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# Bacterial community diversity associated with four marine sponges from the South China Sea based on 16S rDNA-DGGE fingerprinting

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**Abstract**

Culture-independent 16S rDNA-DGGE fingerprinting and phylogenetic analysis were used to reveal the community structure and diversity of the predominant bacteria associated with the four sponges *Stelletta tenui*, *Halichrondria*, *Dysidea avara*, and *Craniella australiensis* from the South China Sea for the first time. Sponge total community DNA extracted with a direct grinding disruption based method was used successfully after series dilution for 16S rDNA PCR amplification, which simplifies the current procedure and results in good DGGE banding profiles. 16S rDNA-V3 fragments from 42 individual DGGE bands were sequenced and the detailed corresponding bacteria were found in sponges for the fist time based on BLAST results. The sponge-associated bacteria are sponge host-specific because each of the tested four sponges from the same geographical location has different predominant bacterial diversity. *Proteobacteria*, e.g. α, β and γ subdivisions, make up the majority of the predominant bacteria in sponges and are perhaps in close symbiotic relationship with sponges. Though similar bacteria with close phylogenetic relationships were found among different sponges, the sponge-associated predominant bacterial community structures differ. Sponge *C. australiensis* has the greatest bacterial diversity, with the four bacteria phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, followed by the sponge *D. avara* with the two phyla *Proteobacteria* and *Bacteroidetes*, and the sponges *S. tenui* and *Halichrondria* with the phylum *Proteobacteria*. DGGE fingerprint-based analysis should ideally be integrated with band cloning and sequencing, phylogenetic analysis and molecular techniques to obtain precise results in terms of the microbial community and diversity.


*Keywords:* Microbial community; Phylogenetic analysis; Sponge; 16S rDNA-DGGE

## 1. Introduction

It has been estimated that less than 1% of the total microbial population in the land environment and even less in the marine environment have been successfully isolated in pure culture (Amann et al., 1995). Many environmental microorganisms cannot be cultured using current culture-based and traditional methods. Fortunately, some molecular approaches have greatly enhanced the knowledge of population structure in natural microbial communities.

In general, all microorganisms found in association with a sponge host are termed "associated microorganisms", including symbionts. The special two-layer structure of outer and inner endosome and special strategy for sequestering food by filtering seawater make sponges an ideal habitat for microorganisms. Sponge-associated microorganisms may contribute up to 40% of sponge body volume, exceeding the numbers of microorganisms in seawater by two to three orders of magnitude (Wilkinson, 1978). To date, the detailed roles that these sponge-associated microorganisms

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play within sponges are unclear, although Müller et al. (1981) first suggested a symbiotic relationship between bacteria and sponges 20 years ago, and various hypotheses have been proposed, including a source of nutrition (Van Roest, 1996), nitrogen fixation and nitrification (Wilkinson and Fay, 1979), participation in the host's chemical defense system (Althoff et al., 1998), protection against UV radiation (Sara, 1971), and secondary metabolite production (Osinga et al., 2001). Little evidence (Schmidt et al., 2000) has been found to prove these hypotheses because of complex microbial diversity, the difficulty of microbial cultivation outside the host, and the lack of suitable physical compartments for microorganisms separated from sponges (Friedrich et al., 2001). Understanding the nature of the interaction between sponge host and associated microorganisms remains a challenging task. In this regard, the documentation of sponge-associated microbial communities and diversity will provide a scientific foundation for understanding sponge–microorganism relationships and their relative functions.

For understanding the community and diversity of sponge-associated microorganisms, electron microscopic techniques are often used (Manz et al., 2000; Usher et al., 2001), but only provide morphological data. Standard isolation and cultivation methods enable the identification of some sponge bacteria (Burja et al., 1999), but only a very small number of microorganisms can be studied because most of the sponge-associated microorganisms are not easily cultivated (Lopez et al., 1999). As with other environmental studies, molecular techniques have provided new tools to genetically identify sponge-associated microorganisms. Methods centered around the 16S rRNA gene make it possible to phylogenetically describe complex communities of unculturable bacteria (Amann et al., 1995). Correlative techniques include fluorescence *in situ* hybridization (FISH) (Friedrich et al., 1999, 2001), amplified rRNA gene restriction analysis (ARDRA) (Webb and Maas, 2002), terminal-restriction fragment length polymorphism (T-RFLP) (Lee et al., 2003) and 16S rRNA sequencing-based phylogenetic analysis (Margot et al., 2002). Although we can obtain information concerning sponge-associated microbial diversity using a 16S rDNA clone library strategy (Webster et al., 2001a), it is time-consuming and unsuitable for comparing microbial communities in different sponges or the dynamics of microbial communities. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified partial 16S rRNA gene sequences, which was introduced by Muyzer et al. (1993, 1998) as a new culture-independent genetic fingerprinting technique for describing microbial community structure and diversity in different samples or changes in microbial communities over time and space, is particularly useful for characterizing sponge-associated predominant microbial communities. DGGE has been used to monitor changes in a sponge-associated microbial community over time (Friedrich et al., 2001) and transplantation (Thoms et al., 2003) based on the difference in DGGE fingerprint profiles. DGGE analysis was also used by Schmidt et al. (2000) to identify idiographic bacterium such as proteobacterium, using antifungal petide. To date, there are no reports using the DGGE technique to compare the predominant bacterial community and diversity in different sponges from the same marine location.

