The Different Potential of Sponge Bacterial Symbionts in N₂ Release Indicated by the Phylogenetic Diversity and Abundance Analyses of Denitrification Genes, nirK and nosZ

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Abstract

Nitrogen cycle is a critical biogeochemical process of the oceans. The nitrogen fixation by sponge cyanobacteria was early observed. Until recently, sponges were found to be able to release nitrogen gas. However the gene-level evidence for the role of bacterial symbionts from different species sponges in nitrogen gas release is limited. And meanwhile, the quantitative analysis of nitrogen cycle-related genes of sponge microbial symbionts is relatively lacking. The nirK gene encoding nitrite reductase which catalyzes soluble nitrite into gas NO and nosZ gene encoding nitrous oxide reductase which catalyzes N₂O into N₂ are two key functional genes in the complete denitrification pathway. In this study, using nirK and nosZ genes as markers, the potential of bacterial symbionts in six species of sponges in the release of N₂ was investigated by phylogenetic analysis and real-time qPCR. As a result, totally, 2 OTUs of nirK and 5 OTUs of nosZ genes were detected by gene library-based saturated sequencing. Difference phylogenetic diversity of nirK and nosZ genes were observed at OTU level in sponges. Meanwhile, real-time qPCR analysis showed that Xestospongia testudinaria had the highest abundance of nosZ gene, while Cinachyrella sp. had the greatest abundance of nirK gene. Phylogenetic analysis showed that the nirK and nosZ genes were probably of Alpha-, Beta-, and Gammaproteobacteria origin. The results from this study suggest that the denitrification potential of bacteria varies among sponges because of the different phylogenetic diversity and relative abundance of nosZ and nirK genes in sponges. Totally, both the qualitative and quantitative analyses of nirK and nosZ genes indicated the different potential of sponge bacterial symbionts in the release of nitrogen gas.

Introduction

The oceans are a central feature of the biosphere with biogeochemical links to atmosphere. The microorganisms in seawater maintain the fertility of the ocean by catalyzing C/N/P/S transformation reactions to provide nutrients for marine organisms. Nitrogen cycle, which is driven by complex biogeochemical transformations, including nitrogen fixation, nitrification and denitrification, and assimilation, mediated by microorganisms, is a critical biogeochemical process of the oceans because it controls the productivity of the oceans and results in production and consumption of greenhouse gases [1].

Marine sponges (Porifera) are thought to have evolved approximately ~580 million years and are among the most ancient metazoa [2,3]. They represent a significant component of the marine biosphere throughout the coral reefs and benthic ecosystem. Sponges are sessile filter feeders that pump large amount of seawater every day (e.g. over many thousands of litres of water per day) [4], through numerous tiny pores on their surface by the flagella motion of their choanocyte cells. The ecological functions of sponges, especially N cycle [5–7], have attracted much more attention to the researchers. For example, sponge Geodia barretti has been reported to carry out nitrification rate of 566 nmol N cm⁻³ sponge day⁻¹ [5], and the majority of the benthic nitrification on the Florida Keys outer reef tract probably occurs in sponges [6]. Sponges are known to harbor phylogenetically complex and abundant microbial communities including bacteria, archaea and fungi [4,8–15]. Recently a great number of investigations have been done to address the functional features of sponge microbes in nitrogen cycle [5,16–21]. Schläppy et al. observed nitrification and denitrification in high and low microbial abundance sponges [17]. Diaz and Ward observed that the nitrification capacity of tropical sponges was related to the association between bacteria and sponges [7]. To date, many achievements in nitrification mediated by sponge microbial symbionts have been made, for example ammonia-oxidizing archaea (AOA) [17,18–20], ammonia-oxidizing Gammaproteobacteria, nitrite-oxidizing Nitrospira and anaerobic ammonia-oxidizing bacteria Planctomycetales [16,18].

Denitrification is a dissipilatory process in which nitrate and nitrite are reduced to gaseous nitric oxide, nitrous oxide and
molecular nitrogen when oxygen is limited, which consists of four reaction steps catalyzed by nitrate reductase (napA or narG), nitrite reductase (nirK or nirS), nitric oxide reductase (nosB or niorB) and nitrous oxide reductase (nosZ). Nitrite reductase is the key enzyme of this respiratory process since it catalyzes the reduction of soluble nitrite into gas NO. Nitrous oxide reductase catalyzes the last step in the complete denitrification pathway. Greenhouse gas N2O, which contribute not only to global warming but also directly to the destruction of the stratospheric ozone layer, will go to the atmosphere without the further reduction by nitrous oxide reductase. Therefore nitrite reductase (cytochrome cd1-dependent nirK or copper-containing nirS) genes and nitrous oxide reductase (nosZ) gene are usually used as genetic markers to investigate denitrifying community [22–26].

