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Molecular detection of *Candidatus* Scalindua flavia, study of anammox bacterial community structure, composition in the sediments of the East China Sea and the Yellow Sea

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In this study, the diversity, community structure and distribution of anammox bacteria in sediments from five sites of the ECS and YS were explored by using both the 16S rRNA and *hzo* functional gene biomarkers. A high diversity of anammox bacteria in the studied sites with *Candidatus* is observed. Scalindua being the dominant group of anammox bacteria. With 16S rRNA gene primers, an inimitable *Candidatus.* Scalindua phylotype was identified, putatively represented a novel species, and tentatively named "*Candidatus.* Scalindua flavia". In addition, three other distinct clades were also detected with a discrete clustering in the consensus tree. Statistical analysis of environmental variables indicated that NH_4^+ and PO_4^{3-} are positively correlated to shape the differential distribution of anammox bacteria in different environments.

[Keywords: Anammox bacterial diversity, Novel *Scalindua* specie, East China Sea and Yellow Sea]

Introduction

Anaerobic ammonium oxidation (anammox) is a newly discovered biochemical pathway involved in the microbial nitrogen cycle, and it allows coupling between ammonium oxidation and nitrite reduction under anoxic conditions[1]. Discovery of anaerobic ammonium oxidizing bacteria has changed the assumption that fixed nitrogen could only be removed by denitrification. The anammox bacteria are chemolithoautotrophic in nature, belonging to *Planctomycetes*[2]. So far, five putative genera of anammox bacteria that are "*Ca.* Kuenenia" "*Ca.* Scalindua" "*Ca.* Anammoxoglobus" "*Ca.* Brocadia" and "*Ca.* Jettenia" have been identified from various wastewater treatment plants and different natural environments[2-7]. In addition, fourteen species have also been delineated, including novel species of genus *Ca.* Scalindua such as "*Ca.* Scalindua zhenhei" I, II, III and "*Ca.* Scalindua sinooilfield", which were recovered from sediments of South China Sea and petroleum reservoirs Yellow River Delta, respectively[8 & 9]. The cells of anammox bacteria are composed of distinctive compartmentalization and consist of specialized structures, such as anammoxosome, riboplasm and paryphoplasm. Moreover, an additional protein surface layer (which is a crystalline array of protein subunits) was recently illustrated in anammox cell[10].

The speculation about the microbially facilitated anammox process was first made in 1977[11], whereas, the process was discovered in a wastewater treatment plant of the Netherland in 1995[12]. Among marine natural environment, anammox bacteria were initially documented in the Black Sea[13]. Afterward, the anammox activity was detected in diverse ecosystems, including freshwater (Lake Tanganyika), estuaries, hypoxic or anoxic water columns, marine sponges, sea ice, sediments, activated sludge, multiyear sea ice and geothermal subterranean oil reservoirs[2, 5-7, 13 & 14]. In congruence to denitrification, anammox bacteria also play a significant contribution in the nitrogen loss in some marine environments i.e. 30-70%[15-17].

The current understanding of anammox community and their impact on marine and global nitrogen production has absolutely changed the views of scientists in terms of their role in different marine ecosystems and the mechanism involved in the process and their evolution[18-20]. Previous studies have revealed that environmental factors had a significant impact on the overall composition, abundance and distribution of anammox bacteria depending on the ecosystem. Similarly, anammox bacteria responded differently to nitrogen loss in these regions accordingly; albeit the exact role is still dubious [17].

Molecular techniques based on 16S rRNA genes, DNA hybridization and functional gene biomarkers are the methods of choice for ecological studies of anammox bacteria, due to the fact that they could not be isolated as a pure culture because of their nutritional complexity and slow growth rate[3]. Hydrazine is an important intermediate product in the anammox process and the genes encoding the synthase and oxidoreductase of hydrazine are present in all cells of anammox bacteria. Hence, both these enzymes can be used as functional biomarkers to investigate the ecological distribution of anammox in natural habitats[21-24]. The utilization of these enzymes is preferable because of their functional implications with a comparison to 16S rRNA gene-based approaches.

In this study, the sediments of two vital Chinese marginal seas, including the East China Sea (ECS) and Yellow Sea (YS) were focused, and anammox bacterial community was investigated. The YS located in the northern part of China and Korean Peninsula is a semi-enclosed sea[25], receiving a great amount of discharge from both the Changjiang River and Yellow River. The ECS is one of the major marginal seas of the North Western Pacific. It receives sizeable input from the Changjiang River as compared to the other rivers[26]. During last few decades, anthropogenic interferences have greatly increased the ecological stresses in the ECS. Moreover, the overall biogeography of ECS has been greatly influenced due to overpopulation in the basin of the Changjiang River, and the coastal areas of ECS[27]. Generally, the ECS has a complex heterogeneous nature, which receives nitrogen elements from multiple sources. Therefore, it is supposed to be a relatively diverse marine environment for anammox bacteria.

