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Cytochrome *cd1*-Containing Nitrite Reductase Encoding Gene *nirS* as a New Functional Biomarker for Detection of Anaerobic Ammonium Oxidizing (Anammox) Bacteria

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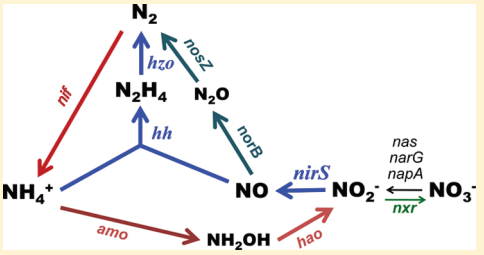
Supporting Information

ABSTRACT: A newly designed primer set (AnnirS), together with a previously published primer set (ScnirS), was used to detect anammox bacterial *nirS* genes from sediments collected from three marine environments. Phylogenetic analysis demonstrated that all retrieved sequences were clearly different from typical denitrifiers' *nirS*, but do group together with the known anammox bacterial *nirS*. Sequences targeted by ScnirS are closely related to *Scalindua nirS* genes recovered from the Peruvian oxygen minimum zone (OMZ), whereas sequences targeted by AnnirS are more closely affiliated with the *nirS* of *Candidatus* 'Kuenenia stuttgartiensis' and even form a new phylogenetic *nirS* clade, which might be related to other genera of the anammox bacteria. Analysis demonstrated that retrieved sequences had higher sequence identities (>60%) with known anammox bacterial *nirS* genes than with denitrifiers' *nirS*, on both nucleotide and amino acid levels. Compared to the 16S rRNA and hydrazine oxidoreductase (*hzo*) genes, the anammox bacterial *nirS* not only showed consistent phylogenetic relationships but also demonstrated more reliable quantification of anammox bacteria because of the single copy of the *nirS* gene in the anammox bacterial genome and the specificity of PCR primers for different genera of anammox bacteria, thus providing a suitable functional biomarker for investigation of anammox bacteria.



INTRODUCTION

Anaerobic ammonium oxidation (anammox) was first described in wastewater treatment plant bioreactors.[1,2] Later studies revealed that the anammox process is likely responsible for 30−70% of global oceanic N_2 production,[3] and thus plays an important role in global nitrogen cycling. To date, anammox has been recorded in various natural ecosystems, including marine,[4−14] freshwater,[15−18] and terrestrial.[15] Most studies of anammox bacterial phylogeny have been based on their 16S rRNA gene sequences. However, the high divergence (<87.1% similarity) among different genera of anammox bacteria makes this approach difficult for detection of anammox bacteria when they are only present at low concentrations.[16] As a result, many specific PCR primers of anammox bacterial 16S rRNA genes have been applied to improve the efficiency of anammox bacterial detection.[17−19] However, one of the main limitations in using the 16S rRNA gene as a molecular marker is that it is not necessarily related to the physiology of the bacteria.[20] Functional biomarkers, such as the genes encoding key enzymes involved in the anammox biochemical metabolism, such as the nitrite reductase (NIR), hydrazine hydrolase (HH), and hydrazine oxidoreductase (HZO), may be better alternatives for the study of anammox bacterial communities.[16,21,22] The first confirmed functional gene biomarker, HZO encoding genes (*hzo*), has been suggested as an efficient tool for investigating these bacteria.[18,23−26] Schmid et al.[25] detected the *hzo* genes in various ecosystems and suggested that *hzo* gene sequences in cluster 1 were most appropriate and feasible for phylogenetic analysis, because they appeared to be functional and present in all anammox bacteria studied. Several studies have successfully used *hzo* genes as functional biomarkers to describe anammox diversity from various environmental samples.[18,27−31] Previous work from our group[18] and Hirsch et al.[30] designed new PCR primers to detect *hzo* genes from various marine sediment samples, and demonstrated that anammox bacterial *hzo* genes are widely distributed in marine ecosystems, and could be used to identify the diversity of anammox bacteria at a high resolution. However, a survey of the genome of the anammox bacteria, *Candidatus* 'Kuenenia stuttgartiensis,' has confirmed the occurrence of the cytochrome cd_1-containing nitrite reductase encoding gene (*nirS*) with one copy number.[22] This gene is proposed to reduce nitrite to nitric oxide in the initial step of the anammox process.[22] Recently, Lam et al.[32] have proposed that *nirS* in the anammox