Early in 1979, the nitrogen fixation by sponge cyanobacteria was observed [27]. To date, using nitrogen cycle-related functional genes as markers, the possible roles of sponge microbial symbionts in nitrogen transformation have been suggested, e.g., nitrogen fixation [nifH gene] [28], ammonia oxidation [amoA gene] [18–20, 29–34], and nitrite reduction [nir gene] [5, 33, 34, 35]. Hoffmann et al. [5] and Schlappey et al. [17] detected the N2 release from the sponges with high microbial abundances (Geodina barrett, Chondrosia reniformis) and low microbial abundance (Dysidea avrica), however no information of nosZ gene (nitrous oxide reduction to N2) was provided in these reports. In the genome analysis of a member of the Porifera from sponge Aplysina aerophoba, nosZ gene was not found [35]. Until recently, Fan et al. detected nosZ gene in the metagenomes of some sponges [36]. Therefore, more gene-level molecular evidence for N2 release by sponges needs to be provided. Meanwhile, at present, quantitative analysis of nitrogen cycle-related genes is relatively lacking. In this study, using two key functional genes as markers, nirK gene encoding nitrite reductase and nosZ gene encoding nitrous oxide reductase, the potential of sponge microbiota in the release of N2 was investigated. Besides the phylogenetic diversity analysis of nosZ and nirK genes, their relative quantification was analyzed by real-time qPCR for the first time. This study provides the further understanding of sponge bacterial denitrification potential by the qualitative and quantitative analyses of nirK and nosZ genes, extending our knowledge of nitrogen cycling process in sponges.

Materials and Methods

Ethics Statement: N/A

This study and the collection of sponges were approved by the ethics committee at School of Life Sciences and biotechnology, Shanghai Jiao Tong University.

No legislation was required for the sampling of sponges around Yongxing island (112° 20′E, 16° 50′N). The government of China permits the sampling of sponge samples around the Yongxing island in the South China Sea for scientific research, and no specific permissions were required for these locations/activities, the location is not privately-owned or protected in any way, the field studies did not involve endangered or protected species. We collected the sponge samples ourselves.

Sponge Sampling

Sponges Iostrochta sp., Xestospongia testudinaria, Cinachyrella australiensis and Cinachyrella sp., were 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. Sponges Amphimedon queenslandica and Sphacelospongea vestiporium were collected from the Linshai port (110° 10′E, 18° 24′N) in Hainan province by diving at a depth of ca. 20 m. For each sponge species, three individual samples were collected. Samples were placed in bags with natural sea water and transported to the laboratory immediately in an ice-cooled box. The microbes from seawater column on the sponge surface and in inner cavity were removed by washing three times with sterile artificial seawater (ASW) (1.1 g CaCl2, 10.2 g MgCl2·6 H2O, 31.6 g NaCl, 0.75 g KCl, 1.0 g Na2SO4, 2.4 g Tris·HCl, 0.02 g NaHCO3, 1 L distilled water, pH 7.6). Then the sponge samples were stored at −20°C before DNA extraction. The sponge samples were identified according to 28S rRNA or 18S rRNA gene with 99% similarity.

DNA Extraction and PCR Amplification

Three replicates for each sponge species were used for DNA extraction and the DNA from triplicates was pooled for PCR amplification. Samples were rinsed 3 times by ASW and then homogenized in 1 ml of TE Buffer (10 mMTris, 1 mM EDTA, pH 8.0), centrifuged at 10,000×g for 3 min and grinded using a mortar containing 600 µl CTAB lysisbuffer (2% CTAB, 1.4 M NaCl, 100 mM Tris, 20 mM EDTA, 1% PVP) at 65°C. The mycelial suspension was transferred into a 1.5 ml eppendorf tube and heated at 65°C for 30 min, extracted twice with an equal volume of phenol/chloroform/isomyl alcohol (25:24:1) and washed with chloroform/isomyl alcohol (24:1). After centrifugation at 10,000×g for 5 min, the supernatant was transferred to a new microtube and precipitated by adding equal volume of isopropanol at −20°C for 1 h. Finally, the DNA pellets were collected by centrifugation (12,000×g, 15 min), washed with 75% ethanol twice and re-suspended in 40 µl TE Buffer. DNA was removed by an incubation period with 2 µl of RNase A (10 mg/ml, Invitrogen) at 37°C for 10 min. The DNA was quantified by spectrophotometry at 260 nm using a Bio-Photometer (Nano Vue plus, USA).

