ENVIRONMENTAL MICROBIOLOGY

Residence of Habitat-Specific Anammox Bacteria in the Deep-Sea Subsurface Sediments of the South China Sea: Analyses of Marker Gene Abundance with Physical Chemical Parameters

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Abstract Anaerobic ammonium oxidation (anammox) has been recognized as an important process for the global nitrogen cycle. In this study, the occurrence and diversity of anammox bacteria in the deep-sea subsurface sediments of the South China Sea (SCS) were investigated. Results indicated that the anammox bacterial sequences recovered from this habitat by amplifying both 16S rRNA gene and hydrazine oxidoreductase encoding *hzo* gene were all closely related to the *Candidatus* Scalindua genus. A total of 96 16S rRNA gene sequences from 346 clones were grouped into five subclusters: two subclusters affiliated with the brodae and arabica species, while three new subclusters named zhenghei-I, -II, and -III showed ≤97.4% nucleic acid sequence identity with other known *Candidatus* Scalindua species. Meanwhile, 88 *hzo* gene sequences

from the sediments also formed five distant subclusters within hzo cluster 1c. Through fluorescent real-time PCR analysis, the abundance of anammox bacteria in deep-sea subsurface sediment was quantified by *hzo* genes, which ranged from 1.19×10^4 to 7.17×10^4 copies per gram of dry sediments. Combining all the information from this study, diverse *Candidatus* Scalindua anammox bacteria were found in the deep-sea subsurface sediments of the SCS, and they could be involved in the nitrogen loss from the fixed inventory in the habitat.

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Introduction

Anaerobic ammonium oxidation (anammox) has been identified as an important pathway for anaerobic N_2 production in marine environment [29, 44]. These initial discoveries have changed the traditional view on the biogeochemical nitrogen cycling and present a new recognition that classical denitrification (heterotrophic process in which nitrogen oxides serve as the terminal electron acceptor for organic carbon metabolism) is not the only pathway for fixed nitrogen loss [12, 21]. The anammox activity in the marine environments was firstly investigated in Danish coastal regions using ^{15}N-labeled NH_4^+ [50]. The activity and diversity of anammox bacteria have been found in various marine environments, and further study has demonstrated that anammox reaction may be responsible for a large part (>50%) of oceanic N_2 production on a global scale [7, 14, 22–24, 34, 50, 52].

Five genera of anammox bacteria have been identified from the surveys of wastewater treatment systems and environmental samples, including *Candidatus* Brocadia [19, 44], *Candidatus* Kuenenia [37], *Candidatus* Anammoxglobus [18], *Candidatus* Scalindua, and *Candidatus*

Jettenia [32, 57], which form a monophyletic branch within the phylum *Planctomycetes*. Although anammox bacteria play an important role in the nitrogen cycle and have a widespread distribution in the marine ecosystem, the phylogenetic diversity of this group is quite low [39]. The anammox bacteria retrieved from the anoxic water columns of Golfo Dulce [7], in Namibian and Peruvian OMZs [13, 14, 24, 51, 58], and a number of temperate estuarine, coastal, and offshore sediments [16, 33, 34, 46, 53] were all affiliated with the *Candidatus* Scalindua genus, where four subgroups in *Candidatus* Scalindua sorokinii [22], *Candidatus* Scalindua brodae, *Candidatus* Scalindua wagneri [38] and *Candidatus* Scalindua arabica [56] were recorded and identified from the marine ecosystem.

Marine subsurface sediment is one of the most extensive microbial habitats on the Earth [10, 11, 49]. It had been estimated that the microbial cells in subseafloor sediments constituted 1/2~5/6 of the earth's microbial biomass and as much as 1/3 of the earth's total living biomass [30, 55]. As an essential metabolic process of microorganisms, it can be inferred that the microbial nitrogen transformation should be essential and active in the deep-sea sediment biosphere. Although microorganisms are ubiquitous in the ocean sediments, little is known about nitrogen-utilizing microorganisms in this environment. The diversity of potential nitrogen fixers inhabiting the ocean sediment and hydrothermal vents, based on the unique nitrogenase (*nifH*) and *nifH*-like genes, provides a basis for active N transformation in the sediment [8, 28]. However, the removal processes of fixed nitrogen by microorganisms are mainly focused on the denitrification [45], and very little information is available on the anammox bacteria. Understanding the role of fixed nitrogen removal by anammox microbial communities is therefore essential for a more comprehensive understanding of the nitrogen biogeochemical cycling in the deep-sea sediment ecosystem.

The South China Sea (SCS) with a maximum depth greater than 5,000 m is the largest marginal sea in Southeast Asia. It covers an area from the equator to 23° N and from 99° E to 121° E, and joins the Pacific Ocean through the Luzon Strait (or named as the Bashi Strait or the Bashi Channel in some literatures) between Taiwan Island and Luzon Island. The SCS is considered an oligotrophic ecosystem; both N and P in its euphotic layer are usually below the detection limits when measured by conventional methods [4]. In the central gyre of the SCS, nitrogen is the limiting factor for phytoplankton growth [58] suggesting that the environment is nitrogen deficient. In the present study, we investigated the diversity and abundance of anammox bacteria in the deep-sea subsurface sediments at different depths in the SCS based on 16S rRNA and hydrazine oxidoreductase encoding gene (*hzo*) analyses.

