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RESEARCH ARTICLE

Exploring the cyanobacterial diversity in Portugal: Description of four new genera from LEGE-CC using the polyphasic approach

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Abstract

Culture collections such as the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) hold approximately 1200 cyanobacterial strains and are critical community resources. However, many isolates in this and other collections have not been described with a polyphasic approach, and this limits further study. Here, we employed a polyphasic methodology that integrates 16S rRNA gene phylogenetic analyses, similarity (*p*-distance), 16S-23S ITS rRNA region secondary structures, morphological analyses, and habitat assessments to describe four novel cyanobacterial genera from the LEGE-CC, Portugal. *Pseudolimnococcus planktonicus* gen. et sp. nov. (Chroococcales) is phylogenetically and morphologically related to *Limnococcus*. The 16S rRNA gene similarity between the types of both genera is only 93.1%. Morphologically, *Pseudolimnococcus* cells do not reach the original spherical shape before the next division or have aerotopes and firm mucilage, while *Limnococcus* cells reach the original shape, lack aerotopes, and have diffluent mucilage. *Eucapsopsis lusitanus* gen. et sp. nov. (Chroococcales) is morphologically similar to *Eucapsis* but differs from it by having aerotopes and diffluent envelope. *Eucapsis* lacks aerotopes and has firm mucilaginous envelopes, rarely diffluent. Both genera are phylogenetically very distant from each other and have only 90.68% 16S rRNA gene similarity. *Pseudoacaryochloris arrabidensis* gen. et sp. nov. (Acaryochloridales) differs from *Acaryochloris* by the lack of mucilaginous envelope, which is present in *Acaryochloris*. Both genera are phylogenetically distant and have only 94.1% 16S rRNA gene similarity. Moreover, *Acaryochloris* is marine (sponge symbiont), while *Pseudoacaryochloris* is from freshwater. *Vasconcelosia minhoensis* gen. et sp. nov. (Nodosilineales) is phylogenetically related to *Cymatolege* but has only 94.3% similarity with this genus. Morphologically both genera are distinct. *Vasconcelosia* has a *Romeria*-like structure, while *Cymatolege* has a *Phormidium*-like structure. In all cases the 16S-23S ITS rRNA region secondary structures are in agreement with the other analyses. These novel genera expand the diversity of cyanobacteria in culture collections.

KEYWORDS
benthic, biodiversity, brackish water, cyanobacteria, freshwater, LEGE-CC, planktonic, polyphasic approach, taxonomy

Abbreviations: ACOI, coimbra collection of algae; BI, bayesian inference; 16S rRNA gene, 16S ribosomal RNA gene; ITS, intergenic spacer region; ML, maximum likelihood; LEGE-CC, blue biotechnology and ecotoxicology culture collection; PCR, polymerase chain reactions.

Flavio Luis de Oliveira, Guilherme Scotta Hentschke and João Morais contributed equally to this article.

INTRODUCTION

Cyanobacteria are gram-negative prokaryotic organisms, which have been playing essential roles in Earth's biogeochemical cycles for billions of years (Couradeau et al., 2011). Despite their ecological significance, cyanobacterial blooms pose notable environmental and socioeconomic challenges due to the production of cyanotoxins, which can adversely impact various organisms, including aquatic life and humans (Carmichael et al., 2001). Additionally, these blooms can lead to diminished oxygen levels in waterbodies through light attenuation (Whitton, 2012). However, cyanobacteria also have substantial biotechnological potential, synthesizing diverse organic compounds with applications ranging from antioxidants to pharmaceuticals. Moreover, they contribute to the production of secondary metabolites, which have applications in aquaculture, wastewater treatment, and food production (Lopes et al., 2022).

Currently, there are more than 4500 validly described cyanobacterial species, and they are classified in 20 orders (Kastovsky, 2024; Strunecký et al., 2023). A great part of this diversity is cryptic, and estimates suggest this includes almost 50% of the species described since January 1, 2000 (Kastovsky, 2024). This notable proportion of cryptic taxa is primarily due to the application of the polyphasic approach in cyanobacterial taxonomy and by the use of 16S rRNA gene analysis, which has split many traditional genera; for example, *Limnococcus* Komarková et al. (Komárková et al., 2010), *Inacoccus* Gama et al., *Cryptococcum* Gama et al., and *Neochroococcus* Geng & Yu (Gama et al., 2019; Geng et al., 2021), were cleaved away from *Chroococcus* Nageli based on the polyphasic approach even though they all are morphologically identical to *Chroococcus*.

Many genera in Portugal, such as *Romeriopsis* Hentschke et al., *Zarconia* Hentschke et al., *Ciimarium* Hentschke et al., *Lusitaniella* Ramos et al., *Geminobacterium* Ramos et al., and *Calenema* Ramos et al. (Brito et al., 2017; Hentschke et al., 2022, 2024) have been described using these methods, but in this country, the cyanobacterial diversity is still underexplored, despite the existence of important culture collections such as the LEGE-CC and the ACOI. Portugal has a diversity of aquatic environments, including freshwater and marine habitats, along with diverse terrestrial habitats, ranging from rivers valleys to high mountains and volcanic islands. These different habitats are promising sources of new cyanobacterial taxa. The LEGE-CC holds approximately 1200 cyanobacterial strains. Based on 16S rRNA gene phylogenies (Ramos et al., 2018), many of them are phylogenetically distant from any other already-described cyanobacterial genera and are potentially available to be described as new taxa. During the work of identification of LEGE-CC strains, we determined, using a polyphasic approach, that LEGE 07310, LEGE 12582, LEGE 18583, LEGE 16640, LEGE 17667, and LEGE 19970 are in fact new genera .

MATERIALS AND METHODS

Sampling and morphological analysis

In 2018, the LEGE-CC acquired the planktonic strains LEGE 12582, LEGE 18583, LEGE 16640, LEGE 17667, and LEGE 19970 from the Doctor Maria de Fátima Santos personal culture collections (Alga2O, Coimbra, Portugal). The planktonic samples had been collected from freshwater environments in different cities in Portugal (Figure 1). Additionally, the benthic strain LEGE 07310, for which the biotechnological potential was studied by Lopes et al. (2012), had been collected from a marine/brackish water environment at the Minho River estuary, Caminha, also in Portugal (Figure 1). The strains are currently maintained in a Z8 liquid medium (Kotai, 1972) under the following conditions: 19°C on a 12:12 h light:dark cycle (25 μmol photons $\cdot m^{-2} \cdot s^{-1}$). The strains were microphotographed, recorded, and analyzed by the LEICA LAS version 4.12.0 image analysis software (Leica Microsystems Limited and CMS GmbH, Switzerland). The form and dimensions of vegetative cells (both intercalary and terminal); the quantity of trichomes per filament; and the existence or lack of sheaths, constrictions at the cross-wall, and necridic cells were documented. To visualize the mucilaginous envelopes, China Ink was used for staining. The measurements were performed for each characteristic of the strain (20 to 30 measurements) and were carried out at various positions of the slide preparation. From these cultures, aliquots were lyophilized and preserved in the University of Porto Herbarium.

DNA extraction, PCR amplification, and sequencing

The cyanobacteria cells were harvested from the received cultures, and the total genomic DNA (gDNA) of the strain was extracted using the PureLink Genomic DNA kit (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions provided for gram-negative bacteria. Specific cyanobacteria primers were used for gene amplification, including primers 27SF (Neilan et al., 1997) and 23SR (Taton et al., 2003).

The PCRs were performed in a Veriti Thermal Cycler (Veriti 9902, Applied Biosystems, Thermo Fisher Scientific, Singapore). The final reaction volume was 20 μL, consisting of 5.9 μL of molecular biology-grade water, 4 μL of Green GoTaq Flexi Buffer, 2 μL of $MgCl_2$, 2 μL of each forward and reverse primer, 1.5 μL of deoxynucleotide triphosphate (dNTPs), 0.5 μL of bovine serum albumin (BSA), 0.1 μL of GoTaq Flexi DNA

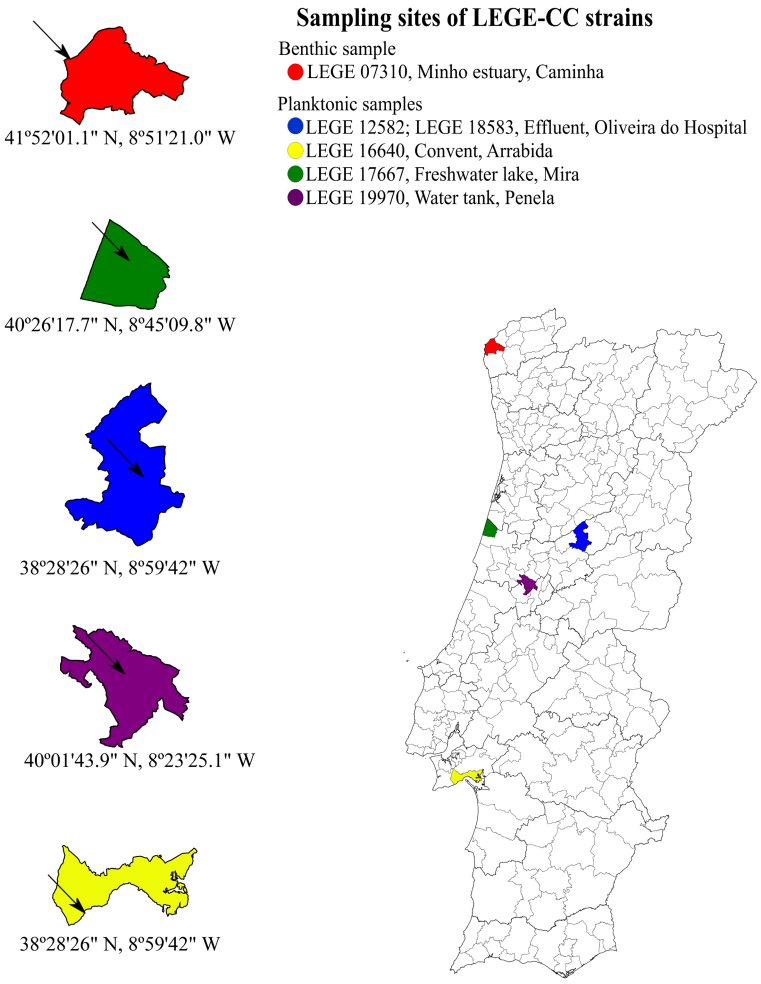

FIGURE 1 Map showing the cyanobacteria sampling sites in Portugal. Different colors indicate cities where samples were collected (Created by mapchart.net). [Color figure can be viewed at wileyonlinelibrary.com]

Polymerase (Promega, USA), and 2 μL of genomic DNA (Katana et al., 2001). The 16S rRNA gene sequence was obtained upon PCR amplification using the following conditions: initial denaturation at 94°C for 5 min, followed by 10 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 2 min. This was followed by an additional 25 cycles of denaturation at 92°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 2 min, with a final extension step at 72°C for 7 min. The PCR products were separated using 1% (w/v) agarose gel stained with SYBR Safe DNA gel stain (Invitrogen by Thermo Fisher Scientific, USA), and DNA gene fragments of the expected size were excised from the gel and purified using the NZYGelpure kit (Nzytech, Portugal), following the manufacturer's instructions. Finally, the purified fragments were sent for sequencing (separately) with primers 359F and 781R (Nübel et al., 1997), 1494R and 27SF (Neilan et al., 1997), 23SR (Taton et al., 2003), and 1114F (Lane, 1991). The sequencing was performed by Sanger dideoxy sequencing at GATC Biotech (Ebersberg, Germany), and the nucleotide sequences obtained were manually inspected for quality and assembled using Geneious Prime 2023.2.1 software (Biomatters Ltd., Auckland, New Zealand). Before phylogenetic analysis, the sequences were checked for possible chimera formation using the DECIPHER software 2.27.2 (Firtrh et al., 2009). To assess the presence and quality of the DNA obtained from extraction and PCR, we performed electrophoresis on a 1% (w/v) and 1.5% (w/v), respectively, agarose gel stained with SYBR Safe DNA gel stain (Invitrogen by Thermo Fisher Scientific, USA). The confirmation of high-molecular-weight DNA was based on the presence of clear bands observed in the gel. The sequences were deposited in GenBank (National Center for Biotechnology Information, NCBI) and the codes are exhibited in the phylogenetic trees.

