Plankton Biol. Ecol. 47 (1): 1–6, 2000

plankton biology & ecology

Genetic typing of a bloom-forming cyanobacterial genus *Microcystis* in Japan using 16S rRNA gene sequence analysis

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Received 28 May 1999; accepted 12 July 1999

Abstract: Portions of the 16S ribosomal RNA genes (16S rDNAs) from 33 strains of a bloom-forming cyanobacterial genus *Microcystis*, which included five 'morphospecies' (*M. aeruginosa*, *M. ichthyoblabe*, *M. novacekii*, *M. viridis* and *M. wesenbergii*) isolated from Japanese freshwater systems, were sequenced and subjected to phylogenetic analysis by comparison with the sequences of five *Microcystis* strains previously deposited in DNA databases, in order to classify the genetic types of this genus. *Microcystis* species used in this study were divided into six genetic types according to the 16S rDNA phylogenetic analysis and sequence signatures. Phylogenetic clusters I, II, III, IV and V included the ribotypes 1, 2, 3, 4 and 5, respectively, except for the ribotype 3 of *M. wesenbergii* NIES111 which clustered with *M. aeruginosa* NIES98 of phylogenetic cluster IV. Cluster VI was further divided into three types, including ribotypes 6, 7, 8 and 9. Although clusters III with *M. wesenbergii* NIES111 and V clustered phylogenetically, mainly with 'morphospecies' of *M. wesenbergii* and *M. novacekii*, respectively, other clusters consisted of mixed 'morphospecies' of *Microcystis*. The results of this study should be more useful for the objective typing of *Microcystis* species than the use of morphological characteristics.

Key words: genetic typing, 16S rDNA, *Microcystis*, cyanobacteria, phylogenetic analysis

Introduction

The cyanobacterial genus *Microcystis* commonly forms blooms in eutrophicated water systems during the warmer seasons. Some members of the genus contain the cyclic heptapeptide hepatotoxin, microcystin. The blooms of this genus present a considerable threat to the public health of humans and contribute to the death of wild and domestic animals.

The genus *Microcystis* is described in the classical botanical sense as a coccoid unicellular cyanobacterium that forms spherical or lens-shaped colonies of net-like or irregularly arranged cells resulting from division in 3 planes (Geitler 1932; Holt et al. 1994; Komárek & Anagnostidis 1986). Further classification of *Microcystis* species is based on morphological features observed microscopically, such as cell size, cell arrangement in colonies, existence of gas vesicles, and characteristics of the mucilage of colonies (Geitler 1932; Komárek 1991). According to Geitler (1932), who established the current systematics of cyanobacteria, there are 32 species in the genus *Microcystis*, including 8 species which have not yet been adequately described. Nine species of *Microcystis*, *M. aeruginosa*, *M. flos-aquae*, *M. ichthyoblabe*, *M. novacekii*, *M. viridis*, *M. wesenbergii*, *M. elabens*, *M. holsatica* and *M. incerta*, have been identified from eutrophic freshwaters in Japan (Ichimura & Itoh 1977; Komárek 1991; Watanabe 1984, 1996). The morphological features of these cyanobacteria under selective culturing conditions, however, are often markedly altered from those in natural environments. Thus, we can not discriminate among species which are single cells and/or from broken up colonies.

Genetic analyses of rRNA gene sequences (Neilan et al. 1994a, b, 1997; Kondo et al. 1998a, b; Otsuka et al. 1998), random amplified polymorphic DNA (RAPD) (Neilan 1995; Nishihara et al. 1997), restriction length polymor-

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phisms (RFLPs) of the phycocyanin intergenic spacer (Neilan et al. 1995), and of allozyme divergence (Kato et al. 1991) have been carried out to create an alternative, molecular genetics-based taxonomy for the cyanobacterial genus *Microcystis*. Most of these genetic analyses indicated that no relationships exist between morphological characteristics and molecular analysis within the genus *Microcystis*.

