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Zarconia navalis gen. nov., sp. nov., *Romeriopsis navalis* gen. nov., sp. nov. and *Romeriopsis marina* sp. nov., isolated from inter- and subtidal environments from northern Portugal

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Abstract

The morphology, 16S rRNA gene phylogeny and 16S–23S rRNA gene ITS secondary structures of three strains of marine Cyanobacteria, isolated from inter- and subtidal environments from north Portugal were studied, resulting in the description of *Zarconia navalis* gen. nov., sp. nov. (Oscillatoriales *incertae sedis*), *Romeriopsis navalis* gen. nov., sp. nov. (Leptolyngbyaceae) and *Romeriopsis marina* sp. nov., named under the International Code of Nomenclature for algae, fungi, and plants. No diacritical morphological characters were found for the new genera and species. The 16S rRNA gene maximum-likelihood and Bayesian phylogenies supported that the genus *Zarconia* is a member of the Oscillatoriales, morphologically similar to the genera *Microcoleus* and *Phormidium*, but distant from them. The genus *Romeriopsis* is positioned within the Leptolyngbyaceae (Synechococcales) and is closely related to *Alkalinema*. The secondary structures of the D1-D1′, Box B, V2 and V3 helices corroborate the phylogenetic results. Furthermore, our study supports previous observations of polyphyletic Oscillatoriales families and reinforces the need for their taxonomic revision.

INTRODUCTION

Cyanobacteria are important primary producers in the world's oceans and shape both planktonic and benthic marine communities [1]. In addition, they produce a plethora of secondary metabolites, such as alkaloids, polyketides and peptides with a great variety of biological activities [2, 3]. Despite their global and biotechnological importance, the diversity of marine Cyanobacteria is still underestimated, and recently, many new genera of Cyanobacteria have been described, such as *Leptothoe* Konstantinou and Gkelis, *Marileptolyngbya* Zhou and Ling, *Salileptolyngbya* Zhou, *Lusitaniella* Ramos *et al*., *Dapis* Engene *et al*., *Capillus* Caires *et al*. and *Neolyngbya* Caires *et al*., for example [4–9].

The growing number of new taxa descriptions, the availability of 16S rRNA gene sequences for type species, and the access to powerful computational tools [10], has brought to light that many cyanobacterial families from the classical, morphological-based taxonomy are polyphyletic, warranting taxonomical revisions [11–13]. Up to now, the only revision at the order level for Cyanobacteria, on the basis of robust phylogenetic analysis and morphological descriptions, concerned the Synechococcales and was carried out by Mai *et al*. [12]. In that paper, two new families and six new genera containing 14 species are described. The authors consider five families in the order, one of which (Trichocoleusaceae) comprises a single genus.

Following this growing number of descriptions of new cyanobacterial taxa, in this paper we describe the new genera *Zarconia* (Oscillatoriales *incertae sedis*) and *Romeriopsis* (Leptolyngbyaceae Komárek *et al*.), from intertidal and subtidal environments sampled in north Portugal.

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LEGE 06013 GenBank accession for the 16S rRNA gene MN537584. LEGE 11480 GenBank accessions: MN758888 (16S rRNA gene) and JADEXQ000000000 (genome). LEGE 11467 GenBank accessions: MN537583 (16S rRNA gene), JADEXN000000000 (genome).
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Two supplementary figures, one supplementary table and one supplementary text are available with the online version of this article.

METHODS

Sampling and sites

Two samples were obtained using 50 ml sterile polypropylene syringes, from a rocky substrate at 13 m depth, in the subtidal zone at 'A Pedra' diving spot (São Francisco Xavier fort, ~200 m off the coast, in the city of Porto, Portugal, 41.185809° N 8.719079° W). The collection date was 3 October 2011. The samples were kept in 50 ml polypropylene tubes until being processed in the laboratory at CIIMAR, Porto, Portugal. These samples led to the isolation of strains LEGE 11467 and LEGE 11480, while strain LEGE 06013 had been previously isolated from a wave-exposed rock in Praia da Foz do Arelho, Caldas da Rainha (39.43327° N 9.230275° W), Portugal, as reported in Brito *et al.* (2012) [14] and obtained from the LEGE Culture Collection (LEGE-CC) at CIIMAR, Porto, Portugal (http://lege.ciimar.up.pt) [15]. The north Portuguese coastal environment where the studied strains were collected are vulnerable to strong tides and waves, especially in the winter [16].

Isolation strategy

After arrival at the laboratory, subtidal samples were observed under a light microscope. The environmental samples were not clearly dominated by cyanobacteria and therefore they were inoculated in liquid Z8 medium, supplemented with 25 g l^{-1} sea salts (Tropic Marine) and 10 µg ml^{-1} vitamin B12. The enrichment cultures were kept under low light conditions <10 µmol m^{-2} s^{-1} under a 14 h light/ 10 h dark regimen and at 19 °C. As soon as consistent growth of cyanobacteria was detected, aliquots were transferred onto solid Z8 medium plates with 1.5% agarose, supplemented with sea salts and vitamin B12 as described above. Liquid and solid cultures were grown at 25 °C, under a 14 h light (approximately 30–40 µmol m^{-2} s^{-1})/10 h dark regimen. When single colonies or filaments were detected, these were picked with an inoculating loop and streaked onto a new medium plate. The streak plate technique was repeated until unicyanobacterial cultures were obtained following inoculation in liquid medium. The resulting unicyanobacterial strains have been deposited and since been kept in LEGE-CC under a controlled temperature between 19 and 21 °C, photoperiod 14 h light/10 h dark, and light intensity 15–25 µmol photons m^{-2} s^{-1}.

Morphological analysis

The morphological plasticity and cell measurements (n=50 cells) of 50 individuals of *Zarconia* (LEGE 11467) and *Romeriopsis* strains (LEGE 06013, LEGE 11480) were examined using a Leica DMLB light microscope (Wetzlar) and images were acquired with an Olympus DP73 camera and the Leica Application Suite version 4 software. The morphological characterization was made according to Komárek and Anagnostidis [17], observing the following characters: (1) macroscopic aspect of the culture; (2) number of cells in trichomes; (3) trichome curvature and constriction; (4) shape of cells and measurements; (5) cell content; (6) presence/absence of aerotopes; (7) presence/absence of filaments sheaths or mucilage (using China Ink); (8) reproduction. The measurements were tabulated, and the cell lengths/width were calculated for each of the new genera.

DNA extraction, PCR amplification and sequencing

Total genomic DNA of the three studied strains (LEGE 06013, LEGE 11480 and LEGE 11467) was isolated using the MOBIO Ultraclean DNA Isolation Kit (Life Technologies). To obtain the 16S rRNA gene and the 16S–23S internal transcribed spacer (ITS) regions of strains LEGE 06013 and LEGE 11467, PCR was performed using primers 27F1 [18] and 23 SR [19] in a Biometra two thermal cycler (Analytik Jena). The reaction contained 13 µl H_2O, 5 µl 5×buffer (Promega), 2 µl $MgCl_2$ (25 mM), 1 µl DNTPs (10 µM), 1.25 µl of each primer (10 µM), 0.3 µl of GoTaq polymerase (Promega). The thermal cycling conditions used were: initial denaturing 94 °C (5 min) followed by 10 cycles of 94 °C (45 s), 57 °C (45 s), 72 °C (2 min), then 25 cycles of 92 °C (45 s), 54 °C (45 s), 72 °C (2 min) before a final elongation step of 72 °C (7 min). The PCR products were cloned using the pGEM-T Easy Vector System (Promega), transformed by heat-shock into *E. coli* cells and plated for blue–white screening [20]. Two colonies were selected for each strain. After growth, plasmids were extracted from white colonies using the NZYTech Miniprep Kit, and were prepared for sequencing using the primers 27F [18], 359F [21], 781R [21], 1114F [22] and 23S30R [18]. The resulting sequences were assembled using the Geneious 8.1.9. software package (Biomatters) and analysed for the presence of chimaeras hidden in the rRNA sequences by the DECIPHER web tool [23].

