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Title; Genotypic composition and the relationship between genotypic composition and geographical proximity of the cyanobacterium *Microcystis aeruginosa* in western Japan

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Abstract

Microcystis aeruginosa is one of the bloom-forming harmful algae in freshwater ecosystems. We genetically characterized *Microcystis* populations during bloom-forming periods in various reservoirs, lakes, and ponds in Japan during 2009. Using phylogenetic analysis, we evaluated the relationship between current genotype expansions and geographic location within western Japan and intraspecific variation. *Microcystis aeruginosa* colonies were isolated at 15 sites and were analyzed by

sequencing the 16S–23S internal transcribed spacer (ITS) region of the ribosomal operon, and the potential to produce toxins was assessed by PCR-based detection of the microcystin synthetase gene *mcyG*. In total, 171 colonies were separated into 41 genotypes. The highest genotypic composition was detected in the south basin of Lake Biwa and the lowest in Lagoon Iba. Cluster analysis indicated no obvious association between genotypic composition and geographic distance. Thus, clear genetic differentiation accompanied by geographic origins was not found in western Japan. The resulting neighbor-joining tree revealed 3 clusters, 2 of which contained strains that showed both nonamplification and amplification of the *mcyG* gene.
Key words: ITS region, genotypic diversity, harmful algae, *Microcystis*.

Introduction

On a global scale, human-induced eutrophication is a threat to the use and sustainability of aquatic ecosystems. Furthermore, global warming will likely promote the expansion of harmful algae (Paerl and Huisman 2008). Cyanobacteria produce various toxins that can be assigned to different substance classes, such as alkaloids, lipopolysaccharides, polyketides, or peptides (Codd 2000; Codd *et al*. 2005). Hepatotoxic microcystins, the predominant toxins in freshwater lakes worldwide have been implicated in several cases of animal and human intoxication (Jochimsen *et al*. 1998; Codd 2000; Dittmann and Wiegand 2006). Therefore, expansion of bloom-forming cyanobacteria may cause serious problems for environmental and human health.

The genus *Microcystis*, which is found in eutrophic lakes and ponds, is a widespread freshwater bloom-forming cyanobacterium that produces hepatotoxin (microcystin). The ability of *Microcystis* to produce microcystin is strain-specific, and many previous

studies have examined the relationship between strain sequence and microcystin production (Otsuka *et al*. 1999; Kondo *et al*. 2000; Gobler *et al*. 2007; Yoshida *et al*. 2008; Davis *et al*. 2009). Recent studies have focused on the genotypic (strain) diversity of several harmful algae in marine and freshwater systems using molecular techniques (Humbert *et al*. 2005; Wilson *et al*. 2005; Briand *et al*. 2009; Sabart *et al*. 2009; Humbert 2010; Otten and Paerl 2011).

High genotypic diversity in *M. aeruginosa* has been observed during bloom seasons using molecular approaches such as multilocus sequence typing analysis (Tanabe *et al*. 2007, 2009, 2011) and 16S-23S ribosomal DNA internal transcribed spacer region (ITS) sequence analysis (Humbert *et al*. 2005; Yoshida *et al*. 2008; Briand *et al*. 2009; Sabart *et al*. 2009; Otten and Paerl 2011; van Gremberghe *et al*. 2011). Those studies focused on changes in strain variation (genotypic composition) of *Microcystis* within a site, or in comparisons between strain intraspecific differences in phylogenetic trees. Some studies have reported a lack of phylogeographic structure in *Microcystis* strains (genotypes) (*e.g.*, Sabart *et al*. 2009, van Gremberghe *et al*. 2011). However, few studies have compared strain variation (genotypic composition) among populations in close proximity within the same blooming season.

In Japan, the frequency of bloom formation in lakes and reservoirs has increased since the 1970s, and *Microcystis* strains have been isolated from lakes and ponds throughout Japan and are now maintained in the microbial culture collection at the National Institute for Environmental Studies (NIES Collection; Microbial Culture Collection at the National Institute for Environmental Studies, 2012). Many of those strains were characterized genetically by sequencing the 16S-23S rDNA ITS region, and this sequence information was obtained from GeneBank (Otsuka *et al*., 1999). Thus,

characterization of the present *M. aeruginosa* genotypes in blooming water will provide information regarding the intraspecific differences among those strains within Japan.

In the present study, we assessed the genotypic composition of *Microcystis* in western Japan, which has received little research attention to date, to examine the expansion and distribution of *M. aeruginosa*. The aim of this study was to evaluate (a) the strain variation (genotypic composition) of *M. aeruginosa* in western Japan, (b) the relationship between genotypic composition and geographic distance, and conduct (c) a phylogenetic analysis of intraspecific variation.