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Table 1. Environmental Characteristics of Sediment Samples

sites	pH	$NO_2^-+NO_3^-$ (μM)	NH_4^+ (μM)	organic matter (%)	salinity (‰)
aquaculture	7.85 ± 0.02	10.4 ± 0.5	220.9 ± 5.2	10.6 ± 0.5	30.61 ± 0.01
Mai Po	7.52 ± 0.02	7.7 ± 0.3	120.2 ± 3.6	10.1 ± 0.4	25.72 ± 0.01
SCS	7.50 ± 0.02	5.9 ± 0.2	47.1 ± 1.2	9.29 ± 0.4	34.58 ± 0.02

bacteria, *Scalindua*, would be an effective functional gene for anammox bacteria in environmental samples. However, research on anammox bacterial *nirS* is quite limited, and more studies are needed to further confirm the application of this new functional biomarker in natural and engineered systems.

In the present study, we extend and confirm the anammox bacterial *nirS* gene as a functional biomarker for investigating the phylogenetic diversity, abundance, and spatial distribution of different genera of anammox bacteria in natural ecosystems. Our results demonstrate that anammox bacterial *nirS* genes could be reliable and effective functional biomarkers; they are well suited for quantitative analysis, and thus provide new tools for studying the diversity and distribution of these bacteria.

MATERIALS AND METHODS

Sample Collection and DNA Extraction. Surface sediments (1−2 cm) were collected from three marine environments representing a gradient of anthropogenic impacts.[18] The sites include a long-term marine aquaculture zone, the coastal wetland of the Mai Po Nature Reserve in Hong Kong, and the deep ocean of the South China Sea (SCS).[18] The concentrations of NH_4^+ and NO_x (NO_3^- + NO_2^-) in pore water of sediment samples were measured with an autoanalyzer (QuickChem, Milwaukee, WI) according to standard methods of the American Public Health Association,[33] and the pH of the sediment samples was measured in situ using an IQ180G Bluetooth Multi-Parameter System (Hach Company, Loveland, CO). The organic matter content of each sediment sample was measured by the loss on ignition, according to the method reported by Heiri et al.[34] Selected characteristics of these sediment samples are shown in Table 1. Further details of these sediment sampling sites and DNA extraction protocols for each sediment sample were described in our previous study, where all samples tested positive for the presence of anammox bacterial 16S rRNA and *hzo* genes after PCR amplification.[18]

PCR Amplification. Two primer sets were used to target *nirS* genes of anammox bacteria from three different marine sediments. The first primer set Scnir372F-Scnir845R (ScnirS) was used to detect *nirS* genes from the genus *Scalindua*, and PCR amplification protocols have been described previously.[32] The second primer set AnnirS379F (5′-TCTATCGTTGCATCGCATTT-3′) and AnnirS821R (5′- GGATGGGTCTTGATAAACA-3′) (AnnirS) was designed according to the sequence alignments of the *nirS* genes from *Candidatus* 'Kuenenia stuttgartiensis' and *Scalindua* bacteria isolated from the Peruvian OMZ and from the SCS, and typical denitrifying bacteria. The new primer set (AnnirS) was used to target *nirS* genes from other genera of anammox bacteria. The expected fragment targeted by this new primer set is about 442 bp, which is overlapped by the fragment (473 bp) targeted by the ScnirS primer set. In a final volume of 50 μL, the PCR reaction mixture contained: 1 μL DNA (30−50 ng mL^{-1}), 5 μL 10 × GoTaq Flexi buffer (Promega, Hong Kong), 2 μL $MgCl_2$ (25 mM, Promega), 1.5 μL of dNTPs (10 mM, Invitrogen, Hong Kong), 1 μL of each forward and reverse primer (20 μM), and 0.25 μL of GoTaq Flexi polymerase (5U μL^{-1}, Promega, Hong Kong). The PCR program was 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 51 °C for 45 s, and 72 °C for 60 s; and finally 72 °C for 10 min. PCR products were checked by electrophoresis on 1% agarose gels and subsequent staining with 0.5 mg mL^{-1}gel red (Figure S-1 of the Supporting Information).

