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NOTES AND SHORT COMMUNICATIONS

Epibiosis of Oxygenic Phototrophs Containing Chlorophylls *a, b, c,* and *d* on the Colonial Ascidian *Cystodytes dellechiajei*

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Abstract The external surfaces of marine animals are colonized by a wide variety of epibionts. Here, we study the phototrophic epibiotic community attached to the colonial ascidian *Cystodytes dellechiajei* collected in the Mediterranean Sea. Epifluorescence microscopy analysis showed abundant filamentous cyanobacteria on the upper and basal parts of the ascidian that displayed autofluorescence, as well as some unicellular cyanobacteria, diatoms, and structures, which could belong to microscopic rhodophyte algae. In addition, high-performance liquid chromatography of the photosynthetic pigments confirmed that the phototrophic epibionts possess chlorophyll (Chl) *d,* as well as Chl *a*, *b,* and *c,* which enable them to use far-red light for photosynthesis in that peculiar microenvironment. Furthermore, laser scanning confocal microscopy showed the presence of a few small patches of cells on the basal part of the ascidian displaying fluorescence between 700 and 750 nm after excitement with a 635-nm red laser, typically within the range of Chl *d.* Denaturing gradient gel electrophoresis of the 16S rRNA gene polymerase chain reaction amplified using specific primers for *Cyanobacteria* detected sequences related with the genera *Planktothricoides*, *Synechococcus*, *Phormidium*, and *Myxosarcina*, as well as sequences of chloroplasts of diatoms and rhodophyte algae. Remarkably, only the sequences related to the filamentous cyanobacteria *Planktothricoides* spp. and some chloroplast sequences were found in almost all specimens collected under different macroecological conditions and geographical areas, suggesting thus certain specificity in the epibiotic association. On the other hand, *Prochloron* spp. and *Acaryochloris marina,* typically associated to tropical ascidians, were not detected by denaturing gradient gel electrophoresis. However, given the low abundance of cells displaying Chl *d* in *C. dellechiajei* and the fact that molecular fingerprinting techniques not always recover low abundance groups, the presence of these cyanobacteria cannot be ruled out. Nevertheless, our data indicate that tropical ascidians and *C. dellechiajei* differ in their phototrophic communities, although Chl *d*-containing cells are present in both microenvironments.

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Introduction

Most biological interactions in the sea are mediated through the body surface of organisms, and often epibionts may affect and even modulate the interaction between the basibiont (substrate organism) and its environment [1–3]. In the present study, we analyze the association between *Cystodytes dellechiajei* and its phototrophic epibiotic community. This marine invertebrate is a colonial ascidian widely distributed around the world that harbors inside its tissue a conserved and stable community composed by

ammonia-oxidizing *Crenarchaeota* and *Alphaproteobacteria* that express the *amoA* and *pufM* genes involved in ammonia oxidation and aerobic anoxygenic photosynthesis, respectively [4, 5]. The inner parts of this animal thus represent a singular ecosystem, in which *Crenarchaeota* could use ammonium excreted by the animal for oxidation reactions and *Alphaproteobacteria* light to generate extra energy. Now, we widen the characterization of interactions of this ascidian with microbes and provide some new insights into the association among marine invertebrates and their oxygenic phototrophic epibionts. For this purpose, several ascidian specimens of *C. dellechiajei* which displayed a great pigmentation variability were collected from different regions in the Western, Central, and Eastern Mediterranean (depth <23 m) between 2003 and 2008. Epifluorescence microscopy analysis showed abundant nonheterocystous filamentous cyanobacteria with red autofluorescence on the surface of the upper and basal parts of the ascidian colony, as well as some unicellular cyanobacteria (Fig. 1). Moreover, diatoms and larger structures, which could belong to microscopic rhodophyte algae, were also detected (Fig. 1). On the other hand, as shown in a previous work [4], no autofluorescent microorganisms were detected inside the colony of *C. dellechiajei*, nor associated to the external surfaces of larvae of this ascidian, indicating thus that phototrophic epibionts were not vertical transmitted to the next generation, but they colonized the animal after the settlement of the larva.

