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Taxonomic investigation using DNA fingerprinting in *Geitlerinema* species (Oscillatoriales, Cyanobacteria)

Maria do Carmo Bittencourt-Oliveira,[1]* **Nelson Sidnei Massola Jr,**[2] **Mariona Hernandez-Marine,**[4] **Susana Romo**[5] **and Ariadne do Nascimento Moura**[3]

Departments of [1]Biological Sciences and [2]Entomology, Plant Pathology and Agricultural Zoology, Luiz de Queiroz School of Agriculture, University of São Paulo, Piracicaba 13418-900, [3]Department of Biology, University Federal Rural of Pernambuco, Avenue Dom Manoel de Medeiros, S/N, Dois Irmãos, Recife, Pernambuco 52171-030, Brazil, [4]University of Barcelona, School of Pharmacy, Laboratory of Botany, Barcelona E-08028, and [5]University of Valencia, School of Biology, Burjasot, Valencia E-46100, Spain

SUMMARY

The taxonomic study of 14 strains of *Geitlerinema amphibium* (Ag. ex Gom.) Anagnostidis and *Geitlerinema unigranulatum* (R.N. Singh) Komárek and Azevedo, coming from several localities was undertaken. Use was made of morphological data and molecular data were obtained by means of the DNA fingerprinting technique using highly iterated palindrome (HIP1) sequences. The employed morphological characteristics were those used for species taxonomic identification belonging to the *Geitlerinema* genus, namely, cell dimensions, shape of the apical cell, motility, number and localization of cyanophycin granules in the cell. The two species revealed as polymorphic were discriminated only by means of the average cellular diameters. In spite of this, minima and maxima values of the cellular diameters overlapped. It was found from molecular analysis that a high genetic diversity and the formation of two clusters consisted of *G. amphibium* and *G. unigranulatum*, plus a sole strain keeping itself isolated from the remaining. Also, these clusters were not related to the geographic location; they encompassed strains from water bodies distant from each other by as much as 3500 km, or Brazilian and Spanish strains. Molecular and morphological data support the possibility that *G. unigranulatum* could be considered a synonym for *G. amphibium*. HIP1 fingerprinting is a powerful tool for the study of genetic of cyanobacteria closely related taxa. This study points to the necessity of using other than morphological data in the taxonomic revision of cyanobacteria, as well as in the proposition of new taxons.

Key words: Cyanobacteria (blue-green algae), DNA fingerprinting, *Geitlerinema*, genetic diversity, highly iterated palindrome sequences.

INTRODUCTION

The subgenus *Geitlerinema* (Oscillatoriales) was established in the genus *Phormidium* in the classification system of Anagnostidis and Komárek (1988), for encompassing some species of *Oscillatoria*. Later on, Anagnostidis (1989) raised this taxon to the Pseudanabaenaceae, and upgraded it to the genera category. Species are commonly found in eutrophic environments, also constituting blooms in reservoirs (Romo & Miracle 1994; Torgan & Paula 1994; Kirkwood *et al*. 2001).

Geitlerinema amphibium (Agardh ex Gomont) Anagnostidis and *Geitlerinema unigranulatum* (R. N. Singh) Komárek and Azevedo are morphologically similar to each other. According to Komárek and Anagnostidis (2005) and Romo *et al*. (1993), they could be differentiated only by their dimensions and the number of cyanophycin granules close to the cross-walls. However, no molecular data have been used to evaluate the delimitation between these two species.

