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Microbial epibionts of the colonial ascidians *Didemnum galacteum* and *Cystodytes* sp.

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Abstract Symbiosis with microorganisms has been well documented for many marine invertebrate taxa. However, knowledge of the diversity of microorganisms associated with ascidians is still limited. This study assessed the microbial epibionts of *Didemnum galacteum* and *Cystodytes* sp., two ascidian species collected from the western coast of Ceará state (Brazil), at Dois Coqueiros beach and the port of Pecém, respectively. The microbiota were examined using optical microscopy, followed by subsequent analysis of fingerprinting profiles obtained by denaturing gradient gel electrophoresis (DGGE) and 16S rRNA clone libraries. The microscopy analysis showed for both ascidians a community comprising cyanobacteria, mainly *Prochloron*-like species, and diatoms. The DGGE results indicated that *D. galacteum* hosts a more diverse microbiota when compared to *Cystodytes* sp. The same analysis also suggested that the diversity of the seawater microbiota was higher than the diversity of the ascidian-associated microbiota. The analysis of the 16S rRNA clone libraries revealed the dominance of Proteobacteria symbionts associated with both ascidians, with Alphaproteobacteria as the major component in *D. galacteum* and Gammaproteobacteria the major component in *Cystodytes* sp. The analysis of the clone libraries also revealed the presence of other taxa such as Bacteroidetes, Planctomycetes, Actinobacteria, Cyanobacteria, and uncultured bacteria in *D. galacteum*, but not in *Cystodytes* sp. Among the bacteria found to be exclusively associated with the ascidians, none were shared by the two studied hosts. The combined results point to a diverse microbiota associated with the external surface of the ascidians, with a mixed composition including organisms typically found in the surrounding seawater, but also a more specific set of taxa.

Keywords Symbiosis · Ascidiacea · Microbiota · Brazil

1 Introduction

Ascidians comprise the most diverse group of tunicates, with an estimated 3,000 living species (Lambert 2005). They are sessile marine filter feeders with a short-lived non-feeding motile larval stage. Ascidians usually attach to a variety of natural and artificial substrates from the intertidal to the deep sea, existing in solitary and colonial forms (Millar 1971; Monniot et al. 1991).

A wide range of bioactive natural compounds of ecological, chemical, and biomedical interest have been isolated from tunicates (Jimenez et al. 2003; Martínez-García et al. 2007a). However, recent studies have reported that the synthesis of these compounds is often attributed to associated microorganisms (Schmidt et al. 2004; Dunlap et al. 2007; Donia et al. 2011; Schmidt et al. 2012; Pérez-Matos et al. 2007; Monniot et al. 1991; Pérez-Matos et al. 2007).

Many microorganisms such as bacteria, cyanobacteria, and diatoms have already been detected in association with

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ascidians (Martínez-García et al. 2007b; Hirose et al. 2009). Several studies have shown that the ascidians belonging to the Didemnidae and Polycitoridae families are hosts for photosymbionts (Lewin et al. 1980; Oka et al. 2005; Hirose and Fukuda 2006; López-Legentil et al. 2011). The ascidian-*Prochloron* symbiosis is a well-known example of a stable photosymbiosis within the Chordata (Monniot et al. 1991). However, few studies have thoroughly examined the presence of associated bacteria (Groepler and Schuett 2003; Schuett et al. 2005; Tait et al. 2007; Menezes et al. 2010). Martínez-García et al. (2011) reported the presence of complex microbiota composing the biofilms of *Cystodytes dellechiajei*, including diatoms, rhodophytes, and four different cyanobacteria genera, in addition to other bacterial taxa. A more thorough assessment by Behrendt et al. (2012) of the species *Lissoclinum patella* revealed that cyanobacteria dominated the assemblage but that other taxa such as Proteobacteria, Bacteroidetes, Verrucomicrobia, and Fusobacteria were also present.

On the Ceará State coastline in Northeast Brazil, ascidians are commonly found on beach rocks at the intertidal zone, especially in shaded areas. In this region, a total of 27 species have been identified (Lotufo and Silva 2006; Lotufo and Dias 2007). The species *Cystodytes* sp. and *Didemnum galacteum* Lotufo and Dias 2007 were found to harbor a conspicuous green microbial biofilm on the tunic surface. The microbial composition of the biofilms in ascidians species have only been investigated in a small number of species (Martínez-Garcia et al. 2011; Behrendt et al. 2012). Therefore, the aim of this study was to assess the microbial community of the outer surface of two ascidian species, using both optical microscopy and molecular approaches.

