Culture-dependent characterization of cyanobacterial diversity in the intertidal zones of the Portuguese coast: A polyphasic study

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Abstract

Cyanobacteria are important primary producers, and many are able to fix atmospheric nitrogen playing a key role in the marine environment. However, not much is known about the diversity of cyanobacteria in Portuguese marine waters. This paper describes the diversity of 60 strains isolated from benthic habitats in 9 sites (intertidal zones) on the Portuguese South and West coasts. The strains were characterized by a morphological study (light and electron microscopy) and by a molecular characterization (partial 16S rRNA, *nifH*, *nifK*, *mcyA*, *mcyE*/*ndaF*, *sxtI* genes). The morphological analyses revealed 35 morphotypes (15 genera and 16 species) belonging to 4 cyanobacterial Orders/Subsections. The dominant groups among the isolates were the Oscillatoriales. There is a broad congruence between morphological and molecular assignments. The 16S rRNA gene sequences of 9 strains have less than 97% similarity compared to the sequences in the databases, revealing novel cyanobacterial diversity. Phylogenetic analysis, based on partial 16S rRNA gene sequences showed at least 12 clusters. One-third of the isolates are potential N_2-fixers, as they exhibit heterocysts or the presence of *nif* genes was demonstrated by PCR. Additionally, no conventional freshwater toxins genes were detected by PCR screening.

Keywords

Cyanobacteria; Marine; 16S rRNA gene; Nitrogen fixation; Phylogeny; Toxins

Introduction

Continental Portugal has an extensive coastline, of about 940 km, facing the North Atlantic Ocean. It is one of the warmest European countries, and its climate is classified as Mediterranean type Csa in the south (C – warm temperate; s – summer dry; a – hot summer) and Csb in the north (C – warm temperate; s – summer dry; b – warm summer), according to the Köppen's scheme [23]. The near-shore wave energy has a strong spatial and seasonal variability [30]. Western and southern coasts are evidently under different wave regimes, with the unsheltered west coast sites experiencing higher wave height and power than southern ones, and a moderate decreasing gradient from north to south can be observed. Wave height and power in the winter are also much higher than in the summer [30], and along the coast it is possible to encounter different topographies and beach morphologies – rocky beaches, beaches with sand and rocks and sandy beaches with dispersed rocks, resulting in diverse levels of exposure to the prevailing wave regimen.

Cyanobacteria are photosynthetic prokaryotes with a wide geographical distribution that are present in a broad spectrum of environmental conditions [49]. Taxonomy of cyanobacteria is a controversial subject, with two prevailing approaches, the "botanical" and "bacterial". The reorganized taxonomic revision based on the botanical code uses morphological, biochemical and molecular characters [19], [20] and [21]. After the recognition of the prokaryotic features of cyanobacteria, Rippka et al. [36] published a bacteriological taxonomy based on morphological, biochemical and genetic characters of axenic cultures, while Bergey's Manual of Systematic Bacteriology [5] uses a phylogenetic approach primarily based on 16S rRNA sequence comparisons. In summary, cyanobacteria can be classified into five Subsections [5] and [36] that broadly coincide with Orders of the botanical approach [19], [20] and [21]. Cyanobacteria belonging to subsections I (Chroococcales) and II (Pleurocapsales) are unicellular, but while the organisms in subsection I divide exclusively by binary fission, the ones from subsection II can also undergo multiple fission producing small easily dispersible cells called baeocytes. The subsection III (Oscillatoriales) includes the filamentous strains without cell differentiation. Subsections IV (Nostocales) and V (Stigonematales) comprise the filamentous strains that are able to differentiate heterocysts and akinetes. In addition, filaments of cyanobacteria from subsection V are able to divide in multiple planes displaying true branching.

Benthic cyanobacteria grow along the shore on different substrata, mainly in the intertidal zones, as part of complex multi-taxa communities, often forming cohesive mats and biofilms. In these habitats, they are exposed to a range of daily stresses such as nutrient limitation, high UV-radiation and desiccation[1] and [6]. The successful adaptation to these harsh environments is largely due to their morphological and functional versatility [31]. Cyanobacteria play a major role in the global carbon cycle as important primary producers performing oxygenic photosynthesis, and the diazotrophic taxa are fundamental to the nitrogen cycle, particularly in oceans [18]. In addition to their ecological importance, cyanobacteria are also recognized as being a prolific source of biologically active natural products; some of these compounds are toxic to a wide array

of organisms [50]. Nevertheless, little is known about the diversity of these organisms along the Portuguese coast with only a few reports published [2] and [9]. Araújo et al. [2] provided an updated checklist of the benthic marine algae of the northern Portuguese coast, including 26 species of cyanobacteria. However, these authors based their work on new records, literature references and herbarium data but did not isolate or maintain cultures of the observed specimens. The aim of this work was to identify the cyanobacteria present in the intertidal zones of the Portuguese coast using a polyphasic approach. The isolated specimens are kept at LEGE Culture Collection, and available for further studies. In addition to the assessment of cyanobacterial diversity, a PCR-based screen for putative diazotrophs and toxin-producers was performed to unveil their role(s) in the ecosystem.

Materials and methods

Sampling sites

The sites (Table 1) were selected in order to represent the diversity of the Portuguese coast. Along the coast it is possible to discriminate between rocky beaches (sampling sites 1 and 8), beaches with sand and rocks (sampling sites 2, 3, 4, 7) and sandy beaches with dispersed rocks (sampling sites 5, 6 and 9) (Table 1). Several relevant variables: wave power [30], sea surface temperature [SST [25]], river runoff and other important climatic variables such as air temperature and precipitation (Instituto de Meteorologia, IP, Portugal) were also taken into account. In brief, West coast sampling sites experience generally higher energetic wave regimes, lower overall mean SSTs and mean air temperatures [40], and higher fresh water inputs, both from river runoff and precipitation, than their South coast counterparts.

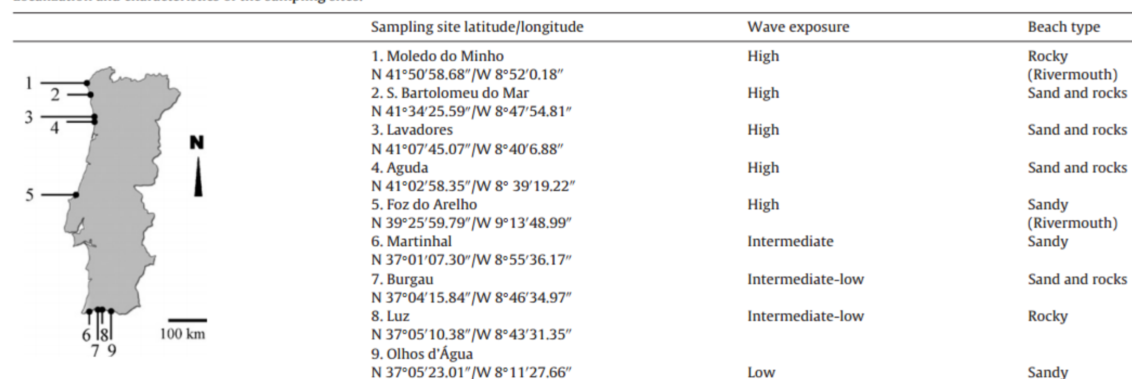
Table 1
Localization and characteristics of the sampling sites.

