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

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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 Prochlorococaceae 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 Thermosynechococaceae, 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 Petrochloraceae fam. nov., along with the description of Petrochloros gen. nov. and Petrochloros 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 2005, 2016), 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 genera or species (Kavlie 1993). Nonetheless, revisionary work has already commenced with the reformation of the Synechococcales by Mai et al. (2018), who split the Leptolyngbyaceae into four families (Leptolyngbyaceae, Oculatellaceae, Prochlorotrichaceae, and Trichocoleaceae). 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 2013), which often lead to biodeterioration and loss of valuable cultural heritage materials (Scheerer 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 – Petrarichlorosaceae – to accommodate Petrarichloris 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 BG11 culture medium (Rippka et al. 1979) and incubated at 20 ± 1°C, under a 16:8 h (light:dark) photoperiod (30–40 μmol photons · m⁻² · 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 unicellular 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%), impregnated, and embedded using an Epoxy Embedding Kit (NUCYTech 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 Cya559/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 # MZ788651 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 # MK249801. 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 (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 1,045 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 v2.4 (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.
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 Thermosynechococcaceae (order Pseudanabaenales), but with Acaryochloris marina MBIC 11017 and Aphanocapsa montana BDKH1210001 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 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).

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 95% to 99% with uncultured cyanobacterial clones, namely Alchichica A01_2 1B (JN825308, 99%; 100% query cover), AL58 2CY 30 (HQ419058, 96%; 92% query cover), VERDEA64 (FJ902632, 94%; 100% query cover), Alchichica AL64 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
**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. kilaeuensis* were used as the outgroups. Support at the nodes represent bootstrap values, and asterisk indicates a value of 100%. ReSeq 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]
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(continued)
universally applicable for family-level assignment due to its highly conservative nature (Mai et al. 2018), Petrichloros still does not match perfectly any family for these positions, therefore highlighting its distinctiveness (Table S1 in the Supporting Information).

*ITS secondary structures.* Petrichloros possessed both tRNA<sup>Ile</sup> and tRNA<sup>Ala</sup> genes (separated by 4 nucleotides) and the ITS secondary structures D1-D1<sub>0</sub>, 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 Petrichloros 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 (Trichocoleaceae), 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<sub>0</sub> 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 Petrichloros 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 Petrichloros 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 Petrichloros 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).

**PETRACLOROSACEAE FAM. NOV.**
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
is distinct from members of Leptolyngbyaceae, Oculatellaceae, Prochlorotrichaceae, and Trichocoleaceae, which are filamentous, whereas *Petrachloros* presents coccolid 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, mucilaginous envelopes.

**ETYMOLOGY:** The genus name was derived from the Greek words *πέτρα, χλόρος* meaning stone/rock and *νεός, κλόρος* 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 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...
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, Dvorák et al. 2017). Mai et al. (2018) revised the Synechococcales by splitting the family Leptolyngbyaceae into four monophyletic families, two newly described, Oculatellaceae and Trichocoleaceae, 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
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), Kaszmerczak 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 Thermosynechococcales, but with the strains *Acaryochloris* marina MBIC 11017 and *Aphanocapsa montana* BDHKU'210001 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 muscicola 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 ITS-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, Hasler 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, Lukesová 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 Trichocoleaceae, as these are filamentous taxa and Petrachloros presents unicellular coccosid forms. On the other hand, Petrachloros presents a morphology similar to members of the Prochlorococcales, which are mainly characterized as unicellular, coccosid, small sized, reproducing by binary fission and with parietal thylakoids. Petrachloros is morphologically most similar to Prochlorococcus and Parasynechococcus but is different from Cyanohium, which presents shortly oval to rod-shaped cells. As Petrachloros is phylogenetically placed distantly from the Prochlorococcales 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 2B, Al58 2CY, and Alchichica AL64 1 1B 03, were found in the alkaline lake Alchichica in Mexico and were isolated from microbiolites predominantly composed of hydromagnesite (Gourdeau et al. 2011, Kazmierczak 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 Prochlorococcales, which are mainly from marine/freshwater environments. Members from other families, namely Oculatellaceae, Prochlorotrichaceae, Leptolyngbyaceae, and Trichocoleaceae, 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 Constantino et al. (2021) in the order Synechococcales (namely Cymatolege, Metis, Rhodoplaca, and
Aegrocostoc) are placed near other Prochlororichiaeae members (i.e., Halomicronema and Nodosilinea). Of these, Halomicronema, Nodosilinea, Cymatoloege, Metis, and Rhodomplaca have filamentous morphotypes, but Aegrocostoc presents a simple cocoid morphology similar to Synechococcus (family Synechococcaceae). Although Konstantinou et al. (2021) placed Aegrocostoc 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 Synechococcales. 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.

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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. Portu
gal: Funding acquisition (lead); Project administration (lead); Resources (supporting); Supervision (lead); Validation (lead); Writing – review & editing (supporting).

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DATA AVAILABILITY STATEMENT

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

Becerra-Absalón, I., Johansen, J. R., Osorio-Santos, K. & Montejo, G. 2020. Two new Oculatella (Oculatellaceae,


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) ≥ 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 *Petrachlorosaceae* family, 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 *Petrachlorosaceae*, or the different positions are in red.