To obtain the 16S rRNA gene sequence of strain LEGE 11480, this gene was amplified from its gDNA using two set of primers: 27F/CYA-781R and CYA-359F/1494R [18, 21] in MyCycler (Bio-Rad laboratories) or T-Professional Standard (Analytik Jena) thermal cyclers, following the methodology previously described [4]. For this set of primers, the PCR reaction contained 7.9 µl H_2O, 4 µl 5×buffer (Promega), 2 µl $MgCl_2$ (25 mM), 1 µl DNTPs (10 µM), 2 µl of each primer (10 µM), 0.1 µl GoTaq polymerase (Promega) and 1 µl template DNA. The thermal cycling conditions were: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. After PCR, to obtain the sequences, the PCR products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Purified PCR products were cloned into pGEM-T Easy Vector (Promega), and transformed into OneShot TOP10 chemically competent *E. coli* cells (Invitrogen) by heat-shock. After blue–white screening, plasmid DNA was isolated using GenElute Plasmid Miniprep Kit (Sigma-Aldrich) and sequenced using the M13 primers: reverse (22mer) – 5′-d(TCACACAG

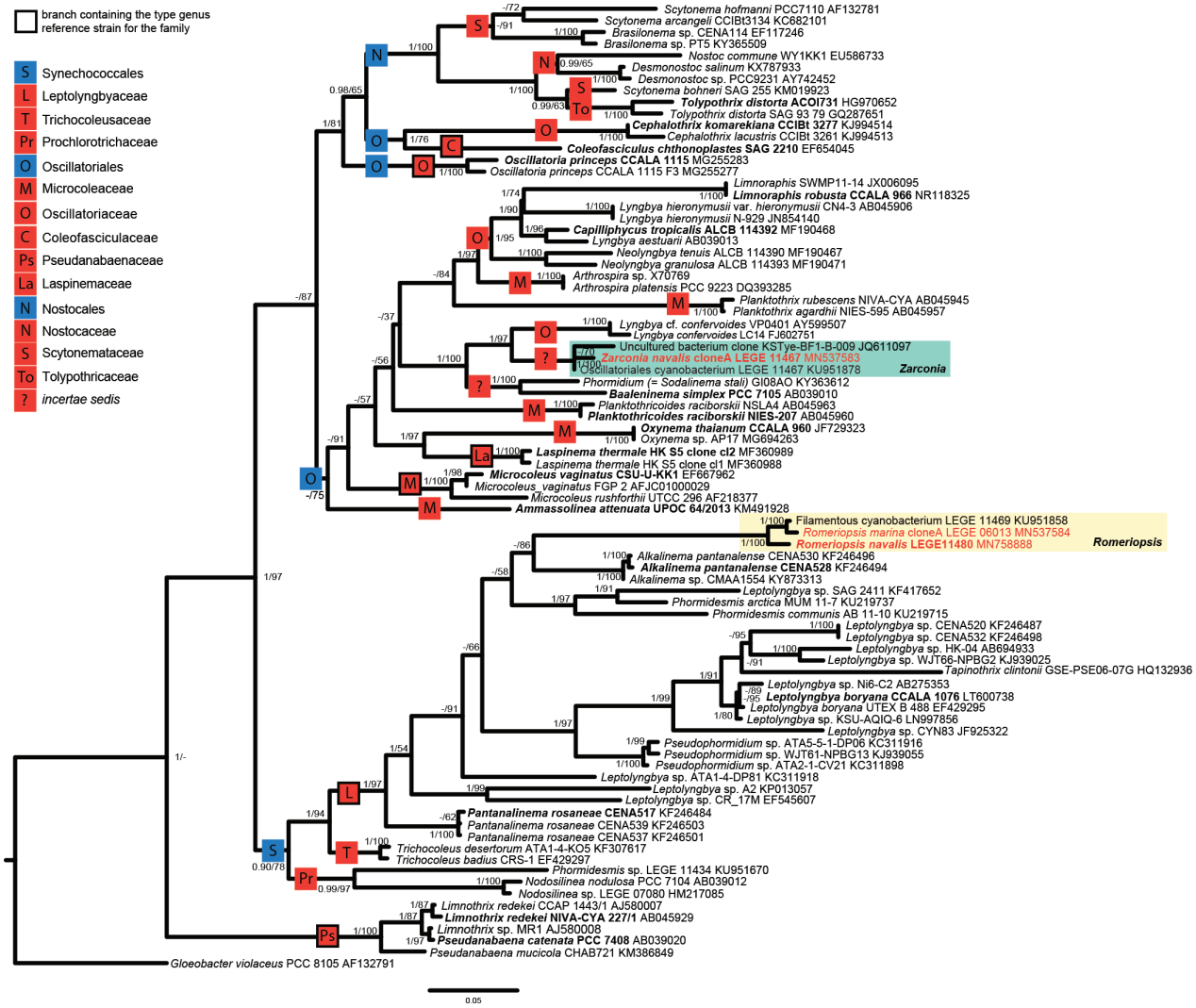

Fig. 1. Maximum-likelihood 16S rRNA gene phylogeny reconstructed with 83 operational taxonomic units and 927 informative sites. Type species are presented in bold. Taxa described in this study are presented in red. Bootstrap value supports are shown at nodes, together with posterior probability values from Bayesian inference analysis. Blue squares represent order level nodes. Red squares represent family-level nodes; those with a thick black border contains the type genus reference strain for that family.

GAAACAGCTATGAC)-3′ and forward (24mer) – 5′-d(CGCCAGGGTTTTCCCAGTCACGAC)-3′. The resulting sequences were assembled using the Geneious 7.0. software package (Biomatters) and analysed for the presence of chimaeras hidden in the rRNA sequences by DECIPHER web tool [23].

16s rRNA gene phylogenetic analysis and 16S–23S rRNA ITS secondary structures

Phylogenies were reconstructed aligning the 16S rRNA gene sequences from *Zarconia* and *Romeriopsis* along with those from their best BLAST hit results as retrieved from GenBank and from a set of selected cyanobacterial strains, including reference strains covering the cyanobacterial diversity particularly from different Oscillatoriales and Synechococcales families. Reference strains were retrieved from CyanoType [15] and from newer taxonomic literature [5, 6, 24, 25]. The total alignment length had 83 sequences and 1721 nucleotide positions with 927 informative sites. Then, to find the phylogenetic positions of our sequences, we performed maximum-likelihood (ML) and Bayesian inference (BI) analyses. A similarity matrix (pairwise identity), calculated using Geneious, was also used to compare taxa.