Sampling site latitude/longitude	Wave exposure	Beach type
1. Moledo do Minho N 41°50′58.68″/W 8°52′0.18″	High	Rocky (Rivermouth)
2. S. Bartolomeu do Mar N 41°34′25.59″/W 8°47′54.81″	High	Sand and rocks
3. Lavadores N 41°07′45.07″/W 8°40′6.88″	High	Sand and rocks
4. Aguda N 41°02′58.35″/W 8° 39′19.22″	High	Sand and rocks
5. Foz do Arelho N 39°25′59.79″/W 9°13′48.99″	High	Sandy (Rivermouth)
6. Martinhal N 37°01′07.30″/W 8°55′36.17″	Intermediate	Sandy
7. Burgau N 37°04′15.84″/W 8°46′34.97″	Intermediate-low	Sand and rocks
8. Luz N 37°05′10.38″/W 8°43′31.35″	Intermediate-low	Rocky
9. Olhos d'Água N 37°05′23.01″/W 8°11′27.66″	Low	Sandy

Cyanobacteria sampling, isolation, and culturing

Biological samples were collected in both summer and autumn of 2006, and spring of 2007. Sample collection was performed by scraping the surface of a wide range of substrates (e.g. seaweeds, rock surfaces, seashells, *Sabellaria alveolata* reefs) present on bare rocks or shallow

puddles tidal pools. For the isolate LEGE 06009 see also [9]. In addition, seawater samples from the surf zone were also collected.
Raw biological samples were screened for cyanobacterial specimens using a light microscope (Leica DMLB), and subsequently subjected to liquid culture enrichment, agar plates streaking or micromanipulation. Whenever possible single cells/filaments were isolated and transferred to liquid or solid medium using a Pasteur pipette [34]. When micromanipulation was found unsuitable, aliquots of the enriched liquid cultures were transferred to liquid medium or streaked on agar plates. Sea water samples were filtered with sterile glass fiber filters (GF/C – Ø 47 mm, Whatman), and subsequently placed on liquid medium until a visible colony appeared. Single colonies were picked and streaked on agar plates. New colonies were transferred into liquid medium. Cultures were maintained using the following media: MN [34], $BG11_0$, BG11 [44], and Z8 [22] supplemented with 25 g L^{-1} NaCl, further named Z8 25‰. The media were supplemented with B12 vitamin, and when necessary with cycloheximide or amphothericin B [34]. The cultures were kept under 14 h light (10–30 µmol photons m^{-2} s^{-1})/10 h dark cycles at 25 °C. Cyanobacterial isolates are deposited at LEGE Culture Collection (Laboratório de Ecotoxicologia, Genómica e Evolução; CIIMAR, Porto, Portugal). Additionally, the isolate LEGE 06123 is also deposited at Culture Collection of Algae and Protozoa (CCAP 1425/1), for details on this organism see [33].

Light and transmission electron microscopy (TEM) and morphological identification

Cells were observed using a Leica DMLB microscope (Leica Microsystems GmbH), images were captured with a Leica ICCA Analogue Camera System, and the cells were measured using Qwin Leica software (Leica Microsystems GmbH). Each morphometric parameter was measured 20 times in different individuals.

For TEM studies, cells were collected, centrifuged and processed as described by Seabra et al. [39]. Sections were examined using a Zeiss EM 902.
The morphological identification was carried out following the criteria of Komárek and Anagnostidis [19],[20] and [21], Waterbury and Stanier [47] and by using Bergey's Manual of Systematic Bacteriology [4], i.e. whenever the identification differs in the two systems ("botanical" and "bacteriological"), the corresponding form-genus in Bergey's classification is also mentioned. For Pleurocapsales Waterbury and Stanier [47]classification was followed.

DNA extraction, purification, PCRs and sequencing

Cells were harvested by centrifugation and DNA was extracted using the Maxwell® 16 System, and the Cell DNA Purification Kit for Gram-negative bacteria (Promega Corporation) according to the instructions of the manufacturer. DNA fragments within the following genes were amplified using the oligonucleotide primers listed on Table 2: 16S rRNA gene (small subunit ribosomal gene), *nifK* (dinitrogenase β subunit), *nifH*(dinitrogenase reductase), *mcyA* and *mcyE* (microcystin

synthetase), *ndaF* (nodularin synthetase), and*sxtI* (saxitoxin biosynthesis). The 16S rRNA gene was amplified using two primer pairs, each including one universal primer and one specific for cyanobacteria [8-27F(universal)/CYA781R(specific), and CYA361F(specific)/1492R(universal)], this allowed us to amplify a longer fragment, and to avoid unspecific amplifications since the cultures are not axenic. For *nifK*, the first group of primer pairs (see Table 2) was designed for filamentous cyanobacteria, whereas the second group was designed for unicellular cyanobacteria. PCR reactions were performed using a thermal cycler MyCyclerTM (Bio-Rad laboratories Inc., Hercules, CA, USA) following [45]. The PCR profiles, after a 2–5 min at 94 °C of denaturation step, were the following: 16S rRNA gene – 35 cycles of 94 °C 1 min, 50 °C 1 min, and 72 °C 1 min 30 s; *nifK* – 30 cycles of 94 °C 1 min, 50 °C 1 min, and 72 °C for 1 min 15 s; *mcyA, mcyE,* and *ndaF* – 30 cycles of 95° C 1 min 30 s, 52° C 30 s, and 72 °C 50 s; *sxtI* – 30 cycles of 94° C 10 s, 52 °C 20 s, and 72 °C 1 min. In all cases a final extension of 7 min at 72 °C was performed. Amplification of the *nifH* fragment was performed according to the methods described previously [29]. The PCR products were separated by agarose gel electrophoresis using standard protocols [38]. DNA fragments were isolated from gels using the NZYGelpure Kit (NZYtech, Lda. INOVISA, Lisbon, Portugal), according to the manufacturer's instructions. Purified PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA), and transformed into *Escherichia coli* DH5α competent cells following the manufacturer's instructions, and the methodology described in [33]. Some purified PCR products were directly sequenced at STAB Vida (Lisbon, Portugal).

Table 2
Target genes and oligonucleotide primers used in this study.

