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Genetic Diversity of Brazilian Cyanobacteria Revealed by Phylogenetic Analysis

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1. Introduction

Cyanobacteria are prokaryotic microorganisms with a long evolutionary history. They are usually aquatic, perform oxygenic photosynthesis and have been responsible for initial rise of atmospheric O₂. Cyanobacteria are predominant in the phytoplankton of continental waters, reaching an ample diversity of shapes due to their morphological, biochemical and physiological adaptabilities acquired all along their evolutionary history. Some cyanobacteria, like the *Microcystis*, *Cylindrospermopsis*, *Anabaena*, *Aphanizomenon* and *Planktothrix*, could give rise to blooms with the liberation of a wide range of toxins.

Cyanobacteria constitute a monophyletic group within the Bacteria domain (Castenholz, 2001). However, the group taxonomy is traditionally based on morphological characters, according to botanic criteria (Anagnostidis & Komárek 1985, 1988, 1990; Komárek & Anagnostidis 1986, 1989, 1999, 2005), and added to ecological data.

The high morphological variability and the low number of phenotypic characters used in the cyanobacterial taxonomy leads to serious identification problems. Several studies using molecular techniques have questioned the use of morphological characteristics for identification at the species, as well as, at the genus level. These studies showed the importance of using molecular tools other than morphology to help their identification and phylogenetic studies (Bittencourt-Oliveira et al., 2007a, 2009b; Bolch et al., 1996, 1999; Margheri et al., 2003; Tomitani et al., 2006; Valério et al., 2009; Wood et al., 2002).

Several techniques based on DNA polymorphism detection have been developed and applied on cyanobacterial phylogenetic studies as, RFLP (*Restriction Fragment Length Polymorphism*) (Bittencourt-Oliveira et al., 2009a; Bolch et al., 1996; Iteman et al., 2002; Lu et al., 1997; Lyra et al., 2001), RAPD (*Random Amplified Polymorphic DNA*) (Casamatta et al., 2003; Nishihara et al., 1997; Prabina et al., 2005; Shalini et al., 2007), STRR (*Short Tandemly Repeated Repetitive*) (Chonudomkul et al., 2004; Valério et al., 2009; Wilson et al., 2000) and HIP1 (*Highly Iterated Palindrome*) sequences (Bittencourt-Oliveira et al., 2007a, 2007b; Neilan et al., 2003; Orcutt et al., 2002; Pomati et al., 2004; Wilson et al., 2005; Zheng et al., 2002).

However, the most important breakthrough in phylogenetical studies took place with the advent of direct sequencing of molecular markers. For cyanobacteria, the phycocyanin operon are among the most used molecular markers. The c-phycocyanin genes (*cpcB* and

cpcA) and the intervening intergenic spacer (IGS) show variations in their sequences which are capable of differentiating genotypes below the generic level. Besides, they are relatively large-sized in comparison with other genes encoding for photosynthetic pigments (~700-800bp), belonging to all cyanobacteria and they are almost totally restricted to this group of organism when in freshwater ecosystems (Barker et al., 2000; Bittencourt-Oliveira et al., 2001, 2009b; Bolch et al., 1996; Dyble et al., 2002; Haverkamp et al., 2008, 2009; Neilan et al., 1995; Tan et al., 2010; Wu et al., 2010).

Among cyanobacteria, several taxa need to be studied in order to clarify their taxonomic position as the *Geitlerinema amphibium* (Agardh ex Gomont) Anagnostidis, *G. unigranulatum* (R.N. Singh) Komárek & Azevedo and *Microcystis panniformis* Komárek et al.

Species of *Geitlerinema* are cosmopolitan and frequently found in different kinds of habitats, as well as in freshwater or marine ecosystems (Kirkwood & Henley, 2006; Margheri et al., 2003; Rippka et al., 1979, Romo et al., 1993; Silva et al., 1996), and they can also form blooms in reservoirs (Torgan & Paula, 1994).

Geitlerinema amphibium and *G. unigranulatum* are morphologically similar to each other. According to Romo et al. (1993) and Komárek & Anagnostidis (2005), they could be differentiated only by their dimensions and the number of cyanophycin granules close to the cross-walls. However, Bittencourt-Oliveira et al. (2009b) used transmission electronic and optical microscopy to study strains of these two morphospecies showing that there is a large overlap between them, in both cell dimensions and the number of granules per cell. Therefore, the authors concluded that it was not possible to distinguish *G. amphibium* from *G. unigranulatum* by means of morphological data.

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. *M. 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 odor problems in lakes and water supply systems (Carmichael, 1996; Codd et al., 1999). However, in tropical regions such as in Brazil, *M. panniformis* is also a potential microcystin-producing morphospecies. It is morphologically characterized by flattened irregular colonies and it is closely related to *M. aeruginosa* morphospecies (Bittencourt-Oliveira et al., 2005).