Transmission electron microscopy (TEM)

The strains were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 72 h and postfixed overnight with 2% osmium tetroxide in 50 mM sodium cacodylate buffer (pH 7.2). Subsequently, the cells were washed three times in sodium cacodylate buffer and stained en bloc overnight with aqueous 2% uranyl acetated

solution, dehydrated, and embedded in Embed-812 resin (#14120; Electron Microscopy sciences). Ultrathin sections (50 nm thickness) were cut on a RMC Ultramicrotome (PowerTome, USA) using Diatome diamond knives, mounted on mesh copper grids (Electron Microscopy Sciences), and stained with uranyl acetate substitute (#11000; Electron Microscopy Sciences) and lead citrate (#11300; Electron Microscopy Sciences) for 5 min each. Samples were visualized on a JEOL JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV, and images were digitally recorded using a CCD digital camera Orius 1100 W (Tokyo, Japan).

Phylogenetic analysis

The phylogenetic analyses were conducted in two rounds. To position our strains among the cyanobacterial families, in the first round, we aligned the 16S rRNA gene sequences of our six strains with those of 437 cyanobacterial reference strains, for a total of 443 sequences. The final alignment contained 724 informative sites. The phylogenetic reconstruction was conducted using the FastTree method (Price et al., 2009), with a bootstrap value set by default to 1000, as per the manual. The command used to run the phylogeny was "FastTree -gtr -nt alignment_file > tree_file." The tree was edited using iTOL (Letunic & Bork, 2021).

For the second round, we selected cyanobacterial sequences more closely related to our strains, such as Chroococcaceae, Cyanotrichaceae, Microcystaceae (Chroococcales), Acaryochloridales, Nodosilineales, and others retrieved from GenBank (NCBI) by BLAST, resulting in 136 sequences. The final alignment contained 951 informative sites. Then, the phylogenetic trees were built using ML and BI analyses. A GTR + G + I evolutionary model was selected by Molecular Evolutionary Genetics Analysis version 11 (MEGA11; Tamura et al., 2021). The robustness of the ML tree was estimated by bootstrap percentages, using 1000 replications using IQ-Tree online version v1.6.12 (Trifinopoulos et al., 2016). The Bayesian tree was constructed in two independent runs with four chains each for 5×10^6 generations, with burn-in fraction set to 0.25 and sample frequency to 1000, using MrBayes (Ronquist et al., 2012) in Cipres Gateway (Miller et al., 2010). The processing and visualization of these trees used FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

For all analyses, the sequences were aligned using MAFFT (Katoh et al., 2002), and the outgroup used was *Gloeobacter violaceus* PCC 8105 (AF132791). A similarity (*p*-distance) matrix was generated using MEGA11 and the secondary structures of the ITS rRNA region between the 16S and 23S rRNA genes (ITS rRNA region) of D1–D1', V2, Box B, and V3 helices were folded using MFold (Zuker, 2003), according to Lukesova et al. (2009).

RESULTS

Description of the new taxa

Order Chroococcales

Family Cyanotrichaceae

Pseudolimnococcus planktonicus gen et sp. nov. G. S. Hentschke, F. Oliveira & J. Morais. Figure 2

Description: Colonies with irregular margins, formed by cells arranged in two to four celled packet-like subcolonies. The subcolonies are irregularly arranged. Cells subspherical or hemispherical, remaining in pairs after division. Cells do not reach the original shape before the next division. Divisions in three planes. Mucilage firm, conspicuous. Cell content granulose, dark green, with aerotopes. Cells dimensions: 13.6–16.8 × 10.2–11.89–13.6 μm.

Etymology: "*Pseudo*" is derived from ancient Greek "*Pseudes*" and means "false." "*Pseudolimnococcus*" refers to "false" *Limnococcus*, due to the similar morphology between these genera. "*planktonicus*" refers to the planktonic habitat.

Holotype: Collected from a freshwater lake, at Mira (40°26′17.7″ N, 8°45′09.8″ W), Portugal in 2017, by Maria de Fátima Santos. Deposited in the University of Porto herbarium, in metabolically inactive state (lyophilized), under the code PO-T4785.

Type strain: LEGE 17667 (PP477468)

Habitat: Planktonic, freshwater lake

Order Chroococcales

Family Microcystaceae

Eucapsopsis lusitanus gen. et sp nov. G. S. Hentschke, F. Oliveira & J. Morais. Figure 3

Description: Colonies with polygonal margins, formed by cells arranged in rows, in quadratic (two-dimensional) or cubic subcolonies. Subcolonies sometimes arranged in rows. Cells spherical, later hemispherical, and sometimes in pairs after division. Cells reach the original spherical shape before the next division. Cell division in three planes. Mucilage diffluent, invisible without staining. Cells with individual envelopes. Cell content light or dark green, with aerotopes. Cells diameter: 4.6–5.2 μm.

Etymology: "*opsis*" is derived from Greek and means "resembling." "*Eucapsopsis*" refers to resembling *Eucapsis*, due to the identical morphology between these genera. "*lusitanus*" refers to Portuguese gentilic.

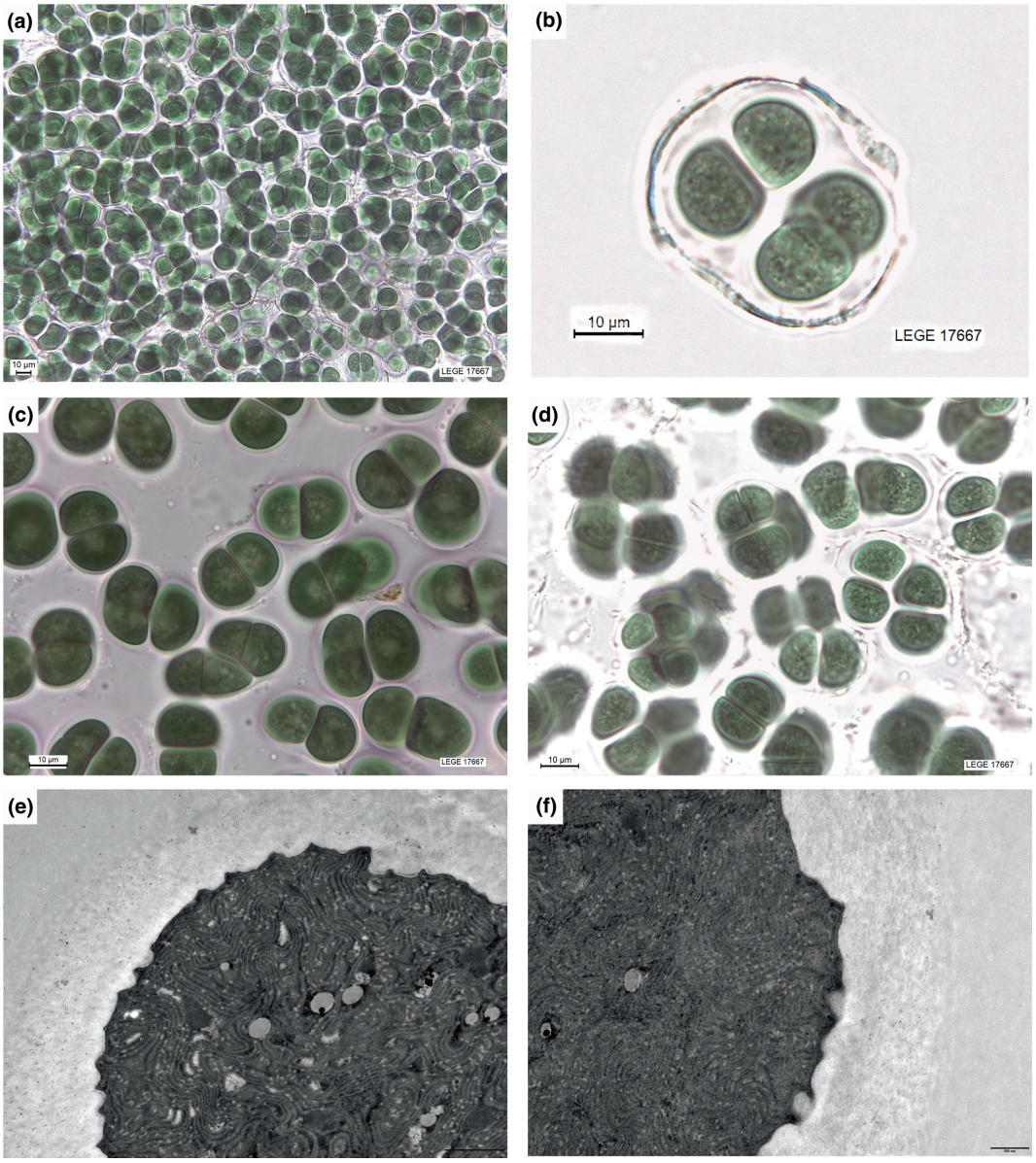

FIGURE 2 *Pseudolimnococcus planktonicus* gen. et sp. nov. LEGE 17667 (Type strain). (a) General overview of colony. (b) Detail of a packet. (c, d) Details of packets with two to four cells, (e, f) Ultrastructure (TEM). [Color figure can be viewed at wileyonlinelibrary.com]

Holotype: Collected from an artificial water tank, at Penela (40°01′43.9″ N, 8°23′25.1″ W), Portugal in 2019, by Maria de Fátima Santos. Deposited in the University of Porto herbarium, in metabolically inactive state (lyophilized), under the code PO-T4786.

Type strain: LEGE 19970 (PP477470)

Habitat: Water tank, freshwater

Order Acaryochloridales

Family Thermosynechococcaceae

Pseudoacaryochloris arrabidensis gen. et sp. nov. G. S. Hentschke, F. Oliveira & J. Morais. Figure 4

Description: Cells solitary, spherical, or subspherical. Sparsely or densely distributed. Not forming colonies in common mucilage. Mucilaginous envelope absent. Cell content homogenous, without aerotopes. Cells diameter: 2.44–2.9 µm.

