WATSON ARANTES GAMA JÚNIOR

Estudo polifásico de cianobactérias cocoides terrestres de áreas da Mata Atlântica, SP, Brasil

Tese apresentada ao Instituto de Botânica da Secretaria do Meio Ambiente, como parte dos requisitos exigidos para a obtenção do título de DOUTOR em BIODIVERSIDADE VEGETAL E MEIO AMBIENTE, na Área de Concentração de Plantas Vasculares em Análises Ambientais.

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Dedicatória

Dedico este trabalho ao saber. Ao individual. Ao coletivo.

gnōthi seauton

Mistério revelado é no fundo, um mistério ampliado (R.J. Lopes).

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Aqueles que nos ensinam, dificilmente caem no esquecimento.

Por isso, sejamos como Cora Coralina nos recomendou: felizes! Sim, felizes, pois 'feliz aquele que transfere o que sabe e aprende o que ensina'. E assim seremos lembrados e lembraremos pela a alegria gratuita de receber e doar o aprendizado. E assim traremos conosco a renovação cintilante do conhecimento adquirido a cada passo dado na evolução da vida. Eu sou imensamente grato a todos que me ensinaram e me deram a oportunidade de ensinar. Vocês me fizeram (e fazem) muito feliz.

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RESUMO

A taxonomia e sistemática das cianobactérias vem passando por grandes alterações após o conceito de taxonomia polifásica ter aberto as portas para um novo entendimento sobre esses organismos. Por muito tempo entendidos com base apenas na morfologia, as cianobactérias passam a ser caracterizadas por novas ferramentas após o avanço tecnológico na ciência. Neste sentido, a morfologia pode parecer obsoleta, mas na verdade é a ferramenta chave para unir o conhecimento preexistente com o novo. Assim, é fundamental estudar a morfologia das cianobactérias de uma maneira precisa, principalmente de grupos como as cocoides, que possuem caracteres morfo-taxonômicos de difícil reconhecimento. Além disso, é primordial a extensão de estudos a *habitats* e regiões ainda pouco conhecidos, como o caso dos ambientes terrestres tropicais. Visando a caracterização de cianobactérias unicelulares e coloniais desses ambientes, o presente estudo analisou linhagens isoladas de ambientes terrestres da Mata Atlântica por meio de uma abordagem polifásica. Foram coletadas amostras em cinco diferentes áreas preservadas do Bioma Mata Atlântica, todas localizadas no estado de São Paulo. Os substratos amostrados foram rochas, solos, cascas de árvore e também alguns artificiais, *e.g.* concreto. Todas as cepas isoladas são mantidas na Coleção de Cultura do Instituto de Botânica (CCIBt) sob condições controladas. Um total de 32 linhagens foram estudadas de acordo com a caracterização morfológica por microscopia ótica e eletrônica de transmissão; pelos marcadores moleculares rDNA 16S e ITS 16S-23S e algumas tiveram o potencial para produção de substâncias antioxidantes e antifúngicas investigado. Dessa forma, foi possível definir e distinguir os termos baeócitos e nanócitos, além de levantar a possibilidade de que as cocoides produtoras de baeócitos formem um grupo monofilético. Isso porque foi evidenciado que o clado formado por *Chroococcidiopsis thermalis* produz nanócitos, não baeócitos. Também foi possível evidenciar a heterogeneidade encontrada no gênero *Chroococcus*, com a descrição de dois novos gêneros a ele relacionados e cujas espécies tipo apresentam grande potencial na produção de antioxidantes e antifúngicos. O gênero *Asterocapsa* foi caracterizado pela primeira vez por marcadores moleculares e mostrou-se amplamente distribuído em ambientes terrestres, principalmente sobre rochas e concreto. Morfotipos similares a *Asterocapsa,* mas encontrados sobre casca de árvores, são filogeneticamente distintos e foram designados como um novo gênero. O gênero *Gloeothece* foi caracterizado e sua separação filogenética de *Aphanothece* confirmada. Além disso, descobriu-se um novo gênero filogeneticamente relacionado a *Gloeothece.* Esse novo gênero e *Gloeothece* foram agrupados na nova família proposta Gloeothecaceae. Assim, o presente estudo confirma o esperado quanto a alta biodiversidade dos ambientes investigados e contribuiu para a caracterização de cocoides não conhecidas por meio de marcadores moleculares. Também demonstrou que associado à biodiversidade de cianobactéria há um grande potencial para produção de substâncias que podem ser exploradas pela indústria farmacêutica.

Palavras-chave: Chroococcales, Pleurocapsales, Synechococcales, RNAr 16S, ITS 16S-23S, diversidade, filogenia.

ABSTRACT

The taxonomy and systematics of cyanobacteria have gone through major changes after the concept of polyphasic taxonomy promoted new understanding of these organisms. Being understood only morphologically for a long time, cyanobacteria are now characterized with new tools brought about by technological advancement in science. In this sense, morphology may seem obsolete, but in fact it is a key tool for joining all preexisting and new knowledge. Therefore, it is essential to study the morphology of cyanobacteria in a precise way, especially in groups such as coccoids, which have morphologic and taxonomic characteristics that are difficult to recognize. In addition, it is essential to extend the studies to little known habitats and regions such as tropical terrestrial environments. Aiming to characterize unicellular and colonial cyanobacteria, the present study analyzed strains isolated from terrestrial environments of the Atlantic Rainforest through a polyphasic approach. Samples were collected in five different preserved areas of the Atlantic Rainforest Biome, all located in São Paulo State, Brazil. The sampled substrates were rocks, soils, tree barks and some were artificial, *e.g.* concrete. All isolated strains are kept in the Culture Collection of Institute of Botany (CCIBt) under controlled conditions. A total of 32 lineages were studied according to the morphological characterization by optical and electronic transmission microscopy and by molecular markers rDNA 16S and ITS 16S-23S, and some strains had their potential for producing antioxidant and antifungal substances investigated. Thus, based on our results, it was possible to distinguish and define the difference between baeocytes and nanocytes, besides considering the possibility that the coccoids producing baeocytes form a monophyletic group. This is based on the evidence that the clade of *Chroococcidiopsis thermalis* produces nanocytes and not baeocytes. It was also possible to show the heterogeneity in the genus *Chroococcus*, which resulted in the description of two new related genera, whose type species showed great potential for the production of antioxidants and antifungals. The genus *Asterocapsa* was characterized for the first time by molecular markers and showed to be widely distributed in terrestrial environments, mainly on rocks and concrete. Morphotypes similar to *Asterocapsa*, but found on tree barks, are phylogenetically different and have been designated as a new genus. The genus *Gloeothece* was characterized and its phylogenetic separation from *Aphanothece* was confirmed. In addition, a new genus phylogenetically related to *Gloeothece* was discovered. This new genus and *Gloeothece* were grouped in the proposed new family Gloeothecaceae. Thus, the present study confirms the expected high biodiversity of the investigated environments and contributes to the characterization of unknown coccoid cyanobacteria by means of molecular markers. It has also shown that associated with the biodiversity of cyanobacteria, there is great potential for the production of substances that can be exploited by the pharmaceutical industry.

Key words: Chroococcales, Pleurocapsales, Synechococcales, RNAr 16S, 16S-23S ITS, diversity, phylogeny.

LISTA DE ABREVIATURAS

16S – gene da subunidade ribossomal 16S

16S rDNA – DNA da subunidade ribossomal 16S

23S – gene da subunidade ribossomal 23S

AR – Atlantic Rainforest

Avg. – average value

CCALA – *Culture Collection of Autotrophic Organisms*

CCD – Cromatografia em Camada Delgada (idem TLC – *Thin Layer Chromatography*)

CCIBt – Coleção de Cultura do Instituto de Botânica

DPPH – 2,2-difenil-1-picrilhidrazila

GTR+G+I – *Generalised time-reversible*+distribuição gama+sítios invariantes

HKY+G+I – Hasegawa, Kishino & Yano+distribuição gama+sítios invariantes

ITS – Espaçador intergênico transcrito (*Internal transcribed spacer*)

Max. – maximum value

Min. – minimum value

ML – máxima verossimilhança (*Maximum likelihood*)

NJ – Neighbor-joining

PEFI – Parque Estadual Fontes do Ipiranga

PESM – Parque Estadual da Serra do Mar

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Introdução Geral/Material & Métodos Gerais/Objetivos

1. INTRODUÇÃO GERAL

As cianobactérias são organismos procariontes que realizam fotossíntese oxigênica. Essa característica as tornam únicas dentre bactérias e as aproximam das algas. Por esta razão, por muito tempo houve o entendimento das cianobactérias como algas e por isso o estudo desses organismos por meio de uma abordagem botânica (Geitler, 1932; Komárek & Anagnostidis, 1998, 2005). Contudo, com a total confirmação da natureza procariótica das cianobactérias na década de 70 (Stanier et al., 1978), houve uma alteração da visão científica sobre esses organismos, o que atingiu desde a nomenclatura até a taxonomia e sistemática das cianobactérias. Nestas duas últimas áreas o impacto foi maior. Isso porque as cianobactérias passaram a ser mais estudadas sob a ótica microbiologista, cujo entendimento taxonômico estava voltado à época para a fisiologia e biologia molecular, enquanto na botânica para a morfologia e ecologia. Com o seguimento dessa ideia, hoje as cianobactérias são identificadas e classificadas segundo uma abordagem polifásica, a qual leva em consideração o conhecimento de várias áreas a fim de melhor caracterizar esses organismos (Komárek, 2016a).

Com essa alteração na taxonomia das cianobactérias, houve um enorme avanço sobre o entendimento desses organismos. Inicialmente, pensava-se que as cianobactérias possuíssem apenas clorofila *a* como principal pigmento fotossintetizante, o mesmo encontrado em plantas e algas (Lee, 2008). Contudo, atualmente sabe-se que há cianobactérias que além da clorofila *a*, também possuem clorofila *b* (Prochlorophyta – Chisholm et al., 1992), *d* (*Acaryochloris* Miyashita et al. – Miyashita et al., 2003) e *f* (estromatólitos – Chen et al., 2010). Essa variação na produção de clorofilas está intimamente ligada a capacidade de absorção de luz em diferentes comprimentos de onda, uma vantagem evolutiva com relação a capacidade fotossintética. Além dessa variação de tipos de clorofila, as cianobactérias possuem ainda as ficobiliproteínas, que também são pigmentos atuantes na captação de luz para a fotossíntese, sendo eles a aloficocianina, ficocianina e ficoeritrina (Lee, 2008). Tais pigmentos estão organizados em estruturas denominados ficobilissomos e há cianobactérias capazes de alterar a concentração de uma ficobiliproteína em detrimento de outra, dependendo da irradiação luminosa a qual está exposta, o que é denominado de Capacidade de Adaptação Cromática (Marsac, 1977).

Como podemos perceber, as cianobactérias possuem uma ampla capacidade fotossintética, o que deve ter tido grande influência na existência delas por cerca de 3,7 bilhões de anos sobre a Terra (Nutman et al., 2016). Devido a essa longa existência, é atribuído às cianobactérias a início da oxigenação atmosférica (Lyons et al., 2014) e ainda hoje são elas o grande protagonista da produção de oxigênio nos oceanos (Flombaum et al., 2013). Em comparação, pode-se atribuir essa mesma importância às cianobactérias terrestres, as quais dominam a maior parte das crostas biológicas (Belnap, 2003). Além disso, também são fixadoras de nitrogênio e exercem grande contribuição do ciclo geológico deste elemento (Belnap, 2002). Esse longo tempo de existência também permitiu às cianobactérias um grande período de evolução e adaptação às mais adversas condições. Prova disso é que podemos encontrar cianobactérias em praticamente todos os lugares onde haja um mínimo de iluminação (Horikoshi et al., 2011). Além da ampla distribuição nos mais diversos ecossistemas terrestres, as cianobactérias também apresentam grande variabilidade morfológica, bem maior se as compararmos com outras bactérias.

Neste sentido, podemos diferenciar as cianobactérias entre unicelulares e coloniais, denominadas cianobactérias cocoides e as filamentosas, as quais podem ser homocitadas ou heterocitadas. Baseando-se na simplicidade *vs.* complexidade morfológica, por muito tempo classificaram-se as cianobactérias cocoides como as mais primitivas, seguidas das homocitadas e por fim as heterocitadas, que possuem células especializadas na fixação de nitrogênio (heterócitos) e de resistência a intempéries (acinetos). Contudo, essa ideia foi confrontada com a descoberta de que a multicelularidade, *i.e.* capacidade de formação de filamentos, é um evento que evoluiu independentemente em várias linhagens (Schirrmeister et al., 2013).

Dessa forma, apesar de morfologicamente mais simples, as cianobactérias cocoides possuem grande diversidade e complexa taxonomia (Komárek & Anagnostidis, 1998). Devido à simplicidade dos caracteres, um dos principais meios utilizados para diferencia-las foi o processo de divisão celular. Apesar de ser comum a todos os grupos de cianobactérias, a fissão binária pode ocorrer de quatro formas nas cocoides, variando quanto ao plano/direção de divisão e retorno ou não das células filhas ao tamanho original antes da próxima fissão (Kováčik, 1988). Além disso, as cocoides também apresentam alternativas à fissão binária, sendo elas a fissão assimétrica, na qual as células produzem exócitos, e fissão múltipla com produção de baeócitos. O tipo de

divisão celular foi o critério utilizado e sustentou a separação dessas cianobactérias em famílias, principalmente (vide Komárek & Anagnostidis, 1986, 1998). Contudo, atualmente sabe-se, com base em estudos do DNAr 16S, que essa característica não reflete grupos monofiléticos na maior parte dos casos (Komárek et al., 2014). Também é uma característica de difícil reconhecimento, podendo em alguns casos estar ligada às variações ambientais (Kováčik, 1988). Além do plano de divisão, a organização das colônias/talo formadas pelas cianobactérias cocoides é um critério adotado para a separação de famílias e gêneros (Komárek & Anagnostidis, 1998). As células podem estar organizadas radialmente, em arranjo sarciniforme, enfileiradas formando talos polarizados, colônias ocas, etc. e dentro de cada família essa característica tem o peso adequado. O tipo de mucilagem das células/colônias também é carácter fundamental para a separação morfológica dos gêneros de cocoides, sendo consideradas a cor, a estrutura (simples, lamelada, verrucosa, etc.), a consistência (gelatinosa ou rígida) e a visibilidade (conspícua ou inconspícua) como principais critérios.

O valor filogenético dessas características ainda é uma incógnita para muitos grupos de cianobactérias cocoides. Contudo, é notável que muitas delas tiveram uma evolução independente, podendo uma mesma característica morfológica ser observada em grupos distantes filogeneticamente. No mais novo sistema de classificação proposto, Komárek et al. (2014), baseado em análise genômica, fica muito clara a diversidade filogenética desse grupo, além de ser confirmada a relação das cianobactérias unicelulares e coloniais com as filamentosas.

Além disso, todas essas características morfológicas mostram-se intimamente ligadas às condições ambientais e por isso são altamente influenciáveis por condições de cultivo. Uma vez que grande parte da taxonomia das cianobactérias foi baseada na caracterização morfológica, e as cocoides não são uma exceção, encontramos aqui um impasse. Com o avanço da taxonomia polifásica, ficou cada vez mais comum o estudo desses organismos em cultivo e em muitos casos as culturas não mantêm as características morfológicas diacríticas necessárias para a correta identificação taxonômica. O resultado disso é um grande volume de culturas e de sequências de DNAr 16S depositadas em bancos públicos que estão mal identificadas. Adicionalmente, o reconhecimento dessas características requer experiência e aqui se torna fundamental o papel do taxonomista: a ligação do conhecimento morfológico junto à caracterização por marcadores moleculares. No caso das cianobactérias cocoides

isso se agrava devido ao reduzido número de especialistas dedicados ao estudo taxonômico destes organismos.

Com o cultivo das cianobactérias, houve um grande avanço na caracterização e investigação do potencial biotecnológico desses organismos. Assim, sabe-se que as cianobactérias produzem diversas substâncias com ação antioxidante, antifúngica, antibacteriana, antiviral, antitumoral, etc. (Vijayakumar & Menakha, 2015). E sabe-se também que cianobactérias isoladas de ambientes extremos tendem a produzir mais desses compostos (Abed et al., 2008), e os ambientes terrestres podem ser considerados ambientes extremos devido às rápidas variações de temperatura e de luminosidade, além da baixa umidade, sendo as cianobactérias que ai habitam consideradas *poikilo-tolerant* (resistentes a estresses diversos) (Gorbushina, 2007).

Como já mencionado, as cianobactérias apresentam ampla distribuição em ambientes terrestres. Nesses ambientes, elas formam os biofilmes, que são comunidades microbiológicas que estão associadas de maneira intrínseca, unidas numa matriz de polissacarídeos sobre uma superfície (Donlan & Costerton, 2002). Fungos, bactérias heterotróficas, algas, protozoários e cianobactérias são os principais componentes dos biofilmes (Gorbushina, 2007), sendo muito comum encontrá-los sobre rochas, árvores, solos e até mesmo substratos artificiais como concreto, paredes e telhados (Gama Jr et al., 2014). Os biofilmes desempenham papel nos ciclos ecológicos e geológicos e as cianobactérias são importantes agentes mantenedores da sua estrutura por produzirem mucilagem, além de atuarem na produção primária (Gorbushina, 2007). É comum encontramos todos os grandes grupos de cianobactérias nos biofilmes, em maior ou menor grau dependendo do tipo de ambiente. Mas é fato que as cocoides estão na maioria deles e sobre rochas elas tendem a ser o grupo mais representativo (Hauer et al., 2015) e essa diversidade mostrou-se muito grande em ambientes terrestres tropicais (Sant'Anna et al., 2011a).

A faixa tropical do planeta abriga a maior parte dos *hotspots* para conservação da biodiversidade do mundo (Myers et al., 2000). Dentre eles, o Brasil abriga dois: a Mata Atlântica e o Cerrado. Esses também são dois dos maiores biomas brasileiros, ou pelo menos eram antes de serem devastados pela ação antrópica (SOS Mata Atlântica & INPE, 2014). Contudo, a Mata Atlântica ainda apresenta consideráveis áreas remanescentes, boa parte delas localizadas no estado de São Paulo (SOS Mata Atlântica

& INPE, 2014). Por ser um bioma de temperaturas amenas e úmido (em sua maior parte), a Mata Atlântica se torna um ambiente extremamente favorável ao crescimento de cianobactérias. Além disso, por possuir ecossistemas muito diversos, com áreas costeiras até montanhosas, há grande variação de *habitats* e se levarmos em conta a influência de microhabitats no crescimento de microrganismos, essa variação se multiplica. Prova disso é alta diversidade de cianobactérias encontrada neste bioma (Rigonato et al., 2012; Rigonato et al., 2016b).

Ao todo, já foram descritos oito novos gêneros e 52 novas espécies para ambientes terrestres da Mata Atlântica, incluindo morfoespécies (Sant'Anna, 1988; Sant'Anna & Silva, 1988; Silva & Sant'Anna, 1988; Azevedo, 1991; Sant'Anna et al., 1991a; Sant'Anna et al., 1991b; Azevedo & Sant'Anna, 1993, 1994a, b; Branco et al., 1994; Azevedo & Kováĉik, 1996; Komárek, 2003; Branco et al., 2006; Fiore et al., 2007; Sant'Anna et al., 2007; Lemes-da-Silva et al., 2010; Sant'Anna et al., 2010; Sant'Anna et al., 2011b; Sant'Anna et al., 2011c; Gama Jr et al., 2012; Komarek et al., 2013; Sant'Anna et al., 2013; Hentschke & Komárek, 2014; Malone et al., 2015; Alvarenga et al., 2016; Hentschke et al., 2016; Martins et al., 2016). Contudo, deste total, apenas um gênero e 12 espécies são de cianobactérias unicelulares ou coloniais. Considerando que as cocoides são muito diversas em ambientes terrestres, provavelmente esses baixos números estejam relacionados à falta de estudos direcionados a esse grupo. Essa ideia é reforçada pelo trabalho de Gama Jr et al. (2014), no qual é mostrada que grande parte da diversidade de cianobactérias unicelulares ou coloniais de ambientes terrestres da Atlântica ainda é desconhecida. Adicionalmente, Nabout et al. (2013) demonstram que cerca de metade da diversidade de cianobactérias ainda é desconhecida e que grande parte do que ainda se tem a conhecer, está nos ambientes terrestres.

2. OBJETIVO GERAL

Estudar e caracterizar linhagens de cianobactérias cocoides isoladas de ambientes terrestres da Mata Atlântica com base numa análise polifásica.

3. OBJETIVOS ESPECÍFICOS

1. Ampliar o conhecimento sobre a biodiversidade e a distribuição geográfica de cianobactérias cocoides terrestres para regiões tropicais e subtropicais;

2. Investigar a variabilidade das características morfológicas utilizadas na taxonomia tradicional das cianobactérias unicelulares e coloniais e a relevância dessas características frente às análises filogenéticas do gene do RNAr 16S e do ITS 16S-23S;

3. Caracterizar e comparar a ultraestrutura das linhagens estudadas em relação ao arranjo dos tilacóides;

5. Selecionar bons marcadores morfológicos e ecológicos que reflitam as análises moleculares dos táxons estudados;

6. Detectar a presença de metabólitos secundários com possíveis atividades antifúngica e antioxidante.

4. MATERIAL E MÉTODOS

4.1. Área de estudo

4.1.1. Mata Atlântica

No presente estudo, a Mata Atlântica que cobria originalmente 69% do estado de São Paulo, atualmente possui apenas 13,9% de áreas florestais (SOS Mata Atlântica & INPE, 2014). Apesar da drástica redução da vegetação, o Estado de São Paulo apresenta uma das maiores porções remanescente deste bioma ao longo do litoral, preservada como parques estaduais e estações ecológicas (SOS Mata Atlântica & INPE, 2014). O Parque Estadual da Serra do Mar (PESM), localizado neste estado, é a maior área de proteção integral do litoral brasileiro e a maior área protegida de Mata Atlântica.

As áreas de estudo realizadas aqui estão detalhas em Gama Jr et al. (2012), Hentschke & Komárek (2014) e Malone et al. (2015). Todas as áreas amostradas estão incluídas no Bioma Mata Atlântica definido pela Lei Federal 11428/2006 e pelo Decreto 6660/2008, constituindo-se basicamente de áreas de Floresta Ombrófila Densa e ecossistemas associados (restinga e mangue) (SOS Mata Atlântica & INPE, 2014). As áreas especificadas abaixo e na Figura 1.

- **Parque Estadual da Serra do Mar, Núcleo Santa Virgínia** (23º17'S a 23º24' S e 45º03W a 45º11' W)
- **Parque Estadual da Ilha do Cardoso** (25°03’S a 25°18’S e 47°53’W a 48°05’W)
- **Estação Ecológica Juréia-Itatins** (24°18'S a 24°37'S e 47°00'W a 47°31'W)
- **Parque Estadual Fontes do Ipiranga** (PEFI) (23º38’S a 23º40’S e 46º36’W a 46º38’W)
- **Gruta-que-chora, Praia do Sununga, Ubatuba** (23°30'15"S 45°8'18"W)

Figura 1: Localização das áreas de coleta referentes ao Bioma Mata Atlântica, Estado de São Paulo. 1. Parque Estadual Fontes do Ipiranga (PEFI); 2. Parque Estadual da Serra do Mar, Núcleo Santa Virgínia; 3. Gruta-que-chora, Praia do Sununga, Ubatuba; 4. Estação Ecológica Juréia-Itatins; 5. Parque Estadual da Ilha do Cardoso.

4.2. Cultivo e Isolamento

As linhagens estudadas da Mata Atlântica foram isoladas em maioria durante o trabalho florístico realizado por Gama Jr. (2012). Todas essas linhagens são mantidas no Banco de Culturas de Cianobactérias pertencente à Coleção de Cultura do Instituto de Botânica (CCIBt) e são mantidas nas seguintes condições: temperatura 23±1 ºC, irradiância 40-50 μmol fótons m^{-2}/s^{-1} e fotoperíodo 14–10 h claro-escuro, sendo as cepas repicadas a cada 30 dias. Além das linhagens da Mata Atlântica, foram adicionadas ao presente estudo linhagens isoladas de ambientes terrestres do Cerrado e que estavam depositadas na CCIBt. Além dessas, também foi adquirida a linhagem CCALA054, originalmente depositada na *Culture Collection of Autotrophic Organisms* (CCALA). Todas essas linhagens foram incluídas no trabalho por serem morfologicamente semelhantes às isoladas da Mata Atlântica (

).

Os meios de cultura utilizados para isolamento e manutenção das linhagens foram BG-11 ou ASM-1 em meio líquido ou sólido (10% de ágar), com ou sem cicloheximida (antibiótico que não permite o desenvolvimento de organismos eucariontes), conforme descrito em Jacinavicius et al. (2013). O pH dos meios de cultura é inicialmente ajustado para 7,4 e, quando necessário, pode ser alterado para possibilitar melhor crescimento dos organismos.

O principal método para isolamento foi o de tomada unitária de células -"pescaria"- a partir do material da natureza. Este método consiste em separar e inocular apenas um organismo de cada espécie por tubo, como descrito a seguir: em uma lâmina flambada coloca-se uma gota ou parte da amostra e várias gotas de meio de cultivo estéril. Ao microscópio óptico (ocular de 10 e 20 ×), com o auxílio de uma micropipeta, retiram-se organismos da amostra, transferindo-os para uma gota com meio de cultivo limpo. Este processo é realizado sucessivamente, até que se obtenha apenas um organismo na gota de meio de cultura. Após este procedimento, o espécime isolado é transferido para um tubo de ensaio contendo meio de cultura. Além do material em cultura, uma alíquota de cada cepa foi preservada em formol 4% e depositada no Herbário do Instituto de Botânica (SP).

Tabela 1. Lista das linhagens estudadas com respectivas informações quanto a localidade e *habitat* de onde foram isoladas, meio de cultura no qual são mantidas e número de exsicata.

Linhagem	Meio de cultura	Localidade	Habitat	Coordenadas	Número herbário
CCALA054	ASM-1	Desconhecida	Fonte termal	-	SP469762
CCIBt 3405	ASM-1	Cerrado - Pirassununga	Rocha	22° 1'14.05"S 47°26'2.66"W	SP469748
CCIBt 3427	ASM-1	Mata Atlântica – Parque Santa Virgínia	Concreto	25º4’8.00”S 47º55’12.24”W	SP469746
CCIBt 3444	ASM-1	Cerrado - São Francisco de Goiás, Goiás	Quartzito	16°0'43.06"S 49°14'30.28"W	SP469747
CCIBt 3504	ASM-1	Cerrado - São Francisco de Goiás, Goiás	Casca de árvore	16°0'43.06"S 49°14'30.28"W	SP469037
CCIBt 3511	ASM-1	Mata Atlântica – Parque Fontes do Ipiranga	Concreto	23°38'32.44"S 46°37'21.59"W	SP469038
CCIBt 3522	BG11	Mata Atlântica – Estação Ecológica da Juréia	Concreto	24°23'56.47"S 47° 7'35.92"W	SP469039
CCIBt 3537	ASM-1	Mata Atlântica – Estação Ecológica da Juréia	Concreto	24°23'56.47"S 47° 7'35.92"W	SP469040
CCIBt3407	ASM-1	Mata Atlântica – Parque Santa Virgínia	Concreto	23º20’36”S 45º07’44”W	SP469749
CCIBt3408	BG-11	Mata Atlântica –	Concreto encoberto com	23º20’10”S	SP427348

Tabela 1. Lista das linhagens estudadas com respectivas informações quanto a localidade e *habitat* de onde foram isoladas, meio de cultura no qual são mantidas e número de exsicata.

Linhagem	Meio de cultura	Localidade	Habitat	Coordenadas	Número herbário
		Parque Santa Virgínia	vegetação	45º08’44”W	
CCIBt3410	ASM-1	Mata Atlântica, Parque da Ilha do Cardoso	Solo	25º01’16”S 47º55’31”W	SP469754
CCIBt3411	ASM-1	Mata Atlântica – Parque Santa Virgínia	Concreto	23º20’36”S 45º07’44”W	SP469755
CCIBt3416	ASM-1	Mata Atlântica – Parque Santa Virgínia	Concreto com vegetação	24°23.311’S 47°00.940’W	SP427303
CCIBt3418	ASM-1	Mata Atlântica – Parque Santa Virgínia	Concreto	23º20’36”S 45º07’44”W	SP469756
CCIBt3419	ASM-1	Mata Atlântica – Parque Santa Virgínia	Concreto	23º20’36”S 45º07’44”W	SP469750
CCIBt3420	BG-11	Mata Atlântica – Parque Santa Virgínia	Concreto	23º20’36”S 45º07’44”W	SP427340
CCIBt3421	BG-11	Mata Atlântica – Parque Santa Virgínia	Concreto	23º20’36”S 45º07’44”W	SP469751
CCIBt3428	ASM-1	Mata Atlântica, Parque da Ilha do Cardoso	Concreto	25º4’8.00”S 47º55’12.24”W	SP469752
CCIBt3431	ASM-1	Mata Atlântica,	Solo	25º4’8.00”S	SP469765

Tabela 1. Lista das linhagens estudadas com respectivas informações quanto a localidade e *habitat* de onde foram isoladas, meio de cultura no qual são mantidas e número de exsicata.

Linhagem	Meio de cultura	Localidade	Habitat	Coordenadas	Número herbário
CCIBt3433	ASM-1	Mata Atlântica, Parque da Ilha do Cardoso	Casca de árvore	25º05’08”S 47º55’30”W	SP469766
CCIBt3470	ASM-1	Cerrado - São Francisco de Goiás, Goiás	Concreto	16°0'43.06"S 49°14'30.28"W	SP469763
CCIBt3475	BG-11	Mata Atlântica, Parque da Ilha do Cardoso	Rocha úmida	25º04’12”S 47º55’27”W	SP469757
CCIBt3494	ASM-1	Mata Atlântica – Estação Ecológica da Juréia	Rocha úmida	24°23.311’S 47°00.940’W	SP469753
CCIBt3505	ASM-1	Mata Atlântica – Estação Ecológica da Juréia	Rocha úmida	24°22.694’ S 47°04.793’W	SP469758
CCIBt3506	ASM-1	Mata Atlântica – Estação Ecológica da Juréia	Rocha úmida	24°22.694’S 47°04.793’W	SP469759
CCIBt3508	ASM-1	Mata Atlântica – Estação Ecológica da Juréia	Solo úmido	24°22.747’S 47°04.729’W	SP469760
CCIBt3513	ASM-1	Mata Atlântica, Parque da Ilha do Cardoso	Solo	25º4’8.00”S e 47º55’12.24”W	SP469768

Tabela 1. Lista das linhagens estudadas com respectivas informações quanto a localidade e *habitat* de onde foram isoladas, meio de cultura no qual são mantidas e número de exsicata.

Linhagem	Meio de cultura	Localidade	Habitat	Coordenadas	Número herbário
CCIBt3534	ASM-1	Mata Atlântica – Estação Ecológica da Juréia	Concreto	S24 23.013 W47 04.836’	SP469769
CCIBt3549	ASM-1	Mata Atlântica – Estação Ecológica da Juréia	Rocha úmido	S24 26.162’ W 47 03.773’	SP469761
CCIBt3601	ASM-1	Mata Atlântica – Estação Ecológica da Juréia	Rocha	S24 22.694’ W 47 04.793’	SP469771
CCIBt3609	ASM-1	Mata Atlântica – Estação Ecológica da Juréia	Rocha	S24 22.694’ W 47 04.793’	SP469764
CCIBt3611	BG-11	Mata Atlântica, Gruta-que-chora	Rocha	23° 30.414 e W 45° 08.055	SP469770
CCIBt3613	BG-11	Mata Atlântica, Gruta-que-chora	Rocha	23° 30.414 e W 45° 08.054	SP469767

4.3. Estudo Morfométrico

A análise qualitativa das linhagens foi realizada sob microscópio óptico binocular Carl Zeiss, modelo Axioplan-2 com câmara clara, ocular de medição, dispositivo de epifluorescência acoplado ao equipamento e sistema de captura de imagem modelo AxioCam MRc Zeiss. O sistema de epifluorescência foi utilizado para evidenciar a presença de ficocianina (filtro verde: excitação na faixa de 546 nm) e clorofila *a* (filtro azul: excitação no faixa de 450-490 nm), que possibilitam a distinção entre cianobactérias (que apresentam clorofila *a* e ficocianina) e eubactérias (que não apresentam ambos os pigmentos).

Durante a análise qualitativa foram observadas as características morfológicas e métricas de no mínimo 50 indivíduos de cada linhagem com objetivo de registrar a variabilidade morfométrica populacional. Nas descrições taxonômicas, os dados métricos são apresentados como valor mínimo-valor médio-valor máximo. A comparação estatística entre os dados morfométricos do material estudado foi realizada por meio de teste T para amostras independentes ou ANOVA com teste a posteriori de Tukey (Callegari-Jacques, 2003). A normalidade dos dados foi verificada através do teste de Komolgorov-Smirnov (Zar, 1999) e as diferenças foram consideradas significativas quando a análise estatística apresentar $p<0,05$. Para todos os cálculos foi utilizado o software GraphPad Prism 5.0.

As culturas foram analisadas em diferentes tempos de cultivo, ou seja, após 20, 30, 40, 60 e 120 dias após inoculação. Além disso, as linhagens também foram expostas a luz solar direta e cultivadas em meio sólido sempre que suspeitas quanto a coloração da bainha e formação de pseudofilamentos fossem levantadas, respectivamente.

4.4. Estudo Taxonômico

A identificação taxonômica foi baseada em literatura especializada (Gardner, 1927; Geitler, 1932; Desikachary, 1959; Komárek & Anagnostidis, 1989, 1998), além de artigos mais recentes e específicos para cada gênero. Sempre que possível, as descrições originais dos táxons também foram analisadas para que o conceito inicial fosse preservado e eventualmente comparado com demais estudos. O sistema de classificação adotado foi o de Komárek et al. (2014).

4.5. Microscopia eletrônica de transmissão

Para observação em Microscopia Eletrônica de Transmissão (MET), as linhagens foram coletadas com cerca de 30 dias de cultivos. Para eliminação de resíduos, o material foi lavado com água ultrapura e centrifugado por 5 minutos e 7.000x. Em seguida, foi fixado com glutaraldeído 2,5% em tampão cacodilato de sódio 0,1M (pH 7,2) mais sacarose 0,2M *overnight* (Schmidt et al., 2009; Schmidt et al., 2010). Esse método permite uma melhor visualização de membranas. O material foi então lavado por três vezes com tampão cocodilato 0,05M e pós-fixado com tetróxido de ósmio 1% por quatro horas. Em seguida as amostras foram submetidas ao contraste por acetato de uranilo 5% *overnight* e desidratadas em uma série graduada de acetona. Após lavar em acetona 100%, as amostras foram pré-infiltradas em resina Spurr/Acetona 100% na proporção de 1:1, mantidas em agitação por 5h. A infiltração seguinte se deu em resina Spurr pura por 12h, tendo sido as amostras polimerizadas a 65°C por 3 dias. Os cortes ultrafinos foram realizados em ultramicrótomo (Leica Ultracut UCT) com lâmina de diamante (450) em seções de 70 nm de espessura, tendo sido montados em telas de cobre 200-mesh. Essas seções foram coradas com solução aquosa de acetato de uranilo e citrato de chumbo. Quatro réplicas serão feitas para cada linhagem, sendo estas analisadas em TEM JEM 1011 (JEOL Ltd., Tokyo, Japan, at 100 kV).

4.6. Estudo de substâncias bioativas

Foi obtida biomassa das linhagens selecionadas de acordo com os procedimentos descritos por Jacinavicius et al. (2013). Após o crescimento das cepas, a biomassa foi coletada por meio de filtragem e em seguida foi liofilizada. Os extratos foram obtidos por meio de extração seriada utilizando-se os solventes puros Hexano seguido por Metanol. Para ambos os solventes utilizados, a extração deu-se até o esgotamento da porção solúvel. As placas de Cromatografia em Camada Delgadas (CCD) foram eluídas de acordo com o tipo de extrato, sendo que extratos metanólicos foi utilizada fase móvel composta por Metanol/Clorofórmio/Água (68/32/8) (v/v/v) e para hexânicos, Diclorometano/Metanol 0,5% (v/v).

4.6.1. Prospecção de metabólitos secundários com atividade antifúngica

Foi empregada a técnica de bioautografia com fungos filamentosos *Cladosporium sphaerospermum* Penzig e *C. cladosporioides* (Fresen) de Vries (Homans & Fuchs, 1970). Amostras dos extratos brutos de cianobactérias foram submetidas à Cromatografia em

Camada Delgada (CCD) com sistemas de eluentes apropriados. Após desenvolvimento da cromatografia e completa evaporação do solvente, as cromatoplacas foram nebulizadas com a suspensão de esporos dos fungos citados e incubadas em câmara úmida a 25ºC, no escuro, por 48 horas, segundo método de Homans & Fuchs (1970). Foi utilizada nistatina como controle positivo. Compostos inibidores formam zonas de inibição claras contra o fundo escuro da placa cromatográfica.

4.6.2. Prospecção de metabólitos secundários com atividade antioxidante

A placa de CCD, desenvolvida com a amostra do extrato analisado, foi nebulizada com uma solução metanólica (2 $mg.mL^{-1}$) do radical 2,2-difenil-1-picrilhidrazila (DPPH) que permite detectar substâncias com atividade antioxidante em extratos vegetais. Essas substâncias aparecem como manchas amareladas sobre fundo violeta (Hostettmann et al., 2003). Foi utilizado catequina como controle positivo.

A mudança de coloração da solução de DPPH do violeta para o amarelo ocorre devido à transferência de elétrons ou átomos de hidrogênios de substâncias antioxidantes para o radical livre (Souza et al., 2007).

