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RESEARCH ARTICLE

The gorgonian coral *Eunicella labiata* hosts a distinct prokaryotic consortium amenable to cultivation

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One sentence summary: In contrast to current understanding, the phylogenetically distinct prokaryotic community inhabiting the gorgonian *Eunicella labiata* is highly amenable to cultivation, opening new avenues to study coral-microbe interactions.

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ABSTRACT

Microbial communities inhabiting gorgonian corals are believed to benefit their hosts through nutrient provision and chemical defence; yet much remains to be learned about their phylogenetic uniqueness and cultivability. Here, we determined the prokaryotic community structure and distinctiveness in the gorgonian *Eunicella labiata* by Illumina sequencing of 16S rRNA genes from gorgonian and seawater metagenomic DNA. Furthermore, we used a 'plate-wash' methodology to compare the phylogenetic diversity of the 'total' gorgonian bacteriome and its 'cultivatable' fraction. With 1016 operational taxonomic units (OTUs), prokaryotic richness was higher in seawater than in *E. labiata* where 603 OTUs were detected, 68 of which were host-specific. *Oceanospirillales* and *Rhodobacterales* predominated in the *E. labiata* communities. One *Oceanospirillales* OTU, classified as *Endozoicomonas*, was particularly dominant, and closest relatives comprised exclusively uncultured clones from other gorgonians. We cultivated a remarkable 62% of the bacterial symbionts inhabiting *E. labiata*: *Ruegeria*, *Sphingorhabdus*, *Labrenzia*, other unclassified *Rhodobacteraceae*, *Vibrio* and *Shewanella* ranked among the 10 most abundant genera in both the cultivation-independent and dependent samples. In conclusion, the *E. labiata* microbiome is diverse, distinct from seawater and enriched in (gorgonian)-specific bacterial phylotypes. In contrast to current understanding, many dominant *E. labiata* symbionts can, indeed, be cultivated.

Keywords: host–microbe interactions; symbiosis; microbiome; microbial cultivation; next-generation sequencing

INTRODUCTION

Metagenomic surveys of the microbiota associated with marine invertebrates have revealed an enormous diversity of symbiotic prokaryotes. Their functional features and genome architectures, however, remain elusive, mainly due to the lack of representative culture collections. Yet, *in vitro* tests of physiological performance and genetic manipulations of cultivated symbionts are necessary to assign new gene clusters and proteins and to understand complex metabolic pathways. Laboratory collections of symbiotic marine microbes can feed the growing demand of the health sector for novel remedies from marine animals, since associated microbes often are the true producers of the bioactive compounds (Piel *et al.* 2004;

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Berrue *et al.* 2011). A holistic understanding of symbiont behaviour can only be achieved through bioassays conducted with cultivated strains. Ultimately, such insights can guide the development of protocols for the application of beneficial symbionts in host-oriented remediation strategies and in the mitigation of host diseases (Peixoto *et al.* 2017).

In temperate marine biomes, gorgonian corals (Cnidaria, Anthozoa, Octocorallia, Gorgoniidae) may contribute to up to 95% of the total biomass (Gili and Garcia 1985; Curdia *et al.* 2013) and determine carbon flux from pelagic to benthic zones (Gili and Coma 1998). As founder species, they increase habitat complexity and the number of available niches, ultimately increasing benthic biodiversity (Carpine and Grasshoff 1975; Cerrano *et al.* 2010; Curdia *et al.* 2013). However, infectious diseases linked to elevated temperatures are a major force of change in coral-dominated ecosystems and have led to mass mortality events in several octocorallian species (Cerrano *et al.* 2000; Hall-Spencer Pike and Munn 2007; Vezzulli *et al.* 2010; Ponti *et al.* 2014). Massive mortalities of gorgonians can alter critical ecosystem processes and result in biodiversity loss in the benthos of temperate zones (Ponti *et al.* 2014).

Corals live in association with complex microbial communities that consist of microalgae, bacteria, archaea, viruses, fungi and alveolates (Knowlton and Rohwer 2003; Rosenberg *et al.* 2007; Siboni *et al.* 2008; Tout *et al.* 2014; Bourne, Morrow and Webster 2016; Thurber *et al.* 2017). The microbial consortium delivers an extra supply of carbohydrates to the coral and participates in nutrient cycling (Raina *et al.* 2009; Lema, Willis and Bourne 2012; Bourne, Morrow and Webster 2016). Other proposed beneficial effects are chemical defence, detoxification, UV protection, antioxidant delivery, genetic exchange, pathogen control and natural phage therapy (Peixoto *et al.* 2017). The plasticity of the coral microbiota, via adaptive responses of prevailing symbionts or shifts in its taxonomic structure, may allow the coral holobiont (the animal host and its microbial symbionts) to more rapidly adapt to changing environmental conditions and thus enhance host fitness (Ainsworth, Thurber and Gates 2010). Intriguingly, some gorgonian species may rely exclusively on prokaryotic partners as they lack the typical carbon-fixing zooxanthella (*Symbiodinium*; Dinoflagellata) symbionts (Gori *et al.* 2012; Ransome *et al.* 2014). Recent 16S rRNA gene-based surveys indicate that prokaryotic communities of temperate gorgonians can be fairly diverse and distinct from the surrounding seawater (Bayer *et al.* 2013; La Riviere *et al.* 2013; van de Water *et al.* 2017). Gorgonian bacterial associates appear to be host species specific (La Riviere, Garrabou and Bally 2015; van de Water *et al.* 2017) and relatively stable throughout different sampling locations and seasons (La Riviere *et al.* 2013; La Riviere, Garrabou and Bally 2015). However, community composition can shift with increased anthropogenic influence and vary according to the health of the host (Hall-Spencer, Pike and Munn 2007; Bayer *et al.* 2013; La Riviere *et al.* 2013; Ransome *et al.* 2014; van de Water *et al.* 2017). *Proteobacteria* appears to be the most abundant bacterial phylum in temperate gorgonians and a core consortium of *Oceanospirillales* phylotypes related to the genus *Endozoicomonas* has been repeatedly described from gorgonian (Bayer *et al.* 2013; Correa *et al.* 2013; La Riviere *et al.* 2013; Ransome *et al.* 2014; La Riviere, Garrabou and Bally 2015; van de Water *et al.* 2017) and calcified coral microbiomes (Bourne, Morrow and Webster 2016; Neave *et al.* 2017b). Although 16S rRNA gene-based metagenomic surveys have explored the taxonomic composition of bacteria associated with several temperate gorgonian species, the cultivability of these communities has rarely been addressed. Collections from the few studies that have attempted cultivation were rather small, of reduced taxonomic diversity (consisting mostly of *Vibrio* spp.) and mainly derived from diseased gorgonians (Martin, Bonnefort and Chancerelle 2002; Hall-Spencer, Pike and Munn 2007; Vezzulli *et al.* 2010).

Here, we present the first report on the diversity, specificity and cultivability of the prokaryotic community inhabiting healthy specimens of the temperate gorgonian *Eunicella labiata*. Like many gorgonians, *E. labiata* is a prolific source of diterpene compounds, including some unusual *E. labiata*-specific amino-diterpenoids (labiatamids) that possess cytotoxic activity against human cancer cell lines (Roussis *et al.* 1996; Berrue and Kerr 2009). To address the cultivable fraction of the bacterial consortium associated with *E. labiata*, we used a lower incubation temperature, a prolonged incubation period and a carbon-reduced medium, to allow slow-growing bacterial symbionts to develop. In addition to the isolation of single colonies, we employed a 'plate-wash' strategy (Hardoim *et al.* 2014) that circumvents the conventional approach and permits direct comparison between bacterial community structures retrieved with cultivation-dependent and cultivation-independent methods. We hypothesised that this strategy would represent the phylogenetic diversity of the bacterial community inhabiting *E. labiata* to a higher extent than expected from colony-forming units/microscopy count ratios or conventional cultivation surveys.

