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Cyanobacterial diversity in the marine sponge *Hymeniacidon perlevis* from a temperate region (Portuguese coast, Northeast Atlantic)

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ABSTRACT: *Cyanobacteria* are commonly associated with marine sponges and are known to be difficult to isolate. In the present study, we used isolation and molecular techniques to investigate the diversity of *Cyanobacteria* associated with the intertidal marine sponge host *Hymeniacidon perlevis*, collected along the coast of Portugal (Northeast Atlantic). Cyanobacterial community profiling and comparison using 16S rRNA gene-sequence-based denaturing gradient gel electrophoresis (DGGE) revealed different banding patterns between the sponge tissue and seawater. We succeeded in isolating *Cyanobacteria* belonging to the genera *Synechococcus*, *Cyanobium*, *Synechocystis*, *Nodosilinea*, *Pseudanabaena* and *Phormidesmis* from the sponge tissues. Chlorophyll *a* concentrations were very low, in spite of the diversity of cyanobacteria identified. DGGE analyses comparing sponge samples and ambient seawater further revealed the presence of *Synechococcus*, *Acaryochloris* and *Prochlorococcus.* Many of the isolated cyanobacteria show a high similarity with previously isolated free-living cyanobacteria from the coast of Portugal, highlighting the advantages of using sponges as a source for obtaining cyanobacteria present only in small amount in seawater.

KEY WORDS: Cyanobacteria · Marine sponges · Diversity · Phylogeny · DGGE · North-eastern Atlantic coast

INTRODUCTION

Sponges are the most primitive multi-celled animals, with fossil records dating back 700 to 800 million years (Belarbi 2003). They are known for harbouring a diversity of symbiotic microorganisms such as bacteria, fungi, unicellular algae and cyanobacteria (Taylor et al. 2007). Based on the abundance and diversity of the microbial community they contain, sponges are classified as being high microbial abundance (HMA) or low microbial abundance (LMA) sponges (Hentschel et al. 2003, Weisz et al. 2007). HMA sponges can contain a concentration of microorganisms 2 to 4 orders of magnitude higher than seawater (Friedrich et al. 2001, Hentschel et al. 2006). LMA sponges are typically smaller (Hentschel et al. 2006), with a smaller mesohyl and simpler aquiferous system but a higher pumping rate (Weisz et al. 2008).

Cyanobacteria, common photosymbionts, form associations with a wide variety of organisms in different habitats. In the marine environment, they are

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known to occur with sponges, ascidians, Echuroid worms, diatoms, dinoflagellates and protozoans (Carpenter & Foster 2002). *Cyanobacteria* are an important group among the photosynthetic symbionts of sponges. Sponges with photosynthetic symbionts can constitute up to 85% of the total intertidal sponge communities in tropical reefs (Steindler et al. 2002) and up to 64% in temperate waters (Lemloh et al. 2009). According to Rützler (1990), unicellular and filamentous cyanobacteria can comprise up to 50% of a sponge's cellular volume. Cyanobacteria contribute to the relationship by transferring nutrients to the sponge, such as glycerol (Wilkinson 1979), organic phosphate and nitrogen (Wilkinson & Fay 1979), which enhances its growth rate and competiveness with other benthic communities (Wilkinson 1980, Arillo et al. 1993). Cyanobacteria also provides UV protection as well as chemical defence through the production of secondary metabolites, as reviewed in Taylor et al. (2007), Usher (2008), and Webster & Taylor (2012). Cyanobacteria can also benefit from the association with sponges, although the mechanisms are not as clear. The host provides shelter (Erwin & Thacker 2007), and higher levels of ammonium and phosphorus than those present in the ocean (Usher 2008). Primary productivity and nutrient cycling in marine ecosystems can also be enhanced by these symbioses (Diaz & Rützler 2001). Vertical transmission of cyanobacterial symbionts (cyanobionts) to new generations has already been reported (Usher et al. 2001, Oren et al. 2005), which is considered to benefit the offspring by giving them photosynthetic energy before they are able to feed (Lemloh et al. 2009), enhancing their competitive fitness (Oren et al. 2005). Maldonado (2007) reported that in some sponges the symbiont is not transmitted to gametes or embryos, but instead they are obtained in each new generation from the environment (i.e. horizontal transmission). According to Schmitt et al. (2007) embryos from LMA sponges are typically microbe-free.

Cyanobacterial associations occur within the sponge classes Calcarea and Demospongiae (Carpenter & Foster 2002). The sponge-associated *Cyanobacteria* identified so far belong to *Aphanocapsa*, *Synechococcus*, *Prochloron*, *Synechocystis* and *Oscillatoria*. Recently, Alex et al. (2012) also reported the presence of *Xenococcus*-like and *Acaryochloris* sp. from the intertidal marine sponge *Hymeniacidon perlevis*. Some unknown species have also been found, as reviewed by Usher (2008). Some of these associations can occur in geographically distinct areas, and it is known that different sponges can have the same symbiont and each one can harbour more than one cyanobacterial species (Usher et al. 2006).

Cyanobacterial diversity in marine sponges has been the focus of many studies, mainly in tropical environments. Approximately 99% of the sponge-associated microorganisms cannot be cultured (Santavy & Colwell 1990, Friedrich et al. 2001, Hentschel et al. 2003, Isaacs et al. 2009) and, allied to the fact that the morphological characteristics are not enough to distinguish the cyanobacterial species (Usher et al. 2006), it is thought that the diversity is being underestimated and many relationships are yet to be discovered. In the last few years, molecular approaches have demonstrated that symbiotic cyanobacteria in sponges differ from those in the seawater communities (Usher et al. 2004, Steindler et al. 2005, Lemloh et al. 2009). These techniques have been able to assess the cyanobacterial diversity among the sponge hosts (Taylor et al. 2007). Denaturing gradient gel electrophoresis (DGGE) has been commonly used to assess the diversity of *Bacteria* associated with marine sponges (Usher et al. 2004, Li et al. 2006, Wichels et al. 2006, Thiel et al. 2007, Lemloh et al. 2009, Anderson et al. 2010, Gerçe et al. 2011) and can provide insights into enrichment of the communities (Hentschel et al. 2003).

