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# Random Amplified Polymorphic DNA (RAPD) Analyses for Discriminating Genotypes of *Microcystis* Cyanobacteria

Hirofumi NISHIHARA, Hiroyasu MIWA, Masayuki WATANABE,* Minoru NAGASHIMA,** Osami YAGI,*** and Yoshichika TAKAMURA†

*Department of Applied Biological Resource Sciences, Ibaraki University, 3–21–1 Chu-ou, Ami-machi, Ibaraki 300–03, Japan*
**Department of Botany, National Science Museum, 4–1–1 Amakubo, Tsukuba-shi, Ibaraki 305, Japan*
***Tsukuba Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 2 Miyukigaoka, Tsukuba-shi, Ibaraki 305, Japan*
****Water and Soil Environment Division, The National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba-shi, Ibaraki 305, Japan*
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**Random amplified polymorphic DNA (RAPD) analysis was used to discriminate genotypes in five species of *Microcystis* cyanobacteria. Strains of each group with the identical allozyme genotype (T. Kato *et al.*, Algol. Stud., 1991, 129–140; M. Watanabe, in "Toxic Microcystis," ed. by M. F. Watanabe *et al.*, CRC Press, Tokyo, 1966, pp. 13–34) gave similar RAPD patterns characterizing the respective group. On the other hand, no similarities in RAPD patterns were observed among strains of which allozyme genotypes were different. A good accordance between the RAPD analysis and allozyme divergence indicated a high reliability of both methods for discrimination of the affiliated groups of *Microcystis*. Several amplified DNA fragments, which were expected to be markers for a particular taxon with identical allozyme genotype, were also observed on the RAPD patterns. Genetic homogeneities of *M. novacekii*, *M. viridis*, and *M. wesenbergii* were shown by RAPD analysis as well as the allozyme genotype. However, significant variations were observed in *M. aeruginosa* and *M. ichthyoblabe* in the levels of DNA and proteins (allozymes).**

**Key words:** RAPD analysis; DNA polymorphism; *Microcystis*; cyanobacterium; allozyme genotype

*Microcystis* is one of the dominant genus of cyanobacteria (blue-green algae) which form water blooms in eutrophic lakes and reservoirs. The progressive deterioration of water quality due to the cyanobacterial bloom has evoked serious problems for water use. Especially, because of the toxicities of some species of *Microcystis*, the extensive growth of *Microcystis* in fresh waters presents a considerable threat to the health of humans and other animals.[1–4] Therefore, the establishment of a rapid and reliable method for identification of the species of *Microcystis* is of great importance for the management of water quality.

The taxonomy of *Microcystis* has been done traditionally by classical phenotypic criteria such as microbial morphologies and cell arrangement of colonies.[5,6] However, the morphological characteristics of cyanobacteria are easily altered by the environmental conditions of the habitat and cultural conditions in a laboratory. To resolve the problems of the traditional taxonomic method, Kato *et al.* applied a molecular taxonomic method based on allozyme divergence to discriminate between three species of *Microcystis*.[7,8] According to their analyses, strains of *M. viridis* and *M. wesenbergii* had a single and unique allozyme genotype for each taxon, and were regarded as well-established species. However, *M. aeruginosa* were highly polymorphic and contained 12 allozyme genotypes from among the 19 strains tested.

New technologies for DNA fingerprinting such as random amplified polymorphic DNA (RAPD)[9,10] and arbitrarily primed PCR (AP-PCR)[11] analyses give direct information on the genetic relatedness of the affiliated group of organisms, allowing reliable identification and discrimination of taxa irrespective of external growth conditions. Restriction fragment length polymorphism (RFLP) analysis[12] and targetted PCR assay have also been reported for studying genetic polymorphism, however, they have some limitations. RFLP analysis requires considerable amounts of genomic DNA and cloned probes specific for a related group of organisms. The targetted PCR assay requires DNA sequence knowledge of the organism under study, although it needs much less genomic DNA. In RAPD and AP-PCR, a single oligonucleotide primer with arbitrary sequence is used for random amplification of DNA by the polymerase chain reaction (PCR). Therefore, polymorphisms can be easily analyzed by small amounts of template DNA, and no DNA sequence information is required. This is advantageous as a taxonomic method for cyanobacteria because growth of some cyanobacteria is poor and the genomic DNA is not easily obtained. Furthermore, not much genetic information is available for cyanobacteria compared to eubacteria. RAPD analysis involves one stage of the PCR reaction and amplification products on gel electropherogram are detected by ethidium bromide.[10] AP-PCR involves two stages of amplification reaction (low stringency and higher stringency annealing stages) in PCR, and radioactive labelling is used for the detection of amplification products.[11] Thus, RAPD seems to be simpler and more convenient than AP-PCR.