All the alignments were performed using ClustalW [26] and manually inspected; ML trees were reconstructed using MEGA version 6 [27], using the GTR+G+I evolutionary model, with 1000 bootstraps. The BI tree was reconstructed using MrBayes on XSEDE 3.2.6 [28], with two runs of 5×10^7 generations and NST=6, sample frequency=1000, on the CIPRES Science Gateway [10]. For all

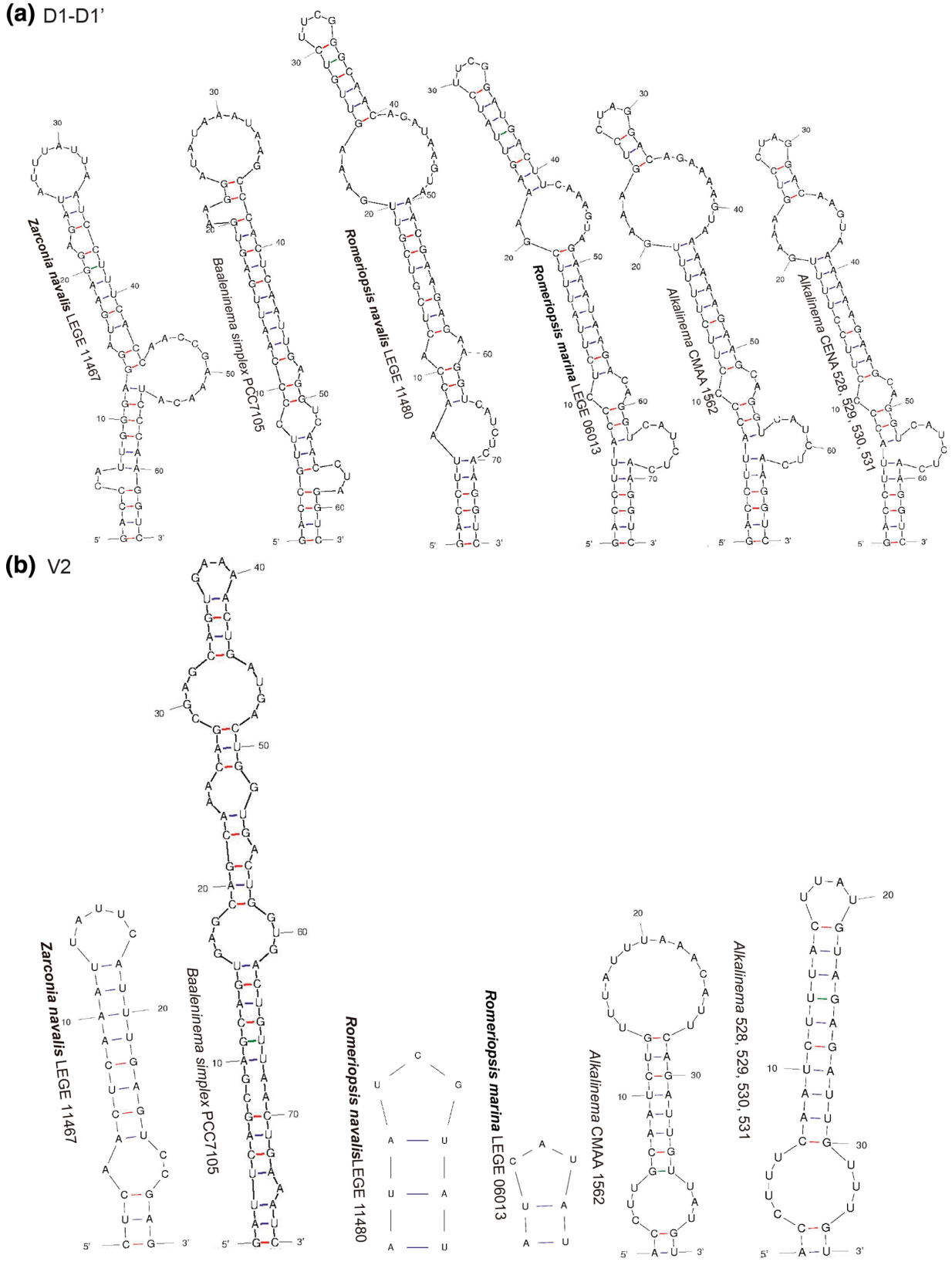

Fig. 2. 16S–23S rRNA gene ITS secondary structures of (a) D1-D1′ helices and (b) V2 helices of *Zarconia*, *Romeriopsis* and phylogenetically related genera.

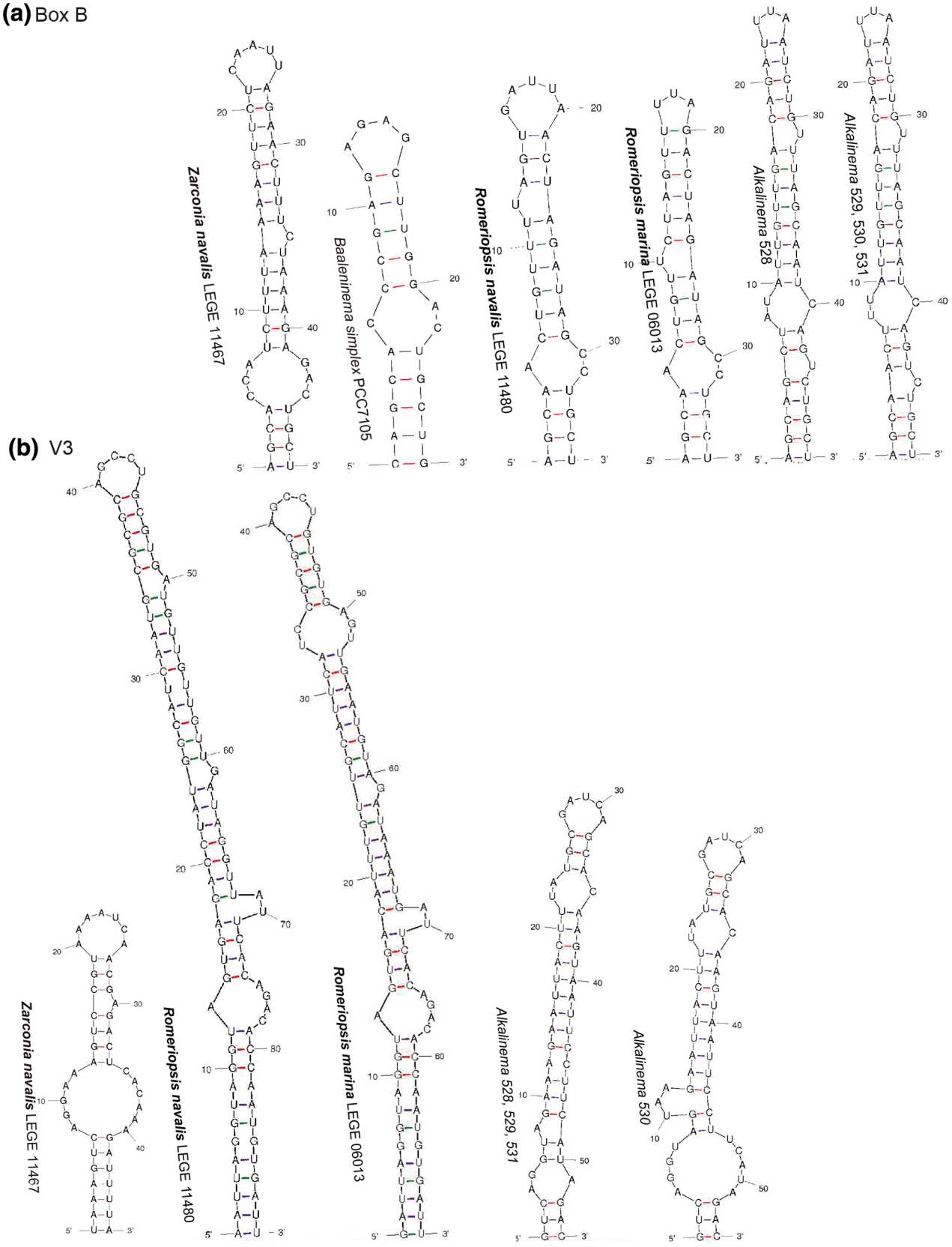

Fig. 3. 16S–23S rRNA gene ITS secondary structures of (a) Box B helices and (b) V3 helices of *Zarconia*, *Romeriopsis* and phylogenetically related genera.