Cloning, Sequencing, and Phylogenetic Analysis. PCR products from the two primer sets for each sediment sample (triplicate for each site) were purified and cloned into the T-vector (Takara, Japan). The appropriate-sized DNA fragment insertion was determined by PCR amplification with the primer set M13F and M13R. Different number clones in each library were randomly selected for sequencing with the Big Dye Terminator kit (Applied Sciences, Foster City, CA) and an ABI Prism 3730 DNA analyzer. DNA sequences were initially examined and edited by *MEGA 4.0* software.[35] Nucleic acid sequences were then translated into amino acids and the resulting sequences were aligned with related sequences in databases using the *ClustalW* program.[36] Phylogenetic trees were constructed using *MEGA 4.0* with the neighbor-joining and maximum parsimony methods, with the 1000 replicate bootstrap resampling method to estimate the confidence intervals of the tree nodes.

Quantitative PCR Assay. The abundance of anammox bacterial *nirS* genes in the three marine sediment samples was determined in triplicate using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Quantification was based on the fluorescent dye SYBR-Green I. Each reaction was performed in a 25 μL volume containing 1 μL of DNA template, 0.5 μL of each primer (ScnirS and AnnirS), and 12.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). PCR conditions were the same as above. Standard plasmid carrying *nirS* gene fragments were generated by amplification from DNA extracted from the Mai Po site sediments, and cloning into the pMD-18 T-Vector (Takara, Japan). The plasmid DNA concentrations were then determined and the copy numbers of target genes were calculated. A 10-fold serial dilution of a known copy number of the plasmid DNA was subjected to a quantitative-PCR assay in triplicate to generate an external standard curve. The quantitative PCR amplification efficiencies ranged from 0.92−0.97 and 0.93−0.98 for ScnirS and AnnirS, respectively; whereas the correlation coefficients (R^2) of the standard curve were greater than 0.99.

Statistical Analysis. Sequences were analyzed by the *DOTUR* (distance-based OTU and richness) program to compare their diversity and richness.[37] Operational taxonomic units (OTUs) for community analysis were defined by a 5% variation in amino acid sequence.[37] Diversity indices, including Chaol, Shannon, and Simpson, were also generated by DOTUR for each clone library. The Coverage index (C) is calculated from the equation $[C = 1 - (n_i/N)]$, in which n_i is the number of clones from the clone library that occurred only once, and N is the total number in the clone library.[38] The geographic distribution of phylogenetic structures for anammox

Table 2. Comparison of Diversity Characteristics of Anammox Bacteria in Each Sampling Site Described by Cytochrome cd1-Containing Nitrite Reductase Encoding Genes[a]

primer sets	sample	clone number	OTUs	Shannon	Simpson	Chaol	coverage
	SCS	33	15	2.27	0.14	24	0.73
	Mai Po	27	5	0.62	0.72	11	0.85
ScnirS	aquaculture	0	0	0	1	0	0
	SCS	0	0	0	1	0	0
	Mai Po	35	4	0.26	0.89	4	0.94
AnnirS	aquaculture	40	7	1.35	0.32	8.5	0.93

[a] The OUT was defined by the *DOTUR* program with 5% amino acid sequence variation, and diversity indices were also generated by the *DOTUR* program.

bacterial *nirS* genes in the three marine environments was analyzed by the online software UniFrac (http://bmf2.colorado.edu/unifrac/index.psp) using the principal coordinates analysis (PCoA) and Jackknife environmental clusters.[39] Sequence similarities, including nucleic acid and amino acid sequences, between anammox bacterial *nirS* genes detected in the present study and other reported *nirS* genes of anammox and typical denitrifying bacteria in the database were compared using the *DNAMAN* program (Lynnon BioSoft, Lynnon Corporation; http://www.lynnon.com).