In this study, we used RAPD analysis for discrimination of five species of *Microcystis* cyanobacteria that have been classified on the basis of morphology, toxicity, and allo-

† Corresponding author.

zyme genotype.[8] A few studies to analyze the genotypic characteristics of cyanobacteria have been attempted,[13-15] however to our knowledge, this is the first report concerning RAPD analyses of cyanobacteria. We showed that there was a good accordance between the analyses of RAPD and allozyme genotypes, and that RAPD analysis was a simple and reliable method for discrimination of *Microcystis* species.

## Materials and Methods

*Strains.* Strains of *Microcystis* cyanobacteria used in this study are listed in Table I. These strains were isolated from Japanese lakes and ponds, and classified in the Tsukuba Algal Collections (TAC), National Science Museum, Tsukuba, Japan according to descriptions of *Microcystis* species.[8] Every strain was purified to a monoclonal culture using the capillary pipette washing method and kept in TAC. When several monoclonal cultures originated from the same culture are kept in TAC, they are distinguished by hyphenated numbers in the strain number. Alloyzmes of isocitrate dehydrogenase (Idh, EC 1.1.1.42), 6-phosphogluconate dehydrogenase (6Pgd, EC 1.1.1.44), glucosephosphate isomerase (Pgi, EC 5.3.1.9), and phosphoglucomutase (Pgm, EC 2.7.5.1) were analyzed electrophoretically by Kato *et al.*,[7] and alphabetized according to their electrophoretic mobilities[7,8] (Allozyme genotype of TAC 140 is unpublished data of Watanabe). For example, *M. aeruginosa* TAC 157 had a genotype of $Idh^K$-$6Pdg^g$-$Pgi^k$-$Pgm^d$, which was abbreviated as Kgkd.

*Cultivation.* The strains were cultivated in 500-ml Erlenmeyer flasks containing 200 ml of medium under 2000 lux of continuous fluorescent light at 30°C. The cultivation medium, pH 8.2, contained 0.40 g of TAPS, 0.15 g of Ca $(NO_3)_2 \cdot 4H_2O$, 0.10 g of $KNO_3$, 40 mg of $MgSO_4 \cdot 7H_2O$, 50 mg of $\beta$-$Na_2$ glycerophosphate, 10 $\mu$g of thiamin, 0.1 $\mu$g of vitamin B-12, 0.1 $\mu$g of biotin, and 3 ml of metal solution, per liter. The metal solution contained 1.0 g of $Na_2EDTA \cdot 2H_2O$, 196 mg of $FeCl_3 \cdot 6H_2O$, 36.0 mg of $MnCl_2 \cdot 4H_2O$, 10.5 mg of $ZnCl_2$, 4.0 mg of $CoCl_2 \cdot 6H_2O$, and 2.5 mg of $Na_2MoO_4 \cdot 2H_2O$, per liter. Cells in early-stationary phase were pressured at 5 kg/cm$^2$ for 5 min to disrupt gas vacuoles, washed with 50 mM Tris–HCl, 1 mM EDTA (pH 8.0) and then harvested by centrifugation. Cell growth was followed turbidimetrically at 660 nm. The cell pellet was stored frozen at −80°C.

**Table I.** Strains of *Microcystis* Species Used in This Study

| Strains | Allozyme genotypes[a] | | | | Collection locality |
|---|---|---|---|---|---|
| | Idh | 6Pgd | Pgi | Pgm | |
| *Microcystis aeruginosa* | | | | | |
| TAC 157 | K | g | k | d | Chiba: Tega-numa Pond |
| 169 | k | b | j | d | Tokyo: Lake Okutama-ko |
| 192 | h | b | k | d | Tokyo: Lake Okutama-ko |
| *M. ichthyoblabe* | | | | | |
| TAC 48-1 | j | a | a | d | Nagano: Lake Suwa-ko |
| 48-3 | j | a | a | d | Nagano: Lake Suwa-ko |
| 51 | j | a | a | d | Nagano: Lake Suwa-ko |
| 91 | b | a | d | f | Hokkaido: Lake Barato-ko |
| 125 | g | d | o | c | Hokkaido: Lake Barato-ko |
| 146 | d | d | f | g | Hokkaido: Lake Oonuma-ko |
| *M. novacekii* | | | | | |
| TAC 65 | g | c | h | d | Nagano: Chikatou-ike Pond |
| 66 | g | c | h | d | Nagano: Rokusuke-ike Pond |
| 75 | g | c | h | d | Shiga: Lake Yogo-ko |
| *M. viridis* | | | | | |
| TAC 45-1 | f | f | i | b | Ibaraki: Lake Kasumigaura |
| 78 | f | f | i | b | Fukui: Lake Mikata-ko |
| 92 | f | f | i | b | Hokkaido: Lake Barato-ko |
| 140 | f | f | i | b | Fukuoka: Yamesyouwa-ike Pond |
| *M. wesenbergii* | | | | | |
| TAC 52-1 | a | f | g | a | Nagano: Lake Suwa-ko |
| 57-1 | a | f | g | a | Nagano: Lake Suwa-ko |

[a] Allozyme genotypes were cited from Kato *et al.*[7] and Watanabe[8] except for TCA 140 (Watanabe, unpublished data).

