SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
ENVIRONMENTAL MICROBIOLOGY
BACTERIA
BIODIVERSITY
ARCHAEA

Received
20 April 2012

Accepted
6 July 2012

Published
24 July 2012

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Spatial distribution of prokaryotic symbionts and ammoxidation, denitrifier bacteria in marine sponge *Astrosclera willeyana*

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The present knowledge of microbial community mainly focus on total sponge, the spatial distribution of microbes in sponges is rarely known, especially those with potential ecological functions. In this study, based on gene library and ∫-LIBSHUFF analysis, the spatial distribution of prokaryotic symbionts and nitrogen cycling genes in the cortex and endosome sections of sponge *Astrosclera willeyana* were investigated. A significance difference of bacterial phylotypes between the cortex and endosome was revealed. For example *Bacteroidetes*, *Frankineae* and *Propionibacterineae* were detected only in the endosome, whereas *Cyanobacteria*, *Planctomycetacia* and *Micrococcineae* were only associated with the cortex. Some branches of α-*Proteobacteria*, γ-*Proteobacteria*, *Corynebacterineae*, *Acidimicobidae*, *Crenarchaeota* and *Euryarchaeota* also showed distribution difference. Bacterial denitrifiers and ammonia oxidizing bacteria (AOB) were observed using *nir*S and *amo*A genes as markers. Particularly, AOB were only associated with the endosome. This study highlighted the spatial distribution of bacterial symbionts especially those with ammonia oxidization function.

Marine sponges are hosts of diverse marine microorganisms[1-3]. The association between microorganisms and their host has been studied firstly using microscopic observation and culture-dependent approach[4,5]. Nucleic acid-based culture-independent molecular techniques have demonstrated the extraordinary microbial diversity associated with sponges[2,3,6,7], and sponge-specific microbes which are different from those in the environmental seawater have been suggested[2,3]. In recent years deep sequencing has demonstrated the presence of as many as 27 bacterial phyla and 8 candidate divisions in sponges[8–10]. However there is only limited information on the spatial distributions of these populations as most studies have focused on the microbial population of the complete sponge.

As sessile filter-feeding organisms, sponges pump large amounts of water through their channel system. As a consequence of seawater pumping in and out the sponge body, an oxygen concentration gradient in sponge body may occur[11,12]. Thus, it can be hypothesized that a spatial distribution of microorganisms in sponges probably exists because of the filtering by canals with different apertures and the different inner-environments *e.g.* oxygen. It is reasonable that the microbial population on the sponge surface is different form that in the sponge mesohyl[13]. But, the microbial spatial distribution in the sponge body remains largely unknown. The exploratory survey of spatial distribution of microorganisms in sponge body was first made in 2000 to investigate the spatial arrangement of *Desulfovibrionaceae*[14]. Since 2007, the microbial spatial distribution in sponges has drawn people's more attention[15–17]. However different results have been observed, for example, the spatial distribution of microorganisms within the body of sponges *Polymastia cf. corticata* and *Tethya aurantium* was found to be specific[15,16], while a homogeneous spatial distribution of microorganisms in sponge *T. californiana* was indicated[17]. Meanwhile, to date, we have no information on the distribution of *Euryarchaeota* in sponges. In addition, though many reports have suggested the nitrogen cycling mediated by the sponge-associated microbes since 2006[18–21], only the spatial distribution of ammonia monooxygenase *amo*A gene in sponge *Polymastia* cf. *corticata* has been investigated[16]. Thus, more investigations need to be carried out on the spatial distribution of microbial symbionts especially those with ecological functions in different sponges to provide more evidence for the spatial distribution hypothesis and increase the understanding of related microbial functions.

The sponge *Astrosclera willeyana* provides an ideal species for microbial spatial distribution investigation because it is characterized by a round sphere shape. In this study, using gene library-based molecular approach and statistical ∫-LIBSHUFF analysis, the spatial distribution of prokaryotic symbionts, especially those with nitrogen-cycling function, in the cortex and endosome of sponge *A. willeyana* were investigated. The spatial distribution difference of microbial symbionts in the cortex and endosome of *A. willeyana*, particularly ammonia oxidizing bacteria (AOB) in the endosome is suggested. This is the first time that the spatial distribution of bacterial denitrifiers with *nir*S gene and *Euryarchaeota* in sponges has been demonstrated.

Results

Four 16S rRNA gene libraries of bacteria, actinobacteria, cyanobacteria and archaea and two functional gene libraries of *nir*S and *amo*A genes were constructed. Diverse bacterial phylotypes including actinobacteria and cyanobacteria, and archaeal phylotypes were detected in sponge *A. willeyana*. The ammonia monooxygenase *amo*A gene and the nitrite reductase *nir*S gene were also observed. In contrast, the bacterial anaerobic ammonia oxidation of bacteria, archaeal ammonium oxidation, nitrite reductase *nir*K, nitric oxide reductase *nor*B, cytochrome nitrite reductase *nrf*, nitrite oxidoreductase *nxr* and nitrogen fixation *nif* genes were not detected (Table 1). A total of 334 clones from these six gene libraries were sequenced successfully, and the number of sequenced sequences was saturated based on rarefaction analysis (Fig. S1). According to Table 1, bacteria including those with *nir*S gene, archaea and actinobacteria were detected in both endosome and cortex samples. Notably, cyanobacteria were only observed in the cortex and the *amo*A gene was only found in the endosome suggesting some extent of spatial distribution. At the level of OTU, these results also indicated different microbial populations between the cortex and endosome of sponge *A. willeyana*.

The spatial distribution of bacteria. Based on the 16S rRNA gene library using universal primer for bacteria (Fig. 1-1), diverse bacterial phylotypes including *Planctomycetes*, *Cyanobacteria*, *Bacteroidetes*, *Firmicutes*, α-*Proteobacteria*, γ-*Proteobacteria* and *Actinobacteria* were observed in the endosome and cortex of sponge *A. willeyana*. γ-*Proteobacteria* dominated in both endosome and cortex samples (51.72% and 30%, respectively) followed by α-*Proteobacteria*. *Firmicutes* were also found in endosome and cortex. Particularly, *Bacteroidetes* (8.62%) was only associated with the endosome (Fig. 1-1A), while *Planctomycetes* (28.33%) and *Cyanobacteria* (10%) were only observed in the cortex (Fig. 1-1B). As shown in Fig 2, 2 of 5 OTUs of γ-*Proteobacteria* were detected only in the cortex, the other 2 OTUs were detected only in the endosome, while the remaining one was overlapped. In the case of α-*Proteobacteria*, 3 of 5 OTUs were detected only in the cortex, 1of 5 was present only in the endosome and the remaining one was overlapped in two sections.

In Fig. 1-1A, actinobacteria were found in the endosome rather than in the cortex. However, using actinobacteria specific primer, a total of 118 actinobacteria 16S rRNA gene sequences were observed in the cortex and endosome sections. *Acidimicrobidae* and *Actinobacteridae* including *Propionibacterineae, Frankineae, Micrococcineae* and *Corynebacterineae* were found (Fig. 1–2). *Acidimicrobidae* dominated the actinobacterial community in the endosome and cortex (73.85% and 77.14%) followed by *Corynebacterineae*. Particularly, *Propionibacterinea* (6.15%) and *Frankineae* (3.08%) were only found in the endosome, while *Micrococcineae* (8.57%) was only found in the cortex. Fig. 3 shows that 6 of 10 OTUs of *Acidimicrobidae* were detected only in the endosome and 2 only in the cortex. The remaining 2 OTUs were overlapped. One OTU of *Propionibacterineae*, one OTU of *Frankineae* and 2 of 3 OTUs of *Corynebacterineae* were only present in the endosome, while 2 OTUs of *Micrococcineae* were only observed in the cortex. Therefore, actinobacteria also exhibited obvious spatial distribution difference.

