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REPORT

# Evidence for host specificity among dominant bacterial symbionts in temperate gorgonian corals

**Marie La Rivière[1] · Joaquim Garrabou[1,2] · Marc Bally[1]**

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**Abstract** Gorgonian corals serve as key engineering species within Mediterranean rocky-shore communities that have recently suffered from repeated mortality events during warm temperature anomalies. Among the factors that may link thermal conditions with disease outbreaks, a number of bacterial pathogens have been implicated; they may take advantage of decreases in the defenses and/or overall health of the gorgonian hosts. Considering the beneficial role of the resident bacteria in tropical coral holobionts, a detailed characterization of the gorgonian-associated microbial populations is required to better understand the relationships among native microbiota, host fitness, and pathogen susceptibility. In this study, the bacterial communities associated with three sympatric gorgonian species, *Eunicella singularis*, *Eunicella cavolini*, and *Corallium rubrum*, were investigated to provide insight into the stability and the specificity of host–microbe interactions. Natural variations in bacterial communities were detected using terminal restriction fragment length polymorphism (T-RFLP) of the 16S ribosomal DNA. No major differences were identified between individual colonies sampled in winter or in summer within each gorgonian species. Although hierarchical cluster analysis of the T-RFLP profiles revealed that the three species harbor distinct communities, comparison of the T-RFLP peaks indicated the presence of common bacterial ribotypes. From phylogenetic analysis of 16S rDNA clone libraries, we identified a bacterial lineage related to the *Hahellaceae* family within the *Oceanospirillales* that is shared among *E. singularis*, *E. cavolini*, and *C. rubrum* and that dominates the communities of both species of *Eunicella*. However, distinct clades of *Hahellaceae* are harbored by various gorgonian species from Mediterranean and tropical waters, suggesting that these bacteria have formed host-specific symbiotic relationships with gorgonian octocorals. In addition, the relatedness of symbionts from host species belonging to the same taxon but occurring in geographically remote areas is consistent with codivergence between gorgonians and their associated bacteria.

**Keywords** Gorgonians · Corals · Bacteria · Hahellaceae · Host specificity · Mediterranean Sea

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✉ Marc Bally
marc.bally@univ-amu.fr

[1] Aix Marseille Université, CNRS, Université de Toulon, IRD, MIO UM 110, 13288 Marseille, France

[2] Institut de Ciències del Mar (ICM-CSIC), 08003 Barcelona, Spain

## Introduction

Gorgonian corals (Cnidaria, Octocorallia) are common members of rocky subtidal communities in the northwestern Mediterranean basin, and they play a prominent role in structuring the emblematic coralligenous assemblages (Ballesteros 2006). Over the past few years, gorgonian populations have been severely affected by repeated mortality outbreaks that coincide with summer temperature anomalies (Cerrano et al. 2000; Garrabou et al. 2009; Crisci et al. 2011). Considering their geographic extent and degree of impact, these events are comparable to the mass bleaching events that have occurred on coral reef areas in tropical waters since the 1980s (Garrabou et al. 2009). Although the causes of these outbreaks are unequivocally

associated with increased seawater temperatures, microbial factors have been implicated in the onset of gorgonian disease lesions (Martin et al. 2002; Bally and Garrabou 2007; Vezzulli et al. 2010). Notably, pathogenic *Vibrio* species that cause bleaching and coral mortality worldwide have been shown to promote necrosis in the red gorgonian *Paramuricea clavata*, suggesting that diseases in Mediterranean gorgonian populations and scleractinian tropical reef corals might be triggered by similar mechanisms under stressful conditions. In this context, a broader understanding of the microbial communities associated with gorgonians may help to determine their role in host health status and to identify the biological basis of increased susceptibility to infection.

Several studies have suggested that the physiology of healthy corals is linked to the presence of diverse assemblages of microorganisms including symbiotic bacteria, i.e., bacteria living in close and enduring association with their coral partners (Rosenberg et al. 2007; Ainsworth et al. 2010; Mouchka et al. 2010). Although their metabolic functions within the coral holobiont are not well understood, bacterial associates are thought to provide the host with protection from pathogens via interspecific competition and via the secretion of antibiotic substances (Rypien et al. 2010; Krediet et al. 2013; Frydenborg et al. 2014). There is also evidence that environmental disturbances, such as thermal stress, can alter the structure of microbial communities prior to the manifestation of disease signs (Bourne et al. 2008; Vega Thurber et al. 2009; Croquer et al. 2013). These findings suggest a pivotal role of host–microbe interactions in the overall physiological health of corals, but the mechanisms by which specific bacteria are stably maintained in the holobiont remain to be identified.

Analyses of healthy coral microbiota have indicated that coral-associated bacteria are distinct from those in the surrounding seawater and exhibit at least some features that are specific to the host. Several studies have found similar communities associated with related coral species from multiple reef sites (Rohwer et al. 2002; Webster and Bourne 2007; Morrow et al. 2012) and distinct bacterial assemblages among unrelated corals living in the same habitat (Rohwer et al. 2002; de Castro et al. 2010; Schöttner et al. 2012). However, there are an increasing number of examples demonstrating that coral-associated bacterial diversity may also be affected by various environmental parameters, such as water quality (Klaus et al. 2005), depth (Lee et al. 2012), and nutrient status (Garren et al. 2009). Therefore, analyzing the diversity of bacterial communities among corals that coexist in close proximity is required to comprehensively investigate species-specific associations.

Although there is currently no evidence that corals harbor obligate bacterial symbionts, recent studies have revealed that specific bacterial groups are common to multiple hosts and may be long-term associates. For example, a group of *Endozoicomonas*-related bacteria from the order *Oceanospirillales* is associated with many corals from diverse habitats (Hansson et al. 2009; Kvennefors et al. 2010; Morrow et al. 2012; Speck and Donachie 2012; Apprill et al. 2013; Jessen et al. 2013) and may be maintained through vertical transmission (Sharp et al. 2012). Whether these bacteria provide the host with beneficial functions is not known, but their identification in various coral lineages suggests common mechanisms for the maintenance of a coral-specific microbiota. Analysis of the phylogenetic relatedness of bacteria associated with various corals may provide further insight into the extent of specificity and may help identify ecologically important players.

