Title:

Detection and identification of potentially toxic cyanobacteria: ubiquitous distribution of *Microcystis aeruginosa* and *Cuspidothrix issatschenkoi* in Japanese lakes

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Abstract

We studied the frequency and composition of potentially toxic cyanobacteria in 30 samples from 25 Japanese lakes using automated rRNA intergenic spacer analysis (ARISA) and eight primer sets for nodularin, microcystin, cylindrospermopsin, anatoxin-a, and saxitoxin synthetase genes. Potential microcystin- and anatoxin-a-producers were detected in 25 and 7 samples, respectively. Cylindrospermopsin- or saxitoxin-producers were not detected. PCR and clone library analyses indicated that *Microcystis* was the sole microcystin-producing genus. Moreover, potential microcystin-producing *Microcystis* strains were detected in 25 of 26 samples which included *Microcystis* ARISA fragments, suggesting that toxic *Microcystis* is ubiquitous. Potential anatoxin-a-producers detected in the samples were estimated to be *Cuspidothrix issatshcenkoi* (synonym: *Aphanizomenon issatschenkoi*) from clone library analyses of the anatoxin-a biosynthesis gene cluster and 16S-23S rDNA intergenic spacer region. Anatoxin-a-producing strains of *C. issatschenkoi* are known from two lakes in New Zealand and Germany. In contrast, *C. issatschenkoi* was the second most common toxic cyanobacteria in our study. This is the first report of potential anatoxin-a-producing *C. issatschenkoi* strains in Asia.

1. Introduction

Cyanobacterial blooms occur in freshwater bodies worldwide and have become more frequent due to cultural eutrophication (Paerl and Huisman, 2008). Many strains of cyanobacteria produce a wide range of secondary metabolites including hepatotoxins and neurotoxins. Cyanobacterial hepatotoxins include microcystins, nodularins, and cylindrospermopsin, and at least 10 genera include microcystin-producers (Codd et al., 2005). The neurotoxins include anatoxin-a, anatoxin-a(s), homoanatoxin-a, and saxitoxins from more than six genera (Codd et al., 2005). Moreover, global warming increases the risks of introducing non-native and toxic cyanobacteria including the apparent invasion of *Cylindrospermopsis raciborskii* into Europe (Wiedner et al., 2007; Paerl and Huisman, 2008: Kaštovský et al., 2010).

In Japan, cyanobacterial blooms and their toxins have been studied extensively for several decades (e.g., Watanabe et al., 1994). However, most studies have been conducted on *Microcystis aeruginosa* and its microcystins, because *M. aeruginosa* blooms have been encountered most frequently (e.g., Park et al., 1993; Ozawa et al., 2005; Yoshida et al., 2005). An anatoxin-a- and homoanatoxin-a-producing strain of *Raphidiopsis mediterranea* Skuja has been isolated from Lake Biwa in Japan (Namikoshi et al., 2003; Watanabe et al., 2003). *R. mediterranea* has not formed a bloom itself in Lake Biwa but frequently appears as a minor component in the blooms of *Microcystis* and *Dolichospermum* (Watanabe et al., 2003), although this species is

well known as a bloom-forming cyanobacterium in other regions (Li et al., 2008; Kaštovský et al., 2010; Moustaka-Gouni et al., 2010). Thus, to reduce the future risk of human exposure to cyanotoxins, careful attention should be paid to all (potentially) toxic cyanobacteria, even those that may be rarely found in some regions at present.

Because the ability to produce cyanotoxins is determined at strain level (Otsuka et al., 1999; Tanabe et al., 2007), and morphological identification is insufficient for monitoring toxigenic cyanobacteria, recent characterizations of the cyanotoxin synthetase gene clusters have provided several molecular markers that target cyanotoxin synthetase genes directly (Pearson and Neilan, 2008; Humbert et al., 2010). These markers permit the detection of potential toxin-producing strains using the polymerase chain reaction (PCR), with quantitative PCR to indicate population densities. In particular, specific primer sets of the *mcy*E gene for *Dolichospermum*, *Microcystis* and *Planktothrix* microcystin biosynthesis have been developed (Rantala et al., 2004; Briand et al., 2008). Thus, seasonal variation in the abundance and/or the geographical distributions of potentially toxic cyanobacteria using molecular markers can be evaluated.

In the present study, we evaluated the distribution of potentially toxic cyanobacteria in the western part of Japan using automated rRNA intergenic spacer analysis (ARISA) and multiple primer sets targeting different cyanotoxin-encoding genes. ARISA is a DNA fingerprinting method that detects the length heterogeneity of

the intergenic spacer (ITS) region between 16S and 23S rDNA (Fisher and Triplett, 1999). Using these methods, we have established a way to evaluate the presence or absence of toxin-producing cyanobacteria using a capillary sequencer. Although ARISA has been applied to analyze cyanobacterial communities using specific primers for cyanobacteria (Wood et al., 2008, 2009; Drakara and Liess, 2010), information about the identity of ARISA fragment lengths in each species or strain remains limited. Therefore, we confirmed the resolution of ARISA using 19 cultured strains. Secondly, we collected water samples from 30 locations in the western part of Japan. We discuss the current distribution of potentially toxic cyanobacteria, particularly for the newly discovered anatoxin-a-producing strains of *Cuspidothrix issatschenkoi* (Usačev) Rajaniemi et al. (Rajaniemi et al. (2005), synonym: *Aphanizomenon issatschenkoi* (Usačev) Proškina-Lavrenko) in Japan.

