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ORIGINAL ARTICLE

Functional Transcripts Indicate Phylogenetically Diverse Active Ammonia-Scavenging Microbiota in Sympatric Sponges

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Abstract

Symbiotic ammonia scavengers contribute to effective removal of ammonia in sponges. However, the phylogenetic diversity and in situ activity of ammonia-scavenging microbiota between different sponge species are poorly addressed. Here, transcribed ammonia monooxygenase genes (*amoA*), hydrazine synthase genes (*hzsA*), and glutamine synthetase genes (*glnA*) were analyzed to reveal the active ammonia-scavenging microbiota in the sympatric sponges *Theonella swinhoei*, *Plakortis simplex*, and *Phakellia fusca*, and seawater. Archaeal *amoA* and bacterial *glnA* transcripts rather than bacterial *amoA*, *hzsA*, and archaeal *glnA* transcripts were detected in the investigated sponges and seawater. The transcribed *amoA* genes were ascribed to two Thaumarchaeota ecotypes, while the transcribed *glnA* genes were interspersed among the lineages of Cyanobacteria, Tectomicrobia, Poribacteria, Alpha-, Beta-, Gamma-, and Epsilonproteobacteria. In addition, transcribed abundances of archaeal *amoA* and bacterial *glnA* genes in these sponges have been quantified, showing significant variation among the investigated sponges and seawater. The transcriptome-based qualitative and quantitative analyses clarified the different phylogenetic diversity and transcription expression of functional genes related to microbially mediated ammonia scavenging in different sympatric sponges, contributing to the understanding of in situ active ecological functions of sponge microbial symbionts in holobiont nitrogen cycling.

Keywords Sponge · Ammonia-oxidizing archaea · Ammonia-assimilating bacteria · Functional transcript · RT-qPCR assays

Introduction

The nitrogen cycle involves the conversion of nitrogen compounds between different chemical forms (Zehr and Kudela 2011). Processes such as ammonotelism, nitrogen fixation, organic nitrogen remineralization, and assimilatory nitrate reduction might influence the accumulation of ammonia in the oceans (Paulot et al. 2015; Regnault 1987). Ammonia can be oxidized into nitrite during the first step of nitrification by ammonia-oxidizing archaea (AOA) of the phylum Thaumarchaeota and by aerobic ammonia-oxidizing bacteria (AOB) of the phylum Proteobacteria (Bouskill et al. 2012), or by some newly discovered *Nitrospira* bacteria with the capacity to catalyze the complete nitrification process in a single microbe (Daims et al. 2015; van Kessel et al. 2015). Ammonia can also be oxidized into nitrogen via the anammox by anaerobic AOB of the phylum Planctomycetes (Kuypers et al. 2003). Additionally, ammonia can be assimilated into organic matter by diverse aerobic or anaerobic prokaryotes (Zehr and Kudela 2011). The occurrences of ammonia oxidation and assimilation in the oceans can be tracked by the detection of the *amoA* gene (encoding the subunit A of ammonia monooxygenase) in AOA and aerobic AOB, the *hzsA* gene (encoding the subunit of hydrazine synthase) in anaerobic AOB, and the *glnA* gene (encoding the subunit of glutamine synthetase) in ammonia-assimilating prokaryotes (Christman et al. 2011; De Corte et al. 2009; Hurt et al. 2001; Kong et al. 2013; Kuypers et al. 2003).

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Marine sponges are widely distributed and play critical ecological roles in benthic ecosystems (McClintock et al. 2005). Sponges often host abundant and diverse microbial communities (Hentschel et al. 2006; Reveillaud et al. 2014; Schmitt et al. 2012; Thomas et al. 2016; Webster and Thomas 2016) that are hypothesized to be involved in carbon, nitrogen, and sulfur transformations (Thacker and Freeman 2012). Microbially mediated nitrogen cycling has important ecological functions for sponges (Hoffmann et al. 2009). Like many marine invertebrates, sponges can excrete ammonia as a waste product which is toxic with its accumulation in sponge tissues (Southwell et al. 2008; Yahel et al. 2007). Ammonia excreted by sponges and other symbionts is presumably metabolized by specific symbiotic ammonia-oxidizing taxa via nitrification strategy. High rates of nitrification in sponges have been detected via physiological experiments in many sponges from different geographic areas (Bayer et al. 2008; Diaz and Ward 1997; Fiore et al. 2013; Hoffmann et al. 2009; Jiménez and Ribes 2007; Radax et al. 2012a; Schlappy et al. 2010; Southwell et al. 2008). Beside nitrite and nitrate production, nitrification can also result in ammonia uptake in sponges. For example, a seasonal switch between ammonia excretion and uptake has been reported in the Mediterranean sponge *Aplysina aerophoba* which hosts diverse ammonia-oxidizing microbes (Bayer et al. 2008).

