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Microbial diversity in the coralline sponge *Vaceletia crypta*

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Abstract Coralline sponges of the genus *Vaceletia* are regarded as 'living fossils', the only recent members of the so-called 'sphinctozoan-type' sponges that contributed to reef-building during the Palaeozoic and Mesozoic eras. *Vaceletia* species were thought to be extinct until the discovery of *Vaceletia crypta* in the 1970s. Here, we used molecular methods to provide first insights into the microbial diversity of these coralline sponges. Both denaturing gradient gel electrophoresis (DGGE) analyses of 19 *Vaceletia* specimens and the analysis of 427 clones from a bacterial 16S rRNA gene clone library of a specimen of *V. crypta* from the Great Barrier Reef (Australia) revealed high diversity and a complex composition with a relatively uniform phylogenetic distribution. Only a single archaeal 16S rRNA phylotype was recovered. The most abundant bacteria were the *Chloroflexi* (35 %). Of the microbial community, 58 % consisted of the *Gammaproteobacteria, Gemmatimonadetes, Actinobacteria, Nitrospira, Deltaproteobacteria*, *Deferribacteres* and *Acidobacteria*, with nearly equal representation. Less abundant members of the microbial community belonged to the *Alphaproteobacteria* (3 %), as well as to the *Poribacteria, Betaproteobacteria, Cyanobacteria, Spirochaetes*, *Bacteroidetes*, *Deinococcus-Thermus* and *Archaea* (all together 4 %). Of the established 96 OTUs, 88 % were closely related to other sponge-derived sequences and thereof 71 OTUs fell into sponge- or sponge-coral specific clusters, which underscores that the "living fossil" coralline sponge *Vaceletia* shares features of its microbial community with other sponges. The DGGE cluster analysis indicated distinct microbial communities in the different growth forms (solitary and colonial) of *Vaceletia* species.

Keywords Microbial diversity · Community composition · Sponge · *Vaceletia crypta*

Introduction

Sponges (phylum Porifera) are arguably the earliest branching Metazoa (Philippe et al. 2009, 2011), with a fossil record dating back nearly 700 million years (Erwin et al. 2011). Sponges harbor rich and diverse microbial communities in their tissues (for a comprehensive review see Taylor et al. 2007). In the so-called 'high microbial abundance sponges' or 'bacteriosponges', microbes can make up to 40 % of the biomass of the host, whereas the

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'low microbial abundance sponges' harbor relatively small numbers of microorganisms (Reiswig 1981). Currently, 30 bacterial phyla, two major lineages of the *Archaea* and several types of eukaryotic microbes associated with sponges have been identified (Hentschel et al. 2002, 2003; Taylor et al. 2007; Hardoim et al. 2009; Webster et al. 2010; Schmitt et al. 2012). Phylogenetic analyses have indicated a low temporal variability in marine sponge-associated microbial communities, which are fairly stable within individuals and through time (Taylor et al. 2004; Webster et al. 2004; Hentschel et al. 2006). Hentschel et al. (2002) established criteria to define monophyletic, sponge-specific "clusters" (SSC): a group of at least three sequences that (i) are found in different host sponge species and/or from different geographical locations, (ii) are more similar to each other than to any other sequence from a non-sponge source and (iii) are grouped together into one clade independent of the tree construction method. This and further studies have shown that taxonomically distantly related sponges with geographically non-overlapping distribution patterns and with host-specific secondary metabolite profiles contain surprisingly uniform microbial signatures (for a review see Taylor et al. 2007). However, a recent pyrosequencing study by Webster and colleagues reported the presence of *Poribacteria* and 17 of the other 33 currently reported sponge-specific groups in seawater (Webster et al. 2010; Taylor et al. 2012). Therefore, this study questions the hypothesis that some groups of microbes are restricted to the sponge host and distinct from those in the surrounding seawater (Taylor et al. 2004).

Coralline sponges, also known as sclerosponges, are unique members of phylum Porifera (Reitner 1992; Wörheide 2008) because they build a solid secondary calcareous skeleton (Reitner 1992; Chombard et al. 1997) in addition to a primary, often spicular, skeleton. During long periods of the Earth's history, sclerosponges were dominant, diverse and abundant reef-building organisms (Vacelet 1985). These organisms were thought to be extinct until their rediscovery in the late 1960s (Hartman 1969). Today, only approximately 15 taxa live, mainly restricted to the cryptic niches of coral reefs with reduced light and oligothrophic conditions, such as caves and deeper fore-reef areas (Reitner 1992; Wörheide 1998). Sclerosponge genera, such as *Acanthochatetes*, *Vaceletia* or *Astrosclera*, are regarded as "living fossils" due to their occupation of the same ecological niches for hundreds of millions of years. In addition, these organisms display very similar morphological characteristics when compared to their fossil relatives that lived millions of years ago (Reitner et al. 2001). Therefore, coralline sponges might provide insight into the evolution of sponge-microbial symbioses. Fossil records from Silurian microbial reefs, with stromatoporoids neighbouring ubiquitous microbial laminae or less commonly encrusted by cyanobacteria, might already indicate those close associations (Soja et al. 2003).

Sponges of the genus *Vaceletia*, which was thought to be extinct until its rediscovery in the 1970s (Vacelet 1977), systematically belong to the Keratosa, a group of sponges devoid of a primary mineralized skeleton (Wörheide 2008). Bacteria may make up more than 50 % of the entire biomass of the sponge (Reitner and Wörheide 2002). *Vaceletia* species occur in two putative sister-species with different growth modes (solitary vs. colonial; Wörheide and Reitner 1996). For the detailed descriptions and definitions of the solitary and colonial forms see Vacelet (1988), Vacelet et al. (1992), Wörheide and Reitner (1996).

The microbial communities in coralline sponges have yet to be investigated in detail. Here, we aimed to perform detailed characterizations and sequenced a 16S rRNA clone library of a randomly picked *Vaceletia crypta* specimen, the only validly described recent species of the genus. To further investigate whether growth mode and/or putative sister-species relationships lead to differences in microbial diversity, we additionally performed denaturing gradient gel electrophoresis (DGGE). We aimed to determine whether the microbial communities of these "living fossil" sponges differ from those reported from other sponges and, by phylogenetic analyses, contribute to the question of the maintenance of sponge-microbe symbioses.

Materials and methods

Sample collection

Seventeen samples were collected by SCUBA diving at depths from 7 to 30 m at several sampling sites in the Coral Sea and Pacific Ocean. Sponges were excised from the substrate using a chisel and a hammer and transferred directly (underwater) to plastic bags. Two samples were collected by a remotely operated vehicle (ROV) at depths from 200 to 250 m. The sampling details for all samples used are listed in Table 1.

After collection, sponge samples were preserved either in silica gel (Erpenbeck et al. 2004), DMSO buffer (20 % DMSO, 0.25 M EDTA and NaCl to saturation, pH 8.0; adapted from Seutin et al. 1991) or 95 % ethanol.

DNA extraction

Samples were rinsed with autoclaved Millipore water and the preserved living tissue was cut and crushed aseptically with a sterile scalpel on a Petri dish. Total DNA was extracted from 3 mg of tissue using a Qiagen DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions.

