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GEITLERINEMA SPECIES (OSCILLATORIALES, CYANOBACTERIA) REVEALED BY CELLULAR MORPHOLOGY, ULTRASTRUCTURE, AND DNA SEQUENCING[1]

Maria do Carmo Bittencourt-Oliveira[2]

Department of Biological Sciences, Luiz de Queiroz College of Agriculture, University of São Paulo, Piracicaba 13418-900, SP, Brazil

Ariadne do Nascimento Moura

Department of Biology, University Federal and Rural of Pernambuco, Recife, Pernambuco 52171-030, Brazil

Mariana Cabral de Oliveira

Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo 05508-900, SP, Brazil

and Nelson Sidnei Massola Jr.

Departments of Entomology, Plant Pathology and Agricultural Zoology, Luiz de Queiroz College of Agriculture, University of São Paulo, Piracicaba 13418-900, SP, Brazil

Geitlerinema amphibium **(C. Agardh ex Gomont) Anagn. and** ***G. unigranulatum*** **(Rama N. Singh) Komárek et M. T. P. Azevedo are morphologically close species with characteristics frequently overlapping. Ten strains of** ***Geitlerinema*** **(six of** ***G. amphibium*** **and four of** ***G. unigranulatum*****) were analyzed by DNA sequencing and transmission electronic and optical microscopy. Among the investigated strains, the two species were not separated with respect to cellular dimensions, and cellular width was the most varying characteristic. The number and localization of granules, as well as other ultrastructural characteristics, did not provide a means to discriminate between the two species. The two species were not separated either by geography or environment. These results were further corroborated by the analysis of the** ***cpc*****B-*****cpc*****A intergenic spacer (PC-IGS) sequences. Given the fact that morphology is very uniform, plus the coexistence of these populations in the same habitat, it would be nearly impossible to distinguish between them in nature. On the other hand, two of the analyzed strains were distinct from all others based on the PC-IGS sequences, in spite of their morphological similarity. PC-IGS sequences indicate that these two strains could be a different species of** ***Geitlerinema*****. Using morphology, cell ultrastructure, and PC-IGS sequences, it is not possible to distinguish** ***G. amphibium*** **and** ***G. unigranulatum*****. Therefore, they should be treated as one species,** ***G. unigranulatum*** **as a synonym of** ***G. amphibium*****.**

Key index words: ***cpc*****BA sequences; cyanobacteria; microscopy; morphology; taxonomy; ultrastructure**

Abbreviations: **BCCUSP, Brazilian Cyanobacteria Collection of University of São Paulo; HIP1, highly iterated palindrome; ML, maximum likelihood; MP, maximum parsimony; PC-IGS,** ***cpc*****B-*****cpc*****A intergenic spacer; TBR, tree-bisection-reconnection**

The high morphological variability and the low number of phenotypic characters used in the cyanobacterial taxonomy lead to serious identification problems. New techniques have, therefore, been used to help with their identification and classification. The application of EM techniques, as well as new biochemical and molecular methods, has improved the characterization of cyanobacteria, elucidating many of their enigmatic characteristics (Rippka et al. 1979, Anagnostidis and Komárek 1985, Castenholz 2001, Komárek 2003, Komárek and Anagnostidis 2005). Cyanobacterial ultrastructure has been used in the last decades to help resolve taxonomic problems (Edwards et al. 1968, Gantt and Conti 1969, Bourrelly and Couté 1975, Guglielmi and Cohen-Bazire 1984, Chou and Huang 1991, Romo et al. 1993a,b, Janson et al. 1995, Komárek and Cépak 1998, Komárek and Azevedo 2000, Komárek and Anagnostidis 2005).

Oscillatoria amphibia C. Agardh ex Gomont 1892 was placed in the genus *Phormidium*, subgenus *Geitlerinema* as *P. amphibium* (C. Agardh ex Gomont) Anagnostidis and Komárek (1988), and was subsequently reclassified as *Geitlerinema amphibium* (C. Agardh ex Gomont) Anagnostidis (1989). *Oscillatoria quadripunctulata* Bruhl et Biswas var. *unigranulata* Rama N. Singh was ranked to the level of species, now belonging to the genus *Geitlerinema* being designated by *Geitlerinema unigranulatum* (Rama N. Singh) Komárek et Azevedo (2000).

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[2]Author for correspondence: e-mail mbitt@esalq.usp.br.

Species of *Geitlerinema* are cosmopolitan and frequently occur in different kinds of habitats, such as freshwater (Rippka et al. 1979, Romo et al. 1993b, Torgan and Paula 1994) and marine environments (Silva et al. 1996, Margheri et al. 2003).

Romo et al. (1993b) and Komárek and Azevedo (2000) used ultrastructure data in the study of cultured strains of *G. amphibium* and *G. unigranulatum.* However, several studies using molecular techniques have questioned the use of morphological characteristics for identification at the species as well as at the genus levels (Lópes-Córtes et al. 2001, Wood et al. 2002, Margheri et al. 2003, Kirkwood and Henley 2006, Bittencourt-Oliveira et al. 2007).

Bittencourt-Oliveira et al. (2007) did not accomplish the differentiation of strains pertaining to these two species by means of molecular data using highly iterated palindrome (HIP1) sequences. They then suggested *G. unigranulatum* as a synonym for *G. amphibium.* In the present study, strains of *G. unigranulatum* and *G. amphibium* were analyzed using cellular morphology, ultrastructure, and DNA sequencing.