Studies of ammonia oxidation in sponges provide an elegant example of the effective revelation of molecular gene-centric, genomic, transcriptomic, and proteomic data. For example, genomic reconstruction of the mesophilic AOA *Cenarchaeum symbiosum* harvested from the sponge *Axinella mexicana* has revealed the key genes including amo*ABC* genes for nitrification (Hallam et al. 2006). Besides, metagenomic reconstruction of a relative high abundant AOA, i.e., *Nitrosopumilus* sp. LS_AOA within the deep-sea sponge *Lophophysema eversa*, has uncovered the *amoABC* gene cluster in the genome which is mostly similar to the homolog in *Nitrosopumilus maritimus* SCM1 (Tian et al. 2016). It is known that most of the detected AOA and AOB within sponges cannot be cultivated. Therefore, their community structure and diversity would be analyzed by 16S rRNA gene assays. For example, 16S rRNA gene sequencing-based analyses have shown that several types of AOB in the genera *Nitrosospira* have been identified from the sponges *Aplysina aerophoba*, *Ircinia strobilina*, and *Mycale laxissima* (Bayer et al. 2008; Mohamed et al. 2010). Phylotypes belonging to the *Nitrosomonas eutropha*-/*europaea*-affiliated ammonia oxidizers have been recovered from various mangrove sponges (Diaz et al. 2004). In addition, the *amoA* gene from AOA or AOB has been commonly used as a phylogenetic marker to reveal the ammonia-oxidizing lineages in sponge microbiomes. For instance, *amoA* gene-based analysis has revealed diverse Thuamarchaeota AOA lineages in the sponge *Phakellia fusca* (Han et al. 2012), and *Nitrosospira* AOB taxa in the sponges *I. strobilina* and *M. laxissima* (Mohamed et al. 2010). Similarly, *amoA* and 16S rRNA gene assays have disclosed the ammonia-oxidizing group consisting of *Nitrosospira* AOB and Thaumarchaeota AOA in the sponge *Aplysina aerophoba* (Bayer et al. 2008). AOA genes have been shown to be vertically transmitted from sponge parental generation to their offspring in various sponges from different geographic locations by *amoA* assays (Steger et al. 2008), indicating the ubiquitous vertical transmission style of AOA in sponges and the close relationship between sponges and their AOA symbionts. In addition, related transcription level studies have shown the metabolic activity potential of the ammonia-oxidizing community in sponges. For example, transcribed *amoA* genes from AOA/AOB have been detected from the cold-water sponges *Phakellia ventilabrum*, *Geodia barretti*, *Antho dichotoma*, and *Tentorium semisuberites*; however, transcribed AOA *amoA* genes were several orders of magnitude higher than the transcribed AOB *amoA* genes in abundance (Radax et al. 2012a), indicating that AOA may be the main functional populations of ammonia oxidation in these sponges. The emerging high-throughput sequencing techniques performed in the sponge microbiology have provided new insights for the revelation of the multiple ecological roles, like the nitrification of sponge symbionts. For instance, 16S rRNA gene pyrosequencing revealed the Nitrosomonadales lineage in the sponge *Arenosclera brasiliensis* (Trindade-Silva et al. 2012) and the *Nitrosopumilus* taxon in the sponges *Xestospongia testudinaria* and *Hyrtios erectus* (Polonia et al. 2015). Metagenomic analyses have revealed the ammonia oxidation-related genes falling into the genus *Nitrosopumilus* in the deep-sea sponge *Neamphius huxleyi* (Li et al. 2014) and the genera *Nitrosopumilus*, *Nitrosococcus*, *Nitrosomonas*, and *Nistrosospira* in the sponges *Didiscus oxeata* and *Scopalina ruetzleri* (Rua et al. 2015). Metagenome comparison of the shallow-water sponge *Theonella swinhoei* and the deep-sea sponge *N. huxleyi* has showed that *amoA* genes related to the genera *Nitrosopumilus* and *Cenarchaeum* were detected in both sponges, yet exhibited a varied composition and diversity (Li et al. 2016). In addition, metatranscriptomic analyses have demonstrated the representative active nitrifying organisms consisted of AOA which have been confirmed by the significantly high expression of archaeal *amoA* transcripts in the sponges *G. barretti*, *Stylissa carteri*, and *Xestospongia muta* (Fiore et al. 2015; Moitinho-Silva et al. 2014; Radax et al. 2012b). Metagenomic and metaproteomic analysis revealed the expression of *amoABC* genes in the sponge *Cymbastela concentrica*; these genes were most closely related to those of the archaeon *Nitrosopumilus maritimus*, showing that aerobic nitrification is being carried out by *Nitrosopumilus* AOA in this sponge species (Liu et al. 2012). These studies demonstrated that AOA and AOB may play an important role in ammonia metabolism of sponge holobionts.

Ammonia excreted by sponges and other symbionts can also be metabolized by anaerobic ammonia-oxidizing taxa via the anammox process. First physiological evidence of anammox activity in sponges was described in the sponge *Geodia barretti* whose symbiotic anammox bacteria were closely related to the Planctomycetes species *Scalindua sorokinii* and *Scalindua brodae* (Hoffmann et al. 2009). There is mounting molecular evidence that anammox bacteria reside in sponges. For example, 16S rRNA gene sequences of anammox bacteria have been confirmed by clone library sequencing or pyrosequencing in different sponge species, including *Discodermia dissoluta* (Schirmer et al. 2005), *Antho chartacea* (Taylor et al. 2007), *Tethya aurantium* (Thiel et al. 2007), *M. laxissima* (Mohamed et al. 2008), *Crambe crambe* (Croue et al. 2013), and *Hymeniacidon heliophila* (Weigel and Erwin 2015). Unlike the ammonia-oxidizing microbes, many Planctomycetes strains can be isolated from sponges. For instance, three Planctomycetes strains were firstly isolated from the sponges *Aplysina* spp., which were closely related to the genus *Pirellula* (Pimentel-Elardo et al. 2003), while 29 species have been isolated from the sponge *Niphates* sp., which are related to the species *Blastopirellula marina*, *Rhodopirellula baltica*, and *Planctomyces brasiliensis* (Izumi et al. 2013). Therefore, the anammox process might be an important pathway for ammonia metabolism in some sponges.

Moreover, some sponge symbionts are able to assimilate ammonia released by their hosts and other symbionts (Freeman et al. 2013; Southwell et al. 2008). The efficient recycling of sponge-excreted nitrogenous wastes is one obvious benefit of the residence of symbionts in sponge tissues. In the metagenome of the sponge *C. concentrica*, abundant bacterial *glnA* genes were detected (Thomas et al. 2010). Metagenome comparison between the sponges *T. swinhoei* and *N. huxleyi* showed varied phylotype composition and diversity of bacterial *glnA* genes in them (Li et al. 2016). Moreover, transcribed bacterial *glnA* genes have been uncovered in the metatranscriptomes of the sponges *Stylissa carteri* (Moitinho-Silva et al. 2014) and *X. muta* (Fiore et al. 2015), demonstrating that some sponge symbionts have great potential for organic nitrogen metabolism through the ammonia assimilation pathway. These findings suggest that the ammonia assimilation process might be important for ammonia utilization by the bacterial community in sponge nitrogen cycling. However, comparison of the community structure and diversity of active ammonia-scavenging microbiota between different sponge species particularly those in the same geographical area, or between sponge and seawater biotopes, is poorly addressed.

We hypothesized that the diversity and expression of ammonia scavenging-related genes in sponge microbiome would be different among divergent sponge species and the seawater. In order to test this hypothesis, the diversity and transcription expression of functional genes involved in ammonia scavenging were qualitatively and quantitatively analyzed in this study to uncover and compare the active ammonia-scavenging microbiota among three sympatric sponge species and the ambient seawater.

Materials and Methods

Sponges and Environmental Seawater Sampling

The sponges *T. swinhoei* (class Demospongiae, order Tetractinellida, family Theonellidae), *Plakortis simplex* (class Homoscleromorpha, order Homosclerophorida, family Plakinidae), and *Phakellia fusca* (class Demospongiae, order Axinellida, family Axinellidae) were collected by SCUBA diving near Yongxing Island (112° 20′ E, 16° 50′ N) in the South China Sea at approximately10 m depth in June 2013. These sponge species were selected because they are abundant and coexist in the same reef. Samples were transferred underwater to sealable plastic bags containing seawater, brought to the boat, and stored on ice. Three individuals per sponge species were sampled. In addition, three seawater samples (2 l for each sample) were collected in close proximity (less than 1 m) before the sponges were collected and were filtered with 0.22-μm micron filters (Millipore, Bedford, USA). The collected sponge and seawater samples were preserved in RNA fixer stabilization solution (YuanPingHao, Beijing, China) to stabilize and protect cellular RNA and DNA. The sponge samples were sliced into 1-cm^3 pieces with sterile scalpels and were transferred into 10 volumes of RNA fixer after they have been rinsed three times with sterile artificial seawater (Mohamed et al. 2010). The time between sample acquisition and fixation was no longer than 20 min (Ozturk et al. 2013). RNA fixer-fixed sponge and filtered seawater samples were stored at − 80 °C before total RNA and DNA extraction within two weeks.

