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1 **Establishment of the family Zarkiaceae (Oscillatoriales, Cyanobacteria) and**
2 **description of the new marine genera *Zarkia* (Zarkiaceae, Oscillatoriales) and**
3 ***Romeriopsis* (Leptolyngbyaceae, Synechococcales), from northern Portugal**

4

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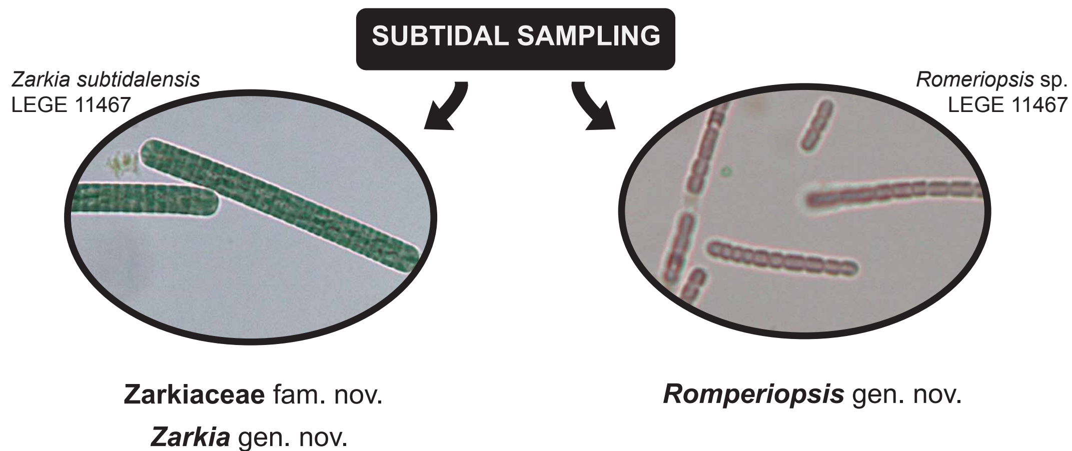
ABSTRACT



The morphology, 16S rRNA gene phylogeny and the 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 *Zarkia subtidalensis* gen. et. sp. nov. (Zarkiaceae fam. nov.) and *Romeriopsis marina* gen. et. sp. nov (Leptolyngbyaceae). No diacritical morphological characters were found either for the new family or for the new genera. The 16S rRNA gene Maximum Likelihood and Bayesian phylogenies supported that *Zarkia* and Zarkiaceae are members of the Oscillatoriales, positioned close to Microcoleaceae genera, but distant from *Microcoleus*. *Romeriopsis* is positioned within the Leptolyngbyaceae and is closely related to *Alkalinema*. The secondary structures of the D1-D1', Box B, V2 and V3 helices corroborate with the phylogenetic results. Furthermore, our study supports previous observations of polyphyletic Oscillatoriales families and reinforces the need for their taxonomical revision.

Keywords: subtidal, intertidal, phylogeny, taxonomy.

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39 **1. Introduction**

40

41 Cyanobacteria are important primary producers in the world's oceans and shape
42 both planktonic and benthic marine communities (Hamilton et al., 2016). In addition,
43 they produce a plethora of secondary metabolites, such as alkaloids, polyketides, and
44 peptides with a great variety of biological activities with some being currently used to
45 treat cancer in the clinic (Leão et al., 2012; Calteau et al., 2014). Despite their global
46 and biotechnological importance, the diversity of marine Cyanobacteria is still
47 underestimated, and recently, many new genera of Cyanobacteria have been described,
48 such as *Leptothoe* Konstantinou et Gkelis, *Marileptolyngbya* Zhou et Ling,
49 *Salileptolyngbya* Zhou, *Lusitaniella* Ramos et al., *Dapis* Engene et al., *Capillus* and
50 *Neolyngbya*, for example (Brito et al., 2017; Caires et al., 2018a; 2018b; Engene et al.,
51 2018; Zhou et al., 2018; Konstantinou et al., 2019).

52 The growing number of new taxa descriptions, the availability of 16S rRNA
53 gene sequences for the respective type species, and the access to computational tools
54 that allow to expeditiously perform extensive phylogenetic reconstructions (Miller et
55 al., 2015), has brought to light that many cyanobacterial families from the classical,
56 morphological-based taxonomy are polyphyletic, warranting taxonomical revisions
57 (Jahodarová et al., 2017; Nowicka-Krawczyk et al., 2018; Mai et al., 2018). Up to now,
58 the only revision at the order level for Cyanobacteria, on the basis of robust
59 phylogenetic analysis and morphological descriptions, concerned the
60 Synechococcacales and was carried out by Mai et al. (2018). In that paper, the authors
61 describe two new families and six new genera containing 14 species. The authors
62 consider five families in the order, one of which (Trichocoleaceae) comprising a single
63 genus. The description of families with a single genus based on a single-gene

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64 phylogenetic analysis is not a novelty (Hentschke et al., 2016) and is necessary for
65 monophyletic clades (Johansen and Casamatta, 2005), which are well supported and not
66 clustered in any already known family.
67 Against this backdrop, in this paper we describe the new family Zarkiaceae to
68 hold the new cyanobacterial genus *Zarkia*, and also describe the new genus *Romeriopsis*
69 (Leptolyngbyaceae), from intertidal and subtidal environments sampled in north
70 Portugal.
71
72
73 **2. Materials and Methods**
74 *2.1. Sampling and sites*
75 Two samples were obtained in the subtidal zone seafloor, at 13 m depth, by
76 collection of rocky substrate surfaces using 50 mL sterile polypropylene syringes, by
77 SCUBA diving at 'A Pedra', a diving spot in front of the São Francisco Xavier fort,
78 ~200 m off the coast, in the city of Porto, Portugal (41.185809 N 8.719079 W). The
79 samples were kept in 50 mL polypropylene tubes until being processed in the laboratory
80 at CIIMAR, Porto, Portugal. These samples led to the isolation of strains *Zarkia*
81 *subtidalensis* LEGE 11467 and *Romeriopsis* sp. LEGE 11480, while strain *Romeriopsis*
82 *marina* LEGE 06013 had been previously isolated from a wave-exposed rock, Praia da
83 Foz do Arelho, Caldas da Rainha (39.43327 N 9.230275 W), Portugal, as reported in
84 Brito et al. (2012) and was obtained from the LEGE Culture Collection (LEGE-CC) at
85 CIIMAR, Porto, Portugal (http://lege.ciimar.up.pt) (Ramos et al., 2017). The north
86 Portuguese coastal environments, a temperate climate region, where the studied strains
87 were collected are vulnerable to wave action, being under a strong tide and wave regime
88 especially in the winter (Coelho et al., 2009).

