Using a polyphasic approach for exploring secondary metabolites from freshwater and thermal cyanobacteria strains from Azores islands

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Tese de Doutoramento submetida como requisito parcial para obtenção do grau de Doutor em Biologia

sleep is overrated

dormir é sobrevalorizado

somnus aestimātus nimium est

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Abstract

Cyanobacteria are photosynthetic microorganisms with a long evolutionary history and high morphological complexity, inhabiting a wide range of habitats, from freshwater to seawater, including extreme environments. This is accompanied by an increasing complexity of its taxonomical system based on ecological, morphological and genetic characters, using single-gene phylogenies, and more recently, a genomic approach, that has contributed to an overall reformulation of the cyanobacteria taxonomical system. The evolutionary history of cyanobacteria allowed them to acquire specific genes that may produce secondary metabolites of interest. Among these, several high-value bioactive compounds have been identified with significant potential in cytotoxicity and the treatment of metabolic diseases.

In this work, a taxonomical review using the most recent literature and a revision on reported cyanobacteria from the Azores was made, contributing to the current knowledge of cyanobacteria diversity in the region. Strains deposited and maintained in the Bank of Algae and Cyanobacteria of the Azores (BACA) from aquatic, terrestrial, thermal and brackish habitats were taxonomically studied. Five new genera and ten new species were described: *Azorothrix ramosa* gen. & sp. nov., *Radiculonema aquaticum* gen. & sp. nov., *Venetifunis florensis* gen. & sp. nov., *Tumidithrix elongata* gen. & sp. nov., *Pseudocalidococcus azoricus* gen. & sp. nov., *Pycnacronema lacustrum* sp. nov., *Pegethrix atlantica* sp. nov., *Kovacikia atmophytica* sp. nov., *Albertania obscura* sp. nov. and *Leptodesmis lacustris* sp. nov.. To describe these new taxa, a polyphasic approach was used based on morphology (light and transmission electron microscopy), ecology, and genetic markers, such as the 16S rRNA gene and 16S-23S rRNA internal transcriber space (ITS). For the description of *Pseudocalidococcus azoricus*, a genomic approach to improve genetic support was applied.

The identified taxonomical diversity of cyanobacteria in the BACA culture collection reinforced the need for a bioactivity search. For that purpose, 56 BACA strains were selected and grown in laboratory conditions, recovered by centrifugation, freeze-dried and secondary metabolites extracted by sonication and methanol. The media used in biomass production was recovered after centrifugation, filtered and freeze-dried. Extracts were tested for cytotoxicity against carcinoma cell lineages, HepG2 and HCT116, and lipid reduction activity using both zebrafish larvae and a steatosis model with fatty acid overloaded HepG2 cells. Several bioactive extracts were identified with promising bioactivities in cytotoxicity (*Scytonematopsis* sp. BACA0005) and lipid reduction assays (*Dulcicalothrix* sp. BACA0344 and *Pseudocalidococcus azoricus* BACA0433), with some extract demonstration high toxicity against zebrafish larvae (*Symphyonema* sp. BACA0090 and *Aliinostoc* sp. BACA0355). The extract's composition revealed

by ESI-HR-LC-MS/MS shows that chemodiversity was mainly determined by the strain's phylogenetic relation than by their original habitat. Metabolomic data was used in a feature-based molecular network to identify several mass peaks potentially linked to the observed bioactivities. This approach highlighted several strains, compound classes, and mass peaks as promising candidates for further bioactive compound research, with potential applications in biotechnology and pharmaceuticals.

Overall, this work reinforces the Azores as a source of unique genetic resources with a high impact on the cyanobacteria taxonomic system and the need to study these novel resources in the biotechnological and pharmaceutical fields. The high taxonomical variability and reported bioactivity support the possible existence of many new high-value biological active compounds.

Keywords: taxonomy, polyphasic approach, novel genera, novel species, chemodiversity, biotechnological applications, lipid-reduction, antisteatosis, metabolomic, genomic

Resumo

As cianobactérias são microrganismos fotossintéticos com uma longa história evolutiva e uma elevada complexidade morfológica, ocupando uma grande diversidade de habitats, desde água doce a marinha, incluindo ambientes extremos. Esta diversidade é acompanhada por uma crescente complexidade do seu sistema taxonómico, baseado em caracteres ecológicos, morfológicos e genéticos, utilizando filogenias baseadas em marcadores genéticos específicos, e, mais recentemente, numa abordagem genómica, que contribuiu para a reformulação geral do sistema taxonómico das cianobactérias. A história evolutiva das cianobactérias permitiu-lhes adquirir genes específicos para a produção de metabolitos secundários de interesse. Entre estes, já foram identificados vários compostos bioativos de elevado valor, com significativo potencial citotóxico e no tratamento de doenças metabólicas.

Neste trabalho foi efetuada uma revisão taxonómica das cianobactérias existentes nos Açores baseada nos registos anteriormente publicados e tendo em consideração a literatura mais recente sobre a taxonomia do grupo, contribuindo, assim, para o conhecimento da diversidade de cianobactérias na região. Para além disso, foi efetuado o estudo taxonómico das estirpes de cianobactérias depositadas no Banco de Algas e Cianobactérias dos Açores (BACA), provenientes de habitats aquáticos, terrestres, termais e salobros. Foram descritos cinco novos géneros e dez novas espécies: *Azorothrix ramosa* gen. & sp. nov., *Radiculonema aquaticum* gen. & sp. nov., *Venetifunis florensis* gen. & sp. nov., *Tumidithrix elongata* gen. & sp. nov., *Pseudocalidococcus azoricus* gen. & sp. nov., *Pycnacronema lacustrum* sp. nov., *Pegethrix atlantica* sp. nov., *Kovacikia atmophytica* sp. nov., *Albertania obscura* sp. nov. e *Leptodesmis lacustris* sp. nov.. Na descrição destes novos taxa foi utilizada uma abordagem polifásica baseada na morfologia (microscopia ótica e eletrónica de transmissão), na ecologia e em marcadores genéticos, nomeadamente, o gene do 16S rRNA e a região do espaço interno de transcrição do 16S-23S rRNA (ITS). Para a descrição da *Pseudocalidococcus azoricus* foi aplicada uma abordagem genómica de modo a melhorar o suporte genético.

A elevada diversidade taxonómica de cianobactérias encontrada na coleção BACA reforçou a necessidade de se proceder uma pesquisa das suas potenciais bioatividades. Para esse efeito, selecionaram-se 56 estirpes do BACA que foram cultivadas em condições laboratoriais, tendo-se recuperado a sua biomassa por centrifugação seguida de liofilização e os seus compostos extraídos por metanol e sonicação. O meio de cultura utilizado na produção de biomassa foi recuperado após centrifugação, tendo sido filtrado e liofilizado. Os extratos foram testados quanto à sua citotoxicidade contra linhagens de células de carcinoma humano, HepG2 e HCT116, e atividade de redução de lípidos, através da utilização de larvas de peixe-zebra e um

modelo de esteatose com células HepG2 sobrecarregadas com ácidos gordos. Foram identificados vários extratos com bioatividades promissoras nos ensaios de citotoxicidade (*Scytonematopsis* sp. BACA0005) e de redução de lípidos (*Dulcicalothrix* sp. BACA0344 e *Pseudocalidococcus azoricus* BACA0433), tendo alguns extratos demonstrado uma elevada toxicidade contra larvas de peixe-zebra (*Symphyonema* sp. BACA0090 e *Aliinostoc* sp. BACA0355). A análise da composição dos extratos por ESI-HR-LC-MS/MS revelou que a quimiodiversidade é determinada principalmente pela relação filogenética das estirpes, mais do que pelo seu habitat original. Os dados da metabolómica foram utilizados numa *feature-based molecular network* para identificação de vários picos de massa potencialmente ligados às bioatividades observadas. Esta abordagem destacou várias estirpes, classes de compostos e picos de massa como candidatos promissores para investigação futura de compostos bioativos, com potenciais aplicações em biotecnologia e produtos farmacêuticos.

No geral, este trabalho reforça a importância dos Açores como uma fonte de recursos genéticos únicos, com um elevado impacto no sistema taxonómico das cianobactérias, e a necessidade de estudar estes novos recursos nos campos da biotecnologia e farmacêutica. A elevada variabilidade taxonómica e as bioatividades relatadas suportam a possível existência de vários novos compostos, biologicamente ativos e de elevado valor.

Palavras-Chave: taxonomia, abordagem polifásica, novos géneros, novas espécies, quimiodiversidade, aplicações biotecnológicas, redução lipídica, anti-esteatose, metabolómica, genómica

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List of Abbreviations

16S rRNA: 16S ribosomal ribonucleic acid

16S-23S rRNA ITS: 16S-23S ribosomal ribonucleic acid internal transcribed spacer

AAI: Average Amino Acid identify

ANI: Average nucleotide identity

ATCC: American Type Culture Collection

AZB: Herbário Ruy Telles Palhinha

BIC: Bayesian Information Criterion

BI: Bayesian inference

BACA: Bank of Algae and Cyanobacteria of the Azores

CHAB: Collection of Harmful Algae Biology

Chl: Chlorophyll

DDH: DNA-DNA hybridization

DIC: Differential interference contrast

DiPaC: Direct pathway cloning

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DwC: Darwin Core

EPS: Exopolysaccharides

FBMN: Feature-based molecular network

FBS: Fetal bovine serum

GBIF: Global Biodiversity Information Facility

HR-ESI-LC-MS/MS : Liquid chromatography-high resolution electrospray ionization tandem mass spectrometry

KABOOM: Prokaryote BUSCO phylogenomics

LM: Light microscope

ML: Maximum likelihood

NAFLD: Nonalcoholic fatty liver disease

NAP: Natural product atlas

NC: Necridia cells

NRPS: Non-ribosomal peptide synthetases

OTU: Operational taxonomic unit

PC: Pearson correlation

PCC: The Pasteur Culture Collection of Cyanobacteria

PCR: Polymerase chain reaction

PKS: Polyketide synthases

PT: Parietal thylakoids

RiPPs: Ribosomally produced and post-translationally modified peptide

REV: Resveratrol

SAR: Species-area relationship

TEM: Transmission electron microscopy

WFD: Water Framework Directive

UV: Ultraviolet

Chapter I

General introduction

Chapter I. General introduction

1.1 Introduction to cyanobacteria

Cyanobacteria are photosynthetic prokaryotes (Whitton & Potts 2012) that emerged around 3.0 Ga ago (Schirrmeister et al. 2015). These microorganisms are pointed out as the main cause of the Great Oxidation Event (Figure 1) in the early Proterozoic, around 2.4-2.3 Ga (Bekker et al. 2004). Their significance stems from their evolutionary development of both oxygenic photosystems, photosystem I and photosystem II, which played a crucial role in the rise of O_2 in the atmosphere (Hohmann-Marriott & Blankenship 2011, Sánchez-Baracaldo & Cardona 2020).

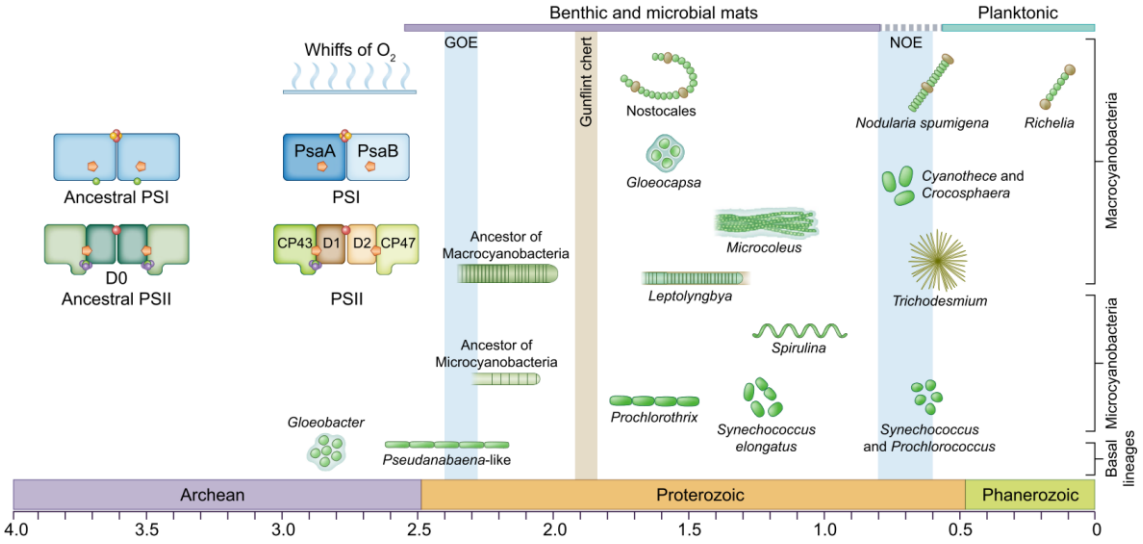

Figure 1. Timeline of the emergence of photosystem I, photosystem II and cyanobacterial lineages (in Ga before present). Adapted from Sánchez-Baracaldo & Cardona (2020).

These photosynthetic microorganisms have adapted to several types of habits from freshwater to seawater, terrestrial and extreme environments of temperature, salinity, among others (Komárek & Johansen 2015). This has made them one of the most distributed worldwide organisms, with a wide geography distribution, from the Antarctic to the Brazilian rainforest, Atacama Desert, oceanic Islands, caves, among many others (Komárek & Johansen 2015). This wide distribution of habitats, coupled with 3.0 Ga years of evolution, has made cyanobacteria one of the most diverse classes of microorganism, not only ecologically but morphologically, as shown in Figure 1, evolving from simple coccoid cyanobacteria (*Gloeobacter* Rippka et al.) to simple filamentous (*Pseudanabaena* Lauterborn) or to more complex forms with specialized cells in the Nostocales Borzì (Sánchez-Baracaldo & Cardona 2020).

In the XXI century, the taxonomy of cyanobacteria has advance quickly with the use of molecular markers and more recently genomes (Komárek et al. 2014, Strunecký et al. 2023). This approach has elucidated several problematic taxonomical clades, known to by polyphyletic and of very difficult taxonomic classification, such as *Lyngbya* C.Agardh ex Gomont and *Nostoc* Vaucher ex Bornet & Flahault (Engene et al. 2012, Bagchi et al. 2017, Lee et al. 2021). This taxonomic revision has also contributed to a better understanding of cyanobacteria known to be prolific producers of bioactive compounds, such as the *Moorena* Engene & Tronholm genus (Engene et al. 2012). This has supported cyanobacteria as one the most promising organisms for the search of novel bioactive natural products (Baunach et al. 2024).

1.1.1 Morphology

Cyanobacteria can grow in different conditions, either as solitary cells or trichomes, or in colonies (Komárek & Anagnostidis 2000, Komárek 2013). Colonies are aggregate group of cells or trichomes that can have different shapes, according to its pattern of cellular division, growth and production of mucilage (Komárek & Anagnostidis 2000). The colony shape varies from irregular to spherical colonies and even flattened plate-like or regularly grouped packs of cells (e.g. 4, 8, 16, 32, 64), and many times these features are characteristic of the genera (Komárek & Anagnostidis 2000). In more complex taxa, cyanobacteria filaments can present true branching or be multicellular (Hammerschmidt et al. 2021). Much of the ways colonies are formed, are due to cyanobacteria cell division, typically occurring through binary fission in a single plane, but also continuously in several planes or by forming endospores, baeocytes, and monocytes, among other strategies (Komárek & Anagnostidis 2000, Berthold et al. 2022). As a strategy of reproduction and dispersion, some cyanobacteria produce hormogonia, short trichomes with motility, which can then grow to fully developed filaments (Komárek & Anagnostidis 2005). Hormogonia can originate through death cells (necridia) that allow for the fragmentation of trichomes and their dispersion (Komárek & Anagnostidis 2005).

The cell morphology of cyanobacteria (Figure 2) can change depending on the genera and even species, with vegetative cells having several shapes (e.g. rectangular, oval, cylindrical), with possible differentiation of vegetative cells in specialized cells, called akinets and heterocytes, in certain genera (Komárek & Anagnostidis 2000, Komárek 2013). The vegetative cells of cyanobacteria have a stable arrangement of organelles, except for some exception where the development of some features is absent in some taxa, such as thylakoids, gas vesicles, and mucilage coats (Komárek & Anagnostidis 2000).

Thylakoids are structures found inside vegetative cells formed by two thylakoid membranes with a translucent lumen, where the phycobilisomes are attached (van de Meene et al. 2006). The absence of phycobilisomes or their modifications can cause thylakoids to stack abnormally, with little or no space between them (Elanskaya et al. 2018). This has been observed in *Spirulina major* Kützing ex Gomont, with both stacked and normally spaced thylakoids (Mareš et al. 2019). In certain phylogenetically distant clades of cyanobacteria, such as *Gloeobacter* and *Anthocerotibacter* Rahmatpour et al., the thylakoids are absent (Rippka et al. 1974, Mareš et al. 2013, Jiang et al. 2023), and phycobilisomes can have different arrangements (Rahmatpour et al. 2021).

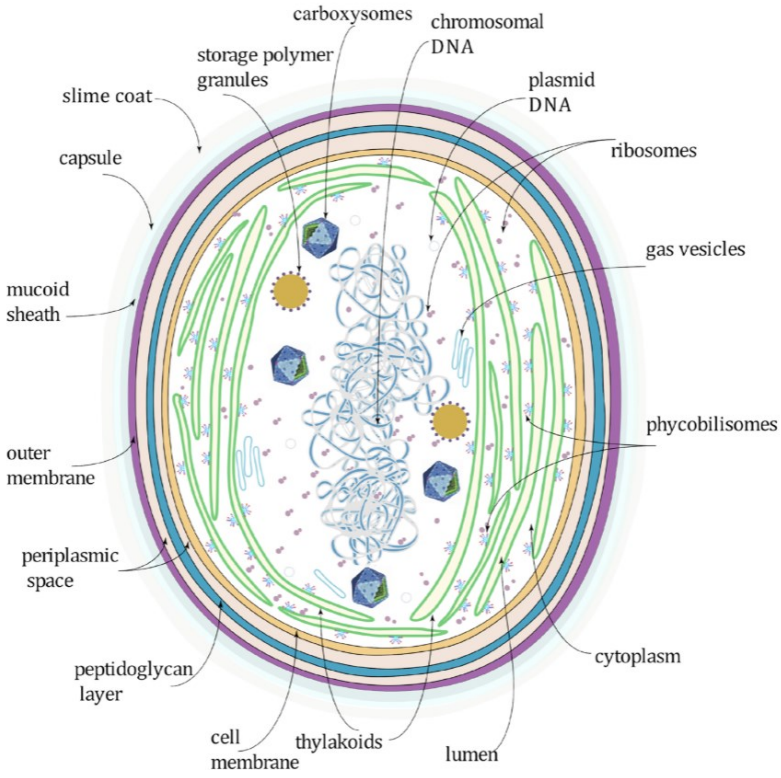

Figure 2. Cellular organization in cyanobacteria. Adapted from Noreña-Caro & Benton (2018).

In cyanobacteria, a large diversity in the architecture of thylakoids has been reported (Mareš et al. 2019), with either completely absent thylakoids (Rippka et al. 1974, Rahmatpour et al. 2021) or different thylakoid arrangements, namely parietal, radial, fascicular, irregular, parallel or *Cyanothece*-like (Komárek & Kaštovský 2003, Mareš et al. 2019). However, their biogenesis in cyanobacteria and the cause of such diversity are not yet fully known (Perez-Boerema et al. 2024). The thylakoid structural arrangement has been used as the basis for the taxonomical organization (Komárek & Kaštovský 2003), however, with increased description of

novel taxa and new published information along the years, its taxonomic significance was restricted to a few cases (Mareš et al. 2019).

Other cyanobacteria cell structures of significant taxonomic relevance have also been studied, including carboxysomes (Rae et al. 2013), cyanophycin granules (Flores et al. 2019, Kwiatos & Steinbüchel 2021), and gas vesicles (Walsby 1994, Hill & Salmond 2020), among others (Kromkamp 1987, Gonzalez-Esquer et al. 2016, Murata et al. 2016). Gas vesicles and aerotopes have gained increasing importance as apomorphic features supporting new taxa, such as *Limnothrix* M.-E.Meffert and *Dolichospermum* (Ralfs ex Bornet & Flahault) P.Wacklin, L.Hoffmann & J.Komárek (Meffert 1988, Komárek & Zapomìlová 2007). Gas vesicles are also crucial for cyanobacteria buoyancy, providing an ecological advantage in less favorable environments (Bouma-Gregson et al. 2017).

Heterocysts are specialized cells found in some cyanobacteria for N_2 fixation (Flores & Herrero 2010) that result from the differentiation of vegetative cells (Haselkorn 1978, Xing et al. 2022). Normally, heterocysts can be distinguished from vegetative cells by their larger size, modified shape, decreased pigmentation, thicker cell envelopes, and presence of cyanophycin granules at the poles adjacent to vegetative cells (Kumar et al. 2009). These can have a fixed position in the trichome, terminal, intercalary, or both, and when intercalary they can have a metameric disposition in the trichome (Komárek 2013). Heterocysts supply the adjacent cells with fixed nitrogen incorporated into glutamine and other amino acids (Wolk et al. 1976) while being fed with carbon from neighbouring cells (Wolk 1968). Nitrogen fixing in the heterocysts is possible by the availability of microoxic sites for N_2 fixing, with conversion to amino acids made by nitrogenases (Flores & Herrero 2010). Despite being unable to develop heterocysts, several cyanobacteria can fix nitrogen, made possible when cyanobacteria are grown under microoxic or anoxic conditions, with few exceptions fixing N_2 aerobically (Bergman et al. 1997).

Cyanobacteria vegetative cells can also differentiate into resting cells with reduced metabolic activity, known as akinets, under grown-limiting conditions (Adams & Duggan 1999, Perez et al. 2016). Akinets are usually larger than vegetative cells, from spherical to cylindrical, with a thickened cell wall and a multilayered extracellular envelope (Sukenik et al. 2018). These spore-like cells can grow back when favorable conditions are met again, developing novel vegetative cells (Flores & Herrero 2010, Kwon et al. 2023). The position of akinets can vary, from subterminal to paraheterocytic (adjacent to the heterocyst toward the middle of the trichome) or apoheterocytic (from the between two heterocysts, in the middle of the trichome, towards the outside) (Komárek 2013). Taxonomically, akinets are of great importance and frequently used as an apomorphic feature (Komárek 2010, Zapomělová et al. 2012).

1.1.2 Photosynthetic apparatus

Cyanobacteria can perform anoxygenic photosynthesis (Hamilton et al. 2018), allowing them to better adapt to several growing conditions than other bacteria. The photosystem used in anoxygenic photosynthesis predates the oxygenic photosystem (Hohmann-Marriott & Blankenship 2011), being considered as an intermediate stage in phototrophic evolution (Oren et al. 1977) and in the development of cyanobacteria (Cardona et al. 2015). In anoxygenic photosynthesis, cyanobacteria use hydrogen sulfate as an electron donor (Cohen et al. 1975), while in the oxygenic photosystem, the presence of chlorophyll enables to use water as the electron donor, leveraging the ability of Chlorophyll (Chl) to both capture light and convert it into chemical energy that is used to split water (Vinyard et al. 2013). Having both types of photosystems, cyanobacteria can switch between them according to the environmental conditions and available resources (Cohen et al. 1975, Klatt et al. 2015).

The phycobilisomes present in cyanobacteria thylakoids work as a light-harvesting antenna for photosystems II and I (Zheng et al. 2021) while also serving as communication between photosystems for energy transfer (Watanabe et al. 2014, Zheng et al. 2021). The phycobilisome (Figure 3) is a molecular complex of phycobiliproteins (Bryant et al. 1979) of hemidiscoidal shape with a triangular core structure (Zheng et al. 2021), constructed by phycobiliproteins, which have covalently attached pigments called bilins, and linker proteins (de Marsac & Cohen-Bazire 1977, Zheng et al. 2023). The main pigments that can be found in the phycobilisome can be categorized as allophycocyanins, phycocyanins and phycoerythrins (Bryant et al. 1979, Maccoll 1998, Domnguez-Martn et al. 2022). However, phycoerythrin cannot always be found in cyanobacteria (Maccoll 1998, Rahmatpour et al. 2021).

Five chemically distinct Chl have been scientifically reported: Chl a, b, c, d and f (Vinyard et al. 2013). Cyanobacteria can naturally produce Chl a (Bullerjahn & Post 1993), Chl b (Whatley 1977, Burher-Wiersma et al. 1989), Chl d (Miyashita et al. 1996) and Chl f (Chen et al. 2010), lacking only the ability to produce Chl c. Specific phylogenetic clusters, such as in *Prochlorococcus* Chisholm et al. ex Komárek et al. has evolved to use divinyl derivatives of chlorophyll a and b, rather than the phycobilisome (Bullerjahn & Post 1993, Roche et al. 1996). These Chl modifications can alter the thylakoid disposition, appearing in stacks, with no evident presence of phycobilisomes (Whatley 1977, Chisholm et al. 1992, Miyashita et al. 1996).

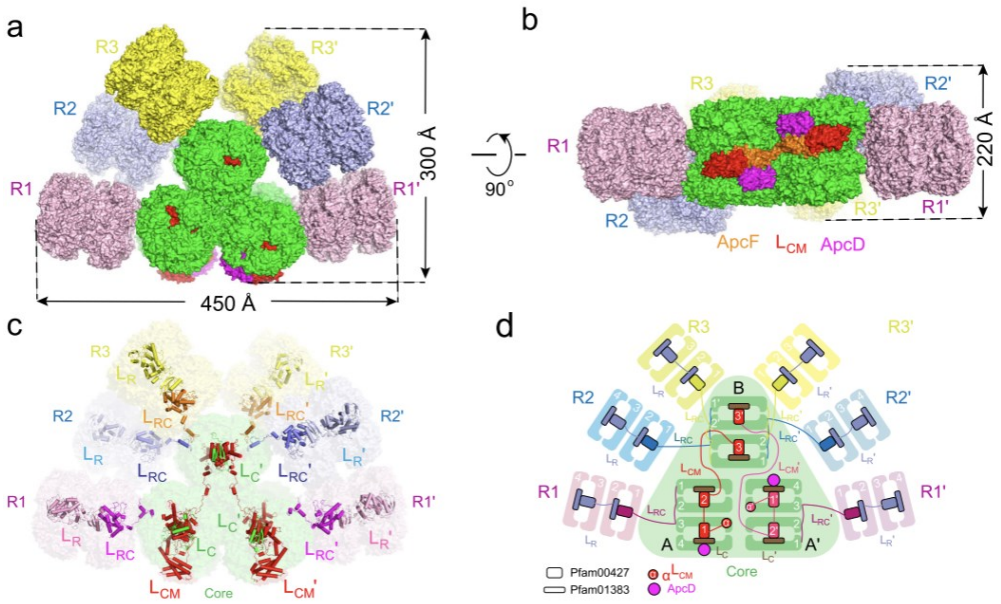

Figure 3. Overall structure of the phycobilisome from *Synechococcus* PCC 7002. a) Side view; b) Bottom view, ApcF, ApcD, and L_{CM} are highlighted in orange, magenta, and red, respectively; c) Distribution of linker proteins; d) Schematic models; a, c, d are colored as follows: allophycocyanin trimer, green; phycocyanin, pink, sky blue, and yellow. Adapted from Zheng et al. (2021).

1.1.3 Ecology

Cyanobacteria are one of the most common microorganisms worldwide (Garcia-Pichel et al. 2003). Ecologically dispersed, cyanobacteria are present in a wide range of ecosystems, either freshwater, marine or thermal (Cordeiro et al. 2020a), proliferating in some extreme conditions of salinity (Oren 2015), temperature (Castenholz 1981) and pH (Steinberg et al. 1998, Zepernick et al. 2021). In some of these habitats, cyanobacteria can be the dominant organism or the only colonizer (Komárek & Johansen 2015).

Cyanobacteria can be found in almost all freshwater aquatic habitats, from lakes to streams, rivers, and small ponds, among others (Whitton & Potts 2012). Cyanobacteria are very well adapted to aquatic environments, where they can be found both in the plankton and in the benthos and are frequently responsible for observed blooms (Santos et al. 2005, Scott & Marcarelli 2012, Scholz et al. 2017). Cyanobacterial blooms can occur due to anthropogenic influences, where planktonic cyanobacteria commonly overgrow competitors (O'Neil et al. 2012). Cyanobacteria are common inhabitants of the periphyton, forming layers in several types of substrates (Scott & Marcarelli 2012). These layers, which can be up to several centimeters long and are common in stromatolites, can detach to form a suspension layer rich in cyanobacteria (Scott & Marcarelli 2012, Stal 2012). In terrestrial habitats, cyanobacteria grow

well due to the development of strategies to resist long periods of desiccation (Potts 1999). These adaptations enabled cyanobacteria to grow in several terrestrial habitats, such as soils, caves (Zammit 2018, Gaysina et al. 2022, Joseph & Ray 2024), deserts (Mühlsteinová et al. 2014, Jung et al. 2021) and polar habitats (Taton et al. 2011, Komárek et al. 2015).

Cyanobacteria proliferate in marine and halophilic habitats, in both plankton and benthos (Sánchez-Baracaldo 2015), tolerating high salinity levels near saturation (Oren 2015). Brackish water, characterized by salinity levels between freshwater and seawater, often experiences salinity stratification and seasonal fluctuations (den Hartog 1967). These dynamic environments provide favorable conditions for cyanobacteria, where they can show great taxonomic diversity. Under certain conditions, some cyanobacteria can produce massive blooms in these ecosystems (Lopes & Vasconcelos 2011).

Thermal habitats harbor a great diversity of microorganisms, including cyanobacteria, which can sustain temperatures above 45 °C and grow successfully up to 73 °C (Castenholz 1981). Despite the several studies that have been carried out in thermal habitats, there is still a great diversity of cyanobacteria to be described (Luz 2018, Cordeiro et al. 2020b, Brenes-Guillén et al. 2021). Geographical distance from the mainland and the age of the island seems to influence the diversity of thermal cyanobacteria, such as in the case of the Azores (Castenholz 1978).

1.2 Cyanobacterial taxonomy

1.2.1 Cyanobacterial species concept

One of the biggest challenges for taxonomists is to achieve a consensus on what can be the definition of species (Dvořák et al. 2015). This has been a subject of great debate among cyanobacteria taxonomists, with several theories proposed over the years. Geitler (1932) used a wide species concept, assuming that cyanobacteria are ubiquitous and cosmopolitan, while others suggested more complex theories like the ecotypic species concept, where ecology drives taxa selection and should be taxonomically considered (Cohan 2001). This has been advocated and widely used in European taxonomical keys (Komárek & Anagnostidis 2000). However, the general use of European epithets has been considered incorrect when studying different biotopes and geographical positions (Johansen & Casamatta 2005). Currently, the most widely accepted species concept in cyanobacteria was proposed by Johansen & Casamatta (2005). This concept brings together morphological, by light and transmission electron microscopy observations (TEM), ecological, and genetic information, where a species is a monophyletic

group of strains that should be distinguished both in culture and in natural populations (Johansen & Casamatta 2005). This concept has been used in the reorganization of the cyanobacteria classification system proposed by Komárek et al. (2014) and more recently by Strunecký et al. (2023).

1.2.2 Taxonomic approaches

The taxonomy of cyanobacteria has changed dramatically over the years (Hoffmann et al. 2005, Komárek et al. 2014, Strunecký et al. 2023), with many novel taxa described in recent years (Kaštovský 2024). Traditionally, this was based mainly on morphological characteristics. However, many studies have long supported the need to use different approaches for taxonomy elucidation, namely through the combination of morphology, ecology, phylogeny, and, more recently, full genomic analysis (Johansen & Casamatta 2005, Johansen et al. 2011, Komárek et al. 2014, Dvořák et al. 2015, 2023). Known as the polyphasic approach, this methodology has revolutionized cyanobacterial taxonomy, relying deeply on phylogenetic analysis and the formation of stable monophyletic clades (Johansen & Casamatta 2005). With the inclusion of genetic markers, 16S rRNA and the 16S-23S rRNA internal transcriber spacer (16S-23S rRNA ITS), several genera and species lacking apomorphic features have been described in well-defined phylogenetic clades (Strunecký et al. 2011, 2014, Engene et al. 2012, Raabová et al. 2019).

Genome characteristics have long been used in the Bacterial System for species delimitation, adopting a 70% DNA-DNA hybridization threshold (Wayne et al. 1987). Although there isn't a clear reason for the selection of the 70% DNA-DNA hybridization threshold value, it was used in several papers to support the creation of new taxa. Several studies have attempted to define a threshold value for the 16S rRNA gene that correlates with the 70% % DNA-DNA. This resulted in the initial p-distance value of 97.5% to the more currently used 98.7% threshold of the 16S rRNA gene for species delimitation (Stackebrandt & Ebers 2006, Kim et al. 2014). In cyanobacteria, genomic studies have used guanine-cytosine percentage (GC%) as support criteria for taxa delimitation (Rippka & Cohen-Bazire 1983), yet this practice is not widely used or recognized.

Despite the general use of bacterial 16S rRNA threshold values in the taxonomy of cyanobacteria, deep taxonomical studies comparing genome and 16S rRNA threshold values for taxa delimitation have never been performed in cyanobacteria. This has led to differently applied values, with considerably higher 16S rRNA p-distance values applied in Nostocales (Kaštovský et al. 2014) and exceptionally in specific non-Nostocales genera like *Pegethrix* Mai, J.R.Johansen & Bohunická (Mai et al. 2018). The different values used for the 16S rRNA gene are

supported using the 16S-23S rRNA ITS region (as a complementary molecular marker), widely used in taxonomic studies of cyanobacteria (Erwin & Thacker 2008, Osorio-Santos et al. 2014, Mai et al. 2018, Pietrasiak et al. 2019, Jung et al. 2020), increasing the genetic support for taxa delimitation (Johansen & Casamatta 2005).

1.2.3 Current taxonomy system

Genomes are powerful sources of information that allow deeper taxonomical studies based on population genomics (Dvořák et al., 2023), integrating all available molecular data for the taxonomy of cyanobacteria. The most recent cyanobacteria reclassification was performed by Strunecký et al. (2023) using a multilocus DNA sequence analysis (MLSA) approach. Considering the available genomic data, the authors were able to perform a phylogenetic analysis, reclassifying cyanobacteria in a total of 20 orders and many new families (Strunecký et al. 2023). This work recognizes a much more complex system than the one proposed by Komárek et al. (2014), that was based on a combination of molecular markers with strong morphological support for a taxonomical organization. Strunecký et al. (2023) didn't disregard the morphological characteristics of the known described cyanobacteria (Figure 4 and Figure 5), but strongly support its results in the phylogenetic results using genomic data and the 16S rRNA gene. This approach supported the separation of similar morphological taxa in different orders such as the case of Oculatellales, Nodosilineales, Leptolyngbyales and Desertifilales.

1.3 Cyanobacteria natural products

Secondary metabolites are compounds not strictly required for cell division or metabolism but can be biologically active and promote an ecological advantage (Carmichael 1992). In cyanobacteria, these natural products have been the focus of several studies, mainly because an important part of them are known toxic compounds (Carmichael 1992, Merel et al. 2013). On the other hand, several have demonstrated a strong potential for biotechnological and/or pharmaceutical applications, with some even reaching commercialization (Baunach et al. 2024, Kallifidas et al. 2024). At least 2071 secondary metabolites from cyanobacteria have been reported (Baunach et al. 2024), but this number is growing. Chemical information on these compounds is deposited in important repositories such as CyanoMetDB (Jones et al., 2021) and the Natural Product Atlas database (van Santen et al., 2022) and is available to the scientific community and the general public.

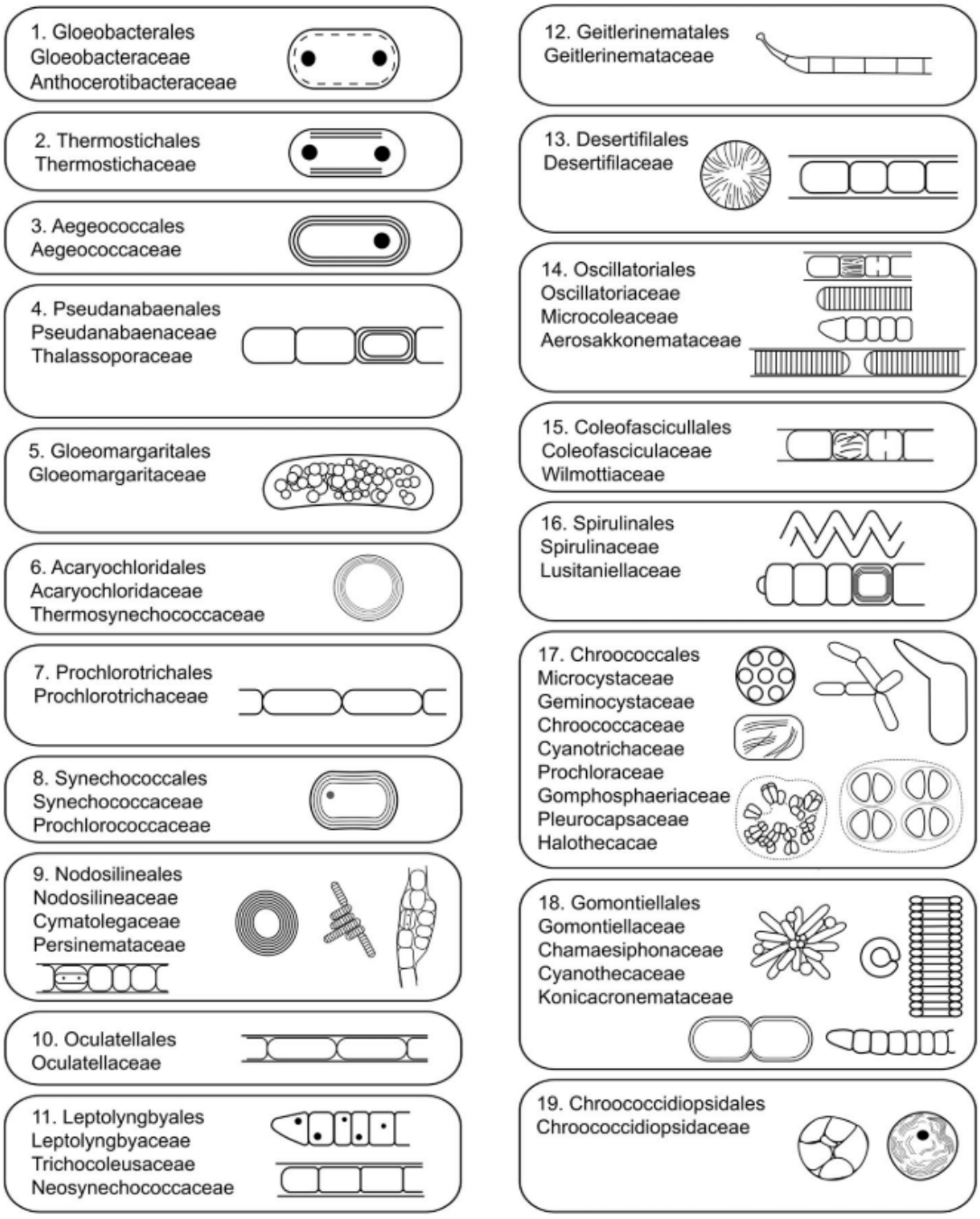

Figure 4. Currently accept orders in the cyanobacteria taxonomic system (except Nostocales). Drawings represent important diacritical characters, including cell shape, distribution and arrangement of thylakoids, presence of sheath and reproduction strategy. Adapted from Strunecký et al. (2023).

Several synthetic pathways allow cyanobacteria to produce a wide array of secondary metabolites (Baunach et al., 2024) classified into several classes of compounds, such as peptides, glycolipids, fatty acids, and polysaccharides, among many others (Demay et al. 2019). Cyanobacterial bioactive natural products are formed through many bioactive synthetic

pathways (Figure 6), such as non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), and ribosomally produced and post-translationally modified peptides (RiPP) (Baunach et al., 2024). The NRPS pathway is a modular enzymatic sequential assembly of amino acids to construct a target molecule (Miller & Gulick, 2016), while the PKS are biosynthesized from acyl CoA precursors by polyketide synthases. PKS are divided into three categories according to their structure: PKS type I, PKS type II, and PKS type III (Shen, 2003). RiPPs are ribosomally synthesized precursors typically consisting of a leader and a core peptide that are post-translationally modified by maturase enzymes and proteolytically released to form the final natural product (Richter & Piel, 2024). Bioactive natural compounds from cyanobacteria are not exclusively bound to NRPS, PKS or RiPP, as cyanobacteria can also produce retinoids, alkaloids, lactones, and phospholipids, among many others (Elersek et al., 2017, Jones et al., 2021).

Several cyanobacterial secondary metabolites have been shown to be bioactive and of interest, such as dolastatin 10, used as an anti-cancer drug (Kallifidas et al. 2024), or cryptophycins, a highly cytotoxic group of metabolites (Borbély et al. 2019). These metabolites or their analogues have reached clinical trials, and some analogues of dolastatin are now in commercial use (Kallifidas et al. 2024). Many secondary metabolites of cyanobacteria are excreted, serving as allelochemicals (Berry et al. 2008, Leão et al. 2009, 2010). Some compounds exudates by cyanobacteria are bioactive, including the portoamides that are cytotoxic (Leão et al. 2010, Ribeiro et al. 2017) and the scytonemin that shows strong anti-UV activity (Garcia-Pichel & Castenholz 1991). Many cyanobacterial compounds are produced by phylogenetically distant taxa (Cirés et al. 2017, Sen & Mallick 2022). On the other hand, the production of different compounds was even reported inside the same species or even at the strain level (Gkelis et al. 2019, Cordeiro et al. 2024).

1.4 Cyanobacteria compounds application on metabolic diseases

The metabolic syndrome is a cluster of conditions that constitutes a major risk factor for several health problems, including cardiovascular disease and type 2 diabetes mellitus (Neeland et al. 2024). Individuals with this syndrome can present several symptoms, such as hyperglycemia, insulin resistance, or obesity, among others (Heindel et al. 2017). This can lead to the development of several metabolic diseases that can have harmful effects on the organism and promote the development of other diseases (Heindel et al. 2017).

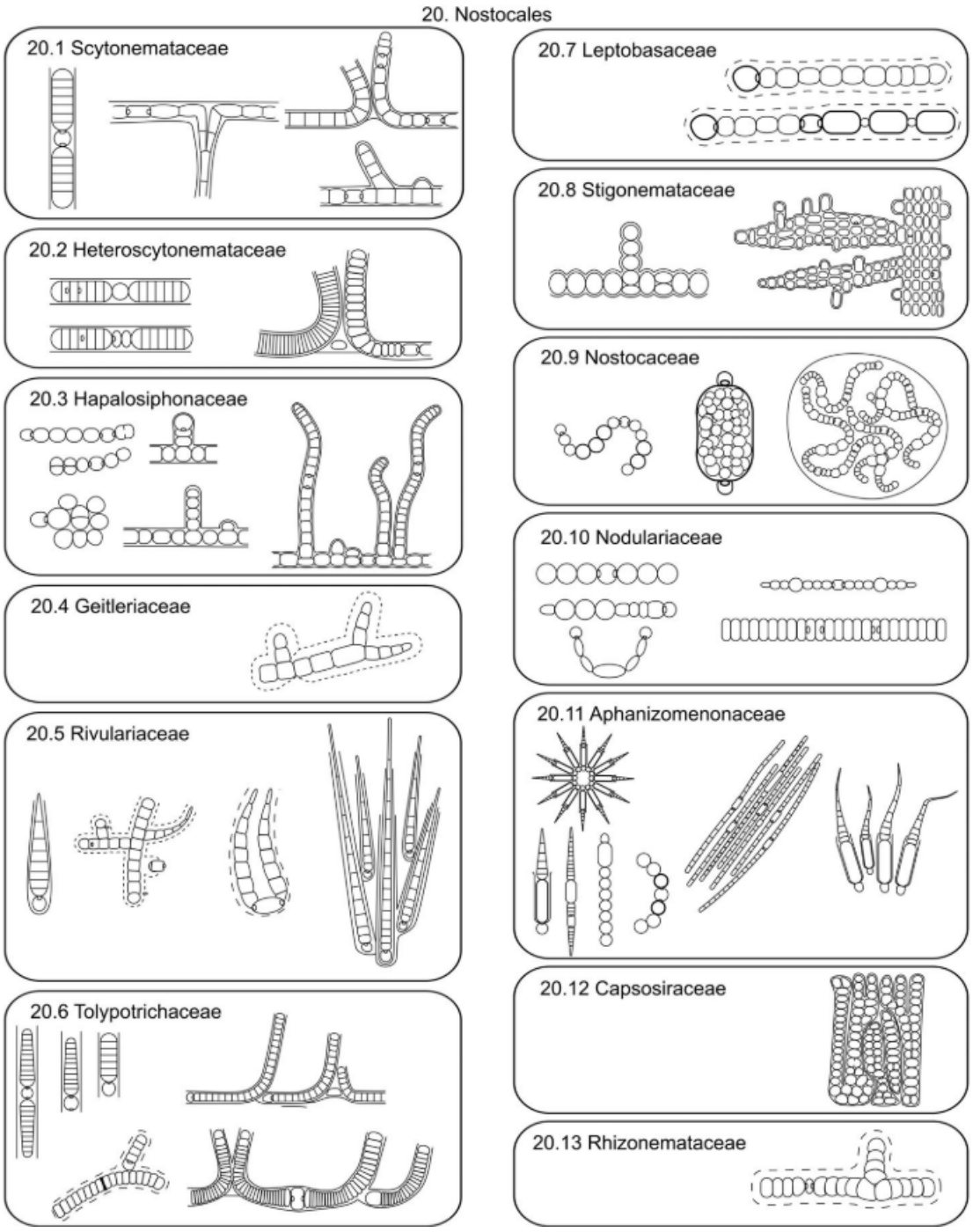

Figure 5. Schematic view of the Nostocales and important morphological features. Drawings represent important diacritical characters, including cell shape, akinets presence and position, presence and type of branching and colony formation. Adapted from Strunecký et al. (2023).

Obesity is an increasing problem in the modern world and is a risk factor to the development of other diseases (Harborg et al. 2024). It is associated with several other pathologies, such as nonalcoholic fatty liver disease (NAFLD) or cancer (Genua & Cusi 2024). NAFLD is a pathological condition characterized by abnormal accumulation of fat in liver tissue, which can lead to the development of cirrhosis or hepatocellular carcinoma (Loomba et al.

2021). This complex group of diseases promotes an increased need for prevention and effective treatments of obesity and its comorbidities (Genua & Cusi 2024). Obesity can cause other diseases, such as cancer (Harborg et al. 2024), one of the common causes of mortality (Schwartz 2024). Cancer is characterized by alterations in cell division control, leading to tumor growth (Recillas-Targa 2022). Many novel drugs that are less invasive than chemotherapy have been developed, with several ways of action (Khader et al. 2022). From inhibitors that can prevent signalling and tumor growth, inhibit growth factors and immune system evasion, or by decreasing the hormone level required for cancer cell growth (Khader et al. 2022). Cyanobacteria have been described to produce cytotoxic compounds (Robles-Bañuelos et al. 2022), some of these have been deeply studied, such as apratoxin A (Luesch et al. 2001) and dolastatin, the latest with highly valuable analogs already commercially used as anticancer drugs (Kallifidas et al. 2024).

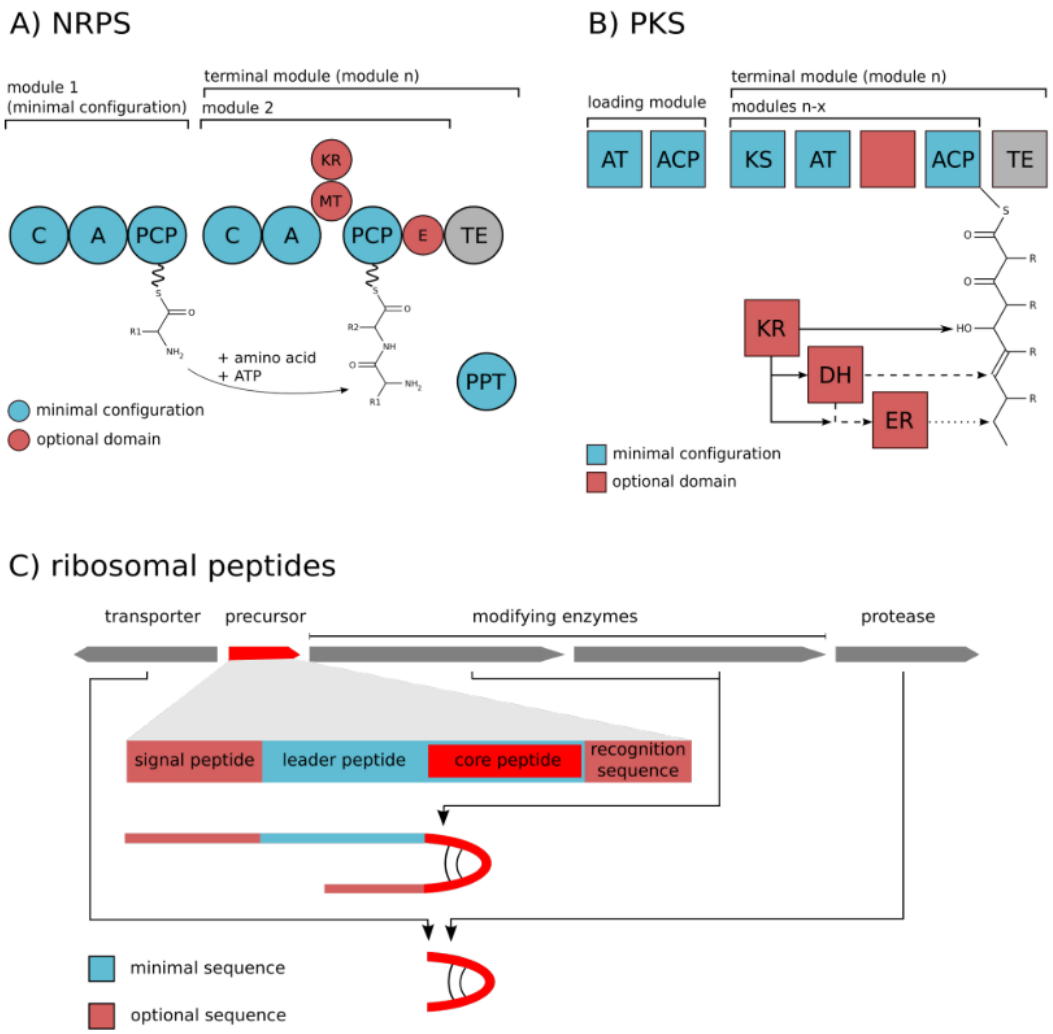

Figure 6. Schematic representation of enzymatic domains. A) nonribosomal peptide synthetases; B) polyketide synthases and C) the typical organization of a ribosomal biosynthetic gene cluster. Adapted from Kehr et al. (2011).

Various model organisms, including mouse and zebrafish, have been used in obesity treatment research to investigate drug efficacy and uncover the functional mechanisms and genes involved (Loos & Yeo 2022). Zebrafish has become an interesting model organism for lipid reduction studies due to its functional conservation in lipid metabolism (Zang et al. 2018).

1.5 OMICs in cyanobacteria

OMICs refers to the study of large datasets derived from biological systems (Dai & Shen, 2022). Different categories of OMICs are distinguished according to the origin of the datasets, such as metabolites (metabolomics), genetic information (genomic), and proteins (proteomic), among others (Dai & Shen 2022). This comprehensive enables a broader understanding by analyzing multiple systems simultaneously.

Metabolomics is the study of all metabolites originating from a biological system, using complex workflows (Figure 7), allowing the study of the endometabolome (intracellular metabolites) and the exometabolome (excreted metabolites produced by the organism). Metabolomics normally follows a mass spectrometry-based approach (Alseekh et al. 2021), which allows for mixture separation, depending on the target, by polarity and/or mass-to-charge (m/z) ratio (Alseekh et al. 2021). Tandem mass spectrometry (MS/MS) allows for a m/z characterization of both the precursor and product ions (Thomas et al. 2022). This advance in technology has been followed by a strong development of new software solutions that allows for the study of such complex mixtures, such as the Global Natural Product Social Molecular Networking (GNPS) platform (Wang et al. 2016). Several tools have been proposed to explore data from liquid chromatography-tandem mass spectrometry (LC-MS/MS), including GNPS Molecular Network (Wang et al. 2016) and SpecXplore (Mildau et al. 2024). The most used metabolomics tool is the GNPS Molecular Network, where mass features are grouped according to spectral similarity (fragmentation patterns), creating spectral feature groups with implied structural similarity (Wang et al. 2016). Posterior works have further developed this approach to integrate bioactivity results in identifying possible bioactive compounds. This method is called the feature-based molecular network (Nothias et al. 2018).

One of the key steps in metabolomics workflows is dereplication. The GNPS platform, through the Molecular Network, can match the mass features of LC-MS/MS runs with open access platforms with the mass features of isolated compounds (Wang et al. 2016). The MS2Query software performs similarly, where mass features such as m/z values and fragment ions are compared to public spectral libraries to identify the compound class (de Jonge et al. 2023). This approach allows the identification of compound analogs, streamlining and increasing

compound discovery works (Wang et al. 2016). However, since most described compounds lack MS2 data in the available spectral library, other software tools, such as molDiscovery, CFM-ID and MetFrag, play a crucial role (Ruttkies et al. 2016, Cao et al. 2021, Wang et al. 2021). These programs perform in-silico fragmentation from the known compound SMILES and compare the results with the obtained spectral data (Cao et al. 2021, Wang et al. 2021). Other approaches can also rely only on the precursor ion data, comparing the obtained m/z value with common public databases, such as LOTUS (Rutz et al. 2022), Natural Product Atlas (van Santen et al. 2022) and, specifically for cyanobacteria, the CyanoMetDB (Jones et al. 2021), within the used mass spectrometer data acquiring error.

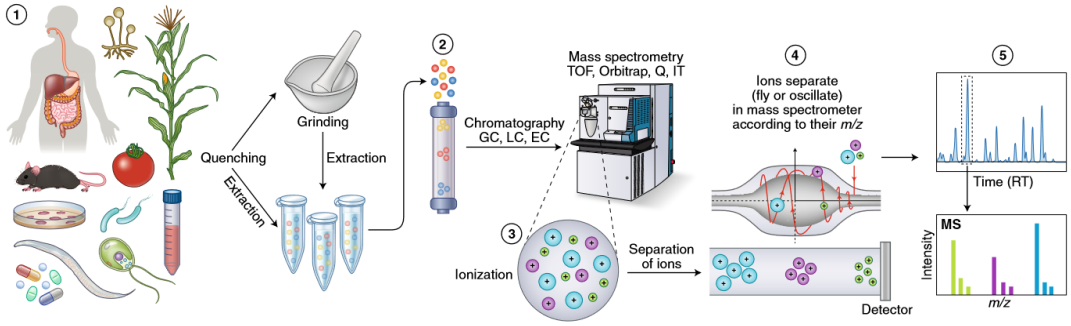

Figure 7. Metabolomics workflow. (1) sample preparation and extraction, (2) metabolite separation, (3) ionization of metabolites, (4) separation by a mass analyzer, (5) detection. TOF, time of flight; Q, quadrupole; IT, ion trap. Adapted from Alseekh et al. (2021).

Genomics uses all genes in a biological system to study the structure, function and expression of genes (Horgan & Kenny 2011). Bacterial whole-genome sequencing (WGS), through modern techniques such as Illumina, PacBio or Nanopore, allows for several studies, from biodiversity, conservation, and evolution to more applied studies in biosynthetic gene clusters and system function (Gavriilidou et al. 2022, Dvořák et al. 2023, Strunecký et al. 2023). The high value of bioactive secondary metabolites of cyanobacteria and its high number of biosynthetic gene clusters makes cyanobacteria a valuable source of information for genomic studies (Baunach et al. 2024, Cameron et al. 2024). Phylogenomics is based on a multilocus approach, giving robustness to the analysis when appropriate ortholog genes and evolutionary models are applied (Lozano-Fernandez 2022). This approach is best used with genomic data and can contribute to a better understanding of the taxonomic system of cyanobacteria (Dvořák et al. 2023, Strunecký et al. 2023).

1.6 Objectives and work overview

Given the uncertainties surrounding cyanobacterial diversity in the Azores, the available strains in the BACA culture collection were subjected to taxonomic study. Building on the taxonomic and diversity knowledge gained, the secondary metabolite diversity and bioactivity were also explored for potential pharmaceutical applications. To achieve the main objective, the following secondary objectives were proposed for the success of this project:

1) Characterize cyanobacteria strains in the BACA culture collection with a modern taxonomic approach and extensively describe cyanobacteria strains that prove to be new to science.
2) Increase the knowledge of the diversity of cyanobacteria from overlooked habitats in the Azores and worldwide through the isolation and maintenance of cyanobacteria strains, contributing to biodiversity conservation.
3) Evaluate the bioactivity and potential applications of secondary metabolites from cyanobacteria strains maintained in the BACA culture collection against metabolic diseases (obesity, NAFLD) and cancer.
4) Promote the value of native cyanobacterial strains and encourage their production for biotechnological applications in the Azores, thereby boosting the local economy.

Considering the work carried out, this thesis has been organized into nine chapters (Table 1). The first chapter is a general introduction to cyanobacteria (morphology, ecology, taxonomy), cyanobacteria natural products, the metabolic diseases that were the subject of the new bioactive compounds screening, and the OMIC tools used in this work.

Chapter II presents a taxonomical review of reported taxa in the Azores. This work compiles previously published work that is not easily accessible in online databases with modern literature, documenting all known observed taxa in the Azores archipelago up to December 31, 2020. The work comprises a distribution analysis of taxa by island and habitat, identifying less known habitats in the Azores archipelago.

In chapter III, *Azorothrix ramosa* gen. & sp. nov. Luz et al., a new Nostocales genus and species, is described. The article presents novel findings on the genetic diversity in the Tolypothrichaceae family, where an exceptionally high p-distance is being used for genera description. The description of the novel genus follows a polyphasic approach, comparing several locally isolated strains from the BACA culture collection using ecology, morphology, and genetic markers, such as 16S rRNA and 16S-23S rRNA ITS.

Table 1. Thesis scheme. Shades represent chapter contents: addressed objectives (grey), main topics (blue), main applied techniques (green) and published article titles (orange).

Chapter 1	Chapter 2	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7	Chapter 8	Chapter 9
	Taxonomy/Diversity					Bioactivity/Applications		
	Objectives 1) and 2)					Objectives 3) and 4)		
	Checklist	New Nostocales taxon	New filamentous aquatic taxa	New filamentous terrestrial taxa	New coccoid taxon	Metabolome, chemodiversity and bioactivity search	Exometabolome and bioactivity search	
	Literature review	Phylogeny	TEM / Cloning	TEM	Genomics	Metabolomics	Metabolomics	
General introduction	Distribution and diversity of cyanobacteria in the Azores Archipelago: An annotated checklist	Description of *Azorothrix ramosa* gen. et sp. nov. (Tolypothrichaceae, Cyanobacteria), a new Tolypothrichaceae from North Atlantic oceanic islands	Description of four new filamentous cyanobacterial taxa from freshwater habitats in the Azores Archipelago	New terrestrial cyanobacteria from the Azores Islands: description of *Venetifunis* gen. nov. and new species of *Albertania*, *Kovacikia* and *Pegethrix*	Description of *Pseudocalidococcus azoricus* gen. sp. nov. (Thermosynechococcaceae, Cyanobacteria), a rare but widely distributed coccoid Cyanobacteria	Metabolite profiling and bioactivity assessment of cyanobacteria from the Azores reveals unique producers of cytotoxic and lipid reducing compounds	Exometabolome from cyanobacteria and analysis of its lipid reducing and cytotoxic activities	General discussion and conclusion

Chapter IV introduces four novel taxa described from the Azores, namely two new genera (*Radiculonema aquaticum* gen. & sp. nov. Luz et al. and *Tumidithrix elongata* gen. & sp. nov. Luz et al.) together with two novel species, *Leptodesmis lacustris* sp. nov. Luz et al. and *Pycnacronema lacustrum* sp. nov. Luz et al. The description of these four novel taxa follows a characterization of aquatic strains from the BACA culture collection. A polyphasic approach was applied based on ecology, genetics (16S rRNA and 16S-23S rRNA ITS genes) and a combination of morphological techniques using bright field and TEM. This article reports a rare organization of thylakoids on the reference strain of *Radiculonema aquaticum*, BACA0731.