Fragments of the nirK(514 bp) and nosZ (454 bp) genes were amplified using the primer pairs nirK1F/nirK5R for nirK gene [37] and nosZ-F/nosZ1622R for nosZ gene [38] in a final volume of 50 µl using KOD FX (TOYOBO) polymerase system. The PCR mixture was composed of 0.2 µM of each primer, 1×PCR buffer (20 mM Tris·HCl, pH 8.8, 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4), 250 µM dNTP, 2.5UKOD FX and 50–100 ng/µl template DNA. PCR was carried out as follows: 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C (nirK-F/nosZ1622R) or 56°C (nirK1F/nirK5R) for 40 s, and elongation at 72°C for 40 s. Cycling was completed by a final elongation step of 72°C for 10 min. The PCR products were examined on 1.2% (w/v) ethidium bromide-stained agarose gels.

Gene Library Construction, Sequencing, and Phylogenetic 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 Zero Cloning kit (TransGen) following the manufacturer’s instructions. The positive recombinants were screened on indicator plates with X-Gal, IPTG and ampicillin by color-based recombinant selection. The positive clones were further identified by sequencing using vector primers M13F/R by Shanghai Majorbio Company. Each clone library has at least 200 positive clones showing the right insert. All DNA sequences were checked online by NCBI BLAST and aligned using software package Clustal W. The diversity was determined by rarefaction analysis using PHYLIP (Version 3.69). Using DOTUR software, sequences of all nirK and nosZ gene fragments with similarities >97% were considered as one operational taxonomic unit (OTU) [39]. One representative clone was selected from each OTU for further phylogenetic analysis.
According to maximum identity and habitat, all of the OTUs' nearest neighbors were determined by BLAST analysis. All OTUs' representative sequences, their nearest neighbors and some reference sequences were imported in MEGA (Version 5) to construct unrooted phylogenetic tree using Neighbor-Joining method.

**Primer Design for Real-time qPCR**

For the preparation of real-time qPCR standards, all OTUs' representative sequences were aligned by software package ClustalW. The real-time qPCR primers were designed using software package Primer Premier 5. Two sets of primers for nirK gene were designed for OTU 1 and OTU 2 (Table 1). While, three sets of primers were designed for 5 OTUs of nosZ gene. The specificity of the primers was tested by PCR, agarose (1.2%) gel electrophoresis and UV transillumination after ethidium bromide staining.

**Standard Curve and Real-time qPCR Assay**

The plasmids containing nirK and nosZ gene fragments were used as standards, which were transformed from Trans1-T1 phage resistant chemically competent cell with specific primers in Table 1. The recombinant plasmids were inoculated into LB broth with ampicillin (100 mg liter\(^{-1}\)) and incubated at 37°C overnight. Plasmid DNA was then extracted using the Mini BEST Plasmid Purification Kit (Ver.3.0, Takara, Dalian) according to the manufacturer's instructions and the plasmid concentrations were determined by spectrophotometry using a BioPhotometer (Nano Vue plus, USA). Standards were prepared from plasmid serial dilutions containing between 10\(^{1}\) to 10\(^{9}\) copies calculated directly from the concentration of extracted plasmid.

The real-time qPCR was performed in 8-Strip Low Profile tubes (TLS-0851; MJ Research, Watertown MA) and covered with Ultra Clear caps (TCS-0803; MJ Research) on the Real Plex 4S (Eppendorf, Germany). The 25 μl reaction mixture contained SYBR green PCR Master Mix (SYBR Premix EX TaqTM kit, Takara, Dalian), 0.2 μM of primer, 12.5 μl of SYBR Premix EX TaqTM (TakaRa, Dalian) and 1 μl of template DNA (at 50–100 ng μl\(^{-1}\)). Thermal cycling conditions for nirK gene were as follows: an initial cycle of 95°C for 5 s, 56°C for 30 s, and 72°C for 30 s. The thermal cycling conditions for the nosZ primers were similar except for the annealing temperature at 57°C. Thermal cycling, fluorescent data collection and data analysis were carried out with monitor software detection system according to the manufacturer's instructions. The real-time qPCR assay of the nosZ and nirK genes were performed independently as described above for different OTU in triplicates (Table 1), the corresponding standard curve was given by specific plasmid. 1 μl of ddH\(_2\)O instead of template DNA was used as negative control.