*Abbreviations*: Idh, isocitrate dehydrogenase; 6Pgd, 6-phosphogluconate dehydrogenase; Pgi, glucosephosphate isomerase; Pgm, phosphoglucomutase.

*Extraction and purification of DNA.* One gram of frozen cells was thawed in 8 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 20 mM EDTA, 50 mM NaCl, and 0.25 M sucrose. Forty mg of lysozyme was dissolved in 1 ml of the same buffer and added to the cell suspension, then incubated at 37°C for 60 min. Five mg of Actinase E (Kaken Seiyaku Co., Ltd., Tokyo, Japan) dissolved in 0.2 ml of the same buffer and 0.25 ml of 20% SDS solution was added to the suspension and incubated at 37°C for 60 min. Subsequently, 0.8 ml of 20% SDS was added and incubated at 60°C for 20–40 min until the suspension became clear. Extracted DNA was purified by chloroform/isoamyl alcohol extraction, phenol extraction, and RNase treatment as described by Dzelzkalns *et al.*[16] DNA was recovered by adding 2.5 vols of ethanol to the solution. The purified DNA was dissolved in 1 ml of water and dialyzed against water. DNA concentration was estimated from the equation: $OD_{260}$ 1.0 = 50 $\mu$g DNA/ml.

*Primer synthesis.* Oligonucleotide primers were synthesized by the phosphoramidite method on a model 392 DNA synthesizer (Perkin Elmer Japan, Applied Biosystems Divison, Chiba, Japan). After completion of the trithyl-on synthesis, the oligonucleotide was purified using an Oligonucleotide Purification Cartridge (Perkin Elmer Japan, Applied Biosystems Division) according to their commercial manual.

*PCR conditions and analysis of amplified DNA fragments.* Primers used for RAPD analyses are listed in Table II. Amplification reactions were done in volumes of 100 $\mu$l containing 10 $\mu$l of 10 × Tth buffer (Toyobo Co., Ltd., Tokyo, Japan), 10 $\mu$l of dNTPs mix (2 mM each of dATP, dCTP, dGTP, and dTTP), 4 $\mu$l of 10 $\mu$M primer, 2 ng of template DNA, and 3 unit of Tth polymerase (Toyobo Co., Ltd.). The reaction was cycled through the following temperature profile on a Program Temp Control System PC-700 (Astec Co., Ltd., Fukuoka, Japan): 1 cycle of 5 min at 94°C, 35 cycles of 50 s at 35°C, 2 min at 72°C, 1 min at 93°C, lcycle of 50 s at 35°C, 7 min at 72°C. Amplification products were analyzed by electrophoresis in 2% agarose gels and detected by staining with ethidium bromide.

## Results

### *Yield of cells and DNA*

Yield of cyanobacterial cells harvested at early-stationary phase of growth and the amounts of purified DNA from 1 g of the cell pellets are shown in Table III.

### *Selection of appropriate primers for RAPD analysis*

Genomic DNA purified from each of two strains of *Microcystis aeruginosa* (TAC 157, TAC 169), *M. ichthyoblabe* (TAC 91, TAC 146), and *M. novacekii* (TAC 65, TAC 75) was amplified using eight kinds of primers listed in Table II to slect suitable primers for RAPD analysis. Agarose gel electropherograms of the amplified DNA fragments (RAPD patterns) are shown in Fig. 1. RAPD patterns showing DNA

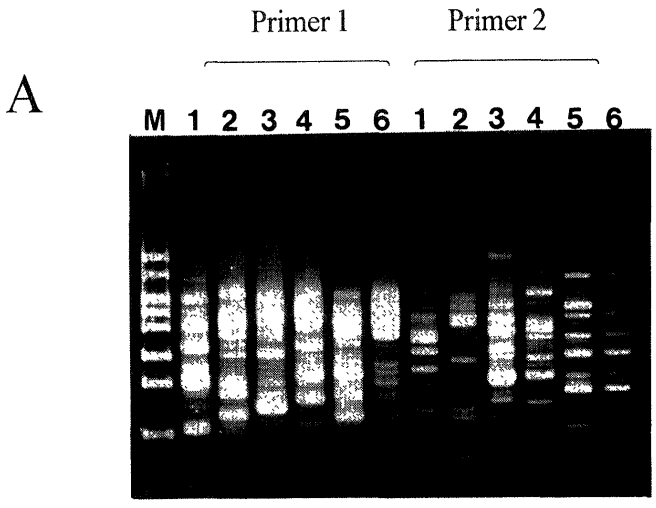
**Table II.** Primer Sequences Used in This Study