PCR amplification and cloning of the 16S rRNA genes of *V. crypta* from Yonge Reef from Great Barrier Reef (GBR), Australia (sample no. GW947)

The bacterial 16S rRNA genes were amplified from the DNA extract obtained by PCR using GoTaq polymerase (Promega GmbH, Mannheim, Germany) and universal bacterial primers (616F: 5′-AGA GTT TGA TYM TGG CTC AG-3′ and 1525R: 5′-AGA AAG GAG GTG ATC CAG CC-3′) (Lane 1991). Cycling conditions for the Biometra thermocycler were as follows: an initial denaturation step (2 min at 95 °C) followed by 35 cycles of denaturation (30 s at 94 °C), primer annealing (1 min at 55 °C), elongation (2 min + 4 s at 72 °C) and a final extension step (5 min at 72 °C). After purification using the mi-PCR Purification Kit (metabion GmbH, Martinsried, Germany), the DNA was subsequently cloned into the plasmid cloning vector using an Invitrogen TOPO® TA Cloning Kit for Sequencing according to the manufacturer's instructions (Life Technologies GmbH, Darmstadt, Germany). The inserts of 427 clones were PCR reamplified using vector specific primers (M13) (Sambrook and Russell 2001) and Promega GoTaq polymerase. Analytical digestions of PCR products of 1,500 base pairs length were performed in single reactions using the restriction enzyme *Msp*I (Fermentas GmbH, St. Leon-Rot,

Table 1 Sample data of investigated *Vaceletia* sp. specimens, with collection sites details

	Sample No.	Location	Site (location)	Depth (m)	Date	Latitude	Longitude
Solitary							
V. crypta	GW947	Coral Sea	Yonge Reef	8	2006	14°57.212 S	145°61.489 E
V. sp.	GW5147.1	Palau	Siaes Tunnel #1	6	2000	7°30 N[a]	134°30 E[a]
V. sp.	GW5147.2	Palau	Siaes Tunnel #2	6	2000	7°30 N[a]	134°30 E[a]
V. sp.	GW5147.3	Palau	Siaes Tunnel #3	6	2000	7°30 N[a]	134°30 E[a]
V. sp.	GW727		Solomon Islands	200	2000	8°00 S[a]	159°00 E[a]
V. crypta	GW5450	Coral Sea	Osprey Reef #1	12	1995	13°53.392 S	146°33.267 E
V. crypta	G 313971	Coral Sea	Osprey Reef #2	10	1999	13°48.063 S	146°32.731 E
V. crypta	G 313989	Coral Sea	Osprey Reef #3	9	1999	13°53.392 S	146°33.267 E
V. crypta	G 316280	Coral Sea	Osprey Reef #4	30	2002	13°50.09 S	146°33.07 E
V. sp.	G 316297	Coral Sea	Holmes Reef	12	2002	16°30.629 S	147°50.400 E
Colonial							
V. sp.	G 318578	Norfolk Ridge	Jumeau-West	240	2001	23°40.766 S	168°00.602 E
V. sp.	G 313956	Coral Sea	Bougainville Reef #1	10	1999	15°28.934 S	147°06.076 E
V. sp.	G 316289	Coral Sea	Bougainville Reef #2	25	2002	15°28.934 S	147°06.076 E
V. sp.	G 316284	Coral Sea	Osprey Reef #5	14	2002	13°53.5 S	146°33.1 E
V. sp.	G 316001	Coral Sea	Osprey Reef #4	8	1999	13°56.594 S	146°35.909 E
V. sp.	G 313993	Coral Sea	Osprey Reef #6	10	1999	13°53.428 S	146°33.300 E
V. sp.	G 313986	Coral Sea	Osprey Reef #7	7	1999	13°49.803 S	146°33.940 E
V. sp.	G 313979	Coral Sea	Osprey Reef #8	15	1999	13°49.744 S	146°33.958 E
V. sp.	G 313972	Coral Sea	Osprey Reef #9	10	1999	13°48.063 S	146°32.731 E

[a] As the exact coordinates for the sampling sites in Palau and Solomon Islands were not available, the given coordinates are based on the Gazetteer of Conventional Names, Third Edition, August 1988, US Board on Geographic Names

Germany) following the manufacturer's instructions. Based on the restriction patterns, similar clones were grouped together and chosen randomly for sequencing. Clones with undefined restriction patterns were additionally taken for sequencing. Prior to sequencing, amplified inserts were purified using a silica-based protocol modified after Boyle and Lew (1995).

For the amplification of archaeal 16S rRNA gene from the DNA extract, touchdown PCR using universal archaeal primers (21F: 5′-TTC CGG TTG ATC CYG CCG GA-3′ and 915R 5′-GTG CTC CCC CGC CAA TTC CT-3′) (DeLong 1992; Raskin et al. 1994) and an annealing temperature decreasing from 60 to 50.5 °C (30 s each) in 0.5 °C increments was employed. The cycling conditions for the Biometra thermocycler using Promega GoTaq were as follows: one cycle of initial denaturation (2 min at 95 °C); 35 cycles of denaturation (30 s at 94 °C), primer annealing (30 s from 60 °C minus 0.5 °C), and elongation (2 min + 4 s at 72 °C) followed by 25 cycles of denaturation (30 s at 94 °C), primer annealing (30 s at 51 °C), and elongation (2 min + 4 s at 72 °C) and a final extension step (5 min at 72 °C). A strong band of approx. 900 base pairs was excised and the DNA was purified from an agarose gel using the E.Z.N.A Gel Extraction Kit (VWR International GmbH, Darmstadt, Germany) following the manufacturer's instructions and subsequently taken for sequencing.

Sequencing

Sequencing was performed by the Genomics Service Unit (Ludwig-Maximilians-Universität München) using the BigDye® Terminator v3.1 on a 48-capillary sequencer (ABI 3730, Applied Biosystems). For the cloned bacterial inserts, the primers: 610RII (5′-ACC GCG/T A/GCT GCT GGC AC-3′) (Dotzauer et al. 2002), 616F and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Lane 1991), or 614F (5′-GTG CAT GGC TGT CGT CAG CTC G-3′) (this study) were used. The archaeal PCR product was sequenced with AR20F primer (5′-TTC CGG TTG ATC CYG CCRG-3′) (Moyer et al. 1998). CodonCode Aligner (http://www.codoncode.com/aligner/) was used for sequence editing and assembly. Sequences were checked for chimeras using the Bellerophon web applications (Huber et al. 2004). Chimera sequences were removed before further analyses.

Phylogenetic analyses

Phylogenetic analyses were performed using ARB (Ludwig et al. 2004). The initial ARB database was constructed from the SILVA Small Subunit rRNA Database (release 96; (Pruesse et al. 2007) and from the database constructed by Taylor and colleagues, which contains sponge–derived sequences along with their closest relatives (Taylor et al. 2007). Our sequences were compared to the sequences available in public databases using BLAST (http://blast.ncbi.nlm.nih.gov/) and the nearest relative sequences obtained from different sponges, corals and non-sponge sources, were also incorporated into the ARB Database. The sequences were aligned using the ARB Integrated Aligner. The alignment was checked and corrected manually for alignment errors. The partial sequences were added to the ARB database using the ARB parsimony "quick add" tool. Initial phylogenetic trees were evaluated using the neighbour-joining algorithm (Jukes–Cantor correction) using ARB. Subsequently, the alignment was exported from the ARB database and maximum likelihood trees were constructed using RAxML (v.7.2.5; (Stamatakis 2006), 1,000 bootstrap replicates and the GTR+ GAMMA model of sequence evolution. The resulting trees were visualized using the program FigTree (v.1.3.1) (http://tree.bio.ed.ac.uk/software/figtree/). Monophyletic, sponge-specific and sponge-coral clusters (SSC/SCC) were defined based on established criteria (Hentschel et al. 2002). Sequences obtained from the sponges and corals that grouped together into one clade independent of the tree reconstruction method (neighbour-joining and maximum likelihood) were regarded as SSC and/or SCC.

Estimation of microbial diversity and statistical analyses

Based on the distance matrix generated by ARB, the sequences were assigned to operational taxonomic units (OTUs) using Mothur (Schloss et al. 2009). The clones that were only analyzed by restriction digestion were assigned to corresponding OTUs based on their restriction patterns. For the analysis of an OTU, a cut-off value of 0.03 was used (Schloss and Handelsman 2005). The rarefaction curves were also calculated using Mothur. The curves were plotted using the R software package (http://www.R-project.org). The

Shannon–Wiener index (Spellerberg and Fedor 2003) was calculated to determine the abundance and richness of the bacterial community associated with *V. crypta*. The Chao1 index (Colwell and Coddington 1994) was employed to estimate total species richness. Calculations were performed using the Mothur software. In order to determine the phylogenetic composition of the clone library constructed from the microbial community associated with *V. crypta*, the percentage for each phylogenetic group was calculated based on the number of clones assigned to the particular group.

Denaturing gradient gel electrophoresis (DGGE)

Nineteen samples of members of the genus *Vaceletia* representing colonial (9 samples) and solitary (10 samples) growth forms were used for DGGE. All of the samples used are listed in Table 1. The bacterial 16S rRNA genes were amplified from the DNA extracts using touchdown PCR, Promega GoTaq polymerase and the universal bacterial primers 341F-GC and 907RC (Muyzer and Smalla 1998; Schäfer 2001). Cycling conditions for the Biometra thermocycler were as follows: one cycle of initial denaturation (2 min at 95 °C); 15 cycles of denaturation (30 s at 94 °C), primer annealing (30 s from 58 °C minus 0.5 °C), and elongation (2 min + 4 s at 72 °C) followed by 25 cycles of denaturation (30 s at 94 °C), primer annealing (30 s from 51 °C minus 0.5 °C), and elongation (2 min + 4 s at 72 °C) and a final extension step (5 min at 72 °C).