We recently demonstrated that the Mediterranean gorgonian *P. clavata* is associated with a specific and dominant bacterial symbiont (La Rivière et al. 2013). In the present study, we investigated the bacterial communities of *Eunicella singularis*, *Eunicella cavolini*, and *Corallium rubrum*, three gorgonian species coexisting in the same environment and at the same location on the coast of Marseilles in the northwestern Mediterranean region. The main objective of our study was to assess whether sympatric gorgonian corals harbor specific bacterial assemblages and share common associates. Using culture-independent approaches based on 16S rDNA analyses, we examined the temporal stability and composition of bacterial communities among individuals of the three species. In addition, we analyzed the phylogeny of a group of prevailing symbionts to evaluate their host specificity across multiple gorgonian taxa. The current gorgonian phylogeny places the three genera *Paramuricea*, *Eunicella*, and *Corallium* in different octocoral families, with *Paramuricea* and *Eunicella* being more closely related to each other than they are to *Corallium* (Bayer 1981; Daly et al. 2007). We thus performed phylogenetic analyses on symbionts of the different host species to investigate the possibility of bacteria–host codivergence. This study is the first to address the potential genetic relatedness of bacterial associates in sympatric populations of octocorals.

## Materials and methods

### Sample collection and DNA extraction

Samples of the gorgonian corals *E. singularis*, *E. cavolini*, and *C. rubrum* were collected from a site on Riou Island (France) (43°10.345′N, 05°23.319′E) in summer (September 2008) and in winter (March 2009; Fig. 1). Seawater temperatures were measured hourly using in situ Stowaway

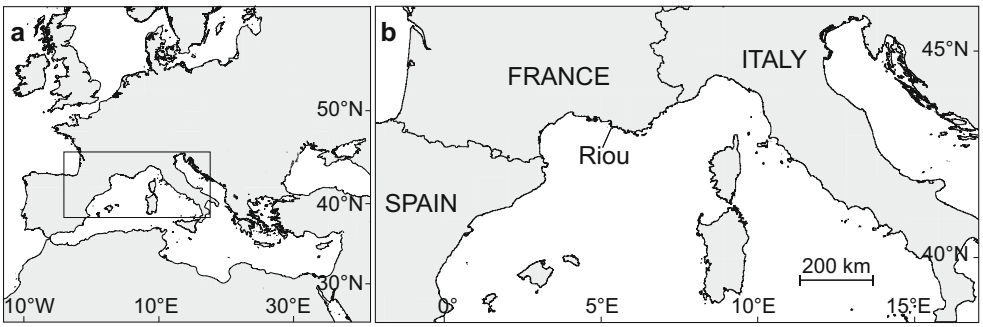


**Fig. 1** Sampling site. Map of the Mediterranean Sea (**a**) with an enlargement of the northwestern Mediterranean basin (**b**) showing the location of the Riou study site on the south coast of France

Tidbits autonomous sensors (precision 0.2 °C, resolution 0.15 °C) positioned at the sampling site in Riou Island (for further information, see http://www.t-mednet.org). From these recordings, the average temperatures at 20-m depth were 17.4 ± 2.2 °C in summer 2008 and 13.8 ± 0.5 °C in winter 2009. The *C. rubrum* population was found on an overhang at a depth of approximately 20 m near a dense population of *P. clavata* living on a vertical wall. This latter population was mixed with a population of *E. cavolini*. The *E. singularis* population was found at the bottom of the wall at a depth of approximately 35 m. During each sampling, apical branch fragments (2-cm length) of randomly chosen, apparently healthy colonies (i.e., with no visible signs of necrosis; $n = 3$) were collected using shears and placed in plastic bags underwater. The collected samples were then transferred to the laboratory within 2 h.

The gorgonian samples were processed according to the protocol described by La Rivière et al. (2013) for the study of *P. clavata*-associated bacterial communities. Samples were rinsed three times with sterile 0.22-µm-filtered seawater. Tissues were then detached from the central axis, homogenized in 3 ml of sterile seawater using a scalpel blade, and aliquoted into three microtubes. Tissue slurries were pelleted by quick centrifugation and stored at −80 °C before subsequent DNA extraction.

The bacterial DNA in the gorgonian tissue samples was extracted following a protocol adapted from Bourne et al. (2008). Briefly, tissue slurries were incubated for 5 min at room temperature in 0.5 ml of extraction buffer (50 mM Tris–HCl pH 8.0, 40 mM EDTA, and 0.75 M sucrose) containing 1 µg of salmon sperm DNA. The samples were then incubated for 1 h at 37 °C with slow agitation in lysozyme solution (75 µl of 100 mg $ml^{-1}$ per sample). After three freeze–thaw cycles, 100 µl of 25 % (w/v) sodium dodecyl sulfate (SDS) was added, and the samples were incubated for 10 min at 70 °C. The samples were digested with proteinase K (20 µl of 20 mg $ml^{-1}$ per sample) for 1 h at 37 °C with slow agitation and again subjected to three freeze–thaw cycles. Tissue lysates were then subjected to a standard phenol–chloroform DNA extraction procedure followed by the addition of an equal volume of isopropanol and 50 µl of 3 M sodium acetate to precipitate the DNA. The extracted total DNA was pelleted by centrifugation (16,000 × *g* for 30 min at 4 °C), washed with cold 70 % ethanol, and suspended in 30 µl of sterile ultra-pure water. The DNA concentration was estimated by spectrophotometry using a 6131 BioPhotometer (Eppendorf, Hamburg, Germany), and the DNA samples were stored at −20 °C until further processing.

## Terminal restriction fragment length polymorphism (T-RFLP) analysis

### *PCR amplification, enzymatic digestion, and T-RFLP*

The universal primers 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′; Marchesi et al. 1998) labeled at the 5′ end with 6-FAM (phosphoramidite fluorochrome 6-carboxyfluorescein; Applied Biosystems, Carlsbad, CA, USA) and 1389R (5′-ACG GGC GGT GTG TAC AAG-3′; Osborn et al. 2000) were used to amplify the bacterial 16S rDNA genes. PCR mixtures contained 1 X Taq buffer with $(NH_4)_2SO_4$ (Fermentas, Burlington, Canada), 200 µM each dNTP, 1 µM each of the forward and reverse primers, 2 mM $MgCl_2$, 0.1 µg $µl^{-1}$ bovine serum albumin (BSA), 0.3-3 µl (approximately 100-300 ng) bacterial DNA template, 1 U native Taq DNA polymerase (Fermentas), and reaction volumes made up to 25 µl with sterile ultra-pure water. PCRs were performed with an initial 5-min denaturation step at 94 °C followed by 35 amplification cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min). The labeled PCR products were purified using the Wizard® PCR Clean-Up System (Promega) following the manufacturer's instructions and eluted in 30 µl of DNase-free distilled water.

