GUILHERME SCOTTA HENTSCHKE

Cyanobacteria heterocitadas de ambientes terrestres da Mata Atlântica no estado de São Paulo, Brasil

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

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“Um passo de cada vez. Não consigo imaginar nenhuma outra maneira de realizar algo.”

Michael Jordan

Apresentação

Esta tese de doutorado está organizada em Introdução, Material e Métodos, Resultados e Discussão e Considerações Finais. Na introdução estão incluídos dados de levantamento bibliográfico, informações sobre os locais de estudo e características gerais das cianobactérias heterocitadas. Os resultados estão divididos em três seções: artigos publicados, manuscritos elaborados e resultados ainda não incluídos em artigos científicos. No final da tese estão incluídos três artigos publicados e seis manuscritos aceitos ou que serão submetidos a revistas científicas que são produtos do presente trabalho e estão referidos no texto na seção de resultados.

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À minha família e aos que convivem junto a mim.

RESUMO

As cianobactérias terrestres heterocitadas são muito abundantes e diversas na Mata Atlântica, ocorrendo principalmente sobre solo, rochas e troncos e folhas de árvores. Têm importante função ecológica, atuando na retenção do solo e adicionando matéria orgânica e nutrientes ao mesmo. Através da atividade da enzima nitrogenase, possuem a capacidade de fixar nitrogênio molecular atmosférico em células diferenciadas chamadas heterocitos. São potencialmente produtoras de cianotoxinas e compostos secundários com variadas atividades biológicas. Considerando os poucos estudos tratando deste grupo de organismos, os objetivos gerais desta tese foram conhecer a biodiversidade e o potencial biotecnológico de cianobactérias terrestres heterocitadas. As coletas foram realizadas em áreas de Mata Atlântica no Estado de São Paulo, especificamente no Parque Estadual da Serra do Mar – Núcleo Santa Virgínia, Estação Ecológica Juréia-Itatins e Parque Estadual da Ilha do Cardoso, raspando-se os substratos com auxilio de espátula. No estudo taxonômico foram utilizados dados morfológicos e filogenéticos para caracterizar as populações encontradas. Para os estudos filogenéticos foram analisadas as regiões do gene 16S rRNA e 16-23S rRNA ITS através dos métodos de máxima verossimilhança, máxima parsimônia e análise Bayesiana, além das estruturas secundárias das hélices d1-d1', Box B, V2 e V3 da região 16-23S rRNA ITS. Os estudos de prospecção de metabolitos secundários foram realizados através de testes de revelação em Cromatografia de Camada Delgada e bioensaios em camundongos. Foram testadas as presenças de microcistinas, saxitoxinas, anatoxina-a, b-metilaminoalanina, além das atividades anticolinesterásica, antifúngica e antioxidante dos extratos metanólicos e de ácido acético. No total das 263 amostras coletadas, 128 continham cianobactérias heterocitadas e 133 populações foram identificadas ao menos em nível genérico. Trinta e cinco linhagens foram isoladas e depositadas na Coleção de Cultura do Instituto de Botânica (CCIBt), sendo que quatorze destas tiveram o gene 16S rRNA sequenciado; adicionalmente sete tiveram o 16-23S ITS sequenciado. Além disso, populações de *Streptostemon lutescens*, *Stigonema jureiensis* e *S. fremyi* também tiveram o gene 16S sequenciado a partir de material proveniente da natureza. Nesta tese ainda são propostos os gêneros *Dapisostemon* e *Komarekiella*, além de sete novas espécies. Todos os extratos testados presentaram pelo menos um tipo de atividade biológica.

ABSTRACT

Heterocytous terrestrial cyanobacteria are very abundant and diverse in the Atlantic rainforest environments, occurring mainly on soil, rocks and tree leaves and barks. They have an important ecological role since they retain the soil and increase its level of organic matter and nutrients. Due to the nitrogenase enzyme, they are able to perform inorganic nitrogen fixation in their heterocytes. Due to the nitrogenase enzyme, they are able to fixate atmospheric molecular nitrogen in their heterocytes. They are potential producers of cyanotoxins and secondary compounds presenting different biological activities. Considering the few studies about this group of organisms, the aim of the present thesis is to know the biodiversity and biotechnological potential of heterocytous cyanobacteria from the Atlantic rainforest terrestrial environments. Samples were collected in areas of the Atlantic rainforest, State of São Paulo, specifically in Parque Estadual da Serra do Mar – Núcleo Santa Virgínia, Estação Ecológica Juréia-Itatins and Parque Estadual da Ilha do Cardoso, by scraping substrates with a spatula. The taxonomic study to characterize the collected populations was based on morphologic and phylogenetic data. For the phylogenetic studies the 16SrRNA and 16-23S rRNA ITS regions were analyzed by Maximum Likelihood, Maximum Parsimony and Bayesian analysis, and also the secondary structures of d1-d1', Box B, V2 and V3 helices of the 16-23S rRNA ITS were analyzed. The prospection studies on secondary metabolites were carried out using Thin-Layer Chromatography and biological tests performed in mice. The presence of microcystins, saxitoxins, anatoxin-a and BMAA in the methanolic and acetic extracts were tested besides the anticholinesterasic, antifungal and antioxidant biological activities. Tests were performed using the methanolic and acetic extracts to determine the presence of microcystins, saxitoxins, anatoxin-a and BMAA as well as the anticholinesterasic, antifungal and antioxidant biological activities. Out of the 263 collected samples, 128 presented heterocytous cyanobacteria and 133 populations were identified at least in generic level. Thirty-five strains were isolated and held in CCIBt and fourteen had the 16S rRNA gene sequenced. Additionally, seven strains had the 16-23S ITS sequenced. Moreover, the 16S gene of *Streptostemon lutescens*, *Stigonema jureiensis*, and *S. fremyi* populations was sequenced directly from nature material. In this thesis the genera *Dapisostemon* and *Komarekiella* are also proposed in addition to seven new species. All of the extracts presented at least one type of biological activity.

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1. INTRODUÇÃO

1.1 Mata Atlântica

A Mata Atlântica é considerada Patrimônio Nacional pela Constituição Federal do Brasil e abrange 18 estados ao longo da cadeia montanhosa desde o litoral do Rio Grande do Sul até o Rio Grande do Norte. Sua área principal localiza-se nas Serras do Mar e da Mantiqueira, nos estados de São Paulo, Minas Gerais, Rio de Janeiro e Espírito Santo (Câmara 1991, Rizzini 1997).

As características do relevo e dos solos têm importância na distribuição da vegetação, modificando localmente as relações de temperatura e umidade. Quanto ao clima, para o estado de São Paulo, a temperatura média é de 16 ºC e as chuvas atingem a média de 2.240 mm anuais no Núcleo Cunha do Parque Estadual da Serra do Mar, distribuídas em um período úmido (outubro a março) e outro pouco úmido (abril a setembro). Nas regiões montanhosas, as precipitações podem atingir média de 4.600 mm por ano e a umidade relativa do ar é superior a 80% (Rizzini 1997, Arcova *et al.* 2003, Ferreira 2008).

Originalmente, a Mata Atlântica ocupava uma área de 1.100.000 Km^2 , o que é equivalente a 12% do território nacional, porém o impacto da ocupação humana resultou em severas alterações de seus ecossistemas devido à fragmentação dos habitats, permitindo a manutenção de apenas 7% desta área (Ministério do Meio Ambiente 2007). No estado de São Paulo, a Mata Atlântica ocupa atualmente cerca de 7% da área do Estado, sendo que a maior parte está disposta ao longo do litoral e preservada como parques estaduais (Câmara 1991). O conjunto de fitofisionomias (floresta pluvial montana, floresta pluvial baixo-montana, floresta de araucária e floresta pluvial ripária em manchas) que formam a Mata Atlântica propiciaram significativa diversificação ambiental criando condições adequadas para a evolução de comunidades com alta riqueza de espécies. Por esse motivo, é considerada atualmente como um dos biomas com os mais altos valores de biodiversidade do planeta (Ministério do Meio Ambiente 2007). No entanto, a biodiversidade da Mata Atlântica permanece ainda extremamente mal conhecida no que se refere aos microorganismos, principalmente de ambientes

terrestres. De acordo com Komárek (2006), menos de 10% da diversidade de microorganismos de ambientes tropicais é conhecida.

1.2. Cianobactérias heterocitadas

As cianobactérias heterocitadas, abordadas neste estudo, são microorganismos muito abundantes nos ambientes terrestres da Mata Atlântica. Possuem talos filamentosos uni ou multiseriados, ramificados ou não, iso ou heteropolares e a reprodução é realizada principalmente por fragmentação dos tricomas (hormogônios e hormocistos). Ocorrem em ambientes úmidos ou áridos, quentes ou frios e nos mais variados tipos de substrato tais como solo, rochas, animais, troncos de árvores, briófitas e pteridófitas (Hoffmann 1989, Sant'Anna *et al.* 2007), além de substratos artificiais como cimento, postes de madeira e telhas. Têm importante função ecológica, atuando na retenção do solo e adicionando matéria orgânica e nutrientes ao mesmo (Branco *et al.* 2009).

Através da atividade da enzima nitrogenase, possuem a capacidade de fixar nitrogênio molecular atmosférico em células diferenciadas chamadas heterocitos. A nitrogenase é sensível ao oxigênio e somente reduz o nitrogênio em meio anaeróbico. Para tanto, os heterocitos apresentam parede espessada, o que impede a entrada de oxigênio e não apresentam o fotossistema II. A ausência deste fotossistema evita a liberação de oxigênio durante a fotossíntese e em consequência mantém o meio redutor dentro da célula. O ATP necessário para os processos metabólicos é produzido pelo fluxo cíclico de elétrons do fotossistema I e pela respiração. Os heterocitos apresentam nos polos zonas altamente diferenciadas denominadas “nódulos polares” que permitem trocas metabólicas com as células vizinhas como entrada de glicídios e saída de glutamina, mas não permitem entrada de oxigênio (Graham & Wilcox 2000, Reviers 2006).

A fixação de nitrogênio atmosférico por cianobactérias atribui vantagens adaptativas ao grupo e é essencial para os processos biológicos dos ecossistemas. Através desse processo, o nitrogênio molecular é reduzido a amônio e consequentemente disponibilizado para o consumo por plantas e animais (Graham &

Wilcox 2000). Em ambientes terrestres naturais, a fixação biológica de nitrogênio por microorganismos em geral compreende 97% da entrada total de nitrogênio (Vitousek *et al.* 2002 *apud* Andreote 2013). Na Mata Atlântica esse processo é ainda mais essencial devido a escassez deste elemento, apresentando apenas a metade do já pouco nitrogênio encontrado em solos da Amazônia por exemplo (1,2 $g.kg^{-1}$) (Martinelli 2008 *apud* Andreote 2013).

1.3. Cianotoxinas e substâncias bioativas

As cianotoxinas, metabólitos secundários das cianobactérias, constituem um grupo heterogêneo tanto em relação às suas propriedades toxicológicas quanto em relação à natureza química, pois pertencem a três classes químicas diferentes (peptídeos, alcalóides e lipossacarídeos) (Sivonen & Jones 1999, Carvalho 2006). Essas substâncias são responsáveis por intoxicações com efeitos agudos e crônicos em animais e em seres humanos (Apeldoorn *et al.* 2007); de acordo com seu modo de ação são denominadas hepatotoxinas, neurotoxinas ou dermatotoxinas (Carvalho *et al.* 2006, Apeldoorn *et al.* 2007) e podem ser produzidas igualmente por cianobactérias de ambientes aquáticos e terrestres (Cox *et al.* 2005).

Os fatores que induzem a produção dessas toxinas não são ainda bem conhecidos, assim como seu papel ecológico e adaptativo para as cianobactérias. No caso das microcistinas, peptídeos com atividade hepatotóxica para mamíferos (Carvalho *et al.* 2006), várias hipóteses tem sido especuladas para o seu papel no meio ambiente. Atualmente a hipótese de que as microcistinas tenham função antipredador é questionada, pois de acordo com Rohrlack *et al.* (1999), as mesmas taxas de predação por *Daphnia* sp. são apresentadas entre uma linhagem ambiental tóxica de *Microcystis* e sua mutante espontânea não produtora de toxinas. É conhecido, porém que a linhagem tóxica apresenta melhor crescimento em condições de baixo CO_2 e é melhor competidora em experimentos de crescimento conjunto (Rantala *et al.* 2004). Outra questão é que de acordo com estudos de relógio molecular, as microcistinas são mais antigas do que as linhagens de eucariotos, refutando a ideia de que tenham evoluído com função antipredador (Rantala *et al.* 2004). A hipótese de que as microcistinas

atuam como sinalizadores químicos de cianobactérias também é aceita, pois a liberação desses compostos no meio ambiente estimula a produção da toxina por outros indivíduos aumentando a competitividade da população frente às outras espécies. Ainda especula-se que as microcistinas tenham papel no metabolismo primário devido a funções intracelulares como a ligação nos ficobilissomos, embora não se conheça os efeitos desse processo (Martins *et al.* 2008, Kaplan *et al.* 2012).

A princípio, todas as cianobactérias são consideradas potencialmente tóxicas (Carvalho *et al.* 2006). Para as heterocitadas terrestres há registros da produção de microcistinas e de β-metil-amino-alanina (BMAA) por cepas de *Nostoc* Born. & Flah e de outros compostos tóxicos por cepas de *Scytonema* Born. & Flah., *Stigonema* Born. & Flah., *Tolypothrix* Born. & Flah e *Fischerella* Born. & Flah. (Ressom *et al.* 1994, Dixit & Suseela 2013). Não há registro da presença de saxitoxinas em cianobactérias terrestres.

Além das toxinas, as cianobactérias são capazes de produzir também outros metabólitos secundários com potencial ação farmacológica, tais como, substâncias antibacterianas, antitumorais, antivirais, antifúngicas, antioxidantes e anticolinesterásicas (Becher *et al.* 2009, Chilpala *et al.* 2009, Dixit & Suseela 2013).

A partir do início dos estudos nos anos 70 (Schlegel *et al.* 2004), pesquisas para o descobrimento de compostos com uso comercial, farmacêutico ou toxicológico em cianobactérias resultaram no descobrimento de um grande número de peptídeos cíclicos, depsipeptideos e alcalóides com propriedades bioativas. As criptoficinas, por exemplo, são uma família de agentes antitumorais obtidas a partir do gênero *Nostoc* (Schwartsmann *et al.* 2001).

De modo geral, a procura de novos compostos bioativos produzidos por cianobactérias, até o momento, privilegiou as espécies de água doce, especialmente *Microcystis aeruginosa* (Kütz.) Kütz., da qual foram extraídos diversos inibidores de proteases e alguns antivirais (Skulberg 2000, Martins *et al.* 2008). Os estudos com espécies marinhas estão restritos às formas filamentosas homocitadas de regiões tropicais (Martins *et al.* 2008). *Lyngbya majuscula* Harv. ex Gom. e *Symploca* spp. foram as mais estudadas e delas foram extraídas diversas substâncias com potencial antitumoral (Harrigan & Goetzl 2002, Martins *et al.* 2008). Considerando-se os grupos

de cianobactérias, os metabólitos secundários já descobertos são provenientes principalmente de espécies das ordens Oscillatoriales (49%) e Nostocales (26%) (Gerwick *et al.* 2008). Para o Brasil, o único trabalho publicado sobre substâncias com atividades biológicas (propriedades farmacêuticas) de cianobactérias é o de Carvalho *et al.* (2013), no qual foi encontrada atividade anticolinesterásica nos extratos brutos de cianobactérias terrestres dos gêneros *Tolypothrix*, *Calothrix* Born. & Flah. e *Phormidium* Gomont.

1.4. Biodiversidade e sistemática de cianobactérias

A grande diferenciação dos habitats e a diversidade ecológica tropical levaram à maior taxa de especiação nessa área (Rejmankova *et al.* 2004). Devido à grande plasticidade morfológica das cianobactérias e sua sensibilidade a diferentes ambientes, muitas espécies ainda devem ser descritas para as regiões tropicais (Komárek & Anagnostidis 1989). Baseando-se nessas afirmativas, juntamente com o conhecimento de que a ocorrência de diferentes genótipos está determinada também por condições ecológicas (Rejmánkova *et al.* 2004), há uma tendência atual de rejeição ao paradigma da ubiquidade das cianobactérias. Segundo a revisão de Martiny & al. (2006), a delimitação das espécies de microorganismos ainda é relativamente imprecisa em comparação com a de animais e plantas. Este fato poderia levar à falsa idéia de espécies cosmopolitas de cianobactérias.

A abordagem taxonômica polifásica, na qual características ecológicas, fisiológicas, ultraestruturais e morfológicas são utilizadas ao lado das análises moleculares para a definição de grandes e pequenos grupos biológicos, tem atualmente papel indispensável na delimitação dos táxons e tem modificado substancialmente a taxonomia das cianobactérias nas últimas décadas (Hoffmann *et al.* 2005). Dentre essas áreas de conhecimento, a biologia molecular se destaca e vem indicando maior diversidade em nível de gênero do que tradicionalmente conhecido, dividindo gêneros como *Aphanizomenon* Born. & Flah., *Anabaena* Born. & Flah. e *Nostoc* Born. & Flah. em vários novos gêneros (Rajaniemi *et al.* 2005, Komárek & Komárková 2006, Komárek & Zapomelová 2007, Komárek 2010, Hrouzek *et al.* 2013).

Para grandes grupos, estudos baseados em biologia molecular sustentam a separação de quatro subclasses de cianobactérias que haviam sido propostas com base na disposição dos tilacóides. As heterocitadas, tradicionalmente classificadas nas ordens Nostocales (ausência de ramificações ou ramificações falsas) e Stigonematales (ramificações verdadeiras) (Komárek & Anagnostidis 1989), apresentam em comum o arranjo irregular dos tilacóides. A tradicional separação dessas ordens não é natural de acordo com estudos filogenéticos e atualmente ambas estão incluídas em uma única ordem monofilética (Nostocales), dentro da subclasse Nostocopycidae (Hoffmann *et al.* 2005). Neste trabalho os autores dividem a ordem em dez famílias (Scytonemataceae, Symphyonemataceae, Borzinemataceae, Rivulariaceae, Microchaetaceae, Nostocaceae, Chlotogloeopsidaceae, Hapalosiphonaceae, Loriellaceae e Stigonemataceae), considerando principalmente estudos moleculares, além de características morfológicas.

De acordo com estudos filogenéticos mais recentes, Komárek (2013) extingue as famílias Borzinemataceae e Loriellaceae e passa a reconsiderar Nostochopsidaceae e Fischerellaceae (Anagnostidis & Komárek 1990), sendo que as últimas estão muito próximas filogeneticamente da família Hapalosiphonaceae. A família Nostochopsidaceae é semelhante à Symphyonemataceae por apresentar heterocitos em posição lateral aos tricomas, porém pode ser diferenciada por apresentar geralmente ramificações verdadeiras em T, enquanto Nostochopsidaceae apresenta principalmente ramificações em Y. A família Fischerellaceae compreende táxons com ramificações verdadeiras e alternativamente falsas. Como citado anteriormente, em termos filogenéticos Fischerellaceae está estreitamente relacionada à Hapalosiphonaceae e Nostocopsidaceae, sendo que no futuro essas famílias podem ser unidas ou novas famílias podem ser separadas dessas à medida que novos táxons forem descobertos (Komárek 2013).

1.5. Biodiversidade de cianobactérias heterocitadas na Mata Atlântica do Estado de São Paulo

Para o estado de São Paulo, os estudos que registram cianobactérias terrestres heterocitadas na Mata Atlântica limitam-se aos de Büdel *et al.* (2002) em ambientes rochosos, no qual são descritas duas espécies de *Camptylonemopsis* Desik.; Fiore *et al.*

(2007) que descreveram o gênero *Brasilonema* e Sant'Anna *et al.* (2007) que apresentaram e descreveram três novas espécies de *Nostoc*. Além destes, há uma monografia de graduação de Murunová & Kastovsky (2007) que apresenta quatorze táxons de cianobactérias heterocitadas para a região de Ubatuba, litoral norte do estado de São Paulo, sendo sete táxons identificados em nível gênero e sete sem confirmação específica. Ainda, o trabalho de Ferreira (2008) registra onze espécies de cianobactérias heterocitadas para o litoral norte paulista. Lemes-da-Silva *et al.* (2010) descreveram *Hapalosiphon santannae* entre outras espécies novas de outros grupos de cianobactérias e Lemes-da-Silva *et al.* (2012), em trabalho com cianobactérias cortícolas encontraram espécies dos gêneros *Hapalosiphon* Born. & Flah., *Hassalia* Born. & Flah., *Nostoc, Scytonema,* and *Stigonema,* no noroeste de São Paulo. Além dos trabalhos resultantes desta tese que serão detalhados posteriormente, os trabalhos mais recentes são o de Ferreira *et al.* (2013), no qual populações de *Hapalosiphon* e *Stigonema* tiveram o 16S rRNA sequenciado diretamente de material da natureza e o de Fiore *et al.* (2013), descrevendo o novo gênero *Dactylothamnos*.

1.6. Objetivos

1.6.1. Objetivos gerais

Conhecer a biodiversidade e o potencial biotecnológico de cianobactérias heterocitadas de ambientes terrestres da Mata Atlântica no Estado de São Paulo.

1.6.2. Objetivos específicos

a) Identificar e caracterizar as populações encontradas com base em análises morfológicas, ambientais e moleculares, sempre que possível.

b) Analisar a produção de saxitoxinas, microcistinas, anatoxina-a e β-metil-amino-alanina por espécies heterocitadas isoladas em cultura.

c) Testar metabólitos secundários com possíveis atividades anticolinesterásica, antifúngica e antioxidante, em cepas selecionadas.

2. MATERIAL E MÉTODOS

2.1. Coleta do material

As áreas de estudo foram selecionadas de forma a abranger regiões preservadas de Mata Atlântica e que contemplassem as faixas tropical e subtropical do estado de São Paulo. Assim, as coletas de cianobactérias terrestres foram realizadas no período de 02/2010 a 08/2011, especificamente nas localidades do Parque Estadual da Serra do Mar – Núcleo Santa Virgínia (23º20'16"S e 45º09'01"O), Estação Ecológica Juréia-Itatins (24 º26'25''S e 47 º04'35''O) e Parque Estadual da Ilha do Cardoso (25º04'12"S e 47º55'27"O) (Figura 1). Com a finalidade de abranger diferentes tipos de vegetação e de substratos, 263 amostras foram coletadas em diversos tipos de ambiente. Utilizando-se espátula, foram feitas raspagens de biofilmes crescendo sobre rochas, solos, troncos de árvores e folhas, em locais como interior de mata, restinga, mangue, praia, áreas desmatadas e paredões rochosos e de terra. Após a coleta, as amostras foram mantidas em sacos de papel, secadas a sombra e transportadas ao Núcleo de Pesquisa em Ficologia do Instituto de Botânica de São Paulo, onde se encontram armazenadas. Alíquotas das amostras citadas nesta tese foram fixadas em formaldeído 4% e incluídas no Herbário Científico do Estado de São Paulo (SP); além disso, todas as amostras coletadas estão mantidas secas em sacos de papel, no Núcleo de Pesquisa em Ficologia do Instituto de Botânica de São Paulo para futuros estudos de cianobactérias em geral.

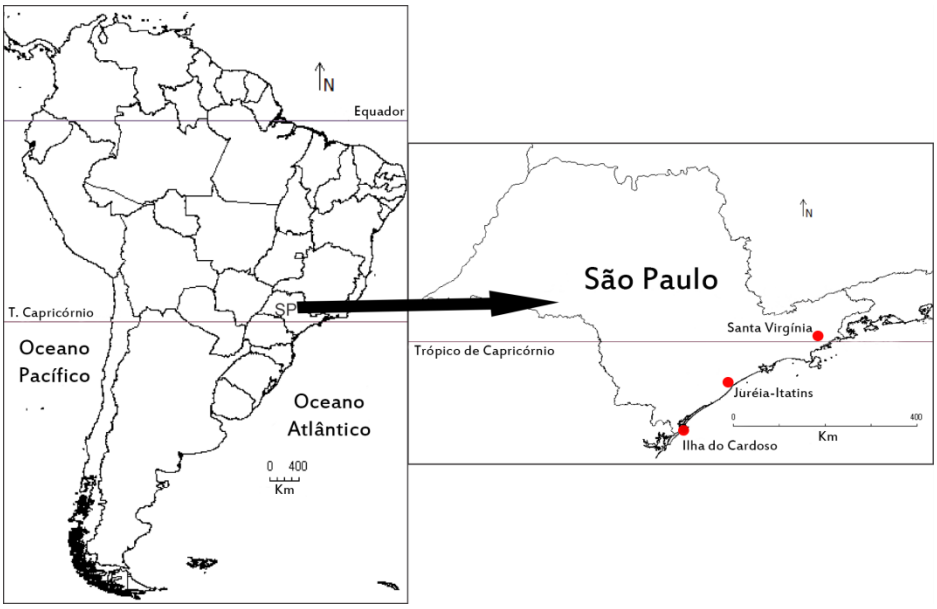

Figura 1. Mapa indicando os locais de amostragem na Mata Atlântica, no Estado de São Paulo.

2.2. Estudos morfológicos e sistema de classificação utilizado

Para os estudos morfológicos das amostras secas, alíquotas das populações foram reidratadas em placas de Petri, com água destilada, por 20 horas antes de serem analisadas. Complementarmente, análises dessas populações foram feitas também em material de cultura, nos casos em que o isolamento das linhagens foi bem sucedido. Algumas linhagens incluídas no presente trabalho não foram encontradas no material da natureza, mas cresceram quando as amostras foram colocadas em cultura.

Os estudos de variabilidade fenotípica foram realizados ao microscópio óptico binocular Zeiss Axioplan 2 com contraste de fase, câmara clara, ocular de medição, câmara fotográfica Zeiss Axiocam MRc e epifluorescência acoplados ao equipamento. Tais estudos basearam-se em um mínimo de vinte indivíduos (cultura e natureza) de cada população analisada, que foram medidos e fotografados. As variáveis morfométricas comprimento (comp.) e largura (larg.) de células vegetativas, heterocitos e acinetos e largura dos filamentos foram medidas utilizando-se fotomicrografias, através do programa ZEISS Axiovision 4.6. O sistema de classificação adotado foi o de Komárek (2013).

2.3. Isolamento e cultura

Para o isolamento e cultura de linhagens, as amostras da natureza foram inoculadas em tubos de ensaio contendo meio líquido BG-11, pH 7,4 (Ripka *et al.* 1979), sem fonte de nitrogênio e mantidas sob temperatura de 22+1^{o}C, irradiância de 40 - 50 μmol.m-2.s-1 e fotoperíodo de 14 - 10h claro-escuro. Quinze dias após a inoculação, foram feitas as triagens das culturas ao microscópio e o isolamento de indivíduos de Nostocales para estudos moleculares e de atividades biológicas. Concomitantemente, populações de interesse taxonômico e bioquímico, previamente observadas no material da natureza, foram selecionadas, isoladas, cultivadas e destinadas aos estudos já citados.

Para o isolamento das linhagens diretamente do material da natureza foi adaptado o método descrito em Jacinavicius *et al.* (2013), que consiste na "pescaria" de indivíduos em lâminas, com auxílio de pipetas Pasteur afiladas ao fogo e observação ao microscópio. Os indivíduos de interesse de cada amostra estudada foram separados manualmente e colocados em áreas isoladas das lâminas utilizando-se as pipetas afiladas. Após o isolamento na lâmina, os indivíduos foram "pescados" com pipetas novas e transferidos para novos meios de cultura.

Todas as etapas do isolamento de material e manutenção das cepas (repicagem a cada 60 dias) foram feitas com material e meios de cultura autoclavados a 120 ^{o}C por 30 minutos, em câmara de fluxo laminar previamente esterilizada por luz ultravioleta e equipada com Bico de Bunsen. Todas as linhagens isoladas foram depositadas no Banco de Culturas de Cianobactérias pertencente à Coleção de Culturas de Algas e Fungos do Instituto de Botânica (CCIBt).

2.4. Estudos filogenéticos

As atividades de extração do DNA, até preparação para o sequenciamento do gene 16S rRNA e 16-23S ITS, foram realizadas nos seguintes laboratórios: Laboratório de Biologia, Ecologia e Taxonomia de Algas do Departamento de Zoologia e Botânica da Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP/Rio Preto), sob a

supervisão dos pesquisadores Dr. Luís Henrique Zanini Branco e Dra. Janaína Rigonato; Laboratorio de Ecologia Molecular de Cianobactérias do Centro de Energia Nuclear na Agricultura da Universidade de São Paulo (CENA - USP/Piracicaba), sob a supervisão dos pesquisadores Dra. Marli de Fátima Fiore e Dra. Janaína Rigonato. As construções de árvores filogenéticas e estruturas secundárias foram realizadas no laboratório de biologia molecular do Departamento de Biologia da John Carroll University (Cleveland, USA), sob a supervisão do pesquisador Dr. Jeffrey R. Johansen.

2.4.1. Extração de DNA e amplificação por PCR dos genes de RNAr 16S e 16-23S rRNA ITS

As extrações e purificações do DNA genômico foram realizadas utilizando-se os kits MOBIO Ultraclean para amostras de cultura e MOBIO PowerSoil para amostras da natureza. A amplificação por PCR das regiões adjacentes RNAr 16S e 16-23S RNAr ITS foi feita utilizando-se os oligonucleotídeos 27F1 (Neilan *et al.* 1997) e 23S30R (Lepère *et al.* 2000). As amplificações foram realizadas em solução contendo: tampão para a reação PCR 1X (20mM Tris HCl pH 8,4; 50 mM KCl); 0,2 mM de cada dNTP; 3 mM $MgCl_2$; 1,5 U de Platinum® Taq DNA Polimerase (Invitrogen, Carlsbad, CA, EUA); 10 ng de DNA; 5 ρmol de cada primer; água ultrapura (Milli-Q, Millipore, EUA), esterilizada, para um volume final de 25 μL. As reações foram realizadas em termociclador "Gene Amp PCR System 2400" (Applied Biosystems, Foster City, CA, EUA). As condições para amplificação do gene RNAr 16S e do ITS 16-23S foram: 95ºC/3 min; 30 ciclos de desnaturação à 94ºC por 10 s, anelamento à 50ºC por 20 s, extensão à 72ºC por 1 min; e extensão final a 72ºC por 7 min.

2.4.2. Clonagem e transformação dos produtos de PCR

Após a amplificação de DNA via PCR das sequências foram realizadas a clonagem e a transformação dos produtos da PCR utilizando o Kit de clonagem "pGEM®-T Easy Vector Systems" (Promega). A presença dos insertos nas células transformadas foi checada através de PCR das colônias e a introdução do vetor

contendo o inserto nas células competentes de *E. coli* DH5α foi feita através de choque térmico (Sambrook & Russel 2001). Inicialmente, alíquotas de 10 μL do produto de ligação e 100 μL de suspensão de células competentes de *E. coli* DH5α foram misturadas em um microtubo esterilizado, o qual foi incubado no gelo durante 30 minutos e após, o microtubo foi transferido imediatamente para banho-maria a 42ºC e mantido por 30 segundos sem agitação. Em seguida o microtubo foi incubado no gelo por 2 minutos. Adicionou-se 250 μL de meio SOC (Sambrook & Russel 2001) a temperatura ambiente e a mistura foi incubada a 37ºC, durante 1 hora, sob agitação de 200 rpm. As células competentes transformadas foram plaqueadas em meio LB sólido contendo ampicilina (USB Corporation, Cleveland, OH, EUA) e X-Gal (Invitrogen), ambos em concentrações finais de 100 μg/mL de meio. Numa próxima etapa, as placas foram incubadas por 15 horas, a temperatura de 37ºC e em seguida, uma colônia de cor branca foi utilizada para nova reação 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 utilizando-se os iniciadores: M13F (5'-GCCAGGGTTTTCCCAGTCACGA-3'); M13R (5'-GAGCGGATAACAATTTCACACAGG-3'). A amplificação foi feita em solução contendo: tampão para reação PCR 10X (Tris HCl 20mM pH 8,4; KCl 50 mM); 0,2 mM de cada dNTP; MgCl2 3 mM; 1,5 U de Platinum® Taq DNA Polimerase (Invitrogen); 10 ng de DNA; 5 pmol de cada iniciador; água ultrapura (Milli-Q) esterilizada, para um volume final de 25 μL. Esta reação foi feita em um termociclador "Gene Amp PCR System 2400" (Applied Biosystems) com condições de amplificação: 94ºC/5 min; 25 ciclos de 95ºC/20 s, 50ºC/15 s, 60ºC/1 min. A verificação do tamanho dos produtos da PCR gerados foi feita em gel de agarose 1,0% - 0,5 X TBE conforme descrito acima.

2.4.3. Extração de DNA plasmidial

A extração de plasmídeos das células de *E. coli* DH5α que continham os insertos foi feita pelo método de preparação de pequena escala de plasmídeo, usando hidrólise alcalina (Birnboim & Doly 1979). As colônias brancas foram transferidas para 4 mL de meio LB contendo ampicilina e cultivadas por 15 horas, a 37ºC, sob agitação de 200

rpm. Em microtubos foram colocados 1,5 mL da cultura de células produzidas e, em seguida, foram centrifugadas a 10.000 × g por 20 segundos, duas vezes. O pélete formado foi ressuspenso em 100 µL de solução I gelada (Tris-HCl 25 mM, pH 8,0, EDTA 10 mM e glucose 50 mM). Em seguida, 200 µL de solução II (NaOH 0,2 N, SDS 1%) foram adicionados e misturados gentilmente por meio da inversão dos microtubos. Após terem sido incubados no gelo por 5 minutos, foram acrescentados 150 µL de solução III gelada (acetato de potássio 3 M e ácido fórmico 1,8 M). Procedeu-se novamente a mistura por inversão e os microtubos foram incubados no gelo por mais 5 minutos. Posteriormente, foram centrifugados a 10.000 × g durante 7 minutos e o sobrenadante foi transferido para um novo tubo. Adicionou-se 270 µL de isopropanol a temperatura ambiente, misturando-se e centrifugando-se conforme descrito anteriormente. Após a eliminação do sobrenadante, o pélete foi lavado uma vez com 250 µL de etanol 70% gelado e centrifugado a 10.000 x g por 2 minutos. O pélete foi secado e ressuspenso em 30 µL de uma solução contendo Tris-HCl 10 mM, pH 8,0, EDTA 0,5 M e 10 mg RNAse/mL. Incubou-se essa mistura a 37ºC por 30 minutos. Os plasmídeos assim extraídos foram armazenados a -20ºC até o próximo uso.

2.4.4. Sequenciamento

A PCR para o sequenciamento dos fragmentos inseridos nos plasmídeos foi feita usando-se o kit "DYEnamic ET Terminator Cycle Sequencing" (Amersham Biosciences, Piscataway, NJ, EUA). Para a reação utilizou-se 200 ng de plasmídeo contendo o inserto, 5 ρmol dos iniciadores externos M13F(5'-GCCAGGGTTTTCCCAGTCACGA-3'), SP6 (5'-ATTTAGGTGACACTATAGAA-3') e 1 µL de "DYEnamic", 2 µL de tampão 2,5 X "Save Money" (protocolo fornecido pelo fabricante) e água ultrapura para volume final de 10 µL. As condições de amplificação foram as seguintes: 25 ciclos de 95ºC/20 s, 50ºC/15 s, 60ºC/1 min. Após a amplificação dos fragmentos, realizou-se a precipitação dos mesmos conforme manual de instruções do kit "DYEnamic ET Terminator Cycle Sequencing". As reações precipitadas foram sequenciadas nos laboratórios de biologia molecular do Centro de Energia Nuclear na Agricultura (CENA/USP) ou na empresa Genomic Engenharia Molecular.

2.4.5. Processamento e análise das sequências

As sequências geradas foram processadas para remoção de bases produzidas com baixa qualidade (índice de qualidade < 20) através do pacote que contém os programas Phred/Phrap/Consed (Ewing & Green 1998, Ewing *et al.* 1998, Gordon *et al.* 1998) em sistema operacional Linux. Após, foram comparadas com outras sequências previamente depositadas no GenBank do National Center for Biotechnology Information (NCBI), utilizando-se a ferramenta Basic Local Aligment Search Tool (BLAST) (Altschul *et al.* 1990). Para a construção das árvores filogenéticas, as sequências obtidas e outras selecionadas de bancos de dados públicos foram alinhadas, os métodos de máxima verossimilhança e máxima parsimônia foram utilizados usando-se o pacote de programa MEGA 5.0 (Tamura *et al.* 2011), com bootstrap de 1000 repetições. A análise bayesiana foi aplicada utilizando-se o programa MrBayes 3.2 (Ronquist & Huelsenbeck 2003), em duas corridas independentes de oito cadeias cada e 20×10^6 gerações. Em todos os casos o modelo GTR+G+I foi utilizado.

As determinações das estruturas secundárias das regiões D1-D1', Box B, V2 e V3 do16-23S ITS foram feitas utilizando-se o Mfold versão 2.3 (Zuker 2003), com temperatura de dobramento definida em 20^{o}C. Para todas as análises de ITS foram utilizadas sequencias com os $tRNA^{Ile}$ and $tRNA^{Ala}$.

2.5. Produção de biomassa para estudos de atividade biológica

A biomassa cianobacteriana foi obtida a partir de repiques de linhagens selecionadas, dentre as isoladas nesse trabalho e que apresentaram rápido crescimento (Komarekiella gen. nov., Calothrix Born. & Flah., Tolypothrix Born. & Flah. e Brasilonema Fiore et al.: CCIBt 3307, 3319, 3320, 3321, 3463, 3481, 3482, 3484, 3485, 3487 e 3551) e/ou de interesse taxonômico (Dapisostemon gen. nov. CCIBt 3318), todas mantidas no Banco de Culturas do Núcleo de Pesquisa em Ficologia. Inicialmente, 5 mL de inóculo foram transferidos para frascos com capacidade de 50 mL contendo meio de cultura BG-11 (Ripka et al. 1979). Após um período de 20 dias, essas culturas foram transferidas para frascos contendo 500 mL de meio sendo então

mantidas até atingirem desenvolvimento satisfatório para a seguinte etapa. Finalmente, esses 500 mL de cultura foram divididos em duas porções iguais, sendo cada uma delas transferida para erlenmeyer contendo 5000 mL cada, que foram mantidos sob aeração e iluminação contínua, por cerca de 20 dias, até atingirem o estádio adequado de crescimento.

2.6. Prospecção de metabólitos secundários com atividade biológica

2.6.1. Preparo das amostras

As biomassas cianobacterianas obtidas em cultura foram liofilizadas e divididas em duas porções. A primeira porção foi submetida à extração com ácido acético 0,1 M (EAA) e a segunda, à extração com metanol 100% (EM). O extrato em ácido acético foi liofolizado e o metanólico foi concentrado a vácuo e, em seguida, liofilizado (Harada *et al.* 1999, Pyo & Shin 2002).

2.6.2. Prospecção de substâncias com atividade anticolinesterásica, por bioautografia, segundo o protocolo de Rhee *et al.* (2001)

Alíquotas de 200 μg de cada extrato seco (EAA e EM) foram dissolvidas em água (EAA) e em metanol (EM), respectivamente e aplicadas à placas cromatográficas de gel de sílica (Silica 60 F_{254}, 10X10cm, espessura 0,2 mm, E. Merck). Os cromatogramas foram desenvolvidos em clorofórmio/metanol/água (64:36:8, v/v/v), secos em capela, em corrente de ar e a seguir, nebulizados com solução da enzima acetilcolinesterase (6,66 U ml), secas perfeitamente e incubadas em atmosfera úmida, à temperatura de 37 °C, por 20 min. Em sequência, os cromatogramas foram nebulizados com solução 0,25% de acetato de 1-naftila em etanol e de solução aquosa 0,25% do sal Fast Blue B. As manchas correspondentes aos potenciais inibidores da acetilcolinesterase foram vistas como manchas claras contra o fundo púrpura (Poon *et al.* 1987, Harada *et al.* 1991).

2.6.3. Prospecção de substâncias com atividade antifúngica, frente ao fungo *Colletotrichum lagenarium* (Agripino *et al.* 2004)

Também neste ensaio, alíquotas de 200 μg de cada extrato seco (EAA e EM) foram dissolvidas em água (EAA) e em metanol (EM), respectivamente e aplicadas à placas cromatográficas de gel de sílica (*Sílica 60 F_{254}*, 10X10cm, espessura 0,2 mm, E. *Merck*). Os cromatogramas foram desenvolvidos na fase móvel constituída por clorofórmio/metanol/água (64:36:8, v/v/v), secos em capela e a seguir, nebulizados com suspensão de conídios. As placas foram incubadas por seis dias, à temperatura de 28°C, em câmara de incubação, após o que foram analisadas e fotografadas**.**

2.6.4. Prospecção de substâncias com atividade antioxidante pelo método do sequestro do radical livre DPPH• (Hostettmann *et al.* 2003)

Para esta avaliação, 200 μg de cada um dos extratos (EAA e EM) foram submetidos à bioautografia, desenvolvida com a fase móvel clorofórmio/metanol/água, 64:36:8 (v/v/v). Como padrão positivo, foi empregada a Quercetina. Após o desenvolvimento, o cromatograma foi seco e nebulizado com solução metanólica ($2mg.ml^{-1}$) do radical 2,2-difenil-1-picrilhidrazila (DPPH), que permite detectar substâncias com atividade antioxidante, em extratos vegetais. Essas substâncias aparecem como manchas amareladas sobre fundo violeta, após a derivatização.

2.6.5. Prospecção de cianotoxinas conhecidas (microcistinas, saxitoxinas, anatoxina-a e β-metil-amino-alanina) nos extratos em ácido acético 0,1 M, das cepas em análise

A pesquisa química de cianotoxinas foi realizada por Cromatografia Planar (CP).

2.6.5.1. Pesquisa cromatográfica de microcistinas (Harada *et al.* 1988, Harada *et al.* 1999)

10 µg de extrato liofilizado de cada cepa foi dissolvido em água e aplicado a placas de gel de Sílica (20x20 cm, 0,25 mm, Kieselgel 60GF_{254}, E.Merck), juntamente com o padrão. O cromatograma foi desenvolvido com a fase móvel clorofórmio/metanol/água, 64:36:8, v/v/v (Poon *et al*., 1987; Harada *et al*., 1991). As placas cromatográficas foram desenvolvidas em atmosfera equilibrada pela colocação do solvente 20 min. antes das placas. Após a corrida cromatográfica, as placas foram secas em correntes de ar, avaliadas sob luz ultravioleta (λ 254nm e 365 nm) e em seguida, derivatizadas com tetrametilbenzidina (TMB) (Pelander *et al*. 2000).

Para essa derivatização, as placas secas ficaram expostas, por 20 minutos, ao gás cloro, gerado pela mistura de volumes iguais de HCl 10% e solução de permanganato de potássio 5%, aeradas por 15 segundos e nebulizadas com TMB.

O reagente TMB para microcistinas. 0,8 mg de TMB foi dissolvido em 1,5 ml de ácido acético glacial ao qual foram acrescentados 25 ml de água deionizada e 50 mg de iodeto de potássio.

2.6.5.2. Pesquisa de saxitoxinas (Buckley *et al.* 1976)

Para essa análise, 10 µg de extrato liofilizado de cada cepa foi dissolvido em água deionizada e aplicado a placas de gel de Sílica (20x20 cm, 0,25 mm, Kieselgel 60GF_{254}, E.Merck). Os cromatogramas foram desenvolvidos no mesmo sistema de eluentes utilizado para a pesquisa de microcistinas. As placas foram secas em correntes de ar por uma hora, nebulizadas com peróxido de hidrogênio 1%, aquecidas à 100 °C por 10 min e observadas sob luz ultra violeta, em comprimento de onda λ 365 nm. As saxitoxinas são visíveis como manchas fluorescentes.

2.6.5.3. Pesquisa de anatoxina-a (Ojanperä *et al.* 1991)

10 µg de extrato liofilizado de cada cepa foram dissolvidos em água deionizada e tiveram seu pH ajustado para 11-11,5, com hidróxido de sódio 1 N, após o que foram

extraídos em funil de separação, com 10 ml de diclorometano/isopropanol 95:5, (v/v). As fases foram separadas por centrifugação. A fase aquosa foi descartada, a fase orgânica foi evaporada e o resíduo, reconstituído com 15 µl de clorofórmio que foram aplicados integralmente à placa cromatográfica de gel de sílica 60 F_{254}. Sobre este material aplicado, foram aplicados 2 µl de solução 0,5 M de bicarbonato de sódio. Após secagem sob corrente de ar, sobre a mesma mancha foram aplicados 3 µl de solução aquosa de Fast Black K Salt (FBK) 1%. O cromatograma foi desenvolvido em tolueno/metanol (90:10) e após secagem em corrente de ar, foi avaliado para a presença de mancha alaranjada, em Rf 0,42, relativa ao derivado de anatoxina-a.

2.6.5.4. Pesquisa do aminoácido β-metilaminoalanina (Merck 1997)

10 µg de extrato liofilizado de cada cepa foi dissolvido em água deionizada e aplicado a placa de papel Watman N^{o} 3, juntamente com o padrão. O cromatograma foi desenvolvido em butanol/ácido acético/água 5:4:1 (v/v/v) e a seguir foi seco em corrente de ar. Após a secagem, o cromatograma foi derivatizado com ninidrina e aquecido a 110 °C, por 15 minutos.

2.6.6. Ensaio de toxicidade aguda (dose única), por via intraperitoneal (i.p.), em camundongos

No presente estudo, foram observados todos os procedimentos e normas éticas relativos ao uso de animais de laboratório, do Instituto Butantan, da Organização Mundial da Saúde e da OECD (Organização para Cooperação Econômica e Desenvolvimento). Nosso projeto foi aprovado pela Comissão de Ética Para Pesquisa Animal do Instituto Butantan, tendo sido protocolado sob o número 385/07.

Nesses ensaios toxicológicos, foram utilizados camundongos Swiss-Webster, machos, com idade entre 30 e 50 dias ou com peso entre 19 e 22 g. Esses animais eram provenientes do Biotério Central do Instituto Butantan.

Para alojá-los, foram usadas caixas de plástico fosco, medindo 30x20x19 cm, dotadas de tampas metálicas. Em cada uma delas, eram colocados, no máximo, três animais. A temperatura e umidade ambientes foram mantidos constantes (21-24°C e 65-

70%, respectivamente)(controle por aparelho de ar condicionado central) assim como o ciclo noite:dia de 12:12 horas, cuja fase clara iniciava às 6:00 h.

Durante o experimento, os animais tiveram livre acesso à água e à ração balanceada para roedores. Também nos dias anteriores aos ensaios, os animais foram mantidos nessas condições.

Nestes testes, realizados em triplicata, cada animal recebeu, por via intraperitoneal, 20 mg dos extratos EAA e EM das cepas em estudo, reconstituídos com até 1 mL de água destilada, seguindo método preconizado pela Organização Mundial da Saúde (Harada *et al*. 1999) para o estudo de cianotoxinas.

Os animais- controle receberam unicamente 1 mL de água destilada, pela mesma via. Os sinais de intoxicação e o tempo de sobrevida (quando pertinente) foram anotados. Os animais foram observados por 7 dias, após os quais foram eutanasiados e submetidos a exame "post-mortem".

3. RESULTADOS

3.1 Resultados gerais

Considerando-se materiais da natureza e cultura, das 263 amostras coletadas, 128 continham cianobactérias heterocitadas, sendo que as espécies presentes em 53 destas amostras já foram publicadas ou estão citadas em manuscritos aceitos ou que serão submetidos a revistas científicas (anexos I a IX). Dentre as restantes amostras de interesse (75 amostras), 40 apresentaram material em mau estado para análise ou muito escasso, impossibilitando estudos morfológicos (Figuras 2 e 3). Apenas 35 das amostras viáveis para estudos apresentaram morfotipos ainda não incluídos em manuscritos, os quais serão apresentados na seção 3.4.1.

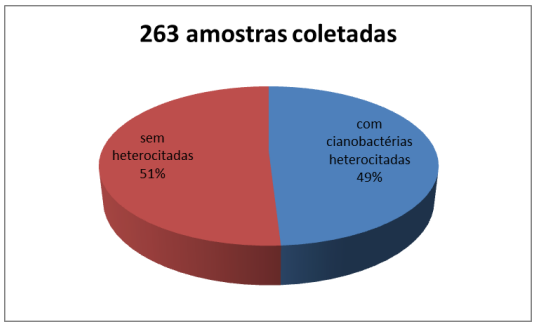

Figura 2. Proporção de amostras com e sem cianobactérias heterocitadas.

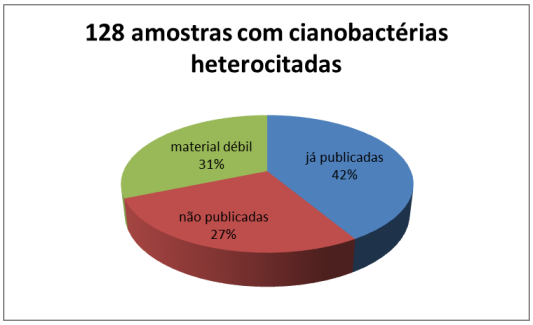

Figura 3. Proporção de amostras contendo táxons já publicados, não publicados e inviáveis para identificação.

No total, 133 populações foram identificadas pelo menos em nível genérico, sendo que quinze táxons foram publicados ou estão incluídos em manuscritos já submetidos a revistas cientificas. Dentre esses, sete são espécies novas e dois constituem gêneros novos. Também, 35 linhagens foram isoladas e depositadas na CCIBt , sendo que 14 destas tiveram o gene 16S rRNA sequenciado e adicionalmente sete tiveram o 16-23S ITS sequenciado (Tab. 1). Além disso, populações de *Streptostemon lutescens* Sant'Anna *et al.* (SP 427505), *Stigonema jureiensis* Hentschke *et al.* sp nov. (SP 427311) e *S. fremyi* Sant'Anna *et al.* (SP 428606) também tiveram o gene 16S sequenciado, desta vez a partir de material proveniente da natureza, originando dois artigos científico (anexos V e VII).

O gênero encontrado com maior frequência nas amostras estudadas e foi *Scytonema* Born. & Flah., presente em 36 amostras, seguido por *Stigonema* Born. & Flah., presente em 33 amostras e *Streptostemon* Sant'Anna *et al.* em 26 (Figura 4). Apesar de *Scytonema* ser o gênero com maior frequência em amostras, comumente as populações encontradas deste gênero apresentaram-se escassas ou em más condições,

impossibilitando suas identificações. Dentre as populações de *Stigonema*, treze espécies foram identificadas, indicando grande diversidade desse grupo na Mata Atlântica. Ao contrário, *Streptostemon* apesar de estar presente em muitas amostras, apresentou pouca variação morfológica sendo que, provavelmente, todas as populações encontradas pertençam à mesma espécie (*S. lutescens* Sant'Anna *et al.*). Ainda, as populações deste gênero apresentaram sempre grande desenvolvimento de biomassa, sendo dominantes nos biofilmes em que estavam presentes.

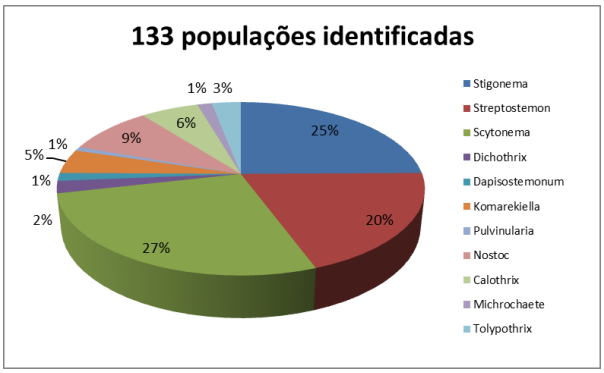

Figura 4. Porcentagens de gêneros identificados nas amostras da natureza e cultura.

Os testes químicos foram realizados com os extratos brutos de 12 linhagens (Tabela 1). Essas linhagens foram selecionadas devido a facilidade de cultura ou por serem novos táxons (*Komarekiella* gen. nov. e *Dapisostemon* gen. nov.). Todos os extratos estudados apresentaram pelo menos um tipo de atividade biológica e estão detalhados na seção 3.4.2.

Finalmente, considerando-se os resultados taxonômicos e bioquímicos, este projeto originou três artigos publicados durante o seu desenvolvimento, além de seis manuscritos que estão aceitos ou serão submetidos a revistas científicas nacionais e internacionais, como explícito nas seções 3.2 e 3.3. Os resultados ainda não publicados estão detalhados na seção 3.4.

Tabela 1. Linhagens de Nostocales isoladas durante o projeto de doutorado e estudos realizados.

N	CCIBt	Local	Gênero	Estudos moleculares	Testes químicos
1	**3319**	Santa Virgínia	*Calothrix*	-----	Testados
2	**3581**	Santa Virgínia	*Calothrix*	-----	
3	**3583**	Santa Virgínia	*Calothrix*	-----	
4	**3585**	Ilha do Cardoso	*Calothrix*	-----	

N	CCIBt	Local	Gênero	Estudos moleculares	Testes químicos
5	**3579**	Santa Virgínia	*Calothrix*	-----	
6	**3582**	Ilha do Cardoso	*Calothrix*	-----	-----
7	**3586**	Santa Virgínia	*Calothrix*	-----	-----
8	**3320**	Santa Virgínia	*Calothrix*	-----	Testados
9	**3321**	Ilha do Cardoso	*Tolypotrhix*	-----	Testados
10	**3587**	Ilha do Cardoso	*Tolypotrhix*	-----	-----
11	**3580**	Ilha do Cardoso	*Tolypotrhix*	-----	-----
12	**3577**	Ilha do Cardoso	*Tolypothrix*	-----	-----
13	**3481**	Santa Virgínia	*Komarekiella*	16S e ITS sequenciados	Testados
14	**3307**	Santa Virgínia	*Komarekiella*	16S sequenciado	Testados
15	**3483**	Santa Virgínia	*Komarekiella*	16S e ITS sequenciados	-----
16	**3486**	Santa Virgínia	*Komarekiella*	16S e ITS sequenciados	-----
17	**3487**	Ilha do Cardoso	*Komarekiella*	16S e ITS sequenciados	Testados
18	**3552**	Ilha do Cardoso	*Komarekiella*	16S e ITS sequenciados	-----
19	**3485**	Santa Virgínia	*Nostoc commune*	16S e ITS sequenciados	Testados
20	**3488**	Ilha do Cardoso	*Nostoc*-like	-----	-----
21	**3489**	Ilha do Cardoso	*Nostoc*-like	16S sequenciado	-----
22	**3553**	Santa Virgínia	*Nostoc*-like	16S sequenciado	-----
23	**3484**	Santa Virgínia	*Nostoc*-like	-----	Testados
24	**3588**	Santa Virgínia	*Nostoc*-like	-----	-----
25	**3576**	Santa Virgínia	*Nostoc*-like	-----	-----
26	**3588**	Santa Virgínia	*Nostoc*-like	-----	-----
27	**3584**	Santa Virgínia	*Nostoc*-like	-----	-----
28	**3482**	Ilha do Cardoso	*Nostoc*-like	-----	Testados
29	**3551**	Ilha do Cardoso	*Brasilonema*	16S sequenciado	Testados
30	**3464**	Ilha do Cardoso	*Brasilonema*	16S sequenciado	-----
31	**3463**	Ilha do Cardoso	*Brasilonema*	16S sequenciado	Testados
32	**3318**	Ilha do Cardoso	*Dapisostemon*	16S sequenciado	Testados
33	**3536**	Ilha do Cardoso	*Dapisostemon*	16S e ITS sequenciados	-----
34	**3578**	Juréia	*Iphinoe*	-----	-----
35	**3575**	Ilha do Cardoso	Stigonematales	-----	-----

----- = não realizado. CCIBt= Coleção de Culturas do Instituto de Botânica

3. 2. Artigos publicados

Komárek, J., Sant´Anna, C. L., Bohunická, M., Mareš, J., Hentschke, G. S., Rigonato, J. & Fiore, M. F. 2013. Phenotype diversity and phylogeny of selected *Scytonema*–species (Cyanoprokaryota) from SE Brazil. Fottea 13(2): 173-200.

Neste artigo são descritas as espécies novas *Scytonema papillicapitatum* Sant´Anna & Komárek e *S. chorae* Sant´Anna & Komárek. Além disso, também são apresentadas caracterizações de outras doze espécies do gênero coletadas a maioria na Mata Atlântica, Estado de São Paulo. Através das análises morfológicas e moleculares destas populações, o gênero foi dividido em cinco grupos (clados filogenéticos) suportados morfologicamente de acordo com características dos hormogônios, ápices dos filamentos, bainhas e ramificações. Ainda não há suficiente suporte molecular para

a divisão formal do gênero, o que depende de estudos mais completos com um maior número de populações analisadas (anexo I).

Sant´Anna, C. L., Kastovsky, J., Hentschke, G. S. & Komárek, J. 2013. Phenotypic studies on terrestrial stigonematacean Cyanobacteria from the Atlantic Rainforest, São Paulo State, Brazil. Phytotaxa 89(1): 1-23.

Neste artigo são descritas as espécies novas *Fischerella clavata* Sant'Anna, Kaštovský, Hentschke & Komárek, *Stigonema fremyi* Sant'Anna, Kaštovský, Hentschke & Komárek, *S. corticola* Sant'Anna, Kaštovský, Hentschke & Komárek e *S. parallelum* Sant'Anna, Kaštovský, Hentschke & Komárek baseando-se apenas em características morfológicas. Os estudos filogenéticos para estes gêneros ainda não são comuns devido à dificuldade de isolamento e cultura de suas populações. A descrição de novos táxons com base na morfologia ainda é aceitável considerando-se a grande variedade morfológica apresentada principalmente pelas populações de *Stigonema*, além da dificuldade de isolamento. Neste artigo são ainda caracterizadas mais cinco espécies dos gêneros citados, comuns na Mata Atlântica (anexo II).

Carvalho, L. R., Costa-Neves, A., Conserva, G. A. A., Brunetti, R., Hentschke, G. S., Malone, C. F., Torres, L., Sant'Anna, C. L., Rangel, M. 2013. Biologically active compounds from cyanobacteria extracts: in vivo and in vitro aspects. Revista Brasileira de Farmacognosia 23(3): 471-480.

Neste artigo a atividade anticolinesterásica de extratos das linhagens *Calothrix* sp. CCIBt 3320, *Tolypothrix* sp. CCIBt 3321, *Phormidium* cf. *amoenum* CCIBt 3412, *Phormidium* sp. CCIBt 3265 e *Geitlerinema splendidum* CCIBt 3223 foram testadas. Para todas as linhagens, os resultados foram positivos para inibição da enzima acetilcolinesterase nos testes *in vitro*; já nos ensaios em camundongos, as linhagens *G. splendidum* e *Tolypothrix* sp. não apresentaram atividade. Como os efeitos dos sintomas produzidos nos estudos *in vivo* foram reversíveis, estes extratos tem potencial para estudos mais aprofundados no sentido de produção de fármacos. Além disso, neste

artigo são apresentados os efeitos dos extratos em órgãos dos camundongos, em nível histológico (anexo III).

3.3. Manuscritos elaborados

Hentschke, G. S. & Komárek, J. 2014. *Scytonema santannae* sp. nov., a new morphospecies of Cyanobacteria from the Atlantic rainforest, southeastern Brazil. Revista Brasileira de Botânica (aceito).

Neste artigo é descrita uma nova espécie de *Scytonema* coletada na Ilha do Cardoso, Estado de São Paulo, baseando-se apenas em dados morfológicos. Esta espécie apresenta todas as características do gênero, é epífita de briófitas e se caracteriza principalmente pela ornamentação incomum da bainha, formada por placas em mosaico. Até o momento nenhuma espécie de *Scytonema* apresenta essa característica, e de acordo com isso, a descrição deste novo táxon sem a utilização de estudos filogenéticos é justificável (anexo IV).

Hentschke, G. S., Rigonato, J., Branco, L. & Sant'Anna, C. L. 2014. Morphological and molecular characterization of *Stigonema jureiensis* sp. nov. and *Stigonema fremyi* (Nostocales, Cyanobacteria). Phycologia (não submetido).

O artigo apresenta uma nova espécie de *Stigonema*, baseando-se em dados morfológicos e moleculares com material sequenciado diretamente da natureza. A espécie é caracterizada pelo tipo especial de bainha ornamentada, ainda não descrita para nenhuma espécie do gênero. Apresenta também a plasticidade morfológica e a confirmação por dados moleculares da morfoespécie *S. fremyi* . Este é o primeiro artigo descrevendo uma espécie nova do gênero abordando conjuntamente filogenia e morfologia (anexo V).

Hentschke, G. S., Johansen, J. R., Pietrasiak, N., Rigonato, J. Fiore, M. F. & Sant'Anna, C. L. 2014. *Komarekiella atlantica* gen. et sp. nov. (Nostocaceae,

Cyanobacteria): a new subaerial taxon from the Mata Atlântica. PlosOne (não submetido).

O artigo trata da descrição de um novo gênero morfologicamente semelhante à *Nostoc* Born. & Flah. encontrado na Mata Atlântica, baseando-se em morfologia, filogenia e estrutura secundária do ITS 16-23S. Nas análises de Máxima Verossimilhança e Bayesiana, o novo gênero apresenta seis linhagens em um clado único bem suportado. As estruturas secundárias das hélices D1-D1', Box B, V2 e V3 reforçam a ideia da descrição de um novo gênero, pois são muito diferentes de quaisquer outras conhecidas. Análises morfológicas também são consideradas, sendo que o novo gênero apresenta uma característica conhecida até o momento apenas para *Chlorogloeopsis* Pandey & Mitra, que consiste na divisão do acineto em duas células: um heterocito significativamente menor que a célula vegetativa originada neste processo (anexo VI).

Hentschke, G. S., Johansen, J. R., Pietrasiak, N., Rigonato, J. Fiore, M. F., Sant'Anna, C. L. & Komárek, J. 2014. Phylogenetic placement of *Dapisostemon* gen. nov. and *Streptostemon*, two tropical microchaetacean genera (Cyanobacteria). Journal of Phycology (submetido).

O artigo trata da descrição de um novo gênero fasciculado de Michrochaetaceae encontrado na Mata Atlântica, baseando-se em morfologia, filogenia e estrutura secundária do ITS 16-23S. As análises de Máxima Verossimilhança e Bayesiana agrupam as duas linhagens do gênero separadamente de quaisquer outras já conhecidas. Além disso, tanto a morfologia como o estudo das estruturas secundárias reforçam a proposição do novo táxon. Neste artigo também está incluída a confirmação filogenética do gênero *Streptostemon* Sant'Anna *et al.*, descrito até então apenas com base na morfologia (anexo VII).

Hentschke, G. S. & Sant'Anna, C. L. 2014. Current trends and perspectives for cyanobacterial taxonomy – are only cultured populations enough? Algological Studies (submetido).

