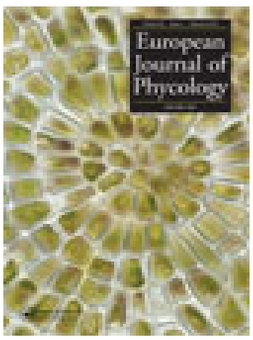


European Journal of Phycology

ISSN: 0967-0262 (Print) 1469-4433 (Online) Journal homepage: https://www.tandfonline.com/loi/tejp20

New insights into *Chroococcus* (Cyanobacteria) and two related genera: *Cryptococcum* gen. nov. and *Inacoccus* gen. nov.

Watson Arantes Gama, Janaina Rigonato, Marli Fátima Fiore & Célia Leite Sant'Anna

To cite this article: Watson Arantes Gama, Janaina Rigonato, Marli Fátima Fiore & Célia Leite Sant'Anna (2019): New insights into *Chroococcus* (Cyanobacteria) and two related genera: *Cryptococcum* gen. nov. and *Inacoccus* gen. nov., European Journal of Phycology, DOI: 10.1080/09670262.2018.1563913

To link to this article: https://doi.org/10.1080/09670262.2018.1563913

EUROPEAN JOURNAL OF PHYCOLOGY
https://doi.org/10.1080/09670262.2018.1563913

New insights into *Chroococcus* (Cyanobacteria) and two related genera: *Cryptococcum* gen. nov. and *Inacoccus* gen. nov.

Watson Arantes Gama [a], Janaina Rigonato [b], Marli Fátima Fiore [b] and Célia Leite Sant'Anna [a]

[a]Institute of Botany, Nucleus of Phycology, Av. Miguel Stéfano, 3687, São Paulo, Brazil, 04301-902; [b]University of São Paulo, Center of Nuclear Energy in Agriculture, CENA-USP, Av. Centenário, 303, Piracicaba, SP, 13400-970

ABSTRACT

Many cyanobacterial morphospecies with hemispherical cells have been assigned to the genus *Chroococcus*, but increasingly genetic information has shown that the genus is polyphyletic. Here we describe two new genera based on two new species, *Inacoccus* gen. nov. based on *Inacoccus carmineus* sp. nov. and *Cryptococcum* gen. nov. based on *Cryptococcum brasiliense* sp. nov. Both genera have morphological features typical of the genus *Chroococcus* and are based on strains isolated from terrestrial habitats of the Brazilian Atlantic Forest. *Inacoccus* differs from *Chroococcus* in forming nanocytes and by producing an intensely red-coloured sheath. *Cryptococcum* is a cryptic genus and two species, *C. brasiliense* and *C. komarkovaum*, have been recognized by a molecular approach (16 rRNA and 16S-23S ITS sequences). The 16S rRNA gene sequence phylogeny indicates that these proposed genera form monophyletic clades apart from the *Chroococcus sensu stricto* cluster. Additionally, the tropical species *Chroococcus subviolaceus* was studied by molecular methods for the first time. The results confirmed a vast phylogenetic diversity hidden under the simple morphology of *Chroococcus* morphotypes, and show that terrestrial environments are a promising field of research.

ARTICLE HISTORY Received 24 September 2017; revised 19 October 2018; received 18 November 2018

KEYWORDS Chroococcaceae; coccoid cyanobacteria; new genus; new species; 16S rRNA phylogeny; 16S-23S ITS

Introduction

The systematics of cyanobacteria have been extensively revised over the last few years. Traditionally identified by morphological features, cyanobacterial classification is now subject to technological advances, particularly in molecular biology. This has led to the rise of polyphasic taxonomy, which uses a pool of information from morphology, ecology, physiology, systematics and molecular biology to infer phylogeny and characterize and separate taxa (Komárek *et al.*, 2014).

As a result of the polyphasic approach, several new genera and species have emerged (e.g. Dvořák *et al.*, 2015; Alvarenga *et al.*, 2016; Berrendero Gomez *et al.*, 2016; Rigonato *et al.*, 2016*a*; Brito *et al.*, 2017; Hašler *et al.*, 2017), providing greater resolution for reconstructing phylogenies and to characterize cyanobacterial systematics. Although morphology might seem relatively uninformative, it is key to linking all previous knowledge to the information provided by this new approach. One of the most important recent discoveries in this field is the revelation that the systematic separation between coccoid and simple filaments, used for centuries, is artificial (Hoffmann *et al.*, 2005; Schirrmeister *et al.*, 2011). However, the amount of polyphasic information differs for each group of cyanobacteria and is still relatively low for the coccoid types (Komárek *et al.*, 2014; Komárek, 2016).

Chroococcus Nägeli (1849) is one of the most commonly recognizable genera among coccoid cyanobacterial morphotypes. The type species, *Chroococcus rufescens* (Kützing) Nägeli, was originally described as occurring on moist substrata, but records from phytoplankton and on submerged rocks are also found (Daily, 1942). So far, no molecular data are available and Komárek & Anagnostidis (1998) recommend the revision of its taxonomic circumscription. *Chroococcus* morphotypes are found in aquatic and terrestrial environments, including marine and thermal waters, hot and cold deserts, growing on soils, wood and rocks (Komárek & Anagnostidis, 1998). In all these different habitats, the morphotypes displayed the diacritical morphological features of the genus, which are spherical or hemispherical cells, dividing only by binary fission in three irregular planes, forming solitary cells (rare) or colonies (Komárek & Anagnostidis, 1998). The typical *Chroococcus* has densely packed cells in a conspicuous firm envelope, which can be simple (one-layered) or intensely lamellate (Komárek & Anagnostidis, 1995): the envelope is the main feature that distinguishes *Chroococcus* from the related genus *Limnococcus* (Komárek & Anagnostidis) Komárková *et al.* (Komárková *et al.*, 2010; Komárek, 2016), which tends to group planktonic species (Komárek, 2016), while the terrestrial ones are assigned to *Chroococcus*.

CONTACT Watson Arantes Gama watsonarantes@gmail.com

Published online 23 Apr 2019

Fifty-six valid morphospecies have been described within the genus *Chroococcus* (Komárek & Hauer, 2013), only four of which have been studied using the polyphasic approach (Komárková *et al.*, 2010; Kováčik *et al.*, 2011). Phylogenetic studies have confirmed that some morphological features used to discriminate *Chroococcus* species, such as the presence and shape of mucilage and cell dimensions, are generally environmentally dependent (Welsh, 1965; Komárková *et al.*, 2010). Considering the lack of phylogenetic information for *C. rufescens*, Komárková *et al.* (2010) and Kováčik *et al.* (2011) identified a clade of strains of *Chroococcus* species as the reference clade for the genus, which is treated here as typical *Chroococcus*. Recently, a new clade of marine *Chroococcus*-like strains close to *Limnococcus* has been found (Wood *et al.*, 2016). These marine strains have wide cells (>38 µm), which is uncommon in *Limnococcus* but common in many terrestrial *Chroococcus* species, so Wood *et al.* (2016) suggested that further studies were required to characterize the clade. This has further highlighted the polyphyletic nature of *Chroococcus sensu lato*, and the marine origin of these strains contrasts with the typical habitat of *Chroococcus* in terrestrial environments, such as soil and rock surfaces.

The Brazilian Atlantic Forest is a hotspot for biodiversity conservation (Myers *et al.*, 2000) and has been shown to support a huge diversity of terrestrial cyanobacteria (Rigonato *et al.*, 2016*b*). To date, six new genera and 50 new species have been discovered (Fiore *et al.*, 2007; Sant'Anna *et al.*, 2011, 2013; Hentschke & Komárek, 2014; Malone *et al.*, 2015; Alvarenga *et al.*, 2016; Hentschke *et al.*, 2016). The majority of these taxa are filamentous, although Gama *et al.* (2014) revealed that the Atlantic Forest has a great variety of coccoid terrestrial taxa too, with many unclassified morphotypes. Therefore, this study aims to characterize by a polyphasic approach some terrestrial *Chroococcus*-like strains isolated from the Brazilian Atlantic Forest.

