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HIP1 DNA fingerprinting in *Microcystis panniformis* (Chroococcales, Cyanobacteria)

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Microcystis panniformis is a planktonic freshwater cyanobacterium that has become increasingly prevalent in tropical water bodies in Brazil. Recently, it has been shown that the strain BCCUSP100 is able to produce microcystins (MCs, especially, MC-LR and [Asp3]-MC-LR), which are toxins that can affect the health of humans and other animals. This study explores genetic diversity among 14 strains of *M. panniformis* isolated from 4 freshwater reservoirs in 2 different Brazilian regions (north-east and south-east). Initially, the strains were morphologically characterized, followed by DNA fingerprint analyses using HIP1 sequences. Few morphological variations were observed and sequences of HIP1 were abundant in all strains studied. Strains isolated from water bodies in the south-east showed more evident genetic variation. Our findings also indicated genetic variation and the presence of different genotypes both between and within populations. The presence of *M. panniformis* in two distinct groups emphasizes the inconsistencies between morphological and molecular analyses as well as indicating the necessity of validating new classification approaches, with more discriminatory power, by using molecular data.

KEY WORDS: Cyanobacteria, HIP1, *Microcystis,* Polymorphism

INTRODUCTION

Cyanobacterial blooms of the genus *Microcystis* (Chroococcales, Cyanobacteria) are of serious ecological and public health concern due to their ability to dominate the planktonic environment and produce toxins. These toxins can affect aquatic and terrestrial organisms and humans. *Microcystis aeruginosa* (Kützing) Kützing, *M. ichthyoblabe* Kützing, *M. novacekii* (Komárek) Compère, *M. flos-aquae* (Wittrock) Kirchner ex Forti and *M. viridis* (A. Braun in Rabenhorst) Lemmermann are commonly reported species causing hepatotoxicity and odour problems in lakes and water supply systems (Carmichael 1996; Codd *et al.* 1999).

However, in tropical regions such as in Brazil, other potential microcystin-producing species of *Microcystis* are more commonly found. Bittencourt-Oliveira et al. (2005) have recently reported for the first time the presence of two microcystins (MCY-LR and [Asp^3]-MCY-LR) in *M. panniformis,* a common species from tropical America.

Microcystis panniformis Komárek *et al.* is a recently described species that is morphologically characterized by flattened irregular colonies generally constituting monospecific blooms in Brazilian eutrophicated reservoirs (Komárek *et al.* 2002).

Colonies forming morphologically different stages during their life cycle can be difficult to define and to establish taxonomic limits for classification (Bittencourt-Oliveira 2000; Komárek & Komarková 2002). In *Microcystis,* previous studies performed on the basis of only morphological data have been found to be inadequate due to large morphologic polymorphism shown by species (see Otsuka *et al.* 1999a, b, 2000; Kurmayer et al. 2003). A limitation of the classic phenotype-based taxonomy is the difficulty of recognizing inherited traits from the environmentally induced ones, and to separate genetic variability (among strains) from residual variability (variations observed among members of the same strain due to nongenetic factors) (López-Rodas *et al.* 2006; Rico *et al.* 2006).

In Brazilian *Microcystis* populations, it has been observed that a genotype could represent more than one morphotype or that distinct morphotypes could represent a single genotype (Bittencourt-Oliveira *et al.* 2001). This was corroborated by other studies in different localities (Kurmayer *et al.* 2003; Wilson *et al.* 2005). Analyses are necessary to investigate the diversity of other populations.

DNA fingerprinting methods have been successfully used to study genetic diversity of cyanobacterial strains, as well as populations from different geographical regions, cryolysates and cyanobionts. The following are the most commonly employed methods: short tandemly repeated repetitive (STRR) (Rouhiainen *et al.* 1995; Rasmussen & Svenning 1998; Wilson *et al.* 2000; Guevara *et al.* 2002; Chonudomkul et al. 2004), random amplification of polymorphic DNA (RAPD) (Nishihara *et al.* 1997; Casamatta *et al.* 2003; Chen *et al.* 2003; Prabina *et al.* 2005) and highly iterated palindrome 1 (HIP1)

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(Saker & Neilan 2001; Orcutt *et al.* 2002; Zheng *et al.* 2002; Neilan *et al.* 2003; Comte *et al.* 2004; Pomati *et al.* 2004; Wilson *et al.* 2005). HIP1 is an octameric palindrome sequence (5′-GCGATCGC-3′) abundant in coding regions of cyanobacterial genomes (Robinson *et al.* 1995).

