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Polyphasic description of *Ciimarium marinum gen. et sp. nov.* (Prochlorococcaceae, Synechococcales): a new picocyanobacterial taxon from the Portuguese coastal ecosystem

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Abstract

In this paper, we describe *Ciimarium marinum gen. et sp. nov*. (Prochlorococcaceae, Synechococcales) based on 16S rRNA gene phylogenies, 16S-23S ITS secondary structures, ultrastructure (TEM), morphological, pigments and ecological data of "*Synechococcus"* sp. LEGE 11466. The strain was isolated from a sample collected at Leixões Harbour, Portugal (41°11'08.1"N 8°43'08.8"W) on September 26th, 2011, by scraping an epilithic biofilm under 10-13m depth, less than 1km offshore. Bayesian Inference and Maximum Likelihood phylogenies demonstrate that the *Ciimarium* sequence is placed in a monophyletic clade with strong phylogenetic support (BI=1, ML=100), within the Prochlorococcaceae cluster. The type strain LEGE 11466 is phylogenetically distant from other reference strains of Cyanobacteria and is grouped with Uncultured "*Synechococcus"* MLS1228cl3 , "*Synechococcus"* MLCB, "*Cyanobium"* ATX 6A2 ATX-6A2-C45 and "*Synechococcus"* MBIC10613.. The secondary structures of 16S-23S ITS corroborate with the separation of *Ciimarium* from all the Prochlorococcaceae genera. Also, they show that MLCB and ATX 6A2 ATX-6A2-C45 may be different species from the type LEGE 11466. The ultrastructure analysis shows parietal thylakoids, in agreement with the family description. Morphologically the genus is indistinguishable from other Prochlorococcaceae genera. The pigments analysis shows Zeaxanthin and β-carotene as the main pigments, and also a low concentration of phycocyanin. Finally, our phylogenies indicate that a revision of the Prochlorococcaceae family is necessary, as there are still many *Synechococcus*-like Cyanobacteria strains that should be described as new genera in the future.

Key words: Taxonomy, New Genus, New Species, Marine, Picoplankton, Phylogeny

Introduction

Marine Cyanobacteria are a great source of bioactive compounds with many biotechnological applications such as cosmetics, anticancer, antioxidant, anti-obesity, anti-inflammatory and antiviral. Also, Cyanobacteria are used as food supplement and can contribute against nutritional insecurity in poor countries, being used as a source of proteins. They contribute in agriculture fixing atmospheric nitrogen and in aquaculture, in fish feeding (Żymańczyk-Duda *et al.* 2022).

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The correct classification and the knowledge of the cyanobacterial biodiversity is essential for the development of these biotechnological studies, being the marine Cyanobacteria a great source of bioactive compounds (Hentschke & Gama-Júnior 2022). According to Hentschke & Gama-Júnior (2022), marine Cyanobacteria are very diverse but still underestimated, with 106 genera described up to 2022. Taxonomically this is proportionally the group with fewer molecular studies, indicating a great potential for new discoveries.

Among marine Cyanobacteria, the *Synechococcus*-like types comprise a picoplanktonic group, which represents a significant portion of the biodiversity and biomass in marine environments. The diversity and success of this group are likely related to their small cell size, allowing them to thrive in oligotrophic conditions and be less affected by sedimentation (Partensky *et al.* 1999; Partensky & Garczarek 2010). Taxonomically, *Synechococcus*-like Cyanobacteria is a polyphyletic group, and their strains are distributed in different cyanobacterial families. For instance, from this group, a subgroup named as *Parasynechococcus* Coutinho *et al.*, within the family Prochlorococcaceae (Synechococcales), was described based solely on molecular data (Coutinho *et al.* 2016; Komárek *et al.* 2020). Subsequently, there was a proposal to split *Prochlorococcus* into five distinct genera and 120 species. However, it's important to note that this proposal does not conform to the rules of the Bacteriological Code of Nomenclature – as well as to the rules of International Code of Nomenclature for algae, fungi, and plants (Turland *et al.* 2018; Dvořák *et al.* 2023).

The Prochlorococcaceae is a monophyletic family, and encompasses coccoid genera, with parietal thylakoids. Within this family, the coccoid genera are picoplanktonic and present elongated cyanobacterial cells, with 1-4 μm long, and are represented by *Prochlorococcus* Chisholm, *Parasynechococcus* Coutinho *et al.*, *Cyanobium* Rippka & Cohen-Bazire and *Anathece* Komárek *et al*. (Strunecký *et al.* 2023).. Most of these genera are known solely from cultures, and due to their simple morphology, they are primarily distinguished through 16S rRNA phylogenetic analysis. Additionally, as mentioned earlier, the morphology of the coccoid group closely resembles that of the genus *Synechococcus* Nägeli (Synechococcaceae), further complicating the identification of strains.

This morphological simplicity makes the differentiation among all *Synechococcus*-like genera very difficult and because of that, according to 16S rRNA gene phylogenetic analyses, there is still a huge number of sequences misidentified as "*Cyanobium"* and *"Synechococcus"*, in GenBank (NCBI). (Ramos *et al.* 2018). These strains form distinct phylogenetic monophyletic clades, distant from any already described cyanobacterial genera, and have to undergo through revisions and described as new taxa in the future (Ramos *et al.* 2018; Strunecký *et al.* 2023).

The Blue Biotechnology and Ecotoxicology Culture Collection – LEGE-CC (CIIMAR, Portugal) is a valuable resource for isolates of *Synechococcus*-like cyanobacteria (Ramos *et al.* 2018). Among them, the strain *"Synechococcus"* sp. LEGE 11466, is phylogenetically positioned in a monophyletic clade, distant from any previously described cyanobacteria. Therefore, this paper presents the description of *Ciimarium marinum gen. et. sp. nov.*, isolated from an epilithic marine environment in Portugal. This description is based on 16S rRNA phylogenies, 16S-23S ITS secondary structure analysis, ultrastructure (TEM), as well as morphological, pigments and ecological analyses.

Material and methods

Sampling, isolation of strains and morphological analysis

The sample was collected at 'Pêlo Negro', diving spot near Leixões Harbour, (41°11'08.1"N 8°43'08.8W), Portugal, in 09/26/2011, by scraping an epilithic biofilm under 10-13m deep, less than 1km off the shore. From this sample, the strain LEGE 11466 was isolated inoculating biomass in $Z8_{25}$ solid medium agar plate (Z8 medium with 25% of Tropical Marine Salt and B12 vitamin in final concentration of 10 μg/L) (Kotai 1972). Then, isolate colonies were transferred to new flasks with $Z8_{25}$ (+B12 vitamin) liquid media and maintained at LEGE-CC (CIIMAR) under the conditions: 19°C, 12:12 hours light/dark cycle (25 μmol photons m^{-2} s^{-1}). The strain was observed and photographed under a Leica DMLB microscope. Measurements of 40 cells were performed using a dedicated software (Leica LAS EZ; Leica Microsystems). From this culture, part of biomass was lyophilized and preserved in the University of Porto Herbarium under the code PO-T4784.

Transmission electron microscopy (TEM)

Cells 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 overnight, dehydrated and embedded in Embed-812 resin (#14120; Electron Microscopy sciences). Ultra-thin sections (50 nm thickness) were cut on a RMC Ultramicrotome (PowerTome, USA) using Diatome diamond knifes, 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 viewed 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 1100W (Tokyo, Japan).