In addition, high-performance liquid chromatography (HPLC) was used to characterize the photosynthetic pigments of the phototrophic epibiotic community attached to the colony of *C. dellechiajei.* For this analysis, tissue samples (approximately 1 g) from external surfaces of the colony, where photosynthetic microorganisms occur, were extracted twice in 2 mL of acetone/methanol (7:2 vol/vol) buffered with 0.3 M $MgCO_3$ to prevent pheophytinization

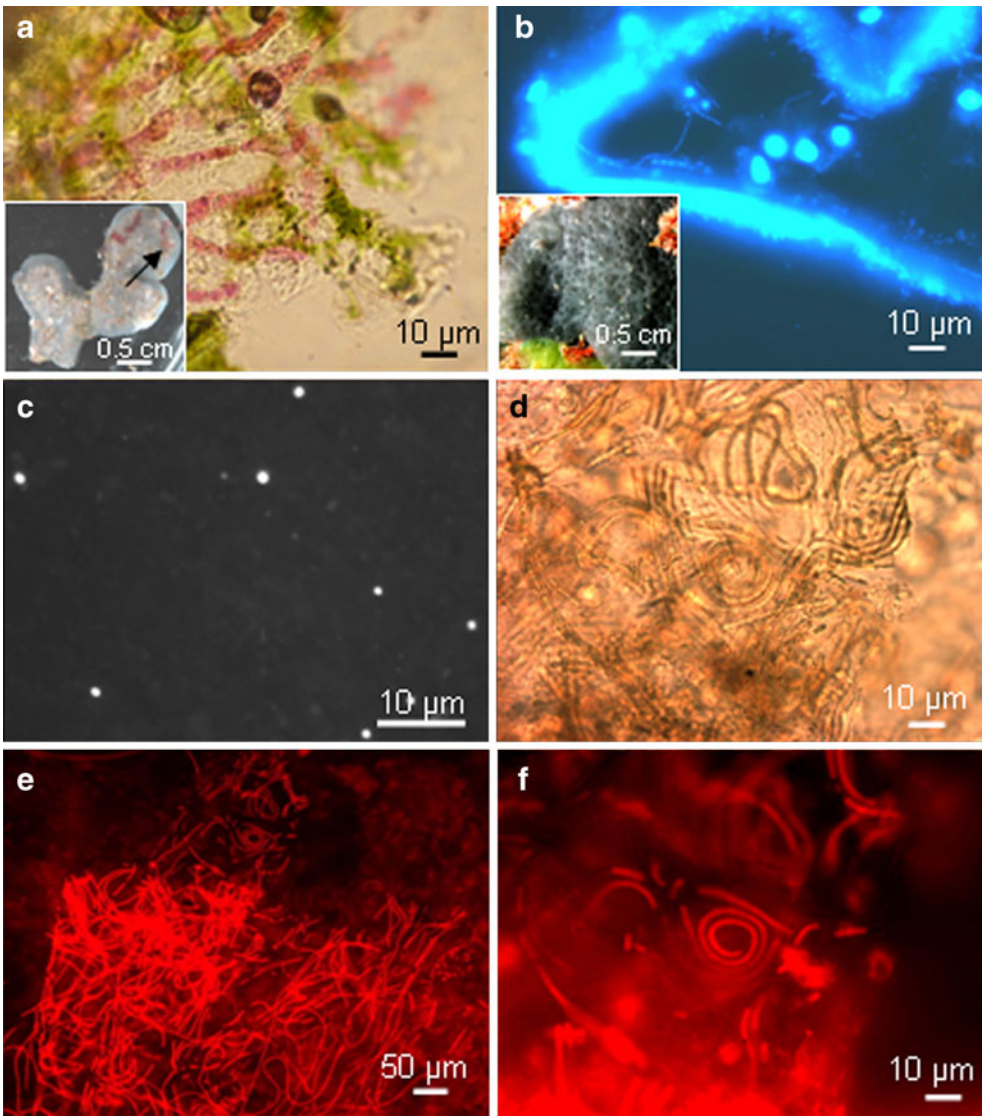

Figure 1 Optic microscopy of the phototrophic epibiotic community of *C. dellechiajei*. **a** Image of the basal part of the colony showing the patches formed by cyanobacteria-like structures and microscopic red algae. **b** 4′-6-Diamidino-2-phenylindole staining of the dense biofilm formed by the epibionts in the upper side of the colony. **c** Epifluorescence image taken with a filter specific for Chl *a* fluorescence showing unicellular cyanobacteria. **d–f** Images of optic and epifluorescence microscopy showing filamentous cyanobacteria, which were the most dominant fraction in the epibiotic community