Many studies on the taxonomy of cyanobacteria have shown the importance of using molecular tools other than morphology and ultra-structure to identify and classify these organisms due to phenotypical variability (Bolch *et al*. 1996, 1999). DNA fingerprinting methods have been successfully used to study genetic diversity of cyanobacterial strains, as well as populations from different geographic regions, cryolysates and cyanobionts. The most commonly used methods are: short tandemly repeated repetitive (STRR) (Wilson *et al*. 2000; Chonudomkul *et al*. 2004), random amplified polymorphic DNA (RAPD) (Nishihara *et al*. 1997; Casamatta *et al*. 2003; Prabina *et al*. 2005) and highly iterated palindrome (HIP1) (Saker & Neilan 2001; Orcutt *et al*. 2002; Zheng *et al*. 2002; Neilan *et al*. 2003; Pomati *et al*. 2004; Wilson *et al*. 2005; Bittencourt-Oliveira *et al*. 2007). HIP1s are octameric

*To whom correspondence should be addressed.
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Table 1. *Geitlerinema amphibium* and *G. unigranulatum* strains used in this study

Strain	Species	Sampling date	Locality
BCCUSP54	*G. amphibium*	Jun/2001	CA
BCCUSP79	*G. amphibium*	Apr/11/2000	JU
BCCUSP80	*G. amphibium*	Oct/2002	TA
BCCUSP85	*G. amphibium*	Nov/11/2000	JU
BCCUSP87	*G. amphibium*	Dec/18/2000	JU
BCCUSP91	*G. amphibium*	Feb/14/2003	USP
BCCUSP95	*G. amphibium*	Mar/04/1998	SR
Albufera	*G. amphibium*	1989	AL
BCCUSP96†	*G. unigranulatum*	May/2001	CA
BCCUSP287	*G. unigranulatum*	Jan/21/1998	SR
BCCUSP350	*G. unigranulatum*	Nov/04/1998	SR
BCCUSP352	*G. unigranulatum*	Dec/21/1998	SR
BCCUSP47	*G. unigranulatum*	Jul/29/1999	CP
BCCUSP94	*G.unigranulatum*	Oct/28/2003	SR

†Strain isolated from bloom. AL, Albufera Lake, Spain (39°20′S and 0°20′W); BCCUSP, Brazilian Cyanobacteria Collection of University of São Paulo (SP); CA, Carpina Reservoir, Pernambuco State (7°52′S and 35°18′W); CP, Capivara Reservoir, Paraná State (22°10′S and 50°80′W); JU, Jucazinho Reservoir, Pernambuco State (7°58′02.4″S and 35°44′33″W); SR, Santa Rita Lagoon, São Paulo (22°36.34′6″S and 50°38.48′6″W); TA, Tapacurá Reservoir, Pernambuco State (08°02′14″S and 35°09′46″W); USP, University of São Paulo lagoon, SP (22°42.47′5″S and 47°37.51′6″W).

palindrome sequences (5′-GCGATCGC-3′) abundant in coding regions of cyanobacterial genomes (Robinson *et al*. 1995).

In the present study, 14 Brazilian and Spanish strains of *G. amphibium* and *G. unigranulatum* were obtained from different reservoirs. These reservoirs exhibit differentiated environmental conditions, and they are very distant from each other. Since these species are polymorphic and the variation intervals of the cellular dimensions are overlapping, genetic diversity of these strains based on the DNA fingerprinting method was used to evaluate the existence of these two species.

MATERIALS AND METHODS

Field sampling, isolation and growth conditions

Fourteen clonal and nonaxenic strains of *Geitlerinema* spp. were used in this study (Table 1). Thirteen strains were isolated from six ecosystems situated in the southern, southeastern and northeastern regions of Brazil and only one from Spain. All Brazilian samples were gathered on the surface of water using a plankton net with a 25 μm mesh size. Part of the environmental samples was preserved in 4% formaldehyde for morphological analysis. Individual trichomes were removed by micromanipulation techniques from environmental samples with Pasteur pipettes at magnifications of 100–400×. Each isolated trichome 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 transferred again 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 h light : dark photoperiod at the Brazilian Cyanobacteria Collection of the University of São Paulo, Brazil (BCCUSP). The *G. amphibium* Spanish strain was isolated from Albufera Lake, Valencia in 1988 and maintained in the Laboratory of Ecology of the University of Valencia, Spain. It was acclimatized by three months in the BCCUSP in the same conditions of the Brazilian strains until the beginning of the experiment.