The aim of the present study was to assess the diversity of the cyanobacterial community in the most common intertidal LMA marine sponge, *Hymeniacidon perlevis* (Demospongiae, Halichondrida), distributed along the western coast of Portugal (NE Atlantic), using culture-based and molecular-based techniques. We also compared the phylogenetic relationships of the cyanobacterial community retrieved from sponge tissues and the water column with other, previously reported sponge-associated and free-living cyanobacteria, and discuss the ecological relevance of this study.

MATERIALS AND METHODS

Sample collection and preparation

Sampling was performed from September 2010 to September 2011 in Portugal (Northeast Atlantic). Specimens of the sponge *Hymeniacidon perlevis* were collected along the western coast of Portugal, during the lowest tide over each month (1.5 to 1.9 m below mean sea level). All selected sampling sites were beaches consisting of a combination of sand and rocks. Sample collection only required a small portion

of the sponge, which did not affect the animals' survival in the natural environment. For molecular purposes, only *H. perlevis* from 3 sampling locations (Memória, Aguda and Porto Côvo) (Fig. 1) were used.

Samples were cleaned of debris and sediment, and placed in sterile 100 ml flasks containing filtered natural seawater from the sampling location. Water samples (150 ml) were also collected from each sampling location to isolate free-living cyanobacteria and for molecular-based analysis. After collection, sponge samples were immediately transported to the laboratory in a cooler on ice. Processing began between 1 and 6 h after sample collection. Samples were divided into 3 parts: one was processed immediately for the isolation of *Cyanobacteria*; one was preserved in 100% ethanol for subsequent genetic analysis; and one was preserved in 70% ethanol for morphological identification. For seawater, 150 ml samples were filtered through a 0.45 µm sterile filter followed by DNA extraction.

Sponges were identified based on the sampling habitat, shape, consistency, texture, colour, smell of the sponge sample and characteristic features (morphology, dimensions) of spicules. All sponge species were confirmed according to Hooper & Van Soest (2002).

Fig. 1. Sampling locations in Portugal (SW Europe) for denaturing gradient gel electrophoresis (DGGE) analysis: Memória (41° 13′ 52.27″ N, 8° 43′ 18.34″ W); Aguda (41° 2′ 58.35″ N, 8° 39′ 19.22″ W); and Porto Côvo (37° 52′ 3.04″ N, 8° 47′ 37.19″ W)

Chlorophyll *a* quantification

We employed the protocol described by Thacker (2005) to extract chlorophyll *a* (chl *a*) from marine sponges, assuming that most of the sponges harbour chl *a*-containing cyanobacteria. Quantification of chl *a* was done in 9 specimens of *H. perlevis* collected from the sampling site at Memória. To summarize, 0.25 g from each sponge (wet weight) was extracted in 10 ml of 90% acetone, and kept overnight at 4°C. Three aliquots from the supernatant were used to determine absorbance at 630, 647, 664 and 750 nm. Chl *a* concentration was calculated using the equations of Parsons et al. (1984) standardized by sponge mass extracted.

Cyanobacteria culture and morphological characterization

To avoid the culturing of superficial bacteria, 1 mm of the exposed sponge surface tissues was removed with a sterile, double-sided razor. The remaining sponge samples were rinsed with distilled water to remove the transient and loosely attached organisms. Sections of the sponge body were used for culturing cyanobacteria. Small fragments (<0.5 cm^3) of sponge tissue were placed in 2 different media: Z8 liquid medium (Kótai 1972) supplemented with 30 g l^{-1} of NaCl, and MN liquid medium (Rippka 1988). The media were supplemented with vitamin B12 and cycloheximide (Rippka 1988). The cultures were kept under 14 h light (10 to 30 µmol photons m^{-2} s^{-1}), 10 h dark cycles at 25°C. When cyanobacterial growth in the liquid was visible, an isolation procedure was done using a micromanipulation technique (Rippka 1988), using a sterile Pasteur pipette to transfer a single cell or filament to liquid medium. Cyanobacteria cultures were achieved after several subcultures, and were unicyanobacterial and non-axenic. Water samples were centrifuged at 16 000 × *g* for 5 min (nSorval Legend RT centrifuge) and the pellet was placed in cyanobacterial culture media and kept under the same conditions as mentioned above.

Morphological cyanobacterial identification was performed following the criteria of Komárek and Anagnostidis (Komárek & Anagnostidis 1998, 2005, Komárek 2013), using Bergey's manual of systematic bacteriology (Castenholz 2001) and Komárek et al. (2014). Pictures were taken using an Olympus BX41 microscope (Olympus Europe) and analysed using Cell^{B} (Olympus Europe). Cyanobacterial isolates were deposited at LEGE Culture Collection (Laboratory of Ecotoxicology, Genomics and Evolution, CIIMAR, Porto, Portugal).

Molecular analyses

DNA extraction

Total genomic DNA (gDNA) was extracted from pure cyanobacterial cultures and sponge tissue, using a commercially available Purelink^{TM} genomic DNA mini kit (Invitrogen) following the protocol described for Gram-negative bacteria in accordance with the manufacturer's recommendations, and stored at −20°C until further analysis. For the water samples, 150 ml was centrifuged at 16 000 × *g* for 8 min followed by DNA extraction from the 'pellet' as described above. gDNA integrity was checked by agarose gel electrophoresis with ethidium bromide staining.

PCR and sequencing of cyanobacterial cultures

Two sets of primers were used for amplification and sequencing of 2 fragments of the partial 16S ribosomal RNA (rRNA) gene sequence, as shown in Table 1. PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 30 s and extension at 72°C for 1 min and a final extension step at 72°C for 5 min. A total of 5 to 10 ng of DNA were used for the PCR amplification. All PCR reactions were prepared in a 50 µl volume containing 1× PCR buffer, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate, 10 pmol of each primer, and 0.5 U of *Taq* DNA polymerase (Bioline). Thermal cycling was carried out using Biometra T-professional standard thermocycler (Biometra). PCR products were separated by 1.5% (w/v) agarose gel in 1× TAE buffer (Invitrogen). The gels were stained with ethidium bromide and photographed under UV transillumination. For DNA sequencing, each amplified product was purified using an Invitrogen PureLink^{TM}QuickGel Extraction and PCR Purification Combo Kit (Invitrogen) according to the manufacturer's protocol, followed by direct sequencing (Macrogen Europe). Sequences were deposited in GenBank database (accession numbers JQ927344, JQ927345, JQ927348, JQ927353 and KX608887 to KX608890).

