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ARTICLE *in* MARINE BIOTECHNOLOGY · FEBRUARY 2012

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ORIGINAL ARTICLE

Bacterial and Archaeal Symbionts in the South China Sea Sponge *Phakellia fusca*: Community Structure, Relative Abundance, and Ammonia-Oxidizing Populations

Minqi Han · Fang Liu · Fengli Zhang · Zhiyong Li · Houwen Lin

Received: 28 July 2011 / Accepted: 16 January 2012

Abstract Many biologically active natural products have been isolated from *Phakellia fusca*, an indigenous sponge in the South China Sea; however, the microbial symbionts of *Phakellia fusca* remain unknown. The present investigations on sponge microbial community are mainly based on qualitative analysis, while quantitative analysis, e.g., relative abundance, is rarely carried out, and little is known about the roles of microbial symbionts. In this study, the community structure and relative abundance of bacteria, actinobacteria, and archaea associated with *Phakellia fusca* were revealed by 16S rRNA gene library-based sequencing and quantitative real time PCR (qRT-PCR). The ammonia-oxidizing populations were investigated based on *amo*A gene and anammox-specific 16S rRNA gene libraries. As a result, it was found that bacterial symbionts of sponge *Phakellia fusca* consist of *Proteobacteria* including *Gamma-*, *Alpha-*, and *Delta-proteobacteria*, *Cyanobacteria* with *Gamma-proteobacteria* as the predominant components. In particular, the diversity of actinobacterial symbionts in *Phakellia fusca* is high, which is composed of *Corynebacterineae*, *Acidimicrobidae*, *Frankineae*, *Micrococcineae*, and *Streptosporangineae*. All the observed archaea in sponge *Phakellia fusca* belong to *Crenarchaeota*, and the detected ammonia-oxidizing populations are ammonia-oxidizing archaea, suggesting the nitrification function of sponge archaeal symbionts. According to qRT-PCR analysis, bacterial symbionts dominated the microbial community, while archaea represented the second predominant symbionts, followed by actinobacteria. The revealed diverse prokaryotic symbionts of *Phakellia fusca* are valuable for the understanding and in-depth utilization of *Phakellia fusca* microbial symbionts. This study extends our knowledge of the community, especially the relative abundance of microbial symbionts in sponges.

Keywords *Phakellia fusca* · Bacteria · Actinobacteria · Archaea · Community structure · Abundance, *amo*A gene

Introduction

Sponges (Porifera), which are known as the most ancient metazoans, have been the focus of much recent interest firstly due to their close associations with a wide variety of microorganisms, e.g., symbiotic relationship. According to Taylor et al. (2007a), the term "symbiont" is used in its loosest possible definition as two or more different organisms that live together over a long period of time. A variety of symbiotic prokaryotic microbes, including two phyla archaea and 30 phyla bacteria, have been discovered in sponges using culture-independent molecular approaches and deep sequencing (Lee et al. 2011; Taylor et al. 2007a; Webster et al. 2010). Diverse fungal symbionts have also been observed in sponges (Gao et al. 2008). Based on the observed similar sponge symbionts from different habitats, sponge-specific symbionts, including candidate phylum, *Poribacteria*, have been suggested (Fieseler et al. 2004;

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Published online: 05 February 2012

Hentschel et al. 2002; Taylor et al. 2007a), which are different from the microbial community of environmental sea water. Another reason for the increasing research interest in sponges is because they are a rich source of biologically active second metabolites (Taylor et al. 2007a; Wang 2006). Nowadays, more and more natural products isolated from sponges are deemed to be synthesized by sponge symbiotic microorganisms (Piel 2004). Therefore, study on the symbiotic microorganisms of sponges is valuable for the revelation of the true origin of sponge-derived natural products and provides evidence for the contribution of microbial symbionts for sponge hosts.

At present, most of the information on the microbial community in sponges is based on qualitative investigation, while the quantitative analysis on the microbial community is rarely carried out, for example, the relative abundance of bacteria, actinobacteria, and archaea in one sponge remains largely unknown. Since sponges account for about one-fifth of marine animals and are able to filter enormous volumes of seawater, they have potential influence on the marine ecosystem. But, compared with sponge-associated microbial diversity (Hentschel et al. 2002, 2003, 2006; Taylor et al. 2007a; Wang 2006; Webster et al. 2004), to date, little is known about the roles of microbial symbionts in sponge biology and ecology and the association of sponge-microbial symbionts (Taylor et al. 2007b; Vogel 2008; Webster and Blackall 2009).

It is estimated that there are several thousand species of sponges in China oceans (Zhang et al. 2003), but compared with sponges from other sea areas, the diversity of China Sea sponge microbial symbionts remain largely unknown (Li et al. 2006; Li and Liu 2006; Li 2009). Sponge *Phakellia fusca* is an indigenous sponge in the South China Sea, from which many natural products with various biological activities have been isolated (Li et al. 2003; Pettit et al. 1993; Pettit et al. 1995), thus *Phakellia fusca* is with great biotechnological value. However, the community structure of microbial symbionts in *Phakellia fusca* remains unknown, let alone the knowledge of their ecological roles. The revelation of microbial community of *Phakellia fusca* is valuable for the understanding and in-depth utilization of *Phakellia fusca* microbial symbionts.

In this study, for the first time, the community structure of prokaryotic microbial symbionts, including ammonia-oxidizing populations in the South China Sea sponge *Phakellia fusca*, was investigated using 16S rRNA and ammonia monooxygenase gene library analysis. Particularly, the relative abundance of bacteria, actinobacteria, archaea, and ammonia-oxidizing archaea was analyzed by quantitative real time PCR (qRT-PCR). It is the first time to reveal the relative abundance of bacteria, actinobacteria, archaea, and ammonia-oxidizing archaea in one sponge as well as *amo*A genes in China Sea sponges.

Materials and Methods

Sponge Sample

Sponge *Phakellia fusca* was collected around Yongxing Island (16°50′N, 112°20′E) in the South China Sea at a depth of ca. 20 m by scuba diving and enclosed in sterile bag immediately. Before DNA extraction, the sponge samples were stored at −20°C. The sponge was identified by Prof. Jinhe Li at the Institute of Oceanology, Chinese Academy of Sciences.

DNA Extraction and PCR Amplification

Total genomic DNA was extracted from 20-mg lyophilized sponge tissue using a DNeasy Blood & Tissue Kit (Qiagen). Then, the extracted total genomic DNA was purified using a MicroElute DNA Clean-Pure Kit (OMEGA). The target genes were amplified using corresponding primers as shown in Table 1 and TransStart™ FastPfu DNA Polymerase (Transgen) in Mastercycler personal (Eppendorf).

PCR conditions for bacterial 16S rRNA genes, archaeal 16S rRNA genes, *amoA* genes of ammonia-oxidizing archaea (AOA), and ammonia-oxidizing bacteria (AOB) were as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, annealing for 20 s at the temperature depending on primers (Table 1), elongation at 72°C for 30 to 50 s, and final elongation at 72°C for 10 min.