The spatial distribution of archaea. A total of 36 archaea 16S rRNA gene sequences were obtained. As shown in Fig. 1–3, *Crenarchaeota* and *Euryarchaeota* were detected in both endosome and cortex sections with *Crenarchaeota* as the predominant group, *i.e.* 88.89% and 61.11% in the endosome and cortex, respectively. As shown in Fig. 4, *Crenarchaeota* and *Euryarchaeota* were observed in both sections, but some OTUs were found only in the cortex.

The spatial distribution of ammonia-oxidizing bacteria and bacterial denitrifiers. As for microbial functional genes, 1 of 2 OTUs of bacterial *nir*S gene was found only in the cortex, while another was overlapped between two samples (Fig. 5). Both OTUs of bacterial *amo*A genes were only found in the endosome indicating their space-specific distribution in this sponge (Fig. 6).

The comparison of prokaryotic populations and nitrogen cycling genes between the cortex and endosome of sponge *Astrosclera willeyana*. Fig. 7 summarizes the spatial distribution of sponge prokaryotic symbionts and their *nir* and *amo*A genes (represented by OTUs). Obviously, different spatial distribution of bacteria and those with *nir*S and *amo*A genes in the cortex and endosome was indicated. For example, *Bacteroidetes*, *Frankineae*, *Propionibacterineae* and AOB (with dotted line box in Fig. 7) are space-specific in the endosome, while *Cyanobacteria*, *Planctomycetacia* and *Micrococcineae* (with solid line box in Fig. 7) are cortex-specific. In addition, some α-*Proteobacteria*, γ-*Proteobacteria*, *Corynebacterineae* and *Acidimicobidae* show spatial distribution difference. In the case of functional genes involved in the ammonia-oxidization and denitrification, the *amo*A gene is only associated with the endosome section.

The result of statistical analysis of bacterial community, archaeal community and *nir*S gene spatial distribution difference between the cortex and endosome by ∫-LIBSHUFF is exhibited in Table S1 (the gene libraries of cyanobacteria and actinobacteria were included in the bacterial library). Because *amo*A gene shows certain spatial specificity, the *amo*A gene sequences were not analyzed by ∫-LIBSHUFF. According to Table S1, a significance difference of bacterial phylotypes between the cortex and endosome is suggested because of the P-value below 0.098 at the 0.05 level. Though some archaeal OTUs are associated only with cortex (Fig. 4), no statistically

Table 1 | The results of OTU analysis based on gene libraries

Target gene	Number of clones sequenced (cortex/endosome)	Sequenced successfully		Number of OTUs			
		cortex	endosome	only cortex	only endosome	both	total
Bacteria 16S rRNA gene	70/65	+	+	11	5	4	20
Actinobacteria 16S rRNA gene	60/58	+	+	5	9	3	17
Cyanobacteria specific 16S rRNA gene	12/-	+	-	2	0	0	2
Archaea 16S rRNA gene	18/18	+	+	4	0	2	6
Ammonium oxidation of bacteria (*amo*A)	-/10	-	+	0	2	0	2
Dissimilatory nitrate reduction and denitrification (*nir*S)	11/12	+	+	1	0	1	2

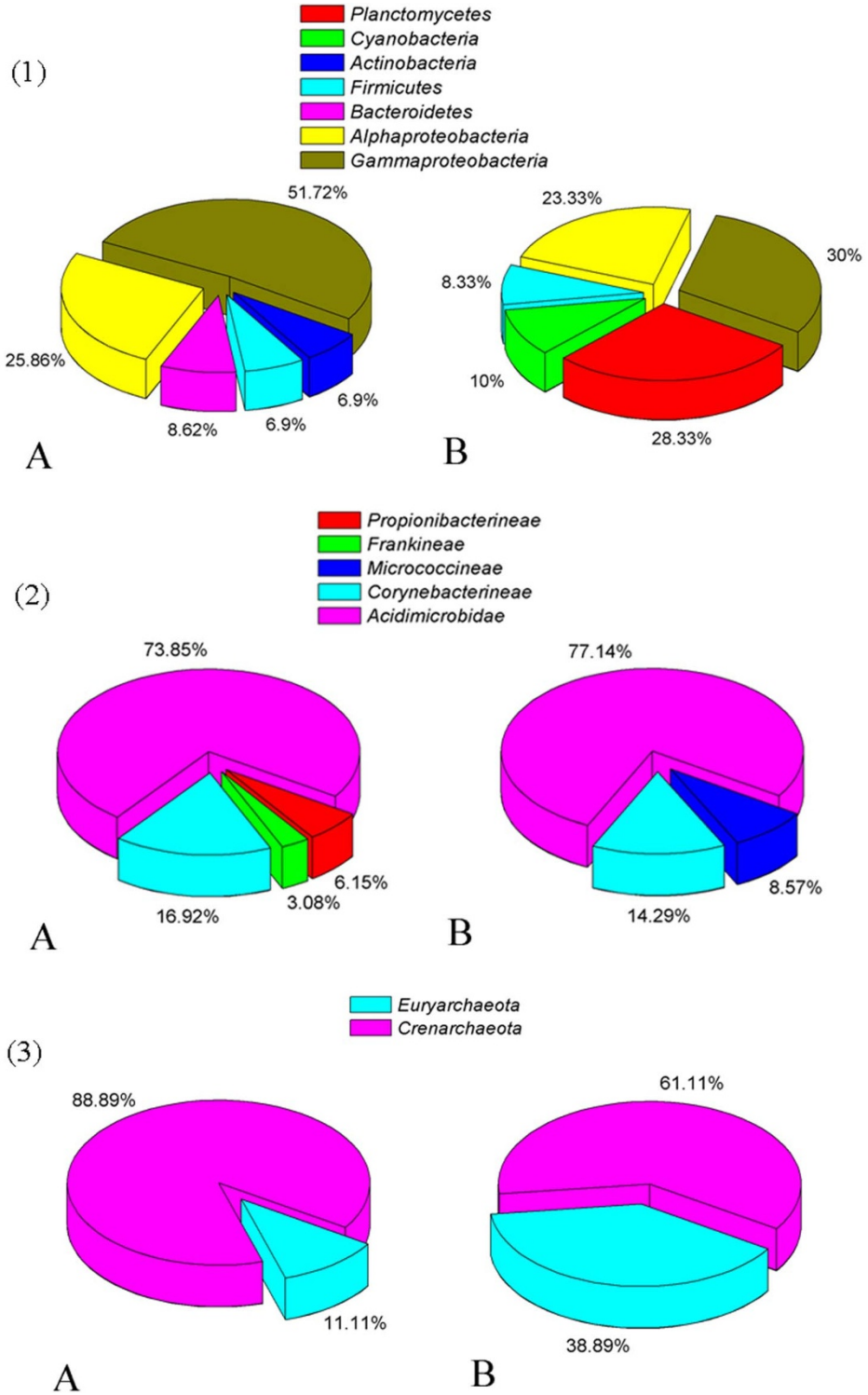

Figure 1 | Diversity of bacteria (part 1), actinobacteria (part 2) and archaea (part 3) in the endosome and cortex of sponge *A. willeyana*. A: endosome, B: cortex.

significant difference of spatial distribution of archaea was suggested according to ∫-LIBSHUFF analysis. Meanwhile, bacterial *nir*S genes in endosome and cortex also did not exhibit a significant difference.