Screening of cyanobacterial community from sponge tissue and water samples

For 16S rRNA gene amplicons, a first round of PCR employing the *Cyanobacteria*-specific primers CYA-106F and CYA781R (described in Table 1) (Nübel et al. 1997) was followed by nested PCR reaction with GC-clamped primers, to amplify a *Cyanobacteria*-specific fragment from the 16S rRNA gene (16S-CYA) with 359F-GC and 781R primers (Nübel et al. 1997). PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 12 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, and a final extension step at 72°C for 4 min.

A total of 20 µl of the PCR products corresponding to the 16S-CYA fragments were loaded into 6% polyacrylamide 1 mm gels, using a 30 to 55% denaturing

Table 1. Primer pairs used in this study. F: forward; R: reverse

Target gene	Primer pair	Sequence (5′ to 3′ determination)	Size (bp)	Reference
16S rRNA	CYA106F	CGG ACG GGT GAG TAA CGC GTG A	675	Nübel et al. (1997)
	CYA781R (A)[a]	GAC TAC TGG GGT ATC TAA TCC CAT T		
	CYA781R (B)[a]	GAC TAC AGG GGT ATC TAA TCC CTT T		
	CYA359F	GGG GAA TYT TCC GCA ATG GG	1135	
	1494R	TAC GGC TAC CTT GTT ACG AC		Neilan et al. (1997)

[a](A) and (B): primers used together in a mixture with equimolar concentration

gradient (100% denaturing conditions correspond to 7 M urea and 40% formamide). One gel was used to accommodate the 8 samples (4 specimens of *H. perlevis* and 4 seawater samples). Electrophoresis was performed using 1% TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), at 60 V for 16 h, in a DCode system (Bio-Rad). The gels were stained with 1× SYBR Gold nucleic acid stain (Invitrogen) and selected DGGE bands were excised using a razor blade and placed in sterile microcentrifuge tubes with 10 µl of sterile Milli-Q H_2O. When bands with the same length appeared in different samples, only one of them was extracted; it was assumed that bands of the same length corresponded to the same cyanobacterial species. A total of 5 µl was used as a template in a new PCR reaction. This re-amplification was performed under the same conditions described in the previous section (see Table 1) for the corresponding fragment type, except that forward primers did not contain a GC-clamp. PCR products were excised from agarose gel and cleaned (Cut&Spin Gel Extraction columns, GRiSP) prior to cloning.

Purified PCR products from DGGE bands were then cloned into pGEM®-T Easy vector (Promega), and transformed into OneShot® TOP10 chemically competent *E. coli* cells (Invitrogen) using standard procedures (Sambrook & Russell 2001) and following the manufacturer's instructions. Plasmid DNA was isolated using GenElute™ plasmid miniprep kit (Sigma-Aldrich) and sequenced (Macrogen Europe) using M13 primers. For sequencing, 2 clones for each DGGE band were selected. Sequences obtained from DGGE clones were deposited in GenBank (accession numbers KC896629 to KC896638).

Phylogenetic analysis

The partial 16S rRNA gene sequences obtained were analysed using Geneious® v.9.1.5 software (www.geneious.com; Kearse et al. 2012). The final sequence length, ranging from 345 to 1373 bp, was used for a similarity search using BLAST and the NCBI nucleotide database (www.ncbi.nlm.nih.gov/BLAST). A chimera check for derived 16S rRNA sequences was performed using Mallard (Ashelford et al. 2006). The sequences used in phylogenetic analyses were chosen to include (1) representatives of cyanobacterium diversity (reference strains), (2) sponge-associated cyanobacteria sequences overlapping with the new 16S rRNA sequences, and (3) representatives of the cyanobacteria–sponge symbionts. BLAST similarity searches were also conducted for each cyanobacterial sequence to retrieve the closely related sequences available in the databank. Chimeras and the DGGE clones that did not retrieve sequences similar to *Cyanobacteria* through the BLASTn search in the NCBI database (March 2013) were not included in the phylogenetic analysis. The sequences were aligned with Clustal Omega (Sievers & Higgins 2014), a multiple sequence alignment program implemented in Sea View v.4.4.2 (Gouy et al. 2010). Ambiguously aligned regions were filtered by Gblocks using less stringent options (Castresana 2000). A final multiple alignment containing 1293 positions was used for the phylogenetic reconstructions of the 16S rRNA nucleotide data set performed using the Maximum Likelihood (ML) approach (Felsenstein 1981) implemented in PhyML (Guindon & Gascuel 2003) with a nearest-neighbor-interchange (NNI) heuristic search method, resampled using 100 bootstrap replicates. Posterior probabilities of branch nodes were calculated in MrBayes (BY) v.3.2.6 (Huelsenbeck et al. 2001) employing the optimal nucleotide substitution model. The best fit evolutionary model — general time-reversible (GTR) plus gamma distributed (G) plus invariant sites (I) (GTR+G+I) — was adopted under Akaike's information criterion with correction (AICc) implemented in MrAIC v.1.4.4 (Nylander 2004).

RESULTS

Isolation of cyanobacteria

To promote growth of the highest diversity possible, 2 isolation media with different compositions were used (MN and Z8 30‰). Eight strains of *Cyanobacteria* were isolated from the sponge tissue (Fig. 2), as well as 1 cyanobacterium from the surrounding waters (*Cyanobium* sp. LEGE10378). These strains belong to the order *Synechococcales* (Table 2). In most cases, morphological characterization based on light microscopy allowed identification to genus level, and in some cases to species level. The *Chroococcales* isolates belong to the genera *Synechocystis*. Partial 16S rRNA gene sequences obtained from the isolates were compared with those available in the NCBI database (June 2016), and the results are shown in Table 2. Similarities above 98% were obtained for all isolates. The molecular analyses were, in most cases, in agreement with the morphological classification previously done.

