Phyllonema aviceniicola gen. nov., sp. nov. and *Foliisarcina bertiogensis* gen. nov., sp. nov., epiphyllic cyanobacteria associated with *Avicennia schaueriana* leaves

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Cyanobacteria dwelling on the salt-excreting leaves of the mangrove tree *Avicennia schaueriana* were isolated and characterized by ecological, morphological and genetic approaches. Leaves were collected in a mangrove with a history of oil contamination on the coastline of São Paulo state, Brazil, and isolation was achieved by smearing leaves on the surface of solid media or by submerging leaves in liquid media. Twenty-nine isolated strains were shown to belong to five cyanobacterial orders (thirteen to *Synechococcales*, seven to *Nostocales*, seven to *Pleurocapsales*, one to *Chroococcales*, and one to *Oscillatoriales*) according to morphological and 16S rRNA gene sequence evaluations. More detailed investigations pointed six Rivulariacean and four Xenococcacean strains as novel taxa. These strains were classified as *Phyllonema* gen. nov. (type species *Phyllonema aviceniicola* sp. nov. with type strain CENA341^T) and *Foliisarcina* gen. nov. (type species *Foliisarcina bertiogensis* sp. nov. with type strain CENA333^T), according to the International Code of Nomenclature for Algae, Fungi, and Plants. This investigation shows some of the unique cyanobacteria inhabiting the phyllosphere of *Avicennia schaueriana* can be retrieved by culturing techniques, improving current taxonomy and providing new insights into the evolution, ecology, and biogeography of this phylum.

Phyllosphere, the external surface of plant leaves, is a habitat that has traditionally received low attention in microbial ecology, with most of the initial research being primarily focused on the study of plant–pathogen interactions in cultures of economic interest (Lindow & Brandl, 2003; Belkin *et al.*, 2010). Though still lagging behind rhizosphere studies, phyllosphere research has been a subject of increased interest in recent years (Vorholt, 2012; Rastogi *et al.*, 2013).

Micro-organisms in the phyllosphere face several challenges. They are in direct contact with the cuticle, a barrier for the release of water, ions and nutrients to the exterior of the plant, and are subject to fluctuations in temperature, UV radiation, wind, moisture and relative humidity varying in scales ranging from seconds to hours (Hirano & Upper, 2000; Schreiber *et al.*, 2004). The phyllosphere from *Avicennia* mangroves presents even more unique conditions. To maintain their osmotic balance, these trees eliminate up to 90 % of the salt absorbed from seawater through a transpiration current carrying it from the roots to glands on the abaxial epidermis of leaves, which in turn release it on the leaf surface, sometimes resulting in crystals visible to the naked eye (Drennan & Pammenter, 1982; Fitzgerald *et al.*, 1992). Moreover, microbial communities in the *Avicennia* phyllosphere may be subjected to volatile organic compounds produced by aerial parts of the hosts, including some with antimicrobial activity (Bobbarala *et al.*, 2009; Junker & Tholl, 2013).

In spite of the unfavourable conditions frequently found in these habitats, a number of micro-organisms are capable of

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CENA315–CENA348 are KT731136–KT731164, respectively.

Two supplementary figures are available with the online Supplementary Material.

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tolerating environmental stress in the phyllosphere and establishing diverse and complex microbiomes, with several consequences for the host plants and their ecosystem (Gau *et al.*, 2002; Peñuelas & Terradas, 2014). Up to 10^7 bacterial cells per cm^2 can be detected in the leaf surface of some plants, and many of them pertain to taxa which have not been studied yet and which may possibly present unique adaptations to survival on this hostile habitat (Lindow & Leveau, 2002). Survival of bacterial communities in the phyllosphere depends mainly on carbon, nitrogen and essential inorganic nutrients released on the leaf surface (Leveau & Lindow 2001; Miller *et al.* 2001). However, as cyanobacteria usually have lower nutritional requirements due to their ability to fix atmospheric carbon and (in some taxa) nitrogen, they are less dependent on the plant exudates. Cyanobacteria have been found to be the main nitrogen-fixing epiphytes in the phyllosphere of some tropical vascular plants, promoting a significant input of bioavailable nitrogen into these environments (Freiberg, 1998; Fürnkranz *et al.*, 2008). This trait constitutes a significant ecological advantage that also facilitates the establishment of heterotrophic and non-diazotrophic organisms in these habitats.

Mangroves host several cyanobacteria with important ecological roles, including a considerable number of undescribed taxa (for a review, see Alvarenga *et al.*, 2015). A study using culture-independent methods to assess the diversity of cyanobacteria inhabiting leaf surfaces of mangrove trees observed the phyllosphere of *Avicennia schaueriana* was colonized by a unique cyanobacterial community (Rigonato *et al.*, 2012). In order to investigate these findings further and to access unknown taxa, the present study was undertaken with the purposes of isolating and characterizing cyanobacteria inhabiting the phyllosphere of *Avicennia schaueriana* trees from a Brazilian mangrove forest.

Leaves of three adult trees identified as *Avicennia schaueriana* Stapf & Leechman were collected at a trunk height of approximately 1.75 m on 25 March 2008 (early Autumn) from trees at the margin of the Iriri river (23° 53′ 50.4″ S 46° 12′ 30.6″ W), along the Bertioga channel, on the coastline of São Paulo, Brazil. After detachment from the trees, leaves were packed in sterile plastic bags and kept at 4 °C until the moment of processing. The Bertioga mangrove is close to a seaside resort, and hence it is subject to the influence of human activities. In addition, the Iriri river mangrove was impacted by an accident during the construction of the SP-55 road on 14 October 1983 when it received a large volume of crude oil from a broken pipeline, an event which added further complexity to its conditions. The isolation of cyanobacteria from the surfaces of the sampled leaves was achieved using the culture medium BG-11 (Allen, 1968) and three modifications of this medium: BG-11_0 (Stanier *et al.*, 1971), lacking nitrogen; SWBG-11 (Castenholz, 1988), simulating salt water; and SWBG-11_0 (nitrogen-free SWBG medium), lacking nitrogen and simulating salt water. Individual leaves were either smeared and placed on the surface of solid media contained within Petri dishes, or were submerged into liquid media within 125 ml Erlenmeyer flasks. Five replicates of each medium were used. The Petri dishes and Erlenmeyer flasks were kept at a temperature of 25 ± 1 °C with 14 : 10 h light/dark cycles using fluorescent light (20 μmol photons·m^{-2}·s^{-1}).

