UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

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DIVERSIDADE E DISTRIBUIÇÃO DE CIANOBACTÉRIAS DE CROSTAS BIOLÓGICAS DO BIOMA CAATINGA COM BASE EM TAXONOMIA POLIFÁSICA E ANÁLISE METAGENÔMICA

SÃO JOSÉ DO RIO PRETO
2020

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

Financiadora: CAPES

Orientador: Prof. Dr. Luis Henrique Z. Branco
Coorientador: Dr Miriam Muñoz Rojas

São José do Rio Preto
2020

L732d

Lima, Náthali Maria Machado de

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175 f. : il., tabs., fotos

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto

Orientador: Luis Henrique Zanini Branco

Coorientadora: Miriam Muñoz Rojas

1. Cianobactérias. 2. Caatinga. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto. Dados fornecidos pelo autor(a).

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30 de setembro de 2020

AGRADECIMENTOS

Agradeço primeiramente à minha mãe Angela Priscila e meu pai João, que com muita luta puderam me proporcionar um ensino de qualidade. Ensino este que me deu a base para que hoje eu pudesse realizar esta defesa. Neste agradecimento incluo todos os colégios que passei, a UNESP e todos os meus professores, desde o ensino básico ao ensino superior, por terem mantido o ânimo e o amor por esta profissão tão desvalorizada em nosso país.

Agradeço à minha família, em especial ao meu avô Sezefredo Machado, por sempre me motivar aos estudos, ao conhecimento, à curiosidade e ao trabalho árduo. Tenho certeza que sua grande sabedoria serviu de exemplo a todos os membros de nossa família e alicerçou grande parte do que sou.

Agradeço os meus amigos Evelise, Ariane, Fábio, Luciana e Marcela por caminharem comigo por tantos anos, me proporcionando momentos leves e alegres durante toda a minha caminhada, além de torcerem por todas minhas realizações.

Agradeço então, ao núcleo mais diretamente relacionado a este trabalho, começando pelo meu orientador Luís Branco, com o qual caminhei por oito anos. Não fosse seu imenso conhecimento, paciência, dedicação e extrema atenção, eu com absoluta certeza, não seria capaz de desenvolver um trabalho como este. Deixo aqui estampada minha imensa gratidão e orgulho por este exímio professor, orientador e ídolo.

Agradeço aos meus colegas de laboratório, todos os que comigo passaram por essa jornada, Mariéllen, Mônica, Nádia, Bruna, Natália e Iago. Deixo então um abraço especial para a Natália, que foi a umas das últimas companheiras de guerra comigo, alegrando todos os meus dias no nosso querido BETA.

Também deixo meus agradecimentos especiais aqueles que participaram do meu último ano de doutorado, à minha coorientadora Miriam, que me deu todo suporte para vir à Austrália e realizar um ano em doutorado sanduíche, enriquecendo todo meu universo cientifico e acadêmico, sendo o melhor exemplo de mulher na ciência com quem eu poderia trabalhar, e me dando a oportunidade de continuar.

Agradeço também aos meus colegas de trabalho do BEES, meus queridos companheiros de trabalho Jana e Fred que tanto me ensinaram e ajudaram.

Por fim, agradeço à família que formei aqui na Austrália, Carol e Lívia, que me deram todo suporte para a redação desta tese, além de todo amor e alegria em todos os meus dias

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001*, à qual agradeço imensamente por ambos os projetos que me foram proporcionados.

Resumo

Crostas biológicas (CBs) consistem em uma comunidade de elementos entrelaçados composta por organismos poiquiloídricos, como cianobactérias, algas verdes, microfungos, bactérias, líquens, musgos e microartrópodes. Estas comunidades exercem funções ecológicas importantes e ocorrem nas camadas superficiais do solo, em uma variedade de regiões climáticas ao redor do globo, principalmente em regiões áridas e semiáridas. As cianobactérias são componentes de extrema importância nessas comunidades, sendo consideradas pioneiras e responsáveis por estruturar a camada superior do solo, tornando possível a colonização por outros grupos. Embora as cianobactérias de CBs venham sendo bem estudadas, ainda há muita diversidade não acessada, principalmente na América do Sul. No Brasil, estes estudos estão praticamente restritos às regiões de Cerrado e os dados que foram encontrados até o momento ressaltam a necessidade de ampliação de estudos no país para que também haja o preenchimento de vazio de conhecimento em outros biomas. Dessa forma, a Caatinga, que é um ambiente restritivo, considerada uma das áreas mais quentes entre as regiões semiáridas do mundo e portanto, propício à presença de crostas biológicas, foi escolhida para ser explorada. As coletas foram feitas nos municípios de Cabaceiras, Barra de Santa Rosa e Pocinhos (Paraíba) em quatro localidades, onde amostras das crostas foram recolhidas segundo à metodologia padrão, ao longo de transeções de 200 m. Foram coletadas 10 amostras de crostas em cada local amostrado e também recolhidas porções de solo para a realização de análises de parâmetros físicos e químicos, como nitrogênio total, fósforo total, ortofosfato, matéria orgânica, pH, carbono orgânico e textura do solo. Temperatura, umidade relativa do ar e irradiância foram parâmetros medidos diretamente em campo. Uma parcela de solo de cada amostra foi separada e analisada quanto à presença de cianobactérias, e para cada população registrada foram feitas análises morfológicas e cultivos. Outra parcela de solo de cada amostra foi inoculada em meio de cultura, visando o estabelecimento de culturas de cianobactérias que não tivessem sido observadas anteriormente. As populações cultivadas e consideradas unicianobacteriais tiveram seus materiais genéticos acessados por meio de extração, amplificação e sequenciamento. A última parcela de solo da amostra seguiu para análises metagenômicas, por meio de extração, amplificação e tecnologia de sequenciamento de próxima geração. Foram estudadas 26 populações, sendo nove a partir de amostras provenientes da natureza e outras 17 provenientes de cultivo e analisadas por critérios morfológicos e moleculares. Em todas as quatro localidades estudadas foram encontradas populações de Scytonema e Microcoleus, além de Nostoc também ter sido registrada em três das quatro regiões. Microcoleus e Nostoc foram analisados separadamente, por meio de estudos detalhados de filogenia e resultaram no descrição de seis novos gêneros e 12 novas espécies. A análise de NGS possibilitou uma investigação ecológica, que relacionou a distribuição dos grupos

de cianobactérias registradas a fatores ambientais, e também a comparação entre Caatinga e outro bioma propício à formação de crostas, o Pampa. A composição de cianobactérias registrada na Caatinga evidenciou a dominância de organismos adaptados à sobrevivência em ambiente com altas temperaturas e níveis de irradiância. Comumente encontrados em crostas em estágio de desenvolvimento avançado e apresentando uma relação positiva com compostos químicos essenciais para o solo, como fósforo e nitrogênio. Enfatizando assim, a importância das cianobactérias na nutrição dos solos e na consequente criação de condições favoráveis para o estabelecimento de organismos superiores. Como apêndice foram apresentados dados de um estudo realizado em parceria com um laboratório na Austrália. Neste trabalho foram aplicadas técnicas recentes que utilizam cianobactérias provenientes de crostas biológicas na conservação e restauração de espécies de plantas nativas.

Palavras-chave: Crostas biológicas. Ambientes restritivos. Diversidade em crostas. Bactérias fotossintetizantes.

Abstract

Biological soil crusts (BSCs or biocrusts) consist of a community of elements that are interwoven and composed of poikilohydric organisms such as cyanobacteria, green algae, bacteria, lichens, mosses and microarthropods. These communities, which inhabit the top few centimeters of the soil, are responsible for important ecological functions. Biocrusts cover large parts of the soil surface in a broad range of habitats from a variety of climatic regions around the globe, mainly in arid and semiarid areas. Cyanobacteria are extremely important components of these communities, being considered their pioneers and responsible for structuring the soil biocrust and preparing it for the colonization by other organisms. Even though cyanobacteria from BSCs have been relatively well studied, a large part of their diversity is still unknown, especially in South America. Studies in Brazil are nearly restricted to the *Cerrado* region and data found so far highlights the need of further research in the country to improve knowledge from other biomes. Therefore, this study focused on the *Caatinga* biome, an extreme environment considered one of the hottest regions among the semiarid regions in the globe, being favorable to the presence of BSCs are broadly present. Sampling was carried out in the three cities: *Cabaceiras*, *Barra de Santa Rosa* and *Pocinhos* (*Paraiba* state) in four different localities, where biocrusts were sampled along 200 m transects following standard protocols. At each locality, a total of 10 samples were sampled, with an extra amount of soil collected for physical and chemical analysis, e.g. total nitrogen, total phosphate, organic matter, pH, organic carbon and soil texture. Temperature, air relative humidity and irradiance were measured in the field. Each soil sample was divided into three subsamples; the first one was used for the analyses of cyanobacteria populations, which were cultured and morphologically examined. A second subsample was inoculated in BG11 medium to promote the development of populations not observed before. The unicyanobacterial cultures had their DNA material extracted, amplified, and sequenced. The last subsample of each soil sample was used for metagenomic analyses, with extraction, amplification and sequencing through next-generation sequence technologies (NGS). We studied 26 populations, nine of which were observed directly after sampling, and 17 were cultivated and analyzed through morphological and molecular methods. *Scytonema* and *Microcoleus* were found in all the four studied localities, while *Nostoc* was registered in three of them. *Nostoc* and *Microcoleus* were submitted to a deep phylogenetic evaluation that resulted in the proposition of six new genera and 12 new species. The NGS analysis allowed an ecological investigation, relating the distribution of the registered cyanobacteria with environmental factors. It made possible the comparison among

Caatinga and another biome suitable to the development of biocrusts, called *Pampa*. The registered cyanobacterial composition in *Caatinga* was evidence that the dominant cyanobacteria were able to activate chemical pathways that allow their survival under high temperatures and high levels of irradiance. This group is common to biocrusts in a late stage of development and presents a positive relation with essential chemical compounds to the soil, e.g. phosphorus and nitrogen. This record highlights the importance of these communities in promoting a nutritional rich soil system able to harbor bigger organisms. The last component of this thesis brings results of a project developed in collaboration with an international research group in Australia. This study applied novel techniques harnessing cyanobacteria from biological soil crusts for promoting the conservation and restoration of native plants.

Keywords: Biological Soil Crusts. Extreme environments. Biocrust diversity. Photosynthetic bacteria.

Sumário

Capítulo I - O que são crostas biológicas e por que estudá-las?

1.1 Introdução geral

As crostas biológicas (CBs) consistem em uma comunidade de elementos entrelaçados composta por organismos poiquiloídricos, como cianobactérias, algas verdes, microfungos, bactérias, líquens, musgos e microartrópodes (BELNAP et al., 2016). São também chamadas de biocrostas, crostas criptogâmicas, criptobióticas, microbióticas (BELNAP, 2002, 2005, 2006a; LANGHANS et al., 2009). Ocorrem nas camadas superficiais do solo, em uma variedade de regiões climáticas ao redor do globo e se estabelecem em áreas onde a cobertura vegetal é escassa o suficiente para que a luz solar atinja a superfície (BELNAP, 2005). Sendo assim, elas são mais comuns em zonas áridas, que são regiões que compreendem mais de 40% da superfície da Terra, o que faz com que as CBs sejam os elementos mais característicos da superfície terrestre (BELNAP et al., 2016).

As CBs também apresentam importantes contribuições ecológicas nos ambientes onde desenvolvem-se, por exemplo:

O entrelaçamento das cianobactérias e microfungos agrega partículas de solo e forma uma crosta firme que dá estabilidade e protege o solo contra forças erosivas, que são causadas pelo vento ou precipitações (BELNAP, 2005).

A estabilização do solo também é favorecida pela presença dos exopolissacarídeos produzidos pelos organismos colonizadores das CBs, resultando, também, na agregação de partículas de solo (GUNDLAPALLY & GARCIA-PICHEL, 2006).

Em desertos frios, a presença das crostas proporciona acúmulo e maior tempo de residência da água no solo, aumentando a quantidade e profundidade da infiltração (BELNAP, 2005).Além disso, as CBs contribuem na fixação de nitrogênio e carbono (BELNAP, 2002, 2005, 2006b).

Sabe-se que, na formação das crostas biológicas, as cianobactérias são os primeiros organismos a colonizar (GARCIA-PICHEL & WOJCIECHOWSKI, 2009) e estabilizar o solo, tornando possível o estabelecimento de outros organismos (GARCIA-PICHEL et al., 2016). Grande parte dos estudos considera como pioneiras as cianobactérias filamentosas, móveis e que se organizam em “formato de corda” (GARCIA-PICHEL & WOJCIECHOWSKI, 2009). Entretanto, em estudo de diversidade feito com crostas do Cerrado brasileiro, cianobactérias que não se organizam em “formato de corda”, como *Leptolyngbya* Anagnostidis et Komárek e *Porphyrosiphon* Kützing ex Gomont foram encontradas como as mais frequentes, indicando que sejam colonizadoras primárias neste ambiente (MACHADO-DE-LIMA et al., 2019).

Belnap & Eldridge (2003) propuseram que a sequência sucessional esperada para crostas

biológicas de regiões áridas e semiáridas tem início com a colonização por cianobactérias e algas, seguidas pela instalação de líquens, e posteriormente, por várias colonizações de briófitas e líquens. Na maioria das sucessões, não é esperada a substituição total de um grupo de organismos por outro, mas a adição de espécies à comunidade com mudança nas abundâncias relativas ao longo do tempo (WEBER et al., 2016).

CBs de desertos quentes são dominadas por cianobactérias, enquanto as de desertos frios são dominadas por líquens e musgos (BELNAP, 2005). Em algumas localidades, as CBs dominadas por cianobactérias podem cobrir até 95% da superfície do solo, como observado no Kalahari (Botsuana), por Thomas & Dougill (2007). Nos desertos, onde a maioria das chuvas cai nas estações frias, predominam cianobactérias do gênero *Microcoleus* Desmazières ex Gomont e, em desertos quentes com verões chuvosos, os gêneros *Scytonema* Agardh ex Bornet & Flahault, *Nostoc* Vaucher ex Bornet & Flahault e *Schizothrix* Kützing ex Gomont são predominantes (BELNAP, 2005). Esses gêneros apresentam adaptações importantes para a sobrevivência neste tipo de ambiente, como por exemplo, a capacidade de movimentação e migração dos tricomas de *Microcoleus* para as camadas inferiores do solo quando a superfície se torna seca (GARCIA-PICHEL et al., 2001) e até mesmo movimentos horizontais (SOROCHKINA et al., 2018). A presença de pigmentação nas bainhas de *Scytonema* também permite aos indivíduos manterem-se como colonizadores de topo de crostas e se protegerem do contato direto com a radiação UV (GARCIA-PICHEL et al., 2003).

Outra contribuição relevante das crostas biológicas está relacionada aos fluxos de nutrientes, em especial de carbono e nitrogênio. A fixação de carbono pelas crostas biológicas é feita por meio do processo de fotossíntese (SANCHO et al., 2016). Esta contribuição atua tanto em escala local quanto global, pois estimativas recentes apontam que coberturas criptogâmicas (crostas que recobrem plantas e rochas, além das de solo) produzem cerca de 6 a 7% da produção anual estimada das vegetações terrestres (ELBERT et al., 2012). Já o processo de fixação de nitrogênio atmosférico, em ambientes áridos, é predominantemente feito por crostas biológicas (EVANS & EHLERINGER, 1993) e diversos estudos têm demonstrado a atuação das cianobactérias na fixação de nitrogênio e na liberação de compostos nitrogenados para o ambiente (BELNAP et al., 2002; YEAGER et al., 2004, 2007; STRAUSS et al., 2012; ABED et al., 2013; RODRIGUES-CABALLERO et al., 2018).

Pelos motivos apresentados, é possível perceber a importância do estudo de cianobactérias nessas comunidades. Nos últimos anos, tem havido grandes investimentos na investigação de crostas biológicas, o que pode ser constatado pela duplicação no número de

gêneros registrados em crostas ao redor do mundo (BÜDEL et al., 2016), além dos novos gêneros e espécies descritos com base em organismos encontrados em CBs (FLECHTNER et al., 2002; ŘÉHÁKOVÁ et al., 2007; FLETCHNER et al., 2008; DADHEECH et al., 2012; MÜHLSTEINOVÁ et al., 2014; BECERRA-ABSALÓN et al., 2018, MARTINS et al., 2018).

Entretanto, embora as crostas biológicas venham sendo bem estudadas, ainda há muita diversidade não acessada, e esta diversidade precisa ser investigada, pois apenas um sólido embasamento a respeito dos organismos presentes nas crostas biológicas permitirá a compreensão das funções dessas comunidades (GARCIA-PICHEL, 2001). Neste sentido, Büdel et al. (2016) fizeram um levantamento das principais regiões que precisam ser exploradas quando à riqueza de cianobactérias e enfatizaram as Américas, em especial a América do Sul, como as regiões mais carentes de investigação.

Com o objetivo de contribuir com o conhecimento na América do Sul, crostas biológicas de regiões de Cerrado brasileiro vêm sendo investigadas desde 2014. Até o momento, os estudos foram feitos em sete áreas localizadas nos estados de Minas Gerais e São Paulo.. O levantamento da diversidade de cianobactérias de crostas registrou grande quantidade de gêneros comuns a crostas biológicas de outras localidades mundiais, como são os casos de *Nostoc*, *Scytonema*, *Stigonema*, *Calothrix*, *Microcoleus*, *Leptolyngbya*, *Schizothrix* e *Phormidium*. Entretanto, muitas populações morfologicamente estudadas não foram identificadas em nível específico e outras foram descritas como gêneros novos, comprovando a presença de grupos ainda desconhecidos para ambientes pouco explorados (MACHADO-DE-LIMA, 2016; MACHADO-DE-LIMA et al. 2019). Análises mais detalhadas, com abordagem metagenômica, em seis regiões de Cerrado e realizadas com base em metodologia de sequenciamento de próxima geração (SPG) provaram que embora no Cerrado haja gêneros comuns a crostas de diferentes localidades, a composição das assembleias de cianobactéria (grupo de gêneros presentes) de cada uma dessas regiões é única. Observou-se, também, que os gêneros *Leptolyngbya* e *Porphyrosiphon* foram os mais frequentes, diferentemente da maioria dos estudos com crostas de outras regiões do mundo onde predominam espécies dos gêneros *Microcoleus* e *Trichocoleus* (MACHADO-DE-LIMA et. al., 2019).

Considerando estes importantes dados registrados no Cerrado e o recente início das investigações com crostas biológicas no Brasil, fica evidente que, para o preenchimento deste vazio de conhecimento, é necessária a ampliação de estudos no país. Dessa forma, a Caatinga, um ambiente restritivo e propício à presença de crostas biológicas, foi um bioma escolhido para ser explorado quanto à diversidade de cianobactérias presentes em tais comunidades.

Devido à importância e recorrência nos ambientes amostrados de populações apresentando características morfológicas de *Microcoleus* e *Nostoc*, estes foram analisadas separadamente, por meio de estudos detalhados de filogenia. O estudo de *Nostoc* incluiu populações reportadas em trabalho anterior (Machado-de-Lima, 2016) e provenientes de crostas biológicas do Cerrado brasileiro. Os resultados relativos a essas abordagens compõem dois capítulos desta tese (Capítulos III e IV).

1.2 Hipóteses

Com base nos dados disponíveis e na expectativa em função das características do grupo e dos ambientes amostrados, algumas hipóteses podem ser formuladas e confrontadas com os dados obtidos ao final do projeto.

1. Será constatada a presença de variados grupos de cianobactérias em crostas biológicas de áreas da Caatinga - cianobactérias apresentam um conjunto de adaptações adequado para se ajustarem a ambientes ocasionalmente extremos quanto às características ambientais e serão encontradas em todas as crostas analisadas.

2. Haverá diferenças na composição florística entre amostras de crostas coletadas na mesma localidade – diferenças ambientais, mesmo em pequena escala (metros), deverão resultar em condições distintas e, portanto, em composições e relações de abundância particulares para cada amostra estudada.

3. Serão registradas novas ocorrências taxonômicas para o Brasil, com probabilidade de existência de novas espécies (ou mesmo novos gêneros) para a Ciência - devido ao tipo de ambiente enfocado, que pode ser bastante seletivo, e também pelos poucos estudos realizados nessa área geográfica, considera-se provável a ocorrência de táxons ainda desconhecidos.

1.3 Objetivos

1.3.1 Objetivos gerais

Realizar o levantamento taxonômico de assembleias de cianobactérias de crostas biológicas em diferentes localidades da Caatinga, por métodos tradicionais e moleculares.

1.3.2 Objetivos específicos

1) Estudar a composição florística de cianobactérias segundo critérios tradicionais (morfológicos) e com base em amostras de campo e cultivo artificial.

2) Realizar sequenciamento de cianobactérias de interesse, incluindo as informações em abordagens filogenéticas.

3) Acessar a composição das assembleias de cianobactérias baseada em sequenciamento de próxima geração (SPG) e relacionar as unidades taxonômicas operacionais (UTOs) encontradas com as variáveis ambientais medidas.

1.4 Material e métodos

1.4.1 Áreas de estudo

Para este projeto foram selecionadas quatro localidades na região da cidade de Cabaceiras/PB (CA, CB, CC, CD), escolhidas como representantes da Caatinga (Figura 1, 2 Tabela 1). As coletas foram realizadas em setembro de 2017.

Este bioma ocupa cerca de 12% do território nacional, estendendo-se por 1.037.517,80 Km^2 e englobando partes dos territórios dos estados do Maranhão, Rio Grande do Norte, Piauí, Ceará, Pernambuco, Alagoas, Sergipe, Bahia, Paraíba e norte de Minas Gerais (NASCIMENTO, 2015). A região apresenta terrenos cristalinos e praticamente impermeáveis, além de terrenos sedimentares com boa reserva de água subterrânea (ALVES et al., 2008). Na maioria dos casos, os solos são mineralmente ricos, mas pedregosos, pouco desenvolvidos e praticamente incapazes de reter água (ALVES et al., 2008). O clima da região é semiárido, sendo a pluviosidade de 400 a 800 mm/ano, com as chuvas distribuídas irregularmente (ECOLOGIA IB-USP, 2019). Alves et al. (2008) afirmam que as condições climáticas da região, fatores ecológicos, como solos e fenômenos de exposição, e abrigo são fatores consideráveis para o estabelecimento do bioma, entretanto, as atividades antrópicas, principalmente a pecuária extensiva, trazem alterações estruturais para a Caatinga.

Figura 1. Localização da região de Cabaceiras (PB), marcada no mapa pelo polígono amarelo, onde se distribuem as áreas de amostragem. (Fonte da imagem: Google Earth; http://www.google.com/earth/index.html).

Tabela 1. Localização das áreas de amostragem, considerando os municípios a que pertencem no estado da Paraíba e as respectivas coordenadas geográficas.

Código	Município/Localidade	Latitude	Longitude
CA	Cabaceiras área 1	07°27’S	36°16’O
CB	Cabaceiras área 2	07°24’S	36°19’O
CC	Pocinhos	07°07’S	36°08’O
CD	Barra de Santa Rosa	06°41’S	36°03’O

Fonte: tabela do autor.

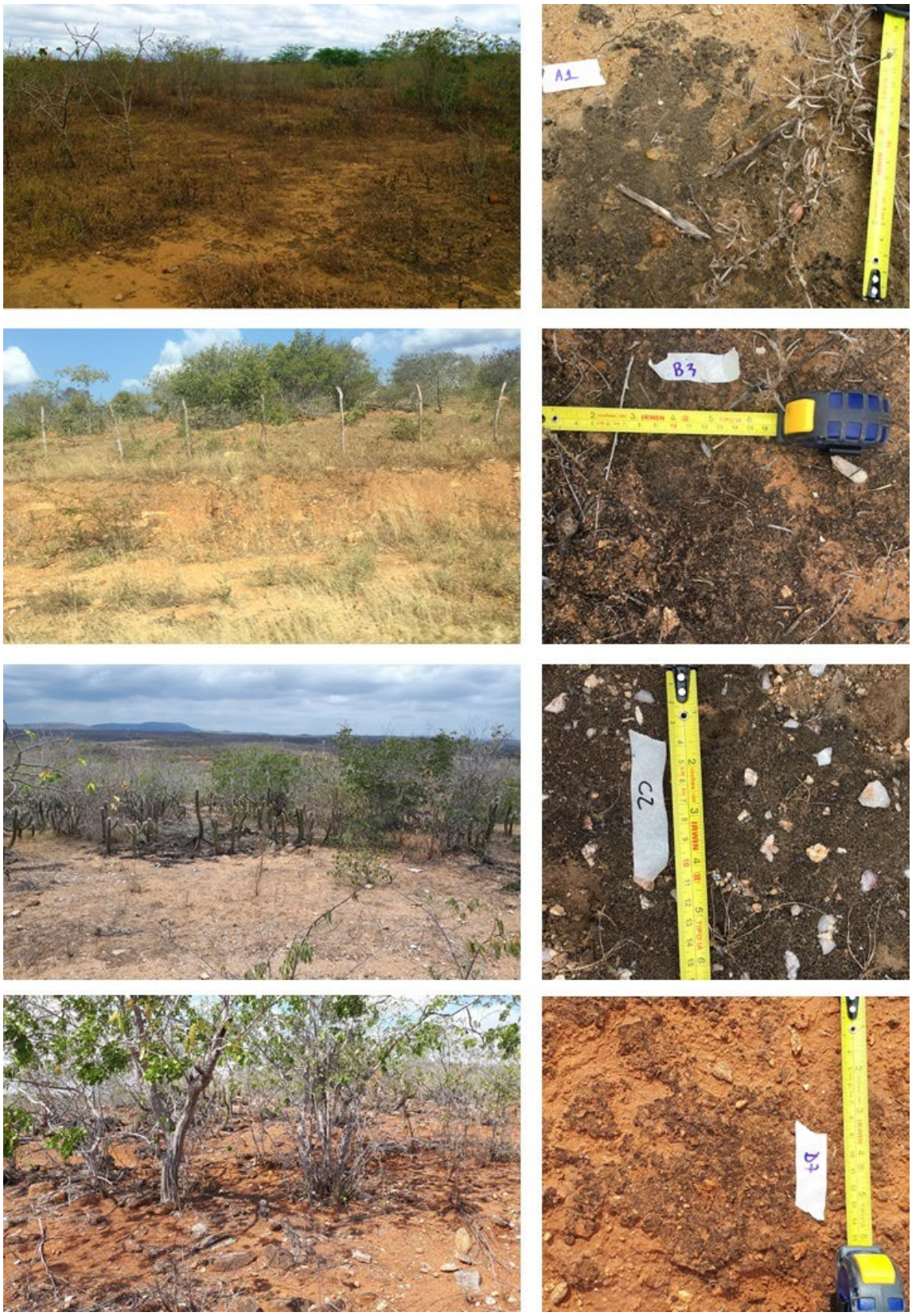

Figura 2. Aspecto das áreas amostradas (CA, CB, CC e CD – nas linhas) e das crostas biológicas encontradas na região do município de Cabaceiras (Paraíba), representando a caatinga. Fonte: figura do autor.

1.4.2 Atividade de coleta

As áreas de amostragem foram escolhidas segundo características de interesse (presença das crostas, condições de preservação ambiental e facilidade de acesso). Em cada localidade, foi demarcada uma transeção com 200 m de comprimento e foram registrados os dados de posicionamento global e altitude (GPS Garmin E-trex Vista). Ao longo da transeção, foram coletadas amostras de CBs a cada 20 m, totalizando 10 amostras para cada local de amostragem.

A coleta das crostas foi feita inserindo-se a parte inferior de uma placa de Petri (55 mm de diâmetro) invertida no solo, excisando a camada superficial e retirando uma amostra de cerca de 1cm de profundidade (GUNDLAPALLY & GARCIA-PICHEL, 2006, ZAADY et al., 2010). O material coletado, após secagem em temperatura ambiente durante o período necessário, foi adequadamente acondicionado, rotulado, identificado e mantido refrigerado.

Nos pontos de coleta de CBs ao longo das transeções, foram feitas leituras de umidade do solo a 1,0 cm e a 5,0 cm de profundidade. Após a medida de umidade, nos mesmos pontos foram recolhidas amostras de solo até 10,0 cm profundidade, com auxílio de uma pá de metal pequena, desprezando a camada superficial (entre 0,5 e 1,0 cm; região com crescimento das CBs). Estas amostras de solo foram enviadas para laboratório comercial para análise de textura da concentração de nitrogênio total e frações, de fósforo total e de ortofosfato, da quantidade de matéria orgânica e dos valores de pH.

Em cada localidade, também foram tomados, diretamente em campo, dados de temperatura e umidade relativa do ar (termo-higrômetro PeakTech 5090) e de irradiância (quantômetro Li-Cor).

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Capítulo II - Cianobactérias de crostas biológicas da Caatinga: identificação a partir de observação de material proveniente da natureza e de cultivo artificial

2.1 Introdução

Os primeiros trabalhos de sistemática de cianobactérias apareceram por volta de 1870, com as publicações de Thuret (1875), Bornet & Flahault (1886-1888) e Gomont (1892). Estes autores consideravam as cianobactérias como sendo um tipo de alga, e as classificavam, exclusivamente, com base em dados morfológicos e/ou reprodutivos. Geitler (1932) é um dos principais trabalhos na Europa Central, apresentando aproximadamente 1300 espécies baseadas em dados morfológicas, métricos e eclógicos. Com metodologia semelhantes, os trabalhos de Frémy (1929-1933, 1930), Elenkin (1935, 1938-1949), Geitler (1942), Desikachary (1959), Starmach (1966) e Bourrelly (1970) também são referência na sistemática do grupo.

Foi apenas em 2005, por meio do trabalho de Hoffman e colaboradores, que houve a junção entre dados genéticos, de ultraestrutura e fenotípicos na composição de um sistema de classificação de cianobactérias. Esta publicação foi um divisor de águas nos estudos de sistemática, permitindo um rearranjo para todo o grupo de cianobactérias.

Estes métodos modernos resultaram na divisão de vários gêneros tradicionais em clados diversos, como ocorrido para *Phormidium*, *Leptolyngbya*, *Microcoleus*, *Lyngbya*, *Nostoc* e outros. Entretanto, as análises moleculares não excluíram a morfologia clássica como ferramenta taxonômica. Estas duas análises, além de outros possíveis critérios, como os bioquímicos, ecológicos e ultraestruturais, têm sido avaliadas conjuntamente, segundo é proposto pela "abordagem polifásica", um conceito aplicado nos trabalhos de Anagnostidis & Komárek (1985, 1988, 1990) e Komárek & Anagnostidis (1986, 1989), para classificação das cianobactérias e que é utilizado até os dias de hoje.

Para os estudos de cianobactérias de crostas biológicas, o que se observa é que muitos gêneros novos foram descritos a partir de análises de observação direta (morfologia e cultivo) seguidas de análises moleculares e ecológicas (ŘÉHÁKOVÁ et al., 2007; FLECHTNER et al., 2008; DADHEECH et al., 2012; MÜHLSTEINOVÁ et al., 2014a, b; BECERRA-ABSALÓN

et al., 2018). Entretanto, mais recentemente essas análises têm dado lugar a outras que sustentam uma visão mais ampla da composição florística, como métodos de análises independentes de cultivo (análises metagenômicas) que resultam na reprodução de milhares de sequências genéticas (e. g. PUSHKAREVÁ et al., 2015; WILLIAMS et al., 2016).

Embora os resultados destas análises gerem muitas sequências, em maioria são sequências curtas, que não permitem análises filogenéticas acuradas. Além disso, essas análises

não descartam a necessidade de utilização dos métodos clássicos de estudos de morfologia, cultivo e extração de DNA a partir de amostras cultivadas. Isso porque parte da diversidade pode deixar de ser identificada nas análises metagênomicas se a biomassa da população/quantidade de DNA for insuficiente para a construção de bibliotecas (KUNIN et al., 2008).

Considerando a importância do emprego de ferramentas clássicas em estudos de diversidade de cianobactérias, este capítulo avaliou amostras de crostas biológicas da Caatinga a partir de métodos como análises morfológicas por observação direta e de análises morfológicas e moleculares de populações cultivadas.

2.2 Material e métodos

2.2.1 Análise e processamento das amostras de campo

As amostras provenientes diretamente de campo foram estudadas segundo o protocolo de Garcia-Pichel et al. (2001). As crostas coletadas foram reidratadas com água destilada e examinadas para verificação e identificação dos organismos presentes. Inicialmente, as amostras foram observadas sob microscópio estereoscópico (Olympus, modelo SZX7) para reconhecimento de morfotipos. As cianobactérias encontradas foram avaliadas segundo os critérios taxonômicos aplicáveis ao grupo (ordem, família e gênero) a que pertencem. O sistema de classificação seguido para posicionamento dos táxons encontrados foi o de Komárek et al. (2014).

A separação de espécimes para cultivo também foi realizada sob microscópio estereoscópico. Porções de crosta, de massas ou indivíduos isolados foram inoculados em diferentes meios de cultura BG11 (RIPPKA et al., 1979) e/ou Bold (NICHOLS, 1973). As condições de crescimento foram temperatura 20ºC ±1ºC, irradiância 50 µmol fótons.m^{-2}.s^{-1} e fotoperíodo de 14h de luz e 10h de escuro. Os cultivos foram inspecionados periodicamente (intervalos de cerca de 20 dias) e, quando necessário, procedimentos de isolamento foram feitos pelo método de "pescaria" (RIPPKA, 1988).

Porções representativas dos organismos identificados foram preservadas com solução de formaldeído 4% ou equivalente e incorporadas no Herbário SJRP (IBILCE/UNESP – campus de São José do Rio Preto – THIERS, 2019) para testemunho.

2.2.2 Estudos moleculares a partir de amostras de cultivo

2.2.2.1 Extração de DNA genômico e amplificação por PCR (Polymerase Chain Reaction) do gene 16S rRNA e da região intergênica 16S-23S (ITS)

Na extração a partir de culturas, o DNA genômico foi extraído utilizando-se o *kit MoBio PowerSoil DNA Isolation* (Laboratories Inc, Carlsbad, CA, EUA) de acordo com instruções do fabricante.

A amplificação dos genes 16S rRNA e ITS foi realizada utilizando-se os *primers* 359F (NÜBEL et al., 1997) e 23S30R (TATON et al., 2003). Cada reação consistiu em 5 µL de tampão de PCR 10X, 2 µL de $MgCl_2$ 50 mM, 1 µL de dNTPs 10 mM, 5 ρmol de cada iniciador, 1,5 U de "Platinum® Taq DNA Polimerase" (Invitrogen), 10 ng de DNA, água ultrapura (Milli-Q, Millipore, EUA), esterilizada, para um volume final de 25 µL. A verificação do tamanho dos *amplicons* resultantes foi realizada por comparação com o padrão de massa molecular "Low DNA Mass Ladder" (Invitrogen).

2.2.2.2 Clonagem, transformação e PCR de colônias

A clonagem das sequências produzidas na PCR foi feita utilizando-se o *kit* de clonagem "pGEM-T Easy Vector System I" (Promega), de acordo com as instruções do fabricante.