Etymology: "*Pseudo*" is derived from the ancient Greek "*Pseudes*" and means "false." "*Pseudoacaryochloris*" refers to "false" *Acaryochloris*, due to the identical morphology between these genera. "*arrabidensis*" refers to the sample location "Serra da Arrábida."

Holotype: Collected from a freshwater channel, at the Convent of Serra da Arrábida, Setúbal (38°28′26″ N, 8°59′42″ W), Portugal in 2016, by Diogo Leão. Deposited in the University of Porto herbarium, in metabolically inactive state (lyophilized), under the code PO-T4787.

Type strain: LEGE 16640 (PP477467)

Habitat: freshwater channel

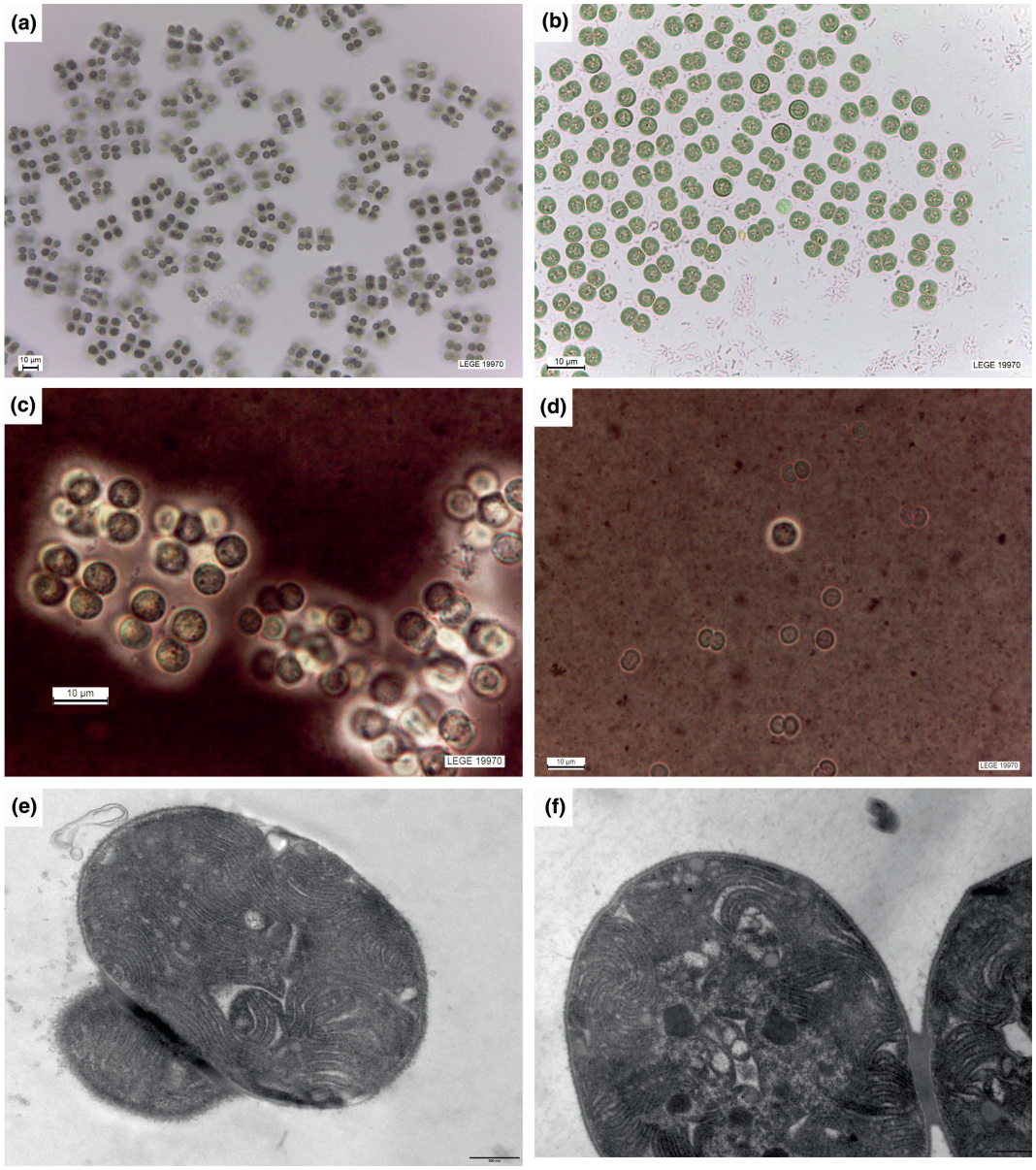

FIGURE 3 *Eucapsopsis lusitanus* gen. et sp. nov. LEGE 19970 (Type strain) (a, b) General overview of flattened or three-dimensional colonies. (c) Detail of three-dimensional colonies. (d) Single cells with thin diffluent mucilage, (e, f) Ultrastructure (TEM). [Color figure can be viewed at wileyonlinelibrary.com]

Order Nodosilineales

Family Cymatolegaceae

Vasconcelosia minhoensis gen. sp. nov. G. S. Hentschke, F. Oliveira & J. Morais. Figure 5

Description: Cells commonly solitary, in pairs or sometimes forming short trichomes with a maximum of eight cells (exceptionally >20). Trichomes irregular, constricted at cross walls, with cells loosely attached (fragmentation). Cells cylindrical, rod shaped or barrel shaped with homogenous content. Apical cells rounded, not attenuated or swollen. Mucilaginous envelope absent. Without aerotopes. Cells dimensions: 1.5–2.5×0.8–1.2 µm.

Etymology: "*Vasconcelosia*" is in honor of Vitor M. Vasconcelos, cyanobacteria researcher, director of CIIMAR and founder of LEGE-CC. "*minhoensis*" refers to Minho River the region where V.M.V. was born, as well as the sample location.

Holotype: Collected from Minho River estuary, at Caminha, (41°52′01.1″ N, 8°51′21.0″ W), Portugal in 2007, by Viviana Lopes. Deposited in the University of Porto herbarium, in metabolically inactive state (lyophilized), under the code PO-T4788.

Type strain: LEGE 07310 (PP477458)

Habitat: benthic, river estuary

In the first-round analysis (Figure S1 in the Supporting Information), the resultant phylogeny revealed that the strains LEGE 12582, LEGE 18583, and LEGE 16640 are related to *Acaryochloris* Miyashita & Chihara and *Thermosynechococcus* Katoh et al. within the Acaryochloridales cluster. Strain LEGE 07310 is related to *Nodosilinea*

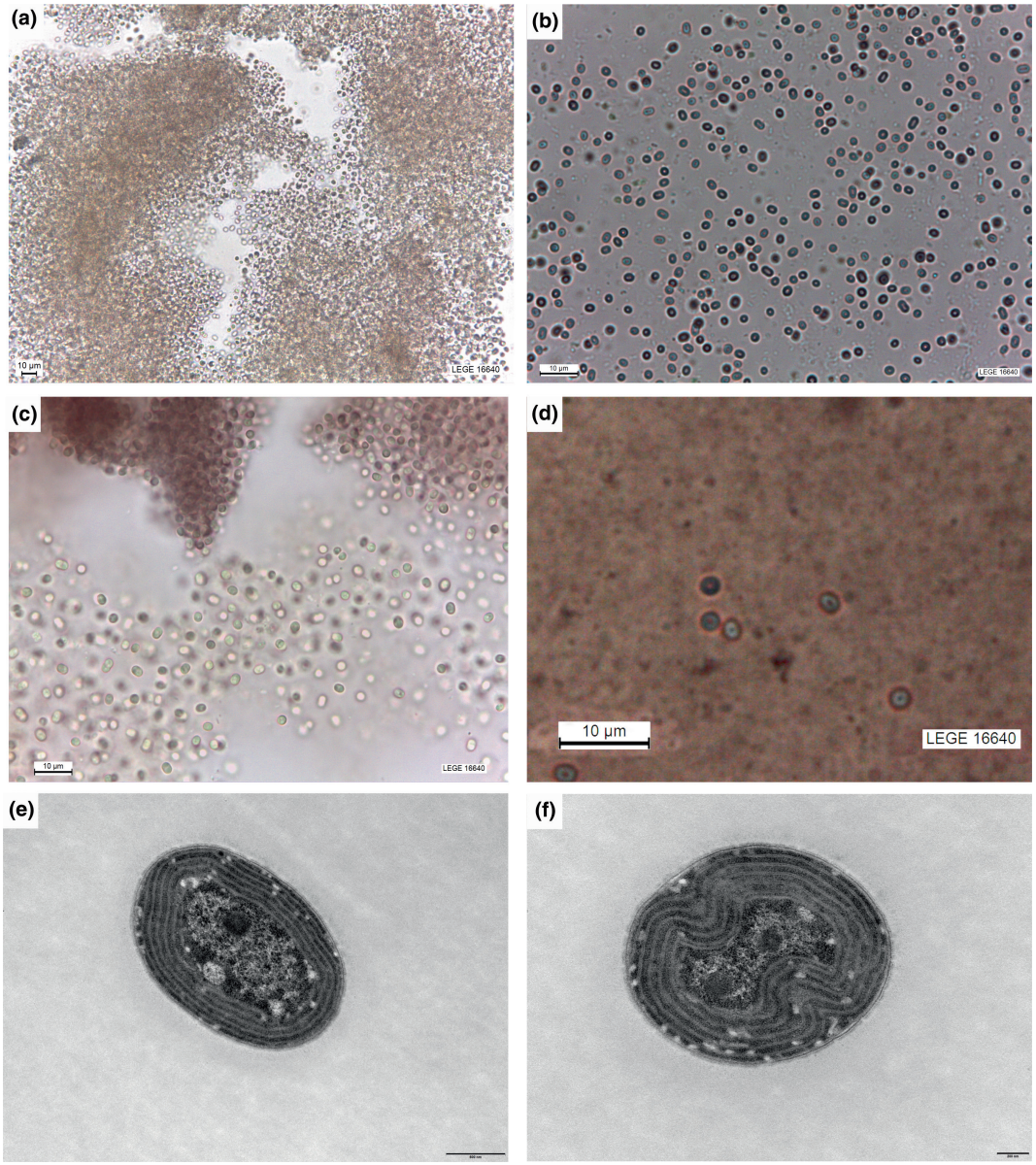

FIGURE 4 *Pseudoacaryochloris arrabidensis* gen. et sp. nov. LEGE 16640 (Type strain) (a–c) General overview of cells agglomerations. (d) Detail of cells without mucilage, (e, f) Ultrastructure (TEM). [Color figure can be viewed at wileyonlinelibrary.com]

Perkerson & Casamatta, *Cymatolege* Konstantinou and *Gibliniella* Strunecky & Raabová, in the Nodosilineales cluster. The strains LEGE 17667 and LEGE 19970 clustered with Chroococcales genera such as *Limnococcus*, *Neochroococcus*, *Inacoccus*, *Cryptococcum*, *Johanseninema* Hasler et al., *Chroococcus*, *Gloeothece* Nageli, *Microcystis* Lemmerman, and others. Filamentous genera such as *Lusitaniella* were also located in the LEGE 17667 and LEGE 19970 cluster. We attribute this to the limitation of the FastTree analysis, which only analyzed 724 nucleotide positions. However, this analysis enabled us to identify the closest related genera to all of our strains and subsequently assign them to their respective orders, which were then confirmed in the second-round analysis.

