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Cultivable Bacterial Community from South China Sea Sponge as Revealed by DGGE Fingerprinting and 16S rDNA Phylogenetic Analysis

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Abstract The cultivable bacterial communities associated with four South China Sea sponges—*Stelletta tenuis*, *Halichondria rugosa*, *Dysidea avara*, and *Craniella australiensis* in mixed cultures—were investigated by microbial community DNA-based DGGE fingerprinting and 16S rDNA phylogenetic analysis. Diverse bacteria such as α-, γ-, δ-*Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were cultured, some of which were previously uncultivable bacteria, potential novel strains with less than 95% similarity to their closest relatives and sponge symbionts growing only in the medium with the addition of sponge extract. According to 16S rDNA BLAST analysis, most of the bacteria were cultured from sponge for the first time, although similar phyla of bacteria have been previously recognized. The selective pressure of sponge extract on the cultured bacterial species was suggested, although the effect of sponge extract on bacterial community in high nutrient medium is not significant. Although α- and γ-*Proteobacteria* appeared to form the majority of the dominant cultivable bacterial communities of the four sponges, the composition of the cultivable bacterial community in the mixed culture was different, depending on the medium and sponge species. Greater bacterial diversity was observed in media C and CS for *Stelletta tenuis*, in media F and FS for *Halichondria rugosa* and *Craniella australiensis*. *S. tenuis* was found to have the highest cultivable bacterial diversity including α-, γ-, δ-*Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, followed by sponge *Dysidea avara* without δ-*Proteobacteria*, sponge *Halichondria rugosa* with only α-, γ-*Proteobacteria* and *Bacteroidetes*, and sponge *C. australiensis* with only α-, γ-*Proteobacteria* and *Firmicutes*. Based on this study, by the strategy of mixed cultivation integrated with microbial community DNA-based DGGE fingerprinting and phylogenetic analysis, the cultivable bacterial community of sponge could be revealed effectively.

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Introduction

Sponges are well known to harbor diverse microorganisms, which can contribute up to more than 40% of the sponge body volume exceeding microorganisms in seawater by two to four orders of magnitude [2]. Studies have suggested the possible role of sponge-associated microorganisms in biologically active metabolite production and host chemical defense [12, 13, 14, 20]. Recently, a great diversity of unique and previously unrecognized microorganisms associated with sponges have been revealed by culture-independent molecular methods such as 16S rDNA library, DGGE, and FISH [7, 8, 21, 22, 26]. The understanding of cultivable sponge-associated microbial diversity is an important basis for microbial isolation, metabolite, and biofunction studies. Although some attempts have been made [3, 6, 9, 13, 25], the cultivable sponge-associated microbial diversity remains very unclear because of the difficulty in sponge-associated microbial isolation and pure cultivation.

The current investigation of cultivable sponge-associated microbial diversity is mainly based on pure

cultivation, single strain is isolated and cultured individually. Obviously, it is difficult to reveal greater cultivable microbial diversity because of the low cultivability out of the sponge host resulting from the change in microbial survival conditions such as nutrition and multiple microbial relationships. In the natural environment, microorganisms use pheromones originating from their neighbors to communicate both within and across species to maintain mixed survivorship, commensalisms, or symbiotic relationship. Microbial cultivability depends on suitable nutrient and a familiar environment. In pure culture, even if appropriate nutrient is provided, some microorganisms will not grow without maintaining multiple microbial relationships. For instance, Kaeberlein et al. [4] found that some marine microorganisms were not able to grow in artificial media alone but could form colonies in the presence of other microorganisms. In this paper, the term "mixed cultivation" means to cultivate one microorganism in the presence of other microorganisms that can maintain a natural community environment, and thus microorganisms that are difficult to grow alone may grow in the mixed cultures.