METHODS

Sampling and sample processing

Eunicella labiata specimens were sampled in the Atlantic Ocean offshore Faro beach, Algarve, Portugal ('Pedra da Greta': Lat. 36° 58′ 47.2N, Long. 7° 59′ 20.8W) on 2 March 2015. *Eunicella labiata* was identified in the field based on its characteristic morphology (large size, typical branch pattern, verrucae all over colony) and colour (cream to dark brown) (Curdia *et al.* 2013). Three branches (~15–20 cm each) from three different *E. labiata* colonies (EL.01–EL.03) were collected by SCUBA diving at a depth of 18 m and placed in 3-L plastic bags (type Ziploc®) filled with surrounding seawater. In addition to the gorgonian samples, four replicates of surrounding seawater (SW.01–SW.04; ca. 2 L each) were collected in separate Ziploc plastic bags. All samples were transported to the laboratory in a cool box within 1.5 h post sampling and processed for total community DNA (TC-DNA) extraction and the cultivation of bacterial symbionts. In the laboratory, the branches of each *E. labiata* colony were rinsed with artificial seawater (ASW: 23.38 g L^{-1} NaCl, 2.41 g L^{-1} $MgSO_4{*}7H_2O$, 1.90 g L^{-1} $MgCl_2{*}6H_2O$, 1.11 g L^{-1} $CaCl_2{*}2H_2O$, 0.75 g L^{-1} KCl and 0.17 g L^{-1} $NaHCO_3$), aseptically cut into pieces and the soft tissue (coenenchyme and polyps) was separated from the inner gorgonin skeleton with a scalpel. The soft tissue was homogenised in sterile Ca^{2+} and Mg^{2+}-free artificial seawater (CMFASW: 27 g L^{-1} NaCl, 1 g L^{-1} $NaSO_4$, 0.8 g L^{-1} KCl and 0.18 g L^{-1} $NaHCO_3$, 1 g of soft tissue per 9 ml CMFASW w/v) using a sterile mortar and pestle. One millilitre of each homogenate was then serially diluted and used for cultivation (see below). The remaining homogenates were centrifuged for 2 min at 500 *g*. The supernatants were transferred into new centrifuge tubes and subjected to a final centrifugation step for 30 min at 10 000 *g*. The resulting coral-derived microbial cell pellets were stored at –80°C until TC-DNA extraction. Each seawater sample (2 L; SW.01–04) was filtered through a sterile 0.22-μm nitrocellulose membrane filter (Millipore, Billerica, MA, USA; 47 mm) using a

vacuum pump. The filters were aseptically cut into small pieces and stored at –80°C until TC-DNA extraction.

Cultivation, isolation and identification of bacterial symbionts from *Eunicella labiata*

Serial dilutions were prepared from the gorgonian soft tissue homogenates and spread onto half-strength Marine Agar 'MA2' (Difco Marine Broth 2216, Carl Roth GmbH + Co. KG; Marine Broth was diluted 1:2 in artificial seawater) plates in duplicates (10^{-3} dilution) or triplicates (10^{-4} and 10^{-5} dilutions). Colony-forming units (CFUs) were counted after 1, 2, 3 and 4 weeks of incubation at 18°C. Carbon reduction (diluted medium) together with a lower (18°C instead of the usual 25°C) incubation temperature and prolonged incubation (4 weeks) was chosen to allow slow-growing bacterial symbionts to develop into CFUs. In total, 416 visible CFUs were obtained over 4 weeks of incubation from EL.01-EL.03. Of these, 175 colonies were picked, streaked to purity on MA2 plates and stocked in 1:2 diluted Marine Broth (MB2) supplemented with 20% glycerol at –80°C. For genomic DNA extraction, 2-ml aliquots of shaken cultures (grown in MB2 medium until stationary phase) of all isolates were centrifuged at 10 000 *g* for 30 min. Genomic DNA was extracted from the resulting cell pellets using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instructions. The purified DNAs of all isolates were then subjected to 16S rRNA gene amplification and Sanger sequencing for identification as previously described (Esteves *et al.* 2013). 16S sequences were trimmed and edited with the Sequence Scanner Software version v1.0 (Applied Biosystems, Foster, CA, USA), resulting in high-quality 16S rRNA gene sequences of 800–1000 bp. Closest matches to sequence queries were identified using the BLAST algorithm of the National Center for Biotechnology Information (NCBI—http://blast.ncbi.nlm.nih.gov/Blast.cgi). Taxonomic assignment of bacterial isolates to the genus level was performed using the classifier tool of the Ribosomal Database Project (RDP, release 11, http://rdp.cme.msu.edu) as described earlier (Esteves *et al.* 2013; Keller-Costa *et al.* 2014). Closest type strains to all sequence queries were determined using the RDP sequence match tool (http://rdp.cme.msu.edu/seqmatch) (Keller-Costa *et al.* 2014).

Assessments of bacterial community diversity and composition in *Eunicella labiata* and seawater

Cultivation-independent TC-DNA was extracted from the three coral (EL.01–EL.03) and four seawater (SW.01–SW.04) samples using the Power Soil® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and as detailed elsewhere (Costa *et al.* 2013). The cultivation-dependent method of 'plate-washing' (see (Hardoim *et al.* 2014) for a detailed description) involved extraction of DNA from washes of the 10^{-3} dilution MA2 culture plates. The bacterial colonies were detached from the agar medium by rinsing the plate with 10 mL of sterile CMFASW and with the help of a Drigalski spatula. The resulting cell suspensions were centrifuged for 30 min at 10 000 *g* to yield the cultivation-dependent 'plate-wash' microbial cell pellets which were then subjected to TC-DNA extraction with the Power Soil DNA Isolation Kit (Mo-Bio Laboratories, Inc.) as explained above. Thus, in total 10 TC-DNA samples (three *E. labiata* replicates handled either with the cultivation-independent or -dependent 'plate-wash'—EL.01.PL–EL.03.PL—method and four seawater replicates) were obtained and subjected to 16S rRNA gene-based next generation sequencing (NGS). The V4 hypervariable region (515–806) of the 16S rRNA gene was amplified at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) using the primers 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) (Caporaso *et al.* 2011) and 806RB (5′-GGA CTA CNV GGG TWT CTA AT-3′) (Apprill *et al.* 2015) with barcodes on the forward primer. The PCR reactions were prepared using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) and carried out under the following conditions: 94°C for 3 min; followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min and, final elongation at 72°C for 5 min. Samples were purified using calibrated Ampure XP beads. The purified PCR products were then used to prepare the DNA library according to the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA on an Illumina MiSeq platform following the manufacturer's guidelines to generate on average 20 000 paired-end sequence reads of ca. 300 bp in length per sample.

Sequence data processing and analysis

Raw sequence data were processed using the MR DNA analysis pipeline (MR DNA). In total, 397 405 raw 16S rRNA (V4 region) gene sequence reads amplified from 10 TC-DNA samples were obtained. All reads were depleted of primers and barcodes and those sequences smaller than 250 bp as well as those with ambiguous base calls were removed. The sequences were de-noised, operational taxonomic units (OTUs) generated and chimeras and singletons (OTUs that only had one single read in the entire dataset) removed using UCHIME (Edgar *et al.* 2011). In total, 1911 OTUs were established at 97% sequence similarity cut-off. We thereafter performed taxonomic classification of OTUs using the SINA sequence alignment tool of the SILVA database (https://www.arb-silva.de/aligner/). Sequences below 70% identity were considered as unclassified at the domain level and rejected from the final set of OTUs. Moreover, OTUs classified as Eukaryota, chloroplasts or mitochondria were removed. The final analytical dataset comprised 1092 archaeal and bacterial OTUs with a total of 256 715 16SrRNA gene reads.

Data analyses comprised (i) calculation and estimation (Chao1) of symbiont richness and diversity (Shannon's index); (ii) assessment of phylum-, class- and genus-level prokaryotic composition in individual samples; (iii) multivariate analysis of OTU data and (iv) assessment of specific and shared bacterial symbionts across sample groups via Venn diagrams (Venni 2.1, BioinfoGP, CSIC). For (i) α-diversity analyses, sample libraries were size-normalised by rarefying all samples down to 10 162 reads (corresponding to sample EL.01 with the lowest number of reads) using the rarefy function in the phyloseq package for R. For (ii) prokaryotic composition, relative abundance data (percentages, non-rarefied) were used. For (iii) multivariate analysis, OTU data (non-rarefied) were Hellinger-transformed and Euclidean distances calculated from the Hellinger-transformed OTU-data table using the vegan package for R. The resulting distance matrix was then used to perform principal coordinate analysis (PCoA) and UPGMA clustering of samples according to their OTU profiles using the R packages phyloseq and pvclust (hclust/vegan-based). For (iv) Venn diagram constructions, the non-rarefied, quality-filtered dataset was used and explored first with all 1092 OTUs and second, after removal of all the OTUs with less than 10 sequence reads (corresponding to 1% of the total number of reads) across the entire dataset.

Statistics

One way ANOVAs followed by Tukey post hoc tests were used to check for significant differences between sample categories for the (i) observed and estimated (Chao1) species richness as well as the Shannon diversity measures. One way repeated measures (RM) ANOVAs followed by Tukey post hoc tests were used to compare the observed with the estimated (Chao1) species richness values obtained for each sample category. All analysed data were normally distributed (Shapiro-Wilk test passed), and equal variance was observed for all species richness measures. One way ANOVAs followed by Tukey post hoc tests were further used to test for differences in the (ii) relative abundance in each of the five most dominant phyla across sample categories. All relative abundance data were normally distributed and of equal variance. PERMANOVA—permutational multivariate analysis of variance- using the (Euclidean) distance matrix was performed with 999 permutations using the adonis function of the vegan package for R to test for overall differences in the (iii) Euclidean distance matrix between sample categories.