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89

90 *2.2. Isolation strategy*

91 After arrival at the laboratory, subtidal samples were observed under a light
92 microscope. The environmental samples were not clearly dominated by cyanobacteria
93 and therefore they were inoculated in liquid medium, supplemented with 25 g L^{-1} sea
94 salts (Tropic Marine) and 10 µg ml^{-1} vitamin B_{12}. The enrichment cultures were kept
95 under low light conditions <10 µmol m^{-2} s^{-1} under a 14 hour light/ 10 hour dark regimen
96 and at 19 ºC. As soon as consistent growth of cyanobacteria was detected, aliquots were
97 transferred onto solid Z8 medium plates with 1.5% agarose, supplemented with sea salts
98 and vitamin B_{12} as described above. Liquid and solid cultures were grown at 25 °C,
99 under a 14 hour light (approximately 30 – 40 µmol m^{-2} s^{-1})/ 10 hour dark regimen.
100 When single colonies or filaments were detected, these were picked with an inoculating
101 loop and streaked onto a new medium plate. The streak plate technique was repeated
102 until unicyanobacterial cultures were obtained following inoculation in liquid medium.
103 The resulting unicyanobacterial strains have been deposited and since been kept in
104 LEGE-CC under controlled temperature between 19ºC and 21ºC, photoperiod 14h
105 light/10h dark, and light intensity 15–25 µmol photons m^{-2} s^{-1}.

106

107 *2.3. Morphological analysis*

108 The morphological plasticity and cell measurements (n = 50 cells) of 50
109 individuals of *Zarkia* (LEGE 11467) and *Romeriopsis* strains (LEGE 06013, LEGE
110 11480) were examined using a Leica DMLB light microscope (Wetzlar, Germany) and
111 micrographs were acquired with an Olympus DP73 camera and the Leica Application
112 Suite V.4 software. The morphological characterization was made according to
113 Komárek and Anagnostidis (2005), observing the following characters: 1) macroscopic

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114 aspect of the culture; 2) number of cells in trichomes; 3) trichome curvature and
115 constriction; 4) shape of cells and measurements; 5) cell content; 6) presence/absence of
116 aerotopes; 7) presence/absence of filaments sheaths or mucilage (using China Ink); 8)
117 reproduction. The measurements were tabulated and the cells length/width were
118 calculated for each of the new genera.
119
120 *2.4. DNA extraction, PCR amplification and sequencing*
121 Total genomic DNA of the three studied strains (LEGE 06013, LEGE 11480 and
122 LEGE 11467) was isolated using MOBIO Ultraclean DNA Isolation Kit (Life
123 Technologies). To obtain the 16S rRNA gene and the 16S-23S Internal Transcribed
124 Spacer (ITS) of the strains LEGE 06013 and LEGE 11467, the PCR was performed
125 using the primers 27F1 (Neilan et al., 1997) and 23SR (Taton et al., 2003) in a Biometra
126 2 thermal cycler (Analytik Jena). The reaction contained 13 µl H_2O, 5 µl 5× Buffer
127 (Promega), 2 µl $MgCl_2$ (25mM), 1 µl DNTPs (10 µM), 1.25 µl of each primer (10 µM),
128 0.3 µl of GoTaq polymerase (Promega). The thermal cycling conditions used were:
129 initial denaturing 94ºC (5 min) followed by 10 cycles of 94 ºC (45 s), 57 ºC (45 s), 72
130 ºC (2 min), then 25 cycles of 92 ºC (45 s), 54ºC (45 s), 72ºC (2 min) before a final
131 elongation step of 72ºC (7min). The PCR products were cloned using the pGEM®–T
132 Easy Vector System (Promega, Madison, WI, USA), transformed by heat–shock into *E.*
133 *coli* cells and plated for blue–white screening (Sambrook & Russel 2001). Two colonies
134 were selected for each strain. After growth, plasmids were extracted from white
135 colonies using the NZYTech Miniprep Kit (NZYTech), and were prepared for
136 sequencing using the primers 27F (Neilan et al., 1997), 359F (Nubel et al., 1997), 781R
137 (Nubel et al., 1997), 1114F (Lane, 1991) and 23S30R (Neilan et al,. 1997). The
138 resulting sequences were assembled using Geneious 8.1.9. software package

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139 (Biomatters) and analyzed for the presence of chimeras hidden in the rRNA sequences
140 by DECIPHER web tool (Wright et al., 2012).
141 To obtain the sequence of the 16S rRNA gene of strain LEGE 11480, this gene
142 was amplified from its gDNA using two set of primers: 27F/781R and 359F/1494R
143 (Neilan et. al, 1997; Nubel et al., 1997) in a MyCycler (Bio-Rad laboratories Inc.,
144 Hercules, CA, USA) or T-Professional Standard (Analytik Jena) thermal cyclers,
145 following the methodology previously described (Tamagnini et al., 1997). For this set of
146 primers, the PCR reaction contained 7.9 µl H_2O, 4 µl 5× Buffer (Promega), 2 µl $MgCl_2$
147 (25mM), 1 µl DNTPs (10 µM), 2 µl of each primer (10 µM), 0.1 µl GoTaq polymerase
148 (Promega) and 1 µl of template DNA. The thermal cycling conditions were: initial
149 denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1
150 min, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, and a final extension
151 step at 72 °C for 5 min. After PCR, to obtain the sequences, the PCR products were
152 purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren,
153 Germany). Purified PCR products were cloned into pGEM®-T Easy Vector (Promega,
154 Madison, WI, USA), and transformed into OneShot TOP10 chemically competent
155 *Escherichia coli* cells (Invitrogen, Carlsbad, CA) by heat-shock. After blue-white
156 screening, plasmid DNA was isolated using GenElute ™ Plasmid Miniprep Kit (Sigma-
157 Aldrich) and sequenced using the M13 primers: reverse (22mer): 5'-d
158 (TCACACAGGAAACAGCTATGAC)-3' and forward (24mer): 5'-d
159 (CGCCAGGGTTTTCCCAGTCACGAC)-3'. The resulting sequences were assembled
160 using Geneious 7.0. software package (Biomatters) and analyzed for the presence of
161 chimeras hidden in the rRNA sequences by DECIPHER web tool (Wright et al., 2012).
162