A "touchdown" protocol was used to amplify actinobacterial 16S rRNA genes and anammox-specific 16S rRNA genes of anaerobic ammonia-oxidizing bacteria (AnAOB) (James et al. 2003; Mohamed et al. 2010; Roux 1995). The actinobacterial 16S rRNA PCR reaction was carried out with 5 min at 95°C, followed by denaturation at 95°C for 20 s, annealing at 72°C for 20 s, and elongation at 72°C for 30 s. From the preceding cycle (annealing temperature was 72°C), ten cycles in which the annealing temperature was decreased by 0.5°C per cycle were carried out and then 25 cycles of 95°C for 20 s, 68°C for 20 s, and 72°C for 30 s, with the last cycle followed by a 10-min elongation at 72°C. The anaerobic ammonia-oxidizing bacteria (AnAOB) PCR reaction was carried out with denaturation at 95°C for 5 min, seven touchdown cycles (denaturation at 95°C for 20 s; annealing at 62°C, 60°C, 59°C, 58°C, 57°C, 56°C, and 55°C for 20 s; and elongation at 72°C for 40 s), 30 consistent cycles (at 95°C for 20 s, at 54°C for 20 s, and at 72°C for 40 s), and final elongation at 72°C for 10 min.

Gene Library Construction and Sequencing

PCR products were purified by electrophoresis on a 1% (wt/vol) agarose gel, and the products were recovered using an AxyPrep™ DNA Gel Extraction Kit (AXYGEN) and

Table 1 Primers used in PCR and qRT-PCR

Primer	Sequence	Reference
27F[1]	5′-AGAGTTTGATCCTGGCTCAG-3′	Universal primer
21F[2]	5′-TTCCGGTTGATCCYGCCGCCGGA-3′	Universal primer
1492R[1,2]	5′-GGTTACCTTGTTACGACTT-3′	Universal primer
S-C-Act-0234-a-S-20[3,9]	5′-CGCGGCCTATCAGCTTGTTG-3′	James et al. 2003
S-C-Act-0878-a-A-19[3]	5′-CCGTACTCCCCAGGCGGGG-3′	
Arch-*amo*AF[4]	5′-STAATGGTCTGGCTTAGACG-3′	Christopher et al. 2005
Arch-*amo*AR[4]	5′-GCGGCCATCCATCTGTATGT-3′	
*amo*A-1F[5]	5′-GGGGTTTCTACTGGTGGT-3′	Mohamed et al. 2010
*amo*A-2R[5]	5′-CCCCTCKGSAAAGCCTTCTTC-3′	
AMX386F[6]	5′-CCTTTCGGGCATTGCGAA-3′	Mohamed et al. 2010
1392R[6]	5′-ACGGGCGGTGTGTAC-3′	Universal primer
341F[7]	5′-CCTACGGGAGGCAGCAG-3′	Muyzer et al. 1993
534R[7]	5′-ATTACCGCGGCTGCTGG-3′	
PARCH340F[8]	5′-CCCTACGGGGYGCASCAG-3′	Ovreas et al. 1997
PARCH519R[8]	5′-TTACCGCGGCKGCTG-3′	
513R[9]	5′-CGGCCGCGGCTGCTGGCACGTA-3′	Heuer and Krsek 1997
*amo*A19F[10]	5′-ATGGTCTGGCTWAGACG-3′	Nakaya et al. 2009
*amo*A643R[10]	5′-TCCCACTTWGACCARGCGGCCATCCA-3′	

[1,2] Amplify bacterial and archaeal 16S rRNA gene, to investigate diversity of bacteria and archaea. Annealing temperatures were 55°C and 52°C, respectively

[3] Amplify actinobacterial partial 16S rRNA gene, to investigate diversity of actinobacteria

[4,5] Amplify archaeal and bacterial *amo*A gene, to investigate diversity of AOA and AOB. Annealing temperatures were 53°C and 60°C, respectively

[6] Amplify anammox-specific 16S rRNA gene, to investigate diversity of AnAOB

[7,8,9,10] To quantify bacteria, archaea, actinobacteria, and AOA. Annealing temperatures were 55°C, 53.5°C, 54°C, and 55°C, respectively

ligated into pEASY-Blunt Simple vector and transformed into Trans1-T1 Phage Resistant Chemically Competent Cells using a pEASY-Blunt Simple Cloning Kit (Transgen). The positive recombinants were screened on indicator plates with X-Gal, IPTG, and ampicillin by color-based recombinant selection. The positive clones were sequenced using universal primers M13F and M13R by Shanghai Invitrogen Company.

Quantitative Real-Time PCR (qRT-PCR)

Total DNA from sponge *Phakellia fusca* was used to generate a plasmid standard containing the target region. Each kind of the qRT-PCR targeted fragment generated by PCR was cloned, and the purified recombinant plasmid DNA concentration was measured fluorimetrically using a Hoefer DyNA Quant 200 apparatus (Amersham Biosciences Corp); calf thymus DNA (Pharmacia Biotech) was used as a standard. The purified recombinant plasmid DNA was serially diluted in ddH_2O (double-distilled water) to a final concentration ranging from 1.5×10^9 to 1.5×10^2 copies/μL. Two-microliter aliquots of each dilution were used for qRT-PCR to generate the standard curve and used as quantization standard.

All qRT-PCR experiments were performed in 8-Strip Low Profile tubes (TLS-0851; MJ Research, Watertown, MA) and closed with Ultra Clear caps (TCS-0803; MJ Research). In the preliminary experiment, the temperatures at which the fluorescent signal was read were determined by using melting curves. This can exclude interference of primer dimmers or unspecific products. The following qRT-PCR program was performed as described by the handbook of SYBR® Premix Ex Taq™ II (Takara): an initial denaturation step at 95°C for 2 min, 35 consistent cycles of 95°C for 20 s, annealing for 30 s at the temperature depending on primers (Table 1), and elongation at 83°C for 10 s. At the end of each cycle, the fluorescent signal was measured at 83~84°C. Total genomic DNA sample was quantified using corresponding primers (Table 1), each reaction was carried out in triplicate in DNA Engine Opticon 2 system (MJ Research), and data were recorded and analyzed by using the corresponding Monitor software (Version 1.1). Each copy number was the average of three parallel samples.

Estimation of Community Richness and Phylogenetic Analysis

Operational taxonomic units (OTUs) were defined as sequence groups in which sequences differed by 3%. Meanwhile ACE, Chao1, Shannon, Simpson, and rarefraction

analysis were performed using DOTUR (Schloss and Handelsman 2005). One representative clone was selected from each OTU for further phylogenetic analysis. According to maximum identity and habitat, all of OTUs' nearest neighbors were determined by BLAST analysis. Neighbor-joining phylogenetic trees were constructed using MEGA4.1 (Tamura et al. 2007). At the same time, based on nomenclatural taxonomy and Bergey's Manual, all sequences were classified using the RDP (http://rdp.cme.msu.edu/index.jsp) classifier with a confidence threshold of 80%.

Nucleotide Sequence Accession Number

All sequences obtained in this study were submitted to GenBank under accession numbers HQ877724–HQ877761 (for bacterial, archaeal, and actinobacterial 16S rRNA gene clone sequences) and HQ877762–HQ877764 (for archaeal *amo*A gene clone sequences).