Enzymatic digestions were performed for 16 h at 37 °C using a 20-µl reaction mixture containing 6 µl of the

purified PCR products, 1 X reaction buffer (Promega), 0.1 μg $\mu l^{-1}$ BSA, and 20 U of *Cfo*I or *Msp*I (Promega). Each digested sample (3 μl) was mixed with 0.3 μl of the GeneScan 600-LIZ® (Applied Biosystems) size standard and 10 μl of Hi-Di® formamide (Applied Biosystems) and then denatured at 90 °C for 3 min before separation of the terminal restriction fragments (TRFs) on an ABI 3130 Genetic Analyzer (Applied Biosystems) with the default fragment analysis parameters. Each sample was run twice to ensure the reproducibility of electrophoresis. The T-RFLP electropherograms were tabulated in GeneMapper® version 4.0 software (Applied Biosystems) using the Local Southern method as the size-calling algorithm (Southern 1979). Only peaks representing TRFs longer than 80 bp and smaller than 600 bp were considered.

### *Statistical analysis of the temporal variability*

Raw data sets were processed for normalization and statistical analysis as described by La Rivière et al. (2013). The average area and size of each TRF peak were calculated from the repeated runs for each sample (TRFs with sizes within 0.5 bp of each other were considered identical, while TRFs that were present in only one of the replicates were removed). The T-RFLP data were then normalized by applying a variable-percentage threshold to eliminate background fluorescence (Osborne et al. 2006). To account for variability of peak size calling in electrophoretic profiles from samples run at different times, peak binning was carried out using binning algorithms (Ramette 2009) implemented in the R programming language (scripts available online at http://www.mpi-bremen.de/en/Software_4.html#Section28343). The automatic binning script was used to determine the optimal window size (WS) and shift (Sh) values. The best binning frame was subsequently identified by using the chosen values (threshold = 3 %; WS = 1; Sh = 0.1) in computation carried out with the interactive binning script. Relationships among normalized T-RFLP profiles were assessed using cluster analysis based on the Bray–Curtis dissimilarity index (sensitive to abundant species) with the single-linkage method (R package vegan; Oksanen et al. 2012). Clustering was displayed on a heatmap representing the relative abundance of each TRF using the R package pheatmap (Kolde 2012).

## Bacterial clone libraries

The construction of a bacterial clone library for each gorgonian species sampled in summer was performed according to the protocol described by La Rivière et al. (2013). Amplification of bacterial 16S rDNA was performed using PCR of extracted DNA samples with the universal primers 63F and 1389R (Osborn et al. 2000). PCR amplifications were performed in a total volume of 25 μl containing 1 X Taq® Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM each primer, 1 U of GoTaq® Flexi DNA polymerase (Promega), and 0.3–3 μl (approximately 100–300 ng) of DNA template. After an initial denaturation step (5 min at 94 °C), 35 cycles of amplification (94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min) were performed, followed by a final elongation step at 72 °C for 10 min. For each library, the DNA extracted from the three sampled colonies of each gorgonian species was PCR-amplified in triplicate. For each species, the PCRs ($n = 9$) were pooled and the amplified products were purified with Wizard® PCR Clean-Up minicolumns (Promega) before being cloned into the pGEM®-T Easy vector (Promega) according to the manufacturer's instructions. After transformation into *E. coli* JM109 competent cells, PCR re-amplification using 63F and M13 reverse primers was used to determine the orientation of the inserted 16S rDNA in each clone. Clones producing a PCR product with the expected size (approximately 1500 bp) were sequenced with a plasmid forward primer (LGC Genomics GmbH, Berlin, Germany).

## Sequencing and phylogenetic analysis

Sequence data were checked for chimeras using Pintail software (Ashelford et al. 2005), and the sequences were trimmed to 750 bp in length to retain only high-quality bases. The cloned 16S rDNA sequences of each library were assigned to the lowest possible taxonomic rank using the Classifier and SeqMatch tools of the Ribosomal Database Project Web site (http://rdp.cme.msu.edu). Sequences were grouped into operational taxonomic units (OTUs), and the Shannon–Weaver diversity indices of the libraries were determined using the FastGroupII algorithm (Yu et al. 2006) with a 97 % similarity threshold value, which is generally accepted for discriminating bacterial species (Stakebrandt and Goebel 1994). To obtain the nearest relatives of each OTU, representative sequences selected by FastGroupII were analyzed by comparison with the GenBank database using the BLAST algorithm (Altschul et al. 1997; http://blast.ncbi.nlm.nih.gov/Blast.cgi). For phylogenetic analysis, sequences were aligned using ClustalW2 (Larkin et al. 2007), which is available at the EBI Web site (http://www.ebi.ac.uk), with the default parameters. Maximum-likelihood phylogenetic trees were constructed using PhyML 3.0 (Guindon et al. 2010), which is available on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr), with a GTR substitution model. The consistency of the tree topology was evaluated using the approximate likelihood-ratio test (aLRT; Anisimova and Gascuel 2006).

The 16S rDNA sequences generated in this study were deposited in GenBank under the accession numbers KP008373-KP008780.