The DNA fingerprinting method used here based on oligonucleotide primers for HIP1 enabled investigation of genetic diversity of 14 strains of *M. panniformis* isolated from south-eastern and north-eastern Brazilian reservoirs. These reservoirs are about 3100 km apart and exhibit quite different temperatures and rain regimes. Moreover, four strains of *M. aeruginosa,* plus one without identification at the species level, were also analyzed for comparative purposes.

This is the first study to analyze the genetic diversity of *M. panniformis,* which is frequently found in Brazilian reservoirs. Considering the increase of blooms in tropical reservoirs, including those of the new, potentially toxin-producing species of *M. panniformis,* this study clarifies the genetic diversity of populations occurring in several water bodies located in different geographical regions in Brazil.

MATERIAL AND METHODS

Study area and field sampling

Water samples from reservoirs in São Paulo State, in south-eastern Brazil, were collected from (1) Barra Bonita Reservoir (22°32′34.5″S, 48°29′26.4″W; area 308 km^2, water volume 5.66×10^8 m^3), which has been used for recreation and to run a hydroelectric plant; (2) Salto Grande Reservoir (22°44′21″S, 47°19′53″W; area 11.5 km^2, water volume 1.06×10^8 m^3) and (3) Garças Lagoon (23°39′S, 46°37′W; area 5.5 km^2). *Microcystis* and, more recently, *Cylindrospermopsis* blooms have often occurred in these reservoirs (Dos Santos & Calijuri 1998; Calijuri *et al.* 1999).

Water samples from Pernambuco State in north-eastern Brazil were collected from Jucazinho Reservoir (07°58′02″S, 35°44′33″W; area 18 km^2, water volume 3.27×10^8 m^3), which has been used as a drinking water supply and for irrigation. Only *Cylindrospermopsis* blooms (> 20,000 cells/ml^{-1}) have been recorded in this water body (Secretaria de Recursos Hídricos de Pernambuco 2000). All samples were collected using Van Dorn bottles or a 25-μm plankton net. Environmental samples of naturally occurring populations were preserved with 4% formaldehyde.

Morphology

Before cultivation, field colonies of *Microcystis* spp. were identified according to morphological criteria (colony shape, cell diameter and extent of mucilage) following the description by Komárek & Anagnostidis (1998), Komárek & Komarková (2002) and Komárek *et al.* (2002). Field colonies and young cultures were photographed with a microscope (Nikon E200, Melville, NY, USA) equipped with a video camera system (Samsung SCC833) using the Imagelab software (Softium, Brazil).

Colonies were identified at the beginning of the isolation procedure to avoid misidentification due to morphological variations in culture. One drop of diluted nankin ink was used on slide preparations to visualise the extent of mucilage.

Measurements of cell diameters (n = 50) of each strain of *Microcystis* spp. in culture were randomly performed during the logarithmic phase of growth. Average cell diameter and standard deviation were calculated.

Strains and growth conditions

Seventeen clonal and nonaxenic strains of *Microcystis* spp. were used in this study (Table 1). One individual colony was removed by micromanipulation techniques with Pasteur pipettes at magnifications of ×100–400. Each isolated colony was washed by transferring it through several consecutive drops of water until all other microorganisms were removed, and subsequently transferred to glass tubes containing 10 ml of BG-11 medium (Rippka *et al.* 1979). After a few weeks of growth an inoculum was transferred to Petri dishes containing bacteriological agar at 1% (w/v) and BG-11 medium, and then after two weeks, the inoculum was again transferred to tubes containing liquid medium. The cultures were examined microscopically to ensure that there were no contaminating organisms. All strains were maintained in incubators at 21°C ± 1°C and 30 ± 5 μmol m^{-2} s^{-1} (photometer Li-Cor mod. 250), under a 14 : 10 hour light : dark photoperiod at the Brazilian Cyanobacteria Collection of the University of São Paulo, Brazil – BCCUSP.