Results

Community Structure of Prokaryotic Microbial Symbionts in Sponge *Phakellia fusca*

Three 16S rRNA gene clone libraries were successfully constructed from the total genomic DNA of bacteria, actinobacteria, and archaea in sponge *Phakellia fusca*, respectively. In all, 104, 90, and 97 clones were generated in three 16S rRNA gene libraries for bacteria, actinobacteria, and archaea, respectively, and 60, 46, and 50 randomly selected clones, respectively, were sequenced. Figure 1 shows that all three rarefaction curves for 16S rRNA gene clones tend to be steady and reach saturation at the phylum level (distance=0.20). At the 97% identity level, 9, 16, and 10 representative OTUs from bacterial, actinobacterial, and archaeal 16S rRNA gene clone libraries were observed, respectively. Phylogenetic trees based on the representative OTUs of bacteria, actinobacteria, and archaea are shown in Figs. 2, 3, and 4, respectively.

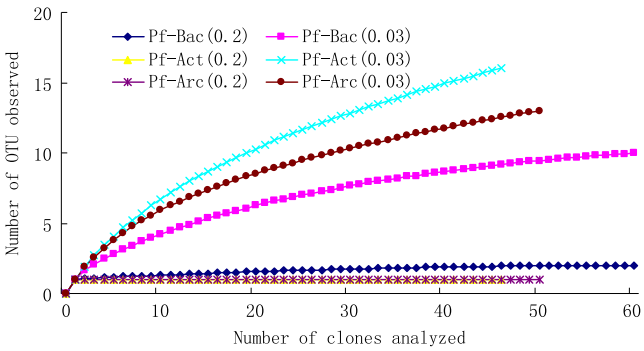


Fig. 1 Rarefaction curves for bacterial, actinobacterial, and archaeal 16S rRNA gene sequences. OTUs were defined at distances of 0.20 and 0.03, respectively

As shown in Fig. 2, the bacterial symbionts of sponge *Phakellia fusca* consist of *Proteobacteria* including *Gamma-*, *Alpha-*, and *Delta-proteobacteria* and *Cyanobacteria* based on RDP analysis. All the *Proteobacteria* clone sequences affiliated with sponge-derived clones except for X1bac5. Meanwhile, four out of five *Gammaproteobacteria* spacer OTUs formed an independent clade. Besides the sponge-derived closest relatives, all the other nearest neighbors are of marine origin, including AM997287 (deep-sea sediment clone), EU925907 (Northern Bering Sea sediment clone), AB015565 (deep-sea sediment clone), EF574483 (Coco's Island marine clone), and FJ155044 (Bohai Bay clone). As shown in Fig. 5a, *Gammaproteobacteria* (78%) are the predominant bacteria in sponge *Phakellia fusca*, followed by *Cyanobacteria* (12%).

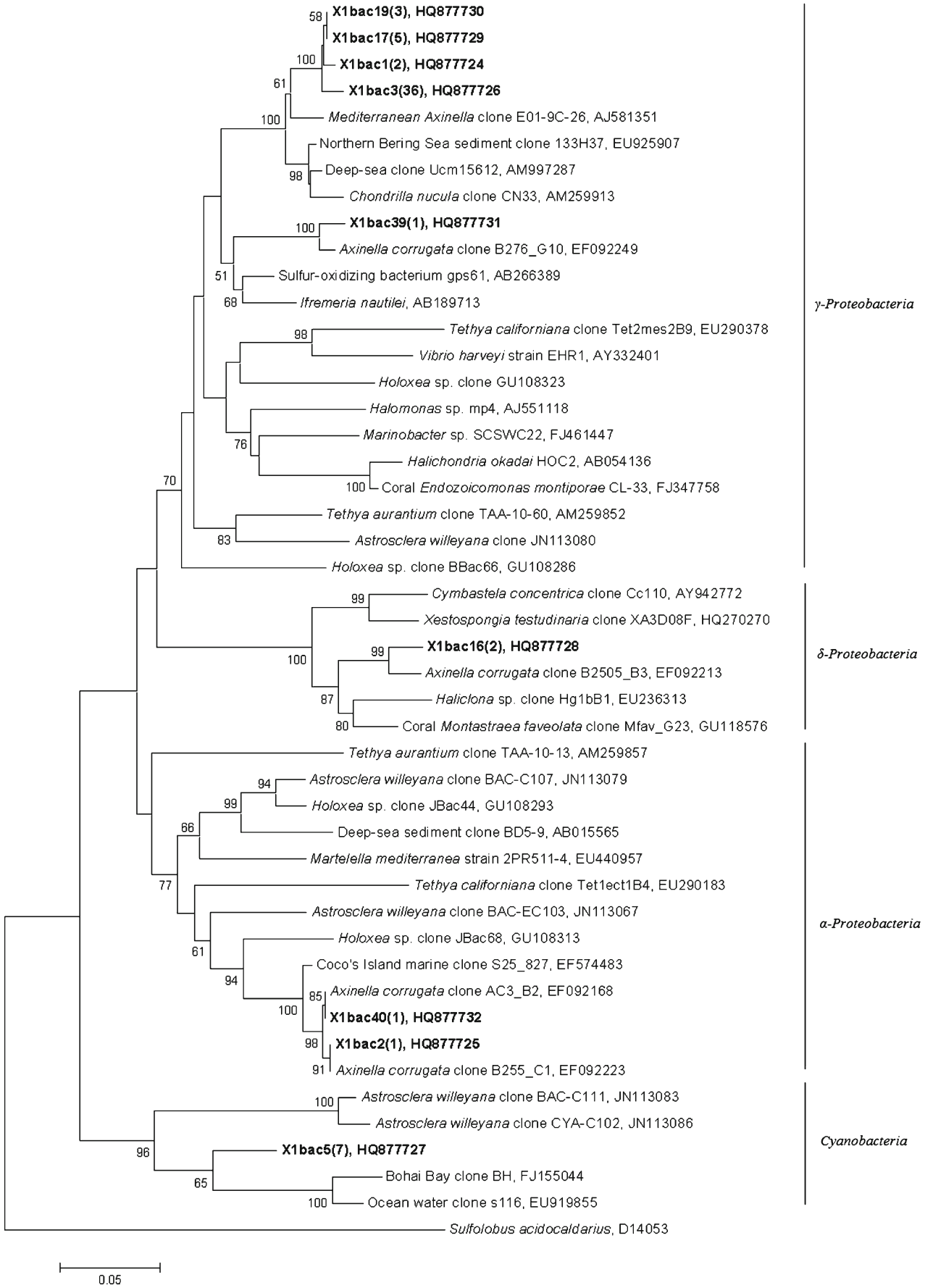
Figures 3 and 6 show that the diversity of actinobacteria in sponge *Phakellia fusca* is high. *Acidimicrobidae*, *Corynebacterineae*, *Micrococcineae*, and some rare actinobacteria, including *Frankineae* and *Streptosporanginea*, were observed based on RDP analysis. As shown in Fig. 5b, *Corynebacterineae* (48%) dominates the actinobacterial community, followed by *Acidimicrobidae* (22%), *Frankineae* (15%), *Micrococcineae* (13%), and *Streptosporangineae* (2%). In Fig. 3, all the *Acidimicrobidae* is grouped into the sponge-references group (FJ229948, FJ229962, and AJ347026). The closest relatives of X1act11 and X1act2, FJ999615, and GQ504245 are also actinobacteria associated with marine sponges.