The in vivo predominant bacterial communities associated with four South China Sea sponges *Stelletta tenuis*, *Halichondria rugosa*, *Dysidea avara*, and *Craniella australiensis* have been investigated by us using culture-independent molecular methods [6–8], but the cultivable microbial communities associated with these sponges remain rarely known. In order to assess greater cultivable sponge-associated bacterial diversity, the cultivable bacterial communities associated with *Stelletta tenuis*, *Halichondria rugosa*, *Dysidea avara*, and *Craniella australiensis* were investigated by a mixed cultivation strategy integrated with microbial community DNA-based DGGE fingerprinting and 16S rDNA phylogenetic analysis in this paper.

Materials and Methods

Sponge Sample

Four South China Sea sponges—*Stelletta tenuis*, *Halichondria rugosa*, *Dysidea avara*, and *Craniella australiensis* as described in Li et al. [7]—were used.

Media

Two basic media F and C, i.e., FL and C-mix in the study of Olson et al. [13] for sponge-associated bacterial isolation, were used in this investigation. Medium F is composed of 23.4 g NaCl, 0.75 g KCl, 7.0 g $MgSO_4{\cdot}7H_2O$, 0.2 g $CaCl_2{\cdot}2H_2O$, 0.015 g KH_2PO_4, 1.0 g mannitol, 1.0 g yeast extract, 1.0 g peptone, 1 mL trace metal solution A, 1 L distilled water, and 10 g agar. Medium C consists of 0.1 g maltose, 0.1 g mannitol, 0.1 g glucose, 0.1 g soluble starch, 0.1 g galactose, 0.1 g peptone, 0.1 g tryptone, 0.1 g yeast extract, 1.0 mL trace metal solution A, 1.0 mL solution B, 1 L filtered seawater, and 10 g agar. Trace metal solution A: 2.86 g H_3BO_3, 1.81 g $MnCl_2{\cdot}4H_2O$, 1.36 g FeEDTA, 0.08 g $CuSO_4{\cdot}5H_2O$, 0.049 g $Co(NO_3)_2{\cdot}6H_2O$, 0.39 g $NaMoO_4{\cdot}2H_2O$, 0.22 g $ZnSO_4{\cdot}7H_2O$,1 L distilled H_2O. Solution B: 5.0 g $NaH_2PO_4{\cdot}H_2O$, 1 L distilled H_2O.

For each species of sponge, a 10-g sample was extracted with 200 mL 75% ethanol for 30 days. After filtration with a 10-μm membrane, ethanol was evaporated under vacuum condition, and the obtained 1.8-g extract was dissolved in 200 mL distilled water. Twenty-milliliter solution of sponge extract was added into 180 mL media F and C to obtain media FS and CS, respectively.

Bacterial Mixed Cultivation

After being washed with sterilized water three times, a 5-g sample was cut from different parts of the sponge body and mixed together, rubbed into slurry, and vibrated aseptically in sterile artificial seawater [6] for 1 day at room temperature to release microorganisms. After settling naturally, the suspension was filtered through filter paper to remove sponge debris, and the filtrate containing microorganisms was transferred to inoculate flasks containing media F, FS, C, and CS at a ratio of 20/200 (v/v, mL), respectively. The mixed cultivation was carried out at 28°C, 200 rpm for 7 days; then bacteria were harvested by centrifugation (10,000 rpm, 5 min) and used for microbial genomic DNA extraction.

Total Microbial Community DNA Extraction and 16S rDNA-V3 PCR Amplification

DNA was extracted with modified phenol-chloroform method and incubated with RNase A (10mg/mL) (Sigma) at 37°C for 30 min to remove RNA as described by Li et al. [7]. The purity of DNA was assessed by electrophoresis in 1% agarose gels, stained with ethidium bromide, and analyzed spectrophotometrically by calculating A260/A280 ratio. DNA was stored at –20°C before 16S rRNA gene polymerase chain reaction (PCR) amplification.