2 Materials and methods

2.1 Sample collection and preparation

Samples of the ascidian *Didemnum galacteum* (Fig. 1a) were manually collected at the intertidal region of the Dois Coqueiros beach (03°41′29″S, 38°36′10″W), and samples of *Cystodytes* sp. (Fig. 1b) were collected at a depth of 6 m by a scuba diver at the port of Pecém (03°32′02″S, 38°47′58″W). Seawater samples were collected at the same localities for a comparative analysis of the microbial profile. Both sampling areas were located on the west coast of Ceará state, northeast Brazil (Fig. 2).

The colonies were removed from the substrate, washed in sterile seawater, and placed in plastic bags containing sterile phosphate-buffered saline (PBS, pH 7.4) in a proportion of approximately 5 mL/g of the animals. The colonies were gently rinsed and maintained in the PBS buffer for about 2 h at room temperature, allowing the transference of the microorganisms from the biofilm to the liquid medium. The transference was confirmed by the disappearance of the green biofilm and the detection of microorganisms in the microscopy analysis.

2.2 DNA extraction

A fraction of the PBS was mounted on glass slides for inspection using optical microscopy. After the microscopy analysis, the saline containing the epibionts was centrifuged (1,500 g, 5 min), and the pellet was used for DNA extraction. The seawater was filtered through a 0.45 μm membrane. The DNA was extracted using CTAB 2X according to the protocol of Roger and Bendich (1985).

2.3 Denaturing gradient gel electrophoresis (DGGE)

The analysis of the microbial profile using DGGE was performed with the hypervariable region V3 of the genes encoding 16S rRNA, amplified with the primers 338 GC F (5′ − CCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGACTCCTACGGGAGGCAGCA − 3′) and 518 R (5′ − ATTACCGCGGCTGCTGG − 3′). Each 30 μl reaction mixture contained 1X buffer, 0.2 mM of each dNTP, 2.5 mM of $MgCl_2$, 0.5 μM of each primer, and 1 U of Taq polymerase. The cycle conditions were programmed with an initial denaturation at 95 °C for 5 min, 30 amplification cycles (92 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min), followed by a final extension of 72 °C for 10 min. The amplification products were examined using 2 % agarose gel electrophoresis

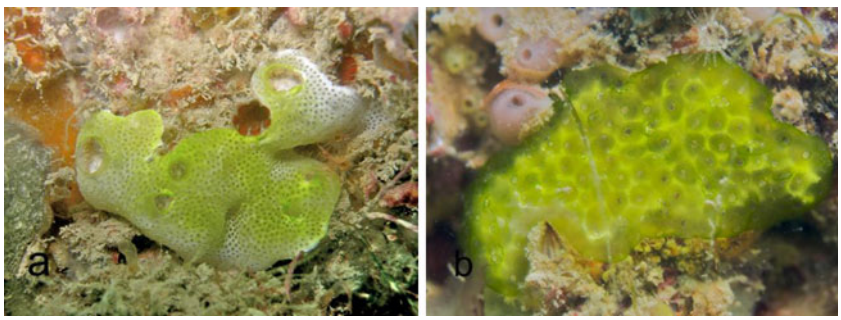

Fig. 1 Photographs of the ascidian species with the green biofilm; **a** *Didemnum galacteum* and **b** *Cystodytes* sp.