Discussion

In common with most sponges, *A. willeyana* contains a large population of *Proteobacteria*, especially γ-*Proteobacteria*, as the dominant group of its bacterial community[3,6,7,10]. However there is only spatial differentiation within a few of its branches. Similarly, *Acidimicrobidae*, which has been grouped into sponge-specific microorganisms[3], shows no spatial specificity. In contrast, *Bacteroidetes*, *Cyanobacteria* and *Planctomycetacia* show obvious spatial distribution specificity. *Cyanobacteria* has been observed widely in sponges[22], its space-specific distribution in the cortex of sponge *A. willeyana* maybe related to its biological functions *e.g.* photosynthesis[23]. Interestingly, *Cyanobacteria* was detected only in the endosome of sponge *Tethya aurantium* Pallas 1766[15], which

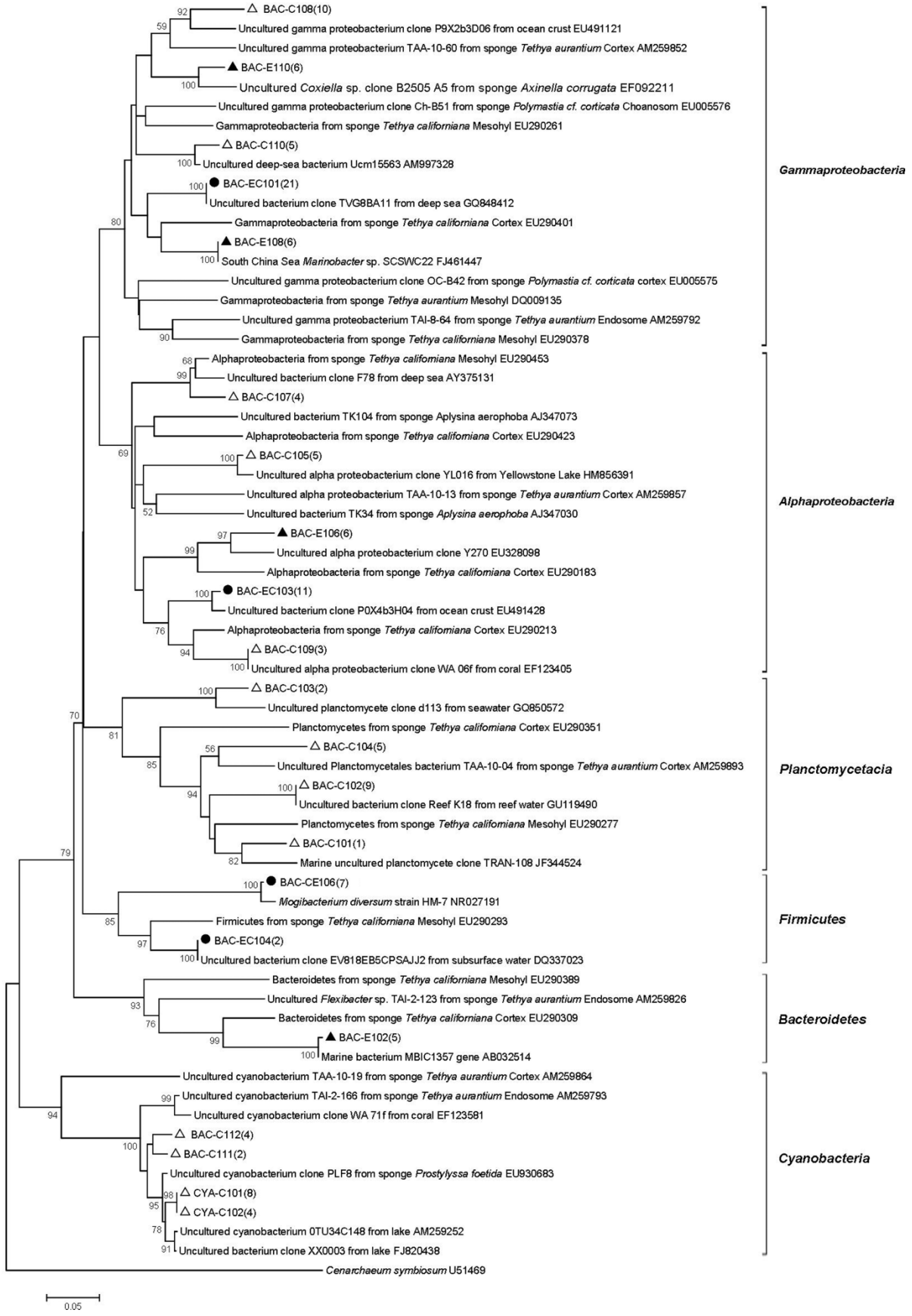

Figure 2 | NJ-phylogenetic tree based on bacteria 16S rRNA gene sequences (1,420–1,520bp) without actinobacteria. Bootstrap values (1,000 replicates) lower than 50% are not shown. △ mark means the OTU only in the cortex, and ▲ mark means the OTU only in the endosome. ● mark means the OTU in both cortex and endosome samples. The number inside the parenthesis means the number of sequences within each OTU.