Phylogenetic analysis

For *Endozoicomonas*-specific phylogenetic inference, all *Endozoicomonas*-like 16S rRNA gene sequences obtained from *E. labiata* were aligned using the SINA/SILVA (online) sequence alignment tool. Further included in the alignment procedure were the 16S rRNA gene sequences of closest matches observed in BLASTN (NCBI), *Endozoicomonas* 16S rRNA sequences derived from temperate gorgonian species (e.g. Bayer *et al.* 2013; La Riviere, Garrabou and Bally 2015; La Riviere *et al.* 2013) and other marine animals and six (until March 2017) described *Endozoicomonas* type strains (RDP) as well as seven *Reinekea* spp. (order *Oceanospirillales*) sequences to form an outgroup. The General Time Reversible (GTR) model with a discrete gamma distribution (five categories (+ G, parameter = 0.2693)) was used as evolutionary model for phylogenetic inference using MEGA7 (Kumar, Stecher and Tamura 2016). A maximum-likelihood tree was then determined with bootstrap support using 100 repetitions. The tree with the highest log likelihood (–1212.7743) was used. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 53 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 254 positions in the final dataset.

RESULTS

Plate counts and taxonomic composition of bacteria isolated from the gorgonian *Eunicella labiata*

The CFU counts of *E. labiata* heterotrophic aerobic bacteria varied between 1.5×10^5 and 1.2×10^6 on MA2 medium (Fig. S1A, Supporting Information). On average, 61% of all CFUs appeared during the first week of cultivation, 29% occurred after two weeks and 9% emerged only in weeks three and four post-plating (Fig. S1B, Supporting Information). In total, 175 colonies were picked and purified from the three *E. labiata* specimens, the three dilutions (10^{-3}, 10^{-4}, 10^{-5}) and the four weeks of cultivation, and identified by 16S rRNA gene Sanger sequencing (Table S1, Supporting Information). The isolates affiliated with three bacterial phyla (*Proteobacteria*, *Bacteroidetes*, *Firmicutes*), seven different orders and 13 classified genera plus two distinct *Rhodobacteraceae* strains not classifiable at genus level (Table S2, Supporting Information). Eighty-two per cent of all isolates were classified as *Alphaproteobacteria*, the most abundant genera being *Ruegeria* (38%), *Labrenzia* (25%), *Roseovarius* (9%) and *Sphingorhabdus* (5%). *Gammaproteobacteria* accounted for 15% of the isolated bacteria with *Vibrio* (9%) as the most abundant genus. The remaining 3% of the isolates affiliated with *Aquimarina* (2%, *Flavobacteriia, Bacteroidetes*) and *Bacillus* (*Firmicutes*). Although the taxonomic composition varied between the three *E. labiata* specimens, the dominant taxa *Ruegeria, Labrenzia* and *Sphingorhabdus* were isolated from all specimens (Fig. 1A). All *Gammaproteobacteria* and *Aquimarina* isolates grew into visible colonies during the first week of cultivation, but dominant *Alphaproteobacteria* taxa such as *Sphingorhabdus* and (most) *Labrenzia* only appeared during the second week and later (Fig. 1B). The 10^{-3} dilution represented best the overall taxonomic composition of the bacterial isolates with all 13 classified plus one unclassified genus being obtained from this dilution (Fig. 1C). On higher dilutions, however, mostly (dilution 10^{-4}) or exclusively (dilution 10^{-5}) *Alphaproteobacteria* of the *Rhodobacteraceae* family were isolated, suggesting dominance of this class/family in the *E. labiata* microbiome.

Metagenome dataset

To assess the structure of the prokaryotic consortium inhabiting *E. labiata*, we employed NGS taxonomic profiling for the three *E. labiata* specimens and compared them with the profiles of surrounding seawater. For direct comparison between bacterial community structures retrieved from *E. labiata* with cultivation-dependent and cultivation-independent methods, NGS taxonomic profiling of the 'plate-wash' samples from the same *E. labiata* specimens was performed, in addition to the isolation of single colonies. In total, 1092 OTUs (20 archaeal and 1072 bacterial OTUs) were obtained from the 10 TC-DNA samples (Table S3, Supporting Information).

Alpha-diversity analyses: species richness and Shannon diversity

The alpha-diversity (species richness and Shannon's diversity) in seawater versus the gorgonian *E. labiata* was explored on the rarefied (size-normalised) dataset. The observed number of prokaryotic phylotypes (OTUs) was significantly higher ($P < 0.001$) in seawater than in *E. labiata* (Fig. S2A, Supporting Information). Surprisingly, bacterial richness in the cultivation-dependent *E. labiata* 'plate-washes' did not differ ($P_{Tukey} = 0.137$) from the cultivation-independent *E. labiata* samples. Similar patterns were seen for the richness estimate Chao1 (Fig. S2B). For all three sample categories, the respective estimated Chao1 richness was somewhat higher than the observed species richness (one way RM ANOVA—seawater: $F_{2,8} = 104.011$, $P < 0.001$; *E. labiata* $F_{2,8} = 26.0338$, $P = 0.005$; 'plate-wash': $F_{2,8} = 9.505$, $P = 0.03$). The Shannon diversity indices—which consider not only the number of bacterial phylotypes (i.e. OTUs) but also their relative abundance in each sample—were highest for seawater (4.21 ± 0.09; mean ± SE), followed by the cultivation-independent *E. labiata* samples (3.4 ± 0.52, mean ± SE) and the cultivation-dependent *E. labiata* 'plate-washes' (2.75 ± 0.09, mean ± SE), although statistical difference was only seen between the seawater and 'plate-wash' samples (Fig. S2C). The Shannon diversity indices of the cultivation-independent *E. labiata* and the cultivation-dependent 'plate-wash' samples were also similar.