The purpose of this study is to document the predominant bacterial community structure and diversity in the sponges *Stelletta tenui*, *Halichrondria*, *Dysidea avara*, and *Craniella australiensis* from a site in the South China Sea using PCR-DGGE fingerprinting and phylogenetic analysis. Furthermore, sponge-specific bacteria and the main predominant bacteria are evaluated, and we suggest appropriate procedures for DGGE fingerprinting analysis. To our knowledge, this is the first investigation of the predominant bacterial community and diversity of the sponges *S. tenui*, *Halichrondria*, *D. avara*, and *C. australiensis*.

## 2. Materials and methods

### 2.1. Sponge samples

Four sponges (Fig. 1) were collected around Sanya Island in the South China Sea and were identified as *S. tenui* (Lindgren, 1897), *Halichrondria* (Ridley and Dendy), *D. avara* (Schmidt) and *C. australiensis* (Carter) by Professor Jin-He Li of the Institute of Oceanology, Chinese Academy of Sciences. Samples were cut into small pieces and immediately stored at −20 °C or conserved in absolute methanol prior to DNA extraction.

### 2.2. Total community DNA extraction

Small, (e.g. 2 $mm^3$), sponge pieces were diped in TE buffer (10 mM Tris and 1 mM EDTA, pH8.0) solution and blended to a slurry. The slurry was ground in a mortar on ice for 6–9 min under sterile condition. Cells were collected by centrifugation at 10 000 rpm for 5 min at 4 °C and resuspended in TE buffer and vibrated for 30 s. After centrifugation (10 000 rpm, 5 min, 4 °C) the sedimentation was

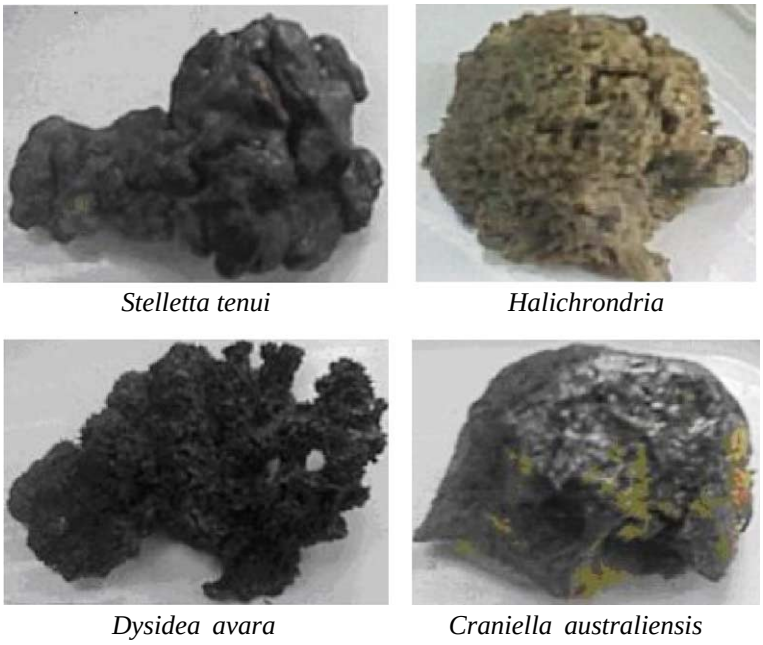


Fig. 1. Sponges from the South China Sea analyzed in this study.

resuspended in TE buffer and incubated with lysozyme (10 mg/ml) (ChemSonic) at 37 °C for 30 min and 10% SDS and proteinase K (10 mg/ml) (Merck) at 55 °C for 30 min. DNA was extracted orderly with tris-phenol (pH 8.0), tris-phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (24:1). Five M NaCl was added in supernatants after centrifugation (12 000 rpm, 5 min, 4 °C) at a final concentration of 0.7 M before DNA precipitation with 2.5 volume of absolute ethyl alcohol at −20 °C for 30 min. The DNA sample was incubated with RNase A (10 mg/ml) (Sigma) at 37 °C for 30 min to remove RNA. The total community DNA was stored at −20 °C before 16S rRNA gene PCR amplification. The purity of DNA was assessed by electrophoresis in 1% agarose gels and ethidium bromide staining and analysed spectrophotometrically by calculating the A260/A280 ratio for protein impurity. Yield of DNA was calculated according to the formula: [DNA] = 50 × $OD_{260}$ ng/μL.