The DGGE analysis was performed with an Ingeny phorU-2 system (Ingeny International), Power Pac 300 (BioRad) as a power supplier, and a denaturing gradient of 30–70 % (urea and formamide) in a 6 % polyacrylamide gel. Gels were run for 16 h at 180 V (60 °C), then stained for 25 min in SYBR Gold (Molecular Probes) and photographed using a RT Color SPOT camera and SPOT advance imaging software (Visitron Systems GmbH).

Gel images were analyzed using Quantity One software (version 4.69, Bio-Rad). Similarities between the DGGE banding patterns were calculated using the band-matching Dice coefficient with an optimization at 0.75 % and a tolerance level of 0.75 %. The unweighted pair-group method with arithmetic averages (UPGMA) was used for cluster analysis to obtain similarity dendrograms.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences representing all OTUs generated in this study have been deposited in EMBL database under the accession numbers HE817775 to HE817870.

Results

Clone library construction and OTU assignment

427 clones were selected from the 16S rRNA clone library amplified from a solitary form specimen of *V. crypta* from Yonge Reef (GBR, Australia), which possessed a high microbial abundance, in parts of the sponge outnumbering sponge cells (Fig. 1). From those clones, 253 were sequenced and the remaining 174 clones were assigned to a particular OTU based on their restriction patterns. A single archaeal 16S rRNA sequence was retrieved, however the multiple genotypes cannot be entirely ruled out. Three sequences were discarded as chimeras. The remaining 250 sequences were clustered into 96 OTUs using a 97 % similarity criterion. From those 96 OTUs, 39 were singletons (Fig. 2). Only 8 OTUs grouped more than 10 clones (two OTUs with 18 and 11 clones and single OTUs with 15, 22, 23 and with 30 clones, respectively).

Phylogenetic analyses

Using BLAST, 88 % of the 96 OTUs (84 OTUs) were found to be closely related with other previously described sponge- or coral-derived sequences. Of the OTUs, 70 % (67 OTUs) were related to other sponge-derived 16S rRNA genes obtained from 22 different sponge species. A further 18 % of the OTUs contained sequences obtained from four different species of corals as closest relatives. Of those OTUs, 14 were related to 16S rRNA sequences obtained from *Montastraea faveolata* and a single OTU was related to sequences obtained from *Oculina patagonica, Diploria strigosa* and *Erythropodium caribaeorum*. Only one OTU was closely related (99 % similarity) to a validly named organism (*Delftia acidovorans*), and one was distantly related (91 % similarity) to a 16S rRNA gene sequence from a chloroplast of a red alga. Further, 7 % of the OTUs had sequences from marine

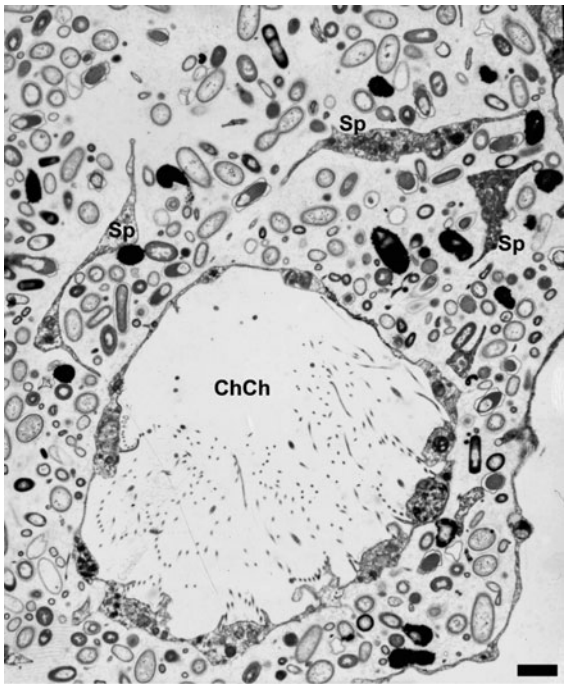


Fig. 1 TEM micrograph of the choansosome of *Vaceletia crypta* with numerous bacterial cells and a few sponge cells (Sp) in the mesohyl, as well as in the choanocyte chamber (ChCh). *Scale bar* 2 μm

environments as next relatives (3 OTUs from seawater, 1 OTU from basaltic glass from a seamount and 3 OTUs from sediment, with one of the 3 from deep sea). Three OTUs contained sequences from the terrestrial subsurface as next relatives. The results of the BLAST search are summarized in the supplementary Table (ESM 1 of the Online Resource).

Fig. 3 Maximum likelihood phylogeny of *V. crypta*-derived 16S rRNA sequences affiliated to the phylum *Chloroflexi* with next similar sequences obtained from other sponges or corals, and from the environment. Reference sequences are listed with their GenBank numbers. *Bold text* signifies clones obtained during this study. *Shaded boxes* represent sponge-specific clusters; the percentage values next to the boxes indicate the similarity between the sequences belonging to the clusters. Bootstrap analysis was based on 1,000 replicates—the support values 70–85 % are indicated by *asterisk*. *Scale bar* signifies 10 % sequence divergence

The phylogeny obtained with ARB showed that the majority of the sponge-derived microbial clones were assigned to the *Chloroflexi* (39 OTUs, number of clones $n = 144$) and *Gammaproteobacteria* (13 OTUs, $n = 46$). Clones affiliated with the *Deltaproteobacteria* (9 OTUs, $n = 29$), *Acidobacteria* (6 OTUs, $n = 24$), *Gemmatimonadetes* (5 OTUs, $n = 46$), and *Alphaproteobacteria* (4 OTUs, $n = 11$) were also observed. Numerous clones affiliated to the *Nitrospirae* and *Actinobacteria* were associated with a single OTU ($n = 30$ clones) or three OTUs ($n = 34$), respectively. The minor components of the clone library were clones affiliated with the *Poribacteria* (3 OTUs, $n = 4$), *Betaproteobacteria* (2 OTUs, $n = 3$), *Cyanobacteria* (2 OTUs, $n = 3$), *Spirochaetes* (1 OTU, $n = 2$), *Deinococcus-Thermus* (1 OTU, $n = 1$), and *Bacteroidetes* (1 OTU, $n = 1$). The single sequence obtained by PCR using universal archaeal primers was affiliated to the *Crenarchaeota*. Over 9 % of the clone sequences (5 OTUs, $n = 30$) were not