Water collection

From July to November 2009, we collected water samples from 14 sites, including two lakes, one lagoon, seven irrigation ponds, and four reservoirs in western Japan (Fig. 1). We collected 10 L of surface water and brought it to the laboratory. The seston was removed from the water samples onto pre-combusted GF/F filters (3 h at 420°C) and preserved at -20°C until DNA extraction. The density of *Microcystis* was very low in water samples collected from the north basin of Lake Biwa; therefore, we first passed the lake water through a 20-μm mesh plankton net. We collected seston samples for chlorophyll *a* measurements by filtering 20 mL of water through GF/F filters, and the filtered samples were stored at -20°C. Chlorophyll *a* concentrations were determined by the method of Welschmeyer (1994) using a spectrofluorophotometer (RF-5300; Shimadzu, Kyoto, Japan) after extraction with 10 mL of *N*, *N*-dimethylformamide (Suzuki and Ishimaru 1990).

Isolates were prepared by randomly picking single colonies from water samples under a microscope using sterile glass Pasteur pipettes with a narrow opening. The

isolates were then washed repeatedly with a small volume of GF/F-filtered sample water to remove debris from around each colony. The colonies were incubated in a cultivation solution (450 µL of GF/F-filtered sample water plus 50 µL of CT medium; Watanabe and Ichimura 1977) for a few days and then transferred to sterilized CT medium. All strains were maintained at 25°C with a continuous illumination of approximately 30 µmol m^{-2} S^{-1}. When the colony size became sufficient for DNA extraction, the colony was trapped on the GF/F filter and preserved at -20°C until DNA extraction.

DNA was extracted from filtered colonies on filters using a modified CTAB method (Doyle and Doyle 1987). Briefly, filters were placed in 2-mL tubes, 1.5 mL of CTAB buffer [2% cetyltrimethylammonium bromide, 1.4 M NaCl, 100 mM DTT, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), and 1% polyvinylpyrrolidone] was added to each tube, and the tubes were incubated at 55°C for 1 h. After sample extraction with chloroform: isoamyl alcohol (24:1), DNA was precipitated using Ethachinmate (Nippon Gene Co. Ltd., Toyama, Japan) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until polymerase chain reaction (PCR) amplification.

Detection of potentially toxin-producing strains and ITS region sequencing of the isolated colonies

Potential toxin-producing strains were identified by PCR-based detection of the microcystin synthetase gene, *mcy*G, which is located in the adenylate-forming domain (Tanabe *et al*. 2007). PCR reactions (10 µL) included 1 µL of template DNA (about 5 ng/µL), 0.2 mM dNTPs, 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4

μM of each primer (primers GF and GR; Table 1), and 0.25 U of Ampli *Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The PCR cycling conditions were as follows: 10 min at 94°C, followed by 35 cycles of 30 s at 90°C, 30 s at 53°C, and 30 s at 72°C, with a final extension for 15 min at 72°C. The PCR products were separated by electrophoresis through a 2.0% agarose gel with TAE buffer and detected by ethidium bromide staining.

ITS sequences of the rDNA operon were amplified for each DNA preparation using a cyanobacteria-specific primer set: 16S rDNA CSIF (forward primer) and 23S rDNA ULR (reverse primer) (Table 1). PCR amplification was performed in a 30-μL reaction volume containing 3 μL of template DNA (about 5 ng/μL), 0.2 mM dNTPs, 15 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 0.5 U of Ampli *Taq* Gold DNA polymerase. The PCR cycling conditions were as follows: 10 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at the annealing temperature (T_a), and 1 min at 72°C, with a final extension for 30 min at 72°C. For the first 20 cycles, T_a was decreased by 1°C after every second cycle, from 62°C to 52°C, according to the touchdown procedure (Don *et al*. 1991). The last 10 cycles were performed at a T_a of 52°C. The PCR products were separated by electrophoresis on a 2.0% agarose gel with TAE buffer and detected by ethidium bromide staining. For samples showing a single band, the PCR product was purified using a Qiagen DNA purification kit (Qiagen, Hilden, Germany) and sequenced using an ABI3130 Genetic Analyzer (Applied Biosystems) and a BigDye Terminator Cycle Sequencing Ready Reaction Kit (ver. 3.1; Applied Biosystems).

Data analysis

We assessed the dissimilarities in genotypic composition among sites using agglomerative hierarchical clustering analysis (XLSTAT, 2011) to clarify the relationship between genetic composition and geographic proximity in *Microcystis* genotypes. If the similarity of genotypic composition was determined by the absolute geographic distance between sampling sites, close populations would show higher similarities (lower dissimilarity) than distant populations. We also analyzed the correlations between geographic distances of collection sites and dissimilarity using Spearman's rank-correlation test (Sokal and Rohlf, 1997). Estimates of nucleotide and gene diversity were calculated using Arlequin ver. 3.5 (Excoffier and Lischer 2010), except for the Ishitekawa, Shinji, and Midorikawa samples, which yielded only a small number of sequences. All analyses were conducted using the 16S-23S rDNA region (~485 bp) and we defined a genotype as a sequence that differs by more than one base pair. All unique sequences are listed in Supplementary Table 1 and were deposited in the DNA Data Bank of Japan. The assigned accession numbers were AB602254–AB602299 and AB686446–AB686455.