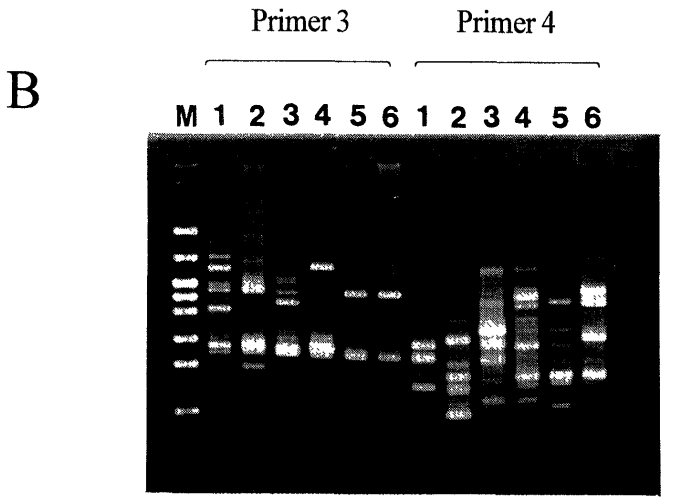
| Primer number | Sequence (5′–3′) |
|---|---|
| 1 | CAATATGGTGCGCGACAAT |
| 2 | TGCCATTCCTATACTAACCA |
| 3 | AGCCATGGC |
| 4 | GCCGTGCTGCCCCTGGTA |
| 5 | TAGATAGATAGATAGA |
| 6 | GACAGACAGACAGACA |
| 7 | TGTGTGTGTGTG TGTGTGCC |
| 8 | CCAGAAATCCAAGAATGTGA |

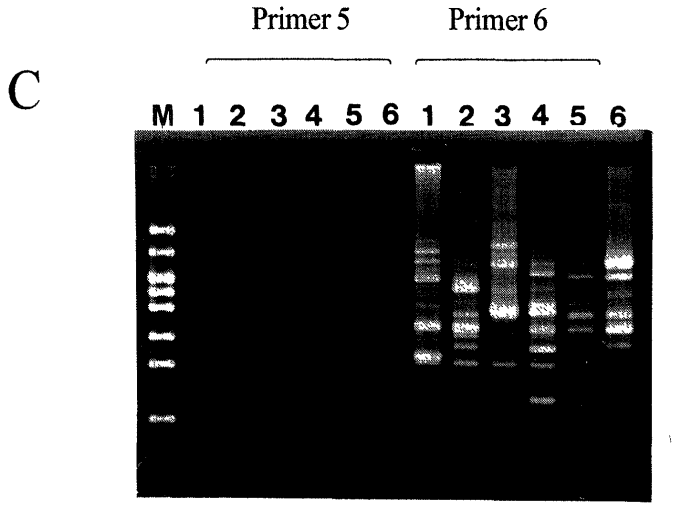
**Table III.** Yield of Cells and Purified DNA from the Cultures of *Microcystis* Species

The cells were cultivated as described in Materials and Methods, and were then harvested at cultivation period shown in the Table. DNA was purified from 1.0 g (wet weight) of the cell pellet.

| Strains | Cultivation period (days) | Cell yield (g wet weight/liter) | Purified DNA ($\mu$g) |
|---|---|---|---|
| *Microcystis aeruginosa* | | | |
| TAC 157 | 23 | 9.47 | 31 |
| 169 | 14 | 8.48 | 43 |
| 192 | 25 | 0.94 | 115 |
| *M. ichthyoblabe* | | | |
| TAC 48-1 | 23 | 0.57 | 109 |
| 48-3 | 23 | 2.11 | 76 |
| 51 | 20 | 1.00 | 464 |
| 91 | 14 | 1.08 | 226 |
| 125 | 14 | 1.01 | 160 |
| 146 | 23 | 2.59 | 104 |
| *M. novacekii* | | | |
| TAC 65 | 21 | 7.23 | 166 |
| 66 | 14 | 0.74 | 8 |
| 75 | 14 | 1.71 | 161 |
| *M. viridis* | | | |
| TAC 45-1 | 20 | 1.14 | 189 |
| 78 | 24 | 1.04 | 189 |
| 92 | 18 | 1.21 | 292 |
| 140 | 23 | 1.70 | 570 |
| *M. wesenbergii* | | | |
| TAC 52-1 | 20 | 1.42 | 325 |
| 57-1 | 23 | 1.63 | 114 |