Materials and Methods

Sampling and Chemical Analyses

Subsurface sediment samples of the South China Sea were collected in 2008 at four sites: E401 (3.7 mbsf, meter below seafloor), E407 (1.0 mbsf), E525 (4.0 mbsf), and CF5 (7.5 mbsf) during South China Sea Open Cruise by R/V Shiyan 3. The details of the sampling sites are presented in Table 1 and Fig. S1. After surface samples of sediment cores were discarded due to exposure to the air, samples were taken and transferred aseptically into plastic bags and frozen immediately at −20°C. Chemical analysis of ammonium-N, nitrate-N, and nitrite-N in the sediment samples was carried out using 2 M KCl as extractant and measured with an autoanalyzer (QuickChem, Milwaukee, WI) according to standard methods [1].

DNA Extraction and PCR Amplification

Total genomic DNA of the sediment samples were extracted using the SoilMaster DNA Extraction Kit (Epicenter Biotechnologies, Madison, WI) according to the manufacturer's instructions. PCR amplifications of 16S rRNA genes were based on previous studies with primers

Table 1 Physical and chemical environmental parameters at each sampling site

	E401	E407	E525	CF5
Sampling position	21°31 N/119°59 E	18°30 N/120°08 E	19°23 N/114°36 E	19°55 N/115°13 E
Depth of seawater (m)	3,300	1,900	1,100	1,153
Depth (mbsf)	3.7	1.0	4.0	7.5
Temperature (°C)	2~4	2~4	2~4	2~4
NH_4^+ (mg kg^{-1})	88.6±11.7	13.8±0.63	33.5±4.0	29.9±7.5
NO_3^- (mg kg^{-1})	234.9±17.6	<0.1	<0.1	<0.1
NO_2^- (mg kg^{-1})	119.8±10.8	110.0±2.2	114.5±1.3	94.2±4.5

mbsf meter below seafloor

Brod541F (5′-GAGCACGTAGGTGGGTTTGT-3′) [31], Amx368F (5′-TTCGCAATGCCCGAAAGG-3′) [38] and Amx820R (5′-AAAACCCCTCTACTTAGTGCCC-3′) [37]. The primers HZOF1 (5′-GTGCATGGTCAATTGAAAG-3′) and HZOR1 (5′-CAACCTCTTCWGCAGGTGCATG-3′) [26] were used for amplification of *hzo* gene. The optimized PCR reaction mixture in a final volume of 50 μl contained the followings: 2 μl DNA (30~50 ng μl^{-1}), 0.5 μl bovine serum albumin (100 mg ml^{-1}, Roche), 10 μl 5×GoTaq Flexi Buffer (Promega) and 4 μl MgCl$_2$ (25 mM, Promega), 1 μl of dNTPs (10 mM, Invitrogen), 1 μl of forward and reverse primers (20 μM), and 0.25 μl of GoTaq Flexi polymerase (5 U μl^{-1}, Promega). PCR programs were as followings: 95°C for 3 min; 30–32 cycles of 95°C for 45 s, 60°C (for Brod541F/Amx820R) or 59°C (for Amx368F/Amx820R) or 53°C (HZOF1/HZOR1) for 1 min, followed by 72°C for 1 min; and finally 72°C for 10 min.

Cloning, Sequencing and Phylogenetic Analysis

The PCR products were purified using the Gel Advance Gel Extraction System (VIOGEME, Taipei) following the manufacturer's instructions, and cloned into the pMD-18T Vector (Takara, Japan). The insertion of an appropriate-sized DNA fragment was determined by PCR amplification with the primer set M13F and M13R. The clones in each library were screened by restriction fragment length polymorphism (RFLP) (with *Sau*3AI and *Nla*III for 16S rDNA, *Eco*RI and *Bam*HI for *hzo* DNA) and selected for sequencing. Sequencing was performed with the BigDye Terminator Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3730 DNA analyzer.

The DNA sequences were examined and edited by MEGA 4.0 software [47] and then checked for chimera formation using the Check Chimera program of Ribosomal Database Project [5] or manually. For the 16S rRNA gene, DNA sequences were manually compiled and aligned using the ClustalW [48]. For *hzo* gene, the nucleic acid sequences were firstly translated into amino acids, and the resulting amino acid sequences of the corresponding proteins were aligned using ClustalW. Phylogenetic trees were constructed by MEGA 4.0 with the neighbor-joining and maximum parsimony methods. Bootstrap resampling analysis on 500 replicates was performed to estimate the confidence of the tree nodes.

Quantitative PCR Assay

The copy numbers of *hzo* gene in all samples were determined in triplicate using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The 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 BSA (0.1%),

0.5 μl of each primer (20 μM, HZOF1 and HZOR1) and 12.5 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The PCR cycle was started with 2 min at 50°C and 10 min at 95°C, followed by a total of 48 cycles of 1 min at 95°C, 1 min at 53°C, and 1.5 min at 72°C. A standard plasmid carrying the *hzo* gene fragment was generated by amplifying the *hzo* gene from the extracted DNA from sediments and cloned into pMD18-T Vector (Takara, Japan). The plasmid DNA concentration was determined on a Biophotometer (Eppendorf, Germany), and the copy number of the target gene was calculated directly from the concentration of the extracted plasmid DNA. Tenfold serial dilutions of a known copy number of the plasmid DNA ($1.83×10^2$ to $1.83×10^{10}$ copies) were subjected to fluorescent real-time PCR assay in triplicate to generate an external standard curve (r^2=0.991).