4.7. Estudo molecular

4.7.1. Extração de DNA e amplificação dos genes de RNAr 16S e ITS 16S-23S

A extração do DNA genômico foi realizada por meio do kit de extração MOBIO Power Soil. Alíquotas (5 μl) dos DNAs extraídos acrescidos de tampão de carregamento (azul de bromofenol 0,25%, glicerol 30% e 0,1% de SYBER® Green I (Eugene, OR, EUA)) para verificação e quantificação do DNA em gel de agarose 1,2%, após corrida eletroforética em tampão 0,5 X TBE (1 X TBE: Tris-borato 45 mM, EDTA 1 mM, pH 8,0). O marcador de tamanho e massa molecular Low DNA Mass Ladder (Invitrogen, Carlsbad, CA, EUA) foi utilizado como referência. A documentação do gel foi feita usando o programa "Kodak MI Application" do "Kodak Gel Logic 212 Imaging System" (Molecular Imaging System Carestream Health, Inc, Rochester, NY, EUA). O DNA extraído foi acondicionado a temperatura de -20°C até o início dos procedimentos seguintes.

A amplificação por PCR do gene do rRNA 16S e da região intergênica ITS 16S-23S foi realizada em tampão para a reação de PCR 1X (20mM Tris HCl pH 8,4; 50 mM KCl); 0,2 mM de cada dNTP; 2 mM de $MgCl_2$; 1,5 U de Platinum® Taq DNA Polimerase (Life

Technologies, Carlsbad, CA, EUA); 10 ng de DNA; 0,5 µM do primer 27F1 (5'-AGAGTTTGATCCTGCTCAG-3') (Neilan et al., 1997), 0,5 µM do primer 23S30R (5'-CTTCGCCTCTGTGTGCCTAGGT -3') (Lepère et al., 2000) e água ultrapura (Milli-Q, Millipore, EUA), esterilizada, para um volume final de 25 µL. A reação foi realizada em termociclador Techne TC-412 Thermal Cycler (Techne, Chelmsford, Essex, England), nas seguintes condições: 94 ºC/5 min; 10 ciclos de 94 ºC/45s, 57 ºC/45s, 72 ºC/2min; 25 ciclos de 94 ºC/45s, 54 ºC/45s, 72 ºC/2min e extensão final a 72 ºC/7min. A verificação do tamanho dos produtos da PCR foi feita em eletroforese de agarose conforme descrito acima.

4.7.2. Clonagem e transformação dos produtos de PCR

A clonagem dos produtos da PCR foi realizada utilizando o Kit de clonagem "pGEM®-T Easy Vector Systems" (Promega, Madison, WI, EUA). A introdução do vetor contendo o inserto nas células competentes de *E. coli* DH5α foi feita através de choque térmico (Sambrook et al., 1989). Alíquotas de 10 µL do produto de ligação e 50 µL de suspensão de células competentes de *E. coli* DH5α foram misturadas em um microtubo esterilizado, o qual foi incubado no gelo durante 30 minutos. O microtubo foi então transferido imediatamente para banho-maria a 42ºC e deixado por 30 segundos, sem agitar. Em seguida o microtubo foi incubado no gelo por mais 2 minutos. Posteriormente 250 µL de meio SOC (Sambrook et al., 1989) foram adicionados à solução de ligação a temperatura ambiente e em seguida foi incubada a 37ºC, durante 1 hora, sob agitação de 200 rpm. As células competentes transformadas foram plaqueadas em meio LB sólido contendo ampicilina (USB Corporation, Cleveland, OH, EUA) e X-Gal (Invitrogen), ambos em concentrações finais de 100 µg/mL de meio. As placas foram incubadas por 15 horas, a temperatura de 37ºC. Em seguida, cinco colônias de cor branca foram selecionadas visando confirmar a presença dos insertos de interesse. Uma pequena quantidade de células de cada clone transformado foi adicionada a reação de *nested*-PCR como descrito por Nubel et al. (1997), utilizando-se os iniciadores CYA359 (5`- GGGGAATTTTCCGCAATGGG – 3`) e CYA781aR (5` - GACTACTGGGGTATCCTAATCCCATT - 3`) e CYA781bR (5` - GACTACAGGGGTATCTAATCCCTTT - 3`) (Nübel et al. 1997). A reação de amplificação foi composta por tampão de PCR 2 X (20 mM Tris HCl pH 8,4; 50 mM KCl); 0,2 mM de cada dNTP; 6 mM MgCl2; 3 U de Platinum® Taq DNA Polimerase (Invitrogen, Brasil); 10 ng de DNA; 0,4 ρmol de cada iniciador e água ultrapura (Millipore) esterilizada, para o volume final de 25 µL. A reação foi realizada no mesmo termociclador acima descrito, nas seguintes condições: 94 ºC/2min; 30 ciclos de 94 ºC/1min, 63 ºC/41min, 72 ºC/1min e

extensão final a 72 ºC/7min. A verificação do tamanho dos produtos da PCR foi feita em eletroforese de agarose conforme previamente descrito.

4.7.3. Extração de DNA plasmidial

A extração de plasmídeos das células de *E. coli* DH5α que contenham os insertos foi feita pelo método de preparação de pequena escala de plasmídeo, usando hidrólise alcalina (Birnboim & Doly, 1979). As colônias brancas foram transferidas para 4 mL de meio LB contendo ampicilina e cultivadas por 15 horas, a 37ºC, sob agitação de 200 rpm. Em microtubos, foram colocados 1,5 mL da cultura de células produzidas e, em seguida, centrifugadas a 10.000 × g por 20 segundos. Esse procedimento repetiu-se por duas vezes. O pélete formado foi ressuspenso em 100 µL de solução I gelada (Tris-HCl 25 mM, pH 8,0, EDTA 10 mM e glucose 50 mM). Em seguida, 200 µL de solução II (NaOH 0,2 N, SDS 1%) foram adicionados e misturados gentilmente por meio da inversão dos microtubos. Após terem sido incubados no gelo por 5 minutos, foram acrescentados 150 µL de solução III gelada (acetato de potássio 3 M e ácido fórmico 1,8 M). Novamente foi realizada a mistura por inversão e os microtubos foram incubados no gelo por mais 5 minutos. Posteriormente, foram centrifugados a 10.000 × g durante 7 minutos e o sobrenadante transferido para um novo tubo. Adicionou-se 270 µL de isopropanol a temperatura ambiente, misturando e centrifugando o tubo conforme descrito anteriormente. Após a eliminação do sobrenadante, o pélete foi lavado uma vez com 250 µL de etanol 70% gelado e centrifugado a 10.000 x g por 2 minutos. O pélete foi então secado e ressuspenso em 30 µL de uma solução contendo Tris-HCl 10 mM, pH 8,0, EDTA 0,5 M e 10 mg RNAse/mL. Essa mistura foi incubada a 37ºC por 30 minutos. Os plasmídeos assim extraídos foram armazenados a -20ºC até o próximo uso.

4.7.4. Sequenciamento

A PCR para o sequenciamento dos fragmentos inseridos nos plasmídeos foi realizada usando-se o kit "ABI Prism Big Dye terminator (Applied Biosystems by Life Techonologies). Para a reação foram utilizados 200 ng de plasmídeo contendo o inserto, 5 ρmol dos iniciadores M13F (5'-GCCAGGGTTTTCCCAGTCACGA-3') ou SP6 (5'-ATTTAGGTGACACTATAGAA-3'). Também foram utilizados outros seis iniciadores (357F, 357R, 704F, 704R, 1114F, 1114R), visando o fechamento interno das sequências de DNAr 16S e do ITS 16S-23S. À reação foram adicionaodos 1 µL de "Big Dye", 2 µL de tampão 2,5 X "Save Money" (protocolo fornecido pelo fabricante) e água ultrapura para volume final de 10 µL. As condições de amplificação foram as seguintes: 25 ciclos de

95ºC/20 s, 50ºC/15 s, 60ºC/1 min. Após a amplificação dos fragmentos, foi realizada a precipitação conforme manual de instruções do kit de sequenciamento. Posteriormente, as reações precipitadas foram inseridas no sequenciador capilar ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems) para o sequenciamento dos fragmentos de DNA. Os dados gerados pelo sequenciador foram coletados e processados pelo programa "ABI PRISM® DNA Sequencing – Analysis Software" versão 3.7 (Applied Biosystems).

4.7.5. Processamento e análise filogenética das sequências

As sequências geradas foram processadas para remoção de bases produzidas com baixa qualidade (índice de qualidade < 20) através do pacote que contém os programas Phred/Phrap/Consed (Ewing & Green, 1998; Ewing et al., 1998; Gordon et al., 1998), em sistema operacional Linux. As sequências obtidas foram comparadas com outras sequências previamente depositadas no GenBank do National Center for Biotechnology Information (NCBI), utilizando-se a ferramenta Basic Local Aligment Search Tool (BLAST) (Altschul et al., 1990). Para a construção da árvore filogenética, as sequências obtidas e outras selecionadas de bancos de dados públicos, foram alinhadas, editadas usando-se o pacote de programa MEGA 6.0 (Tamura et al., 2013) (método de Clustal W para rDNA 16S e Muscle para ITS 16S-23S). Sempre que necessário, e principalmente para as sequências de ITS, o alinhamento foi ajustado manualmente a fim de fazer-se coincidir as mesmas regiões (no caso do ITS, seguiu-se o proposto por Iteman et al. (2000)). O teste de melhor modelo evolutivo foi realizado por meio do programa jModelTest 2.1.1 (Darriba et al., 2012). As análises filogenéticas utilizadas foram *Neighbor Joining* e Máxima Verossimilhança (*Maximum Likelihood*), com *bootstrap* de 1.000×, realizadas no programa MEGA 6.0 (Tamura et al., 2013). A análise Bayesiana foi realizada no programa MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2005) em 5×10^6 gerações. Cadeias foram amostradas a cada 100 ciclos e 25% dos registros foram descartados como *burn-in*. A convergência do algoritmo MCMC foi monitorada pela média do desvio padrão das frequências (<0.003). A similaridade entre as sequências foi calculada pela fórmula *[(1-p) × 100]*, sendo que '*p'* corresponde à matriz de *p-distance*, calculada no programa MEGA 6.0 (Tamura et al. 2013).

A partir do alinhamento do ITS e comparação com o proposto por Iteman et al. (2000), foram identificadas as regiões D1-D1' e BoxB, além dos RNA transportadores e outras regiões do ITS, essenciais para o correto alinhamento das sequências. Em relação à estrutura secundária,

as regiões selecionadas foram moldadas no programa Mfold (Zuker, 2003), seguindo configurações padrões do software.

Capítulo II

Chroococcus/ Inacoccus/ Cryptococcum

NEW INSIGHTS ABOUT THE GENUS CHROOCOCCUS (CYANOBACTERIA) AND DESCRIPTION OF ITS TWO RELATED NEW GENERA: *CRYPTOCOCCUM* AND *INACOCCUS*

1. INTRODUCTION

The taxonomy and systematic of cyanobacteria have been changing abruptly. Traditionally classified by morphological features, the cyanobacterial classification is now facing the advances of technology in science, mainly by molecular biology. This has emerged the polyphasic taxonomy, which holds information from different knowledge areas to characterize and separate taxa (Komárek et al., 2014).

As a result of polyphasic approach, several new genera and species have raised (Fiore et al. 2007; Malone et al. 2015; Alvarenga et al. 2016; Hentschke et al. 2016; Martins et al. 2016), and it improves the evolutionary information about cyanobacteria. Morphology may seem obsolete in this scenario, while indeed it is the most important tool to adequate and links all information of the new approach to previously existent knowledge. One of the most important recent discoveries is the close relationship between coccoid and simple filaments, what revealed that their systematic separation, used for centuries, is artificial (Hoffmann et al.; Schirrmeister et al., 2011). Improving the chances of novelties, unmapped environments have been explored, where a great diversity of cyanobacteria has confirmed to be hidden and unknown (Nabout et al., 2013; Gama Jr et al., 2014). However, the amount of polyphasic information differs according to each cyanobacterial group, and the coccoid types are among those with lowest knowledge up to date (Komárek et al., 2014; Komárek, 2016a).

Chroococcus Nägeli was described by Nägeli (1849), and it is one of the most common recognizable genus among coccoid cyanobacterial morphotypes. It is often found in aquatic and terrestrial environments, including marine and thermal waters, hot and cold deserts, growing on soils, woods and rocks (Komárek & Anagnostidis, 1998). In all different habitats, the morphotypes kept the diacritical morphological features of the genus, which are spherical or hemispherical cells, dividing only by binary fission in three irregular plans, forming solitaries (rare) or joined colonies (Komárek & Anagnostidis, 1998). Moreover, the typical *Chroococcus* has densely packed cells in a conspicuous and firm envelope, which can be

simple or intensely lamellate (Komárek & Anagnostidis, 1995) and it is the main feature to differ *Chroococcus* from its related genus *Limnococcus* Komárková et al. (Komárková et al., 2010; Komárek, 2016b).

From the 56 valid morphospecies traditionally described as typical *Chroococcus* (Komárek & Hauer, 2013), just four were studied using a polyphasic approach (Komárková et al., 2010, Kováčik et al., 2011). This was enough to confirm that some morphological features used to discriminate typical *Chroococcus* species are generally environment dependent, as the presence/shape of mucilage and cells dimension (Welsh, 1965; Komárková et al., 2010). Recently, Wood et al. (2016) proposed a new clade of marine *Chroococcus*-like lineages close to *Limnococcus*. However, these marine strains have a wide range of cells diameter (>38 µm), what is not common to *Limnococcus*, but there is many terrestrial *Chroococcus* species described with this diameter range. These authors let the resolution of this relationship open and suggest further studies to better characterize the clade, what proves that *Chroococcus* morphotypes are more phylogenetic complex than first thought. Thus, the species of *Chroococcus* have great possibility to be heterogeneous, and the terrestrial environment is the most probable place to find them, once typical *Chroococcus* tends to grow on soils and rock surfaces.

The Atlantic Rainforest is considered a hotspot for biodiversity conservation (Myers et al., 2000), and it hides a huge diversity of terrestrial cyanobacteria (Rigonato et al., 2016b). Up to date, six novel genera and 50 new species have been found on there (Sant'Anna et al., 2011b; Sant'Anna et al., 2013; Hentschke & Komárek, 2014; Malone et al., 2015; Alvarenga et al., 2016; Hentschke et al., 2016). Even the majority of these taxa are of filamentous types, Gama Jr. et al. (2014) revealed that the Atlantic Rainforest has a great variety of coccoid terrestrial species, and this discrepancy in the number of new taxa is mostly due to the lack of specific studies with this group.

It is also known that cyanobacteria from tropical areas has a large potential in production of secondary metabolites, with potential uses in pharmacology (Silva-Stenico et al., 2011; Shishido et al., 2015). Carvalho et al. (2013) showed that nostocalean and oscillatorean strains from the Atlantic Rainforest terrestrial habitats have anticholinesterase activity; but we still have little knowledge about biotechnological potential of coccoid types from this environment (Silva-Stenico et al., 2013; Sanz et al., 2015). Thus, we intend to characterize terrestrial

Chroococcus-like lineages isolated from the Atlantic Rainforest by a polyphasic approach, including their potential in production of antifungal and antioxidant compounds.

2. MATERIAL AND METHODS

The lineages were isolated in liquid medium following standard techniques (Jacinavicius et al., 2013) from samples collected on terrestrial habitats in the Atlantic Rainforest (Table 1). They are all stored in CCIBt (Culture Collection of Institute of Botany) under controlled conditions, as following: temperature of 23±1°C, irradiance of 40-50 µmol photons $m^{-2}.s^{-1}$ (provided by daylight fluorescent lamps and measured with a Li-COR quanta meter sensor) and photoperiod of 14–10h light-dark cycle. The lineage CCALA054 was bought from CCALA collection and herein studied. An aliquot of each lineage was preserved in formaldehyde 4% and deposited in the Herbarium of the Institute of Botany (SP), Brazil.

Table 1. List of studied lineages with details of their culture medium, habitat, and Herbarium access number.

Culture number	Culture Media	Origin	Habitat	Coordinates	Exsiccate number
CCIBt3410	ASM-1	Ilha do Cardoso Park, Brazil	Dried soil	S25º01'16" W47º55'31"	SP469754
CCIBt3411	ASM-1	Santa Virgínia Park, Brazil	Concrete	S23º20'36" W45º07'44"	SP469755
CCIBt3418	ASM-1	Santa Virgínia Park, Brazil	Concrete	S23º20'36" W45º07'44"	SP469756
CCIBt3475	BG-11	Ilha do Cardoso Park, Brazil	Wet Rock	S25º04'12" W47º55'27"	SP469757
CCIBt3505	ASM-1	Ecological Station of Juréia-Itatins, Brazil	Wet Rock	S24°22.694' W47°04.793'	SP469758
CCIBt3506	ASM-1	Ecological Station of Juréia-Itatins, Brazil	Wet Rock	S24°22.694' W47°04.793'	SP469759
CCIBt3508	ASM-1	Ecological Station of Juréia-Itatins, Brazil	Wet soil	S24°22.747 W47°04.729	SP469760
CCIBt3549	ASM-1	Ecological Station of Juréia-Itatins, Brazil	Wet Rock	S24°26.162' W47°03.773'	SP469761
CCALA054	ASM-1	Unknown	Thermal spring	-	SP469762

The morphological study was carried out from culture material using a Zeiss Axioplan 2 optical microscope and the identification and cell measurements were based on at least 50

individuals. The metric information is expressed as minimum-average-maximum values in the taxonomic description. To confirm the distinctiveness among species, the significance of cell diameter differences was tested by ANOVA-one way using GraphPad Prism 6, with Tukey as posteriori test and $\alpha = 0.05$. The construction of life cycle was made by cells observation at five days after culture replication, and then at 30, and 120 days after. The organisms were documented by digital photographs taken with a Zeiss Axiocam MRc digital camera. The main morphological features observed were: color and disposition of sheaths, type of cell division, cell diameter, color and uniformity of cell content, and organization of cells inside the colonies. All new taxa were described in accordance to the International Code of Nomenclature for algae, fungi, and plants (McNeill et al. 2012).

Cellular ultrastructure (position of thylakoids and mucilage arrangement) was studied from cultures by transmission electron microcopy. The cells were centrifuged for concentration and the medium was eliminated. After, each sample was fixed with Karnovsky plus sucrose 0.2M solution for 24h, washed three times (10 min each) with 0.05M cacodylate buffer and post-fixed with 1% osmium tetroxide for 1 h. For a better contrast, the samples were kept overnight in uranyl acetate 5%. Then, they were dehydrated in an acetone series of increasing concentration and after washing with 100% acetone, samples were submitted to pre-infiltration with 1:1 Spurr resin/100% acetone for 5 h with agitation. The subsequent infiltration of the samples was done 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 using an ultramicrotome (Leica Ultracut UCT) with a diamond blade (450) into 70 nm thick sections and mounted on uncoated 200-mesh copper grids, and stained with uranyl acetate and lead citrate (Reynolds, 1963). Visualization and microphotography were performed on a Jeol Jem 1011 transmission electron microscope (JEOL, Tokyo, Japan) at 100 kVA.

Total genomic DNA was isolated from a liquid culture of the cyanobacterial strains using the Ultra Clean Soil DNA Isolation Kit (MOBIO). The partial 16S-ITS-23S rRNA sequence was amplified by PCR using the 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, 200µm of each dNTP, 3.0 mM $MgCl_2$, 1 × PCR buffer and 1.0 U Platinium *Taq* DNA polymerase (Life Techonologies, Grand Island, NY, USA) under conditions according to Taton et al. (2003). The resulting PCR product was cloned into a pGEM®-T Easy Vector System (Promega, Madison, WI, USA) and inserted into

chemiocompetent *E. coli* DHG5α cells by a heat-shock method (Sambrook et al., 1989). After growth in LB plates containing 5 µl X-Gal (50 µg.mL^{-1}) (Life Techonologies) and 10 µl of ampicillin sodium salt (100 µl. mL^{-1}) (Sigma-Aldrich Co., St. Louis, MO, USA), recombinant plasmids were purified from white colonies by the alkaline lysis method (Birnboim & Doly, 1979). The cloned gene fragment was sequenced using the 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 the sample was placed in an ABI PRISM 3500 Genetic Analyzer (Life Technologies). The sequenced fragments were assembled with the Phred/Phrap/Consed software package (Ewing & Green, 1998; Ewing et al., 1998; Gordon et al., 1998).

The phylogenetic analyses were based on 16S rRNA gene and 16S-23S internal transcribed spacer (ITS) sequences obtained in this study and reference sequences retrieved from GenBank. The General Time Reversible (GTR) evolutionary model of substitution with Gamma distribution (G) and with an estimate of proportion of invariable (I) sites was selected as the best fit for the 16S rDNA alignment and Hasegawa, Kishino and Yano (HKY) model + G +I for 16S-23S ITS using jModelTest (Posada, 2008). Evolutionary analyses based on the Neighbor Joining and Maximum Likelihood methods were conducted in Mega 6.0 (Tamura et al., 2013) and estimations of the robustness of the tree were tested by bootstrap with 1,000 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 using p-distance method in Mega 6.0 (Tamura et al., 2013) and the percentage was given by the formula *[1–(p-distance)]×100*. The mean similarity was estimated by the average of similarity value of each sequence from the two compared clades.

Antifungal and antioxidant tests were performed with the hexane and methanol extracts of CCIBt3410 and CCIBt3411 strains. Both extracts were obtained from 30 days cultures, with a serial an exhaustive extraction; first hexane and then methanol. After, the extracts were dried in rotary evaporator and ran in TLC plates with dichloromethane/methanol (0.5%) and methanol/chloroform/water (68:32:8) as mobile phase, respectively. For antioxidant activity,

the plates were derivatized with DPPH methanolic solution (2 $g.l^{-1}$), with catechin as positive control and the activity was revealed by a yellow color in a purple background. The antifungal activity was tested by spraying spores within 30% glucose saline solution (10^8 $spores.mL^{-1}$ concentrated) of *Cladosporium cladosporioides* (Fresen.) de Vries and *C. sphaerospermum* Penz. over the plates, which were incubated for three days at 27°C to allow fungal growth (Homans & Fuchs, 1970). The activity was revealed as a light spot in a dark background, and nystatin was used as positive control.

3. RESULTS AND DISCUSSION

The eight studied strains were morphologically similar to *Chroococcus* genus. However, genetic analyses revealed that they are separated in three different groups. Some lineages (CCIBt 3505, 3506, 3508, 3549) were close to typical *Chroococcus*, as proposed by Komárková et al. (2010) and Kováčik et al. (2011). Other three (CCIBt 3411, 3418 3475) formed a very distinct clade, related to the typical *Chroococcus* but not enough to be kept on the same genus. The same occurred to CCIBt 3410. These two last clades are herein designed as new genera: *Inacoccus* gen. nov. and *Cryptococcum* gen. nov., respectively (Figure 1). All groups were high supported by NJ, ML and BA phylogenetic analyses of 16S rDNA and 16S-23S ITS (see nodes values in Figure 1-2 and similarity in Table 2**Erro! Fonte de referência não encontrada.**-3).

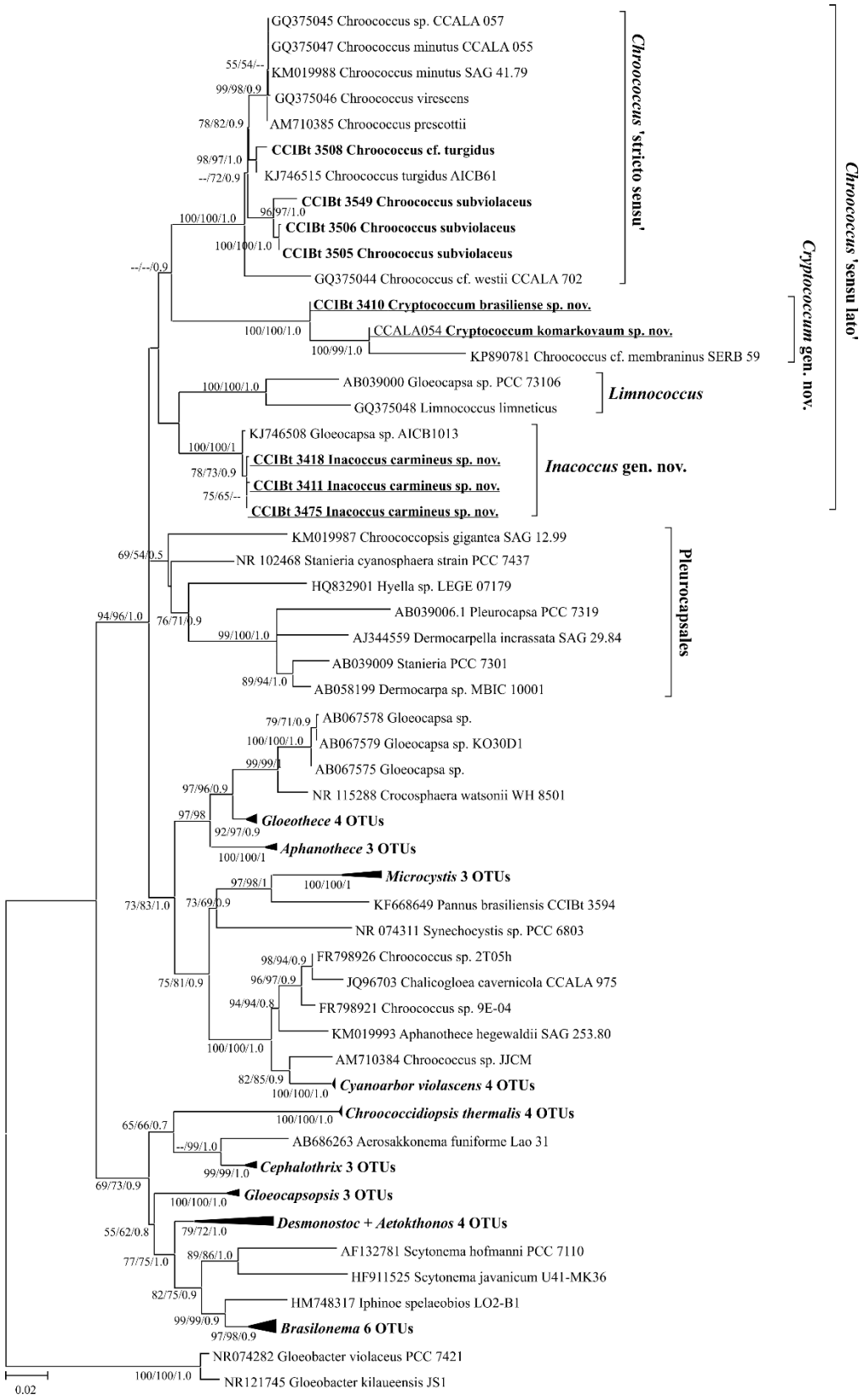


Figure 1: Phylogenetic tree based on 16S rRNA gene sequences and reconstructed using the maximum-likelihood (ML) analysis of evolutionary distances determined by the GTR + G + I model. NJ and ML bootstrap (1,000×) values (above 50 %) and Bayesian posterior probabilities are provided for each node, respectively.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

Table 2. 16S rDNA similarity matrix of studied lineages and their close relatives, conducted using the p-distance model with a total of 1,369 positions.

	Sequences identification	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	GQ375047 *Chroococcus minutus* CCALA 055																					
2	GQ375046 *Chroococcus virescens*	99.5																				
3	GQ375044 *Chroococcus* cf. *westii* CCALA 702	96.3	96.3																			
4	AM710385 *Chroococcus prescottii*	99.7	99.2	96.3																		
5	GQ375045 *Chroococcus* sp. CCALA 057	99.9	99.4	96.3	99.6																	
6	KM019988 *Chroococcus minutus* SAG 41.79	100	99.5	96.3	99.7	99.9																
7	KJ746515 *Chroococcus turgidus* AICB61	98.7	98.5	96.1	98.4	98.6	98.7															
8	CCIBt 3508 *Chroococcus* cf. *turgidus*	98.3	98	95.8	98	98.2	98.3	99.3														
9	CCIBt 3505 *Chroococcus subviolaceus*	97.1	97	95.9	97	97	97.1	96.9	96.9													
10	CCIBt 3506 *Chroococcus subviolaceus*	97.1	97	95.9	97	97	97.1	96.9	96.9	99.9												
11	CCIBt 3549 *Chroococcus subviolaceus*	96.7	96.9	95.8	96.7	96.6	96.7	96.5	96.2	98.2	98.2											
12	CCIBt 3411 *Inacoccus carmineus*	92.7	92.8	92.5	92.6	92.6	92.7	92.5	92.4	93.1	93.1	92.8										
13	CCIBt 3418 *Inacoccus carmineus*	92.8	92.9	92.6	92.8	92.8	92.8	92.6	92.5	93.3	93.3	92.9	99.8									
14	CCIBt 3475 *Inacoccus carmineus*	92.8	92.9	92.6	92.8	92.8	92.8	92.6	92.5	93.3	93.3	92.9	99.9	99.9								
15	KJ746508 *Gloeocapsa* sp. AICB1013	92.6	92.6	92.5	92.5	92.5	92.6	92.3	92.4	93.3	93.3	92.8	99.1	99.1	99.2							
16	CCIBt 3410 *Cryptococcum brasiliense*	92	92.2	91.9	92	92	92	92.5	92.4	91.9	91.9	91.5	92.6	92.8	92.8	92.5						
17	CCALA054 *Cryptococcum komarkovaum*	92	91.8	90.8	91.7	91.9	92	92.1	92.3	91.7	91.7	90.9	91.7	91.8	91.8	92	97.8					
18	KP890781 *Chroococcus* cf. *membraninus* SERB 59	88.1	87.9	86.9	87.9	88	88.1	88.2	88.5	88	88	87.3	88.1	88.1	88.1	88.2	93.4	95.5				
19	FR798926 *Chroococcus* sp. 2T05h	91.2	91.2	91.5	90.9	91.1	91.2	91.6	91.4	91.5	91.5	91.1	91.5	91.7	91.7	91.5	91.5	90.7	86.9			
20	AB039000 *Gloeocapsa* sp. PCC73106	91.8	91.5	91.2	91.8	91.7	91.8	91.9	91.9	91.8	91.8	91.4	92	92.2	92.2	92	91.2	90.9	87.3	90.7		
21	GQ375048 *Limnococcus limneticus*	91.7	91.7	91.4	91.7	91.6	91.7	91.9	92	91.3	91.3	90.9	92.3	92.4	92.4	92.2	91.9	91.4	87.4	91.3	94.7	
22	FJ589716 *Gloeocapsopsis crepidinum* LEGE 06123	90.9	90.9	91.3	90.9	90.9	90.9	91.3	91.5	90.8	90.8	90.2	91.3	91.5	91.5	91.5	90.9	90.3	86.4	90.2	90.1	90.5

3.1. Chroococcus

The four strains that grouped with typical *Chroococcus* in the phylogenetic tree were identified as two distinct species: *Chroococcus* cf. *turgidus* (CCIBt 3508) and *Chroococcus subviolaceus* (CCIBt 3505, 3506, 3549). CCIBt3508 morphology better fits in *C. turgidus* (Kützing) Nägeli original description (Kützing, 1843; Nägeli, 1849). However, this species has gained a very broad morphological range, with reports from different places and habitats, what created a ubiquity that could not belong to this species. Besides, the original habitat of *C. turgidus* is not clear (Kützing, 1843; Nägeli, 1849). Palinska et al. (2006) sequenced a *Chroococcus turgidus* exsiccate (HUW 799), but only a short length sequence and its positioning in phylogenetic trees is not accurate (Kováčik et al., 2011). In Figure 1, CCIBt 3508 is grouped with AICB61, identified as *C. turgidus*, with 99.3% of similarity. However, there are no published taxonomic data about AICB61 lineage, what turns its present identification unverifiable. Also, further investigations are needed to confirm if this 16S rRNA similarity value is strong enough to place these lineages in the same species. Moreover, CCIBt 3508 revealed cells dividing unilaterally with asymmetrical invagination, turning cells into kidney shape (Figure 5B-C). This type of division was never reported before to *Chroococcus* and it is rarely reported in literature to cyanobacteria; what we found to occur only in *Synechococcus nidulans* (Pringsheim) Komárek in Bourrelly (Allen, 1968). In CCIBt3508, this type of division was commonly found in cultures 60 days old, but not in younger cultures, like in those with 30 days old.

The other three *Chroococcus* lineages, CCIBt 3505, 3506 and 3549, morphologically and ecologically, match with *Chroococcus subviolaceus* (Wille) Gama-Jr. et al. The first two strains were isolated from the same substrate/sample, but different inoculums. *C. subviolaceus* occurred in different samples from the Atlantic Rainforest (Gama Jr et al., 2014), though it was originally found in Samoa Island; a tropical forest too. The present study is the first molecular characterization of *C. subviolaceus*, what confirms its phylogenetic distinctiveness from other *Chroococcus* species, in accordance to morphology (Figure 3) and 16S rRNA phylogeny. The ITS phylogenetic tree likewise endorsed this relation among the studied *Chroococcus* species (Figure 2).

3.2. Inacoccus

Three analyzed lineages (CCIBt 3411, 3418 and 3475) formed a distinctive clade, phylogenetic related but apart from *Chroococcus* (91.2% of mean similarity), which we described as the new genus *Inacoccus* gen. nov. Morphologically, the strains were very similar to *Chroococcus* species, mainly due the cells shape and organization. However, they all produce a high amount of intense red-colored sheath, which is not often recorded in *Chroococcus*, which species have hyaline or, at most, yellowish mucilage (Komárek & Anagnostidis, 1998). The pigment is produced by lineages in a very high quantity, enabling the formation of lumps which turn dark the culture medium (Figure 4). This was observed when lineages were growing in ASM-1 medium after 30 days and was more pronounced to CCIBt 3411. Besides it, nanocytes were eventually observed in *Inacoccus* strains. This kind of cell division generates small densely colony-packet cells by quick and successive divisions, what differs it from baeocytes (formed by simultaneous divisions). Additionally, *Inacoccus* is phylogenetic far enough from baeocyte-forming cyanobacteria, as the members from Pleurocapsales and Chroococcidiopsidales orders (Figure 1).

Chroococcus can divide irregularly in different planes, but never forming nanocytes (Komárek and Anagnostidis 1998). On the other hand, *Cyanosarcina* Kováčik, a Chroococcaceae family's member, forms nanocytes but it differs from *Inacoccus* by presenting only hyaline mucilage and cells constantly in sarcinoid arrangement, while in *Inacoccus* this structure only occurs during nanocytes development (Figure 4), which is rare. So far, there are no *Cyanosarcina* molecular data available to confirm and better resolve the phylogenetic proximity between these taxa. *Gloeocapsopsis* Geitler ex Komárek is also another genus morphologically close to *Inacoccus*, but nanocytes production is an opening question in this genus and, as *Cyanosarcina*, its colonies are constantly in sarcinoid form. Furthermore, *Gloeocapsopsis* is phylogenetically unrelated with *Inacoccus* (Figure 1). Thus, the production of intense colored mucilage, loosely cells arrangement and formation of nanocytes can be considered as *Inacoccus* autapomorphic characters.

The three studied *Inacoccus* strains belong to the same species: *Inacoccus carmineus* sp. nov. This certainty came from ITS analyzes and cells diameter (Figure 2 and Figure 3), where it is possible to see that they clearly differ from *Chroococcus*. CCIBt 3411 and 3418 strains were isolated by single cell from the same sample, what represent population variability and explain their equality. In contrast, CCIBt 3475 was collected in Ilha do Cardoso Park, about

355 km linear distance from Santa Virgínia Park, where the first two strains were collected. The ANOVA analysis revealed that there was no difference in cell diameter for these strains (*F=1.097, p=0.3365*), although some dimensions were discrepant, as those observed in CCIBt 3411 (Table 4).

The ultrastructure confirmed the presence of a very thick layer of mucilage around the cells (Figure 6). Also, the position of thylakoids revealed to be fasciculate, as the same as found in *Chroococcus* species.

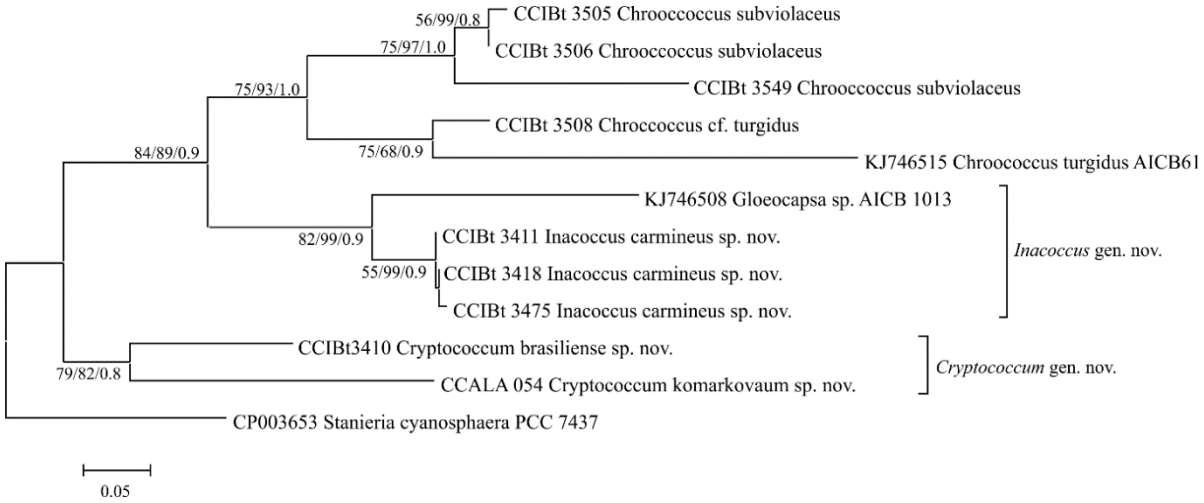


Figure 2: Phylogenetic tree based on 16S-23S ITS sequences and reconstructed using the maximum-likelihood (ML) analysis of evolutionary distances determined by the HYK + G + I model. NJ and ML bootstrap (1,000×) values (above 50 %) and Bayesian posterior probabilities are provided for each node, respectively.

Table 3. 16S-23S ITS similarity matrix of studied lineages and their close relatives, conducted using the p-distance model with a total of 150 positions.

