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Diversity of Repetitive Sequences in Toxigenic Cyanobacteria Detected by Repetitive Oligonucleotides-Primed PCR

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반복염기 프라이머 PCR에 의해 탐색된 독성 남조류에 분포한 반복염기의 다양성. 최창원* · 구정모 · 유순애 · 박상호 (배재대학교 생물학과, 바이오의약연구센터 RRC)

남조류 분리균주들은 주어진 배양조건에 따라서 특징적인 세포모양이 결핍되거나 형태가 변형되기 쉽기 때문에, 형태학적으로 종을 정확하게 구분하기 어렵다. 형태학적 지표대신에 단일 혹은 조합된 반복염기를 프라이머로 이용한 repetitive oligonucleotides-primed PCR (ROP-PCR)을 수행하여 담수계 오염을 일으키는 독성 남조류 *Anabaena*와 *Oscillatoria* 속의 구성원들의 DNA 밴드 양상을 구별하였다. 그람음성 세균에 빈번하게 분포한 것으로 알려진 ERIC 및 REP 반복염기, 남조류의 게놈으로부터 파생된 STRR1A와 LTRR 반복염기, 그리고 진핵생물의 반복염기를 이용하여 수행한 ROP-PCR은 남조류 분리균주들의 특이적이고 반복적인 DNA 지문 및 뚜렷한 유전형을 동정하게 하였다. 분리균주의 그룹분석은 ROP-PCR에 이용된 프라이머에 따라서 유의성있는 차이를 나타내었다.

Key words : Morphological parameters, *Anabaena*, *Oscillatoria*, Repetitive oligonucleotides, ROP-PCR

INTRODUCTION

Highly conserved inverted repeat sequences constitute an important part of the prokaryotic genome. Two classes of short intergenic repeated sequences, the repetitive extragenic palindromic (REP) and repetitive intergenic consensus (ERIC) sequences, have been found in enteric bacteria as well as in gram-negative bacteria (Stern *et al.*, 1984; Hulton *et al.*, 1991; Versalovic *et al.*, 1991). Another distinct repetitive sequences, the short tandemly repeated repetitive (STRR) sequences, have been identified in a number of heterocystous cyanobacterial genera and species (Mazel *et al.*, 1990; Rasmussen and Svenning, 1998), while a 37-bp long tandemly repeated repetitive (LTRR) sequence in both heterocystous and non-heterocystous cyanobacteria (Masepohl *et al.*, 1996). The multi-function of the repeated sequences has been suggested that they may regulate transcription termination or be the target of DNA-binding proteins responsible for chromosomal maintenance in the cell (Stern *et al.*, 1988; Mazel *et al.*, 1990; Shyamala *et al.*, 1990; Hulton *et al.*, 1991; Haselkorn and Buikema, 1992), however, the actual function of these highly repeated and conserved elements is still unclear. Such repeated sequences dispersed in the genome of bacterial species have been used as primers to differentiate closely related strains (Louws *et al.*, 1994).

Cyanobacteria are an ancient group of oxygen-evolving and photosynthetic microorganism having the general characteristics of gram-negative bacteria. Members of the cyanobacterial genera

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Anabaena, *Oscillatoria* and *Microcystis* are commonly present as a mixed population during cyanobacterial mass occurrence, rapidly form nuisance blooms of their freshwater habitat, resulting in significant effects on aquatic environments (Baker and Humpage, 1994; Rapala *et al.*, 1997). However, it is poorly understood what causes a water bloom to be toxic from co-existing cyanobacterial population in the water blooms. Traditional taxonomic parameters based on observed morphological characteristics, do not distinguish potential-bloom inducing or toxigenic strains.

The final goal of our work is to apply a reliable detection system of potential toxic cyanobacteria in environmental water. To do this, we compared several fingerprint methods for cyanobacteria with different sets of repetitive oligonucleotides as primers derived from various organisms in the PCR (ROP-PCR). We chose, as model organisms, *Anabaena* sp. and *Oscillatoria* sp., respectively, being differentiated and undifferentiated cyanobacteria. The former commonly associated with toxic water blooms in the Daechung reservoir for the source of drinking water supply of Taejon city in Korea.