**Nucleotide Sequence Accession Number**

All representative sequences were deposited in GenBank under the accession numbers: JQ823133 and JQ823134 for nirK genes, JQ823135, JQ823136, JQ823138, JQ823139 and JQ965748 for nosZ genes. The 28S rRNA and 18S rRNA genes of sponge samples were also deposited in GenBank under the accession numbers: KC762728, KC762706, KC762714, KC763778, KC762736, KC774024.

**Results**

**Phylogenetical Diversity of Bacterial nirK and nosZ Genes in Sponges**

Two nirK and nosZ gene clone libraries were successfully constructed. As a result, based on the 23 and 34 clones sequenced, 2 and 5 OTUs were obtained for nirK and nosZ genes, respectively, at 97% identity level (Table 2; Figure S1). The mean estimations of OTU richness using Chao estimators were 2 and 5.5 OTUs for nirK and nosZ genes, respectively. Meanwhile, the analysis in Table S1 also suggested the sequencing is nearly saturated, for example the coverage of nirK and nosZ reached 95.7%, 94.1%, respectively. All the information indicated that sequencing more clones could not greatly increase the actinobacterial diversity because of the conservatism of nirK and nosZ genes.

As shown in Table 2, OTU1 of nirK gene was predominated in the gene library with 22 sequences (95.6% of the clones sequenced) from 5 species of sponges except for X. testudinaria, while the OTU2 of nirK gene was found only one in sponge S. aequianthus, and particularly, nirK gene was not detected in X. testudinaria. For nosZ genes, A. queenslandica and S. aequianthus had OTU1 and OTU2. X. testudinaria had OTU1, 2 and OTU4, C. australiensis sp. had OTU2 and OTU5, whereas OTU3 was observed in C. australiensis. In particular, nosZ gene was not detected in I. crotonotus sp. The OTU2 of nosZ gene was dominated in the gene library with a highest ratio

**Table 1. Primer specificity and quantification of standard plasmids.**

| Target gene | Primer | Nucleotide sequence (5′–3′) | Reference | Annealing temperature C | Expected size (bp) | Quantification of standard plasmids copies/μl | OTU |
|-------------|--------|-----------------------------|-----------|-------------------------|-------------------|---------------------------------------------|-----|
| nirK        | nirK1f | GGMATGTTGCCCCTGGGCA         | [37]      | 55.5                    | 127               | 4.8E+10                                     | OTU1|
|             | nirK127r | CCTGCTCACCAGCATAATAGA       | This study |                        |                   |                                             |     |
|             | nirK1f | GGMATGTTGCCCCTGGGCA         | [37]      | 56                      | 208               | 3.5E+10                                     | OTU2|
|             | nirK208R | CCGAAACCCCATCTCTGCT         | This study |                        |                   |                                             |     |
| nosZ        | nosZ130F | CRATGGGTGGAAAACCAAGA        | [37]      | 55.2                    | 145               | 3.4E+10                                     | OTU1,2|
|             | nosZ2275S | ATGGACACCCCTTATTTCCG        | This study |                        |                   |                                             |     |
|             | nosZ-F | CGYGTTCMTCGACGCAGCAG       | [38]      | 57                      | 156               | 3.9E+10                                     | OTU3 |
|             | nosZ156R | GTTCGCGCCTCTGGTCTC          | This study |                        |                   |                                             |     |
|             | nosZ-F | CGYGTTCMTCGACGCAGCAG       | [38]      | 56                      | 201               | 2.8E+10                                     | OTU4,5|
|             | nosZ201R | GGWYACCGTCCCTGGAGAAT       | This study |                        |                   |                                             |     |

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of 67.6% and it was detected in four species of sponges, *A. queenslandica*, *S. vesparium*, *X. testudinaria* and *Cinachyrella* sp.