Sequences identification		1	2	3	4	5	6	7	8	9	10	11
1	CCIBt3410 *Cryptococcum brasiliense*											
2	CCALA054 *Cryptococcum komarkovaum*	86.1										
3	CCIBt3411 *Inacoccus carmineus*	68	68									
4	CCIBt3418 *Inacoccus carmineus*	68	68									
5	KJ746508 *Gloeocapsa* sp. AICB1013	63.9	62.3	82.8	82.8							
6	CCIBt3475 *Inacoccus carmineus*	67.2	67.2	99.2	99.2	82.8						
7	CP003653 *Stanieria cyanosphaera* PCC7437	67.2	68.9	75.4	75.4	70.5	74.6					
8	CCIBt3505 *Chroococcus subviolaceus*	67.2	64.8	74.6	74.6	73.8	73.8	70.5				
9	CCIBt3506 *Chroococcus subviolaceus*	68	65.6	75.4	75.4	74.6	74.6	71.3	99.2			
10	CCIBt3549 *Chroococcus subviolaceus*	68.9	65.6	73.8	73.8	73	73	71.3	95.9	96.7		
11	CCIBt3508 *Chroococcus* cf. *turgidus*	69.7	68	74.6	74.6	68.9	73.8	68.9	83.6	84.4	84.4	
12	KJ746515 *Chroococcus turgidus* AICB61	69.7	68	67.2	67.2	62.3	66.4	60.7	70.5	71.3	71.3	86.9

3.3. Cryptococcum

The lineage CCIBIt 3410 showed to be very peculiar by grouping with CCALA 054 and SERB59 in a separated clade. Despite of having three sequences, the group formed by these lineages is very consistent and share only 90,9% of mean similarity with typical *Chroococcus*. However, they are morphologically very close from each other and to *Chroococcus* species; it is not possible to differentiate them only by morphological characters. Based on these, we decided to describe the clade formed by these three lineages as a separated genus, herein designated as *Cryptococcum* gen. nov., which is characterized as a cryptic genus, meaning it is phylogenetically distinct from *Chroococcus*, but morphologically very similar. Komárková et al. (2010) already indicated that CCALA 054 had unclear taxonomic and phylogenetic positions, and could not be included in *Chroococcus*. In spite of joining this lineage in *Limnococcus* clade, these authors put a *conferatur* behind the name, and suggested that CCALA 054 could be a *Gloeocapsa* Kützing, probably due its similarity with *Gloeocapsa* 2SD05bisS08 sequence. However, the morphology of CCALA 054 does not correspond to *Gloeocapsa*, according to Komárek (1993). Furthermore, even though *Gloeocapsa* has genetic sequences available, its phylogenetic position is not yet confirmed due strains misidentifications, and naming CCALA 054 into this genus will just move the problem to another place, not solving it in fact.

Whereas CCIBt 3410 and CCALA 054 are members of the same genus and have quite same morphology, they could be considered as different species based on 16S-23S ITS and cell diameter (Figure 3). Also, the places where these lineages were found (the Atlantic Rainforest soil and thermal spring) suggest a different ecology and physiology and reinforce their distinctiveness. Based on this, we here propose two species: *Cryptococcum brasiliense* sp. nov. (CCIBt3410) and *Cryptococcum komarkovaum* sp. nov. (CCALA054). Transmission microscopy revealed the CCIBt 3410 thylakoids in fasciculate arrangement (Figure 6), like it is found in typical *Chroococcus.*

Cryptic genera already exist in cyanobacteria (Dvořák et al., 2015), and it is predicted that more will emerge, since cyanobacterial morphology is attested to be very simple to express the total diversity. *Cryptococcum* proves it. Of course, there are some groups, like Nostocales (e.g. *Cylindrospermum* Kützing ex É.Bornet & C.Flahault Johansen et al., 2014), where morphology matches with species phylogeny. However, simpler morphologic groups, like

coccoid cyanobacteria, fall into the first case, what does not mean that morphology is a useless marker; it is just necessary to adjust its weight in cyanobacterial systematics.

Table 4. Cells dimensions of analyzed strains. Max.= maximum value; Avg.= average value; Min.= minimum value

Culture number	Taxonomic identification	Diameter (µm)		
		Avg.	Max.	Min.
CCALA054	*Cryptococcum komarkovaum* gen. et sp. nov.	10.8	13.9	8.7
CCIBt3410	*Cryptococcum brasiliense* gen. et sp. nov.	11.6	15.5	8.3
CCIBt3411	*Inacoccus carmineus* gen. et sp. nov.	6.9	11.9	4.7
CCIBt3418	*Inacoccus carmineus* gen. et sp. nov.	6.8	8.9	5.8
CCIBt3475	*Inacoccus carmineus* gen. et sp. nov.	6.6	8.4	4.9
CCIBt3505	*Chroococcus subviolaceus* (Wille) Gama-Jr. *et al.*	7.2	8.9	4.9
CCIBt3506	*Chroococcus subviolaceus* (Wille) Gama-Jr. *et al.*	7.3	9.5	6.0
CCIBt3508	*Chroococcus* cf. *turgidus* (Kützing) Nägeli	7.9	11.4	6.4
CCIBt3549	*Chroococcus subviolaceus* (Wille) Gama-Jr. *et al.*	8,43	10,47	6,32

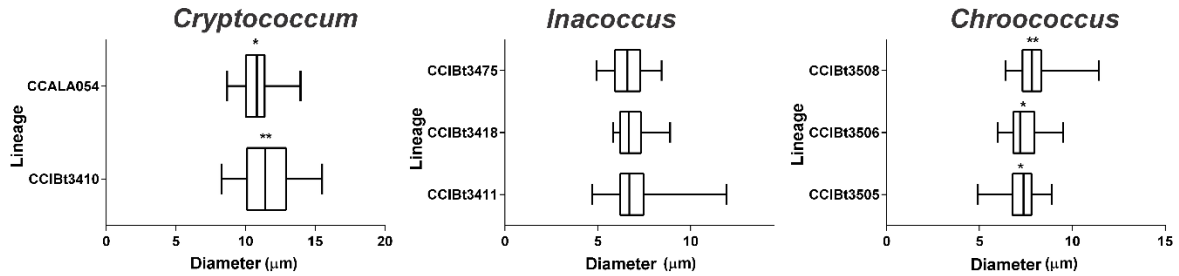


Figure 3: Box-plot of minimum, average and maximum cells diameter values together with statistical analyses. Different signs (*) represent significant differences tested by ANOVA.

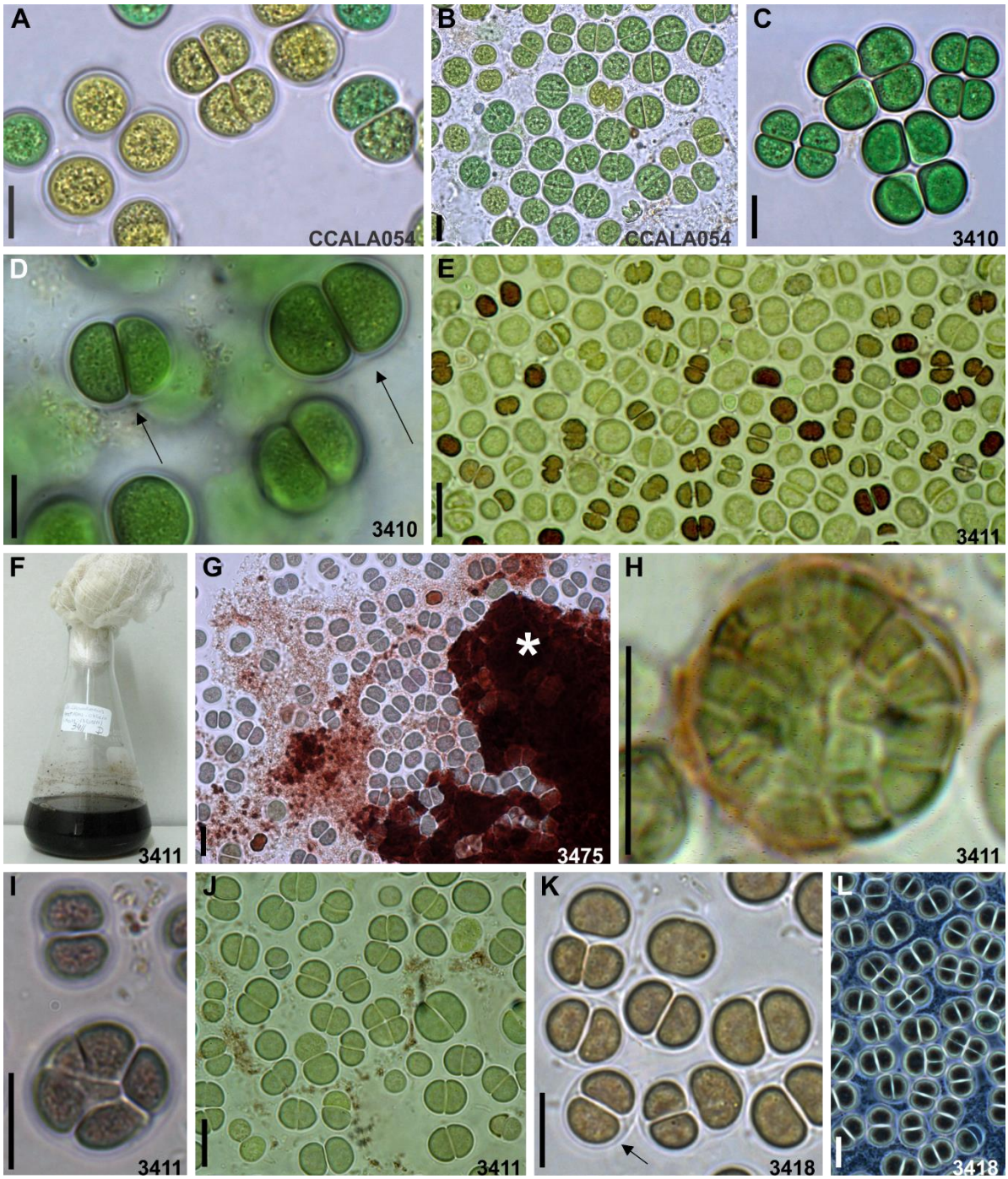


Figure 4: A-B: *Cryptococcum komarkovaum*; C-D: *Cryptococcum brasiliense* (arrows indicate the sheaths); E-L: *Inacoccus carmineus*; E: cells with intense red sheaths (dark ones) and cells without it (light ones); F: culture medium colored by sheath production; G: sheath block (*) released in medium culture; H: nanocyte formation; I: nanocyte development; J: heterogeneity of cells dimensions; K: cells with hyaline sheath (arrow); L: cells under phase contrast microscope. Scale bars = 10 µm. The strains number are on the right of each picture.

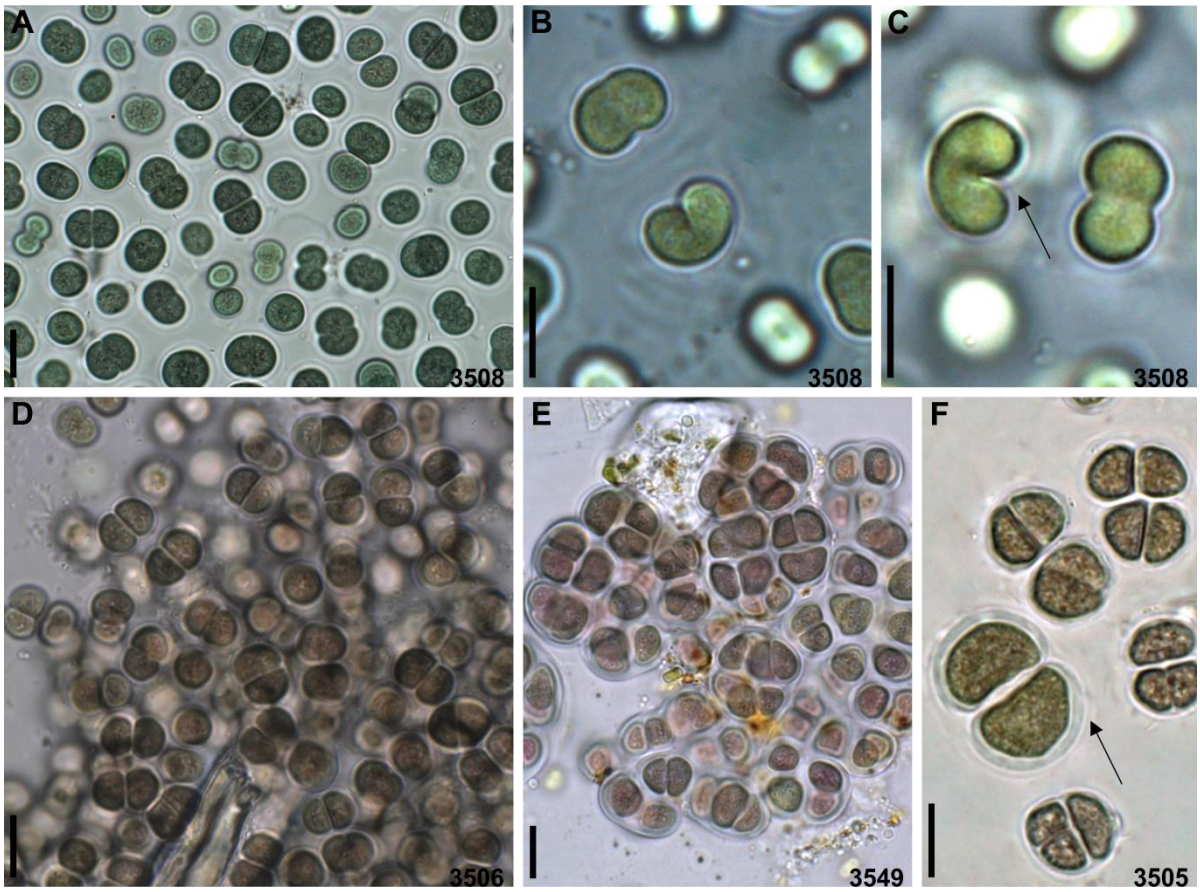


Figure 5: A-C: *Chroococcus* cf. *turgidus*. B-C: kidney-shape cells representing unilateral invagination during binary fission. D-F: *Chroococcus subviolaceus*. D-E: variation of brown to purple color of cell content and presence of large colonies (E). F: dense mucilaginous sheath around cells (arrow). Scale bars = 10 µm. The strains number are on the right of each picture.

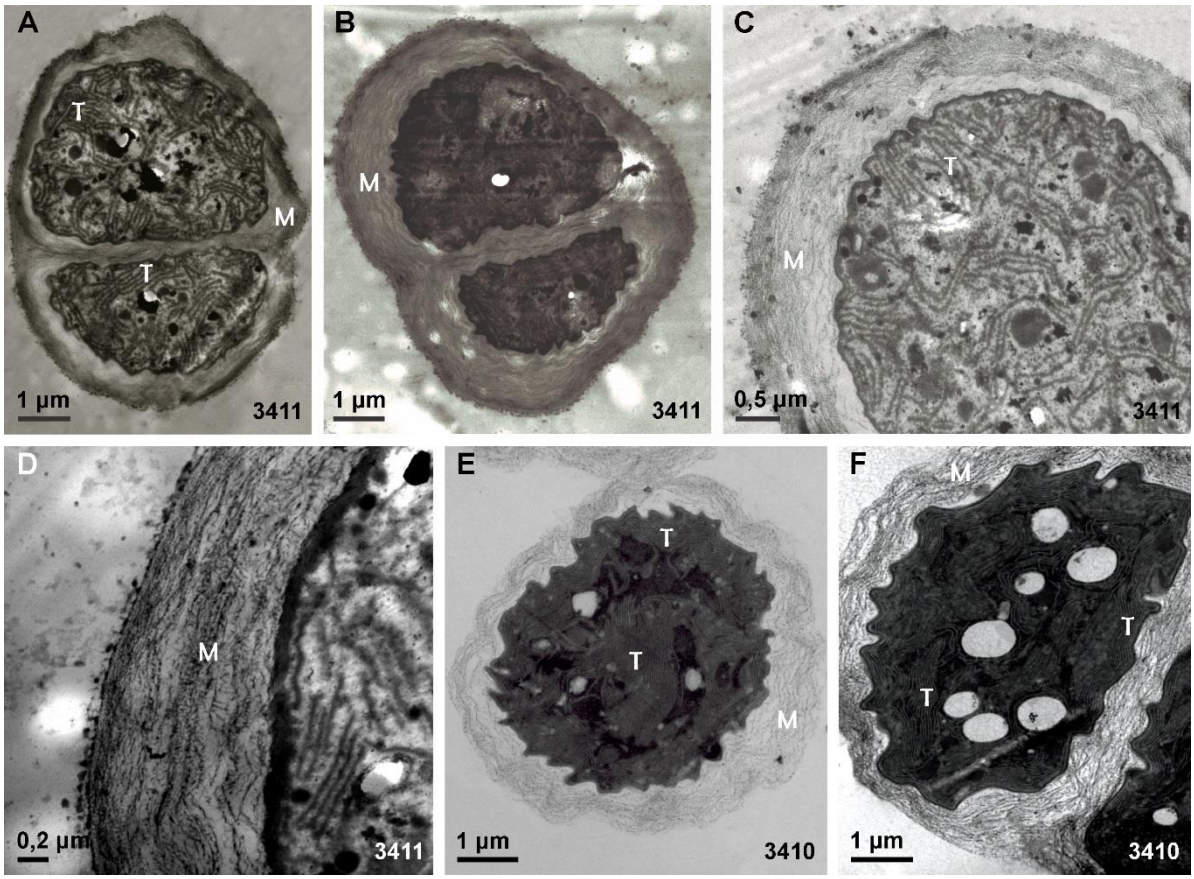


Figure 6: A-D: *Inacoccus carmineus*; B,D: dense mucilaginous surrounding the cells; E-F: *Cryptococcum brasiliense*. Caption: M: mucilaginous sheath; T: thylakoids. The strains number are on the right of each picture.

3.4. Taxonomic descriptions

***Inacoccus* gen. nov.**

Cells hemispherical to rounded, solitary or rarely grouped in few-celled colonies. Cell content green olive to purple brownish, homogenous to granulated, fasciculate thylakoids. Hyaline to generally intense red sheaths surrounding cells, simple to lamellate. Reproduction by binary fission in three irregular planes and nanocyte formation.

Type species: *Inacoccus carmineus* (see below)

Etymology: genus named in honor to Dr. Ina de Souza Nogueira for her contributions to Brazilian Phycological Research.

***Inacoccus carmineus* sp. nov.**

Cells hemispherical to rounded, 4.7–(6.7)–11.9 μm diam., solitary or rarely grouped in few-celled colonies. Hyaline to intense red-colored sheaths surrounding cells and colonies, smooth or rarely lamellate. Cell content finely granulated, purple-brownish to green-brownish. Nanocytes presented, formed by polygonal cells surrounded by red-colored sheaths.

Holotypus: exsiccate access number SP469755, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt 3411

Habitat: terrestrial, growing on rocks and concrete.

Etymology: from Latin '*carmineus*' meaning carmine, vivid red color, in reference to the sheath pigment produced by this species.

***Cryptococcum* gen. nov.**

Cells hemispherical to rounded, solitary or rarely grouped in few-celled colonies. Cell content green, blue green to olive green, homogenous to granulated, fasciculate thylakoids. Sheaths hyaline surrounding the cells, simple to lamellate. Reproduction only by binary fission in three irregular planes.

Type species: *Cryptococcum brasiliense* (see below)

Etymology: from Greek c*rypto*- (hidden) and *coccus* s.m. II (coccus).

***Cryptococcum brasiliense* sp. nov.**

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 granulated, green to blue-green. Nanocytes never observed.

Holotypus: exsiccate access number SP469754, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt3410

Habitat: terrestrial, growing on dry soil among pebbles.

Etymology: in reference to Brazil, from where the type strain was isolated.

***Cryptococcum komarkovaum* sp. nov.**

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.

Holotypus: exsiccate access number SP469762, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCALA054

Habitat: aquatic, thermal spring.

Etymology: species named in honor to Dr. Jaroslava Komárková for her contributions to Cyanobacterial Research.

3.5. Bioactivity compounds

The potential of cyanobacteria in production of bioactivity compounds is known from isolates of different places, but in terrestrial and extreme environments this production showed to be higher (Abed et al., 2008). This was confirmed by the TCL plates of methanol and hexane extracts from CCIBt 3411 and 3410, since both revealed antioxidant activity. Also, it is possible to see that these same hexane soluble compounds have antifungal activity for the two tested fungi species. The methanol extracts did not show antifungal activity (Figure 7).

The shape of the spot let by hexane extracts of these strains, and the polarity of the solvent, suggest that the antioxidant and antifungal compounds could be a fat acid. Piccardi et al. (2000) also found antifungal activity compounds in lipophilic extracts from *Nostoc* isolates and showed that these extracts have more propensity to have antifungal activity than the hydrophilic ones. Also, many of described compounds regard to nostocalean and oscillatorean types (Mukherjee et al., 2013), what makes the coccoid cyanobacteria a very promisor source for new compounds.

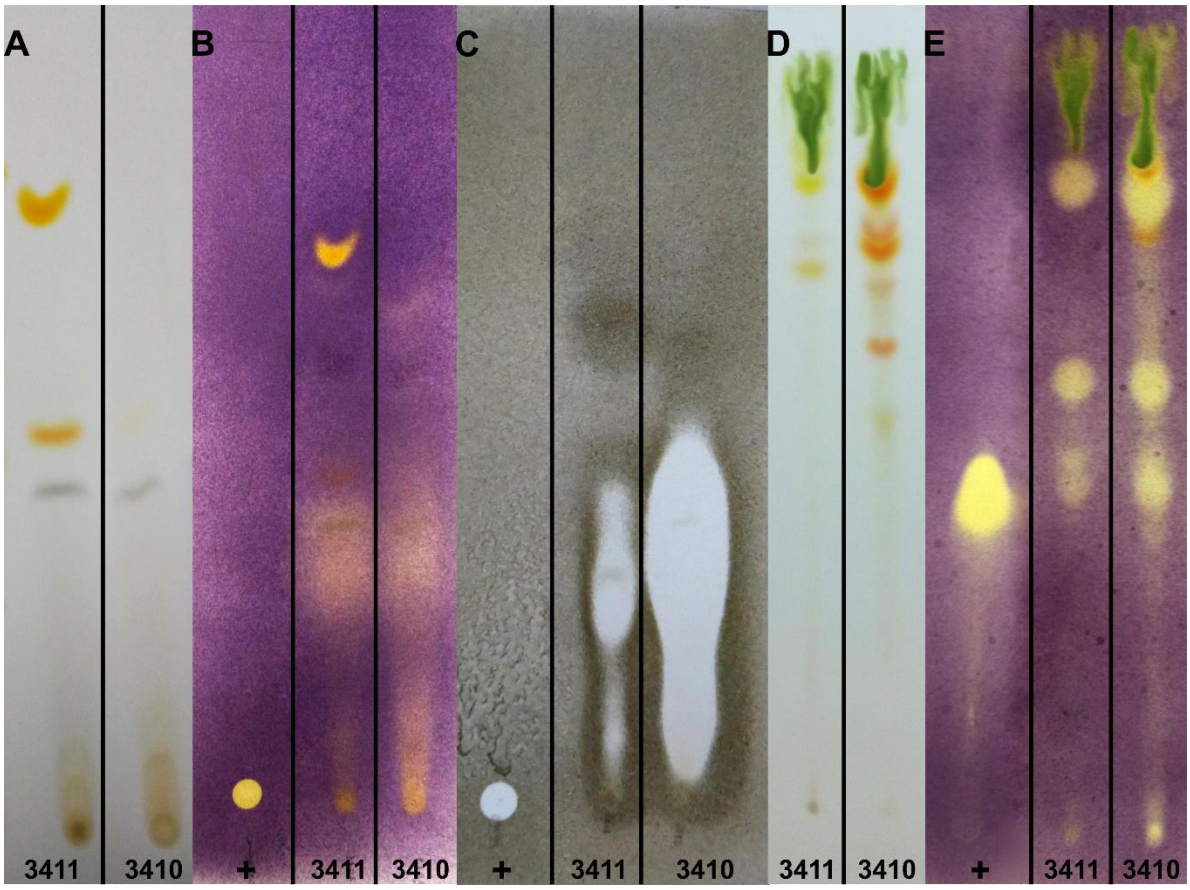


Figure 7: TLC plates of hexanoic (A, B, C) and methanolic (D, E) extracts showing DPPH antioxidant (B, E) and antifungal (*C. cladosporium*) (C) activities. +: catechin (B, E) and nystatin (C) positive control.

4. CONCLUSIONS

The presented data confirmed the heterogeneity of *Chroococcus*, with the description of its two sister genera *Inacoccus* and *Cryptococcum*. Even morphology has thought to be exhausted known in *Chroococcus*, the discovery of unilateral invagination capacity in CCIBt 3508 proves that we are still able to find novelties. The production of nanocytes by *Inacoccus* is the same case. Rarely formed, these both structures could not be accessed without extensive studies of lineages under microscope, including cultures in different times. The morphology of 'hamburger-shaped' loose cells showed to be conserved in the whole *Chroococcus* 'sensu lato' clade, which also includes the genus *Limnococcus*. As a lot of species are described in *Chroococcus* and the majority has no polyphasic data, it is probable that some of them are heterogeneous, and will result in new genera or split up in different species. Besides taxonomy and phylogeny, *Inacoccus* and *Cryptococcum* have shown a promisor potential for

antioxidant and antifungal compounds, what highlight that novelties are beyond taxonomic area and also reach biotechnology.

Capítulo III

Asterocapsa: a puzzling genus

ASTEROCAPSA: A PUZZLING, BUT WIDESPREAD CYANOBACTERIAL GENUS IN TERRESTRIAL ENVIRONMENTS

1. INTRODUCTION

The cyanobacterial taxonomy has been gone through a transition time. Almost entirely based on morphoecological characters, the systematics and taxonomy of cyanobacteria are facing the advances of molecular and genomic Era. As result, a lot of changes have been done, what is necessary to combine all data improving the understanding and classification of these organisms. However, there are many genera and species which are not well defined even in relation to morphology, being *Asterocapsa* Chu one of the best example (Komárek 1993, Komárek et al. 2014).

Described by Chu (1952), the genus *Asterocapsa* remained obscured up to date. This author designated as its diacritical features the verrucous mucilage envelope, the spherical shape of the mature colonies and the non-confluent lamellae of the individual cells. However, nowadays is known that these features are deeply linked with environmental conditions (Komárková et al. 2010), and they are similarly found in other genera, as *Gloeocapsa* Kützing and *Gloeocapsopsis* Geitler ex Komárek (Komárek 1993). Trying to solve this problematic, Komárek (1993) constructed the morphological life cycle of these three genera based on environmental material, giving to each one the diacritical characteristics. Other authors also tried to circumscribed the life cycle and better characterize different *Asterocapsa* species (Lederer, 2000; Hindák & Smarda, 2006; Montejano et al., 2008). However not much advances were made since then, and nowadays *Asterocapsa* is very widely conceived and evidently heterogeneous, with 36 valid described morphospecies (in terms of Botanical's Code rules). Most of them (28 spp.), were described to China (genus type locality) and just one to South America (*Asterocapsa submersa* Azevedo et al.), being 35 spp. originally found growing on terrestrial habitats. As the International Code of Nomenclature for algae, fungi, and plants does not require isolated cultures and molecular information for description of new taxa, none of these species were characterized by genetic data, only morphoecological ones. This runs off the recent tendency of cyanobacterial taxonomy,

which is based on a polyphasic approach, meaning a combination of various data characterizing the taxa.

Nevertheless, there is a huge problem among the described species, once the majority is published in Chinese local journals, and many of them are written in Chinese (Liu, 1985; Xiao, 2000; Xiao & Cai, 2001). Thus, the access to these species descriptions is complicated, what turns them unknown to the rest of scientific world. Probably, this is the reason for *Asterocapsa* species have only few and seldom citations in literature. Even then, due to the intersection of this genus with *Gloeocapsa* and also its taxonomic irresolution, the occurrence of *Asterocapsa* is probably wider than we already know. Lots of paper registered the occurrence of *Asterocapsa* close morphotypes from different parts of the Globe (Gardner, 1927; Aboal et al., 2003; Gaylarde & Gaylarde, 2005; Gaylarde et al., 2005; Hauer & Pažoutová, 2009), mainly when they are mixed with *Gloeocapsa* types. Thus, our goal is to characterize and define the cyanobacterial genus *Asterocapsa* in face to morphological studies on isolated strains and ultrastructure and phylogenetic data.

2. MATERIAL AND METHODS

The strains were isolated in liquid medium following standard techniques (Jacinavicius et al. 2012) from samples collected on terrestrial habitats in two different Brazilian Biomes: Cerrado (Brazilian Savannah) and the Atlantic Rainforest (Table 5). They are all stored in CCIBt (Culture Collection of Institute of Botany) under controlled conditions, as following: temperature of 23±1°C, irradiance of 40-50 mmol photons m^{-2}/s^{-1} (provided by daylight fluorescent lamps and measured with a Li-COR quanta meter sensor) and photoperiod of 14–10h light-dark cycle. An aliquot of each lineage was preserved in formaldehyde 4% and deposited in the Herbarium of the Institute of Botany (SP), Brazil.

Table 5. List of studied lineages with details of their culture medium, habitat, Biome and Herbarium access number.

Lineage	Culture medium	Habitat	Biome/Locality	Herbarium access number
CCIBt 3504	ASM-1	Tree Bark	Cerrado - São Francisco de Goiás, Goiás State	SP469037
CCIBt 3511	ASM-1	Concrete	Atlantic Rainforest - Fontes do Ipiranga Park, São Paulo State	SP469038
CCIBt 3522	BG11	Concrete	Atlantic Rainforest - Juréia Ecological Station	SP469039
CCIBt 3537	ASM-1	Concrete	Atlantic Rainforest - Juréia Ecological Station, São Paulo State	SP469040
CCIBt 3427	ASM-1	Concrete	Altantic Rainforest - Santa Virgínia Park, São Paulo State	SP469746
CCIBt 3444	ASM-1	Quartzite	Cerrado - São Francisco de Goiás, Goiás State	SP469747
CCIBt 3405	ASM-1	Rock	Cerrado - Pirassununga, São Paulo State	SP469748

The morphological study was carried out from culture material using a Zeiss Axioplan 2 optical microscope and the identification and cell measurements were based on at least 30 colonies. The metric information is expressed as minimum-average-maximum values in the taxonomic description. The construction of life cycle was made by observation of colonies in culture during five days after replication, and then 30, 60 and 120 days after.

The link between one cycle's states to the follow was based on morphotypes in transition. The organisms were documented by digital photographs taken with a Zeiss Axiocam MRc digital camera.

Cellular ultrastructure (position of thylakoids and mucilage characteristics) was studied from cultures by transmission electron microcopy. The cells were centrifuged for concentration and the medium was eliminated. After, each sample was fixed with Karnovsky solution for 24h, washed three times (10 min each) with 0.05M cacodylate buffer and post-fixed with 1% osmium tetroxide for 1 h. After that, samples were overnight submitted to contrasting in uranyl acetate 5% and dehydrated in an acetone series of increasing concentration. After washing with 100% acetone, the samples were proceeded to pre-infiltration with 1:1 Spurr resin/100% acetone for 5 h with agitation. The subsequent infiltration of the samples was done with pure resin for 12 h. The samples were then shaped in Spurr resin and polymerized at 65°C for 3 days. The blocks were cut on an ultramicrotome (Leica Ultracut UCT) with a diamond blade (450) into 70 nm thick sections and mounted on uncoated 200-mesh copper grids, and stained with uranyl acetate and lead citrate (Reynolds 1963). Visualization and microphotography were performed on a Jeol Jem 1011 transmission electron microscope (JEOL, Tokyo, Japan) at 100 kVA.

Total genomic DNA was isolated from a liquid culture of the cyanobacterial strains using the Ultra Clean Soil DNA Isolation Kit (MOBIO). The partial 16S-ITS-23S rRNA sequence was amplified by PCR using the 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, 200µm of each dNTP, 3.0 mM $MgCl_2$, 1 × PCR buffer and 1.0 U Platinium *Taq* DNA polymerase (Life Techonologies, Grand Island, NY, USA) under conditions according to Taton et al. (2003). The resulting PCR product was cloned into a pGEM®-T Easy Vector System (Promega, Madison, WI, USA) and inserted into chemiocompetent *E. coli* DHG5α cells by a heat-shock method (Sambrook et al., 1989). After growth in LB plates containing 5 µl X-Gal (50 $\mu g.mL^{-1}$) (Life Techonologies) and 10 µl of ampicillin sodium salt (100 $\mu l.\ mL^{-1}$) (Sigma-Aldrich Co., St. Louis, MO, USA), recombinant plasmids were purified from white colonies by the alkaline lysis method (Birnboim & Doly, 1979). The cloned gene fragment was sequenced using the 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 the sample was placed in an ABI PRISM 3500 Genetic Analyzer (Life Technologies). The sequenced fragments were assembled with the Phred/Phrap/Consed software package (Ewing & Green, 1998; Ewing et al., 1998; Gordon et al., 1998).

The phylogenetic analyses were based on 16S rRNA gene and 16S-23S internal transcribed spacer (ITS) sequences obtained in this study and reference sequences retrieved from GenBank. The General Time Reversible (GTR) evolutionary model of substitution with Gamma distribution (G) and with an estimate of proportion of invariable (I) sites was selected as the best fit for the 16S rDNA alignment and Hasegawa, Kishino and Yano (HKY) model + G +I for 16S-23S ITS using jModelTest (Posada, 2008). Evolutionary analyses based on the Neighbor Joining and Maximum Likelihood methods were conducted in Mega 6.0 (Tamura et al., 2013) and estimations of the robustness of the tree were tested by bootstrap with 1,000 replications. The Bayesian analysis was inferred by MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2005) in 5 $\times 10^6$ generations. Chains were sampled every 100^{th} 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 using p-distance method in Mega 6.0 (Tamura et al., 2013) and the percentage was given by the formula *[1–(p-distance)]×100*. The mean similarity was estimated by the average of similarity value of each sequence from the two compared clades.

The 16S-23S ITS sequence obtained was compared within the obtained sequences. RNAmmer 1.2 (Lagesen et al. 2007) was used for the elimination of ribosomal RNA from the sequences. tRNA sequences were identified with tRNAscan-SE 1.21 (Lowe & Eddy 1997). The sequences were aligned with Clustal W 2.1 (Larkin et al. 2007) for the detection of conserved regions as identified by Iteman et al. (2000). D1-D1', Box B and V3 were identified and their secondary structure was predicted with mfold (Zuker, 2003) using a temperature set of 25°C and 10% of thermodynamic suboptimality after van Gremberghe et al. (2011). Diagram were created with RNAViz (De Rijk et al. 2003) and Inkscape (http://www.inkscape.org/).

3. RESULTS AND DISCUSSION

The present study analyzed seven strains morphologically related to the genus *Asterocapsa*. Due the complexity of this genus, we adopted the presence of wart-like/spinous ornamentation on sheaths as *Asterocapsa* diacritical feature, which was the same idea gave by Chu (1952) in the original description of the genus. Wart-like ornamentation is also described in *Gloeocapsa*, but only in adaptation to adverse environmental conditions (Komárek, 1993). All herein analyzed material often showed this feature when in culture, what confirms its stabilization during the life cycle of strains. Based on this, it is possible to consider this ornamentation as an adaptation to the aerophytic way of life and not an adaptation to a restrictive environment (see also Montejano et al., 2008 and Aboal et al., 2003).

In the literature, it is common to see these ornamented cells to be called as arthocytous/arthropores (Komárek, 1993; Hindák & Smarda, 2006; Montejano et al., 2008), which refers to a spore, a resistant phase. Based on our results, the mucilage decoration is a stable and regular feature present in cultured colonies; a non-pressure environment. So, we suggest that those cells with wart-like/spine structures cannot be called as spores or arthrocytous/arthropores when referring to the genus *Asterocapsa*; they should be interpreted as a regular and diacritical feature to morphologically characterize this genus.

When the 16S rDNA phylogenetic tree with all strains morpho-related to *Asterocapsa* is seen (Figure 8), we can observe the placement of studied lineages in two different clades, with low similarity between them but very well bootstrapped supported. This is an indicative that even the decoration on cells is stable, it is not enough to support a monophyletic clade.

Six linages were more relative among themselves and they grouped in one clade. We denoted it as the true *Asterocapsa* group, once all strains are morphologically close to the original description (Chu, 1952) and they were all collected from rocks and concrete; a similar environment to the original place of the type species. When compared with sequences from GenBank, none identified organism was close enough to these lineages to be considered in the same genus. The closest ones were all from uncultured and unidentified bacteria, what proved *Asterocapsa* to be unknown by molecular assignment. Moreover, among these *Asterocapsa* relatives there are some

from China, collected on carbonate surface of Yunnan Stone Forest (Figure 8). This geographic proximity between China sequences and those from the Atlantic Rainforest strengthen the identification and the phylogenetic placement of *Asterocapsa* genus herein proposed.