O artigo é em forma de carta de opinião, discutindo a tendência cada vez mais forte na taxonomia de cianobactérias de publicação de novos táxons sem dados morfológicos de populações da natureza. Atualmente, diversos novos táxons deste grupo de organismos tem sido propostos e as descrições morfológicas estão ficando cada vez mais pobres, principalmente no que diz respeito às populações da natureza. O artigo questiona se publicar novos táxons baseando-se apenas em dados moleculares e, no máximo, dados morfológicos de material de cultura não acarretaria problemas na resolução e delimitação de táxons em níveis genérico e específico (anexo VIII).

Hentschke, G. S & Sant'Anna, C. L. 2014. Three new morphospecies of *Stigonema* (Nostocales, Cyanobacteria) from the rainforest Mata Atlantica, Southeastern, Brazil. Acta Botanica Brasilica (não submetido).

Este manuscrito trata da descrição de três novas espécies de *Stigonema* com base em dados morfológicos. Muitas populações de *Stigonema* dentro da mesma espécie apresentam com frequência diferenças morfométricas em características como largura e comprimento das células e largura dos filamentos. Neste trabalho são utilizadas análises estatísticas para diferenciação de populações dentro das espécies e formulação de hipóteses para sua separação (anexo IX).

3.4. Resultados não incluídos em artigos científicos

3.4.1. Táxons identificados

Família Scytonemataceae

Scytonema siculum Bornet & Flahault, Annales des Sciences Naturelles 5: 96. 1887.

(Figuras 5, 6)

Filamentos frouxamente emaranhados, 25-35 µm larg., ramificações raras, às vezes formando feixes. Tricomas cilíndricos, 20-27 µm larg., levemente constritos apenas nos ápices, às vezes atenuados. Células mais largas que longas, 4,5-8 µm comp.

Bainha amarelada, raramente incolor, lamelas paralelas. Heterocitos quadráticos, mesmas medidas das células vegetativas.

Hábitat: sobre rocha na foz de um rio, às vezes submersa.

Material examinado: BRASIL, São Paulo, Estação Ecológica Juréia-Itatins, 16/08/2011, *Guilherme Hentschke, Célia Sant'Anna, Watson Gama-Júnior, Camila Malone* (SP 428562).

Scytonema guyanense Bornet & Flahault, Ann. Sci. Nat. Bot., ser. 7, 5: 94. 1887.

(Figuras 7, 8)

Filamentos pouco emaranhados, 15,5-18,8 µm larg. Ramificações unidas pela base, depois divergentes. Tricomas cilíndricos 9,5-15,1 µm larg, levemente constritos ou não. Células quadráticas ou mais curtas que largas, 5,9-9 µm comp. Bainha hialina, amarelada, espessa, lamelas paralelas ou irregulares. Heterocitos quadráticos ou mais curtos que largos, mesmas medidas das células vegetativas.

Hábitat: epífitas em briófita crescendo sobre rocha úmida, próximo a rio.

Material examinado: BRASIL, São Paulo, Parque Estadual da Ilha do Cardoso, 30/06/2010, *Watson Gama-Júnior, Camila Malone* (SP 401436).

Brasilonema epidendron Sant'Anna & Komárek, Brazilian Journal of Botany 34: 52. 2011.

(Figuras 9, 10)

Filamentos emaranhados na base 10-13 µm larg., tornando-se paralelos em direção aos ápices. Tricomas cilíndricos 7-11 µm larg. Células quadráticas 5-10 µm comp., mais curtas próximo aos ápices; conteúdo celular verde acastanhado. Bainha

amarelada, lamelas paralelas. Heterocitos quadráticos, mesmas medidas das células vegetativas.

Habitat: sobre rocha no interior da mata.

Material examinado: BRASIL, São Paulo, Parque Estadual da Ilha do Cardoso, 30/06/2010, *Watson Gama-Júnior, Camila Malone* (SP 428554).

***Brasilonema* sp.**

(Figura 11)

Filamentos emaranhados, podendo formar feixes eretos, 6,6-12,9 µm larg. Tricomas não constritos ou apenas levemente, 4,6-10,3 µm diam. Células quadráticas ou mais curtas que largas, 3,5-6,5 µm comp.; conteúdo celular homogêneo, verde brilhante. Bainha aderida ao tricoma, homogênea, amarelada. Heterocitos quadráticos, mesmas medidas das células vegetativas.

O morfotipo apresenta as características diagnósticas do gênero *Brasilonema* Fiore *et al.*, apresentando filamentos isopolares organizados paralelamente, porém devido ao pouco material encontrado, não foi possível a identificação da espécie. A partir do material disponível, o morfotipo não se assemelha a nenhuma das espécies conhecidas para o gênero, porém mais indivíduos devem ser observados para a confirmação ou não de uma nova espécie.

Hábitat: epífita em briófita crescendo sobre tronco seco em mangue, exposto ao sol.

Material examinado: BRASIL, São Paulo, Parque Estadual da Ilha do Cardoso, 30/06/2010, *Watson Gama-Júnior, Camila Malone* (SP 428556).

Família Stigonemataceae

Stigonema informe Bornet & Flahault, Annales des Sciences Naturelles, Botanique 5: 75. 1886.

(Figura 12)

Filamentos crostosos 35-60 µm diâm., multisseriados, intensamente ramificados, afilados. Ramificações geralmente em um dos lados do filamento principal. Ápices unisseriados ou com longo hormogônio. Células arredondadas, dispostas em fileiras transversais ao filamento, 7-11 µm diâm. Bainha irregular, lamelada, hialina ou amarelada. Heterocitos arredondados, com as mesmas medidas das células.

Hábitat: Rocha no interior de mata.

Material examinado: BRASIL, São Paulo, Parque Estadual da Serra do Mar – Núcleo Santa Virgínia, 23/02/2010, *Watson Gama-Júnior, Ewerton Manarin* (SP 428558).

Stigonema cf. ***mamilosum*** Bornet & Flahault, Annales des Sciences Naturelles, Botanique 5: 62. 1886.

(Figura 13)

Filamentos crostosos, multisseriados, 29-61 µm diâm, intensamente ramificados, ápices arredondados, às vezes levemente atenuados. Ramificações dos dois lados do filamento principal. Ápices com células apicais amareladas e hemisféricas. Células arredondadas, 8-14 µm diâm. Bainha homogênea, amarelada. Heterocitos arredondados, mesmas medidas das células.

O morfotipo está de acordo com a descrição de *Stigonema mamilosum*, porém difere ligeiramente pela ecologia, forma dos ápices e medidas menores. De acordo com Geitler (1932), *S. mamilosum* é típico de regiões temperadas, apresenta ápices unisseriados atenuados e filamentos com diâmetro 65-90 µm diâm. O nosso material é tropical, apresenta medidas menores e os ápices dos filamentos são apenas eventualmente ramificados. Apesar dessas diferenças, ainda não é possível separar os dois morfotipos até que estudos filogenéticos sejam realizados.

Hábitat: sobre rocha eventualmente submersa em uma queda d'água.

Material examinado: BRASIL, São Paulo, Parque Estadual da Serra do Mar – Núcleo Santa Virgínia, 23/02/2010, *Watson Gama-Júnior, Ewerton Manarin* (SP 401418).

Stigonema sp1

(Figuras 14, 15)

Filamentos crostosos 18-28 µm diâm., predominantemente bisseriados, inicialmente unisseriados, cilíndricos, às vezes levemente atenuados ou alargados nos ápices Ramificações geralmente em um dos lados do filamento principal, às vezes atenuadas na base. Ápices geralmente terminando em uma célula hemisférica. Células esféricas, dispostas diagonalmente ao pares 6,7-14 µm diâm. Bainha amarelada, às vezes lamelada. Heterocitos arredondados ou hemisféricos, com as mesmas medidas das células vegetativas.

O morfotipo apresenta as características diagnósticas de *Stigonema*, apresentando filamentos ramificados e plurisseriados. Assemelha-se à *S. fremyi*, porém difere desta por apresentar no máximo duas séries de células, enquanto *S. fremyi* apresenta tipicamente três ou mais séries de células.

Hábitat: Sobre costão de terra.

Material examinado: BRASIL, São Paulo, Parque Estadual da Serra do Mar – Núcleo Santa Virgínia, 23/02/2010, Watson Gama-Júnior, Ewerton Manarin (SP 428561); Estação Ecológica Juréia-Itatins, 16/08/2011, *Guilherme Hentschke, Célia Sant'Anna, Watson Gama-Júnior, Camila Malone* (SP 428564).

Pulvinularia suecica Borzi, Nuovo Giorn. Bot. Ital., ser. 2, 23: 574. 1916.

(Figuras 16, 17)

Indivíduos prostrados, formados por filamentos dispostos radialmente com ramificações pseudodicotômicas, 6-8 µm larg. Tricomas unisseriados ou raramente bisseriados, constritos, 4,1-6 µm larg. Células irregularmente arredondadas, quadráticas ou mais curtas que largas 3-5,5 µm comp. Bainha hialina e homogênea. Heterocitos não encontrados.

Hábitat: Sobre briófita em rocha.

Material examinado: BRASIL, São Paulo, Parque Estadual da Serra do Mar – Núcleo Santa Virgínia, 23/02/2010, *Watson Gama-Júnior, Ewerton Manarin* (SP 428557).

Família Nostocaceae

Nostoc commune Bornet & Flahault, Annales des Sciences Naturelles, Botanique 7: 181. 1886.

(Figura 18)

Colônias mucilaginosas, macroscópicas, verde escuras, formadas por tricomas dispersos em ampla mucilagem. Tricomas tortuosos, moniliformes, intensamente emaranhados, 4,2-6,5 µm larg.. Células em forma de barril 4-6 µm comp., com conteúdo celular verde-oliva. Heterocitos intercalares ou terminais, esféricos ou levemente alongados, do mesmo tamanho ou ligeiramente maiores que as células vegetativas.

Hábitat: Solo (terra e britas) exposto ao sol, grande massa perto de área de mata densa.

Material examinado: BRASIL, São Paulo, Parque Estadual da Serra do Mar – Núcleo Santa Virgínia, 23/02/2010, *Watson Gama-Júnior, Ewerton Manarin* (SP 428555).

Família Rivulariaceae

Dichotrhix orsiniana Bornet & Flahault, Ann. Sci. Nat. Bot., ser. 7, 3: 376. 1886.

(Figuras 19, 20)

Filamentos heteropolares, 8-13 µm diâm., formando tufos presos ao substrato pela base, ramificações falsas, isoladas, frequentes. Tricomas atenuados nos ápices, constritos, 2,8-6 µm larg. Células quadráticas ou mais largas que longas, 2,7-4,8 µm comp., tornando-se alongadas e hialinas nas terminações em forma de pelo. Bainha abundante, amarelada, fortemente lamelada. Heterocitos cônicos, 3-4,5 µm comp. 4-5,5 µm larg., sempre basais.

Hábitat: Solo (terra) no interior de mata, rochas eventualmente submersas em uma queda d'água.

Material examinado: BRASIL, São Paulo, Parque Estadual da Serra do Mar – Núcleo Santa Virgínia, 23/02/2010, *Watson Gama-Júnior, Ewerton Manarin* (SP 428559, 401418, 428551, 428560).

***Calothrix* sp.**

(Figuras 21, 22)

Filamentos alargados ou não na base, 10-13,6 µm larg, evidentemente longos, afilados, formando pequenos tufos, comumente ramificados. Tricomas constritos, 7-9,2 µm larg na base. Células mais curtas do que largas, 3,5-4,5 µm comp, tornando-se mais alongadas em direção aos ápices hialinos e fortemente atenuados, conteúdo celular verde-oliva. Bainha hialina ou amarelada, lamelada. Heterocitos basais cônicos ou hemisféricos; intercalares quadráticos, 4-5 µm comp., 7-9 µm larg.

Este morfotipo apresenta todas as características de *Calothrix* e difere de qualquer espécie conhecida do gênero devido aos seus filamentos significativamente longos, semelhantes aos de *Scytonema*, porém são heteropolares e afilados com estrutura em forma de pelo nos ápices. A única espécie de *Calothrix* com filamentos

equivalentemente longos é *C. gloeocola* Skuja, porém esta vive na mucilagem de outras cianobactérias enquanto o material estudado é rupícola. Provavelmente este material constitua uma nova espécie.

Habitat: sobre rochas em água corrente, próximas à borda de mata.

Material examinado: BRASIL, São Paulo, Estação Ecológica da Juréia-Itatins, 16/08/2011, *Guilherme Hentschke, Célia Sant'Anna, Watson Gama-Júnior, Camila Malone* (SP 428563).

Família Michrochaetaceae

***Michrochaete* sp1**

(Figura 23)

Filamentos curtos, tortuosos, 14,9-21,8 µm larg. Tricomas levemente constritos, 9,4-10,8 µm larg. Células mais curtas que largas, 3-5,7 µm comp., conteúdo celular granuloso, verde-azulado. Bainha com lamelas divergentes, espessa, amarelada. Heterocitos mais curtos que largos, 5,1-6,3 µm comp., 9,4-11,5 µm larg.

O morfotipo possui as características diagnósticas de *Michrochaete* Born. & Flah. por apresentar indivíduos isolados, heteropolares e raramente ramificados. Porém, difere de qualquer espécie do gênero pelas características da bainha. As espécies de *Michrochaete* apresentam bainha geralmente fina, hialina e às vezes lamelada paralelamente. Nosso material apresenta bainha muito espessa, amarelada e com lamelas divergentes, constituindo provavelmente uma nova espécie. A bainha espessa e colorida provavelmente proporciona maior proteção à dessecação e fotoinibição em maré baixa.

Hábitat: Casca de árvore em mangue, submersa quando a maré sobe.

Material examinado: BRASIL, São Paulo, Parque Estadual da Ilha do Cardoso, 30/06/2010, *Watson Gama-Júnior, Camila Malone* (SP 428552).

***Michrochaete* sp2**

(Figura 24)

Filamentos curtos, tortuosos, 10,2-12,4 µm larg. Tricomas constritos, afilados ou não em direção aos ápices, 8-10 µm larg. Células mais curtas que largas, 3-8 µm comp., conteúdo celular homogêneo, verde escuro. Bainha irregularmente lamelada, hialina, com estria mediana longitudinal conspícua. Heterocitos quadráticos, 7,3-8,4 µm comp., 9,2-10,2 µm larg.

O morfotipo possui as características diagnósticas de *Michrochaete* por apresentar indivíduos isolados, heteropolares e raramente ramificados, porém difere de qualquer espécie do gênero de acordo com as características da bainha. Não há nenhuma espécie para o gênero que apresente a estria longitudinal descrita para este morfotipo, que provavelmente constitui uma nova espécie.

Hábitat: Tronco de árvore em mata densa, úmido e sombreado.

Material examinado: BRASIL, São Paulo, Parque Estadual da Ilha do Cardoso, 30/06/2010, *Watson Gama-Júnior, Camila Malone* (SP 428553).

Tolypothrix byssoidea (Berk.) Kirchner, Krypt-Fl. Schles: 80. 1878.

(Figuras 25, 26)

Filamentos heteropolares, 9,2-15 µm larg., tortuosos, às vezes emaranhados, comumente ramificados. Ramificações falsas isoladas ou raramente duplas. Tricomas levemente constritos, cilíndricos 7,4-12,3 µm larg. Células mais largas que longas, conteúdo verde-oliva, 3,8-9,3 µm comp. Bainha hialina, homogênea ou levemente lamelada. Heterocitos quadráticos, mesmas medidas das células vegetativas.

Habitat: sobre árvore

Material examinado: BRASIL, São Paulo, Parque Estadual da Ilha do Cardoso, 30/06/2010, *Watson Gama-Júnior, Camila Malone* (SP 401444).

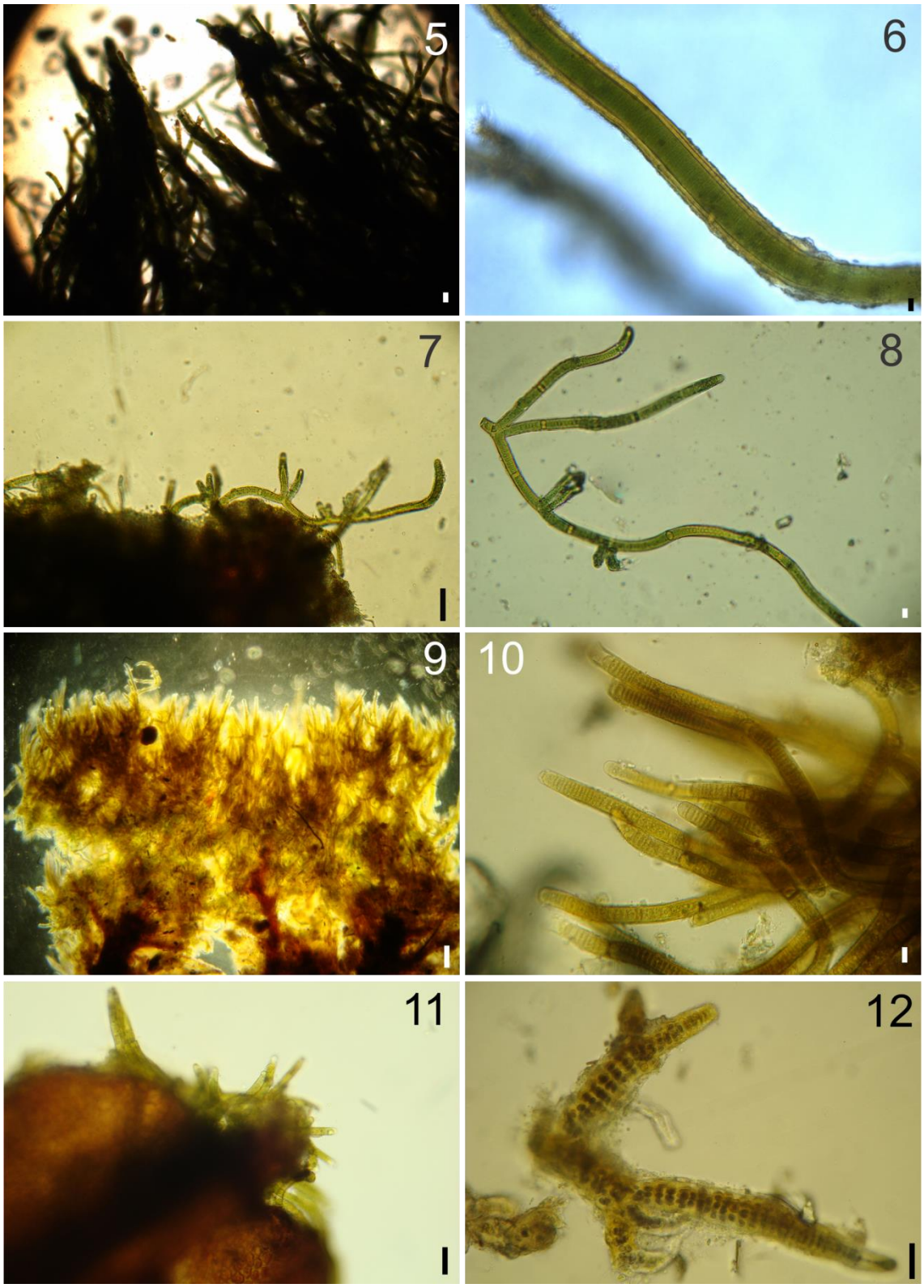

Figura 5-12. 5. Talo fasciculado em *Scytonema siculum*; **6.** Detalhe do fila mento de *S. siculum*; **7.** Talo de *S. guyanense* como epífita de briófita; **8.** Detalhe do filamento de *S. guyanense*; **9.** Filamentos paralelos de *Brasilonema epidendron*; **10.** Detalhe de filamentos de *B. epidendron*; **11.** Talo fasciculado de *Brasilonema* sp. como epífita de briófita; **12.** Detalhe de filamento de *Stigonema informe*. Escalas: 5, 7, 9, 11, 12 = 50 µm; 6,8,10 = 10 µm.

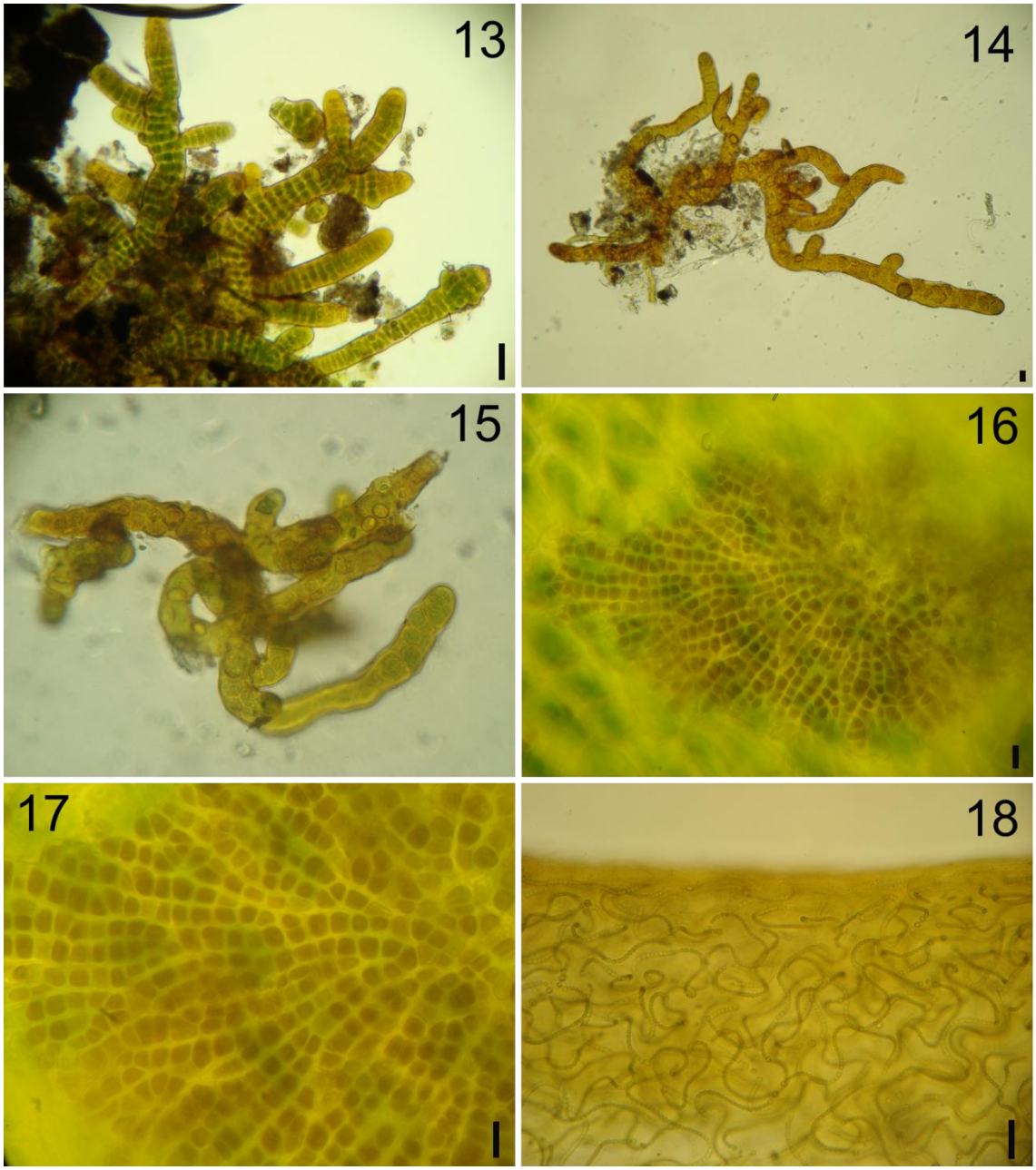

Figura 13 -18. 13. Talo de *Stigonema* cf. *mamilosum*; **14, 15.** Filamentos uni ou bisseriados de *Stigonema* sp.; **16, 17.** Talo de *Pulvinularia suecica* como epífita de briófita; **18.** Detalhe de filamentos imersos na mucilagem de *Nostoc commune*. Escalas: 13, 18 = 50 µm; 14, 15, 16, 17 = 10 µm.

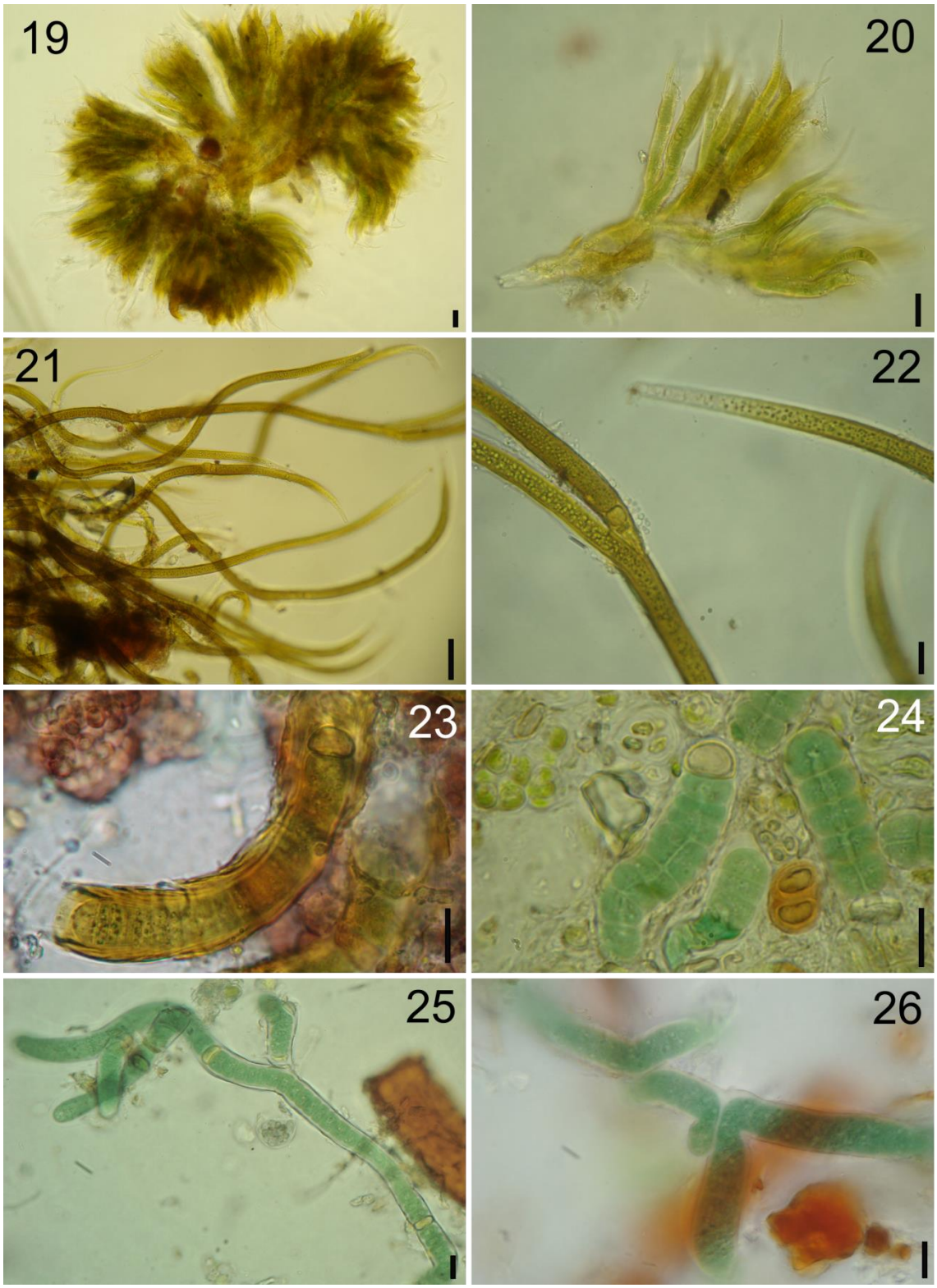

Figura 19-26. 19, 20. Talos ramificados de *Dichothrix orsiniana*; **21.** Ápices de filamentos de *Calothrix* sp. **22.** Ramificação de *Calothrix* sp.; **23.** Filamento de *Michrochaete* sp1 com bainha evidente; **24.** Filamento de *Michrochaete* sp2 com estria mediana longitudinal na bainha; **25.** Filamento de *Tolypothrix byssoidea* com ramificações simples; **26.** Filamento de *T. byssoidea* com ramificação dupla. Escalas: 19, 21 = 50 µm; 20, 22-26 = 10 µm.

3.4.2. Prospecção de substâncias com atividades anticolinesterásica, antifúngica e antioxidante

3.4.2.1. Atividade Anticolinesterásica

Em todas as cepas foram detectadas substâncias com atividade anticolinesterásica, em pelo menos, um de seus extratos (tabela 2). As Cianobactérias sintetizam uma quantidade expressiva de substâncias ativas, tóxicas ou com propriedades terapêuticas (Botos & Swolodawer 2003, Garrido *et al.* 2007), porém grande número desses compostos apresenta atividade antienzimática, dos quais são exemplos as microcistinas e a anatoxina-a(S), potentes inibidores das fosfatases 1 e 2A e da acetilcolinesterase, respectivamente (Apeldoorn *et al.* 2007). As substâncias com ação anticolinesterásica devem sua particular importância ao fato de pertencerem ao grupo de potenciais fármacos, passíveis de serem empregados no tratamento da Doença de Alzheimer, aumentando os níveis de acetilcolina no cérebro (Yánez & Viña 2013). Essa condição neurodegenerativa tem tratamento sintomático com a restauração da função colinérgica, pela inibição da acetilcolinesterase (Trevisan *et al.* 2003, Khoobi *et al.* 2013).

3.4.2.2. Atividade antifúngica (frente ao fungo *Colletotrichum lagenarium*)

A atividade antifúgica foi detectada em seis das cepas testadas (Tabela 2). A importância do descobrimento de novos fungitóxicos está relacionada à alta taxa de mutação a que estão sujeitos os fungos e ao consequente aumento da resistência que apresentam aos antifúngicos em uso.

Esses extratos ativos serão futuramente submetidos a fracionamento para isolamento e caracterização estrutural dos bioativos, estudo detalhado de seu modo de ação e de sua viabilidade econômica.

3.4.2.3. Atividade antioxidante

Nenhum dos extratos apresentou atividade antioxidante, em ensaios realizados segundo (Hostettmann *et al.* 2003) (tabela 2).

Para complementar estes ensaios, os extratos ativos foram submetidos à pesquisa de fenóis, por Cromatografia em Camada Delgada (CCD) (Brito *et al.* (2001). Substâncias fenólicas ativas são comuns em cianobactérias (Singh *et al.* 2011, Dixit & Suseela 2013), porém esta classe de compostos não foi detectada nos extratos cianobacterianos em questão.

Tabela 2. Compilação dos dados obtidos na prospecção de substâncias com atividades anticolinesterásica, antifúngica e antioxidante, nos extratos das cepas em estudo

Cepa	Gênero	Solvente	Anticolinesterásica	Antifúngica	Antioxidante
3307	*Komarekiella*	MeOH	Positivo	Negativo	Negativo
		HAc	-----	Negativo	Negativo
3481	*Komarekiella*	MeOH	Negativo	Negativo	Negativo
		HAc	Positivo	Negativo	Negativo
3487	*Komarekiella*	MeOH	--------	Negativo	Negativo
		HAc	Positivo	Negativo	Negativo
3551	*Brasilonema*	MeOH	Negativo	Positivo	Negativo
		HAc	Positivo	Negativo	Negativo
3463	*Brasilonema*	MeOH	Negativo	Positivo	Negativo
		HAc	Positivo	Negativo	Negativo
3484	*Nostoc*-like	MeOH	-------	Negativo	Negativo
		HAc	Positivo	Negativo	Negativo
3482	*Nostoc*-like	MeOH	Negativo	Positivo	Negativo
		HAc	Positivo	Negativo	Negativo
3485	*Nostoc commune*	MeOH	-------	Negativo	Negativo
		HAc	Positivo	Negativo	Negativo
3320	*Calothrix*	MeOH	Positivo	Negativo	Negativo
		HAc	Positivo	Positivo	Negativo
3319	*Calothrix*	MeOH	Positivo	Negativo	Negativo
		HAc	Negativo	Negativo	Negativo
3321	*Tolypothrix*	MeOH	Positivo	Negativo	Negativo
		HAc	Positivo	Positivo	Negativo
3318	*Dapisostemon*	MeOH	-----	-----	-----
		HAc	Positivo	Positivo	Negativo

----- = não realizado.

3.4.2.4. Prospecção de cianotoxinas conhecidas (microcistinas, saxitoxinas, anatoxina-a e β-metil-amino-alanina) nos extratos em ácido acético 0,1 M, das cepas em análise

No estudo realizado por Cromatografia Planar, não foram detectadas as cianotoxinas conhecidas, que possuem de efeito agudo, nas 12 cepas estudadas; entretanto, 09 dessas cepas (tabela 3) apresentaram resultado positivo, quanto à presença do aminoácido β-metilaminoalanina, toxina de efeito crônico neurodegenerativo, presente em muitos gêneros de cianobactérias (Cox *et al.* 2005). Essa substância que está sendo relacionada à Esclerose Lateral Amiotrófica, Doença de Parkinson e de Alzheimer (Bannack *et al.* 2007, Bhattacharjee & Lukiw 2013), acumula-se em organismos, sendo transferida pela cadeia alimentar (Cox *et al.* 2003). A frequente presença de BMAA nos extratos das linhagens estudadas alerta para necessidade de mais estudos sobre a concentração e os efeitos dessa substância nos organismos, devido a sua importância na saúde pública.

Tabela 3. Resultados dos testes cromatográficos para a detecção de cianotoxinas conhecidas, por Cromatografia em Camada Delgada.

Cepa	Gênero	Extrato	Microcistinas	Saxitoxinas	Anatoxina-a	BMAA
3307	*Komarekiella*	MeOH	----	----	----	-----
		HAc	Negativo	Negativo	Negativo	Positivo
3481	*Komarekiella*	MeOH	----	----	----	-----
		HAc	Negativo	Negativo	Negativo	Negativo
3487	*Komarekiella*	MeOH	----	----	----	-------
		HAc	Negativo	Negativo	Negativo	Positivo
3551	*Brasilonema*	MeOH	----	----	----	-----
		HAc	Negativo	Negativo	Negativo	Positivo
3463	*Brasilonema*	MeOH	----	----	----	------
		HAc	Negativo	Negativo	Negativo	Positivo
3484	*Nostoc*-like	MeOH	----	----	----	-------
		HAc	Negativo	Negativo	Negativo	Positivo
3482	*Nostoc*-like	MeOH	----	----	----	------
		HAc	Negativo	Negativo	Negativo	Positivo
3485	*Nostoc commune*	MeOH	----	----	----	-----
		HAc	Negativo	Negativo	Negativo	Negativo
3320	*Calothrix*	MeOH	----	----	----	------
		HAc	Negativo	Negativo	Negativo	Positivo
3319	*Calothrix*	MeOH	----	----	----	-----
		HAc	Negativo	Negativo	Negativo	Positivo
3321	*Tolypothrix*	MeOH	----	----	----	-----
		HAc	Negativo	Negativo	Negativo	Positivo
3318	*Dapisostemon*	MeOH	----	----	----	-----
		HAc	Negativo	Negativo	Negativo	Positivo

3.4.2.5. Ensaio de toxicidade aguda (i.p.), em camundongos

Para a pesquisa de toxinas desconhecidas, foram realizados bioensaios em camundongo (n=3) com extratos selecionados entre os que apresentaram resultados negativos para a presença de cianotoxinas conhecidas (pesquisa por Cromatografia Planar) (tabela 3). As respostas fisiológicas observadas, o tempo decorrido até o óbito e as alterações macroscópicas anotadas em exame post-mortem estão na Tabela 4.

Tabela 4. Compilação das respostas fisiológicas observadas, tempo decorrido até o óbito e alterações macroscópicas apresentadas pelos camundongos após administração i.p. dos extratos das cepas em estudo

Cepa	Extrato	Sinais de intoxicação	Tempo decorrido até a morte	Principais achados post-mortem
Nostoc like CCIBt 3282	MeOH	Contração abdominal, rubor, perda de reflexos, ptose palpebral, andar cambaleante.	Eutanásia após 07 dias da administração.	Sem alterações macroscópicas visíveis.
	HOAc	Não submetido a teste.	---	---
Komarekiella CCIBt 3307	MeOH	Contração abdominal, rubor, perda de reflexos, ptose palpebral, andar cambaleante.	Eutanásia após 07 dias da administração.	Sem alterações macroscópicas visíveis.
	HOAc	Não submetido a teste.	---	---
Dapisostemon sp. CCIBt 3318	MeOH	Não submetido a teste.	---	---
	HOAc	Ausência de sinais.	Eutanásia após 07 dias da administração.	Fígado com manchas brancas.
Calothrix CCIBt 3319	MeOH	Piloereção, ptose palpebral, contrações abdominais, paralisia do trem posterior.	Eutanásia após 07 dias da administração.	Sem alterações macroscópicas visíveis.
	HOAc	Ausência de sinais.	Eutanásia após 07 dias da administração.	Pulmões hemorrágicos.
Calothrix CCIBt 3320	MeOH	Contrações abdominais, contrações do dorso, coceira, piloereção, ptose palpebral, dispnéia.	Eutanásia após 07 dias da administração.	Manchas negras entre os lobos do fígado.
	HOAc	Contrações abdominais, piloereção, paralisia do trem posterior, desequilíbrio, dispnéia, prostração, dificuldade de locomoção.	Eutanásia após 07 dias da administração.	Manchas brancas no fígado.
Tolypothrix CCIBt 3321	MeOH	Agitação intensa, coceira, incontinência fecal, dificuldade de locomoção, edema escrotal.	Eutanásia após 07 dias da administração.	Sem alterações macroscópicas visíveis.
	HOAc	Diminuição de atividade, paralisia no trem posterior, contração abdominal, dispnéia.	Eutanásia após 07 dias da administração.	Sem alterações macroscópicas visíveis.
Brasilonema	MeOH	Dispneia, diminuição de atividade.	Morte após 24 horas (n=1), da	Pulmões hemorrágicos.

Cepa	Extrato	Sinais de intoxicação	Tempo decorrido até a morte	Principais achados post-mortem
CCIBt 3463			administração.	
	HOAc	Diminuição de atividade.	Morte após 24 horas (n=3), da administração.	Pulmões hemorrágicos.
Komarekiella CCIBt 3481	MeOH	Diminuição de atividade, dispneia.	Eutanásia após 07 dias da administração.	Lesões no fígado.
	HOAc	Não submetido a teste.	---------	---------
Nostoc-like CCIBt 3482	MeOH	Agitação intensa, cauda em Straube.	Eutanásia após 07 dias da administração.	Sem alterações macroscópicas visíveis.
	HOAc	Não submetido a teste.	---------	---------
Nostoc-like CCIBt 3482	MeOH	Agitação.	Eutanásia após 07 dias da administração.	Sem alterações macroscópicas visíveis.
	HOAc	Não submetido a teste.	---------	---------
Brasilonema CCIBt 3551	MeOH	Ausência de sinais.	Eutanásia após 07 dias da administração.	Figado com manchas e coloração alterada.
	HOAc	Micção intensa, cauda em Straube.	Eutanásia após 07 dias da administração.	Sem alterações macroscópicas visíveis.

A completa regressão dos sinais clínicos, nos animais que sobreviveram, ocorreu após 2 – 3 horas, da administração.

Apenas a cepa *Brasilonema* sp. CCIBt 3463 causou a morte dos animais. Nesta cepa não foram detectadas, por CCD, microcistinas, saxitoxinas ou anatoxina-a, cianotoxinas de efeito agudo. O tempo decorrido até a morte exclui a ação das toxinas neurotóxicas (saxitoxinas e anatoxinas), que levam o animal a óbito em poucos minutos e não causam lesões; a ausência de lesões no fígado, a presença de hemorragia nos pulmões e o tempo decorrido até o óbito (maior do que 24 horas) corroboram o resultado negativo do teste químico, para microcistinas.

Os sinais de intoxicação apresentados pelos animais, após a administração de ambos os extratos (dispnéia e diminuição de atividade), não são as respostas características à ação de substâncias anticolinesterásicas (Andrade Filho & Romano 2001, Nair & Hunter 2004).

O conjunto dessas observações permite considerar a presença de substância(s) com estrutura(s) e atividades ainda não determinadas, na cepa *Brasilonema* sp. CCIBt 3463.

As respostas apresentadas, pelos animais, à administração dos extratos das cepas CCIBt 3282, 3307 3319, 3320, 3321, que são dispnéia, ptose, contrações abdominais, incontinência fecal (manifestações muscarínicas), paralisia, piloereção, contrações musculares (manisfestações da superestimulação dos receptores nicotínicos) e dificuldade de caminhar (manifestação do Sistema Nervoso Central) estão relacionadas aos efeitos causados, em mamíferos, por substâncias com ação anticolinesterásica. Como esses efeitos foram transitórios, as substâncias ativas contidas nestes extratos ligaram-se, de maneira reversível, à enzima acetilcolinesterase. A reversibilidade desta reação é uma característica indispensável para que uma substância possa ser considerada para pesquisa de potenciais agentes terapêuticos a serem empregados no controle da Doença de Alzheimer (Nair & Hunter 2004, Yoon *et al.* 2008). Substâncias que se ligam irreversivelmente à enzima actilcolinesterase são consideradas tóxicas, pois levam à morte, caso da anatoxina-a(S) (Nair *et al*., 2004).

Os extratos das cepas *Nostoc*-like CCIBt 3482 e *Brasilonema* sp. CCIBt 3551 podem conter substâncias com ação sobre o Sistema Nervoso Central, uma vez que os animais, após administração, apresentaram sinais característicos de intoxicação por substâncias neurotóxicas que são agitação intensa e cauda em Straube (Andrade Filho & Romano, 2001).

Assim sendo, do total de cepas (12) estudadas quanto a atividades biológicas, 8,33% possui toxina de efeito agudo, 83,33% são potencialmente causadoras de efeitos neurodegenerativos, por produzirem BMAA, 100% têm atividade anticolinesterásica e 50% têm atividade antifúngica.

4. CONSIDERAÇÕES FINAIS

As cianobactérias heterocitadas terrestres mostraram-se bastante diversas nos ambientes estudados. Os estudos das populações amostradas, realizados através da taxonomia polifásica permitiram a redação de oito manuscritos contendo gêneros e espécies novas e um manuscrito com resultados provenientes dos estudos químicos. A partir dos resultados dos estudos químicos será possível futuramente isolar moléculas

com atividades biológicas e, além disso, outras atividades biológicas como anticâncer e antiviral poderão ser testadas.

Todos os táxons novos encontrados foram incluídos em artigos científicos. As populações identificadas apenas em nível genérico (não incluídas em manuscritos) são significativamente diferentes de quaisquer espécies conhecidas e provavelmente são espécies ou gêneros novos. Futuramente, tentativas serão feitas para os estudos desses táxons em nível molecular. Táxons identificados em nível espécifico e que não constituíam novos táxons não foram incluídos em artigos científicos, com exceção de *Streptostemon lutescens*, o qual foi sequenciado a partir de material da natureza. Até a realização deste projeto, o gênero *Streptostemon* era considerado um morfotipo e atualmente já está confirmado pelos estudos moleculares resultantes desta tese. As espécies não publicadas poderão ser utilizadas em artigos de flora das regiões estudadas ou de variabilidade morfológica.

Os estudos moleculares do gene 16S rRNA permitiram que novos táxons fossem publicados, mas também corroboraram com a possibilidade de que muitos dos táxons identificados nos bancos de dados moleculares estão mal identificados e que a utilização exclusiva deste marcador não é suficiente para solucionar as relações filogenéticas entre os gêneros de cianobactérias. Isso se deve principalmente ao fato de que a utilização de métodos diferentes de análises de populações leva a resultados igualmente diferentes em relação ao que se trata da mono- ou polifilia de gêneros como é o caso de *Calothrix* e *Tolypothrix* (Berrendero *et al.* 2011). De acordo com isso, são inevitáveis os questionamentos se a maioria dos gêneros seria realmente polifilética ou se os resultados filogenéticos do gene 16S por si só seriam definitivos e refletiriam a divergência evolutiva em nível genérico. No manuscrito "Current trends and perspectives for cyanobacterial taxonomy – are only cultured populations enough?" são questionados os métodos de publicação de novos táxons sem os cuidados necessários na avaliação morfológica das populações. Historicamente, além do fato de o gene 16S ser suficientemente conservado para estudos filogenéticos, um dos motivos da sua utilização para a taxonomia de cianobactérias é que as árvores geradas a partir de suas sequencias estariam de acordo com a separação tradicional dos gêneros. Com o decorrer do tempo esse cenário se inverteu e atualmente os mesmos gêneros estão sendo divididos de acordo com os estudos deste gene. Por isso, com a utilização restrita do gene 16S, atualmente há uma crise no reconhecimento de características morfológicas

diagnósticas de gêneros, sendo mais acentuada em espécies. A associação dos resultados de análises de características morfológicas como forma, largura e cor de bainhas mucilaginosas, frequência e tipos de ramificação e posição dos heterocitos são precariamente compatíveis com as análises do gene 16S. Neste cenário, futuramente seria interessante a utilização de mais genes para os estudos filogenéticos ao lado dos estudos morfológicos, ultraestruturais e bioquímicos para que as características diagnósticas de gêneros e espécies possam ser definidas com base em análises evolutivas. Os marcadores morfológicos devem ser considerados, porém ainda devem ser "testados" através de diferentes estudos para que sejam realmente consolidados. Outra forma de pensamento seria desconsiderar o sistema de gêneros e espécies adotando apenas linhagens de cianobactérias sem dar valor a aspectos morfológicos ou ecológicos, porém a contribuição disso para o conhecimento da evolução desses organismos seria reduzida.

Por fim esta tese contribui para o conhecimento e conservação da biodiversidade de cianobactérias em ambientes tropicais e subtropicais, através do descobrimento e descrição de novos táxons e dos estudos de atividades biológicas. No total, sete espécies novas e dois gêneros novos foram descritos e os estudos químicos revelaram alto potencial para estudos mais aprofundados, visto que todos os extratos de cianobactérias estudados apresentaram ao menos um tipo de atividade biológica. A alta frequência de atividades anticolinesterásica nos extratos de cianobactérias abre caminho para estudos no tratamento da doença de Alzheimer e Esclerose Lateral Amiotrófica. Além disso, a tese teve contribuição mundial através dos artigos publicados em revistas internacionais que apresentaram muitas informações sobre a morfologia, biologia molecular e filogenia das cianobactérias heterocitadas.

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Anexo I

Phenotype diversity and phylogeny of selected *Scytonema*–species (Cyanoprokaryota) from SE Brazil

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Abstract: Members of the genus *Scytonema* belong to prominent components of microflora of tropical and subtropical soils, but their diversity and taxonomic classification is still little known. Molecular analyses of isolated strains, but also the morphological and ecological examination of natural populations are important for the starting revisions. Here we present phenotype characteristics of fourteen morphotypes of the cyanobacterial genus *Scytonema* from the SE Brazil, mostly from the state São Paulo and from the Atlantic Rainforest (ecosystem of "Mata Atlântica"). The populations of studied *Scytonema* species are ecologically significant, as they hold the dominant position in the microvegetation communities of lateritic and forest soils and of stony substrates (less frequently also aquatic forms). Species commonly distributed throughout the tropical regions (such as *S. guyanense, S. javanicum, S. stuposum*), as well as the types with restricted geographic distribution in S America are included in the study. Two new species from a little known subgenus *Myochrotes,* in which the cultivation is difficult, are described: *S. papillicapitatum* Sant´Anna et Komárek and *S. chorae* Sant´Anna et Komárek. Our analyses support the principle of congruency of morphological characters with phylogenetic position of the studied species.

Key words: Cyanobacteria, *Scytonema*, taxonomy, new species, polyphasic approach, ecology, Brazil (SP)

Introduction

The statement that cyanobacteria have a cosmopolitan distribution is valid only for a few types from this group of organisms and most species have specific ecological demands according to our results. In consequence, cyanobacterial species typically occur in ecologically specific and often also geographically restricted habitats. The genus *Scytonema* is generally considered to be cosmopolitan, however, it contains numerous species, which grow only in tropical and ecologically distinctly delimited habitats, such as lateritic soils, dripping rocks and reservoirs with water vegetation. The knowledge of their morphological variability is important for their identification, but still little known, and their supposed restricted areas of distribution require thorough revision. We have found several *Scytonema* populations in the important global biodiversity hotspot area (cf. Myers et al. 2000) in the tropical/subtropical Atlantic Rainforest ("Mata Atlântica") during our studies of cyanobacteria in the last years in the state of São Paulo (SE Brazil). The observed populations formed a substantial component of the abundant subaerophytic microphyte vegetation of this ecosystem.

The traditional taxonomic description of the genus *Scytonema* was summarized by Geitler (1932), Desikachary (1959), Starmach (1966), Bourrelly (1970) and Komárek & Anagnostidis (1988), but the modern revision based on polyphasic approach is still in the beginning. As follows from the current molecular data, *Scytonema* is polyphyletic (similarly to other traditional cyanobacterial genera, see e.g., Boyer et al. 2002; Berrendero et al. 2008; Zapomělová et al. 2011, etc.) and it will evidently be divided into several generic entities (Bohunická et al. 2012). The genus sensu stricto has to be based on the type species *S. hofmannii* C. Agardh ex Bornet et Flahault, morphologically characterized by cylindrical trichomes along the whole length, more or less quadratic cells in the main trichomes, and relatively narrow sheaths (Komárek & Anagnostidis 1988). A conspicuous part of the genus, distinctly different in terms of morphology from the former typical group, is the subg. *Myochrotes,* which has, however, not yet been sequenced. This applies also to the genus *Petalonema,* sometimes also included into *Scytonema.* Both these taxa (genera) seem to be morphologically more related one to another than to

the typical *Scytonema* (comp. Table 3). *Brasilonema* is a recently described genus (FIORE et al. 2007) that is morphologically similar to *Scytonema*, but was clearly separated from *Scytonema* after polyphasic evaluation. Moreover, there are several other characteristic morphological groups within *Scytonema sensu lato* that can be separated by careful examination, and we comment these groups in the results of our study.

The modern cyanobacterial taxonomy is mainly based on the polyphasic approach (JOHANSEN & CASAMATTA 2005; KOMÁREK 2010), in which the molecular sequencing and phylogenetic comparisons belong to the basic techniques. Unfortunately, *Scytonema* species are among those cyanobacteria (together with numerous other genera) which frequently resist isolation into cultures. Thus, the transfer of our studied populations into monospecific cultures was sometimes difficult and we were able to support our taxonomic decisions by molecular sequencing only in few cases. Although the molecular evaluation of species within the genus *Scytonema* is apparently essential, we consider a good characterization of the variation in different morphological markers to be equally important for the overall biological knowledge of this group of cyanobacteria. Nowadays, the importance of cyanobacteria in the ecosystems is increasingly acknowledged and the correct identification of the dominant populations in common habitats (such as various types of soils, forests, lakes, reservoirs, etc.) is particularly desirable according to recognizable markers. Therefore, the knowledge of morphological variability of various eco– and morphotypes (and species) in natural populations is extremely beneficial.

In this study, we present a thorough phenotypic analysis of the dominant *Scytonema* populations from one of the most important hotspots of biodiversity of the world, Atlantic Rainforest in SE Brazil, supplemented with an evolutionary reconstruction showing phylogenetic position of some of the studied *Scytonema* species.

MATERIAL, LOCALITIES AND METHODS

The studied material was collected from different localities in São Paulo and Minas Gerais States, Brazil, mainly in Atlantic Rainforest (Table 1). Majority of the samples were collected from terrestrial habitats, where they grew on periodically or irregularly wetted surface; only few types originated from water biotopes. Details are described in the individual species. Terrestrial samples were collected scraping the substrate (soil, rock, concrete and wood), then kept in dried state in paper envelopes. The aquatic material was collected passing the plankton net among the macrophytes.

The morphological study was carried out using optical microscopes, type Leitz Dialux 22 and Olympus BX 51, equipped with Nomarski DIC and brightfield optics. The identification of taxa was done based on at least 20 specimens of each population. The species were documented by drawings and digital photographs. First subsample of each of the samples was preserved in formaldehyde and held in the Herbarium of the Institute of Botany (SP), Brazil (Table 1). In dried samples, the second subsample was placed into a liquid Z8 medium (KOTAI 1972) to recover from a dried state for 24 h. The *Scytonema* species were then isolated into unicyanobacterial clonal strains using dilution plating on agar–solidified Z8 medium. Cultures are maintained in Z8 agar slants in ambient light and temperature of 16 °C and are available at Culture Collection of Autotrophic Organism (CCALA), Institute of Botany of the AS CR, Třeboň, Czech Republic and Collection of the Botanical Institute, São Paulo, Brazil.

Molecular and phylogenetic analyses. Total genomic DNA of *Scytonema* strains was isolated from biomass dried over silica gel and pulverized using Retsch MM200 laboratory mill with wolfram carbide beads (3 minutes, 30.s^{-1}), following the modified xanthogenate–SDS buffer extraction protocol with addition of 3% PVPP and PEG–$MgCl_2$ precipitation (YILMAZ et al. 2009). Partial 16S rRNA gene and the adjacent ITS region were amplified following BOYER et al. (2001), using 10 ng of template DNA and 2×conc. Plain PP Master Mix (Top Bio, Czech Republic). The PCR products were cloned using the standard pGEM®–T Easy vector system, and sequenced on ABI PRISM 3130xl automated sequencer with plasmid primers T7f (5′–TAA TAC GAC TCA CTA TAG GG–3′) and SP6r (5′–TAT TTA GGT GAC ACT ATA G–3′). Obtained sequences were aligned by MAFFT v. 6 (KATOH et al. 2009) together with 141 OTUs derived from GenBank, representing the current variability of the family Scytonemataceae and the whole group of heterocytous cyanobacteria, and two outgroup taxa (*Gloeobacter violaceus* PCC 7421 and *Chroococcidiopsis thermalis* PCC 7203). The resulting alignment was corrected manually to remove ambiguous gap regions. The final phylogenetic tree was constructed by the ML method via PhyML v. 3.0 (GUINDON et al. 2010) run on the MetaCentrum computer cluster (www.metacentrum.cz), using the generalized time–reversible (GTR) substitution model with discrete gamma distribution in six categories. The gamma shape parameter α as well as the proportion of invariable sites were estimated from the data set. The GTR + Γ + I model was chosen by Modeltest 3.7 (POSADA & CRANDALL 1998) using Akaike information criterion (AIC). One thousand bootstrap replicates were executed to evaluate the relative support of branches. A maximum parsimony (MP) analysis was run using the same alignment as previously: one hundred replicate searches with starting tree obtained by random stepwise addition were performed using the tree bisection–reconnection (TBR) branch swapping algorithm in TNT v. 1.1. (GOLOBOFF et al. 2008); one thousand nonparametric bootstrap replications were run with default settings to evaluate the relative branch support. All bases and base changes were weighted equally, and gaps were coded as missing data. A neighbor–joining analysis using HKY model upon default parameters with 1000 bootstrap replicates was run via SeaView v. 4 (GOUY et al. 2010). Phylogenetic trees were drawn and edited using FigTree v. 1.3. (http://tree.bio.ed.ac.uk/software/figtree/). The sequences of strains CCIBt were deposited in NCBI GenBank under accession numbers KC682101–KC682103 and CCALA strains under accession numbers HF911525–HF911528.

Table 1. Sampling localities, coordinates and herbarium numbers of the examined material of *Scytonema*-species from SE Brazil; (N.A.) not available.

Locality	Coordinates	Date	Species	Herbarium number
Cantareira Ridge (Horto Florestal)	23°27'35''S 46°37'52''W	10/1996	*S. guyanense*	SP 427934
			S. javanicum	
			S. ocellatum	
			S. chorae	
Campos do Jordão Municipality (Horto Florestal)		11/2002	*S. guyanense*	N.A.
	22°50'06''S 45°37'18'W	11/2002	*S. javanicum*	SP427509
	22°58'30''S 45°38'44''W	11/2002	*S. ocellatum*	SP 427507 SP 427933
			Scytonema sp.	SP 427935
			S. cf. *bohneri*	
			S. guyanense	SP 427938
Ecological Station Juréia-Itatins	24°31'48''S 47°11'27''W	09/2002	*S. guyanense*	N.A.
			S. hyalinum	N.A.
			S. cf. *longiarticulatum*	SP 427936
Ubatuba Municipality (Gruta que Chora)	23°24'05''S 45°0'07''W	01/1986	*S. chorae*	SP 188482
		11/2002	*S. javanicum*	SP 427934
		11/2004	*S. crispum*	N.A.
Onda Verde (Santa Irene Stream)	20°38'40"S 49°18'29"W	11/2002	*S. guyanense*	SP 427941
Ecological Station of Paranapiacaba	23°46'43''S 46°18'18''W	11/2004	*S. hyalinum*	SP 427939
			S. cf. *bohneri*	
		11/2004	*S. stuposum*	N.A.
Mogi Guaçu Municipality (Jacaré Lake)	22°22'15''S 46°56'16''W	06/2002	*S. papillicapitatum*	SP 188495
Viçosa Municipality	20°45'26''S 42°52'30''W	06/2004	*S. arcangelii*	SP 427940
Bertioga Municipality	23°48'12''S 46°08'81''W	11/2002	*S. schmidtii*	SP 371429
State Park of Ilha do Cardoso	25°04'08"S 47°55'88"W	06/2010	*S. schmidtii*	SP 401438
			S. hyalinum	SP 427937
		06/1989	*S. chorae*	SP 187567 SP 238953 SP 238956 SP 238973
		04/1988		SP 238978
Nucleous Santa Virginia	23°20'35"S 45°08'17"W	02/2010	*S.* cf. *bohneri*	SP 401449

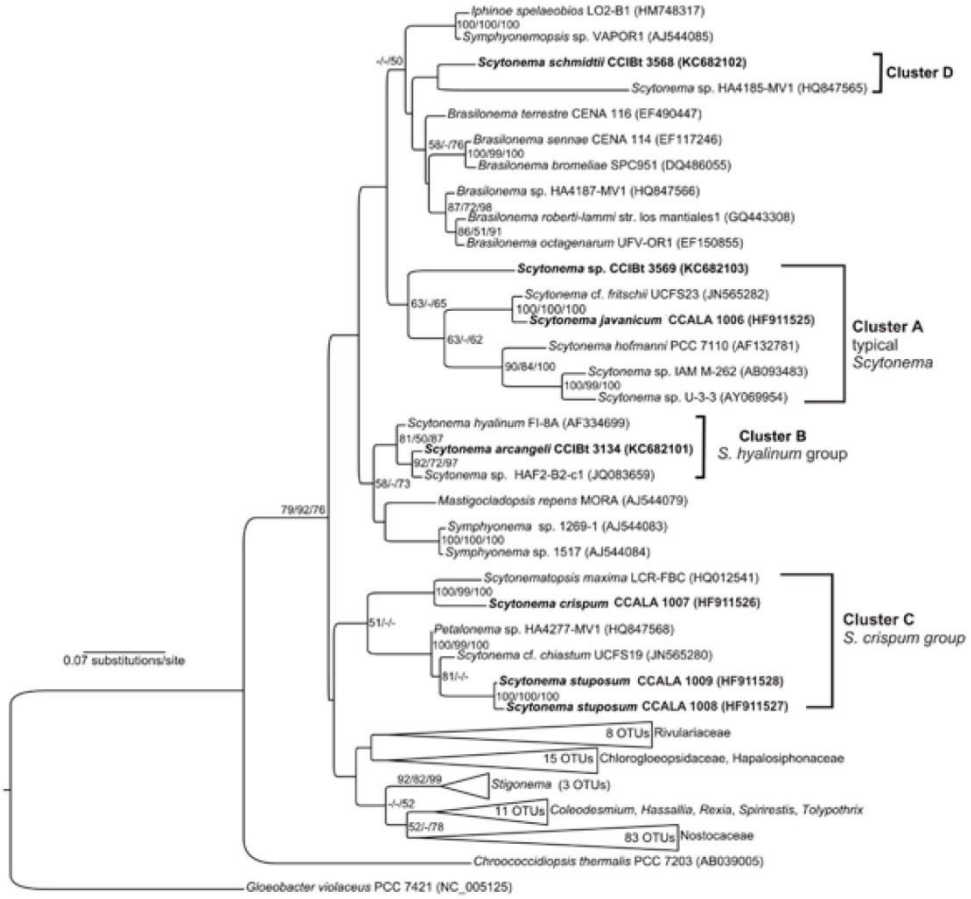


Fig. 1. Maximum likelihood (ML) phylogenetic tree inferred from 148 sequences (partial 16S rDNA) representing the current GenBank data on heterocytous cyanobacteria, focusing on the family Scytonemataceae. Bootstrap values (1000 replicates for ML, maximum parsimony – MP, and neighbour–joining – NJ) equal to 50 or higher are given at the nodes in this form: ML/MP/NJ. Our original sequences are printed in bold font. In the tree, strains of the genus *Scytonema* formed four separate clusters (A–D) correspoding to the respective morphological groups A–D described in the text.

RESULTS

We have analysed 28 populations from natural samples (mainly from aerophytic habitats), and also 7 isolated strains. From this material, we have recognized 13 *Scytonema*–taxa on the specific level according to the phenotype criteria, presented in Tables 2, 3. Characters of Brazilian specimens are described in the paragraph designated "Description".

Phylogenetic analysis

The final alignment of the 16S rRNA gene was 1320 bp long. Within the alignment, 750 sites (56.8%) were conservative, and 336 sites (25.5%) were parsimony informative. A single tree (–lnL = 18728.37) was recovered in the ML analysis, while 288 most parsimonous trees (l = 3,313) were collapsed into a strict consensus tree. In all trees obtained, included sequences of the family Scytonemataceae formed four lineages (Fig. 1): (A) the cluster including the type species *S. hofmanni*, *Scytonema* sp. CCIBt 3569 and *S. javanicum* CCALA 1006 from Brazil; (B) the group of *S. arcangelii* (Brazil) and *S. hyalinum*; (C) a separate lineage of three *Scytonema* isolates from Brazil (*S. crispum* CCALA 1007, *S. stuposum* CCALA 1008 and CCALA 1009) together with possible *Petalonema* sp. from Hawaii, *S.* cf. *chiastum* and *Scytonematopsis maxima* (both from the New Zealand); (D) the cluster consisiting of *Scytonema schmidtii* CCIBt 3568 from Brazil and *Scytonema* sp. isolated from a wet wall in Hawaii – this group forms a sister clade to *Brasilonema* in our tree. Other genera and families (Rivulariaceae, Hapalosiphonaceae, Nostocaceae, etc.) clustered separately from the family Scytonemataceae,

with exception of several representatives of true–branching genera, which were closely related to *Brasilonema* (*Symphyonemopsis*, *Iphinoe*) and the *S. hyalinum* lineage (*Mastigocladopsis*, *Symphyonema*). The results consistently showed the traditional family Scytonemataceae and the genus *Scytonema* itself to be polyphyletic.