We determined the 16S rDNA genetic types among the major *Microcystis* species isolated from Japanese freshwater systems by partial 16S rDNA sequence analysis in order to get information useful for species or strain identification instead of (or in addition to) identification by morphological characteristics, which are inconstant. The partial 16S rDNAs from 33 strains of the genus *Microcystis* were sequenced and subjected to phylogenetic analysis by comparison with the sequences of the five *Microcystis* strains previously deposited in DNA databases. The partial 16S rDNA sequences corresponding to *Escherichia coli* base pair numbers 135–629 have been found to be useful for phylogenetic analysis, giving results equivalent to those obtained by complete sequencing (Kondo et al. 1998a, b). The results of this study suggest that the Japanese *Microcystis* species can be divided into six 16S rDNA genotypes.

Materials and Methods

Strains and growth conditions

The *Microcystis* species used in this study are listed in Table 1. The strains whose designations begin with NIES and TAC were obtained from the National Institute for Environmental Studies, Environmental Agency, Japan and Tsukuba Algal Collection, National Science Museum, Japan, respectively. All strains were cultured in MA medium (Ichimura & Itoh 1977) at 25°C under illumination of approximately 20 $\mu E\,m^{-2}\,s^{-1}$ with a 12 h : 12 h light–dark cycle.

DNA extraction and PCR amplification

PCR templates were prepared by the rapid DNA extraction method using the InstaGene™ Matrix (Bio-Rad Laboratories, USA) described elsewhere (Kondo et al. 1999). PCR amplifications were performed using a single set of MAF and MAR primers, which are specific for the major *Microcystis* species, corresponding to *Escherichia coli* positions 135–157 and 610–629, respectively (Kondo et al. 1998b). The thermal cycling conditions were denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and polymerization at 72°C for 3 min, with a final elongation step of 7 min at 72°C. DNA was amplified using a DNA Thermal Cycler PJ2000 (Perkin–Elmer, Co., USA) for a total of 25 cycles.

16S rDNA sequencing and phylogenetic analysis

After PCR amplification of the DNA, unpurified 16S rDNA PCR products were cloned using a TA Cloning® Kit (Invitrogen, USA) with pCR™ II vector and INV′α competent cells of *E. coli*, according to the manufacturer's instructions. DNA sequencing of the cloned PCR amplicons was performed with a DNA sequencer model 373A (Perkin–Elmer, Co., USA) using a *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit. The M13 universal forward and reverse primers were employed for sequencing.

The partial 16S rDNA sequences corresponding to *E. coli* positions 135–629 were determined in this study and aligned with those of other cyanobacteria by taking into account their sequence similarities. Evolutionary distance values were calculated according to the two-parameter model of Kimura (1980). Unrooted phylogenetic trees were reconstructed using the neighbor-joining method (Saitou & Nei 1987) as implemented in the program CLUSTAL W version 1.7 developed by Thompson et al. (1994). Confidence limits on tree topology were estimated by bootstrap analysis (Felsenstein 1985) with 1000 replicates.

The EMBL/GenBank/DDBJ accession numbers for the 16S rDNA sequences used in this phylogenetic analysis are shown in Table 1.

Results and Discussion

DNAs of all 33 strains tested were amplified with the single set of MAF and MAR primers. Analysis of the PCR amplification products by 2% agarose-gel electrophoresis and staining with ethidium bromide solution resulted in a single band of about 450 bp, which corresponded to the predicted size of the partial 16S rDNA of the cyanobacterial genus *Microcystis*.