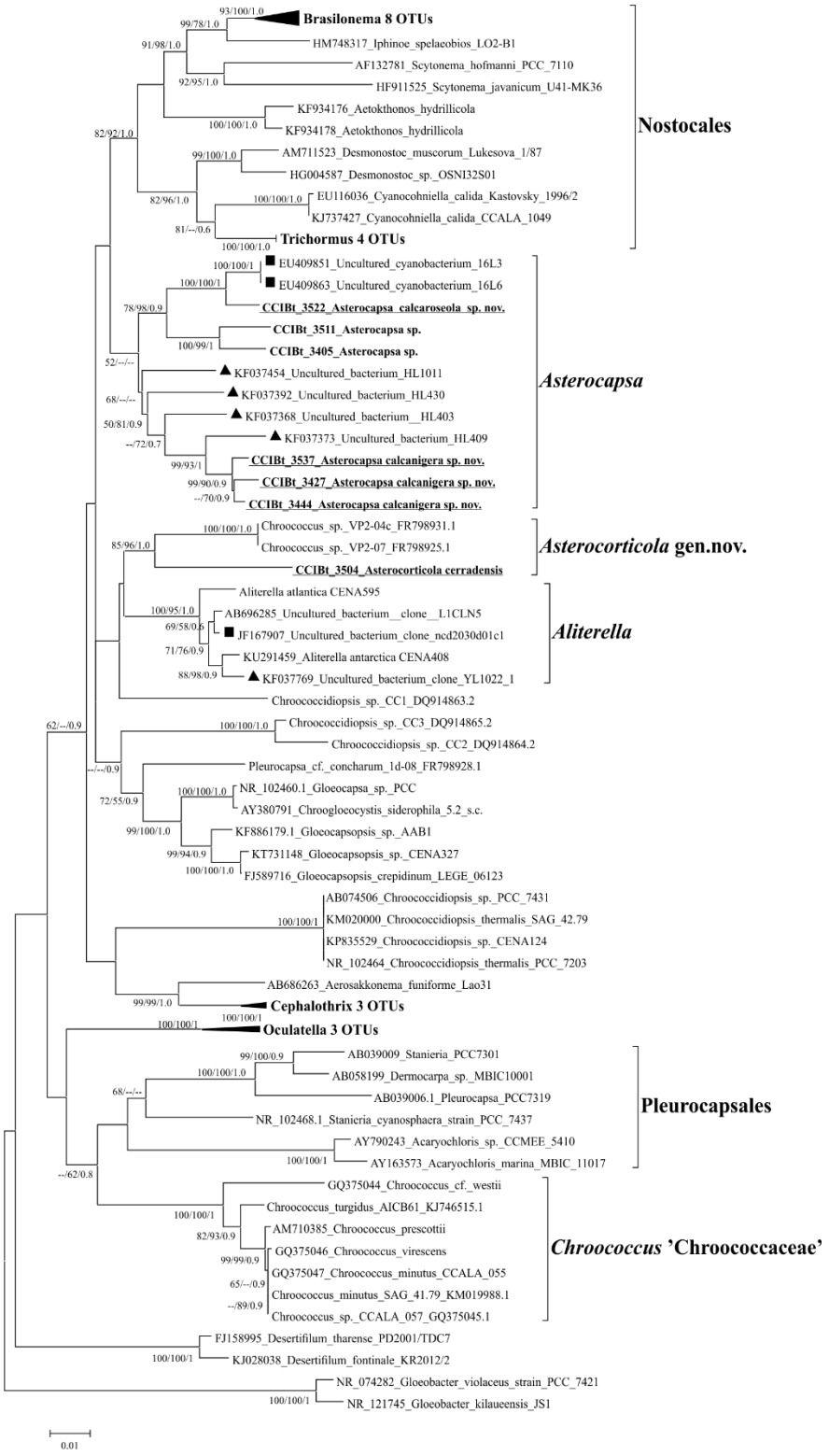


Figure 8: Phylogenetic tree based on 16S rRNA gene sequences and reconstructed using the maximum-likelihood (ML) analysis of evolutionary distances determined by the GTR + G + I model. NJ and ML bootstrap (1,000×) values (above 50 %) and Bayesian posterior probabilities are provided for each node, respectively. Sequences determined in this work are indicated in bold and new taxa are underlined. Squares indicate sequences from USA (concrete) and triangles from China (rocks covered with plants).

Table 6. 16S rDNA similarity matrix of studied lineages and their close relatives. conducted using the p-distance model with a total of 1,031 positions.

	Sequences identification	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	CCIBt 3427 *Asterocapsa calcanigera*																					
2	CCIBt 3444 *Asterocapsa calcanigera*	99.2																				
3	CCIBt 3537 *Asterocapsa calcanigera*	98.9	99.3																			
4	CCIBt 3504 *Asterocorticola cerradensis*	92	92	91.9																		
5	CCIBt 3522 *Asterocapsa calcaroseola*	95.3	95.8	95.6	92																	
6	CCIBt 3511 *Asterocapsa* sp.	95.2	95.2	95.2	92	96.1																
7	CCIBt3405 *Asterocapsa* sp.	94.5	94.7	94.8	92	95.7	97.9															
8	KF037368 Uncultured bacterium HL403	96.4	96.9	96.8	92.9	95.3	96	95.6														
9	EU409851 Uncultured cyanobacterium 16L3	94.9	95.3	95.2	92.2	98.5	95.9	95.7	95.2													
10	KF037392 Uncultured bacterium HL430	95.6	96.2	96	92.6	94.9	95.1	94.7	97.2	95.1												
11	KF037454 Uncultured bacterium HL1011	95.4	95.9	95.8	93.3	95.6	95.9	95.3	97.4	95.2	96.8											
12	CENA595 *Aliterella atlantica*	95.1	95.2	95.1	93.1	93.6	93.8	93.9	95.1	93.7	94.6	94.7										
13	CENA408 *Aliterella antarctica*	94.1	94.3	94.4	93.3	93.5	93.9	93.8	95.2	94	94.2	94.7	98.4									
14	AM710385 *Chroococcus prescottii*	91.6	92	91.6	91.5	90.7	90.4	90.9	91.7	90.9	91.9	91.6	92.5	91.8								
15	*Chroococcus* sp. CCALA 057 GQ375045.1	91.6	92	91.6	91.6	90.7	90.4	90.9	91.7	90.9	91.9	91.6	92.5	91.8	99.8							
16	GQ375046 *Chroococcus virescens*	91.5	91.9	91.5	91.5	90.6	90.3	90.8	91.6	90.8	91.8	91.5	92.4	91.7	99.7	99.9						
17	*Chroococcus* sp. VP2-04c FR798931.1	93.7	94.1	94.2	94.9	93.6	92.4	92.5	93.9	93.2	94	93.6	95.4	94.6	91.6	91.7	91.6					
18	*Chroococcus* sp. VP2-07 FR798925.1	93.7	94.1	94.2	94.9	93.6	92.4	92.5	93.9	93.2	94	93.6	95.4	94.6	91.6	91.7	91.6	100				
19	AF132781 *Scytonema hofmanni* PCC 7110	92	92	92	91	91.9	91.3	91	92.2	91.9	93.4	92.2	91.3	91.6	90.5	90.5	90.5	91.9	91.9			
20	KF934176 *Aetokthonos hydrillicola*	93.1	93.2	93.4	91.3	93.2	94.1	94	93.5	93.3	93.7	93.9	92.7	92.7	90.6	90.6	90.5	91.9	91.9	93.5		
21	DQ486055 *Brasilonema bromeliae* SPC 951	92.1	92.1	92.3	91.6	92.2	92.2	92	92.8	92.1	93.5	94	91.4	91.5	90	90	90	91.3	91.3	94.5	94.5	
22	EU116036 *Cyanocohniella calida* Kastovsky 1996/2	92.5	92.6	92.7	90.7	92.9	93.9	94.3	93.4	93.5	93.5	93.2	93.1	93.1	89.8	89.9	89.8	92.4	92.4	91.9	94.7	

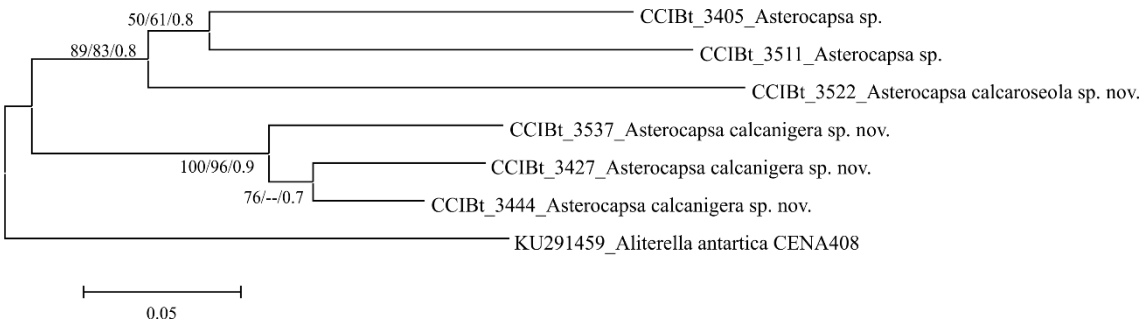


Figure 9: Phylogenetic tree based on 16S-23S ITS sequences and reconstructed using the maximum-likelihood (ML) analysis of evolutionary distances determined by the HYK + G + I model. NJ and ML bootstrap (1,000×) values (above 50 %) and Bayesian posterior probabilities are provided for each node, respectively.

Table 7. 16S-23S ITS similarity matrix of studied lineages and their close relatives, conducted using the p-distance model with a total of 388 positions.

Sequences identification	1	2	3	4	5	6	7
1 CCIBt3405 *Asterocapsa* sp.							
2 CCIBt3522 *Asterocapsa calcaroseola*	79.4						
3 CCIBt3511 *Asterocapsa* sp.	82.8	77.2					
4 CCIBt3427 *Asterocapsa calcanigera*	78.7	77.7	79.4				
5 CCIBt3444 *Asterocapsa calcanigera*	77.0	78.4	77.7	89.7			
6 CCIBt3537 *Asterocapsa calcanigera*	76.0	77.0	76.0	85.8	88.5		
7 KU291459 *Aliterella antartica* CENA408	76.5	73.7	75.0	77.8	79.4	77.3	

Inside *Asterocapsa* clade herein proposed, we can distinguish three different species based on the studied strains; two of them were designed as new taxa and the third one was kept as *Asterocapsa* sp. All the clades were well distinguishable and strongly supported by morphology and 16S rDNA and 16S-23S ITS analyses (Table 6 and Table 7).

A. submersa is a reddish colored and the unique species of *Asterocapsa* described to Brazil (Azevedo et al. 2003). As its name already suggests, it was originally found in periodically flooded mats of benthic *Phormidium* Kützing ex Gomont in shallow channels near Atibainha reservoir (Cantareira reservoirs system). Two analyzed strains, CCIBt3405 and CCIBt3511, have a high morphological resemblance with *A. submersa*

and they also found to be very close between themselves by molecular similarity (97.7% of 16S rDNA) (Table 6), even they came from different Biomes (Table 5).

Asterocapsa was described as an aerophytic genus (Chu 1952) and *A. submersa* was the first aquatic species to be included on it. However, the habitat where CCIBt3405 and CCIBt3511 were isolated is very different from that descried for *A. submersa* (Table 5). Besides, these strains did not show the reddish colored sheaths in culture, what can be an environment dependent feature, once we can still recognize the wart-like projections on the hyaline sheaths (Figure 11). Nevertheless, the confirmation of the relationship between these strains and *A. submersa* will be truly elucidated only with the polyphasic study of a strain isolated from a similar and close environment where this species was found. Since this and the molecular sequence of *A. submersa* are non-available now, we decided to kept these strains as *Asterocapsa* sp.

The identity between CCIBt3405 and CCIBt3511 is also confirmed by the high similarity of their ITS sequences, which is 82.8%. However, when we look to the secondary structure of the ITS regions D1-D1' and BoxB, it is possible to note great differences between the strains (Figure 10). These divergences in the secondary structure from a same species is already reported to other cyanobacterial genera. Dvořák et al. (2015) described *Pinocchia polymorpha* Dvořák et al. (Pseudanabaenaceae) and found dissimilar secondary structures of D1-D1' and BoxB from clones of the same *P. polymorpha* strain. Probably this happen due the variation in the number of copies of the ribosome operon, what showed to be very variable among cyanobacteria (Schirrmeister et al. 2012). This reveals that the draw of such ITS regions can be very flexible inside the same taxa and other methodologies should be taken into account to distinguish species, as the similarity and phylogenetic tree of ITS.

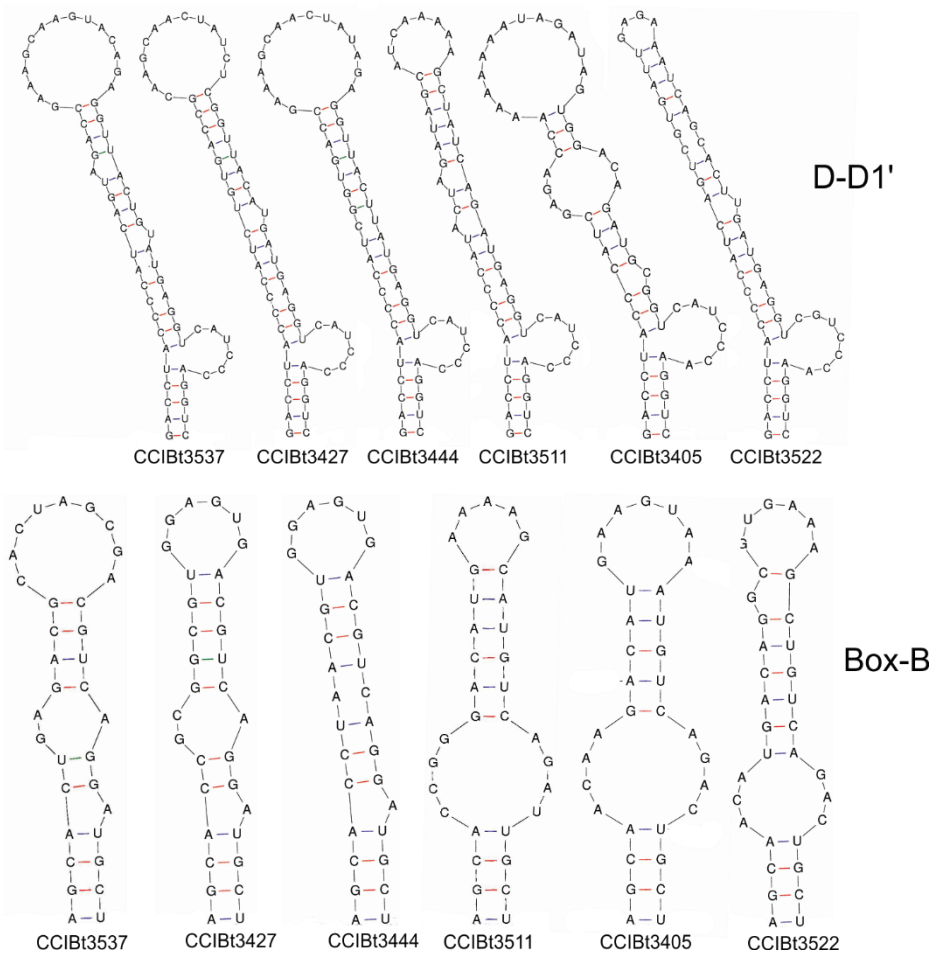


Figure 10: Secondary structure of the regions D1-D1' and BoxB from the 16S-23S ITS of *Asterocapsa* analyzed strains.

CCIBt3522 was the phylogenetic closest strain to *Asterocapsa* sp. clade, but not enough to be kept on the same species, as revealed by the 16S rDNA and 16S-23S ITS similarity (Table 6 and Table 7). Its ITS secondary structure was very different of all analyzed *Asterocapsa* strains, what reinforces the identity of CCIBt3522 as distinct species. An interesting fact is that all strains present a lower loop in D1-D1' structure, which is identical in all of them, including from different species, except for one base substitution in CCIBt3522. This loop, formed by the sequence TCATCCA, showed to be very conserved in the *Asterocapsa* strains, and this base alteration in CCIBt3522 reinforces its individuality at specific level. Morphologically, CCIBt3522 has the reddish-colored sheaths, as well as *A. submersa*, but differently from CCIBt3405 and CCIBt3511, CCIBt 3522 displayed the colored mucilage when in culture (Figure 12). However, the colored mucilage was very rare in submersed colonies, being more frequent and intense in colonies growing over the culture flask wall, in the interface of

air and liquid medium (Figure 12). Surely, this pink pigment is produced in response to an aerophytic condition, but more studies should be done in face to elucidate which parameter may interfere on its production.

Still referring to morphology, there are some *Gloeocapsa* species that are morphologically close to *Asterocapsa* species. One of them is *Gloeocapsa novacekii* Komárek & Anagnotidis, which is quite alike to CCIBt3522. This species was described to Czech Republic and its description matches with CCIBt3522 (Komárek & Anagnostidis, 1998; Hauer, 2007), including characteristics of the habitat. Gama Jr et al. (2014) also morpho-identified *G. novacekii* in samples from the Atlantic Rainforest. However, the identity between CCIBt3522 and *G. novacekii* would be confirmed only with molecular studies with Czech material and up to date there is none strains or molecular information about this species. Thus, we prefer to designate CCIBt3522 as the new species *Asterocapsa calcaroseola* sp. nov. in reference to its habitat and pink sheath color. Two sequences from uncultured bacteria grouped with CCIBt3522 forming a clade with 100% of bootstrap value (Figure 8). These both sequences were collected on concrete walls from USA (Giannantonio et al., 2009), what sustains the habitat specificity and confirms the broad distribution of *Asterocapsa calcaroseola*.

The third species recognizable inside the *Asterocapsa* clade is herein represented by three different strains CCIBt 3427, CCIBt 3444 and CCIBt 3537. Morphologically, all these cultures have in common the black color of mucilage, besides the habitat and equality of cells morphology and dimensions (Figure 13). Among the described species of *Asterocapsa*, none of them have this color of mucilage. On the other hand, we can find several *Gloeocapsa* species with sheaths varying between black to violet/blue color. Rabenhorst (1865), the original work for the majority of them, lists 10 species with this color of sheaths, like *Gloeocapsa nigrescens* Nägeli and *Gloeocapsa violacea* Kützing. Another ancient genus, *Anacystis sensu* Gardner, also has species similar to *Asterocapsa*, e.g. *Anacystis nigropurpurea* Gardner and *Anacystis nigroviolacea* Gardner, both described by Gardner (1927) from Puerto Rico. However, the great majority of these species has never been revised and they were only studied based on morphology of natural populations. There are sequences identified as *Gloeocapsa* available in GenBank. However, they appear in different position in a phylogenetic tree, what suggests their misidentification or the high heterogeneity of *Gloeocapsa*. Anyway, none of these sequences was designated as the taxonomic reference for *Gloeocapsa*. So,

even it is possible that the strains herein analyzed are one of these species, it would be arbitrary to identify them as such ones. For this reason, we proposed *Asterocapsa calcanigera* sp. nov. as a new species, represented by the strains CCIBt3427, 3444, 3537, which is highly supported by analyzes of 16S rDNA and 16S-23S ITS (Figure 8 and Figure 9). The ITS secondary structure showed to be different among the strains (Figure 10). The BoxB basal stem of Atlantic strains (CCIBt3227 and 3537) were identic, but the two subsequent loops were very distinct among them. On the other hand, CCIBt3427 had the same BoxB upper loop when compared to CCIBt3444, which was isolated from Cerrado Biome. This reinforce that the secondary structure of ITS inside one species can be very variable in the genus *Asterocapsa*, as observed in *Asterocapsa* sp. representatives.

Comparing 16S rDNA sequences of *Asterocapsa calcanigera* with those from GenBank, we found an uncultured bacterium sampled at China. Moreover, two of analyzed strains are from the Atlantic Rainforest and one from the Cerrado Biome, what reveals the wide occurrence of this species and confirms the worldwide distribution of *Asterocapsa*.

The last studied strain, CCIBt3504, also morphologically fits in the genus *Asterocapsa* according to Komárek (1993) (Figure 14). However, its 16S rDNA sequence revealed that it concerns into a genus phylogenetically apart. When compared with the sequences from the GenBank, CCIBt3504 was closer from the recent described genus *Aliterella* Rigonato et al. than to *Asterocapsa*, although they are not morphologically related (Rigonato et al., 2016b). This can be another evidence that the decoration of cells mucilage is not a good morphological marker in the phylogeny of this group of cyanobacteria, even it is stable on culturable strains. *Aliterella* has two different species, one from Antarctica and another from the Atlantic Ocean, habitats also very distinct from CCIBt3504. However, this strain also is the only herein investigated which was isolated from tree bark, instead of rock or concrete, besides being from the Cerrado Biome. This can be an evidence that *Asterocapsa* morpho-related genera should be separated by the ecology and habitat of strains. Also, CCIBt3504 produces nanocytes regularly (Figure 14), what was not seen in the *Asterocapsa* strains when cultured on the same conditions and can be a morphological difference to distinguish them. Moreover, this strain showed the mucilage in purple-brown color during the culture, which is very different from the color saw on *Asterocapsa* strains. Thus, based on morpho-ecological

and molecular features, we proposed CCIBt3504 as the new genus *Asterocorticola* gen. nov., in reference to its occurrence on tree bark and resemblance to *Asterocapsa*; and *Asterocorticola cerradensis* sp. nov. as its type species, due to the location of its first find. CCIBt 3504 sequence was also close to two *Chroococcus* sp. strains VP2-04c and VP2-07. For sure, these *Chroococcus* Nägeli strains are misidentified, once they are not phylogenetically close to true-*Chroococcus* clade (Komárková et al., 2010). Nevertheless, it is necessary more information about the morphology and other molecular markers of these lineages to better elucidate the relation of them with the genus *Asterocorticola*, particularly because they seem to be clones and have 94.5% of similarity with CCIBt3504 by the 16S rDNA comparison.

Based on our data, we evidently see *Asterocapsa* and *Asterocorticola* to show the ornamented sheaths regularly when in culture, never disposing lamellate mucilage, even the cells have individual envelopes, which are not obligatory. The cells on these genera can have a regular division during the first steps of colonies development. However, in mature colonies the cells are clearly widespread with no pattern, what reveals the latter irregular division of cells. These features surely distinguish *Asterocapsa* and *Asterocorticola* from *Gloeocapsa*, which has regular cell division with cells surrounded by layered sheaths (Komárek, 1993). On the other side, the morphological distinction between *Asterocapsa* and *Asterocorticola* can be not very obvious. Although we have seen the regular production of nanocytes by *Asterocorticola* strain, we analyzed just one of exemplar of this genus, opposing to six of *Asterocapsa*, which never produce such structures. Thus, we suggest that *Asterocapsa* do not divide cell into nanocytes, in contrast to *Asterocorticola* that is able to produce it.

In spite of suggesting the production of nanocytes in *Asterocapsa divina* Komárek, Montejano et al. (2008) just find these structures in natural population, growing on *Scytonema* filaments. As these authors never found nanocytes in their cultures, it can be the case of those observed structures in natural conditions were not produced by *Asterocapsa*, but by other cyanobacteria presented in the sample. One evidence to support this is the non-production of nanocytes by all herein analyzed *Asterocapsa* strains, even when they were cultured for a long time.

Another interesting fact about all analyzed strains is the color of mucilage. Some authors have suggested that the color of mucilage is pH dependent when gloeocapsin is

present, varying from the blackish-purple to reddish-pink (Storme et al., 2015). However, it is very common to see both color on different colonies from the same environment sample; here we have the case of CCIBt 3522 and CCIBt3537 which were both isolated from the same environment sample. Besides, the analyzed strains kept the color of mucilage constant under the same cultivation conditions, except from CCIBt3405 and CCIBt3511, as already mentioned. So, to *Asterocapsa* species it is possible to say that the color of mucilage is probably gave by different pigments and they are stable enough to be used as a marker in the taxonomy of the genus. The ITS phylogenetic tree reveals it (Figure 9), where the strains with blackish color are clearly separated from those with reddish ones. Also, the electron microscopy revealed that even in hyaline mucilage it is possible to detect an electron dense compound comparable to those found in the colored sheaths (Figure 15). Probably, these substances have similar activities and function, even some of them has no visible color.

Keeping looking to the electron microscopic pictures, we see that the thylakoids arrangement is similar to those found in Nostocales (Jensen & Bowen, 1970; Komárek & Kaštovský, 2003), with vortex and irregular disposition (Figure 15). This resemblance with Nostocales is also visible in the phylogenetic analyzes. Fewer et al. (2002) presented the group composed by *Chroococcidiopsis thermalis* Geitler as a close Nostocales relative clade and Rigonato et al. (2016a) showed that family Aliterellaceae is another one. Here, we can see that *Asterocapsa* is the closest cyanobacterial outgroup of Nostocales, actually it can be considered a sister clade, achieving 93-94% of 16S rDNA similarity to some heterocytous species, against an average of 91% to the typical *Chroococcus* clade. This is a strong indicative that the genus *Asterocapsa* does not belong to the family Chroococcaceae, where it was classified in accordance to the morphology. Its phylogenetic position indicates that a new family should be proposed to better fit it, but to be in agreement with the newest cyanobacterial system of classification proposed by Komárek et al. (2014), we suggest a genomic study with at least one lineage, what will give further elucidations about the relationship between *Asterocapsa* and the Nostocales cluster. Due the high similarity with the genus *Aliterella* it is possible to already classify *Asterocorticola* inside the family Aliterellaceae, which together with *Asterocapsa* family form the closest Nostocales relatives.

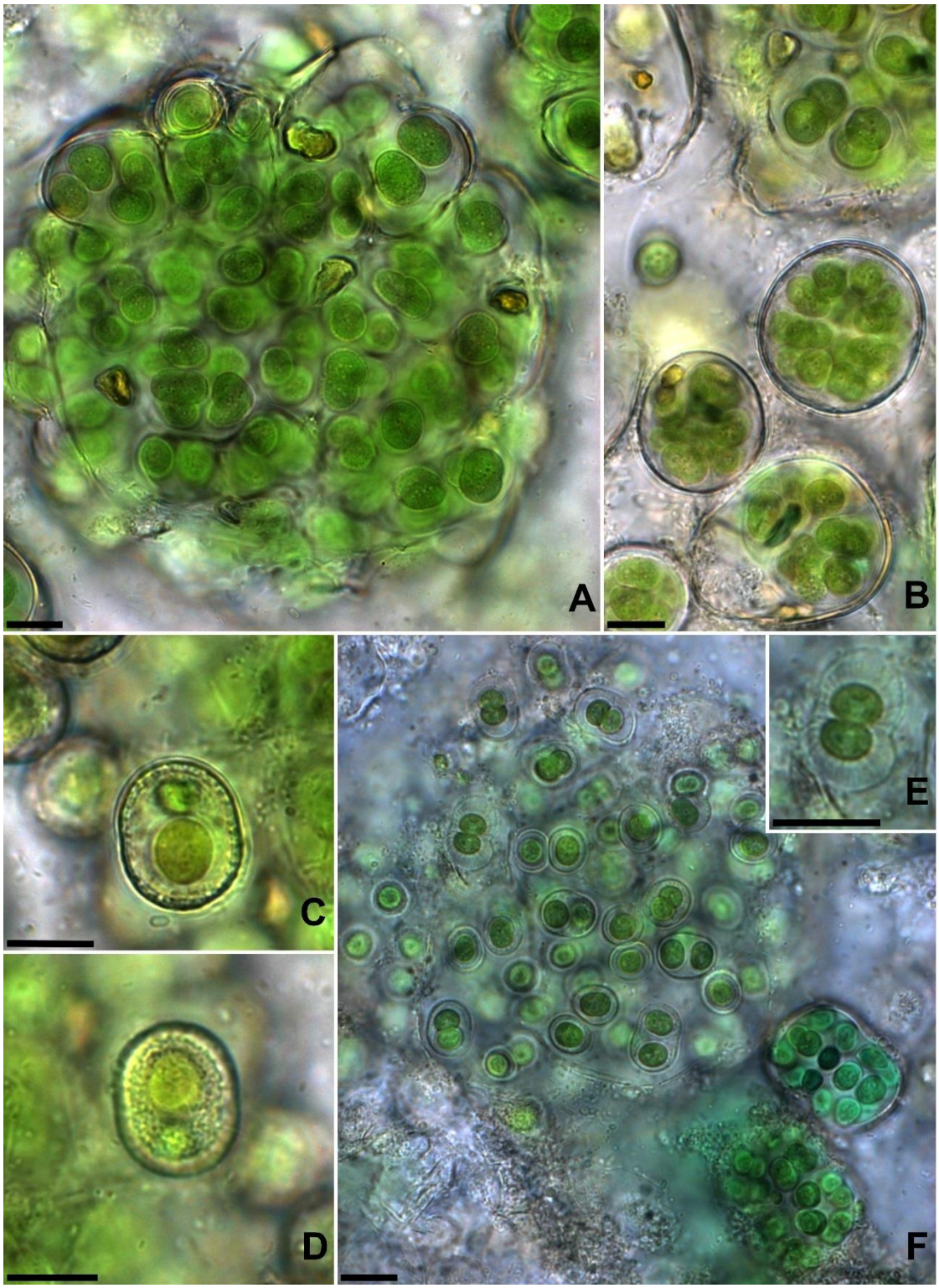


Figure 11: *Asterocapsa* sp.; A-D: CCIBt3405; A-B: general aspect of colonies. C-D: detail of granulations on sheaths; E-F: CCIBt3511; E: colony disintegration with cells surrounded by their own mucilage envelope; F: detail of cell ornamentation. Scale bars: 10 µm.

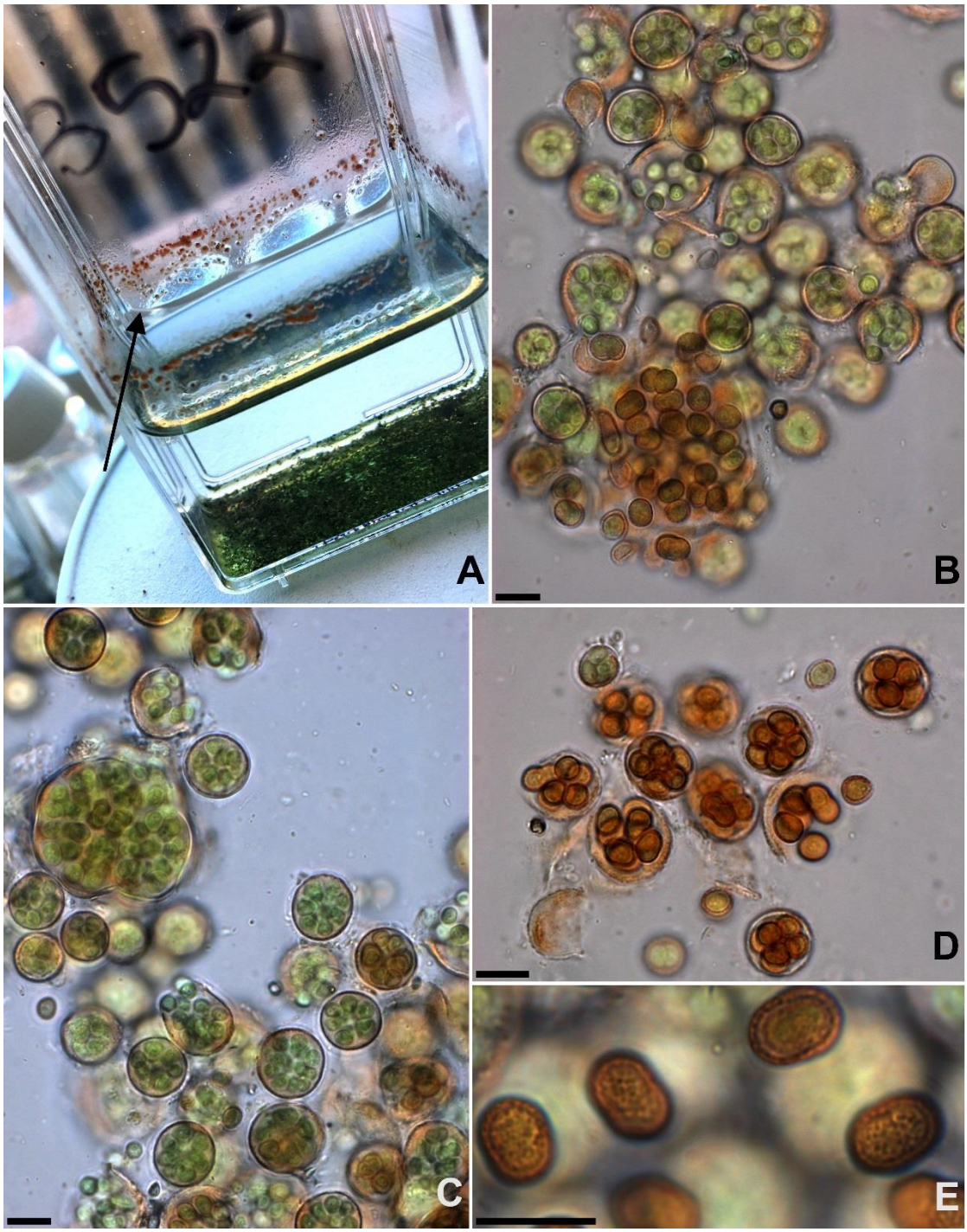


Figure 12: *Asterocapsa calcaroseola* (CCIBt3522); A: culture flask with the detail of colonies growing over the wall (arrow) and their intense reddish color; B-D: colonies in general aspect at different stages of development; E: detail of ornamented cells. Scale bars: 10 µm.

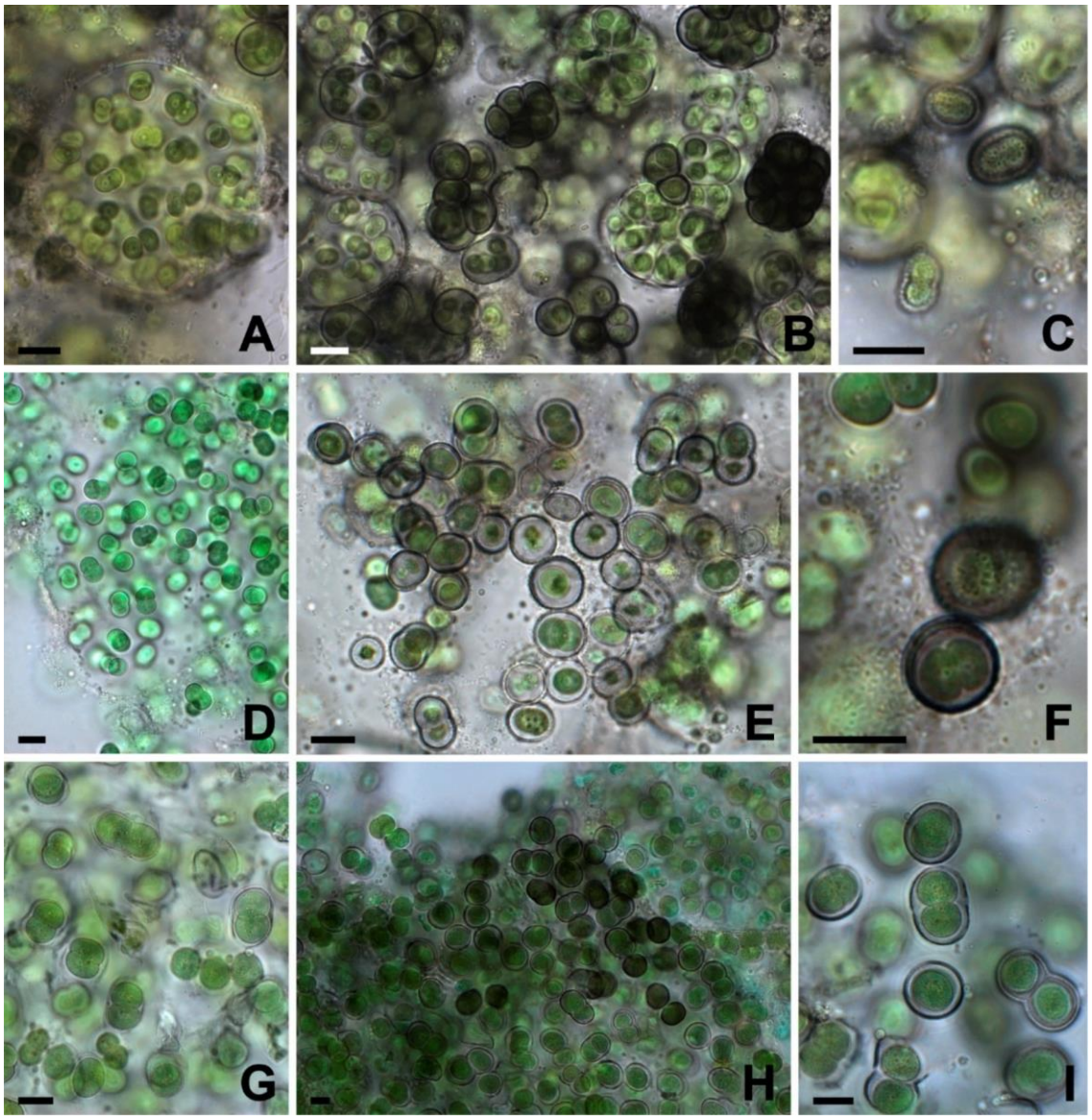


Figure 13: *Asterocapsa calcanigera*; A-C: CCIBt3537; A: colony with cells loosely arranged; B: aggregated of colonies and subcolonies; C: detail of cell ornamentation; D-F: CCIBt3427; D: colonies with hyaline mucilage; F: detail of cell ornamentation; G-I: CCIBt3444. Scale bars: 10 µm.

