INTRODUCING PETRACHLOROSACEAE FAM. NOV., *PETRACHLOROS* GEN. NOV. AND *PETRACHLOROS MIRABILIS* SP. NOV. (SYNECHOCOCCALES, CYANOBACTERIA) ISOLATED FROM A PORTUGUESE UNESCO MONUMENT[1]

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The Synechococcales is a large cyanobacterial order comprising both unicellular and filamentous forms, with parietal thylakoid arrangement. Previously, this order has been the subject of taxonomic revisions with new families being erected. During studies of the phototrophic communities on the limestone walls of the Old Cathedral of Coimbra (UNESCO monument), a coccoid *Aphanocapsa*-like cyanobacterium was isolated. It was characterized using a polyphasic approach, based on morphology, 16S rRNA phylogenetic and phylogenomic analyses, internal transcribed spacer (ITS) secondary structure, and ecology. The 16S rRNA phylogenetic analyses showed that this strain is placed in a separate and highly supported family-level clade, as part of a large group comprising the families Prochlorococcaceae and Prochlorotrichaceae, with *Lagosinema* as the closest (although quite distant) taxon. Additionally, the phylogenomic analysis also placed this strain in a separate lineage, situated distantly apart from the family Thermosynechococcaceae, but with strains assigned to Acaryochloris marina MBIC 11017 and *Aphanocapsa montana* BDHKU210001 as the closest taxa. Based on these data, as well as on the results from the secondary ITS structure, morphology, and ecology, we here propose the establishment of Petrachlorosaceae fam. nov., along with the description of *Petrachloros* gen. nov. and *Petrachloros mirabilis* sp. nov. We also address additional considerations regarding some cyanobacterial taxa within the order Synechococcales, which we believe deserve further revisions.

Key index words: cyanobacteria; novel taxa; phylogeny; Synechococcales; taxonomy

Abbreviations: BS, Bootstrap values; MCMC, Markov chain Monte Carlo; PP, Posterior Probabilities

Cyanobacteria are prokaryotic microorganisms with a significant evolutionary role in Earth's history, as they are considered to be responsible for the accumulation of oxygen in the primitive atmosphere, thanks to their ability to perform oxygenic photosynthesis (Bekker et al. 2004, Schirrmeister et al. 2013, Dvořák et al. 2017, Demoulin et al. 2019). They are one of the most important microbial primary producers (Tomitani et al. 2006, Dvořák et al. 2017) and one of the most morphologically diverse groups of prokaryotic organisms (Schirrmeister et al. 2013, Demoulin et al. 2019). Although, traditionally, their classification relied mostly on morphological data (Komárek 2016, Dvořák et al. 2017, Komárek et al. 2020), such criteria cannot be applied alone (Castenholz 1992, Komárek 2005, 2016), as some morphological and physiological characteristics can change during cultivation under laboratory conditions (Komárek et al. 2014, Komárek 2016). Additionally, such an approach may lead to taxonomic confusion when dealing with morphologically indistinguishable but molecularly unrelated strains (i.e., cryptotaxa; Komárek et al. 2014, Komárek 2016, Dvořák et al. 2017). As such, a polyphasic approach that combines morphological, molecular, and ecological data is currently considered the best methodology to correctly classify cyanobacteria (Komárek 2016).

Cyanobacterial taxonomy is evolving rapidly with new taxa described each year (Dvořák et al. 2017). There are currently over 300 genera, of which more than 50 were described since 2000 (Komárek et al. 2014, Mai et al. 2018, Akagha et al. 2019). Despite such progress, at the family level, taxonomic revisions are still scarce (Mareš 2018). This is because the majority of the families are described using morphology, due to the lack of sequence data for generitypes (Komárek et al. 2014, Mai et al. 2018). Nonetheless, revisionary work has already commenced with the reformulation of the Synechococcales by Mai et al. (2018), who split the Leptolyngbyaceae into four families (Leptolyngbyaceae, Oculatellaceae, Prochlorotrichaceae, and Trichocoleusaceae). More recently, using a polyphasic approach, Komárek et al. (2020) also performed a taxonomic revision and classification of *Synechococcus*-like cyanobacteria, validly describing distinct genera and correcting their taxonomic position.

Cyanobacteria are known to cause severe aesthetic and physicochemical alterations to stone monuments (Macedo et al. 2009, Sterflinger and Piñar 2013), which often lead to biodeterioration and loss of valuable cultural heritage materials (Scheerer et al. 2009). In 2013, the Old Cathedral of Coimbra was awarded UNESCO classification and, due to its deteriorating status, has been the subject of studies regarding the microorganisms that inhabit its limestone walls (Soares et al. 2019a, Trovão et al. 2019a, Coelho et al. 2021). Some novel taxa have already been isolated and described (Soares et al. 2019b, 2020b, Trovão et al. 2019b), and their biodeterioration status was characterized (see Trovão et al. 2020, 2021). During these ongoing studies, we isolated an *Aphanocapsa*-like cyanobacterium (Soares et al. 2019a) and have sequenced its genome (Soares et al. 2020a). The aim of the present study was to proceed with its characterization using a polyphasic approach based on morphology, phylogenetic and phylogenomic analyses, internal transcribed spacer (ITS) secondary structures, and ecology. We propose the establishment of a new Synechococcales family – Petrachlorosaceae – to accommodate *Petrachloros mirabilis* gen. et sp. nov.

MATERIALS AND METHODS

Site description and sampling. The Old Cathedral of Coimbra (40°12′32″ N, 8°25′38″ W) was one of the monuments within the city of Coimbra nominated as a UNESCO site in 2013. This emblematic monument from the *Reconquista* times was constructed during the 12th and 13th centuries and encompasses a single cloister floor surrounded by five lateral chapels carved in yellow dolomitic limestone.

The strain used in this study was collected by scraping the center of a biofilm from the limestone walls of the Chapel of São Miguel. The collected sample was inoculated into flasks containing liquid BG_{11} culture medium (Rippka et al. 1979) and incubated at $20 \pm 1°C$, under a 16:8 h (light:dark) photoperiod (30–40 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}), for 4 weeks. The isolation of the strain from the liquid enrichment cultures was then performed by means of micromanipulation under an inverted microscope (Andersen and Kawachi 2005). Successive isolations were conducted until a unicyanobacterial culture was obtained, following inoculation and incubation under the same conditions as above.

Morphological analysis. Periodic light microscope observations of the culture evaluated cell shape, size, and reproduction. At least 50 cells were examined and cell dimensions are expressed as minimum and maximum values. Photomicrographs were captured with a Leica microscope Model DM4000B (Leica, Germany) coupled to a camera. For TEM analysis, strain samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2.5 h and post-fixed using 2% osmium tetroxide for 2 h. Cells were then dehydrated in a graded ethanol series (70–100%), embedded in 2% molten agar, re-dehydrated in ethanol (70–100%), impregnated, and embedded using an Epoxy Embedding Kit (Fluka® Analytical, USA). Ultrathin sections (70 nm) were obtained with an ultramicrotome (Leica EM UC6), mounted on copper grids, and observations were carried out on an FEI-Tecnai G2 Spirit Bio Twin transmission microscope at 100kV (iLAB – Bioimaging Lab, iCBR – Coimbra Institute for Clinical and Biomedical Research, Faculty of Medicine, University of Coimbra).