Morphologic analysis

The Brazilian strains were identified by morphological characteristics according to Komárek and Anagnostidis (2005), and the Spanish strain was identified by Romo *et al*. (1993). Measurements of cell diameters ($n = 50$) of each strain of *Geitlerinema* spp. in culture were randomly carried out during the logarithmic phase of growth. Average cell diameter, length and standard deviation were calculated using the statistical routine (SAS Institute Inc., version 8.0, Cary, North Carolina, USA, 1999). Cellular diameter and length were obtained by measurements with an ocular coupled to the microscope. Trichomes were photographed with a microscope (Nikon E200, Melville, NY, USA) equipped with a video camera system (Samsung SCC833, Tokyo, Japan) using the software Imagelab (Softium, São Paulo, Brazil).

The cell numbers were determined by calculating the sampling sufficiency as a function of the variance coefficient of cell diameters and length within 5% (Scheaffer *et al*. 1979). Observation of morphological variations were carried out both in populations kept in cultivation, during the exponential growth phase, and in those from natural samples.

Molecular analysis

Total genomic DNA was prepared using the hexadecyltrimethylammonium bromide (CTAB) (Doyle & Doyle 1990). 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). The DNA extraction of BCCUSP94 took place immediately after the strain isolation, without exceeding 30 days. Remaining strains from the same place were in cultivation controlled conditions at least for five years.

Polymerase chain reactions (PCR) were carried out in 25 μL volumes in a GeneAmp 2400 thermocycler (Perkin-Elmer, Foster City, CA, USA) according to Bittencourt-Oliveira *et al*. (2007). All primers were synthesized by IDT (Medley, FL, USA). Agarose gels were recorded using Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290) (Kodak, Melville, NY, USA) associated with Kodak 1D Image Analysis Software. HIP1 amplified fragments obtained from four primers were carried out in triplicate to confirm the DNA profiles, and doubtful bands were disregarded according to Bittencourt-Oliveira *et al*. (2007). The visible brightness bands were assembled and converted into binary codes based on presence (1) and absence (0) data. It was used in the analysis 14 operational taxonomic units (OTUs) and 364 characters. The similarity matrix was calculated by the Jaccard coefficient (Jaccard 1901) using the (NTSYS software, version 2.1, Metagraphics Software Corporation, Palo Alto, CA 2002) and the unweighted pair group method with arithmetic mean (UPGMA) algorithm (Sneath & Sokal 1973) for phenogram construction.

RESULTS

The investigated strains were identified as *G. unigranulatum* (Fig. 1a,b) and *G. amphibium* (Fig. 1c,d), and their cellular measurements and standard deviation are in Table 2. The natural populations and observed cultivated strains exhibited intense blue-green (cultivation) or pale (environmental samples) thallus, flexuous to straight, solitary trichomes, rarely attenuated at the extremities (only BCCUSP94) and not constricted at the cross-walls (for strains other than Albufera); conic-rounded apical cell, rarely bent with a granule of cyanophycin in the apical cell, and cylindrical cells with two or more granules of cyanophycin, one of them always close to the cross-wall. Also it was also frequently observed that straight to bent apical cells in cultivation presented intense motility through gliding in the direction of longitudinal axis, a slightly trembling oscillation, and reproduction by trichome disintegration.

Through electrophoretic profiles (Figs 2,3) it was observed that the analyzed populations exhibited high genetic diversity with similarity values varying from 0.40 to 0.86 (Table 3). The calculated correlation coefficient (*r*) was equal to 0.815. The dendrogram showed two clusters: I (0.51) and II (0.57), besides a sole non-clustered strain (BCCUSP96) (Fig. 4). The cluster I exhibited itself subdivided into three (Ia, Ib, Ic).