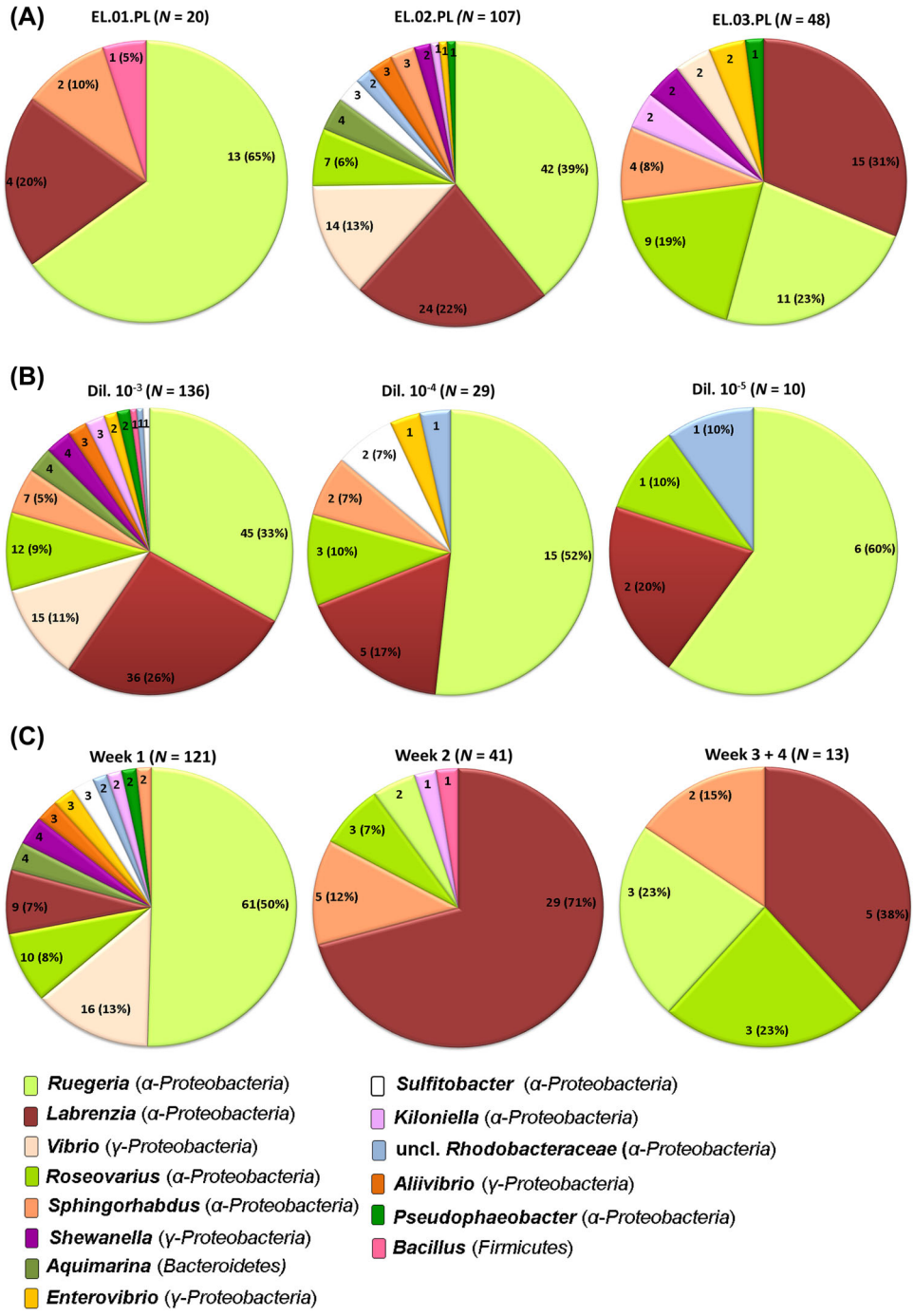

Figure 1. Genus-level taxonomic composition of 175 strains isolated from *E. labiata*. Bacterial composition is compared **(A)** between gorgonian specimens (EL.01; EL.02; EL.03), **(B)** between the dilutions (10^{-3}; 10^{-4}; 10^{-5}) of the microbial suspensions that were spread on the plates and **(C)** between the weeks of incubation (week 1; week 2; week 3 + 4). Values in the pie charts represent the number of isolated strains per genus, with their respective relative abundances (if ≥5%) given in brackets. Taxonomic assignment at the genus level (based on Sanger sequencing of 16S rRNA genes) was achieved using the 'Classifier' tool of the RDP (release 11) at 80% confidence threshold.

Prokaryote community composition

Proteobacteria was the most abundant phylum in all three sample categories, although their relative abundance changed significantly between categories (Table 1; one way ANOVA, $F_{2,9} = 107.51$, $P < 0.001$). While in seawater *Proteobacteria* represented between 44% and 53% of the total community, they comprised between 78% and 91% of the reads in the cultivation-independent *E. labiata* samples and 98% or more in the cultivation-dependent 'plate-washes'. *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Euryarchaeota* and *Marinimicrobia* (in this order) were the five most dominant phyla in the entire dataset. Archaeal populations had about 0.6% relative abundance in the cultivation-independent *E. labiata* microbiome, with most OTUs (N = 18) belonging to the phylum *Euryarchaeota* and two OTUs belonging to the phylum *Thaumarchaeota* (Marine

Table 1. Number of OTUs and sequence reads per prokaryote phylum across sample categories.

	Seawater			*Eunicella labiata*			*Eunicella labiata* 'plate-wash'		
Phylum	OTUs	Seqs	%Seqs	OTUs	Seqs	%Seqs	OTUs	Seqs	%Seqs
Acidobacteria	6	15	0.010	7	66	0.213	1	1	0.001
Actinobacteria	60	37336	25.95	30	2064	6.676	29	273	0.333
Bacteroidetes	197	23415	16.28	71	979	3.167	44	139	0.170
Chlamydiae	0	0	0.000	1	8	0.026	0	0	0.000
Chlorobi	1	9	0.006	1	1	0.003	1	1	0.001
Chloroflexi	1	5	0.003	4	23	0.074	0	0	0.000
Cyanobacteria	9	1120	0.779	7	129	0.417	2	8	0.010
Euryarchaeota	18	4590	3.191	6	142	0.459	3	8	0.010
Fibrobacteres	2	6	0.004	0	0	0.000	0	0	0.000
Firmicutes	5	108	0.075	8	352	1.139	6	337	0.411
Fusobacteria	2	14	0.010	1	1	0.003	0	0	0.000
Gemmatimonadetes	7	31	0.022	2	8	0.026	3	3	0.004
Lentisphaerae	2	21	0.015	0	0	0.000	1	1	0.001
Marinimicrobia	18	3850	2.676	11	273	0.883	12	28	0.034
Nitrospirae	0	0	0.000	1	13	0.042	0	0	0.000
Parcubacteria	3	10	0.007	2	18	0.058	0	0	0.000
Planctomycetes	24	886	0.616	11	146	0.472	6	10	0.012
Proteobacteria	633	71833	49.934	427	26339	85.193	403	81123	99.002
SBR1093	1	25	0.017	0	0	0.000	1	1	0.001
Thaumarchaeota	2	255	0.177	1	30	0.097	1	1	0.001
TM6 *(Dependentiae)*	1	3	0.002	0	0	0.000	0	0	0.000
Unclassified *Bacteria*	1	18	0.013	0	0	0.000	1	1	0.001
Verrucomicrobia	23	307	0.213	12	325	1.051	6	6	0.007
Total	1016	143857	100	603	30917	100	520	81941	100

Values correspond to quality-filtered OTUs and sequences across the full data set (non-rarefied libraries).
Seqs—number of sequence reads per phylum. %Seqs—relative abundance of each phylum.
The five most dominant phyla of the entire dataset are highlighted in bold. Significant differences in the relative abundances of these five phyla were observed between seawater and the *E. labiata* samples. Between the cultivation-independent and dependent *E. labiata* samples, however, only *Proteobacteria* relative abundance differed, the other four phyla were similar (one way ANOVAs followed by Tukey tests: *Proteobacteria*—$F_{2,9} = 107.51$, $P < 0.001$; *Actinobacteria*—$F_{2,9} = 49.112$, $P < 0.001$; *Bacteroidetes*—$F_{2,9} = 78.712$, $P < 0.001$; *Euryarchaeota*—$F_{2,9} = 32.977$, $P < 0.001$; *Marinimicrobia*—$F_{2,9} = 11.110$, $P = 0.007$.)

Group I). Class-level taxonomic composition was most diversified in seawater with *Alphaproteobacteria*, *Acidimicrobiia*, *Flavobacteriia*, *Gammaproteobacteria* and *Thermoplasmata* (in this order) being the most abundant classes (Fig. 2A). Compared to seawater, *Gammaproteobacteria* seemed to be enriched in the (cultivation-independent) *E. labiata* samples, although considerable variability of *Gammaproteobacteria* relative abundance was noted between gorgonian individuals. The cultivation-dependent *E. labiata* 'plate-washes' consisted of mainly *Alpha-* and *Gammaproteobacteria*, with *Alphaproteobacteria* being significantly enriched via cultivation (one way ANOVA $F_{2,9} = 6.535$, $P = 0.025$). The most drastic shifts in taxonomic composition were seen at the genus level (Fig. 2B). The 10 most dominant genera in seawater all represent as yet unclassified, uncultured or poorly cultured genera. The candidate genus *Actinomarina* (*Actinobacteria*) was dominant in seawater (20% of the reads) and with 21 OTUs also rich in candidate species. Genus-level taxonomic composition in the cultivation-independent gorgonian samples differed sharply from seawater, with *Endozoicomonas* (28.5%, *Gammaproteobacteria*) and *Ruegeria* (23%, *Alphaproteobacteria*) being clearly the dominant genera, followed by *Candidatus Actinomarina* (6%) and *Sphingorhabdus* (5%; *Alphaproteobacteria*). In the cultivation-dependent 'plate-wash' samples, however, *Endozoicomonas* was only detected at low abundance (0.12%), while *Ruegeria* (48%), *Vibrio* (17%; *Gammaproteobacteria*), *Sphingorhabdus* (16%), unclassified *Rhodobacteraceae* (6%) and *Shewanella* (5%; *Gammaproteobacteria*) were enriched on the culture plates.