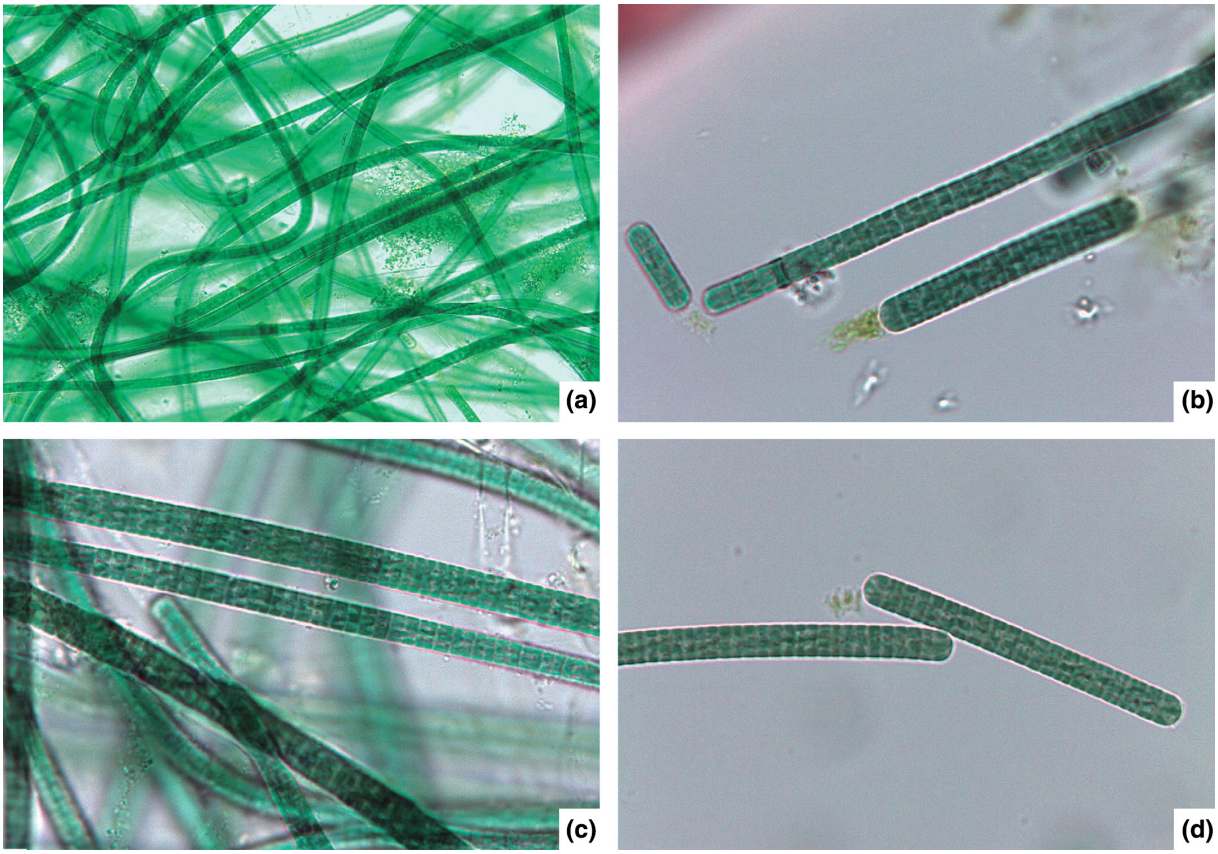

Fig. 4. Microscope images of *Zarconia navalis* sp. nov. (a) General view of the culture. (b)–(d) Details of hormogonia and trichomes showing shorter than wide cells. Magnification: (a) ×4; (b)–(d) ×1000.

parameters in BI, the minimal estimated sample size was above 100 and the potential scale reduction factor ranged from 0.999 to 1.006. The standard deviation of split frequencies was 0.009. The secondary structures of the D1-D1′, Box B, V2 and V3 helices of 16S-23S rRNA ITS were folded using Mfold [29] using default parameters.

Analysis of genomic data

Genome sequencing of *Zarconia navalis* LEGE 11467 (G+C content 49.0mol%; 435 contigs; N50, 10186 bp; genome coverage, 30×; completeness, 92.5%) and *Romeriopsis navalis* LEGE 11480 (G+C content 49.0mol%; 435 contigs; N50, 10186 bp; genome coverage, 30×; completeness, 99.3%) had been carried out as part of a different study (CIT) [30]. Phylogenomic trees supporting the proposal of the new genera (despite low coverage of the phylum Cyanobacteria) were calculated and provided in the supporting information along with methodological details on their reconstruction.

RESULTS

Phylogenetic analysis and placement of the Genera

The ML (Fig. 1) and BI (Fig. S1, available in the online version of this article) phylogenies (83 operational taxonomic units, 927 informative sites) show strong statistical support in the backbones. Both trees show Synechococcales (with ML and BI support values of 78 and one, respectively) as a monophyletic order, although Pseudanabaenaceae (traditionally Synechochoccales [31]; is positioned at the base of both trees, outside of Oscillatoriales or Synechococcales.

Both phylogenies confirm *Romeriopsis* as a monophyletic clade (ML=100, BI=1) in the family Leptolyngbyaceae (Synechococcales) [31], being more closely related to *Alkalinema* Vaz *et al.* The new genus *Zarconia* is also monophyletic, with strong phylogenetic support (ML=100, BI=1), and is clustered with *Lyngbya* cf. *confervoides* (ML=97, BI=1), '*Phormidium*' (=*Sodalinema stali*) GI08AO and *Baaleninema* clades [25] in the Oscillatoriales. In these phylogenetic trees, the order Synechococcales presents well-supported monophyletic families. The order Oscillatoriales is polyphyletic and presents polyphyletic families, such as Microcoleaceae Komárek *et al.* and Oscillatoriaceae (S. F. Gray) Harvey ex Kirchner [31].