Statistical Analysis

The Distance-based OTU and Richness (DOTUR) program [36] was employed to compare diversity for anammox 16S rRNA and *hzo* gene sequences from each sampling site. Operational taxonomic units (OTUs) for community analysis were defined by 3% differences in nucleic acid or protein sequences, as determined by using the furthest neighbor algorithm in DOTUR [36]. DOTUR was also used to generate diversity indices, such as Shannon and Simpson for each clone library. The correlation analysis between diversity and environmental variables was conducted using Microsoft Excel. To examine the geographic distribution of the phylogenetic structure of anammox bacteria in deep-sea subfloor sediments, 16S rRNA gene and Hzo protein sequences were analyzed with the online software UniFrac (http://bmf2.colorado.edu/unifrac/index.psp) by using the principal coordinates analysis (PCoA) as suggested previously [27].

Nucleic Acid Sequence Accession Numbers

The GenBank accession numbers for the 16S rRNA gene sequences reported here are GQ331139 to GQ331201 (with primer pair Brod541F/Amx820R) and GQ1202 to GQ331244 (with primer pair Amx368F/Amx820R), and the GenBank accession numbers for the *hzo* gene sequences are GQ331245 to GQ331332.

Results

Detection of Anammox Bacteria and Phylogenetic Analysis with 16S rRNA Gene

Anammox bacterial communities in the deep-sea subsurface sediments of the SCS were detected successfully with

Table 2 Diversity characteristics of 16S rRNA gene and deduced Hzo protein sequences recovered from each sampling site

Biomarkers	site	Number of screened clone	Number of marker gene detected (%)	OTUs	Shannon	Simpson	Chao
16S rRNA	E401	82	48 (58.5)	4	0.93	0.50	4
	E407	105	20 (19.0)	3	0.60	0.65	3
	E525	60	16 (26.7)	3	1.02	0.33	3
	CF-5	99	12 (12.1)	7	1.53	0.26	10
hzo	E401	30	30 (100)	6	1.50	0.24	6
	E407	20	20 (100)	5	1.26	0.30	5.5
	E525	21	21 (100)	5	1.32	0.28	5
	CF-5	17	17 (100)	6	1.68	0.15	6