DNA extraction

Total genomic DNA was prepared using the commercial kit Genome DNA (BIO 101, Vista, CA, USA). DNA samples were purified with a polymerase chain reaction (PCR) purification kit (QIAquick, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA concentrations were estimated directly from ethidium bromide fluorescence in agarose gel images against standard quantities of DNA (Low DNA mass, Invitrogen, Carlsbad, CA, USA).

PCR amplification

Amplifications were carried out in 25-μl volumes in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). All primers were synthesized by IDT (Medley, FL, USA). PCRs were performed using extended HIP1 primers: HIP1-TG (5′-GCGATCGCTG-3′), HIP1-GC (5′-GCGATCGCGC-3′), HIP1-AT (5′-GCGATCGCAT-3) and HIP1-CA (5′-GCGATCGCCA-3′) as described by Smith *et al.* (1998), containing 2 μl of extracted DNA (approximately 1.0 ng of DNA), 5 μM of each primer, 2.5 U of *Taq* polymerase (Amersham Pharmacia Biotech, Piscataway, NJ) with buffer containing 1.5 mM $MgCl_2$ and 200 μM of each dNTP (Boehringer-Mannheim, Mannheim, Germany). The following cycling parameter conditions were employed: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s; 36°C for 1 min; 72°C for 2 min; followed by a final extension at 72°C for 5 min. Amplification products were visualized by electrophoresis on 1.5% agarose gels added with ethidium bromide (0.2 μg · ml^{-1}) and run for 4 h in 1× TBE running buffer (pH 8.0, 89 mM Tris, 89 mM boric acid and 2 mM EDTA) at a constant voltage of 7.5 V · cm^{-1}. Agarose gels were recorded using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290) (Kodak, Melville, NY, USA) associated with Kodak 1D Image Analysis Soft-

Table 1. Cell dimensions of Brazilian strains of morphospecies of *Microcystis* used in this study.

Morphospecies	Strain[1]	Sample location[2]	Sample date	Bloom	Season[3]	Cell diameter		
						Mean (μm)	*s*	Min–Max (μm)
South-east Brazil (22°32′34.5″–22°44′21″S, 46°37′–48°29′26.4″W)								
M. aeruginosa	BCCUSP3	GA	Dec. 1996	Yes	Rainy	4.14	0.314	4.0–5.9
Microcystis sp.	BCCUSP16	BB	Nov. 1999	Yes	Rainy	4.58	0.347	3.75–5.0
M. aeruginosa	BCCUSP30	GA	Nov. 1996	Yes	Rainy	2.92	0.370	2.5–4.0
M. panniformis	BCCUSP97	BB	Apr. 2000	Yes	Drought	4.10	0.790	3.0–5.25
M. panniformis	BCCUSP98	BB	Apr. 2000	Yes	Drought	2.66	0.306	2.5–3.75
M. panniformis	BCCUSP99	BB	Apr. 2000	Yes	Drought	3.14	0.648	2.5–5.0
M. panniformis	BCCUSP100	BB	Apr. 2000	Yes	Drought	4.34	0.600	3.5–5.8
M. aeruginosa	BCCUSP158	GA	Dec. 1996	Yes	Rainy	4.34	0.646	3.9–5.9
M. panniformis	BCCUSP200	SG	Aug. 1996	Yes	Drought	3.64	0.490	3.0–4.9
M. aeruginosa	BCCUSP310	GA	Apr. 1997	Yes	Drought	3.14	0.388	2.4–4.0
North-east Brazil (07°58′02″S, 35°44′33″W)								
M. panniformis	BCCUSP22	JU	Jan. 2001	Not	Drought	4.06	0.500	3.0–5.0
M. panniformis	BCCUSP23	JU	Jan. 2001	Not	Drought	4.32	0.592	3.0–5.0
M. panniformis	BCCUSP25	JU	Feb. 2001	Not	Drought	4.02	0.458	3.2–5.0
M. panniformis	BCCUSP26	JU	Feb. 2001	Not	Drought	4.42	0.540	3.0–5.1
M. aeruginosa	BCCUSP28	JU	Jun. 2001	Not	Rainy	4.72	0.429	3.75–6.25
M. panniformis	BCCUSP29	JU	Jun. 2001	Not	Rainy	4.40	0.493	3.8–5.2
M. panniformis	BCCUSP40	JU	Nov. 2001	Not	Drought	4.08	0.410	3.5–5.0