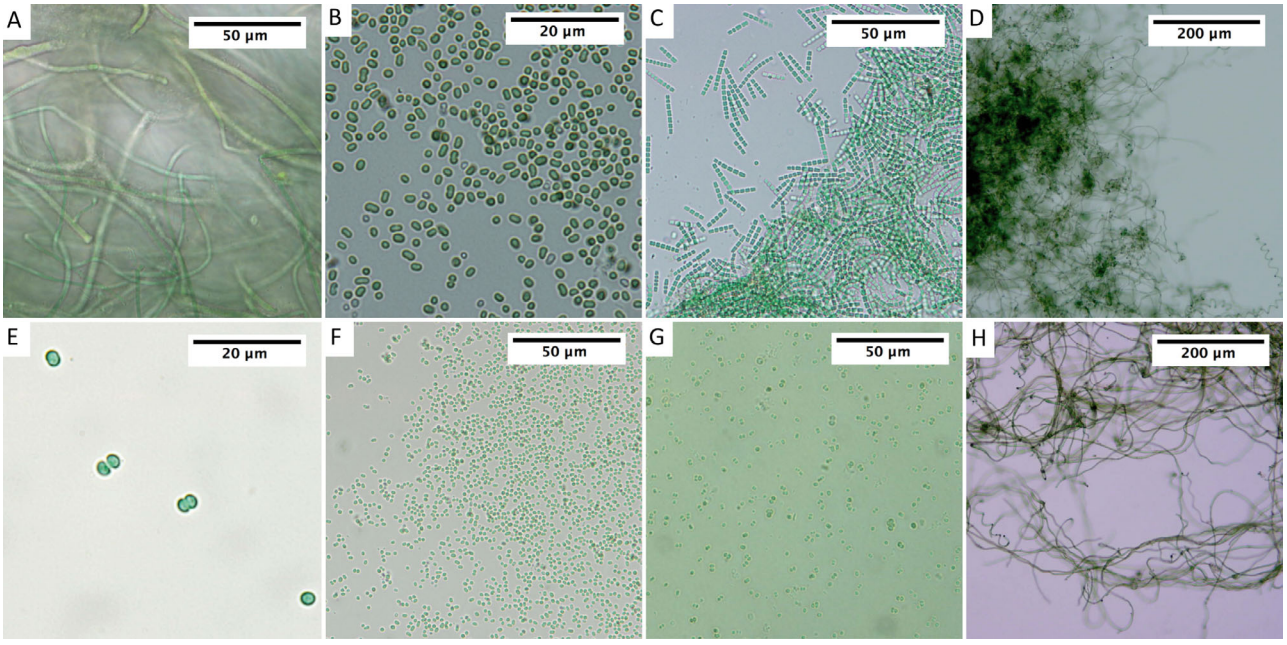

Fig. 2. *Cyanobacteria* isolated from *Hymeniacidon perlevis*. Identification was done based on morphological characters; accordingly, strains were classified as: (A) *Phormidesmis* sp. LEGE 10370, (B) *Cyanobium* sp. LEGE 11382, (C) *Pseudanabaena* cf. *curta* LEGE 10371, (D) *Nodosilinea* cf. *nodulosa* LEGE 10376, (E) *Synechocystis* sp. 12A210hp, (F) *Synechococcus* sp. 12A10hp, (G) *Cyanobium* sp.19B10hp and (H) *Nodosilinea* sp. 19D10hp

Table 2. Morphological identification and molecular analysis of the cyanobacterial isolates

Strain reference	*Cyanobacteria* species	Accession no.	Source	Sampling location	Best hit indicated by BLAST		
					Accession no.	Molecular analysis	% max. identity
LEGE 10370	*Phormidesmis* sp.	JQ927344	Sponge	Memória	AY493587	*Pseudophormidium* sp. ANT.LPE.3	98
LEGE 10371	*Pseudanabaena* cf. *curta*	JQ927345	Sponge	Angeiras	AB039018	*Pseudanabaena* sp. PCC7367	98
LEGE 10376	*Nodosilinea* cf. *nodulosa*	JQ927348	Sponge	Porto côvo	EF122600	*Nodosilinea nodulosa* UTEX 2910	99
					HM217060	*Leptolyngbya* sp. LEGE 06308	99
LEGE 10378	*Cyanobium* sp.	JQ927350	Seawater	Aguda	AY172837	*Cyanobium* sp. NS01	99
					HM217069	*Cyanobium* sp. LEGE 06068	99
LEGE 11382	*Cyanobium* sp.	JQ927353	Sponge	Memória	AY172837	*Cyanobium* sp. NS01	100
					HM217069	*Cyanobium* sp. LEGE 06068	100
12A210hp	*Synechocystis* sp.	KX608887	Sponge	Memória	GQ131855	*Synechocystis* sp. *CK5*	98
12A10hp	*Synechococcus* sp.	KX608888	Sponge	Memória	AY172800	*Synechococcus* sp. *ALMO3*	99.8
19B10hp	*Cyanobium* sp.	KX608889	Sponge	Aguda	KC469573	*Cyanobium* sp. *LEGE 06134*	100
19D10hp	*Nodosilinea* sp.	KX608890	Sponge	Aguda	LN849925	*Nodosilinea* sp. *LD14*	98.6

Chl *a* quantification

The method we used for chl *a* quantification in marine sponges has been used by different authors (Becerro & Paul 2004, Thacker 2005, Erwin & Thacker 2007, Thacker et al. 2007, Erwin et al. 2012, Pita et al. 2013, Burgsdorf et al. 2014). Chl *a* quantification was done in sponges collected from different light intensity locations. Chl *a* varied from 6.04 to 17.35 µg g^{-1}, averaging 9.41 ± 3.66 (SD) µg

g^{-1} of wet sponge. Organic solvents, such as acetone, can interfere with chlorophyll quantification. Chl *d* is a red-shifted chlorophyll, but when extraction is done with an organic solvent it shows a minor red-shift (Li et al. 2012) and the peaks of chl *a* and chl *d* overlap. This makes it impossible to distinguish between chl *a* and *d*.

