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Diversity, abundance, and distribution of NO-forming nitrite reductase–encoding genes in deep-sea subsurface sediments of the South China Sea

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ABSTRACT

In marine ecosystems, both nitrite-reducing bacteria and anaerobic ammonium-oxidizing (anammox) bacteria, containing different types of NO-forming nitrite reductase–encoding genes, contribute to the nitrogen cycle. The objectives of study were to reveal the diversity, abundance, and distribution of NO-forming nitrite reductase–encoding genes in deep-sea subsurface environments. Results showed that higher diversity and abundance of *nirS* gene than *nirK* and *Scalindua-nirS* genes were evident in the sediments of the South China Sea (SCS), indicating bacteria containing *nirS* gene dominated the NO-forming nitrite-reducing microbial community in this ecosystem. Similar diversity and abundance distribution patterns of both *nirS* and *Scalindua-nirS* genes were detected in this study sites, but different from *nirK* gene. Further statistical analyses also showed both *nirS* and *Scalindua-nirS* genes respond similarly to environmental factors, but differed from *nirK* gene. These results suggest that bacteria containing *nirS* and *Scalindua-nirS* genes share similar niche in deep-sea subsurface sediments of the SCS, but differed from those containing *nirK* gene, indicating that community structures of nitrite-reducing bacteria are segregated by the functional modules (NirS vs. NirK) rather than the competing processes (anammox vs. classical denitrification).

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INTRODUCTION

Dissimilatory nitrate reduction to dinitrogen, the major microbial nitrogen removal processes in marine ecosystems, occurs in two major pathways under anoxic conditions, classical denitrification and anaerobic ammonium oxidation (anammox) (Klotz & Stein 2010). In the former process, nitrite reduction to nitric oxide is the rate-limiting step that is catalyzed by either copper-containing NirK or cytochrome *cd*-1 NirS nitrite reductases (Zumft 1997). The corresponding genes encoding the catalytic subunits (*nirK* and *nirS*) have been used extensively as biomarkers to elucidate community structures of nitrite reducers in marine, estuarine, and groundwater environments, and these studies were often amended with probing for genes encoding nitric oxide (i.e., *norB, norZ*) and/or nitrous oxide (*nosZ, nosW*) reductases to identify the classical denitrifying community (Braker *et al.*, 1998, 2001; Santoro *et al.*, 2006; Oakley *et al.*, 2007; Smith *et al.*, 2007; Tamegai *et al.*, 2007; Cao *et al.*, 2008). For a long time, classical denitrification has been considered the only pathway for nitrogen removal from marine ecosystems before discovery of anammox as another competing process (Strous *et al.*, 1999; Schmidt *et al.*, 2003; Francis *et al.*, 2007). Putative *nirS* genes have been identified in the genomes of anammox bacteria (Strous *et al.*, 2006). Due to the significant

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differences in the protein sequences of nitrite reductases in classical denitrifiers and anammox bacteria, Lam *et al*., (2009) proposed *Scalindua-nirS* as a candidate biomarker for the detection of anammox bacteria of the *Scalindua* genus in marine environments. Li *et al*., (2011) further confirmed the *nirS* as a useful biomarker for detection of anammox bacteria in marine sediments.

Because the oxidant for ammonium oxidation in the anammox process is nitrite-derived nitric oxide (Jetten *et al*., 2009), both anammox and denitrification pathways compete for available nitrite (Kartal *et al*., 2007). Furthermore, in addition to *nirK*, *nirS,* and *Scalindua-nirS* gene-encoded NO-forming enzymes in the nitrite-reducing denitrifiers and anammox bacteria, nitrite is also a substrate to respiratory and assimilatory ammonia-forming nitrite reductases active in ammonification, indicating another competition for substrate by a wide variety of nitrite-reducing micro-organisms. Because ammonification does not remove nitrogen from the marine ecosystem, an investigation of abundance and distribution of *nirK*, *nirS,* and *Scalindua-nirS* genes may reveal the interactions of different nitrogen removal microbial populations and the environmental conditions anammox bacteria can successfully compete *nirK* and *nirS* type denitrifiers for the available nitrite. Here, we report the molecular diversity, abundance, and distribution of *nirK*, *nirS,* and *Scalindua-nirS* genes in subsurface sediments of the South China Sea, the largest marginal sea in southeast Asia.

MATERIALS AND METHODS

Sampling and site description

Deep-ocean subsurface sediment samples were collected at different depths from four sites (401, 407, 525, and CF5) of the Northern quadrant of the South China Sea during the South China Sea Open Cruise by R/V Shiyan 3 in 2008. A detailed description of sampling sites and their physiochemical characteristics is provided in Fig. 1 and in our previous research (Hong *et al*., 2011). The geographical locations and physiochemical characteristics of the four sampling sites represented some niche differences for anammox bacteria (Hong *et al*., 2011) and ammonia oxidizers (Cao *et al*., 2012).