DNA extraction, PCR amplification, and sequencing. Molecular analyses were performed according to Soares et al. (2019b, 2020b). Briefly, genomic DNA was extracted using NZY Microbial gDNA Isolation kit NZYTech (NZYTech™, Portugal), following the manufacturer's protocol, and 16S rRNA gene amplified with primer pairs Cya106F/Cya781R and Cya359/Cya1494R (Neilan et al. 1997, Nübel et al. 1997), and the entire 16S-23S rRNA ITS region amplified with primer pair P2/P1 (Boyer et al. 2001). PCR products were purified using the NZYGelpure DNA purification kit (NZYTech™, Portugal) and sequenced with the same primers described above using an ABI 3730xl DNA Analyzer system (96 capillary instruments) at STABVIDA, Portugal.

16S rRNA gene phylogenetic analyses. The obtained sequences were trimmed and assembled using Geneious® 10.2.2 software (https://www.geneious.com). Sequences were deposited in GenBank: 16S rRNA gene accession # MW172408; 16S-23S ITS accession # MZ788631 and compared with available GenBank sequences by BLASTN (Altschul et al. 1990). A preliminary analysis based on a BLAST search showed that the isolated strain belonged to the order Synechococcales, as it shared 93% similarity with a GenBank sequence identified as *Aphanocapsa* sp. GenBank # KM350248 and 92% similarity with *Oculatella leona* GenBank # MK248001. For this reason, a 16S rRNA gene sequence dataset was created to include representatives of most of the cyanobacteria taxa within the Synechococcales, and it was based on sequences used in the studies of Mai et al. (2018), Akagha et al. (2019), Komárek et al. (2020), and Konstantinou et al. (2021). Alignment was performed using the online version of MAFFT (https://mafft.cbrc.jp/alignment/server/; Katoh and Standley 2013), and UGENE 1.26.3 (Okonechnikov et al. 2012) was used to visually check and correct the resulting alignment (if applicable). The final aligned dataset (182 sequences for a total of 1,043 nucleotide positions) was used to conduct both Bayesian (BI) and Maximum Likelihood (ML) phylogenetic analyses. The Bayesian inference (BI) was conducted with MrBayes XSEDE V3.2.6 through the CIPRES Science Gateway (Miller et al. 2010), and the best fit model, previously calculated using MrModeltest v.2.4 (Nylander 2004), was set to be GTR + I + G. The analysis resulted in an estimated sample size (ESS) above 200 for all parameters, a value typically accepted as sufficient by phylogeneticists

(Drummond et al. 2006). The final average standard deviation of split frequencies was 0.009 and the potential scale reduction factor (PSRF) approached 1, indicating the convergence of the MCMC analysis. The convergence was also confirmed in Tracer v.1.5 software (Rambaut and Drummond 2007). The ML analysis was performed using the IQTree online version (Trifinopoulos et al. 2016) with 1,000 ultrafast bootstrap replicates and the selected option "approximate Bayes test." For both analyses, *Gloeobacter violaceus* PCC 8105 was used as the outgroup and FigTree v.1.1.2 (Rambaut and Drummond 2008) was used for tree visualization. Both analyses resulted in the same tree topology; only one tree with both Bayesian posterior probabilities and maximum likelihood bootstrap values (BI/ML, respectively) is shown. In addition, we constructed a second 16S rRNA gene tree based on the previous one but including the environmental sequences present among the first BLAST hits (from 92% to 98% similarity). The alignment and both BI and ML analyses were conducted as previously mentioned, and the resulting tree is provided in the supplementary materials (Fig. S1 in the Supporting Information). For the calculation of the 16S rRNA gene p-distance matrix, we used sequences of representative taxa from families within the order Synechococcales and other closely related taxa that were selected on the basis of the results from the phylogenetic and phylogenomic analyses. Multiple sequence alignment and p-distance calculation were performed using MUSCLE in MEGA X (Kumar et al. 2018).

Phylogenomic analysis. The whole-genome phylogeny was assessed by using bcgTree v1.1.0 (Ankenbrand and Keller 2016) that extracts 107 essential single-copy genes (Dupont et al. 2012) from amino acid sequences of whole-genome data and reconstructs a phylogenetic tree using a partitioned ML analysis. A total of 101 available and annotated genomes of cyanobacteria within Synechococcales and the genome of the isolated strain (see Soares et al. 2020a for details; NCBI accession number PRJNA596374) were used as the final dataset.

ITS secondary structures. The secondary structures D1-D1′, Box-B, and V3 of the ITS regions were found using LocARNA-Alignment and Folding (Will et al. 2007, 2012, Smith et al. 2010) and were folded individually using the Mfold WebServer 3.5 with default conditions, except for the application of the structure draw mode with "untangle loop fix" (Zuker 2003). The tRNA genes were found using the tRNAscan-SE 2.0 webserver (Lowe and Chan 2016).

RESULTS

Phylogenetic and phylogenomic analyses. Analysis using BLAST (Altschul et al. 1990) showed that the newly isolated *Petrachloros* strain shared 16S rRNA gene sequence identities of 93% (100% query cover) and 92% (100% query cover) with GenBank sequences identified as *Aphanocapsa* sp. BDU 130052 (KM350248) and *Oculatella leona* ATE710 (MK248001), respectively. In addition, it also showed identities ranging from 93% to 99% with uncultured cyanobacterial clones, namely Alchichica AQ1 2 1B (JN825308, 99%; 100% query cover), AL58 2CY 30 (HQ419058, 96%; 92% query cover), VERDEA64 (FJ902632, 94%; 100% query cover), Alchichica AL64 1 1B (JN825309, 93%; 100% query cover), and YM-4 (JQ769946, 93%; 100% query cover).

Both BI and ML analyses placed the *Petrachloros* strain in a well-separated and highly supported (1/99%; PP/BS) family-level clade encompassing sequences identified as *Aphanocapsa* sp. BDU 130052, with *Lagosinema* and sequences assigned to *Limnothrix* as the closest, although quite distant, taxa (Fig. 1). The same topology was obtained for the supplementary BI and ML analyses, which also showed that *Petrachloros* was placed in a well-differentiated clade (1/99%; PP/BS) encompassing the sequence assigned to *Aphanocapsa* sp. BDU 130052 and the uncultured cyanobacterial clones (with the exception of clone YM-4), with *Lagosinema* and *Limnothrix* as the closest (but quite distant) taxa (Fig. S1).

In the phylogenomic analysis, the *Petrachloros* strain was placed in a separate lineage, apart from the family Thermosynechoccaceae (order Pseudanabaenales), but with Acaryochloris marina MBIC 11017 and *Aphanocapsa montana* BDHKU210001 as the closest, although quite distant, taxa (Fig. 2). To the best of our knowledge, Acaryochloris marina MBIC 11017 is currently an invalid genus and species and for this reason, this name is not italicized (Hauer and Komárek 2022).

For the calculation of the 16S rRNA p-distance matrix, we used sequences of representative taxa from families within the order Synechococcales, in addition to the sequences of *Aphanocapsa* sp. BDU 130052, *Aphanocapsa muscicola* 5N-04, Acaryochloris marina MBIC 11017, and *Thermosynechococcus vestitus*. The sequence of the *Petrachloros* strain shared 93% sequence similarity with *Aphanocapsa* sp. BDU 130052; 92% with *Lagosinema tenuis*; 91% with Acaryochloris marina; and 90% with *Aphanocapsa montana, A. muscicola,* and both species of *Limnothrix* (*L. redekei* and *L. planktonica*). When compared to the remaining taxa, *Petrachloros* shared 88–91% sequence similarities with members of Oculatellaceae (*Oculatella subterranea* and *Droutiella lurida*); 90% with members of Leptolyngbyaceae (*Leptolyngbya boryana* and *Alkalinema pantanalense*) and Trichocoleusaceae (*Trichocoleus desertorum*); 89–90% with members of Prochlorotrichaceae (*Prochlorothrix hollandica, Halomicronema excentricum,* and *Nodosilinea nodulosa*); 89% with members of Pseudanabaenaceae (*Pseudanabaena* sp.); and 88–89% with members of Prochlorococcaceae (*Prochlorococcus* sp., *Parasynechococcus* sp., and *Cyanobium gracile* [see Table 1]).