Materials and methods

The CCIBt (Culture Collection of Institute of Botany, São Paulo, Brazil) strains were isolated from samples collected in terrestrial habitats in the Brazilian Atlantic Forest (Table 1), according to standard techniques (Jacinavicius *et al.*, 2013), and strain CCALA054 was obtained from the Culture Collection of Algae at the Laboratory of Algology, Třeboň, Czech Republic. Initial isolation of the Brazilian strains was on solid medium (1% agar). After transfer to liquid medium, no decrease in cell growth or variation in cell morphology was observed. Since then, they have been maintained in BG-11 or ASM-1 liquid media (Jacinavicius *et al.*, 2013) under controlled conditions: 23±1°C, 40–50 µmol photons m^{-2} s^{-1} and 14–10 h light-dark photoperiod. An aliquot of each strain was preserved in 4% formaldehyde and deposited in the Herbarium of the Institute of Botany (SP), Brazil (Table 1).

Morphological study was carried out on liquid culture material using a Zeiss Axioplan 2 optical microscope. The identification and cell measurements were based on at least 50 individuals and expressed as minimum-average-maximum values in the taxonomic description (Supplementary table 3). Differences in cell diameter were tested by one-way ANOVA with GraphPad Prism 6, and Tukey *posteriori* test with $\alpha = 0.05$. Life cycle was inferred after 5, 30 and 120 days of culturing. The organisms were documented using a Zeiss Axiocam MRc digital camera and the measurements were made with AxioVision SE64 Rel 4.9 software. The main morphological features evaluated were: colour and disposition of sheaths, type of cell division, cell diameter, colour and uniformity of cell content and cell organization inside the colonies. The new taxa were described in accordance with the International Code of Nomenclature for algae, fungi and plants (McNeill *et al.*, 2012).

Cell ultrastructure (position of thylakoids and mucilage arrangement) was visualized by transmission electron microscopy. The cells were collected by centrifugation and each sample was fixed with

Table 1. List of studied strains with details of their culture medium, habitat and herbarium accesion number.

Culture number	Culture Medium	Origin	Habitat	Coordinates	Exsiccatum number
CCIBt3410	ASM-1	Ilha do Cardoso State Park, Brazil	Dried soil	25°01′16″S, 47°55′31″W	SP469754
CCIBt3411	ASM-1	Santa Virgínia Park, Brazil	Concrete	23°20′36″S, 45°07′44″W	SP469755
CCIBt3418	ASM-1	Santa Virgínia Park, Brazil	Concrete	23°20′36″S, 45°07′44″W	SP469756
CCIBt3475	BG-11	Ilha do Cardoso State Park, Brazil	Wet Rock	25°04′12″S, 47°55′27″W	SP469757
CCIBt3505	ASM-1	Ecological Station of Juréia-Itatins, Brazil	Wet Rock	24°22.694′S, 47°04.793′ W	SP469758
CCIBt3506	ASM-1	Ecological Station of Juréia-Itatins, Brazil	Wet Rock	24°22.694′S, 47°04.793′ W	SP469759
CCIBt3508	ASM-1	Ecological Station of Juréia-Itatins, Brazil	Wet soil	24°22.747′S, 47°04.729′ W	SP469760
CCIBt3549	ASM-1	Ecological Station of Juréia-Itatins, Brazil	Wet Rock	24°26.162′S, 47°03.773′ W	SP469761
CCALA054	ASM-1	Unknown	Thermal spring	-	SP469762

Karnovsky plus 0.2 M sucrose for 24 h, washed three times (10 min each) with 0.05 M cacodylate buffer and post-fixed with 1% osmium tetroxide for 1 h. The samples were maintained overnight in 5% uranyl acetate then dehydrated in an acetone series of increasing concentration and after being washed with 100% acetone, submitted to pre-infiltration with 1:1 Spurr resin/100% acetone for 5 h with agitation. The subsequent infiltration of the samples was with pure resin for 12 h. The samples were shaped in Spurr resin and polymerized at 65°C for 3 days. The blocks were cut with an ultramicrotome (Leica Ultracut UCT) with a diamond blade (450) in 70 nm-thick sections, mounted on uncoated 200-mesh copper grids, and stained with uranyl acetate and lead citrate (Reynolds, 1963). Visualization and microphotography were performed with a JEOL JEM-1011 (JEOL, Tokyo, Japan) at 100 kVA.

Total genomic DNA was extracted from liquid culture of the cyanobacterial strains with the Ultra Clean Soil DNA Isolation Kit (MOBIO, Carlsbad, California, USA). The partial 16S-23S rRNA internal transcribed spacer (ITS) sequence was amplified by PCR with primers 27F (Neilan *et al.*, 1997) and 23S30R (Lepère *et al.*, 2000) on a Techne TC-412 thermocycler (Bibby Scientific Ltd, Stone, Staffordshire, UK) with 10 ng of genomic DNA, 5 µmol of each primer, 200 µm of each dNTP, 3.0 mM $MgCl_2$, 1× PCR buffer and 1.0 U Platinum *Taq* DNA polymerase (Life Technologies, Grand Island, New York, USA) according to Taton *et al.* (2003). The resulting PCR product was cloned into a pGEM®-T Easy Vector System (Promega, Madison, Wisconsin, USA) and inserted into chemocompetent *E. coli* DH5α cells by the heat-shock method (Sambrook *et al.*, 1989). After growth on LB plates containing 0.5 µl ml^{-1} X-Gal (50 µg ml^{-1}) (Life Technologies) and 1 µl ml^{-1} of ampicillin sodium salt (100 µl ml^{-1}) (Sigma-Aldrich Co., St. Louis, Missouri, USA), recombinant plasmids were purified from white colonies by the alkaline lysis method (Birnboim & Doly, 1979). The cloned gene fragment was prepared with BigDye XTerminator kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with the pGEM®-T Easy Vector-anchored primers M13F and M13R and the internal 16S rRNA primers 357F/357R, 704F/704R and 1114F/1114R (modified from Lane, 1991). The purified pellets were re-suspended in HiDi formamide (Life Technologies), and sequenced on an ABI PRISM 3500 Genetic Analyzer (Life Technologies). The sequenced fragments were assembled with the Phred/Phrap/Consed software package (Ewing *et al.*, 1998; Gordon *et al.*, 1998).

The phylogenetic analysis was based on 16S rRNA gene and ITS sequences obtained in this study and reference sequences retrieved from GenBank. The evolutionary models were tested with jModelTest (Posada, 2008). The best models were the General Time Reversible (GTR) substitution with Gamma distribution (G) and estimate of proportion of invariable sites (I) for 16S rDNA alignment and the Hasegawa, Kishino and Yano (HKY) model + G +I for ITS. Evolutionary analysis based on Neighbour joining and Maximum likelihood methods was conducted in Mega 6.0 (Tamura *et al.*, 2013) and estimations of the robustness of the tree were tested by bootstrap with 1000 replications. The Bayesian analysis was inferred by MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2005) in 5×10^6 generations. Chains were sampled every 100th cycle and 25% of the records were discarded as burn-in. Convergence of the MCMC algorithm was monitored by the average standard deviation of split frequencies (<0.003). The similarity among sequences was calculated with a p-distance method in Mega 6.0 (Tamura *et al.*, 2013) and the percentage was provided by the formula [1–(p-distance)]×100. The 16S rRNA gene cut-off proposed by Stackebrandt & Goebel (1994) and revised by Stackebrandt & Ebers (2006) for prokaryotic taxa was used to differentiate genera (when lower than 95%) and as an indication to distinguish species (when lower than 98.7%). The mean similarity was estimated by the average similarity value of each sequence from the two compared clades and is shown in Supplementary tables 1 and 2.