Average number of marker genes obtained from 16s RNA and hzo gene abundances

16S rRNA gene. From the eight clone libraries (two clone libraries were constructed for each sampling site with anammox-specific primer pairs Brod541F/Amx820R and Amx368F/Amx820R), a total of 346 clones (from more than 1,000 clones with RFLP) were sequenced. The BLAST results revealed that 96 clones (27.8%) were closely affiliated with known anammox bacteria. Frequencies of positive clones for anammox bacterial 16S rRNA gene were highly variable and site dependent (Table 2). The positive clone (affiliated into the anammox cluster in phylogeny) frequency was 59%, 19%, 27%, and 12% for site E401, E407, E525, and CF5 in this study respectively.
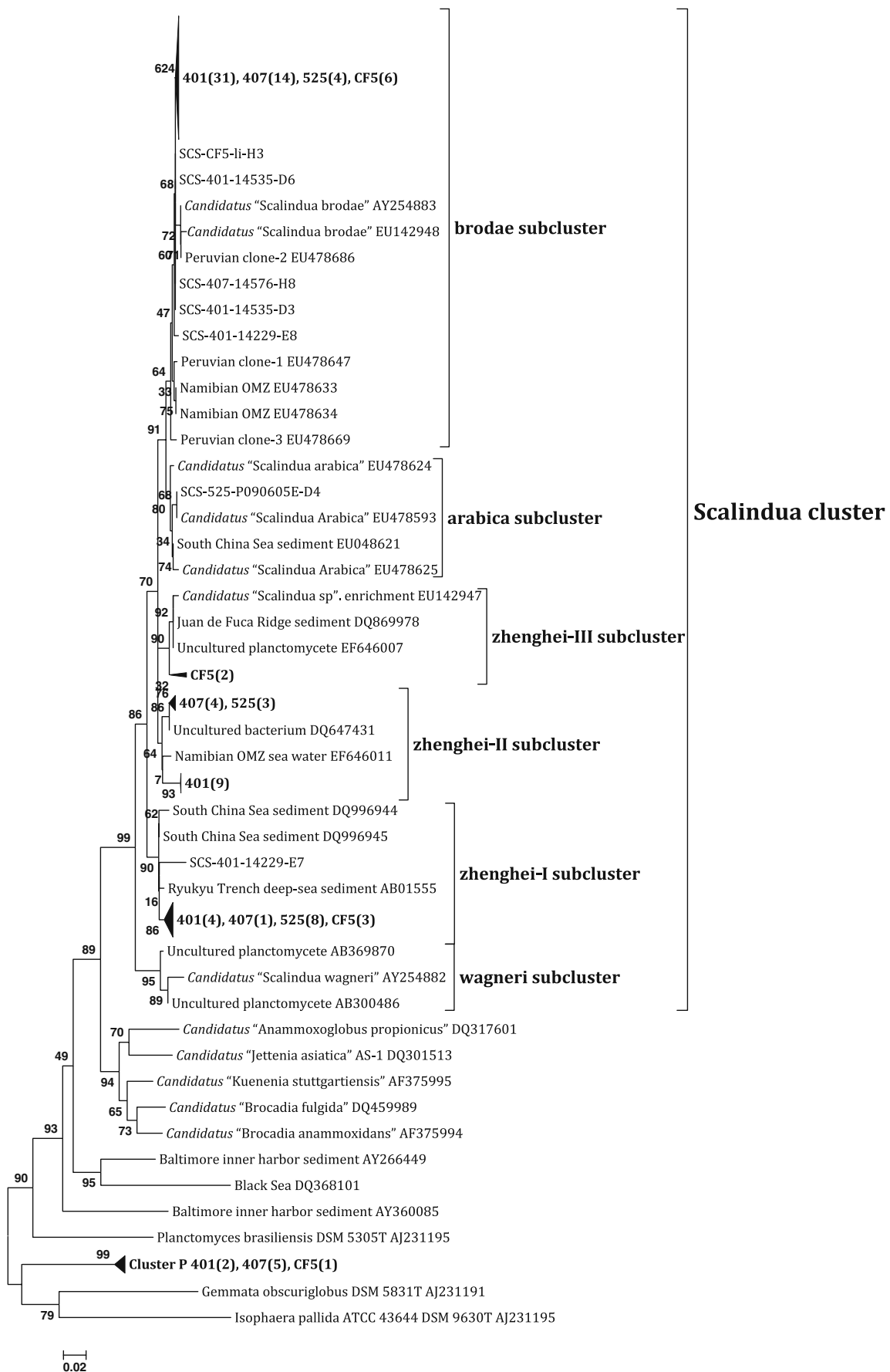
The phylogenetic tree was built with the 96 positive anammox clones and other known anammox 16S rRNA gene sequences using neighbor-joining and maximum parsimony algorithms (Fig. 1). The tree revealed that all anammox bacterial-like sequences of this study were affiliated with the genus Candidatus Scalindua. Two phylogenetic trees constructed using different algorithms supported the grouping of anammox bacterial sequences into six coherent subclusters: brodae subcluster, arabica subcluster, wagneri subcluster, zhenghei-I subcluster, zhenghei-II subcluster, and zhenghei-III subcluster. The 96 anammox-like sequences identified from the deep-sea subsurface sediments of SCS were distributed into five of the six subclusters; none of the sequences that were identified were affiliated with the wagneri group. The percentage of anammox-like sequences affiliated with the brodae subcluster was 62.5%. This subcluster includes sequences from all four sampling sites. In addition, this subcluster contains identified anammox bacteria Candidatus Scalindua brodae, Candidatus Scalindua sorokinii as well as some clones from Peruvian and Namibian OMZs. Only one clone from the E525 sampling site was included within the arabica subcluster, which is a newly proposed anammox bacteria phylotype [56].

When all subclusters were compared with each other, zhenghei-I, zhenghei-II, and zhenghei-III (named after He Zheng (also known as Cheng Ho) for his contribution to maritime navigation seven times to the Indian Ocean between 1405–1433 in China) were apparently distant from the others. The zhenghei-I subcluster (Fig. S3-A), containing 17 sequences from all our sampling sites, was most similar to sequences from SCS sediment [42] and Ryukyn Trench deep-sea sediment (AB015552) [25]. The zhenghei-I subcluster showed only 92~96.6% identity with other subclusters within the Candidatus Scalindua genus (Table 2). The zhenghei-II subcluster (Fig. S3-B), which contained 16 sequences from E407, E525, and E401, showed 94.7–96.6% identity with other subclusters within the Candidatus Scalindua genus (Table 2). The zhenghei-II subcluster is the most similar to a clone from Namibian OMZ sea water (EF646011) [57] and an uncultured bacterium in freshwater sediments of the Xinyi River (DQ647431) [59]. Only two clones affiliated with the zhenghei-III subcluster (Fig. S3-C) were related to sequences from Juan de Fuca Ridge sediment (DQ869978) [31] and Candidatus Scalindua sp. enrichment culture (EU142947) [54]. This subcluster has 93.6~97% identity with other subclusters within Candidatus Scalindua genus (Table 2).

Interestingly, nine of the 16S rRNA gene sequences of this study (cluster P) (Fig. S3-D) were grouped into the Planctomycetes but are not closely related to any known anammox bacteria or other described Planctomycetes genera. The sequence identities ranged from 77.7% to 82.2% compared with the known anammox bacteria and from 74.1% to 78.4% with non-anammox Planctomycete genera (Table S1). These nine sequences from the four sampling sites shared 97.3~100% identity to each other.