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163 *2.5. 16S rRNA gene phylogenetic analysis and 16S-23S rRNA intergenic spacer (ITS)*
164 *secondary structures*

165 Phylogenies were constructed aligning *Zarkia* and *Romeriopsis* 16S rRNA gene
166 sequences with a set of 85 sequences of homocytous cyanobacterial strains retrieved
167 from the GenBank using BLAST. Also, we added reference strains of Synechococcales
168 and Oscillatoriales genera and families. The total alignment length had 2384 nucleotide
169 positions with 936 informative sites. Then, to find the phylogenetic positions of our
170 sequences, we performed Maximum Likelihood (ML) and Bayesian Inference (BA)
171 analysis. A similarity matrix (p-distance) was also generated to compare taxa, using
172 MEGA version 6 (Tamura et al., 2013).

173 All the alignments were performed using ClustalW (Thompson et al., 1994); ML
174 trees were performed using RAxML-HPC2 on XSEDE 8.2.10 (Stamatakis, 2014) with
175 bootstrap = 1000; BA tree was performed using MrBayes on XSEDE 3.2.6 (Ronquist et
176 al., 2012), with two runs of 5×10^7 generations and NST = 6. The other parameters were
177 left as defaults. The standard deviation for split frequencies was 0.01. All of those were
178 ran on CIPRES Science Gateway (Miller et al., 2015).

179 The secondary structures of the D1-D1', Box B, V2 and V3 helices of 16S-23S
180 rRNA ITS were folded using Mfold (Zuker, 2003) using default parameters. The V2
181 helices were not found in *Pantanalinema* Vaz et al. sequences, because these ITS
182 sequences do not present tRNAs.

183

184

185 **3. RESULTS**

186 *3.1. Phylogenetic analysis*

187

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188 The BA (Fig. 1) and ML (Fig. S1) phylogenies (91 OTUs, 936 informative sites)
189 show very identical topologies and strong statistical support in the backbones. Both
190 trees show Oscillatoriales (BA 0.99, ML 78) and Synechococcales (BA 1, ML 37) as
191 monophyletic orders, although Pseudanabaenaceae (traditionally Synechochoccales) is
192 positioned at the base of both trees, outside of Oscillatoriales or Synechococcales.
193 The phylogenies confirm *Romeriopsis* as a monophyletic clade (ML=100,
194 BA=1) in family Leptolyngbyaceae (Synechococcales), being closely related to
195 *Alkalinema*. The new genus *Zarkia* is also monophyletic, with strong phylogenetic
196 support (ML=100, BA=1), and is clustered with *Lyngbya cf. confervoides* (ML=79,
197 BA=0.8), "*Phormidium*" GI08AO and *Oxynema* clades in the Oscillatoriales. In these
198 phylogenetic trees, the order Synechococcales presents well-supported monophyletic
199 families. The order Oscillatoriales presents polyphyletic families, such as
200 Microcoleaceae and Oscillatoriaceae.
201 Although *Zarkia* is clustered with the Microcoleaceae genus *Oxynema* (ML=72,
202 BA=1), the new genus is phylogenetically distant to *Microcoleus* and cannot be
203 included in Microcoleaceae. Furthermore, *Zarkia* is not clustered with any other
204 reference strain of any already known family (e. g. *Oscillatoria princeps* CCALA 115
205 for Oscillatoriaceae). These observations, taken together with the polyphyletic
206 Oscillatoriales families, preclude the inclusion of the new genus *Zarkia* in any
207 previously described family. Consequently, we describe below the new monophyletic
208 family Zarkiaceae to encompass *Zarkia*, *Oxynema* Chatchawan and related clades.
209 The similarity matrix (p-distance) (Table S1) corroborates the phylogenetic
210 inferences and confirms *Romeriopsis* and *Zarkia* as new genera. This analysis shows
211 *Romeriopsis* with 98.4-99.7% of intra-clade similarity and only 89.6% of similarity to
212 *Alkalinema*, its phylogenetically most closely-related clade. The similarity between