16S rDNA amplification was carried out in a Touchdown Thermal Reactor (Hybaid, U.K.) according to Li et al. [7]. In the first-round PCR, the 50-μL PCR mixture consisted of 10 pM primer pairs 8f (5′-GGA GAG TTT GAT CA/CT GGC T-3′ and 798r (5′-CCA GGG TAT CTA ATC CTG TT-3′), which were designed to amplify

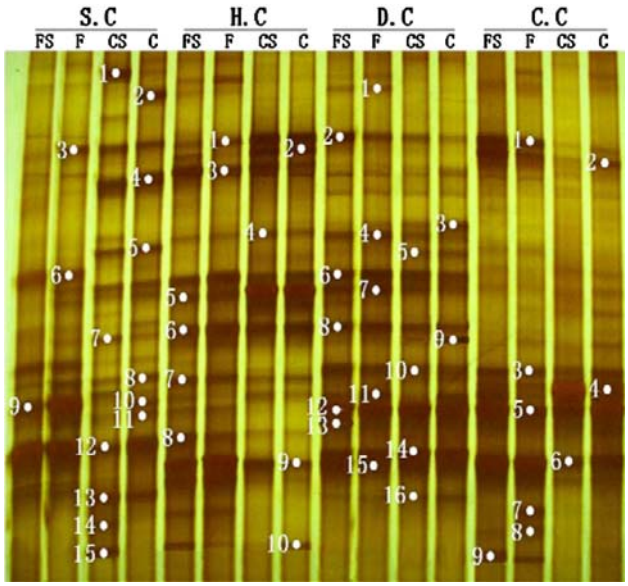

Fig. 1 16S rDNA-V3 DGGE fingerprints of sponge-associated cultivable bacteria. S.C, H.C, D.C and C.C are derived from cultivable bacteria associated with sponges *Stelletta tenuis*, *Halichrondria rugosa*, *Dysidea avara*, and *Craniella australiensis*, respectively. F and C are media FL and C-mix [13], FS and CS are media F and C with sponge extract, respectively

approximate 800 bp 16S rDNA fragments from all members of the bacteria [18], 9 μL 10× PCR solution (50 mmol/L Tris-HCl (pH 8.2), 18 mmol/L $MgCl_2$, 500 mmol/L KCl, 0.1% glycerol, 1% Triton X-100), 2 μL 10 mmol/L dNTP, 1 μL DNA sample, and 2.5 U pfu DNA polymerase (Shenergy Biocolor Bioscience & Technology Company, China) and ddH_2O. The PCR amplification consisted of 5 min at 94°C, a hold temperature of 80°C during which time pfu DNA polymerase was added, 25 cycles of 1 min denaturation at 94°C, 1 min annealing at 57°C, 2 min extension at 72°C, and a final step at 72°C for 2 min. Second-round PCR was performed with primers 341F (5′-CC TAC GCG AGG CAG CAG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) to amplify bacterial 16S rDNA-V3 segments corresponding to nucleotides 341–534 of the *Escherichia coli* sequence. A 40-bp GC-clamp cgc ccg ccg cgc gcg gcg ggc ggg gcg ggg gca cgg ggg g was added to primer 341F in order to increase the separation of DNA bands in DGGE gel [11]. The PCR mixture contained 0.1 μM each primer, 5 μM of DNA sample, and ddH_2O in a total reaction volume of 50 μL. The entire amplification program consisted of 5 min at 94°C, a hold temperature of 80°C during which time pfu DNA polymerase was added, 1 min at 60°C; 21 cycles of 30 s at 94°C, 30 s at 65°C (1°C decrease for every two cycles), 1 min at 72°C; 5 cycles of 30 s at 94°C, 30 s at 55°C, and 3 min at 72°C. PCR products were analyzed by electrophoresis in 1.5% agarose gels and stained with ethidium bromide.

Denaturing Gradient Gel Electrophoresis, Sequencing and Phylogenetic Analysis

Denaturing gradient gel electrophoresis, sequencing, and phylogenetic analysis were carried out according to Li et al. [7]. DGGE was performed with a Mutation Detection System (Bio-Rad, USA). Electrophoresis was performed at a constant voltage of 150 V and 60°C for 4.5 h using 30–50% denaturant gradient. DGGE gels were stained with a routine silver staining protocol [18]. The stable bands in DGGE gel verified to be a single band were excised, and eluted in 30 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). As for the bands in the same position in DGGE gels of four media for each sponge, only one band was excised.