The second-round analyses were based on the 16S rRNA gene on ML and BI trees (Figure 6). These phylogenies, along with similarity, ITS rRNA region secondary structures, morphological and habitat analyses permitted us to describe *Pseudolimnococcus planktonicus* gen. et sp. nov. (LEGE 17667), *Eucapsopsis lusitanus* gen. et sp. nov. (LEGE 19970), *Pseudoacaryochloris arrabidensis* gen. et sp. nov. (LEGE 16640) and *Vasconcelososia minhoensis* gen. et sp. nov. (LEGE 07310). *Pseudolimnococcus planktonicus* gen et sp. nov. (LEGE 17667) is positioned at the top of our tree (Figure 6), strongly supported (ML = 100, BI = 1) as a sister clade of *Limnococcus* (Cyanotrichaceae). Both clades are within a bigger cluster composed of *Choroccoccus*, *Neochorococcus*, *Inacoccus*, and *Cryptococcum* (Chroococcaceae, Chroococcales).

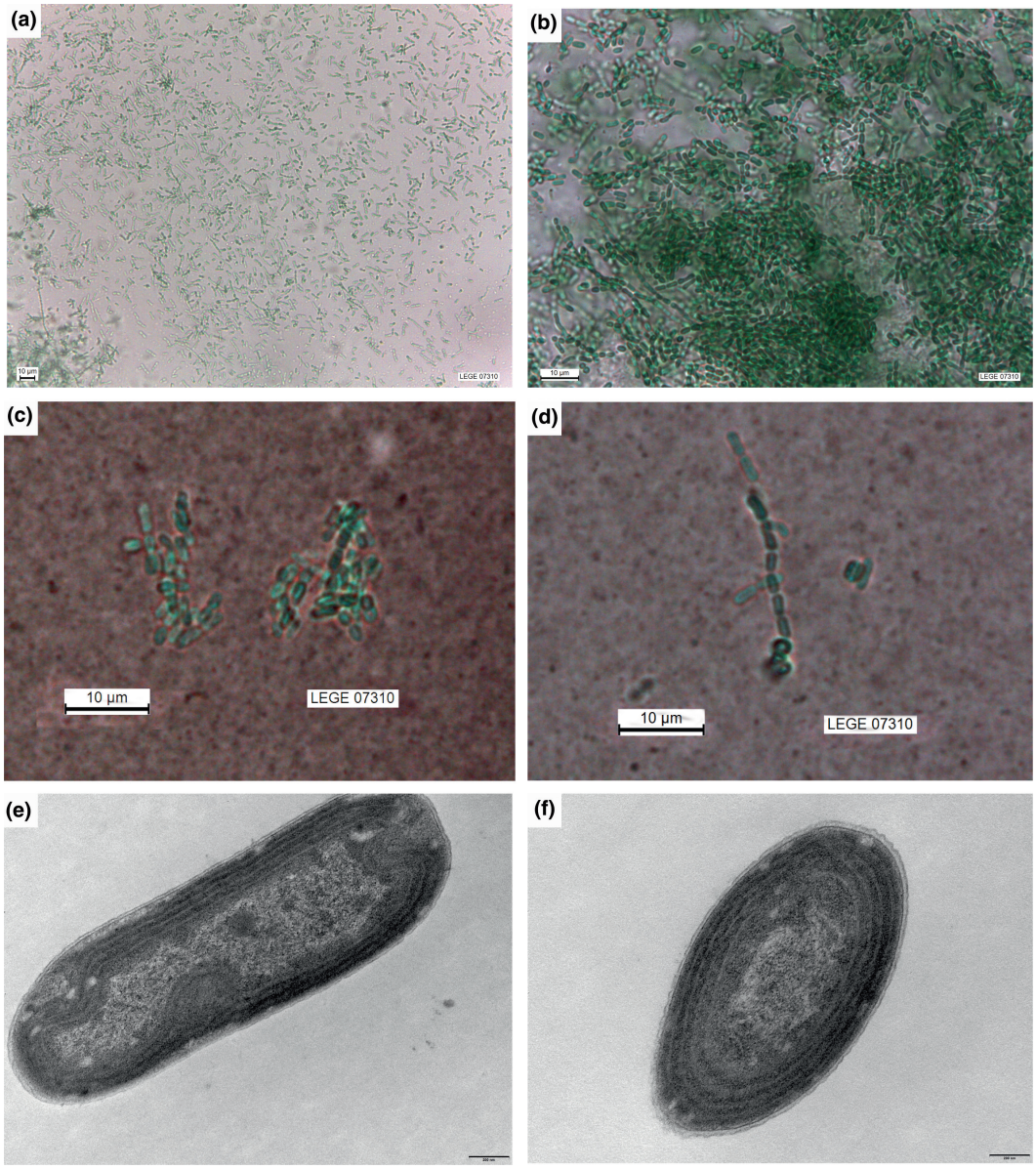

FIGURE 5 *Vasconcelosia minhoensis* gen. et sp. nov. LEGE 07310 (Type strain) (a–c) General overview of trichomes arrangement. (d) Details of trichomes without mucilage, (e, f) Ultrastructure (TEM). [Color figure can be viewed at wileyonlinelibrary.com]

Although there is a close phylogenetic relationship with *Limnococcus*, the 16S rRNA gene similarity (*p*-distance) between *Pseudolimnococcus* gen. nov. and the *Limnococcus* reference strain *L. limneticus* Svet06 (GQ365048; Komárková et al., 2010) is only 93.1% (Table S1 in the Supporting Information). The similarity between *Pseudolimnococcus* gen. nov. and the other strains of the *Limnococcus* clade reaches a maximum of only 94.7%. These findings indicate that *Pseudolimnococcus* gen. nov. is a different taxon from *Limnococcus*.

Morphologically, *Pseudolimnococcus* gen. nov. is a coccoid genus characterized by the formation of packet-like colonies, with subspherical or hemispherical cells after division. The division is in three planes, and the cells do not reach the original spherical shape before the next division. The mucilaginous envelope is firm and conspicuous without staining. The habit is planktonic in freshwater. These characters make *Pseudolimnococcus* gen. nov. similar to *Limnococcus*, *Choroccoccus*, *Neochorococcus*, *Inacoccus*, and *Cryptococcum*. The differences from those genera are shown in Table 1 and noted in the "Discussion" section. Primarily, *Pseudolimnococcus* gen. nov. differs from *Limnococcus*, the phylogenetically closest related genus, due to the lack of aerotopes and firm envelope in the latter. Moreover, *Pseudolimnococcus* gen. nov. cells don't reach the original spherical shape before the next division, while in *Limnococcus* the cells reach the original shape.

For *Pseudolimnococcus* gen. nov., the 16S-23S ITS rRNA regions D1–D1', V2, and V3 are present, but the

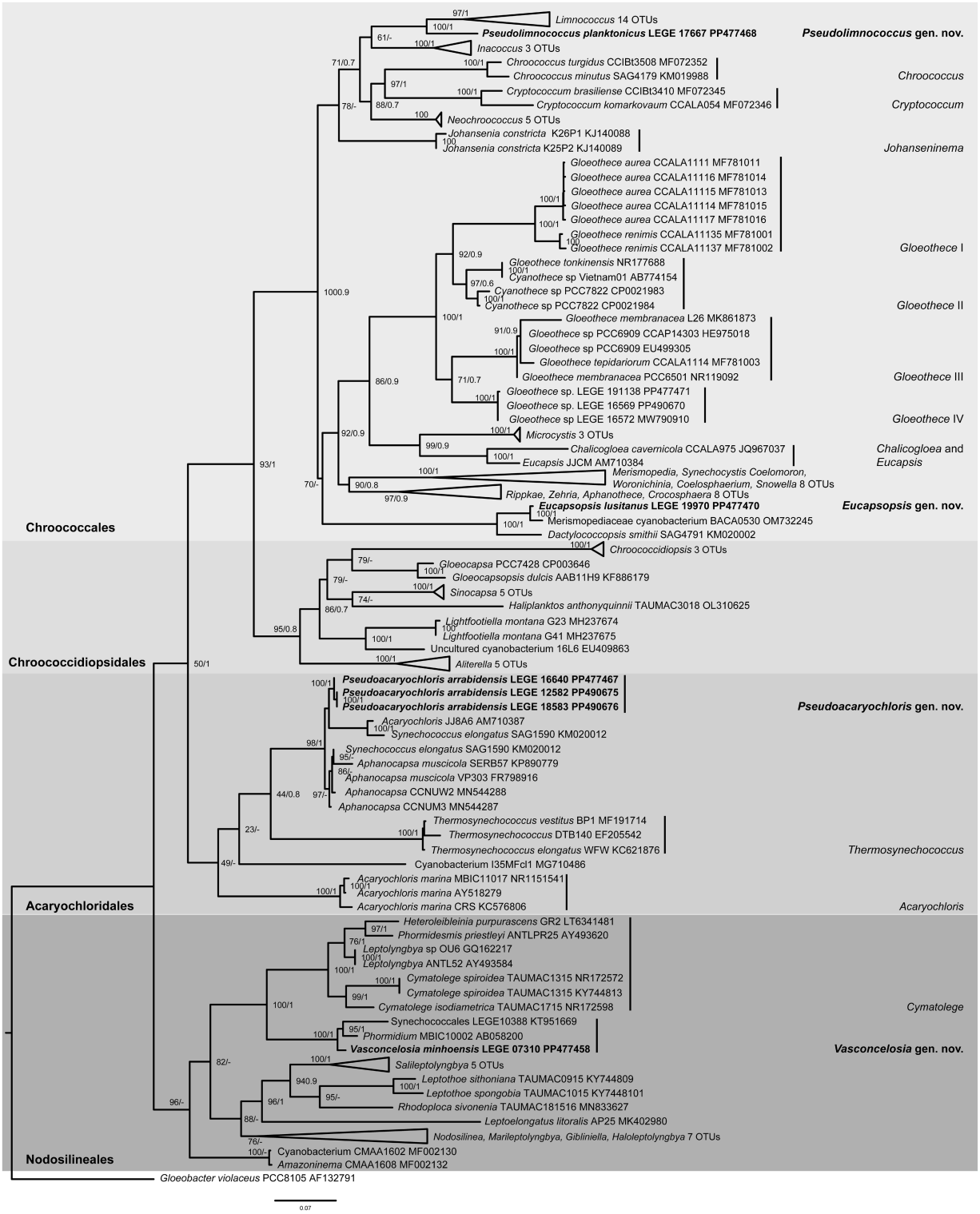

FIGURE 6 Second-round analysis. 16S rRNA gene ML tree. The bootstrap values and the BI posterior probabilities are, respectively, indicated at the nodes. The newly described taxa are in bold.