### 2.3. PCR amplification of 16S rDNA

Nested PCR and touchdown PCR with hot start were used for 16S rDNA-V3 amplification using a MasterCycler Gradient (HYBAID, UK). In the first round PCR, the mixture consisted of the 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 approximate 800 bp 16S rDNA fragments from all members of the bacteria (Rochelle et al., 1995). The 50 μL PCR mixture consisted of 9 μL 10 × PCR solution (50 mmol/L Tris–HCl (pH8.2), 18 mmol/L $MgCl_2$, 500 mmol/L KCl, 0.1% glycerol, 1% TritonX-100), 2 μL 10 mmol/L dNTP, 1 μL DNA sample and 2.5 U Pfu DNA Polymerase (Shenergy Biocolor Bioscience and Technology Company, China) and $ddH_2O$. The entire 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 to increase the separation of DNA bands in DGGE gel (Muyzer et al., 1993). The PCR mixture contained 0.1 μM of each primer, 5 μM of the DNA sample, and $ddH_2O$ in a total reaction volume of 50 μL. In touchdown PCR the annealing temperature was set at 65 °C and decreased by 1 °C every second cycle until 55 °C. 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 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 ethidium bromide staining.

To reduce possible inter-sample PCR variation, all PCR reactions were run in triplicates and pooled together before loading on DGGE gel. In DNA extraction and PCR amplification, genomic DNA of *E. coli* was used as positive control and PCR mixture without DNA template was used as negative control.

### 2.4. Denaturing gradient gel electrophoresis

DGGE was performed with the Dcode™ Universal Mutation Detection System (BIO-Rad, USA), according to manufacturer instructions. Equal amounts of PCR products were loaded onto 8% (wt/vol) polyacrylamide gels in 1 × TAE (40 mM Tris, 20 mM acetate, 1.0 mM $Na_2$-EDTA) with denaturant-gradient of 30–50% (100% denaturant was 7 M urea and 40% (wt/vol) deionized formamide). The gels were electrophoresed at a constant voltage of 150 V and 60 °C for 4.5 h. After electrophoresis, DGGE gels were stained with a routine silver staining protocol as described by Rochelle et al. (1995). Cluster analysis of the DGGE fingerprints was performed with the software UVI Band Map V.99 (UVItec, Cambridge, UK).

### 2.5. Sequencing and phylogenetic analysis

Stable single bands in DGGE gel, verified by DGGE three times to ensure a single band at the same location, were signed with A1–10, B1–8, C1–14, and D1–10, where A, B, C, and D represent the sponges *S. tenui* (Lindgren, 1897), *Halichrondria* (Ridley and Dendy), *D. avara* (Schmidt), and *C. australiensis* (Carter), respectively. The bands were excised from gel and eluted in 30 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH8.0). The supernatant after centrifugation (12 000 rpm, 5 min, 4 °C) was used as a template for 16S rDNA-V3 sequence amplification using the same primer pairs without GC-clamp. The 50 μL reaction mixture consisted of 5 μL 10 × PCR solution (50 mmol/L Tris–HCl (pH 9.0), 15 mmol/L $MgCl_2$, 500 mmol/L KCl, 0.1% glycerol, 1% TritonX-100), 1.75 μL 10 mmol/L dNTP, 4 μL DNA supernatants, 0.5 μL 2.5 U Pfu DNA Polymerase and $ddH_2O$. The PCR reaction program was as follows: 20 cycles of denaturization at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 3 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels and DGGE gel to ensure the same location as before.

The amplified 16S rDNA-V3 segment was cloned into PUCm-T Vector after being purified with a PCR purification mini kit (Shenergy Biocolor Bioscience and 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. PCR products were affirmed in DGGE gel to confirm its position. Positive recombinants were then submitted for sequencing using an ABI3730 DNA Sequencer (USA) with T7 primer at the Bioasia Biotechnology Company, China. Sequences were compared to those in the GENBANK database by BLAST algorithm to identify sequences with a high degree of similarity. 16S rDNA sequences were aligned using CLUSTALX software, while the phylogenetic tree was generated using the neighbor-joining algorithms in Mega II software. The level of support for the phylogenies derived from neighbor-joining analysis was gauged by 1000 bootstrap replicates.