Beta-diversity analyses: ordination and clustering of phylotypes (OTUs)

PCoA allowed the visualisation of the community dissimilarity between samples at the OTU level (Fig. 3A). Overall, the PCoA revealed a separate grouping of habitats (i.e. seawater versus gorgonian). A high similarity was observed within the four seawater replicates which clustered away from all *E. labiata* samples with statistical support (PERMANOVA: $F = 6.832$, $P = 0.002$). The *E. labiata* samples, in contrast, showed a higher individual-to-individual variation, and discrete grouping of the cultivation-independent versus dependent samples was not as evident. Plotting of the 20 most abundant phylotypes (OTUs) into the PCoA diagram demonstrated their correspondences with the different microhabitats: *Endozoicomonas* OTU 6 was prevalent in the cultivation-independent *E. labiata* samples, while bacterial genera such as *Ruegeria*, *Sphingorhabdus* and *Labrenzia* were shared between the cultivation-independent and dependent *E. labiata* samples. The abundance of *Planktomarina* and many unclassified taxa related to *Rhodobacteraceae*, *Marinimicrobia*, SAR11, SAR116, SAR86 and NS7 correlated with the seawater samples (Fig. 3A). UPGMA (Fig. 3B) and bootstrap analysis also revealed two distinct groups, one comprising all seawater replicates and the other all gorgonian samples, confirming the PCoA results (Fig. 3A) and the trends revealed by genus-level assessment of taxonomic composition (Fig. 2B). Clustering of the cultivation-dependent *E. labiata* 'plate-washes' together with cultivation-independent *E. labiata* samples and their long distance from all seawater replicates indicates the successful cultivation of a

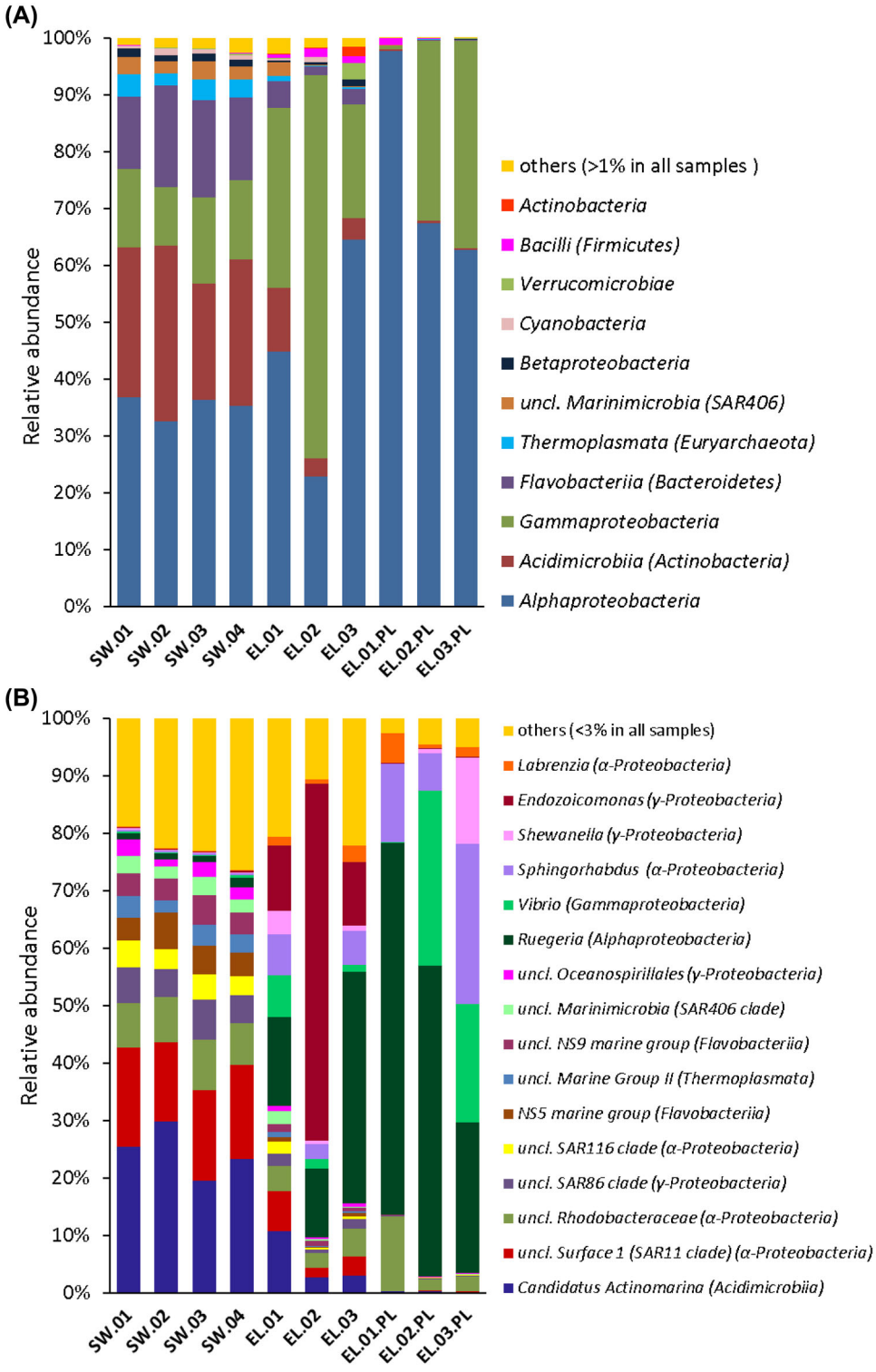

Figure 2. Prokaryotic community composition at (A) class- and (B) genus-level in seawater and the gorgonian *E. labiata*. Results obtained for each replicate sample across all sample categories are shown. Relative abundances are displayed for taxa representing more than 1% **(A)** or 3% **(B)** of the total dataset reads. Taxa with abundances below 1% (A) or 3% (B) across the data are collectively labelled as 'rare classes' or 'rare genera'.

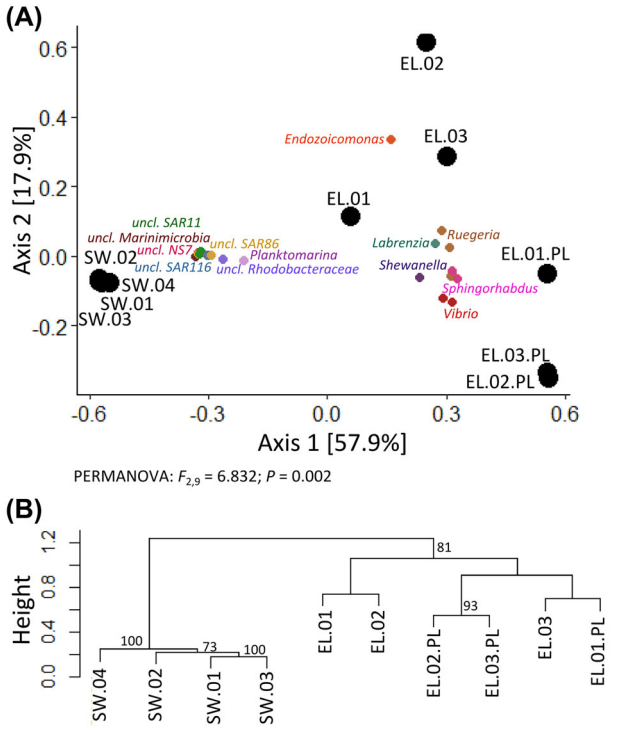

Figure 3. Ordination of prokaryote community profiles at the phylotype (OTU) level. **(A)** PCoA was performed using the Euclidean distance matrix calculated from Hellinger-transformed sequence libraries. Samples are represented by large, black circles: SW.0n—seawater samples; EL.0n—cultivation-independent *E. labiata* samples; EL.0n.PL—cultivation-dependent *E. labiata* 'plate-washes'. The 20 most dominant phylotypes (OTUs) of the entire dataset are plotted as coloured dots in the diagram, and the genus-level taxonomic identity of these OTUs (same colouring) is presented next to the dots. Discrete grouping of the three sample categories was statistically supported by a PERMANOVA test with 999 permutations: $F_{2,9} = 6.832$; $P = 0.002$. Grouping of samples was further inspected by **(B)** cluster analysis using the UPGMA algorithm on the same Euclidean distance matrix. Robustness of the clusters was assessed by bootstrap analysis (1000 repetitions). Bootstrap values above 70% are presented next to their respective cluster nodes.

significant portion of the symbiotic community inhabiting *E. labiata*.

Exclusive and shared OTUs between sample categories

To determine how many and which OTUs were common or specific to each sample category, the full (non-rarefied) quality-filtered dataset was explored (Fig. 4). A total of 1016, 603 and 515 OTUs were detected in seawater, in the culture-independent and culture-dependent ('plate-wash') *E. labiata* samples, respectively. Although a considerable number of prokaryotic OTUs (32%: 352 out of 1092) was shared between all three sample categories, seawater also harboured a large number (350) of bacterioplankton-specific OTUs (Fig. 4A). The cultivation-independent *E. labiata* samples had 62% (376 out of 603) of OTUs in common with the cultivation-dependent 'plate-washes' and 11% (68) of these OTUs were unique to the *E. labiata* microbiome. Thirty-six per cent (187 of 515) of all OTUs detected in the *E. labiata* 'plate-wash' samples were represented only by a single sequence read, likely derived from non-viable cells. The 'plate-wash' method further led to the detection of eight 'cultivation-specific' OTUs not seen in any of the cultivation-independent datasets and affiliating with *Vibrio*, *Shewanella*, *Ruegeria*, *Endozoicomonas*, *Kiloniella* and an unclassified *Rhodobacteraceae*.

