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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, performe oxigenic 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; Itelman 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; Haerkamp 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 Geitlerinena amphibia (Agardh ex Gomont) Anagnostidis, G. unigranulatum (R.N. Singh) Komárek & Azevedo and Microcystis panniformis Komárek et al.

Species of Geitlerinena 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).

Geitlerinena amphibia 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. amphibia 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. amphibia 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°C ± 1°C and 30 ± 5 µmol photons·m⁻²·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 |
|--------------------|---------|-------------|----------------|-----------------------|
| G. amphibium a     | BCCUSP31| SP          | 143 293 198    | FJ545649              |
| G. amphibium a     | BCCUSP79| BR          | 143 293 198    | FJ545644              |
| G. amphibium b     | BCCUSP80| BR          | 143 86 198     | JN578762              |
| G. amphibium b     | BCCUSP84| BR          | 143 296 198    | JN578760              |
| G. amphibium a     | BCCUSP85| BR          | 143 291 198    | FJ545646              |
| G. amphibium b     | BCCUSP86| BR          | 143 294 198    | JN578759              |
| G. amphibium a     | BCCUSP87| BR          | 143 293 198    | FJ392653              |
| G. unigranulatum a | BCCUSP91| BR          | 143 292 198    | FJ545650              |
| G. amphibium b     | BCCUSP94| BR          | 143 83 198     | FJ545642              |
| G. amphibium a     | BCCUSP338| BR        | 143 295 198    | JN578761              |
| G. amphibium b     | BCCUSP54| BR          | 143 293 198    | FJ545654              |
| G. unigranulatum c | SC2     | VT          | 143 296 169    | EF680778              |
| G. unigranulatum a | BCCUSP350| BR        | 143 292 198    | FJ545647              |
| G. unigranulatum a | BCCUSP352| BR        | 143 83 198     | FJ545643              |
| G. unigranulatum a | BCCUSP356| BR        | 143 292 198    | FJ545648              |
| Oscillatoria sp. c | CYA479  | UG          | 143 296 169    | AM048624              |
| Oscillatoria sp. c | CYA469  | UG          | 143 296 169    | AM048623              |
| Planktothrix sp. d | FP1     | IT          | 143 298 169    | AF212923              |
| M. aeruginosa e    | BCCUSP09| BR          | 200 69 277     | AF385375              |
| M. aeruginosa e    | BCCUSP30| BR          | 200 69 277     | AF385376              |
| M. panniformis e   | BCCUSP200| BR        | 200 69 277     | AF385374              |
| M. aeruginosa e    | BCCUSP232| BR        | 200 69 277     | AF385372              |
| M. aeruginosa e    | BCCUSP262| BR        | 200 69 277     | AF385369              |
| M. panniformis e   | BCCUSP298| BR        | 200 69 277     | AF385370              |
| M. aeruginosa e    | BCCUSP299| BR        | 200 69 277     | AF385371              |
| M. aeruginosa e    | BCCUSP03| BR          | 200 69 277     | AF385380              |
| M. aeruginosa e    | BCCUSP158| BR        | 200 69 277     | AF385381              |
| M. panniformis b   | BCCUSP100| BR        | 200 69 277     | JN578763              |
| M. aeruginosa e    | BCCUSP310| BR        | 200 69 277     | AF385378              |
| M. panniformis b   | BCCUSP28 | BR          | 200 69 277     | JN578764              |
| M. panniformis b   | BCCUSP22 | BR          | 200 69 277     | JN578765              |
| M. panniformis b   | BCCUSP25 | BR          | 200 69 277     | JN578766              |
| M. panniformis b   | BCCUSP26 | BR          | 200 69 277     | JN578767              |
| M. panniformis b   | BCCUSP23 | BR          | 200 69 277     | JN578768              |
| M. panniformis b   | BCCUSP29 | BR          | 200 69 277     | JN578769              |
| M. aeruginosa e    | NIES-98 | JP          | 200 69 277     | AF385384              |
| M. aeruginosa e    | PCC7820 | SC          | 200 69 277     | AF385387              |
| M. aeruginosa e    | FACHB 978| CH         | 200 69 277     | AY566862              |
| M. aeruginosa e    | UTEX 1939| UN        | 200 69 277     | AY566898              |
| M. aeruginosa e    | PCC 7806| NE          | 200 69 277     | AF385386              |
| M. aeruginosa e    | 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 μM of each oligonucleotide primer in a total volume of 25 μL and 2.5 U of Taq DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ) with buffer containing 1.5 mM MgCl$_2$ and 200 μM of each dNTP (Boehringer-Mannheim, Mannheim, Germany).

Amplifications for the intergenic spacer and flanking regions from cpcBA-phycocyanin operon were accomplished with the primers PCβ-F and PCα-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 Pure Link 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.](image)

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*-phycoerythrin 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 them 70. *Cyanothecae* 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-phyocyanin 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 show 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

This research was supported by grants from FAPESP (State of São Paulo Research) Foundation Proc. 2004/01749-6 and 2008/56534-5, CNPq (Brazilian Council for Research and Development Proc. 300112/2008-4

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