Box B is lacking (Figures 7–10). The $tRNA^{Ile}$ is complete, but the $tRNA^{Ala}$ is very short, with only 29 nucleotides. For *Limnococcus* the $tRNA^{Ala}$ and the V2 helix are lacking, but Box B is present. The complete ITS rRNA region sequences are shown in Table S2 and the presence or absence of the helices are shown in Table S3, both in the Supporting Information. The D1–D1' helix of *Pseudolimnococcus* gen. nov. basal stem starts with 5′GACC3′ and is characterized by the presence of a stem originating from the 5′ side of the middle loop (Figure 7). For *Limnococcus*, the D1–D1′ basal stem is composed by 5′CCC3′, and the stem in

TABLE 1 Morphological and habitat comparisons between *Pseudolimnococcus* gen. nov., *Eucapsopsis* gen. nov. and their morphologically and phylogenetically closest related genera in the order Chroococcales.

Family	Genus	Colonies	Envelopes	Cell shape	Cell content	Cell dimensions (μm)	Habitat
Cyanotricaceae	*Pseudolimnococcus* gen. nov. LEGE 17667	Irregular. Cells irregularly arranged. 2–4 celled packet-like subcolonies	Firm. Delineating cells margins	Spherical or hemispherical after division. Do not reach original shape before next division	Granulose, dark green. with aerotopes	13.6–16.8×10.2–11.89–13.6	Freshwater
Cyanotricaceae	*Limnococcus* (Komárková et al., 2010)	Irregular. Cells irregularly arranged	Diffluent	Cells spherical, after division subspherical. Reach original shape before next division	Without aerotopes	Up to 22 diameter	Freshwater
Microcystaceae	*Eucapsopsis* gen. nov. LEGE 19970	Polygonal, flattened or three dimensional. Cells in cubic subcolonies, not densely arranged in rows. Subcolonies sometimes in rows	Diffluent. Invisible without staining. Cells with individual envelopes	Spherical or hemispherical in pairs after division. Reach original shape before next division	Light green, with aerotopes	4.6–5.2 diameter	Freshwater
Microcystaceae	*Eucapsis* (Komárek & Anagnostidis, 1999)	Cubic. Cells arranged in three-dimensional perpendicular rows	Delimited, rarely diffluent. Individual envelopes facultative	Spherical or widely oval. Reach original form before next division	Without aerotopes	2–11 diameter	Freshwater, terrestrial
Microcystaceae	*Zehria* (Mares et al., 2019)	Not forming colonies. Cells solitary or in pairs	Absent	Short bacilloid to spherical	Without aerotopes	2–8 (1–2 times as long as wide)	Marine
Microcystaceae	*Rippkaea* (Mares et al., 2019)	Not forming colonies. Cells solitary	Absent	Broadly oval to cylindrical. Rounded ends	Homogenous or finely granular. Without aerotopes	4–9×3–5	Soil of rice field
Microcystaceae	*Aphanothece* (Komárek & Anagnostidis, 1999)	Spherical or amorphous. Cells arranged irregularly	Diffluent or firm. Cells with individual envelopes	Widely oval to cylindrical (rod-shaped) with rounded ends	Facultative aerotopes	1–2.8×0.8–1.6 (2 times as long as wide)	Freshwater
Microcystaceae	*Crocosphaera* (Mares et al., 2019)	Not forming colonies. Cells solitary or in pairs	Large diffluent mucilage	Spherical to elongate	Without aerotopes	2.5–6.7 diameter	Marine
Chroococcaceae	*Chroococcus* (Komárek & Anagnostidis, 1999)	Irregular or more or less spherical. Cells never in rows or regular arrangements. 2–8 celled packet-like subcolonies	Firm or diffluent. Usually concentrically lamellate	Cells at first subsphaerical, widely oval or spherical, later hemispherical or in the form of a segment of sphere. do not reach original shape before next division	Without aerotopes	3–50 diameter	Terrestrial, freshwater
Chroococcaceae	*Neochroococcus* (Geng et al., 2021)	Cubic colonies with 8–16 cells, sometimes aggregated in large colonies	Firm. Cells without individual envelopes	Spherical or hemispherical after division	Without aerotopes	8.4–12.3 diameter	Freshwater
Chroococcaceae	*Inacoccus* (Gama et al., 2019)	Cells solitary or rare in few-celled colonies	Firm. Delineating cells margins	Spherical or hemispherical after division	Without aerotopes	4.7–11.9 diameter	Terrestrial
Chroococcaceae	*Cryptococcum* (Gama et al., 2019)	Cells solitary or rare in few celled colonies	Firm. Delineating cells margins	Spherical or hemispherical after division	Without aerotopes	8.3–15. diameter	Terrestrial, thermal springs
Pleurocapsaceae	*Chondrocystis* (Komárek & Anagnostidis, 1999)	Irregular or irregular-polygonal, packet-like, composed by subcolonies. Cells irregularly arranged	Subcolonies and cells with individual delimited envelope	Spherical	Without aerotopes	1.5–6 diameter	Terrestrial, saline water

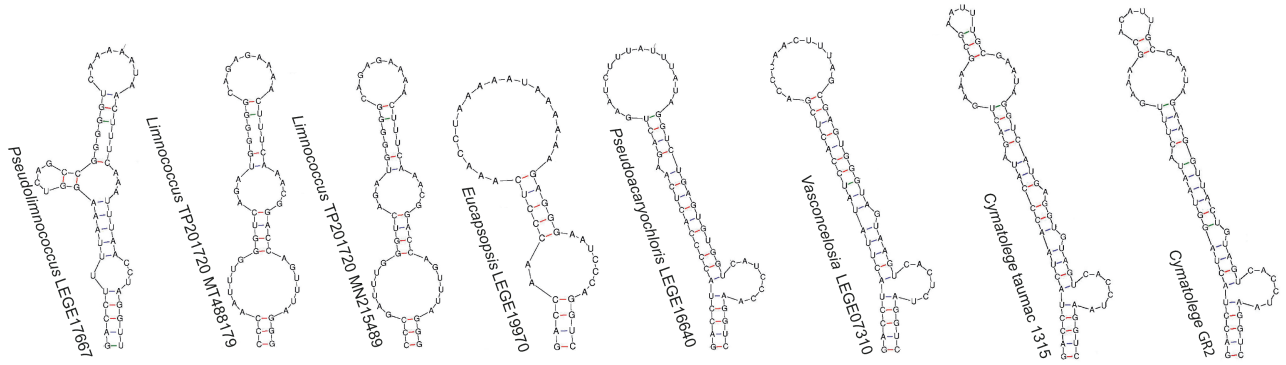

FIGURE 7 ITS rRNA region D1–D1' helices of the newly described genera and their closest related taxa. [Color figure can be viewed at wileyonlinelibrary.com]

the middle loop is lacking. The V3 helices are also very different in length and sequence between both genera (Figure 10, Table S2).

Eucapsopsis lusitanus gen. et sp. nov. (LEGE 19970) is positioned at the base of a big cluster containing Microcystaceae (Chroococcales) genera, such as *Gloeothece*, *Microcystis*, *Chalicogloea* Roldán et al., *Eucapsis* Clements & Shantz, *Snowella* Elenkin, *Coelosphaerium* Lemmerman, *Woronichinia* Elenkin, *Coelomoron* Buell, *Merismopedia* Meyen, *Synechocystis* Sauvageau, *Rippkaea* Johansen & Mares, *Zehria* Johansen & Mares, *Aphanothece* Nageli and *Crocosphaera* Zehr et al. This cluster is sister to *Neochroococcus*, *Chroococcus*, *Cryptococcum*, *Inacoccus*, *Pseudolimnococcus* gen. nov. and *Limnococcus*. The type strain LEGE 19970 is not closely related to those genera and is clustered with "Merimopediaceae" BACA0530 and the strain SAG 47.91, which is identified as "*Dactylococopsis smithii*" (Figure 6). The 16S rRNA gene similarity analysis (Table S4 in the Supporting Information) confirms the phylogenetic results, separating *Eucapsopsis* gen. nov. from the other cyanobacterial genera. Comparing *Eucapsopsis* gen. nov. with the other clades, these values from range from 84.9% to a maximum of only 91.4% (vs. *Zehria*).

Morphologically, *Eucapsopsis* gen. nov. is characterized by polygonal colonies, flattened (young) or three dimensional, forming by cubic subcolonies, often organized in rows. The cells within the subcolonies are also organized in rows. Cells are spherical and hemispherical after the division, with aerotopes and individual mucilaginous envelopes. The divisions are in three planes and the cells reach the original spherical shape before the next divisions. The habitat is planktonic in freshwater. These characters make *Eucapsopsis* gen. nov. similar to *Eucapsis*, *Chondrocystis*, *Cryptococcum*, *Inacoccus*, *Neochroococcus*, *Limnococcus*, and *Chroococcus*. The differences among those genera are summarized in Table 1 and discussed in the "Discussion" section.

Regarding the ITS rRNA region secondary structures, we could retrieve the D1–D1' helix and the $tRNA^{Ile}$ regions. The $tRNA^{Ala}$ and the next regions were not present in the sequencing results. The D1–D1' helix is short (45 bases) and has two residues opposing the first lateral bulge and a large terminal loop (Figure 7). These characters are unique among the taxa studied by us and also support the separation of *Eucapsopsis* gen. nov. as a new cyanobacterial genus.

Pseudoacaryochlorys arrabidensis gen. et sp. nov. (LEGE 16640) is in a monophyletic strongly supported clade (ML = 98, BI = 1) with 10 strains, positioned within the Acaryochloridales cluster. It is a sister clade of *Thermosynechococcus* but with low phylogenetic support (ML = 44, BI = 0.8; Figure 6). These results suggest that *Pseudoacaryochloris* gen. nov. should be described as a new cyanobacterial genus. At the base of the Acaryochloridales is the type species *Acaryochloris marina* Miyashita & Chihara. The 16S rRNA gene similarity matrix shows *Pseudoacaryochloris* gen. nov. intrageneric values ranging from 97.5% to 100%. The values comparing *Pseudoacaryochloris* gen. nov. to *Thermosynechococcus* and *Acaryochloris*, the genera, range from 92.8% to 94.2% and from 92.8% to 94.1%, respectively. The similarity between *Thermosynechococcus* and *Acaryochloris* ranges between 90.5% and 91.2% (Table S5 in the Supporting Information).

Morphologically, *Pseudoacaryochloris* gen. nov. is different from its phylogenetically closest related genus, *Thermosynechococus*. *Pseudoacaryochloris* gen. nov. has spherical or subspherical cells, while *Thermosynechococcus* has oval or cylindrical cells that can be straight or curved. *Pseudoacaryochloris* gen. nov. is a freshwater genus, while *Thermosynechococcus* is from thermal and saline environments (Table 2). *Pseudoacaryochloris* gen. nov. morphologically differs from *Acaryochloris* by the lack of mucilaginous envelope in *Pseudoacaryochloris*. These genera are phylogenetically distant and have low 16S rRNA gene similarity (92.8%–94.2%). Furthermore, *Acaryochloris* is marine and an Ascidian symbiont genus.