DNA extraction and PCR amplification

Genomic DNA was extracted from all sediment samples (collected in triplicate) using the SoilMaster DNA Extraction kit following the manufacturer's instructions (Epicentre Biotechnologies, Madison, WI, USA). PCR amplifications were performed in 50-μL-volume assays containing 10 μL of 5 × PCR GoTaq Flexi buffer (Promega, Madison, WI, USA), 3 μL of $MgCl_2$ buffer (25 mM, Promega), 1 μL bovine serum albumin (100 mg mL^{-1}, Roche, Hong Kong), 1 μL dNTPs (10 mM, Invitrogen, Carlsbad, CA, USA), 0.3 μL of GoTaq Flexi polymerase (5 U μL^{-1}, Pro-

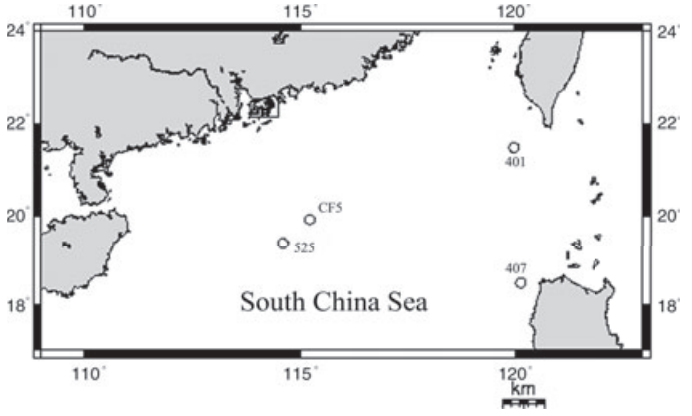

Fig. 1 Descriptions of four sampling sites at deep-sea subseafloor of the South China Sea in this study.

	401	407	525	CF5
Sampling Position	21°31N/119°59E	18°30N/120°08E	19°23N/114°36E	19°55N/115°13E
Depth of sea water (m)	3300	1900	1100	1153
depth (mbsf)	3.7	1.0	4.0	7.5
Temperature (°C)	2~4	2~4	2~4	2~4
NH_4^+ (mg /kg)	88.6 ± 11.7	13.8 ± 0.63	33.5 ± 4.0	29.9 ± 7.5
NO_3^- (mg /kg)	234.9 ± 17.6	< 0.1	< 0.1	< 0.1
NO_2^- (mg /kg)	119.8 ± 10.8	110.0 ± 2.2	114.5 ± 1.3	94.2 ± 4.5

mbsf, meters below the sea floor (Hong *et al*., 2011).

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mega), 1 μL of each primers (25 mM), and 2 μL (30–50 ng μL^{-1}) of the isolated genomic DNA as the template. Amplicons of *nirK* genes were obtained using the primer set nirK1F-nirK5R according to Braker *et al.*, (1998), and *nirS* genes were targeted with primer pair cd3aF (Michotey *et al.*, 2000) and R3cd (Throback *et al.*, 2004). The modified 'touchdown' protocols for *nirK* and *nirS* genes' PCR amplifications were performed. After an initial denaturing step at 95 °C for 5 min, 15 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min were carried out, with a 0.5 °C step down in annealing temperature of each cycle. This was followed by 20 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min and a final extension at 72 °C for 7 min. Primer sets Scnir372F-Scnir845R developed by Lam *et al.*, (2009) were used to detect *Scalindua-nirS* genes, and the primer set AnnirS379F-AnnirS821R reported by Li *et al.*, (2011) was also used to detect other anammox bacterial *nirS* gene by the same PCR protocols showed in previous studies (Lam *et al.*, 2009; Li *et al.*, 2011). The PCR products were checked by electrophoresis on 1% agarose gels and subsequent staining with 0.5 mg ml^{-1} gel red. All amplicons for each of the targeted genes showed a single band on the gel except no PCR products could be obtained using the primer set AnnirS379F-AnnirS821R.

Sequencing and phylogenetic analysis

Amplicons resulting from PCR performed in triplicate for each site were captured (pMD18-T; Takara, Japan), and the resulting clone libraries were prepared for insert sequencing. DNA sequences were determined (ABI Prism 3730 DNA analyzer; Applied Sciences, Foster City, CA, USA) and translated using MEGA 4.0 software (Tamura *et al.*, 2007), and the resulting protein sequences were aligned using CLUSTALW (Thompson *et al.*, 1994). These alignments and the MEGA 4.0 software (neighbor-joining algorithm with 1000 times bootstrap resampling) were used to construct phylogenetic trees. Operational taxonomic units (OTUs) were identified using a cutoff of 5% difference in compared protein sequences, and the DOTUR program was employed to compare the diversity of *nirK, nirS,* and *Scalindua-nirS* gene sequences for each sampling site (Schloss & Handelsman 2005). Correlation analysis between diversity and environmental variables was conducted using Microsoft Excel.