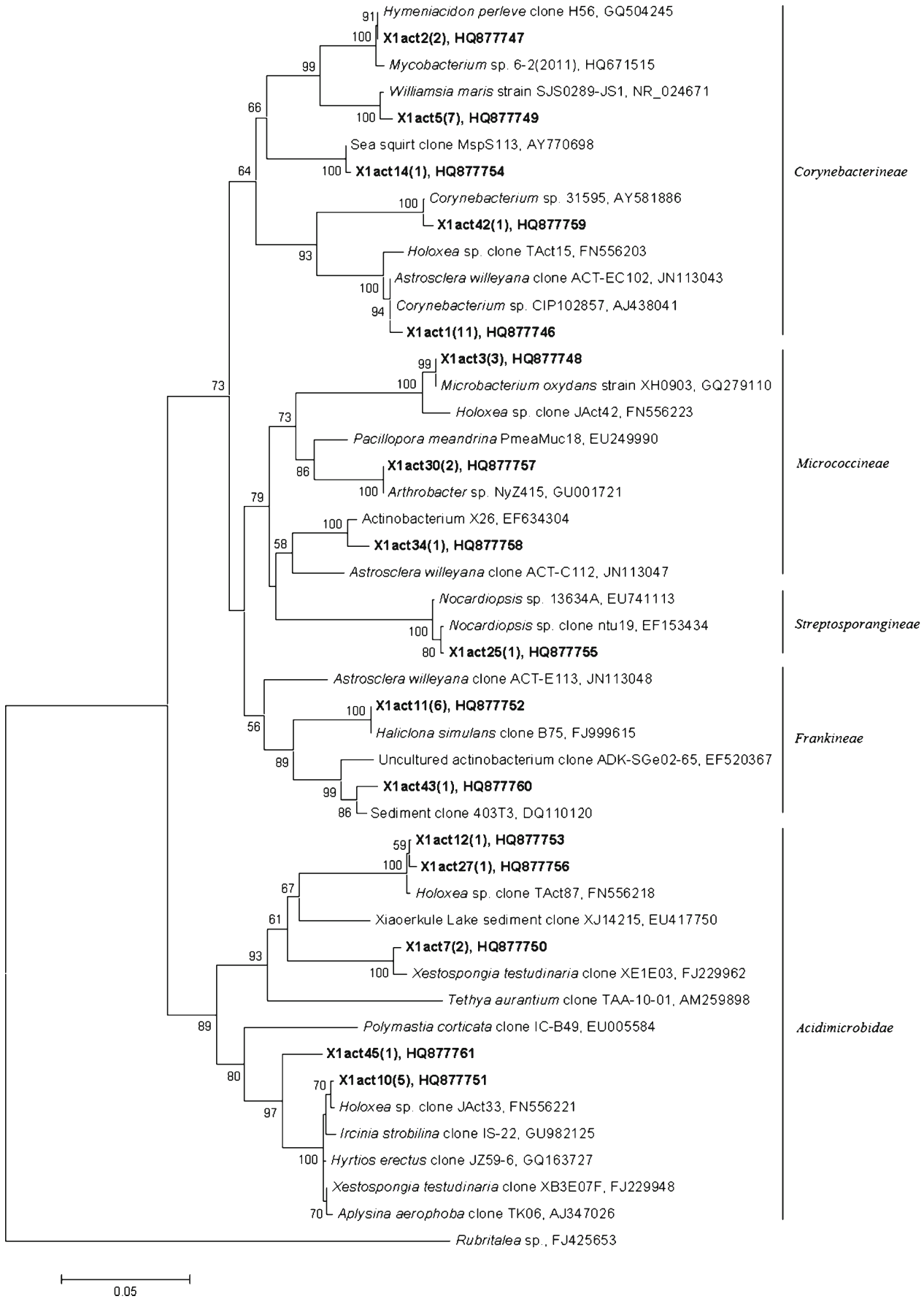
All of the observed archaeal sequences showed high similarity to uncultured crenarchaeote clones at 90–99% based on BLAST analysis. They were grouped together and related to uncultured marine sponges *Crenarchaeote* clones (AF420236, AF421159, AF420237, DQ299277, DQ299280, DQ424908, EU005641, and AY192629) in Fig. 4.

Ammonia-Oxidizing Populations in Sponge *Phakellia fusca*

Based on the PCR screening using primer set *amo*A-1F/*amo*A-2R for ammonia-oxidizing bacteria (AOB) and primer set AMX386F/1392R for anaerobic ammonia-oxidizing bacteria (AnAOB), AOB and AnAOB were not found in sponge *Phakellia fusca*. The *amo*A gene clone library of AOA was constructed successfully after archaeal *amo*A gene fragments were amplified from the total genomic

Fig. 2 Phylogenetic neighbor-joining tree based on ca. 1,500 bp of ▶ bacterial 16S rRNA gene sequences. Root was D14053 (*Sulfolobus acidocaldarius*). Bootstrap values <50% were hidden. The number of replicates was 1,000. The *scale bar* represents 0.05 substitutions per nucleotide position. The number in brackets showed the number of sequences in each OTU


X1bac19(3), HQ877730
X1bac17(5), HQ877729
X1bac1(2), HQ877724
X1bac3(36), HQ877726
Mediterranean Axinella clone E01-9C-26, AJ581351
Northern Bering Sea sediment clone 133H37, EU925907
Deep-sea clone Ucm15612, AM997287
Chondrilla nucula clone CN33, AM259913
X1bac39(1), HQ877731
Axinella corrugata clone B276_G10, EF092249
Sulfur-oxidizing bacterium gps61, AB266389
Ifremeria nautilei, AB189713
Tethya californiana clone Tet2mes2B9, EU290378
Vibrio harveyi strain EHR1, AY332401
Holoxea sp. clone GU108323
Halomonas sp. mp4, AJ551118
Marinobacter sp. SCSWC22, FJ461447
Halichondria okadai HOC2, AB054136
Coral Endozoicomonas montiporae CL-33, FJ347758
Tethya aurantium clone TAA-10-60, AM259852
Astrosclera willeyana clone JN113080
Holoxea sp. clone BBac66, GU108286
Cymbastela concentrica clone Cc110, AY942772
Xestospongia testudinaria clone XA3D08F, HQ270270
X1bac16(2), HQ877728
Axinella corrugata clone B2505_B3, EF092213
Haliclona sp. clone Hg1bB1, EU236313
Coral Montastraea faveolata clone Mfav_G23, GU118576
Tethya aurantium clone TAA-10-13, AM259857
Astrosclera willeyana clone BAC-C107, JN113079
Holoxea sp. clone JBac44, GU108293
Deep-sea sediment clone BD5-9, AB015565
Martelella mediterranea strain 2PR511-4, EU440957
Tethya californiana clone Tet1ect1B4, EU290183
Astrosclera willeyana clone BAC-EC103, JN113067
Holoxea sp. clone JBac68, GU108313
Coco's Island marine clone S25_827, EF574483
Axinella corrugata clone AC3_B2, EF092168
X1bac40(1), HQ877732
X1bac2(1), HQ877725
Axinella corrugata clone B255_C1, EF092223
Astrosclera willeyana clone BAC-C111, JN113083
Astrosclera willeyana clone CYA-C102, JN113086
X1bac5(7), HQ877727
Bohai Bay clone BH, FJ155044
Ocean water clone s116, EU919855
Sulfolobus acidocaldarius, D14053
γ-Proteobacteria
δ-Proteobacteria
α-Proteobacteria
Cyanobacteria
0.05