A introdução do vetor contendo o inserto nas células competentes de *E. coli* DH5α foi feita por meio de choque térmico, segundo as orientações de Sambrook et al. (1989). Após o plaqueamento em meio de cultivo LB contendo ampicilina e X-Gal, cinco colônias de cor branca foram selecionadas e utilizadas para novas reações de PCR, visando confirmar a presença dos insertos de interesse. Uma pequena quantidade de células de cada clone transformado foi adicionada a 25 µL de reação de PCR (conforme descrito no item 5.4.1.) contendo os iniciadores CYA359F, CYA781a e CYA781b (NÜBEL et al., 1997).

2.2.2.3 Extração do DNA plasmidial

As colônias brancas selecionadas foram transferidas para 3 mL de meio LB líquido e cultivadas por 15 horas a 37ºC, sob agitação de 180 rpm. Após este período de incubação, foi iniciada a extração de plasmídeo das células de *E. coli* DH5α com o *kit* "ZR Plasmid Miniprep" (Zymo Research) de acordo com as instruções do fabricante.

2.2.2.4 Sequenciamento e processamento das sequências

A PCR para o sequenciamento dos fragmentos dos genes 16S rRNA e ITS foi feita usando-se o *kit* "Big Dye Terminator" versão 3.0 (Applied Biosystems). Os *primers* utilizados foram 359F (NÜBEL et al., 1997), 704R (LANE, 1991), 1114F, 1494R (NEILAN et al., 1997) e 23S30R (TATON et al., 2003).

Posteriormente, os microtubos com as reações precipitadas foram enviadas para sequenciamento em empresa comercial.

2.2.3 Análises filogenéticas do gene 16S rRNA

As sequências de 16S rRNA obtidas foram comparadas com outras sequências previamente depositadas no *GenBank* do *National Center for Biotechnology Information* (NCBI), utilizando-se a ferramenta *Basic Local Alignment Search Tool* (BLAST) (ALTSCHUL et al., 1990).

2.3 Resultados e discussão

Foram estudadas 26 populações, sendo nove a partir de amostras provenientes da natureza e outras 17, de cultivo (Tabela 1).

Das 17 amostras provenientes de cultivo e analisadas por critérios morfológicos e moleculares, 13 serão descritas nos capítulos III e IV, pois foram identificadas como pertencentes aos gêneros *Microcoleus* e *Nostoc*. Estes gêneros são extremamente comuns em crostas biológicas (ROSENTRETER & BELNAP, 2003; BELNAP, 2005; GUNDLAPALLY & GARCIA-PICHEL, 2006; BÜDEL et al., 2009; GARCIA-PICHEL & WOJCIECHOWSKI, 2009; HAGEMANN et al., 2015; PUSHKAREVÁ et al., 2015; ROSSI & DE PHILIPPIS, 2015;

SCHULZ et al., 2016; DULIC et al., 2016; WILLIAMS et al., 2016). *Microcoleus* é considerado colonizador primários das crostas (BÜDEL et al., 2009; GARCIA-PICHEL & WOJCIECHOWSKI, 2009), enquanto *Nostoc* tem características de colonizador tardio (GARCIA-PICHEL et al., 2016).

As amostras tratadas neste capítulo totalizaram 13 populações, sendo quatro provenientes de cultivo e nove da natureza. Destas, nove foram identificadas em nível específico e quatro, apenas em nível genérico por diferirem em algumas características morfométricas e/ou quanto ao habitat das espécies já descritas (Tabela 1).

	Organismo	Código da Amostra	Localidade
CAPÍTULO II	*Microcrocis*	M2	CB
	Microcoleus steenstrupii	CC4	CC
	Porphyrosiphon notarisii notarisii	X1	CC
	Nostoc sp. 1	CC9	CC
	Nostoc sp. 2	CC10	CC
	Nostoc sp. 3	CD4	CD
	Calothrix sp.	V1	CC
	Scytonema guyanense	CC1, CA9, CB8	CA, CB, CC
	Scytonema hyalinum	U1	CD
	Scytonema javanicum	CD2	CD
	Scytonema millei	CA7	CA
CAPÍTULO III *Nostoc-like*	Nostocaceae 4 sp.	CATCC2	CC
	Nostocaceae 3 sp.	CATCB5.1	CB
	Nostocaceae 3 sp.	CATCB5.2	CB
	Nostocaceae 2 sp. 1	CATCB4	CB
	Nostocaceae 2 sp.2	CATCC10	CC
CAPÍTULO IV *Microcoleus-like*	*Pycnacronema edaphica*	CATCA7	CA
	Pycnacronema caatingensis	CATCC2	CC
	Gracilinea arenicola	CATCC10	CC
	Gracilinea arenicola	CATCB9	CB
	Marmoreocelis xerophila	CATCB5	CB
	Konicacronema caatingensis	CATCB1	CB
	Konicacronema caatingensis	CATCB6	CB
	Trichocoleus caatingensis	CATCD2	CD

Fonte: tabela do autor.

Descrição dos táxons encontrados

CHROOCOCCALES

***cf. Microcrocis* sp.**

Figura 1.

Colônias microscópicas a macroscópicas, com células densamente empacotadas em um plano. Células esféricas, e quando agregadas em formato poligonal, azul-esverdeadas, 4,0-6,0 µm diâm., 4,0-7,0 µm compr. Divisão celular em dois planos.

Comentários: A população estudada não se enquadrou em nenhuma das descrições feitas por Komárek e Anagnostidis (1999) para espécies de *Microcrocis* Richter. As distinções se deram, principalmente, quanto ao tamanho celular e ao habitat (espécies em literatura sempre descritas como aquáticas). No entanto, o formato das colônias, das células, a forma

como se agregam, e os planos de divisão se assemelharam à descrição do gênero.

Observação: espécimes encontrados apenas em cultivo.

Ocorrência nas áreas estudadas: Cabaceiras (Amostra M2).

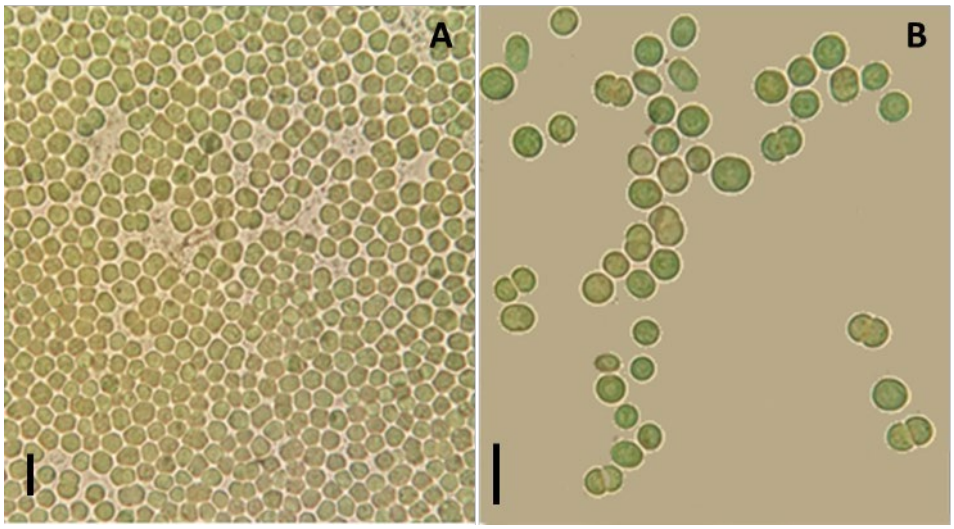

Figura 1. *Microcrocis* sp. A) Aspecto geral. B) Detalhe das células e divisões. Escalas: 10µm. Fonte: figura do autor.

A sequência molecular do gene 16S rRNA desta amostra, com 1162 pares de base, mostrou relação com inúmeras sequências de cianobactérias cocoides não identificadas do banco de dados, ainda assim com valores de similaridade inferiores a 95%. Este fato pode ser explicado por não haver sequências de *Microcrocis* depositadas no NCBI. De qualquer forma, as cianobactérias cocoides não identificadas que mais se relacionaram à amostra estudada têm origem em Mojave, na Califórnia, em solo, mostrando, portanto, um habitat comum à presença de crostas biológicas (e. g. ROSENTRETER & BELNAP, 2003).

OSCILLATORIALES

***Microcoleus steenstrupii* J.B. Petersen**

Arbejder fra den Botaniske have i København. 101(7): 292, 1923.

Figura 2.

Filamentos esparsos entre os grãos de areia, contendo mais de cinco tricomas por bainha, 48 µm diâm. Bainha incolor, aberta na extremidade. Tricomas não constritos, azul-esverdeados 4,0-4,5 µm diâm.. Células, na maioria das vezes, isodiamétricas, de a 0,9 a 1,2 vez mais longas que largas, 4,0-6,0 µm compr., conteúdo celular granuloso. Células apicais cônicas. **Comentários:** *Microcoleus steenstrupii* é um dos colonizadores primários de crostas biológicas (BOYER et al., 2002), tendo sido encontrado como dominante no Deserto de Sonora, no Arizona (NAGY et al., 2005). Atualmente sabe-se que este táxon é polifilético, representando muitas espécies e até mesmo gêneros separados (MÜHLSTEINOVÁ et al., 2014; PARZELT et al. 2014, PIETRASIAK et al., 2014).

Observação: espécimes encontrados apenas na natureza.

Ocorrência nas áreas estudadas: Pocinhos (Amostra CC4)

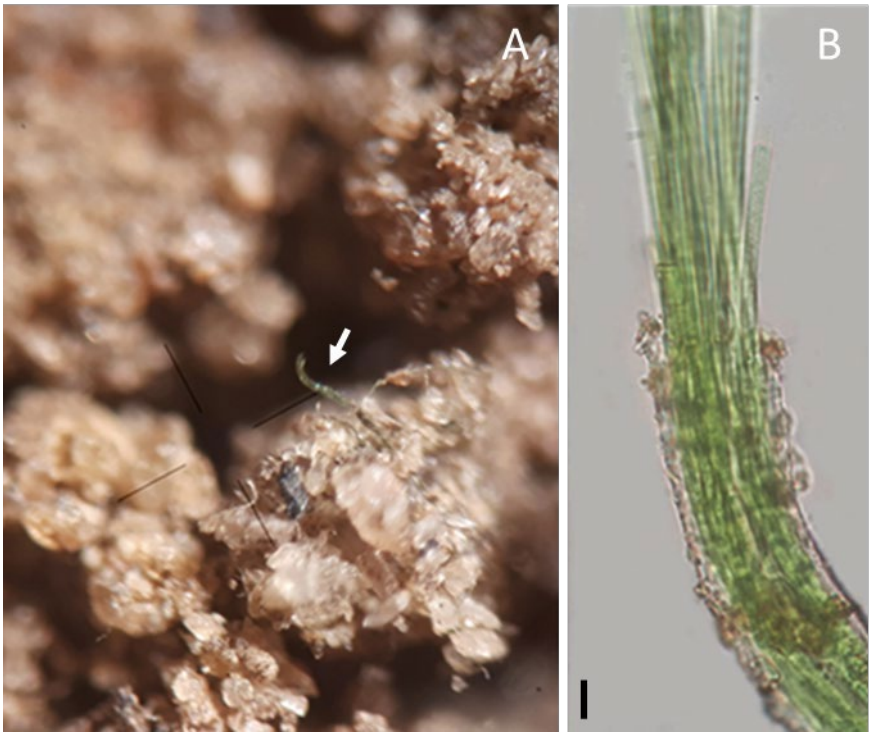

Figura 2. *Microcoleus steenstrupii*. A) Filamento entre os grãos de areia (seta). B) Detalhe do filamento multitricoma. (dir.). Escala: 10µm. Fonte: figura do autor.

***Porphyrosiphon notarisii* Kützing ex Gomont**

Ann. Sci. Nat., Bot, Sér. 7, 15: 331, 1892.

Figura 3.

Filamentos emaranhados, ocasionalmente constituindo massas expandidas, avermelhadas. Filamentos superficiais ou entremeando-se no solo, 28,0-34,0 µm diâm. Quando em cultura, filamentos verde escuros crescendo soltos no meio, 21,0-33,0 µm diâm. Bainha lamelada e incolor nas regiões mais internas da crosta e quando em cultivo; e espessada, lamelada e alaranjada nas regiões superficiais da crosta. Tricomas azul-esverdeados, 13,0-18,0µm diâm. (natureza), 16,0-22,0 µm diâm. (cultura), constritos nas paredes transversais. Células isodiamétricas, 0,3 a 0,7 vez mais longas que largas, 4,0-10,0 µm compr. (natureza), 5,0-11,0 µm compr. (cultura). Células apicais hemisféricas a cônico-arredondadas.

Comentários: Os espécimes observados estão de acordo com a descrição morfológica, métrica e ecológica da espécie feita por Gomont (1892) e Komárek & Anagnostidis (2005), distinguindo levemente quanto ao diâmetro de filamento, que na literatura não ultrapassou 28µm.

Porphyrosiphon notarisii ocorre em ambientes aéreos e provavelmente é cosmopolita (KOMÁREK & ANAGNOSTIDIS, 2005). A espécie foi registrada em crostas biológicas da savana africana (ULLMAN & BÜDEL, 2003) e em diferentes regiões da Mulga lands, na Austrália (WILLIAMS & BÜDEL, 2012). No Brasil, teve ocorrência registrada sobre rochas e solos em diferentes áreas no estado de São Paulo (SANT'ANNA & AZEVEDO, 1995; BRANCO et al., 2009; MACHADO-DE-LIMA, 2016).

A intensa coloração da bainha em regiões mais expostas das crostas pode indicar uma estratégia de proteção contra radiação ultravioleta. Esta estratégia já foi descrita anteriormente para cianobactérias de outros gêneros, como *Scytonema*, p.ex., e é atribuída à deposição de pigmentos, denominado *scytonemina*, nas bainhas mucilaginosas (GARCIA-PICHEL & CASTENHOLZ, 1991). Embora não se trate do mesmo pigmento presente nas bainhas dos filamentos de *P. notarisii,* a estratégia pode ser semelhante.

Observação: espécimes encontrados na natureza e com crescimento em cultivo.

Ocorrência nas áreas estudadas: Pocinhos (Amostra X1).

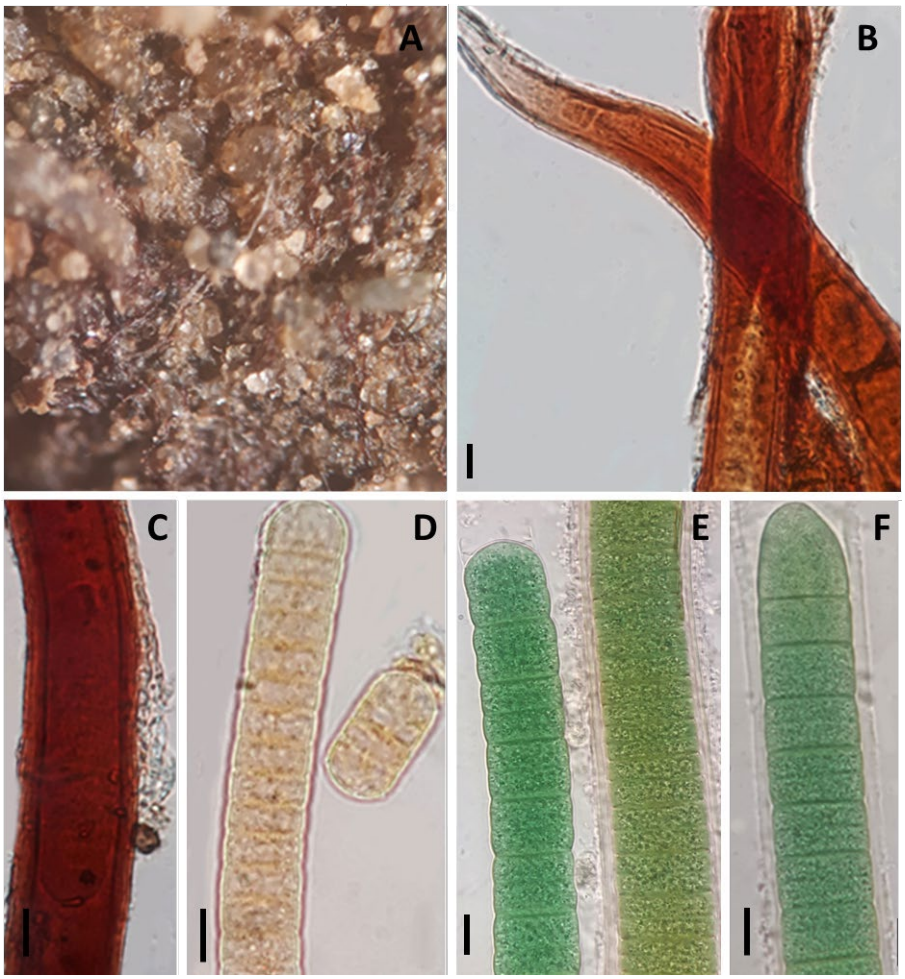

Figura 3. *Porphyrosiphon notarisii*. A) Aspecto geral quando na crosta. B-D) Detalhe tricomas quando em natureza. E-F) Tricomas quando em cultivo. Escalas: 10µm. Fonte: figura do autor.

A sequência molecular do gene 16S rRNA desta amostra contou com 1161 pares de bases e mostrou alta identidade com quatro sequências de bactérias não cultivadas originárias da Austrália. Estas bactérias foram caracterizadas como hipolíticas e coletadas em regiões do semiárido, portanto, apresentando semelhanças ecológicas com a população estudada. Outro indicador de que a população estudada foi corretamente identificada é o fato de *Porphyrosiphon* já ter sido registrado na Austrália em crostas biológicas e como organismo dominante (WILLIAMS & BÜDEL, 2012).

A amostra estudada se assemelhou em 98% à linhagem de *Porphyrosiphon notarisii* 9FUR do banco de dados (Tabela 2). Esta última tem origem em crostas biológicas deCerrado brasileiro, indicando, além da relação molecular, a proximidade ecológica entre as duas populações.

Tabela 2. Correspondência da sequência do 16S rRNA da linhagem X1 comparada com sequências de cianobactérias do GenBank (NCBI).

I (%)	C (%)	Organismos mais próximo (nº de acesso)	Habitat da Amostra
98,6	99	Uncultured bacterium (FJ230804.1)	Hipolítico (Semiárido)
98,4	99	Uncultured bacterium (FJ230801.1)	Hipolítico (Semiárido)
98,4	99	Uncultured bacterium (FJ230800.1)	Hipolítico (Semiárido)
98,3	99	Uncultured bacterium (FJ230826.1)	Hipolítico (Semiárido)
98,0	92	*Porphyrosiphon notarisii* (MF109120.1)	Aéreo (Semiárido)

I = Identidade; C= Cobertura. Fonte: tabela do autor.

NOSTOCALES

Nostocaceae

***Nostc* sp.1**

Figura 4.

Colônias macroscópicas, gelatinosas, firmes, irregularmente esféricas, hemisféricas, lobadas, com superfície verrucosa e periderme distinta, marrom-escuras. Tricomas flexuosos, emaranhados entre hifas de fungos, 5,0-6,0 µm diâm. Células curtas, em formato de barril ou quase esféricas, esféricas ou levemente alongadas, 3,0-7,0 µm compr. Heterócitos e acinetos não observados.

Comentários: A população estudada é semelhante à *Nostoc commune* Vaucher ex Bornet & Flahault quanto à morfologia da colônia, morfologia e disposição dos tricomas e morfologia da célula descritas para a espécie por Komárek (2013 – 4,0-5,0 µm compr.). Outra característica comum entre a população estudada e *N. commune* é a interação com fungos, muitas vezes formando o líquen *Collema* (YEAGER et al., 2007), frequente em crostas biológicas (LANGE et al., 1998). Além disso, *N. commune* é considerada como provável colonizadora primária de crostas, assim como *Microcoleus*, havendo registros de ocorrência em crostas de todo globo (BÜDEL et al., 2016).

Porém, mesmo considerando todas as semelhanças entre a população estudada e *N. commune* a identificação específica é inviável pela falta de dados sobre heterócitos e acinetos, estruturas ausentes na amostra analisada.

Observação: espécimes encontrados em amostra da natureza

Ocorrência nas áreas estudadas: Pocinhos (Amostra CC9).

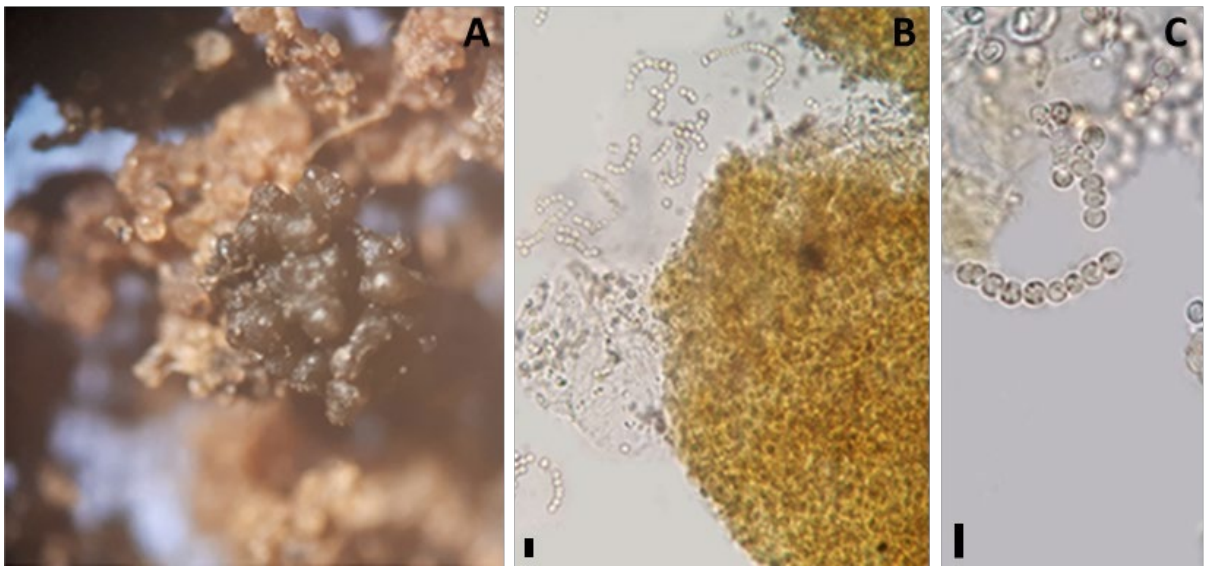

Figura 4. *Nostoc* sp.1. A) Aspecto geral da colônia quando na crosta. B-C) Detalhes de tricomas e células. Escalas: 10µm. Fonte: figura do autor.

***Nostoc* sp.2**

Figura 5.

Colônias gelatinosas, alongadas verticalmente, macroscópicas, verrucosas, macias, facilmente soltas do substrato, amarronzadas. Tricomas flexuosos, emaranhados, 5,0 µm diâm. Células em formato de barril, verde-oliva, 4,0-,8,0 µm compr. Não foram observados heterócitos e acinetos. **Comentários:** A população estudada é semelhante à *Nostoc flagelliforme* Berkeley et Curtis ex Bornet e Flahault quanto à morfologia da colônia, disposição dos tricomas e morfologia da célula, diferindo levemente quando ao comprimento celular descrito para a espécie por Komárek (2013 - 4-5 µm compr.).

Os registros de *N. flagelliforme* para solos de deserto e montanhas também são notáveis semelhanças com a população estudada, entretanto, esta não foi identificada em nível específico devido à não observação de heterócitos e acinetos, importantes características para diferenciação das espécies neste gênero.

Observação: espécimes encontrados em amostra da natureza

Ocorrência nas áreas estudadas: Pocinhos (Amostra CC10).

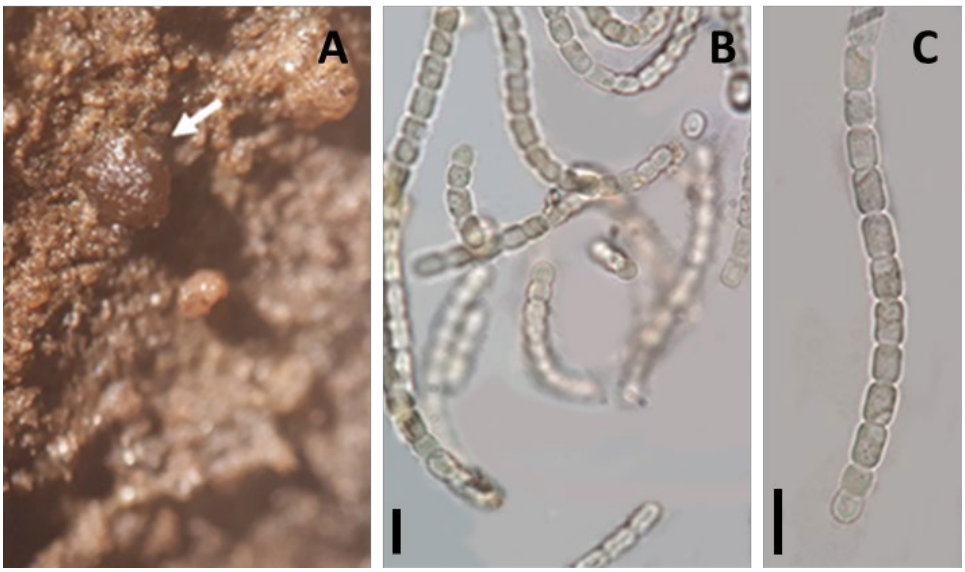

Figura 5. *Nostoc* sp.2. A) Aspecto geral das colônias quando na crosta. B) Colônia arredondada. C) Tricomas e células. Escalas: 10µm. Fonte: figura do autor.

***Nostoc* sp.3**

Figura 6.

Colônias macroscópicas, gelatinosas, firmes, esféricas, com superfície lisa e periderme distinta, marrom-amareladas Tricomas flexuosos, emaranhados, 4,0-5,0 µm diâm. Células esféricas ou em formato de barril, 3,0-6,0 µm compr.

Comentários: A população estudada é semelhante à *Nostoc commune* Vaucher ex Bornet & Flahault quanto à morfologia da colônia e morfologia e disposição dos tricomas, diferindo levemente quando ao comprimento mínimo das células vegetativas. Como já citado anteriormente, *N. commune* é muito comum em crostas biológicas, havendo registros ao redor de todo globo (ROSENTRETER & BELNAP, 2003; BÜDEL, 2003a; ROUSSOMOUSTAKAKI, 1983; BÜDEL, 2003b; BÜDEL et al., 2016).

A identificação da população estudada como *Nostoc commune* é inviável devido à ausência de dados sobre heterócitos e acinetos.

Esta população também é muito semelhante à população de *Nostoc* sp. 1 descrita anteriormente, no entanto, foram consideradas como populações distintas por diferirem quanto ao aspecto geral das colônias.

Observação: espécimes encontrados em amostra da natureza

Ocorrência nas áreas estudadas: Barra de Santa Rosa (Amostra CD4).

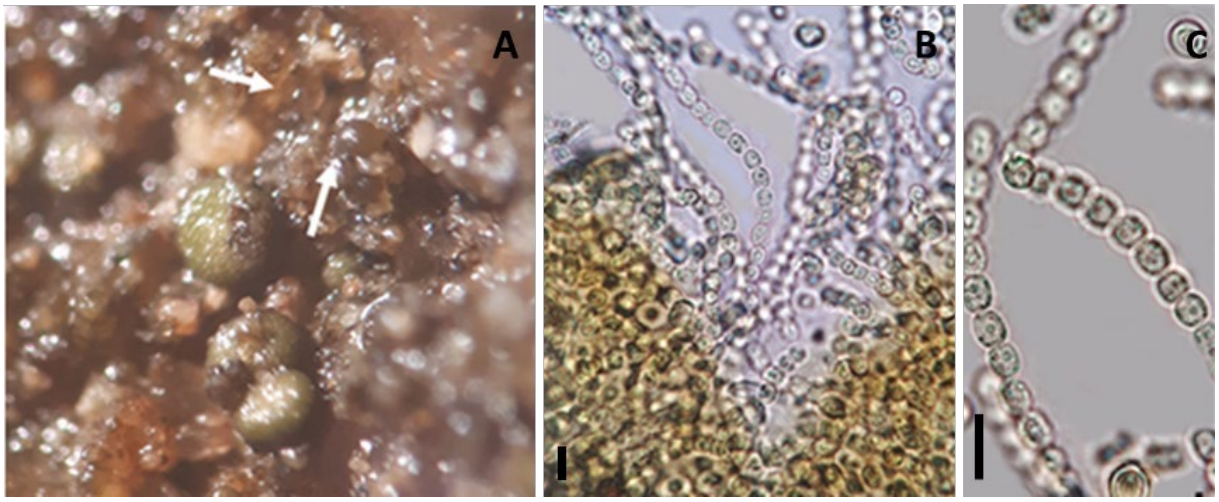

Figura 6. *Nostoc* sp.3. A) Aspecto geral da colônia quando na crosta (setas brancas). B-C) Tricomas e células. Escalas: 10µm. Fonte: figura do autor.

Rivulariaceae

***Calothrix* sp.**

Figura 7.

Filamentos em cultura formando tufos de feixes eretos ou emaranhados e presos à parede do tubo, 7,2-12,0 µm diâm. próximo à base. Ramificações falsas, unilaterais. Bainha ampla, fechada, incolor. Tricomas curtos, até 150 µm compr., constritos nas paredes transversais, 6,8- 12,0µm diâm. próximo à base, afilando até o ápice, sem formação de pelos hialinos. Células, em maioria, mais curtas que largas, 2,4-9,6 µm compr. Conteúdo celular granuloso com coloração acinzentada. Heterócitos basais, isolados, amarelados, esféricos até aproximadamente triangulares, 5,5-10,0 µm diâm, 4.8-9,6 µm compr.

Comentários: A população estudada é semelhante à *Calothrix parietina* Thruret ex Bornet et Flahault quanto à morfologia, diferenciando-se com relação à formação de pelo hialino (presente em *C. parietina*), à coloração das células (verde azuladas) e ao habitat (geralmente descrito como aquático). Entretanto, Komárek & Anagnostidis (2005) reconheceram diferentes expressões da espécie e algumas foram registradas em ambiente aéreo. *Calothrix parietina* é uma espécie comumente registrada ocorrendo em crostas biológicas de vários continentes e em numerosas regiões dos Estados Unidos (ROSENTRETER & BELNAP, 2003; BÜDEL et al., 2016).

Observação: espécimes encontrados apenas em cultivo.

Ocorrência nas áreas estudadas: Pocinhos (Amostra V1).

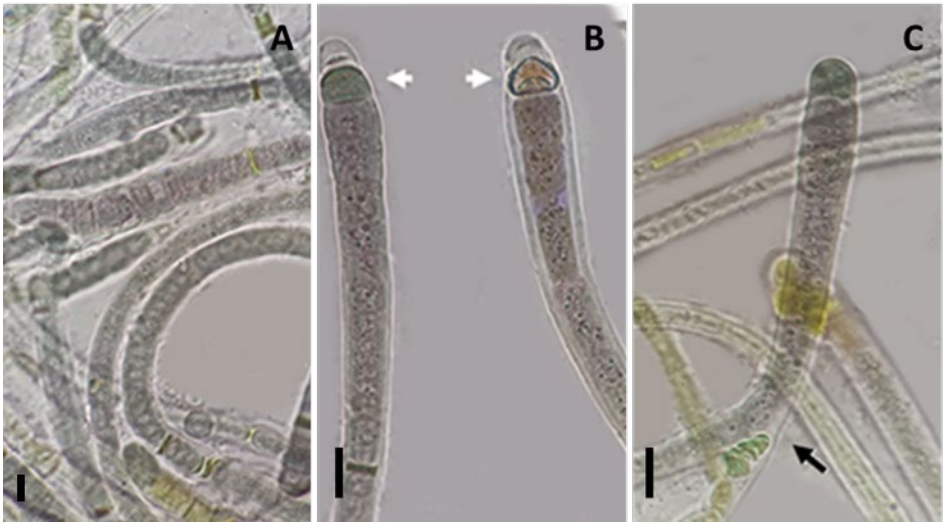

Figura 7. *Calothrix* sp. A) Filamentos emaranhados. B-C) Tricomas, heterócitos apicais (setas brancas), ramificação falsa (seta preta). Escalas: 10µm. Fonte: figura do autor.

A sequência molecular do gene 16S rRNA de *Calothrix* sp., de 1161 pares de base, mostrou alta identidade com 29 sequências do banco de dados identificadas como *Calothrix* sp. e uma identificada como *Microchaete* sp. (Tabela 3). Estas relações comprovam a correta identificação genérica da amostra estudada, mesmo as sequências do banco de dados, sendo em maioria, de hábito aquático. Já a linhagem *Microchaete* sp. (KY488002) provavelmente foi identificada incorretamente, sendo *Calothrix* e *Microchaete* muito semelhantes morfologicamente.

Tabela 3. Correspondência da sequência do 16S rRNA da linhagem V1 comparada com sequências de cianobactérias do GenBank (NCBI).

I (%)	**C (%)**	**Organismos mais próximo (nº de acesso)**	**Habitat da Amostra**
98.5	100	*Calothrix* sp. (HQ847573.1)	Aquático (cachoeira)
98.9	98	*Microchaete* sp. (KY488002.1)	Aéreo (estufa)
98.3	100	*Calothrix* sp. (HQ847579.1)	Aquático (cachoeira)
98.2	100	*Calothrix* sp. (KY863521.1)	Aquático

I = Identidade; C= Cobertura. Fonte: tabela do autor.

Scytonemataceae

***Scytonema guyanense* (Montagne) Bornet et Flahault**

Ann. Sci. Nat., Bot., Sér. 5, 51: 129, 1886

Figura 8.

Filamentos marrom escuros, entrelaçados, formando feixes eretos, 15,0-20,0 µm diâm. Ramificações falsas, na maioria das vezes duplas, ramos com a mesma morfologia do filamento principal. Bainha espessa, marrom-amarelada, lamelada paralelamente. Tricomas constritos no nível das paredes transversais, azul esverdeados ou marrons claros, 11,0-15,0 µm diâm. Células cilíndricas, quadráticas ou mais curtas que largas, 4,0-13,0 µm compr. Heterócitos solitários, cilíndricos ou quadráticos, 10,0-16,0 compr.

Comentários: Os espécimes observados estão de acordo com a descrição morfológica da espécie feita por Komárek (2013), embora esta não cite a morfologia dos heterócitos. Ademais, há uma pequena distinção quanto ao habitat, porque a espécie é descrita como habitando solos molhados, enquanto a população estudada foi coletada em solo seco.

Esta espécie é muito comum no Brasil, tendo sido registrada em diferentes ambientes do estado de São Paulo, incluindo Serra da Cantareira e outras regiões de Mata Atlântica (KOMÁREK et al., 2013). Também há registro desta espécie em crostas biológicas na Argentina (DE HALPERIN et al., 1976).

Observação: espécimes encontrados em amostra da natureza.