RNA, DNA Extraction and cDNA Synthesis

RNA fixer-fixed sponge and filtered seawater samples were ground in liquid nitrogen with a mortar and pestle. Both RNA and DNA were extracted from ground powders using the PrepRNA/DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. RNA and DNA were separately extracted from three individuals of each sponge and seawater sample. RNase-free DNase I (Fermentas, Hanover, USA) was used to digest the residual genomic DNA at 37 °C for 60 min. RNA quality and integrity were checked by gel electrophoresis and by examining the A260/A280 ratio (ranging from 1.97 to 2.02) using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The final RNA and DNA concentration and purity were quantified using the

Qubit system (Invitrogen, Darmstadt, Germany). First-strand cDNA synthesis was performed using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, USA). Each reaction volume was10 μl containing 100 ng RNA, 0.5 μl random hexamer primer (50 ng μl^{-1}), 5 μl cDNA Synthesis Mix, and proper RNase-free water. This reaction system was incubated at 25 °C for 10 min and then 50 °C for 50 min and terminated at 85 °C for 5 min. All cDNA aliquots were stored at − 80 °C before PCR amplification.

Amplification and Clone Library Construction

Both cDNA and DNA templates were used for PCR amplification of *amoA*, *hzsA*, and *glnA* gene fragments with the primers in Table 1. Primers targeting thaumarchaeotal *glnA* gene were designed in this study. In detail, GlnA amino acid sequences from different Thaumarchaeota species and unclassified thaumarchaeotal lineages were aligned; two conserved protein regions, i.e., "HHEAV" and "VPGVEAP," were identified from these peptide sequences and were used to design the primer pair TglnAF/TglnAR (Supplementary Fig. S1) by using the CODEHOP tool (Rose et al. 2003). The primer pair TglnAF/TglnAR amplify a 369-bp size fragment and the specificity of these primers was assessed using BLAST searches against the NCBI nucleotide database. PCR amplifications were performed in a total volume of 40 μl containing 2 μl cDNA or 2 ng DNA, 0.1 μM of each primer, and 20 μl TaqMasterMix (CoWin Biotech, Beijing, China) on a Thermocycler (Eppendorf, Hamburg, Germany) according to the following procedures: 95 °C for 5 min; followed by 30 cycles at 95 °C for 40 s, annealing (temperature referring to Table 1) for 1 min and 72 °C for 1 min; and finally 72 °C for 15 min. For negative control, similar procedure was carried out using purified RNA to ensure that there was no genomic DNA contamination. cDNAs of three individuals for each sponge or seawater sample were PCR-amplified, and three technical replicates per individual were processed. PCR products of technical triplicates were pooled to reduce potential amplification bias. Presence and sizes of these amplification products were estimated by gel electrophoresis (1.5% agarose gel). DNA-based PCR products were only used as control to detect the presence of the targeted genes in the investigated samples, which were not sequenced. cDNA-based PCR products were gel-purified with MinElute Gel Extraction Kit (Qiagen), cloned with TA-Cloning Kit (CoWin Biotech), and transformed to the *Escherichia coli* DH5α competent cells (CoWin Biotech) according to the standardized instructions. The positive clones were screened by ampicillin resistance and identified by PCR screening with vector-specific M13 primers. A variable number of clones (30–60) from each clone library were sequenced on an ABI 3100 capillary sequencer (Sangon Corp., Shanghai, China).

Diversity Estimation and Phylogenetic Analysis

All the obtained nucleotide sequences were trimmed manually by using ClustalW implemented in MEGA 6 with default settings (Tamura et al. 2013). The trimmed sequences were used to perform BLAST searches against the NCBI Nucleotide database. Operational taxonomic unit (OTU) was defined as sequence groups in which nucleotide sequences differed by ≤5% using the Mothur package (Schloss et al. 2009). This sequence dissimilarity cutoff has been used for OTU classification of *amoA* sequences (Damashek et al. 2014) and represented at least one amino acid substitution in the GlnA peptide sequences of this study. Sobs (number of the

Table 1 Primers for PCR amplification of partial *amoA*, *hzsA*, and *glnA* genes

Primer	Sequence (5′-3′)	Target	Tm. (°C)	Approach	Reference
amo111F	TTYTAYACHGAYTGGGCHTGGACATC	Archaea *amoA*	55	PCR	Treusch et al. (2005)
amo643R	TCCCACTTWGACCARGCGGCCATCCA	Archaea *amoA*			
amo196F	GGWGTKCCRGGRACWGCMAC	Archaea *amoA*	53	qPCR	Treusch et al. (2005)
amo277R	CRATGAAGTCRTAHGGRTADCC	Archaea *amoA*			
amoA 1F	GGGGTTTCTACTGGTGGT	Bacteria *amoA*	50	PCR	Rotthauwe et al. (1997)
amoA 2R	CCCCTCKGSAAAGCCTTCTTC	Bacteria *amoA*			
amoA 3F	GGTGAGTGGGYTAACMG	Bacteria *amoA*	45	PCR	Purkhold et al. (2000)
amoB4R	GCTAGCCACTTTCTGG	Bacteria *amoB*			
hzoF1	TGTGCATGGTCAATTGAAAG	Planctomycetes *hzsA*	50	PCR	Li et al. (2010)
hzoR1	CAACCTCTTCWGCAGGTGCATG	Planctomycetes *hzsA*			
hzocl2aF1	GGTTGYCACACAAGGC	Planctomycetes *hzsA*	48	PCR	Schmid et al. (2008)
hzocl2aR2	ATATTCACCATGYTTCCAG	Planctomycetes *hzsA*			
GS2γ	AAGACCGCGACCTTYATGCC	Bacteria *glnA*	47	PCR, qPCR	Hurt et al. (2001)
GS1β	GATGCCGCCGATGTAGTA	Bacteria *glnA*			
TglnAF	CAYCAYCAYGARGTDGCT	Thaumarchaeota *glnA*	55	PCR	This study
TglnAR	GGHGCYTCRTAICCIGG	Thaumarchaeota *glnA*			

observed OTUs), Chao1 richness estimator, Inverse Simpson diversity index, and Shannon diversity index were calculated using Mothur package (Schloss et al. 2009) to reflect the richness and diversity of the transcribed gene markers. Rarefaction curves and Good's coverage estimators were determined using the Mothur package (Schloss et al. 2009) to estimate whether the sequencing depth is enough to cover most of the transcribed genes in each clone library. One representative sequence from each OTU and its closest sequence retrieved from the NCBI Nucleotide database were aligned using Clustal W implemented in the MEGA 6. Maximum-likelihood (ML) tree was constructed by using the MEGA 6 with the Kimura-2 parameter model according to a published guideline (Hall 2013), since the ML tree is a better estimate of the true phylogenetic tree than those produced by other methods (Morrison 2007). Bootstrap analysis was used to estimate the reliability of phylogenetic reconstructions (1000 replicates). Representatives of the *amoA* and *glnA* OTUs were deposited in GenBank/EMBL/DDBJ under the accession numbers KP056353–KP056361and LN897349–LN897375, respectively.