MATERIALS AND METHODS

Field sampling, isolation, and growth conditions. Fourteen clonal and nonaxenic strains of *Geitlerinema* spp. were used in this study (Table S1 in the supplementary material). These strains were isolated from seven aquatic habitats situated in the southern, southeastern, and northeastern regions of Brazil and from Spain (Table S1). All samples were gathered on the surface of water bodies using a handmade 25 μm mesh plankton net. Individual trichomes were removed by micromanipulation techniques from environmental samples using Pasteur pipettes. Each isolated trichome was washed in several consecutive drops of water until all other algae were removed, then 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 again transferred to tubes containing liquid medium. The cultures were examined microscopically (mod. E200; Nikon Instruments Inc., Melville, NY, USA) to ensure that there were no contaminating organisms. All strains were maintained in incubators (mod. 122FC; EletroLab, São Paulo, Brazil) at 21°C ± 1°C and 30 ± 5 μmol photons$\cdot m^{-2} \cdot s^{-1}$ (photometer, Li-Cor mod. 250; Li-Cor Corporate Headquarters, Lincoln, NE, USA), under a 14:10 light:dark (L:D) photoperiod. The cultures are maintained 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 for 3 months in the BCCUSP in the same conditions as the Brazilian strains before the beginning of the experiment.

Abiotic variables. Some abiotic variables were determined in situ. Water temperature and dissolved oxygen were determined using an Oximeter Handylab OX1/Set® (Schott-Geräte, Hofheim, Germany); electric conductivity, with a conductivimeter (handylab LF1; Schott Glaswerke, Mainz, Germany); and pH, using a potentiometer (Digimed, Campo Grande, SP, Brazil, mod. DMPH-20). Water transparency was obtained with a Secchi disk. The analysis of nitrite ($\mu g \cdot L^{-1}$), total phosphorus ($\mu g \cdot L^{-1}$), and orthophosphate ($\mu g \cdot L^{-1}$) was in accordance with the procedure described by Mackereth et al. (1978), Valderrama (1981), and Strickland and Parsons (1965), respectively. For the trophic characterization of the ecosystems, we employed the Carlson trophic state index, adapted by Toledo et al. (1983) for tropical regions. Calculations were based on total phosphorus content, where the ultra-oligotrophic (≤20), oligotrophic (21–40), mesotrophic (41–50), eutrophic (51–60), and hypertrophic (≥61) conditions were then determined (Kratzer and Brezonik 1981).

Morphological analysis. The Brazilian strains were identified as *G. amphibium* and *G. unigranulatum*, according to morphological characteristics given by the system of Komárek and Anagnostidis (2005), such as cellular dimensions, the shape of trichome and apical cell, occurrence and number of granules per cell, arrangement of thylakoids, and trichome movement. Observations of morphological variations were performed in populations kept in cultivation, during the exponential growth phase. For cellular measurements, an ocular micrometer coupled to the microscope (Nikon Instruments Inc.) was used. The number of cells to be measured (n) was determined by calculating the sampling sufficiency as function of the variance coefficient of cell width and length within 5% (Scheaffer et al. 1979). Measurements of cell width and cell length (n = 50) of each strain of *Geitlerinema* spp. in culture were randomly performed. Mean and standard deviation of cell widths and lengths were calculated using the statistical routine SAS software, version 8.0 (1999) (SAS Institute, Cary, NC, USA). To distinguish the two species from each other, the cellular length and diameter dimensions were used (*G. amphibium* n = 350 and *G. unigranulatum* n = 300). The Kruskal–Wallis variance analysis was employed at 5% significance level, using Statistica software 2004 (StatSoft Inc., Tulsa, OK, USA). For a better visualization of the differences and similarities between the strains being compared, we employed the method used by Golubic and Focke (1978).

Trichomes were photographed with a light microscope (Nikon E200) equipped with a video camera system (Samsung SCC833, Tokyo, Japan) using Imagelab software (Softium, Fortaleza, CE, Brazil).

Ultrastructural analysis. Samples of *Geitlerinema* strains in exponential growth phase were centrifuged (mod. U-32R; Boeco, Hamburg, Germany) at 21,000*g* for 10 min. The pellets were fixed in a solution of modified Karnovsky (2.5% glutaraldehyde; 2.5% paraformaldehyde in cacodylate buffer 0.05 M pH 7.0 with added 0.001 M $CaCl_2$ solution) for at least 1 h, then rinsed three times (10 min each) with cacodylate buffer. The samples were postfixed in 1% osmium tetroxide in the same buffer for 2 h. After en bloc staining with 0.5% uranyl acetate overnight at 4°C, they were dehydrated in acetone (30, 50, 70, 90, and 3 × 100%) for 10 min each, embedded in Spurr's Low Viscosity epoxy resin (Electron Microscope Science, Hatfield, PA, USA), and polymerized at 60°C for 48 h. Ultrathin sections were prepared with a Leica Ultracut UCT (Leica Microsystems GmbH, Wetzlar, Germany) microtome with a diamond knife. After staining with uranyl acetate and lead citrate, sections were examined in a Zeiss EM900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

DNA extraction. DNA was extracted from fresh cells that were harvested at exponential phase. Total genomic DNA was prepared using the commercial kit Gnome DNA (BIO 101, Vista, CA, USA), according to the manufacturer's instructions. DNA concentrations were estimated directly from ethidium bromide fluorescence in 0.7% agarose gel images against standard quantities of DNA by using Kodak Eletrophoresis Documentation and Analysis System 290 (EDAS 290; Kodak, Melville, NY, USA) associated with Kodak 1D Image Analysis Software.