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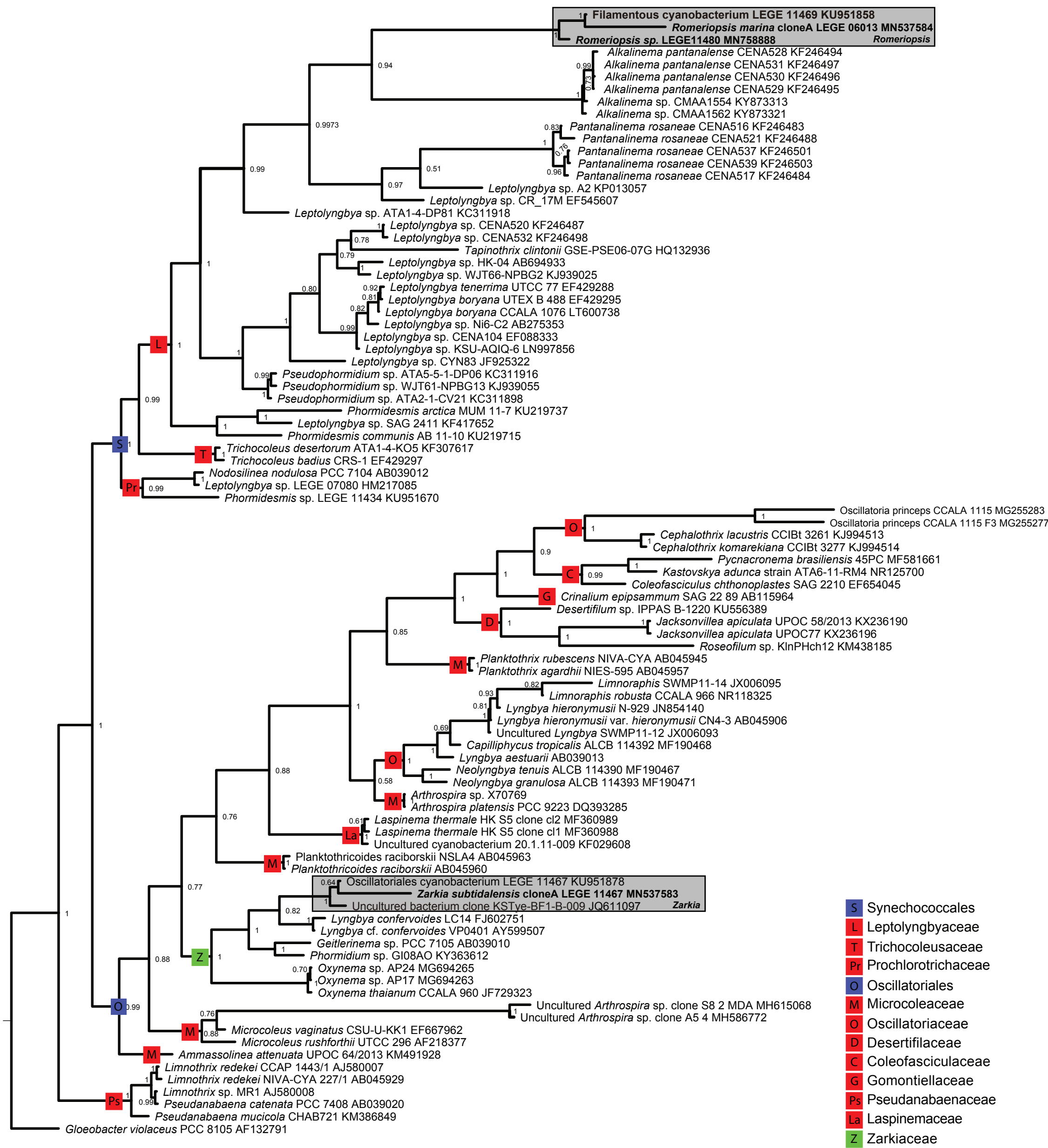
213 *Zarkia* sequences is 99.1% and the similarity between this clade and *Lyngbya*

214 *confervoides*, its most closely-related clade, is only 86.3%. The similarity between

215 *Zarkia* and the clade of *Phormidium* GI08AO and *Geitlerinema* sp. PCC 7105 ranges

216 between 93.6-94.5%.

217



218 **Fig. 1.** Bayesian 16S rRNA gene phylogeny constructed with trees 91 OTUs and 936 informative
219 sites. Nodes support are presenting BA posterior probabilities. The strains sequenced for this study
220 are in bold. Blue squares represent order level nodes. Red squares represent family level nodes.

221

222

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223 *3.2. 16S-23S rRNA intergenic spacer (ITS) analysis*

224 *3.2.1. Zarkia*

225 We compared the ITS secondary structure of *Zarkia* with that of its
226 phylogenetically closest related described genus with available ITS data, *Oxynema* (Fig.
227 2). It was possible to compare only the D1-D1' helix, because of the short length of the
228 *Oxynema* CCALA 960 JF729323 sequence. Between both genera, this helix is variable
229 in sequence, length and structure. *Zarkia* D1-D1' helix presents a long basal stem with
230 10 bp plus two residues, while *Oxynema* presents the typical 4 bp ($^{5'}$GACC$^{3'}$ /$^{5'}$GGTC$^{3'}$)
231 D1-D1' cyanobacterial basal stem. The long basal stem of *Zarkia* is unique among
232 Cyanobacteria. Furthermore, the 5' side of *Zarkia*'s molecule presents no residues
233 opposing the first lateral bulge, while *Oxynema* presents three residues in this position.
234 These differences support the separation of both genera, which is in line with our
235 phylogenetic analysis. It was not possible to compare *Zarkia* with *Lyngbya*
236 *confervoides,* "*Phormidium*" GI08AO and "*Geitlerinema*" PCC7105, because of the
237 lack of ITS data for these strains.

238

239 *3.2.2. Romeriopsis*

240 We compared the ITS secondary structures of *Romeriopsis* with those of its
241 phylogenetically closest related genera, *Alkalinema* and *Pantanalinema* (Fig. 2).
242 *Pantalinema*'s ITS sequences do not present the tRNAs, and because of that, the
243 comparisons among the V2 helices were not possible with this genus. The D1-D1' helix
244 of *Romeriopsis* and *Alkalinema* are identical in the basal stem, lateral bulge and first
245 loop, making the differentiation between genera impossible by this helix region. It is
246 possible to differentiate both genera by the terminal loop. Although *Alkalinema*'s D1-
247 D1' helices are variable in the genus, the terminal loop is conserved ($^{5'}$CUAG$^{3'}$) and

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248 different from *Romeriopsis* ($^{5'}$UUCG$^{3'}$). The Box B, V2 and V3 helices are very

249 different in length, sequence and structure, supporting the separation of both genera.

250 Comparing *Romeriopsis* with *Pantanalinema*, the D1-D1' helix presents small

251 variations among the strains of the latter genus. Even so, these helices are different from