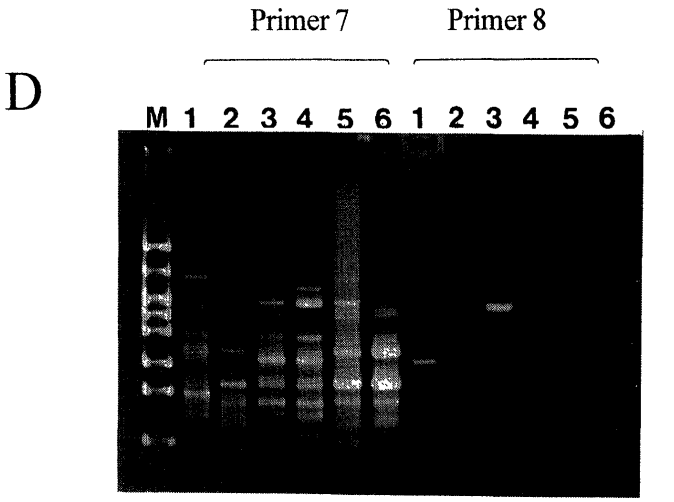




**Fig. 1.** RAPD Patterns for *Microcystis* Cyanobacteria with Primers 1, 2 (A), 3, 4 (B), 5, 6 (C), 7, and 8 (D).

Lanes are as follows: 1, DNA from *M. aeruginosa* TAC 157; 2, TAC 169; 3, *M. ichthyoblabe* TAC 91; 4, TAC 146; 5, *M. novacekii* TAC 65; 6, TAC 75; M, markers of molecular size (pHY marker, Takara Shuzo Co., Ltd., Kyoto, Japan). Size (bp) of molecular markers are as follows from the upper fragment: 4870, 2016, 1360, 1107, 926, 658, 489, and 267.

polymorphism were obtained when Primers 1, 2, 4, 6, and 7 were used. RAPD pattern using Primer 3 was not suitable for the analysis of DNA polymorphism because not a few amplified fragments were observed to be commonly present in all strains tested (Fig. 1B). No or few amplified fragments were observed when Primers 5 or 8 was used (Figs. 1C and 1D). Primers 1, 6, and 7 were used for the following experiments for discriminating 5 species of *Microcystis*.

*RAPD analysis of each group with an identical allozyme genotype*

Genomic DNAs from 3 strains of *M. ichthyoblabe* (allozyme genotype, jaad), 3 strains of *M. novacekii* (allozyme genotype, gchd), 4 strains of *M. viridis* (allozyme genotype, ffib), and 2 strains of *M. wesenbergii* (allozyme genotype, afga) were subjected to RAPD analyses using Primers 1, 6, and 7. Strains of each group with the same allozyme genotype gave similar and unique RAPD patterns to the respective group (Fig. 2).