2. Materials and methods

2.1 Culture strains and environmental samples

Samples from 30 locations and 25 water bodies in the western part of Japan were collected from August to November 2009 and from October to November 2010 (Fig. 1, Table 1). Water samples were collected at shorelines using a bucket, although offshore surface water was collected from shipboard using a 20-μm mesh plankton net only for Station No. 1. Phytoplankton cells from 20–100 ml water samples were

collected onto pre-combusted (3 h at 420°C) Whatman GF/F filters for DNA extraction by filtration. The samples were stored at -20°C until DNA extraction.

We also used 19 cultured strains of cyanobacteria to collect basic information about the ITS fragment lengths and sequences (Table 2). Culture conditions of each strain are described in Table 2, and cells in the logarithmic growth phase were collected by filtration. The samples were stored at -20°C until used for DNA extraction.

2.2. DNA extraction and PCR amplification of the ITS region and cyanotoxin synthetase genes

Cells retained on filters were immersed in 1 ml modified cetyltrimethylammonium bromide (CTAB) buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA, 2% [w/v] CTAB, 100 mM DTT, 1% [w/v] polyvinylpyrrolidone) and heated in a water bath at 58°C for 1 h. After the heat treatment, DNA was extracted with chloroform:isoamyl alcohol (24:1) and then washed and precipitated twice with ethanol. Extracted DNA was resuspended in 30 μL TE buffer and quantified with a NanoDrop 1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

We used nine primer sets for ARISA and multiple toxityping (Table 3). For ARISA, the cyanobacteria-specific CSIF 16S rDNA and the ULR 23S rDNA primers were used to amplify the ITS region (Neilan et al., 1997; Janse et al., 2003). The ULR

23S rDNA primer was labeled at the 5′ end with fluorescent dye 6-FAM (Drakara and Liess, 2010). PCR amplification was performed in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with a 10 μL reaction mixture containing 100 ng template DNA, 0.2 mM of each dNTP, 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 0.5 μM of each primer. After preincubation at 94°C for 10 min, 30 cycles were performed at 94°C for 1 min, followed by annealing temperature for 1 min and 72°C for 1 min. In the first 20 cycles, the annealing temperature was reduced by 1°C after every two cycles, from 62°C in the first cycle to 52°C in the twentieth. During the last 10 cycles, the annealing temperature was 52°C, followed by a final extension for 30 min at 72°C.

We applied eight specific primer sets to analyze cyanotoxin-encoding genes in different cyanobacteria (Table 3). All PCR amplifications were performed with a 10 μL reaction mixture containing 20 ng template DNA, 0.2 mM of each dNTP, 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 U AmpliTaq Gold DNA polymerase, 0.5 μM of specific primer for each toxin, and the primer-specific $MgCl_2$ concentration (Table 3). The 5′ ends of the forward primers were also labeled with fluorescent dye (NED, VIC, or PET; Applied Biosystems) for detection by the capillary auto sequencer. To prevent additional adenine (A), the 5′ end of each reverse primer was also tailed with seven specific bases (Applied Biosystems).The PCR cycling conditions were as follows: 9 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at the primer-specific annealing

temperature (Table 3), and 1 min at 72°C; and a final extension step at 72°C for 15 min.

2.3. Fragment analysis of the ITS region and cyanotoxin synthetase genes

0.3 μL of all PCR products were added to 12 μL Hi-Di formamide (Applied Biosystems) and the mixture heated at 95°C for 5 min to denature the PCR products. Fragment lengths of all PCR products were determined using an ABI3100-*Avant* Capillary Auto Sequencer (Applied Biosystems) with a 0.5 μL internal size standard (GeneScan^{TM} 1200LIZ, Applied Biosystems). Running conditions were a 60°C separation temperature, 10 kV run voltage, and a 120 min separation time. Law data were analyzed by GeneMapper ver 4.0 (Applied Biosystems).

Peaks of ARISA fragment lengths from 350–1000 bp with an area of more than 500 fluorescence units were read and the percentage of each peak to total peak area of the sample was calculated. Sensitivity of ARISA was also assessed by dilution of template DNA using 4 strains (NIES-40, 230, 843, and 1645; Table 2). The amounts of template DNA in peak areas at 500 fluorescence units ranged from 15.3 to 18.0 pg (17.2 ± 1.3 pg) and accounted for 0.017 ± 0.001% of the template DNA used in ARISA. These amounts of DNA could be extracted from 3147–28,809 cells (11,417 ± 11,824 cells) per sample by the aforementioned DNA extraction method (data not shown). ARISA fragments that differed in length by ≤2 bp were considered identical (Wood et al., 2008, 2009). All peaks in each sample were divided into four levels by proportion

and were represented by the following symbols: r (<5%), + (<20%), ++ (<50%), and +++ (≥50%).

Since cyanotoxin-producing strains usually coexist with non-toxic ones, threshold values were set lower for multiple toxityping than for ARISA, and the presence or absence of cyanotoxin synthetase genes was judged by the peaks, which yielded more than 50 fluorescence units around the correct positions (Table 3). Because cyanotoxin synthetase gene fragment lengths can differ to some degree between strains (e.g., Rantala *et al*., 2004), all peaks located within 5 bp of known positions were counted (Table 3).

2.4. Cloning and sequences

To identify the toxin-producing cyanobacteria in detail, clone library analyses of the environmental samples were also conducted for the aminotransferase domain on the *mcy*E and *nda*F modules of the microcystin and nodularin synthetase enzyme complexes (HEP), for the polyketide synthase domain on the anatoxin-a biosynthesis gene cluster (ATX), and for the cyanobacteria-specific 16S-23S rDNA ITS region (Cya-ITS). PCR amplification was performed under the same conditions as fragment analysis except for the PCR scale (50 μL) and the use of non-labelled forward and reverse primers (Table 3). After electrophoresis on a 2% (w/v) agarose gel with TAE buffer, PCR products were purified with the QIAquick PCR Purification Kit (Qiagen,

Valencia, CA, USA) and cloned using a pT7 Blue Perfectly Blunt Cloning Kit (Novagen Gibbstown, NJ, USA), according to the manufacturer's instructions. More than 24 positive colonies were picked for each sample and the inserted DNA fragments amplified using T7 and U19 primers. The HEP and ATX PCR products included unspecific PCR products. The amplified fragments were verified with 2% agarose gel electrophoresis and colonies with an insert of the correct size were sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI3100-*Avant* Capillary Auto Sequencer. At least eight sequences were determined for each sample.