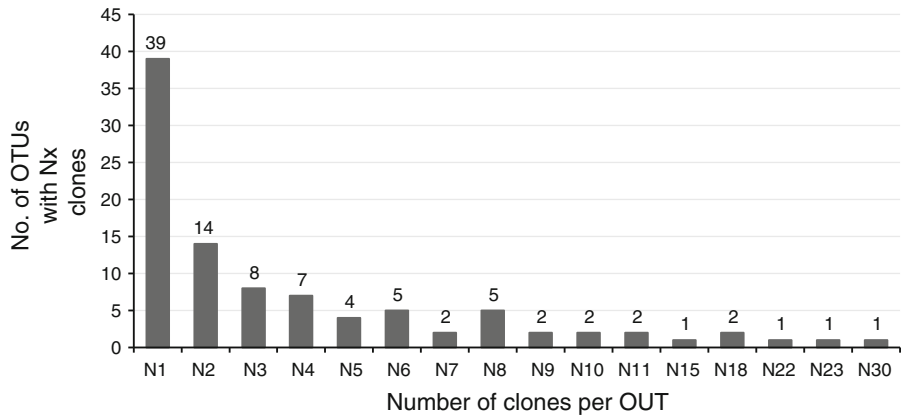
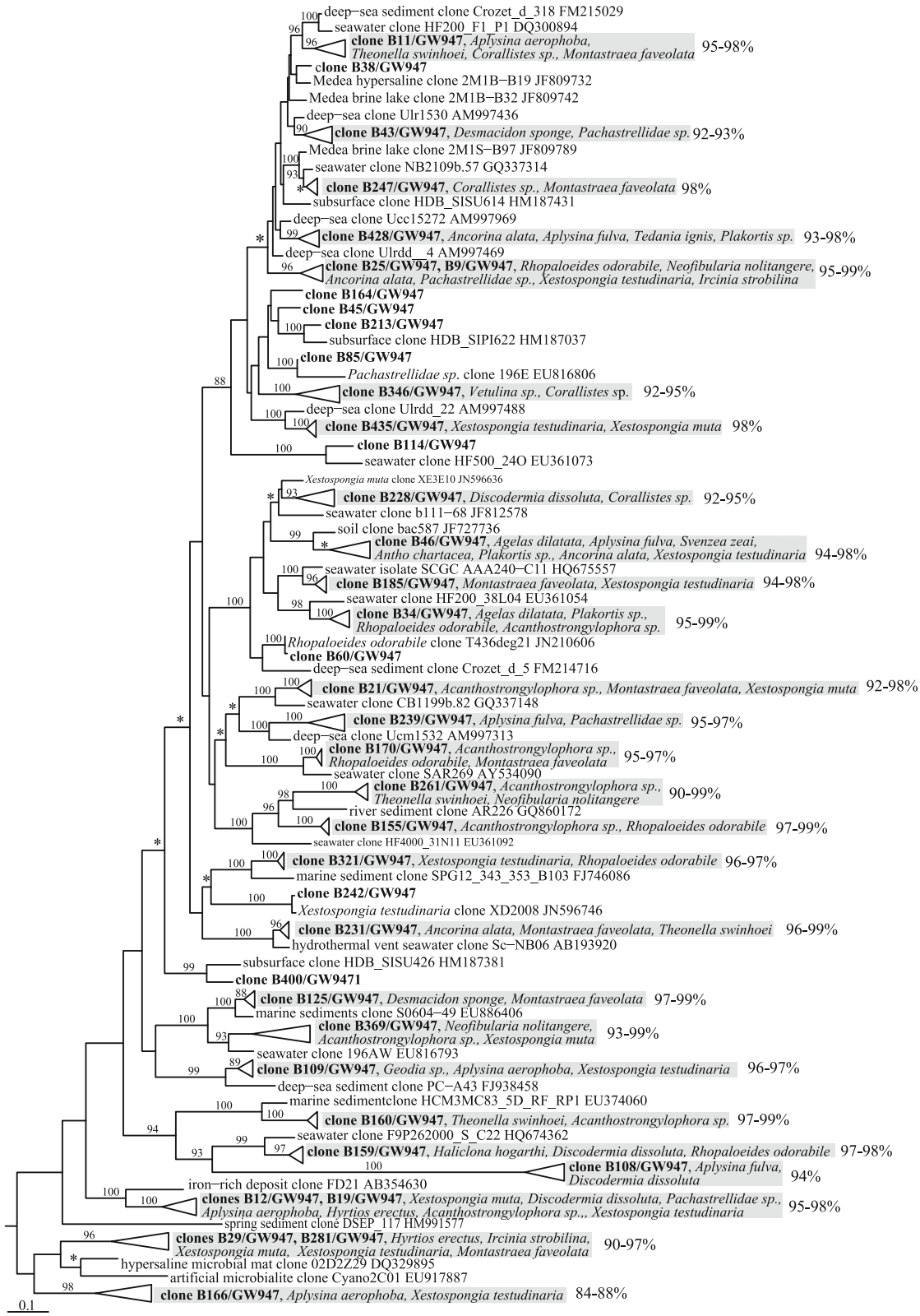


Fig. 2 Distribution of 16S rRNA gene clones among the OTUs defined at the 97 % similarity criterion. * *N1* number of singletons, *N2* number of doubletons, etc


deep−sea sediment clone Crozet_d_318 FM215029
seawater clone HF200_F1_P1 DQ300894
clone B11/GW947, Aplysina aerophoba, Theonella swinhoei, Corallistes sp., Montastraea faveolata 95-98%
clone B38/GW947
Medea hypersaline clone 2M1B−B19 JF809732
Medea brine lake clone 2M1B−B32 JF809742
deep−sea clone Ulr1530 AM997436
clone B43/GW947, Desmacidon sponge, Pachastrellidae sp. 92-93%
Medea brine lake clone 2M1S−B97 JF809789
seawater clone NB2109b.57 GQ337314
clone B247/GW947, Corallistes sp., Montastraea faveolata 98%
subsurface clone HDB_SISU614 HM187431
deep−sea clone Ucc15272 AM997969
clone B428/GW947, Ancorina alata, Aplysina fulva, Tedania ignis, Plakortis sp. 93-98%
deep−sea clone Ulrdd__4 AM997469
clone B25/GW947, B9/GW947, Rhopaloeides odorabile, Neofibularia nolitangere, Ancorina alata, Pachastrellidae sp., Xestospongia testudinaria, Ircinia strobilina 95-99%
clone B164/GW947
clone B45/GW947
clone B213/GW947
subsurface clone HDB_SIPI622 HM187037
clone B85/GW947
Pachastrellidae sp. clone 196E EU816806
clone B346/GW947, Vetulina sp., Corallistes sp. 92-95%
deep−sea clone Ulrdd_22 AM997488
clone B435/GW947, Xestospongia testudinaria, Xestospongia muta 98%
clone B114/GW947
seawater clone HF500_24O EU361073
Xestospongia muta clone XE3E10 JN596636
clone B228/GW947, Discodermia dissoluta, Corallistes sp. 92-95%
seawater clone b111−68 JF812578
soil clone bac587 JF727736
clone B46/GW947, Agelas dilatata, Aplysina fulva, Svenzea zeai, Antho chartacea, Plakortis sp., Ancorina alata, Xestospongia testudinaria 94-98%
seawater isolate SCGC AAA240−C11 HQ675557
clone B185/GW947, Montastraea faveolata, Xestospongia testudinaria 94-98%
seawater clone HF200_38L04 EU361054
clone B34/GW947, Agelas dilatata, Plakortis sp., Rhopaloeides odorabile, Acanthostrongylophora sp. 95-99%
Rhopaloeides odorabile clone T436deg21 JN210606
clone B60/GW947
deep−sea sediment clone Crozet_d_5 FM214716
clone B21/GW947, Acanthostrongylophora sp., Montastraea faveolata, Xestospongia muta 92-98%
seawater clone CB1199b.82 GQ337148
clone B239/GW947, Aplysina fulva, Pachastrellidae sp. 95-97%
deep−sea clone Ucm1532 AM997313
clone B170/GW947, Acanthostrongylophora sp., Rhopaloeides odorabile, Montastraea faveolata 95-97%
seawater clone SAR269 AY534090
clone B261/GW947, Acanthostrongylophora sp., Theonella swinhoei, Neofibularia nolitangere 90-99%
river sediment clone AR226 GQ860172
clone B155/GW947, Acanthostrongylophora sp., Rhopaloeides odorabile 97-99%
seawater clone HF4000_31N11 EU361092
clone B321/GW947, Xestospongia testudinaria, Rhopaloeides odorabile 96-97%
marine sediment clone SPG12_343_353_B103 FJ746086
clone B242/GW947
Xestospongia testudinaria clone XD2008 JN596746
clone B231/GW947, Ancorina alata, Montastraea faveolata, Theonella swinhoei 96-99%
hydrothermal vent seawater clone Sc−NB06 AB193920
subsurface clone HDB_SISU426 HM187381
clone B400/GW9471
clone B125/GW947, Desmacidon sponge, Montastraea faveolata 97-99%
marine sediments clone S0604−49 EU886406
clone B369/GW947, Neofibularia nolitangere, Acanthostrongylophora sp., Xestospongia muta 93-99%
seawater clone 196AW EU816793
clone B109/GW947, Geodia sp., Aplysina aerophoba, Xestospongia testudinaria 96-97%
deep−sea sediment clone PC−A43 FJ938458
marine sedimentclone HCM3MC83_5D_RF_RP1 EU374060
clone B160/GW947, Theonella swinhoei, Acanthostrongylophora sp. 97-99%
seawater clone F9P262000_S_C22 HQ674362
clone B159/GW947, Haliclona hogarthi, Discodermia dissoluta, Rhopaloeides odorabile 97-98%
clone B108/GW947, Aplysina fulva, Discodermia dissoluta 94%
iron−rich deposit clone FD21 AB354630
clones B12/GW947, B19/GW947, Xestospongia muta, Discodermia dissoluta, Pachastrellidae sp., Aplysina aerophoba, Hyrtios erectus, Acanthostrongylophora sp.,, Xestospongia testudinaria 95-98%
spring sediment clone DSEP_117 HM991577
clones B29/GW947, B281/GW947, Hyrtios erectus, Ircinia strobilina, Xestospongia muta, Xestospongia testudinaria, Montastraea faveolata 90-97%
hypersaline microbial mat clone 02D2Z29 DQ329895
artificial microbialite clone Cyano2C01 EU917887
clone B166/GW947, Aplysina aerophoba, Xestospongia testudinaria 84-88%
0.1

classified using ARB database to any described phylum. However, based on the EMBL phylogeny the available sequences that were most similar implied an affiliation of these sequences with the phylum *Deferribacteres*. The phylogenetic trees present the OTUs with nearest similar sequences assigned to the *Chloroflexi* (Fig. 3), *Proteobacteria* (Fig. 4) and to all other phyla (Fig. 5).