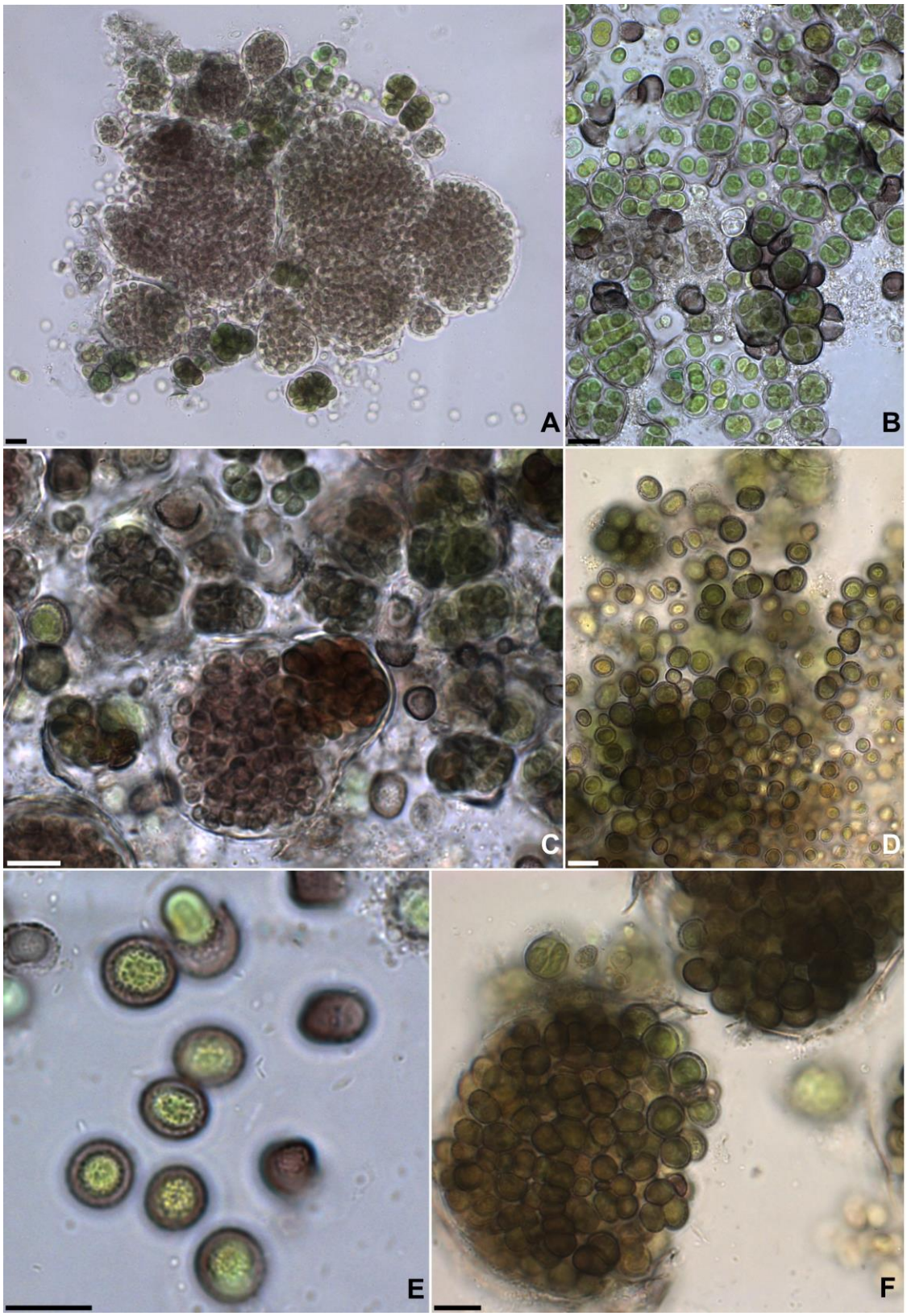


Figure 14: *Asterocorticola cerradensis* (CCIBt3504); A-B: general aspect of colonies. C: colony of nanocytes starting the development and pigmentation of internal cells mucilage; D: releasing of the cells by disintegration of colony; E: detail of cells showing mucilage ornamentation; F: colony with all cells surrounded by their own colored mucilage envelope. Scale bars: 10 µm.

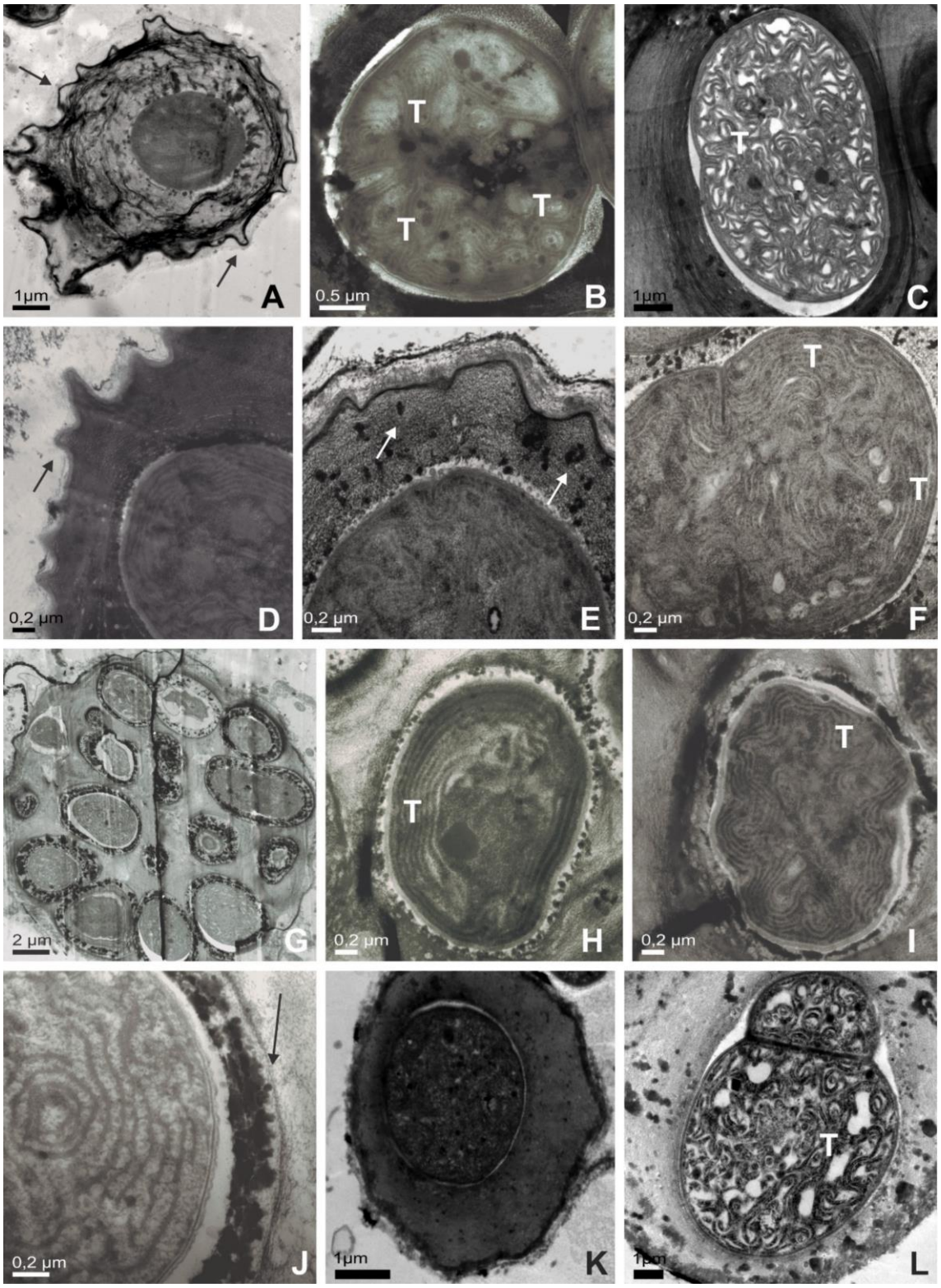


Figure 15: A-C: *Asterocapsa* sp.; A-B: CCIBt3511; A: detail of sheaths ornamentations (arrow); B: thylakoids arrangement; C: CCIBt3405, detail of thylakoids disposed in vortex arrangement; D-F: *Asterocapsa calcaroseola*, arrows point the mucilage decorations; G-J: *Asterocorticola cerradensis*, the cells arrangement inside the colony (G) and detail of electron dense compounds (J – arrow); K-L: *Asterocapsa calcanigera* (CCIBt3547). Scale bars: 10 µm.

3.1. Taxonomic descriptions

Here we detail and descript the new taxa found. The cells dimensions of all strains are specified in Table 8.

Table 8. Cells dimensions of analyzed strains. Max.= maximum value; Avg.= average value; Min.= minimum value.

Strains	Cells diameter (μm)		
	Max.	Avg.	Min.
CCIBt3427 *Asterocapsa calcanigera*	8.0	6.4	4.9
CCIBt3444 *Asterocapsa calcanigera*	10.4	9.2	7.4
CCIBt3537 *Asterocapsa calcanigera*	5.8	4.9	4.1
CCIBt3522 *Asterocapsa calcaroseola*	6.3	4.2	3.3
CCIBt3405 *Asterocapsa* sp.	9.5	7.1	5.1
CCIBt3511 *Asterocapsa* sp.	5.8	5.0	4.1
CCIBt3504 *Asterocorticola cerradensis*	6.9	4.9	3.6

***Asterocorticola* gen. nov.**

Colonies rounded up to irregular (rare), surrounded by a simple or two-layers firm sheath, with cells densely arranged. Sheaths are hyaline to colored, smooth or ornamented with wart-like projections. Cells spherical to subspherical, surrounded by individual sheaths. Nanocytes commonly present.

Type species: *Asterocorticola cerradensis* (see below)

Etymology: *corticalis* (Latin, adj. B) bark

***Asterocorticola cerradensis* sp. nov.**

Colonies rounded up to irregular (rare), surrounded by a firm sheath, with cells densely arranged. Sheaths are hyaline to dark purple-brownish, smooth or ornamented with wart-like projections. Cells subspherical, 3.5-(4.9)-6.8 μm of diameter, surrounded by individual sheaths. Cell content olive-green to purple-brown, granulated. Nanocytes present, surrounded by individual sheath or not, 2.2-(2.8)-3.5 μm of diameter.

Holotypus: exsiccate access number SP469037, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt3504

Habitat: terrestrial, growing on tree bark.

Etymology: in reference to the Biome Cerrado (Brazilian Savannah), from where the type strain was isolated.

***Asterocapsa calcanigera* sp. nov.**

Colonies rounded, surrounded by a firm sheath, with cells densely to loosely arranged. Sheaths are hyaline to dark bluish-black, smooth or ornamented with wart-like projections. Cells subspherical, 4.0-(4.9)-5.7μm of diameter, surrounded by individual sheaths. Cell content blue-green, granulated. Nanocytes absents.

Holotypus: exsiccate access number SP469040, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt3537

Habitat: terrestrial, growing on concrete and rocks.

Etymology: *calcareus* (Latin, adj.A, limestone) + *niger* (Latin, adj.A, black).

***Asterocapsa calcaroseola* sp. nov.**

Colonies rounded, surrounded by a firm sheath, with cells densely to loosely arranged. Sheaths are hyaline to pink-reddish, smooth or ornamented with wart-like projections. Cells subspherical 3.3-(4.2)-6.2 μm of diameter, surrounded by individual sheaths. Cell content blue-green, granulated. Nanocytes absents.

Holotypus: exsiccate access number SP469039, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt3522

Habitat: terrestrial, growing on concrete.

Etymology: *calcareus* (Latin, adj.A, limestone) + *roseolus* (Latin, adj.A, pink).

4. CONCLUSIONS

The genus *Asterocapsa* showed to be heterogenous in relation to its morphological circumscription. The wart-like ornamentation was found to be very stable on cultured strains, what confirms that it is not formed in an adverse condition. However, the strains with such characteristic did not grouped all together and they can be found in two unrelated clades: one is the typical *Asterocapsa* and the other one is described as the new genus *Asterocorticola*. Six strains grouped forms the *Asterocapsa* clade in accordance to 16S rDNA and they have as morpho-diacritic features the wart-like ornamentation on sheaths, non-production of nanocytes neither layered mucilage sheaths. Inside this proposed clade, we can distinguish three different species: *Asterocapsa* sp., *Asterocapsa calcaroseola* sp. nov. and *Asterocapsa calcanigera* sp. nov. The first has morphological resemblance to *Asterocapsa submersa* but they substantially differ but the kind of habitat. The last two species were designed as new, even they have some morpho-correspondents in the genera *Gloeocapsa* and *Anacystis sensu* Gardner. This option was take due to the lack of information about the species of these genera, but further studies regarding their life cycle in pure cultures and molecular characterization should confirm their relationship with the proposed species.

The genus *Asterocapsa* showed to be very well distributed over the World. All close sequences that matched in Blast with those from *Asterocapsa* were from uncultured material. Some of them were from China, where *Asterocapsa* was first described and has a lot of occurrences registered; a reinforcement to the identification of analyzed strains into this genus. Also, the isolation of *Asterocapsa* strains from different Brazilian Biomes, but from similar habitats, highlights this wide occurrence and the specificity of the genus with rocky environments. It is more evident when we analyzed *Asterocorticola cerradensis*, the *Asterocapsa* morpho-related new genus which mainly differs by growing on tree barks.

It is possible that both genera *Asterocapsa* and *Asterocorticola* occur in more parts of the Globe; but we suggest that they are habitat specifics. With the molecular and phylogenetic characterization of these genera it will be possible to confirm this, as the studies keep going forward. Also, the analyses of 16S rDNA reveal the close relationship between Nostocales group and *Asterocapsa*, what should be better clarify with a genomic approach.

Capítulo IV

Gloeothece/ Aphanothece

REVEALING THE RELATIONSHIP BETWEEN THE CYANOBACTERIAL GENERA *APHANOTHECE* AND *GLOEOTHECE* BASED ON TERRESTRIAL TROPICAL STRAINS

1. INTRODUCTION

The tropical zone of the Globe is known to centralize most part of the hotspots for biodiversity conversation on Earth (Myers et al., 2000). The Atlantic Rainforest is among them and in spite of its large diversity of plants and animals, the knowledge about its microorganisms is still little, mainly that concerning to the cyanobacteria. Even then, the literature confirms the Atlantic Rainforest to very diverse, with several new cyano-taxa have been described to it. However, more than 50% of them concern of heterocytous types (Sant'Anna et al., 2011c; Hentschke & Komárek, 2014; Hentschke et al., 2016). It is expected that Nostocales group has a huge biodiversity in terrestrial habitats, since they are very-well adapted to this type of environment, but Gama Jr et al. (2014) showed that there are also a lot of coccoid types living on there and many of them have no correspondents in the literature and should be described as new taxa.

Coccoid cyanobacteria are a complex non-monophyletic group with mostly of its genera designated by morphology (Komárek et al., 2014). In spite of being morphologically simple, compared to others cyanobacterial groups, the coccoid types can have a very complex life cycle and some of their taxonomic diacritical features are environment dependent, what turns their taxonomy very complex and difficult. This is the case of *Aphanothece* Nägeli and *Gloeothece* Nägeli genera, differentiated by the type and production of the mucilaginous envelope. Both described by Nägeli (1849), these colonial genera have in common the elongated shape of cells, with division in only one plan. On the other hand, they differ basically by the constant presence of lamellate sheaths in *Gloeothece* and the absence of such lamellas in *Aphanothece* (Komárek & Anagnostidis, 1998). Considering these circumscriptions, there are 36 spp. taxonomically accepted in *Gloeothece* and 62 in *Aphanothece*, what is raised to 52 spp. and 116 spp., respectively, if we include the unclear taxa and synonymized names (Guiry & Guiry, 2017). This shows how big these genera are, even they have a simple morphology; actually, the species are mostly differentiated by the habitat, color of cellular content and mucilage and cell dimensions.

Based on this, Komárek & Anagnostidis (1998) morpho-distinguished three subgenera inside *Aphanothece*: 1) *Anathece* (Komárek & Anagnostidis) Komárek, Kastovsky & Jezberová, chiefly formed by planktonic species, with small cells and slime mucilage; 2) *Aphanothece*, where the typical species were placed, with colonies well delimited by sheaths and cells at the margin often show their own envelopes, usually benthic or subaerophytic; 3) *Cyanogastrum Schiller*, represented by colonies with a firm and sharp delimitation and cells densely grouped in their center (composed of freshwater tropical species only). Komárek et al. (2011) already recognized *Anathece* as an independent genus based on a phylogenetic approach. These authors also addressed the clade of true *Aphanothece*, which possess its type species *Aphanothece microscopica* Nägeli. However, none *Aphanothece* terrestrial strain was included in the phylogenetic tree of this study and species from this habitat have not been revised yet. Also, the position of *Cyanogastrum*-like species is still an open question, once all them have only being morphologically characterized.

Differently from *Aphanothece*, *Gloeothece* has not been phylogenetic positioned and some sequences identified as *Gloeothece* appear grouped in different clades mixed with others genera. This may occur because many cyanobacterial strains can completely change their morphology when in culture, what difficult their taxonomic identification and frequently allows mislabels. The production of mucilage is one these culture changeable features and a *Gloeothece* strain without mucilage can be easily identified as a *Cyanothece*, a unicellular genus mainly characterized by its keritomized cell content (Komárek et al., 2004). Moreover, the type species of *Gloeothece*, *Gloeothece linearis* Nägeli, was recently synonymized in the genus *Gloeobacter* Rippka, J.B.Waterbury & Cohen-Bazire, and the genus was retyped; *Gloeothece fuscolutea* (Nägeli ex Kützing) Nägeli is the proposed new type to *Gloeothece* based on its morphology only (for details see Mareš et al., 2013a; Mareš et al., 2013b; Mareš et al., 2013c).

The systematics of *Aphanothece* and *Gloeothece* need revision that includes investigation of terrestrial strains, mainly those from tropical regions. Here, we intend to characterize strains morpho-related to *Aphanothece* and *Gloeothece* isolated from different terrestrial habitats of the Atlantic Rainforest based on a polyphasic approach.

2. MATERIAL AND METHODS

The studied lineages were isolated following standard techniques as described in Jacinavicius et al. (2012), from samples collected on terrestrial habitats in the Atlantic Rainforest and

Brazilian Savanah (Cerrado) (Table 9). They are kept in CCIBt (Culture Collection of Institute of Botany) under controlled conditions, as following: temperature of 23±1°C, irradiance of 40-50 µmol photons $m^{-2}.s^{-1}$ (provided by daylight fluorescent lamps and measured with a Li-COR quanta meter sensor) and photoperiod of 14–10h light-dark cycle. An aliquot of each lineage was preserved in formaldehyde 4% and deposited in the Herbarium of the Institute of Botany (SP), Brazil.

Table 9. List of studied lineages with details of their culture medium, habitat, and Herbarium access number.

Strain	Medium	Locality	Substrate	Exsiccate number
CCIBt3431	ASM-1	Atlantic Rainforest, Cardoso Island Park, São Paulo State	Soil	SP469765
CCIBt3433	ASM-1	Atlantic Rainforest, Cardoso Island Park, São Paulo State	Tree bark	SP469766
CCIBt3470	ASM-1	Atlantic Rainforest, Cerrado - São Francisco de Goiás, Goiás State	Concrete	SP469763
CCIBt3513	ASM-1	Atlantic Rainforest, Cardoso Island Park, São Paulo State	Soil	SP469768
CCIBt3534	ASM-1	Atlantic Rainforest, Jureia Ecological Station, São Paulo State	Concrete	SP469769
CCIBt3601	ASM-1	Atlantic Rainforest, Jureia Ecological Station, São Paulo State	Rock	SP469771

Table 9. List of studied lineages with details of their culture medium, habitat, and Herbarium access number.

Strain	Medium	Locality	Substrate	Exsiccate number
CCIBt3609	ASM-1	Atlantic Rainforest, Jureia Ecological Station, São Paulo State	Rock	SP469764
CCIBt3611	BG-11	Atlantic Rainforest, Gruta-que-chora cave, São Paulo State	Rock	SP469770
CCIBt3613	BG-11	Atlantic Rainforest, Gruta-que-chora cave, São Paulo State	Rock (on the entrance of the cave)	SP469767

All strains had their morphology evaluated under a Zeiss Axioplan 2 optical microscope and the identification and cell measurements were based on at least 50 individuals. To confirm the differences among species, the significance of cell dimensions was tested by ANOVA-one way using GraphPad Prism 6, with Tukey as posteriori test and $\alpha = 0.05$. The construction of life cycle was made by cells observation at five days after culture replication, and then at 30, and 60 days after. The metric information is expressed as minimum-average-maximum values in the taxonomic description. The organisms were documented by digital photographs taken with a Zeiss Axiocam MRc digital camera. The main morphological features observed were: type of cell division, cell diameter and length, structure and color of sheaths and color and uniformity of cell content. All new taxa were described in accordance to the International Code of Nomenclature for algae, fungi, and plants (McNeill et al. 2012).

Cellular ultrastructure was studied from cultures by transmission electron microcopy. The cells were centrifuged for concentration and the medium was eliminated. After, each sample was fixed with Karnovsky plus sucrose 0.2M solution for 24h, washed three times with 0.05M cacodylate buffer and post-fixed with 1% osmium tetroxide. After that, samples were overnight submitted to contrasting in uranyl acetate 5% and dehydrated in an acetone series of increasing concentration and infiltrated in Spurr resin. The blocks were cut on an ultramicrotome (Leica Ultracut UCT) with a diamond blade (450) into 70 nm thick sections

and mounted on uncoated 200-mesh copper grids, and stained with uranyl acetate and lead citrate (Reynolds 1963). Visualization and microphotography were performed on a Jeol Jem 1011 transmission electron microscope (JEOL, Tokyo, Japan) at 100 kVA.

Total genomic DNA was isolated from a liquid culture of the cyanobacterial strains using the Ultra Clean Soil DNA Isolation Kit (MOBIO). The partial 16S-ITS-23S rRNA sequence was amplified by PCR using the 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, 200µm of each dNTP, 3.0 mM $MgCl_2$, 1 × PCR buffer and 1.0 U Platinium *Taq* DNA polymerase (Life Techonologies, Grand Island, NY, USA) under conditions according to Taton et al. (2003). The resulting PCR product was cloned into a pGEM®-T Easy Vector System (Promega, Madison, WI, USA) and inserted into chemiocompetent *E. coli* DHG5α cells by a heat-shock method (Sambrook et al., 1989). After growth in LB plates containing 5 µl X-Gal (50 $\mu g.mL^{-1}$) (Life Techonologies) and 10 µl of ampicillin sodium salt (100 $\mu l.\ mL^{-1}$) (Sigma-Aldrich Co., St. Louis, MO, USA), recombinant plasmids were purified from white colonies by the alkaline lysis method (Birnboim & Doly, 1979). The cloned gene fragment was sequenced using the 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 the sample was placed in an ABI PRISM 3500 Genetic Analyzer (Life Technologies). The sequenced fragments were assembled with the Phred/Phrap/Consed software package (Ewing & Green, 1998; Ewing et al., 1998; Gordon et al., 1998).

The phylogenetic analyses were based on 16S rRNA gene and 16S-23S internal transcribed spacer (ITS) sequences obtained in this study and reference sequences retrieved from GenBank. The General Time Reversible (GTR) evolutionary model of substitution with Gamma distribution (G) and with an estimate of proportion of invariable (I) sites was selected as the best fit for the 16S rDNA alignment and Hasegawa, Kishino and Yano (HKY) model + G +I for 16S-23S ITS using jModelTest (Posada, 2008). Evolutionary analyses based on the Neighbor Joining and Maximum Likelihood methods were conducted in Mega 6.0 (Tamura et al., 2013) and estimations of the robustness of the tree were tested by bootstrap with 1,000 replications. The Bayesian analysis was inferred by MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2005) in 5×10^6 generations. Chains were sampled every 100^{th} 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 using p-distance method in Mega 6.0 (Tamura et al., 2013) and the percentage was given by the formula *[1–(p-distance)]×100*. The mean similarity was estimated by the average of similarity value of each sequence from the two compared clades.

3. RESULTS AND DISCUSSION

The morphological characterization is the first step in an attempt to circumscribe a group of cyanobacteria strains taxonomically related. Following this idea, among our 10 studied strains, it is possible to recognize two main groups: I) one is morpho-related to the genus *Aphanothece*, where are grouped the mucilage producer strains, with cells frequently loose arranged or rarely forming irregular colonies, but lamellate sheaths never show up. Here we have CCIBt3609, CCIBt3470, CCIBt3431, CCIBt3433, CCIBt3513 and CCIBt3613. II) In the second group are assembled the lineages similar to *Gloeothece*, which also produce mucilage, but their cells are surrounded by concentrically lamellate sheaths (CCIBt3534, CCIBt3601 and CCIBt3611). As we can see, all studied strains are mucilage producers, what is a diacritic feature that already differs them from the genus *Cyanothece* Komárek, in comparison with its type species (details in Komárek & Cepák, 1998).

The characterization based on the 16S rDNA analyzes reveals that these morphological separation of the strains is supportable by molecular approach (Figure 16 and Figure 17, Table 10 and Table 11). In the phylogenetic tree, we can see that these two main groups are related, but they have an average similarity of 91% between themselves, what confirms their definition as two distinct genera.

After comparing our sequences with GenBank, those from the Group II joined with sequences identified as *Gloeothece* from the strains PCC6501 and PCC6909 (Figure 16). According to Weckesser et al. (1987) and Micheletti et al. (2008), these lineages have the typical concentric lamellate sheaths, what confirms their morphological identification as *Gloeothece*. However, we can recognize in the tree another clade also identified as *Gloeothece*, which is close to the typical *Aphanothece*, as defined by Komárek et al. (2011). This second clade mostly contains strains isolated from different habitats of Singapore coast, including some from seawater (Ohki et al., 2008). Based on the pictures showed in Ohki et al. (2008), we can see that even these strains developed colonies, they are not surrounded by lamellate sheaths. Besides, they were isolated from an environment very distinct from that where *Gloeothece fuscolutea* was

found, the type species of *Gloeothece* (Mareš et al., 2013a). On the other side, we have all strains from the Atlantic Rainforest (AR) isolated from a similar habitat where *G. fuscolutea* occurs and they also fitted in all morphological features of *Gloeothece*. Besides, they group with morpho-similar lineages also identified as *Gloeothece* in the phylogenetic tree, as we could see. Based on this, we suggest the clade formed by the strains of Group II to be recognized as the typical *Gloeothece* clade. As the group of strains showed by Ohki et al. (2008) does not matches with the morphologic description of *Gloeothece*, neither was isolated from a similar habitat, they should be described as a separated genus, all distinct from *Gloeothece*, *Aphanothece* and *Cyanothece*.

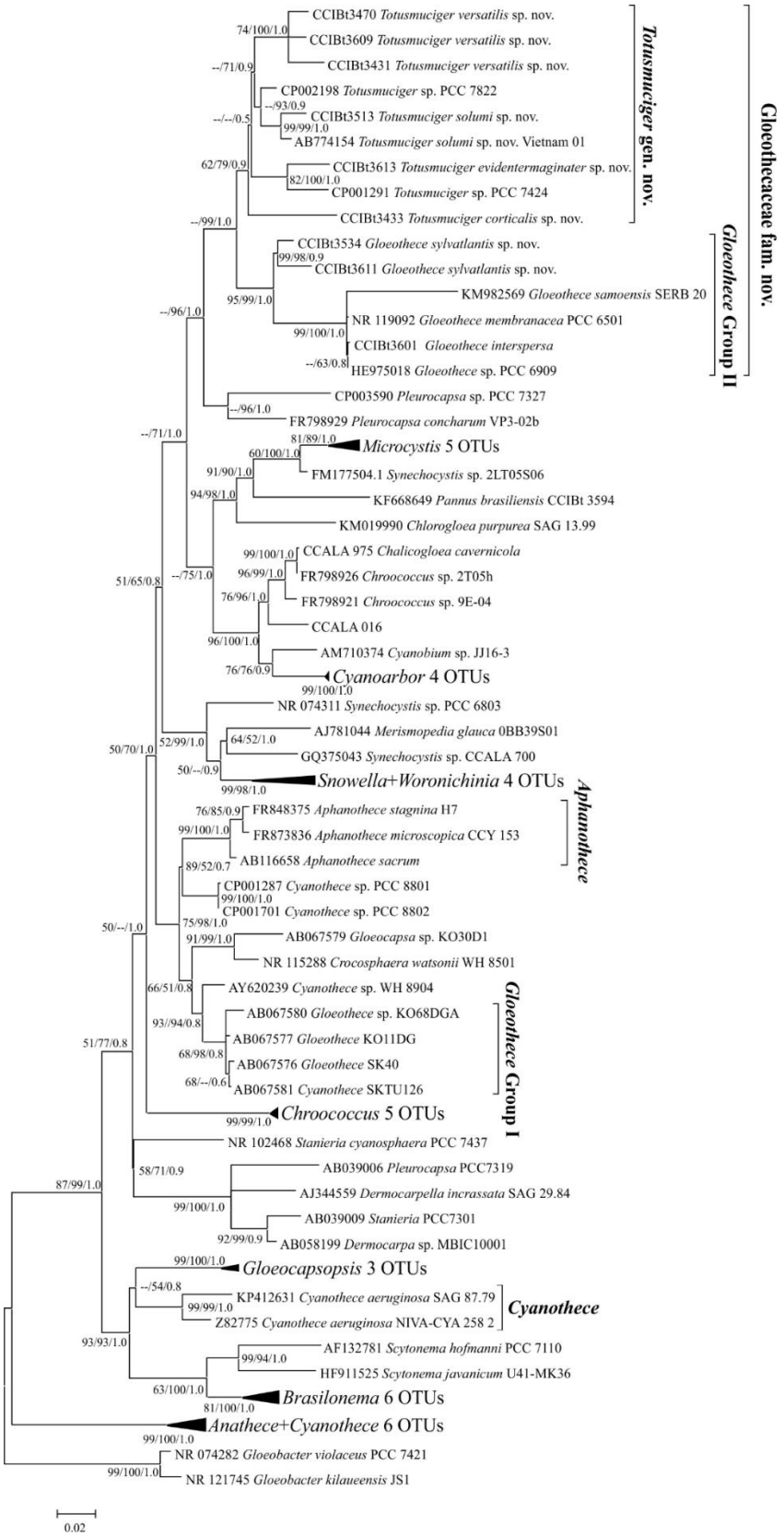


Figure 16. Phylogenetic tree based on 16S rRNA gene sequences and reconstructed using the maximum-likelihood (ML) analysis of evolutionary distances determined by the GTR + G + I model. NJ and ML bootstrap (1,000×) values (above 50 %) and Bayesian posterior probabilities are provided for each node, respectively.

Table 10. 16S rDNA similarity matrix of studied lineages and their close relatives, conducted using the p-distance model with a total of 920 positions.

	Taxa identification	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	CCIBt3431 *Totusmuciger versatilis*																				
2	CCIBt3470 *Totusmuciger versatilis*	97.8																			
3	CCIBt3609 *Totusmuciger versatilis*	97.7	99.2																		
4	CCIBt3513 *Totusmuciger solumi*	94.9	96.3	96.2																	
5	AB774154 *Totusmuciger solumi* Vietnam01	95.2	96.5	96.4	99.1																
6	CP002198 *Totusmuciger* sp. PCC7822	96.0	97.5	97.1	98.2	98.3															
7	CCIBt3613 *Totusmuciger evidentermarginater*	95.2	95.0	95.0	94.3	94.3	94.8														
8	CP001291 *Totusmuciger* sp. PCC7424	94.2	94.8	94.7	94.7	94.9	95.1	97.1													
9	CCIBt3433 *Totusmuciger corticalis*	95.5	96.2	96.3	95.1	95.2	95.8	94.8	94.5												
10	CCIBt3611 *Gloeothece sylvatlantis*	92.1	93.6	93.7	94.6	94.8	94.5	92.1	93.3	93.4											
11	CCIBt3534 *Gloeothece sylvatlantis*	93.4	94.6	94.7	94.8	95.1	95.4	93.3	93.7	94.2	98.0										
12	CCIBt3601 *Gloeothece interspersa*	92.4	93.8	93.9	94.5	95.0	94.5	92.1	92.4	93.5	96.6	96.4									
13	KM982569 *Gloeothece samoensis* SERB20	89.0	90.0	90.1	90.7	91.2	90.7	88.8	89.0	90.3	92.8	92.6	95.7								
14	NR119092 *Gloeothece membranacea* PCC6501	92.5	93.8	93.9	94.6	95.1	94.5	92.2	92.5	93.6	96.8	96.4	99.8	95.9							
15	HE975018 *Gloeothece* sp. PCC6909	92.5	93.9	94.0	94.6	95.1	94.6	92.2	92.5	93.6	96.7	96.5	99.9	95.8	99.9						
16	AB067576 *Gloeothece* SK40	92.9	92.9	93.0	92.0	92.2	92.5	92.1	92.2	92.4	91.1	92.0	92.4	88.5	92.2	92.3					
17	AB067580 *Gloeothece* sp. KO68DGA	92.6	92.6	92.7	92.0	92.1	92.5	92.0	92.2	92.3	90.4	92.0	92.0	88.3	91.7	91.8	99.0				
18	AB116658 *Aphanothece sacrum*	90.8	91.6	91.7	92.0	92.1	92.0	90.8	91.5	91.5	91.4	91.5	93.2	89.5	93.2	93.3	95.9	95.7			
19	CP001287 *Cyanothece* sp. PCC8801	93.7	94.2	94.2	93.4	93.5	93.9	92.6	92.7	93.4	91.7	92.0	92.6	88.8	92.6	92.7	96.2	95.5	96.3		
20	KP412631 *Cyanothece aeruginosa* SAG87.79	89.8	89.6	90.0	89.7	89.8	90.4	89.3	90.0	90.0	88.8	89.6	90.2	87.1	90.2	90.3	92.3	92.7	92.4	92.4	

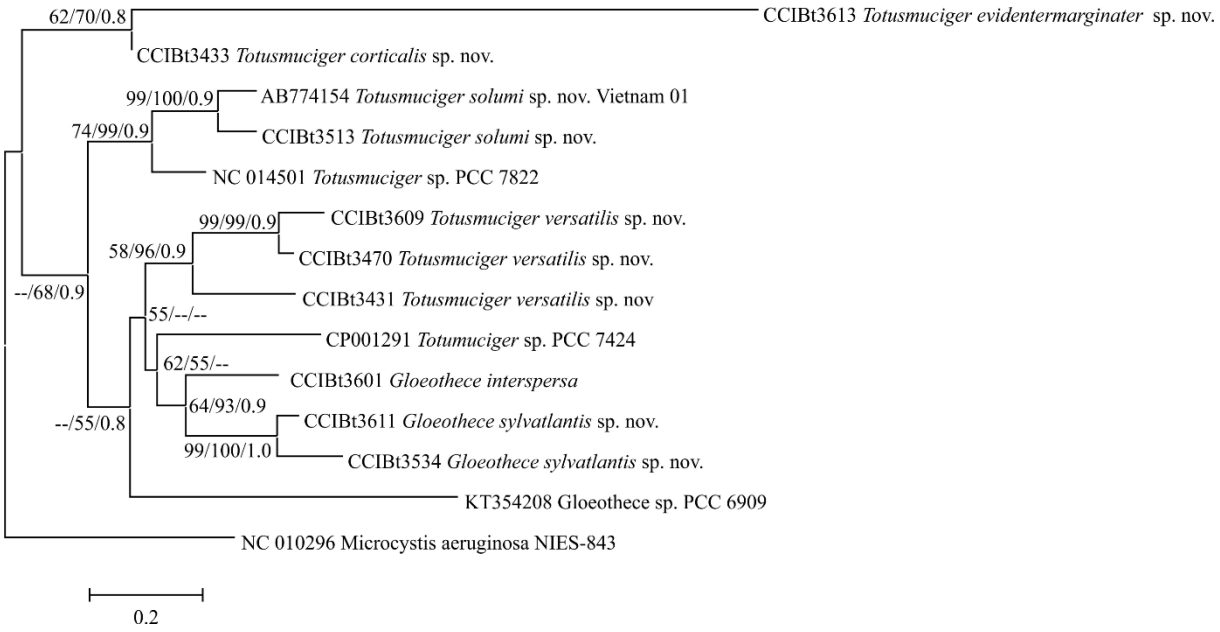


Figure 17: Phylogenetic tree based on 16S-23S ITS sequences and reconstructed using the maximum-likelihood (ML) analysis of evolutionary distances determined by the HYK + G + I model. Bootstrap 1,000×.

Table 11. 16S-23S ITS similarity matrix of studied lineages and their close relatives, conducted using the p-distance model with a total of 216 positions.

	Species identification	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	CCIBt3470 *Totusmuciger versatilis* sp. nov.														
2	CCIBt3431 *Totusmuciger versatilis* sp. nov.	85.2													
3	CCIBt3609 *Totusmuciger versatilis* sp. nov.	93.1	82.9												
4	AB774154 *Totusmuciger solumi* Vietnam01	76.9	78.2	77.8											
5	CCIBt3513 *Totusmuciger solumi* sp. nov.	77.8	78.7	77.3	94.9										
6	NC014501 *Totusmuciger* sp. PCC7822	77.8	79.6	77.8	90.3	89.4									
7	CCIBt3613 *Totusmuciger evidentermarginater* sp. nov.	62.5	65.3	61.6	66.2	64.8	69.0								
8	CCIBt3433 *Totusmuciger corticalis* sp. nov.	78.2	80.6	78.2	80.6	79.6	84.7	70.4							
9	CP001291 *Totusmuciger* sp. PCC7424	77.8	79.2	79.6	79.2	76.4	77.8	63.0	79.2						
10	CCIBt3534 *Gloeothece sylvatlantis* sp. nov.	81.5	78.7	79.6	73.6	72.7	75.0	63.4	76.4	80.1					
11	CCIBt3601 *Gloeothece interspersa*	81.5	80.6	79.6	81.5	81.0	82.4	64.8	78.7	82.9	83.8				
12	CCIBt3611 *Gloeothece sylvatlantis* sp. nov.	79.6	79.6	79.2	75.5	75.5	76.9	64.4	76.4	81.9	91.2	86.1			
13	KT354208 *Gloeothece* sp. PCC6909	76.9	76.4	74.5	73.1	73.1	75.5	59.7	75.9	76.9	74.1	75.0	74.5		
14	NC010296 *Microcystis aeruginosa* NIES-843	76.9	78.2	74.5	74.5	73.6	76.9	64.4	82.4	75.9	73.6	75.5	72.7	73.6	

With the generic identification confirmed, we can see that the three AR strains grouped in *Gloeothece* clade are differently related and can be considered as two species. The strain CCIBt3601 showed to be morphologically close to *Gloeothece interspersa* Gardner, described by Gardner (1927) to Puerto Rico. This strain has the intense lamellate sheaths and cells dimensions similar to that described to *G. interspersa* (Figure 18 and Figure 25). Also, the habitat is very close and Gama Jr et al. (2014) identified this species from samples of the Atlantic Rainforest. Based on this, we herein proposed the first molecular characterization and phylogenetic placement of *G. interspersa*, which showed to be a very good species since its sequence is not close related to any other available.