2.5. Phylogenetic analysis

All sequences were aligned using BioEdit ver 7.0.9 and all unique sequences were deposited at the DNA Data Bank of Japan. The assigned accession numbers were AB638213 to AB638267. All unique sequences obtained by clone library analysis were checked for sequence similarity using the BLAST search programme (Altschul et al., 1997), and sequences found to share a high level of similarity were used for phylogenetic studies. Phylogenetic analysis of HEP, ATX and Cya-ITS partial sequences was performed using Phylip ver. 3.69 (Felsenstein, 2009). Phylogenetic trees were constructed using the neighbour-joining algorithm and confidence levels calculated with 1000-fold bootstrap analysis.

3. **Results**

3.1. ARISA fragment lengths of cultured strains and environmental samples

ARISA fragment lengths of five strains of *Chroococcales*, three strains of *Oscillatoriales*, nine strains of *Nostocales* and two strains of *Pseudanabaenales* were determined (Table 2). All of the *Chroococcales* and *Pseudanabaenales* showed one ARISA fragment, and all of the *Nostocales* and two *Oscillatoriales* had more than one ARISA fragment with different lengths. Strains within the same genus showed similar fragment lengths, at least for shorter fragments (ITS 1), and most genera had unique fragment lengths, except for *Dolichospermum* and *Anabaenopsis* (Table 2). ARISA fragment lengths of *Leptolyngbya* sp. and *Oscillatoria neglecta* were 657 and 677 bp, respectively and overlapped with the lengths of longer fragments (ITS 2) of *Nostocales* and *Planktothrix* spp. (Table 2).

3.2. ARISA of a natural cyanobacterial community

Twenty-three unique fragment lengths in 30 environmental samples were detected by ARISA (Table 1), and 14 of the 23 unique fragments could be assigned to the ARISA fragment lengths obtained from known cultured strains (Table 2). *Microcystis* was the most common genus and was found in 26 out of 30 samples. The fragments derived from *Dolichospermum* and *Anabaenopsis* spp. were also detected in

16 out of the 30 samples. Although the third most commonly found fragment was that at 411 and 412 bp in 7 samples, we were unable to assign these to the known taxa in Table 2.

3.3. Multiple toxityping of a natural cyanobacterial community

Eight specific primer sets for four cyanotoxin-encoding genes were tested (Table 3). No samples gave positive PCR products for cylindrospermopsin (CLC) or saxitoxin (SxtA) genes. However, PCR products related to microcystin- (HEP, UME and MIC) and anatoxin-a (ATX) biosynthesis were found (Table 1). Moreover, for PCR products related to microcystin-specific primer sets, no *Dolichospermum*- or *Planktothrix*-specific PCR products were detected in any samples (ANA and PLA; Table 3). In contrast, positive HEP, UME, and MIC PCR products were detected in 25, 22, and 25 samples, respectively. These samples generally corresponded to the ones in which the fragments were presumably derived from *Microcystis* as detected by ARISA (Table 1). Only one sample was found by ARISA which included putative *Microcystis* fragments but showed negative HEP, UME, and MIC PCR products (no. 13, Lake Ibanaiko, Table 1). Specific primer set for anatoxin-a biosynthesis gene (ATX) was also positive in 7 samples (Table 1). Although these samples had multiple ARISA fragments, 6 out of 7 samples had the unassigned fragment at 411 and 412 bp (Table 1).

3.4. Phylogenetic analysis for HEP, ATX, and Cya-ITS

To clarify the presence or absence of microcystin-producing species other than *M. aeruginosa*, and of possible nodularin-producers, clone library analyses of the HEP region were conducted on 11 samples (nos. 4, 7, 9, 12, 14, 15, 18, 21, 24, 25, and 30 in Table 1). In total, 109 sequences were analyzed and further distinguished as 32 unique sequences. A phylogenetic tree was constructed including the sequences with high similarity in the BLAST searches (Fig. 2). All sequences could be divided into four distinct clusters. However, all sequences obtained from the clone library analysis showed 97.8–100% similarity to the *M. aeruginosa* sequences and fell into cluster I (Fig. 2). No sequences fell into other clusters including the *Oscillatoriales* (clusters II and III) and *Nostocales* sequences (cluster IV).

Clone library analyses were also conducted on 7 samples which showed positive PCR products for ATX (nos. 7, 11, 12, 15, 16, 21 and 22). Seventy sequences were analyzed and distinguished as four unique sequences (Fig. 3). All of the unique sequences found in the present study showed 99–100% similarity to the polyketide synthase domain on the ATX biosynthesis gene of *C. issatschenkoi* (synonym: *A. issatschenki*) SP 33 and clustered together with it (Fig. 3).

To gain further information about the anatoxin-a-producing strains, clone library analyses of Cya-ITS were conducted for the 7 ATX-positive samples. Because

many sequences from the clone libraries showed high similarity to known *M. aeruginosa* ITS sequences, we omitted these sequences and constructed a phylogenetic tree including known sequences from the BLAST searches and newly determined the sequences of 8 strains (Fig. 4). Five unique sequences from 5 samples showed 95.8–97.9% similarity to the *C. issatschenkoi* 16-1 sequence and clustered together with it. These sequence fragment lengths ranged from 414 to 415 bp and corresponded to the ARISA fragments detected at 411 and 412 bp (Table 1). The other 4 sequences from 2 samples were of 446 bp in length and showed the highest similarity to *Dolichospermum flos-aquae* NIES-1669 (Fig. 4).