Target genes	Primer pair [a]	Sequence 5′ → 3′	PCR product expected size (bp)	Reference
16S rRNA	8-27F	AGAGTTTGATCCTGGCTCAG		[48]
	CYA781R (A) [b]	GACTACTGGGGTATCTAATCCCATT	773	[28]
	CYA781R (B)	GACTACAGGGGTATCTAATCCCTTT		[28]
	CYA361F	GGAATTTTCCGCAATGGG	1131	[27]
	1492R	GGTTACCTTGTTACGACTT		[48]
nifK	nifK0F (A)	CAAGGTTCTCAAGGTTGCGTT	1139	[33]
	nifK1F (B)	CAAGGTTCTCAAGGTTGTGTG		[33]
	nifK4R	TGTAAGTGGTGGCGATCAAAGA		[33]
	nifK0F (A)/nifK1F (B)	See above	407	[33]
	nifK3′R	GGGATGAAGTTGATTTTGCCGTT		This work
	nifk146F	CCTGGGAATATCGGGAA	319	This work
	nifk465R	GCCATACAAGTTGTACAGACA		This work
	nifk310F	CGTCACTTCAAAGAGCCTT	1084	This work
	nifk1394R	GGCGATCAAAGATAGGATA		This work
	nifH4	TTYTAYGGNAARGGNGG		[29]
nifH	nifH3	ATRTTRTTNGCNGCRTA	361	[29]
	nifH2	ADNGCCATCATYTCNCC		[29]
	nifH1	TGYGAYCCNAARGCNGA		[29]
mcyA	*mcyA*-Cd1F	AAAAGTGTTTTATTAGCGGCTCAT	297	[14]
	mcyA-Cd1R	AAAATTAAAAGCCGTATCAAA		[14]
mcyE/ndaF	HEPF	TTTGGGGTTAACTTTTTTGGGCATAGTC	472	[16]
	HEPR	AATTCTTGAGGCTGTAAATCGGTTT		[16]
sxtI	sxtI-F2	GGATCTCAAAGAAGATGGCA	991	This work
	sxtI-R	GGTTCGCCGCGGACATTAAA		[17]

[a] F (forward) and R (reverse).
[b] (A) and (B) – primers used together in a mixture with equimolar concentration.

Nucleotide sequence accession numbers

Novel sequences associated with this study are available in GenBank under the accession numbers FJ589716, HQ832895–HQ832951, JF708120 and JF708121.

Phylogenetic analysis

In order to integrate the cyanobacterial diversity along the Portuguese coast into a broader phylogenetic context, the 16S rRNA gene sequences were analyzed, compared with the currently available databases and used to construct phylogenetic trees. Each sequence was independently used as the query in a BLAST search against the non-redundant nucleotide database of the National Centre for Biotechnology Information (NCBI, March 2011). In order to include full-length sequences and to obtain a reliable overall picture of the cyanobacterial diversity, a number of reference strains from each subsections/orders represented in our samples were retrieved from GenBank and were included in the following phylogenetic analyses. The reference strains were selected according to the Bergey's Manual of Systematic Bacteriology (2001), and from those the ones closely related to our samples were used. A multiple alignment encompassing 16S rRNA gene sequences from the isolates, the reference strains and *Chloroflexus aurantiacus* J-10-fl as the outgroup was performed using the ClustalW2 algorithm [24], with all the default parameters. Due to the size differences in the amplification products of each isolate, and to avoid the consequent bias effect on the tree construction, this alignment was pruned to an internal core of 655 nucleotides present in all sequences – corresponding to nucleotides 519 to 1172 in *E. coli*. The phylogenetic tree was computed using the Maximum-likelihood (ML) methodology [8]. The alignment was imported to Geneious Pro [7], and the tree was build with the PhyML [12] plugin, using the HKY85 as the substitution model and 1000 pseudo-replicates for the bootstrap analysis, and allowing the software to estimate the transition/transversion ratio, the proportion of invariable sites and the gamma distribution parameter with 4 substitution rate categories.

Results and discussion

This work presents a polyphasic study of cyanobacterial isolates from selected intertidal zones along the Portuguese coast, combining the isolation of strains, and their characterization by microscopic observations and molecular analysis.

Morphological characterization

To evaluate the diversity of cyanobacteria, nine beaches that reflect the heterogeneity of habitats present along the continental Portuguese coast were selected (Table 1). Approximately 100 cyanobacterial isolates were retrieved, from which 60 were characterized and shown to belong to 35 different morphotypes. Fifteen genera and 16 species belonging to four cyanobacterial orders/subsections were identified based on morphological characters (Table S1, supplementary material). In terms of the isolate's spatial distribution no clear pattern was observed, i.e. the different cyanobacterial groups are distributed by all the different beaches types. However, one must take into account that the number of isolates of certain genera is much higher than others, and that the different number of isolates obtain for each genus can be due to their ubiquity or to the fact that they are easier to isolate. The isolation media were selected due to their different composition in order to expose the highest possible diversity. MN medium revealed the highest

diversity, Z8 25‰ was particularly successful for coccoids and *Leptolyngbya* spp., while $BG11_0$and BG11 did not contribute to a higher diversity than the Z8 25‰, with the exception of *Nostoc* sp. LEGE 06158. The most representative group among the isolates comprises the nonheterocystous forms, notably the filamentous Oscillatoriales and the unicellular Chroococcales. Representatives from the unicellular Pleurocapsales were also found, as well as heterocystous taxa: Nostocales (Fig. 1). No true-branching filamentous, Stigonematales, were found. The nonheterocystous cyanobacteria are, indeed, reported as the predominant forms in marine environments [3], [6], [43] and [46]. This seems to be also the case for Portugal, the *Checklist of benthic marine algae and cyanobacteria of northern Portugal* ("Checklist") reported 26 species of cyanobacteria, 15 of which are Oscillatoriales [2]. Strains belonging to the genera*Hyella*, *Myxosarcina*, *Lyngbya*, *Pseudophormidium* and *Calothrix* were found in the same area (north of Portugal) as reported in the Checklist. *Cyanobium*, *Leptolyngbya* and *Pseudanabaena* are the most numerous among our isolates (Table S1 and Table 3). Some genera described by [2] were not found in our study (*Chamaecalyx*, *Dermocarpella*, *Entophysalis*, *Hydrococcus*, *Trichocoleus*, *Porphyrosiphon*,*Sirocoleum*, and *Spirocoleus*), whereas *Xenococcus*, *Microcoleus*, *Spirulina* morphotypes were present in our field samples but its isolation was not successful. On the other hand, genera that are not described by these authors were present in our samples (*Aphanothece*, *Cyanobium*, *Synechocystis*, *Chroococcidiopsis*,*Chroococcopsis*, *Leptolyngbya, Pseudanabaena*, *Romeria*, *Schizothrix*, *Nostoc*, and *Scytonema*), some of them also confirmed by a recent studies on the Portuguese coast: *Synechocystis*, *Cyanobium*, and*Leptolyngbya* [9] and [26]. However, Araújo et al. [2] based their work not only on new records but also on literature references and herbarium data, and did not isolate the observed specimens. Representatives of the order Stigonematales were not found in this study nor reported by Araújo et al. [2]. This was expected since it is known that these organisms are poorly represented in marine environments [15], and mainly encountered in the flowing waters of hot springs and soils [11], [36] and [52].

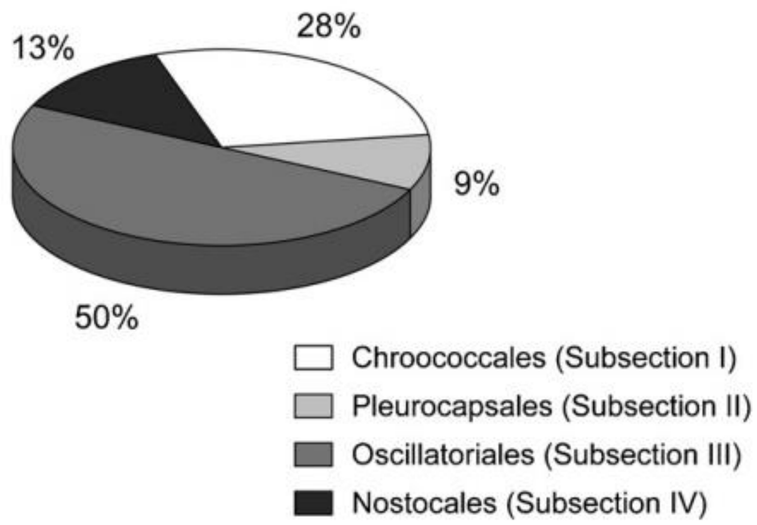

Fig. 1. Distribution of the isolates per taxonomic group.