DNA extraction, PCR amplification and sequencing

Genomic DNA isolation was performed using NZY Microbial gDNA Isolation Kit (NzyTech), following the manual specifications. The 16S rRNA gene amplification was performed using the primers 27F1 (5'-AGAGTTTGATCCTGCTCAG-3') and 1494R (5'-TACGGCTACCTTGTTACGAC-3') (Neilan *et al.* 1997), under the following conditions: initial denaturation step at 94ºC for 4 min, followed by 30 cycles of DNA denaturation at 94ºC for 20s, primers annealing at 50ºC for 30 s, strand extension at 72ºC for 2 min, and a final extension step at 72ºC for 7 min. The inserts were cloned into "pGEM®-T Easy Vector Systems" (Promega, Madison, WI, EUA), pGEM®–T Easy Vector System (Promega, Madison, WI, USA) according to the supplier's manual, inserted by heat–shock in *E. coli* DH5α cells and plated for blue–white selection (Sambrook *et al.* 1989). Three white colonies for each strain were selected and the plasmid extractions were performed by the alkaline lysis method (Birnboim & Doly 1979). The gene of the three plasmids were sequenced using "Big Dye Terminator" version 3.0 (Applied Biosystems) with the plasmid primers T7 and M13 and the internal primers 357F/357R, 704F/704R and 1114F/1114R (Lane 1991). This sequence is deposited in GenBank (NCBI) under the code KU951807.

To obtain the 16S-23S ITS region, firstly we performed an amplification using the primers 27F and 23SR (Neilan *et al.* 1997; Nübel *et al.* 1997) under the conditions: initial denaturation step at 95ºC for 5 min; 10 cycles of denaturation at 94ºC at 45s, primers annealing at 57ºC for 45s, strand extension at 72ºC for 2min; 25 cycles of denaturation at 92ºC for 45s, primers annealing at 54ºC for 45s, strand extension at 72ºC for 2min and a final elongation step at 72ºC for 7min. The PCR product was purified using the NZYGelpure kit (NZYTech), according to the fabricant instructions, and sequenced using the primers 27F, 359F and 23SR (Neilan *et al.* 1997a; Nübel *et al.* 1997). This sequence was aligned with our previous (cloned) 16S rDNA sequence and both shared 99,9% of similarity. The 16S-23S ITS sequence is deposited in GenBank under de code OR772944.

Phylogenetic analysis

To determine the position of our strains among other Cyanobacteria, we aligned the 16S rRNA nucleic acid sequences obtained in this study with those of reference strains of Nodosilineaceae, and coccoid genera from Synechococcaceae and Prochlorococcaceae families based on Coutinho *et al.* (2016), Forrest *et al.* (2023) and Komárek *et al.* (2020). Also, other sequences were retrieved from GenBank (NCBI) using BLAST. The sequences were aligned using ClustalW, in MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura *et al.* 2021), and the final dataset contained 60 sequences with 1293 informative sites. The phylogenetic trees were built using Maximum Likelihood and Bayesian Inference analysis. GTR+G+I evolutionary model was selected by MEGA 11. The robustness of ML tree was estimated by bootstrap percentages, using 1000 replications using IQ-Tree online version v1.6.12 (Trifinopoulos *et al.* 2016). Bayesian trees were constructed in two independent runs, with four chains each, for 5×10^6 generations, burnin fraction set to 0.25, sample frequency 1000, using MrBayes (Ronquist *et al.* 2012) in Cipres Gateway (Miller *et al.* 2010). Also, a similarity (p-distance) matrix was generated. The 16S-23S ITS secondary structures of D1-D1', V2, Box B and V3 helices were folded using MFold (Zuker 2003), according to (Lukesova *et al.* 2009).

Pigments extraction

A sequential extraction was carried out from the dry biomass using solvents of different polarities. Acetone was selected to extract low polar compounds (carotenoids and chlorophylls), while water was employed for polar ones (phycobiliproteins, PBPs). The acetone extract was prepared by suspending 0.5 g of dry pulverized biomass in 10 mL of HPLC-grade acetone (100%), in a glass Erlenmeyer. The suspension was sonicated at room temperature, in an ultrasonic bath (Fisherbrand®-FB15053), for 5 minutes, in order to help breaking the cell walls and release the cell content. The mixture was centrifuged (Thermo ScientificTM HERAUS MegafugeTM 16R; 10000 Gs, 5 min,

4ºC) and the extraction process was repeated 3 times in the same biomass. After completing 4 extraction sequences, the supernatants were combined into a round-bottomed flask, and evaporated under reduced pressure (BUCHI R-210 Rotary Evaporator; 200 mBar; condenser temperature -8ºC). The pellet resulting from the acetone extraction was left to dry in the fume hood overnight. After completely dry, the biomass was extracted with 10 mL of distilled water, following the same procedure as before. The supernatants were combined, frozen and lyophilized. The dry extracts were kept at -20 ºC until chemical analysis.

Carotenoid and chlorophyls profiling by HPLC-PDA

Ciimarium LEGE 11466 carotenoid and chlorophylls profile was determined using a Waters Alliance 2695 high-performance liquid chromatography (HPLC) system equipped with a photodiode array (PDA) detector (USA), following a previously reported protocol (Morone *et al.* 2022). The stationary phase was an YMC Carotenoid C30 (250 × 4.6 mm; 5 µm) column, kept at a constant temperature (25 ºC) with a column heater (Waters Corporation, Milford, CT, USA). The mobile phase was composed of 2 solvents: methanol (A) and methyl tert-butyl ether (B) (VWR Prolabo), starting with 95% A and installing a gradient to obtain 10% B at 5 min, 18% B at 20 min, 30% B at 28 min, 50% B from 31 to 37 min, 80% B from 38 to 47 min, and 5% B from 48 to 50 min. The flow rate was 0.90 mL/min and the injection volume was 5 µL. Data was processed with Empower® chromatography software (Waters, USA). Spectra data from all peaks were collected in the range 250 to 750 nm, and compounds were tentatively identified by comparing their retention times and UV-Vis spectra with those of authentic standards. Carotenoids quantification was achieved by measuring the absorbance recorded in the chromatograms relative to external standards at 450 nm.

Lutein, chlorophyll-*a*, zeaxanthin, β-carotene (Extrasynthese, Genay, Fance) and myxoxantophyll (DHI, Horsholm, Denmark) were quantified with the authentic standards. The β-carotene oxygenated derivative was quantified as zeaxanthin (the most abundant xanthophyll), β-carotene derivatives as β-carotene (the most representative carotene), and the chlorophyll-*a* derivative as chlorophyll-*a*. Calibration curves were performed with five different concentrations of standards, selected as representative of the range of compounds concentration in the samples. The calibration plots and r^2 values for the analysed carotenoids and chlorophyll-*a* are shown in Table 1.

TABLE 1. Calibration curves of authentic standards used for pigments quantification.

Standards	Calibration Curve	r^2
Lutein	$y = 93629588x + 13548$	0.9999
Chlorophyll-*a*	$y = 83010783x + 144987$	0.9985
Zeaxanthin	$y = 80009404x + 19635$	0.9944
Myxoxantophyll	$y = 30518380x + 5976$	0.9993
β-Carotene	$y = 29605275x + 36236$	0.9982

PBPs quantification

PBPs are water-soluble proteins able of being quantified spectrophotometrically in cyanobacteria aqueous extracts. The aqueous extract was resuspended in water to a final concentration of 0.5 mg/mL. PBPs were determined by measuring the absorbance of the resulting solutions at different wavelengths (562, 615, and 652 nm), using a cell with 1 cm of optical path. This quantification was performed in duplicate and calculated using the following formulas:

$$Phycocyanin(PC) = \frac{A_{615\,nm} - 0.474 \times A_{652\,nm}}{5.34}$$

$$Allophycocyanin(APC) = \frac{A_{652\,nm} - 0.208 \times A_{615\,nm}}{5.09}$$

$$Phycoerythrin(PE) = \frac{A_{562\,nm} - 2.41 \times PC - 0.849 \times APC}{9.62}$$

The results were expressed in µg of the respective phycobiliprotein per mg of dry extract.

