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Comparative Analysis of Cyanobacterial Communities from Polluted Reservoirs in Korea

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Cyanobacteria are the dominant phototrophic bacteria in water environments. Here, the diversity of cyanobacteria in seven Korean reservoir waters where different levels of algal blooms were observed during the summer of 2002, was examined by T-RFLP analysis. The number of T-RF bands in the *Hae*III T-RFLP profiles analyzed from those water samples ranged from 20 to 44. Of these, cyanobacteria accounted for 6.1 to 27.2% of the total bacteria. The water samples could be clustered into 2 groups according to the Dice coefficient of the T-RF profiles. The eutrophic Dunpo and oligotrophic Chungju reservoirs were selected, and several representative clones from both reservoir waters analyzed for the nucleotide sequences of their 16S rDNA. The major clones were found to belong to the *Microcystis* and *Anabaena* species in the waters from the Dunpo and Chungju reservoirs, respectively, which was in agreement with the T-RFLP result. That is, the *Microcystis* and *Anabaena* species were dominant in the eutrophic and polluted Dunpo and oligotrophic Chungju reservoir waters, respectively. These results indicated that there is a correlation between prevalence of cyanobacterial species and levels of pollution in reservoir waters.

Key words: cyanobacteria, community analysis, water pollution, reservoirs in Korea

Cyanobacteria are recognized as the dominant phototrophs in fresh water and marine ecosystems. Bloom-forming cyanobacteria can be a serious problem, because they produce a wide range of toxic compounds, including hepatotoxins and neurotoxins, such as microcystins (Humpage *et al.,* 2000) and saxitoxins (Sivonen, 1996; Baker *et al.,* 2002), respectively. In order to study the characteristics of the toxin producers, numerous attempts have been made to identify cyanobacteria using conventional methods, such as enrichment cultivation and microscopy (Ferris *et al.,* 1996), and more recently by specific gene analysis, which include PCR-RFLP (Neilan *et al.,* 1995; Bolch *et al.,* 1996) and 16S to 23S rRNA operon spacer region analysis (Neilan *et al.,* 1997; Otsuka *et al.,* 1999). The PCR technique has recently been adapted to allow for the direct analysis of environmental samples, with the advantage of being specific for cyanobacteria in the presence of other organisms (Baker *et al.,* 2001).

Cyanobacteria are known to proliferate in nitrogen and phosphorus rich water environments; and therefore, to be a clear sign of eutrophication. In addition, evidence exists that many phototrophic cyanobacteria are actively involved in the degradation of pollutants, such as polychlorinated biphenyls, polycyclic aromatic hydrocarbon and crude oil (Macnaughton *et al.,* 1999; Kanaly *et al.,* 2000; Nogales *et al.,* 2001). Some studies have demonstrated the ability of cyanobacterial isolates to degrade hydrocarbons (Cerniglia *et al.,* 1980a; 1980b; Al-Hasan *et al.,* 1998), and the bacterial diversity and community changes in various pollutant-degrading ecosystems have been examined using culture-independent molecular techniques (Abed *et al.,* 2002a; 2002b). The *pcbC* genes responsible for the *meta*-cleavage degradation of aromatic hydrocarbons were identified from metagenomic DNA library derived from the Dunpo reservoir in Korea (Moon, 2004). However, comparatively little research has been performed on the microbial communities dominated by photosynthetic organisms in relation to the levels of pollution.

In this study, the cyanobacterial diversity of mixed bloom waters during the summer of 2002 were studied in several Korean reservoirs, including the Dunpo reservoir, using polymerase chain reaction (PCR), terminal restriction fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis (ARDRA) and nucleotide sequencing, in order to examine for any correlation

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between the cyanobacterial community and the levels of pollution. It was found that these molecular techniques were able to identify cyanobacterial strains. In particular, the dominant species in two representative reservoirs, with different water qualities, were analyzed. The predominance of the *Microcystis* sp. and *Anabaena* sp. distributed in both reservoir waters were positively correlated with the water quality of these eutrophic and oligotrophic reservoirs.