Results and discussion

Genetic analysis of the eight *Chroococcus*-like strains revealed that they are separated into three different groups (Figs 1, 2), in agreement with the morphometric analyses (Fig. 3). CCIBt 3505, 3506, 3508, 3549 grouped within the *Chroococcus* typical clade, as identified by Komárková *et al.* (2010) and Kováčik *et al.* (2011). Here, we term this clade *Chroococcus sensu stricto*(Figs 1, 4–9). The other strains were placed in two distinct groups (Fig. 1): (1) encompassing the strains CCIBt 3410 and CCALA054, designated as *Cryptococcum* gen. nov. (Figs 10–13) and (2) CCIBt 3411, 3418 3475, here designated as *Inacoccus* gen. nov. (Figs 14–21). All three clades had high similarities (Supplementary tables 1, 2) and were well-supported by bootstrap values and/or posterior probabilities (Figs 1, 2) for 16S rDNA and ITS sequences. The transmission electron microscopy reveals that all new genera have thylakoids arranged typically for the Chroococcaceae (Figs 22–27).

Chroococcus

The CCIBt strains placed in the *Chroococcus sensu stricto*clade in the phylogenetic tree were identified as two distinct taxa, *Chroococcus turgidus* (CCIBt 3508) and *Chroococcus subviolaceus* (Wille) Gama-Jr., Laughinghouse IV & Sant'Anna (CCIBt 3505, 3506, 3549).

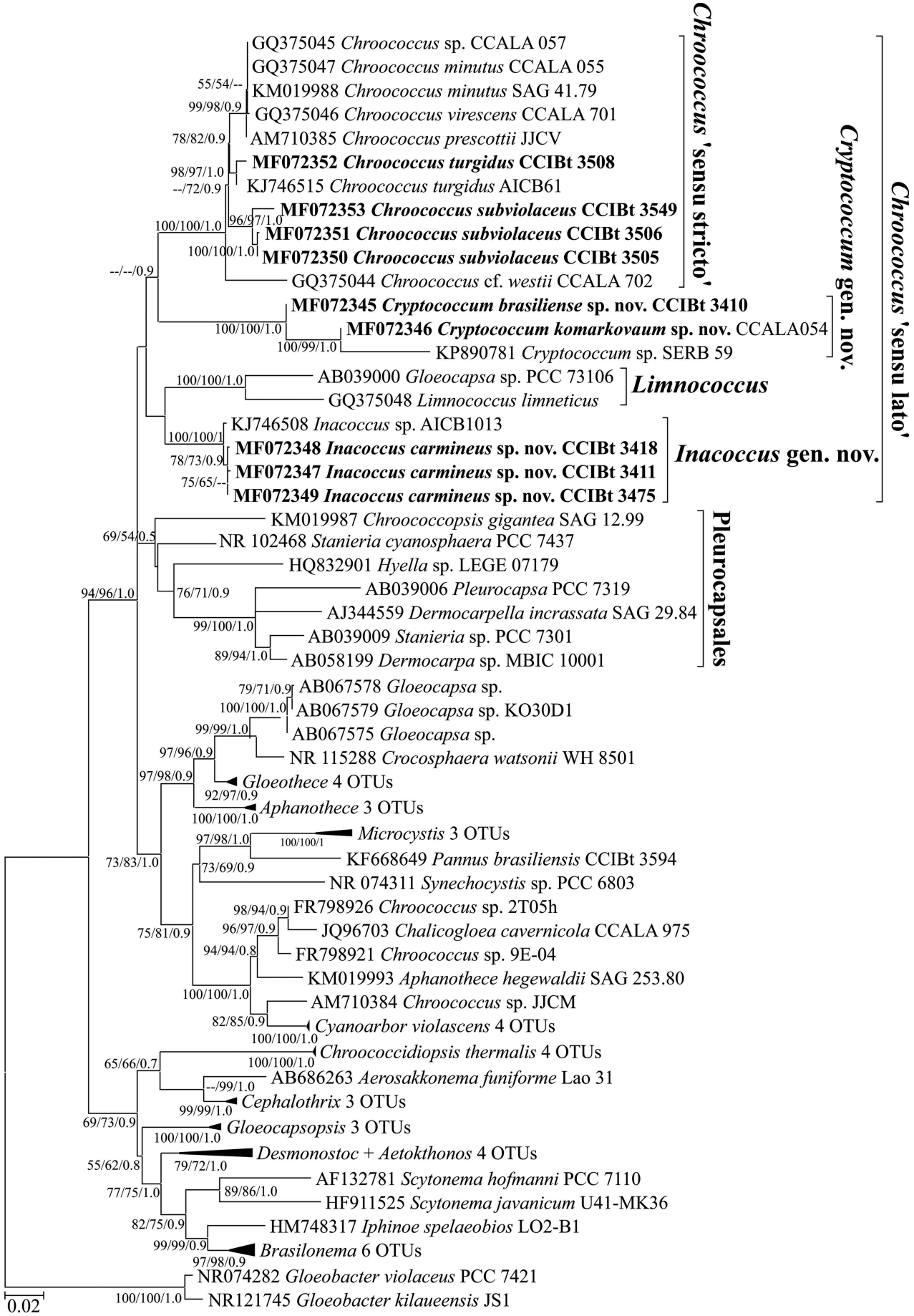


Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences and reconstructed with the maximum-likelihood (ML) analysis of evolutionary distances determined by the GTR + G + I model. NJ and ML bootstrap (1,000×) values (>50%) and Bayesian posterior probabilities are provided for each node, respectively. Sequences determined in this work are indicated in bold.

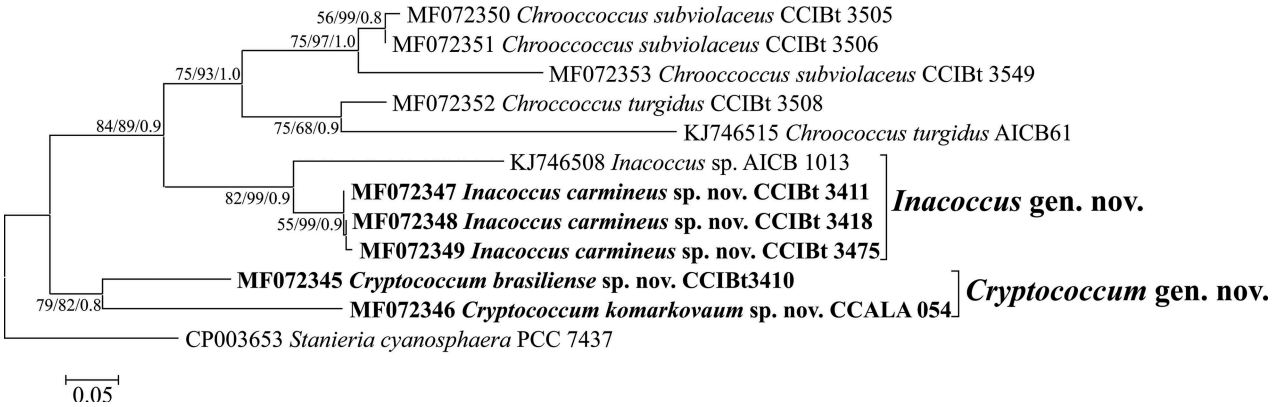


Fig. 2. Phylogenetic tree based on 16S-23S ITS sequences and reconstructed with the maximum-likelihood (ML) analysis of evolutionary distances determined by the HYK + G + I model. NJ and ML bootstrap (1,000×) values (>50 %) and Bayesian posterior probabilities are provided for each node, respectively.

