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Bacterial Community Composition Associated with Freshwater Cyanobacterial Blooms of Intensive Culture Ponds

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ABSTRACT

To determine the composition of the bacterial communities associated with cyanobacteria in the freshwater ponds of *Channa argus* in intensive culture in the late culture period, we examined the bacterial community of non-cyanobacterial blooms freshwater ponds (C) and cyanobacterial blooms freshwater ponds (CB) using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and 16S rDNA clone library. DGGE profiles showed that *Microcystis* sp. (JX391871.1), *Leptospira* sp. (JX067673.1) and *Verrucomicrobium* sp. (FN668203.2) existed only in CB, whereas *Sphingomonas* sp. (AB681542.1) and *Paenibacillus* sp. (JQ659563.1) were the C-specific bacteria. The 16S rDNA clone libraries of pond water showed that Actinobacteria (15 OTUs, 31.9%), Bacteroidetes (13 OTUs, 28.7%), Fibrobacteres (7 OTUs, 14.9%) and Proteobacteria (5 OTUs, 10.0%) were the dominant groups in C; the four dominant groups in CB were Bacteroidetes (17 OTUs, 34.7%), Actinobacteria (9 OTUs, 18.4%), Fibrobacteres (6 OTUs, 12.2%) and Proteobacteria (8 OTUs, 16.2%). In the clone library, distribution of sequences belonging to *Fibrobacter* in CB (22.2% of the 99 clones) was twice as that of C (11.1% of the 99 clones). In both DGGE map and 16S rDNA clone library the Firmicutes group (JQ659563.1 in DGGE and 3 OTUs in C library) existed only in C. These results suggested that the composition of bacterial communities changed with the cyanobacterial blooms in *Channa argus* intensive culture freshwater ponds, and whether the change of the Firmicutes-to-Bacteroidetes ratio could be used to predict cyanobacterial blooms in these types of ponds required further validation.

INTRODUCTION

The production of hybrid snakehead (*Channa argus*) in China reached 0.5 million tons in 2014 (Yearbook of Fishery Statistics 2014, China). The breeding density of hybrid snakehead is about 10000 kg ha^{-1}. Hybrid snakehead is a carnivorous freshwater fish, which can be fed the pellet extruded feed by weaning, protein level of feed is about 40%. High-yielding intensive culture ponds of hybrid snakehead are typically characterized by high density and high input. The large amount of residual feeds, excrement of cultured species, and dead organic debris entering into the aquaculture water can easily lead to nitrogen pollution and eutrophication, and deterioration of aquaculture ecological environment, finally resulting in frequent cyanobacterial blooms, threatening fish health and bringing harmful effects on water around, which are not conducive to aquaculture development (Zhong et al. 2011). The blooms of mucilaginous Cyanobacteria will limit light penetration below water surface (Carey et al. 2012). The death and degradation of Cyanobacteria will cause a decline in dissolved oxygen (DO) in water (Li et al. 2011a), resulting in surface scum and smell. Moreover, some species may produce cyanobacterial toxins, posing a serious threat to aquatic organisms and human health (Wu et al. 2010). Therefore, controlling cyanobacterial blooms has become one of the key water-control techniques in pond culture. In-depth research of the mechanism and dominant contributing factors of cyanobacterial blooms is the key to solving this problem.

Previous studies have focused on Cyanobacteria species composition and physical and chemical factors affecting cyanobacterial growth (Zhang et al. 2016, Lv et al. 2014). In recent years, research has also been performed to explore the relationship between Cyanobacteria and heterotrophic bacteria in the environment, in order to better understand the processes and mechanisms of cyanobacterial bloom formation (Cai et al. 2014, Berg et al. 2009). Aquatic hypoxia caused by cyanobacterial blooms and secondary pollution by algal toxins (Li et al. 2011b) can be repaired by heterotrophic bacteria. Some heterotrophic bacteria are capable of degrading organic matter produced by cyanobacterial blooms to avoid decreased DO and death of cultured species (Havens 2007), for example, *Legionella pneumophila* can utilize the organic matter produced by cyanobacterial blooms (Berendt 1981). Some other heterotrophic bacteria