In the following text, we categorized all observed populations from SP into five groups corresponding to the phylogenetic clusters A–D and the cluster "*Myochrotes*" (of which we have no strains; the main morphological features of all clusters are summarized also in Table 3). Where available, these groups are based on a combination of phylogenetic clustering and morphological markers; species that could not be sequenced were classified to the individual groups based solely on their morphological similarity. Group A involves part of *Scytonema*–taxa (*S. guyanense, S. javanicum*), which could be assigned to the typical subgenus *Scytonema* (morphologically and genetically close to *S. hofmannii*, Fig. 1, A). Group B is another special cluster, including species similar to *S. hyalinum* (*S. arcangelii*, *S. ocellatum*), as follows from the combined morphological and phylogenetic analyses (Fig. 1B). Group C is the next phylogenetically separated cluster of *Scytonema*–like morphotypes (Fig. 1C), containing species with more or less cylindrical filaments, relatively thick sheaths and constricted cylindrical trichomes with cells always distinctly shorter than wide (cluster of *S. crispum*, *S. stuposum* and *S. bohneri*). One of the sequenced strains, *Scytonema schmidtii* CCIBt 3568 (Fig. 1D), seems to cluster separately from all remaining members of the genus (group D). All four mentioned *Scytonema*–clusters (and corresponding morphotypes) are recognizable in our samples. Remaining three populations corresponding to the subgenus *Myochrotes* are not in the phylogenetic tree, since no representative strain was isolated. The members of the genus *Brasilonema* from SP region were described recently in papers of Fiore et al. (2007) and Sant´Anna et al. (2011). We have not studied populations morphologically corresponding to the traditional genus *Petalonema.*

Morphological descriptions

Group A: Subg. *Scytonema*

1. *Scytonema guyanense* [Montagne] Bornet et Flahault 1887 (Fig. 2)

One of the most common tropical *Scytonema*–species, widely distributed and probably with pantropical distribution. There are also a few records from the temperate zone, but usually lacking satisfactory documentation; all these data are problematic and should be revised. The substantial variability is connected with the wide distribution and existence of several subspecific taxa. The correct area of distribution of this species and the variability of the genotype could be solved only by detailed molecular analyses of populations from different tropical regions with simultaneous morphological examination. – Characteristic species of the soil surface (common on wet lateritic soils), less frequently occurs on other subaerophytic substrates (stones, rocks and wood). – Quite common in Brazil. We have studied seven, morphologically almost identical populations from different localities near Cantareira, from other different places of Mata Atlântica, and from the vicinity of Rio Preto (Santa Maria) in the NW of the SP State. All our populations possessed very similar morphology.

Description of our material: Mats on wet soils and rocks, common. Filaments slightly flexuous and entangled, cylindrical, with rarely solitary, mostly binary false branches, which soon divaricate one from another, 14 – 21 μm wide. Sheaths firm, relatively thin or slightly thickened (especially in old filaments), laminated, yellow–brown. Trichomes cylindrical and more or less of the same width along the whole length, not or slightly constricted at cross walls, cylindrical up to the ends, (9)11–13(15) μm wide. Cells in trichomes cylindrical and ± isodiametric or slightly longer or shorter than wide, but towards the ends clearly shortened and forming terminal meristematic zones. Hormogonia composed of 3–12 cells, liberate from the end of filaments often in series. Heterocytes intercalary, usually solitary, hemispherical, short barrel–shaped, up to cylindrical and up to 2× longer than wide, 8–24 × 11–15 μm.

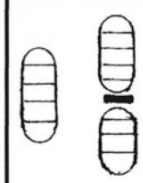
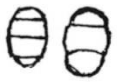
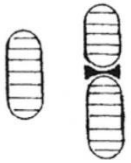
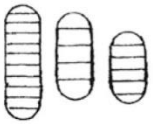
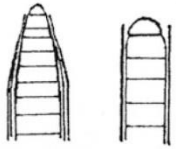
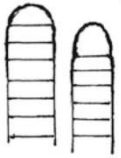
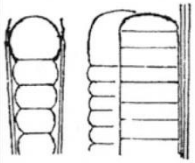
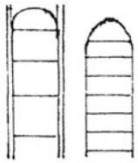
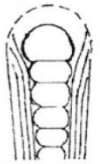
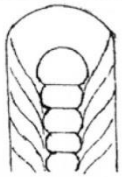
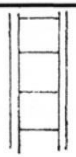
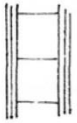
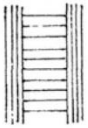
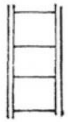
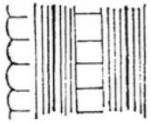
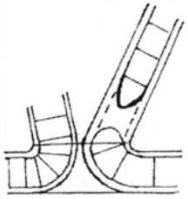
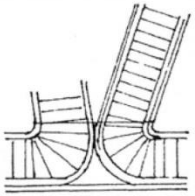
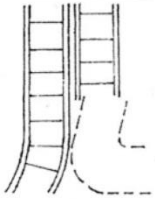
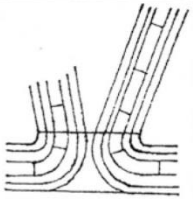
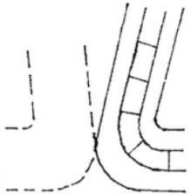
2. *Scytonema javanicum* [Kützing] Bornet et Thuret ex Bornet et Flahault 1887 (Fig. 3)

Species with wide geographic distribution, but known mainly from tropical areas (pantropical distribution). It is sometimes recorded also from temperate zones, but the identity of such various populations from climatically very distant regions was never confirmed and should be studied in future. The records from the temperate regions are also distinctly scarcer (Europe, Japan, N America, temperate S America) and often relate to glasshouses. The species has colorless up to yellow–brown, not distinctly lamellate sheaths, and characteristic, shortly connected bases of branches (parallely arranged). – *S. javanicum* is a typical soil species, inhabiting mainly wet lateritic soils, but occurs also on leaves and on trunks of trees. The few records from aquatic habitats are not well documented and must be considered as mistakes. – In SE Brazil, it is commonly distributed in soil habitats and it forms relatively morphologically unique populations here. Four populations from different localities in Mata Atlântica were used for our analyses, one from the region of Cantareira, two from different localities in Campos de Jordão, and one from Ubatuba. The last one was isolated into a strain CCALA 1006.

Table 2. Key to the studied *Scytonema*–taxa with the main morphological diagnostic characters.

<table>
<tr><td>1. S. guayanense</td><td colspan="2">filaments 14–21 µm wide; old sheaths yellow–brown; branches usually completely divaricated</td><td rowspan="2">trichomes usually compact; hormogonia separate, solitary</td><td rowspan="4">cells mostly isodiametric; trichomes usually unconstricted or rarely slightly constricted at cross–walls</td><td rowspan="8">sheaths mostly smooth from outside</td><td rowspan="10">trichomes ± of the same width along the whole length (Scytonema)</td></tr>
<tr><td>2. S. javanicum</td><td colspan="2">filaments 12–15, trichomes 6–10(14) µm wide; sheaths colorless to yellow; bases of branches usually shortly parallel</td></tr>
<tr><td>3. S. arcangelii</td><td colspan="2">aquatic; filaments 9–14 µm wide</td><td rowspan="3">filaments often disintegrate; hormogonia often connected in short rows</td></tr>
<tr><td>4. S. hyalinum</td><td>young branches usually constricted at cross–walls; trichomes mostly 4.6–12 µm wide</td><td rowspan="2">aerophytic; filaments 9–19 µm wide</td></tr>
<tr><td>5. S. ocellatum</td><td>young branches not constricted at cross–walls; trichomes 10–15 µm wide</td></tr>
<tr><td>6. S. cf. bohneri</td><td colspan="2">filaments 7.5–15, trichomes 2.5–9 µm wide; cells shorter than wide up to rarely isodiametric</td><td rowspan="2">sheaths smooth from outside; cells shorter than wide, rarely up to isodiametric</td><td rowspan="3">cells shorter than wide, very rarely up to isodiametric; usually clearly constricted at cross–walls</td></tr>
<tr><td>7. S. stuposum</td><td colspan="2">filaments 12–24, trichomes 8–18 µm wide; cells always shorter than wide</td></tr>
<tr><td>8. S. crispum</td><td colspan="3">sheaths smooth or slightly irregularly granulated; cells always distinctly shorter than wide; trichomes 8–17 µm wide</td></tr>
<tr><td>9. S. schmidtii</td><td colspan="4">sheaths irregularly lengthwise structured; cells quadratic; trichomes 7–18 µm wide, constricted or rarely unconstricted at cross–walls</td><td rowspan="2">sheaths structured on the surface</td></tr>
<tr><td>10. S. sp. („verrucosum“)</td><td colspan="4">sheaths with lengthwise rows of irregular warts; cells isodiametric or mostly shorter than wide; trichomes 7–11(13) µm wide, mostly unconstricted at cross–walls</td></tr>
<tr><td>12. S. papillicapitatum</td><td colspan="5">sheaths terminate sometimes by capitate formations with distinct central papilla; only aquatic</td><td rowspan="3">trichomes narrowed in the middle (Myochrotes)</td></tr>
<tr><td>11. S. cf. longiarticulatum</td><td colspan="3">filaments 15–20 µm wide; yellow–brown sheaths darkest in outer layer; mostly on lateritic soils</td><td colspan="2" rowspan="2">sheaths without terminal capitate formations; mostly terrestrial</td></tr>
<tr><td>13. S. chorae</td><td colspan="3">filaments 12–15 µm wide; yellow–brown sheaths darkest mostly in inner layers; mostly on wet rocks</td></tr>
</table>

Table 3. The main morphological differences between clusters separated from the traditional genus Scytonema (according to Geitler 1932), in consequence with molecular analyses (16S rRNA gene sequencing) and morphology. The generic status and autapomorphic characters must be confirmed by further combined molecular and phenotypic analyses.

Position in phylogenetic tree	Cluster A	Cluster B	Cluster C	Cluster D	*Brasilonema*	Not yet sequenced	Not yet sequenced
Type species	*Scytonema hofmannii*	*Scytonema* (type) *hyalinum*	*Scytonema* (type) (?) *crispum*	*Scytonema* (type) *schmidlei*	*Brasilonema bromeliae*	*Scytonema myochrous*	*Petalonema alatum*
Hormogonia							
Ends of filaments and trichomes							
Sheaths and form of cells in the middle part of trichomes							
Branching and form of old trichomes and filaments							
Valid generic name	*Scytonema*				*Brasilonema*	(*Myochrotes*)	*Petalonema*

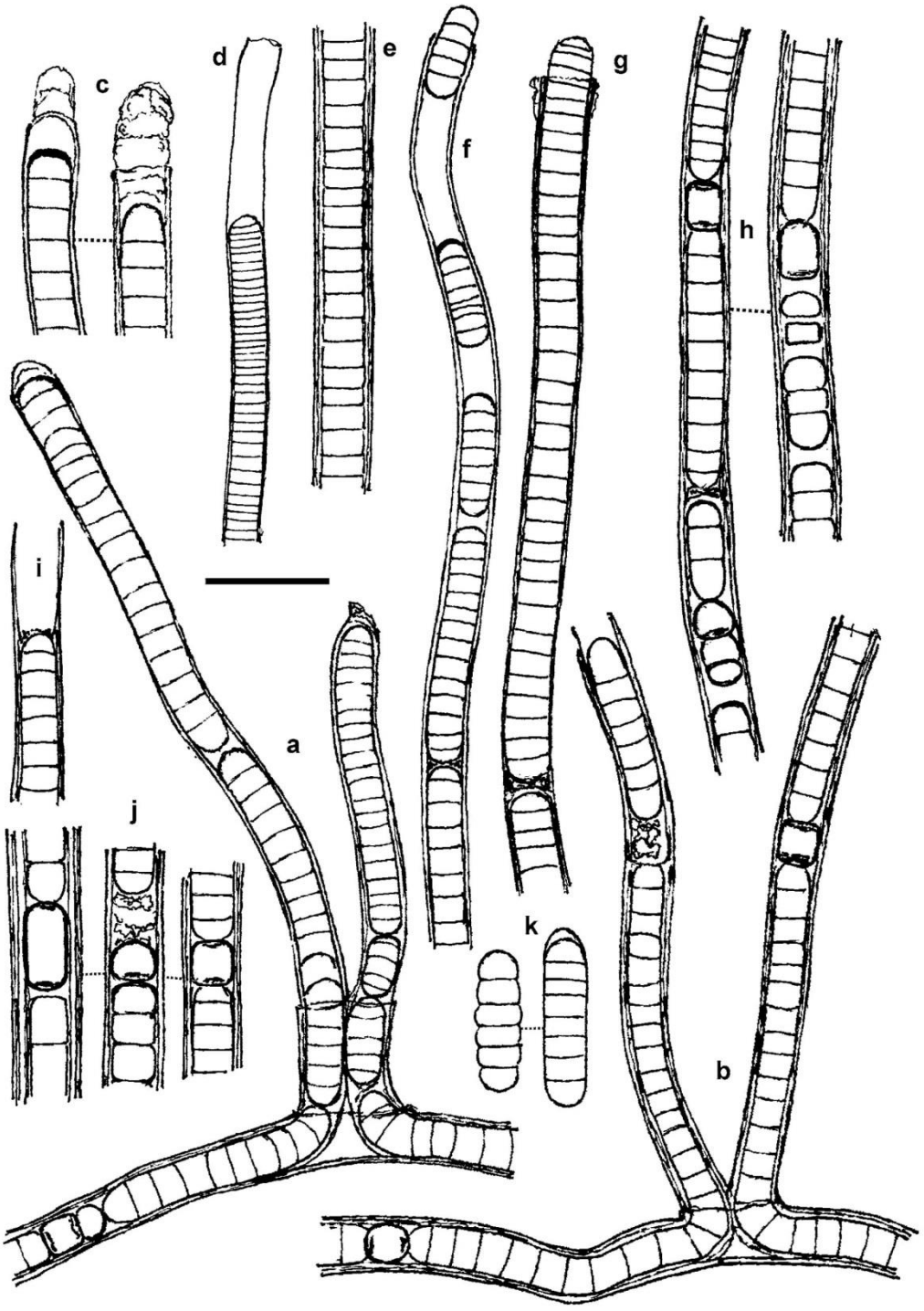


Fig. 2. *Scytonema guyanense* from natural conditions (Brazil, SP). Scale bar 20μm. Orig.

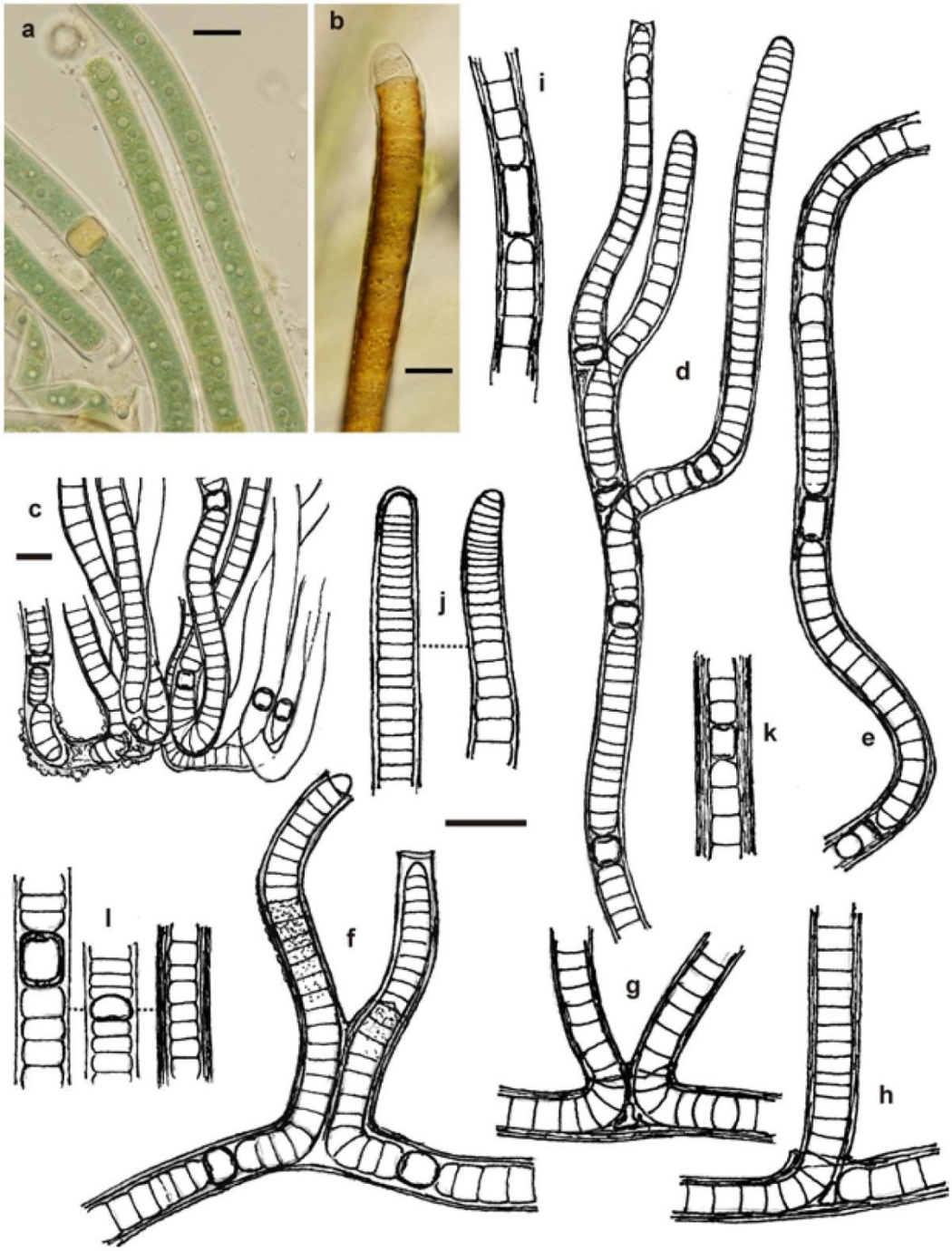


Fig. 3. *Scytonema javanicum, line drawings from natural material (Brazil, SP), light micrographs from strain CCALA 1006.* Scale bars (a–b) 10 µm; (c, d–l) 20 µm. Orig.

Description: Dark mats, mostly on soils, common on wet lateritic substrates in tropical regions, densely entangled together in developed colonies. Filaments cylindrical, with common, especially binary false branchings, sometimes slightly narrowed towards the ends and rounded, usually 12–15 μm wide; the binary branches are usually connected parallelly together for a short segment after branching. Sheaths firm, thin to slightly widened, laminated and colorless to yellow, especially in older filaments. Trichomes cylindrical, of the same width along the whole length, not or slightly constricted at cross–walls, usually slightly narrowed and rounded at the ends, 6–10(14) μm wide. Cells with green or blue–green content, cylindrical, rarely ± isodiametric, usually shorter than wide, shortened especially in mostly subterminal meristematic zones. Heterocytes basal or intercalary, solitary, cylindrical or short hemispherical, barrel–shaped, up to cylindrical, mostly ± isodiametric, less frequently longer than wide, 6–15 × 8–12 μm.

Group B: Cluster of „*Scytonema hyalinum*"

3. *Scytonema arcangelii* BORNET et FLAHAULT 1887 (Fig. 4)

A commonly cited species, mainly from tropical regions, particularly known from Central and South Americas. The populations from Africa show some morphological deviations (FRÉMY 1930; SILVA & PIENAAR 2000). Recorded rarely also from Italy, Japan and North America, but these populations should be revised. – It is an aquatic species, occurs on submerged plants and stones, mostly in stagnant and slowly streaming waters, where it forms up to macroscopically visible clusters. – We have studied one population from the unnamed small lake in the area of the University Federal in Viçosa, Minas Gerais State in detail. This population was isolated in culture (CCIBt 3134, collection of the Botanical Institute, São Paulo) and its 16S rRNA gene was sequenced, confirming its phylogenetic position sister to *S. hyalinum* (Fig. 1).

Description: Aquatic, thallus in form of floating or sessile clusters. Filaments cylindrical, at the ends not or very rarely slightly widened (only in sheaths), sometimes slightly and shortly narrowed, (9)10 – 13(14) μm wide. Sheaths thin, firm, colorless. Trichomes cylindrical, not or very slightly constricted at cross walls (higher magnifications!), ± 10 μm wide, at the ends up to 8 μm wide, blue–green or greyish green. Cells cylindrical, ± quadratic, isodiametric or often shorter than wide, especially at the ends of branches; narrower cells particularly in the terminal parts of branches. Terminal cells widely rounded, or slightly narrowed and rounded. Meristematic zones occur rarely at the ends of branches, hormogonia liberate singly. Heterocytes shortly oval or cylindrical with rounded ends, very short up to 2× longer than wide, of the same width as trichomes.

4. *Scytonema hyalinum* GARDNER 1927 (Fig. 5)

S. hyalinum was described by Gardner from China. It is morphologically and ecologically well characterized, particularly by colorless sheaths, dimensions and numerous necridic cells. The ends of branches are often terminated by cup–shaped formations. This species was later recorded from more places and it seems that it has really wide distribution, especially in tropical Central and S America. However, it is recorded also from desert crusts in N America, the records from hot springs are probably mistaken. The final position and taxonomic delimitation must be supported by further studies. – According to the description and also according to our results, *S. hyalinum* is a characteristic aerophytic species, growing on periodically wetted rocks, among mosses and in places with detritus. – It occurs in similar localities in Brazil. We have studied in detail the species morphology and variability in samples collected from two localities (rocky and concrete walls) in Mata Atlântica, in the vicinity of Juréia, in Paranapiacaba and one sample form an island of Ilha do Cardoso.

Description: Aerophytic, blackish mats, mostly on stony substrates, less on wet lateritic soils. Filaments relatively densely entangled together, cylindrical, sometimes slightly widened or narrowed at the ends, (9)10–15(18) μm wide; branches often entangled and intensely coiled and crossed. Sheaths firm, thin, only slightly lamellate, colorless, slightly yellowish or brownish only when old. Trichomes cylindrical, sometimes disintegrating and divided in isolated segments, usually not constricted, only short segments of old trichomes constricted at cross walls, of different and variable width along the whole trichome length, 6–12(14) μm wide. Meristematic zones in terminal parts, hormogonia separate sometimes in short rows (up to 8 together), (3–)8–16–celled. Cells barrel–shaped to cylindrical, very short in hormogonia and in terminal parts of trichomes, up to isodiametric or rarely slightly longer than wide in middle parts, usually 4.6–8 × ± 9 μm, greyish–blue or bright blue–green, terminal cells yellowish. Heterocytes cylindrical with rounded ends, shorter than wide up to almost 2× longer than wide, 5–18 × 6–12 μm.

5. *Scytonema ocellatum* [DILLWYN] LYNGBYE ex BORNET et FLAHAULT 1887 (Fig. 6)

An interesting species, supposedly with cosmopolitan distribution, widely cited, but not yet exactly taxonomically defined. It occurs in several morphotypes and several subspecific taxa were described particularly from tropical regions. Majority of the described populations require revision. Typically, trichomes are cylindrical with simple rounded terminal cells and ± quadratic cells. Sheaths are thin, firstly hyaline, later

yellow–brown. There is considerable similarity with *S. javanicum,* and both species can be misinterpreted. Especially the character of facultative rough surface of sheaths is problematic; it is not clearly explained and a study was published (Tilden 1910), where the sheath surface was described as covered with „detritus particles". – *S. ocellatum* forms dark blackish, flat mats on the moist soil, rocks, walls, flower pots and possibly also bark of trees (similarly as e.g. *S. guayanense* or *S. javanicum*). – In the State of São Paulo, *S. ocellatum* is widely distributed. We studied five populations from Mata Atlântica, one from the area of Cantareira, and four populations from distant localities in Horto forrestal, Campos de Jordão.

Description: Colonies on wet, mostly lateritic soils, not very common. Filaments slightly flexuous, strictly cylindrical, (10)15–19 μm wide, usually with binary false branching, branches of the same morphology as the main filaments. Sheaths firm, distinct, colorless, yellowish up to yellow–brown, slightly laminated, smooth from outside. Trichomes cylindrical, of the same width along the whole length, mostly 10–15 μm wide, not constricted at cross walls, ends not attenuated, end cells rounded. Cells ± isodiametric or slightly longer or shorter than wide, usually 6–15 μm wide, greyish–blue, only in terminal parts and in hormogonia distinctly shortened. Hormogonia cylindrical, with short cells, 8–18–celled. Heterocytes hemispherical to barrel–shaped up to cylindrical with rounded ends, intercalary, solitary, mostly 8–12 × 8–15 μm.

Group C: Cluster of "*Scytonema crispum*"

6. *Scytonema* cf. *bohneri* Schmidle 1902 (Fig. 7)

S. bohneri is an exclusively tropical species and is known mostly from aquatic localities, mainly from stony and rocky streams. It is described from Africa (Cameroon), but known also from tropical Asia (India, Myanmar), Seychelles Islands and from Brazil. Rare reports from temperate areas are not well documented and relate probably to other species. – Also our specimens do not correspond exactly to the characteristics of this species, mainly in ecology. We have studied two populations from Mata Atlântica, both from aerophytic habitats (Paranapiacaba, coll. from a wet concrete wall; Campos de Jordão, coll. from bark of trees). Both of these populations correspond morphologically well with the description of *S. bohneri,* but they differ slightly by colored, yellow sheaths, and by ecology. A little different concept of *S.bohneri* was suggested by Starmach (1975) from Seychelles Islands (± isodiametrical cells, widened trichomes towards ends).

Description: Mats on wet soils and rocks, less frequently on wet wooden substrates. Filaments nearly straight to flexuous, with mostly binary false branchings, sometimes slightly widened or narrowed toward the ends, (7.5)10–12(15) μm wide. Sheaths slightly thickened, with ± parallel layers, yellowish or yellow–brown with colorless ends; often with inner yellow–brown layer, outside almost colorless. Trichomes cylindrical, sometimes slightly narrowed in various parts, not or distinctly constricted at cross walls, especially in old trichomes, short segments sometimes distinctly constricted and composed of barrel–shaped cells, (2.5)4.8–6(9) μm wide. Meristematic zones terminal or subterminal. Cells mostly cylindrical, usually shorter than wide (sometimes only 3 μm long) rarely up to isodiametric and sometimes in old parts of trichomes long cylindrical, up to 1.8× longer than wide, blue–green. Heterocytes intercalary, solitary or very rarely in pairs, mostly cylindrical or short barrel–shaped, rarely rounded or hemispherical, (4)6–10 × 5–8.5 μm.

7. *Scytonema stuposum* [Kützing] Bornet ex Bornet et Flahault 1887 (Fig. 8)

A species commonly distributed over tropical regions, considered to have pantropical distribution. It is recorded also from regions with mediterranean climate and from warmer areas within temperate zones. In S America , it probably belongs to the commonest species. It has quite characteristic morphology; the cylindrical filaments, colourless and relatively thin sheaths (to 4 μm thick), trichomes constricted at cross–walls and short cells belong to the main diagnostic characters. Terminal cells are usually larger, yellowish and rounded. *S. stuposum* is aerophytic and subaerophytic, occurs on wet soils, on rocks and among mosses. – It was recorded several times from Brazil (Sant´Anna & al. 1978, 1983, 1991; Senna & Ferreira 1987; Sant´Anna 1988, and others). We have had the opportunity to study two strains, CCALA 1008 and CCALA 1009, isolated from aerial localities in Ubatuba, São Paulo. Their position in the phylogenetic tree was confirmed by molecular 16S rRNA gene sequencing (Fig. 1).

Description: Colony flat spreading, dark green; in old cultures forms greyish or brownish mats. Filaments short, densely arranged, single or double false branched, 12–20(24) μm wide. Sheaths colourless, thin, attached or up to 4 μm wide, unstructured, closed at the trichome ends. Trichomes distinctly irregularly constricted at cross–walls, wider in the middle part and slighly tapered towards the ends, 8–18 μm wide. Cells blue–green or green, finely granulated, cylindrical to disc–like or irregularly compressed, shorter than wide, 2–6 μm long; incomplete cross–walls in meristematic zones distinctly visible. End cells rounded or slightly conical, green–tan coloured; a row of cells at the growing ends usually distinctly constricted (later released as hormogonia?). Heterocytes abundant, single or in pairs, mostly cylindrical, intercalary, beige

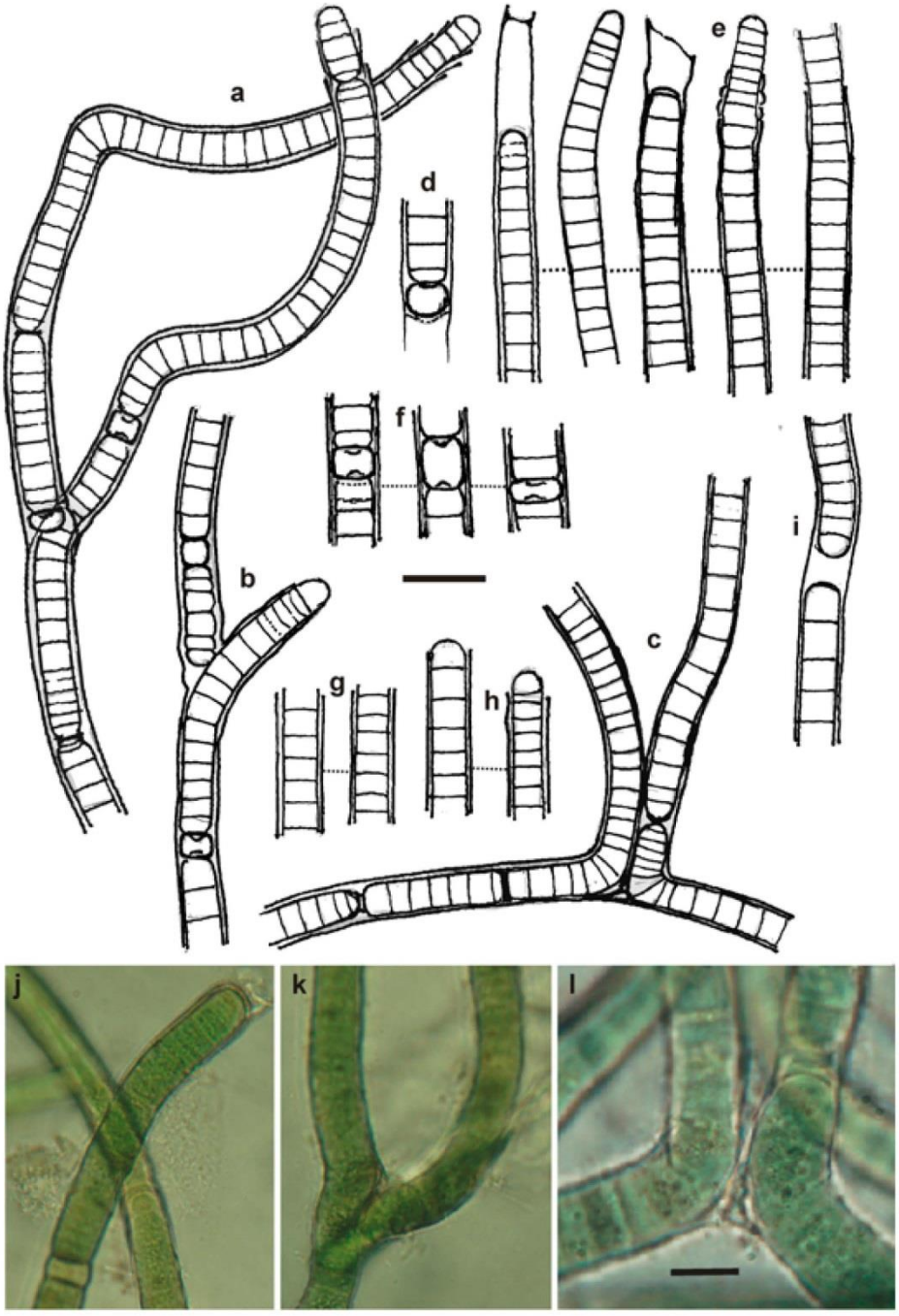


Fig. 4. *Scytonema arcangelii* (strain CCIBt 3134) from culture conditions. Scale bars (a–k) 20 µm; (l) 10 µm. Orig.

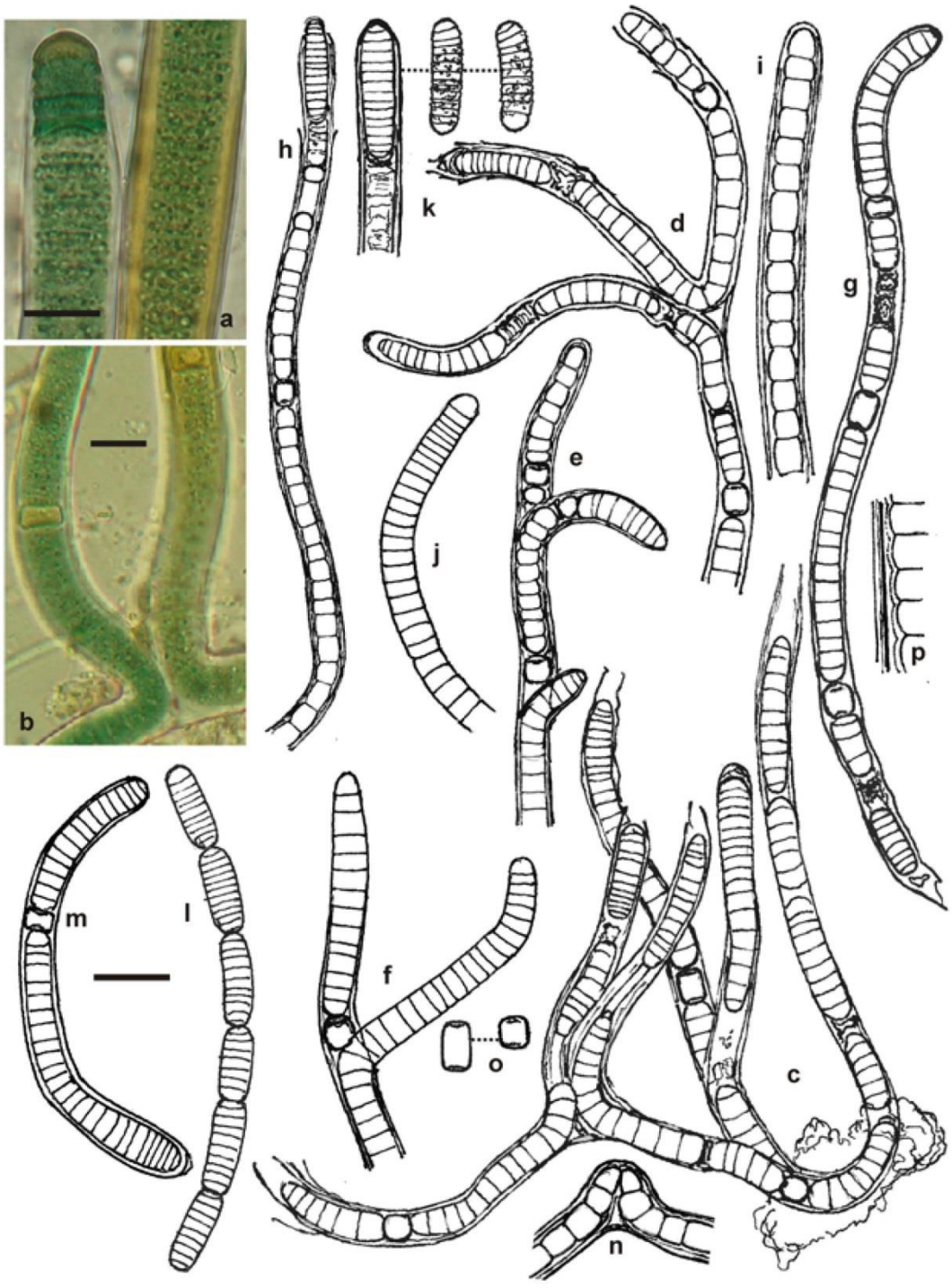


Fig. 5. *Scytonema hyalinum* from natural material (Brazil, SP). Scale bars (a–b) 10 µm; (c–o) 20 µm. Orig.

to yellow, with sap vesicle, 4–10 × 10–15 μm. Necridic cells and hormogonia present.

8. *Scytonema crispum* [Agardh] Bornet 1889 (Fig. 9)

This primarily aquatic species is probably quite common in Brazil, but we studied only one strain, CCALA 1007, isolated from aerial localities in Ubatuba, São Paulo. The distribution of this species, which was described from Europe, is considered cosmopolitan (with exception of subpolar regions). However, the species is variable and different populations, corresponding more or less to the original description, should be compared by genetic methods. The dimensions of our specimen (e.g. trichome width) were near the lower size limit for *S. crispum*, which was previously reported in several populations of this taxon described from South America (e.g. Montoya et al. 1998 from Peru, but isolated from desert soils). This fact might imply that the populations found in S America belong to different, previously not described taxon.

Description (mainly from isolated cultures): Colonies crumbly, velvet–like, dark green. Filaments short to long, isopolar, easily fragmentating, in shorter parts, singly or binarily false–branched. Trichomes cylindrical, sometimes slightly narrowed towards both ends and towards ends of branches, constricted at cross–walls, with distinct necridic cells, (8)10–17 μm wide. Sheaths relatively thick, colorless to yellow, orange to brownish, smooth or finely granulated at the surface, slightly and sometimes indistinctly lamellated, closed at the ends. Cells inside coarsely granulated, blue–green, usually shorter than wide, barrel–shaped to disc–like, 2–10 μm long; end cells widely rounded. Heterocytes solitary, yellow–green, of various shape, abundant, intercalary or basal (branches), of the same width as vegetative cells, usually flattened (shorter than wide). Common reproduction by hormogonia consisting of 1–8 cells.

Group D: cluster of "*Scytonema schmidtii*"

9. *Scytonema schmidtii* Gomont 1901 (Fig. 10)

Scytonema schmidtii is an old described species, which is, however, up to now little known and recorded in different concepts. The unclear description follows also from the fact that the usually known documentation (a later drawing of Frémy 1930 from Africa) is very schematic and does not illustrate the variability and main characters of this species well. The characteristic morphology of the cells, constrictions at cross–walls, characteristic terminal parts of branches and the sheath morphology seem to be typical for this taxon. The sheath is relatively thin, but later slightly thickened, yellow–brown, laminated and closed in young branches, open after hormogonia release. In old, thickened sheaths characteristic lengthwise irregular striation on the surface (see Fig. 9), unusual in other species, was found. – The species grows on aerophytic localities and occurs usually among other algae and cyanobacteria on wet soils, rocks and bark of trees in humid forests. It has evidently only tropical distribution; it was described from Africa, but recorded also from S and SE Asia, New Caledonia and Brazil. There is also a report of the occurrence in marine habitat in Mozambique (Silva & Pienaar 1997), but it seems to be very unusual for this species and probably doubtful. – We studied material from two distant localities, from the area of Botanical Garden in São Paulo from concrete walls covered with mosses and other cyanobacteria in Ilha do Cardoso; we isolated a strain CCIBt 3568 of this species.

Description: Thallus in form of mats on stony and concrete substrates and wet soils. Filaments cylindrical, relatively short, slightly flexuous, with simple or binary false branchings, (8)13–15(25) μm wide. Branches usually divaricate soon one from another, more or less of the same morphology as the main filaments. Sheaths firstly thin, later thickened, laminated, yellow–brown, often more dark in outer layers, from outside lengthwise rugulose. Trichomes cylindrical, not constricted or constricted at cross–walls; constricted particularly at the ends (with very short cells), 7–12(18) μm wide. Meristematic zones terminal, hormogonia liberate from the ends of branches, usually solitary, 4–10–celled. Cells in old trichomes ± isodiametric, in branches and especially at the ends shortened, end cells rounded. Heterocytes mostly intercalary, ± of the same width like the trichome, short oval, hemispherical, barrel–shaped, usually shorter, rarely slightly longer than wide.

Notes: It seems that there is a wide variability in dimensions of the otherwise very similar morphological types collected from various localities. We have found populations with different variation in width of filaments and trichomes (up to 45 μm), but with other characters almost identical (morphology of trichomes, terminal cells, sheaths, heterocytes etc.) (Fig. 11). It seems that all these populations are closely related one to another, but the correctness of this hypothesis must be supported by molecular analyses. For instance, a similar type (particularly in the special morphology of sheaths) was found in specimens from mats of periodically wetted rocks in Mata Atlântica near Bertioga. The main differences from typical *S. schmidtii* were found only in quantitative markers, particularly in the greater width of filaments and trichomes, less frequent and less intense constrictions at cross walls and more distinct, laminated and more intensely coloured sheaths. The intense surface striation of the sheaths occurred distinctly in the whole population, also in young filaments and branches. It was not possible to decide, if it belongs into the range of variability of *S. schmidtii,* or if this type represents a specific taxon. The description of this population follows.

10. *Scytonema* sp. (Fig. 12)

In Mata Atlântica in Campos de Jordão (Horto Forrestal) on bark of trees, populations of *Scytonema*–like cyanobacteria, not identifiable according to existing literature and identification keys, were found. It is probably an up to now unknown *Scytonema* species, but we were not able to isolate it in a culture and the amount of the material available is insufficient for a proper taxonomic description. The main characters of this species are the always colorless, relatively thick and in outer layers irregularly laminated sheaths, which are on the surface ornamented by a dense rows of small, colorless warts, evidently composed of the same material as the sheaths. Also the ecology seems to be restricted only in the epiphytic habitat on stems of trees and possibly other wooden substrates. The variability of this taxon and especially the curious morphology of sheaths need further studies.

Description: Thallus in form of mats on bark of trees. Filaments cylindrical, binary false branches usually rare, branches divaricate soon one from another. Filaments and branches ± straight, slightly curved or rarely sligthly flexuous, 15–17(18) μm wide. Sheaths relatively thick, colorless up to slightly yellowish, slightly lamellated and on the surface sculptured by lengthwise, densely arranged lines of small, irregular verrucae. Trichomes cylindrical up to the ends, not constricted at cross walls, only in few segments slightly constricted, 7–11(13) μm wide. Cells cylindrical, usually shorter than wide, rarely up to isodiametric, blue–green, terminal cells widely rounded. Heterocytes solitary, intercalary, usually ± barrel–shaped up to rounded cylindrical, rarely slightly longer than wide, 7–10(12) μm broad.

Group E: subg. *Myochrotes* (not sequenced)

11. *Scytonema* cf. *longiarticulatum* Gardner 1927 (Fig. 14)

Originally described from rocks (aerophytic) in Puerto Rico. It is characterized by relatively thick, laminated and partly yellowish sheaths, and by very narrow trichomes with cylindrical, long cells and continually widened terminal parts. Our population is a little different from the original description. The cells in central trichomes are not so long as described by Gardner. There is also wider variability in constrictions at cross walls between the cells and in formation of meristematic parts and hormogonia in apical parts. However, both populations are morphologically and ecologically similar in such a degree, that they can belong to the same genotype. – In Brazil (SP), it grows mostly in lateritic soils, our studied material was collected in Jureia (SP 427936).

Description: Clusters of filaments on wet rocks and lateritic soils. Filaments cylindrical, slightly flexuous, rounded at the ends, 15–20 μm wide, usually with binary false branches. Sheaths thick, laminated, yellow–brown, darkest in outer layers, rough from outside. Trichomes cylindrical, narrow in the middle and ± 5 μm wide, gradually widened towards the ends, up to 11 μm wide at the ends, slightly constricted at cross–walls. Meristematic zones terminal. Cells in middle narrow parts long cylindrical, up to 3× longer than wide, towards the ends shortened, ± isodiametric up to very short at the ends; terminal cells often yellowish. Heterocytes intercalary, solitary, usually wider than trichomes, very polymorphic, hemispherical, cylindrical.

12. *Scytonema papillicapitatum* Sant´Anna et Komárek sp. nov. (Fig. 13)

A morphotype similar to *S. longiarticulatum* from the *Myochrotes* section, i.e. with narrow middle part of trichomes. This delicate and fine species occurs as more or less monospecific submersed clusters in aquatic localities, and have several characteristic features, such as a sheath with pale yellow coloration in the inner layers (outer layers are colorless) and occasional development of mucilaginous cup–shaped, hemispherical formation with one central prominent calyptra at the ends of the filaments with finger-like projection (characteristic for this species). This formation is not present in the whole population and occurs only locally, but it is very distinct. The apices of the thick sheaths can be terminated also by other morphological modifications. Because we did not found a similar morphotype in literature, we describe this taxon as a new species. – Aquatic up to subaerophytic species, which was collected in the lake Lagoa de Jacaré (type locality), Mogi Guaçu, sample SP 188495.

Description: Clusters of filaments. Filaments cylindrical, flexuous, sometimes slightly, continually widened towards ends, 13–16 μm wide; well developed filaments have hemispherical, cup–shaped cell–wall formation with terminal, finger–like processus. False branching relatively rare, usually with two branches (binary branching). Sheaths thick, slightly lengthwise laminated (particularly in old parts), yellow (mostly only internal layers of sheaths), rarely up to brownish. Trichomes thin, 2–3 μm thick, with long cells, not or very slightly constricted at cross–walls, widened towards the ends, 5–7(8) μm wide, usually distinctly constricted. Meristematic zones terminal or subterminal, usually with constrictions at the cross–walls. Cells in older trichomes long cylindrical, up to 5× longer than wide, in terminal parts distinctly shortened, cylindrical to barrel–shaped, up to 2–3× shorter than wide. Heterocytes intercalary, solitary, sometimes slightly wider than trichomes, cylindrical, 10–15 × 6–6.5 μm.

Diagnosis: *Strato pannoso, tomentoso, viridi, cum filis paucim tortuosis intricatis. Filamenta cylindrica, flexuosa, praecipue paucim vel distincte et gradatim dilatata ad apices, 13–16 µm lata, pseudo–ramosa. Vaginis latis, longitudinem lamellosis, luteis (praecipue in partes internis), rare ad fuscis. Ramificatio rara, plerumque cum ramis binis. Trichoma tenues, 2–3 µm lata, cum cellulis longis, ad dissepimenta not vel paucim constricta, ad apices dilatata, ad 5–8 µm lata, distincte constricta. Partes meristematicae terminales vel subterminales. Cellulae cylindricae in trichomatibus adulties, ad 5× longior quam latae, in partes terminalibus curtae, cylindricae vel barriliformes, ad 2–3× brevior quam latae, cellula apicalis saepe cum calyptra membranosa, cum papilla cetrali externa. Heterocytae intercalares, solitariae, subcylindricae, 10–15 × 6–6.5 µm. – Habitatio: in piscinis, stagnis, paludibusque cum plantis aquaticis natans, vel subaerophytice; locus classicus: lacus Lagoa de Jacaré, Mogi Guaçu, provincia São Paulo, Brasilia. – Typus: SP 188495, deposited in Herbarium of the Institute of Botany in São Paulo, Brazil.*

13. *Scytonema chorae* SANT´ANNA et KOMÁREK sp. nov. (Fig. 15)

This morphotype is similar to the previous ones, but with characteristic morphology, from aerophytic localities (granitic rocks). This species also shows the diacritical features of *Myochrotes,* but is distinct by thick lamellated sheaths which are mostly closed at the apex and together with widened trichome form slightly club–shaped rounded ends. The striation is distinct through the layers with changing intensity of the yellow or yellow brown and brown coloration. The layers of the sheath are sometimes fan–like widened towards the ends (inside of the sheath). The dimensions are very uniform and one of the characteristical features are cylindrical heterocytes, usually 2–times wider than vegetative cells. Because this species was impossible to fit into any previously described species (particularly in respect to morphology of filaments and trichomes) and because we had an opportunity to study its populations with the same morphology and ecology from two distant localities, we describe it as a new species. Its name was selected according to the name of characteristic locality. – We studied intensely two populations of this species from stony walls in the region of Cantareira, from the Gruta que Chora near Ubatuba (type locality) and from State Park of Ilha do Cardoso.

Description: Subaerophytic mats on stones. Filaments cylindrical, slightly continually club–shaped, widened towards ends, with rare binary false branching, slightly flexuous, 12–14 µm, towards the ends up to 15 µm wide. Sheaths thick, firm, distinctly laminated with layers, at the ends funnel–like divaricated (inside the outer sheath margin), yellow–brown to brown, especially in inner layers. Trichomes in the middle parts cylindrical, thin, not constricted at cross–walls, 3–4 µm wide, at the ends club–shaped widened with shortened cells, up to 8–9 µm wide. Meristematic zones ± terminal. Cells in middle filaments long cylindrical, up to 3× longer than wide, up to 15 µm long, at the terminal parts short cylindrical, up to distinctly shorter than wide. End cells rounded to conical–rounded. Heterocytes distinctly wider than neighboring vegetative cells, intercalary, solitary, relatively rare, mostly short up to long cylindrical, to 8 × 5–6 µm.

Diagnosis: *Strata epilithica, pannosa. Filamenta cylindrica, flexuousa, paucim gradatim dilatata ad apices ad paucim clavata, 12–14 µm, ad apices ad 15 µm lata, rare pseudoramosa. Vaginis latis, firmis, distincte lamellosis, lamellis divergentibus in partes apicalis, luteo–fuscis vel fuscis, praecipue in partes internis. Rami longi, filamentis principalibus similares. Trichoma tenues et cylindricae in partes adultis, ad dissepimenta not constricta, 3–4 µm lata, ad apices paucim clavata. Partes meristematicae, plus minusve terminales. Cellulae cylindricae, longae, ad 3x longior quam latae, ad 15 µm longae, in partes terminalibus curtae, ad brevior quam latae; cellula apicalis rotundata vel conice–rotundata. Heterocytae distincte latior quam cellulae vegetativae, solitariae, intercalares, plus minusve cylindricae ad 8 × 5–6 µm. – Habitatio: Subaerophytice ad rupes humidas; locus classicus: caverna Gruta que Chora dicta, prope oppido Ubatuba, provincia São Paulo, Brasilia. – Typus: SP 427934 deposited in Herbarium of the Institute of Botany in São Paulo, Brazil.*

DISCUSSION

The genus *Scytonema* is an important component of microphyte communities in terrestrial (aerophytic) habitats, particularly in tropical regions. Many species seem to have pantropical distribution, but the populations from various distant regions need taxonomic revision and their areas of distribution must be checked. The molecular determination of different populations must be preferred in taxonomic evaluation of the samples, but the precise reassessment of their morphology and study of life cycles is still an essential part of the modern polyphasic analyses. Relatively broad diversity of *Scytonema*–like types over the tropical countries was documented in numerous articles (e.g. GARDNER 1927; SKUJA 1949; DESIKACHARY 1959; SANT'ANNA 1978; COUTÉ et al. 1999, and others). These records need comparative analysis and further revision according to the modern criteria, both molecular and morphological, before a new taxonomic system can be accomplished.

The revision of the populations belonging to the traditional genus *Scytonema* in tropical regions and their taxonomic evaluation according to the currently accepted criteria must be therefore based on determination of phylogenetic relationships between various populations and taxa, which usually results in recognition of a wider diversity of genera and species. This complex study and reorganization of the old system was already started. The genus *Brasilonema,*

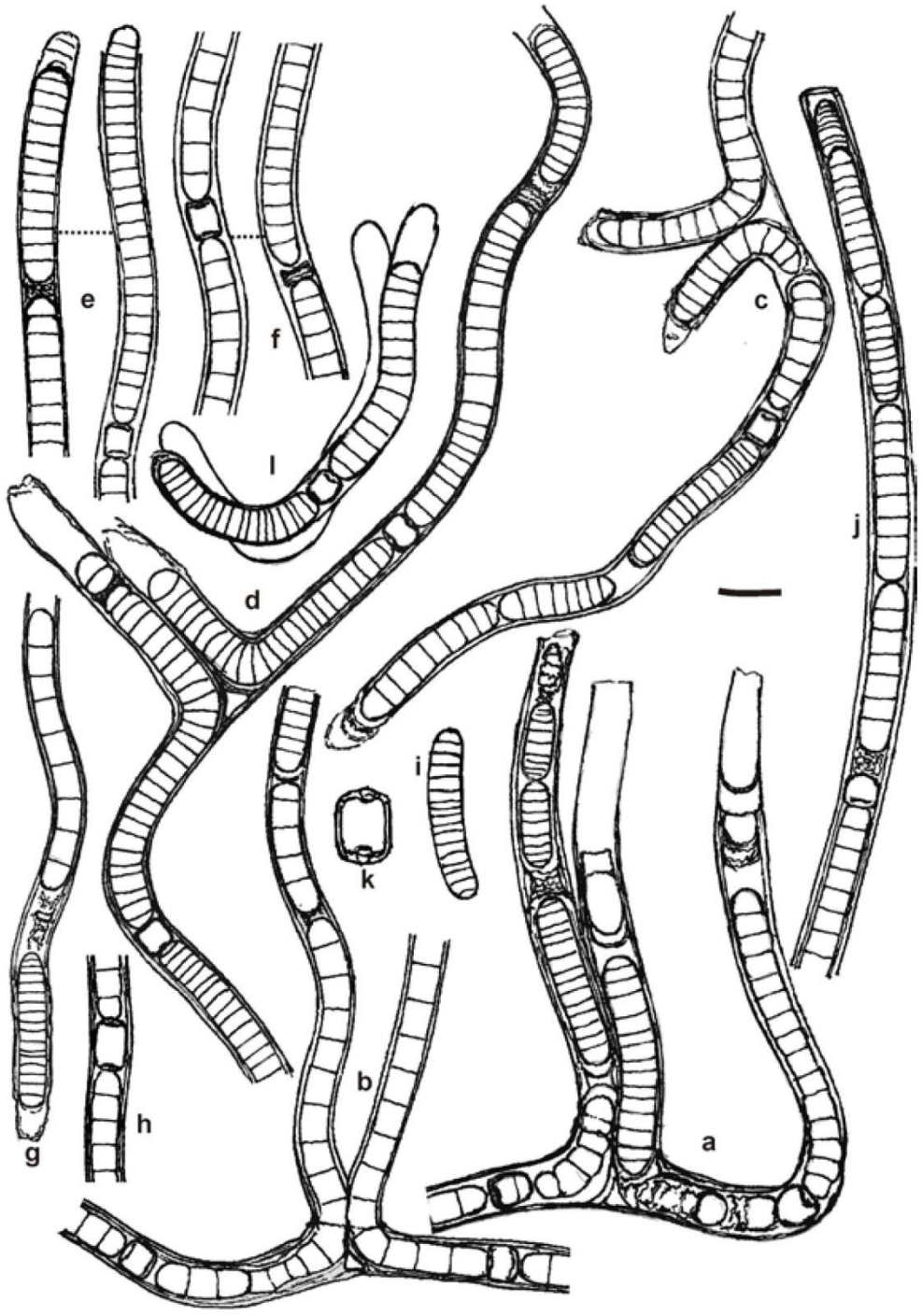


Fig. 6. *Scytonema ocellatum* from natural material (Brazil, SP). Scale bar (a–i) 20 µm. Orig.

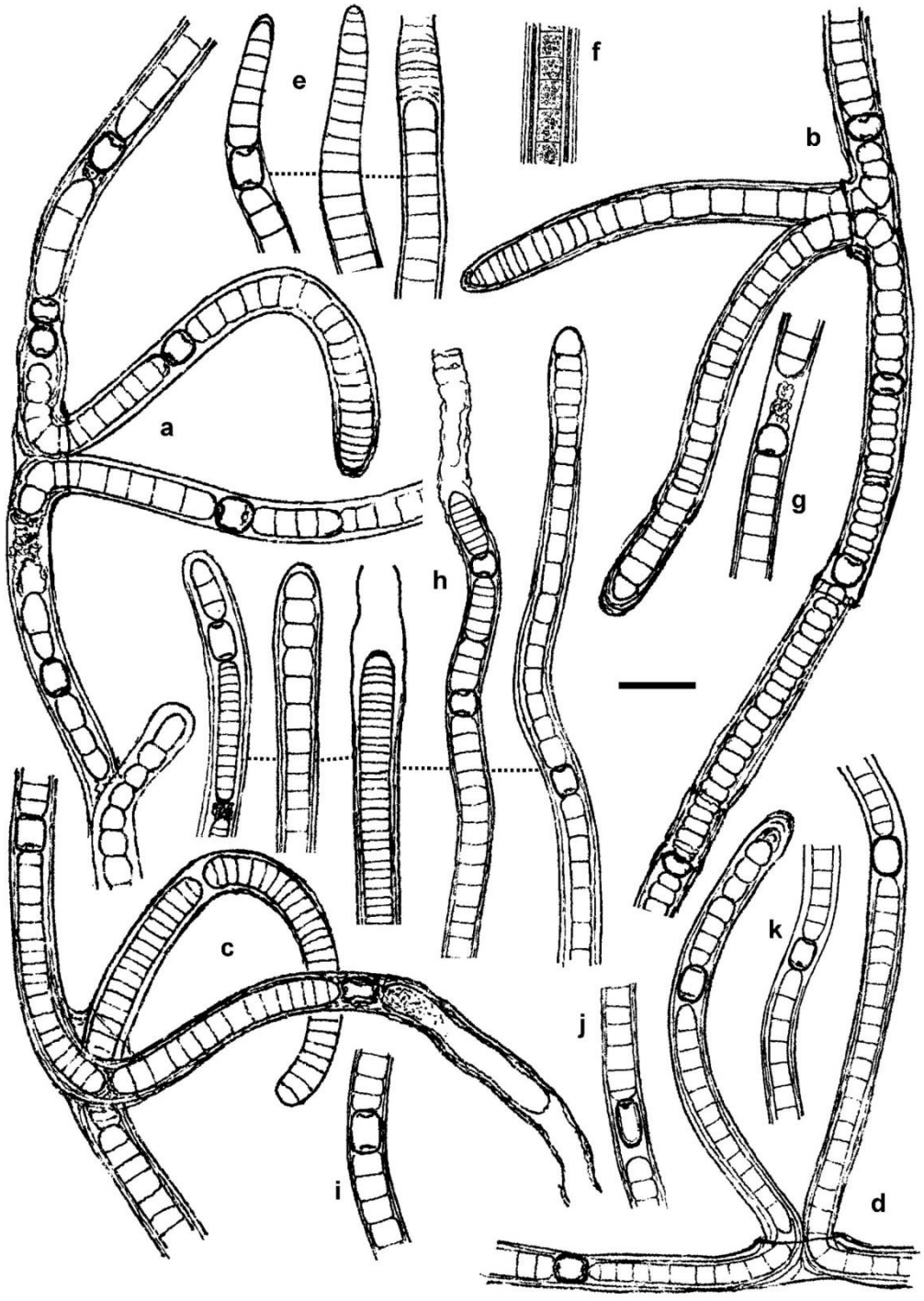


Fig. 7. *Scytonema* cf. *bohneri* from natural material (Brazil, SP). Scale bar 20 µm. Orig.

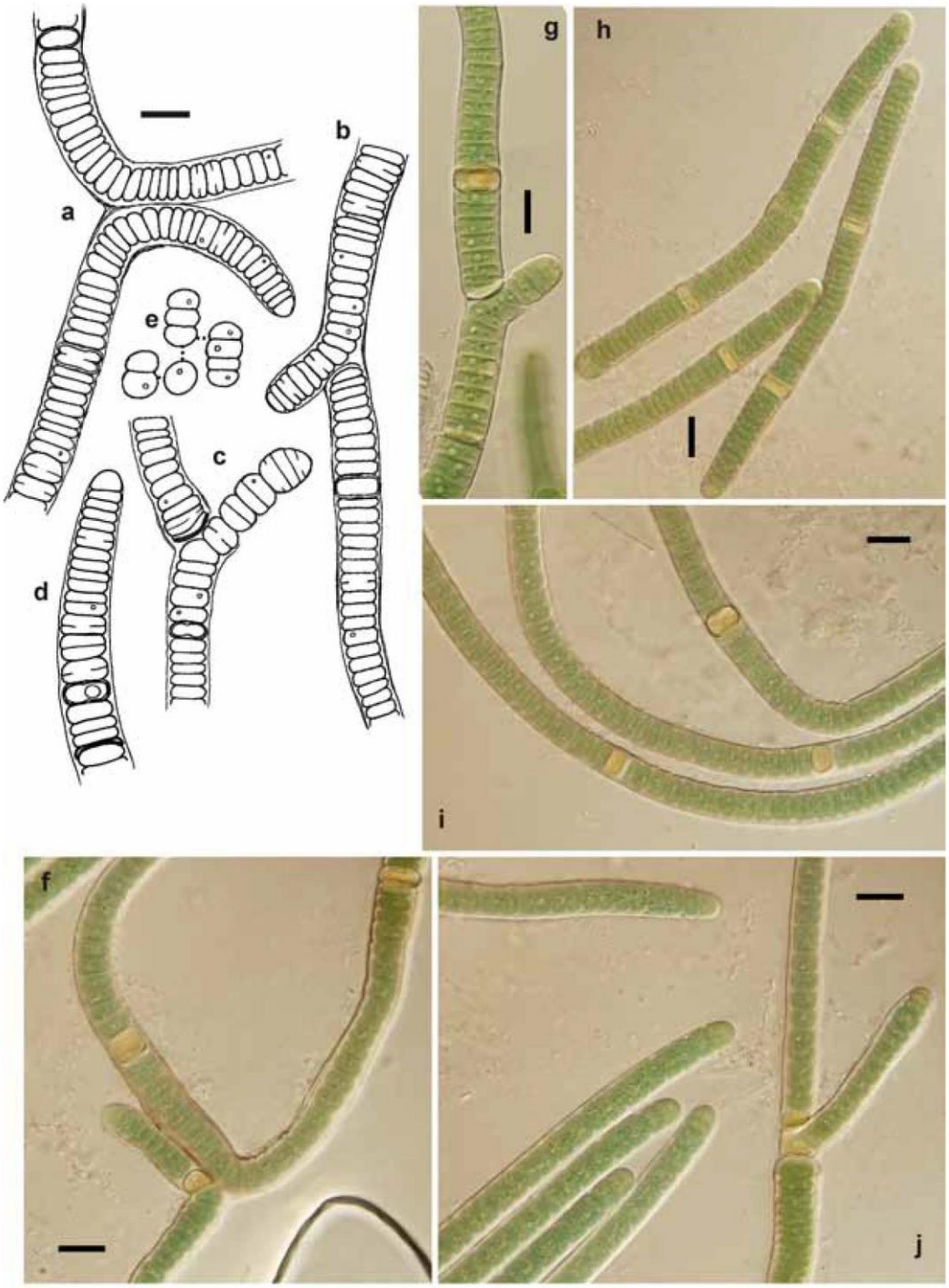


Fig. 8. *Scytonema stuposum* from cultures CCALA 1008 and CCALA 1009. Scale bars 20 µm. Orig.