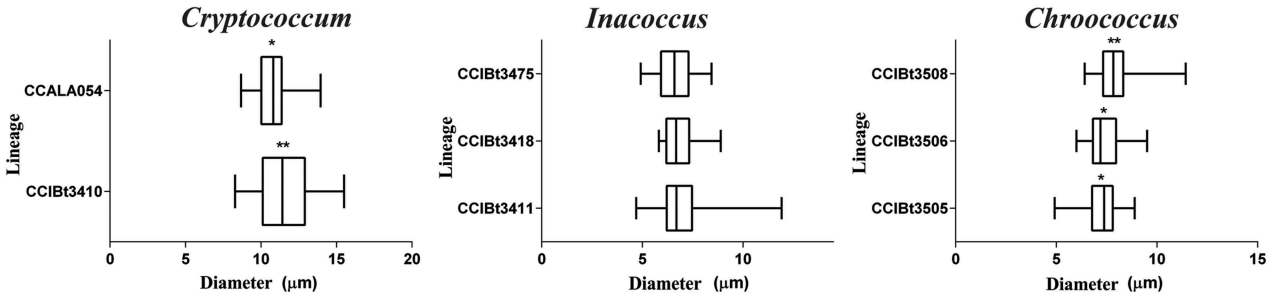


Fig. 3: Box-plot of minimum, average and maximum cell diameter values together with statistical analysis. Different signs (*) represent significant differences tested by ANOVA.

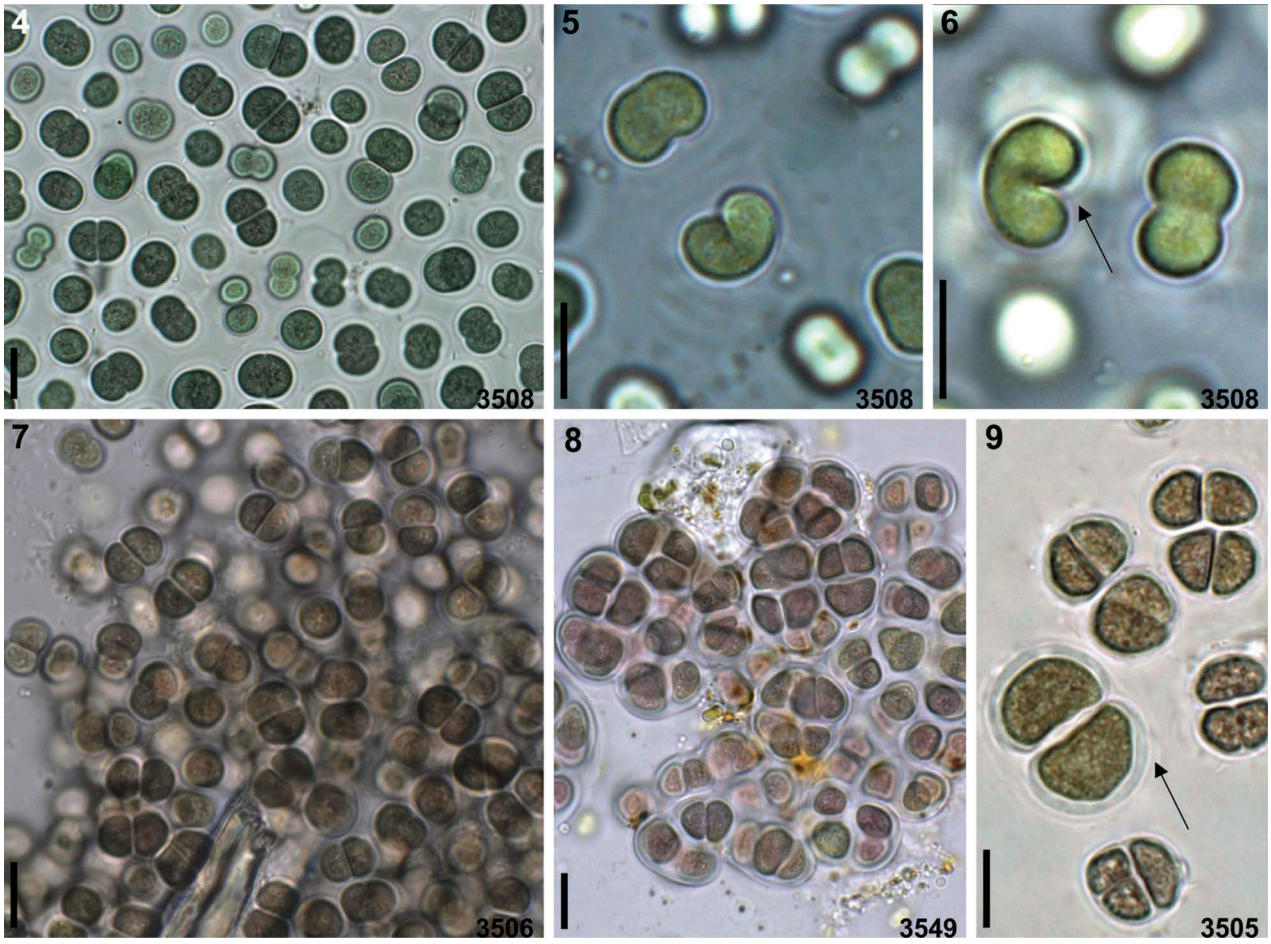
The morphology of CCIBt 3508 corresponds with the original description of *Chroococcus turgidus* (Kützing) Nägeli (Kützing, 1843; Nägeli, 1849) and it is grouped with AICB61 (Fig. 1), named as *C. turgidus*, with 99.3% 16S rRNA sequence similarity (Supplementary table 1). Here we present morphological and molecular data confirming the position of *C. turgidus* within the *Chroococcus* clade. Several studies have reported this species in different localities and habitats with a wide range of morphological variation (Komárek & Anagnostidis, 1998), suggesting wide dispersal and generalist habitat. Since the habitat of the type of *C. turgidus* is unknown (Kützing, 1843; Nägeli, 1849), the analyses of more sequences from *C. turgidus* morphotypes may elucidate whether this species is truly generalist, as reported in the literature (Daily, 1942).

CCIBt 3508 strain also showed unilateral cell division with asymmetrical invagination, giving cells a kidney shape (Figs 5, 6) after 60 days of cultivation. This asymmetrical invagination is rarely reported in cyanobacteria, e.g. in *Synechococcus nidulans* (Pringsheim) Komárek in Bourrelly (Allen, 1968), and further investigations are needed to confirm it as a regular feature rather than an artefact resulting from the culture conditions.

The remaining strains (CCIBt 3505, 3506 and 3549) in the *Chroococcus* clade were morphologically and ecologically identified as *Chroococcus subviolaceus*. CCIBt 3505 and 3506 were isolated from the same substrate/sample, but from different inocula. *Chroococcus subviolaceus* has already been reported from different habitats in the Brazilian Atlantic Forest (Gama *et al.*, 2014), and the type originated from a tropical forest in the Samoan Islands. Here, we phylogenetically characterize *C. subviolaceus*, confirming its distinctiveness from other *Chroococcus* species, in accordance with morphology (Fig. 3) and 16S rRNA gene and ITS phylogenies (Figs 1, 2).