DGGE analysis

A 16S rRNA DGGE analysis was done followed by cloning of the extracted bands and sequencing. In this analysis, both sponge tissues and water samples from the same locations and dates were analysed (Memória, September 2010; Aguda, October 2010; Porto Côvo, November 2010; Memória, September 2011). To analyse the banding pattern, we assumed that bands in the same position on the gel represented the same organism. We were able to determine the presence of 24 unique bands from DGGE (Fig. 3). Ten of them were present in all samples, both from sponge tissues and water samples (stars in Fig. 3). Another 10 were found exclusively in water samples (triangles in Fig. 3) and 4 in sponge tissue only (filled circles in Fig. 3). Because a single DGGE band can represent more than one strain, we cloned each extracted DGGE band twice. From the initial 24 bands, we successfully identified 10 clones corresponding to 9 different bands (numbered 1 to 9 in Fig. 3). The closest representatives of the clones retrieved through the BLASTn search are shown in Table 3. DGGE banding patterns revealed higher species richness in the seawater compared to the sponge samples (Fig. 3). DGGE clone 1_1, derived from a band only present in sponge tissue, showed molecular similarity with other *Cyanobacteria* identified in the sponge *Hymeniacidon heliophila*. The same similarity was found in DGGE clone 7_1, extracted from a band only present in water samples. Two of them (DGGE clones 4_1 and 5_1) seem to belong to the genus *Acaryochloris*, and clones 2_1, 6_1, 8_2 and 9_1 to the genus *Synechococcus*.

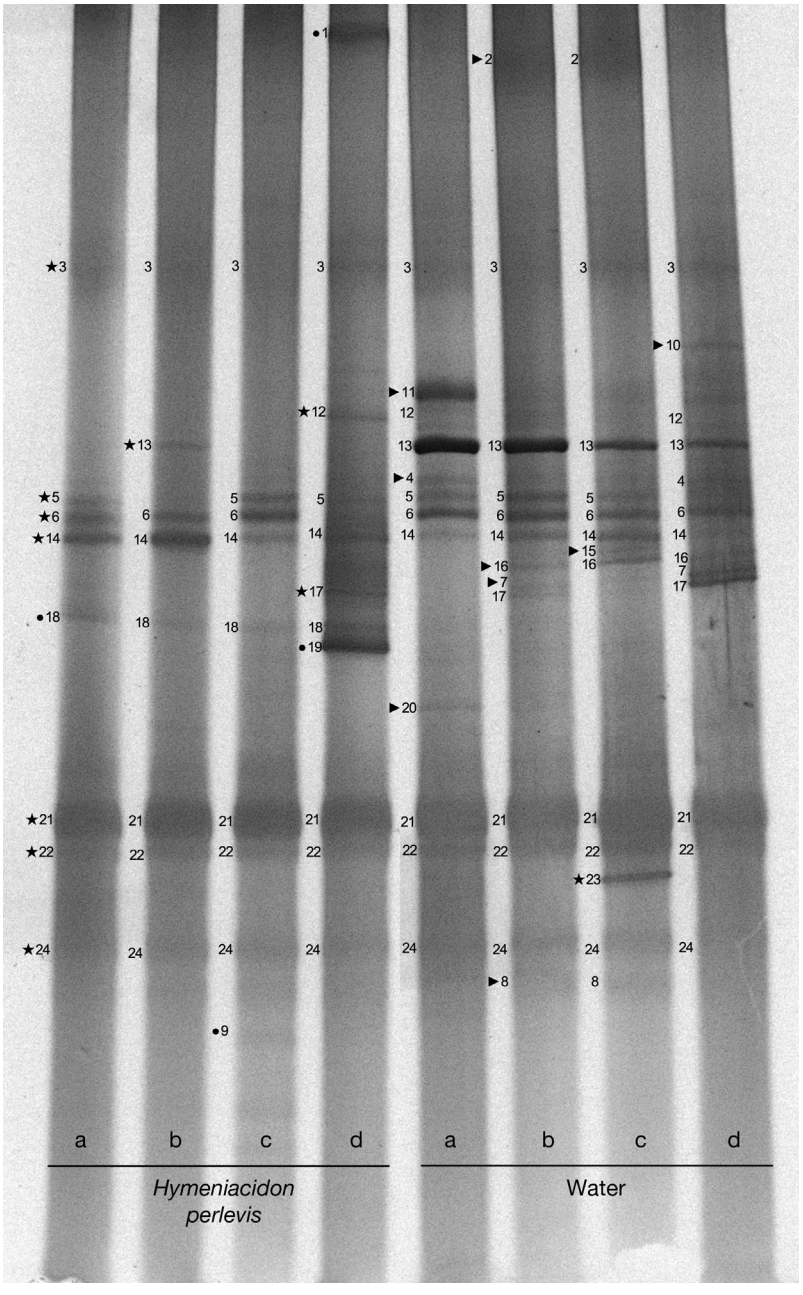

Fig. 3. Denaturing gradient gel electrophoresis (DGGE) banding profiles of cyanobacterial 16S rRNA genes PCR-amplified from the tissue of the marine sponge *Hymeniacidon perlevis* tissue in comparison to samples of seawater from same locations and dates (a–d). (a) Memória (September 2010); (b) Aguda (October 2010); (c) Porto Côvo (November 2010); (d) Memória (September 2011). Individual bands are labelled on the left-hand side of the lane numbered from 1 to 24. (▶) bands present only in water samples; (●) bands present only in sponge samples; (★) bands present both in water and sponge samples

Table 3. Phylogenetic affiliations of 16S rRNA gene clones obtained from denaturing gradient gel electrophoresis (DGGE) bands from *Hymeniacidon perlevis* and seawater