MATERIALS AND METHODS

The *Anabaena* isolates N1444, PC1, PC2, PC9 and PC16, *Oscillatoria* isolates PC22 and PC24, were collected from the Daechung reservoir, or lakes in the province of Chungnam, segregated by micromanipulation, and made axenic by selective culturing. Strains with IAM numbers, originally isolated in Japan, were obtained from the Institute of Applied Microbiology, University of Tokyo. All cyanobacteria used in this study were grown in BG-11 medium (Stanier *et al.*, 1971) at 28°C under continuous shaking and cool white fluorescent light. Microscopic examinations were made by the cell size, shape and the nature of colonial sheath, and the cell arrangement in the colony. The following bacteria were included as gram negative and gram positive bacteria controls: *Escherichia coli*, and *Streptomyces* sp.

Total genomic DNA was extracted by a following method. A 100 ml culture of late-log-phase was pelleted by centrifugation, and the resulting pellet was resuspended in 50 mM Tris-HCl (pH 8.0) containing 5 mM EDTA and 50 mM NaCl. After adding lysozyme (1 mg/ml), proteinase K (10 mg/ml) and 10% sodium dodecyl sulfate, the mixture was incubated at 55°C for 30 min. The solution was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1), and repeated several times until white interfaces removed. The resulting supernatant was precipitateded by an equal volume of 4 M ammonium acetate, and 2 volumes of isopropanol (v/v) followed by centrifugation for 10 min at room temperature. The precipitate was washed with cold 70% (v/v) ethanol, dried and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or distilled water.

For ROP-PCR, primers were derived from the repeat sequences, synthesized commercially (Bioneer Co. Korea). The REP (REPIR-I: 5′-IIIICGICGICATCIGGC-3′; REP2-I: 5′-ICGICTTATCIGGCCTAC-3′) and ERIC (ERICIR: 5′-ATGTAAGCTCCTGGGGATTCAC-3′; ERIC2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′) primer sets were used (Versalovic *et al.*, 1991), and the PCR conditions for these primers were as specified by de Bruijn (1992). Secondly, we applied the STRR 1A primer (5′-CCARTCCCCARTCCCC-3′) and LTRR primer set (LTRR 1: 5′-GGATTTTTG TTAGTTAAAAC-3′; LTRR 2: 5′-CTATCAGGGATTGAAAG-3′) derived from cyanobacterial geneome (Rasmussen and Svenning, 1998). In addition, eukaryotic repetitive oligonucleotides (Freeman *et al.*, 1996), $(CAG)_5$, $(GACAC)_3$, and $(GACA)_4$, were applied to discriminate the cyanobacterial sp. Each PCR was performed in a total volume of 20 μl, containing 50 ng of genomic DNA; 10 mM Tris-HCl; 50 mM KCl; 0.25 mM each dATP, dCTP, dGTP and dTTP; 1.5 mM $MgCl_2$; 1 U of *Taq* DNA polymerase (Takara); and 1~3 μM primer. Buffers supplied with the respective enzymes were used according to the manufacturer's directions. The DNA amplification was performed in a Gene Cycler (Bio-Rad, USA), starting with a denaturation at 95°C. For the ERIC primers, the cycles were as follows: 1 cycle at 95°C for 7 min; 30 cycles of 94°C for 1 min, 46°C for 1 min, 65°C for 8 min; and 1 cycle at 65°C for 15 min. For the REP primers, the program was the same except that the annealing temperature was optimized to 40°C for 1 min. For the STRR primers, 1 cycle at 95°C for 6 min; 30 cycles of 94°C for 1 min, 46°C for 1 min, and 65°C for 5 min; and 1 cycle at 65°C for 16 min. For the LTRR primers, the program was the same except that the annealing temperature was optimized to 42°C for