PCR amplification. Amplifications were carried out in 25 μL volumes in a GeneAmp 2400 thermocycler (Applied Biosystems, Foster City, CA, USA). All the primers were synthesized

by IDT (Medley, FL, USA). Amplification for the PC-IGS was accomplished with the primers PCβ-F: 5′-GGC-TGCTTGTTTACGCGACA-3′ and PCα-R: 5′-CCAGTACCAC-CAGCAACTAA-3′. Reactions contained ~2–5 ng of DNA, 5 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with buffer containing 1.5 mM $MgCl_2$ and 200 μM of each dNTP (Boehringer-Mannheim, Mannheim, Germany). The following cycling parameters and conditions for PC-IGS were used: 92°C for 2 min, followed by 40 cycles of 92°C for 40 s; 55°C for 50 s, and 72°C for 2 min, followed by a final extension at 72°C for 8 min. 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 reactions were repeated at least five times. Amplification products were visualized by electrophoresis on agarose gels as described earlier and were estimated against standard DNA (Low DNA mass, Invitrogen, Carlsbad, CA, USA). PCR products were purified through a PCR purification kit (QIAquick, Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

PC-IGS sequencing. The PC-IGS and flanking coding region on the phycocyanin operon were directly sequenced using the forward and reverse primers with ABI Prism® Big Dye® Terminator Cycle Sequencing Ready Kit (Applied Biosystems) and 3100 ABI Sequencher (Applied Biosystems), according to the manufacturer's instructions. To avoid errors by PCR, at least four separated amplification reactions were pooled for sequencing. The sequencing was repeated on independent PCR products. The PCRs 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 Sequencher program (version 3.0, Applied Biosystems) to establish a consensus sequences for each strain.

Matrix. Consensus sequences were obtained for the *cpc*BA region for the strains listed in Table S1, encompassing 144 bp from gene *cpc*B, from 95 to 293 bp from the spacer region, and 169 bp from gene *cpc*A. The sequences were aligned using ClustalW in BioEdit program (Hall 1999) and were manually inspected, including the sequences from GenBank AM048623, AM048624, and AF212923. The sequence AY575949 (*Spirulina subsalsa* PD2002) was used as outgroup for the rooted analysis based on sequence similarity. The alignment matrix contained 13 taxa, without the outgroup, and 606 positions.

Construction of phylogenetic trees construction. Phylogenetic analyses were performed with PAUP* version 4.0b10 (Swofford 2000) and Mr. Bayes v3.1.2 (Ronquist and 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 4,000,000 generations, sampling every 100 generations, were employed. The initial 20,000 generations were discarded as burn-in.

The neighbor-joining (NJ) tree (Saitou and Nei 1987) was built with the Tamura and Nei (1993) substitution model. A maximum-parsimony (MP) tree was inferred by heuristic search, with starting trees obtained by stepwise addition, with random sequence addition (10 replicates) using the tree-bisection-reconnection (TBR) branch-swapping algorithm. In both NJ and MP trees, gaps were treated as missing data, and all sites were weighted equally. Bootstrap analyses (Felsenstein 1985) were performed with 2,000 replicates for the methods described earlier. Maximum-likelihood (ML) analysis was performed with heuristic search using the TBR algorithm, with starting trees obtained via stepwise addition as described for the MP tree. Bootstrap resampling was performed for 100 replicates due to computational limitations. For all analyses, bootstrap values were considered low up to 70%, moderate from 71% to 90%, and high above 90%.

RESULTS

Morphological data. The trophic characterization of the environments is presented in Table 1. The measurements including the number of cyanophycin granules in Brazilian strains of *Geitlerinema* are found in Table 2. The trichomes varied in shape from flexuous to straight; they were not constricted at the cross walls and were rarely attenuated toward their ends (only BCCUSP96). Trichome cells were cylindrical with one or more granules of cyanophycin, one of them always close to the cross wall (Fig. 1, a–c). Apical cells were rounded-conical with a granule of cyanophycin; a hooked apical cell was frequently observed in cultures. The trichomes showed mobility through their sliding and moderate creeping at the end.

The variation in size under defined culture conditions was lower within each strain than between strains. The means of the studied populations formed two distinct clusters (A and B in Fig. 2), although the maximum values for the smaller species overlapped with the minimum values for the larger one, leaving no separation in cell width

Table 1. Physical and chemical variables at the localities sampled.

Water bodies	SR	LE	TA	JU	CA	CP[a]	AL[b]
Water temperature (°C)	22.00	27.00	26.00	27.40	27.00	19.00	19.00
Dissolved oxygen ($mgO_2 \cdot L^{-1}$)	14.40	3.92	4.00	6.83	7.20	2.00	8.50
pH	8.27	9.48	6.30	8.54	7.50	7.50	8.70
Electric conductivity ($\mu S \cdot cm^{-1}$)	183	327	584	17.90	ND	ND	1780
Water transparency (m)	0.57	0.30	0.85	1.85	1.30	ND	0.21
Total phosphorus ($\mu g \cdot L^{-1}$)	13.00	180.00	253.00	493.11	147.00	60.00	ND
Orthophosphate ($\mu g \cdot L^{-1}$)	0.24	0.33	ND	ND	1.82	ND	ND
Nitrite ($\mu g \cdot L^{-1}$)	10.36	6.27	64.40	0.01	ND	ND	155.00
Trophic index state	Oligotrophic	Eutrophic	Hypertrophic	Hypertrophic	Eutrophic	Eutrophic	Hypertrophic

SR, Santa Rita Lagoon; LE, Engenharia Lagoon; TA, Tapacurá Reservoir; JU, Jucazinho Reservoir; CA, Carpina Reservoir; CP, Capivara Reservoir; AL, Albufera Lake; ND, not determined.

[a]Data from Kamogae (2002).

[b]Data from Romo and Miracle (1994).

Table 2. Cellular width, cellular length, and number of cyanophycin granules per cell in *Geitlerinema amphibium* and *G. unigranulatum* strains used in this study.