In the same way that happens to other species of the genus, *M. panniformis* colonies can have morphologically different stages during their life cycle, which make difficult to define and to establish taxonomic limits for its identification (Bittencourt-Oliveira, 2000; Otsuka et al., 2000, 2001). Furthermore, it has been observed that in *Microcystis* populations a genotype could represent more than one morphotype, or that distinct morphotypes could represent a single genotype (Bittencourt-Oliveira et al., 2001; Hannde et al., 2007).

HIP1 sequences is a powerful tool to study genetic diversity of cyanobacteria strains or closely related taxa, and it was used by Bittencourt-Oliveira et al. (2007a, 2007b) to investigate *M. panniformis*, *G. amphibium* and *G. unigranulatum*. However, it is recommended that studies on molecular phylogeny do confirm, by the use of DNA sequences, previous findings which have used fingerprinting techniques like HIP1.

The goal of this study was the investigation of *Geitlerinema amphibium*, *G. unigranulatum* and *Microcystis panniformis* taxonomic position, using the phycocyanin gene partial sequencing for the build up of phylogenetic trees.

2. Material and methods

2.1 Field sampling, isolation and growth conditions

Sequences from 14 clonal and non-axenic strains of *Geitlerinema* and 17 of *Microcystis* from the Brazilian Cyanobacteria Collection of the University of São Paulo (BCCUSP; previously named FCLA), were used in this study (Figure 1, Table 1). These strains were isolated from aquatic habitats situated in localities of Brazil. One strain of *Geitlerinema amphibium* (BCCUSP31) was donated by Dr. Romo from University of Valencia, Spain. For isolation purpose one individual colony or thricome was removed by micromanipulation techniques with Pasteur pipettes at magnifications of 100 – 400X. Each isolate was washed, by transferring it through several consecutive drops of water until all other microorganisms be removed, and subsequently transferred to glass tubes containing 10 ml of BG-11 medium (Rippka et al., 1979). All strains were maintained in incubators at $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $30 \pm 5 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (photometer Li-Cor mod. 250), under a 14:10 hour light:dark photoperiod. The cultures are maintained at the Brazilian Cyanobacteria Collection of the University of São Paulo, Brazil (BCCUSP). The Spanish strain (BCCUSP 31) was acclimatized for three months in the BCCUSP at the same conditions as for those before the beginning of the experiment.