Following the work of Mai et al. (2018), who proposed single nucleotide variations in five helices of the 16S rRNA gene (helices 18, 20, 23, 27, and 34) as indicative of family-level clades, we have searched for those in the 16S rRNA gene sequence of *Petrachloros. Petrachloros* had identical sequences for three helices indicative of the Prochlorotrichaceae (helices 18, 23, and 34) and Trichocoleusaceae (helices 18, 20, and 27); for two helices characteristic of the Leptolyngbyaceae (helices 20 and 23), Oculatellaceae (helices 20 and 27), and Pseudanabaenaceae (helices 18 and 34); and none for the Gloeobacteraceae. Although the nucleotide variations within 16S rRNA gene helices are not considered
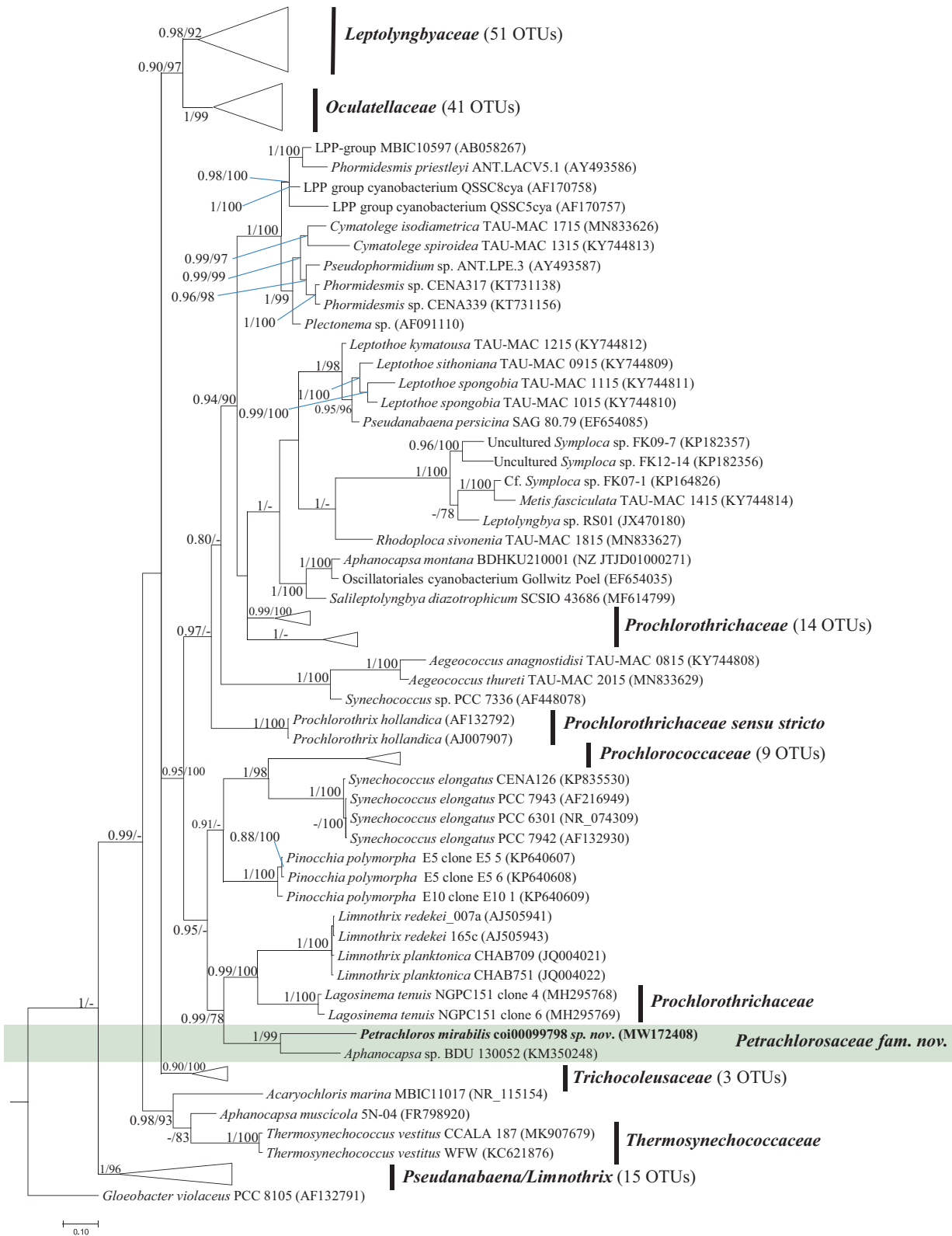
FIG. 1. Phylogenetic tree based on the Bayesian Inference and Maximum Likelihood analyses from partial 16S rRNA gene sequences. Support at the nodes represents posterior probabilities and bootstrap values (PP/ML, respectively) ≥ 75. *Petrachloros mirabilis* is shown in bold and *Gloeobacter violaceus* PCC 8501 was used as the outgroup. The first combination of letters and numbers after each taxon name corresponds to cyanobacterial strains, whereas the numbers given in parentheses () are GenBank accession numbers. The scale bar specifies 0.10 expected changes per site. [Color figure can be viewed at wileyonlinelibrary.com]

FIG. 2. Phylogenomic tree based on 107 essential single-copy genes from amino-acid sequences. *Petrachloros mirabilis* is shown in bold, and *Gloeobacter violaceus* and *G. kilaueensis* were used as the outgroups. Support at the nodes represent bootstrap values, and asterisk indicates a value of 100%. RefSeq assembly accession numbers are given for each taxon. The scale bar specifies 0.10 expected changes per site. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1. Similarity matrix based on the 16S rRNA gene sequence data from *Petrachloros mirabilis* (MZ788631) and closely related taxa from families within the Synechococcales

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. *Petrachloros mirabilis* (MZ788631)																							
2. *Aphanocapsa* sp. BDU 130052 (KM350248)	93%																						
3. *Lagosinema tenuis* NGPC151 clone 4 (MH295768)	92%	92%																					
4. *Acaryochloris marina* MBIC11017 (NR_115154)	91%	90%	90%																				
5. *Oculatella subterranea* VRUC135/Albertano 1985 (X84809)	91%	92%	92%	91%																			
6. *Aphanocapsa montana* BDHKU210001 (NZ_JTJD01000271)	90%	92%	93%	91%	92%																		
7. *Aphanocapsa muscicola* 5N-04 (FR798920)	90%	91%	90%	94%	91%	90%																	
8. *Leptolyngbya boryana* PCC 6306 (EF429290)	90%	90%	90%	89%	91%	89%	90%																
9. *Trichocoleus desertorum* WJT46-NPBG1 (KF307608)	90%	92%	91%	92%	92%	91%	94%	89%															
10. *Thermosynechococcus vestitus* WFW (KC621876)	90%	90%	89%	91%	91%	90%	94%	89%	93%														
11. *Halomicronema excentricum* TFEP1 (AF320093)	90%	92%	92%	90%	91%	93%	93%	90%	92%	92%													
12. *Limnothrix redekei* 007a (AJ505941)	90%	90%	93%	90%	90%	91%	90%	89%	91%	90%	90%												
13. *Limnothrix planktonica* CHAB709 (JQ004021)	90%	90%	93%	90%	90%	91%	90%	89%	91%	90%	90%	0%											
14. *Nodosolinea nodulosa* UTEX 2910 (EF122600)	90%	90%	90%	91%	91%	92%	92%	88%	92%	91%	93%	90%	90%										
15. *Alkalinema pantanalense* CENA528 (KF246494)	90%	90%	90%	91%	91%	90%	91%	93%	91%	91%	90%	90%	90%	89%									
16. *Aggeococcus thureti* TAU-MAC (MN833629)	90%	90%	88%	89%	89%	88%	90%	88%	89%	90%	91%	87%	87%	90%	88%								
17. *Prochlorothrix hollandica* (AF132792)	89%	91%	91%	91%	89%	92%	91%	88%	92%	91%	91%	91%	91%	91%	90%	89%							