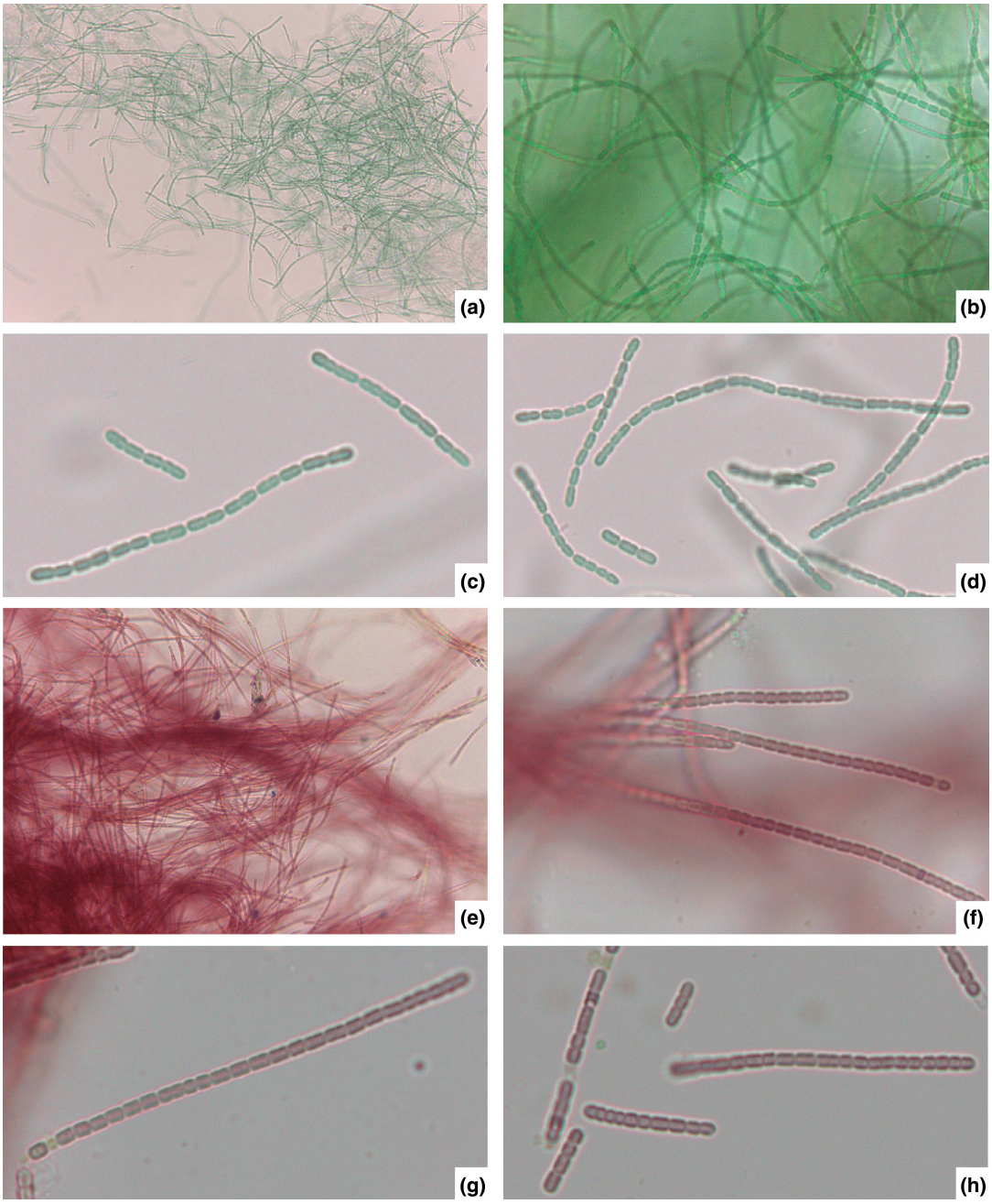

Fig. 5. Microscope images of *Romeriopsis marina* sp. nov. (a–d) and *Romeriopsis navalis* sp. nov. (e–h). (a) and (b) General view of the culture showing long and short trichomes. (c) and (d) Details of short trichomes. (e) General view of the culture showing long and short trichomes. (f)–(h) Details of short trichomes. Magnification: (a) and (e) ×40; (b)–(d) and (f)–(h) ×1000.

Although *Zarconia* presents typical Microcoleaceae trichomes and morphologically fits in this family, the newly proposed genus is phylogenetically distant from *Microcoleus* Gomont. Also, *Zarconia* has morphological similarities (see the 'Taxa description' subsection) to some *Phormidium* species (Oscillatoriaceae), namely those belonging to group V in Komárek and Anagnostidis [17]. Considering that Microcoleaceae and Oscillatoriaceae are polyphyletic, we chose to keep *Zarconia* as Oscillatoriales *incertae sedis* until more taxonomical studies on this order can clarify the systematics of the group.

The similarity matrix (pairwise identity; Table S1) corroborates the phylogenetic inferences and confirms *Romeriopsis* and *Zarconia* as new genera. This analysis shows *Romeriopsis* with 98 % of intra-clade similarity and only 90–91% of similarity to *Alkalinema*, its phylogenetically most closely related clade. The similarity between *Zarconia* sequences is 99% and the similarity

between this clade and *Lyngbya confervoides*, its most closely related clade (Fig. 1), is 95%. The similarity between *Zarconia* and the clade comprising '*Phormidium*' (=*Sodalinema stali*) GI08AO and *Baaleninema simplex* PCC 7105 is of 94%, two strains belonging to recently erected marine genera [25].

16S–23S rRNA ITS analysis

We compared the ITS secondary structure of *Zarconia* with that of its phylogenetically closest related described genus with available ITS data, *Baaleninema* (Figs 1–3). Comparing those genera, the D1-D1′ helix is variable in sequence, length and structure. The *Zarconia* D1-D1′ helix presents a long basal stem with 10 bp plus two residues, while *Baaleninema* presents the typical 4 bp (5′-GACC-3′/5′-GGTC-3′) D1-D1′ cyanobacterial basal stem. The long basal stem of *Zarconia* is unique among Cyanobacteria. Furthermore, the 3′ side of *Zarconia*'s molecule presents a long first lateral bulge (10 nucleotides), while *Baaleninema* presents three nucleotides in this position. These differences support the separation of both genera, which is in line with our phylogenetic analysis. V2 and Box B helices are also very different in length and sequence among these genera. We could not find any data about the *Baaleninema* V3 helix. It was also not possible to compare *Zarconia* with *Lyngbya confervoides* (strains VP0401 and LC14) and '*Phormidium*' (=*Sodalinema stali*) GI08AO, due to the absence of ITS data for these strains.

The ITS secondary structures of both *Romeriopsis* species were compared with each other and with those of its phylogenetically closest related genus, *Alkalinema* (Figs 1–3). The D1-D1′ helix of *Romeriopsis* and *Alkalinema* are identical in the basal stem. The lateral bulge of *R. marina* LEGE 06013 is also identical to *Alkalinema*, but *R. navalis* LEGE 11480 differs from *Alkalinema* by the presence of one residue opposing this bulge. The internal loops are variable among these genera strains, but it is possible to differentiate both genera mainly by the terminal loop. Although *Alkalinema*'s D1-D1′ helices are variable within the genus, the terminal loop is conserved (5′-CUAG-3′) and different from *Romeriopsis* (5′-UUCG-3′). The Box B, V2 and V3 helices are very different in length, sequence and structure, supporting the separation of both genera.

The helices among *Romeriopsis* sequences support the separation of the genus in two species. In D1-D1′, both species present identical basal stem and lateral bulge, but *R. navalis* presents a residue (A) opposing the lateral bulge (5′-UCAUCUCA-3′), while *R. marina* lacks a residue in this position. These helices also present same terminal loop but are very different comparing internal stems and loops. The basal stem of Box B helices and the mismatches in the first internal loop are identical in both species, but these helices are different in length, sequence and structure of the terminal portion. The V2 helices of both species are notably short and present different sequences between each other. The V3 helices are very long (90 nucleotides) and similar, but although the terminal loop is identical (5′-CAGCCUG3-′), the helices present different sequences and terminal stems structures. Beyond that, *R. marina* presents one internal loop, which is lacking in *R. navalis*.

DESCRIPTIONS OF THE NEW TAXA

The newly proposed taxa were named under the International code of nomenclature for algae, fungi, and plants [32].

DESCRIPTION OF *ZARCONIA* GEN. NOV.