Materials and Methods

Study site and sample collection

Seven water reservoirs, located in mid South Korea, were selected for this study, and are listed in Table 1. These reservoirs suffer from cyanobacterial blooms in both the summer and autumn each year. In particular, the Dunpo and Namil reservoirs are relatively small, with surface areas of about 0.5 to 1.0 km^2, and are affected by both point and non-point pollution sources. Conversely, the Chungju and Daecheong reservoirs are relatively large with surface areas of about 60 to 80 km^2. These reservoirs were constructed about 20 years ago as multipurpose dams to supply drinking, agricultural and industrial waters, and to control summer flooding. Water samples (10 liter) were collected from each reservoir during September and October 2002, when an algal bloom was observed in the Dunpo reservoir, and kept on ice until processed for bacterial community characterization. Physical measurements, including pH and temperature, were taken in triplicate at the times of sampling. Chemical analyses, such as chlorophyll-a, BOD and total nitrogen and phosphate quantifications, were performed at the Chungbuk Institute for Public Health and Environment.

DNA extraction

The bacterial genomic DNAs from the reservoir waters were prepared using the protocol described by Rhochelle *et al.*, (1992). The samples were centrifuged at 14,000×g and 4°C for 15 min, and the bacterial cell pellets obtained resuspended in 300 μl of lysozyme solution (0.15 M NaCl, 0.1 M EDTA, pH 8.0, lysozyme 15 mg/ml) and incubated for 1 h at 37°C, with gently mixing at 15 min intervals. After the addition of 300 μl SDS buffer (0.1 M NaCl, 0.5 M Tris-HCl, pH 8.0, 4% SDS), the samples were incubated for 10 min at -70°C and then for 10 min at 65°C. This freezing-thawing process was repeated three times. Bacterial genomic DNA was then extracted and purified from the cell lysates by two sequential phenol-chloroform extractions, and the precipitated with isopropanol. The DNA pellets obtained were washed with 70% ethanol and dissolved in sterile TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). These genomic DNAs were then purified using an UltraClean™ kit (MoBio, USA), electrophoresed in a 0.8% agarose gel, and stored at -20°C.

PCR amplification for T-RFLP

16S rDNA fragments were amplified by PCR using the two eubacterial primers 27FB (*E. coli* numbering 8-27: 5'-AGAGTTTGATCMTGGCTCAG-3') and 785R (*E. coli* numbering 785-804: 5'-ACTACCRGGGTATCTAATCC-3'). The 27FB was biotinylated at the 5'-end to separate the terminal restriction fragments (T-RFs) from the other digested fragments (Kim *et al.*, 2001). PCR was carried out in a 50 μl reaction mixture containing 1×PCR buffer (100 mM Tris-HCl, pH 8.3, 400 mM KCl, 1.5 mM $MgCl_2$ and 500 μg/ml BSA), 160 μM of each dNTP, 0.3 μM of each primer, 1.5 unit of *Taq* polymerase (Genenmed, Korea) and 10-15 ng/μl of template DNA. An initial denaturation step (3 min at 95°C) by 30 cycles of denaturation (30 sec at 95°C), annealing (30 sec at 58°C) and extension

Table 1. Diversity statistics calculated from *Hae*III T-RF profiles of reservoir waters

Reservoir	Total T-RF band	Proportion of *Microsystis* (%)[1]	Proportion of *Anabaena* (%)[2]	Proportion of Cyanobacteria (%)[3]	Diversity[4]
Keumma	44	5.9	1.1	12.0	5.052
Namil	31	11.4	1.5	17.2	4.528
Daecheong	36	2.3	0	6.1	4.776
Dunpo	24	16.3	3.5	27.4	4.082
Yanggu	28	3.1	1.8	14.2	4.482
Chungju	32	1.1	12.0	17.7	4.493
Paroho	20	6.4	0	10.6	4.009