Chapter V presents the characterization results of four terrestrial strains deposited in the BACA culture collection. Based on this work, a new genus, *Venetifunis florensis* gen. & sp. nov. Luz et al., is described together with three novel species, *Albertania obscura* sp. nov., *Kovacikia atmophytica* and *Pegethrix atlantica* sp. nov. Luz et al. The description of these taxa followed a polyphasic approach using morphological (bright field and TEM), genetic (16S rRNA and 16S-23S rRNA ITS genes) and ecological information. Strains used in this work were isolated from several terrestrial habitats, including aerophytic and atmospheric habitats on caves, bridges, and other less common habitats present in the Azores.

In Chapter VI, a taxonomic revision of four coccoid strains present in the BACA culture collection was conducted using the polyphasic approach as previously described, together with a complementary analysis of the genome of BACA0444, the reference strain of the newly described genus *Pseudocalidococcus azoricus* gen. & sp. nov. Luz et al. The genomic approach

used to describe the new taxon improved the robustness of phylogenetic analysis and comparison with close phylogenetic taxa. Genomic comparison with close phylogenetic taxa on the same genus revealed an inconsistency between the currently used p-distance of 16S rRNA and the average amino acid identification used for species delimitation in cyanobacteria compared to the general bacterial values.

Chapter VII presents a bioactivity search on a diverse array of cyanobacterial taxa from the BACA culture collection. The bioactivity search was conducted in methanolic extracts together with a comprehensive metabolomic analysis. This consisted of a comparison of the chemodiversity of the strains using ESI-HR-LC-MS/MS data and correlation with bioactivity results from zebrafish larvae for lipid reduction and carcinoma cell lines for anticancer and antisteatosis activity, using a feature-based molecular network approach.

Chapter VIII presents the analysis of a normally ignored fraction of cyanobacteria production, the exometabolome. In this work, the bioactivity of exudate secondary metabolites was screened in combination with a metabolomic analysis. Using a feature-based molecular network approach, several mass peaks were identified as possibly responsible for the bioactivity found in the zebrafish larvae lipid reduction assay in Chapter VII.

Chapter IX integrates and discusses the main results of all chapters, relating previous knowledge with novel taxa. In addition, taxa diversity is related to the bioactivity found in several strains from the BACA culture collection. Autochthonous strains with high biotechnological or pharmaceutical potential are highlighted.

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Chapter II

Distribution and diversity of cyanobacteria in the Azores Archipelago: An annotated checklist

Luz, R., Cordeiro, R., Fonseca, A., Raposeiro, P. M., & Gonçalves, V. (2022). Distribution and diversity of cyanobacteria in the Azores Archipelago: An annotated checklist. Biodiversity Data Journal, 10, e87638. https://doi.org/10.3897/BDJ.10.e87638

Chapter II. Distribution and diversity of cyanobacteria in the Azores Archipelago: An annotated checklist

Background

Knowledge about cyanobacteria diversity in the Azores is spread over several publications, dating from 1874, with some of them not generally available to the scientific community due to their restricted access. The dispersion and sometimes inaccessibility of this information hinder a deeper analysis and a better understanding of the biodiversity of the Azores Islands and more general ecological processes in oceanic islands. Here we present the first checklist of cyanobacteria for the Azores Archipelago with updated taxonomy of all recorded taxa.

New information

This work provides a compiled and annotated checklist of all known cyanobacteria from the Azores Archipelago with morphological identification from preserved samples and cultures, based on published literature. All records of taxa known to occur in the Azores were taxonomically updated. The present checklist comprises 225 taxa distributed by six orders (Chroococcales, Nostocales, Oscillatoriales, Pleurocapsales, Spirulinales, Synechococcales). Our literature review reveals that the Azores Archipelago hosts a high diversity of cyanobacteria, despite several overlooked habitats that may present great potential regarding cyanobacteria diversity. Increasing efforts to study these neglected habitats could contribute to the knowledge of cyanobacteria taxonomy. This checklist provides the basis for future works on the taxonomy and taxa richness of cyanobacteria in the Azores and the Atlantic Islands, as also for understanding and monitoring nonindigenous and invasive species.

Keywords: Oceanic islands, biodiversity, Cyanophyceae, Macaronesia, Atlantic Ocean

2.1 Introduction

Cyanobacteria are gram-negative photosynthetic prokaryotes that developed around 3500 million years ago (Schirrmeister et al. 2015). As one of the most primitive organisms on earth (Mareš et al. 2013), they successfully occupy various habitats in terrestrial and aquatic ecosystems, both marine and freshwater (Whitton & Potts 2012). Cyanobacteria diversity amongst these systems is unbalanced, being larger in freshwater and terrestrial ecosystems (Komárek & Johansen 2015). They persist in almost all types of illuminated habitats, with optimum growing temperatures generally higher than microalgae, which enable them to

support a wide array of stress conditions, including extreme habitats (Komárek & Johansen 2015).

Freshwater cyanobacteria are commonly present in wetlands, lakes, rivers and streams, both in benthic (Scott & Marcarelli 2012) and planktonic (e.g. Stockner et al. 2000, Oliver et al. 2012) habitats. Benthic cyanobacteria are commonly found solitary or forming mats in the various stream and river substrates, such as rocks, sand, plants and many others (Casamatta & Hašler 2016). In shallow lakes and littoral zones of deep lakes, benthic species of cyanobacteria can also occur if enough light reaches the substrates (Scott & Marcarelli 2012). However, cyanobacteria are mostly known from the plankton of lentic waters, where they can grow in high abundance, usually known as blooms, especially in eutrophic lakes (Paerl et al. 2011, Carmichael & Boyer 2016). Cyanobacteria blooms negatively affect the ecosystems and services they provide (Carmichael & Boyer 2016) as most bloom-forming species produce toxins that can be accumulated at the water surface, causing unpleasant surface scums.

Extreme habitats, such as thermal springs, are successfully occupied by Cyanobacteria, where they are often the main and/or sole autotrophic organisms inhabiting these environments (Komárek & Johansen 2015). In marine systems, cyanobacteria are found in a wide array of habitats, including benthos, plankton, associated with other organisms, amongst others (Golubic et al. 2010, Konstantinou et al. 2018).

In the last ten years, cyanobacteria taxonomy has changed dramatically with the use of new techniques, mainly through 16S rRNA sequencing, contributing to a taxonomic reassessment of the group (e.g. Wacklin et al. 2009, Komárek et al. 2011, 2014, Zapomělová et al. 2011, Strunecký et al. 2013, 2017, Mai et al. 2018). In the Azores, a remote oceanic archipelago located in the middle of the North Atlantic Ocean, the first work to be published on cyanobacteria taxonomy came from the Challenger expedition that occurred from 1872 to 1876 and had a brief passage in São Miguel Island from 3 July to 9 July 1873 (Brock & Brock 1967). Some members of the Challenger expedition visited the Furnas Village and later Moseley (1874) and Archer (1874), who received samples from Moseley, published the first records. Later, Trelease (1897) and Bohlin (1901) contributed with several cyanobacteria records from several islands. In the 20 century, more biologists visited the Islands contributing considerably to the knowledge of the microalgae and cyanobacteria of the Azores. First by Krieger (1931), with a small contribution and after with the valuable works of Cedercreutz (1941), Bourrelly & Manguin (1946) and Johansson (1976), contributing with several detailed descriptions of the cyanobacteria flora in several islands of the Azores. The later works contributed with the highest number of known species for the Azores. Bourrelly and Manguin (1946) also describes a new form *Oscillatoria geitleri f. major* Bourrelly in Bourrelly & Manguin (1946), which is the first and

only known endemic cyanobacteria to the Azores. After 1980, works were mainly focused on planktonic freshwater species due to the rise of lake eutrophication signs. Important contributions to the known cyanobacteria flora have been provided after the implementation of the Water Framework Directive (WFD) in the Azores, with regular monitoring programmes since 1994 (Santos et al. 2005, 2012, Luz et al. 2020). The more recent works on cyanobacteria were based on cultured strains isolated from freshwater lakes (Cordeiro 2015), which provide the addition of new species. Several works performed on thermal, terrestrial, brackish and marine habitats, which were overlooked in previous studies, contributed to several new cyanobacteria species records (Luz 2018, Cordeiro et al. 2020b)

Despite the increased research efforts, especially in the last decade, the knowledge of the diversity and distribution of Cyanobacteria in the Azores Archipelago is not consistently organised and a local checklist has never been published. This study aims to present an updated checklist of cyanobacteria present in the Azores, based on a taxonomically updated list of previously reported species from preserved samples and based on cultured strains.

2.2 Materials and Methods

2.2.1 Study area

The Azores are an oceanic group of islands located in the middle of the North Atlantic Ocean, roughly 1500 km from Europe and 1900 km from America (Fig. 1). The Archipelago is made up of nine islands roughly aligned along 615 km in a WNW-ESE trend, that are divided into three groups according to their geographical position. Although they are in geographical proximity, the Islands present unique features differentiating themselves from each other (Table 1), with different amounts of annual rainfall (Secretaria Regional do Ambiente e do Mar 2011) and distinct geological settings (e.g. Moore 1990, Azevedo & Portugal Ferreira 2006, Cole et al. 2008).

The western group includes the Islands of Flores and Corvo, which are amongst the smallest islands of the Archipelago. Corvo and Flores are very rich in aquatic habitats despite their small size due to their higher annual precipitation (Secretaria Regional do Ambiente e do Mar 2011). The central group (Graciosa, Faial, Pico, São Jorge and Terceira Islands) comprise the youngest in the Archipelago (Ávila et al. 2016). The islands in the central group include a high diversity of inland aquatic habitats, including freshwater and saline lakes, streams and thermal waters (Morton et al. 1997, Porteiro 2000, Cruz & França 2006, Morton 2014). The eastern group, Santa Maria and São Miguel, includes the Archipelago's oldest (Santa Maria) and the

largest (São Miguel) islands. São Miguel is the island with most of the lakes and the larger area of water bodies (Porteiro 2000), whereas Santa Maria is the driest island of the Archipelago, with only 775.2 mm mean annual precipitation and no significant inland water habitats (Secretaria Regional do Ambiente e do Mar 2011).

Table 1. Island characterisation. [1] Ávila et al. (2016), [2] Secretaria Regional do Ambiente e do Mar (2011), [3] Porteiro (2000), [4] Morton (2014), [5] Cruz & França (2006), [6] Secretaria Regional da Agricultura e Ambiente – Direção Regional do Ambiente (2015), [7] Ramsar (2013), [8] Morton et al. (1997).

Group	Island	Age (Ma)[1]	Area (Km^2)[2]	Freshwater lakes (N)[3]	Permanent Streams (N)[6]	Coastal lakes (N)[4, 7, 8]	Thermal waters (N)[5]	Total lake area (km^2)[3, 4, 7, 8]	Annual precipitation (mm)[2]
Eastern	São Miguel	4.00	744.6	33	6	-	14	8.34	1027.1
	Santa Maria	6.30	96.9	-	1	-	-	-	775.2
Central	Terceira	0.40	400.3	18	-	3	-	0.36	1125.6
	Pico	0.27	444.8	28	-	-	-	0.16	956.3
	Faial	0.85	173.1	-	-	-	2	-	974.0
	São Jorge	1.32	243.7	-	-	2	-	0.86	1194.3
	Graciosa	0.70	60.7	-	-	-	1	-	918.4
Western	Flores	2.16	141.0	8	2	-	2	0.72	1716.1
	Corvo	1.50	17.1	1	-	-	-	0.24	1144.6

The Azores are particularly rich in freshwater systems, with 88 lakes (Porteiro 2000), nine permanent streams, five saline lakes and several thermal springs (Table 1). Lakes are located between 230 and 1.050 m altitude and, according to Gonçalves (2008), could be classified into two main lake types: shallow lakes, with a maximum depth below five metres; and (ii) deep lakes, with maximum depths, greater than five metres. The insular lotic systems are small, narrow, with steep watersheds and are fed by lakes or springs, most of them having torrential or seasonal flowing regimes (Raposeiro et al. 2013).

2.2.2 Checklist production

The checklist was based on all known literature mentioning cyanobacteria from the Azores with morphological identification, published until 2020. The nomenclature was revised according to Guiry & Guiry (2022). The complete taxonomic list (taxon data table and occurrence data table) is published in DwC (Suppl. material 1) in GBIF, the Global Biodiversity Information Facility (Luz et al. 2022). Taxa identified only to the family level or above were not included in the discussed taxonomic list.

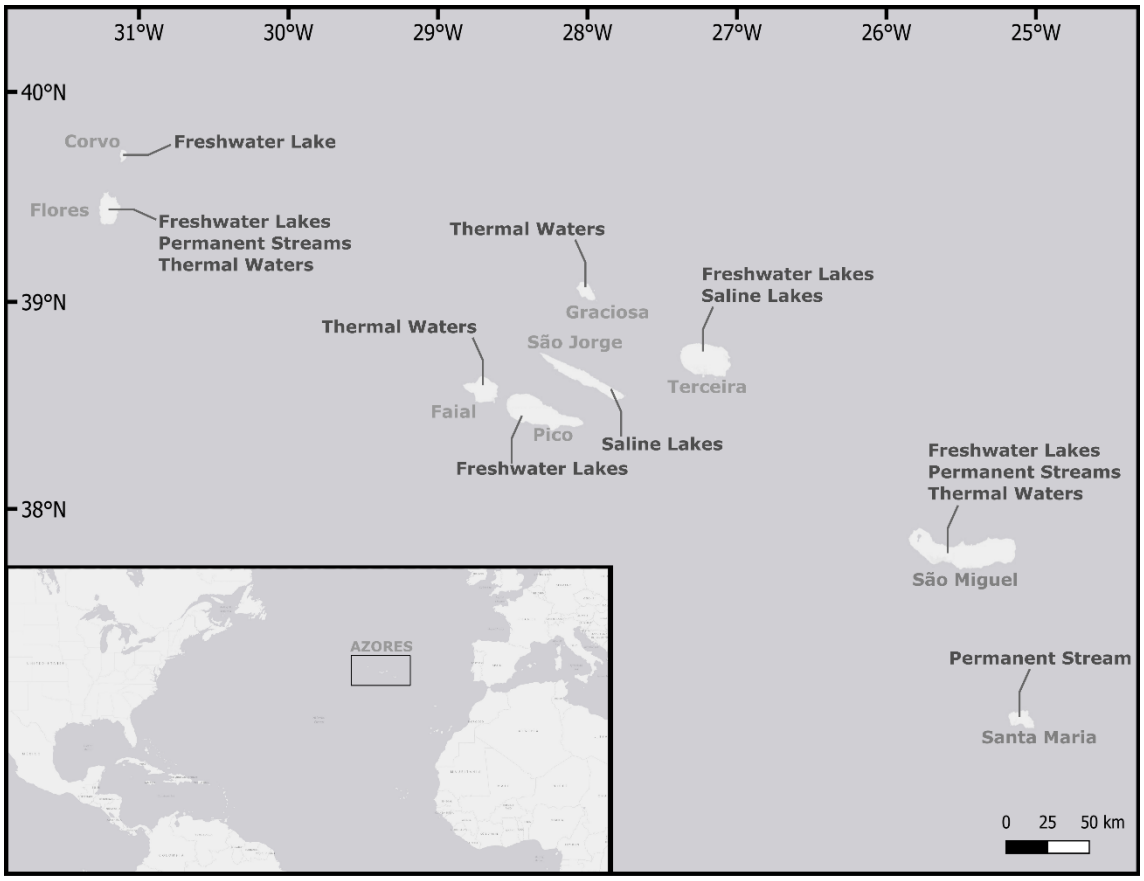

Figure 1. Azores Archipelago location with an indication of the most represented aquatic habitats on each island.

2.2.3 Data resources

Cyanobacteria occurrence in the Azores

Data set name: Cyanobacteria Checklist of the Azores Archipelago, Portugal – Occurrence data table

Data format: Darwin Core

Description: Cyanobacteria occurrence records in the Azores Archipelago, dating from 1874 to 2020, with 2838 records (Luz et al. 2022). Used Darwin Core terms are described in Table 2.

Cyanobacteria checklist from the occurrence

Data set name: Cyanobacteria Checklist of the Azores Archipelago, Portugal - Taxon data table

Data format: Darwin Core

Description: Cyanobacteria taxa recorded in the Azores Archipelago, based on the occurrence data table, with a total of 229 taxa (Luz et al. 2022). Used Darwin Core terms are described in Table 3.

Table 2. Darwin Core terms used in the occurrence data table.

Column label	Column description
id	Identifier.
type	The nature of the resource.
collectionCode	Acronym identifying the collection from which the record was derived.
basisOfRecord	Specific nature of the data record.
occurrenceID	Occurrence identifier.
catalogNumber	Identifier for the record within the collection.
associatedReferences	Literature associated with the occurrence.
eventDate	Date-time or interval during which the event was recorded.
continent	Name of the continent in which the occurrence location occurs.
waterBody	Name of the water body in which the occurrence location occurs.
islandGroup	Name of the island group in which the occurrence location occurs.
island	Name of the island on which the occurrence location occurs.
country	Name of the country in which the occurrence location occurs.
countryCode	Standard code for the country in which the occurrence location occurs.
municipality	Name of the municipality in which the occurrence location occurs.
locality	Name of the locality in which the occurrence location occurs.
decimalLatitude	Geographic latitude in which the occurrence location occurs.
decimalLongitude	Geographic longitude in which the occurrence location occurs.
geodeticDatum	Geodetic datum upon which the geographic coordinates given are based.
taxonID	Taxon identifier.
scientificName	The full scientific name including author.
acceptedNameUsage	The full scientific name including author currently accepted.
kingdom	Kingdom name in which the taxon is classified.
phylum	Phylum name in which the taxon is classified.
class	Class name in which the taxon is classified.
taxonRank	Lowest taxonomic rank of the taxon.

Table 3. Darwin Core terms used in the taxon data table.

Column label	Column description
id	Identifier.
taxonID	Taxon identifier.
scientificName	The full scientific name including author.
kingdom	Kingdom name in which the taxon is classified.
phylum	Phylum name in which the taxon is classified.
class	Class name in which the taxon is classified.
order	Order name in which the taxon is classified
family	Family name in which the taxon is classified.
genus	Genus name in which the taxon is classified.
specificEpithet	Species epithet name in which the taxon is classified.
infraspecificEpithet	Infraspecific epithet name in which the taxon is classified.
taxonRank	Lowest taxonomic rank of the taxon.
scientificNameAuthorship	Authorship information for the scientific name.

2.3 Cyanobacteria checklist from Azores islands

Genus *Anabaena* Bory ex Bornet & Flahault, 1886

Distribution: Corvo (INOVA 1996), Flores (Bourrelly & Manguin 1946), Pico (Luz et al. 2020), São Miguel (Bohlin 1901), Terceira (Luz et al. 2020)

Notes: Freshwater (lake), thermal (pool)

***Anabaena aspera* Frémy, 1930**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

***Anabaena augstumalis* Schmidle, 1900**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

***Anabaena cylindrica* Lemmermann, 1896**

Distribution: São Miguel (Oliveira 1989)

Notes: Freshwater (lake)

***Anabaena inaequalis* Bornet & Flahault, 1886**

Distribution: Flores (Luz et al. 2020), Pico (Luz et al. 2020), São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

***Anabaena torulosa* Lagerheim ex Bornet & Flahault, 1886**

Distribution: Corvo (Trelease 1897)

Notes: Freshwater

Genus *Anabaenopsis* V.V.Miller, 1923

Distribution: Corvo (Cordeiro et al. 2020b), Flores (Luz et al. 2020)

Notes: Freshwater (lake)

***Anabaenopsis circularis* (G.S.West) Woloszynska & V.Miller in V.Miller, 1923**

Distribution: Flores (Gonçalves 2008)

Notes: Freshwater (lake)

***Anagnostidinema amphibium* (C.Agardh ex Gomont) Strunecký, Bohunická, J.R.Johansen & J.Komárek, 2017**

Distribution: Flores (Bourrelly & Manguin 1946), São Miguel (Cedercreutz 1941), Terceira (Cedercreutz 1941)

Notes: Brackish (lake), freshwater (lake)

***Anathece clathrata* (W.West & G.S.West) Komárek, Kastovsky & Jezberová, 2011**

Distribution: São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake)

***Anathece minutissima* (West) Komárek, Kastovsky & Jezberová, 2011**

Distribution: São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

Genus *Aphanizomenon* A.Morren ex É.Bornet & C.Flahault, 1886

Distribution: São Miguel (Santos et al. 2001)

Notes: Freshwater (lake)

***Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault, 1886**

Distribution: Corvo (INOVA 1996), Flores (INOVA 1996), Graciosa (Azevedo et al. 2005), Pico (INOVA 1996), São Miguel (Krieger 1931)

Notes: Freshwater (lake)

***Aphanizomenon gracile* Lemmermann, 1907**

Distribution: Flores (Luz et al. 2020), Pico (Luz et al. 2020), São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

***Aphanizomenon manguinii* Bourrelly in Bourrelly & Manguin, 1952**

Distribution: Pico (Luz 2018)

Notes: Freshwater (lake)

Genus *Aphanocapsa* Nägeli, 1849

Distribution: São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake)

***Aphanocapsa delicatissima* West & G.S.West, 1912**

Distribution: Pico (Santos & Santana 2009a), São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake)

***Aphanocapsa elachista* West & G.S.West, 1894**

Distribution: São Jorge (Cedercreutz 1941), São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake), terrestrial

***Aphanocapsa grevillei* (Berkeley) Rabenhorst, 1865**

Distribution: São Jorge (Johansson 1977)

Notes: Freshwater

***Aphanocapsa incerta* (Lemmermann) G.Cronberg & Komárek, 1994**

Distribution: São Miguel (Santos & Santana 2009a)

Notes: Freshwater (lake)

Genus *Aphanothece* Nägeli, 1849

Distribution: Flores (Luz et al. 2020), São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

***Aphanothece castagnei* (Kützing) Rabenhorst, 1865**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Terrestrial

***Aphanothece microscopica* Nägeli, 1849**

Distribution: São Miguel (Bohlin 1901)

Notes: Freshwater (lake), terrestrial

***Aphanothece naegelii* Wartmann in Rabenhorst, 1865**

Distribution: São Miguel (Bohlin 1901)

Notes: Freshwater

***Aphanothece nidulans* P.Richter, 1884**

Distribution: São Miguel (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

***Aphanothece pallida* (Kützing) Rabenhorst, 1863**

Distribution: São Miguel (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

***Aphanothece saxicola* Nägeli, 1849**

Distribution: São Miguel (Bohlin 1901)

Notes: Terrestrial

***Aphanothece stagnina* (Sprengel) A.Braun in Rabenhorst, 1863**

Distribution: Pico (Johansson 1977)

Notes: Freshwater

Genus *Arthrospira* Sitzenberger ex Gomont, 1892

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

Genus *Calothrix* C.Agardh ex Bornet & Flahault, 1886

Distribution: Flores (Cordeiro et al. 2020b), São Jorge (Luz 2018), Santa Maria (Cordeiro et al. 2020b), São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake, stream)

***Calothrix breviarticulata* West & G.S.West, 1897**

Distribution: Flores (Luz 2018)

Notes: Freshwater (lake)

***Calothrix castellii* Bornet & Flahault, 1886**

Distribution: Pico (Luz 2018), São Miguel (Luz 2018)

Notes: Freshwater (lake)

***Calothrix parietina* Thuret ex Bornet & Flahault, 1886**

Distribution: São Miguel (Bohlin 1901)

Notes: Terrestrial

***Chlorogloeopsis fritschii* (A.K.Mitra) A.K.Mitra & D.C.Pandey, 1967**

Distribution: São Miguel (Luz 2018)

Notes: Thermal (stream)

Genus *Chroococcus* Nägeli, 1849

Distribution: São Miguel (Moseley 1874)

Notes: Freshwater (lake), thermal (spring)

***Chroococcus dispersus* (Keissler) Lemmermann, 1904**

Distribution: Pico (Santos & Santana 2009a), São Miguel (Santos & Santana 2009b)

Notes: Freshwater (lake)

***Chroococcus membraninus* (Meneghini) Nägeli, 1849**

Distribution: São Miguel (Bohlin 1901)

Notes: Thermal (stream)

***Chroococcus minor* (Kützing) Nägeli, 1849**

Distribution: São Miguel (Archer 1874)

Notes: Freshwater (lake)

***Chroococcus minutus* (Kützing) Nägeli, 1849**

Distribution: Flores (Luz et al. 2020), Pico (Johansson 1977), São Miguel (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

***Chroococcus tenax* (Kirchner) Hieronymus, 1892**

Distribution: São Miguel (Oliveira 1989)

Notes: Freshwater (lake)

***Chroococcus turgidus* (Kützing) Nägeli, 1849**

Distribution: Corvo (Cedercreutz 1941), Faial (Johansson 1977), Flores (Cedercreutz 1941), Pico (Johansson 1977), São Jorge (Johansson 1977), São Miguel (Bohlin 1901), Terceira (Cedercreutz 1941)

Notes: Freshwater (lake), terrestrial

***Chroococcus turicensis* (Nägeli) Hansgirg, 1887**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Freshwater

***Chroococcus westii* J.B.Petersen, 1923**

Distribution: São Miguel (Oliveira 1989)

Notes: Terrestrial

Genus *Coelosphaerium* Nägeli, 1849

Distribution: São Miguel (Santos et al. 2001)

Notes: Freshwater (lake)

***Coelosphaerium kuetzingianum* Nägeli, 1849**

Distribution: São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake)

Genus *Coleospermum* Kirchner ex Frank, 1886

Distribution: Flores (Luz 2018), Pico (Luz 2018), São Miguel (Luz 2018)

Notes: Freshwater (lake), thermal (pool, spring)

***Coleospermum goeppertianum* Kirchner ex Frank, 1886**

Distribution: São Miguel (Krieger 1931)

Notes: Freshwater (lake)

***Cyanobacterium synechococcoides* Komárek 1999**

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

Genus *Cyanobium* R.Rippka & G.Cohen-Bazire, 1983

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

***Cyanobium plancticum* (G.Drews, H.Prauser & D.Uhlmann) Komárek, J.Kopecký & Cepák, 1999**

Distribution: São Miguel (Xavier et al. 2018)

Notes: Freshwater (lake)

Genus *Cyanosaccus* K.J.Lukas & S.Golubic, 1981

Distribution: Faial ((Wisshak et al. 2011)

Notes: Marine (intertidal)

Genus *Cylindrospermum* Kützing ex Bornet & Flahault, 1886

Distribution: Pico (Cedercreutz 1941), São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake, stream)

***Cylindrospermum licheniforme* Kützing ex Bornet & Flahault, 1886**

Distribution: São Miguel (Trelease 1897)

Notes: Freshwater

***Cylindrospermum majus* Kützing ex Bornet & Flahault, 1886**

Distribution: Faial (Johansson 1977), Flores (Bourrelly & Manguin 1946), São Jorge (Johansson 1977), São Miguel (Trelease 1897)

Notes: Freshwater (lake)

***Dichothrix baueriana* Bornet & Flahault, 1886**

Distribution: Corvo (Trelease 1897)

Notes: Freshwater

***Dichothrix orsiniana var. africana* Frémy, 1924**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

Genus *Dolichospermum* (Ralfs ex Bornet & Flahault) P.Wacklin, L.Hoffmann & J.Komárek, 2009

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

***Dolichospermum affine* (Lemmermann) Wacklin, L.Hoffmann & Komárek, 2009**

Distribution: Pico (Santos & Santana 2009a), São Miguel (Oliveira 1989)

Notes: Freshwater (lake)

***Dolichospermum circinale* (Rabenhorst ex Bornet & Flahault) Wacklin, L.Hoffmann & Komárek, 2009**

Distribution: São Miguel (Cordeiro 2015)

Notes: Freshwater (lake)

***Dolichospermum delicatulum* (Lemmermann) Wacklin, L.Hoffmann & Komárek, 2009**

Distribution: Corvo (Luz et al. 2020), Flores (Luz et al. 2020), Pico (Luz et al. 2020), São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

***Dolichospermum flos-aquae* (Brébisson ex Bornet & Flahault) Wacklin, L.Hoffmann & Komárek, 2009**

Distribution: São Miguel (INOVA 1996)

Notes: Freshwater (lake)

***Dolichospermum planctonicum* (Brunnthaler) Wacklin, L.Hoffmann & Komárek, 2009**

Distribution: Pico (Luz et al. 2020), São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

***Dolichospermum scheremetieviae* (Elenkin) Wacklin, L.Hoffmann & Komárek, 2009**

Distribution: Corvo (Luz et al. 2020), Flores (Luz et al. 2020), Pico (Santos & Santana 2009a), São Miguel (Oliveira 1989)

Notes: Freshwater (lake)

***Dolichospermum sigmoideum* (Nygaard) Wacklin, L.Hoffmann & Komárek, 2009**

Distribution: Pico (Santos & Santana 2009a), São Miguel (Cordeiro 2015)

Notes: Freshwater (lake)

***Dolichospermum solitarium* (Klebahn) Wacklin, L.Hoffmann & Komárek, 2009**

Distribution: Flores (Luz et al. 2020), Pico (Luz et al. 2020), São Miguel (Bourrelly & Manguin 1946), Terceira (Luz et al. 2020)

Notes: Freshwater (lake)

***Dolichospermum spiroides* (Klebhan) Wacklin, L.Hoffmann & Komárek, 2009**

Distribution: São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

***Eucapsis alpina* F.E.Clements & H.L.Schantz, 1909**

Distribution: Corvo (Luz et al. 2020), Flores (Luz et al. 2020), Pico (Luz et al. 2020), São Miguel (Luz et al. 2020), Terceira (Luz et al. 2020)

Notes: Freshwater (lake)

***Eucapsis minuta* F.E.Fritsch, 1912**

Distribution: São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

Genus *Fischerella* (Bornet & Flahault) Gomont, 1895

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Thermal (pool)

Genus *Fortiea* De Toni, 1936

Distribution: Pico (Luz 2018)

Notes: Freshwater (lake)

***Fortiea striatula* (F.C.Hy) De Toni, 1936**

Distribution: Pico (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

***Geitlerinema ionicum* (Skuja) Anagnostidis, 1989**

Distribution: Santa Maria (Bourrelly & Manguin 1946)

Notes: Terrestrial

***Geitlerinema splendidum* (Greville ex Gomont) Anagnostidis, 1989**

Distribution: Pico (Luz et al. 2020), São Miguel (Cedercreutz 1941)

Notes: Freshwater (lake)

Genus *Gloeocapsa* Kützing, 1843

Distribution: São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

***Gloeocapsa atrata* Kützing, 1843**

Distribution: São Miguel (Cedercreutz 1941), Terceira (Johansson 1977)

Notes: Freshwater, terrestrial

***Gloeocapsa caldariorum* Rabenhorst, 1865**

Distribution: Terceira (Johansson 1977)

Notes: Freshwater

***Gloeocapsa compacta* Kützing, 1847**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

***Gloeocapsa gelatinosa* Kützing, 1843**

Distribution: São Jorge (Johansson 1977), Terceira (Johansson 1977)

Notes: Freshwater

***Gloeocapsa quaternata* Kützing, 1846**

Distribution: São Miguel (Johansson 1977)

Notes: Freshwater

***Gloeocapsa rupestris* Kützing, 1847**

Distribution: São Jorge (Johansson 1977), Terceira (Johansson 1977)

Notes: Freshwater

***Gloeocapsa thermalis* Kützing, 1843**

Distribution: São Miguel (Johansson 1977)

Notes: Thermal

***Gloeocapsopsis dvorakii* (Novácek) Komárek & Anagnostidis ex Komárek 1993**

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Thermal (pool)

***Gloeocapsopsis magma* (Brébisson) Komárek & Anagnostidis ex Komárek, 1993**

Distribution: São Miguel (Bohlin 1901)

Notes: Terrestrial

***Gloeothece cystifera* (Hassall) Rabenhorst, 1865**

Distribution: São Miguel (Bohlin 1901)

Notes: Terrestrial

***Gloeothece rupestris* (Lyngbye) Bornet in Wittrock & Nordstedt, 1880**

Distribution: Flores (Bourrelly & Manguin 1946), São Miguel (Cedercreutz 1941)

Notes: Freshwater (lake)

***Gloeotrichia pisum* Thuret ex Bornet & Flahault, 1886**

Distribution: São Miguel (Bohlin 1901)

Notes: Terrestrial

***Goleter apudmare* Miscoe & J.R.Johansen, 2016**

Distribution: Flores (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

Genus *Gomphosphaeria* Kützing, 1836

Distribution: São Miguel (Santos et al. 2001)

Notes: Freshwater (lake)

***Hapalosiphon hibernicus* West & G.S.West, 1896**

Distribution: Corvo (Cedercreutz 1941), Flores (Cedercreutz 1941), São Miguel (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

***Hapalosiphon intricatus* West & G.S.West, 1894**

Distribution: São Jorge (Cedercreutz 1941), Terceira (Johansson 1977)

Notes: Freshwater (lake, stream)

***Hapalosiphon pumilus* Kirchner ex Bornet & Flahault, 1887**

Distribution: Flores (Bourrelly & Manguin 1946), Santa Maria (Luz 2018)

Notes: Freshwater (stream), terrestrial

Genus *Hapalosiphon* Nägeli ex É.Bornet & C.Flahault, 1886

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Terrestrial

***Heteroleibleinia kuetzingii* (Schmidle) Compère, 1985**

Distribution: São Jorge (Cedercreutz 1941)

Notes: Freshwater

***Homoeothrix africana* G.S.West, 1912**

Distribution: São Jorge (Johansson 1977)

Notes: Freshwater

***Hydrocoryne spongiosa* Schwabe ex Bornet & Flahault 1887**

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

Genus *Hyella* É.Bornet & C.Flahault, 1888

Distribution: Faial (Wisshak et al. 2011)

Notes: Marine (intertidal)

***Hyella caespitosa* Bornet & Flahault, 1888**

Distribution: Faial (Wisshak et al. 2011)

Notes: Marine (intertidal)

***Hyella gigas* Lukas & Golubic, 1983**

Distribution: Faial (Wisshak et al. 2011)

Notes: Marine (intertidal)

***Isocystis planctonica* Starmach 1962**

Distribution: Flores (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

Genus *Kamptonema* O.Strunecký, J.Komárek & J.Smarda, 2014

Distribution: Flores (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

***Kamptonema formosum* (Bory ex Gomont) Strunecký, Komárek & J.Smarda, 2014**

Distribution: Santa Maria (Trelease 1897), São Miguel (Bohlin 1901), Terceira (Cedercreutz 1941)

Notes: Freshwater, brackish (lake), thermal (stream)

***Kyrtuthrix dalmatica* Ercegovic, 1929**

Distribution: Faial (Wisshak et al. 2011)

Notes: Marine (intertidal)

Genus *Leptodesmis* Raabová, Kovacik & Strunecký, 2019

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

Genus *Leptolyngbya* Anagnostidis & Komárek, 1988

Distribution: São Jorge (Luz 2018), São Miguel (Luz 2018)

Notes: Freshwater (lake), marine (lake), thermal (pool, stream)

***Leptolyngbya gelatinosa* (Woronichin) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Luz 2018)

Notes: Thermal (stream)

***Leptolyngbya granulifera* (J.J.Copeland) Anagnostidis 1936**

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Thermal (pool, spring)

***Leptolyngbya laminosa* (Gomont ex Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Trelease 1897)

Notes: Thermal (spring)

***Leptolyngbya nostocorum* (Bornet ex Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Bohlin 1901)

Notes: Freshwater (lake)

***Leptolyngbya ochracea* (Thuret ex Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Bohlin 1901)

Notes: Thermal (pool)

***Leptolyngbya rivulariarum* (Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Bohlin 1901)

Notes: Freshwater (lake)

***Leptolyngbya subuliformis* (Gomont) Anagnostidis 2001**

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Thermal (spring)

***Leptolyngbya valderiana* (Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Bourrelly & Manguin 1946)

Notes: Terrestrial

Genus *Limnothrix* M.-E.Meffert, 1988

Distribution: Flores (Cordeiro et al. 2020b), São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

***Limnothrix planctonica* (Woloszynska) Meffert, 1988**

Distribution: São Miguel (Santos & Santana 2009b)

Notes: Freshwater (lake)

Genus *Lyngbya* C.Agardh ex Gomont, 1892

Distribution: São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake)

***Lyngbya lutea* Gomont ex Gomont, 1892**

Distribution: Terceira (Neto et al. 2009)

Notes: Marine

***Lyngbya martensiana* Meneghini ex Gomont, 1892**

Distribution: São Miguel (Bohlin 1901), Terceira (Johansson 1977)

Notes: Freshwater, thermal, terrestrial

Genus *Mastigocladus* Cohn ex Kirchner, 1898

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Thermal (spring, stream)

***Mastigocladus laminosus* Cohn ex Kirchner, 1898**

Distribution: São Miguel (Bohlin 1901)

Notes: Thermal (spring, pool, stream)

***Mastigocoleus testarum* Lagerheim ex Bornet & Flahault, 1886**

Distribution: Faial (Wisshak et al. 2011)

Notes: Marine (intertidal)

Genus *Merismopedia* Meyen, 1839

Distribution: São Miguel (Archer 1874), Pico (Luz et al. 2020)

Notes: Freshwater (lake)

***Merismopedia glauca* (Ehrenberg) Kützing, 1845**

Distribution: São Miguel (Cedercreutz 1941), Terceira (Cedercreutz 1941)

Notes: Freshwater (lake)

***Merismopedia tenuissima* Lemmermann, 1898**

Distribution: Graciosa (Azevedo et al. 2005), São Miguel (Oliveira 1989), Terceira (Luz et al. 2020)

Notes: Freshwater (lake)

***Microchaete bulbosa* J.Copeland, 1936**

Distribution: São Miguel (Luz 2018)

Notes: Thermal (spring)

***Microchaete tenera* Thuret ex Bornet & Flahault, 1886**

Distribution: Pico (Luz 2018), São Miguel (Bohlin 1901)

Notes: Freshwater (lake), terrestrial

***Microcoleus amoenus* (Gomont) Strunecky, Komárek & J.R.Johansen, 2013**

Distribution: Flores (Bourrelly & Manguin 1946), São Miguel (Cedercreutz 1941)

Notes: Freshwater (lake)

***Microcoleus autumnalis* (Gomont) Strunecky, Komárek & J.R.Johansen, 2013**

Distribution: Flores (Cedercreutz 1941), São Miguel (Cedercreutz 1941)

Notes: Freshwater (stream), terrestrial

***Microcoleus lyngbyaceus* Kützing ex Forti, 1907**

Distribution: Terceira (Neto et al. 2009)

Notes: Marine

Genus *Microcystis* Lemmermann, 1907

Distribution: São Miguel (INOVA 1996)

Notes: Freshwater (lake)

***Microcystis aeruginosa* (Kützing) Kützing, 1846**

Distribution: Corvo (Luz et al. 2020), Flores (INOVA 1996), São Miguel (Vasconcelos et al. 1994)

Notes: Freshwater (lake)

***Microcystis flos-aquae* (Wittrock) Kirchner, 1898**

Distribution: Flores (Luz et al. 2020), São Miguel (Oliveira 1989)

Notes: Freshwater (lake)

***Microcystis pulverea* (H.C.Wood) Forti, 1907**

Distribution: Pico (Santos & Santana 2009a), São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake)

***Microcystis robusta* (H.W.Clark) Nygaard, 1925**

Distribution: São Miguel (Santos & Santana 2009b), Terceira (Santos & Santana 2009a)

Notes: Freshwater (lake)

***Nodularia harveyana* Thuret ex Bornet & Flahault, 1886**

Distribution: Corvo (Trelease 1897), Terceira (Cedercreutz 1941)

Notes: Brackish (lake), marine

Genus *Nostoc* Vaucher ex Bornet & Flahault, 1886

Distribution: Corvo (Cedercreutz 1941), Flores (Cedercreutz 1941), Santa Maria (Bourrelly & Manguin 1946), São Miguel (Moseley 1874)

Notes: Freshwater (lake, stream), terrestrial

***Nostoc carneum* C.Agardh ex Bornet & Flahault, 1886**

Distribution: Faial (Johansson 1977)

Notes: Freshwater

***Nostoc commune* Vaucher ex Bornet & Flahault, 1886**

Distribution: Pico (Johansson 1977)

Notes: Freshwater (lake)

***Nostoc ellipsosporum* Rabenhorst ex Bornet & Flahault, 1886**

Distribution: Corvo (Trelease 1897), São Miguel (Bohlin 1901)

Notes: Freshwater

***Nostoc paludosum* Kützing ex Bornet & Flahault, 1886**

Distribution: Corvo (Cordeiro et al. 2020b), Santa Maria (Bourrelly & Manguin 1946), São Miguel (Bohlin 1901)

Notes: Freshwater (lake)

***Nostoc punctiforme* Hariot, 1891**

Distribution: São Miguel (Bohlin 1901)

Notes: Freshwater (lake), terrestrial

***Nostoc sphaericum* Vaucher ex Bornet & Flahault, 1886**

Distribution: Faial (Johansson 1977), Flores (Bourrelly & Manguin 1946), São Jorge (Johansson 1977), São Miguel (Bourrelly & Manguin 1946)

Notes: Freshwater (lake), terrestrial

***Nostoc sphaeroides* Kützing ex Bornet & Flahault, 1886**

Distribution: Faial (Johansson 1977), São Jorge (Johansson 1977)

Notes: Freshwater

***Nostoc verrucosum* Vaucher ex Bornet & Flahault, 1886**

Distribution: São Miguel (Cedercreutz 1941), Terceira (Trelease 1897)

Notes: Freshwater (stream)

***Nostochopsis lobatus* H.C.Wood ex Bornet & Flahault, 1886**

Distribution: São Miguel (Bohlin 1901)

Notes: Terrestrial

Genus *Oscillatoria* Vaucher ex Gomont, 1892

Distribution: Flores (Luz et al. 2020), Pico (INOVA 1996), São Miguel (Moseley 1874), Terceira (Neto et al. 2009)

Notes: Freshwater (lake), marine

***Oscillatoria geitleri f. major* Bourrelly in Bourrelly & Manguin, 1946**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Freshwater (stream)

***Oscillatoria planctonica* Woloszynska, 1912**

Distribution: São Miguel (Oliveira 1989)

Notes: Freshwater (lake)

***Oscillatoria princeps* Vaucher ex Gomont, 1892**

Distribution: Terceira (Neto et al. 2009)

Notes: Marine

***Oscillatoria sancta* Kützing ex Gomont, 1892**

Distribution: São Miguel (Bohlin 1901)

Notes: Terrestrial

***Oscillatoria tenuis* C.Agardh ex Gomont, 1892**

Distribution: Corvo (Cedercreutz 1941), Faial (Cedercreutz 1941), Flores (Cedercreutz 1941), Graciosa (Cedercreutz 1941), Pico (Luz et al. 2020), São Miguel (Bohlin 1901), Terceira (Luz et al. 2020)

Notes: Freshwater (lake, stream), terrestrial

Genus *Pegethrix* Mai, J.R.Johansen & Bohunická, 2018

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

***Petalonema velutinum* Migula, 1907**

Distribution: Flores (Cedercreutz 1941)

Notes: Freshwater

Genus *Phormidium* Kützing ex Gomont, 1892

Distribution: Corvo (Luz et al. 2020), Faial (Cedercreutz 1941), Pico (Luz et al. 2020), São Jorge (Luz 2018), São Miguel (Fish & Codd 1994)

Notes: Freshwater (lake), marine (lake), thermal

***Phormidium aerugineo-caeruleum* (Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Bohlin 1901)

Notes: Freshwater (lake)

***Phormidium allorgei* (Frémy) Anagnostidis & Komárek, 1988**

Distribution: Terceira (Johansson 1977)

Notes: Freshwater

***Phormidium breve* (Kützing ex Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Bohlin 1901)

Notes: Marine, thermal

***Phormidium durum* N.L.Gardner, 1927**

Distribution: São Jorge (Johansson 1977)

Notes: Freshwater

***Phormidium irriguum* (Kützing ex Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Jorge (Johansson 1977), Terceira (Bohlin 1901)

Notes: Freshwater (stream)

***Phormidium pachydermaticum* Frémy, 1930**

Distribution: São Jorge (Johansson 1977), Terceira (Johansson 1977)

Notes: Freshwater

***Phormidium retzii* Kützing ex Gomont, 1892**

Distribution: Faial (Johansson 1977), São Jorge (Johansson 1977), São Miguel (Cedercreutz 1941)

Notes: Freshwater

***Phormidium rotheanum* Itzigsohn in Rabenhorst, 1865**

Distribution: São Jorge (Johansson 1977)

Notes: Freshwater

***Phormidium terebriforme* (C.Agardh ex Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Bohlin 1901)

Notes: Thermal

Genus *Planktolyngbya* Anagnostidis & Komárek, 1988

Distribution: Flores (Luz et al. 2020), Pico (Luz et al. 2020), São Miguel (Cordeiro 2015)

Notes: Freshwater (lake)

***Planktolyngbya limnetica* (Lemmermann) Komárková-Legnerová & Cronberg, 1992**

Distribution: Pico (Luz et al. 2020), São Miguel (Cedercreutz 1941)

Notes: Freshwater (lake)

***Planktothrix agardhii* (Gomont) Anagnostidis & Komárek, 1988**

Distribution: São Miguel (Santos & Santana 2009b)

Notes: Freshwater (lake)

***Plectonema endolithicum* Ercegovic, 1932**

Distribution: Faial (Wisshak et al. 2011)

Notes: Marine (intertidal)

***Plectonema terebrans* Bornet & Flahault ex Gomont, 1892**

Distribution: Faial (Wisshak et al. 2011)

Notes: Marine (intertidal)

Genus *Pseudanabaena* Lauterborn, 1915

Distribution: Corvo (Luz et al. 2020), Flores (Luz et al. 2020), Pico (Luz et al. 2020), São Miguel (Santos et al. 2005)

Notes: Freshwater (lake)

***Pseudanabaena catenata* Lauterborn, 1915**

Distribution: São Miguel (Oliveira 1989)

Notes: Freshwater (lake)

***Pseudanabaena limnetica* (Lemmermann) Komárek, 1974**

Distribution: Corvo (Luz et al. 2020), Flores (Luz et al. 2020), Pico (Luz et al. 2020), São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

***Pseudanabaena minima* (G.S.An) Anagnostidis, 2001**

Distribution: Pico (Luz 2018)

Notes: Freshwater (lake)

***Pseudanabaena mucicola* (Naumann & Huber-Pestalozzi) Schwabe, 1964**

Distribution: São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake)

***Pseudophormidium pauciramosum* (Anissimova) Anagnostidis, 2001**

Distribution: Santa Maria (Luz 2018)

Notes: Brackish

***Raphidiopsis curvata* F.E.Fritsch & M.F.Rich, 1930**

Distribution: Corvo (INOVA 1996), Flores (INOVA 1996), São Miguel (INOVA 1996)

Notes: Freshwater (lake)

Genus *Rivularia* C.Agardh ex Bornet & Flahault, 1886

Distribution: São Miguel (Cordeiro et al. 2020b), Terceira (Neto et al. 2009)

Notes: Freshwater, marine

***Rivularia biasolettiana* Meneghini ex Bornet & Flahault 1886**

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

***Rivularia bullata* Berkeley ex Bornet & Flahault, 1886**

Distribution: São Miguel (Trelease 1897)

Notes: Freshwater

***Rivularia nitida* C.Agardh ex Bornet & Flahault, 1886**

Distribution: Flores (Trelease 1897)

Notes: Freshwater

Genus *Schizothrix* Kützing ex M.Gomont, 1892

Distribution: Terceira (Johansson 1977)

Notes: Freshwater

***Schizothrix cuspidata* (West & G.S.West) West & G.S.West, 1896**

Distribution: Faial (Bourrelly & Manguin 1946)

Notes: Terrestrial

***Schizothrix fuscescens* Kutzing ex Gomont, 1892**

Distribution: Terceira (Johansson 1977)

Notes: Freshwater

***Schizothrix lacustris* A.Braun ex Gomont, 1892**

Distribution: São Jorge (Johansson 1977)

Notes: Freshwater

***Schizothrix pallida* (Kützing ex Forti) Geitler, 1932**

Distribution: Terceira (Neto et al. 2009)

Notes: Marine

***Schizothrix symplocoides* (N.L.Gardner) Geitler, 1932**

Distribution: Terceira (Johansson 1977)

Notes: Freshwater

***Schizothrix telephoroides* Gomont, 1890**

Distribution: Faial (Johansson 1977)

Notes: Freshwater

***Schizothrix vaginata* Gomont, 1890**

Distribution: São Jorge (Johansson 1977)

Notes: Freshwater

Genus *Scytonema* C.Agardh ex É.Bornet & C.Flahault, 1886

Distribution: Flores (Cedercreutz 1941)

Notes: Terrestrial

***Scytonema amplum* West & G.S.West, 1895**

Distribution: São Miguel (Bourrelly & Manguin 1946)

Notes: Terrestrial

***Scytonema dilatatum* Bharadwaja, 1934**

Distribution: Terceira (Johansson 1977)

Notes: Freshwater

***Scytonema guyanense* Bornet & Flahault, 1888**

Distribution: Flores (Bourrelly & Manguin 1946), São Miguel (Bourrelly & Manguin 1946)

Notes: Terrestrial

***Scytonema hofmannii* C.Agardh ex Bornet & Flahault, 1886**

Distribution: São Jorge (Johansson 1977), São Miguel (Cedercreutz 1941)

Notes: Freshwater (lake)

***Scytonema javanicum* Bornet ex Bornet & Flahault, 1886**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

***Scytonema mirabile* Bornet, 1889**

Distribution: Pico (Cedercreutz 1941), São Jorge (Cedercreutz 1941), São Miguel (Cedercreutz 1941), Terceira (Cedercreutz 1941)

Notes: Freshwater (lake, stream), terrestrial

***Scytonema stuposum* Bornet ex Bornet & Flahault, 1887**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Freshwater (lake)

Genus *Scytonematopsis* E.I.Kiseleva, 1930

Distribution: Flores (Cordeiro et al. 2020b), Pico (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

Genus *Snowella* A.A.Elenkin, 1938

Distribution: São Miguel (Luz et al. 2020)

Notes: Freshwater (lake)

***Snowella lacustris* (Chodat) Komárek & Hindák, 1988**

Distribution: São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake)

Genus *Sphaerospermopsis* Zapomelová, Jezberová, Hrouzek, Hisem, Reháková & Komárková, 2010

Distribution: Pico (Luz 2018)

Notes: Freshwater (lake)

***Sphaerospermopsis aphanizomenoides* (Forti) Zapomelová, Jezberová, Hrouzek, Hisem, Reháková & Komárková, 2010**

Distribution: Pico (Santos & Santana 2009a)

Notes: Freshwater (lake)

***Spirulina subsalsa* Oersted ex Gomont, 1892**

Distribution: São Jorge (Luz 2018)

Notes: Marine (lake)

Genus *Stenomitos* Miscoe & J.R.Johansen, 2016

Distribution: Pico (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

***Stigonema hormoides* Bornet & Flahault, 1886**

Distribution: Flores (Bourrelly & Manguin 1946), São Jorge (Johansson 1977), São Miguel (Cedercreutz 1941), Terceira (Cedercreutz 1941)

Notes: Freshwater (lake), terrestrial

***Stigonema informe* Kützing ex Bornet & Flahault, 1886**

Distribution: São Jorge (Johansson 1977), Terceira (Johansson 1977)

Notes: Freshwater

***Stigonema mamillosum* C.Agardh ex Bornet & Flahault, 1886**

Distribution: São Jorge (Johansson 1977), São Miguel (Bourrelly & Manguin 1946)

Notes: Freshwater, terrestrial

***Stigonema minutum* Hassall ex Bornet & Flahault, 1886**

Distribution: Flores (Bourrelly & Manguin 1946), São Jorge (Johansson 1977), São Miguel (Bohlin 1901), Terceira (Johansson 1977)

Notes: Freshwater (lake)

***Stigonema multipartitum* N.L.Gardner, 1927**

Distribution: São Jorge (Johansson 1977)

Notes: Freshwater

***Stigonema ocellatum* Thuret ex Bornet & Flahault, 1886**

Distribution: Flores (Cedercreutz 1941), São Miguel (Cedercreutz 1941), Terceira (Johansson 1977)

Notes: Freshwater (lake)

***Stigonema panniforme* Bornet & Flahault, 1886**

Distribution: São Miguel (Bourrelly & Manguin 1946)

Notes: Freshwater (stream)

***Stigonema robustum* N.L.Gardner, 1927**

Distribution: São Jorge (Johansson 1977)

Notes: Freshwater

***Stigonema tomentosum* Hieronymus, 1895**

Distribution: São Jorge (Johansson 1977), São Miguel (Cedercreutz 1941)

Notes: Freshwater, terrestrial

***Symploca dubia* Gomont, 1892**

Distribution: São Miguel (Cedercreutz 1941)

Notes: Thermal (pool)

***Symploca thermalis* Gomont, 1892**

Distribution: São Miguel (Bohlin 1901)

Notes: Thermal

Genus *Synechococcus* Nägeli, 1849

Distribution: São Miguel (Luz et al. 2020), Terceira (Luz et al. 2020)

Notes: Freshwater (lake)

***Synechococcus nidulans* (Pringsheim) Komárek, 1970**

Distribution: São Miguel (Xavier et al. 2018)

Notes: Freshwater (lake)

Genus *Synechocystis* C.Sauvageau, 1892

Distribution: São Miguel (Luz et al. 2020), Pico (Luz et al. 2020)

Notes: Freshwater (lake)

***Tildeniella torsiva* Mai, J.R.Johansen & Pietrasiak, 2018**

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

Genus *Tolypothrix* Kützing ex É.Bornet & C.Flahault, 1886

Distribution: São Miguel (Archer 1874)

Notes: Freshwater

***Tolypothrix distorta* Kützing ex Bornet & Flahault, 1886**

Distribution: São Miguel (Bohlin 1901)

Notes: Freshwater

***Tolypothrix helicophila* Lemmermann, 1910**

Distribution: Pico (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

***Tolypothrix lanata* Wartmann ex Bornet & Flahault, 1886**

Distribution: São Miguel (Cedercreutz 1941)

Notes: Freshwater (lake)

***Tolypothrix tenuis* Kützing ex Bornet & Flahault, 1886**

Distribution: Flores (Bourrelly & Manguin 1946)

Notes: Freshwater

***Trichormus variabilis* (Kützing ex Bornet & Flahault) Komárek & Anagnostidis, 1989**

Distribution: São Miguel (Bohlin 1901)

Notes: Thermal (pool)

Genus *Tychonema* K.Anagnostidis & J.Komárek, 1988

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake)

Genus *Westiellopsis* Janet, 1941

Distribution: São Miguel (Cordeiro et al. 2020b)

Notes: Freshwater (lake), thermal (stream)

***Woronichinia naegeliana* (Unger) Elenkin, 1933**

Distribution: Corvo Luz et al. 2020), Flores (Luz et al. 2020), Pico (Luz et al. 2020), São Miguel (Santos & Santana 2004)

Notes: Freshwater (lake)

2.4 Analysis

The present work comprises 225 taxa, 179 identified species and 11 only to genus level, distributed by six orders (Chroococcales, Nostocales, Oscillatoriales, Pleurocapsales, Spirulinales and Synechococcales), 30 families and 79 genera (Table 4). Most species belong to the Nostocales (43.0%) and Synechococcales (21.2%) orders. Chroococcales and Oscillatoriales orders contributed almost with the same number of species (17.3% and 16.8%, respectively), despite their different genera contributions.

Table 4. Cyanobacteria taxa richness of the Azores Archipelago.

Order	Taxa	Family	Genus	Species		Habitat (by taxa)			
				Nº	%	Freshwater	Thermal	Brackish	Marine
Chroococcales	36	5	8	31	17.3	34	2	-	-
Nostocales	95	11	34	77	43.0	85	9	1	4
Oscillatoriales	36	4	14	30	16.8	27	7	2	2
Pleurocapsales	4	1	2	2	1.1	-	-	-	4
Spirulinales	1	1	1	1	0.6	-	-	-	1
Synechococcales	53	8	20	38	21.2	47	5	-	2
Total:	225	30	79	179	100	193	23	3	13

A summary of cyanobacteria species richness found in the Azores and on each of the nine islands in the different types of habitats is given in Table 5. The number of recorded species was highest on São Miguel Island (115) and lowest on Graciosa Island (3). Freshwater systems were the most diverse habitats, comprising 193 taxa (85.7%), followed by thermal, with 23 species (10.2%), marine (13 species, 5.8%) and brackish systems (3 species, 1.3%).

A positive Pearson correlation coefficient (r = 0.86, n = 9, P = 0.003) was evident between species richness (S) and island area. This correlation is best described by a linear relationship (Fig. 2), where Pico and Flores seem to be outliers. Flores presented higher, while Pico has lower than expected species richness concerning its surface area.

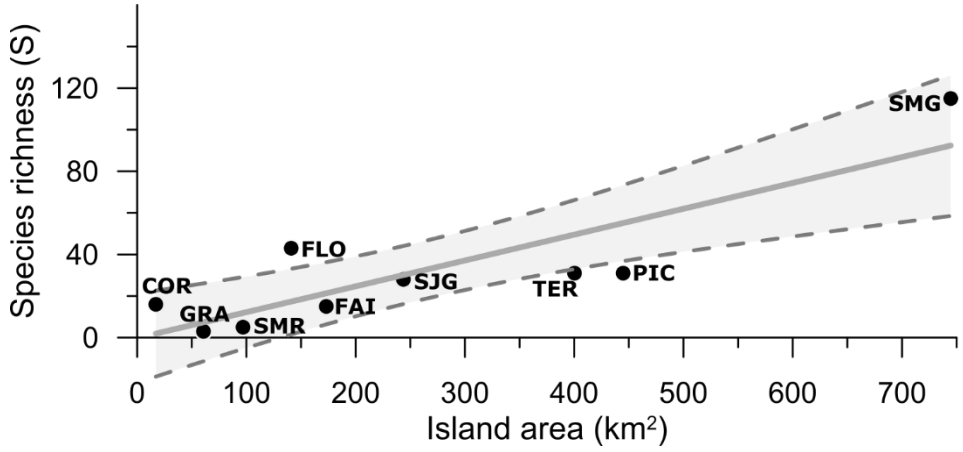

Figure 2. Species-area relationship. Regression line indicates a significant linear relationship with P = 0.003 and R^2 = 0.86 (Pearson correlation). Dashed lines represent 95% interval confidence. COR: Corvo, FAI: Faial, FLO: Flores, GRA: Graciosa, PIC: Pico, SMR: Santa Maria, SJG: São Jorge, SMG: São Miguel, TER: Terceira.