Quantitative analysis of *nirK*, *nirS,* and *Scalindua-nirS* genes

The abundance of *nirK*, *nirS,* and *Scalindua-nirS* genes in sediment samples was quantified in triplicate using an ABI 7000 Sequence Detection System and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The real-time fluorescent PCR (qPCR) protocols for *nirK*, *nirS,* and *Scalindua-nirS* genes used were the same ones as above for endpoint PCR with exception of the following: (i) the initial denaturing step was at 95 °C for 10 min; (ii) there was no final extension; (iii) the total number of cycles was 48. A 10-fold serial dilution (1.02×10^2–1.02×10^8) of pMD18 T-plasmids (Takara, Japan) carrying *nirK*, *nirS,* and *Scalindua-nirS* gene insert fragments selected from our clone libraries was used as standards. qPCR amplification efficiencies were 0.94–0.96, 0.95–0.97, and 0.93–0.95 for *nirK*, *nirS,* and *Scalindua-nirS*, respectively; the correlation coefficients (R^2) of the standard curves were >0.99.

Nucleic acid sequence accession numbers

The GenBank accession numbers for the *nirK*, *nirS,* and *Scalindua-nirS* gene sequences reported here are GQ443760–GQ443888, GQ443889–GQ444002, and GQ444003–GQ444135, respectively.

RESULTS AND DISCUSSION

Phylogenetic analysis of *nirK* and *nirS* genes

A total of 129 *nirK* gene sequences obtained from the clone libraries were grouped into three clusters in the phylogenetic tree (Fig. 2). Cluster I, containing 44.4% of all *nirK* sequences from station CF5, 5.7% from station 407, and 4.3% from station 401, is affiliated with *Alphaproteobacteria*, including *Rhizobium* sp. (82.6% protein sequence identity), *Sinorhizobium* sp. (81.8% protein sequence identity), *Ochrobactrum* sp. (80.5% protein sequence identity), and *Paracoccus* sp. (76.7% protein sequence identity. Sequences in cluster II are closely related to those from *Gammaproteobacteria* (*Pseudomonas* sp. 85% protein sequence identity), representing a minor group with only 30.4% of the sequences from station 401, 17.1% from station 525, and 2.8% from station 407 (Fig. 2). Cluster III represents the majority of sequences, containing 65.2%, 91.4%, 82.8%, and 55.5% of the *nirK* sequences recovered from stations 401, 407, 515, and CF5, respectively. Within cluster III, sequences in two subclusters are more closely related to those found in Pacific Northwestern sediments (Jayakumar *et al.*, 2004; Lam *et al.*, 2009) and the corresponding protein sequence identities ranged from 88.0% to 97.3%. The third subcluster, including only a few clones from stations CF5 and 401, may be considered novel because no sequence-similar to *nirK* sequences could be retrieved from the sequence databases (Fig. 2) and share less than 30% identity to amino acid sequence of KSU-1.

The 114 identified *nirS* gene sequences were clustered in eight phylogenetic groups (Fig. 3). Sequences in cluster I (with two subclusters) are affiliated with *Pseudomonas* and clones retrieved from Arabian seawater column and marine

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sediment (Dang *et al.*, 2009), and the relative sequence proportions are 21.4%, 24.0%, 6.9%, and 9.4% of those obtained from stations 401, 407, 525, and CF5, respectively. Sequences in clusters II and IV are affiliated with the clones found in Jiaozhou Bay, Changjiang Estuary, and California coastal sediments, while those in cluster III containing nine sequences from station 407 are affiliated with the clones found in Hai River sediments and *Pseudomonas* sp. Sequences in cluster V affiliated with uncultured bacteria of soil, sediment–water interface, Baltic Sea sediment, even the clones obtained from activated sludge, represent <10% of the clone libraries obtained from stations 401, 407, and CF5. Cluster VI, including 71.4%, 32.0%, 37.9%, and 28.2% of the sequences obtained from stations 401, 407, 525, and CF5, respectively, is the most common *nirS* gene sequences from South China Sea (SCS) subseafloor sediments, and sequences in this cluster are affiliated with the *Betaproteobacteria* (*Alcaligenes faecalis*, *Dechloromonas* sp.) and uncultured bacteria from activated sludge. Sequences in clusters VII (two sequences from station 525 and one sequence from station CF5) are related to the clones from sediments of estuary, Huntigton Beach, and water column