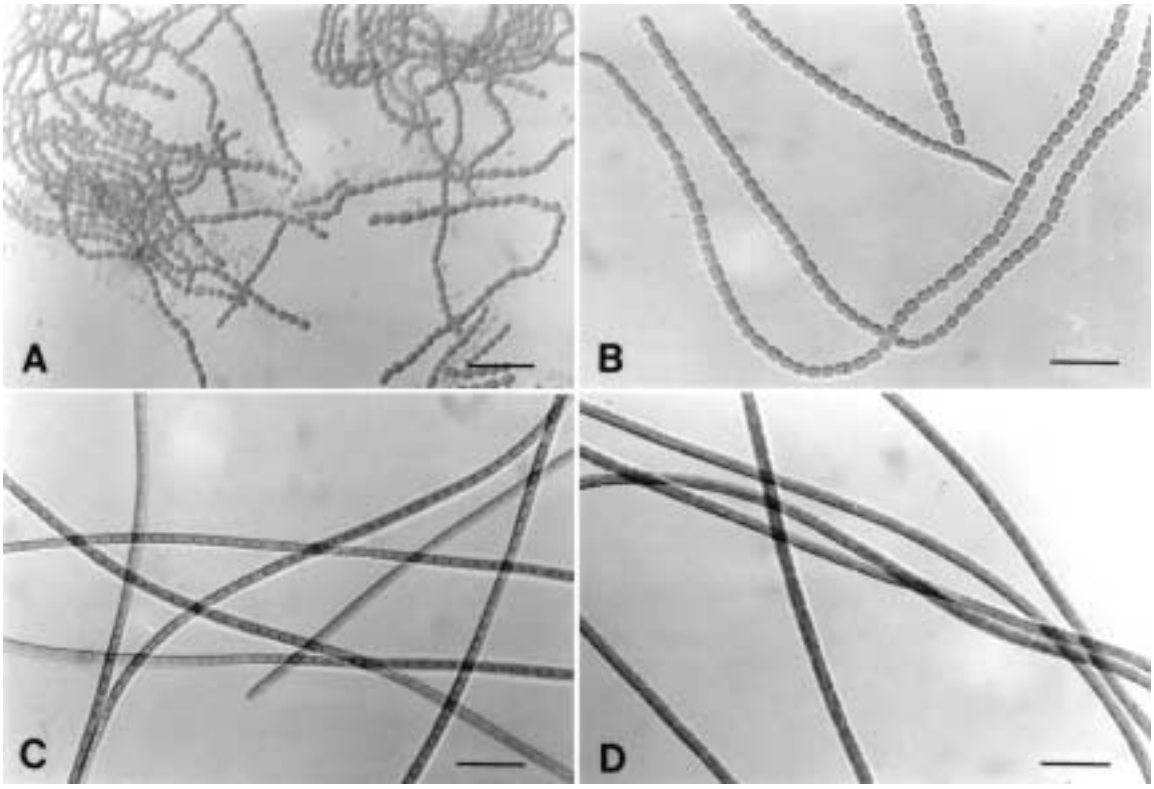

Fig. 1. A, *Anabaena* sp. PC9 collected from Daechung reservoir; B, *Anabaena* sp. PC16 from Kongju; C, *Oscillatoria* sp. IAM35 from foreign source; D, *Oscillatoria* sp. PC22 from Taejon. Scale bars = 40 μm

1 min. For the $(CAG)_5$, $(GACAC)_3$, and $(GACA)_4$ primers, 30 cycles consisting of 30 s at 95°C, 30 s either 45°C for $(CAG)_5$, 48°C for $(GACAC)_3$, or 40°C for $(GACA)_4$, and 1.5 min at 72°C. All PCRs were performed at least three independent times with identical results being obtained from each. Percentage similarity of amplified DNA products among the cyanobacterial isolates, based on the presence or absence of amplified bands (1/0), was calculated by pairwise comparisons with the SIMQUAL program of NTSYS-pc software version 1.80 (Rohlf, 1993).