The supernatant after centrifugation (12,000 rpm, 5 min, 4°C) was used for 16S rDNA-V3 amplification with the V3 primers 341F and 518R without GC-clamp using the same program as Li et al. [7]. The amplified 16S rDNA-V3 segments were cloned into PUCm-T Vector after being purified with a PCR product purification mini kit (Shenergy Biocolor Bioscience & Technology Company, China). The positive recombinants were screened on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-IPTG (isopropyl-β-D-thiogalactopyranoside)-ampicillin-tetracycline indicator plates by color-based recombinant selection. Positive clones were identified by PCR amplification with PUCm-T Vector primer pairs T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) and M13 (5′-CAG GAA ACA GCT ATG ACC-3′) using the same program as 16S rDNA-V3 amplification. Subsequently, positive recombinants were submitted for sequencing using an ABI3730 DNA Sequencer (USA) with T7 primer at the Bioasia Biotechnology Company, China.

As for the obtained 50 DGGE bands, 15, 10, 16, and 9 bands for sponges *S. tenuis*, *H. rugosa*, *D. avara*, and *C. australiensis*, respectively, were sequenced and registered in the GenBank nucleic acid sequence database with 50 accession numbers DQ180068–DQ180117. Sequences were compared to those in GenBank database by BLAST algorithm to identify sequences similarity. 16S rDNA sequences were aligned using Clustal X software, and the phylogenetic tree was generated using the neighbor-joining algorithms in Mega II software according to Li et al. [7]. The bacterial percentage was calculated according to the total number of DGGE bands representing different bacteria obtained from the mixed cultures derived from each sponge species.

Results

According to the band patterns of 16S rDNA-DGGE fingerprint in Fig. 1, the composition of the cultivable bacterial community in the mixed culture was different,

Table 1 16S rDNA-V3 sequence similarities to the closest relatives of DNA derived from the respective bands in DGGE gels from mixed cultures