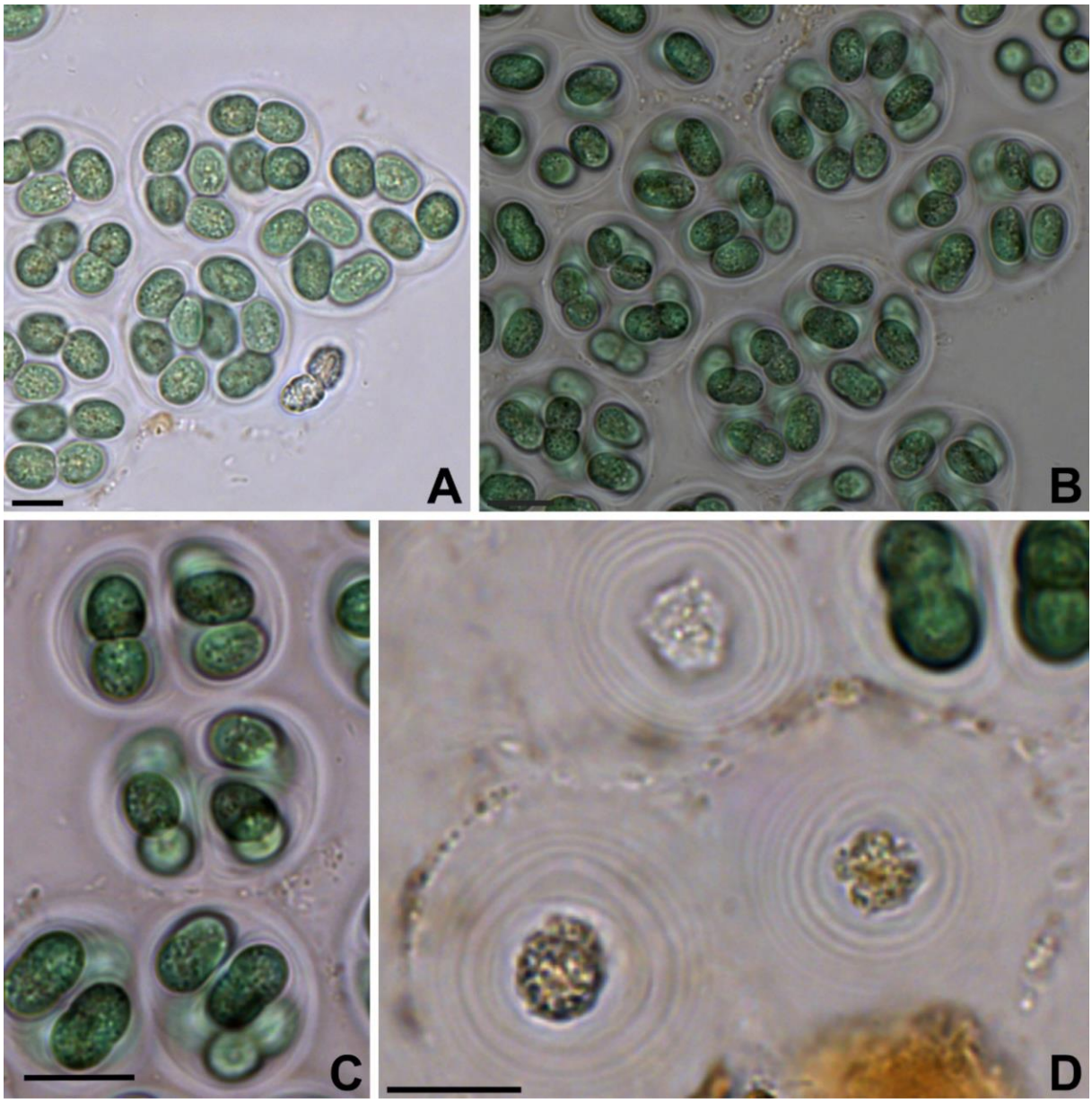


Figure 18: *Gloeothece interspersa*. A-B: general view of colonies; C: colonies showing lamellate sheaths; D: detail of senescent cells surrounded by intense lamellate sheaths. Scale bars = 10 µm.

The other two strains, CCIBt3534 and CCIBt3611, showed to be very close to each other and possess 98% of 16S rDNA similarity. Moreover, the ITS also showed a high value of similarity between these strains: 91.2%. This confirms their identification as the same species, even they were isolated from different places and slightly different habitats (Table 9). Regarding the morphology, these strains are similar to the species *Gloeothece fuscolutea* and *Gloeothece palea* (Kützing) Nägeli. With this last species, the AR strains have the cells dimensions in common. Sant'Anna et al. (1991) found a population morphologically close to our strains and identified them as *G. palea.* Also, the population studied by these authors was

found in the same locality where CCIBt3611 was isolated, which are rocks on the entrance of the cave Gruta-que-Chora (São Paulo, State) (Table 9). This reinforces that CCIBt3611 represents the same species showed by Sant'Anna et al. However, Kützing (1843), the original description of *G. palea*, is very clear in saying that this species has a mucilaginous sheath not very conspicuous, which is not the case of the analyzed strains, neither is the material from Sant'Anna et al. (1991). So, we discard the possibility of these strains being identified as *G. palea.*

Regarding to *G. fuscolutea*, Mareš et al. (2013a) analyzed its type material and based on the picture showed by these authors we can check its morphological identity. Comparing it with the strains from AR we can see that the shape of the cells and the dimensions are different between them (Figure 20). CCIBt3534 and CCIBt3611 showed to have cells cylindric/elongated and longer, while *G. fuscolutea* has elliptical/oblong and shorter cells, what can confirm that the strains from AR are not *G. fuscolutea*. Gama Jr et al. (2014) identified this species to the Atlantic Rainforest, but these authors based their identification only in the original description of this species (Nägeli, 1849) and it was not very detailed. With the access to the type material picture we can affirm that the material studied by Gama Jr et al. (2014) is not *G. fuscolutea* and it matches with the strains herein presented. Actually, the strain CCIBt3534 was isolated from the same sample and population that these authors studied and formerly identified as *G. fuscolutea*. In face that the lineages from the Atlantic Rainforest have no matches in the literature, we proposed the new species *Gloeothece sylvatlantis* sp. nov. (Figure 19), which is represented by the strains CCIBt3534 (type strain) and CCIBt3611 and is named in reference to the Atlantic Rainforest. Additionally, we propose the synonymizing of *Gloeothece fuscolutea sensu* Gama Jr. et al. (2014) and *Gloeothece palea sensu* Sant'Anna et al. (1991) into this new species.

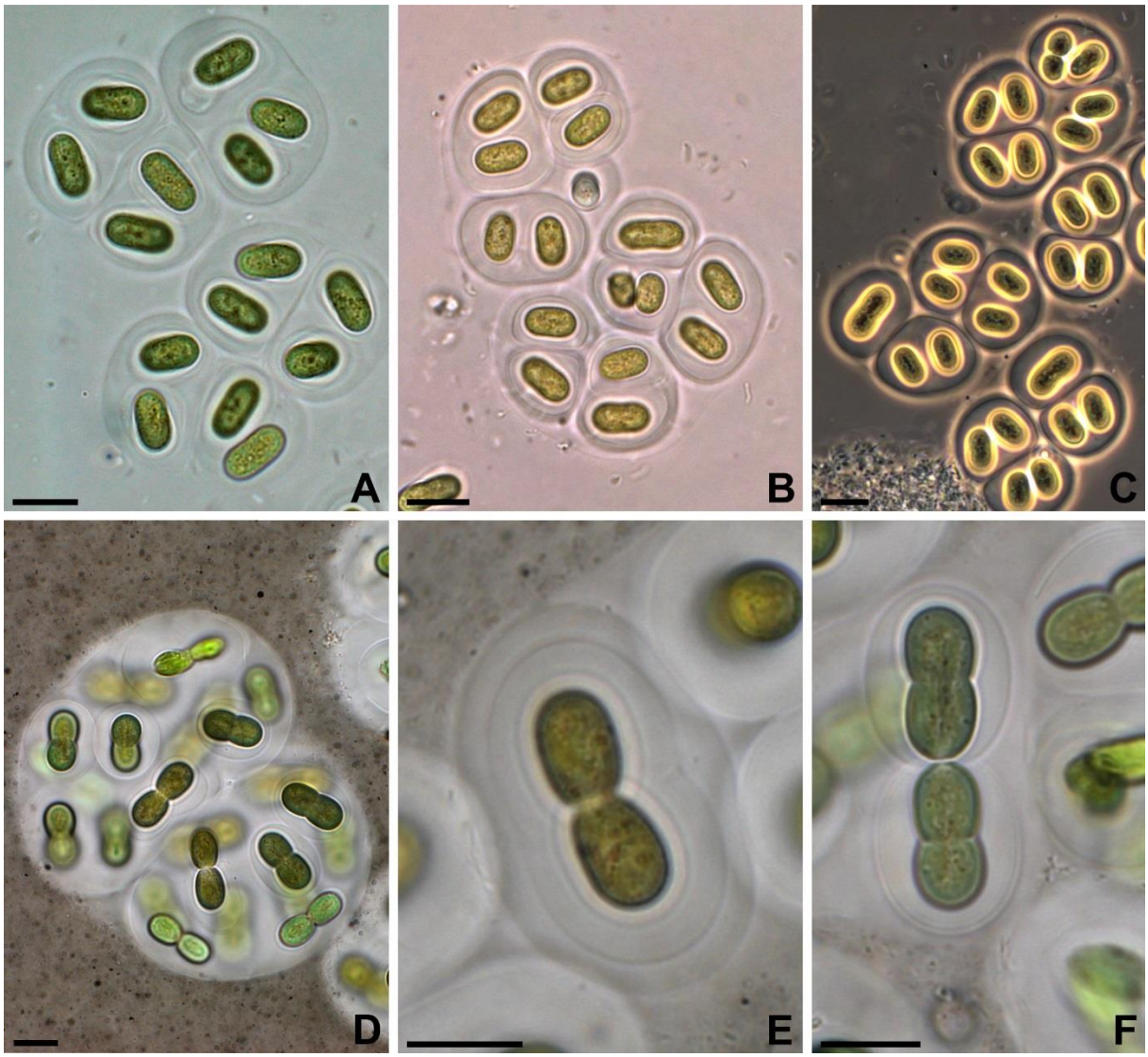


Figure 19: A-F: *Gloeothece sylvatlantis*. A-C: CCIBt3435 general view; D-F: CCIBt3611. E-F: detail cells with lamellate sheath. Scale bars = 10 µm.

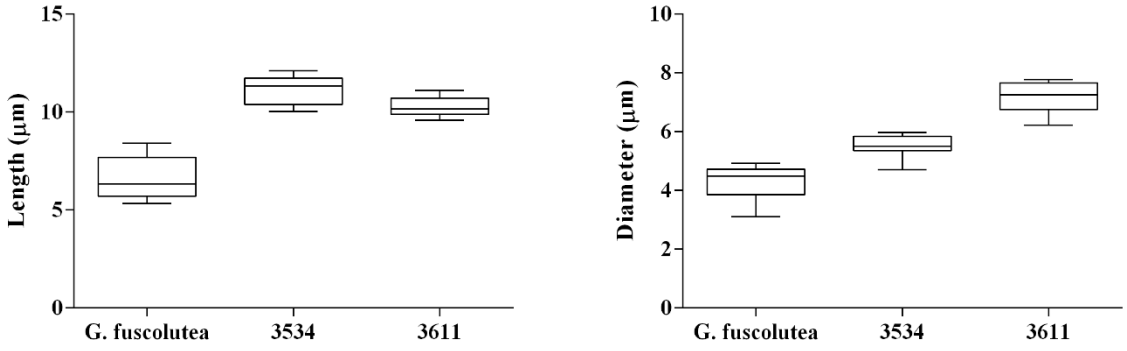


Figure 20: Box-plot (maximum-average-minimum values) showing the difference of cells length and diameter among the type material of *Gloeothece fuscolutea* and the *Gloeothece* strains isolated from the Atlantic Rainforest.

The other group of studied strains (Group I), that similar to *Aphanothece*, were placed together with sequences identified as *Cyanothece* (PCC7822, PCC7424 and Vietnan01). Surely, these strains are misidentified, since they are not related to type clade of *Cyanothece*, as we can see in the tree (Figure 16). Besides, in the literature it is possible to know that all these strains are mucilage producers, what already differ them from truly *Cyanothece,* and they also have in common the fact of being isolated from soil at rice fields (see Ohki et al., 2014 for Vietnan01 and Rippka, 1979 for PCC7822 and PCC7424). Actually, Rippka et al., 1979 empathize that these PCC strains used to produce an abundant mucilage, but this property is no longer expressed by them probably due their long time of culturing. Nevertheless, this high production of mucilage, with cells barely organized, is the common feature to all strains inside this clade, including those herein analyzed. Morphologically, this feature could associate these strains to the genus *Aphanothece*, but as we can see they are very phylogenetic far from typical *Aphanothece*. Thus, we propose the new genus *Totusmuciger* gen. nov. in order to encompasses all these strains and its name refers to the abundant mucilage produced by its representatives. In spite of being well separated from *Aphanothece* by molecular approach, *Totusmuciger* is not entirely distinguishable from *Aphanothece* by morphology sole, what can make it a cryptic genus in a certain way. Nevertheless, as Komárek et al. (2011) did not include any truly terrestrial strains in their analyzes, we suggest that *Totusmuciger* encloses the aerophytic *Aphanothece*-like species. This kind of habitat can also justify the high production of mucilage by its species, what can be and adaption to the arid conditions, like is described to *Nostoc* Vaucher ex Bornet & Flahault (Gao et al., 2012).

Analyzing the strains grouped inside *Totusmuciger* it is possible to note that they can be split into four different species, in accordance to 16S rDNA and ITS sequences (Table 10 and Table 11). The first to be discussed and the most pronounced one is formed by the strains CCIBt3431, CCIBt3470 and CCIBt 3609. In spite of being isolated from different localities (CCIBt3470 was isolated from the Brazilian Cerrado Biome and CCIBt 3431 and 3609 from the Atlantic Rainforest), they all have similarity higher than 97.8% of 16S rDNA, reaching 99.2% between CCIBt3470 and 3609. The ITS also shows a high value of similarity among these strains (Table 16). Morphologically, they all have the same kind of cells shape and the cells dimensions do not differ significantly (Figure 21). Moreover, we point out the presence of pseudofilaments as a diacritical feature of this species, which can also differentiate them from *Aphanothece* and *Gloeothece* taxa.

All these features confirm the identification of these strains into a same species. However, going in the opposite way of the majority of cyanobacteria species, the strains herein analyzed were isolated from different habitats. CCIBt3609 and CCIBt3470 have a more similar origin: wet rock and concrete, respectively. On the other hand, CCIBt3431 was isolated from a wet soil sample. This put in question the selectivity of cyanobacteria species to the type of habitat, what is so well confirmed to other species. But as this event showed here is isolate from what is most common and two of the analyzed strains have a more related habitat, it could be the case of a potential flora. This means that a vial organism came from another place and was present in the soil sample which once in culture conditions, make its growing favorable. These suppositions have no definitive answer right now and only further studies with strains morpho-related to those herein presented, but from different habitats, can better elucidate it. In relation to species already described in literature, none was found to have the morpho-characters close to the lineages analyzed (we compared to *Aphanothece* and *Gloeothece* species). Thus, we proposed the new species *Totusmuciger versatilis* sp. nov. (Figure 22) based on these three lineages and we also designated it as the type species of *Totusmuciger*. The name is a reference to its broad distribution, both related to geographic occurrence and type of habitat.

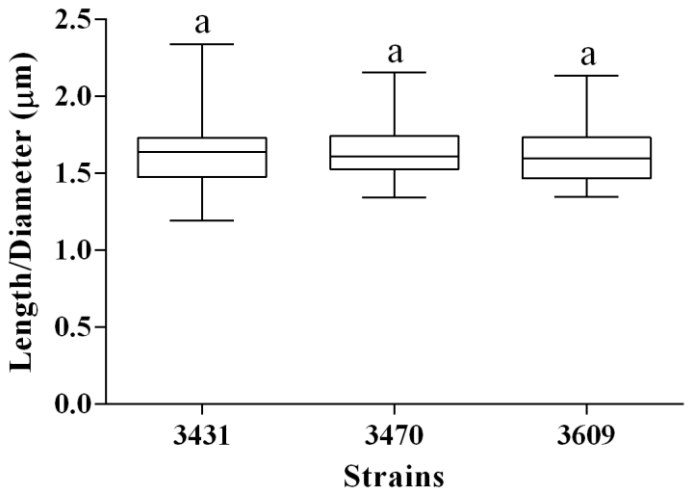


Figure 21: Box-plot (maximum-average-minimum values) showing the difference of cells length/diameter rate among the strains identified as *Totusmuciger versatilis*. Different letters represent significant differences tested with ANOVA one-way.

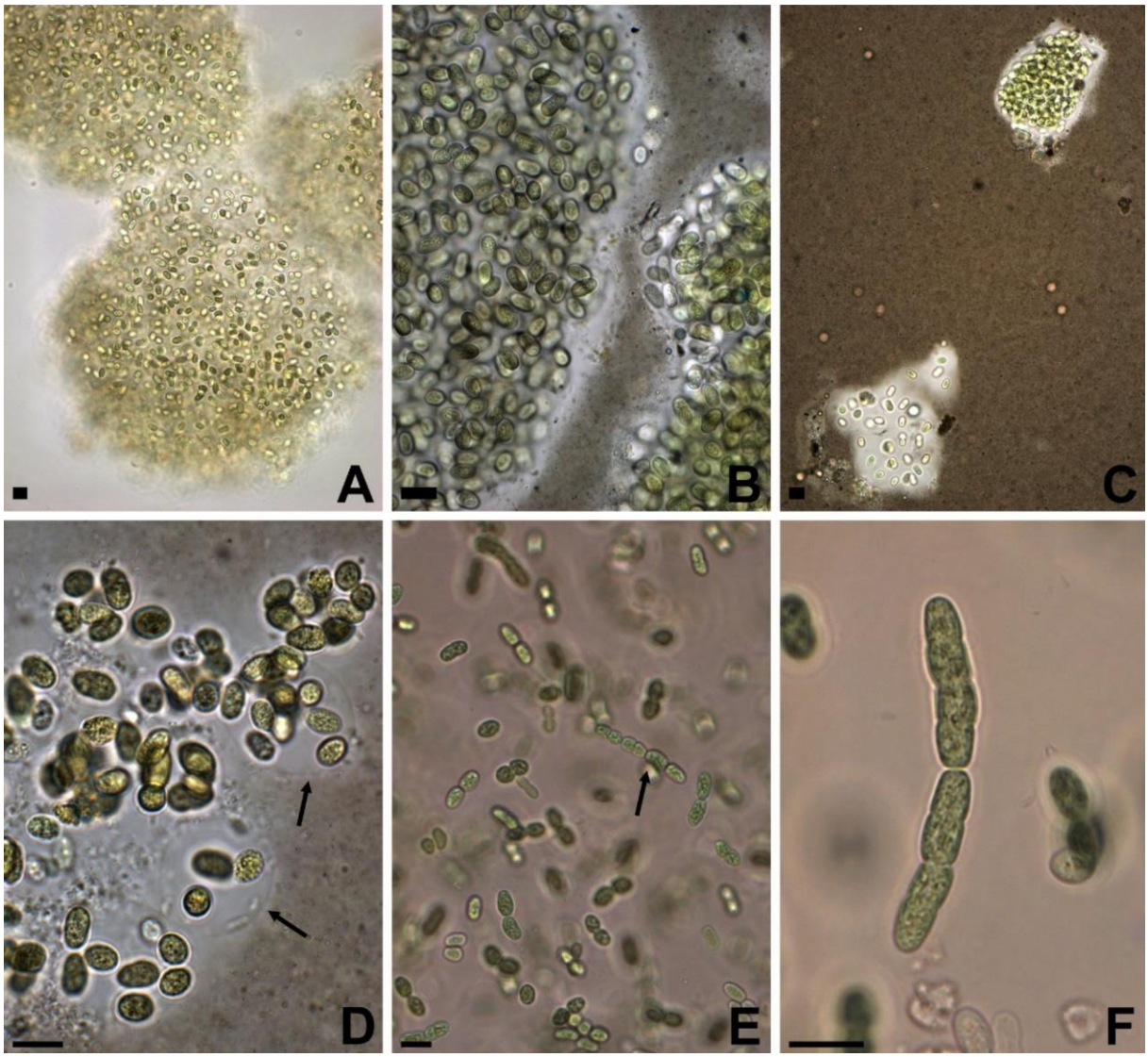


Figure 22: *Totusmuciger versatilis*. A: general aspect of rounded colony (CCIBt3609); B: colonies mucilage highlighted by China Ink (CCIBt3609); C: colonies with densely and loosely cells arranged with mucilage delimitation highlighted by China Ink (CCIBt3470); D: detail of conspicuous mucilage (arrow) (CCIBt3470); E-F: pseudofilaments detailed (arrow in E) (CCIBt3431). Scale bars = 10 µm.

The lineage CCIBt3513 is also a distinctive species inside *Totusmuciger* and it is also a very close to the strain *Cyanothece* sp. Vietnam 01. This lineage was isolated from soil of rice fields in Vietnam (Ohki et al., 2014) and the morphology matches with the strain isolated from the Atlantic Rainforest, which was also isolated from wet soil. Besides, they have 99.1% of 16S rDNA similarity and 94.9% related to the ITS sequences. These values are high enough to consider them as the same species. As well as we observed to *Totusmuciger versatilis*, CCIBt3513 has no morphological correspondence in the species already described, what allows us to propose another new species, *Totusmuciger solumi* sp. nov., named in

relation to the occurrence of this species on soils, represented by CCIBt3513 (type strain) and Vietnan01. Differently from *T. versatilis* and *T. solumi*, the other two species recognized in *Totusmuciger* clade are represented by one strain each. However, they have morphological features that sustain them as independent taxa, besides the molecular support.

As common feature shared only by these two strains, CCIBt3433 and CCIBt3613 have colonies more often formed than in the other *Totusmuciger* strains. Also, the cells are more or less densely agglomerated in the colony center, surrounded by a thick mucilage. This feature can morphologically link CCIBt3433 and CCIBt3613 to *Cyanogastrum*. However, this subgenus is composed only by freshwater species and as we saw to *Aphanothece*, it is probable that the aquatic species consist in a genus apart from the terrestrial ones. Besides, the strains from AR also showed the loose cells and their 16S rDNA similarity with *Totusmuciger* strains is higher than 95% (Table 10). This confirms their maintenance inside this genus.

Regarding their similarity with other strains, CCIBt3613 showed to be close to PCC7424 according to 16S rDNA. The morphology between them cannot be comparable, once we do not have access to PCC7424. However, it is possible to affirm they do not belong to the same species by ITS analyzes (Table 11 and Figure 17). Also, CCIBt3613 was isolated from the interior of the cave Gruta-que-chora, which is very different from the origin of PCC7424 (soil of rice field). In relation to its morphology, CCIBt3613 was the only strain from *Totusmuciger* to show a very conspicuous envelope, but this feature was observed only in cultures over 60 days of cultivation. Pseudofilaments were also presented in aged cultures. These features make CCIBt3613 the unique exemplar very distinct species inside *Totusmuciger*, what we consider enough to endorse its proposal as the new species *Totusmuciger evidentermarginater* sp. nov., in reference to its well-delimited sheath. Following this same way, CCIBt3433 is also an insulated representative of a new species from *Totusmuciger*, herein designated as *Totusmuciger corticalis* sp. nov. As its name suggests, this strain is the only one isolated from tree bark, what ecologically distinguish it from the other species. Even so, it has a 16S rDNA average similarity of 95% with *Totusmuciger* type species (*T. versatilis*), what confirms its generic identity. Besides the ecology, *T. corticalis* is also separated from the other *Totusmuciger* species by the cell content color and the presence of a deltoid grain in the center of the cells, which refers to a carboxysome according to electron microcopy (Figure 26). This structure was observed only in this strain and it was commonly present during the whole time of cultivation.

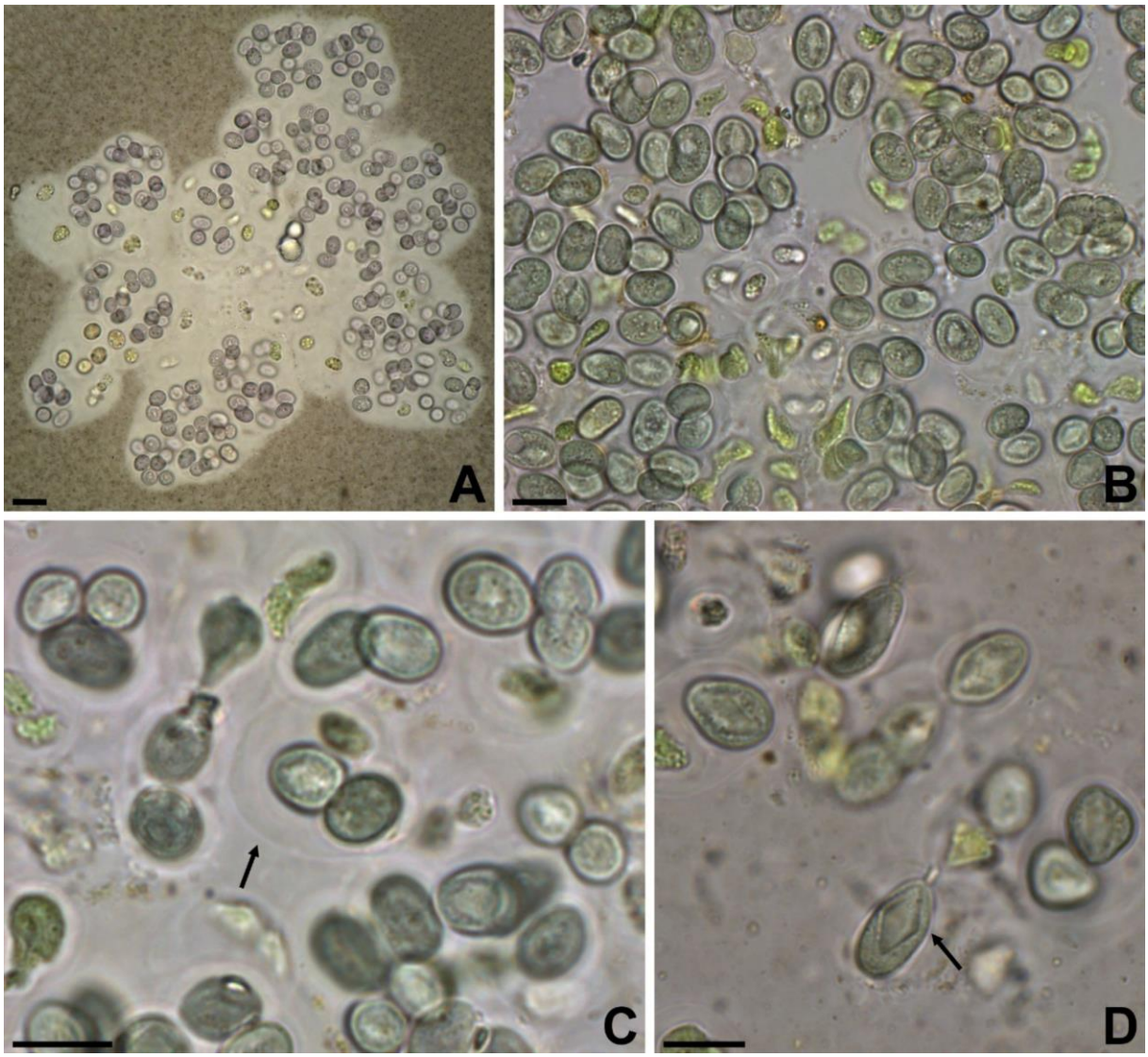


Figure 23: *Totusmuciger corticalis* (CCIBt3433). A: general aspect of colonies with mucilage highlighted by China Ink; B: cells in general aspect; C: detail of cells surrounded by conspicuous mucilage (arrow); D: cell with rhomboid carboxysome grain (arrow). Scale bars = 10 µm.

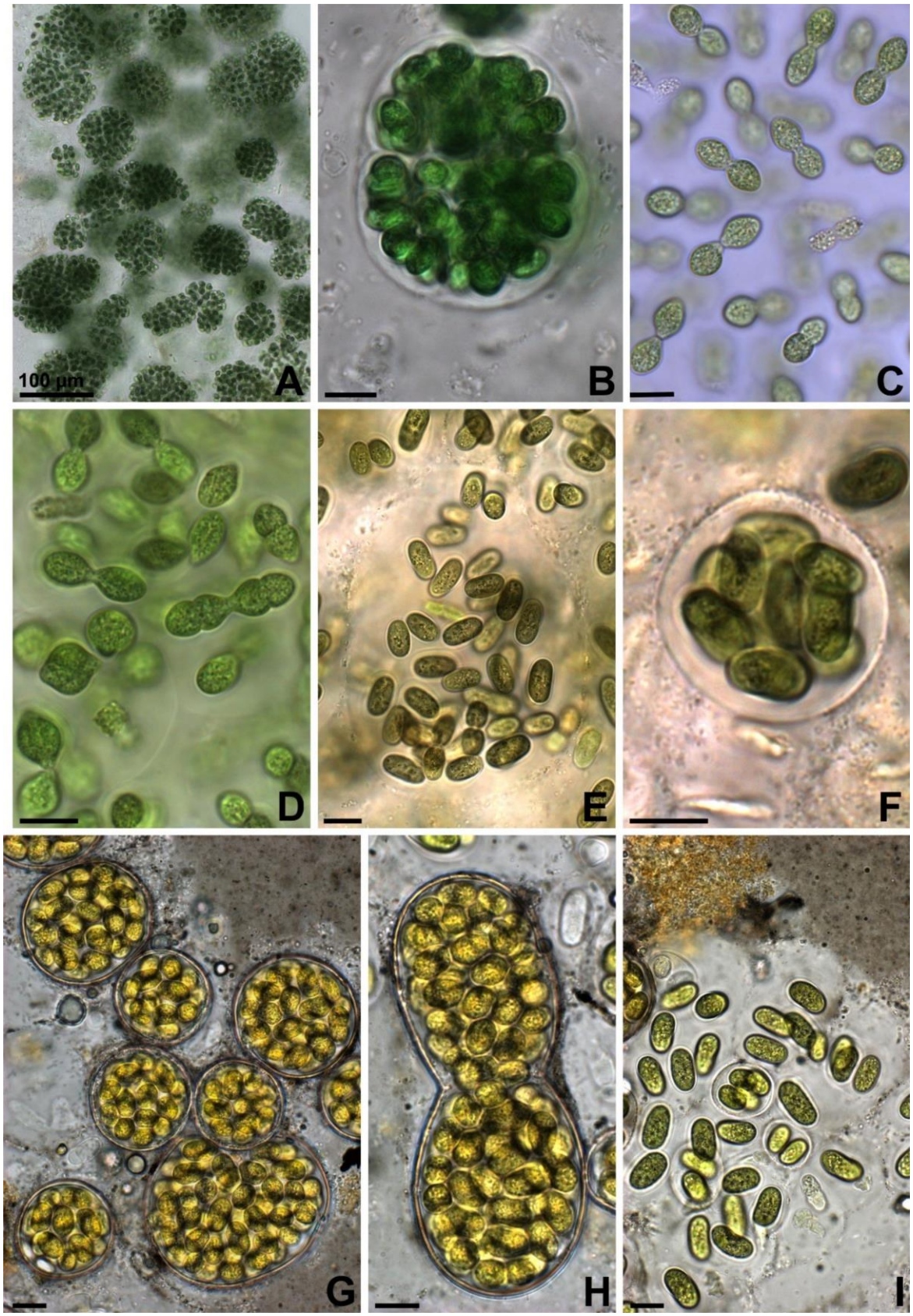


Figure 24: A-D: *Totusmuciger solumi* (CCIBt 3513); A: general aspect of colonies; B: colony with evident mucilage; C-D: detail of cells and pseudofilaments. E-I: *Totusmuciger evidentermarginater* (CCIBt 3613); E-F: young colonies; G: mature colonies; H: colony propagation. Scale bars = 10 µm.

The electron microscopy also reveals the arrangement of thylakoids in fasciculus organized in loops, which showed by *Gloeothece* and *Totusmuciger* (Figure 25). This organization is the same showed by Komárek et al. (2011) to the typical-*Aphanothece*, what reveals that they are related and can be classified under the same order, Chroococcales (according to Komárek et al. 2014). However, the bigger clade formed by the genera *Gloeothece* and *Totusmuciger* are not close enough to *Aphanothece* to be included into its family, Aphanothecaceae, neither to the typical *Cyanothece* and its family Cyanothecaceae. Thus, we suggest the new family Gloeothecaceae fam. nov. in order to accommodate the genus *Gloeothece* and *Totusmuciger*. The description of a new family is suggested also to emphasize the complexity of coccoid cyanobacteria systematics, which are morphologic simple but have a diverse and complex evolution history.

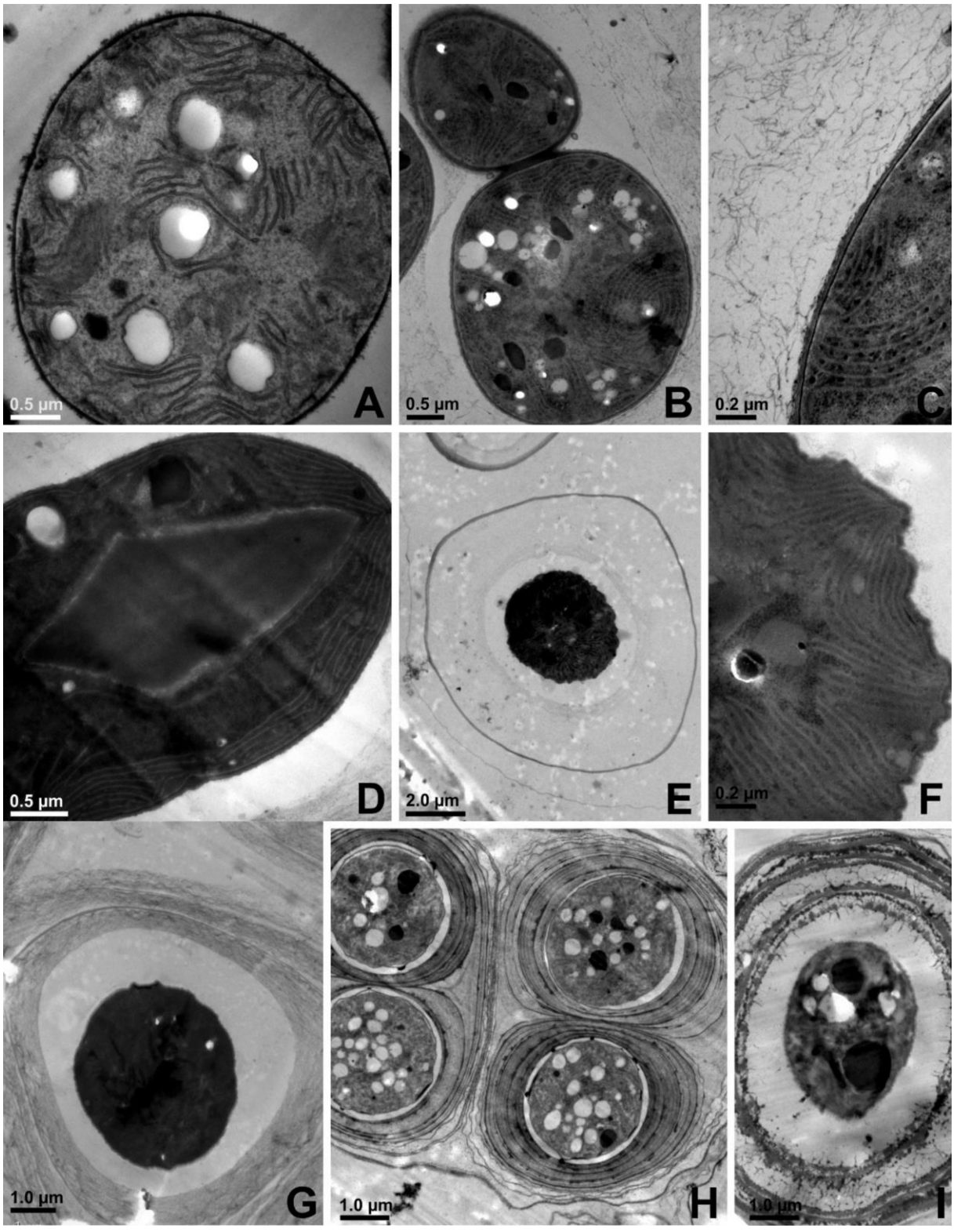


Figure 25: A-C: *Totusmuciger versatilis*, A: CCIBt3470, B-C: CCIBt3431; C: detail of mucilage matrix. D: *Totusmuciger corticalis* (CCIBt3433), detail of rhomboid carboxysome grain. E-G. *Gloeothece sylvatlantis*, E: CCIBt3534, F-G: CCIBt3611. H-I: *Gloeothece interspersa* (CCIBt3601) showing the intense lamellate sheaths.

4. TAXONOMIC DESCRIPTIONS

Here we detail and descript the new taxa found. The cells dimensions of all strains are specified in Table 12

Table 12. Cells dimensions of analyzed strains. Max.= maximum value; Avg.= average value; Min.= minimum value; L/D: ratio between length and diameter.

Strains	Cells dimension (µm)								
	Length			Diameter			L/D		
	Min.	Avg.	Max.	Min.	Avg.	Max.	Min.	Avg.	Max.
CCIBt3470 *Totusmuciger versatilis*	5.5	7.4	8.8	5.2	4.6	3.8	2.2	1.6	1.3
CCIBt3609 *Totusmuciger versatilis*	8.6	7.0	5.4	4.9	4.3	3.5	2.1	1.6	1.3
CCIBt3431 *Totusmuciger versatilis*	7.7	6.2	4.6	4.7	3.8	2.8	2.3	1.6	1.2
CCIBt3433 *Totusmuciger corticalis*	11.8	9.5	7.1	8.3	6.8	5.5	1.8	1.4	1.1
CCIBt3513 *Totusmuciger solumi*	10.7	8.0	5.4	6.5	4.9	3.9	2.1	1.6	1.1
CCIBt3613 *Totusmuciger evidentermarginater*	12.5	9.6	7.7	8.1	6.8	5.6	1.8	1.4	1.1
CCIBt3534 *Gloeothece sylvatlantis*	13.5	10.4	8.4	6.1	5.3	4.3	2.4	2.0	1.5
CCIBt3611 *Gloeothece sylvatlantis*	13.3	10.2	6.9	7.8	6.9	5.8	2.2	1.5	1.0
CCIBt3601 *Gloeothece interspersa*	9.1	7.4	5.3	6.1	5.2	4.1	1.8	1.4	1.0

***Totusmuciger* gen. nov.**

Colonies spherical to subspherical when young, after irregular, composed with cells densely to loosely aggregated, which are surrounded by a gelatinous mucilage. The sheaths can be conspicuous or inconspicuous, not lamellate. Cells cylindrical, oblong to elliptic, with color content varying from blue-green to greyish-green. Pseudofilaments may be present.