Table 3
Morphological identification (for details see Table S1) and molecular analysis of the cyanobacterial isolates.

ORDER (Subsection) Genus and species	LEGE code	Site[a]	Accession number	Best hit indicated by BLAST 16S rRNA gene	% Max. Identity	Closest known relative	% Max. Identity
CHROOCOCCALES (I)							
Aphanothece cf. *salina* (*Synechococcus*)	**06149**	1	HQ832898	*Synechococcus* sp. G2.1 (AY054298)	99		
Cyanobium sp.	**06137**	3	HQ832914	*Cyanobium* sp. NS01 (AY172837)	100		
Cyanobium sp.	**06109**	4	HQ832920	*Cyanobium* sp. NS01 (AY172837)	100		
Cyanobium sp.	**06140**	4	HQ832922	*Cyanobium* sp. NS01 (AY172837)	100		
Cyanobium sp.	**06012**	5	HQ832926	*Cyanobium* sp. NS01 (AY172837)	99		
Cyanobium sp.	**06097**	6	HQ832928	*Cyanobium* sp. NS01 (AY172837)	100		
Cyanobium sp.	**07153**	6	HQ832931	*Synechococcus* sp. HOS (AF448064)	98		
Cyanobium sp.	**06127**	7	HQ832935	*Cyanobium* sp. NS01 (AY172837)	100		
Cyanobium sp.	**06143**	7	HQ832936	Cyanobium sp. NS01 (AY172837)	100		
Cyanobium sp.	**06184**	7	HQ832940	*Synechococcus* sp. PS838 (AF448068)	99		
Cyanobium sp.	**06130**	9	HQ832946	*Cyanobium* sp. NS01 (AY172837)	99		
Gloeocapsopsis cf. *crepidinum*	**06123**	8	FJ589716	Unicellular thermophilic cyanobacterium tBTRCCn 23 (DQ471449)	97	*Pleurocapsa* cf. *concharum* 1d-08 (FR798928)	94
Synechococcus nidulans	**07171**	7	HQ832939	Uncultured cyanobacterium clone SP1_1B_30 (GQ325753)	97	*Aphanocapsa muscicola* 5N-04 (FR798920)	95
Synechococcus sp.	**07172**	9	HQ832950	*Synechococcus* sp. MBIC10613 (AB183569)	99		
Synechocystis salina	**06099**	1	HQ832895	*Synechocystis* sp. PCC 6803 (BA000022)	99		
Synechocystis salina	**06155**	2	HQ832911	*Synechocystis* sp. LMECYA 68 (EU078508)	99		
Synechocystis salina	**07173**	9	HQ832951	*Cyanobium* sp. NS01 (AY172837)	99		
PLEUROCAPSALES (II)							
Chroococcidiopsis sp.	**06174**	4	HQ832924	*Pleurocapsa* sp. CALU 1126 (DQ293994)	99		
Chroococcopsis sp. (*Myxosarcina* & *Pleurocapsa*-group)	**07187**	1	HQ832904	*Myxosarcina* sp. PCC 7325 (AJ344562)	99		
Chroococcopsis sp. (*Myxosarcina* and *Pleurocapsa*-group)	**07161**	6	HQ832932	Uncultured cyanobacterium clone AO26 (FJ358912)	96	*Cyanobacterium* sp. MBIC10216 (AB058249)	94
Hyella sp. (*Pleurocapsa-group*)	**07179**	1	HQ832901	*Chroococcidiopsis* sp. PCC 6712 (AJ344557)	95		
Myxosarcina sp. (*Chroococcidiopsis*)	**06146**	1	HQ832897	*Chroococcidiopsis* sp. CCMP1489 (AJ344556)	98		
OSCILLATORIALES (III)							
Leptolyngbya cf. *halophila*	**06110**	1	HQ832896	*Leptolyngbya* sp. 0BB19S12 (AJ639895)	99		
Leptolyngbya cf. *halophila*	**06102**	2	HQ832906	*Leptolyngbya nodulosa* UTEX 2910 (EF122600)	99		
Leptolyngbya cf. *halophila*	**06152**	3	HQ832915	Uncultured bacterium clone ncd516h07c1 (JF046736)	98	*Leptolyngbya nodulosa* UTEX 2910 (EF122600)	98
Leptolyngbya fragilis	**07167**	3	HQ832917	Uncultured cyanobacterium clone SC3-19 (DQ289927)	94	Cyanothece sp. PCC 8802 (CP001701)	93
Leptolyngbya fragilis	**07176**	4	HQ832925	*Gloeothece* sp. KO11DG (AB067577)	94		
Leptolyngbya minuta	**07181**	1	HQ832903	*Phormidium* sp. MBIC10070 (AB058219)	99		
Leptolyngbya mycoidea	**07157**	3	HQ832916	Uncultured bacterium clone CI5cm.45 (EF208676)	93	*Cyanothece* sp. PCC 8802 (CP001701)	93
Leptolyngbya mycoidea	**06009**	5	JF708121	*Leptolyngbya nodulosa* UTEX 2910 (EF122600)	98		
Leptolyngbya mycoidea	**06126**	7	HQ832934	*Pseudanabaenaceae cyanobacterium* DPG1-KK5 (EF654067)	99	*Leptolyngbya* sp. 0BB24S04 (AJ639893)	99
Leptolyngbya mycoidea	**06108**	8	HQ832942	*Leptolyngbya* sp. PCC 9221 (AF317507)	100		
Leptolyngbya mycoidea	**06118**	8	HQ832943	*Leptolyngbya* sp. PCC 9221 (AF317507)	100		
Leptolyngbya saxicola	**07132**	8	HQ832944	*Leptolyngbya* sp. ITAC101 (GU220365)	100		
Leptolyngbya saxicola	**07170**	9	HQ832949	Uncultured bacterium clone Mfra_H11 (GU118858)	98	*Leptolyngbya antarctica* ANT.FIRELIGHT.1 (AY493590)	95
Leptolyngbya sp. 1	**06121**	9	HQ832945	*Leptolyngbya* sp. 0BB24S04 (AJ639893)	98		
Leptolyngbya sp. 2	**06188**	3	HQ832918	Uncultured cyanobacterium clone VERDEA95 (FJ902665)	95	*Leptolyngbya* sp. 1T12c (FR798935)	94
Lyngbya cf. *aestuarii*	**07165**	2	HQ832912	Uncultured cyanobacterium clone Z4MB30 (FJ484830)	98	*Lyngbya majuscula* CCAP 1446/4 (HQ419207)	95
Phormidium laetevirens (*Oscillatoria*)	**06103**	8	JF708120	Uncultured cyanobacterium, clone 27 T9d+oil (FM242382)	95	*Planktothricoides raciborskii* OR1-1 (AB045964)	95
Phormidium sp. 1 (*Leptolyngbya*)	**06111**	6	HQ832929	Uncultured bacterium clone SHFH692 (FJ203604)	95	*Cyanothece* sp. PCC 8802 (CP001701)	94
Phormidium sp. 2 (*Oscillatoria*)	**07162**	1	HQ832899	Uncultured bacterium clone CI5cm.45 (EF208676)	93	*Stanieria cyanosphaera* PCC 7437 (AF132931)	92
Plectonema cf. *radiosum* (*Scytonema*)	**06105**	8	HQ832941	*Calothrix* sp. CCMEE 5085 (AY147030)	99		
Pseudanabaena aff. *curta*	**07169**	4	HQ832923	Uncultured cyanobacterium, clone UMAB-cl-31 (FN811215)	97	*Synechococcus* sp. PCC 7335 (AB015062)	96
Pseudanabaena aff. *curta*	**07160**	9	HQ832948	*Synechococcus* sp. PCC 7335 (AB015062)	98		
Pseudanabaena aff. *persicina*	**07163**	1	HQ832900	*Pseudophormidium* sp. ANT.PENDANT.3 (AY493587)	97		
Pseudanabaena cf. *frigida*	**06144**	7	HQ832937	*Leptolyngbya* sp. FLKBBD1 (EF110975)	98		
Pseudanabaena sp. 2	**06129**	4	HQ832921	Oscillatoriales cyanobacterium 2Dp86E (GU265558)	98	*Leptolyngbya* sp. 0BB24S04 (AJ639893)	98
Pseudanabaena sp. 3	**07190**	3	HQ832919	Uncultured cyanobacterium clone R8-B31 (DQ181689)	98	*Phormidium pristleyi* ANT.ACEV5.1 (AY493586)	97
Pseudanabaena sp. 3	**06116**	6	HQ832930	LPP-group MBIC10086 (AB058224)	98	*Lyngbya* sp. A09DM (HM446280)	97
Pseudophormidium sp.	**06125**	2	HQ832909	*Leptolyngbya foveolarum* PMC302.07 (GQ859653)	99		
Romeria sp. (*Pseudanabaena*)	**06013**	5	HQ832927	*Synechococcus* sp. G2.1 (AY054298)	99		
Schizothrix aff. *septentrionalis*	**07164**	1	HQ832902	Uncultured cyanobacterium clone DPC044 (DQ269094)	98	*Leptolyngbya* sp. FLKBBD1[a] (EF110975)	92
NOSTOCALES (IV)							
Calothrix sp. 1 (*Rivularia*)	**06100**	3	HQ832913	Uncultured bacterium clone GBII-17 (GQ441291)	98	*Rivularia atra* BIR KRIV1 (AM230674)	97
Calothrix sp. 2 (*Rivularia*)	**06122**	2	HQ832908	Uncultured bacterium clone GBI-7 (GQ441198)	99	*Calothrix* sp. XP9A (AM230670)	97
Calothrix sp. 2 (*Rivularia*)	**07177**	6	HQ832933	Uncultured bacterium clone GBI-7 (GQ441198)	99	*Calothrix* sp. XP9A (AM230670)	97
Nostoc sp. 1	**06106**	2	HQ832907	*Nostoc* sp. 1189P (GU062468)	98		
Nostoc sp. 1	**06150**	2	HQ832910	*Nostoc* sp. 1189P (GU062468)	98		
Nostoc sp. 2	**06158**	9	HQ832947	*Nostoc* sp. 'Collema crispum cyanobiont' (DQ185216)	99		
Rivularia sp.	**07159**	7	HQ832938	Uncultured bacterium clone GBI-7 (GQ441198)	98	*Calothrix* sp. PCC 7507 (AM230678)	97
Scytonema sp.	**07189**	1	HQ832905	Uncultured bacterium clone GBI-7 (GQ441198)	98	*Calothrix* sp. XP9A (AM230670)	98