In this study, five different stations from the ECS and YS were chosen to determine the diversity, distribution and community structure of anammox bacteria by using 16S rRNA and *hzo* gene clone libraries. In addition, various environmental parameters were measured and used to analyze their possible impacts on the distribution and composition of anammox bacterial community in the connecting seas.

Materials and Methods

Five sediments samples were collected from four different mud areas of East Chinese marginal seas (ECMS) using a stainless steel box-sampler during cruise *R/V 'Dong Fang Hong 2'*in July 2013. The water depth at sampling sites ranged from 24 to 83 m.

Sampling was performed carefully by inserting two PVC tube of 7.5 cm diameter from inside into the boxsampler in order to collect sub-cores and to avoid surface sediments intact. Steel cutter was used to section 1 cm of PVC containing samples immediately, and to store at -20°C onboard and later at -80°C in the laboratory before initiating DNA extraction and organic carbon analysis. At every cm distance across the whole of the sub-core, the pore waters were collected by using Rhizon samplers injected through the pre-drilled holes in the core tube, which was connected to vacuum test tubes. The $HgCl_2$ treatment of collected pore water was done and stored at refrigeration temperature for further analysis of dissolved inorganic nutrients.

Total organic carbon (TOC), total nitrogen (TN), stable carbon and nitrogen isotopes ($\delta^{13}C$ and $\delta^{15}N_{TN}$) were studied by the method described previously [28]. Dissolved inorganic nutrients, i.e. nitrate, nitrite, ammonia, phosphate and silicate (NO_3^-, NO_2^-, NH_4^+, $PO_4^{3-}$and SiO_3^{2-}) in sediment pore waters were analyzed by the calorimetric method by a nutrient autoanalyzer (AA3, Seal Analytical Ltd., UK). Detection limits for NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}, SiO_3^{2-} were 0.02, 0.01, 0.04, 0.02, 0.01 μM L^{-1}, respectively. Analytical uncertainty for all dissolved nutrients in replicate samples was < 5-10%.

Sediment samples from five different sites of ECS and YS were selected for anammox bacterial studies. Two sampling sites C05 and L06 in the YS are located closer to Korean peninsula northward and Shandong peninsula southward and on east towards the Yellow River. Therefore, it gives us an evidence that both the studied sites (C05 and L06) could possibly be influenced by these surrounding areas (Fig. 1). The other three sites were located at the ECS, P01 is adjacent to the estuary of Changjiang River and ME3 links to Zhejiang coastal areas and Changjiang River from where they receive the bulk of nutrients along with water inflow and sediments. Sampling site MT3 is closer to highly populated Cheju Island of the Korean peninsula. All the 3 sites were linked to coastal areas, and therefore, they would have some possible environmental disturbances (Due to limited environmental parameters from the site (MT1), it is not included in the statistical analysis).

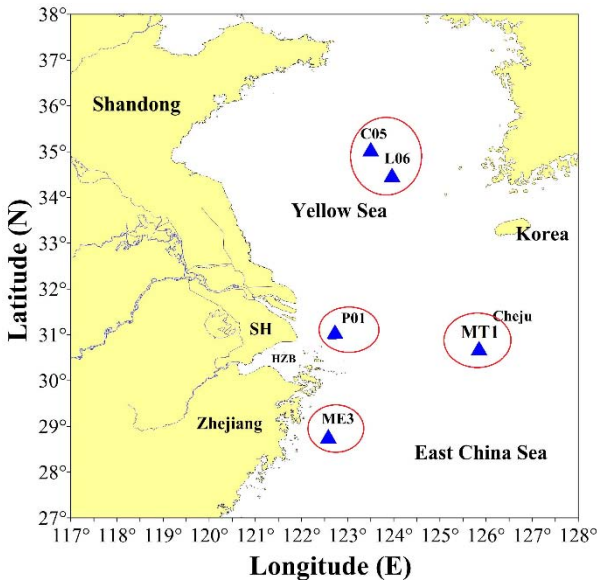

Fig. 1. Map indicating the sampling sites across the Yellow Sea and the East China Sea.