2.5 Discussion

The cyanobacteria diversity in the Azores Archipelago is understudied compared to other European regions (Gkelis et al. 2016), despite being one of the best studied archipelagos in the North Atlantic (Cordeiro et al. 2020a). With its first records of cyanobacteria in Furnas, São Miguel Island, under the Challenger expedition, Moseley (1874) identified three genera: *Chroococcus* Nägeli, 1849, *Nostoc* Vaucher ex Bornet & Flahault, 1886 and *Oscillatoria* Vaucher ex Gomont, 1892. After that, as seen in Fig. 3, the contributions of the Cyanobacteria flora were sporadic, but significant, mainly with Bohlin (1901), Cedercreutz (1941), Bourrelly & Manguin (1946) and Johansson (1977). Their contributions were important, but geographically restricted to the larger islands, such as São Miguel and Terceira. From thereon, the number of recorded cyanobacteria species in the Azores has risen throughout the end of the 20th century and the 21st century and presently stands at 225 taxa with 179 identified species (Figure 3). This boost in the 21st century is mainly due to the implementation of the WFD (e.g. Santos et al. 2005, Santos et al. 2012, Luz et al. 2020) with 39 new described species, which makes 21.8% of the total described species. This programme has helped the continuous study of freshwater cyanobacteria present in the major lakes of the Azores, in Pico, Flores and São Miguel. The works

of Luz (2018), Xavier et al. (2018) and Cordeiro et al. (2020b) significantly contributed to the records of cyanobacteria for the Azores, reporting 19 new species, 10.6% of all taxa recorded only through in-vitro cultivation methods. Using a cultivation approach, these studies were able to isolate strains from lakes, terrestrial and thermal habitats, increasing the ability to identify small or rare species normally not detected in regular monitoring works.

Table 5. Cyanobacteria taxa richness in the Azores by island.

	Island	Taxa	Taxonomy				Habitat			
			Order	Family	Genus	Species	Freshwater	Thermal	Brackish	Marine
Eastern	São Miguel	151	4	25	59	115	133	20	1	1
	Santa Maria	7	2	5	6	5	5	-	2	-
Central	Terceira	37	4	14	21	31	28	1	2	4
	Pico	43	4	16	27	31	43	-	-	-
	São Jorge	31	5	14	15	28	28	-	-	3
	Graciosa	3	3	3	3	3	3	-	-	-
	Faial	18	5	8	11	15	10	-	-	8
Western	Flores	56	4	18	33	43	56	-	1	-
	Corvo	21	4	11	16	16	20	-	-	1

Freshwater cyanobacteria are the most represented taxa in the Azores records, mainly from lakes. Although this result may reflect the abundance of this type of habitat in the Azores, it may also denote the less effort on diversity studies in other types of habitats. A much lower percentage of cyanobacteria was identified from thermal, marine and brackish habitats (10%, 6% and 1%, respectively), probably due to low sampling efforts. The availability of freshwater habitats in the Azores favours the establishment of incoming cyanobacteria in São Miguel, Flores, Terceira and São Jorge. These Islands have permanent streams, lakes, peat bogs and wetlands, providing highly diverse habitats for incomers, while these are absent in Faial, Graciosa and Santa Maria.

Several islands of the Azores have active volcanoes and present high numbers of fumarolic fields, geysers and hot springs (Cruz & França 2006) creating conditions for the growth of thermophilic cyanobacteria. Nevertheless, the current knowledge about thermal cyanobacteria in the Azores is low with few published works focusing on this habitat (Moseley 1874, Bohlin 1901, Luz 2018, Cordeiro et al. 2020b). The recent works by Luz (2018), with morphological identifications and Cordeiro et al. (2020b), that used both morphological and genetic characters for its identification, contributed to several new cyanobacteria taxa reports for the Azores from thermal habitats in São Miguel. The cyanobacteria diversity and distribution in lotic systems in

the Azores are much less known compared to other sites (Branco et al. 2001, Casamatta & Hašler 2016). Only a few works are available addressing this type of habitat in the Azores, with contributions mainly by Cedercreutz (1941), Bourrelly & Manguin (1946) and Johansson (1977), with no relevant works in the latest years. This is unusual as, in lotic systems, cyanobacteria are easily identified and sometimes even the dominant taxa (Schultz et al. 2013, Casamatta & Hašler 2016).

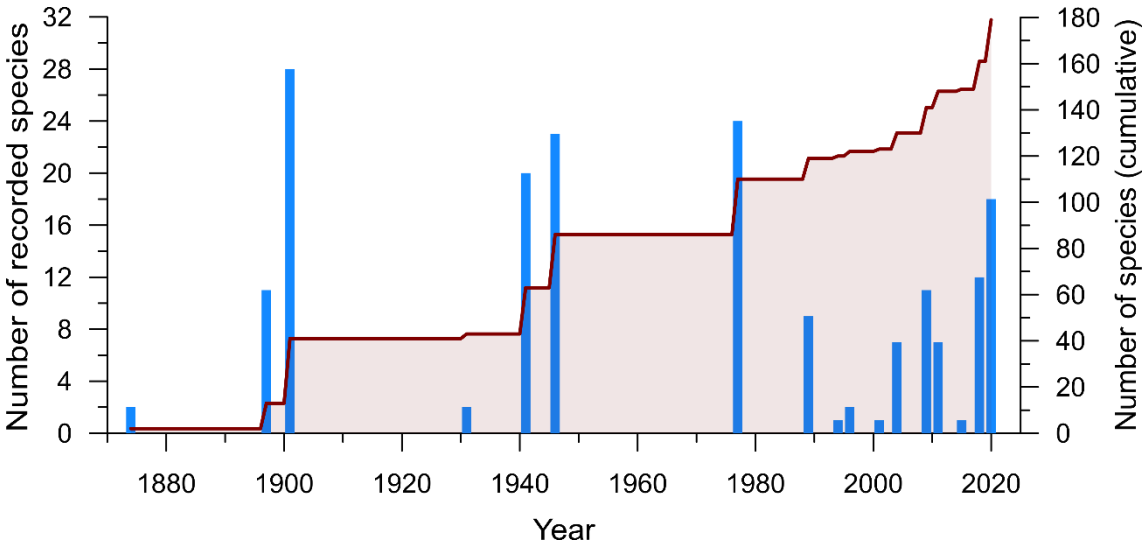

Figure 3. The number of described species through time from 1874 to 2020. Bars represent the number of species described per year, the line represents the cumulative number of described species.

One of the most accepted explanations for regional biodiversity is the species-area relationship (SAR), according to which the number of species along the spatial scale increases with the area (e.g. Rosenzweig 1995, Drakare et al. 2006). This pattern was thoroughly studied on islands (e.g. Lomolino & Weiser 2001, Whittaker & Fernandez-Palacios 2007, Triantis et al. 2012), where the number of species from different taxonomic groups increases with the increase in island size (Triantis et al. 2012). In our data, a positive relationship between the island area and the number of species was observed (Fig. 3). This increase in cyanobacteria species richness with increasing island area in the Azores is consistent with the work of (Borges et al. 2005), for arthropods and bryophytes and Raposeiro et al. (2009) for chironomids with an exception from Flores and Corvo Islands. A possible explanation for these exceptions is the higher percentage that water bodies represent in the total island area (Porteiro 2000) and also the higher precipitation (Secretaria Regional do Ambiente e do Mar 2011, Reichwaldt & Ghadouani 2012, Haakonsson et al. 2017). Compared to Pico Island, the percentage of land covered with water is double in Corvo and almost six times higher in Flores (Porteiro 2000). This suggests that, for cyanobacteria in the Azores, habitat diversity is an important factor in

determining the SAR, as shown for other taxonomic groups and islands (Hortal et al. 2009, Chase et al. 2019).

Compared to other North Atlantic islands, the Azores present the highest species richness (Table 6). The overall distribution of species richness in the different cyanobacteria orders on the North Atlantic archipelagos generally follows the same pattern as the total world species, with Nostocales and Oscillatoriales being the richest orders. However, Nostocales represents a much higher contribution to the regional species richness in the Azores and Madeira Archipelagos, which could reflect their longer dispersion capabilities (Ribeiro et al. 2018). Nostocacean cyanobacteria are able to produce akinetes that can resist long periods of unfavourable conditions (Sarma 2013), enabling them to survive during long dispersion routes and colonise remote oceanic archipelagos, such as the Azores and Madeira. The absence of some orders, such as the Chroococcidiopsidales and Thermostichales, in the Azores and the other Macaronesia Archipelagos, suggests that they probably have a more restricted geographic distribution. Although biological, geographical and climatic factors may contribute to cyanobacteria regional species richness (Moreira et al. 2013, Walter et al. 2017, Ribeiro et al. 2018), the differences amongst islands are most probably related to different sampling efforts (Cordeiro et al. 2020b) and, between Azorean Islands, the distribution of planktic cyanobacteria seems to be mainly related to lake typology rather than environmental parameters (Cordeiro et al. 2020c). For instance, the reduced richness of Oscillatoriales in the Azores could be related to their preference for terrestrial and benthic habitats that are less studied in this Archipelago. With the increase in sampling campaigns covering all types of habitats, the reports of new cyanobacteria in the Azores are expected to increase and the representation of the different orders can become similar to the global pattern.

The hereby presented taxonomic list of cyanobacteria in the Azores represents a valuable resource for biodiversity research and awareness of described cyanobacteria tracked through years that, in the future, will allow the identification of possible invader species and studies of the influence of temperature changes in the World. Besides that, knowing the biodiversity of a specific archipelago enriches its value and allows future works in ecology and, in a more practical way, in biotechnology or pharmaceutical if found to be of increased value.

Table 6. Cyanobacteria species richness in the Azores compared to world-known species richness (World's order and species number retrieved from Guiry & Guiry (2022); Canary Islands, Madeira and Cuba numbers retrieved from Cordeiro et al. (2020a)).

Order	Azores		Madeira		Canary Islands		Cuba		World	
	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%
Chroococcales	31	17.32	2	8.33	4	6.35	28	19.05	649	13.20
Chroococcidiopsidales	0	0	0	0	0	0	2	1.36	37	0.75
Gloeobacterales	0	0	0	0	0	0	0	0	3	0.06
Gloeomargaritales	0	0	0	0	0	0	0	0	1	0.02
Nostocales	77	43.02	11	45.83	20	31.75	44	29.93	1547	31.46
Oscillatoriales	30	16.76	8	33.33	18	28.57	30	20.41	1397	28.41
Pleurocapsales	2	1.12	1	4.17	6	9.52	3	2.04	223	4.53
Spirulinales	1	0.56	0	0	4	6.35	5	3.40	56	1.14
Synechococcales	38	21.23	2	8.33	11	17.46	35	23.81	995	20.23
Thermostichales	0	0	0	0	0	0	0	0	10	0.20
Total:	179		24		63		147		4918	

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2.7 Supplementary material

Suppl. material 1: Cyanobacteria checklist of the Azores Archipelago, Portugal

Authors: Luz R, Cordeiro R, Fonseca A, Gonçalves V

Data type: Darwin Core Archive (.zip) of cyanobacteria taxa and occurrence data used for the presented and analysed checklist.

Brief description: Published Darwin Core Archive with two data tables in GBIF (doi: 10.15468/bfktqo) about the reports of cyanobacteria in the Azores Archipelago. The taxon (core) data table contains 229 records of cyanobacteria (from class to species level). One extension data table exists, with a total of 2838 occurrence records of cyanobacteria found in literature. The taxon data table is constructed, based on the occurrence data table.

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Chapter III

Description of *Azorothrix ramosa* gen. et sp. nov. (Tolypotrichaceae, Cyanobacteria), a new Tolypotrichaceae from Atlantic oceanic islands

Luz, R., Hentschke, G. S., Cordeiro, R., Fonseca, A., Urbatzka, R., Vasconcelos, V., & Gonçalves, V. (2024). Description of *Azorothrix ramosa* gen. et sp. nov. (Tolypotrichaceae, Cyanobacteria), a new Tolypotrichaceae from Atlantic oceanic islands. Fottea, 24(1), 99-108. https://doi.org/10.5507/fot.2023.014

Chapter III. Description of *Azorothrix ramosa* gen. et sp. nov. (Tolypotrichaceae, Cyanobacteria), a new Tolypotrichaceae from Atlantic oceanic islands

Abstract

The Tolypotrichaceae is a well–defined family within the filamentous heterocyst–forming cyanobacteria. However, the morphological and genetic similarity of some of its genera is very high, making the taxonomic assessment and the description of new taxa in this family challenging. Here we describe six strains of *Tolypothrix*–like cyanobacteria that were isolated from freshwater lakes and streams from São Miguel Island (Azores Archipelago, Portugal), and deposited in the BACA collection. All strains showed morphological similarities, restricting them to the same taxa, with the phylogenetic analysis revealing a distinct position in the Tolypotrichaceae clade. A deeper analysis of the morphological, ecological, and genetic (16S rRNA and 16S–23S rRNA internal transcribed spacer) data, and comparison with known genera in the Tolypotrichaceae, allowed the description of the taxon as *Azorothrix ramosa* gen. et sp. nov. in the Tolypotrichaceae family.

Keywords: Atlantic Ocean, Azores, Freshwater, New genus, Nostocales, Oceanic islands, Phylogeny, 16S rRNA, 16S–23S rRNA ITS

3.1 Introduction

Cyanobacteria in the North Atlantic islands have been studied since the 19th century (Cordeiro et al. 2020a), starting in 1874 in the Azores Archipelago by Moseley (1874). However, cyanobacteria diversity in the Azores is still poorly known despite the long history of cyanobacteria records and the habitat diversity of these islands (Luz et al. 2020, 2022). The recently created Bank of Algae and Cyanobacteria of the Azores (BACA) holds a significant collection of cultured cyanobacteria strains from different ecosystems (lakes, streams, terrestrial and thermal) of the Azores, allowing their detailed morphological and genetic characterization (Luz 2018, Xavier et al. 2018, Cordeiro et al. 2020b). Recent results by Cordeiro et al. (2020b) revealed that several strains in the BACA collection could be new genera, mostly for the Nostocales order, three of them forming a monophyletic clade phylogenetically related to the Tolypotrichaceae Hauer et al. (Cordeiro et al. 2020b).

The Tolypotrichaceae family was proposed by Hauer et al. (2014) based on morphological characters and phylogenetic analysis of several cultured strains. Their work addresses a

previously known problem of wrongly identified cyanobacteria such as *Tolypothrix* Kützing ex Bornet et Flahault, which are taxonomically problematic and misinformative in phylogenetic studies. Hauer et al. (2014) demonstrated that the Tolypotrichaceae could be distinguished as a unique family with several well–characterized genera, such as *Spirirestris* Flechtner et Johansen, *Rexia* Casamatta, Gomez et Johansen, *Coleodesmium* Borzì et Geitler, and more recently, *Dactylothamnos* Komárek et al. and *Kryptousia* Alvarenga et al. concerning morphology, ecology, and genetics. Nevertheless, *Tolypothrix* is still a polyphyletic genus with high morphological polymorphism among identified strains (Hauer et al. 2013, 2014).

As for all Cyanobacteria, the major challenge of the Tolypotrichaceae genera is their morphological polymorphism, making morphological identification of both genera and species problematic. Also, the DNA similarity (p–distance) among morphologically close genera in the Tolypotrichaceae is quite high, making phylogenetic analysis more difficult (Flechtner et al. 2002). The same can be applied to *Dactylothamnos* (Komárek et al. 2015) or *Kryptousia* (Alvarenga et al. 2017). Indeed, Komárek et al. (2015) suggested that the strains used to describe *Kryptousia microlepis* could belong to *Dactylothamnos*, something that was never validly published and was later discarded by Alvarenga et al. (2017) and described as *Kryptousia* species. Alvarenga et al. (2017) supported this assessment on the phylogenetic position between *Kryptousia* strains and the distinctive ecology of *Dactylothamnos* and *Kryptousia* species. The role of *Streptostemon* Sant'Anna, Azevedo, Kaštovský et Komárek in the Tolypotrichaceae family is not considered here due to its problematic position in the family (Hentschke et al. 2016).

In this work, we applied a polyphasic approach, considering morphological, genetic, and ecological characteristics of six cultured cyanobacterial strains from freshwater ecosystems (lakes and streams) from the Azores to propose a new genus, *Azorothrix* gen. nov., and one species, *Azorothrix ramosa* sp. nov. All taxonomic treatment was made according to the International Code of Nomenclature for algae, fungi, and plants.

3.2 Materials and Methods

3.2.1 Site description and sampling

The Azores archipelago is an oceanic group of islands in the middle of the North Atlantic Ocean, roughly 1500 km from Europe and 1900 km from America (Fig. 1), with an oceanic temperate climate (Hernández et al. 2016). The six studied strains were isolated from five locations in São Miguel Island, four from lakes and two from a stream (Table 1).

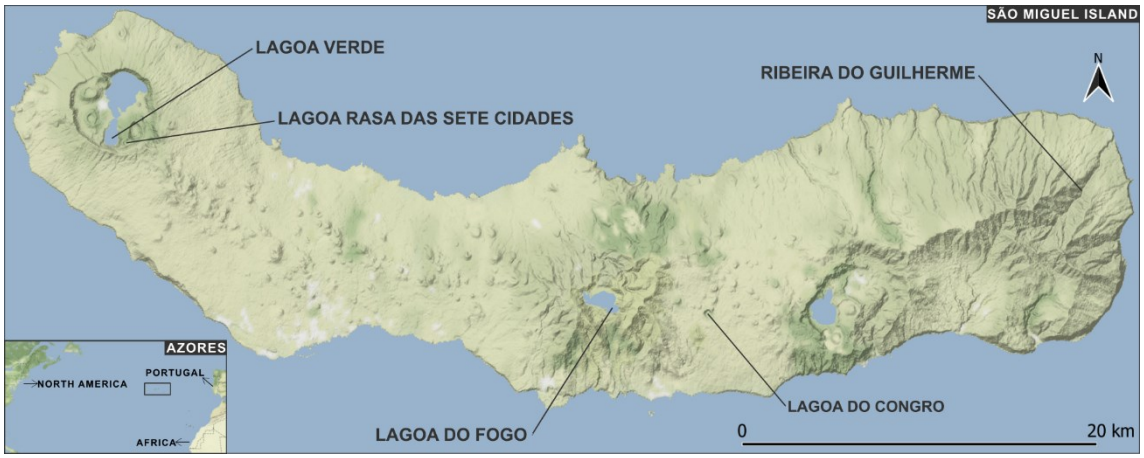

Figure 1. Sample site locations of the six isolated strains in São Miguel Island in the Azores Archipelago, Portugal. Lagoa Verde (BACA0043), Lagoa Rasa das Sete Cidades (BACA0028), Lagoa do Fogo (BACA0147), Lagoa do Congro (BACA0066), Ribeira do Guilherme (BACA0093 and BACA0098). Base map retrieved from OpenStreetMap®, licensed under the Open Data Commons Open Database License by the OpenStreetMap Foundation.

Environmental data to characterize sampling sites was obtained during sampling or retrieved from previous studies (Table 1). Temperature (°C), pH, conductivity (µS cm^{-1}), and dissolved oxygen (mg.L^{-1}) were recorded in situ using a multiparameter probe Horiba U–52 (Horiba, Pasadena, TX, USA). The trophic state of the lakes follows (Cordeiro et al. 2020c), and hydromorphological data was retrieved from (Pereira et al. 2014).

Table 1. Sampled locations with hydromorphological and physicochemical characterization of the studied strains of *Azorothrix ramosa*.

Strain	Location	Coordinates	Elev. (m)	Type	Sampling	T (ºC)	pH	C (µS cm^{-1})	O_2 (mg/L^{-1})	Trophic state
BACA0028 ***A. ramosa***	Lagoa Rasa das Sete Cidades	37°50'33.72''N 25°46'48.04''W	545	Lake	2016-05-19	18	6.8	45	9.4	Oligotrophic
BACA0043 ***A. ramosa***	Lagoa Verde	37°50'34.41''N 25°47'19.37''W	260	Lake	2016-09-06	23	9	130	9	Eutrophic
BACA0066 ***A. ramosa***	Lagoa do Congro	37°45'22.31''N 25°24'29.55''W	420	Lake	2017-01-31	13	7.3	100	9.5	Eutrophic
BACA0093 ***A. ramosa***	Ribeira do Guilherme	37°48'43.09"N 25°10'27.76"W	260	Stream	2017-05-25	15	7.7	93	10.4	-
BACA0098 ***A. ramosa***	Ribeira do Guilherme	37°47'54.50"N 25°12'11.38"W	573	Stream	2017-05-12	13	7.4	89	10.5	-
BACA0147 ***A. ramosa***	Lagoa do Fogo	37°45'53.31''N 25°28'26.79''W	574	Lake	2017-07-31	22	7.9	36	8.7	Mesotrophic

3.2.2 Isolation and culture

Field samples were grown on BG–11_0 (BG–11 medium without nitrogen) (Allen 1968) for two weeks in 50 mL Erlenmeyer Flasks under 14:10 light:dark photoperiod. Growing colonies

were then picked by pipette and transferred to BG–11_0 agar plates (1% agar). The target cyanobacteria were then isolated by repeating streaks in new agar plates (Rippka 1988). Isolated strains were deposited in BACA, maintained in a 14:10 light:dark cycle (under 10–40 µmol photons m^{-2} s^{-1}) photoperiod at 19 °C.

3.2.3 Morphological characterization

Strains were characterized using a Leica DM4 B microscope with Digital Camera Leica MC 190 HD (Leica, Germany). At least 50 trichomes and three cells per trichome were examined for cell dimensions and descriptions. The cultures were examined at two weeks and two months of growth for morphology and branching characteristics description. Two–month agar streak cultures were photographed and examined using a Stemi 2000–C with an Axiocam 105 color using the ZEN 3.0 (blue edition) (Carl Zeiss™, Germany).

3.2.4 DNA extraction, 16S rRNA and 16S–23S rRNA ITS gene amplification, and sequencing

Fresh cultures were used for DNA extraction with the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), following the protocol recommended by the manufacturer for gram–negative bacteria. For the 16S rRNA and 16S–23S rRNA ITS region amplification, the primers 27F and 23S30R (Table 2) were used in a Polymerase chain reaction (PCR) with a total volume of 25 µL containing 1× PCR Buffer, 2 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (Thermo Fisher, Waltham, MA, USA), 0.5 pmol of each primer, 5–10 ng of DNA and 1.5 U of Supreme NZYTaq II DNA polymerase (Nzytech, Portugal). PCR conditions followed Taton et al. (2003), and thermal cycling was carried out in a ProFlex™ 3 × 32–well PCR System (Thermo Fisher, USA). PCR amplification products were visualized by electrophoresis in 0.8% agarose gel, in 0.5 × TBE (Tris–Borate–EDTA) buffer and stained with SYBR™ SAFE. Amplified bands were removed from the gel using a clean scalpel and then purified using NZYGelpure (Nzytech, Portugal). Sequencing of the 16S rRNA and 16S–23S rRNA ITS region was done by Macrogen Ltd. (Madrid, Spain) using the 27F, 781F, 781R, NITS_F, and 23S30R primers (Table 2).

3.2.5 Phylogenetic analysis of the 16S rRNA

The sequences of the new genera were aligned with 198 sequences retrieved from GenBank using BLAST, with the addition of reference strains of cyanobacterial genera. The sequences were aligned using MAFFT v7.520 with the G–INS–i method (Katoh & Standley 2013),

with a final alignment containing 1107 informative sites. The best–fit nucleotide model was assessed using ModelFinder (Kalyaanamoorthy et al. 2017), according to BIC, with the selection of the K3P+I+G4. Phylogenetic trees were constructed using Bayesian inference (BI) with MrBayes v3.2.7a on XSEDE (Ronquist et al. 2012) through the CIPRES Science Gateway, and Maximum likelihood (ML) using IQ–Tree online version v1.6.12 (Trifinopoulos et al. 2016), respectively, using *Chroococcidiopsis thermalis* PCC 7203 and *Oscillatoria princeps* CCALA 1115 as outgroups. The BI was carried out with 1.0×10^7 generations, with two runs of four Markov chains, with custom parameters (temp=0.0175), sampling every 1000 generations and a 0.25 burn–in rate (the final average standard deviation of split frequencies was 0.037568) using the GTR+GAMMA+I model. The ML analysis was carried out using the K3P+I+G4 model with 1000 ultrafast bootstrap replicates (Hoang et al. 2017). Trees were visualized using FigTree v1.4.4 (Rambaut 2012), and the final composite tree from Maximum likelihood with posterior probabilities values for BI was re–drawn using Inkscape v1.2.

Table 2. Primer sequences used for PCR and sequencing.

Primer	Sequence (5'-3')	Reference
27F	AGA GTT TGA TCC TGG CTC AG	Neilan et al. (1997)
781F[a]	GGG ATT AGA TAC CCC TGT AGT C	This study
781R	GAC TAC TGG GGT ATC TAA TCC CAT T	Nübel et al. (1997)
NITS_F	GAA TTC GTT CCC GGG CCT TG	This study
23S30R	CTT CGC CTC TGT GTG CCT AGG T	Lepère et al. (2000)

[a]Direct reverse complement of the 781R primer by Nübel et al. (1997).

3.2.6 P–distance calculations

For the 16S rRNA p–distance, Tolypotrichaceae genetic information from type species was selected (only one sequence was chosen if more than one was available, choosing the first made available in GenBank). Some OTUs outside the Tolypotrichaceae were selected for a broader analysis of the 16S rRNA p–distance delimitations values currently used in the Nostocales. Sequences were aligned using MAFFT v7.520 with the G–INS–i method (Katoh & Standley 2013), and p–distance calculation for both regions was done using MEGA 11.0.13 (Tamura et al. 2021).

3.2.7 Analyses of 16S–23S rRNA ITS region

The 16S–23S rRNA ITS secondary structures of D1–D1', Box–B, and V3 helices were identified following (Iteman et al. 2000), and secondary structures were predicted using M–fold (Zuker 2003). Final composite images were re–drawn in Inkscape v1.2.

3.3 Results

***Azorothrix* R.F.S.Luz, G. S. Hentschke et V.Gonçalves gen. nov.**

Description: Thallus fasciculated forming irregular mats when old, cushion–like with erect filaments. Filaments solitary or fasciculated, later irregularly entangled, up to 1.5 mm. Trichomes heteropolar, unconstricted or constricted at cross walls, repeatedly false branched. False branches unilateral and/or bilateral, never more than one per filament, divaricated or ± parallelly arranged. False branches of the "scytonematoid" type are very rare. Sheaths are thin, firm, colourless, open at the apex. Cells isodiametric to shorter than wide. Terminal cells rounded. Heterocytes basal, hemispherical, cylindrical, conical rounded or discoid, solitary, or seriated up to 5 in a row, rarely intercalary (before disintegration?). Akinetes not observed. Reproduction by hormogonia and disintegration of trichomes (necridia present).

Type species: *Azorothrix ramosa* R.F.S. Luz, G.S. Hentschke et V. Gonçalves

Etymology: *Azorothrix* = Azores (site of origin) + thrix (for its filamentous structure and resemblance to *Tolypothrix*).

Remark: Phylogenetic distinct and placed in the Tolypotrichaceae family by the 16S rRNA. Morphologically similar to *Tolypothrix*, but with a distant phylogenetic placement.

***Azorothrix ramosa* R.F.S.Luz, G.S. Hentschke et V.Gonçalves sp. nov. (Fig. 2)**

Description: Thallus fasciculate, forming erect cushion–like aggregations when old. Filaments false branched, unilateral, and bilateral, later growing in a predominantly parallel orientation. Filaments commonly very long, reaching more than 1 mm in length (1.5 mm), 10.2–18.4 µm in diameter. Trichomes uniseriate, cylindrical slight constricted at cross–walls, more visible in the trichome terminal part, not tapering. Trichomes with isolated heterocytes or in rows up to five, one or two pored, very rarely without basal heterocytes. Sheaths are thin, firm, colourless, and distinct. Cells isodiametric to shorter than wide, 8.1–12.1 µm wide and 2.4–10.7 µm long, blue green or olive green, end cells rounded and sometimes larger. Necridia present. Basal heterocytes variable in shape, hemispherical, cylindrical, conical rounded or discoid 6.7–14.2 µm wide 4.4–13.2 µm long. Intercalary heterocytes are very rare, probably developing before disintegration. Akinets not found in culture, reproduction by trichome fragmentation.

Holotype: Dried material preserved in a permanently inactive state at Herbário Ruy Telles Palhinha, University of Azores, Portugal, under the AZB 3833 code.

Type locality: Lagoa do Congro, São Miguel Island, Azores, Portugal (37°45' 22.3092"N, 25°24'29.5488"W).

Habitat: Found in lakes with very different trophic states (oligotrophic, mesotrophic, and eutrophic) and freshwater streams on the surface of submerged stones (epilithon).

Etymology: ramosa = having many branches.

Reference strain: BACA0066 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal).

Gene Sequences: GenBank accession number MT176722 for the 16S rRNA and 16S–23S rRNA ITS region.

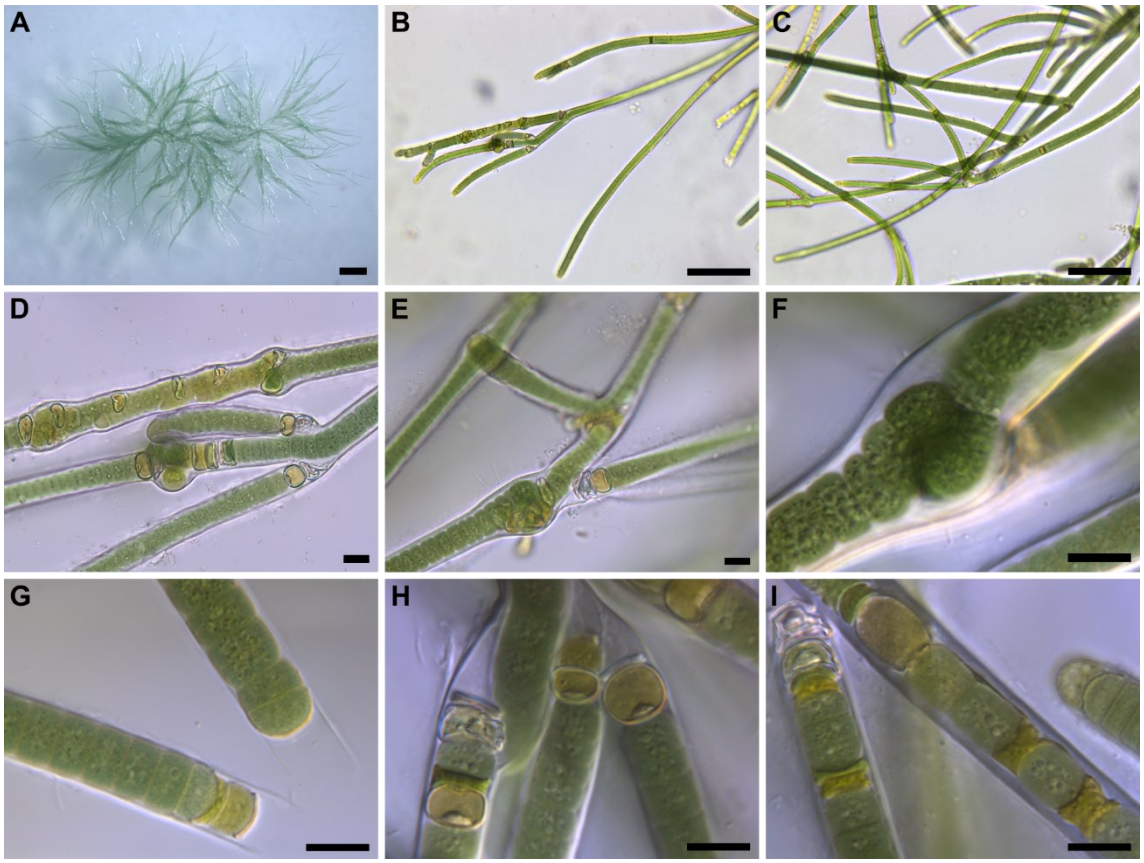

Figure 2. *Azorothrix ramosa* BACA0066 morphology: (A) colony grown in agar; (B-F) filaments ramification; (G) end cells, (H-I) heterocytes and necridic cells in liquid media. Scale bars 100 µm (A-C), 10 µm (D-I).

3.3.1 Morphological analysis

Azorothrix is positioned in the Tolypotrichaceae, according to its characteristic isolate false branching (Fig. 2A–C and Fig. 3A–C) and its phylogenetic position (Fig. 4). This new genus is phylogenetically closely related to *Kryptousia*, from which it differs in morphological traits and ecology. *Azorothrix* presents fasciculate or parallel–oriented thallus, with erect filaments when older, while *Kryptousia* presents interwoven filaments, which are never parallel–oriented or erect. These two genera also differ in their ecology. *Azorothrix* is found in the epilithon of

freshwater lakes and streams, while *K. macronema* is from mangroves and is epiphytic on *Avicennia schaueriana*, a plant known for salt–secreting glands in its leaves (Fitzgerald et al. 1992).

Compared to *Coleodesmium*, *Azorothrix* presents only one trichome per sheath, whereas *Coleodesmium* always has more than one. *Dactylothamnos* differs from *Azorothrix* by presenting narrowed branches, while *Azorothrix* trichomes are cylindrical. Morphologically, *Azorothrix* presents isodiametric to shorter than wide cells, while in *Hassallia* Berkeley ex Bornet et Flahault cells are always shorter than wide. *Azorothrix* presents morphological features common to *Tolypothrix* from which can only currently be separated by molecular analysis.

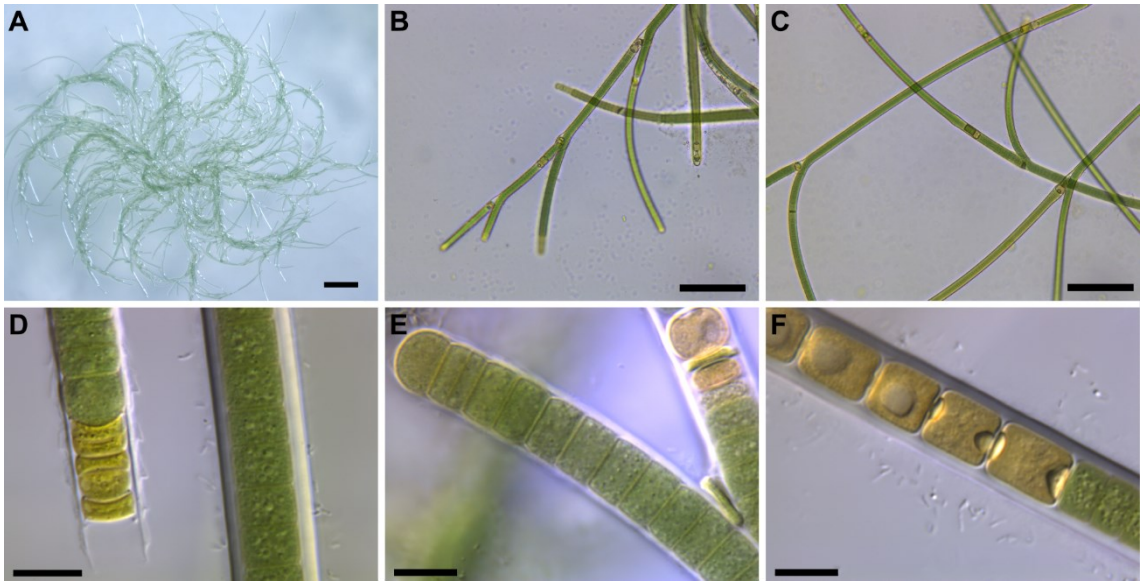

Figure 3. *Azorothrix ramosa* BACA0028 morphology: (A) colony grown in agar; (B-C) filaments ramification; (D-E) end cells and necridic cells; (E-F) heterocytes in liquid media. Scale bars 100 µm (A-C), 10 µm (D-F).

3.3.2 16S rRNA phylogenetic and 16S–23S rRNA ITS analysis

All six strains showed only one band in the electrophorese gel following PCR. Sequencing of the obtained bands showed high quality when using the new designed primer (NITS_F) together with the remaining used primers (Table 2), with the presence of both $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in the sequenced operons.

The ML and BI trees were built with 198 operational taxonomic units (OTUs) and 1107 analysed positions of the 16S rRNA gene. The new genus is positioned in the Tolypotrichaceae clade, along with *Tolypothrix* (HG970652), *Hassallia* (AM905327), *Spirirestis* (AF334690), *Rexia* (KF934181), *Dactylothamnos* (KM199732), and *Coleodesmium* (AF334703). The new genus *Azorothrix* is supported as a sister clade of a cluster containing identified *Hassallia* strains,

including the type species *Hassallia byssoidea*. Other Tolypotrichaceae genera are closely placed, namely *Kryptousia*, *Rexia*, *Tolypothrix*, and *Dactylothamnos*. The genus *Kryptousia* is polyphyletic as *Kryptousia microlepis* is positioned close to *Dactylothamnos* with good support (ML 93, BI 0.90) and far from the type species *K. macronema*. In both ML and BI trees, the family Tolypotrichaceae is formed by a monophyletic clade, although the placement of OTUs within the family is not the same. Among the few clades that seem more stable is the one formed by *Dactylothamnos*, *K. microlepis* and *T. distorta*, where a good support value is observed (ML 93, BI 0.90), while the remaining OTUs placement changes frequently (Fig. 4; Fig. S1).

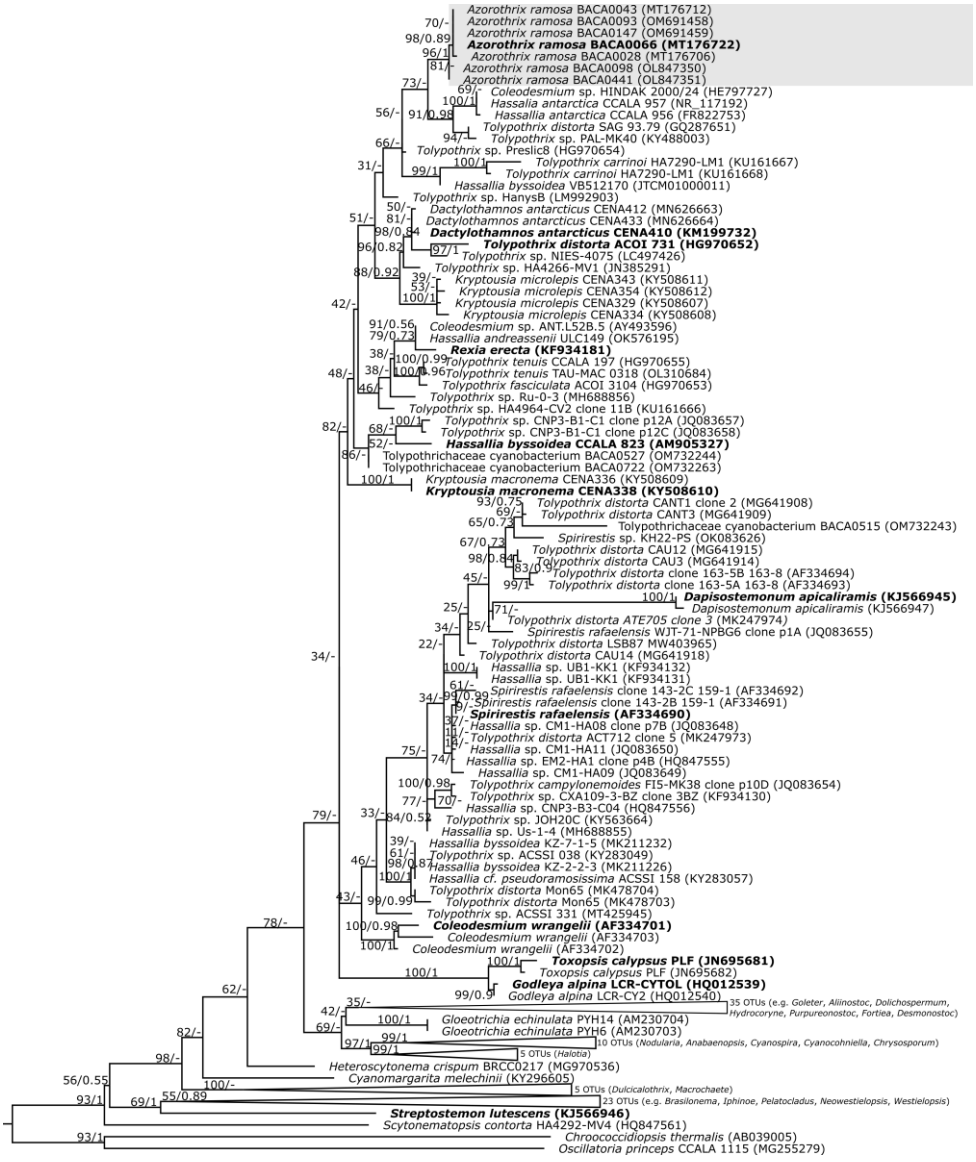

Figure 4. Maximum Likelihood (ML) phylogenetic tree based on 16S rRNA sequences (1107bp) of *Azorothrix* and other cyanobacterial strains. Bootstrap values for maximum likelihood and posterior probabilities for Bayesian Inference are indicated on the tree. The novel genus is in grey shade and type species of the Tolypotrichaceae are in bold font. Outgroups: *Chroococcidiopsis thermalis* (AB039005) and *Oscillatoria princeps* CCALA 1115 (MG255279).

Regarding the 16S rRNA, there is a high similarity among some Nostocales genera, with a p–distance as low as 2.18% among phylogenetic distant species such as *Goleter apudmare* HA4356–MV2 and *Tolypothrix fasciculata* ACOI 3104. These occur between almost all Tolypotrichaceae genera, with p–distance close to 2%. Although all these genera present high 16S rRNA gene similarity (Table S1), the obtained phylogeny supports the new genus as an independent and monophyletic taxon in the Tolypotrichaceae.

The 16S–23S rRNA ITS region was aligned among Tolypotrichaceae genera and 16S–23S rRNA ITS secondary structures folded after the identification of the D1–D1' (Fig. 5), Box–B (Fig. 6) and V3 helix (Fig. 7). It is possible to identify conserved motifs among all compared genera, such as the basal stem and the lateral bulge in the D1–D1'. Indeed, the D1–D1' region presents similar sequences and structures among *Azorothrix*, *Dactylothamnos*, *Tolypothrix*, *Coleodesmium*, *Rexia*, and *Kryptousia microlepis*. The differences among these genera are found only in a few nucleotide substitutions in the 3' side of the molecule between the lateral bulge and the mid internal loop. *Kryptousia macronema* helices present very distinct stems, on both sequence and structure, above the basal lateral bulge. The Box–B (Fig. 6) and V3 helix (Fig. 7) have a higher variability than the D1–D1', showing high variability in the sequence and structure among them. The Box–B has a common basal stem and common internal loop, except for *Azorothrix*, which significantly differs from the remaining by a much larger internal loop. All analysed strains share a terminal hair pin, although the sequence and structure differ among all. The V3 helix has the most significant difference among strains where the common structure is the terminal hair loop, except for *Azorothrix ramosa* and *Dactylothamnos antarcticus* that share the same V3 helix sequence and structure.

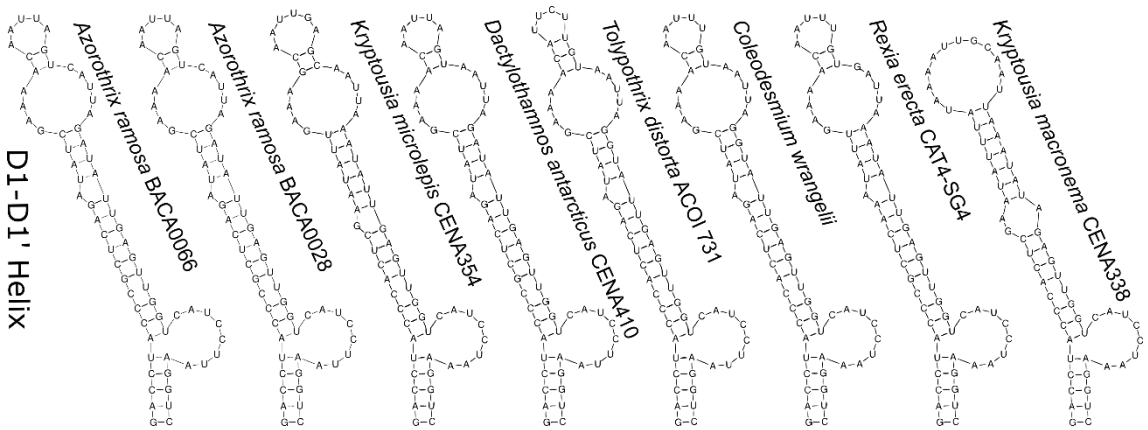

Figure 5. Secondary structure of the D1–D1' helix in *Azorothrix ramosa* and representative Tolypotrichaceae genera.

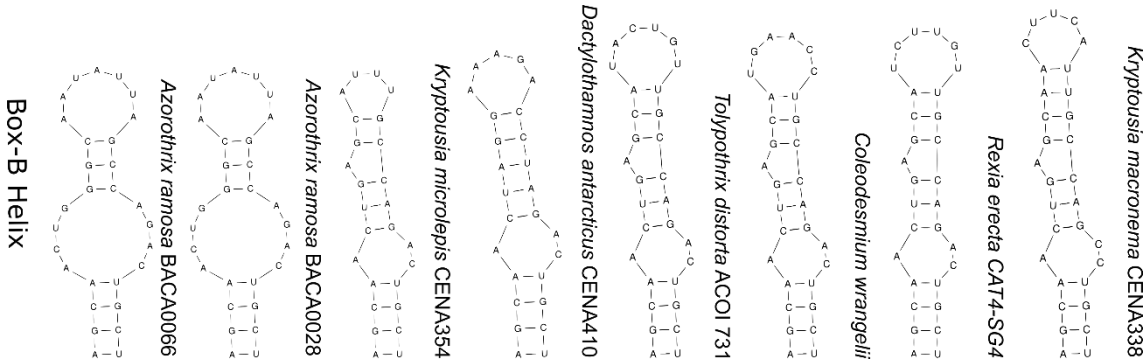

Figure 6. Secondary structure of the Box-B' helix in *Azorothrix ramosa* and representative Tolypotrichaceae genera.

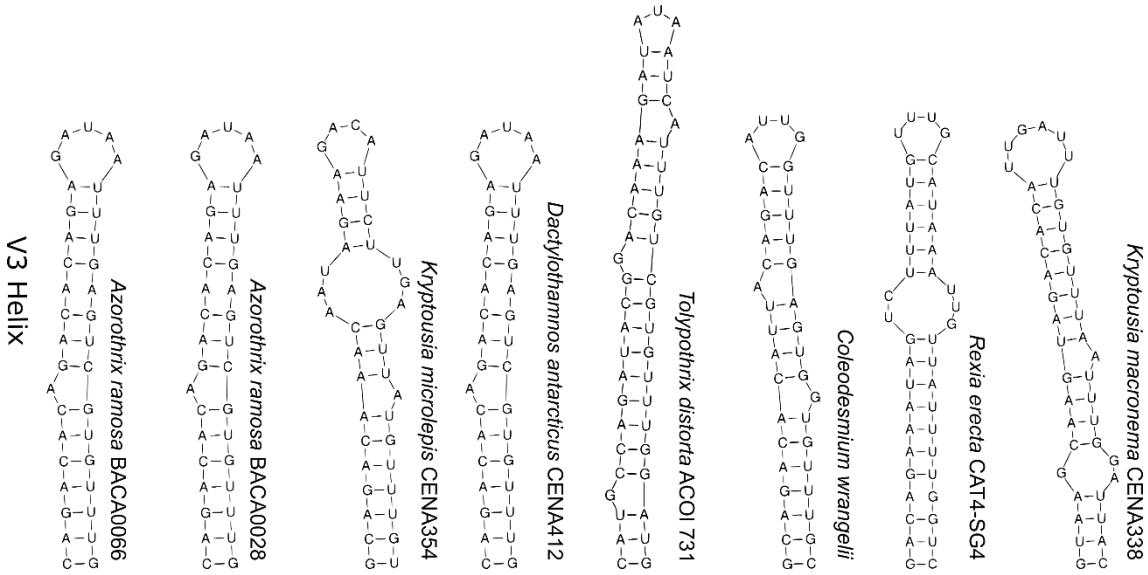

Figure 7. Secondary structure of the V3 helix in *Azorothrix ramosa* and representative Tolypotrichaceae genera.

3.4 Discussion

The six studied BACA strains, previously identified as *Tolypothrix*, present a distinct phylogenetic position, which, combined with morphological and ecological features, allowed the description of a new genus. Morphologically very similar to the *Tolypothrix* and *Hassallia* genera (*Tolypothrix distorta* ACOI 731, *Hassallia byssoidea* CCALA 823) but distant phylogenetically and in p–distance (Table S1), *Azorothrix* is a new genus genetically distinct from *Tolypothrix* and *Hassallia*. Although the current known geographic distribution of *Azorothrix* is restricted to the Azores Islands, it will probably be found in other areas, and the description of new species or strains will help better characterize this genus. All strains are identical when compared with cell size and form along the trichome and hormogonia, but some differences were noticed, namely in the type of branching that influences the formation of the colonies in agar plates (Fig. 2 and

Fig. 3). In BACA0066 (Fig. 2A), bilateral branching is more frequent than unilateral, with branches staying close to the main filament (later fasciculated), while in BACA0028 (Fig. 3A), false branches are widely divaricated and less frequent. These traits seem to be stable as they are maintained by each strain on long–term maintenance culture.

All six strains showed only one band in the electrophoresis gel following PCR, something unusual in the Nostocales as normally these present up to five operons (Kaštovský et al. 2014, Johansen et al. 2017). However, this seems common in the Tolypotrichaceae family, as only one operon was found in *Spirirestis*, *Tolypothrix* and *Coleodesmium* (Flechtner et al. 2002).

The *Azorothrix* strains are phylogenetically positioned close to *Kryptousia*, *Dactylothamnos*, and *Coleodesmium*. Together with the phylogenetic distinction, these genera can also be distinguished morphologically. Although similar to *Coleodesmium*, *Azorothrix* does not have more than two trichomes per sheath, which is a diacritical feature in *Coleodesmium* (Komárek 2013). Both *Kryptousia* and *Dactylothamnos*, possess similar colony formation with interwoven or flake–like groups (Komárek et al. 2015, Alvarenga et al. 2017), different from *Azorothrix* that follows a more organized (fasciculate) growth. A distinguishable trait is also the longer trichomes found in *Azorothrix* against the short trichomes found in *K. microlepis* and *Dactylothamnos* (Komárek et al. 2015, Alvarenga et al. 2017). In terms of ecology, these genera are all found in freshwaters, but they were described from very different geographic and climate zones. *Dactylothamnos* was originally found in the Antarctic (Komárek et al. 2015), *Kryptousia* from South American Atlantic Forest (Alvarenga et al. 2017), *Coleodesmium* from Europe (Bornet & Flahault 1888), and *Azorothrix* from the Azores.

The folding of the 16S–23S rRNA ITS shows a high similarity of the D1–D1' region among the phylogenetic cluster made by *Azorothrix*, *K. microlepis*, *Dactylothamnos*, *Coleodesmium*, and *Rexia*, in contrast with *K. macronema*. Their differences rely mainly on nucleotide sequence on the 3' side between the main lateral bulge and the first internal loop. In the Box–B and V3 Helix, a more diverse folding was found, as expected (Johansen & Casamatta 2005). In Box–B, although *Azorothrix* has a more distinct fold with a larger mid internal loop, the remaining analysed strains have a common secondary structure, varying mostly in the length and sequence of the hair pin. The V3 secondary structure is the most distinct among the studied strains, with the presence/absence of some internal loops, except for *Azorothrix* and *Dactylothamnos* where the V3 is equal in structure and sequence. The highly conserved D1–D1' structure among *Azorothrix*, *Kryptousia*, *Dactylothamnos*, *Coleodesmium*, and *Rexia*, and the similarity in the V3 region of *Azorothrix* and *Dactylothamnos* is an unusual feature when comparing so many different genera, however with such a conserved 16S rRNA as seen in the Tolypotrichaceae, is not surprising that

all the 16S–23S rRNA operon would also be somehow conserved, and variability of the 16S–23S rRNA ITS is in concordance with the variability of the 16S rRNA.

Hauer et al. (2014) elevated the former subfamily Tolypothrichoidae Komárek and Anagnostidis to the family level. Our results support this classification with the monophyletic nature of the Tolypotrichaceae (Fig. 4). Nevertheless, some uncertainties remain due to the morphological polymorphism of *Tolypothrix* species and distant phylogenetic placement of some strains in phylogenetic trees (Hauer et al. 2014). In our tree (Fig. 4), the distant position between *Tolypothrix distorta* (type species) and the clade composed by *Tolypothrix fasciculata* and *Tolypothrix tenuis* strains suggests that the latter could be a new undescribed genus. The identification of close genetic strains that are either distinct morphologically, as seen in *Spirirestis* (Flechtner et al. 2002) and *Dactylothamnos* (Komárek et al. 2015), or ecologically, as seen in *Kryptousia* (Alvarenga et al. 2017), seems to be common in the Tolypotrichaceae.

The phylogenetic analysis is important for genera and species delimitation in the Tolypotrichaceae and in the Nostocales in general (Strunecký et al. 2023). The 16S rRNA in the Nostocales is highly conserved (Kaštovský et al. 2014), but at the same time, it shows a high phylogenetic divergence among the studied OTUs. This, combined with the high morphological similarities that many of the described genera and species have, is challenging for a correct taxonomic assessment. Although this work uses detailed morphological characterization, ecology, geographical distribution and genetics for genus and species distinction, it relies strongly on the phylogenetic analysis for the new taxa description, especially the current phylogenetic separation of previously described genera, such as *Tolypothrix*, *Coleodesmium*, *Rexia*, *Dactylothamnos* and *Kryptousia*. Taking into account the morphological similarities with *Tolypothrix*, *Azorothrix ramosa* could be described as a new *Tolypothrix* species, however this would only create more confusion in an already problematic clade. Besides, if that would be done, we would be recognizing only morphology, and ignoring genetic data and the existence of cryptic taxa, failing to follow the recommended polyphasic approach in the taxonomic assessment (Komárek et al. 2014). Furthermore, the adoption of such approach would mean that the broader clade containing *Tolypothrix* / *Hassallia* / *Rexia* / *Dactylothamnos* / *Kryptousia* / *Azorothrix* type species should be collapsed into a single genus.

Presently, Tolypotrichaceae is one of the most problematic cyanobacteria families, with a low 16S rRNA p–distance threshold (close to 2%) for genera delimitation. This situation extends to several other genera of the Nostocales (e.g. *Cyanocohniella*, *Goleter*, *Halotia*; Table S1) that are placed in distant phylogenetic clades and genomically recognized families (Kaštovský et al. 2014, Strunecký et al. 2023). Thus, phylogeny is of paramount importance in taxonomic studies in the Nostocales. Furthermore, in such clades as the Tolypotrichaceae, where 16S rRNA

threshold values are low, and the morphology is similar, it is urgent to include genomic characterization to resolve their taxonomic situation. A taxonomic assessment using a much more robust analysis with genomes and phylogenomics will help clarify the status of these genera, supporting their distinction or collapse to the original *Tolypothrix* genus.

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3.6 Supplementary material

The following supplementary material is available for this article: Table S1. 16S rRNA p-distance percentage of *Azorothrix* and close phylogenetic genera. (Red: < 2%; Yellow: >2% and <3%; Green: >3%). Type species of the Tolypotrichaceae genera are in bold.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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Chapter IV

Description of four new filamentous cyanobacterial taxa from freshwater habitats in the Azores Archipelago

Luz, R., Cordeiro, R., Kaštovský, J., Johansen, J. R., Dias, E., Fonseca, A., Urbatzka R., Vasconcelos V. & Gonçalves, V. (2023). Description of four new filamentous cyanobacterial taxa from freshwater habitats in the Azores Archipelago. Journal of Phycology, 59(6), 1323-1338. https://doi.org/10.1111/jpy.13396

Chapter IV. Description of four new filamentous cyanobacterial taxa from freshwater habitats in the Azores Archipelago

Abstract

Simple filamentous cyanobacteria comprise a diverse and polyphyletic group of species, primarily in the orders Leptolyngbyales and Oscillatoriales, that need more sampling to improve their taxonomy. Oceanic islands, such as the Azores archipelago, present unique habitats and biogeographic conditions that harbor an unknown range of diversity of microorganisms. Filamentous cyanobacteria isolated from aquatic habitats in the Azores and maintained in the BACA culture collection were described using morphology, both light and transmission electron microscopy, ecology, and genetic data of the 16S rRNA gene sequences and 16S–23S Internal Transcribed Spacer (ITS) rRNA region secondary structure. Our analyses revealed two new monophyletic genera: *Tumidithrix elongata* gen. sp. nov. (Pseudanabaenaceae) and *Radiculonema aquaticum* gen. sp. nov. (Leptolyngbyaceae). In addition, two new species *Leptodesmis lacustris* sp. nov. (Leptolyngbyaceae) and *Pycnacronema lacustrum* sp. nov. (Wilmottiaceae) are reported as the first aquatic species for these genera. The description of these new taxa and the genetic study of an isolate of *Leptodesmis alaskaensis* from the Azores followed the polyphasic approach, identifying diacritical features. Our results reinforce the need for taxonomic studies on cyanobacteria from less-studied habits and geographic regions, which have a potential for new taxa description.

Keywords: 16S rRNA gene, lakes, Leptolyngbyales, Oscillatoriales, phylogeny, Polyphasic approach, streams

4.1 Introduction

The taxonomy of cyanobacteria has been rapidly changing, with many newly published taxa in the last few years based on morphologic, genetic, and ecological data (Komárek et al. 2020, Lima & Branco 2020, Soares et al. 2020, Zimba et al. 2020, Johansen et al. 2021, Moro et al. 2021, Cai et al. 2022, Tang et al. 2022, Strunecký et al. 2023). The use of this combined set of data is important, as many traditional genera are considered polyphyletic, such as the character-poor genus *Leptolyngbya* (Johansen et al. 2011, Komárek et al. 2014). The polyphasic approach has been crucial in some of the most recently described taxa, such as *Leptodesmis* (Raabová et al. 2019) for which molecular methods (e.g., the 16S rRNA gene phylogeny and 16S–23S ITS rRNA region) were important for taxon description. The study of other genera such as *Plectolyngbya*

(Taton et al. 2011), *Kamptonema* (Strunecký et al. 2014), *Pegethrix* (Mai et al. 2018), *Pycnacronema* (Martins et al. 2019), and *Tenebriella* (Hauerová et al. 2021), among many others, has been aided by the use of molecular methods for the description of new genera or species with dubious morphologic identification.

For cyanobacteria, habitat has always been important for genus or species description and identification. Several studies of polyphyletic taxa show a clear ecological restriction among morphologically similar cyanobacteria from marine and freshwater habitats (Engene et al. 2012, González-Resendiz et al. 2018). Within the freshwater taxa, cyanobacteria that inhabit thermal habitats represent a special cluster of understudied taxa with a clear phylogenetic separation (Cordeiro et al. 2020). The same can be seen between aquatic and terrestrial cyanobacteria (Komárek & Zapomìlová 2007, Wacklin et al. 2009), with recent studies having identified clear genome speciation related to habitat origin (Chen et al. 2021). Thus, the use of a polyphasic approach is of great importance in the study of simple filamentous cyanobacteria that comprise a diverse and polyphyletic group of species, primarily in the orders Leptolyngbyales and Oscillatoriales, with few morphologic diacritical characters (Strunecký et al. 2017). Furthermore, it has been recognized that this group of cyanobacteria needs more sampling to improve its taxonomy (Komárek et al. 2014).