Ocorrência nas áreas estudadas: Pocinhos (Amostra CC1); Cabaceiras área 1 (Amostra CA9), Cabaceiras área 2 (Amostra CB8).

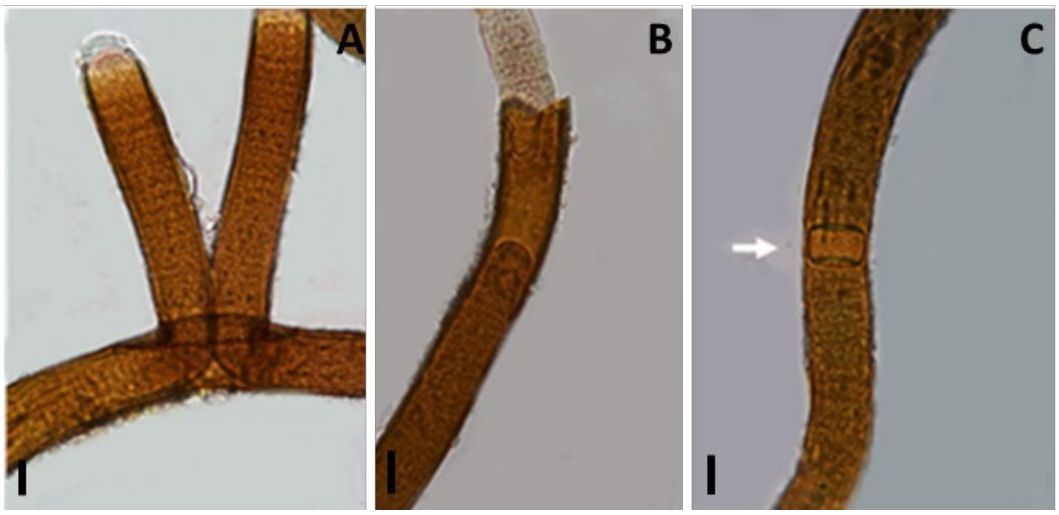

Figura 8. *Scytonema guyanense*. A) Ramificação falsa. B) Tricoma saindo da bainha. C) Heterócito (seta branca). Escalas: 10µm. Fonte: figura do autor.

***Scytonema javanicum* (Kützing) Bornet ex Bornet et Flahault**

Ann. Sci. Nat., Bot., Sér. 5, 51: 129, 1886.

Figura 9.

Filamentos marrons escuros ou esverdeados, entrelaçados, formando feixes eretos, 11,0-14,0 µm diâm. Ramificações falsas duplas e simples, ramos com a mesma morfologia do filamento principal. Bainha levemente espessa, transparente ou marrom-amarelada e lamelada paralelamente. Tricomas constritos no nível das paredes transversais, azul esverdeados 7,0-12,0 µm diâm. Células quadráticas ou mais curtas que largas, sempre mais curtas nas extremidades dos ramos, 3,0-14,5 µm compr. Heterócitos solitários, cilíndricos ou quadráticos, 10,0-15,0 µm compr.

Comentários: Os espécimes observados estão de acordo com a descrição morfológica da espécie feita por Komárek (2013), apenas diferindo quanto à constrição dos tricomas, quanto ao comprimento celular (3,0-13,8 µm compr.) e quanto ao habitat. A espécie é descrita como habitando solos molhados, entre musgos, também folhas ou troncos de árvores, enquanto a população estudada foi coletada em solo seco, entre musgos que pertencem ao gênero *Syntrichia* (muito comum em crostas biológicas – BÜDEL, 2003c; SEPPELT et al., 2016).

Entretanto, *S. javanicum* é um organismo comumente registrado em crostas biológicas, até mesmo sendo considerado um estruturador (RIVERA-AGUILAR et al., 2006) e também muito comum em solos brasileiros, já tendo sido registrado no sudoeste do país (KOMÁREK et al., 2013).

Observação: espécimes encontrados em amostra da natureza

Ocorrência nas áreas estudadas: Barra de Santa Rosa (Amostra CD1).

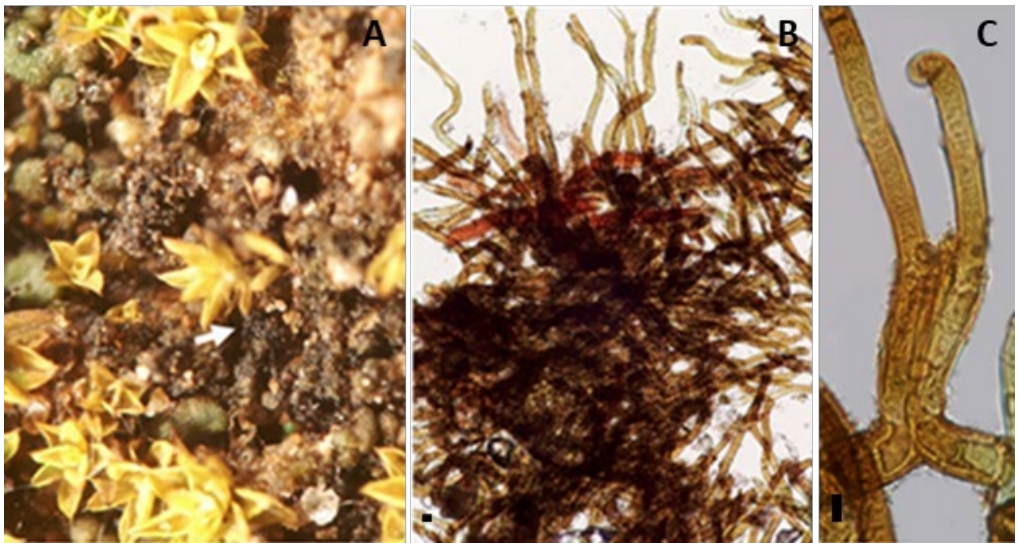

Figura 9. *Scytonema javanicum*. Aspecto geral dos filamentos quando na crosta (a); seta preta: filamentos. Disposição dos filamentos (b). Ramificação falsa (c). Escalas: 10µm. Fonte: figura do autor.

***Scytonema millei* Bornet et Flahault**

Ann. Sci. Nat., Bot., Sér. 5, 51: 129, 1886

Figura 10.

Filamentos marrom escuros, entrelaçados, prostrados, 19,0-24,0 µm diâm. Ramificações falsas, na maioria das vezes duplas, ramos com a mesma morfologia do filamento principal. Bainha espessa, marrom-amarelada, lamelada paralelamente. Tricomas constritos no nível das paredes transversais, azul esverdeados ou marrom claros, 12,0-15,0 µm diâm. Células isodiamétricas ou mais curtas que largas, 3,0-13,0 µm compr. Heterócitos solitários, cilíndricos ou quadráticos, 12,0-15,0 compr.

Comentários: Os espécimes observados estão de acordo com a descrição morfológica da espécie feita por Komárek (2013). A espécie é descrita como habitando rochas, solos úmidos e troncos de árvores, e embora a população estudada seja de crosta biológica, a massa observada estava sobre pequenas pedras.

Scytonema millei também foi registrada em crostas biológicas, no Deserto da Namíbia e em Savana seca no Kalahari, África (BÜDEL et al., 2009).

Observação: espécimes encontrados em amostra da natureza.

Ocorrência nas áreas estudadas: Cabaceiras (amostra CA7).

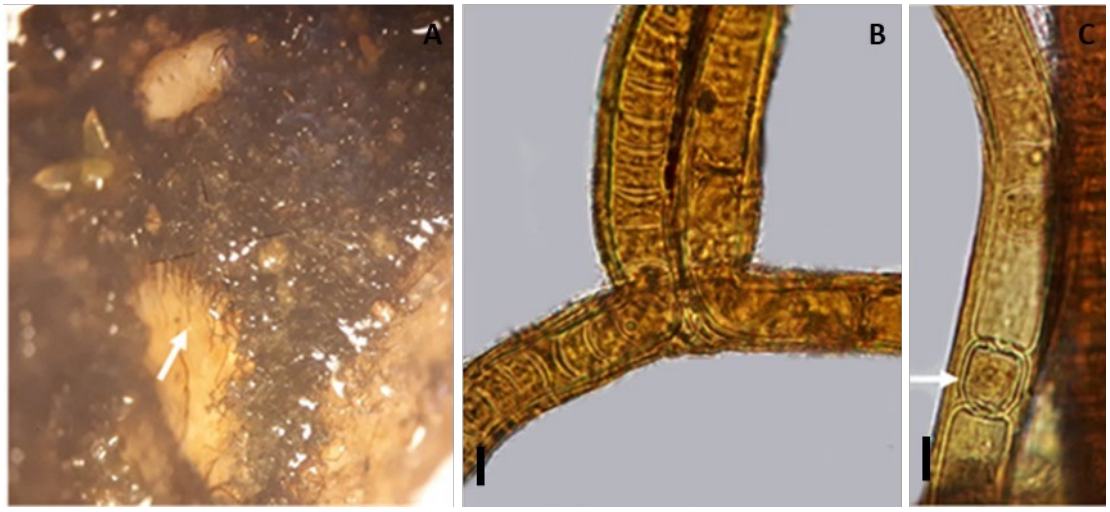

Figura 10. *Scytonema millei*. A) Aspecto geral dos filamentos quando em partícula de rocha na crosta. B) Ramificação falsa. C) Heterócito (seta branca). Escalas: 10µm. Fonte: figura do autor.

***Scytonema hyalinum* Gardner**

Mem. N. Y. Bot. Gard. 7: 1-144. 1927

Figura 11.

Filamentos em cultura formando tufos, emaranhados, 13,0-16,0 µm diâm. Ramificações falsas, na maioria das vezes duplas, ramos com a mesma morfologia do filamento principal. Bainha fina, hialina. Tricomas constritos no nível das paredes transversais, azul esverdeados, 12,0-14,0

µm diâm. Células quadráticas ou mais curtas que largas, 4,0-11,0 µm compr. Heterócitos solitários ou em dois, cilíndricos, amarelados, 7,0-13,0 compr.

Comentários: Os espécimes observados estão de acordo com a descrição morfológica da espécie feita por Komárek (2013), entretanto, não apresentaram cápsulas espessas ou lameladas nas células terminais.

Segundo Komárek (2013), a espécie ocorre em rochas, entre musgos e em águas termais na China, mas devem ter distribuição mais ampla, incluindo regiões tropicais. Já as populações registradas em regiões desérticas foram consideradas como prováveis espécies diferentes

Observação: espécimes encontrados em amostra de cultivo.

Ocorrência nas áreas estudadas: Área CD (amostra U1).

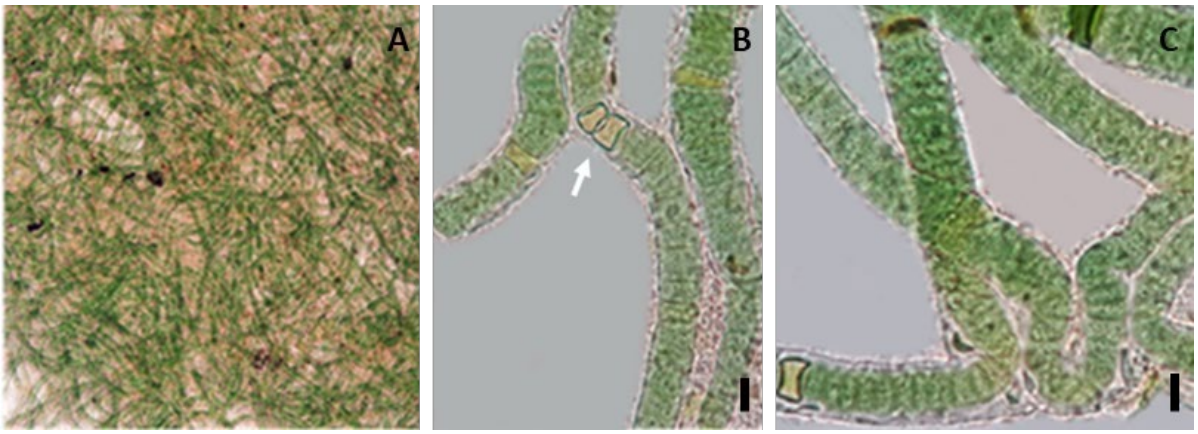

Figura 11. *Scytonema hyalinum*. A) Aspecto geral dos filamentos. B) Heterócito (seta branca). C) Ramificação falsa. Escalas: 10µm. Fonte: figura do autor.

A sequência molecular do gene 16S rRNA de *Scytonema hialynum* contou com 1162 pares de base, mostrando alta similaridade com sequências do banco de dados identificadas como *Scytonema* sp. e *Scytonema hyalinum.* Estas relações comprovam a identificação da espécie, principalmente por todas as amostras relacionadas terem origem em solos do Novo México, uma região rica em crostas biológicas (HOUSMAN et al., 2006; FERNANDES et al., 2017).

Tabela 4. Correspondência da sequência do 16S rRNA da linhagem U1 comparada com sequências de cianobactérias do GenBank (NCBI).

I (%)	**C (%)**	**Organismos mais próximo (nº de acesso)**	**Habitat da Amostra**
99,8	100	*Scytonema* sp. (HQ847553.1)	Aéreo (solo)
99,8	99	*Scytonema hyalinum* (KY365485.1)	Aéreo (solo)
99,7	100	*Scytonema* sp. (HQ847552.1)	Aéreo (solo)
99,7	99	*Scytonema hyalinum* (MF574180.1)	Aéreo (solo)

I = Identidade; C= Cobertura. Fonte: tabela do autor.

2.4 Conclusão

Em todas as quatro localidades estudadas foram encontradas populações de *Scytonema* e *Microcoleus,* além de *Nostoc* também ter sido registrada em três das quatro regiões. Estes gêneros são muito comuns em crostas biológicas, sendo *Microcoleus*, *Scytonema* e *Nostoc* registrados em crostas de pelo menos quatro continentes (BÜDEL et al., 2017). *Microcoleus* é o principal colonizador primário em crostas (GARCIA-PICHEL, 2001; GUNDLAPALLY & GARCIA-PICHEL, 2006; BÜDEL et al., 2009; HAGEMANN et al., 2015) enquanto *Scytonema* e *Nostoc* aparecem em estádios mais tardios do desenvolvimento (YEAGER et al, 2004, 2007; FERNANDES et al., 2017).

Assim sendo, pode-se concluir que a diversidade de cianobactérias da Caatinga é semelhante, em nível genérico, à diversidade de crostas ao redor do globo, principalmente das de regiões áridas dos Estados Unidos (GARCIA-PICHEL, 2001; BELNAP, 2002; ROSENTRETER & BELNAP, 2003; YEAGER et al., 2004; FERNANDES et al., 2017).

Porém, é diferenciada da diversidade de crostas do próprio Cerrado brasileiro, em que, por exemplo, não foi encontrado um grande número de populações relacionadas a *Microcoleus* (MACHADO-DE-LIMA, 2016).

A quantidade de espécies identificadas apenas em nível genérico comprova a complexidade desses grupos e a presença de morfotipos ainda desconhecidos para ambientes pouco explorados.

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Capítulo III – New cyanobacterial genera found in biological soil crusts of savanna and semi-arid environments from South America

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3.1 Abstract

Nostoc is one of the most well-known genera within the cyanobacteria phylum, being widely distributed around the globe. Recently, investigations applying the polyphasic approach have shown that *Nostoc* is polyphyletic, and that its morphological traits, besides overlapping among its own species, are also present in many other genera. In this way, the genus requires further investigation to trace its phylogenetic history. To contribute to these investigations, this work studied populations from biological soil crusts of two Brazilian biomes, *Caatinga* and *Savanna.* The ten studied populations presented *Nostoc*-like morphology, but the phylogenetic analyses based on 16S rRNA gene and 16S-23S ITS sequences showed that they represent four new genera, here designed Nostocaceae 1, Nostocaceae 2 and Nostocaceae 3, and six new species. This studied contributed substantially to the systematic of cyanobacteria, showing the fragility of a taxonomy based just on morphological characters and emphasizing the importance of the molecular analyses. It also highlighted the importance of studying biological soil crusts and how diverse is the composition of cyanobacteria in these communities.

3.2 Introduction

The systematic revision presented fourteen years ago by Hoffmann et al. (2005) was the first attempt to link available genetic, ultrastructural, and phenotypic data to reevaluate the cyanobacterial taxonomy. Since then, the molecular data started to be even more requested as part of the polyphasic approach and resulted in a considered number of new genera and species inside cyanobacteria (FIORE et al., 2007; SIEGESMUND et al., 2008; STRUNECKÝ et al., 2011; MACHADO-DE-LIMA et al., 2017; HENTSCHKE et al., 2017).

However, even with this advent and the proposition of some taxa, many of the genera considered as polyphyletic are far to have their evolutionary relationships defined. These are the cases of *Phormidium* Kützing ex Gomont (STRUNECKÝ et al., 2011; MARTINS et al., 2016a,b, 2018; BUCH et al., 2017), *Leptolyngbya* Anagnostidis & Komárek (ZAMMIT et al., 2012; VAZ et al., 2015; BECERRA-ABSALÓN et al., 2018), *Lyngbya* Agardh ex Gomont (ENGENE et al. 2010; 2012), *Calothrix* Agardh ex Bornet & Flahault (BERRENDERO et al., 2011; VACCARINO & JOHANSEN, 2011) and *Nostoc* Vaucher ex Bornet & Flahault (ŘEHÁKOVÁ et al., 2007; HROUZEK et al., 2013).

The present work has focused on *Nostoc*, a genus referred since Paracelsus (1493-1541) and probably the first cyanobacterial genus to be named (OREN & VENTURA, 2017). *Nostoc* is the type genus of all the Nostocales, it is widely distributed, occurring in aquatic, terrestrial and some species live in symbiosis with fungi, mosses, liverworts, ferns and vascular plants (KOMÁREK, 2013). Commonly, their filaments are aggregated and entangled irregularly in rounded gelatinous colonies, that can be or not enveloped by a peripherical periderm, or forming amorphous, flattened mucilaginous mat. The trichomes of specimens of *Nostoc* are uniseriated, not branched, moniliform, isopolar and present differentiated cell, as akinetes and heterocytes (KOMÁREK, 2013).

The genus *Nostoc* is one of the most devoid of morphological synapomorphies, not presenting true branching, false branching, special positioning of the heterocytes, specialized end cells, tapering of the trichomes, heteropolarity, nor aerotopes, although, as apomorphic character, it presents the production of a mucilaginous investment (ŘEHÁKOVÁ et al., 2007). *Nostoc* contains 226 species and some of them show a considerable overlap in the morphological characters (GUIRY & GUIRY, 2020).

Through modern investigations applying the polyphasic approach, *Nostoc* was found to be polyphyletic (RAJANIEMI et al., 2005; HROUZEK et al., 2005; 2013; PAPAEFTHIMIOU et al., 2008; LUKEŠOVÁ et al., 2009; GENUÁRIO et al., 2010;

SILVA et al., 2014), but a well-supported cluster appears in many of the phylogenetic investigations. This cluster is composed of the type species of *Nostoc, N. commune* Vaucher ex Bornet et Flahault, and some other related species as *N. punctiforme* Hariot PCC 73102, *N. desertorum* Reháková & Johansen, *N. lichenoides* Reháková & Johansen, *N. indistinguendum* Reháková & Johansen and

N. edaphicum Kondrateva, whose all samples were collected from soils, leading to the proposition of the called "*Nostoc sensu stricto*" cluster (HROUZEK et al., 2005; ŘEHÁKOVÁ et al., 2007; LUKEŠOVÁ et al., 2009; PAPAEFTHIMIOU et al., 2008).

The occurrence of many different clades with *Nostoc*-like strains, distant from *Nostoc sensu stricto*, motivated deeper investigations and some of those resulted in the proposal of new genera. *Mojavia* Reháková & Johansen (REHÁKOVÁ et al., 2007) described based on soil populations, most from deserts, and presenting morphological differences from *Nostoc*, as significantly longer and wider heterocytes and vegetative cells. *Desmonostoc* Hrouzek & Ventura (HROUZEK et al., 2013) forms long vegetative filaments embedded in diffluent mucilaginous envelopes, but never forming a firm periderm, except in primary stages and never presenting filaments densely coiled with compact trichomes as found in *Nostoc*. *Halotia* Genuário et al. (GENUÁRIO et al., 2015) was described as saline tolerant, differing from *Nostoc* by ecological, physiological and genetic aspects. The genus *Komarekiella* Hentschke, Johansen & Sant'Anna (HENTSCHKE et al., 2017) is morphologically similar to *Desmonostoc*, *Halotia*, *Mojavia* and indistinguishable from *Chlorogloeopsis* Mitra et Pandey, being considered a cryptogenus. The genera *Allinostoc* Bagchi et al. (BAGCHI et al., 2017) and *Compactonostoc* Cai & Li (CAI et al., 2019) were recently described and both are morphologically *Nostoc*-like, but they are genetically and phylogenetically distant from the *Nostoc sensu stricto* clade.

In this way, although *Nostoc* is a well-known group, it requires a continue investigation to trace its phylogenetic story. Aiming to contribute with this investigation, this work studied ten samples from biological soil crusts of Brazilian biomes, two of them placed in well-established groups and other eight formed four new genera. All the new genera were morphologically similar to *Nostoc*, considering the aspect of colonies, trichomes and the presence of specialized cells. Therefore, the effective proposition of new taxa was just based on the genetic distinction found, with Nostocaceae 1, Nostocaceae 2, Nostocaceae 3 and Nostocaceae 4 placing distantly from *Nostoc sensu stricto* cluster and other groups.

3.3 Materials and methods

3.3.1 Sampling sites

The ten populations evaluated in this study were isolated from biological soil crust samples of Brazilian savanna and *Caatinga* localities (Table 1).

The Brazilian savanna, also known as *Cerrado*, is the second largest biome in Brazil, being present in 11 states of the country (SANO et al., 2007). It is characterized by a dry season over 5-6 months. Temperature ranges from 22°C to 27°C, while the rainfall varies from 750-800 mm (northeast side) to 2,000 mm (west side). Soils in this biome are mainly Oxisols, Ultisols and Entisols, which are poor in nutrients (LOPES & GUILHERME, 2016). Vegetation cover includes open grasslands, shrub lands, open woodlands and closed canopy woodlands (CARDOSO-DA-SILVA & LACHER-JR, 2019).

The *Caatinga* is one the hottest areas of the semi-arid regions around the world (AB'SABER, 1974). It covers 11% of the national territory of Brazil (IBGE, 2019), spanning nine states along the country. The temperatures range from 27°C to 29°C, while the irregular precipitations vary from 400 to 800 mm (AB'SABER, 1974). Soils are stony, shallow and with low capacity for retaining water (ALVES et al., 2009). The vegetation is characterized by seasonally dry tropical forest (PENNINGTON et al., 2009).

Table 1. Geographic origin of *Nostoc*-like strains studied.

Sample Code	**Coordinates**	**Origin**	**Biome**
2FUR	20º14’S, 47º27’W	State Park of *Furnas do Bom Jesus*, São Paulo State	Savanna
1CIP	19°20'S, 43°34'W	National Park of *Serra do Cipó*, Minas Gerais State	Savanna
2CAN	20°21'S, 46°38'W	National Park of *Serra da Canastra*, Minas Gerais State	Savanna
3FUR	20º14’S, 47º27’W	State Park of *Furnas do Bom Jesus*, São Paulo State	Savanna
2GO	17°47’S, 48°42’W	Caldas Novas, Goias State	Savanna
CATCC2	07°07’S, 36°08’W	Pocinhos, Paraiba State	Caatinga
CATCB5.1	07°24’S, 36°19’W	Cabaceiras, Paraíba State	Caatinga
CATCB5.2	07°24’S, 36°19’W	Cabaceiras, Paraíba State	Caatinga
CATCB4	07°24’S, 36°19’W	Cabaceiras, Paraíba State	Caatinga
CATCC10	07°07’S, 36°08’W	Pocinhos, Paraiba State	Caatinga

Table of the own author.

3.3.2 Cultivation and morphological evaluation

Each cyanobacterial strain was isolated after cultivation of crust portions in BG11 medium (RIPPKA et al., 1979) followed by repeated inoculations of colonies, pieces of biomass, or even bundles. The unicyanobacterial cultures were maintained under 20 ± 1ºC, 60 µmol photons m^{-2} s^{-1} of irradiance and a 14:10 h light-dark cycle in the culture collection ofIBILCE/UNESP.

Morphological variability of populations was evaluated from cultured samples by using an Olympus BH2 microscope. Taxonomic features, such as morphology of colonies, width, length and shape of vegetative cells, akinetes and heterocytes were analyzed in at least 30 trichomes for each sample.

3.3.3 Molecular analyses

DNA was obtained from non-axenic unicyanobacterial cultures. The filaments were washed with deionized water, several times through the centrifugation process, eliminating or reducing the mucilage and growth medium substances attached. Thereafter, the DNA was

extracted using the 'PowerSoil DNA Isolation' kit (MO BIO Laboratories) according to the manufacturer's protocol. The step of lyse was performed by "Precellys 24 Tissue Homogenizer", a homogenizer based on bead beating technology.

The 16S rRNA partial gene and 16S-23S internal transcribed spacer (ITS) markers were amplified by polymerase chain reaction (PCR) with the primers CYA 359 (NÜBEL et al., 1997) and 23S30R (TATON et al., 2003), which was performed in a 'Techgene TC-512' thermal cycler (Techne) using 25 µL of total reaction volume containing 5 µL 10X PCR buffer, 2 µL 50 mM MgCl2, 1 µL 10 mM dNTP mix, 1.25 µL of each primer (5 ρmol), 14.2 µL 'Milli-Q' water, 1.5 U 'Platinum Taq DNA polymerase' (Life Technologies) and 10 ng genomic DNA. Thermal cycling was 94°C for 5 min, followed by ten cycles of 94ºC for 45 s; 57ºC for 45 s and 72ºC for 2 min; 25 cycles of 92ºC for 45 s and 54ºC for 45 s; and one final cycle of 72ºC for 7 min. The PCR products were analyzed on 1% agarose gels stained with 'GelRed 0.6X' (Biotium) and viewed on 'Mini Bis Pro' transilluminator (Micro Photonics). The positive products were cloned using the 'pGEM-T Easy Vector System I' (Promega) following the supplier's manual. Competent *Escherichia coli* DH5α cells were transformed by heat shock and recombinant plasmids were isolated by the 'ZR Plasmid Miniprep' kit (Zymo Research). Sequencing was conducted by an 'ABI 3130 sequencer', using 'Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction' kit (Applied Biosystems) following the manufacturer's protocol. Primers 359F (NÜBEL et al., 1997), 704R, 1114F, 1494R (NEILAN et al., 1997) and 23S30R (TATON et al., 2003) were used for sequencing. The DNA fragments were assembled into contigs using the 'Phred/Phrap/Consed' software (EWING & GREEN,1998, EWING et al., 1998, GORDON et al., 1998) and only bases with quality higher than 20 were considered.

3.3.4 Phylogenetic analyses

A database including sequences produced in this study and their closely related ones previously published in NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) as indicated by BLAST (http://www.ncbi.nlm.nih.gov/BLAST) was constructed for the phylogenetic analyses. Additional sequences from NCBI, closely related to *Nostoc*, were incorporated to the data matrix to structure the analyses. The alignment was carried out using the software 'ClustalW 1.8' (THOMPSON et al., 1994) in 'MEGA 6' (TAMURA et al., 2013) and inspected and refined manually. The unicellular cyanobacteria *Gloeobacter violaceus* and *Synechococcus*

elongatus (GenBank access number NR074282, NR074309) were designated as the evolutionary outgroup. Phylogenetic analyses were based on partial 16S rRNA gene sequences (1105 bp). The appropriate nucleotide substitution models were selected in 'jModelTest 2.1.1' (DARRIBA et al., 2012). Maximum-likelihood (ML) inferences were performed using 'MEGA 6' software (TAMURA et al., 2013) and Kimura-2p model was applied in the last method assuming heterogeneous substitution rate and gamma substitution of variable sites. Bootstrap resampling was performed on 1000 replicates. Sequence similarity matrix/nucleotide divergence was calculated in 'Geneious 7' (Kearse et al., 2012) considering the alignment in all positions, including gaps.

3.3.5 Secondary structure of conserved ITS regions and p-distance among ITS sequences

The conserved domains D1-D1', Box-B and V3 regions (ITEMAN et al., 2000) were identified and their secondary structures were determined using 'Mfold Web 2.3' (ZUKER, 2003). The tRNA sequences were identified with 'tRNAscan-SE 1.21' (LOWE & EDDY, 1997). Sequence alignment was performed using the software "ClustalW 1.8" (THOMPSON et al., 1994) in "MEGA 7" (KUMAR et al., 2016) and the p-distance was also generated by "MEGA 7".

3.4 Results

3.4.1 Morphological evaluations

Ten populations were isolated from the BSCs, starting their development with the formation of colonies thereafter becoming amorphous mats.

The studied populations presented the typical *Nostoc* morphology for colonies or amorphous mats, filaments and their mucilaginous sheaths and the different cell types (vegetative cells, heterocytes, and akinetes). However, despite the observation of a general pattern in common for all strains, the populations could be separated in eight morphotypes (Table 2).

The first morphotype was characterized by the presence of microscopic colonies containing loosely entangled filaments and barrel-shaped cells and heterocytes, with general morphology in accordance with the description of *N. commune*. The second morphotype shared morphological traits with *Desmonostoc muscorum*. The samples 2CAN, 2GO and 3FUR were very similar, but could be separated in two morphotypes due the difference in the length of the vegetative cells, in relation to the measurements of the heterocytes and in the measurements and content of the akinetes. The fifth morphotype was represented by CATCC2, showing trichomes isolate or few together or cells/trichomes in microscopic colonies, which were grouped and forming floccose aggregates. CATCC10 and CATCB4 presented similar morphology, but they differed in relation to the diameter of their colonies, and by the length of the vegetative cells. The last morphotype was characterized by microscopic colonies, nearly spherical to irregular forming dark green mass aggregates and its vegetative cells could have many different shapes.

3.4.2 Molecular analyses and phylogeny – 16S rRNA

Phylogenetic analyses disposed the Nostocaceae family intermixed with other families, Aphanizomenonaceae, Coleochaetaceae, Fortiaceae and Tolypothricaceae, and *Nostoc* strains were spread in many different clades. Some of recent genera, derived from *Nostoc*, placed separated and in well supported nodes, is the case of *Desmonostoc*, *Halotia*, *Komarekiella*, *Aliinostoc* and *Compactonostoc.* Seven strains identified as *Calothrix* and two as *Scytonema* maintained well separated and as a more external group in comparison with the other sequences of heterocytous cyanobacteria (Figure 26). The ten studied sequences were placed in six different clades, in the same way the similarity matrix based on 16S rRNA had separated them. 2FUR was closed related with other *Nostoc* sequences including the type species, *Nostoc*

commune, forming a well-supported clade (87% of bootstrap). The partial 16S rRNA (1098 pb) of the sequence was compared with the other sequences within the clade and revealed similarity of 95.3 to 97.65%. Therefore, 2FUR was considered as a new species for the genera, *Nostoc savannensis*.

1CIP placed with many other *Desmonostoc* strains from GenBank (92% of bootstrap), showing similarity of 16S rRNA higher than 99% with many *Desmonosto*c sp. strains, being considered the same species, here named as *Desmonostoc brasiliensis*. CATCC2 grouped with two other strains from GenBank, both identified as *N. calcicole* Brébisson ex Bornet & Flahault with 98% of bootstrap, presenting high similarity of 16S rRNA sequence (99.4%), composing the separated genus Nostocaceae 1.

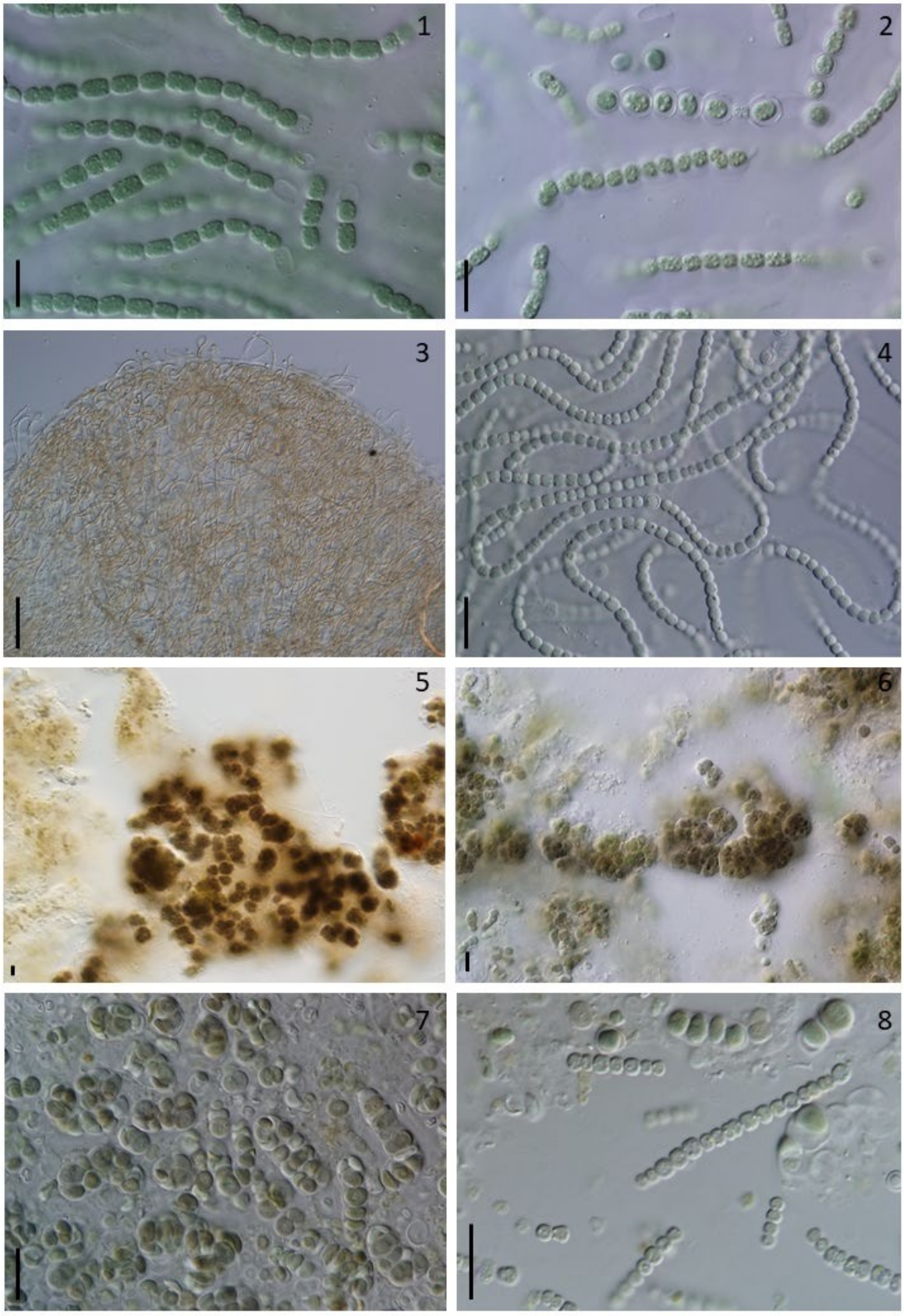
2CAN, 2GO and 3FUR grouped together with good support (78% of bootstrap), showing to be a separated genus, here named Nostocaceae 2. 2GO and 3FUR presented similarity of 16S rRNA of 99%, being considered as Nostocaceae 2 sp. 1, while in comparison with 2CAN the similarity varied from 97.74 to 97.92, therefore, being separated in Nostocaceae 2 sp. 2. CATCB5.1, CATCB5.2 and *Nostoc* sp. CENA239 from GenBank grouped with 91% of bootstrap, forming a separated new genus, Nostocaceae 3. The similarity of 16S rRNA among CATCB5.1 and CATCB5.2 was 99.28%, while varied of 97.74-97.92 in relation to *Nostoc* sp. CENA239.