The extraction of genomic DNA from 0.3 g of sediment sample by Power Soil DNA Kit (Mol Bio Laboratories, Inc., Carlsbad, CA) was performed as described by manufacturer protocol with brief alterations. The extracted DNA concentration was measured by a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA) spectrophotometer. For both 16S rRNA and functional *hzo* genes, nested PCR method was used as described previously[22; 29]. Initial amplification of 16S rRNA gene was done by primer, Pla46f (5´-GACTTGCATGCCTAATCC-3´) Pla1390r (5´-CGACAAGGAATTTCGCTAC-3´) with thermal profile described before[4]. The resulting product was used as a template for the second round of PCR amplification of anammox specific fragment with another set of primers Amx368f (5-TTCGCAATGCCCGAAAGG-3´) and Amx820r (5-AAAACCCCTCTACTTAGTGCCC-3´) with a thermal profile as described previously[30]. In PCR reaction, the quantity of reactants used included: 5 µl of 5× Go Taq Flexi buffer (Promega, USA), 4 µl of dNTPs (2mM, TOYOBO CO., Japan), 4 µl of $MgCl_2$ (25mM, Promega), 4 µl of DNA (15-25 ng µl-1), 1 µl of BSA (100 mg ml-1, Takara), 0.5 µl of each primer (50mM), 0.5 µl of Go Taq DNA Polymerase (5 U µl-1, Promega).

For the amplification of *hzo* gene fragment, the analogous methodology was employed with the primers AB1F/R and AB4F/R[22]. The final volume of 50 µl PCR mixture contained 5 µl of 5×Go Taq Flexi buffer (Promega, USA), 5 µl of dNTPs (2mM, TOYOBO CO., Japan), 4 µl of $MgCl_2$ (25mM, Promega), 4 µl of DNA (15-25 ng µl-1), 2 µl of BSA (100 mg ml-1, Takara), 0.5 µl of each primer (50mM), 0.5 µl of Go Taq DNA Polymerase (5 U µl-1, Promega). PCR schemes were applied as described previously[22].

1% agarose gel was used for further confirmation and separation of the final product, which was in turn purified by Agarose Gel Recovery Kit (Biomed Co., Beijing, China) via recommended protocol. Ligation of the purified fragment with pUCm-T vector (Sangon biotech Co., Shanghai, China) was carried out and further transformed into competent *Escherichia coli* JM-109 strain prepared in the lab. The positive clones were picked up in the selection process by using X-Gal-IPTG LB indicator plates modified with 100 µg/ml ampicillin and re-confirmed further by performing colony PCR technique using M13F/R vectors. Similarly, the PCR product was run on 1% agarose gel and rechecked to avoid inappropriate fragment selection for sequencing.

The 16S rRNA gene and Hzo amino acid sequences after translation were aligned by Clustal-X (version 2.1)[31]. Both the gene sequences were grouped into operational taxonomic units (OTUs) by 3% cutoff value using Dotur program[32]. Phylogenetic tree was constructed by the neighbor-joining algorithm

with Kimura 2-paramters and P-distance method, respectively[33] with MEGA software (version 5.2)[34] and bootstrap values (n=1,000 replicates). The phylogenetic analysis was performed by previously applied method[35].

All the sequences obtained in this study are available in National Centre for Biotechnology Information (NCBI) GenBank database under the accession number of KP126678-KP126723 and KP273970- KP274016 for 16S rRNA gene and Hzo amino acid sequences respectively.

Dotur (program) was used to estimate the diversity of anammox bacteria using Chao 1, Shannon indices, Simpson, rarefaction analysis and numbers of observed OTUs were calculated for each gene library. Topographical distribution of the phylogenetic structure of anammox bacterial assemblages and their correlations to ecological parameters were analyzed through an online software, Fast UniFrac, by using Jackknife environment clustering and principal coordinate analysis (PCoA)[36]. Canonical correspondence analysis (CCA) was performed by using CANOCO software (version 4.5) to test the correlations of phylogenetic structure and ecological parameters.

Results

Sediment samples from five different stations of the YS and ECS were selected, and ecological factors were analyzed (Table 1).

Comparatively, most of the detected environmental parameters in this study were scarce in magnitude, particularly, the dissolved inorganic nitrogen (NO_3^-, $NO_2^-$and NH_4^+) as compared to analysis in some other areas. As high quantities of the dissolved inorganic nitrogen were determined from Jiaozhou Bay and Bohai Sea[21 & 37].

Sediment pore water ammonium concentration was 12 to 140μM. However, silicate and phosphate were relatively found in elevated quantity; the concentrations of sediment pore water phosphate and silicate ranged from 0.90 to 8.40μM and 115 to 259μM, respectively. Most of the environmental parameters were reported abundantly in the YS; whereas, only ME3 site of ECS showed a high concentration of ammonium (140.5μM). No significant differences in TOC, TN, C/N, δ^{13}Cand δ^{15}N were detected among the samples.