[a]1- Moledo do Minho, 2- S. Bartolomeu do Mar, 3- Lavadores, 4- Aguda, 5- Foz do Arelho, 6- Martinhal, 7- Burgau, 8- Luz, 9- Olhos d'Água; for details see Table 1.

The diversity of our cyanobacterial isolates is depicted in Fig. 2 and Fig. 3 (as well as in Figs. S1 and S2), where it is possible to observe genera belonging to the four orders. The Chroococcales *Synechocystis* and*Cyanobium* dividing in one plane (Fig. 2a and b, Fig. 3a and b), the Pleurocapsales *Chroococcopsis*,*Myxosarcina* and *Hyella* dividing in more than one plane (Fig. 2c–g, Fig. 3d) and producing baeocytes (Fig. 2f), the Oscillatoriales *Romeria*, *Phormidium*, *Leptolyngbya*, *Pseudophormidium*, *Lyngbya* and*Plectonema* (Fig. 2h–q, Fig. 3e–f), with *Leptolyngbya* cf. *halophila* exhibiting nodule-like structures (Fig.

2m, Fig. 3f) and *Plectonema* cf. *radiosum* geminate false branches (Fig. 2q), and the Nostocales genera*Nostoc*, *Calothrix* and *Scytonema* (Fig. 2r–u, Fig. 3g), with intercalar (Fig. 2t) or terminal (Fig. 2s) heterocysts. In *Calothrix* sp. LEGE 06100 hormogonia were found (Fig. 2u). Additionally, the ultrastructural images provided information on the size and shape of the sheath (Fig. 3e, f and i), and the different arrangement of the thylakoids (Fig. 3a–c, e, g and h). It was also possible to observe the disruption of the mother sheath leading to disintegration of the colonies after cell division (Fig. 3c), and a nodule detail of *Leptolyngbya* cf. *halophila* LEGE 06102 (Fig. 3f). A brief description of each of the isolates and the morphological characteristics used to taxonomically affiliate them is depicted in Table S1, as well as their distribution and habitat description.