CATB4, CATCC10 and other sequences from GenBank identified as *Nostoc* grouped with 78% of bootstrap with other two genera, *Desikacharya* Saraf & Prashant Singh and *Minunostoc* F.Cai & R.Li forming the clade The similarity of16S rRNA among all the strains in the clade varied from 93.5 to 100%, and of 97.1% between the samples CATCB4 and CATCC10, which were considered two different species.

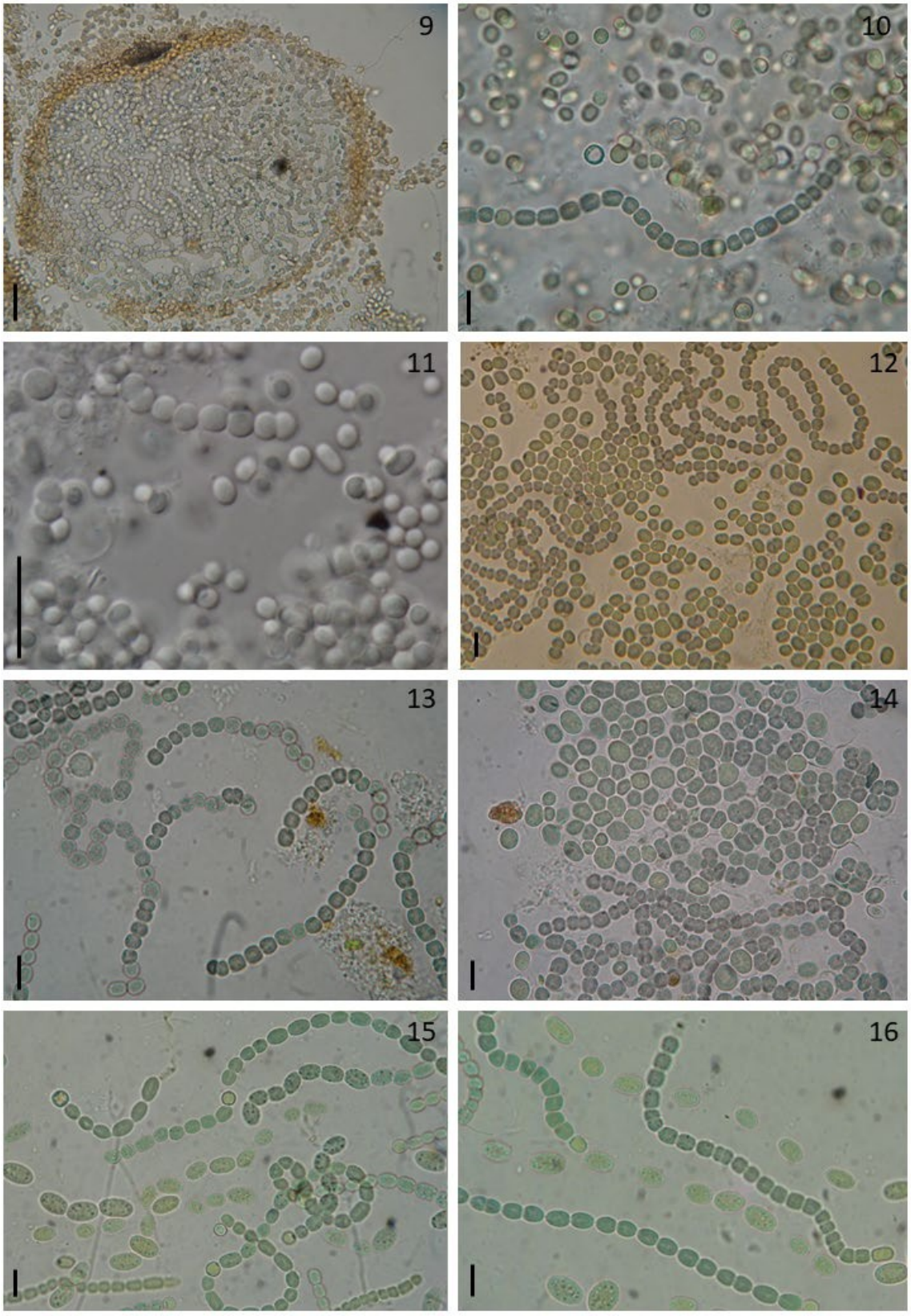
Table 2. Morphological comparison among *Nostoc*-like strains. Dimensions (µm) represent full ranges observed.

Population	Veg. cell length	Veg. cell width	Veg. cell length/ width	Het. length	Het. width	Het.	Akinete length	Akinete width	Akinete length/width
2FUR	2.0-5.0 (3.0)	3.0-5.0 (4.0)	0.4-1.2	4.0-6.0 (4.9)	4.0-6.0 (5.3)	0.9-1.2	N.O.	N.O.	N.O.
1CIP	2.9-6.5 (4.6)	2.5-4.0 (3.0)	0.7-2	4.0-8.0 (5.7)	5.0-7.0 (5.7)	0.8-1.3	7-8 (7.5)	4.0-5.5 (4.8)	1.4-2.0
2CAN	3.0-9.0 (5.5)	3.0-6.0 (4.1)	0.7-2.2	2.1-2.6 (2.4)	4.0-6.0 (5.3)	-	9.0-12.0 (10.6)	6.0-7.0 (6.3)	1.4-1.8
3FUR/2GO	3.0-6.0 (4.7)	3.0-5.4 (3.85)	0.75-1.7	1.8-2.8 (2.3)	2.1-3.1 (2.6)	1.8-2.8	6.5-8.0 (7.3)	5.0-5.5 (5.1)	0.9-1.6
CATCC2	1.3-4.0 (1.9)	1.8-3.0 (2.4)	0.5-1.4	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.
CATCB5.1/ CATCB5.2	4.0-8.8 (6.1)	3.6-7.2 (5.2)	1.0-1.6	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.
CATCB4	1.7-4.5 (3.1)	1.8-2.9 (2.5)	0.7-1.8	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.
CATCC10	1.4-3.1 (2.4)	1.7-2.9 (2.3)	0.7-1.4	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.

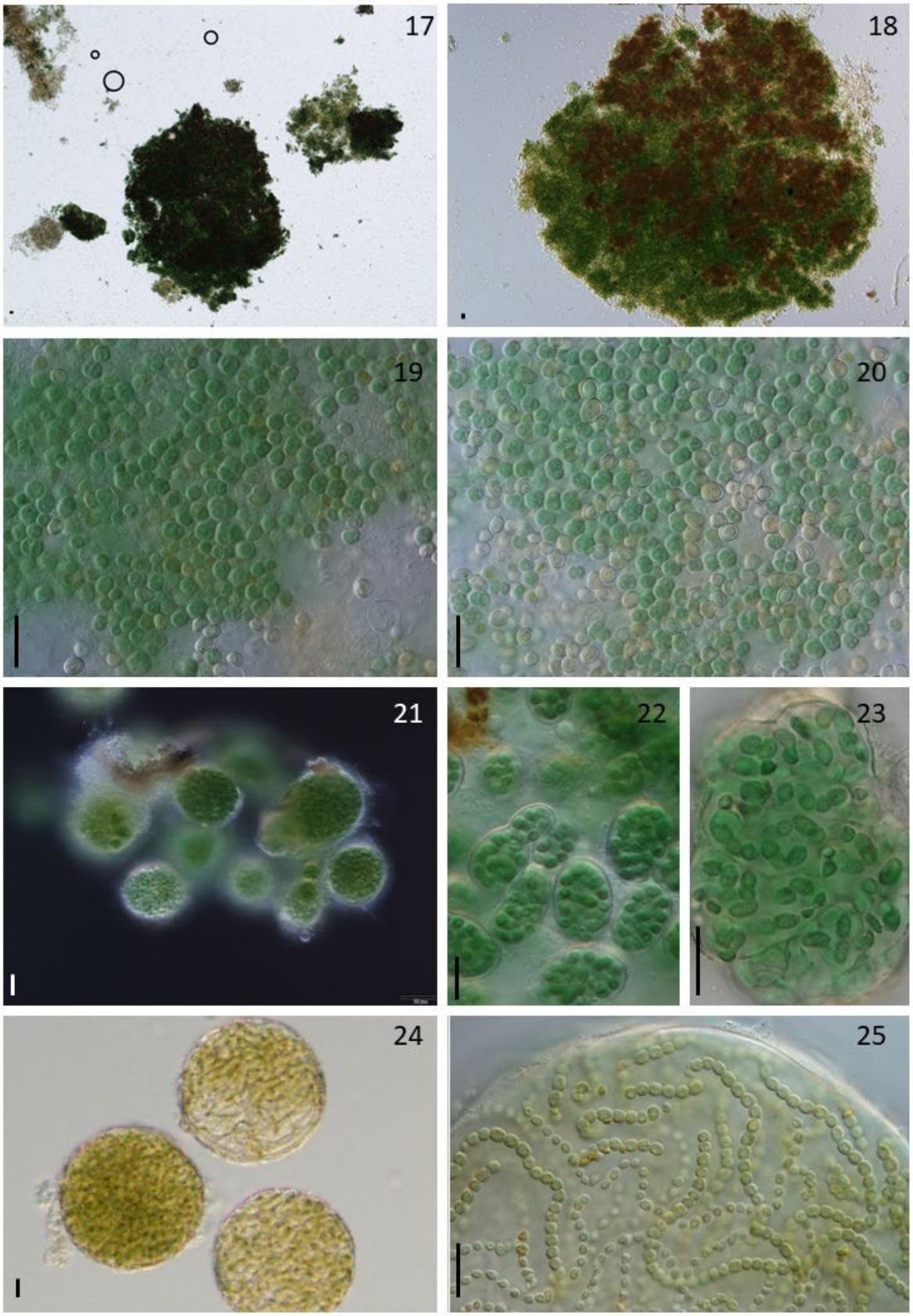
Veg. = Vegetative; Het. = Heterocytous. Table of the own author.



Figures 1-8: 1, 2: *Nostoc savannensis* (FA2)*;* 3, 4: *Desmonostoc brasiliensis* (1CIP); 5-8: Nostocaceae 1 sp. (CATCC2). Bars = 10 µm. Figure of the own author.



Figures 9-16: 9-14 Nostocaceae 2 sp. 1 (3FUR, 2GO); 15, 16 Nostocaceae 2 sp.2 (2CAN). Bars = 10 µm. Figure of the own author.



Figures 17-25: 17-20 Nostocaceae 3 sp. (CATCB5.1); 21-23 population CATCB4; 24, 25 population CATCC10. Bars = 10 µm. Figure of the own author.

3.4.3 Molecular analyses – 16S-23S ITS

The lengths of 16S-23S ITS regions were analyzed comparing the strains which were close related according to 16S rRNA (Tables 3 and 4). The sample *Nostoc savannensis* 2FUR and strains *Nostoc commune* (AY579904) and *N. lichenoides* (AY579895) presented similar lengths for the different regions. *Nostoc* sp. KC854771 showed a total length much longer and including the presence of tRNAs. *Nostoc savannensis* 2FUR presented secondary structures different of other *Nostoc sensu stricto* strains, with an exception for Box-B structure that was the same of *Nostoc lichenoides* (AY579895 - Figures 26, 27).

No variations in ITS was observed between *Desmonostoc brasiliensis* 1CIP and *Desmonostoc* sp. (KF761564), both having identical lengths for each region and identical secondary structures for all the three domains.

The strains pertaining to the new genus Nostocaeae 2 had similar total length, tRNAs and were identical in most of the regions, except by Pre-Box-B and V3 with spacer. The D1-D1', Box-B and V3 domains reaffirmed the results based on 16S rRNA, showing the same structures between Nostocaceae 2 sp.1 2GO and Nostocaceae 2 sp. 1 3FUR, and very similar to Nostocaceae 2 sp.2 2CAN. The p-distance between Nostocaceae 2 sp.1 2GO and Nostocaceae 2 sp. 1 3FUR was 0.006, while higher than 0.13 when in comparison with Nostocaceae 2 sp.2.

Nostocaceae 3 strains presented identical total length, tRNAs and identical secondary structures for all the main regions. They could not be compared with *Nostoc* sp. CENA 239, because its whole ITS region is not available in GenBank.

CATCB4 and CATCC10 showed identical length to D1-D1'and D2, but the other regions could not be compared because CATCC10 did not have the 16S-23S ITS complete sequenced. All the other *Nostoc* sp. CENA strains of this clade presented tRNAs.

Nostocaceae 1 CATCC2 could not be compared with the *Nostoc calcicola* strains from GenBank because there are no 16S-23S ITS published sequences for the species.

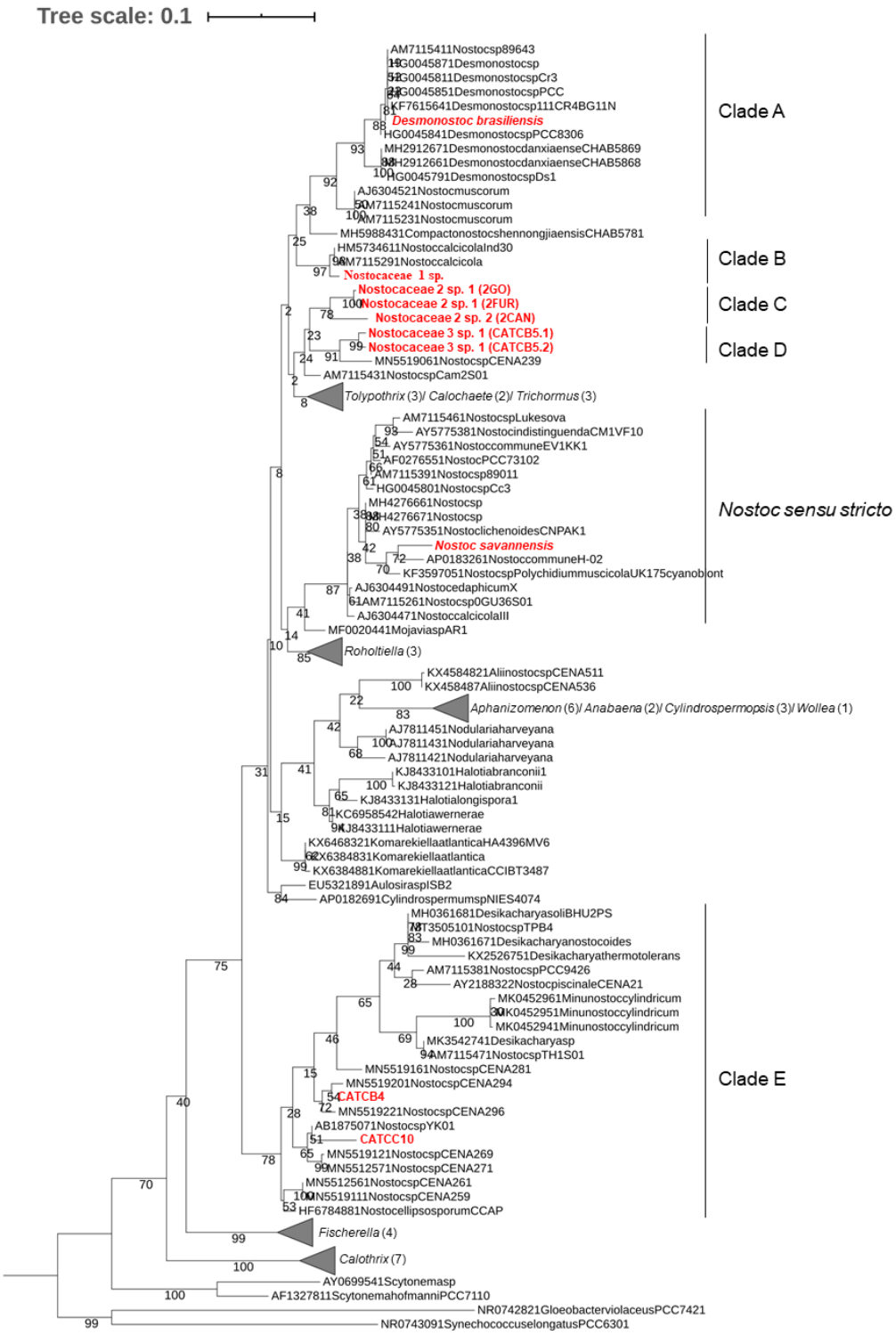


Figure 26. Maximum Likelihood tree based on 16S rRNA gene sequences of Nostocales strains. Codes in red boldface represent the strains analyzed in this study. Bootstrap values (ML >70%) shown at nodes. GenBank accession numbers before sample identification. Bar represents 0.1 substitutions per nucleotide position. Fonte: figura do autor. Figure of the own author.

Table 3. 16S rRNA identity (I), query cover (C) and most related organism of the studied strains according to GenBank.

Strain	**Length (bp)**	**I (%)**	**C (%**	**Most related organism (accession number)**
Nostoc savannensis (2FUR)	1166	98	100	*Nostoc* sp. (KF359705.1)
Desmonostoc brasiliensis (1CIP)	1166	99	100	*Desmonostoc* sp. (KF761564.1)
Nostocaceae 1 sp. (CATCC2)	1165	99	99	*Nostoc calcicola* (HM573461.1)
Nostocaceae 2 sp. 1 (3FUR)	1108	97	99	*Nostoc* sp. (AM711543.1)
Nostocaceae 2 sp. 1 (2GO)	1165	98	99	*Nostoc* sp. (AM711543.1)
Nostocaceae 2 sp. 2 (2CAN)	1165	97	100	*Cylindrospermum* sp. (AP018269.1)
Nostocaceae 3 sp. 1 (CATCB5.1)	1165	97	99	*Nostoc* sp. (AM711543.1)
Nostocaceae 3 sp. 1 (CATCB5.2)	1167	97	99	*Nostoc* sp. (AM711543.1)
CATCB4	1163	98	97	*Nostoc* sp. (AB187507.1)
CATCC10	1165	98	99	*Nostoc ellipsosporum* (HF678488.1)

Fonte: tabela do autor.

Table 4. Lengths of 16S-23S ITS regions (number of nucleotides) in the analysed sequences

Strain	ITS until V3	Leader	D1-D1' helix	D2 with spacer	D3 with spacer	$tRNA^{Ile}$ gene	$tRNA^{Ala}$ gene	Pre-Box-B spacer	Box-B helix	Post-Box-B spacer	Box-A	D4	V3
Nostoc savannensis (2FUR)	267	6	68	31	43	-	-	-	28	17	12	13	39
AY579904 *N. commune*	274	5	68	32	44	-	-	-	31	9	12	13	43
AY579895 *N. lichenoides*	274	5	68	32	48	-	-	-	28	17	12	13	39
KC854771 *Nostoc* sp.	533	8	66	41	17	73	72	47	34	17	12	13	40
Desmonostoc brasiliensis (1CIP)	259	5	66	30	39	-	-	-	27	17	12	13	42
KF761564 *Desmonostoc* sp.	259	5	66	30	39	-	-	-	27	17	12	13	42
Nostocaceae 1 sp. (CATCC2)	497	5	65	32	18	73	72	31	31	18	12	13	36
Nostocaceae 2 sp. 2 (2CAN)	509	5	66	33	16	73	72	38	36	16	12	13	35
Nostocaceae 2 sp. 1 (3FUR)	524	5	66	33	15	73	72	37	37	17	12	13	50
Nostocaceae 2 sp. 1 (2GO)	524	5	66	33	15	73	72	37	37	17	12	13	50
Nostocaceae 3. sp. 1 (CATCB5.1)	257	5	66	29	31	-	-	-	34	18	12	13	35
Nostocaceae 3 sp. 1 (CATCB5.2)	257	5	66	29	31	-	-	-	34	18	12	13	35
CATCB4	259	5	62	29	39	-	-	-	28	16	12	13	35
CATCC10	*	5	62	29	*	*	*	*	*	*	*	*	*
Nostoc sp. CENA294	*	5	63	31	10	73	72	11	50	18	12	13	*

Table fo the own author.

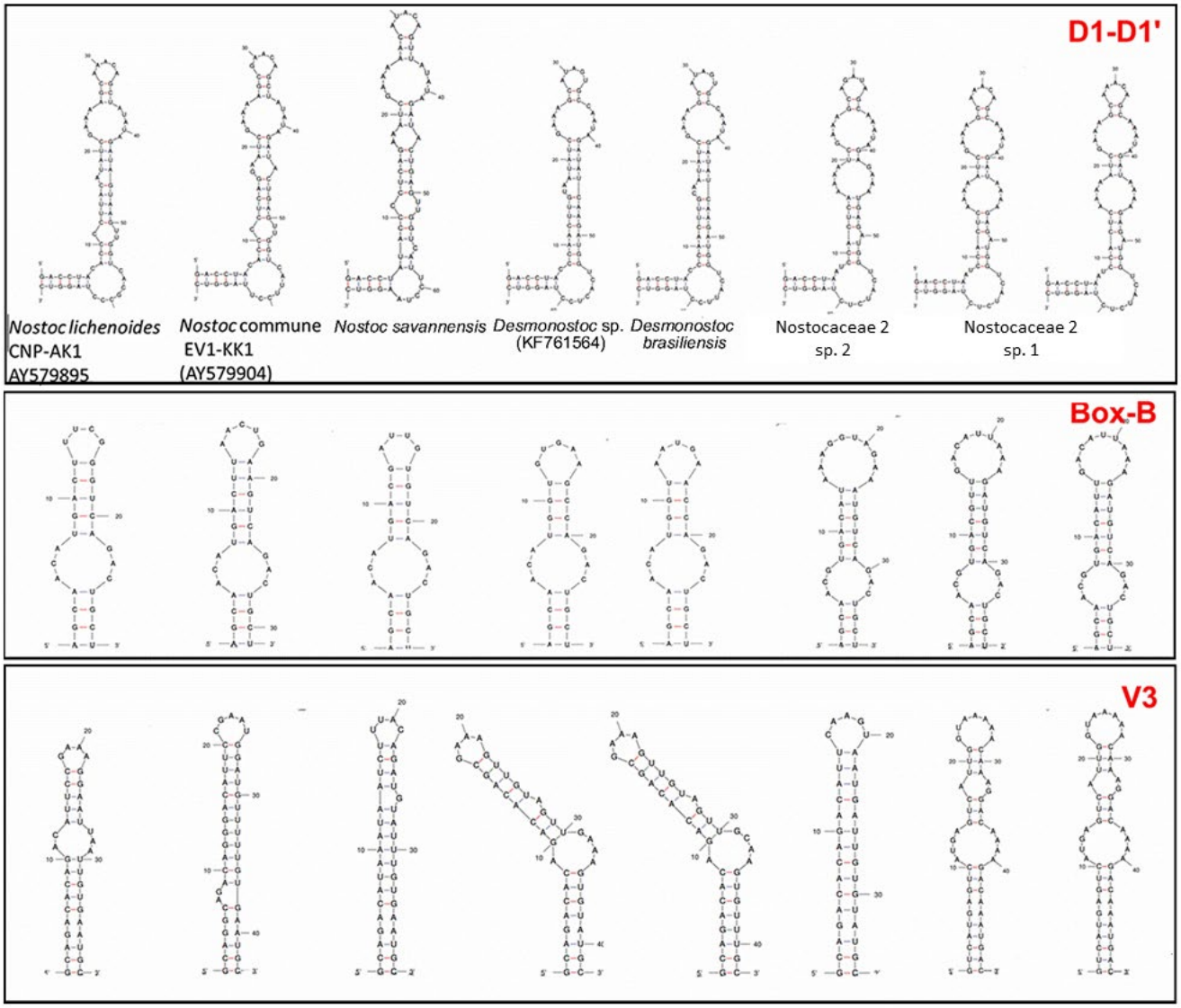


Figure 26. Secondary structure of conserved regions of 16S-23S ITS of *Nostoc*-like strains. D1-D10 helices, Box-B helices and V3 helices.

Figure of the own author.

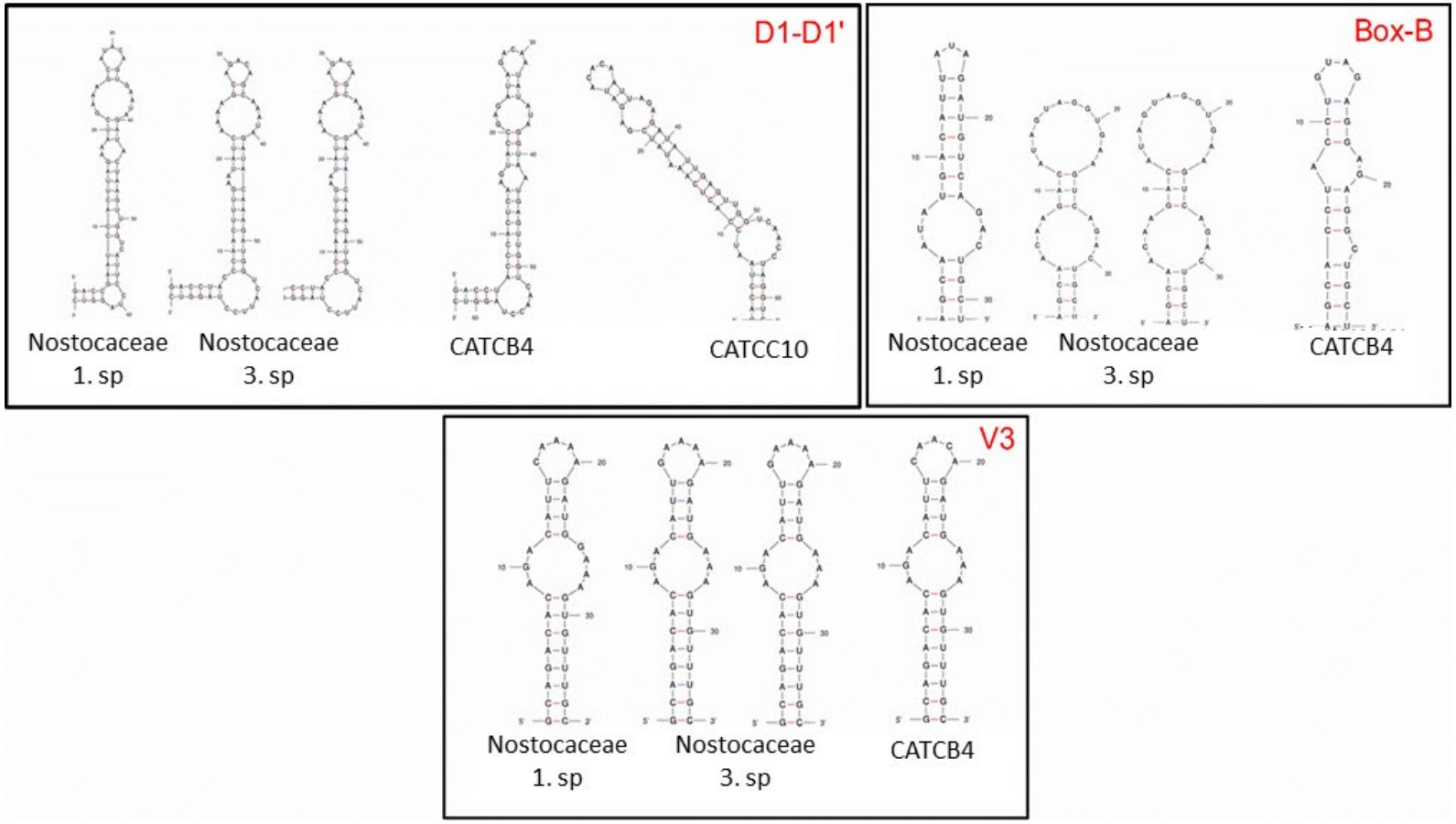


Figure 27. Secondary structure of conserved regions of 16S-23S ITS of *Nostoc*-like strains. D1-D10 helices, Box-B helices and V3 helices. Figure of the own author.

3.5 Discussion

Nostoc morphospecies are very common with occurrence records in many different habitats, but a special attention is given when it is found in BSC (ROSENTRETER & BELNAP, 2003; BÜDEL, 2003a; b; BÜDEL et al., 2016). The presence of the specimens pertaining to this genus is easily notable by the black color given to the dark crust and such coloring also permits the estimation of the crust development stage as mature (YEAGER et al., 2004; FERNANDES et al., 2017). Hence, investigations about *Nostoc* are essential for works on crusts, not only for describing the diversity of the genus, but also for providing basis to future applied works as restoration practices.

The genus presents similar morphology to many genera recently described (HROUZEK et al., 2013; GENUÁRIO et al., 2015; HENTSCHKE et al., 2017; BAGCHI et al., 2017; CAI
et al., 2019), having with them a very clear genetic distinction that characterized the genus as polyphyletic (RAJANIEMI et al. 2005; HROUZEK et al. 2005, 2013; PAPAEFTHIMIOU et al. 2008; LUKEŠOVÁ et al. 2009; GENUÁRIO et al. 2010; SILVA et al. 2014. This morphological similarity not supported by genetic distance was also observed in all the new genera proposed in this study.

The *Nostoc*-like strains were separated in new genera based on their distant phylogenetic position, according to 16S rRNA gene sequences, in relation to the *Nostoc sensu stricto* clade, the well-established cluster that represents *Nostoc* as long it harbors the type species of the genus (HROUZEK et al., 2005; ŘEHÁKOVÁ et al., 2007; PAPAEFTHIMIOU et al., 2008; LUKEŠOVÁ et al., 2009).

The seven new described species were proposed in accordance with the 16S rRNA gene similarity threshold (98.65% - KIM et al., 2014), supported by the p-distance values among 16S-23S ITS, that it was already used in past works to separate species (<0.03 p-distance values to infer strains pertaining to the same species and >0.07 values to separate species - ERWIN & THACKER 2008, OSORIO-SANTOS et al., 2014; PIETRASIAK et al., 2014; 2019).

The new species *Desmonostoc* brasiliensis could be considered the same species represented by sequences of several strains named *Desmonostoc* sp. (KF761564, HG004587, HG004581, HG004585, HG004584) and *Nostoc* sp. 8964 (AM71154) in the GenBank. However, despite the genetic similarity among the strains, this is not the unique point to be considered. Strains *Desmonostoc* sp. HG004584 and HG004587 are from Europe and from a completely different habitat in comparison with biocrusts.

Nostocaceae 1 sp. showed a strong relation with two strains designed as *Nostoc calcicola* in the GenBank (AM711529, HM573461), but the proposition of the three populations as pertaining to the same species is unsustainable. The two populations from GenBank do not have available morphological information and are from different geographical regions, aside from presenting habitat not similar to biocrusts. The morphology of Nostocaceae 1 sp. is also not in accordance with the described for *Nostoc calcicola*.

The populations CATCB4 and CATCC10, together with the Brazilian samples from the Genbank that also grouped in their clade, may pertain to the same genus. These samples were sampled in the same geographical region and present the same ecological habitat. However, their relationship with other strains from the Genbank, also with *Desykacharia* and *Minunostoc* strains could not be solved using the 16s rRNA. The determination of the correct phylogenetic position of these strains requires studies with the addition of new sequences.

The evaluation of the ten samples contributed with the systematic of cyanobacteria, showing the fragility of a taxonomy based just on morphological characters and emphasizing the molecular data analysis through the proposition of four new genera. This study shows how diverse and poorly known is the cyanobacterial taxonomic composition of biocrusts and suggests different ecological roles being played by the different components of such communities. The present results also reinforce the need for more detailed studies on biological soil crusts worldwide.

Description of the new genera and species

Nostoc savannensis N.M. Machado-de-Lima et Branco, *sp. nov.*

Figs.: 1, 2.

Colonies microscopic, irregular to spherical, mucilaginous envelope hyaline. Trichomes long, flexuous, constricted, cylindrical, loosely entangled. Cells barrel-shaped, 2.0-5.0 µm long (3.0 µm in average), 3.0-5.0 µm diam. (4.0 µm in average), mostly short, occasionally isodiametric or longer, length/width ratio 0.4 to 1.2. Cell content granulated, periplasm and centroplasm distinguishable, blue-green to green. Heterocytes barrel-shaped, squarish or nearly spherical, 4.0-6.0 µm long (4.9 µm in average), 4.0-6.0 µm diam. (5.3 µm in average), content yellowish. Akinetes not observed.

Habitat: topsoil.

Code: 2FUR

Desmonostoc brasiliensis N.M. Machado-de-Lima et Branco, *sp. nov.*

Figs.: 3, 4.

Colonies microscopic, irregular to spherical, mucilaginous envelope hyaline. Filaments flexuous, long, loosely entangled, commonly regularly arranged within the colonies, 5.5-9.2 µm diam. (7.8 µm in average). Sheath hyaline, lamellate, constricted, consistent. Trichomes constricted, cylindrical. Cells barrel-shaped, 2.9-6.5 µm long (4.6 µm in average), 2.5-4.0 µm diam. (3.0 µm in average), shorter, isodiametric or longer than broad, length/width ratio 0.9 to

2.4 (1.6 in average). Cell content granulated, periplasm and centroplasm distinguishable, blue-green to green. Heterocytes barrel-shaped, elliptic, conical rounded or nearly spherical, 4.0-8.0

µm long (5.7 µm in average), 5.0-7.0 µm diam. (5.7 µm in average), content yellowish. Akinetes in rows (up to 15 cells), paraheterocytic, elliptical to cylindrical, with prominent granules. 7.0-8.0 µm long (7.5 µm in average), 4.0-5.5 µm diam. (4.8 µm in average), epispore thick, hyaline.

Habitat: topsoil.

Code: 1CIP

Nostocaceae 1 N.M. Machado-de-Lima et Branco, *gen. nov.*

Figs.: 9-16.

Colonies microscopic, irregular to spherical, mucilaginous envelope hyaline. Trichomes long, flexuous, sometimes circinate, constricted, cylindrical, loosely entangled. Cells barrel-shaped, isodiametric or longer/shorter. Cell content homogeneous, periplasm and centroplasm distinguishable, light to dark blue-green. Heterocytes mainly hemispherical, content yellowish. Akinetes in rows, paraheterocytic, mostly elliptical, epispore not visible.

Type species: Nostocaceae 1 sp. 1 N.M. Machado-de-Lima et Branco, *sp. nov.*

Nostocaceae 1 sp. 1 N.M. Machado-de-Lima et Branco, *sp. nov.*

Figs.: 9-14.