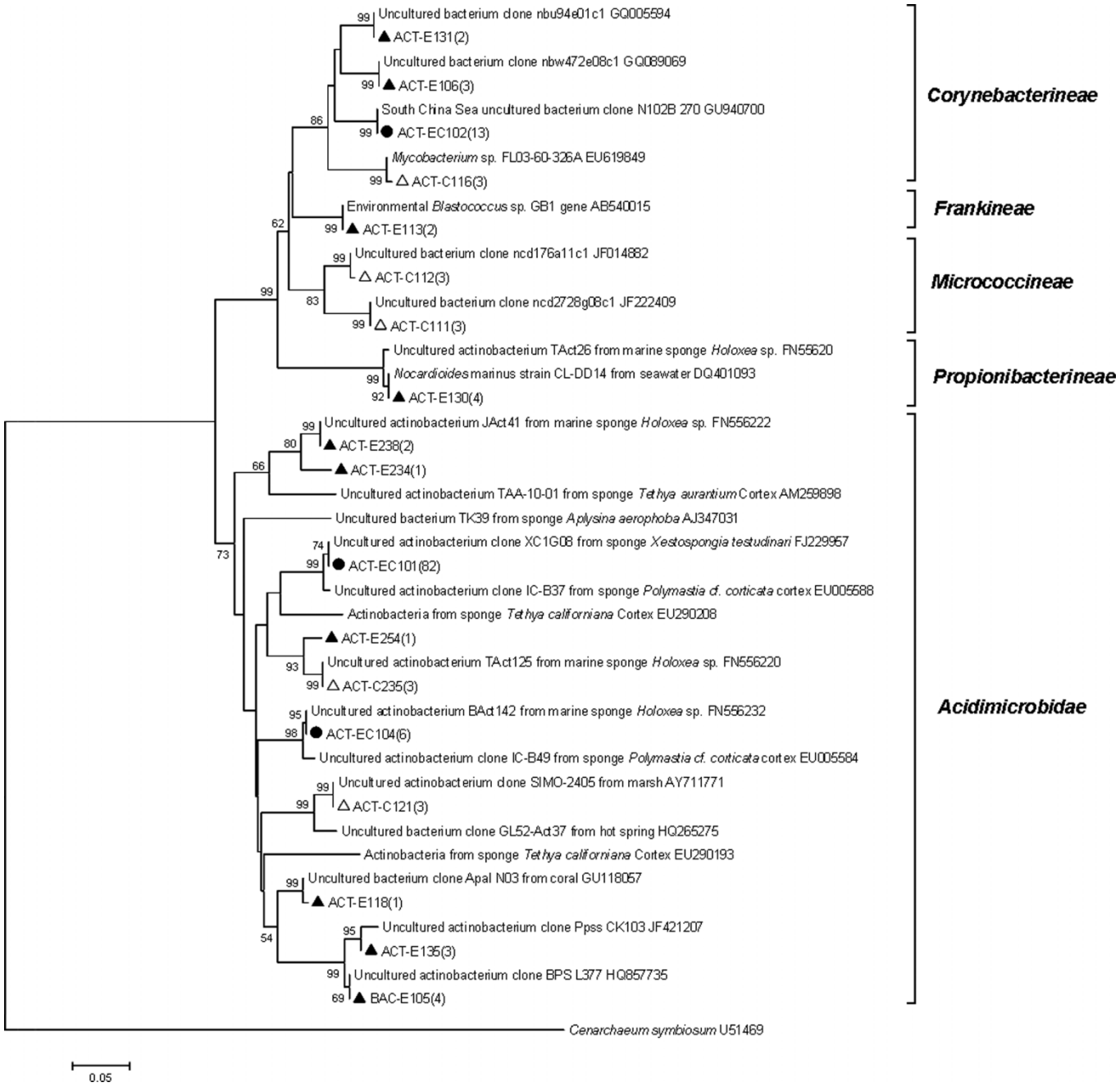

Figure 3 | **NJ-phylogenetic tree based on actinobacteria 16S rRNA gene sequences (639 bp).** Bootstrap values (1,000 replicates) lower than 50% are not shown. △ mark means the OTU only in the cortex, and ▲ mark means the OTU only in the endosome. ● mark means the OTU in both cortex and endosome samples. The number inside the parenthesis means the number of sequences within each OTU.

indicated that microbial spatial distribution maybe sponge species-dependent.

Diverse sponge-associated actinobacteria have been revealed[24–26]. In this study actinobacteria did show a strong selectivity in the spatial location, for example *Frankineae*, *Propionibacterineae* in the endosome, and *Micrococcineae* in the cortex. Meanwhile, the abundance of actinobacteria in the endosome is greater than that in the cortex. It is worth mentioning that, in this study, a significant difference of DNA specificity between bacterial 16S rRNA gene universal primer and actinobacteria 16S rRNA gene specific primer was observed. Actinobacterial 16S rRNA gene specific primer is much more sensitive to sponge-associated actinobacteria DNA than bacterial 16S rRNA gene universal primer. For instance, using bacteria 16S rRNA gene universal primer only one actinobacteria OTU was detected. In contrast, *Acidimicrobidae*, *Corynebacterineae*, *Propionibacterineae*, and *Micrococcineae* were detected using actinobacterial specific primer. This result suggests that the species-specific primers are very important for the diversity study of sponge microbial symbionts.

Group I *Crenarchaeota* are widely distributed in the marine environment[27]. Almost all sponge-associated archaea belong to this group[28-31], while *Euryarchaeota* appeared in few sponges[8]. In this study, both *Crenarchaeota* and *Euryarchaeota* were detected in sponge *A. willeyana*. It was the first time to reveal the homogeneous spatial distribution of *Euryarchaeota* in sponges. Though some archaeal OTUs were only observed in the cortex, archaea show no significant distribution difference between the cortex and endosome of sponge *A. willeyana*.

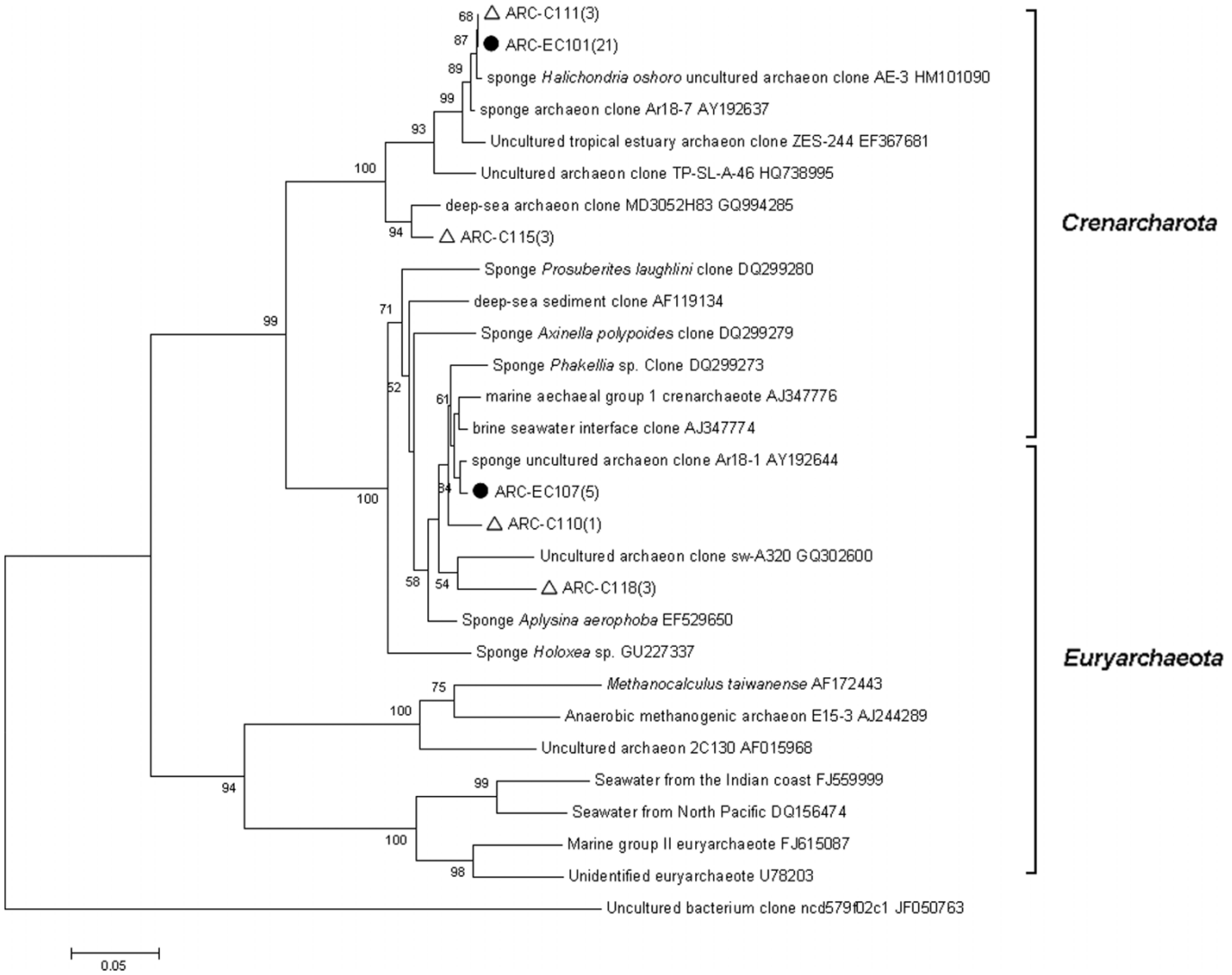

Figure 4 | NJ-phylogenetic tree based on archaea 16S rRNA gene sequences (913bp). Bootstrap values (1,000 replicates) lower than 50% are not shown. △ mark means the OTU only in the cortex, and ● mark means the OTU in both cortex and endosome samples. The number inside the parenthesis means the number of sequences within each OTU.