4. Discussion

ARISA is a method which detects length heterogeneity of the 16S-23S rDNA ITS region and has been applied to evaluate cyanobacterial community structure (Wood et al., 2008, 2009; Drakara and Liess, 2010). In the present study, since fragment analysis by the capillary sequencer not only provides high accuracy for the measurement of DNA fragment length, but can also detect several PCR products by simultaneous fluorescence labelling, attempts were made to combine ARISA and multiple toxityping to detect and monitor potentially toxigenic cyanobacteria. It was possible to detect the presence or absence of target cyanotoxin-encoding genes, even for samples containing many unspecific PCR products. Other methods, such as

quantitative PCR and DNA microarray, have also been developed and used for detection of potentially toxigenic genera (e.g., Rinta-Kanto et al., 2005; Rantala et al., 2008; Briand et al., 2009). These methods are more sensitive and specific than that used here, but may not be suitable for comprehensive monitoring of toxic cyanobacteria, since the availability of specific primers and probes remains limited. Moreover, conventional agarose gel electrophoresis can also be applied for detection of cyanotoxin-encoding genes. However, fragment lengths of cyanotoxin-encoding genes can differ by a few base pairs among toxin-producing strains (e.g., Rantala et al., 2004), and so agarose gel electrophoresis may be unable to discriminate several base pair differences in fragment length. This is also a potential benefit of using fragment analysis to directly identify potentially toxic cyanobacteria.

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are also well-established fingerprinting methods for analyses of microbial communities (Muyzer, 1999; Kumari et al., 2009; Saker et al., 2009). In contrast to ARISA, these methods have the advantage of identifying unknown community members due to the direct sequencing of excised target bands. However, DGGE and TGGE seem to be unsuitable for monitoring on a massive scale, since inter-gel comparison is difficult (Schäfer and Muyzer, 2001), and sequencing of the bands may be needed in every analysis even to identify bands of common species. Moreover, DGGE and TGGE can be applied to DNA fragments with short and usually

equal sequence lengths (up to *ca*. 500 bp, Schäfer and Muyzer, 2001), and the amount of sequence information is insufficient to identify a broad range of genera down to the species level. On the other hand, ARISA uses internal size standards for each sample and does not require comparable sample numbers, and the 16S-23S rDNA ITS region is highly polymorphic (e.g., Gugger et al., 2002; Saker et al., 2009). Therefore, ARISA is a more useful method for monitoring a variety of genera with previously identified fragment lengths, although identification of unassigned fragments still requires cloning and sequencing of PCR products.

Despite the utility of ARISA for analyses of cyanobacterial communities, the differences in fragment number between strains appeared to complicate the interpretation of the data (Table 2). This problem arises from interoperonic differences in spacer lengths within genomes (Nagpal et al., 1998) and the resulting shorter and longer fragments have sometimes been called ITS 1 and ITS 2 or ITS-S and ITS-L, respectively (Gugger et al., 2002; Wood et al., 2009). Although strains with multiple fragment lengths can be identified more specifically in some cases (Gugger et al., 2002; Wood et al., 2009), ITS 2 fragments make it rather difficult to identify other strains with longer ITS 1 fragments such as *Oscillatoria* and *Leptolyngbya* spp. (Table 2). Therefore, to directly discriminate between the longer ITS 1 fragments and ITS 2 fragments and obviate the need for additional clone library analysis, construction of an ARISA fragment length library for target strains is necessary.

PCR methods have been used to detect potentially toxic cyanobacteria, though only a few studies have addressed multiple types of cyanotoxins or potentially toxic genera simultaneously. Toxin-producing cyanobacterial strains belonging to different genera frequently coexist within the same bloom (Vezie et al., 1997, 1998). In a study on Finnish lakes, 54% of the lake samples contained more than one potential microcystin-producing genus (Rantala et al., 2006). Moreover, *Dolichospermum* and *Microcystis* are common microcystin-producers (Sivonen et al., 1990; Rantala et al., 2006), and their frequency of coexistence in multiple microcystin-producing genera is high in eutrophic lakes (Rantala et al., 2006). In contrast, the multiple toxityping assay used in the present study indicated that *Microcystis* was the sole microcystin-producing genus in the western part of Japan, even though other potential microcystin-producing genera, including *Dolichospermum* and *Planktothrix* coexisted (Table 3). Interestingly, the sequences that can be related to the aminotransferase domain for the *mcy*E gene (HEP) of *Microcystis* and *Dolichospermum* have been detected from Kenyan lakes, but do not appear to coexist within the same lakes (Dadheech et al., 2009). Jungblut and Neilan (2006) studied HEP sequences in 5 samples from Australia, Germany, and Italy and detected only one sequence with a high similarity to the HEP sequences of *Microcystis*, *Planktothrix* or *Nodularia* in each sample. Therefore, it appears that dominant producers and the coexistence of multiple cyanotoxin-producers vary regionally.

Despite the low diversity of potential microcystin-producing genera, these were present in 83% of the samples when studied with an HEP primer set (Table 1). This result supports those of previous studies (range, 25–92%; Sivonen et al., 1990; Rantala et al., 2006), although it was generated in the Japanese waterbodies solely by microcystin-producing *Microcystis* strains, which was detected in 25 out of the 26 samples which included *Microcystis* ARISA fragments. The wide distribution of potential microcystin-producing *Microcystis* strains may be empirically predictable, at least in Japan, as toxic *Microcystis* strains comprise more than one-third (104/293) of all strains listed in the microbial culture collection of the Natioral Institute of Environmental Studies of Japan (National Institute for Environmental Studies, 2011). Moreover, *Microcystis* populations usually consist of multiple strains (Yoshida et al., 2005; Kardinaal et al., 2007; Briand et al., 2009; Sabart et al., 2009), and *Mirocystis* might have a cosmopolitan distribution (van Gremberghe et al., 2011). Therefore, it is conceivable that microcystin-producing *Microcystis* strains are ubiquitous.