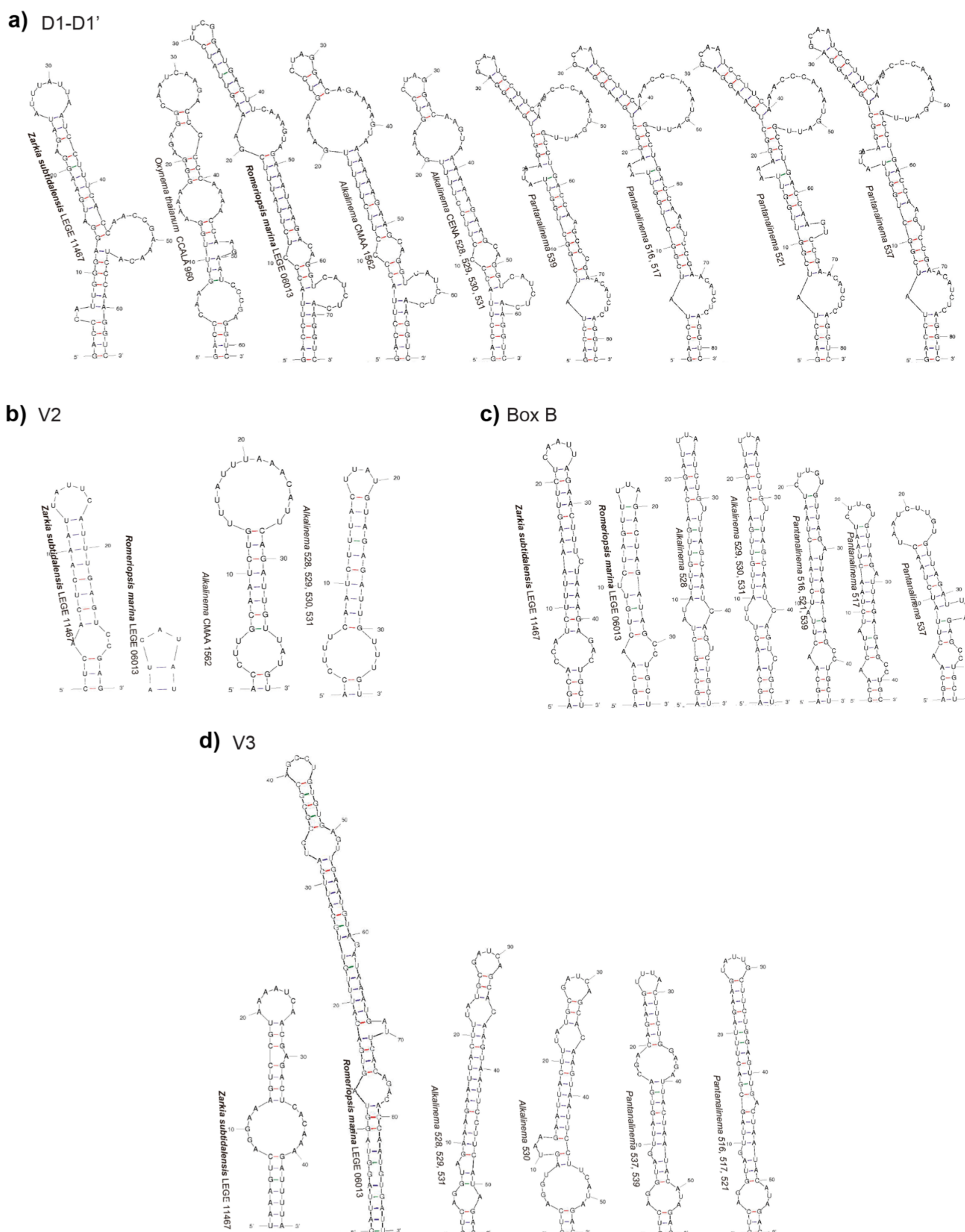

Fig. 2. 16S-23S rRNA gene ITS secondary structures of a) D1-D1' helices, b) V2 helices, c) Box B helices and d) V3 helices of *Zarkia*, *Romeriopsis* and phylogenetically related genera.

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252 *Romeriopsis* helices by length, sequence and structure, remarkably by the presence of an
253 adenine opposing the first lateral bulge in *Pantanalinema* and absence of residues in this
254 position in *Romeriopsis*. The Box B, V2 and V3 helices are very different in length,
255 sequence and structure, also corroborating the separation of both genera.
256
257 *3.3. Descriptions of the new taxa*
258 **Oscillatoriales** Schaffner
259 **Zarkiaceae** fam. nov. G.S. Hentschke & P. N. Leão
260
261 Filaments isopolar, solitary or entangled. Sheaths present, firm or diffluent,
262 homogenous, opened at the ends, hyaline. Trichomes straight or wavy, cylindrical or
263 narrowed at the ends, constricted or not constricted at cross-walls, facultatively motile.
264 Cells isodiametric, longer than wide, shorter than wide, or discoid. Cell content
265 sometimes granulated, with or without aerotopes. Reproduction by hormogonia.
266
267 Type: *Zarkia subtidalensis* G.S. Hentschke, A. Pinheiro, V. Ramos & P. N. Leão
268
269 ***Zarkia subtidalensis*** gen. et sp. nov. G.S. Hentschke, A. Pinheiro, V. Ramos & P. N.
270 Leão
271 Fig. 3
272
273 In culture, forming mats attached to the flask walls. Filaments isopolar, solitary
274 or entangled, straight or wavy. Sheaths firm, thin, homogenous and colourless.
275 Trichomes cylindrical, not constricted or slightly constricted (shorter cells) at cross-
276 walls, sometimes motile. Cells shorter than wide, rarely isodiametric or, rarely discoid

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277 (only after division), 2.7-4 µm length, 5.6-7.6 µm wide, ratio l/w 1.4-2.3 for adult cells.

278 Apical cells rounded. Cell content homogenous, dark green, with aerotopes.

279

280 **Holotype**: PO-T4766 (unialgal population preserved lyophilized), University of Porto

281 Herbarium.

282 **Type locality:** 'A Pedra', diving spot in front of the fort 'Castelo do Queijo', Portugal:

283 (41.185809 N 8.719079 W)