In the process of nitrification, ammonia monooxygenase (*amo*) is an important enzyme for oxidizing ammonia to hydroxylamine. The process of turning nitrite to nitrogen oxide catalyzed by nitrite reductase is an important limiting step of denitrification. Nitrite reductase can be classified into two types, copper-type nitrite reductase (the product of the *nir*K) and cytochrome-type (the product of the *nir*S)[32]. In the absence of appropriate 16S rRNA gene to look for specific ammonia-oxidizing bacteria and denitrifying bacteria, genes encoding ammonia monooxygenase (*amo*A) and nitrite reductase (*nir*K and *nir*S) are often used as molecular markers to study homologous microbial diversity[33–35]. In this study, bacterial *nir*S and *amo*A genes were found in the South China Sea sponge *A. willeyana*, suggesting the presence of bacterial denitrifiers and ammonia-oxidizing bacteria in this sponge.

It should be mentioned that, the *amo*A gene of β-*Proteobacteria* showed no spatial distribution difference in sponge *Polymastia* cf. *corticata*[16]. However in this study, the bacterial *amo*A genes were found only in the endosome of sponge *A. willeyana* exhibiting spatial distribution specificity. This maybe a result of different *Proteobacteria* groups in these different two sponges, *e.g.* α-*Proteobacteria* and γ-*Proteobacteria* rather than β-*proteobacteria* were observed in sponge *A. willeyana*. It is suggested that ammonia-oxidizing archaea (AOA) dominate the process in low ammonia nitrogen level area, while in an area of high level ammonia nitrogen, AOB may take on the dominator role instead of archaea[36]. In this study AOB was found in sponge *A. willeyana*, which is in consist with the results of sponge *Polymastia* cf. *corticata*[16], indicating the important nitrification role of AOB in sponges because of high level of ammonia nitrogen released by the host as a metabolic waste.

Denitrification is generally considered as a process medicated by bacteria under low oxygen concentration[37–39]. During sponge's filter-feeding, the internal oxygen concentration changes because of the water pumping[11,40]. If the oxygen concentration reduces to a low level when water pumping out, it just creates a condition for denitrification process. In this study, *nir*S gene was detected in cortex and endosome sections. It was the first time to investigate the spatial distribution of bacterial denitrifiers with *nir*S gene in sponges.

Sponge microbial symbionts are proposed to be captured by a combination of horizontal and vertical transmission[2]. As filter feeders, sponges are suggested to select microorganisms in the different areas of their bodies[41]. The microbial spatial distribution hypothesis is helpful to explain the different microbial community in different spatial locations within one sponge, and then understand the related microbial functions. The revealed complex but unique microbial communities in the cortex and endosome of sponge *A. willeyana* in this study provide new evidence for the spatial distribution

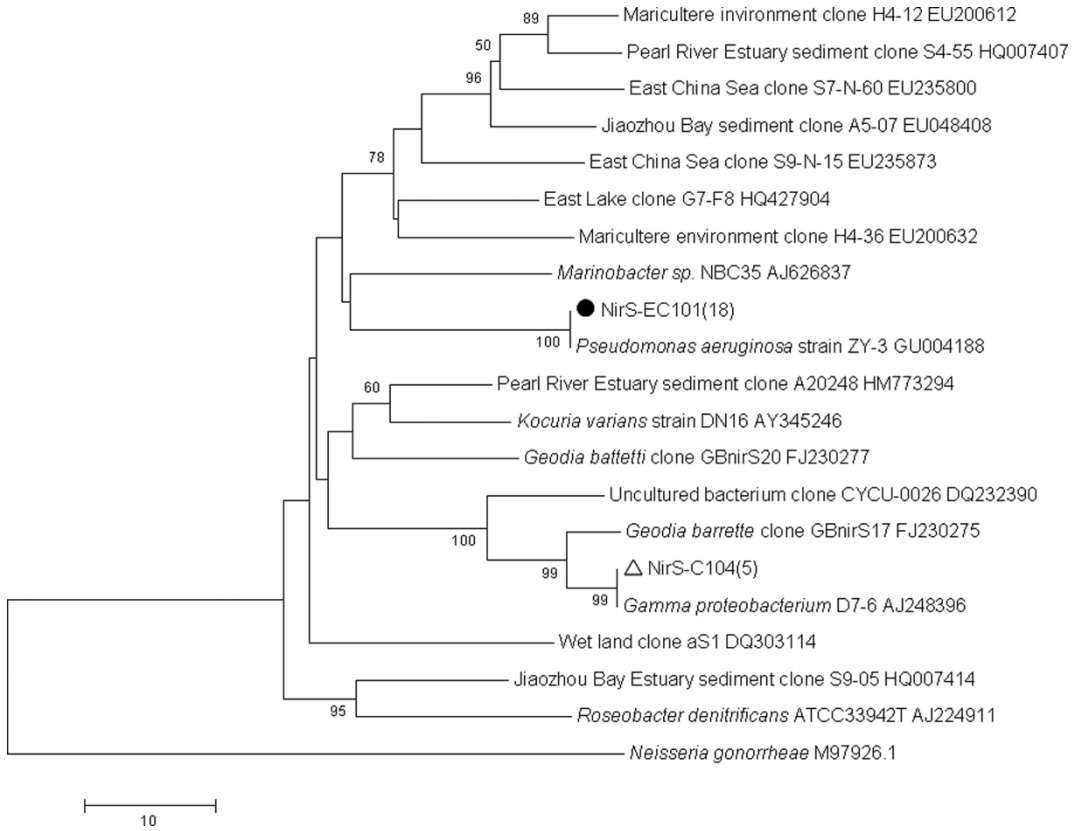

Figure 5 | NJ-phylogenetic tree of *nir*S (290 amino acids). Bootstrap values (1,000 replicates) lower than 50% are not shown. △ mark means the OTU only in the cortex, ● mark means the OTU in both cortex and endosome samples. The number inside the parenthesis means the number of sequences within each OTU.