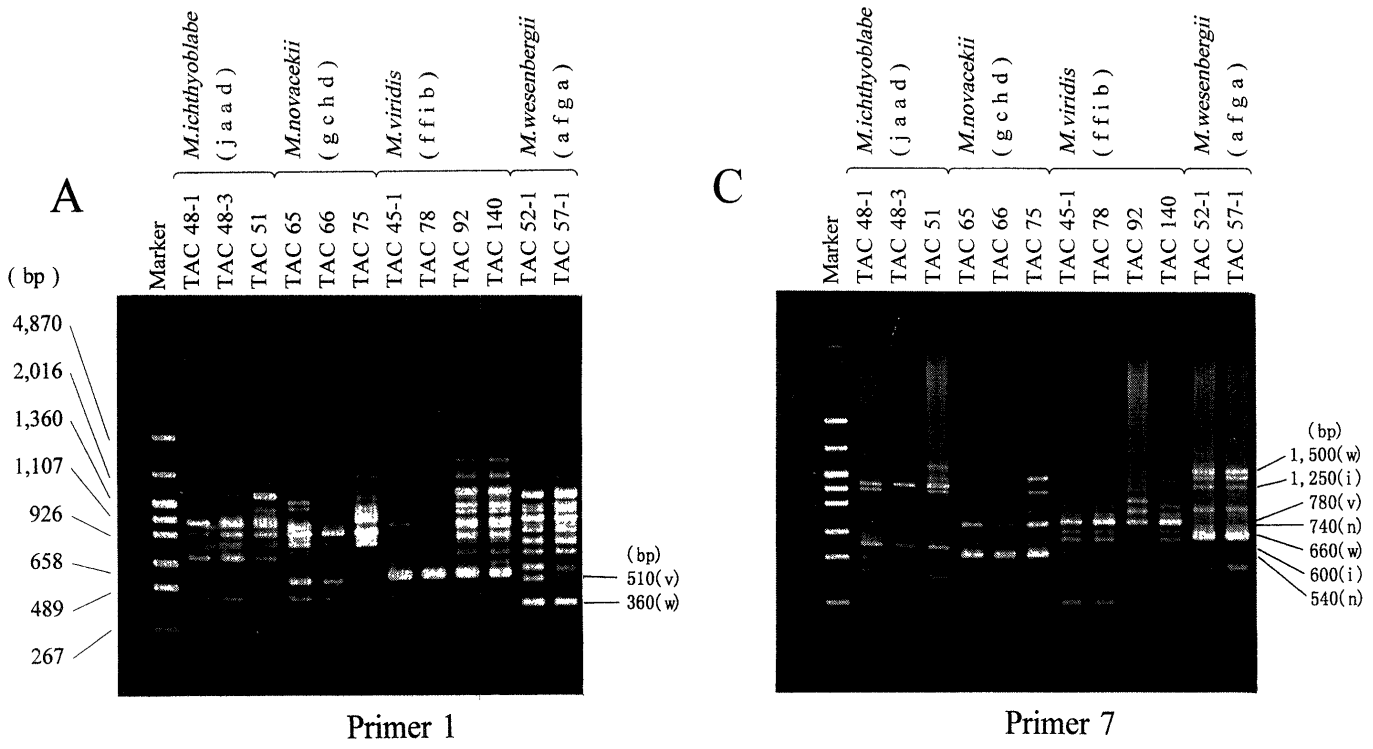



**Fig. 2.** RAPD Patterns for *Microcystis* Cyanobacteria Possessing Identical Allozyme Genotype to Each Group with Primers 1 (A), 6 (B), and 7 (C).

Alloyzme genotype of each taxon is noted in parenthesis below the species name. Size of molecular markers (pHY marker, Takara Shuzo Co., Ltd.) are indicated on the left (A). Positions of RAPD markers are indicated on the right with molecular size, and abbreviations of species names to which RAPD markers are specific are also indicated in parenthesis (A, C). Abbreviations are as follows: i, *M. ichthyoblabe*; n, *M. novacekii*; v, *M. viridis*; w, *M. wesenbergii*.

When Primer 1 was used, RAPD patterns for two strains of *M. wesenbergii* are almost identical. A major fragment of 360 bp seemed to distinguish *M. wesenbergii* from others shown in Fig. 2A. RAPD patterns for *M. viridis* TAC 45-1 and TAC 78 gave simple patterns compared to TAC 92 and TAC 140, though a major fragment of 510 bp was commonly observed in all strains of this species examined (Fig. 2A).

When Primer 6 was used, RAPD patterns of strains belonging to *M. ichthyoblabe*, *M. viridis*, and *M. wesenbergii* were almost identical in each species, although some variations were observed in *M. viridis* when Primer 1 was used (Fig. 2B).

Primer 7 gave rather simple RAPD patterns, but this primer was quite useful to discriminate among the four species shown in Fig. 2C. RAPD pattern for *M. ichthyoblabe* gave major bands of 600 bp and 1250 bp which seemed to characterize this species. Likewise, the other species also showed characteristic major fragments for each species (*i.e.*, *M. novacekii*, 540- and 740-bp fragments; *M. viridis*, 780-bp fragment; and *M. wesenbergii*, 660- and 1500-bp fragments).

However, it should be noticed that some of these characteristic fragments were near other fragments from strains with different allozyme genotypes in the mobilities on RAPD pattern. For instance, fragments close to the 510-bp frament of *M. virids* and 360-bp fragment of *M. wesenbergii* (Fig. 2A) were recognized in RAPD patterns from *M. ichtyoblabe* TCA 125 and TAC 91, respectively (Fig. 3A). Likewise, the 1250-bp fragment of *M. ichthyoblabe*, 780-bp fragment of *M. viridis*, and 540-bp fragment of *M. novacekii* (Fig. 2C) were in need of minute comparison with *M. wesenbergii* (TAC 52-1 shown in Figs. 2C and 3C, and TAC 57-1 in Fig. 2C). *M. aeruginosa* TAC 192 (Fig. 3C), and *M. aeruginosa* TAC 169 (Fig. 3C), respectively.