		Width (μm)			Length (μm)				Granules				
Strain	Species	Min–Max.	Mean	SD	Min–Max.	Mean	SD	L:W	1	2	3	4	*n*G
BCCUSP54	*G. amphibium*	1.3–2.3	1.91	0.22	4.0–7.0	5.19	0.96	2.7	10	10	–	–	20
BCCUSP79	*G. amphibium*	1.5–2.0	1.79	0.15	5.0–6.0	5.62	0.37	3.1	11	10	–	–	21
BCCUSP80	*G. amphibium*	1.5–2.2	1.87	0.19	2.2–4.3	3.25	0.60	1.7	8	11	8	–	27
BCCUSP85	*G. amphibium*	1.0–1.9	1.43	0.21	3.0–6.0	4.31	0.96	3.0	12	8	1	–	21
BCCUSP87	*G. amphibium*	1.0–2.0	1.57	0.27	3.0–5.0	4.11	0.69	2.6	9	10	–	–	19
BCCUSP91	*G. amphibium*	1.1–1.8	1.49	0.22	3.2–4.0	3.74	0.31	2.5	6	10	–	–	16
BCCUSP95	*G. amphibium*	1.1–2.0	1.49	0.24	4.5–6.2	5.28	0.63	3.5	4	12	7	–	23
BCCUSP31	*G. amphibium*	1.1–1.8	1.49	0.32	2.9–4.0	3.54	0.34	2.4	11	14	–	–	25
BCCUSP96	*G. unigranulatum*	1.0–1.3	1.12	0.09	4.5–6.5	5.09	0.50	4.5	11	7	1	1	20
BCCUSP287	*G. unigranulatum*	1.0–1.1	1.02	0.04	3.2–4.0	3.79	0.33	3.7	10	10	4	–	24
BCCUSP350	*G. unigranulatum*	1.0–1.2	1.08	0.07	4.0–5.0	4.53	0.47	4.2	10	11	5	–	26
BCCUSP352	*G. unigranulatum*	1.0–1.2	1.04	0.06	3.2–5.0	4.23	0.67	4.1	10	10	1	–	21
BCCUSP47	*G. unigranulatum*	1.0–1.3	1.05	0.09	3.0–6.5	4.10	0.94	3.9	11	10	–	–	21
BCCUSP94	*G. unigranulatum*	1.0–1.1	1.02	0.04	3.2–4.0	3.90	0.18	3.8	10	10	2	–	22

BCCUSP, Brazilian Culture Collection of University of São Paulo; SD, standard deviation, $n = 50$; L:W, length to width ratio; *n*G, total number of granules.

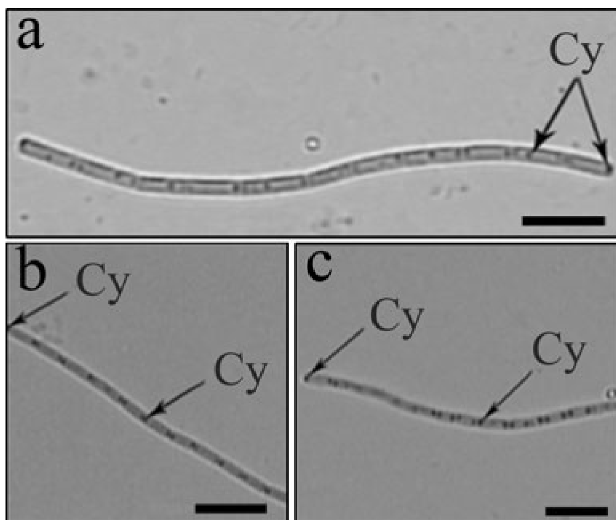

Fig. 1. Brazilian strains of *Geitlerinema* in exponential growth phase showing cyanophycin granules close at the cross walls and apical cells. (a) *G. unigranulatum* (BCCUSP95), (b) *G. amphibium* (BCCUSP85), (c) *G. unigranulatum* (BCCUSP352). Cy, cyanophycin granule. Scale bar, 10 μm.

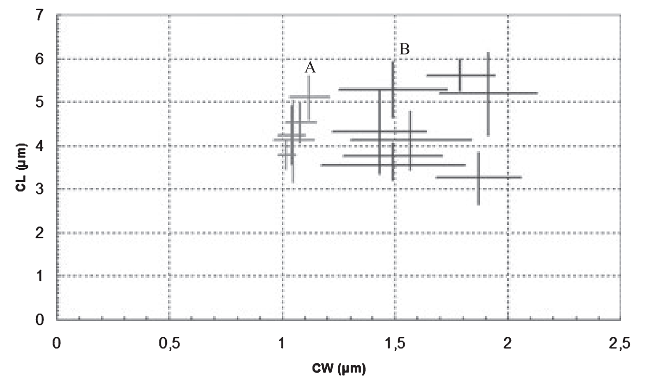

Fig. 2. Cell size variation (mean values and standard deviations) in the Brazilian strains of *Geitlerinema*. The clusters A and B encompass, respectively, *G. unigranulatum* and *G. amphibium* strains. CL, cell length; CW, cell width.

between the two taxa. The variation intervals of cellular diameters measured in the studied strains of *G. amphibium* and G. *unigranulatum* ranged from 1.0 to 2.3 μm and 1.0 to 1.3 μm, respectively (the lowest values are less reliable due to possible shrinkage under unfavorable conditions). The measurements of cell lengths in strains attributable to *G. amphibium* and *G. unigranulatum* show complete overlap of maximum, minimum, and average values. Strains of *G. amphibium* exhibited a wider variation interval for cellular length (2.2 to 7.0 μm) compared with the studied strains of *G. unigranulatum* (3.0 to 6.5 μm). Accordingly, the cell length/width ratio expressed the distinction between the two clusters (Fig. 2). The variance analysis revealed that *G. amphibium* and *G. unigranulatum* significantly differ in length ($H = 8.820$, $P = 0.003$ when $P < 0.01$) as well as in cellular width ($H = 441.999$, $P = 0.000$ when $P < 0.001$).