Sequence	Accession no.	Closest relative and its accession number	Similarity (%)
S.C1	DQ180078	*Oleiphilus messinensis* (AJ295154)	97
S.C2	DQ180079	*Bdellovibrio* sp. JS10 (AF084863)	94
S.C3	DQ180080	Uncultured alpha proteobacterium clone IAFDn48 (AY090123)	95
S.C4	DQ180081	*Bdellovibrio* sp. JS10 (AF084863)	92
S.C5	DQ180082	*Idiomarina* sp. NT N118 (AB167034)	99
S.C6	DQ180083	*Brevundimonas vesicularis* (AY456200)	100
S.C7	DQ180084	*Brevundimonas vesicularis* (AY456200)	99
S.C8	DQ180085	*Halomonas* sp. J21.8 (AJ717724)	100
S.C9	DQ180086	*Brevundimonas vesicularis* (AY456200)	100
S.C10	DQ180087	*Halomonas* sp. SP3 (AY780448)	99
S.C11	DQ180088	Uncultured bacterium clone B-10 (AY604019)	99
S.C12	DQ180089	*Halomonas* sp. SP3 (AY780448)	100
S.C13	DQ180090	*Idiomarina* sp. ARD M28 (AB167076)	99
S.C14	DQ180091	Uncultured Clostridia bacterium clone SIMO-2479 (AY711845)	98
S.C15	DQ180092	*Vitellibacter vladivostokensis* (AB071382)	99
H.C1	DQ180068	*Halomonas* sp. K354 (AY368511)	99
H.C2	DQ180069	Marine bacterium KMM 3937 (AF536386)	99
H.C3	DQ180070	*Idiomarina* sp. NT N118 (AB167034)	100
H.C4	DQ180071	*Halomonas* sp. Claire (AJ969933)	100
H.C5	DQ180072	Uncultured bacterium clone CLB-18 (DQ068740)	97
H.C6	DQ180073	Bacterium K2-57B (AY345411)	100
H.C7	DQ180074	*Rhodobacteraceae* bacterium JC2049 (AY442178)	100
H.C8	DQ180075	Uncultured bacterium clone CLB-18 (DQ068740)	97
H.C9	DQ180076	Uncultured bacterium clone F1 (AY375107)	98
H.C10	DQ180077	*Halomonas* sp. Claire (AJ969933)	99
D.C1	DQ180093	*Staphylococcus epidermidis* strain EIB 7-1 (AY458861)	99
D.C2	DQ180094	*Idiomarina* sp. NT N118 (AB167034)	99
D.C3	DQ180095	*Idiomarina* sp. NT N118 (AB167034)	100
D.C4	DQ180096	*Idiomarina* sp. NT N118 (AB167034)	100
D.C5	DQ180097	*Flavobacteriaceae str.* SW058 (AF493683)	95
D.C6	DQ180098	*Flavobacteriaceae str.* SW058 (AF493683)	94
D.C7	DQ180099	*Brevundimonas vesicularis* (AY456200)	100
D.C8	DQ180100	*Brevundimonas vesicularis* (AY456200)	100
D.C9	DQ180101	*Alpha proteobacterium* UMB1A (AF505720)	100
D.C10	DQ180102	*Marinobacter* sp. NT N148 (AB167047)	100
D.C11	DQ180103	*Marinobacter* sp. NT N148 (AB167047)	97
D.C12	DQ180104	*Brevundimonas vesicularis* (AY456200)	99
D.C13	DQ180105	Uncultured proteobacterium clone BMS32 (AY193223)	99
D.C14	DQ180106	*Alpha proteobacterium* UMB1A (AF505720)	98
D.C15	DQ180107	*Halomonas* sp. Claire (AJ969933)	100
D.C16	DQ180108	*Bellia baltica* (AJ564643)	96
C.C1	DQ180109	*Halomonas* sp. K354 (AY368511)	99
C.C2	DQ180110	*Halomonas* sp. Claire (AJ969933)	100
C.C3	DQ180111	*Brevundimonas vesicularis* (AY456200)	99
C.C4	DQ180112	*Roseovarius crassostreae* strain CV919-312 (AF114484)	100
C.C5	DQ180113	*Halomonas* sp. Claire (AJ969933)	97
C.C6	DQ180114	*Halomonas* sp. Claire (AJ969933)	100

Table 1 continued

Sequence	Accession no.	Closest relative and its accession number	Similarity (%)
C.C7	DQ180115	Unidentified bacterium (Z94008)	95
C.C8	DQ180116	*Ochrobactrum anthropi* (U88442)	96
C.C9	DQ180117	Uncultured gram-positive bacterium (AB116389)	100

depending on the medium and sponge species. For *S. tenuis*, higher bacterial diversity was observed in media C and CS, whereas for *H. rugosa* and *C. australiensis*, higher bacterial diversities were found in media F and FS.

Based on the BLAST analysis shown in Table 1, some previously uncultivable bacteria such as S.C3, S.C11, S.C14, H.C5, H.C8, H.C9, D.C13, and C.C9 were found in the mixed cultures. Meanwhile, some potential novel strains such as S.C2, S.C3, S.C4, D.D5, and D.C6 with similarity less than 95% to their closest relatives were found. Among the 50 relatives in Genbank, *Halomonas* sp. SP3 (AY780448) and *Halomonas* sp. K354 (AY368511) corresponding to sequences S.C10, H.C12, and C.C1 were previously found in sponge; the other 47 strains were not previously cultured from sponge, although the same phyla bacteria such as *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* have been found.