(continued)

TABLE 1. (continued)

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
18. *Synechococcus elongatus* PCC 6301 (NR_074309)	89%	91%	92%	90%	90%	90%	91%	89%	90%	90%	90%	90%	90%	90%	90%	87%	92%						
19. *Parasynechococcus* sp. WH8016 (AY172834)	89%	90%	90%	89%	88%	89%	89%	88%	90%	89%	89%	89%	89%	89%	89%	87%	90%	91%					
20. *Pseudanabaena* sp. PCC 7367 (AB039018)	89%	88%	89%	91%	89%	89%	91%	89%	91%	91%	89%	89%	89%	90%	89%	88%	90%	88%	87%				
21. *Prochlorococcus* sp. MIT9312 (AF053398)	88%	90%	89%	90%	88%	88%	89%	88%	90%	89%	89%	89%	89%	89%	89%	87%	90%	91%	97%	87%			
22. *Drouetiella lurida* 1986/6 (HM018690)	88%	88%	88%	88%	92%	88%	89%	88%	90%	87%	89%	87%	87%	88%	88%	86%	88%	87%	87%	87%	87%		
23. *Cyanobium gracile* PCC 6307 (NR_102447)	88%	90%	89%	90%	88%	89%	89%	87%	91%	90%	89%	91%	91%	89%	89%	87%	91%	91%	96%	87%	96%	87%	87%

universally applicable for family-level assignment due to its highly conservative nature (Mai et al. 2018), *Petrachloros* still does not match perfectly any family for these positions, therefore highlighting its distinctiveness (Table S1 in the Supporting Information).

ITS secondary structures. *Petrachloros* possessed both tRNA[Ile] and tRNA[Ala] genes (separated by 4 nucleotides) and the ITS secondary structures D1-D1′, Box-B, and V3. At the primary structure level, a BLASTn analysis with the option megablast did not yield any significant similarity for the portions of ITS sequences before and after the two conserved tRNA genes. When a discontinuous megablast was used, some similarity was only found for a short stretch of sequence (40–60 bp) after the two tRNA genes, including the conserved Box A and D4 (Iteman et al. 2000). The ITS secondary structures of the isolated *Petrachloros* strain were compared with the ITS structures of representative taxa from four families within the Synechoccales, namely species *Lagosinema tenuis*, *Oculatella subterranea* (Oculatellaceae), *Trichocoleus desertorum* (Trichocoleusaceae), *Leptolyngbya boryana* (Leptolyngbyaceae), and *Prochlorotrix hollandica* (Prochlorotrichaceae; Figs. 3–5). We were not able to predict the ITS structures for the family Prochlorococcaceae and, therefore, they are not shown. The D1-D1′ helix of all studied strains differed from one another, with distinct helix shapes and lengths. Helix lengths varied from 51 nt (*L. boryana*) to 107 (*P. hollandica*). All strains presented a similar basal unilateral bulge, except for the isolated *Petrachloros* strain, which showed two unpaired nucleotides opposing the basal unilateral bulge on the 5′ side of the helix (Fig. 3). The Box-B helices of the studied strains were relatively similar to each other, with lengths varying from 30 nt (*L. tenuis*) to 37 nt (*T. desertorum*). The helices in species *L. boryana*, *P. hollandica*, *T. desertorum*, and *Petrachloros* presented an unpaired nucleotide opposing the basal unilateral bulge on the 5′ side, contrasting with *L. tenuis*, which showed three unpaired nucleotides on the 5′ side. All strains showed almost identical basal clamps and terminal hairpins, with the exception of the *Petrachloros* strain, which showed a different terminal hairpin (Fig. 4). As to the V3 region, all strains possessed highly variable helices, sharing no similar patterns between each other. Lengths varied between 23 nt (*L. boryana*) and 116 nt (*P. hollandica*; Fig. 5).

Descriptions of the new taxa. The following descriptions of new cyanobacterial taxa are proposed under the provisions of the International Code of Nomenclature for Algae, Fungi, and Plants (ICN; Turland et al. 2018).

Petrachlorosaceae F. Soares, Trovão & Portugal, fam. nov. (Figs. 6 and 7).