[1] BCCUSP: Brazilian Cyanobacteria Collection of the University of São Paulo.
[2] GA, Garças Reservoir, São Paulo, SP; BB, Barra Bonita Reservoir, Barra Bonita, SP; SG, Salto Grande Reservoir, Americana, SP; JU, Jucazinho Reservoir, Surubim, PE.
[3] The south-east Brazil drought and rainy seasons are characterized by low (16–22°C) and high (22–29°C) temperatures, respectively. In north-east Brazil, temperatures are high in both seasons.

ware. All reactions were repeated at least three times to confirm the same results (data not shown).

Molecular analysis

HIP1-amplified fragments obtained from four primers were assembled and converted to binary codes based on the presence (1) and absence (0) of data. Doubtful bands were disregarded and, in this way, the equivalent remaining bands from other strains with the same molecular weight were also discarded. The similarity matrix was calculated by the Jaccard coefficient (Jaccard 1901) using NTSYS (NTSYS-PC 2002) software version 2.1 and the unweighted pair group method with arithmetic mean (UPGMA) algorithm (Sneath & Sokal 1973) for phenogram construction.

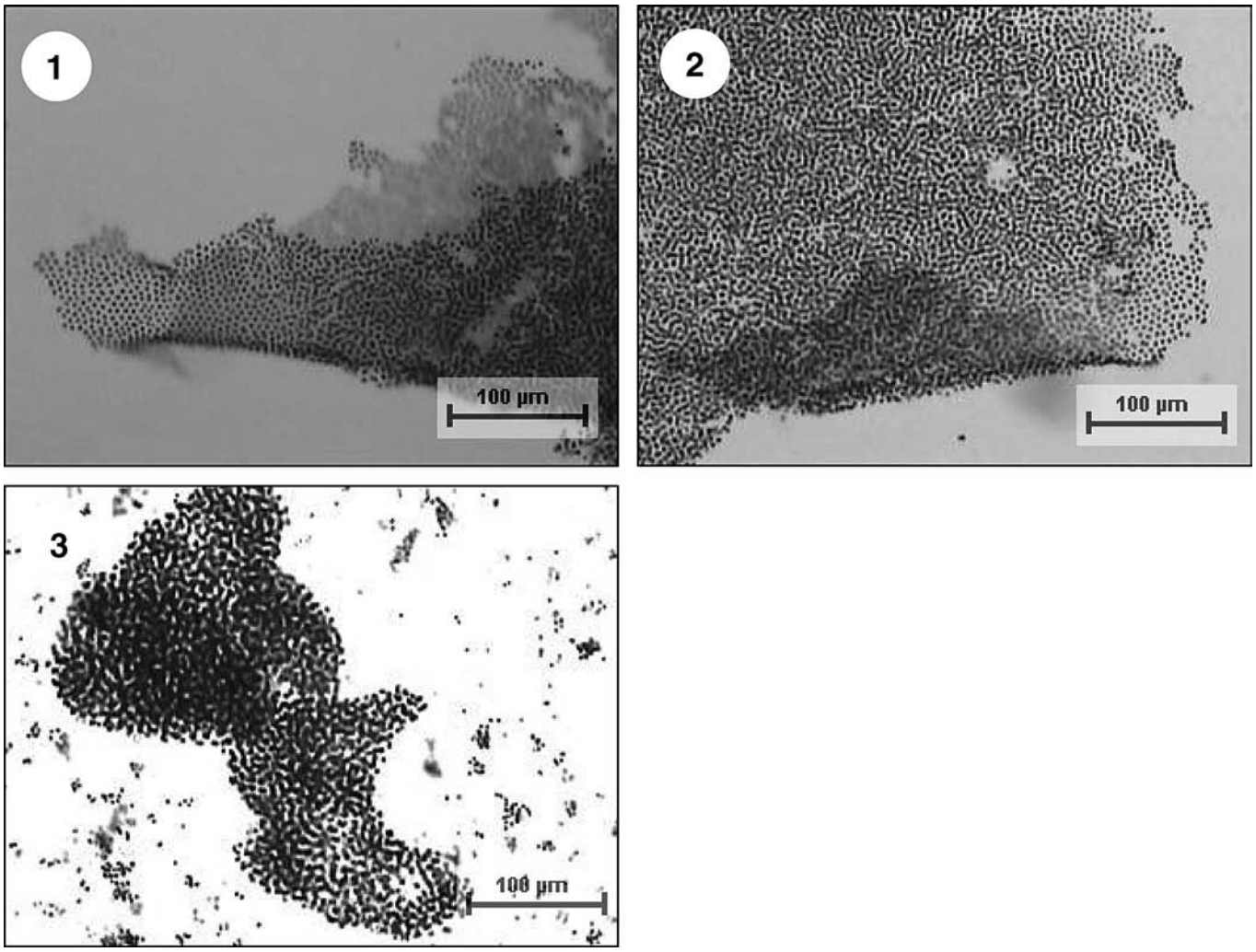
RESULTS

Microcystis panniformis strains from south-eastern Brazil displayed colonies with cells homogeneously distributed at the periphery, sometimes in a single layer, inside elongate, ribbon-like and flat colonies; mature colonies had diffluent and narrow mucilage, and mean cell diameters varied from 2.26 μm (BCCUSP98) to 4.34 μm (BCCUSP100). Colonies collected in north-eastern Brazil were predominantly longer and also flattened with cells closely arranged and homogeneously distributed; mature colonies had diffluent and narrow mucilage and cells had mean diameters from 4.02 μm (BCCUSP25) to 4.72 μm (BCCUSP28) (Table 1, Figs 1–3). Colonies isolated and cultured kept the same morphology as those found in environmental samples for up to 2–3 months. Subsequently, colonial morphology was lost due to complete mucilage dissolution, which released single cells. Mean, standard deviation, minimum and maximum values of cell diameters are presented in Table 1.