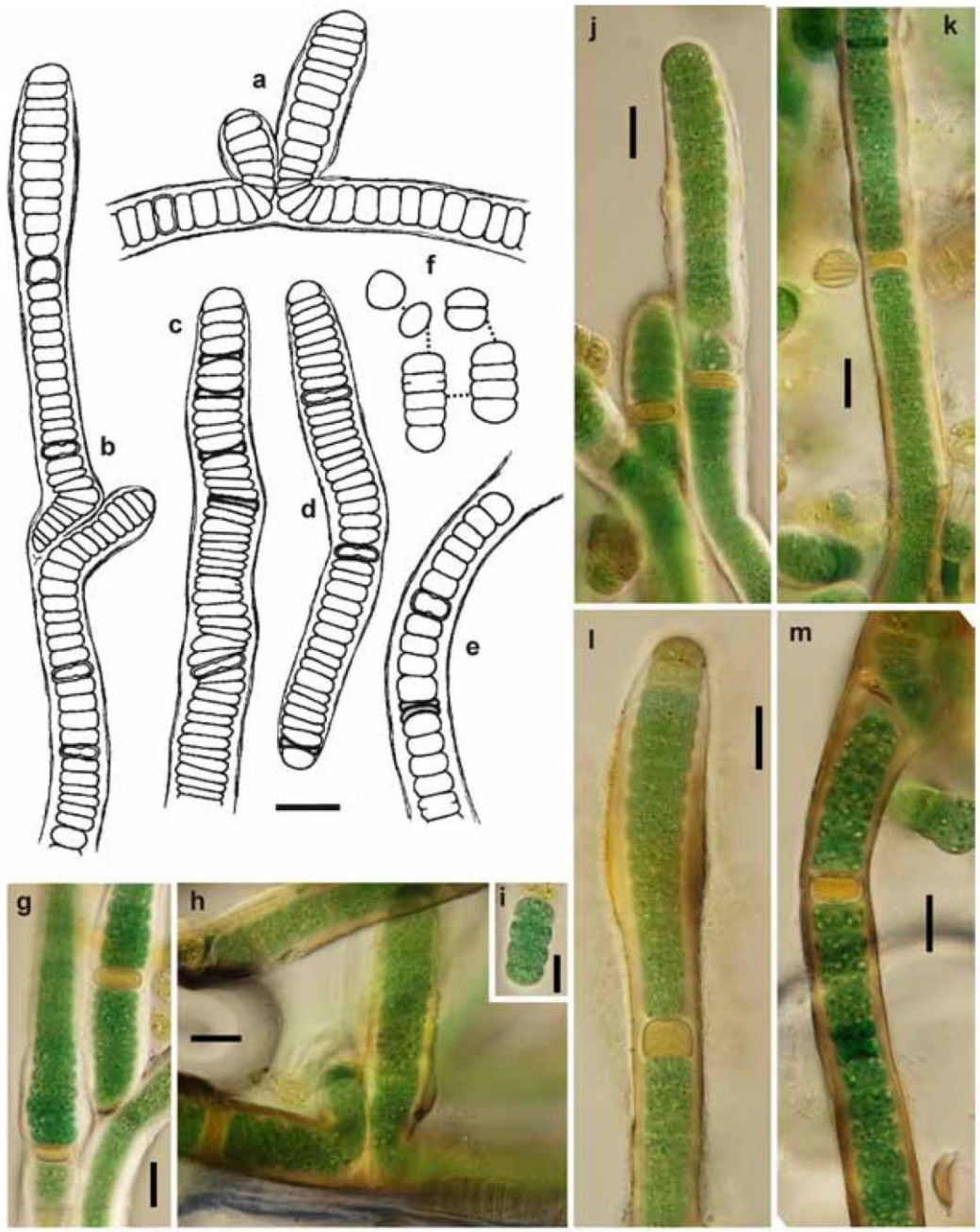


Fig. 9. *Scytonema crispum* from cultured material (Brazil, SP), strain CCALA 1007. Scale bars 20 µm. Orig.

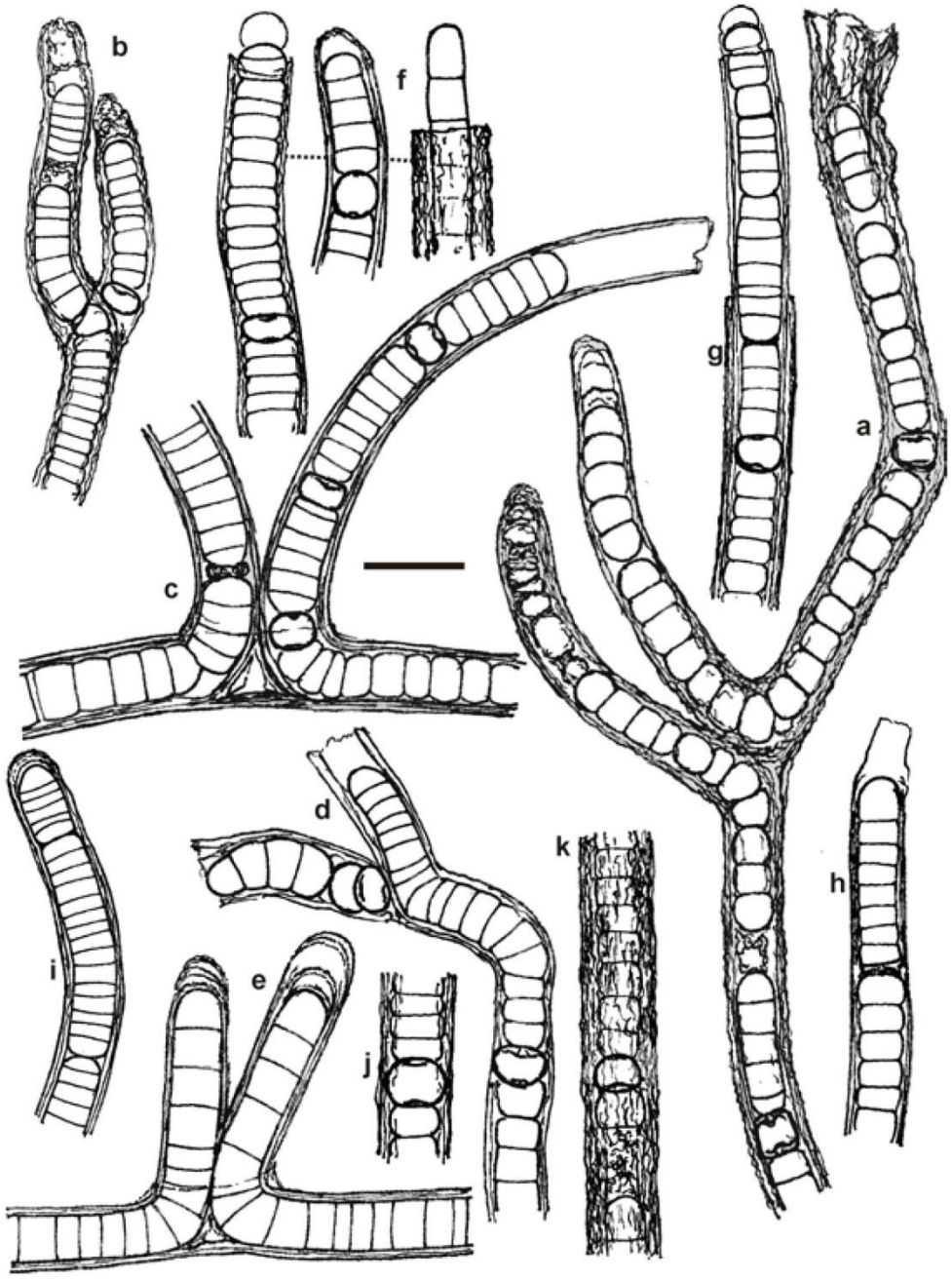


Fig. 10. *Scytonema schmidtii* from the culture (strain CCIBt 3568). Scale bar 20 µm. Orig.

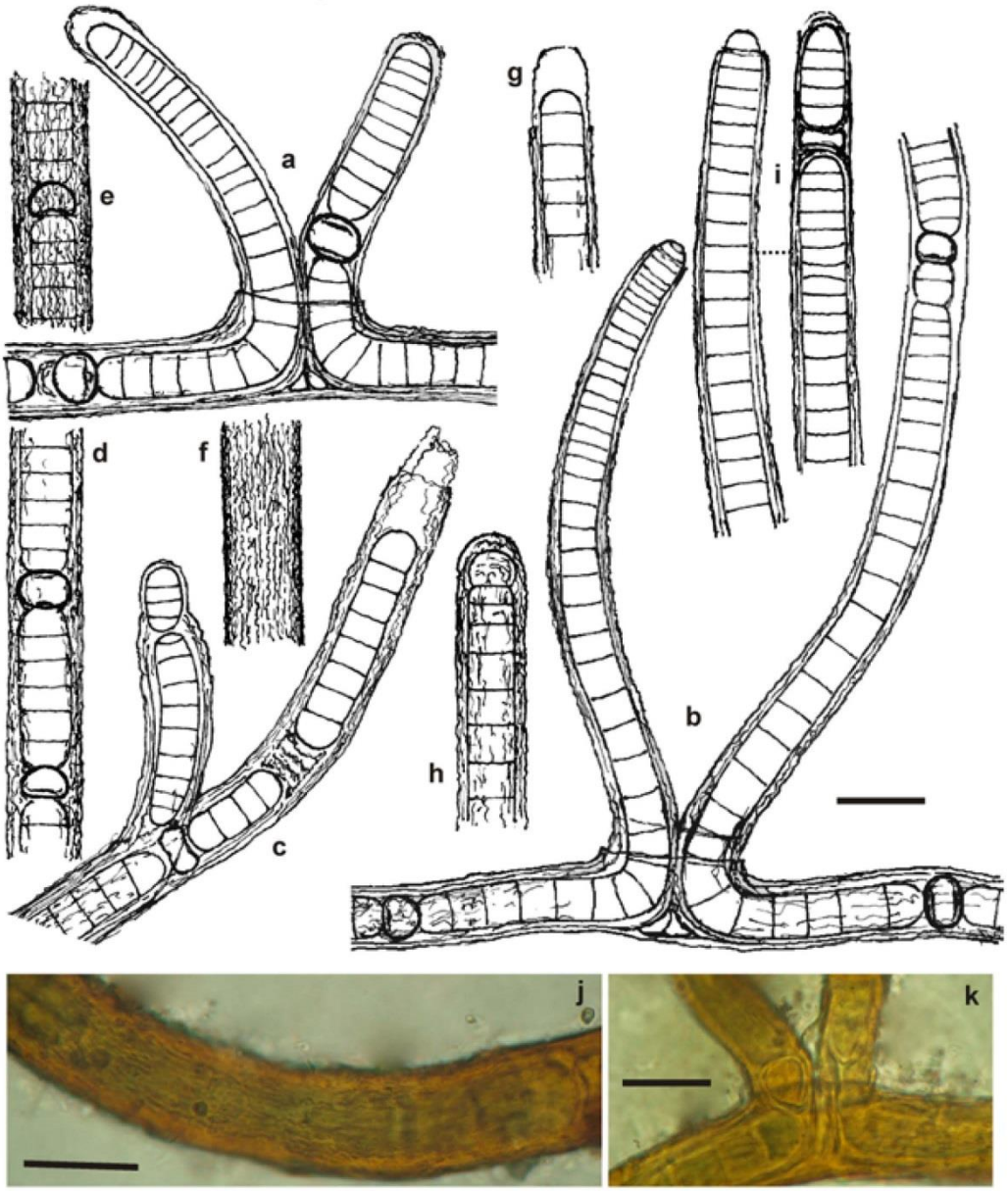


Fig. 11. *Scytonema* cf. *schmidtii* from natural material (Brazil, SP). Scale bars 40 μm.

close to the traditional genus *Scytonema* (FIORE et al. 2007; AGUIAR et al. 2008; SANT' ANNA et al. 2011; VACCARINO & JOHANSEN 2012; BECERRA–ABSALÓN et al. 2013) was recently defined. Further separation and definition of several other generic units from this cluster, which are distinguishable genetically and phenotypically, are expected (Fig. 1, Table 3).

The species included in our paper do not represent the whole diversity of the scytonematacean vegetation neither from Brazil, nor from São Paulo State (cf. SENNA & FERREIRA 1987; SANT'ANNA 1988; SENNA 1994). BÜDEL et al. (2002) recorded *Scytonema myochrous* and *S. ocellatum* from the epilithic cyanobacterial communities of Inselbergs in the Atlantic Rainforest in eastern Brazil. However, we tried to describe precisely most common selected populations in respect to modern taxonomic concepts of the traditional genus *Scytonema sensu lato*, although the examination of natural variability of *Scytonema* populations was not easy. The great diversity of habitats in tropical ecosystems favored the development of numerous differently modified populations and this

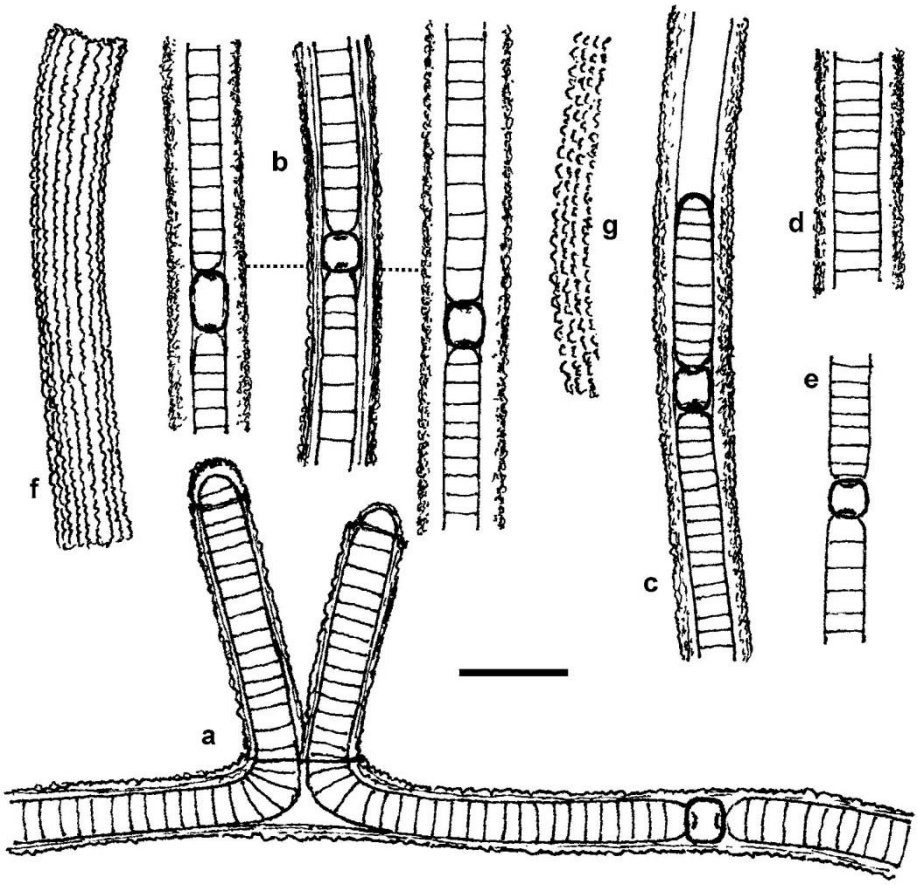


Fig. 12. *Scytonema* sp. ("*verrucosum*") from natural material (from bark of trees in Campos de Jordão, Mata Atlântica; Brazil, SP); g = detail of outside sculpture of a sheath. Scale bar (a–f) 20 μm. Orig.

diversity made then difficult to find two quite identical populations or strains from the same species (i. e. genetically uniform clusters).

The molecular evaluation of the species within the genus *Scytonema* is essential, but the limits in variation of different morphological markers are important for the common knowledge of such cyanobacterial species as well. We present therefore in this article our phenotypical analysis of several dominant *Scytonema* populations from Atlantic Rainforest in SE Brazil. Separation of the traditional genus *Scytonema* in different clusters, which probably represent separate genera according to the modern classification criteria for cyanobacteria (Johansen & Casamatta 2005; Komárek 2010) is continually being confirmed just by molecular sequencing. All the up to now recognized clusters (A, B, C, D), which follow from the phylogenetic tree (based on our own strains and strains included in GenBank), are morphologically recognizable, their autapomorphic characters can be defined and they can be designated as different morphotypes (Bohunická et al. 2012). Members of all four mentioned clusters (morphotypes) were identified during our studies in Brazil primarily according to the morphological markers. The following phenotypic markers are characteristic for *Scytonema* groups, studied in this paper:

(1) Group **A**: It contains the type species of the genus *Scytonema, S. hofmannii.* The characteristic features are (i) cylindrical filaments and cylindrical trichomes of the same width along the whole length up to the ends, (ii) firm and slightly lamellated, but relatively thin, colorless or yellowish sheaths, (iii) ± quadratic cells in old trichomes, and (iv) terminal cells are widely rounded at the ends and do not differ distinctly from other vegetative cells. From our samples, *S. guyanense* and *S. javanicum* belong to this morphotype.
(2) Group **B**: Contains three species (*S. arcangelii, S. hyalinum, S. ocellatum*) with colorless, firm sheaths, usually indistinctly narrowed trichomes in the middle parts, mostly not constricted at cross–walls, and with tendency to fragmentation in shorter segments of trichomes. In our populations characteristical

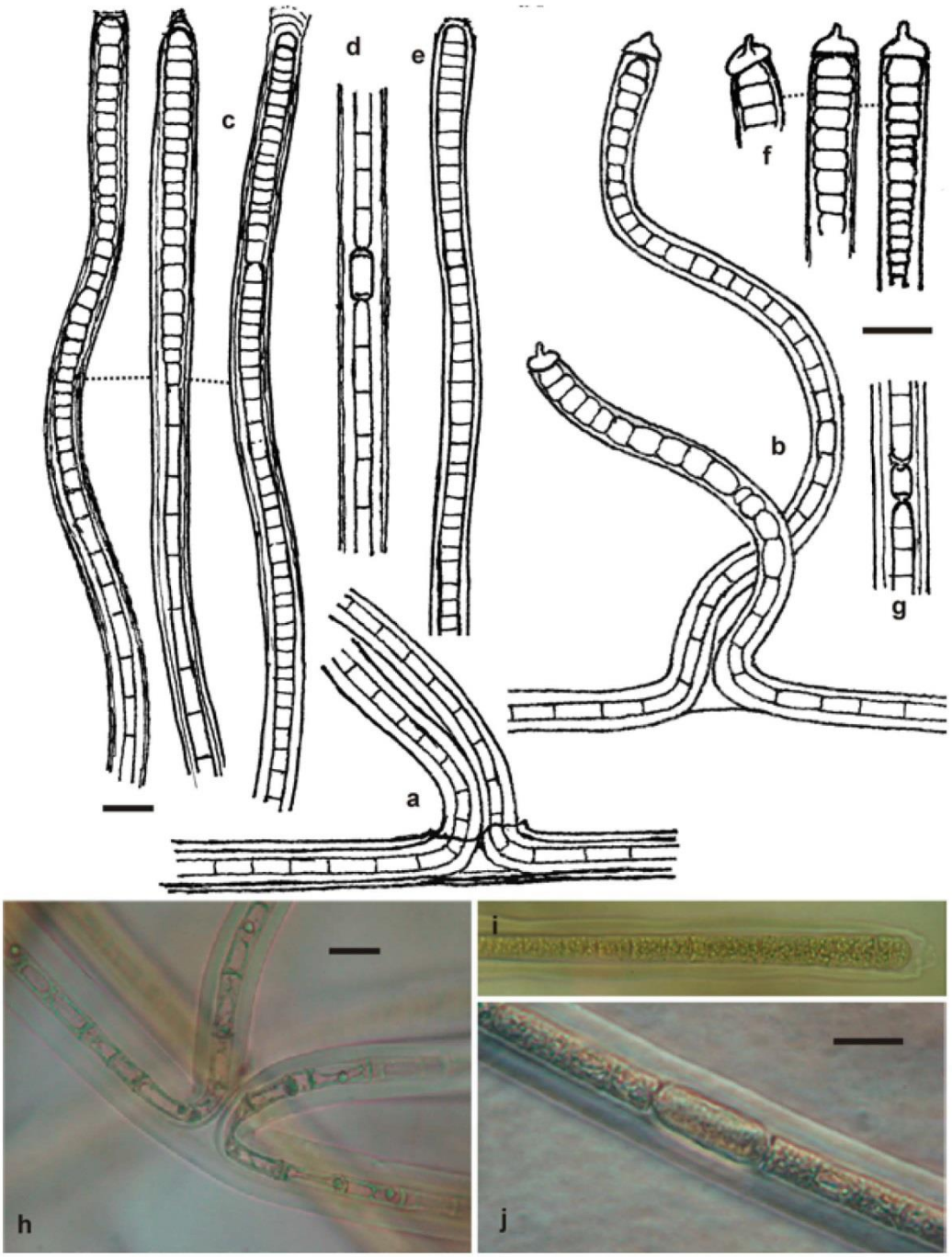


Fig. 13. *Scytonema (Myochrotes) papillicapitatum* from natural material (aquatic, from lake Lagoa de Jacaré, Moji Giuani; Brazil, SP); from type material (SP 188495). Scale bars 20 µm. Orig.

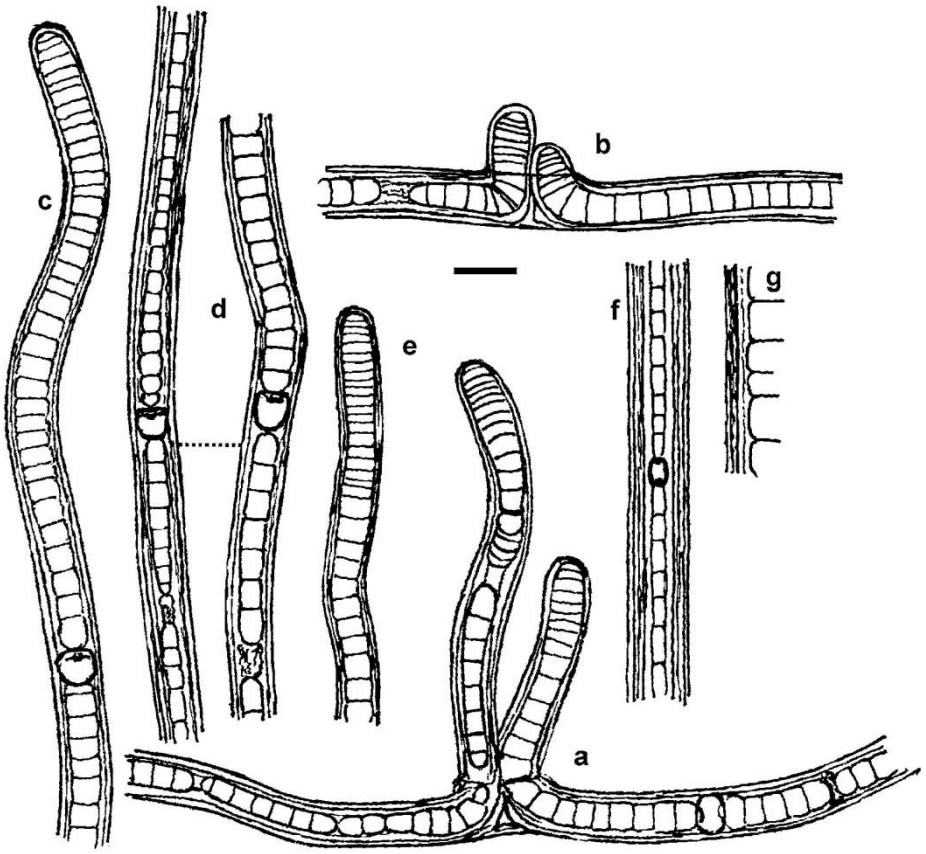


Fig. 14. *Scytonema (Myochrotes)* cf. *longiarticulatum* from natural material (lateritic soils in Jureia; Brazil, SP); (g) detail of a sheath. Scale bar 20 μm. Orig.

terminal meristematic zones were observed, resulting in two or more separated hormogonia with short cells, developing often in rows. Terminal cells are widely rounded.

(3) Group **C**: Included populations have mostly relatively thick, cylindrical filaments and trichomes, not narrowed in the middle and not distinctly widened at the ends, often constricted (facultatively) at cross–walls. The trichomes are composed only of relatively uniform short cells, usually distinctly shorter than wide, rarely up to isodiametric in short segments. Sheaths are firm, thickened, lamellate and often structured from outside when old, frequently of yellow–brown color. Terminal cells in developed filaments are usually little longer than vegetative cells, up to isodiametric or longer than wide and remarkably yellowish. Based on morphology, *S. crispum, S. millei* and *S. polycystum* belong to this cluster; of our materials we assigned *S. bohneri*, *S. stuposum* and *S. crispum* to this group.

(4) Group **D**: This cluster is represented mostly by *S. schmidtii*, which is, however, very variable and forms several slightly different types, the taxonomic evaluation of which is not clear. The thickened, laminated, yellow–brown and from outside usually rugulose sheaths belong to the main characters of this group. Cells are mostly isodiametric in old trichomes and short in terminal parts of branches.

(5) The morphotype of *Myochrotes* (with the type species *S. myochrous*) was not yet sequenced. However, this cluster is distinctly phenotypically different from the typical *Scytonema* and was classified as a separate subgenus or section already by old authors. The trichomes are clearly narrowed in the middle (older) parts, usually with distinctly long (often to 2–4× longer than wide), narrow and cylindrical cells; the cells are shortened and widened only in terminal parts, up to shortly barrel–shaped form. Sheaths are firm, wide, lamellate and often colored (yellow–brown). They resemble therefore rather the genus *Petalonema.* We never have found *S. myochrous* in the material of São Paulo State, but *S.* cf. *longiarticulatum* and the two new species *S. chorae* and *S. papillicapitatum* from the same subgenus were registered in our aquatic, epilithic and soil samples.

Our results must be further checked and confirmed

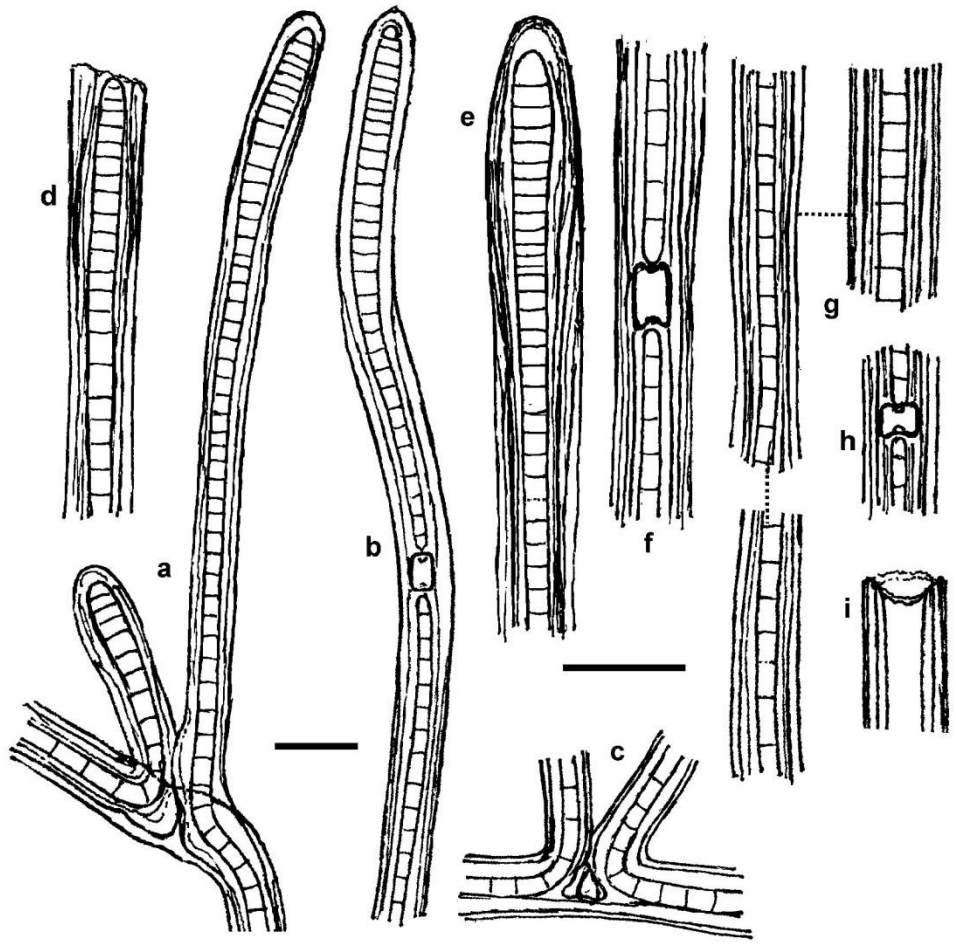


Fig. 15. *Scytonema (Myochrotes) chorae* from natural material (aerophytic, Gruta que Chora near Ubatuba; Brazil, SP); type material (SP 427934). Scale bars 20 μm. Orig.

by more comprehensive molecular analysis in future studies, and folowed by a decisive taxonomical and nomenclatural revision. Especially, the relatively small number of sequenced taxa, and the use of a single molecular marker (16S rRNA gene) did not provide convincing statistical support for higher phylogenetic clustering. A multi–locus evolutionary reconstruction of a wider selection of scytonematacean strains is in progress and should soon bring a better picture of the actual taxonomy within this family of cyanobacteria. However, for now, the detailed phenotypic analyses indicate the probable conformity of revised morphological characters with natural clusters, following from the recent phylogenetic analyses (Table 3). Our results also yield further contribution to the ecological significance and phytogeographical distribution of important terrestrial cyanobacteria in tropical and subtropical regions.

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Anexo II

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Phenotypic studies on terrestrial stigonematacean Cyanobacteria from the Atlantic Rainforest, São Paulo State, Brazil

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Abstract

The terrestrial cyanobacterial microflora of the Atlantic Rainforest is very rich and particular, occurring among numerous epiphytic plant communities, but also growing on mosses, tree barks, various wooden substrates, rocks, and on soil (mainly lateritic). The diversity of terrestrial populations from the genera *Fischerella* (2 species) and *Stigonema* (7 species), collected in different tropical and subtropical zones of the Atlantic Rainforest is described in this article. One of the *Fischerella* morphotypes was not recognizable according to the available literature, and it was described as a new species (*Fischerella clavata*). Several *Stigonema* taxa are morphologically characterized and further data on their variability and diversity are provided. Three new species are described for the genus *Stigonema* (*S. fremyi*, *S. corticola*, and *S. parallelum*), and one is raised to species level (*S. crassivaginatum*).

Key words: biodiversity, Stigonemataceae, new species, Mata Atlântica

Introduction

The Atlantic Rainforest is one of the most important biodiversity hotspots worldwide considering its high number of endemic taxa and high level of impact (Myers *et al.* 2000). This forest was originally distributed along the entire Brazilian coast, but is now restricted only to fragments preserved in National or State Parks, mainly in southeastern Brazil and includes tropical and subtropical zones (Rizzini 1997). The fragmentation of this biome is related to the intense deforestation brought by urbanization, agriculture, pasture, extractivism, and large tourism companies (Leal & Câmara 2005). Certainly, several habitats had already been destroyed before their biodiversity could be studied.

The cyanobacterial diversity of this biome still remains poorly studied, despite recent species and genera of Cyanobacteria that have been described for this area over the past years (Branco *et al.* 2006, Fiore *et al.* 2007, Sant'Anna *et al.* 2011). However, these new taxa belong to cyanobacterial groups other than the stigonematacean types and only one species of *Stigonema* Bornet & Flahault (1886: 62) (*S. gracile* Silva & Sant'Anna 1988: 393) and one of *Hapalosiphon* Bornet & Flahault (1886: 54) (*H. santannae* Lemes-da-Silva, Branco & Necchi-Júnior 2010: 920) have been described for the Atlantic Rainforest.

During our studies on the terrestrial cyanobacteria from the Atlantic Rainforest, we observed that the genus *Stigonema* and related morphotypes are highly diversified in this area. The knowledge of this diversity, including several possible endemic morphotypes, is very important for future phylogenetic studies on this group in tropical and subtropical regions. Obtaining cultures of these organisms is difficult (Hoffmann *et al.* 2005), and this is the main reason why the phylogenetic relationships among the *Stigonema*-like types are still

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poorly understood. In this context, phenotypic studies based on material from nature are appropriate to understand the basic diversity of this group of cyanobacteria. Moreover, defining the species concept of Cyanobacteria is a major problem because of their wide morphological and genetic variability, which complicates cyanobacterial taxonomy. Nowadays, the tendency is to consider as species within a single genus those strains or populations which belong to the "same genotype and morphotype with stable phenotypic features, and more or less stable and distinct ecological limits" (Komárek & Mareš 2012). According to these authors, "a single species must be genetically, ecologically, and morphologically uniform".

Thus, our aim is to show the phenotypic and ecological characterization of eleven stigonematacean Cyanobacteria from tropical and subtropical areas of the Atlantic Rainforest, as a basis for future molecular studies.

Material and Methods

In the State of São Paulo, the Atlantic Rainforest used to cover 68% of the state territory, but nowadays the forest is restricted to only 10% of its original size (SOS Mata Atlântica & INPE 2011). This Biome is composed of distinct types of vegetation disposed on mountain chains or along the littoral zone. In general, the climate of this Biome is warm and humid, but in high mountains (up to 2800 m) negative temperatures can occur. The mean annual temperature is 16 °C and the pluviosity can range from 1800 mm to 4200 mm. In general the mountains are formed by granitic rocks, and the soil is shallow, acidic, nutrient-poor, and always humid.

The samples were collected in different terrestrial habitats in the Atlantic Rainforest (Fig. 1), particularly in protected areas such as the State Park of "Serra do Mar" (Santa Virginia) (23º 20' S, 45º 09' W), State Park

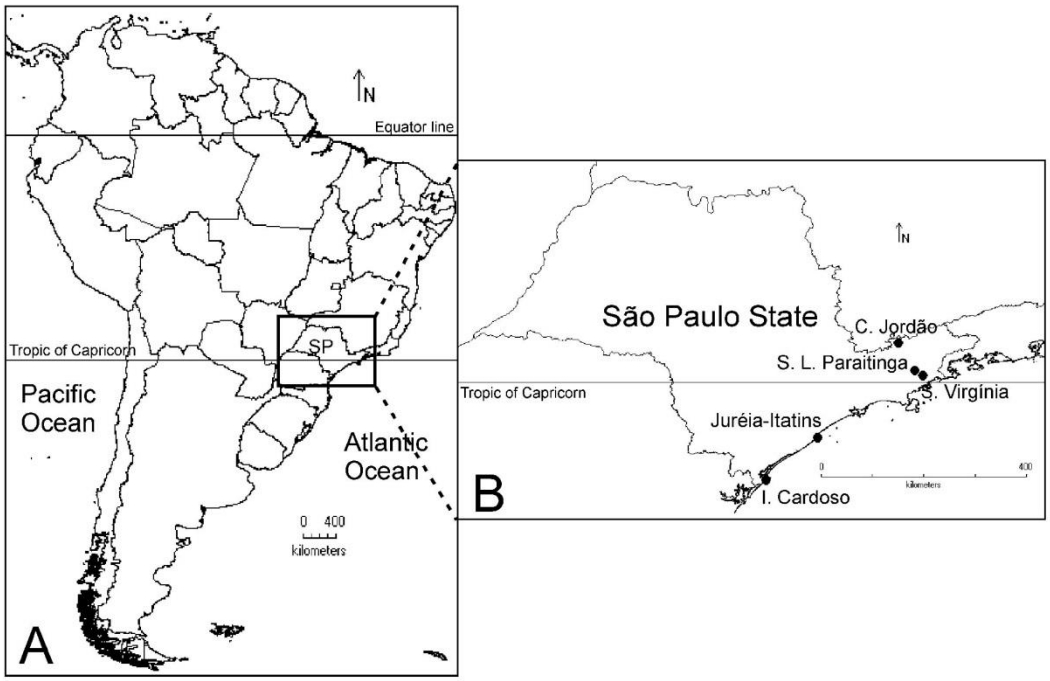

FIGURE 1. A, B. A. Map of South America; São Paulo State (SP) is indicated in Southeast Brazil. **B.** Map of São Paulo State; indication of the studied areas.

of "Ilha do Cardoso" (25º 04' S, 47º 55' W), and the Ecological Station "Juréia-Itatins" (24º 22' S, 47º 04' W). Besides, samples were also collected in the vicinities of the towns of São Luiz do Paraitinga (23º 13' S, 45° 18' W) and Campos do Jordão (22° 44' S, 45° 35' W) where there are still areas of well-preserved forest. Biofilms were scraped on soils, rock surfaces and wood, and were then kept under dried condition inside paper bags. The morphological study was carried out using an optical microscope Zeiss Axioplan 2 after the rehydration of a part of the collected material. Identification of taxa was undertaken based on at least 20 specimens of each population. The species were documented by drawings and digital photographs, which were taken with a Zeiss Axiocam MRc digital camera. Part of the dried samples were preserved in formaldehyde and stored in the Herbarium of the Institute of Botany (SP), Brazil.

Results

Fischerella clavata Sant'Anna, Kaštovský, Hentschke & Komárek, *sp. nov.* (Fig. 2)

Strato expanso, tenui, atrato. Fila primaria repentia, 10–13 µm crassa, praecipue uniseriata, rare cum cellulis binis, plus minusve torulosa, terminaliter et latere ramosissima; vagina firma, tenuis, paucim lamellosa in partes adulties, brunneola. Ramae erectae, distincte tenuior, 5–6 µm latae, cylindricae, ad apices sparsim inflatae et 7–8 µm latae, cum vagina sine colore, tenui. Cellulae subsphaericae vel barriliformes in filis primariis, 4–5.8 × 5.2–9 µm, in ramis cylindricae, 3.9–6.5 × 3.9–5.2 µm. Heterocytae solitariae, intercalares, ovales, semiglobosae, barriliformes ad cylindricae, dimensionibus similaris quam cellulae vegetativae. Reproductio hormogoniis terminalibus, ad apices ramis separantur et filis fragmentatione.—Habitatio: ad solis plus minusve siccis in sylvis humidis montanosis, Mata Atlantica dictis, prope Campos do Jordão, provincia São Paulo, Brasilia.

Type:—BRAZIL. São Paulo: Campos do Jordão, Horto Florestal, preserved sample collected in November 2002, *C.L. Sant'Anna, M.T.P. Azevedo* and *J. Komárek* (Holotype SP 427513!).

Main filaments creeping on the substrate, from which the erect branches grow more or less perpendicular. Main filaments 10–13 µm wide, mostly uniseriate, rarely with two cells parallely arranged. Sheaths more or less firm, thin, in oldest parts slightly lamellate and brownish. Branches distinctly thinner than main filaments, 5–6 µm wide, claviform at the ends, up to 7–8 µm wide. Sheaths in young filaments and in branches thin, colourless. Cell content greyish blue-green, at the ends brownish or yellow-brown (also sheaths). Cells of main trichomes rounded ("moniliform" trichomes) 4.0–5.8 µm long, 5.2–9.0 µm wide, in cylindrical branches 3.9–6.5 µm long, 3.9–5.2 µm wide. Branches are only slightly and sometimes indistinctly constricted at cross-walls. Relatively long, claviform hormogonia separate from ends of branches . The creeping filament can arise again from this separated part of the branch. The creeping filaments can produce also a narrower, cylindrical, branch-like filament at their ends. Heterocytes semiglobose, oval to spheroidal in old trichomes, sometimes lateral, cylindrical in branches, usually of the same size as the neighbouring vegetative cell.

Habitat:—On dry soil.

Etymology:—Species named according to characteristically widened ends of long, thin branches.

Notes:—This morphospecies was not identifiable according to the literature available. It has typical characteristics for *Fischerella* Bornet & Flahault (1886: 100), especially morphologically diverse main filaments and branches, which grow laterally from cells in the main trichomes or from their ends. It is most similar to *F. major* Gomont (1902: 299), but differs by dimensions (branches 6–12 µm wide, trichomes in branches 4–10 µm wide), by cell morphology of the branches, and by ecology. The branches of *F. major* are wider and present barrel shaped cells, while *F. clavata* has narrower branches with elongated cells.

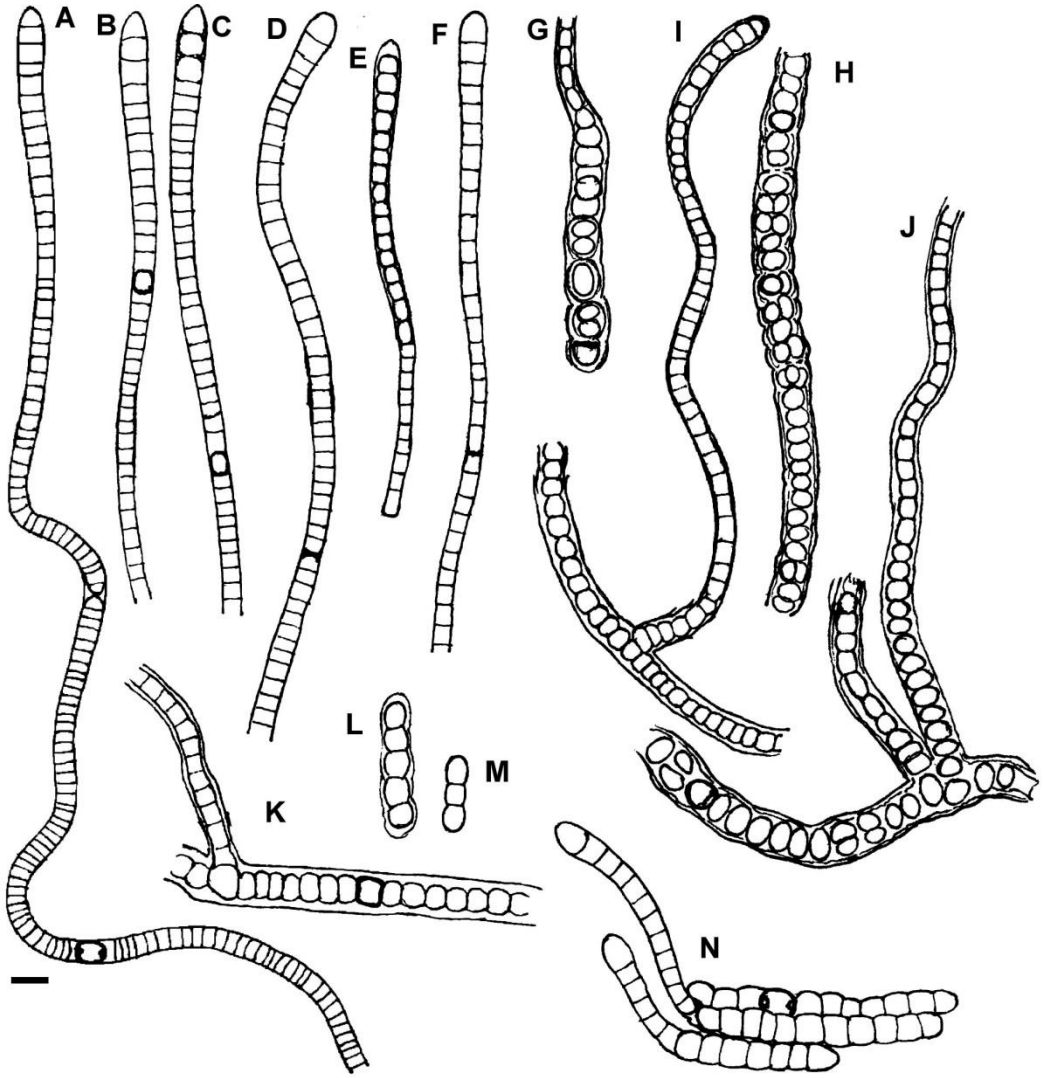

FIGURE 2 A–N: *Fischerella clavata*. A–F: Details of widened apices of branches. G: Uniseriate main filament. H: Main filament with biseriate parts. I–K: Branching in main filaments. L–M: Hormogonia. N: Creeping filaments producing branch-like terminal filaments. Scale: 10 μm in A (use for A–N) .

Fischerella letestui Frémy (1930: 446) (Fig. 3)

Main filaments creeping, more or less cylindrical, mostly monoseriate up to multiseriate or forming basal cell clusters, sometimes fasciculate. Old monoseriate filaments 7–8 μm wide, polyseriate up to 15–20 μm wide. True branches are formed from creeping filaments, usually perpendicular to the main filament, more or less with the same morphology as the monoseriate main filaments, cylindrical, 5–6 μm wide. Trichomes cylindrical, slightly to intensely constricted at the cross walls, 4–6 μm wide. Sheaths thin, distinct, colourless or yellowish in old parts. The cells are cylindrical to barrel-shaped or rounded, more or less isodiametric or slightly longer or shorter than wide. Heterocytes hemispherical, barrel-shaped, cylindrical or cylindrical-rounded, of the same size as the neighbouring vegetative cells. Hormogonia develop at the ends of branches, with distinctly shorter cells, 6–7 μm wide, mostly by the help of necridic cells.

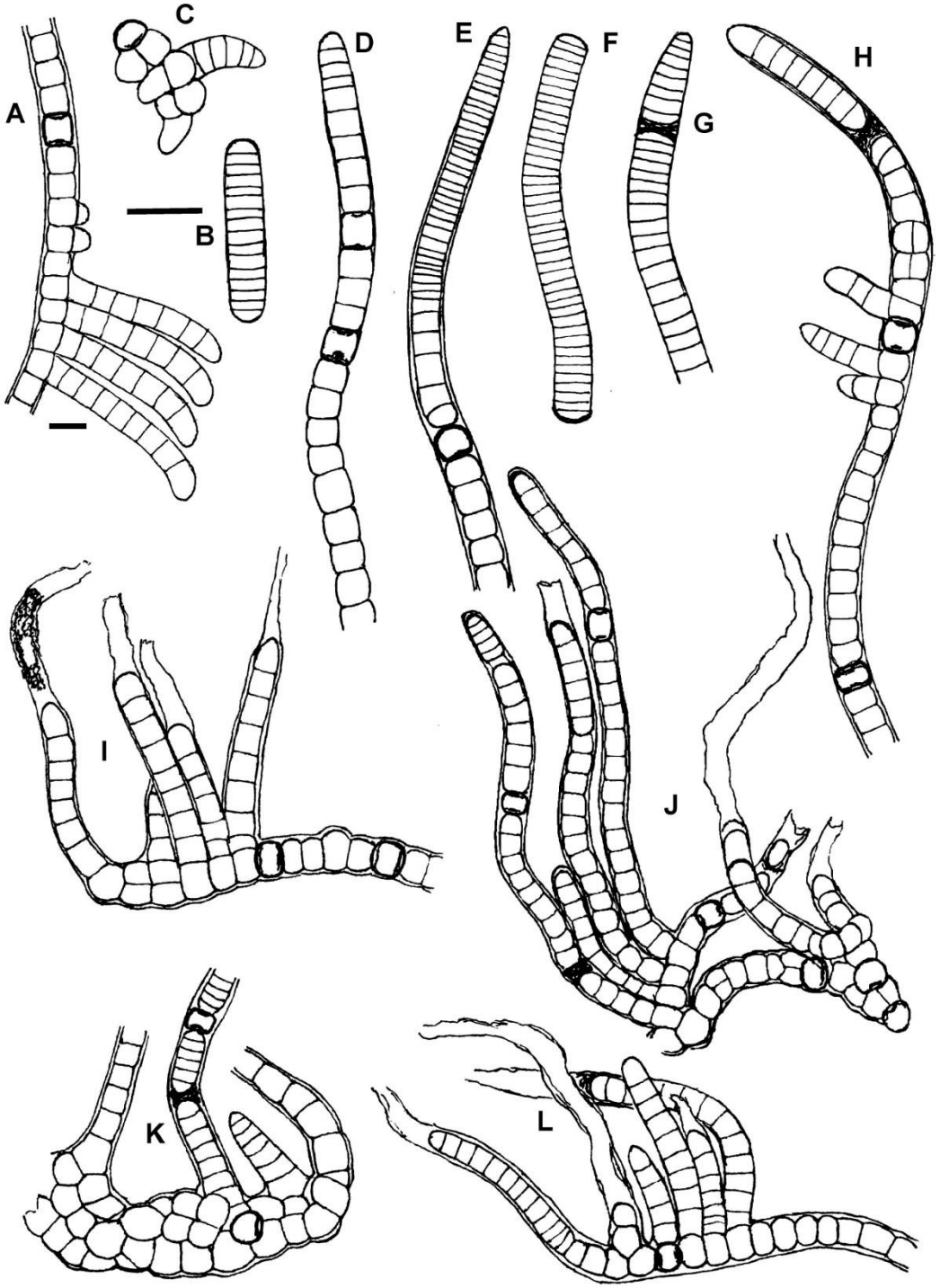

FIGURE 3 A–L: *Fischerella letestui.* A: Detail of branching. B: Hormogonium. C: Initial stage of development. D–G: Details of the apices of branches. H–L: Branched main filaments. Scales: 10 μm in A (use for A, C–E, H–L) and B (use for B, F–G).

Habitat:—On wood found on soil, next to a waterfall in the forest.

Samples examined:—BRAZIL. São Paulo: Campos do Jordão, Horto Florestal, 8 November 2002, *C.L. Sant'Anna, M.T.P. Azevedo* and *J. Komárek* (SP 427512).

Notes:—This species corresponds exactly to the description of *F. letestui* from Africa (Frémy 1930), except for shorter cells in the branches (in original description of *F. letestui* these are 1.5–2.0 × longer than wide). The polyseriate parts of the main filaments are rare and correspond rather to irregular groups of cells. Similar groups also occur in initial stages after germination from hormogonia.

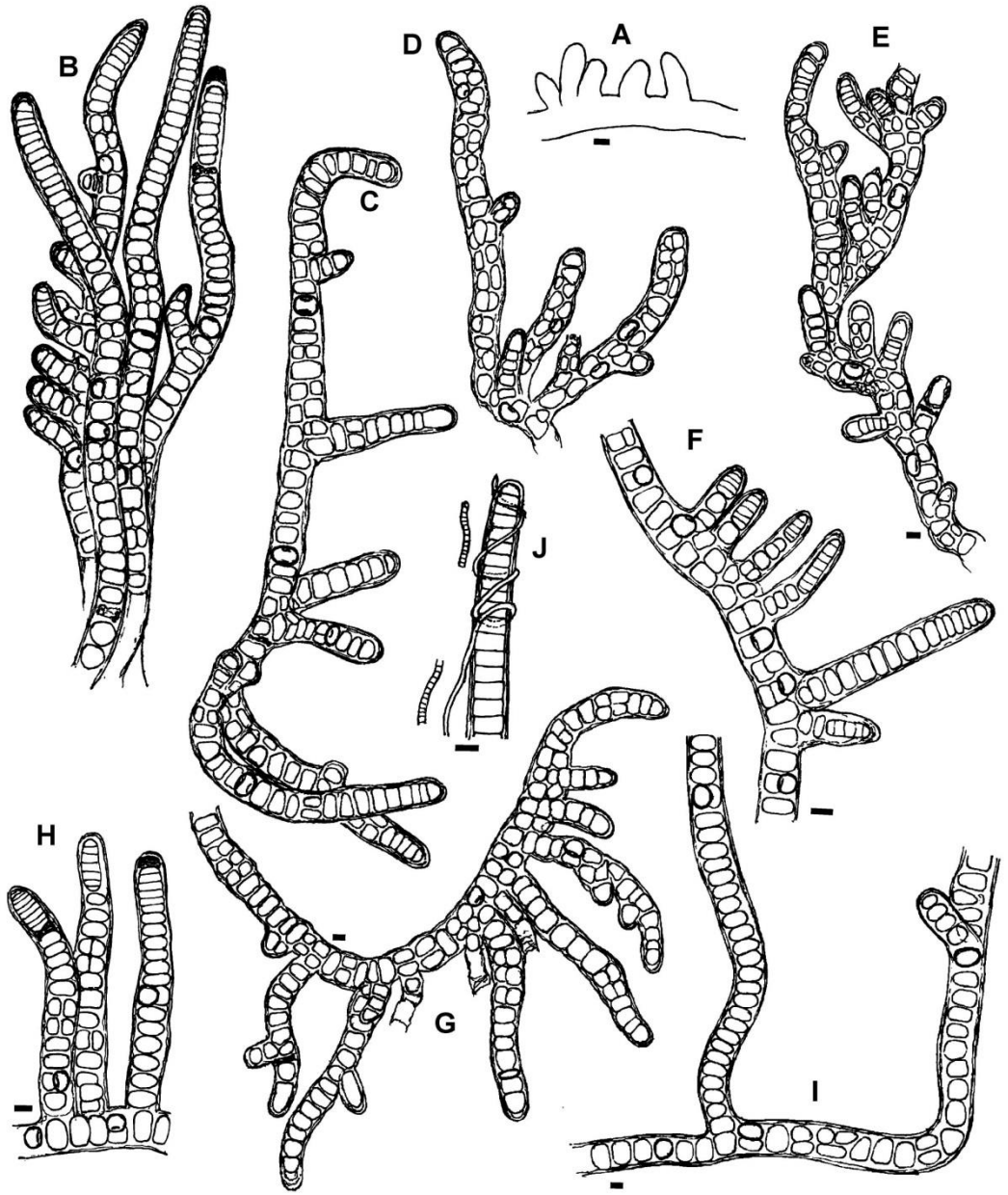

FIGURE 4 A–J: *Stigonema cf. minutum*. A–G: General aspect of a thallus. H: Branches forming hormogonia at the ends. I: Young filament. J.: Detail of apex with epiphytic filament of *Heteroleibleinia* sp.. Scales: 10 µm A (use for A–D) and E–J.

Stigonema cf. minutum Hassall ex Bornet & Flahault (1886: 72) (Figs 4, 5)

Main filaments mono or biseriate, composed by rows of cells, 15–35 µm wide, with firm, delimited, brownish sheaths. Branches are morphologically the same as the main filaments, slightly and continually narrowed at the end, often ending in monoseriate parts, 15–30 µm wide. Trichomes moniliform, 12–30 µm wide, with compressed or irregularly rounded cells, 6.5–10.0 µm long, terminal cells sometimes slightly elongated and rounded at the end. Lateral heterocytes rounded, intercalary compressed, with the same dimensions as the cells. Hormogonia develop at the ends of branches, usually 4–8 cells.

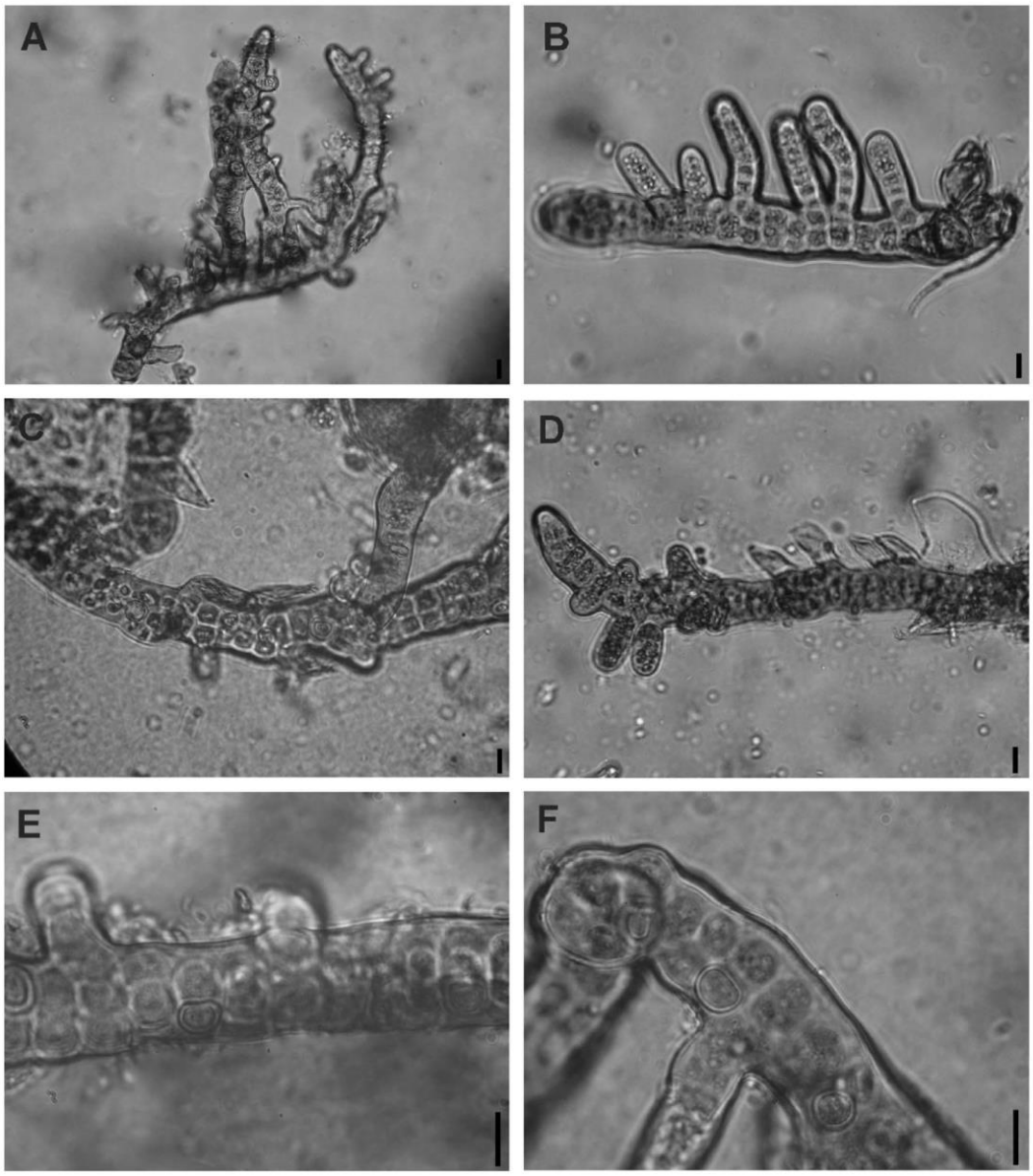

FIGURE 5 A–F: *Stigonema cf. minutum.* A: General aspect of a thallus. B: Unilateral branching. C–D: Branches after releasing hormogonia. E–F: Details of main filaments. Scales: 10 µm.

Habitat:—On lateritic surface of termites nests, on the soil and on wet rocky surfaces.

Samples examined:—BRAZIL. São Paulo: Campos do Jordão, Horto Florestal, 9 November 2002, *C. L. Sant'Anna, M.T P. Azevedo* and *J. Komárek,* (SP 427507, SP 427509, SP 427511); State Park of "Ilha do Cardoso", 29 June 2010, *W.A. Gama Jr.* and *C.F.S. Malone* (SP 401436).

Notes:—This species is considered cosmopolitan in unpolluted soil habitats and on rocky substrates (Geitler 1932). As other commonly distributed species, it has a great morphological variability and has been described under various concepts. We identify this species in the original sense, corresponding to Geitler (1932). The Brazilian populations are similar to *S. minutum* Bornet & Flahault (1886: 72) according to descriptions in the literature, but there are doubts about their identity in European populations. Even within the Brazilian populations, there are slight differences, indicating that this group requires a revision comparing the tropical/subtropical populations with those from temperate regions.

Stigonema fremyi Sant'Anna, Kaštovský, Hentschke & Komárek, *sp. nov.* (Fig. 6)

Strata subaerophytica, fasciculata vel aggregata, tomentosa, atro-olivacea. Fila ramique similares, multiseriata, 18–50 µm lata; rami de filis principalis plus minusve ad angulum 90º divaricati, ad apices gradatim attenuati, cum segmentis curtis terminalibus uniseriatis, ad 25 µm latis, apice rotundati. Vaginae firmae, distinctae, paucim laminosae, luteo-fuscae. Cellulae irregulariter sphaericae, 11–20 µm diametro. Heterocytae solitariae, intercalares, dimensionibus similaris cellulis vegatativis. Hormogonia curta, praecipue uniseriata, terminaliter separantur.—Habitatio: Aerophytice ad saxa et rupes facultative madidas, in montibus "Mata Atlantica" dictis, prope Campos de Jordão, provincia São Paulo, Brasilia.

Type:—BRAZIL. São Paulo: Campos do Jordão, preserved sample collected on 9 November 2002, *C.L. Sant'Anna, M.T.P. Azevedo* and *J. Komárek* (Holotype SP 427508!).

Filaments forming dense clusters, morphologically similar to the branches which separate them from the main filaments in right angles; both polyseriate, (18)28–45(50) µm wide. Branches narrowed at the base and toward the ends, with short uniseriate segments in terminal parts, ends rounded, up to 25 µm. Sheaths firm, limited, mostly yellow-brown. Cells irregulary sphaerical, 11–20 µm diameter. Heterocytes numerous, intercalar or lateral, with the same dimensions as the cells.

Habitat:—On rocky walls.

Etymology:—Species is named to the honor of the famous French specialist in Cyanophyceae (Cyanobacteria) Abbé Pierre Frémy, who first described and documented similar *Stigonema* populations from African tropical localities.

Notes:—The Brazilian population with polyseriate filaments and more or less morphologically identical branches (only slightly narrower) corresponds well to the concept of *S. minutum sensu* Frémy (1930). However, these specimens clearly differ from the original description of *S. minutum* (Geitler 1932) by the morphology of polyseriate filaments, dimensions, ecology, and also by the occurrence in tropical habitats. The type of branching is very characteristic in the studied material as well as in Frémy´s material from Africa: branches are often characteristically narrowed at the initial point from the main filaments. Based on our studies of populations corresponding to the original description of *S. minutum,* we could confirm that our material, and also that of *S. minutum sensu* Frémy are specifically tropical species, clearly different from the typical *S. minutum* described in temperate regions. Thus, we named the new species *S. fremyi*, which includes the material from Africa identified by Frémy (1930) as *S. minutum* and the Brazilian material.