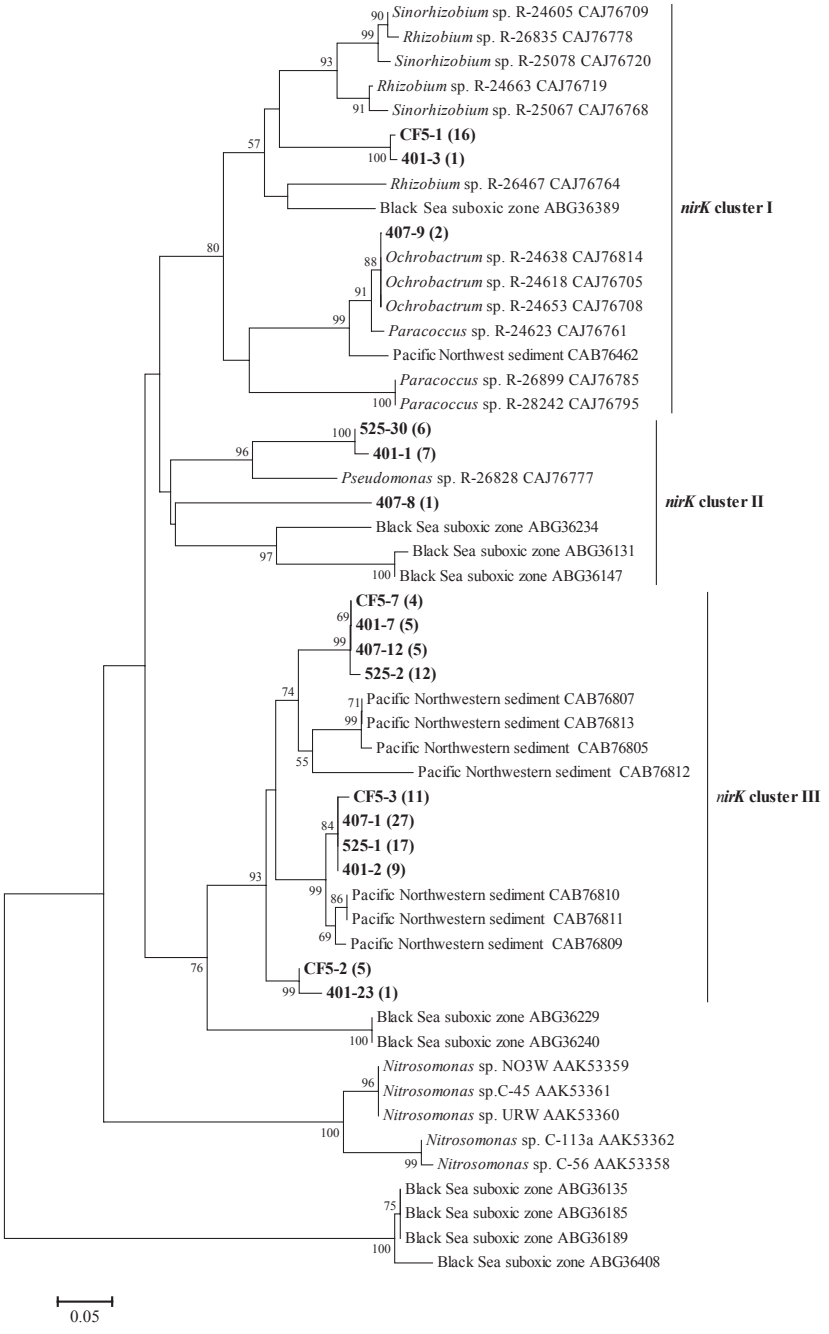

Fig. 2 Phylogenetic relationship of *nirK* in the South China Sea deep-sea subseafloor sediments. Bootstrap values represent 1000 replicates, and only values above 50% are shown. Branch lengths correspond to sequence differences as indicated by the scale bar. Numbers in parenthesis refer to how many clones were assigned to a phylotypes.

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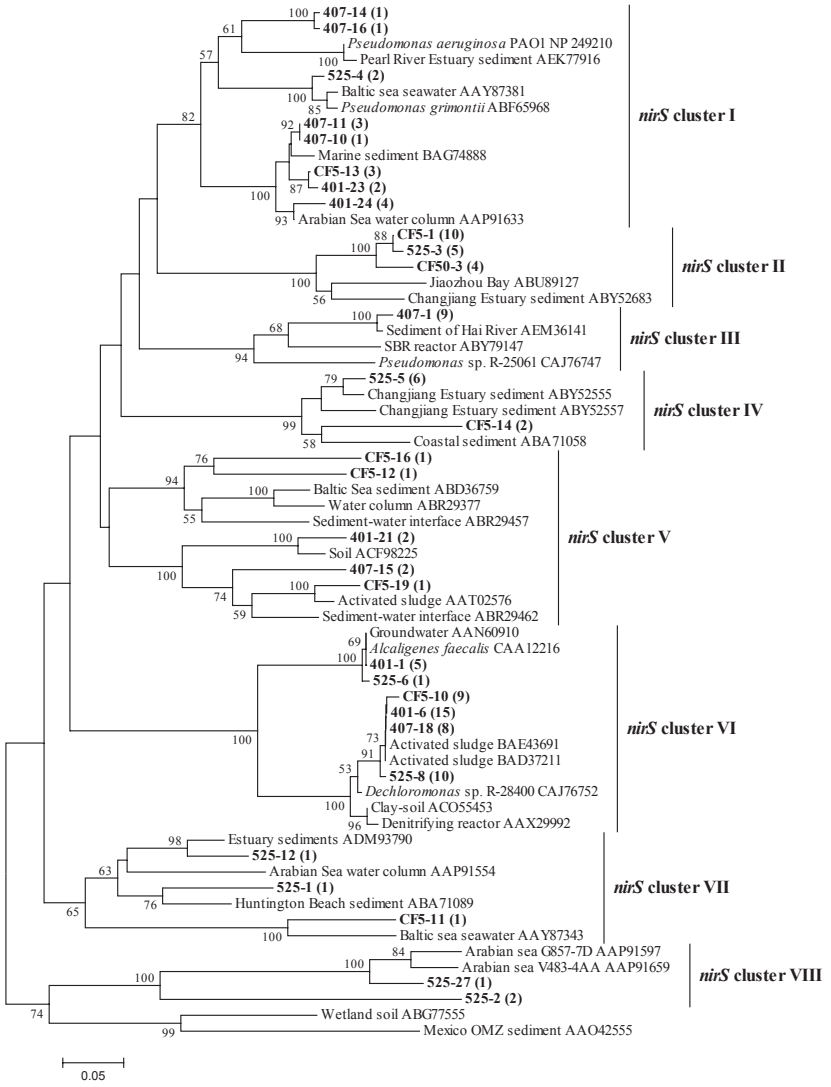