The protocol described previously[22 & 29], was used to detect 16S rRNA gene sequences with a final

Table 1-Physical and chemical properties across all samples from East China Sea and Yellow Sea

Name	Longitude	Latitude	$N0_3^-$ μM	NO_2^- μM	NH_4^+ (μM)	SiO_3^{2-} (μM)	PO_4^{3-} (μM)	TOC %	TN%	C/N	δ^{13}C ‰	δ^{15}N%
C05-2	123.50º	35.002º	2.10	0.42	47.1	259.01	3.13	0.97	0.14	8.31	-22.2	5.61
L06-2	123.960º	34.440º	1.41	0.36	66.5	181.57	8.41	0.79	0.13	7.32	-22.1	3.86
ME3-2	122.58º	28.732º	0.78	0.19	140.5	115.07	0.90	0.66	0.12	6.47	-22.6	2.57
P01-2	122.7º	31.042º	0.82	0.27	12.4	196.48	2.71	0.83	0.14	6.95	-21.5	3.80
MT1-2	125.50º	31.99º	ND	ND	ND	ND	ND	0.43	0.07	7.64	-21.7	5.72

Table 2 Diversity features and estimated richness by using 16S rRNA gene and *hzo* gene sequences recovered from sampling sites of the Yellow Sea and East China Sea

Station	No. of Sequences		No, of OTUs		Shannon		Chao 1		Simpson	
	16S	*hzo*	16S	*hzo*	16S	*hzo*	16S	*hzo*	16S	*hzo*
C05	89	83	13	12	1.98	1.49	16.3	40	0.168	0.319
L06	96	110	10	6	1.81	1.31	10	6	0.182	0.310
ME3	110	100	14	21	1.77	2.285	29	34.75	0.254	0.174
P01	89	84	13	15	1.76	1.765	14	33	0.257	0.252
MT1	110	98	19	16	1.725	2.083	45	18.5	0.279	0.167

amplification product of 477bp (Fig 1S, Electronic Supplementary Material). Five gene clone libraries were constructed from distinct sampling stations. Total of 500 sequences with 3% cutoff value of the nucleotide variation were used to define the OTUs and 46 distinct phylotypes were obtained. From each gene clone library, variable numbers of OTUs were estimated to be 9 to 20, indicating incongruent diversities which accounted for 19.6%, 19.2% and 6% of the total sequences, respectively. Hence, it can be assumed that this is possibly the most prevalent and abundant anammox bacterial group present across all the studied sites of ECS and YS.

Phylogenetic analysis of the retrieved representative sequences of the 46 OTUs indicated that sediment samples of both the seas were comprised of diverse groups of anammox