Nucleotide Sequence Accession Numbers. The GenBank accession numbers for the *Sc-nirS* and *An-nirS* gene sequences reported here are HQ265300 to HQ265345 and HQ265346 to HQ265376, respectively.

RESULTS

Detection of Anammox Bacterial *nirS* Genes from Three Marine Sediments Using Two Primer Sets. To detect the *nirS* genes in different genera of anammox bacteria from marine sediment samples, the primer set (ScnirS) was used to detect the *Scalindua nirS* gene, whereas the new primer set (AnnirS) was selected for other genera of anammox bacteria. Anammox bacterial putative *nirS* clone libraries were initially constructed for the three marine sediments using these two primer sets. The details of the results, including the selected clone numbers, operational taxonomic units (OTUs, defined by 5% amino acid cutoff), Shannon, Simpson, Chaol and Coverage indices of the four clone libraries are shown in Table 2. Results indicated that all anammox bacterial *nirS* genes from the SCS deep-ocean sediments were recovered with the ScnirS primer set, and sequences retrieved from aquaculture zone sediments were targeted by the AnnirS primer set. In the Mai Po Nature Reserve sediments, anammox bacterial *nirS* gene sequences could be detected by both primer sets, indicating a more complex community structure. Although rarefaction analysis curves might suggest that more OTUs could be detected if more clones were selected from the SCS samples (Figure 1), the coverage index indicates that sequenced clone numbers have covered most of the anammox bacterial *nirS* gene OTUs in the clone libraries constructed by the selected PCR primers (>0.85).

Phylogenetic and Homogeny Comparison of Anammox Bacterial *nirS* Genes from Three Marine Environmental Sediments. Phylogenetic analysis demonstrated that all anammox bacterial *nirS* gene sequences retrieved in the present

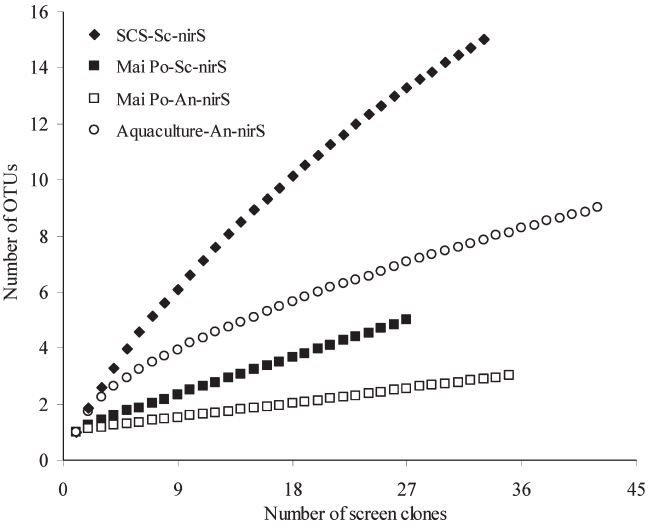

Figure 1. Rarefaction analysis of potential anammox bacterial *nirS* targeted by the respective primer sets (ScnirS and AnnirS) from three marine environmental sediments. The *DOTUR* program was used with 5% amino acid sequence variation. aquaculture: Hong Kong Deep Bay aquaculture sediment; Mai Po: Hong Kong Mai Po Nature Reserve coastal sediment; SCS: South China Sea sediment.