The Azores is an oceanic archipelago of nine volcanic islands located in the North Atlantic Ocean, roughly 1500 km from Europe and 1900 km from North America. Despite their geographic proximity, each island is unique in habitat diversity, such as in having freshwater and saline lakes, thermal springs, and several permanent or temporary streams (Luz et al. 2022). Regardless of this broad array of suitable habitats for cyanobacteria, their diversity is still poorly known (Luz et al. 2022). The high diversity of cyanobacteria in the Bank of Algae and Cyanobacteria of the Azores (BACA) culture collection (Cordeiro et al. 2020), combined with the polyphasic approach, can contribute to the taxonomic knowledge of cyanobacteria.

In this work, we describe four new taxa from aquatic habitats, including two new monospecific genera, *Tumidithrix elongata* gen. sp. nov., a morphologically similar genus to *Arthronema* but with an evident distinct ecology, and *Radiculonema aquaticum* gen. sp. nov., a new genus with false branching and distinct morphologic characters. The other two taxa are *Leptodesmis lacustris* sp. nov. and *Pycnacronema lacustrum* sp. nov., isolated from aquatic habitats, both with unique morphology and phylogenetic placement relative to previously described species. In the *Leptodesmis* clade, a newly isolated strain of *Leptodesmis alaskaensis* from the entrance of a cave in Pico Island was also identified. This strain has been fully characterized, contributing to the full 16S–23S ITS rRNA region description, which had been missing from some published *Leptodemis* species.

4.2 Materials and Methods

Seven filamentous cyanobacterial strains isolated from samples collected in different islands of the Azores were retrieved from BACA culture collection for genetic and morphologic characterization (Table 1). These strains are maintained in the BACA collection with a 14:10 light:dark cycle (under 10–40 µmol photons · m^{-2} · s^{-1}) photoperiod at 19°C.

Table 1. Strain sampling location in the Azores archipelago, Portugal, and GenBank accession codes.

Strain	Taxonomy	Locality	Sampling date	GPS-coordinates	GenBank
BACA0078	*Pycnacronema lacustrum*	Capitão Lake, Pico Island	01-06-2017	38°29'12.8"N 28°19'05.7"W	OM732220
BACA0141	*Tumidithrix elongata*	Paul Lake, Pico Island	20-07-2016	38°25'43.7"N 28°13'56.2"W	MT176747
BACA0202	*Leptodesmis lacustris*	Caldeirão Pequeno Sul Lake, São Miguel Island	01-06-2013	37°49'23.5"N 25°45'01.9"W	OM732227
BACA0203	*Leptodesmis lacustris*	Peixe Lake, São Miguel Island	01-06-2013	37°49'07.3"N 25°44'10.9"W	MT176753
BACA0204	*Leptodesmis lacustris*	São Brás Lake, São Miguel Island	01-06-2013	37°47'35.0"N 25°24'36.6"W	OM732228
BACA0748	*Leptodesmis alaskaensis*	Torres Cave, Pico Island	25-06-2019	38°29'39.6"N 28°30'08.5"W	OP508344
BACA0731	*Radiculonema aquaticum*	Salto Stream, Santa Maria Island	30-10-2019	36°58'54.4"N 25°03'09.2"W	OM732264

Strains were morphologically characterized using a Leica DM4 B microscope with Digital Camera Leica MC 190 HD (Leica, Germany) using bright field and differential interference contrast. At least 25 trichomes and 50 cells were examined for each species to obtain cell dimensions and descriptions. The cultures were observed at different phases of growth for morphologic description. Holotypes were deposited in the Herbário Ruy Telles Palhinha (AZB), University of Azores, Portugal (Thiers 2023).

For DNA extraction, fresh cultures were extracted using the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol for Gram-negative bacteria. For the 16S rRNA gene and 16S–23S ITS rRNA region amplification, primers 27F (Neilan et al. 1997) and 23S30R (Lepère et al. 2000) were used in a polymerase chain reaction (PCR) with a total volume of 25 µL containing 1× PCR Buffer, 2 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (Thermo Fisher, Waltham, MA, USA), 0.5 pmol of each primer, 5–10 ng of DNA, and 1.5 U of Supreme NZYTaq II DNA polymerase (Nzytech, Portugal). Polymerase chain reaction conditions followed Taton et al. (2003), and thermal cycling was carried out in a ProFlex™ 3 × 32-well PCR System (Thermo Fisher, USA). Polymerase chain reaction amplification products were visualized by electrophoresis in 0.8% agarose gel and in 0.5 × TBE (Tris-Borate-EDTA)

buffer, and stained with SYBR™ SAFE. Amplified bands were removed from the gel using a clean scalpel and then purified using NZYGelpure (Nzytech, Portugal). Strains that produced one band were sent directly for sequencing. Strains with more than one band amplified had the PCR product cloned using the NZY-A PCR cloning kit (Nzytech, Portugal), following the manufacturer's protocol. Sequencing of the 16S rRNA gene and 16S–23S ITS rRNA region was conducted using the 27F (Neilan et al. 1997), 781F (Cordeiro et al. 2021), 781R (Nübel et al. 1997), CSIF (Janse et al. 2004), and 23S30R primers (Lepère et al. 2000).

The sequences of the studied strains were aligned with 281 sequences retrieved from GenBank using BLAST and reference strains of cyanobacteria species from previously published papers. The retrieved sequences represented most of the genetically and morphologically well-classified filamentous genera from the Leptolyngbyales and Oscillatoriales. The sequences were aligned in MAFFT v7.490 (Katoh & Standley 2013) using the G-INS-i algorithm, with the final alignment containing 1042 columns. The best-fit nucleotide model was assessed using ModelTest-NG (Darriba et al. 2020) in the raxmlGUI (Edler et al. 2021), with the selection of the TIM+G4+I evolution model. Phylogenetic trees were constructed using Bayesian inference (BI) with MrBayes v3.2.7a (Ronquist et al. 2012) on XSEDE through the CIPRES Science Gateway and maximum likelihood (ML) with the IQ-Tree online version v1.6.12 (Trifinopoulos et al. 2016), using *Gloeobacter violaceus* PCC 8105 as an outgroup. The BI was carried out with 5.0×10^6 generations, with two runs of four Markov chains, with custom parameters (nswaps = 4; temp = 0.01), sampling every 1000 generations, with a 0.25 burn-in rate (the final average standard deviation of split frequencies was less than 0.05) with the GTR+GAMMA+I model. The ML analysis was conducted using the TIM+G4+I model with 1000 ultrafast bootstrap replicates (Hoang et al. 2017). Trees were visualized using FigTree v1.4.4 (Rambaut 2012), and the final composite trees from maximum likelihood with bootstrap values for BI were re-drawn using Inkscape v1.2.

For the 16S rRNA gene p-distance, genetic information from phylogenetically relevant or morphologically relevant strains was selected. Sequences were aligned using MAFFT v7.490 with the G-INS-i method (Katoh & Standley 2013), and p-distance was calculated using MEGA 11.0.13 (Tamura et al. 2021). Values were transformed into percentages for easier reading (p-distance*100).

The 16S–23S ITS rRNA region secondary structures of the D1–D1', Box-B, and V3 helices were identified (Iteman et al. 2000), and secondary structures were predicted using M-fold (Zuker 2003) and re-drawn in Inkscape v1.2.

4.3 Results

The taxonomic analysis combining genetic, morphologic, and ecological data provided for the description of two new genera and four new species, all with strong genetic support either phylogenetically (Figure 1) or by p-distance (Table S1). Two new monospecific genera, *Tumidithrix elongata* gen. sp. nov. (Pseudanabaenaceae) and *Radiculonema aquaticum* gen. sp. nov. (Leptolyngbyaceae), and two new species, *Leptodesmis lacustris* sp. nov. (Leptolyngbyaceae) and *Pycnacronema lacustrum* sp. nov. (Wilmottiaceae), were identified.

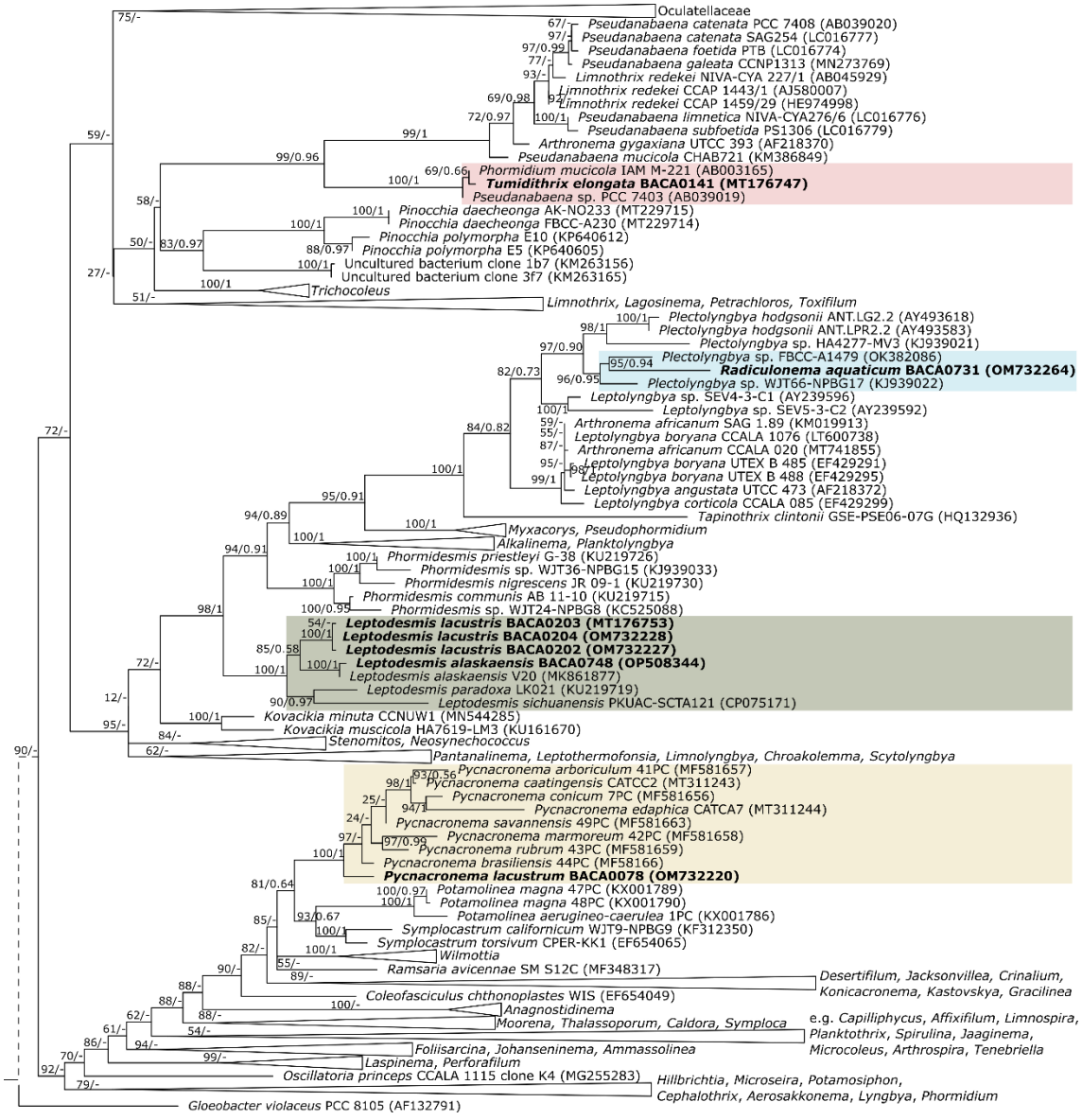

Figure 1. Partial maximum likelihood (ML) phylogenetic tree based on 16S rRNA genes. Bootstrap values for maximum likelihood and posterior probabilities for Bayesian inference are indicated on the tree. The studied strains are in bold font.

Within the Pseudanabaenaceae (Figure 1), *Tumidithrix elongata* gen. sp. nov. BACA0141 (Figure 2) was in a clade with *Pseudanabaena* sp. PCC 7403 (AB039019) and *Phormidium mucicola* IAM M-221 (AB003165). The morphologic differences with the enlarged and elongated cells present in *Tumidithrix elongata* corroborated the new genus description when compared to the closest phylogenetic genus *Pseudanabaena*. The phylogeny positioned the new genus in a separate and unique clade (Figure 1), and the p-distance analysis strongly supported the new genus with a high value (Table S1 in the Supporting Information; >7.9%) along with the 16S–23S ITS rRNA region secondary structure that showed several differences in both sequence and structure (Figure 3).

In the phylogenetic tree (Figure 1), *Radiculonema aquaticum* gen. sp. nov. BACA0731 (Figure 4) as within the Leptolyngbyaceae and closely related to *Leptolyngbya* sensu stricto and *Plectolyngbya hodgsonii* ANT.LPR2.2 (AY493583). In terms of morphology, *Plectolyngbya* and *Radiculonema* shared the trait of both tolypotrichoid and scytonematoid types of false branching. However, *Radiculonema* differed from *Plectolyngbya* in the formation of hemispherical colonies and divaricated trichomes and rounded wide apical cells. The p-distance analysis with the closest genera was superior to 4.4% (Table S1), and the phylogeny positioned the new genus in a separated clade with strong support (ML, 97; BI: 0.90). The 16S–23S ITS rRNA region analysis of *Radiculonema*, *Leptolyngbya*, and *Plectolyngbya* revealed some structural similarities, but their sequences were quite different (Figure 5).

In the 16S rRNA gene phylogeny of the genus Leptodesmis (Figure 1), three BACA strains formed a clade that was distinct from the other described species such that *L. lacustris* sp. nov. was identified (Figure 6). This species differed morphologically from the remaining *Leptodemis* species by cell size and genetically by its 16S rRNA gene p-distance (Table S1), phylogenetic placement, and different 16S–23S ITS rRNA region secondary structures (Figure 7). In the *Pycnacronema* clade (Figure 1), the new species *P. lacustrum* sp. nov. (Figure 8) was distinguished from the remaining species by its wider filaments and trichomes, genetic difference (Table S1), and different 16S–23S ITS rRNA region secondary structures (Figures 9–11).

A full comparison of described taxa and close phylogenetic and morphologically similar taxa is shown in Table S2 in the Supporting Information.

4.3.1 Taxonomic descriptions

Tumidithrix elongata **R.F.S.Luz, Kaštovský, J.R.Johans., V.Gonçalves gen. sp. nov. (Figure 2).**

Diagnosis: Phylogenetically distinct and placed in the Pseudanabaenaceae with distinct swollen or elongated cells, differentiating it from morphologically similar genera like *Pseudanabaena* and *Arthronema*.

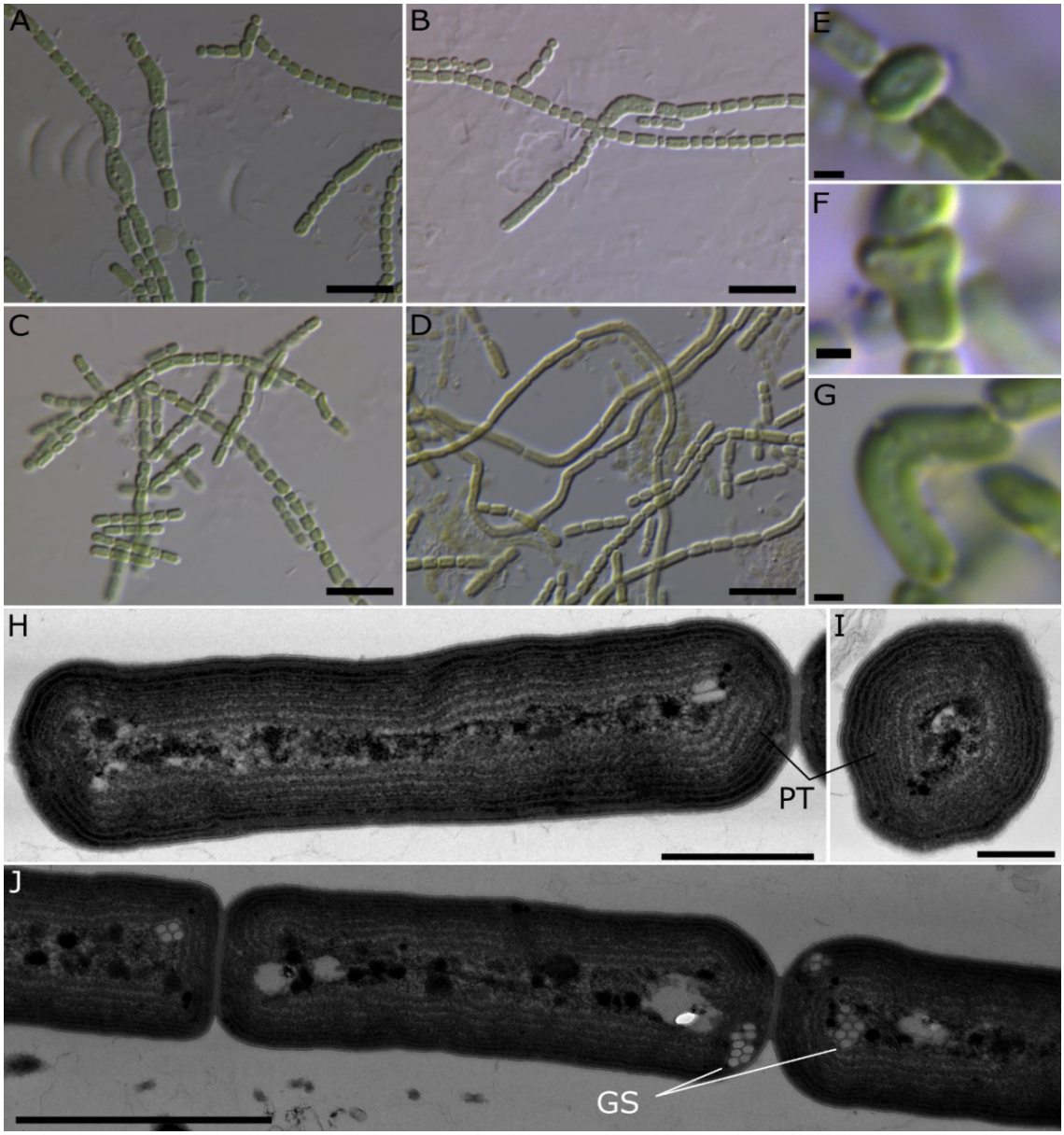

Figure 2. *Tumidithrix elongata* BACA0141 morphologic features in light microscopy (a–g) and TEM (h–j). (a, b) Swollen/involution cells; (c) Hormogonia; (d) Elongated cells; (e, f) different cell morphologies found in culture; (h, j) Longitudinal cut with visible gas vesicles; (i) Transversal cut; Scale for (a–d) 10 µm; scale for (e–h) 1 µm, scale for (i) 500 nm; scale for (j) 2 µm. GS, gas vesicles; PT, parietal thylakoids.

Description: Colony dark blue-green. Trichomes uniseriate, straight, slightly irregularly waived or bent, without sheaths. Hormogonia few celled, from two to eight cells. Cells cylindrical or barrel-shaped, constricted at cross walls normally with one or two polar granules, with small clusters of gas vesicles near the cross walls and parietal thylakoids. Cells mostly longer than wide, 1.2–2.0 µm wide (mean = 1.47 µm) and 1.3– 6.3 µm long (mean = 3.00 µm), with a length/width ratio of 0.8–4.6 (mean = 2.07). Cells sometimes irregularly swollen, straight to deeply bent, 1.75–3.77 µm wide and 4.0–21.0 µm long, elongated cells up to 30 µm, without necridia. End cells rounded.

Holotype: AZB 3908, type strain-dried material preserved in a permanently inactive state.

Type locality: Lagoa do Paul, Pico Island (Azores), Portugal (38°25'43.7" N 28°13'56.2" W). Collected by the MONITAIA project team on July 20, 2016, in a shallow freshwater lake.

Etymology: *Tumidithrix* – Tumidus (swollen) + thrix (hair), for its swollen cells, feminine; elongata—for its characteristic elongated cells.

Type strain: BACA0141 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal).

Gene Sequences: GenBank accession number MT176747 for the 16S rRNA gene and 16S–23S ITS rRNA region.

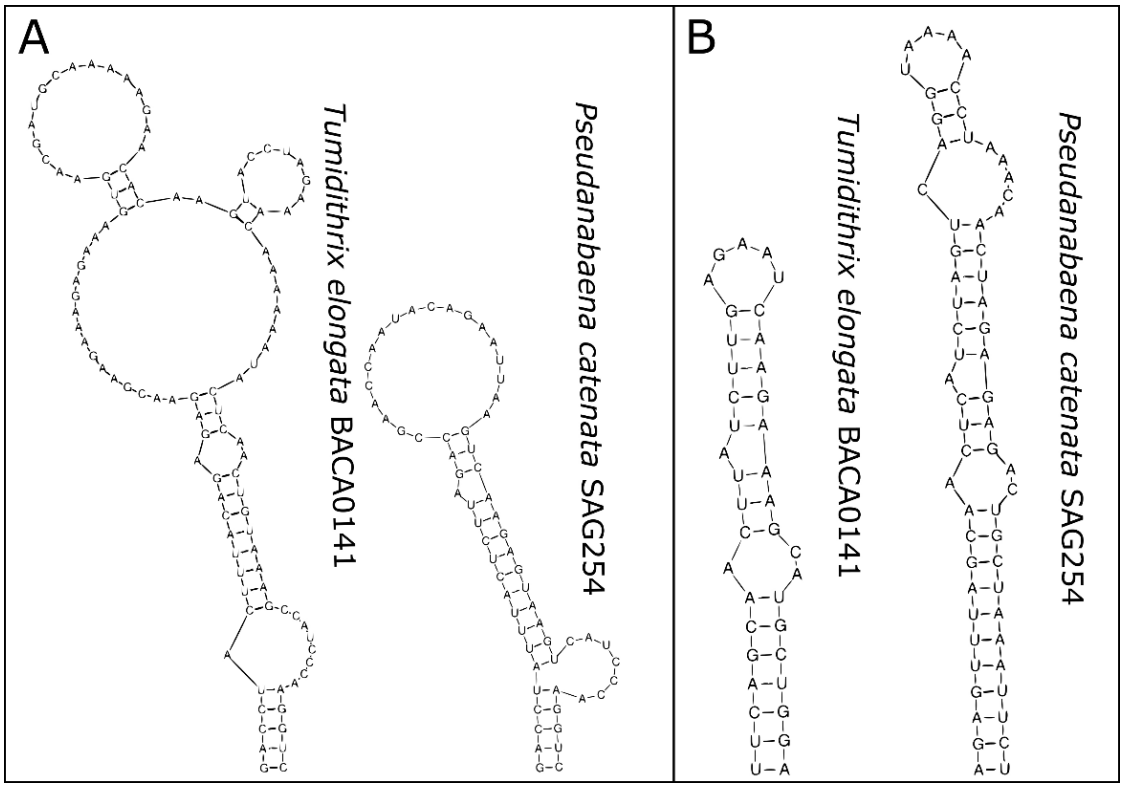

Figure 3. Secondary structure of the 16S–23S ITS rRNA region D1–D1' helix (a) and Box-B helix (b) in *Tumidithrix elongata* and *Pseudanabaena* type species.

Radiculonema aquaticum **R.F.S.Luz, Kaštovský, J.R.Johans., V.Gonçalves gen. sp. nov. (Figure 4).**

Diagnosis: Phylogenetically distinct and placed in the Leptolyngbyaceae by the 16S rRNA gene. Morphologically different from *Leptolyngbya* in branching and from *Plectolyngbya* in the formation of colonies and phylogenetic placement.

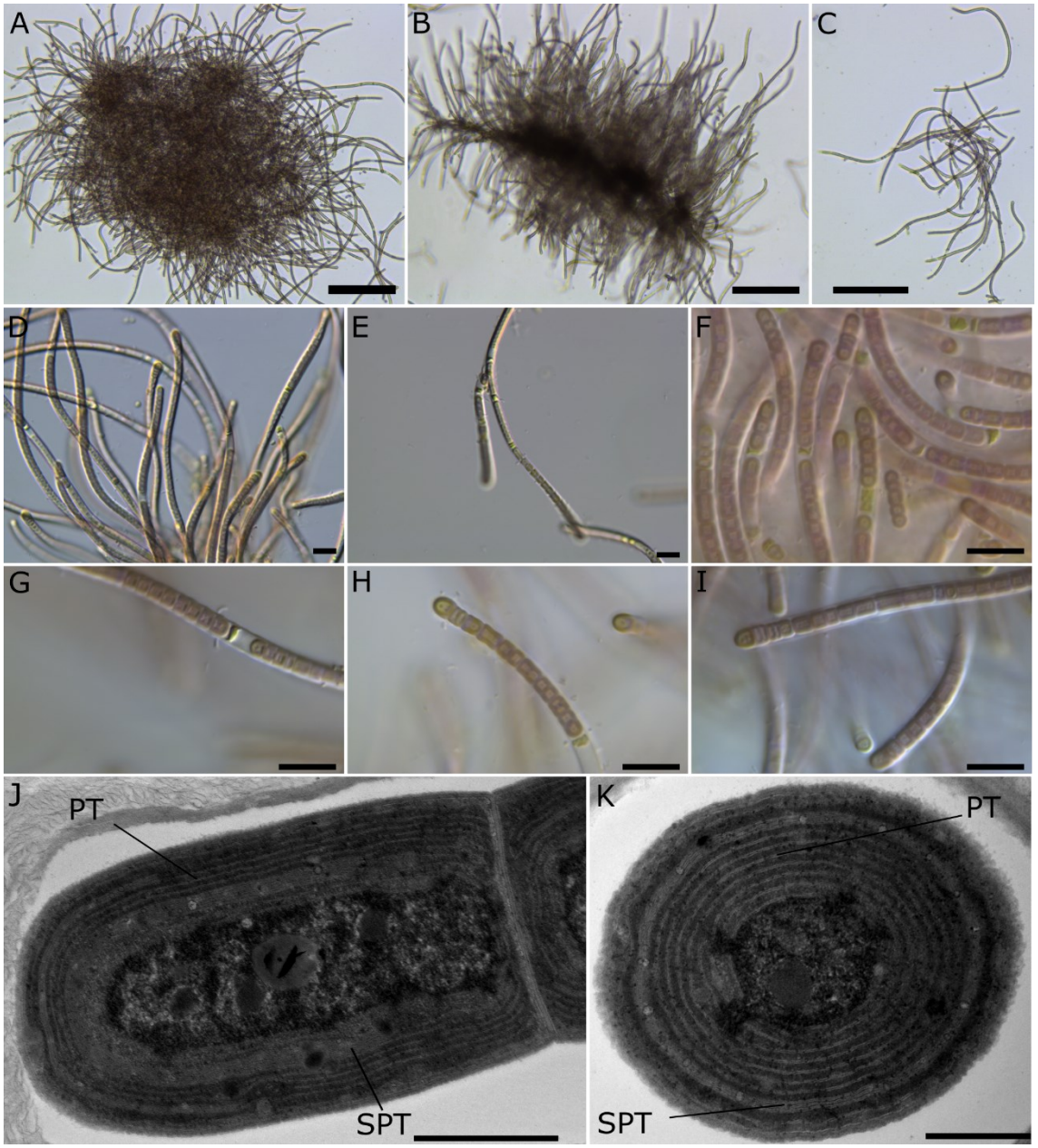

Figure 4. ***Radiculonema aquaticum*** **BACA0731 morphologic features under light microscopy (a–i) and TEM (j–k). (a–c) Formation of colonies; (d) Divaricated trichomes; (e) Trichome branching of tolypotrichoid type; (f–h) Necridia and fragmented trichomes; (h, i) Terminal cells; (j) Longitudinal cut; (k) Transversal cut. Scale for (a–c) 100 µm; scale for (d–i) 10 µm, scale for (j) 1 µm; scale for (k) 500 nm. PT, parietal thylakoids; SPT, subperipheral layer of parietal thylakoids.**

Description: Hemispherical colony greenish-brown, brown to purplish, growing as the culture ages with deeply entangled filaments. Filaments flexuous, false branched, 3.3–5.7 µm wide (mean = 4.10 µm). Sheath firm, colorless, attached to the trichome. Trichomes are untapered, constricted at cross walls. Hormogonia few celled. Cells shorter than wide, isodiametric, or longer than wide, 2.5–3.8 µm wide (mean = 3.05 µm) and 1.2–7.4 µm long (mean = 3.02 µm), with a length/width ratio of 0.4–2.3 (mean = 0.99). Thylakoids are parietal, somewhat visible in bright-field microscope, stacked in some cells, probably without phycobilisomes, creating a clear thickened layer of thylakoids when observed under TEM. Terminal cells are normally rounded wide. Necridia are common along the trichome.

Holotype: AZB 4485 type strain-dried material preserved in a permanently inactive state.

Type locality: Ribeira do Salto, Santa Maria Island (Azores), Portugal (36°58'54.4" N 25°03'09.2" W). Collected by Rúben Luz and Rita Cordeiro on October 30, 2019, attached to a submerged rock in a freshwater stream.

Etymology: *Radiculonema* – Radicula (small root) + nema (filament), for the grown shape of the colony, neutral gender; aquaticum—from an aquatic environment.

Type strain: BACA0731 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal).

Gene Sequences: GenBank accession number OM732264 for the 16S rRNA gene and 16S–23S ITS rRNA region.

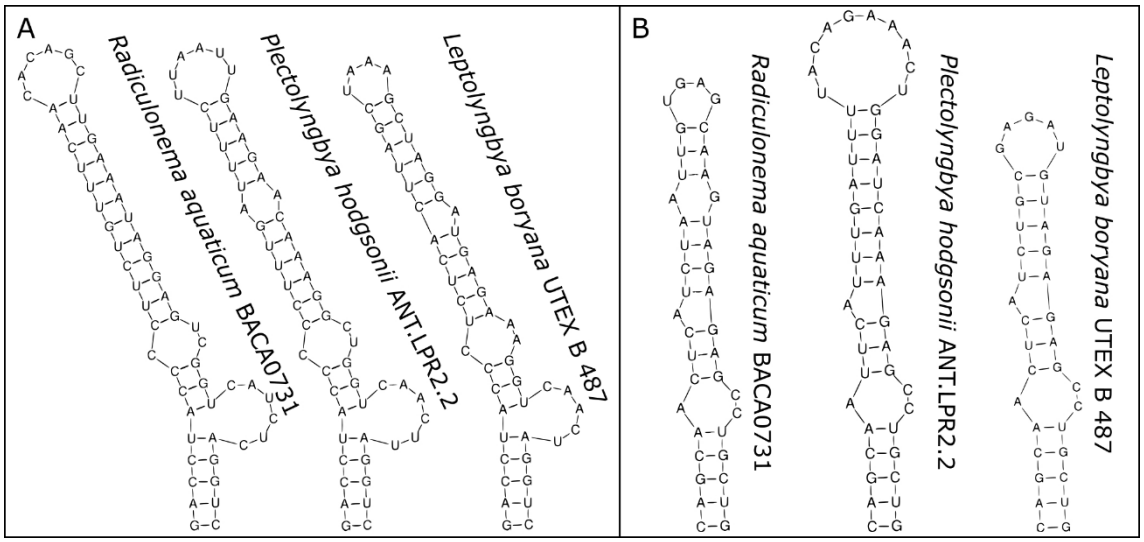

Figure 5. Secondary structure of the 16S–23S ITS rRNA region D1–D1' helix (a) and Box-B helix (b) in *Radiculonema aquaticum*, *Plectolyngbya*, and *Leptolyngbya* type species.

***Leptodesmis lacustris* R.F.S.Luz, Kaštovský, J.R.Johans., V.Gonçalves. sp. nov. (Figure 6).**

Diagnosis: It differs from *Leptodesmis paradoxa* by its narrower cells and from *L. alaskaensis* and *L. sichuanensis* mostly by its longer than wider cells (8-week cultures). *Leptodesmis lacustris* is also distinguished by its phylogenetic position.

Description: Colony blue-green to greenish, flat, with deeply entangled filaments. Filaments straight or flexuous, rarely coiled, sometimes parallel arranged, 1.8– 2.5 µm wide (mean = 2.15 µm). Sheath firm, colorless, attached to the trichome. Trichomes are untapered, not or slightly constricted at the visible cross walls, flexuous, without motility. Hormogonia few celled. Cells mostly longer than wide, even when in older cultures, commonly granulated, 1.1–2.1 µm wide (mean = 1.56 µm) and 1.2–4.3 µm long (mean = 2.10 µm), with a length/width ratio of 0.8–2.4 (mean = 1.35) with parietal thylakoids. Necridia are present occasionally. End cells rounded.

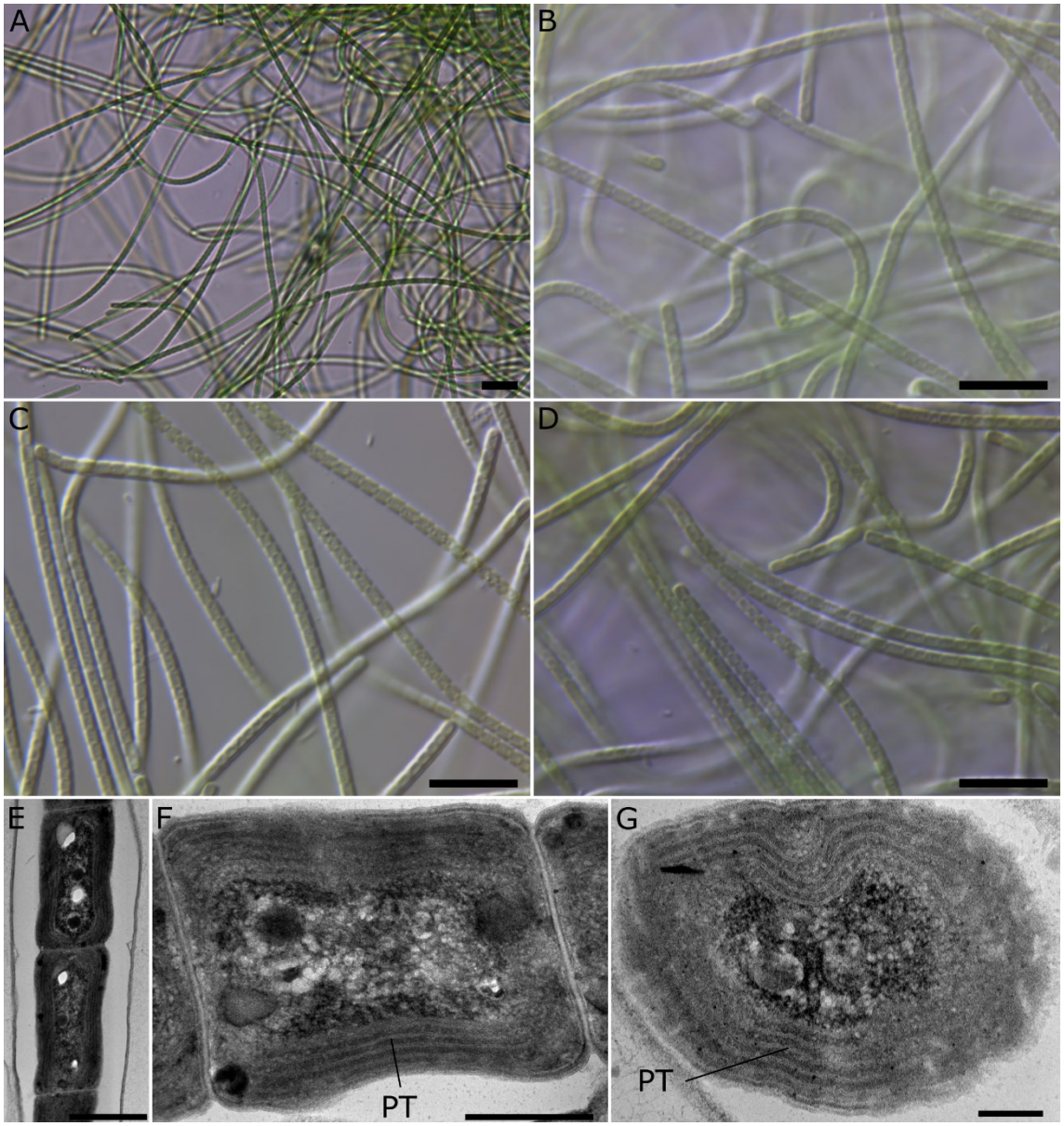

Figure 6. *Leptodesmis lacustris* morphologic features. (a, b) Randomly entangled trichomes; (c, d) Cell morphology and parallel arranged trichomes. (d, e) Longitudinal cut; (g) Transversal cut. Scale for (a) 100 µm, scale for (b–d) 10 µm, scale for (e) 1 µm, scale for (f) 500 nm, scale for (g) 200 nm. PT, parietal thylakoids.

Holotype: AZB 3969, type strain-dried material preserved in a permanently inactive state.

Type locality: Lagoa do Peixe, São Miguel Island (Azores), Portugal (37°49'07.3" N 25°44'10.9" W). Collected by Emanuel Xavier on June 1, 2013, in freshwater lakes.

Etymology: lacustris—as all strains were isolated from lakes.

Type strain: BACA0203 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal).

Gene sequences: GenBank accession number MT176753 for the 16S rRNA gene and 16S–23S ITS rRNA region.

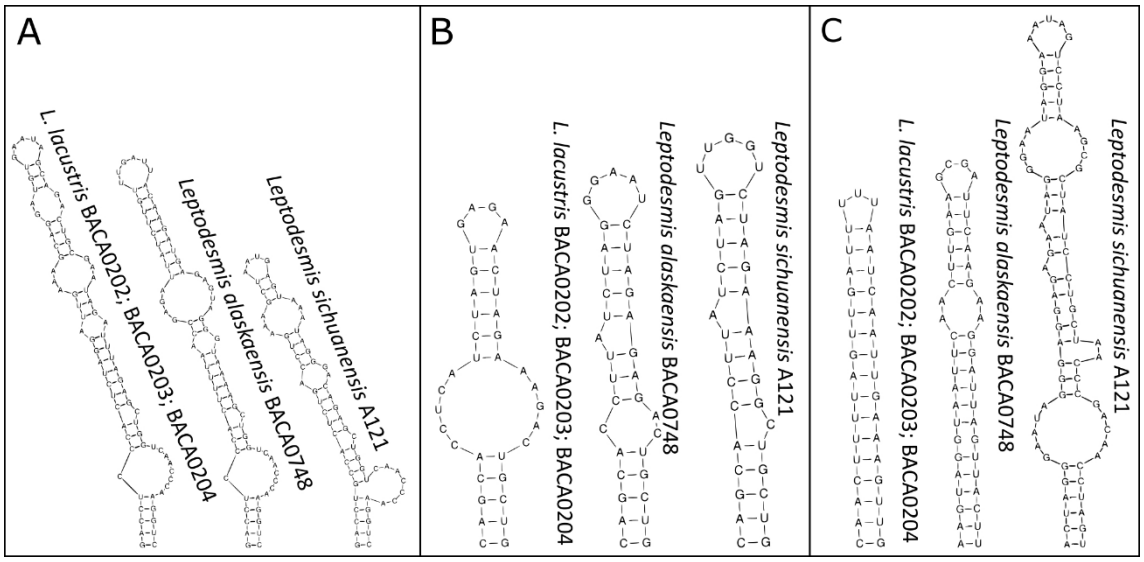

Figure 7. Secondary structure of the 16S–23S ITS rRNA region D1–D1' helix (a), Box-B helix (b), and V3 helix (c) of *Leptodesmis* spp.

***Pycnacronema lacustrum* R.F.S.Luz, Kaštovský, J.R.Johans., V.Gonçalves sp. nov. (Figure 8).**

Diagnosis: Distinguished by its wider filaments and trichomes and by the presence of stratified sheath in older filaments. The 16S rRNA gene phylogeny and 16S–23S ITS rRNA region results also strongly supported this new taxon.

Description: Colony blue-green tufted mat. Filaments long straight, sometimes slightly bent, entangled, 11.0–17.0 µm wide (mean = 14.12 µm). Sheaths normally firm, colorless, thin, or stratified up to 3 µm in older filaments. Trichomes untapered, slightly to distinctly constrict at cross walls, without motility. Cell content is blue-green, sometimes granulated. Cells are mostly shorter than wide, 6.6–14.4 µm wide (mean = 11.27 µm) and 2.5–12.2 µm long (mean = 7.05 µm), with a length/ width ratio of 0.2–1.4 (mean = 0.66), with parietal and fascicular thylakoids. Hormogonia not observed. Apical cells rounded, isodiametric, sometimes slightly longer than wide. Necridia present.

Holotype: AZB 3845, type strain-dried material preserved in a permanently inactive state.

Type locality: Lagoa do Capitão, Pico Island (Azores), Portugal (38°29'12.8" N 28°1'05.7" W). Collected by the MONITAIA project team on June 1, 2017, in a freshwater eutrophic lake.

Etymology: lacustrum—as the strain was isolated from a lake.

Type strain: BACA0078 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal).

Gene sequences: GenBank accession number OM732220 for the 16S rRNA gene and 16S–23S ITS rRNA region.

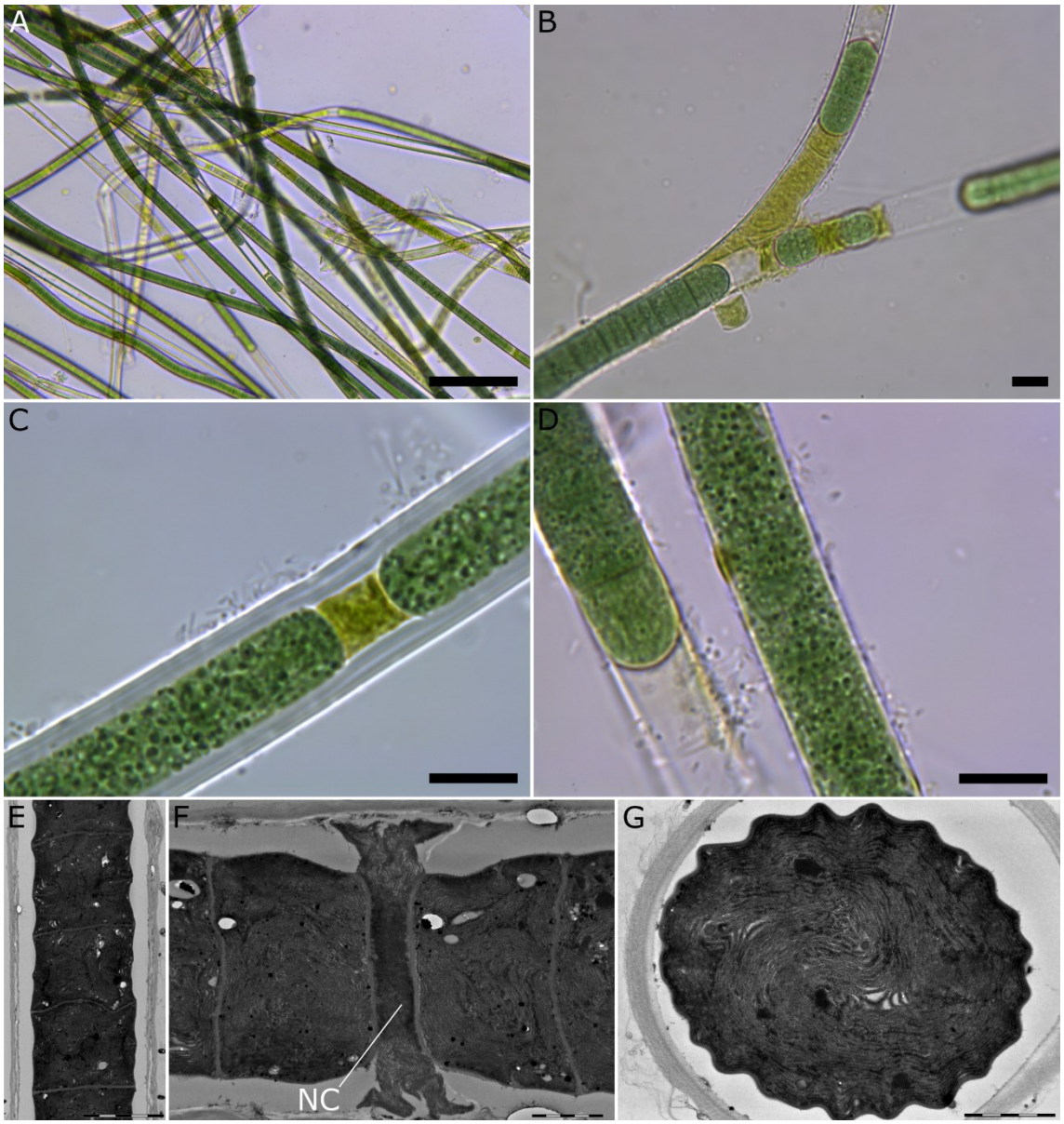

Figure 8. *Pycnacronema lacustrum* morphologic features. (a) Entangled trichomes forming a mat; (b) Fragment trichomes with false branching; (c) Necridia and thick sheath; (d) Rounded terminal cell. (e, f) Longitudinal cut; (g) Transversal cut; scale for (a) 100 µm, scale for (b–d) 10 µm, scale for (e) 5 µm, scale for (f, g) 2 µm. NC, Necridia.

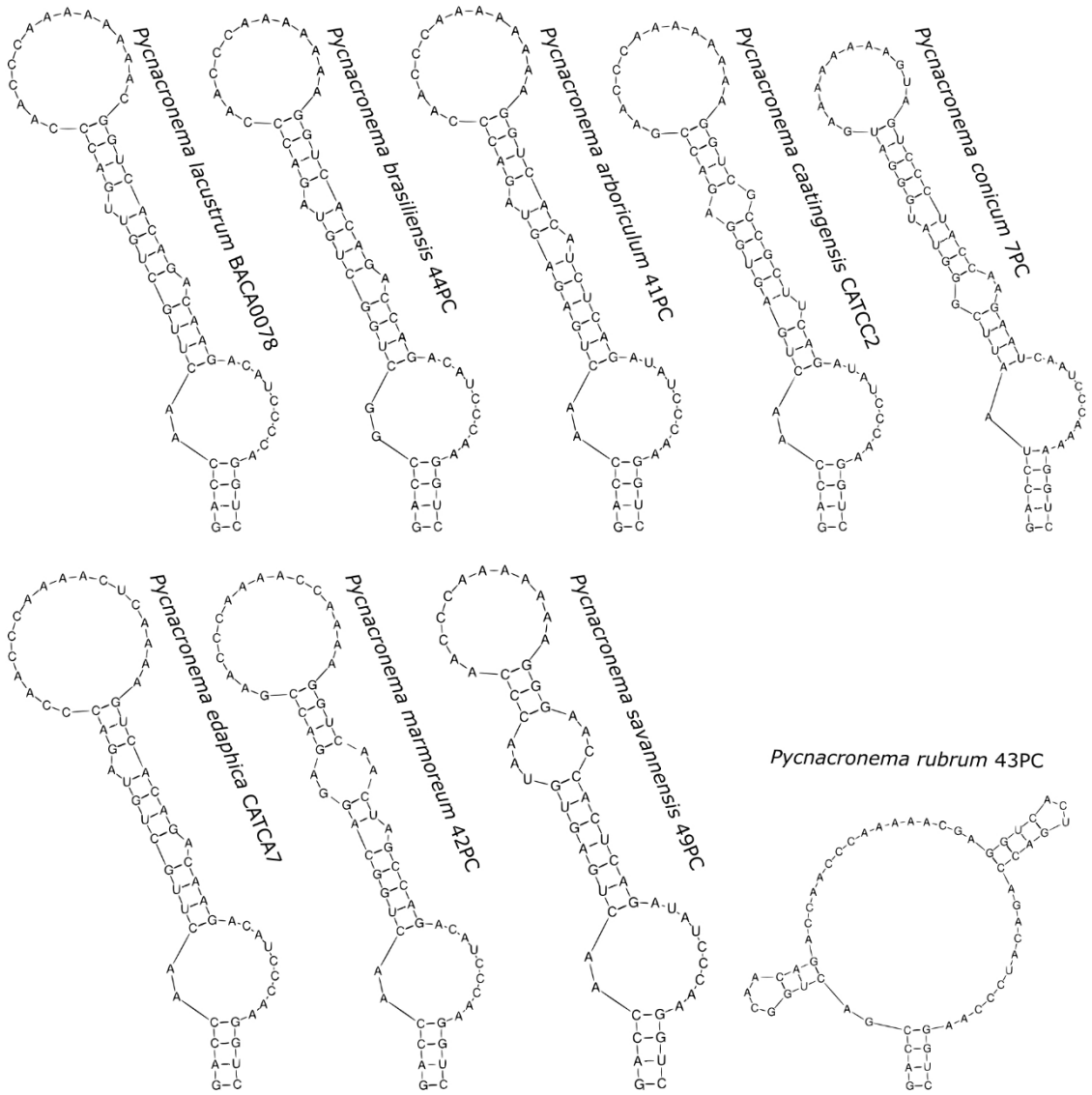

Figure 9. Secondary structure of the 16S–23S ITS rRNA region D1–D1' helix in *Pycnacronema* species.

4.4 Discussion

The Bank of Algae and Cyanobacteria of the Azores has a diverse culture collection of cyanobacteria isolated from several terrestrial and aquatic habitats (Cordeiro et al., 2020). Following a taxonomic characterization using morphology, molecular data, and ecology of selected strains enabled us to describe two new genera and four new species of filamentous cyanobacteria. The new taxa are phylogenetically well supported by 16S rRNA gene sequence data. The threshold used for species separation, 98.7% similarity in the 16S rRNA gene (Yarza et al., 2014), is a well supported value for all the new species (Table S1). For the distinction of new genera, the 16S rRNA gene 94.5% similarity threshold, suggested by Komárek et al. (2014) and Yarza et al. (2014), was surpassed, with a similarity to the closest phylogenetic and morphologic genera of *Tumidithrix* below 94.5%. This is not the case for *Radiculonema*, as the similarity values

are close to the generic threshold with *Leptolyngbya* and *Plectolyngbya*. Nevertheless, the similarity threshold values cannot be seen as a rule but only as an indication when clear discontinuities in morphology and ecology are identifiable (Komárek et al. 2014, Yarza et al. 2014).

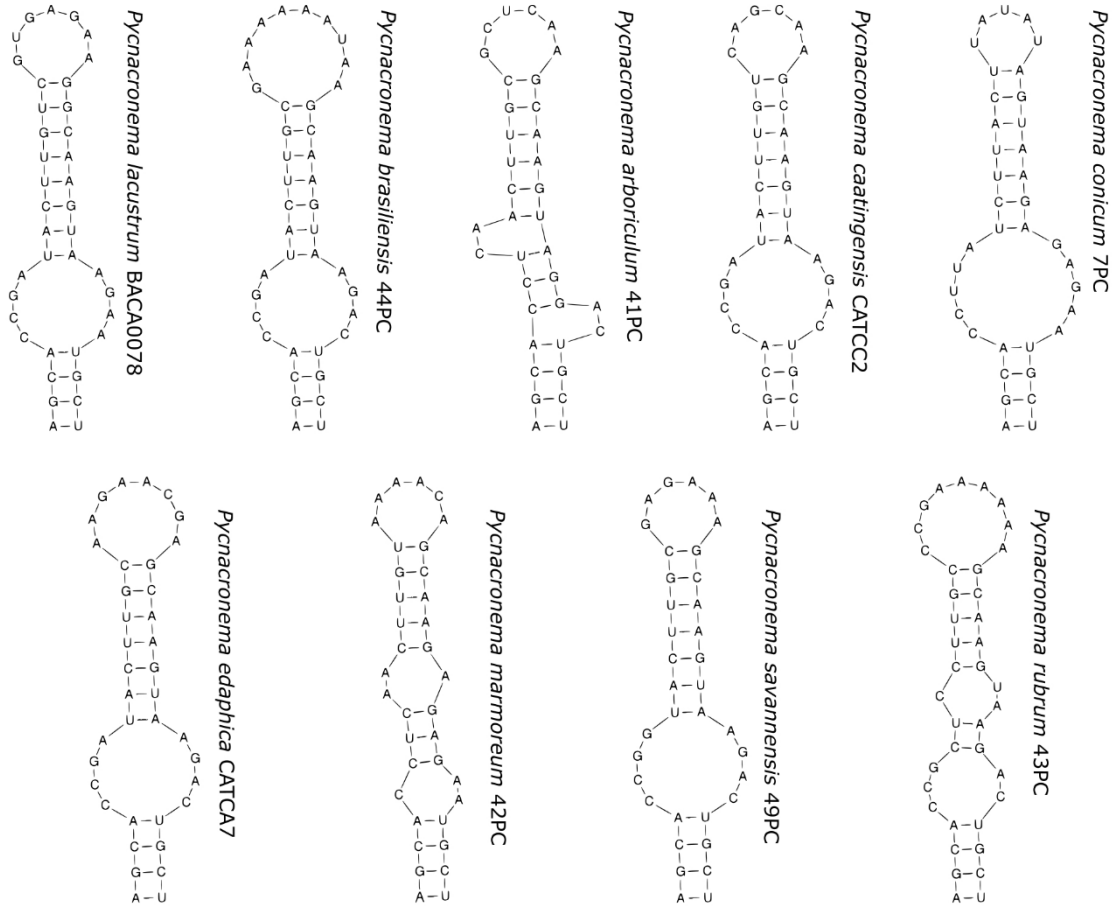

Figure 10. Secondary structure of the Box-B helix in *Pycnacronema* species.

The closest phylogenetic clade to *Tumidithrix* is *Pseudanabaena*, but *Tumidithrix* show a distinct phylogenetic placement within the Pseudanabaenaceae, and the 16S–23S ITS rRNA region structures revealed distinctive folding. Morphologically, *Tumidithrix* differs greatly from *Pseudanabaena* by the presence of elongated or swollen cells and gas vesicles. The strains *Pseudanabaena* sp. PCC 7403 and *Phormidium mucicola* IAM M-221, which are phylogenetically positioned close to *Tumidithrix elongata*, probably belong to *Tumidithrix*. However, as key information (e.g., 16S–23S ITS rRNA region and morphology) is missing, they should be treated as unknown species of *Tumidithrix* until a more detailed analysis is possible.

Tumidithrix elongata gen. sp. nov. morphologically resembles *Arthronema africanum* (Komárek & Lukavský 1988). However, these species have distinct geographic distribution and ecological preferences. *Arthronema africanum* was initially reported by Schwabe & Simonsen

(1961) from the Wau en-Namus brackish volcano lake (Libya); the CCALA 020 strain was isolated from within the sand of a dry lake (Kuwait), where crystalline salts could be found, whereas *Tumidithrix elongata* comes from a shallow freshwater lake in Pico Island (Azores) in the North Atlantic region. Furthermore, the new genus is phylogenetically distant from the strain used to describe the genus *Arthronema* (CCALA 020). The phylogenetic position of known *Arthronema* strains, the closest morphologic genus, presents some challenges: (1) *A. africanum* SAG 12.89 formed a separate and distinct clade in the Leptolyngbyaceae; (2) *A. gygaxiana*, described in Casamatta et al. (2005) depicting the characteristic involution cells, was positioned within the *Pseudanabena* spp. clade; and (3) *A. africanum* SAG 1.89 and *A. africanum* CCALA 020 were placed in the *Leptolyngbya* sensu stricto clade, a well-defined and recognized clade containing the type species *L. boryana*. This raises some concerns, as *Arthronema* was described taking into account the description of *Pseudanabaena africana* but based on the strain *A. africanum* LUKAVSKY 1980/1 by Komárek and Lukavský (1988) later maintained in the CCALA collection as CCALA 020. Since *A. africanum* CCALA 020 is phylogenetically positioned in the *Leptolyngbya* sensu stricto clade, the current validity of the genus is doubtful.

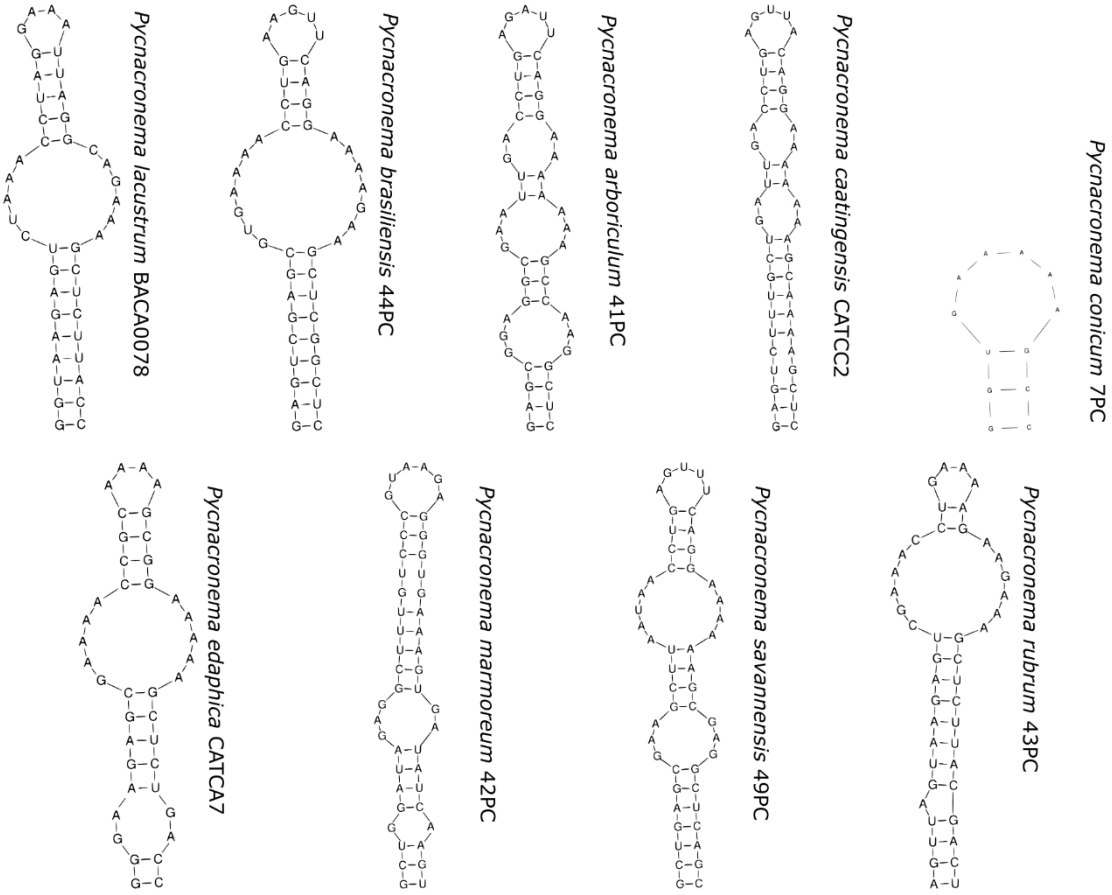

Figure 11. Secondary structure of the V3 helix in *Pycnacronema* species.

In the simple filamentous cyanobacteria, there are several examples of genera depicting false branching, such as *Plectonema* from the Oscillatoriales and, more recently, *Plectolyngbya* (Taton et al. 2011) from the Leptolyngbyales. *Radiculonema aquaticum* has false branching and forms compact hemispherical colonies. This branching feature, already seen by Taton et al. (2011) in *Plectolyngbya*, is a crucial characteristic for distinguishing *Radiculonema* from *Leptolyngbya* sensu stricto. Despite their similar branching, *Plectolyngbya* and *Radiculonema* can clearly be distinguished by the formation of hemispherical colonies, divaricated trichomes and the presence of wide rounded apical cells in *Radiculonema*. The thylakoids in the cells of *R. aquaticum* present a parietal distribution, with the absence of phycobilisomes in some of the thylakoids, or at least a modification in the phycobilisomes position, that allows a reduction in the space between thylakoids. This particular feature had already been observed in *Spirulina major* PCC 6313 (Mareš et al. 2019). The phylogenetic placement of this genus is clearly in the Leptolyngbyaceae, close also to the morphologically similar genera *Plectolyngbya* and *Leptolyngbya* sensu stricto. The 16S–23S ITS rRNA region secondary structures of the D1–D1' helix are somehow conserved in their secondary structure but with a different genetic sequence.