Growth of inoculated material was constantly monitored with an Axiostar Plus light microscope (Zeiss). After confirmation of cyanobacterial growth, colonies were purified by constant transfers to fresh sterile solid media and incubated under the aforementioned conditions, until each culture was free of other cyanobacteria and eukaryotic organisms. Cycloheximide (Sigma-Aldrich) was added to media at a final concentration of 75 mg·ml^{-1} to inhibit growth of eukaryotes. Whenever possible, the leaf side of origin of the isolate was noted. Cyanobacterial isolates were studied under the Axioskop 40 light microscope (Zeiss) for the evaluation of morphological features of taxonomic interest and comparison to previously described taxa (Komárek & Anagnostidis, 1998, 2005; Komárek, 2013). Detailed descriptions of novel genera and species were produced and their taxonomic placement was determined according to the classification system proposed by Komárek *et al.* (2014). Isolates were photographed by an Olympus BX53 optical microscope equipped with differential interference contrast and imaging systems (Olympus). Afterwards, cells were fixed with 1 ml modified Karnovsky solution for 64 h at 4 °C (Karnovsky, 1965) and post-fixed with 1 % osmium tetroxide for 1 h at room temperature. Fixed cells were subjected to pre-staining with 2.5 % uranile acetate for 18 h at 4 °C, followed by dehydration with acetone solutions at increasing concentrations. Spurr resin (Electron Microscopy Sciences) was used for the infiltration and polymerization of samples (Spurr, 1969). Resin blocks were cut into 600–1000 μm ultrathin sections in a Porter Blum MT-2 ultramicrotome (Sorvall Instruments), which were collected with 200-mesh copper grids covered with 5 % colodium. After staining with uranile acetate and lead citrate, the samples were observed and photographed in a Zeiss EM 900 electron transmission microscope at 50 kV.

DNA extraction was carried out according to Fiore *et al.* (2000). The extracted DNA was used for PCR amplification of the 16S rRNA gene under previously described conditions using the primers 27F1 and 1494Rc (Neilan *et al.*, 1997) in a Techne TC-412 thermocycler (Bibby Scientific). Amplicons were ligated into pGEM-T Easy Vector Systems (Promega), inserted into *Escherichia coli* DH5α chemocompetent cells, and plated for blue-white colony screening (Sambrook & Russell, 2001). Sequencing according to the method of Sanger *et al.* (1977) was performed in the ABI PRISM 3100 Genetic Analyzer (Life Technologies). Consensus sequences were generated with the Phred/Phrap/Consed software package (Ewing & Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998) and only gene regions with base-calling qualities over Phred 20 were considered.

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Multiple sequence alignments were performed by CLUSTAL W (Thompson *et al.*, 1994) and evolutionary models were estimated with jModelTest 2.1.7 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012). Reconstruction of phylogenetic trees by the maximum-likelihood algorithm (Felsenstein, 1981) was performed with RaxML 8.2.3 (Stamatakis *et al.*, 2005), which was tested by a bootstrap value of 1000 (Felsenstein, 1985). Bayesian inference (Mau *et al.*, 1999) was performed with MrBayes 3.2.5 (Ronquist & Huelsenbeck, 2003) using two separate runs, four chains and 5 000 000 Markov Chain Monte Carlo generations. The tree was visualized with Figtree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree) and edited with Inkscape 0.48.5 (https://inkscape.org).

Twenty-nine cyanobacterial strains were isolated. The majority of the strains (25) were obtained using liquid media, while only four strains grew on solid media (Table 1). Seven strains were obtained with the BG-11 medium, nine with SWBG-11, four with BG-11_0, and nine with SWBG-11_0. Eleven strains were isolated from the adaxial side of leaves and eight from the abaxial side, but no correlation between taxon and leaf side was observed; the location for the ten remaining strains could not be determined. According to morphological criteria and the cyanobacterial classification system proposed by Komárek *et al.* (2014), the strains represented five different orders: thirteen belonged to *Synechococcales*, seven to *Nostocales*, seven to *Pleurocapsales*, one to *Chroococcales* and one to *Oscillatoriales*, which were distributed among six families. Eleven strains had their genera identified as *Gloeocapsopsis*, *Nodosilinea*, *Microcoleus*, *Phormidesmis* or *Brasilonema*. Identification of the remaining 18 strains was feasible at the family level, but their genera could not be determined. Overall, phylogenetic analyses showed agreement with morphological identifications (Fig. 1).

Seven unicellular baeocyte-producing cyanobacteria were isolated in this study (CENA315, CENA331, CENA333^T, CENA337, CENA345, CENA346 and CENA348) and identified as members of the family *Xenococcaceae*. This family encompasses genera whose morphology is usually not informative since their morphological traits are unstable and their genetic diversity exceeds the described morphological diversity (Ishida *et al.*, 2001). As expected, phylogenetic reconstruction showed several genera from this cyanobacterial family are polyphyletic and require revision (Fig. 1A). Although some of these strains seem related to cyanobacteria

Table 1. Cyanobacterial strains isolated from the leaf surface of *Avicennia schaueriana*