RT-qPCR Assays

RT-qPCR assays were performed using an ABI 7500 Fast Real-time qPCR platform (Applied Biosystems, Foster, USA), following the approaches of Radax et al. (2012a). Gene expression was tested using biological triplicates and technical triplicates for each sponge or seawater sample. For transcribed archaeal *amoA* or bacterial *glnA* genes, the PCR was performed in a total volume of 25 μl containing 12.5 μl of SYBR Premix Ex Taq™ II (Takara, Dalian, China), 1 μl of cDNA template (tenfold serial dilution), and 0.1 μM of each primer (Table1). PCR thermocycling steps were set as follows: 95 °C for 5 min and 40 cycles at 95 °C for 45 s, 54 °C (for *amoA*) or 53 °C (for *glnA*) for 45 s, and 72 °C for 45 s. For quantification, standard curves (log-linear $R^2 > 0.99$) were generated using purified and quantified plasmids containing 123-bp archaeal *amoA* fragment (partial sequence of the representative clone amoATS3 in Fig. 2) or 152-bp bacterial *glnA* fragment (sequence of the representative clone Ths3 in Fig. 3) in a dilution series that spanned from 10^1 to 10^7 gene copies per reaction. All standard dilutions were prepared in 10 ng μl^{-1} aqueous tRNA solution (Sigma-Aldrich, Steinhiem, Germany). Plasmid DNA was extracted using the PurePlasmid 96 Kit (CoWin Biotech), and the plasmid concentration was measured using Qubit system (Invitrogen). Since the sequences of the vector and PCR insert are known, copy numbers of transcribed *amoA* or *glnA* genes were directly calculated according to the reported formula: copy numbers $\mu l^{-1} = (A \times 6.022 \times 10^{23}) \times (660 \times B)^{-1}$, where A is the plasmid concentration (g μl^{-1}), B is the recombinant plasmid length (bp) containing the *amoA* or *glnA* fragment, 6.022×10^{23} is the Avogadro's number, and 660 is the average molecular weight of 1 bp (Perini et al. 2011). Copy numbers of *amoA* or *glnA* transcripts in 1 cm^3 wet sponge or in 1 ml seawater sample were calculated according to the formula: $(M \times 100^{-1}) \times 10 \times 1^{-1} \times 10 \times N$. In turn, M is the yield of purified RNA from 1 cm^3 wet sponge or 1 ml seawater (ng), 100 is the RNA used in each reverse-transcription reaction (ng), 10 is the obtained cDNA volume in each reverse-transcription reaction (μl), 1 is the cDNA (tenfold serial dilution) volume used in each qPCR reaction (μl), 10 is the dilution ratio, and N is the copy number of *amoA* or *glnA* obtained in each qPCR reaction. For negative control, similar procedure was performed using purified RNA to ensure that there was no genomic DNA contamination. After qPCR assay, the specificity of amplification was verified by generation of melting curves (in steps of 0.5 °C for 5 s, with temperatures ranging from 60 to 95 °C) and the qPCR product size was checked by 2% agarose gel electrophoresis.

Statistical Analysis

Data acquisition of the qPCR assay was performed using the 7500 System SDS Software Version 1.2 (Applied Biosystems). Two-tailed independent-sample t test was performed to evaluate the abundance variations of the transcribed *amoA* or *glnA* genes among the investigated sponge and seawater samples. Statistical differences were determined at the level of $\alpha = 0.01$ and all statistical analyses were conducted using the commands in Microsoft Excel 2007.

Results

Diversity of Transcribed *amoA* and *glnA* Genes

Archaeal *amoA* and bacterial *glnA* fragments from both DNA and cDNA templates of *T. swinhoei*, *P. simplex*, *P. fusca*, and seawater were detected by PCR. Bacterial *amoA*, *hzsA* and archaeal *glnA* genes, and their transcripts were not detected. Although some rarefaction curves seemed not to reach an asymptote (Fig. 1a, b), the Good's coverage estimator of each archaeal *amoA* or bacterial *glnA* transcript clone library ranged from 96 to100% at 5% nucleotide cutoff (Table 2), indicating that enough clones were sequenced to represent the diversity of corresponding clone library. In total, 152 high-quality transcribed archaeal *amoA* gene sequences were grouped into nine unique OTUs, including seven unique OTUs to sponges and two unique OTUs to seawater, and no OTU was shared between sponge and seawater samples (Fig. 1c). In addition, 27 OTUs were obtained from 155 transcribed bacterial *glnA* gene sequences, containing 19 unique OTUs to sponges, three unique OTUs to seawater, three shared OTUs between sponges (one *P. fusca*-/*P. simplex*-/*T. swinhoei*-derived OTU,