The description of *Leptodesmis* follows the review of *Phormidesmis* and is considered a cryptic genus to both *Phormidesmis* and *Leptolyngbya*, from which it is distinguished mostly by its 16S rRNA gene (Raabová et al. 2019). *Leptodemis lacustris* sp. nov. is identified by its phylogenetic position in the *Leptodesmis* clade but with different cell morphology and the presence of necridia. The complete 16S–23S ITS rRNA region sequence is not available from the type strain, and only a small part is available from *L. alaskaensis* (Strunecký et al. 2019). The full 16S–23S ITS rRNA region sequence presented here for *L. alaskaensis* BACA0748 allowed the folding of all the conserved 16S–23S ITS rRNA region structures, increasing the genetic knowledge of the genus. In the 16S–23S ITS rRNA region analysis, *L. lacustris* is more similar to *L. alaskaensis* in secondary structures but quite different from *L. sichuanensis*, which is not surprising due to the high phylogenetic distance observed in the 16S rRNA gene phylogeny.

Pycnacronema is a recent genus with eight described species, all from terrestrial habitats in Brazil (Martins et al. 2019, Lima & Branco 2020). *Pycnacronema lacustrum* sp. nov. is easily distinguishable morphologically from the other species of the genus by its wider trichomes and filaments. Phylogenetically, the new species is distinguished by the position at the base of the *Pycnacronema* clade in the 16S rRNA gene tree. When comparing the 16S–23S ITS rRNA region secondary structures among all *Pycnacronema* species, *P. lacustrum* shows the same type of structures and loop, though it is different in the genetic sequence.

4.5 Conclusions

This work contributes to clarifying the current taxonomic status of cyanobacteria with the description of the morphologically and genetically well-defined new genera *Tumidithrix* and *Radiculonema*. The description of these new taxa will help to improve the cyanobacteria taxonomy with its identification of diacritical morphologic attributes and new genetic information. Our study also suggests there are many species still to uncover globally and in the Azores. The discovery of two species of *Leptodesmis* in the Azores has increased the biogeographic distribution of this genus, and the enlargement of the ecological distribution of *Pycnacronema* to aquatic habitats confirms the plasticity of cyanobacteria and their ability to adapt to very different habitats and to "travel" across the globe. Further genomic work must be done, especially on such well-characterized strains, that can serve as base for further studies with more robust genetic analyses. In this case, a genomic approach, through ANI and phylogenomics, would help to clarify genera and species delimitation and should soon become the standard for new taxa description.

4.6 Acknowledgements

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4.7 Supporting Information

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. 16S rRNA gene p-distance of all studied strains and closest taxa. In red is a comparison between new genera and the type species of the closest genera. In green shade is highlighted the 16S rRNA gene p-distance between the type strains of the newly described species and related taxa.

Table S2. Morphological comparison of *Tumidithrix*, *Radiculonema*, *Pycnacronema*, *Leptodesmis* and close phylogenetic and morphological genera.

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Chapter V

New terrestrial cyanobacteria from the Azores Islands: description of *Venetifunis* gen. nov. and new species of *Albertania*, *Kovacikia* and *Pegethrix*

Luz, R., Cordeiro, R., Kaštovský, J., Johansen, J. R., Dias, E., Fonseca, A., Urbatzka R, Vasconcelos V. & Gonçalves, V. (2023). New terrestrial cyanobacteria from the Azores Islands: Description of *Venetifunis* gen. nov. and new species of *Albertania*, *Kovacikia* and *Pegethrix*. Phycologia, 62(5), 483-498. https://doi.org/10.1080/00318884.2023.2259243

Chapter V. New terrestrial cyanobacteria from the Azores Islands: description of *Venetifunis* gen. nov. and new species of *Albertania*, *Kovacikia* and *Pegethrix*

Abstract

The taxonomy of cyanobacteria has advanced quickly with the use of molecular methods in combination with well-defined morphological and ecological characters. Using this approach, many taxonomical changes have occurred in the Synechococcales and Oscillatoriales, with the description of new families, genera and species. Here we present the study of four cyanobacterial strains from the Bank of Algae and Cyanobacteria of the Azores (BACA) culture collection, all isolated from the Azores Archipelago. The strains were genetically characterized through the amplification of the 16S rRNA gene and 16S-23S rRNA internal transcribed spacer (ITS) region, as well as morphologically by light and transmission electron microscopy. One of the strains presented a high genetic divergence from known genera of Synechococcales and was described as *Venetifunis florensis* gen. & sp. nov. (Trichocoleusaceae, Cyanobacteria). The remaining three strains were found to be new species of the genera *Albertania*, *Kovacikia* and *Pegethrix*. *Venetifunis florensis* was isolated from the shores of Lake Rasa in Flores Island, *Kovacikia atmophytica* sp. nov. is a new species mainly distinguished genetically from other *Kovacikia* species from Terceira Island. *Albertania obscura* sp. nov. was isolated from inside a volcanic cave in Terceira Island and is distinct both morphologically and genetically from the already described species. *Pegethrix atlantica* sp. nov. is a new morphologically and genetically different species isolated from São Miguel Island. The description of these new taxa increases the biogeographic knowledge of the four mentioned cyanobacterial genera to remote oceanic islands and new terrestrial habitats.

Keywords: Aerophytic, Atlantic islands, Atmophytic, *Venetifunis minimus*, Leptolyngbyaceae, Oculatellaceae, Trichocoleusaceae

5.1 Introduction

Cyanobacteria occur in several types of habitats in aquatic and terrestrial ecosystems (Whitton & Potts 2012). Compared to aquatic habitats, cyanobacteria in terrestrial habitats are still relatively understudied (e.g. Garcia-Pichel et al. 2001, Hauer et al. 2015, Cordeiro et al. 2020), and the number of known species from these habitats is expected to increase in the future (Hauer et al. 2015), especially through the application of molecular methods (e.g.

Komárek et al. 2014, Sherwood et al. 2015). The use of a polyphasic approach allowed the description of several new genera and species, such as the aerophytic *Pegethrix* T. Mai, J.R. Johansen & Bohunická (Mai et al. 2018), *Pycnacronema* M. D. Martins & Branco (Martins et al. 2019), *Albertania* Zammit (Zammit 2018) and *Oculatella* Zammit, Billi & Albertano (Zammit et al. 2012). Although not so common, several reports are found in the literature for atmophytic cyanobacteria, growing on several types of substrates, such as the recently described genus *Cyanocohniella* Kaštovský, Berrendero, Hladil & J.R. Johansen, found in thermal springs from Karlovy Vary in Czech Republic (Kaštovský et al. 2014).

Albeit the use of a polyphasic approach was a breakthrough in the taxonomy of cyanobacteria, traditionally described taxa need to be sequenced so that this method can be fully applied (Komárek 2018). One of the greatest difficulties when describing new taxa using a polyphasic approach is the lack of genetic information for less-known genera and species, especially for type species of these genera. For instance, *Schizothrix* Kützing ex Gomont is a well-defined genus morphologically, now placed in the Trichocoleusaceae T. Mai & J.R. Johansen (Strunecký et al. 2023), but there is no molecular data for the type species, and the species for which data exist are phylogenetically positioned within the Leptolyngbyaceae (Osorio-Santos et al. 2014).

Of the recently described aerophytic genera, *Pegethrix* (Oculatellaceae; Mai et al. 2018, Shen et al. 2023, Strunecký et al. 2022) has five described species, all morphologically, genetically and ecologically characterized. Four of them are geographically restricted to the USA, with one undescribed species from continental east Antarctica (Mai et al. 2018) and one recently described from China (Shen et al. 2023). The speciation is not completely clear in some *Pegethrix* species, as *Pegethrix indistincta* T. Mai, J.R. Johansen & Bohunická is morphologically similar to *Pegethrix convoluta* T. Mai, J.R. Johansen & Bohunická, and p-distance of the 16S rRNA is low (below 1.3%), with the authors making use of 16S–23S rRNA ITS as the criterium for the separation of these species (Mai et al. 2018).

The genus *Albertania*, placed in the Leptolyngbyaceae, has two species described so far (Zammit 2018). *Albertania skiophila* Zammit was reported from several catacombs in Malta and Italy, within very humid and low-light habitats (Zammit 2018). The second species, *Albertania alaskensis* Strunecký, Raabova, Bernardova, Ivanova, Semanova, Crossley & Kaftan, was reported from the Alaska North Slope, USA, growing periphytically on roots or in snow, and showed significant morphological and phylogenetic distance from *A. skiophila* (Strunecký et al. 2019). The presence of the two species on different continents and rather distinct habitats reveals the wide geographical distribution and ecological adaptations of the genus, anticipating the possibility of its presence in other locations and habitats.

Kovacikia Miscoe, Pietrasiak & J.R. Johansen is currently a two-species genus with its type species *K. muscicola* Miscoe, Pietrasiak & J.R. Johansen reported from the Waikapala'e Cave in Hawaii, associated with moss growing on the cave walls (Miscoe et al. 2016). The other species was described from the Wuhan Botanical Garden in the Hubei Province in China as *K. minuta* L.Q. Shen, Renhui Li & B.S. Qiu (Shen et al. 2022), underneath macrophytes in a shaded shallow pond. These two species of *Kovacikia* are morphologically similar but quite distant genetically (Shen et al. 2022).

The present study describes four new taxa of Cyanobacteria, one taxon in the Leptolyngbyaceae, *Kovacikia atmophytica* sp. nov., two taxa in the Oculatellaceae, *Albertania obscura* sp. nov. and *Pegethrix atlantica* sp. nov., and one in the Trichocoleusaceae, *Venetifunis florensis* gen. & sp. nov. The characterization of these new cyanobacterial strains was based on a polyphasic approach, using the genetic markers 16S rRNA and 16S–23S rRNA ITS, morphologic characters assessed by light microscopy and transmission electron microscopy (TEM), as well as ecological preferences. The description of all four new taxa follows the International Code of Nomenclature for algae, fungi, and plants (Turland et al. 2018).

5.2 Material and Methods

5.2.1 Strains and grown conditions

Four filamentous cyanobacterial strains (BACA0077, BACA0587, BACA0619 and BACA0713) were retrieved from the Bank of Algae and Cyanobacteria of the Azores (BACA) for genetic and morphological characterization. The selected strains were all isolated from samples collected in the Azores islands between 2017 and 2020 (Table 1), and are maintained in the BACA collection with a 14:10 h light:dark cycle (under 10–40 µmol photons m^{-2} s^{-1}) at a temperature of 19°C.

Table 1. Location of sampling sites of studied strains in the Azores archipelago, Portugal.

Strain	Taxonomy	Locality	Habitat	Sampling Date	Coordinates
BACA0077	*Pegethrix atlantica*	Sete Cidades, São Miguel Island	Aerophytic	13 Jul 2017	37°51.308'N, 25°47.178'W
BACA0587	*Venetifunis florensis*	Lagoa Rasa, Flores Island	Aerophytic	29 Apr 2019	39°24.565'N, 31°13.456'W
BACA0619	*Kovacikia atmophytica*	Furnas do Enxofre, Terceira Island	Atmophytic	04 Mar 2020	38°43.733'N, 27°13.892'W
BACA0713	*Albertania obscura*	Algar do Carvão, Terceira Island	Aerophytic	13 Nov 2019	38°43.657'N, 27°12.908'W

5.2.2 Morphological characterization

All strains were characterized using a Leica DM4 B microscope with Digital Camera Leica MC 190 HD (Leica, Wetzlar, Germany) using bright field and differential interference contrast (DIC). At least 25 filaments or trichomes and 50 cells were examined from each strain to obtain cell dimensions and descriptions from cultures with two weeks and two months growth. The cultures were examined at different ages of growth for morphological description.

5.2.3 DNA extraction, 16S rRNA and 16S–23S rRNA ITS gene amplification, and sequencing

Fresh cultures were used for DNA extraction with the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, California, USA), following the protocol suggested by the manufacturer for Gram-negative bacteria.

For the 16S rRNA gene and 16S–23S rRNA ITS amplification, the primers 27F and 23S30R (Table 2) were used in a polymerase chain reaction (PCR) with a total volume of 25 µl containing 1× PCR Buffer, 2 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (Thermo Fisher, Waltham, Massachusetts, USA), 0.5 pmol of each primer, 5–10 ng of DNA and 1.5 U of Supreme NZYTaq II DNA polymerase (Nzytech, Portugal). PCR conditions followed Taton et al. (2003), and thermal cycling was carried out in a ProFlex™ 3 × 32-well PCR System (Thermo Fisher). PCR amplification products were visualized by electrophoresis in 0.8% agarose gel, in 0.5 × TBE (Tris-Borate-EDTA) buffer, stained with SYBR™ SAFE.

Table 2. Primers used for PCR and sequencing.

Name	Sequence	Reference
27F	AGA GTT TGA TCC TGG CTC AG	Neilan et al. (1997)
359F	GGG GAA TYT TCC GCA ATG GG	Nübel et al. (1997)
781R	GAC TAC TGG GGT ATC TAA TCC CAT T	Nübel et al. (1997)
781F	GGG ATT AGA TAC CCC TGT AGT C	Cordeiro et al. (2021)
CSIF	G(T/C)C ACG CCC GAA GTC (G/A)TT AC	Janse et al. (2004)
23S30R	CTT CGC CTC TGT GTG CCT AGG T	Lepère et al. (2000)

Amplified bands were removed from the gel using a clean scalpel and then purified using NZYGelpure (Nzytech). Strains that produced just one sized band were sent directly for sequencing. PCR products from strains that had more than one sized band amplified were cloned using the NZY-A PCR cloning kit (Nzytech), following the manufacturer's protocol. Sequencing of

the 16S rRNA and 16S–23S rRNA ITS region was conducted using the 27F, 781F, 781R, CSIF and 23S30R primers (Table 2).

5.2.4 Phylogenetic analysis

The sequences of the new taxa were aligned with 262 sequences retrieved from GenBank using BLAST, and reference strains of cyanobacterial species from previously published papers. The retrieved sequences represent most of the filamentous genera from the Synechococcales and Oscillatoriales that are well-classified genetically and morphologically. The sequences were aligned in MAFFT v7.490 (Katoh & Standley 2013) using the G-INS-i algorithm, with the final alignment containing 1,055 columns.

Phylogenetic trees were constructed using Bayesian inference (BI) with MrBayes v3.2.7a (Ronquist et al. 2012) on XSEDE through the CIPRES Science Gateway, and Maximum likelihood (ML) with the IQ-Tree online version v1.6.12 (Trifinopoulos et al. 2016), using *Gloeobacter violaceus* PCC 8105 as an outgroup. The BI was carried out with 5.0×10^6 generations, with two runs of four Markov chains, with custom parameters (temp = 0.015), sampling every 1,000 generations and a 0.25 burn-in rate (the final average standard deviation of split frequencies was lower than 0.05) with the GTR+GAMMA +I model. The ML analysis was carried out using the best-fit model TVMe+I+G4, chosen according to the Bayesian Information Criterion in ModelFinder (Kalyaanamoorthy et al. 2017), with 1,000 ultrafast bootstrap replicates (Hoang et al. 2017) and final Bootstrap correlation coefficient of split occurrence frequencies of 0.993. Trees were visualized using FigTree v1.4.4 (Rambaut 2012) and the final composite trees from Maximum likelihood with prior probabilities values for BI were re-drawn using Inkscape v1.2.

5.2.5 Analyses of the 16S–23S rRNA ITS region

The 16S–23S rRNA ITS secondary structures of the D1–D1', Box-B and V3 helix were identified following the obtained results at M–fold, using the default parameter settings (Zuker 2003), in combination with previously published 16S–23S rRNA ITS secondary structures, and re-draw in Inkscape v1.2.

5.3 Results

Analysis of the 16S rRNA gene sequencing results (Fig. 1) reveals the phylogenetic positioning and distinction of the studied strains. *Venetifunis florensis* BACA0587 (Figs 2–9) formed a distinct clade with two unidentified OTUS in the Trichocoleusaceae near *Pinocchia* P.

Dvořák, Jahodářová et P. Hašler and *Trichocoleus* Anagnostidis, with a p-distance higher than 5.5% to both (Table S1), a high value supporting the new genus description as well. The described species *Pegethrix atlantica* BACA0077 (Figs 10–16), *Albertania obscura* BACA0713 (Figs 17–23) and *Kovacikia atmophytica* BACA0619 (Figs 24–29) clustered well within the assigned genera, all with a 16S rRNA p-distance equal or higher than 1.3% (Table S1) and the 16S–23S rRNA ITS p-distance values for the reported species all higher than 7%, namely more than 14% for *Pegethrix atlantica* (Table S2), more than 11% for *Albertania obscura* (Table S3) and more than 11% for *Kovacikia atmophytica* (Table S4), supporting genetically the description of the new species.

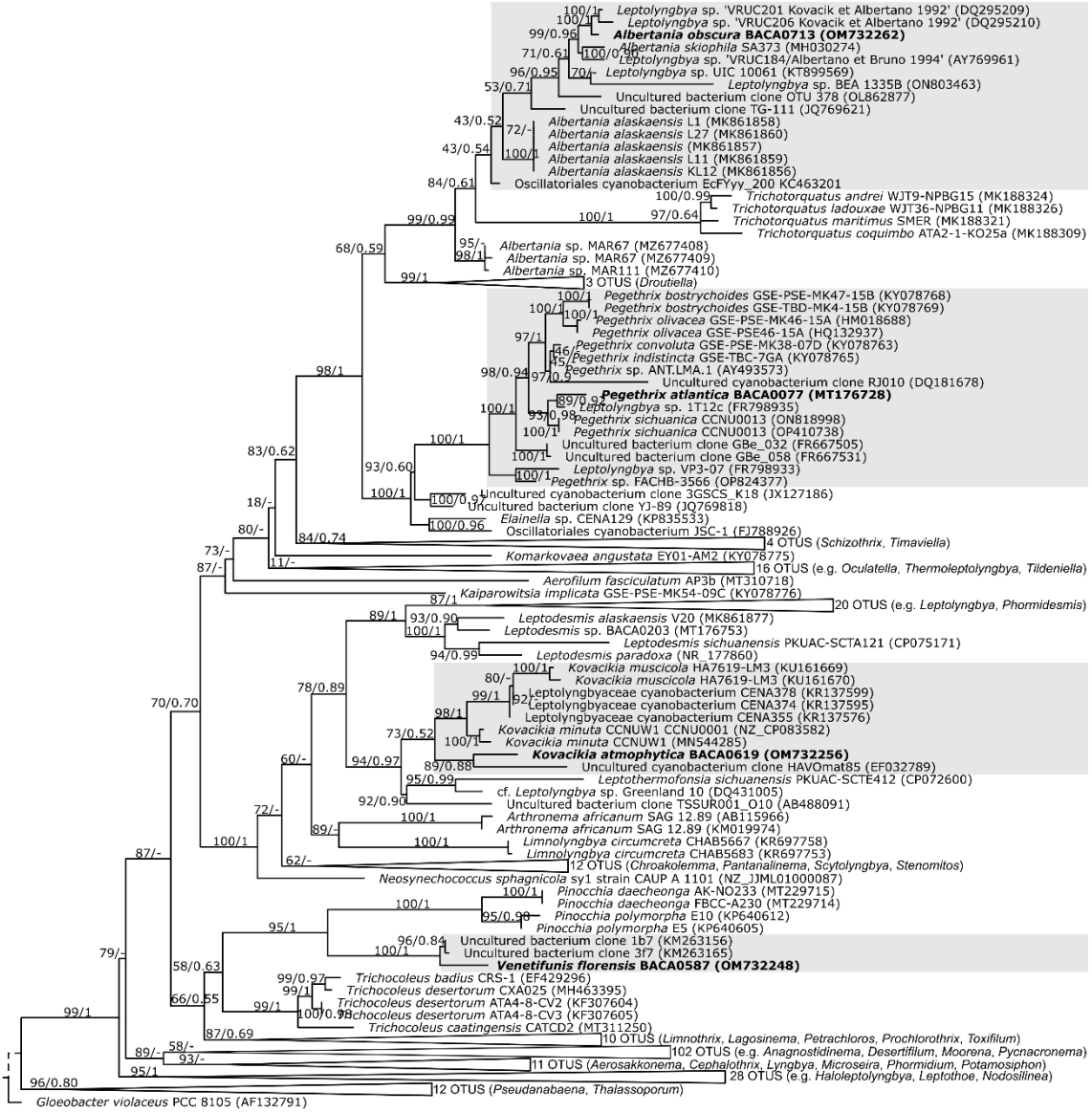


Figure 1. Partial Maximum Likelihood (ML) phylogenetic tree based on 16S rRNA sequences depicting *Albertania* spp., *Venetifunis florensis*, *Kovacikia* spp., *Pegethrix* spp. and other cyanobacterial strains. Bootstrap values for maximum likelihood and posterior probabilities for Bayesian Inference are indicated on the tree. The novel species are in boldface and studied genera are shaded in grey.

The complete 16S–23S rRNA ITS sequence was retrieved from all studied strains, with both $tRNA^{Ile}$ and $tRNA^{Ala}$ within the sequence. All conserved regions (e.g. D1–D1', Box-B and V3 helix) were identified in the sequences. In all described species, the 16S–23S rRNA ITS sequences were aligned, with some conservation of the regions both in sequence length and secondary structure observed, supporting the assignment to the phylogenetic and morphological identified genus. The 16S–23S rRNA ITS secondary structure of *Venetifunis florensis* varies greatly from the closest phylogenetic taxa to the point where the Box-B alignment was not possible.

Morphologically the new genus and species are distinct with clear diacritical morphological features that allow for its delimitation from close taxa, apart from the new *Kovacikia* species that is mainly genetically and ecologically differentiated. A schematic morphological comparison is presented in Table S5.

***Venetifunis* R.F.S. Luz, Kaštovský, J.R. Johansen & V. Gonçalves gen. nov.**

Diagnosis: Distinguishable genetically and morphologically from all remaining genera. Positioned in the Trichocoleusaceae with a clear phylogenetic position, creating a new genetic clade with a 16S rDNA p-distance greater than 5.5% from closely related taxa (*Pinocchia* and *Trichocoleus*). Morphologically, *Venetifunis* is described with an always diffluent mucilage and rounded terminal cells, distinguished from similar taxa like *Schizothrix*, which has clear pointed and common delimited sheaths with trichomes enveloped in a single sheath, and *Trichocoleus*, with its delimited, diffluent and open sheaths, and commonly with conical terminal cells.

Description: Colonies dense, blue-green, greenish, of irregular shape with diffluent mucilage. Filaments with diffluent mucilage containing several trichomes in parallel arrangement and/or with a rope-like organization. Trichomes uniseriate, straight, irregularly waived, entangled, with motility. Cells longer than wide, constricted at cross walls, sometimes with one polar granule at cross walls, with parietal thylakoids. End cells rounded.

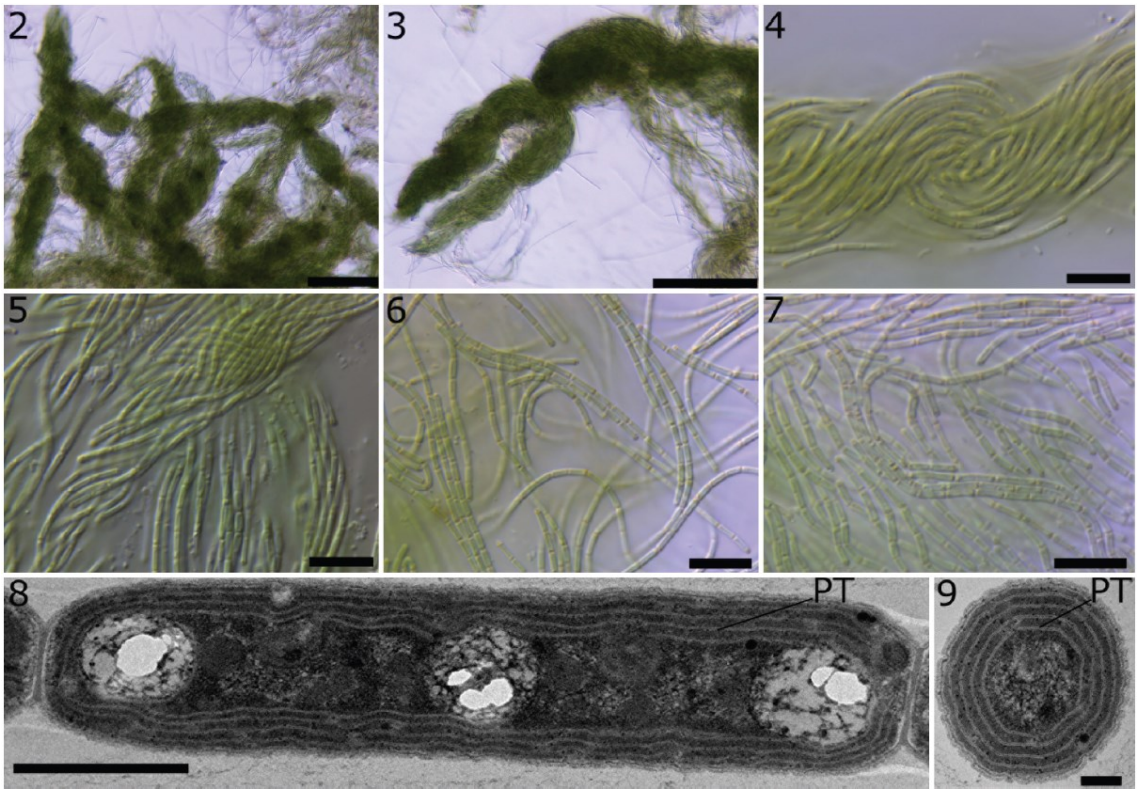
Type species: *Venetifunis florensis* R.F.S. Luz, Kaštovský, J.R. Johansen & V. Gonçalves

Etymology: From Latin venetus, blueish-green and Latin funis, rope, alluding to the interwoven arrangement of the blue-green trichomes in a filament. Masculine gender.

***Venetifunis florensis* R.F.S. Luz, Kaštovský, J.R. Johansen & V. Gonçalves sp. nov. Figs 2–9**

Description: Colonies dense, dark blue-green to greenish, of irregular shape with diffluent mucilage. Filaments with diffluent mucilage always with several trichomes in parallel arrangement, sometimes with a rope-like organization. Trichomes uniseriate, straight, irregularly waived, entangled, motile. Hormogonia few-celled. Cells are always longer than wide, constricted at cross walls, 0.7–1.2 µm wide (mean = 1.05), 2.1–5.8 µm long (mean = 3.41), with

a length: width ratio of 2.0–6.8 (mean = 3.30), sometimes with one polar granule at cross walls and parietal thylakoids. Necridia not observed. End cells rounded.



Figures 2-9. *Venetifunis florensis* BACA0587 morphological features in LM and TEM. Fig. 2. Colonies of *Venetifunis florensis*, with several different fascicles of trichomes arranged in parallel, and loose trichomes. Scale bar = 100 µm. Fig. 3. Trichomes arranged in parallel. Scale bar = 100 µm. Fig. 4. Trichomes arranged in parallel in a common diffluent mucilage with rope-like organization. Scale bar = 10 µm. Fig. 5. Trichomes arranged in parallel in a common diffluent mucilage with entangled rope-like organization. Scale bar = 10 µm. Fig. 6. Loose trichomes with the visible longer than wide cells and rounded end cells. Scale bar = 10 µm. Fig. 7. Loose trichomes with the visible longer than wide cells and rounded end cells. Scale bar = 10 µm. Fig. 8. Longitudinal section of vegetative cell (TEM), with parietal thylakoids and cell constrictions, and no individual sheath. Scale bar = 1 µm. Fig. 9. Transverse section of a vegetative cell (TEM) with parietal thylakoids (PT). Scale bar = 200 nm.

Holotype: Dried material preserved in a metabolically inactive state at Herbário Ruy Telles Palhinha, University of Azores, Portugal, with the code AZB 4344.

Type locality: Rocky wet substrate in the shores of Lagoa Rasa in Flores Island, Azores archipelago, Portugal, 39°24.565'N, 31°13.456'W. Collected 29 April 2019 by Rúben Luz and Rita Cordeiro.

Habitat: Aerophytic in rock substrate on the lake shores.

Etymology: Epithet florensis, -e, referring to the inhabitants of Flores Island, in the Azores.

Reference strain: BACA0587 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal), isolated by Rúben Luz.

Gene sequences: GenBank accession number OM732248 for the 16S rRNA and 16S–23S rRNA ITS genes.

***Venetifunis minimus* (Frémy) R.F.S. Luz, Kaštovský, J.R. Johansen & V. Gonçalves comb. nov.**

Basionym: *Microcoleus minimus* Frémy 1930, Archives de Botanique, Mémoires 3 (Mémoire 2): 82, fig. 83.

Homotypic synonym: *Trichocoleus minimus* (Frémy) Anagnostidis (2001, p. 369).

***Pegethrix atlantica* R.F.S. Luz, Kaštovský, J.R. Johansen & V. Gonçalves sp. nov. Figs 10–16**

Diagnosis: Distinguishable genetically and morphologically from all species. Phylogenetically positioned in the *Pegethrix* clade, with 16S rRNA p-distance greater than 1.3% and a 16S–23S rRNA ITS p-distance greater than 14% to all described species. Morphologically closer to *P. indistincta* T. Mai, J.R. Johansen & Bohunická, but always without common sheaths enclosing trichomes and instead with very fine and delimited ones.

Description: Colony blue-green to greenish, with long fasciculate filaments and sometimes entangled. Filaments straight or loosely coiled, very rarely with single false branching and without nodule formation, 2.4–3.7 µm wide (mean = 3.11 µm). Sheath firm, colourless, attached to the trichome. Trichomes untapered, not or slightly constricted at the visible cross-walls, flexuous, with abundant necridia in mature trichomes. Hormogonia few celled. Cells mostly shorter than wide, sometimes with a unique central granule, with parietal thylakoids, 1.8–3.0 µm wide (mean = 2.25 µm) and 1.0–2.4 µm long (mean = 1.55 µm), with a length:width ratio of 0.4–1.1 (mean = 0.69). End cells rounded.

Holotype: Dried material preserved in a metabolically inactive state at Herbário Ruy Telles Palhinha, University of Azores, Portugal, with the code AZB 3844.

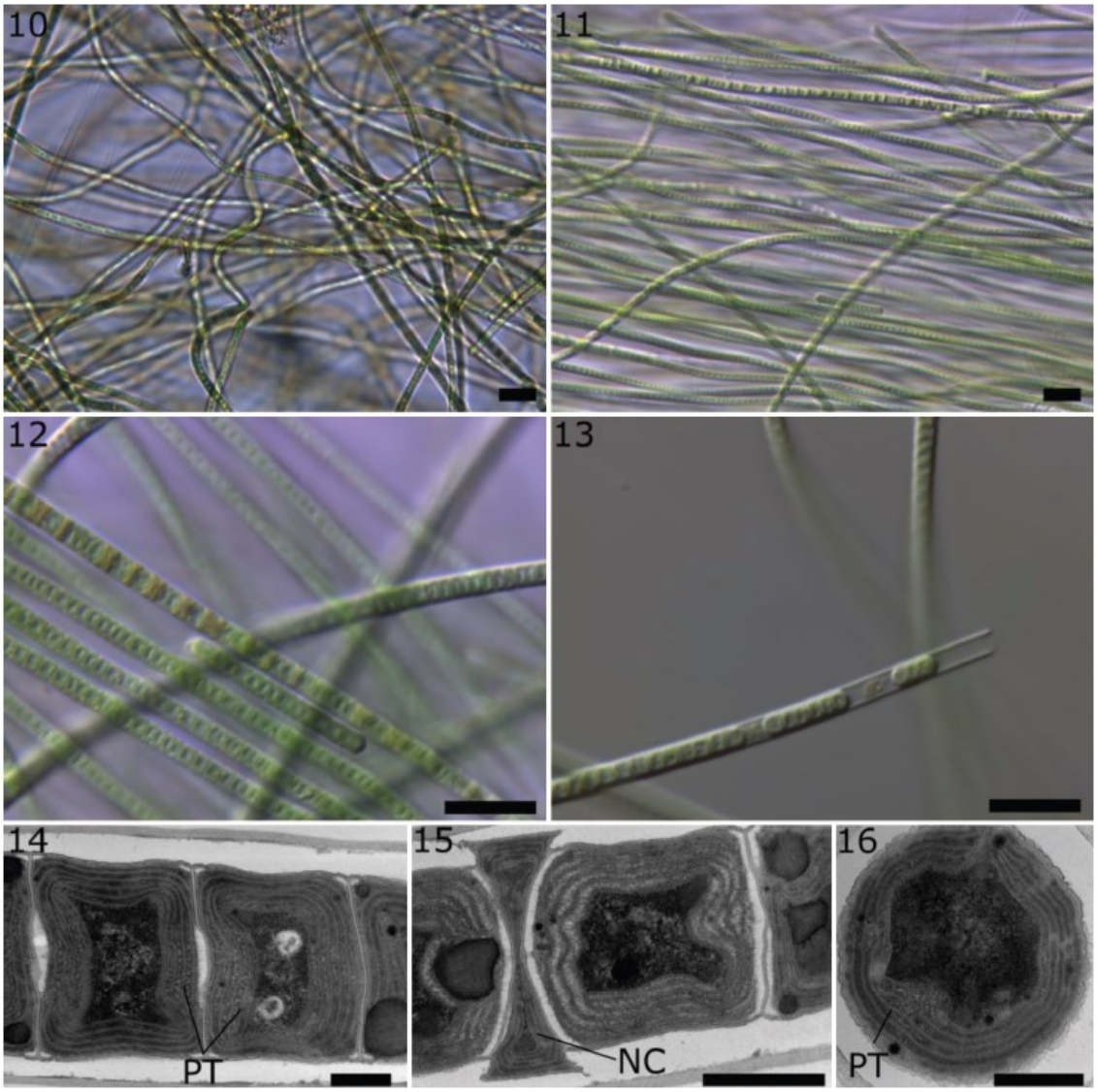
Type locality: A bridge that separates the two Lakes Lagoa Azul and Lagoa Verde in Sete Cidades, São Miguel Island, Azores archipelago, Portugal, 37°51.308'N, 25°47.178'W. Collected 13 July 2017 by the MONITAIA team project.

Habitat: Aerophytic in rocky substrate, under low/indirect sunlight under a bridge between two lakes).

Etymology: Epithet atlantica, named for its place of origin.

Reference strain: BACA0077 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal), isolated by Rita Cordeiro.

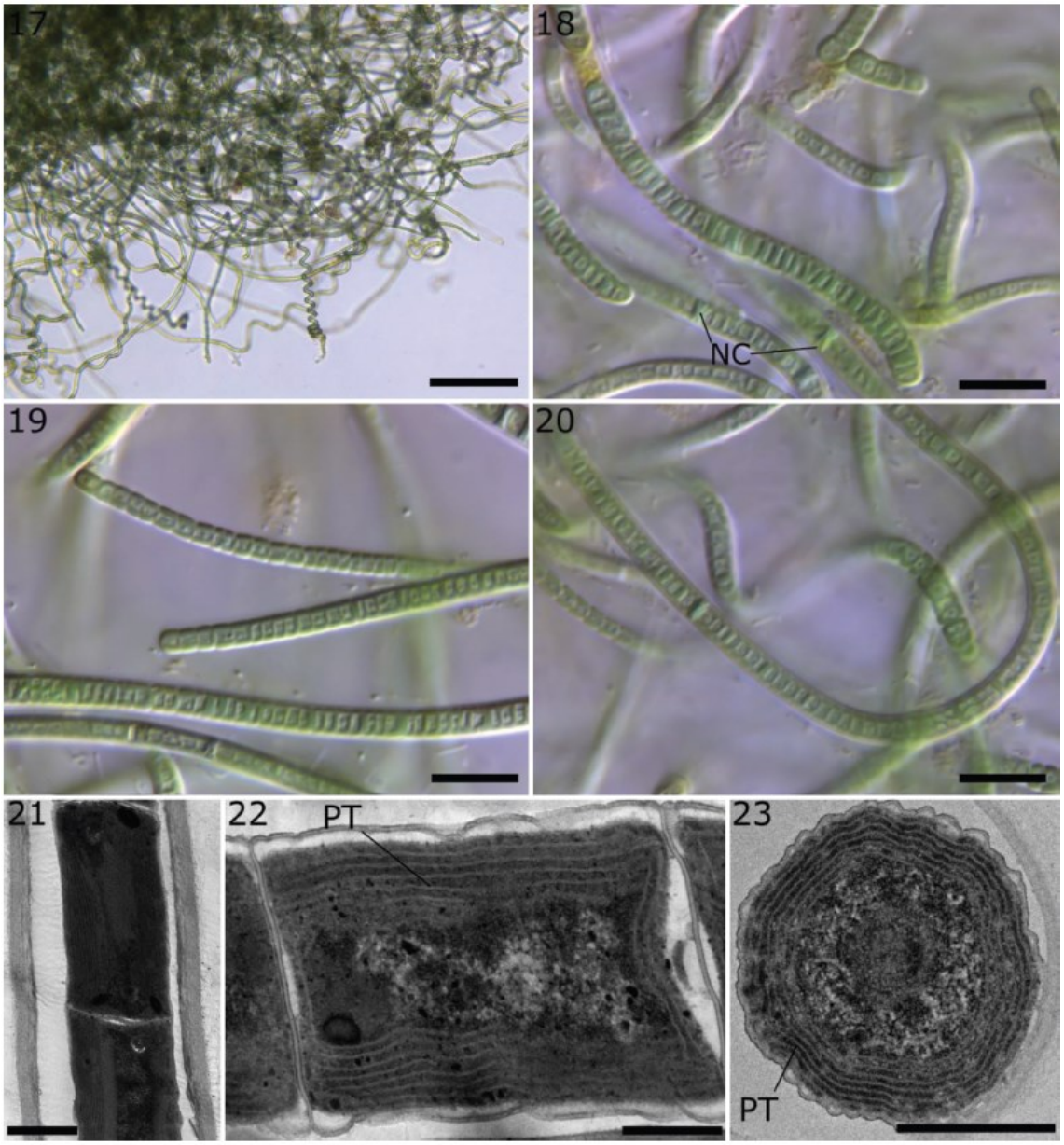
Gene sequences: GenBank accession number MT176728 for the 16S rRNA and 16S–23S rRNA ITS genes.



Figures 10–16. *Pegethrix atlantica* BACA0077 morphological features in LM and TEM. Fig. 10. Colony organization with visible trichomes, either coiled or straight. Scale bar = 10 µm. Fig. 11. Straight trichomes arranged in parallel. Scale bar = 10 µm. Fig. 12. Visible multiple necridia cells in a single trichome and hormogonia. Scale bar = 10 µm. Fig. 13. Trichome fragmentation and hormogonia formation inside a single firm sheath. Scale bar = 10 µm. Fig. 14. Longitudinal section of a vegetative cell (TEM), with parietal thylakoids with no cell constriction and evident mucilage layer. Scale bar = 500 nm. Fig. 15. Longitudinal section of vegetative cells (TEM), showing parietal thylakoids (PT), no cell constriction, mucilage layer one necridium (NC). Scale bar = 1 µm. Fig. 16. Transversal cut of a vegetative cell under TEM with parietal thylakoids. Scale bar = 500 nm.

***Albertania obscura* R.F.S. Luz, Kaštovský, J.R. Johansen & V. Gonçalves sp. nov. Figs 17–23**

Diagnosis: Distinguishable genetically and morphologically from all species. Phylogenetically positioned in the *Albertania* genus clade, with 16S rRNA p-distance greater than 1.3% and a 16S–23S rRNA ITS p-distance greater than 11% from all described species. Morphologically closer to *A. skiophila* and distinguish by its wider cells, mainly shorter than wide cells and with diffluent sheaths when older, in contrast to the mostly isodiametric and firm sheaths of *A. skiophila*.



Figures 17–23. *Albertania obscura* BACA0713 morphological features in LM and TEM. Fig. 17. Colony formation with spirally coiled and wavy trichomes. Scale bar = 100 µm. Fig. 18. Trichomes with cells broader than long, parietal thylakoids (inferred by the more intense colour at the cell periphery), rounded end cells, and necridia (NC), with visible differences in trichome width. Scale bar = 10 µm. Fig.

19. Trichomes with cells broader than long, parietal thylakoids (inferred by the more intense colour at the cell periphery), end cells and necridia. Scale bar = 10 µm. Fig. 20. Trichomes with cells broader than long, a more intense colour at the cell periphery, necridia and a thickened sheath. Scale bar = 10 µm. Fig. 21. Longitudinal section of vegetative cell (TEM), showing parietal thylakoids, a lack of cell constriction and evident mucilage layer. Scale bar = 1 µm. Fig. 22. Longitudinal section of vegetative cell (TEM), showing parietal thylakoids (PT), and a lack of cell constriction. Scale bar = 500 nm. Fig. 23. Transverse section of vegetative cell (TEM), showing parietal thylakoids (PT) and a visible individual sheath. Scale bar = 1 µm.

Description: Tufted colonies blue-green, with entangled filaments. Filaments normally straight or loosely coiled, rarely tightly coiled, very rarely with false branching, 3.3–7.9 µm wide (mean = 5.36 µm). Sheaths firm, colourless, attached to the trichome to slightly widened in older filaments. Trichomes untapered, not constricted at cross walls or rarely slightly constricted at cross walls, with necridia (several in the same trichome), without motility. Hormogonia few-celled. Cells mostly shorter than wide, with parietal thylakoids, 1.7–4.8 µm wide (mean = 3.38 µm) to 1.1–3.7 µm long (mean = 2.10 µm), with a length:width ratio of 0.3–1.5 (mean = 0.65). End cells rounded.

Holotype: Dried material preserved in a metabolically inactive state at Herbário Ruy Telles Palhinha, University of Azores, Portugal, under the AZB 4467 code.

Type locality: Algar do Carvão in Angra do Heroísmo, Terceira Island, Azores archipelago, Portugal, 38°43.657'N, 27°12.908'W. Collected 13 November 2019 by Rúben Luz and Joana Vilaverde.

Habitat: Aerophytic, in rocky substrate, in a biofilm in a low light (indirect) zone inside a natural volcanic cave.

Etymology: Epithet obscura, in reference to the dark or low-light habitat.

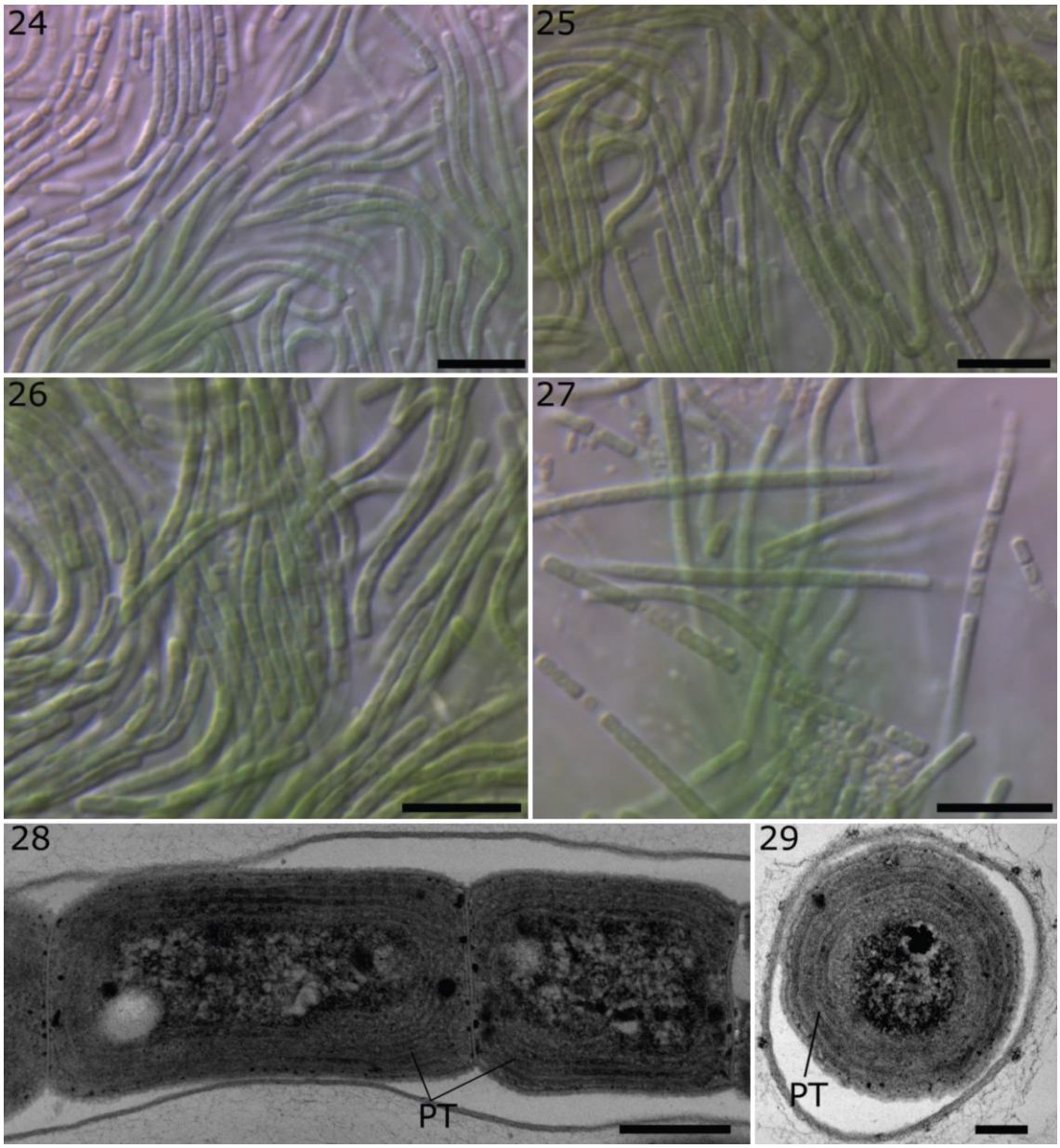
Reference strain: BACA0713 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal), isolated by Rúben Luz.

Gene sequences: GenBank accession number OM732262 for the 16S rRNA and 16S–23S rRNA ITS genes.

***Kovacikia atmophytica* R.F.S. Luz, Kaštovský, J.R. Johansen & V. Gonçalves sp. nov. Figs 24–29**

Diagnosis: Distinguishable mainly genetically and by its unique habit preference (atmophytic) in the *Kovacikia* clade. Phylogenetically positioned in the *Kovacikia* clade, with a high 16S rRNA p-distance greater than 2.9% and a 16S–23S rRNA ITS p-distance greater than 11% from all described species. Morphologically similar to both described *Kovacikia* species,

overlapping both in cell width and cell length, although with longer cells than both species, about 0.7 µm.



Figures 24–29. *Kovacikia atmophytica* BACA0619 morphological features in LM and TEM. Fig. 24. Filaments with fragmented trichomes and hormogonia with visible firm sheaths. Scale bar = 10 µm. Figs 25, 26. Trichomes arranged in parallel, with cells longer than wide and rounded apical cells. Scale bars = 10 µm. Fig. 27. Fragmented filaments with visible firm sheaths, necridia and hormogonia. Scale bar = 10 µm. Fig. 28. Longitudinal section of vegetative cell (TEM), with parietal thylakoids (PT), slight cell constriction. Scale bar = 500 nm. Fig. 29. Transverse section of vegetative cell (TEM), with parietal thylakoids (PT) and visible individual sheath. Scale bar = 200 nm.

Description: Colony a flat mat, with entangled filaments, in young cultures blue green, and greenish to brownish when old. Filaments normally straight or slightly waived, without false

branching, 1.4–2.0 µm wide (mean = 1.68 µm). Sheaths thin, colourless, attached to the trichome. Trichomes untapered, slightly constricted at cross walls, without necridia, without motility. Hormogonia few celled, with motility. Cells mostly longer than wide or almost isodiametric, sometimes varying within the same trichome, with parietal thylakoids, 1.0–1.6 µm wide (mean = 1.30 µm) to 1.1–2.9 µm long (mean = 1.94 µm), with a length:width ratio of 0.8–2.1 (mean = 1.49). End cells rounded.

Holotype: Dried material preserved in a metabolically inactive state at Herbário Ruy Telles Palhinha, University of Azores, Portugal, under the code AZB 4375.

Type locality: At or near the top of a deep fumarole at Furnas do Enxofre, Terceira Island, Azores archipelago, Portugal, 38°43.733'N, 27° 13.892'W. Collected 4 March 2020 by Martin Souto, Pedro R. Raposeiro and Ana Balibrea.

Habitat: Freshwater, atmophytic among other cyanobacteria and microalgae.

Etymology: Epithet atmophytica, referring to the habitat of the original sample.

Reference strain: BACA0619 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal), isolated by Rúben Luz.

Gene Sequences: GenBank accession number OM732256 for the 16S rRNA and 16S–23S rRNA ITS genes.

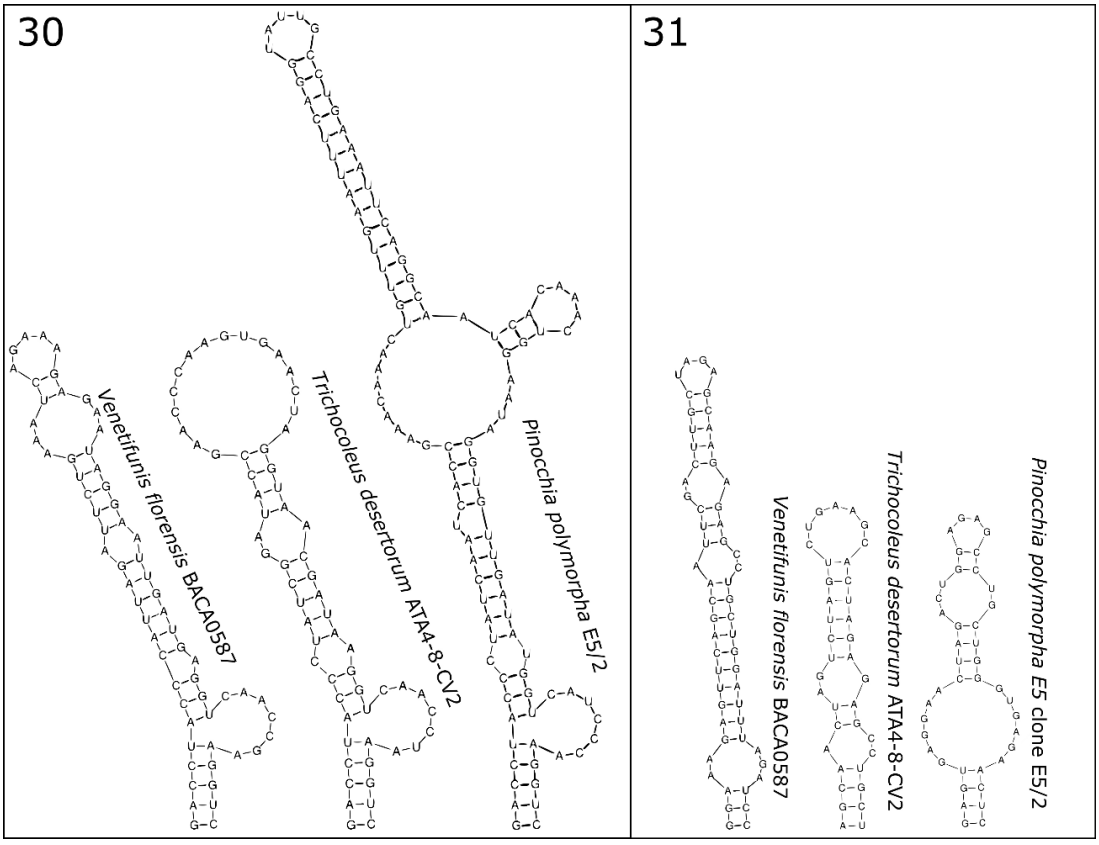
5.4 Discussion

The descriptions of the new genus and the three new species are based on the strains *Venetifunis florensis* BACA0587, *Albertania obscura* BACA0713, *Kovacikia atmophytica* BACA0619 and *Pegethrix atlantica* BACA0077, all phylogenetically well supported by the 16S rRNA, 16S–23S rRNA ITS secondary structure and both genes p-distance analysis. The threshold for species distinction of 98.7% similarity using the 16S rRNA (Yarza et al. 2014) is well supported for all new species (Table S1) as well as the 16S–23S rRNA ITS dissimilarity above 7% (Tables S2, S3, S4), a widely used criterium for species distinction (Erwin & Thacker 2008, Osorio-Santos et al. 2014, Mai et al. 2018, Mareš et al. 2019, Pietrasiak et al. 2019, 2021, Jung et al. 2020) The 16S rRNA 5.5% p-distance threshold suggested for genus distinction (Komárek et al. 2014, Yarza et al. 2014) is surpassed (>6.0%) relative to the phylogenetically and morphologically closest genera. Morphologically, the new species *Pegethrix atlantica* BACA0077, *Albertania obscura* BACA0713 and *Venetifunis florensis* BACA0587 show differences from closely related taxa, while *Kovacikia atmophytica* BACA0619 is mainly distinguished by genetic and ecological characteristics.

Venetifunis florensis BACA0587 is positioned near species of *Pinocchia* and *Trichocoleus*, with a good phylogenetic support (Fig. 1), in the Trichocoleusaceae (Guiry et al. 2018, Mai et al. 2018, Strunecký et al. 2023). Pinocchia is a mat-forming, unbranched genus, with solitary trichomes (Dvořák et al. 2015, Kim et al. 2021), morphologically very different from *Venetifunis*. *Trichocoleus* has firm, often diffluent, wide and open sheaths with several trichomes per filament, commonly with acute conical terminal cells (Anagnostidis 2001, Mühlsteinová et al. 2014). *Venetifunis* can be morphologically distinguished from *Trichocoleus* by its always diffluent sheaths, high phylogenetic distance and high 16S rRNA p-distance, above the 5.5% threshold (Table S1). *Schizothrix* sensu Kutzking ex Gomont is morphologically close to *Venetifunis*, with very distinctly pointed, closed sheaths, with trichomes enclosed in individual sheaths (Gomont 1892), the latter a diacritical feature absent in *Venetifunis*. The type species of *Schizothrix*, *S. fuscescens* Kutzing ex Gomont, has not been sequenced yet, so the exact phylogenetic position of this genus is not certain. However, if the holotype is still available, efforts to sequence its genetic information would be of great importance to the taxonomy of this genus and related genera. The same is true of *Trichocoleus*, as the type species, *T. delicatulus* (West & G.S. West) Anagnostidis, has not been sequenced yet, and was poorly described (West & West 1896). For this reason, we used *Trichocoleus desertorum* Mühlsteinová, J.R.Johansen & Pietrasiak as the genetic reference for the genus (Mühlsteinová et al. 2014).

The morphological and genetic results support the description of *Venetifunis* gen. nov., although the review of the morphologically closest genus (*Trichocoleus*) reveals some species with shared characteristics. The sheaths in *Venetifunis* are always completely diffluent and apical cells rounded, and this has been clearly described in at least three species of *Trichocoleus*. *Trichocoleus wuitneri* (Frémy) Anagnostidis has a very similar morphology to *V. florensis*, but has a different colour and habitat, as *T. wuitneri* was described from a marine habitat, a very distant ecology and a diacritical feature (Frémy 1932, Komárek et al. 2014). *Trichocoleus voukii* (Frémy) Anagnostidis also shared completely diffluent sheaths and similar morphological description with *V. florensis* but is also a marine species (Frémy 1932). From freshwater habitats, *Trichocoleus minimus* is the only species that features always diffluent sheaths and shares many other morphological features with the newly described genus, such as visible cell constriction and hemispherical/rounded apical cells (Frémy 1930). This suggests that *T. minimus* belongs to *Venetifunis* and, therefore, is now transferred to *Venetifunis*. *Venetifunis minimus* comb. nov. is distinguished from *V. florensis* based on cell morphology. Cells of *V. minimus* are mostly isodiametric, 1.2–1.5 µm wide, while *V. florensis* has narrower cells (0.7–1.2 µm wide), at least twice as long as wide. Two species of *Microcoleus* also have some resemblance with *Venetifunis*. *Microcoleus sampaianus* Sampaio, described from Portugal, has a diffluent mucilage according

to Komárek & Anagnostidis (2005), although in the original publication the species is described with a firm mucilage that becomes diffluent (Sampaio 1941). *Microcoleus violaceus* Frémy is described as sometimes having an entirely diffluent sheath but others with thin sheaths, as is clearly illustrated in the original publication (Frémy 1930). Finally, *Trichocoleus erectiusculus* (Starmach) Anagnostidis & Komárek was described with facultative diffluent sheaths (Starmach 1960).



Figures 30, 31. 16S–23S rRNA ITS secondary structures of *Venetifunis florensis*, the type species of *Pinocchia*, *P. polymorpha*, and *Trichocoleus desertorum*. Fig. 30. Secondary structures of the D1–D1' helix. Fig. 31. Secondary structures of the Box-B helix.

In addition to the two *Venetifunis* species, there are two sequenced OTUS of uncultured cyanobacteria, collected from a biofilm grown in dolomite in Scotland, that is perhaps one undescribed species based on its phylogenetic placement (Feder 2014). *Venetifunis* appears to have wide geographical distribution, with *V. florensis* described from Flores Island in the Azores archipelago, Portugal, and *V. minimus* reported (as *Trichocoleus minimus*) in several places other than the type locality in the shore of a lake in Gaban (Frémy 1930), including an alkaline freshwater stream over decomposing *Potamogeton* sp. (Aboal 1988) in Spain, over wet rocks by a paved road in North Carolina (Johansen et al. 2007), in Greece (Anagnostidis et al. 1981) and in Israel (Barinova & Smith 2022).

Pegethrix is a recently described genus with five described species (Mai et al. 2018, Shen et al. 2023), four of them known from Utah, USA (Mai et al. 2018) and one from Sichuan, China (Shen et al. 2023). *Pegethrix atlantica* sp. nov. was collected from a mat under a bridge that crosses a lake in São Miguel Island (Azores), an ecology that fits all previously described species (Mai et al. 2018, Shen et al. 2023). Genetically the species is well delimited presenting >1.3% of dissimilarity for the 16S rRNA and >7% dissimilarity for the 16S–23S rRNA ITS when compared to sister taxa. Even from the closest phylogenetic taxon (*P. sichuanica* Li-Qin Shen, Renhui Li & B.S. Qiu) it has 1.3% 16S rRNA dissimilarity, which is considered the threshold for genetic delimitation (Yarza et al. 2014); and with the highest 16S-23S rRNA dissimilarity (18.8%; Table S2), reinforcing their genetic difference. The 16S–23S rRNA ITS secondary structures show some similarity among species, even for the D1–D1' helix (Fig. 32) and Box-B helix (Fig. 33), with the highest variability found in the V3 region (Fig. 34). *Pegethrix atlantica* shares a similar basal lateral bulge in the D1–D1' helix (Fig. 32) with *P. bostrychoides*, a particular feature only present in these species, although with some differences in the mid-internal loops. The terminal hairpin of *Pegethrix* species has a common four-nucleotide residue (5'-GCGA-3') in *P. atlantica*, *P. bostrychoides* and *P. olivacea*, with equal ones for *P. convoluta* an *P. indistincta* (5'-GAGA-3') and a unique one in *P. sichuanica* (5'-GGAA-3'). Box-B is in some way structurally conserved, with the same mid internal loops but varying in size, with the V3 presenting the highest variability, with a common basal stem and a small basal lateral bulge but with some unique mid-internal loops to each species, and a high variable sequence terminal hairpin.