Figure 1 Phylogenetic tree based on an alignment of 16S rRNA gene sequences showing the phylogenetic affiliations of anammox bacterial sequences from the different subseafloor sediment of South China Sea. The *numbers in parentheses* refer to how many clones retrieved by the amplification with the primer sets Brod541F/Amx820R and Amx368/Amx820R were assigned to an individual phylotype. The consensus phylogenetic tree constructed based on neighbor-joining analysis. The *numbers* at the nodes are percentages that indicate the levels of bootstrap support based on 500 resampled data sets. Branch lengths correspond to sequence differences as indicated by the *scale bar*

624 **401(31), 407(14), 525(4), CF5(6)**

SCS-CF5-li-H3

68 SCS-401-14535-D6

Candidatus "Scalindua brodae" AY254883

72 *Candidatus* "Scalindua brodae" EU142948   **brodae subcluster**

60 **71** Peruvian clone-2 EU478686

SCS-407-14576-H8

47 SCS-401-14535-D3

SCS-401-14229-E8

64 Peruvian clone-1 EU478647

33 Namibian OMZ EU478633

75 Namibian OMZ EU478634

91 Peruvian clone-3 EU478669

Candidatus "Scalindua arabica" EU478624

68 SCS-525-P090605E-D4

80 *Candidatus* "Scalindua Arabica" EU478593   **arabica subcluster**

34 South China Sea sediment EU048621

74 *Candidatus* "Scalindua Arabica" EU478625

70 *Candidatus* "Scalindua sp". enrichment EU142947

92 Juan de Fuca Ridge sediment DQ869978

90 Uncultured planctomycete EF646007   **zhenghei-III subcluster**

CF5(2)

32 **76** **407(4), 525(3)**

86 Uncultured bacterium DQ647431

64 Namibian OMZ sea water EF646011   **zhenghei-II subcluster**

7 **401(9)**

93

62 South China Sea sediment DQ996944

99 South China Sea sediment DQ996945

SCS-401-14229-E7

90 Ryukyu Trench deep-sea sediment AB01555   **zhenghei-I subcluster**

16

86 **401(4), 407(1), 525(8), CF5(3)**

89 Uncultured planctomycete AB369870

95 *Candidatus* "Scalindua wagneri" AY254882   **wagneri subcluster**

89 Uncultured planctomycete AB300486

70 *Candidatus* "Anammoxoglobus propionicus" DQ317601

49 *Candidatus* "Jettenia asiatica" AS-1 DQ301513

94 *Candidatus* "Kuenenia stuttgartiensis" AF375995

65 *Candidatus* "Brocadia fulgida" DQ459989

73 *Candidatus* "Brocadia anammoxidans" AF375994

93 Baltimore inner harbor sediment AY266449

95 Black Sea DQ368101

90 Baltimore inner harbor sediment AY360085

Planctomyces brasiliensis DSM 5305T AJ231195

99 **Cluster P 401(2), 407(5), CF5(1)**

Gemmata obscuriglobus DSM 5831T AJ231191

79 Isophaera pallida ATCC 43644 DSM 9630T AJ231195

Scalindua cluster

0.02

Rarefaction analysis indicated that site CF5 showed the greatest anammox-16S rRNA gene diversity whereas sites E407 and E525 had the lowest OTUs, which was consistent with the values of Simpson and Shannon indices (Table 2). The OTU numbers in CF5 may have been higher if more clones were sequenced based on non-asymptotic rarefaction curves (Fig. S2).

Phylogenetic Analysis Based on an Alignment of *hzo* Gene Sequences

Using our new primer pair HZOF1/HZOR1, the PCR amplification products targeting *hzo* gene were amplified from the deep-sea sediments and subsequently *hzo* gene libraries were constructed for each sample. A total of 88 clones screened from the libraries showed a high similarity of their deduced protein sequences (Table S2) with those published in previous studies [32, 40, 41]. A phylogenetic analysis of experimental- and GenBank-retrieved *hzo* gene-deduced protein sequences resulted in a tree, in which the experimental Hzo protein sequences from our study were grouped into hzo cluster 1 defined by previous study (Fig. 2) [40]. Compared to the hzo clusters 1a and 1b, sequences obtained in this study all fell into a distant cluster within hzo cluster 1, for which hzo cluster 1c was proposed. A *Candidatus* Scalindua sp. enrichment culture (CAQ57909) [40] is also included in the hzo cluster 1c, indicating that the *hzo* sequences recovered from deep-sea subsurface sediments are closely related to *Candidatus* Scalindua bacteria. Further cladistic analysis revealed that five distinct subclusters were embedded within the hzo cluster 1c, designated as subclusters 1c-1 to 1c-5. Sequences in subclusters 1c-3 and 1c-5 were obtained from all four sampling sites; sequences in subclusters 1c-2 and 1c-4 were recovered only from the E525 and CF5 sites. To date, no published sequences were found to be closely related to any of the sequences in subclusters 1c-2, 1c-3, 1c-4, and 1c-5. The Hzo protein sequences of the subcluster 1c-1 are all from the E401 site and are most similar to Hzo protein sequence of *Candidatus* Scalindua sp. enrichment culture (CAQ57909) and two uncultured *Planctomycete* (CAQ57913 and CAQ 57914) [39]. The sequence identities of subclusters ranged from 83.0% to 94.0% when compared with each other and 77.2% to 83.5% with hzo clusters 1a and 1b, respectively but for other hzo clusters, including clusters 2a, 2b and 3, the identity was less than 50% (Table S2).

Using a 3% cutoff at the amino acids sequence variation to define an OTU, the OTU numbers were almost the same at all four study sites of this study. The values of Simpson and Shannon indices were consistent with OTU richness analysis (Table 2). The non-asymptotic rarefaction curves also showed the OTU numbers in CF5 might have been higher if more clones were sequenced (Fig. S2).