Strain	Family	Genus	Isolation medium	Leaf side
CENA315	*Xenococcaceae*	–	liquid SWBG-11	adaxial
CENA316	*Leptolyngbyaceae*	*Phormidesmis*	liquid SWBG-11_0	unidentified
CENA317	*Leptolyngbyaceae*	*Phormidesmis*	liquid SWBG-11_0	unidentified
CENA318	*Leptolyngbyaceae*	*Phormidesmis*	liquid SWBG-11	adaxial
CENA319	*Leptolyngbyaceae*	–	liquid SWBG-11	adaxial
CENA320	*Leptolyngbyaceae*	–	liquid BG-11	adaxial
CENA321	*Leptolyngbyaceae*	–	liquid BG-11	abaxial
CENA322	*Leptolyngbyaceae*	*Nodosilinea*	liquid SWBG-11_0	unidentified
CENA323	*Leptolyngbyaceae*	*Nodosilinea*	liquid SWBG-11_0	abaxial
CENA324	*Rivulariaceae*	*Phyllonema* gen. nov.	liquid BG-11	unidentified
CENA325	*Rivulariaceae*	*Phyllonema* gen. nov.	solid BG-11_0	abaxial
CENA326	*Rivulariaceae*	*Phyllonema* gen. nov.	liquid SWBG-11_0	unidentified
CENA327	*Chroococcaceae*	*Gloeocapsopsis*	liquid SWBG-11_0	abaxial
CENA328	*Rivulariaceae*	*Phyllonema* gen. nov.	liquid BG-11	abaxial
CENA330	*Rivulariaceae*	*Phyllonema* gen. nov.	liquid SWBG-11	adaxial
CENA331	*Xenococcaceae*	*Foliisarcina* gen. nov.	liquid BG-11	abaxial
CENA332	*Leptolyngbyaceae*	*Phormidesmis*	solid SWBG-11	adaxial
CENA333^T	*Xenococcaceae*	*Foliisarcina* gen. nov.	solid SWBG-11	adaxial
CENA335	*Leptolyngbyaceae*	*Phormidesmis*	liquid SWBG-11	unidentified
CENA337	*Xenococcaceae*	*Foliisarcina* gen. nov.	liquid SWBG-11_0	abaxial
CENA339	*Leptolyngbyaceae*	*Phormidesmis*	liquid SWBG-11	unidentified
CENA340	*Leptolyngbyaceae*	–	liquid BG-11_0	adaxial
CENA341^T	*Rivulariaceae*	*Phyllonema* gen. nov.	solid BG-11_0	abaxial
CENA342	*Leptolyngbyaceae*	–	liquid BG-11	unidentified
CENA344	*Microcoleaceae*	*Microcoleus*	liquid SWBG-11	adaxial
CENA345	*Xenococcaceae*	–	liquid SWBG-11_0	unidentified
CENA346	*Xenococcaceae*	*Foliisarcina* gen. nov.	liquid SWBG-11_0	unidentified
CENA347	*Scytonemataceae*	*Brasilonema*	liquid BG-11_0	adaxial
CENA348	*Xenococcaceae*	–	liquid BG-11	adaxial

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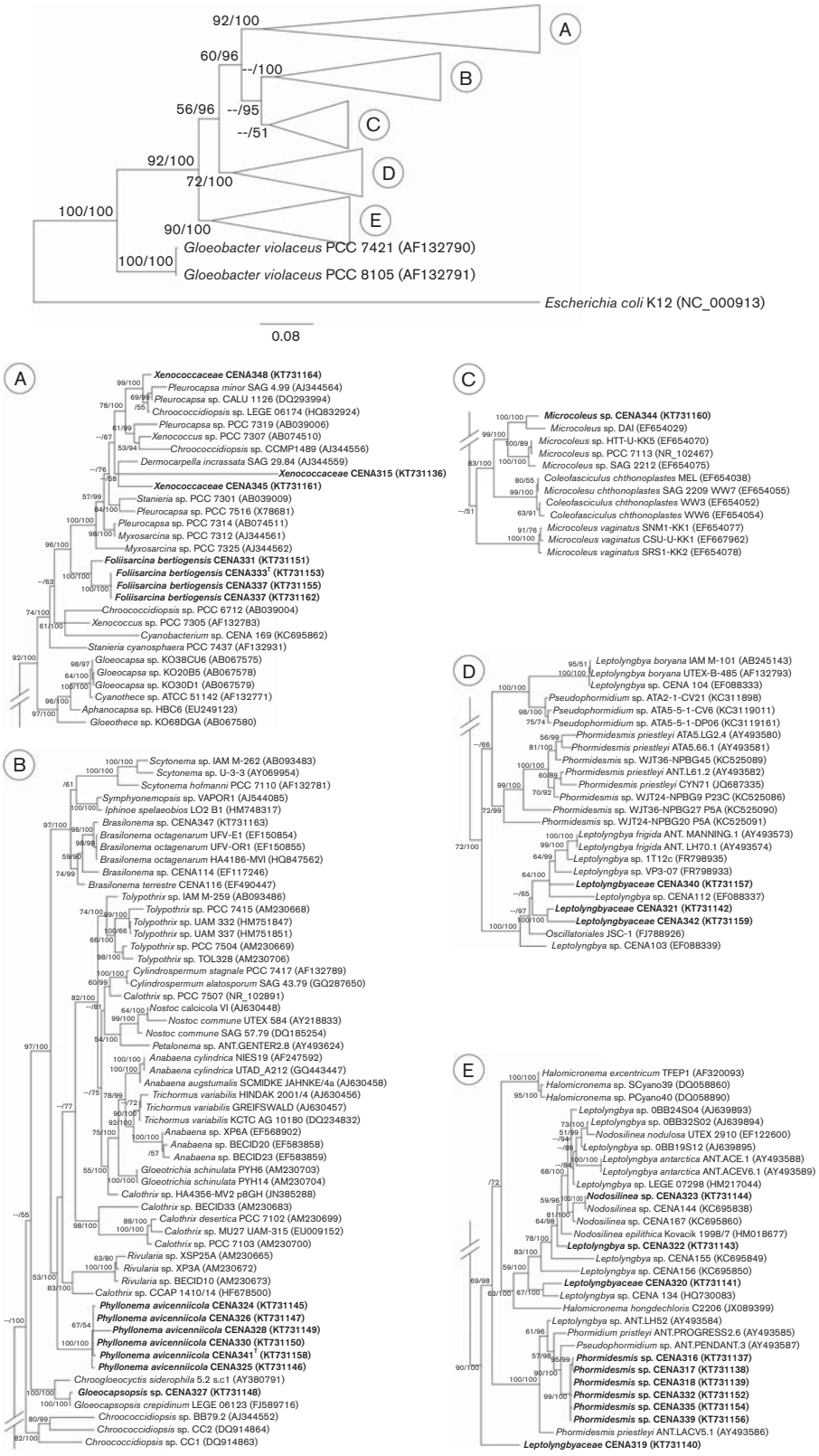

Fig. 1. Phylogenetic tree reconstructed by Bayesian inference. Strains isolated in the present work are highlighted in bold. Values>50 for bootstraps observed in maximum-likelihood topologies followed by Bayesian posterior probabilities are illustrated in the tree knots. Bar, 0.08 substitutions per nucleotide position.