A single granule per cell was observed in 41% of *G. amphibium* strains and in 46% of those of *G. unigranulatum*. Two and three granules were observed in 48% and 11% of the *G. amphibium* cells, and in 43% and 10% of *G. unigranulatum* cells, respectively. Both the number and dimensions of granules were variable during the exponential growth phase, but no change in these characteristics was observed when cultures entered the stationary phase (data not shown).

Transmission electron micrographs of the 13 *Geitlerinema* Brazilian strains revealed variations in the presence, number, dimension, and distribution of cyanophycin granules (Figs. 3–6). All strains

analyzed showed a thin cell wall with a slight constriction at the cross walls and absence of mucilage. The number of peripherally arranged thylakoids varied from two to five. When observed in transverse sections, these thylakoids exhibited a triangular or polyhedral arrangement (Figs. 3, g–i; 4, e and f; 5, g and i; 6d). The strains showed one or two (rarely three to four) cyanophycin granules in all strains, situated close to the cross walls (Figs. 3, a–e; 4, a, b, and d; 5b; 6a,b). Only the strain BCCUSP96 had some trichomes with four granules per cell (Table 2) and was also characterized by the highest cell length to width ratio (L:W = 4.5). Carotenoid inclusions were observed near the cross walls. Polyphosphate bodies in the studied strains were rare. When present, they were variable in size (Figs. 3, c, d, and f; 4, a–c; 5c; 6, a–d) and distributed irregularly in the cell. The strain BCCUSP96 showed in some trichomes cells with some peculiarities, such as slightly thickening of the apical cell similar to a calyptra (Fig. 5, d, e), a wavy cell wall (Fig. 5h), and unidentified cytoplasmic inclusions similar to polysaccharide granules distributed all over the cell (Fig. 5, f, g).

Molecular data. The sequences for *cpc*BA region were obtained for 10 isolates of *Geitlerinema* (Table S1). Of those, the spacer region between *cpc*B and *cpc*A genes had from 291 to 293 bp for seven isolates from Brazil, plus one from Spain and three sequences obtained from GenBank (AM048623, AM048624, and AF212923). For two isolates from Brazil, *G. unigranulatum* BCCUSP352 and BCCUSP94, the spacer region was much shorter, presenting 95 bp. The sequence identity among the Brazilian isolates with the longer spacer region was between 96.5% and 100%, and among the Brazilian isolates with the longer spacer region and the three sequences in the GenBank, the identity was between 94.5% and 97.8%. The sequence identity between the isolates with the short spacer region (*G. unigranulatum* BCCUSP352 and BCCUSP94) and the isolates with the longer spacer region, not considering the indel, was from 90.1% to 92.4%.