of the pigments. The joint extracts were clarified by centrifugation (2,000×g for 2 min) and supernatants concentrated by N_2 gas bubbling until reaching a volume of 200 μL. The extracts were analyzed by the HPLC using the Agilent 1100 Series system (Agilent Technologies Inc., Palo Alto, CA) equipped with the UV-VIS diode-array detector (Agilent DAD 61315B). Pigments were separated using a modified method of Van Heukelem and Thomas [6] on the heated (35°C) Phenomenex Luna 3 μ C8(2) 100-Å column with binary solvent system (0 min 100% A, 20 min 100% B, 25 min 100% B, 27 min 100% A, 30 min 100% A; A: 70% methanol + 28 mM ammonium acetate, B: methanol). The solvent flow rate was 0.8 mL min^{-1}. The peak assignment and characterization of photosynthetic pigments were based on the retention times and acquired absorption spectra. HPLC analysis from the external tissue fractions of the colony confirmed that phototrophic epibionts possessed chlorophyll (Chl) *d* (Fig. 2). Chl *b* and Chl *d* were coeluted but clearly distinguished by their absorption peaks as shown in the insert depicted in Fig. 2, where the characteristic absorbance peak of Chl *d* between 700 and 720 was obtained [16]. The coelution phenomenon for both Chl *b* and *d* has been described in other studies, recently in Kashiyama and collaborators [11]. Thus, HPLC analysis indicates that Chl *d*-containing oxygenic phototrophs would be located on the external surface of the ascidian. In addition, HPLC data demonstrate that the

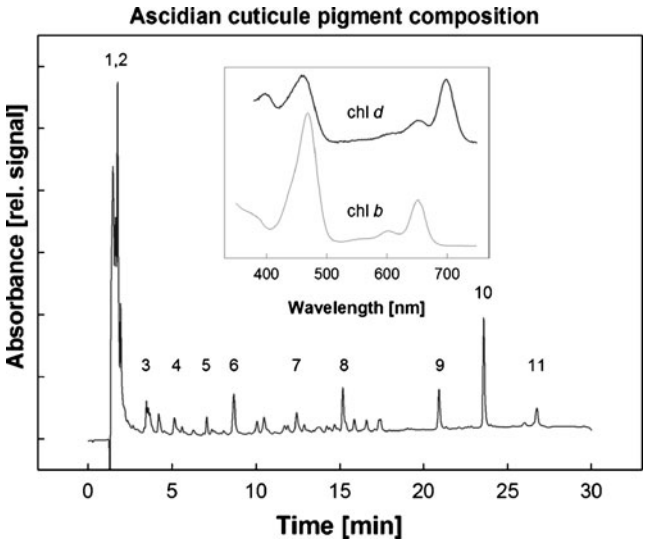

Figure 2 HPLC chromatogram of pigments extracted from external surface of *C. dellechiajei* colony, where the epibionts occur, recorded at 440 nm. *Numbers* indicate the main peaks: 1 and 2—polar violet–blue pigments, 3—Chl degradation products, 4—Chl *c*, 5—unidentified carotenoid, 6—fucoxanthin-like, 7—unidentified carotenoid, 8—zeaxanthin, 9—Chl *d* and *b*, 10— Chl *a*, and 11—β-carotene. The *insert* depicts the online absorption spectra of Chl *d* and Chl *b* obtained from the external tissue fractions, which migrated at 20.8 and 21.0 min forming a joint peak 9. The characteristic absorbance peak of Chl *d* at 700–720 nm can be observed in the *insert panel*