The PCR products were cloned in the pCR™ II vector and sequencing was carried out on at least two clones of each strain. The five sequences from *Microcystis aeruginosa* NIES87, NIES89, NIES98, *M. wesenbergii* NIES111 and *M. viridis* NIES102, which had been previously deposited in the DNA databases as almost-complete sequences, were obtained from the EMBL/GenBank/DDBJ databases. The 16S rDNA partial sequences determined resulted in sequences 447–459 base pairs long, corresponding to *E. coli* positions 135–629. *M. aeruginosa* NIES89, TAC157–2, TAC169, *M. viridis* TAC92 and *M. wesenbergii* TAC38 had identical sequences, as did *M. wesenbergii* NIES104, NIES105, NIES106, NIES108, NIES110, NIES112, TAC52–1 and *M. viridis* TAC140. *M. aeruginosa* NIES100, *M. novacekii* TAC65–2, TAC66 and TAC75 also shared 100% similarity, as did *M. aeruginosa* NIES91, NIES101 and *M. ichthyoblabe* TAC125. *M. aeruginosa* NIES99 and *M. ichthyoblabe* TAC51 were also identical. The 16S rDNA sequences determined here were compared to each other, and to those deposited previously in DNA databases. Sequence similarity values and evolutionary dis-

Table 1. *Microcystis* species used in this study, and summary of genotyping determined by 16S rDNA clustering from phylogenetic analysis and ribotyping.

Strain	Isolation locality	16S rDNA cluster	Sequence signature corresponding to *E. coli* position				Ribotype[a]	DDBJ/GenBank accession number
			187–188	223	263–264	602		
Microcystis aeruginosa								
NIES87	Lake Kasumigaura	I	AA	A	AG	C	1	D89031
NIES88	Lake Kawaguchi	VI	CG	A	GA	T	9	AB023255
NIES89	Lake Kawaguchi	II	CG	A	AG	T	2	U03403
NIES90	Lake Kawaguchi	VI	CA	A	GA	T	8	AB023256
NIES91	Lake Kasumigaura	VI	AA	A	GA	C	1	AB023257
NIES98	Lake Kasumigaura	IV	CG	A	GG	C	4	D89032
NIES99	Lake Suwa	VI	CA	A	GA	C	7	AB023258
NIES100	Lake Suwa	V	CA	A	AA	C	5	AB023259
NIES101	Lake Suwa	VI	AA	A	GA	C	6	AB023260
NIES298	Lake Kasumigaura	VI	CA	A	GA	C	7	AB023261
NIES299	Lake Kasumigaura	VI	AA	A	GA	C	6	AB023262
TAC157–2	Teganuma Pond	II	CG	A	AG	T	2	AB023263
TAC169	Lake Okutama	II	CG	A	AG	T	2	AB023264
TAC192	Lake Okutama	VI	CG	A	GA	T	9	AB023265
Microcystis wesenbergii								
NIES104	Chiyoda-ku	III	CA	G	AG	C	3	AB023266
NIES105	Lake Kasumigaura	III	CA	G	AG	C	3	AB023267
NIES106	Lake Kasumigaura	III	CA	G	AG	C	3	AB023268
NIES108	Lake Suwa	III	CA	G	AG	C	3	AB023269
NIES109	Lake Yogo	VI	CG	A	GA	C	9	AB023270
NIES110	Lake Kasumigaura	III	CA	G	AG	C	3	AB023271
NIES111	Lake Kasumigaura	III'	CA	G	AG	C	3	D89034
NIES112	Lake Suwa	III	CA	G	AG	C	3	AB023272
NIES604	Lake Kasumigaura	VI	CG	A	GA	T	9	AB023273
TAC38	Lake Kasumigaura	II	CA	A	AG	T	2	AB023274
TAC52–1	Lake Suwa	III	CA	G	AG	C	3	AB023275
TAC57–1	Lake Suwa	III	CA	G	AG	C	3	AB023276
Microcystis viridis								
NIES102	Lake Kasumigaura	II	CG	A	AG	T	2	D89033
TAC78	Lake Mikata	VI	CG	A	GA	T	9	AB023277
TAC92	Lake Barato	II	CG	A	AG	T	2	AB023278
TAC140	Tameshowa Pond	III	CA	G	AG	C	3	AB023279
Microcystis ichthyoblabe								
TAC48–1	Lake Suwa	VI	CA	A	GA	C	7	AB023280
TAC51	Lake Suwa	VI	CA	A	GA	C	7	AB023281
TAC91	Lake Barato	I	AA	A	AG	C	1	AB023282
TAC125	Lake Barato	VI	AA	A	GA	C	6	AB023283
TAC146	Kashima-Onuma	I	AA	A	AG	C	1	AB023284
Microcystis novacekii								
TAC65–2	Chikazu Pond	V	CA	A	AA	C	5	AB023285
TAC66	Rokusuke Pond	V	CA	A	AA	C	5	AB023286
TAC75	Lake Yogo	V	CA	A	AA	C	5	AB023287