BLAST and phylogenetic analyses indicated that all the detected *nirK* and *nosZ* genes were probably of *Proteobacteria* origin (Fig. 1, 2). The *nirK* OTU1 (95.6% of the clones sequenced) was closely related to *nirK* of an uncultured bacterium with in *Alphaproteobacteria* group in an acidic nitrate and uranium contaminated aquifer in Oak Ridge of USA [40]. Meanwhile, *nirK* OTU1 was closely related to *nirK* of *Ochrobactrum anthropi* isolated from a Paddy Soil [41]. The *nirK* OTU2 contained only one sequence, which belonged to *Betaproteobacteria*. OTU2 was closely related to *nirK* of *Psillimonas* sp. T7–7 isolated from the Bohai Sea in China [42].

The *nosZ* OTU1 and OTU2 were grouped together with an uncultured bacterium in a bioreactor treating piggery waste [43]. All *nosZ* gene sequences from sponge *C. australiensis* formed an independent group of OTU3, which had 90% similarity with *Polyphorium gilvum* isolated from oil-polluted saline soil [44]. *NosZ* OTU4 was detected only one from *X. testudinaria*. All the above four *nosZ* OTUs were showed closest similarity with the nitrous oxide reductase of *Alphaproteobacteria* (Fig. 2). Only one sequence from sponge *Cinachyrella* sp. formed OTU5, which showed 96% similarity with the nitrous oxide reductase from an uncultured bacterium of *Gammaproteobacteria* group in permafrost affected peat soil in arctic tundra [45].

### Table 2. Distribution of clone numbers in *nirK* and *nosZ* gene libraries.

| Gene | Family          | Reference                     | Accession number | Similarity % | OTU | Sponge* | Total clone |
|------|-----------------|-------------------------------|------------------|--------------|-----|---------|-------------|
|      |                 |                               |                  |              |     |         |             |
| nirK | *Alphaproteobacteria* | Uncultured bacterium from contaminated aquifer | ABP33197         | 100          | OTU1 | 1 2 3 4 5 6 | 22          |
|      | *Betalproteobacteria* | *Psillimonas* sp. T7–7 from Bohai Sea | YP004418446     | 88           | OTU2 | / 1 / / / / | 1           |
| *nosZ* | *Alphaproteobacteria* | Uncultured bacterium from bioreactor treating piggery waste | ACH69029         | 99           | OTU1 | / 1 / 1 / / | 2           |
|      | *Alphaproteobacteria* | Uncultured bacterium from coastal marine sediment to Arable Land | ACJ002259       | 81           | OTU3 | / / / 7 / 7 | 7           |
|      | *Gammaproteobacteria* | Uncultured bacterium from mesotidal sediment in France: Arcachon Bay | CBI71158         | 88           | OTU4 | / / / 1 / / | 1           |
|      |                  | Uncultured bacterium from permafrost affected peat soil in arctic tundra | CCA94823         | 96           | OTU5 | / / / / / / | 1           |

Note: *Sponge: 1 is *A. queenslandica* from the Linshui port; 2 is *S. vesparium* from the Linshui port; 3 is *Iotrochota* sp. from Yongxing Island; 4 is *X. testudinaria* from Yongxing Island; 5 is *Iotrochota* sp. from Yongxing Island; 6 is *Cinachyrella* sp. from Yongxing Island. The BLAST analysis was based on amino acid sequences. doi:10.1371/journal.pone.0065142.t002

#### Discussion

The Phylogenetic Diversity and Abundance of *nirK* Gene and *nosZ* Genes in Sponges

Phylogenetic analysis revealed that most of the *nirK* genes were probably of *Alphaproteobacteria* origin. Only one sequence belonged to *Betaproteobacteria* (Fig. 1). These results agree with Heylen et al. who indicated that *nirK* genes were prevalent in *Alphaproteobacteria* [46]. Unlike *nirK* genes, *nosZ* genes were distinctively distributed in different sponges. According to this study, none sponges have sequences in all *nosZ* OTUs and none *nosZ* OTU exists in all sponges. Some sponges only have unique OTU, such as *C. australiensis* has sequences belonging to OTU3 and this OTU does not appearing in other sponges.