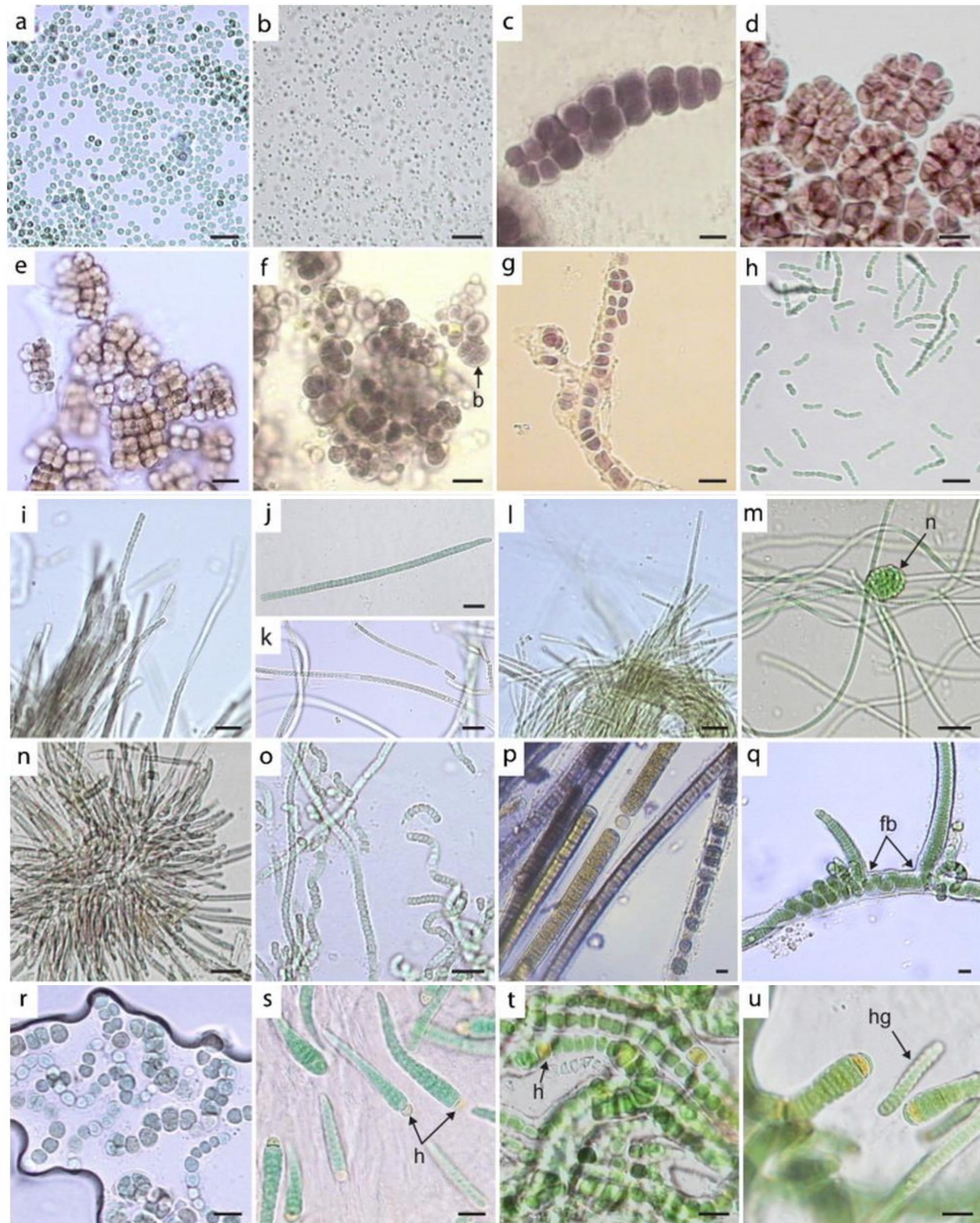

Fig. 2.

Light micrographs illustrating the diversity of cyanobacterial morphotypes isolated from the intertidal zones of nine beaches on the Portuguese coast. (a) *Synechocystis salina* LEGE 06099, (b) *Cyanobium* sp. LEGE 06184, (c) *Chroococcopsis* sp. LEGE 07168, (d) *Chroococcopsis* sp. LEGE 07187, (e) *Myxosarcina* sp. LEGE 06146, (f) *Chroococcopsis* sp. LEGE 07161, (g) *Hyella* sp. LEGE 07179, (h) *Romeria* sp. LEGE 06013, (i) *Phormidium* sp. 1 LEGE 06111, (j) *Phormidium laetevirens* LEGE 06103, (k) *Leptolyngbya mycoidea* LEGE 06126, (l) *Leptolyngbya mycoidea* LEGE 06118, (m) *Leptolyngbya* cf. *halophila* LEGE 06102, (n) *Leptolyngbya* sp. LEGE 06188, (o) *Pseudophormidium* sp. LEGE 06125, (p) *Lyngbya* cf. *aestuarii* LEGE 07165, (q) *Plectonema* cf. *radiosum* LEGE 06105, (r) *Nostoc* sp. 1 LEGE 06106, (s) *Calothrix* sp. LEGE 06122, (t) *Scytonema* sp. LEGE 07189, (u) *Calothrix* sp. LEGE 06100. b – baeocytes; fb – false branching; hg – hormogonia; h – heterocysts; n – nodule. Scale bars – 10 µm.

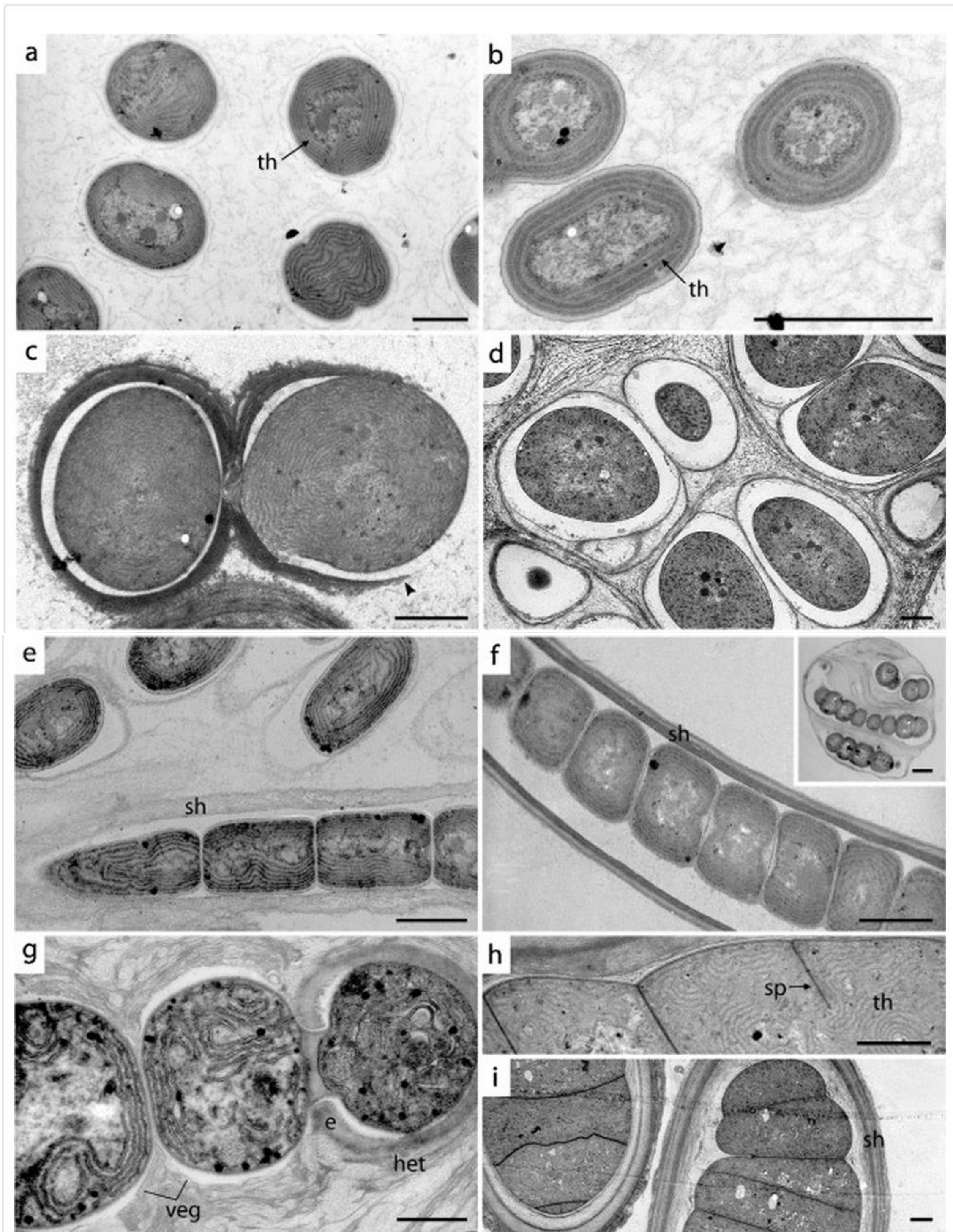

Fig. 3.