Type species: *Totusmuciger versatilis* sp. nov. (see below)

Etymology: *totus* (Latin, adj.A, entire) + *muciger* (Latin, adj.A, mucus-producing)

Totusmuciger versatilis sp. nov.

Cells cylindrical to oblong, 4.6-(6.9)-8.8µm length, 2.8–(4.2)–5.2µm diam., 1.2-(1.6)-2.3 × longer than larger, densely or loosely grouped in rounded colonies. Mucilage surrounding the colonies, inconspicuous to conspicuous (young colonies), hyaline, not lamellate. Cell content granulated, green-brownish. Pseudofilaments formed by 4-8 cells aligned in a chain.

Holotypus: exsiccate access number SP469765, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt3431.

Habitat: terrestrial, growing on rocks, soil and concrete.

Etymology: *versatilis* (Latin, Adj.B., versatile)

Totusmuciger solumi sp. nov.

Cells elliptical to rhomboid, 5.4-(8.0)-10.7 µm length, 3.9–(4.9)– 6.5 µm diam., 1.1-(1.6)-2.1 × longer than larger, densely or loosely grouped in rounded colonies. Mucilage surrounding the colonies, inconspicuous to conspicuous (young colonies), hyaline, not lamellate. Cell content granulated, dark green to olive-green. Pseudofilaments formed by 4-6 cells aligned in a chain.

Holotypus: exsiccate access number SP469768, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt3513

Habitat: terrestrial, growing on soil.

Etymology: *solum* (Latin, s.n.II, soil).

Totusmuciger evidentermarginater sp. nov.

Cells cylindrical to oblong, 7.7–(9.6)–12.5 μm length, 5.6–(6.8)– 8.1 μm diam., 1.1-(1.4)-1.8× longer than larger, densely or loosely grouped in rounded colonies. Mucilage surrounding the colonies, conspicuous, hyaline, not lamellate. Cell content granulated, dark green to yellow-green. Pseudofilaments not observed.

Holotypus: exsiccate access number SP469767, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt3613

Habitat: terrestrial, growing on rocks.

Etymology: *evidenter* (Latin, Adv., evident) + *marginatus* (Latin, Adj.A), margined).

Totusmuciger corticalis sp. nov.

Cells cylindrical to oblong, 7.1–(9.5)–11.8 μm length, 5.5–(6.8)–8.3 μm diam., 1.1-(1.4)-1.8× longer than larger, densely or loosely grouped in rounded to irregular colonies. Mucilage surrounding the colonies, inconspicuous to conspicuous, hyaline, not lamellate. Cell content granulated, purple to greyish-green. Pseudofilaments not observed.

Holotypus: exsiccate access number SP469766, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt3433

Habitat: terrestrial, growing tree bark.

Etymology: *corticalis* (Latin, Adj.B, bark).

Gloeothece sylvatlantis sp. nov.

Synonym: *Gloeothece fuscolutea sensu* Gama Jr. et al. (2014) and *Gloeothece palea sensu* Sant'Anna et al. (1991).

Cells cylindrical, 6.9–(10.3)–13.5 μm length, 4.3–(6.1)–7.8 μm diam., 1.0-(1.5)- 2.2 × longer than larger, 2-4-6 grouped in rounded colonies. Mucilage surrounding the colonies, conspicuous, hyaline, lamellate, 2-4 layers. Cell content granulated, yellowish-green.

Holotypus: exsiccate access number SP469769, Herbarium of Institute of Botany, São Paulo, Brazil

Type strain: CCIBt3534

Habitat: terrestrial, growing on rocks and concrete.

Etymology: *sylva* (Latin, s.f.I, forest) + *atlantis* (in reference to the Atlantic Ocean coast).

5. CONCLUSIONS

The genera *Aphanothece* and *Gloeothece* showed to be phylogenetic distinguishable, however *Aphanothece* is heterogenic in relation to its morphological definition. This was clearly revealed when we molecularly analyzed six strains morpho-related to it but isolated from different terrestrial environments. All these lineages formed a concise clade not phylogenetic so close to typical *Aphanothece* or other cyanobacterial genus, what fundaments their description as the new genus *Totusmuciger*. This new genus showed to be widespread on terrestrial habitats, what can be a recognizable feature to differentiate it from the genus *Aphanothece*, which shows to contain only the aquatic species. All *Totusmuciger* strains, recognizable in four new species, have in common the production of an abundant mucilage and they never show lamellate sheaths. This last feature, distinguish this new genus from its closest relative *Gloeothece*. Here we analyzed three different strains morphological identified as *Gloeothece* and they showed to form a very consistent clade together with other strains also identified as *Gloeothece*. So, we designed the group formed by them as the typical *Gloeothece* clade and proposed the new species *Gloeothece sylvatlantis* based on two strains isolated from the Atlantic Rainforest. Also, we first phylogenetic place the species *Gloeothece interspersa*.

The results shown here highlight the accentuated cyanobacterial diversity present on tropical terrestrial environments and how little we know about it. Also, they reinforce the phylogenetic divergence between aquatic and terrestrial cyanobacteria; the genera that possess species from both these environments, like *Aphanothece* and *Gloeothece*, are probably heterogenous and should be revised with this idea in mind. This divergence showed to be so high that species from these environments can be not together even at family level.

Capítulo V

Pleurocapsales

BAEOCYTES, NANOCYTES AND THEIR RELATION WITH THE MONOPHYLY OF PLEUROCAPSALES (CYANOBACTERIA)

1. INTRODUCTION

Multiple fission is a cyanobacterial cell division defined as a rapid succession of many fission rounds that occurs in an enlarged multi-nucleoid spherical cell (Angert, 2005). This process results in diminutive cells, which are released by the rupture of the mother cell and will grow to their former size to restart the cycle. The products of multiple fission have been called by different words, as endospores (Gardner, 1927), baeocyte (Waterbury & Stanier, 1978) and nanocytes (Komárek & Anagnostidis, 1986). Endospore is a bacteriological term, which represents a specialized dormant cell highly resistant to agents that would normally harm a vegetative cell (Angert, 2005). This is not the case of the small cells formed by multiple fission in cyanobacteria and to differentiate them from bacterial endospores, Waterbury & Stanier (1978) created the term baeocyte (Greek, small cells). Nanocytes also refers to small cells produced by cyanobacteria and the term is widely used in the botanic literature (Komárek & Anagnostidis, 1998). However, differently from baeocytes, it is more often applied in reference to the small cells formed by Chroococcaceae family representatives (*sensu* Komárek & Anagnostidis, 1998). Even so, this term has never been well-defined and some authors considered it as a synonym of baeocytes (Komárek & Anagnostidis, 1986) and others not (Waterbury & Stanier, 1978).

The order Pleurocapsales traditionally englobed all cyanobacteria which divide by multiple fission (Komárek & Anagnostidis, 1986). Very widely used in the Botanical classification of cyanobacteria (Desikachary, 1959; Starmach, 1966; Bourrelly, 1970, 1985), this order felt in disuse when Komárek & Anagnostidis suggested the grouping of coccoid types into a unique order, Chroococcales, differentiating them in families (Komárek & Anagnostidis, 1988, Komárek & Anagnostidis, 1998). Following this classification system, the representatives of Pleurocapsales order were split into the families Dermocarpellaceae, Xenococcaceae and Hyellaceae and the main features to distinguish them were based on morphology. On the order hand, in the Bergey's Manual all cyanobacteria which cells show multiple fission are grouped in the Section II (Rippka et al., 2001) and they are referred as pleurocapsalean cyanobacteria. Waterbury & Stanier (1978) pointed out another common thing to these

cyanobacteria: the presence of a fibrous layer (F-layer) covering the cells, which is closely appressed to the external surface of outer membrane layer (OM-layer).

As we can see, the pleurocapsalean cyanobacteria have enough morphological features that could group them in a monophyletic clade. However, different authors demonstrated the opposite. Ishida et al. (2001) showed the close relationship between them and oscillatorean cyanobacteria, revealing that the Pleurocapsales is polyphyletic by proving the polyphyly of the genera *Pleurocapsa* Thuret and *Chroococcidiopsis* Geitler. Fewer et al. (2002) also reinforce the heterogeneity of *Chroococcidiopsis* by showing that the group formed by its type species, *Chroococcidiopsis thermalis* Geitler (PCC7302), is closer to Nostocales clade than to typical coccoid types (like Chroococcaceae members). In the proposal of the classification system and based on genomic approach, Komárek et al. (2014) created the order Chroococcidiopsidales to classify this clade, and reborn the order Pleurocapsales, where is kept the majority of multiple fission cyanobacteria. However, it is frequently seen that *Chroococcidiopsis* sequences are overspread in the phylogenetic tree and grouped in different clades when they are compared by 16S rDNA (Cumbers & Rothschild, 2014).

Here, we should highlight that part of these *Chroococcidiopsis* strains are probably misidentified, what is a very common situation in culturable cyanobacteria, since they can change their morphology under culture conditions (Komárková et al., 2010; Singh & Montgomery, 2011). Also, lineages that produce dense cubic (sarcinoid) colonies are conventionally designated as *Chroococcidiopsis* (Cumbers & Rothschild, 2014). However, genera like *Gloeocapsopsis*, *Cyanosarcina* Kováčik and *Pseudocapsa* also have this type of colonies (Komárek & Anagnostidis, 1998) and, except for *Gloeocapsopsis* Geitler ex Komárek, there are no sequences available for these genera. Thus, the probability that some of *Chroococcidiopsis*-like strains are one of these Chroococcaceae genera, or even something else, is high.

Independently of the classification system adopted, or the sense of the genera used, pleurocapsalean cyanobacteria showed to be very widespread. Mostly found in terrestrial environments, these organisms grown over rocks and soils, including those extreme habitats where they play fundamental roles as primary producers (Bahl et al., 2011). This can explain the difficulties in find the best conditions to cultivate them, what resulted in lacks about their knowledge from many parts of the world. Thus, we intend to discuss the formation of baeocytes and nanocytes and figure out the phylogenetic relationship of multiple fission

strains isolated from the Atlantic Rainforest, a tropical hotspot for biodiversity conservation (Myers et al., 2000).

2. MATERIAL AND METHODS

All lineages were isolated in liquid medium following standard techniques (Jacinavicius et al. 2012) from samples collected on terrestrial habitats in the Atlantic Rainforest (Table 13). They are all stored in CCIBt (Culture Collection of Institute of Botany) under controlled conditions, as following: temperature of 23±1°C, irradiance of 40-50 µmol photons $m^{-2}.s^{-1}$ (provided by daylight fluorescent lamps and measured with a Li-COR quanta meter sensor) and photoperiod of 14–10h light-dark cycle. An aliquot of each lineage was preserved in formaldehyde 4% and deposited in the Herbarium of the Institute of Botany (SP), Brazil.

Table 13. List of studied lineages with details of their culture medium, habitat, and Herbarium access number.

Strain	Medium	Locality	Substrate	Exsiccate number
CCIBt3407	ASM-1	Santa Virgínia Park, São Paulo State	Concrete	SP469749
CCIBt3408	BG11	Santa Virgínia Park, São Paulo State	Concrete covered with vegetation	SP427348
CCIBt3416	ASM-1	Santa Virgínia Park, São Paulo State	Concrete covered with vegetation	SP427303
CCIBt3419	ASM-1	Santa Virgínia Park, São Paulo State	Concrete	SP469750
CCIBt3420	BG11	Santa Virgínia Park, São Paulo State	Concrete	SP427340
CCIBt3421	BG11	Santa Virgínia Park, São Paulo State	Concrete	SP469751
CCIBt3428	ASM-1	Cardoso Island Park, São Paulo State	Concrete	SP469752
CCIBt3494	ASM-1	Jureia Ecological Station, São Paulo State	Wet Rock	SP469753

The morphology of strains was evaluated under a Zeiss Axioplan 2 optical microscope and the identification and cell measurements were based on at least 50 individuals. To confirm the differences among species, the significance of cell dimensions was tested by ANOVA-one way using GraphPad Prism 6, with Tukey as posteriori test and $\alpha = 0.05$. The construction of life cycle was made by cells observation at five days after culture replication, and then at 30, and 120 days after. The organisms were documented by digital photographs taken with a Zeiss Axiocam MRc digital camera. The main morphological features observed were: type of cell division, including the direction of the fission, cells shape and diameter (including nanocytes and baeocytes), structure of sheaths and color and uniformity of cell content.

Cellular ultrastructure was studied from cultures by transmission electron microcopy. The cells were centrifuged for concentration and the medium was eliminated. After, each sample was fixed with Karnovsky plus sucrose 0.2M solution for 24h, washed three times with 0.05M cacodylate buffer and post-fixed with 1% osmium tetroxide. After that, samples were overnight submitted to contrasting in uranyl acetate 5% and dehydrated in an acetone series of increasing concentration and infiltrated in Spurr resin. The blocks were cut on an ultramicrotome (Leica Ultracut UCT) with a diamond blade (450) into 70 nm thick sections and mounted on uncoated 200-mesh copper grids, and stained with uranyl acetate and lead citrate (Reynolds 1963). Visualization and microphotography were performed on a Jeol Jem 1011 transmission electron microscope (JEOL, Tokyo, Japan) at 100 kVA.

Total genomic DNA was isolated from a liquid culture of the cyanobacterial strains using the Ultra Clean Soil DNA Isolation Kit (MOBIO). The partial 16S-ITS-23S rRNA sequence was amplified by PCR using the 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, 200µm of each dNTP, 3.0 mM $MgCl_2$, 1 × PCR buffer and 1.0 U Platinium *Taq* DNA polymerase (Life Techonologies, Grand Island, NY, USA) under conditions according to Taton et al. (2003). The resulting PCR product was cloned into a pGEM®-T Easy Vector System (Promega, Madison, WI, USA) and inserted into chemiocompetent *E. coli* DHG5α cells by a heat-shock method (Sambrook et al., 1989). After growth in LB plates containing 5 µl X-Gal (50 $\mu g.mL^{-1}$) (Life Techonologies) and 10 µl of ampicillin sodium salt (100 $\mu l.\ mL^{-1}$) (Sigma-Aldrich Co., St. Louis, MO, USA), recombinant plasmids were purified from white colonies by the alkaline lysis method (Birnboim & Doly, 1979). The cloned gene fragment was sequenced using the 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 the sample was placed in an ABI PRISM 3500 Genetic Analyzer (Life Technologies). The sequenced fragments were assembled with the Phred/Phrap/Consed software package (Ewing & Green, 1998; Ewing et al., 1998; Gordon et al., 1998).

The phylogenetic analyses were based on 16S rRNA gene and 16S-23S internal transcribed spacer (ITS) sequences obtained in this study and reference sequences retrieved from GenBank. The General Time Reversible (GTR) evolutionary model of substitution with Gamma distribution (G) and with an estimate of proportion of invariable (I) sites was selected as the best fit for the 16S rDNA alignment and Hasegawa, Kishino and Yano (HKY) model + G +I for 16S-23S ITS using jModelTest (Posada, 2008). Evolutionary analyses based on the Neighbor Joining and Maximum Likelihood methods were conducted in Mega 6.0 (Tamura et al., 2013) and estimations of the robustness of the tree were tested by bootstrap with 1,000 replications. The Bayesian analysis was inferred by MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2005) in 5×10^6 generations. Chains were sampled every 100^{th} 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 using p-distance method in Mega 6.0 (Tamura et al., 2013) and the percentage was given by the formula *[1–(p-distance)]×100*. The mean similarity was estimated by the average of similarity value of each sequence from the two compared clades.

3. RESULTS AND DISCUSSION

A total of eight strains were isolated from different areas and terrestrial substrates of the Atlantic Rainforest. Among them, four phylogenetically grouped with *Chroococcidiopsis thermalis* (PCC7203), and are representatives of Chroococcidiopsidales, and the other four were similar to strains from the order Pleurocapsales (orders adopted according to Komárek et al., 2014). Analyzing they morphology, we found circumstantial differences in the process of cell division of these strains, mainly regarding those grouped with *C. thermalis*. Also, the morphology of pleurocapsalean strains showed to be very plastic and different from their phylogenetic relatives. The cells dimensions of all analyzed strains are in the Table 14.

Table 14. Cells dimensions (μm) of analyzed strains. Max.= maximum value; Avg.= average value; Min.= minimum value.

Strains	Cells diameter (μm)		
	Max.	Avg.	Min.
CCIBt3419 *Chroococcidiopsis* sp.	4.9	4.0	3.1
CCIBt3407 *Chroococcidiopsis* sp.	4.5	3.4	2.3
CCIBt3421 *Chroococcidiopsis* sp.	8.1	4.7	2.9
CCIBt3428 *Chroococcidiopsis* sp.	5.1	4.3	3.5
CCIBt3416 *Stanieria cyanosphaera*	14.9	11.1	7.7
CCIBt3494 *Stanieria cyanosphaera*	18.5	11.2	5.3
CCIBt3408 *Pleurocapsa* sp.	15.1	9.9	5.9
CCIBt3420 *Pleurocapsa* sp.	12.4	8.7	6.7

3.1. Baeocytes *vs.* Nanocytes

As said before, these terms were found to be very close related but here we suggest they denominate two distinct things. They both designate small cells originated from a bigger mother cell, but we observed that the processes in which they are formed are unrelated. Basically, multiple fission originates baeocytes and successive fission, nanocytes. Kunkel (1984) showed the process of baeocytes formation and it is possible to see that multiple fission is not a series of repetitive binary fissions, but a multiple partitioning of the protoplast. In this process, firstly the cells have their nuclear regions condensed and segregated; usually you can recognize more than three regions and they are together with a carboxysome grain (Waterbury & Stanier, 1978). After this, radial ingrowth of cytoplasmic membrane and cell wall created a septum and tangential ingrowth and constriction begins to occur in different plans, resulting in the formation of many baeocytes (Kunkel, 1984). This process occurs very fast and baeocytes are all produced at once, having a spherical or sub-spherical cell shape and similar dimensions (Pinevich et al., 2008). The F-layer, the most external cells envelope, does not grow around baeocytes and only starts being formed after they are released by mechanical rupture of the mother cell wall (Waterbury & Stanier, 1978). Moreover, the baeocytes are known to be motile and have cell composition different from the mother cell (Pinevich et al., 2008); although Waterbury & Stanier (1978) showed that only baeocytes not F-layer surrounded are able to have movement.

This process is well described to strains that phylogenetically group in the Pleurocapsales order, like PCC7301, PCC7302, PCC7305, PCC6712, PCC7437 and CALU1126 (Waterbury & Stanier, 1978, Pinevich et al., 2008). However, our strains related to *Chroococcidiopsis*

thermalis showed to have a different process of division. Based on our material, it is possible to see the all *Chroococcidiopsis thermalis* related strains have F-layer around the daughter cells while they still inside of the mother cell wall (Figure 26). Besides, we can recognize the different steps of cell division many times, both by electronic and light microscopy pictures, what is not common to see in baeocytes formation, since they are very quickly formed. The cells also show only two (rarely three) nuclear regions (Figure 26), not more than this, what is an indicative that the full offspring of these lineages are not formed by a unique cell division event. Instead, it is formed by a serial chain of cell divisions occurring in different plans/directions in the cells, in which the daughter cells do not grow to their original size before the next division. Also, they produce their own F-layer after each step of fission, what is an indicative that the division do not occur at once, but one after the other. The F-layer has a fibrous and rigid composition (Waterbury & Stanier, 1958), what compresses all the daughter cells into a tiny space and shapes them irregularly; usually gives a sarcinoid aspect to the colony too (Figure 26). At the end of this process, the daughter cells have a polygonal or irregular shape (Figure 27) and do not show any kind of movement after their liberation. This type of cell division is described in details by Kováčik (1988) to strains from the genus *Cyanosarcina* and *Pseudocapsa* Ercegovic, Chroococcaceae member's family. In these genera, the cells go through a successive series of division in different directions, and the cells do not grow to their original size before to the next fission, what results in smaller cells at each step: the nanocytes. This was exactly what we observed in *C. thermalis* related strains. So, we consider all these features to differentiate baeocytes from nanocytes, defining these terms as:

- Nanocytes: small polygonal or irregular cells formed by a chain of successive fissions that occur in different plans/directions in the cell. The daughter cells do no return to their original size before the next division, what results in smaller cells at each step of fission. The cells produce by their own F-layer (an external rigid layer around the cells) before each division; nanocytes do not show movement.
- Baeocytes: small spherical to sub-spherical cells formed by multiple fission event which occurs at once in the cell. The daughter cells are not surrounded by F-layer after the division process and start forming it after being released by mechanical rupture of mother cell wall; baeocytes shown movement as soon as they are released.

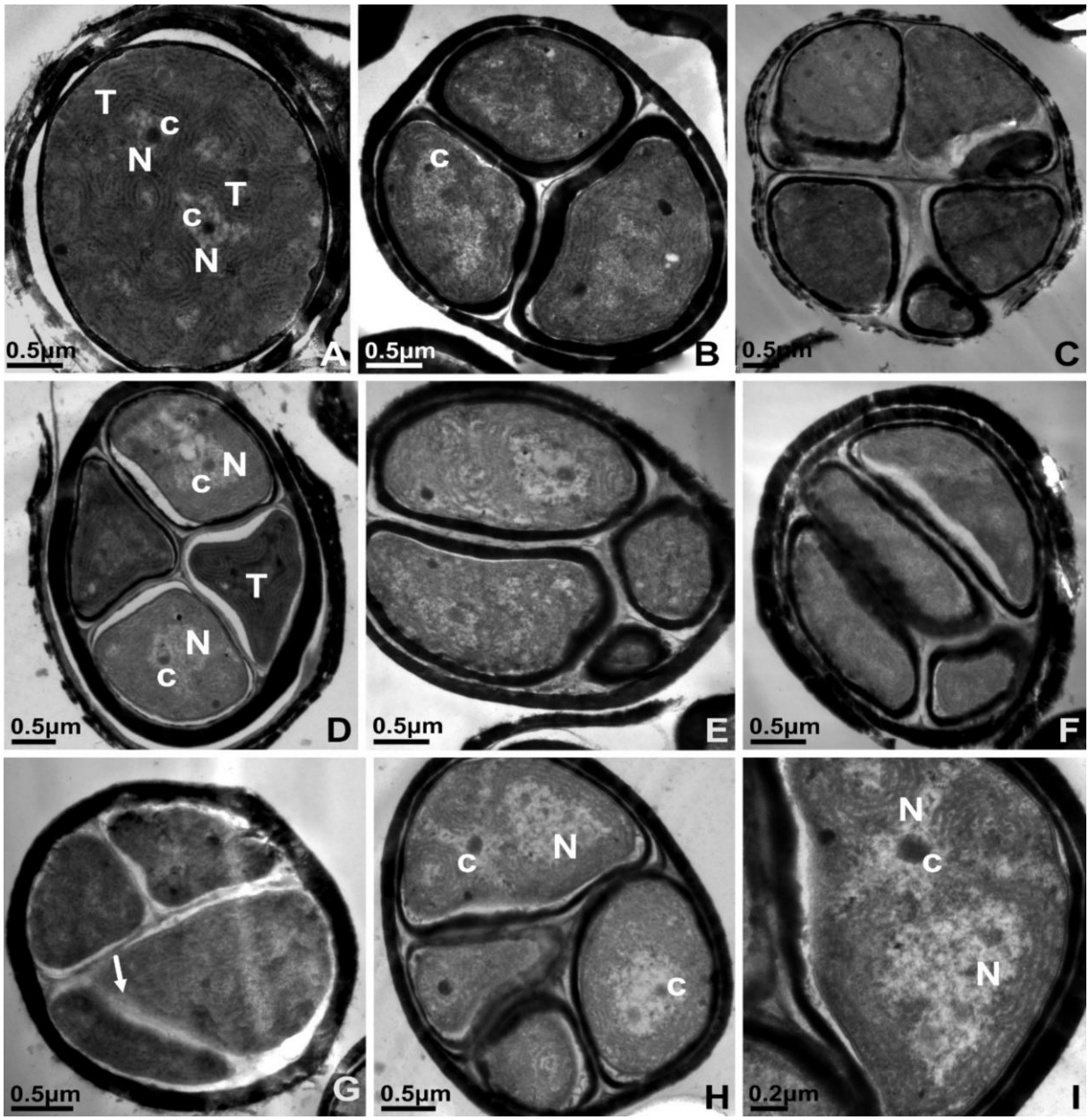


Figure 26: Transmission Electron Microscopy of CCIBt3421, Chroococcidiopsis sp.; A: cell with two nuclear regions; B-D: cells in different stages of division and in all the daughter cells are surrounded by F-layer; F-H: irregular cells formed by division in different cells and compression caused by F-layer surrounding; G: shows the irregular plan of cell division (arrow); I: detail of cell nuclear region associated with a carboxysome grain. T=thylakoids; C=carboxysome grain; N=nuclear region.

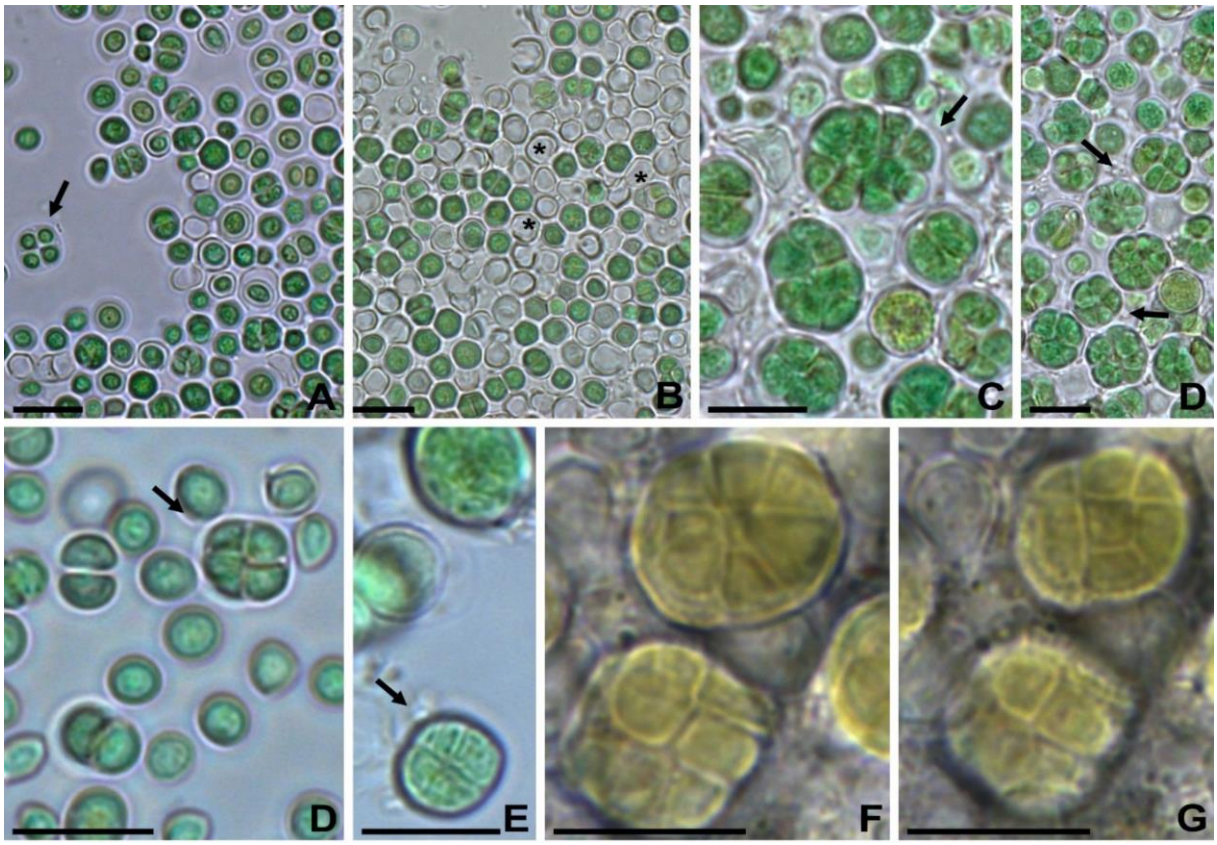


Figure 27: *Chroococcidiopsis* sp. A: CCIBt3407, general aspect of colonies disposition and detail of tetrad colony (arrow); B: CCIBt3419, showing empty sheaths after nanocytes releasing (asterisks); C-D: CCIBt3421, colonies at different stages of nanocytes development. Arrow highlight the directions of divisions; D-G: CCIBt3428, colonies in different stages of division and nanocytes a serial fission in different plans (arrows; F-G). Scale bars: 10 µm.

Based on this, *C. thermalis* and its relatives do not produce baeocytes, like the true pleurocapsalean cyanobacteria, but nanocytes. However, the opposite is not true. Among the pleurocapsalean we can recognize the successive chain of cell division too (Figure 28 and Figure 29), mainly in the genus *Myxosarcina* H.Printz and *Xenococcus* Thuret. This was also described to the species *Chroococcidiopsis kashaii* Friedmann (1961) and this author classified its life cycle in accordance to the number of generations of cells division. When the cells go straightly to the production of smaller cells, in one event, they went through multiple fission and result in true baeocytes (scheme A, *sensu* Friedmann, 1961). However, the cells can also go through a division process of variable and sequential generations, like one generation (scheme B, *sensu* Friedmann, 1961), two (scheme C) or three (scheme D) (Figure 30). In the process described in B, C and D the resulting cells are called 'pseudoendospore' by Friedmann (1961), but as we see they are actually nanocytes. This was also observed to the strain PCC7307 (*Xenococcus*) by Waterbury & Stanier (1978) and in our strains CCIBt3416

and CCIBt3494, confirming that nanocytes production is a possibility to true pleurocapsalean cyanobacteria.

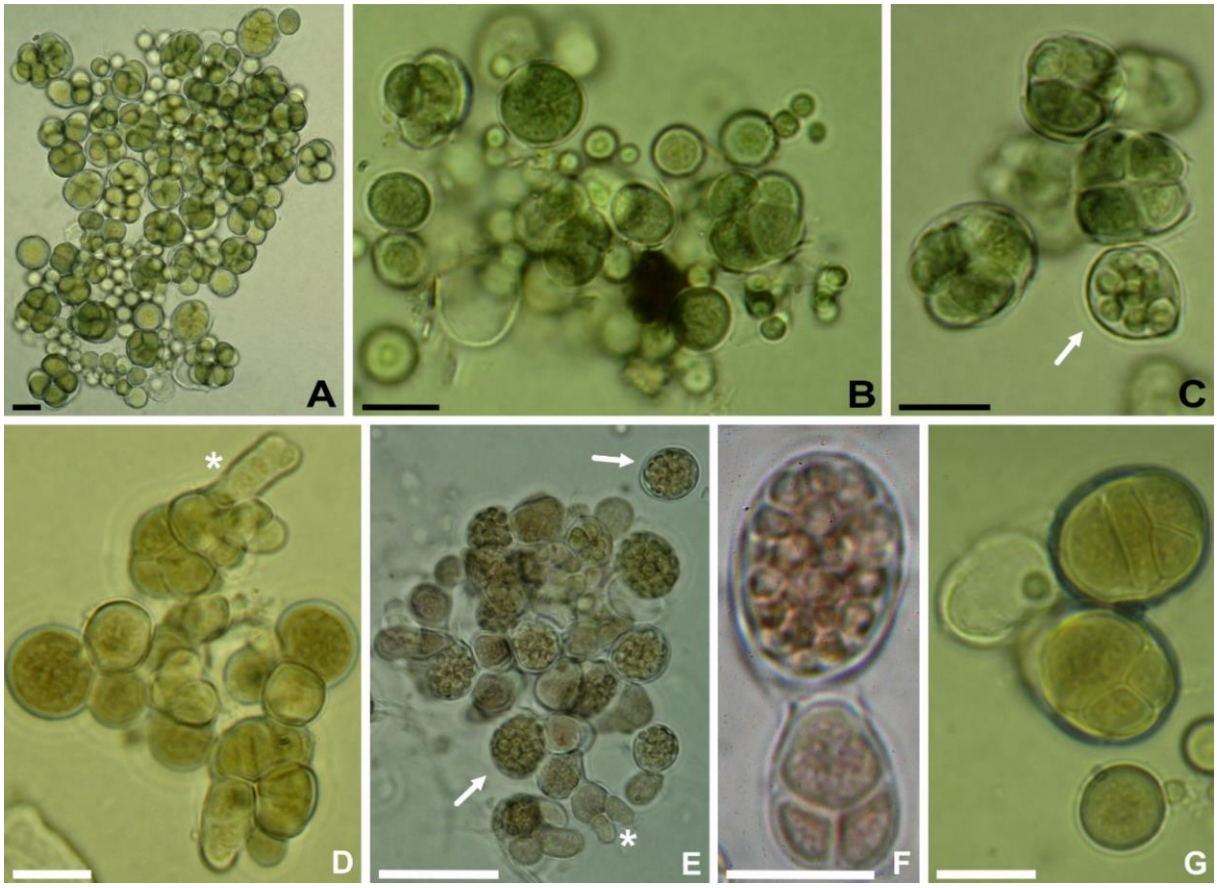


Figure 28: A-C: CCIBt3408 *Pleurocapsa* sp.; A: general arrangement of colonies; B-D: colonies in baeocytes production and baeocytes release (arrow); D-G: CCIBt3420 *Pleurocapsa* sp.; D-E: cells aggregated and pseudofilament (asterisk) and baeocyte (arrow) formation; F-G: cells division pattern typical of pseudofilaments and baeocyte of group I (Waterbury & Stanier, 1978). Scale bars: 10 µm.

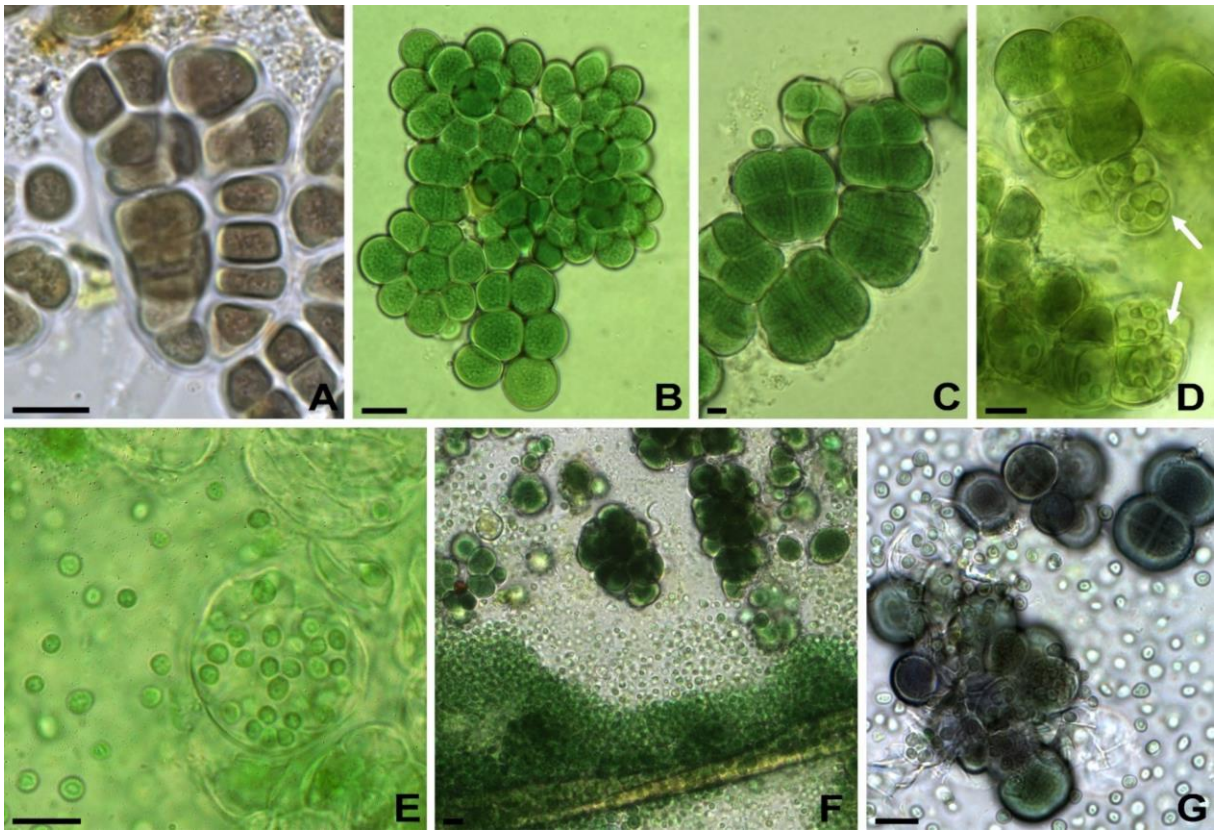


Figure 29: A: CCIBt3549 *Chroococcus* sp., anomaly colony with resemblance to the pseudofilaments observed in the genus *Pleurocapsa*; B-E: CCIBt3416 *Stanieria cyanosphaera*; B-C: general aspect of solitary cells (B) and sarcinoid colonies (C); D-F: baeocytes formation and liberation by the rupture of the mother cell; F: baeocytes agglomeration in a sterilized cotton yarn; G: CCIBt3494 *Stanieria cyanosphaera*, colonies releasing their baeocytes. Scale bars: 10 µm.