Fig. 3 Phylogenetic relationships of *nirS* in the South China Sea deep-sea subseafloor sediments. Bootstrap values represent 1000 replicates, and only values above 50% are shown. Branch lengths correspond to sequence differences as indicated by the scale bar. Numbers in parenthesis refer to how many clones were assigned to a phylotypes.

of Arabian Sea and Baltic Sea. Interestingly, three *nirS* sequences from station 525 appear to form a novel group, cluster VIII, which is distinctively different from deposited and known *nirS* gene sequences (<58.1% protein sequence identity), but share 60.2–63.4% and 59.0–60.9% protein sequence identities with *Scalindua-nirS* sequences detected in the Peruvian OMZ and identified in the genome of anammox bacterium *Candidatus* 'Kuenenia stuttgartiensis', respectively.

Comparing the diversity of *nirS*- and *nirK*-type denitrifiers, three major groups of *nirK*-type nitrite reducers were detected, including *Alphaproteobacteria*, *Gammaproteobacteria,* and uncultured bacteria recovered from Pacific Northwestern sediments, while *nirS*-type nitrite reducers not only affiliate with known denitrifying *Betaproteobacteria* (*Alcaligenes faecalis* and *Dechloromonas* sp.) and *Gammaproteobacteria* (*Pseudomonas aeruginosa* and *Pseudomonas grimontii*), but also with uncultured bacterial *nirS* clones retrieved from seawater column, coastal and estuary sediments, soils, activated sludge, and SRB reactors. Thus, it appears that the *nirS*-type nitrite-reducing bacteria are more abundant than *nirK*-encoding bacteria in deep-sea subseafloor sediments. On the other hand, it has been suggested that the diversity of nitrite reducers at a given location may be high during the initial stage of nitrite removal (high ratio of nitrate/nitrite), but lower in later stages of the process when most of the available nitrate/nitrite is exhausted (Jayakumar *et al.*, 2009). If this holds true for deep-ocean subseafloor sediments, more nitrite reducers are expected at site 401, where the nitrate/nitrite ratio was the highest among all sites in this study (Fig. 1). To our surprise, this general pattern was applicable only to *nirK* gene abundance and diversity, not to *nirS*. Our results are in agreement with those by Oakley *et al.*, (2007) for the Black Sea suboxic zone, showing a clear difference between *nirK*- and *nirS*-encoding nitrite reducers due to physical and chemical differences in these sampling stations.

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Phylogenetic analysis of *Scalindua-nirS* gene

A previous study (Oakley *et al.*, 2007) focused on anammox bacterial *nirS* gene abundance and distribution in the Black Sea suboxic zone, but failed to retrieve any due to mismatches with the employed primers. Only recently, two primer sets have been developed for targeting *nirS* genes in anammox bacteria (Lam *et al.*, 2009; Li *et al.*, 2011). Using the two new primer sets, anammox bacterial related *nirS* gene sequences were only obtained using the primer set Scnir372F-Scnir845R, which is consistent with our previous results (Li *et al.*, 2011). A total of 133 clones in the *Scalindua-nirS* group with high protein sequence identities (80.0–91.7%) to *Scalindua-nirS* gene sequences retrieved from the water column of Peruvian OMZ (Lam *et al.*, 2009) and sediments of the SCS and Mai Po mangrove wetland (Li *et al.*, 2011). These retrieved sequences also showed high protein sequence identity (60.0–61.4%) with the *nirS* gene in the genome of anammox bacterium *Candidatus* 'Kuenenia stuttgartiensis', but lower identity (<58%) with *nirS* genes from known denitrifiers (Fig. 4). In addition, all sequences grouped phylogenetically in a distinct clade separate from the group of *nirS* gene from classical denitrifiers. The clade contains five clusters, of which *Scalindua-nirS* cluster I to III includes 70.9%, 84.3%, 68.6%, and 77.1% of the sequences obtained from stations 401, 407, 515, and CF5, respectively. While sequences in cluster V were closely related to the sequences of the SCS surface sediment, sequences in both clusters IV and V formed unique groups clearly different from those found in the Peruvian OMZ, thereby indicating presence of a SCS site-specific sequence cluster (Fig. 4). To our knowledge, this is the first report of *Scalindua-nirS* gene sequences from the deep-ocean subseafloor biosphere, which extends our knowledge about anammox and denitrifying processes in natural ecosystem. In our previous study, a variety of *Scalindua*-like anammox bacteria was detected in these samples, including five different 16S rRNA and hydrazine oxidoreductase (*hzo*) gene subclusters (Hong *et al.*, 2011). Our current results in this study are