[a] Ribotype consists of nine types derived from the sequence signatures of positions 187–188, 223, 263–264 and 602, corresponding to *E. coli* numbering.

tances between the partial 16S rDNA sequences of *Microcystis* species are shown in Table 2. Overall, sequence similarities among *Microcystis* strains were high, 98% on average, corresponding to an evolutionary distance of 0.0095. The levels of sequence similarity were slightly lower than those obtained by analysis of the almost-complete sequence of 16S rDNA (Neilan et al. 1997; Otsuka et al. 1998). This may be because highly conserved regions in the full-length 16S rDNA were not included in our partial 16S rDNA.

We found four variable regions, corresponding to *Escherichia coli* positions 187–188, 223, 263–264 and 602, in the partial 16S rDNA of the *Microcystis* species. In the first

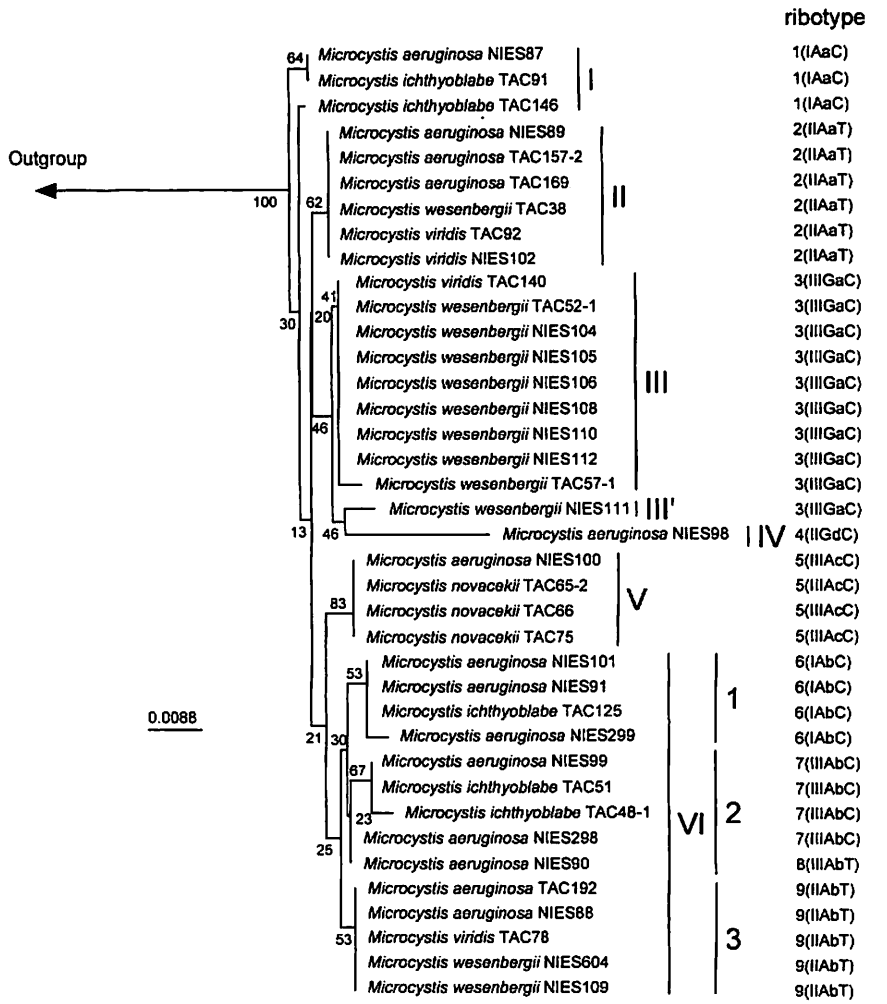

Fig. 1. Phylogenetic relationships within the genus *Microcystis* derived from the partial 16S rDNA sequences between *Escherichia coli* positions 135 and 629, using the neighbor-joining method. Numbers on the branches represent percentage of 1000 bootstrap repetitions. The distance scale indicates the expected number of changes per sequence position. Outgroup represents *Synechococcus* sp. PCC6301 (accession number X03538), *Phormidium minitum* D5, *P. ectocarpi* CCAP14625, N182 and PCC7373 of which sequence data were obtained from the DDBJEMBLGenBank databases or the Ribosomal Database Project (Maidak et al. 1999).

position, there were three types of sequence signatures, AA, CG and CA, which were designated as I, II and III, respectively, and four types, AG, GA, AA and GG in the third position, which were designated as a, b, c and d, respectively. Sequence signatures of *E. coli* positions 223 and 602 were A or G and C or T, respectively. Combining these types of sequence signatures, nine ribotypes were recognized: ribotype 1, IAaC; ribotype 2, IIAaT; ribotype 3, IIIGaC; ribotype 4, IIGdC; ribotype 5, IIIAcC; ribotype 6, IAbC; ribotype 7, IIIAbC; ribotype 8, IIIAbT; ribotype 9, IIAbT (Table 1).