Real-time qPCR, is not limited by the cultivability of bacteria and has been successfully applied for the quantification and identification of enteroviruses from surface waters and sponge
tissue from the Florida Keys [47]. It was also applied to quantify the relative abundance of unculturable bacterial members from a marine sponge *Vetulina* [48], and to assess *Pseudoalteromonas* species in other marine samples [49]. The abundance of denitrifying bacteria was assessed by quantifying functional genes such as *nirK* and *nosZ* genes from grassland [50,51], agricultural soil [24,52], forest [53], glacier foreland [54], riparian [55], arctic soils [45], ocean shore transect [56], and seawater [57]. Using real-time

**Figure 1. Phylogenetic tree based on amino acid sequence (171 aa) translated from partial gene fragment of *nirK*.** The tree is reconstructed using the neighbor-joining method and bootstrap analysis is carried out with 1,000 replicates. Bootstrap values <50% are hidden. The scale bar represents 0.1 AA substitutions per site. The number in parentheses shows the number of sequences in each OTU. ● means sequences obtained in this study.
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**Figure 2. Phylogenetic tree based on amino acid sequence (151 aa) translated from partial gene fragment of *nosZ*.** The tree is reconstructed using the neighbor-joining method and bootstrap analysis is carried out with 1,000 replicates. Bootstrap values <50% are hidden. The scale bar represents 0.1 AA substitutions per site. The number in parentheses shows the number of sequences in each OTU. ● means sequences obtained in this study.
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qPCR, among the tested six sponges from two different sea areas, each sponge was found to possess specific abundance of \( \text{nirK} \) and \( \text{nosZ} \) genes.

### Table 3. Quantification of \( \text{nirK} \) and \( \text{nosZ} \) genes by qRT-PCR.

| Sponge           | OTU1 of \( \text{nirK} \) gene copies/µg | OTU2 of \( \text{nirK} \) gene copies/µg | OTU1,2 of \( \text{nosZ} \) gene copies/µg | OTU3 of \( \text{nosZ} \) gene copies/µg | OTU4,5 of \( \text{nosZ} \) gene copies/µg |
|------------------|------------------------------------------|------------------------------------------|-------------------------------------------|------------------------------------------|-------------------------------------------|
| \( \text{A. queenslandica} \) | 1630.2                                   | /                                        | 27.8                                     | /                                        | /                                        |
| \( \text{S. vesparium} \)       | 1246.1                                   | 278.4                                   | 2.71E+05                                 | /                                        | /                                        |
| \( \text{Iotrochota sp.} \)     | 1.12E+04                                 | /                                        | /                                        | /                                        | /                                        |
| \( \text{K. testudinaria} \)    | /                                        | /                                        | 393.1                                    | /                                        | 610                                       |
| \( \text{C. australiensis} \)   | 5.35E+04                                 | /                                        | /                                        | 1.44E+04                                 | /                                        |
| \( \text{Cinachyrella sp.} \)   | 6.21E+04                                 | /                                        | 1.15E+04                                 | /                                        | 1584.1                                   |

Note: ‘/’ means no gene copies were detected. The data were gene copies/µg sponge tissue.

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The Potential of Sponge Bacterial Symbionts in Marine Nitrogen Balance

In 1979, the potential to fix nitrogen by symbiotic cyanobacteria was demonstrated in Red Sea sponges with the acetylene reduction test [58]. Wilkinson et al. demonstrated nitrogen fixation in the Indo-Pacific coral reef sponge \( \text{Callyspongia maricata} \) by the incorporation of \( ^{15}\text{N}_2 \) into the amino acids of glutamine, glutamate.

![Figure 3. The \( \text{nirK} \) and \( \text{nosZ} \) gene copies in sponges.](https://example.com/figure3.png)

Sponge names are shown in abscissa axis ordinate shows gene copies treated with log\(_{10}\) for per microgramme sponge. The \( \text{nirK} \) gene (OTU1 and OTU2 together) is shown in pink column, while \( \text{nosZ} \) gene (OTU1, OTU2, OTU3, OTU4 and OTU5 together) is shown in green column.

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and aspartate [59,60]. In 2008, Mohamed et al. proved the nitrogen fixation by sponge bacterial symbionts based on the expression analysis of nitrogenase gene nifH [28]. Therefore, nitrogen fixation by sponge symbionts is possibly an important source of new nitrogen to the reef environment.