study were fairly diverse, but all clustered with the *nirS* gene present in the *Candidatus* 'Kuenenia stuttgartiensis' genome, the *Scalindua nirS* gene clones recovered from Peruvian OMZ, and the two sequences obtained from the Arabian Sea. However, they are clearly different from the typical denitrifiers' *nirS* gene sequences, forming a relative new anammox bacterial *nirS* gene clade where three major clusters are observed (Figure 2). The first clade, *Sc-nirS*, is composed of *Scalindua nirS* sequences from the present study and from the Peruvian OMZ, targeted by the same primer set. The four subclusters include: the *Sc-nirS* Peruvian OMZ cluster; the *Sc-nirS* cluster 1, which includes parts of the SCS deep-ocean *Scalindua nirS*-like sequences; and the *Sc-nirS* clusters 2 and 3, composed of sequences retrieved from the SCS deep-ocean and the Mai Po Nature Reserve. The other two major anammox bacterial *nirS* gene clades, *An-nirS* clade and *Kuenenia nirS* clade, are composed of sequences detected mostly by the new primer set, AnnirS. The *An-nirS* clade forms a new phylogenetic group in the phylogenetic tree, with the *An-nirS* cluster 1 containing sequences recovered from Mai Po Nature Reserve and the aquaculture zone, whereas the *An-nirS* clusters 2 and 3 are composed of sequences from the aquaculture zone. The *Kuenenia nirS* clade contains the *nirS* in the genome of *Candidatus* 'Kuenenia stuttgartiensis,' and one sequence recovered from the aquaculture zone by the AnnirS primer set (Figure 2). To further confirm these phylogenetic group relationships between and within each clade, sequence similarities based on nucleotide and amino acid levels were calculated using the sequences obtained in this study, together with reference sequences from previous studies (Table S-1 of the Supporting Information). Except for one detected sequence, the *Kuenenia nirS* clade is closely related to the *nirS* in the genome of *Candidatus* 'Kuenenia stuttgartiensis' (sharing 60.7% and 60.8%) identities for nucleotides and amino acids, respectively. All targeted anammox bacterial *nirS* gene sequences in the present study share up to 60% sequence identities with *Scalindua nirS* sequences recovered from the Peruvian OMZ, but less than