To evaluate *Microcystis* intraspecific variation, we used ITS sequences reported by Otsuka *et al*. (1999), which are listed in Table 3 (~360 bp of 16-23S rDNA, 74 bp of which corresponded to the tRNA-Ile gene) and confirmed each strain collection origin using NIES Collection Information HP (Microbial Culture Collection at the National Institute for Environmental Studies, 2012). Our ITS sequences and those of Otsuka *et al*. (1999) were aligned using MEGA5 (Tamura *et al*. 2011). A phylogenetic tree was generated using a neighbor-joining method with 2000 bootstrap replicates. A phylomonogram was rooted with a microcystin-producing strain of *Planktothrix rubescens* (NIES-928 strain, AB686456). For comparison, additional phylograms were

created using maximum-likelihood methods with 500 bootstrap replicates using a Kimura two-parameter model with a gamma-distributed rate. Since both phylograms were congruent, we show only the result of the neighbor-joining tree.

Results

Genotypic composition in western Japan

In total, 171 cultivated strains from 15 sites were discriminated into 55 ITS genotypes (Supplementary Table 1 and Table 2). Average sequence length was 486.6 ± 2.2 (standard deviation, SD) base pairs (minimum: 480 bp; maximum: 492 bp). Based on the *mcy*G gene amplification, 26 ITS genotypes represented potentially toxin-producing strains, 29 genotypes were potentially non-toxin-producing strains, and three of the 55 ITS genotypes included both toxin-producing and non-toxin-producing strains. Some genotypes were repeatedly detected within the same sites, and some were detected among different sites (Supplementary Table 1). The total number of unique genotypes was 41, excluding those ITS genotypes detected repeatedly among collection sites. The number of genotypes was highest in the south basin of Lake Biwa (63.5%) and lowest in Lagoon Iba (8.3%) (Table 2). The south basin of Lake Biwa also exhibited the highest genetic diversity and Lagoon Iba the lowest genetic diversity, since one genotype dominated the site.

Relationship between genotypic composition and geographic distance

The results of the cluster analysis are shown in Fig. 2. If the similarity of genotypic composition were determined by geographic closeness, close populations would cluster together. No tendencies between clusters and observed genotypes were identified, and

the genotypes were well-mixed, irrespective of geographic proximity (Fig. 2a). No significant relationship between geographic proximity and genotypic dissimilarities was detected (Fig. 2b, Spearman's rank-correlation, $\rho = 0.051$, $p = 0.60$, n.s.). Thus, we observed no clear relationship between genotypic composition and geographic proximity.

Phylogenetic analysis of intraspecific variation

The phylogenetic trees were divided into three clusters (Fig. 3). Clusters I and II consisted of both potentially toxin and non-toxin-producing strains. Cluster III included only potentially non-toxin-producing strains. Some genotypes had an identical ITS region to those of strains isolated previously (Fig. 3).

Discussion

In the present study, 171 strains were discriminated into 41 unique *Microcystis* genotypes based on the 16S-23S rDNA ITS region. Current genotypes from Lake Biwa strains appeared in all clusters (clusters I, II, and III and other clusters; Fig. 3) and the genotypic diversity of Lake Biwa was the highest in western Japan. Therefore, Lake Biwa, which is the largest and oldest lake in Japan, held a large amount of *M. aeruginosa* genotypic diversity. Two genotypic composition patterns were observed during bloom formation: one type consisted of various genotypes (high genotypic diversity) and the other consisted of a few dominant genotypes (low genotypic diversity); this tendency varied among collection sites. The genotypic composition of *M. aeruginosa* was expected to differ with water-body type (*e.g.*, reservoir, pond, or lake) and with the amount of time that has elapsed since the beginning of a bloom based

on local adaptation (Tanabe *et al*., 2011). Yoshida *et al*. (2005) reported 16S rDNA ribotype variation in the *Microcystis* during one bloom season in Lake Mikata, Japan. Their results suggested that genotypic composition and the dominant *Microcystis* genotype differed according to sampling date.

The genotypic diversity of cyanobacteria is a hot topic and it is interesting that some cyanobacteria show geographic differentiation and some do not (Gugger *et al*. 2005; Haande *et al*. 2008; Otten and Paerl 2011; Piccini *et al*. 2011; Tanabe *et al*., 2009, 2011). The toxic cyanobacterium *Cylindrospermopsis raciborskii* shows three typical clusters according to the geographic origins of the African-Australian, European, and American populations (Gugger *et al*. 2005; Haande *et al*. 2008; Piccini *et al*. 2011). Additonally, the phenotypic and genetic variability in *C. raciborskii* populations has been proposed to be linked to different ecotypes that have local environmental adaptations (Piccini *et al*. 2011). In contrast, *M. aeruginosa* seems not to exhibit such tendencies. Tanabe *et al*. (2009) using multilocus sequence genotyping reported that fine-scale temporal genetic differentiation in *M. aeruginosa* is determined by genetic drift or other evolutionary forces rather than geographic distance. No consistent geographical pattern of genetic differentiation was identified in our cluster analysis. Therefore, we did not detect a relationship between genotypic composition and geographic proximity. Previous studies that used the 16S-23S rDNA ITS region reported the same tendency (Janse *et al*. 2004; Sabart *et al*. 2009; Van Gremberghe *et al*. 2011).