RESULTS AND DISCUSSION

The morphological variation of cultured cyanobacteria in media frequently occurrs from the original morphology of natural isolates, and some strains may not be cultured under selective culturing conditions (Doers and Parker, 1988; Ward *et al.*, 1990). Two kinds of cultured filamentous cyanobacteria, *Anabaena* sp. and *Oscillatoria* sp., were examined by microscope. Under our culture condition, the former shows spherical vegetative cells (Fig. 1A, B), having sometimes released trichome fragments (data not shown), but lacking of characteristic specialized cells such as heterocysts and alkinetes. The latter shows constant cell diameter and shape within individual organisms, but among isolates cell diameters vary from 6~8 μm, having either chain-shaped (Fig. 1C) or disk-shaped without sheath (Fig. 1D). Since morphological variation exists even in a single isolate, the morpho-taxonomic criteria are not accurate enough to discriminate between species. In addition, the biochemical analysis of metabolic pigment content or isozyme variation may also lead to false results because some cyanobacterial gene products can be differentially expressed depending on the culture condition (Kato *et al.*, 1991).

ROP-PCR performed on 10 isolates of the cyanobacteria with ERIC and REP sequences from gram-negative bacteria, STRR1A and LTRR sequences from cyanobacterial genome, and eukaryotic repetitive sequences, led to the identifica-

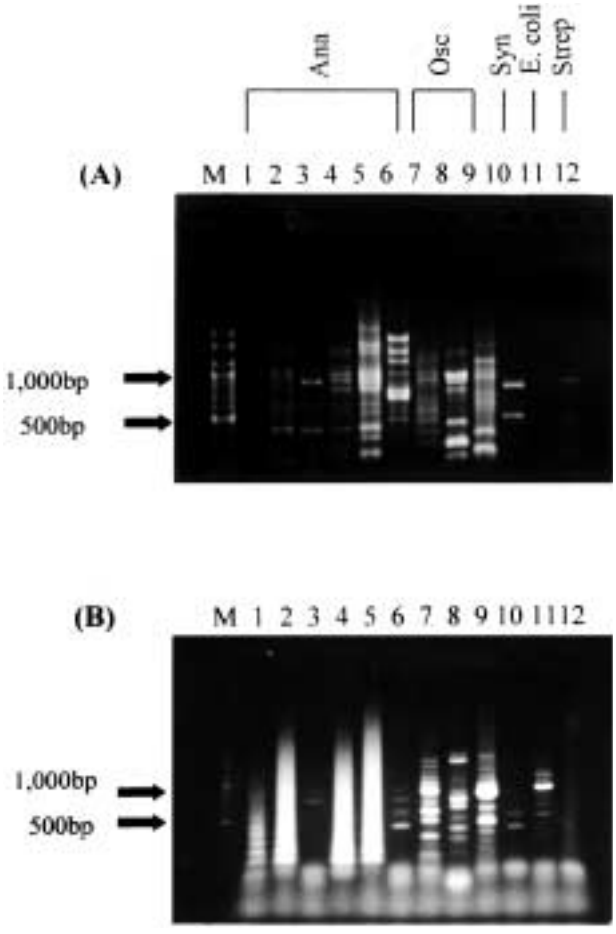

Fig. 2. ROP–PCR of cyanobacterial strains and electrophoresis for phylogenetic analysis. PCR was performed at an annealing temperature of 45°C with primers ERIC (A) and REP (B). M, 100 bp DNA ladder marker. Lanes 1 to 6 (Ana), *Anabaena* sp. N1444; *A. flos–aquae* PC1; *A. cylindrica* IAM1; *Anabaena* sp. PC2; *Anabaena* sp. PC9; and *Anabaena* sp. PC16. Lanes 7–9 (Osc), *Oscillatoria* sp. IAM35; *Oscillatoria* sp. PC22; and *Oscillatoria* sp. PC24. Lane 10 (Syn), *Synechocystis lepoliensis* IAM6. Lane 11 (E. coli), *Escherichia coli* DH5. Lane 12 (Strep), *Streptomyces* sp. 1080.