are capable of degrading cyanobacterial toxins produced by cyanobacterial blooms (Ho et al. 2007); for example, *Sphingomonas* sp. which belongs to α-Proteobacteria, has a strong ability to degrade microcystin (MC) (Li et al. 2011a). Some other heterotrophic bacteria are also capable of degrading cyanobacterial biomass directly; for example, novel *Clostridium* can directly degrade *Microcystis* cells under hypoxic conditions (Xing et al. 2011).

Therefore, we aimed to determine the composition of bacterial communities associated with cyanobacteria in the freshwater ponds of *Channa argus* in intensive culture of late culture periods using the denaturing gradient gel electrophoresis (DGGE) technology and 16S rDNA gene clone libraries. To the best of our knowledge, this was the first attempt to investigate the composition of bacterial communities in such intensive culture ponds. An effective way for blooms prevention and controlling of these water bodies might be found in this study.

MATERIALS AND METHODS

Sampling and chemical analysis: *Channa argus* (body weight of 11.36 ± 0.57 g) was fed with 42% protein feeds at a density of 225,000 fingerlings/ha. The feed coefficient was 1.1-1.2, and the final yield of *Channa argus* was 75,000 kg/ha. Water samples were collected from six ponds located in Sanshui, Guangdong Province (China) on October 26, 2012. These six ponds were divided into two groups: the control group containing three non-cyanobacterial bloom ponds (C1, C2 and C3), and the experimental group containing three cyanobacterial bloom ponds (CB1, CB2 and CB3). Table 1 shows the physical and chemical characteristics of pond water. These samples were placed into sterile bottles and transported to the laboratory in a mini-icebox. Then, 200 mL of each sample was immediately filtered with a 0.2 μm filter (PALL, Supor-200, 47 mm, USA), and filters with microorganisms were stored at -40°C until further analysis.

DNA extraction and polymerase chain reaction (PCR) amplification: Frozen filters with microorganisms were cut into small pieces (1-2 mm^2) using a sterile scalpel, and then DNA was extracted using E.Z.N.A.™ water DNA kit (OMEGA, USA) according to the manufacturer's instructions. The isolated DNA was purified using an E.Z.N.A.™ microelute DNA cleanup kit (OMEGA, USA) according to the manufacturer's instructions. PCR amplifications were performed in a 25 μL reaction system containing 0.5 μL of forward and reverse primers (10 pmol of each), 1.0 μL of DNA template, 0.5 μL of dNTPs (2.5 mM of each, TaKaRa, Japan), 2.5 μL of Ex-Taq buffer (TaKaRa, Japan), 0.2 μL of Ex-Taq polymerse (TaKaRa, Japan) and a certain amount of sterile water. The primers used for DGGE were as follows: GC-338F (52 -CGCCC GCCGC GCGCG GCGGG CGGGG CGGGG GCACG GGGGG ACTCC TACGG GAGGC AGCAG-32) consisting of primers 338F attached to the 32 -end of a GC clamp and 534R (ATTAC CGCGG CTGCT GG). The PCR amplification was performed with a PCR System 9700 (Foster, CA, USA). Touchdown PCR was performed as previously described (Muyzer et al. 1993). In order to establish the 16S rDNA gene clone libraries, 16S rDNA gene in each group was mixed thoroughly and was amplified using broad-range bacteria-specific primers 27F (52 -AGAGT TTGAT CCTGG CTCAG-32) and 1492R (52 -TACGG CTACC TTGTT ACGAC TT-32). Briefly, after a denature step of 94°C for 5 min, amplifications were carried out with 35 cycles at a melting temperature of 94°C for 30 sec, an annealing temperature of 58°C for 30 sec, and an extension temperature of 72°C for 1.5 min. Finally, an extra extension step of 72°C for 10 min was performed. PCR products were purified using E.Z.N.A.™ gel extraction kit (OMEGA, USA).