## Results

### Interspecific and seasonal comparison of bacterial communities

The bacterial community patterns of three gorgonian species, *E. singularis*, *E. cavolini*, and *C. rubrum*, were investigated using T-RFLP analysis. The profiles produced from a total of 18 individual colonies sampled during summer and winter revealed 19 and 24 unique TRFs after digestion with *Cfo*I and *Msp*I, respectively. Clustered heat maps of TRF abundance were constructed to analyze the similarities among the bacterial communities (Fig. 2). Hierarchical clustering of the *Cfo*I and *Msp*I profiles clearly indicated that the communities associated with *C. rubrum* were grouped into a cluster distinct from that of the two *Eunicella* species. Within the *Eunicella* cluster, all but one of the *E. singularis* samples clustered together in both the *Cfo*I and the *Msp*I analysis. The remaining *E. singularis* samples (Es-S1 and Es-S2 in the *Cfo*I and *Msp*I restriction analyses, respectively) grouped with the *E. cavolini* profiles. Overall, no seasonal pattern could be observed in the composition of associated bacteria. Although the bacterial communities sampled from *E. cavolini* during winter and summer clustered separately after *Msp*I digestion, this seasonal clustering was not supported upon *Cfo*I digestion.

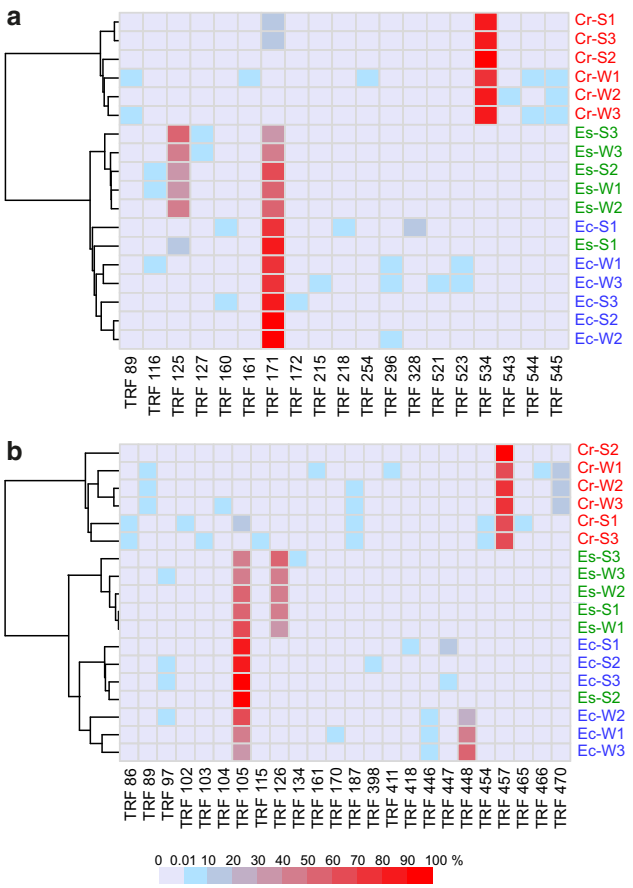


**Fig. 2** Bacterial community similarities among gorgonian samples analyzed using T-RFLP. The cluster analysis and heatmap are based on the relative fluorescence of the TRFs generated by *Cfo*I (**a**) and *Msp*I (**b**) digestion of PCR amplicons from three replicate samples of each gorgonian species collected in summer (S) and winter (W). The analyzed colonies of *Eunicella singularis* (Es), *Eunicella cavolini* (Ec), and *Corallium rubrum* (Cr) are indicated in *green*, *blue*, and *red text*, respectively. The TRFs are designated by their size (in bp) after binning

We found that none of the TRFs was shared by all of the samples in the *Cfo*I or *Msp*I profiles. Only one TRF (TRF-171) was common to all the profiles of the *Eunicella* spp. samples digested with *Cfo*I, and this TRF dominated 11 of the 12 corresponding bacterial communities (Fig. 2a). The same trend was observed upon *Msp*I digestion, with a single TRF (TRF-105) detected in all the *Eunicella* spp. samples and dominant in ten of them (Fig. 2b). Only these two TRFs were shared between the *Eunicella* spp. and *C. rubrum* profile clusters. Specifically, the *Cfo*I TRF-171 was found in two *C. rubrum* summer samples (Cr-S1 and Cr-S3), and the *Msp*I TRF-105 was also detected in one of these samples (Cr-S1). Two other notable TRFs were observed in the profiles from the *E. singularis* communities: the *Cfo*I TRF-125, which was common to all the sampled colonies (Fig. 2a), and the *Msp*I TRF-126, which was found in all but one of the profiles (Es-S2; Fig. 2b). The bacterial profiles from *C. rubrum* were dominated by two TRFs (*Cfo*I TRF-534 and *Msp*I TRF-457) that were not detected in the *Eunicella* samples.

### Diversity and taxonomic affiliation of gorgonian-associated bacteria

Because the dominant TRFs common to *E. singularis* and *E. cavolini* were also detected in *C. rubrum* samples collected during the summer but not the winter, 16S rDNA clone libraries were constructed using the DNA extracted from the colonies sampled during the summer season to identify the corresponding bacterial associates of the three gorgonian species. A total of 408 sequences were obtained from the libraries (*E. singularis*, 168 clones; *E. cavolini*, 126 clones; *C. rubrum*, 114 clones) and then assembled into 25 OTUs of 97 % similarity (Table 1). Notably, 18 of the 25 OTUs were represented by three or fewer sequences, and the communities exhibited markedly low Shannon–Weaver diversity index values (*E. singularis*, 0.34; *E. cavolini*, 0.71; *C. rubrum*, 1.33).

**Table 1** Taxonomic affiliations and relative abundances of bacterial groups from gorgonian clone libraries ($n$ = number of clones sequenced)

