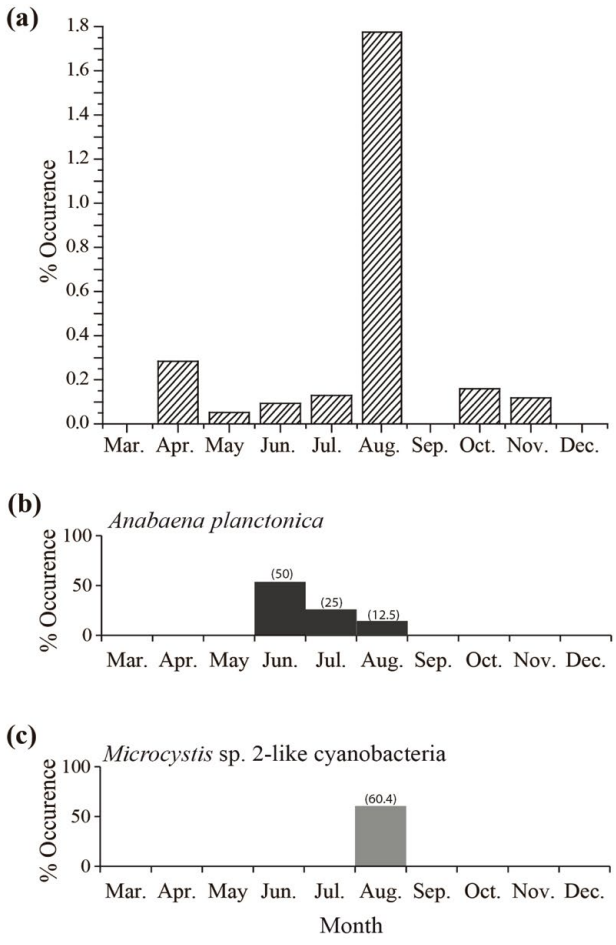

Fig. 7. Relative abundances of phylum cyanobacteria (a) and dominant phylotypes [(b): *Anabaena planctonica*; (c): *Microcystis* sp. 2-like uncultured cyanobacteria] from the total 16S rRNA gene sequences at Paldang Reservoir from March−December 2012.

August month samples, which were in accordance with morphological observation. However, we also found that the uncultured cyanobacteria were present in most of the samples. In particular, they are predominant in the August month sample; however, these reads were closely related to *Microcystis* sp. 2 (see Fig. 5).

Shannon-Weaver diversity index (H') is generally used to describe the species diversity in a community. In the present study, H' scores varied between 0 (May) to 3.13 (August) as listed in Table 3, showing that the cyanobacterial diversity in August was comparatively higher than the other months. In addition, the Chao-1 species richness estimator values were ranged between 1 (May) to 351 (August), and they are also congruent with the Shannon-Weaver diversity index values.

Further analysis was carried out to find the relationship between microscopic and molecular diversity of cyanobacteria. Pyrosequencing analysis revealed that DNA affiliations (8 genera, 13 species and 26 unidentified species) were a greater diversity rather than microscopical observation (8 genera). In pyrosequence data, we did not detect three genera, such as *Dactylococcopsis*, *Oscillatoria* and *Phormidium*, although they were observed by microscopy. This kind of incomparable diversity pattern between microscopic and molecular methods was previously reported by many others (Taton *et al*. 2003; Lopes *et al*. 2012; Eiler *et al*. 2013). As noted, this may be caused by misidentification of cyanobacteria, due to microscopic ambiguities, taxonomic experiences and skills. Even, certain cyanobacteria (*e.g*. single Microsystis, *Prochlorococcus* and *Synechococcus* spp.) resemble in size and morphology to green algae (*Chlorella* spp.). In addition, we could explore low number of reads belong to cyanobacteria in pyrosequencing, when compared to total cyanobacterial cell numbers detected microscopically, but still with higher diversity which necessitate more research on standardizing both microscopic and molecular methods to obtain comparable results. However, we cannot neglect any of the methods but combining the results will be helpful to resolve the complete picture of the cyanobacterial diversity as much as possible. Moreover, our results were similar to the microscopical surveillance of cyanobacteria in the Paldang Reservoir during July to December, 1997 (Park *et al*. 2000). It was found that cyanobacterial species were dominant during summer and autumn and diatoms in the winter and spring in the Paldang Reservoir (Park *et al*. 2000). *Microcystis* was a dominant genus during summer time. They also found that the water temperature has played a major role in cyanobacterial succession and also the dam gate operation affected the abundance and dominance of cyanobacterial genera in the Paldang Reservoir (Park *et al*. 2000). In addition, similar results were recorded in the Nakdong River in South Korea using pyrosequencing which showed that the relative abundance of *Microcystis* and *Anabaena* were increased with increase in water temperature (Hur *et al*. 2013).

Table 3. Distribution of cyanobacterial species in samples from Paldang Reservoir. Shannon-Weaver diversity index (H') and Chao-1 estimator values given for all the months