Denitrification, as a reverse process of nitrogen fixation and nitrification plays an important role in the balance of marine nitrogen. According to Schlappert et al. [17], sponges can change the internal environment such as hypoxia/anoxia or normoxia by water pumping. When sponges stop pumping, their tissues become hypoxic/anoxic and create the microenvironment for denitrification. In general, in the surface water of the oceans, nitrate concentration is very low and nitrite probably comes from the nitrification e.g. ammonia oxidation [61,62]. The surface waters of the oceans are slightly supersaturated with N₂O [63], because some nitrite is reduced to N₂O as the final product. Nitrite and N₂O in sponge body might come from the nitrification and denitrification of sponge microbial symbionts. Inside the oxygen minimum zone in sponge body, nitrite could be reduced to NO and N₂O and further to N₂ by denitrifying bacteria with nirK or nosZ genes, preventing nitrite and N₂O from accumulating in sponge body. Based on this study, A. queenslandica, S. vesparium, C. australiensis, Cinachyrella sp. and X. testudinaria could reduce the greenhouse gas N₂O by denitrifying bacteria with nosZ genes, which is accompanied by the release of the final product N₂. The results from this study, together with that from Hofmann et al. [5], Schlappert et al. [17] and Fan et al. [36], indicate the bacterial symbionts of some sponges have similar potential in N₂ production by denitrification. On the other hand, Fan et al. found the denitrification process was not complete for some sponges because nosZ gene was lack [36]. Similarly, in this study, nosZ was not detected in sponge Lotrochota sp. So, if there are alternative pathways of nitrous oxide reduction needs further investigation. In addition, this study also suggests the denitrification potential of bacteria varies among sponges because of the different phylogenetic diversity and relative abundance of nosZ and nirK genes. Ribes et al. [64] suggested that unique metabolic pathways were mediated in each sponge species by a different, and host specific, microbial community. The observed different diversity and abundance of nosZ and nirK genes in this study might come from the different sponge-specific microbes. However, because we did not compare the nosZ and nirK genes among different samples of one sponge species, so the difference of nosZ and nirK genes among different individuals of the same sponge species could not be revealed.

Phylogenetic diversity and quantification of denitrification genes are important for a better understanding of denitrifying activity of sponge microbial symbionts in the marine environment. Based on the the qualitative and quantitative analyses of two key functional genes in the complete denitrification process, nosZ and nirK genes, the different potential of sponge bacterial symbionts in nitrogen gas release was suggested. To our knowledge, this is the first qRT-PCR approach enabling a rapid quantification of functional genes of the denitrifiers in sponges. The limitation of this study lied in the DNA-based approach, only the potential of sponge bacterial symbionts was suggested. The in-depth investigation, at RNA level or combined with nitrogen gas detection, will give more details about the ecological roles of sponge bacterial symbionts in nitrogen gas release.

Supporting Information

Figure S1 Rarefaction curves for nirK and nosZ gene sequences. OTUs are defined at distances of 0.03.
(DOC)

Figure S2 Quantification of nirK and nosZ genes by Real time qPCR. Standard curve was constructed with plasmid containing nirK or nosZ gene sequence for respective OTUs. A: Quantification PCR for OTU1 of nirK gene, 5 species of sponges were measured. R² = 0.999 and E = 0.98; B: Quantification PCR for OTU2 of nirK gene, 1 species of sponge was measured. R² = 0.999 and E = 0.99; C: Quantification PCR for OTU1 and OTU2 of nosZ gene, 4 species of sponges were measured. R² = 0.998 and E = 1.07; D: Quantification PCR for OTU3 of nosZ gene, 1 species of sponge was measured. R² = 0.999 and E = 1.03; E: Quantification PCR for OTU4 and OTU5 of nosZ gene, 2 species of sponges were measured. R² = 0.998 and E = 1.00.
(DOC)

Table S1 Phylogenetic information of nirK and nosZ genes libraries.
(DOC)

Table S2 Real time qPCR. A: Real time qPCR of nirK gene for OTU1; B: Real time qPCR of nirK gene for OTU2; C: Real time qPCR of nosZ gene for OTU1 and OTU2; D: Real time qPCR of nosZ gene for OTU3; E: Real time qPCR of nirK gene for OTU4 and OTU5.
(DOC)

Author Contributions

Conceived and designed the experiments: XZ ZL. Performed the experiments: XZ LH. Analyzed the data: XZ ZL. Contributed reagents/materials/analysis tools: WS FZ. Wrote the paper: XZ ZL.
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