| OTU no. | Number of clones | | | Affiliation | | Closest relative | | |
|---|---|---|---|---|---|---|---|---|
| | *Eunicella singularis* | *Eunicella cavolini* | *Corallium rubrum* | Class | Genus or family | Accession no. | % Identity | Source |
| 1 | 0 | 0 | 4 | Unknown Bacteria | Unknown | DQ395417 | 99 | Deep-sea octocoral |
| 2 | 0 | 0 | 2 | Unknown Bacteria | Unknown | HM173261 | 95 | Octocoral |
| 3 | 2 | 0 | 0 | *Alphaproteobacteria* | *Rhodobacteraceae* sp. | FJ202909 | 93 | Scleractinian coral |
| 4 | 2 | 0 | 0 | *Alphaproteobacteria* | * | JQ287355 | 99 | Marine sediment |
| 5 | 2 | 0 | 0 | *Alphaproteobacteria* | * | DQ395495 | 99 | Deep-sea octocoral |
| 6 | 0 | 0 | 2 | *Alphaproteobacteria* | * | DQ395662 | 98 | Deep-sea octocoral |
| 7 | 1 | 0 | 0 | *Alphaproteobacteria* | *Kordiimonas* sp. | HE663284 | 93 | Mollusk |
| 8 | 0 | 1 | 0 | *Alphaproteobacteria* | *Erythrobacteraceae* sp. | JF514248 | 99 | Seawater |
| 9 | 0 | 1 | 0 | *Alphaproteobacteria* | *Rhizobiales* sp. | KF180028 | 99 | Scleractinian coral |
| 10 | 0 | 1 | 0 | *Alphaproteobacteria* | *Ruegeria* sp. | FJ695550 | 99 | Scleractinian coral |
| 11 | 0 | 1 | 0 | *Alphaproteobacteria* | * | FJ425643 | 90 | Scleractinian coral |
| 12 | 0 | 0 | 1 | *Alphaproteobacteria* | * | GU799621 | 84 | Sponge |
| 13 | 161 | 105 | 0 | *Gammaproteobacteria* | *Hahellaceae* sp. 1 | JQ691574 | >99 | Octocoral |
| 14 | 0 | 0 | 79 | *Gammaproteobacteria* | * | DQ917857 | 92 | Octocoral |
| 15 | 0 | 13 | 0 | *Gammaproteobacteria* | * | JX874599 | 96 | Octocoral |
| 16 | 0 | 0 | 7 | *Gammaproteobacteria* | *Hahellaceae* sp. 2 | JX280191 | 97 | Sponge |
| 17 | 0 | 0 | 5 | *Gammaproteobacteria* | *Neptunomonas* sp. | FJ403078 | 99 | Scleractinian coral |
| 18 | 0 | 0 | 4 | *Gammaproteobacteria* | * | AM503922 | 98 | Mollusk |
| 19 | 0 | 3 | 0 | *Gammaproteobacteria* | * | JQ691580 | 99 | Octocoral |
| 20 | 0 | 0 | 3 | *Gammaproteobacteria* | * | KF741496 | 94 | Marine sediment |
| 21 | 0 | 0 | 2 | *Gammaproteobacteria* | *Hahellaceae* sp. 3 | JX280191 | 98 | Sponge |
| 22 | 0 | 0 | 2 | *Gammaproteobacteria* | *Shewanella* sp. | FM878646 | 99 | Fish |
| 23 | 0 | 0 | 2 | *Gammaproteobacteria* | *Vibrio* sp. | KF577063 | 99 | Scleractinian coral |
| 24 | 0 | 1 | 0 | *Gammaproteobacteria* | * | AM259847 | 99 | Sponge |
| 25 | 0 | 0 | 1 | *Gammaproteobacteria* | *Hahellaceae* sp. 4 | DQ917877 | 96 | Octocoral |
| n | 168 | 126 | 114 | | | | | |

The distribution of the OTUs among the clone libraries from *Eunicella singularis*, *Eunicella cavolini*, and *Corallium rubrum* is indicated. The taxonomic affiliation of 16S rDNA sequences at the family or genus level, the most closely related hit in GenBank, and the corresponding sample source are shown for each identified OTU