Inacoccus

The CCIBt 3411, 3418 and 3475 strains were separated from the *Chroococcus sensu stricto* clade in the phylogenetic tree and the 16S rRNA gene similarities between the Brazilian strains and *Chroococcus sensu stricto*were low (Supplementary table 1), supporting their description as *Inacoccus* gen. nov. Morphologically, these strains were similar to *Chroococcus* species in cell shape and organization. However, they produced a large volume of intensely red-coloured sheaths (Figs 14–17) not often recorded in *Chroococcus*, which usually has hyaline or yellowish mucilage (Komárek & Anagnostidis, 1998). The

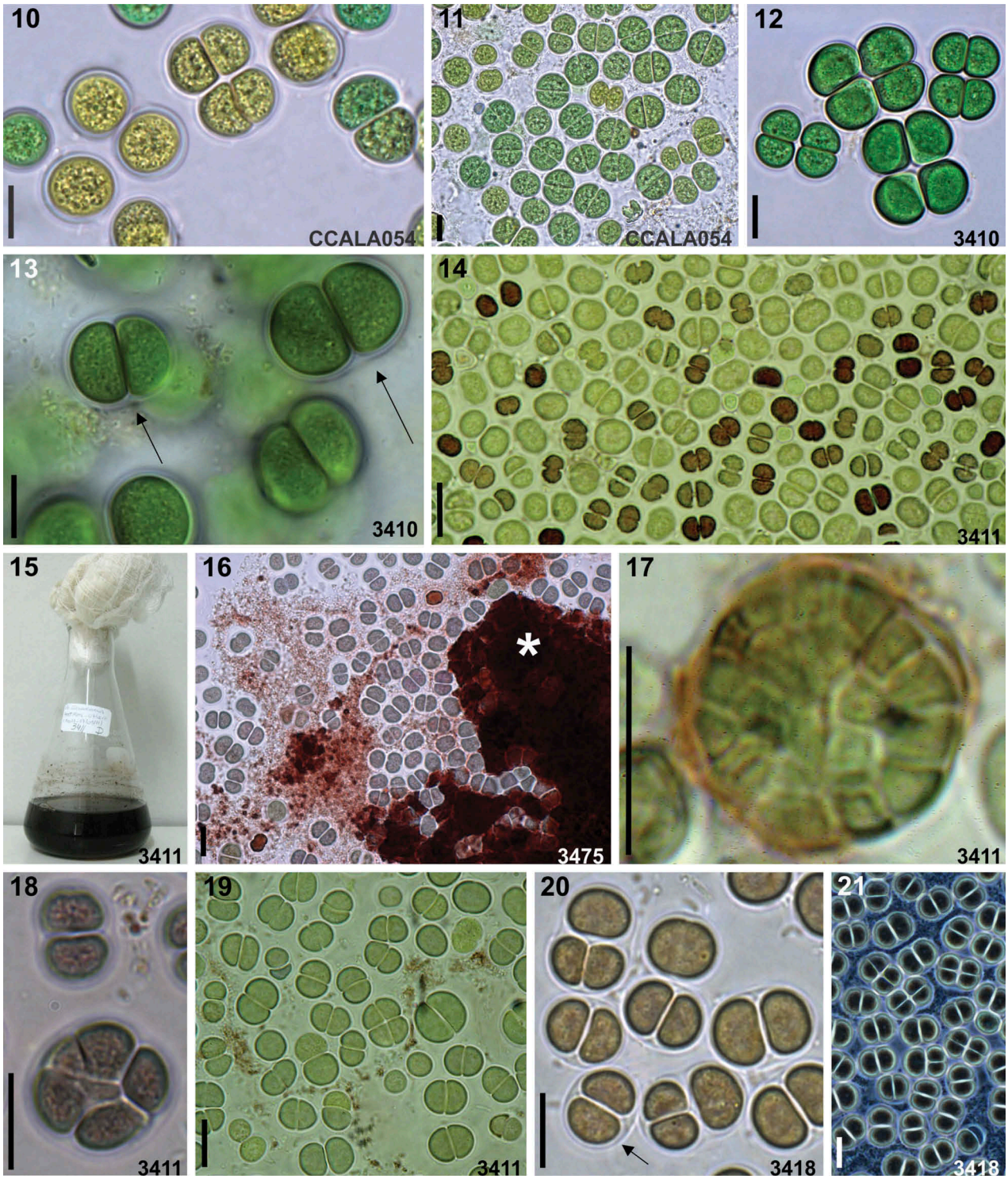


Figs 4–9. Morphology of cyanobacterial strains. **Figs 4–6**. *Chroococcus turgidus*. Kidney-shaped cells (5–6) representing unilateral invagination during binary fission. **Figs 7–9**. *Chroococcus subviolaceus* showing variation of brown to purple colour of cell content (7–8), presence of large colonies (8) and dense mucilaginous sheath around cells (9, arrow). Scale bars = 10 μm. The strain numbers are on the right of each picture.

Inacoccus strains produce large amounts of this red pigment, forming lumps which darkened the culture medium (Figs 15, 16). This was observed in 30-day cultures in ASM-1 medium and was more intense in CCIBt 3411. In early cultures, this pigment was present only around the cells in BG-11 medium. In the *Inacoccus* strains nanocytes were occasionally observed – this type of quick and successive cell division generates small densely packed colonial cells, differing from baeocytes (formed by simultaneous divisions).

Chroococcus can divide irregularly in different planes, but it never forms nanocytes (Komárek & Anagnostidis, 1998). The nanocyte-forming *Cyanosarcina* Kováčik, a member of the Chroococcaceae, differs from *Inacoccus* by the presence of hyaline mucilage and cells are always in a sarcinoid arrangement. Colony formation only occurs during nanocyte development in *Inacoccus* representatives (Figs 17, 18). No molecular data for *Cyanosarcina* are available to confirm the phylogenetic relationship between these taxa. *Gloeocapsopsis* Geitler *ex* Komárek is also morphologically similar to *Inacoccus*, but nanocyte production in this genus is doubtful and like *Cyanosarcina*, its colonies are always sarcinoid in form; it is not closely related to *Inacoccus* (Fig. 1). Thus, the production of intensely coloured mucilage, a loose cell arrangement and nanocyte formation can be considered as autapomorphic characters of *Inacoccus*. The strain AICB1013, previously identified as *Gloeocapsa*, is also placed in the *Inacoccus* clade. It was probably identified as *Gloeocapsa* based on the old concept of this genus in Kützing (1843) rather than its revised circumscription proposed by Komárek (1993), who defined *Gloeocapsa* as forming centric lamellate colonies, the spherical cells having individual mucilaginous involucres, which clearly distinguishes *Gloeocapsa* from *Inacoccus*. Since AICB1013 16S and ITS sequences are similar to those of CCIBt *Inacoccus* strains (Supplementary tables 1, 2), and its morphology is incompatible with *Gloeocapsa*, we here classify AICB1013 as *Inacoccus*.