Hymeniacidon perleve clone H56, GQ504245
X1act2(2), HQ877747
Mycobacterium sp. 6-2(2011), HQ671515
Williamsia maris strain SJS0289-JS1, NR_024671
X1act5(7), HQ877749
Sea squirt clone MspS113, AY770698
X1act14(1), HQ877754
Corynebacterium sp. 31595, AY581886
X1act42(1), HQ877759
Holoxea sp. clone TAct15, FN556203
Astrosclera willeyana clone ACT-EC102, JN113043
Corynebacterium sp. CIP102857, AJ438041
X1act1(11), HQ877746
X1act3(3), HQ877748
Microbacterium oxydans strain XH0903, GQ279110
Holoxea sp. clone JAct42, FN556223
Pacillopora meandrina PmeaMuc18, EU249990
X1act30(2), HQ877757
Arthrobacter sp. NyZ415, GU001721
Actinobacterium X26, EF634304
X1act34(1), HQ877758
Astrosclera willeyana clone ACT-C112, JN113047
Nocardiopsis sp. 13634A, EU741113
Nocardiopsis sp. clone ntu19, EF153434
X1act25(1), HQ877755
Astrosclera willeyana clone ACT-E113, JN113048
X1act11(6), HQ877752
Haliclona simulans clone B75, FJ999615
Uncultured actinobacterium clone ADK-SGe02-65, EF520367
X1act43(1), HQ877760
Sediment clone 403T3, DQ110120
X1act12(1), HQ877753
X1act27(1), HQ877756
Holoxea sp. clone TAct87, FN556218
Xiaoerkule Lake sediment clone XJ14215, EU417750
X1act7(2), HQ877750
Xestospongia testudinaria clone XE1E03, FJ229962
Tethya aurantium clone TAA-10-01, AM259898
Polymastia corticata clone IC-B49, EU005584
X1act45(1), HQ877761
X1act10(5), HQ877751
Holoxea sp. clone JAct33, FN556221
Ircinia strobilina clone IS-22, GU982125
Hyrtios erectus clone JZ59-6, GQ163727
Xestospongia testudinaria clone XB3E07F, FJ229948
Aplysina aerophoba clone TK06, AJ347026
Rubritalea sp., FJ425653
Corynebacterineae
Micrococcineae
Streptosporangineae
Frankineae
Acidimicrobidae
0.05

◀ **Fig. 3** Phylogenetic neighbor-joining tree based on about 640 bp of actinobacterial partial 16S rRNA gene sequences. Root was FJ425653 (*Rubritalea* sp.). Bootstrap values <50% were hidden. The number of replicates was 1,000. The *scale bar* represents 0.05 substitutions per nucleotide position. The number in brackets showed the number of sequences in each OTU

DNA of sponge *Phakellia fusca* using primers Arch-*amo*AF and Arch-*amo*AR. Fifteen randomly selected clones from AOA *amoA* gene clone library were sequenced, and three OTUs were observed. All the detected *amoA* gene sequences of AOA were 82% identical to an uncultured marine sponge clone (EU049840) at phylum level and 80% identical to GU270252 (Okhotsk Sea deep-sea sediment clone),

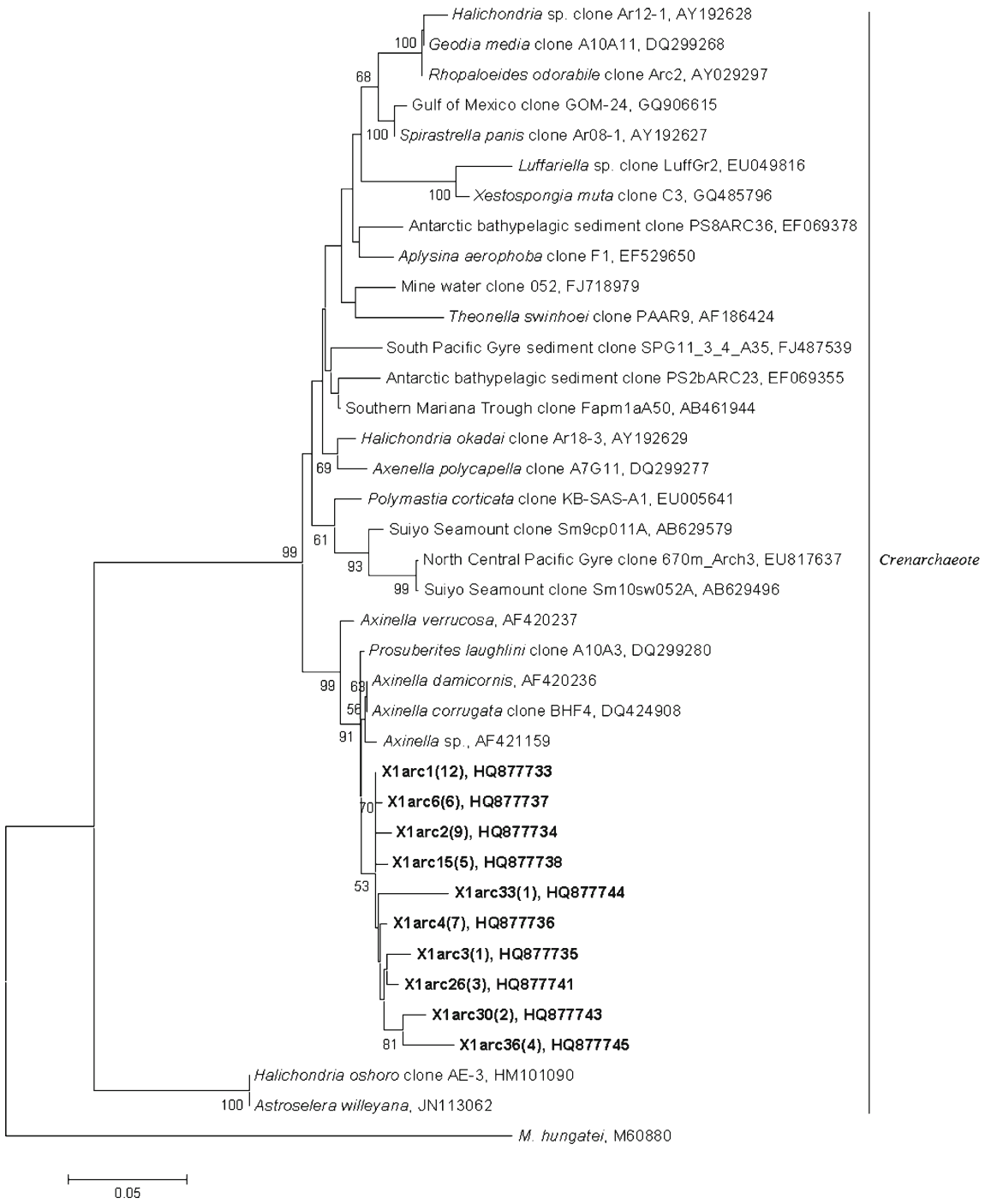


Fig. 4 Phylogenetic neighbor-joining tree based on about 1,500 bp of archaeal 16S rRNA gene sequences. Root was M60880 (*M. hungatei*). Bootstrap values <50% were hidden. The number of replicates was 1,000. The *scale bar* represents 0.05 substitutions per nucleotide position. The number in brackets showed the number of sequences in each OTU