Zarconia (Zar.co'ni.a. N.L. fem. n. *Zarconia*, named after J.G. Zarco, a Portuguese maritime explorer that discovered the Madeira archipelago, and who gave the name to the square with the fort located near the collection site; zarco is also an Arabic-hispanic word that means blue, the colour of the ocean).

In culture, forming mats attached to the flask walls. Filaments isopolar, solitary or entangled, straight or wavy. Sheaths firm, thin, homogenous and colourless. Trichomes cylindrical, not constricted or slightly constricted (shorter cells) at cross-walls, sometimes motile. Cells shorter than wide, rarely isodiametric or, rarely discoid (only after division), 2.7–4.0 µm long, 5.6–7.6 µm wide, ratio length:width 1.4:2.3 for adult cells. Apical cells rounded. Cell content homogenous, dark green, with aerotopes (Fig. 4). Reproduction occurs by disintegration of the trichomes into motile hormogonia, with the help of necridic cells (Fig. 4). Phylogenetically, the genus is *incertae sedis*. The type species is *Zarconia navalis*.

DESCRIPTION OF *ZARCONIA NAVALIS* SP. NOV.

Zarconia navalis (na'va.lis. L. gen. n. *navalis*, naval, referring to the navigation and maritime services arising from the seaport located near the sampling spot).

Same characteristics detailed above in the genus description. The reference strain, LEGE 11467, was isolated from a subtidal environment – 'A Pedra', a diving spot in front of the fort 'Castelo do Queijo', Portugal (41.185809° N 8.719079° W). The habitat is marine, subtidal sample, epilithic (13 m depth), about 200 m off the shore. Holotype: PO-T4766 (unialgal population preserved lyophilized), University of Porto Herbarium. GenBank accession: MN537583 (16S rRNA gene), JADEXN000000000 (genome).

DESCRIPTION OF *ROMERIOPSIS* GEN. NOV.

Romeriopsis (Ro.me.ri.op'sis. N.L. fem. n. *Romeriopsis*, similar to *Romeria*, due to the short trichomes).

In culture, trichomes solitary or forming fluffy clusters not attached to the flask walls. Trichomes cylindrical, constricted, slightly curved or wavy, few celled (more common) or long (>50 cells). With very thin sheaths (visible only in broken filaments) or without. Without mucilaginous envelope. Adult cells longer than wide, cylindrical or rarely barrel-shaped, 1.6–3 µm long, 1.5–2 µm wide, ratio length:width 1.2:1.9. Terminal cells rounded. Cell content olive-green or reddish, not granulated, without aerotopes (Fig. 5). Reproduction by fragmentation of trichomes. Similar to *Romeria* by the presence of constricted trichomes, often few cylindrical cells, longer than wide. Differs from *Romeria* by the common presence of long trichomes (>50 cells), which do not disintegrate easily, by the presence of sheaths and by the absence of diffluent mucilaginous envelopes. Indistinguishable in morphology from *Pantanalinema* Vaz *et al.* and *Alkalinema*. Phylogenetically, the genus is a member of the Leptolyngbyaceae. The type species is *Romeriopsis navalis*.

DESCRIPTION OF *ROMERIOPSIS NAVALIS* SP. NOV.

Romeriopsis (na'va.lis. L. gen. n. *navalis*, naval, referring to the navigation and maritime services arising from the seaport located near the sampling spot).

Same characteristics detailed above in the genus description, but the cells contents are exclusively reddish. The reference strain, LEGE 11480, was isolated from same location and habitat as the subtidal *Zarconia navalis* LEGE 11467, on a rocky substrate at 13 m depth and 200 m off the shore. Holotype: PO-T4783 (unialgal population preserved lyophilized), University of Porto Herbarium. GenBank accession: MN758888 (16S rRNA gene), JADEXQ000000000 (genome).

DESCRIPTION OF *ROMERIOPSIS MARINA* SP. NOV.

Romeriopsis marina (L. fem. n. *marina*, marine, referring to the marine habitat from where this species was collected).

Same characteristics detailed above in the genus description, but the cells contents are exclusively olive-green. The reference strain, LEGE 06013, was isolated from a wave-exposed rock in a marine, intertidal habitat at Praia da Foz do Arelho, Caldas da Rainha, Portugal (39.43327° N 9.230275° W). Holotype: PO-T4767 (unialgal population preserved lyophilized), University of Porto Herbarium. GenBank accession for the 16S rRNA gene: MN537584.

NOTES

The genus reference strains LEGE 11480 (*R. navalis*) and LEGE 06013 (*R. marina*) share only 98.2% 16S similarity (p-distance), are in separate subclades in the phylogenetic tree and present very distinct 16S–23S ITS secondary structures, considering length and structure (Figs 1–3). Because of that we separate these strains into two species. Beyond these differences, in culture LEGE 11480 (*R. navalis*) presents reddish cells content while LEGE 06013 (*R. marina*) presents olive-green cells content, probably a result of adaptation to light intensity and quality prevailing in their original ecological niches (LEGE 11480 is subtidal while LEGE 06013 is intertidal). However, we cannot ascertain if this can be used as a distinctive morphological taxonomic feature, or if it is just an effect of culturing.

DISCUSSION

The wave energy action and related environmental conditions from the north continental Portuguese coast [16], where the studied marine strains were collected, are known drivers shaping the coastal biodiversity, including that of microbial communities [33]. Along with the sampling and isolation efforts undertaken for this specific environment, this may partially explain why this marine temperate region is being a rich source of novel cyanobacteria taxa, already described [4] or at least phylogenetically highlighted (see [4]).

In this paper we describe the new genera *Romeriopsis* and *Zarconia* supported by 16S rRNA gene phylogenetic analysis. The genus *Zarconia* is clustered with *Lyngbya confervoides* LC14 (FJ602751), *Lyngbya* cf. *confervoides* VP0401 (AY599507), '*Phormidium*' (=*Sodalinema lapsi*) GI08AO (KY363612) and *Baaleninema simplex* PCC 7105 (AB039010), but cannot be assigned to any of these genera considering molecular and morphological analysis. The strains identified as *Lyngbya confervoides* were previously used as reference for *Lyngbya* by Komárek *et al.* [34] and Caires *et al.* [5], but although they are morphologically and ecologically similar to the type species description, they are not well characterized [34] and cannot be surely assigned to this genus, because of limited available data (only one photo and inconclusive phylogeny in [35]. Many other papers depict *Lyngbya* Gomont as a polyphyletic genus [6, 7, 36] and to identify the 'true' *Lyngbya* clade, a robust revision of the genus and of its type species *Lyngbya confervoides* Gomont is needed. This has been done, for example, for *Oscillatoria* Gomont by Mühlsteinová *et al.* [36], which is the type genus of the family Oscillatoriaceae, and subsequently of the order Oscillatoriales. Indeed, by exposing the 'true' *Oscillatoria* clade through