Brazilian *M. panniformis* strains exhibited high genetic diversity when using HIP1 primers with similarity values of 0.425 to 1.000. However, south-eastern strains displayed genetic diversity greater than those from the north-eastern region (Fig. 4). *Microcystis panniformis* strains isolated from south-east and north-east water bodies showed similarity values ranging from 0.425 to 0.806 and 0.513 to 1.000, respectively.

Analysis of strain similarity from banding patterns obtained by gel electrophoresis with primers HIP (Figs 5–8) allowed the delineation of three clusters of strains of Brazilian *Microcystis* spp. (Fig. 4). Strain *M. aeruginosa* BCCUSP30 was not included in any clusters and exhibited low values of similarity, 0.293–0.545 when compared with other strains. Cluster I consisted of two strains of *M. aeruginosa* (BCCUSP3 and BCCUSP158) and *Microcystis* sp. (BCCUSP16) isolated during the season characterized by rainfall and high temperatures (22–29°C) in south-eastern Brazil. Cluster II contained *M. panniformis* strains from south-eastern Brazil (BCCUSP97, BCCUSP98 and BCCUSP99) collected during periods of lower rainfall and temperatures, and from north-eastern Brazil at high temperatures in both drought (BCCUSP40) and rainy seasons (BCCUSP28) and one strain of *M. aeruginosa* (BCCUSP310).

Cluster III included only strains of *M. panniformis* from the north-east collected during both high and low rainfall seasons, and two from the south-east during drought seasons (Table 1). Although they belong to cluster III, BCCUSP100 and BCCUSP200 showed low similarity values, 0.513 to 0.556 and 0.658 to 0.703, respectively, relative to other strains of this group. All clusters had low internal similarity (I = 0.45, II = 0.49 and III = 0.55) (Fig. 4).