To explore whether the specific OTUs of each sample category belong to rare or abundant OTUs, we removed all those OTUs represented by less than 10 sequence reads across the entire dataset. Although the number of removed reads (2033) corresponded to only 1% of the total number of reads (257 615) in the non-rarefied dataset, they represented 36% (397 OTUs) of the total prokaryotic richness in this study. The removed (rare) OTUs mainly belonged to habitat-specific OTUs (present only in seawater or only in *E. labiata*; Fig. 4B), indicating that the differences between these two habitats were mainly due to oscillations in OTU relative abundances rather than absolute presence/absence of OTUs. Of the 10 most abundant OTUs of seawater, only two (OTU 1—surface I group, SAR11 and OTU 3—Candidatus *Actinomarina*) were also among the top 10 OTUs in the cultivation-independent *E. labiata* samples (Table 2). In contrast, all top 10 OTUs of the cultivation-independent *E. labiata* samples were present in the 'plate-wash' samples (with >12 reads per replicate, Table S3, Supporting Information), and 7 of these OTUs also ranked among the 10 most dominant OTUs in the 'plate-washes' (Table 2); they affiliated with the genera *Ruegeria*, *Vibrio*, *Shewanella* and *Sphingorhabdus*.

Phylogenetic analyses

The most abundant phylotype (OTU 6) retrieved from the *E. labiata* microbiome by cultivation-independent means affiliated with the animal-associated genus *Endozoicomonas* (*Oceanospirillales, Gammaproteobacteria*) and contributed to up to 59% (in EL.02) of the gorgonian community. This OTU was deselected in the cultivation-dependent *E. labiata* 'plate-washes' as well as in seawater. The sequences most similar to OTU 6 represented exclusively uncultured clones retrieved from other gorgonian hosts that, together with other *E. labiata*-derived, *Endozoicomonas*-like OTUs, formed a separate cluster in the phylogenetic analysis (Fig. 5). This cluster (cluster II) shared an average sequence similarity of only 90.9% (between groups $p_{distance} = 0.091$) with cluster I that contained all so-far described *Endozoicomonas* type strains and isolates (Fig. 5). Likewise, the RDP sequence match results revealed that all type strains and cultivates (which mostly affiliated with *Endozoicomonas*) closest to OTU 6 shared <93% 16S rRNA gene sequence similarity with it.

DISCUSSION

Structure and uniqueness of the *Eunicella labiata* microbiome

This study represents the first structural description of prokaryotic communities in the gorgonian species *E. labiata*. With 18 phyla detected, this symbiotic consortium was found to be rich at the phylum level, although the predominance of *Proteobacteria, Actinobacteria* and *Bacteroidetes* was seen, congruent with previous studies on coral microbiomes (reviewed in Bourne, Morrow and Webster 2016) and other marine environments (e.g. Hardoim *et al.* 2014; Briand *et al.* 2017). Furthermore, the prokaryotic communities inhabiting *E. labiata* were unique and highly distinct from the seawater community. PCoA and Venn diagram analyses made clear that the difference between the prokaryotic communities of the two habitats is caused by strong oscillations in species' relative abundance rather than an absolute presence or absence of species. Thus, distinction of the gorgonian

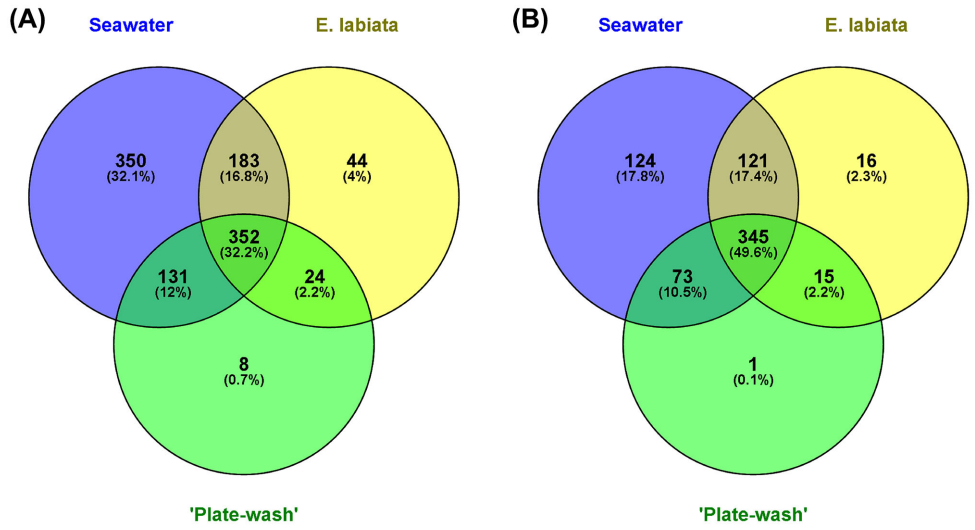

Figure 4. Shared and specific prokaryote phylotypes in *E. labiata* and in seawater samples. Venn diagrams were constructed with non-rarefied libraries. Replicate samples were pooled to portray the total and relative (percentages) number of prokaryote OTUs recovered within each sample category. **(A)** All detected OTUs common and exclusive to seawater (blue) and the gorgonian *E. labiata* using cultivation-independent (yellow) and dependent 'plate-wash' (green) methods, respectively. **(B)** Shared and exclusive OTUs with at least 10 or more sequence reads across the whole dataset.

Table 2. The 10 most abundant prokaryotic OTUs of each sample category.

	Seawater			*Eunicella labiata*			*Eunicella labiata* 'plate-wash'		
	Classification	OTU	Seqs	Classification	OTU	Seqs	Classification	OTU	Seqs
1	Cand. *Actinomarina* (*Actinobacteria*)	OTU_1*	28654	*Endozoicomonas* (*Proteobacteria*)	OTU_6	8362	*Ruegeria* (*Proteobacteria*)	OTU_4#	16232
2	uncl. Surface 1 (SAR11, *Proteobacteria*)	OTU_3*	11747	*Ruegeria* (*Proteobacteria*)	OTU_11#	3006	*Ruegeria* (*Proteobacteria*)	OTU_11#	9644
3	uncl. Surface 1 (SAR11, *Proteobacteria*)	OTU_148	7032	*Ruegeria* (*Proteobacteria*)	OTU_4#	2204	*Sphingorhabdus* (*Proteobacteria*)	OTU_8#	9020
4	uncl. *Rhodobacteraceae* (*Proteobacteria*)	OTU_10	6848	Cand. *Actinomarina* (*Actinobacteria*)	OTU_1*	1521	*Vibrio* (*Proteobacteria*)	OTU_5#	8893
5	uncl. SAR86 (*Proteobacteria*)	OTU_15	4071	*Sphingorhabdus* (*Proteobacteria*)	OTU_8#	1342	*Ruegeria* (*Proteobacteria*)	OTU_123#	7993
6	uncl. SAR116 (*Proteobacteria*)	OTU_17	3318	*Ruegeria* (*Proteobacteria*)	OTU_123#	881	*Vibrio* (*Proteobacteria*)	OTU_192	2842
7	NS5 marine group (*Bacteroidetes*)	OTU_13	3160	*Vibrio* (*Proteobacteria*)	OTU_5#	766	*Shewanella* (*Proteobacteria*)	OTU_9#	2580
8	Marine group II (*Euryarchaeota*)	OTU_12	2871	uncl. Surface 1 (SAR11, *Proteobacteria*)	OTU_3*	704	*Ruegeria* (*Proteobacteria*)	OTU_1217#	2540
9	NS9 marine group (*Bacteroidetes*)	OTU_22	2686	*Ruegeria* (*Proteobacteria*)	OTU_1217#	605	uncl. *Rhodobacteraceae* (*Proteobacteria*)	OTU_67	1975
10	SAR406 (*Marinimicrobia*)	OTU_21	2664	*Shewanella* (*Proteobacteria*)	OTU_9#	450	*Sphingorhabdus* (*Proteobacteria*)	OTU_117	1906

*Asterisks indicate OTUs shared between seawater and *Eunicella labiata*. # Hash keys indicate OTUs shared between cultivation-independent and -dependent ('plate-wash') *E. labiata* samples. Seqs—number of sequence read obtained per sample category.

microbial community from the bacterioplankton is indeed driven by the dominant phylotypes of each habitat. Taxa such as *Endozoicomonas*, *Ruegeria* and *Sphingorhabdus* were particularly enriched in the gorgonian host, suggesting a selection of the symbiotic community. Although the overall observed and estimated species richness values were higher for seawater than for the gorgonian coral, the Shannon diversity indices of both habitats were comparable, implicating higher equitability (the different species being represented by similar numbers of individuals) in the *E. labiata* community. Yet the *E. labiata* samples exhibited a higher individual-to-individual variation in their prokaryotic community structures than the seawater replicates. Variability in microbial community composition between different individuals of the same gorgonian species has been noted earlier (Bayer