Description: Microscopic; spherical to hemispherical cells, solitary or forming colonies; reproduction by binary fission; thylakoids arranged parietally;
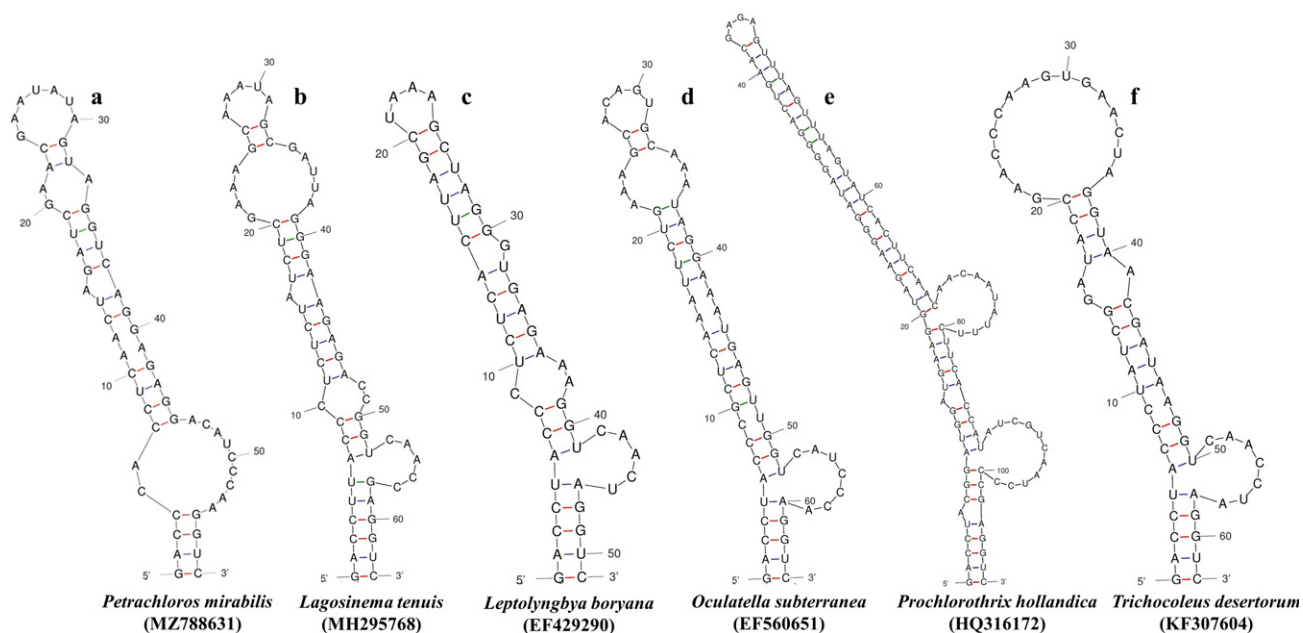
FIG. 3. D1-D1′ helix of the secondary structure of 16S-23S ITS region. (a) *Petrachloros mirabilis* (MZ788631); (b) *Lagosinema tenuis* (MH295768); (c) *Leptolyngbya boryana* (EF429290); (d) *Oculatella subterranea* (EF560651); (e) *Prochlorothrix hollandica* (HQ316172); (f) *Trichocoleus desertorum* (KF307604). [Color figure can be viewed at wileyonlinelibrary.com]
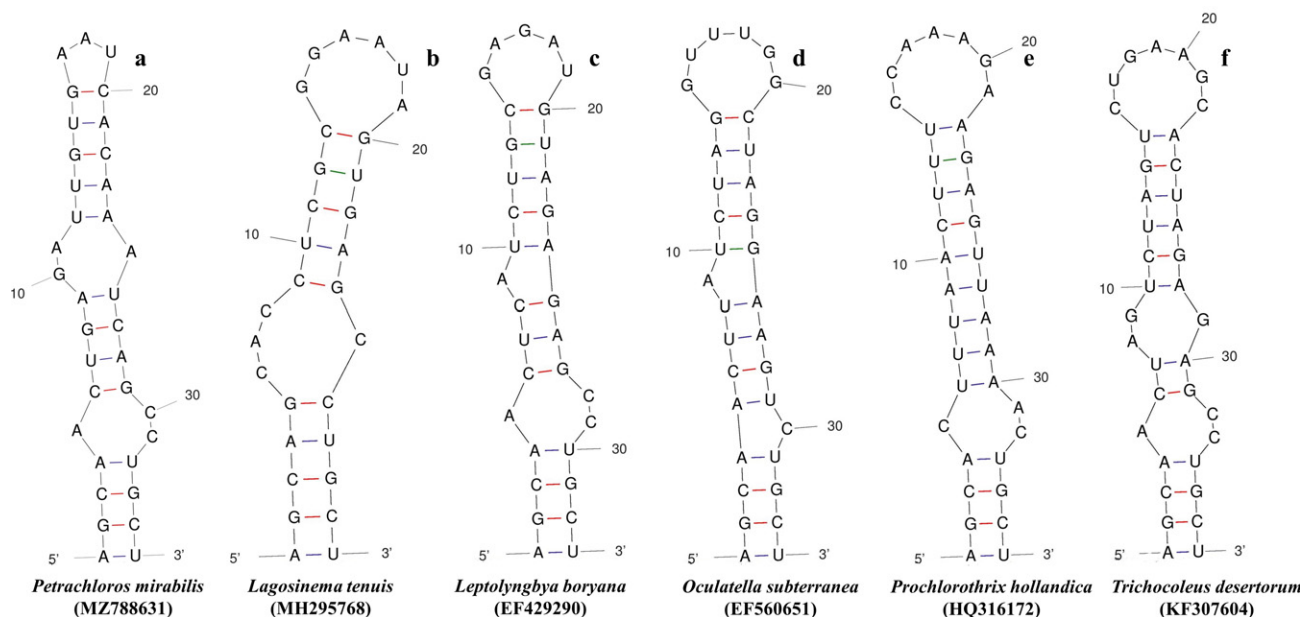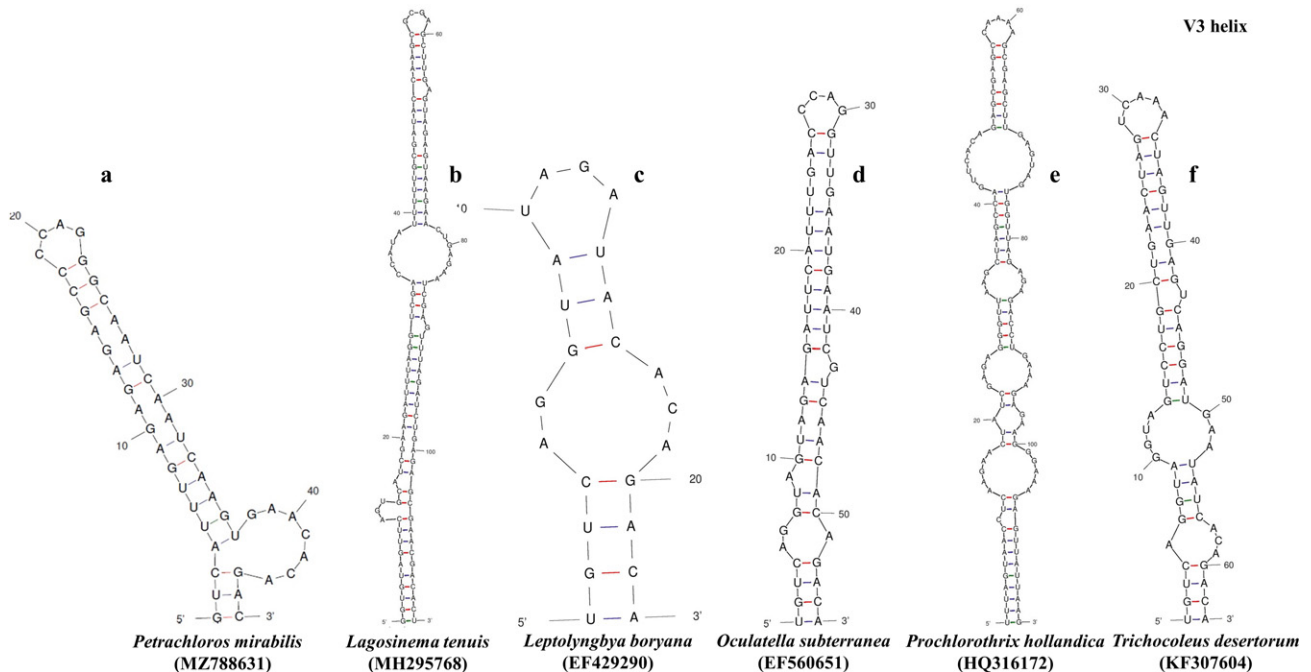

FIG. 4. Box-B helix of the secondary structure of 16S-23S ITS region. (a) *Petrachloros mirabilis* (MZ788631); (b) *Lagosinema tenuis* (MH295768); (c) *Leptolyngbya boryana* (EF429290); (d) *Oculatella subterranea* (EF560651); (e) *Prochlorothrix hollandica* (HQ316172); (f) *Trichocoleus desertorum* (KF307604). [Color figure can be viewed at wileyonlinelibrary.com]

individual mucilaginous envelopes facultative, rarely present.