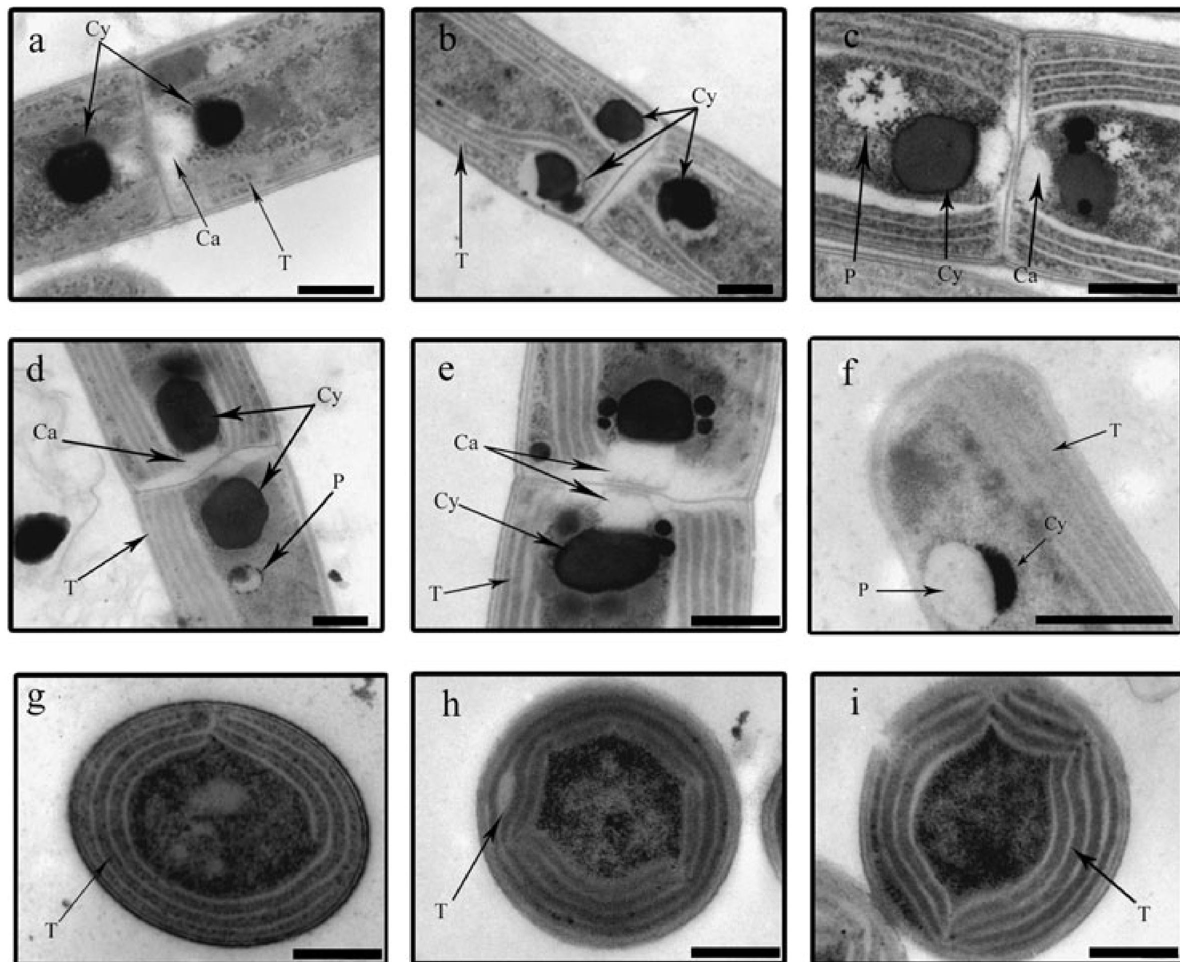

Fig. 3. Brazilian strains of *Geitlerinema amphibium* in longitudinal (a–f) and transverse (g–i) sections of trichomes. Thylakoids are peripheral (parietal) with longitudinal folds involving several laminae, appearing triangular to polygonal in transverse sections; cyanophycin and carotenoid granules are close to the cellular cross walls. (a) Strain BCCUSP95; (b, c, and g) strain BCCUSP54; (d) strain BCCUSP85; (e, h, i) strain BCCUSP91; (f) strain BCCUSP54 showing apical cell. Ca, carotenoid granule; Cy, cyanophycin granule; P, polyphosphate body; T, thylakoid. (a–e and g–i) Scale bar, 500 nm. (f) Scale bar, 1 μm.

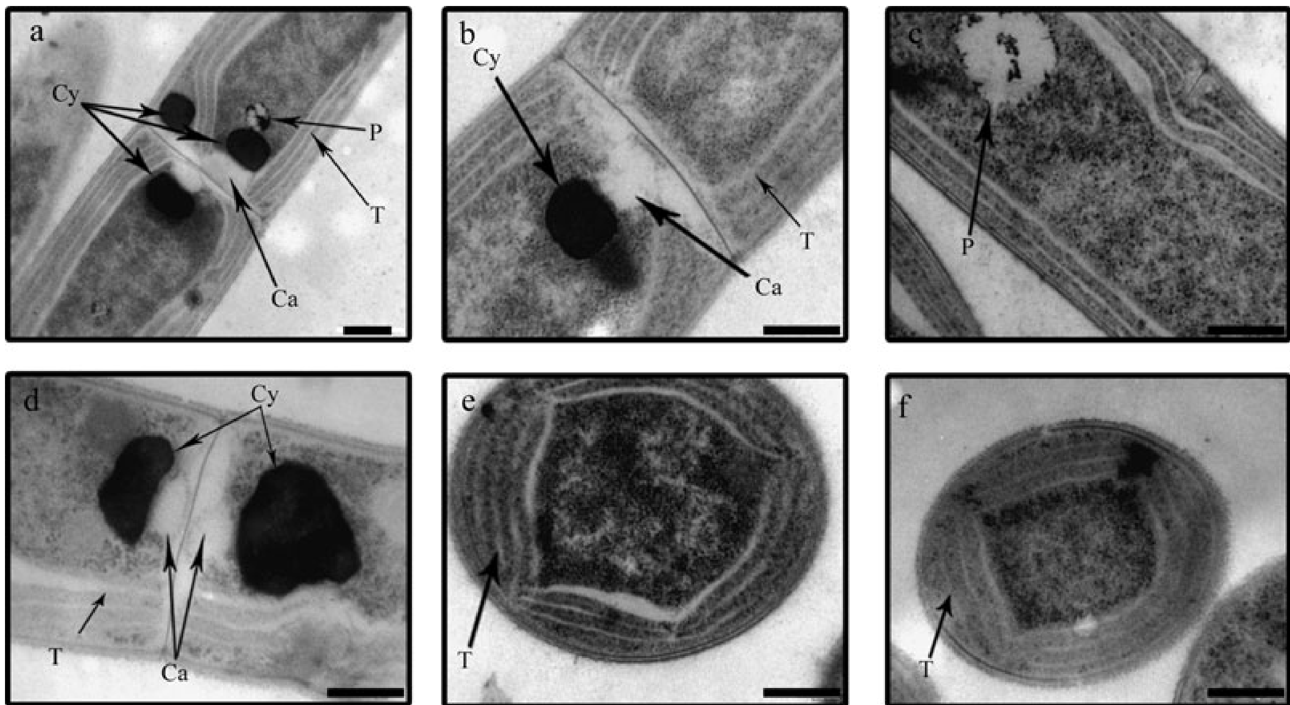

Fig. 4. Brazilian strains of *Geitlerinema unigranulatum* in longitudinal (a–d) and transverse (e–f) sections of trichomes, showing peripheral thylakoids and granular inclusions similar in structure and distribution to *G. amphibium* (a) strain BCCUSP350; (b, c, and e) strain BCCUSP47; (d and f) strain BCCUSP350. Ca, carotenoid granule; Cy, cyanophycin granule; P, polyphosphate body; T, thylakoid. Scale bar, 500 nm.

