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Cyanobacteria Phylogenetic Studies Reveal Evidence for Polyphyletic Genera from Thermal and Freshwater Habitats

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Abstract: Cyanobacteria are among the most diverse morphological microorganisms that inhabit a great variety of habitats. Their presence in the Azores, a volcanic archipelago of nine islands in the middle of the North Atlantic Ocean, has already been reported. However, due to the high diversity of cyanobacteria habitats, their biodiversity is still understudied, mainly in extreme environments. To address this, a total of 156 cyanobacteria strains from Azores lakes, streams, thermal and terrestrial habitats were isolated. Identification was made based on a polyphasic approach using classical taxonomy (morphological characteristics and environmental data) and phylogeny among 81 strains assessed by maximum likelihood and Bayesian analysis of 16S rDNA partial sequences. The 156 isolates showed a high genera diversity (38) belonging to the orders Chroococcales, Nostocales, Oscillatoriales, and Synechococcales. Eleven new genera for the Azores habitats are here reported, reinforcing that cyanobacteria biodiversity in these islands is still much understudied. Phylogenetic analysis showed 14 clusters associated with these cyanobacteria orders, with evidence for six new genera and valuable information towards *Microchaete/Coleospermum* taxonomic revision that better reflects species environmental distribution. These results emphasize the need for cyanobacteria taxonomy revisions, through polyphasic studies, mainly in Synechococcales order and in the *Microchaete/Coleospermum*, *Nostoc*, and *Anabaena* genera.

Keywords: 16S rRNA; freshwater; thermal; oceanic islands; Cyanophyceae; polyphyletic genera; Azores

1. Introduction

Cyanobacteria are photoautotrophic prokaryotes with high morphological and ecological diversity, that are able to produce specialized cells (e.g., heterocysts and akinetes), which makes them unique organisms, allowing them to survive and endure in unfavorable and extreme environments (e.g., lack of light or nutrients, high salinity and/or extreme oscillations of temperature) [1,2]. Cyanobacteria are more frequently reported from freshwater, brackish water, marine, and terrestrial ecosystems [1,3,4], but reports on their presence in extreme environments are rather scarce [4]. In the Azores, a remote Atlantic Ocean archipelago of volcanic origin, the study of cyanobacteria on different types of ecosystems is also biased. Although cyanobacteria have been reported in these islands since 1874 [5,6], most reports

are from freshwater lakes (e.g., [5,7–9]), due to high abundance of these ecosystems (88) [10] and to the occurrence of cyanobacteria blooms associated with lake cultural eutrophication [8,11]. Despite the abundance of thermal sites [12] and other environments suitable for cyanobacteria inhabitance [13], only a few studies on cyanobacteria diversity in these habitats were done [9,13]. In fact, a recent work by Luz et al. [6] gathers almost 2.000 cyanobacteria occurrences (42 different taxa) only from freshwater lakes.

Morphological and ecological features are crucial for cyanobacteria taxonomic identification [1,3,14]. However, recent contributions from molecular studies challenged the traditional classification based on morphological and ecological features. Taxonomic identification should preferably be based not only on morphological features but also on genetic and biochemical characters [14–20]. Cyanobacteria classification is in constant change, and the most used is the latest taxonomic approach proposed by Komárek et al. [14], using morphological, ecological and genetic data, that classified cyanobacteria into eight orders: Gloeobacterales (cyanobacteria without thylakoids), Synechococcales and Spirulinales (unicellular and filamentous genera with parietal thylakoids), Chroococcales and Pleurocapsales (coccoid), Oscillatoriales (filamentous without cell differentiation and the coccoid *Cyanothece*), Chroococcidiopsidales (mostly from extreme habitats) and Nostocales (filamentous with heterocysts and akinetes) [14].

A common taxonomic misconception is the identification of genera/species regardless of their ecology, despite several recognized polyphyletic genera containing species from different habitats [21,22]. This problem was resolved, for instance, in the *Lyngbya* genus, where marine species were genetically recognized as distinct (*Moorea*) [23] and more recently in the *Calothrix* cluster genus with the description of freshwater/terrestrial genus *Dulcicalothrix* [24]. Many other studies address this problem, but most of them do not consider other habitats, such as brackish or thermal habitats, mainly due to the lack of available cultured strains. More complex genera are e.g., *Nostoc* and *Leptolyngbya* where the lack of diacritical features has been of great concern for taxonomic identification in cultured strains with difficult taxonomic identification [20,25]. Contributions to this problematic are being addressed mainly by genetic techniques with the characterization of several *Nostoc* alike genera (e.g., *Desmonostoc*, *Halotia*) [16,20] and *Leptolyngbya* alike genera (e.g., *Pegethrix*, *Cartusia*) [17].