Transmission electron micrographs of selected isolates from the sampling sites. (a) *Synechocystis salina* LEGE 06099, (b) *Cyanobium* sp. LEGE 06097, (c) *Gloeocapsopsis* cf. *crepidinum* LEGE 06123, (d) *Chroococcopsis* sp. LEGE 07187, (e) *Leptolyngbya mycoidea* LEGE 06118, (f) *Leptolyngbya* cf. *halophila* LEGE 06102, insert – nodule detail (g) *Nostoc* sp. 2 LEGE 06158, (h) *Calothrix* sp. 2 LEGE 06122, (i) *Rivularia* sp. LEGE 07159. het – heterocyst; sh – sheath; sp – septum; th – thylakoid; veg – vegetative cell. arrow head – disruption of the mother sheath. Scale bars – 1 μm.

DNA sequence and phylogenetic analysis

Partial 16S rRNA gene sequences were obtained for all the isolates. These sequences were compared with the ones available in the NCBI database (March 2011) using BLASTn, and the results are shown inTable 3. There was a quite good correlation between the phenotypic and genotypic based identifications, for more than one-third of the isolates (Table 3, highlighted in grey). For the remaining isolates discrepancies were observed between the two identifications, possibly resulting from limitations of the databases and/or taxonomical constraints – it is well recognized that cyanobacterial taxonomy faces several problems, and is currently under revision [46]. One should point out that 9 of our isolates have less than 97% similarity to the 16S rRNA gene sequences in the database, emphasizing the presence of novel cyanobacterial diversity in Portuguese shore waters (Table 3, dashed boxes).

Phylogenetic analyses were performed to assess the relative positioning of the cyanobacteria isolated in this study. An ML algorithm was applied to a multiple alignment of partial 16S rRNA gene sequences (655 bp) of all isolates, reference strains, and *C. aurantiacus* J-10-fl as the outgroup. The resulting tree revealed 12 consistent clusters (A–L), which were defined as monophyletic groups with bootstrap values equal or higher than 70% (Fig. 4). The heterocystous types form a coherent genetic cluster, whereas unicellular and filamentous nonheterocystous forms were intermixed and dispersed throughout the tree, as previously described [10] and [51]. In general, the phylogenetic distribution is congruent with the classification results based on the morphology. Moreover, the species or genus attributed to the isolates in the morphological analysis is in agreement with the species/genus of the reference strains they cluster with. Cluster A includes two isolates identified as *Leptolyngbya mycoidea* supported by a bootstrap value of 100%. Cluster B comprises one isolate identified as *Chroococcopsis* sp. and the reference strain*Synechocystis* sp. PCC 6308. Cluster C includes the *Chroococcidiopsis* sp. PCC 6712 and an isolate identified as *Hyella* sp. Cluster D includes 3 isolates belonging to Pleurocapsales, and the reference strains*Pleurocapsa* sp. PCC 7516, *Pleurocapsa* sp. PCC 7319, and *Myxosarcina* sp. PCC 7325, corroborating the close relationship between these two genera. This is in agreement with previous findings, where*Myxosarcina* sp. PCC 7325 clusters with other members of *Pleurocapsa*-group: *Pleurocapsa* sp. PCC 7516 and PCC 7321 [37]. *Chroococcidiopsis* PCC 7203, *Chroococcidiopsis* PCC 6712 and *Stanieria cyanosphaera* PCC 7437, appear distant from all the other baeocyte-forming cyanobacteria, which has been previously reported for *Chroococcidiopsis* PCC 7203 [37]. Cluster E comprises isolates identified as*Leptolyngbya* and *Phormidium* and, although no reference strain is included, it is supported by a bootstrap value of 100%. Cluster F contains three *Synechocystis*: *Synechocystis* sp. PCC 6803, and two isolates identified as *Synechocystis salina*. The reference strain S*ynechocystis* PCC 6308, is unrelated to the above isolates. The scattered distribution of *Synechocystis* strains has been described previously; Wilmotte and Herdman [51] showed that *Synechocystis* PCC 6308 did not group with *Synechocystis* PCC 6803 and *Synechocystis* PCC 6909. Cluster G includes the genus reference strain *Lyngbya* sp. PCC

7419, and one isolate identified as *Lyngbya* cf. *aestuarii* (*Lyngbya* PCC 7419 was originally identified as *Lyngbya aestuarii*) [5]. Cluster H contains the heterocystous cyanobacteria, and within it is possible to distinguish two sub-clusters: the *Nostoc* and *Calothrix*/*Rivularia*. *Scytonema hofmanni* PCC 7101 is not included in this cluster due to a low bootstrap value (48.5) but forms a monophyletic group with the heterocystous types, which is in agreement with previous reports [10], [32] and [51]. This cluster also contains an isolate identified as *Plectonema* cf. *radiosum*. As it described earlier, members of this “botanical” genus might be *Scytonema* individuals that do not exhibit heterocysts even in natural samples [35]. Cluster I, constituted mainly of Oscillatoriales morphotypes, contains isolates identified as *Leptolyngbya*, *Pseudophormidium*, *Pseudanabaena* and the reference strain *Leptolyngbya* PCC 7104. Cluster J contains the *Leptolyngbya* sp. PCC 7375 and the isolate identified as *Pseudanabaena* cf. *frigida*. Cluster K comprises isolates identified as *Pseudanabaena* and the marine unicellular *Synechococcus* PCC 7335. This has been previously observed by Wilmotte and Herdman [51] who reported a close relationship between filamentous cyanobacteria and *Synechococcus* PCC 7335. Cluster L includes cyanobacteria belonging to Chroococcales and Oscillatoriales. Within this cluster it is possible differentiate a *Cyanobium* and a *Synechococcus* sub-cluster. Previously, Herdman et al. [13] reported that *Synechococcus* WH 8103 was included in the *Cyanobium* spp. clade, corroborating the data presented here. The same 16S rRNA gene alignment was analyzed by Neighbor-Joining (NJ) (Fig. S3), and the computed tree supported all the 12 clusters, therefore validating the ML approach and reinforcing the described phylogenetic analysis.