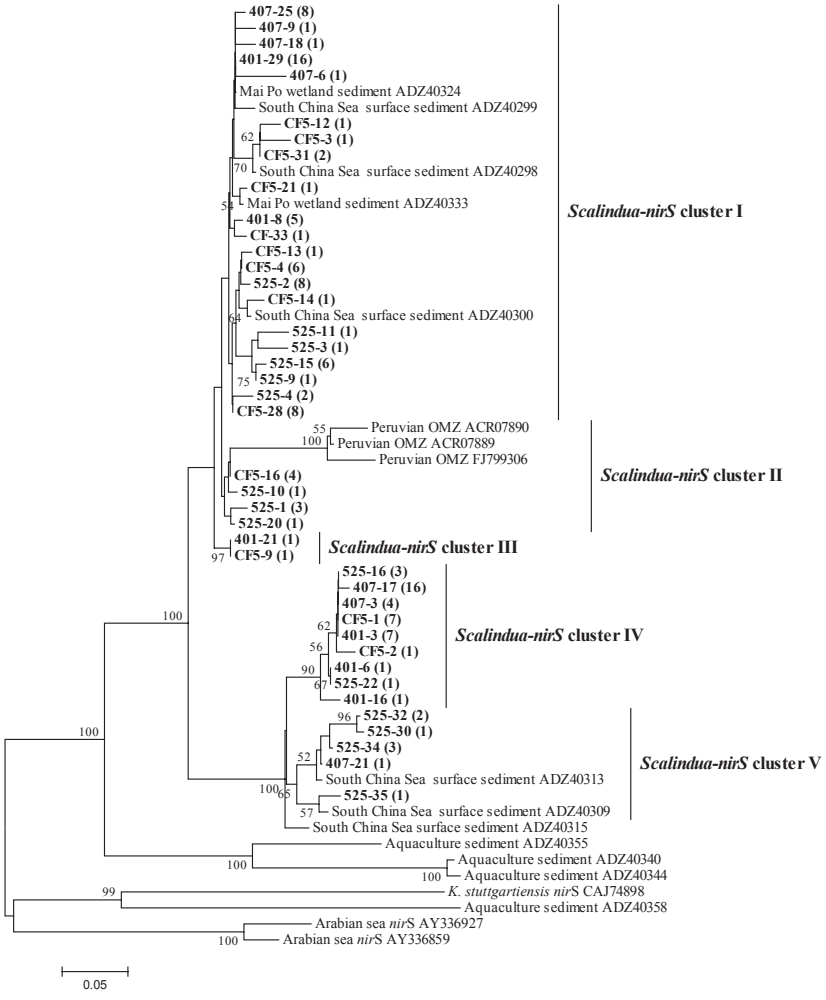

Fig. 4 Phylogenetic relationships of *Scalindua-nirS* in deep-ocean subseafloor sediments of the South China Sea. Bootstrap values represent 1000 replicates, and only values above 50% are shown. Branch lengths correspond to sequence differences as indicated by the scale bar. Numbers in parenthesis refer to how many clones were assigned to a phylotypes.

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consistent with the previous ones, further confirm the *Scalindua-nirS* as an effective functional biomarker for detection and analysis of marine anammox bacteria. Although the five different clusters of *Scalindua-nirS* gene could not be perfectly matched with those of anammox bacteria 16S rRNA and *hzo* genes as shown in our previous study (Hong *et al.*, 2011), *Scalindua-nirS* gene provides an additional biomarker and more comprehensive analysis of the phylogenetic diversity and composition of anammox bacteria in the marine ecosystem.

Abundance of *nirK*, *nirS*, and *Scalindua-nirS* genes

The *nirK* gene abundance in the four sampling sites ranged from 1.74 ($\pm$0.73) $\times$ 10^5 to 1.30 ($\pm$0.01) $\times$ 10^3 copies/g (dry mass sediments), for which site 525 had the lowest. The *nirS* gene abundance varied from 2.06 ($\pm$0.26) $\times$ 10^6 to 4.29 ($\pm$0.81)$\times$$10^5$ copies/g, and *Scalindua-nirS* ranged from 2.34 ($\pm$0.24) $\times$ 10^5 to 2.06 ($\pm$0.21) $\times$ 10^5 copies/g (Table 1). Interestingly, both *nirS* and *Scalindua-nirS* genes showed similar abundance distribution patterns and the highest abundance of *nirS* and *Scalindua-nirS* genes was detected at station 525, followed by station CF5, with the lowest abundance at station 407. The *nirK* gene followed a reverse distribution pattern, in which site 407 had the highest abundance and station 525 had the lowest (Table 1). However, comparing with our previous results of anammox bacteria described by 16S rRNA and *hzo* genes at these stations, abundance of *Scalindua-nirS* in this study was much higher and relatively even, possibly due to the low qPCR efficiency with a longer *hzo* gene amplicons (>1000 bp) (Hong *et al.*, 2011); thus, new PCR primers for the qPCR of *hzo* genes are needed in the future.