Isolation of cyanobacteria from different ecosystems allows multidisciplinary and more complex studies, thus the creation and preservation of cyanobacteria isolates in culture collections has increased recently [26,27]. Isolated strains allow intricate studies to understand the detailed characteristics of their morphology, genetics, physiology, and biochemistry [28,29]. Genetic studies using axenic cultures are essential for cyanobacteria polyphasic taxonomic classification studies, especially for understudied or unresolved polyphyletic families/genera/species [14]. In fact, in recent studies, many of the newly described genera and species are based exclusively on cultivated strains [17,20,30,31].

Taking this into consideration, we aimed to increase cyanobacteria richness in our collection, the Bank of Algae and Cyanobacteria of the Azores (BACA), and increase knowledge on cyanobacteria diversity, by isolating cyanobacteria from various habitats from the Azores islands, as well as to infer phylogenetic relationships among them.

2. Materials and Methods

2.1. Study Site and Sample Collection

The Azores archipelago located in the Northeast Atlantic Ocean is composed of nine volcanic islands divided into three groups following a Southeast-Northwest alignment: Eastern (Santa Maria and São Miguel), Central (Terceira, Graciosa, São Jorge, Pico, and Faial) and Western (Corvo and Flores). For this study five of the nine islands were sampled, comprising a total of 47 sample sites from 25 volcanic lakes, one artificial lake, 12 thermal sites, four streams, and five terrestrial sites (detailed in Table S1).

Phytoplankton samples were collected from surface waters with a 10 µm mesh plankton net, while biofilm samples were collected by scraping rocks, sediment, algae, or plants. Sampling was carried out seasonally between 2016 and 2017. During cyanobacteria sampling, environmental variables were taken *in situ* with the multiparameter probe Horiba U-52 (Horiba, Pasadena, TX, USA).

2.2. Strains Isolation, Morphological Characterization and Culture Conditions

Primarily, ca. 1 mL of environmental sample was added to ca. 20 mL of liquid BG-11 media with and without combined nitrogen (for aerobic N_2-fixing cyanobacteria) [32] and subjected to an adaptation phase for about two weeks. Freshwater and terrestrial samples stayed in a climate-controlled room with a 14:10 h light:dark (10–40 µmol photons m^{-2} s^{-1}) photoperiod at 19 °C [32,33]. Thermal samples stayed in a climate-controlled chamber (POL-EKO APARATURA®, Wodzisław Śląski, Poland) in a 14:10 h light:dark (30 µmol photons m^{-2} s^{-1}) photoperiod at 35 °C [34]. After adaptation and visual growth, isolation was made by several replicates either in liquid media or agar plates using an inverted microscope Leica DMi1 (Leica, Germany).

Species identification was performed by optical microscopy, with the microscope Leica DM4 B with Digital Camera Leica MC 190 HD (Leica, Germany), following specific floras based on morphological and ecological characterization (e.g., [35–37]). Isolated strains are maintained in unicyanobacterial cultures in the Bank of Algae and Cyanobacteria of the Azores (BACA), created in the framework of the REBECA project (MAC/1.1a/060). Strains BACA0203 and BACA0224 previously isolated by Xavier et al. [38] under the codes MIA-SMG-2013-13 and MIA-SMG-2013-48, respectively, and later deposited in BACA, were also included in the phylogenetic analysis.

2.3. DNA Extraction, Polymerase Chain Reaction (PCR) Amplification, and Sequencing

Total genomic DNA was extracted from the pellet of centrifuged 2–5 mL of fresh cultures with the PureLinkTM Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), following the protocol recommended by the manufacturer for Gram-negative bacteria. DNA samples were stored at −20 °C.

For 16S rRNA gene (partial) amplification and sequencing the following primers were used: CYA106F [39] and CYA785R [40]. Polymerase chain reactions (PCR) were performed with a total volume of 50 µL containing 1× PCR Buffer, 1.25 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (Thermo Fisher, Waltham, MA, USA), 10 pmol of each primer, 5–10 ng of DNA and 1 U of Taq DNA Polymerase (Thermo Fisher, USA). Thermal cycling was carried out in a ProFlex™ 3 × 32-well PCR System (Thermo Fisher, USA). PCR conditions were as follows: initial denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 60 s, and a final extension step at 72 °C for 6 min. PCR amplification products were visualized by electrophoresis in 1.5% agarose gel, in 0.5× TBE (Tris-Borate-EDTA) buffer, stained with SYBR™SAFE (0.2 g mL^{-1}) and gel image captured with Molecular Imager® Gel Doc™ XR+ (BioRad, Hercules, CA, USA).