* OTUs that cannot be affiliated below class level

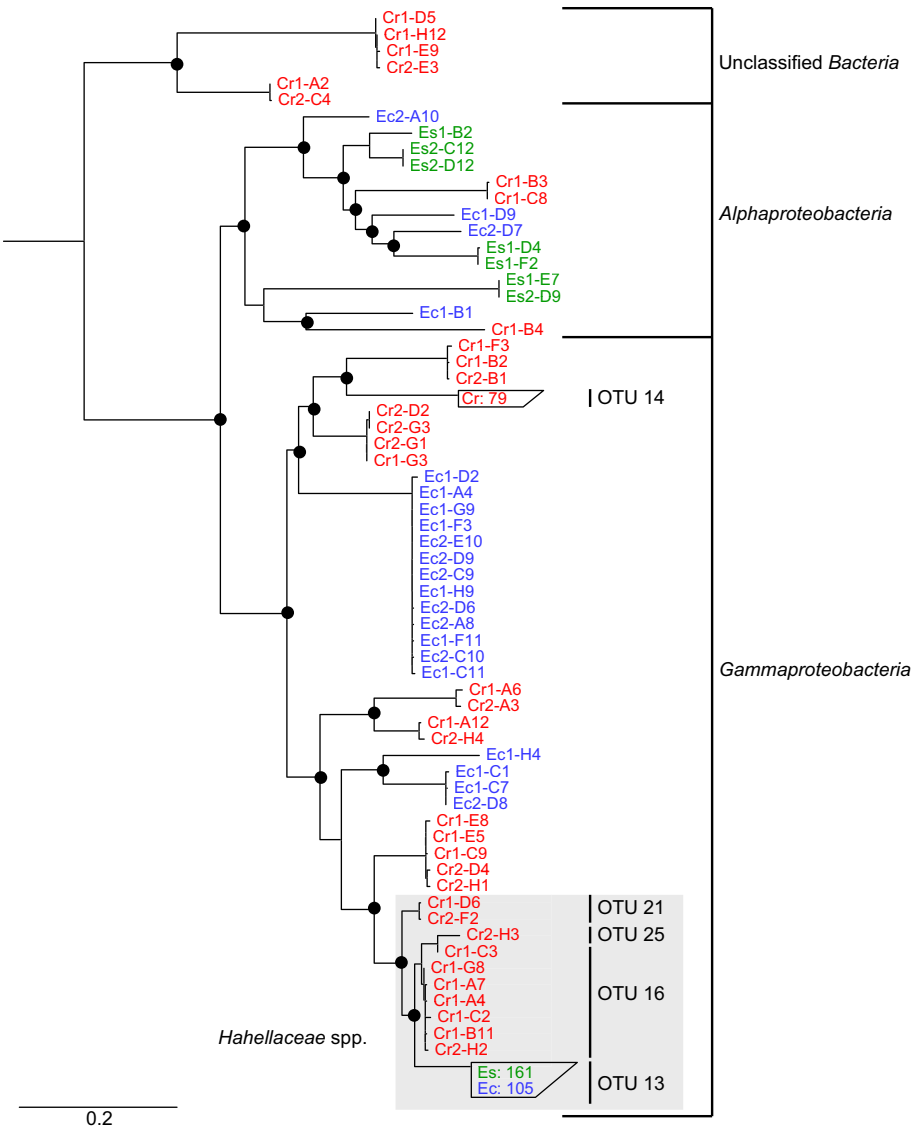


**Fig. 3** Phylogenetic analysis of 16S rDNA sequences from gorgonian-associated bacteria. The sequences recovered from clone libraries of *Eunicella singularis*, *Eunicella cavolini*, and *Corallium rubrum* are indicated in *green*, *blue*, and *red text*, respectively. *Light-gray-shadowed* phylotypes indicate *Hahellaceae*-affiliated sequences in the gorgonian libraries. For the dominant OTUs (OTU 13 and OTU 14), the number of sequences from the *Eunicella singularis* (Es), *Eunicella cavolini* (Ec), and *Corallium rubrum* (Cr) libraries is shown. The archeon *Sulfolobus acidocaldarius* U05018 was used as an outgroup, and branch points with support values >75 % are indicated by *black circles*. The *scale bar* represents 0.2 changes per nucleotide

At the class level, the libraries were dominated by *Gammaproteobacteria*, which represented 95.8, 96.8, and 92.1 % of the retrieved sequences from *E. singularis*, *E. cavolini*, and *C. rubrum*, respectively (Table 1). The remaining sequences were affiliated with *Alphaproteobacteria* and constituted 2.6–4.2 % of each library, while a small proportion (5.3 %) of the *C. rubrum* bacterial sequences could not be assigned to any known phylum. Within the *Alphaproteobacteria*, members of an order (*Rhizobiales*) and of multiple families (*Rhodobacteraceae*, *Erythrobacteraceae*) and genera (*Ruegeria*, *Kordiimonas*) were identified, but none of these bacterial groups were shared among the studied gorgonian species. In contrast, we found that most of the *Gammaproteobacteria* sequences (86–100 %) in the *E. cavolini* and *E. singularis* libraries were affiliated with the *Hahellaceae* family within the order *Oceanospirillales*. In *C. rubrum*, a small proportion (9.5 %) of *Hahellaceae* sequences was also identified among the *Gammaproteobacteria*, while the majority (81.9 %) of the sequences within this class could not be affiliated to a lower taxonomic level (Table 1). Representative sequences of *Vibrio*, *Shewanella*, and *Neptunomonas* spp. were also found in the *C. rubrum* library and accounted for 1.9–4.8 % of the *Gammaproteobacteria*. Notably, most of the bacterial sequences from the three gorgonians were related to marine invertebrate-associated bacteria.

To further characterize the diversity of gorgonian-associated bacteria, a phylogenetic analysis was performed on the pooled set of sequences from the three libraries ($n = 408$). As expected from the bacterial lineages identified above, the main clusters corresponded to *Alphaproteobacteria* and *Gammaproteobacteria* (Fig. 3). Within *Gammaproteobacteria*, the *Hahellaceae* ribotypes from

*E. singularis* and *E. cavolini* grouped tightly into a single cluster of 266 sequences corresponding to OTU 13. Pairwise comparisons revealed low levels of divergence (<1.9 %) among the 16S rDNA sequences in this cluster. According to BLAST analysis, the closest relatives were uncultured *Hahellaceae* bacteria previously retrieved from a distinct population of *E. cavolini* (>98.5–100 % identity; Bayer et al. 2013a), from colonies of the red gorgonian *P. clavata* (>94.3 % identity; La Rivière et al. 2013) and from the Caribbean gorgonian *Gorgonia ventalina* (>96.5 % identity; Sunagawa et al. 2010). The *Hahellaceae* ribotypes from *C. rubrum* were less tightly grouped, in accordance with a lower identity level (>94.4 %) within this group, which encompassed three distinct OTUs (OTU 16, OTU 21, and OTU 25). Most of the *C. rubrum*-associated *Gammaproteobacteria* formed a single cluster of 79 highly similar sequences that shared >99.1 % identity (OTU 14).

In silico *Cfo*I and *Msp*I digestions of the 266 *Hahellaceae*-related sequences from *E. singularis* and *E. cavolini* predicted two unique TRFs that matched the sizes of the dominant peaks observed in the T-RFLP profiles obtained for both species (*Cfo*I TRF-171 and *Msp*I TRF-105; Fig. 2). Notably, these peak sizes correspond to the TRFs generated from the dominant *Hahellaceae* ribotype previously identified in *P. clavata* (La Rivière et al. 2013). A total of eight *Hahellaceae*-affiliated sequences from the *C. rubrum* library were also predicted to generate the same TRFs, in agreement with detection of the corresponding *Cfo*I and *Msp*I peaks in the summer profiles (Fig. 2). The predicted sizes of the TRFs for the major *Gammaproteobacteria* ribotype found with *C. rubrum* matched the dominant *Cfo*I TRF-534 and *Msp*I TRF-457 observed in the profiles from this gorgonian. However, the *Cfo*I TRF-125 and *Msp*I TRF-126 found in most of the *E. singularis* profiles could not be attributed to any cloned sequence, suggesting that inefficient cloning of the corresponding 16S rDNA prevented its recovery in the library.

### Phylogenetic comparison of gorgonian-associated *Hahellaceae*

To better understand the relationship between the diversity of *Hahellaceae*-related bacteria and the lineages of their gorgonian hosts, we compared the phylogenetic positioning of 16S rDNA sequences isolated from *E. singularis*, *E. cavolini*, and *C. rubrum* (in the present work), to that from *P. clavata* (La Rivière et al. 2013) and *G. ventalina* (Sunagawa et al. 2010). To the best of our knowledge, sequences from *G. ventalina* constitute the only available data set of full-length 16S rDNA sequences from a healthy tropical gorgonian. Among the 211 bacterial sequences from *G. ventalina* deposited in GenBank by Sunagawa et al. (2010), we used BLAST searches to identify a subset ($n = 71$) of 16S rDNA sequences from *Hahellaceae*-related bacteria, which we then included in the phylogenetic analysis (Fig. 4).