Abundance of the *hzo* Gene in Deep-Sea Subsurface Sediment

The copy numbers of the *hzo* gene in all the samples were determined using the fluorescent real-time PCR method with the same primer pair HZOF1/HZOR2 (Fig. 3). The abundance of the *hzo* gene in CF5 was the highest, up to 7.17×10^4 copies per gram of dry sediments. In E407 and E525, the abundances of the *hzo* gene were 4.54×10^4 and 5.44×10^4 copies per gram of dry sediments, respectively. The lowest *hzo* gene copy number of 1.19×10^4 copies per gram of dry sediments was determined at the sampling site E401, where the water column is the deepest (3,300 m below sea surface).

Community Structures of Resident Anammox Bacteria

A significant geographical distribution difference of the anammox community structure based on 16S rRNA and *hzo* genes was determined by the weighted UniFrac PCoA analysis. For 16S rRNA gene, the anammox communities in all four sampling sites of the SCS deep-sea sediment formed a unique group which separated from that of Antarctic sediment, Namibian and Peruvian OMZ (Fig. 4a), indicating a specific niche of SCS for the anammox bacteria, but four sampling sites are indistinguishable (Fig. 4a), The Hzo-based analysis also showed that the anammox communities in all four sampling sites of the SCS were separated from that of deep-sea Tephra deposits, hydrothermal Vent, Jiaozhou Bay, North Carolina groundwater, Upper Cape Fear River sediment and Black River sediment [9, 15]. The weighted UniFrac PCoA analysis for Hzo supported that the diverse distribution pattern is consistent with their actual geographical locations and the community between E401 and E407 as well as between E525 and CF5 was very similar (Fig. 4b).

Relationship Between Nitrogen and the Diversity of Anammox Bacteria

Variations in the concentration of ammonium and nitrate were observed at the four sampling sites. The concentration of ammonium varied from 13.8 mg kg^{-1} sediment in the E407 sample to 88.6 mg kg^{-1} sediment at the E401 site. The concentration of nitrate at E401 was as high as 235 mg kg^{-1} sediment but below detection level at the

Figure 2 Phylogenetic tree constructed from alignment of amino acid sequences of deduced Hzo protein from deposited *hzo* gene sequences in the GenBank and those obtained by amplifying the community DNA samples from the subseafloor sediment of the South China Sea at different depths. The *numbers in parentheses* refer to *hzo* gene clones retrieved using the respective primer set were assigned to an individual phylotype. The *numbers* at the nodes are percentages that indicate the levels of bootstrap support based on 500 resampled data sets. Branch lengths correspond to sequence differences as indicated by the *scale bar*. Major clades were named following a proposal of Schmid et al. [39]
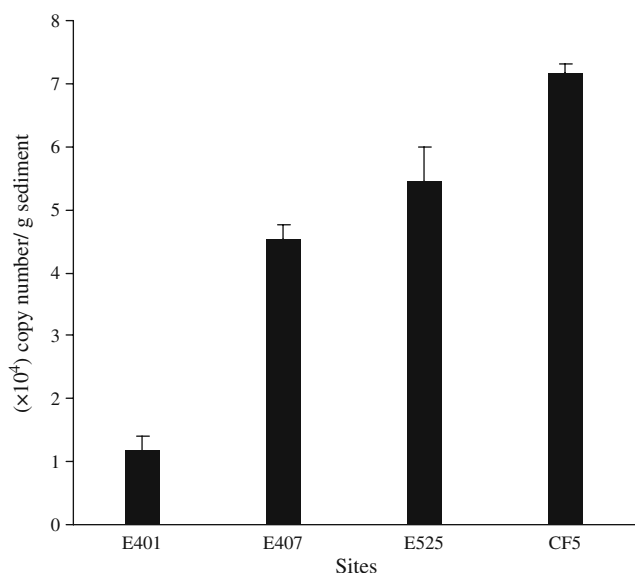
Figure 3 Abundance of *hzo* gene of anammox bacteria in sediments of selective sampling sites at the South China Sea. The experiment was performed in triplicate. *Error bars* represent standard deviations of triplicate analysis

other three sampling sties (Table 1). However, the concentration of nitrite was fairly consistent at all four sites (Table 1). A Pearson moment correlation analysis for physicochemical parameters and anammox bacteria diversity showed that only the Chao index of Hzo protein sequences at each site significantly correlated with nitrite concentration according to the statistical algorithm (Table 3).

Discussions

In our study, we detected the presence of anammox bacteria with both 16S rRNA gene and *hzo* functional gene. The results showed that 96 of the 346 screened 16S rRNA gene sequences fell into known anammox species, indicating the presence of anammox bacteria in the deep-sea subsurface sediment of the SCS. In addition, all the 16S rRNA gene sequences from the deep-sea subsurface sediments were closely related to the *Candidatus* Scalindua cluster. We presumed that the non-selective amplification might have resulted from two possibilities: (1) PCR bias and (2) only *Candidatus* Scalindua genus anammox bacteria are present predominantly in this environment.