Four different phylogenetic inference methods were used to generate both rooted and unrooted trees. The trees recovered by MP, NJ, ML, and Bayesian analysis without the addition of an outgroup were very similar and had no significant differences (Fig. 7), as were the rooted trees (Fig. 8). When an outgroup was added, the clades remained the same compared with the unrooted tree (Fig. 8). The two isolates with the shorter spacer region (*G. unigranulatum* BCCUSP352 and BCCUSP94) were basal to all the other sequences with the longer spacer region. The sequence AF212923 from Italy appears basal to the other long-spacer sequences. The two sequences from Africa (AM048623 and AM048624) are more closely related to the Brazilian isolates (Figs. 7 and 8). The two taxa *G. amphibium* and *G. unigranulatum* are mixed in the trees and could not be distinguished as monophyletic entities.

DISCUSSION

Morphological data. G. amphibium and *G. unigranulatum* show morphological properties with overlapping characteristics, which make taxonomic discrimination difficult. According to Komárek and Anagnostidis (2005), the variation interval of the *G. amphibium* cellular width ranges from (1.0) 1.8 to 3–4 μm, whereas for *G. unigranulatum,* it ranges from 0.8 to 2.4 μm. The means of the studied populations formed two clusters, although the maximum values for the smaller species overlapped with the minimum values for the larger one, thus leaving no gap in cell width between the two taxa. 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 is the distinction more accentuated. One or two cyanophycin granules per cell (less frequently three) were positioned near the cross walls in strains attributable to both species. 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.

Our EM observations of studied strains showed structures that are not different from those found in other oscillatorialean species (e.g., cyanophycin granules, carotenoids, polyphosphate bodies, polysaccharides, and thylakoids; Bourrelly and Couté 1975, Guglielmi and Cohen-Bazire 1984). The

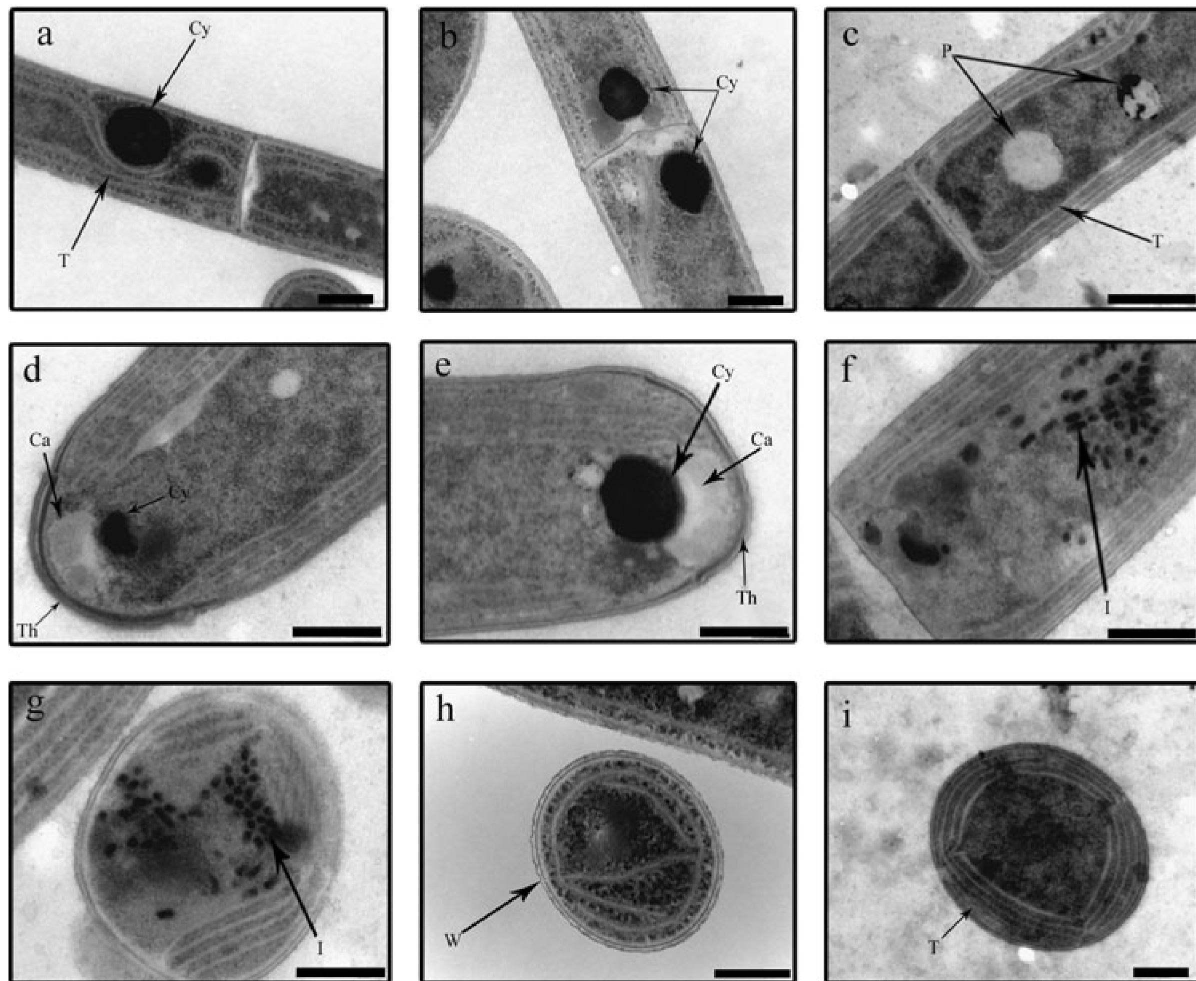

Fig. 5. Strain BCCUSP96 of *Geitlerinema unigranulatum* in longitudinal (a–f) and transverse (g–i) sections of trichomes, showing peripheral thylakoids and granular inclusions arranged similar to other strains, but also peculiar distinctive properties, especially a thickening of the apical cell wall (d and e), unidentified cell inclusions similar to polysaccharide granules distributed throughout the cells (f and g), and undulating external wall layer (h). Ca, carotenoids; Cy, cyanophycin granule; I, inclusions; P, polyphosphate body; T, thylakoid; Th, thickening in the apical cell; W, cell wall. Scale bar, 500 nm.

abundance of polyphosphate bodies and the presence of extended thylakoids observed in Romo et al. (1993b) and Komárek and Azevedo (2000) have not been found in our study. The low incidence of polyphosphate bodies might be related to the exponential growth phase and with the concentration of nutrients used in our study.