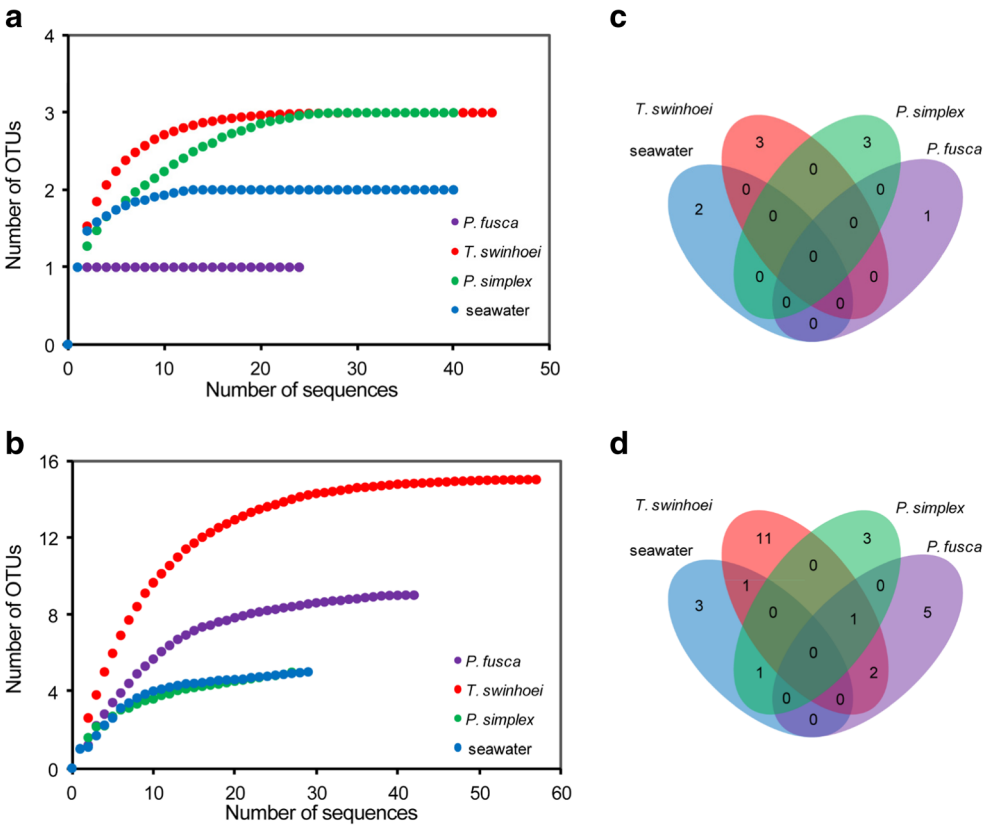

Fig. 1 Rarefaction curves of transcribed archaeal *amoA* (**a**) and bacterial *glnA* (**b**) genes and Venn description of transcribed archaeal *amoA* (**c**) and bacterial *glnA* (**d**) OTUs from the sponges *T. swinhoei*, *P. fusca*, and *P. simplex*, and seawater, with OTU calculation at 5% nucleotide sequence dissimilarity cutoff

two *T. swinhoei*-/*P. fusca*-derived OTUs), and two shared OTUs between sponge and seawater samples (one *T. swinhoei*-/seawater-derived OTU and one *P. simplex*-/seawater-derived OTU) (Fig. 1d). As shown in Table 2, the maximum *amoA* diversity in terms of Shannon index and inverse Simpsons index was found in *T. swinhoei* (Shannon index = 0.9 and inverse Simpsons index = 1.5) and *P. simplex* (Shannon index = 0.7 and inverse Simpsons index = 1.3), while the minimum diversity was found in *P. fusca* (Shannon index = 0.8 and inverse Simpsons index = 1) (Table 2). In addition, the maximum *glnA* diversity was found in *T. swinhoei* (Shannon index = 3.8 and inverse Simpsons

Table 2 Richness and diversity estimates for transcribed archaeal*amoA* and bacterial *glnA* gene clone libraries from *T. swinhoei*, *P. simplex*, *P. fusca*, and seawater

	No. of seqs	Sobs[a]	Good's coverage[b]	Chao1[c]	Inverse Simpson[d]	Shannon[e]
Archaeal*amoA*						
T. swinhoei	47	3	1	3	1.5	0.9
P. simplex	40	3	1	3	1.3	0.7
P. fusca	25	1	1	1	1	0.8
Seawater	40	2	1	2	1.2	0.6
Bacterial *glnA*						
T. swinhoei	57	15	0.98	16	5.2	3.8
P. simplex	29	5	0.96	6	2.1	1.4
P. fusca	42	8	1	8	2.8	2.1
Seawater	27	5	0.97	5	2.4	1.5

[a] Number of the observed OTUs based on the 95% nucleotide sequence similarity

[b] Percentage of observed number of OTUs divided by Chao1 estimate

[c] Nonparametric statistical predictions of total richness of OTUs based on distribution of singletons and doubletons

[d] Reciprocal of Simpson's diversity index. A higher number represents more diversity

[e] Shannon diversity index. A higher number represents more diversity

index = 5.2) and *P. fusca* (Shannon index = 2.1 and inverse Simpsons index = 2.8), while the minimum diversity was found in *P. simplex* (Shannon index = 1.4 and inverse Simpsons index = 2.1) (Table 2). Diversity of the transcribed genes related to ammonia scavenging showed significant difference among these sympatric sponges and seawater, since no *amoA* OTU and very few *glnA* OTUs were shared among these niches.

Phylogenetic Analysis of Active Archaeal Ammonia Oxidizers

All nine transcribed *amoA* OTUs fell into two Thaumarchaeota ecotype clusters, i.e., the HAC-AOA and LAC-AOA (high- and low-ammonia-concentration AOA) ecotype clusters (Fig. 2a), according to the reported AOA ecotype classification (Steger et al. 2008). As shown in Fig. 2a, five sponge-derived OTUs and two seawater-derived OTUs fell into the HAC-AOA ecotype cluster, and two sponge-derived OTUs were gathered into the LAC-AOA ecotype cluster. Phylogenetic analysis suggested that six of seven sponge-derived OTUs were closely related to the reported sponge-derived sequences; the remaining one was similar to the sequences from salt-water aquaria. Two seawater-derived sequences were affiliated with the sequence from sediment and the sequence of *Nitrosopumilus maritimus* SCM1 (Supplementary Table S1). These OTUs showed 84–98% nucleotide sequence similarity with their closest relatives (Query coverage, 94–100%) (Supplementary Table S1). The different percentage of each transcribed *amoA* OTU (Supplementary Table S2) in corresponding clone library is displayed in Fig. 2b. This finding showed the phylogenetically diverse transcribed archaeal *amoA* phylotypes in the investigated sympatric sponges and seawater.

Phylogenetic Analysis of Active Bacterial Ammonia Assimilators

Phylogenetic analysis demonstrated that all 27 transcribed bacterial *glnA* OTUs were grouped into the Cyanobacteria, Tectomicrobia, Poribacteria, Alpha-, Beta-, Gamma-, and Epsilonproteobacteria lineages (Fig. 3a). Most of these OTUs were closely related to the sequences of identified isolates, except for OTU-Sew59, which was related to the reported seawater-derived sequence (Supplementary Table S2). These OTUs shared 69–96% nucleotide sequence similarity with their closely matched sequences (query coverage, 97–100%) (Supplementary Table S1). Different proportion of the transcribed bacterial *glnA* OTUs (Supplementary Table S2) in corresponding clone library is demonstrated in Fig. 3b. These results displayed the phylogenetically divergent transcribed bacterial *glnA* phylotypes in the investigated sympatric sponges and seawater.

Quantification of the Transcribed *amoA* and *glnA* Genes

qPCR was employed to quantitatively test the community-wide transcription expressions of archaeal *amoA* and bacterial *glnA* genes in three sympatric sponges and their environmental seawater (Supplementary Table S3). The copies of archaeal *amoA* transcripts in *T. swinhoei* were not statistically different from those in *P. simplex* ($P = 0.017 > 0.01$, two-tailed independent-sample *t* test, similarly hereinafter), but their abundances in *T. swinhoei* and *P. simplex* were statistically higher than those in *P. fusca* and seawater ($P = 1.7 \times 10^{-6}$–$1.2 \times 10^{-3} < 0.01$) (Fig. 4). Likewise, the copies of bacterial *glnA* transcripts in *T. swinhoei* were not statistically different from those in *P. simplex* ($P = 0.047 > 0.01$), but their abundances in *T. swinhoei* and *P. simplex* were statistically higher than those in *P. fusca* and seawater ($P = 2.0 \times 10^{-7}$–$9.9 \times 10^{-3} < 0.01$) (Fig. 4). These results discerned the varied abundances of transcribed archaeal *amoA* and bacterial *glnA* genes in the sympatric sponges which were higher than those in seawater.