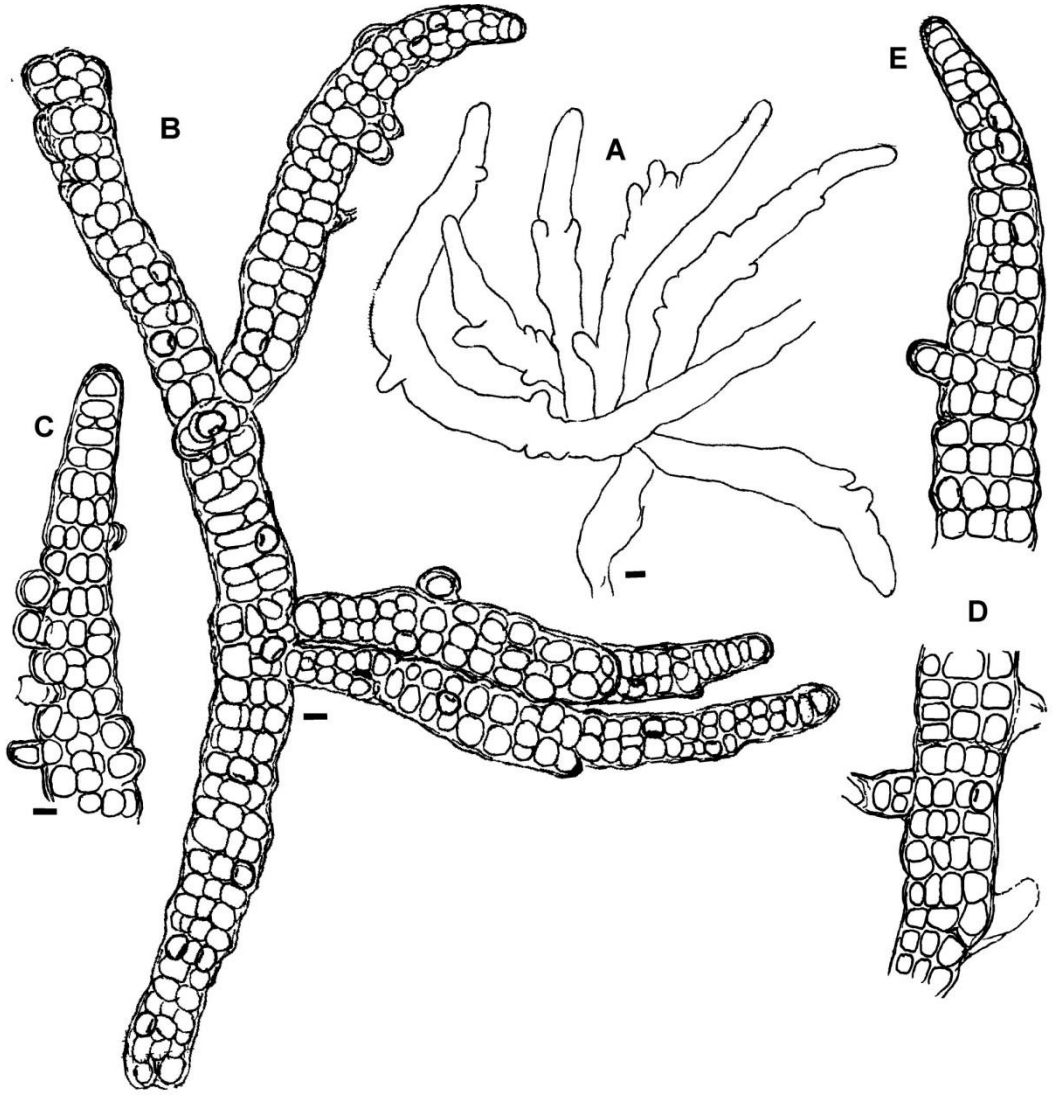

FIGURE 6 A–E: *Stigonema fremyi.* A, B: General aspects of thallus. C–E: Details of attenuated branches. Scales: 10 µm in A, B, C (use for C–E).

Stigonema flexuosum West & West (1897: 293) (Fig. 7)

Filaments entangled, mostly creeping on the substrate, uniseriate, only occasionally with two cells aside, more or less cylindrical, 15–20(22) µm wide. Morphology of the main filaments and branches is almost the same, branches sometimes slightly narrower, rounded at the ends. Sheaths slightly lamellate, mostly slightly brownish. Trichomes moniliforms, 13–17 µm wide. Cells rounded, usually shorter than wide, after lengthwise division almost isodiametric, 9–15 µm long. Heterocytes numerous, mostly lateral 10–13 µm wide, less frequently intercalar, with the same dimensions as the cells. Hormogonia short, cylindrical, composed of shortened cells, uniseriate, separated from sheaths at the ends of branches.

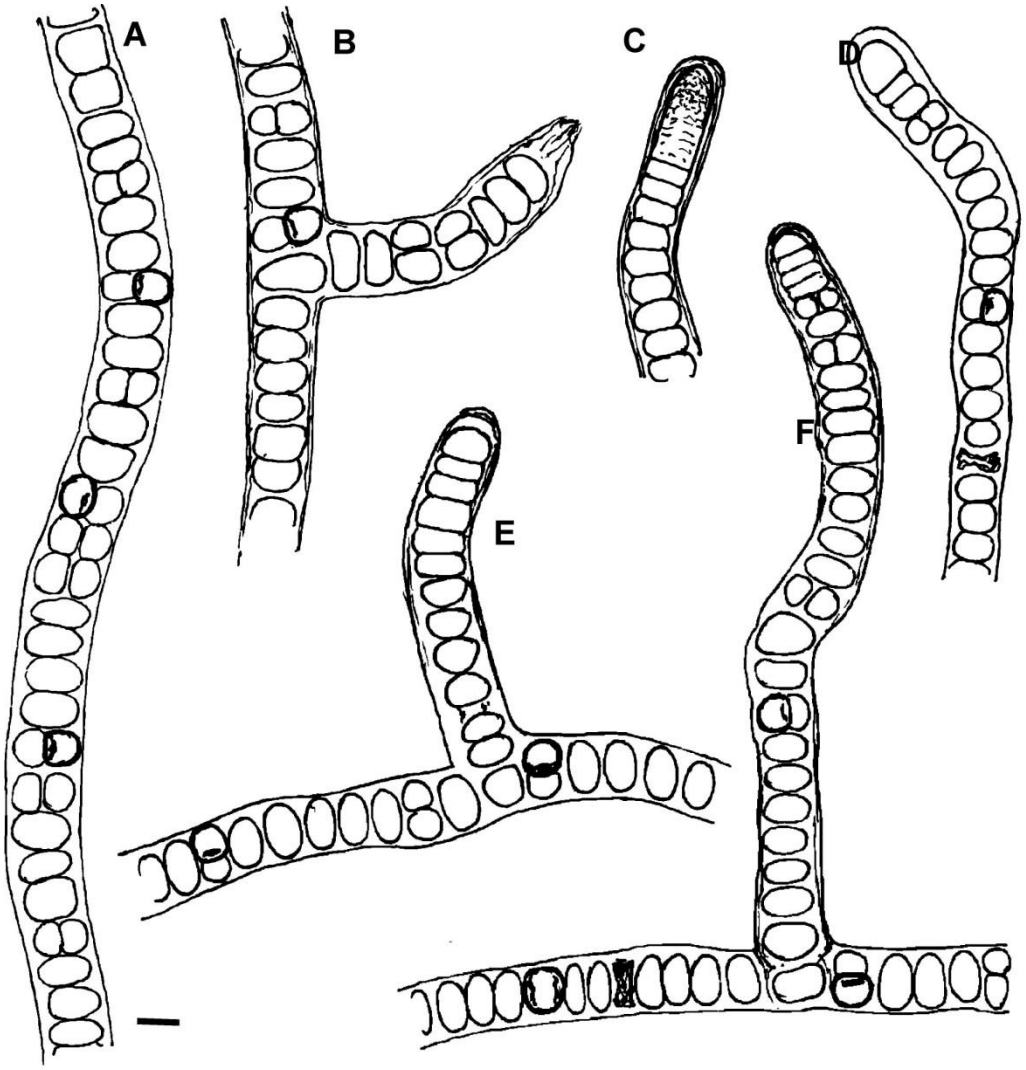

FIGURE 7 A–F: *Stigonema flexuosum*. A: Detail of a biseriate filament. B: Branch after the release of a hormogonium. C: Branch forming a terminal hormogonium. D: Detail of a branch. E, F: Branched filaments with heterocytes. Scale: 10 µm in A (use for A–F).

Habitat:—On wet soil.

Samples examined:—BRAZIL. São Paulo: Campos do Jordão, Mata Atlântica, 9 November 2002, *C.L. Sant'Anna, M.T.P. Azevedo* and *J. Komárek* (SP 427510); State Park of "Ilha do Cardoso", 29 June 2010, *W.A. Gama Jr.* and *C.F. da S. Malone* (SP 401439).

Notes:—The Brazilian populations correspond almost exactly to the original description of *S. flexuosum,* and the ecology is not very different (wet soils, rocky substrates, stagnant waters). However, very similar populations were often recorded from tropical regions under different names (*S. ocellatum* Bornet & Flahault 1886: 69*, S. hormoides* Bornet & Flahault 1886: 68 or *S. hormoides* var. *africanum* Fritsch 1923: 370). The taxonomy of these similar types is still unclear and should be resolved with culture studies and experimental methods.

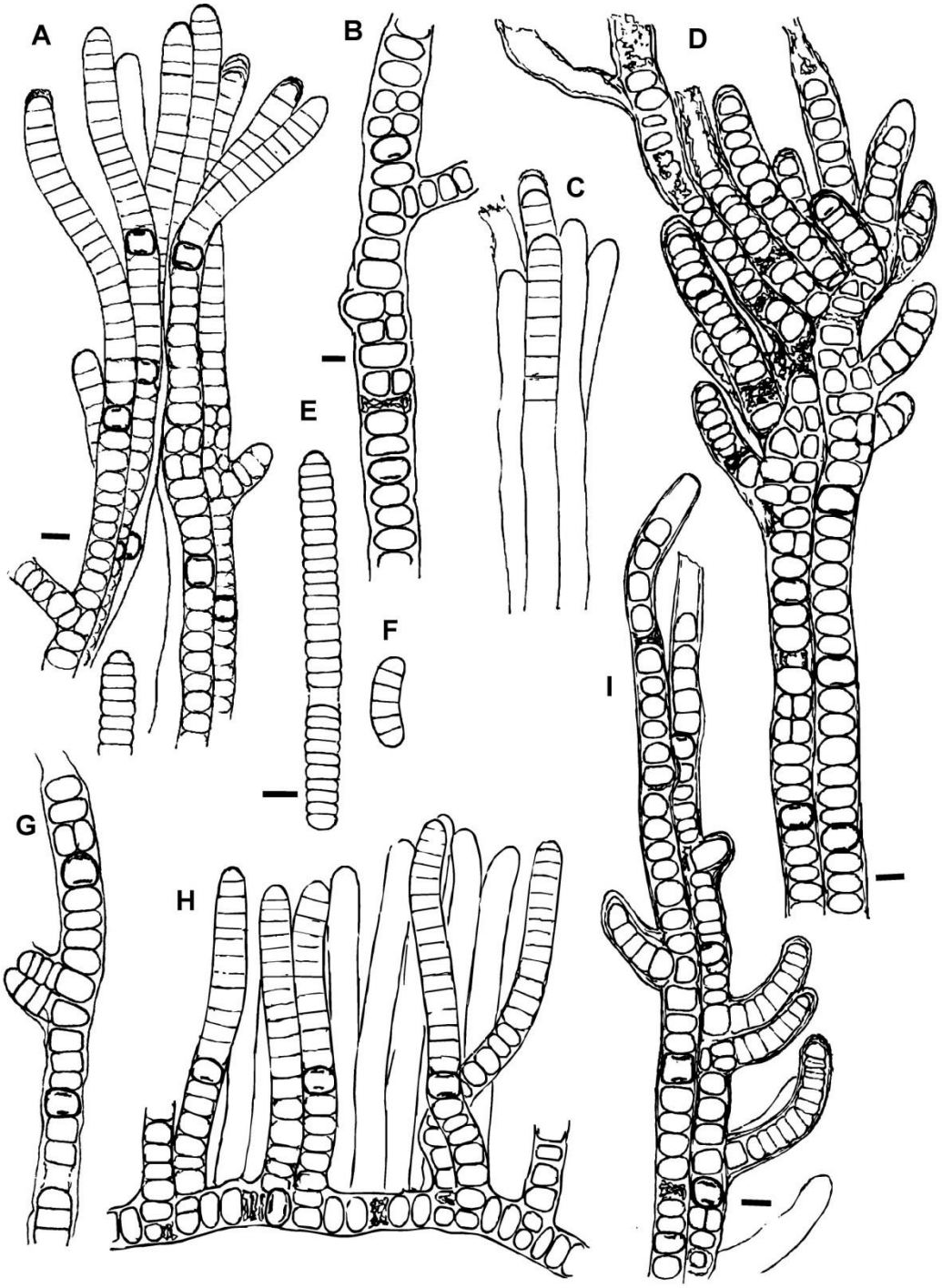

FIGURE 8 A–I: *Stigonema tomentosum*. A: Fasciculated filaments. B: Detail of biseriate filament. C: Detail of apex of branches. D: Branched filaments. E, F: Hormogonia. G: Initial stage of branching. H: Parallel erect branching. I: Branched uniseriate filaments. Scales: 10 μm A, B (use for B, C), D, E (use for E–H) and I.

Stigonema tomentosum (Kützing) Hieronymus (1895: 166) (Fig. 8)

Thallus forming a black thin layer on the substrate, slightly woolly surface. Creeping filaments from which grow numerous fasciculated, more or less erect branches, similar in morphology. Creeping filaments cylindrical, uniseriate to biseriate, 15–20 µm wide. Branches rise perpendicular from the main filaments, often near one another, 12–17 µm, forming erect fascicles, usually divaricated at the apical region. Sheaths yellow-brown. Trichomes moniliform in the main filament, 12–15 µm wide, with rounded and compressed cells 6–10 µm long; and almost not constricted in the apical parts of the branches. Branches 11–15 µm wide, with shorter than wide cells, 4–10 µm long. Heterocytes intercalary arranged, short barrel-shaped up to rounded-cylindrical, generally 5–8 µm wide, 10.5–11.5 µm long. Hormogonia formed at the ends of branches, composed of 5–18 short cells, distinctly constricted at cross walls.

Habitat:—On wet wooden substrate.

Samples examined:—BRAZIL. São Paulo: Campos do Jordão, Horto Florestal, 9 November 2002, *C. L. Sant'Anna, M.T P. Azevedo* and *J. Komárek* (SP 427513).

Notes:—This species is not very common and is rarely recorded, probably in different senses according to various authors. For this reason, the identification of this species is unclear. The fasciculation of trichomes is its main characteristic. *Stigonema tomentosum* grows on wet rocks and wood, but it has been recorded from very distant areas worldwide, both from temperate and tropical regions. The material from the Atlantic Rainforest corresponds quite well to the original description of this species, mainly in relation to large, flat, blackish woolly mats on a wet wooden desk. The principal characteristics of this species are the simple, densely arranged, fasciculated trichomes. In basal parts numerous creeping main filaments occur, from which parallel branches grow erect.

Stigonema crassivaginatum (Geitler) Sant'Anna, Kaštovský, Hentschke & Komárek, *comb. et stat. nov.* (Figs 9, 10)

Basionym:—*Stigonema hormoides* var. *crassivaginatum* Geitler, Arch. Hydrobiol. Suppl. 12: 629, 1933 (Geitler 1933).
Type:—BRAZIL. São Paulo State: São Luiz do Paraitinga, 23º13'S, 45°18'W, 9 November 2002, *C.L. Sant'Anna, M.T.P. Azevedo & J. Komárek* (holotype: SP 427514).

Filaments creeping, more or less straight, uniseriate, narrowed towards the ends (both trichomes and filaments), 20–25 (40) µm wide. Branching relatively rare, branches short, usually conical, 12–15 µm wide at the end. Sheaths relatively thick, distinct, delimited, stratified, yellow-brown, colorless at the end and in young branches, closed and round. Trichomes constricted at cross walls, attenuated, composed of near-regular rows of slightly compressed round cells, 12–14 µm wide, at the ends 7–8 µm. Heterocytes rarely hemispherical, usually barrel-shaped or shortly barrel-shaped, of the same size as the neighbouring vegetative cells. Hormogonia are formed at the end of narrowed branches, cylindrical, almost without constrictions at cross-walls.

Habitat:—On rocks.

Samples examined:—BRAZIL. São Paulo: Ecological Station "Juréia-Itatins", 15 August 2011, *C.L. Sant'Anna* (SP 427307).

Notes:—*Stigonema hormoides* is a very variable species and is recorded under different concepts (Frémy 1930, Silva & Sant'Anna 1996). According to Bornet & Flahault (1886), *S. hormoides* var. *hormoides* presents subglobose cells and rarely branched subtorulose filaments that can be uni- or biseriate The tropical material from Brazil and from the Sunda Islands presents uniseriate and non-subtorulose filaments with frequent branching and compressed cells. Moreover, the typical populations of *S. hormoides* grow in peaty, acidic habitats in temperate regions, where this species was described, while the tropical populations were found on wet rocks. The concept of subspecific taxa in cyanobacterial taxonomy is unclear, and the taxon from Brazil and from Sunda Islands should be recombined to a status of species rank.

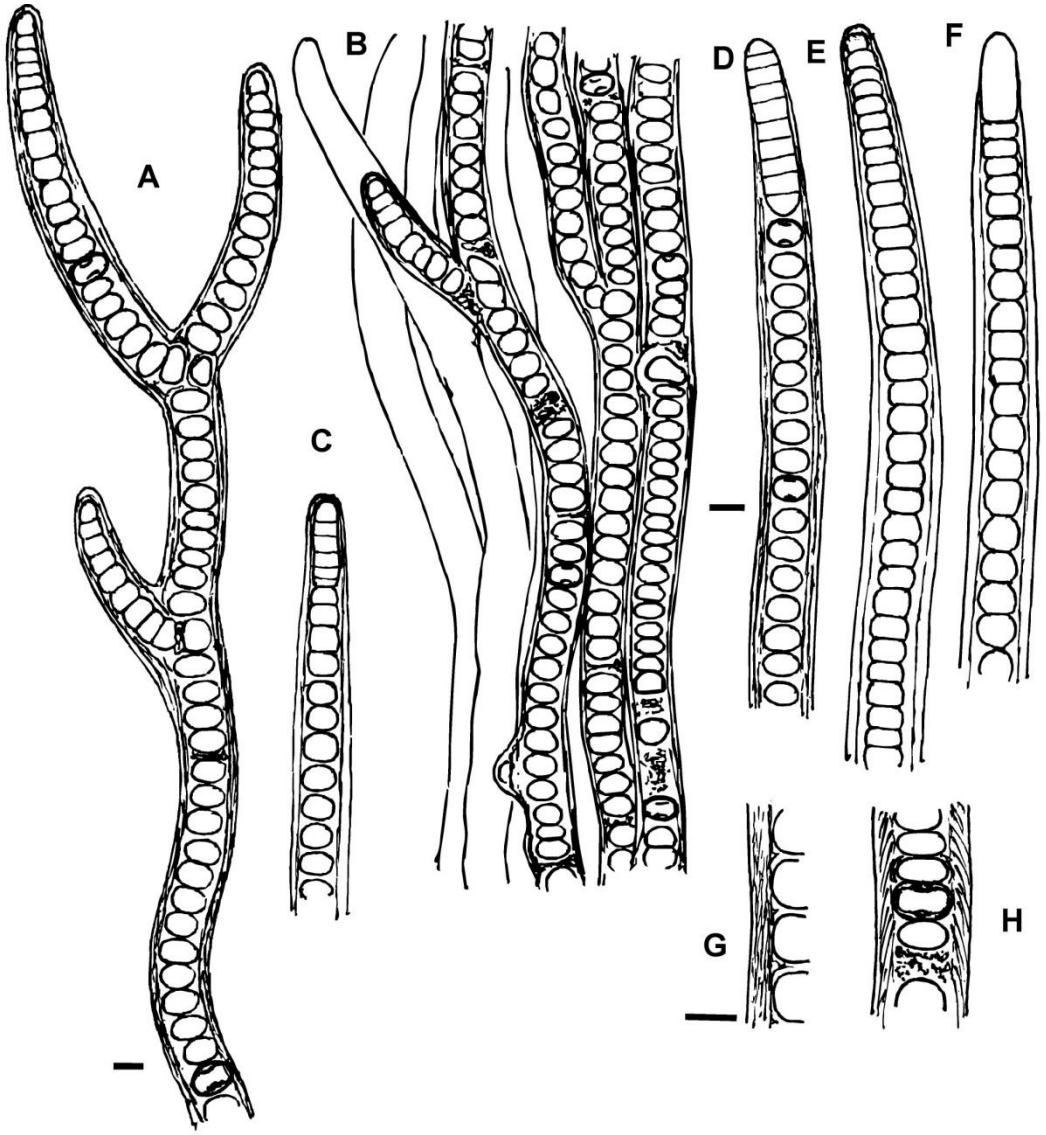

FIGURE 9 A–H: *Stigonema crassivaginatum*. A: Branched filament with attenuated ends. B: Strictly uniseriate filaments. C: Formation of a hormogonium. D–F: Morphological variability of cells and apex. G: Sheaths with parallel lamellae. H: Sheaths with divergent lamellae. Scales: 10 μm in A (use for A–C), D (use for D–F) and G (use for G, H).

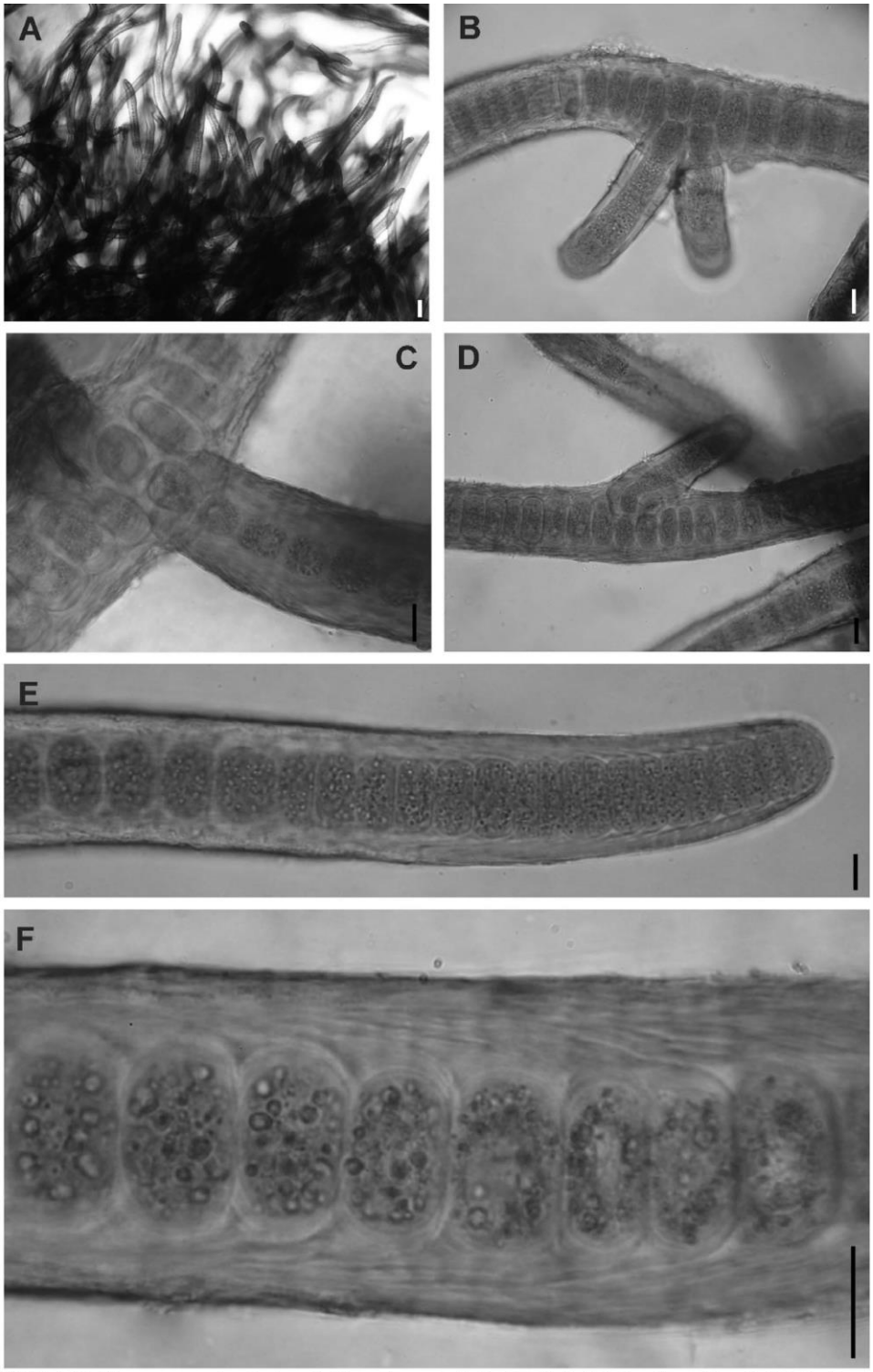

FIGURE 10 A–F: *Stigonema crassivaginatum*. A: General aspect of a thallus. B–D: Details of branching. E: Detail of an apex. F: Lamellated sheaths. Scales: 50 μm in A and 10 μm in B–F.

Stigonema corticola Sant'Anna, Kaštovský, Hentschke & Komárek, *sp. nov.* (Figs 11–13)

Strata aerophytica, fasciculata cum filis irregulariter et dense aggregatis, praecipue nigro-fusca. Fila irregulares, flexuosa, multiseriata, rarissime uniseriata, saepe in agglomerationes cellulis transientes, cum ramis numerosis curtis, plus minusve filis principalis similares, apice rotundatae, saepe cum cellula solitaria, irregulariter rotundata terminatae; fila 12–40 µm lata, ad apices 10–15 µm lata. Numerosae agglomerationes cellularum irregularium. Vaginae firmae, distinctae, homogeneae vel lammelosae, sine colore vel luteo-fuscae, apice clausae. Cellulae irregulariter sphericae, ad 10 µm in diametro, contentu fuscescente. Heterocytae solitariae, intercalares, praecipue laterales, hemisphaericae 5–6 × 6–7 µm. Hormogonia curta, uniseriata, cylindrica, cum cellulis curtis, apice separantur.—Habitatio: Aerophytice vel epiphytice, praecipue ad cortice arborum, minus quam superficie solis praesens, in sylvis in montibus "Mata Atlantica" dictis, provincia São Paulo, Brasilia.

Type:—Brazil. São Paulo: State Park of "Serra do Mar" (Santa Virginia), preserved sample collected on 22 February 2010, *W.A. Gama Jr.* and *E.M. Caltran* (Holotype SP 427515!); Ubatuba, 9 november 2002, *C.L. Sant'Anna, M.T.P. Azevedo* and *J. Komárek* (Isotype SP 427511!).

Clusters of densely agglomerated filaments, not distinctly separated in the main filaments and branches. Filaments are mono- to polyseriate, relatively short, flexuous, sometimes wart-like, irregular, usually from 12 (monoseriate) to 40 (polyseriate) µm wide, ends of branches about 10–15 µm wide. Numerous stages with irregular cell agglomerations. Sheaths distinct, homogeneous or lamellate, hyaline to yellow-brown, closed at the ends. Cells irregularly round, up to 10 µm in diameter, apical cells usually solitary, larger and rounded from outside, cell content brownish. Heterocytes mainly hemispherical, usually lateral, rarely intercalar, 5–6 µm long, 6–7 µm wide.

Habitat:—On tree barks or in soil among mosses.

Etymology:—Species named according to the main occurrence on bark of trees.

Notes:—*Stigonema corticola* is an interesting stigonematacean type with closely agglomerated and frequently branched, uni-to multiseriate, isopolar filaments. The main filaments are only slightly recognizable and are very similar to the branches. *Stigonema corticola* probably belongs to the vicinity of *S. minutum,* but differs in morphology mainly because of the presence of an amorphous stage (agglomeration of cells with lateral heterocytes) in young individuals of *S. corticola*. In addition, both types differ in ecology, since *S. minutum* from northern Europe is typically epilithic, while the tropical *S. corticola* typically grows on tree barks or less frequently on soils.

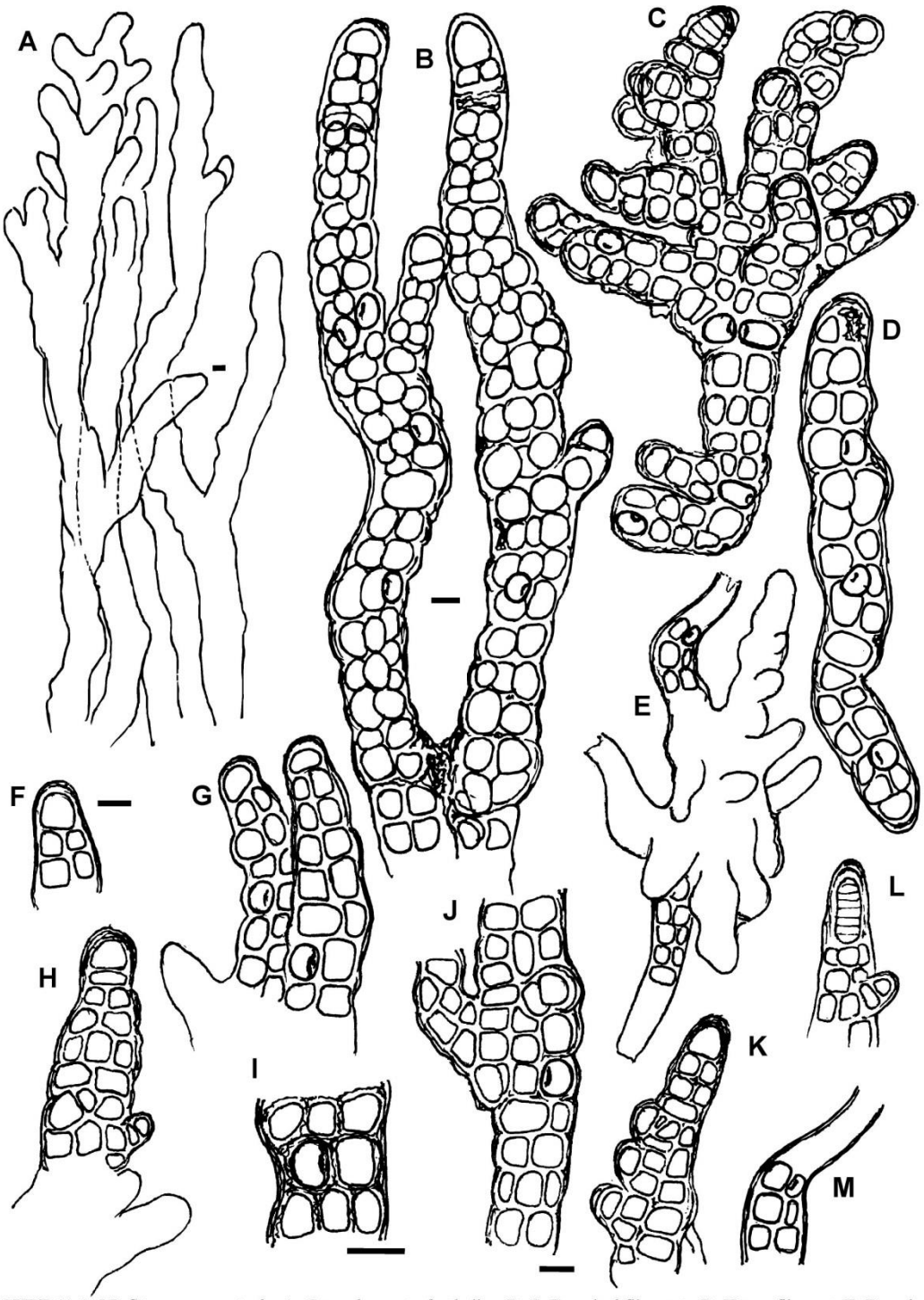

FIGURE 11 A–M: *Stigonema corticola*. A: General aspect of a thallus. B, C: Branched filaments. D: Young filament. E: Branches with agglomerated cells. F–H: Details of apex. I: Filament with a lateral heterocyte. J: Detail of a multiseriate filament. K: Initial stages of branching near the apex. L: Formation of hormogonium. M: Apex after releasing hormogonium. Scales: 10 μm in A (use for A, E), B (use for B–D), F (use for F–H), I and J (use for J–M).

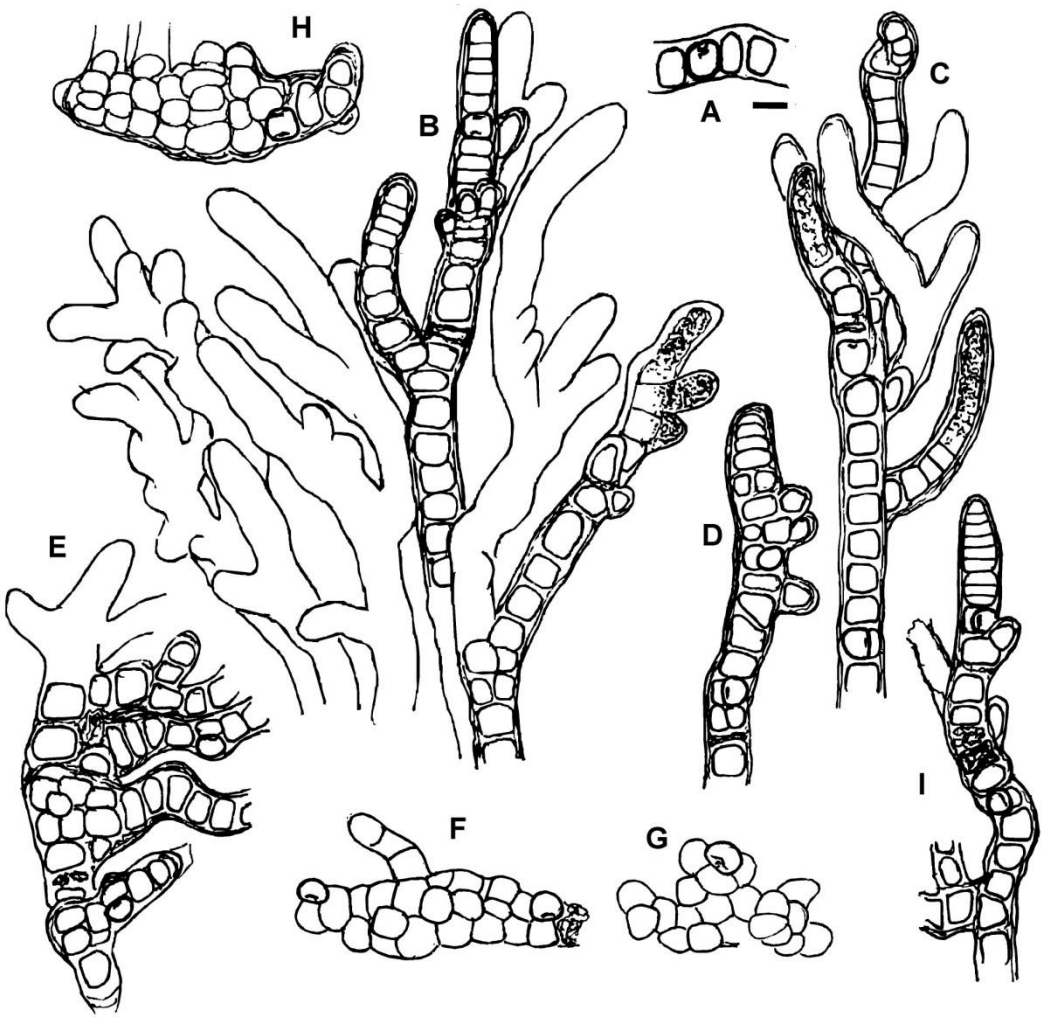

FIGURE 12 A–I: *Stigonema corticola*. A: Uniseriate filament with lateral heterocyte. B–D: Mostly uniseriate filaments. E: Richly branched filaments. F–H: Agglomerated cells. I: Formation of a hormogonium. Scales: 10 µm in A (use for A–I).

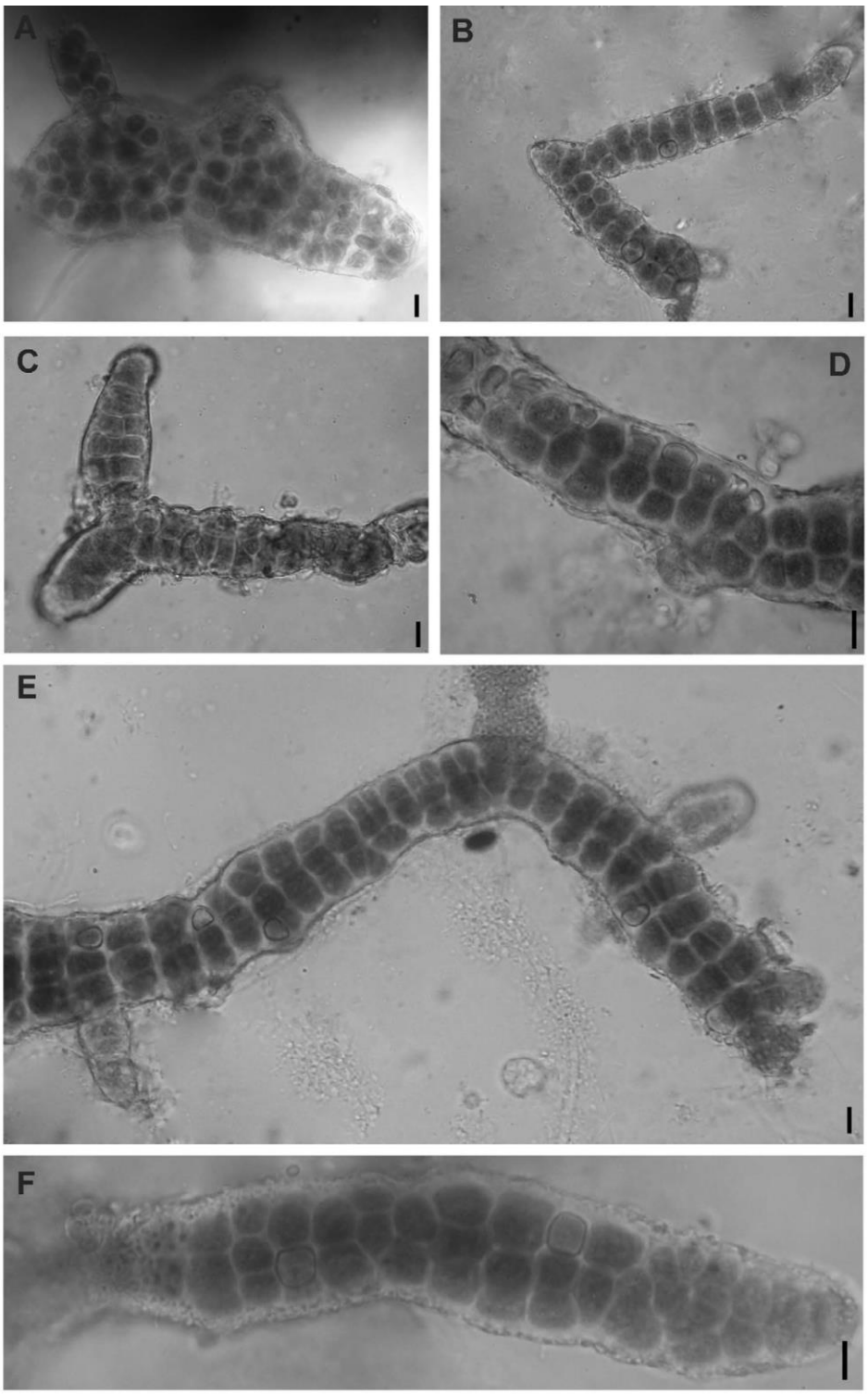

FIGURE 13 A–F: *Stigonema corticola*. A: Agglomerations of cells with branches. B, C: Branched filaments. D, E: Details of multiseriate filaments with heterocytes. F: Detail of apex. Scales: 10 µm.

Stigonema parallelum Sant'Anna, Kaštovský, Hentschke & Komárek, *sp. nov.* (Fig. 14, 15)

Strata aerophytica, fusco-atrata, tenues, atteram adhaerentes, de filis curtis, erectis, parallele agglomeratis composita. Fila bipolares, praecipue uniseriata rarisime ad tri-seriata, apud basis 10–18 µm lata, ad apices rotundatis attenuata ad 8–10 µm, rarissime elongata, flexuosa cum ramis lateralis. Rami curti, ad apices rotundati. Vaginae firmae, lamellosae, luteo-fuscae. Cellulae irregulariter rotundatae, saepe brevior quam latae vel ad isodiametricae, contentu griseo-aeruginosae vel olivaceae. Heterocytae solitariae, intercalares vel laterales subsphaericae vel irregulariter ovales. Hormogonia curta, 4–12-cellularia, praecipue 7–8 µm lata, cylindrica vel ellipsoidea, cum cellulis curtis, barriliformis composita, apice de filis ramisque separantur.—Habitatio: Aerophytice in solis latericis humidis in sylvis montium "Mata Atlantica" dictis, provincia São Paulo, Brasilia.

Type:—Brazil. São Paulo: Ecological Station "Juréia-Itatins", preserved sample collected on 19 September 2002, *C.L. Sant'Anna* (Holotype SP 427516!, Isotype SP 427517!).

Thallus forming flat mats on the substrate, composed of relatively short, parallel and densely arranged filaments, perpendicular to the substrate. Filaments mostly uniseriate, only rarely with 2(3) cells aside, wider near the bases (10–18 µm), narrowed toward the ends (8–10 µm). Branches lateral, very short, narrowed towards the ends, rounded at the apex. From the layer of short, parallel oriented filaments sometimes solitary longer, flexuous filaments grow, also with short branches, up to 3 times longer than other filaments. Hormogonia 4–12-celled, mostly 7–8 µm wide, cylindrical or ellipsoid in outline, composed of short barrel-shaped cells, constricted at the cross-walls. Hormogonia are formed also at the end of elongated filaments. Sheaths slightly up to intensely lamellate, yellow-brown. Cells irregularly rounded, mostly shorter than wide, rarely isodiametric. Cell content grey-blue-green, olive green, or pale blue-green.

Habitat:—On wet lateritic soil.

Etymology:—Specific name refers to the parallel organization of filaments in thalli.

Notes:—This species represents a morphologically very distinct and characteristic stigonematacean type with heteropolar filaments, attached by one end to the substrate and very characteristic life cycle. Short, mostly uniseriate filaments are round at the apical ends, attached firmly to the substrate, and arranged very densely and parallel in erected fascicles. The short hormogonia liberate individually from the sheaths in the apical part of filaments attached to the substrate, representing the main mode of reproduction (see Fig. 15). The only stigonematacean species with a similar morphology and life cycle is *Stigonema compactum* Gardner (1927: 10), described from China, but with a distinctly different cell morphology. Since there are no other similar known stigonematacean taxa, we describe this as a new *Stigonema* species. The biology and morphology of this Brazilian morphotype are so specific that the separation at the generic level might be possible in the future.

Discussion

The cyanobacterial microflora from various tropical and subtropical habitats is more diverse than previously thought according to literature, and the tropical forests belong to these ecosystems where numerous and unidentifiable morpho- and ecospecies play an important ecological role. Sampling in poorly studied regions often results in additions of new taxa, as demonstrated by Novis & Visnovsky (2011) for New Zealand and Lemes-da-Silva *et al.* (2010) for Brazil.

Traditionally, authors lumped the tropical morphotypes to the most similar European species, such as Frémy (1930) with African material. The exception was the work of Gardner (1927) who described several new species of Cyanobacteria from Puerto Rico that have been widely accepted up to date. These morphotypes of Gardner (1927) and Frémy (1930), as well as the Brazilian material from the Atlantic Rainforest, are similar to the species from temperate regions, however, they do not correspond to their original descriptions.

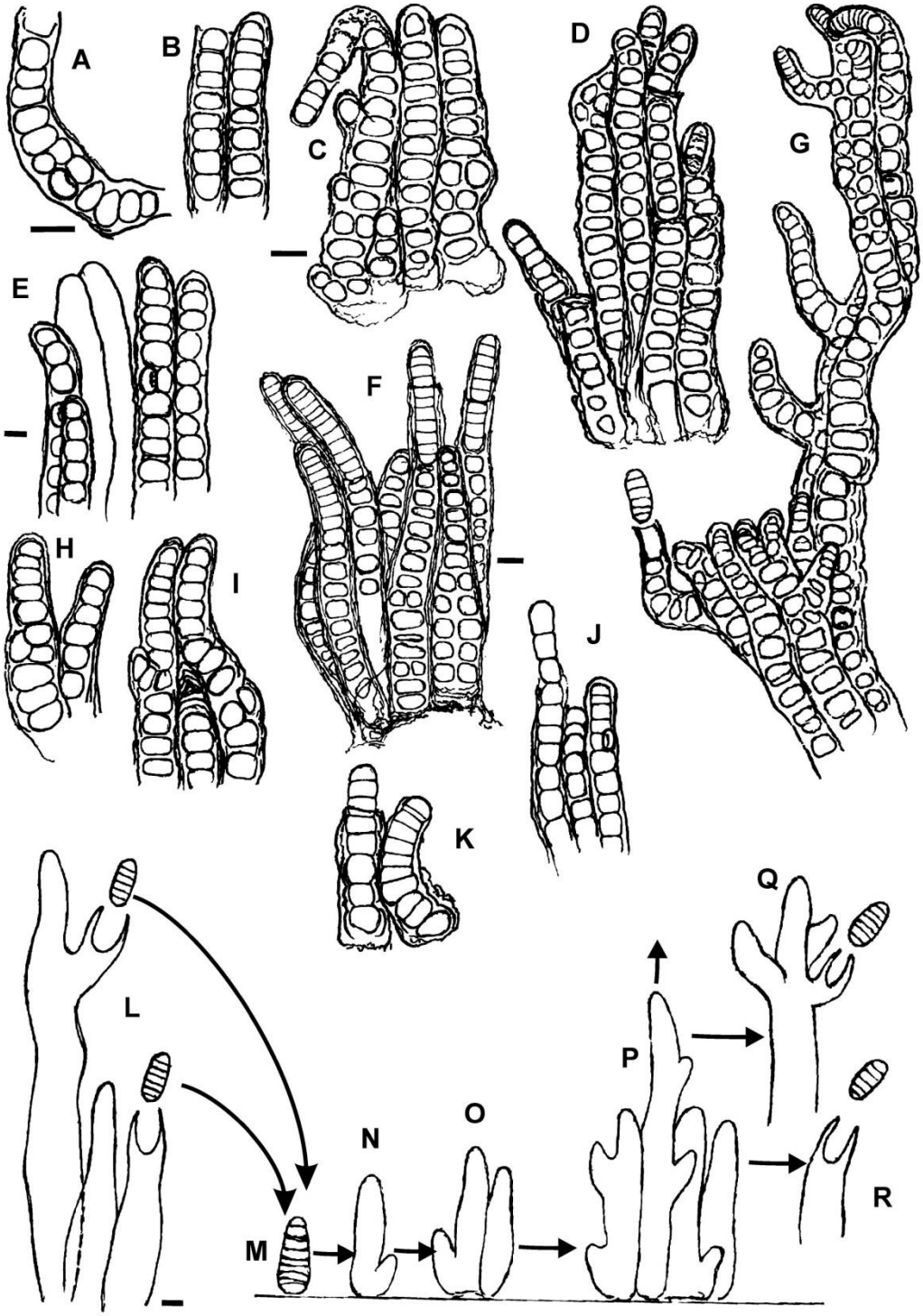

FIGURE 14 A–R: *Stigonema parallelum*. A: Filament with a lateral heterocyte. B: Detail of filaments with lamellated sheaths. C–F: Parallel filaments forming hormogonia. G: Long filament with short branches. H–J: Details of apices. K: Germinating hormogonia. L–R: Development of filaments and releasing of hormogonia (life cycle). Scales: 10 µm in A (use for A, B), C (use for C–D), E, F (use for F–K) and L (use for L–R).

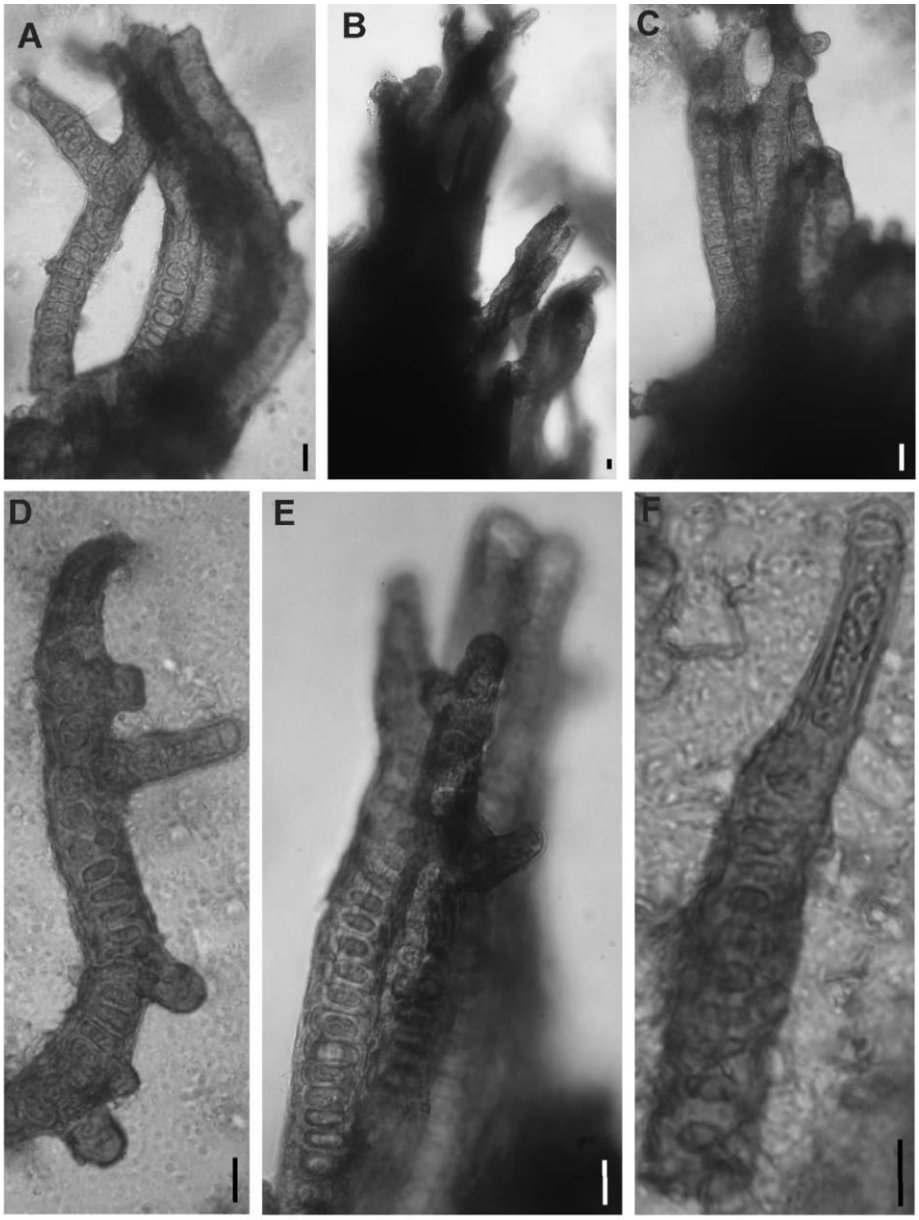

FIGURE 15 A–F: *Stigonema parallelum*. A: General aspect of a thallus. B–D: Parallel filaments. E: Branched filament. F: Filament forming a hormogonium. Scales: Scales: 10 μm.

Stigonematacean cyanobacteria are very common in the Atlantic Rainforest and contain numerous ecologically distinct types (Silva & Sant'Anna 1996). They also belong to multicellular cyanobacteria with diversified and functionally specialized thalli, and must be defined urgently so that the characterization of many terrestrial microphyte communities all over the world can be carried out. In spite of the great diversity of this cyanobacterial group in the Atlantic Rainforest, only two stigonematacean morphotypes have been described to date. These results are much lower than expected, especially if compared with the 3 new genera

and 15 new species described recently for the other heterocytous Cyanobacteria (Fiore *et al.* 2007, Sant'Anna *et al.* 2010, 2011). These facts together with our results highlight the lack of studies on stigonematacean taxa and the high potential for the discovery of new taxa in the Atlantic Rainforest.

Specifically, *Stigonema* is a morphologically complex and heterogeneous genus (Drouet 1981). There are large differences between uni- and multiseriate species, among types with different kinds of branching, and especially among types with isopolar and heteropolar thalli. All these differences are also recognizable in the Brazilian material described here. The majority of the species form isopolar filaments, which creep on the substrate and originate new branches, sometimes erect and fasciculate. Both *Fischerella* species, *Stigonema minutum* and related taxa (*S. fremyi, S. flexuosum*, and *S. corticola*) belong to these morphotypes. However, *S. parallelum* is a typical heteropolar species with morphologically different basal and apical parts of the filaments. This different type of thallus seems to be very important for phylogeny, but in order to have a deeper knowledge of the real taxonomic value of this character, it is necessary to analyze a larger number of populations, also applying molecular methods.

Acknowledgments

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Anexo III

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Biologically active compounds from cyanobacteria extracts: *in vivo* and *in vitro* aspects

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Abstract: An investigation was directed towards the antiacetylcholinesterase activity of the acid aqueous and methanolic extracts of five cyanobacterial taxa, which encompasses an enzymatic inhibition essay and the evaluation of the physiological responses of mice to cyanobacterial extracts along with toxicological observations. The strains *Calothrix* sp. CCIBt 3320, *Tolypothrix* sp. CCIBt 3321, *Phormidium* cf. *amoenum* CCIBt 3412, *Phormidium* sp. CCIBt 3265, and *Geitlerinema splendidum* CCIBt 3223 were from the São Paulo Botanical Institute Cyanobacterial Culture Collection and all of them showed inhibitory effect on acetylcholinesterase activity (*in vitro*) and caused systemic effects similar to those described for anticholinesterase drugs (*in vivo*). With the exception of *G. splendidum* and *Tolypothrix* sp. strains, all extracts produced reversible antiacetylcolinesterase effects in mice. Complementary histopathological studies were carried out on tissues from animals administered with *Phormidium* sp. and *P.* cf. *amoenum*.

Article

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Tolypothrix sp.

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Introduction

Cyanobacteria are common members of microscopic populations of freshwater lakes and reservoirs worldwide. They are capable of forming blooms and producing potent toxins, which can present serious human and animal health problems (Van Apeldoorn et al., 2007, Pearson et al., 2010). Besides cyanotoxins, these organisms have the ability to synthesize considerable amounts of structurally distinct compounds that can be used as food and feed, fuel, dyes, sunscreen agents, as well as therapeutic drugs (Abed et al., 2009).

In scientific literature there is an appreciable amount of studies on the potential use of cyanobacterial compounds as medication, along with cyanotoxin poisoning cases in humans by ingesting contaminated water and food or by accidental administration during dialysis treatment. Some of these compounds are already being employed in anal fissures and common fistula treatment, as well as anti-HIV drugs (Botos & Swlodawer, 2003; Garrido et al., 2007), however, one of the most interesting set of activities displayed by cyanobacterial metabolites is their inhibitory action on certain enzymes (Grainger et al., 1989; Chen et al., 2007; Zelik et al., 2009). The cyanotoxins microcystins and anatoxin-a(S) have anti-phosphatase and anticholinesterase effects, respectively (Van Apeldoorn et al., 2007). However, there are other antienzymatic activities described for compounds synthesized by these organisms (Radau, 2000; Sisay et al., 2009; Matthew et al., 2010).

Compounds bearing antiacetylcholinesterase action play a very important role in the search for potential drug candidates against Alzheimer disease (AD); this neurodegenerative condition is associated with brain neurotransmitter deficits and its symptomatic treatment is the restoration of cholinergic function by inhibiting acetylcholinesterase (Francis et al., 1999; Trevisan et al., 2003).

Therefore, the effect of an antiacetylcholinesterase drug is a long-lasting and more effective stimulation of the cholinergic system, which results in responses from autonomic effector organs, autonomic ganglia, as well as skeletal muscles, and from cholinergic receptors in the Central Nervous System. According to its molecular structure, each antiacetylcholinesterase compound

has its own chemical characteristics that determine its reactivity. As a consequence, the binding between the antiacetylcholinesterase compound and the enzyme shall be either a short, medium (reversible), or long-term (irreversible) association, being considered as potential therapeutic drugs only the ones which form reversible bonds (Nair et al., 2004).

There is an ongoing search for new bioactive compounds in cyanobacterial extracts from the São Paulo Institute of Botany Cyanobacterial Culture Collection strains, where some caused similar physiological responses in the mouse bioassays, which relate to responses demonstrated by antiacetylcholinesterase compounds. Such extracts were evaluated for AChE inhibition and short-term toxicity to mammals; a first step for further studies.

Materials and Methods

The organisms and extract preparation

Five different cyanobacterial strains were studied: *Calothrix* sp. CCIBt 3320, *Tolypothrix* sp. CCIBt 3321, and *Phormidium* cf. *amoenum* CCIBt 3412, isolated from soil samples in the Atlantic Rainforest, State Park of the Serra do Mar, SP (23°24' S and 45°11'06'' W); *Phormidium* sp. CCIBt 3265, isolated from an alkaline lake from the Pantanal, MS (18°57'42'' and 56°37'26''); and *Geitlerinema splendidum* CCIBt 3223, from Guarapiranga Reservoir waters, SP (23°43' S and 46°32') (Figure 1). The strains were cultured under the following conditions: ASM-1 medium, temperature 23±1 °C, and continuous irradiance 40-50 Mmol/m^{-2} s^{-1} (Azevedo & Sant'Anna, 2003).

For each strain, the biomass obtained was freeze-dried, divided into two halves: one of them subjected to ultrasound-assisted extraction (5x, 30 s, 100 W) with 0.1 M aqueous acetic acid (AAE) and the other, with methanol 100% (ME). After centrifugation (1,750 x *g*, 50 min), the aqueous supernatants were lyophilized and the methanolic ones were dried via speed-vac centrifugation. The dried samples were stored in hermetically sealed vials, at -20 °C, until analysis (Conserva et al., 2011).

In vitro *assay: qualitative evaluation of acetylcholinesterase inhibitory activity*

This *in vitro* assay was accomplished following Rhee et al. (2001) TLC autographic protocol: aliquots of 100 µg of each dried extract were dissolved and spotted on a pre-coated plate (Silica gel 60 F_{254}, 10x 10 cm, layer thickness 0.2 mm, E. Merck, Germany). The chromatogram was developed with mobile phase $CHCl_3$: MeOH:H_2O (64:36:8, v/v/v), dried and sprayed with the enzyme

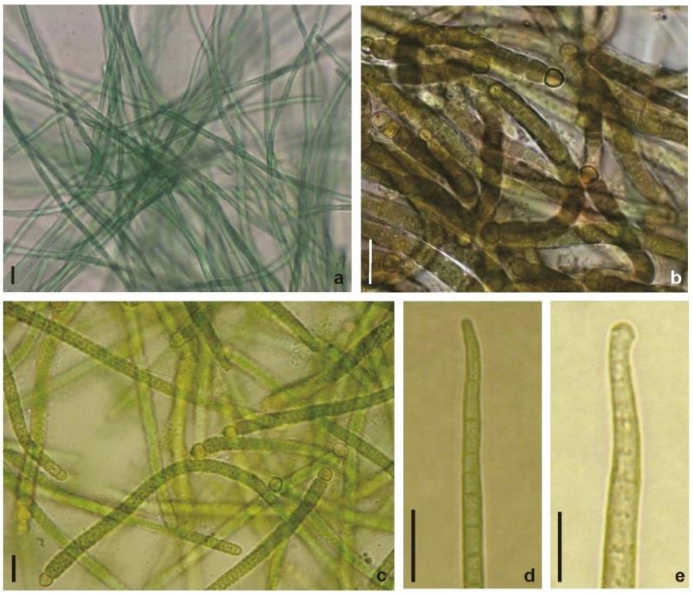

Figure 1. a. *G. splendidum* CCIBt 3223; b. *Calotrix* sp.CCIBt 3320; c. *Tolypothrix* sp. CCIBt 3321; d. *Phormidium* sp. CCIBt 3265; e. CCIBt 3412 *Phormidium* cf. *amoenum*.

solution (6,66 U mL), thoroughly dried and incubated in a humid atmosphere, at 37 °C, for 20 min. Subsequently, the plate was sprayed with a 0.25% 1-naphthylacetate in ethanol plus 0.25% aqueous Fast Blue B salt solution. The spots corresponding to potential acetylcholinesterase inhibitors were unambiguously identified as clear zones against a purple background. The Electric eel AChE type V (Product no C 2888, 1000 U) was purchased from Sigma as well all analytical grade reagents.

The retention factors (R_f) of the compounds that positively reacted against the enzyme were also calculated.

In vivo *assay: acute toxicity study* (i.p.)

Toxicological assays were performed in triplicate on each crude extract by using mice of the same sex, which simultaneously allowed the achievement of reliable data and the use of a minimum number of animals (Rangel et al., 2012). The procedures were carried out according to the WHO guidelines (Harada et al., 1999) and a single dose of 1,000 mg dried cells/kg body weight was used, which enabled extracts to be ranked as low toxicity, if animal death was caused (Lawton et al., 1994). Ethical clearance was obtained from the Ethical Committee for Animal Research of Butantan Institute - Protocol No. 385/07.

Male Swiss mice (19-21 g, 50 days) were intraperitoneally (*i.p.*) treated with dried aqueous acetic acid or methanolic cyanobacterial extracts dissolved in Milli-Q water; the control animals received only the vehicle (Milli-Q water), according to WHO protocol. Any changes in the skin, fur, eyes and respiratory, autonomic and central nervous system, somamotor activity and behavior pattern were observed, and signs of tremors, convulsions, salivation, diarrhea and lethargy and coma were noted as well. The animals were observed for 8 days following administration because, after an extended observation time, notable findings on tissue lesions can be observed (Rangel et al., 2012). Surviving animals were euthanized with CO_2, necropsy findings were recorded and tissue samples were taken.

Results and Discussion

Extract preparation and qualitative evaluation of AChE inhibitory activity

The freeze-dried cyanobacterial biomass and the dried extract weights are displayed in Table 1, along with the qualitative results of the antiacetylcholinesterase bioautographic assay (Figure 2) and the R_f (retention factors) of spots corresponding to antiacetylcholinesterase compounds.

All extracts were previously analyzed for the presence of microcystins (Conserva et al., 2011).

In vivo *assays: acute toxicity study* (i.p.)

In acute toxicity testing, among the treated animals, the ones that received the AAE *G. splendidum* CCIBt 3223 and only one administered with AAE *Tolypothrix* CCIBt 3321 died, while all the others showed mild and transient physiological effects, which can be associated with the intrinsic side effects induced by anti-AChE drugs (McGleenon et al., 1999; Xavier et al., 2007; 2008).

The physiological responses, time to death, and gross changes seen post-mortem are compiled in Chart 1.

Similar responses were observed in animals treated with extracts of all studied strains, which are also very similar to those observed in the mouse studies on anti-AChE drugs (McGleenon et al., 1999; Xavier, 2008) and such responses are related to muscarinic and nicotinic

Table 1. Biomass and extract dry weights, qualitative anticholinesterase assay results and Rf of the AntiAChE compounds.