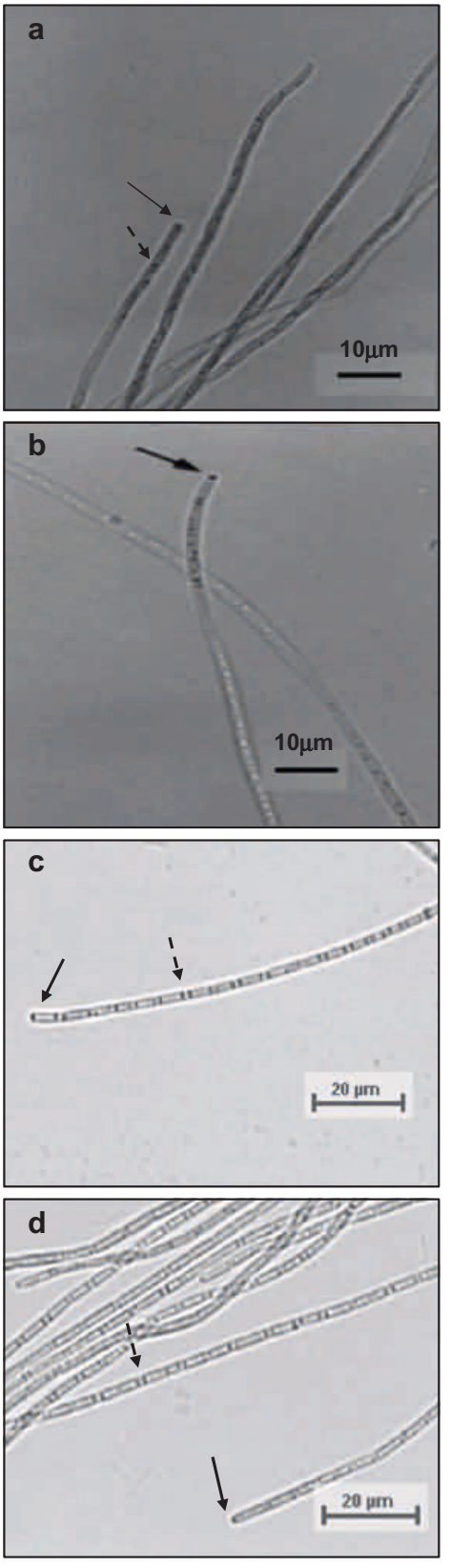

Fig. 1. (a,b) *Geitlerinema unigranulatum*. (a) BCCUSP287. (b) BCCUSP 352. (c,d). *G. amphibium*. (c) BCCUSP54. (d) BCCUSP79. Granules of cyanophycin in cellular tip (full arrow) and at the cross-walls (dashed arrow).

Table 2. Cellular measurements of *Geitlerinema amphibium* and *G. unigranulatum*

Strain	Species	Cellular diameter (μm)			Length (μm)		
		Min–Max	Average	SD	Min–Max	Average	SD
BCCUSP 54	*G. amphibium*	1.3–2.3	1.91	0.22	4.0–7.0	5.19	0.96
BCCUSP 79	*G. amphibium*	1.5–2.0	1.79	0.15	5.0–6.0	5.62	0.37
BCCUSP 80	*G. amphibium*	1.5–2.2	1.87	0.19	2.2–4.3	3.25	0.60
BCCUSP 85	*G. amphibium*	1.0–1.9	1.43	0.21	3.0–6.0	4.31	0.96
BCCUSP 87	*G. amphibium*	1.0–2.0	1.57	0.27	3.0–5.0	4.11	0.69
BCCUSP 91	*G. amphibium*	1.1–1.8	1.49	0.22	3.2–4.0	3.74	0.31
BCCUSP 95	*G. amphibium*	1.1–2.0	1.49	0.24	4.5–6.2	5.28	0.63
Albufera	*G. amphibium*	1.1–1.8	1.49	0.32	2.9–4.0	3.54	0.34
BCCUSP 96	*G. unigranulatum*	1.0–1.3	1.12	0.09	4.5–6.5	5.09	0.50
BCCUSP 287	*G. unigranulatum*	1.0–1.1	1.02	0.04	3.2–4.0	3.79	0.33
BCCUSP 350	*G. unigranulatum*	1.0–1.2	1.08	0.07	4.0–5.0	4.53	0.47
BCCUSP 352	*G. unigranulatum*	1.0–1.2	1.04	0.06	3.2–5.0	4.23	0.67
BCCUSP 47	*G. unigranulatum*	1.0–1.3	1.05	0.09	3.0–6.5	4.10	0.94
BCCUSP 94	*G. unigranulatum*	1.0–1.1	1.02	0.04	3.2–4.0	3.90	0.18