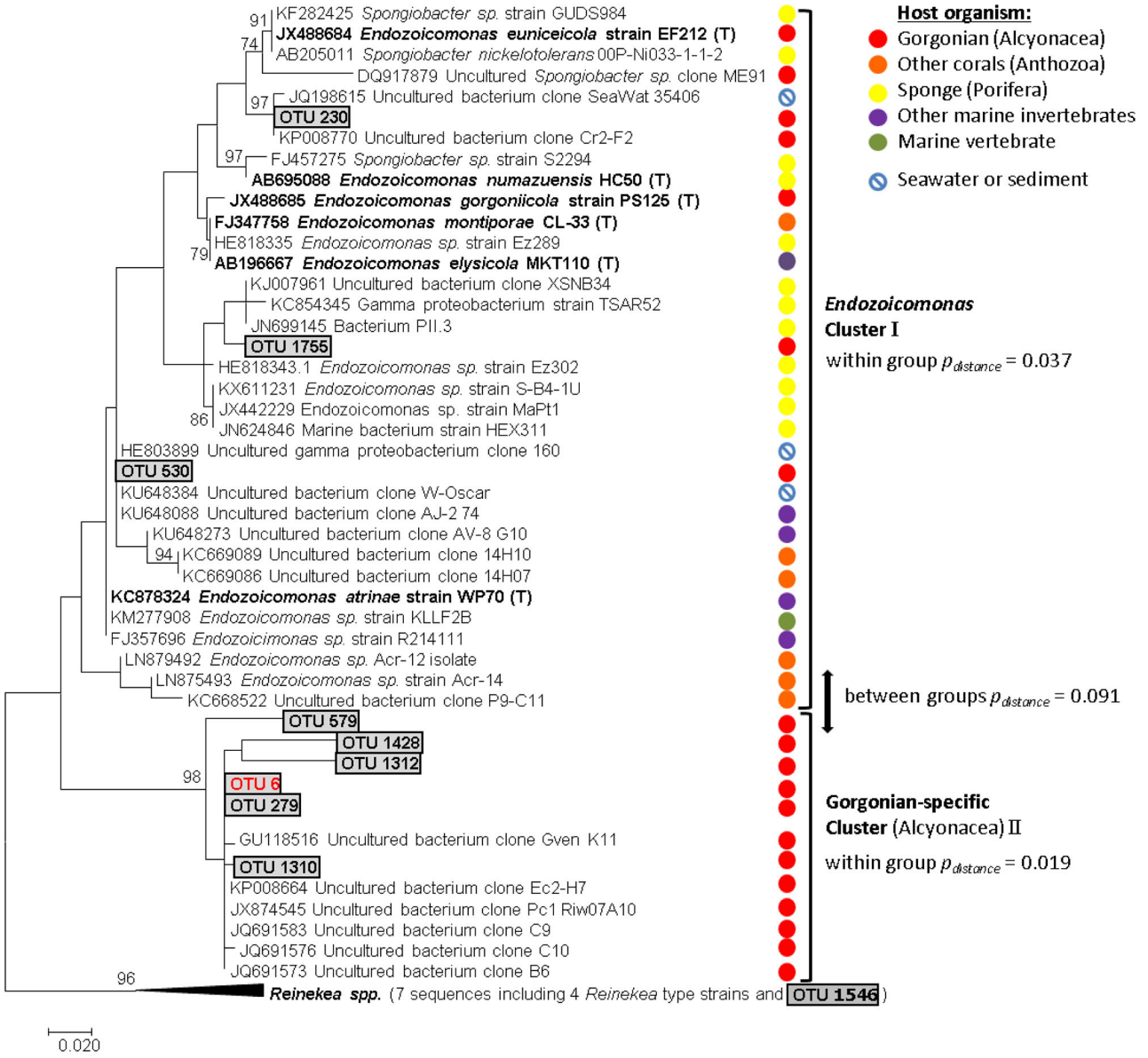

Figure 5. Phylogenetic inference of *Endozoicomonas*-like OTUs (16S rRNA gene reads sharing ≥97% homology) obtained from the gorgonian *E. labiata* (grey highlights) and their closest relatives. A maximum-likelihood tree constructed using the GTR model (with discrete Gamma distribution) to infer evolutionary relationships among sequence entries is shown. The most dominant *E. labiata* OTU (OTU 6) is highlighted in red. Closest NCBI BlastN hits and *Endozoicomonas* type strains ((T), *in* bold) are shown on the tree. Coloured circles indicate the source/host of each sequence (red: gorgonian; orange: other coral; yellow: marine sponge; purple: other marine invertebrate; green: marine vertebrate, blue: seawater or sediment). The percentage of trees in which the associated taxa clustered together is shown next to the branches (70% cut-off). The *Oceanospirillales* genus *Reinekea* was used to root the tree.

et al. 2013) and may be the result of varying physiological performances of the different host individuals.

Endozoicomonas was the dominant genus of the *E. labiata* microbiome, followed by *Ruegeria* and *Actinomarina*, the latter being albeit more abundant in seawater. Members of the recently described (2007) *Endozoicomonas* genus (order *Oceanospirillales*) (Kurahashi and Yokota 2007) associate with a range of marine invertebrates and vertebrates, but are most frequently detected as coral (Bourne, Morrow and Webster 2016; Neave *et al.* 2016), particularly gorgonian coral, symbionts (Bayer *et al.* 2013; Correa *et al.* 2013; Ransome *et al.* 2014; La Riviere, Garrabou and Bally 2015; van de Water *et al.* 2017). In general, their abundance seems to be positively correlated with coral health, while anthropogenic pollution, habitat degradation and diseases seem to impact *Endozoicomonas* abundance negatively (Neave *et al.* 2016). Catalysed reporter deposition–fluorescence in situ hybridisation analysis with *Endozoicomonas*-specific fluorescent probes demonstrated that these bacteria form dense, cyst-like aggregates deep inside the coral endoderm and reside in the polyps' tentacles (Neave *et al.* 2017b). Genome sequencing data obtained from cultured representatives, single-cell genomics and metagenomics suggest a role of *Endozoicomonas* in nutrient acquisition and provision for their hosts, including carbon and nitrogen cycling and the synthesis of amino acids, B vitamins and other molecules (Neave *et al.* 2016, 2017a). Yet an experimental proof of this interaction is still lacking. Interestingly, the *E. labiata*-derived *Endozoicomonas* OTUs obtained in this study clustered together with uncultured clones from other gorgonian species

(Sunagawa, Woodley and Medina 2010; Bayer *et al.* 2013; La Riviere *et al.* 2013; La Riviere, Garrabou and Bally 2015) and apart from *Endozoicomonas* strains derived, for example, from scleractinian corals and all the currently described type strains of this genus. The average sequence similarity between the two phylogenetic clusters was <91%. This indicates that the gorgonian phylotypes are probably not *Endozoicomonas sensu stricto* and should rather be considered as a new, undescribed genus. A similar observation was made in a recent study on the bacterial communities associated with three *Octocorallia* species from the Mediterranean Sea (La Riviere, Garrabou and Bally 2015).

The second most abundant prokaryotic genus of *E. labiata* was the alphaproteobacterium *Ruegeria* that forms part of the extremely versatile marine *Roseobacter* clade (family *Rhodobacteraceae*, order *Rhodobacterales*). The *Rhodobacteraceae* family associates frequently with adult scleractinian corals (Rohwer *et al.* 2001; Cooney *et al.* 2002; Rothig *et al.* 2016), and *Rhodobacterales* bacteria were also found to be consistent symbionts of the gorgonian *Paramuricea clavata* obtained from different geographic locations and depths (Vezzulli *et al.* 2013). Other two studies conducted in different seasons and at different locations have reported *Ruegeria* associates in the gorgonian *E. cavolini* (Bayer *et al.* 2013; La Riviere, Garrabou and Bally 2015). However, *Rhodobacteraceae* seem to dominate the early life stages of corals. They can either be transmitted vertically as in the larvae-brooding coral *Porites astreoides* (Sharp, Distel and Paul 2012) or acquired horizontally as in the spawning *Pocillopora meandrina*, where *Roseobacter* bacteria are the main primary colonisers of their planula larvae (Apprill *et al.* 2009). The genomic blueprints of host-associated *Ruegeria* reveal advantageous traits for symbiotic lifestyles, including genes encoding for chemical communication and attachment (LuxRI quorum-sensing system, type IV secretion system and pili), defence (polyketide synthases; non-ribosomal peptide synthetases, bacteriocin), vitamins (e.g. biotin) synthesis and a potential role in sulphur cycling (sulphur oxidation and degradation of dimethylsulfoniumpropionate) (Newton *et al.* 2010; Collins *et al.* 2015). Nevertheless, associations with *Ruegeria* and other *Rhodobacteraceae* may not be as consistent as with *Endozoicomonas* and the types of symbiotic interactions may be more variable. Analysis of five sympatric Mediterranean gorgonian species did not, for example, find *Rhodobacteraceae* bacteria to be abundant members of their microbiomes (van de Water *et al.* 2017), and other studies found an increased abundance of specific *Ruegeria* and *Roseobacter* phylotypes in diseased corals (Sekar *et al.* 2006; Apprill, Hughen and Mincer 2013). However, the *E. labiata* specimens of this study appeared healthy with no macroscopic signs of necrosis or disease.