DGGE clone	Accession no.	Source	Best hit indicated by BLAST		
			Accession no.	Molecular analysis	% max. identity
1_1	KC896629	Sponge	JF824768	Uncultured cyanobacterium from *Hymeniacidon heliophila*	99
2_1	KC896630	Seawater	AY172835	*Synechococcus* sp. WH8020	99
3_1	KC896631	Seawater	JN825316	Uncultured cyanobacterium	99
4_1	KC896632	Seawater	NR_074407	*Acaryochloris marina* MBIC11017	99
5_1	KC896633	Sponge	NR_074407	*Acaryochloris marina* MBIC11017	99
6_1	KC896634	Sponge	HE687328	Uncultured *Synechococcus*	99
7_1	KC896635	Seawater	AM259807	Uncultured cyanobacterium from *Thethya aurantium*	99
8_1	KC896636	Seawater	JX255822	Uncultured cyanobacterium	99
8_2	KC896637	Seawater	FJ903249	Uncultured *Synechococcus*	99
9_1	KC896638	Sponge	HE687328	Uncultured *Synechococcus*	99

Phylogenetic analysis

Phylogenetic analysis was performed to assess the relative positioning of the isolated cyanobacteria and DGGE clones from the present study with free-living and previously reported sponge-associated cyanobacteria. The phylogenetic tree (Fig. 4) revealed a well-supported topology, both by ML and Bayesian tree-reconstruction approaches, showing a heterogeneous diversity among our sequences, clearly forming 3 distinct clusters. DGGE clones and most of the cyanobacterial isolates from the sponges grouped in cluster A, which was mainly comprised of unicellular cyanobacteria and the filamentous *Pseudanabaena* genus. The isolate from seawater (*Cyanobium* sp. LEGE 10378) was also placed in this cluster. DGGE clones in cluster A showed similarity both with previously reported sponge-associated cyanobacteria and free-living strains. Two DGGE clones (clone 4_1 and clone 5_1) showed similarity with *Acaryochloris* sp., one (clone 8_2) with *Prochlorococcus* sp., 5 (clones 1_1, 2_1, 6_1, 7_1 and 9_1) with *Synechococcus* sp., and the remaining 2 (clones 3_1 and 8_1) had affiliation with *Synechocystis* sp. Clusters B and C were mainly comprised of filamentous species. Cluster B grouped *Phormidesmis* sp. (LEGE10370) with different filamentous cyanobacteria, as well as a *Synechococcus* species. Cluster C only contained *Nodosilinea* species.

DISCUSSION

Hymeniacidon perlevis is one of the most common sponge species along the rocky intertidal shore of Portugal. The presence of photosymbionts such as *Cyanobacteria* may be beneficial for the survival and growth of this sponge. In this study, we assessed the cyanobacterial diversity contained in *H. perlevis* sampled from the coast of Portugal (Fig. 1), using culture-dependent and culture-independent approaches. Although photosynthetic microorganisms are usually present in the outer layers (which are more exposed to sunlight) while the inner layers (mesohyl) are populated by heterotrophic and autotrophic bacteria (Hentschel et al. 2003, Kennedy et al. 2007), *Cyanobacteria* are distributed throughout the whole sponge, as the mesohyl provides higher quantities of nutrients than the surrounding waters (Hentschel et al. 2006, Kennedy et al. 2007). Hence, the whole sponge tissue was used to isolate and assess cyanobacterial diversity.

We succeeded in characterizing 8 isolates, 7 of them from sponge tissue using phenotypic characteristics (Fig. 2) and molecular features. Phylogenetic analysis of the cyanobacterial isolates from this study (Fig. 4) were in agreement with the morphological characters validating their taxonomic affiliation (Komárek et al. 2014).

Erwin & Thacker (2007) classified sponges according to their photosynthetic community through chl *a* quantification. The sponges from the present study might harbour a small photosynthetic community, as the values determined for all 9 specimens were below 50 µg g^{-1}. As previously noted, the results for chl *a* quantification may also be incorporating chl *d* (Li et al. 2012). Through DGGE analysis, we were not able to identify *Synechococcus spongiarum*, known to be a true sponge cyanobiont. Erwin & Thacker (2007) reported that low chl *a* sponges did not harbour *S. spongiarum*. DGGE analysis only showed