Microcystis aeruginosa is well known to have a variety of morphological traits. Because the variance in morphology is not correlated with differences in 16S rDNA sequences (sequence divergences are low), all of these morphological types are called *M.*

aeruginosa (Otuka *et al*. 1998, 2001). Otsuka *et al*. (1999) assessed the relationships among microcystin production ability, morphological traits and ITS genotype. The results showed three distinct clusters; cluster I contained various morphological traits and both non- and microcystin-producing strains, cluster II consisted of three morphological traits and contained only microcystin-producing strains, and cluster III included one morphological species, *M. wesenvergii* and only non-microcystin-producing strains. Our phylomonograph also consisted of three main clusters. These corresponded to those reported by Otsuka *et al*. (1999). However, cluster II, which contained two strains that showed both non- and amplification of the *Mcy*G gene region, was included in our analysis. Although we did not measure microcystin production in each strain, clusters I and II showed both non- and amplification of the *Mcy*G gene, while cluster III strains did not. Humbert *et al*. (2005) suggested that cluster III may exist only in the Asian region based on a comparison of ITS regions in organisms isolated from French and Japanese lakes. Relationships among genotypic variation, genetic environmental adaptations, and ecophysiological phenotypes in *M. aeruginosa* have not been clarified. Further studies are needed to elucidate these relationships.

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Figures

Fig. 1. Map of the collection sites. Please refer to Table 2 for site identification.