the epitypification of *O. princeps*, Mühlsteinová *et al.* [36] showed that the reference strain *O. princeps* CCALA 1115 is phylogenetically apart from most of the genera currently assigned to Oscillatoriaceae, being placed at the base of the Nostocales (Fig. 1). This particular phylogenetic placement of *O. princeps*/strain CCALA 1115 has been already noticed by other studies, including Suda *et al.* [37], Komárek *et al.* [31]. The monophyletic subclade containing '*Phormidium*' (=*Sodalinema stali*) GI08AO and *Baaleninema simplex* PCC7105 strains was recently described [25]. These are currently the closest known genera to *Zarconia* and to the '*L. confervoides* subclade', all forming a clade with high ML and BI support (Figs 1 and S1). Samylina and co-authors [25] did not assign the novel genus *Baaleninema* (and, implicitly, *Sodalinema*) to any existing Oscillatoriales family, thus having an uncertain position within the order. Morphologically, *Zarconia* is similar to *Microcoleus* or to *Phormidium* Gomont 'group V' according to Komárek and Anagnostidis [17] ("trichomes cylindrical along their whole length; apical cells widely rounded or obtuse…"), presenting terminal rounded cells and isodiametric or slightly shorter than wide adult cells, but no diacritical morphological markers can be distinguished for *Zarconia*. Despite this, according to our phylogenetic analysis, we could not assign *Zarconia* to any previously described genus or family, considering that it is phylogenetically distinct to any reference strain of Oscillatoriales, namely to the Type genus of Microcoleaceae, *Microcoleus*, or to the type genus of Oscillatoriaceae, *Oscillatoria*. *Zarconia* is also morphologically and phylogenetically dissimilar to *Lyngbya* (traditionally assigned to Oscillatoriaceae), *Sodalinema* Cellamare *et al.* and *Baaleninema* (*incertae sedis*). Moreover, along with these three phylogenetically close genera, there is considerable phylogenetic distance from *Oscillatoria*. Also, LEGE 11467 is morphologically and/or ecologically different from marine/brackish morphospecies from *Phormidium* 'group V', namely to *P. bulgaricum* Anagnostidis and Komárek, *P. crassior* Anagnostidis and *P. gracile* Lindstedt [17]. There are no sequences publicly available for these species, but the 16S rRNA gene sequence from LEGE 11467 shares only 90.5% identity with strain CCALA 759, which represent the epitype for the genus, *Phormidium irriguum* Sciuto *et al.* [38]. Altogether, and by acknowledging that the Oscillatoriales families need a robust taxonomic revision, we could not unequivocally assign *Zarconia* to any existent cyanobacterial family.

Indeed, our phylogenies clearly expose that Oscillatoriales families are polyphyletic. Our trees show seven clades containing Microcoleaceae genera, six of which not related to *Microcoleus*. Likewise, there are four additional Oscillatoriaceae clades not related to *Oscillatoria*. Our phylogenies agree with those from Ishida *et al.* [39], Jahodarová *et al.* [11] and Nowicka-Krawczyk *et al.* [13], which also shown the polyphyly of several Oscillatoriales families, including Microcoleaceae and Oscillatoriaceae. We highlight also that the family Pseudanabaenaceae (traditionally Synechococcales [31]) is not included in Oscillatoriales or Synechococcales clades, indicating that the phylogenetic position and taxonomy of this family must be revised, something already noticed by Mai *et al.* [12].

In this study we delimit genera on the basis of their monophyletic placement [12, 31, 40]. For higher levels, as stated by Komárek *et al.* [31], "morphological characters used to define higher taxa … have apparently arisen and/or been lost several times during the evolution of modern species and genera". Considering the many polyphyletic families within Oscillatoriales and Synechococcales, we believe that in the near future, after taxonomical revisions based on 16S rRNA gene, or preferably on concatenated multi-gene or genome-based phylogenies, cryptic cyanobacterial families will be commonly described. Indeed, multiple-gene based phylogenies should translate to more robust analyses, which is essential for any taxonomic revision.

In the Synechococcales clade, our phylogenetic analysis (Fig. 1) is in agreement with the revision of Mai *et al.* [12] for the order, showing monophyletic families. *Romeriopsis* is clearly monophyletic (ML=100, BI=1) and positioned in Leptolyngbyaceae, clustered with *Alkalinema*. The 16S–23S rRNA gene ITS secondary structures are also in agreement with these findings, as detailed in the results section.

The herein described new genera, *Zarconia* and *Romeriopsis*, are supported by 16S rRNA phylogenies, 16S–23S rRNA gene ITS secondary structures and morphological analysis. *Zarconia* is grouped with high phylogenetic support with *Lyngbya* cf. *confervoides*, but these taxa are morphologically very different. *Zarconia* presents adult cells mainly smaller than wide (ratio length:width 1.4:2.3), discoid only after division, while *Lyngbya* cf. *confervoides* presents the typical *Lyngbya* discoid adult cells. The 16S–23S rRNA gene ITS secondary structures of the D1-D1′ helix of *Zarconia* are unique among Cyanobacteria. *Romeriopsis* is morphologically similar to *Romeria* regarding a generally short trichome length but differs by the common presence of long trichomes (>50 cells), which do not disintegrate easily as in *Romeria*, by the presence of sheaths and the absence of diffluent mucilaginous envelopes. Up to now, there is no available 16S rRNA gene sequence clearly assigned to *Romeria* in public databases. For this reason, we have not included this genus in our phylogeny. *Romeriopsis* is morphologically indistinguishable from *Pantanalinema* and *Alkalinema,* but the phylogenies and the 16S–23S rRNA gene ITS secondary structures in the current study confirm that these are three separate genera.

CONCLUSION

The genera *Zarconia* and *Romeriopsis* are described on the basis of 16S rRNA gene phylogenies. Considering that many cyanobacterial orders and families are polyphyletic, a global effort from the research community is required to allow the reconstruction of the evolutionary history of these photosynthetic micro-organisms. Traditional, morphological-based family taxonomy is in conflict with present molecular and phylogenetic data. This work is an additional contribution towards the

long journey of resolving Cyanobacteria systematics. Based on the phylogenetic data presented herein, we also re-emphasize the need for a taxonomic revision of several Oscillatoriales families and genera.