Distribution of *nirK*, *nirS*, and *Scalindua-nirS* genes and their relationships with environmental factors

The geographical distribution of *nirK*, *nirS*, and *Scalindua-nirS* genes in subsurface sediments of the SCS was investigated and compared with other marine ecosystems using the Unifrac package (Figs S1–S3) (Lozupone *et al.*, 2006). Jackknife environmental clustering and principal coordinate analysis (PCoA) identified that *nirS* and *Scalindua-nirS* genes share a similar geographic distribution pattern and similar structures of *nirS*- and *Scalindua-nirS*-encoding microbial communities among the four stations, but not for *nirK* genes. The *nirS* gene sequences recovered from the SCS subseafloor were clearly different from those retrieved from Pacific Northwestern sediments (Braker *et al.*, 2001), the Black Sea suboxic zone (Oakley *et al.*, 2007), the Baltic Sea (Hannig *et al.*, 2006), the Arabian Sea (Jayakumar *et al.*, 2004), and the Peruvian OMZ (Lam *et al.*, 2009), and *Scalindua-nirS* gene sequences of this study were different from Mai Po wetland, marine aquaculture zone (Li *et al.*, 2011), and the Peruvian OMZ (Lam *et al.*, 2009), while the *nirK* gene distribution reflected similar community structures as found in the Pacific Northwestern sediments (Braker *et al.*, 2001), suggesting that the SCS subsurface sediments serve as a site-specific niche for *nirS* and *Scalindua-nirS* communities (Figs S1–S3). The correlation analysis was further used to identify the relationships among different physiochemical parameters with the diversity and abundance of the three targeted genes (Table 2). Results indicated that diversity of *nirK* gene was positively correlated with the seawater depth of the sampling stations, while the *nirS* and *Scalindua-nirS* genes were negatively correlated, especially for the *nirS* gene, showing a very significant negative relationship with seawater depth. However, when comparing the relationship of abundance of the three genes with environmental factors, *nirS* and *Scalindua-nirS* genes were positively correlated with the ratio of $NH_4^+/(NO_2^-+ NO_3^-)$, while the *nirK* gene abundance was very negatively correlated with the ratio of $NH_4^+/(NO_2^-+ NO_3^-)$. These results further indicated that *nirS* and *Scalindua-nirS* genes were similarly distributed with similar responses to the environmental factors in deep-ocean subsurface sediments, which differed from that of *nirK*. Furthermore, the significant correlation between the *Scalindua-nirS* abundance and seawater depth also indicated that the seawater depth might strongly affect

Table 1 Diversity characteristics and abundance of *nirK*, *nirS*, and *Scalindua-nirS* genes in subseafloor sediments of the South China Sea

Groups	Sites	Clone numbers	OTUs	Shannon	Simpson	Chaol	Coverage	Abundance ($\times10^4$ g^{-1})
nirK	E401	23	5	1.33	0.26	6	0.91	5.03 $\pm$ 0.58
	E407	35	5	1.07	0.27	5	0.97	17.34 $\pm$ 7.33
	E525	35	3	1.02	0.36	3	1	0.13 $\pm$ 0.01
	CF5	36	4	1.24	0.30	4	1	1.153 $\pm$ 0.56
nirS	E401	28	5	1.30	0.33	5	1	144.21 $\pm$ 29.43
	E407	25	7	1.58	0.23	8.5	0.88	42.90 $\pm$ 8.05
	E525	29	11	2.17	0.11	12.5	0.86	206.42 $\pm$ 25.66
	CF5	32	11	2.07	0.13	14.3	0.84	145.20 $\pm$ 24.12
Scalindua-nirS	E401	31	4	1.11	0.36	4	0.97	20.64 $\pm$ 2.14
	E407	32	6	1.29	0.33	9	0.91	21.25 $\pm$ 3.48
	E525	35	9	2.01	0.12	12	0.91	23.41 $\pm$ 2.42
	CF5	35	6	1.49	0.24	7	0.94	23.10 $\pm$ 2.50

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Table 2 Statistical analysis of physiochemical parameters with diversity and abundance of *nirK*, *nirS*, and *Scalindua-nirS* genes