TABLE 2. Morphological, ecological and pigment composition comparisons among Prochlorococcaceae genera.

	C. marinum LEGE 11466	*Ciimarium sp. MLCB* (Budinoff & Hollibaugh 2007)	*Anathece* (Komárek *et al.* 2011)	*Cyanobium* (Komárek & Anagnostidis 1988, Komárek 2020)	*Parasynechococcus* (Komárek *et al.* 2020)	*Prochlororoccus* (Komárek *et al.* 2020)
Colony formation	Common, amorphous. Cells loosely aggregate, irregularly arranged	Rare (aggregates with 5–20 cells)	Amorphous, rarely spherical. Micro- to macroscopic. Cells irregularly arranged, loosely or densely aggregate	Absent	Absent	Absent (or in pairs)
Mucilage	Facultative. Diffluent, narrow. Visible only when stained. Colorless. Cells without own sheaths	Unknown	Diffluent. Colourless or (rarely) yellowish. Cells with own sheaths.	Facultative. Diffluent, narrow. Visible only when stained.	Facultative. Diffluent, narrow.	Absent
Cell shape	Slightly elongate or rod-shaped.	Elongate	Widely oval to cylindrical. Straight or slightly arcuate	Oval, ellipsoid or rod-shaped	Elongate	Rod-shaped
Cell measurement	1–1.5 x 0.75–1.2 µm (ratio l/w 1.3–1.9) µm long	Up to 10 µm long (ratio l/w =2)	(2) 3–9 (12) x (1) 2.5–5 (8) µm	1–4 x 1–3 µm	(0.4)0.61.2(2.1) x 0.4–0.8 µm	1.2–1.6 x 0.6–0.8 µm
Thylakoids	Three to four parietal layers	Two layers. Glycogen deposit between thylakoids and cell wall.	Two to four layers. Parietal	Parietal	One to four parietal layers. Polyphosphate granules between thylakoids.	Parietal
Habitat	Marine. Portuguese coast. Subtidal, epilithic (10–12 m deep). Less than 1km from shore	Hypersaline lake in Sierra Nevada, USA. Benthic or planktonic	Freshwater (benthic, metaphytic) or subaerophytic	Marine or freshwater	Marine/oceanic. Picoplanktonic	Marine. Warm waters.
Tolerance to salinity in culture	Maintained in culture with 25% NaCl	10% Nacl	Unknown	Unknown	Not described	Not described
Pigments	90% carotenoids, 10% chlorophylls. Low phycocyanin concentration. High Zeaxanthin and β-carotene concentration	Not described	Not described	Presence of Zeaxanthin, α–carotene	Not described	Divinyl chlorophylls–a and –b as principal photosynthetic pigments

Statistical analysis

Statistical analysis for comparison of carotenoids was performed using IBM® SPSS® STATISTICS software, version 23, IBM Corporation, New York, NY, USA (2015). Data were analysed for normality and homogeneity of variances and then submitted to one-way ANOVA, with Tukey's HSD (honest significant difference) as a post hoc test.

Results

Phenotypic analysis

The strain "*Synechococcus"* LEGE11466 (*Ciimarium*) presents small elongate cells (maximum 1.5 µm long), solitary or irregularly arranged in amorphous colonies, with facultative diffluent mucilage. The thylakoids arrangement is parietal. Based on these characters, it is impossible to distinguish *Ciimarium* from the other Prochlorococcaceae genera (Table 2).

Considering habitats, it is possible to distinguish *Ciimarium*, which is marine and benthic, from *Cyanobium*, which is planktonic in freshwater environments. It is also possible to distinguish *Ciimarium* from *Parasynechococcus* and *Prochlorococcus*, which are also marine, but planktonic (Table 2).

Within *Ciimarium* circumscription, the strain "*Synechococcus"* MLCB (*Ciimarium* sp.) differs from the type strain LEGE 1466 by presenting longer cells (up to 10 µm) and by presenting glycogen deposits between the thylakoids and the cell wall. Also, the formation of colonies is rare in MLCB, while are very common in LEGE 11466. These characters indicate that MLCB may be a different species from *C. marinum* (LEGE 11466) (Table 2). There are no available data for the other strains within *Ciimarium* clade.

Description

Ciimarium marinum gen et. sp. nov. G.S. Hentschke, J. Morais & Vitor Vasconcelos
Figures 1, 2.

Only in culture, cells solitary or in clusters. Cells slightly elongated or rod-shaped, 1–1.5 µm long, 0.75–1.2 µm wide (ratio l/w 1.3–1.9), with or without a thin layer of diffluent mucilage. Cell content homogenous, pale blue-green. In colonies, cells irregularly arranged, forming amorphous clusters, loosely aggregate, within diffluent mucilage. Two to four layers of parietal thylakoids.

Diagnosis: Monophyletic clade, phylogenetically close related to *Cyanobium* and *Anathece* cluster in family Prochlorococcaceae, by 16S rRNA analysis. The D1-D1' helix from the 16S-23S ITS rRNA region presents three adenines (5'AAA3'), opposing the first lateral bulge.

Type:—PORTUGAL. Matosinhos, Leixões Harbour, marine on submersed rock, 41°11'08.1"N 8°43'08.8"W, 10–13m deep, 26 September 2011, *Pedro Leão et Aldo Barreiro.* Lyophilized biomass deposited in University of Porto Herbarium (holotype PO-T4784!).

Habitat: Type species is from a subtidal sample, epilithic (10–13m deep), less than 1 km off the shore.

Reference strain: "*Synechococcus"* sp. LEGE 11466 (KU951807)

Etymology: "*Ciimarium"* is honor of Interdisciplinary Centre of Marine and Environmental Research of the University of Porto (CIIMAR); "*marinum"* is for the marine habitat (marine) where the species was sampled.

Molecular analysis

Our phylogenetic BI (Average Standard Deviation of Split Frequencies <0,001) (Fig. 3) and ML (Suppl. 1) trees show similar backbone topology. The Nodosilineaceae strains are at the base of the phylogenies and there is strong backbone support separating Nodosilineaceae and Synechococcaceae from Prochlorococcaceae, in the Bayesian tree (BI=1) (Fig. 3). These three families are also separated in the ML tree (Suppl. 1), but in this case, the Synechococcaceae strains are more related to Prochlorococcaceae. The studied strain LEGE 11466 is in the monophyletic cluster containing other coccoid Prochlorococcaceae genera as *Cyanobium*, *Anathece*, *Parasynechococcus*, along with the type *Prochlorococcus* (Strunecký *et al.* 2023). All these genera are monophyletic and present strong phylogenetic

support, with exception of the *Cyanobium*/*Anathece* paraphyletic clade. Both of these genera must undergo through a revision to re-evaluate their taxonomic status and probably will be merged in a single one in further studies, due to their intermixing strains in the phylogenetic trees.