Pegethrix atlantica presents clear morphological differences with the closest phylogenetic taxa, including wider and longer cells than *P. sichuanica*, and always very fine and delimited sheath and never with common sheaths to multiple trichomes as in *P. indistincta* (Figs 12, 13).

Two species of *Albertania* were previously known, one subaerophytic (Zammit 2018) and the other in the periphyton of freshwater (Strunecký et al. 2019). The new *Albertania* species here described was found living aerophytically in a lava tunnel (with a collapsed and small opening on the top), a habitat similar to the type species (Zammit 2018). Phylogenetically the species are well separated, with *A. obscura* phylogenetically closer to *A. skiophila*, and with a good genetic delimitation with 1.3% 16S rRNA p-distance to *A. skiophila* and 2.5% to *A. alaskaensis*. *Albertania obscura* is on the limited threshold of similarity with *A. skiophila* for the 16S rRNA (98.7%; Yarza et al. 2014), but the 16S–23S rRNA ITS dissimilarity values are all well above 11% (Table S3), which is strong enough to support the new taxa (Jung et al. 2020, Mai et al. 2018, Mareš et al. 2019, Osorio-Santos et al. 2014, Pietrasiak et al. 2019, 2021). This high p-distance value for the 16S–23S rRNA ITS is not properly revealed in the 16S–23S rRNA ITS secondary structures, with a well-conserved secondary structure in the Box-B and V3 helix.

Structurally, the D1–D1' helix (Fig. 35) is the most variable one, with a common basal stem and a common basal lateral bulge, with the bigger changes in the mid internal loops in *A. skiophila*. The terminal hairpin has the same length in these three *Albertania* species (five nucleotides), although with slightly different sequences (one nucleotide between *A. obscura* and *A. skiophila* and two nucleotides between *A. obscura* and *A. alaskaensis*). The Box-B secondary structures (Fig. 36) are only available to *A. obscura* and *A. skiophila*, but it is possible to observe the conservation of the secondary structures, with the second mid internal loop larger in the *A. skiophila* (by one nucleotide), and the terminal hairpin larger in the *A. obscura* (by one nucleotide), and with a different sequence. The V3 helix (Fig. 37) is totally conserved both structurally and in sequence.

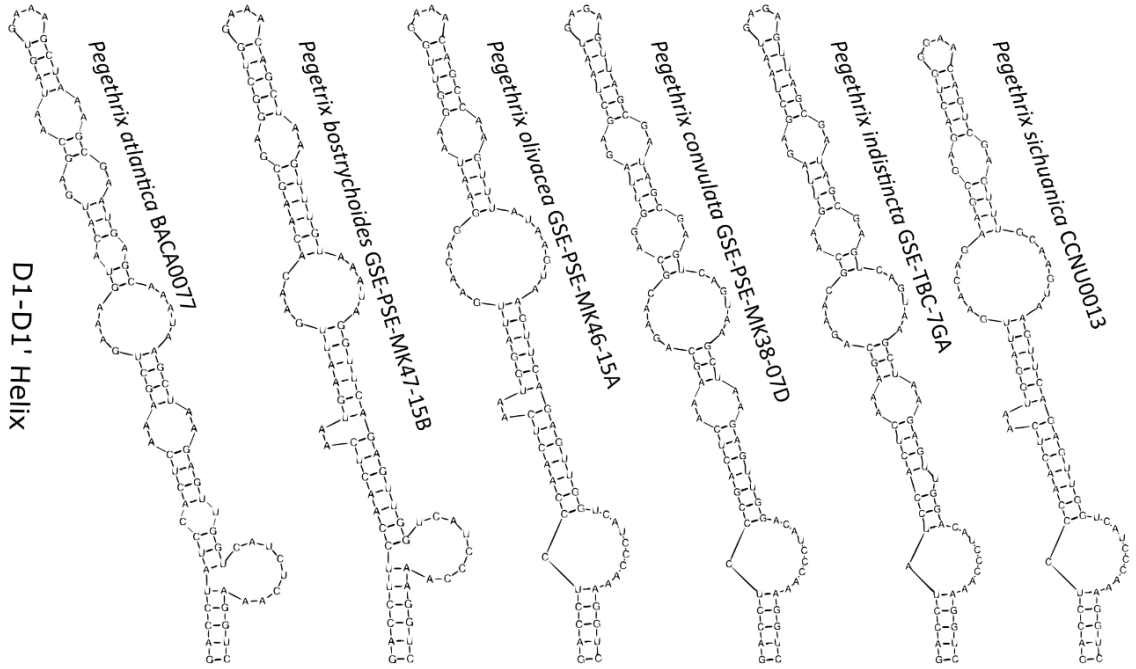


Figure 32. Secondary structures of the D1–D1' helix in *Pegethrix* species.

All described *Albertania* species present coiled trichomes; although this was not stated in the description of previous species, it is clearly observed in photographs of both species (see Fig. 2 in Zammit (2018), and Fig. 6D in Strunecký et al. (2019)), and have firm, colourless sheaths (Strunecký et al. 2019, Zammit 2018). The main morphological features that distinguish *A. obscura* from *A. alaskaensis* is cell morphology, with *A. obscura* cells mostly shorter than wide, 1.7–4.8 µm wide and 1.1–3.7 µm long, and *A. alaskaensis* with slightly narrower cells 1.8–3 µm wide, 1.9–4 µm long and mostly isodiametric, or longer than wide before division (Strunecký et al. 2019), the opposite of cell form of *A. obscura*. Similarly, relative to *A. skiophila*, *A. obscura* has wider cells (up to 1.8 µm more), commonly with most cells shorter than wide and with diffluent sheaths when in older stages, in contrast to mostly isodiametric and firm sheaths in *A.*

skiophila (Zammit 2018). In this case, with similar morphological characters, it is important the genetic analysis that supports the description of *A. obscura* as a new taxon based on the 16S rRNA and 16S–23S rRNA ITS.

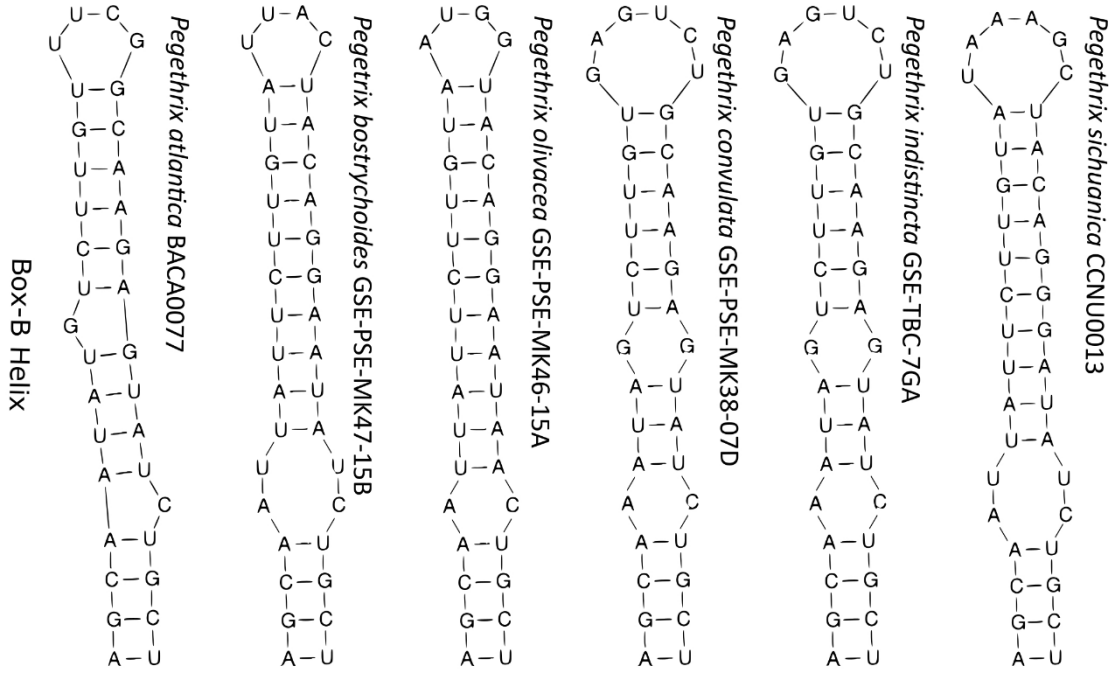


Figure 33. Secondary structures of the Box-B helix in *Pegethrix* species.

Two morphologically similar species of the genus *Kovacikia* have previously been described, with overlapping cell length but different cell width. The new species *K. atmophytica* is morphologically similar to the previous species, overlapping both in cell length (up to 0.7 µm more) and width, being almost morphologically indistinguishable. However, the new species is quite different genetically, both in 16S rRNA (Table S1) and 16S–23S rRNA ITS p-distance (Table S4). Regarding the habitat, all described species were collected among other algae/mosses, although *K. atmophytica* was collected near the proximity of a deep fumarole emitting hot vapor, a clearly distinct ecological environment. Nevertheless, the species seems to be thermotolerant rather than strictly thermal, as despite being isolated from a natural sample placed to grow at 35°C, it is maintained successfully in BACA at 19°C. Phylogenetically, *K. atmophytica* resolves far from the remaining described species, supported by the high 16S rRNA p-distance with (2.9%–3.2%) and high 16S–23S rRNA ITS p-distance (11.2%–16.2%; Table S4). The 16S–23S rRNA ITS secondary structures between the *Kovacikia* species are similar, with the D1–D1' Helix (Fig. 38) the more similar one with a common basal steam, basal lateral bulge and the stem loops, with *K. atmophytica* and *K. minuta* sharing a common paired stem before the hairpin, a feature absent from *K. muscicola*. The Box-B helix (Fig. 39) also shares a similar secondary structure, although with more variability in loop formation (size and sequence), and

the V3 helix shares an equal basal stem with a higher variability in sequence and structure. Particularly this species also shares high similarity with '*Leptothermofonsia sichuanensis'*, nom. inval. (4.3%) a value low enough to consider this species a possible member of the same genus, following the 16S rRNA p-distance criteria for genus delimitation (Yarza et al. 2014). However, the phylogenetic result, the morphological similarity, the 16S–23S rRNA ITS secondary structures (high secondary structure similarity with *Kovacikia*) and the p-distance value closer to *Kovacikia* do not support that hypothesis.

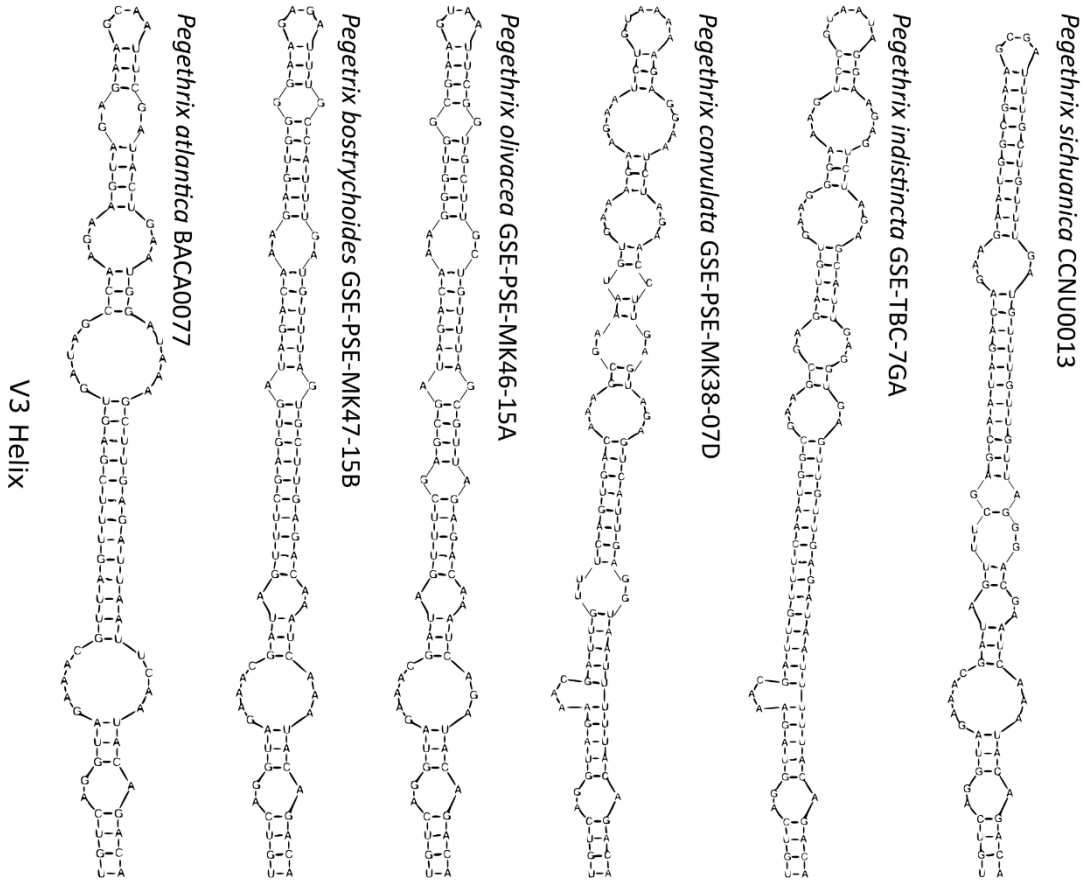
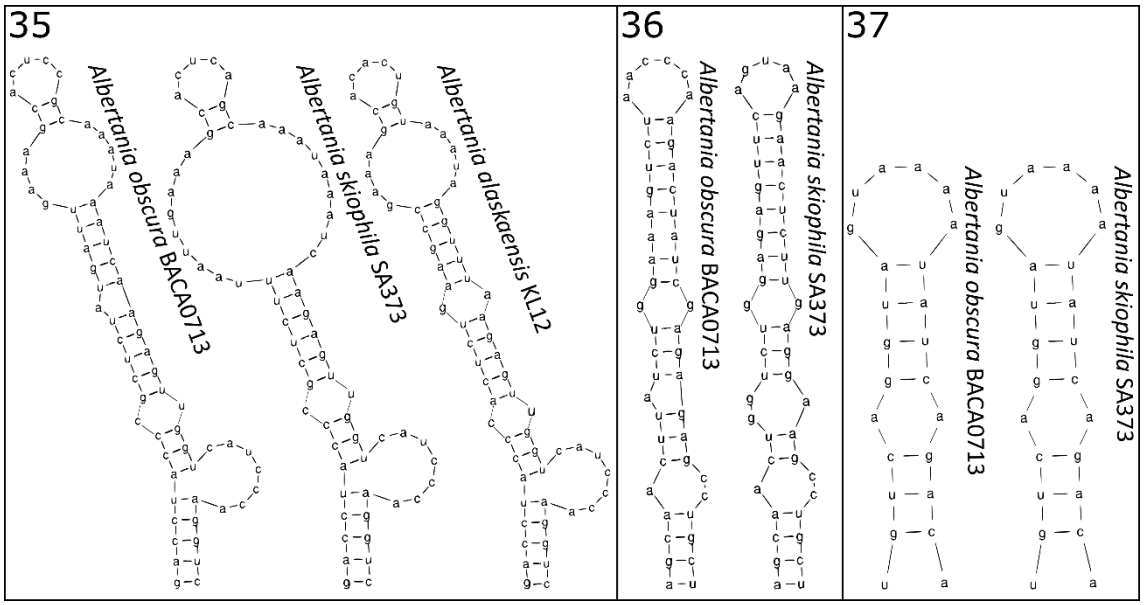


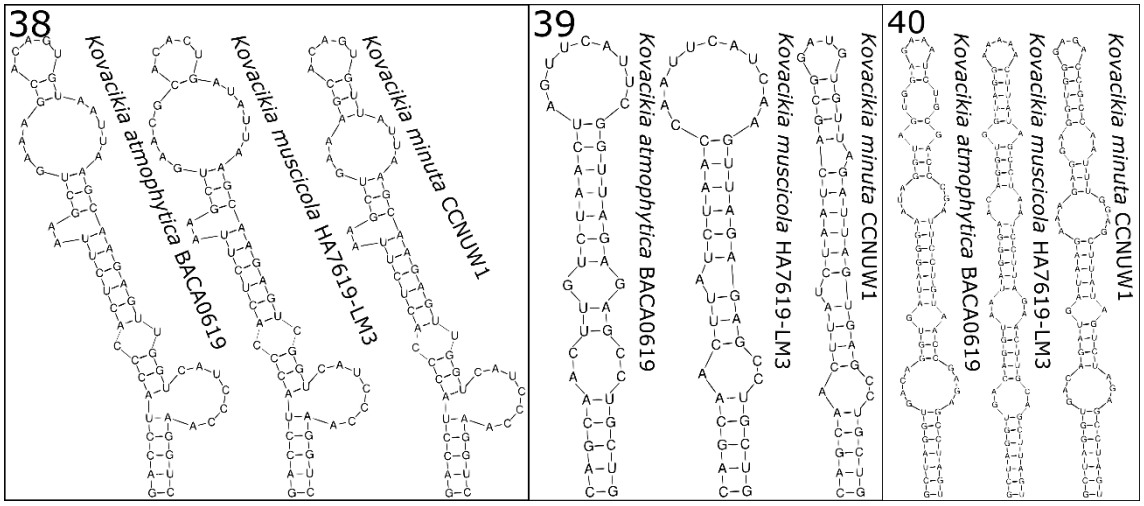
Figure 34. Secondary structures of the V3 helix in *Pegethrix* species.

The description of all new taxa follows a polyphasic approach with a morphological description and comparison with taxa phylogenetically and morphologically closely related, a genetic analysis using 16S rRNA and 16S–23S rRNA, phylogenetics with the 16S rRNA, and ecology. The polyphasic approach is essential in some morphologically uniform taxa, such as *Kovacikia*, where genetics and ecology were of utmost importance, and for the reinforcement of new taxa description where there is a morphological similarity with published taxa, such as *Venetifunis* vs *Trichocoleus*. This work increases not only the knowledge of three previously described genera, extending their geographical distribution to the Azores archipelago and the

North Atlantic Ocean, but also of one new genus with two species, one newly described and the other a new combination.



Figures 35–37. 16S–23S rRNA ITS secondary structure of *Albertania* spp. For *A. alaskaensis* the full 16S–23S rRNA ITS is not available. Fig. 35. Secondary structures of the D1–D1' helix. Fig. 36. Secondary structures of the Box-B helix. Fig. 37. Secondary structures of the V3 helix.



Figures 38–40. 16S–23S rRNA ITS secondary structure of *Kovacikia* spp. Fig. 38. Secondary structures of the D1–D1' helix. Fig. 39. Secondary structures of the Box-B helix. Fig. 40. Secondary structures of the V3 helix.

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5.6 Disclosure statement

No potential conflict of interest was reported by the authors.

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Chapter VI

Description of *Pseudocalidococcus azoricus* gen. sp. nov. (Thermosynechococcaceae, Cyanobacteria), a rare but widely distributed coccoid Cyanobacteria

Luz, R., Cordeiro, R., Kaštovský, J., Fonseca, A., Urbatzka, R., Vasconcelos, V. & Gonçalves, V. (2023). Description of *Pseudocalidococcus azoricus* gen. sp. nov. (Thermosynechococcaceae, Cyanobacteria), a Rare but Widely Distributed Coccoid Cyanobacteria. Diversity, 15(12), 1157. https://doi.org/10.3390/d15121157

Chapter VI. Description of *Pseudocalidococcus azoricus* gen. sp. nov. (Thermosynechococcaceae, Cyanobacteria), a rare but widely distributed coccoid Cyanobacteria

Abstract

Coccoid cyanobacteria represent an important part of cyanobacterial freshwater diversity, with many studied strains in public databases identified as *Synechococcus*. This is a diverse genus, both morphologically and ecologically, with a global distribution. However, many of the so-called *Synechococcus*-like cyanobacteria strains could represent several independent genera that require further studies. In this work, four strains of a *Synechococcus*-like cyanobacteria isolated from freshwater lakes and terrestrial atmophytic habitats on São Miguel and Flores Islands (Azores archipelago) were studied genetically using the 16S rRNA and 16S–23S rRNA ITS, morphologically with light and transmission electron microscopy, and ecologically. A draft genome was produced from the reference strain by Illumina sequencing, which allowed a more complete phylogenetic study and a deeper taxonomic analysis, revealing a divergent phylogenetic evolution and low ANI and AAI values (69.4% and 66.3%, respectively) to *Thermosynechococcus*, the closest phylogenetic genus. Although morphologically similar to *Synechococcus*, the 16S rRNA and genome phylogenetic analysis placed the studied strains in a clade sister to *Thermosynechococcus*, inside the Thermosynechococcaceae. Thus, *Pseudocalidococcus azoricus* gen. sp. nov. is described as a new coccoid freshwater genus and species from the Azores archipelago. A detailed comparison with similar morphological taxa is provided, supporting the separation of the new genus. The 16S rRNA with a high genetic similarity to other strains from several continents identified as *Synechococcus* sp. suggests that the new genus probably has a worldwide distribution. Future studies should be performed to clarify the taxonomic identity of those strains.

Keywords: AAI, ANI, Azores, coccoid cyanobacteria, DDH, genome, new genus, phylogeny, *Synechococcus*, 16S rRNA

6.1 Introduction

Cyanobacteria are one of the most ancient organisms (Mareš et al. 2013), which arose around 3500 million years ago (Schirrmeister et al. 2015). They are present in a wide diversity of habitats, in terrestrial and aquatic ecosystems (Scott & Marcarelli 2012, Whitton & Potts 2012, Komárek & Johansen 2015), and are common inhabitants of extreme environments (Komárek &

Johansen 2015, Cordeiro et al. 2020, Luz et al. 2022). Recent studies on cyanobacteria diversity in the Azores Islands, a remote oceanic archipelago with a great variety of suitable habitats (Luz et al. 2020, 2022), allowed the description of several new taxa (Luz et al. 2023b, 2023a). Therefore, many cyanobacteria species may still be unknown, and increased sampling efforts should be taken in these remote areas, especially in less-studied habitats such as terrestrial atmophytic habitats.

The morphology of coccoid cyanobacteria is poorly characterized, compromising their taxonomical classification, which was until recently based mainly on morphological characteristics (Mareš et al. 2019, Komárek et al. 2020). In recent years, a significant effort to improve the coccoid cyanobacteria taxonomy has been made using a polyphasic approach with molecular and morphological data for the description of new taxa or its reassessment (Jung et al. 2021, Pokorný et al. 2023), and more recently using genomic data (Komárek et al. 2020, Pessi et al. 2023).

Synechococcus Nägeli represents a large role, with a recognized polyphyletic nature from the most commonly studied cyanobacteria (Dvořák et al. 2014). However, many strains lack apomorphic features that could aid in the morphologic identification, which often resulted only in the classification of *Synechococcus*-like cyanobacteria (Komárek et al. 2014). *Synechococcus* was traditionally classified as a benthic freshwater rod-like cyanobacteria, according to Nägeli (1849), yet with several described species over the years with an increasing ecological range. Recent molecular studies have confirmed the polyphyletic nature of the genus (Callieri 2017, Komárek et al. 2020), such as *Thermosynechococcus* Katoh, Itoh, Shen, and Ikeuchi (Katoh et al. 2001, Komárek et al. 2020), which was initially based on its ecophysiological and biochemical features, since all its strains were thermophilic (Katoh et al. 2001), and was recently validated (Komárek et al. 2020).

An important shift in cyanobacteria taxonomy was observed in the twentieth century, with many authors relying more on genetic data (Mai et al. 2018, Mareš et al. 2019, Kaštovský et al. 2023, Luz et al. 2023b). More recently, the fast growth of cyanobacteria genomic data has allowed for a more robust analysis (Komárek et al. 2020, Stanojković et al. 2022, Cai et al. 2023, Strunecký et al. 2023), not only for taxonomic resolution (Willis & Woodhouse 2020, Dvořák et al. 2023) but also for ecological studies (Chen et al. 2021, Dick et al. 2021). The relevance of this new taxonomical tool is well represented in the last cyanobacterial order and family classification update, with its results based on the available genomic data (Strunecký et al. 2023).

In this work, we applied a polyphasic approach to study four *Synechococcus*-like cyanobacteria strains from BACA (Bank of Algae and Cyanobacteria of the Azores), assigning *Pseudocalidococcus azoricus* gen. sp. nov. to the Thermosynechococcaceae Komárek, Strunecký,

and Johansen, according to its phylogenetic placement. More importantly, *Pseudocalidococcus azoricus* was defined as a new genus through a combination of molecular data (genomic data, 16S rRNA, and 16S–23S rRNA ITS), morphological characters (by light and transmission electron microscopy), and ecological data. The obtained draft genome and the taxonomic analysis allowed for a better knowledge of the *Synechococcus* polyphyletic nature. The description of the new taxa followed the International Code of Nomenclature for algae, fungi, and plants (Turland et al. 2018).

6.2 Materials and Methods

6.2.1 Strains and morphological characterization

The studied strains (Table 1) were isolated from lakes and a rock wall in São Miguel Island and Flores Island (Azores archipelago), and maintained in BG11 medium at 19 °C and a 14/10 h photoperiod in the BACA culture collection. For the morphological descriptions, at least 50 cells per strain were examined using a Leica DM4 B microscope equipped with a digital camera, the Leica MC 190 HD (Leica, Wetzlar, Germany). Morphological data from the four strains were combined for taxa description.

Table 1. Strain location of the sampling in the Azores archipelago, Portugal, and GenBank accession codes of the *Pseudocalidococcus* strains.

Strain	Taxonomy	Local	Sampling Date	Habitat	Coordinates	GenBank
BACA0433	*P. azoricus*	Furnas, São Miguel Island	1 August 2017	Aquatic	37°46'18.3" N 25°18'42.7" W	OM732237
BACA0444	*P. azoricus*	Lagoa Comprida, Flores Island	27 September 2017	Aquatic	39°26'26.1" N 31°13'19.0" W	OM732240 GCA_031729055
BACA0446	*P. azoricus*	Lagoa das Empadadas Norte, São Miguel Island	12 July 2017	Aquatic	37°49'32.5" N 25°44'54.9" W	OM732241
BACA0781	*P. azoricus*	Ribeira Grande, São Miguel Island	6 September 2022	Atmophytic	37°47'35.9" N 25°29'05.7" W	OR725120

The transmission electron microscopy (TEM), biomass was preserved in 2.5% glutaraldehyde and 0.1 M cacodylate buffer, and postfixed with 2% osmium tetroxide; then, it was dehydrated in an acetone series (30%, 50%, 70%, 80%, 90%, 95%, and 100%) and embedded in Spurr's resin (Spurr 1969). Ultra-thin sections (70 nm) were placed on formvar-coated grids, contrasted by uranyl acetate and lead citrate, and analyzed with a JEOL JEM 1010 microscope.

6.2.2 DNA extraction, gene amplification, and sequencing

The PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) was used for the DNA extraction following the protocol recommended by the manufacturer for Gram-negative bacteria. For the 16S rRNA and 16S–23S rRNA ITS region amplification, the primers 27F and 23S30R (Neilan et al. 1997, Lepère et al. 2000) were used in a polymerase chain reaction following the protocol described by Luz et al. (2023). Thermal cycling reactions were carried out in a ProFlex™ 3 x 32-well PCR System (Thermo Fisher, Waltham, Massachusetts, USA) using the same conditions as Taton et al. (2003). Visualization and purification of the amplified sequences followed Luz et al. (2023), and amplicon sequencing was conducted as a commercial service at MACROGEN (Madrid, Spain) using the 27F, 781F, 781R, CSIF, and 23S30R primers (Neilan et al. 1997, Nübel et al. 1997, Lepère et al. 2000, Janse et al. 2004, Cordeiro et al. 2021).

6.2.3 Genome sequencing and assembly

The chosen reference strain (BACA0444) was produced in 50 mL cultures in BG11 medium for three weeks, and biomass was recovered through centrifugation at 7000 RCF. The same kit was used to extract DNA as for the 16S rRNA and 16S–23S rRNA ITS amplicons. However, for the elution, DNAse- and RNAse-free water was used. Sequencing was performed on an Illumina platform in NovoGene (Cambridge, UK) using NovaSeq, producing 1 G of data output.

The draft genome was assembled using the GEN-ERA assembly pipeline 2.0 (Cornet et al. 2023) with SPAdes v3.15.3 (Nurk et al. 2017) and with the metagenomics option selected. Binning was performed by CONCOCT v1.1.0 (Alneberg et al. 2014), and the produced binned genome was assessed for quality using CheckM v1.2.2 (Parks et al. 2015), BUSCO v5.5.0 (Manni et al. 2021), and CheckM2 v1.0.2 (Chklovski et al. 2023).

6.2.4 16S rRNA phylogenetic analysis

The sequences of the studied strains were aligned with 96 sequences from other cyanobacteria retrieved from GenBank using BLAST or from the literature. Sequence alignment was carried out using MAFFT v7.520 with the G-INS-i method (Katoh & Standley 2013). The final alignment contained 1076 columns. The best-fit nucleotide model was assessed using ModelFinder (Kalyaanamoorthy et al. 2017), with the selection of the GTR + G4 + I + F evolution model according to the Bayesian information criterion. Phylogenetic trees were constructed using Bayesian inference (BI) with MrBayes v3.2.7a (Ronquist et al. 2012) on XSEDE through the CIPRES Science Gateway, and maximum likelihood (ML) using the IQ-Tree online version v1.6.12

(Trifinopoulos et al. 2016). *Gloeobacter violaceus* PCC 8105 was used as an outgroup. The following conditions were used in the BI analysis: 2.5 x 10^6 generations, with two runs of four Markov chains, custom parameters (temp = 0.01), sampling every 1000 generations, and a 0.25 burn-in rate (the final average standard deviation of split frequencies was equal to 0.004500). The ML analysis was carried out using the GTR + G4 + I + F model with 1000 ultrafast bootstrap replicates (Hoang et al. 2017). Trees were visualized in FigTree v1.4.4 (Rambaut 2012), and the final composite tree from the maximum likelihood with the addition of the posterior probabilities values from the BI was redrawn with Inkscape v1.3.

6.2.5 Genome analysis

Genomes were selected following a literature review and their availability in GenBank. For quality control, an analysis using BUSCO v5.5.0 (Manni et al. 2021) was performed in all retrieved genomes with the cyanobacteria_odb10 dataset lineage selected (date: 23 February 2021; number_of_BUSCOs: 773) and CheckM v1.2.2. The number of identified genes was analyzed, and genomes with low-conserved genes were removed, along with genomes with fragment genes (5 >; BUSCO analysis) and less than 95% completeness (CheckM analysis). A final dataset of 114 genomes was then used for further analysis.

An adapted and custom python pipeline based on Jamie McGowan (https://github.com/jamiemcg/BUSCO_phylogenomics.git, accessed on 31 August 2023) was used for the phylogenomic analyses, modified to better fit a prokaryote analysis and taking into account updated software, here presented as KABOOM (https://github.com/rubenluz/KABOOM.git, accessed on 31 August 2023). This python pipeline, working in a Conda environment, takes assembled genomes as input (.fasta and .fna), performs BUSCO analyses for the identification of conserved BUSCO genes, trims them, concatenates common and single copy genes, and performs a phylogenetic analysis based on nucleotides or amino acid sequences. Briefly, common genes to all the selected genomes were retrieved and then aligned using MAFFT v7.520 (Katoh & Standley 2013), trimmed using trimAl v1.4.1 (Capella-Gutiérrez et al. 2009), and concatenated. Phylogenetic analysis was performed on the final concatenated alignment of 217 genes, with 65,463 columns of amino acids, using IQTREE 2.2.3 (Minh et al. 2020) with the automatic selection of the LG + F + I + R10 best-fit model according to the Bayesian information criterion by ModelFinder (Kalyaanamoorthy et al. 2017) and 1000 ultra-fast bootstrap (Hoang et al. 2017). The final bootstrap correlation coefficient of split occurrence frequencies was 1 after 102 iterations. The same approach was applied using the nucleotide option for the phylogenomic inference, with a final concatenated alignment of 217

genes with 198,274 columns of nucleotides. The model was selected by ModelFinder (Kalyaanamoorthy et al. 2017), with the best-fit model SYM + I + R10 chosen according to Bayesian information criterion. The final bootstrap correlation coefficient of split occurrence frequencies was 0.998 after 200 iterations. Trees were visualized in FigTree v1.4.4 (Rambaut 2012) and redrawn with Inkscape v1.3.

The average nucleotide identity (ANI) and the average amino acid identity (AAI) to the closest phylogenetic and morphological taxa were calculated using orthoANI (Lee et al. 2016) and EzAAI (Kim et al. 2021), respectively. Digital DNA–DNA hybridization (DDH) was calculated using the genome-to-genome distance calculator (Meier-Kolthoff et al. 2022).

6.2.6 Analyses of the 16S–23S rRNA ITS region

For the 16S–23S rRNA ITS secondary-structure identification, M-fold was used, applying the default parameter settings (Zuker 2003). The D1–D10, Box-B, and V3 helix sequences were identified after the M-fold results and the published literature. Final structures were redrawn with Inkscape v1.3.

6.3 Results

***Pseudocalidococcus azoricus* R.F.S. Luz, J. Kaštovský, V. Gonçalves gen. sp. nov. (Figure 1).**

Diagnosis: Morphologically similar to *Synechococcus*, but with a distinct phylogenetic placement in the Thermosynechococcaceae by the 16S rRNA phylogeny and phylogenomic analysis. Differs from *Thermosynechococcus* ecologically, as *Pseudocalidococcus* is a freshwater and *Thermosynechococcus* is strictly thermal, and genomically, both in its phylogenetic position and low AAI (66.3%) and ANI (69.4%).

Description: Cells solitary or arranged in small clusters. Without mucilage or any evident envelops. Cells blue-green, cylindrical, rod-shaped (sometimes slightly arcuate) to elongated cylindrical, and occasionally slightly widened at both ends. Cells 1.6–6.5 µm in length (mean = 2.9 µm) and 0.8–2.0 µm wide (mean = 1.4 µm), with a length/width ratio of 1.1–6.3 (mean = 2.2). Observed elongated cells were up to 45 µm in length. Cell division perpendicular to the long axis of the cells. Thylakoids present in a parietal arrangement; up to five.

Holotype: Dried material preserved in a permanently inactive state at Herbário Ruy Telles Palhinha, University of Azores, Portugal, under the AZB 4202 code.

Type Locality: Lagoa Comprida, Lajes, Flores Island (Azores archipelago, Portugal); 39°26’26.052” N 31°13’19.0128” W, collected by the MONITAIA team project.

Habitat: Aquatic in freshwater

Etymology: *Pseudocalidococcus*: Pseudo (fake)—calidum (hot)—coccus: fake thermal cyanobacteria, as it is positioned inside a supposedly thermal family; masculine gender. azoricus: isolated from the Azores archipelago.

Reference Strain: BACA0444 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal), isolated by Rita Cordeiro.

Gene Sequences: GenBank accession number OM732240 for the 16S rRNA and 16S–23S rRNA ITS genes and GenBbank accession number GCA_031729055 for the genome assembly.

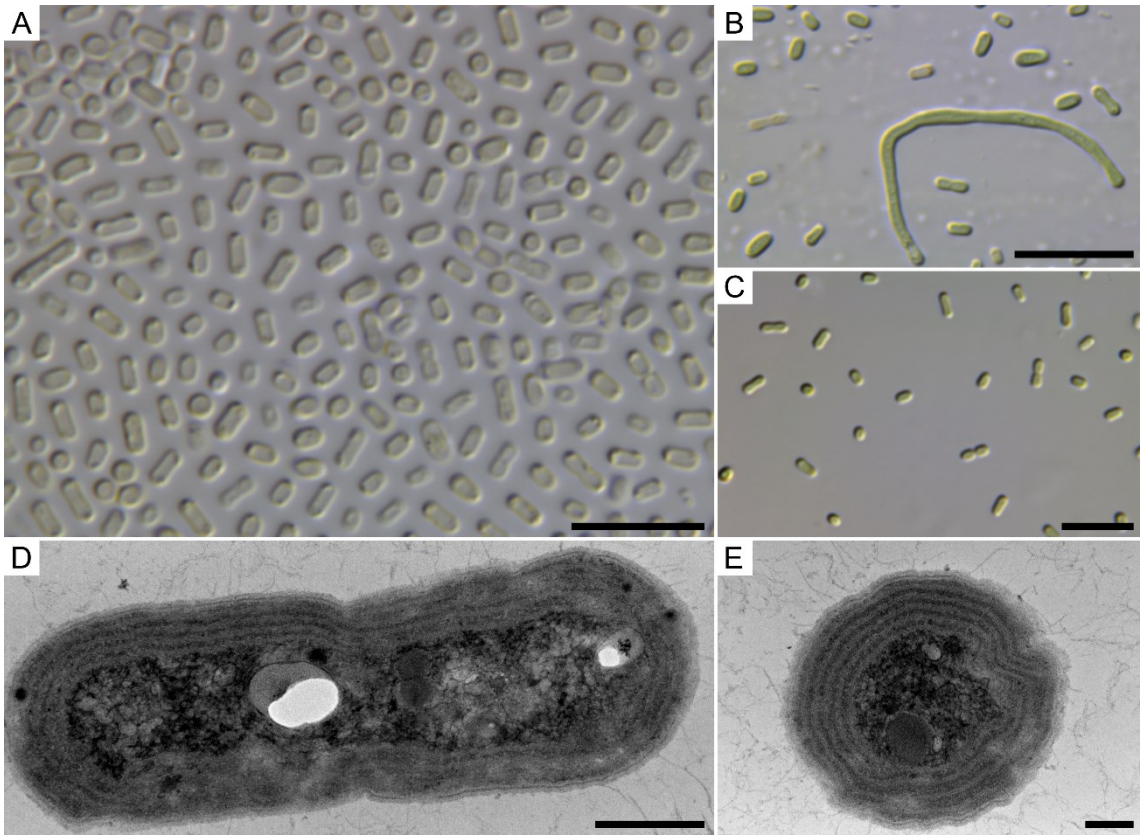

Figure 1. Morphology of *Pseudocalidococcus azoricus* BACA0444 under light microscope and TEM. (A) Different cell morphologies in DIC; (B) Normal and elongated cells in DIC; (C) Cells with incomplete binary fission; (D) Transversal cut showing four parietal thylakoids; (E) Longitudinal cut showing five parietal thylakoids in the cell. Scale bars 10 µm (A–C), 500 nm (D), and 200 nm (E).

6.3.1 Morphological analysis

The four strains studied in this work have very similar morphological characteristics (Table 2) despite originating from different ecosystems in the Azores; namely, from a small lake in Furnas village (São Miguel Island), Lake Empadadas Norte (São Miguel Island), Lake Comprida (Flores Island), and an atmophytic site in Ribeira Grande (São Miguel Island). This represents a

large geographical distance of separated populations from where the strains of *P. azoricus* were isolated.

Table 2. Cell dimensions in the four studied strains, with the minimum, maximum, and arithmetic mean of the length and width in micrometers. The *P. azoricus* cell dimensions correspond to the combined values of the four strains.

	Length			Width			Ratio (Length/Width)		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
BACA0433	1.8	6.5	3.0	0.8	1.4	1.1	1.7	6.3	2.6
BACA0444	1.6	4.0	2.6	1.0	1.6	1.3	1.1	3.4	2.0
BACA0446	1.8	5.1	3.0	1.0	1.9	1.4	1.2	4.0	2.2
BACA0781	2.0	4.6	3.0	1.1	2.0	1.5	1.3	3.4	2.0
P. azoricus	1.6	6.5	2.9	0.8	2.0	1.4	1.1	6.3	2.2

The four strains presented the same morphological cell characteristics, but not all presented the elongated cells, as seen in Figure 1B, with up to five parietally arranged thylakoids (Figure 1E). An attempt was made to compare the morphological characteristics of our strains with the strains that fall within the *Pseudocalidococcus* phylogenetic clade, but no description was found in the literature.

6.3.2 16S rRNA phylogeny and 16S–23S ITS secondary structures

The four Azorean strains were grouped together with other *Synechococcus* sp. strains (EO68, CHAB TP201738, IPPAS B-1202, PCC 6312, and PCC 6603) in the 16S rRNA phylogenetic analysis (Figure 2), near *Thermosynechococcus*, with strong support (100 ML, 1 BI), suggesting the position of *Pseudocalidococcus* in the Thermosynechococcaceae. Furthermore, the *Synechococcus* sp. strains positioned in the cluster of *Pseudocalidococcus azoricus* are genetically closely related and must all belong to the genus *Pseudocalidococcus*. Therefore, *Pseudocalidococcus* has a wide geographical distribution, being present at least in the United States of America (a freshwater pond in California, PCC 6603 strain) and Kazakhstan (Issyk Lake, IPPAS B-1202 strain), besides the Azores archipelago.

The secondary structure of the D1-D1' and Box-B helix of the 16S–23S rRNA ITS is shown in Figure 3. As expected and reinforcing the distance of the *Pseudocalidococcus* genus to *Thermosynechococcus*, a large difference was observed between the folded structures in both the sequence and folding. A marked difference can be seen in the formation of the different lateral bulges in both genera in the D1-D1' helix and in the Box-B helix in the mid-internal loop of *Pseudocalidococcus*, in contrast with its absence in *Thermosynechococcus*.

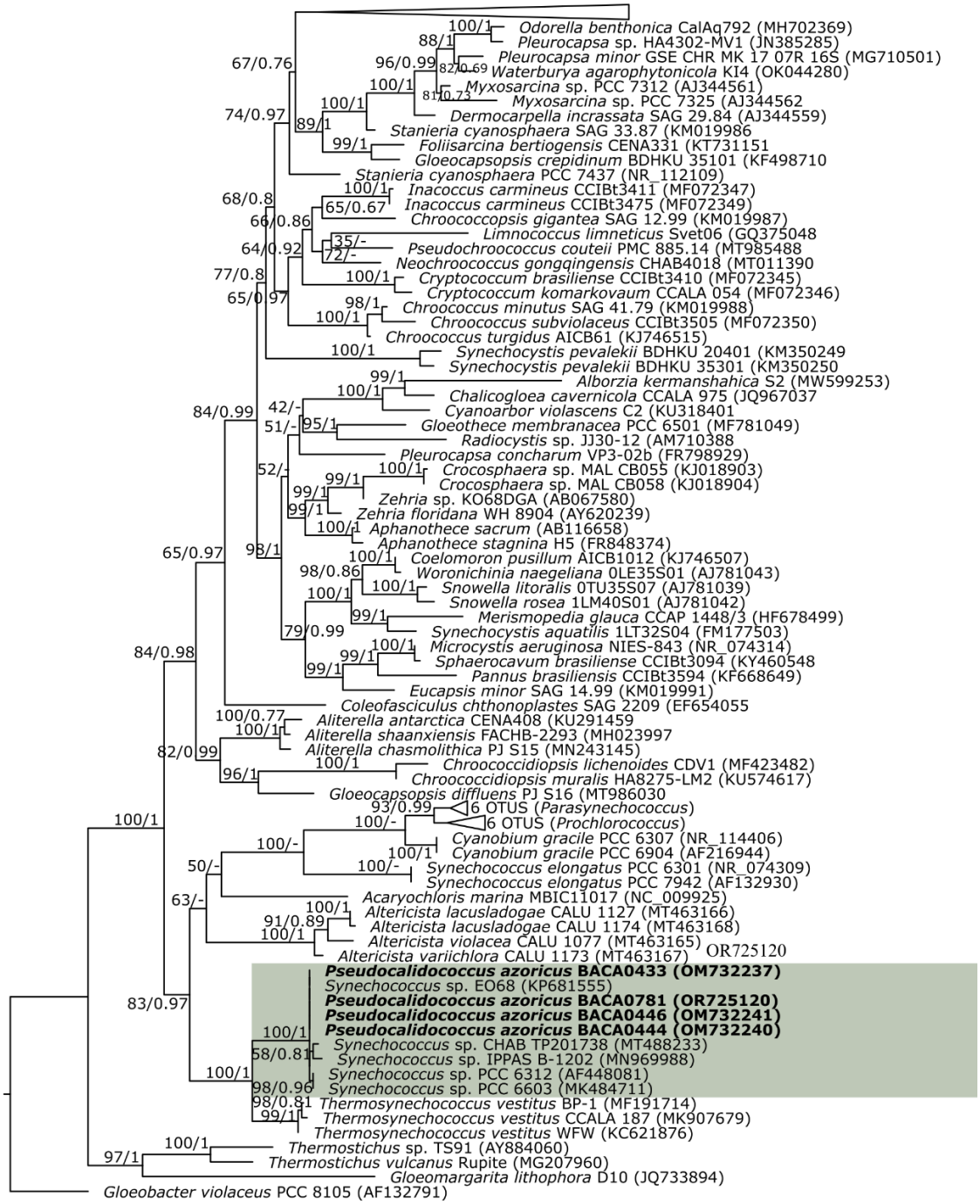

Figure 2. 16S rRNA partial maximum likelihood (ML) phylogenetic tree. Bootstrap values for the maximum likelihood and posterior probabilities for the Bayesian inference are indicated on the tree. The studied strains are in bold font. The new genus is in the green shade.

6.3.3 Genomic analysis

The produced genome is of high quality, with 34 contigs, a size of 3,463,985 base pairs (Figure 4), and a GC content of 48.7%. Quality control showed a 99.53% completeness and a 0.12% contamination according to CheckM v1.2.2, a 99.8% completeness and a 0.0%

contamination according to CheckM2 v1.0.2, and a 98.4% completeness according to BUSCO v5.5.0. Assembly data and annotation statistics to the closest phylogenetic genera are presented in Table 3.

The phylogenomic analysis placed the new genus in the same position as the 16S rRNA phylogenetic analysis, confirming its similarity with *Thermosynechococcus*, with a good bootstrap support of 100 (Figure 5). The ANI and AAI analysis supported the gene separation against *Thermosynechococcus*, with low values (below 70%). The ANI, AAI, and DDH supported the presence of at least two species in the genus, following the genomic analysis of the available data.

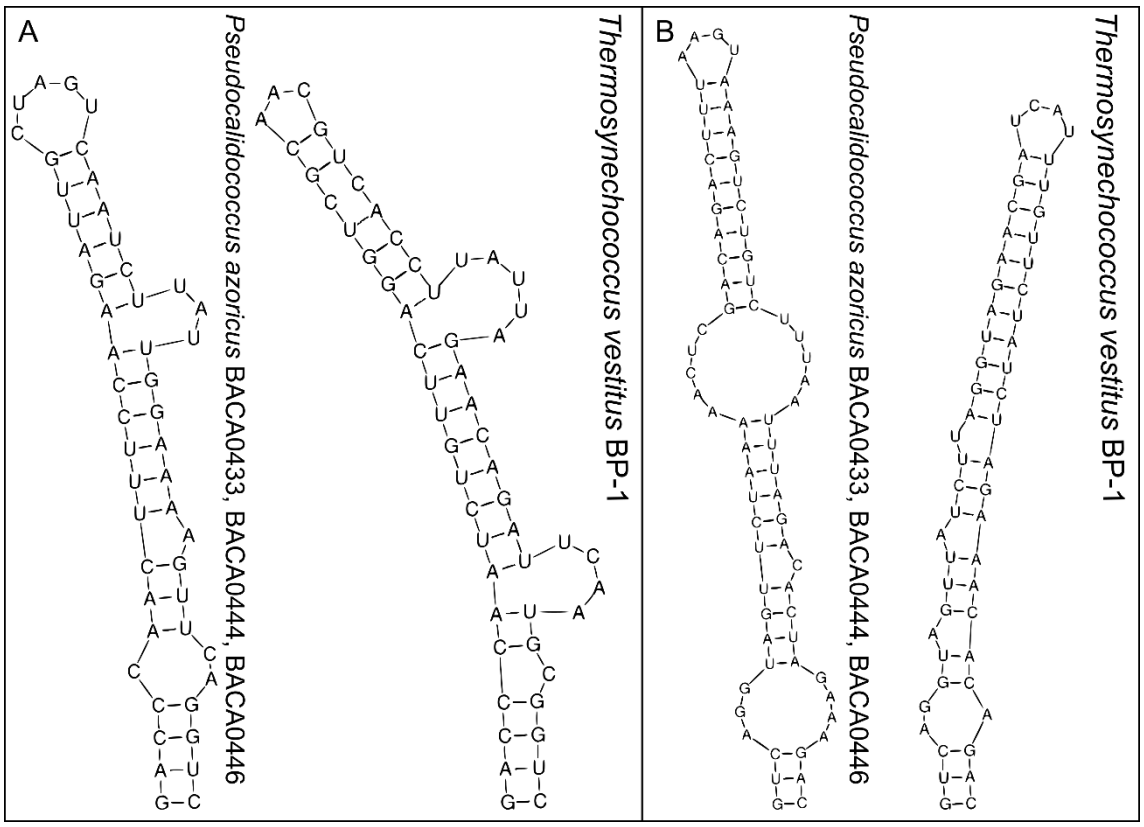

Figure 3. The 16S–23S rRNA ITS secondary structures of the D1-D1' (A) and Box-B (B) region of *Pseudocalidococcus azoricus* BACA0444 and the closest phylogenetic species of *Thermosynechococcus vestitus* BP-1.

6.4 Discussion

The new genus *Pseudocalidococcus* is phylogenetically closely related to *Thermosynechococcus*. However, it is noteworthy that *Thermosynechococcus* is recognized as a genus strictly associated with thermal environments (Katoh et al. 2001, Komárek et al. 2020), and *Pseudocalidococcus* strains have been isolated, not only from freshwater lakes, but also

from an atmophytic habitat on a rock wall. This habitat distribution suggests that *Pseudocalidococcus* is primarily thermotolerant rather than thermophilic. Genetically, the 16S rRNA pairwise distance (Table 4) is slightly above (94.8%) the recommended minimum threshold values of 94.5% for the 16S rRNA (Yarza et al. 2014). However, the combination of the 16S rRNA phylogenetic distance (Figure 1), the genomic analysis, the ANI and AAI values, and the phylogenomic analysis strongly support the creation of the new genus, *Pseudocalidococcus*.

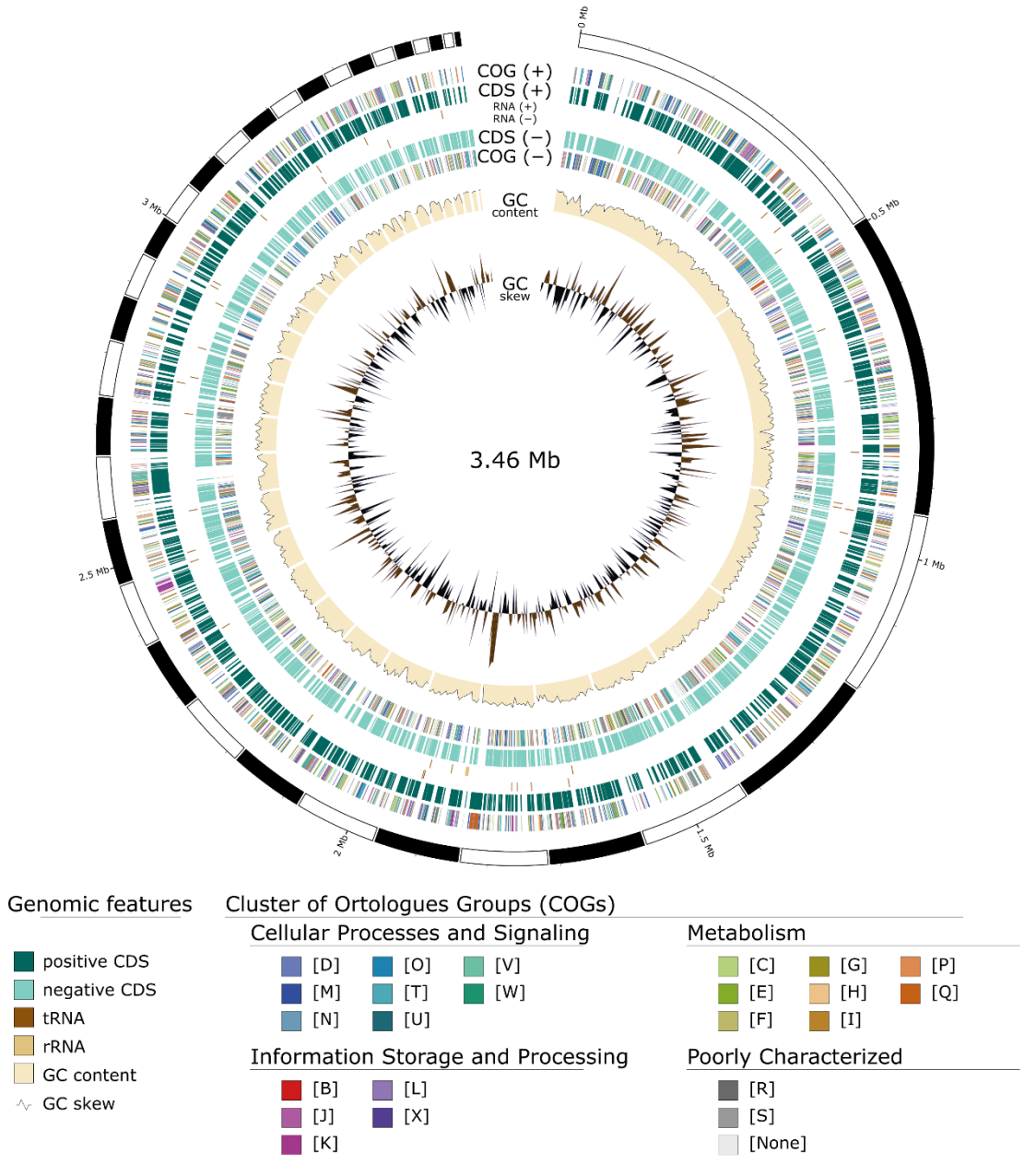

Figure 4. Circular genome representation of *Pseudocalidococcus azoricus* BACA0444 using GenoVi (Cumsille et al. 2023).

Compared to *Synechococcus*, *Pseudocalidococcus* is morphologically very similar. *Pseudocalidococcus* present the same type of involution/elongated cells when in culture, as

described for the type species *Synechococcus elongatus* (Komárek 1970, Komárek & Anagnostidis 2000). However, phylogenetically, it is distantly placed from *S. elongatus* PCC 6301, the currently accepted reference strain. The phylogenetic distance provides strong support for recognizing the difference between these genera.

Pseudocalidococcus azoricus falls within the general morphological cell description of *Synechococcus nidulans* (Komárek & Anagnostidis 2000). However, this can be very problematic, as the validity of the latter is questionable. *Synechococcus nidulans* is a comb., cited in (Bourrelly 1970), with the basionym of *Lauterbornia nidulans* (Richter) Pringsheim (Bourrelly 1970), and *L. nidulans* with the basionym of *Aphanothece nidulans* Richter (Pringsheim 1968). Thus, *Aphanothece nidulans* and *Synechococcus nidulans*, both currently considered valid, have the same holotype, which is not taxonomically acceptable. In the same year, Komárek (1970) describes *Synechococcus leopoliensis* comb. nov., arguing that *Aphanothece nidulans* and *Lauterbornia nidulans* are different taxa, including the latter as a synonym of *Synechococcus leopoliensis* (Komárek 1970). To increase the complexity of the subject, the strain used by Pringsheim (1968) for *Lauterbornia* description (Pringsheim 1968) was Kratz-Allen/Bloom 625, which are synonyms of PCC 6301, CCAP 1405/1, and SAG 1402-1 (Komárek 1970, Rippka & Cohen-Bazire 1983). PCC 6301 is the reference strain of *Synechococcus* (Rippka et al. 1979), and the currently accepted neotype of *Synechococcus elongatus* (Stanier et al. 1971, Rippka & Cohen-Bazire 1983).

Table 3. Genome data from *Pseudocalidococcus azoricus* and the closest phylogenetic genera. Statistical data of assembly were retrieved from CheckM v1.2.2 and Bakta v1.8.2.

Species	*P. azoricus*	*Pseudocalidococcus* sp.	*T. vestitus*	*T. vestitus*	*Thermosynechococcus* sp.	*A. marina*	*S. elongatus*
Strain	BACA0444	PCC 6312	E542	BP-1	HN-54	MBIC11017	PCC 7942
Origin	Azores, Portugal	California, USA	Ganzi, China	Beppu, Japan	Hunan, China	Republic of Palau	California, USA
Habitat	Freshwater	Freshwater	Thermal	Thermal	Thermal	Marine	Freshwater
GenBank accession	GCA_031729055	GCA_000316685	GCA_003555505	GCA_000011345	GCA_023650955	GCA_000018105	GCA_000012525
Number of contigs	34	2	1	1	1	10	2
Completeness	99.53	99.29	100.0	99.76	100.0	99.53	100.0
Contamination	0.12	0.0	0.12	0.12	0.12	5.07	0.0
N50	125,609	3,697,276	2,650,294	2,593,857	2,705,963	6,503,724	2,695,903
Genome size (bp)	3,463,985	3,720,499	2,650,294	2,593,857	2,705,963	8,361,599	2,742,269
G + C content (%)	48.7	48.5	53.3	53.9	53.1	47.0	55.4
Coding density (%)	86.7	87.4	92.7	90.3	91.8	84.3	89.5
Nº of rRNA genes	3	3	3	3	3	6	6
Nº of tRNA genes	40	41	42	42	43	76	45
Nº of protein-coding genes	3386	3699	2541	2514	2610	7760	2720
Nº of pseudogenes	15	4	1	0	2	14	0
Nº of hypotheticals genes	356	132	41	81	102	424	63

Therefore, *Synechococcus nidulans* is a nom. inval., as it has no valid holotype (it is invalid under the International Code of Nomenclature for algae, fungi, and plants), and only *Aphanothece nidulans* is still valid, as previously confirmed (Komárek 1970). Under these terms, *Lauterbornia nidulans* should be considered as a synonym for *Synechococcus elongatus*, as they are all based on the same strain, with morphological differences probably related to culture conditions and/or long-term maintenance (Lopez-Igual et al. 2022). This approach allows the separation of *Synechococcus leopoliensis*, which should be regarded as a synonym of *Romeria leopoliensis*, as suggested by Komárek & Anagnostidis (2005). Considering that *Synechococcus nidulans* is a nom. inval., we disregard this taxon for the proposal of the new species. However, as there are many reports of this taxon in the literature, its validity should be reassessed as soon as possible.

Table 4. The 16S pairwise distance similarity, the 16S rRNA ITS pairwise distance similarity, and the ANI, AAI, and DDH (identities/HSP length) percentages.