Sponge-specific and sponge-coral clusters

From the 84 OTUs that contained sequences similar to sequences obtained from other sponges or corals, 71 OTUs (85 %) were assigned to 63 SSC or SCC. The largest number of clusters belonged to the phylum *Chloroflexi* (27 clusters with 30 OTUs). A further 15 clusters were defined among the *Proteobacteria* (15 clusters with 16 OTUs). The SSC and SCC are indicated with grey-shaded boxes in the phylogenetic trees (Figs. 3, 4, 5).

Estimation of microbial diversity and statistical analyses

The microbial community composition was calculated for all clones affiliated to each phylogenetic group and revealed a high diversity with a complex composition (Fig. 6). The most abundant taxa were the *Chloroflexi* (35 %). Due to the complexity, variety and diversity of the phylum *Proteobacteria,* the proteobacterial classes were treated as separate phylogenetic groups (*Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria*) and as separate groups for the estimations of the microbial community composition. If, for the calculation of community composition, the *Proteobacteria* were regarded as one single group (phylum), it would be the second most abundant group in the community (22 %) behind the *Chloroflexi*.

A rarefaction analysis was used to assess whether the number of clones sequenced from the library represented the full diversity of the microbial community. The rarefaction curves calculated using 97 % and 95 % cut-off criteria for grouping OTUs at the "species" and "genus" levels as well as 90 % did not reach a clear saturation (Fig. 7). However, according to the Chao1 index (Table 2), we sequenced over 70 % of the predicted number of microbial species, which provides a representative picture of the core microbial community of *V. crypta*. Using Sanger sequencing to cover the remaining 30 % of the community would be costly and time-consuming (Schmitt et al. 2012).

Denaturing gradient gel electrophoresis

DGGE analysis of nine colonial and ten solitary forms of individuals from genus *Vaceletia* indicated that the diversity of all the bacterial communities was very high. The number of bands ranged from 27 to 35 per sample. The lowest number of bands was obtained from the bacterial community associated with a solitary form of *Vaceletia* from Palau, Siaes Tunnel (sample #3, ESM 2 of the Online Resource) and the largest number of bands was obtained from a colonial form from Bougainville Reef (sample #2, ESM 2 of the Online Resource). The banding patterns displayed numerous co-occurring bands; however, only four bands were found in all samples from both growth forms. 18 bands were specific to the samples from the solitary *Vaceletia* form and 19 to the samples from the colonial form. A cluster analysis showed that the microbial communities appear to be growth-form specific (Fig. 8); however, the solitary specimens from Palau, which clustered together, displayed a higher affiliation to the cluster of colonial samples. The bacterial profiles for the samples obtained from Norfolk Ridge (colonial form) and from Solomon Island (solitary form), both from deeper sampling zones, did not cluster with the other samples.

Discussion

To our knowledge, this is the first study assessing the phylogenetic diversity of *Bacteria* and *Archaea* in coralline sponges using molecular approaches. The 16S rRNA gene-based diversity analysis of *V. crypta* revealed that its associated microbial community is phylogenetically complex and diverse because it is composed of representatives of the *Archaea* and 13 bacterial phyla. The distribution of the sequences was relatively even between phylogenetic groups, however the largest number of sequences was affiliated with the *Chloroflexi*, which have frequently been reported as members of sponge-associated microbial communities and often as the predominant group (Hentschel et al. 2002; Webster et al. 2004; Thiel et al. 2007). In a recent work Schmitt and colleagues (2011) showed

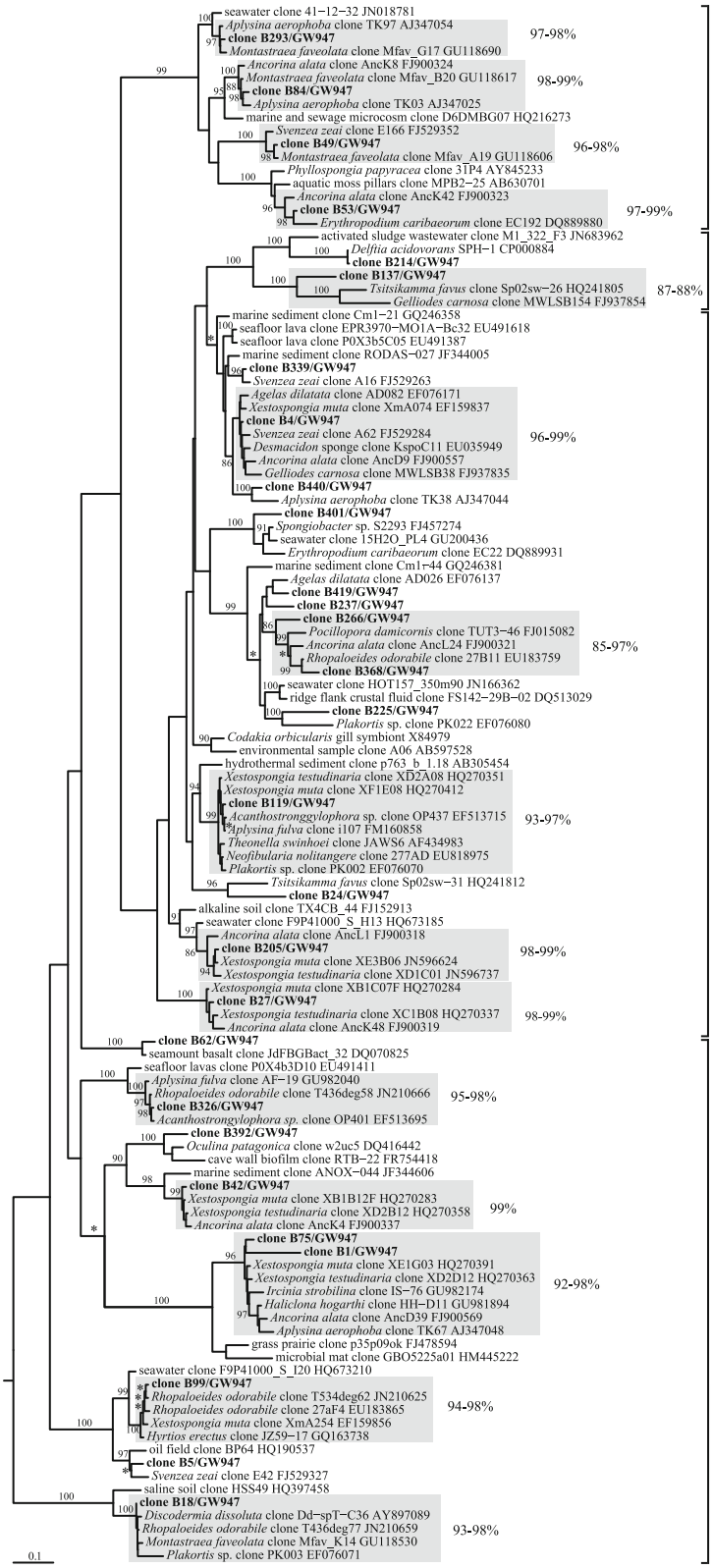
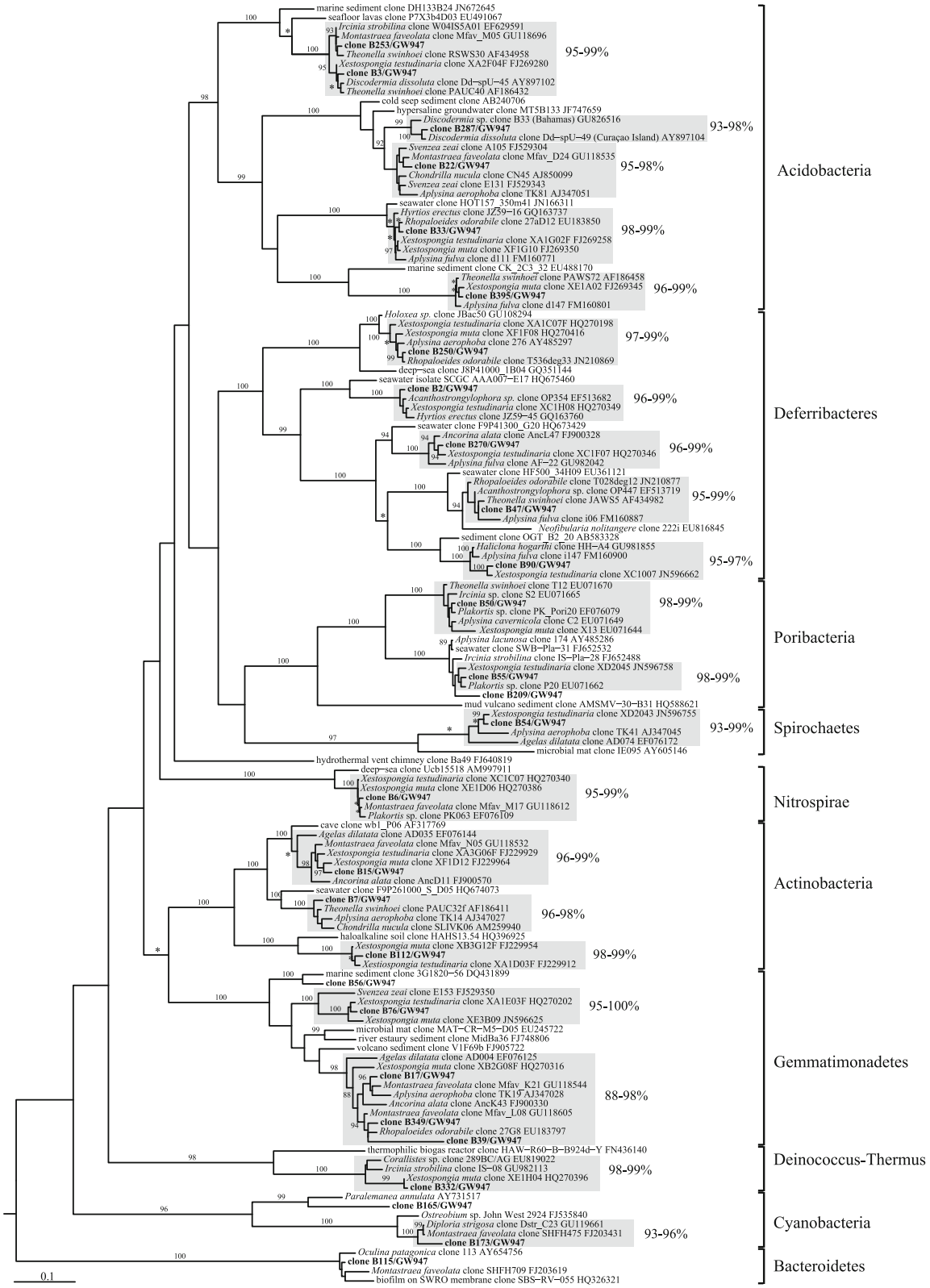