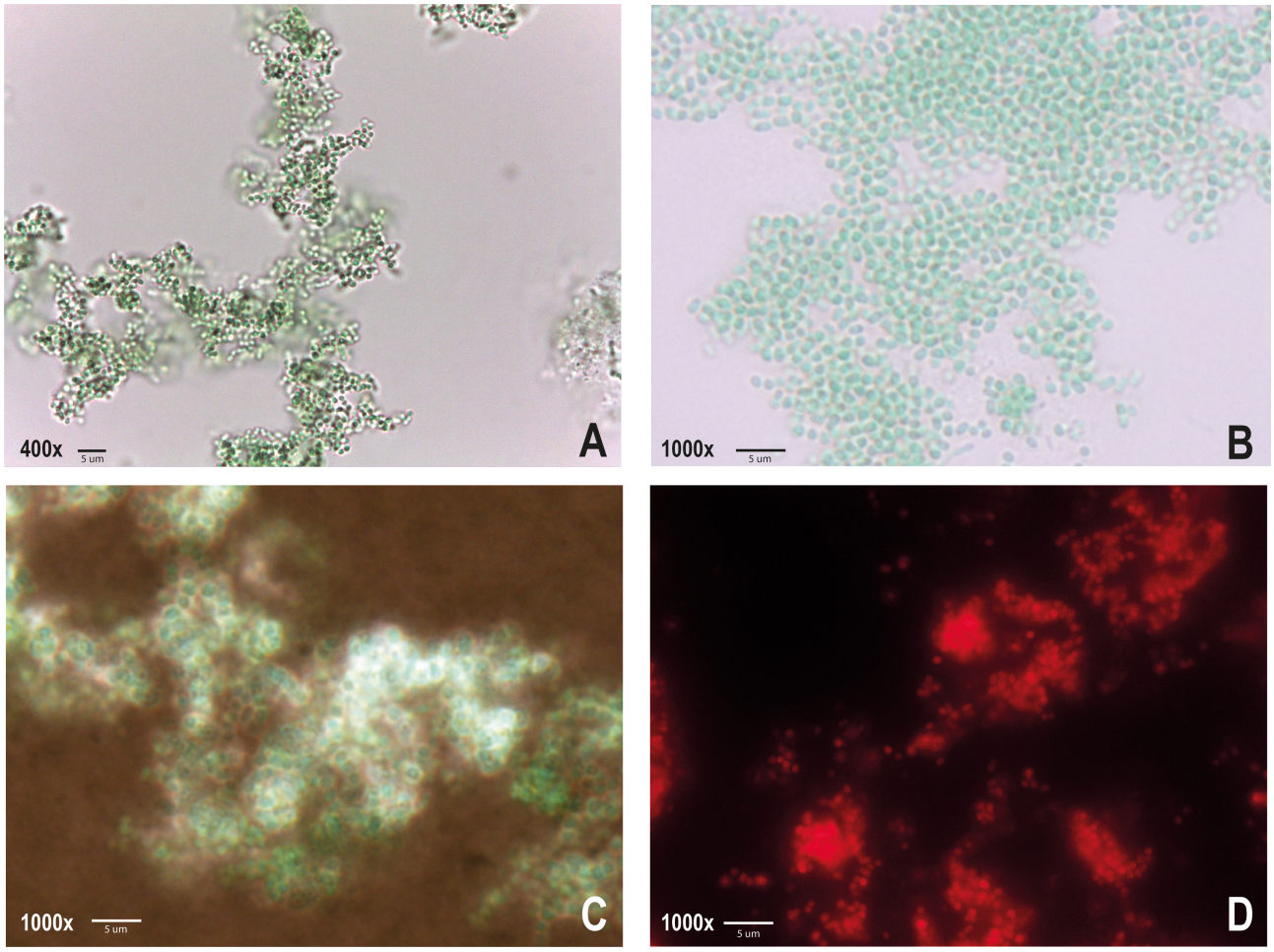

FIGURE 1. *Ciimarium marinum*. **A**. General view of cell cluster. **B**. Details of cells. **C**. Slide with China Ink, showing diffluent mucilage around cell clusters. **D**. Cluster view with phycocyanin lens filter (Excitor 525/45nm; Emmiter 595/60 nm).

In both trees, *Ciimarium* clade is monophyletic, with strong phylogenetic support (BI=1, ML=100). In the BI phylogeny, it is sister to the *Cyanobium/Anathece* clade, at the top of the tree. In the ML tree, it is placed in a basal position within Prochlorococcaceae, also sister to *Cyanobium/Anathece* cluster. The type strain LEGE 11466 is phylogenetically distant from any other cyanobacterial reference strain, indicating that it is a separate taxon. The *Ciimarium* type strain is grouped with Uncultured "*Synechococcus"* MLS1228cl3, "*Cyanobium"* ATX 6A2 ATX-6A2-C45, "*Synechococcus"* MBIC10613 and "*Synechococcus"* MLCB, which must be considered within the genus circumscription based on phylogenetic, 16S-23S ITS rRNA secondary structures and (similarity) p-distance analyses, as discussed below. .

The 16S rRNA gene similarity matrix (p-distance) (Tab.3, Suppl. 1, 2) corroborates with the phylogenetic results and also support the description of the new genus. The intrageneric similarity within the studied genera in our phylogenies ranged from 96.1% (within *Parasynechococcus*) to 100% (within *Parasynechococcus,Synechococcus* and *Prochlorococcus*). Across genera, the similarities spanned from a minimum of 88.7%, when comparing *Synechococcus* with *Prochlorococcus*, to a maximum of 98%, when comparing *Parasynechococcus* with *Prochlorococcus* and comparing *Cyanobium* with *Anathece*. *Cyanobium* and *Anathece* reference sequences, are in the same phylogenetic clade andthe high similarity between them corroborates with the idea that these both genera should be merged in one in the future.

The sequences of *Ciimarium* conform to these similarity patterns within Prochlorococcaceae, exhibiting intrageneric similarity ranging from 98.2% to 99.9%. When compared to other genera, the similarities range between 89% (in comparison to *Synechococcus*) and 96.8% (in comparison to *Prochlorococcus* and *Parasynechococcus*). Notably, these values are lower than the maximum similarity observed when comparing *Parasynechococcus* with *Prochlorococcus* (98%).

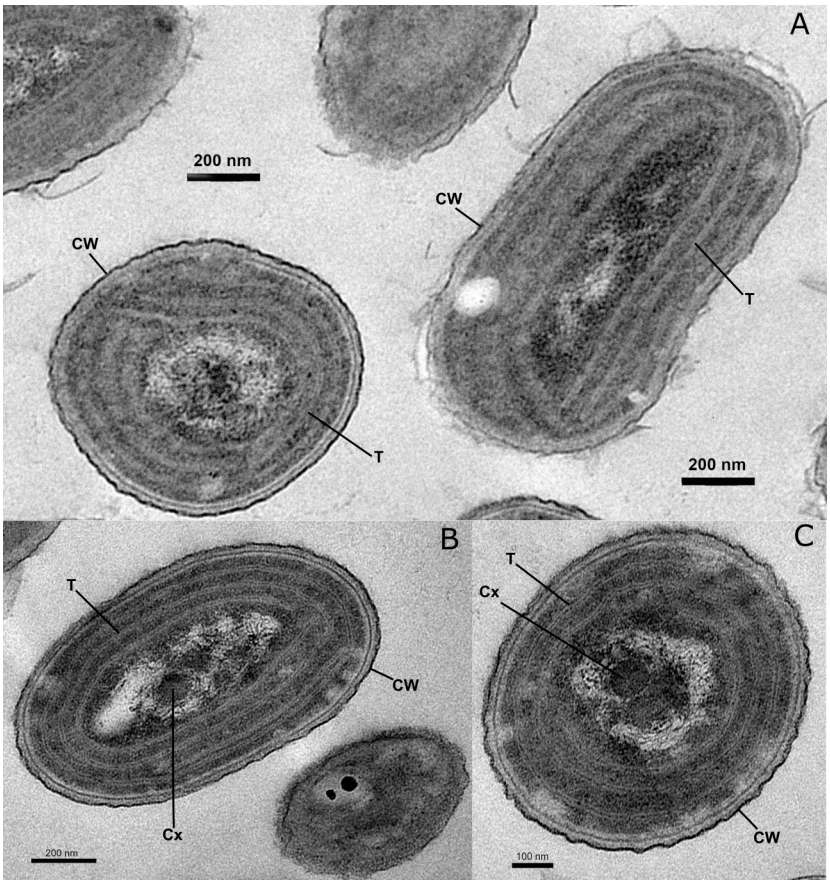

FIGURE 2. Transmission electron microscopy (TEM) of *Ciimarium marinum* LEGE 11466 analyzed using an electron microscope. Ultrastructural features: CW—cell wall, T—thylakoids with parietal arrangement, Cx—carboxysome.