Groups	Environmental factors	OTUs	Shannon	Simpson	Chaol	Abundance
nirK	Seawater depth	0.76	0.65	−0.75	0.93	0.29
	Sediment depth	−0.44	0.43	0.28	−0.36	−0.33
	NH_4^+	0.29	0.76	−0.35	0.59	−0.33
	NO_x	0.51	0.71	−0.53	0.76	−0.06
	NO_2^-	0.18	−0.05	−0.08	0.37	0.15
	$NH_4^+/NO_2^{\dagger}$	0.24	0.76	−0.30	0.54	−0.39
	$NH_4^+/NO_x^{\dagger}$	−0.77	0.14	0.67	−0.56	**−0.99***
nirS	Seawater depth	**−0.95***	**−0.95***	**0.98***	**−0.95***	−0.25
	Sediment depth	0.60	0.54	−0.46	0.63	0.57
	NH_4^+	−0.59	−0.59	0.69	−0.65	0.35
	NO_x	−0.79	−0.78	0.86	−0.83	0.10
	NO_2^-	−0.63	−0.55	0.59	−0.77	0.11
	$NH_4^+/NO_2^{\dagger}$	−0.53	−0.53	0.64	−0.60	0.40
	$NH_4^+/NO_x^{\dagger}$	0.60	0.60	−0.49	0.51	**0.96***
Scalindua-nirS	Seawater depth	−0.81	−0.79	0.81	−0.78	**−0.92***
	Sediment depth	0.03	0.23	−0.36	−0.20	0.60
	NH_4^+	−0.59	−0.43	0.39	−0.72	−0.51
	NO_x	−0.69	−0.60	0.59	−0.75	−0.54
	NO_2^-	−0.08	−0.11	0.19	−0.05	−0.54
	$NH_4^+/NO_2^{\dagger}$	−0.55	−0.39	0.34	−0.70	−0.45
	$NH_4^+/NO_x^{\dagger}$	0.39	0.60	−0.69	0.12	0.68

Pearson moment correlation (r) was determined using the following equation: $r = \frac{n(\sum xy)-(\sum x)(\sum y)}{\sqrt{[n\sum x^2-(\sum x)^2][n\sum y^2-(\sum y)^2]}}$. Asterisks denote a P of <0.05, which is typically regarded as significant, as determined by Excel function TDIST from the t value given by the following equation: $t = r \times \sqrt{\frac{n-2}{(1-r)^2}}$. The number of samples is given by n. †Ratios of ammonium to oxidized inorganic species (nitrite or nitrate plus nitrite).

the distribution of anammox bacteria, which has also been reported in other studies (Engstrom *et al.*, 2009; Glud *et al.*, 2009; Trimmer & Nicholls 2009).

By combining these results, it can be concluded that the distribution of *nirS*- and *Scalindua-nirS*-encoding communities is likely controlled by the same set of environmental factors or that they might respond similarly to the environmental gradients in the subsurface ecosystem of the SCS (Dang *et al.*, 2010). Because nitrite reduction is dependent on the presence of active nitrite reductases (Zumft 1997), it is reasonable to propose that a similar distribution pattern within bacteria containing *nirS* genes, different from that of *nirK*-type nitrite reducers, is due to the different types of nitrite reductase–encoding genes in these microbes and the different regulation of metabolic mechanism in the context of their nitrite reduction. Similar gene distribution patterns of denitrifier-*nirS* and *Scalindua-nirS* were also reported in the Peruvian OMZ (Lam *et al.*, 2009). Furthermore, the report that *nirS* communities responded differently to environmental gradients than *nirK* communities in diverse ecosystems (Jones & Hallin 2010) supports our finding in that coexisting populations of *nirS*-type nitrite reducers and anammox bacteria follow similar or identical community assembly rules imposed by their surrounding environment, which is different from *nirK* nitrite reducer communities.

In conclusion, the *nirS*-encoding nitrite-reducing bacteria showed much higher diversity and abundance than the *nirK*-encoding nitrite reducers in subseafloor sediments of the SCS, indicating that *nirS*-encoding communities may play a more important role in contributing to the overall nitrogen removal in deep-sea subsurface sediments. The *nirS*-type nitrite reducers and *Scalindua* anammox bacteria share similar distributions, different from that of *nirK*-type nitrite reducers, proposing different responses of these three groups of micro-organisms to environmental gradients in the deep-sea subseafloor sediments.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Jackknife Environmental Clusters (a) and PCoA (b) for the South China Sea deep-ocean subseafloor sediments *nirK* protein sequences and representative data from Pacific Northwestern sediments, Black Sea suboxic zone and California coastal sediments.

Fig. S2 Jackknife Environmental Clusters (a) and PCoA (b) for the South China Sea deep-ocean subseafloor sediments *nirS* protein sequences and representative data from Pacific Northwestern sediments, Black Sea suboxic zone, Peruvian OMZ, Arabian OMZ, and Baltic Sea.

Fig. S3 Jackknife Environmental Clusters (a) and PCoA (b) for the South China Sea deep-ocean subseafloor sediments Scalindua-*nirS* protein sequences and representative data from Peruvian OMZ, Marine aquaculture zone, Mai Po Natural Reserve.

ADDENDUM

Recently, Hu *et al.* (2012) reported the function of copper-nitrite reductases (NirK) in anammox bacteria, which allowed for a new perspective on the evolution of nitrite reduction in the anammox process (*Front. Microbio.* 3:366. doi:10.3389/fmicb.2012.00366).

van de Vossenberg *et al.* (2012) showed the functional replacement of the likely ancient multi-heme cytochrome c reductase (reverse HAO) by NirK in Jettenia asiatica and KSU-1 (*Environ Microbiol.* doi:10.1111/j.1462-2920.2012.02774.x.

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