Discussion

In this study, we analyzed the active ammonia-scavenging microbiota in the sympatric sponges *T. swinhoei*, *P. simplex*, and *P. fusca* and the surrounding seawater. Since aerobic and anaerobic AOB for ammonia oxidation were not detected, AOA are likely the dominant ammonia oxidizers in the investigated samples. Thus, the dominant Thaumarchaeota AOA in sponges may contribute to efficient ammonia oxidation in these niches. AOA rather than AOB was identified as the dominant ammonia oxidizers from metatranscriptome data of different sponges (Fiore et al. 2015; Moitinho-Silva et al. 2014; Radax et al. 2012b). Compared to AOB, AOA often show a lower half saturation constant and substrate threshold (Horak et al. 2013; Stark and Firestone 1996).

Although the investigated sponges were collected from the same location, this research clearly demonstrated that ammonia-scavenging microbiota with transcriptional activity varied significantly among these sympatric sponges and the surrounding seawater since few OTUs were shared among these niches (Fig. 1). Likewise, significantly different archaeal *amoA* phylotypes were detected from the sympatric Mediterranean sponges *Ircinia fasciculata* and *I. oros*, Caribbean sponges *I. strobilina* and *M. laxissima*, and the surrounding seawater (Zhang et al. 2014a, b). This might be caused by a long evolutionary divergence among different sponge species, with concomitant selective enrichment of diverse AOA lineages by each sponge species (Taylor et al. 2007). Taken together, these findings suggested the specific composition of the dominant ammonia-oxidizing community

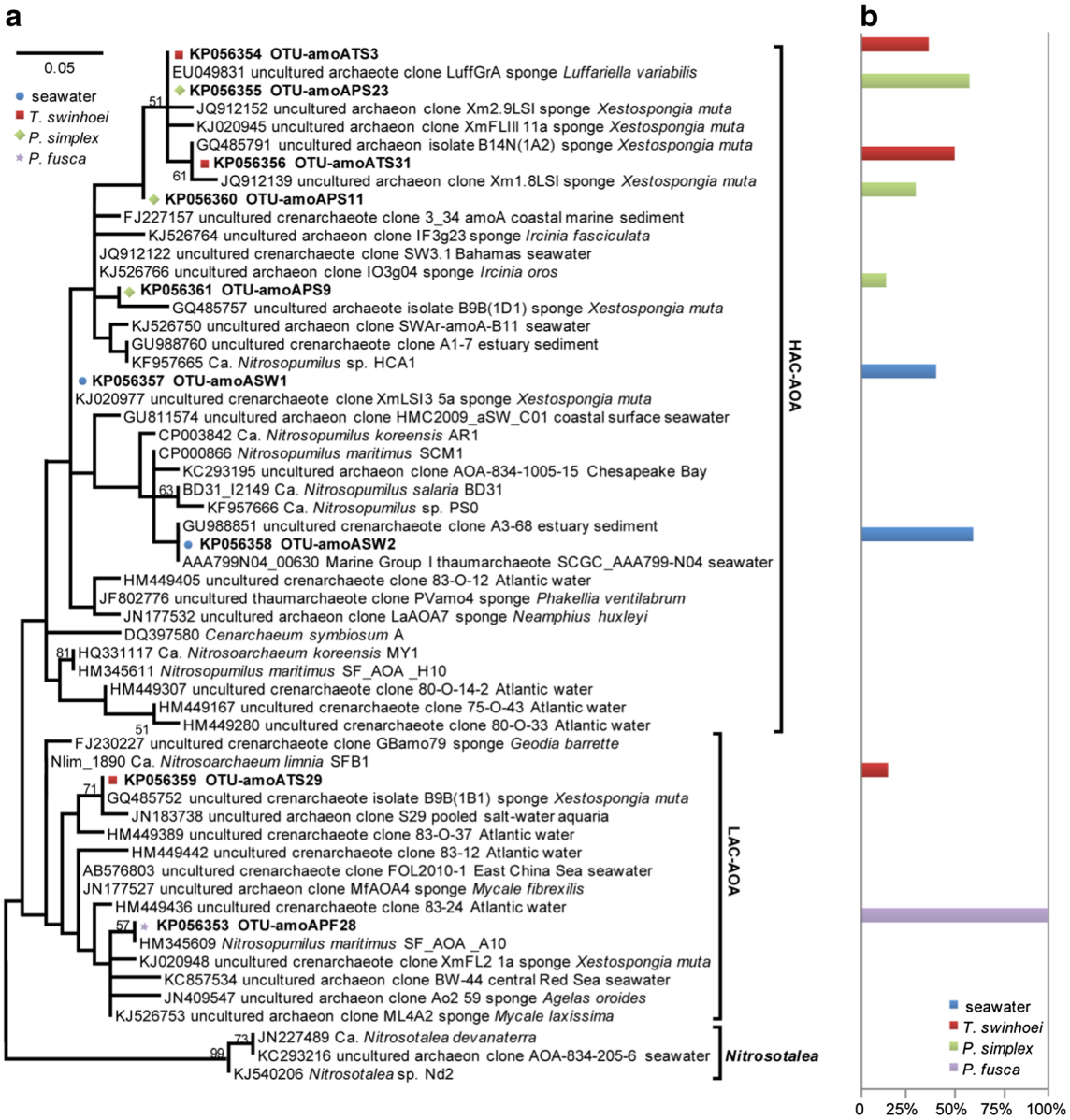

Fig. 2 **a** Phylogenetic maximum-likelihood tree of transcribed archaeal *amoA* OTUs retrieved from the microbiome of the sponges *T. swinhoei*, *P. fusca*, and *P. simplex*, and seawater. OTU representatives are highlighted. Scale bar represents 5% sequence divergence per homologous position. Bootstrap values more than 50% of 1000 replicates are shown. In the research of Sintes et al. (2013), sequences with GenBank accession number HM449167, HM449280, and HM449307 belonged to the HAC-AOA ecotype, while HM449389, HM449436, and HM449442 belonged to the LAC-AOA ecotype. **b** Percentage of sequences belonging to a particular transcribed *amoA* OTU in corresponding clone library

among the sympatric sponge species and the surrounding seawater.

Previous investigation implied that marine AOA could be grouped into two ecotypes, i.e., HAC-AOA and LAC-AOA, according to their different efficiencies of ammonia availability (Sintes et al. 2013). HAC-AOA ecotype often appears in regions with relatively high ammonia concentrations, while LAC-AOA ecotype appears in ammonia concentrations below the detection limit of conventional methods (Sintes et al. 2013). Remarkably, the differentiation into these two ecotypes extends to the ammonia monooxygenase subunit A at the amino acid level; i.e., some amino acid substitutions are particularly significant (Sintes et al. 2016). In this study, both HAC-AOA and LAC-AOA ecotypes were detected in *T. swinhoei*, whereas HAC-AOA and LAC-AOA ecotypes were found in *P. simplex* and *P. fusca*, respectively (Fig. 2). Since symbiotic AOA in sponges are considered stable and host-specific in community structure (Zhang et al. 2014a, b), HAC-AOA or/and LAC-AOA ecotype(s) within a particular sponge may imply the adaptation of AOA group to their hosts. This would contribute to the effective ammonia scavenging in sponge tissues.