Colonies microscopic, irregular to spherical, mucilaginous envelope hyaline. Trichomes long, flexuous, sometimes circinate, constricted, cylindrical, loosely entangled. Cells barrel-shaped, 3.0-6.0 µm long (4.7 µm in average), 3.0-6.0 µm diam. (4.1 µm in average), isodiametric or longer, occasionally shorter (just after cell division), length/width ratio 0.7 to 1.7. Cell content homogeneous, periplasm and centroplasm distinguishable, light to dark blue-green. Heterocytes elliptical, barrel-shaped, cylindrical, conical rounded, hemispherical or spherical, 1.8-2.8 µm long (2.3 µm in average), 2.1-3.1 µm diam. (2.6 µm in average), content yellowish. Akinetes in rows, paraheterocytic, mostly elliptical, homogeneous content, 6.5-8.0 µm long (7.3 µm in average), 5.0-5.5 µm diam. (5.1 µm in average), epispore not visible.

Habitat: topsoil.

Code: 2GO, 3FUR

Nostocaceae 1 sp. 2 N.M. Machado-de-Lima et Branco, *sp. nov.*

Figs.: 15, 16.

Colonies microscopic, irregular to spherical, mucilaginous envelope hyaline. Trichomes long, flexuous, sometimes circinate, constricted, cylindrical, loosely entangled. Cells barrel-shaped, 3.0-9.0 µm long (5.5 µm in average), 3.0-6.0 µm diam. (4.1 µm in average), mostly short, occasionally isodiametric or longer, length/width ratio 0.7 to 2.2. Cell content homogeneous, periplasm and centroplasm distinguishable, light to dark blue-green. Heterocytes mainly hemispherical, 2.1-2.6 µm long (2.4 µm in average), 4.0-6.0 µm diam. (5.3 µm in average), content yellowish. Akinetes in rows (up to 12 cells), paraheterocytic, mostly elliptical, with prominent granules, 9.0-12.0 µm long (10.6 µm in average), 6.0-7.0 µm diam. (6.3 µm in average), epispore not visible.

Habitat: topsoil.

Code: 2CAN

Nostocaeae 2 N.M. Machado-de-Lima et Branco, *gen. nov.*

Figs.: 21-25.

Colonies microscopic, mucilaginous envelope hyaline. Trichomes flexuous, constricted, loosely entangled. Cells elliptical to barrel-shaped. Cell content homogeneous or granulate, periplasm and centroplasm usually distinguishable, blue-green to green. Heterocytes and

akinetes not observed.

Type species: Nostocaceae 2 sp. 1 N.M. Machado-de-Lima et Branco, *sp. nov.*

Nostocaeae 2 sp. 1 N.M. Machado-de-Lima et Branco, *sp. nov.*

Figs.: 21, 23.

Colonies microscopic, long, irregular, mucilaginous envelope hyaline, 83-155 µm long (104 µm in average), 48-90 µm diam. (69 µm in average). Trichomes flexuous, constricted, loosely entangled inside the colonies. Cells elliptical to barrel-shaped, 1.7-4.5 µm long (3.1 µm in average), 1.8-2.9 µm diam. (2.5 µm in average), mostly longer, occasionally isodiametric or shorter, length/width ratio 0.7 to 1.8 (1.2 in average). Cell content homogeneous or granulate, commonly with some prominent granules, periplasm and centroplasm usually distinguishable, blue-green to green. Heterocytes and akinetes not observed.

Habitat: topsoil.

Code: CATCB4

Nostocaeae 2 sp. 2 N.M. Machado-de-Lima et Branco, *sp. nov.*

Figs.: 24, 25.

Colonies microscopic, spherical or nearly spherical when young, latter irregular, mucilaginous envelope hyaline or yellowish, no distinct periderm, up to 350 µm diam. Trichomes of variable length, flexuous, constricted, cylindrical, loosely entangled. Cells elliptical to barrel-shaped, 1.4-3.1 µm long (2.4 µm in average), 1.7-2.9 µm diam. (2.3 µm in average), mostly short or isodiametric, occasionally longer, length/width ratio 0.7 to 1.4 (1.0 in average). Cell content homogeneous or granulate, commonly with one central prominent granule, periplasm and centroplasm usually distinguishable, blue-green. Heterocytes and akinetes not observed.

Habitat: topsoil.

Code: CATCC10

Nostocaceae 3 N.M. Machado-de-Lima et Branco, *gen. nov.*

Figs.: 17-20.

Colonies microscopic, nearly spherical to irregular forming dark green mass aggregates. Vegetative cells mostly cylindrical, but also quadratic, triangular and irregular, 4.0-8.8 µm long (6.1 µm in average), 3.6-7.2 µm diam. (5.2 µm in average), length/width ratio 1.0 to 1.6. Heterocytes and akinetes not observed.

Type species: Nostocaceae 3 sp. N.M. Machado-de-Lima et Branco, *sp. nov.*

Nostocaceae 3 sp. N.M. Machado-de-Lima et Branco, *sp. nov.*

Figs.: 17, 20.

Microscopic colonies, nearly spherical to irregular, forming dark green mass aggregates. Vegetative cells mostly cylindrical, but also quadratic, triangular and irregular, 4.0-8.8 µm long (6.1 µm in average), 3.6-7.2 µm diam. (5.2 µm in average), length/width ratio 1.0 to 1.6. Heterocytes and akinetes not observed.

Habitat: topsoil.

Code: CATCB5.1, CATCB5.2

Nostocaeae 4 N.M. Machado-de-Lima et Branco, *gen. nov.*

Figs.: 5-8.

Trichomes isolate or few together or cells/trichomes in microscopic colonies, nearly spherical to irregular, grouped forming floccose aggregates. Trichomes short, constricted, cylindrical. Cells of trichomes barrel-shaped to elliptical, short, occasionally isodiametric or longer. Colonial cells bigger than cells of trichomes, spherical, elliptical, cylindrical or irregular. Cell content homogeneous or granulate, periplasm and centroplasm mostly indistinguishable, light green, dark blue-green or brownish green.

Type species: Nostocaceae 4 sp. N.M. Machado-de-Lima et Branco, *sp. nov.*

Nostocaeae 4 sp. N.M. Machado-de-Lima et Branco, *sp. nov.*

Fig: 5-8.

Trichomes isolate or few together or cells/trichomes in microscopic colonies, nearly spherical to irregular, grouped forming floccose aggregates. Trichomes short, constricted, cylindrical. Cells of trichomes barrel-shaped to elliptical, 1.3-4.0 µm long (1.9 µm in average), 1.8-3.0 µm diam. (2.4 µm in average), short, occasionally isodiametric or longer, length/width ratio 0.5 to

1.4 (0.8 in average). Colonial cells bigger than cells of trichomes, (1.4-)2.1-4.2(-4.8) μm long (3.3 μm in average), 2.4-4.6 μm diam. (3.4 μm in average), spherical, elliptical, cylindrical or irregular, length/width ratio 0.4 to 1.4 (1.0 in average). Cell content homogeneous or granulate, periplasm and centroplasm mostly indistinguishable, light green, dark blue-green or brownish green.

Habitat: topsoil.

Code: CATCC2

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Capítulo IV – Biological soil crusts: new genera and species of Cyanobacteria from Brazilian semi-arid regions

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Accepted: Phytotaxa (8th October 2020)

4.1 Abstract

In the uppermost millimeters of soils is commonly found a thin layer of cryptobiotic organisms, including cyanobacteria, microalgae, lichens, mosses, fungi, bacteria and archaea. These communities are called Biological Soil Crusts (BSCs) or biocrusts and perform important ecological functions, mainly attributed to their capacity for providing soil stability and incorporating nutrients into the soil through nitrogen and carbon fixation. Among all the organisms found in the biocrusts, the filamentous cyanobacteria *Microcoleus vaginatus* and *M. steenstrupii* are the best studied soil colonizers. The genus *Microcoleus* is considered complex and has shown close relation with some species of *Phormidium*. The poor understanding of these two genera is a limit to the description of the real composition of biocrusts and can generate underestimations in the diversity community and the use of incorrect organisms in applied projects (e.g. environmental restoration). This work studied eight cyanobacterial populations from Brazilian BSCs sampled in the *Caatinga* biome. The populations presented *Microcoleus*-like and *Phormidium*-like morphologies, but the phylogenetic analyses based on 16S rRNA gene sequences showed that they represent three new genera and six new species of filamentous cyanobacteria associated to the cryptic genera, they are *Pycnacronema caatingensis sp. nov., Pycnacronema edaphica sp. nov., Gracilinea arenicola gen. et sp. nov., Marmoreocelis xerophila gen. et sp. nov., Konicacronema caatinguensis gen. et sp. nov. and Trichocoleus caatingensis sp. nov.* The generic name and specific epithets of the new taxa are proposed according to the provisions of the International Code of Nomenclature of algae, fungi, and plants.

4.2 Introduction

Biological soil crusts (BSCs) are communities that can be composed of cyanobacteria, microalgae, lichens, mosses, fungi, bacteria and archaea which are aggregated with soil particles. They occur in a variety of climatic regions, having their most conspicuous development in drylands and arid areas (Belnap, 2005). Their ecological importance is mainly attributed to the capacity of joining soil particles, providing soil stability and protecting against erosion (Belnap, 2005). They also can perform nitrogen and carbon fixation, resulting in energy and nutrient inputs to the soil system (Dojani et al., 2007; Schmdt et al., 2008; Bowker et al., 2011).

Cyanobacteria are usually the main components of these communities. They are the first colonizers of soil surfaces (Garcia-Pichel & Wojciechowki, 2009), mainly the filamentous forms and those which create soil stabilization and provide conditions to the arrival of the next organisms (Garcia-Pichel et al., 2016). In most cases, they can form bundles, limited by exopolysaccharide sheath, capable of attach to the solid substrate and connect the sand grains (Garcia-Pichel & Wojciechowki, 2009).

The fundamental requirement to understand BSCs' functions is a solid base about which organisms compose them (Garcia-Pichel et al., 2001). Consequently, research in this area is dependent on previous evaluations of diversity, including refined phylogenetic analyses.

Microcoleus vaginatus Gomont ex Gomont (1892:352) and *M. steenstrupii* Petersen (1923:292) are the best studied soil colonizers (Garcia-Pichel et al., 2001; Gundlapally & Garcia-Pichel, 2006; Büdel et al., 2009; Hagemann et al., 2017; Rossi & De Philippis, 2015; Schulz et al., 2016; Dulic et al., 2016). These two species are distributed worldwide, having been recorded in the seven continents and used in many experimental studies, including those of cultivations aiming future environmental restorations (Zheng et al., 2011; Ayuso et al., 2017).

Microcoleus is considered a complex and possibly polyphyletic genus with its phylogeny being deeply investigated since the publication of Boyer et al. (2002). It is morphologically similar to *Phormidium* Kützing ex Gomont (1892: 156), and, for a long time, the shape of the colonies and the organization of the filaments were their only distinction traits. Strunecký et al. (2013) discussed about the similarity between the two genera and also established that *Microcoleus vaginatus* and *Phormidium autumnale* Gomont (1892:187) pertained to the same genus, proving their intermixed relation.

Microcoleus steenstrupii is a questionable species itself. Many studies argued it can no longer be considered a single species, instead it is probably a taxonomic complex to which even several genera may pertain (Mühlsteinová et al., 2014; Patzelt et al. 2014, Pietrasiak et al., 2014). Those new data and interpretations change several taxonomic and ecological understandings, including considerations about the geographic and ecological distribution of *Microcoleus vaginatus* and *M. steenstrupii* (e.g., Garcia-Pichel et al., 2013).

The complexities about *Microcoleus* and its relationship with *Phormidium* emphasize the necessity of detailed phylogenetic investigations about the two genera. The importance of *Microcoleus* species in the establishment and maintenance of soil biocrusts make these investigations even more necessary.

Therefore, this work aimed to study (taxonomically and phylogenetically) *Microcoleus*- like and *Phormidium*-like populations found in Brazilian BSCs from neotropical regions, contributing to more in depth investigations about cyanobacterial composition of biological crusts and serving as basis for further applied initiatives.

4.3 Materials and methods

4.3.1 Sampling sites

The cyanobacterial populations studied were isolated from biological soil crust samples found in Brazilian semi-arid areas (Table 1), distributed along a biome locally named *caatinga* and considered exclusive to Brazil. The vegetation of the area is a type of steppic savanna adapted to low and irregular water availability.

The *caatinga* ranges nine states in Brazil, representing 11% of the national territory, covering about 844,000 Km^2 (IBGE, 2019). The soils are mostly poorly developed, with high mineral content, stony, shallow and with weak water retention capacity (Alves et al. 2009). Total precipitation varies from 300 to 800 mm/year, with irregular rainfall distribution along the time (Barbosa et al., 2019).

TABLE 1. Geographic origin of *Microcoleus*-like strains studied in the state of Paraíba, Brazil.

Code	GPS Coordinates	Municipality	Altitude (m)
CATCC2, CATCC10	07°07'S; 36°08'W	Pocinhos	559
CATCB6, CATCB9, CATCB5	07°24'S; 36°19'W	Cabaceiras	420
CATCA7	07°27'S; 36°16'W	Cabaceiras	433
CATCD2, CATCB1	06°41'S; 36°03'W	Barra de Santa Rosa	458

Table of the own author.

4.3.2 Cultivation and morphological evaluation

Each cyanobacterial strain was isolated after cultivation of filamentous bundles, or even portions of crusts, in BG11 growth medium (Rippka et al. 1979). Trichomes of each cultivated sample were repeatedly separated from others and successively transferred to clean drops of deionized water using Pasteur pipette under an inverted light microscope (Leica DMIL LED) and the isolated trichomes were then inoculated in tubes with BG11 medium. This process was repeated as many times were necessary until the culture became unicyanobacterial. The cultured specimens were maintained under 20ºC ± 1ºC, 60 µmol of photons m^{-2} s^{-1} of irradiance and a 14:10 h light-dark cycle.

Morphological variability of populations was analyzed from cultured samples by using an Olympus BH2 microscope coupled with differential interference contrast (DIC)

device. Taxonomic features, such as vegetative cell width, cell length, apical cell shape and dimensions and attenuation of trichomes, were analyzed in at least 15 trichomes from each sample, summarizing 50 cells.

4.3.3 Molecular analyses

DNA extraction was carried out according to Martins et al. (2018). The 16S rRNA partial gene and 16S-23S internal transcribed spacer (ITS) markers were amplified by polymerase chain reaction (PCR) using primers CYA 359 (Nübel et al., 1997) and 23S30R (Taton et al., 2003), and performed in a "Techgene TC-512" thermal cycler (Techne) with 25 µL of total reaction volume, containing 5 µL 10X PCR buffer, 2 µL 50 mM MgCl2, 1 µL 10 mM dNTP mix, 1.25 µL of each primer (5 ρmol), 14.2 µL 'Milli-Q' water, 1.5 U "Platinum Taq DNA polymerase" (Life Technologies) and 10 n g genomic DNA. Thermal cycling was 94oC for 5 min, followed by ten cycles of 94ºC for 45 s; 57ºC for 45 s and 72ºC for 2 min; 25 cycles of 92ºC for 45 s and 54ºC for 45 s; and one final cycle of 72ºC for 7 min. The PCR products were analyzed on 1% agarose gels stained with "GelRed 0.6X" (Biotium) and viewed on "Mini Bis Pro" transilluminator (Micro Photonics). Amplicons were ligated using the "pGEM-T Easy Vector System I" (Promega) following the supplier's manual, and inserted in competent *Escherichia coli* DH5α cells, which were growth and plated for blue-white colony screening (Sambrook & Russel, 2001). Sequencing was performed in the "ABI 3130 sequencer", using "Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction" kit (Applied Biosystems) and following the manufacturer's protocol. Primers 359F (Nübel et al., 1997), 704R, 1114F, 1494R (Neilan et al., 1997) and 23S30R (Taton et al., 2003) were used for sequencing. Consensus sequences were assembled into contigs using the "Phred/Phrap/Consed" software (Ewing & Green 1998, Ewing et al., 1998, Gordon et al., 1998) and only bases with Phred over 20 were considered.

4.3.4 Phylogenetic analyses

The dataset constructed for the phylogenetic analyses included sequences produced in this study and their closest related sequences previously published and available in NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) as determined by BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Additional sequences of *Microcoleus* related genera available in NCBI were also incorporated to the data matrix to provide structure for the analyses. Sequence alignment was performed using the software

"ClustalW 1.8" (Thompson et al., 1994) in "MEGA 7" (Kumar et al., 2016) and manually inspected and refined. The unicellular cyanobacteria *Gloeobacter violaceus* and the filamentous *Pseudanabaena* sp. (GenBank access numbers NR074282, GU935357) were designated as the evolutionary outgroups. Phylogenetic analyses were based on partial 16S rRNA gene sequences (1080 bp). Bayesian inference (BI) and a maximum-likelihood (ML) were performed using MrBayes (Ronquist et al., 2012) Workflow on XSEDE 3.2.6 and RAxML-HPC2 (Stamatakis, 2014) Workflow on XSEDE (8.2.12), both on the CIPRES Science Gateway (Miller et al., 2010). The ML + thorough bootstrap workflow was used with the following modified parameters: 1000 bootstraps (-N 1000) and the GTRGAMMA model, while for BI, Markov chain was run for 800,000,000 generations. All other parameters were left at default values. Sequence similarity matrix/nucleotide divergence was calculated in "Geneious 7" (Kearse et al., 2012) considering the alignment in all positions, including gaps.

4.3.5 Secondary structure of conserved ITS regions

D1-D1', box-B and V3 regions (Iteman et al., 2000) were identified and their secondary structures were determined using "Mfold Web 2.3" (Zuker, 2003). The tRNA sequences were identified with "tRNAscan-SE 1.21" (Lowe & Eddy, 1997). Sequence alignment was performed using the software "ClustalW 1.8" (Thompson et al., 1994) in "MEGA 7" (Kumar et al., 2016) and the p-distance was also generated by "MEGA 7".

4.4 Results

Description of the new genera and species

Pycnacronema caatingensis N.M. Machado-de-Lima et Branco, *sp. nov.*

Fig: 1a,b.

Thallus dark blue-green. Filaments entangled, 7.0-11.0 µm wide. Sheaths firm, thin, homogeneous, colorless. Trichomes cylindrical, not attenuated, constricted at the ungranulated cross walls, 5.5-8.0 µm wide. Cells cylindrical, isodiametric, shorter or longer than wide, 3.0-

7.5 µm long. Cell content blue-green, granulated. Apical cells slightly conical-rounded, with thickened cell wall, sometimes with yellow cell content, 6.0 µm long.

Representative sequences (GenBank codes – *Pycnacronema caatingensis*, strain CATCA7): MT311243 – partial 16S rRNA.

Etymology: L. adj. *caatingensis* = from Brazilian biome Caatinga.

Habitat: topsoil.

Holotype: formaldehyde-fixed sample of strain CATCB1 deposited in Herbarium SJRP (IBILCE/UNESP), Brazil, voucher number SJRP 32601, collected by Branco L. H. Z et al. in September, 2017.

Pycnacronema edaphica N.M. Machado-de-Lima et Branco, *sp. nov.*

Thallus dark blue-green. Filaments with many trichomes per sheath, 8.0-32.0 µm wide. Sheaths firm, thin, homogeneous, colorless. Trichomes cylindrical, not attenuated, constricted at the ungranulated cross walls, 4.8-6.40 µm wide. Cells cylindrical, isodiametric, shorter or longer than wide, 3.2-8.8 µm long. Cell content blue-green, granulated. Apical cells slightly conical- rounded, with thickened cell wall, sometimes with yellow cell content, 8.0-8.8 µm long.

Representative sequences (GenBank codes – *Pycnacronema edaphica*, strain CATCA7): MT311244 – partial 16S rRNA.

Etymology: G. n. *edaphos* = soil, ground; *edaphica* = from soils.

Type locality: municipality of Cabaceiras, Paraíba State, Brazil (07°27'S, 36°16'W).

Habitat: topsoil.

Holotype: formaldehyde-fixed sample of strain CATCA7 deposited in Herbarium SJRP

(IBILCE/UNESP), Brazil, voucher number SJRP 32604, collected by Branco L. H. Z et al. in September, 2017.

Gracilinea N.M. Machado-de-Lima et Branco, *gen. nov.*

Filaments entangled among soil granules. Sheaths firm, thin, colorless, hyaline. Trichomes isopolar, not attenuated at the ends, unconstricted at the cross walls. Cells cylindrical, isodiametric to longer than wide. Cell content finely granular. Apical cell conical-rounded to conical-obtuse, without calyptra. Reproduction via hormogonia.

Etymology: (L. adj. *gracilis* simple, modest; L. noun fem. *linea* line/filament; *Gracilinea* = simple filament).

Type species: *Gracilinea arenicola* N.M. Machado-de-Lima et Branco, *sp. nov.*

Gracilinea arenicola N.M. Machado-de-Lima et Branco, *sp. nov.*

Fig: 1e-g.

Filaments entangled among soil granules, 4.0-7.0 µm wide. Sheaths firm, thin, colorless, hyaline. Trichomes isopolar, not attenuated at the ends, unconstricted at the cross walls, 4.0-5.0 µm wide. Cells cylindrical, isodiametric to longer than wide, 4.0-10.0 µm long. Cell content finely granular. Apical cell conical-rounded to conical-obtuse, without calyptra, 7.5-10.0 µm long. Reproduction via hormogonia.

Representative sequences (GenBank codes – *Gracilinea arenicola*, strain CATCC10): MT311245– 16S rRNA.

Etymology: L. n. *arena* = sand; L. suffix *cola* = inhabitant; *arenicola* = inhabiting sandy substrates.

Habitat: topsoil.

Holotype: formaldehyde-fixed sample of strain CATCC10 deposited in Herbarium SJRP (IBILCE/UNESP), Brazil, voucher number SJRP 32602, collected by Branco L. H. Z et al. on September, 2017.

Marmoreocelis N.M. Machado-de-Lima et Branco, *gen. nov.*

Filaments entangled among soil granules. Sheaths firm, thin, colorless, hyaline. Trichomes isopolar, not attenuated at the ends, constricted at the cross walls. Cells cylindrical, shorter than wide. Cell content blue-green to dark green, keratomized. Apical cell conical-rounded,

without calyptra. Reproduction via hormogonia.

Etymology: G. noun. *marmor* = marble; L. noun. cella = room, cell; *Marmoreocelis* - referring to the cell with marble aspect due to keritomy.

Type species: *Marmoreocelis xerophila* N.M. Machado-de-Lima et Branco, *sp. nov.*

Marmoreocelis xerophila N.M. Machado-de-Lima et Branco, *sp. nov.*

Fig: 2a-d.

Filaments entangled among soil granules, 6.8-8.0 µm wide. Sheaths firm, thin, colorless, hyaline. Trichomes isopolar, not attenuated at the ends, constricted at the cross walls, 6.0-7.2 µm wide. Cells cylindrical, shorter than wide, 2.4-6.2 µm long. Cell content blue-green to dark green, keratomized. Apical cell conical-rounded, without calyptra, 5.2-11.2 µm long. Reproduction via hormogonia.

Representative sequences (GenBank codes – *Marmoreocelis xerophila* strain CATCB5): MT311247– 16S rRNA.

Etymology: G. adj. *xeros* = dry; G. noun. *phílos* = friend; *xerophila* = occurring in dry, xeric habitat.

Habitat: topsoil.

Holotype: formaldehyde-fixed sample of strain CATCB5 deposited in Herbarium SJRP (IBILCE/UNESP), Brazil, voucher number SJRP 32603, collected by Branco L. H. Z et al. in September, 2017.

Konicacronema N.M. Machado-de-Lima et Branco, *gen. nov.*

Filaments entangled among soil granules. Sheaths firm, thin, colorless, hyaline. Trichomes isopolar, not attenuated at the ends, constricted at the cross walls. Cells cylindrical, shorter than wide. Cell content blue-green, granulated. Apical cell conical to pointed, without calyptra. Reproduction via hormogonia.

Etymology: G. adj. *konikó* = conical; G. noun. *akro* = tip; G. *nema* = filament; *Konicacronema* = filament with conical apical cell.

Type species: *Konicacronema caatingensis* N.M. Machado-de-Lima et Branco, *sp. nov.*

Konicacronema caatingensis N.M. Machado-de-Lima et Branco, *sp. nov.*

Fig: 2e-g.

Filaments entangled among soil granules, 4.0-7.0 µm wide. Sheaths firm, thin, colorless,

hyaline. Trichomes isopolar, not attenuated at the ends, constricted at the cross walls, 4.0-6.0 μm wide. Cells cylindrical, shorter than wide to isodiametric, 2.4-5.0 μm long. Cell content blue-green with brown/yellow granules. Apical cell conical to pointed, without calyptra, 4.0- 9.0 μm long. Reproduction via hormogonia.

Representative sequences (GenBank codes – *Konicacronema caatingensis* strain CATCB1): MT311248 – 16S rRNA.

Etymology: L. adj. *caatingensis* = from Brazilian biome Caatinga.

Habitat: topsoil.

Holotype: formaldehyde-fixed sample of strain CATCB1 deposited in Herbarium SJRP (IBILCE/UNESP), Brazil, voucher number SJRP 32601, collected by Branco L. H. Z et al. in September, 2017.

Morphological evaluation and ecological aspects

Eight populations of homocytous cyanobacteria morphologically similar to *Microcoleus* and *Phormidium* were cultivated from BSCs samples. In the nature they were observed as solitary bundles entangled among soil granules, but in culture conditions the filaments entangled and formed mats. Some populations presented only one trichome within a sheath while some of them formed multrichome filaments (the capacity to form multi-trichome filaments was usually lost during cultivation).

The populations could be separated in five morphotypes (Table 2). The first morphotype was represented by the populations CATCC2 and CATCA7. Specimens of CATCC2 (Fig. 1a,b) had trichomes constricted at the cross walls, isodiametric or shorter than wide cells, cell content blue-green and granulated and apical cell cylindrical, with rounded apex. Those characteristics correspond to the genus *Pycnacronema* Martins et al. (Martins et al. 2018:153). The individuals of population CATCA7 presented trichomes with the same characteristics of CATCC2 but formed mostly fasciculate filaments in a common sheath (Fig. 1c,d).

Specimens of CATCC10 and CATCB9 presented trichomes unconstricted at the cross- walls, isodiametric to longer than wide cells, cell content blue-green and apical cells conical and elongated (Fig. 1e-g). These trichome characteristics are related to *Microcoleus steenstrupii* as described in Komárek & Anagnostidis (2005).

Morphology of individuals from population CATCB5 was distinct from those of the CATCC10 and CATCB9 populations. Trichomes are constricted at the cross-walls, the

cells are isodiametric or more commonly they are shorter than wide, the cell content is blue green to dark green and keratomized and the apical cells are rounded or conical (Fig. 2a-d).

The fourth morphotype was found in populations CATCB6 and CATCB1 which filaments are characterized by having more than one trichome per sheath and the trichomes are constricted at the cross-walls, the cells are shorter than wide, the cell content is blue-green and the apical cells are conical-obtuse (Fig. 2e-g).

The last morphotype was observed in CATCD2 population with the trichomes constricted at the cross-walls, the cells isodiametric, the cell content blue-green and the apical cells conical-obtuse to occasionally sharply pointed (Fig. 2h).

TABLE 2. Morphological comparison among simple filamentous cyanobacteria from Caatinga soils. Dimensions (µm) represent full ranges observed. L/W = cell length/cell width ratio; *with thickened cell wall. Fonte: tabela do autor.

Species/strain	Cell content	Apical cell	Apical cell length	Filament width	Trichome width	Cell length	L/W
Pycnacronema caatingensis CATCC2	Blue-green, homogeneous	Conical-rounded	6.0	7.0-11.0	5.5-8.0	3.0-7.5	0.4-1.1
Pycnacronema edaphica CATCA7	Blue-green, homogeneous	Conical-rounded*	8.0-8.8	8.0-32.0	4.8-6.4	3.2-8.8	0.5-1.4
Gracilinea arenicola CATCC10	Blue-green, homogeneous	Conical-rounded	7.5-10.0	6.0-7.0	4.0-5.0	4.0-10.0	1.0-2.5
Gracilinea arenicola CATCB9	Blue-green, homogeneous	Conical-rounded	8.0-9.0	4.0-6.0	4.0-.5.0	4.0-9.0	0.8-1.7
Koniacronema caatingensis CATCB1	Blue-green, homogeneous	Conical-obtuse	4.0-9.0	4.0-4.8	4.0-4.8	2.4-4.0	0.6-1.0
Koniacronema caatingensis CATCB6	Blue-green, homogeneous	Conical-obtuse	5.0-7.0	5.0-7.0	4.0-6.0	1.5-5.0	0.3-1.0
Marmoreocelis xerophila CATCB5	Blue-green to dark green, keratomized	Conical-rounded	5.6-11.2	6.8-8.0	6.0-7.2	2.4-6.2	0.4-0.9
Trichocoleus caatingensis CATCD2	Blue-green, homogeneous	Conical-obtuse	4.0-5.0	2.0	2.0	2.0-4.0	1.0-2.0

Table of the own author.

FIGURE 1. (a-b) *Pycnacronema caatingensis,* strain CATCC2; (c-d) *Pycnacronema edaphica*, strain CATCA7; (e-g) *Gracilinea arenicola*, strain CATCC10. Bars = 10 µm. Figure of the own author.

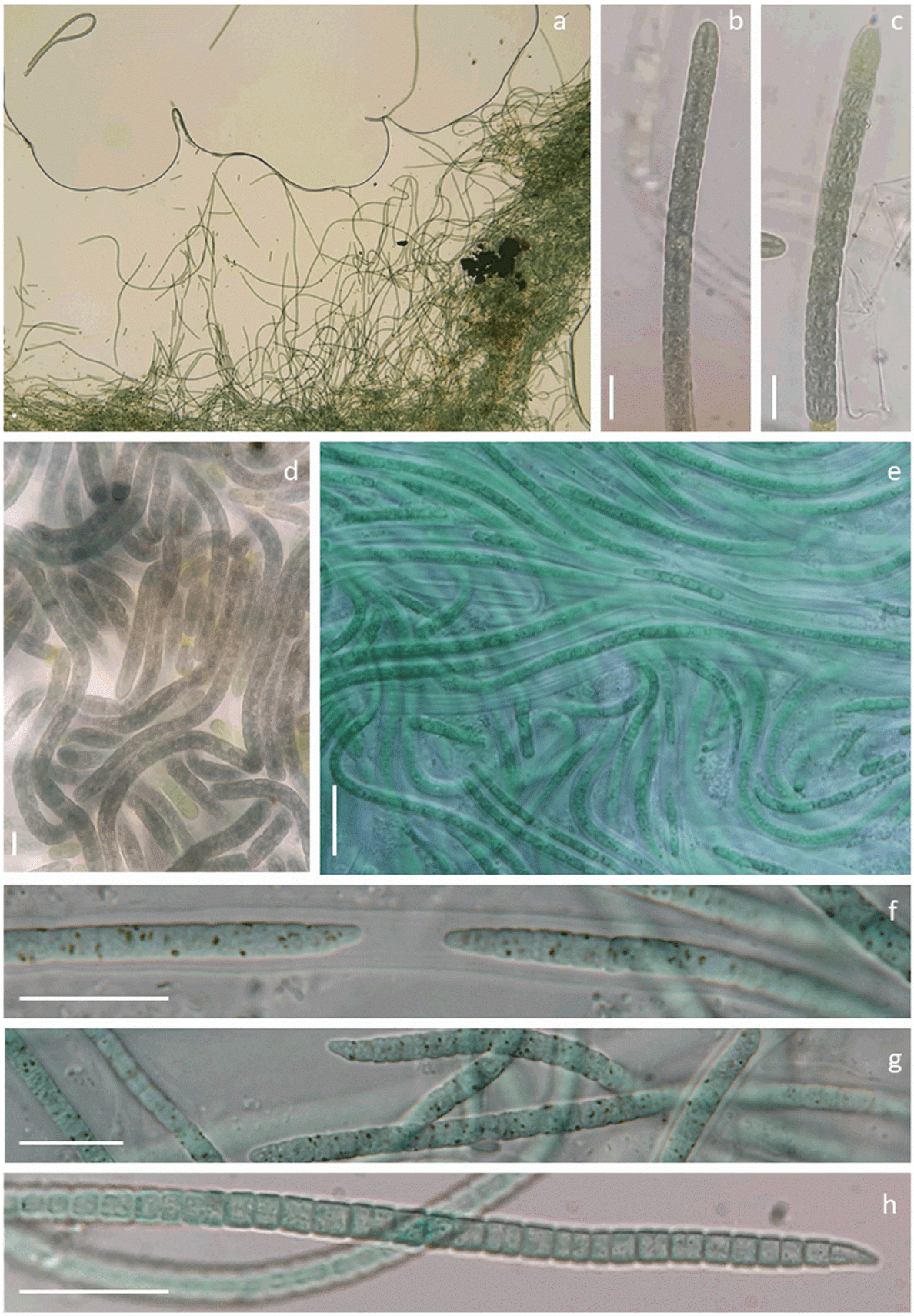


FIGURE 2. (a-d) *Marmoreocelis xerophila*, strain CATCB5; (e-g): *Konicacronema caatinguensis*, strain CATCB1; (h) *Trichocoleus caatingensis*, strain CATCD2. Bars = 10 µm. Figure of the own author.

4.4.1 Molecular analyses and phylogeny - 16s rRNA

The RAxML analysis intermixed the members of the families Coleofasciculaceae, Phormidiaceae and Microcoleaceae, but the recently erected taxa *Wilmottia* Strunecký et al. (Strunecký et al. 2011: 62), *Kamptonema* Strunecký et al. (Strunecký et al. 2014:203), *Ancylothrix* Martins et Branco (Martins *et al.* 2016: 2402), *Cephalothrix* Malone *et al.* (Malone *et al*. 2015: 3001), *Potamolinea* Martins et Branco (Martins & Branco 2016: 3640), *Pycnacronema*, *Kastovskya* Mühlsteinová et al. (Mühlsteinová et al. 2014:219), *Coleofasciculus* Siegesmund et al. (Siegesmund et al. 2008: 1575) and *Trichocoleus desertorum* Mühlsteinová, J.R.Johansen & N.Pietrasiak 2014, all derived from *Phormidium* and *Microcoleus*) were placed in well supported clades (Fig. 3).

The eight studied sequences were distributed through five highly supported clades. CATCD2 clustered with *Trichocoleus* strains, indicating its identity with the group (from 95.6 to 98.2%), but it positioned more distantly from the other populations studied. *Trichocoleus* belongs to the family Trichocoleusaceae (order Synechococcales) while the other strains evaluated pertain to Coleofasciculaceae (Oscillatoriales), ~~what~~ which explains its positioning in a more distant clade. The similarity below the 98.65% (threshold to separate species proposed by Kim et al., 2014) indicates CATCD2 as a new species, *Trichocoleus caatingensis*.