	1	2	3	4	5	6
1. *Pseudocalidococcus azoricus* BACA0444						
2. *Pseudocalidococcus* sp. PCC 6312	99.8|93.4|90.0 |92.0|39.5					
3. *Thermosynechococcus vestitus* E542	94.8|77.5|69.4 |66.4|22.9	94.6|76.9|69.4 |66.3|22.8				
4. *Thermosynechococcus elongatus* BP-1	94.8|80.6|69.5 |66.4|19.5	94.8|80.5|69.5 |60.9|21.9	99.7|96.5|87.6 |91.6|33.3			
5. *Thermosynechococcus* sp. HN-54	94.9|79.6|69.5 |66.3|21.6	94.8|78.9|69.4 |66.3|23.0	99.4|91.5|88.2 |93.1|34.5	99.3|95.1|86.5 |90.9|30.9		
6. *Acaryochloris marina* MBIC11017	90.0|70.5|67.1 |61.8|24.1	90.1|71.2|67.2 |61.7|25.8	90.7|72.3|67.1 |61.9|25.8	90.6|74.6|67.1 |61.7|29.3	90.7|72.3|67.2 |61.8|30.1	
7. *Synechococcus elongatus* PCC 7942	90.6|67.3|66.5 |60.7|34.4	90.5|65.3|66.5 |60.7|39.4	90.4|66.2|67.2 |61.3|20.3	90.5|68.8|67.3 |61.3|28.8	90.5|64.9|67.2 |61.3|20.3	90.2|64.9|66.1 |60.2|24.4

In the *Pseudocalidococcus* clade, genomic data are only available for the strains *P. azoricus* BACA0444 and *Pseudocalidococcus* sp. PCC 6312. The 16S rRNA similarity of these two strains is quite high, with only a 0.2% difference, indicating that these strains belong to the same species, following the criteria of 98.7% for species delimitation in bacteria (Stackebrandt & Ebers 2006, Kim et al. 2014). However, the ANI and AAI values (90.0% and 92.0%, respectively) are well below the recommended 95% threshold for species separation (Varghese et al. 2015, Jain et al. 2018), supporting the possible separation of these strains in two distinct species. This hypothesis was also supported by the DDH analysis, with a value of 39.5%, considered a good support value for species distinction (Tindall et al. 2010, Meier-Kolthoff et al. 2013). These contradictory results can be problematic, as the 16S rRNA similarity has been used as a reference for species delimitation (Mai et al. 2018). The 98.7% recommended threshold value (Stackebrandt & Ebers 2006, Kim et al. 2014) is widely used, and this value is based on DNA–DNA hybridization and the

correlation that exists between the 98.7% 16S rRNA values and the 70% DNA–DNA hybridization value, which is the gold standard for microbial species delimitation (Tindall et al. 2010, Meier-Kolthoff et al. 2013). This pattern was also observed in the *Thermosynechococcus* strains, as the 16S rRNA similarity (recommended 98.7%) does not match with the ANI and DDH similarities (suggested as 95% and 70%, respectively); instead, a difference of only 0.4–0.7% 16S rRNA similarity corresponds to a more than 10% divergence in the ANI values, and much lower than 70% in the DDH.

To our knowledge, no generic value is accepted for genera distinction using genomic ANI or AAI criteria. Based on the 16S rRNA, a 94.5% similarity is suggested as the threshold for genera separation (Yarza et al. 2014). In the Cyanophyceae, these values are often not followed, e.g., in Nostocales phylogenetic studies, as different values are applied, resulting in some confusion (Kabirnataj et al. 2020). The sole use of general genetic threshold values from bacterial broad studies, which normally do not even include cyanobacteria data due to the lack of available genomes, must be avoided. Future cyanobacteria taxonomic studies should adopt a heuristic approach, integrating traditional markers (morphological and amplicons) and genomic data. The 16S rRNA analysis must be complemented with a deep genomic approach, including phylogenomic, ANI, AAI, DDH, and other criteria that might support the new taxa (e.g., GC content, coding density, and number of genes), and through the use of a pangenome analysis (Stanojković et al. 2022, Dvořák et al. 2023).

6.5 Conclusions

This work provided a concise description of a new coccoid cyanobacteria, *Pseudocalidococcus azoricus* gen. sp. nov., using a polyphasic approach. This approach allowed the separation of what would appear to be a *Synechococcus nidulans* strain to a new and well-defined genus that probably has a global distribution. With the predictable future increase in genomic data, this study provides a new perspective on the values that should be applied in cyanobacteria taxonomy. The growing accessibility of genomic data and the increase in available software or pipelines, such as KABOOM, that facilitate the recovery and use of genomes or metagenomes should be considered in new taxa descriptions, as they bring important insights when discussing closely related taxa with few differentiating morphological characteristics. Our results reinforce the need for deeper studies in cyanobacteria taxonomy, with larger datasets to clarify if the minimum values suggested for species and genera delimitation can be blindly applied. Using such criteria in cyanobacteria may be too conservative and undermine the knowledge of cyanobacterial diversity.

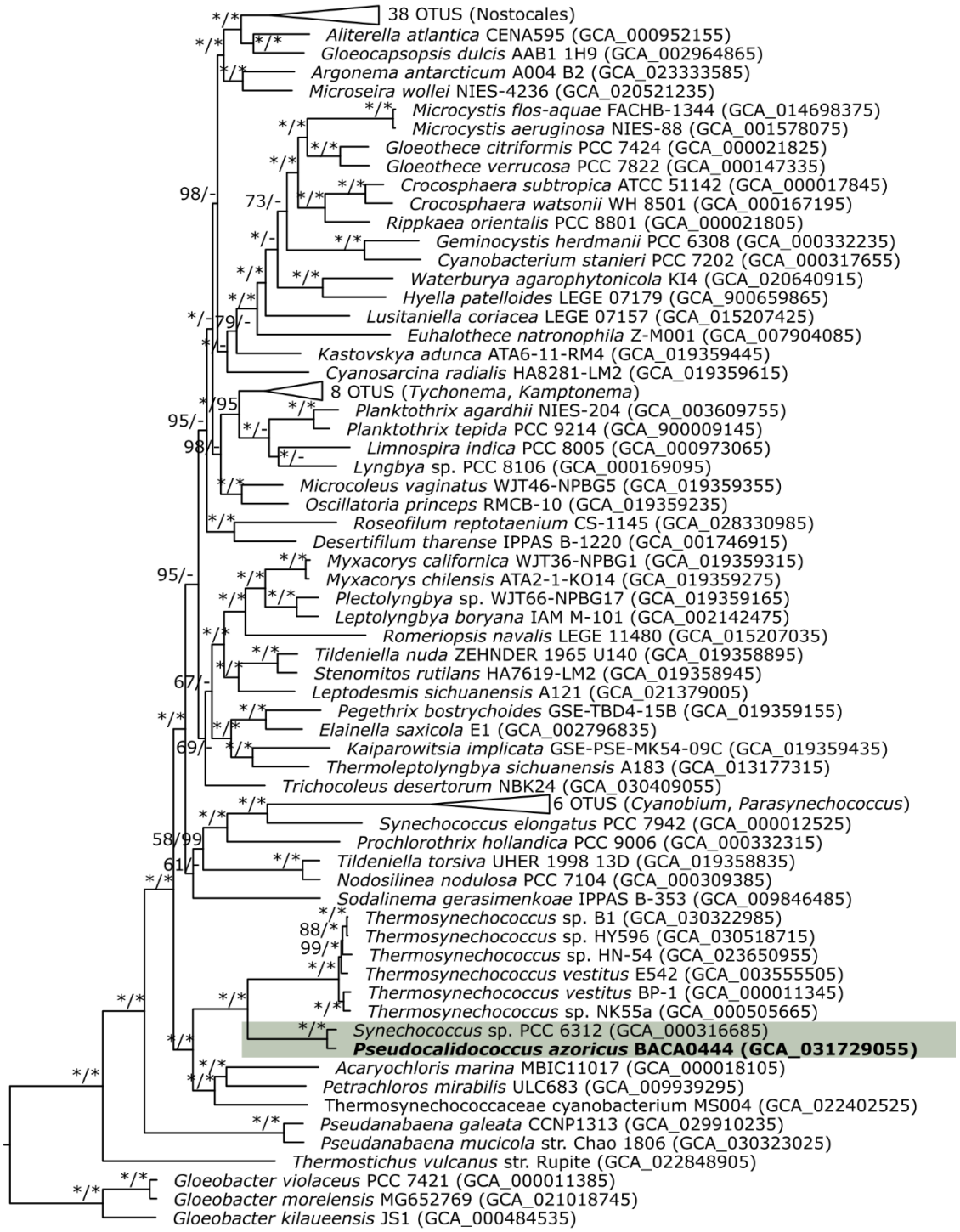

Figure 5. Amino acid maximum likelihood phylogenomic tree with 114 OTUS of 217 concatenated BUSCO genes. Bootstrap values for the maximum likelihood based on amino acids and nucleotides are indicated on the tree. The new genus is in the green shade. * 100% bootstrap.

6.6 Author contributions

Conceptualization, R.L. and V.G.; methodology, R.L., R.C. and J.K.; software, R.L.; formal analysis, R.L., J.K. and R.C.; data curation, R.L. and R.C.; writing—original draft preparation, R.L.;

writing—review and editing, R.L., R.C., J.K., A.F., R.U., V.V. and V.G; funding acquisition, J.K. and V.G. All authors have read and agreed to the published version of the manuscript.

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6.8 Data availability statement

KABOOM is written in python and is freely available at GitHub (https://github.com/rubenluz/KABOOM.git, accessed on 31 August 2023).

6.9 Conflicts of interest

The authors declare no conflict of interest.

6.10 References

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Chapter VII

Metabolite profiling and bioactivity assessment of cyanobacteria from the Azores reveals unique producers of cytotoxic and lipid reducing compounds

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Chapter VII. Metabolite profiling and bioactivity assessment of cyanobacteria from the Azores reveals unique producers of cytotoxic and lipid reducing compounds

Abstract

Cyanobacteria are a group of photosynthetic bacteria and a rich secondary metabolites source. The Bank of Algae and Cyanobacteria of the Azores (BACA) culture collection holds a significant number of strains, including many novel genera and species. 56 strains from freshwater, brackish, and thermal habitats were selected, and grown under standard conditions. Biomass was extracted with methanol, and cytotoxicity was assessed on two carcinoma cell lines, HepG2 and HCT116. The reduction of lipids was tested in zebrafish larvae, and in a steatosis model with fatty acid overloaded human liver cells. The cyanobacterial metabolome was analyzed by HR-ESI-LC-MS/MS and compared using CompareMS2. High similarities were observed in strains of the same genus when isolated from similar habitats, clustering in concordance to the taxonomical order, while no relation could be observed between strains from different genera originated from the same habitat. The extracts of *Cyanobium* sp. BACA0019, *Pseudocalidococcus azoricus* BACA0433 and *Pegethrix atlantica* BACA0077 reduced neutral lipids >40 % in zebrafish at 25 µg/mL, while from *Symphyonema* sp. BACA0090 and *Aliinostoc* sp. BACA0355 induced mortality. Lipid reduction in the steatosis model was observed in many strains, with significant results varying from 50 % to 100 %. Several strains reduced cell viability with the strongest effects from *Scytonematopsis* sp. BACA0005 (HepG2, 59.8 % and HCT116, 68.1 %), *Aliinostoc* sp. BACA0035 (HepG2, 43.3 %, and HCT116, 59.4 %) and *Aliinostoc* sp. BACA0355 (HepG2, 46.2 %, and HCT116, 75.5 %). The feature-based molecular networking identified several cluster of mass peaks related to the observed bioactivities. Chlorophyll derivatives and glycerolipids from *Cyanobium* sp. BACA0019, *Pseudocalidococcus azoricus* BACA0433 and *Pegethrix atlantica* BACA0077 were correlated with the reduction of lipids in zebrafish larvae, while several oligopeptides and fatty amides of *Symphyonema* sp. BACA0090 and *Aliinostoc* sp. BACA0355 with toxicity. Many clusters associated to the bioactivities remained unidentified, which may represent novel compounds, highlighting the chemodiversity of the BACA culture collection.

Keywords: Cyanobacteria, Zebrafish, Cytotoxicity, Anti-steatosis, Lipid reduction, Metabolome

7.1 Introduction

Cyanobacteria comprise a wide range of photosynthetic bacteria that can inhabit and survive in several habitats, ranging from freshwater to brackish and thermal conditions (Cordeiro et al. 2020). This diversity is related to its continuously growing and complex taxonomy with a fast-increasing number of genera and species described in the last years (Luz et al. 2023, Strunecký et al. 2023). Taxonomic studies on cyanobacteria are therefore important to identify genera/species and its potentialities, increasing work reproductivity and impact (Engene et al. 2012).

Cyanobacteria are known to produce a wide array of secondary metabolites (Baunach et al. 2024). The best-known cyanobacterial compounds are toxins, due to its harmful impacts in the ecosystem (Backer et al. 2015, Cirés et al. 2017), with different compound production by distinct strains of the same species (Gkelis et al. 2019, Cordeiro et al. 2024). Many cyanobacteria secondary metabolites show important bioactivities, such as dolastatin 10 (Kallifidas et al. 2024) and cryptophycins (Borbély et al. 2019), with a few already in clinical uses for cancer treatment (Khalifa et al. 2021). This high value of bioactive secondary metabolites of cyanobacteria, and its increasing number of biosynthetic gene cluster (Gavriilidou et al. 2022) reinforce the importance of screening of cyanobacteria from novel microbiological resources centers. The Bank of Algae and Cyanobacteria of the Azores (BACA) is a rich source of unstudied and novel genetic resources (Cordeiro et al. 2020, Luz et al. 2023a, 2023b, 2023c, 2024), and could be a resource for novel bioactive biosynthetic gene cluster and bioactive compounds.

Obesity and its comorbidities are one of the biggest problems for healthcare worldwide (Harborg et al. 2024). Obesity leads to other associated pathologies, as the nonalcoholic fatty liver disease, defined by the accumulation of fat in the liver tissue that may cause cirrhosis or cancer (Genua & Cusi 2024). For the study of lipid reduction activity, several works have used zebrafish larvae as a model organism, considering its functional conservation in the lipid metabolism (Zang et al. 2018). This model organism was also frequently used to test compounds of cyanobacterial origin (Freitas et al. 2019, Martelli et al. 2024, Silva et al. 2024), such as 13–2–hydroxypheophytine a or a vitamin K1-analogue, both reported with lipid reduction activity (Freitas et al. 2019, Silva et al. 2024). Cancer is one of the most common causes of mortality (Schwartz 2024), and some cancer types may be caused by underlying diseases as obesity (Harborg et al. 2024). Cyanobacteria is well described to produce cytotoxic compounds (Robles-Bañuelos et al. 2022), such as dolastatin (Kalemkerian et al. 1999) and apratoxin A (Luesch et al. 2001) among many others (Robles-Bañuelos et al. 2022), and has long been studied for the search of novel, anticancer compounds.

The metabolite profiling using LC-MS/MS data allows the identification of mass peaks in promising extracts, which may be related to the observed bioactivities. The feature-based molecular network (FBMN) is a statistical approach that allows faster and easier compound discovery (Nothias et al. 2018, 2020) on the Global Natural Product Social Molecular Networking (GNPS) platform (Wang et al. 2016). Molecular networks groups the mass spectra (MS2) of metabolites, that can be enriched using the GNPS public library and third party programs such as MS2Query (de Jonge et al. 2023), giving supporting information that can be further used for cluster class prediction with NPClassifier (Kim et al. 2021). This approach has been adopted in many works (Jung & Kim 2023, Selegato et al. 2023, Zwerger et al. 2023), and can be used to streamline further research, reducing costs, and improving metabolite characterization.

The aim of this work was to screen cyanobacteria strains from the BACA culture collection for their cytotoxicity against liver carcinoma (HepG2) and colon carcinoma (HCT116) cell lines, and for the reduction of lipids in zebrafish larvae in vivo and in a steatosis model in HepG2 cells in vitro. Toxicity in zebrafish was also evaluated, which could indicate further hits for the search of anticancer compounds (Shen et al. 2023). Secondary metabolites were extracted using methanol and characterized by HR-ESI-LC-MS/MS and metabolite profiling on the GNPS platform. The metabolome of all strains was compared to their taxonomic clustering, and feature-based molecular networking revealed several mass peaks related to the bioactivities.

7.2 Materials and Methods

7.2.1 Biomass, extract production, and metabolome comparison

Fifty-six cyanobacteria strains from the BACA culture collection were selected (Table S1) to cover the major taxonomic groups present in BACA and understudied taxa. Strains were maintained in the BACA culture collection at 19 °C and a photoperiod of 14 h of light and 10 h of dark, except thermal strains that were kept at 35 °C. Cyanobacteria strains were maintained in the same standard media used during isolation (BG-11, BG-11(N), Z8 and Waris-H; Table S1).

Biomass was produced in 1 L flasks with aeration for four weeks with a photoperiod of 14 h of light and 10 h of dark at 25 °C for freshwater/ terrestrial/brackish strains and at 35 °C for thermal strains. Biomass production was repeated under the same conditions until one gram of freeze-dried biomass was obtained.

One gram of freeze-dried biomass was extracted using an ultrasound bath (120 W) in a 5-min cycle at 1:50 (weight/volume) in methanol and the temperature maintained at a maximum

of 30 °C. Five extraction cycles were performed, pooled, and filtered in 1.2 µm glass fibres filters. Extracts were dried using a rotavapor at 40 °C under vacuum and kept at -20 °C until use.

The dried extracts were resuspended at 1 mg/mL using LC-MS grade methanol and analyzed using liquid chromatography-high resolution electrospray ionization tandem mass spectrometry (HR-ESI-LC-MS/MS) on a Q Exactive Focus Orbitrap LC-MS/MS system in positive mode at the Materials Centre of the University of Porto, as described by Ferreira et al. (2021). Raw data was converted to .mgf format files using MSConvert (Chambers et al. 2012) and the extracts compared using CompareMS2 v2.0.1 (Marissen et al. 2022) using 0.005 m/z as the maximum difference in precursor mass, the minimum intensity of the base peak of 1.0×10^6 and 1.0×10^3 noise, with the remaining options at default.

7.2.2 Phylogenetic analysis

The sequences of the strains were recovered from GenBank and aligned using MAFFT v7.520 with the G-INS-i method (Katoh & Standley 2013). The final alignment contained 1042 columns. The best–fit nucleotide model was evaluated using ModelFinder (Kalyaanamoorthy et al. 2017), with the selection of the evolution model GTR +F +I +G4 according to the Bayesian information criterion.

The phylogenetic tree was constructed using the IQ-Tree online version v1.6.12 (Trifinopoulos et al. 2016) using *Gloeobacter violaceus* PCC 8105 as an outgroup. The maximum likelihood analysis was carried out using the GTR +F +I +G4 model with 1000 ultrafast bootstrap replicates (Hoang et al. 2017). The produced tree was visualized in FigTree v1.4.4 (Rambaut 2012), and the final one redrawn with Inkscape v1.3.

7.2.3 Bioactivity assays

HepG2 and HCT116 cells from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were used for cytotoxicity assessment, and HepG2 for the anti-steatosis assays. Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (Gibco, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10 % (v/v) fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 1 % penicillin/streptomycin (100 IU/mL and 10 mg/mL, respectively) (Biochrom) and 0.1 % amphotericin B (GE Healthcare, Little Chalfont, UK).

Anti-steatosis and cytotoxicity evaluation was performed as described by Costa et al. (2019). Briefly, for anti-steatosis screening, cells were seeded at 1×10^5 cells/well and after 24 h the medium was changed to DMEM without FBS, supplemented with 62 µM sodium oleate (Sigma-Aldrich, St. Louis, MO, USA) and exposed to extracts at 25 µg/mL. The solvent control

was exposed to DMEM without FBS, supplemented with 62 µM sodium oleate and 0.5 % DMSO. After 6 h, 75 ŋg/ mL Nile red (Sigma-Aldrich) and 10 µg/mL Hoechst 33342 (HO-33342) (Sigma-Aldrich) were added and incubated for 15 min at 37 °C in the dark. Fluorescence images were acquired with a 20× objective on the BioTek Cytation 5 Cell Imaging Multimode Reader (Santa Clara, United States of America) at 531/593 nm (RFP) excitation/emission for Nile Red (lipids) and 377/447 nm (DAPI) excitation/emission for HO-33342 (nuclei). DAPI and RFP images were overlapped, and Gen5+ software (Biotek) was used to detect the nucleus and cytoplasm area. Fluorescence intensities in cells were quantified and lipid level were used for threshold analysis, to separate cells into low and high lipid level. The percentage of cells with high lipid level per well was used as 1 replicate value for the analyzes and normalized to the solvent control.

For cytotoxicity, cells were seeded at 3.3 $\times 10^4$ cells per well. After 24 h, the medium was renewed, and the cells were exposed to the extracts at 25 µg/mL for 48 h. Final toxicity was analyzed using the MTT assay (Ferreira et al. 2021).

The lipid reduction in zebrafish larvae was carried out using a Nile red fat metabolism assay, as described by Costa et al. (2019). Briefly, larvae 3 days post fertilization (DPF), raised in E3 medium with 200 µM 1-phenyl-2-thiourea (PTU), were exposed to the cyanobacterial extracts at a concentration of 25 µg/mL. Dimethyl sulfoxide (DMSO, 0.5 %) and resveratrol (REV, at 50 µM) were used as solvent and positive controls respectively. After 36 h, the larvae were stained overnight with Nile red, at 10 ng/mL, the next day anesthetized and imaged with BioTek Cytation 5 Cell Imaging Multimode Reader (Santa Clara, United States of America). The fluorescence intensity was quantified at 531/593 nm (RFP) excitation/emission for Nile Red in each individual zebrafish larvae using ImageJ (Schneider et al. 2012).

All screenings were performed in two independent assays in triplicates per sample.

7.2.4 Feature-based molecular network analysis

The feature based molecular network (FBMN) analysis followed the conditions described by Nothias et al. (2018) and was analyzed using Cytoscape v3.10.2 (Shannon et al. 2003). The higher the difference between the peak intensity of a certain mass peak in an extract with activity compared to an extract without activity, the higher is the correlation and significance values, visualized by size in the molecular networks. For enrichment and cluster class classification, the FBMN output .mgf file was analyzed using MS2Query v1.3.0 (de Jonge et al. 2023), with analog identification considered when MS2Query score was above 0.7. Analog class was based on NPClassifier (Kim et al. 2021).

Statistical analysis followed the script made available by Nothias et al. (2018) (v1.1 for R) for calculation of the p-value (after Bonferroni correction) and Pearson correlation (PC) between feature intensity and bioactivity level. Mass peaks with $p < 0.01$ and correlation >0.6 were matched to the Natural Product Atlas (NPA) database v2024_03 (van Santen et al. 2022) within 5 ppm for a putative identification, with compound class based on NPClassifier (Kim et al. 2021).

7.3 Results

7.3.1 Phylogenetic diversity of cyanobacteria

The 56 studied strains represent 44 genera from the BACA culture collection (Fig. 1) and have a wide representation of cyanobacterial orders and classes, ranging from simple coccoid genera (e.g. *Cyanobium*) to more complex genera (e.g. *Neowestiellopsis*), isolated from freshwater, brackish and thermal habitats. A high diversity of commonly culturable genera is represented, as well as some rarer or understudied ones (e.g. *Symphyonema*, *Scytonematopsis*). Many recently described genera are included, such as *Tumidithrix elongata* BACA0141, *Radiculonema aquaticum* BACA0731, and *Pseudocalidococcus azoricus* BACA0433, or novel species such as *Pegethrix atlantica* BACA0077, increasing the set of chemical diversity for bioactivity search.

7.3.2 Bioactivity of the methanolic extracts

A set of 56 cyanobacterial methanolic extracts were used for the bioactivity screening using in vitro and in vivo approaches. The extracts were analyzed for cytotoxic effects against cancer cell lines and for lipid reduction in hepatosteatosis and zebrafish larvae.

Twelve extracts significantly reduced cell viability in the HepG2 cell line, 22 in the HCT116 cell line, and 10 were active in both cell lines (Fig. 2). The strongest reduction against HepG2 cell line were observed in *Scytonematopsis* sp. BACA0005 (59.8 %), *Aliinostoc* sp. BACA0355 (46.2 %) and *Aliinostoc* sp. BACA0035 (43.3 %). Regarding cell viability reduction in HCT116 cells, nine strains strongly reduce cell viability, *Aliinostoc* sp. BACA0355 (75.5 %), *Scytonematopsis* sp. BACA0005 (68.1 %), *Aliinostoc* sp. BACA0035 (59.4 %), *Dulcicalothrix* sp. BACA0344 (50.3 %), *Mastigocladus laminosus* BACA0121 (49.2 %), *Leptolyngbya* sp. BACA0112 (48.9 %), Rivulariaceae BACA0345 (47.9 %), Rivulariaceae BACA0345 (47.9 %), *Desertifilum* sp. BACA0127 (47.4 %) and *Pegethrix atlantica* BACA0077 (44.2 %). *Scytonematopsis* sp. BACA0005, *Aliinostoc* sp. BACA0035 and *Aliinostoc* sp. BACA0355 were the only strains to strongly reduce cell viability in both cell lines.

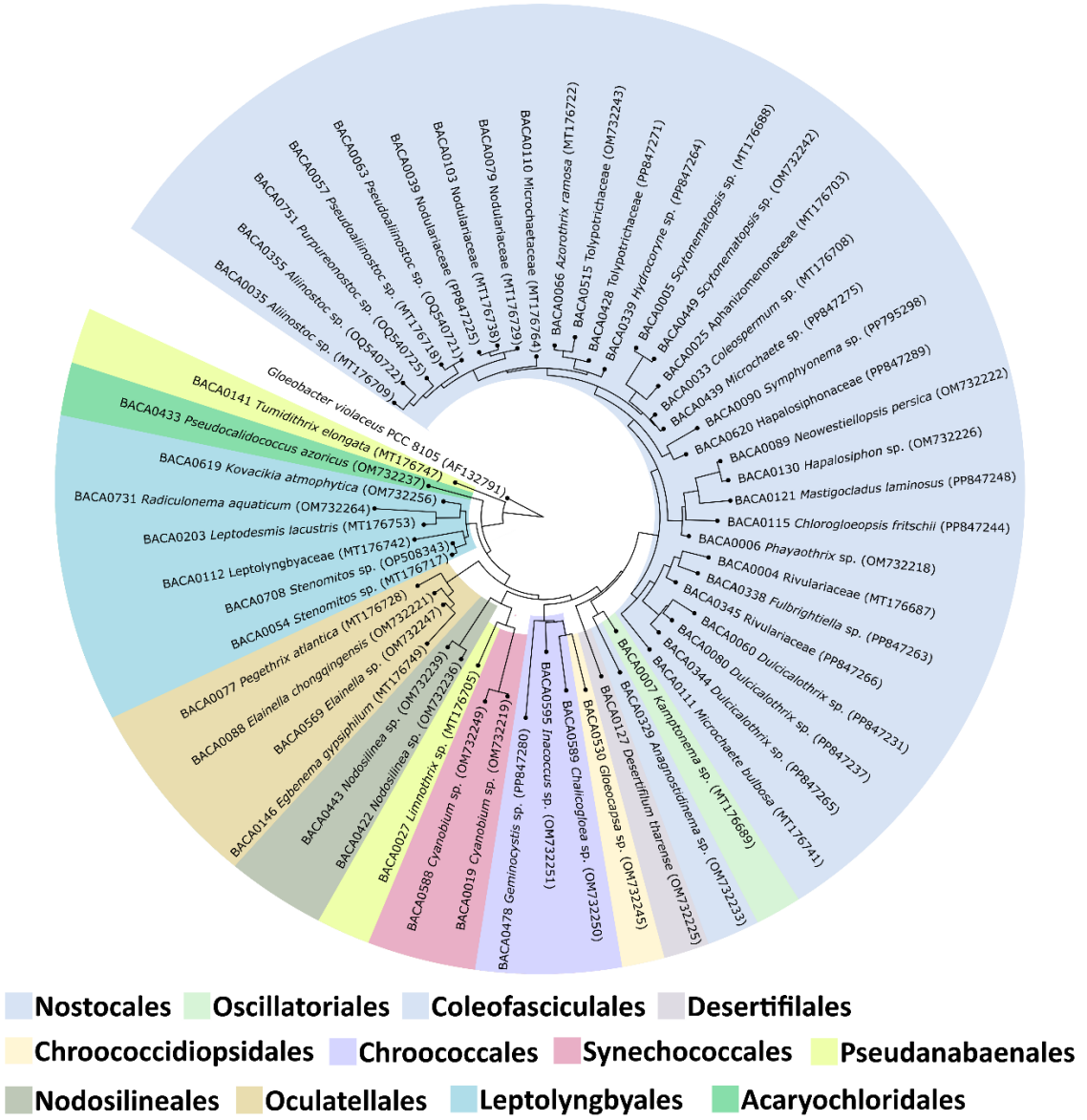

Figure 1. Phylogenetic relationship of the 56 cyanobacterial taxa used from the BACA culture collection.

For hepatosteatosis, 19 extracts significantly reduced the lipid content (Fig. 3). The strongest reductions were observed from six extracts ($p < 0.001$; Kruskal-Wallis test), namely with Nostocaceae BACA0103 (81.0 %; Fig. 4C), Rivulariaceae BACA0345 (80.6 %; Fig. 4F), *Mastigocladus laminosus* BACA0121 (74.2 %; Fig. 4D), *Hapalosiphon* sp. BACA0130 (72.5 %; Fig. 4E) *Dulcicalothrix* sp. BACA0060 (71.2 %) and *Elainella* sp. BACA0569 (70.2 %). For the in vivo assay in zebrafish, significant reduction was observed in three strains, namely, *Cyanobium* sp. BACA0019 (39.1 %), *Pegethrix atlantica* BACA0077 (40.4 %), and *Pseudocalidococcus azoricus* BACA0433 (50.7 %). No congruence has been observed between both assays for lipid reduction. In the in vivo assay with zebrafish larvae, no activity is presented for *Symphyonema* sp. BACA0090 and *Aliinostoc* sp. BACA0355, due to mortality of the larvae prior to analysis after 48 h.

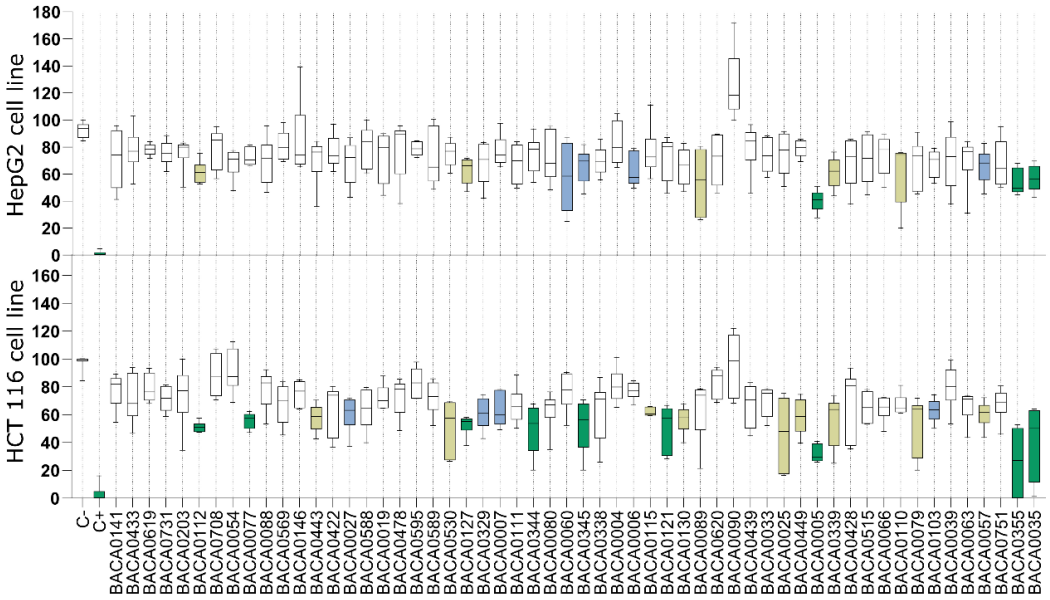

Figure 2. Cell viability after 48 h of exposure to cyanobacterial extracts at 25 µg/mL in the HepG2 and HCT116 cell lines. Strains are phylogenetically sorted as presented in Fig. 1. Green: $p < 0.001$; yellow: $p < 0.01$; blue: $p < 0.05$, according to Kruskal-Wallis test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

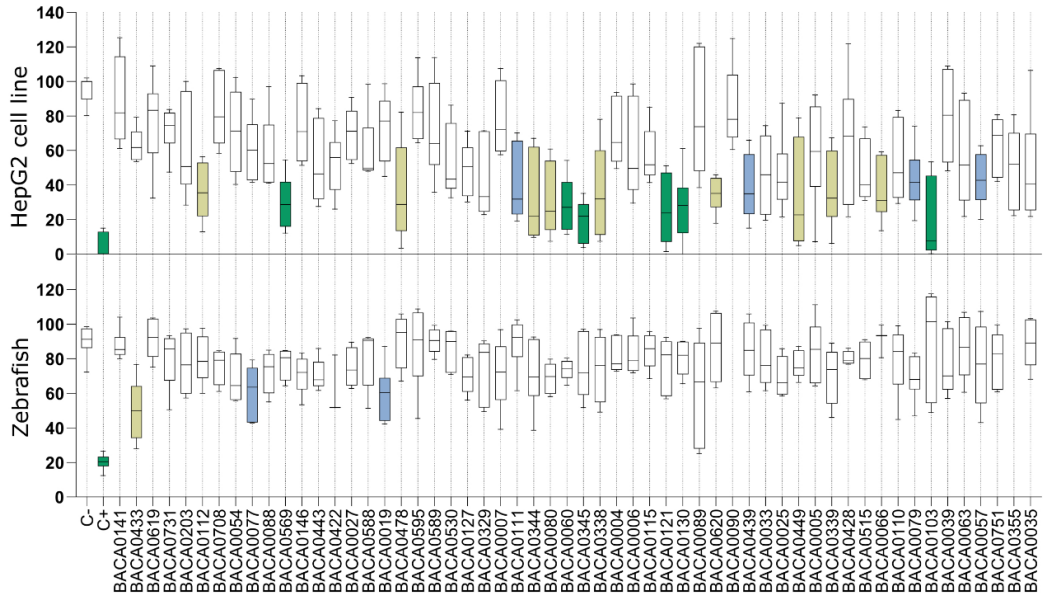

Figure 3. Lipid reduction in the steatosis model with fatty acid overloaded HepG2 cells and in zebrafish larvae exposure to cyanobacterial extracts at 25 µg/mL. Strains are phylogenetically sorted as presented in Fig. 1. Green: $p < 0.001$; yellow: $p < 0.01$; blue: $p < 0.05$, according to Kruskal-Wallis test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

7.3.3 Metabolomic diversity

For the metabolomic analysis the CompareMS2 software was used (Fig. 5). The phylogenetic distribution is not clearly reflected when using this approach, since heterocystous cyanobacteria (Nostocales) are widely distributed through the tree, though with a bigger cluster in the top middle part. In contrast, the bottom middle part mostly comprises coccoid and simple filamentous strains. Several exceptions were observed in the distribution of strains in the tree, namely the placement of several filamentous genera between Nostocales strains in the middle top part of the tree.

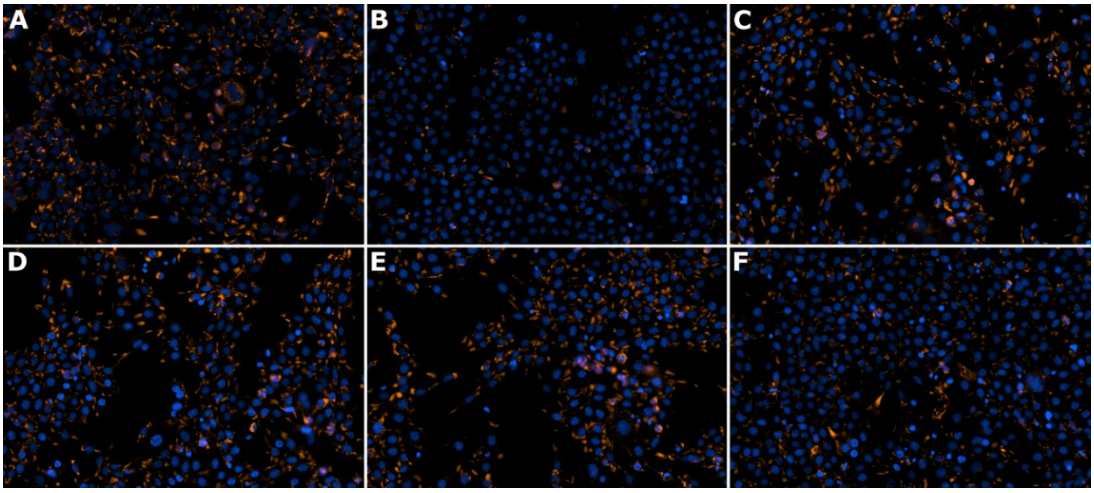

Figure 4. Reduction of lipids in the steatosis model using fatty acid overloaded HepG2 cells. Cells were stained with Nile red for lipidic content (orange) and with HO-33342 for nucleus (blue). A: Fatty acid overloaded cells (using oleic acid); B: Cells without oleic acid; C-F: Fatty acid overloaded cells exposed to methanolic extracts; (C) BACA0103, (D) BACA0121, (E) BACA0130 and (F) BACA0345. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As expected, closely related strains at species level cluster together (*Dulcicalothrix* clade), however, this was not observed for *Aliinostoc* strains, which are distantly placed. No distribution pattern could be observed for the habitat of strains, for example, the selected thermal strains (BACA0110, BACA0111, BACA0112, BACA0127, BACA0146) are placed together with morphologically similar strains.

At the bioactivity level, a high concentration of strains with antisteatosis activity was seen in the top part of the tree, belonging to the heterocystous cyanobacteria (Nostocales). Lipid reduction in zebrafish formed a very small cluster of bioactive strains.

This analysis visualized the chemodiversity of cyanobacterial strains, which did not allow to observe strong clusters regarding phylogeny, habitat or bioactivity, which in turn signifies that

bioprospecting of cyanobacteria still needs a strain-by-strain approach. However, this strategy may help for prioritizing strains for compound isolations, focusing on those that cluster differently, hence having a distinct chemodiversity.

7.3.4 Feature-based molecular network analysis

Results from the feature-based molecular network analysis were considered relevant, if mass peaks were statistically significant ($p < 0.01$) and correlation >0.6 (Fig. S1).

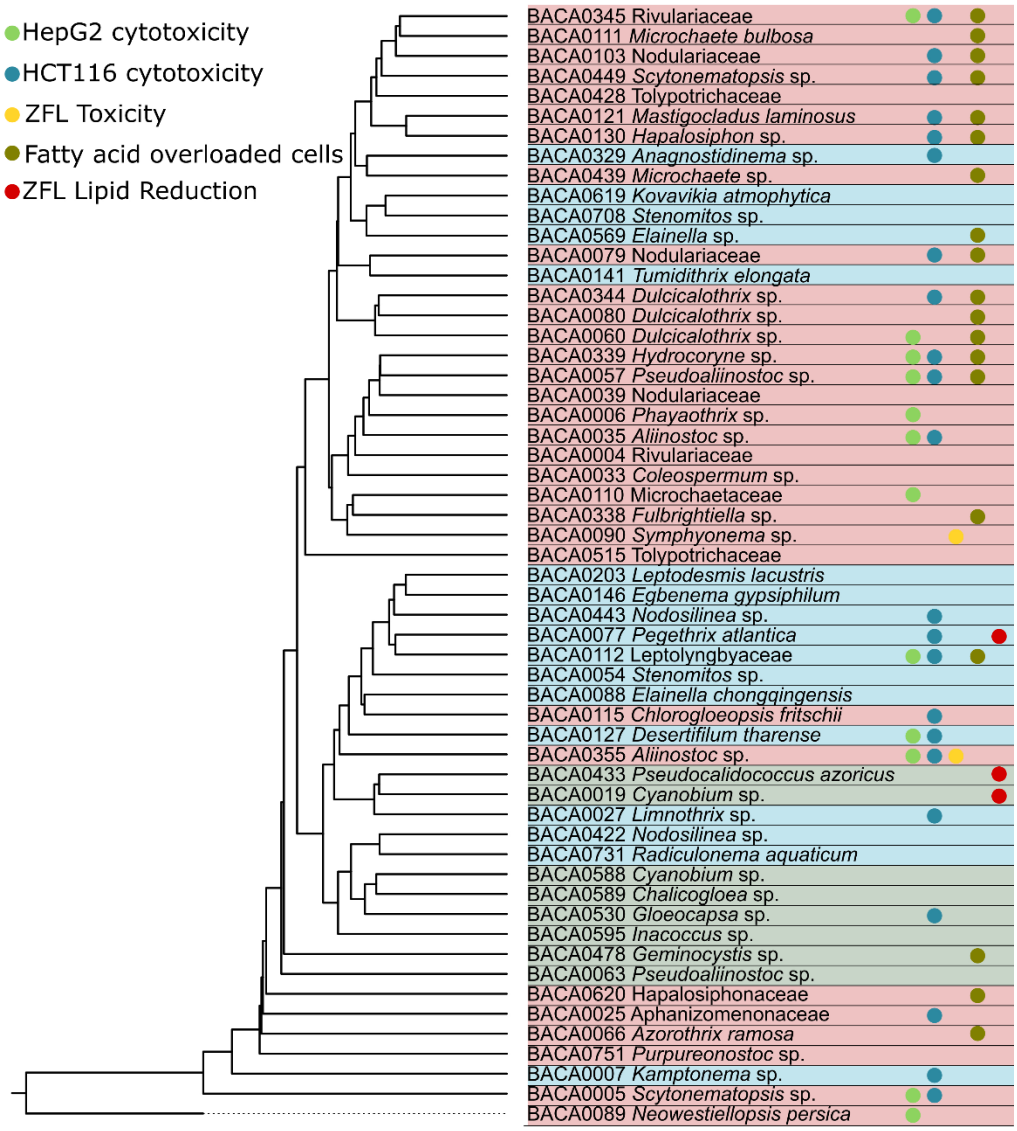

Figure 5. CompareMS2 output tree using metabolomic data from the methanolic extracts with associated statistically significant bioactivities (colored dots). Shades are placed according to general morphology; red: heterocystous cyanobacteria, blue: simple filamentous cyanobacteria, green: coccoid cyanobacteria. ZBL toxicity: zebrafish larvae toxicity; ZBL Lipid reduction: zebrafish larvae lipid reduction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Following these conditions, masses were identified, which correlated with the cytotoxicity in HepG2 and HCT116 cells. Regarding the lipid reduction in zebrafish, 60 masses were associated to this bioactivity (Fig. 6, Table S2). Most of them were produced by both bioactive strains BACA0019 and BACA0433, but in higher quantities than in non-active strains (based on mass peak intensities). Very few masses were unique to one of the strains. Lipid reduction was mostly associated with peptides (oligopeptides), fatty acids (glycerophospholipids and glycerolipids), chlorophyll derivates (pheophytins), and unidentified compounds.

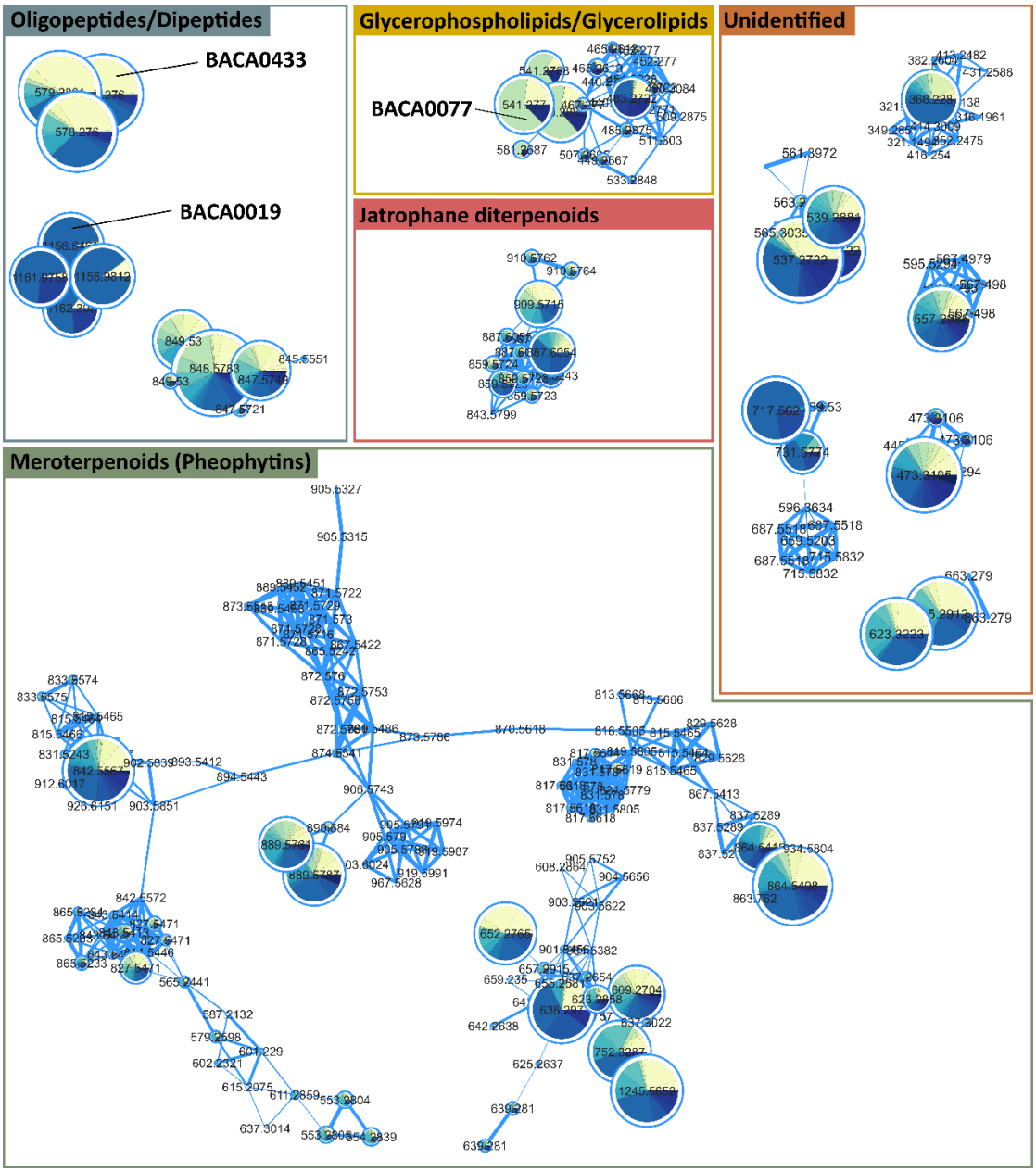

Figure 6. Clusters from the metabolite profiling with FBMN for lipid reduction activity in zebrafish larvae. Significantly correlated nodes are highlighted with size and colour (BACA0019, yellow; BACA0077, green; BACA0433, blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The FBMN for toxicity against zebrafish larvae identified 146 compounds, which correlated to the observed activity (Fig. 7, Table S3). Contrary to what was observed in the lipid reduction in zebrafish analysis, the putative bioactive compounds were predominantly produced in the bioactive strains, with much lower peak intensities in nonactive strains. Bioactivity was highly related with peptides, fatty acids (glycerolipids and fatty amides) and many unidentified compounds with high correlation to the bioactivity.

The FBMN for lipid reduction in the steatosis model in vitro identified 18 compounds with potential activity (Fig. 8, Table S4). Bioactivity was highly related with coumarins and many unidentified class compounds, however, peak intensity differences between bioactive and nonactive strains were not very pronounced.

7.4 Discussion

Several prolific genera regarding their secondary metabolite production were described in cyanobacteria, many related to *Nostoc*-like and *Lyngbya*-like cyanobacteria (Baunach et al. 2024). However, these genera are highly polyphyletic, and similar morphological taxa are distantly placed in phylogenetic analysis (Cordeiro et al. 2020). For work reproductivity, all studied strains have a published 16S rRNA sequence (Fig. 1), which allows for further studies in close genetic strains. Forty-four strains were identified to genera (with 35 different genera) and 11 strains (Cordeiro et al. 2020, Luz et al. 2023b, 2023c) were only identified at the family level. This diversity was chosen to reproduce the large cyanobacterial diversity identified in the BACA culture collection (Cordeiro et al. 2020, Luz et al. 2023c, 2023b), but also reinforces the current literature, where microbiological resource centres possess a high diversity of unexplored genetic resources, and a high potential for the discovery of novel metabolites (Freitas et al. 2016, Ferreira et al. 2021).

A metabolomic analysis was performed for strain comparison using HR-LC-MS/MS data and CompareMS2 v2.0.1 (Fig. 5). In axenic cultures of prokaryotes, the identification or distinction of species is common using metabolomic data (Singhal et al. 2015), however, limited in certain scenarios, especially for novel species (Mortier et al. 2021). The use of metabolomics tools for cyanobacteria distinction is not currently used, but recently, some works used HRLC-MS/MS metabolomic data to support species distinction (Teneva et al. 2023), which reported high metabolomic differences even for same species strains (Racine et al. 2019, Cordeiro et al. 2024). The present work compared a high spectrum of strains regarding their phylogenetic and metabolomic position. No clear relation was found between the chemodiversity and the phylogenetic tree in genera position, however, cluster existed with respect to the position of

strains from similar morphology (coccoid, simple filamentous, heterocytous cyanobacteria) or, in one case, from strains of the same genus. This can be verified for the *Dulcicalothrix* strains (all freshwater), where all strains are phylogenetic positioned in the same clade, and metabolomic positioned near each other. Nonetheless, this is not seen in the *Aliinostoc* strains; albeit of the same genus, the isolated strains originate from different habitats (*Aliinostoc* sp. BACA0035: freshwater; *Aliinostoc* sp. BACA0355: brackish) or *Nodosilinea* strains (*Nodosilinea* sp. BACA0422: brackish, *Nodosilinea* BACA0433: freshwater), probably influencing metabolite production in long-term maintenance cultures, as seen with other strains (Cordeiro et al. 2024). Also, genera isolated from closely related habitats, such as thermal, don't represent any special clade, with metabolome similarity mostly related with genetic evolution, though with several exceptions.

Cyanobacteria were described as prolific producers of bioactive secondary metabolites (Baunach et al. 2024). Previous works identified lipid-reducing activity from cyanobacteria on zebrafish larvae at similar concentrations (Costa et al. 2019, Freitas et al. 2019, Ribeiro et al. 2023, Silva et al. 2024). In the present work, three new strains were reported from three different genera that significantly reduced neutral lipids in zebrafish larvae. One of these strains belong to *Cyanobium*, a previously reported genus (Freitas et al. 2019) with the production of lipid-reduction compounds (Carrasco del Amor et al. 2023). The analysis of lipid reduction in a steatosis model in human liver cells has been widely used and is a well-established methodology (Gómez-Lechón et al. 2007). Few studies have been published using cyanobacteria for anti-steatosis screening (Costa et al. 2019, Ribeiro et al. 2023), which identified some promising strains with potential activity in accordance with our results, reinforcing cyanobacteria as a potential source of bioactive compounds for the prevention and treatment of steatosis.

Several cyanobacteria have been reported as producers of cytotoxic secondary metabolites (Hrouzek et al. 2015, Robles-Bañuelos et al. 2022). A third of our tested strains presented cytotoxicity, which aligns with other studies where many cyanobacterial strains had cytotoxic effects in vitro (Hrouzek et al. 2015, Shishido et al. 2019). For general toxicity assessment, zebrafish larvae is a good in vivo model (Berry et al. 2007). Cyanobacteria are known to produce neurotoxins, such as saxitoxins and anatoxins (Pearson et al. 2010), however, many extracts not related to known cyanotoxins presented toxic effects on zebrafish larvae (Berry et al. 2009, Jonas et al. 2015) even at low extract concentrations of 10 µg/mL (Jacinavicius et al. 2023). The observed toxicity from *Symphyonema* in our study was already reported in Wright et al. (2006), and albeit no toxicity in zebrafish has been reported from *Aliinostoc*, this genus is known to produce cyanotoxins and several bioactive secondary metabolites (Jokela et al. 2017).

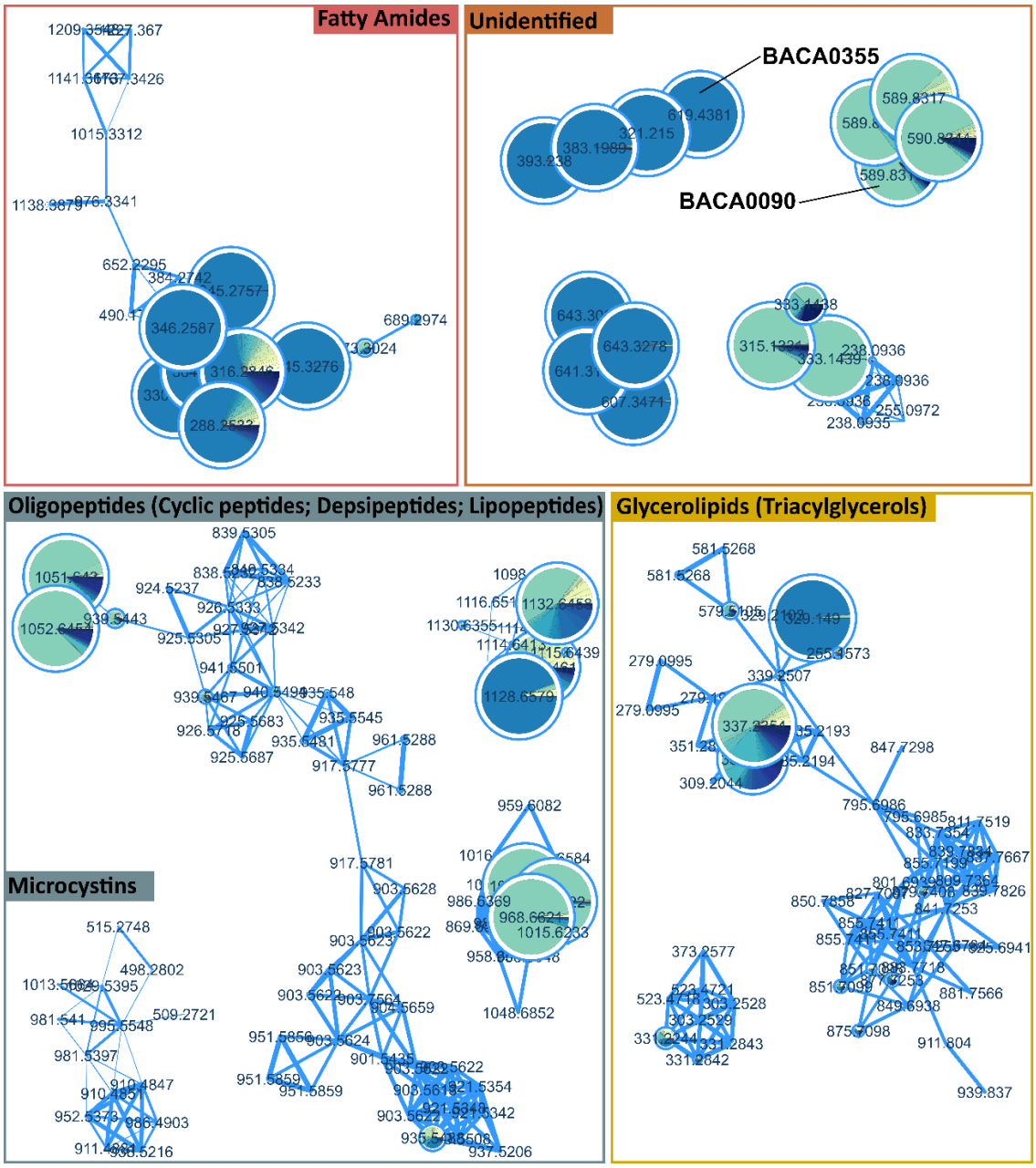

Figure 7. Clusters from the metabolite profiling with FBMN for zebrafish larvae toxicity. Significantly correlated nodes are highlighted with size and colour (BACA0090, green; BACA0355, blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For the feature-based molecular network, *Cyanobium* sp. BACA0019 and *Pseudocalidococcus azoricus* BACA0433 have statistically significant lipid reduction bioactivity (Fig. 3) and are closely positioned in the same clade in the metabolomic tree (Fig. 5). This supports a possible common production of bioactive compounds, also corroborated by the FBMN analysis (Fig. 6), which identified many correlated compounds with the bioactivity, commonly produced by *Cyanobium* sp. BACA0019 and *Pseudocalidococcus azoricus* BACA0433.

In the literature, the lipid reduction activity of *Cyanobium* strains were mainly related to chlorophyll derivates (Freitas et al. 2019, Carrasco del Amor et al. 2023). In Fig. 6, two meroterpenoids masses are shown that have a high bioactivity-feature correlation, 889.5787 m/z (p-value <0.001 and PC: 0.64), mainly produced by *Cyanobium* sp. BACA0019 and *Pseudocalidococcus azoricus* BACA0433, putatively identified as Bacteriopheophytin with MS2Query, and 609.2704 m/z (p-value: 0.001 and PC: 0.60), identified as 132-hydroxypheophytine a by GNPS (Freitas et al. 2019). Some oligopeptide clusters were identified, commonly produced by *Cyanobium* sp. BACA0019 and *Pseudocalidococcus azoricus* BACA0433 or exclusively produced by *Cyanobium* sp. BACA0019. Several peptides have been reported to efficiently reduce lipid content in zebrafish larvae, either exposed or injected (Mohammed-Geba et al. 2016, Guru et al. 2022, 2023). A β-lactoglobulin-derived peptide significantly reduced total lipids (Mohammed-Geba et al. 2016), and the RF13 peptide derived from vacuolar protein sorting associated protein 26B, reduced total lipids in exposed larvae at 5 µM (Guru et al. 2022). The mass peak 848.5783 m/z (p- value <0.001 and PC: 0.88) was putatively identified as Viridamide B, using MS1 data and the NPA database with an error of 4.126 ppm. However, viridamides have not been described with lipid reduction activity, but rather with antitrypanosomal and antileishmanial activity (Simmons et al. 2008).

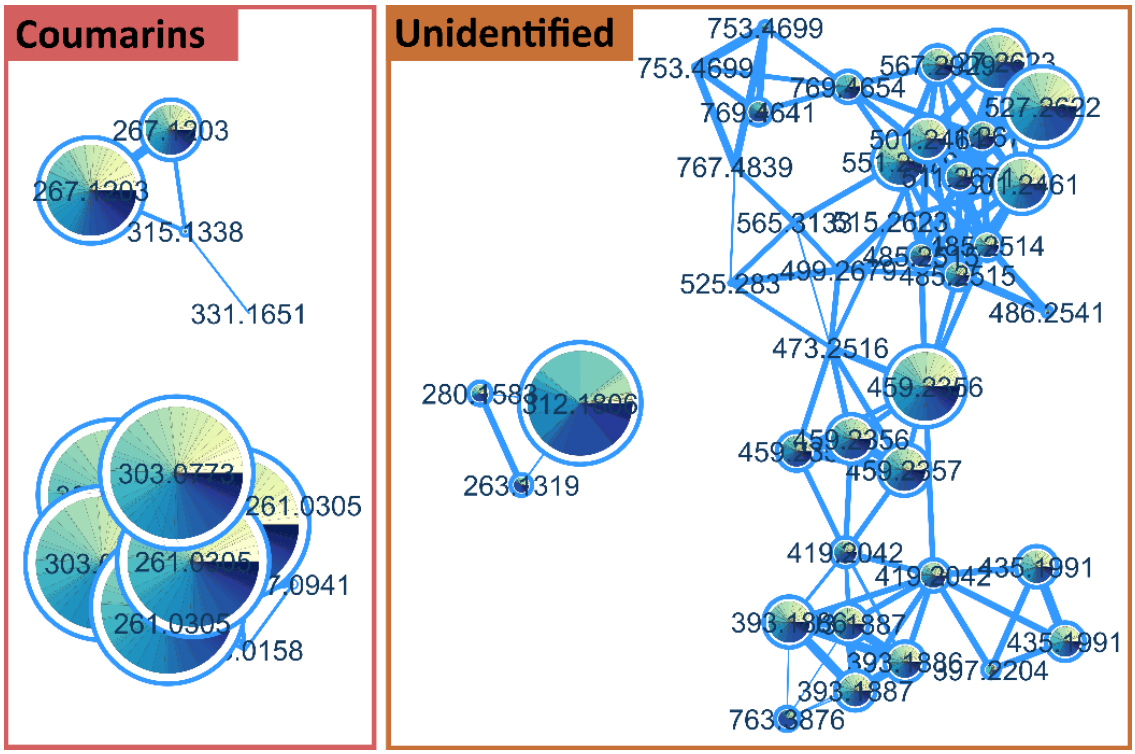

Figure 8. Clusters from the metabolite profiling with FBMN for hepatosteatosis. Nodes significant correlated are highlighted with size, and colour.