Three well-supported, monophyletic clusters of *Hahellaceae* sequences were recovered: a large cluster that included all of the sequences from the congeneric species *E. singularis* and *E. cavolini* and two other clusters corresponding to sequences from *P. clavata* and *G. ventalina*. The *Hahellaceae* sequences recovered from *C. rubrum* grouped into a distinct cluster that included *Endozoicomonas* and *Spongiobacter* bacterial type strains isolated from various marine invertebrates. Overall, the phylogenetic relationships of the *Hahellaceae*-affiliated sequences from the five gorgonians correspond to the current systematic classification of their respective hosts (Bayer 1981; Daly et al. 2007). *C. rubrum* belongs to the Corallidae family within the Scleraxonia suborder of Alcyonaceae, while the four other species are classified in the suborder Holaxonia. Within this lineage, *E. singularis*, *E. cavolini*, and *G. ventalina* belong to the Gorgoniidae family, while *P. clavata* is a member of the Plexauridae family.

## Discussion

This study provides the first comparison among the bacterial communities associated with three sympatric gorgonian species in the Mediterranean Sea. Analysis of the T-RFLP fingerprint data demonstrated that the dominant bacteria associated with *E. singularis*, *E. cavolini*, and *C. rubrum* are conserved between individual colonies and are maintained throughout the year in each gorgonian species. In addition, a comparison of community profiles revealed the presence of TRF peaks common to all three species, indicating that gorgonians share certain specific groups of bacterial associates.

The 16S rDNA clone libraries constructed from tissue samples revealed a high relative abundance of *Gammaproteobacteria* sequences, which represented >92 % of the libraries. This abundance is consistent with the observed predominance of *Gammaproteobacteria* in the microbial communities of a variety of hard and soft corals (Rohwer et al. 2002; Bourne and Munn 2005; Webster and Bourne 2007; Lee et al. 2012). A small proportion of *Alphaproteobacteria* sequences were also retrieved from the libraries.

Bacterial assemblages associated with *E. singularis* and *E. cavolini* were dominated by sequences affiliated with the *Hahellaceae* family within the *Oceanospirillales* order of *Gammaproteobacteria*. Sequences belonging to *Hahellaceae* were also found in the *C. rubrum* library. The

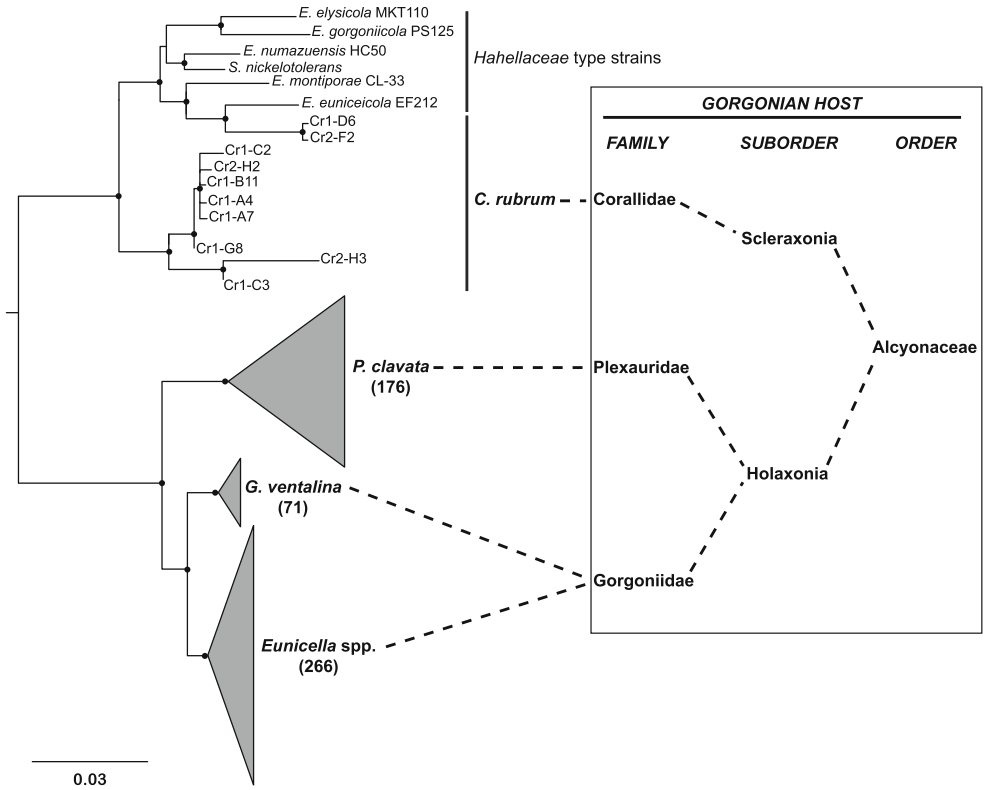


**Fig. 4** Phylogeny of *Hahellaceae*-affiliated bacteria recovered from various gorgonian host species. The maximum-likelihood tree is based on the 16S rDNA sequences, filtered to approximately 750 aligned nucleotides. The phylogenetic positions of the closest cultured relatives (type strains) within the *Hahellaceae* bacterial family are shown (*Endozoicomonas elysicola* MKT110 (AB196667), *Endozoicomonas euniceicola* EF212 (JX488684), *Endozoicomonas gorgoniicola* PS125 (JX488685), *Endozoicomonas montiporae* CL-33 (FJ347758), *Endozoicomonas numazuensis* HC50 (AB695088), and *Spongiobacter nickelotolerans* (AB205011)). The total number of sequences in the condensed clades (*gray triangles*) is indicated in *parentheses*. The current classification of host taxa in which *Hahellaceae* are found is summarized in the right frame. Branch points with support values >75 % are indicated by *black circles*, and the *scale bar* represents 0.03 changes per nucleotide. The gammaproteobacterium *Pseudomonas aeruginosa* ATCC 10145 was used as an outgroup

closest relatives of the *Hahellaceae* ribotypes from the *Eunicella* spp. were found in the microbiota of two other gorgonians, the sympatric species *P. clavata* (La Rivière et al. 2013), and the Caribbean species *G. ventalina* (Sunagawa et al. 2010). Moreover, the *Hahellaceae* ribotypes from *E. cavolini* are virtually indistinguishable from those associated with other samples of this gorgonian species taken more than 180 km from our study site (Bayer et al. 2013a), supporting the existence of a consistent and specific bacteria–host interaction. All together, the *Hahellaceae*-related bacteria associated with the five gorgonian species shared >95 % 16S rDNA sequence identity (except for a few *C. rubrum*-associated sequences with a slightly lower similarity level), which exceeds the cutoff value suggested for bacterial genus definition (Ludwig et al. 1998). The most closely related cultivated bacterial strains were *Endozoicomonas* and *Spongiobacter* spp., with >92 % identity to this group; this similarity level is significant but insufficient to tentatively classify the gorgonian associates within one of these genera. Thus, the *Hahellaceae* ribotypes revealed in this study may be considered as members of a unique yet undescribed bacterial genus.