Figs 1–3. The morphospecies of Brazilian *M. panniformis* Komárek *et al.* from the Jucazinho Reservoir, PE, north-eastern Brazil.
Figs 1–2. Flat colonies with cells arranged in a single plane in environmental samples. Scale bar = 100 µm.
Fig. 3. Colony of isolated strain after one week in culture. Scale bar = 100 µm.

DISCUSSION

In a previous study, Bittencourt-Oliveira *et al.* (2001) observed that strain FCLA200 (currently designated BCCUSP200) was morphologically similar to the genus *Pannus* Hindák. The authors determined that it belonged to the '*M. aeruginosa* complex'. Komárek *et al.* (2002) described a new species, *M. panniformis,* using the same field sample (Salto Grande Reservoir, Americana, São Paulo State, August 1996). This description agreed with the data reported by Bittencourt-Oliveira *et al.* (2001) for FCLA200 (BCCUSP200). Therefore, in this study, this strain was considered as belonging to the morphospecies *M. panniformis.*

There were few morphological variations among investigated populations. Those from Barra Bonita (south-east) and Jucazinho (north-east) reservoirs showed elongated and flat colonies with densely arranged cells in agreement with the report by Komárek & Komarková (2002). All *M. panniformis* strains showed smaller cell diameters (Table 1) than those presented in the original description (3.9–6.4 µm diameter) and those in the population from Elphinstone Lake (4.5–6.0 µm diameter; White *et al.* 2003). This low variation in strains from distinct regions could be analogous to that shown by HIP1 alignment. When compared with natural populations, changes in banding patterns observed in cultured strains may be indicative of gene rearrangements (Robinson *et al.* 1995). Consequently, interpretation of results should be conducted carefully after use of simple algorithms such as UPGMA in fingerprinting techniques.

Genetic diversity in strains isolated from south-eastern water bodies with blooms was more pronounced than in north-eastern samples (Figs 5–8). Although there were many *Microcystis* colonies, no bloom was observed in the Jucazinho Reservoir. The presence of different genotypes within and among populations of bloom-forming *M. aeruginosa* has recently been found at several localities (Bittencourt-Oliveira 2003; Kurmayer & Kutzenberg 2003; Janse *et al.* 2004; Wilson *et al.* 2005). Similarly, *M. panniformis* presented substantial genetic intrapopulation variation, particularly in Barra Bonita (April 2000 – BCCUSP97, BCCUSP98, BCCUSP99 and BCCUSP100) and Jucazinho reservoirs (June 2001 –