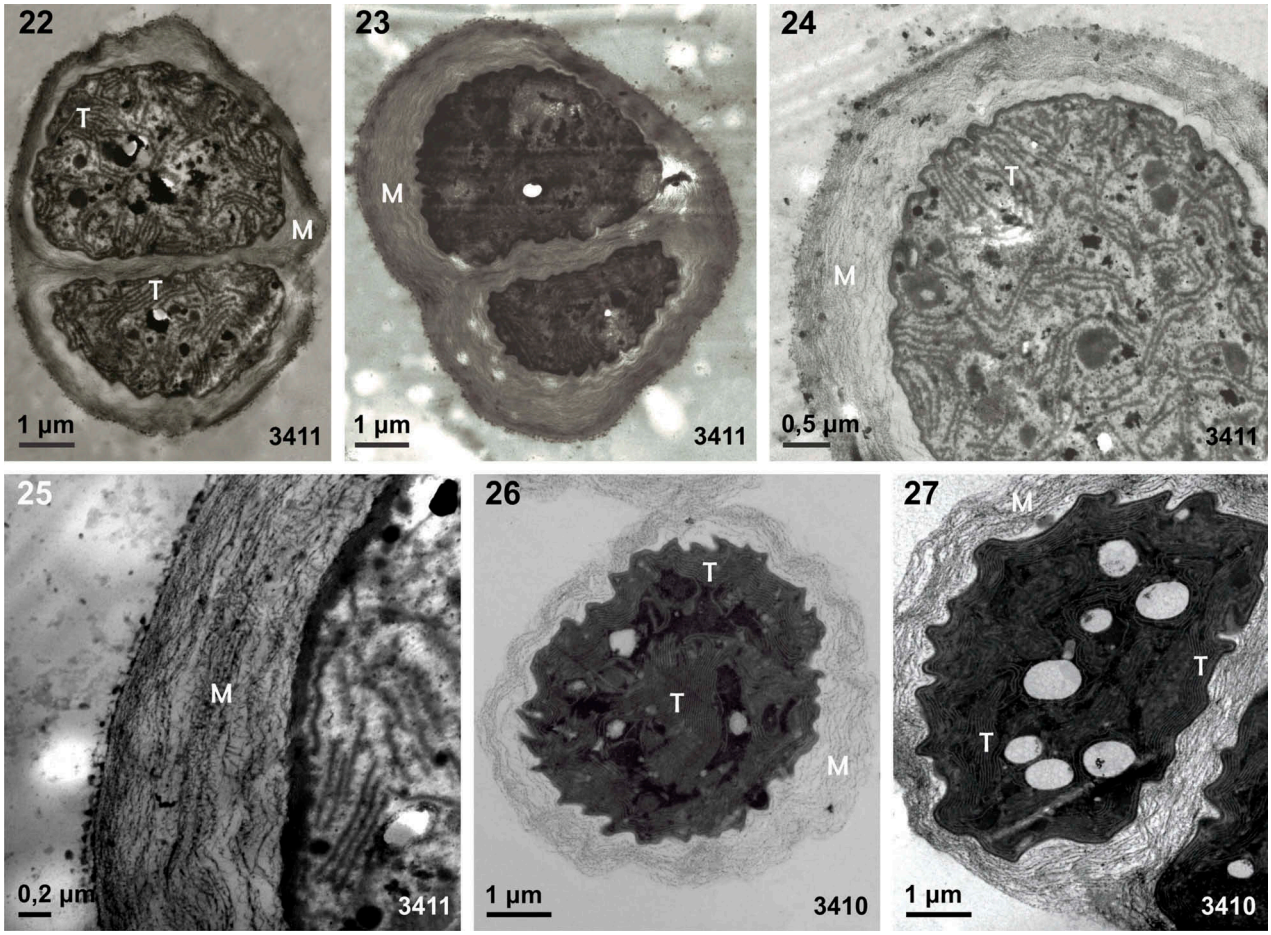
The three studied *Inacoccus* strains belong to the same species, *Inacoccus carmineus* sp. nov. The ITS sequence analysis and cell diameters (Figs 2, 3) reinforce this taxonomic placement. The CCIBt 3411 and 3418 strains were isolated as single cells from the same environmental sample collected in Santa Virgínia Park, representing population variability, while CCIBt 3475 was isolated



Figs 10–21. Morphology of cyanobacterial strains. **Figs 10–11**. *Cryptococcum komarkovaum*. **Figs 12–13**. *Cryptococcum brasiliense* (arrows indicate the sheaths). **Figs 14–21**. *Inacoccus carmineus*. Fig. 14, cells with intense red sheaths (dark ones) and cells without sheaths (light ones); Fig. 15, culture medium coloured by sheath production; Fig. 16: sheath block (*) released in culture medium Fig. 17: nanocyte formation; Fig. 18: nanocyte development; Fig. 19: heterogeneity of cell dimensions; Fig. 20: cells with hyaline sheath (arrow); Fig. 21: cells under phase contrast microscope. Scale bars = 10 μm. The strain numbers are on the right of each figure.

from a sample collected in Ilha do Cardoso State Park, about 355 km from Santa Virgínia. The ANOVA analysis revealed that there was no significant difference in cell diameter between these strains (F=1.097, p=0.3365).

The TEM ultrastructure confirmed the presence of a very thick layer of mucilage around the cells (Figs 22–25). Thylakoids were fasciculate, as found in *Chroococcus* species.



Figs 22–27. Morphology of cyanobacterial strains. **Figs 22–25**. *Inacoccus carmineus*, showing dense mucilage surrounding the cells (23, 25). **Figs 26–27**. *Cryptococcum brasiliense* (M, mucilaginous sheath; T, thylakoids). The strain numbers are on the right of each figure.

Cryptococcum

The CCIBIt 3410 strain is placed together with CCALA 054 and SERB59 strains in a highly supported clade separated from *Chroococcus sensu stricto* in the phylogenetic tree (Figs 1, 2). Although similarities between the 16S rRNA gene sequences and ITS of these three strains and *Chroococcus sensu stricto*are low (Supplementary tables 1, 2), it is not possible to distinguish them by morphological characters. Even so, the phylogenetic results indicated and supported the need for the description of a new genus, herein designated as *Cryptococcum*, which is characterized as a cryptic genus of *Chroococcus sensu lato*. The CCALA 054 strain has already been reported to be divergent from *Chroococcus* but its taxonomic and phylogenetic positions were unclear (Komárková *et al*., 2010). Despite resolving this strain in the *Limnococcus* clade, Komárková *et al.* (2010) did not confirm this identification, and suggested that CCALA 054 could be a *Gloeocapsa* Kützing. However, the morphology of the CCALA 054 strain does not match *Gloeocapsa*, according to Komárek (1993). SERB59 is identified as *Chroococcus* cf. *membraninus*, but as it is positioned in the *Cryptococcum* group, we identify it as *Cryptococcum* sp., since we do not have enough information to describe it at species level.

Whereas CCIBt 3410 and CCALA 054 are members of the same genus and have similar morphology, based on ITS (Fig. 2) and cell diameter (Fig. 3, Supplementary table 3), they are different species. Also, the localities where these strains were found (the Brazilian Atlantic Forest soil and a thermal spring, respectively) suggest a different ecology and physiology, reinforcing their distinctiveness. Therefore, we here propose two species: *Cryptococcum brasiliense* sp. nov. (CCIBt3410) and *Cryptococcum komarkovaum* sp. nov. (CCALA054). These strains were studied in both liquid and solid media but no diacritical features were observed. TEM ultrastructure revealed the CCIBt 3410 thylakoids to be in a fasciculate arrangement (Figs 26, 27), as found in typical *Chroococcus*.

The typical morphology of *Chroococcus* is seen in the clade labelled *Chroococcus sensu lato*. However, our data confirmed that morphology has limitations for inferring evolutionary variability, and some cyanobacterial groups can be truly distinguished only by robust molecular characterization since morphology alone is not enough, as shown in the genus *Cryptococcum* described here. Many

cryptic genera have been recently described in cyanobacteria based on phylogenetic reconstructions, especially from homo and heterocystous types (Dvořák *et al.*, 2015; McGregor & Sendall, 2015; Shalygin *et al.*, 2017). Regarding coccoid morphotypes, the cryptic genera formally described are uncommon. However, special attention should be given to this cyanobacterial group as many of its genera are differentiated by morpho-ecological features that can be minor, or sometimes environmentally dependent (Komárek & Anagnostidis, 1998). In addition, many of these characteristics are widely used in classical taxonomy but have never been phylogenetically tested or endorsed (e.g. number of division planes in binary fission, radial display of cells, hollow colonies). The recent confirmation that, according to the phylogenetic approach, *Sphaerocavum* belongs to the *Microcystis* clade (Rigonato *et al.*, 2018) demonstrates the need for morphological markers to be reviewed. Therefore, it is predicted that cryptic genera will emerge with the advance of phylogenetic studies, in the same way that distinct genera like *Sphaerocavum* and *Microcystis* are fusing.

Taxonomic descriptions

Inacoccus gen. nov. (Figs 14–21)

Cells hemispherical to rounded, solitary or rarely grouped in few-celled colonies. Cell content green olive to purple brownish, homogeneous to granulate, fasciculate thylakoids. Hyaline to generally intense red sheaths surrounding cells, simple to lamellate. Reproduction by binary fission in three irregular planes and nanocytes present.
TYPE SPECIES: *Inacoccus carmineus*
ETYMOLOGY: Genus named in honour of Dr Ina de Souza Nogueira for her contributions to Brazilian phycological research.