Extract strains	Freeze-dried biomasses (g)	Dried extracts (g)	AntiAChE activity	Rf of antiAChE compounds
AAE *G. splendidum* CCIBt 3223	0.7847	0.3941	+	0.28; 0.66
ME *G. splendidum* CCIBt 3223	0.6675	0.2308	+	0.26; 0.58
AAE *Calotrix* sp.CCIBt 3320	0.4937	0.1888	+	0.64
EM *Calotrix* sp.CCIBt 3320	0.2242	0.1135	+	0.37
AAE *Tolypothrix* sp. CCIBt 3321	0.4050	0.1868	+	0.62
EM *Tolypothrix* sp. CCIBt 3321	0.3813	0.1144	+	0.38
AAE *Phormidium* sp. CCIBt 3265	0.4871	0.1727	+	0.66
EM *Phormidium* sp.CCIBt 3265	0.6398	0.1875	+	0.45; 0.59; 0.66; 0.73
AAE *Phormidium* cf. *amoenum* CCIBt 3412	0.5052	0.1485	+	0.66
EM *Phormidium* cf. *amoenum* CCIBt 3412	0.8941	0.2129	+	0.55; 0.59; 0.58

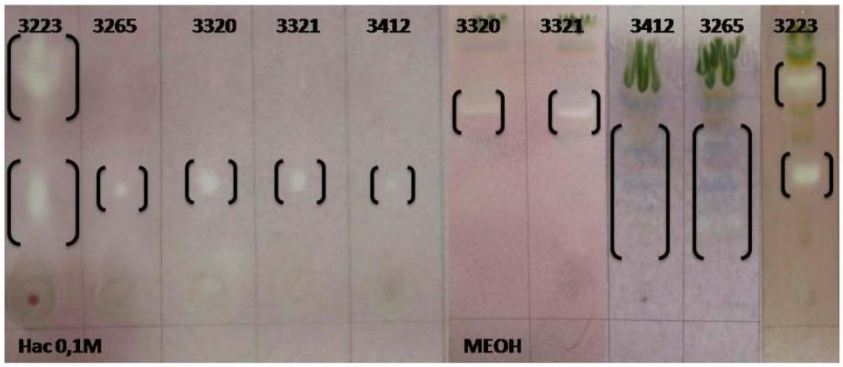

Figure 2. AE CCIBt 3223, 3265, 3320 and 3421 TLC qualitative antiacetylcholinesterase assay (a, b, c, d and e); ME CCIBt 3223, 3265, 3320 and 3421 TLC qualitative antiacetylcholinesterase assay (f, g, h, i, and j). Brackets indicate the anticholinesterase compounds.

Chat 1. Physiological responses, observation time or time to death, and macroscopic lesions observed *post-mortem*.

Strains/extract	Physiological responses	Observation time	Main necropsy findings
AAE- *G. splendidum* CCIBt 3223	Dyspnea, loss of reflexes, and prostration	Deaths two hours after extract administration	With no apparent macroscopic changes
ME- *G. splendidum* CCIBt 3223	Abdominal contractions, loss of reflexes, and agitation	Euthanasia seven days after administration	Hemorrhagic lungs
AAE- Calotrix sp. CCIBt 3320	Piloerection, dyspnea, abdominal contractions, eyebrow ptosis, loss of reflexes and transitory paralysis	Euthanasia seven days after administration	White spots on the liver; liver adhered to the intestines.
ME- *Calotrix* sp. CCIBt 3320	Loss of reflexes, transitory paralysis, intense abdominal contractions, dyspnea and eyebrow ptosis	Euthanasia seven days after administration	Black spots located between the liver lobes (N=1)
AAE- *Tolypothrix* sp. CCIBt 3321	Transitory paralysis, abdominal contractions, piloerection, dyspnea, and diarrhea	Death four days after administration (N=1); euthanasia seven days after administration (N=2)	Diminished liver size, fusion of lobes and white spots on the liver. (N=1)
ME- *Tolypothrix* sp. CCIBt 3321	Agitation, itch, scrotal edema, and abdominal contractions.	Euthanasia seven days after administration	With no apparent macroscopic changes
AAE- *Phormidium* sp. CCIBt 3265	Dyspnea, abdominal contractions, and loss of reflexes.	Euthanasia seven days after administration	Hemorrhagic lungs, white spots on the liver; abnormalities in gallbladder morphology
ME- *Phormidium* sp. CCIBt 3265	Abdominal contractions, piloerection, prostration, eyebrow ptosis transitory paralysis, and dyspnea	Euthanasia seven days after administration	Hemorrhagic lungs (N=2), and degraded lungs (N=1); green spot on the stomach
AAE- *Phormidium* cf. *amoenum* CCIBt 3412	Prostration, piloerection abdominal contractions, and loss of reflexes	Euthanasia seven days after administration	Hemorrhagic lungs (N=1); fusion of liver lobes; increased peritoneal thickness, with hemorrhagic área (N=1)
ME- *Phormidium* cf. *amoenum* CCIBt 3412	Muscle spasms, abdominal contractions, eyebrow ptosis, dyspnea, and loss of reflexes	Euthanasia 7 days after administration	Hemorrhagic lungs; diminished liver size and presence of black spots; bubble on the left kidney (N=2); green spot on the stomach

actions as well as on the central nervous system.

Among the muscarinic manifestations are dyspnea, abdominal cramps and diarrhea; among those resulting from overstimulation of the nicotinic receptors are muscle cramps, motor weakness, paralysis, tachycardia, and piloerection, and among those due to Central Nervous System are tremors, ataxia, and walking difficulty. The extent, the progression and the persistence of clinical observations depend on administration route, bioactive compound structure, and exposure magnitude (Andrade Filho & Romano, 2001). The compound structure defines the bond nature to the enzyme, which can be reversible, or irreversible, being the intermediate compound short, medium (reversible), or long acting (irreversible), respectively. The long acting intermediates are considered to be toxic (Nair et al., 2004).

The clinical signs complete regression ranged between two to three hours in three out of five groups of mice tested; that is, AAE *G. splendidum* CCIBt 3223 caused acute intoxication and only one animal that received AAE *Tolypothrix* sp. CCIBt 3321 died after four days from administration.

The results also showed that the AAE of the strain *G. splendidum* CCIBt 3223 possessed a lethal anti-AChE activity, most likely by the presence of long acting inhibitory substances to the enzyme (Nair et al., 2004), but there is insufficient information on the AE *Tolypothrix* sp. effects.

Anatoxin-a(S), anticholinesterase with potent lethal effect, is the only organophosphate produced by Cyanobacteria (Van Apeldoorn et al., 2007); meanwhile, great part of the compounds considered highly toxic are synthetic, such as carbamate Aldicarb (Cazenave et al., 2005) and of the organophosphate Parathion (Bardin et al., 1994).

Our results of the *in vitro* anti-AChE assay and clinical observations indicate that the methanolic and aqueous extracts of *Calothrix* sp. CCIBt 3320, *Tolypothrix* sp. CCIBt 3321, *Phormidium* sp. CCIBt 3265, *Phormidium* cf. *amoenum* CCIBt 3412, and *G. splendidum* CCIBt 3223 contain compounds which inhibit the enzyme acetylcholinesterase in a transient or reversible way.

Compounds which act as transient anticholinesterase drugs should be evaluated as potential therapeutic drugs because the current cholinesterase inhibitors prescribed for the treatment of AD are tacrine, donepezil, rivastigmine, galantamine, and serine which present several adverse side-effects such as hepatotoxicity, gastrointestinal disturbance and depression (Yoon et al., 2008). Due to these limiting factors for their use, new anticholinesterase drugs will be well received.

Aside from allowing a detailed observation of the biological responses to active compounds, an important outcome of the mouse bioassay is to determine the nature and extense of the adverse effects to a single dose or an overdose of a toxic or a therapeutic compound (Xavier, 2008). In our studies, with the exception of the acetic acid extract from *G. splendidum* CCIBt 3223 and *Tolypothrix* sp. CCIBt 3321, all other extracts are not lethal and caused physiological responses associated with the pharmacological actions of antiacetylcholinesterase compounds.

Complementary histopathological studies were carried out on liver, kidney, and lung tissues of animals intoxicated with extracts of *Phormidium* sp CCIBt 3265 and *P.* cf. *amoenum* CCIBt 3412.

Previous studies have described and illustrated the microscopic lesions caused by CCIBt strain 3223 (Rangel et al., 2012). The mice lungs were severely affected, presenting hemorrhage focuses, edema, alveolar collapse, and hyperplasia, due to an increase in the number of immune system cells (macrophages). Disorganization of the hepatic parenchyma, necrosis, loss of vein endothelim, and presence of giant multinuclear cells and polymorphonuclear cells in the liver were observed. Finally, the kidneys of mice intoxicated with AAE CCIBt 3223 (*G. splendidum*) presented alterations in the convoluted tubules and necrotic areas (Rangel et al., 2012).

Histological sections from control animal are shown in Figure 3. The main alterations observed in the organs of animals tested with AAE CCIBt 3265 were: in the lungs, edema, hemorrhage, and alveolar collapse and in the liver, areas of necrosis and steatosis (Figure 4).

In the animals that received ME CCIBt 3265, necrosis, steatosis, damage in endothelial cells of central lobular veins, enlarged sinusoids, inflammatory infiltrate close to bile duct, and giant multinuclear cells were observed in the liver; in the lungs, hemorrhage and alveolar collapse; and in the kidneys, the interstitial space between tubules was greater than normal and there was increase in light of convoluted tubules (Figure 5).

The microscopic lesions provoked by AAE CCIBt 3412 were shown in the Figure 6: granulomatous foci, hemorrhage, and alveolar collapse were seen in the lungs, large amounts of inflammatory infiltration close or not to bile duct cells were seen in the liver, and enlarged convoluted tubules were seen in the kidneys.

In the only study concerning antiacetylcholinesterase effects on mice, the microscopic lesions observed were: in the lungs, hemorrhage, edema and congestion; in the liver, vacuolar degeneration and in the kidneys, hemorrhage, congestion and tubular degeneration (Xavier, 2008). Comparison between the findings on AAE and ME CCIBt 3265 microscopic lesions showed similarity with the results obtained by Xavier (2008). Other histological findings could be attributed to unknown substances in the extracts.

In conclusion, the present study identifies *G. splendidum* CCIBt 3223 as a producer of a toxin, which administered as a single dose of 1,000 mg dried cells/kg

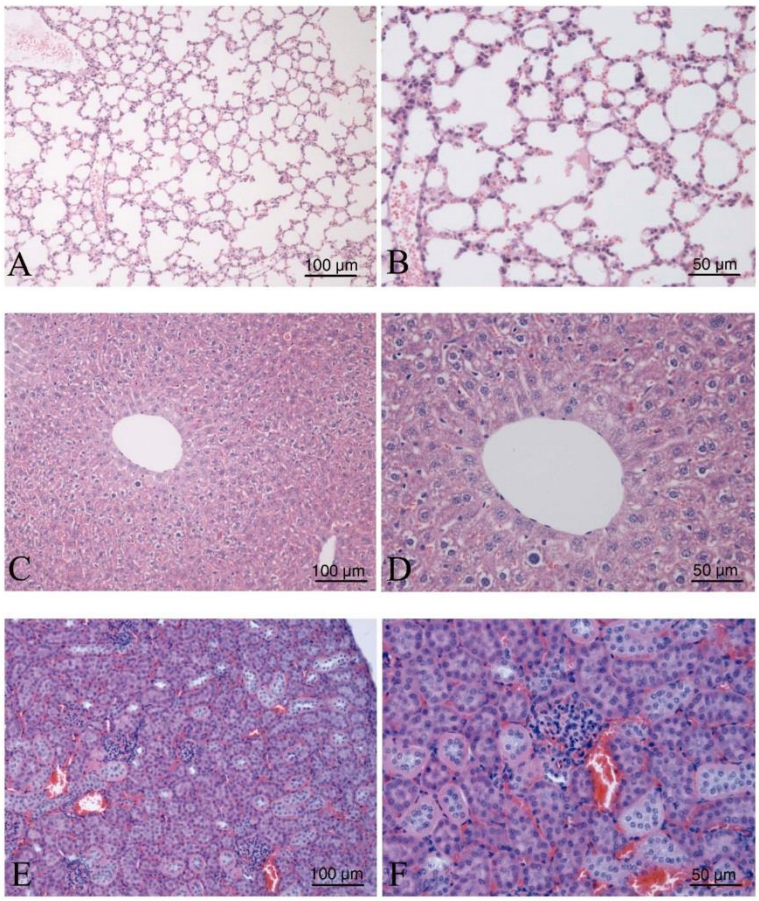

Figure 3. Histological sections from control animals (HE). A and B: Lung (100x, 200x). C and D: Liver (100x, 200x). E and F: Kidney (100x, 200x).

body weight, causes animal death within 2 h and indicates ME- *Tolypothrix* sp. CCIBt 3321 as a candidate for further studies for potential anticholinesterase drugs.

Acknowledgments

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Author's contributions

LRC designed the study, supervised the laboratory work, contributed to analysis of the data and drafted the paper and wrote the final manuscript; ACN contributed to histological studies and to critical reading of the manuscript; GAAC contributed in running the laboratory work and to chemical and biological studies; RLB contributed to toxicological analysis; GSH and CFSM contributed to cyanobacterial collection, identification and culture. LMBT contributed to biological analysis and to

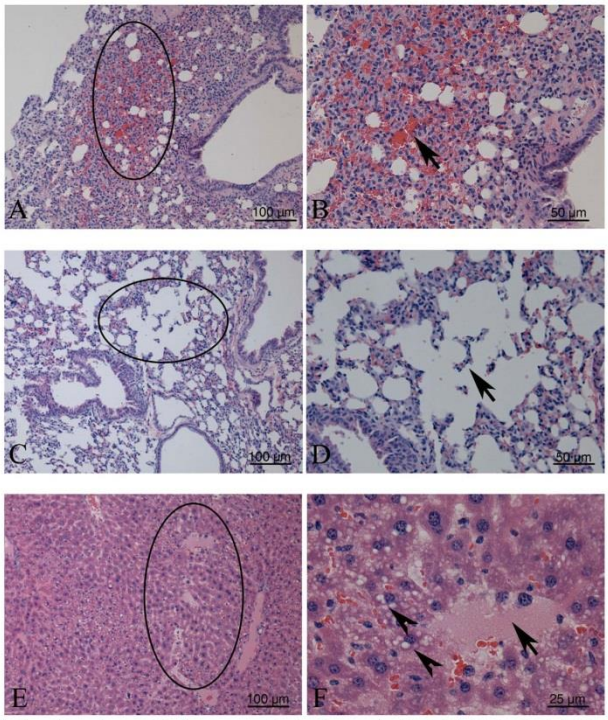

Figure 4. Histological alterations observed in the organs of animals tested with AAE CCIBt 3265 (HE). A, B, C and D: Lung. A: Hemorrhagic area and alveolar collapse (circle) (100x). B: Erythrocytes extravasation (arrow) (200x). C: Edema (circle) (100 x). D: Disruption the wall of a pulmonary alveolus (200x). E and F: Liver. A: Areas of necrosis and steatosis (100x). B: Necrotic area (arrow), steatotic cells (arrow heads) (400x).

critical reading of the manuscript; CLS contributed to cyanobacterial identification and to critical reading of the manuscript and MR contributed to toxicological studies and to critical reading of the manuscript. All the authors have read the final manuscript and approve the submission.

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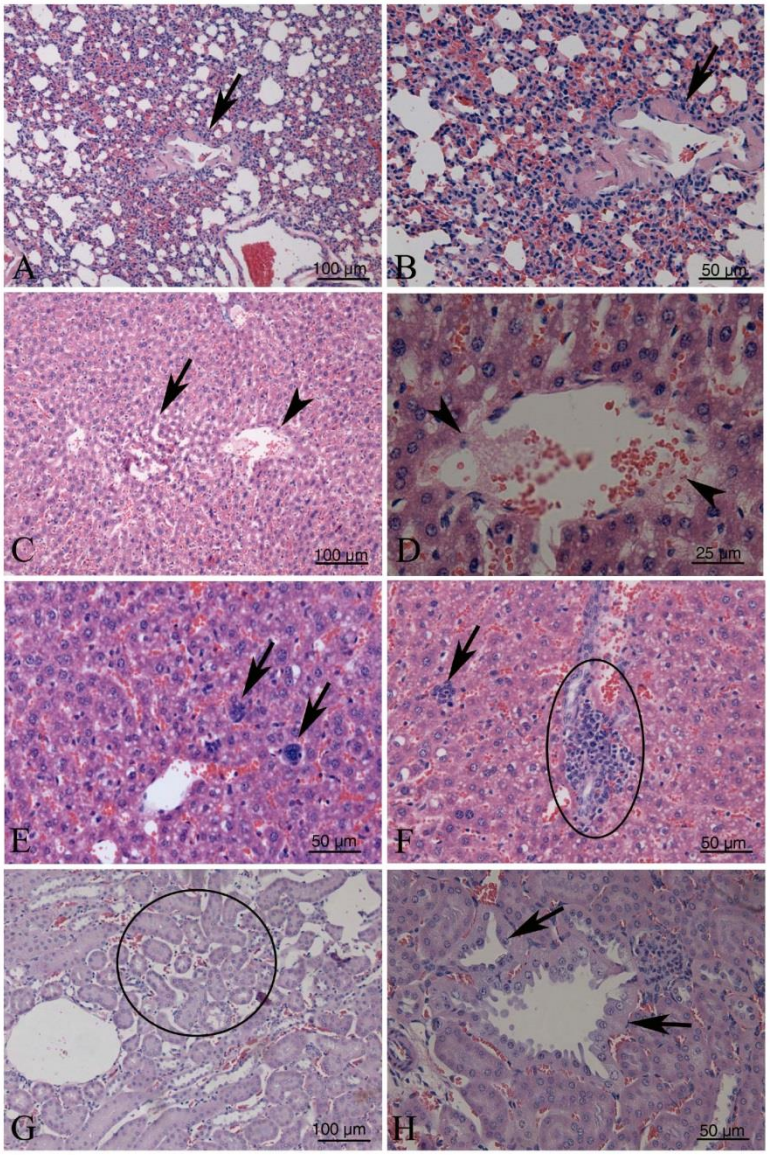

Figure 5. Histological alterations observed in the organs of animals tested with ME CCIBt 3265. A and B: Lungs. Hemorrhage and alveolar collapse. Collapse of an artery. (arrow) (100x, 200x). C, D, E and F: Liver. C and D: Enlarged sinusoids (arrow) and damage in endothelial cells of central lobular veins (arrow head) (100x, 400x), E: Giant multinuclear cells (200x), F: Mononuclear inflammatory infiltrate (arrow) inflammatory infiltrate close to bile duct (circle) (200X). G and H: Kidney, G: Interstitial space between tubules was greater than normal (circle) (100x). H) Increase in light of convolute tubules (arrow) (200x).

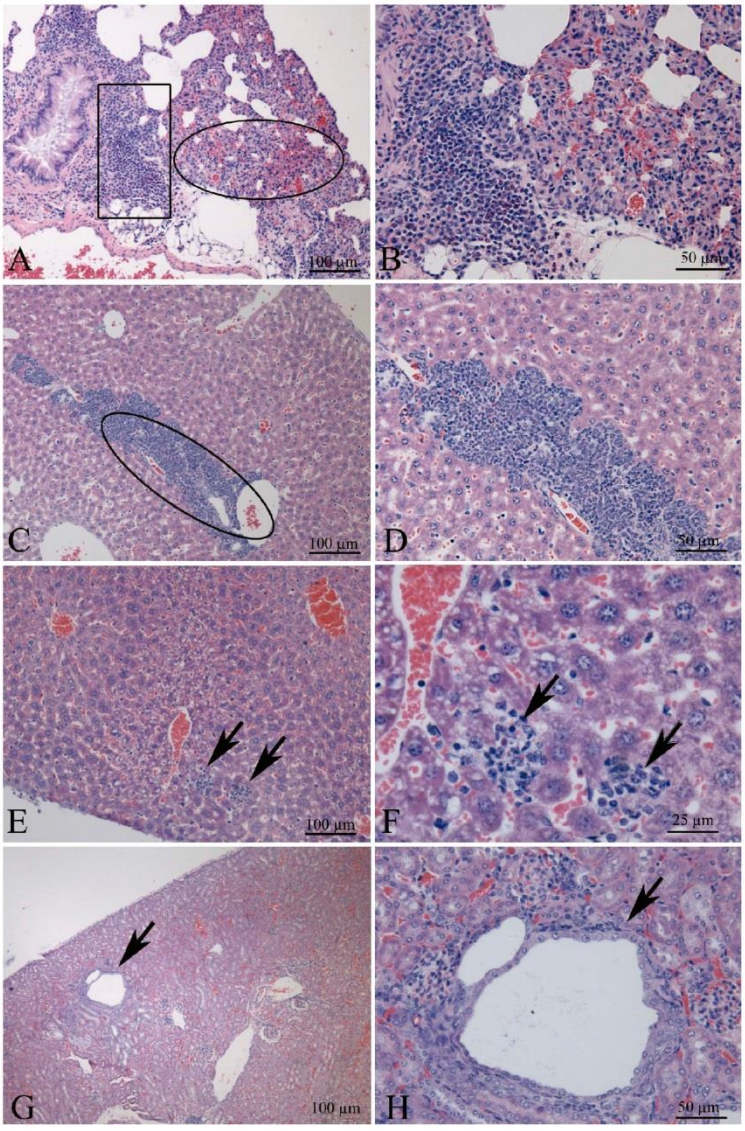

Figure 6. Histological alterations observed in the organs of animals tested with AE CCIBt 3412. A and B: Lungs. Granulomatous foci (rectangle), hemorrhage and alveolar collapse (circle) (100x, 200x), C, D, E and F: Liver. C and D) Large amounts of inflammatory infiltration close to bile duct (100X, 200X). E and F) Mononuclear inflammatory infiltrate (arrows) (100X, 400X). G and H) Kidney. Enlarged convoluted tubules (arrow) (100X, 200X).

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Anexo IV

Brazilian Journal of Botany

Scytonema santannae sp. nova, a new morphospecies of Cyanobacteria from "Mata Atlântica"

--Manuscript Draft--

Manuscript Number:	BRJB-D-13-00058R3
Full Title:	Scytonema santannae sp. nova, a new morphospecies of Cyanobacteria from "Mata Atlântica"
Article Type:	Original Research
Abstract:	ABSTRACT - (Scytonema santannae sp. nova, a new morphospecies of Cyanobacteria from "Mata Atlântica") The genus Scytonema (Cyanobacteria) is one of the most common components of terrestrial biofilms in tropical and subtropical areas, mainly because its nitrogen fixation capability and wide diversity of species. In the Brazilian rainforest "Mata Atlântica", Scytonema populations are frequently found growing on rocks, bark of trees, soil and among mosses, but are still underestimated and poorly characterized. In this article, we describe a new terrestrial morphospecies of Scytonema collected in "Ilha do Cardoso", an island located in the subtropical area of "Mata Atlântica". The main features of the new species are remarkable mosaic-ornate sheaths and typical epiphytic growth on mosses on wooden substrates. The building process of mosaic-ornate sheaths is not yet clarified, and up to now, their occurrence were known only for two types (several populations of S. cf. stuposum and Brasilonema ornatum).
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Scytonema santannae, a new morphospecies of Cyanobacteria from the Atlantic rainforest, southeastern Brazil.

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ABSTRACT – (*Scytonema santannae*, a new morphospecies of Cyanobacteria from the Atlantic rainforest, southeastern Brazil.) The genus *Scytonema* (Cyanobacteria) is one of the most common and important components of terrestrial biofilms in tropical and subtropical areas, mainly because of its nitrogen fixation capability and wide diversity of species. In the Brazilian Atlantic rainforest, *Scytonema* populations are frequently found growing on rocks, bark of trees, soil and among mosses, but are still underestimated and poorly characterized. In this article, we describe a new terrestrial morphospecies of *Scytonema* collected in the State Park of Ilha do Cardoso, an island located in the subtropical area of the Atlantic rainforest, State of São Paulo. The main features of the new species are remarkable mosaic-ornate sheaths and typical epiphytic growth on mosses on wooden substrates. The developlemental process of mosaic-ornate sheaths has not yet been clarified, and hitherto, their occurrence was known only for two taxa (several populations of *S.* cf. *stuposum* and *Brasilonema ornatum*).

Key words - Nostocales, Scytonemataceae, biodiversity, subtropical, aerophytic habitat.

INTRODUCTION

The genus *Scytonema* was created in 1887 by Bornet and Flahault for filamentous Cyanobacteria with obligatory geminate false branching and intercalary heterocytes. Later, after the description of the genera *Scytonematopsis* Kiseleva (tapering trichomes) and *Brasilonema* Fiore *et al.* (cylindrical filaments in fascicles, rare pseudobranching), *Scytonema* came to be accepted as a genus with mainly scytonematoid branching and irregularly entangled filaments with cylindrical trichomes. However, according to recent results, several other related, but distinct clusters will probably be separated from *Scytonema*, as special clades at the generic level (subg. *Myochrotes, Petalonema* and possibly several others).

Currently, the genus *Scytonema* includes approximately 95 species divided into the subgenera *Euscytonema* (trichomes mostly cylindrical along the whole length, sheaths homogenous or parallel lamellate) and *Myochrotes* (thin filaments with widened ends, divergent lamellae) (Bornet & Flahault 1887, Komárek & Hauer 2011). Because of the difficulties in isolating these organisms and the lack of available molecular data, the phylogeny of the species within the genus and their relationships are still unclear.

For Brazil, the only two specific studies for Scytonemataceae (Sant'Anna 1988, Komárek *et al.* 2013) described four new *Scytonema* species and otherwise reported about 20 morphotypes collected in different habitats from the southeastern Brazil, showing how diverse and poorly studied the genus is in these regions. Herein, we describe a new morphospecies of typical *Scytonema* collected from unusual habitats (epiphytic on mosses and growing on bark of trees and woody substrates) in the subtropical area of the Brazilian Atlantic rainforest, in the coastal region of southeastern Brazil.

MATERIAL AND METHODS

The sample was collected by scraping the biomass growing on a dead branch of a tree in the subtropical area of the Atlantic rainforest, State Park of Ilha do Cardoso, Municipality of Cananéia, State of São Paulo, Brazil (25º04'12"S and 47º55'27"W). The material was kept in dried conditions in a paper bag and a small amount was rehydrated for 20 hours before morphological analysis in the laboratory using a light microscope. Morphological analyses were carried out on at least 20 filaments, and characteristics such as arrangement of filaments, tapering of trichomes, ornamentation of sheaths, dimensions and ecological aspects were evaluated. Part of the collected material was preserved in formaldehyde and deposited in the Herbarium of the Institute of Botany (SP), Brazil.

RESULTS

In the subtropical Atlantic rainforest in SE Brazil (State of São Paulo) we found an interesting filamentous cyanobacterial morphotype, growing subaerophytically on mosses and dead woody substrates. The main characters correspond to the traditional genus *Scytonema*, but it was not possible to identify it with any known and validly described species of this generic entity. Therefore, we described it as a new morphospecies.

Scytonema santannae Hentschke & Komárek sp. nova

Figures 1-15

Filaments mostly cylindrical, entangled, 13-20 μm diam. Trichomes constricted, mostly cylindrical, 4.5- 13 μm wide, rarely tapering toward the ends in older filaments. Binary branches frequently connected together for a short part; isolated branching rare. Cells shorter than wide, 3-6 μm long; cell content homogeneous, dark green. Sheaths firm, with wavy margins and irregular mosaic-like ornamentation on the surface. Heterocytes subspherical to quadrate, 3.6-9.5 μm length, 5.1-9.2 μm wide.

Habitat: Epiphytic on mosses growing on a dead branch of a tree in the subtropical part of the Atlantic rainforest.

Type material: BRAZIL. São Paulo: State Park of Ilha do Cardoso, preserved sample collected on VI-29-2010, *Watson A. Gama Jr. and Camila F. da S. Malone*, Herbarium of the Institute of Botany, São Paulo State (Holotype SP 427914).

Etymology: The species is named in honor of Dr. Célia Leite Sant'Anna, Brazilian specialist in Cyanobacteria.

DISCUSSION

This morphospecies differs from any other validly described *Scytonema* species, because of the special type of surface sculpture of the sheaths, rarely occurring within

scytonemataceae genera. Up to now, mosaic-ornate sheaths were reported only for *Brasilonema ornatum* Sant'Anna *et al*. (Sant´Anna *et al.* 2011) and *Scytonema stuposum* sensu Sant'Anna *et al.* (Sant'Anna *et al.* 1983). However, the type reported in Sant'Anna *et al.* (1983) also exhibits biseriate trichome parts and because of that, probably concerns a taxon different from *Scytonema*. Although both *S. santannae* and *B. ornatum,* share the same kind of sheath, they differ in diagnostic characters at the generic level, as in the presence of parallel filaments in *B. ornatum*, which are untangled in *S. santannae.* The process of formation of the mosaic-ornate sheaths is as yet unknown. The study of Bharadwaja (1933) clarified how lamellate sheaths are formed in Scytonemataceae, but did not include the still undiscovered mosaic-ornate surface type. Sant'Anna *et al.* (1983) suggested that mosaic-ornate sheaths could be the initial stages of lichen formation. Indeed, we found hyphae growing together with Cyanobacteria in our sample, just as described for *S. stuposum* sensu Sant'Anna *et al.* (1983). Also, we observed that the three types cited above are corticolous and two of them (*Scytonema santannae* and *Brasilonema ornatum*) were found growing on or among mosses. Nevertheless, it is not yet possible to correlate these facts, because mosaic-ornate sheaths are not often reported in morphotypes growing under those conditions. In our opinion, more experimental studies are needed to evaluate how this kind of sheath is formed, as well as its taxonomic importance at the generic level.

The studied population features cylindrical trichomes, as described in the traditional concept of the genus (Bornet & Flahault 1887), but sometimes exbibits trichomes which are slightly and gradually narrowed toward the ends. This morphological variation has already been reported for many *Scytonema* morphotypes and it is quite common in species of the genus (Desikachary 1959). The problem is that

the presence of tapered trichomes is the diagnostic characteristic of *Scytonematopsis* and due to this morphological variation in some *Scytonema* species, the current separation of these genera based only on the slight tapering of trichomes must be revised in the future in the light of molecular analysis.

Currently, the taxonomic divergence between *Scytonema* and *Scytonematopsis* cannot be confirmed by 16S rDNA data because of the lack of molecular data available. Only few 16S rRNA gene sequences of *Scytonema* strains have been published (Komárek *et al.* 2013), and the species relationships within the genus are not yet clear, indicating a polyphyletic genus. In the near future, this genus will be split into four or more genera, based on 16S analysis, with morphological circumscription for each genus, using characters such as sheath type and trichome terminations (Komárek *et al.* 2013). For the heterogeneous genus *Scytonematopsis*, the only species sequenced is *S. contorta* Vaccarino & Johansen (Vaccarino & Johansen 2011), a taxon morphologically very different from the type-species *S. woronichinii* Kisseleva. Furthermore, the genus *Scytonematopsis* is evidently also heterogeneous in phenotypic characters. It is divided in three distinct morphological groups: (i) marine forms with short cells and relatively short "hairs" at the filament ends, (ii) forms with trichomes and branches narrowed into long hair-like ends and (iii) forms with trichomes only narrowed at the ends, but without the markers described in the type species; our new species is similar only to this last group of *Scytonematopsis*. However, the concept of this genus and/or the separation of these groups into two or more genera must be revised in the light of molecular analyses and the classification of our species into the genus *Scytonema* is more acceptable according to current taxonomy.

The new taxon is remarkable also in its ecology. It was found on woody substrates and as epiphyte on *Cyclolejeunia peruviana* (Lehm. & Lindemb.) A. Evans, a widely distributed bryophyte in tropical/subtropical America; in Brazil it has been recorded in the States of São Paulo, Bahia, Pernambuco and Amazonas (Gradstein 2003, Yano 2008). Cyanobacterial species living as epiphytes of bryophytes are diverse, but are still a neglected component in biodiversity studies, even in those dealing with terrestrial habitats. In Brazil, the only specific study on Cyanobacteria growing on bryophytes is that of Sant'Anna (1984), which reported five *Scytonema* morphotypes among 23 species of Cyanobacteria.

Scytonema species are commonly found growing in terrestrial habitats in tropical and subtropical areas, but *S. santannae* is only the fifth species of this genus described in Brazil. This shows how much cyanobacterial biodiversity is underestimated in different heterogeneous Brazilian areas (Lemes-da-Silva *et al.* 2010, Sant'Anna *et al.* 2010) and highlights the importance of floristic studies, since many species, and probably as yet unknown ones,are being lost due fragmentation of their habitats.

Finally it is uncommon to describe new Cyanobacteria species without molecular analysis, but unfortunately, after many attempts, it was not possible to isolate *S. santannae* or even carry out molecular studies based on the environmental material. Nevertheless, in our opinion, describing new taxa without molecular analysis remains necessary, in these cases, for the purpose of improving knowledge on cyanobacterial diversity. Failure to do this implies an underestimation of diversity.

ACKNOWLEDGEMENTS

The first author works in the framework of the program "*Programa de Pós-Graduação em Biodiversidade Vegetal e Meio Ambiente"*. The authors thank Dr[a]. Olga Yano and Dr. Denílson Peralta, from Institute of Botany of São Paulo, Brazil, for helping with identification of *Cyclolejeunia peruviana* data, and CNPq (562213/2010-4) for financial support. Jiří Komárek was supported by the grant P506/12/1818.

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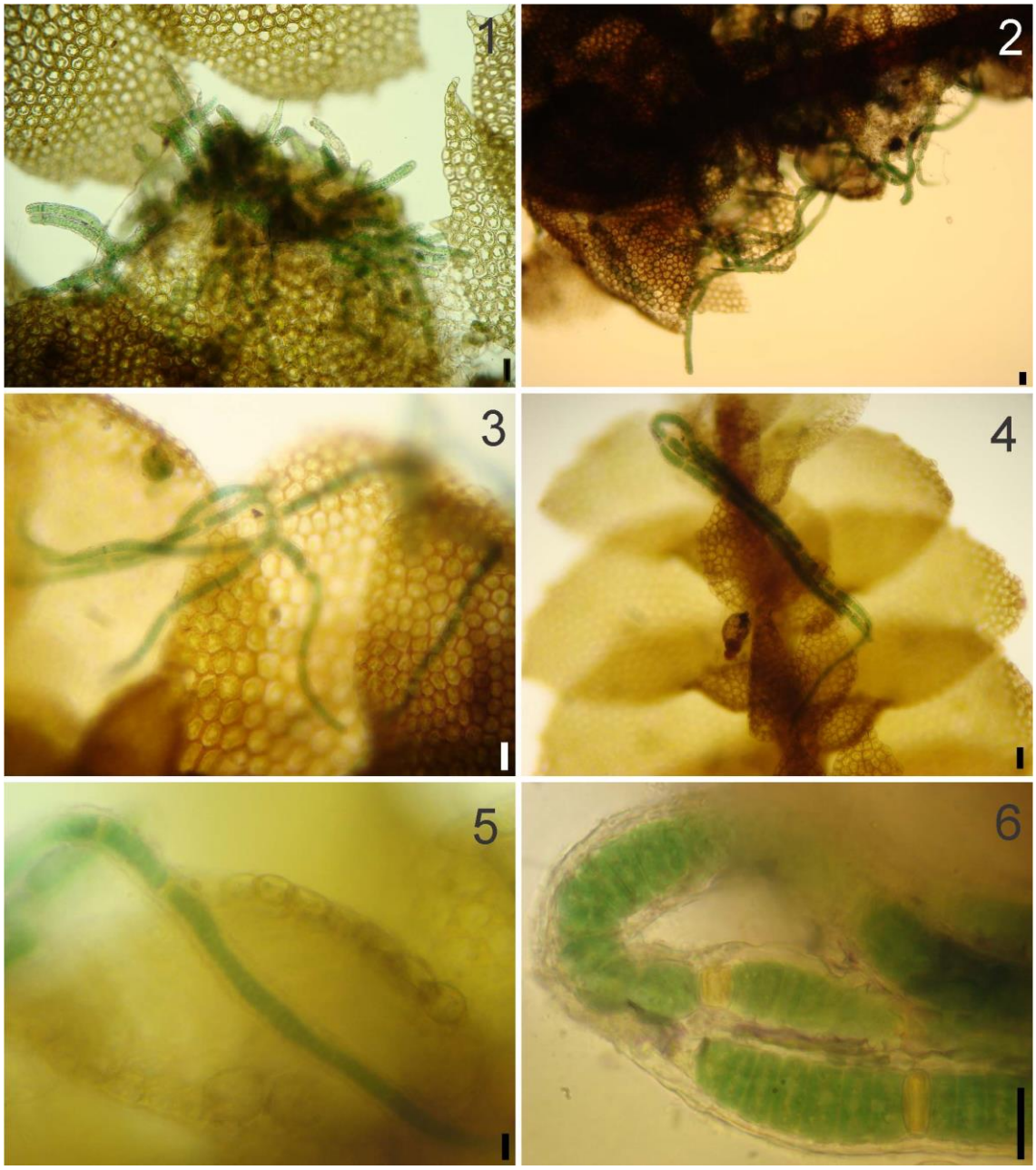
Figs. 1-6. 1-4. General aspect of thallus growing on mosses. 5. Slightly tapered filament. 6. False branching and details of cells. Bars = 30 μm in 1-4; 10 μm in 5 and 6.

Figs. 7-12. 7-9. False branching. 10. Detail of a filament. 11. Mosaic-ornate sheaths (arrow). 12. Tapered filament. Bar = 10 μm

Figs. 13-15. 13. Tapered filament. 14. Surface of mosaic-ornate sheath. 15. Detail of geminate false branching. Bar = 10 μm.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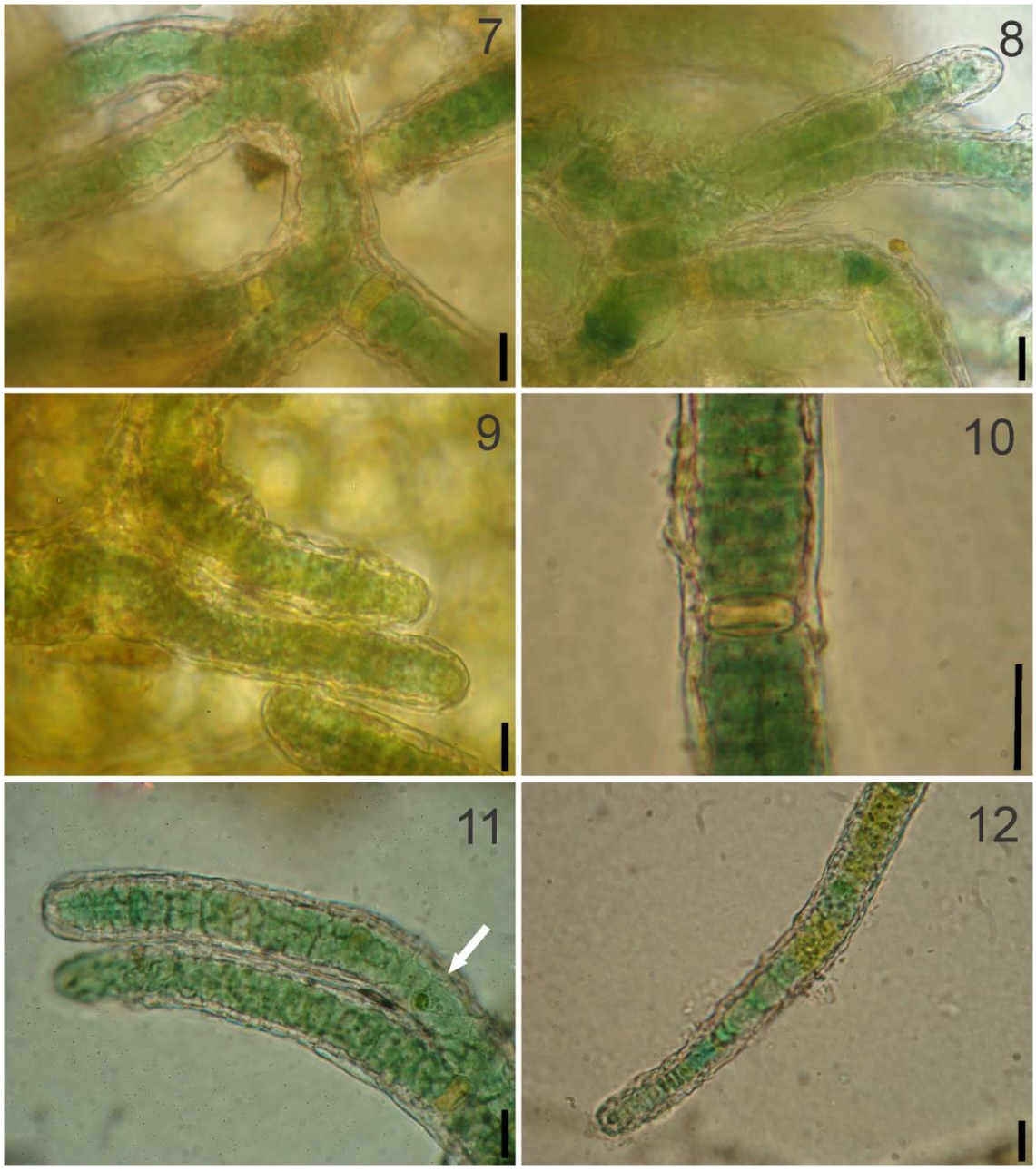
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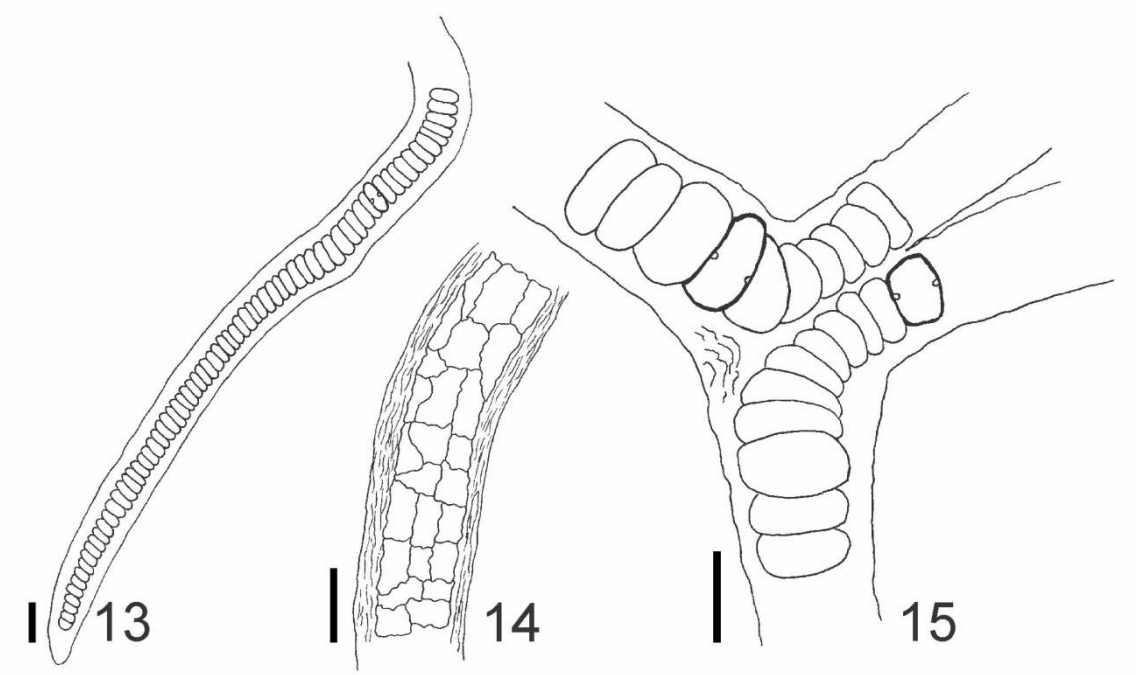
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Anexo V

Morphological and molecular characterization of *Stigonema jureiensis sp. nova* and *Stigonema fremyi* (Nostocales, Cyanobacteria)

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ABSTRACT

Key words: terrestrial habitats, tropical environments,

INTRODUCTION

The nostocalean genus *Stigonema* Bornet & Flahault is abundantly distributed worldwide (Cyanodb), but studies on these types are getting rare when compared to other Cyanobacteria, mainly due to the difficulties to carry out phylogenetic studies on this genus. Up to now, for *Stigonema*, the sequencing of the 16S rRNA gene and phylogenetic evaluations are conditioned to the achievement of isolation of strains, which is a very difficult task and perhaps impossible for some populations. In these cases, the sequencing of the 16S rRNA gene from not cultured individuals (from nature material) is necessary, and more precise technics to perform this must be developed, leading to a better comprehension of the phylogeny of Nostocales in the future. The amplification of 16S gene by PCR, from single filaments from nature is widely used for other cyanobacterial genera, but for *Stigonema* this method seems to be not efficient.

Because of that, the majority of the important taxonomical studies comprising *Stigonema* species are restricted to morphological evaluations presented in classic works as Bornet & Flahault (1889), Gardner (1927), Fremy (1930) and Geitler (1933). Recently, Sant'Anna *et al*. (2013) described three new species of the genus and reported other four types commonly found in Mata Atlântica, southeastern Brazil. Since the

describing of a few Chinese species of *Stigonema* in 2006, these are the only species described for the genus in the last years. Considering specifically the Mata Atlântica, other important *Stigonema* studies are Silva & Sant'Anna (1988), which described *S. gracile* from Ilha do Cardoso and Silva & Sant'Anna (1996), which revised the genus for the São Paulo State. Unfortunately, as for all *Stigonema* reports, no one of these papers cited above present new species characterized by both morphology and phylogeny together.

According to this, in the present study we sequenced the 16S rRNA gene from two natural populations of *Stigonema* sampled in the Mata Atlântica, São Paulo State, Southeastern Brazil. One of them was identified as *S. fremyii* Sant'Anna *et al.* and we present here the morphological plasticity of this species, besides its confirmation by phylogenetic data. The other population is quite different from any other species of the genus and then, we described it as a new species named *Stigonema jureiensis sp. nov.* based on phylogenetic and morphological data. This is the first paper describing a species of *Stigonema* based on both morphological and molecular data.

MATERIAL AND METHODS

Samples were collected using spatula, by scrapping biofilms growing on rocks in the tropical (Ecological Station Juréia-Itatins, 24º24'76''S and 47º01'15''W) and subtropical (State Park of Ilha do Cardoso, 25º04'12"S 47º55'27"W) areas of the Brazilian rainforest Mata Atlântica. The material was kept dry into paper sacks and aliquots of the samples were rehydrated during 20 hours, in distilled water, for morphological analysis in laboratory. At least, 20 individuals of each studied population were evaluated and photographed using microscope Zeiss Axioplan 2 equipped with Zeiss Axiocam MRc digital camera. Part of the samples were also preserved in formaldehyde (4%) and deposited in Herbarium of Institute of Botany (SP).

Total genomic DNA was isolated from the environmental populations using MOBIO Ultraclean DNA Isolation Kit. For that, the naturally isolated populations were manually purified, using microscope and point-tapered Pasteur pipets, for debris and other organisms removal. The PCR amplification of 16S rRNA gene and sequencing methods were described before in Fiore *et al.* (2007) and Hentschke *et al.* (2014).

The 16S rRNA gene sequences obtained in this study and reference sequences retrieved from GenBank were aligned by ClustalW and used to generate the phylogenetic trees. The trees were constructed based mainly in the stigonematacean tree of Lamprinou *et al.* (2011), using the Maximum Likelihood (ML) and Maximum Parcimony (MP) methods implemented by MEGA version 5.0 program package (Tamura *et al.* 2011) and Bayesian (BA) criteria using MrBayes 3.2 (Ronquist &

Huelsenbeck 2003) in two independent runs, with four chains each, for 5×10^5 generations. The GTR+G+I evolutionary model was applied and the robustness of the trees were estimated by bootstrap percentages using 1.000 replications for ML and MP, and posterior probability for BA. A similarity matrix (p-distance) was also generated for related taxa. The 16S rRNA gene sequence of the *Stigonema* new species were deposited in the NCBI GenBank database under accession numbers (…).

RESULTS

In tropical and subtropical areas from Mata Atlântica in southeastern Brazil, state of São Paulo, we found a new terrestrial stigonematacean species named *Stigonema jureiensis sp. nov.*, supported by morphological and molecular data. Also, we discuss here the morphological plasticity of *S fremyi* and present the phylogenetic position of this species.

Both taxa presented in this paper fit very well in the morphological circunscription of *Stigonema* Bornet & Flahault, featuring true branches (T-type), plurisseriate trichomes and thallus not diversified in distinct mains filaments and branches. The populations identified as *S. fremyi* are in total agreement with the description of the species (Sant'Anna *et al.* 2013), although they present higher morphological plasticity as the presence of alternative parallel branches. The populations described here as *S. jureiensis* sp. nov. differs from all other already known *Stigonema* types, due to a special kind of mosaic-like sheaths, never described before for the genus.

Phylogenetic analysis showed that the 16S rRNA gene sequences of *S. fremyi* and *S. jureiensis* are not close related to any other sequence available in GenBank. The calculated similarity values (p-distance) presented in Table 1 indicated that the sequences of our populations share up to 96% of similarity with two *Stigonema ocellatum* (SAG 4890 AJ544082 and SAMA 35 GQ354275); thereafter the values were always lower than 94% when compared to the other strains sequences available in GenBank. The sequences of *S. jureiensis sp. nov.* and *S. fremyi* present 99% of similarity between each other (Table 1), but according to the morphological results they cannot be united in a single species. In the ML, MP and BA trees (Fig 1), our populations were always in well supported clades together with the type species of *Stigonema* (*S. ocellatum* strains), indicating that the genus is probably monophyletic, although there are scarce molecular data available to assert that. These strains together with Stigonematales AEL (AY785313) form a sister group of two well supported Hapalosiphonaceae clades and *Chlorogloeopsis fritschii* PCC6912 (AB093489).

Description of species

Stigonema jureiensis Hentschke, G. S. & Sant´Anna, C. L. **sp.nov.**

(Figures 2-8)

Filaments forming a tree-like thallus, erect, more or less parallel, tortuous 23-33 µm diam., sometimes slightly tapered toward the ends and intensely branched. Branches identical to the main filament, bending toward the thallus apex. Trichomes with uni and plurisseriate parts 16-25 µm diam, composed by dark-green compressed cells 5-11 µm long. Apex rounded, usually unisseriate, slightly tapered or not. Sheats abundant, hyaline or yellowish, mosaic-like sculptured and with irregular margins. Heterocytes lateral subspherical.

Holotype: BRAZIL, São Paulo State, Ecological Station Jureia-Itatins, 08/16/2011, *Guilherme S. Hentschke, Watson A. Gama Jr., Camila F. da S. Malone* and *Célia L. Sant'Anna* (SP 427311), Herbarium of São Paulo State, São Paulo, Brazil.

Habitat: On humid rocks in the limit of a dense rainforest area.

Etymology: The name is after the Ecological Station Juréia-Itatins.

Stigonema fremyi Sant'Anna *et al*., Phytotaxa 89(1): 8. 2013.

Main filaments creeping, cylindrical and intensely branched 23-31 µm diam. Branches similar to the main filaments, erect, long, sometimes parallel and with less frequent shorter branches. Apex and bases of branches slightly tapered or not. Trichomes initially unisseriate, than plurisseriate composed by usually up to 3 rows of ovoid or subspherical bright green cells. Cells 9-14 µm diam. Sheats hyaline or yellowish, homogenous forming a lanceolate structure after releasing of hormogonia. Hormogonia very common, usually formed in lateral or alternatively at apex of branches and main filament. Heterocytes with the same shape and size of cells.

Studied material: BRAZIL, São Paulo State, State Park of Ilha do Cardoso, 06/29/2010, *Watson A. Gama Jr. and Camila F. da S. Malone* (SP 401447), Ecological Station Jureia-Itatins, 08/16/2011, *Guilherme S. Hentschke, Watson A. Gama Jr., Camila F. da S. Malone* and *Célia L. Sant'Anna* (SP 428606, 427310).

Habitat: On rocks by the sea in the limit of dense rainforest.

DISCUSSION

The populations identified as *S. fremyi* are in agreement with the original description of this species, although they present higher morphological plasticity. The main *S. fremyi* features are "Filaments forming dense clusters, morphologically similar to the branches which separate them from the main filaments in right angles…" and "…Branches narrowed at the base and toward the ends, with short uniseriate segments in terminal parts, ends rounded…" (Sant'Anna *et al.* 2013). Our populations present all these described characteristics for the taxon and beyond that, we found some individuals with erect parallel branching, branches not in right angles, not tapered and with rich hormogonia formation. Besides that, we also observed unisseriate initial stages of the life cycle. This morphological plasticity is explained by considering that all studied populations were found by the sea in the border of the rainforest and exposed to the sea spray, while originally, this species was described based on a population growing in Mata Atlântica, but on rocks on the top of mountain, over 1.600 meters high. This fact strongly reinforces the idea that cyanobacterial species can present certain morphological variations when growing in different habitats and still belong to the same taxon.

S. jureiensis is a very special type and not morphological related to any other species of the genus, because its unusual mosaic sculptured sheaths. These kind of sheaths were already found in *Scytonema stuposum*, *S. santannae* and *Brasilonema ornatum* (Hentschke & Komárek 2014, Sant'Anna *et al*. 2011) in the Brazilian Mata Atlântica, but was never reported for stigonematacean types. Up to now, all populations with mosaic sculptured sheaths were found growing together with fungi hyphae (Hentschke & Komárek 2014), but we did not observed this for *S. jureiensis*. Probably this characteristic was developed independently in different lineages, as an adaptation to still unknown environmental factors. This feature together with molecular data strongly indicates that our population is a new species of *Stigonema*.

The only paper considering morphological and molecular characterization of a *Stigonema* species is the one of Ferreira *et al*. (2013), in which a population of *S. ocellatum* is reported. Our paper is the first to describe a new species of the genus by molecular and morphological data. The difficulties to isolate strains of this genus complicate the molecular studies, and we just could sequence our population due to the huge naturally isolate biomasses, which we found on nature. Because of that, we think that describing new species based strictly in morphological evaluations is still important for these uncultivable groups, since they can be validated later as we did for *S. fremyi*. The option of don´t describe such types, in this cases, implies in underestimation of morphological and species diversity. In order to validate the extant known types, evaluate the main morphological markers for identification of species and improve the

knowledge of the phylogeny of *Stigonema*, more methods for sequencing populations directly from nature or for culturing these organisms must be developed.

Beyond describing a new species, this study combining molecular and morphological data of environmental populations, aggregate knowledge about the important morphological markers for species level for *Stigonema*. Recently, a morphological and molecular study was done for *Scytonema* strains (Komárek *et al.* 2013) and further, the same must be done for *Stigonema* populations sequenced from nature material, since more data gets generated and compiled. Finally, as reported for *Stigonema corticola* Sant'Anna *et al.* (Sant'Anna *et al.* 2013) we also found and unisseriate stage for *S. fremyi*. Consequently, more studies in this level are necessary to the better understanding of the phylogenetic relationships within Nostocales clades and to the clarification of whether the unisseriate *Stigonema* are only life cycle stages of plurisseriate ones or are true different types.

Table 1. Similarity matrix for ten strains comparing 16S rRNA gene sequences.

Figure 1. Molecular Phylogenetic anaylsis by Maximum Likelihood method. Bootstrap values ML/MP/BA are indicated at nodes. The analysis involved 28 nucleotide sequences. There were a total of 1246 positions in the final dataset. -=no support.

Figures 2-8. *Stigonema jureiensis.* **2-4.** General view of thallus. **5.** Detail of Apex. **6.** Detail of branching and mosaic-like sheats. **7.** Mosaic-like sheats. **8.** Filament with uni- and polyseriate parts. Scales: 2 = 50 µm, 3-8 = 10 µm.

Figures 9-15. *Stigonema fremyi.* **9, 10.** Parallel branching arising from the same side of the main filament. **11.** Hormogoniferous branching. **12.** Branch slightly tapered on the basis. **13.** Branch not tapered on the basis. **14.** Uni- and polyseriate filaments. **15.** Detail of filament. Scales: 9, 10 = 50 µm, 11-15 = 10 µm.

Table 1.