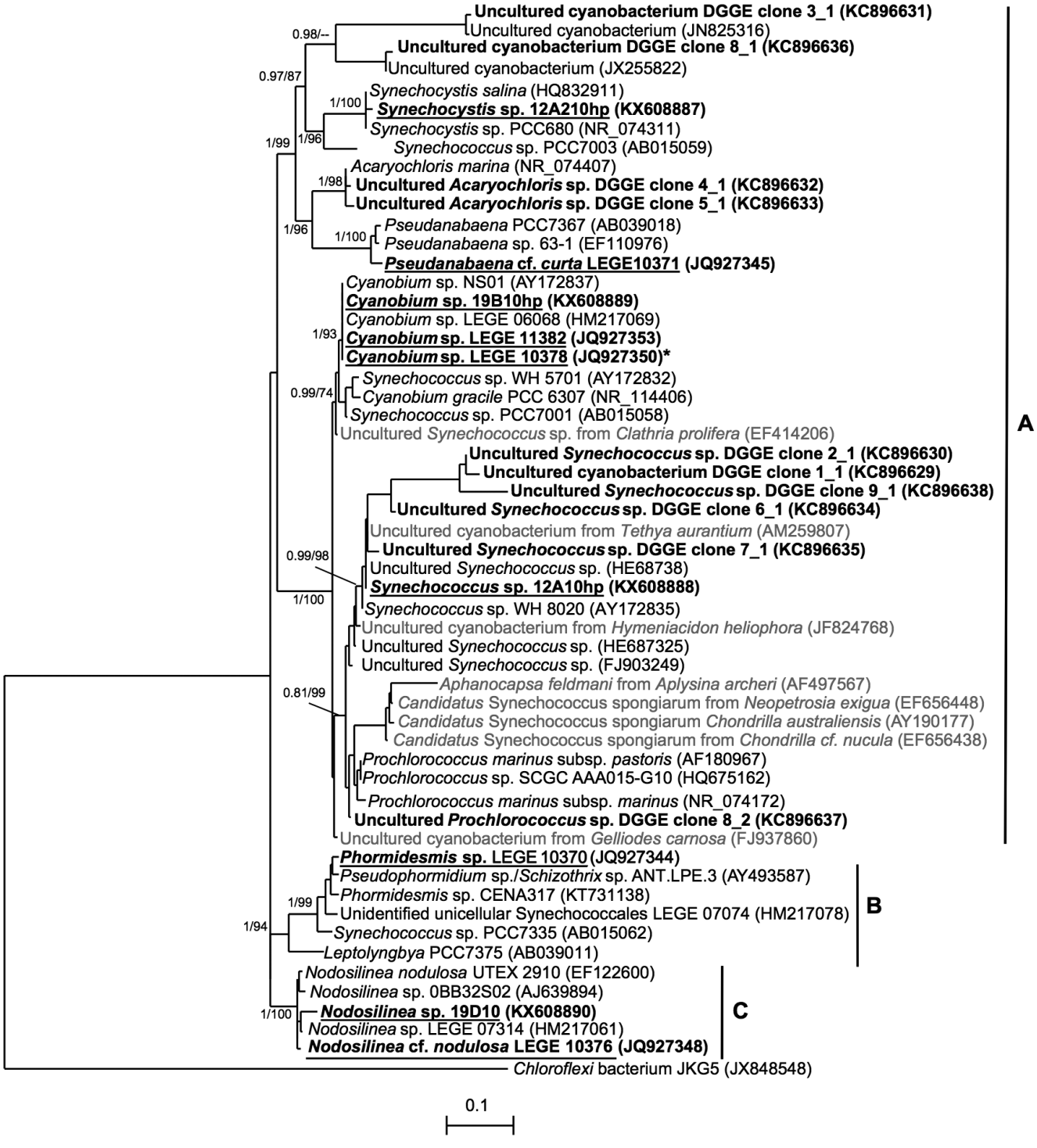

Fig. 4. Maximum likelihood (ML) phylogenetic tree based on the 16S rRNA sequences. The isolates from the present study are in bold and underlined. Isolate with an asterisk (*) was isolated from water sample. Denaturing gradient gel electrophoresis (DGGE) clones obtained from the present study are in bold. The different cyanobacterial clusters are represented with letters from A to C. 16S rRNA sequences obtained from marine sponges are in grey with information of the host sponge species. The retrieved sequences of GenBank were selected based on being the reference strains and the best match for BLASTn analysis GenBank accession numbers are given in parentheses. The tree was rooted using *Chloroflexi* bacterium JKG5. Bayesian posterior probabilities and ML bootstrap support values are represented at the nodes. Only bootstrap values greater than 50% are given. The scale bar at the bottom represents 10% sequence divergence

the presence of unicellular cyanobacteria, indicating that these are likely more abundant than filamentous forms.

Molecular analysis revealed different DGGE banding patterns between seawater and sponge samples (Fig. 3), suggesting the presence of sponge-associated cyanobacterial communities that are distinct from the seawater. Interestingly, the 16S rRNA-DGGE fingerprint from *H. perlevis* samples a, b and c (Fig. 3), sampled from different geographical locations within an interval of 3 mo, revealed a similar banding pattern, further pointing to a consistency in sponge-associated cyanobacteria, even though there was a slight change in the banding pattern from the seawater. However, we observed an enrichment of the cyanobacterial community, represented by the presence of more bands, in sponge-d compared to sponge-a, which were collected from the same location at times 1 yr apart. This observed trend of inconsistency among the sponge-associated bacteria has been previously reported, suggesting the possibility of temporary association or host-switching (Alex et al. 2012), or part of the sponges' dietary supply (Sipkema et al. 2015). It is known that irradiance conditions may influence the photosynthetic activity of sponge-associated cyanobacteria (Erwin et al. 2012), which could explain the differences in the banding patterns between sponges a and d.

Not all filtered bacteria are ingested. They can survive and grow in the mesohyl tissue, becoming part of the sponge microbial community (Kennedy et al. 2007). It is known that only the most common and abundant organisms of the main populations are displayed in the banding pattern, and that organisms representing <1% of the community will not be identified (Muyzer et al. 1993), resulting in an underestimation of the bacterial community. Many bands that were present in sponge samples were not detected in the water samples, and recent studies have shown that most of the sponge-specific 16S rRNA gene sequence clusters are also present in the seawater but in smaller amounts (Taylor et al. 2013). The absence of a band in the DGGE does not necessarily mean the absence of that species; rather, it could mean that the organism was present at the moment of collection, but in an amount below the detection limit of the method. Banding pattern shifts cannot be analysed in terms of diversity, only abundance. Some bands, more evident in the sponge tissues than water samples, may show a selective uptake of the cyanobacterium.

From the 24 bands present in DGGE, only 10 were successfully sequenced. This can explain why filamentous cyanobacteria were not identified. Also, filamentous cyanobacteria may exist in smaller amounts than the detection limit of the method. In the future, it would be interesting to clone more bands, as well as to pick more clones from each band, to assess cyanobacterial diversity.

The phylogenetic analysis with partial 16S rRNA gene sequences from the isolates and DGGE band clones show 3 different clusters. Clusters A and C were comprised of only *Synechococcales*, and cluster B of *Synechococcales* and an *Oscillatoriales* (Fig. 4). Sponge-associated cyanobacteria from our study resulted in polyphyletic clusters, which is a common phenomenon according to previous reports (e.g. Steindler et al. 2005). Although we failed to detect *S. spongiarum*, in accordance with a previous study our phylogenetic reconstruction showed a clear distinction between free-living cyanobacteria and the cyanobionts (*S. spongiarum*) (cluster A; Fig. 4) (Erwin & Thacker 2007). Cluster A was represented by *Synechococcus* and *Prochlorococcus* species, an association that has been widely described in 26 Demospongiae and 17 Calcarea families (Li et al. 2011). An earlier study also showed that the marine sponge *Clathria prolifera* harboured cyanobacteria belonging to the genus *Pseudanabaena* (Isaacs et al. 2009). All DGGE clones are represented in cluster A.