## 3. Results

### 3.1. Primary comparison of the predominant bacterial communities in sponges based on DGGE fingerprints

We compared ultrasonic and grinding approaches for sponge tissue disruption based on DNA yield and purity using the sponge *S. tenui*. According to the DNA extraction results for the sponges *Halichrondria, D. avara*, and *C. australiensis*, using the same procedure as for the sponge *S. tenui*, we established approach based on direct grinding disruption combined with the phenol/chloroform method for sponge total community DNA extraction. Because the $OD_{260}/OD_{280}$ of extracted total DNA samples were in the range of 1.5–1.7, 10, 20, and 30-fold dilutions of crude total community DNA were used in 16S rDNA amplification to avoid any remaining traces of inhibitors (e.g. proteins) in the samples. As a result, the approximate bacterial 800 bp 16S rDNA segment was successfully amplified from a 1:20 diluted DNA sample. Consequently, an approximate 194 bp 16S rDNA-V3 segment of bacterial DNA

was successfully obtained by nest-PCR using the 800 bp 16S rDNA segment as a template.

The DGGE fingerprints of the predominant bacteria associated with the four sponges are displayed in Fig. 2. The DGGE fingerprints have good banding patterns; every sponge sample gave rise to different community profiles. Cluster analysis indicates a clear difference in community structure in each of the four sponges.

### 3.2. Identification of the predominant bacteria by sequencing analysis

Sequencing analysis of the predominant bacteria that represent the DGGE bands of sponges *S. tenui*, *Halichrondria*, *D. avara*, and *C. australiensis* is summarized in Table 1. All the sequences obtained in this study have been assigned to the GenBank nucleic acid sequence database with accession numbers AY947721–AY947762.

Sequence analysis shows that none of the sponge-associated bacteria in Genbank are closest relatives to the 42 sequences. The 42 predominant bacteria associated with the 42 bands in DGGE gel were found in sponges for the first time. It was interesting to find *Adellie penguin guano bacterium,* uncultured Arctic sea ice bacterium, and uncultured Antarctic sea ice bacterium in sponges from the South China Sea. According to Table 1, most of the predominant bacteria in the sponge *S. tenui* are the genus *Psychrobacter*. For the sponge *Halichrondria*, the predominant bacteria composition is different to sponge *S. tenui*. Uncultured *Bacteroidetes* bacteria associated with bands C7, C8 and *Biziolla paragoriae* bacteria associated with bands C4, C9 were found in the sponge *D. avara*. In the sponge *C. australiensis*, we found *Actinobacterium* associated with band D10, *Staphylococcus* sp. and *Planococcus* sp. associated with D3, D6 band sequences.

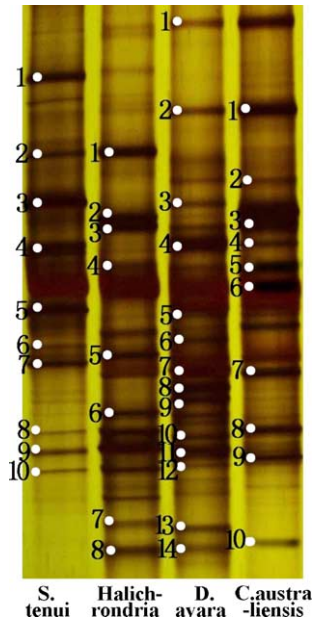


Fig. 2. DGGE fingerprints of sponges based on 16S rDNA-V3.

Based on Table 1, the predominant bacterial strains that correlate with the 16S rDNA-V3 sequences recovered from the respective 42 bands in DGGE gel of the four sponges are different. Sponge *C. australiensis* has the highest diversity of bacteria of the tested four sponges, followed by the sponges *D. avara*, *Halichrondria*, and *S. tenui*. The above results indicate that each of the four sponges, collected from the same sea location, has sponge-specific predominant bacteria.

### 3.3. Phylogenetic analysis of the predominant bacteria based on 16S rDNA-V3 sequences

Fig. 3 shows the phylogenetic relationship of all the partial 16S rDNA-V3 sequences representing the respective excised DGGE bands. The neighbor-joining analysis divided the 42 bacterial sequences into four main groups: *Proteobacteria* (total 33 sequences: 23 of γ subdivision, 7 of α subdivision, and 3 of β subdivision), *Bacteroidetes* (6 sequences), *Firmicutes* (2 sequences), *Actinobacteria* (1 sequence). The majority of the 42 sequences clustered with *Proteobacteria*, especially γ-*Proteobacteria*. Similar bacteria with close phylogenetic relationship (e.g. C1 and D8, A1 and C5, and D7) were found among the different sponges (Fig. 3).

The bacterial community structure of the four different sponges is compared in Fig. 4, according to the ratio percent of different groups of bacteria in each sponge. All the predominant bacteria in the sponges *S. tenui* and *Halichrondria* belong to the phylum *Proteobacteria*, especially the γ and α subdivisions. It is clear that γ-*proteobacteria* are the most common bacteria in the four sponges, with α-*proteobacteria* the most common bacteria in the three sponges *S. tenui*, *Halichrondria*, and *D. avara*, and β-*proteobacteria* the most common bacteria in the two sponges *Halichrondria* and *D. avara*.