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identified in previously described genera, the current polyphyletic state of a considerable number of Xenococcacean genera allowed identification only at the family level. Strains CENA331, CENA333^T, CENA337 and CENA346 had morphological traits typical of the family Xenococcaceae (Fig. 2 and Fig. S1, available in the online Supplementary Material), and grouped in a cluster close to, but phylogenetically distinct from, Xenoccocacean genera with high support from both maximum-likelihood (100 % bootstrap) and Bayesian inference (100 % posterior probability) trees (Fig. 1A). Ultrastructural analyses of strain CENA333^T revealed typical Xenococcacean thylakoid arrangements (Fig. 3a, b). Similarities between 16S rRNA gene sequences from these strains and their closest relatives are found in Table 2. The combination of ecological, morphological, ultrastructural and molecular data of these four strains allowed the proposal of

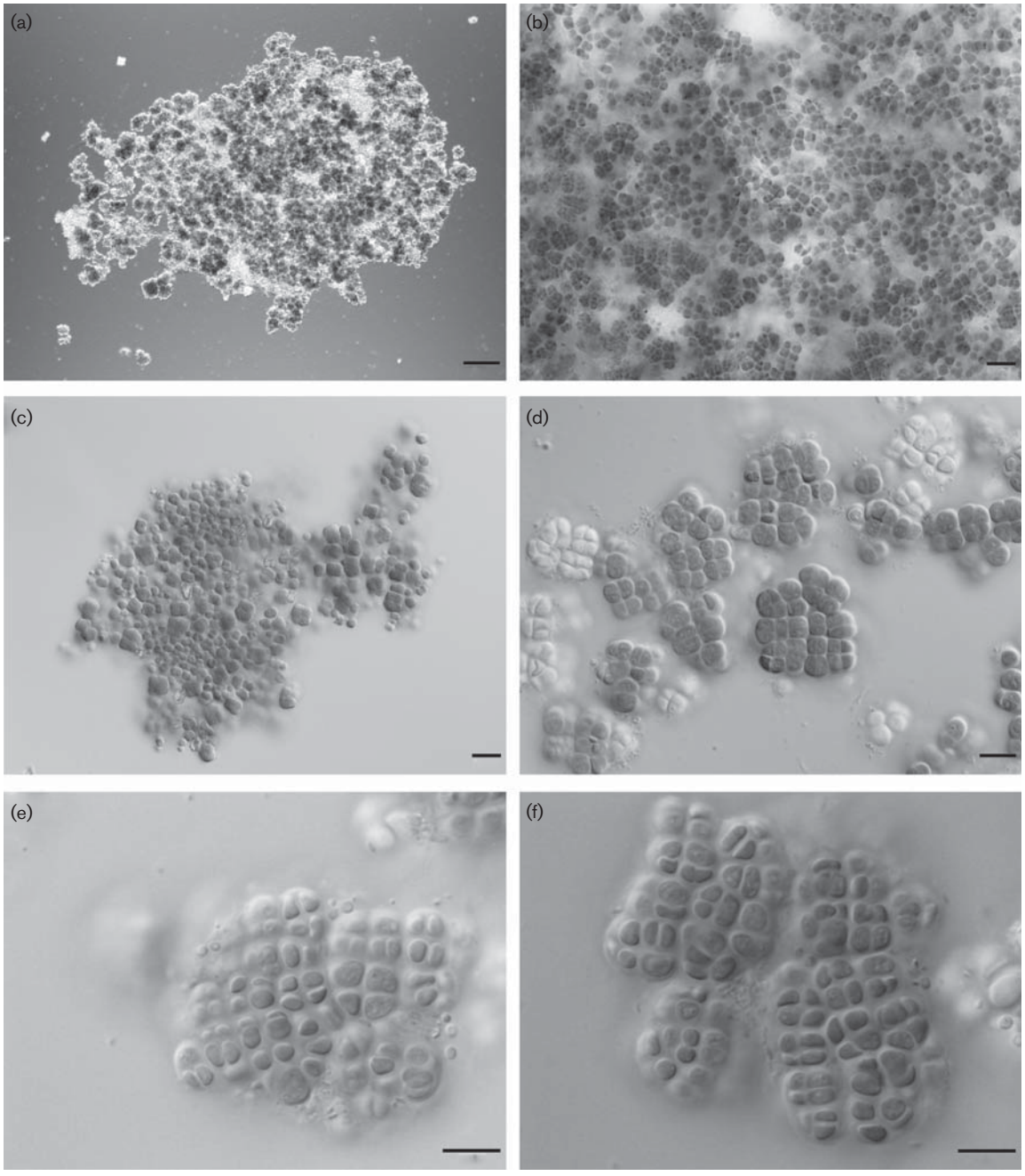

Fig. 2. Photomicrographs of *Foliisarcina bertiogensis* gen. nov., sp. nov. strains CENA337 (a, d), CENA333^T (b), CENA331 (c), and CENA346 (e, f). Bars, 100 μm (a), 20 μm (b, c, e, f), 10 μm (d). A coloured version of this figure is available as Fig. S1 in the online Supplementary Material.