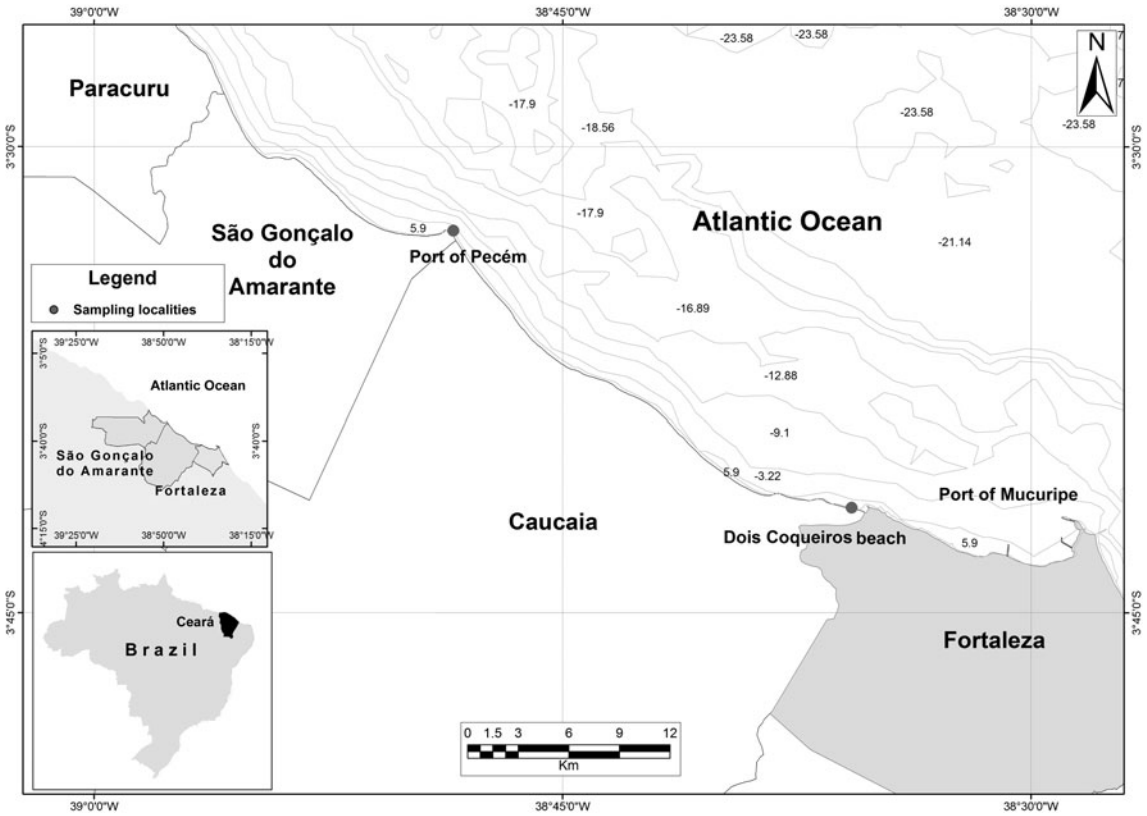

Fig. 2 Map of the region indicating the sampling localities

before the DGGE was performed, according to the protocol of Øvreas et al. (1997). The DGGE was run on a 8 % polyacrylamide gel with a 35–65 % denaturing gradient for 4 h at 60 °C at 200 V in a 0.5X TAE buffer. Subsequently, the gels were stained with SYBR Green I (Molecular Probes) for 1 h, visualized, and photographed. The DGGE images were analyzed using the software BioNumerics (Applied Mathematics) for band detection and construction of the data matrix.

2.4 Construction of clone libraries and sequencing analysis

For the construction of the 16S rRNA clone library, genomic DNA was extracted from the PBS from each species and purified for use in PCR amplification using 27 F (5′ − AGAGTTTGATCCTGGCTCAG − 3′) and 1494R (5′ − TACGGCTACCTTGTTACGAC − 3′). The PCR amplification was performed under the following conditions: 95 °C for 5 min, followed by 34 cycles of 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 7 min. The PCR products were cloned into the pGEM-T Easy cloning vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA) using electroporation according to the manufacturer's protocol. DNA sequencing was performed using purified plasmid DNA, the T7 primer (5′ − TAATAC GACTCACTATAGGG − 3′), and the ABI PRISM BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The fluorescent-labeled fragments were purified using ethanol precipitation. The samples were resuspended in distilled water and analyzed in an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). The chromatograms were trimmed and selected for high-quality sequences at the Ribosomal Database Project. Sequences longer than 300 bp, where the quality was 20 or more, were selected to constitute the final dataset for further analysis. The sequences were assigned to operational taxonomic units (OTU), which were determined at 97 % similarity using DOTUR (Schloss and Handelsman 2005). Rarefaction analysis was performed to determine OTU richness at 97 % and Shannon-Wiener diversity. In addition, one sequence from each OTU was compared for similarity using the Blastn program from NCBI (http://www.ncbi.nlm.nih.gov/blast).

2.5 Nucleotide sequence

The 16S rRNA sequences obtained in this study were deposited in GenBank under accession numbers JN 637373 − JN 637445.

3 Results

3.1 Microscopy

The analysis using optical microscopy showed the presence of a diverse microbial assemblage associated with the ascidians comprising diatoms and cyanobacteria. The diatoms found were assigned to the genera *Amphora* and *Cylindrotheca* (Fig. 3a) and *Nitzschia*, (Fig. 3b). The most abundant component of the biofilm was composed of green spherical coccoid cells measuring, 12–18 μm (Fig. 3c–d), pointing to Group I *Prochloron* species (Cox 1986).