Inacoccus carmineus sp. nov. (Figs 14–21)

Cells hemispherical to rounded, 4.7–(6.7)–11.9 µm diam., solitary or rarely grouped in few-celled colonies. Hyaline to intense red-coloured sheaths surrounding cells and colonies, smooth or rarely lamellate. Cell content finely granulated, purple-brownish to green-brownish. Nanocytes present, formed by polygonal cells surrounded by red-coloured sheaths.
HOLOTYPE: Exsiccatum accession number SP469755, Herbarium of Institute of Botany, São Paulo, Brazil.
TYPE STRAIN: CCIBt 3411.
TYPE LOCALITY: Terrestrial, growing on rocks and concrete.
ETYMOLOGY: From Latin '*carmineus*' meaning carmine, vivid red colour, in reference to the sheath pigment produced by the species.

Cryptococcum gen. nov. (Figs 10-13)

Cells hemispherical to rounded, solitary or rarely grouped in few-celled colonies. Cell content green, blue green to olive green, homogeneous to granulate, fasciculate thylakoids. Sheaths hyaline surrounding the cells, simple to lamellate. Reproduction only by binary fission in three irregular planes.
TYPE SPECIES: *Cryptococcum brasiliense.*
ETYMOLOGY: From Greek *crypto-* (hidden) and *coccus* s.m. II (rounded).

Cryptococcum brasiliense sp. nov. (Figs 12, 13)

Cells hemispherical to rounded, 8.3–(11.6)–15.5 µm diam., solitary or rarely grouped in few-celled colonies. Sheaths hyaline surrounding cells and colonies, smooth or rarely lamellate. Cell content granulate, green to blue-green. Nanocytes never observed.
HOLOTYPE: Exsiccatum accession number SP469754, Herbarium of Institute of Botany, São Paulo, Brazil.
TYPE STRAIN: CCIBt3410.
TYPE LOCALITY: Terrestrial, growing on dry soil among pebbles.
Etymology: In reference to Brazil, from where the type strain was isolated.

Cryptococcum komarkovaum sp. nov. (Figs 10, 11)

Cells hemispherical to rounded, 8.7–(10.8)–13.9 µm diam., solitary or rarely grouped in few-celled colonies. Sheaths hyaline surrounding cells and colonies, smooth or rarely lamellate. Cell content intensely granulated, blue-green to yellowish-green. Nanocytes never observed.
HOLOTYPE: Exsiccatum accession number SP469762, Herbarium of Institute of Botany, São Paulo, Brazil.
TYPE STRAIN: CCALA054.
TYPE LOCALITY: Aquatic, thermal spring.
ETYMOLOGY: Species named in honour of Dr Jaroslava Komárková for her contributions to cyanobacterial research.

Acknowledgements

The authors wish to thank Dr Tarciso Filgueiras for Latin support. WAG and JR were supported by the São Paulo Research Foundation (FAPESP 2012/16430-1) and Brazilian Federal Agency for the Support and Evaluation of Graduate Education (CAPES-PNPD20131744 USP/CENA program) graduate and postdoctoral fellowships,

respectively. The molecular studies were supported by a grant from FAPESP to MFF (2013/50425-8).

Disclosure statement

No potential conflict of interest was reported by the authors.

Supplementary Information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at https://doi.org/10.1080/09670262.2018.1563913

Supplementary table 1. 16S rDNA similarity matrix of studied strains and their close relatives using the p-distance model with a total of 1369 positions.

Supplementary table 2. 16S-23S ITS similarity matrix of studied strains and their close relatives, using the p-distance model with a total of 150 positions.

Supplementary table 3. Cell dimensions and GenBank accession number of analysed strains.

Author contributions

W. Gama: conceived the study, field sampling, strains isolation, electron microscopy, morphological, molecular and phylogenetic analysis; J. Rigonato: molecular and phylogenetic analysis; M. Fiore: molecular and phylogenetic analysis; C. Sant'Anna: conceived the study, morphological analysis, culture conditions supplied. All authors were involved in drafting and editing the paper and had final approval of the manuscript.

ORCID

Watson Arantes Gama http://orcid.org/0000-0002-0458-5005
Janaina Rigonato http://orcid.org/0000-0002-1937-7030
Marli Fátima Fiore http://orcid.org/0000-0003-2555-7967
Célia Leite Sant'Anna http://orcid.org/0000-0001-7706-756X

References

Allen, M.M. (1968). Simple conditions for growth of unicellular blue-green algae on plates. *Journal of Phycology*, **4**: 1–13.

Alvarenga, D.O., Rigonato, J., Branco, L.H., Melo, I.S. & Fiore, M.F. (2016). *Phyllonema aviceniicola* gen. et sp. nov. and *Foliisarcina bertiogensis* gen. et. sp. nov., novel epiphyllic cyanobacteria associated with *Avicennia schaueriana* leaves. *International Journal of Systematic and Evolutionary Microbiology*, **66**: 689–700.

Berrendero Gomez, E., Johansen, J.R., Kaštovský, J., Bohunická, M. & Čapková, K. (2016). *Macrochaete* gen. nov. (Nostocales, Cyanobacteria), a taxon morphologically and molecularly distinct from *Calothrix*. *Journal of Phycology*, **52**: 638–655.

Birnboim, H.C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, **7**: 1513–1523.

Brito, A., Ramos, V., Mota, R., Lima, S., Santos, A., Vieira, J., Vieira, C.P., Kastovsky, J., Vasconcelos, V.M. & Tamagnini, P. (2017). Description of new genera and species of marine cyanobacteria from the Portuguese Atlantic coast. *Molecular Phylogenetics and Evolution*, **111**: 18–34.

Daily, W.A. (1942). The Chroococcaceae of Ohio, Kentucky, and Indiana. *American Midland Naturalist*, **27**: 636–661.

Dvořák, P., Jahodářová, E., Hašler, P., Gusev, E. & Poulíčková, A. (2015). A new tropical cyanobacterium *Pinocchia polymorpha* gen. et sp. nov. derived from the genus *Pseudanabaena*. *Fottea*, **15**: 113–120.

Ewing, B., Hillier, L., Wendl, M.C. & Green, P. (1998). Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research*, **8**: 175–185.

Fiore, M.F., Sant'Anna, C.L., Azevedo, M.T.P., Komárek, J., Kaštovský, J. & Sulek, J. (2007). The cyanobacterial genus *Brasilonema*, gen. nov., a molecular and phenotypic evaluation. *Journal of Phycology*, **43**: 789–798.

Gama, W.A., Laughinghouse IV, H.D. & Sant'Anna, C.L. (2014). How diverse are coccoid cyanobacteria? A case study of terrestrial habitats from the Atlantic Rainforest (São Paulo, Brazil). *Phytotaxa*, **178**: 61.

Gordon, D., Abajian, C. & Green, P. (1998). Consed: a graphical tool for sequence finishing. *Genome Research*, **8**: 195–202.

Hašler, P., Casamatta, D., Dvořák, P. & Poulíčková, A. (2017). *Jacksonvillea apiculata* (Oscillatoriales, Cyanobacteria) gen. & sp. nov.: a new genus of filamentous, epipsamic cyanobacteria from North Florida. *Phycologia*, **56**: 284–295.

Hentschke, G.S. & Komárek, J. (2014). *Scytonema santannae*, a new morphospecies of Cyanobacteria from the Atlantic rainforest, southeastern Brazil. *Brazilian Journal of Botany*, **37**: 293–298.

Hentschke, G.S., Johansen, J.R., Pietrasiak, N., Fiore, M.F., Rigonato, J., Sant'Anna, C.L. & Komárek, J. (2016). Phylogenetic placement of *Dapisostemon* gen. nov. and *Streptostemon*, two tropical heterocytous genera (Cyanobacteria). *Phytotaxa*, **245**: 129–143.