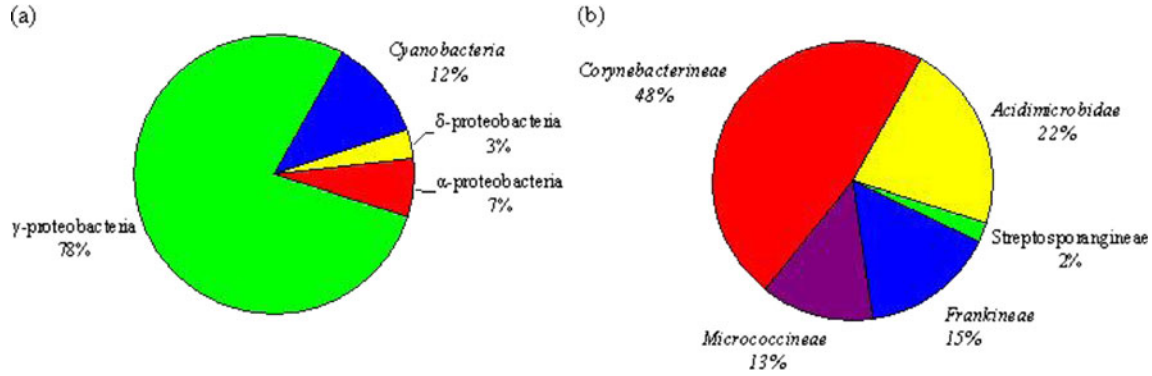


Fig. 5 **a** Bacterial community in sponge *Phakellia fusca*. **b** Actinobacterial community in sponge *Phakellia fusca*. Sequences in corresponding library were classified using the Classifier Tool provided by the Ribosomal Database Project. Hierarchical taxa assignment was based on RDP naive Bayesian rRNA Classifier. Percentage represented specific value between the number of clones in each taxon and the number of all clones in 16S rRNA gene clone libraries

EU885652 (deep-sea sediment clone), DQ148666 (ocean sediment clone), and EU125519 (coral reef sediment clone). As shown in Fig. 6, all AOA *amoA* gene sequences obtained in this study gathered with each other and were close to sponge-derived sequences (EU049829, EU049831, EU049840, GQ485687, GQ485795, and EF529657) (Bayer et al. 2008; López-Legentil et al. 2010; Steger et al. 2008).

Relative Abundance of Bacteria, Actinobacteria, Archaea, and Ammonia-Oxidizing Archaea in Sponge *Phakellia fusca* Based on qRT-PCR

Copy numbers of bacteria, actinobacteria, archaea, and ammonia-oxidizing archaea (AOA) were analyzed based on the corresponding standard curve and average value of three parallel samples. They were 2.3×10^6 copies/ng genomic DNA, 1.2×10^4 copies/ng genomic DNA, 1.2×10^5 copies /ng genomic DNA, and 1.6×10^3 copies/ng genomic DNA, respectively, and their standard errors of value of $C(t)$ were 0.18, 0.06, 0.17, and 0.15, respectively. In prokaryotic microbes, 16S rRNA may be multiple copies, but this will not affect the trend of abundance. Accordingly, the copy number of archaea was about one-twentieth of that of bacteria, the copy number of actinobacteria was less than one-hundredth of that of bacteria, and AOA accounted for 1.3% of the archaea (Fig. 7).

Discussion

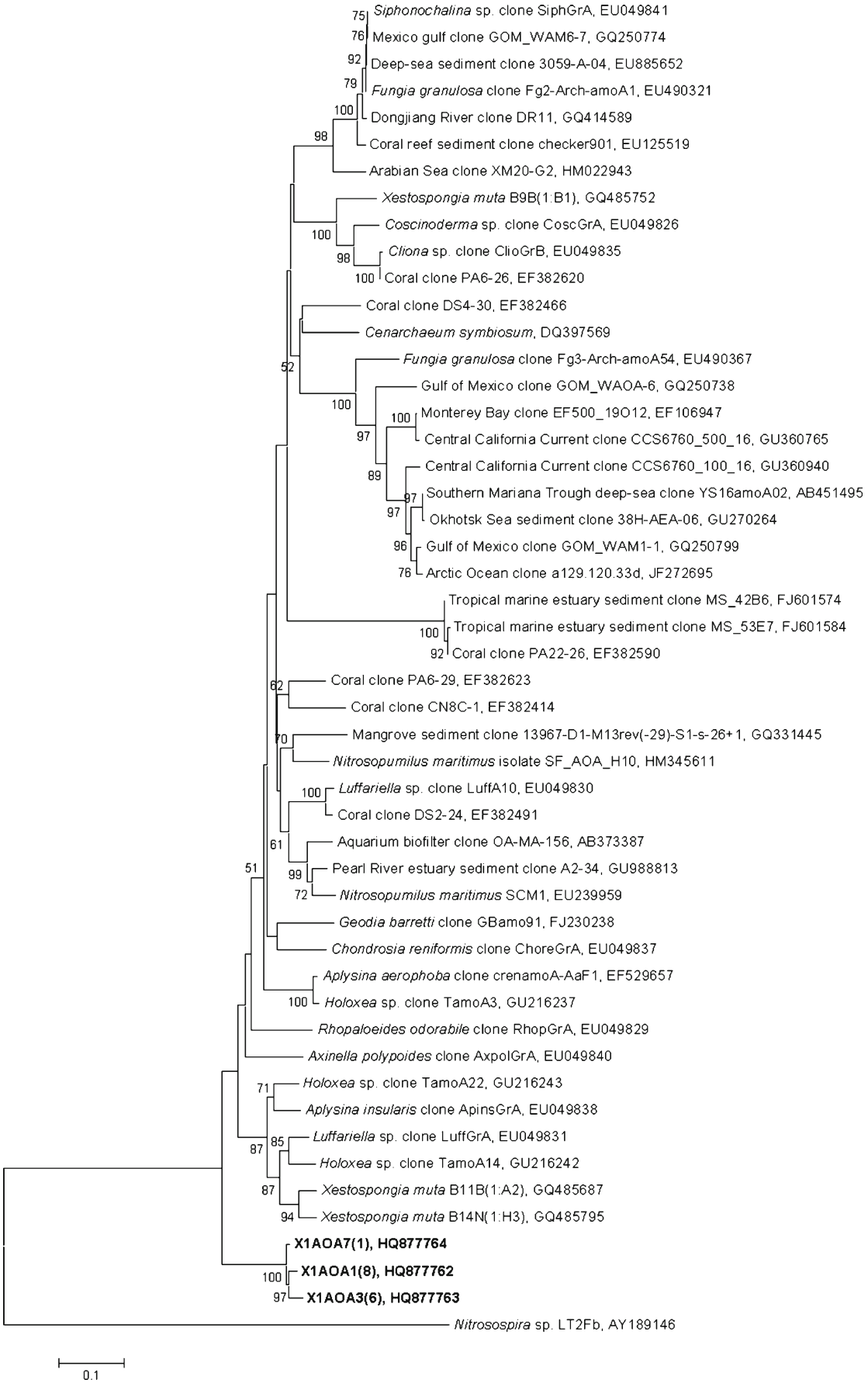
In the case of gene library-based microbial diversity analysis, fragment length polymorphism (RFLP) has been used in the selection of representative clones for sequencing; however, it is greatly limited by the selected endonuclease. At present, direct sequencing of gene library combined with rarefraction analysis is widely adopted in the microbial phylogenetic diversity. Though, for bacteria, actinobacteria, and archaea, only 60, 46, and 50 clones were randomly sequenced, respectively, their rarefraction curves were nearly saturated, so no more clones were sequenced. For 16S rRNA gene analysis, RDP can provide detailed information in microbial classification as a supplement to the BLAST analysis. By this approach, diverse microbial symbionts were revealed in this study.