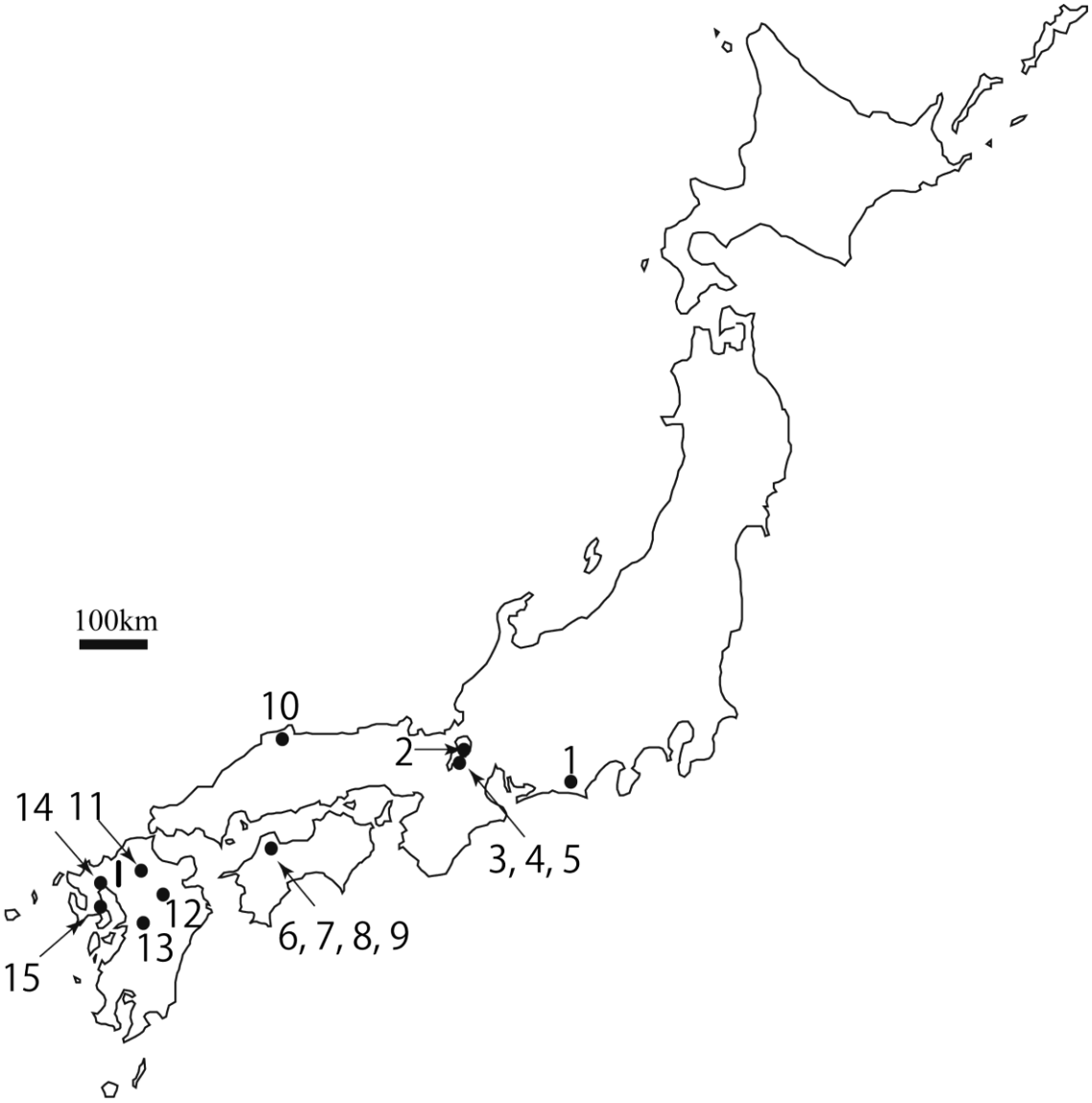

Fig. 2. (a) Cluster analysis results. The x axis indicates collection sites (as shown in Fig. 1); the y axis indicates genotypic dissimilarity. (b) Relationship between geographic proximity and genotypic dissimilarity at each site (log transformed).

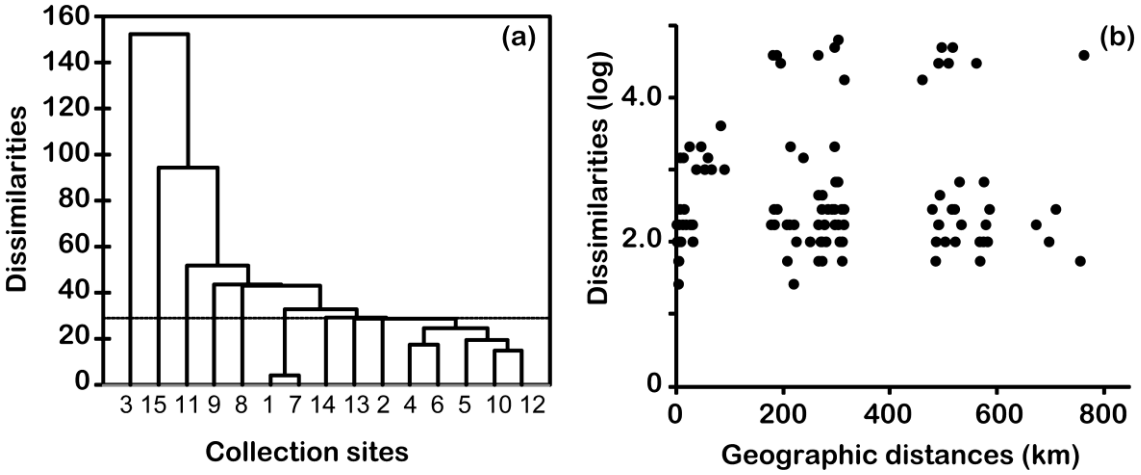

Fig. 3. Neighbor-joining tree based on the internal transcribed sequence (ITS) region (about 360 bp) with NIES strains (italicized). The outgroup (*Planktothrix rubescens* NIES928) is omitted. Strain abbreviations are shown in Table 3 and Supplementary Table 1. Strains in bold were positive for amplification of the *mcy*G gene (present study) or microcystin production (National Institute for Environmental Studies 2012).

Bootstrap values are indicated at nodes (>50%) and the distance scale indicates the expected number of changes per sequence position.