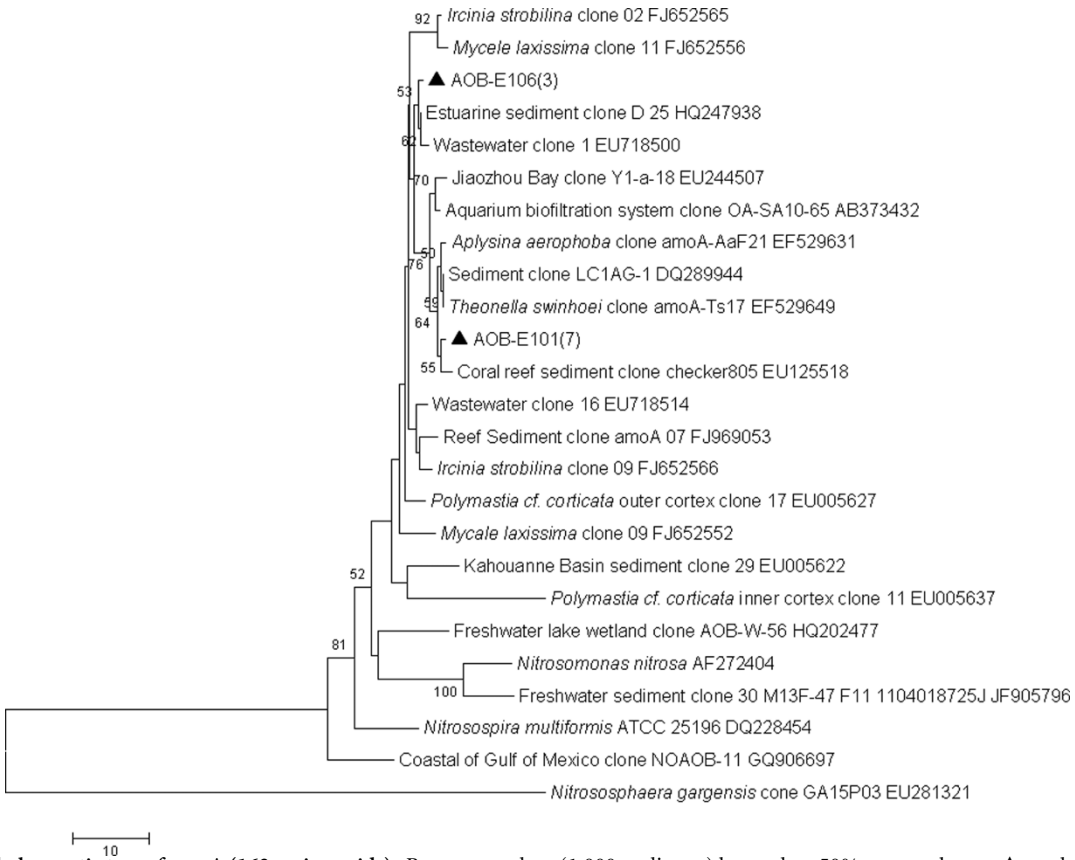

Figure 6 | NJ-phylogenetic tree of *amo*A (163 amino acids). Bootstrap values (1,000 replicates) lower than 50% are not shown. ▲ mark means the OTU only in the endosome. The number inside the parenthesis means the number of sequences within each OTU.

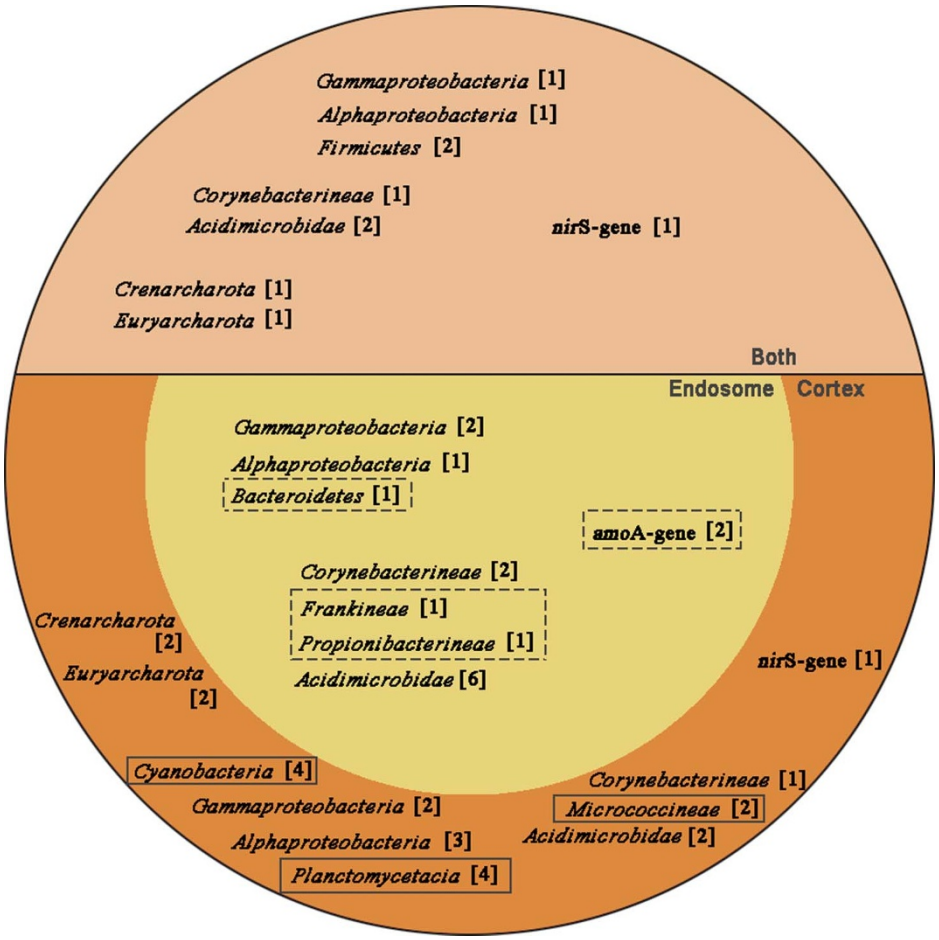

Figure 7 | Spatial distribution of the prokaryotic symbionts and functional genes in the South China Sea sponge *A. willeyana*. The outer orange circle of the lower half represents the cortex sample. The inner yellow circle represents the endosome sample. The upper half of the figure means the shared microbes or genes in two samples. The phylum which was detected only in the cortex sample or only in the endosome sample was surrounded by solid line box or dotted line box. Number refers to the number of OTUs.

hypothesis on sponge microbial symbionts. It is suggested that more sponge species with different shape and different microbial community should be investigated to provide more information for the microbial spatial distribution in sponges. Meanwhile, further research should be undertaken to confirm that whether there is a particular selective mechanism in sponges resulting in the spatial distribution of microbial community.

Methods

Sponge sampling. Sponge was collected from the Yongxing island (112°20′E, 16°50′N) in the South China Sea by diving at a depth of *ca*. 20 m. It was placed in an ice-cooled box and transported to the laboratory immediately. The sponge sample was identified as *Astrosclera willeyana* according to 28S rRNA gene with 99% identify.

DNA extraction and PCR amplification. Sponge was rinsed 3 times by artificial seawater (ASW) (1.1 g $CaCl_2$, 10.2 g $MgCl_2{\cdot}6H_2O$, 31.6 g NaCl, 0.75 g KCl, 1.0 g Na_2SO_4, 2.4 g Tris-HCl, 0.02 g $NaHCO_3$, 1 L distilled water, pH 7.6). A-endosome sample and B-cortex sample were obtained from the cortex and endosome sections of sponge *A. willeyana*, respectively (six parallel samples from each section, Fig. S2). The spatial samples were washed 3 times with ASW and separately grinded using sterilized mortars and pestles. Then the genomic DNA was extracted and purified using the QIAGEN DNeasy Tissue Kit following the manufacturer's protocol. The mixed DNA from three parallel samples for each section was used for PCR.