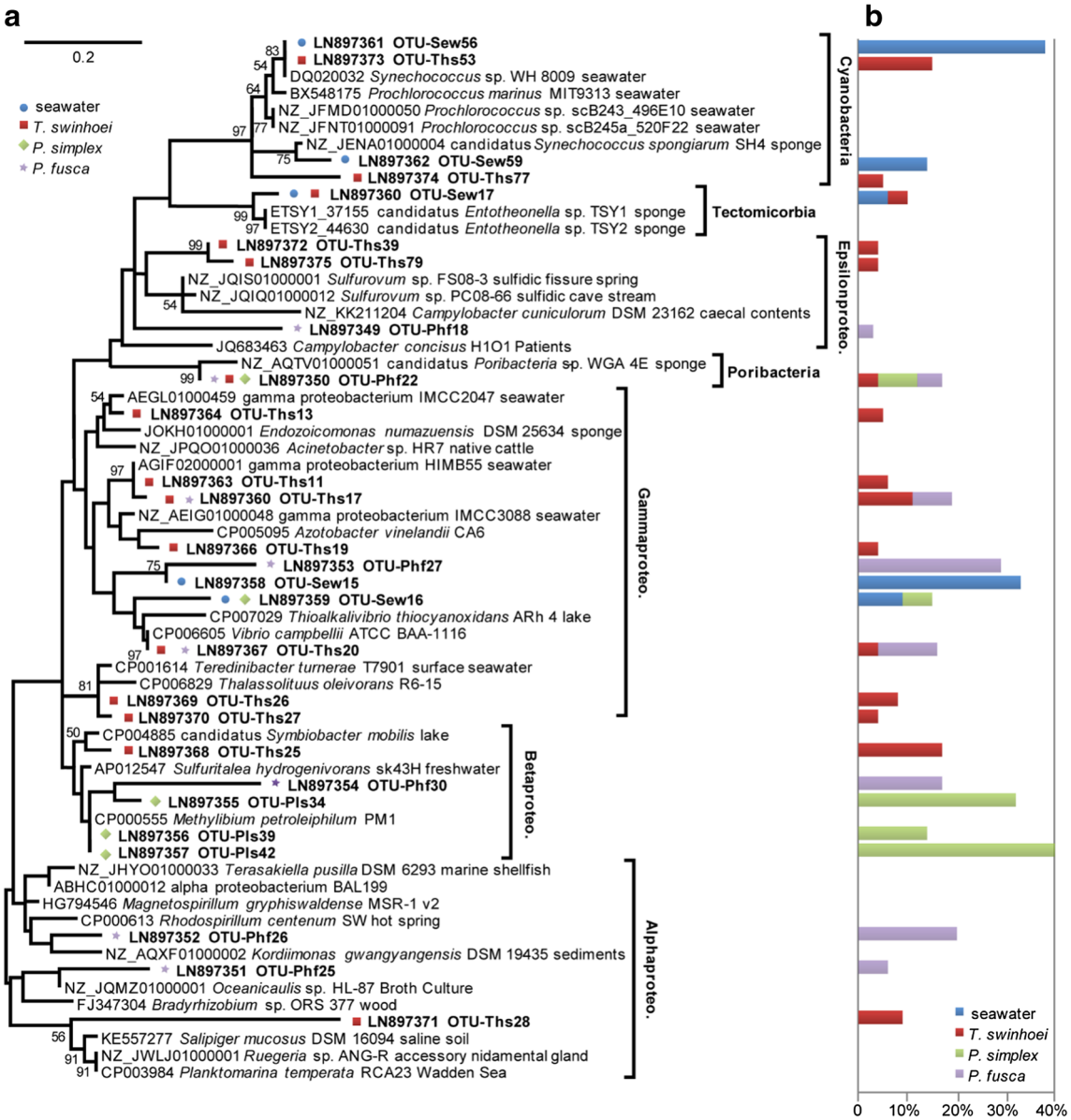

Fig. 3 **a** Phylogenetic maximum-likelihood tree of transcribed bacterial *glnA* OTUs retrieved from the microbiome of the sponges *T. swinhoei*, *P. fusca*, and *P. simplex*, and seawater. OTU representatives are highlighted. Scale bar represents 20% sequence divergence per homologous position. Bootstrap values more than 50% of 1000 replicates are shown. **b** Percentage of sequences belonging to a particular transcribed *glnA* OTU in corresponding clone library

In the case of ammonia-assimilating bacteria, transcribed bacterial *glnA* genes fell into seven phylum-/class-level lineages, indicating phylogenetically diverse bacterial ammonia assimilators with transcriptional activity in these sympatric sponges (Fig. 3). However, our data might underestimate the diversity of the active ammonia-assimilating bacteria because of the wide distribution and deep phylogenetic branches of bacterial *glnA* genes (Forchhammer 2007) and the inherent biases of PCR-based methods (von Wintzingerode et al. 1997). Our study implied that ammonia assimilation via symbiotic bacteria might be a necessary pathway contributing to microbially mediated ammonia removal in the investigated niches. Furthermore, similar to the revealed AOA population, significant diversity in bacterial ammonia assimilators with transcriptional activity was detected among the sympatric sponges, since very few transcribed *glnA* OTUs were shared among these biotopes (Fig. 1). This phenomenon may be explained by the hypothesis that a certain microbial metabolic process in different sponges might be performed by phylogenetically divergent but functionally equivalent microbial lineages (Bayer et al. 2014; Fan et al. 2012).

Currently, gene-centric high-throughput sequencing analysis has been performed to reveal the diversity of certain functional genes, such as *nifH* (encoding the subunit of