autotrophic epibiotic phototrophs also possessed Chl *a, b*, *c* and accessory pigments, like β-carotene, zeaxanthin, fucoxanthin, and other unidentified carotenoid pigments (Fig. 2). The detection of Chl *a*, *b*, and *c* is consistent with the epifluorescence microscopy results, since it is well known that some of these chlorophylls are present in the photosynthetic reaction center of cyanobacteria, dinoflagellates, algae, and diatoms [7, 8], which have been detected in *C. dellechiajei* by epifluorescence microscopy. So far, *Acaryochloris marina* is known to be the only oxygenic photoautotroph described that uses Chl *d* as the predominant photosynthetic pigment, which has its maximum absorption peak in the near-infrared region [9–12]. Furthermore, in the present study, laser scanning confocal microscopy (LSCM) was used in order to study the fluorescent emission of cells attached to *C. dellechiajei.* LSCM has shown to be an extremely useful method to examine and identify the pigment composition and fluorescence from chlorophylls and phycobiliproteins (PBPs) in different cyanobacteria, including Chl *d*-containing cells, like *A. marina* [13]. For this purpose, thin tissue sections from basal and upper parts of the colony were cut using a sterile razor blade and transferred to a microscope well slide with a single depression filled with seawater and sealed with a cover slip. Samples were inspected with a Leica SP2 confocal microscope by using two excitation lasers, HeNe (543 nm) and red (635 nm). Fluorescence was detected between 555 and 700 nm for Chl *a* and *b*, 700 and 750 nm for Chl *d*, and 640 and 670 nm for PBP [13]. As expected according to epifluorescence microscopy and HPLC data, the photoautotrophic community attached to the upper and most of basal parts of the colony was composed mainly by filamentous cyanobacteria-like cells that displayed fluorescence typically within the range of Chl *a, b*, and other PBPs (fluorescence emission between 555 and 700 after excitement with HeNe laser 543 nm; data not shown). In addition, LSCM analysis revealed the presence of small patches of cells on the basal section of the ascidian that emitted fluorescence between 640 and 670 nm, and 700 and 750 nm, within the range of PBPs and Chl *d,* respectively [13, 16], after excitement the sample with a 633-nm red laser (Fig. 3). The diameter and length of the Chl *d*-containing cells were similar to that described for *A. marina* by Miyashita et al. [10] (1–1.5 μm in diameter and 1.5–3 μm in length). Thus, both HPLC and LCMS data indicate that *Acaryochloris*-like cells are present in the ascidian *C. dellechiajei* and use Chl *d* and PBPs to harvest the light energy in the microenvironment of the underside of the colony.

On the other hand, polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene amplified by using cyanobacteria-specific primers Cya359-GC and Cya781R [14] for *Cyanobacteria* was used to

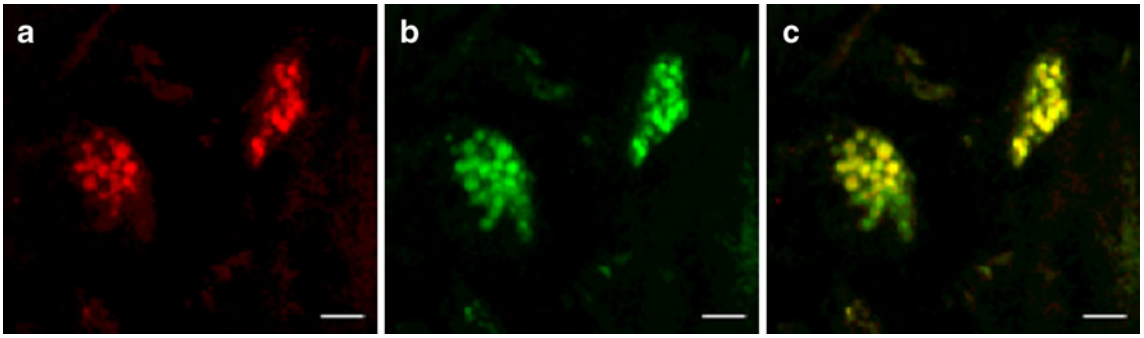

Figure 3 LSCM images of *Acaryochloris*-like cells in the colonial ascidian *C. dellechiajei*. Cells were excited with a 635-nm red laser, and fluorescence was detected between 700 and 750 nm for Chl *d* (**a**), and 640 and 670 nm for PBP (**b**). **c** Composite image from combining the Chl *d* (*red*) and PBP (*green*) channels. Each image is a maximum intensity projection of five sections collected at 0.25-μm steps. Scale bar = 6 μm