We sequenced the complete ITS rRNA region of LEGE 16640, which contains both tRNAs, along with

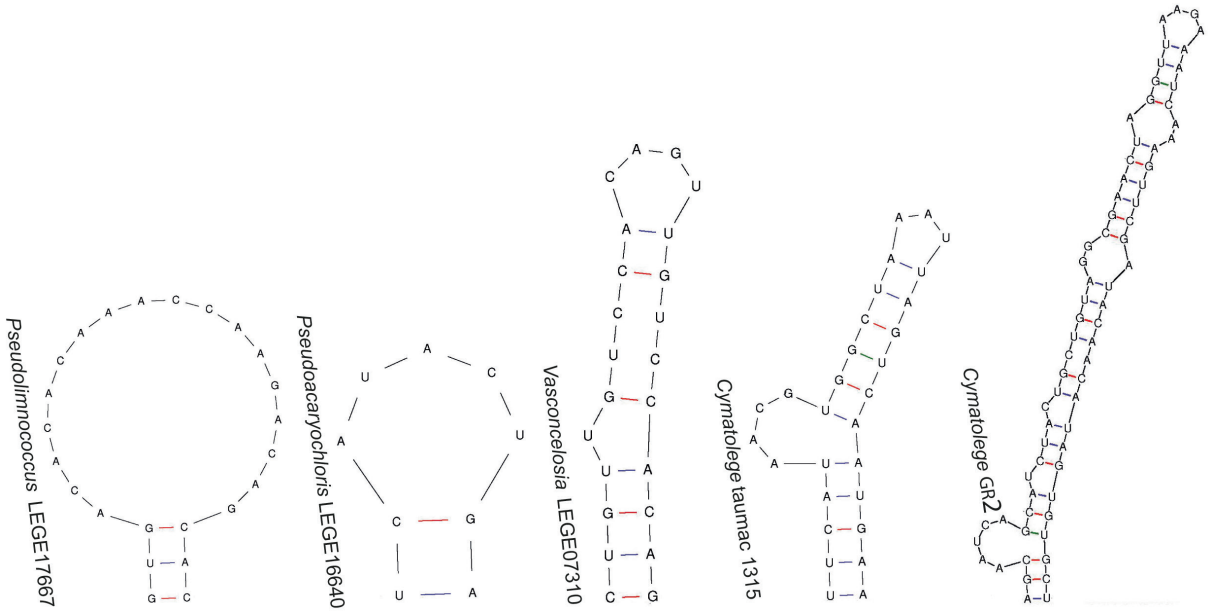

FIGURE 8 ITS rRNA region V2 helices of the newly described genera and their closest related taxa. [Color figure can be viewed at wileyonlinelibrary.com]

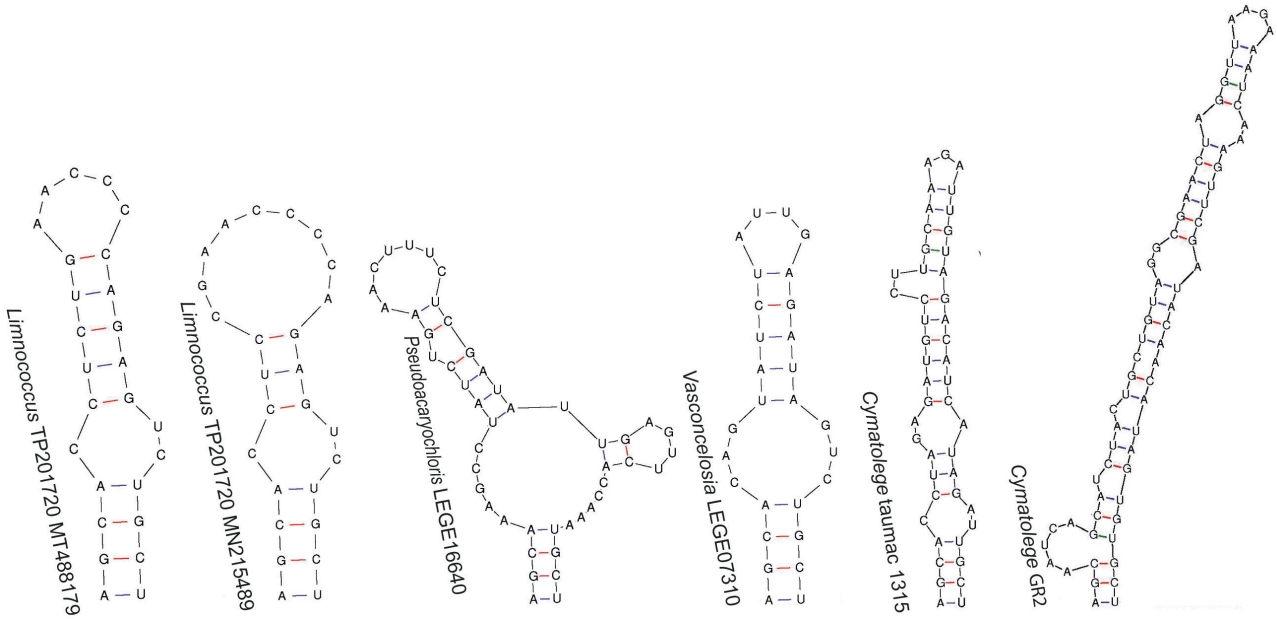

FIGURE 9 ITS rRNA region Box B helices of the newly described genera and their closest related taxa. [Color figure can be viewed at wileyonlinelibrary.com]

the D1–D1', V2, Box B, and V3 helices (Figures 7–10). The LEGE 16640 D1–D1' helix is characterized by the absence of residues opposing the first lateral bulge and the absence of a loop in the middle stem. The Box B helix is characterized by the presence of a stem in the 3′ side of the middle loop. The V2 has a very short stem compared to the other analyzed genera, only two base pairs long. The V3 is very long compared to the other genera. We could not compare these helices with *Thermosynechococcus*, the phylogenetically closest related genus, because there are no available 16S ITS rRNA region data for this genus. Nevertheless, the ITS rRNA region helices of LEGE 16640 are different in sequence, length, and structure from the other studied genera, supporting the separation of *Pseudoacaryochloris* gen. nov. as a new genus.

Vasconcelosia minhoensis gen. et sp. nov. is positioned at the base of our phylogeny (Figure 6) within the Nodosilineales clade. The Type strain LEGE 07310 forms a strongly supported separated monophyletic clade along with "*Phormidium*" MBIC10002 and "Synechococcales" LEGE 10388 (ML = 100, BI = 1

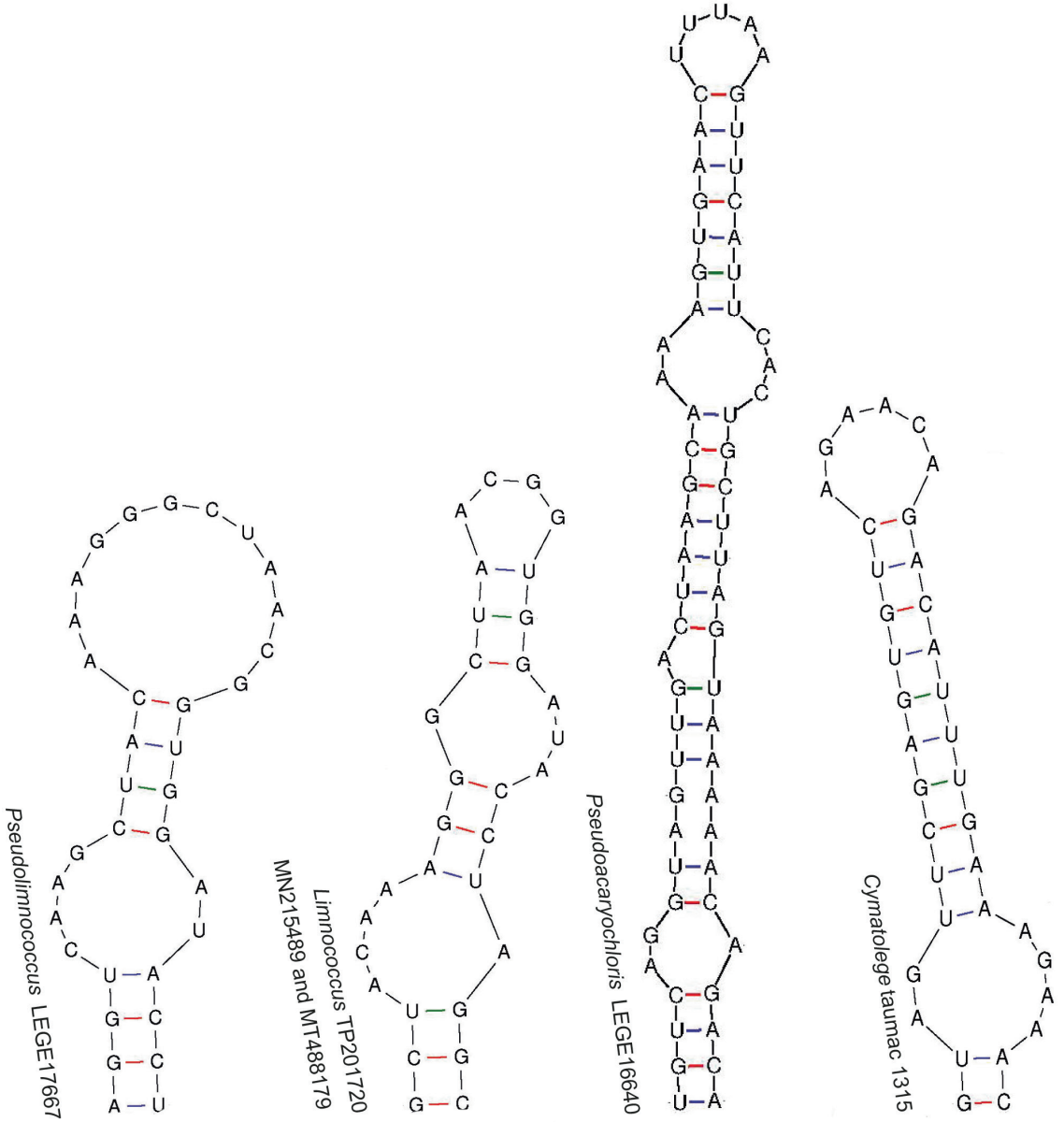

FIGURE 10 16S-23S ITS rRNA region V3 helices of the newly described genera and their closest related taxa. [Color figure can be viewed at wileyonlinelibrary.com]

and ML = 100, BI = 1, respectively), which is sister to *Cymatolege* (ML = 100, BI = 1; Cymatolegaceae). This whole cluster is sister to *Salileptolyngbya*, *Leptothoe*, *Rhodoploca*, *Leptoelongatus*, *Haloleptolyngbya*, *Nodosilinea*, *Marileptolyngbya*, and *Gibliniella*. The 16S rRNA gene similarity matrix (Table S6 in the Supporting Information) shows that the *Vasconcelosia* gen. nov. intrageneric similarity values range from 97.3% to 98.8%. Comparing *Vasconcelosia* gen. nov. to the Phylogenetically closest related genus *Cymatolege*, the similarity values range from 92.1% to 94.3%.