In addition to the microcystin-producing *Microcystis* strains, potential anatoxin-a producers, presumed to be *C. issatschenkoi*, were also detected in 7 samples (Table 3, Figs. 3, 4). *C. issatschenkoi* is known as an alien and expansive cyanobacterium in Europe (Kaštovsky et al., 2010). Anatoxin-a-producing strains have also been discovered in two lakes in New Zealand and Germany (Wood et al., 2007; Ballot et al., 2010a). In Japan, *C. issatschenkoi* was first observed in 1976 in eutrophic

Lake Kasumigaura in central Japan (Watanabe, 1985). However, *C. issatschenkoi* seems to be much less common in Japan, as there are few authoritative data on this species except for in Lake Kasumigaura and the Teshio River in northern Japan (Watanabe, 1985, 1991). Isolation and analysis of *C. issatschenkoi* toxicity has also been conducted, although no toxin-producing strains were confirmed (Park et al., 1993). Therefore, anatoxin-a-producing strains of *C. issatschenkoi* may have expanded in distribution for several decades to become the second most common toxin-producing cyanobacterium in western Japan. This study is the first report of potential anatoxin-a-producing strains of *C. issatschenkoi* in Asia.

C. issatschenkoi morphologically resembles other species and has sometimes been misidentified (Li et al., 2003; Wood et al., 2007). For example, trichomes of *Raphidiopsis mediterranea* Skuji var. *grandis* Hill are similar to those of *C. issatschenkoi*, except for the absence of heterocysts in *R. mediterranea* (Hill, 1970; Watanabe, 1985). Microscopic identification of the two species has been conducted routinely based on the presence of heterocysts (Wood et al., 2007). However, phylogenetic analysis of the 16S rRNA gene sequence has demonstrated that the anatoxin-a-producing strain previously identified as *R. mediterranea* Skuja CAWBG02 is a heterocyst-lacking *C. issatschenkoi*, indicating uncertainty in identifying the two species based on the presence of heterocysts (Wood et al., 2007). Additionally, *R. mediterranea* can be distinguished from *C. issatschenkoi* by apical cell shape, and only

R. mediterranea Skuji var. *grandis* Hill is morphologically difficult to distinguish from *C. issatschenkoi* (Moustaka-Gouni et al., 2010). Interestingly, an anatoxin-a- and homoanatoxin-a-producing strain of *R. mediterranea* Skuji var. *grandis* Hill was isolated from Lake Biwa (Watanabe et al., 2003; Namikoshi et al., 2003), and the presence of potential anatoxin-a producers has been found in the present study. Indeed, we also have found morphologically indistinguishable heterocysts lacking trichomes that resemble *R. mediterranea* Skuji var. *grandis* Hill and *C. issatschenkoi* in some samples by microscopic observation (data not shown). Therefore, further studies are needed to understand the ecological and phylogenetic relationships between *C. issatschenkoi* and *R. mediteranea* Skuji var. *grandis* Hill.

5. Conclusion

This study combined ARISA with multiple toxityping to monitor toxic cyanobacteria. The sensitivity of ARISA was demonstrated and it was shown to possess the advantage of producing a mass of information on the composition of potentially toxic cyanobacteria. On the other hand, some genera exhibited more than one ARISA fragment of different lengths (ITS 1 and ITS 2), which makes detection of strains with longer ITS 1 fragments problematic. As clone library analyses are also needed to identify unidentified ARISA fragments, construction of an ARISA fragment length library is vital to facilitate accurate and effective use of this method. Using this method,

we found that microcystin-producing *Microcystis* strains and anatoxin-a-producing strains of *Cuspidothrix issatshcenkoi* were ubiquitous in the western part of Japan. Other potentially toxin-producing strains were not detected and seemed to be quite rare. Although information on the global distribution of anatoxin-a-producing strains of *C. issatschenkoi* remains limited, this strain of *C. issatschenkoi* was the second most-common toxic cyanobacteria in Japan.

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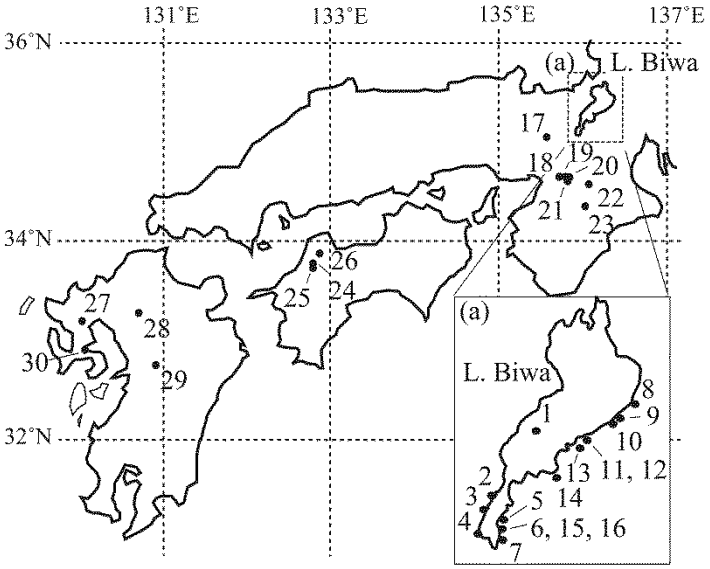

Fig. 1. Location of study sites in western Japan, and a magnified figure of Lake Biwa (a). Numbers on the map indicate sites and correspond with the location numbers in Table 1.