BCCUSP, Brazilian Cyanobacteria Collection of University of São Paulo; SD, Standard Deviation.

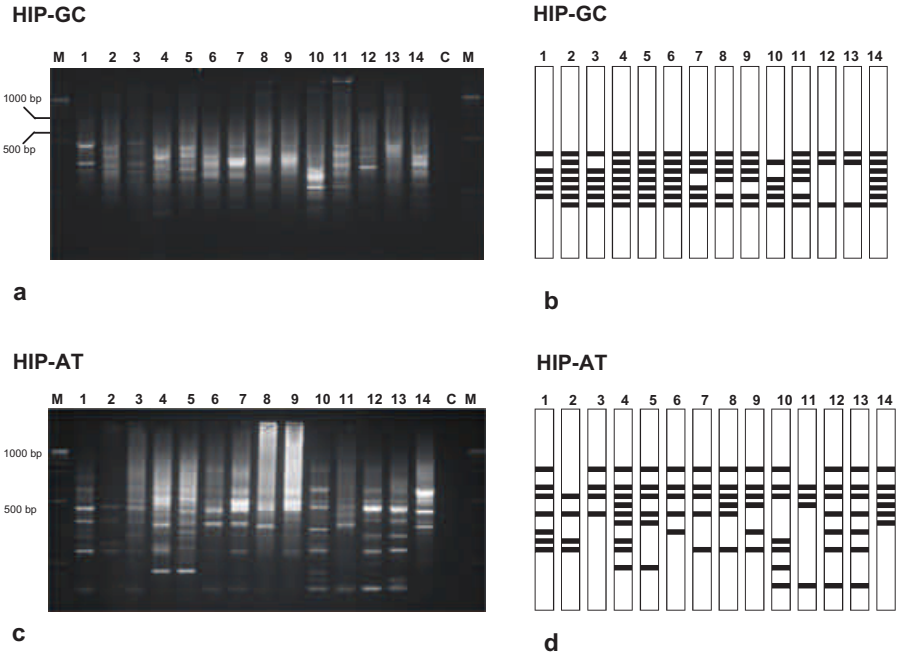

Fig. 2. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified highly iterated palindrome (HIP1). Fingerprint patterns of *Geitlerinema amphibium* and *G. unigranulatum*. (a) HIP1-GC and, (b) discrimination of the DNA fingerprinting profiles obtained in the HIP1-GC and used to elaborate data sheets for presence and absence. (c) HIP1-AT and, (d) discrimination of the DNA fingerprinting profiles obtained in the HIP1- AT and used to elaborate data sheets for presence and absence. Lanes that are labeled M are DNA molecular mass standards (1 KB ladder). Each lane corresponds to one strain in the order: 1. BCCUSP287, 2. BCCUSP95, 3. BCCUSP350, 4. BCCUSP352, 5. BCCUSP94, 6. BCCUSP85, 7. BCCUSP87, 8. BCCUSP79, 9. BCCUSP54, 10. BCCUSP96, 11. BCCUSP91, 12. Albufera, 13. BCCUSP47, 14. BCCUSP80; C, control without DNA.

Strains of *G. unigranulatum* and *G. amphibium* from the southeast and northeast regions showed up in subgroup Ia. *G. amphibium* strains were present in subgroup Ib, with those belonging to the southeast and northeast regions. Subgroup Ic was constituted by two strains of *G. unigranulatum*, belonging to the same reservoir but was isolated in intervals greater than five years. Cluster II encompassed the *G. amphibium* strain from Albufera Lake, Spain and another *G. unigranulatum* from South Brazil (BCCUSP47).