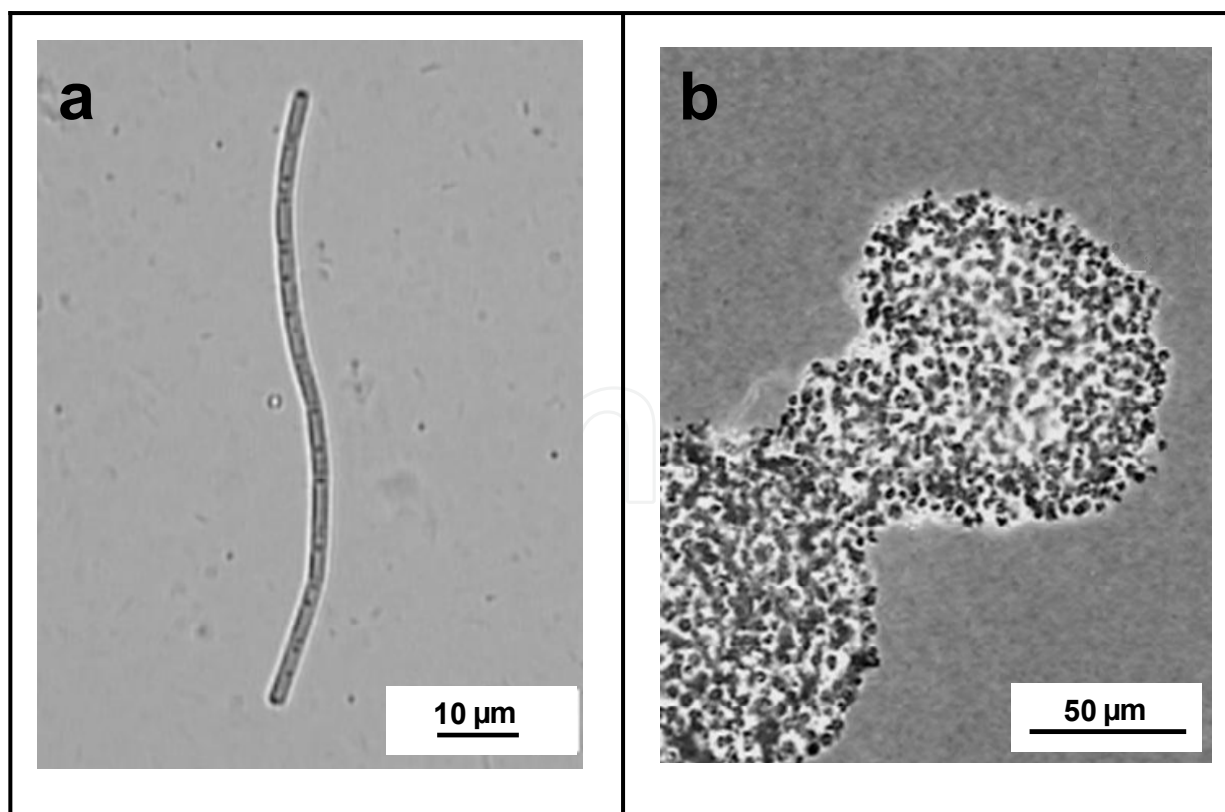


Fig. 1. The morphospecies a) *G. amphibium* BCCUSP85 and b) *M. panniformis* from Jucazinho reservoir, Northeast Brazil.

Morphospecies	Code	Origin site	cpcBA-IGS (bp)			GenBank Access Number
			cpcA	IGS	cpcB	
<i>G. amphibium</i> ^a	BCCUSP31	SP	143	293	198	FJ545649
<i>G. amphibium</i> ^a	BCCUSP79	BR	143	293	198	FJ545644
<i>G. amphibium</i> ^b	BCCUSP80	BR	143	86	198	JN578762
<i>G. amphibium</i> ^b	BCCUSP84	BR	143	296	198	JN578760
<i>G. amphibium</i> ^a	BCCUSP85	BR	143	291	198	FJ545646
<i>G. amphibium</i> ^b	BCCUSP86	BR	143	294	198	JN578759
<i>G. amphibium</i> ^a	BCCUSP87	BR	143	293	198	FJ392653
<i>G. amphibium</i> ^a	BCCUSP91	BR	143	292	198	FJ545650
<i>G. unigranulatum</i> ^a	BCCUSP94	BR	143	83	198	FJ545642
<i>G. amphibium</i> ^b	BCCUSP338	BR	143	295	198	JN578761
<i>G. amphibium</i> ^a	BCCUSP54	BR	143	293	198	FJ545645
<i>G. amphibium</i> ^c	SC2	VT	143	296	169	EF680778
<i>G. unigranulatum</i> ^a	BCCUSP350	BR	143	292	198	FJ545647
<i>G. unigranulatum</i> ^a	BCCUSP352	BR	143	83	198	FJ545643
<i>G. unigranulatum</i> ^a	BCCUSP96	BR	143	292	198	FJ545648
" <i>Oscillatoria</i> sp." ^c	CYA479	UG	143	296	169	AM048624
" <i>Oscillatoria</i> sp." ^c	CYA469	UG	143	296	169	AM048623
" <i>Planktothrix</i> sp." ^d	FP1	IT	143	298	169	AF212923
<i>M. aeruginosa</i> ^e	BCCUSP09	BR	200	69	277	AF385375
<i>M. aeruginosa</i> ^e	BCCUSP30	BR	200	69	277	AF385376
<i>M. panniformis</i> ^e	BCCUSP200	BR	200	69	277	AF385374
<i>M. aeruginosa</i> ^e	BCCUSP232	BR	200	69	277	AF385372
<i>M. aeruginosa</i> ^e	BCCUSP262	BR	200	69	277	AF385369
<i>M. aeruginosa</i> ^e	BCCUSP298	BR	200	69	277	AF385370
<i>M. aeruginosa</i> ^e	BCCUSP299	BR	200	69	277	AF385371
<i>M. aeruginosa</i> ^e	BCCUSP03	BR	200	69	277	AF385380
<i>M. aeruginosa</i> ^e	BCCUSP158	BR	200	69	277	AF385381
<i>M. panniformis</i> ^b	BCCUSP100	BR	200	69	277	JN578763
<i>M. aeruginosa</i> ^e	BCCUSP310	BR	200	69	277	AF385378
<i>M. panniformis</i> ^b	BCCUSP28	BR	200	69	277	JN578764
<i>M. panniformis</i> ^b	BCCUSP22	BR	200	69	277	JN578765
<i>M. panniformis</i> ^b	BCCUSP25	BR	200	69	277	JN578766
<i>M. panniformis</i> ^b	BCCUSP26	BR	200	69	277	JN578767
<i>M. panniformis</i> ^b	BCCUSP23	BR	200	69	277	JN578768
<i>M. panniformis</i> ^b	BCCUSP29	BR	200	69	277	JN578769
<i>M. aeruginosa</i> ^e	NIES-98	JP	200	69	277	AF385384
<i>M. aeruginosa</i> ^e	PCC7820	SC	200	69	277	AF385387
<i>M. aeruginosa</i> ^f	FACHB 978	CH	200	69	277	AY568682
<i>M. aeruginosa</i> ^f	UTEX 1939	UN	200	69	277	AY568698
<i>M. aeruginosa</i> ^e	PCC 7806	NE	200	69	277	AF385386
<i>M. aeruginosa</i> ^g	UWOCC 017	AS	200	69	277	AF195170