Notes: Members of Petrachlorosaceae currently encompass coccoid forms, solitary or forming aggregates of many cells; reproduction by binary fission and parietally arranged thylakoids. Petrachlorosaceae differs from the other families within the Synechococcales on the basis of the 16S rRNA gene phylogeny, *p*-distance, the secondary structures of the 16S-23S ITS region, and ecology. *Petrachloros*

Fig. 5. V3 helix of the secondary structure of 16S-23S ITS region. (a) *Petrachloros mirabilis* (MZ788631); (b) *Lagosinema tenuis* (MH295768); (c) *Leptolyngbya boryana* (EF429290); (d) *Oculatella subterranea* (EF560651); (e) *Prochlorothrix hollandica* (HQ316172); (f) *Trichocoleus desertorum* (KF307604). [Color figure can be viewed at wileyonlinelibrary.com]

is distinct from members of Leptolyngbyaceae, Oculatellaceae, Prochlorotrichaceae, and Trichocoleusaceae, which are filamentous, whereas *Petrachloros* presents coccoid forms.

TYPE GENUS: *Petrachloros*

Petrachloros F. Soares, Trovão & Portugal, gen. nov. (Figs. 6 and 7).

Description: Microscopic; when in nature, growing on limestone surfaces. In liquid media, growing on the bottom of test tubes, sometimes forming thin biofilms attached to test tube walls. Color of cultures green, olivaceous-green, or yellow; cells spherical to hemispherical, solitary or forming colonies with a variable number of cells in compacted aggregates; reproduction by binary fission; thylakoids arranged parietally; with facultative, rarely present, individual mucilaginous envelopes.

ETYMOLOGY: The genus name was derived from the Greek words πέτρα, *Pétra*, meaning stone/rock, and χλωρός, chlōros/khlorōs, meaning green.

TYPE SPECIES: *Petrachloros mirabilis*

Petrachloros mirabilis F. Soares, Trovão & Portugal, sp. nov. (Figs. 6 and 7).

Description: Microscopic, growing on limestone surfaces. Color of cultures green, olivaceous-green, or yellow. Cells spherical to hemispherical, solitary or forming colonies with a variable number of cells in compacted aggregates; young cells rounded, 0.5–1.7 μm in diameter; mature cells enlarged, 1.3–1.9 μm; elongated cells before division, 1.3–3 μm in diameter; reproduction by binary fission in one

plane parallel to the short axis; thylakoids arranged parietally; facultative, rarely present, mucilaginous envelopes.

HOLOTYPE (here designated): COI00099798 (deposited at the Herbarium of the University of Coimbra, Portugal), 22 November 2016, Igor Tiago. Algal cultural material preserved in 4% formaldehyde.

ISOTYPE: COI00099799, deposited at the Herbarium of the University of Coimbra, Portugal, preserved in 4% formaldehyde; COI00103200, deposited as dried material at the Herbarium of the University of Coimbra, Portugal.

TYPE LOCALITY: Portugal. Old Cathedral of Coimbra, Almedina, Coimbra, (40°12′32″ N, 8°25′38″ W), attached to the limestone walls of the Chapel of São Miguel. REFERENCE STRAIN: ULC683, deposited at the BCCM/ULC culture collection, Liège, Belgium.

ETYMOLOGY: The species name refers to the royal charter "*Scientiae thesaurus mirabilis*" signed by King D. Dinis in 1290 when the University of Coimbra, the first University in Portugal, was created.

DNA SEQUENCES: Sequences were deposited in GenBank with accession numbers MW172408 and MZ788631.

Ecology. The strain was collected from the limestone walls of Chapel of São Miguel, situated on the eastern area of the Old Cathedral of Coimbra. Due to its architectural structure, the chapel is not exposed to direct sunlight. During the sampling
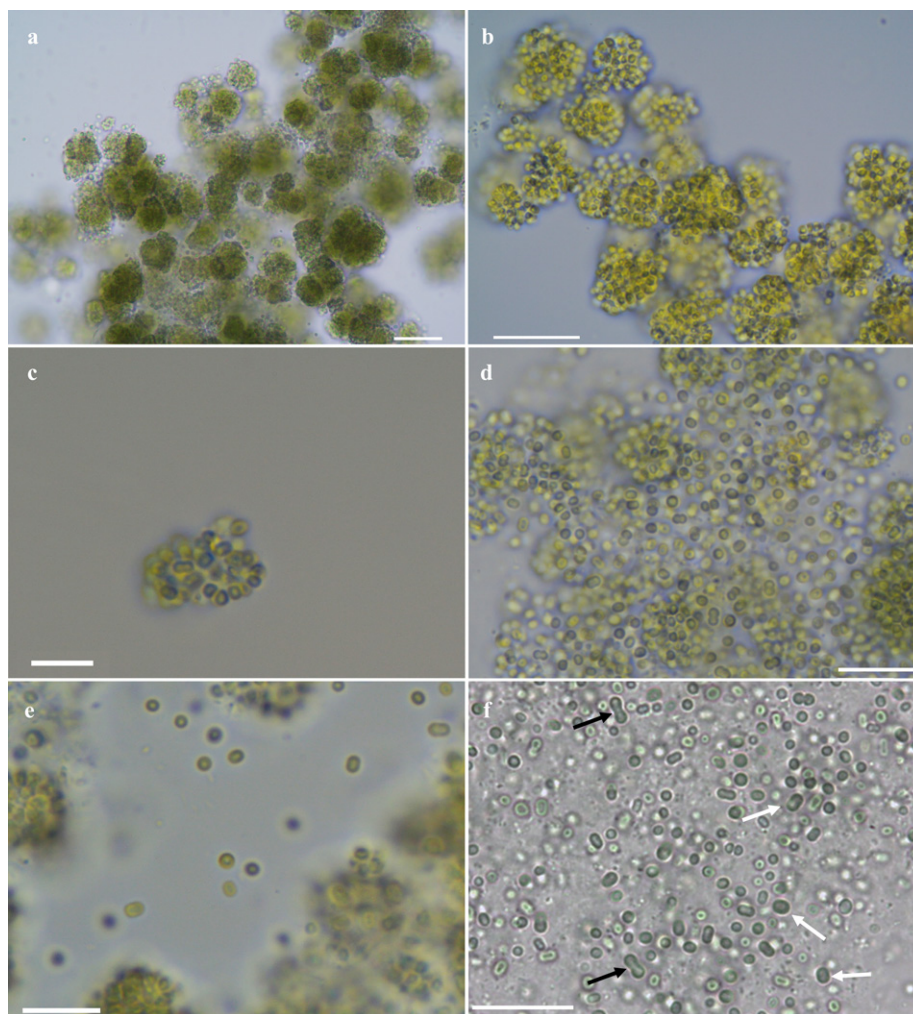
Fig. 6. Photomicrographs of *Petrachloros mirabilis*. General characteristics showing: colonies of many cells in compacted aggregates (a, b, c); solitary, spherical to hemispherical cells (d, e, f); elongated cells prior to division (f; black arrows); enlarged cells (f); white arrows. Scale bars: (a, b) = 20 μm; (c) = 5 μm; (d, e, f) = 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

day, the temperature was ~11–12°C and the relative humidity inside the chapel was 56%. The strain was collected from a wet dark-green biofilm with salt efflorescence where other phototrophic and heterotrophic organisms (fungi) were also observed, namely the unicellular chlorophytes *Pseudochloris* sp. and *Bracteacoccus* sp., and the fungal genera *Aspergillus*, *Cladosporium*, *Mortierella*, and *Parengyodontium* (see Soares et al. 2019a and Trovão et al. 2019a).