Although *Pegethrix atlantica* BACA0077 had the best bioactivity results for lipid reduction, the FBMN results were less clear and fewer mass hits were obtained. These were mainly related to unidentified glycerophosphoethanolamines, however, many fatty acids have been associated to lipid reduction (Siroma et al. 2022). A cluster of Jatrophane diterpenoids, a class of diterpenes with multidrug resistance-reversing activity (Fattahian et al. 2020), was also related to lipid-reduction, however with lower statistical significance and a more general production by the bioactive strains.

In Fig. 5 a high concentration of strains with anti-steatosis bioactivity can be observed in the top portion of the tree. These strains are mostly from the Nostocales order, together with the non-bioactive strains of other taxonomical orders. This leads to the hypothesis of a common production of an antisteatotic active compound with a common evolutionary production in the Nostocales. Most significant results were obtained from unidentified clusters, except for an unidentified mass in the meroterpenoids cluster and several masses associated with the bioactive coumarin compounds (Flores-Morales et al. 2023). Coumarins have been associated with antimicrobial and antiviral bioactivities (Flores-Morales et al. 2023), especially pyranocoumarins (Kumar et al. 2009, Khandy et al. 2022), but also lipid lowering activity in in vivo studies in rats (Sashidhara et al. 2013, Taşdemir et al. 2017).

In the toxicity analysis for zebrafish larvae, 146 mass peaks have a high bioactivity-feature correlation, and many of them are distributed in single clusters and small cluster of unidentified masses, uniquely produced by *Symphyonema* sp. BACA0090 or *Aliinostoc* sp. BACA0355, with a high correlation to toxicity. The FBMN analysis for *Aliinostoc* sp. BACA0355 revealed a quite varied cluster of fatty amides, with high correlation. The cluster has several masses uniquely related to BACA0355, with a putative identification of the mass of 288.2533 m/z, which was identified as lauryl diethanolamide by MS2Query (p-value <0.001 and PC: 0.70). However, this type of compounds is considered safe, without the description of detected toxicity (Fiume et al. 2013). Few masses with high correlation were observed in the glycerolipids cluster, however, no putative identification was obtained by searching in any of the used databases. Microcystin are the best-known peptides from cyanobacteria with hepatotoxic activity (Chen et al. 2015), however, the producing strain did not present any mortality in zebrafish larvae. On the one hand, the absence of in vitro toxicity by microcystins has been already reported in cells lines (Brózman et al. 2020), and on the other hand, the microcystin concentration is unknown in the tested strain. Many of *Symphyonema* sp. BACA0090 masses related to the oligopeptide cluster have a high correlation with toxicity, such as 1132.6458 m/z, identified as Stenothricin D by MS2Query (p-value <0.001 and PC: 0.66), only reported with antimicrobial activity (Liu et al. 2014).

Symphyonema sp. BACA0090, is closely related to *Symphyonema bifilamentatum* 97.28, previously known as *Fischerella ambigua* (Jung et al. 2021), a highly studied strain with several described compounds (Falch et al. 1993, Wright et al. 2005). Further works described toxicity against zebrafish larvae, using cell-free culture media, yet, with unreported toxic compounds (Wright et al. 2006). Although the present work only uses methanol extracts from the biomass, the presence of such compounds cannot be excluded. A search in current data using NPA did not match with any of the putative identifications by Wright et al. (2006), remaining unclear what is causing the toxic effects.

7.5 Conclusion

From the BACA culture collection, 56 cyanobacterial strains were screened for cytotoxicity against HepG2 and HCT116 cell lines, and lipid reduction in zebrafish and an in vitro model for steatosis in HepG2 cells. Cytotoxicity was observed for *Scytonematopsis* sp. BACA0005, *Aliinostoc* sp. BACA0035 and *Aliinostoc* sp. BACA0355. However, no relation with any compounds or classes of compounds could be identified using the FBMN approach. For general toxicity on zebrafish larvae, *Symphyonema* sp. BACA0090 and *Aliinostoc* sp. BACA0355 were identified and the FBMN associated oligopeptides, glycerolipids, and fatty amides as compound families. Three strains demonstrated high potential for lipid reduction in zebrafish larvae, namely *Cyanobium* sp. BACA0019, *Pseudocalidococcus azoricus* BACA0433, and *Pegethrix atlantica* BACA0077, and the responsible compounds were mostly related to meroterpenoids, peptides, and unidentified compounds. Several strains showed bioactivity on the steatosis assay in vitro, mainly of the Nostocales order, although most of the correlated compounds were unidentified. Promising strains were identified from the BACA culture collection, which had strong bioactivities for lipid reduction in zebrafish larvae and in the steatosis model in vitro, which reinforces that the high biodiversity and potential of microbial resource centres such as BACA possess a huge potential for the discovery of novel, bioactive compounds.

7.6 Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103703.

7.7 CRediT authorship contribution statement

Rúben Luz: Writing – original draft, Investigation, Formal analysis, Conceptualization. Vitor Gonçalves: Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Vitor Vasconcelos: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. Ralph Urbatzka: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

7.8 Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7.9 Data availability

Data will be made available on request.

7.10 Acknowledgements

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Chapter VIII

Screening of lipid-reducing activity and cytotoxicity of the exometabolome from Cyanobacteria

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Chapter VIII. Screening of lipid-reducing activity and cytotoxicity of the exometabolome from Cyanobacteria

Abstract

Cyanobacteria are rich producers of secondary metabolites, excreting some of these to the culture media. However, the exometabolome of cyanobacteria has been poorly studied, and few studies have dwelled on its characterization and bioactivity assessment. In this work, exometabolomes of 56 cyanobacterial strains were characterized by HR-ESI-LC-MS/MS. Cytotoxicity was assessed on two carcinoma cell lines, HepG2 and HCT116, while the reduction in lipids was tested in zebrafish larvae and in a steatosis model with fatty acid-overloaded human liver cells. The exometabolome analysis using GNPS revealed many complex clusters of unique compounds in several strains, with no identifications in public databases. Three strains reduced viability in HCT116 cells, namely Tolypotrichaceae BACA0428 (30.45%), Aphanizomenonaceae BACA0025 (40.84%), and Microchaetaceae BACA0110 (46.61%). Lipid reduction in zebrafish larvae was only observed by exposure to *Dulcicalothrix* sp. BACA0344 (60%). The feature-based molecular network shows that this bioactivity was highly correlated with two flavanones, a compound class described in the literature to have lipid reduction activity. The exometabolome characterization of cyanobacteria strains revealed a high chemodiversity, which supports it as a source for novel bioactive compounds, despite most of the time being overlooked.

Keywords: zebrafish, anti-steatosis, lipid reduction, exudate, extracellular compounds, flavonoids, chemodiversity

8.1 Introduction

Cyanobacteria are rich producers of bioactive secondary metabolites (Baunach et al. 2024). The best-known metabolites are toxins due to their environmental damage associated with cyanobacterial blooms (Paerl & Otten 2013). However, many other metabolites of interest are produced by cyanobacteria, such as dolastatin (Kallifidas et al. 2024), cryptophycin (Eggen & Georg 2002), and even toxins, such as saxitoxins (Schantz & Johnson 1992), that have long been recognized for their high biotechnological potential. The high chemical diversity and their genomic potential (Calteau et al. 2014, Popin et al. 2021, Gavriilidou et al. 2022) make cyanobacteria an optimal model for the search for new compounds.

From the wide array of secondary metabolites produced by cyanobacteria, many have been shown to be secreted into the external environment. Examples include polysaccharides

(Mota et al. 2013, Wang et al. 2014), proteins (Oliveira et al. 2015), glycolipids (Moslavac et al. 2007), or fatty acids (Bellefleur et al. 2019). This secretion mechanism plays a role in granting an ecological advantage to cell survival against foreign agents, protecting against desiccation (Potts 1999), UV radiation (Proteau et al. 1993), reactive oxygen species (Shirkey et al. 2000, Wang et al. 2014, Quan et al. 2015), as well as aiding in motility and/or adhesion (Hoiczyk & Baumeister 1999, Khayatan et al. 2015, Rossi & De Philippis 2015) or in metal-deficient environments by the production of siderophores (Årstøl & Hohmann-Marriott 2019). Cyanobacteria is also known to produce allelochemicals with toxic effects (Leão et al. 2009b) or as phytohormones, specifically growth promoters (Toribio et al. 2020). The transfer of compounds to the extracellular media is mediated through secretory portals or extracellular vesicles (Lima et al. 2020).

The exometabolome of cyanobacteria is a group of metabolites produced by cells and expelled to the media. It is mainly composed of exopolysaccharides (EPS), which play an important role in biofilm production, providing protection against environmental agents such as UV radiation, desiccation, and predators (Cruz et al. 2020). Many of the compounds found in the exometabolome are bioactive and may have potential biotechnological applications (Li et al. 2001, Cruz et al. 2020), but toxic and teratogenic effects have also been observed in zebrafish (*Danio rerio*) larvae (Jonas et al. 2014, 2015). For exopolysaccharides, mainly antioxidant and anti-inflammatory activities were described (Wang et al. 2014, Hussein et al. 2015, Zampieri et al. 2020). For non-EPS compounds, several studies have reported toxic effects, such as for portoamides (Leão et al. 2009a, 2010), antifungal activity for tolybyssidins (Jaki et al. 2001), and unidentified compounds with possible anticancer activity (Nováková et al. 2013).

There is a worldwide need to address the increasing incidence of obesity and its comorbidities (Harborg et al. 2024). Obesity is associated with increased morbidity and mortality (Sarma et al. 2021), and it is a leading factor in the emergence of chronic diseases that cause severe health risks, such as cardiovascular diseases, diabetes (Piché et al. 2020), and cancer (Harborg et al. 2024, Schwartz 2024). Another related pathology is nonalcoholic fatty liver disease, which causes an abnormal accumulation of fat in liver tissue that can lead to cirrhosis or hepatocellular carcinoma (Loomba et al. 2021). This fosters an increasing need for treatments to prevent the worsening of obesity-associated pathologies (Genua & Cusi 2024). There has been a significant rise in anti-obesity drugs (Müller et al. 2022), with numerous studies highlighting the potential of cyanobacteria for obesity treatment (Mcfarlane et al. 2002) through both in vitro (Imai et al. 2007, Liu et al. 2011, Fan et al. 2018) and in vivo approaches (Ran et al. 2017, Sadek et al. 2017, Carrasco del Amor et al. 2023). In human trials, the commonly consumed cyanobacteria Spirulina (*Limnospira* spp.) has been associated with the reduction in triglycerides and total cholesterol (Bohórquez-Medina et al. 2021).

One of the most problematic comorbidities of obesity is cancer (Harborg et al. 2024). Cyanobacteria have long been associated with cytotoxic activity (Martins et al. 2008, Hrouzek et al. 2015, Robles-Bañuelos et al. 2022), with many identified cytotoxic compounds from cyanobacteria (e.g., cryptophycin (Eggen & Georg 2002), dolastatin (Kallifidas et al. 2024), portoamides (Ribeiro et al. 2017), and leptochelins (Avalon et al. 2024)); many of these are already in commercial use as anticancer drugs, such as dolastatin (Gao et al. 2021). However, only a few compounds can also be found in the cyanobacterial exometabolome (for example, portoamides) (Leão et al. 2009a), with few works focusing on these extracts (Volk & Mundt 2007) and a small subset of them using purified compounds (Stevenson et al. 2002, Wright et al. 2006, Zhang et al. 2013, Ribeiro et al. 2017).

This work focuses on a seldom-investigated aspect of cyanobacteria secondary metabolites, the secreted compounds known as exometabolomes. The main aim of this work was to explore the chemodiversity of the cyanobacterial exometabolome of 56 cyanobacterial strains from the Bank of Algae and Cyanobacteria of the Azores (BACA) culture collection and to identify possible bioactive strains. Exudate extracts were tested for cytotoxic effects against two cancer cell lines, HepG2 and HCT116, to reveal potential anticancer activities. In addition, exudates were tested for their reduction in lipids in an antisteatosis model using the HepG2 cell line in vitro and in the fat metabolism assay in zebrafish larvae. Metabolomic profiling of the cyanobacterial extracts was performed on the Global Natural Product Social Molecular Networking (GNPS) platform to characterize the chemodiversity and to identify unique mass features in the bioactive strains, which could be related to the observed bioactivity.

8.2 Results

8.2.1 Bioactivity results

All 56 cyanobacteria produced extracellular compounds; however, in very different amounts, ranging from around 0.05 g to 1.5 g of dry weight of the exudates from each 400 mL of media (Table S1). The extracts were applied in in vitro assays for cytotoxicity in HepG2 and HCT116 cell lines, lipid reduction using the HepG2 cell line as an antisteatosis model, and zebrafish larvae for the in vivo model.

In the cytotoxicity assays (Figure 1), none of the extracts significantly reduced the viability of the HepG2 cells. On the contrary, exposure to extracts of three strains resulted in low cellular viability on the HCT116 cell line, namely: Tolypotrichaceae BACA0428 (30.45%), Aphanizomenonaceae BACA0025 (40.84%), and Microchaetaceae BACA0110 (46.61%).

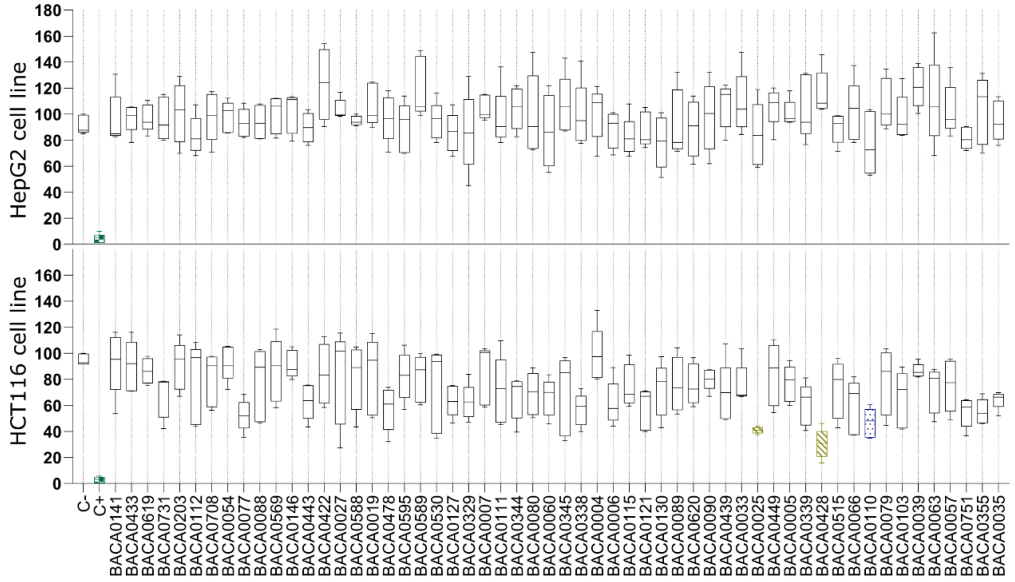

Figure 1. Cell viability after 48 h of exposure to cyanobacterial exudate extracts at 25 µg/mL in the HepG2 and HCT116 cell lines. Strains are taxonomically sorted. Statistical differences vs. the solvent control are indicated in color: green: p < 0.001; yellow: p < 0.01; blue: p < 0.05, Kruskal–Wallis test followed by Dunn's posthoc test.

In the antisteatosis model, no statistically significant differences were observed for any strain vs. the negative control group (Figure 2). In contrast, exposure to the exudate extract from *Dulcicalothrix* sp. BACA0344 resulted in significantly less fluorescence in zebrafish larvae when compared to the negative control (Figure 3), supporting a strong reduction in neutral lipids.

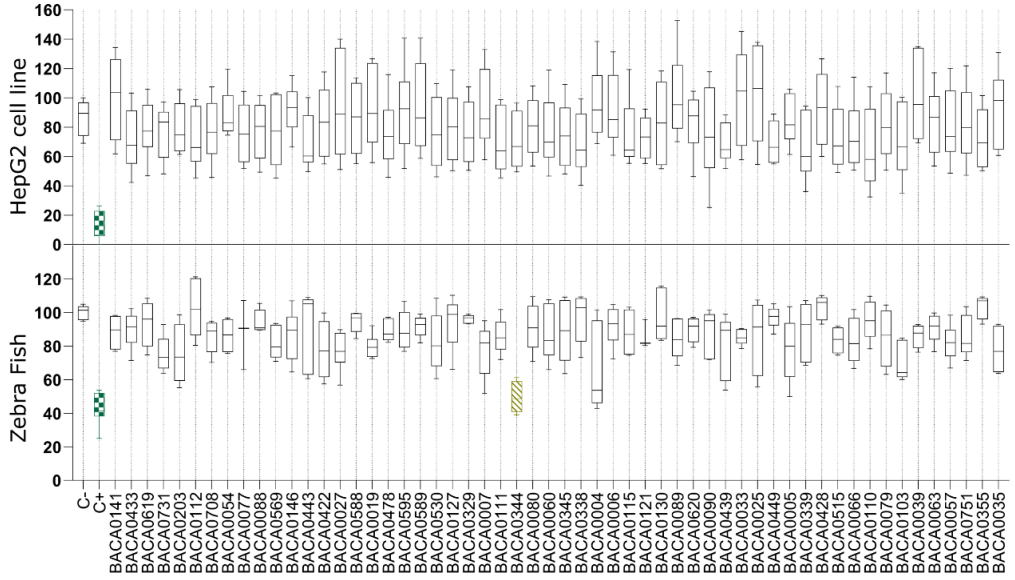

Figure 2. Lipid reduction in the steatosis model with fatty acid overloaded HepG2 cells and in zebrafish larvae exposed to cyanobacterial exudate extracts at 25 µg/mL. Strains are taxonomically sorted. Statistical differences vs. the solvent control are indicated in color, green: p < 0.001; yellow: p < 0.01, according to the Kruskal–Wallis test followed by Dunn's posthoc test.

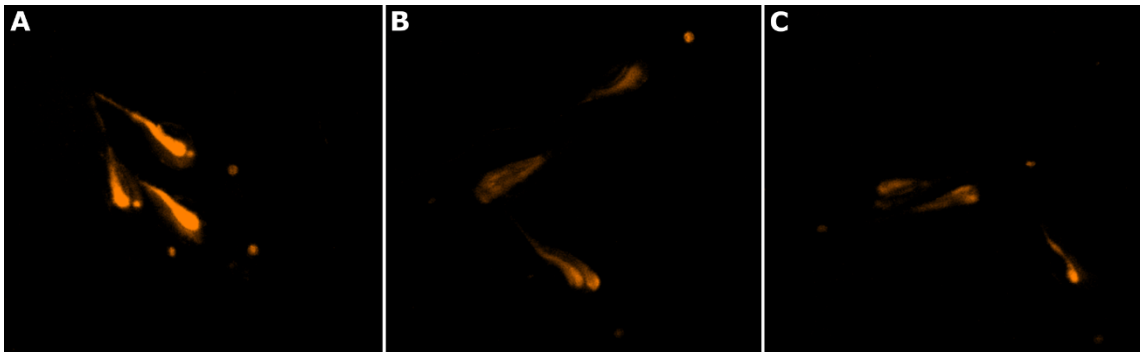

Figure 3. Reduction in neutral lipids in zebrafish larvae, stained with Nile red (orange), by the exudate extract. (A) zebrafish larvae from the negative control group (water); (B) zebrafish larvae exposed to resveratrol at 50 µM (positive control); (C) zebrafish larvae exposed to *Dulcicalothrix* sp. BACA0344 exudate extract.

8.2.2 Feature-based molecular network analysis

The metabolite network (1843 compounds) of the 56 studied strains is presented in File S1. The FBMN revealed several clusters to be produced by just one strain (Figure 4). No putative identifications were obtained for such clusters, which could represent novel compounds. The biggest clade was produced by *Neowestiellopsis persica* BACA0089, and although no bioactivity was identified, it contributed the most to the molecular network (Figure 4). Other strains creating relevant clades of secondary metabolites (≥ 4 compounds) are *Phayaothrix* sp. BACA0006, *Kamptonema* sp. BACA0007, *Aliinostoc* sp. BACA0035, *Pegethrix atlantica* BACA0077, *Symphyonema* sp. BACA0090, and Hapalosiphonaceae BACA0620.

The FBMN analysis for cytotoxicity against the HCT116 cell line identified 51 compounds (Table S2) with significant correlation ($p < 0.05$, correlation > 0.5). The identified clusters consisted of fatty amides, glycerophosphocholines, and many unidentified clusters of compounds (Figure 5), mainly produced by Tolypotrichaceae BACA0428 or Aphanizomenonaceae BACA0025. Microchaetaceae BACA0110 did not have correlations in any cluster, but some single nodes with low correlation and no putative identifications.

For lipid reduction in the zebrafish larvae, only the strain *Dulcicalothrix* sp. BACA0344 demonstrated activity. The FBMN analysis identified 18 compounds (Figure 6; Table S2) correlated with lipid-reducing activity, and 14 of them with a very high correlation (>0.9). Although many compounds are represented by unclassified single nodes in GNPS or MS2Query, two compounds related to the bioactivity were present in a cluster and identified as flavanones.

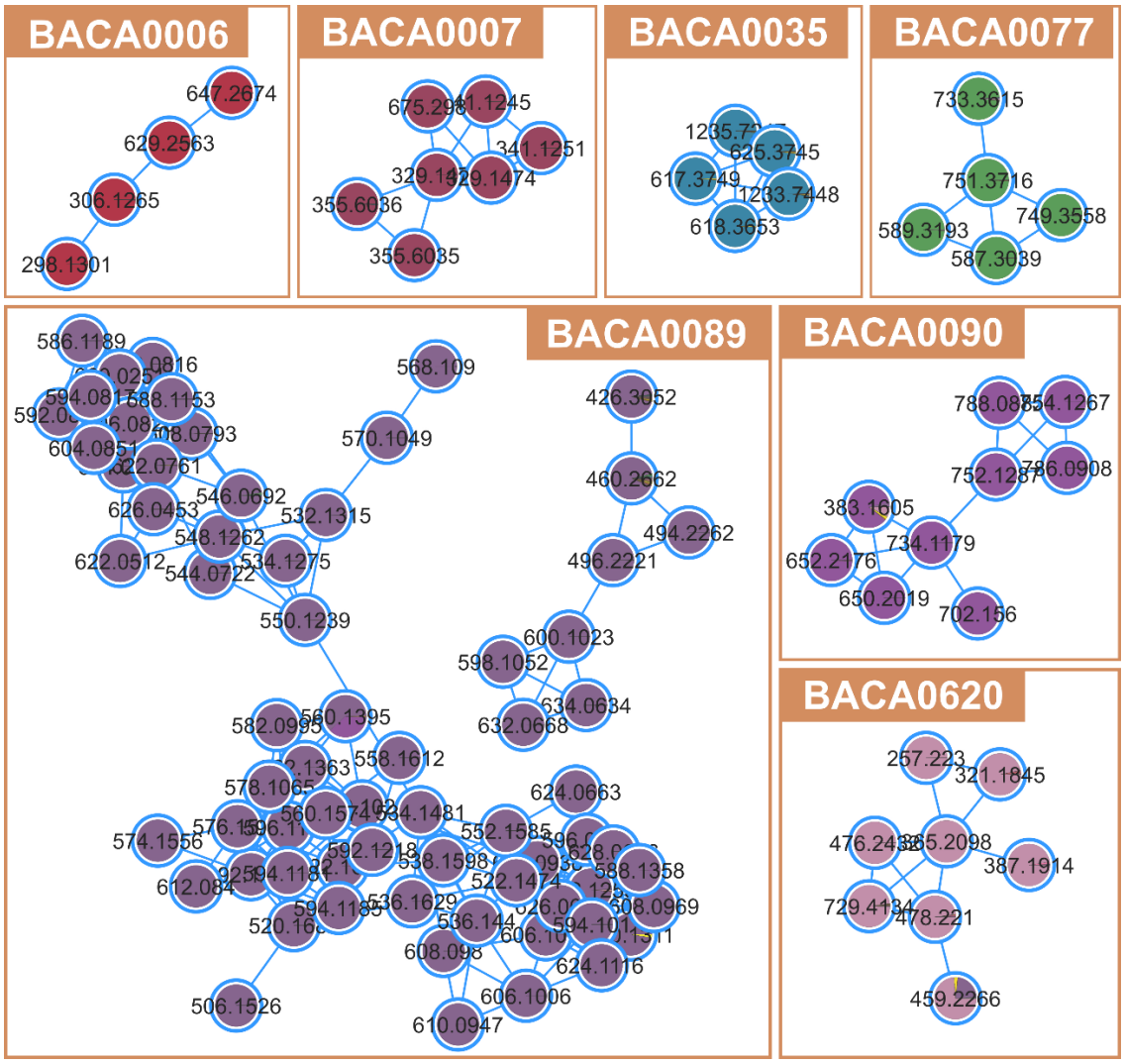

Figure 4. Unidentified clusters from FBMN belonging to non-bioactive strains.

8.3 Discussion

The BACA culture collection is a highly taxonomically diverse culture collection (Cordeiro et al. 2020), containing many newly described genera and species (Luz et al. 2023b, 2023c, 2023a, 2024). Focusing on the exometabolome, 56 strains of cyanobacteria from this collection were studied, offering a vast array of genetic resources for this research. Cyanobacteria culture medium is normally rich in EPS (Laroche 2022), characterized by a high variability of excreted compounds (Scholz & Liebezeit 2006, Laroche 2022) and as a source of bioactive metabolites (Volk 2005, Leão et al. 2009a, 2010, Mutalipassi et al. 2022). However, extracts from supernatants are less complex than biomass-derived extracts, which provides advantages for isolating possible new compounds. The studied extracts resulted in a total of 1843 mass features with MS2 data. From these, using the MS2 data, 61 (3%) were identified using the GNPS, and class prediction was made for 203 (11%) using MS2Query. Using only MS1 (m/z), it was possible

to identify 85 (5%) masses using the CyanoMetDB and 519 (28%) using the NPA both within a deviation of <5 ppm. Although identifications using NPA or CyanoMetDB must be taken with caution, the low percentage of identified metabolites highlights the high number of compounds still to be characterized.

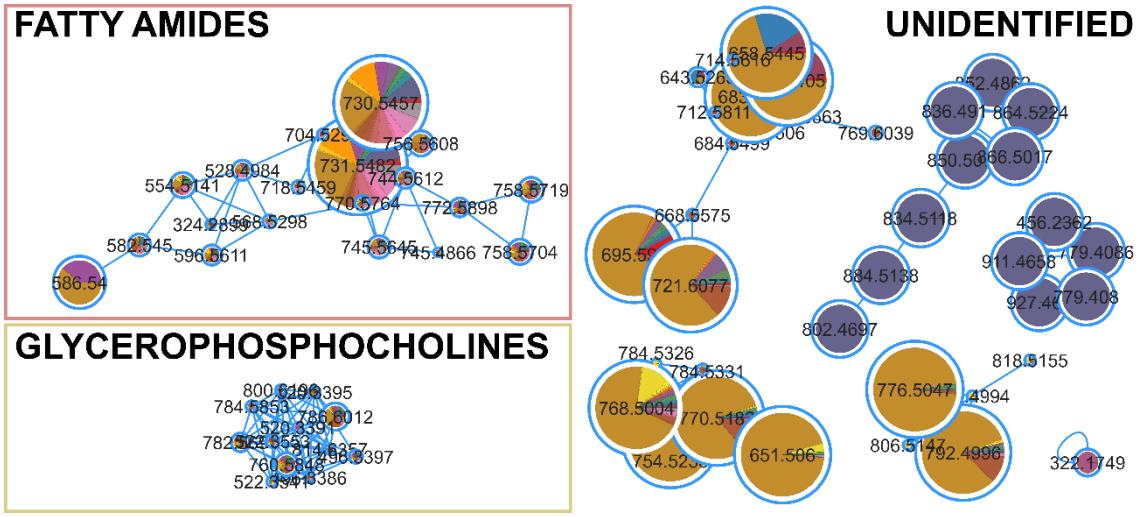

Figure 5. Clusters from the FBMN for cytotoxic activity against HCT116 cell lines. Significantly correlated nodes are highlighted with size and color (Aphanizomenonaceae BACA0025 in purple; Microchaetaceae BACA0110 in pink; Tolypotrichaceae BACA0428 in dark yellow).

A high variability in exudate production (Table S1) was observed, ranging from around 0.05 g to 1.5 g. Strongly bioactive strains had a low production of exudate (0.05–0.1 g), which could be related to a lower production of EPS. Albeit EPS are often mentioned in the literature with several bioactivities (Salimi & Farrokh 2023), it can be produced in very high amounts and conceal non-EPS bioactive compounds when used in low extract concentrations, as performed in this work. This can be observed in Figure 4, where bigger compound clusters were only detected from strains with low exudate production. Several BACA strains presented complex unclassified clusters of compounds (e.g., *Neowestiellopsis persica* BACA0089 and *Symphyonema* sp. BACA0090), which represent a big diversity of the cyanobacterial exometabolome still to characterize. Also, the observed clusters in Figure 4 are related to the presented strain, meaning an exclusive compound production, reinforcing the high chemodiversity of cyanobacteria at the strain level. Compound production appears to be more influenced by taxonomy than by the habitat of origin, with Nostocales strains showing the greatest compound diversity, regardless of the biotopes from where they were isolated.

The BACA0025 strain is a novel yet undescribed genus known for producing cylindrospermopsin (Cordeiro et al. 2021). However, this compound was not detected in the analyzed exometabolome. In the FBMN (Figure 5), BACA0025 exhibited several clusters unique

to the strain. One of these clusters had a putative identification from NPA on the mass feature 866.5017 m/z as Anabaenopeptin T, a carboxypeptidase A inhibitory compound, with an error margin of 1.20 ppm (Murakami et al. 2000, Leunda-Esnaola et al. 2024). Despite GNPS and MS2Query not identifying any similar compounds in the clade, this might be an Anabaenopeptin-related clade. However, none of the other masses in the cluster were matched with any known compound using either NPA or CyanoMetDB. The other putative identification, also in the cluster presented in Figure 5, is the mass of 927.4609 m/z, putatively identified as Nodularin-R-phenylglyoxal (IIa/b) with an error of 0.74 ppm. Nodularin-R-phenylglyoxal (IIa) is a Nodularin-R synthetic derivative that induced apoptosis in microinjected Swiss 3T3 fibroblasts (Herfindal et al. 2009), although the capacity of the strain to produce this analog is uncertain. However, no microcystins/nodularin genes were amplified, nor was the toxin identified, in this case MC-LR (Cordeiro et al. 2021). The presence of a complete cluster produced exclusively by BACA0025, which was correlated with the bioactivity, supports the use of this strain in the search for new bioactive extracellular compounds. BACA0428 showed the strongest cytotoxicity against HCT116. The metabolite profiling by FBMN showed many metabolites, mostly produced by this strain evidencing specific clusters with several compounds, though none had putative identifications. Nonetheless, this suggests that cyanobacterial exudate might be a rich source of bioactive secondary metabolites. Considering the diversity of cyanobacteria in the BACA culture collection, many new compounds could be discovered.

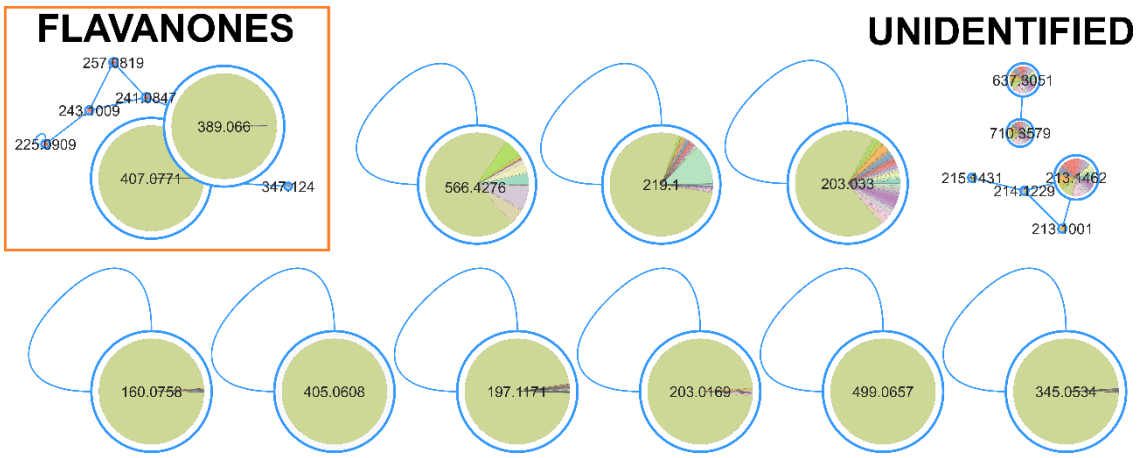

Figure 6. Clusters from the FBMN for lipid reduction activity in zebrafish larvae. Significantly correlated nodes are highlighted with size and color (*Dulcicalothrix* sp. BACA0344 in green).

Lipid reduction in zebrafish larvae is used as a model for metabolomic diseases, such as obesity, due to the functional conservation in lipid metabolism and adipose biology, among others (Zang et al. 2018). There are many known natural compounds with lipid reduction

potential (Russo et al. 2023), such as resveratrol, a well-characterized polyphenol (Pardal et al. 2014, Ran et al. 2017), used as the positive control in this work. A cluster of flavanones was identified from *Dulcicalothrix* sp. BACA0344 (Figure 6), having two masses of 389.066 m/z and 407.0771 m/z with high correlation, though none matches known compounds in the searched databases. Flavanones are a class of flavonoids derived from the chalcone structure, containing three rings (Martens & Mithöfer 2005). Flavonoids have been related to several bioactivities, mainly antioxidant and anti-UV protector (Chen et al. 2012, Abbate et al. 2021). In the zebrafish larvae model, several commercially used flavonoids (e.g., flavone, kaempferol, quercetin, among others) presented a strong reactive oxygen species scavenging rate at a concentration of 20 ppm (Chen et al. 2012). In lipid-reducing assays using zebrafish larvae exposed to extracts of wine lees with a high concentration of flavonoids (mainly rutin and quercetin), at a concentration of 100 µg/mL, it proved to have a 40% reduction with significant effects on expression of lipid metabolism key genes, such as FASN and CPT1B (Caro et al. 2017). Reduction in lipids was also observed in zebrafish larvae fed with a high-fat diet from 5 dpf to 20 dpf while exposed to 20 µM kaempferol (Lee et al. 2015). Similarly, zebrafish exposed to 6.25 uM Baicalein, grown under the same conditions, exhibited a 30% reduction in lipid accumulation (Seo et al. 2014). The described bioactivity of flavonoids in the literature supports the search for new bioactive flavanones excreted from the culture medium. Cyanobacteria, and in this case *Dulcicalothrix* sp. BACA0344, have demonstrated to be a promising source for novel lipid-reducing compounds. However, this bioactivity appears to be species- or strain-dependent, as the other two studied *Dulcicalothrix* sp. strains (BACA0060 and BACA0080) did not yield positive results. Therefore, further research should focus on BACA0344.

8.4 Materials and methods

8.4.1 Biomass and extract production

Fifty-six uni-cyanobacterial strains from the BACA culture collection were selected according to habitat and phylogenetic diversity (Figure S1), including freshwater (aquatic and terrestrial), brackish, and thermal cyanobacteria from 11 taxonomic orders (Table S1). For the phylogenetic analysis, the 16S rRNA sequences were selected according to the current literature and retrieved from GenBank together with published BACA sequences. These were aligned using MAFFT v 7.520 (Katoh & Standley 2013) and phylogenetic analysis using maximum likelihood, with 1000 ultrafast bootstrap (Hoang et al. 2017), in the IQ-Tree online version v1.6.12

(Trifinopoulos et al. 2016) with the model, TVMe + I + G4 chosen according to the Bayesian Information Criterion, using ModelFinder (Kalyaanamoorthy et al. 2017).

Cyanobacteria cultures were grown in 1 L flasks with aeration for four weeks with a 14:10 h light:dark cycles, at an intensity of approximately 110 µmol/s/m^2 of light and temperature of 25 °C. Biomass was centrifuged at 15,000× g for 10 min. A total of 400 mL of supernatant was recovered and filtered with 1.2 µm glass fiber filters, except for BACA0019 and BACA0588, when the supernatant was filtered with 0.7 µm glass fiber filters due to the lower cell size. The supernatant was frozen and freeze-dried at −45 °C and 0.1 mBar. The produced freeze-dried extracts were resuspended in ultra-pure water for further use.

8.4.2 Bioactivity assays

For the cytotoxicity assessment, HepG2 and HCT116 cells were used from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (100 IU/mL and 10 mg/mL, respectively) (Biochrom), and 0.1% amphotericin B (GE Healthcare, Little Chalfont, UK).

The phenotypic antisteatosis assay was performed as described by Costa et al. (2019). Briefly, HepG2 cells were seeded at 1 × 10^5 cells/well, and after 24 h, the medium was changed to DMEM without FBS, supplemented with 62 µM sodium oleate (Sig-ma-Aldrich, St. Louis, MO, USA), and exposed to extracts at 25 µg/mL. Positive control was exposed to DMEM without FBS, supplemented with 62 µM sodium oleate, and negative control to DMEM without FBS and sodium oleate supplementation. After 6 h, 75 ηg/mL Nile red (Sigma-Aldrich) and 10 µg/mL Hoechst 33342 (HO-33342) (Sigma-Aldrich) were added and incubated for 15 min at 37 °C in the dark. Fluorescence images were taken with a BioTek Cytation 5 Cell Imaging Multimode Reader (Santa Clara, CA, USA) at 531/593 nm (RFP) excitation/emission for Nile Red and 377/447 nm (DAPI) excitation/emission for HO-33342. DAPI and RFP images were overlapped, and threshold analysis was carried out to determine the percentage of lipid-positive cells compared to the negative control. For the cytotoxicity analysis, the MTT assay was used as previously described (Ferreira et al. 2021). Both HCT116 and HepG2 cells were seeded at 3.3 × 10^4 cells per well with medium renewal at 24 h, and cells exposed to the extracts at 25 µg/mL for 48 h.

The lipid reduction in zebrafish (*Danio rerio*; WT AB strain) larvae was carried out as described by Costa et al. (2019) using the Nile red fat metabolism assay. Larvae were raised in E3 medium at 28 °C with 200 µM 1-phenyl-2-thiourea (PTU). At 3 days post-fertilization (DPF),

the larvae were exposed to extracts at a concentration of 25 µg/mL. Ultra-pure water and resveratrol (REV, at 50 µM) were used as solvents and positive controls, respectively. For imaging, the larvae were stained overnight with Nile red at 10 ng/mL. Imaging was performed with the BioTek Cytation 5 Cell Imaging Multimode Reader (Santa Clara, CA, USA), and the fluorescence intensity was quantified at 531/593 nm (RFP) excitation/emission in each individual zebrafish larvae using ImageJ v1.54d (Schneider et al. 2012).

8.4.3 Statistical analysis

All screenings were performed in two independent assays in triplicates per sample (n = 6). The Gaussian distribution of data was tested by a Kolmogorov–Smirnov normality test ($p < 0.05$). One-way ANOVA was used followed by Kruskal–Wallis with Dunn's post hoc test (non-parametric distribution). Statistically significant differences were considered with $p < 0.05$. All statistical analyses were carried out in GraphPad Prism v9.5.0.

8.4.4 Exometabolome and feature-based molecular networking

The dried extracts were desalted using Pierce™ C18 Tips, following the recommended protocol for LC-MS/MS analysis, with a final elution made with 0.1% formic acid in 95% methanol LC-MS/MS grade. Extracts were then analyzed using liquid chromatography-high resolution electrospray ionization tandem mass spectrometry (HR-ESI-LC-MS/MS) on a Q Exactive Focus Orbitrap LC-MS/MS system (Thermo Fisher) in the positive mode at the Materials Centre of the University of Porto, as previously described in detail (Ferreira et al. 2021).

The feature-based molecular network (FBMN) analysis followed previously described conditions (Nothias et al. 2018). Briefly, raw data were converted to .mzML format files using MSConvert (Chambers et al. 2012), and data were processed with MZmine v2.53 (Pluskal et al. 2010). The FBMN workflow ran on the GNPS platform (Wang et al. 2016) with default parameters after MZmine processing. Data were visualized using Cytoscape v3.10.2 (Shannon et al. 2003).

Molecular class enrichment and cluster class classification were conducted using MS2Query (de Jonge et al. 2023) run locally and molDiscovery (Cao et al. 2021) on the GNPS platform. For the MS2Query, the MS2 data (.mgf file output from FBMN) was analyzed using MS2Query v1.3.0 (de Jonge et al. 2023), in positive mode, using the available pre-trained embeddings and models (de Jonge 2024), with analogs considered only with ≥0.7 MS2QueryScore as thresholds with compound class prediction by NPClassifier (Kim et al. 2021). The FBMN output was also used for molDiscovery v1.0.0, which ran on the GNPS platform with

the default database. The workflow ran with a custom value of precursor ion mass tolerance set to 5 ppm and fragment ion mass tolerance set to 0.02 Da. Compound class prediction was made using NPClassifier (Kim et al. 2021).

Statistical analysis followed the script made available by Nothias et al. (2018) (v1.1 for R) for calculation of the p-value (after Bonferroni correction) and Pearson correlation (PC) between feature intensity and bioactivity level. Mass peaks with $p < 0.05$ and correlation > 0.50 were matched to the Natural Product Atlas (NPA) database v2024_03 (van Santen et al. 2022) and CyanoMetDB v0.2.0 (Jones et al. 2021) within 5 ppm for a putative identification, using a custom-made R script with compound class prediction based on NPClassifier (Kim et al. 2021).

8.5 Conclusions

This work comprised a wide bioactivity prospection on cultured cyanobacteria from the Azores Islands and identified several bioactive strains from the BACA culture collection with the production of extracellular bioactive compounds. The large unidentified chemodiversity on the cyanobacterial exometabolome supports the need for further studies. Strong cytotoxic activity from Aphanizomenonaceae BACA0025, Microchaetaceae BACA0110 and Tolypotrichaceae BACA0428 was observed, and from the analyzed FBMN, several unique clusters of compounds were identified, mainly fatty amides. However, the majority are unidentified clusters, reinforcing the presence of possible novel bioactive compounds. In the lipid reduction assay with zebrafish larvae, the exudates of *Dulcicalothrix* sp. BACA0344 had strong activity, and several mass peaks were correlated and putatively identified as flavanones. Cyanobacteria have not been extensively studied for their flavonoid production and diversity, suggesting the potential discovery of new bioactive flavanones. This reinforces the importance of exploring cyanobacteria for novel lipid-reduction compounds. The presence of many bioactive compounds on the cyanobacteria exometabolome also supports future biorefinery approaches, allowing full use of cyanobacterial cultures, from biomass to the waste media of cyanobacterial production.

8.6 Supplementary materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md22090412/s1, File S1: Cytoscape file with the feature-based molecular network with annotated clusters; Figure S1: Phylogenetic analysis of the 16S rRNA gene of the studied strains and sequences retrieved from the current literature. Used BACA strains are presented in bold; Table S1: List of used cyanobacteria strains from the Bank of Algae and Cyanobacteria of the Azores culture collection with taxonomic data, habitat

description and extracts yield; Table S2: List of statistically significant compounds associated with lipid reduction in zebrafish larvae and cytotoxicity. Match in GNPS, MS2Query, molDiscovery, CyanoMetDB and Natural Product Atlas are presented for each compound.

8.7 Author contributions

Conceptualization, R.L., V.G., V.V. and R.U.; formal analysis, R.L. and R.U.; investigation, R.L. and R.C.; resources, V.G., V.V. and R.U.; data curation, R.L.; writing—original draft preparation, R.L., R.C., V.G., V.V. and R.U.; writing—review and editing, R.L., R.C., V.G., V.V. and R.U.; visualization, R.L.; supervision, V.G., V.V. and R.U.; funding acquisition, V.G., V.V. and R.U. All authors have read and agreed to the published version of the manuscript.

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8.9 Institutional review board statement

Not applicable.

8.10 Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors on request.

8.11 Conflicts of interest

The authors declare no conflicts of interest.

8.12 References

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Chapter IX

General discussion and conclusions

Chapter IX. General discussion and conclusions

Considering all the conducted work, two main contributions stand out: advancement of the current understanding of cyanobacterial diversity in the Azores and expansion of the knowledge of the chemodiversity of natural products from cyanobacteria present in the BACA culture collection. After reviewing all cyanobacteria records to the Azores published until 2020 (Chapter II), a detailed taxonomic analysis of all cyanobacteria strains present in the BACA culture collection (Chapter III to VI) allowed the description of five novel genera and ten novel species. In the search for new bioactive natural products from cyanobacteria, 56 cyanobacteria were screened for lipid-reducing and cytotoxic compounds. This, together with a metabolomic characterization, allowed the identification of several putative bioactive compounds and highlighted the high chemodiversity present in the endometabolome (biomass methanolic extracts) and exometabolome (exudate extracts) of cyanobacteria. Furthermore, these works highlighted the great potential that culture collections, such as BACA, and the Azores Islands have for taxonomic studies and the discovery of novel bioactive products.

9.1 Diversity of cyanobacteria and novel taxa from the Azores

The Azores have a high diversity of habitats suitable for the growth of photosynthetic microorganisms. Several publications have dwelled on the freshwater and terrestrial algal diversity of the Azores, with many focusing on cyanobacteria (Bohlin 1901, Cedercreutz 1941, Bourrelly & Manguin 1946, Johansson 1976, Cordeiro et al. 2020a). However, detailed taxonomical works on cyanobacteria diversity were lacking (Cordeiro et al. 2020b). As demonstrated in Chapter II, several taxonomical orders are underrepresented in the Azores, despite the high availability of habitats in the Azores archipelago. As discussed in Chapter II, this is mainly due to the lack of sampling. Results from chapters III, IV, V and VI support this conclusion since sampling from different habitats, such as aquatic from lakes and lotic systems, terrestrial, atmophytic, and thermal habits, complemented by detailed taxonomical analyses of cultured specimens deposited in the BACA culture collection allowed for the description of five new genera and ten new species.

Many novel genera are described for freshwater aquatic systems, from lakes to streams and small freshwater ponds. From lakes are described *Tumidithrix elongata* gen. sp. nov., *Pycnacronema lacustrum* sp. nov., and *Leptodemis lacustris* sp. nov. For *Pycnacronema* this represents a new habitat description for a mainly terrestrial genus (Jusko & Johansen 2024). In addition to *L. lacustris* sp. nov., we also found *L. alaskaensis* isolated from a terrestrial habitat (see Chapter IV). The genus *Leptodesmis* now includes four species, with two reported for the

Azores. Other new genera and species isolated from lakes were *Azorothrix ramosa* gen. sp. nov. (Chapter IV) and *Pseudocalidococcus azoricus* gen. sp. nov. (Chapter VI). These last two species seem to have a broader ecological distribution as they were described based on multiple strains deposited in BACA from different sampling sites. *Azorothrix ramosa* was isolated from both lakes and streams, while *Pseudocalidococcus azoricus* was isolated from lakes and an atmophytic site. *Radiculonema aquaticum* gen. sp. nov. has only been found in a stream from Santa Maria Island. The reports of *Azorothrix ramosa* and *Radiculonema aquaticum* contribute to the poorly known diversity of cyanobacteria in lotic systems reported in Chapter II. Interestingly, *Pseudocalidococcus azoricus* was found in two very distinct environments, freshwater lakes and atmophytic, demonstrating a good adaptation to a wide range of environmental conditions. These adaptations to two distinct ecological habitats and their report are relevant in the discussion and the definition of the cyanobacteria species concept, and strong evidence for the need of a polyphasic approach for cyanobacteria description. The description of P*seudocalidococcus azoricus* can be of significant importance as it could correspond to an important component of Azorean lakes' plankton. Recent papers have shown that small coccoid cyanobacteria can represent a significant part of lake phytoplankton biomass (Lefler et al. 2023), although they are frequently overlooked due to their reduced size, which makes them difficult to observe.

For terrestrial environments, Chapter V describes four new taxa, all from very different habits. *Venetifunis florensis* gen. sp. nov. was isolated from a sample collected on the shore of a lake on Flores Island. As for *Pegethrix atlantica* sp. nov. the studied strain came from a sample collected from a wall beneath the bridge that separates lakes Azul and Verde in Sete Cidades (São Miguel Island). *Albertania obscura* sp. nov. was isolated from a sample collected inside the Algar do Carvão (Terceira Island), an open volcano cave with low light. Finally, *Kovacikia atmophytica* sp. nov. was isolated from an atmophytic site, similar to one of the strains of *Pseudocalidococcus azoricus*. This is one of the first works to focus on cyanobacteria from terrestrial habits in the Azores archipelago, complementing the known diversity of an important ecological habitat for cyanobacteria (Joseph & Ray, 2024).

The known cyanobacterial diversity of the Azores is relatively low, considering recent phylogenetic work using BACA strains (Cordeiro et al. 2020a). Chapter VII published a phylogenetic tree of the 56 strains used for a bioactivity study. This genetic analysis, based on the 16S rRNA data, revealed the existence of several taxa unknown for the Azores, such as *Phayaothrix* sp., *Fulbrightiella* sp., *Dulcicalothrix* sp., *Symphyonema* sp., *Neowestiellopsis persica*, *Egbenema* sp., among many others. As Cordeiro et al. (2020a) stated, there is a high potential for cyanobacteria taxonomy studies in the Azores, with many species still to be

described. Despite not being the main goal of the work, Chapter VII results clearly show that many strains deposited in the BACA culture collection are genetically different from all known species, which need to be described, originating novel taxa.

9.2 Cyanobacterial novel products and potential applications

This work performed a screening for novel natural products on 56 cyanobacteria strains deposited in the BACA culture collection, including some of the novel genera and species discussed in Chapters III to VI, to promote the value of BACA's unique genetic resources. While Chapter VII reports a comprehensive metabolome analysis and a chemodiversity comparison, Chapter VIII analyses the exometabolome of the same strains.

Chapter VII presents a phylogenetic analysis of the studied strains and a metabolomic comparison between them using CompareMS2. The results revealed a similarity of the metabolome in the most closely related phylogenetic strains, especially within the Nostocales. In addition, many cyanobacteria with antisteatosis activity were found in a confined cluster, which suggests that this bioactivity is probably related to common produced compounds.

Some of the identified bioactivity found to be common to several strains is lipid-reduction bioactivity in zebrafish larvae, with reports for *Cyanobium* sp. BACA0019, *Pegethrix atlantica* BACA0077, *Dulcicalothrix* sp. BACA0344 and *Pseudocalidococcus azoricus* BACA0433. Contrary to *Cyanobium* spp., which was already known for its lipid-reduction activity (Freitas et al. 2019, Carrasco del Amor et al. 2023), *Pegethrix*, *Dulcicalothrix* and *Pseudocalidoccus*, the last, one of the new described genera in this work (Chapter VI), are here firstly reported as bioactive for lipid-reduction. Interestingly, bioactivity was found in the two types of assayed extracts (endo- and exometabolome), but no strain showed positive results for both. The highest lipid-reduction effect was found for the exometabolome of *Dulcicalothrix* sp. BACA0344, with a very strong reduction of neutral lipids. Natural compounds of cyanobacteria with lipid reduction activity were already reported (Freitas et al. 2019, Silva et al. 2024), as well as the existence of bioactive extracellular compounds (Leão et al. 2009). This work confirms that bioactivity is not restricted to intracellular compounds and that the cyanobacteria exometabolome is a potential source for many novel bioactive compounds.

Antisteatotic activity was reported for several Nostocales strains, with a group of bioactive antisteatotic strains found closely positioned in Figure 5 of Chapter VII. However, using the same strains with the exudate extracts (Chapter VIII), no positive results were obtained. Methanolic extract of *Dulcicalothrix* sp. BACA0344 showed a strong lipid reduction in the HepG2 cell model, while the exudate extract of the same strain revealed a strong lipid reduction in zebrafish larvae.

This strain also showed a strong reduction in cell viability against HepG2 cell lines using the MTT assay. Further work is needed for bioactive compound elucidation and fractioning steps are needed to fully explore the possible use of this strain as both a lipid-reduction and an antisteatosis compound source. The positive results obtained with both the biomass methanolic extracts and the exudate of *Dulcicalothrix* sp. BACA0344 supports the need for biorefinery approaches for efficient valorization of the biomass and the culture media.

Several strains demonstrated cytotoxic effects against HepG2 and HCT116 cancer cell lines and toxic effects against zebrafish larvae (Chapter VII and VIII). One of the strains with the strongest cytotoxic activity was *Aliinostoc* sp. BACA0355, which was also one of the strains that showed toxicity against the zebrafish larvae. Zebrafish toxicity could indicate the presence of a neurotoxic compound (Jonas et al. 2015). However, the metabolomic analysis did not reveal any known toxins in the extract using MS1 data when matched to public databases, such as CyanoMetDB (Jones et al. 2021), which suggests that *Aliinostoc* sp. BACA0355 might be producing an undisclosed toxic compound. Also, the detection of toxicity effects of *Symphyonema* sp. BACA0090, a genus already reported as toxic (Wright et al. 2006), reinforces the need to study less known taxa that might contribute to harmful situations in supposably safe environments.

9.3 Main conclusions

This work proposed four main objectives, regarding taxa characterization and diversity analysis from overlooked habitats, biological activity evaluation using BACA strains and valorization of native strains for biotechnological applications in the Azores.

From the BACA culture collection around 200 cyanobacteria strains were characterized following the polyphasic approach that allowed the description of 10 new species, namely, *Azorothrix ramosa* gen. & sp. nov., *Radiculonema aquaticum* gen. & sp. nov., *Venetifunis florensis* gen. & sp. nov., *Tumidithrix elongata* gen. & sp. nov., *Pseudocalidococcus azoricus* gen. & sp. nov., *Pycnacronema lacustrum* sp. nov., *Pegethrix atlantica* sp. nov., *Kovacikia atmophytica* sp. nov., *Albertania obscura* sp. nov. and *Leptodesmis lacustris* sp. nov. Still many new taxa are still to be described, as most of them remain unidentified.

Most represented habitats of the Azores had cyanobacteria isolated and maintained in the BACA culture collection, including freshwater and brackish lakes, terrestrial and thermal (atmophytic) that were studied in this work. The novel described taxa are new reports to the Azorean habitats, with two reports to the poorly known lotic systems, four to terrestrial habitats and the remaining to aquatic environments. However, many other genera are reported to the

Azores following the strains used in the bioactivity search, in this case, *Aliinostoc*, *Chalicogloea*, *Desertifilum*, *Dulcicalothrix*, *Egbenema*, *Elainella*, *Fulbrightiella*, *Inacoccus*, *Neowestiellopsis*, *Nodosilinea*, *Phayaothrix*, *Pseudoaliinostoc*, *Purpureonostoc* and *Symphyonema*, from terrestrial, freshwater, thermal and brackish habitats. Most of these, without specific epithet.

The bioactivity of 56 strains were tested in metabolic diseases. The strongest activity for lipid-reduction was reported from the exudate of *Dulcicalothrix* sp. BACA0344 and the methanolic extracts of *Pseudocalidococcus azoricus* BACA0433. Strong toxicity effects against zebrafish larvae were identified from *Aliinostoc* sp. BACA0355 and *Symphyonema* sp. BACA0090 and high reduction of cell viability was reported from *Scytonematopsis* sp. BACA0005.

All reported bioactive strains are native from the Azores, with promising applications, however some with a higher potential due to biorefinery production feasibility and possible reduction production costs associated with cyanobacteria biomass production. *Dulcicalothrix* sp. BACA0344 exudate has a high value for both pharmaceutical and cosmeceuticals products, with anti-obesity applications that together with a biorefinery approach, can bring value to the biomass by the possible presence of other bioactive compounds or direct application in animal feed. BACA0344 can also be a sustainable source of nitrogen for fertilizers, as it is grown under BG-11_0 media (without nitrogen) and develops heterocytes that fix aerobic N_2. The report of high cytotoxic strains, such as *Scytonematopsis* sp. BACA0005 and *Aliinostoc* sp. BACA0355, support the high potential in pharmaceuticals, to be researched and used as novel cancer drugs. In the case of BACA0355, the high toxicity in zebrafish larvae, might suggest potential use as pesticide, antifouling, among others. In these a directed extraction of the compounds of interest can leave a high value residue (nutritionally or chemically) that can be highly valorized, supporting a circular economy under a no waste approach if a biorefinery approach is applied.

9.4 Future research

Despite several recent advances and robust approaches using genomics (e.g. Willis & Woodhouse 2020, Strunecký et al. 2023), further research on cyanobacteria taxonomy is needed. A contribution to the genome-based taxonomy was given in this work (Chapter VI) in the description of *Pseudocalidococcus azoricus*. However, more work is still needed, and the new genera and species described here must have their genome sequenced and published shortly, contributing to the increase in high-quality studies and genomic approaches for taxonomy, as recommended by Dvořák et al. (2023).

For the identification of the reported bioactive mass features by the FBMN approach, further research is needed. Alongside the traditional fractioning and bioactivity guided assays for compound elucidation, this can be made by including genomic data that allows a paired-OMICs approach, that can contribute to a faster identification of a compound production pathways and possible further biotechnological and/or pharmaceutical applications (Eldjarn et al. 2021, Schorn et al. 2021). Furthermore, with knowledge of the BGC composition, further strains producing the compound can be searched, as analogs may have increased bioactivity (Eggen & Georg 2002, Kallifidas et al. 2024).

A complete characterization of the production of a compound from both a genomic and chemical point of view can be extremely valuable. There are novel compound modification approaches to increase bioactivity or identify the minimum chemical structure for bioactivity retaining, using the direct pathway cloning (DiPaC) approach (D'Agostino & Gulder 2018, Greunke et al. 2018). DiPaC allows the identification of key genes in the production of a compound using heterologous expression in bacterial vectors (D'Agostino & Gulder 2018). However, this can be leveraged by combining different genes in biosynthetic pathways to increase chemical diversity and bioactivity (Alam et al. 2021).

9.5 References

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Annex I – CrediT author statement

Author contributions to the published scientific work, following the Contributor Roles Taxonomy (CRediT).

Chapter II. Luz, R., Cordeiro, R., Fonseca, A., Raposeiro, P. M., & Gonçalves, V. (2022). Distribution and diversity of cyanobacteria in the Azores Archipelago: An annotated checklist. Biodiversity Data Journal, 10, e87638. https://doi.org/10.3897/BDJ.10.e87638

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Chapter III. Luz, R., Hentschke, G. S., Cordeiro, R., Fonseca, A., Urbatzka, R., Vasconcelos, V., & Gonçalves, V. (2024). Description of *Azorothrix ramosa* gen. et sp. nov. (Tolypotrichaceae, Cyanobacteria), a new Tolypotrichaceae from Atlantic oceanic islands. Fottea, 24(1), 99-108. https://doi.org/10.5507/fot.2023.014

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Chapter IV. Luz, R., Cordeiro, R., Kaštovský, J., Johansen, J. R., Dias, E., Fonseca, A., Urbatzka R., Vasconcelos V. & Gonçalves, V. (2023). Description of four new filamentous cyanobacterial taxa from freshwater habitats in the Azores Archipelago. Journal of Phycology, 59(6), 1323-1338. https://doi.org/10.1111/jpy.13396

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Chapter V. Luz, R., Cordeiro, R., Kaštovský, J., Johansen, J. R., Dias, E., Fonseca, A., Urbatzka R, Vasconcelos V. & Gonçalves, V. (2023). New terrestrial cyanobacteria from the Azores Islands: Description of *Venetifunis* gen. nov. and new species of *Albertania*, *Kovacikia* and *Pegethrix*. Phycologia, 62(5), 483-498. https://doi.org/10.1080/00318884.2023.2259243

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