Since the 16S rRNA gene is not specific enough in detecting the relevant anammox community in the environmental samples, the *hzo* gene was used for further more specific detection. HZO, a member of the octaheme cytochrome *c* hydroxylamine oxidoreductase protein family and a key step in the anammox biochemical process, dehydrogenates the unique anammox intermediate, hydrazine, to N_2 [17, 35]. So far, the *hzo* gene has been isolated
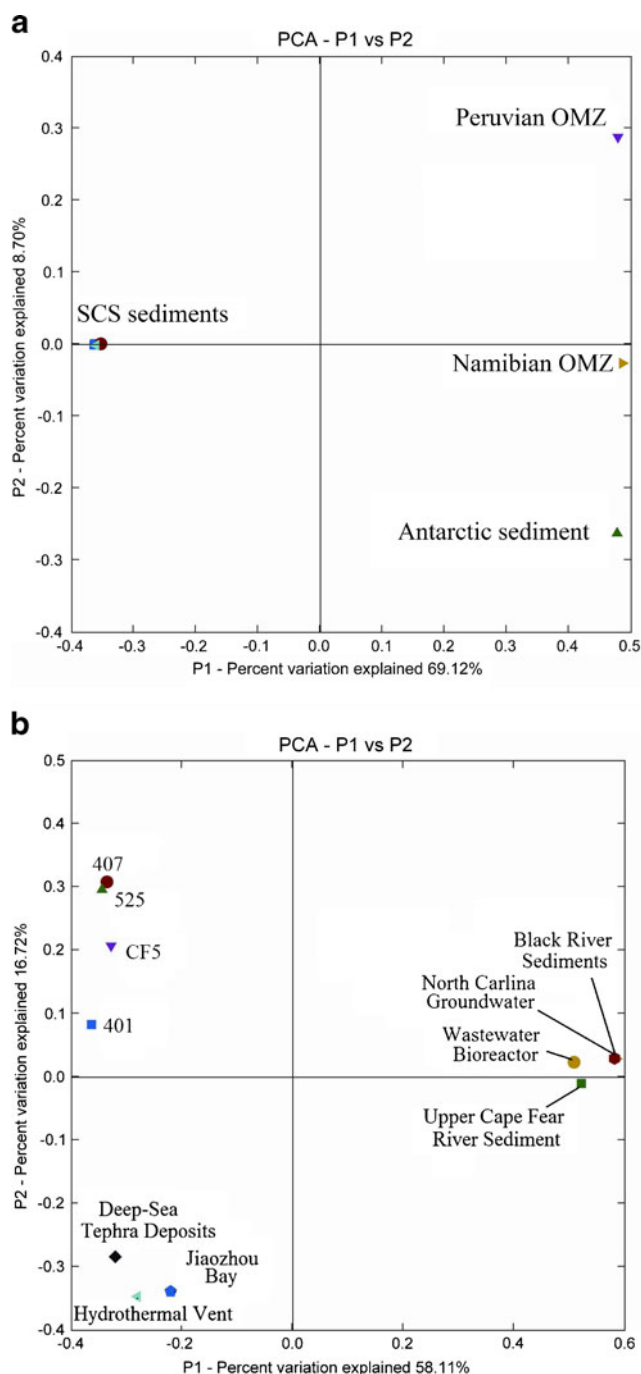

Figure 4 Weighted UniFrac PCA analyses for (**a**) 16S rRNA gene sequences and (**b**) deduced amino acid sequences of Hzo protein in the context of physical and chemical conditions

from several anammox enrichment cultures [35, 41] including a new genus of anammox bacteria *Candidatus* Jettenia sp. enrichment culture [32] and corresponding sequences were identified in the genome of *Candidatus* Kuenenia stuttgartiensis [43]. Recently, Klotz and Stein [20] proposed that genes encoding hydrazine oxidoreductase (Hzo) can be used as a functional phylogenetic marker. Schmid et al. [40] employed several degenerate primer sets for detecting *hzo*

Table 3 Statistical analysis of physicochemical parameters in context with the diversity and abundance of anammox bacteria

Parameter	Pearson moment correlation[a]								Abundance
	OTU		Shannon index		Simpson index		Chao		
	16S rRNA gene	Hzo	16S rRNA gene	Hzo	16S rRNA gene	Hzo	16S rRNA gene	Hzo	hzo gene
NH_4^+	0.08	0.69	0.09	0.40	-0.01	−0.27	−0.03	0.54	−0.77
NO_2^-	0.47	0.79	0.11	0.57	0.27	−0.49	0.40	0.96*	−0.43
NO_x	−0.04	0.61	−0.13	0.26	0.26	−0.11	−0.15	0.58	−0.89
NH_4^+/NO_2^- [b]	0.04	0.65	0.10	0.37	−0.06	−0.24	−0.06	0.46	−0.76
NH_4^+/NO_x [b]	0.39	0.34	0.75	0.50	−0.92	−0.52	0.38	−0.09	0.27

*$p < 0.05$, 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.