TABLE 3. 16S rRNA gene similarity (p-distance) between Prochlorococcaceae and Synechococcaceae studied genera. The values are expressed as percentages.

		1	2	3	4	5	6
1	*Cyanobium*	#					
2	*Anathece*	98	#				
3	*Ciimarium*	95.5–95.9	95.8–96.4	98.2–99.8			
4	*Parasynechococcus*	95.4–96.4	96–96.7	95.8–96.8	96.1–100		
5	*Prochlorococcus*	95.4–96	95.4–96.1	95.8–96.8	95.5–98	97.6–100	
6	*Synechococcus*	89.4–89.5	89.2–89.3	89–89.8	88.9–90.2	88.7–90.5	99.8–100

= Genus with only one analyzed strain. Cells shaded in grey represent intrageneric variation.

The 16S-23S ITS sequence of *Ciimarium* LEGE11466 contained both $tRNA^{Ile}$ and $tRNA^{ale}$ regions. We compared the helices D1-D1', Box B, V2, and V3 of the new genus with those of other members of Prochlorococcaceae, such as *Cyanobium, Parasynechococcus*, and *Prochlorococcus*. It is evident that among these genera, all helices differ significantly in terms of both structure and length (Figs 4–7, Suppl. 3). It's important to highlight that among these helices, the more conserved region is located at the D1-D1' helix, opposing the first lateral bulge. For *Ciimarium*, this region is unique among the family genera and is characterized by three adenines (5'AAA3'), whereas for the other genera, the region presents a mutation (5'AAC3') (Fig. 4). We also noticed that for all Prochlorococcaceae genera, the region between the two tRNAs is very short, consisting of only 9 nucleotides. From this region, notably, only *Ciimarium* presented the V2 helix (Fig. 5). The remaining genera of the family, *Cyanobium, Parasynechococcus* and *Prochlorococcus* presented sequences which cannot be folded in helices. The Box B helix of *Ciimarium* also significantly differ from the other Prochlorococcaceae genera. *Ciimarium marinum* LEGE11466 Box B helix begins with 5'GCAG3', and *"Synechococcus"* MLCB helix begins with 5'GAU3'. The other family genera presented the typical basal Box B stem composed by 5'AGCT3'. The V3 helices also displayed considerable variation in structure, length and sequence across all family genera. These findings concerning the secondary structures of the 16S-23S ITS further support the differentiation of *Ciimarium* from the other taxa within Prochlorococcaceae.

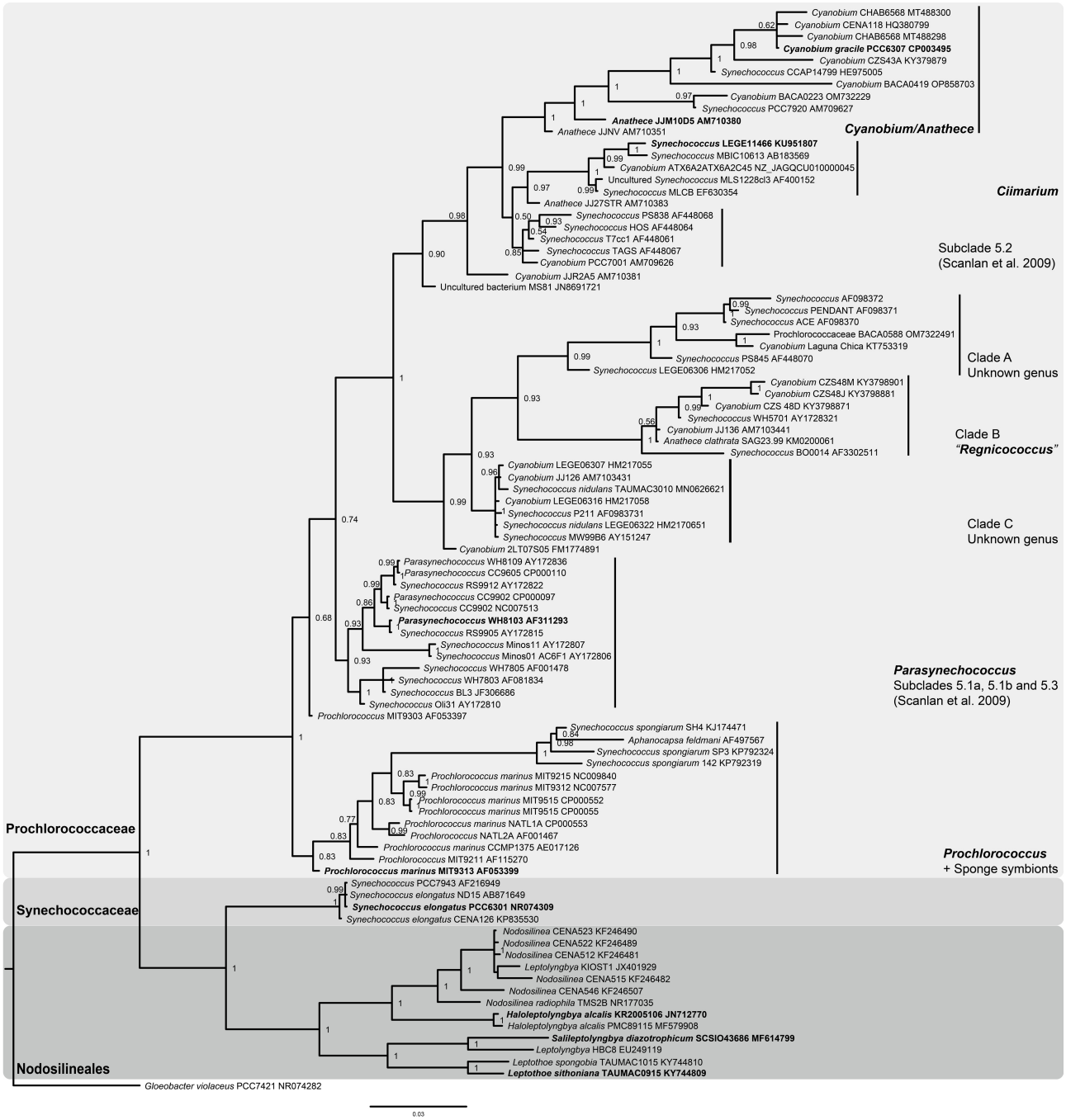

FIGURE 3. 16S rRNA gene Bayesian Inference Phylogeny. Clade B represents the invalid described genus "*Regnicococcus*". Clades A, and C are unknown taxa which must be described as new genera in the future. Clades numbered "5" were retrieved from Scanlan *et al.* (2009). The clades 5.1a, 5.1b and 5.3 are *Parasynechococcus.* The clade 5.2 must be described as a new genus. Reference strains are in bold.