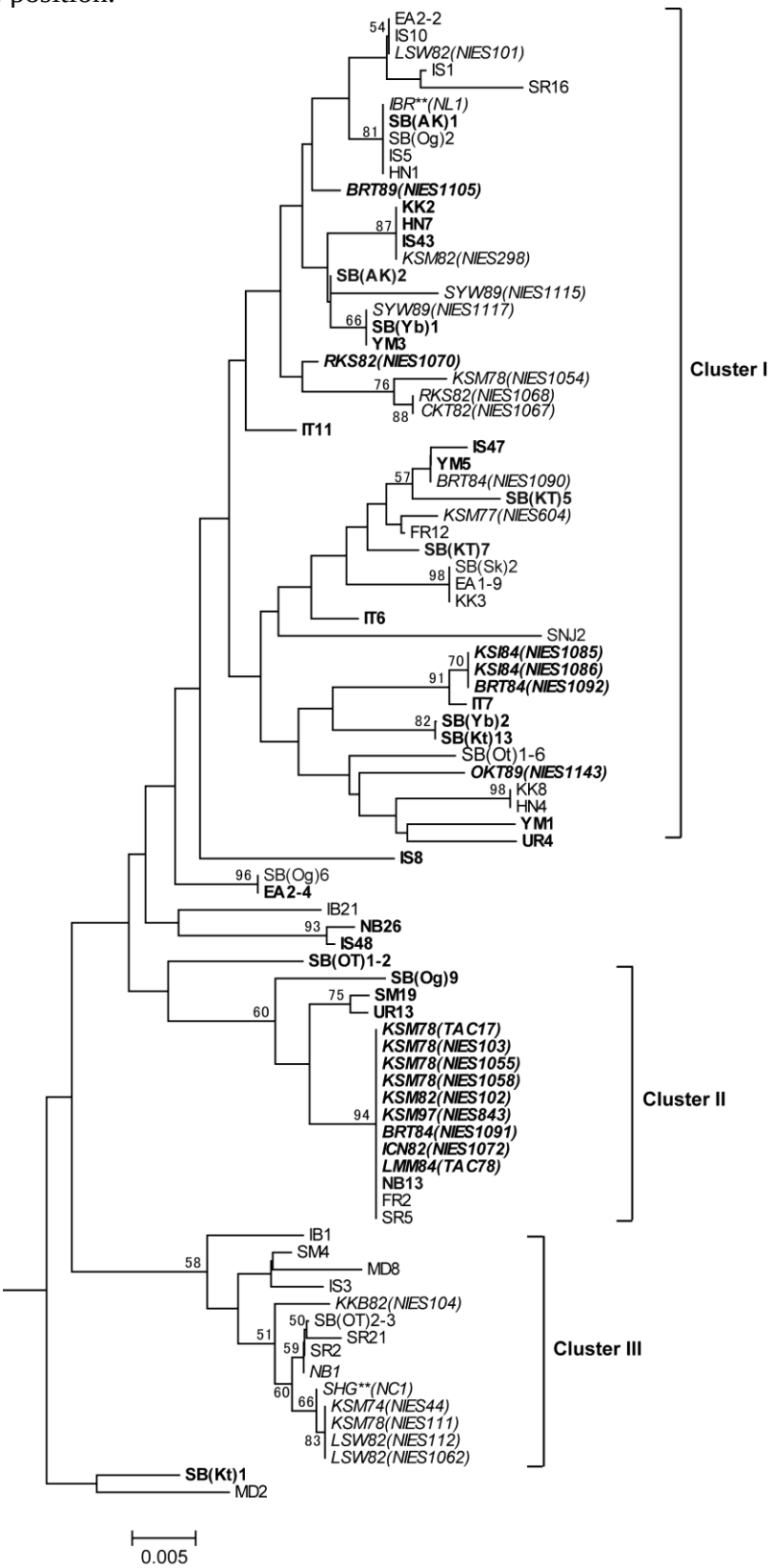

Table 1. Primers used in this study.

Gene region and primers	Sequences (5' to 3')	References
Microsystin synthetase gene *:mcy* G		
GF	GGA AAA TTT TAG ACA ATC CCT TGA T	Tanabe et al (2004)
GR	AAT TTC TAA GAA TAG GCG AAT CGT T	
Cyanobacteria 16S-23S rDNA region		
CSIF (16S region)	G(T/C)C ACG CCC GAA GTC (G/A) TT AC	Janse et al (2003)
ULR (23S region)	CCT CTG TGT GCC TAG GTA TC	

Table 2. The genotypic composition of *Microcystis*.

Collection site	#	Prefecture	Type	Strain	Chl*a* (μg/L)	Strains	Seq.	Genotypes	*mcyG*	Gene diversity	Nucleotide diversity
Kakegawa Pond	1	Shizuoka	Irrigation pond	KK	220.5	10	9	3	1	0.72 ± 0.10	0.03 ± 0.02
Lagoon Iba	2	Shiga	Lagoon	IB	-	24	24	2	0	0.08 ± 0.07	0.00 ± 0.00
South basin of Lake Biwa	3	Shiga	Lake	SB	-	22	22	14	9	0.94 ± 0.07	0.03 ± 0.02
North basin of Lake Biwa	4	Shiga	Lake	NB	-	9	9	3	2	0.56 ± 0.17	0.02 ± 0.01
Experimental Pond at Kyoto Univ.	5	Shiga	Irrigation pond	EA	278.6	6	5	3	1	0.80 ± 0.16	0.02 ± 0.02
Furuike	6	Ehime	Irrigation pond	FR	87.7	6	5	2	0	0.40 ± 0.24	0.02 ± 0.01
Handaji	7	Ehime	Irrigation pond	HN	88.1	9	8	3	1	0.75 ± 0.10	0.02 ± 0.01
Ishitekawa	8	Ehime	Reservoir	IT	99.6	4	3	3	3	-	-
Yamada	9	Ehime	Irrigation pond	YM	56.7	13	13	3	3	0.60 ± 0.09	0.02 ± 0.01
Lake Shinji	10	Shimane	Lagoon Lake	SNJ	-	6	6	1	0	-	-
Shirouzu	11	Fukuoka	Irrigation pond	SR	24.7	18	18	4	0	0.31± 0.14	0.01 ± 0.00
Shimouke	12	Oita	Reservoir	SM	306.6	7	5	2	1	0.40 ± 0.24	0.01 ± 0.01
Midorikawa	13	Kumamoto	Reservoir	MD	49.2	3	2	2	0	-	-
Ureshino	14	Saga	Irrigation pond	UR	53.8	11	9	2	1	0.39 ± 0.16	0.02 ± 0.01
Isahaya	15	Nagasaki	Reservoir	IS	123.6	33	33	8	4	0.71 ± 0.07	0.01 ± 0.01

Note: No., corresponds to collection site number in Fig. 1; Strain, corresponds to strain abbreviation in Supplementary Table 1; Chl *a*, chlorophyll a concentration; Strains, number of isolated strains; Seq., number of sequenced strains; Genotype, number of genotypes; *mcy*G, number of strains that were positive for *mcy*G gene amplification.