DISCUSSION

The latest classification system advocated by Komárek et al. (2014) recognized the Synechococcales as a large order with 74 genera. According to Akagha et al. (2019), 18 additional genera subsequently have been described. Recently, Konstantinou et al. (2021) established five additional new genera. This order encompasses both unicellular and filamentous forms with a parietal thylakoid

arrangement. *Leptolyngbya*, *Prochlorococcus*, *Synechococcus*, and *Pseudanabaena* are considered the most commonly encountered genera (Whitton and Potts 2000, Komárek et al. 2014, Dvořák et al. 2017). Mai et al. (2018) revised the Synechococcales by splitting the family Leptolyngbyaceae into four monophyletic families, two newly described, Oculatellaceae and Trichocoleusaceae, and two traditional families redefined, Leptolyngbyaceae and Prochlorotrichaceae. The Prochlorotrichaceae, first described by Burger-Wiersma et al. (1989) and only comprising the genus *Prochlorothrix*, was extended by Mai et al. (2018) to accommodate other genera, namely *Halomicronema* and *Nodosilinea*. Later, Akagha et al. (2019) described the filamentous genus *Lagosinema*, placing it in the Prochlorotrichaceae. However, both Mai et al. (2018) and Akagha et al. (2019) noted that the Prochlorotrichaceae could possibly be split in the future, thus requiring further revisions. In our 16S rRNA phylogenetic analyses, *Petrachloros* is
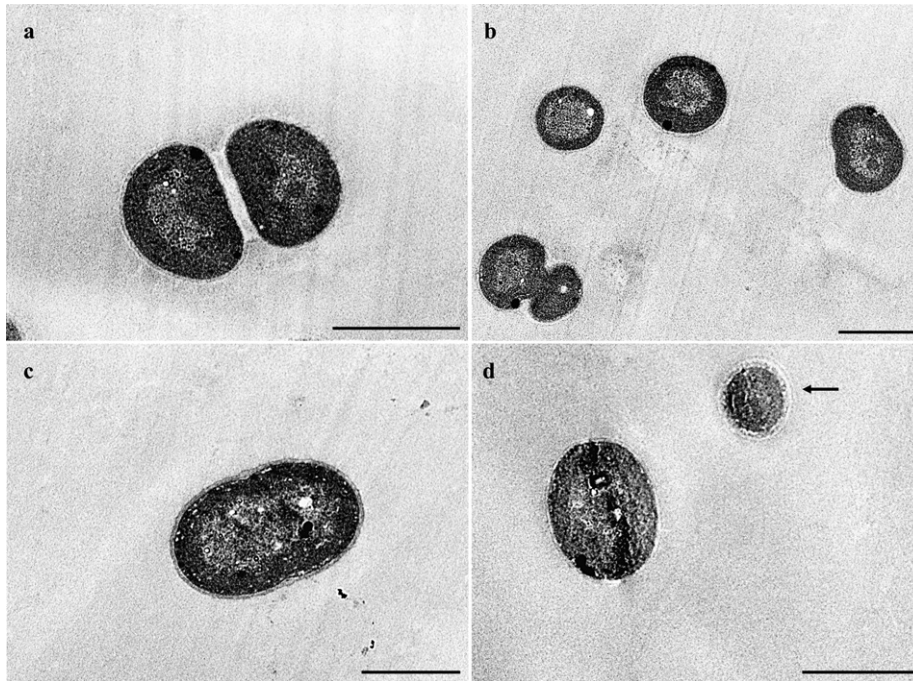
Fɪɢ. 7. Transmission electron micrographs of cells of *Petrachloros mirabilis* coi00099798. Photomicrographs showing cell division in one plane (a, b); parietal thylakoids (c); mucilage layer (d; black arrow). Scale bars: 1 μm. [Color figure can be viewed at wileyonlinelibrary.com]

placed in a separated and highly supported clade, as part of a large clade that comprises the families Prochlorococcaceae and Prochlorotrichaceae, with *Lagosinema* as the closest taxa. However, in our phylogenetic analyses, *Lagosinema* clusters separately outside the Prochlorotrichaceae. This separation was also observed by Komárek et al. (2020). In addition, Akagha et al. (2019) stated that the low genetic identity between *Lagosinema* and other members of the Prochlorotrichaceae was a sufficient justification to separate *Lagosinema* from that family. Similarly, considering that our analyses placed *Petrachloros* apart from the Prochlorotrichaceae but with *Lagosinema* as the closest relative (although the p-value is 92%), placing *Petrachloros* in that family would create a polyphyletic taxon. According to Komárek et al. (2014), it is preferable to have well-defined monophyletic genera composed by few species, rather than large polyphyletic genera containing many unrelated species. Therefore, we support the statements of Komárek et al. (2014), Mai et al. (2018), and Akagha et al. (2019) about the need to revise the Prochlorotrichaceae and other families within the order Synechococcales to create well-defined and monophyletic taxonomic groups.

The 16S rRNA gene phylogenetic tree (Fig. S1) also showed that *Petrachloros* forms a cluster with environmental sequences isolated during the studies of Couradeau et al. (2011), Kaźmierczak et al. (2011), and Sahl et al. (2010) and a GenBank 16S rRNA gene sequence identified as *Aphanocapsa* sp. BDU 130052 (Bhuvaneshwari et al. 2017). However, to the best of our knowledge, none of latter strains were formally described. On the other hand, the genomic analysis revealed that *Petrachloros* falls within a separated lineage, distant from the Thermosynechococcaceae, but with the strains Acaryochloris marina MBIC 11017 and *Aphanocapsa montana* BDHKU210001 as the closest taxa, although they are distant. However, and as observed in the online cyanobacteria database CyanoDB 2.0 (Hauer and Komárek 2022), Acaryochloris marina is an invalid genus and species, as it does not meet the requirements of article 8.4 of the ICN. Thus, we propose a new family, Petrachlorosaceae, to accommodate the new genus and species *Petrachloros mirabilis*. However, it is important to recognize that, although the use of genomes in higher level taxonomy is desirable, the low number of genomes currently available in databases hinders the use of phylogenomics as the unique criterion on which to confidently base a new family. Indeed, a number of closely related taxa based on the 16S rRNA gene sequence similarity do not have sequenced genomes, which influences the tree topology. In this work, the phylogenetic and phylogenomic analyses generated different groupings. For example, *Limnothrix* was the closest taxon to *Petrachloros* in the 16S rRNA gene phylogenetic analysis but was placed distantly in the phylogenomic analysis. Similarly, Acaryochloris marina and *Aphanocapsa montana* were distant in the 16S rRNA gene phylogenetic analysis but appeared closely related to *Petrachloros* in the phylogenomic tree.

Yarza et al. (2014) recommended a cut-off value of less than 86.5% for the recognition of different bacterial families and less than 94.5% for different genera. However, this criterion alone would be unhelpful in our case, given that the calculated p-distance based on the 16S rRNA gene between *Petrachloros* and the type species (or representative strains) of genera from families within the Synechococcales varied between 88% and 92%. We do not take into account the calculated p-distances between sequences belonging to *Petrachloros* and the strains *Aphanocapsa* sp. BDU 130052 (93%), *Aphanocapsa montana* BDHKU210001 (90%), and *Aphanocapsa muscicola* 5N-04 (90%) strains, as we are not certain that these sequences belong to *Aphanocapsa*, given that the type species is not yet sequenced and its current taxonomic placement is still unknown. Nonetheless, Mai et al. (2018) discussed that the use of cut-off values has some limitations, and that cyanobacterial families in the Synechococcales are recognized because "*they form highly supported phylogenetic clusters of genera, recognized by morphology or using a combination of morphology and phylogeny.*" We do agree with these authors, since using the cut-off values suggested by Yarza et al. (2014) in such a strict way would result in placing *Petrachloros* as *incertae familiae*. In addition, Yarza et al. (2014) listed some bacterial families with minimal similarity values well above 86.5% (e.g., Shewanellaceae with 91%; Nitrosomonadaceae with 93%; Dietziaceae with 95%).