We constructed the phylogenetic tree shown in Fig. 1 using the neighbor-joining method (Saitou & Nei 1987). *Microcystis* species were phylogenetically divided into six clusters. Cluster I consisted of ribotype 1: *M. aeruginosa* NIES87 and *M. ichthyoblabe* TAC91. *M. ichthyoblabe* TAC146 also belonged to cluster I, because it had the sequence signature of ribotype 1 (Table 1, Fig. 1) and shared higher similarity (99.5%) with *M. aeruginosa* NIES87 and *M. ichthyoblabe* TAC91 than with *Microcystis* group II and *M. viridis* NIES102 (99.3%) (Table 2). Clusters II, III and V also consisted only of ribotypes 2, 3 and 5, respectively. Phylogenetic cluster IV included the ribotype 3 of *M. wesenbergii* NIES111 and ribotype 4 of *M. aeruginosa* NIES98. Sequence similarity between *M. wesenbergii* NIES111 and *M. wesenbergii* of group III was 98.5%, higher than that between *M. wesenbergii* NIES111 and *M. aeruginosa* NIES98 (94.5%) (Table 2). Thus, *M. wesenbergii* NIES111 is considered to belong to cluster III, indicated as III'. Cluster VI was further divided into three clusters which included the four ribotypes 6, 7, 8 and 9. Clusters VI–1 and VI–3 were constructed with ribotypes 6 and 9, respectively. Cluster VI–2 included ribotypes 7 and 8, of which the difference in sequence was only C or T in the position corresponding to *E. coli* position 602.

As shown by Otsuka et al. (1998), *Microcystis* species have high sequence similarities of the almost-complete 16S rDNA, and phylogenetic analysis reveals no clear divisions in the 'major' *Microcystis* cluster. Our partial 16S rDNA sequence analysis also indicated high sequence similarities, but six smaller clusters within the 'major' *Microcystis* cluster were clearly recognized from the phylogenetic analysis and sequence signatures. Clusters III (including cluster III') and V consisted phylogenetically mainly of the 'morphospecies' of *M. wesenbergii* and *M. novacekii*, respectively. The genetic homogeneities of *M. novacekii*, *M. wesenbergii*, and *M. viridis* have also been shown by RAPD analysis (Nishihara et al. 1997) as well as allozyme geno-

Table 2. Levels of sequence similarities and evolutionary distances for 16S rDNA sequences of the genus *Microcystis*.