Besides the predominant phylum *Proteobacteria*, the second most abundant bacteria are the phylum *Bacteroidetes*, which were found in the sponges *D. avara* and *C. australiensis*. We note that *Actinobacterium*, which correlates with band D10, *Firmicutes* bacteria (e.g. *Staphylococcus* sp.) that correlates with band D3 and *Planococcus* sp. that correlate with band

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

| Band | Accession no. | Closest relative and its accession number | % Identity |
|---|---|---|---|
| *Stelletta tenui* A1 | AY947753 | Adelie penguin guano bacterium 253 (AY377478) | 100 |
| A2 | AY947754 | Uncultured Arctic sea ice bacterium clone ARKXVI/2-136 (AY165598) | 98 |
| A3 | AY947755 | *Psychrobacter* sp.AI-2 (AY437611) | 100 |
| A4 | AY947756 | *Psychrobacter glacincola* strain ANT9276b (AY167310) | 100 |
| A5 | AY947757 | Uncultured Antarctic sea ice bacterium clone ANTXI/4-14-26 (AY165583) | 100 |
| A6 | AY947758 | *Psychrobacter maritimus* strain IH2-12 (AY789538) | 100 |
| A7 | AY947759 | *Psychrobacter maritimus* strain IH2-12 (AY789538) | 100 |
| A8 | AY947760 | *Psychrobacter maritimus* strain IH2-12 (AY789538) | 99 |
| A9 | AY947761 | *Psychrobacter* sp.AI-2 (AY437611) | 98 |
| A10 | AY947762 | Uncultured alpha-Protebacterium clone 80H (AY466776) | 100 |
| *Halichrondria* B1 | AY947721 | Acinetobacter johnsonii (AB099655) | 100 |
| B2 | AY947722 | Rainbow trout intestinal bacterium A75 (AY374112) | 100 |
| B3 | AY947723 | Uncultured bacterium clone P4-29 (AY119401) | 100 |
| B4 | AY947724 | Uncultured gamma-Proteobacterium (AB074626) | 100 |
| B5 | AY947725 | *Acidovorax* sp.Fon15 (AY788961) | 100 |
| B6 | AY947726 | *Brevundimonas vesicularis* (AY456200) | 99 |
| B7 | AY947727 | *Brevundimonas vesicularis* (AY456200) | 100 |
| B8 | AY947728 | *Stenotrophomonas maltophilia* (AY803989) | 100 |
| *Dysidea avara* C1 | AY947739 | *Klebsiella pneumoniae* (AY369139) | 100 |
| C2 | AY947740 | *Alcaligenes* sp.R-21939 (AJ786800) | 93 |
| C3 | AY947741 | *Erythobacter lutedus* strain SW-109 (AY739662) | 99 |
| C4 | AY947742 | *Biziolla paragoriae* strain KMM6029 (AY651070) | 100 |
| C5 | AY947743 | *Psychrobacter* sp.HS5323 (AY443042) | 100 |
| C6 | AY947744 | *Psychrobacter* sp.HS5323 (AY443042) | 97 |
| C7 | AY947745 | Uncultured bacteroidetes bacterium clone ML617.5J-5 (AF507867) | 95 |
| C8 | AY947746 | Uncultured bacteroidetes bacterium clone CFB3 (AY494676) | 96 |
| C9 | AY947747 | *Biziolla paragoriae* strain KMM6029 (AY651070) | 100 |
| C10 | AY947748 | Rhizobiaceae bacterium Cpub4 (AY178080) | 97 |
| C11 | AY947749 | *Acidovorax* sp.12M7 (AB120338) | 98 |
| C12 | AY947750 | Marine bacterium SIMO-IS105 (AF460869) | 100 |
| C13 | AY947751 | Uncultured alpha-Proteobacterium (AJ505786) | 100 |
| C14 | AY947752 | *Oceanisphaera* sp.MaT12 (AY690695) | 96 |
| *Craniella australiensis* D1 | AY947729 | *Cytophaga* sp.41-DBG2 (AF427479) | 96 |
| D2 | AY947730 | *Psychrobacter* sp.Mp9 (AJ551122) | 98 |
| D3 | AY947731 | *Staphylococcus* sp.10 (AY269873) | 100 |
| D4 | AY947732 | *Psychrobacter* sp.ANT9171 (AY167289) | 99 |
| D5 | AY947733 | Marine bacterium KMM3937 (AF536386) | 99 |
| D6 | AY947734 | *Planococcus* sp.D36 (AY582938) | 100 |
| D7 | AY947735 | *Psychrobacter glacincola* strain ANT9276b (AY167310) | 99 |
| D8 | AY947736 | *Photorhabdus luminescens* (AY444555) | 100 |
| D9 | AY947737 | *Halomonas* sp.MI2-20A (AY730254) | 99 |
| D10 | AY947738 | Actinobacterium MWH-HuqW11 (AJ630368) | 100 |

D6, were only found in the sponge *C. australiensis*. Thus, the bacteria of the sponge *C. australiensis* are the most diverse of the four tested sponges, whereas the bacteria of the sponge *S. tenui* is the least diverse. The bacterial community structures of the sponges are therefore different.