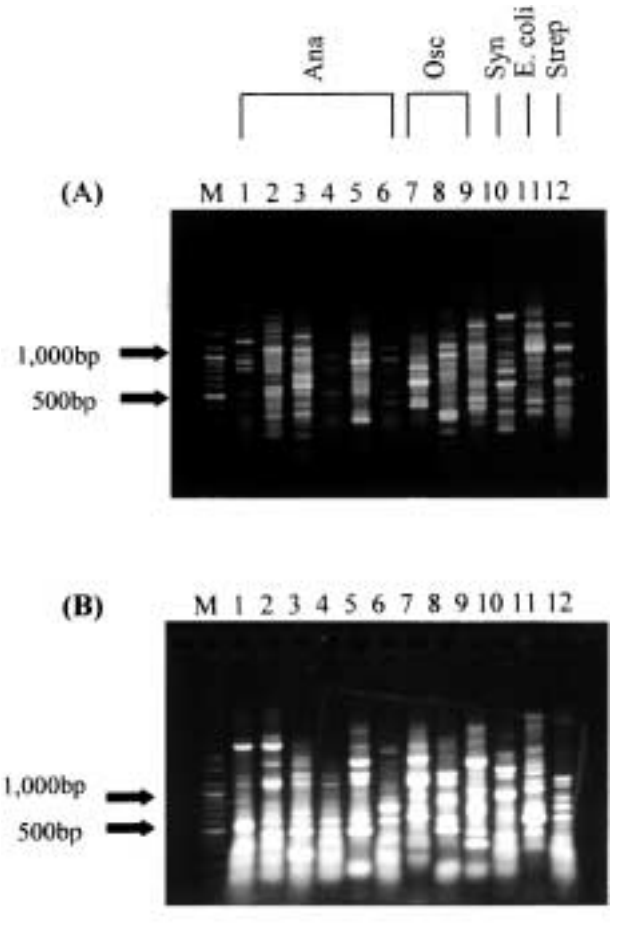

Fig. 3. ROP–PCR with primers STRR 1A (A) and LTRR (B). M, 100 bp DNA ladder marker. Lanes 1 to 6 (Ana), *Anabaena* sp. N1444; *A. flos–aquae* PC1; *A. cylindrica* IAM1; *Anabaena* sp. PC2; *Anabaena* sp. PC9; and *Anabaena* sp. PC16. Lanes 7–9 (Osc), *Oscillatoria* sp. IAM35; *Oscillatoria* sp. PC22; and *Oscillatoria* sp. PC24. Lane 10 (Syn), *Synechocystis lepoliensis* IAM6. Lane 11 (E. coli), *Escherichia coli* DH5. Lane 12 (Strep), *Streptomyces* sp. 1080.

tion of 10 distinct genotypes by the distinctive polymorphic patterns. The use of ERIC and REP sequences as primers shows that both ERIC and REP sequences generated more multiple distinct DNA profiles than any other primers, of sizes ranging from about 200 to 5,000 bp. Among isolates of *Anabaena* sp., isolates PC2 and PC16 were observed as a same group by the REP primers (Fig. 2A), however, more produced DNA fragments of isolate PC16 were distinguished from isolate PC2 by the ERIC primers (Fig. 2B). Unexpectedly, the gram–positive bacterium *Streptomyces* sp. 1080 also produced multiple bands by both primers, indicating that the taxonomic application of these primers should be considered. Both REP and ERIC sequences gave distinct reproducible PCR profiles but less recommended as primers for genomic fingerprinting of cyanobacteria, due to the common presence of these sequences in many prokaryotes and the use of both primers can be limited in nonaxenic cultures.