The Community Structure and Relative Abundance of Prokaryotic Microbial Symbionts in Sponge *Phakellia fusca*

The phylogeny analysis based on randomly sequencing and rarefraction analysis shows that the prokaryotic microbial community of the South China Sea sponge *Phakellia fusca* mainly includes proteobacteria, cyanobacteria, actinobacteria, and archaea, which is relatively simple compared with other sponges (Hentschel et al. 2002, 2003, 2006; Imhoff and Stohr 2003; Lee et al. 2006; Lee et al. 2009; Li et al. 2006; Li and Liu 2006; Radwan et al. 2010; Webb and Maas 2002; Wang 2006). All of the bacterial representatives were related to uncultured clone sequences at low similarity of 89–96%. *Gammaproteobacteria* dominate the microbial symbionts in sponge *Phakellia fusca*, and archaea are mainly composed of *Crenarchaeota*, which consists with the present knowledge of sponge microbial symbionts (Taylor et al. 2007a).

Microbes found in sponges are suggested to include sponge specialists, associates, and generalists (Hentschel et al. 2002; Taylor et al. 2004). *Acidimicrobidae* associated with sponge was first found in sponge *Xestospongia* spp.

Fig. 6 Phylogenetic neighbor-joining tree based on 635 bp of archaeal *amo*A gene sequences. Root was AY189146 (*Nitrosospira* sp. LT2Fb). Bootstrap values <50% were hidden. The number of replicates was 1,000. The *scale bar* represents 0.1 substitutions per nucleotide position. The number in brackets showed the number of sequences in each OTU ▶


Siphonochalina sp. clone SiphGrA, EU049841
Mexico gulf clone GOM_WAM6-7, GQ250774
Deep-sea sediment clone 3059-A-04, EU885652
Fungia granulosa clone Fg2-Arch-amoA1, EU490321
Dongjiang River clone DR11, GQ414589
Coral reef sediment clone checker901, EU125519
Arabian Sea clone XM20-G2, HM022943
Xestospongia muta B9B(1:B1), GQ485752
Coscinoderma sp. clone CoscGrA, EU049826
Cliona sp. clone ClioGrB, EU049835
Coral clone PA6-26, EF382620
Coral clone DS4-30, EF382466
Cenarchaeum symbiosum, DQ397569
Fungia granulosa clone Fg3-Arch-amoA54, EU490367
Gulf of Mexico clone GOM_WAOA-6, GQ250738
Monterey Bay clone EF500_19O12, EF106947
Central California Current clone CCS6760_500_16, GU360765
Central California Current clone CCS6760_100_16, GU360940
Southern Mariana Trough deep-sea clone YS16amoA02, AB451495
Okhotsk Sea sediment clone 38H-AEA-06, GU270264
Gulf of Mexico clone GOM_WAM1-1, GQ250799
Arctic Ocean clone a129.120.33d, JF272695
Tropical marine estuary sediment clone MS_42B6, FJ601574
Tropical marine estuary sediment clone MS_53E7, FJ601584
Coral clone PA22-26, EF382590
Coral clone PA6-29, EF382623
Coral clone CN8C-1, EF382414
Mangrove sediment clone 13967-D1-M13rev(-29)-S1-s-26+1, GQ331445
Nitrosopumilus maritimus isolate SF_AOA_H10, HM345611
Luffariella sp. clone LuffA10, EU049830
Coral clone DS2-24, EF382491
Aquarium biofilter clone OA-MA-156, AB373387
Pearl River estuary sediment clone A2-34, GU988813
Nitrosopumilus maritimus SCM1, EU239959
Geodia barretti clone GBamo91, FJ230238
Chondrosia reniformis clone ChoreGrA, EU049837
Aplysina aerophoba clone crenamoA-AaF1, EF529657
Holoxea sp. clone TamoA3, GU216237
Rhopaloeides odorabile clone RhopGrA, EU049829
Axinella polypoides clone AxpolGrA, EU049840
Holoxea sp. clone TamoA22, GU216243
Aplysina insularis clone ApinsGrA, EU049838
Luffariella sp. clone LuffGrA, EU049831
Holoxea sp. clone TamoA14, GU216242
Xestospongia muta B11B(1:A2), GQ485687
Xestospongia muta B14N(1:H3), GQ485795
X1AOA7(1), HQ877764
X1AOA1(8), HQ877762
X1AOA3(6), HQ877763
Nitrosospira sp. LT2Fb, AY189146
0.1

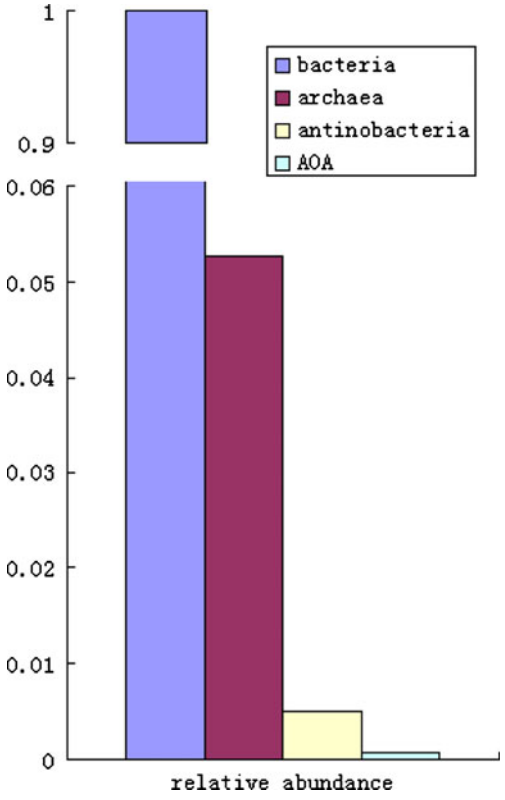


Fig. 7 The relative abundance of prokaryotic symbionts in sponge *Phakellia fusca*, with the copy number of bacteria as 1. Each copy number was the average of three parallel samples