[1]Proportion of *Microsystis* is the density of T-RFs (182 bp) related to *Microsystis*. Refer to reference (Lee *et al.*, 2000).
[2]Proportion of *Anabaena* is the density of T-RFs (290 bp) related to *Anabaena*. Refer to reference (Lee *et al.*, 2000).
[3]Proportion of cyanobacteria is the total density of T-RFs (179, 182, 224, 226, 272, 290, 292, 294 bp) related to cyanobacteria. Refer to reference (Lee *et al.*, 2000).
[4]Diversity was calculated as Shannon-Weiner index (H), $H=-\Sigma(P_i)(\log_2 P_i)$, P_i is the proportion of an individual band relative to the sum of all band density.

(1 min at 72°C), and then a final 10 min extension at 72°C. The PCR products were purified using an UltraClean™ kit (MoBio, USA) and electrophoresed in a 0.8% agarose gel. The bands derived from cyanobacteria were confirmed using the *Hae*III T-RF database.

16S rDNA T-RFLP

Purified PCR products were digested with 5 units of restriction endonuclease *Hae*III (TaKaRa, Japan) for 5 h at 37°C. The biotinylated T-RFs were selectively isolated from the digested fragments using Streptavidin Magne-Sphere paramagnetic particles (SA-PMPs) and a magnetic separation stand (Promega, USA), in accordance with the manufacturer's instructions (Lee *et al.*, 2000; Kim *et al.*, 2001). To denature double stranded T-RFs, the samples were soaked in 0.2 M NaOH for 5 min at room temperature. Biotinylated and single stranded T-RFs were mixed with 25% NH_4OH and incubated at 65°C for 10 min to separate T-RFs from SA-PMPs. The NH_4OH was removed by vacuum microcentrifugation and the T-RF pellets were resuspended in distilled water. T-RFLP patterns were analyzed by electrophoresis on a 6% polyacrylamide gel (acrylamide:bisacrylamide=19 : 1; 7.0 M urea; 1×TBE). After mixing the DNA samples with an equal volume of loading dye buffer (95% formamide, 10 mM NaOH, 20 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol FF), they were heated for 3 min at 95°C and then chilled on ice prior to electrophoresis at 1900 V and 50 W for 3 h. Silver staining was performed according to the manufacturer's instructions (Bioneer, Korea).

To compare the community structures of the different samples, the *Hae*III T-RF bands from the T-RF profiles were converted into binary data (i.e., presence or absence of a band). A Dice coefficient of similarity (SD) matrix was then calculated for the set of samples (Lee *et al.*, 2000). In the diversity statistics, the proportion of each cyanobacterial species out of the total cyanobacteria was analyzed for the diversity of the T-RF related to each cyanobacterial strain using an HP-6200C scanner (Hewlett Packard, USA) and Image II system (Bioneer, Korea) (Lee *et al.*, 2000). The SD was calculated as 2NAB/(NA+NB), where NAB is the number of common bands in samples A and B, and NA and NB are the total number of bands in samples A and B, respectively. A dendrogram was generated using the UPGMA method (unweighted pair-group method using arithmetic averages) in the neighbour-joining program. Finally, the Shannon-Weiner diversity index (Dunbar *et al.*, 2000) was calculated as follows: $H=-\Sigma(P_i)(\log_2 P_i)$, where P_i is the quotient of the individual peak heights and the sum of all peak heights.

Cloning of 16S rDNA and ARDRA

Two universal bacterial primers, 27F and 785R, were used to amplify the 16S rDNA from the extracted DNA to construct a bacterial clone library. PCR was performed as described above. The PCR products were ligated into the pGEM-T vector according to the manufacturer's instructions (Promega, USA), the ligated DNAs were used to transform competent *Escherichia coli* XL1-Blue and recombinant transformants were selected by blue and white screening.