3.2 Denaturing gradient gel electrophoresis (DGGE)

The analysis of the gel showed multiple bands for all samples, characterizing the diversity profile of the microbial community (Figs. 4 and 5). The largest number of OTUs was detected in the water samples from Dois Coqueiros beach (22 OTUs), followed by the biofilm of *D. galacteum* (18 OTUs), the water samples from Pecém (15 OTUs), and the biofilm of *Cystodytes* sp. (9 OTUs). The similarity cluster analysis grouped the water samples with the biofilms from the same localities at less than 40 % distance. The Venn diagram (Fig. 6) shows a large number of shared OTUs and other OTUs that were exclusive to each sample. None of the OTUs exclusively associated with the ascidians were shared by the two species.

3.3 Clone library

Two libraries of 16S rRNA were constructed for the bacterial community of the tunic surface of the ascidians *D. galacteum* and *Cystodytes* sp. A total of 192 clones were sequenced (96 for each sample). A total of 89 sequences were used in the analysis of the bacterial community of the ascidian *D. galacteum*, representing 62 OTUs, and 68 sequences for the ascidian *Cystodytes* sp., representing 11 OTUs.

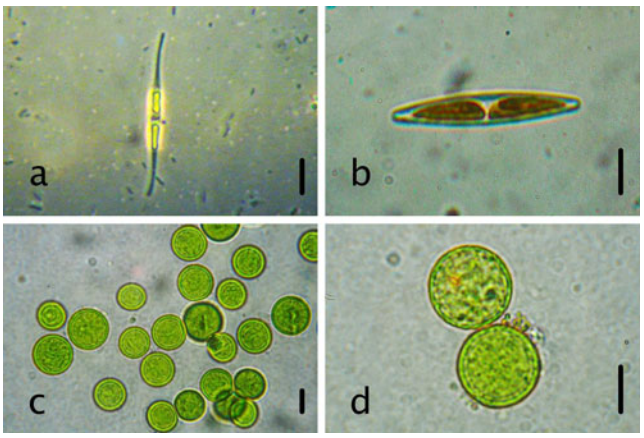

Fig. 3 Microphotographs of some ascidian epibionts. **a** *Cylindrotheca*; **b** *Nitzschia*; **c** *Prochloron*-like cells; **d** Detail of the *Prochloron*-like cells, which reveals the lack of cell organelles. Scale bars=10 μm

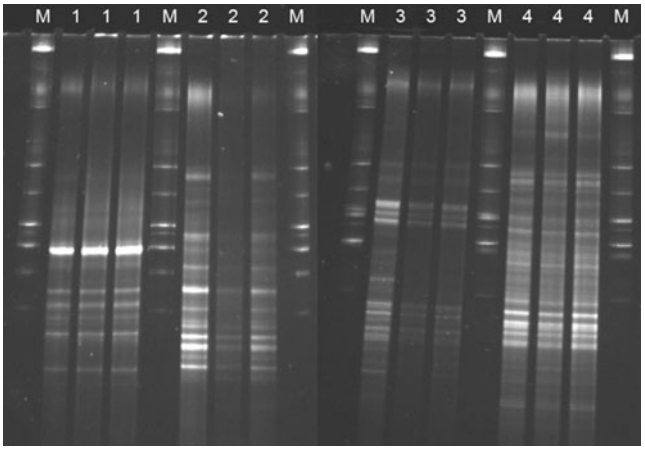

Fig. 4 Images of the two DGGE gels with samples in triplicates. M−1 Kb marker; 1) *Cystodytes* sp.; 2) seawater sample from Pecém; 3) *Didemnum galacteum*; 4) seawater sample from Dois Coqueiros beach

The bacterial community was analyzed using the 16S rRNA clone library. The results revealed the dominance of Proteobacteria in the two libraries, with 62 % of the clones comprising Alphaproteobacteria for the ascidian *D. galacteum*, and 90 % of the clones comprising Gammaproteobacteria for the *Cystodytes* sp. (Fig. 7). Other taxa such as Bacteroidetes, Planctomycetes, Actinobacteria, Cyanobacteria, and other unidentified bacteria were found in the microbiota of *D. galacteum*, but not in the microbiota of *Cystodytes* sp.

4 Discussion

This work reveals the presence of a diverse microbiota associated with the external surface of *D. galacteum* and *Cystodytes* sp. Overall, the microscopy analysis pointed to *Prochloron* (Group I) as the main microbial component in the green biofilm of both ascidians.