Within the *Ciimarium* clade, based on the 16S-23S ITS secondary structure analysis, we could identify that the strain *"Synechococcus"* MLCB may be a different species from *C. marinum*. The D1-D1', Box B and V3 helices of MLCB differ from the type species considering sequence and structure. Regarding the strain *Cyanobium* sp. ATX 6A2 ATX-6A2-C45, there´s no available sequences for the V2, Box B and V3 helices, but we could retrieve the D1-D1' region. This helix significantly differs from the type considering sequence and structure.

Pigments profile

Due to their low polar nature, carotenoids and chlorophylls are effectively extracted with acetone. The HPLC-PDA analysis of the acetone extract allowed the quantification of 9 carotenoids, chlorophyll-*a*, and one chlorophyll-*a* derivate, with a good chromatographic resolution, as illustrated in Figure 8.

Carotenoids were predominant when compared with chlorophylls, accounting 75.69 mg/g of the dry extract, which represented more than 90% of the pigments identified in the acetone extract (Table 4). Of them, β-carotene (10) stood out, representing more than 50% of the total carotenoids (39.21 mg/g), followed by the xanthophyll zeaxanthin (4), accounting 17% of the total (13.25 mg/g) (p<0.05). The chlorophylls represented about 10% of the pigment composition. Regarding phycobiliproteins, the phycocyanin was dominant, with 6.80 ± 0.39 µg/mg of dry extract, followed by allophycocyanin, with 4.01 ± 0.34 µg/mg and finally by phycoerythrin, with 3.04 ± 0.22 µg/mg.

TABLE 4. Carotenoid and chlorophylls content (mg/g of dry extract) in the acetonic extract of the cyanobacteria *C. marinum*. LEGE 11466, determined by HPLC-PDA.

Peak	Compound	RT (min)	Concentration
1	Lutein	13.3	1.59 ± 0.04 [f]
2	β -Carotene oxygenated derivative	14.7	4.15 ± 0.03 [d]
3	Chlorophyll-*a*	15.5	5.72 ±0.02 [c]
4	Zeaxanthin	16.1	13.25 ± 0.39 [b]
5	Chlorophyll-*a* derivative	17.1	1.69 ± 0.01 [f]
6	Myxoxantophyll	20.0	2.59 ± 0.01 [e]
7	β-Carotene derivative	21.5	4.22 ± 0.03 [d]
8	β-Carotene derivative	24.6	2.26 ± 0.05 [e,f]
9	β-Carotene derivative	27.0	2.37 ± 0.22 [e,f]
10	β-Carotene	33.0	39.21 ± 0.51 [a]
11	β-Carotene derivative	34.3	6.04 ± 0.19 [c]
	Total Carotenoids		75.69 ± 12.08
	Total Chlorophylls		7.41 ± 2.85

[1] Values are expressed as mean ± SD of three determinations. Different superscript letters denote statistical differences at $p < 0.05$ (ANOVA, Tuckey HSD)

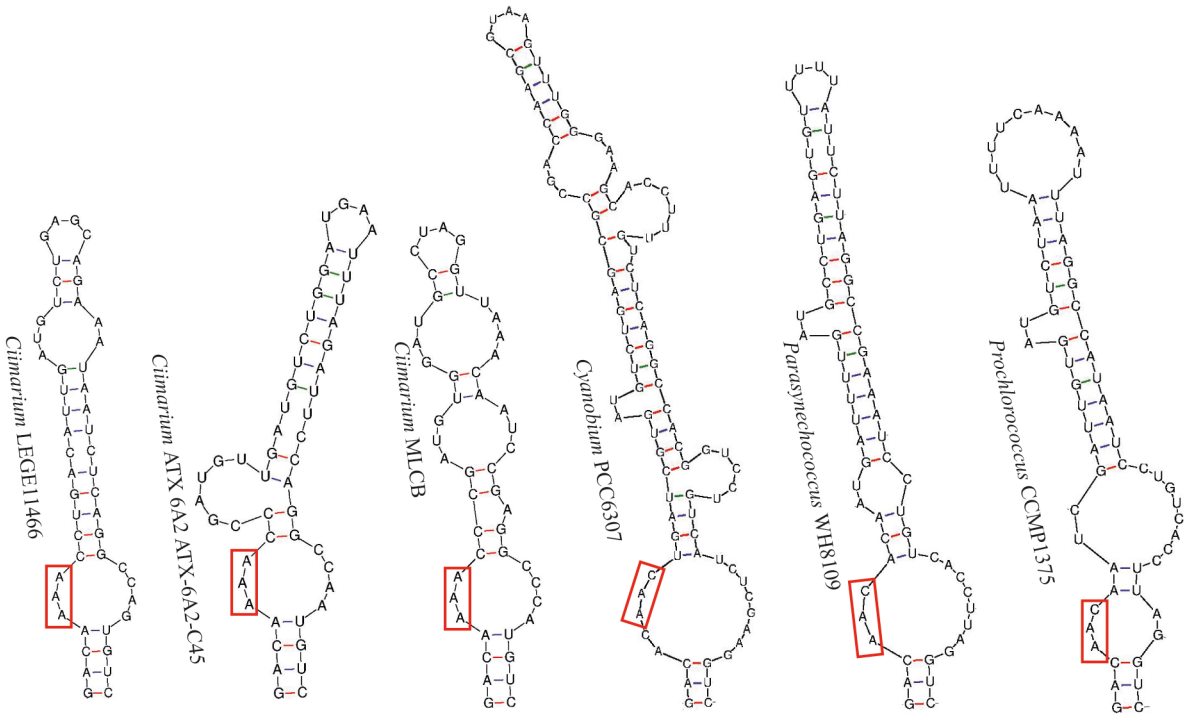

FIGURE 4. Secondary structures of D1–D1` helices (16–23S ITS) of related Prochlorococcaceae genera. Marked region is the most conserved among the structures.

Discussion

Our 16S rRNA gene phylogenies are in agreement with the *Synechococcus*-like genera revision (Komárek *et al.* 2020), showing *Synechococcus* as a sister group of the clade containing *Cyanobium*, *Anathece, Parasynechococcus* and *Prochlorococcus* (Prochlorococcaceae). The same topology is found in Coutinho *et al.* (2016) and Strunecký *et al.*

(2023). Beyond that, in our phylogenies we also found the clades 5.1a, 5.1b, 5.2 and 5.3, which were named by Scanlan *et al.* (2009). We found that the clade 5.1a, 5.1b and 5.3 are in the *Parasynechococcus* circumscription. The clade 5.2 should be described as a new genus in the future. The clades named by us as A and C, also should be described as new genera. The clade B is correspondent to the invalidly described genus "*Regnicococcus"* (Walter *et al.* 2007).

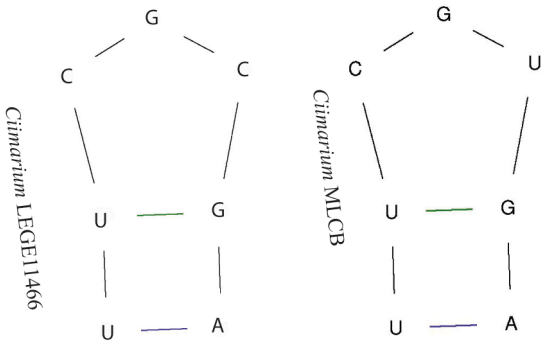

FIGURE 5. Secondary structure of V2 helix (16–23S ITS) of *Ciimarium*. Other genera didn´t present this structure.