DGGE analysis: DGGE was performed using a gradient of denaturing agents (35-55%) in an Ingeny phorU2 (Ingeny, Netherlands). Electrophoresis was performed at 60°C for 12h under a constant voltage of 110 V in a DGGE chamber containing approximately 17 L of 1×TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). After the electrophoresis, the gels were stained with silver nitrate as previously described (Taketani et al. 2010). DGGE gel was then scanned using an HP Colour LaserJet CM1312nfi MFP. Banding patterns of DGGE profile were analysed by software Quantity One-1-D (version 4.5) (BIO-RAD Laboratories).

The total number of bands (N) in a gel track was first

Table 1: Description of the physical and chemical characteristics of pond water.

Treatment	Mean depth(m)	pH	Water Temp (°C)	NH_3-N (mg L^{-1})	NO_2-N (mg L^{-1})	NO_3-N (mg L^{-1})	Secchi disc depth(cm)	ORP	Oxygen (mg L^{-1})
C	1.56±0.55	7.75±0.11	27.2±0.29	2.41±0.27	0.20±0.03	2.10±1.10	14.57±0.51a	157.67±6.11	17.04±2.53
CB	1.55±0.95	8.05±0.26	27.5±0.14	2.61±0.26	0.18±0.04	3.67±1.45	6.67±1.53b	133.67±16.29	19.68±1.82

Notes: C: ponds without cyanobacterial blooms, CB: ponds with cyanobacterial blooms. Different superscript letters in the same column denote significant differences between treatments ($p<0.05$).

corrected and transformed into richness estimates (R) as previously described (Nubel et al. 1999). Approximately, the maximum number of visible bands in a gel track was 100 in our experiment. Accordingly, crowding was likely to affect the results when N >10. After matching bands from independent tracks, pairwise Dice's coefficients of similarity were determined as follows: $S_D = 2n_{AB}/(n_A+n_B)$, where n_A and n_B were the total number of bands in the tracks A and B, respectively, and n_{AB} was the number of bands common to tracks A and B. Moreover, the binary data representing banding patterns were used to generate a pairwise dice distance matrix. A dendrogram was obtained by unweighted pair group mean average (UPGMA) cluster analysis using XLSTAT Pro 7.5 software.

In order to investigate the multiple aspects of microbial diversity, DGGE patterns were examined using two indexes. The Shannon-Weaver index of diversity, H (Shannon & Weaver 1963), and the equitability index, E (Pielou 1975), were calculated for each sample as follows: $H = -\Sigma(Pi \times \ln Pi)$, where Pi was the importance probability of the band i in a track, and H was calculated based on the bands on the gel tracks using the band intensity as judged by peak density. The importance probability P was calculated as $Pi = n_i/\Sigma n_i$, where n_i was the peak density of band i in a track. $E = H/\ln N$, where N was the sum of all the surfaces for all bands in a given sample (used as an estimate of species abundance) (Moura et al. 2009).

Sequencing of excised bands and phylogenetic analysis: Fig. 1 shows that DGGE bands with higher intensity and frequency were selected for excision. The gel was excised from the middle of the band using a sterile razor, the gel slice was placed in a sterile 1.5 mL micro-centrifuge tube, and then 1×TE buffer (20 µL; pH 8.0) was added to the tube. The gel slice was crushed with a pipette tip and stored at 4°C overnight. Subsequently, 6 µL of eluted DNA was used as the template for the second round of PCR amplification under the above-mentioned conditions with the primers of 338F (no GC-clamp) and 534R. The resulting PCR products were examined using a 1% agarose gel and purified using E.Z.N.A.™ gel extraction kit (OMEGA, USA). Purified PCR products were cloned into the pMD19-T vector (Takara, Japan) and transformed into *E. coli* (Takara, Japan). Transformants were screened on agar broth under ampicillin (100 mg/mL) selection. Single colony was then transferred to fresh liquid medium and incubated overnight. Then, four positive recombinant clones for each band were further analysed by sequencing (Invitrogen, USA). Sequences containing no chimera were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1997). Sequences obtained in this study are available in the GenBank database under accession numbers from KC494209 to KC494224.