Amplified rDNA restriction analysis (ARDRA) was performed to analyze for clone diversity. The amplification products generated using the vector-specific primers T7 (5'-TAATACGACTCACTATAGGGCGA-3') and prGTr (5'-CTCAAGCTATGCATCCA ACGC-3') were obtained from clones and digested with 3 units of the restriction endonuclease *Hae*III (TaKaRa, Japan) for 5 h at 37°C. The restriction fragments from each clone were then electrophoresed in a 3.5% NuSieve 3 : 1 agarose gel (BMA, USA) using 0.5×TBE buffer for 2 h at 100 V.

Sequencing and phylogenetic analysis

The ARDRA patterns were compared, and clones with identical patterns considered as the same group. Partial sequences of 16S rDNAs from representatives of each group were determined. The nucleotide sequences of the cloned PCR products (about 800 bp) were obtained using the BaseStation™ DNA Fragment Analyzer (MJ Research, USA). All sequences were checked for chimera formation using the CHECK-CHIMERA software developed by the Ribosomal Database Project (Cole *et al.*, 2003), and the phylogenetic affiliations of their 5' and 3' ends compared. Sequences were submitted to the BLAST search program (Altschul *et al.*, 1997) at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The closest relative 16S rDNA sequences of the clones were identified from database searches, and the appropriate strains aligned using CLUSTAL W (Thomson *et al.*, 1994). Finally, phylogenetic trees were constructed using the Jukes & Cantor distance coefficient (Jukes and Cantor, 1969) and neighbor-joining tree algorithm (Saitou and Nei, 1987) contained within the PHYLIP package v3.6a3 (Felsenstein *et al.*, 2002).

Results and Discussion

Cyanobacterial diversity in the reservoir waters

The water samples taken from the 7 Korean reservoirs were analyzed by T-RFLP to determine the diversity of the microbial communities, and the *Hae*III T-RF profiles are shown in Fig. 1. The total numbers of T-RF bands between 150 and 350-bp were 44 and 20 for the Keumma and Parch reservoirs, respectively. The water samples from the other reservoirs had between 24 and 36 T-RF bands. The 182-bp T-RF band putatively derived from *Micrococcus* and *Synechococcus* were predominately detected in the waters of the Keumma, Namil and Dunpo reservoirs. However, the same band was not detected at Chungju reservoir, where the 292 and 294-bp bands puta-