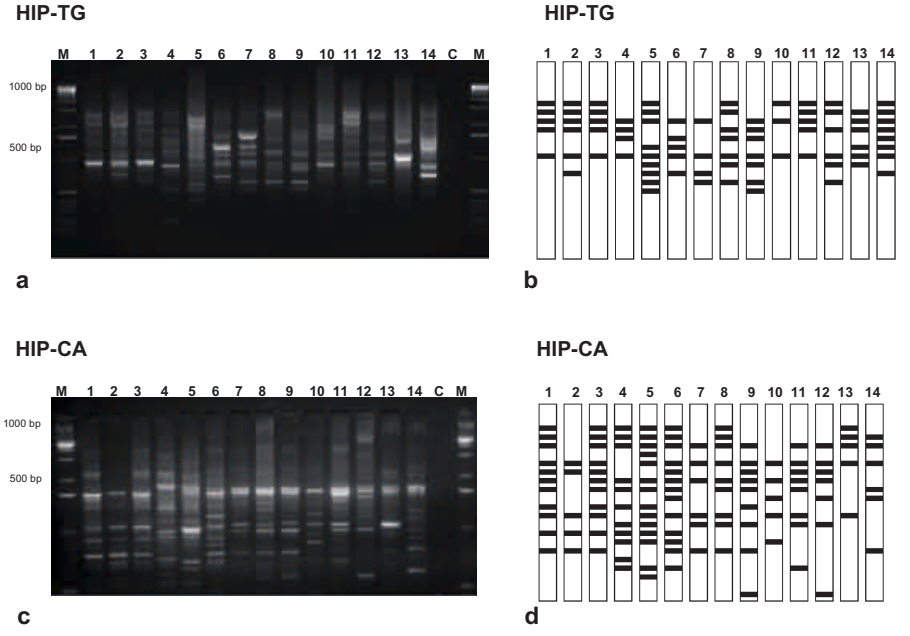

Fig. 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified highly iterated palindrome (HIP1). Fingerprint patterns of *Geitlerinema amphibium* and *G. unigranulatum*. (a) HIP1-TG and (b) Discrimination of the DNA fingerprinting profiles obtained in the HIP1-TG and used to elaborate data sheets for presence and absence. (c) HIP1-CA and (d) Discrimination of the DNA fingerprinting profiles obtained in the HIP1- CA and used to elaborate data sheets for presence and absence. Lanes that are labeled M are DNA molecular mass standards (1 KB ladder). Each lane corresponds to one strain in the order: 1. BCCUSP287, 2. BCCUSP95, 3. BCCUSP350, 4. BCCUSP352, 5. BCCUSP94, 6. BCCUSP85, 7. BCCUSP87, 8. BCCUSP79, 9. BCCUSP54, 10. BCCUSP96, 11. BCCUSP91, 12. Albufera, 13. BCCUSP47, 14. BCCUSP80; C, control without DNA.

Table 3. Similarity matrix generated from polymerase chain reaction (PCR) fingerprinting using highly iterated palindrome (HIP1) primers (TG, GC, AT and CA) based on Jaccard's coefficient

Strain	287	95	350	352	94	85	87	79	54	96	91	Alb.	47	80
287	1.000													
95	0.561	1.000												
350	0.862	0.561	1.000											
352	0.509	0.509	0.509	1.000										
94	0.509	0.509	0.509	0.509	1.000									
85	0.606	0.561	0.606	0.509	0.522	1.000								
87	0.522	0.522	0.522	0.509	0.522	0.526	1.000							
79	0.522	0.522	0.552	0.509	0.522	0.526	0.647	1.000						
54	0.522	0.522	0.522	0.509	0.509	0.522	0.526	0.647	1.000					
96	0.403	0.403	0.403	0.403	0.403	0.403	0.403	0.403	0.403	1.000				
91	0.522	0.522	0.522	0.509	0.509	0.522	0.526	0.569	0.569	0.403	1.000			
Albufera	0.433	0.433	0.433	0.433	0.433	0.433	0.433	0.433	0.433	0.403	0.433	1.000		
47	0.433	0.433	0.433	0.433	0.433	0.433	0.433	0.433	0.433	0.403	0.433	0.571	1.000	
80	0.606	0.561	0.606	0.509	0.509	0.647	0.522	0.522	0.522	0.403	0.522	0.433	0.433	1.000