The comparison of 16S rRNA gene sequences of the remaining studied strains with other sequences from GenBank revealed relatively low similarity with strains of the genera *Phormidium* and *Microcoleus*, especially with their type species (from 90.4 to 93.2% with *Phormidum* cf. *irriguum* CALA 759 and *P. irriguum* f. *minor* ETS-02 – type of *Phormidium* – and from 89.5 to 93.6% with *Microcoleus vaginatus* – type of *Microcoleus*).

Clade A grouped CATCA7 and CATCC2 populations with eight *Pycnacronema* strains which 16S rRNA gene sequences were retrieved from GenBank. Sequence of CATCC2 was highly similar to *P. savannensis* 49PC (MF58163 – 99% of similarity), also with *P. conicum* 7PC (MF581656 – 98.9% of similarity) and *P. arboriculum* 41PC (MF581657 - 98.9% of similarity), forming with this last a well-supported clade. However, CATCA7 population sequence presented only 97.1% of 16S rRNA similarity with its sister sequence (*Pycnacronema conicum* 7PC – MF581656), this similarity places CATCA7 as a distinct and new species referred from here on as *Pycnacronema edaphica*.

CATCC10 and CATCB9 populations' sequences were 99.7% similar each other and were placed together in the Clade B. Due to their genetic distance to other sequences, they are considered to pertain to a separated genus named *Gracilinea* and to the same species, *G.*

arenicola.

Clade C was composed of CATCB5 population sequence and one *Phormidium* sp. sequence (KM438197 - from hot springs in Greece) from GenBank, but they share only 95.9% of similarity. The genetic distance of CATCB5 from other taxa, the distinct habitat and its morphological traits (especially the keritomy) support its classification in a separate new genus, *Marmoreocelis*, and species, *M. xerophila*.

Sequences of CATCB6 and CATCB1 populations and six sequences from GenBank (EF654061, KC463190, KJ489356, MG874712, KY283061, KJ939036) were grouped in the Clade D and shared 16S rRNA similarity from 98.1 to 100%. This clade is apart from other oscillatorialeans and it is strongly supported by bootstrap, allowing the erection of the new genus *Konicacronema.*

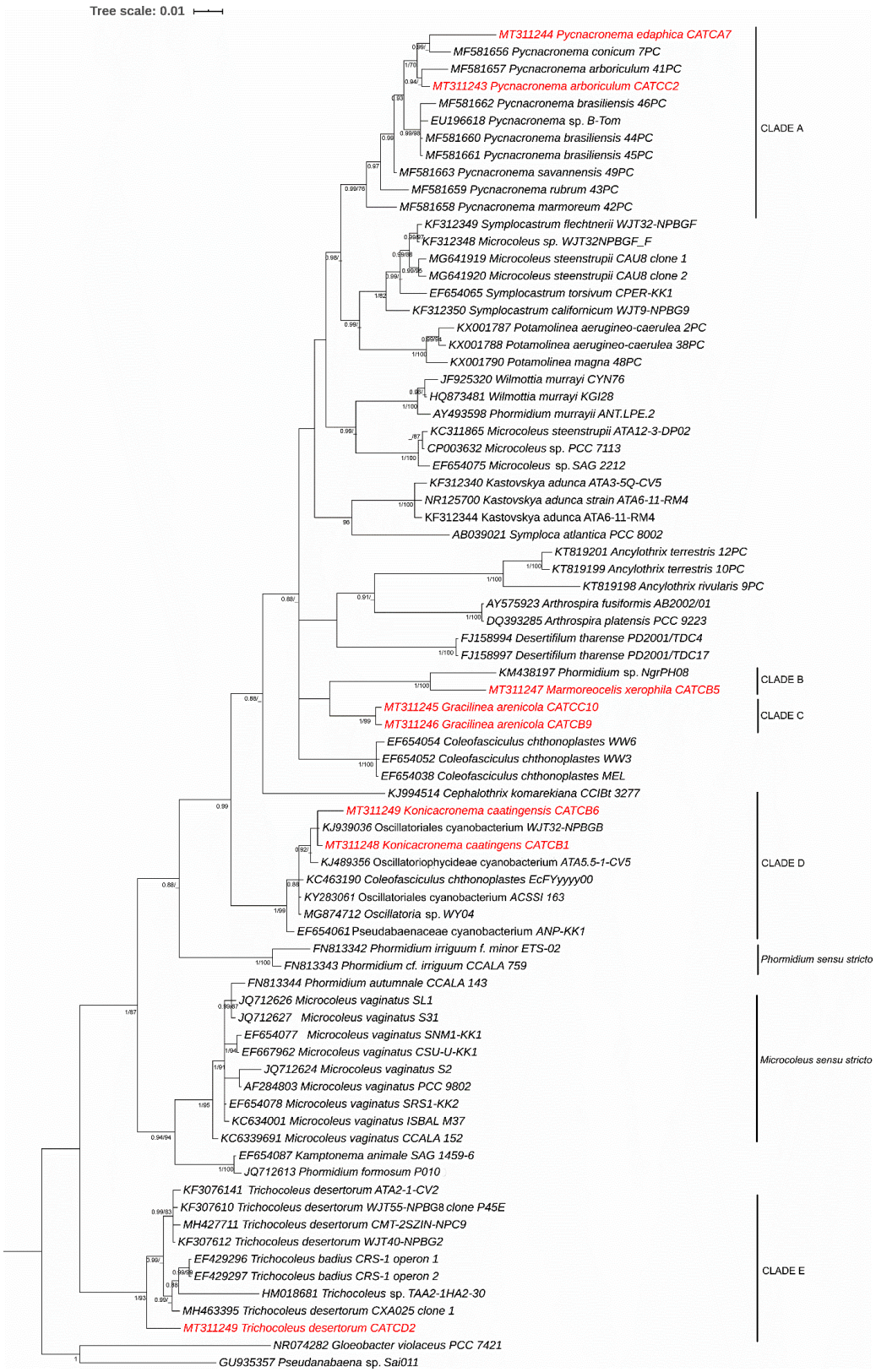


FIGURE 3. Bayesian and maximum likelihood (ML) analysis of the 16S rRNA partial gene alignment of sequences of cyanobacteria strains. Codes in red boldface represent the strains analyzed in this study. Support values are Bayesian posterior probabilities with bootstrap values from ML mapped on to the tree where above 70% (shown at nodes). GenBank accession numbers before sample identification. Bar represents 0.1 substitutions per nucleotide position. Figure of the own author.

4.4.2 Molecular analyses – 16S-23S ITS

16S-23S ITS region lengths were analyzed comparing the strains which were closely related according to 16S rRNA (Table 3). The 16S-23S ITS secondary structures, in general, showed similarities among strains grouped by 16s rRNA gene analysis. The region D1-D1' was the most conserved structure of the 16S-23S ITS region.

The samples CATCA7 (*Pycnacronema edaphica*), CATCC2 and other three *Pycnacronema* populations from GenBank presented similar lengths for all the regions. The secondary structures of the region D1-D1' for these five sequences were very related, with identical basal portion and small differences along the stem and final portion. The Box-B separated them into two groups, being *P. arboriculum* more related to *P. conicum* M.D.Martins & Branco, and *P. savannensis* M.D.Martins, N.M.Machado-de-Lima & Branco 2019 identical to CATCC2 and similar to *P. edaphica*. The V3 structures were different among all the strains (Fig. 4). The p-distance between all the sequences of *Pycnacronema* varied from 0 to 0.26, with CATCC2 sample presenting p-distance from 0.084 to 0.258 in relation to the others (Table S1) allowing to consider it a distinct and new species, *Pycnacronema caatingensis*.

The ITS sequences of the strains CATCC10 and CATCB9 (both *Gracilinea arenicola*) had identical lengths and secondary structures for all the conserved regions, while CATCB5 (*Marmoreocelis xerophila*) and *Phormidium* sp. (KM438197) sequences presented similar lengths for many the regions and close related D1-D1', Box-B and V3 secondary structures (Fig. 5).

Representative ITS sequences of CATCB6 and CATCB1 populations (both *Konicacronema*) revealed the same structures for D1-D1' and Box-B, but they differed of Oscillatoriophycideae cyanobacterium (KJ489356), sequence from the same clade (Fig. 6). The p-distance among these three ITS sequences varied of 0.004 to 0.079.

The strain CATCD2, *T. desertorum* KF307610 and *T. desertorum* KF307612 presented very similar lengths for all their regions except the Pre-Box-B spacer. Their D1-D1' were identical at the basal portion and fairly different at the final portion, while Box-B and V3 were more differentiated (Fig. 7). The p-distance among the samples CATCD2 and all the other *Trichocoleus* samples varied from 0.096 to 0.108 (Table S2).

TABLE 3. Lengths of 16S-23S ITS regions (number of nucleotides) in the analyzed *Microcoleus*-like

strains.

Strain	ITS to end V3	Leader	D1-D1' helix	D2 with spacer	D3 with spacer	$tRNA^{Ile}$ gene	V2	$tRNA^{Ala}$ gene	Pre-Box-B spacer	Box-B helix	Post-Box-B spacer	Box-A	D4	V3
P. caatingensis CATCC2	408	5	60	31	12	73			84	36	18	12	13	47
P. edaphica CATCA7	414	5	61	31	12	73			80	38	18	12	13	40
P. savannensis (MF581663)	403	5	57	32	14	73			81	36	18	12	13	50
P. arboriculum (MF581657)	401	5	59	32	12	73			77	36	18	12	13	47
G. arenicola CATCC10	371	5	56	31	11	73			9	40	70	12	13	36
G. arenicola CATCB9	371	5	56	31	11	73			9	40	70	12	13	36
M. xerophila CATCB5	347	5	58	31	12	72			28	37	19	12	13	53
Phormidium sp. (KM438197)	*	7	59	33	14	73			29	38	19	12	13	*
K. caatingensis CATCB1	444	6	57	32	13	73			118	41	20	12	13	44
K. caatingensis CATCB6	443	6	57	32	13	73			117	41	20	12	13	44
T. caatingensis CATCD2	485	6	61	36	13	73	54	72	35	36	17	12	13	40
T. desertorum (KF307612)	434	6	62	36	14	73	25	72	14	37	17	12	13	36
T. desertorum (KF307610)	435	6	62	38	14	72	25	72	14	37	18	12	13	35

* Incomplete sequencing. Table of the own author.

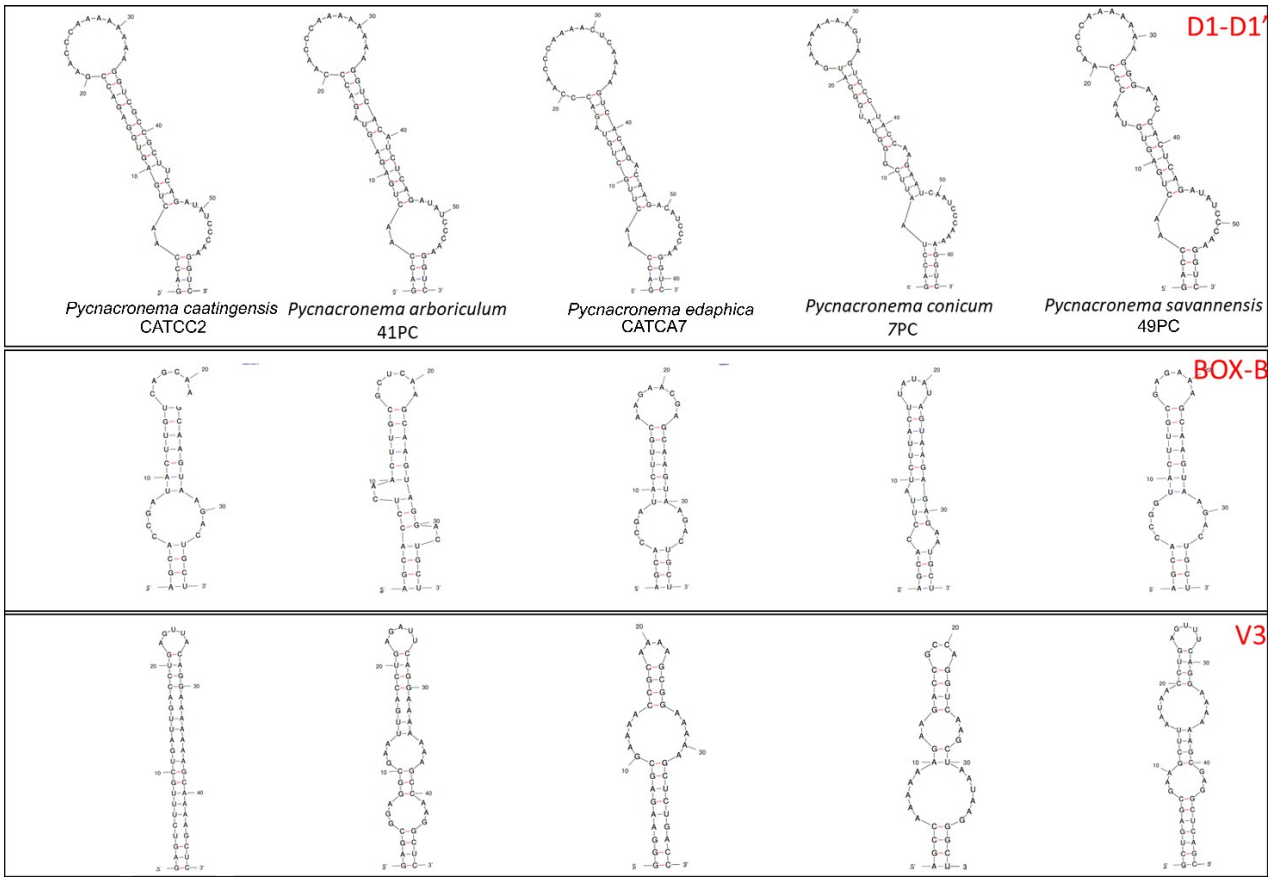


FIGURE 4. Secondary structure of conserved regions of 16S-23S ITS of *Pycnacronema* strains. D1-D1' helices, Box-B helices and V3 helices. Figure of the own author.

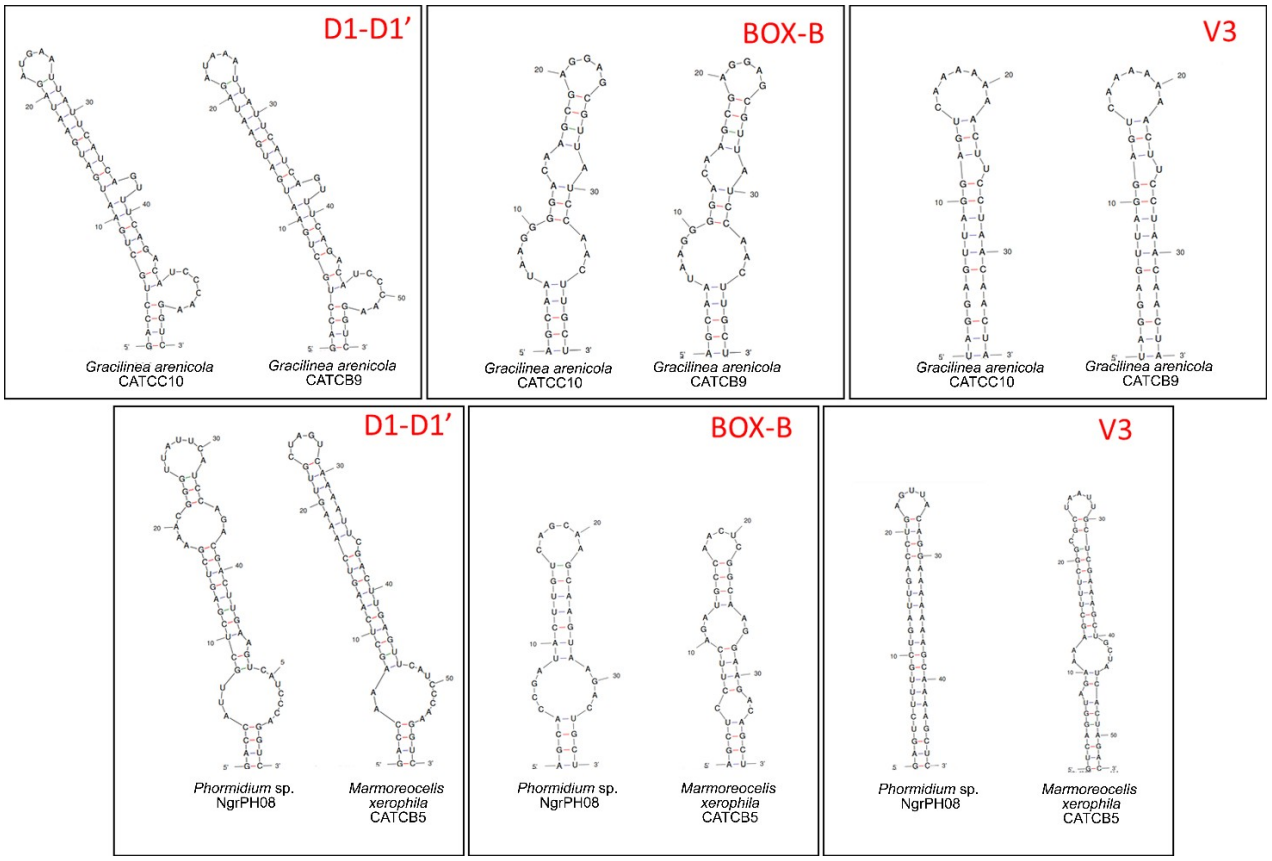
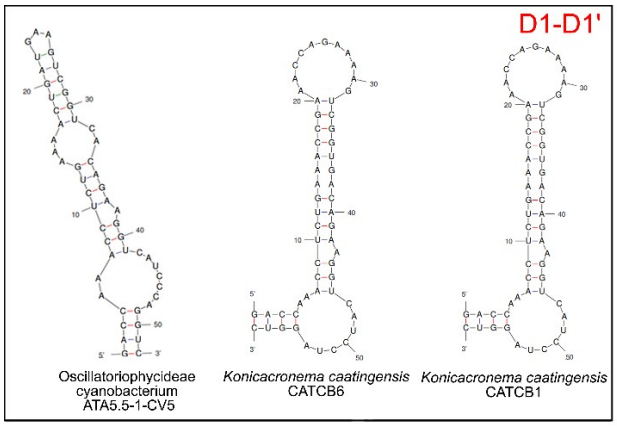
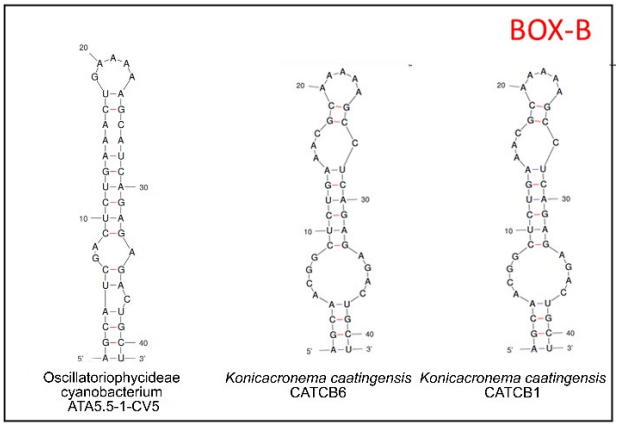


FIGURE 5. Secondary structure of conserved regions of 16S-23S ITS of studied strains. D1-D1' helices, Box-B helices and V3 helices. Figure of the own author.





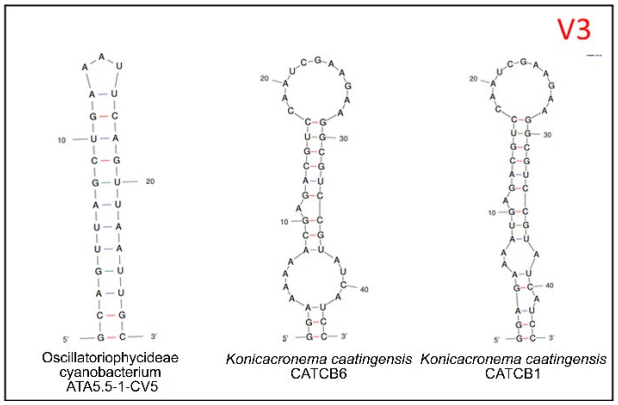


FIGURE 6. Secondary structure of conserved regions of 16S-23S ITS of studied strains. D1-D1' helices, Box-B helices and V3 helices. Figure of the own author.

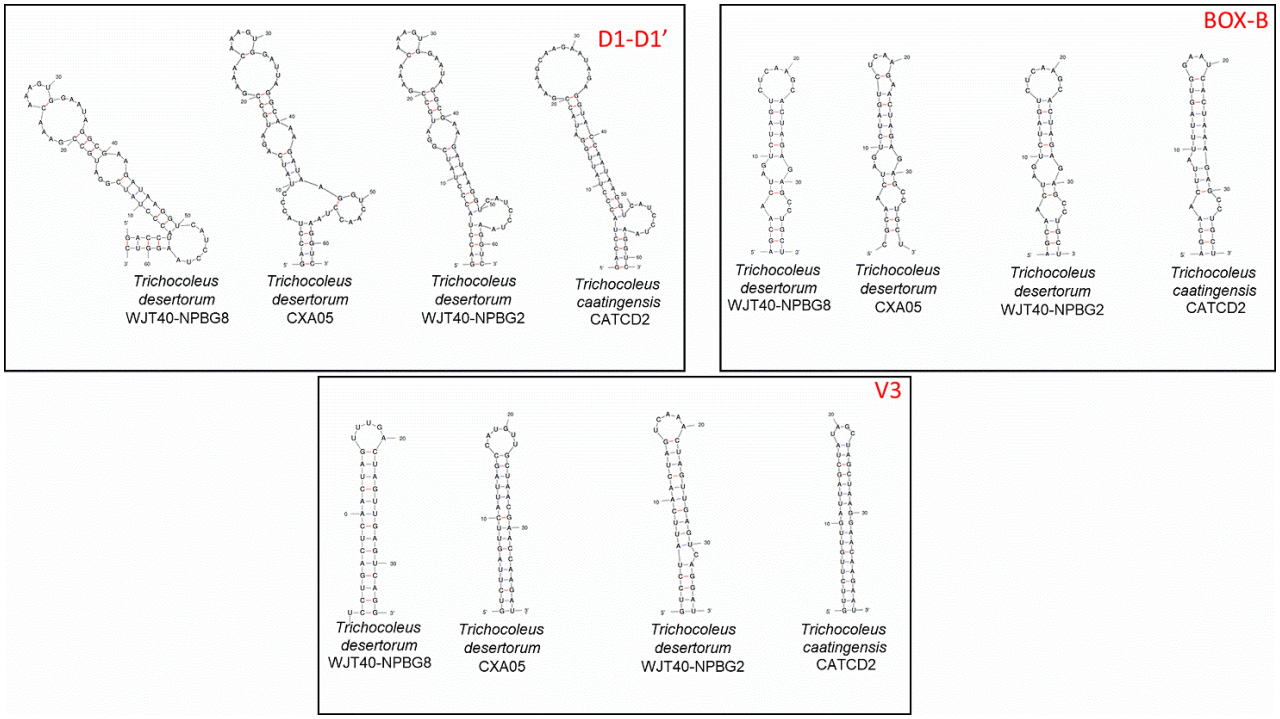


FIGURE 7. Secondary structure of conserved regions of 16S-23S ITS of *Trichocoleus* strains. D1-D1' helices, Box-B helices and V3 helices. Figure of the own author.

4.5 Discussion

The introduction of genetic methods brought significant changes in the cyanobacteria systematics and more than 140 new genera have been defined in the last twenty years (Komárek 2020). The classification system of the group is continuously being updated through the discoveries of new groups or the reinterpretation of some already known.

Microcoleus has been considered to be polyphyletic since Boyer et al. (2002) with many of its strains not being really related to its type species *M. vaginatus*. The erection of the genera *Coleofasciculus* and *Trichocoleus* reinforced the complexity of the genus, while the proven relation of *Microcoleus vaginatus* with *Phormidium autumnale* also showed the complex relationship between the two genera (Strunecký et al., 2013).

This study focused on eight populations from biological soil crusts that presented morphology compatible to the traditional genera *Microcoleus* and *Phormidium*. Considering the similar morphology between both genera and their confusing phylogenetic relationship, it became impossible to study them independently. Therefore, the separation of both genera in this work was accepted by the assumption of *Phormidium irriguum* (Kützing ex Gomont) Anagnostidis & Komárek clade representing *Phormidium* (Sciuto et al., 2012), and *Microcoleus vaginatus* clade representing *Microcoleus* (Boyer et al., 2002).

The determination of the familial placement of most of the studied samples was not possible since the systematic classification of Cyanobacteria at family level is still in flux. The families Coleofasciculaceae, Phormidiaceae and Microcoleaceae stayed intermixed in the phylogenetic tree, allowing only superficial conclusions regarded to this issue. From the evidence at hand, it appears likely that the new genera *Gracilinea*, *Marmoreocelis* and *Konicacronema* are more closely related to the family Coleofasciculaceae than to Microcoleaceae.

The recognition of samples CATCC2 and CATCA7 as pertaining to *Pycnacronema* emphasizes the non-phylogenetical correspondence with the morphological multitrichome disposition for filaments, which for many times was a considerable character for separating some groups. The determination of *P. caatingensis* was based on the p-distance of its ITS sequences in relation to the other sequences. This separation in based on past workers that have used <0.03 p-distance values to infer strains pertaining to the same species and >0.07 values to different species (Erwin and Thacker 2008, Osorio-Santos et al., 2014; Pietrasiak et al., 2014; Becarra-Absalon et al., 2018, and Pietrasiak et al., 2019).

Populations CATCB9 and CATCC10 of *Gracilinea arenicola* presented morphology

similar to *Microcoleus steenstrupii* populations as described by some authors (e.g., Mühlsteinová et al., 2014; Patzelt et al., 2014). However, the low similarity of 16S rRNA gene sequences does not allow identifying these strains as *Microcoleus* species. CATCB9 and CATCC10 were not raised as possible representatives of the real *M. steenstrupii,* since the species is considered a taxonomic complex that possibly includes many genera. The real identity of *M. steenstrupii* awaits sequencing of strains fitting this morphospecies for at least the general location of the type locality (soils of Iceland). This species is certainly not in the genus *Microcoleus*.

The *Marmoreocelis xerophila* population caught our attention by its morphologically distinct kerotomized cytoplasm. *Phormidium* sp. (KM438197) was the more similar 16S rRNA gene sequence, but lower than 96%. In addition, *M. xerophila* occurred in BSCs in South America, while *Phormidium* sp. (KM438197) was found inhabiting a hot spring in Greece. There is no morphological information for this *Phormidium* strain, but it is distant from *Phormidium irriguum*, which is likely more correctly identified as *Phormidium*.

The two strains of *Konicacronema* CATCB1 and CATCB6 presented apical cells typical of Group I of *Phormidium* (Komárek & Anagnostidis, 2005), however, they were genetically not closely related with any already described and sequenced *Phormidium* species. Four strains belonging to their clade, Oscillatoriales cyanobacterium (KJ939036), Oscillatoriophycideae cyanobacterium (KJ489356), Pseudanabaenaceae cyanobacterium (EF654061) and *Coleofasciculus chthonoplastes* (KC463190) also have origin in drylands (Mojave Desert and Colorado Plateau in North America, the Succulent Karoo in South Africa, the Atacama Desert in Chile), emphasizing the correct recognition of the strains as pertaining to the same genus. Only the strain Oscillatoriophycideae cyanobacterium (KJ489356) could be compared to the strains *Konicacronema* CATCB1 and CATCB6 in relation to the ITS, for being the unique sequence with one tRNA available in GenBank. The p-distance between the three ITS sequences showed that *Konicacronema* CATCB1 and CATCB6 pertain to the same species, referred from here on as *Konicacronema caatingensis.* However, Oscillatoriophycideae cyanobacterium (KJ489356) is a different species (p-distance >0.007). Besides, *Coleofasciculus chthonoplastes* (KC463190) was certainly misidentified in GenBank, as it was not grouped inside *Coleofasciculus chthonoplastes* clade.

The proposition of these three new genera and four new species of filamentous cyanobacteria associated with the cryptic genera *Microcoleus* and *Phormidium* reinforces the unknown genetic diversity hidden in groups of organisms with simple morphology and the

importance of phylogenetic studies to reveal it.

This work also contributed to the description of the real composition of the biocrusts presenting in the Brazilian *Caatinga,* highlighting the importance of systematic studies in poorly investigated areas. The understanding of this diversity implies in the knowledge of its roles, which is one of the key factors for success in applied works (e.g. soil restoration works).

Acknowledgements

We are grateful to the anonymous reviewer for the contributions that improved the paper. LHZB thanks to the São Paulo Research Foundation (FAPESP, proc. 2017/11126-6) and to the National Council for Scientific and Technological Development (CNPq, proc. 306851/2017-2) for the financial support to the project and NMML thanks the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil* (CAPES, Finance Code 001) for the fellowship.

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TABLE S1. P-distance among *Pycnacronema* ITS sequences for operons containing $tRNA^{Ile}$.

	1	2	3	4	5	6	7	8
1. *Pycnacronema conicum* 7PC (MF581656)	-							
2. *Pycnacronema arboriculum* 41PC (MF581657)	0.246	-						
3. *Pycnacronema edaphica* CATCA7 (MT311244)	0.261	0.123	-					
4. *Pycnacronema caatingensis* CATCC2 (MT311243)	0.258	0.084	0.129	-				
5. *Pycnacronema marmoreum* 42PC (MF581658)	0.286	0.171	0.162	0.174	-			
6. *Pycnacronema rubrum* 43PC (MF581659)	0.263	0.132	0.134	0.0140	0.176	-		
7. *Pycnacronema brasiliensis* 45PC (MF581661)	0.255	0.104	0.078	0.106	0.157	0.132	-	
8. *Pycnacronema brasiliensis* 44PC (MF5816600)	0.255	0.104	0.078	0.106	0.157	0.132	0.000	-

Table of the own author.

TABLE S2. P-distance among *Trichocoleus* ITS sequences for operons containing both tRNAs.

	1	2	3	4	5	6	7
1. *Trichocoleus caatingensis* CATCD2 (MT311249)	-						
2. *Trichocoleus desertorum* (MH463395)	0.096	-					
3. *Trichocoleus desertorum* (KF307612)	0.106	0.068	-				
4. *Trichocoleus badius* (EF429296)	0.104	0.086	0.096	-			
5. *Trichocoleus badius* (EF429297)	0.108	0.080	0.096	0.008	-		
6. *Trichocoleus desertorum* (KF307610)	0.108	0.066	0.012	0.100	0.094	-	
7. *Trichocoleus desertorum* (KF307614)	0.100	0.056	0.074	0.044	0.040	0.076	-

Table of the own author.

Capítulo V – Biocrust cyanobacteria composition, diversity, and environmental drivers in two contrasting climatic regions in Brazil

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Submitted: Geoderma - Special Issue 'Biocrusts: Engineers and Architects of Surface Soil Properties, Functions, and Processes in Dryland Ecosystems' (4th September 2020)

5.1 Abstract

Biological soil crusts or biocrusts have critical ecological roles in dryland ecosystems including soil stabilization, erosion control and nutrient cycling. Global environmental changes are expected to impact terrestrial ecosystems, including biocrust communities. Thus, a growing number of studies have focused on investigating the diversity of biocrust-forming organisms including cyanobacteria. Despite the increasing interest in understanding biocrust cyanobactria, there are still several knowledge gaps, particularly in areas from South America, where studies are limited. Here, we studied the composition, abundance, and environmental drivers of cyanobacterial biocrust from two important biomes in Brazil, i.e. *Caatinga* and *Pampa,* which are subject to desertification and anthropogenic pressures, respectively. Ten locations at three sites were explored at each biome (n=60) and cyanobacterial biocrust community composition and diversity was analyzed through morphological evaluation and Next Generation Sequencing targeting 16S rRNA. Soil and environmental variables, e.g. organic matter, soil texture, nitrogen and phosphorus content, temperature, and irradiance, were also determined at each site. Our results showed that biocrusts cyanobacteria had distinct taxonomic compositions and assemblages at each biome. Differences across sites within each biome also differed substantially. Soil temperature, solar irradiance, and phosphorous content were identified as the main factors explaining such biotic structures. *Caatinga* sites, characterized by more arid environments, presented a higher abundance of N-fixing cyanobacteria, e.g. *Scytnema*. In contrast, Pampa, with higher rainfall regimes, showed a larger abundance of biocrust-forming bacteria such as *Microcoleus* and *Leptolyngbia*. The outcomes of this research are expected to provide a basis to enhance biocrusts and conservation efforts and promote the use of biocrust cyanobacteria as global change indicators

Keywords: Biological soil crust, cyanobacterial diversity, 16S rRNA, *Scytonema, Caatinga*, *Pampa*.

5.2 Introduction

In dryland regions, where water availability is limited and the development of vascular plants is restricted, the top few centimeters of the soil surface provide a suitable habitat for poikilohydric organisms, like cyanobacteria, algae, fungi, bacteria, lichens, mosses and microarthropods (Belnap, 2016; Bowker et al., 2011). The interwoven community formed by these organisms is known as biological soil crust, biocrust, cryptogamic, cryptobiotic, microbiotic or microphytic soil crusts (Belnap 2005). Biocrusts constitute up to 12% of the Earth's terrestrial surface (Rodriguez-Caballero et al., 2018), and are responsible for many important ecological functions in semiarid and arid ecosystems including soil stabilization, erosion reduction (Gundalapally & Garcia-Pichel, 2006, Chamizo et al., 2018), increased water retention, and nitrogen and carbon fixation (Belnap, 2005, Chamizo et al., 2012).

Given their key ecological functions, biocrusts can be crucial to reverse desertification, and consequently many studies have focused on enhancing biocrust inocula for their recovery (Ayuso et al., 2017; Bethany et al., 2019; Giraldo-Silva et al., 2019, Muñoz-Rojas et al., 2018; Román et al., 2020). Cyanobacteria are the first colonizers of soil biocrusts (Garcia-Pichel & Wojciechowsky, 2009) allowing their succession through soil stabilization, nutrition and increased moisture content (Rossi et al., 2017). Therefore, many studies aimed to assess the diversity of cyanobacteria communities, with detailed investigations becoming possible through non-culture-dependent techniques, e.g. Denaturing Gradient Gel Electrophoresis (DGGE, e. g. Garcia-Pichel et al., 2001) and Next Generation Sequencing (NGS, e. g. Machado- de-Lima et al., 2019). These techniques have allowed the generation of a massive quantity of sequences and also their association with environmental variables, resulting in detailed descriptions (Steven et al., 2013; Büdel et al., 2014; Pushkarevá et al., 2015; Williams et al., 2016; Fernandes et al., 2017; Machado-de-Lima et al., 2019).