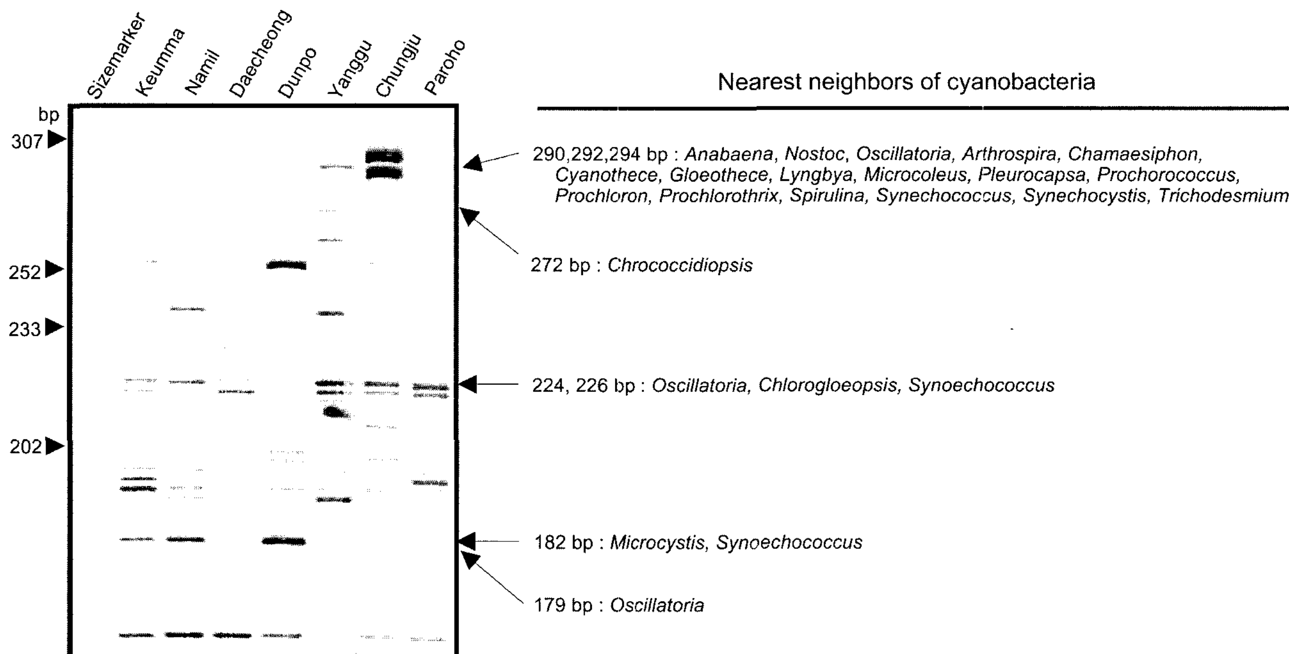

Fig. 1. T-RFLP of 16S rDNA from the reservoir waters in Korea. Bands were assigned to particular cyanobacterial groups, according to their size, as described in Materials and Methods.

tively derived from *Anabaena* predominated. In the work presented here, PCR and T-RFLP assays proved to be as sensitive as the methods used by Baker *et al.*, (2002) and Bolch *et al.*, (1996).

On the basis of the *Hae*III T-RF profiles obtained from the 7 reservoir waters, their bacterial diversities were calculated, and are shown in Table 1. In the reservoir waters examined in this study, putative cyanobacteria were found to range from 6.1 to 27.2% of the total bacteria. The water from Keumma reservoir showed the highest number (44) of T-RF bands among the 7 waters tested, indicating the highest diversity of bacterial species. The Shannon-Weiner index (H) of the Keumma water was highest at 5.052, whereas those of the other water samples were lower, in the range 4.01 to 4.78. It is possible to conclude that the genetic diversity did not differ significantly among the different reservoirs.

When considering single taxonomic groups, the sample from Chungju reservoir was shown to putatively include 12.0% *Anabaena* species, whereas the other samples, particularly that from the Dunpo reservoir, showed a higher content of *Microcystis* like sequences. These results agree with the different amounts of chlorophyll-a found in the waters of those reservoirs (data not shown). The dice coefficients (SD) of the 7 water samples were also calculated from the *Hae*III T-RF profiles. The results shown in Fig. 2 indicated that the reservoirs could be clustered into two groups. The waters of the Dunpo, Namil, Keumma and Daechung reservoirs showed about 59 to 66% similarity. The other cluster was formed by Chungju, Paroho and Yanggu reservoirs, with similarities of 55 to

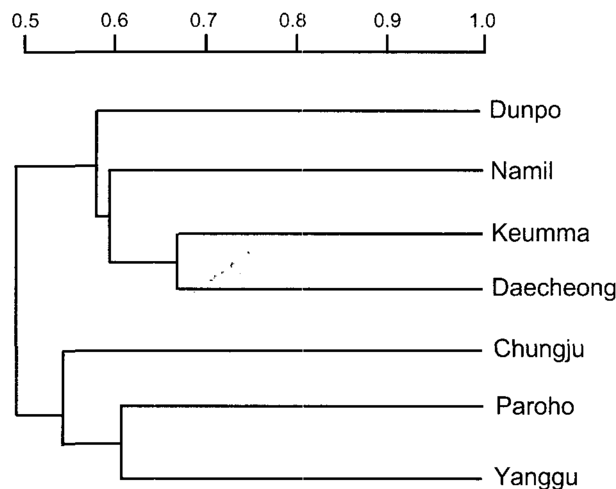

Fig. 2. Dendrogram showing the genetic relationship of the total DNA from the reservoir waters. Dice similarity coefficients were calculated, as described in Materials and Methods, from the T-RF profiles of the 16S rDNA reported in Fig. 1.