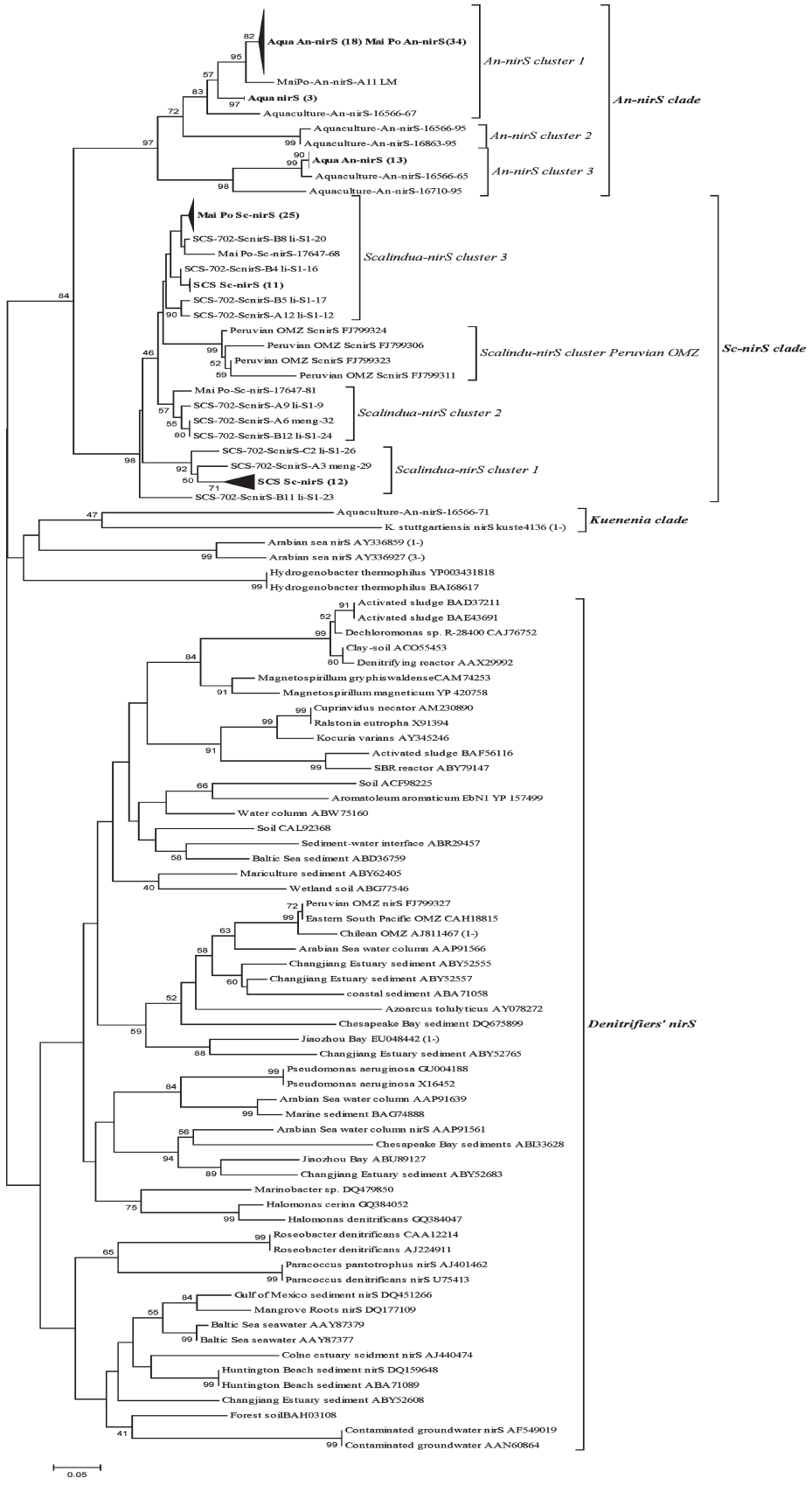

Figure 2. Consensus phylogenetic tree constructed after subjecting an alignment of deduced experimental and retrieved nirS sequences to neighbor-joining analysis. Numbers in parentheses refer to how many *nirS* gene clones retrieved using the respective primer sets (ScnirS and AnnirS) were assigned to an individual sequence target type. The numbers at the nodes are percentages that indicate the levels of bootstrap support based on 1000 resampled data sets (only values greater than 50% are shown). Branch lengths correspond to sequence differences as indicated by the scale bar. Other information is identical to Figure 1.

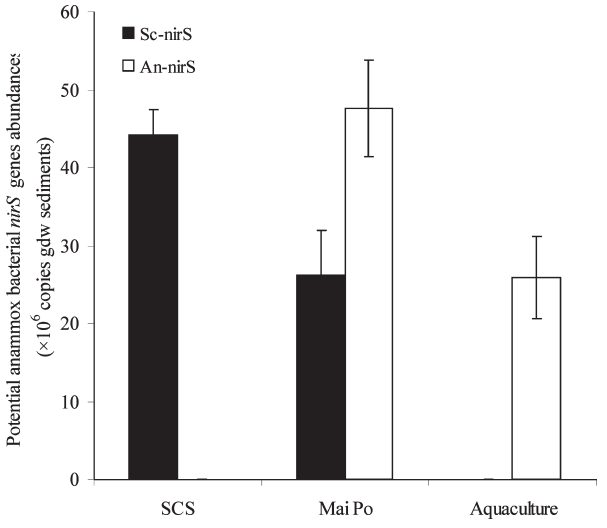

Figure 3. Abundances of potential anammox bacterial *nirS* targeted by the respective primer sets (ScnirS and AnnirS) from three marine environmental sediments. Other information is identical to Figure 1.

60% identity with the typical denitrifiers' *nirS*, in both nucleotide and amino acids.