Many studies have reported the unique symbiotic association between the ascidians and the *Prochloron* cyanobacteria (Kott 1984; Kott et al. 1984; Hirose et al. 2006) and the coexistence with other cyanobacteria (Hirose et al. 2009). *Prochloron* is a symbiont commonly associated with ascidians in obligatory or non-compulsory relationships. For both *D. galacteum* and *Cystodytes* sp., the colonies without the green layer of *Prochloron* are much more common (personal observation), which indicates that the association is probably non-obligatory.

The analysis of the profile of the microbial community revealed a large number of microbial taxa associated with the ascidians. The DGGE results were consistent with the data obtained from the analyses of the 16S rRNA gene clone

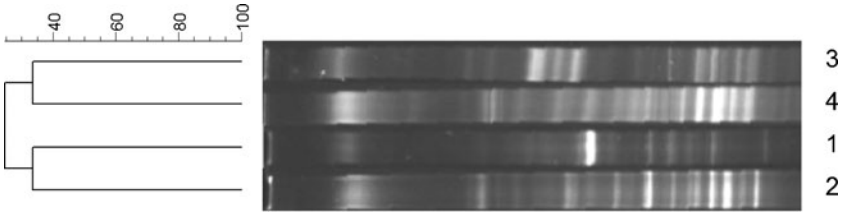

Fig. 5 Hierarchical cluster based on Jaccard distances and UPGMA amalgamation rule for the DGGE results. 1) *Cystodytes* sp.; 2) seawater sample from Pecém; 3) *Didemnum galacteum*; 4) seawater sample from Dois Coqueiros beach

library. While the ascidian *D. galacteum* harbored a diverse microbial community on its tunic surface, the microbial community of *Cystodytes* sp. was less diverse. The assessment of bacterial diversity using DGGE revealed 18 OTUs for *D. galacteum* and 9 OTUs for *Cystodytes* sp. These results are in contrast with the results of Tait et al. (2007) who identified only two OTUs for the ascidian *Didemnum* sp. They attributed this low diversity to the acidic conditions on the tunic surface. These somewhat divergent results may indicate that environmental and biological factors may also influence the composition of the microbiota on the surface of the host organisms, as shown by Behrendt et al. (2012). Differences in the methods employed in the studies may also explain the incongruence.

To evaluate to what extent the seawater microbial community may influence the epibiotic composition, a comparative analysis of the bacterial community of the tunic surface of the ascidians and the seawater where the ascidians were collected was conducted using DGGE. The results showed 22 OTUs for the samples collected at Dois Coqueiros beach and 15 OTUs for the samples from the Pecém port, suggesting that the intertidal region has greater bacterial richness compared with the port area, which was characterized by lower richness and higher dominance.

Interestingly, as shown in the Venn diagram (Fig. 6), the outer surfaces of the ascidians harbor not only a number of shared OTUs, but also exclusive OTUs, which are not shared with the surrounding seawater. The results presented here indicate that the environment contributes to the microbial community via horizontal transmission, but they also suggest the possibility of vertical transmission of specific components from generation to generation (Moss et al. 2003; Groepler and Schuett 2003; Schuett et al. 2005; Bright and Bulgheresi 2010). Concerning the vertical transmission, a study of *Cystodytes dellechiajei* and its larvae reported 95 % similarity between the microbial communities of these two stages (Martínez-García et al. 2007b). For the ascidians investigated here, there seems to be a component from the environment and another specific to the host.

The data from the analysis of the clone library indicated that the two species have a unique bacterial profile, with Alphaproteobacteria dominating in *Didemnum galacteum* (51 %) and Gammaproteobacteria in *Cystodytes* sp. (90 %). Interestingly, there were no dominant taxa in the *D. galacteum* microbiota. However, there was a clear dominance of the *Pseudomonas* genus in the *Cystodytes* sp. The genus *Pseudomonas* has been extensively reported to be associated with the degradation of hydrocarbons in marine environments (Kostka et al. 2011). Nogales et al. (2007) observed the dominance of Alphaproteobacteria and Gammaproteobacteria when evaluating the composition of bacteria in coastal areas. They reported that the Alphaproteobacteria were associated with non-impacted areas, and that the Gammaproteobacteria were associated with areas containing high nutrient concentrations from polluted sites. Thus, the difference in dominance identified here might be related to anthropogenic impacts at the Pecém port.