Table 3. Strains used in the phylogenetic analysis.

Strain No.	Origin	Abbribiation[a]	DDBJ accession #	Microcystin
NIES-44	Lake Kasumigaura, Ibaraki	KSM74	AB015361	-
NIES-101	Lake Suwa, Nagano	LSW82	AB015366	-
NIES-102	Lake Kasumigaura, Ibaraki	KSM82	AB638216	+
NIES-103	Lake Kasumigaura, Ibaraki	KSM82	AB015399	+
NIES-104	Kikkyo-bori, Tokyo	KKB82	AB015387	-
NIES-111	Lake Kasumigaura, Ibaraki	KSM78	AB015388	-
NIES-112	Lake Suwa, Nagano	LSW82	AB015390	-
NIES-298	Lake Kasumigaura, Ibaraki	KSM82	AB254442	+
NIES-604	Lake Kasumigaura, Ibaraki	KSM77	AB333817	-
NIES-843	Lake Kasumigaura, Ibaraki	KSM97	AP009552	+
NIES-1054	Lake Kasumigaura, Ibaraki	KSM78	AB015374	-
NIES-1055	Lake Kasumigaura, Ibaraki	KSM78	AB015389	+
NIES-1058	Lake Kasumigaura, Ibaraki	KSM78	AB015400	+
NIES-1062	Lake Suwa, Nagano	LSW82	AB015391	-
NIES-1067	Chikato-ike, Nagano	CKT82	AB015375	-
NIES-1068	Rokusuke-ike, Nagano	RKS82	AB015376	-
NIES-1070	Rokusuke-ike, Nagano	RKS82	AB333808	+
NIES-1072	Ichinomiya, Ishikawa	ICN82	AB015362	+
NIES-1085	Koshi-ike, Simane	KSI84	AB015363	+
NIES-1086	Koshi-ike, Simane	KSI84	AB015364	+
NIES-1090	Barato, Hokkaido	BRT84	AB015367	-
NIES-1091	Barato, Hokkaido	BRT84	AB015402	+
NIES-1092	Barato, Hokkaido	BRT84	AB015403	+
NIES-1105	Barato, Hokkaido	BRT89	AB015368	+
NIES-1115	Syowa-ike, Fukuoka	SYW89	AB015369	-
NIES-1117	Syowa-ike, Fukuoka	SYW89	AB015370	-
NIES-1143	Lake Okutama, Tokyo	OKT89	AB015365	+
TAC17	Ibaraki	KSM78	AB015398	+
TAC78	Lake Mikata, Fukui	LMM84	AB015401	+
NC1	Shiga	SHG**	AB015393-AB015397	-
NL1	Ibaraki	IBR**	AB015371	-

Note: Information about origin and microcystin-producing ability of the strains were obtained from NIES collection HP (National Institute for Environmental Studies 2012). Sequence information of the strains were from Otsuka et al. (1999). Abbreviations of strain names are used in Fig. 3; Microcystin, microcystin production.
[a]The numbers refer to the year the strain was collected. For those marked with an asterisk (*), the year is unknown.

Supplementary Table 1.The strains isolated in the present study. Site, collection site in Fig. 1; *mcyG* amplification, the result of *mcyG* amplification; The same genotype(sequence)as, If one genotype had been identified previously, this column contains the name of the strain.

Isolated strains		Site	DDBJ Accession#	*mcyG* gene amplification	The same genotype (sequence) as
KK	2	1	AB602274	+	
KK	3	1	AB602275	-	
KK	4	1		+	KK2
KK	6	1		+	KK2
KK	8	1	AB602276	-	
KK	9	1		-	KK8
KK	10	1		+	KK2
KK	11	1		-	KK3
KK	12	1		-	KK8
Iba	1	2	AB602282	-	
Iba	2	2		-	IB1
Iba	3	2		-	IB1
Iba	4	2		-	IB1
Iba	5	2		-	IB1
Iba	6	2		-	IB1
Iba	7	2		-	IB1
Iba	8	2		-	IB1
Iba	9	2		-	IB1
Iba	10	2		-	IB1
Iba	11	2		-	IB1
Iba	13	2		-	IB1
Iba	15	2		-	IB1
Iba	16	2		-	IB1
Iba	17	2		-	IB1
Iba	18	2		-	IB1
Iba	19	2		-	IB1
Iba	20	2		-	IB1
Iba	21	2	AB602283	-	
Iba	22	2		-	IB1
Iba	23	2		-	IB1
Iba	24	2		-	IB1
Iba	25	2		-	IB1
Iba	26	2		-	IB1
SB(Ot)	1-2	3	AB602254	+	
SB(Ot)	1-6	3	AB686448	-	
SB(Ot)	2-3	3	AB686449	-	
SB(Og)	2	3	AB602255	-	