The composition of biocrust communities has been investigated in recent years across several regions, e.g. North America (Fernandes et al., 2018; Pombubpa et al., 2020), Asia (Zhang et al., 2016, Li et al., 2020), Australia (Büdel et al., 2018), Europe (Williams et al., 2016; Mikhailyuk et al., 2019), Africa (Venter, et al., 2017) and South America (Samolov et al., 2020). Some studies described well-known components of biocrusts, e.g. the common genera *Microcoleus*, *Nostoc* and *Scytonema* in areas like Central Spain (Cano-Díaz et al. 2017). Other studies identified filamentous non-heterocytous cyanobacteria as the major components in the Mediterranean crusts (Muñoz-Martin et al., 2018). Uncommon

cyanobacterial groups such as unicellular/colonial cyanobacteria were found as one of the dominants in a semidesert in Central Mexico (Becerra-Absalón et al., 2019). Yet, there are several knowledge gaps about the composition of these communities in soils from many regions. Improving our knowledge about the structure of the biocrust communities, particularly in underrepresented areas, is critical for a broader understanding of their functions across terrestrial ecosystems (Bowker et al., 2011).

In this research, we expanded from previous studies describing cyanobacterial biodiversity of biocrusts in the biome *Cerrado* in Brazil (Machado-de-Lima et al., 2019) to assess the diversity and composition of communities from the Brazilian biomes *Caatinga* and *Pampa*. Although it is considered one of the more diverse drylands (Silva et al., 2017), *Caatinga* is under an intense risk of desertification (Vieira et al., 2015) with changes in the natural xeromorphic vegetation due to burnings caused by human activities, and the development of pastures and agricultural fields (Althoff et al., 2018). *Caatinga* region is particularly susceptible to changes in climate (Seddon et al., 2016) and ecosystems in this region have being poorly investigated (Santos et al., 2011), counting to our knowledge, with only one biocrusts' survey (Szyja et al., 2019). In *Pampa*, despite being one of the most species-rich grasslands in the world (Overbeck et al., 2007), land has been extensively replaced by agricultural activities (Oliveira et al., 2017). Although biocrusts in this region have being previously analyzed (Webber, 2014, 2016), detailed descriptions are not available. Importantly, this region has been identified as the biome with the highest biodiversity conservation gap among the Brazilian protected area network (Fonseca et al., 2017).

The overall objective of this study was to investigate the cyanobacterial composition of biocrusts from the biodiverse *Caatinga* and *Pampa* biomes through next-generation-sequencing combined with morphological and molecular analyses. We also evaluated environmental and soil factors as explanatory variables of the spatial distribution of cyanobacterial communities in these regions. These climatically contrasting biomes have important ecological features and conservation value (Silva et al., 2017; Menezes et al., 2018) and knowledge about their biocrust's composition is limited (Szija et al., 2019; Webber, 2014, 2016). The final aim of this research was to provide an essential baseline for future applications in environmental preservation and restoration of biocrust communities, soils, and ecosystems in these biodiverse areas and elsewhere.

5.3 Materials and Methods

5.3.1 Study areas

This study was conducted in six sites within the *Caatinga* and *Pampa* biomes (Table 1). *Caatinga* lies in the semi-arid section of the country and covers 11% of the national territory, equivalent to about 844,000 km^2, and nine states (IBGE, 2019). Vegetation in the area is dominated by xeric shrublands (Pennington et al., 2009) adapted to long periods of drought conditions (Silva et al., 2017). Total annual precipitation ranges from 200 to 800 mm (Barbosa et al., 2019). Soils derive from different types of rocks, with limestones covering a wide percentage of the biome (Eiten & Goodland, 1979). These soils are generally shallow with no more than 1 m of depth, which can restrict the water supply during the dry season (Eiten & Goodland, 1979).

Pampa is located in the south of Brazil and covers an area of 178,243 km^2 shared among Brazil, Argentina and Uruguay, representing 63% of the Rio Grande do Sul state's area (IBGE, 2004). Climate types in this biome are mainly subtropical and temperate with four marked seasons, and annual precipitation ranging between 1,200–1,600 mm (Roesch et al., 2009). Vegetation is composed by native grasslands, sparse shrubs, and tree formations (Berreta, 2001). Soils originate from sedimentary rocks and are prompt to wind and water erosion processes, since there is no vegetal cover to avoid erosion, the soil sediments keep highly moving (Roesch et al., 2009).

Table 1. Geographic origin of the biological soil samples in Brazil. PB: *Paraíba* state; RS: *Rio Grande do Sul* state.

Sample Code	**GPS Coordinates**	**Municipality/State**	**Biome**	**Altitude (m)**
CA	07°27'S; 36°16'W	*Cabaceiras*/PB	*Caatinga*	433
CB	07°24'S; 36°19'W	*Cabaceiras*/PB	*Caatinga*	420
CC	07°07'S; 36°08'W	*Pocinhos*/PB	*Caatinga*	559
PA	29°53'S; S 55°35'W	*Alegrete*/RS	*Pampa*	114
PB	29°43'S; 55°32'W	*Alegrete*/RS	*Pampa*	102
PC	29°28'S; 55°28'W	*Manoel Viana*/RS	*Pampa*	143

Figure of the own author.

5.3.2 Field survey and soil and biocrust sampling

Biocrust and soil (upper 10 cm) samples were collected from three sites at each of the two studied biomes (Table 1). Ten samples distributed equidistantly were collected throughout a 200 m long transect at each site (total n=60). Samples were stored in Petri dishes, transported to the lab and kept frozen at −20°C until processing. Soil temperature and irradiance were measured in the field using Thermal-Hygrometer (PeakTech 5090) and Quantameter (Li-Cor). Precipitation, temperature, air humidity and insolation were calculated based on the annual average for the year 2017, obtained from the public database available at INMET Portal (2020). Weather stations used were #82795 in the city of *Campina Grande* (*Paraíba state*) for *Caatinga,* and #83927 in the city of *Uruguaina* (*Rio Grande do Sul state*) for *Pampa*.

5.3.3 Morphological evaluation of cyanobacteria from the biocrusts

For the morphological evaluation, one site representative of each biome was selected for analysis, i.e. CC and PC (n=20; Table 1 and Table 2). Biocrust samples from these locations were analyzed following the protocol of Garcia-Pichel et al. (2001). Cyanobacterial populations were identified through a stereoscopic microscope (Olympus, model SZX7) and the morphological variability of populations was evaluated using an Olympus BH2 microscope.

Filamentous bundles and portions of crusts were cultivated in BG11 medium (Rippka et al., 1979), and isolated after repeated inoculation in tubes with BG11 growth medium. Cultures were maintained under 20 ± 1ºC, 60 µmol photons m^{-2} s^{-1} of irradiance and a 14:10 h light-dark cycle. We analyzed the morphological characteristics of the cultivated samples and identified them according to Komárek and Anagnostidis (1999, 2005) and Komárek (2013).

5.3.4 Library Preparation and Illumina Sequencing of 16S rRNA Gene

For DNA extraction, 1 g of soil was separated from each sample (n=60) avoiding soil grains, rocks, plant roots and leaves, and extracted with the MoBio Powersoil kit (Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The V3-V4 region from 16S rRNA gene (420 bp) was amplified by PCR using the CYA 359 and 781a/b primers as described in Nübel et al. (1997). The PCR reaction contained a final

volume of 20 µL, with 10 µL de GoTaq® Colorless Master Mix 2x (Promega, USL), 1 µM of forward oligonucleotide, 0,5 µM of reverse oligonucleotide, 2.2 µl of genomic DNA and ultrapure water to complete 20 µL. PCR products were purified using Agencourt AMPure XP kit (Beckman Coulter).

Illumina sequencing adapters and dual-index barcodes were added to the amplicon target following the protocol of Nextera XT Index Kit (Illumina, USA). The product was purified using Agencourt AMPure XP kit (Beckman Coulter) and quantified with QuantStudio 3 Real Time (Applied Biosystems) and Kit KAPA-KK4824 (Library Quantification Kit- Illumina/Universal), according to the manufacturer's instructions. The preparation of the samples followed the Illumina guidelines for sequencing in a MiSeq platform (Illumina) using MiSeq Reagent kit v2 500 cycles × 150 cycle. All sequence datasets were submitted to NCBI under the project "Cyanobacteria of biological soil crusts from Brazilian dry tropical forest (caatinga) and southern grasslands (pampa): metagenomics, taxonomic characterization and relationships with physical and chemical parameters" (NCBI submission number: SUB8092820).

5.3.5 Data analysis pipeline and Operational Taxonomic Unit Taxonomic assignment

Raw sequencing data was processed with the *OTUreporter v1.0.0-beta (9b72c8e*) pipeline (https://bitbucket.org/xvazquezc/otureporter) based on Mothur (v1.39.5, http://www.mothur.org/ - Schloss et al., 2009). Briefly, the reads were quality-filtered and assigned to their respective samples. Sequences were trimmed according to the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP) and only those with a length between 251 and 473 bp were retained. Samples with homopolymers longer than 8 bp were removed. Chimeric sequences were removed using the *chimera.vsearch* script included in Mothur (Rognes et al., 2016). The sequences were aligned and classified against the SILVA database v132 (Quast et al., 2013), and high-rank lineages not targeted by the primers (i.e. Archaea, Chloroplast, Eukaryota, Mitochondria, unknown) were removed. Sequences were grouped into OTUs based on 97% similarity using the OptiClust algorithm (Westcott and Schloss, 2017) and subsampled based on the sample with the lowest number of sequences, i.e. 10000 sequences.

Representative sequences for each OTU based on the most abundant sequence were selected with the *get.oturep* command from Mothur. Representative sequences from OTUs with at least 10 sequences across all samples were placed in the reference Cyanobacteria phylogenetic tree with Cydrasil v1.5 (https://zenodo.org/record/1409274). The

representative sequences were also searched against NCBI's database (version June 2019) using BLAST+ v2.9.0 (-max_hsps 1 -evalue 1e-5 -max_target_seqs 20) (Altschul et al., 1997). OTUs that were at least 95% similar to sequences assigned in NCBI were recognized in genera level, while those with similarities below this value were not identified. The taxonomical classification for polyphyletic genera were done as in Machado-de-Lima et al. (2019).

5.3.6 Analyses of soil variables

Soil samples were analysed for physicochemical, characteristics following the methods by the Instituto Agronômico, State of São Paulo, Brazil (Camargo et al., 2009). Total nitrogen and fractions were measured by Kjeldahl's procedure, total phosphorous through *sulfuric* acid *digestion and* orthophosphate was determined by ion-exchange resin. Organic matter was measured by chromic acid wet oxidation and pH was determined in $CaCl_2$ solution. Soil texture was obtained by chemical dispersion with NaOH, physical dispersion by vertical agitation for 16 hours. Sand content was determined by sieving and clay content by sedimentation (pipette method).

5.3.7 Statistical analyses

All the statistical analyses were performed using R version 4.0.2 (R Core Team 2020). The package "Phyloseq" (McMurdie & Holmes, 2013) was used to rarefy all the sites and prepare the data for diversity analyses. Alpha diversity analysis was performed using the package "Microbiome" (Lahti et al., 2017). The significance of the differences between the biomes was assessed through ADONIS test performed on Bray-Curtis distance matrices of relative abundance obtained from sequencing, using the function "adonis2" in the package "vegan" (Dixon, 2003). Non-metric multidimensional scaling (NMDS) was used to illustrate the community composition.

The environmental dataset (Table S1) was correlated to the distribution and abundance of OTUs, through the packages "vegan" (Oksanen et al., 2019), "ggplot2" (Wickham, 2018), and "gridBase" (Murrell, 2015), written in R language (R Core Team, 2020). A redundancy analysis (RDA) was run as a constrained ordination method to show the spatial patterns of the cyanobacteria database.

5.4 Results

5.4.1 Morphological evaluation of biocrust cyanobacteria

The CC site at *Caatinga* showed five different species associated to the genera *Microcoleus* Desmazières ex Gomont, *Nostoc* Vaucher ex Bornet & Flahault and *Scytonema* Agardh ex Bornet & Flahault (Table 2). We found *Microcoleus* bundles among soil grains, mainly in the bottom parts of the biocrust. *Nostoc* populations were observed in the top part of the biocrusts in the form of irregular to spherical colonies, and *Scytonema* filaments were found entangled and forming big dark biomasses (Fig. 1). At the PC site in *Pampa*, seven species were identified distributed across the genera *Scytonema, Stigonema* Agardh ex Bornet & Flahault*, Schizothirx* Kützing ex Gomont*, Leptolyngbya* Anagnostidis et Komárek and *Porphyrosiphon* Kützing ex Gomont (Table 2)*. Scytonema* filaments were also found entangled and forming hairy dark biomasses, *Stigonema* filaments also were found in the top of the biocrust forming a hairy yellowish biomass, while *Porphyrosiphon* and *Schizothirx* formed a reddish biomass. *Leptolyngbya* presented filaments entangled inside a colorless mucilaginous biomass (Fig. 2).

5.4.2 Biocrust cyanobacteria composition, diversity, and abundance

The phylogenetic placements of the OTU's in Cydrasil, and the recognitions of the exact clade where the OTU was placed, allowed the exact delimitation of the main taxonomic units, even for those OTUs related to cryptical genera, i.e. *Microcoleus* and *Leptolyngbya. Leptolyngbya* was separated in Clades I, II, III and IV and their delimitation can be observed in Supplementary Fig. 1.

The first clade included many strains identified as *L. frigida,* the type species of *Leptolyngbya*, and here considered as the *Leptolyngbya sensu stricto* clade. All the other three clades placed other strains were identified as *Leptolyngbya, Alkalinema* Vaz et al.*, Phormidesmis* Turicchia, Ventura, Komárková & Komárek*, Elainella* Jahodárová, Dvorák & Hasler*, Oculatella* Zammit, Billi & Albertano, which are genera recently separated and morphologically similar to *Leptolyngbya. Microcoleus* was also divided in the *Microcoleus vaginatus* Gomont ex Gomont clade, which is a well delimitated clade, considered the *Microcoleus sensu stricto* clade (Boyer et al., 2002), and *Microcoleus* I clade, which grouped not only strains identified as *Microcoleus steenstrupii,* but also recently separated genera, as *Potamolinea* Martins & Branco and *Pycnacronema* Martins & Branco. These last two

present *Microcoleus*-like morphology and the exact delimitation of most of the OTUs placed in this big clade was not possible, because of its close relation and the short length of the OTUs evaluated.

Table 2. Cyanobacteria observed in intact field samples. Sample codes numbers refer to the number of the biocrust sampled for each site.

Taxon	Sample code	Origin	Biome	Figure
Microcoleus steenstrupii	CC4	Pocinhos/PB	*Caatinga*	Figure 1
Nostoc sp. 1	CC9	Pocinhos/PB	*Caatinga*	Figure 1
Nostoc sp. 2	CC10	Pocinhos/PB	*Caatinga*	Figure 1
Porphyrosiphon	CC2	Pocinhos/PB	*Caatinga*	Figure 1
Scytonema guyanense	CC1	Pocinhos/PB	*Caatinga*	Figure 1
Leptolyngbya sp.	PC5	Manuel Viana/RS	*Pampa*	Figure 2
Porphyrosiphon	PC5	Manuel Viana/RS	*Pampa*	Figure 2
Schizothrix acutissima	PC8	Manuel Viana/RS	*Pampa*	Figure 2
Schizothix telephoroides	PC5	Manuel Viana/RS	*Pampa*	Figure 2
Scytonema javanicum	PC6, PC8	Manuel Viana/RS	*Pampa*	Figure 2
Scytonema ocellatum	PC8	Manuel Viana/RS	*Pampa*	Figure 2
Stigonema ocellatum	PC2, PC3, PC7,	Manuel Viana/RS	*Pampa*	Figure 2

Table of the own author.

A total of 16,382 OTUs were obtained through the NGS analysis. Those OTUs with at least 10 counts were considered for the abundance plot, representing 3,878 OTUs, 1,329,420 reads and 96% of the total diversity. The 100 most abundant OTUs summarized 1,095,712 reads, accounting for 82,4% of the diversity (considering the 3,878 OTUs). These OTUs had their taxonomy assigned and at least 80% of them were identified (mostly at genus level – Table S2). At this level of identification, the community composition was relatively homogeneous in *Caatinga* and heterogeneous in *Pampa*. *Microcoleus* I, *Chroococidiopsis* Geitler and *Scytonema* were the most abundant genera in *Caatinga,* while *Leptolyngbya* I, *Microcoleus* I, *Potamosiphon* McGregor & Sendall and *Porphyrosiphon* were the most abundant in *Pampa* (Fig. 3).

Figure 1. Cyanobacteria observed in intact field samples from *Caatinga*. *Microcoleus steenstrupii* (a,b); *Nostoc* sp. 1, (c,d); *Nostoc* sp. 2 (e-f); *Scytonema guyanense* (g-h); *Porphyrosiphon notarisii* (i-j). Bars = 10 µm. Figure of the own author.

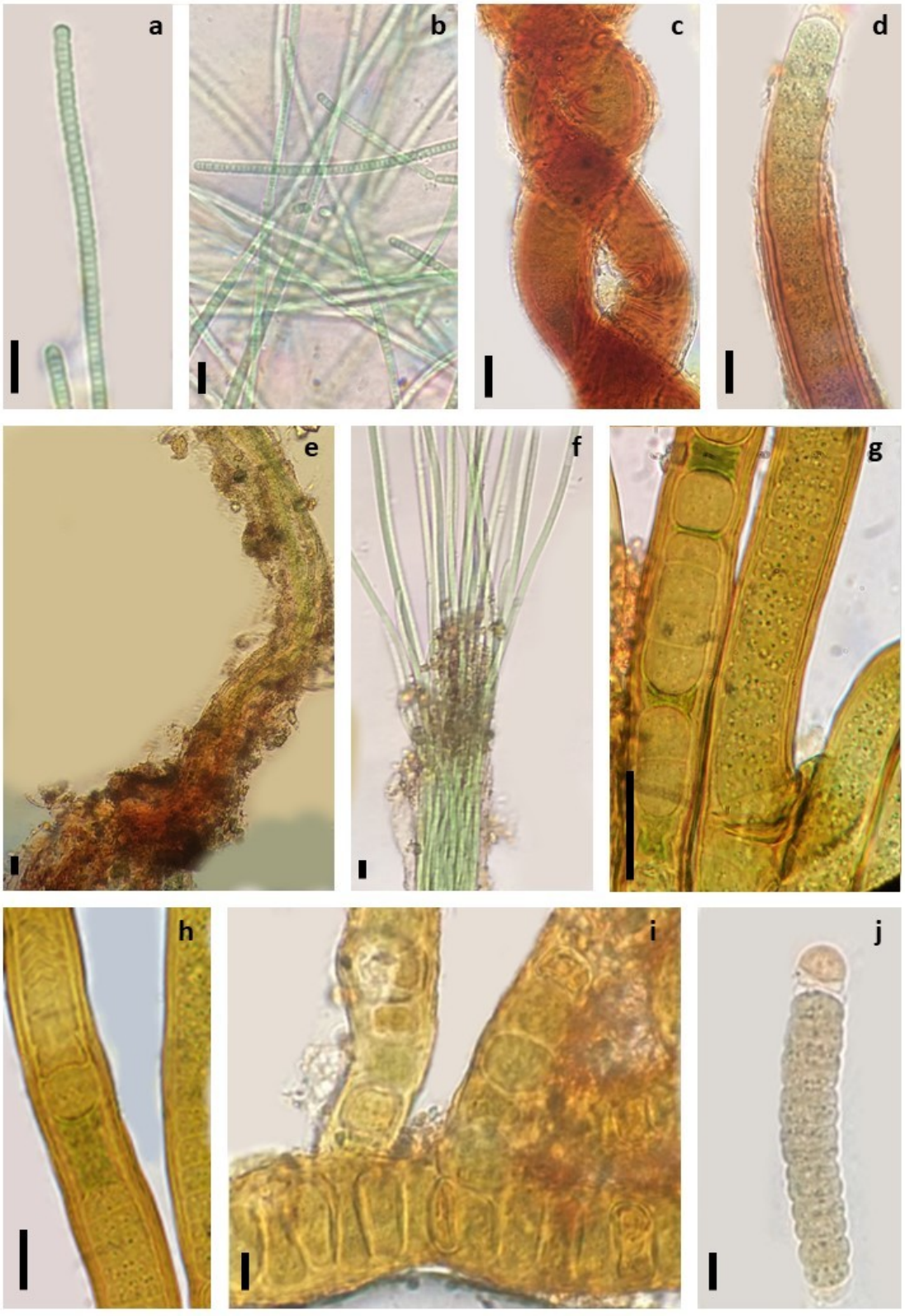

Figure 2. Cyanobacteria observed in intact field samples from *Pampa*. *Leptolyngbya* sp. (a-b); *Porphyrosiphon notarisii (*c-d); *Schizothrix acutissima* (e), *Schizothix telephoroides*(f); *Scytonema javanicum*(g); *Scytonema ocellatum* (h); *Stigonema ocellatum* (i-j). Bars = 10 µm. Figure of the own author..

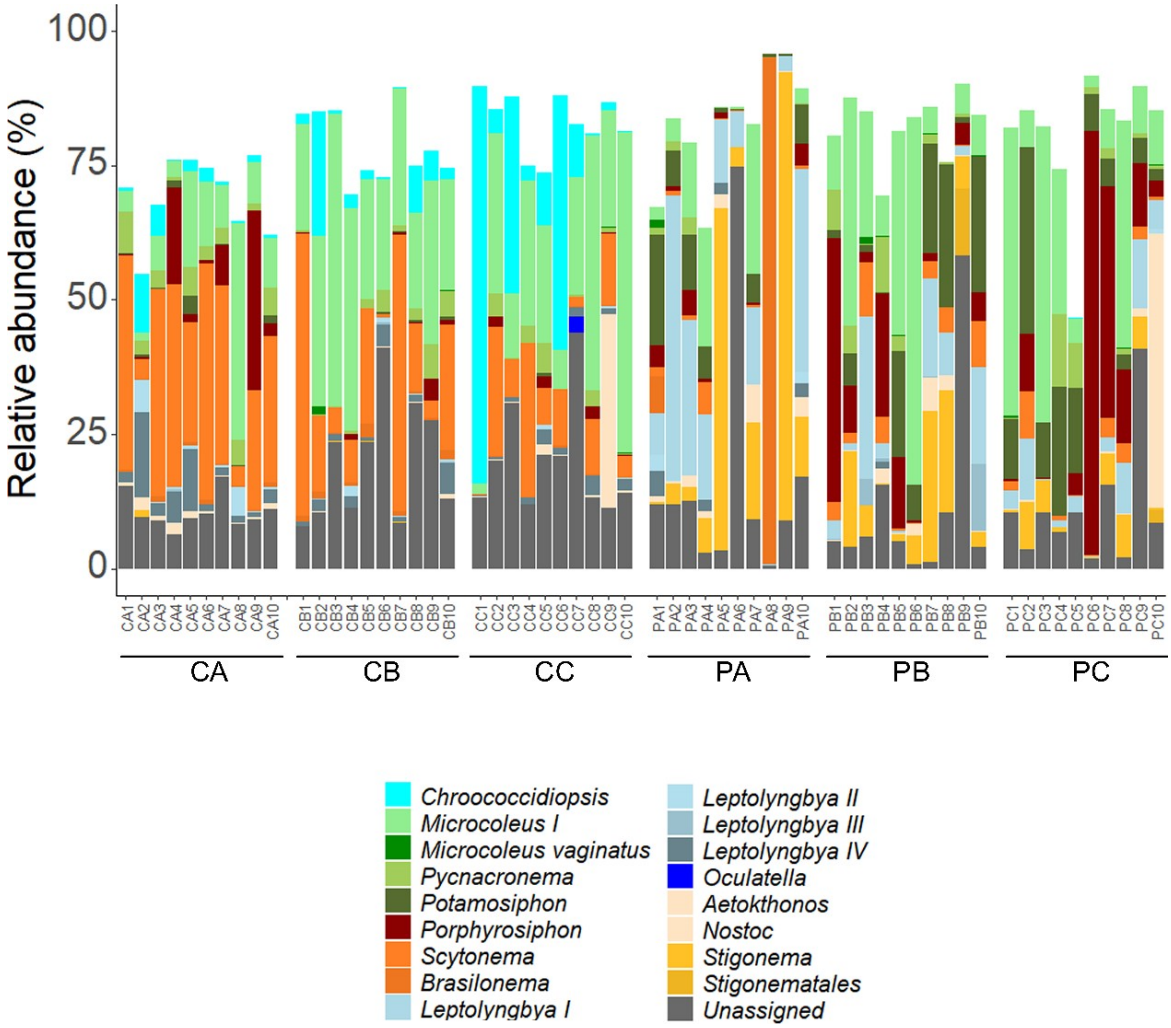

Figure 3. Relative cyanobacterial abundance by proportion of 16S rRNA gene sequence reads showing assignable, unassignable taxa level and not evaluated OTUs in *Caatinga* and *Pampa* biocrusts. CA, CB, and CC refer to the three sites of *Caatinga*, while PA, PB and PC are sites of *Pampa* (see Table 1). Figure of the own author.

Our results showed that the composition of cyanobacteria differed significantly (PERMANOVA, F. Model = 4.1586, df = 5, $P < 0.001$) across the two biomes (Fig. 4). Difference among sites within *Caatinga* and *Pampa* biomes were also significantly different Table 4). The taxonomic composition found at *Caatinga* showed the homocytous-filamentous cyanobacteria as the most common components, with 32% of relative abundance (RA). Heterocytous cyanobacteria and unicellular/colonial cyanobacteria accounted for 19% and 12% of the RA, respectively.

At *Pampa,* the cyanobacterial group with the highest RA was also the homocytous filamentous forms, but in a higher proportion than *Caatinga* (55%), while the heterocytous forms complemented the diversity with 21% of RA (Table 3).

In *Caatinga, Scytonema* (29% of the RA) was dominant in CA and *Microcoleus* in CB (29%), and CC (28%). In CC, the unicellular genus *Chroococidiopsis* followed in abundance with 16% of the RA. In the *Pampa* biome, PB and PC showed similar composition in relation to the two most abundant genera (*Microcoleus* ~ 20%, *Porphyrosiphon* ~ 10%), while *Stigonema w*as the most abundant in PA (22% of the RA) (Table 3).

Table 3. Differences among sites and their dominant composition based on the relative abundance. CA, CB and CC refer to the three *Caatinga* sites, and PA, PB and PC to the *Pampa* sites.

Sites	Most abundant genera per site (percentage of RA)	Most abundant genera per location	PERMANOVA
CA	*Scytonema* (29%) *Microcoleus* (11%)	*Microcoleus* I, *Chroococidiopsis* *Scytonema,*	F Model=2.3264 df=2 *P*<0.001
CB	*Microcoleus* 29%) *Scytonema* (20%)		
CC	*Microcoleus* (28%) *Chroococidiopsis* (16%)		
PA	*Stigonema* (22%) *Leptolyngbya* (18%)	*Leptolyngbya* I, *Microcoleus* I, *Potamosiphon*, *Porphyrosiphon*	F Model=1.6261 df=2 *P*<0.01
PB	*Microcoleus* (20%) *Porphyrosiphon* (10%)		
PC	*Microcoleus* (23%) *Porphyrosiphon* (12%)		

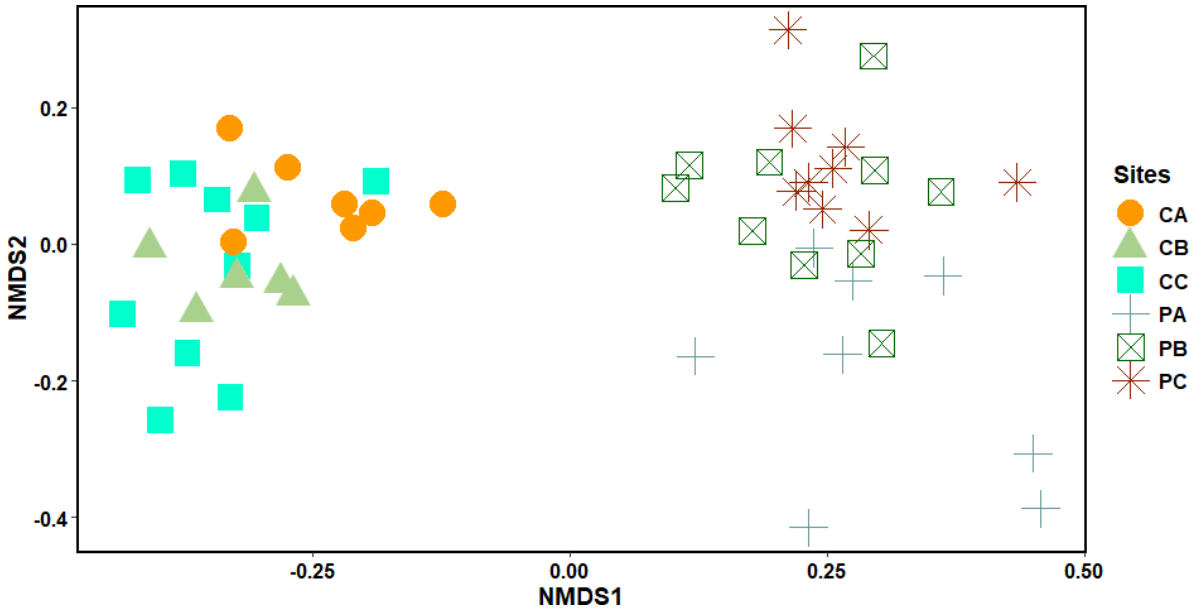

Figure 4. Non-metric Multi-Dimensional Scaling (NMDS) of the biocrust cyanobacterial community composition at the OTU level, based on Bray-Curtis similarity including *Caatinga* and *Pampa.* 2D stress: 0.13. CA, CB and CC refer to the three sites of *Caatinga*, while PA, PB and PC are sites of *Pampa* (see Table 1). Figure of the own author.

5.4.3 Correlation between Operational Taxonomic Units and Environmental Variables

The Redundancy Analysis (RDA) showed significant ($P < 0.0001$) relationships between the soil and environmental variables, and the cyanobacteria composition (Fig. 5). These variables explained 42% of the community distribution. The first two axes explained 18% of the cyanobacteria composition variability, and vectors of soil temperature, orthophosphate, total phosphate content and silt were the most important factors driving this variation, (explaining 30% of their variability). Soil temperature, chemical variables, e.g. N and P and organic matter, and irradiance at the crusts and in the air, were positively related to all the sites of *Caatinga*, and negatively to all the sites of *Pampa*. *Pampa* showed a strong relationship with clay and sand contents, while *Caatinga* was positively related to silt content (Fig.5).

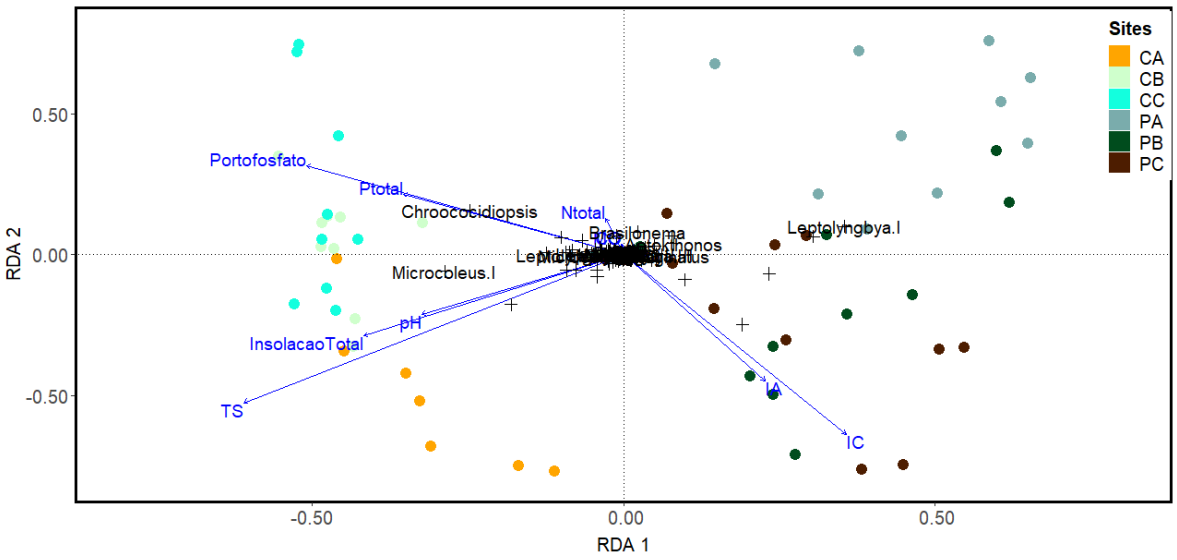

Figure 5. Redundancy analysis (RDA) relating climatic variables with cyanobacterial diversity in 60 sites from *Caatinga* (CA, CB, CC) and *Pampa* (PA, PB, PC) biomes. Ntotal = Total Nitrogen; OC = Organic Carbon; OM = Organic Matter CI = Solar Irradiance at the crust spot; AI = Solar irradiance at nearest spot of soil without vegetation cover; ST = Soil temperature. Figure of the own author.

5.5 Discussion

Overall, our results showed that biocrusts of the *Caatinga* and *Pampa* biomes clearly differed in the taxonomic compositions of their cyanobacterial assemblages and that different sites within each biome had also distinct assemblages. These differences were largely driven by the studied soil and environmental variables, particularly soil temperature, orthophosphate, and total phosphate contents.

The morphological evaluation of the cyanobacteria populations from *Caatinga* and *Pampa* revealed many of the well-known biocrusts colonizers, i.e. *Microcoleus*, *Nostoc* and *Scytonema* (Büdel et al., 2009). These cyanobacteria genera are commonly found in biological crusts from several locations and have been well investigated (Garcia-Pichel & Wojcichowski, 2009, Garcia-Pichel et al., 2016, Yeager et al. 2004, 2007).

The metagenomic analysis, allowed us further access to a portion of the diversity not recognizable by morphological evaluations. Thus, the high throughput sequencing showed a considerable percentage of the filamentous non-heterocytous cyanobacteria *Microcoleus, Leptolyngbya* and *Porphyrosiphon*, commonly considered biocrust colonizers (Büdel, 2009; Garcia-Pichel & Wojciechowski 2009; Péli et al., 2011), but also revealed *Stigonema, Scytonema* and *Nostoc*, which represent late stages of colonization in crusts (Maier et al., 2016). *Chroococidiopsis*, *Porphyrosiphon* and *Brasilonema*, which are genera not commonly recorded in these communities, were also identified.

In a previous study in the Brazilian savanna or *Cerrado* (Machado-de-Lima et al., 2019), the dominant cyanobacteria found on biocrusts samples were substantially different to the ones in the biomes studied here. Cyanobacterial composition in *Cerrado* also presented significative variations across sites, similar to our findings in *Caatinga* and *Pampa*. These variation across sites with similar environmental conditions evidence that cyanobacteria diversity and composition are also influenced by local characteristics, e.g. soil texture as much as by the broad-spectrum environmental variables (e. g. temperature and precipitation).