Correlation coefficient (r) = 0.815.

DISCUSSION

The *G. amphibium* and *G. unigranulatum* populations found in nature or kept in cultivation were polymorphic, with variation in the apical cell, and number and dimensions of granules, as well. The variation in dimensions of cyanophycin granules is related, perhaps, to the nutritional stage of this organism (Romo *et al.* 1993). The trichome tip and the granules dimensions in cyanophycin were shown to be the most evident variations. On the other hand, dimensions of cellular diameter, motility, trichome shape, thallus color and granules localization in the cross-walls were all kept invariable in both nature and cultivation samples.

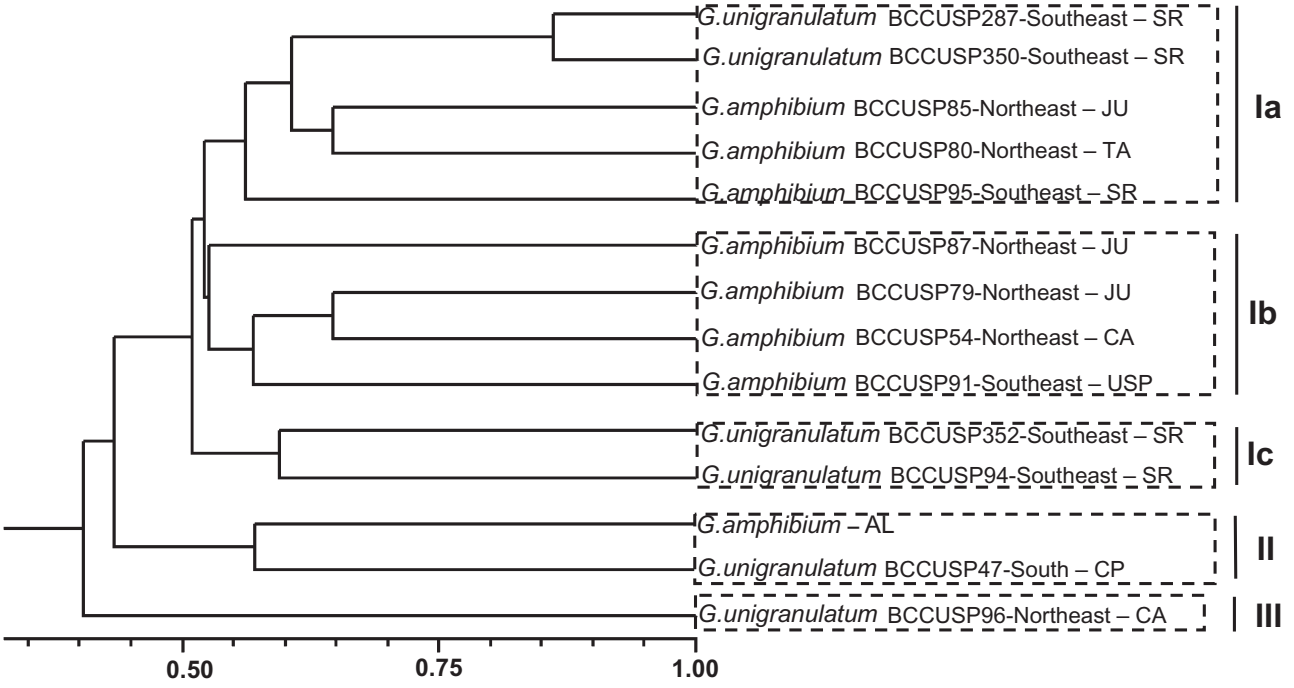

Fig. 4. Clustering dendrogram of *Geitlerinema amphibium* and *G. unigranulatum* strains by an unweighted pair group method with arithmetic mean (UPGMA) algorithm analysis of similarity matrix data using 14 operational taxonomic units and the Jaccard coefficient. The numerical scale indicates the level of similarity at which clusters are formed, according to the Jaccard Coefficient. I = 0.51, II = 0.57. $r = 0.815$. AL, Albufera Lake, Spain; BCCUSP, Brazilian Cyanobacteria Collection of University of São Paulo (SP); CA, Carpina Reservoir, PE; CP, Capivara Reservoir, Paraná State (PR); JU, Jucazinho Reservoir, Pernambuco State (PE); SR, Santa Rita Lagoon, SP; TA, Tapacurá Reservoir, PE; USP, University of São Paulo lagoon, SP.

These results agree with those obtained by Romo *et al*. (1993) in cultured and natural populations, where the author identified *G. amphibium* (Agardh *ex* Gomont) Anagnostidis. *G. amphibium* showed cellular diameters varying from 1.0 to 2.3 μm, while for *G. unigranulatum* the variation ranged from 1.0 to 1.3 μm. However, the variation intervals of the averages of cellular diameters for *G. unigranulatum* and *G. amphibium* were, respectively, 1.02–1.12 and 1.49–1.91 μm. Therefore, it was found by morphological analysis that the unique characteristic, capable of distinguishing the two morphospecies, was the average of cellular diameters, in spite of the overlap among minima and maxima interval values. The number of granules, which is considered a taxonomic characteristic, was revealed to be variable and is an indication of physiological or environmental conditions.