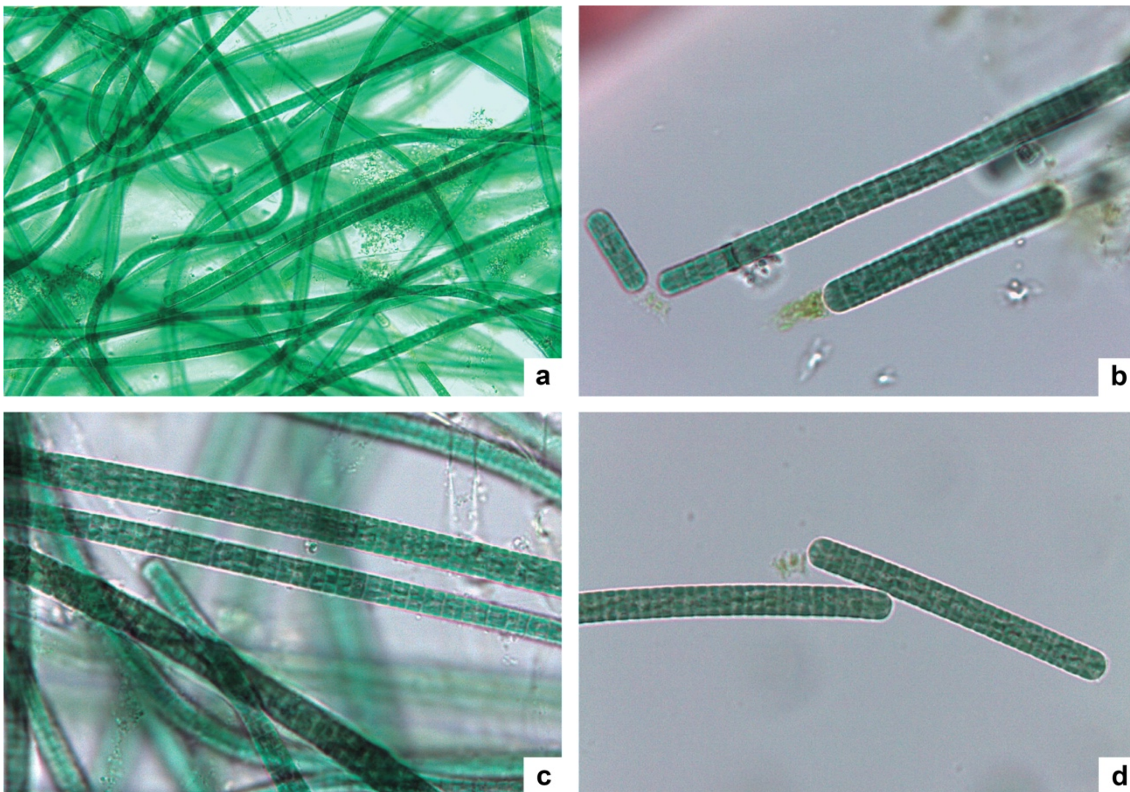
284 **Habitat:** marine, subtidal sample, epilithic (13 m depth), about 200 m off the shore.

285 **Etymology:** *Zarkia,* from Arabic-hispanic "zarco" means blue, the color of the ocean,

286 and for Gonçalves Zarco Square, location of the fort near the collection site;

287 *subtidalensis* is for subtidal.

288 **Reference strain:** *Zarkia subtidalensis* LEGE 11467 (MN537583)



289

290 **Fig. 3.** Microphotographs of *Zarkia subtidalensis*. **a.** General view of the culture; **b-d.** Details of
291 hormogonia and trichomes showing shorter than wide cells. Magnification: **3a** = 40×, **3b-d** = 1000×.
292

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293 Synechococcales Hoffmann et al.

294 **Leptolyngbyaceae** (Komárek et Anagnostidis) Komárek et al. 2014

295 ***Romeriopsis marina*** gen. et sp. nov. G.S. Hentschke, A. Pinheiro, V. Ramos et P. N.

296 Leão

297 Fig. 4a-d

298 In culture, trichomes solitary or forming fluffy clusters not attached to the flask

299 walls. Trichomes cylindrical, constricted, slightly curved or wavy, few celled (more

300 common) or long (>50 cells check other species). With very thin sheaths (visible only in

301 broken filaments) or without. Without mucilaginous envelope. Adult cells longer than

302 wide, cylindrical or rarely barrel shaped, 1.6-3 μm length, 1.5-2 μm wide, ratio

303 length/width 1.2-1.9. Terminal cells rounded. Cell content olive-green, not granulated,

304 without aerotopes. Reproduction by fragmentation of trichomes.

305

306 **Holotype:** PO-T4767 (unialgal population preserved lyophilized), University of Porto

307 Herbarium.

308 **Type locality:** Praia da Foz do Arelho, Caldas da Rainha, Portugal, (39.43327 N

309 9.230275 W)

310 **Habitat:** marine, intertidal zone, wave-exposed rock

311 **Etymology:** *Romeriospsis,* similar to *Romeria*, due to the short trichomes; *marina* is for

312 marine.

313 **Reference strain:** *Romeriopsis marina* LEGE 06013 MN537584

314

315 Notes

316 Differs from *Romeria* by the common presence of long trichomes (>50 cells),

317 which do not disintegrate easily, by the presence of sheaths and by the absence of

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318 diffluent mucilaginous envelopes. Indistinguishable in morphology from

319 *Pantanalinema* and *Alkalinema.*

320 *Romeriopsis* sp. LEGE 11480 (Fig. 4, e-h) was identified only in generic level.