The primers and PCR conditions used to amplify 16 S rRNA gene and functional gene fragments are shown in Table S2[33,34,42–52]. The PCR mixture (50 μL) contained 200 ng template DNA, 250 μM dNTPs, 0.2 μM of each primer, 2.5 U of TransStart Fastpfu DNA polymerase (TransGen) and 1×TransStart FastPfu Buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$).

Gene library construction, phylogenetic and ∫-LIBSHUFF analysis. The amplified products were recovered and purified using Agarose Gel DNA Purification Kit (Takara, Dalian). Purified PCR products were cloned with the pEASY-Blunt Cloning kit (TransGen) following the manufacturer's instructions. The positive recombinants were screened on X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside)-IPTG (isopropyl-β-D-thiogalactopyranoside) -ampicillin plates by color-based recombinant selection. The positive clones were further identified by vector primers M13F/R. Sequencing of plasmids was performed using vector primers on ABI 3730xl capillary sequencers (Applied Biosystems).

Using DOTUR software, sequences of all 16S rRNA genes with similarities > 97% were considered as one operational taxonomic unit (OTU), while functional gene sequences with similarities > 92% were considered as one OTU[53]. The diversity was determined by rarefaction analysis using OriginPro (Version 8). DNA sequences were aligned with Clustal W and classified using the RDP (http://rdp.cme.msu.edu/index.jsp) classifier with a confidence threshold of 70%. All OTUs' representative sequences, their nearest neighbors and some reference sequences were imported in MEGA (Version 5). Neighbor-Joining phylogenetic tree was constructed using software package PHYLIP[54]. The difference analysis between the two libraries of cortex and endosome samples was carried out with ∫-LIBSHUFF[55].

Nucleotide sequence accession number. All representative sequences were deposited in Genbank under accession numbers: JQ362353 (sponge *Astrosclera willeyana* 28S rRNA gene), JN113042-JN113058 (actinobacteria 16S rRNA gene), JN113059-JN113064 (archaea 16S rRNA gene), JN113065-JN113084 (bacteria 16S rRNA gene), JN113085-JN113086 (cyanobacteria 16S rRNA gene), JN113087-JN113088 (*nir*S gene) and JN113089-JN113090 (*amo*A gene).

1. Friedrich, A. B., Fischer, I., Proksch, P., Hacker, J. & Hentschel, U. Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiol. Ecol.* **38**, 105–113 (2001).
2. Hentschel, U., Hopke, J., Horn, M., Friedrich, A. B., Wagner, M. *et al.* Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl. Environ. Microbiol.* **68**, 4431–4440 (2002).