Figure 2 shows the phylogenetic relationship of all the 16S rDNA-V3 sequences representing the respective excised 50 DGGE bands. The 50 bacterial sequences were grouped into 5 clusters: *γ-Proteobacteria* (21/50), *α-Proteobacteria* (17/50), *δ-Proteobacteria* (3/50), *Bacteroidetes* (5/50) and *Firmicutes* (4/50). The comparison of cultivable bacterial community among the four sponges is made in Fig. 3. Obviously, the bacterial community distribution is different on each case, which suggests that the cultivable bacteria are sponge specific. Among the four sponges, *Stelletta tenuis* was found to have the highest cultivable bacterial diversity including α-, *γ-*, *δ-Proteobacteria*, *Bacteroidetes* and *Firmicutes*, followed by sponge *Dysidea avara* without *δ-Proteobacteria*, sponge *Halichondria rugosa* with only α-, *γ-Proteobacteria* and *Bacteroidetes*, and sponge *Craniella australiensis* with only α-, *γ-Proteobacteria* and *Firmicutes*. According to Fig. 3, α-, *γ-Proteobacteria* appear to form the majority of the cultivable bacterial communities of the four sponges, e.g. 90% for sponge *Halichondria rugosa*, 78% for sponge *Craniella australiensis*, 75% for sponge *Dysidea avara* and 67% for sponge *Stelletta tenuis*.

Discussion

Compared with other molecular methods for microbial diversity analysis such as 16S rRNA gene library [6, 8], a major advantage of DGGE fingerprinting is the relative ease for the qualitative comparison among different samples, for example, different sponges and different media in this study. The partial sequence-based phylogenetic analysis is largely consistent with that of using a long near-complete 16S rRNA gene fragment [17]. V3 region fragments of approximately 190 base-pairs of 16S rRNA gene are able to distinguish most phylotypes from their relatives and determine phylogenetic relationships among the clones [11]. So, the phylogenetic analysis based on V3 region of 16S rRNA gene is credible, which has also been shown by the previously published reports [1, 7].

Based on our previous study on the microbial distribution in sponge with a transmission electron microscope [10], microorganisms mainly distribute in sponge mesohyl, cells, and the inner cavity. In this study, by the pretreatment of rubbing and vibrating, the released cultivable bacteria should mainly come from the mesohyl matrix of sponge. Mesohyl is a stable and nutritional habitat for microorganisms and is suggested to be selective for microbial existence [2, 25]. Thus, it is reasonable that there is a cultivable bacterial community difference among these sponges.

Sponge-associated microorganisms can obtain rich nutrition from the mesohyl of sponge, which is different from that of the free-living microorganisms in the sea water column [2]. Olson et al. [13] attempted to isolate sponge *Discodermia* sp.-associated microorganisms using different media based on counting plate colonies, where high nutrient media resulted in higher recoverability. Therefore, media C and F as C-mix and FL used by Olson et al. [13] containing abundant inorganic and organic materials, respectively, were used in this study. As a result, higher cultivable bacterial diversity was achieved for each sponge (Fig. 1). In the study of Olson et al. [13], the effect of sponge extract on bacterial recovery was not significant in high nutrient media, which is inconsistent with our results. The addition of sponge extract in C and F could not change the cultivable bacterial community significantly.

In the study of Webster et al. [24], marine agar 2216 incorporating with sponge extract was used to isolate bacteria from sponge *Rhopaloeides odorabile*; some bacteria not previously recovered were obtained including *γ-* and *δ-Proteobacteria*, *Actinobacteria*, and green nonsulfur bacteria. Meanwhile, sponge extract was found to decrease the total number of cultured bacteria showing a greater