In order to draw a more specific conclusion

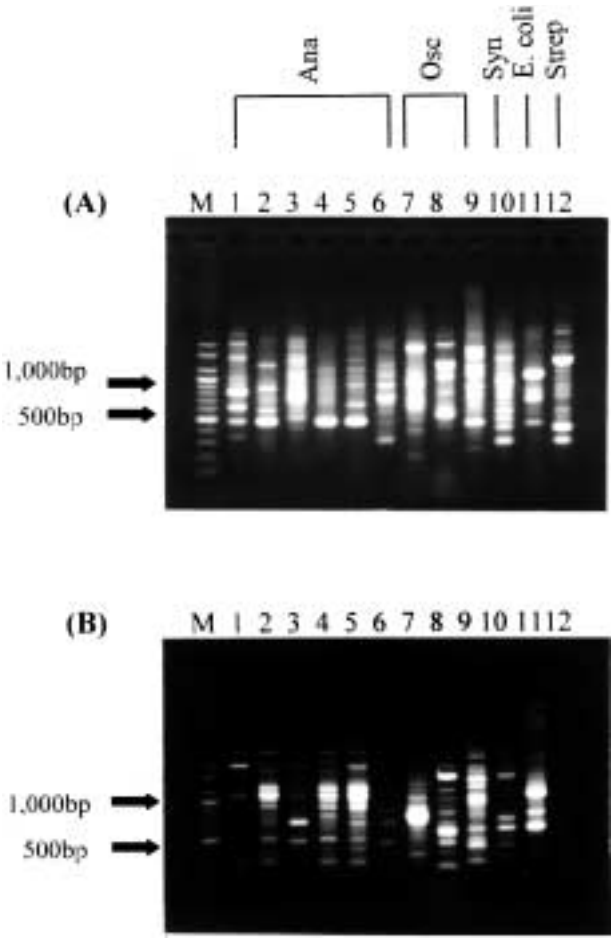

Fig. 4. ROP-PCR profiles using primers $(CAG)_5$ and $(GACA)_4$. M, 100 bp DNA ladder marker. Lanes 1 to 6 (Ana), *Anabaena* sp. N1444; *A. flos-aquae* PC1; *A. cylindrica* IAM1; *Anabaena* sp. PC2; *Anabaena* sp. PC9; and *Anabaena* sp. PC16. Lanes 7-9 (Osc), *Oscillatoria* sp. IAM35; *Oscillatoria* sp. PC22; and *Oscillatoria* sp. PC24. Lane 10 (Syn), *Synechocystis lepoliensis* IAM6. Lane 11 (E. coli), *Escherichia coli* DH5. Lane 12 (Strep), *Streptomyces* sp. 1080.

about diversity or similarity among closely related isolates, different primers have to be included in the PCR analysis, having a different resolution. The STRR 1A sequence generated more multiple DNA profiles than the LTRR ones but the characteristic fragment patterns obtained by the LTRR primers could divide *Anabaena* sp. into two groups, group I (N1444, PC1, PC2, and PC9), and II (IAM1 and PC16) (Fig. 3B). Of the tested *Anabaena* species, isolates PC1 and PC2 are the closest by analysis of combined STRR and LTRR profiles. In *Oscillatoria* sp., each isolate showed quite different band patterns by both STRR and LTRR primers. The gram-negative bacterium, *E. coli*, could be detected by the LTRR primers, but not by the STRR primer. In contrary, the gram-positive bacterium, *Streptomyces* sp., could be detected by the STRR primer, but not by the LTRR primers. Among the primers we tested, the cyanobacterial STRR and LTRR sequences proved their efficiency to identify and characterize cyanobacteria.

By primers $(GACAC)_3$ (data not shown) and $(GACA)_4$, common grouping could be observed in *Anabaena* sp., PC1, PC2, and PC9 as one group (Fig. 4B), but no such common grouping was obtained by $(CAG)_5$. Interestingly, $(CAG)_5$ produced clear amplified DNA patterns of both gram-negative and -positive bacteria (Fig. 4A). The repetitive primers derived from eukaryotes demonstrated to be the valuable tool for identification, but more tests with a variety of samples should be required before their application.

Table 1 shows the similarity of cyanobacterial isolates based on the PCR analysis with STRR1A, LTRR, ERIC, REP, $(CAG)_5$, $(GACAC)_3$, and $(GACA)_4$ primers. Among six isolates of *Anabaena* sp., each shared a range of similarity with others (17.2~50.1%) and all of them are considered to be genetically variable. The isolate N1444 shared 33.9~35.6% similarity with isolates PC1, PC2 and PC9. The isolate, PC16, collected from Kongju, was the lowest similarity among *Anabaena* sp. The highest similarity group ranging by 49.3~50.1% was observed among *A. flos-aquae* PC1, *Anabaena* sp. PC2, and *Anabaena* sp. PC9 and thus they are considered to be closely related isolates belonging to *A. flos-aquae*. The amplified DNA profiles observed among the *Anabaena* species might suggest that genetically various isolates were present in the Daechung reservoir, causing severe eutrophication every year. Of three isolates of *Oscillatoria* sp., IAM35, PC22, and PC24, showed quite different banding patterns with 7.2~13.9% similarity, found to be genetically unrelated. In addition, it was found that distinct polymorphism existed between six isolates of *Anabaena* and three isolates of *Oscillatoria* with intergenus percentage similarity ranging from 5.5 to 12.3%.