(Montalvo et al. 2005) and suggested to be sponge specific (Taylor et al. 2007a). In this study, most of the nearest neighbors of *Acidimicrobidae* were actinobacteria derived from sponges indicating that they are potential sponge specialists. In 1996, a kind of *Crenarchaeota* was first found in sponge (Preston et al. 1996). Besides *Euryarchaeota* (Webster et al. 2001), *Crenarchaeota*, mainly Group I, have been found in sponges as sponge-specific associations (Holmes and Blanch 2007; Lee et al. 2003; Margot et al. 2002). In sponge *Phakellia fusca*, archaea, mainly *Crenarchaeota*, represent the second predominant prokaryotic symbionts, which may also belong to potential sponge-specific cluster.

The Potential Function of Prokaryotic Microbial Symbionts in Sponge *Phakellia fusca*

Nitrification is an indispensable step in the marine nitrogen cycle (Herbert 1999); among which, ammonia oxidization is the first and rate-limiting step of chemoautotrophic nitrification. Ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) are two participators in this process, which can carry out the oxidation of ammonia to nitrite. Recent studies have showed that the role of AOA in marine nitrification is greater than AOB (Beman et al. 2008; Delong 1992; Lam et al. 2007; Wuchter et al. 2006). Sponges have been suggested to be involved in the marine nitrification process (Clay et al. 1999; Corredor et al. 1988; Delong 1992; Diaz and Ward 1997; Jimenez and Ribes 2007). AOA and AOB have already been explored in several sponges (Bayer et al. 2008; Hoffmann et al. 2009; López-Legentil et al. 2010; Mohamed et al. 2010; Preston et al. 1996; Steger et al. 2008). Based on the genomic analysis of sponge symbiont, *Cenarchaeum symbiosum* genotypes (Hallam et al. 2006), homologues of genes potentially associated with chemolithotrophic ammonia oxidation, including ammonia monooxygenase, ammonia permease, urease, a urea transport system, putative nitrite reductase, and nitric oxide reductase accessory protein, are represented in the *C. symbiosum* genome. Recently, anaerobic ammonia-oxidizing bacteria (AnAOB) have also been detected in sponge by using a specific genetic marker (Jimenez and Ribes 2007; Mohamed et al. 2010). Besides these, microbes with functional genes, such as nitrogen fixation and denitrification, have already been found in sponges (Jimenez and Ribes 2007; Mohamed et al. 2008).

Ammonia monooxygenase genes can be used as a functional marker to detect AOA and AOB (Hooper et al. 1997; Sinigalliano et al. 1995); amplification of the gene for ammonia monooxygenase may provide a method to estimate the distribution and relative abundance of chemolithotrophic ammonium-oxidizing microbes in the environment (Sinigalliano et al. 1995). Rotthauwe et al. (1997) suggested that *amo*A represents a very powerful molecular tool for analyzing indigenous ammonia-oxidizing communities due to (1) its specificity, (2) its fine-scale resolution of closely related populations, and (3) the fact that a functional trait rather than a phylogenetic trait is detected. Therefore, in this study, ammonia-oxidizing populations were mainly detected by using an *amo*A gene PCR-based assay targeting a partial stretch of the gene, which encodes the active-site polypeptide of ammonia monooxygenase (*amo*A). As we know that AOB includes *Beta-* and *Gamma-proteobacteria* (Rotthauwe et al. 1997), based on the bacterial 16S rRNA gene library from sponge *Phakellia fusca*, *Gamma-proteobacteria* were found in sponge *Phakellia fusca*, while *amo*A genes of bacteria were not observed, which indicated that the detected *Gamma-proteobacteria* may not process nitrifying function. The presence of AOA and the lack of AOB and AnAOB indicate that AOA are the main ammonia-oxidizing microbes in sponge *Phakellia fusca* though they account for only a small fraction of archaeal community in sponge *Phakellia fusca*. Ammonia released by sponge hosts is toxic to sponges. As a nitrifier, ammonia-oxidizing archaea (AOA) in sponges may oxidize ammonium to nitrate which can be used as nutrient for sponges.

Cyanobacteria have been found widely in sponges (Webb and Maas 2002) and proved to be beneficial to hosts, for example, they can provide saccharide to hosts through

photosynthesis (Wilkinson 1979) and affect the health of hosts (Thacker 2005). In addition, some *cyanobacteria* are regarded as nitrogen-fixing bacteria, such as *Trichodesmium* spp. (Jonathan and Larry 1989). According to the classification of bacteria in sponge *Phakellia fusca*, clone pfbac5 (12%) was classified as *cyanobacteria*. According to the result of classification using RDP, the observed *cyanobacteria* can further be classified as *Cryptomonadaceae*, which may play an important role in the CO_2 fixing by photosynthesis. Additionally, based on RDP analysis, at the confidence threshold of 80%, most of the *Gammaproteobacteria* belong to *Chromatiales*, which are able to photosynthesize and oxidize hydrogen sulphide to granular sulfur.

Sponge-associated actinobacteria represent one important resource for natural products which may be involved in the chemical defense for sponge host. The diversity of actinobacteria associated with sponges has been revealed by culture-independent and culture dependent strategies (Jiang et al. 2007; Li and Liu 2006; Zhang et al. 2006). It has long been noted that different primers have varied annealing efficiencies with the same PCR template. This bias of primers must surely impact the results of the diversity study of bacterial communities in natural environments. In this study, a pair of specific primers for actinobacteria with higher coverage rate and better selectivity was used to amplify actinobacterial 16S rRNA genes (James et al. 2003). As a result, diverse actinobacteria were observed by library construction using specific primers for actinobacteria, while no actinobacteria were detected in the library constructed by bacterium-universal primer. Of course, if more clones, for example more 200 clones, were sequenced, actinobacteria would be observed in the bacterial library. In sponge *Phakellia fusca*, though actinobacteria do not represent the predominant microbial symbionts, its diversity is high, including *Acidimicrobidae*, *Corynebacterineae*, *Micrococcineae*, *Frankineae*, and *Streptosporanginea* (Figs. 3 and 6). The existence of diverse actinobacteria in sponge *Phakellia fusca* may be responsible for the production of bioactive natural products such as cyclic peptides found in sponge *Phakellia fusca* (Li et al. 2003; Pettit et al. 1993; Zhang et al. 2010). Further culture-based investigation of natural products is very helpful for the evaluation of the roles of actinobacteria in *Phakellia fusca*.

Acknowledgements This work was supported by the National Natural Science Foundation of China (NSFC) (41076077, 41176127, 81072573).

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