study the oxygenic phototrophic community composition attached to *C. dellechiajei* colonies. For this analysis, DNA extractions and PCR–DGGE analysis were performed from colony samples without zooids as previously described [4, 5]. Since epibionts formed a complex biofilm strongly attached to the cuticle of the ascidian (see Fig. 1), prior to DNA extractions, samples were rinsed three times in sterile seawater in order to avoid contamination by transient seawater microbes that could be loosely attached to the external surfaces. Each PCR reaction contained 2.25 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 200 μM of each dNTP, 1.75 U *Taq* I DNA polymerase (Invitrogen, California, USA), 0.4 μM of each primer, and 100 ng of DNA. PCR cycling conditions were as described Nübel and colleagues [14]. PCR product concentration and quality were determined using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Approximately 300–400 ng of PCR-amplified products from each sample was separated by DGGE using a denaturing gradient of 40%–65% (100% was defined as 7 M urea and 40% deionized formamide) as previously described [4]. DGGE gels were stained with 100 μL/L SYBR Green (Molecular Probes, Eugene, USA) in 0.5× Tris–acetic acid–EDTA buffer, for 25 min, rinsed with 1× Tris–acetic acid–EDTA buffer for 20 min, and visualized and photographed with Typhoon 9410 (Amersham Biosciences, Buckinghamshire, UK). DGGE bands were excised and incubated overnight at 4°C in 20 μL ultrapure sterile water for 16 h. The eluent was used as template DNA for reamplification with the same conditions. PCR products from each band were again analyzed by DGGE in order to check for the presence of a single band and sequenced using an ABI PRISM TM310 DNA sequencer (Applied Biosystems, Cheshire, UK). 16S rRNA gene sequences retrieved from the DGGE bands were analyzed by Mallard 1.02 and Pintail 1.1 software in order to detect chimeras and other artifacts [5]. Phylogenetic analysis was carried out with the software program ARB [15]. 16S rRNA gene sequences retrieved were aligned by using the ARB editor alignment, and TREE-PUZZLE software package implemented in ARB was used to calculate the maximum-likelihood core tree with the closest complete 16S rRNA gene sequences from the ARB SILVA database (http://www.arb-silva.de), and then the partial DGGE sequences were introduced into the tree by using the ARB parsimony tool. As shown in Fig. 4, a total of 25 DGGE bands were obtained. Remarkably, some of these DGGE bands (1, 3, 4–6, and 25) were shared by almost all specimens collected from the Western, Central, and Eastern Mediterranean between 2003 and 2008. Phylogenetic analysis by maximum likelihood showed that the shared DGGE bands 1, 3, and 4–6 were phylogenetically related to the 16S rRNA gene of chloroplasts of the diatom *Haslea* sp. and the rhodophyte algae *Chondrus crispus*, while the DGGE band 25 was related to the cyanobacterium *Planktothricoides* spp. (DGGE band 25) belonging to the filamentous Oscillatoriales group (Fig. 5). All the shared DGGE bands showed, moreover, high phylogenetic distance with their closest relatives. Since both data microscopy and molecular results confirmed the presence of abundant filamentous cyanobacteria that display same morphotype (see Fig. 1d–f), diatoms, and rhodophyte algae in all specimens analyzed, which were, moreover, collected under different macroecological conditions (time and seasons), geographical areas (some of them at 3500 km of distance), and different microenvironmental conditions (like sea grass of *Posidonia oceanica* for Central Mediterranean samples or rocky substratum for Western Mediterranean samples), it seems reasonable to conclude that there is some specificity in the epibiotic association of oxygenic phototrophs with *C. dellechiajei*. Therefore, these oxygenic phototrophs could form the "core" of the epibiotic microbial community in *C. dellechiajei* and play a significant role in the biology of this ascidian.

Conversely, the rest of sequences detected only in some specimens of *C. dellechiajei* collected in the Balearic and Alboran Seas (see Fig. 4) could correspond either to