Morphologically, *Vasconcelosia* gen. nov. is characterized by primarily cylindrical cells that can be commonly solitary, in pairs, or sometimes forming short trichomes with a maximum of eight cells. The trichomes easily disintegrate, due to the weak cell connections. The mucilage envelope is lacking. The habitat is benthic in estuary waters. These morphological and ecological characters of *Vasconcelosia* gen. nov. are similar to *Romeriopsis* and *Romeria* (Table 3) and are discussed in the "Discussion" section.

The LEGE 07310 ITS rRNA region has both tRNA^{Ile} and tRNA^{Ala}, the D1–D1', V2, and Box B helices (Figures 7–9). The V3 helix could not be retrieved. The D1–D1' helix is characterized by the lack of any residues opposing the first lateral bulge and by the lack of any loop in the middle stem (Figure 7). This structure is similar to very distant genera, such as *Pseudoacaryochloris* gen. nov. and *Pseudolimnococcus* gen. nov. For *Cymatolege*, the *Vasconcelosia* gen. nov. phylogenetically closest related genus, the D1–D1' helix has a middle loop positioned very close to the terminal loop, being different

TABLE 2 Morphological and habitats comparisons between *Pseudoacaryochloris* gen. nov. and its morphologically and phylogenetically closest related genera.

Family	Genus	Colonies	Envelopes	Cell shape	Cell content	Cell dimensions (μm)	Habitat
Thermosynechococcaceae	*Pseudoacaryochloris* LEGE 16640	Absent. Cells sparsely or densely distributed in the culture	Absent	Spherical or subspherical	Homogenous, without aerotopes	2.44–2.9 diameter	Freshwater, planktonic
Thermosynechococcaceae	*Thermosynechococcus* (Komárek et al., 2020)	Absent. Forming irregular groups. Sometimes filamentous-like formations	Absent	Oval. Cylindrical, straight or curved. Rounded or truncate ends	Homogenous	(3.0) 4.0–17.0 (32.0) × 1.1–3.0 (4.8)	Thermal localities, periphytic or benthic
Acaryochloridaceae	*Acaryochloris* (Miyashita et al., 2003)	Absent	Present	Spherical or ellipsoidal	Without aerotopes	1.0–3.0 diameter	Marine, sponge symbiont

in structure and sequence from *Vasconcelosia* gen. nov. The Box B and V2 helices of *Vasconcelosia* gen. nov. are also different in length, sequence, and structure from *Cymatolege* (Figures 8 and 9). The polyphasic approach comprising phylogenetic, similarity, and morphological analysis, along with the ITS rRNA region secondary structures, are in agreement, separating *Vasconcelosia* gen. nov. as a new cyanobacterial genus within the family Cymatolegaceae (Nodosilineales).

DISCUSSION

Culture collections such as Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC), contain valuable isolates for phycological research, but many of the available cyanobacterial strains have not been characterized with a polyphasic approach. Here, we used a polyphasic approach to screen the taxonomic designations for cyanobacterial isolates in the LEGE-CC. The first taxon described in this paper is *Pseudolimnococcus planktonicus* gen. et sp. nov. (LEGE 17667). Although there is a close phylogenetic relation with *Limnococcus*, *Pseudolimnococcus* gen. nov. is morphologically clearly different from that genus. *Pseudolimnococcus* gen. nov. has aerotopes and a firm mucilaginous envelope, and the cells do not reach the original spherical shape before the next division. *Limnococcus* lacks aerotopes and has diffluent mucilage; the cells reach the original spherical shape before the next division (Table 1).

Other important evidence separating*Pseudolimnococcus* gen. nov. from *Limnococcus* includes the low 16S rRNA gene similarity between both genera, which ranges from 93.1% to 94.7%. These values are lower than or very close to the minimum threshold of 94.5% similarity within a genus stated by Yarza et al. (2014) Phylogenetically closest related. Strains with less than 94.5% of 16S rRNA gene similiarity should not belong to the same genus. Similar values were observed when comparing *Pseudolimnococcus* gen. nov. with phylogenetically more distant genera, such as *Neochroococcus* (93.2%–93.4%) and *Johanseninema*, which reached almost 93% of similarity with *Pseudolimnococcus* gen. nov. (Table S1). Another finding that corroborates the separation of *Pseudolimnococcus* gen. nov. from *Limnococcus* is that the 16S rRNA gene similarity between both genera was lower than the values determined when comparing other well-established and very different genera. For instance, *Neochroococcus* and *Inacoccus* have 94.3% 16S rRNA gene similarity between them, while the similarity between *Pseudolimnococcus* gen. nov. and *L. limneticus* Svet06 was only 93.1%.

The ITS rRNA region secondary structures support the separation of *Pseudolimnococcus* gen. nov. and *Limnococcus*. For *Pseudolimnococcus* gen. nov. in the D1–D1' helix, the presence of a middle stem originating

TABLE 3 Morphological and habitats comparisons between *Vasconcelosia* gen. nov. and its morphologically and phylogenetically closest related genera.

Family	Genus	Thallus	Trichome	Cells shape	Envelope	Cell dimensions (μm)	Habitat
Cymatolegaceae	*Vasconcelosia* gen. nov. LEGE 07310	Commonly isolate cells, or in pairs. Sometimes forming short trichomes (easily disintegrating)	1–8 cells (Exceptionally > 20). Irregular with cells very loosely attached. Constricted	Cylindrical or barrel-shaped. Rounded at the apices	Absent	1.5–2.5 × 0.8–1.2	Estuary (brackish water), benthic
Cymatolegaceae	*Cymatolege* (Konstantinou et al., 2021)	Trichomes in sense irregular clusters	Straight, curved or more or less coiled. Cylindrical, slightly constricted, attenuated or not. Occasional false branching	Isodiametric, slightly shorter than wide. Apical cells rounded	Sheaths firm, thin, lamellated containing one or few trichomes	1.3–2.5 wide	Marine or freshwater. Benthic, epizoic, epiphyllic or epilithic
Cymatolegaceae	*Romeria leopoliensis* (Komárek & Anagnostidis, 2005)	Trichomes solitary, rarely isolated cells	Up to 8 cells. Constricted at cross-walls. Slightly curved or sigmoid	Cylindrical	Fine, diffluent	1–12 × 0.6–3	Freshwater. Planktonic
Cymatolegaceae	*Rhabdogloea* (Komárek & Anagnostidis, 1999)	Coccoid. Oriented in one direction in the colony	Absent	Elongate, ellipsoidal or cylindrical with acute ends or at least attenuated	Fine. Diffluent	2.3–20 × 0.8–5	Freshwater, brackish water (endogloeic) or terrestrial
Cymatolegaceae	*Rhabdoderma* (Komárek & Anagnostidis, 1999)	Coccoid, occasionally forming pseudofilament rows. Orientated in one direction in the colony	Pseudofilaments	Cylindrical. Straight, arcuate, or sigmoid with rounded ends	Fine, diffluent	4–12 × 1–3.5	Freshwater. Planktonic, metaphytic or terrestrial
Leptolyngbyaceae	*Romeriopsis* (Hentschke et al., 2022)	Trichomes in irregular clusters	Usually few-celled or more rarely >50 cells. Constricted	Cylindrical or barrel-shaped	Absent	1.6–3 × 1.5–2	Marine. On rocky substrates at 13m depth or intertidal

from the middle loop is very particular among cyanobacteria. Comparing both genera, all helices were very different in length and structure. Furthermore, the D1–D1' helix of *Limnococcus* does not have the stem originating from the middle loop. *Pseudolimnococcus* gen. nov. has a short $tRNA^{Ala}$ sequence, with only 29 nucleotides (Table S2), instead of the typical ~73 nucleotides (Lukesova et al., 2009), and the lack of the Box B needs to be studied more. Additional strains within this clade must be sequenced to confirm if these findings are stable for the genus. We could not compare *Pseudolimnococcus* gen. nov. with the reference *L. limneticus* Svet06, because there is no ITS rRNA region data for this strain.

Pseudolimnococcus gen. nov. is morphologically similar to *Chroococcus*, *Neochroococcus*, *Inacoccus*, and *Cryptococcum*. All had three planes of cell division and firm envelopes, and the cells did not reach the original shape before next divisions. However, *Pseudolimnococcus* differed from these genera by the presence of aerotopes, whereas these structures are lacking in the others. In our analyses, these genera all proved to be distinct monophyletic taxa and are currently considered distinct genera, as already demonstrated in previous studies (Gama et al., 2019; Geng et al., 2021). *Pseudolimnococcus* gen. nov. is phylogenetically distant from all of them, with low 16S rRNA gene similarity values, ranging from a minimum of 90.6% (vs. *Chroococcus*) to a maximum of 93.5% (vs. *Inacoccus*). In sum, this polyphasic characterization suggests separating *Pseudolimnococcus* gen. nov. as a new cyanobacterial genus within the family Cyanotrichaceae (Chroococcales).

Another genus described in this paper is *Eucapsopsis* (LEGE 19970). The phylogenetic position of *Eucapsopsis* gen. nov. included this genus in the family Microcystaceae (Chroococcales). The *Eucapsopsis* gen. nov. clade is divergent from all the other cyanobacterial genera that have been validated by molecular analysis, supporting taxonomic separation. The strain identified as "*Dactylococcopsis smithii*" SAG 47.91 was observed within the *Eucapsopsis* gen. nov. clade. The species *D. smithii* is currently accepted as a synonym for *Rhabdogloea smithii*, but this latter genus has not been validated by molecular analysis (Komárek & Anagnostidis, 1999). *Rhabdogloea* is a coccoid morphotype with elongate cells, which are attenuated at the apices, dividing in two planes and oriented in one direction within their colonies (Table 1). These characters make the genus distinct from *Eucapsopsis* gen. nov. Moreover, there are no published ecological or morphological data from SAG 47.91, and because of that, we could not compare it to LEGE 19970. However, LEGE 19970 and SAG 47.91 are phylogenetic strongly related and share 97.3% 16S rRNA gene similarity. We cannot explain why or how such morphologically different genera could be in the same clade sharing high 16S gene similarity, but surely, *Eucapsopsis* cannot be considered under *Rhabdogloea* circumscription due to the remarkable morphological differences. We believe that SAG 47.91 is misidentified. Accordingly, we describe *Eucapsopsis* gen. nov. as encompassing the strains LEGE 19970 and BACA0530. The taxonomic status of SAG 47.91 remains unclear and needs to be revised.

The type strain *Eucapsopsis* LEGE 19970 is morphologically similar to *Eucapsis* but may be differentiated from it via its the presence of aerotopes and a diffluent envelope, while *Eucapsis* lacks aerotopes and has firm mucilaginous envelopes, rarely diffluent (Table 1). Moreover, these genera are phylogenetically distant from each other and share only 90.68% 16S rRNA gene similarity. *Eucapsospsis* gen. nov. differs from *Chrooccocus*, *Neochroococcus*, *Limnococcus*, *Inacoccus*, and *Cryptococcum*; it has cubic subcolonies with cells organized in rows. It also differs from these genera and *Chondrocystis* by having a diffluent mucilaginous envelope (Table 1).