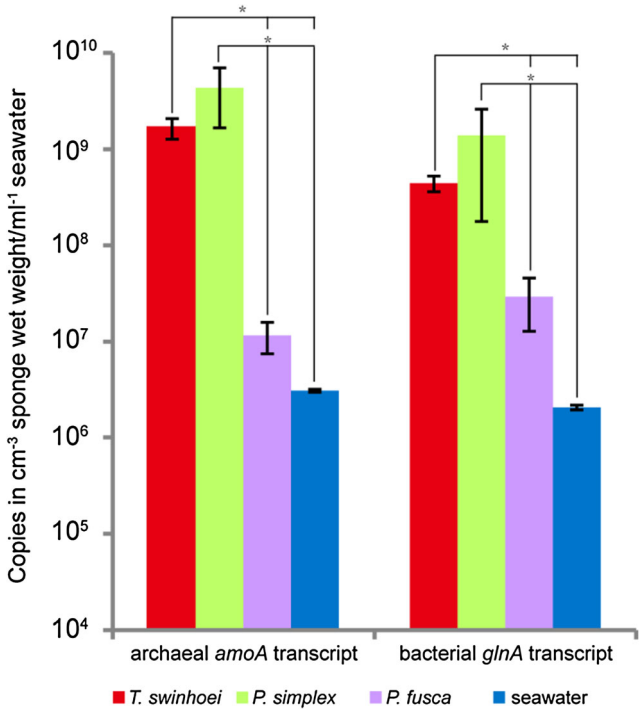

Fig. 4 Abundance of transcribed archaeal *amoA* and bacterial *glnA* genes estimated by qPCR for the sponges *T. swinhoei*, *P. fusca*, and *P. simplex*, and the environmental seawater. Individual triplicates and technical triplicates were performed for each sample. The depicted values are the means of triplicate biological samples, and the error bars indicate the standard error. Two-tailed independent-sample *t* test was performed on the quantitative data at a significance level of $\alpha = 0.01$. The statistically significant difference ($P < 0.01$) of transcribed archaeal *amoA* or bacterial *glnA* gene copies among the sponges and seawater is indicated with an asterisk

dinitrogenase reductase) genes (Jing et al. 2015). However, the advent of high-throughput sequencing techniques is not the finality for Sanger sequencing applications, since Sanger sequencing methodology still retains usefulness in screening assays of a handful of genes (Wiley et al. 2014). In addition, it is not easy to the design of high-effective primers for gene-centric high-throughput sequencing surveys of amplification products (Dowle et al. 2016). Therefore, traditional PCR approach still has some advantages in conducting censuses of environmental microbial functional genes (Lynn et al. 2017; Tahon et al. 2016). The primers selected in this study have been commonly used to detect the archaeal and bacterial *amoA*, bacterial *hzsA*, and bacterial *glnA* gene fragments from various niches, e.g., sponges, seawater, soils, and sediments (Bayer et al. 2008; De Corte et al. 2009; Ganesh et al. 2015; Hurt et al. 2001; Jouett et al. 2015; Li et al. 2010; Purkhold et al. 2000; Rotthauwe et al. 1997; Schmid et al. 2008; Treusch et al. 2005). In this study, bacterial *amoA* and *hzsA* genes and thaumarchaeotal *glnA* genes, and their transcribed counterparts were not detected. One reason might be that these genes and their transcripts may be absent or with very low copies that are below PCR sensitivity threshold. A second possibility is that amplification of the functional genes in a given environment is highly dependent on the primer preference and on the composition of the sampled environmental community (Fredriksson et al. 2013). In addition, failure of amplification of the thaumarchaeotal *glnA* gene fragments from the investigated samples may also be due to the excessive numbers of mismatches of the newly designed primers to combine the target sequences in samples. Given the vast geographic distribution and the important ecological value of Thuamarchaeota, further work should focus on developing alternative primers for thaumarthaeota *glnA* gene sequences, because PCR amplification could not be achieved with the primers in this study. Euryarchaeota have been confirmed in sponges (Reveillaud et al. 2014; Yang and Li 2012); however, no euryarchaeotal *glnA* gene fragments from sponges or seawater have been reported so far. Therefore, degenerate primers covering Euryarchaeota *glnA* genes in these niches were not designed in this study. Overall, the detection of the transcription of microbial ammonia metabolism-related genes would contribute to our knowledge into metabolic activity of microbially mediated ammonia scavenging in the investigated samples, though PCR-based assay may not capture all types of ammonia-scavenging microbiota (von Wintzingerode et al. 1997).

qPCR is commonly used to quantify the abundances of transcripts in a complex microbial community (Aoi et al. 2004; Wang et al. 2014). Transcribed *amoA* genes were either exclusively detectable from archaea or were higher in abundance than their bacterial counterparts by orders of magnitude in the cold-water sponges *A. dichotoma*, *G. barretti*, *P. ventilabrum*, and *T. semisuberites* (Radax et al. 2012a). In another study, higher abundance of *amoA* transcripts from ammonia-oxidizing *Nitrosopumilus* than *nxrB* (encoding the subunit of nitrite oxidordeuctase) transcripts from nitrite-oxidizing *Nitrospira* was detected in the microbiome of the sponge *T. swinhoei* (Feng et al. 2016). In our research, we have used the primer pairs amo196F/amo277R and GS2γ/GS1β (Pal et al. 2015; Treusch et al. 2005) to separately quantify the transcribed *amoA* and *glnA* genes in the sympatric sponge and seawater samples. The abundance of transcribed *amoA* and *glnA* genes significantly differed among *T. swinhoei*, *P. simplex*, *P. fusca*, and between these sponges and the surrounding seawater (lower abundance in the latter), which may be related to the divergent metabolic activity of ammonia-scavenging microbiota consisting of ammonia-oxidizing archaea and ammonia-assimilating bacteria in these niches.

The proposed microbially mediated ammonia scavenging via ammonia oxidation and ammonia assimilation processes by phylogenetically divergent prokaryotic lineages in the investigated sympatric sponges contributes to our understanding of the roles played by sponge symbiotic microbes in ocean nitrogen cycling. Certainly, some aspects need further study, for example, more sponge species or

measurements of respective ammonia-scavenging rate of ammonia oxidation and ammonia assimilation. Additionally, the investigated sponges and seawater samples were only collected at one timepoint rather than at serial timepoints and variability of gene expression is possible, such as the *nifH* transcripts of planktonic diazotrophs in the ocean (Church et al. 2005). Few studies have addressed temporal changes in expression genes involved in nitrogen cycling in bacterial communities of sponges. Temporal changes in gene expression of diazotrophic bacteria were reported in the sponges *I. strobilina* and *M. laxissima* (Zhang et al. 2014a, b). Therefore, more data from different sponges or time series will give us a better insight in the investigated processes.

Conclusions

In this study, the diversity and abundance of active ammonia-scavenging microbiota in the sympatric sponges were investigated using transcriptome-based strategies. Ammonia-oxidizing Thaumarchaeota and ammonia-assimilating Cyanobacteria, Proteobacteria, Tectomicrobia, and Poribacteria were detected by targeting the ammonia scavenging-related microbial genes. Community structure and diversity of the active ammonia-scavenging microbiota showed significant variations among the sympatric sponges and the surrounding seawater. qPCR data indicated the different transcribed activity of archaeal *amoA* and bacterial *glnA* genes in the investigated biotopes. In summary, qualitatively and quantitatively analyses of transcribed ammonia scavenging-related genes indicated the in situ active ammonia-scavenging microbes in different sympatric sponges. Our results indicated that the diversity and expression of ammonia scavenging-related genes in sponge microbiome were different among divergent sponge species and surrounding seawater. The presented results extend our understanding of sponge-microbe association and the ecological functions of sponge microbial symbionts in holobiont nitrogen cycling.

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Compliance with Ethical Standards

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

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