The secondary structure of the 16S–23S ITS region offers several advantages as a taxonomic criterion, visually displaying how the semiconservative motifs of the ITS are related (Johansen et al. 2011). Secondary structure has been used to distinguish taxa at the species level (Boyer et al. 2001, Johansen et al. 2011, Hašler et al. 2014), but some authors have also found that these regions can present genus-specific structures that help with the separation of deep genetic lineages (Mai et al. 2018). The D1-D1′ helix of *Petrachloros* presented two unpaired nucleotides on the 5′ side of the helix, a characteristic not observed in any other genera studied here, reinforcing its taxonomic distinctiveness. On the other hand, the Box-B helices of all studied strains were very similar to one another in shape and length. The only difference between *Petrachloros* and the other strains was in the terminal hairpin, where it only possessed three nucleotides. As to the V3 helix, it appeared very variable and unspecific as already reported in other species and genera (e.g., Řeháková et al. 2007, Lukešová et al. 2009, Shalygin et al. 2020).

Morphologically, *Petrachloros* is very similar to *Aphanocapsa*. Cuzman et al. (2010) isolated two strains of *A. muscicola* (*A. muscicola* 5N-04 (FR798920) and *A. muscicola* VP3-03 (FR798916)), but no details regarding their morphological characteristics were published. Therefore, we were not able to compare them. *Petrachloros* is morphologically similar to the strain *Aphanocapsa* sp. BDU 130052 isolated and characterized by Bhuvaneshwari et al. (2017). These authors morphologically characterized 12 marine unicellular cyanobacteria and investigated their phylogenetic positions. However, they did not use the data from their polyphasic approach to propose a formal and valid taxonomic description. The similarities between *Petrachloros* and this *Aphanocapsa* sp. strain resided mostly in the color, spherical shape of the cells, type of reproduction (binary fission), and parietal thylakoid arrangement. The morphology of *Petrachloros* is very distinct from members of Leptolyngbyaceae, Oculatellaceae, Prochlorotrichaceae, and Trichocoleusaceae, as these are filamentous taxa and *Petrachloros* presents unicellular coccoid forms. On the other hand, *Petrachloros* presents a morphology similar to members of the Prochlorococcaceae, which are mainly characterized as unicellular, coccoid, small sized, reproducing by binary fission and with parietal thylakoids. *Petrachloros* is morphologically most similar to *Prochlorococcus and Parasynechococcus* but is different from *Cyanobium*, which presents shortly oval to rod-shaped cells. As *Petrachloros* is phylogenetically placed distantly from the Prochlorococcaceae and since its morphology is also very different from the other remaining families, it reinforces the need to create a new family to correctly accommodate this strain.

Ecologically, *Petrachloros* occurs on limestone walls. This strain was isolated from a wet dark-green biofilm with salt efflorescence in one of the chapels of the Old Cathedral of Coimbra (Soares et al. 2019a). These efflorescences are believed to be due to the solubilization of salts (mainly gypsum) by water (Trovão et al. 2019a). The fact that *Petrachloros* was isolated from this type of biofilm may indicate that this strain has the ability to withstand briny conditions, probably giving it enough phenotypic plasticity to be able to inhabit other extreme environments. The three environmental clones that cluster with *Petrachloros*, namely Alchichica AQ1 2 1B, AL58 2CY, and Alchichica AL64 1 1B 03, were found in the alkaline lake Alchichica in Mexico and were isolated from microbialites predominantly composed of hydromagnesite (Couradeau et al. 2011, Kaźmierczak et al. 2011), reinforcing our assumptions. In general, the ecology of *Petrachloros* differs from that of the strain *Aphanocapsa* sp. BDU 130052 and members of Prochlorococcaceae, which are mainly from marine/freshwater environments. Members from other families, namely Oculatellaceae, Prochlorotrichaceae, Leptolyngbyaceae, and Trichocoleusaceae, have a more diverse ecology, inhabiting terrestrial and aquatic environments.

Other taxonomic considerations. Considering the 16S rRNA gene phylogenetic analyses presented in this work, four of the new genera described by Konstantinou et al. (2021) in the order Synechococcales (namely *Cymatolege, Metis, Rhodoploca*, and

Aegeococcus) are placed near other Prochlorotrichaceae members (i.e., *Halomicronema* and *Nodosilinea*). Of these, *Halomicronema*, *Nodosilinea*, *Cymatolege*, *Metis*, and *Rhodoploca* have filamentous morphotypes, but *Aegeococcus* presents a simple coccoid morphology similar to *Synechococcus* (family Synechococcaceae). Although Konstantinou et al. (2021) placed *Aegeococcus* within the Synechococcaceae, they have pointed out that this genus diverges at the base of this family, a peculiarity also observed in our 16S rRNA phylogenetic analyses. Similarly, *Pinocchia* is placed outside the family Leptolyngbyaceae (where it originally belonged) in our study, but also in the recent works of Becerra-Absalón et al. (2020) and Kim et al. (2021). Considering all the information available, we believe these taxa deserve some attention, and their phylogenetic positions should be reconsidered in the future.

In the present work, we proposed the establishment of a new family – Petrachlorosaceae – to correctly accommodate the new genus and species *Petrachloros mirabilis* within the order Synechoccocales. The data gathered in this study strongly emphasize the need for a taxonomic revision of several cyanobacterial taxa within this order, and we hope to have presented a valuable contribution to this ongoing revision work. We also encourage other authors that may have isolated *Aphanocapsa*-like microorganisms to describe them in order to better understand the phylogenetic position of this classical genus.

AUTHOR CONTRIBUTIONS

F. Soares: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Software (lead); Visualization (lead); Writing – original draft (lead). **J. Trovão:** Formal analysis (supporting); Investigation (supporting); Software (supporting); Writing – review & editing (supporting). **A. C. Ahn:** Investigation (supporting); Methodology (supporting); Writing – review & editing (supporting). **A. Wilmotte:** Investigation (supporting); Methodology (supporting); Writing – review & editing (supporting). **S. M. Cardoso:** Funding acquisition (supporting); Supervision (supporting); Validation (supporting); Writing – review & editing (supporting). **I. Tiago:** Funding acquisition (supporting); Software (supporting); Supervision (supporting); Validation (supporting); Writing – review & editing (supporting). **A. Portugal:** Funding acquisition (lead); Project administration (lead); Resources (supporting); Supervision (lead); Validation (lead); Writing – review & editing (supporting).

DATA AVAILABILITY STATEMENT

The obtained 16S rRNA and 16S-23S rRNA sequences were submitted to GenBank (accession numbers MW172408 and MZ788631, respectively).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Expanded phylogenetic tree based on the Bayesian Inference and Maximum Likelihood analyses from partial 16S rRNA gene sequences. Support at the nodes represents posterior probabilities and bootstrap values (PP/ML, respectively) $\geq$ 75. The scale bar specifies 0.05 expected changes per site.

Table S1. Sequence comparisons for the five helix sequences indicative of family-level clades following Table 4 of Mai et al. (2018). For the *Petrachloro*s sequence, it is written in bold and the parsimony-informative nucleotides are in red. The family-level sequences are in bold when they are identical to *Petrachloro*s, or the different positions are in red.