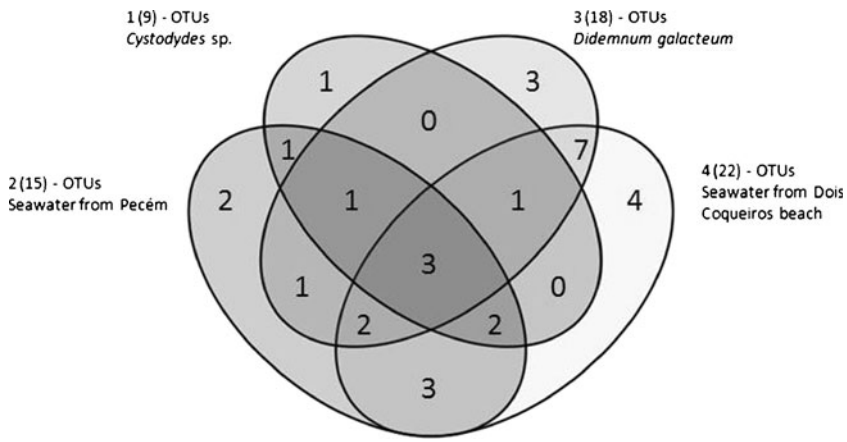

Fig. 6 Venn diagram showing exclusive and shared OTUs in each sample

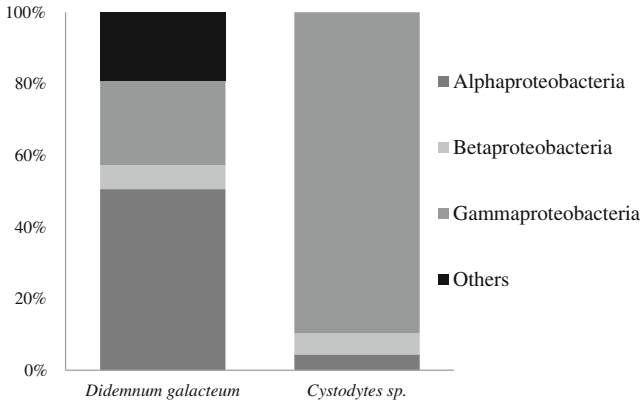

Fig. 7 Microbial diversity of clones for the ascidian *Didemnum galacteum* and *Cystodytes* sp.

In terms of phylogenetic groupings, the bacterial community of *D. galacteum* also comprised Cyanobacteria, Bacteroidetes, Planctomycetes, Actinobacteria, and uncultured bacteria, consistent with previously reported results for marine environments (Pérez-Matos et al. 2007; Tait et al. 2007; Martínez-García et al. 2010). Four OTUs showed similarity with the cyanobacteria sequences deposited in GenBank: *Synechococcus* sp. (99 %), *Symploca* sp. (96 %), *Cyanobium* (99 %), and *Stigonema ocellatum* (96 %). None of the OTUs exhibited similarity with the *Prochloron*-like cyanobacteria, a major component of the biofilm, as revealed in the microscopy analysis. This may be due to the inability of the method employed in the present study to detect some important taxa. Similarly, Martínez-Garcia et al. (2011) studied microbial phototrophs on the surface of *Cystodytes dellechiajei* and detected the presence of four different genera of Cyanobacteria but did not detect *Prochloron* and *Acaryochloris*.

An analysis of organisms on the surface of *Lissoclinum patella* from Heron Island revealed a diverse assemblage, where the dominant taxa were also Cyanobacteria and Proteobacteria (Behrendt et al. 2012). The strong influence of environmental conditions on the microbiota of the superficial biofilm was also revealed by their study, where a comparison of samples from different depths revealed contrasting assemblage profiles. Behrendt et al. (2012) also showed marked differences between biofilms growing over and under the colony. In the present study, this aspect was not taken into account. Thus, microorganisms from the whole biofilm were present in the PBS sample. Another study on the microbiota associated with marine organisms in southeastern Brazil, revealed that the two *Didemnum* species studied exhibited a diverse community, both in terms of filamentous fungi and bacteria (Menezes et al. 2010).

Future studies with other ascidian species and other parts of the colonies will be important to obtain a better insight into the relevance of the microbial component for the ascidians themselves, but also as components of the local biodiversity. As technology evolves and becomes less expensive and more widely available, it will be possible to apply thorough metagenomic approaches to assess better the microbial diversity associated with marine invertebrates and help determine the role they play in the ascidian symbiosis.

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