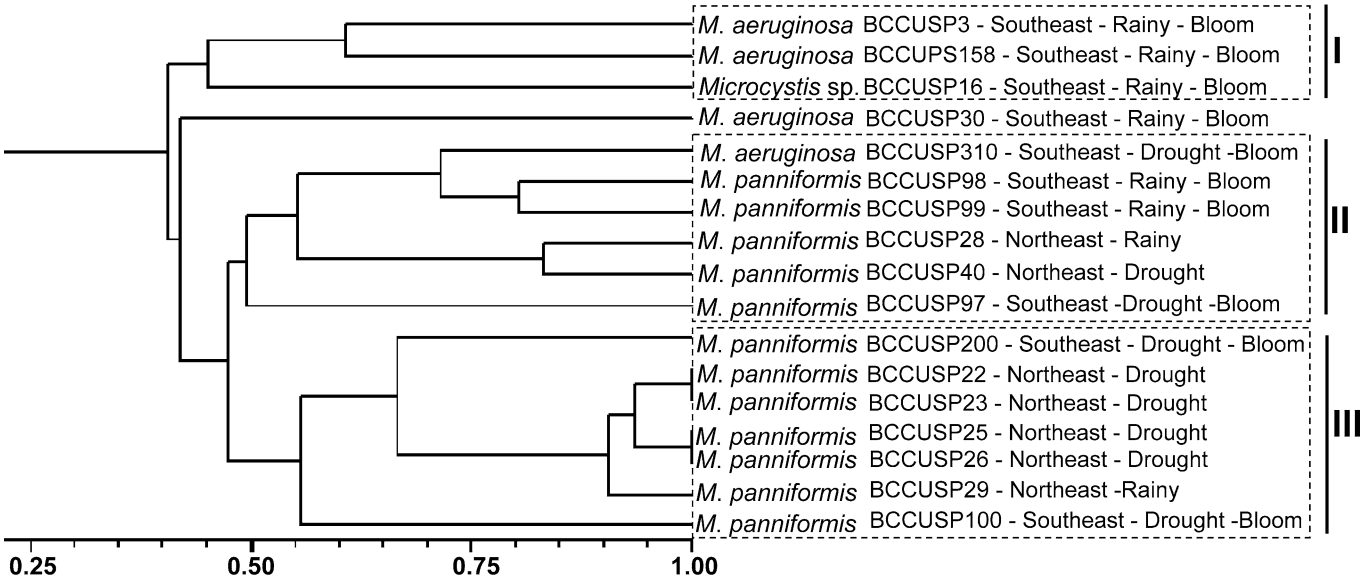
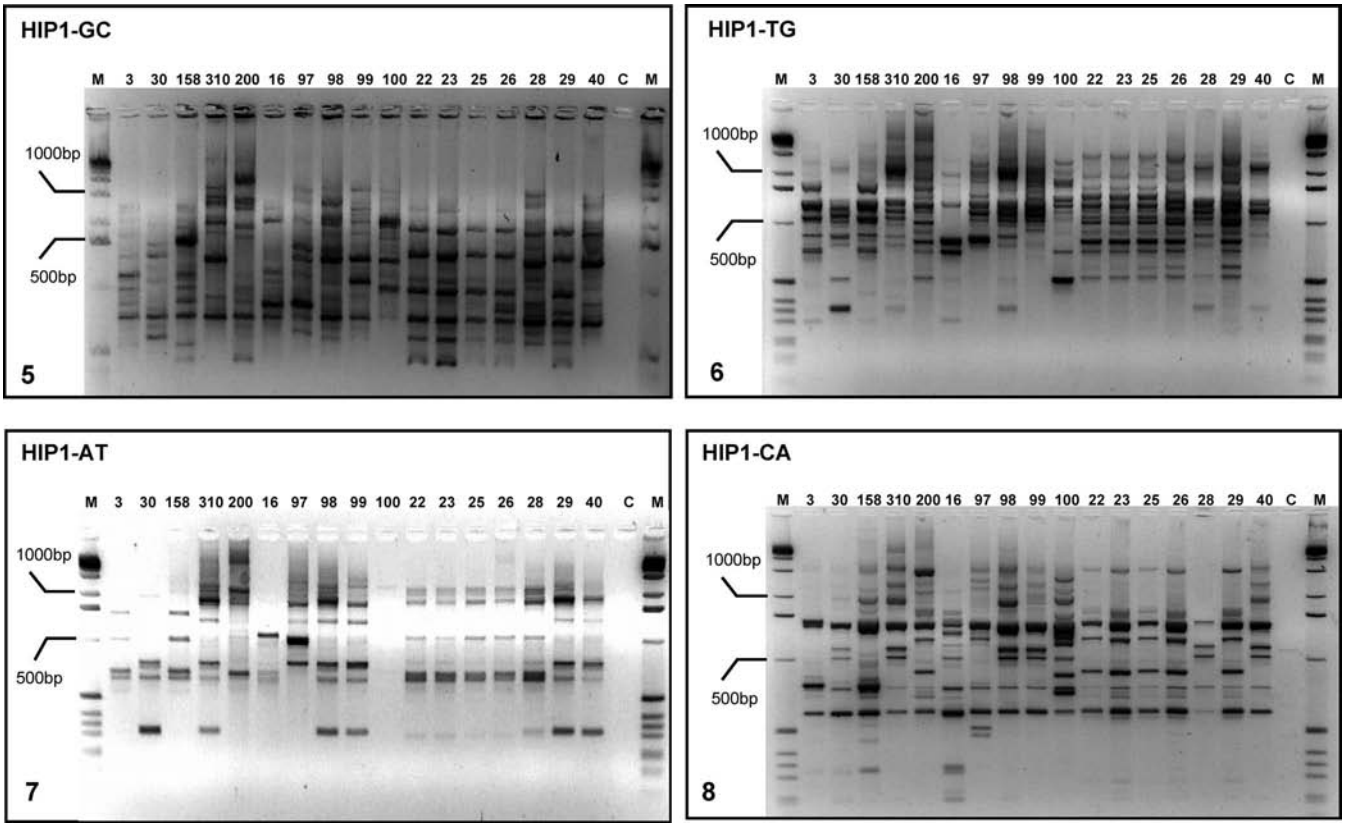