Tab. 1. Morphospecies, code and origin site of the used cyanobacteria strains and the GenBank access number of the sequences present in this study. *cpcBA*-IGS. c-phycocyanin genes *cpcB*, *cpcA* and the intervening intergenic spacer (IGS) (bp). BCCUSP - Brazilian Cyanobacteria Collection of University of São Paulo. SP Spain; BR Brazil; UG Uganda; IT Italy; JP Japan; SC Scotland; CH China; UN Unknown; NE Netherlands; SA South Africa. VT Vietnam; ^a Bittencourt-Oliveira et al (2009b); ^b This work; ^c Unpublished; ^d Pomati et al. (2000); ^e Bittencourt-Oliveira et al. (2001); ^f Wu et al. (2007); ^g Tillett et al. (2001).

Another ten sequences selected from the GenBank were also included in the analysis.

2.2 DNA extraction

DNA was extracted from fresh cells harvested at the exponential phase. Total genomic DNA was extracted using the commercial kit Gnome DNA (BIO 101, Vista, CA, USA) according to the manufacturer's instructions or according to the procedures described in Bittencourt-Oliveira et al. (2010).

2.3 PCR amplification

Amplifications were carried out in the thermocycler GeneAmp 2400 or GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction were performed using 2.5 to 10 ng of DNA, 20 μ M of each oligonucleotide primer in a total volume of 25 μ L and 2.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ) with buffer containing 1.5 mM $MgCl_2$ and 200 μ M of each dNTP (Boehringer-Mannheim, Mannheim, Germany).

Amplifications for the intergenic spacer and flanking regions from *cpcBA*-phycocyanin operon were accomplished with the primers PC β -F and PC α -R described by Neilan et al. (1995) using the same cycling parameters conditions according to Bolch et al. (1996). Control reactions were carried out by using the same reaction conditions and primer without DNA, and no PCR products were detected on agarose electrophoresis. All PCR reactions were repeated at least five times.

Amplification products were visualized by electrophoresis on 0,7% agarose gels stained with ethidium bromide. PCR products were purified using the Purelink Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. When necessary, bands were extracted and purified from the gel using the QIAquick kit (Qiagen, Hilden, Germany) as recommended by the manufacturer.

2.4 Sequencing and phylogenetic analyses

The amplified fragments were directly sequenced using the forward and reverse primers with ABI Prism® Big Dye® Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, CA, USA) and 3100 ABI sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. To avoid errors from PCR at least four separated amplification reactions were pooled for sequencing. The sequencing was repeated on independent PCR products. The PCR products were sequenced on both strands at least three times.

Automated base calls for both strands were checked by manual inspection and ambiguous calls and conflicts resolved by alignment and comparison using BioEdit program (Hall, 1999) to establish a consensus sequence for each strain. Consensus sequences were aligned using ClustalW in BioEdit program (Hall, 1999) and were manually inspected. *Spirulina subsalsa* PD2002/gca (accession number AY575949) and *Cyanothece* sp. ATCC51142 (accession number CP000806) were used as outgroups for *Geitlerinema* and *Microcystis* analysis respectively.

Evolution distances between the sequences were calculated by *P distance* in the program MEGA 4.0.2 (Tamura et al., 2007).

Phylogenetic analyses were performed with MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). An appropriate evolution model was selected using MrModeltest 2.2 (Nylander, 2004) under the Akaike Information Criterion. For the Bayesian analysis two runs of four Markov chains over 5,000,000 generations sampling every 100 generations was employed. The initial 2,500 generations were discarded as burn-in. For all analyses, posterior probability values were considered low up to 70%, moderate from 71% to 90%, and high above 90%.

3. Results and discussion

3.1 *Geitlerinema amphibium*

The phylogenetic tree including *Geitlerinema* species showed that *Geitlerinema amphibium* and *G. unigranulatum* are not genetically separated from each other (Figure 2).

The phylogenetic tree showed two Clades (I and II) (Figure 2). The Clade I encompasses three strains, one of *G. amphibium* separated from the other two of *G. unigranulatum*. These strains showed the region corresponding to the IGS with 83bp, shorter than the remaining sequences which contained up to 298bp. The evolutionary distance calculated by *P distance* between the strains from Clade I and II was up to 0.098, while among strains included on Clade II it did not exceed 0.056. This is a demonstration that the strains included in Clade I were genetically diverse from the others.