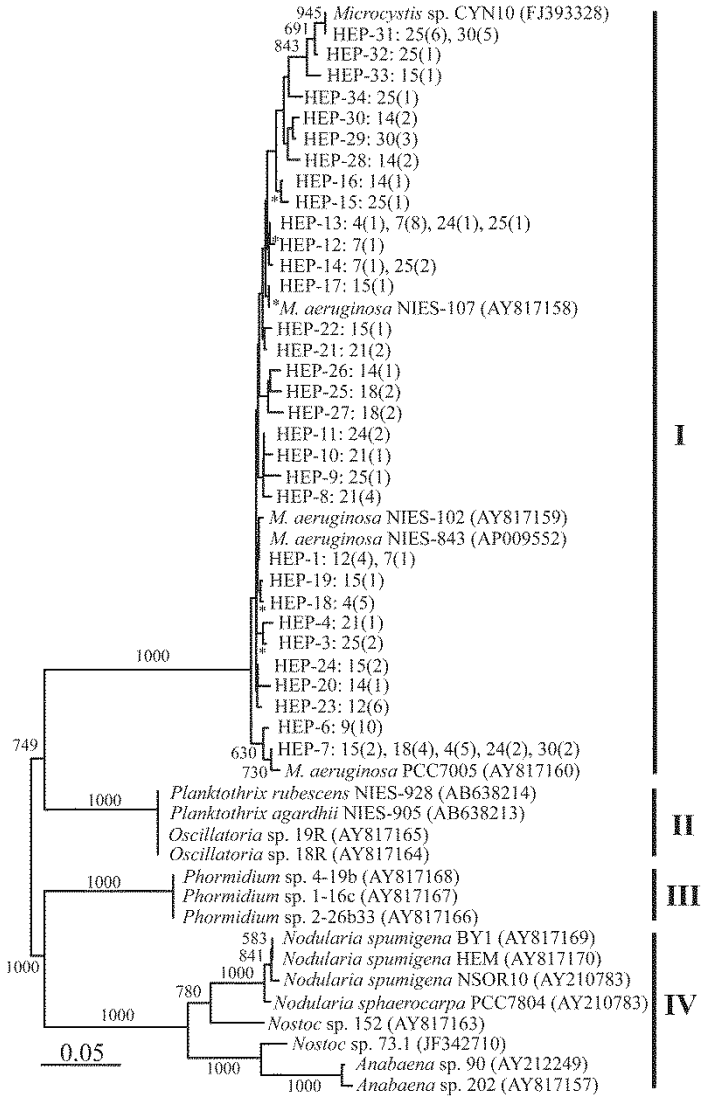

Fig. 2. Neighbour-joining tree based on partial nodularin synthetase enzyme complex (HEP) sequences (413–416 bp) from 11 collected samples, with reference sequences obtained through BLAST analysis and NIES collections. Thirty-five unique sequences obtained by clone library analyses are expressed as HEP. Numbers indicate the location numbers listed in Table 1. Numbers in parentheses indicate the same sequence numbers observed within a sample. Bootstrap values

>50% are indicated near the node. Asterisks indicate nodes with bootstrap values >50%.

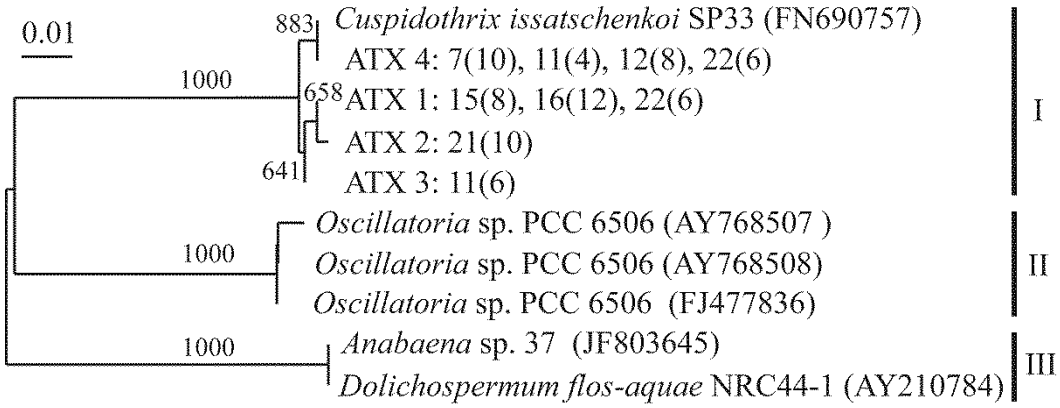

Fig. 3. Neighbour-joining tree based on partial sequences (417 bp) of the anatoxin-a (ATX) biosynthesis gene cluster from seven environmental samples with reference sequences obtained through BLAST analysis. Bootstrap values from 1000 replicates of the sequence data are shown. Four unique sequences obtained by clone library analyses are expressed as ATX. Numbers indicate the location numbers listed in Table 1, and the numbers in parentheses indicate the same sequence number observed within a sample.