Fig. 4. Clustering dendrogram of *Microcystis* spp. strains by UPGMA analysis of similarity matrix data using 17 operational taxonomic units and the Jaccard coefficient. The numerical scale indicates the level of similarity at which clusters are formed, according to the Jaccard coefficient. I = 0.45, II = 0.49 and III = 0.55. r = 0.9357.



Figs 5–8. PCR fingerprint patterns of *M. panniformis*. Lanes M are DNA molecular mass standards (1 kb ladder). Each lane corresponds to one strain in the order: BCCUSP3, BCCUSP30, BCCUSP158, BCCUSP310, BCCUSP200, BCCUSP16, BCCUSP97, BCCUSP98, BCCUSP99, BCCUSP100, BCCUSP22, BCCUSP23, BCCUSP25, BCCUSP26, BCCUSP28, BCCUSP29, BCCUSP40. C: control without DNA.

Fig. 5. HIP1-GC.
Fig. 6. HIP1-TG.
Fig. 7. HIP1-AT.
Fig. 8. HIP1-CA.

BCCUSP29 and BCCUSP40) (Fig. 2). Genetic interpopulation variation also showed up (clusters II and III).

In environmental samples this genetic variation could be much higher than that found in strains in culture, because the isolation procedures and culture medium may select some genotypes (Whitton & Potts 2000; Welker *et al.* 2003; Wilson *et al.* 2005) resulting in an underestimation of actual genetic diversity present in field populations. This selection may contribute to isolation of nontoxic strains despite the presence of toxic ones, because only one microcystin-forming genotype (BCCUSP100) was found in the water bodies studied. Before this study, a mixture of genotypes had been found in field samples (Vezie *et al.* 1997; Bittencourt-Oliveira *et al.* 2001; Kurmayer & Kutzenberger 2003; Welker *et al.* 2003; Janse *et al.* 2004; Wilson *et al.* 2005).

Traditional taxonomy of *Microcystis* on the basis of morphologic criteria is complex and questionable. Earlier infraspecific studies of *Microcystis* concluded that morphology does not correlate with molecular data (Otsuka *et al.* 1999a, b, 2000, 2001; Bittencourt-Oliveira *et al.* 2001; Kurmayer *et al.* 2003; Mikalsen *et al.* 2003) because phenotypical variation was observed in samples from nature as well as in strains grown under cultivation. According to Rico *et al.* (2006), genetic interstrain differences are the important cause of the observed variability in cells, whereas residual variance (i.e. nongenetic factors) is the essential cause of the observed variability in colonies.

In this study, *Microcystis* spp. strains were not grouped according to either water bodies, distance between water bodies, seasons or cell dimensions. Although morphotypes were morphologically similar, the presence of *M. panniformis* strains in two distinct groupings (II and III) reinforces the inconsistencies between molecular and morphological approaches.

Also, these results reinforce the necessity of validating newly described cyanobacterial taxa according to molecular data, and not just on the basis of morphology. Because phenotypic variations are common in this genus (Otsuka *et al.* 2000), other DNA sequences might also be very important for confirming or modifying classification within the genus. HIP1 fingerprinting is a powerful tool for studying genetic diversity of cyanobacteria because it differentiates closely related strains. It can be used to choose strains for sequencing because the latter is still a difficult, time-consuming and expensive technique.

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