321 This strain is morphologically identical to *Romeriospsis marina* LEGE 06013 but

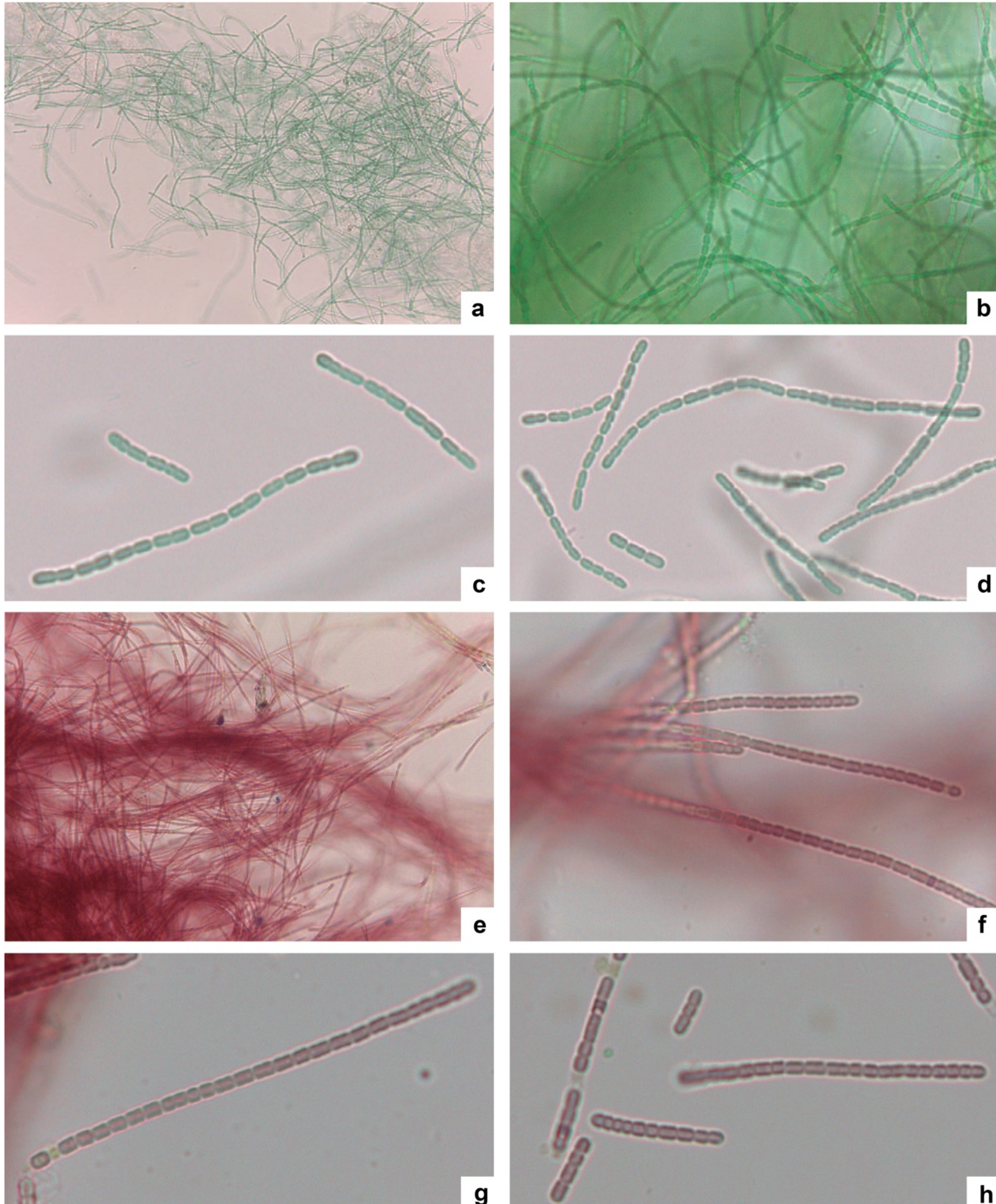

Fig. 4. Microphotographs of *Romeriopsis marina* (**a-d**) and *Romeriopsis* sp. (**e-h**). **a, b** – General view of the culture showing long and short trichomes; **c, d** – Details of short trichomes. **e** – General view of the culture showing long and short trichomes; **f-h** – Details of short trichomes. Magnification: **4a, 4e** = 40×; **4b-d** and **4f-h** = 1000×.

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322 presents reddish cell content and was collected in a different habitat. *Romeriopsis* sp.
323 LEGE 11480 was collected at the same location and habitat as *Zarkia subtidalensis*
324 LEGE 11467, on a rocky substrate at 13m depth and 200 m off the shore, being
325 subtidal, while *R. marina* LEGE 06013 was collected on a wave-exposed rock in the
326 intertidal zone. Although the 16S rRNA gene tree indicates that *Romeriopsis* sp. LEGE
327 11480 could be another species in *Romeriopsis* clade, we could not describe it, because
328 of the lack of ITS data and any morphological diagnostic character.
329

330 **4. Discussion**

331 The wave energy action and related environmental conditions from the north
332 continental Portuguese coast (Coelho et al., 2009), where the studied marine strains
333 were collected, are known drivers shaping the coastal biodiversity, including that of
334 microbial communities (Witt et al., 2012). Along with the sampling effort, this may
335 partially explain why this marine temperate region is being a rich source of novel
336 cyanobacteria taxa, already described (Brito et al., 2017) or at least phylogenetically
337 highlighted (Ramos et al., 2018).
338 In this paper we described the new genera *Romeriopsis* and *Zarkia*, and the new
339 family Zarkiaceae supported by 16S rRNA gene phylogeny and 16S-23S rRNA ITS
340 secondary structures. The family Zarkiaceae was named after *Zarkia* and encompasses a
341 monophyletic clade containing *Zarkia*, as well as the strains *Lyngbya confervoides*
342 LC14 FJ602751, *Lyngbya* cf. *confervoides* VP0401 AY599507, *Phormidim* sp. GI08AO
343 KY363612, *Geitlerinema* sp. PCC 7105 AB039010 and the genus *Oxynema*. The strains
344 identified as *Lyngbya confervoides*, were previously used as reference for *Lyngbya* by
345 Komárek et al. (2013) and Caires et al. (2018a), but although they are morphologically
346 and ecologically similar to the type species description, they are not well characterized

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347 (Komárek et al., 2013) and cannot be surely assigned to this genus, because of limited
348 available data (only one photo and not conclusive phylogeny in Sharp et al., 2009).
349 Many other papers show *Lyngbya* as a polyphyletic genus (Engene et al., 2018; Caires
350 et al., 2018b; Mühlsteinová et al., 2018) and to identify the true *Lyngbya* clade, a robust
351 revision of the genus is needed. This has been done, for example, by Mühlsteinová et al.
352 (2018) to establish the true *Oscillatoria* clade. According to this, we didn´t include the
353 genus *Lyngbya* in Zarkiaceae and chose to consider the *Lyngbya* cf. *confervoides* clade
354 as still undefined, worthy to be revised., The monophyletic clade containing
355 "*Phormidium*" GI08AO and "*Geitlerinema*" PCC 7105 strains is another genus that has
356 to be described in the future.
357 Morphologically, Zarkiaceae is similar to Microcoleaeceae, presenting, in the
358 case of *Zarkia*, isodiametric or slightly shorter than wide adult cells, and is it also
359 similar to Oscilatoriaceae presenting discoid cells, in the case of *Lyngbya* cf.
360 *confervoides* strains. As for the descriptions of Ocullatelaceae and Trichocoleaceae
361 (Synechococcales) (Mai et al., 2018), no diacritical morphological markers were found
362 for Zarkiaceae. Although this lack of morphological diacritic characters, according to
363 our trees, we could not assign *Zarkia* to any already described family, considering that
364 this genus is phylogenetically unrelated to any reference strains for previously-described
365 families represented here by *Desertifilum* sp. IPPAS B-1220 KU556389
366 (Desertifilaceae), *Coleofasciculus chthonoplastes* SAG 2210 EF654045
367 (Coleofasciculaceae), *Crinalium epipsammum* SAG 22.89 NR_112218
368 (Gomontiellaceae), *Microcoleus vaginatus* CSU-U-KK1 EF66796 (Microcoleaceae)
369 and *Oscillatoria princeps* CCALA 1115 F3 MG255277 (Oscillatoriaceae). Furthermore,
370 it is evident that Oscillatoriales families are polyphyletic. Our trees show six clades
371 containing Microcoleaceae genera, five of which not related to *Microcoleus*. Likewise,