Abundance and Community Structure of Anammox Bacteria in Three Marine Sediments Estimated by *nirS* Genes. The abundance of anammox bacteria in the three marine environments was also estimated by presence of the anammox bacterial *nirS* gene with the primer sets described above. The anammox bacterial *nirS* gene abundance estimated by primer set ScnirS ranged from below detection in the aquaculture zone to $44.1(\pm 3.3) \times 10^6$ copies per gram of sediments (dry weight) in the SCS deep-ocean. Using the primer set AnnirS, gene abundance ranged from below detection in the SCS deep-ocean to $47.6(\pm 6.2) \times 10^6$ copies per gram of sediments (dry weight) in the Mai Po Nature Reserve. Mai Po Nature Reserve sediments contained the highest anammox bacterial *nirS* gene copy numbers $73.7(\pm 12.1) \times 10^6$ copies (ScnirS + AnnirS) per gram of sediments (dry weight), followed by the SCS deep-ocean and the marine aquaculture zone (Figure 3). To analyze the community structure of anammox bacteria in the three marine environments, all retrieved anammox bacterial *nirS* gene sequences (amino acid sequences) by the two PCR primer sets were analyzed by Unifrac principal coordinates analysis (PCoA) and Jackknife environment clusters analysis.[39] Both PCoA (Figure S-2 of the Supporting Information) and Jackknife environment clusters analysis (data not shown here) classified these anammox bacterial *nirS* gene sequences into three different groups corresponding to the three marine environments, reflecting a site-specific, distinct anammox bacterial community structure within each marine ecosystem, where the concentrations of NH_4^+ and NO_x ($NO_2^- + NO_3^-$) showed a strong gradient (Table 1).

DISCUSSION

NirS, responsible for nitrite reduction to nitric oxide in the denitrification process, has been used as one of the most important functional biomarkers to investigate denitrifying communities in various environments.[40] In anammox bacteria, NirS encoding genes have been confirmed in the genomes of *Candidatus* 'Kuenenia stuttgartiensis'[22] and *Candidatus* 'Scalindua sp. T23',[32,41] which are believed to be responsible for the initial nitrite reduction to nitric oxide in the anammox process.[22] Lam et al. [32] found that *Scalindua nirS* gene abundance significantly correlated with that of 16S rRNA genes, and was strongly expressed in the upper part of the Peruvian OMZ, where anammox rates were high. Furthermore, these expressed *Scalindua nirS* genes were fairly diverse, but all clustered with the *nirS* gene present in the *Candidatus* 'Scalindua sp. T23' and in 2 sequences obtained from the Arabian Sea; however, they were clearly different from the typical denitrifiers' *nirS* genes.[32] On the other hand, most of the reported anammox bacteria in marine ecosystems are all closely related to the *Candidatus* Scalindua genus.[42−44] Thus, *Scalindua nirS* is proposed as a new functional gene biomarker for anammox bacteria in environmental samples.[20,32] In the present study, we used both the *Scalindua nirS* targeted primer ScnirS, and a newly designed primer set AnnirS to target anammox bacterial *nirS* from three different marine environments that represent a gradient of known anthropogenic impacts (Table 1). From the phylogenetic analysis, all sequences in the present study group together with the *Scalindua nirS* gene clones recovered from the Peruvian OMZ, the *nirS* gene in *Candidatus* 'Kuenenia stuttgartiensis,' and two sequences obtained from Arabian Sea. These sequences are clearly different from the typical denitrifiers' *nirS* gene sequences. Furthermore, the higher nucleotide and amino acid sequence identities of the targeted *nirS* genes are consistent with anammox bacterial *nirS* rather than with the typical denitrifiers' *nirS*, further indicating that all sequences obtained in the present study are closely related to anammox bacterial *nirS* genes. Interestingly, the retrieved sequences also show primer set specificity, with the sequences obtained by the ScnirS primer closely related to the *Scalindua nirS* gene clones recovered from Peruvian OMZ, whereas the sequences recovered by the new primer set AnnirS associated with both the *nirS* gene in *Candidatus* 'Kuenenia stuttgartiensis' and with a new anammox bacterial *nirS* gene clade, including *An-nirS* clusters 1, 2, and 3. Thus, compared with previous studies, a much higher diversity of anammox bacterial *nirS* genes can be detected, including *nirS* genes of *Scalindua*, *Kuenenia* and a new anammox bacterial *nirS* gene group which might be related to other genera of anammox bacteria, such as *Brocadia*, *Jettenia*, and *Anammoxoglobus*. Because of the limited anammox *nirS* gene sequences in the database, the PCR primers designed in this study might not be the ideal primers for all *nirS* genes in other genera of anammox bacteria; however, more sequences obtained by the new primers in the present study will be useful for determining future PCR primers for anammox bacterial *nirS* gene sequences.