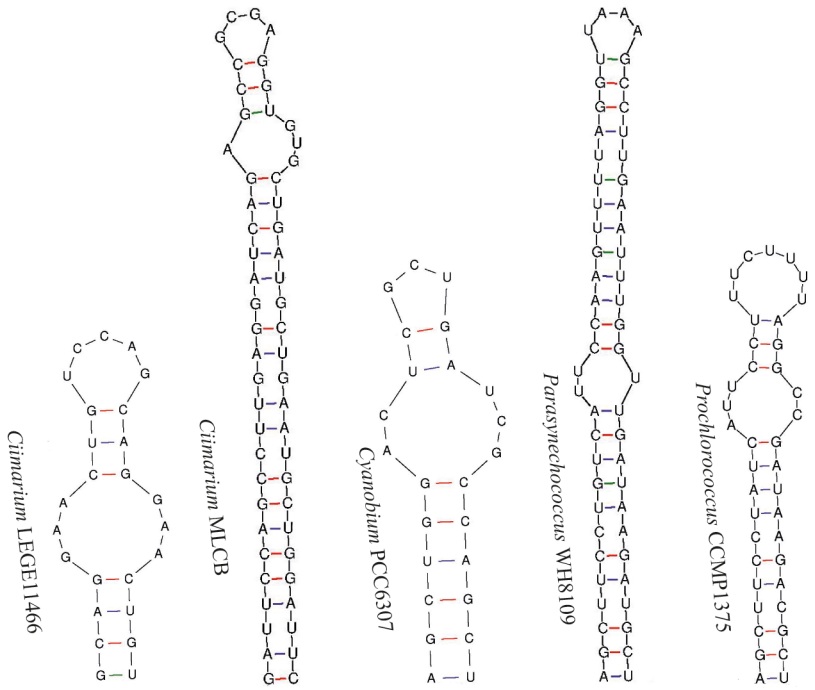

FIGURE 6. Secondary structures of Box B helices (16–23S ITS) of related Prochlorococcaceae genera.

The phylogenetic and similarity (p-distance) analyses of LEGE 11466 strain support the description of the new genus *Ciimarium.* Our phylogenies include the coccoid Prochlorococcaceae reference strains, and *Ciimarium* is in a strong supported monophyletic clade phylogenetically distant from all of them. The similarity (p-distance) pattern found comparing Prochlorococcaceae sequences indicates that the maximum value between different genera is under or equal to 8% (*Parasynechococcus* vs *Prochlorococcus*) (Table 3, Suppl. 2). In the family, above this limit, currently taxa may be considered the same genus. *Cyanobium* and *Anathece* reference sequences also present 98% of similarity.. This value is very close to the genetic identity threshold to separate species (<98.7), established by Yarza *et al.* (2014), reinforcing the idea that they can belong to the same genus. Beyond that, in our phylogenies *Cyanobium* and *Anathece* strains are clustered in a monophyletic clade (BI=1, ML=98). This is in agreement with Komárek *et al.* (2011) phylogeny, in which *Anathece* was described. Currently, there´s no morphological, ecological or molecular markers to separate these genera (Table 2, Fig. 3, Suppl. 1, 2) and in the future both genera may be merged in a single one after a needed taxonomical review.

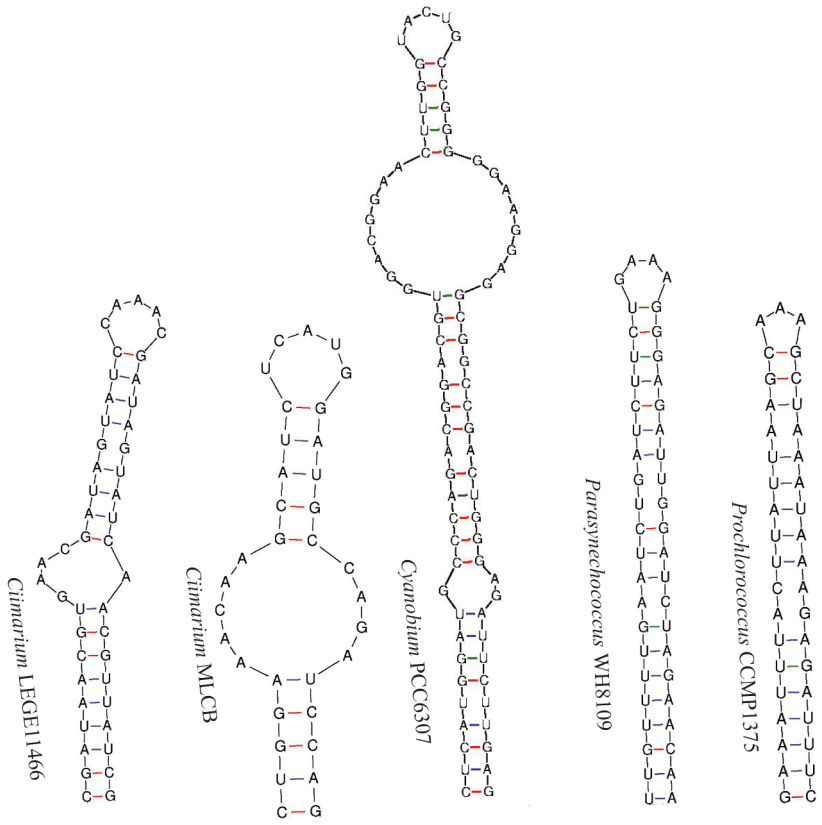

FIGURE 7. Secondary structures of V3 helices (16–23S ITS) of related Prochlorococcaceae genera.

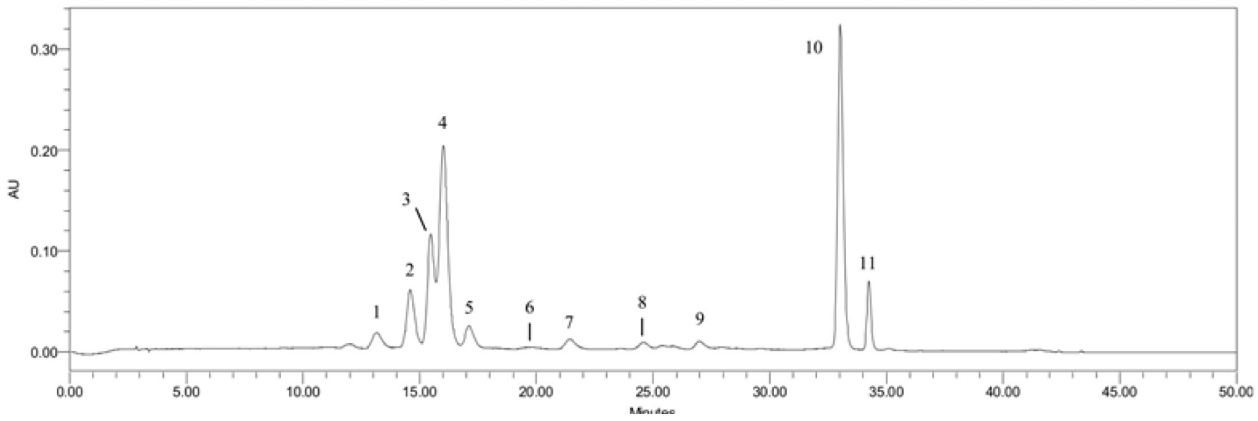

FIGURE 8. Carotenoid and chlorophylls profile of the acetonic extract of the cyanobacteria *C. marinum* LEGE 11466. HPLC-PDA recorded at 450 nm. Lutein (1), β-carotene oxygenated derivative (2), chlorophyll-*a* (3), zeaxanthin (4), chlorophyll-*a* derivate (5), myxoxantophyll (6), β-carotene derivatives (7–9, 11) and β-carotene (10).