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372 there is one additional Oscillatoriaceae clade (*Capilliphycus* and *Neolyngbya* clade) not
373 related to *Oscillatoria*. Although we find Oscillatoriales as a monophyletic order, our
374 phylogenies are in agreement with Ishida et al. (2001), Jahodarová et al. (2017) and
375 Nowicka-Krawczyk et al. (2018), which also show many polyphyletic Oscillatoriales
376 families, including Microcoleaceae and Oscillatoriaceae. We highlight also that the
377 family Pseudanabaenaceae (traditionally Synechococcales) is not included in
378 Oscillatoriales or Synechococcales clades, indicating that the phylogenetic position and
379 taxonomy of this family must be revised. The same result was found by Mai et al
380 (2018).

381 In this study we use the monophyletic species concept (Johansen and Casamatta,
382 2005) for delimitation of genera and families (Komárek et al., 2014; Mai et al., 2018).
383 As stated by Komárek et al. (2014), "morphological characters used to define higher
384 taxa…have apparently arisen and/or been lost several times during the evolution of
385 modern species and genera". Considering this, we believe that in the near future, after
386 taxonomical revisions based on 16S rRNA gene, cryptic cyanobacterial families will be
387 more common. Mai et al. (2018) highlight the importance of finding new markers for
388 higher taxonomical levels; in order to describe Ocullatelaceae and Trichocoleaceae,
389 they have used molecular markers in the 16S rRNA gene secondary structures and
390 *rpco1* gene phylogenies, which, however were not always in agreement with the 16S
391 rRNA gene phylogenies. Due to this, we considered the 16S rRNA gene phylogenies as
392 the current instrument to separate families, which, combined with the monophyletic
393 nature of Zarkiaceae, led to the description of a new family. The 16S-23S rRNA gene
394 ITS secondary structures support our proposal, since *Zarkia* presents a unique D1-D1'
395 helix among Cyanobacteria.

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396 In the Synechococcales clade, our phylogenetic analysis (Fig. 1) is in agreement
397 with the revision of Mai et al. (2018) for the order, showing monophyletic families.
398 *Romeriopsis* is clearly monophyletic (ML=100, BA=1) and positioned in
399 Leptolyngbyaceae, clustered with *Alkalinema* and *Pantanalinema*. A smaller related
400 clade contains strains assigned to *Leptolyngbya* (*Leptolyngbya* sp. A2 KP01305 and
401 *Leptolyngbya* sp. CR_17M EF545607), which must be described as a new genus in the
402 future. The 16S-23S rRNA gene ITS secondary structures are also in agreement with
403 these findings, as explained in the results section.
404 The herein described new genera, *Zarkia* and *Romeriopsis*, are supported by 16S
405 rRNA phylogenies, 16S-23S rRNA gene ITS secondary structures and morphological
406 analysis. *Zarkia* is grouped with high phylogenetic support with *Lyngbya* cf.
407 *confervoides*, but these taxa are morphologically very different. *Zarkia* presents adult
408 cells mainly smaller than wide (ratio l/w 1.4-2.3), discoid only after division, while
409 *Lyngbya* cf. confervoides presents the typical *Lyngbya* discoid adult cells. The 16S-23S
410 rRNA gene ITS secondary structures of the D1-D1' helix of *Zarkia* are unique among
411 Cyanobacteria, as commented above. *Romeriopsis* is morphologically similar to
412 *Romeria* regarding a generally short trichome length but differs by the common
413 presence of long trichomes (>50 cells), which do not disintegrate easily as in *Romeria*,
414 by the presence of sheaths and the absence of diffluent mucilaginous envelopes. Up to
415 now, there is no available 16S rRNA gene sequence clearly assigned to *Romeria* in
416 public databases. For this reason, we have not included this genus in our phylogeny.
417 *Romeriospsis* is morphologically indistinguishable from *Pantanalinema* and
418 *Alkalinema,* but the phylogenies and the 16S-23S rRNA gene ITS secondary structures
419 in the current study confirm that these are three separate genera.
420

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421

422 **5. Conclusion**

423 The 16S rRNA gene is still the most reliable molecular marker for taxonomical
424 studies at the genus level and is proving to be useful also for family delimitation of
425 Cyanobacteria, although additional markers must be tested. For that reason, a global
426 effort from the research community is required to allow the reconstruction of the
427 evolutionary history of these photosynthetic microorganisms. Traditional,
428 morphological-based family taxonomy is in conflict with present molecular and
429 phylogenetic data. By proposing the erection of a new family, this work is an additional
430 contribution towards the long journey of resolving Cyanobacteria systematics. Based on
431 the phylogenetic data presented herein, we also emphasize the need for a taxonomic
432 revision of several Oscillatoriales families and genera.

433

434 **Declaration of interest**

435 All authors declare no conflict of interest.

436

437 **Statement of informed consent**

438 No conflicts, informed consent, human or animal rights applicable.

439

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444

445 **Declaration of the contributions of the authors**

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446 GSH worked in collection and assembly of data, analysis and interpretation of the data
447 and drafting the article. AP, VR, AB, MSC, SB, VV, PNL worked in conception and
448 design, collection and assembly of data, obtaining of funding, analysis and
449 interpretation of the data and critical revision of the article for important intellectual
450 content. All authors read and approved the final version of the manuscript.
451

452 **Supporting Information**

453 **Fig. S1.** Maximum Likelihood 16S rRNA gene phylogeny performed with 91 OTUs
454 and 936 informative sites.
455 **Table S1.** Similarity matrix (p-distance) comparing the 16S rRNA gene of *Zarkia,*
456 *Romeriopsis* and related strains.
457
458

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