Hoffmann, L., Komárek, J. & Kaštovský, J. (2005). System of Cyanoprokaryotes (Cyanobacteria) – state in 2004. *Algological Studies*, **117**: 95–115.

Huelsenbeck, J.P. & Ronquist, R. (2005). Bayesian analysis of molecular evolution using MrBayes. In *Statistical Methods in Molecular Evolution* (Nielsen, R., editor), 183–232. Springer-Verlag, Berlin.

Jacinavicius, F.R., Gama-Jr, W.A., Azevedo, M.T.P. & Sant'Anna, C.L. (2013). *Manual para cultivo de cianobactérias*. Instituto de Botânica, São Paulo.

Komárek, J. (1993). Validation of the genera *Gloeocapsopsis* and *Asterocapsa* (Cyanoprocaryota) with regard to species from Japan, Mexico and Himalayas. *Bulletin of the National Science Museum Series B: Botany*, **19**: 19–37.

Komárek, J. (2016). Review of the cyanobacterial genera implying planktic species after recent taxonomic revisions according to polyphasic methods: state as of 2014. *Hydrobiologia*, **764**: 259–270.

Komárek, J. & Anagnostidis, K. (1995). Nomenclatural novelties in chroococcalean cyanoprokaryotes. *Preslia*, **67**: 15–24.

Komárek, J. & Anagnostidis, K. (1998). Cyanoprokaryota. Teil 1: Chroococcales. In *Süsswasserflora von Mitteleuropa*, (19) (Ettl, H., Gärtner, G., Heynig, H. & Mollenhauer, D., editors), 548. Springer Spektrum, Gustav Fischer, Jena.

Komárek, J. & Hauer, T. (2013). CyanoDB. cz. – Online database of cyanobacterial genera. University of South Bohemia and Institute of Botany AS CR. http://www.cyanodb.cz.

Komárek, J., Kaštovský, J., Mareš, J. & Johansen, J.R. (2014). Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach. *Preslia*, **86**: 295–335.

Komárková, J., Jezberová, J., Komárek, O. & Zapomělová, E. (2010). Variability of *Chroococcus* (Cyanobacteria) morphospecies with regard to phylogenetic relationships. *Hydrobiologia*, **639**: 69–83.

Kováčik, L., Jezberová, J., Komárková, J., Kopecký, J. & Komárek, J. (2011). Ecological characteristics and polyphasic taxonomic classification of stable pigment-types of the genus *Chroococcus* (Cyanobacteria). *Preslia*, **83**: 145–166.

Kützing, F.T. (1843). *Phycologia generalis: oder Anatomie, Physiologie und Systemkunde der Tange*. F. A. Brockhaus, Leipzig.

Lane, D.J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt, E. & Goodfellow, M., editors), 115–175. Wiley, Chichester.

Lepère, C., Wilmotte, A. & Meyer, B. (2000). Molecular diversity of *Microcystis* strains (Cyanophyceae, Chroococcales) based on 16S rDNA sequences. *Systematics and Geography of Plants*, **70**: 275–283.

Malone, C.F.S., Rigonato, J., Laughinghouse IV, H.D., Schmith, E.C., Bouzon, Z.L., Wilmotte, A., Fiore, M.F. & Sant'Anna, C.L. (2015). *Cephalothrix* gen. nov. (Cyanobacteria): towards an intraspecific phylogenetic evaluation by multi-locus analyses. *International Journal of Systematic and Evolutionary Microbiology*, **65**: 2993–3007.

McGregor, G.B. & Sendall, B.C. (2015). Phylogeny and toxicology of *Lyngbya wollei* (Cyanobacteria, Oscillatoriales) from north-eastern Australia, with a description of *Microseira* gen. nov. *Journal of Phycology*, **51**: 109–119.

McNeill, J., Barrie, F., Buck, W., Demoulin, V., Greuter, W., Hawksworth, D., Herendeen, P., Knapp, S., Marhold, K. & Prado, J. (2012). International Code of Nomenclature for algae, fungi and plants. *Regnum Vegetabile*, 154.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A. & Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature*, **403**: 853–858.

Nägeli, C. (1849). Gattungen einzelliger Algen, physiologisch und systematisch bearbeitet. Neue Denkschriften der Allg. *Schweizerischen Gesellschaft für die Gesammten Naturwissenschaften*, **10**: 1–139.

Neilan, B.A., Jacobs, D., Del Dot, T., Blackall, L.L., Hawkins, P.R., Cox, P.T. & Goodman, A.E. (1997). rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. *International Journal of Systematic Bacteriology*, **47**: 693–697.

Posada, D. (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, **25**: 1253–1256.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology*, **17**: 208.

Rigonato, J., Gama, W.A., Alvarenga, D.O., Branco, L.H.Z., Brandini, F.P., Genuário, D.B. & Fiore, M.F. (2016a). *Aliterella atlantica* gen. nov., sp. nov., and *Aliterella antarctica* sp. nov., novel members of coccoid Cyanobacteria. *International Journal of Systematic and Evolutionary Microbiology*, **66**: 2853–2861.

Rigonato, J., Gonçalves, N., Andreote, A.P.D., Lambais, M.R., & Fiore, M.F. (2016b). Estimating genetic structure and diversity of cyanobacterial communities in Atlantic forest phyllosphere. *Canadian Journal of Microbiology*, **62**: 953–960.

Rigonato, J., Sant'Anna, C.L., Giani, A., Azevedo, M.T.P., Gama, W.A., Viana, V.F., Fiore, M.F. & Werner, V.R. (2018). *Sphaerocavum*: a coccoid morphogenus identical to *Microcystis* in terms of 16S rDNA and ITS sequence phylogenies. *Hydrobiologia*, **811**: 35–48.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sant'Anna, C.L., Azevedo, M.T.P., Fiore, M.F., Lorenzi, A. S., Kaštovský, J. & Komárek, J. (2011). Subgeneric diversity of *Brasilonema* (Cyanobacteria, Scytonemataceae). *Brazilian Journal of Botany*, **34**: 51–62.

Sant'Anna, C.L., Kaštovský, J., Hentschke, G.S. & Komárek, J. (2013). Phenotypic studies on terrestrial stigonematacean cyanobacteria from the Atlantic Rainforest, São Paulo State, Brazil. *Phytotaxa*, **89**: 1–23.

Schirrmeister, B.E., Antonelli, A. & Bagheri, H.C. (2011). The origin of multicellularity in cyanobacteria. *BMC Evolutionary Biology*, **11**: 45.

Shalygin, S., Shalygina, R., Johansen, J.R., Pietrasiak, N., Berrendero, E., Bohunická, M., Mareš, J. & Sheil, C.A. (2017). *Cyanomargarita* gen. nov. (Nostocales, Cyanobacteria): convergent evolution resulting in a cryptic genus. *Journal of Phycology*, **53**: 731–915.

Stackebrandt, E. & Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiology Today*, **33**: 152–155.

Stackebrandt, E. & Goebel, B.M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology*, **44**: 846–849.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA 6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, **30**: 2725–2729.

Taton, A., Grubisic, S., Brambilla, E., De Wit, R. & Wilmotte, A. (2003). Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo Dry Valleys, Antarctica): a morphological and molecular approach. *Applied and Environmental Microbiology*, **69**: 5157–5169.

Welsh, H. (1965). A contribution to our knowledge of the blue-green algae of South West Africa and Bechuanaland. *Nova Hedwigia*, **9**: 131–162.

Wood, S.A., Rhodes, L., Smith, K., Lengline, F., Ponikla, K. & Pochon, X. (2016). Phylogenetic characterisation of marine *Chroococcus*-like (Cyanobacteria) strains from the Pacific region. *New Zealand Journal of Botany*, **55:** 1–9.