Fig. 4 Maximum likelihood phylogeny of *V. crypta*-derived 16S rRNA sequences affiliated to the phylum *Proteobacteria* with next similar sequences obtained from other sponges or corals, and from the environment. Reference sequences are listed with their GenBank numbers. *Bold text* signifies clones obtained during this study. *Shaded boxes* represent sponge-specific clusters; the percentage values next to the boxes indicate the similarity between the sequences belonging to the clusters. Bootstrap analysis was based on 1,000 replicates—the support values 70–85 % are indicated by *asterisk*. *Scale bar* signifies 10 % sequence divergence


marine sediment clone DH133B24 JN672645
seafloor lavas clone P7X3b4D03 EU491067
Ircinia strobilina clone W04IS5A01 EF629591
Montastraea faveolata clone Mfav_M05 GU118696
clone B253/GW947
Theonella swinhoei clone RSWS30 AF434958
Xestospongia testudinaria clone XA2F04F FJ269280
clone B3/GW947
Discodermia dissoluta clone Dd−spU−45 AY897102
Theonella swinhoei clone PAUC40 AF186432
95-99%
cold seep sediment clone AB240706
hypersaline groundwater clone MT5B133 JF747659
Discodermia sp. clone B33 (Bahamas) GU826516
clone B287/GW947
Discodermia dissoluta clone Dd−spU−49 (Curaçao Island) AY897104
93-98%
Svenzea zeai clone A105 FJ529304
Montastraea faveolata clone Mfav_D24 GU118535
clone B22/GW947
Chondrilla nucula clone CN45 AJ850099
Svenzea zeai clone E131 FJ529343
Aplysina aerophoba clone TK81 AJ347051
95-98%
Acidobacteria
seawater clone HOT157_350m41 JN166311
Hyrtios erectus clone JZ59−16 GQ163737
Rhopaloeides odorabile clone 27aD12 EU183850
clone B33/GW947
Xestospongia testudinaria clone XA1G02F FJ269258
Xestospongia muta clone XF1G10 FJ269350
Aplysina fulva clone d111 FM160771
98-99%
marine sediment clone CK_2C3_32 EU488170
Theonella swinhoei clone PAWS72 AF186458
Xestospongia muta clone XE1A02 FJ269345
clone B395/GW947
Aplysina fulva clone d147 FM160801
96-99%
Holoxea sp. clone JBac50 GU1108294
Xestospongia testudinaria clone XA1C07F HQ270198
Xestospongia muta clone XF1F08 HQ270416
Aplysina aerophoba clone 276 AY485297
clone B250/GW947
Rhopaloeides odorabile clone T536deg33 JN210869
97-99%
deep−sea clone J8P41000_1B04 GQ351144
seawater isolate SCGC AAA007−E17 HQ675460
clone B2/GW947
Acanthostrongylophora sp. clone OP354 EF513682
Xestospongia testudinaria clone XC1H08 HQ270349
Hyrtios erectus clone JZ59−45 GQ163760
96-99%
Deferribacteres
seawater clone F9P41300_G20 HQ673429
Ancorina alata clone AncL47 FJ900328
clone B270/GW947
Xestospongia testudinaria clone XC1F07 HQ270346
Aplysina fulva clone AF−22 GU982042
96-99%
seawater clone HF500_34H09 EU361121
Rhopaloeides odorabile clone T028deg12 JN210877
Acanthostrongylophora sp. clone OP447 EF513719
Theonella swinhoei clone JAWS5 AF434982
clone B47/GW947
Aplysina fulva clone i06 FM160887
Neofibularia nolitangere clone 222i EU816845
95-99%
sediment clone OGT_B2_20 AB583328
Haliclona hogarthi clone HH−A4 GU981855
Aplysina fulva clone i147 FM160900
clone B90/GW947
Xestospongia testudinaria clone XC1007 JN596662
95-97%
Theonella swinhoei clone T12 EU071670
Ircinia sp. clone S2 EU071665
clone B50/GW947
Plakortis sp. clone PK_Pori20 EF076079
Aplysina cavernicola clone C2 EU071649
Xestospongia muta clone X13 EU071644
98-99%
Aplysina lacunosa clone 174 AY485286
seawater clone SWB−Pla−31 FJ652532
Ircinia strobilina clone IS−Pla−28 FJ652488
Xestospongia testudinaria clone XD2045 JN596758
clone B55/GW947
Plakortis sp. clone P20 EU071662
clone B209/GW947
98-99%
Poribacteria
mud vulcano sediment clone AMSMV−30−B31 HQ588621
Xestospongia testudinaria clone XD2043 JN596755
clone B54/GW947
Aplysina aerophoba clone TK41 AJ347045
Agelas dilatata clone AD074 EF076172
93-99%
Spirochaetes
microbial mat clone IE095 AY605146
hydrothermal vent chimney clone Ba49 FJ640819
deep−sea clone Ucb15518 AM997911
Xestospongia testudinaria clone XC1C07 HQ270340
Xestospongia muta clone XE1D06 HQ270386
clone B6/GW947
Montastraea faveolata clone Mfav_M17 GU118612
Plakortis sp. clone PK063 EF076109
95-99%
Nitrospirae
cave clone wb1_P06 AF317769
Agelas dilatata clone AD035 EF076144
Montastraea faveolata clone Mfav_N05 GU118532
Xestospongia testudinaria clone XA3G06F FJ229929
Xestospongia muta clone XF1D12 FJ229964
clone B15/GW947
Ancorina alata clone AncD11 FJ900570
96-99%
seawater clone F9P261000_S_D05 HQ674073
clone B7/GW947
Theonella swinhoei clone PAUC32f AF186411
Aplysina aerophoba clone TK14 AJ347027
Chondrilla mucula clone SLIVK06 AM259940
96-98%
haloalkaline soil clone HAHS13.54 HQ396925
Xestospongia muta clone XB3G12F FJ229954
clone B112/GW947
Xestiospongia testudinaria clone XA1D03F FJ229912
98-99%
Actinobacteria
marine sediment clone 3G1820−56 DQ431899
clone B56/GW947
Svenzea zeai clone E153 FJ529350
Xestospongia testudinaria clone XA1E03F HQ270202
clone B76/GW947
Xestospongia muta clone XE3B09 JN596625
95-100%
microbial mat clone MAT−CR−M5−D05 EU245722
river estuary sediment clone MidBa36 FJ748806
volcano sediment clone V1F69b FJ905722
Agelas dilatata clone AD004 EF076125
Xestospongia muta clone XB2G08F HQ270316
clone B17/GW947
Montastraea faveolata clone Mfav_K21 GU118544
Aplysina aerophoba clone TK19 AJ347028
Ancorina alata clone AncK43 FJ900330
Montastraea faveolata clone Mfav_L08 GU118605
clone B349/GW947
Rhopaloeides odorabile clone 27G8 EU183797
clone B39/GW947
88-98%
Gemmatimonadetes
thermophilic biogas reactor clone HAW−R60−B−B924d−Y FN436140
Corallistes sp. clone 289BC/AG EU819022
Ircinia strobilina clone IS−08 GU982113
Xestospongia muta clone XE1H04 HQ270396
clone B332/GW947
98-99%
Deinococcus-Thermus
Paralemanea annulata AY731517
clone B165/GW947
Ostreobium sp. John West 2924 FJ535840
Diploria strigosa clone Dstr_C23 GU119661
Montastraea faveolata clone SHFH475 FJ203431
clone B173/GW947
93-96%
Cyanobacteria
Oculina patagonica clone 113 AY654756
clone B115/GW947
Montastraea faveolata clone SHFH709 FJ203619
biofilm on SWRO membrane clone SBS−RV−055 HQ326321
Bacteroidetes
0.1