Microcystis strain	% similarity and evolutionary distance[a]																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 *M. aeruginosa* NIES87		99.3	99.3	98.7	98.5	98.9	98.9	97.2	93.9	98.7	98.9	98.7	98.5	98.5	98.3	98.3	98.0	98.0	98.3	97.8	98.5
2 *M. ichthyoblabe* TAC91	0.000		99.6	98.9	98.9	99.1	98.9	97.4	94.1	98.9	99.1	98.9	98.7	98.7	98.5	98.5	98.3	98.3	98.5	98	98.7
3 *M. ichthyoblabe* TAC146	0.002	0.002		99.3	99.1	99.6	99.3	97.8	94.5	99.3	99.6	99.6	99.1	99.1	99.1	98.9	98.7	98.7	99.1	98.5	99.1
4 *Microcystis* group II[b]	0.009	0.009	0.007		99.8	99.3	99.1	97.6	95.0	99.1	98.9	98.9	98.9	98.9	98.9	99.1	98.9	99.3	99.6	99.1	99.1
5 *M. viridis* NIES102	0.012	0.012	0.009	0.002		99.1	98.9	97.4	94.5	98.9	98.7	98.7	98.7	98.7	98.5	98.9	98.7	99.1	99.3	98.9	99.1
6 *Microcystis* group III[c]	0.007	0.007	0.005	0.007	0.009		99.8	98.2	95.0	99.3	99.1	98.9	99.1	99.1	98.9	98.9	98.7	98.7	98.9	98.7	99.1
7 *M. wesenbergii* TAC57–1	0.009	0.009	0.007	0.009	0.012	0.002		98.0	94.7	99.1	98.9	98.7	98.9	98.9	98.7	98.7	98.5	98.5	98.7	98.2	98.9
8 *M. wesenbergii* NIES111	0.012	0.012	0.009	0.012	0.014	0.005	0.007		94.5	97.6	97.4	97.2	97.4	97.6	97.2	97.2	96.9	96.9	97.2	96.7	97.4
9 *M. aeruginosa* NIES98	0.030	0.030	0.028	0.026	0.028	0.023	0.026	0.023		94.3	94.5	94.5	94.5	94.5	94.3	94.7	94.5	94.5	94.7	94.3	94.5
10 *Microcystis* group V[d]	0.009	0.009	0.007	0.009	0.012	0.007	0.009	0.012	0.03.0		99.3	99.1	99.3	99.3	99.1	99.1	98.9	98.9	99.1	98.7	99.3
11 *Microcystis* group VI 1[e]	0.007	0.007	0.005	0.012	0.014	0.009	0.012	0.014	0.028	0.007		99.8	99.6	99.6	99.3	99.3	99.1	99.1	99.3	98.9	99.6
12 *M. aeruginosa* NIES299	0.009	0.009	0.007	0.014	0.016	0.012	0.014	0.016	0.03.0	0.009	0.002		99.3	99.3	99.3	99.1	98.9	98.9	99.1	98.7	99.3
13 *M. aeruginosa* NIES298	0.012	0.012	0.009	0.012	0.014	0.009	0.012	0.014	0.028	0.007	0.005	0.007		99.6	99.3	99.3	99.1	99.1	99.1	98.9	99.6
14 *Microcystis* group VI-2[f]	0.012	0.012	0.009	0.012	0.014	0.009	0.012	0.014	0.028	0.007	0.005	0.007	0.005		99.8	99.3	99.1	99.1	99.3	99.1	99.6
15 *M. ichthyoblabe* TAC48–1	0.014	0.014	0.012	0.014	0.016	0.012	0.014	0.016	0.03.0	0.009	0.007	0.009	0.007	0.002		99.1	98.9	98.9	99.1	98.7	99.3
16 *M. wesenbergii* NIES109	0.014	0.014	0.012	0.009	0.012	0.012	0.014	0.016	0.026	0.009	0.007	0.009	0.007	0.007	0.009		99.8	99.8	99.6	99.3	99.3
17 *M. wesenbergii* NIES604	0.014	0.014	0.012	0.009	0.012	0.012	0.014	0.016	0.026	0.009	0.007	0.009	0.007	0.007	0.009	0.000		99.6	99.6	98.9	99.1
18 *M. viridis* TAC78	0.016	0.016	0.014	0.007	0.009	0.014	0.016	0.018	0.028	0.012	0.009	0.012	0.009	0.009	0.012	0.002	0.002		99.8	99.3	99.6
19 *M. aeruginosa* TAC192	0.014	0.014	0.012	0.005	0.007	0.012	0.138	0.016	0.026	0.009	0.007	0.009	0.007	0.007	0.009	0.005	0.005	0.002		99.6	99.8
20 *M. aeruginosa* NIES88	0.018	0.018	0.016	0.009	0.012	0.016	0.018	0.021	0.03.0	0.014	0.012	0.014	0.012	0.012	0.014	0.009	0.009	0.007	0.005		99.3
21 *M. aeruginosa* NIES90	0.012	0.012	0.009	0.007	0.009	0.009	0.012	0.014	0.028	0.007	0.005	0.007	0.005	0.005	0.007	0.007	0.007	0.005	0.002	0.007	