[a] Pearson moment correlation (r) was determined by using the following equation: $r = \frac{n(\sum XY) - (\sum X)(\sum Y)}{\sqrt{\left[n\sum X^2 - (\sum X)^2\right]\left[n\sum Y^2 - (\sum Y)^2\right]}}$

[b] Ratios of ammonium to oxidized inorganic species (nitrite or nitrate plus nitrite)

genes in various ecosystems and suggested that the *hzo* gene sequences in cluster 1 (targeted by two primer sets hzocl1F1-hzocl1R2 and hzocl1F1l-hzoclR2) were most appropriate and feasible for phylogenetic analysis because these genes appeared to be functional and present in all anammox bacteria studied. In addition, Quan et al. [32] amplified *hzo* genes from anammox granular biomass using another degenerate primer set (Ana-hzo1F/Ana-hzo2R). The primer pair HZOF1/HZOR1, which has been successfully used to amplify *hzo* gene sequences of multitype anammox genera in a previous study [26], was used to further detect anammox bacteria in the deep-sea subsurface sediment. Surprisingly, the sequences obtained with primer pair HZOF1/HZOR1 were also closely related to the *Candidatus* Scalindua cluster the closest. Thus, the results of 16S rRNA and *hzo* gene analyses support that the *Candidatus* Scalindua genus bacteria are the only anammox species present in the deep-sea subsurface sediments of the SCS.

Previous studies showed that anammox bacteria in the marine environments mainly belonged to the *Candidatus* Scalindua genus. However, in some marine environments strongly affected by the anthropogenic input of wastewater, such as the Cape Fear River Estuary [6, 15], Jiaozhou Bay [9], Japan coastal marine sediments [2], and in the intertidal wetland of Hong Kong [26], genera other than *Candidatus* Scalindua, including *Candidatus* Kuenenia sp., and *Candidatus* Brocadia sp. and *Candidatus* Jettenia sp. were also found. Several anammox species existed concurrently in the deep-sea hydrothermal vent [3]. Based on the available information and our analyses, the anammox bacteria of *Candidatus* Scalindua genus were found in the low-temperature environment of the sea water column, within the surface and subsurface of the ocean sediment. The diversity of the anammox bacteria is much higher in the marine habitat of a hydrothermal vent or anthropogenic impacted environments. The *Candidatus* Scalindua species

may dominate the anammox bacterial community because most marine environments are low in temperature.

An analysis of both 16S rRNA and *hzo* genes revealed a high diversity of *Candidatus* Scalindua bacteria in the deep-sea subsurface sediment of the SCS. As the *Candidatus* Scalindua sorokinii has high identity (98.6%) with *Candidatus* Scalindua brodae, the two species generally fell into one subcluster in the phylogenic analysis. In this study, the 16S rRNA gene sequences from SCS deep-sea subsurface sediment were distributed into five distant subclusters in the phylogenetic tree, which included three new subclusters. Moreover, the new subclusters showed less than 97.4% sequence identities with other identified *Candidatus* Scalindua phylotypes. The results in this study (Fig. 1 and Table S1) suggest that the subclusters "zhenghei-I," "zhenghei-II," and "zhenghei-III" comprise three individual phylotypes within the *Candidatus* Scalindua genus. Thus, the three specific clusters of the anammox bacteria found in the SCS deep-sea sediment provide new information about anammox diversity within a natural ecosystem. An analysis of the clone library composition showed that the *Candidatus* Scalindua brodae/sorokinii species was still the dominant group present and the *Candidatus* "Scalindua zhenghei-I" phylotypes were the second most abundant group in the sediment.

The Hzo protein sequences were used for a further differentiation of closely related clones and to measure the diversity of anammox bacteria in the deep-sea sediment. A total of 88 Hzo sequences from SCS formed five distant subclusters in Hzo cluster 1c, which generally confirms the corresponding 16S rRNA gene phylogeny. However, because there are limited number of *hzo* sequences available at present, including only two *Candidatus* Scalindua enrichment cultures [40], these hzo subclusters cannot be further classified. Under this condition, it is difficult to match the subcluster in hzo cluster 1c to that in the 16S rRNA gene

Scalindua cluster one to one. Further investigation is needed for identifying the *hzo* sequences more clearly.

The sequences in cluster P formed a separated branch in the 16S rRNA gene phylogenic tree, showing clearly low identities with known anammox bacteria though they have a closer relationship with the known anammox clade than other non-anammox *Planctomycetes*, which might comprise a new potential *Planctomycetes* genus which remains to be elucidated further.

The PCoA analysis showed that anammox bacteria have obvious geographical distribution characteristics. It can be concluded that the special geographical conditions of subsurface sediment in the SCS could be a factor that influenced the evolution of diverse *Candidatus* Scalindua anammox bacteria different from other marine environment.

A series of Integrated Ocean Drilling Programs demonstrated that the subsurface deep-sea biosphere harbors a huge biomass and complex physical–ecological processes [49]. As the most important elements for life, the nitrogen biogeochemical cycle is an essential process in this system. According to our data, inorganic nitrogen is not limited for microbial metabolism in the deep-sea sediment of the SCS. The removal of inorganic N is an important step in maintaining N cycling in this ecosystem. With the amplification of 16S rRNA and *hzo* genes, anammox bacteria were detected in the subsurface sediments of the SCS and *hzo* gene abundance was $1.19\sim7.17\times10^4$ copies per gram of dry sediments, suggesting that anammox could be involved in the nitrogen removal process. Furthermore, the correlations of anammox bacterial diversity and abundance with the different nitrogen substrates provided further evidence to the potential activity of anammox bacteria in deep-sea biosphere. However, the exact roles of anammox in the subsurface ecosystem of the SCS remain unknown and require further investigation in the future.

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