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Author contributions
G.S.H. and V.R. worked on collection and assembly of data, analysis and interpretation of the data and also in the drafting of the article together with P.N.L. A.P. worked on conception and design, collection, analysis and assembly of data with contributions from A.B., M.S.C. and S.B. A.R. performed genomic and phylogenomic analyses. V.V. and P.N.L. obtained funding, carried out analysis and interpretation of the data and critical revision of the article for important intellectual content. All authors read and approved the final version of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Hamilton TL, Bryant DA, Macalady JL. The role of biology in planetary evolution: cyanobacterial primary production in low-oxygen Proterozoic oceans. *Environ Microbiol* 2016;18:325–340.
2. Calteau A, Fewer DP, Latifi A, Coursin T, Laurent T, *et al*. Phylum-wide comparative genomics unravel the diversity of secondary metabolism in Cyanobacteria. *BMC Genomics* 2014;15:977.
3. Leão PN, Engene N, Antunes A, Gerwick WH, Vasconcelos V. The chemical ecology of cyanobacteria. *Nat Prod Rep* 2012;29:372–391.
4. Brito Â, Ramos V, Mota R, Lima S, Santos A, *et al*. Description of new genera and species of marine cyanobacteria from the Portuguese Atlantic coast. *Mol Phylogenet Evol* 2017;111:18–34.
5. Caires TA, Lyra G de M, Hentschke GS, Silva AMS da, Araújo VL de, *et al*. Polyphasic delimitation of a *Filamentous marine* genus, *Capillus* gen. nov. (*Cyanobacteria, Oscillatoriaceae*) with the description of two Brazilian species. *ALGAE* 2018;33:291–304.
6. Caires TA, de Mattos Lyra G, Hentschke GS, de Gusmão Pedrini A, Sant'Anna CL, *et al*. *Neolyngbya* gen. nov. (*Cyanobacteria, Oscillatoriaceae*): a new filamentous benthic marine taxon widely distributed along the Brazilian coast. *Mol Phylogenet Evol* 2018;120:196–211.
7. Engene N, Tronholm A, Paul VJ. Uncovering cryptic diversity of Lyngbya: the new tropical marine cyanobacterial genus *Dapis* (Oscillatoriales). *J Phycol* 2018;54:435–446.
8. Konstantinou D, Voultsiadou E, Panteris E, Zervou S-K, Hiskia A, *et al*. *Leptothoe*, a new genus of marine cyanobacteria (Synechococcales) and three new species associated with sponges from the Aegean Sea. *J Phycol* 2019;55:882–897.
9. Zhou W-G, Ding D-W, Yang Q-S, Ahmad M, Zhang Y-Z, *et al*. *Marileptolyngbya sina* gen. nov., sp. nov. and *Salileptolyngbya diazotrophicum* gen. nov., sp. nov. (*Synechococcales, Cyanobacteria*), species of cyanobacteria isolated from a marine ecosystem. *Phytotaxa* 2018;383:75.
10. Miller MA, Schwartz T, Pickett BE, He S, Klem EB, *et al*. A RESTful API for access to phylogenetic tools via the CIPRES science gateway. *Evol Bioinform Online* 2015;11:43–48.
11. Jahodářová E, Dvořák P, Hašler P, Holušová K, Poulíčková A. *Elainella* gen. nov.: a new tropical cyanobacterium characterized using a complex genomic approach. *Eur J Phycol* 2017;53:39–51.
12. Mai T, Johansen JR, Pietrasiak N, Bohunická M, Martin MP. Revision of the *Synechococcales (Cyanobacteria*) through recognition of four families including *Oculatellaceae* fam. nov. and *Trichocoleaceae* fam. nov. and six new genera containing 14 species. *Phytotaxa* 2018;365:1.
13. Nowicka-Krawczyk P, Mühlsteinová R, Hauer T. Detailed characterization of the *Arthrospira* type species separating commercially grown taxa into the new genus *Limnospira (Cyanobacteria*). *Sci Rep* 2019;9:694.
14. Brito A, Ramos V, Seabra R, Santos A, Santos CL, *et al*. Culture-dependent characterization of cyanobacterial diversity in the intertidal zones of the Portuguese coast: a polyphasic study. *Syst Appl Microbiol* 2012;35:110–119.
15. Ramos V, Morais J, Vasconcelos VM. A curated database of cyanobacterial strains relevant for modern taxonomy and phylogenetic studies. *Sci Data* 2017;4:170054.
16. Coelho C, Silva R, Veloso-Gomes F, Taveira-Pinto F. Potential effects of climate change on northwest Portuguese coastal zones. *ICES J Mar Sci* 2009;66:1497–1507.
17. Komárek J, Anagnostidis K. Cyanoprokaryota 2. Teil Oscillatoriales. In: Büdel B, Krienitz L, Gärtner G and Schagerl M (eds). *Süsswasserflora von Mitteleuropa*, vol 19/2. München: Elsevier Spektrum Akademische; 2005. pp. 1–759.
18. Neilan BA, Jacobs D, Del Dot T, Blackall LL, Hawkins PR, *et al*. rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. *Int J Syst Bacteriol* 1997;47:693–697.
19. Taton A, Grubisic S, Brambilla E, De Wit R, Wilmotte A. Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo Dry Valleys, Antarctica): a morphological and molecular approach. *Appl Environ Microbiol* 2003;69:5157–5169.
20. Sambrook J, Russell DW. *Molecular Cloning, Volume 1: A Laboratory Manual*. New York: CSHL Press; 2001.
21. Nübel U, Garcia-Pichel F, Muyzer G. PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl Environ Microbiol* 1997;63:3327–3332.
22. Lane, D. J., 1999. 16S/23S rRNA sequencing, in: Stackebrandt, E., Goodfellow M. (Eds.), *Nucleic Acid Techniques in Bacterial Systematics*. Wiley, Chichester, pp. 115–175.
23. Wright ES, Yilmaz LS, Noguera DR. DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Appl Environ Microbiol* 2012;78:717–725.
24. Heidari F, Zima J, Riahi H, Hauer T. New simple trichal cyanobacterial taxa isolated from radioactive thermal springs. *Fottea* 2018;18:137–149.
25. Samylina OS*et al*. Ecology and biogeography of the 'marine geitlerinema' cluster and a description of *Sodalinema orleanskyi* sp nov., *Sodalinema gerasimenkoae* sp. nov., *Sodalinema stali* sp. nov. and *Baaleninema simplex* gen. et sp. nov. (oscillatoriales, cyanobacteria). *FEMS Miocrbiol Ecol* 2021;97(8):104.
26. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–4680.
27. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 2013;30:2725–2729.
28. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, *et al*. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 2012;61:539–542.

29. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003;31:3406–3415.
30. Eusebio N, Rego A, Glasser NR, Castelo-Branco R, Balskus EP, *et al*. Distribution and diversity of dimetal-carboxylate halogenases in cyanobacteria. *BMC Genomics* 2021;22:633.
31. Komárek J, Kastovsky J, Mares J, Johansen JR. Taxonomic classification of cyanoprokaryotes (cyanobacterial genera), using a polyphasic approach. *Preslia* 2014;86:295–335.
32. Turland NJ, Wiersema JH, Barrie FR, Greuter W, Hawksworth DL, *et al*. *International code of nomenclature for algae, fungi, and plants (Shenzhen Code) adopted by the Nineteenth International Botanical Congress Shenzhen*. Koeltz Botanical Books, 2018.
33. Witt MJ, Sheehan EV, Bearhop S, Broderick AC, Conley DC, *et al*. Assessing wave energy effects on biodiversity: the wave hub experience. *Philos Trans A Math Phys Eng Sci* 2012;370:502–529.
34. Komárek J, Zapomělová E, Šmarda J, Kopecký J, Rejmánková E, *et al*. Polyphasic evaluation of *Limnoraphis robusta*, a water-bloom forming cyanobacterium from Lake Atitlán, Guatemala, with a description of *Limnoraphis* gen. nov. *Fottea* 2013;13:39–52.
35. Sharp K, Arthur KE, Gu L, Ross C, Harrison G, *et al*. Phylogenetic and chemical diversity of three chemotypes of bloom-forming lyngbya species (Cyanobacteria: Oscillatoriales) from reefs of southeastern Florida. *Appl Environ Microbiol* 2009;75:2879–2888.
36. Mühlsteinová R, Hauer T, De Ley P, Pietrasiak N. Seeking the true *Oscillatoria*: a quest for reliable phylogenetic and taxonomic reference point. *Preslia* 2018;90:151–169.
37. Suda S, Watanabe MM, Otsuka S, Mahakahant A, Yongmanitchai W, *et al*. Taxonomic revision of water-bloom-forming species of oscillatorioid cyanobacteria. *Int J Syst Evol Microbiol* 2002;52:1577–1595.
38. Sciuto K, Andreoli C, Rascio N, La Rocca N, Moro I. Polyphasic approach and typification of selected Phormidium strains (Cyanobacteria). *Cladistics* 2012;28:357–374.
39. Ishida T, Watanabe MM, Sugiyama J, Yokota A. Evidence for polyphyletic origin of the members of the orders of *Oscillatoriales* and *Pleurocapsales* as determined by 16S rDNA analysis. *FEMS Microbiol Lett* 2001;201:79–82.
40. Johansen JR, Casamatta DA. Recognizing cyanobacterial diversity through adoption of a new species paradigm. *archiv_algolstud* 2005;117:71–93.