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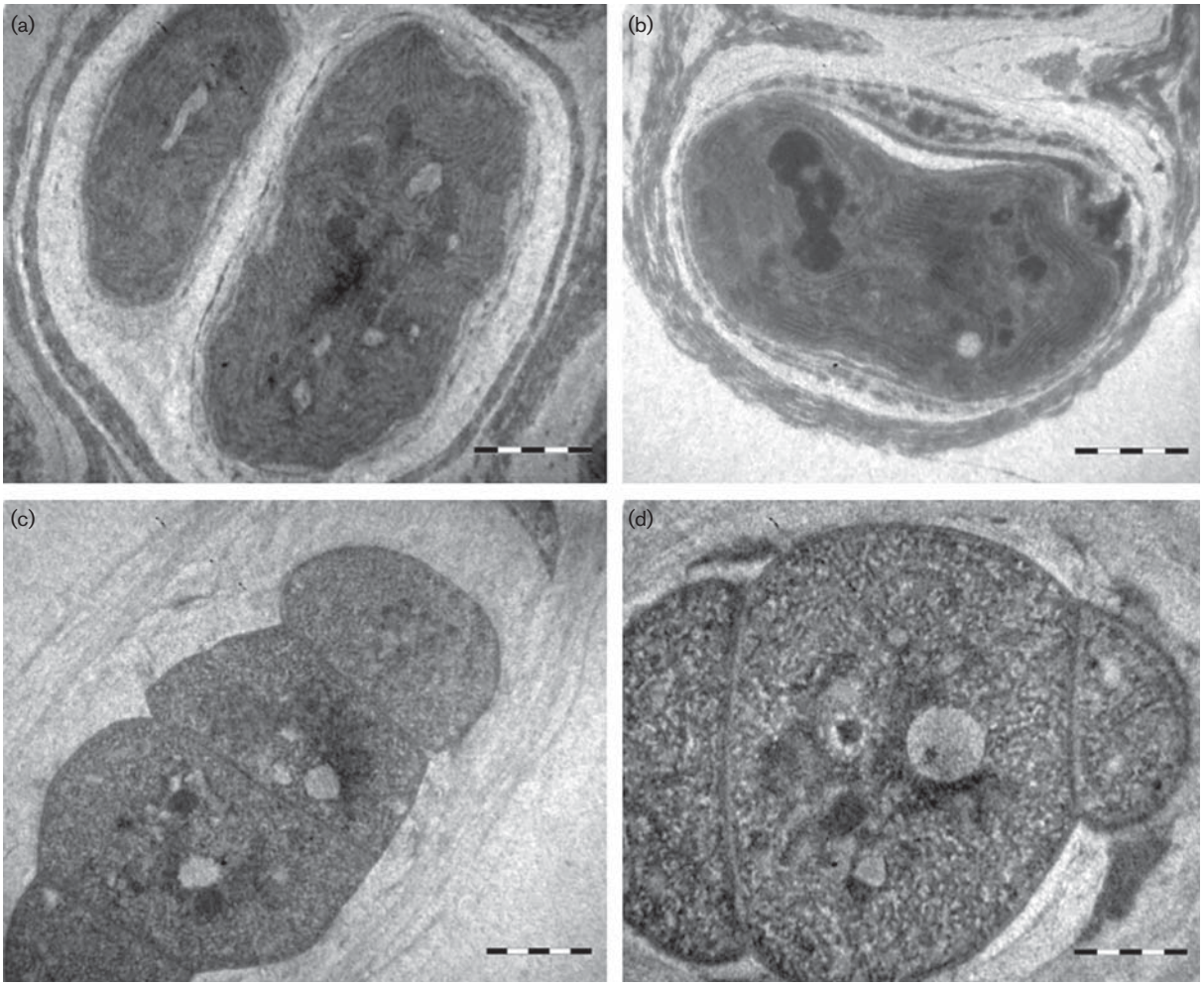

Fig. 3. Ultrastructures from strains CENA333^T (a, b) and CENA341^T (c, d) cells, highlighting thylakoid arrangements typical of the family *Xenococcaceae* and the order *Nostocales*, respectively, as proposed by Hoffmann *et al.* (2005). Bars, 1 μm (a, b, d), 2 μm (c).

Foliisarcina gen. nov. with type species *Foliisarcina bertiogensis* gen. nov., sp. nov., according to the International Code of Nomenclature for Algae, Fungi, and Plants.

Description of *Foliisarcina* gen. nov.

Etymology: *Foliisarcina* (Fo.li.i.sar.ci′na L. neut. n. *folium* a leaf; L. fem. n. *sarcina* a package; N.L. fem. n. *Foliisarcina* a package-like organism from a leaf; referring to the colony morphology of this cyanobacterium).

Description: Cells isolate or forming regular (young, with few cells) or irregular (older, with more cells) colonies with variable number of cells (usually up to 64 cells), sometimes several colonies grouped; colonial envelope thin, firm, colourless; cells usually close to each other within the colony, variable in shape; cell division by binary fission, in two or three planes in successive generations; reproduction by baeocyte formation.

Type species: *Foliisarcina bertiogensis*.

Description of *Foliisarcina bertiogensis* sp. nov.

Etymology: *Foliisarcina bertiogensis* (ber.ti.o.gen′sis N.L. fem. adj. *bertiogensis* from the Bertioga municipality, São Paulo, Brazil).