Cyanobacteria	Apr.	May	Jun.	Jul.	Aug.	Oct.	Nov.
Anabaena planctonica 1	−	−	+	−	+	−	−
Anabaena planctonica 2	−	−	−	+	−	−	−
Anabaena cf. *scheremetievi*	−	−	−	−	+	−	−
Anabaena sphaerica	−	−	−	−	+	−	−
Aphanizomenon flos-aquae	+	−	+	−	−	−	−
Aphanizomenon issatschenkoi	−	−	−	−	−	+	−
Cyanobium sp.	+	−	−	−	−	−	−
Limnothrix sp.1	−	−	−	+	−	−	−
Limnothrix sp.2	−	−	−	−	−	−	+
Merismopedia sp.	−	−	−	+	−	−	−
Microcystis sp.1	−	−	−	−	+	−	−
Microcystis sp.2	−	−	−	−	+	−	−
Sphaerospermopsis reniformis	−	−	−	−	+	−	−
Synechococcus sp.	−	−	−	−	−	−	+
Uncultured cyanobacterium 1	+	−	−	−	−	−	−
Uncultured cyanobacterium 2	+	−	−	−	−	−	−
Uncultured cyanobacterium 3	−	+	−	−	−	−	−
Uncultured cyanobacterium 4	−	−	−	+	−	−	−
Uncultured cyanobacterium 5	−	−	−	−	+	−	−
Uncultured cyanobacterium 6	−	−	−	−	+	−	−
Uncultured cyanobacterium 7	−	−	−	−	+	−	−
Uncultured cyanobacterium 8	−	−	−	−	+	−	−
Uncultured cyanobacterium 9	−	−	−	−	+	−	−
Uncultured cyanobacterium 10	−	−	−	−	+	−	−
Uncultured cyanobacterium 11	−	−	−	−	+	−	−
Uncultured cyanobacterium 12	−	−	−	−	+	−	−
Uncultured cyanobacterium 13	−	−	−	−	+	−	−
Uncultured cyanobacterium 14	−	−	−	−	+	−	−
Uncultured cyanobacterium 15	−	−	−	−	+	−	−
Uncultured cyanobacterium 16	−	−	−	−	+	−	−
Uncultured cyanobacterium 17	−	−	−	−	+	−	−
Uncultured cyanobacterium 18	−	−	−	−	+	−	−
Uncultured cyanobacterium 19	−	−	−	−	+	−	−
Uncultured cyanobacterium 20	−	−	−	−	+	−	−
Uncultured cyanobacterium 21	−	−	−	−	+	−	−
Uncultured cyanobacterium 22	−	−	−	−	+	−	−
Uncultured cyanobacterium 23	−	−	−	−	+	−	−
Uncultured cyanobacterium 24	−	−	−	−	+	−	−
Uncultured cyanobacterium 25	−	−	−	−	−	+	−
Uncultured cyanobacterium 26	−	−	−	−	−	−	+
Shannon-Weaver Diversity Index	1.39	0	0.69	1.39	3.26	0.69	1.09
Chao1	10	1	3	10	351	3	6

5. Implications of molecular cyanobacterial detection

The global warming has effects on hydrological parameters which affect physiochemical and biological processes in the environment, particularly bloom formation. In general, the growth of cyanobacteria has been promoted selectively by the temperature raise rather than other bloom forming higher algae (Park *et al*. 2004; Kosten *et al*. 2012; Paerl and Paul 2012). This trend insists the need of more attention in monitoring cyanobacteria in aquatic environment. It is also found that the increase in the temperature of

surface waters increases the vertical stratification in aquatic ecosystems. In addition, seasonal warming also extends the period of stratification (Paerl and Paul 2012). Eiler *et al*. (2013) studied the phytoplankton diversity in various freshwater lakes using 16S rRNA, found that the NGS derived phytoplankton composition differed significantly among lakes with different trophic status and suggested the use of this technique to monitor the phytoplankton communities for the better ecosystem management.

Unlike other bacteria, identification of cyanobacteria by using microscopical methods requires extraordinary skills and rich experience which majorly hamper the monitoring process in freshwater resources where the occurrence of bloom is a major problem. In addition, it was assumed that major portion of cyanobacterial strains in the culture collections are misidentified (Komárek and Anagnostidis 1989). Phylogenetic analysis using 16S rRNA gene of cyanobacteria provides taxonomic resolution at best classification to the genus level (Eiler *et al*. 2013). NGS-based characterization of 16S rRNA genes can be promising tool for monitoring inland waters in a high-throughput, reproducible and cost-efficient manner (Eiler *et al*. 2013). However, in NGS analysis, biases may be introduced by DNA extraction and PCR procedures (Martin-Laurent *et al*. 2001; Acinas *et al*. 2005). In the present scenario, the use of NGS analysis in continuous monitoring of freshwater ecosystems is merely possible, but requires extensive research on optimization and standardization of all steps from sample collection to the analysis. In addition, the controversies between cyanobacterial phylogeny and various morphological classification systems need to be addressed by improving the taxonomic frameworks in future (Gugger *et al*. 2002; Zapomělová *et al*. 2009). Moreover, the automation of the procedures can lead to precise and constant results. The future research should be focused to address aforesaid problems to the effective use of pyrosequencing method in environmental monitoring.

In conclusions, pyrosequencing analysis using 16S rDNA enabled us to identify the seasonal variation of cyanobacteria present in the Paldang Reservoir, highlighting significant increase in cyanobacterial diversity during summer months. The pyrosequencing analysis also resolved more cyanobacterial phyla when compared to microscopical studies. This study can be a valuable reference for comparing the cyanobacterial diversity in future studies in the Paldang Reservoir. In addition, it is suggested that the cyanobacteria can be used as a bioindicator organism in monitoring the trophic status of freshwater resources. In the context of monitoring cyanobacteria in inland freshwater more research should be initiated to improve the sampling strategies, processing steps and comparable sequence databases for microbes from freshwater. The NGS approach may be useful in providing support to the various monitoring programs to maintain superior water quality in aquatic resources.

ACKNOWLEDGEMENTS

We thank J.-Y. Cheon for water sampling in environments. This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (2016R1D1A1A09920198) and by a grant from the National Institute of Fisheries Science, Korea (R2018043) funded to J.-S. Ki.

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Received: 31 July 2018
Revised: 11 September 2018
Revision accepted: 12 September 2018