*Comparison of the RAPD patterns among strains with different allozyme genotypes*

RAPD patterns of 10 strains having different allozyme genotypes are shown in Fig. 3. Every RAPD pattern showed different fingerprints which were well consistent with the diverse allozyme genotypes of these strains. Any similarities characterized by strains of *M. aeruginosa* (TAC 157, TAC

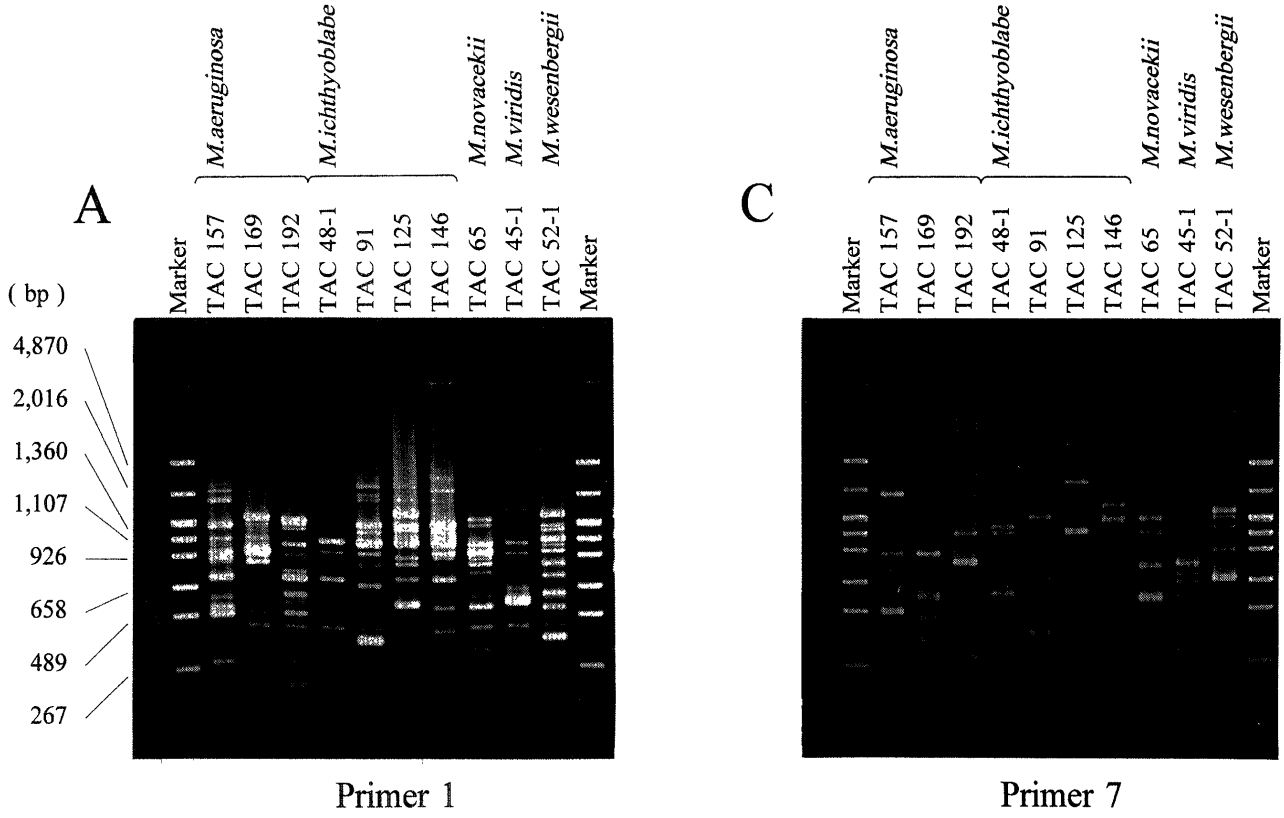



**Fig. 3.** RAPD Patterns for *Microcystis* Cyanobacteria Possessing a Different Allozyme Genotype Obtained with Primers 1 (A), 6 (B), and 7 (C).

Size of molecular markers (pHY marker, Takara Shuzo Co., Ltd.) are indicated on the left (A).

169, and TAC 192) were not observed in RAPD patterns even if any primer was used. This was also the same case for the strains of *M. ichthyoblabe* (TAC 48-1, TAC 91, TAC 125, and TAC 146). These results indicate that the two species are varied in the levels of DNA and protein (allozyme), and further taxonomical revisions might be necessary.

## Discussion

RAPD analysis was applied to *Microcystis* cyanobacteria, which is one of the common water bloom formers. To our knowledge, this is the first report concerning RAPD analysis of cyanobacteria. The growth of some cyanobacteria is poor, and their cultivation in the laboratory is considerably difficult. This is one of the reasons why the usual bacterial taxonomic techniques are not applicable to cyanobacteria. RAPD analysis requires only a small amount of DNA, and sufficient amounts of cyanobacterial DNA for RAPD analysis were obtained in this study.