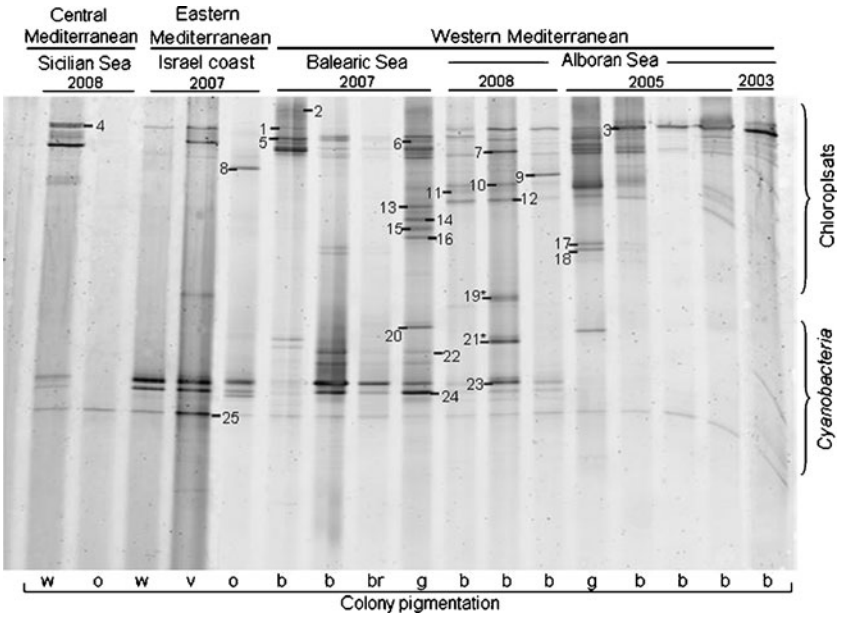

Figure 4 DGGE analysis of 16S rRNA genes from phototrophic epibiotic communities attached to different *C. dellechiajei* colonies collected from Western, Central, and Eastern Mediterranean between 2003 and 2008. Pigmentation of the colony is indicated above each lane: white (w), orange (o), violet (v), green (g), blue (b), and brown (br). Labeled DGGE bands were cut from the gel, PCR reamplified, checked again by DGGE for single band, and sequenced. DGGE bands 19 and 21 (indicated by *asterisks*) were not included in the phylogenetic analysis since they were related to *Proteobacteria*

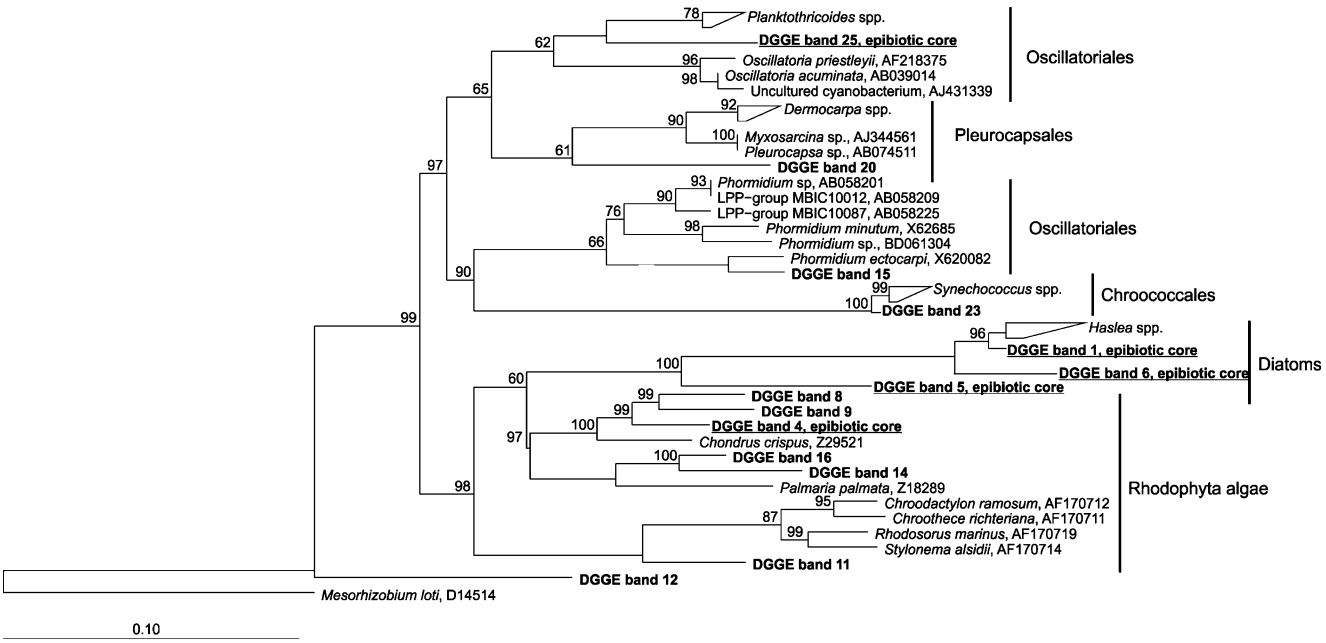

Figure 5 Phylogenetic analysis of ascidian-associated phototrophic epibiotic communities. Maximum likelihood tree based on the 16S rRNA gene sequences from DGGE analysis. The tree was calculated with the nearly complete reference sequences using TREE-PUZZLE program of the ARB package. Sequences from DGGE bands (*capital letters* and *underlined*) that displayed ≥97% of 16 S rRNA gene similarity were grouped into the same phylotype. Each one of the following sequence groups formed a phylotype: bands 1, 3, and 4; bands 7–8; bands 10 and 11; bands 12–14; bands 16–18; and bands 22–23–24. Only a sequence of each phylotype is shown in the tree (DGGE band sequences 1, 8, 11, 14, 16, and 23). Phylotypes present in almost all samples analyzed, suggesting some specificity in the association, are indicated as "epibiotic core." GenBank accession numbers for the sequences analyzed are GQ405186–GQ405198. Quartet puzzling support values for each branch are shown at the branch nodes. The archaeon, *Mesorhizobium loti*, was used as an outgroup