Retrieval of DGGE clones from sponge tissue with significant similarity to *Acaryochloris marina* further validated *Acaryochloris* as a *H. perlevis*-associated cyanobacterium, which has been reported previously in sponges (Alex et al. 2012) and sea-squirts (López-Legentil et al. 2011). *Acaryochloris* is the only known producer of chl *d*, a red-shift chlorophyll. Chl *d* was first identified in red algae (Manning & Strain 1943), then in *A. marina* (Miyashita et al. 1996); eventually, *Acaryochloris* was confirmed as the only chl *d* producer (Murakami et al. 2004). Chl *d* in this cyanobacterium accounts for 95 to 99% of all chlorophyll content (Miyashita et al. 1996), replacing all function of chl *a* and allowing it to exploit light environments depleted of visible radiation. Due to its unique use of far-red light, *Acaryochloris* can live in niches in coastal waters (Murakami et al. 2004), and therefore its presence in intertidal marine sponges is expected, due to their filtration capability. The association of sponges with *Acaryochloris* can be beneficial due to this red-shift chlorophyll. In order to confirm the presence of *Acaryochloris* in *H. perlevis*, in the future, it would be interesting to quantify both chl *a* and chl *d* using the methods described by Li et al. (2012).

Cluster B comprised species from the genera *Leptolyngbya*, *Phormidesmis* and *Pseudophormidium*, as

well as a strain from *Synechococcus* and a former *Synechococcus*, now identified as unicellular *Synechococcales*. The clustering of *Synechococcus* sp. PCC 7335 with filamentous non-heterocystous cyanobacteria from the genus *Leptolyngbya* has been previously reported (Honda et al. 1999, Castenholz 2001, Wilmotte & Herdman 2001). Cluster C formed a well-supported group, only containing *Nodosilinea* species.

The presence of sponge-associated cyanobacteria from seawater samples supports the hypothesis of procurement of symbionts through the environment, i.e. horizontal transmission (Maldonado 2007), apart from the commonly accepted vertical mode of transmission. Furthermore, it indicates the ability of sponge-associated cyanobacteria to survive outside the host tissue (Taylor et al. 2013). According to Alex et al. (2013), *H. perlevis* from the Portuguese coast is a LMA sponge, and it has been suggested by Giles et al. (2013) that LMA sponges may acquire bacteria mainly from ambient seawater. In addition, sponges are filter-feeding animals that use picoplanktonic cyanobacteria as a source of food. Due to the close phylogenetic relationship to planktonic *Synechococcus* strains, a seawater origin for the *H. perlevis* cyanobacterial clones cannot be excluded. But it can also point to the existence of a community shared between sponges and the surrounding waters, because it is known that the sponge microbial community is a mixture of organisms acquired both from the water column and by vertical transmission (Hentschel et al. 2003). Usher et al. (2001) observed *Cyanobacteria* in only 25% of sponge larvae, suggesting that vertical transmission is not the only mode of symbiont procurement. Bacterial profile assessment and comparison using adults and embryos could further validate the mode of symbiont transmission among the intertidal sponge *H. perlevis*.

As has been previously reported (Steindler et al. 2002), the presence of *Cyanobacteria* can be very important for the survival of intertidal marine sponges. For instance, these sponges are prone to air exposure, leading to fluctuations in temperature and irradiance, and lack of filter feeding opportunities (Steindler et al. 2002). During these conditions, the photosymbionts play an important role, providing the sponge hosts with nutrient uptake for their survival and the production of potential UV-screening substances (Steindler et al. 2002). Although we employed a relatively inexpensive technique (DGGE) to profile the microbial diversity, it provided a first glimpse of the cyanobacterial community, allowing visualization and monitoring of changes directly from the banding patterns. This method, when used for a long period can also allow differentiation between transient and permanent communities (Hentschel et al. 2003). Further determination of the origin and diversity of sponge-associated cyanobacteria in comparison to their free-living counterparts can be achieved using advanced next-generation sequencing techniques.

Many previous studies have reported the presence of a huge diversity of marine cyanobacteria isolated from the Portuguese coast (e.g. Brito et al. 2012, Leão et al. 2013). Brito et al. (2012) reported that *Cyanobium*, *Leptolyngbya* and *Pseudanabaena* were the most abundant genera among isolates. Strains from the same genera obtained in the present study, also collected from the coast of Portugal, were found to be a source of bioactive compounds (Leão et al. 2013, Costa et al. 2014, Brito et al. 2015, Costa et al. 2015), namely strains from the genera *Cyanobium* (Costa et al. 2015), *Leptolyngbya*, *Synechocystis*, *Nodosilinea* and *Pseudanabaena* (Costa et al. 2014). Isolation and growth of these species under laboratory conditions would be necessary to obtain sufficient quantities of these natural compounds for their detailed chemical characterisation and production. Also, due to the negligible amount of some cyanobacteria in seawater, they could easily be missed when isolating and culturing, and hence their bioactive potential would remain unexploited. Sponges are filter-feeders capable of pumping 24 cm^3 of seawater per day, per kg of sponge (Vogel 1977), with very efficient filtration systems and a clearance rate of up to 61% (Stabili et al. 2006). In this way, sponges could be used as a natural filtration and concentration mechanism to obtain new cyanobacterial strains with pharmaceutical potential.

The present study shows, for the first time, the diversity of cyanobacteria associated with marine sponges from the intertidal area of the Portuguese coast (NE Atlantic) using both culture- and molecular-based methods, and the comparison of the sponges' cyanobacterial community to that present in seawater. Even although the true cyanobacterial diversity might be underestimated, culture-dependent and culture-independent methods showed that some sponge-associated cyanobacteria were detected in the surrounding waters, suggesting temporary or selective uptake. Nevertheless, we argue that the recurrent presence of a cyanobacterial community at different spatial and temporal scales could be indicative of environmental acquisition of *Cyanobacteria* by the intertidal marine sponge *H. perlevis*. Finally, the isolation technique employed here could be used to isolate new cyanobacteria that are only present in small amounts in the water column.

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