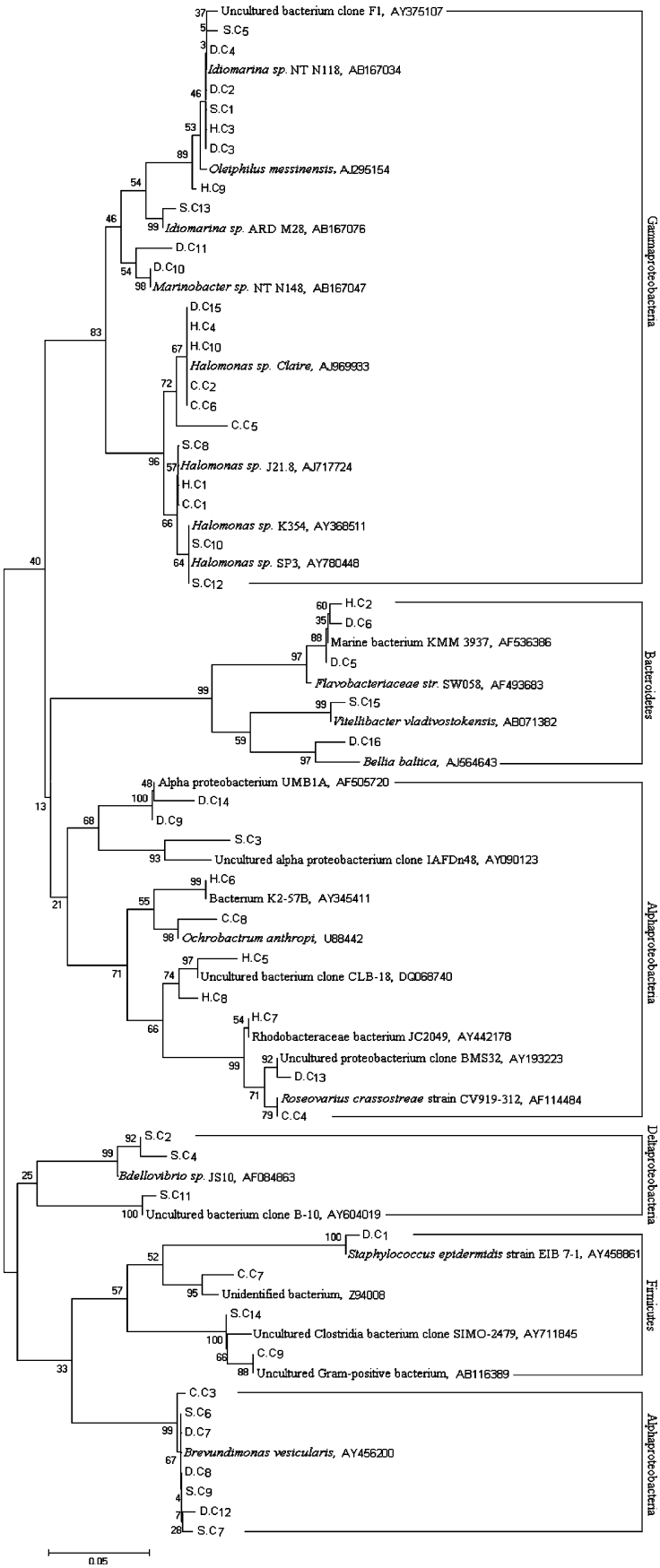

Fig. 2 Phylogenetic tree based on 16S rDNA-V3 sequences representing the respective DGGE bands in Fig. 1. Bootstrap analysis is based on 1000 replicates. Scale indicates 5% sequence divergence

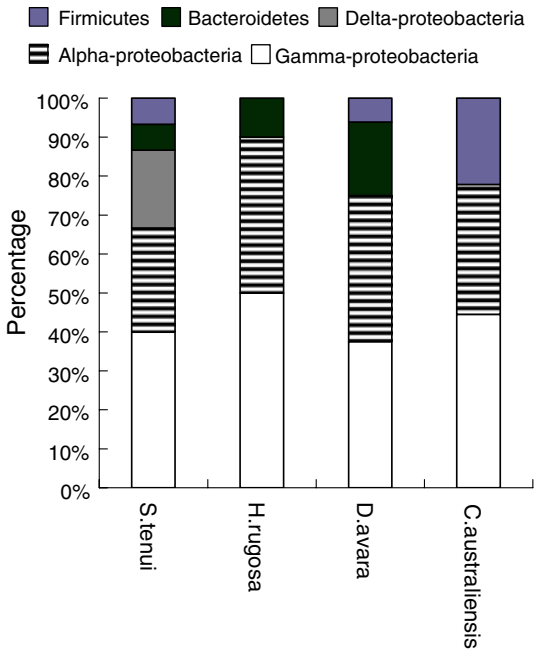

Fig. 3 Comparison of the cultivable bacterial community among sponges based on 16S rDNA-V3. Percentage was calculated according to the total number of DGGE bands representing different bacteria obtained from mixed cultures derived from each sponge species

effect on the cultivable bacterial diversity because marine agar 2216 is a lower nutrient medium than media C and F. In this study, S.C2, C.C7 disappeared when sponge extract was incorporated in media C and F. Particularly, S.C14 and S.C15 growing only in medium CS could be sponge *Stelletta tenuis* symbionts. Thus, the selective pressure of sponge extract on the cultured bacterial species was suggested.