## 4. Discussion

Analysis of sponge-associated microbial community structure using a culture-independent nucleic acids based technique such as DGGE requires rapid and efficient unbiased DNA extraction procedures. To identify the complex, nonculturable microbial communities of sponge-associated microorganisms, the first and very important step is the extraction of microbial community DNA. The quantity of the extracted nucleic acids is obviously very important in terms of obtaining satisfactory community profiles.

There are two main approaches to the extraction of sponge total DNA: (i) the cell extraction method (Webb and Maas, 2002), where free sponge and microorganism cells need to be isolated and a special kit and equipments are required; (ii) the direct lysis method, which is based on mechanical or chemical methods. The present methods are time-consuming, inconvenient to carry out,

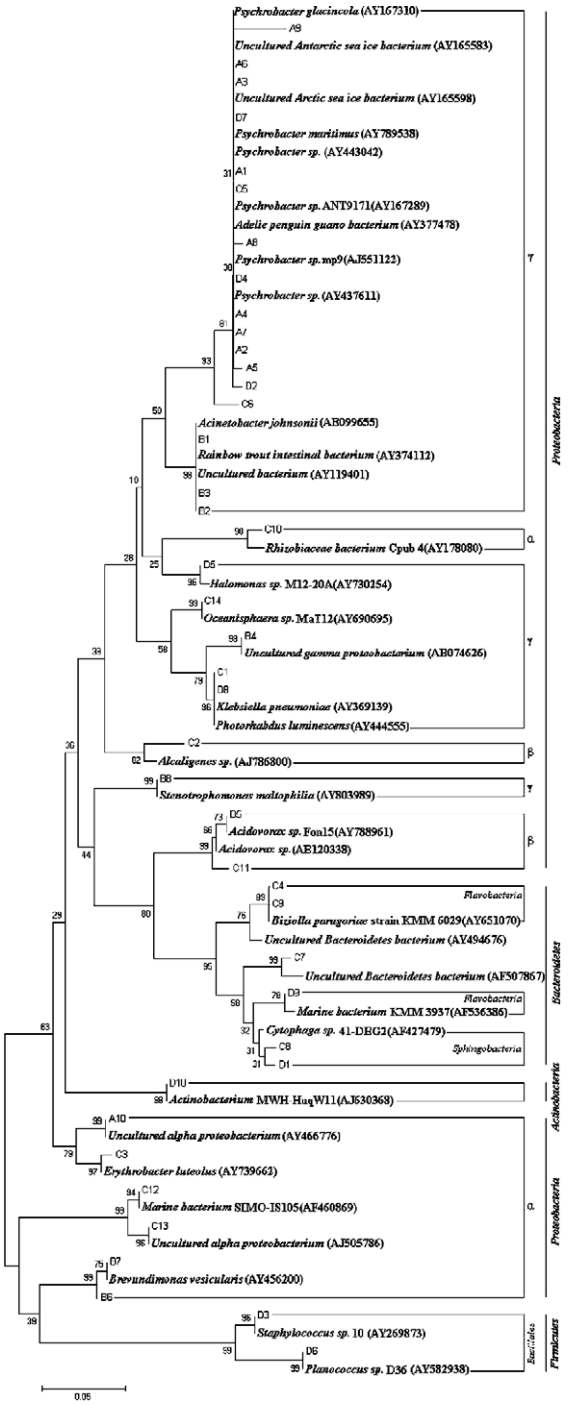


Fig. 3. Unrooted phylogenetic tree based on 16S rDNA-V3 sequences representing the respective DGGE bands in Fig. 2. Bootstrap analysis was based on 1000 replicates. Scale indicates 5% sequence divergence.

and special kit and reagents are needed (Althoff et al., 1998; Erpenbeck et al., 2002; Friedrich et al., 2001; Margot et al., 2002; Thoms et al., 2003; Webster et al., 2001b). The method used in the current paper is mainly based on direct physically grinding disruption, combined with the traditional phenol/chloroform method. The obtained total community can be used as a templet directly for 16S rDNA-PCR amplification after series dilution; there is good reproducibility between triplicate experiments with this method (data not shown). This simplifies the currently used method for DNA-based PCR analysis of sponge-associated microorganisms.