3. Taylor, M. W., Radax, R., Steger, D. & Wagner, M. Sponge-associated microorganisms: Evolution, ecology, and biotechnological potential. *Microbiol. Mol. Biol. Rew.* **71**, 295–324 (2007).
4. Vacelet, J. & Donadey, C. Electron microscope study of the association between some sponges and bacteria. *J. Exp. Mar. Biol. Ecol.* **30**, 301–314 (1977).
5. Webster, N. S. & Hill, R. T. The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium. *Mar. Biol.* **138**, 843–851 (2001).
6. Hentschel, U., Usher, K. M. & Taylor, M. W. Marine sponges as microbial fermenters. *FEMS Microbiol. Ecol.* **55**, 167–177 (2006).
7. Hardoim, C. C., Costa, R., Araújo, F. V., Hajdu, E., Peixoto, R. *et al*. Diversity of bacteria in the marine sponge *Aplysina fulva* in Brazilian coastal waters. *Appl. Environ. Microbiol.* **75**, 3331–3343 (2009).
8. Webster, N. S., Taylor, M. W., Behnam, F., Lücker, S., Rattei, T. *et al*. Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environ. Microbiol.* **12**, 2070–2082 (2010).
9. Lee, O. O., Wang, Y., Yang, J. K., Lafi, F., Al-Suwailem, A. & Qian, P. Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. *ISME J.* **5**, 650–664 (2011).
10. Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M. *et al*. Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. *ISME J.* **6**, 564–576 (2012).
11. Schönberg, C. H. L., Hoffmann, F. & Gatti, S. Using microsensors to measure sponge physiology. *Boll. Mus. Ist. Biol. Univ. Genova* **68**, 593–60 (2004).
12. Schläppy, M., Weber, M., Mendola, D., Hoffmann, F. & de Beer, D. Heterogeneous oxygenation resulting from active and passive flow in two Mediterranean sponges, *Dysidea avara* and *Chondrosia reniformis*. *Limnol. Oceanogr.* **55**, 1289–1300 (2010).
13. Gerce, B., Schwartz, T., Syldatk, C. & Hausmann, R. Difference between bacterial communities associated with the surface or tissue of Mediterranean sponge species. *Microb. Ecol.* **61**, 769–782 (2011).
14. Manz, W., Arp, G. & Schumann-Kindel, G. Widefield deconvolution epifluorescence microscopy combined with fluorescence in situ hybridization reveals the spatial arrangement of bacteria in sponge tissue. *J. Microbiol. Meth.* **40**, 125–134 (2000).
15. Thiel, V., Neulinger, S. C., Staufenberger, T., Schmaljohann, R. & Imhoff, J. F. Spatial distribution of sponge-associated bacteria in the Mediterranean sponge *Tethya aurantium*. *FEMS Microbiol. Ecol.* **59**, 47–63 (2007).
16. Meyer, B. & Kuever, J. Phylogenetic diversity and spatial distribution of the microbial community associated with the Caribbean deep-water sponge *Polymastia cf. corticata* by 16S rRNA, *apr*A, and *amo*A gene analysis. *Microb. Ecol.* **56**, 306–321 (2008).
17. Sipkema, D. & Blanch, H. W. Spatial distribution of bacteria associated with the marine sponge *Tethya californiana*. *Mar. Biol.* **157**, 627–638 (2010).
18. Hallam, S. J., Mincer, T. J., Schleper, C., Preston, C. M., Roberts, K. *et al*. Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine *Crenarchaeota*. *PLoS Biol.* **4**, 520–536 (2006).
19. Steger, D., Ettinger-Epstein, P., Whalan, S., Hentschel, U., de Nys, R. *et al*. Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges. *Environ. Microbiol.* **10**, 1087–1094 (2008).
20. Bayer, K., Schmitt, S. & Hentschel, U. Physiology, phylogeny and in situ evidence for bacterial and archaeal nitrifiers in the marine sponge *Aplysina aerophoba*. *Environ. Microbiol.* **10**, 2942–2955 (2008).
21. Mohamed, N. M., Saito, K., Tal, Y. & Hill, R. T. Diversity of aerobic and anaerobic ammonia-oxidizing bacteria in marine sponges. *ISME J.* **4**, 38–48 (2010).
22. Webb, V. L. & Maas, E. W. Sequence analysis of 16S rRNA gene of cyanobacteria associated with the marine sponge *Mycale (Carmia) hentscheli*. *FEMS Microbiol. Lett.* **207**, 43–47 (2002).
23. Zehr, J. P. & McReynolds, L. A. Use of degenerate oligoncleotides for amplification of the *nif*H gene from the marine cyanobacterium *Trichodesmium-thiebautii*. *Appl. Environ. Microbiol.* **55**, 2522–2526 (1989).
24. Li, Z. Y. & Liu, Y. Marine sponge *Craniella austrialiensis*-associated bacterial diversity revelation based on 16S rDNA library and biologically active actinomycetes screening, phylogenetic analysis. *Lett. Appl. Microbiol.* **43**, 410–416 (2006).
25. Zhang, H., Lee, Y. K., Zhang, W. & Lee, H. K. Culturable actinobacteria from the marine sponge *Hymeniacidon perleve*: isolation and phylogenetic diversity by 16S rRNA gene-RFLP analysis. *Anton. Leeuw. Int. J G* **90**, 159–169 (2006).
26. Jiang, S., Sun, W., Chen, M., Dai, S., Zhang, L. *et al*. Diversity of culturable actinobacteria isolated from marine sponge *Haliclona* sp. *Anton. Leeuw. Int. J G* **92**, 405–416 (2007).
27. Karner, M. B., DeLong, E. F. & Karl, D. M. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409**, 507–510 (2001).
28. Preston, C. M., Wu, K. Y., Molinski, T. F. & DeLong, E. F. A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen nov, sp, nov. *Proc. Natl. Acad. Sci. USA* **93**, 6241–6246 (1996).
29. Margot, H., Acebal, C. & Toril, E. Consistent association of crenarchaeal archaea with sponges of the genus *Axinella*. *Mar. Biol.* **140**, 739–745 (2002).
30. Lee, E. Y., Lee, H. K., Lee, Y. K., Sim, C. J. & Lee, J. H. Diversity of symbiotic archaeal communities in marine sponges from Korea. *Biomol. Eng.* **20**, 299–304 (2003).
31. Holmes, B. & Blanch, H. Genus-specific associations of marine sponges with group I crenarchaeotes. *Mar. Biol.* **150**, 759–772 (2007).
32. Heylen, K., Gevers, D., Vanparys, B., Wittebolle, L., Geets, J., Boon, N. & De Vos, P. The incidence of *nir*S and *nir*K and their genetic heterogeneity in cultivated denitrifiers. *Environ. Microbiol.* **8**, 2012–2021 (2006).
33. Rotthauwe, J. H., Witzel, K. P. & Liesack, W. The ammonia monooxygenase structural gene *amo*A as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* **63**, 4704–4712 (1997).
34. Braker, G., Fesefeldt, A. & Witzel, K. P. Development of PCR primer systems for amplification of nitrite reductase genes (*nir*K and *nir*S) to detect denitrifying bacteria in environmental samples. *Appl. Environ. Microbiol.* **64**, 3769–3775 (1998).
35. Braker, G., Zhou, J. Z. & Wu, L. Y. Nitrite reductase genes (*nir*K and *nir*S) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment sommunities. *Appl. Environ. Microbiol.* **66**, 2096–2104 (2000).
36. Di, H. J., Cameron, K. C., Shen, J. P., Winefield, C. S., O'Callaghan, M. *et al*. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nat. Geosci.* **2**, 621–624 (2009).
37. Zumft, W. G. Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rew.* **61**, 533–545 (1997).
38. Hoffmann, F., Radax, R., Woebken, D., Holtappels, M., Lavik, G. *et al*. Complex nitrogen cycling in the sponge *Geodia barretti*. *Environ. Microbiol.* **11**, 2228–2243 (2009).
39. Fiore, C. L., Jarett, J. K., Olson, N. D. & Lesser, M. P. Nitrogen fixation and nitrogen transformations in marine symbioses. *Trends Microbiol.* **18**, 455–463 (2010).
40. Sarà, M. A study on the genus *Tethya* (Porifera, Demospongiae) and new perspectives in sponge systematics. in: Vacelet, J., Boury-Esnault, N. (eds.). Taxonomy of Porifera from the N.E. Atlantic and Mediterranean Sea. NATO ASI Ser, Ser G: Ecol Sci 13. Springer: Berlin. pp. 205–225 (1987).
41. Wehrl, M., Steinert, M. & Hentschel, U. Bacterial uptake by the marine sponge *Aplysina aerophoba*. *Microb. Ecol.* **53**, 355–365 (2007).
42. Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. *et al*. Rapid determination of 16S ribosomal-RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. USA* **82**, 6955–6959 (1985).
43. Delong, E. F. Archaea in coastal marine environment. *Proc. Natl. Acad. Sci. USA* **89**, 5685–5689 (1992).
44. Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J. C. *et al*. DNA extraction from soils: Old bias for new microbial diversity analysis methods. *Appl. Environ. Microbiol.* **67**, 2354–2359 (2001).
45. Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S. & Germon, J. C. Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. *Appl. Environ. Microbiol.* **68**, 6121–6128 (2002).
46. Mohan, S. B., Schmid, M., Jetten, M. & Cole, J. Detection and widespread distribution of the *nrf*A gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. *FEMS Microbiol. Ecol.* **49**, 433–443 (2004).
47. Stach, J. E., Maldonado, L. A., Ward, A. C., Goodfellow, M. & Bull, A. T. New primers for the class actinobacteria: application to marine and terrestrial environments. *Environ. Microbiol.* **5**, 828–841 (2003).
48. Casciotti, K. L. & Ward, B. B. Phylogenetic analysis of nitric oxide reductase gene homologues from aerobic ammonia-oxidizing bacteria. *FEMS Microbiol. Ecol.* **52**, 197–205 (2005).
49. Francis, C. A., Roberts, K. J., Beman, J. M., Santoro, A. E. & Oakley, B. B. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc. Natl. Acad. Sci. USA* **102**, 14683–14688 (2005).
50. Vanparys, B., Bodelier, P. & De Vos, P. Validation of the correct start codon of *nor*X/*nxr*X and universality of the *nor*AXB/*nxr*AXB gene cluster in *Nitrobacter* species. *Curr. Microbiol.* **53**, 255–257 (2006).
51. Clementino, M. M., Vieira, R. P., Cardoso, A. M., Nascimento, A. P. & Silveira, C. B. Prokaryotic diversity in one of the largest hypersaline coastal lagoons in the world. *Extremophiles* **12**, 595–604 (2008).
52. Jetten, M. S., Niftrik, L., Strous, M., Kartal, B., Keltjens, J. T. *et al*. Biochemistry and molecular biology of anammox bacteria. *Crit. Rev. Biochem. Mol. Biol.* **44**, 65–84 (2009).
53. Schloss, P. D. & Handelsman, J. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl. Environ. Microbiol.* **71**, 1501–1506 (2005).
54. Felsenstein, J. *PHYLIP: phylogeny inference package* (version 3.5c). University of Washington, Seattle (1993).
55. Schloss, P. D., Larget, B. R. & Handelsman, J. Integration of microbial ecology and statistics: a test to compare gene libraries. *Appl. Environ. Microbiol.* **70**, 5485–5492 (2004).

Acknowledgments

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

Author contributions

ZY and ZL conceived and designed the experiments. ZY performed the experiments. ZY and ZL analyzed the data and wrote the paper.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Yang, Z. & Li, Z. Spatial distribution of prokaryotic symbionts and ammoxidation, denitrifier bacteria in marine sponge *Astrosclera willeyana*. *Sci. Rep.* **2**, 528; DOI:10.1038/srep00528 (2012).