◀ **Fig. 5** Maximum likelihood phylogeny of *V. crypta*-derived 16S rRNA sequences affiliated to several phyla with next similar sequences obtained from other sponges or corals, and from the environment. Reference sequences are listed with their GenBank numbers. *Bold text* signifies clones obtained during this study. *Shaded boxes* represent sponge-specific clusters; the percentage values next to the boxes indicate the similarity between the sequences belonging to the clusters. Bootstrap analysis was based on 1,000 replicates—the support values 70–85 % are indicated by *asterisk*. *Scale bar* signifies 10 % sequence divergence

that high microbial abundance sponges host more diverse, abundant, and similar *Chloroflexi* bacteria then low microbial abundance sponges. Of the sequences belonging to the *Chloroflexi*, 91 % of these *V. crypta*-associated sequences fell into sponge- or sponge/coral clusters (Fig. 3), which is consistent with the results of Schmitt et al. (2011).

The second most abundant group of *V. crypta* symbionts belonged to the *Proteobacteria,* which are commonly found and often predominant in microbial consortia associated with different sponges from different marine sites (Friedrich et al. 1999, 2001; Schmidt et al. 2000; Burja and Hill 2001; Webster and Hill 2001; Webster et al. 2001, 2004; Hentschel et al. 2002; Li et al. 2006). *D. acidovorans,* an obligate aerobe able to grow in 1.5 % NaCl (Wen et al. 1999), was the only validly named next relative for one of the *V. crypta* betaproteobacterial clones. *D. acidovorans* has been found in several habitats, such as soil, sediment, activated sludge, crude oil, fresh water and various clinical samples, and Kennedy et al. have reported that it is a sponge-associated bacterium (Kennedy et al. 2008).

Actinobacteria from the microbial communities of sponges have been the focus of natural product screenings (see the review by Taylor et al. 2007), since members of this phylum display the most promising biosynthetic potential for secondary metabolite production (Schneemann et al. 2010). Approximately half of the bioactive secondary metabolites that have been currently discovered in bacteria are attributed to the *Actinobacteria* (Lam 2006) and many new chemical entities and bioactive metabolites have been reported from marine members of this phylum (Blunt et al. 2004; Salomon et al. 2004; Fiedler et al. 2005; Jensen et al. 2005). In this study, two OTUs belonged to the family *Acidimicrobiaceae*, which might be involved in secondary metabolite production; however, secondary metabolites are, at present, unexplored from *Vaceletia* sponges.

Investigations on prokaryotic diversity provide first hypotheses into the putative functions of the microbial communities associated with these sponges. The presence of some clades of the ammonia-oxidizing *Beta-* and *Gammaproteobacteria* or some genera of the nitrite-oxidizing *Deltaproteobacteria/Nitrospina* and *Nitrospirae* in the community suggests that pathways for nitrogen metabolism (Bayer et al. 2008) are also present in *V. crypta*. The ammonia-oxidizing bacteria (AOB) were represented here by

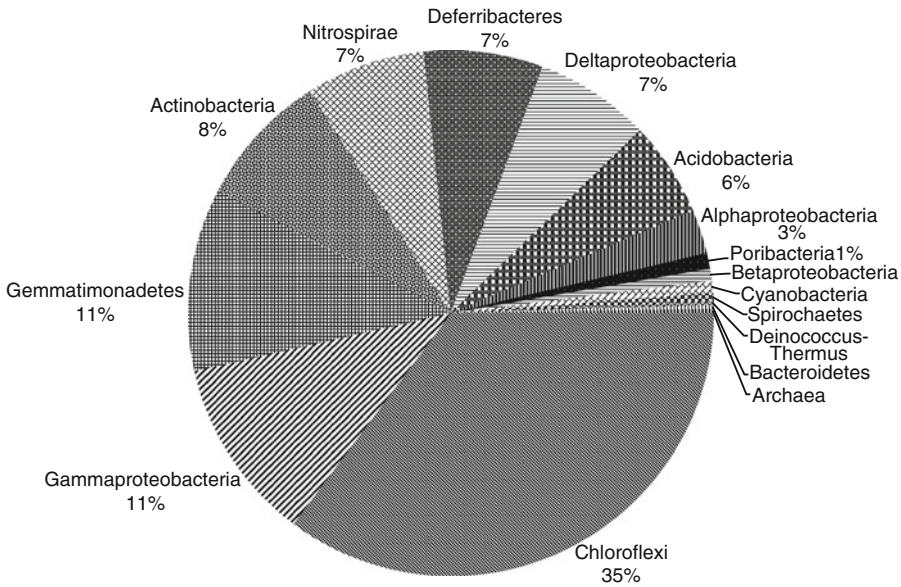


Fig. 6 Distribution of the 16S rRNA clones among particular phylogenetic groups in the clone library obtained from the *V. crypta*

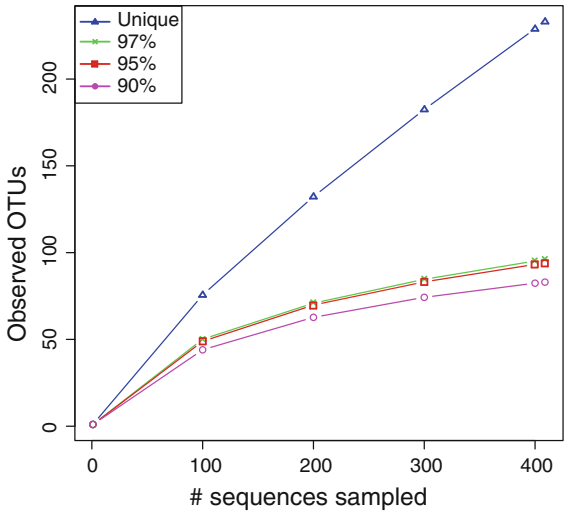


Fig. 7 Rarefaction curves for the 16S rRNA sequences obtained from *V. crypta*. Operational taxonomic units (OTU) were defined at the 97, 95 and 90 % similarity criterion