According to bacterial community investigations using molecular methods such as DGGE, 16S rRNA gene sequencing, and fluorescence in situ hybridization [2, 5], the sponge-associated bacterial community consists of at least nine different bacterial phyla *Proteobacteria*, *Nitrospira*, *Cyanobacteria*, *Bacteriodetes*, *Actinobacteria*, *Chloroflexi, Planctomycetes*, *Acidobacteria*, *Poribacteria*, as well as members of the domain *Archaea*. In general, the cultivable sponge-associated microorganisms mainly consist of *Proteobacteria*, *Firmicutes*, *Bacteriodetes*, and *Actinobacteria* [2]. For instance, *Proteobacteria*, especially α- and *γ-Proteobacteria*, have been previously found to comprise the majority of the cultivable bacteria from sponges such as Mediterranean sponges [23, 25], *Aplysina aerophoba*, and *Aplysina cavernicola* [3]. In addition, *δ-Proteobacteria* have been isolated by Schmidt et al. [19] and Webster et al. [24]. *Bacillus* sp. was found to be the main cultivable species of phylum *Firmicutes* [9, 16]. *Actinobacteria* have been cultured from sponges *Craniella australiensis* [6] and *R. odorabile* [24]. Pimentel-Elardo et al. [15] cultured *Planctomycete* bacteria from Aplysina sponge. Basically, the above pure culture-based bacterial diversity investigations may retrieve specific species of bacteria from sponge because of the shortcoming of pure cultivation in maintaining multiple microbial relationships. In contrast, in this study, a various bacterial diversity including *α-Proteobacteria*, *γ-Proteobacteria*, *δ-Proteobacteria*, *Bacteroidetes*, and *Firmicutes* growing together were observed in the mixed cultures.

By the comparison with in vivo bacterial community structure revealed by us using the culture-independent DGGE approach [7], some dominant in vivo bacteria such as α-, *γ-Proteobacteria* for the four sponges, and *Firmicutes* for sponges *D. avara* and *C. australiensis* were cultured in vitro. Meanwhile, some bacteria that could not be detected in vivo by DGGE [7] were obtained in the mixed cultures, for example, *δ-Proteobacteria*, *Firmicutes*, and *Bacteroidetes* for sponge *Stelletta tenuis*, *Bacteroidetes* for sponges *H. rugosa*, and *D. avara* and *α-Proteobacteria* for sponge *C. australiensis*. Therefore, various bacterial diversity is able to be assessed by mixed cultivation.

At present, the cultivable bacteria from sponges represent only a small fraction of the total microbial community [5]. Most of the sponge-associated microorganisms identified by molecular approaches are still difficult for cultivation in vitro, especially the sponge-specific bacteria. As shown in Fig. 1, the revealed cultivable bacteria are dependent on media. Therefore, novel media should be designed to reveal a greater proportion of the cultivable bacterial community. For example, the optimized medium F will be helpful for sponge *S. tenuis*-associated bacterial diversity revelation. Meanwhile, with the guide of 16S rRNA gene information, retrieval of higher cultivable bacterial diversity can be achieved by the selection of suitable medium and cultivation strategy [6].

Although the method used by Olson et al. [13] belongs to solid mixed cultivation, it cannot optimally reveal bacterial diversity by counting colonies. As shown in this study, by the strategy of mixed cultivation incorporating with 16S rDNA-based DGGE fingerprinting and phylogenetic analysis, diverse cultivable bacterial communities including potential novel strains, unidentified bacteria, possible sponge symbionts, and previously uncultivable bacteria were observed, suggesting a higher efficiency in sponge-associated cultivable bacterial diversity investigation.

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