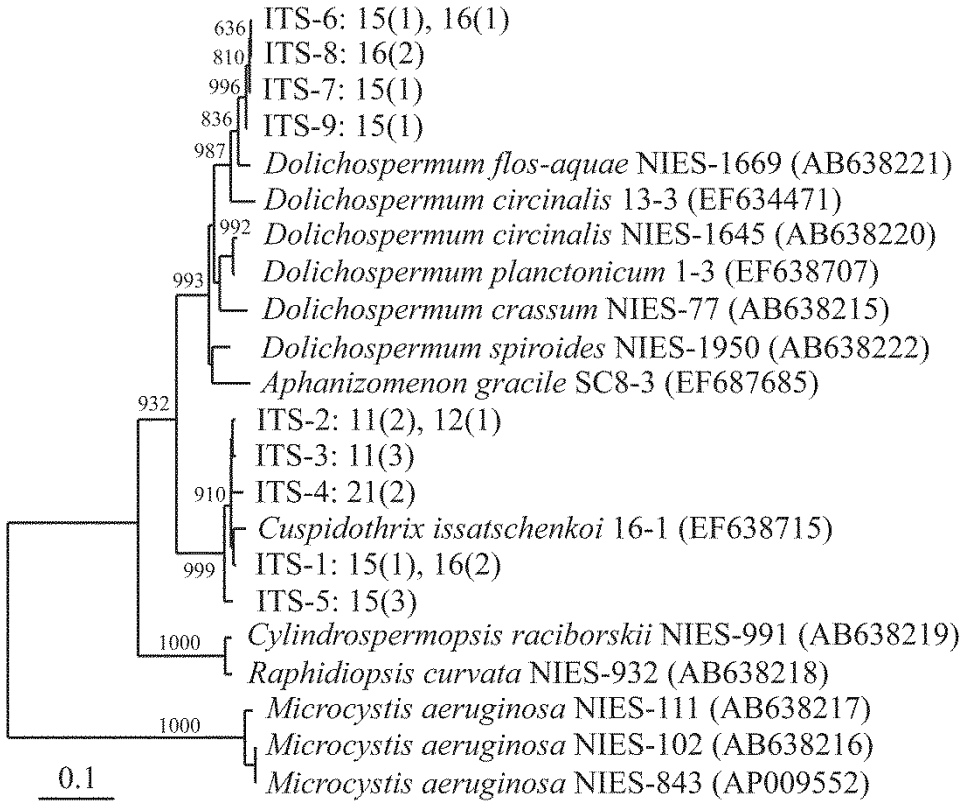

Fig. 4. Neighbour-joining tree based on the partial sequences of 16S rRNA and 16S-23S rRNA intergenic spacer (ITS) region (374–487 bp) from 7 collected samples, with reference sequences obtained through BLAST analysis and NIES collections. Bootstrap values from 1000 replicates of the sequence data are shown. Nine unique sequences obtained by clone library analyses are expressed as ITSs. Numbers indicate the location numbers listed in Table 1, and numbers in parentheses indicate the same sequence number observed within a sample.

Table 1. Study sites and results of automated rRNA intergenic spacer analysis (ARISA) and multiple toxityping of cyanotoxins.

No.	Location	Type[c]	Cyanotoxins[d]				Potential taxonomical groups and their fragment lengths (nm)[e]																						
							UAF	*CR*	UAF		*Anabaena* &*Anabaenopsis*					UAF	*P*	*Microcystis*				UAF		ITS 2			UAF		
			HEP	UME	MIC	ATX	393-394	398-399	405-407	411-412	420-421	428-429	433-434	438-439	442-443	451-452	492-493	519-520	521-522	524-525	538-539	598-599	638-639	655-656	657-658	664-665	734-735	843-844	857-858
1	NBLB[a]	L	+	+	+	-													+++										
2	Ogoto (SBLB[b])	L	+	+	+	-						++							+++										
3	Sakamoto (SBLB[b])	L	+	+	+	-													+++								+	+	
4	Otsu (SBLB[b])	L	+	+	+	-									+					+++									
5	Akanoi (SBLB[b])	L	+	+	+	-						+			+++				+								+	+	
6	Kitayamada (SBLB[b])	L	+	+	+	-						++							r				++	+					
7	Yabase (SBLB[b])	L	+	+	+	+				r				+++	++					r									
8	Hasuike	L	-	-	-	-												*N.D.*											
9	Nodanuma	L	+	-	+	-				+									++	++									++
10	Sonenuma	L	-	-	-	-	++	++	++																				
11	Koyabanuma	L	+	+	+	+				++									++	++									++
12	Jinjyounuma	L	+	+	+	+				+									++	++									++
13	Ibanaiko	L	-	-	-	-									+++				+										
14	Kitazawanuma	L	+	+	+	-									++				++					++	++				
15	Hirako	L	+	+	+	+				+					+++			+								+			
16	Yanagihirako	L	+	-	+	+				+					+++			+											
17	Hirosawa	P	-	-	-	-												*N.D.*											
18	Hajikami	P	+	+	+	-					++							++		+	++								
19	Uwanabe	P	+	-	+	-													+++										
20	Kouno	P	+	+	+	-													+++										
21	Sarusawa	P	+	+	+	+				r		r		r							+++	++							
22	Kamituko	R	+	+	+	+											+	+++											
23	Muroo	R	+	+	+	-													+++	+									
24	Furuike	P	+	+	+	-					++	r	+					+++		r									
25	Handaji	P	+	+	+	-						r							++	+++								++	
26	Ishitekawa	R	-	-	-	-						+++												+					
27	Ureshino	R	+	+	+	-													+++	+									
28	Shimouke	R	+	+	+	-												+++											
29	Midorikawa	R	+	+	+	-						+				++		+++								r			
30	Isahaya	R	+	+	+	-										+			+++										
	Total		25	22	25	7	1	1	1	7	2	8	1	2	7	2	1	8	16	9	2	1	1	2	1	2	2	3	3

a, North basin of Lake Biwa; b, South basin of Lake Biwa; c, Types of the sites (L, lake; P, pond; R, reservoir); d, cyanotoxins specific primers (HEP, all microcystin and nodularin producers; UME, all microcystin producers; MIC, microcystin producing *Microcystis*; ATX, anatoxin-a producers); e, (UAF, unassigned fragment; *CR*, *Cylindrospermopsis* & *Raphidiopsis*; *P*, *Planktothrix*; ITS 2, intergenic spacer 2 frangents of *Nostocales* & *Oscillatoriales*; r, <5%; +, <20%; ++, <50%; +++, >50%).