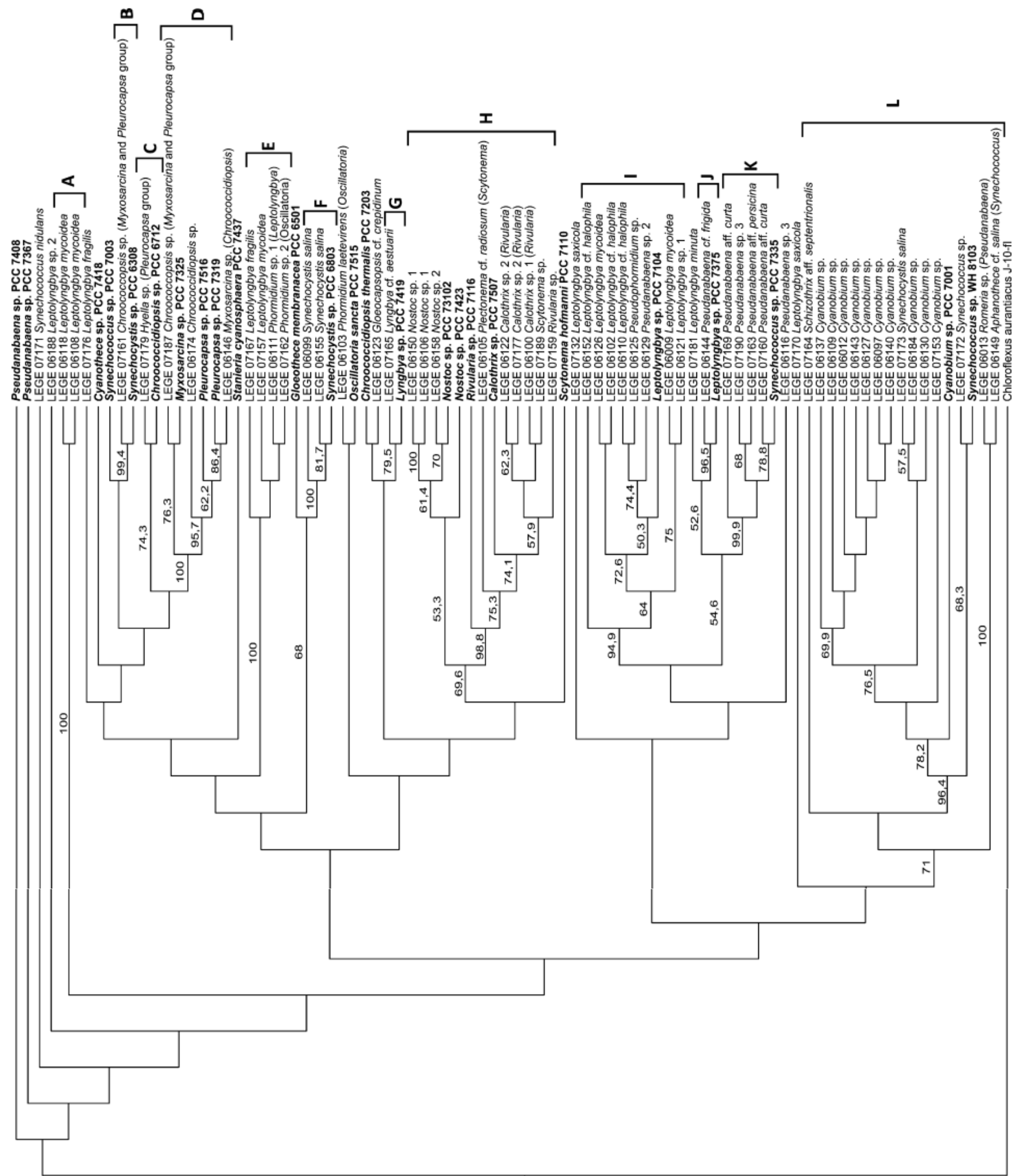

Fig. 4. Maximum-likelihood phylogenetic tree of partial 16S rRNA gene sequences from the isolates, reference cyanobacterial strains and the outgroup *Chloroflexus aurantiacus* J-10-fl. Numbers along branches indicate the percentage of bootstrap support considering 1000 pseudo-replicates: only those equal or higher than 50% are indicated. Isolates are referred by their culture collection code and morphological identification, whereas reference strains are indicated in bold. The 12 recognized clusters (A–L) are indicated along the tree (see text for details).

It is important to notice that some isolates, identified as the same taxon (*S. salina*, *L. mycoidea*,*Leptolyngbya fragilis*), appear in different branches along the tree, indicating that the phylogenetic analyses of small coccoids and some filamentous cyanobacteria offered a higher resolution than the morphological identification.

Screening for putative N_2 fixers and toxin producers

A survey to evaluate the presence of putative N_2 fixers and toxin producers was carried out. Isolates were screened for the presence of the genes *nifK*, *nifH*, *mcyA*, *mcyE*, *ndaF* and *sxtI* (see “Materials and methods” section). With the exception of the filamentous heterocystous strains, it is not possible to identify N_2-fixing cyanobacteria based on morphology; therefore targeting the

structural genes encoding the nitrogenase enzymatic complex is a common approach [53] and [54]. In this work, the presence of *nifH*,*nifK* and/or heterocysts were taken into account to determine the organism's potential for nitrogen fixation. 33% of the isolates (20 out of 60, see Table S1) qualify as potential diazotrophs, suggesting that cyanobacteria may play an important role in N_2 fixation in these intertidal zones. Intertidal ecosystems are harsh and often nutrient-limited environments, so the presence of nitrogen fixers is expected and this ability may confer cyanobacteria a competitive advantage [6] and [41]. Indeed, cyanobacteria are the most important N_2-fixing organisms in the majority of the marine microbial mats, and high rates of nitrogen fixation have been observed [42]. Concerning the genes encoding proteins involved in toxin production, no amplification was obtained indicating the absence, among the isolates, of cyanobacteria producing the conventional freshwater toxins: microcystin, nodularin and saxitoxin. One should bear in mind that a negative PCR result does not exclude the presence of the gene and, conversely, the presence of the gene does not translate into expression and activity (also valid for the *nif* genes screening), but the primer pairs used in this study have been proven to work with a wide range of cyanobacterial strains [14], [16] and [17]. Martins et al. [26] demonstrated that extracts of marine *Synechocystis* and *Synechococcus* strains isolated from the Portuguese coast were toxic to marine invertebrates possibly implicating the presence of other toxic compounds.

Conclusions

This study unveils the cultivated diversity of cyanobacteria present in the intertidal zones of the continental Portuguese coast. The isolated cyanobacteria belong to 35 different morphotypes and comprise members of all the cyanobacterial orders/subsections, with the exception of Stigonematales/subsection V. The predominant forms among the isolates were nonheterocystous and were particularly represented by the filamentous Oscillatoriales. The 16S rRNA gene sequences of 9 strains have less than 97% similarity compared to the sequences in GenBank, revealing unreported cyanobacterial diversity. The phylogenetic distribution, based on the 16S rRNA gene, is congruent with the results obtained with the morphology-based classification. As expected, the heterocystous cyanobacteria form a coherent genetic cluster, whereas the unicellular and filamentous nonheterocystous were intermixed. Most of the isolates cluster with reference strains of the same species or genus assigned to the isolates in the morphological identification. One-third of the isolated organisms are putative diazotrophs, suggesting that cyanobacteria may play an important role in N_2 fixation along the continental Portuguese coast, as it is common in the marine environment. Additionally, no conventional freshwater toxins genes were detected by PCR screening, indicating a low probability for the occurrence of producers of these cyanotoxins in the analyzed zones.

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