Clone library construction and phylogenetic analysis: Equal quantities of purified PCR products from the same group were mixed for clone library construction. The length of PCR fragments was about 1.5 kb, and the cloning procedure was the same as mentioned above. For each group, 120 positive recombinant clones were further sequenced. Operational taxonomic units (OTUs; phylotypes) were defined with similarity matrices and a filter of 1,253 nucleotide positions using 97% similarity as a cutoff, masking out the hypervariable regions.

Statistical analysis of clone libraries: The Shannon-Weaver index of diversity, H (Shannon & Weaver 1963), the equitability index, E (Pielou 1975), and the coverage were calculated for each library. In the present study, the Shannon-Weaver index was calculated according to the equation $H = -\Sigma(Pi \times \ln Pi)$, where Pi was the clone number in each OTU group divided by the total clone number in each library. The Shannon evenness index was calculated using the formula $E = H/\ln S$, where S was the total OTU number in each library. Coverage was derived from the equation Coverage=1-(n1/N), where n1 was the number of singleton clones, and N was the total number of observed clones (Hughes et al. 2001). The Chao estimator is used to indicate the nonparametric estimation of species richness in a population (Chao 1984), while the rarefaction method is used to compare the number of species found in different regions when the sampling strategies are different (Simberloff 1972). In this study, species richness was estimated according to the clone distribution in different OTUs and determined using the Chao estimator $S^* = S + (A^2/2B)$, where S was the number of observed 16S rDNA clones, A was the number of singleton clones, and B was the number of clones observed twice (Chao 1984).

RESULTS

Analysis of DGGE profiles: Fig. 1 shows the structure of the bacterial community, which was determined by DGGE banding patterns of partial 16S rDNA amplified with the primers GC-338 and 534R. In general, DGGE patterns from different water samples were similar, and many bands (marked with red arrows) were common (such as bands 2, 3, 5, 8, 9, 10, 11, 12, 13, 14, 15). However, some bands were only detected in non-cyanobacterial blooms freshwater ponds or cyanobacterial blooms freshwater ponds (such as bands 1, 4, 6, 7, 16). A total of 47 bands was detected in non-cyanobacterial blooms freshwater ponds using Quantity One 4.3.0 software, and the number of bands detected from cyanobacterial blooms freshwater ponds was 48.

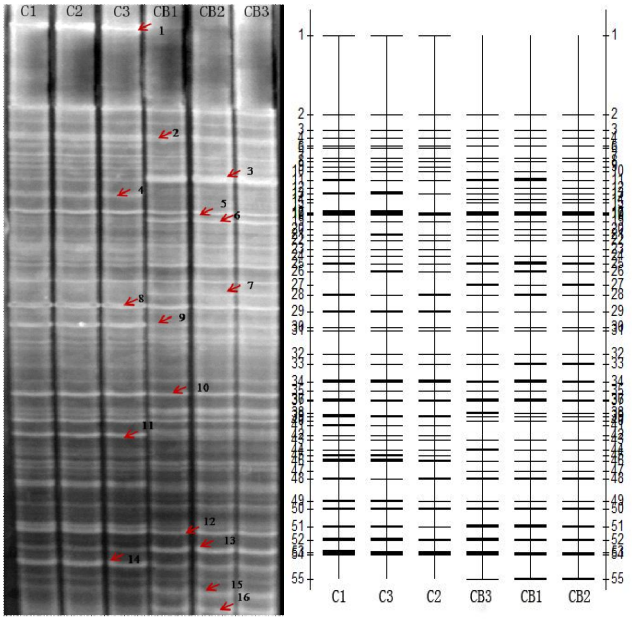

Fig. 1: DGGE band patterns of no cyanobacteria bloom ponds C (C1, C2 and C3) and cyanobacteria bloom ponds CB (CB1, CB2 and CB3). Each number indicates an excised band listed in Table 3.