60%. Such a distinction might be attributed to the water qualities; however, it is even possible that other factors may be involved such as the geographic location or the endemism of cyanobacteria in terrestrial water environments (Al-Hasan *et al.*, 1998; Lopez-Cortes *et al.*, 2001; Taton *et al.*, 2003).

Relationship between cyanobacterial species and water pollution

The Dunpo and Chungju reservoirs were selected, as rep-

resentative examples of each cluster, for further cyanobacterial species analysis in order to investigate any correlations with the different levels of pollution. The environmental conditions at both reservoirs are shown in Table 2. In general, the Dunpo reservoir has a smaller surface area and greater levels of pollution than the Chungju reservoir in terms of the total nitrogen and phosphate, and BOD, which can result in severe algal blooms.

Table 2. Environmental conditions of Dunpo and Chungju reservoir waters

Parameter	Dunpo	Chungju
Location	Asan, Chungnam	Chungju, Chungbuk
Surface area (km^2)	0.5	67.5
Depth (m)	3.0	97.5
Temperature (°C)	24.5	25.7
pH	7.34	7.48
Chlorophyll-a (mg/l)	68.88	1.14
Total nitrogen (mg/l)	5.635	1.469
Total phosphate (mg/l)	3.864	0.034
BOD (mg/l)	8.5	1.4

About 1,000 clones from the two reservoirs were analysed by ARDRA, as described in the Materials and Methods, and 11 and 9 clones were selected from the water samples of the Dunpo and Chungju reservoirs, respectively. The nucleotide sequences of the 16S rDNAs of these representative clones were determined, and the nearest neighbor cyanobacteria identified on the basis of the RFLP patterns of their 16S rDNA sequences, as shown in Table 3. The ARDRA pattern of clone DP1, with 99% similarity to *Microcystis aeruginosa*, represented 80.2% of the cyanobacterial clones. In addition, 6 of the 11 cyanobacterial clones from the Dunpo reservoir were closely related to *Microcystis aeruginosa*.

Conversely, the ARDRA pattern of the CJ1 clone from the Chungju reservoir, with 98% sequence similarity to *Anabena* sp., was found in 33.3% of the cyanobacteria; the CJ2 clone showed high similarity to uncultured bacteria. Five of the 9 sequenced clones from the Chungju reservoir were found to be closely related to *Anabaena* species. The dominant cyanobacterial species differed

Table 3. Closest relatives of the representative clones obtained from Dunpo and Chungju reservoirs on the basis of 16S rDNA sequences

Reservoir	Clone No.	Closest relatives based on partial sequence homology	Similarity (%)	Distribution* (%)
Dunpo	DP1	*Microcystis aeruginosa* (D89031)**	99.2	80.2
	DP2	*Microcystis aeruginosa* (D89031)	99.2	3.7
	DP3	*Microcystis aeruginosa* (D89031)	99.2	1.2
	DP4	uncultured marine eubacterium HstpL83 (AF159642)	94.5	2.4
	DP5	uncultured marine eubacterium HstpL83 (AF159642)	94.5	1.2
	DP6	*Microcystis aeruginosa* (D89031)	99.1	1.2
	DP7	*Anabaena flos-aquae* AWQC112D (AF247590)	97.3	1.2
	DP8	*Microcystis* sp. (AJ133170)	99.7	1.2
	DP9	uncultured bacterium (AY135907)	98.6	2.4
	DP10	*Microcystis aeruginosa* PCC7005 (U40338)	98.4	1.2
	DP11	uncultured bacterium clone CC4 (AY187881)	99.5	1.2
Chungju	CJ1	*Anabaena* sp. (AJ133152)	98.3	33.3
	CJ2	uncultured bacterium, Antarctica (AY218705)	95.0	29.6
	CJ3	*Anabaena* sp. (AJ133152)	98.3	7.4
	CJ4	*Microcystis aeruginosa* (AF139315)	99.5	7.4
	CJ6	*Anabaena* sp. (AJ133152)	99.0	3.7
	CJ7	*Anabaena* sp. (AJ133152)	99.1	3.7
	CJ8	uncultured bacterium (AY135907)	98.6	3.7
	CJ9	*Anabaena* sp. (AJ133152)	98.8	3.7