[a] The values above the diagonal are percentages of sequence similarity, and those below the diagonal are corrected evolutionary distances (Knuc).

[b] *Microcystis* group II consists of the identical sequences of *M. aeruginosa* NIES89, TAC157–2, TAC169, *M. viridis* TAC92 and *M. wesenbergii* TAC38, which belong to phylogenetic cluster II.

[c] *Microcystis* group III consists of the identical sequences of *M. wesenbergii* NIES104, NIES105, NIES106, NIES108, NIES110, NIES112, TAC52–1 and *M. viridis* TAC140, which belong to phylogenetic cluster III.

[d] *Microcystis* group V consists of the identical sequences of *M. aeruginosa* NIES100, *M. novacekii* TAC65–2, TAC66 and TAC75, which belong to phylogenetic cluster V.

[e] *Microcystis* group VI–1 consists of the identical sequences of *M. aeruginosa* NIES91, NIES101 and *M. ichthyoblabe* TAC125, which belong to phylogenetic cluster VI–1.

[f] *Microcystis* group VI–2 consists of the identical sequences of *M. aeruginosa* NIES99 and *M. ichthyoblabe* TAC51, which belong to phylogenetic cluster VI–2.

typing (Kato et al. 1991). However, *Microcystis* species from Japanese culture collections, which were identified by microscopically observable morphological characteristics, were not distinguishable at the 'morphospecies' level by our partial 16S rDNA sequencing, and there was no relationship between morphological species and the ribotypes or the phylogenetic clustering of most *Microcystis* species. Most of the molecular methods such as rDNA sequencing (Neilan et al. 1994a, b, 1997; Kondo et al. 1998a, b; Otsuka et al. 1998), RAPD (Neilan 1995), RFLPs of the phycocyanin intergenic spacer (Neilan et al. 1995) also indicate that no relationships exist between morphological characteristics and molecular types within the genus *Microcystis*. The similarities of the partial 16S rDNA sequence determined here were 94% or greater, sometimes identical, showing that DNA relatedness may be higher than 70% within a species (Stackebrandt & Goebel 1994). DNA–DNA reassociation in the cyanobacterial genus *Microcystis* has to be observed to demonstrate genetic relatedness according to bacterial criteria. The results of this study should be useful for the objective typing and identification of *Microcystis* species, as a superior alternative to the use of morphological characteristics.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research (Nos. 07760189 and 08760186) from the Ministry of Education, Science, Sports and Culture of Japan to RK.

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