SB(Og)	6	3	AB686450	+	
SB(Og)	8	3		-	SB(Og)2
SB(Og)	9	3	AB602256	+	
SB(Yb)	1	3	AB602257	-	
SB(Yb)	2	3	AB686447	+	SB(Kt)13
SB(Ak)	1	3	AB686453	+	
SB(Ak)	2	3	AB686454	+	
SB(Kt)	1	3	AB602258	+	
SB(Kt)	5	3	AB686451	+	
SB(Kt)	6	3		+	SB(Kt)1
SB(Kt)	7	3	AB686452	+	
SB(Kt)	9	3		+	SB(Kt)1
SB(Kt)	10	3		+	SB(Kt)13, SB(Yb)2
SB(Kt)	13	3	AB602259	+	SB(Yb)2
SB(Sk)	2	3	AB602260	-	
SB(Sk)	3	3		-	SB(Sk)2
SB(Sk)	4	3		-	SB(Sk)2
SB(Sk)	7	3		-	SB(Sk)2
NB	1	4	AB602264	-	
NB	3	4		-	NB1
NB	13	4	AB686455	+	
NB	14	4		+	NB13
NB	19	4		-	NB1
NB	21	4		-	NB1
NB	22	4		-	NB1
NB	23	4		-	NB1
NB	26	4	AB602265	+	
EA	1-9	5	AB602261	-	
EA	2-2	5	AB602262	-	
EA	2-4	5	AB602263	+	
EA	2-7	5		+	EA2-4
EA	2-9	5		-	EA1-9

FR	2	6	AB602286	-	
FR	3	6		-	FR2
FR	10	6		-	FR2
FR	11	6		-	FR2
FR	12	6	AB602287	-	
HN	1	7	AB602288	-	
HN	2	7		-	HN1
HN	3	7		-	HN1
HN	4	7	AB602289	-	
HN	5	7		-	HN4
HN	7	7	AB602290	+	
HN	10	7		+	HN7
HN	15	7		+	HN7
IT	6	8	AB602291	+	
IT	7	8	AB602292	+	
IT	11	8	AB602293	+	
YM	1	9	AB602279	+	
YM	2	9		+	YM1
YM	3	9	AB602280	+	
YM	5	9	AB602281	+	
YM	6	9		+	YM5
YM	10	9		+	YM1
YM	11	9		+	YM5
YM	12	9		+	YM1
YM	14	9		+	YM5
YM	15	9		+	YM1
YM	16	9		+	YM1
YM	17	9		+	YM5
YM	21	9		+	YM1
SNJ	2	10	AB686446	-	
SNJ	6	10		-	SNJ2
SNJ	9	10		-	SNJ2
SNJ	10	10		-	SNJ2
SNJ	13	10		-	SNJ2
SNJ	15	10		-	SNJ2
SR	2	11	AB602296	-	
SR	4	11		-	SR2
SR	5	11	AB602297	-	
SR	6	11		-	SR2
SR	9	11		-	SR2
SR	10	11		-	SR2
SR	11	11		-	SR2
SR	12	11		-	SR2
SR	13	11		-	SR2
SR	14	11		-	SR2
SR	15	11		-	SR2
SR	16	11	AB602298	-	

SR	21	11	AB602299	-	
SR	24	11		-	SR2
SR	33	11		-	SR2
SR	37	11		-	SR2
SR	39	11		-	SR2
SR	40	11		-	SR2
SM	4	12	AB602277	-	
SM	17	12		-	SM4
SM	18	12		-	SM4
SM	19	12	AB602278	+	
SM	21	12		-	SM4
MD	2	13	AB602294	-	
MD	8	13	AB602295	-	
UR	4	14	AB602284	-	
UR	5	14		-	UR4
UR	6	14		-	UR4
UR	8	14		-	UR4
UR	9	14		-	UR4
UR	10	14		-	UR4
UR	11	14		-	UR4
UR	13	14	AB602285	+	
UR	14	14		+	UR13
IS	1	15	AB602266	-	
IS	2	15		-	IS1
IS	3	15	AB602267	-	
IS	5	15	AB602268	-	
IS	6	15		-	IS5
IS	8	15	AB602269	+	
IS	9	15		-	IS5
IS	10	15	AB602270	-	
IS	11	15		-	IS1
IS	12	15		-	IS5
IS	13	15		-	IS5
IS	14	15		-	IS5
IS	16	15		-	IS5
IS	17	15		-	IS1
IS	18	15		-	IS5
IS	21	15		-	IS1
IS	22	15		-	IS5
IS	25	15		-	IS5
IS	28	15		-	IS1
IS	29	15		-	IS5
IS	31	15		-	IS5
IS	33	15		-	IS10
IS	34	15		-	IS5
IS	36	15		-	IS5
IS	38	15		-	IS1

IS	42	15		-	IS1
IS	43	15	AB602271	+	
IS	44	15		-	IS5
IS	45	15		-	IS5
IS	46	15		+	IS8
IS	47	15	AB602272	+	
IS	48	15	AB602273	+	
IS	49	15		+	IS48