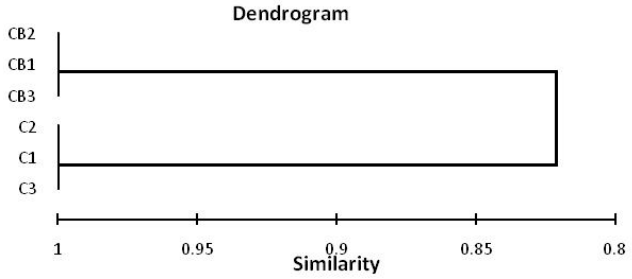

Fig. 2: Analysis of DGGE patterns from Fig. 1. Similarities were calculated using the unweighted pair group method.

Dice coefficients on the DGGE patterns of Fig. 1 were determined using XLSTAT Pro 7.5. A cluster analysis based on these obtained values was performed using the UPGMA cluster analysis and visualized in a dendrogram (Fig. 2). Both Dice coefficients of water samples from non-cyanobacterial bloom ponds and cyanobacterial bloom ponds were 100%. However, the Dice coefficient between non-cyanobacterial bloom ponds and cyanobacterial bloom ponds was only 82%, indicating that cyanobacterial blooms affected water bacterial species to some extent.

Table 2 shows the water bacterial community diversity, as determined by the number of DGGE bands, Shannon's diversity index and equitability index. The results revealed that there was no significant difference between non-cyanobacterial bloom ponds and cyanobacterial bloom ponds in terms of the richness, diversities and evenness of the bacterial communities.

Table 2: Specific richness (R), Shannon's diversity index (H) and equitability index (E) from the digitized DGGE patterns shown in Fig. 1.

Sites	R	H	E
C	47±0.0	3.73±0.18	0.97±0.005
CB	48±0.0	3.72±0.16	0.96±0.004

Notes: C: ponds without cyanobacterial blooms, CB: ponds with cyanobacterial blooms.

DGGE and sequencing analysis: The phylogenetic affiliation of microorganisms was determined by sequencing DGGE bands. A total of 16 completely unambiguous sequences (corresponding bands are indicated in Fig. 1) were obtained from non-cyanobacterial bloom ponds and cyanobacterial bloom ponds. Each sequence was subject to a BLAST search. Their closest results were similar to 16S rDNA sequences reported for uncultured organisms obtained from environmental samples. Sequenced fragments were clustered into seven major phylogenetic groups, including the β- (bands 3 and 5), α-Proteobacteria group (bands 4 and 15), Firmicutes group (band 1), Bacteroidetes group (bands 8 and 9), Cyanobacteria group (band 6), Spirochaetes group (band 7), Actinobacteria group (bands 2, 10, 11, 14), Verrucomicrobia group (band 16) and an unknown group (bands 12 and 13). Among these sequences, *Microcystis* sp., *Leptospira* sp. and *Verrucomicrobium* sp. existed only in CB, whereas *Sphingomonas* sp. and *Paenibacillus* sp. were the C-specific bacteria (Table 3).

Bacterial diversity and species richness comparison: A total of 99 sequences from 120 positive were obtained in each clone library. With 97% similarity as a cutoff, 47 OTUs were identified among the 99 screened clones from the C library, whereas 49 OTUs were identified for the CB library.

The Shannon-Weaver index between the two libraries showed that the diversity index of CB library (3.37) was higher than that of C library (3.18); and the equitability index of CB library (0.87) was also higher than that of C library (0.83) (Table 4). The coverage analysis indicated that C and CB libraries shared similar coverage (65.7% and 63.7%), reflecting the diversity of most bacteria in water samples. The Chao-1 richness estimator of CB library was higher than that of C library (Table 4). However, all these estimates of total sequence richness were underestimated since only about 35% of the total bacterial 16S rDNAs were included in our libraries, as the library screening suggested (Table 4).