*The proportion of the clones having the same RFLP was calculated from the total clone library.
**Genbank accession number

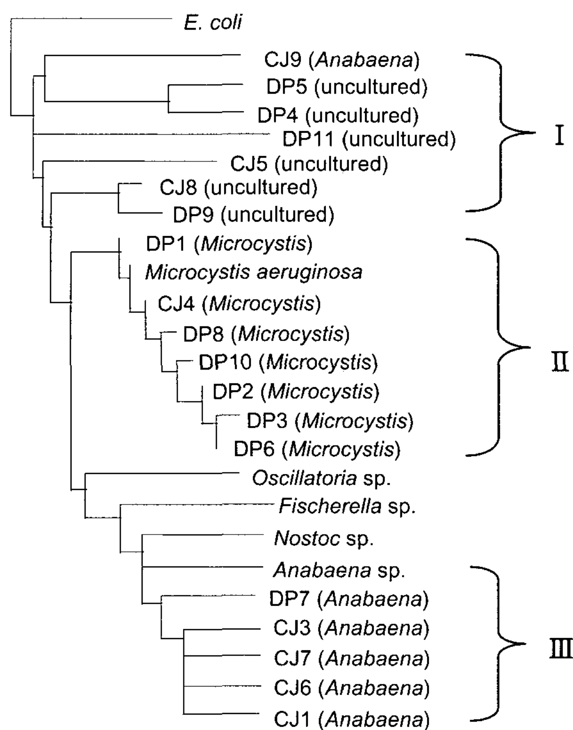

Fig. 3. Phylogenetic tree of the cyanobacteria from the Dunpo and Chungju reservoirs, constructed on the basis of the 16S rDNA sequence data. Group I consists of the different cyanobacteria obtained from both the Dunpo and Chungju reservoirs. Groups II and III show the *Microcystis* and *Anabaena* spp. mainly from the Dunpo and Chungju reservoirs, respectively.

between the two reservoirs, based on their 16S rDNA sequences, which were very similar to the T-RFLP results reported in Fig. 1. *Microcystis* sp. and *Anabaena* sp. were the dominant in the eutrophic Dunpo and oligotrophic Chungju reservoirs, respectively. Vasconcelos and Fereira (2001) studied diversity and toxicity of cyanobacterial in ponds receiving organic wastewaters. They found that *Microcystis aeruginosa* was the dominant species in eutrophic ponds. Such changing levels of cyanobacterial species (*Microcystis* and *Anabaena*) were reported to occur over the course of blooming in water supply reservoirs (Baker *et al.*, 2002).

The cyanobacterial species in the Dunpo and Chungju reservoirs could be phylogenetically clustered into 3 groups, as shown in Fig. 3. Group I was a mixture of the cyanobacterial clones obtained from the Dunpo and Chungju reservoirs. Group II was composed of *Microcystis aeruginosa*, and were mainly isolated from the more polluted Dunpo reservoir, with group III composed of *Anabaena* sp., found mainly in the less polluted Chungju reservoir. These results indicated that there is a clear correlation between the prevalence of cyanobacterial species and the levels of pollution in reservoir waters.

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