Ciimarium sequences fit in those family boundaries of the generic differentiation, presenting a maximum similarity of 96.8% with *Prochlorococcus* and *Parasynechcoccus.* Historically, 95% or superior values of 16S rRNA gene similarity (Stackebrandt & Ebers 2006; Stackebrandt & Goebel 1994) were considered to indicate that the strains would belong to the same genus, but according to our results this is not true for Prochlorococcaceae. This happens also in other cyanobacterial families, such as Tolypothricaceae and Nostocaceae, in which *Spirirestis* Flechtner *et al.*, *Dapisostemon* Hentschke *et al*. and *Desmonostoc* Hrouzek & Ventura (Flechtner *et al.* 2002; Hentschke *et al.* 2016; Hrouzek *et al.* 2013) were described, sharing more than 95% of similarity with other taxa.

The 16S-23S ITS secondary structures analysis corroborates with the phylogenetic analysis and similarity (p-distance) data, separating these genera in different taxonomical units. The conserved region (5'AAA3' or 5'AAC3') in opposition to the first lateral bulge, can potentially be studied as a family character for taxonomical analysis. Another

region that has the potential to be used as a taxonomical marker is the one between the tRNAs, which is very short and commonly lacks the V2 helix in Prochlorococcaceae genera.

Morphologically, *Ciimarium* is indistinguishable from other coccoid Prochlorococcaceae genera, due to their small sized cells and simple cell morphology. Other characters, as the formation of colonies, mucilage and thylakoids arrangement neither can separate *Ciimarium* from other *Prochlorococcaceae*. Beyond molecular methods, the only characters that can distinguish *Ciimarium* from other Prochlorococcaceae are the ecological adaptations. *Ciimarium* is marine and benthic, while *Parasynechococcus* and *Prochlorococcus* are also marine, but planktonic. *Cyanobium* is planktonic from freshwater environments.

This is in agreement with Strunecky *et al.* (2023), which states that there is a huge 16S rRNA diversity within the family and many new cryptic genera must be described in the future based on molecular analyses. The cryptic status of the Prochlorococcaceae genera is probably due to the fact that many of these genera are known only in culture conditions and their very simple morphology.

Regarding the sequences within *Ciimarium* clade, Uncultured "*Synechococcus"* MLS1228cl3 (AF400152) and "*Synechococcus"* MLCB (EF630354) are from a hypersaline lake in California, USA The strain "*Synechococcus"* MLCB (*Ciimarium* sp.) differs from the type strain LEGE 11466 by presenting longer cells (up to 10 μm) and by presenting glycogen deposits between the thylakoids and the cell wall. Also, the formation of colonies is rare in MLCB, while are very common in LEGE 11466. These characters indicate that MLCB may be a different species from *C. marinum* (LEGE 11466) (Table 2).Currently, there´s not enough morphological, or molecular data of Uncultured "*Synechococcus"* MLS1228cl3 to compare with *C. marinum*, but it is probably the same species of MLCB strain, considering that are from the same environment and are phylogenetically very close related. There are no available data for the strains "*Cyanobium"* ATX 6A2 ATX-6A2-C45 and "*Synechococcus"* MBIC10613.

The pigment analysis results corroborate previous ones on the pigments profile of cyanobacteria from different genera, showing a similar pattern of carotenoid synthesis in these microorganisms (Morone *et al.* 2020, Morone *et al.* 2022). In fact, of the strains analysed so far, there is a clear dominance of chlorophyll-*a*, zeaxanthin and β-carotene. In a quantitative way, acetone has been the most effective solvent for carotenoids extraction, resulting in higher extraction yields and cleaner chromatograms. For instance, Morone and co-workers have analysed the carotenoids profile of aqueous, ethanol and acetone extracts of cyanobacteria of different genera, observing a clear predominance of these terpenoids in lower polar solvents. Regarding acetone extracts, the authors reported values between 104.71 and 150.15 mg/g (for the Synechococcales cyanobacterium LEGE 181157 and *Leptothoe* sp. LEGE 181156, respectively) which is in line with the values obtained herein. Another work undertaken by Lopes *et al.* reported the same tendency, with total carotenoids varying between 33.1 (for the freshwater *Alkalinema aff. pantanalense* LEGE15481) and 63.9 mg/g (for the terrestrial *Nodosilinea (Leptolyngbya) antarctica* LEGE13457) (Lopes *et al.* 2020). The major difference between the present work and the mentioned ones regards chlorophylls, which represent about 10% of the total pigments of *C. marinum* LEGE 11466, being significantly higher in the previous studies. This difference can be justified, at least in part, by the origin and morphological features of cyanobacteria strains. Of the cyanobacteria mentioned before, *C. marinum* LEGE 11466 is the one that originates at greater depth, being less exposed to light and therefore having a lower photosynthetic rate, which requires less chlorophyll than the strains that originate more at the surface.

Of the carotenoids identified herein, lutein, zeaxanthin and β-carotene account to 72% of the total pigments (Table 4). Regarding phycobiliproteins, *C. marinum* LEGE 11466 is a phycocyanin-producing strain. Phycocyanin was the dominant phycobiliprotein, with 6.80 ± 0.39 μg/mg of dry extract, followed by allophycocyanin, with 4.01 ± 0.34 μg/mg and finally by phycoerythrin, with 3.04 ± 0.22 μg/mg. Contrary to other cyanobacteria strains originated from intense-light exposed habitats, where the phycocyanin content may range between 18 and 222 mg/g (Morone *et al.* 2022), *C. marinum* LEGE 11466 presented a low amount. The quality and intensity of light significantly influences the light-harvesting system of cyanobacteria, affecting the accumulation of various photosynthetic pigments. In this regard, taking into account that *C. marinum* LEGE 11466 is a strain of relative depth, from a habitat with low light penetration, a low photosynthetic rate is expected. In line with the results, and considering that phycocyanin is an accessory pigment of chlorophyll, a low amount of this phycobiliprotein was also expected (Hsieh-Lo *et al.* 2019).

Comparing *Ciimarium* with other Prochlorococcaceae genera, it is possible to differentiate it from *Cyanobium*. *Ciimarium* presents β-carotene along with Zeaxanthin as main pigments, while *Cyanobium* presents α–carotene and Zeaxanthin.

In conclusion, in this paper we described the new genus and species *Ciimarium marinum* based on polyphasic approach. Within *Ciimarium* circumscription we found that the strains "*Synechococcus"* MLCB and "*Cyanobium"* ATX 6A2 ATX-6A2-C45 differs from the type by morphological, ecological and molecular analyses, and should be described as two different species, in the future. Moreover, in our trees, beyond *Ciimarium*, *Cyanobium, Anathece,*

Parasynechococcus and *Prochlorococcus*, we found three additional clades that should be described as new genera (Subclade 5.2 and Clades A and C). Moreover the genus "*Regnicoccus"* (*Synechococcus* sp. WH 5701, AY172832, clade B) (Walter *et al.* 2017) must be taxonomically validated under both Bacteriological Code of Nomenclature and the International Code of Nomenclature for algae, fungi, and plants. Finally, beyond contributing with Prochlorococcaceae diversity, our paper highlights the urgency of studies in order to review the phylogenetic status of the family .

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