Amplified products were cleaned using the EXTRACTME® DNA clean-up kit (Blirt, Gdańsk, Poland) following the manufacturer's protocol and sent directly to Macrogen Ltd. (Madrid, Spain) for sequencing.

All nucleotide sequences were submitted to the NCBI (National Center for Biotechnology Information) GenBank database under the accession numbers MT176684 to MT176764 (Table S3).

2.4. Phylogenetic Analysis

To verify if the sequences had cyanobacterial origin a BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/; accessed 24/02/2020) was performed running program "blastn" in the "Nucleotide collection (nr/nt)" database. The database was constructed with the sequences from this study and type species sequences retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/; accessed 12/07/2020). All sequences were assembled and trimmed using BioEdit 7.0.5.3 software [41] and aligned using MUSCLE [42] in Version 10.0.5 of MEGA software [43]. The sequence data matrix with a final of 629 bp length was used to infer phylogenetic distances.

16S rRNA gene phylogeny relations were calculated using Maximum Likelihood (ML) and Bayesian inference (BI). The general time-reversible evolutionary model of substitution with gamma-distributed evolutionary rates and with an estimated proportion of invariable sites (GTR+G+I) was selected based on jModelTest 2.1.7 [44]. ML was calculated using the software RAxML 8.2.0 [45] and the graphical interface raxmlGUI 2.0.0 [46], with the thorough bootstrap option (1000 replicates) and general time-reversible with gamma model of rate heterogeneity (GTRGAMMA). BI was calculated with MrBayes 3.2 [47] with GTR+G+I model, applying two separate runs with four chains each and 3,000,000 Markov chain Monte Carlo generations (the first 300,000 sampled trees were discarded as burn-in).

The tree was drawn with FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree) and Inkscape 0.92.4 (https://inkscape.org/pt/). Only the BI tree is presented, with bootstrap percentages (ML) and BI probabilities for branch support, since ML and BI methods resulted in similar trees. Only bootstrap percentages above 50 and probabilities above 0.9 are shown at the branch nodes of the phylogenetic distance trees. *Gloeobacter violaceus* PCC 721 (NR_074282.1) was used as the out group.

3. Results

3.1. Sampling Site Features

Water temperature varied between 12–23 °C in lakes, 14–15 °C in streams, and from 28 °C to over 100 °C in thermal sites. Lake water pH varied from slightly acid to alkaline (6–9), while in streams it was mainly neutral (around 7), and more acidic in thermal sites (5.9–6.9). Dissolved oxygen was similar in lakes and streams (9–12 mg L^{-1}), however lower in thermal sites (2–6 mg L^{-1}), while electric conductivity was higher in thermal sites (477–2440 µS cm^{-1}), and streams (93–380 µS cm^{-1}), and lower in lakes (30–148 µS cm^{-1}). The environmental characteristics of all sampling sites are presented in Table S1.

3.2. Cyanobacterial Isolation and Morphological Identification

A total of 156 cyanobacteria strains were deposited in BACA from the 47 sampled environments (Figure 1; Table 1), presented in detail in supplementary materials (Table S2). Isolated cyanobacteria belonged to orders Chroococcales, Nostocales, Oscillatoriales, and Synechococcales with most of the isolated cultures belonging to Nostocales (118), mainly *Nostoc* species (32) (Table 1).

Most strains were isolated from lakes (99 strains, 28 genera), with eight isolated strains from streams and 12 from terrestrial environments. Cyanobacteria from terrestrial environments, such as trees and caves (Tables S1 and S2), belonged to genera *Hapalosiphon*, *Coleospermum* (Figure 1L), *Cylindrospermum*, *Nostoc*, *Lyngbya* (Figure 1K), *Pegethrix* (Figure 1G), and *Cyanobium*. From the 156 strains, 37 were isolated from thermal environments belonging to the genera *Gloeocapsopsis*, *Chlorogloeopsis* (Figure 1M), *Mastigocladus*, *Coleospermum*, *Microchaete,* and *Leptolyngbya* (Table 1).

3.3. Phylogeny Analysis

A total of 97 OTUs (operational taxonomic units) were used for the phylogenetic analyses, 79 from isolated strains during this study, two retrieved from the BACA collection, BACA0203 and BACA0224, (Table S3) and 16 from type species available in the literature. Blastn results showed that most of the strains had a good correlation between the phenotypic and genotypic identifications. Nonetheless, 49 strains had less than 98.5% similarity and within these 12 were below 95% similarity in Blastn results (Table S3), which suggest that they belong to novel genera and/or species.