Bittencourt-Oliveira et al. (2009b) had shown that strains BCCUSP352 and BCCUSP94, belonging to Clade I, did not exhibit differences regarding cellular morphology and ultrastructure which would allow distinguish them from the other strains of *Geitlerinema*. The inclusion of *G. amphibium* BCCUSP80 in the present study reinforced the argument that these taxons are distinct species, maybe even of distinct genera because of the IGS size difference taxa.

The Clade II encompassed all the remaining strains and could be subdivided into two smaller groups (A and B). The group A included only strains of the morphospecies *G. amphibium*, while the group B was equally constituted by strains from *G. amphibium* or from *G. unigranulatum*, besides two strains of "*Oscillatoria* sp." (AM048623 and AM048624) identified as *Geitlerinema* sp. in Bittencourt-Oliveira et al. (2009b). The sequence "*Planktothrix* sp." (AF212923), also identified as *Geitlerinema* sp. (we refer the reader to Bittencourt-Oliveira et al. 2009b for details), appears basal to the other sequences included in Clade II. By the inclusion of four new sequences from strains of the morphospecies *G. amphibium* alterations in the tree topology did not show up, in agreement therefore with the findings of Bittencourt-Oliveira et al. (2009b).

G. amphibium and *G. unigranulatum* show overlapping morphological characteristics which make difficult the taxonomic discrimination (in the identifying sense). According to Komárek & Anagnostidis (2005), the variation interval of the *G. amphibium* cellular width ranges from (1) 1.8 to 3 (3.5-4) μm , whereas for *G. unigranulatum*, it ranges from 0.8 to 2.4 μm . The means of the studied populations by Bittencourt-Oliveira et al. (2009b) formed two distinct clusters, although the maximum values for the smaller species overlapped with the minimum values for the larger one, thus leaving no hiatus in cell width between the two taxa.