Over the past few years, members of *Hahellaceae* have been detected in many coral species worldwide, and they have been described as a major component of the associated bacterial communities of several corals (Hansson et al. 2009; Kvennefors et al. 2010; Morrow et al. 2012; Speck and Donachie 2012; Bayer et al. 2013b). However, the relationship between the genetic diversity of these bacteria and their host specificity across multiple coral lineages has not yet been investigated. Our current phylogenetic analysis of the *Hahellaceae*-related sequences retrieved from four Mediterranean species (*E. singularis*, *E. cavolini*, *C. rubrum*, and *P. clavata*) and from the tropical gorgonian *G. ventalina* revealed that they form distinct monophyletic clusters in relation to their respective host; the sequences from a particular gorgonian genus clearly clustered together. The two congeneric species *E. cavolini* and

*E. singularis* harbored *Hahellaceae* from the same cluster, suggesting that common selection factors might promote or maintain conserved associations at the host genus level. Unexpectedly, the *Eunicella*-associated sequences were more closely related to sequences isolated from *G. ventalina* than to *Hahellaceae* sequences recovered from the sympatric species *P. clavata* and *C. rubrum*. This finding further strengthens the idea that host-dependent mechanisms act to select specific components of the microbiota regardless of the geographic location or vicinity of gorgonians (La Rivière et al. 2013). It is therefore conceivable that the distinct phylotypes of *Hahellaceae* identified here may represent specialized, host-adapted symbiont clades. Moreover, the *Hahellaceae* ribosomal sequences isolated from *E. singularis*, *E. cavolini*, *P. clavata*, and *G. ventalina* formed monophyletic groups that apparently partitioned according to the systematic classification of their hosts (Bayer 1981; Daly et al. 2007). In other words, the bacterial phylotypes from closely related gorgonian taxa at the family and suborder levels tended to cluster together. However, the *Hahellaceae* sequences recovered from *C. rubrum* appear to cluster with *Hahellaceae* strains associated with various hosts belonging to different phyla of marine invertebrates including sponges, corals, and mollusks. This suggests the existence of ecologically distinct *Hahellaceae* lineages that may differ in their level of specificity and their phylogenetic partitioning among host species.

Taken as a whole, these observations may be consistent with the possibility of coevolution over a long-term symbiotic association between some *Hahellaceae* clades and gorgonians. However, molecular phylogenetic analyses have not yet provided a clear reconstruction of the evolutionary relationships within the Octocorallia and have lacked resolution at the subordinal level (Sanchez et al. 2003; McFadden et al. 2006). Thus, a phylogeny-based revision of the octocoral taxonomy is required to increase our understanding of the relationship between host classification and bacterial phylotype clustering observed in this study.

Various scenarios related to the mode of symbiont transmission may explain the partitioning of *Hahellaceae* phylotypes among their gorgonian hosts. First, the vertical transmission of bacterial communities to offspring can generate species-specific associations and codiversification of symbiosis partners (Moran et al. 2008). Vertically transmitted symbionts have been identified in various associations between bacteria and marine invertebrates, including sponges, bryozoans, and bivalves, and several studies have indicated that specific bacterial symbionts may have coevolved with their hosts (Erpenbeck et al. 2002; Taylor et al. 2004; Roeselers and Newton 2012). There is evidence for vertical transmission to offspring in the brooding tropical coral *Porites astreoides* (Sharp et al. 2012). In contrast, studies of bacterial transmission in spawning corals have suggested that several species acquire their microbiota by horizontal uptake from the surrounding seawater (Apprill et al. 2009; Sharp et al. 2010), demonstrating that various modes of transmission exist in cnidarians. Future studies of the onset of bacterial associations in the brooding species *E. singularis*, *E. cavolini*, and *C. rubrum* (Vighi 1972; Weinberg and Weinberg 1979) and in the spawning species *P. clavata* (Coma et al. 1995) will bolster our understanding of the potential inheritance of *Hahellaceae*. Interestingly, a recent report highlighted that both spawning and brooding coral species release bacteria into the water column that might then be taken up by their larvae (Ceh et al. 2013). Thus, the intergenerational transfer of stable bacterial associates may rely on mechanisms other than vertical transmission in coral holobionts.

Considering the possibility that gorgonians may reacquire their *Hahellaceae* associates from the environment at each generation, there must be robust selection mechanisms in place to drive this level of host specificity. Assuming that *Hahellaceae* recruitment might occur through a specific mode of partner recognition and/or niche selection, subtle differences in physiological function among gorgonian host species could result in colonization by distinct bacterial lineages. Thus, the *Hahellaceae* clades identified in our study may represent bacterial ecotypes that have specifically adapted to their respective host species. Notably, this hypothesis of highly specific symbiont recruitment is compatible with the recovery of the original *P. clavata*-associated clade observed after a unique compositional shift of bacterial communities in the summer of 2007 (La Rivière et al. 2013).

Taken together, our data suggest that the present pattern of bacteria–host specificity does not result from geographic isolation of the gorgonian lineages but rather from long-term partnership and potential coadaptation between host and symbiont. Additional studies including gorgonian populations from different geographic areas will be needed to confirm the generality of this association pattern. The characterization of the functional profiles of *Hahellaceae* associates should also help to unravel the genetic basis of bacterial divergence between host species. Identifying the mechanisms that determine the gorgonian microbiota and maintain the host-specific *Hahellaceae* population may be important for investigating the microbial factors that underlie overall holobiont fitness and the transition to unhealthy states under stressful environmental conditions.

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