AJ544077_Fischerella_muscicola_strain_SAG2027	1.00									
AJ544076_Fischerella_strain_1711	0.99									
Fischerella_sp_MV11_DQ78617116S	0.95	0.95								
AJ544083_Symphyonema_12691	0.93	0.93	0.92							
AJ544084_Syphyonema_1517	0.93	0.94	0.92	0.99						
AJ544082_Stigonema_ocellatum_SAG_4890	0.94	0.94	0.92	0.93	0.94					
GQ354275_Stigonema_ocellatum	0.93	0.93	0.92	0.93	0.94	0.99				
AY785313_Stigonematales_AEL	0.93	0.93	0.92	0.93	0.93	0.95	0.94			
Stigonema fremyi	0.93	0.93	0.92	0.94	0.94	0.96	0.96	0.94		
Stigonema jureiensis	0.93	0.92	0.91	0.94	0.94	0.96	0.96	0.93	0.99	1.00
	1	2	3	4	5	6	7	8	9	10

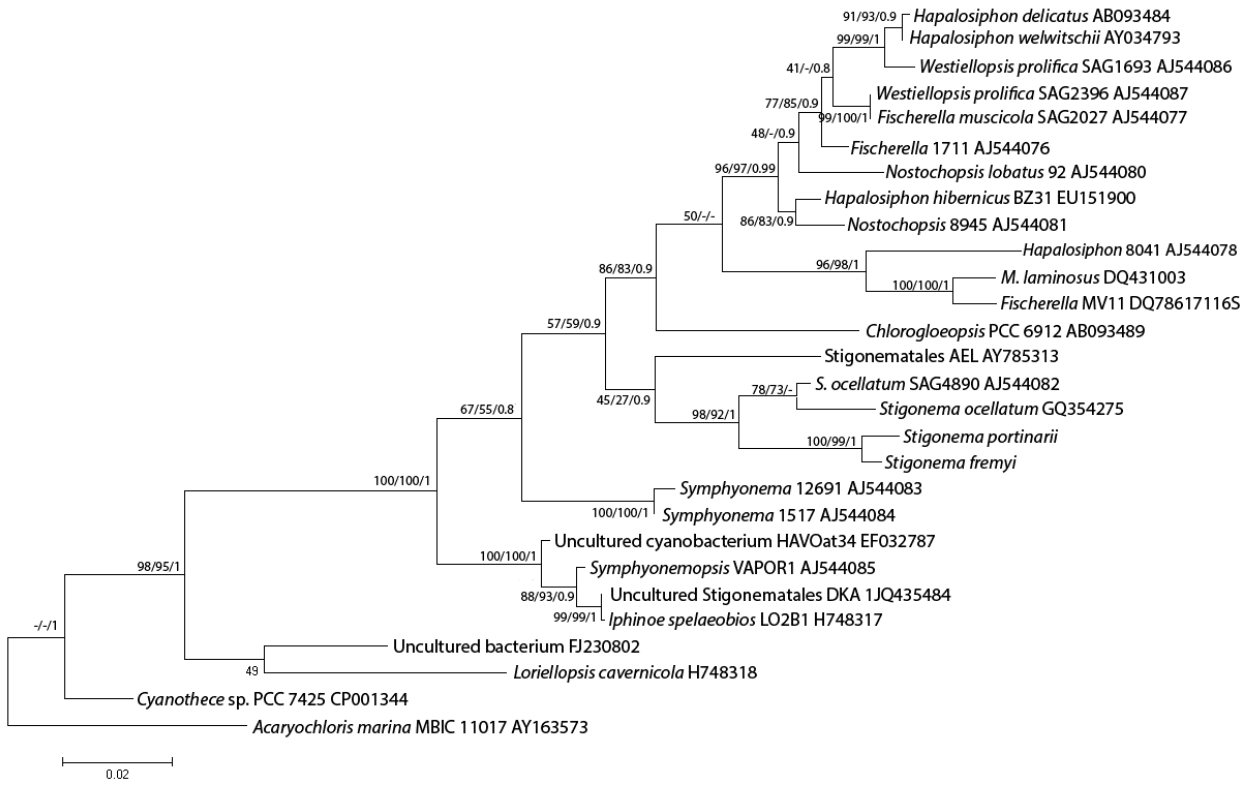
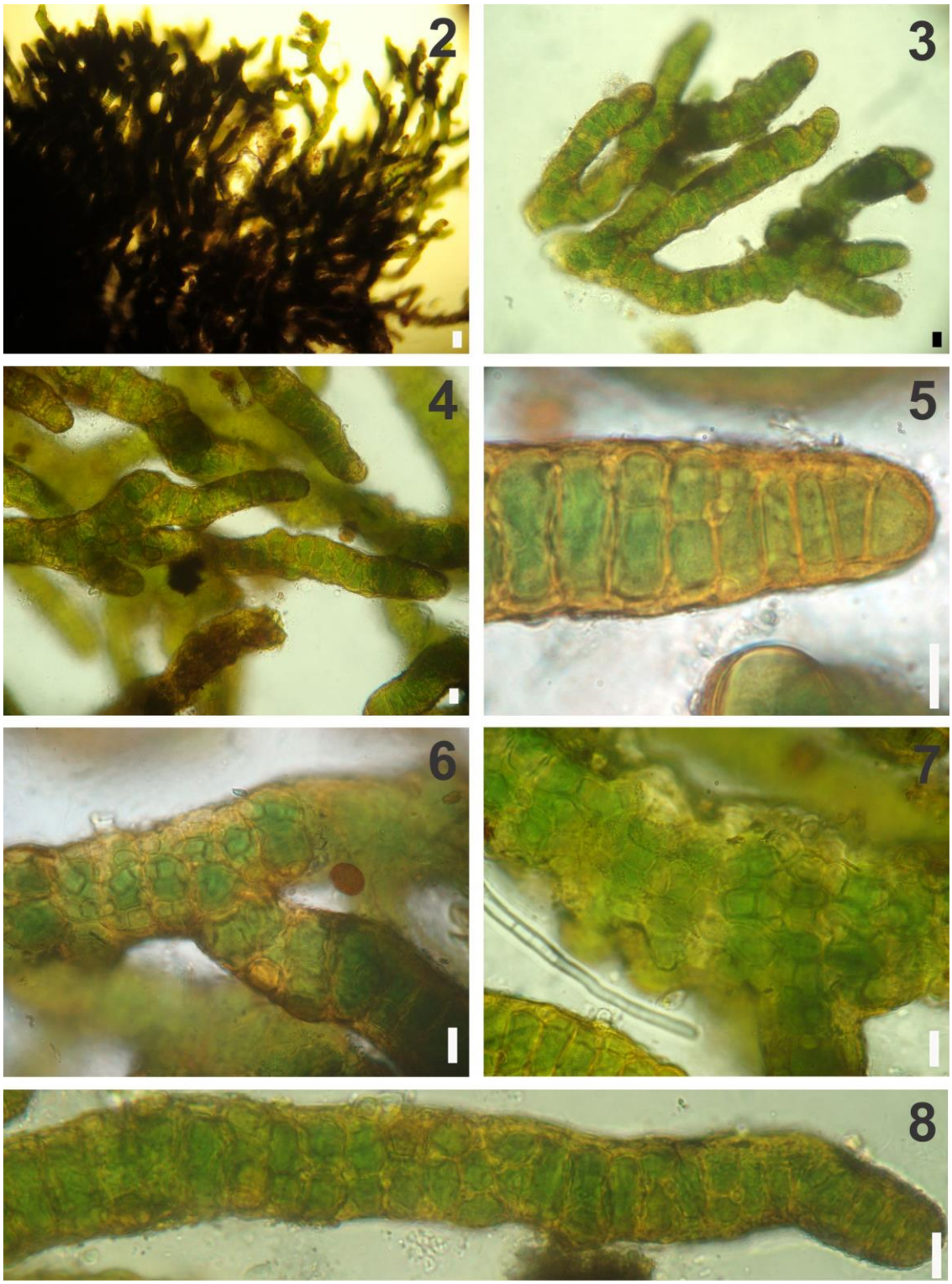
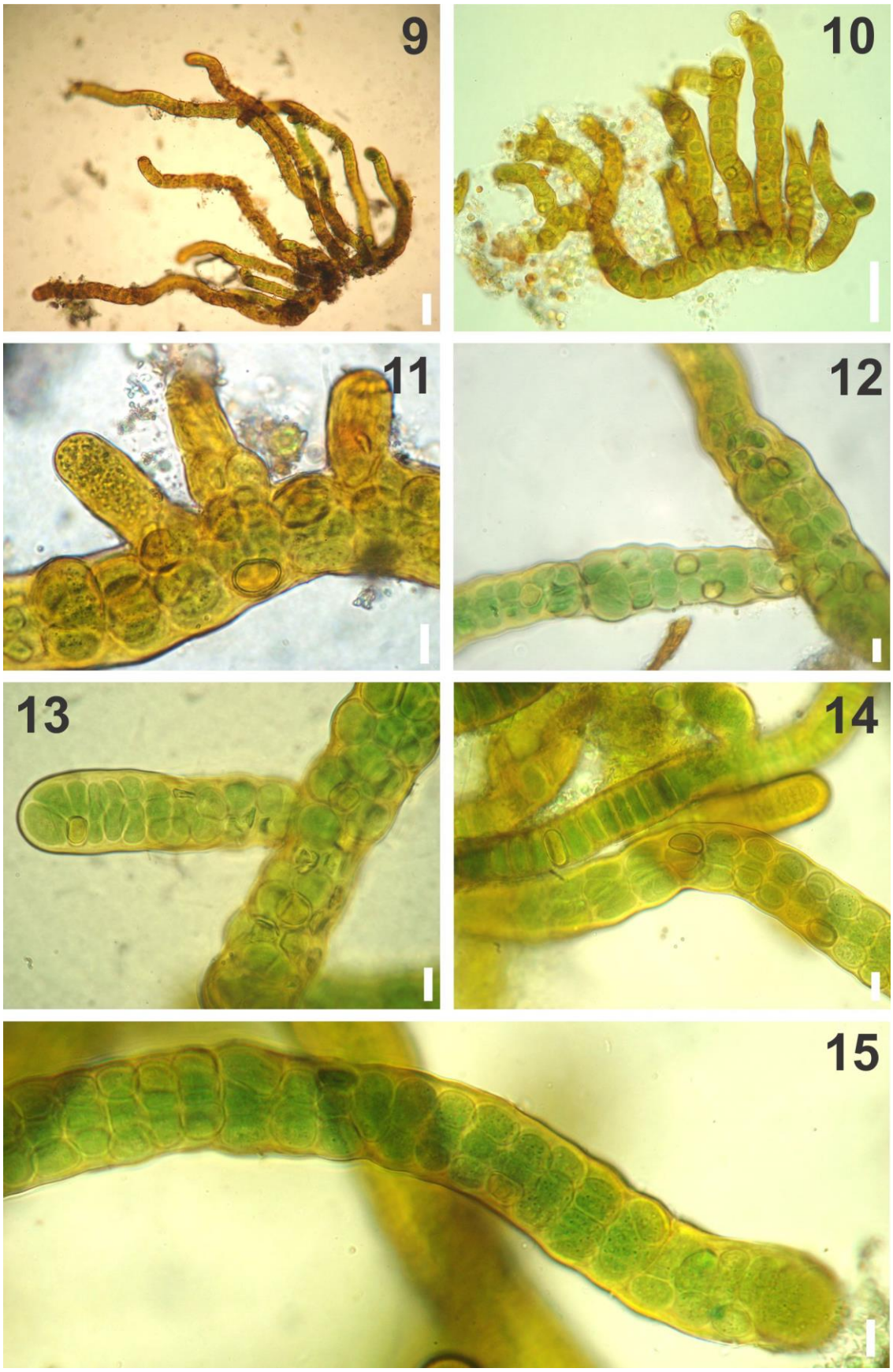

91/93/0.9
Hapalosiphon delicatus AB093484
99/99/1
Hapalosiphon welwitschii AY034793
41/-/0.8
Westiellopsis prolifica SAG1693 AJ544086
77/85/0.9
Westiellopsis prolifica SAG2396 AJ544087
99/100/1
Fischerella muscicola SAG2027 AJ544077
48/-/0.9
Fischerella 1711 AJ544076
96/97/0.99
Nostochopsis lobatus 92 AJ544080
Hapalosiphon hibernicus BZ31 EU151900
50/-/-
86/83/0.9
Nostochopsis 8945 AJ544081
Hapalosiphon 8041 AJ544078
86/83/0.9
96/98/1
M. laminosus DQ431003
100/100/1
Fischerella MV11 DQ78617116S
57/59/0.9
Chlorogloeopsis PCC 6912 AB093489
Stigonematales AEL AY785313
78/73/-
S. ocellatum SAG4890 AJ544082
45/27/0.9
67/55/0.8
98/92/1
Stigonema ocellatum GQ354275
100/99/1
Stigonema portinarii
Stigonema fremyi
100/100/1
Symphyonema 12691 AJ544083
100/100/1
Symphyonema 1517 AJ544084
Uncultured cyanobacterium HAVOat34 EF032787
100/100/1
98/95/1
Symphyonemopsis VAPOR1 AJ544085
88/93/0.9
Uncultured Stigonematales DKA 1JQ435484
99/99/1
Iphinoe spelaeobios LO2B1 H748317
-/-/1
Uncultured bacterium FJ230802
49
Loriellopsis cavernicola H748318
Cyanothece sp. PCC 7425 CP001344
Acaryochloris marina MBIC 11017 AY163573
0.02

Figure 1.



Figures 2-8.



Figures 9-15.

Anexo VI

Komarekiella atlantica gen. et sp. nov. (Nostocaceae, Cyanobacteria): a new subaerial taxon from the Mata Atlântica.

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Abstract

Six strains of nostocacean Cyanobacteria sampled in tropical and subtropical environments of Mata Atlantica, São Paulo, Brazil, were studied by morphological and molecular methods. The populations were not found in nature material, but grew vigorously when inoculated in culture media. Morphologically they are similar to *Nostoc, Desmonostoc* and *Mojavia* and indistinguishable from *Chlorogloeopsis*. The phylogeny of 16S and ITS regions, besides ITS secondary structures analysis show that these strains are close to nostocacean strains, in a supported clade and separated from the other genera, although the backbone of the trees were not resolved. The new type *Komarekiella atlantica gen. et sp. nov* described here is one of the many genera which will be described in the short run, in result specially by the splitting of the genus *Nostoc.*

Introduction

Nostoc Vaucher ex Bornet et Flahault is a widespread, commonly encountered genus of cyanobacteria, with 82 relatively well-defined species and many other poorly described species not in current usage (Komárek 2013). Phylogenetic studies based on 16S rRNA gene sequence have shown that the strains assigned to *Nostoc* are not a monophyletic group (Hrouzek *et al*. 2005, Řeháková *et al*. 2007, Kaštovský &Johansen 2008, Lukešová *et al*. 2009, Johansen *et al*. 2013, and recently several *Nostoc*-like taxa have been described, including *Desmonostoc* Hrouzek et Ventura in Hrouzek *et al*. (2013), *Mojavia* Řeháková et Johansen in Řeháková et al. (2007) and *Trichormus* (Ralfs ex Bornet et Flahault) Komárek et Anagnostidis (1989). Despite this progress, further revisionary work is expected, such as the unlikely placement of aerotope-producing taxa such as *Nostoc kihlmannii* Lemmermann in *Nostoc sensu stricto*.

One of the morphotypes of *Nostoc* commonly encountered from terrestrial habitats is *N. punctiforme* var. *populorum* Geitler, which has tightly compressed trichomes in a common sheath which often appear to have division in multiple planes. A genus with division in multiple planes similar to this form was described as *Chlorogloeopsis fritschii* Mitra et Pandey. Phylogenetic analysis of *Chlorogloeopsis* places this taxon sister to the clade containing true-branching genera such as *Hapalosiphon* Bornet et Flahault, *Fischerella* Bornet et Flahault, *Mastigocladus* Bornet et Flahault, *Nostochopsis* Bornet et Flahault, and *Westelliopsis* Janet (Kaštovský and Johansen 2008), while the *N. punctiforme* var. *populorum*-like morphotypes which are nearly indistinguishable from *Chlorogloeopsis* fall clearly into the *Nostoc sensu stricto* clade (see *N. indistinguendum* Řeháková et Johansen and *N. desertorum* Řeháková et Johansen in Řeháková et al. 2007).

We recently isolated six strains of a C*hlorogloeopsis/N. puntiforme* var. *popularum*-like taxon growing epiphytically on trees in subtropical and tropical regions of the Mata Atlântica (the coastal forest of Brazil). These isolates came up in culture from samples in which *Nostoc*-like species could not be detected, and thus are only known from culture. The strains produce multiseriate filaments in a common mucilage, and appear to have cell division in more than one plane, like *Chlorogloeopsis*. They also have a life-cycle identical to that seen in *Chlorogloeopsis* (Hindák 2008). Some stages of their life cycle include filaments of microcolonies, like *Mojavia* (Řeháková et al. 2007). Phylogenetic analyses show they likely belong to the Nostocaceae, but in a clade separate from *Nostoc*, *Desmonostoc*, *Mojavia* and *Trichormus.* This paper will present evidence that establishes the uniqueness of these strains and will describe these isolates as *Komarekiella atlantica* gen. et sp. nov.

Material and Methods

Studied area, sampling and isolation of strains

Samples were collected by scraping biofilms growing on the bark of trees, wood poles and concrete walls in the tropical and subtropical areas of the Brazilian rainforest "Mata Atlântica", specifically in the State Parks of Santa Virginia (23º20'S and 45º09'W) and Ilha do Cardoso (25º04'12"S and 47º55'27"W) and in the Ecological Station Juréia-Itatins (24 26.481' S and 47 04.660' W). Material was kept dry in paper sacks, and subsequently inoculated in the laboratory onto BG-11 nitrogen-free (Rippka *et al.* 1979) agar media for enrichment and isolation of strains. Isolates were transferred into respective test tubes containing the same type of liquid media and kept under 14 h:10 h (light:dark) cycle with white fluorescent light (30 µmol photons m^{-2} s^{-1}), at a temperature of 23 (±2) ^{o}C, and held in the Culture Collection of the Institute of Botany, São Paulo, Brazil (CCIBt 3307, 3481, 3552, 3483, 3487, 3486 and 3485). Environmental samples and strain clones were also preserved in formaldehyde (4%) and deposited in the Herbarium of Institute of Botany (SP).

Morphological and life cycle studies

Morphological analysis was performed using a Zeiss Axioplan 2 microscope equipped with Zeiss Axiocam MRc digital camera and inverted microscope Zeiss Axiovert 25.

In order to report morphological plasticity and life cycle stages, all strains were observed in the microscope every day for 7 days, then once a week for a month and finally after 3 months, starting 2 weeks after inoculation into new liquid BG11-nitrogen free media. Also, the strain CCIBt 3307 was inoculated onto agar with BG11 nitrogen-

free media in an Üthermohl chamber and observed following the same frequency cited above, using an inverted microscope.

For the morphological comparisons between the macroscopic growing patterns of one strain in the new genus (CCIBt 3307) and a typical macroscopic *Nostoc commune* strain (CCIBt 3485), colonies of both strains were inoculated onto fresh Petri dishes with solid BG11 nitrogen-free media and observed for two months.

In a search for the natural populations, all dry environmental samples were rehydrated with distilled water for 20 hours before being analyzed using the microscope specified above.

DNA extraction, PCR amplification and sequencing:

Total genomic DNA was isolated from liquid cultures of all cyanobacterial strains using MOBIO Ultraclean DNA Isolation Kit. Nearly complete 16S rRNA gene fragments were amplified by PCR using the primers 27F1 (Neilan et al. 1997) and 23S30R (Taton et al. 2003) in a Techne TC-412 thermocycler (Bibby Scientific). The reaction contained 10 ng of genomic DNA, 0.5 µM of each primer, 200 µM of dNTPs, 2.0 mM of MgCl2, l × PCR buffer and 1.5 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), in a final volume of 25 µL. The PCR cycle had initial denaturation at 94 ºC for 5 min, followed by 10 cycles of 94 ºC for 45 s, 57 ºC for 45 s, 72 ºC for 2 min, another 25 cycles of 94 ºC for 45 s, 57 ºC for 45 s, 72 ºC for 2 min and a final extension step at 72 °C for 7 min. The resulting PCR product was cloned into a pGEM®-T Easy Vector System (Promega, Madison, WI, USA) according to the supplier's manual, cloned by heat-shock in E. coli DH5α cells and plated for blue-white selection (Sambrook & Russel 2001). After growth, recombinant plasmids were extracted from white colonies by the alkaline lysis method (Birnboim & Doly 1979). The cloned gene fragment was sequenced using "Big Dye Terminator" version 3.0 (Applied Biosystems) with the plasmid primers T7 and M13 and the internal primers 357F/357R, 704F/704R and 1114F/1114R (Lane 1991). The cycle sequencing reaction was performed as follow: 25 cycles of 95 ºC for 20 s, 50 ºC for 15 s and 60 ºC for 1 m. the DNA was precipitated using 2 µL of sodium acetate buffer (1.5 M sodium acetate - pH 9.0 and 250 mM EDTA- pH 8.0) and 60 µL of 100% ethanol. The tubes were centrifuged at 4 ºC for 15 min at 12,000 × g and the supernatants were discarded. The DNA pellets were washed with 150 µL of 70% ethanol, centrifuged for 5 min and the supernatants removed. The pellets were air-dried overnight in the dark and at room temperature. The purified pellets were resuspended in HiDi formamide (Applied Biosystems), and the sample placed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequenced fragments were assembled into contigs using the software package Phred/Phrap/Consed (Philip Green, Univ. of Washington, Seattle, USA) and only bases with a quality >20 were considered.

Phylogenetic analyses

The 16S rRNA gene sequences obtained in this study and reference sequences retrieved from GenBank were manually aligned, and used to generate the phylogenetic trees. The trees were constructed by Maximum Parsimony (MP) method, using a heuristic search without steepest descent, SWAP=TBR, and 1000 replicates implemented by PAUP version 4.0b10, and Bayesian (BA) criteria using MrBayes 3.2 (Ronquist & Huelsenbeck 2003) in two independent runs, with four chains each, for 5×10^6 generations. The best-fitting evolutionary model GTR+G+I was selected for 16S rRNA gene sequences using ModelTest under the Akaike information criterion (Posada & Crandall 1998).

To determine the position of our strains in the first round of phylogenetic analysis, MP and BA trees were constructed with 454 OTU´s represented by Nostocales and Stigonematales (Komárek & Anagnostidis 1989) sequences. Then, we chose related taxa and constructed smaller trees based on 213 OTU´s. The outgroups used for all 16S rDNA phylogenetic analysis were represented by *Gloeobacter violaceus* PCC 8105, *Chroococcidiopsis* 9E-07, *Chroococcidiopsis cubana* SAG 39-79, *Chroococcidiopsis thermalis* PCC 7203 and *Chroococcidiopsis* BB 3 SAG 2024. A similarity matrix (p-distance) was also generated for related taxa.

For the phylogenetic analysis of the 16S-23S ITS region, we chose closely related taxa based on the 16S rRNA MP and BA trees, and constructed MP trees with 40 OTU´s. The secondary structures of the stems D1-D1´, Box B, V2 and V3 were determined separately using Mfold version 2.3 (Zuker 2003), with folding temperature set at 20^oC for sequences. For all ITS analyses, we used sequences containing both $tRNA^{Ile}$ and $tRNA^{Ala}$.

The 16S rDNA and 16S-23S ITS rDNA sequences of the cyanobacterial strains CCIBt 3307, 3481, 3552, 3483, 3487, 3486 and 3485, isolated in this study were deposited in the NCBI GenBank database under accession numbers (…).

Results

Komarekiella G.S. Hentschke, J.R. Johansen et C.L. Sant'Anna gen. nov. Figs X-XX.

In nature, growing on the bark of trees, wood, or concrete. Thallus microscopic in nature and in culture, in culture primarily growing attached to the glass walls of the test tubes in liquid media, and released as tufts which detach and settle to the bottom of the test tube as they grow, on agar growing radially from the center via hormogonia release, forming a dense creeping mat. Colonies spherical when developing from akinetes,

elongate when developing from hormogonia, eventually aggregated loosely into macroscopic thalli through release of diffluent mucilage. Akinetes apoheterocytic, spherical or subspherical, granulated, developing from vegetative cells in trichomes, germinating into two equal vegetative cells or alternatively into a large vegetative cell and a smaller heterocyte, either of which develops into spherical colonies with a firm mucilage layer, which eventually become larger colonies enclosed within a diffluent mucilage. Hormogonia developing from older more diffuse colonies; when first formed motile, short, lacking firm mucilage, later immotile with heterocytes and akinetes, developing into multiseriate filaments, which eventually become elongate colonies enclosed in a firm mucilage. Cells quadratic in young hormogonia, becoming spherical or subspherical as filament matures, dividing perpendicular or longitudinal to the main axis of filament. Heterocytes spherical, compressed subspherical or oval.

Type species: *Komarekiella atlantica*.

Etymology: Named in honor of Professor Jiří Komárek.

***Komarekiella atlantica* G.S. Hentschke, J.R. Johansen et C.L. Sant'Anna sp. nov.**

(Figure 1)

Thallus at first bright blue-green, becoming a darker duller blue-green with age, and consisting of many subspherical colonies or aggregations of filaments enclosed in a common mucilage, up to 1 cm long. Colonies with compact aggregations of cells, at first with filamentous arrangement not visible, later more diffuse with trichomes apparent. Hormogonia released within a few days of transfer to fresh media, motile, 3 μm wide. Filaments at first uniseriate, later multiseriate, up to 10 μm wide. Vegetative cells in hormogonia quadratic, isodiametric to longer than wide, 3-5 μm long, becoming spherical or compressed subspherical in mature filaments, dividing in more than one plane, 3.4-5.5μm in diameter. Heterocytes developing in germinating akinetes 2-3 μm in diameter, later 3-5.5 μm in diameter. Akinetes 3.5-6 μm in diameter.

Holotype here designated: Brazil, State of São Paulo, State Park of Serra do Mar, Nucleus Santa Virgínia, 02/11/2010, *Watson A. Gama Jr. e Ewerton C. Manarin* (SP 428462)

Type location: growing on a wooden fence post in tropical rainforest, State Park of Santa Virginia (23º20'S, 45º09'W).

Reference strain: CCIBt 3307.

Etymology: Named after the Mata Atlântica

Studied material: Brazil, State of São Paulo, State Park of Ilha do Cardoso, 06/29/2010, *Watson A. Gama Jr. and Camila F. da S. Malone*, (SP 428466); State Park of Serra do

Mar, Nucleus Santa Virgínia, 02/11/2010, *Watson A. Gama Jr. e Ewerton C. Manarin* (SP 428462, SP 428463, SP 428465, SP 428467); Ecological Station Jureia-Itatins, 08/16/2011, *Guilherme S. Hentschke, Watson A. Gama Jr., Camila F. da S. Malone* and *Célia L. Sant'Anna* (SP 428464).

Komarekiella could not be found in the environmental samples that served as the source material for strains CCIBt 3307, 3481, 3552, 3483, 3487 and 3486, but the taxon grew vigorously when inoculated into culture media and was easily isolated from enrichment cultures.

Morphologically *Komarekiella* is close to *Chlorogloeopsis*, *Mojavia*, *Desmonostoc*, and microscopic species of *Nostoc*, although they differ from each other in some aspects. The life cycle of *Komarekiella* is indistinguishable from that of *Chlorogloeopsis*, especially with reference to the germination of the akinetes, which serves as the diagnostic feature of *Chlorogloeopsis*. Without phylogenetic or molecular evidence, we would conclude that our strains belong to *Chlorogloeopsis*. *Komarekiella* also shares characteristics with other genera in the Nostocaceae. It has the aggregated filaments of microcolonies like *Mojavia,* the absence of a firm outer layer of the colonial mucilage like *Desmonostoc*, and stages in its life cycle which resemble some species of microscopic *Nostoc* (e.g. *N. punctiforme* var. *populorum*). If only isolated stages of the life cycle of *Komarekiella* were observed, it would be possible to incorrectly assume that those stages were one or more of these other taxa. A full summary(table 1) of the comparison of these genera illustrates the difficulty of using morphology alone to define these genera.

Evaluation of the macroscopic growth form showed that the strains of *Komarekiella* grow in a substantially different way from *N. commune* isolated from soils in the Mata Atlântica. Macroscopic mucilaginous colonies are diagnostic in *N. commune*. In nature *N. commune* was macroscopic, and in our laboratory trials, it typically formed well-defined spherical macroscopic mucilaginous colonies on solid media. *Komarekiella* grew radially creeping under the same conditions.

Molecular Characterization

Maximum Parsimony and Bayesian analysis based on 16S rRNA sequence data from 213 OTU's were largely in agreement, and only the parsimony analysis is shown (Fig. 2). Both analyses showed *Komarekiella* as a highly supported clade (100, 1.0 bootstrap support and posterior probability, respectively) in an unsupported position within the Nostocaceae proximal to *Mojavia.* The backbone of both trees was unsupported, such that the phylogenetic relationship of *Nostoc, Mojavia, Aulosira bohemensis, Cylindrospermum, Trichormus, Desmonostoc, Calochaete, Fortiea,* and

Komarekiella was poorly resolved. These genera had good support, except for *Nostoc.* Both trees showed *Komarekiella* distant from *Chlorogloeopsis fritschii* (Fig. 2). Phylogenetic analyses with greater taxon sampling (466 OTU's including the set of 213 OTU's as well as taxa from other genera and families of heterocytous cyanobacteria) had less bootstrap support and poorer resolution, although placement of *Komarekiella* was still in the Nostocaceae distant from all true branching taxa (tree not shown).

An alignment of the 16S-23S ITS region was possible among the taxa most closely related to *Komarekiella*. A parsimony analysis of this alignment was conducted with gaps coded as a fifth base to preserve the signficance of indels (Fig. 3). This phylogeny had better support than the 16S rRNA gene phylogeny, although the backbone of the phylogeny was still not well supported. *Komarekiella* was sister to *Desmonostoc* and also close to an undescribed *Nostoc*-like genus (Nostocaceae NC1, NC1-10). *Mojavia* could not be included in the alignment as only operons containing no tRNA genes have been recovered from that genus. The lengths of *Komarekiella* ITS regions were quite conserved among the genus strains and distinctly different from the other genera (Table 2). This basic analysis is in agreement with the 16S rRNA phylogeny: *Komarekiella* is a distinct, supported clade separate from its closest neighbors.

The analysis of the secondary structures of the 16-23S ITS region showed that the regions D2, D3, Box A and D4 were conserved among *Komarekiella, Desmonostoc* and *N. commune* strains. Among the *Komarekiella* strains, the Box B, V2 and V3 helices were identical, but the D1-D1' helix presented three slightly different patterns in the central helix, specifically on the downstream side. CCIBt 3486 D1-D1' helix differs from the others owing to a mismatch in the stem below the upper bulge caused by a change of the 42nd nucleotide (A for C). The folded D1-D1' helices for the strains CCIBt 3483 and 3487 differs from the ones for CCIBt 3481 and 3552, by the insertion of an adenine after nucleotide 45, forming a bulge instead of the mismatch presented in the same position for the latter strains (Fig. 4). However, the D1-D1' helix of all *Komarekiella* strains showed a very distinct synapomorphy: the basal unilateral bulge was opposed by three unpaired nucleotides on the 5' side of the helix, a feature not observed in any cyanobacterial taxon to date. They are also united by having only two pairs of bases between the terminal and subterminal loops, a characteristic shared with *Desmonostoc, Trichormus Mojavia,* and genera in the Microchaetaceae. *Nostoc sensu stricto* always has three pairs of bases in this position.

Comparing the ITS structures between *Komarekiella*, *Desmonostoc*, an undescribed Nostocaceae, and *N. commune*, the V2, V3, and Box B helices were highly variable. No consistent patterns were found for these helices among the strains studied, although other studies have shown high consistency of these structures within *Nostoc sensu stricto* (Lukešova et al. 2009). *Komarekiella* was distinctly different in the structure of the V2 and V3 helices from all other taxa, and had a Box B helix that shared relatively high similarity to the Box B helices of the other taxa (Fig. 4).

Discussion

The five studied populations are morphologically very similar, all of them presenting poliseriate trichomes and the unequal division of akinetes. Despite of some color variations on cells content, it is not possible to separate them as different species by morphology, neither molecular data.

Morphological analysis also showed that the genus *Komarekiella* is closely related to *Chlorogloeopsis Mojavia*, *Desmonostoc* and microscopic *Nostoc*, because of their overlapping features and the almost total lack of derived characters in all these genera. *Komarekiella* clearly differs from the typical *Nostoc*, because it does not form the macroscopic mucilaginous colonies, found in the latter genus. It differs from the other microscopic *Nostoc*, *Desmonostoc* and *Mojavia*, because *Komarekiella* features a special kind of germination of akinetes, originating a bigger vegetative cell and a smaller heterocyte.

When compared to *Chlorogloeopsis*, it is noticed that both genera share the same types of cell division in trichomes, colonies wrapped by mucilaginous firm and diffluent sheaths, apoheterocytic akinetes and the same type of akinetes germination. Before this paper, this special type of akinetes germination, originating a bigger vegetative cell and a smaller heterocyte, was the main diacritic character for *Chlorogloeopsis*. However, the phylogenetic analysis of the 16S rDNA and the 16S-23S ITS rDNA, show that *Komarekiella* and the polyphyletic *Chlorogloeopsis* are genetically not close related (Fig. 2) and this is problably the only way how to differentiate these genera. The fact that *Mojavia*, *Chlorogloeopsis* and *Komarekialla* are still known only for culture conditions makes the comparisons between them even more difficult, meaning that morphological studies based only in culture material should be looked carefully due to the effects of culturing in morphology.

Ecologically, these genera differ from each other (Tab. 1) although *Mojavia*, *Komarekiella* and *Chlorogloeopsis* were never found in their environmental samples and maybe, they could be just opportunist populations from elsewhere, which grow from propagules in culture In the other side, the fact that all of the *Mojavia* and *Komarekiella* strains were found strictly in the habitats where they were originally described is an evidence of the environmental dependence. We found five populations of *Komarekiella* from close related environments in Mata Atlantica, and *Mojavia* has been found repeatedly in desert soils (type environment), than the probability that these strains are growing as opportunists in culture is virtually refuted.

In the case of the polyphyletic *Chlorogloeopsis*, the type *C. fritschii* was originally described for wheat fields in India and reported later to many different environments as thermal springs in Greenland (Reference DQ430996) and Slovakia (Hindák 2008) and in a dry salt lake in Tunisia (Sahara desert) (Hindák 2008). Our,

phylogenetic analysis (Fig. 2) shows that the Indian type *C. fritschii* PCC 6912 is in the same clade of the Greenland strains with 100% of bootstrap support, in the base of Nostocaceae clade. Unfortunately, there´s no molecular data for the Tunisian and Slovakian ones, but probably those two clades can be different species of *Chlorogloeopsis*, from very different environments and that's an example of genus with no environmental dependence. According to this, we treat *Komarekiella* and *Mojavia* as endemic genera, considering that the habitats are important for their taxonomy for now, but we agree that further studies have to be done to confirm this, since they are recent genera and it is still unknown if they will be found in other habitats or not.

The 16S and ITS phylogenies, besides the high variability of the secondary structures and the lengths of ITS regions among *Desmonostoc* strains indicate that this genus can be polyphyletic. Specially in the 16S tree, five *Desmonostoc* clades are shown, three of them with bootstrap support above 81% (Fig. 2). For the ITS tree, three *Desmonostoc* clades are found. In agreement with this, the secondary structures, the *Desmonostoc* CCIBt 3489 presents two bases opposing the first side bulge in the D1-D1' helix while all the other *Desmonostoc* present only one base in this position. This could be an evidence of divergence, since *Komarekiella* is separated from the other nostocacean genera cited here, by presenting three bases opposing the side bulge. The phylogeny also groups CCIBt 3489 with a hawaian strain misidentified as *Chlorogloeopsis* and, as the other *Desmonostoc* clades, could be a new genus.

The genus *Komarekiella* is only one of the early *Nostoc*-like genera that eventually will be described in the short run. *Nostoc* is known as a polyphyletic genus (Hrouzek et al 2005), but the morphological markers to distinguish its clades are not yet known. We still don´t know if these clades are really morphologically indistinguishable or if these morphological incongruences are due to the lack of life cycle studies and nature material observations. In our opinion, efforts must be done in this way, besides phylogenetic and secondary studies analyzes, in intent to help solving nostocacean taxonomical problems. Finally, our paper also contributes to the knowledge of tropical and subtropical biodiversity, as well as for the taxonomy of Cyanobacteria.

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Table 1. Morphological comparisons among *Komarekiella* and related genera.

Table 2. Nucleotide lengths of the regions of the 16S-23S of some studied strains.

Figure 1. a-p. Life cycle of *Komarekiella*; arrows indicate the developmental directons. **a, b.** General view of macrocolonies. **c-h, k.** Unequal division of an akinete in a bigger vegetative cell and a smaller heterocyte and subsequent divisions. **i, j.** equal division of an akinete in two vegetative cell and subsequent division. **l-p.** Developmental stages of hormogonia; longitudinal divisions of cells are shown in n-p. Scales: a = 10 μm; b = 50 μm; c, d, e-g, i = 1 μm; h, l = 3 μm; k, m, n-p = 5 μm.

Figure 2. Phylogenetic relationships between *Komarekiella atlantica*, strains and related cyanobacteria based on 16S rRNA gene sequences resulting from Maximum Parsimony (MP) method.

Figure 3. Phylogenetic relationships between *Komarekiella atlantica*, strains and related cyanobacteria based on 16S rRNA ITS gene sequences resulting from Maximum Parsimony (MP) method.

Figure 4. Comparisosn of secondary structures of the 16S rRNA ITS (D1-D1', Box B, V2 and V3 helices) between *Komarekiella* and Nostocales genera.

Table. 1

Genera/characters	Thallus	Sheats	Germination of akinetes (originating)	Width of vegetative cells (μm)	Habitat
Nostoc Bornet & Flahault	Macroscopic	Firm	Two vegetative cells	4.5-6	Cosmopolitan mainly on unshaded and temporary humid soils
Desmonostoc Hrouzek & Ventura	Microscopic	Firm	Two vegetative cells	3.5 or wider in avarage	Cosmopolitan, mainly on meadow soils.
Mojavia Rehakova & Johansen	Microscopic	Firm	Two vegetative cells	4-10	Soils of Mojave desert, USA
Chlorogloeopsis Mitra & Pandey	Microscopic	Firm or sometimes diffluent	Commonly a larger vegetative cell and a small heterocyte	(2)3-6(8)	Cultivated soil, India
Komarekiella Hentschke et al.	Microscopic	Firm or sometimes diffluent	Commonly a larger vegetative cell and a small heterocyte	3.4-5.5	Wood and concrete, "Mata Atlantica" rainforest, Brazil.

Table 2.

Strain	Leader	D1-D1' helix	Spacer+D2	Spacer+D3+spacer	tRNA Ile gene	Space+V2+spacer	rRNAAla gene	Spacer+BoxB+spacer	Box A	D4	Spacer+V3
K. atlantica CCIBt 3481	9	65	34	8	73	83	72	85	12	11	62
K. atlantica CCIBt 3486	9	65	34	8	73	83	72	85	12	11	62
K. atlantica CCIBt 3483	9	66	34	8	73	83	72	85	12	11	62
Desmonostoc sp. CCIBt 3489	8	69	34	8	73	85	72	83	12	10	33
Desmonostoc sp. 81 NMI ANAB 7A	9	65	35	9	74	79	73	103	11	9	41
Desmonostoc sp. HA4236 MV1 p1	8	65	31	9	74	55	73	152	11	9	82
N. commune CCIBt 3485	8	67	41	9	73	89	72	92	11	11	48

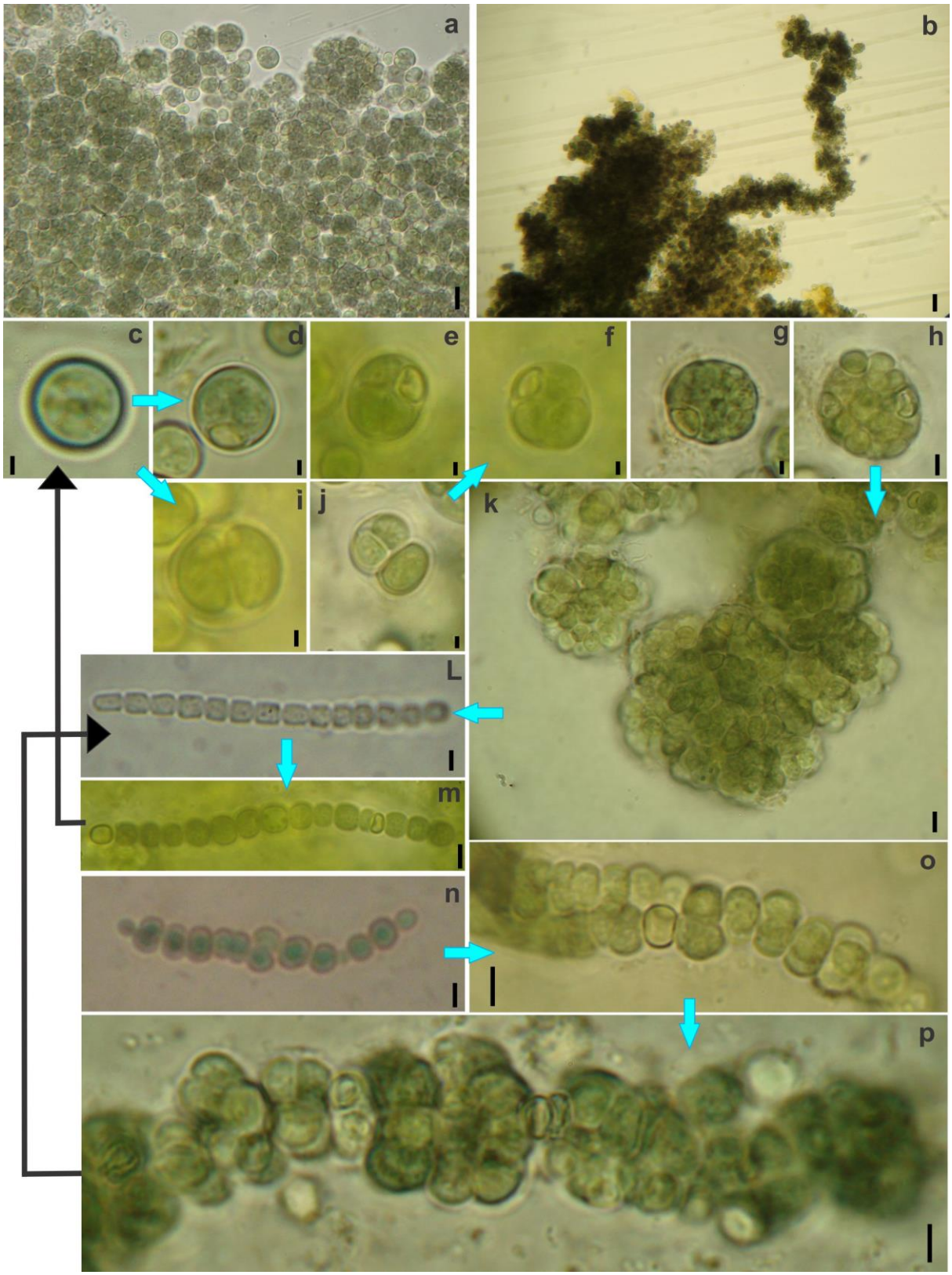

Figure 1.

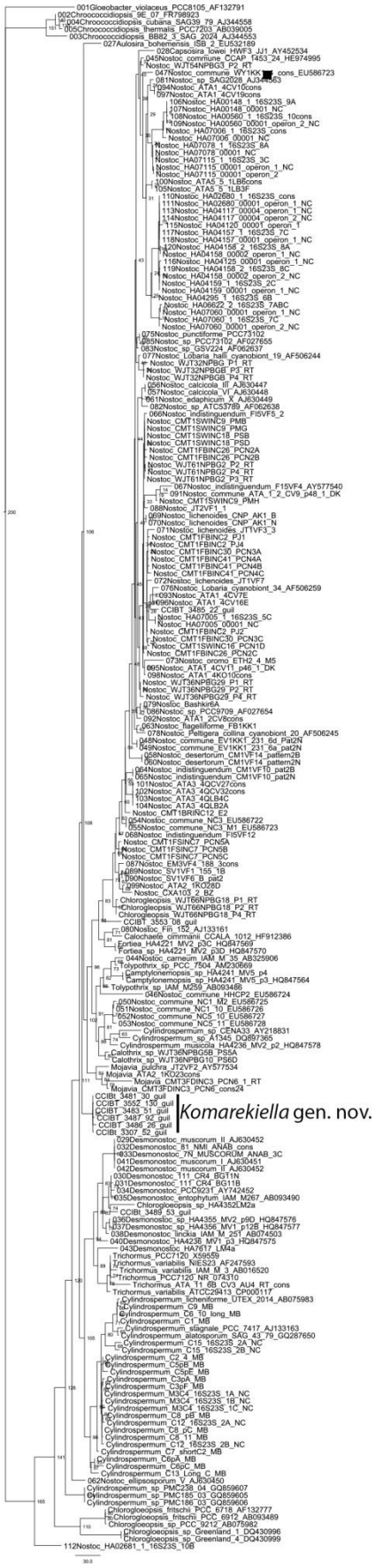

Figure 2.

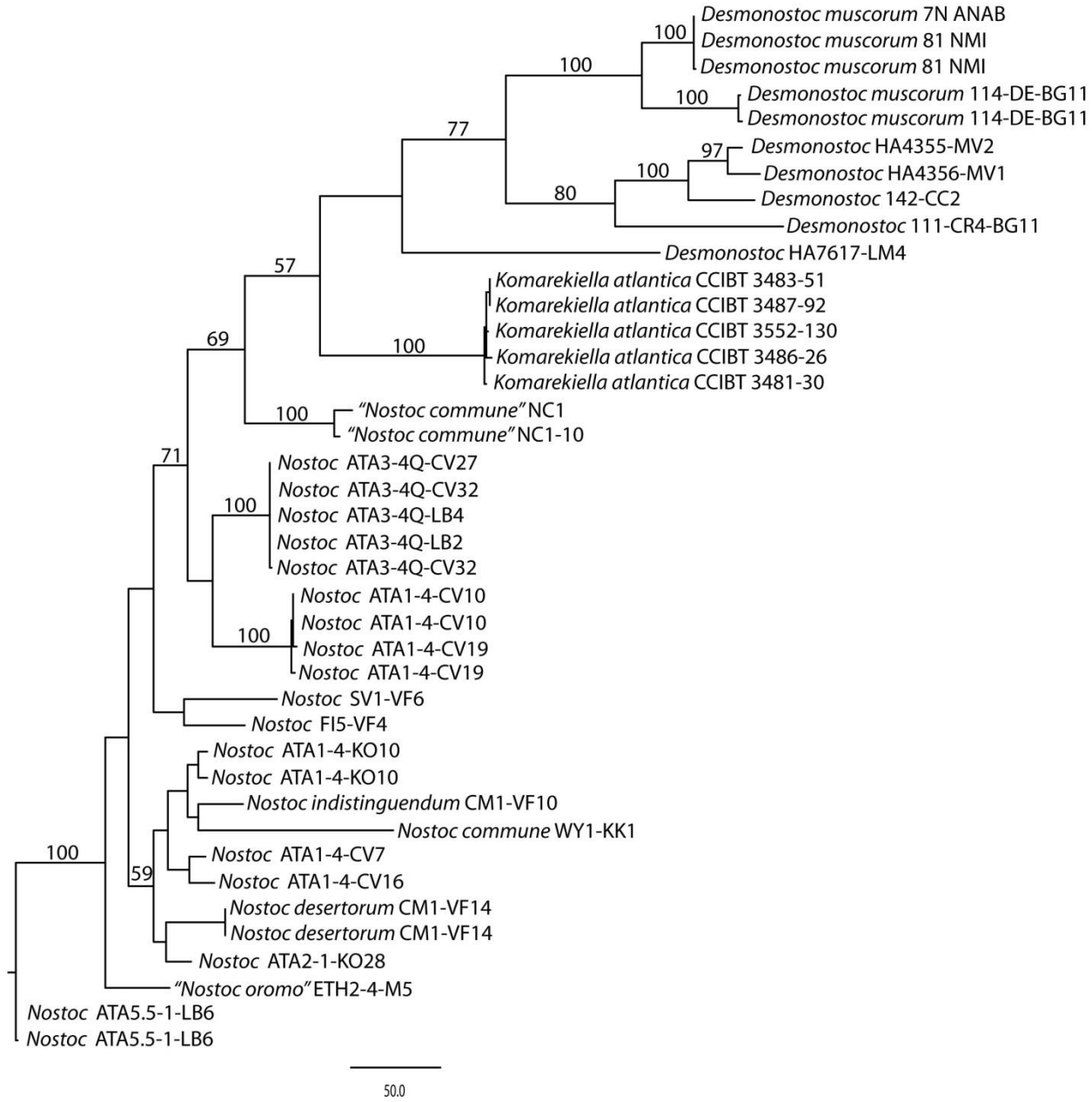
Desmonostoc muscorum 7N ANAB
Desmonostoc muscorum 81 NMI
Desmonostoc muscorum 81 NMI
Desmonostoc muscorum 114-DE-BG11
Desmonostoc muscorum 114-DE-BG11
Desmonostoc HA4355-MV2
Desmonostoc HA4356-MV1
Desmonostoc 142-CC2
Desmonostoc 111-CR4-BG11
Desmonostoc HA7617-LM4
Komarekiella atlantica CCIBT 3483-51
Komarekiella atlantica CCIBT 3487-92
Komarekiella atlantica CCIBT 3552-130
Komarekiella atlantica CCIBT 3486-26
Komarekiella atlantica CCIBT 3481-30
"Nostoc commune" NC1
"Nostoc commune" NC1-10
Nostoc ATA3-4Q-CV27
Nostoc ATA3-4Q-CV32
Nostoc ATA3-4Q-LB4
Nostoc ATA3-4Q-LB2
Nostoc ATA3-4Q-CV32
Nostoc ATA1-4-CV10
Nostoc ATA1-4-CV10
Nostoc ATA1-4-CV19
Nostoc ATA1-4-CV19
Nostoc SV1-VF6
Nostoc FI5-VF4
Nostoc ATA1-4-KO10
Nostoc ATA1-4-KO10
Nostoc indistinguendum CM1-VF10
Nostoc commune WY1-KK1
Nostoc ATA1-4-CV7
Nostoc ATA1-4-CV16
Nostoc desertorum CM1-VF14
Nostoc desertorum CM1-VF14
Nostoc ATA2-1-KO28
"Nostoc oromo" ETH2-4-M5
Nostoc ATA5.5-1-LB6
Nostoc ATA5.5-1-LB6
100
100
100
100
77
97
100
80
57
69
100
100
71
100
100
100
59
50.0

Figure 3.

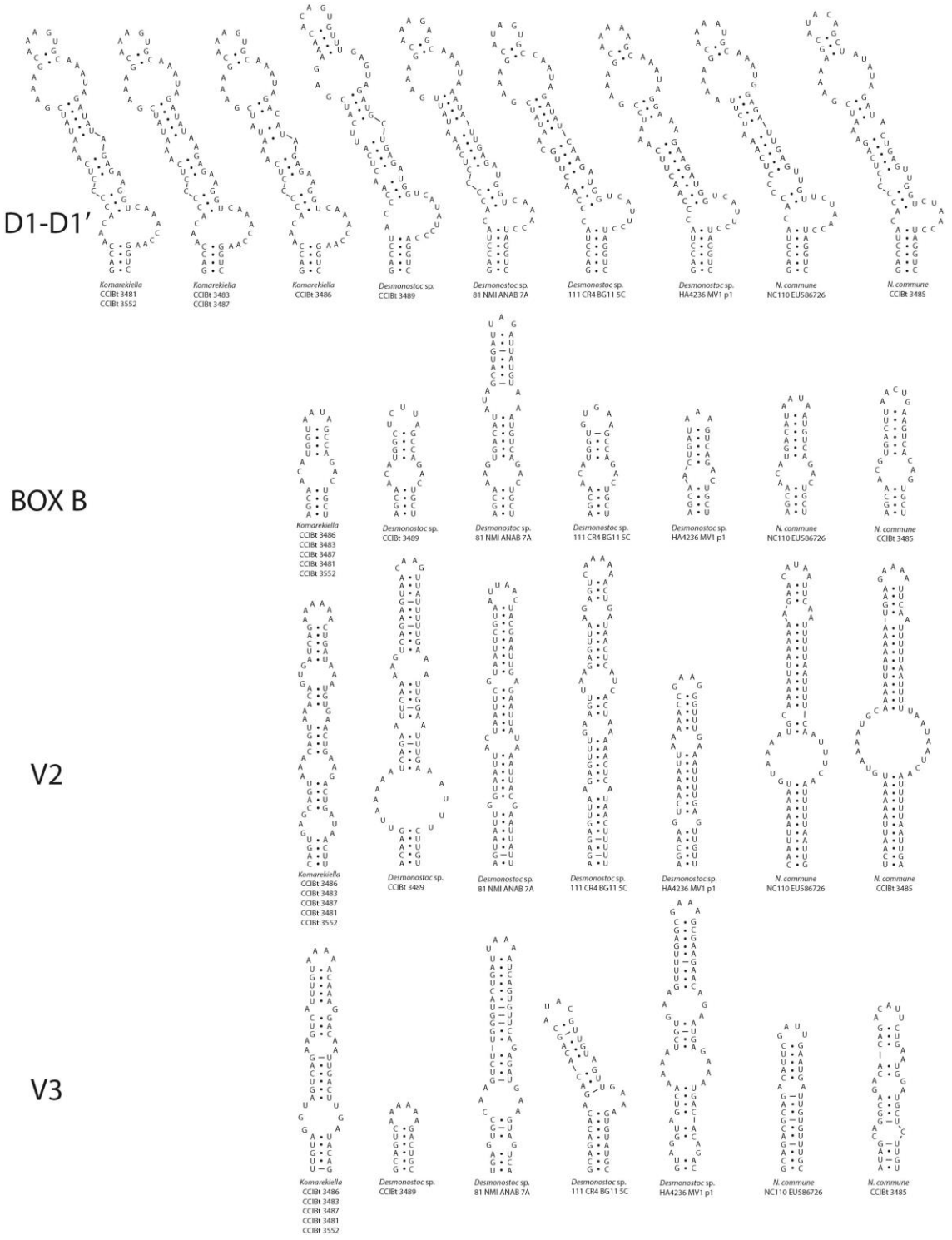
D1-D1′
BOX B
V2
V3
Komarekiella CCIBt 3481 CCIBt 3552
Komarekiella CCIBt 3483 CCIBt 3487
Komarekiella CCIBt 3486
Desmonostoc sp. CCIBt 3489
Desmonostoc sp. 81 NMI ANAB 7A
Desmonostoc sp. 111 CR4 BG11 5C
Desmonostoc sp. HA4236 MV1 p1
N. commune NC110 EU586726
N. commune CCIBt 3485

Figure 4.

Anexo VII

PHYLOGENETIC PLACEMENT OF *DAPISOSTEMON* GEN. NOV. AND *STREPTOSTEMON*, TWO TROPICAL MICROCHAETACEAN GENERA (CYANOBACTERIA)[1]

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ABSTRACT

Two species of cyanobacteria with microchaetacean characteristics from the Mata Atlântica of Southeastern Brazil were studied intensively to determine if they were congeneric or different genera, as well as their affinity to other established genera in the Microchaetaceae such as *Hassallia* Bornet & Flahault and *Tolypothrix* Bornet & Flahault. *Dapisostemon apicaliramis* gen. et sp. nov. isolated from a wooden bridge in a mangrove swamp was found to be associated with the clade of terrestrial Microchaetaceae (but in an unsupported position) by the means of 16S rRNA gene sequence analyses, and had a 16S-23S ITS region similar in length, sequence, and secondary structure to the terrestrial Microchaetaceae. *Streptostemon* Sant'Anna *et al.* was placed in the Microchaetaceae at the time of its description based on unsequenced natural material, but in this study it was found to be phylogenetically distant from the Microchaetaceae based on 16S rRNA gene sequence, presenting also a unique 16S-23S ITS region. Its higher level classification is uncertain at this time, but it likely will not remain in the Microchaetaceae as the Nostocales undergo future revision.

Key index words: *Dapisostemon apicaliramis, Streptostemon lutescens,* Microchaetaceae, Mata Atlântica, 16S-23S ITS secondary structure, biodiversity, genetic diversity.

Running title: *DAPISOSTEMON* AND *STREPTOSTEMON*

INTRODUCTION

In tropical and subtropical forests, terrestrial cyanobacteria, along with other organisms, compose extensive biofilms, which grow on a wide variety of substrates such as wood, soil and rocks (Büdel 2002, Gorbushina 2007, Sant'Anna *et al.* 2013). Ecologically these biofilms play important roles in ecosystems, especially as carbon sinks, decomposers, and sources of soil fertility, mainly due to the nitrogen (N_2) fixation activity of cyanobacteria and phosphorus mobilization by mycorrhizae (Vitouzek *et al.* 2002, Belnap *et al.* 2003). However, our knowledge of these microrganisms, particulary cyanobacteria, is still poor for tropical and subtropical forests. Currently, it is only known that they perform lower rates of nitrogen fixation in old-growth forests than in disturbed environments under regeneration (Barron *et al.* 2011, Pons *et al.* 2006, Gehring *et al.* 2005). Also, in terms of biodiversity, these microbial communities are still misunderstood and often neglected in ecological studies.

We embarked on an extensive study of cyanobacterial diversity in Mata Atlântica (Atlantic Rainforest), one of the most important hotspots of biodiversity in the world which covers both tropical and subtropical areas along the Brazilian coast (Rizzini 1997, Myers *et al.* 2000). Due to human pressure, today it retains only in 7% of its original coverage (Rizzini 1997) and its fragmented remnants are almost totally encompassed by protected areas, from which many new cyanobacterial taxa (genera, species) have been described during the last decade (Fiore *et al.* 2007, Lemes-da-Silva 2010, Sant'Anna *et al.* 2013).

Among the new taxa, fasciculate filamentous forms have received special attention, since two genera and seven species of fasciculate Nostocales were described for this biome based on morphological data. In addition, the monophyletic genus *Brasilonema* Fiore *et al.* was described based on both morphological and molecular data

(Fiore *et al.* 2007). The genus *Streptostemon* (Microchaetaceae) was erected by Sant'Anna *et al.* (2010) for michrochaetacean cyanobacteria with fasciculate filaments without branching. Morphologically, it is a well delimited genus, but up to now it could not be confirmed by molecular analysis owing to difficulties in culturing its populations. Recently, we have isolated, characterized and sequenced another apparent member of the Microchaetaceae similar to *Streptostemon* but differing in keys characters. This new species differs from *Streptostemon* in that it has more common false branching and is less fasciculate. However, morphologically the two genera appear more closely related to each other than to other Microchaetaceae, and molecular evidence is required to determine whether or not they are congeneric.

We sequenced the 16S rRNA gene and the 16S-23S ITS of an environmental population of *Streptostemon* (*S. lutescens*) and the isolate of the putative new species, also from Mata Atlântica. The purpose of this paper is to establish that these two species comprise different genera, and distinguish them from other microchaetacean genera. The new taxon is described as *Dapisostemon apicaliramis gen. et sp. nov.*, and will be referred to under this epithet in the remainder of the paper.

MATERIAL AND METHODS

Sampling and morphological analysis

The studied populations of the new genus *Dapisostemon* gen. nov. were collected by scraping biofilms from terrestrial habitats in the subtropical area of Mata Atlântica (State Park of Ilha do Cardoso - 25º04'12"S, 47º55'27"W). One of them (CCIBt 3318) was isolated from a unispecific biofilm growing on a wood bridge in a mangrove and the other one (CCIBt 3536) grew in culture from an inoculated sample collected on a riverside rock, but it could not be found in natural material.

Both strains were maintained in liquid BG11 nitrogen-free medium (Ripka *et al.* 1979) under 14:10 hr (light:dark) cycle with white fluorescent light (30 µmol photons m^{-2} s^{-1}) and 23 (±2) ^{o}C, and deposited in the Culture Collection of the Institute of Botany, São Paulo, Brazil. The studied environmental populations of *S. lutescens* were collected also from biofilms growing abundantly on wood, rocks or epiphytic on bryophytes in areas of Mata Atlântica: Nucleus Santa Virginia (23º20'16"S, 45º09'01"W), Ecological Station Jureia-Itatins (24 24'36''S, 47 01'15''W) and State Park of Ilha do Cardoso (25º04'12"S, 47º55'27"W), but even after many attempts could not be isolated.

After sampling, dry material was kept in paper bags and parts of the samples were rehydrated for morphological analysis in the laboratory. At least, 30 individuals of each studied population were evaluated and photographed using a Zeiss Axioplan 2 photomicroscope equipped with a Zeiss Axiocam MRc digital camera. Samples were also preserved in formaldehyde (4%) and deposited in the Herbarium of the Institute of Botany (SP).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was isolated from liquid cultures of the cyanobacterial strains CCIBt 3318, CCIBt 3536 and from an unispecific environmental sample of *S. lutescens* using MOBIO Ultraclean DNA Isolation Kit. Specially for *S. lutescens*, sample preparation for DNA extraction was conducted by the manual separation and cleaning of filaments from debris, using tapered Pasteur micropipetes and microscope.

Almost the complete 16S rRNA gene plus the 16S-23S ITS region was amplified by PCR using the primers 27F1 (Neilan *et al.* 1997) and 23S30R (Lepère et al., 2000) in a Techne TC-412 thermocycler (Bibby Scientific). The reaction contained 10 ng of genomic DNA, 0.5 µM of each primer, 200 µM of dNTPs, 2.0 mM of $MgCl_2$, l

× PCR buffer and 1.5 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), in a final volume of 25 µL. The PCR cycle had initial denaturation at 94 ºC for 5 min, followed by 10 cycles of 94 ºC for 45 s, 57 ºC for 45 s, 72 ºC for 2 min, another 25 cycles of 94 ºC for 45 s, 57 ºC for 45 s, 72 ºC for 2 min and a final extension step at 72 ºC for 7 min. The resulting PCR product was cloned into a pGEM®-T Easy Vector System (Promega, Madison, WI, USA) according to the supplier's manual, cloned by heat-shock in *E. coli* DH5α cells and plated for blue-white selection (Sambrook & Russel 2001). After growth, recombinant plasmids were extracted from white colonies by the alkaline lysis method (Birnboim & Doly 1979). The cloned gene fragment was sequenced using "Big Dye Terminator" v. 3.0 (Applied Biosystems) with the plasmid primers T7 and M13 and the internal primers 357F/357R, 704F/704R and 1114F/1114R (Lane 1991). The cycle sequencing reaction was performed as follow: 25 cycles of 95 ºC for 20 s, 50 ºC for 15 s and 60 ºC for 1 m. the DNA was precipitated using 2 µL of sodium acetate buffer (1.5 M sodium acetate - pH 9.0 and 250 mM EDTA- pH 8.0) and 60 µL of 100% ethanol. The tubes were centrifuged at 4 ºC for 15 min at 12,000 × *g* and the supernatants were discarded. The DNA pellets were washed with 150 µL of 70% ethanol, centrifuged for 5 min and the supernatants removed. The pellets were air-dried overnight in the dark and at room temperature. The purified pellets were resuspended in HiDi formamide (Applied Biosystems), and the sample placed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequenced fragments were assembled into contigs using the software package Phred/Phrap/Consed (Philip Green, Univ. of Washington, Seattle, USA) and only bases with a quality >20 were considered.

Phylogenetic analyses

The 16S rRNA gene sequences obtained in this study and reference sequences retrieved from GenBank were aligned manually and used to generate the phylogenetic

trees. The trees were reconstructed using the Maximum Parsimony (MP) method implemented by the MEGA version 5.0 program package (Tamura *et al.* 2011) with 1000 bootstrap replicates and Bayesian (BA) criteria using MrBayes 3.2 (Ronquist & Huelsenbeck 2003) in two independent runs, with eight chains each, for 20×10^6 generations. In both cases, the best-fitting evolutionary model GTR+G+I was applied.

To determine the position of our strains in the first round of phylogenetic analysis, MP and BA trees were constructed with 466 OTU´s (operational taxonomic units) represented by Nostocales sequences (data not shown). Then, we chose the more related taxa and constructed smaller trees based on 210 OTU´s. A similarity matrix (p-distance) was also generated.

For the analysis of 16S-23S ITS region, we compared the lengths of this region and the secondary structures of our strains, with related taxa based on our 16S rRNA trees. The secondary structures of helices D1-D1´, Box B, V2 and V3 were determined separately using Mfold version 2.3 (Zuker 2003), with folding temperature set at 20^{o}C.

The 16S rRNA gene and the 16S-23S ITS sequences of the cyanobacterial strains *D. apicaliramis* CCIBt 3318 and *D. apicaliramis* CCIBt 3536 isolated in this study, and of the *S. lutescens* natural population were deposited in the NCBI GenBank database under accession numbers KJ566947, KJ566945, and KJ566946, respectively.

RESULTS

Dapisostemon was found growing on a wooden bridge above a mangrove swamp in the Mata Atlântica in subtropical southeastern Brazil. Morphologically these populations are similar to *Streptostemon* and *Hassallia* Bornet & Flahault, but differ in many aspects (Table 1). The thallus consisted of a mat of upright heteropolar filaments that were commonly apically branched but not often arranged in fascicles, with

yellowish sheaths and isodiametric cells. The fascicles, when present, lacked the well-developed lanceolate structure of the fascicles of *Streptostemon.* While *Hassallia* has false branching and upright branches, it does not form the carpet-like layer seen in *Dapisostemon,* and has discoid cells. Hence, the population of *Dapisostemon* from nature and isolates made from that collection could not be placed with confidence in any previously described genus. However, enough similarity existed with other Microchaetacean genera that molecular confirmation of the divergence of *Dapisostemon* was considered necessary before it was described.

The studied populations of *Streptostemon* were identified based on nine samples and were in agreement with the original description of the genus (Sant'Anna *et al.* 2010), although they presented more intercalary heterocytes than previously described. We characterized the populations and succeeded in sequencing one of them.

The blast analysis showed that the 16S rRNA gene sequences from *Dapisostemon* (CCIBt 3318 and CCIBt 3536) were most closely related to *Tolypothrix* sp. UAM 332 (HM751847) with 96% and 95% identity and 100% and 98% of coverage, respectively. The calculated similarity values (p-distance) presented in Table 2 indicated that the phylogenetic relationships among the new genera and many polyphyletic genera like *Hassallia*, *Tolypothrix* Bornet & Flahault, *Calothrix* Bornet & Flahault and unexpectedly *Nostoc* Bornet & Flahault ranged from 94 to 96% of identity. For *Streptostemon* the similarity values reached 95% with *Campylonemopsis* (HQ847564) and then lower values compared to sequences of other strains (Table 2).

Phylogenetic analysis using both MP and BA methods produced trees with similar terminal topology, but only MP trees are shown. The phylogenetic trees had no bootstrap support in the backbone (Fig 1), so it is not possible to determine the precise relationships between our strains and other genera. Considering that *Dapisostemon* and

Streptostemon are in well supported clades and diverging from the main branch of the tree, we can state that our populations are phylogenetically very distant from all other nostocalean genera. *Dapisostemon* (clade 2) diverged very early from the outgroup (clade 4), close to more derived Michrochaetaceae strains (clades 1, 18) in agreement with its morphological michrochaetacean condition. According to both morphology and phylogeny, *Dapisostemon* must be classified in the Microchaetaceae until more extensive studies are done for this taxonomic group. The genus *Streptostemon* is also in an early divergent clade (clade 17), but in a half-way position between Michrochaetaceae and Scytonemataceae strains, so it is not possible to determine yet to which family it belongs, considering again the weak support in the backbone of the tree.

The results for the 16S-23S ITS analyses were congruent with morphological and 16S rRNA studies. The lengths of this region were similar between *Dapisostemon* and *Hassallia* (Table 3) as well as the secondary structures. *Dapisostemon* D1-D1', BoxB and V2 stems were similar to the helices in *Hassallia* (Figs 2 a-k) although the V3 was very different, considering sequence and structure (Figs 2 n, o). Although the association of *Dapisostemon* with the Microchaetaceae was not supported with strong bootstrap support, the ITS secondary structures provide strong evidence that it is in that family. Shared features among all Microchaetaceae include the absence of a solitary nucleotide opposite the basal unilateral bulge of the D1-D1' helix and only two paired nucleotides between the terminal loop and subterminal loop (Fig. 2 c) and the basal structure and sequence of the Box-B helix (Figs 2 g, h). *Dapisostemon* has these features, except that it has three paired nucleotides between the terminal loop and the subterminal loop formed at the expense of the size of the subterminal loop (Fig. 2 d). The Nostocaceae typically have three paired nucleotides between the terminal loop and the subterminal loop, and a subterminal loop that is equal in size to the typical loop of

Microchaetaceae (Lukešová *et al.* 2009). All Nostocaceae also have a solitary nucleotide opposite the unilateral bulge in the D1-D1' helix (Lukešová *et al.* 2009).