Table 2 Sample diversity

Label	OTUs	Chao estimate (95 % confidence interval)	Shannon diversity index (95 % confidence interval)
Unique	233	957 (676–1,417)	5.04 (4.93–5.15)
0.03	96	137 (114–189)	4.04 (3.94–4.14)
0.05	94	127 (108–170)	4.01 (3.91–4.11)
0.10	83	101 (90–130)	3.80 (3.69–3.91)

four OTUs. One was associated with the *Betaproteobacteria/Nitrosospira* and the others with the *Gammaproteobacteria/Nitrososcoccus*. In addition, numerous 16S rRNA gene sequences for nitrite-oxidizing bacteria (NOB) were found in our clone library. Three OTUs, representing 5 % of the community, were affiliated with the *Deltaproteobacteria/Nitrospina*, and 30 sequences were recognized as belonging to the *Nitrospira*. All of the sequences affiliated with the phylum *Nitrospira* (9 % of the community) were defined as a single OTU.

The *V. crypta* microbial community also contains microorganisms which show high sequence homologies to known sulfur-metabolizing bacteria, indicating their possible role in the sulfur cycle. One clone indicated the presence of sulfate-reducing bacteria belonging to the *Desulfurellaceae/Deltaproteobacteria*. Hoffmann et al. (2005) provided evidence that anaerobic sulfate reduction occurs in *Geodia barretti* tissue in zones of hypoxia and anoxia, which are created by changes in sponge pumping activity. Sulfate-reducing bacteria (SRB) were also detected by fluorescence in situ hybridization (FISH) in the Mediterranean sponges *Chondrosia reniformis* and *Petrosia ficiformis* (Schumann-Kindel et al. 1997; Manz et al. 2000).

Coralline sponges of the genus *Vaceletia* are representatives of the keratose sponges (Wörheide 2008), which form an early-branching lineage in the Demospongiae (Philippe et al. 2009; Pick et al. 2010), with their earliest fossil record most likely in the late Proterozoic era (Reitner and Wörheide 2002). Our results demonstrate that the complex microbial communities associated with *V. crypta* are very similar to the microbiota found in other sponges (Taylor et al. 2007). An overwhelming majority of the OTUs were very closely related to other sponge- or coral-derived sequences and moreover fell into SSC/SCC, which underscores that this "living fossil" sponge shares features of its microbial community with other sponges. Such a relatively small divergence between the 16S rRNA gene sequences obtained from different sponges might suggest an environmental acquisition of symbionts (Hentschel et al. 2002; Taylor et al. 2007). If we assume that 50 million years of evolution corresponds to an ~1–2 % 16S rRNA sequence difference (Ochman et al. 1999), then a greater discrepancy should occur if these bacteria had been living separately within their host sponges for 600 million years (Taylor et al. 2007). Moreover, small populations of endosymbiotic microorganisms enhance the fixation of mutations and are, therefore, believed to evolve more rapidly (Ochman et al. 1999). Several studies have shown that sponges from different oceans and with distant taxonomic origins harbor specific microbial consortia (Hentschel et al. 2002; Taylor et al. 2007). Our study is consistent with that pattern because the sponges and corals that contain the microorganisms that are the closest relatives to those associated with *V. crypta* were collected from different, mostly tropical, geographic regions. In contrast, our DGGE analysis of the 16S rRNA genes of symbionts obtained from a further nine solitary and nine colonial specimens of *Vaceletia*, reveal that solitary and colonial growth forms appear to harbor distinct communities and suggest a closer relationship between the microbial communities from the same

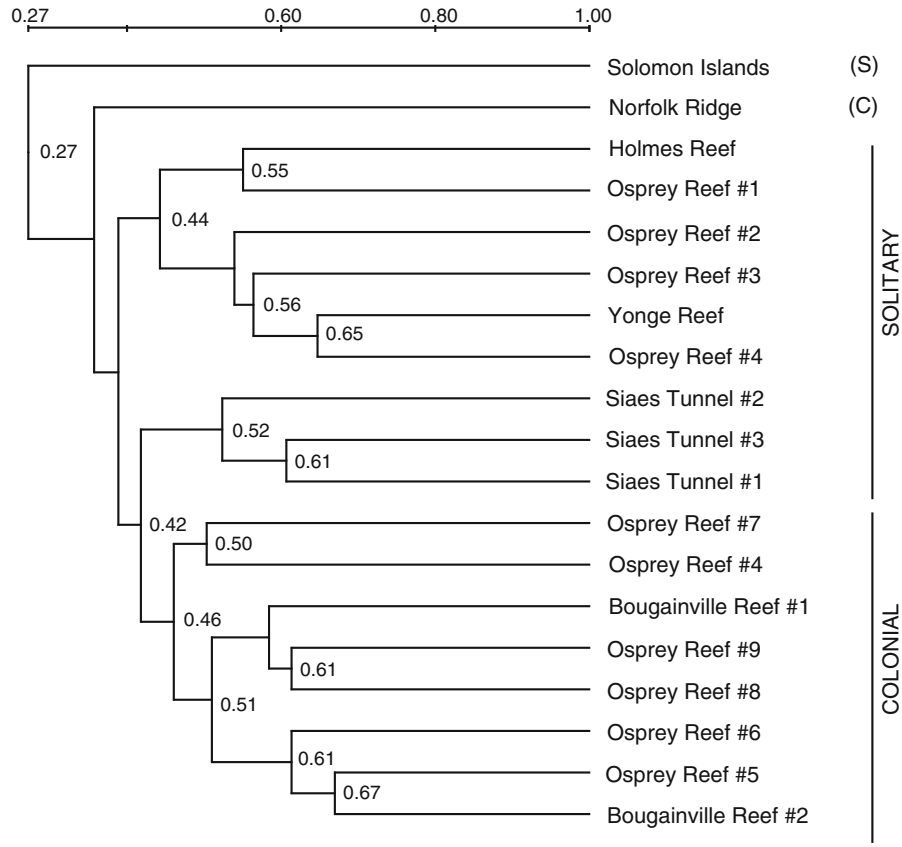


Fig. 8 UPGMA dendrogram constructed from DGGE profiling of PCR-amplified bacterial 16S rRNA genes of microbial community associated with *S* solitary and *C* colonial forms of *Vaceletia* sponges from different locations. Sample names according to the Table 1 (column: Site)

growth form (solitary vs. colonial) than from the same geographic origin (Fig. 8). This observation suggests that the bacterial community might have been achieved not through an environmental acquisition, but through a different mechanism of the transmission followed by successive bacterial speciation within the sponge hosts. Erwin et al. (2012) categorized numerous factors (environmental and host related) which could affect the structure of the microbial communities and noted that factors specific to different host species might have influenced the differences between the *Ircinia*-associated symbiotic communities. The number of SSC/SCC (63) and the proportion of sequences within SSC or SCC (88 %) for *V. crypta* appear to be the highest ever reported, indicating a particularly tight sponge-microbe association, which might be related to the evolutionary age of the host species. In addition, some symbionts were specific for *V. crypta* because they were absent in the microbial community analyzed from another coralline sponge, *Astrosclera willeyana*, which co-occurs at, and was sampled from, the same site (Karlińska-Batres and Wörheide, in review). A similar trend was observed in sympatric *Ircinia* species from Mediterranean Sea, which harbored different symbiont communities (Erwin et al. 2012).

This work on sclerosponges from genus *Vaceletia* enhances our knowledge about microbial communities in sponges and further provides initial insights into the diversity, structure, and composition of the microbiota of these unique sponges. Further research using deeper sequencing, FISH probes and/or specific primers designed for genes involved in denitrification, anammox or particular microbial groups (e.g. SRB and SOB) might reveal these processes in *V. crypta* providing a clearer picture of the metabolism of this sponge's microbial community. Future studies might aim to examine if other coralline sponges harbor such diverse communities of symbionts and how much those communities differ from each other and between different geographical locations.

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Conflict of interest The authors declare that they have no conflict of interest.

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