*Proteobacteria* are common in marine environments (Sekiguchi et al., 2002; Wagner-Döbler et al., 2002; Imhoff, 2001) and are always associated with marine plants (Weidner et al., 2000) or animals (Lau et al., 2002). *Proteobacteria* including α, β and γ subdivisions have been found in many sponges from different marine locations, e.g. *Aplysina cavernicola* from the coast of Elba in the Mediterranean Sea (Thoms et al., 2003) and Banyuls-sur-Mer on the coast of Marseille in France (Friedrich et al., 2001), *Rhopaloeides odorabile* from Davies Reef in Australia (Webster et al., 2001b; Webster and Hill, 2001), *Theonella swinhoei* from the western Caroline islands in the Republic of Palau (Schmidt et al., 2000), *Halichrondria panicea* from the Adriatic Sea (Croatia), the North Sea near Helgoland (Germany), the Baltic Sea near Kiel (Germany) (Althoff et al., 1998), and the sponges *Jaspis johnstoni* and *Plakortis lita* (Guan et al., 2000). Based on our results, phylum *Proteobacteria* are the predominant bacteria in the four sponges from the South China Sea, which is in agreement with the above published results; our results are also in agreement with the DGGE-based study of Webster et al. (2004), in which α- and γ-*protebacteria* and *Bacteroidetes* were found in the sponges *Kirkpatrickia varialosa*, *Latrunculia apicalis*, *Homaxinella balfourensis*, *Mycale acerata* and *Sphaerotylus antarcticus*. The uniform bacterial community of the sponges *S. tenui* and *Halichrondria* is consistent with Hentschel et al. (2002). The phylum *Proteobacteria* are always found in different sponges from the same or different geographic location.

*Proteobacteria* have been suggested to have varied effects on sponge hosts such as nitrogen fixation (Burnett and Mckenzie, 1997) and manipulating host reproduction (Stouthamer et al., 1999). Kalinovskaya et al. (2004) demonstrated that *Proteobacteria* produce low-molecular-weight biological active compounds with antimicrobial and surface-active properties. *Proteobacteria* were also found to produce enzymes at high levels for degrading protein and polysaccharides (Groudieva et al., 2004). In addition, a sulfate-reducting function of *Proteobacteria* was suggested by Hayes and Lovely (2002), and bioactive compounds have been found in *Cytophaga* isolated from the marine environment

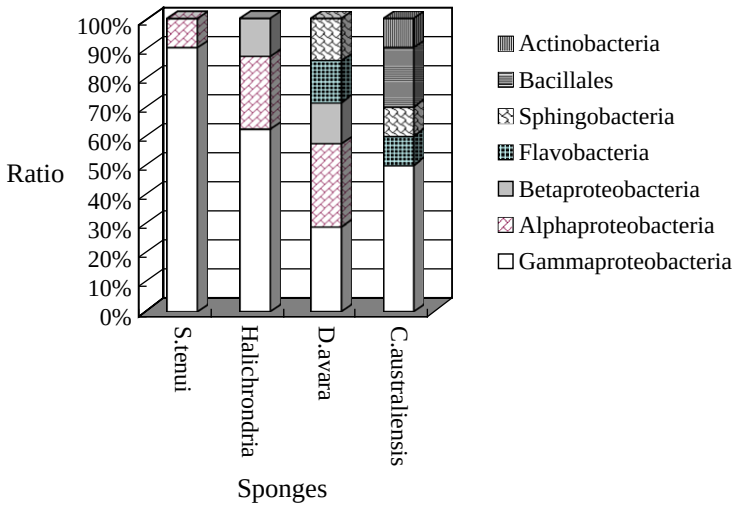


Fig. 4. Comparison of predominant bacterial community among sponges based on 16S rDNA-V3.

(Wagner-Döbler et al., 2002). As one of the characteristics of symbiotic microorganisms is that symbionts are always found in association with the same host species (Osinga et al., 2001), and all the above studies indicate an association of sponge bioactivity with *Proteobacteria* within the sponge, we propose that *Proteobacteria* are in close symbiotic relationship with sponges.

We found *Bacteroidetes* in the sponges *D. avara* and *C. australiensis*. Thoms et al. (2003) and Friedrich et al. (1999, 2001) found *Bacteroidetes* in the sponge *A. cavernicola* from the Mediterranean Sea and the sponge *Aplysina aerophoba* from Banyuls-sur-Mer on the coast of Marseille, France. *Actinobacteria* have been found previously by Hentschel et al. (2002) and Montalvo et al. (2005). In the sponge *C. australiensis* from the South China Sea, we found *Actinobacterium* and now more than twenty strains of *Actinobacteria* have been isolated from the sponge *C. australiensis* and proved to have anti-microbial and enzymatic activity (data not shown); this indicates the potential value of sponge-associated bacteria in active compound production. Although *Staphylococcus* is a typical inhabitant of human skin, the possibility of contamination can be eliminated because of the axenic experimental conditions and the fact that *Staphylococcus* was found in the sponge *C. australiensis*. The possibility of detecting *Staphylococcus* contaminants by DGGE from sponge is small because DGGE is generally suitable only to detect predominant microorganisms with abundances above 1%.