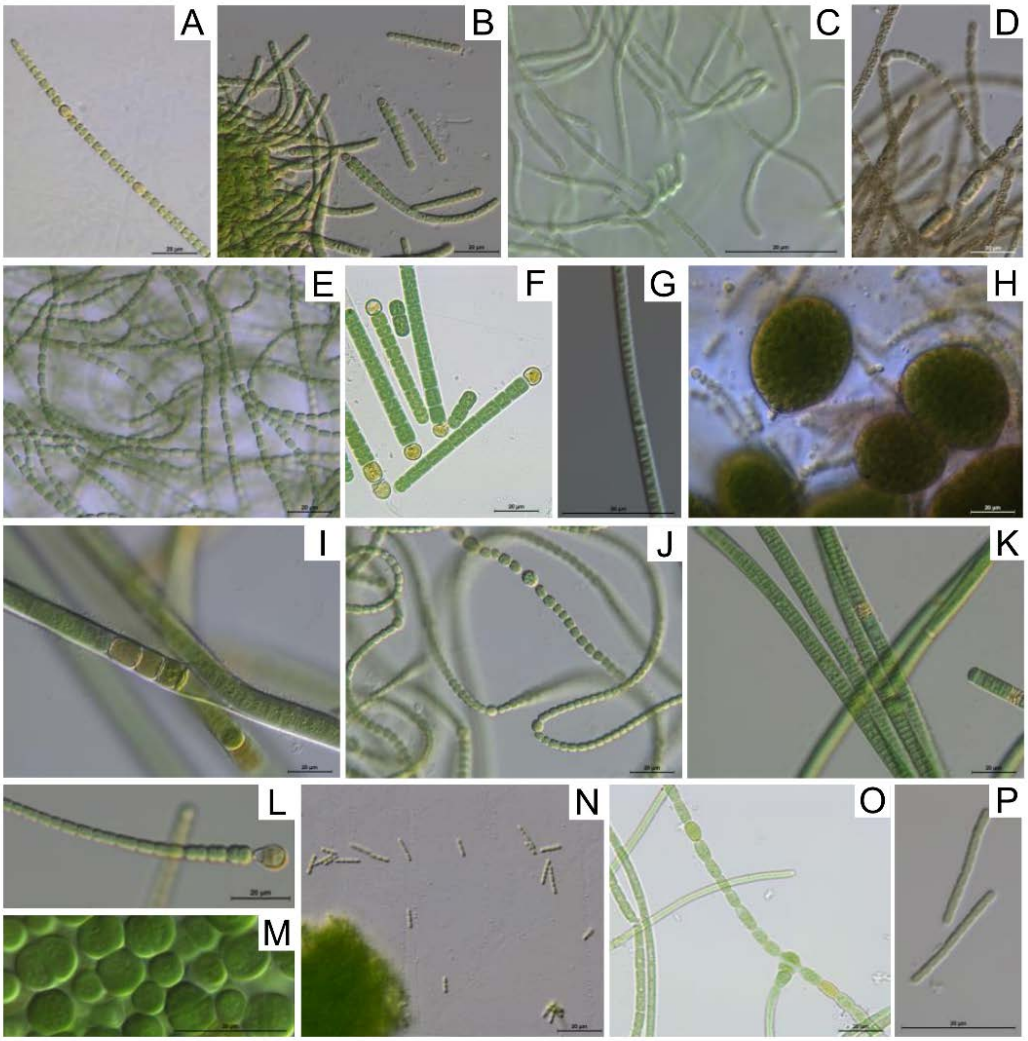

Figure 1. Selected strains photomicrographs of cyanobacteria from the Azores islands. (**A**) *Anabaena* sp. BACA0003; (**B**) *Calothrix* sp. BACA0004; (**C**) *Tildeniella* sp. BACA0012; (**D**) *Aphanizomenon* sp. BACA0293; (**E**) Nostocaceae BACA0013; (**F**) *Microchaete tenera* BACA0017; (**G**) *Pegethrix* sp. BACA0077; (**H**) *Nostoc* sp. BACA0238; (**I**) *Tolypothrix* sp. BACA0028; (**J**) *Nostoc* sp. BACA0096; (**K**) *Lyngbya martensiana* BACA0108; (**L**) *Coleospermum* sp. BACA0101; (**M**) *Chlorogloeopsis fritschii* BACA0136; (**N**) *Pseudanabaena* sp. BACA0141; (**O**) *Westiellopsis* sp. BACA0150; (**P**) *Leptolyngbya* sp. BACA0229. Scale bars-20 µm.