Description: Solitary cells or, more commonly, microscopic colonies (stratified) with several cells (up to 64 or more), forming multicolonial groups, irregular to squarish in outline, up to 44.2 μm (longer axis: mean 27.1 μm; n=34), with dense, more or less regularly arranged cells, clusters of four cells are recognizable within the older colonies or groups. Colonial envelope sometimes visible, thin, usually tightly surrounding the aggregates, hyaline. Cells rounded when isolate, hemispherical when in pairs, nearly squarish when in dense colonies (mutual pressure) or irregular when dividing or forming baeocytes. Isolate cells 2.1–5.7 μm diameter without sheath (mean 3.7 μm;

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Table 2. Similarity between 16S rRNA gene sequences obtained from strains of *Foliisarcina bertiogensis* gen. nov, sp. nov. and the closest relatives deposited in the NCBI GenBank database

Strain	Size (bp)	Closest sequence (NCBI access number)	Sequence similarity (%)	Coverage (%)
CENA331	1416	Unidentified cyanobacterium GI-1 (JN202625)	98	99
CENA333^T	1418	Unidentified cyanobacterium GI-1 (JN202625)	98	99
CENA337	1418	Unidentified cyanobacterium GI-1 (JN202625)	98	99
CENA346	1418	Unidentified cyanobacterium GI-1 (JN202625)	98	99

n=37), 3.6–7.1 μm diameter with sheath (mean 5.4 μm; *n*=37), cells in colonies 1.3–5.2 μm diameter. Cell content usually brownish, sometimes bright green, with few large granules or finely granular. Baeocytes are short cylindrical rounded or more or less spherical, from 1.7 μm diameter when young, with thin, hyaline sheath when a little older, content is brownish to light green.

Holotypus: SP 429275, dried material deposited at the herbarium of the São Paulo Institute of Botany, São Paulo, Brazil.

Type strain: CENA333^T.

GenBank accession number for 16S rRNA gene sequence of type strain: KT731153.

Filamentous false-branched and heterocyted strains CENA324, CENA325, CENA326, CENA328, CENA330 and CENA341^T grouped in a well-supported cluster in both phylogenetic analyses (100% bootstrap from maximum-likelihood and 100% posterior probability from Bayesian inference), clearly distinguished from clusters composed of already described genera (Fig. 1B). These cyanobacterial strains, although morphologically identified as *Rivulariaceae*, did not cluster with known genera of this family. Furthermore, they do not present terminal hairs, even in salt-less media (Figs 4 and S2). Although terminal hairs are not universally found in genera of the family *Rivulariaceae*, they are a common characteristic for members of this family. Since the terminal hair structures may be inhibited not only by phosphatase activity, but also by high salinity, as observed in a Cuban mangrove strain (Mahasneh *et al.*, 1990), it is possible their absence in the isolated strains is the result of an adaptation to the leaf surface of *Avicennia schaueriana*. The ultrastructural analysis of a representative of this novel taxon, CENA341^T, exhibited thylakoid arrangements typically observed in the order *Nostocales* (Fig. 3c, d). Similarities between 16S rRNA gene sequences from these strains and their closest relatives are found in Table 3. Therefore, the combination of ecological, morphological, ultrastructural and molecular data allowed the proposal of the new Rivulariacean genus *Phyllonema* gen. nov. with the type species *Phyllonema aviceniicola* gen. nov., sp. nov., according to the International Code of Nomenclature for Algae, Fungi, and Plants.

Description of *Phyllonema* gen. nov.

Etymology: *Phyllonema* (Phyl.lo.ne′ma Gr. neut. n. *phyllon* leaf; Gr. neut. n. *nema* thread; N.L. neut. n. *Phyllonema* a thread from a leaf; reference to the typical habitat of this filamentous cyanobacterium).

Description: Filaments in groups not surrounded by abundant mucilage or gelatin, without radial disposition, entangled, long; branches rare; sheath present, usually thin and hyaline, opened at the apex; trichomes heteropolar, usually constricted; cells of variable length, shorter or longer than wide; heterocytes basal or intercalary, single or in pairs, variable in shape; necridia present; akinetes absent; phyllosphere inhabitant.

Type species: *Phyllonema aviceniicola*.

Description of *Phyllonema aviceniicola* sp. nov.

Etymology: *Phyllonema aviceniicola* [a.vi.cen.ni.i′co.la N.L. fem. n. *Avicennia* a tree genus; L. suff. *-cola* (from L. n. *incola*), inhabitant dweller; N.L. n. *avicenniicola* inhabiting *Avicennia* trees; in reference to the host of the species].

Description: Filaments in groups (culture), up to 535 μm long, 3.2–13.0 μm diameter (mean 6.49 μm; *n*=112),

Table 3. Similarity between 16S rRNA gene sequences from strains of *Phyllonema aviceniicola* gen. nov., sp. nov. and the closest relatives

Strain	Size (bp)	Closest sequence (NCBI access number)	Sequence similarity (%)	Coverage (%)
CENA324	1414	Uncultured bacterium clone YF930 (KF037928)	94	100
CENA325	1415	*Calothrix* sp. 336/3 (CP011382)	94	100
CENA326	1414	*Calothrix* sp. 336/3 (CP011382)	94	100
CENA328	1401	*Scytonema* sp. '*Coccocarpia* sp. kj30 cyanobiont' (KF359679)	94	96
CENA330	1414	*Calothrix* sp. 336/3 (CP011382)	94	100
CENA341^T	1414	*Calothrix* sp. 336/3 (CP011382)	94	100