Taxonomic groups and phylogenetic distribution: Phylogenetic analysis revealed that 96 full-length 16S rDNA

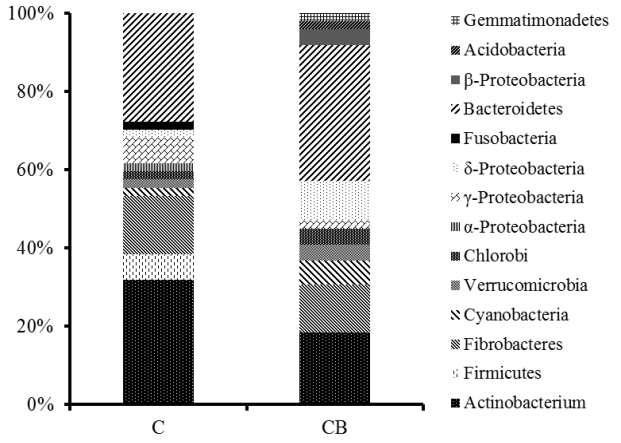

Fig. 3: 16S rDNA phylotype distribution and comparison of the clone libraries at C and CB.

sequences were mainly categorized into Actinobacterium, Bacteroidetes, Fibrobacteres, Firmicutes, Cyanobacteria, Verrucomicrobia, Chlorobi, γ- and δ-Proteobacteria. The following phyla were the major components of each library, since they constituted at least 10% of the total OTUs of a given library: Actinobacteria (15 OTUs, 31.9%), Bacteroidetes (13 OTUs, 28.7%), Fibrobacteres (7 OTUs, 14.9%) and Proteobacteria (5 OTUs, 10.0%) might be the most dominant groups in C library. The four lineages in CB library were Bacteroidetes (17 OTUs, 34.7%), Actinobacteria (9 OTUs, 18.4%), Fibrobacteres (6 OTUs, 12.2%) and Proteobacteria (8 OTUs, 16.2%). Firmicutes and γ-Proteobacteria appeared at C library with a few OTUs (3 OTUs, 6.4%) and Cyanobacteria appeared at CB library with a few OTUs (6.1%) (Fig. 3).

DISCUSSION

Our results of both PCR-DGGE and 16S rDNA clone libraries showed that Firmicutes group existed only in the non-cyanobacterial bloom ponds surveyed, but they disappeared in the cyanobacterial bloom ponds. Vieira and colleagues (2008) reported that in estuary with different levels of eutrophication, the composition of Firmicutes increases with the increase of the nutrient concentration. The Firmicutes almost does not exist in the oligotrophic water bodies (Sun et al. 2009). The cyanobacterial bloom ponds had a 6.4% reduction of OTUs in Firmicutes and a 6.0% increase of OTUs in Bacteroidetes in this study. Some studies in regard to the obesity suggested that Firmicutes increases and Bacteroidetes decreases in the GI tract of obese bodies (Wolf & Lorenz 2012). In general, the Firmicutes-to-Bacteroidetes ratio is regarded to be of significant relevance in human gut microbiota composition (Ley et al. 2006). The mice fed with a high-fat (HF) diet showed an increased ratio of Firmicutes: Bacteroidetes (Murphy et al. 2010, Hildebrandt et al. 2009). Firmicutes appeared to be more adaptive at breaking down otherwise indigestible carbohydrates, converting them into absorbable energy products (de La Serre et al. 2010). Intensive ponds are enriched with inorganic nitrogen, lowering the C/N ratio in pond (Avnimelech & Ritvo 2003). In a late culture period, the decreased organic carbon is the limiting factor for propagation of heterotrophic bacteria, and high concentration of inorganic nitrogen is the main factor causing eutrophication in the pond water (Luo et al. 2011). Decline of the Firmicutes-to-Bacteroidetes ratio would imply a carbon shortage in ponds. These results implied that the decline of carbon and Firmicutes-to-Bacteroidetes ratio may be a predictor of cyanobacterial blooms in freshwater ponds.