The phylogenetic tree based on the 16S rRNA gene presents 14 clusters that include four cyanobacteria orders: Nostocales (clusters I to VIII), Synechococcales (clusters IX, XI, XIII, and XIV), Oscillatoriales (cluster X) and Chroococcales (cluster XII) (Figure 2).

In the Nostocales order, cluster I has two clades, one with thermal strains of *Coleospermum* genus and the other of *Scytonematopsis* strains (Figure 2); clusters II, III, and IV are mainly composed of Nostocaceae taxa (*Anabaena*, *Isocystis*, and *Nostoc*), also with one clade of *Tolypothrix* genus (cluster III). Cluster V contains *Nostoc* type species *Nostoc commune* (BACA0023; UTEX 504), *Nostoc punctiforme* (BACA0026), and thermal species *Westiellopsis* sp. (BACA0114 BACA0150). Cluster VI contains Aphanizomenaceae taxa with *Aphanizomenon*, *Dolichospermum,* and *Sphaerospermopsis* genera. Cluster VII consists of Microchaetaceae cultures, *Chlorogloeopsis fritschii* (BACA0136), and *Anabaena* sp. (BACA0003). Cluster VIII consists of Rivulariaceae genera with *Rivularia* and *Calothrix*, together with one thermal species, *Microchaete bulbosa* (BACA01111).

Table 1. Isolated cyanobacteria (number) by order, family, and genera. Strains isolated from different types of ecosystems.

Order	Family	Genera	No. [1]	Ecosystem
Chroococcales	Chroococcaceae	*Gloeocapsopsis*	1	Th
Chroococcales	Cyanobacteriaceae	*Cyanobacterium*	1	L
Chroococcales	Microcystaceae	*Microcystis*	2	L
Nostocales	Aphanizomenonaceae	*Anabaenopsis*	1	L
Nostocales	Aphanizomenonaceae	*Aphanizomenon*	4	L
Nostocales	Aphanizomenonaceae	*Dolichospermum*	3	L
Nostocales	Aphanizomenonaceae	*Sphaerospermopsis*	1	L
Nostocales	Chlorogloeopsidaceae	*Chlorogloeopsis*	5	Th
Nostocales	Fortieaceae	*Fortiea*	2	L
Nostocales	Hapalosiphonaceae	*Fischerella*	1	Th
Nostocales	Hapalosiphonaceae	*Hapalosiphon*	1	Tr
Nostocales	Hapalosiphonaceae	*Mastigocladus*	8	Th
Nostocales	Hapalosiphonaceae	*Westiellopsis*	2	Th
Nostocales	Microchaetaceae	*Coleospermum*	19	L, Th, Tr
Nostocales	Microchaetaceae	*Goleter*	1	L
Nostocales	Microchaetaceae	*Microchaete*	2	L, Th
Nostocales	Nostocaceae	-	1	L
Nostocales	Nostocaceae	*Anabaena*	5	L
Nostocales	Nostocaceae	*Cylindrospermum*	2	S, Tr
Nostocales	Nostocaceae	*Hydrocoryne*	1	L
Nostocales	Nostocaceae	*Isocystis*	1	L
Nostocales	Nostocaceae	*Nostoc*	32	L, S, Tr
Nostocales	Rivulariaceae	*Calothrix*	11	L, S
Nostocales	Rivulariaceae	*Rivularia*	3	L
Nostocales	Scytonemataceae	*Scytonematopsis*	2	L
Nostocales	Tolypothrichaceae	*Tolypothrix*	9	L, S
Oscillatoriales	Oscillatoriaceae	*Kamptonema*	1	L
Oscillatoriales	Oscillatoriaceae	*Lyngbya*	1	Tr
Oscillatoriales	Oscillatoriaceae	*Phormidium*	2	L, Th
Oscillatoriales	Oscillatoriaceae	*Tychonema*	1	L
Oscillatoriales	Microcoleaceae	*Arthrospira*	3	L
Synechococcales	Leptolyngbyaceae	*Leptolyngbya*	12	L, Th
Synechococcales	Leptolyngbyaceae	*Stenomitos*	1	L
Synechococcales	Oculatellaceae	*Pegethrix*	1	Tr
Synechococcales	Oculatellaceae	*Tildeniella*	1	L
Synechococcales	Pseudanabaenaceae	*Pseudanabaena*	5	L
Synechococcales	Pseudanabaenaceae	*Limnothrix*	3	L
Synechococcales	Synechococcaceae	*Cyanobium*	3	L, Tr

[1] Number of cyanobacterial strains isolated and deposited in BACA. L: lake, S: stream, Tr: terrestrial; Th: thermal.