The results obtained did not show ultrastructural differences between *G. amphibium* and *G. unigranulatum* strains, except for the BCCUSP96, which in some trichomes exhibited slight thickening of the apical cell, slightly folded cellular wall, thylakoids with invaginations, and unidentified granules. The BCCUSP96 strain was unique in having four granules per cell in some trichomes and the highest cell length-to-width ratio.

Molecular data. The sequences of *G. amphibium* and *G. unigranulatum*, eight strains together with three sequences obtained from GenBank (AF212923, AM048623, AM048624), formed a monophyletic group. The high similarity of the *cpc*BA sequences indicates that the sequences in GenBank were misidentified as *Planktothrix* sp. or *Oscillatoria* sp. For the *Planktothrix* sp. FP1 (AF212923), this misidentification is supported by the micrographs of the trichomes presented in Pomati et al. (2000, p. 555, fig. 18). The sequences AM048623 and AM048624 were identified as ''*Oscillatoria* sp.,'' strains CYA469 and CYA479, respectively, are only cited in GenBank and are probably *Geitlerinema* sp. due to their sequence similarity.

The observed characteristics of morphometry, cytology, geography, or environment were not sufficient to distinguish the two taxa. And these results were further corroborated by the analysis of the *cpc*BA sequences. The overlapping similarities of both taxa is corroborated in part by data presented in Bittencourt-Oliveira et al. (2007) using the fingerprint molecular technique using HIP1 sequences.

Fig. 6. Strain BCCUSP352 of *Geitlerinema unigranulatum* in transverse (a–b) and longitudinal (c–d) sections of trichomes, showing peripheral thylakoids, polyphosphate body, cyanophycin and carotenoid granules close to the cellular cross wall and apical cell. Ca, carotenoid granule; Cy, cyanophycin granule; P, polyphosphate body; T, thylakoid. (a, b, and d) Scale bar, 500 nm. (c) Scale bar, 1 μm.

Fig. 7. Consensus unrooted maximum-likelihood (ML) tree based on *cpc*BA region. Bootstrap values for the different analyses are plotted on the branches: neighbor joining (NJ, first line, normal typeface) and maximum parsimony (MP, second line, italic typeface) were performed for 2,000 replicates; maximum likelihood (ML, third line, bold typeface) was performed for 100 replicates. The posterior probabilities (when >95%) inferred from Bayesian analysis are shown as thicker branches. For sample identification, see Table S1 in the supplementary material. AF212923 (''*Planktothrix* sp.'' FP1), AM048623 (''*Oscillatoria* sp.'' CYA469), AM048624 (''*Oscillatoria* sp.'' CYA479), AY575949 (*Spirulina subsalsa* PD2002/gca).

Strains of different origins, such as *G. amphibium* (BCCUSP31 strain) from Spain and BCCUSP79 from Brazil, present high sequence similarity. For *G. unigranulatum* BCCUSP96, despite the presence

Fig. 8. Consensus neighbor-joining (NJ) tree based on *cpc*BA region. Bootstrap values for the different analyses are plotted on the branches: neighbor joining (NJ, first line, normal typeface) and maximum parsimony (MP, second line, italic typeface) were performed for 2,000 replicates; maximum likelihood (ML, third line, bold typeface) was performed for 100 replicates. The posterior probabilities (when >95%) inferred from Bayesian analysis are shown as thicker branches. For sample identification, see Table S1 in the supplementary material. AF212923 (''*Planktothrix* sp.'' FP1), AM048623 (''*Oscillatoria* sp.'' CYA469), AM048624 (''*Oscillatoria* sp.'' CYA479), AY575949 (*Spirulina subsalsa* PD2002/gca).

of trichomes with cytomorphological differences, such as the presence of calyptra and four cyanophycin granules per cell, the sequences are highly similar to *G. amphibium* strain BCCUSP85, differing from what was reported in Bittencourt-Oliveira et al. (2007).

The fingerprint molecular technique using HIP1 (Bittencourt-Oliveira et al. 2007) corroborated the grouping of BCCUSP94 and BCCUSP352, and BCCUSP87, BCCUSP79, and BCCUSP54.

G. unigranulatum BCCUSP94 and BCCUSP352 sequences are the most distinct from all other strains, even if the indel in the spacer region is not considered. In spite of the morphological similarity of those two strains with the other ones, the molecular data based on PC-IGS sequences indicate that these strains could be a different species of *Geitlerinema*. Six et al. (2007) and Haverkamp et al. (2008) presented evidence for lateral transfer in *Synechococcus* strains based on the analysis of PC and 16S rRNA genes. Lateral transfer of genes can be mediated by natural transformation or virus infection. The photosynthetic genes are frequently found in cyanophages (Lindell et al. 2004). Therefore, it is premature to move the strains BCCUSP352 and BCCUSP94 into a new species without analyzing the ribosomal genes to confirm their identity.

Using morphology, cell ultrastructure, and sequences, it is not possible to distinguish *G. amphibium* and *G. unigranulatum*. Therefore, they should be treated as one species, *G. unigranulatum* as a synonym of *G. amphibium*.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Strains of *Geitlerinema amphibium* and *G. unigranulatum* used in this study.

This material is available as part of the online article.

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