Out of eight kinds of primers tested for 6 strains of three species of *Microcystis*, five primers (Primers 1, 2, 4, 6, and 7) gave suitable RAPD patterns for analysis of DNA polymorphism. Furthermore, three different types of primers (Primers 1, 6, and 7) were chosen to investigate the applicability of the RAPD analysis for discriminating genotypes of *Microcystis* cyanobacteria including 18 strains from 5 species. Primer 1 gave the most abundant amplified fragments. On the contrary, Primer 7 gave rather simple RAPD patterns. Primer 6 was chosen from the other three primers which gave a medium number of amplified fragments. Primers 2 and 4 are also thought to be useful when more additional primers become necessary for further study. The RAPD pattern using Primer 3 did not show reliable DNA polymorphism for assessing genetic variation because most of the amplification fragments were commonly present in all strains. The amplification reaction did not occur when Primer 5 or 8 was used.

Allozyme divergency of the strains used in this study were

previously analyzed as a molecular taxonomic method.[7,8] Using either one of three selected primers, RAPD patterns from strains belonging to a group with the same allozyme genotype had similar patterns (Fig. 2). Especially, RAPD patterns obtained using Primers 6 and 7 were highly similar and unique in each group. On the other hand, no similarity was observed if the alloyzme genotypes of strains were varied (Fig. 3). Our results showed that there was a good accordance between the analyses of RAPD and allozyme divergency, suggesting a high reliability of the RAPD analysis for the easy and rapid discrimination of cyanobacteria.

The taxonomy of *Microcystis* has been mainly done by morphological characteristics which easily vary by the surrounding conditions. Allozyme study of the genus *Microcystis* showed that some species were highly polymorphic in allozyme genotypes.[7] This result indicates that rearrangement of classification is necessary for the genus. The group of *Microcystis* with identical allozyme genotypes will be considered as a taxon with high reliability. Therefore, we tried to find the major amplified fragments on the RAPD patterns which might be useful as markers for distinguishing such a taxon from the others (Fig. 2). The strains of the taxon with the same allozyme genotype also had similar RAPD patterns as mentioned before. It should be noticed, however, the amplification of several minor fragments on RAPD patterns was affected by PCR conditions such as ambient temperature where the thermal cycler was run and the type of the thermal cycler, even though the composition of the reaction mixture and the cycle program for PCR were not changed (data not shown). Thus, major bands constantly appeared on the RAPD patterns from the taxon were expected to be candidates for markers.

Such fragments were observed when Primer 1 (Fig. 2A) or Primer 7 (Fig. 2C) was used. Comparing with Primer 1, Primer 7 gave simple and characteristic RAPD patterns for the respective taxon. Primer 7 gave characteristic 600-bp and 1250-bp fragments for *M. ichthyoblabe* (allozyme genotype, jaad), 540-bp and 740-bp fragments for *M. novacekii* (allozyme genotype, gchd), 780-bp fragment for *M. viridis* (allozyme genotype, ffib), and 660-bp and 1500-bp fragments for *M. wesenbergii* (allozyme genotype, afga). However, major fragments similar to some of these marker candidates in sizes were observed in RAPD patterns from other strains with different allozyme genotypes. A possibility is also considered that another DNA fragment was immigrating with the marker fragment on the RAPD pattern. Thus, there are two problems to be solved to apply these fragments as markers; (1) it should be clarified whether these fragments are specific to the corresponding taxon, (2) it is difficult to identify the marker fragment on RAPD patterns obtained from strains to be classified only from mobilities on agarose gel electropherograms. The cloning of the DNA fragment expected as a marker, and specific hybridization of the cloned fragment should be confirmed to clarify the first problem. The use of the cloned marker DNA as a probe will resolve the second problem. The presence of the marker fragment on RAPD patterns will be specifically detectable by the hybridization of the cloned marker DNA even if other fragments with the same mobilities exist.

The amplified DNA fragment that has been confirmed to be specific to a particular taxon is qualified as a RAPD marker. A RAPD marker is a genetic marker that can discriminate between the affiliated groups as described by Williams *et al.*[10] The cloned RAPD marker would also allow the application of the RAPD analysis to field samples which were collected from water blooms in the eutrophic lakes and reservoirs. The detection of a particular species in such a crude sample could be performed by southern hybridization of the RAPD marker to the RAPD patterns obtained from the sample. This will make RAPD analysis a quite useful tool for the management of water quality. Cloning of the DNA fragments expected as markers is now under the way.

Three species of *Microcystis* with single and unique allozyme genotype to each species, *i.e.*, *M. novacekii*, *M. viridis*, and *M. wesenbergii*, were shown to have their genetic homogeneity by the RAPD analysis as well as allozyme genotype. On the other hand, some genetic variations were observed among the strains of *M. aeruginosa* and *M. ichthyoblabe* accoding to analyses of RAPD and allozyme divergency. Further taxonomic revisions are still needed for these two species.

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