Cluster X includes the Oscillatoriales taxa from the genera *Arthrospira*, *Tychonema*, *Kamptonema*, and *Lyngbya*. Cluster XII consists of Chroococcales species *Microcystis aeruginosa* (BACA0148) and *Microcystis flos-aquae* (BACA0230). Clusters IX, XI, XIII, and XIV represent Synechococcales strains, with clusters IX and XI including Leptolyngbyaceae, cluster XIII Pseudanabaenaceae and in cluster XIV the coccoid *Cyanobium* genus.

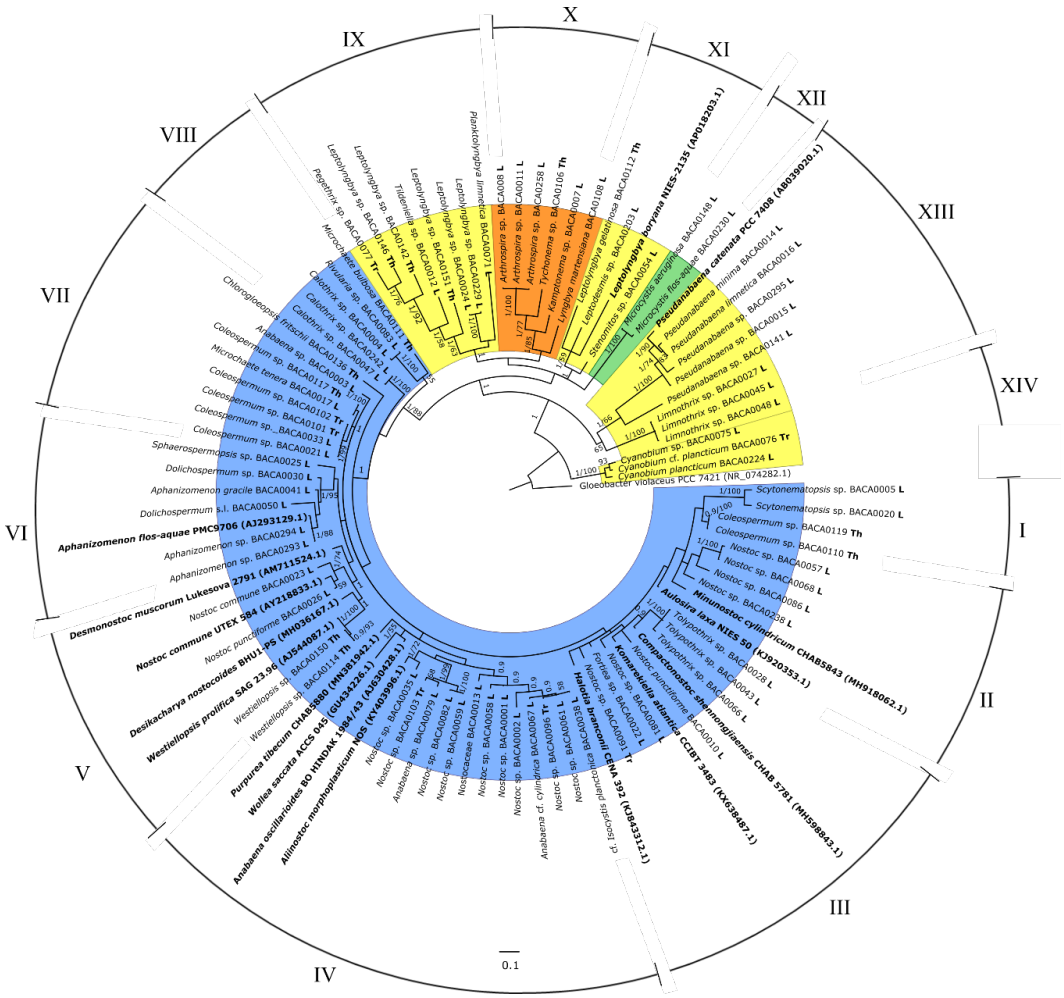

Figure 2. Bayesian inference phylogenetic tree based on the partial 16S rRNA gene sequences. A bootstrap test involving 1000 pseudo-replicates was performed. Bootstrap (greater than 50%) and probabilities values (greater than 0.9) are displayed in front of the relevant nodes obtained from ML and Bayesian methods, respectively. Colors represent cyanobacteria orders: Nostocales (blue), Synechococcales (yellow), Oscillatoriales (orange), and Chroococcales (green). The 14 identified clusters (I–XIV) are indicated along the tree and described in the text. L: lake, S: stream, Tr: terrestrial; Th: thermal.