specific epibionts from a particular geographical area and would be better suited for these microenvironmental conditions or just transitory epibionts. Most of 16S rRNA sequences detected only in some samples were related to the 16S rRNA gene of chloroplasts of other *Rhodophyta* algae (DGGE bands 8–18), as well as to the cyanobacteria *Synechococcus* sp. (DGGE bands 22–24), *Phormidium* sp. (DGGE band 15), and *Myxosarcina* sp. (DGGE band 20).

Other studies carried out with tropical didemnid ascidians have shown that the cyanobacteria *Prochloron* spp. and *A. marina* are the main components of their oxygenic phototrophic communities [16]. In these tropical didemnids, *Prochloron* sp. and some other unicellular cyanobacteria live inside their tunic tissue forming a dense biofilm easily discernible [16], while *A. marina* thrives forming green–yellow patches on the lower surface of the ascidian. Chl *d*-containing organisms like *A. marina* can thus exploit light environments depleted of visible radiation and enhanced in near-infrared region, like the underside of tropical colonial ascidians [16]. *Acaryochloris* spp. have been detected from different benthic and planktonic environments [8, 11, 17, 18]. Recently, Kashiyama and colleagues [19] proved global distribution of Chl *d* in planktonic oceanic and lacustrine environments, showing that the viable habitat for Chl *d*-producing phototrophs extends across different environmental conditions of salinity and temperature, suggesting that other unknown taxa could be involved in the production of Chl *d* as well. Here, we have found significant differences between Mediterranean *C. dellechiajei* and tropical ascidians regarding the oxygenic phototrophic community composition and the location of these phototrophic epibionts. First, oxygenic phototrophs were only located forming a biofilm on the external surface of the tunic tissue of *C. dellechiajei,* while inner parts of the ascidian tunic were totally devoid of cyanobacteria or any other autofluorescent microorganisms. Second, 16S rRNA gene sequences phylogenetically related to *Acaryochloris* spp. or *Prochloron* spp., typically associated with tropical ascidians, were not detected here, while other cyanobacteria like those related to the filamentous *Planktothricoides* spp. were widely detected in *C. dellechiajei*. However, despite all data, the presence of both *Acaryochloris* sp. and *Prochloron* sp. cannot be ruled out in *C. dellechiajei.* Different reasons could explain the lack of detection of these groups by DGGE. First, unicellular cyanobacteria and cells displaying Chl *d* and PBP are rare in *C. dellechiajei*; second, molecular fingerprinting techniques like DGGE are not always able to detect low-abundant groups [21]; and finally, the primer set used may not be specific to detect the *Acaryochloris*-like cells found on the underside of *C. dellechiajei*. It is worth mentioning that the same primers used here for PCR–DGGE analysis were previously tested successfully for *Acaryochloris* spp. [20] and that all the GenBank complete 16S rRNA gene sequences of *Acaryochloris* spp., including the whole genome of *A. marina* strain MBIC 1107, have shown to have the target sequence for the primers used here (data not shown). Although *Acaryochloris* spp. or *Prochloron* may be present in *C. dellechiajei,* both microscopy and molecular data suggest that these microbes would be probably a fraction of the long tail of rare taxa, forming likely the "seed bank" in *C. dellechiajei,* according to Pedrós-Alió (2006) [22], while the sequences detected in all specimens would belong to the most abundant taxa, forming the core of the community [22]. Nevertheless, although tropical ascidians and *C. dellechiajei* from the Mediterranean Sea differ in their photoautotrophic community composition, both share some features, such as the presence of Chl *d*-containing cells in the underside of the colony, where a unique microenvironment is found.

Taken together, data obtained in this study widen the current knowledge on the distribution of Chl *d* within invertebrate animals from marine ecosystems and provide some insights into the epibiotic association among marine invertebrates and oxygenic phototrophs in ascidians.

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