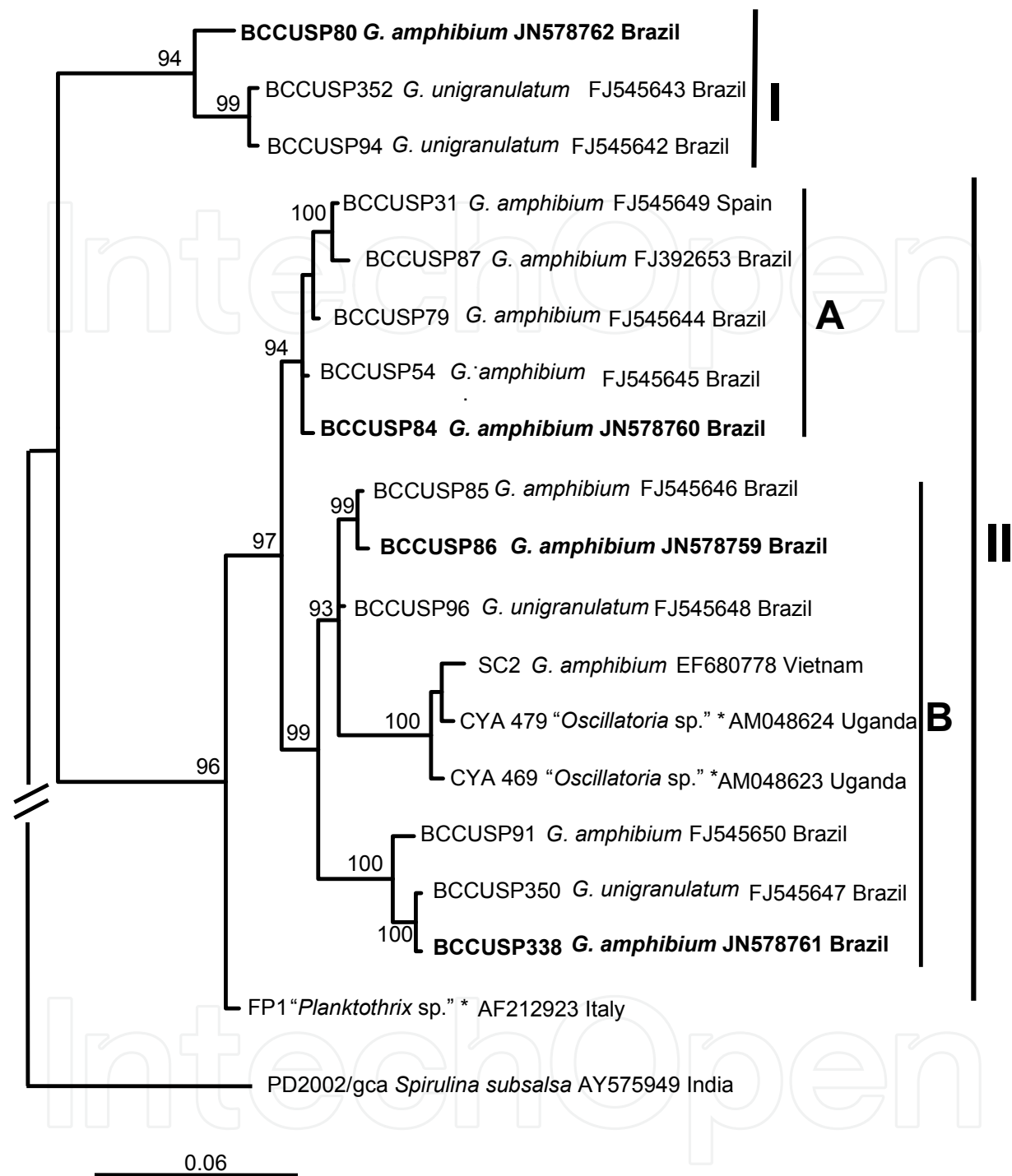


Fig. 2. Bayesian phylogenetic tree with *Geitlerinema* strains. The tree was generated using intergenic spacer and flanking regions from *cpcBA*-phycocyanin operon. For the Bayesian analysis two runs of four Markov chains over 5,000,000 generations sampling every 100 generations was employed. The initial 2,500 generations were discarded as burn-in. Posterior probability (x100) is shown on each branch when higher than 70. *Spirulina subsalsa* PD2002/gca (AY575949) used as outgroup. The bar represents 0.06 substitutions. Strains in boldface were sequenced in this work. * Strains identified as *Geitlerinema* sp. in Bittencourt-Oliveira et al. (2009b).

According to the data taken by Bittencourt-Oliveira et al. (2009b), the measurements of cell lengths in strains attributable to *G. amphibium* and *G. unigranulatum* show complete overlap of maximum, minimum, and mean values. Accordingly, only when the length by width ratio (L:W) is taken into account the distinction is more accentuated.

One or two cyanophycin granules per cell (less frequently three) were positioned near the cross walls in strains attributed to both species (Figure 3). Therefore, based on cellular morphology, only through the length to width ratio was it possible to differentiate *G. amphibium* from *G. unigranulatum*. Given their quite uniform morphology and the occurrence of these taxa in the same habitat, it would be nearly impossible to distinguish them in nature. The localization and number of granules, as well as ultrastructural data, did not aid in species discrimination either. Therefore, those characteristics could not be used as diacritical features. The obtained results by Bittencourt-Oliveira et al. (2009b) did not show ultrastructural differences between *G. amphibium* and *G. unigranulatum* strains, except for the BCCUSP96 which in some trichomes exhibited slightly thickening of the apical cell, slightly folded cellular wall, thylakoids with invaginations and unidentified granules. BCCUSP96 strain was unique in having four granules per cell in some trichomes and the highest cell length-to-width ratio (more details, see Bittencourt-Oliveira et al. 2009b).