4. Discussion

Cyanobacteria studies in the Azores have been reported since 1874 [48], mainly from freshwater habitats (e.g., [5,8,13]) with only a few references to terrestrial and thermal environments [13] despite the abundance of thermal habitats in these islands [12]. With this work, we report 11 genera that have not been identified in the Azores archipelago following the published checklist by Luz [13], namely, *Cyanobacterium*, *Fischerella*, *Westiellopsis*, *Goleter*, *Isocystis*, *Scytonematopsis*, *Tychonema*, *Arthrospira*, *Stenomitos*, *Pegethrix* and *Tildeniella* here reported for the first time in the Azores.

Nostoc is a polyphyletic genus, with a wide genetic diversity, that lacks morphological diacritical features making its identification based on morphological data problematic, thus the necessity of genetic information [14,25]. Our results show that *Nostoc* is widely spread, among clusters II to V, separated with high phylogenetic distances (Figure 2), which is in accordance with previous studies [18,19,49–53]. The positions of *Nostoc* strains from this study, *Nostoc* type species *Nostoc commune* UTEX 584 (AY218833.1) and *Nostoc* alike type species such as *Halotia branconii* CENA 392 (KJ843312.1), *Aliinostoc morphoplasticum* NOS (KY403996.1) and *Desmonostoc muscorum* Lukesova 2/91 (AM711524.1)

highlights *Nostoc* polyphyletic status. It has been suggested previously that the polyphyletic separation of *Nostoc* is not related to habitats [18,52], which is in accordance with our results as terrestrial strains are positioned near freshwater strains (Figure 2). Unlike thermal, terrestrial habitat does not seem to be a feature that separates strains in our phylogenetic tree (Figure 2).

Nostoc taxonomy has undergone several revisions with the creation of new monophyletic genera [14,25], such as *Aliinostoc* [54], *Desmonostoc* [16], *Halotia* [20], and *Mojavia* [55]. Our results support *Nostoc* as a polyphyletic genus and highlights the important need for further taxonomical revision.

Besides *Nostoc* other genera are reported as polyphyletic such as *Anabaena*, *Rivularia*, *Tolypothrix*, and *Westiellopsis* [25,56–58]. Our results are not enough to support this assumption, however, the three strains of *Anabaena* (BACA0003, BACA0067, and BACA0079) in the phylogeny analysis are separated and have high phylogenetic distances. The blastn results showed that strains *Anabaena* cf. *cylindrica* BACA0067 and *Anabaena* sp. BACA0079, in cluster IV near Nostocaceae strains (Figure 2) and closer to *Anabaena* type species *A. oscillatorioides* BO HINDAK 1984/43 (AJ630428.1), are related to *Nostoc* strains with similarities between 98.5% and 97.4% (Table S3). However, strain *Anabaena* sp. BACA0003, with similarities close to 99% with *Anabaena* sp. PCC 7108 (AJ133162.1) (Table S3), is in cluster VII closer to Aphanizomenaceae strains (Figure 2) and more distant from *A. oscillatorioides* BO HINDAK 1984/43 (AJ630428.1). In fact, previous studies have reported that some *Anabaena* strains are closer to Aphanizomenaceae than to Nostocaceae [56,58].

Westiellopsis is a widespread genus first described in terrestrial habitats [59] but also known to withstand high temperatures [60,61]. Strains BACA0114 and BACA0150 isolated from a thermal stream, reinforce the idea of plasticity and adaptation to several habitats among *Westiellopsis* species. This pattern is also observed in BACA0135 a *Fischerella* strain (Table S2), a genus with close phylogenetic distance to *Westiellopsis* [60].

Not well described in the literature is the phylogenetic distance between *Microchaete*/*Coleospermum*. The *Microchaete* genus has been revised, separating freshwater and marine species in different phylogenetic clades [18,30], with *Microchaete* freshwater species (*Coleospermum*) positioned in the Microchaetaceae family and marine species in the Rivulariaceae family [18,30]. Our work reinforces the different phylogenetic positions of thermal species, with two clades, one in the Rivulariaceae family and another near *Scytonematopsis*, a genus with unclear family position [62]. Thermal *Microchaete*/*Coleospermum* strains form a well-supported clade in cluster I (Figure 2), while strains from lakes and terrestrial habitats form two clades in cluster VII (Figure 2). Except for *Coleospermum* sp. BACA0117 which was isolated from a thermal stream; however, this can be supported by the fact that this stream had temperatures around 27.7 °C which may indicate that this species is not really an extremophile but a tolerant species that survive at these temperatures such as already explained previously with *Westiellopsis* [60,61]. *Microchaete bulbosa* BACA0111 and *Microchaete tenera* BACA0017 strains are also separated in the phylogeny tree, the thermal strain is in cluster VIII, closer to Rivulariaceae strains, and the freshwater strain is in cluster VII, between other freshwater and terrestrial strains (Figure 2). Our results support the polyphyletic status of *Microchaete*/*Coleospermum*, namely in habitat differentiation where there is a clear genetic separation in genera between freshwater, thermal and marine species, providing useful information for the taxonomic revision of these genera.