Bacteroidetes was regarded as the most significant lineage (17 OTUs, 34.7%) in the CB library. It is not similar to most of the freshwater lakes associated with cyanobacterial blooms, in which the most significant lineage is Actinobacteria (Sekiguchi et al. 2002, Wu et al. 2007). Usually, Actinobacteria resist protistan predation (Hahn et al. 2003). A previous study (Sun et al. 2009) on the planktonic bacteria diversity in the eutrophic zone of Lake Wuliangsuhai revealed that rise of Bactoridetes group and decline or disappear of some Proteobacteria groups are the sign of water-quality decline. In Swedish lakes associated with cyanobacterial blooms, the Bacteriodetes presented high frequencies in the clone libraries (Eiler & Bertilsson 2004), which is consistent with the present study. We speculated that Bacteroidetes was closely related to extreme eutrophication in the freshwater ponds of intensive polyculture. The Proteobacteria group (8 OTUs, 16.2%) was mainly in oligotrophic water bodies, and they were drastically reduced in these freshwater ponds of *Channa argus* in intensive polyculture during the late culture period. The phylum Proteobacteria contains the majority of the characterized strains in association with cyanobacterial blooms water by culturing on the growth media (Berg et al. 2009). Due to differences of research methods, most microorganisms in aquatic ecosystems still cannot be obtained by culturing methods (Zwart et al. 2002).

In this study, the OUTs of Fibrobacteres were evenly distributed among the two libraries (7 and 6 OTUs, respectively), but the distribution of sequences belonging to Fibrobacteres in CB (22.2% of the total clones) was twice as that of C (11.1% of the total clones). Actinobacteria, Bacteroidetes, Fibrobacteres and Proteobacteria were the most dominant groups in the two libraries we examined. The three lineages in Lake Taihu March library were Proteobacteria, Actinobacteria and Bacteroidetes (Wu et al. 2007). This observation was in line with the limnic ecosys-

Table 3: Comparison of genomic sequences in dominant DGGE bands by sequencing and BLAST analysis.

Band number	Sample ID	Organism	Phylogenetic affiliation	Similarity (%)	Accession no.
1	C	*Paenibacillus* sp. (JQ659563.1)	Firmicutes	98	KC494209
3	C,CB	Uncultured beta proteobacterium (FJ916623.1)	β-Proteobacteria	100	KC494211
5	C,CB	Uncultured *Acidovorax* sp.(JQ723680.1)	β-Proteobacteria	99	KC494213
		Uncultured *Albidiferax* sp. (JQ723658.1)		99	
4	C	*Sphingomonas* sp.(AB681542.1)	α-Proteobacteria	93	KC494212
15	C,CB	*Roseomonas* sp. (HE984358.1)	α-Proteobacteria	100	KC494223
8	C,CB	Uncultured Bacteroidetes bacterium (GU257572.1)	Bacteroidetes	100	KC494216
9	C,CB	Uncultured Bacteroidetes bacterium (JN371451.1)	Bacteroidetes	98	KC494217
6	CB	Uncultured *Microcystis* sp. (JX391871.1)	Cyanobacteria	100	KC494214
7	CB	Uncultured *Leptospira* sp. (JF925243.1)	Spirochaetes	94	KC494215
2	C,CB	*Microbacterium* sp. (JX843382.1)	Actinobacteria	100	KC494210
		Leifsonia sp. (JX067673.1)		100	
10	C,CB	*Agrococcus casei* strain(JX490100.1)	Actinobacteria	99	KC494218
		Microbacterium sp. (AY368526.1)		99	
11	C,CB	*Microbacterium* sp.(JX082295.1)	Actinobacteria	99	KC494219
14	C,CB	Uncultured actinobacterium (FR648178.1)	Actinobacteria	99	KC494222
16	CB	Uncultured *Verrucomicrobium* sp. (FN668203.2)	Verrucomicrobia	94	KC494224
12	C,CB	Uncultured bacterium (GQ359052.1)	Unknown	100	KC494220
13	C,CB	Uncultured bacterium clone(JQ958645.1)	Unknown	99	KC494221
		Uncultured bacterium clone (HQ904633.1)		100	

C: ponds without cyanobacterial blooms, CB: ponds with cyanobacterial blooms.