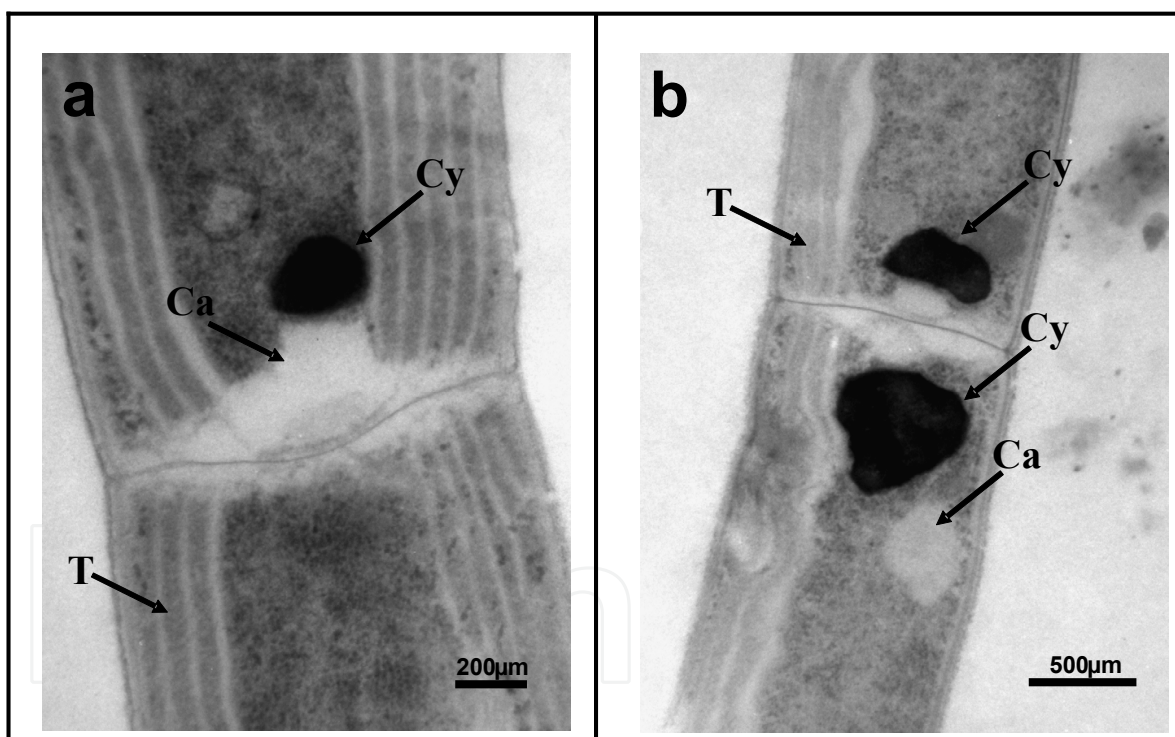


Fig. 3. Brazilian strains of *Geitlerinema* in longitudinal section of trichomes. a) *G. amphibium* BCCUSP91 and b) *G. unigranulatum* BCCUSP350, showing one and two cyanophycin granules (Cy) near the cross walls. Ca carotenoid granules. T thylacoids.

We have observed in the present study that *G. amphibium* and *G. unigranulatum* are not genetically separated from each other. The morphospecies are mixed in the phylogenetic tree and they could not be distinguished as monophyletic entities. Our findings reinforce that they should be considered synonyms as previously stated in Bittencourt-Oliveira et al. (2009b).