Phylogenetic distances are bigger in the Synechococcales clades (IX, XI, XIII, and XIV; Figure 2) which suggests fewer similarities between these strains. Synechococcales is a polyphyletic group of both unicellular and filamentous cyanobacteria that needs revision, even at the family level [14,25]. The threshold accepted for distinguishing cyanobacteria genus is 95% for sequence similarity based on the 16S rRNA gene [63]. Blastn results show that strains BACA0024/BACA0229, BACA0112, BACA0142, BACA0146, and BACA0151, in cluster IX, morphological identified as *Leptolyngbya* sp. have very low similarities, between 91% and 95%, with the sequences available in NCBI (Table S3), indicating that these are probably novel genera. This is also supported by the phylogenetic distance to *Leptolyngbya* type species *L. boryana*_NIES-2135 (AP018203.1) positioned in cluster XI (Figure 2). Most of these strains are from thermal environments (Table S2), where there is a lack of studies and knowledge of

cyanobacteria [4], reinforcing the suggestion that these strains can be novel cyanobacteria. *Leptolyngbya* is a polyphyletic genus recently divided into more genera such as *Leptodesmis* [31], *Pegethrix*, and *Tildeniella* [17]. This study provides evidence for cyanobacteria taxonomic identification of novel genera in thermal habitats.

In the well-supported clade of *Pseudanabaena* strains (Figure 2), BACA0141 has a higher phylogenetic distance supported by the blastn results, with the closest match to *Pseudanabaena* sp. PCC 7403 strain (AB075995.1) with a similarity of 96.72% (Table S3). *Pseudanabaena* is not a well-defined genus [64], with three different morphological groups [35] that probably can be genetically separated. The results from BACA0141 blastn indicates a possible new genus, which is also supported by significant morphological differences (Figure 1N) and by the phylogenetic distance (cluster XIII; Figure 2), in respect to *Pseudanabaena* holotype species *P. catenata* (PCC7408; AB039020.1). Contrarily to the *Pseudanabaena* genus description, BACA0141 is organized in dense and irregular mats, with small trichomes, generally with 3–5 cells per trichome (8.82 ± 1.81 µm long). This differs significantly from *P. catenata* as it is characterized by bigger trichomes 40–200 µm long (up to 1 mm) that are solitary or arranged in small mats [35]. The combination of morphological and phylogenetic (16S rRNA) results supports the establishment of a new possible genera to include BACA0141 and supports the polyphyletic nature of the genus *Pseudanabaena*.

Cyanobacteria taxonomy, as reported by numerous authors (e.g., [14,18,25]), still has several problems and needs further revision. Our work supports this statement, reporting valuable information for genera and species restructuration based on their phylogenetic, morphological, and ecological characteristics. This work enhanced cyanobacteria knowledge in the Azores, mainly in thermal habitats, supporting the assumption that cyanobacteria biodiversity in these islands is still understudied. This is especially true for extreme habitats, where diversity has already been shown to be higher than previously thought [53]. We report 11 new genera for this archipelago, as well as indications for six novel cyanobacteria genera, mainly from thermal environments.

The isolation and deposition of these cyanobacteria strains in a culture collection (BACA) will allow future studies regarding cyanobacteria taxonomical classification following a modern polyphasic approach. Furthermore, these strains can also be used for biotechnological applications for natural compounds investigation or to respond to cyanobacteria related issues such as blooms developments, cyanotoxin occurrence, and toxicity risk assessment.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-2818/12/8/298/s1: Table S1: Sample sites, and environmental variables (sample sites with mean and standard deviation had multiple samplings, samples without mean and standard deviation were sampled once). Table S2: Isolated cyanobacteria from different types of ecosystems. Table S3: Sequence identity (%) of 16S rRNA gene fragment between BACA strains and other cyanobacterial sequences available in GenBank (NCBI).

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