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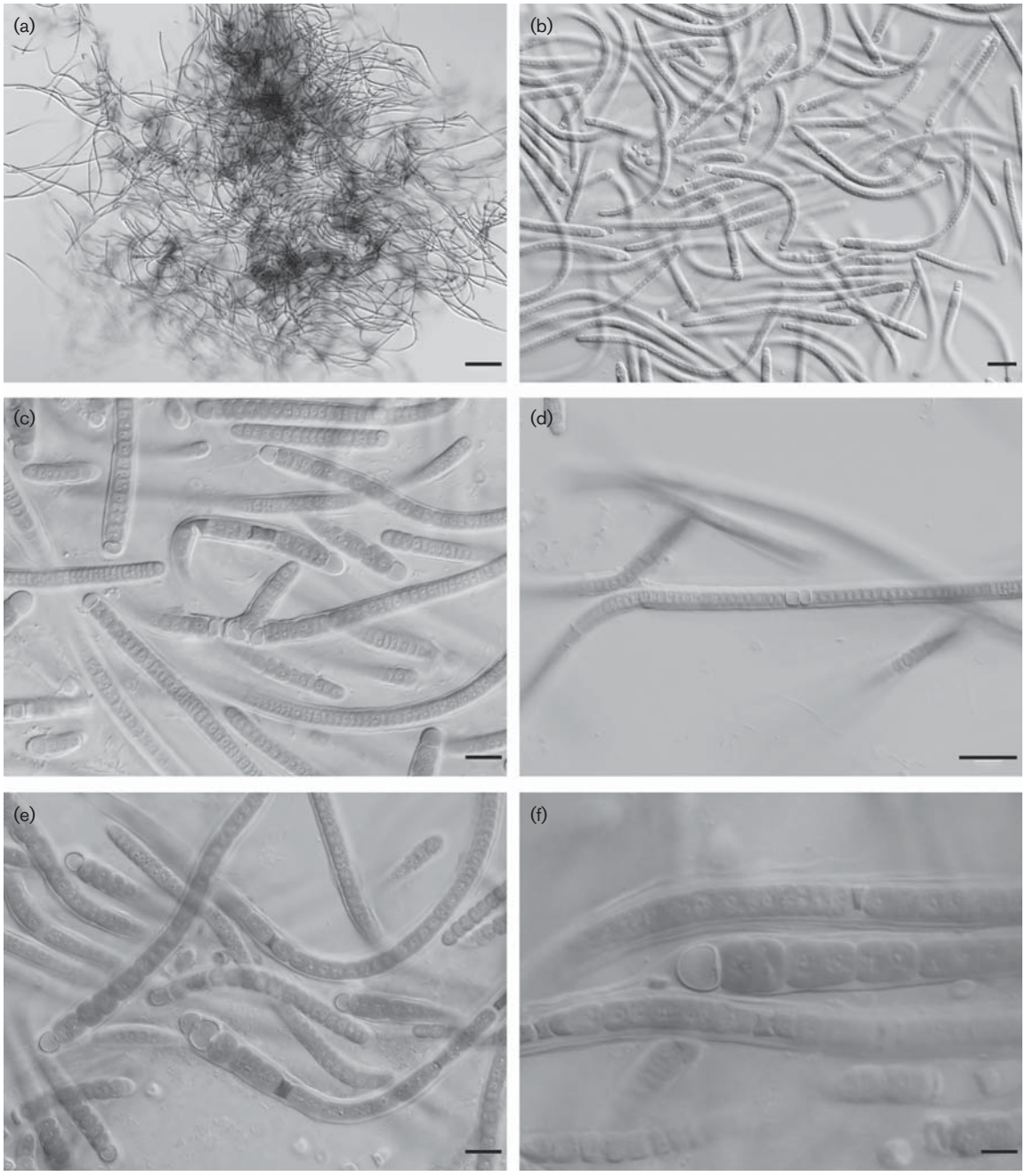

Fig. 4. Photomicrographs of *Phyllonema aviceniicola* gen. nov., sp. nov. strains CENA326 (a), CENA324 (b), CENA328 (c), CENA340 (d), and CENA341^T (e, f). Bars, 100 μm (a), 20 μm (b, d), 10 μm (c, e), 5 μm (f). A coloured version of this figure is available as Fig. S2 in the online Supplementary Material.

commonly narrowed towards the ends, rarely branched. Sheath usually thin (widened and lamellate sheaths occasionally found), firm, colourless, lamellate, opened at the apex of the adult filament. Trichomes long and sharply narrowed towards the ends in older trichomes, short but not so evidently attenuated in young ones (cylindrical trichomes are not rare), distinctly constricted at cross-walls, without hairs. Cells 1.7–8.1 μm long (mean 4.6 μm; n=138), 4.1–8.5 μm diameter (mean 6.1 μm), length/diameter ratio from 0.3 to 1.3 at swallowed region; 1.5–6.0 μm long (mean 3.0 μm), 3.5–6.1 μm diameter (mean 4.7 μm), length/diameter ratio from 0.3 to 1.2 at the middle of trichomes. Cell content blue-green to light green, centroplasm and chromatoplasm evident, large granules present.

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Heterocytes basal, single or in pairs, occasionally in the middle of the trichome (when intercalary and in pairs possibly a trichome fragmentation spot), very variable in shape and size, usually conical, but also rounded, hemispherical or cylindrical, 3.6–9.1 (–11.8) μm long, 4.1–9.0 μm diameter at the longer axis.

Holotypus: SP 429276, dried material deposited at the herbarium of the São Paulo Institute of Botany, São Paulo, Brazil.

Type strain: CENA341^{T}.

GenBank accession number for 16S rRNA gene sequence of type strain: KT731158.

Strains CENA319, CENA320, CENA321, CENA340 and CENA342 presented morphological traits similar to those traditionally described for *Leptolyngbya*, which are usually characterized for their morphological simplicity. Notwithstanding, this simplicity makes it difficult to assure their identification in this genus since *Leptolyngbya* is a polyphyletic genus (Taton *et al.*, 2006; Johansen *et al.*, 2011). Sequences of *Leptolyngbya*-like strains were scattered in the phylogenetic tree, far from the *Leptolyngbya boryana* clade (Fig. 1D, E), the type species of this genus. The most divergent sequence observed was CENA319, which behaves as an outgroup to the *Nodosilinea*, *Leptolyngbya*, and *Phormidesmis* clades (Fig. 1E). These observations led us to maintain identification at the family level for these strains.

Phylogenetic analyses indicate it is possible that at least some of the strains isolated in this work whose genera could not be determined (CENA315, CENA319, CENA 320, CENA321, CENA340, CENA342, CENA345 and CENA348), might also be representatives of novel cyanobacterial taxa. However, most of these strains are not closely related. With single representatives, the description of novel taxa is more difficult, and therefore these strains will be further explored elsewhere.

Historically, cyanobacterial systematics has been through several modifications, which were intensified after the adoption of polyphasic approaches. In this approach, classical morphological analyses are supplemented by other methods that provide alternative views on variation between organisms, and a consensus between them is achieved (Vandamme *et al.*, 1996; Komárek, 2006; Komárek *et al.*, 2014). This approach allowed to assert with higher confidence, the relationship between the members of the phylum Cyanobacteria and to produce more solid taxonomic revisions. The present work describes two novel cyanobacterial genera that, despite presenting subtle morphological variation when compared to previously described genera, are clearly distinguished from known taxa when additional factors are also taken into consideration.