To further confirm the anammox bacterial *nirS* gene as a suitable functional phylogenetic biomarker for anammox bacteria, the phylogeny of anammox bacterial *nirS* was compared with that of 16S rRNA and *hzo* genes from our previous study,[18] where the same sediment samples were used. The results show that the anammox bacterial *nirS* phylogeny is consistent with that of 16S rRNA and *hzo* genes. The available anammox bacterial 16S rRNA gene targeting primers usually result in a large number of nonplanctomycete sequences, as anammox bacterial 16S rRNA gene sequences are not well conserved. Furthermore, a recent study also showed that the quantification of anammox bacterial 16S rRNA genes underestimates the anammox abundance in marine sediment samples.[45] In addition, different *hzo* gene copy numbers are present in different anammox bacterial genera.[22,25] These factors make it difficult to quantify the exact anammox bacterial abundance in the environment using 16S

rRNA and *hzo* genes. In contrast, the anammox bacterial *nirS* gene sequence is specifically targeted by the PCR primer sets, and only one copy of the *nirS* gene has been confirmed in different genera of anammox bacteria. The anammox bacterial *nirS* gene would therefore appear to be a better functional biomarker for anammox bacteria. Our results have demonstrated that anammox bacteria in the genus of *Scalindua* are the most abundant group in the SCS deep-ocean sediment, whereas the Mai Po Nature Reserve sediments have the highest abundance of total anammox bacteria, including *Scalindua, Kuenenia* and other genera of anammox bacteria. Furthermore, the aquaculture sediment contained *Kuenenia* and other genera of anammox bacteria, indicating that anthropogenic impacts may have a strong influence on anammox bacterial diversity (Figure 3). In addition to the present study, several previous studies have reported that anthropogenic or terrestrial inputs can contribute significantly to the uniqueness of anammox bacterial assemblages in coastal environments, such as estuaries and internal bays.[31, 46−49]

PCoA and Jackknife environmental clusters classified the anammox bacterial *nirS* gene sequences into three different groups, consistent with our previous results, and showed a similar distribution pattern to the 16S rRNA and *hzo* gene analysis in our earlier study.[18] In the SCS deep-ocean sediments, the predominant anammox bacteria are the genus *Scalindua*, thus only sequences affiliated with *Scalindua nirS* could be detected, whereas the aquaculture zone is dominated by the new anammox bacterial *nirS* group, which might be related to other genera of anammox bacteria. The anammox community composition in Mai Po Nature Reserve is the most complex, not only containing diverse *Scalindua nirS* genes but also the new anammox bacterial *nirS* groups. The site-specific anammox bacterial *nirS* gene community structure within each marine ecosystem supports our previous hypothesis[18,49] that anammox bacterial community structure in marine environments might be shaped by human impacts on the environment.

In conclusion, the present study shows that the anammox bacterial *nirS* gene can be used as a new functional biomarker to investigate phylogenetic diversity, distribution, and abundance of anammox bacteria in marine sediments. The phylogeny of the anammox bacterial *nirS* gene is consistent with that of the 16S rRNA and *hzo* genes, but the *nirS* genes are more suitable for quantitative analysis of anammox bacteria. In addition, different genera of anammox bacterial *nirS* genes can be detected by specific PCR primer sets, allowing anammox bacterial community dynamics to be resolved at a higher resolution than previously possible. Thus, use of the anammox bacterial *nirS* gene as a new functional biomarker provides us with a new tool to study anammox bacteria in different marine ecosystems.

■ ASSOCIATED CONTENT

Supporting Information. Figures of PCR products, tables of nucleotide sequences, and other figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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