It has been known that the genomes of prokarotic microorganisms contain numerous classes of short repeated sequences (Mazel *et al.*, 1990; Hulton *et al.*, 1991; de Bruijn, 1992). In this report, we have successfully characterized the genetic

Table 1. Matrix of similarity (%) of cyanobacterial isolates based on Rop–PCR.

Isolate #	Similarity (%) to isolate[a]											
	Anabaena Sp.						*Oscillatoria* Sp.			*Synecho-cystis*	*E.coli*	*Strepto-myces*
	N1444	PC1	IAM1	PC2	PC9	PC16	IAM35	PC22	PC24	IAM6	DH5	1080
N1444	–	35.6	21.9	35.3	33.9	19.6	10	6.2	7.3	5.4	4.0	1.8
PC1		–	26.1	50.1	49.3	17.2	9.6	12.3	5.5	9.2	8.7	7.6
IAM1			–	29.1	22.8	25.2	10.5	9.4	5.9	10.5	12.3	3.1
PC2				–	47.3	20.9	7.5	7.3	6.6	6.9	6.2	4.0
PC9					–	28.9	8.7	12.2	7.2	6.1	5.7	1.4
PC16						–	9.5	8.5	7.3	8.9	6.2	3.3
IAM35							–	13.9	9.9	6.6	5.4	6.8
PC22								–	7.2	5.7	5.4	4.2
PC24									–	7.0	6.6	4.0
IAM6										–	8.9	8.1
DH5											–	5.9
1080												–

[a]Percentage similarity is the average calculated from pairwise comparisons done by using SIMQUAL of NTSYS among representative isolates of cyanobacteria from *Anabaena* sp. N1444, *A. flos–aquae* PC1, *A. cylindrica* IAM1, *Anabaena* sp. PC2, *Anabaena* sp. PC9, *Anabaena* sp. PC16, *Oscillatoria* sp. IAM35, *Oscillatoria* sp. PC22, *Oscillatoria* sp. PC24, and *Synechocystis lepoliensis* IAM 6, with primers STRR 1A, LTRR, ERIC, REP, $(CAG)_5$, $(GACAC)_3$, and $(GACA)_4$.

diversity within the limited isolates of *Anabaena* sp. and *Oscillatoria* sp. with different sets of PCR primers. Since the DNA profiles produced for the cyanobacteria provide a rapid and reliable basis for the genetic types of clonal and axenic cultures, the primers used in our experiments might be useful to detect and classify the species diversity of cyanobacteria in lakes or the reservoirs of Korea.

ABSTRACT

Since some cyanobacterial isolates under selective culturing conditions are lacking of characteristic specialized cells or showing altered morphology, the morpho–taxonomic criteria are not accurate enough to discriminate between species. Instead of morphological parameters, a method based on the single or the combination of repetitive oligonucleotides in a single PCR, repetitive oligonucleotides–primed PCR (ROP–PCR), was applied to generate DNA profiles for members of the cyanobacterial genera *Anabaena* and *Oscillatoria*, both of which are responsible for causing poisonous blooms in various freshwater systems. ROP–PCR performed on 10 isolates of the cyanobacteria with ERIC and REP sequences from gram–negative bacteria, STRR1A and LTRR sequences derived from cyanobacterial genome, and eukaryotic repetitive sequences, led to the identification of distinct genotypes, and provided specific and repeatable DNA fingerprints for cyanobacterial isolates. Grouping analysis of cyanobacterial isolates showed a significant difference depending on the primer used in PCR.

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