Table 4: Diversity indices of bacterial 16S rDNA clone libraries from C and CB.

Treatment	Screened clones	OTUs	Shannon Indices	Evenness Indices	Chao-1 estimator	Coverage (%)
C	99	47	3.18	0.83	129.57	65.7
CB	99	49	3.37	0.87	141.57	63.7

Notes: C: ponds without cyanobacterial blooms, CB: ponds with cyanobacterial blooms.

tem during the period that was absence of cyanobacterial blooms, but the Fibrobacteres did not appear in the clone libraries (Trusova & Gladyshev 2002). The phylum Fibrobacteres currently comprises of one formal genus, *Fibrobacter* (Ransom-Jones et al. 2012). Historically, members of the genus *Fibrobacter* were thought to exclusively occupy gut environments (Montgomery et al. 1988). However, a small number of sequenced belonging to the Fibrobacteres phylum or described as *Fibrobacter* related has been reported in general bacterial 16S rDNA gene clone libraries generated from landfill sites and freshwater lakes (Macalady et al. 2008, Percent et al. 2008), and these organisms may contribute to the primary degradation of plant and algal biomass in freshwater lake ecosystems under anoxic conditions (McDonald et al. 2009). The Fibrobacteres might contribute to the degradation of cyanobacteria in these freshwater ponds.

We studied the bacterial diversity of freshwater ponds of *Channa argus* in intensive polyculture with cyanobacterial blooms using the methods of PCR-DGGE and 16S rDNA clone library. Our results indicated no significant differences of the Shannon's diversity index (H) and equitability index (E) between cyanobacterial bloom freshwater ponds and non-cyanobacterial bloom freshwater ponds. However, Trusova et al. (2002) reported that the bacterial community composition became more varied with cyanobacterial blooms. Since molecular biological methods are well known to have several biases (Vallaeys et al. 1997), community structure or dynamics cannot be evaluated by a single method. Therefore, it is important to do a cross-check using several methods. Only a few reports have compared the two methods: Ogino et al. (2001) reported that during a biostimulation process, the succession of microbial communities suggested similar results in the two methods. In contrast, Watanabe et al. (2000)) reported that in underground crude oil storage cavities, the ratio of a dominant population to the total population differed in the two methods. In this study, we obtained no sequences affiliated with Fibrobacteres, Chlorobi, γ- and δ-Proteobacteria using the PCR-DGGE technology, but they were present as above 1% groups by the method of 16S rDNA clone libraries. Not all of the DGGE bands were

recovered in this study: we usually selected bands of a higher intensity and with a more noticeable frequency for excision. But some information about the DGGE pattern was missing. In addition, the estimated numbers (129~141) of OTUs were three times as the actual obtained OTUs, and the bacterial diversity integrity was low. So we did not detect the presence of some common bacteria in a clone library, e.g., the α-Proteobacteria in CB library and β-Proteobacteria in C library. It will be important to expand the size of these two clone libraries.

We firstly focused on the structure of the bacterial communities in the freshwater ponds of intensive polyculture with cyanobacterial blooms using DGGE and 16S rDNA clone library methods. We found that the studied ponds were characterized by dominance of the Actinobacterium, Bacteroidetes, Fibrobacteres and Proteobacteria groups. Fibrobacteres group was characteristic bacteria of these intensive polyculture freshwater ponds. In future process of aquaculture, the organic carbon and Firmicutes group in intensive freshwater ponds might be an early warning factor for cyanobacterial blooms.

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