Table 2. Strains used to determine automated rRNA intergenic spacer analysis (ARISA) fragment lengths and their culture conditions.

Algae[a]	Strain No.	ITS 1 (bp)	ITS 2 (bp)	Culture conditions		
				Medium	Temp. (°C)	Light intensity (μmol m^{-2} s^{-1})
Dolichospermum affine (Lemmermann) Wacklin, Hoffmann et Komárek	NIES-40	443	664	CT	25	70
Dolichospermum circinalis (Rabenhorst ex Bornet et Flahault) Wacklin, Hoffmann et Komárek	NIES-1645	429	667	CB	20	30
Dolichospermum crassum (Lemmermann) Wacklin, Hoffmann et Komárek	NIES-77	443	655, 658	CT	25	70
Dolichospermum flos-aquae ([Lyngbye] Brebisson ex Bornet et Flahault) Wacklin, Hoffmann et Komárek	NIES-1669	441	663	MA	20	30
Dolichospermum spiroides (Klebahn) Wacklin, Hoffmann et Komárek	NIES-1950	423	655	CB	20	5
Anabaenopsis sp.	NIES-1698	433	764, 769	CB	20	30
Cylindrospermopsis raciborskii (Woloszynska) Seenayya et S. Raju	NIES-991	398	553	CT	23	20
Leptolyngbya sp.	NIES-30	657	-	MDM	20	10
Merismopedia tenuissima Lemmermann	NIES-230	855	-	C	20	20
Microcystis aeruginosa (Kützing) Lemmermann	NIES-843	520	-	MA	25	30
Microcystis aeruginosa (Kützing) Lemmermann	NIES-102	520	-	MA	25	30
Microcystis aeruginosa (Kützing) Lemmermann	NIES-111	523	-	CT	25	30
Oscillatoria neglecta Lemmermann	NIES-2116	677	-	MDM	20	10
Planktothrix agardhii (Gomont) Anagnostidis et Komárek	NIES-905	492	652	CT	20	30
Planktothrix rubescens (DC. ex Gomont) Anagnostidis et Komárek	NIES-928	492	653	CT	20	30
Pseudanabaena galeata Böcher	NIES-512	714	-	CT	20	10
Raphidiopsis curvata F. E. Fritsch et F. Rich	NIES-932	399	562	CT	25	30
Raphidiopsis sp.	NIES-1729	399	562	MG	20	20
Synechococcus sp.	NIES-937	994	-	CB	15	20

a, all species name were followed by Wacklin *et al.* (2009).

Table 3. Primer set characteristics used in this study.

Maker	Cyanotoxins	Genes[a]	Specificity	Fowerword & Reverse Primers	Sequence (5'-3')	*Tm* (°C)[b]	$MgCl_2$ (mM)[b]	PCR (bp)[b]	References
HEP	microcystin nodularin	*mcyE* *nduF*	All producers	HEP-F HEP-R	*NED*-TTTGGGGTTAACTTTTTTGGGCATAGTC AATTCTTGAGGCTGTAAATCGGGTTT	52	2.5	472	Jungblut and Neilan (2006)
UME	microcystin	*mcyE*	All producers	mcyE-F2 mcyE-R4	*NED*-GAAATTTGTGTAGAAGGTGC AATTCTAAAGCCCAAAGACG	56	1.5	809-812	Rantala et al. (2004)
MIC	microcystin	*mcyE*	*Microcystis*	mcyE-F2 MicmcyE-R8	*NED*-GAAATTTGTGTAGAAGGTGC CAATGGGAGCATAACGAG	58	1.5	247	Rantala et al. (2004) Vaitomaa et al. (2003)
ANA	microcystin	*mcyE*	*Anabaena*	mcyE-F2 AnamcyE-R8	*VIC*-GAAATTTGTGTAGAAGGTGC CAATCTCGGTATAGCGGC	58	1.5	247	Rantala et al. (2004) Vaitomaa et al. (2003)
PLA	microcystin	*mcyA*	*Planktothrix*	MAPF MAPR	*NED*-CTAATGGCCGATTGGAAGAA CAGACTATCCCGTTCCGTTG	60	2.0	140	Briand et al. (2008)
ATX	anatoxin-a homoanatoxin-a	*pks*[c]	All producers	atoaf atxar	*PET*-TCGGAAGCGCGATCGCAAATCG GCTTCCTGAGAAGGTCCGCTAG	55	2.0	470	Ballot et al. (2010a)
CLS	cylindrospermopsin	*pks*[c]	All producers	k18 m4	*VIC*-CCTCGCACATAGCCATTTGC GAAGCTCTGGAATCCGGTAA	45	3.0	422	Rasmussen et al. (2008)
SxtA	saxitoxin	*pks*[c]	All producers	sxtaf sxtar	*VIC*-GCGTACATCCAAGCTGGACTCG GTAGTCCAGCTAAGGCACTTGC	55	2.0	600	Ballot et al. (2010b)
Cya-ITS	-	16S-23SrDNA	cyanobacteria	CSIF 23ULR	G(T/C)C ACG CCC GAA GTC (G/A)TT AC *6FAM*-CCT CTG TGT GCC TAG GTA TC	-	1.5	-	Janse et al. (2003) Neilan et al. (1997)

a, Genes targeted in the toxin cluster; b, the valuses from the references; c, poyketide synthase domain.