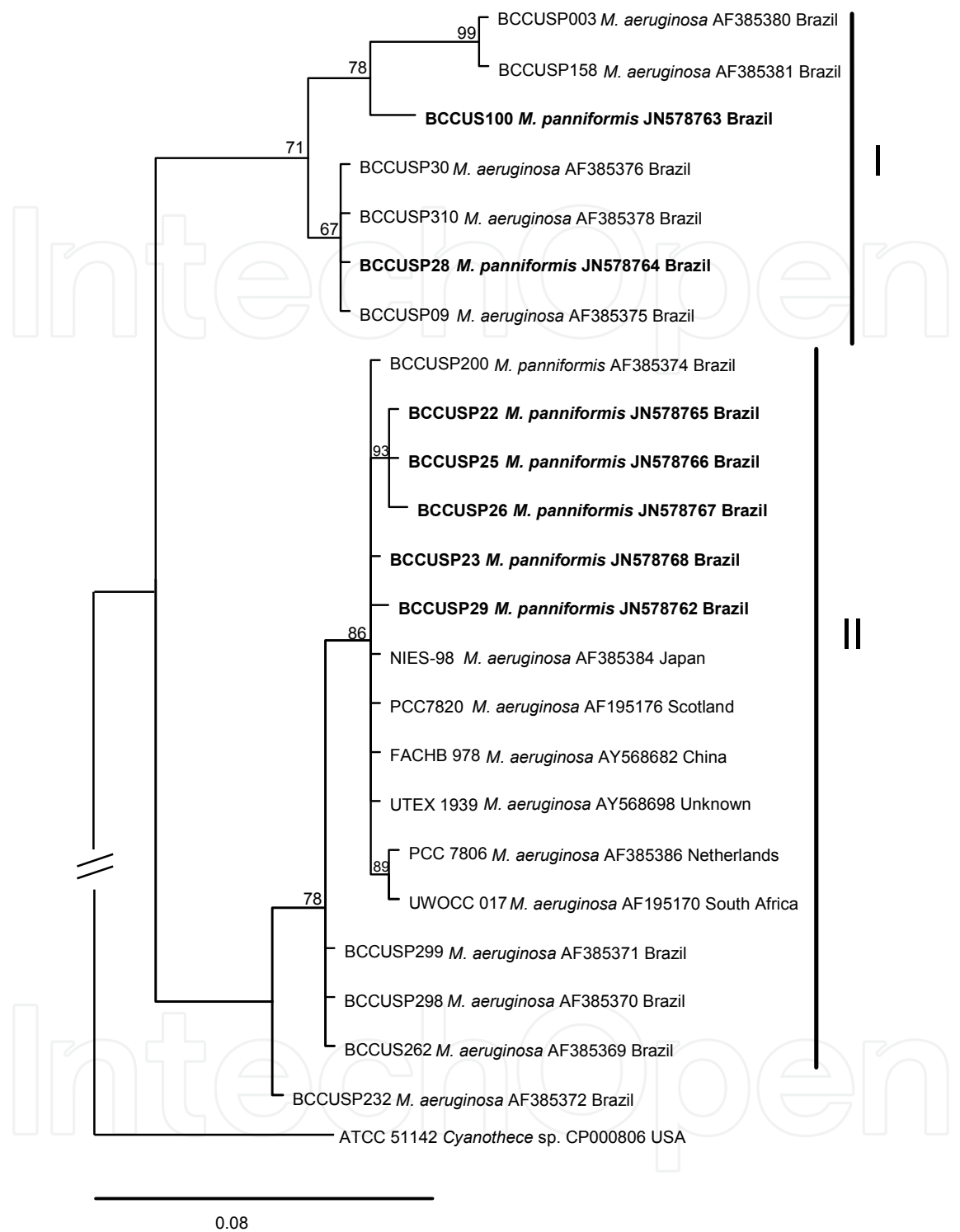


Fig. 4. Bayesian phylogenetic tree with *Microcystis aeruginosa* and *M. panniformis* strains. The tree was generated using intergenic spacer and flanking regions from *cpcBA*-phycocyanin operon. For the Bayesian analysis two runs of four Markov chains over 5,000,000 generations sampling every 100 generations was employed. The initial 2,500 generations were discarded as burn-in. Posterior probability (x100) is shown on each branch when higher than 70. *Cyanothece* sp. ATCC 51142 (CP000806) used as outgroup. The bar represents 0.08 substitutions. Strains in boldface were sequenced in this work.

3.2 *Microcystis panniformis*

Similarly as in the strains of *Geitlerinema*, *Microcystis panniformis* strains did not form a Clade isolated from *M. aeruginosa* in the phylogenetic tree generated by means of the c-phycocyanin genes *cpcB*, *cpcA* sequences and the intervening intergenic spacer (*cpcBA*-IGS) (Figure 4).

Two major groups in the *Microcystis* phylogenetic tree were formed (Clade I and II), constituted by strains of *M. panniformis* and *M. aeruginosa*. We observed from the topology tree that some strains of *M. panniformis* are genetically more close to those identified as *M. aeruginosa* than others from their same morphospecies.

The posterior probability was moderate for both Clades and only the *M. aeruginosa* BCCUSP232 strain, situated as sister group of Clade II, did not shown relevant Bayesian support value for its position.

The Brazilian strains BCCUSP23 and BCCUSP200, both belonging to the morphospecies *M. panniformis*, revealed 100% identity with *M. aeruginosa* strains isolated in Japan (NIES98), China (FACHB978) or Scotland (PCC7820) as well.

Traditional taxonomy of *Microcystis* based on morphologic criteria has been questioned by several authors because of the gap lack in the variations observed in the nature and in laboratory conditions. Earlier infraspecific studies of *Microcystis* concluded that morphology does not correlate with molecular data (Bittencourt-Oliveira et al., 2001; Kurmayer et al., 2003; Otsuka et al., 1999a, 1999b, 2000, 2001; Wu et al., 2007).

Our results indicated that *M. panniformis* could also be considered synonym of *M. aeruginosa*. The similarity found between sequences of *M. panniformis* and *M. aeruginosa* coming from diverse regions all over the world, led us to stress the cosmopolitan character of the species, with strains showing ample geographical distribution in both South and North hemispheres. These results corroborate similar previous findings (Bittencourt-Oliveira et al., 2001, 2007b; Otsuka et al., 1999a, 1999b, 2000, 2001).

4. Conclusion

We conclude that *G. unigranulatum* and *M. panniformis* should be considered as synonyms of *G. amphibium* and *M. aeruginosa*, respectively, since they do not represent genetically isolated Clades inside those genera.

5. Acknowledgements

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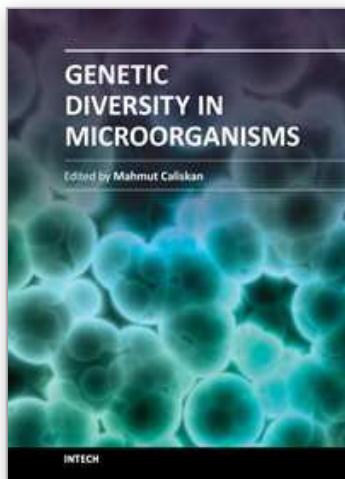
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Genetic Diversity in Microorganisms presents chapters revealing the magnitude of genetic diversity of microorganisms living in different environmental conditions. The complexity and diversity of microbial populations is by far the highest among all living organisms. The diversity of microbial communities and their ecologic roles are being explored in soil, water, on plants and in animals, and in extreme environments such as the arctic deep-sea vents or high saline lakes. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in microorganisms. The purpose of the book is to provide a glimpse into the dynamic process of genetic diversity of microorganisms by presenting the thoughts of scientists who are engaged in the generation of new ideas and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of microbial phylogeny, genetic diversity, and molecular biology.

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