Highly iterated palindrome DNA fingerprinting has previously been shown to be effective at distinguishing between strains from a range of water bodies in northeastern Australia, whose similarity values to 16SrRNA gene sequences were larger than >99.8% (Saker & Neilan 2001). Through HIP1 profiles, Neilan *et al*. (2003) were capable to distinguish strains of *Cylindrospermopsis raciborskii* from Australia, Brazil, Germany, Hungary, Portugal and United States of America. Notwithstanding, interpretations of results coming from the use of simple algorithms such as UPGMA should be carried out carefully when using banding patterns.

Geitlerinema amphibium and *G. unigranulatum* populations exhibited high genetic diversity. However, the clusters formation was not associated with their taxonomic identity, or any morphological characteristics, and much lesser with the geographic origin. Our findings agree with those reported by Margheri *et al*. (2003), which observed that the genetic distance among different *Geitlerinema* clusters can be nearly as large as the one found between a *Geitlerinema* cluster and another genus. Castenholz (2001) emphasizes that *Geitlerinema* is a genus 'Pro tem' because of its ecologic diversity, and that it may require more than one generic subdivision.

The BCCUSP352 and BCCUSP94 strains, both identified as a *G. unigranulatum* morphospecies isolated from the same aquatic ecosystem, but in a five-year interval, were grouped in the same cluster with high similarity value. This fact indicates that, although they were kept under cultivation conditions for a long time, the strains maintained their genetic identity. The changes in banding patterns observed in culture isolates when compared to natural populations may be

indicative of gene rearrangements, which can occur as a result of laboratory maintenance in culture (Robinson *et al*. 1995). However, such a circumstance was not verified in the present study.

Bittencourt-Oliveira *et al*. (2007) showed that the HIP1 fingerprinting is a powerful tool to study genetic diversity of cyanobacteria strains or closely related taxa. It can be used to choose strains for sequencing, since the latter is still a difficult, time-consuming and expensive technique.

From the present study we came to the conclusion that the genus *Geitlerinema* should be revised because of its lower similarity values. The molecular and morphological data support the idea that *G. unigranulatum* should be considered as a synonym for *G. amphibium*.

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