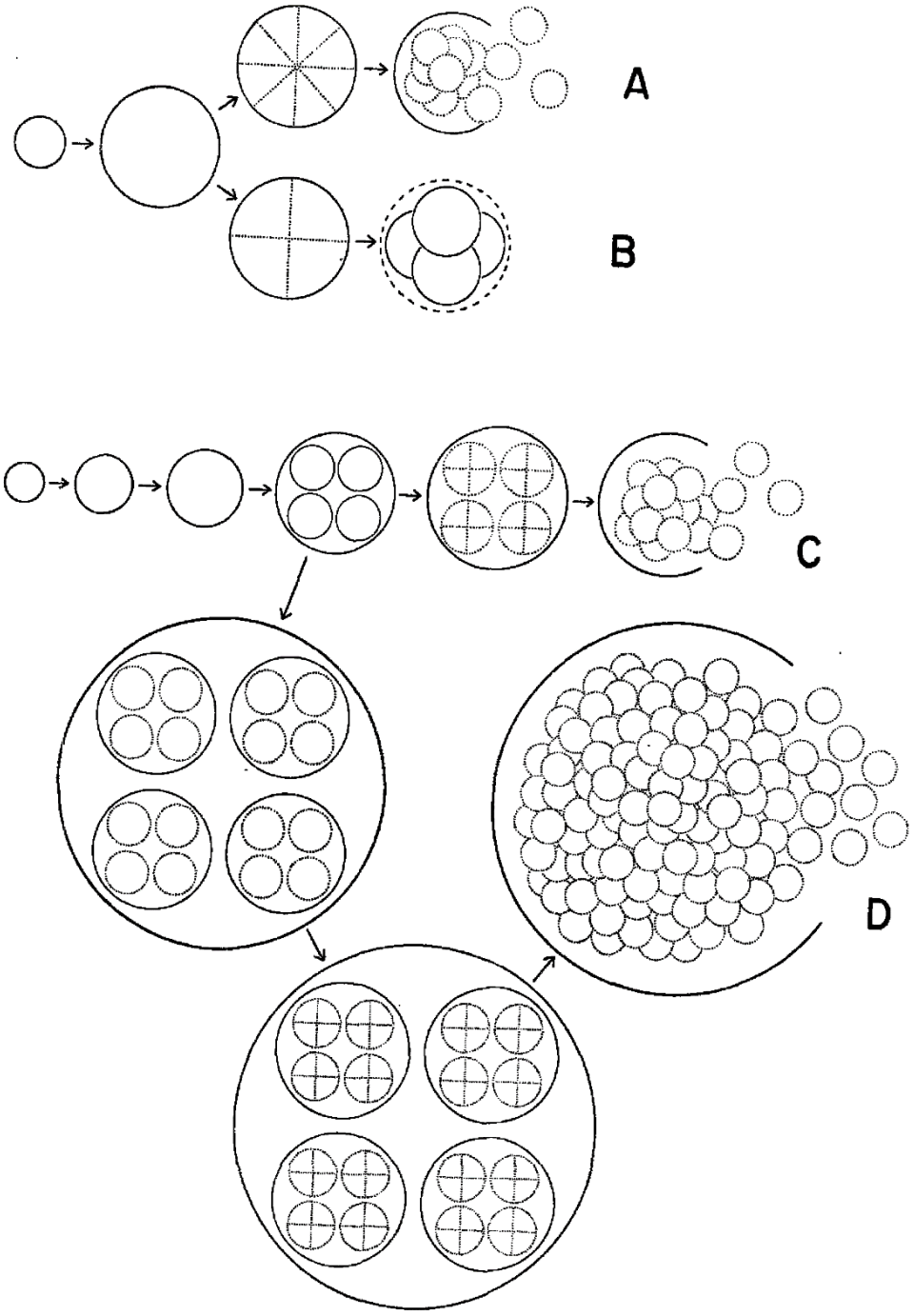


Figure 30: Cell division process described to *Chroococcidiopsis kashaii*. A: represent the baeocytes formation. B, C and D are nanocytes production by one, two or three generations, respectively. Originally schematized by Friedmann (1961).

3.2. *Chroococcidiopsis thermalis* clade

As we already known, *Chroococcidiopsis thermalis* and its phylogenetic relatives produce nanocytes. Once *C. thermalis* is the type species of the genus, we can assume that the genus *Chroococcidiopsis* is characterized by the production of nanocytes, what makes necessary the

change of its description and understanding. Moreover, this feature turns it morphologically close to the genus *Cyanosarcina* and *Pseudocapsa*. Actually, taking into account only the morphology, it is not possible to differentiate *Chroococcidiopsis*, in this new sense, from these genera, mainly from *Cyanosarcina*. However, as no *Cyanosarcina*, neither *Pseudocapsa*, sequences are available and these genera have not been phylogenetic characterized, we cannot confirm their true relationship with the genus *Chroococcidiopsis*, once the nanocytes formation showed to be a character common to different cyanobacterial clades.

Analyzing the group of *C. thermalis* and the relationship among its representatives, we see that they are very similar by 16S rDNA sequences analyzes. Their similarity range is 97-100%, what could be enough to consider all them as the same species. However, the 16S-23S ITS analyzes reveal the establishment of three different clades: one is formed by the four strains isolated from the Atlantic Rainforest, with 100% of similarity among them. The second clade has *C. thermalis* PCC7302 only and it has 91.1% of ITS similarity with the Atlantic Rainforest strains (Figure 32 and Table 16). The third clade is composed by *C. muralis* and *Chroococcidiopsis* sp. SAG 2025 (Figure 31). Even the studied strains represent a different species from *C. thermalis*, what could be plausible due the difference of habitat (Table 13), and from the clade formed by *C. muralis* (Lagerheim) Miscoe & J.R.Johansen, we cannot affirm this based only on 16S rDNA and 16S-23S ITS sequences, once they are all highly similar by these markers. Thus, we prefer to keep all strains from the Atlantic Rainforest identified at generic level, *Chroococcidiopsis*, and we highlight that they belong to same species.

3.3. Pleurocapsales representatives

About the other four studied strains, we can separate them in pairs based on phylogenetic affinity. CCIBt3494 and 3416 are both morphologically and phylogenetic very close. Looking at the morphology only, it would be possible to affirm that these lineages belong to the genus *Myxosarcina*, once they fit in all features described to this genus. Producing typical sarcinoid colonies, these strains have cell division both by multiple and successive fissions (Figure 29), and their baeocytes are motile. However, the phylogenetic analyzes of the 16S rDNA revealed a high similarity between these strains and the type of the genus *Stanieria* Komárek & Anagnotidis, a genus known for its cells to have multiple fission sole. The similarity of 16S rDNA among them ranges from 99.3% to 100%, and that regarding to ITS is 95.9-99.0%

(Table 15 and Table 16). Looking to these high values, it is conclusive that the strains isolated from the Atlantic Rainforest and *Stanieria cyanosphaera* (Komárek & Hindák) Komárek & Anagnostidis (PCC7437=Hindák 1965/25 – the type strain of the genus; (Rippka et al., 1979) belong to the same genus and species. Thus, we conclude that the genus *Stanieria* is not well defined morphologically and should be better characterized based on its representatives variability.

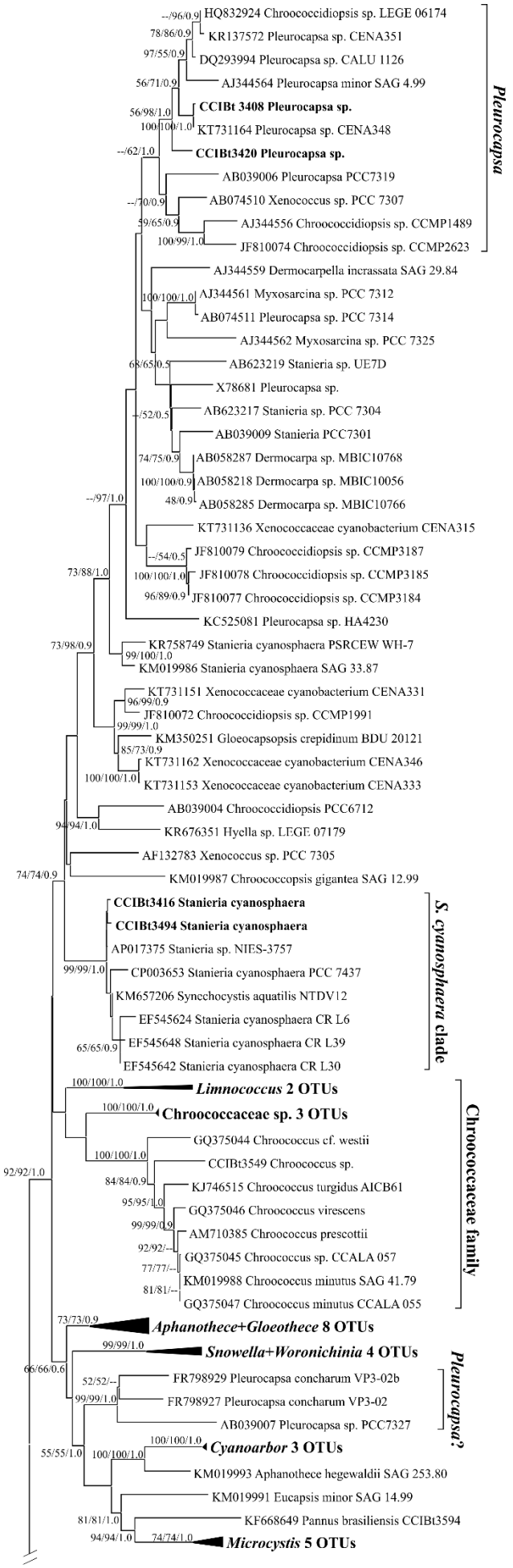
The second pair of strains is formed by CCIBt3408 and 3420. Between themselves there is a 16S rDNA similarity of 98.1%. The ITS sequences are not available for these strains. However, we can affirm based on 16S rDNA that CCIBt3408 and 3420 belong to the same genus. These lineages grouped with sequences identified as *Pleurocapsa* and *Chroococcidiopsis*, what already predicts that their clade shows heterogeneity. In dead, analyzing the morphology of strains we found that CCIBt3420 has cell division very similar to *Pleurocapsa*, in accordance to the life cycle I described by Waterbury and Stanier (1978). On the other hand, CCIBt3408 never showed any kind of pseudofilaments and has the morphology in agreement with *Chroococcidiopsis*, in its old circumscription. This strain has also 98.6% of 16S rDNA similarity with the lineage CALU1126, which is identified as *Pleurocapsa* sp., but Pinevich et al. (2008) analyzed it and confirmed that this lineage does not produce pseudofilaments and it is also very morpho-similar to *Chroococcidiopsis*. However, we observed the growing of CCIBt3420 at different times and pseudofilaments appeared only when it was growing on liquid medium (pseudofilaments were never observed in plates) and they were produced sole during the firsts months after the isolation of the strain. Two years past it, pseudofilaments were not observed anymore. This raises the idea that pseudofilaments are cultured conditions dependent and even we never observed them in CCIBt3408, it is not ruled out its capacity of pseudofilaments production.

The strain PCC7319 is one which Waterbury and Stanier (1978) based on to describe the life cycle of *Pleurocapsa* type I and PCC7516, to group II. These strains have 96.6% and 98.4/98.1% of 16S rDNA similarity with CCIBt 3408 and 3420 (Table 15), respectively, what is considered enough to group them in the same genus. So, based on these similarities and on the idea that pseudofilaments are culture conditions dependent, we proposed that all these strains should belong to same genus: *Pleurocapsa*. We also propose this clade to be considered the true *Pleurocapsa*, since it has the *Pleurocapsa* group I and II representatives (in accordance to Waterbury & Stanier,1978).

3.4. The monophyly of Pleurocapsales

The monophyly of the order Pleurocapsales was questioned mainly due to the consistent clade formed by *Chroococcidiopsis thermalis* (Fewer et al., 2002). However, as we confirm that these species and its close relatives do not produce baeocytes, but nanocytes, the idea of monophyly of multiple fission cyanobacteria is raised again. This is also reinforced by the well supported clade formed by the pleurocapsalean, with high 16S rDNA similarity showed by its representatives, which ranges from 92% to 99.9% (average of 94.5%). These high values are similar to those found among Nostocales, which are, up to now, the only monophyletic big group among the cyanobacteria (Rajaniemi et al., 2005; Howard-Azzeh et al., 2014; Johansen et al., 2014). These evidences highlight the possibility of, but still not confirm, the Pleurocapsales monophyly.

There are some strains groups identified as pleurocapsalean members out of the Pleurocapsales clade. The most prominent is formed by the strain PCC7327, identified as *Pleurocapsa* sp. This strains was studied by Waterbury and Stanier (1978) and Kunkel (1984), but these authors do not show pictures or commentaries regarding the baeocytes production by this strain, so we cannot confirm with they are baeocytes or not. However, these authors confirm the differentiation of PCC7327 cells into pseudofilaments. As we have seen, pseudofilaments can be culture conditions dependent and many phylogenetic unrelated cyanobacteria showed to produce them (Komárek & Anagnostidis, 1998). Also, we highlight the case of strain CCIBt3549, which is phylogenetically related to *Chroococcus* Nägeli, but its culture presented abnormality cells that could be misunderstood as *Pleurocapsa* pseudofilaments (Figure 28). This illustrates the vast and complex field that is the morphological plasticity of cyanobacteria, and lights an alert to the importance of correct taxonomic identification of strains based on an accurate study about their morphological variation. As the most of strains deposited in GenBank does not have this kind of taxonomic support, we suggest a revision of the pleurocapsalean strains to truly defined its monophyly. Moreover, we point out the needed of detailed characterization of cell division in face to recognize and differentiate baeocytes from nanocytes.


HQ832924 Chroococcidiopsis sp. LEGE 06174
KR137572 Pleurocapsa sp. CENA351
DQ293994 Pleurocapsa sp. CALU 1126
AJ344564 Pleurocapsa minor SAG 4.99
CCIBt 3408 Pleurocapsa sp.
KT731164 Pleurocapsa sp. CENA348
CCIBt3420 Pleurocapsa sp.
AB039006 Pleurocapsa PCC7319
AB074510 Xenococcus sp. PCC 7307
AJ344556 Chroococcidiopsis sp. CCMP1489
JF810074 Chroococcidiopsis sp. CCMP2623
Pleurocapsa
AJ344559 Dermocarpella incrassata SAG 29.84
AJ344561 Myxosarcina sp. PCC 7312
AB074511 Pleurocapsa sp. PCC 7314
AJ344562 Myxosarcina sp. PCC 7325
AB623219 Stanieria sp. UE7D
X78681 Pleurocapsa sp.
AB623217 Stanieria sp. PCC 7304
AB039009 Stanieria PCC7301
AB058287 Dermocarpa sp. MBIC10768
AB058218 Dermocarpa sp. MBIC10056
AB058285 Dermocarpa sp. MBIC10766
KT731136 Xenococcaceae cyanobacterium CENA315
JF810079 Chroococcidiopsis sp. CCMP3187
JF810078 Chroococcidiopsis sp. CCMP3185
JF810077 Chroococcidiopsis sp. CCMP3184
KC525081 Pleurocapsa sp. HA4230
KR758749 Stanieria cyanosphaera PSRCEW WH-7
KM019986 Stanieria cyanosphaera SAG 33.87
KT731151 Xenococcaceae cyanobacterium CENA331
JF810072 Chroococcidiopsis sp. CCMP1991
KM350251 Gloeocapsopsis crepidinum BDU 20121
KT731162 Xenococcaceae cyanobacterium CENA346
KT731153 Xenococcaceae cyanobacterium CENA333
AB039004 Chroococcidiopsis PCC6712
KR676351 Hyella sp. LEGE 07179
AF132783 Xenococcus sp. PCC 7305
KM019987 Chroococcopsis gigantea SAG 12.99
CCIBt3416 Stanieria cyanosphaera
CCIBt3494 Stanieria cyanosphaera
AP017375 Stanieria sp. NIES-3757
CP003653 Stanieria cyanosphaera PCC 7437
KM657206 Synechocystis aquatilis NTDV12
EF545624 Stanieria cyanosphaera CR L6
EF545648 Stanieria cyanosphaera CR L39
EF545642 Stanieria cyanosphaera CR L30
S. cyanosphaera clade
Limnococcus 2 OTUs
Chroococcaceae sp. 3 OTUs
GQ375044 Chroococcus cf. westii
CCIBt3549 Chroococcus sp.
KJ746515 Chroococcus turgidus AICB61
GQ375046 Chroococcus virescens
AM710385 Chroococcus prescottii
GQ375045 Chroococcus sp. CCALA 057
KM019988 Chroococcus minutus SAG 41.79
GQ375047 Chroococcus minutus CCALA 055
Chroococcaceae family
Aphanothece+Gloeothece 8 OTUs
Snowella+Woronichinia 4 OTUs
FR798929 Pleurocapsa concharum VP3-02b
FR798927 Pleurocapsa concharum VP3-02
AB039007 Pleurocapsa sp. PCC7327
Pleurocapsa?
Cyanoarbor 3 OTUs
KM019993 Aphanothece hegewaldii SAG 253.80
KM019991 Eucapsis minor SAG 14.99
KF668649 Pannus brasiliensis CCIBt3594
Microcystis 5 OTUs

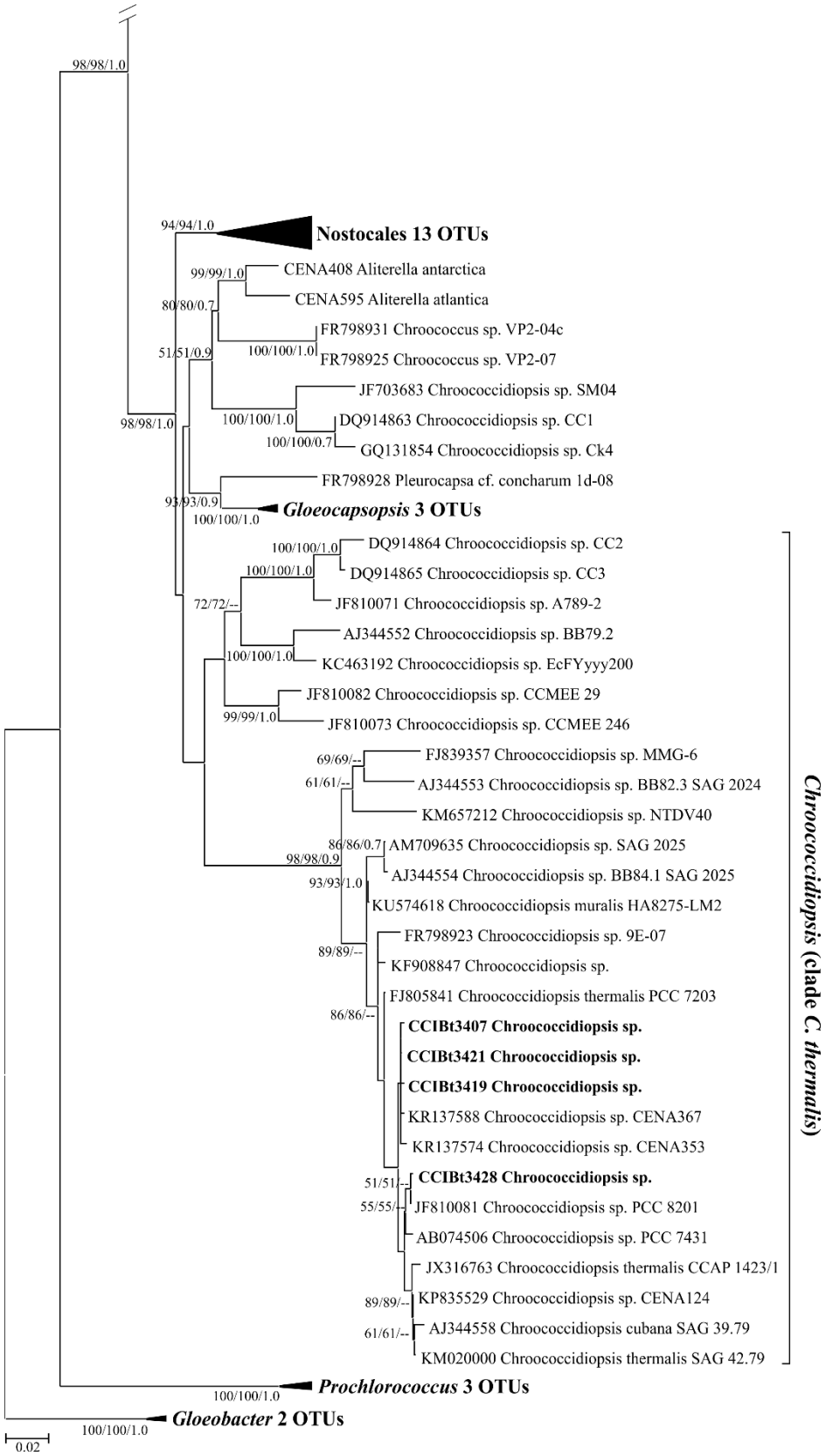


Figure 31: Phylogenetic tree based on 16S rRNA gene sequences and reconstructed using the maximum-likelihood (ML) analysis of evolutionary distances determined by the GTR + G + I model. NJ and ML bootstrap (1,000×) values (above 50 %) and Bayesian posterior probabilities are provided for each node, respectively. Sequences determined in this work are indicated in bold and new taxa are underlined.

Table 15. 16S rDNA similarity matrix of studied lineages and their close relatives, conducted using the p-distance model with a total of 1,066 positions.

Sequences identification	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 CCIBt3407 *Chroococcidiopsis* sp.																					
2 CCIBt3416 *Stanieria cyanosphaera*	90.1																				
3 CCIBt3419 *Chroococcidiopsis* sp.	99.8	90.1																			
4 CCIBt3420 *Pleurocapsa* sp.	88.5	92.6	88.5																		
5 CCIBt3421 *Chroococcidiopsis* sp.	99.8	90.1	99.8	88.5																	
6 CCIBt3428 *Chroococcidiopsis* sp.	99.9	90.2	99.9	88.6	99.9																
7 CCIBt3494 *Stanieria cyanosphaera*	89.9	99.6	89.9	92.6	89.9	90															
8 AB039006 *Pleurocapsa* sp. PCC7319	88.4	92.8	88.4	96.2	88.4	88.5	92.8														
9 AJ344558 *Chroococcidiopsis cubana* SAG 39.79	99.2	89.9	99.2	88.2	99.2	99.3	89.7	88.2													
10 AP017375 *Stanieria* sp. NIES-3757	90.1	99.8	90.1	92.8	90.1	90.2	99.8	93	89.9												
11 CCIBt3408 *Pleurocapsa* sp.	87.7	92.5	87.7	97.9	87.7	87.8	92.5	96.5	87.6	92.7											
12 CP003653 *Stanieria cyanosphaera* PCC7437	89.7	99.2	89.7	92.8	89.7	89.8	99.2	92.9	89.5	99.3	92.4										
13 DQ293994 *Pleurocapsa* sp. CALU1126	88.1	92.4	88.1	98.5	88.1	88.2	92.4	96.4	87.8	92.6	98.2	92.3									
14 FJ805841 *Chroococcidiopsis thermalis* PCC7203	99.6	90.2	99.6	88.6	99.6	99.7	90.1	88.6	99.6	90.2	88	89.9	88.2								
15 KM020000 *Chroococcidiopsis thermalis* SAG42.79	99.6	90.2	99.6	88.6	99.6	99.7	90.1	88.6	99.6	90.2	88	89.9	88.2	100							
16 KP835529 *Chroococcidiopsis* sp. CENA124	99.6	90.2	99.6	88.6	99.6	99.7	90.1	88.6	99.6	90.2	88	89.9	88.2	100	100						
17 KR137572 *Pleurocapsa*_sp. CENA351	87.8	92.2	87.8	98.3	87.8	87.9	92.2	96.2	87.5	92.4	98.3	92.3	99.2	87.9	87.9	87.9					
18 KR137574 *Chroococcidiopsis* sp. CENA353	99.6	90.1	99.6	88.3	99.6	99.7	89.9	88.2	99.1	90.1	87.5	89.7	87.9	99.4	99.4	99.4	87.6				
19 KR137588 *Chroococcidiopsis* sp. CENA367	99.9	90.2	99.9	88.6	99.9	100	90	88.5	99.3	90.2	87.8	89.8	88.2	99.7	99.7	99.7	87.9	99.7			
20 KU574618 *Chroococcidiopsis muralis* HA8275-LM2	98.9	89.9	98.9	88.5	98.9	99	89.7	88.5	98.8	89.9	88.1	89.7	88	99.2	99.2	99.2	87.7	98.7	99		
21 JX316763 *Chroococcidiopsis thermalis* CCAP 1423/1	99.5	90.1	99.5	88.4	99.5	99.6	89.9	88.3	99.3	90.1	87.7	89.7	88	99.7	99.7	99.7	87.7	99.3	99.6	98.9	

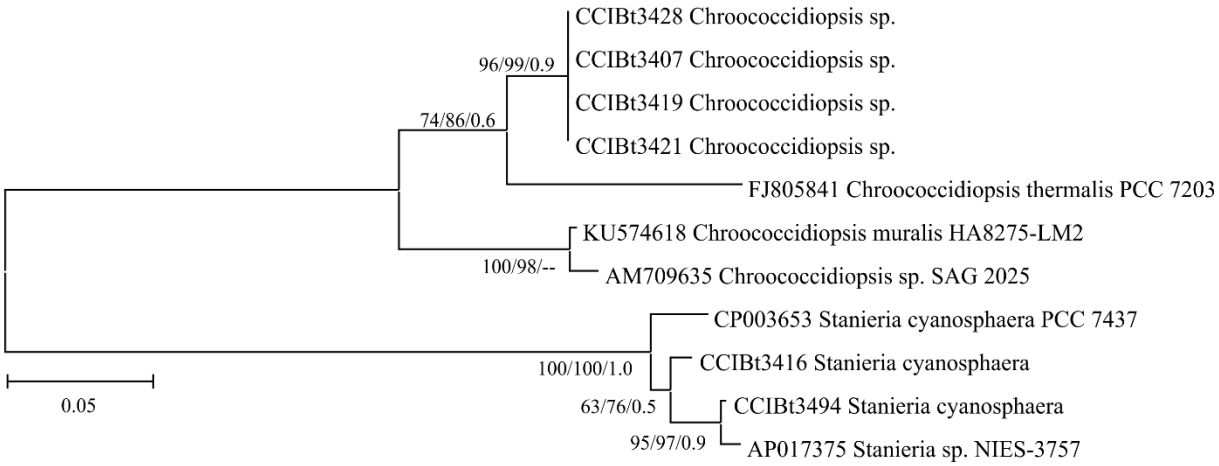


Figure 32: Phylogenetic tree based on 16S-23S ITS sequences and reconstructed using the maximum-likelihood (ML) analysis of evolutionary distances determined by the HYK + G + I model. Bootstrap 1,000×.

Table 16. 16S-23S ITS similarity matrix of studied lineages and their close relatives, conducted using the p-distance model with a total of 417 positions.

Sequences identification		1	2	3	4	5	6	7	8	9	10
1	CCIBt3494 *Stanieria cyanosphaera*										
2	CCIBt3416 *Stanieria cyanosphaera*	97.4									
3	AP017375 *Stanieria* sp. NIES-3757	99	96.9								
4	CP003653 *Stanieria cyanosphaera* PCC 7437	95.9	95.9	95.9							
5	FJ805841 *Chroococcidiopsis thermalis* PCC7203	70.3	70.5	70	70.3						
6	KU574618 *Chroococcidiopsis muralis* HA8275-LM2	72.9	73.1	72.4	73.6	88.5					
7	AM709635 *Chroococcidiopsis* sp. SAG2025	72.4	72.7	71.9	73.1	88.7	98.8				
8	CCIBt3428 *Chroococcidiopsis* sp.	72.9	73.6	72.7	72.7	91.1	89	88.5			
9	CCIBt3407 *Chroococcidiopsis* sp.	72.9	73.6	72.7	72.7	91.1	89	88.5	100		
10	CCIBt3421 *Chroococcidiopsis* sp.	72.9	73.6	72.7	72.7	91.1	89	88.5	100	100	
11	CCIBt3419 *Chroococcidiopsis* sp.	72.9	73.6	72.7	72.7	91.1	89	88.5	100	100	100

4. CONCLUSIONS

Nanocytes and baeocytes were showed to differ based on their development process. Basically, nanocytes are originated by a chain of successive fission and the cells do not return to their original size before the next division; nanocytes showed to be common to different and phylogenetically unrelated cyanobacteria. On the other hand, baeocytes are created by a unique event of multiple fission and they exhibit movement as soon as they are released from the mother cell; baeocytes showed to occur only in pleurocapsalean cyanobacteria. The clade formed by *Chroococcidiopsis thermalis* and its relatives strains showed to produce nanocytes, not baeocytes, what raises the idea of monophyly among the multiple fission cyanobacteria.

However, more studies need to be done regarding the morphological plasticity of these cyanobacteria and their morpho-similar strains, once pleurocapsalean showed to be very affected by culture conditions. Moreover, due this morphologic heterogeneity and the high affinity both by 16S rDNA and 16S-23S ITS among true multiple fission cyanobacteria, we suggest their characterization by other molecular markers in face to better elucidated their genera and species circumscription.

Considerações Finais

CONSIDERAÇÕES FINAIS

Ao concluirmos este trabalho pudemos compreender que as cianobactérias cocoides terrestres são grandes contribuintes da diversidade. Não apenas biodiversidade, mas também de biocompostos produzidos, sejam eles com bioatividade comprovada ou em potencial. Ademais, podemos considerar que:

- A variabilidade morfológica não é capaz de sustentar ou refletir a diversidade filogenética por si só, considerando-se o grande grupo das cianobactérias unicelulares e coloniais. Contudo, a morfologia é de suma importância por duas razões principais:
    - I) é a ligação, por meio da identificação, entre todo o conhecimento taxonômico já estabelecido para as cianobactérias e as novas informações advindas da abordagem atual, como a utilização de métodos moleculares na filogenia;
    - II) diante de gêneros e espécies tem-se mostrado uma ferramenta útil, o que deve ser considerado caso a caso. Adicionalmente, cabe aqui recomendar a avaliação da estabilidade dos caracteres morfológicos adotados na diferenciação dos táxons, o que deve ser feito em cultivos de longo prazo e sob diferentes condições.
- Uma vez que a variabilidade morfológica é insuficiente para expressar a filogenia das cianobactérias cocoides, é esperada a descrição de gêneros e espécies crípticas relacionadas ao grupo. Esse é o caso do gênero *Chroococcus*. De morfologia muito simples, os remanescentes desse gênero ainda formam um grupo heterogêneo, mesmo após a descrição dos gêneros a ele morfo-relacionados (*Cryptococcum* gen. nov. e *Inacoccus* gen. nov). Dessa forma, é necessária uma revisão mais ampla das espécies que compõe *Chroococcus* e é provável que algumas constituam gêneros a parte;
- A filogenia com as sequências de DNAr 16S e ITS 16S-23S, cumpriram a função de separar e agrupar as linhagens estudadas quanto ao gênero e as espécies, respectivamente. Contudo, ressalva-se a falta de sequências e estudos que suportem a comparação do ITS de espécies de cocoides. Além disso, as sequências de ITS desses organismos diferem muito entre si quanto provindas de grupos filogeneticamente não relacionados, o que dificulta a comparação entre elas;
- Devido a essa alta variabilidade, a comparação do ITS deve ser feita dentro de cada espécie e atentando-se sempre ao alinhamento para compararem-se regiões similares, principalmente quanto à presença/ausência de RNAt;

- A análise de microscopia eletrônica de transmissão das linhagens revelou que os tilacóides apresentam um padrão em sua distribuição na célula. Contudo, variações desse padrão foram frequentemente comuns numa mesma linhagem, o que leva a entender que a distribuição dos tilacóides pode ser variável e por isso deve ser analisada com cautela;
- Em geral, as cocoides isoladas de ambientes terrestres apresentam crescimento lento quando em cultivo. Essa característica foi um dos principais entraves para a obtenção de culturas monoespecíficas, uma vez que espécies oportunistas tendem a sobrepujar o crescimento das espécies letárgicas. Neste sentido, o isolamento por meio de tomada unitária de células - 'pescaria' - a partir do material da natureza mostrou-se mais vantajoso; o tempo mínimo de 30 dias também deve ser respeitado antes à avaliação da cultura;
- A análise das linhagens relacionadas aos gêneros *Aphanothece* e *Gloeothece* demonstrou haver uma separação filogenética entre aquelas espécies descritas para ambientes aquáticos e terrestres. Isso é evidente com a descrição do gênero *Totusmuciger* gen. nov., o qual é morfologicamente semelhante à *Aphanothece* (exceto pela formação de pseudofilamentos), mas difere por possuir apenas espécies aerofíticas;
- Em geral, pôde-se notar que as espécies analisadas possuem uma especificidade com o tipo de *habitat* e substrato no qual ocorrem. Isso é bem evidente em *Asterocapsa* e *Asterocorticola* gen. nov. Contudo, não é regra geral, uma vez que a espécie *Totusmuciger versatilis* sp. nov. ocorreu em solo, concreto e rocha. Essa habilidade de *T. versatilis* pode estar ligada a duas possibilidades: I) à característica generalista inerente à espécie; ou II) ao fino relacionamento que essa espécie apresenta com um micro-habitat e microclima específicos, uma vez que todos os substratos em que ela ocorreu possuem origem mineral. Independente de qual possibilidade se adequa a *T. versatilis*, esse caso destaca a necessidade de estudos mais detalhados sobre a relação das cianobactérias com o tipo e condições do *habitat*, principalmente numa escala microcósmica;
- O estudo em cultura das linhagens aqui apresentadas permitiu a elucidação sobre algumas características morfológicas das cianobactérias cocoides, sendo:
    - I) a coloração da bainha: quando avermelhada ou enegrecida, havia a ideia de que se tratasse do mesmo composto (gloeocapsina), o qual sob efeito do pH mudava de cor. Com os estudos das espécies *Asterocapsa calcaroseola* sp.

nov. e *Asterocapsa calcanigera* sp. nov. confirmamos que mesmo que a gloeocapsina esteja presente em algumas bainhas e sofra alteração de cor, há outras substâncias envolvidas na pigmentação da mucilagem de cianobactérias, as quais são estáveis em condições regulares de cultivo;

- II) a presença de granulação na bainha: o gênero *Asterocapsa* caracteriza-se principalmente pela decoração da mucilagem, o que pensava-se estar relacionado a formação de esporos. Contudo, a análise de linhagens relacionadas a este gênero demonstrou que este tipo de decoração é um carácter estável em condições de cultivo, ou seja, que não exercem pressão ambiental sob o crescimento das cepas. Por esse motivo, não devem ser considerados esporos, mas uma provável adaptação às condições ambientais originais desses organismos;
- III) nanócitos *vs.* baeócitos: o complexo processo de divisão celular estudado em linhagens relacionadas à formação de baeócitos e nanócitos revelou serem essas estruturas diferentemente formadas. Enquanto nanócitos são produzidos numa cadeia de divisões sucessivas em diferentes planos/direções, nas quais a células mãe não retorna ao tamanho original antes da próxima fissão, os baeócitos são produzidos num único evento de divisão celular. Outra diferença é em relação ao movimento das células-filhas: nanócitos são revestidos por uma camada externa fibrosa e por isso não apresentam movimento, enquanto os baeócitos não a possuem e somente a formam após a liberação da célula-mãe, o que os possibilita movimentar-se;
- IV) variabilidade em cultura: praticamente todas as linhagens estudadas apresentaram alguma variação morfológica frente às condições de cultura. Até mesmo o tempo da cultura revelou ser um fator interferente na morfologia das cepas, seja na produção de mucilagem, coloração e até dimensões. Essa característica é um empecilho à correta identificação taxonômica e pode explicar a topologia dispersa de alguns gêneros e espécies nas árvores filogenéticas: erros de identificação. Por isso salienta-se a importância do estudo detalhado da morfologia das cocoides em diferentes condições de cultivo.

- O gênero *Chroococcidiopsis* foi originalmente descrito com células produtoras de baeócitos. Contudo, analisando sequências de linhagens filogeneticamente muito próximas a *Chroococcidiopsis thermalis*, espécie tipo do gênero, constatamos que na

verdade as células produzem nanócitos. Dessa forma, o gênero *Chroococcidiopsis* necessita ser resinificado e sua relação com *Cyanosarcina* e *Pseudocapsa* elucidada, uma vez que esses gêneros apresentam padrão de divisão similar ao observado para *Chroococcidiopsis*. Todavia, com essa descoberta, uma das maiores barreiras para considerar-se a monofilia das cianobactérias produtoras de baeócitos foi esclarecida, o que traz de volta a ideia de monofilia dessas cianobactérias;

- O grande grupo formado pelas cianobactérias capazes de se dividirem por fissão múltipla, as pleurocapsales, mostrou-se enormemente diverso quanto a morfologia de seus representantes. Duas das linhagens estudadas (CCIBt3416; 3494) são filogeneticamente muito próximas à linhagem tipo do gênero *Stanieria*, sendo consideradas representantes desse táxon. Mas diferentemente do descrito para *Stanieria cyanosphaera* (tipo), as linhagens analisadas possuem células que se dividem não apenas por fissão múltipla, mas também por fissão binária. Isso torna *Stanieria* morfologicamente próximo ao gênero *Myxosarcina* e demonstra a necessidade de estudar a estabilidade dos caracteres morfológicos dentro do grupo das pleurocapsales;
- Os testes de atividade antifúngica e antioxidantes com as linhagens tipo dos gêneros novos *Cryptococcum* e *Inacoccus* demonstram o grande potencial das espécies cocoides terrestres para a indústria farmacêutica. Ademais, evidenciam que há um promissor recurso biotecnológico associado à biodiversidade; ambos ainda muito pouco conhecidos com relação às cianobactérias cocoides;
- O estudo sobre as cianobactérias terrestres cocoides, em maioria da Mata Atlântica e algumas do Cerrado, revelou uma elevada biodiversidade, condizendo com o esperado para estes Biomas. Um total de 32 cepas foi analisado e a partir do estudo polifásico delas, destacamos:
    - A definição e proposição de uma nova família, Gloeothecaceae;
    - A descoberta de quatro novos gêneros: *Asterocorticola*, *Totusmuciger*, *Cryptococcum* e *Inacoccus*;
    - A descrição de dez novas espécies: *Asterocorticola cerradensis*, *Totusmuciger versatilis*, *T. corticalis*, *T. solumi*, *T. evidentermarginater*, *Gloeothece sylvatlantis*, *Cryptococcum brasiliense*, *Inacoccus carmineus*, *Asterocapsa calcaroseola* e *Asterocapsa calcanigera*.

- A primeira caracterização com base no DNAr 16S e ITS 16S-23S do gênero *Asterocapsa* e das espécies *Chroococcus subviolaceus* e *Gloeothece interspersa*.

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