Some recently described bacterial taxa not yet typically known for their association with gorgonian corals were also detected in *E. labiata*. For example, the third most abundant *E. labiata*-associated genus was Candidatus *Actinomarina*, a small, rhodopsin-containing photoheterotroph actinobacterium. Other examples include *Marinimicrobia* (formerly SAR406) and *Parcubacteria* (formerly OD1), although these were only observed at low abundance. Ecogenomic techniques are now providing insights into potential metabolic functions and lifestyles of these phyla. Reconstructed genomes of fermenting *Parcubacteria* reveal symbiotic lifestyle signatures, including adhesion proteins, type IV secretion systems and the lack of certain biosynthetic capabilities (Nelson and Stegen 2015). *Marinimicrobia* are (potentially synthrophically and fermentatively) degrading amino acids by detritus proteolysis (Nobu *et al.* 2015), and could contribute to carbon- and nitrogen cycling in their hosts. Detection of the ammonium-oxidising Thaumarchaeon *Nitrosopumilus* and nitrite-oxidising bacterium *Nitrospira* in *E. labiata* suggests that nitrification processes are occurring, while the abundance of a wide range of heterotrophic, nitrate-reducing *Alpha-* and *Gammaproteobacteria* (e.g. certain species of *Endozoicomonas*, *Shewanella*, *Labrenzia* and *Kiloniella)* indicates complementary denitrification processes.

Cultivability of *Eunicella labiata* symbionts

This study provides the first comprehensive assessment of the cultivability of bacterial symbionts from a healthy, temperate gorgonian. Understanding whether the dominant members of a community or preferentially the rare ones can be cultivated, or whether communities resulting from cultivation-dependent and cultivation-independent methods share a majority of microbial phylotypes, is instrumental to exploit and manipulate the metabolic breadth encrypted in natural microbial consortia. Employing a similar 'plate-wash' strategy to that used in the current study, an investigation into the cultivability of bacterial communities associated with two marine sponges (*Sarcotragus spinosulus* and *Ircinia variabilis*) revealed that between 3% and 10% of the prokaryotic diversity residing in these sponges could be cultivated (Hardoim *et al.* 2014). Here, we show that as much as 62% of the *E. labiata*-associated phylotypes may be cultivated. This is in sharp contrast to the widespread 'great plate count anomaly' dogma of 0.1%–1.0% (Staley and Konopka 1985) and well above the previous study using sponges as host models (Hardoim *et al.* 2014). More importantly, all of the top 10 most abundant OTUs detected in *E. labiata* by cultivation-independent means were present in the 'plate-wash' samples (with >12 reads per sample) and 7 out of these top 10 cultivation-independent *E. labiata* phylotypes were also among the 10 most abundant phylotypes on the culture plates. These seven dominant OTUs affiliated with the genera *Ruegeria*, *Vibrio*, *Shewanella* and *Sphingorhabdus,* whereby the development of *Sphingorhabdus* into visible CFUs was, in particular, favoured by the longer incubation. Therefore, many dominant *E. labiata* symbionts can be readily cultivated, and lower incubation temperatures and limited carbon, along with longer incubation, are an adequate methodology to enlarge the phylogenetic spectrum of symbiotic bacteria amenable to cultivation, especially if the consortium in question is represented by numerous *Alphaproteobacteria* species. Our 'plate-wash' results are consistent with those from conventional isolation of single colonies that also retrieved many *Ruegeria* (37%), *Vibrio* (9%) and *Sphingorhabdus* (5%) isolates. However, with 515 OTUs identified in the 'plate-wash' samples (versus 37 OTUs obtained by single-colony isolation), the 'plate-wash' methodology scored a much higher bacterial richness than the conventional approach. The 'plate-wash' analysis also detected eight 'cultivation-specific' OTUs not seen in any of the cultivation-independent datasets. It has been noted earlier that cultivation-independent methods failed to detect bacteria isolated with culture-dependent procedures (Donachie, Foster and Brown 2007; Hardoim *et al.* 2014), showing that cultivation-independent studies do not always represent community structures exactly, either because of (still) somewhat low sequencing efforts (Donachie, Foster and Brown 2007; Hardoim *et al.* 2014) or stochastic/systematic amplicon sequencing biases (Kircher, Heyn and Kelso 2011); moreover, most cultivation conditions tend to enrich low-abundant symbiont community members (Hardoim *et al.* 2014).

About one third (36%) of the phylotypes found in the *E. labiata* 'plate-washes' were only represented by a single sequence read. Possibly, these were not derived from living colonies but from dead cells present in the initial microbial cell suspension, or cells not cultivatable under the conditions used here, but whose DNA—resistant to degradation—remained on the plate. On the other hand, some of the so-called difficult-to-cultivate phylotypes (e.g. members of candidate *Actinomarina*, SAR11, SAR86, SAR116) were unexpectedly detected in the 'plate-wash' samples with considerable sequence read numbers, suggesting that they may have been growing on the plates for some time. This emphasises that our cultivation strategy is not only able to cultivate many of the dominant *E. labiata* phylotypes but may also be useful for the cultivation of novel bacteria taxa usually difficult to cultivate. Among these unexpected phylotypes could also be syntrophic microorganisms, or those that only develop microcolonies invisible to the naked eye but that can be captured by the 'plate-wash' procedure employed here. This could explain why these taxa were not among those 175 strains that were isolated using conventional colony purification procedures. Interestingly, 89% of the isolates had their closest NCBI BlastN hits associated with marine host organisms, mostly marine invertebrates (particularly sponges), and to a lesser extent with marine vertebrates, algae and seaweed. This suggests that the bacterial isolates obtained here are generally well adapted to a symbiotic lifestyle and possess a high genome plasticity that allows them to reside in a multitude of marine hosts. Yet the most dominant *Endozoicomonas*-like phylotype detected in the cultivation-independent *E. labiata* samples was not among the 175 isolates retrieved in this study, and was found only at low abundance (0.12%) in the cultivation-dependent 'plate-washes', indicating that these symbionts may have specific metabolic requirements that need to be clarified.

In conclusion, the direct comparison between cultivation-dependent and independent communities shows that simple modifications to standard cultivation protocols (lower incubation temperatures, diluted medium, longer incubation) allow the cultivation of prevalent bacterial associates of temperate gorgonians. In addition to the evidence that the microbial community associated with *E. labiata* is distinct from the surrounding bacterioplankton community, and possibly selected by the host, this consortium is structured by many symbionts amenable to cultivation which can now undergo genome sequencing and comprehensive metabolic tests in the laboratory. Therefore, *E. labiata* and the here cultivated bacterial associates form an excellent model system for future studies on the mechanisms of gorgonian-microbe functioning and the stability and plasticity of these interactions in controlled mesocosms.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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ETHICAL STATEMENT

This article does not contain any studies with human participants performed by any of the authors. This study was exempt from ethical approval procedures according to the current Portuguese legislation. All procedures involving animals were in accordance with the ethical standards of the institution (Centre of Marine Sciences (CCMAR), Faro, Portugal) at which the study was conducted. This study did not involve endangered or protected gorgonian species (according to the IUCN red list of threatened species, 22/06/2017: http://www.iucnredlist.org/searc). The sampling of gorgonians did not occur within privately owned or protected areas. Sampling procedures were minimally intrusive and preserved gorgonian colonies at the field site.

ACCESSION NUMBERS

16S rRNA gene Sanger sequences of the *Eunicella labiata* bacterial isolates have been deposited at NCBI GenBank under the accession numbers [MF461358-MF461394]. This study was registered at the European Molecular Biology Laboratory (EMBL) under the study accession number [PRJEB21720]. Illumina sequences of all replicate samples were deposited at EMBL under the sample accession numbers [ERS1816551–ERS1816560].

AUTHOR CONTRIBUTIONS

AL-L, NCMG and RC secured funding and provided reagents and materials; TK-C and RC designed the study; JMSG collected the samples; TK-C and RC performed the experiments; TK-C and DE analysed the data and prepared figures; TK-C wrote the first manuscript draft; all authors revised and commented on the manuscript.

Conflicts of interest: None declared

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