The lengths of the ITS region and the secondary structures of all *Streptostemon* helices were very different from all other sequences, in agreement with its isolated phylogenetic position based on 16S rRNA gene sequence data and morphological characterization. While the D1-D1' helix has similarities to the same helix in other Nostocales (Fig. 2 a), the Box-B, V2, and V3 helices are all unique (Fig. 2 e, i, l). *Scytonema* Bornet & Flahault had distinctly different ITS secondary structures. *Streptostemon* is unique among all the Nostocales sequenced thus far in having an ITS region with only a single tRNA gene ($tRNA^{Ile}$), with all other Nostocales having at least two operons, one with two tRNA genes ($tRNA^{Ile}$ and $tRNA^{Ala}$) and one or more with no tRNA genes (Iteman *et al.* 2000, Boyer *et al.* 2001, Flechtner *et al.* 2002, Řeháková *et al.* 2007, Lukešová *et al.* 2009, Vaccarino & Johansen 2011, 2012, Johansen *et al.* 2014). The long spacer between the D3 region and the $tRNA^{Ile}$ is also highly unusual and represents a unique multi-nucleotide insertion. Based on secondary structure of the ITS conserved domains, *Streptostemon* is better placed in the Microchaetaceae than in any other family, which is congruent to the conclusions made by Sant'Anna *et al.* (2010) based on morphological evidence alone.

Dapisostemon G. S. Hentschke, C. L. Sant'Anna *et* J. Komárek *gen. nov.*

Thallus forming mats composed by creeping entangled filaments in basal part, which become erect and parallel/interwoven toward the apex forming a caespitose *stratum*, 11-14.5 µm wide. Filaments cylindrical, with tolypotrichoid branching, sometimes clustered together in short related fascicles. Trichomes heteropolar, constricted or not at the cross walls, frequently attenuated in the middle, 5.6-9 µm wide.

Heterocytes basal and intercalary, 7.3-11.2 µm wide, Cells and heterocytes ± isodiametric or slightly longer or shorter than wide, cells shorter at the apex. Sheaths hyaline to brownish, lamellate or homogenous.

Type species: *Dapisostemon apicaliramis*

Etymology: Dapis (Gr.) = carpet, stemon (Gr.) = threads (carpet of threads)

Dapisostemon apicaliramis G. S. Hentschke, C. L. Sant'Anna *et* J. Komárek *sp. nov.* (Figs. 4-7)

Thallus forming black mats. Filaments creeping and entangled in the basal part, becoming parallel and erect, occasionally clustered together in closely related fascicles. Fascicles mostly short and truncate, composed by densely arranged parallel/interwoven filaments. Filaments 11-14.5 µm wide, often with short tolypotrichoid branches in the apical portion. Trichomes heteropolar, cylindrical or slightly attenuated in the middle. Cells ± isodiametric, 6-10.8 µm long; 5.6-9 µm wide; shortened at the apex. Cell content homogenous or with vacuole-like structures, pale or dark blue-green. Sheaths brownish-yellow, with parallel or divergent lamella or rarely homogenous. Basal heterocytes hemispherical, isodiametric or cylindrical when intercalary, 5.1-11.7 µm long, 7.3-11.2 µm wide. Hormogonia heteropolar, short (5-10 cells), constricted, dark blue-green.

Holotype: Brazil, State of São Paulo, State Park of Ilha do Cardoso, 06/29/2010, *Watson A. Gama Jr. and Camila F. da S. Malone* (SP 401441) (herbarium preparation of natural material), Herbarium of São Paulo State, São Paulo, Brazil.

Reference strain: CCIBt 3318

Type locality: State Park of Ilha do Cardoso, State of São Paulo, Brazil.

Etymology: apicale (L.) = apically, ramis (L.) = branched

Streptostemon lutescens Sant'Anna *et al.* 2010 (Figs. 8, 9)

Filaments entangled and creeping in the basal part, than joined together in one to several dense, erect and irregularly separated fascicles. Fascicles mostly lanceolate. Filaments 6.7-14 µm µm wide, not branched or rarely with scytonematoid or tolypotrichoid branches. Sheaths hyaline or yellowish, homogenous or with parallel lamella. Tricomes heteropolar, cylindrical or slightly attenuated in the middle. Cells ± isodiametric, 3-12 µm long; 5.5-8.8 µm wide; shortened at the apex. Cell content homogenous, pale or dark blue-green. Basal heterocytes hemispherical, intercalary isodiametric or cylindrical, 4.3-14 µm long, 6.4-11.1 µm wide. Hormogonia mostly heteropolar , containing 7-18 cells, slightly constricted, cells very short with pale blue-green or yellowish granular content. Habitat: Wet rocks, bark of trees, artificial wood substrata or epiphytic on bryophytes.

Studied material: Brazil, State of São Paulo, State Park of Ilha do Cardoso, 06/29/2010, *Watson A. Gama Jr. and Camila F. da S. Malone*, (SP 427336, SP 427501); State Park of Serra do Mar, Nucleus Santa Virgínia, 02/11/2010, *Watson A. Gama Jr. e Ewerton C. Manarin* (SP 427502); Ecological Station Jureia-Itatins, Guilherme S. Hentschke, 08/16/2011, *Watson A. Gama Jr., Camila F. da S. Malone* and *Célia L. Sant'Anna* (SP 427304, SP 427320, SP427505).

DISCUSSION

The new genus *Dapisostemon* is morphologically similar to *Streptostemon* Sant'Anna *et al.*, but differs mainly by the presence of more common tolypotrichoid branching and the parallel arrangement of erect filaments composing a caespitose, but

not commonly fasciculated layer. The genus *Streptostemon* features filaments joined in dense erect fascicles but lacks branching or quite extraordinarily possesses tolypotrichoid or scytonematoid branching. *Dapisostemon* differs also from *Tolypothrix* and *Hassallia,* other caespitose genera, mainly by the lack of the typical repeated branching of the dendroid filaments observed in these former taxa (Tab. 1). The complete morphological comparisons among these genera are detailed in Sant'Anna *et al.* (2010).

Despite the high similarity (96%) among 16S rRNA gene sequences with other genera retrieved from GenBank, the MP and BA phylogenetic trees combined with morphological and 16S-23S ITS data, strongly support the new genus description. As already shown for *Spirirestis* Fletchner & Johansen (Fletchner *et al.* 2002, Komárek 2011), the "95% similarity between strains rule" to aggregate genera cannot be strictly applied for Nostocales and the morphology of populations together with the phylogenetic tree topologies must be considered. This is endorsed based on the high similarity of *Dapisostemon* and *Streptostemon* when compared to *Hassallia*, *Tolypothrix* and also morphologically distinct genera like *Nostoc* and *Cylindrospermum* Bornet & Flahault (Table 2). In fact, within the Nostocaceae, distinct clades with internal morphological congruence, tend to diverge from each other (Fig. 1), even though the similarity among their strains (>95%) would suggest they are congeneric (Novis & Smissen 2006, Hrouzek *et al.* 2013).

In the previously unsequenced genus *Streptostemon*, although it is confirmed as a monophyletic taxon by 16S rRNA and 16S-23S ITS data, in our analysis the genus has an uncertain phylogenetic position and it is not possible to determine to which family it belongs based on the 16S rRNA phylogeny alone. Relevant to this problem, we found an intermediate morphological condition between the Microchaetaceae and

Scytonemataceae, with *Streptostemon* presenting both intercalary heterocytes and isopolar trichomes (Scytonemataceae characters) and basal heterocytes in heteropolar trichomes (Michrochaetaceae characters). Indeed, in the illustrations for the genus in Sant'Anna *et al.* (2010), intercalary heterocytes are also very common. The phylogenetic relevance of the heterocyte position for determination of family level was already discussed by Vaccarino & Johansen (2011, 2012), in which the authors described two species of Scytonemataceae (*Scytonematopsis contorta* and *Brasilonema angustatum*), with isopolar hormogonia becoming heteropolar after breakage. Hence, we believe that the position of heterocytes is important in classification at the family level, but more studies must be done to clarify these morphological and genetic anomalies more precisely.

The taxonomic placement of *Streptostemon* is further complicated by the fact that the Scytonemataceae are not currently monophyletic (Komárek *et al.* 2013). Our phylogeny shows four clusters of Scytonemataceae, including three putatively being "*Scytonema*". Based on our phylogeny and the finding of others (Vaccarino & Johansen 2011, Johansen *et al.* 2014, Komárek *et al.* 2013) the Scytonemataceae must be revised. *Streptostemon* is unique in the secondary structures of the 16S-23S ITS region, and this consequently fails to align it with existing families. For now it remains in a basal position in all phylogenies and cannot be placed in any family without considerable uncertainty.

The presence of erect fascicles is a remarkable characteristic for both genera presently studied and probably this character was developed independently in many cyanobacterial genera like *Brasilonema*, *Tolypothrix* and *Stigonema* Bornet & Flahault. However their ecological role is not yet understood. The parallel filaments of creeping rope-building cyanobacteria like *Microcoleus* Gomont and *Hydrocoleum* Gomont

(Oscillatoriales) allows them to colonize physically unstable sedimentary environments (Garcia-Pichel &Wojciechowski 2009), but unlike them, the erect fasciculate types of Nostocales grow commonly on stable substrates like rocks, tree bark or bryophytes (Sant'Anna *et al.* 2010, 2011, 2013). Hypothetically, the erect fasciculate thalli could be an advantage to improve light harvesting into the dense and species-specific diverse biofilms found in forests like the Atlantic Rainforest, or a self-shading strategy to avoid extreme solar radiation.

Finally, based on our sampling and previous data (Sant`Anna *et al.* 2010), the genera *Dapisostemon* and *Streptostemon* are widespread in tropical and subtropical Mata Atlântica from São Paulo State, as other fasciculate types of *Brasilonema* and *Stigonema* (Sant'Anna *et al.* 2011, 2013). Many of these taxa were described recently as a result of the increasing number of taxonomic studies in these areas, meaning that the cyanobacterial diversity is still underestimated due to poor sampling or misidentification of the species based on European types. Considering recent biogeographic studies (Bahl *et al.* 2011) we conclude that the terrestrial cyanobacterial diversity composition in tropical areas is very different from temperate zones and the current tendency of describing these tropical populations as new species using polyphasic characterization is valid. In this paper, we describe the new genus *Dapisostemon*, confirm *Streptostemon* as a special genus and highlight the potential of Mata Atlântica as a discovery source of new cyanobacterial taxa, as well as the importance of conservation of this biome in order to preserve its high biodiversity.

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Table 1. Comparison among several tolypotrichoid genera and *Dapisostemon*.

Table 2. The 16S rRNA gene sequence similarity between *Streptostemon lutescens*, *Dapisostemon apicaliramis,* and sequences of related cyanobacterial strains.

Table 3. Nucleotide lengths of the regions of the 16S-23S of the studied strains.

Figure 1. Phylogenetic relationships between *Streptostemon lutescens*, *Dapisostemon* strains and related cyanobacteria based on 16S rRNA gene sequences (1223 bp) resulting from Maximum Parsimony (MP) method. Bootstrap values above 70 are displayed as colored circles on nodes. Bayesian probabilities above 0.8 are shown as numers inside the nodes circles.The name of strains in each branch are detailed in Table S1 and correspond to the numbers labeling the clade**s.**

Figure 2. Secondary structures in representative Nostocales genera. D1-D1` helix (a-d), Box B helix (e-h), V2 helix (i-l), V3 helix (m-o).

Figure 3. *Dapisostemon apicaliramis*. General view of thalli with parallel filaments (a), detail of apices (b), thallus forming fascicle (c), tolypotrichoid branching (d), details of different filaments apices. Scales: a. 50 µm; b,e,f. 10 µm ; c. 30 µm; d. 10 µm

Figure 4. Cultured *Dapisostemon apicaliramis*. General view of thalli (a-b), young culture with older sheats (c). tolypotrichoid branching (d). Scales: a. 100 µm; b,c,f.

Figure 5. *Streptostemon lutescens.* Thalli forming fascicles (a-b), releasing of hormogonia (c), scytonematoid branching (d), tolypotrichoid branching (e), detail of filament with intercalary heterocyte (f). Scales: a, b. 100 µm, c-f.10 µm.

Genera	Heterocytes	Cells	Thallus	Fascicles	Branching
Dapisostemon	Basal and intercalary	Isodiametric or slightly shorter or longer than wide	Filaments densely entangled in the base, with erect parallel, filaments arranged in a carpet-like layer	Rare, erect, compact, short, truncate (not tapered)	Common, tolypotrichoid, apical, parallel to the main filament
Streptostemon			Filaments loosely entangled in the base, apically fasciculate	Obligate, erect, clearly separated, elongated, frequently tapered	Absent or quite extraordinary scytonematoid or tolypotrichoid
Tolypothrix			Filaments attached basally, free apical ends. Sometimes forming woolly mats	Erect, frequent	Dendroid, tolypotrichoid, rarely geminate
Hassallia		Discoid		Erect or crustaceous, frequent	Dendroid, tolypotrichoid, usually arcuated, rarely geminate

Table 2.

	1	2	3	4	5	6	7	8	9	10	11
1. *Dapisostemon* CCIBt 3318											
2. *Dapisostemon* CCIBt 3536	0.99										
3. *Streptostemon lutescens*	0.94	0.94									
4. *Tolypothrix* HM751847	0.96	0.96	0.95								
5. *Tolypothrix distorta* GQ287651	0.95	0.96	0.94	0.96							
6. *Tolypothrix* AB093486	0.96	0.96	0.94	0.98	0.96						
7. *Hassallia byssoidea* AM905327	0.95	0.95	0.93	0.96	0.99	0.95					
8. *Fortiea* HQ847569	0.94	0.94	0.94	0.96	0.95	0.97	0.95				
9. *Calothrix* AB325535	0.96	0.96	0.94	0.98	0.95	0.98	0.95	0.96			
10. *Camptylonemopsis* HQ847564	0.95	0.95	0.94	0.97	0.95	0.98	0.94	0.96	0.98		
11. *Cylindrospermum* DQ897365	0.95	0.95	0.94	0.97	0.95	0.97	0.95	0.96	0.96	0.96	
12. *Nostoc commune* AB428654	0.94	0.95	0.94	0.96	0.94	0.95	0.94	0.95	0.95	0.95	0.94

Table 3.

Strain	Leader	D1-D1' helix	Spacer+D2	Spacer+D3+spacer	tRNA Ile gene	Space+V2+spacer	rRNAAla gene	Spacer+BoxB+spacer	Box A	D4	Spacer+V3
Dapisostemon apicaliramis	10	63	36	14	74	48	73	68	12	10	56
Streptostemon lutescens	10	60	38	58	74	109	--	46	11	9	90
Hassallia CM1 HA11	8	65	35	14	74	53	73	75	12	10	53
Scytonema hyalinum clone 96 3B	7	97	33	16	74	84	73	163	12	10	55

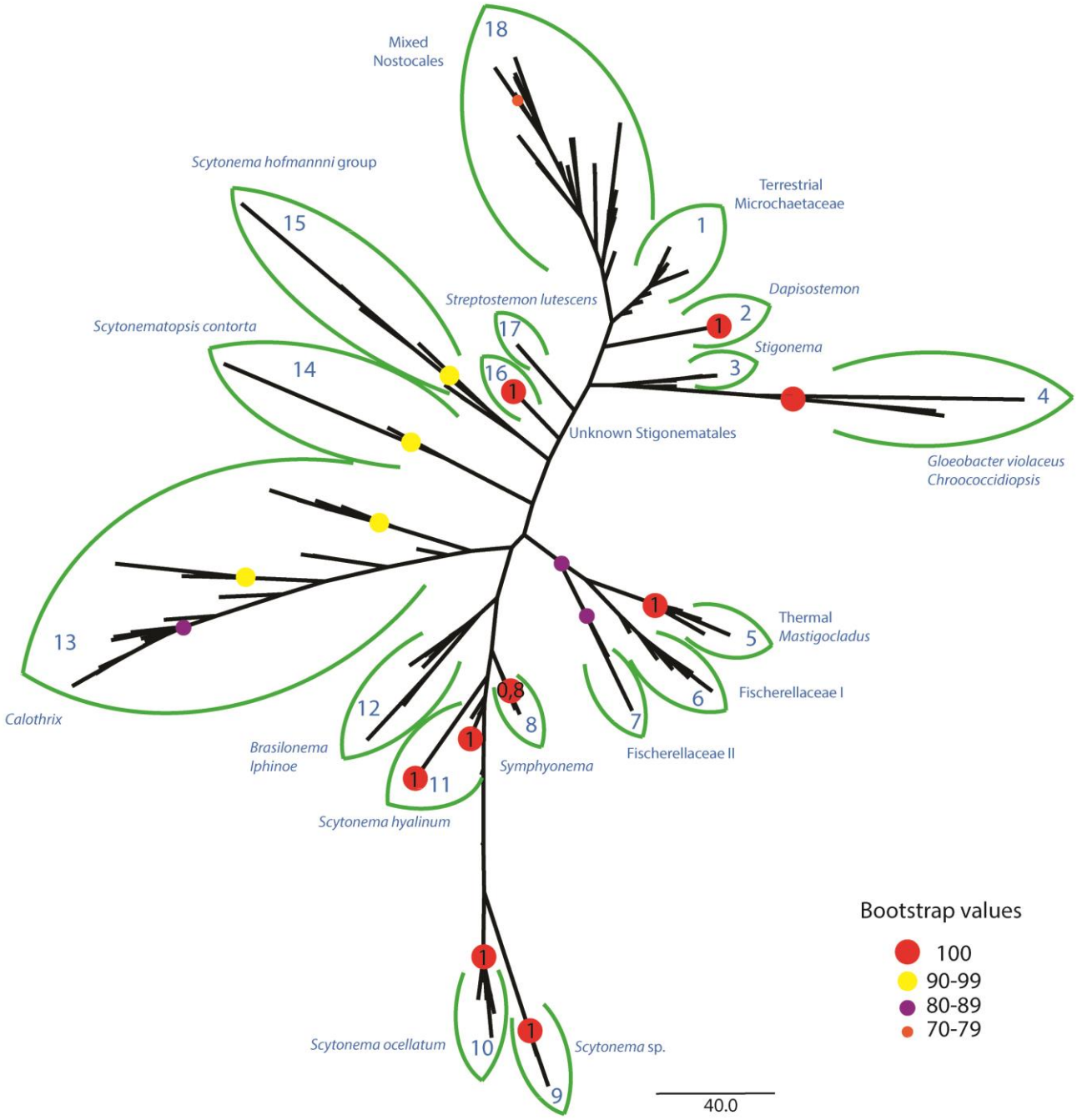
Mixed
Nostocales
18
Scytonema hofmannni group
15
Terrestrial
Microchaetaceae
1
Dapisostemon
2
Streptostemon lutescens
17
Scytonematopsis contorta
14
16
Stigonema
3
4
Unknown Stigonematales
Gloeobacter violaceus
Chroococcidiopsis
Thermal
Mastigocladus
5
Fischerellaceae I
6
13
12
0.8
8
7
Fischerellaceae II
Calothrix
Brasilonema
Iphinoe
Symphyonema
11
Scytonema hyalinum
Bootstrap values
100
90-99
80-89
70-79
10
Scytonema ocellatum
Scytonema sp.
9
40.0

Figure 1.

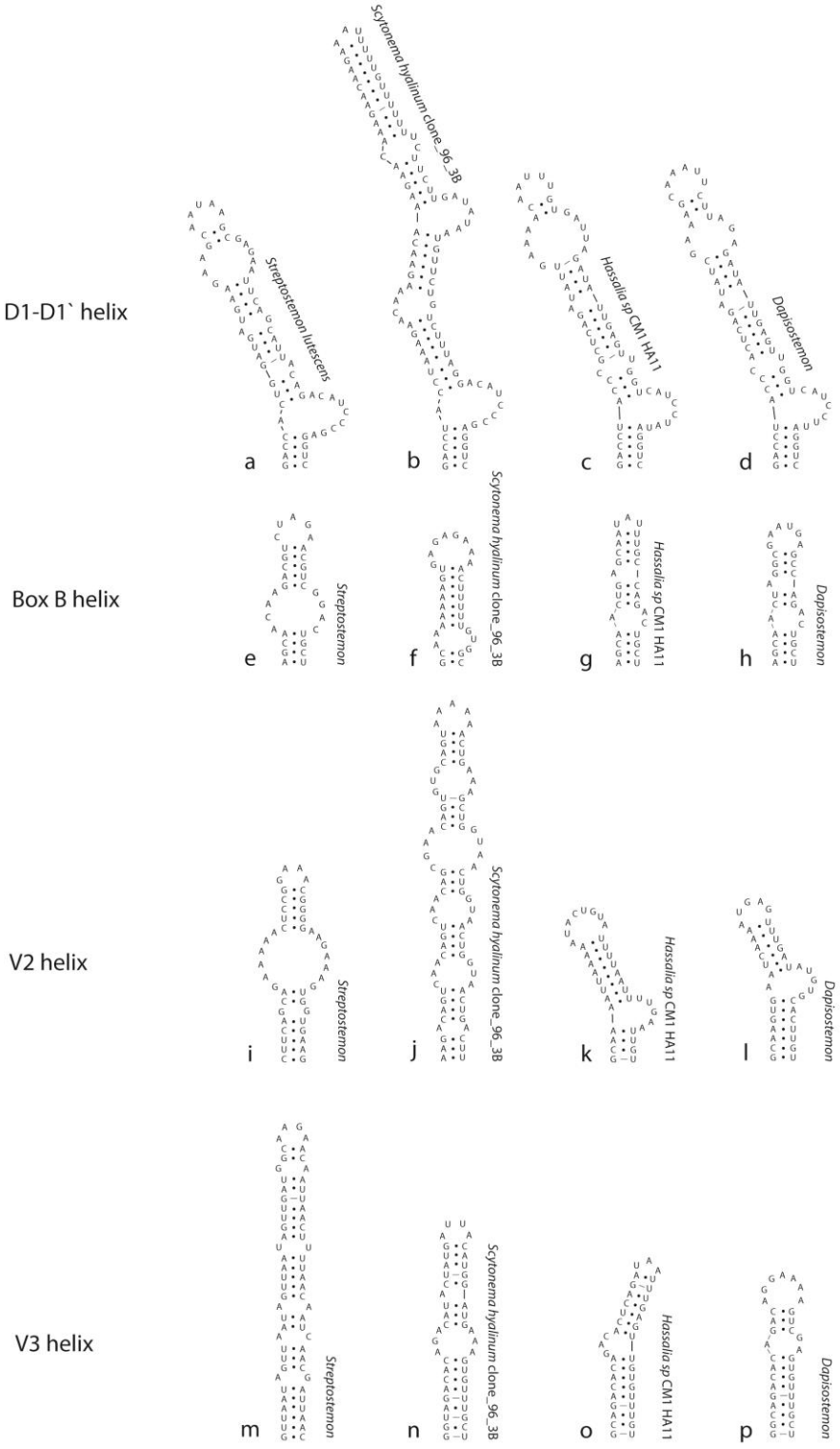
D1-D1` helix
Box B helix
V2 helix
V3 helix
Streptostemon lutescens
Scytonema hyalinum clone_96_3B
Hassalia sp CM1 HA11
Dapisostemon
Streptostemon
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

Figure 2.

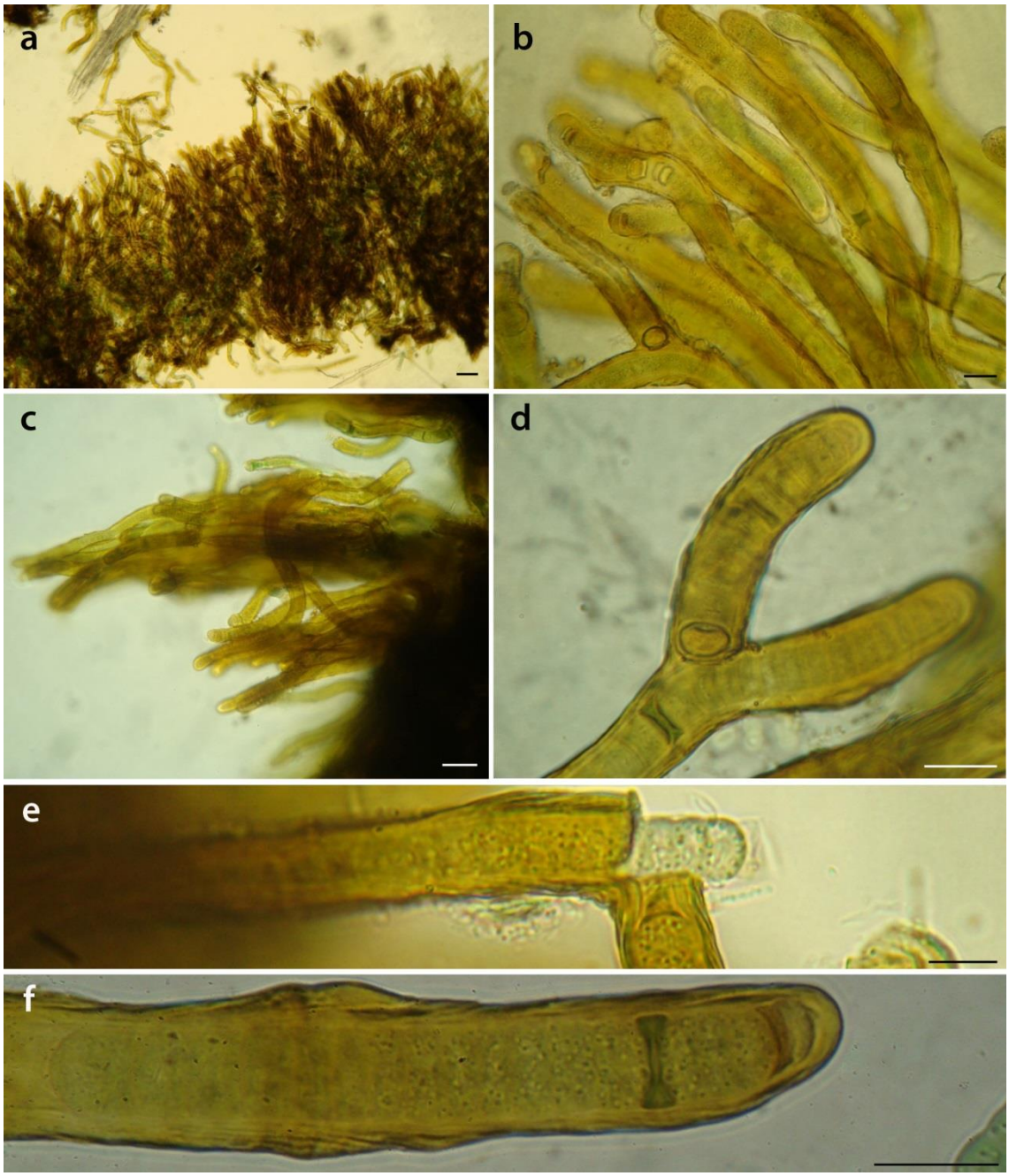

Figure 3.

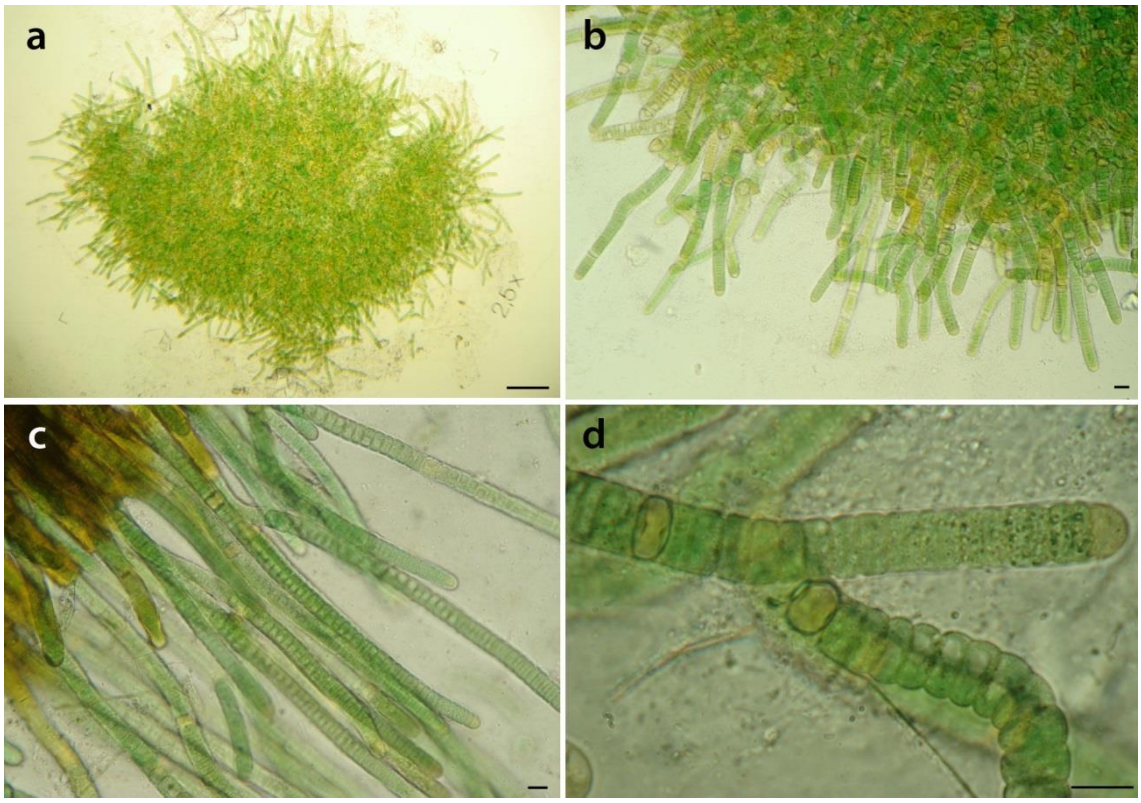

Figure 4.

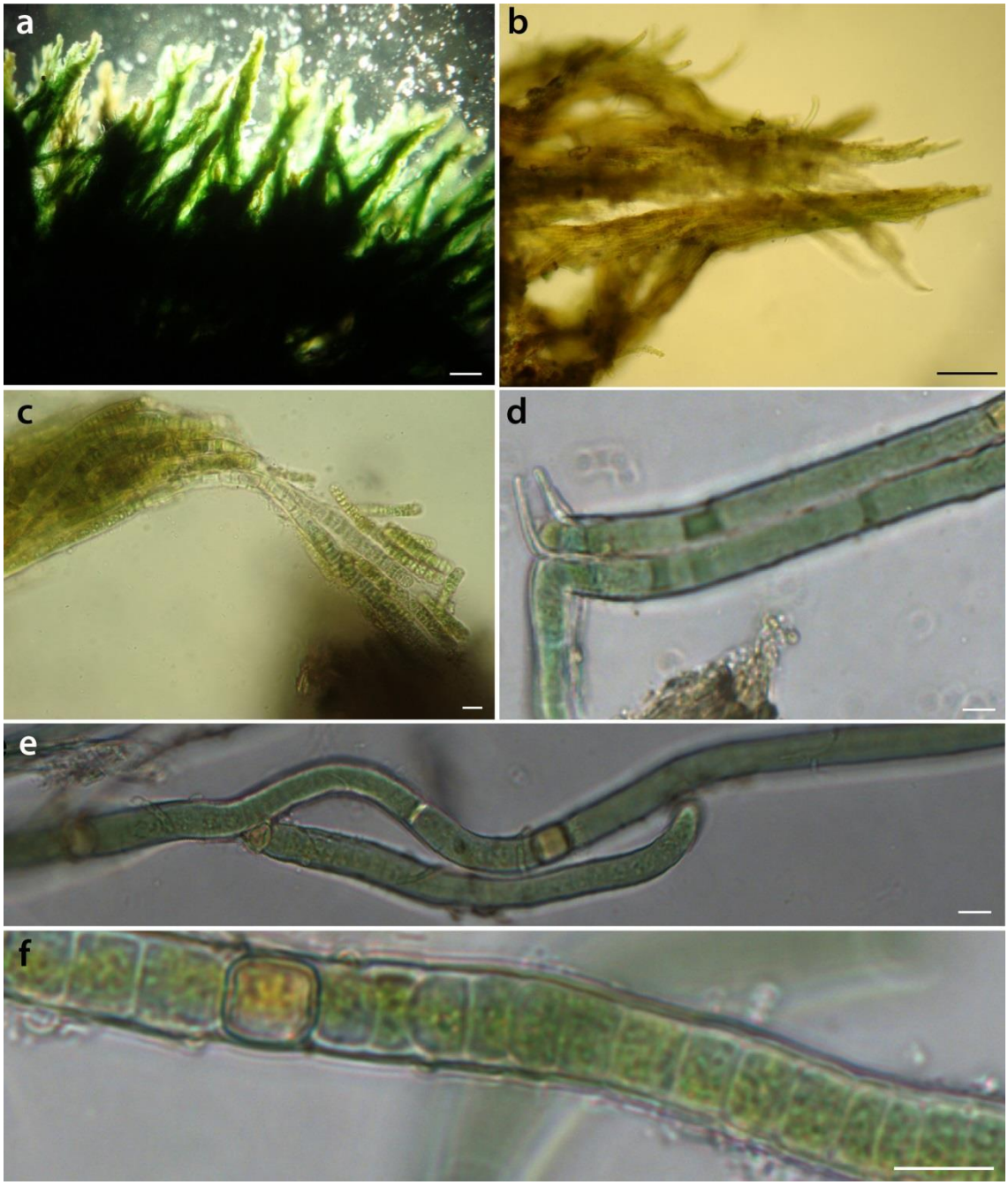

Figure 5.

Anexo VIII

Scientific letter

Current trends and prospects for cyanobacterial taxonomy – are only cultured populations enough?

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Abstract: Describing new taxa for Cyanobacteria is becoming more common in the last years following the increase of taxonomical studies using the polyphasic approach. The improvement of the phylogenetic analysis led to more precise knowledge about the relationships between taxa, but besides this, authors are neglecting the morphological data of populations from environmental samples, describing organisms growing only under culture conditions. Polyphasic approach considers the 16S rRNA gene phylogeny as the main criterion to distinguish taxa for Cyanobacteria, but there´s not enough data to consider that the morphological evaluations from environmental populations are not relevant.

Key words: biodiversity, polytaxonomy, phylogeny, morphology, new taxa.

Since the 16S rRNA molecular analysis was performed for Cyanobacteria, it has been clear that morphology alone is not enough for distinguishing species and genera. That's why, not only modern concepts of species have been created using polytaxonomy but also molecular analysis has become the most important criterion to delimit genera (Johansen & Casamatta 2005). These analyses permitted access to a not yet known biodiversity and, in the last ten years, for example, fifteen genera of Nostocales alone have been described based on this polyphasic approach. Taking into account a small time scale, one week, more than twelve new cyanobacterial genera were presented during the 19th International Association for Cyanophyte Research Symposium (IAC), held in July/2013 in Cleveland, Ohio, USA.

In the past few years, describing new genera using molecular results has become increasingly more common and necessary. However, the morphological description of these new genera has become poorer, especially in the characterization of environmental populations. We are now essentially describing strains which frequently grow as opportunists in culture and are possibly represented only by propagules or akinetes in their natural sites, instead of natural populations with their particular evolutionary adaptations to their environments. It is still unknown how these new exclusively cultured genera look like in natural conditions or even if they are developed in the respective sample sites. On the one hand, we have reported increase of 16S rRNA

diversity of Cyanobacteria, but on the other, the natural morphological diversity has been neglected in relation to genetic diversity, because of the severe changes in the morphology of cultured organisms and the lack of morphological evaluations on field samples. Obviously, culture is important for life cycle studies but, according to our habitual comparisons between nature and cultured populations, we are sure that many natural populations lose their natural morphological adaptations under controlled conditions. We consider that the genetic diversity is much bigger than morphological diversity and although cryptic taxa exist, we think that in many cases, more detailed morphological studies on nature material can be very useful to better understand the phylogenetic trees. We can cite here, as examples, some good papers, in which the morphological approach is used, as in the description of the genera *Brasilonema*, *Iphinoe* and *Loriellopsis* (Fiore *et al*. 2007, Lamprinou *et al.* 2011). Both papers describe these genera from nature material, considering also phylogeny and Transmission Electron Microscopy. Another paper is Ramirez-Reinat & Garcia-Pichel (2012), in which the endolithic genus *Mastigocoleus* is redescripted based on the polyphasic approach, including the morphological variations in simulated natural habitats.

The importance of studies on nature populations in addition to molecular and culture evaluations was also highlighted by Berrendero *et al.* (2011), who compared natural populations and cultured strains of *Tolypothrix* and *Calothrix* and showed that because some *Calothrix* strains in culture go through changes, they may be misidentified as *Tolypothrix*. In this aspect, the importance of the trichomes attenuation and also the validity of the heterocyte position for diagnosing families Scytonemataceae, Microchaetaceae *sensu lato* and Rivulariaceae (Vaccarino & Johansen 2011, 2012) has been intensely discussed. Recently, some scytonematoid species as *Brasilonema angustatum* Vaccarino & Johansen and *Scytonematopsis contorta* Vaccarino & Johansen have been described featuring mainly basal heterocytes and heteropolar hormogonia. Molecular analysis has shown that these are good species, but unfortunately their description is solely based on cultured material. The report on this morphological plasticity for these genera is very important, but it is impossible to be sure whether the natural populations are really attenuated or heteropolar, or if these features are developed only under culture conditions.

However, apart from these issues, there are some taxa described from environments without abundant populations, desert soils for instance (Rehakova et al 2007). It is always very difficult to access them in natural samples, since they cannot be observed by naked eye or are found just as small dry crusts, making the preparation of good slides for microscope observations almost impossible. Nevertheless, in many other cases we believe that natural populations can be found and reported in addition to cultured strains. Although at the present time it is still difficult to be sure whether the best option is to study cyanobacteria based only on molecular data and on culture evaluations, we can assert that many taxonomical problems occasioned by morphological incongruences could be avoided in the short run, if researchers looked more carefully into the environmental samples, searching for natural populations, before culturing.

As one of the consequences of this scenario, we can mention the lack of studies on *Stigonema* Bornet & Flahault and other complex Stigonemataceae, for instance. Only few papers have been published addressing this group in the last years. Surely, the

diversity of these cyanobacteria is underestimated and the relations of species within the genera are still not known due to the difficulties to isolate and cultivate them. In the case of *Scytonema*, which has been correctly split by molecular studies (Komárek *et al.* 2013), the precise morphological pattern for each clade is still not very clear, maybe because not all of the studied populations could be morphologically evaluated from nature material, at that time. This genus has been continually studied and soon more papers will be published splitting it formally in many genera, using the polyphasic approach. Up to now, it is not yet possible to know whether these new genera can be cryptic, reason why more morphological studies on natural populations can greatly contribute in deeper discussions.

Finally many questions remain: Is it possible to morphologically identify these exclusively cultured new genera if they are found in nature? How is their "true" morphology like? Will they be morphologically revaluated in the future? Is the high frequency of misidentified sequenced strains in GenBank related to the lack of morphological studies on their environmental samples? Besides, environmental studies could contribute by providing more morphological support to the currently existing clades and would help to clarify cyanobacterial taxonomy. We are seeking to rectify our mistakes from the past on cyanobacterial taxonomy, but we are not concerned about minimizing our mistakes for the future.

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Anexo IX

Three new morphospecies of *Stigonema* (Nostocales, Cyanobacteria) from the rainforest Mata Atlantica, Southeastern, Brazil

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Abstract

Three new species of the genus *Stigonema* Bornet & Flahault named *S. opaca*, *S. nematoidea* and *S. densa* are described and one new combination (*S. africanum*) is proposed, based on morphological evaluations. Furthermore, statistical analysis of variance was applied in order to distinguish morphologically very similar populations. These results indicate that different populations of a single species, with overlapping dimensions among them, can have statistical differences considering their morphometric parameters. Samples were collected by scrapping biofilms, growing on terrestrial habitats of tropical and subtropical regions of the Brazilian rainforest Mata Atlântica and statistical analysis was carried out considering cells length, cells diameter, filaments diameter, ratio cells length/cells diameter and ratio cells diameter/filament diameter (n=30).

Key Words: Cyanobacteria, terrestrial habitats, populations, statistical analyses.

Introduction

Recently many papers were published reporting the cyanobacterial diversity of the Brazilian Mata Atlantica (Lemes-da-Silva *et al.* 2010, Sant'Anna *et al.* 2010, Komárek *et al.* 2013, Sant'Anna *et al.* 2013, Hentschke & Komárek 2014). All of them describe new species or genera, not only due to the using of molecular data, but also because of the application of a concept which is strongly in evidence in the last years and was already used by (Gardner 1927), considering that tropical cyanobacterial diversity is very typical and different from temperate environments. This concept is in agreement with the cyanobacterial taxonomical polyphasic approach (Hoffmann *et al.* 2005, Johansen & Casamatta 2005) and evolutionary concepts of speciation, considering that geographic isolation, besides environmental differences between different places, contribute to diversification in Cyanobacteria. Thus, accepting the climatic differences between tropical and temperate environments, mainly the European continent, from where most of Cyanobacteria were described in the traditional sense, it is deductible that tropical cyanobacterial components are endemic and lodge many unknown species, which must be described.

Considering specifically the genus *Stigonema* Bornet & Flahault, four species were described to the Brazilian Mata Atlantica since 2013 (Sant'Anna *et al.* 2013, Hentschke *et al.* 2014c). Furthermore the species *S. gracile* was described by Silva & Sant'Anna (1988). These studies highlight the need for papers reporting cyanobacterial diverstity in tropical regions, even if these new descriptions were based only in morphological studies. Currently, molecular studies are almost not in use for this genus, mainly because its difficulties of strains isolation. Efforts are being made to sequence the 16S rRNA gene from single *Stigonema* filaments, but without success up to now. The only paper describing a new species of the genus considering morphology and phylogeny is the one of Hentschke *et al.* (2014c), in which the 16S rRNA gene was amplified from a natural population. This paper highlights the importance of morphological evaluations and the describing of new tropical taxa even based only in morphological studies, because it confirms the former morphospecies *S. fremyi* (Sant'Anna *et al.* 2013) as a good taxon, by phylogenetic evaluation.

Based on these statements, here we describe three new species of *Stigonema* from the Brazilian Mata Atlantica, based on morphological evaluations. Statistical analysis were applied in order to differentiate similar populations within species and elaborate hypothesis of separation of them, which must be tested by molecular studies in future studies.

Samples were collected by scraping biofilms growing on terrestrial habitats of tropical and subtropical regions of the Brazilian Mata Atlântica, respectively Nucleus Santa Virginia - 23º20'16"S, 45º09'01"W and State Park of Ilha do Cardoso - 25º04'12"S, 47º55'27"W.

After sampling, dry material was kept in paper bags. For morphological analysis in laboratory, parts of the samples were rehydrated with distilled water for 20 hours. Aliquots were also preserved in formaldehyde (4%) and deposited in the Herbarium of the Institute of Botany (SP), Brazil. Morphological analysis were carried out based on fresh material, using microscope Zeiss Axioplan 2 photomicroscope equipped with a Zeiss Axiocam MRc digital camera. Measurements were performed using the *software* Carl Zeiss Axiovision 4.6.

Statistical analysis were carried out in intent to distinguish morphologically similar populations by cells length, cells diameter, filaments diameter, ratio cells length/cells diameter and ratio cells diameter/filament diameter. Mean values and standard deviations (SD) were calculated for each group. We assumed normal distribution of variables by the Shapiro-Wilk normality test. Data were compared among groups by One Way ANOVA followed by Bonferroni post hoc test. For that, two measurements of each variable were performed in different cells of 15 random filaments per group (n=30).

Results and discussion

Three new species of *Stigonema* were described based on morphological data. *Stigonema opaca* sp. nov. is a well delimited species, slightly similar to *S. lineare* Gardner, but the author describe this species presenting very thin sheats (Gardner 1297), which is relatively wider in *S. opaca* sp. nov.. We found two populations of *S. opaca* sp. nov., from two different habitats, both presenting the same morphological markers, but statistically different in relation to dimensions. While morphometric data indicate the

overlaying between the dimensions of these populations, statistical significant differences were found comparing their cells and filaments diameters ($p < 0,05$) (Table 1). The biological implications of these results and the separation of the populations in two different species cannot be evaluated here, because of the lack of molecular analyzes. Nevertheless, this could be the base for the separation of these populations in two species, which must be tested in the future by molecular studies.

Stigonema nematoidea sp. nov. is a very typical species, but can be compared to *S. crassivaginatum* Sant'Anna *et al*. Both species present wide lamellate yellowish sheats and similar ends on hormogoniferous branches. In spite of that, the margins and lamellation of the sheats in *S. nematoidea* sp. nov. are usually wavy, while in *S. crassivaginatum*, sheaths margins are straight, well delimited and lamellation is divergent (Sant'Anna *et al.* 2013).

Stigonema densa sp. nov. is similar to *S. parciramosum* Gardner. These two species differ basically because of the very frequent branching in *S. densa* sp. nov, which are very sparse in *S. parciramosum*. Also, the last features longer filaments, which are not that entangled as in *S. densa* sp. nov..

We found other two populations (SP 401439 and A33) very similar to *S. parciramosum*, including dimensions, but with filaments sometimes narrowed toward the apices, characteristic which is not present in the original description. We could consider this as morphological plasticity for this species, but additionally the studied populations present more than "…branches..very sparse…", as described for *S. parciramosum* (Gardner 1927). We are not sure what "very sparse" exactly means, but the Brazilian populations surely present branches more frequent than that, although they cannot be considered much frequent. We identified our populations as *S.* cf. *flexuosum*, because they fit very well in the description of this taxon (Sant'Anna *et al.* 2013). The only difference among our population and the type species are the filaments and trichomes diameters, which are larger in our material. These differences in dimensions could lead to the description of a new taxon, but it must be tested by molecular studies too, in order to evaluate whether these populations consist in different species or the differences in dimensions are just morphometrical variations.

Statistically, our populations of *S.* cf. *flexuosum* differ from each other by cell length and diameter ($p<0.05$) (Table 1). These differences could indicate that the two populations possibly consist in different species, but again, the biological importance of these results and whether this is an effect of the high number of measurements (n=30) or if they really are different species, must be tested by phylogenetic studies.

S. hormoides var. *africanum* was recombined to *S. africanum* comb. et stat. nov., because of the relevant differences between these populations and the type of *S. hormoides* var. *hormoides* Bornet & Flahault. The population from Mata Atlântica fits perfectly the description of var. *africanum* (Geitler 1932) and differs from *S. hormoides* by presenting intensely branched unisseriate and non subtorulose filaments. *S. hormoides* var. *hormoides* presents subglobose cells and rarely branched subtorulose filaments that can be uni- or biseriate (Sant'Anna *et al.* 2013)

The statistical differences between morphologically very different populations (different species) were not conclusive and obviously cannot be used alone to distinguish species (Table 1). In this paper, we conclude that statistical differences are important to distinguishing very similar populations, but they must be considered combined with ecological and morphological markers as cells shapes, types of sheaths, arrangement of filaments and obviously phylogenetic studies. Also, we agree that genetic studies are necessary to the better understanding of the Stigonemataceae phylogeny, but we are still describing new species based on morphology, due to the great differences between the Brazilian material and the already known traditional species. The most of the already described Cyanobacteria are from Europe and consequently, tropical types are still almost unknown, as shown by Frémy (1930). Considering the climatic differences between these regions, in our opinion, tropical morphotypes probably consist in a large number of new taxa, which will be described in the short run, associated with the increasing number of taxonomic studies in tropical regions, specially Brazil (Lemes-da-Silva *et al.* 2010, Komárek *et al.* 2013, Genuário *et al.* 2014, Hentschke *et al.* 2014a, Hentschke *et al.* 2014b, Hentschke & Komárek 2014, Hentschke *et al.* 2014c). The describing of new taxa based only in morphology is still valid and totally necessary for types which do not grow under culturing conditions because is the only way to know this biodiversity that in future could be proved by molecular analysis (Hentschke *et al.* 2014c).

Desctiptions

Stigonema opaca G. S. Hentschke & C. L. Sant'Anna **sp nov.**

(Figures 1-3)

Main filaments creeping, unisseriate, very rarely bisseriate, 6.9-15.5 µm diam., loosely arranged, crooked or tortuous, sometimes widened or attenuated in the ends. Branches common, identical to main filaments. Cells subesferical 2.6-8.3 µm long, 4.4-11.7 µm diam. Cell content pale green, often granulate. Sheaths homogenous, hyaline or sometimes yellowish in older filaments. Heterocytes with the same shape and size of the cells.

Holotype: Brazil, State of São Paulo, State Park of Ilha do Cardoso, 06/29/2010, *Watson A. Gama Jr. and Camila F. da S. Malone* (SP 427937), Herbarium of São Paulo State, São Paulo, Brazil.

Type habitat: Growing on humid rocks inside the Atlantic rainforest.

Studied material: Brazil, State of São Paulo, State Park of Serra do Mar, Nucleus Santa Virgínia, 02/11/2010, *Watson A. Gama Jr. and Ewerton C. Manarin* (SP 428609).

Etymology: Opacus (Lat.) = opaque.

Stigonema nematoidea G. S. Hentschke & C. L. Sant'Anna **sp. nov.**

(Figures 4-8)

Main filaments short, tortuous, unisseriate, except on the arising of branches, 14.5-21.9 µm diam., intensely entangled. Branches similar to the main filament, cylindrical, sometimes widened at the ends or attenuated on hormogoniferous ones. Cells ellipsoid compressed 3.8-8.6 µm long, 8.9-12.2 µm diam., shorter at the apices. Cell content olive green, granulate. Sheaths yellowish, hyaline in young filaments and intensely lamellate. Hormogonia long, not constricted. Heterocytes with the same shape and size of the cells.

Holotype: Brazil, State of São Paulo, State Park of Ilha do Cardoso, 06/29/2010, *Watson A. Gama Jr. and Camila F. da S. Malone* (SP 428608), Herbarium of São Paulo State, São Paulo, Brazil.

Type habitat: On humid soil walls.

Etymology: Nematoideus (Lat.) = nematoid.

Stigonema densa G. S. Hentschke & C. L. Sant'Anna **sp. nov.**

Main filaments unisseriate, except on the arising of branches 22.2-30.8 µm diam, intensely entangled and branched. Branches similar to the main filament, often widened at the apices or cylindrical, sometimes parallel. Cells ellipsoid compressed or rarely quadratic, 7.8-17,8 µm long, 16.3-24.5 µm diam., shorter at the apices. Cell content dark green, granulate. Sheaths yellowish or hyaline in younger filaments and lamellate. Heterocytes with the same shape and size of the cells.

Holotype: Brazil, State of São Paulo, State Park of Ilha do Cardoso, 06/29/2010, *Watson A. Gama Jr. and Camila F. da S. Malone* (SP 428607), Herbarium of São Paulo State, São Paulo, Brazil.

Type habitat: On soil (sand), exposed to sunlight.

Etymology: Densus (Lat.) = dense.

Stigonema africanum (F.E.Fritsch) G. S. Hentschke & C. L. Sant'Anna **comb. et stat. nov.**

Basionym: Stigonema hormoides var. *africanum* F.E.Fritsch, Transactions of the Royal Society of South Africa 11: 370. 1923.

Main filaments creeping, unisseriate 9.2-15.5 µm diam., sometimes slightly attenuated, intensely branched. Branches frequently parallel and erect, in the same side of main filament. Cells elliptic 4.1-9.7 µm long, 7.6-14.4 µm diam., frequently overlaying in ¼ of the length, sometimes with individual sheaths conspicuous. Cell content brownish. Sheaths yellowish, homogenous or lamellate. Heterocytes with the same shape and size of the cells.

Habitat: On a wood pole.

Studied material: Brazil, State of São Paulo, State Park of Serra do Mar, Nucleus Santa Virgínia, 02/11/2010, *Watson A. Gama Jr. and Ewerton C. Manarin* (SP 401421).

Stigonema cf. ***flexuosum*** West & West, Journal of Botany 35: 293. 1897.

Main filaments long, almost straight, 19.6-31.4 µm diam, unisseriate except on the arising of branches, sparsely branched. Branches similar to main filaments, but thinner, with hyaline sheats. cylindrical or rarely slightly tapered. Cells ellipsoid compressed, sometimes quadratic 7-23 µm long, 14.6-24.4 µm diam, shorter in the apices. Cell content olive green, granulated. Sheats homogeneous, hyaline, rarely yellowish in older filaments. Heterocytes with the same shape and size of the cells.

Habitat: growing on soil, exposed to sunlight.

Studied material: Brazil, State of São Paulo, State Park of Serra do Mar, Nucleus Santa Virgínia, 02/11/2010, *Watson A. Gama Jr. e Ewerton C. Manarin* (SP a33).

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Table 1. Comparisons among studied populations dimensions. Values are means ± SD. Clength = Cell length, Cdiam = Cell diameter, Fdiam = Filament diameter, R = Ratio

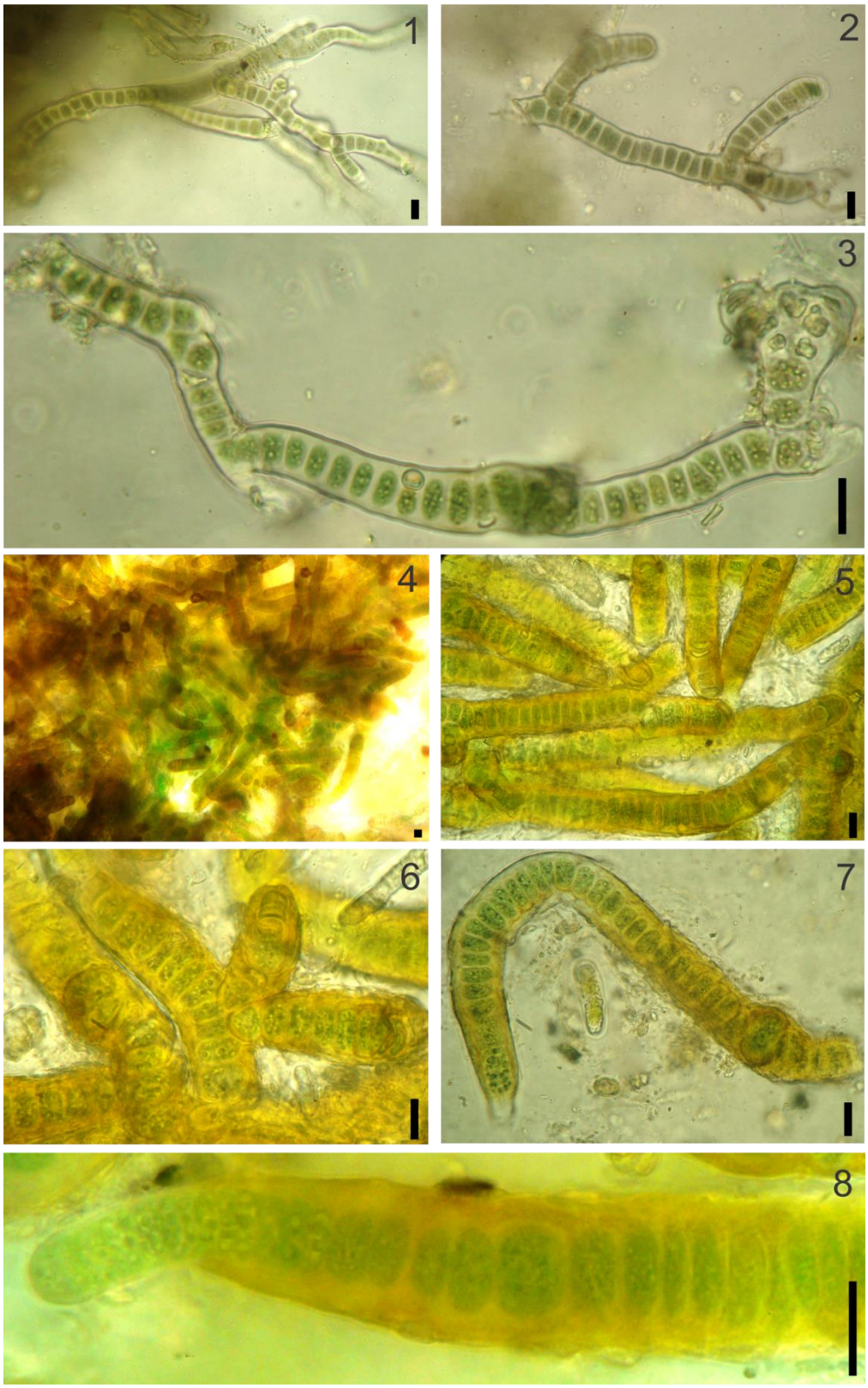
Figures 1-8. 1-3. *Stigonema opaca*; **4, 5.** General view of *S. nematoidea*; **6.** Branching in *S. nematoidea*; **7, 8.** Hormogoniferous branches of *S. nematoidea.* Scales: 10 µm.

Figures 9-17. 9, 10. General view of *Stigonema densa*; **11.** Detail of branching of *S. densa*; **12.** Details of apices of *S. densa*; **13.** General view of parallel filaments of *S. africanum*; **14.** Detail of filament of *S. africanum*,; **15.** Branched filaments of *S.* cf. *flexuosum*; **16.** Branching of *S.* cf. *flexuosum***; 17.** Young branch of *S.* cf. *flexuosum*. Scales: 9, 13, 15 = 50 µm; 10-12, 14, 16, 17 = 10 µm.

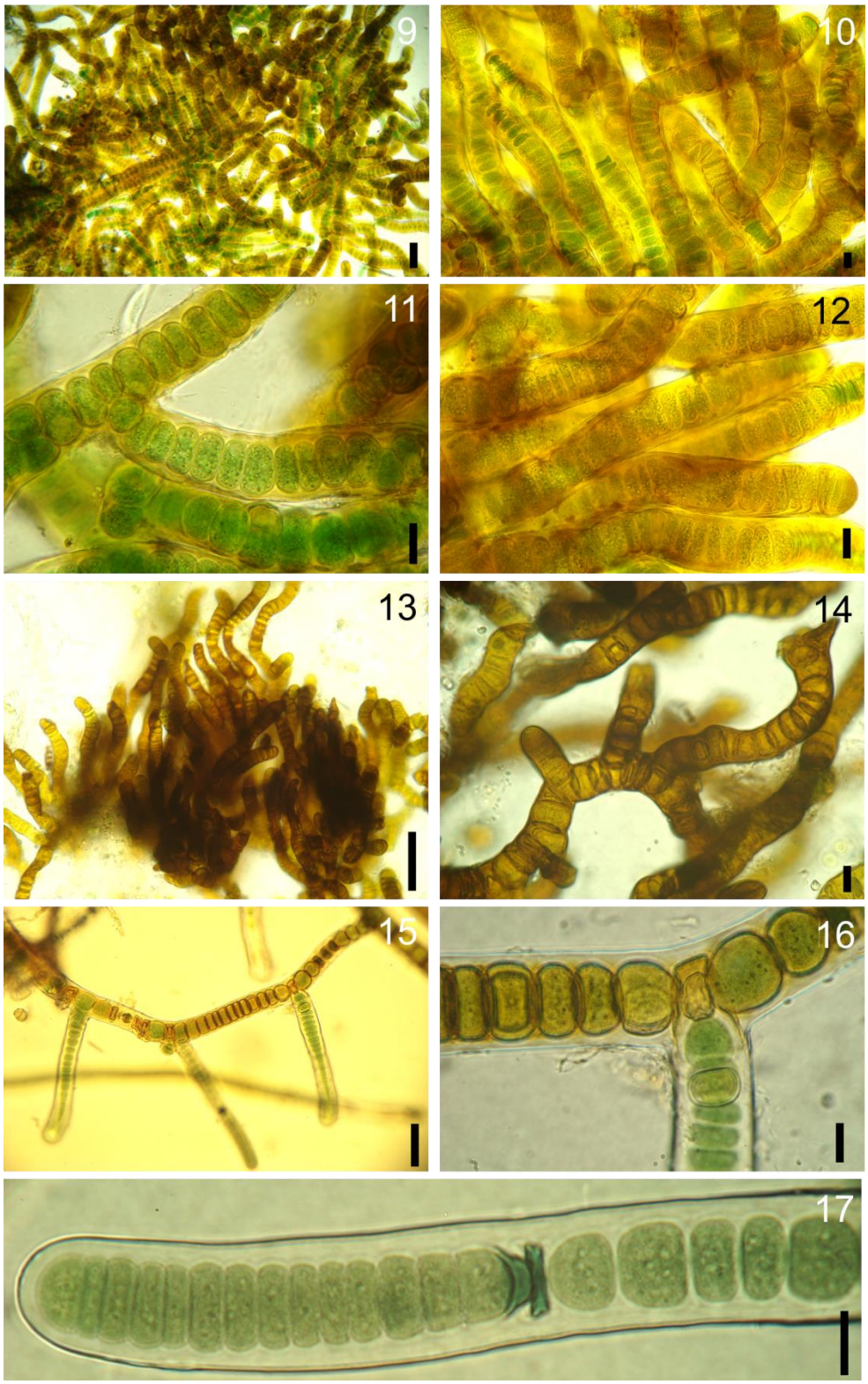
Table 1.

	S. opaca SP 428609	*S. africanum* SP 401421	*S.* cf. f*lexuosum* A33	*S. opaca* SP 427937	*S. densa* SP 428607	*S.* cf. *flexuosum* SP 401439	*S. nematoidea* SP 428608
Clength	3.87±0.69[cf]	6.8±1.4[c]	12.96±3.09[g]	5.53±1.10[c]	11.06±2.4[a]	14.89±3.72[g]	5.50±1.12
Cdiam.	6.01±0.80[g]	10.12±1.35[bce]	19.54±2.35[abd]	9.55±1.08[bc]	20.43±2.11[a]	20.90±2.01[a]	10.31±0.85
Fdiam.	8.56±0.73[g]	11.83±1.20[bce]	26.15±2.39[d]	12.70±1.29[bc]	26.85±2.2	27.87±1.90	16.83±1.47[g]
R Clength/Cdiam	0.64±0.11[ac]	0.66±0.08[ac]	0.66±0.13[ac]	0.58±0.09[b]	0.54±0.10[b]	0.71±0.16[a]	0.53±0.09
R Cdiam / Fdiam.	0.71±0.11	0.85±0.05[g]	0.74±0.04	0.75±0.05	0.76±0.08	0.76±0.06	0.61±0.03[g]

[a] $p<0.05$ vs *S. nematoidea*; [b] $p<0.05$ vs *S.* cf. *flexuosum* SP 401439; [c] $p<0.05$ vs *S. densa*; [d] $p<0.05$ vs *S. opaca* SP 427937; [e] $p<0.05$ vs *S.* cf. *flexuosum* A33; [f] $p<0.05$ vs *S. africanum*; [g] $p<0.05$ vs all the others.



Figures 1-8.



Figures 9-17.