The 16S rRNA gene similarity analysis corroborates the phylogenetic and morphological results, separating *Eucapsopsis* as a new genus. The maximum similarity between LEGE 19970 and any other cyanobacterial genera in our phylogenies is to *Zehria* (91.4%). TheIT rRNA region secondary structure of the D1–D1' helix is remarkably shorter and different from the Chroococcales genera included in our analysis (Figure 7). The lack of the other ITS rRNA region helices does not affect the comparisons with the other genera because the most conserved helix (D1–D1'; Lukesova et al., 2009) supports the separation with *Eucapsopsis* gen. nov. In brief, the aspects of the polyphasic approach comprising phylogenetic, sequence similarity, ITS rRNA region secondary structures, and morphological analyses, are in agreement and support *Eucapsopsis* gen. nov. as a new cyanobacterial genus within the order Chroococcales.

The phylogenetic analysis of *Pseudoacaryochloris* gen. nov. (LEGE 16640) (Figure 6), indicates that the genus is in the Thermosynechococcaceae family. The support values between *Pseudoacaryochloris* gen. nov. and *Thermosynechococcus*, the closet genus, are only 44 for ML and 0.8 for BI. The 16S rRNA gene similarity analysis corroborates the separation of *Pseudoacaryochloris* gen. nov., considering that the genus has similarity values lower than 94.5% (Yarza et al., 2014) with all the other cyanobacterial genera examined here. Morphologically, *Pseudoacaryochloris* gen. nov. is different from *Thermosynechococcus* (Table 2), but it is a very simple unicellular genus, and thus it is not possible to differentiate it from *Acaryochloris*. However, *Pseudoacaryochloris* gen. nov. is planktonic in freshwater, and *Acaryochloris* is marine (endosymbiont in Ascidians), so the phylogenetic distance, the 16S rRNA gene similarity analysis,

and the very different habitats of the two genera clearly separate them into two distinct taxa.

The ITS rRNA region analysis showed that *Pseudoacaryochloris* gen. nov. has different helices compared to the other cyanobacterial genera, supporting the description of this new genus. Although the D1–D1' helix of *Pseudoacaryochloris* gen. nov. is similar to *Vasconcelosia* gen. nov., these genera are in different orders and are also morphologically very different. This fact that different taxa, in different orders, showed similar D1–D1' helices is interesting and raises some questions about the employment of this analysis in the description of new genera. Although secondary structures are employed in this paper and we agree that they can aggregate information, it is important to note that comparisons involving secondary structures do not rely on statistical methods, unlike phylogenetic and similarity analyses, which use statistical algorithms. In our opinion, it makes the analysis subjective, and this leads to results that might be biased to agree with the phylogenies. The questions "How much secondary structures can vary within a genus? How different must they be to separate two genera? Is it possible for secondary structures to not be in agreement with the phylogenetic and similarity results?" have no answer. To answer them, a detailed revision must be performed to check whether the ITS rRNA region secondary structures are really important for taxonomy. If the questions above have no answer, secondary structures should be not mandatory for publishing new genera. Moreover, it is possible that different operons from the same strain may have different ITS rRNA region secondary structures, complicating comparisons with other strains. The very similar D1–D1' helices between *Pseudoacaryochloris* gen. nov. and *Vasconcelosia* gen. nov. may set a precedent for that, and this observation also highlights the need to review the inclusion of ITS rRNA region secondary structures in polyphasic analyses. Nevertheless, the polyphasic approach here resulted in all the evaluation metrics being in agreement, separating *Pseudoacaryochloris* gen. nov. as a new cyanobacterial genus within the order Acaryochloridales.

Within the *Pseudoacaryochloris* gen. nov. clade, strains 5 N04 and VP303, identified as "*Aphanocapsa*," are also from freshwater artificial fountains in Europe (Cuzman et al., 2010) but can be considered different species from *P. arrabidensis* sp. nov., due to the phylogenetic separation. The strains CCNUW2 and CCNUM3 are from a humid Chinese forest and may also be different species from *P. arrabidensis* sp. nov. (Zhang et al., 2019). The strains LEGE 12582 and LEGE 18583 have 99.9% 16S rRNA gene similarity with the type LEGE 16640 and are within the limits of *P. arrabidensis* sp. nov. These strains are also from Portugal, but the precise location of sampling is unknown.

The last genus described in this paper is *Vasconcelosia* gen. nov. (LEGE 07310). Although there is a close phylogenetic relation with *Cymatolege*, the polyphasic analyses showed that strain LEGE 07310 in a monophyletic clade, confirming a separate cyanobacterial genus. The 16S rRNA gene similarity analysis showed only 92.1%–94.3% similarity between both genera. These values are lower than the minimum threshold of 94.5% within a genus, as stated by Yarza et al. (2014). When comparing *Vasconcelosia* gen. nov. with any other cyanobacterial genus herein, the values never reached 94.5%. The phylogenetic position of LEGE 07310 among Cymatolegaceae was previously confirmed by Strunecký et al. (2023). The secondary structures corroborate these results. All LEGE 07310 helices were very different in sequence, length, and structure from the other genera compared here (Figures 7–10). However, it is worth noting that the very long Box B in *Cymatolege* is not common for cyanobacteria. The lack of V3 did not affect the analysis, considering the remarkable differences between the other helices.

Morphologically and ecologically, *Vasconcelosia* gen. nov. is different from the closest genus (*Cymatolege*) in the phylogenetic analysis. *Vasconcelosia* gen. nov. is a benthic genus from the Minho River estuary in Portugal. It has solitary cells, of very short trichomes, in which the cells are very loosely attached to each other and are easily separated by the fragmentation of the trichome. The trichomes are markedly constricted, and the mucilaginous envelope is lacking. In contrast, *Cymatolege* is a false-branched filamentous genus, with long trichomes. The trichomes are slightly constricted, and the cells are strongly attached to each other and not easily fragmented. The sheaths of *Cymatolege* are firm. It is typically from marine habitats (sponge-associated or epilithic) or isolated from Antarctic lakes and Brazilian mangroves (Konstantinou et al., 2021). *Vasconcelosia* gen. nov. is morphologically very similar to *Romeriopsis*, but this latter genus does not commonly have isolated cells, or cells in pairs, and sometimes has longer trichomes (>50 cells). Moreover, *Romeriopsis* is within the family Leptolyngbyaceae, phylogenetically closely related to *Alkalinema* (Hentschke et al., 2022). The common presence of isolate cells in *Vasconcelosia* gen. nov. resembles the coccoid genera *Rhabdogloea* and *Rhabdoderma*, but those genera differ from *Vasconcelosia* gen. nov. by the organization of the cells in one direction in the colony and the very rare formation of pseudofilaments in *Rhabdoderma*. *Rhabdogloea* never form pseudofilaments.

Vasconcelosia gen. nov. is similar to *Romeria leopoliensis*, the lectotype of the genus, but they differ in many aspects. *Vasconcelosia* gen. nov. has a marine origin (river estuary), is benthic, has the common presence of isolate cells (or cells in pairs), and does not have a mucilaginous envelope. The cells length reaches a maximum

2.5 μm. These characteristics differ from *Romeria leopoliensis*, which is planktonic, from freshwater, rarely has isolate cells, and has a diffluent mucilaginous envelope. The cells lengths vary from 3 to 6 μm, larger than *Vasconcelosia* gen. nov. These differences between *Vasconcelosia* gen. nov. (LEGE 07310) and *Romeria* were previously reported by Strunecký et al. (2023), and these authors also commented on the important morphological discrepancy between them. Currently, there is no 16S rRNA gene data for *Romeria*, and because of that, we could not include it in our phylogenies. However, according to our morphological and ecological analysis, LEGE 07310 cannot be identified with *Romeria*, and consequently, we describe here the new genus *Vasconcelosia* gen. nov. The phylogenetic status and morphological circumscription of *Romeria* will remain unclear until *R. leopoliensis* is isolated and sequenced.

As described above, the results of polyphasic approach employed here are in agreement, separating *Vasconcelosia* gen. nov. as a cyanobacterial genus within the family Cymatolegaceae (Nodosilineales). We note that the type strain of this genus (LEGE 07310) has toxicity against hepatocellular carcinoma, osteosarcoma, breast adenocarcinoma, and neuroblastoma cell lines (Costa et al., 2014), making its systematic classification relevant for those working in biotechnology as well as other disciplines. This ongoing requirement for the definition of new taxa is significant, since it results in new systematic studies, which provides opportunities for novel discoveries with scientific applications.

Beyond the description of the new genera discussed above, our phylogenies suggested that *Gloeothece* was divided into four distinct clades and that the 16S rRNA gene similarity values among them were, at times, lower than 94.5% (Table S7 in the Supporting Information). For instance, the clade named *Gloeothece* I had maximum similarity values of 93.3% and 93.6% when compared to clades III and IV, respectively. However, the similarity values between clades I and II was 95.44%. Clades I and III were 95.4% similar. Clades III and IV, in which LEGE 191138 and LEGE 16569 are included, shared also more than 96% 16S rRNA gene similarity. With these similarities, we have not described LEGE 191138 and LEGE 16569 (clade IV) as a new genus, but we highlight the need of a revision of *Gloeothece*, which can probably be separated into different genera.

In conclusion, our paper describes four new cyanobacterial genera from Portugal in the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC). These descriptions of previously deposited isolates support the idea that there are cryptic cyanobacterial types present in culture collections. Application of a polyphasic approach has led to new genera descriptions and our findings suggest that the cyanobacterial diversity is still underestimated. Regarding the ITS rRNA region secondary structures, the similarity among LEGE 16640 and LEGE 07310 D1–D1' helices serves to stimulate a reconsideration about the employment of this analysis in the description of new genera.

AUTHOR CONTRIBUTIONS

Flavio Luis de Oliveira: Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **Guilherme Scotta Hentschke:** Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **João Morais:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **Raquel Silva:** Data curation (supporting); investigation (supporting); writing – review and editing (supporting). **Pedro Cruz:** Data curation (supporting); investigation (supporting); writing – review and editing (supporting). **Vitor M. Vasconcelos:** Funding acquisition (lead); project administration (lead); resources (lead); supervision (lead); writing – review and editing (supporting).

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SUPPORTING INFORMATION

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

Figure S1. First-round analysis. 16S rRNA gene FastTree phylogenetic analysis of the studied strains and reference strains of Cyanobacteria. The strains highlighted in green represent newly described taxa. The condensed clades are shaded in gray.

Table S1. 16S rRNA gene similarity (*p*-distance) matrix among *Pseudolimnococcus* gen. nov. and phylogenetically related genera.

Table S2. ITS rRNA regions of the studied strains. The conserved domains are highlighted in different colors.

Table S3. Presence and absence of the ITS rRNA region helices in the newly described genera.

Table S4. 16S rRNA gene similarity (*p*-distance) matrix among *Eucapsopsis* gen. nov. and phylogenetically related genera.

Table S5. 16S rRNA gene similarity (*p*-distance) matrix among *Pseudoacaryochloris* gen. nov. and phylogenetically related genera.

Table S6. 16S rRNA gene similarity (*p*-distance) matrix among *Vasconcelosia* gen. nov. and phylogenetically related genera.

Table S7. 16S rRNA gene similarity (*p*-distance) matrix among *Gloeothece* strains.

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