The different phylum *Proteobacteria* strains in the four sponges and specific *C. australiensis*-associated phylums *Actinobacteria* and *Firmicutes* reflect host particularity; this is the basis of host-specificity of sponge-associated bacteria despite the similarity in predominant bacteria in these sponges which may result from the same seawater environment (Fig. 3). Taylor et al. (2004) also studied host-specificity because some bacteria were found only in one host species.

In this paper, the result of DGGE fingerprints is based on approximate 194 bp 16S rDNA-V3 fragments; this is a major limitation of the phylogenetic analysis shown in Fig. 3. DGGE fingerprinting analysis provides an appropriate culture-independent approach for the rapid detection of the predominant microbial species and enables the simultaneous analysis of multiple samples. Based on published results (Cebron et al., 2004), phylogenetic analysis based on small 16S rDNA fragment can be useful for understanding the basic relationship among strains.

16S rDNA-DGGE fingerprinting is particularly useful as an initial investigation for distinguishing between communities and identifying the numerically dominant community members. DGGE can separate sequences of the same length with only a base difference. For example, there is only a G base difference in sequence A8 instead of an A base in the A6 and A7 sequences and a T base difference between sequences B6 and B7. Based on our study, attention should be given to 16S rDNA-DGGE fingerprinting analysis. Firstly, bands at the same position in the DGGE gel theoretically contain DNA fragments with identical sequences (e.g. *Gammaproteobacteria* A4 and D4), but in some situations this is incorrect because bands at the same position in the gel have the same melting behavior that is mainly based on GC-content difference (Muzyer et al., 1998) rather than gene sequence. Secondly, although

the ideal case is a DGGE band that represents a single 16S rDNA sequence, some bands cannot be separated; bands that appear in the same location in DGGE gel may give rise to different sequences. Thirdly, the same sequence may have different bands in DGGE gel (e.g., bands A6, A7 have the same 16S rDNA-V3 sequence but different locations). This may result from the different conformation of one sequence and will provide an over-estimate of microbial diversity. This phenomenon of one sequence with multiple bands in DGGE gel has also been found by Fasoli et al. (2003).

Finally, we cannot draw a final conclusion on sequence similarity according to band locations in DGGE gels. For instance, the locations of A1, A2, A3, A6, and A7; C5 and D7; B1 and B2; B3, C1 and D8; and C4 and C9 are obviously different, but were grouped in one cluster with high similarity. Although bands C4 and D4 have a similar location in DGGE gel, their phylogenetic relationship is weak. Cluster analysis based mainly on DGGE profiles can give rise to questionable results. DGGE fingerprinting should therefore be combined with other molecular methods such as gene sequencing and phylogenetic analysis in microbial diversity analysis and community comparisons.

DGGE-based methods provide information on species only when the populations are equally abundant. In general, a 16S rDNA library strategy can reveal much more bacterial diversity than a DGGE-based strategy (Hentschel et al., 2002), especially when the bacteria are not predominant. FISH strategy is suitable for some special strains in terms of their distribution in sponges. If we take the detailed characteristics of different techniques into account, the differences between our results and those of Hentschel et al. (2002) and Webster et al. (2001b) using 16S rDNA library and FISH methods can be easily understood.

## 5. Conclusions

The method used in this paper for total community DNA extraction from sponges and 16S rDNA-PCR analysis simplifies currently used procedures. The extracted DNA can be used directly as a templet in 16S rDNA-PCR amplification after series dilution and meets the demands of sponge-associated dominant microbial community analysis by PCR-DGGE fingerprinting with good DGGE banding patterns.

The phylum *Proteobacteria*, which are perhaps symbionts to sponges, are the main sponge-associated bacteria in the four analyzed sponges, followed by *Bacteroidetes*. *Gammaproteobacteria* in all four species of sponges are the most predominant *Proteobacteria*. The bacterial communities in the sponges *S. tenui* and *Halichrondria* are similar, with all the predominant bacteria belonging to the phylum *Proteobacteria*. Sponge *C. australiensis* has the greatest bacterial diversity of the four sponges with the four bacteria phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, followed by the sponge *D. avara* with the two phyla *Proteobacteria* and *Bacteroidetes*; only *Proteobacteria* were found in the sponges *S. tenui* and *Halichrondria*. Though similar bacteria with close phylogenetic relationships are found in different sponges, the sponge-associated predominant bacterial community structures are different. The sponge-associated bacteria are host-specific because the detailed predominant bacterial diversity is different. The different 42 predominant bacteria that correlate with the 42 bands in DGGE gel were found in sponges for the first time in this study.

DGGE fingerprint-based analysis should be combined with band cloning and sequencing, phylogenetic analysis, and molecular techniques to obtain precise results for the microbial community and diversity revelation.

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