Foliisarcina morphologically resembles *Cyanosarcina* Kováčik and *Chroococcidiopsis* Geitler. Despite morphological similarities, *Foliisarcina* differs from *Cyanosarcina* due to the production of baeocytes, since *Cyanosarcina* does not form baeocytes or nanocytes. The distinction between *Foliisarcina* and *Chroococcidiopsis* is more complicated or even not possible by using morphological markers alone, considering that the characteristics of colonies and cells, growth patterns and reproduction strategies are quite similar between species of both genera. Although there are several coincidences in morphology, there is a strong background for erecting *Foliisarcina* as a new genus based on ecological and molecular characteristics.

Morphologically, *Phyllonema* is very close to *Hassalia* Berkeley ex Bornet et Flahault. These genera share features such as short cells, firm, conspicuous and sometimes lamellate sheath, and filaments that are not very long and slightly attenuated to the apex. The two genera differ by the frequency of branching, which is more frequent in *Hassalia* than in *Phyllonema*; however, considering that *Phyllonema* is described based on cultured material, this characteristic cannot be assured in samples obtained directly from nature. Once again, DNA sequences are determining in the distinction between *Phyllonema* and other genera.

A previous study reporting the cyanobacterial diversity in the phyllosphere of *Avicennia schaueriana* and other mangrove trees using culture-independent analyses uncovered a considerable number of unknown cyanobacteria in these habitats (Rigonato *et al.*, 2012). This finding was furthered in the present study by methods providing direct access to part of this diversity, as evidenced by the isolation of *Phyllonema aviceniicola* and *Foliisarcina bertiogensis* strains, herein described for the first time. Therefore, this study confirmed some of the unique cyanobacteria inhabiting the phyllosphere of *Avicennia schaueriana* can be retrieved by the use of culturing techniques, a fact that raises several new possibilities for cyanobacterial taxonomy and mangrove microbiological research.

To our knowledge, with the exception of *Avicennia*, only two other trees with salt-excreting leaves have had their phyllosphere microbial communities studied to date, *Atriplex halimus* (Simon *et al.*, 1994) and *Tamarix* sp. (Qvit-Raz *et al.*, 2008, 2012; Finkel *et al.*, 2011). However, no cyanobacteria were detected in the samples evaluated. The results of culturing and culture-independent studies on *Avicennia schaueriana* trees suggest this absence could be a consequence of lower abundance or methodological bias, since cyanobacteria are able to withstand and thrive on the conditions found in at least some salt-rich leaf surfaces.

Bacteria are found mainly at the epidermal cell-wall junctions, the stomata and the base of trichomes (Whipps *et al.*, 2008). The diversity of microbial communities in the phyllosphere may also sometimes be positively correlated with herbivory by insects (Humphrey *et al.*, 2014). Usually, higher bacterial abundance in the phyllosphere is found in contact with the abaxial leaf surface

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due to a higher density of stomata and trichomes, and to the presence of a thinner cuticle in this leaf side (Whipps *et al.*, 2008). Although *Avicennia schaueriana* excretes a higher amount of salt on the abaxial leaf epidermis due to its higher concentration of salt glands (Fitzgerald *et al.*, 1992), it seems the salt concentration did not influence the distribution of cyanobacteria on the leaves. However, this observation may not be accurate since the origin of some strains could not be determined and due to the known bias inherent to cultivation techniques (Ward *et al.*, 1998; Furtado *et al.*, 2009).

The use of at least two different media for the isolation of cyanobacteria from extreme habitats is recommended – one with characteristics emulating the conditions naturally found in the studied environment, and another with moderate characteristics (Waterbury *et al.*, 2006). In this study, the main variable tested was salinity, and a significant variation of isolated genera was not verified between isolations performed in media with and without the addition of sodium, magnesium and potassium. The division of micro-organisms from saline environments into specialist/stenohaline (with a narrow range of tolerance to alteration of salinity levels) and generalist/euryhaline (tolerant to wide ranges of salt concentrations) has been proposed (Golubič, 1980). Hence, the isolated cyanobacteria most likely are generalists.

Similar micro-habitats separated by obstacles that hinder dispersion may be considered islands, and they may favour the survival of organisms that would otherwise be eliminated by ecological interactions in richer environments (Simberloff, 1974). Thus, microbial dynamics in the phyllosphere could be understood in the framework of island biogeography, in which the leaf community is viewed as analogous to communities in oceanic islands (Kinkel *et al.*, 1987). Plant specificity may have a major influence on the composition of the phyllosphere microbial community, but climatic factors and geographical isolation may also be significantly correlated with community dissimilarity, surpassing the role of tree species in the determination of epiphyllic microbial taxa in some trees (Redford *et al.*, 2010; Finkel *et al.*, 2011). In Brazilian mangroves, an evaluation of the bacterial communities on the leaf surfaces of *Rhizophora mangle*, *Laguncularia racemosa* and *Avicennia schaueriana* observed community specificity to each tree (Dias *et al.*, 2012). Nevertheless, a study focusing on cyanobacteria have concluded that, although the tree species had some influence on the composition of the cyanobacterial community, the location and environmental conditions were their main driver on the phyllosphere of the mangrove trees evaluated (Rigonato *et al.*, 2012).

Even though no definitive influencing factor on the composition of bacteria in leaf surfaces has been found, there is evidence of an interconnection between the evolutionary adaptation of species and the ecosystem functioning processes (Seehausen, 2009), and of a co-evolutionary relationship between organisms and their environment (Knoll, 2009). Most likely, the combination of the environmental characteristics of mangrove ecosystems, the physiological characteristics of the host plant and the natural conditions of the phyllosphere provide a unique scenario for the occurrence of cyanobacterial taxa. Despite the usual low plant diversity, mangroves are an important area of study for cyanobacterial diversity, and trees such as *Avicennia schaueriana* may host yet-unknown strains that can provide new insights into the evolution, ecology and biogeography of this phylum.

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