The cyanobacterial composition of the two biomes showed a completely separated spatial distribution, and an inverse correlation with the environmental variables. As expected, *Pampa sites,* characterized by higher rainfall regimes, showed stronger relation to lower temperatures and solar irradiances, opposite to *Caatinga* where more extreme environmental conditions prevail. *Caatinga* sites presented a large abundance of *Scytonema* and *Chroocodidiopsis*, and were positively related to high temperatures and solar irradiance. The dominance of *Scytonema* in *Caatinga* can be explained by their ability to synthesize and

accumulate scytonemin, a UV-sunscreen compound that prevent the cyanobacteria cell from damages caused by the excessive radiation (Garcia-Pichel & Castenholz, 1991). The presence of *Chroococcidiopsis* was unexpected as these phylotypes do not produce protectant pigment and commonly live in caves or under rocks where they are relatively protected from strong UV radiation (Péli et al., 2011).

Climate has been reported as one of the main factors explaining the cyanobacterial distribution (Becerra-Absalón et al., 2019; Muñoz-Martin et al., 2018). Similarly, our results pointed to temperature and irradiation as major drivers of these communities 'assemblages at large scale. However, we showed that soil characteristics are the most important factors at local scales, as previously highlighted by Cano-Diaz et al. (2018). In particular, phosphorus (phosphate and orthophosphate) were key variables determining the distribution of cyanobacteria in the *Caatinga's* biocrusts. This element is considered essential for the growth of phototropic organisms; however, its availability is limited (Schmidt et al., 2012). This limitation can be partially suppressed by biological soil crusts and their high capability of bio weathering (Baumann et al., 2018). *Caatinga* sites correlated positively with total soil nitrogen and exhibited high abundance of N2-fixing organisms, e.g., Nostoc and Scytonema (Rosentreter et al., 2016). These communities, also called 'dark' or 'mature' biocrusts, fix larger amounts of nitrogen than light crusts through N "leakage" (Barger et al., 2013), i.e. by exporting ammonium, nitrate and organic N to the surrounding soil (Johnson et al., 2007).

We found a marked distinction across biomes regarding soil texture, with *Pampa's* cyanobacterial communities particularly associated to sandy-rich soils. Previous studies have pointed to soil texture as the main determinant of biocrust cover (Belnap et al., 2014; Rozenstein et al., 2014) and have indicated a dominance of bundle-forming cyanobacteria (i.e. *Microcoleus*) in bare soils (Garcia-Pichel & Wojciechowski 2009). Our study followed that trend showing a higher abundance of *Microcoleus* and *Leptolyngbia* in Pampa, both filamentous, non-heterocystous cyanobacteria capable to colonise and stabilise soils (Muñoz- Martin et al., 2018).

Overall, the biocrusts' composition in the semi-arid *Caatinga* evidenced the dominance of cyanobacteria genus able to activate chemical pathways that allow their survival under high temperatures and levels of irradiance. Cyanobacterial composition found at *Pampa* revealed the occurrence of genera mostly acting as primary colonizers. Although some of these communities also possess strategies to avoid strong solar radiation and desiccation (e.g., vertical migration in *Microcoleus*, Garcia-Pichel & Pringault, 2001), these

cannot synthetize many pigments that would confer protection against radiation (Belnap et al., 2004). The strong positive relation of *Caatinga*'s biocrusts with important chemical compounds to the soil (e.g. phosphorous, nitrogen), and also the presence of cyanobacteria common to dark biocrusts, highlight the importance of these communities for promoting a nutritional rich soil system able to harbor bigger organisms.

Current and future global environmental changes are expected to have a critical impact on biocrust communities in drylands across the globe (Rodriguez-Caballero et al.,2018). These changes can result in a redistribution of biocrust organisms and communities, that will adapt to more extreme conditions (Couradeau et al., 2016; Reed et al., 2019). Given the importance of biological soil crusts in terrestrial ecosystems, research addressing their composition and biodiversity is largely needed (Fernandes et al., 2017; Rutherdord et al., 2017). Here, we highlighted the importance of soil and climatic factors in the composition and diversity of cyanobacterial biocrust communities. This information can provide a basis to enhance biocrusts and conservation efforts (Ayuso et al., 2017; Bethany et al., 2019; Giraldo-Silva et al., 2019) and promote the use of biocrust cyanobacteria as global change indicators (Muñoz-Martin et al., 2018).

5.6 Conclusion

In conclusion, this work described the cyanobacterial composition of biological soil crusts from *Caatinga* and *Pampa*, two previously understudied biodiverse biomes with contrasting climates and important ecological value. The results revealed that the biocrust's cyanobacteria communities from both biomes had distinctive taxonomic compositions and assemblages. Soil temperature, solar irradiance, and phosphorous content were identified as the main environmental drivers of such biotic structures. *Caatinga* sites, characterized by more arid environments, presented a higher abundance of N-fixing bacteria. In contrast, *Pampa* showed a larger abundance of biocrust-forming cyanobacteria. This research advances our knowledge about cyanobacterial assemblages in biocrusts and indicates environmental and soil factor driving these formations. These research outcomes can provide an important baseline for future ecological conservation and restoration in dryland regions in Brazil and elsewhere.

Funding: This work was supported by the São Paulo Research Foundation (FAPESP, [proc. 2017/11126-6]; and by Coordination for the Improvement of Higher Education Personnel.

MMR acknowledges support from the Australia Research Council Discovery Early Career Research Award (DE180100570).

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Supplementary Material Supplementary Table

Table S1. Measurements of environmental variables for each sample. TotalN = Total Nitrogen (mg/kg); TotalP = Total Phosphate (mg/kg); OM = Organic Matter (g/kg); OC = Organic Carbon (g/kg); ST = Soil Temperature (°C); CI = Solar Irradiance at the crust spot (W/m²); AI = Solar Irradiance at nearest spot of soil without vegetation cover (W/m²).

SITE	TotalN	TotalP	Orthophosphate	OM	OC	TS	CI	AI
CA1	516.7486	339.7	129.6	9.9	5.74	36	540	1015
CA4	760.7688	260.8	48.0	14.5	8.41	36	700	1399
CA5	502.3945	216.8	56.2	13.9	8.06	38	950	1450
CA6	703.3523	539.0	124.7	13.6	7.89	43	1130	1650
CA7	1234.455	425.4	29.0	14.1	8.18	37	930	1580
CA8	789.4771	397.6	94.8	15.9	9.22	38	475	965
CA9	617.2275	420.8	77.2	19.2	11.14	38	710	1300
CB2	551	386.0	55.1	12.4	7.19	42	2515	2900
CB3	393.1144	233.0	37.6	8.7	5.05	40	825	1400
CB4	340.9688	163.5	33.1	7.4	4.29	41	790	1160
CB7	360	409.5	38.4	8.0	4.64	44	2100	2100
CB8	348.9912	281.7	40.3	7.6	4.41	43	990	1400
CB9	697.9656	374.4	61.2	16.3	9.45	45	2400	2900
CC1	489.3832	193.6	6.6	11.1	6.44	37	2190	2190
CC2	477	126.4	12.2	11.1	6.44	39	1970	1970
CC3	677.9096	161.2	8.0	15.8	9.16	40	1450	1450
CC4	518	133.3	13.6	11.7	6.79	40	2700	2700
CC5	497	107.8	2.9	10.8	6.26	43	2230	2230
CC6	427	256.2	14.0	9.8	5.68	41	1880	1880
CC7	565.596	214.5	12.6	13.0	7.54	39	1930	2140
CC8	580	200.6	8.7	13.0	7.54	36	2300	3500
CC9	549.5512	89.3	10.5	12.6	7.31	39	3270	3650
CC10	1150	205.2	30.6	27.5	15.95	43	915	1450
PA1	279.9055	117.1	7.3	8.8	5.1	31	750	2000
PA2	394.7385	119.4	5.3	6.7	3.89	33	550	2140
PA3	236.8431	126.4	7.7	7.6	4.41	33	250	1910
PA5	423.4468	138.0	10.2	9.0	5.22	31	178	1750
PA6	294.2596	142.6	4.9	6.5	3.77	36	3	1700
PA7	279.9055	128.7	4.5	8.3	4.81	33	132	349
PA9	208.1349	124.0	2.2	5.1	2.96	32	107	414
PA10	193.7807	123.0	2.7	5.3	3.07	30	420	1042
PB1	538.2798	117.1	6.5	11.8	6.84	19	284	640
PB2	322.9679	84.7	5.9	11.1	6.44	18	508	1235
PB3	509.5716	103.2	3.1	10.2	5.92	20	450	1035
PB4	222.4890	80.0	4.3	8.1	4.70	20	432	990
PB5	351.6761	128.7	3.3	10.9	6.32	20	760	1600

Table of the own author.

Table S1. Cont.

SITE	TotalN	TotalP	Orthophosphate	OM	OC	TS	CI	AI
PB6	322.9679	128.2	4.5	9.9	5.74	22	780	1245
PB7	495.2174	110.2	3.9	10.6	6.15	21	431	1004
PB8	480.8633	142.6	4.1	7.2	4.18	21	981	1980
PB9	581.3422	135.7	2.9	9.7	5.63	22	750	1180
PB10	566.9881	154.2	4.3	7.6	4.41	22	1862	2665
PC1	480.8633	117.1	3.1	12.2	7.08	28	1809	2460
PC2	566.9881	138.0	3.9	11.8	6.84	31	1885	2430
PC3	566.9881	135.7	3.3	9.5	5.51	31	1723	2417
PC4	437.8009	184.3	2.9	11.3	6.55	31	1600	2359
PC6	509.5716	154.2	2.6	9.0	5.22	32	1700	2426
PC7	638.7587	138.0	3.5	14.5	8.41	33	1500	2424
PC8	653.1128	151.9	3.3	14.1	8.18	34	1630	2460
PC9	681.8211	149.6	2.9	12.5	7.25	36	1892	2408
PC10	638.7587	161.2	3.9	16.2	9.40	31	1515	2410

Table of the own author.

Table S2. Twenty more abundant OTUs taxonomically assigned.

OTUs	**Number of reads**	**Best Match (GenBank accession number)**
OTU000001	114598	*Microcoleus* sp. clone OTU_53 (MF527189.1)
OTU000002	83639	*Scytonema hyalinum* BRAZIL-74LC (KY365461.1)
OTU000003	77021	*Porphyrosiphon notarisii* 9FUR (MF109120.1)
OTU000004	56390	*Chroococcidiopsis* sp. BB96.1 (AJ344555.1)
OTU000005	50687	Uncultured *Microcoleus* sp. clone JJD57
OTU000006	49245	*Leptolyngbya* sp. CENA387 (KR137608.1)
OTU000007	44733	*Aetokthonos hydrillicola* (KF934178.1)
OTU000009	35082	Uncultured *Microcoleus* sp. clone JJD57
OTU000010	33057	*Potamosiphon austaliensis* (MH047862.1)
OTU000011	32052	*Nostoc commune* HK-02 (AB251859.1)
OTU000012	28252	Uncultured *Microcoleus* sp. (MF527188.1)
OTU000013	24546	*Stigonema elegans* WYS5-2 (KT867211.1)
OTU000014	24317	Uncultured *Microcoleus* sp. (MF527188.1)
OTU000015	17336	*Pycnacronema savannensis* 49PC (MF581663.1)
OTU000016	16346	*Potamosiphon austaliensis* (MH047862.1)
OTU000017	15269	*Stigonema panniforme* LS21-42 (KT867101.1)
OTU000020	13540	*Chroococcidiopsis* sp. BB79.2 (AJ344552.1)
OTU000021	13207	*Scytonema* sp. Leucking_16561 (EU818966.1)
OTU000026	8953	*Scytonema* sp. CMT-1BRIN-NPC32 (KF934155.1)
OTU000028	8602	*Chroococcidiopsis* sp. NTRI15 (KF752441.1)

Table fo the own author.

Supplementary Figure

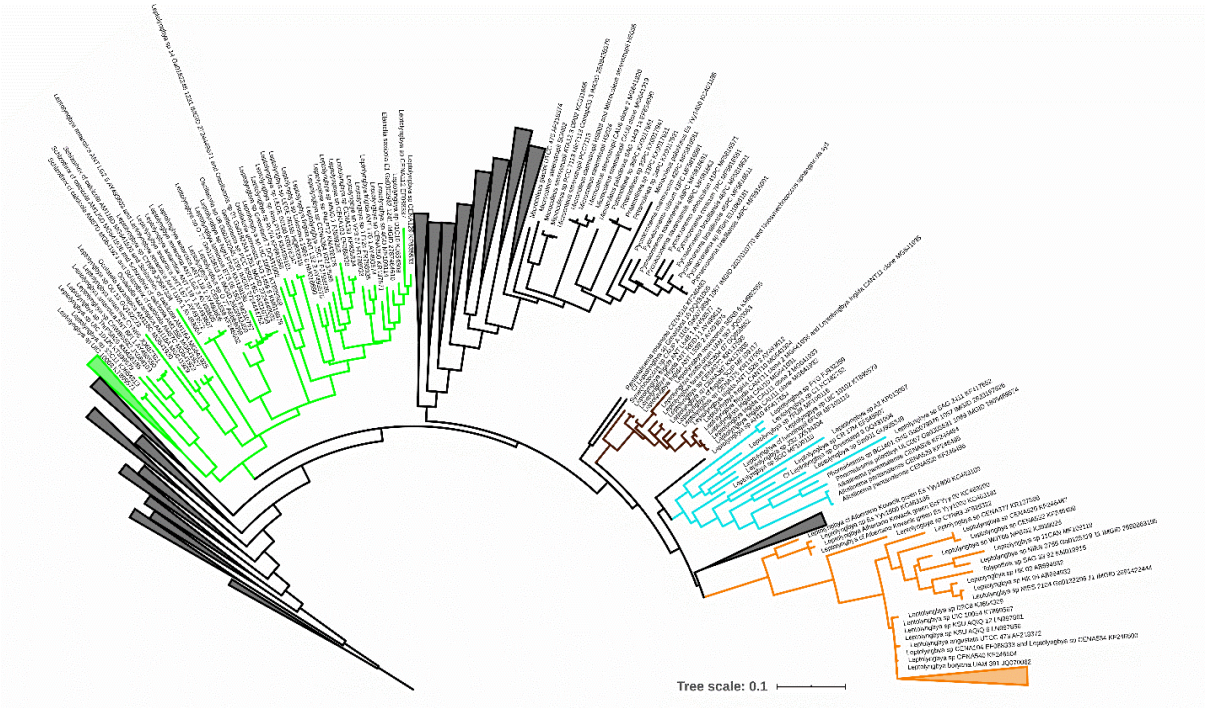

Figure S1. Phylogenetic Tree based on the Cydrasil database and the clades of *Leptolyngbya* and *Microcoleus* where our OTUs were placed. Figure of the own author.

Considerações Finais

Esta investigação contou com metodologias dependentes e independentes de cultivo e enfatizou como ambas são complementares, trazendo diferentes resultados e, assim, explorando melhor a diversidade do ambiente.

Comprovando a primeira hipótese deste trabalho, os resultados obtidos a partir das técnicas dependentes de cultivo demonstraram o quanto a flora de cianobactérias dos solos da Caatinga é diversificada e ainda desconhecida, principalmente pelo grande número de gêneros e espécies novas apresentados ao longo do trabalho. Estes resultados juntamente com dados de Cerrado, levaram à produção de dois trabalhos a serem publicados, que validam a terceira hipótese desta tese. O primeiro capítulo trouxe a proposição de três novos gêneros e seis novas espécies de cianobactérias filamentosas morfologicamente similares a *Microcoleus* e *Phormidium* e o segundo a descrição de três novos gêneros e seis novas espécies de cianobactérias similares a *Nostoc*.

Confirmando a segunda hipótese deste trabalho, os dados obtidos por Sequenciamento de Próxima Geração trouxeram uma investigação mais profunda a respeito da composição de cianobactérias da Caatinga, permitindo a comparação com o bioma Pampa. Esta comparação mostrou um posicionamento distante da Caatinga em relação ao Pampa, e descreveu três composições diferentes, uma para cada região analisada dentro do bioma. Como um segundo resultado desta análise, foram identificadas as variáveis ambientais que mais influenciam na distribuição de cianobactérias de ambos os biomas.

A ciência básica traduzida no levantamento sistemático das cianobactérias dos solos da Caatinga, juntamente com a descrição de novas espécies e gêneros e o conhecimento de como a comunidade de cianobactérias está sendo dirigida, permitirá o desenvolvimento de projetos voltados à aplicação de cianobactérias em solo, com fins de conservação e restauração ambiental.

Apêndice A - Conservation of native plants with cyanobacteria from biological soil crusts

Justificativa

Este estudo é parte de um projeto em parceria com o laboratório de Munõz-Rojas na Universidade de New South Wales, Austrália. O projeto foi desenvolvido durante o período de um ano e financiado pela CAPES através do Programa de Doutorado-sanduíche no Exterior 2019.

O objetivo maior do desenvolvimento deste projeto foi a aprendizagem de técnicas novas e abordagens ainda não desenvolvidas no Brasil, e que portanto pudessem trazer aplicações práticas ao país. O bioma Caatinga, foco desta tese, é um dos ambientes mais afetados negativamente no Brasil (Vieira et a., 2015), sendo que o Ministério do Meio Ambiente classifica 15% do território nacional como áreas susceptíveis à desertificação (MMA, 2018).

Assim, este projeto visou trazer ao Brasil o conhecimento de técnicas utilizando microrganismos de crostas biológicas, que venham a permitir que a ciência básica feita no país até agora venha a ser efetivamente aplicada a programas de restauração e conservação ambientais.

Introduction

Many native plant communities around the world are seriously threaten by land degradation, e.g. land clearing, extreme fire and overgrazing (Broadhurst and Coates, 2017). In Australia, more than 1,300 species are listed in the Act List of Threatened Flora, with 37 being considered extinct. Among the current conservation tools, "translocations" is one of the most commonly used. It consists of transferring plants or regenerative plant material from an ex situ collection or natural population to a new location, usually in the wild. Although, this technique had obtained success in some cases, the success rates are considered low (Plein et al., 2016).Thus, finding solutions for increasing the capacity of native plants to survive and grow is key to improving the success of conservation and restoration programs.

Cyanobacteria are the primary componenets of biological soil crusts and are able to survive in extreme environmental conditions such as high temperatures (Pointing & Belnap, 2012). Two cyanobacteria genera were previously used as bio-fertilizers showing positive abilities for promoting germination and enhancing seedling growth of native Australian plants (Muñoz-Rojas et al., 2018). These genera are *Microcoleus* Desmazières ex Gomont, a filamentous cyanobacteria capable of secrete extracellular polysaccharides (EPS) for primary physiological activity and *Nostoc* Vaucher ex Bornet & Flahault, a cyanobacteria that contains heterocytes, which are oxygen-excluding cells for fixing nitrogen (Garcia-Pichel, 2013).

Despite the known roles of certain cyanobacteria species, the specific species producing bio-active biological compounds or metabolites involved in the germination and growth of plants, have not been studied yet. Therefore, this study aimed to investigate the capacity of Australian indigenous cyanobacteria to promote germination and growth of native plants and to improve cyanobacteria inoculants for application in large-scale conservation and restoration programs.

Objectives

Assess:

(i) The most adequate concentrations of cyanobacteria inoculants,

(ii) The most suitable indigenous cyanobacteria species to compose these inoculants, and

(iii) Develop suitable cyanobacteria inoculants composed of single strains or consortia for large-scale restoration programs.

Materials and Methods

Cyanobacteria strains and culturing

Cyanobacteria from Biological Soil Crusts in the Pilbara, region in Western Australia, were sampled. The inoculation of portions of crusts generated three different populations, representing three different genera (*Leptolyngbya* Anagnostidis & Komárek, *Nostoc* and *Microcoleus*).

The populations were cultivated in with BG11 medium (Cyanobacteria BG11 Freshwater Solution, Merck) and maintained under 24ºC ± 1ºC, 80 µmol of photons m-2 s-1 of irradiance and a 16:8 h light-dark cycle.

Seeds preparation and cyanobacteria bio-priming of seeds

Seeds of *Triodia wiseana* C.A.Gardner*, T. epactia* S.W.L.Jacobs, *Senna notabilis* (F.Muell.) Randell and *Grevillea wickhamii* Meisn. collected in the Pilbara region and stored in a controlled environment room at 15° and 15% relative humidity, were used in this study. These native species, representative of Australian arid environments, have been identified as crucial in restoration programs (Bateman et al., 2018).

Seeds were checked for viability by microscopy and surface sterilized in 1% (w/v) calcium hypochlorite solution for 30 min, being washed with autoclaved milli-Q water.

The cyanobacterial biomass was filtered and resuspended in milli-Q water, generating inoculants in concentrations of 1g/L and 5g/L. Biomass concentration for each genus was determined based on its dry weight, recognized after filtering and drying at 60° for two hours. Therefore, *Nostoc*, *Microcoleus* and *Leptolyngbya* from Pilbara generated three different inoculants, with one more addition containing a mix of all of them (Figure 1).

Seeds were then grouped in separated plastic tubes for each species and treatment. The inoculants were added to the plastic tubes considering both concentrations (1g/L and 5g/L), except for the control treatment, where it was added milli-Q water.

Plastic tubes were then agitated on an orbital shaker (Ratek EOM5) for 20 h at a low speed at 25°C. After agitation, seeds were transferred to Petri dishes for the germination step.

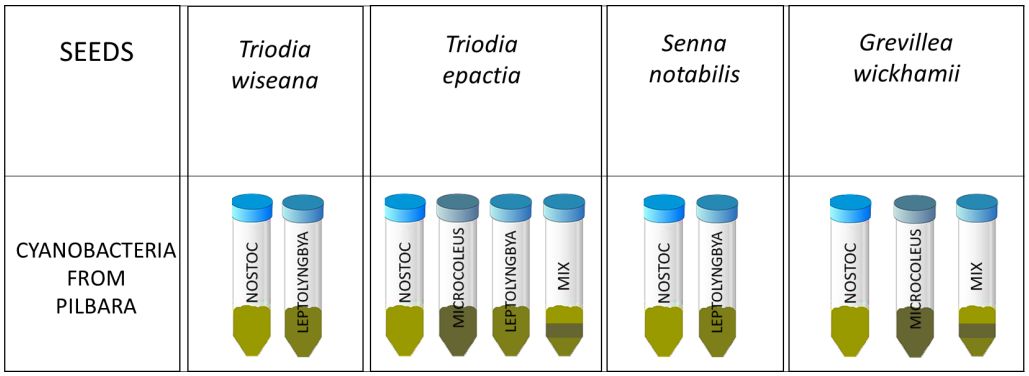

Figure 1. Experimental design for the cyanobacteria bio-priming of native plant's seeds. Figure of the own author.

Seed germination and early seedling growth

Bio-primed seeds and control seeds of all species were plated in 90 mm Petri with agar 5g/L (n= 4 P dishes per treatment; 25 seeds per dish) and incubated under 16/8 h alternating temperature (20/29°C) and light:dark cycles in the laboratory.

Germination was scored as emergence of radicle and recorded daily for around 14 days. After that, randomly chosen seedling radicals were analyzed and their roots and shoots were measured.

Results and Discussion

Overall, our results showed that lower concentrations of cyanobacteria used as seed bio- inoculants (1 g l^{-1}) had a similar or even stronger effect in seedling growth compared to highly concentrated inoculum (5 g l^{-1}).The effects of the cyanobacterial inoculants were specific to each plant species.

For *Senna notabilis* seeds, *Leptolyngbya* inoculant showed an inhibitor effect while *Nostoc* inoculant did not affect seedlings' growth (Figure 2). For *Triodia wiseana* both inoculants, *Leptolyngbya* and *Nostoc* showed negative effects (Figure 3). However, seedlings of *Grevillea wickhamii* showed much longer roots when bioprimed with *Nostoc*. The same species did not show any improvement when treated with *Microcoleus*, which means that the positive results of the consortia or mix were related only to *Nostoc* (Figure 4).

Seeds of *Triodia epactia* bioprimed with the cyanobacteria mix resulted in three times larger roots compared to the control treatment (Figure 5).

The positive effects of the indigenous soil cyanobacteria inoculants on native plants could be related to their ability of promoting nutrient bioavailability, improving stress resistance, protection against other microbes, and production of substances that may act as hormones. Further analyses such as metabolomics may be able to reveal these bioactive components. The findings of this research provide an important baseline for selecting the most effective bio-active inoculants for application in seed-based land rehabilitation programs.

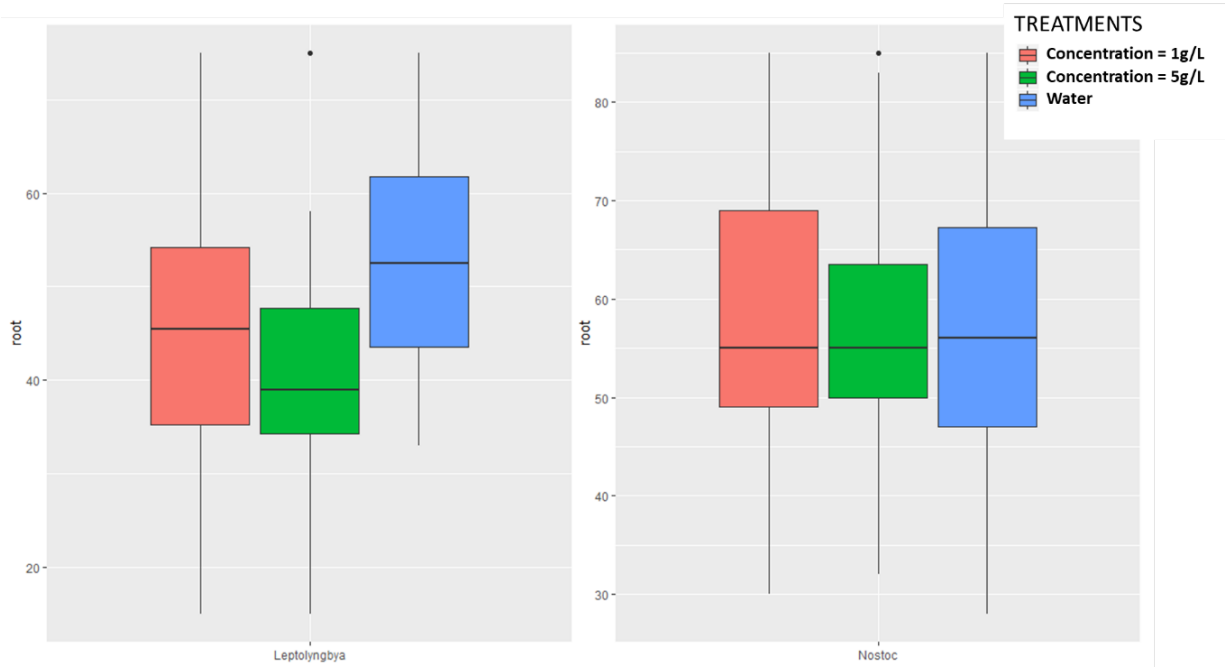

Figure 2. Root's measurements of *Senna notabilis* seedlings around 10 days since biopriming. Representing the inoculants *Leptolyngbya* and *Nostoc*. Figure of the own author.

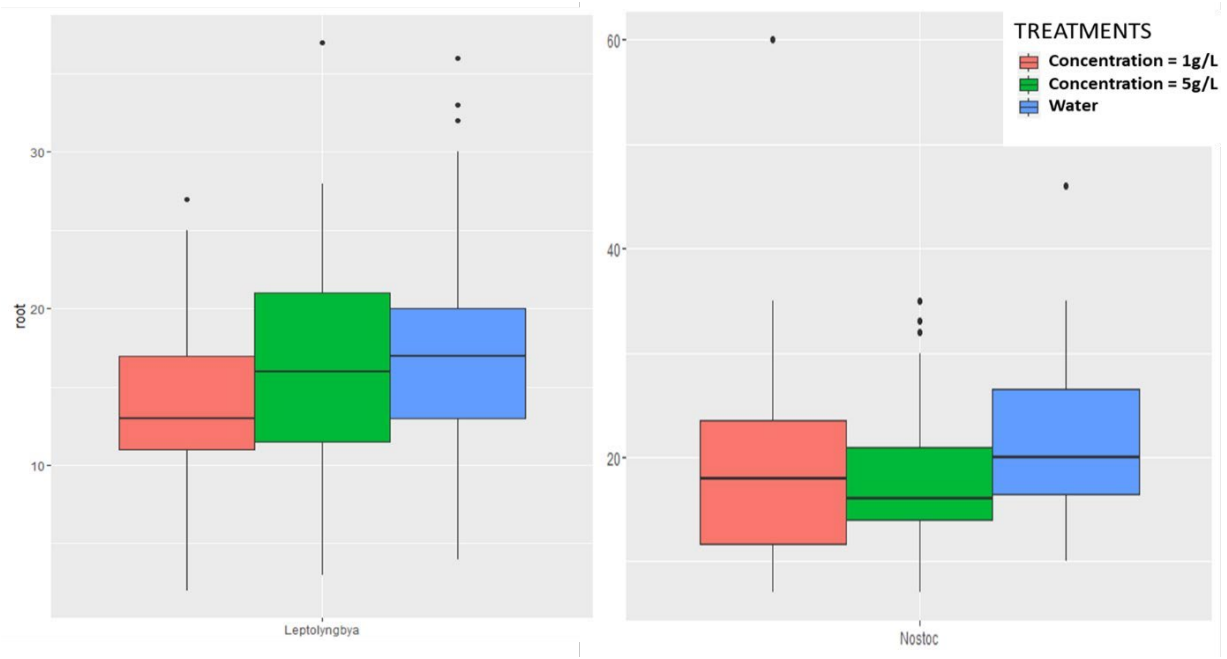

Figure 3. Root's measurements of *Triodia wiseana* seedlings around 10 days since biopriming. Representing the inoculants *Leptolyngbya* and *Nostoc*. Figure of the own author.

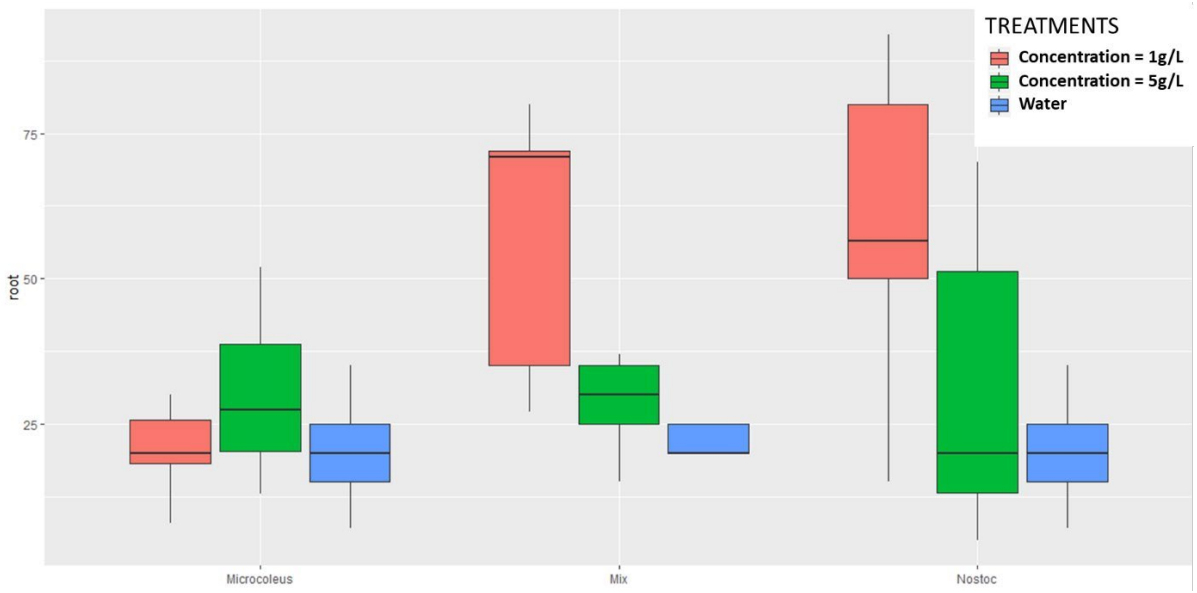

Figure 4. Root's measurements of *Grevilea wickhamii* seedlings around 10 days since biopriming. Representing the inoculants *Microcoleus*, *Nostoc* and Mix (Microcoleus+Nostoc). Figure of the own author.

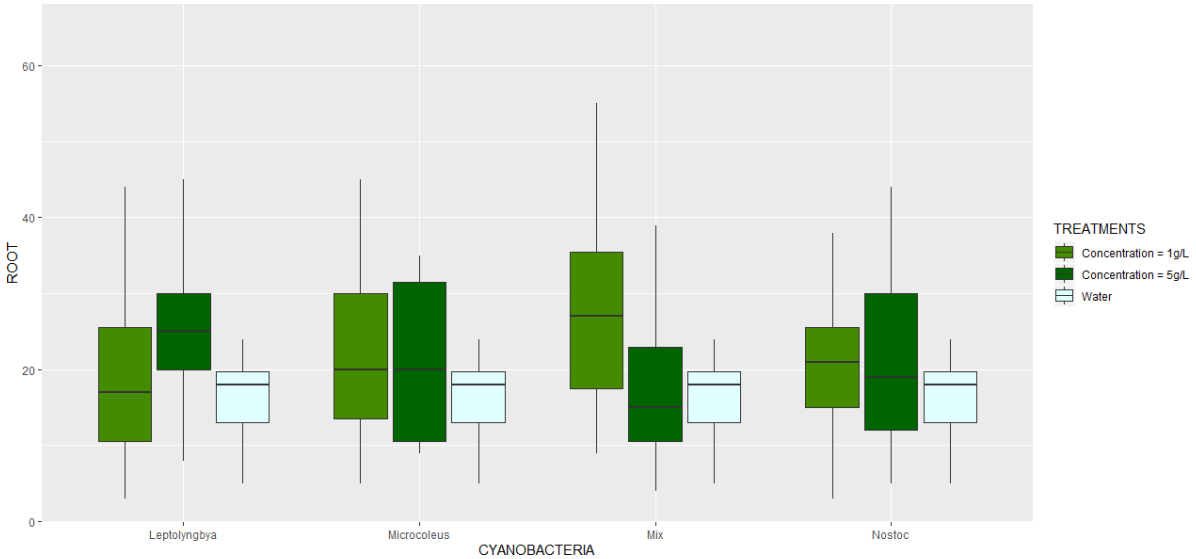

Figure 5. Root's measurements of *Triodia epactica* seedlings around 10 days since biopriming. Representing the inoculants *Leptolyngbya*, *Microcoleus, Nostoc* and Mix (*Leptolyngbya*+ Microcoleus+Nostoc). Figure of the own author.

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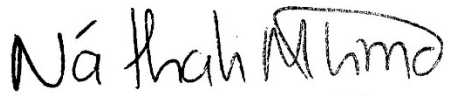
TERMO DE REPRODUÇÃO XEROGRÁFICA

Autorizo a reprodução xerográfica do presente Trabalho de Conclusão, na íntegra ou em partes, para fins de pesquisa.

São José do Rio Preto, 29/10/2020



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Assinatura do autor