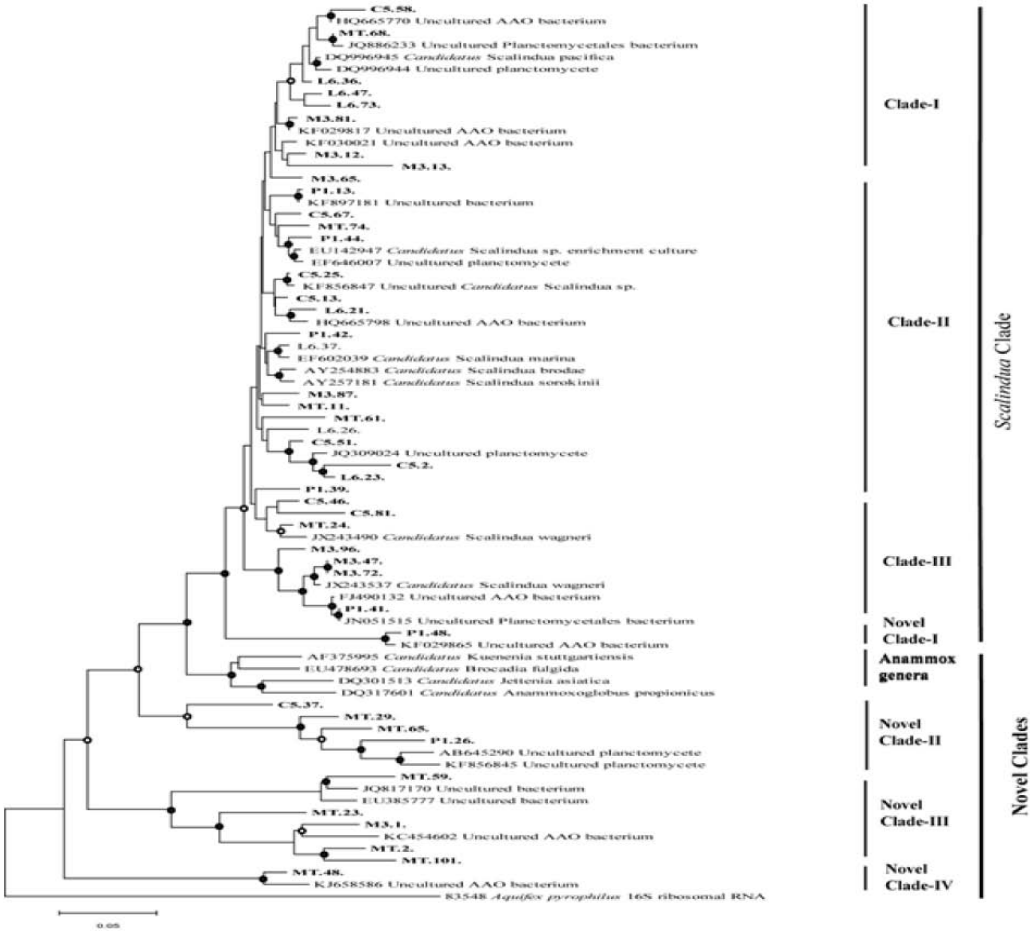

Fig. 2-Phylogenetic tree of the anammox bacterial 16S rRNA gene sequences recovered from five selected sites of the East China Sea and the Yellow Sea. The open and solid circles on each node represent ≥50 but <70, and ≥70 bootstrap value respectively. The substitution rate of nucleotide is characterized by a distance of tree branch, and the expected number of changes per homologous position is signified by the scale bar. The16SrRNA genes of *Aquifex pyrophilus* (M83548) were used as out-groups. (MT-9 monophyletic OTU was excluded from phylogenetic analysis due to doubtful results).

(Table 2). Based on diversity estimators, richness indices and number of OTUs, the highest diversity was observed at the MT1 site situated in the south of the ECS close to the Cheju Island, whereas the lowest diversity was noticed from the L06 site located in the YS (Table 2, Fig. 1 and Fig. 4a).

Three OTUs that were M3-12, L6-37 and C5-51 were found in all five gene libraries generated, bacteria. More than 95% of the sequences belonged to the *Ca.* Scalindua group and the rest represented the members of *Planctomycetes* with an unknown identity. Seven distinct clades of anammox bacteria, which included almost all the putative *Ca.* Scalindua species, were classified by phylogenetic analysis. One novel species of *Ca.* Scalindua lineage (Fig 2; 2S, 3S, 4S-Electronic Supplementary Material), and three unknown anammox members were also detected (Fig 2). All

the retrieved 16S rRNA gene sequences used in the phylogenetic analysis had similarity levels of 76 to 100%. However, they shared 91-100% identity with the top-hit sequences in the NCBI GenBank database. The clade-I which was thesecond dominant part of the tree had the identities of 96-98.7%, 97.3-99% and 96.9-99.2%, respectively with sequences recovered from sediments of the South China Sea, marine sediment of Zhoushan Island, and freshwater wetland sediments respectively[24, 37-39].

Largest fraction of the phylogenetic tree was composed of clade-II and its sequences showed similarity levels of 96-99.6%, 96.9-99.6%, 95.4-99.1%, 95.4-99.1% and 88-97.7% with sequences recovered from sediments of Arabian Sea, marine Sediments of the Gullmar fjord, Sweden, sediment of Black Sea, wastewater treatment plant landfill leachate in Pitsea and Peru Margin sediments[5, 13, 40 & 41].

However, sequences of *Ca.* Scalindua wagneri recovered from the intertidal sediments of the Yangtze estuary shared similarity level of 96.7-97.3% with sequences representing the clade-III in the consensus tree [42]. Inimitable novel clade-I exhibited affiliation with the sequences retrieved from marine sediments of Zhoushan Island and shared an identity of 96-98.7%. The sequences of the novel clade (OTU-P1.48) shared less than 95% resemblance with the closest matched sequences used in the study. In addition, the distinct topology of the monophyletic cluster in the tree, high bootstrap value and distance based similarity with all the known members of anammox bacteria used in the tree also validate its novelty. As the novel cluster was also distantly related, i.e. <95% sequences similarity with all the candidate sequences previously used in the study[37 & 43](Fig 2S, 3S, 4S–Electronic Supplementary Material), thus confirmed as novel *Ca. Scalindua* species, and provisionally suggested the name as "*Ca.* Scalindua flavia".

The novel clade-II situated on a distinct position in the phylogenetic tree had no association with known genera of anammox bacteria, therefore, indicating that they might be

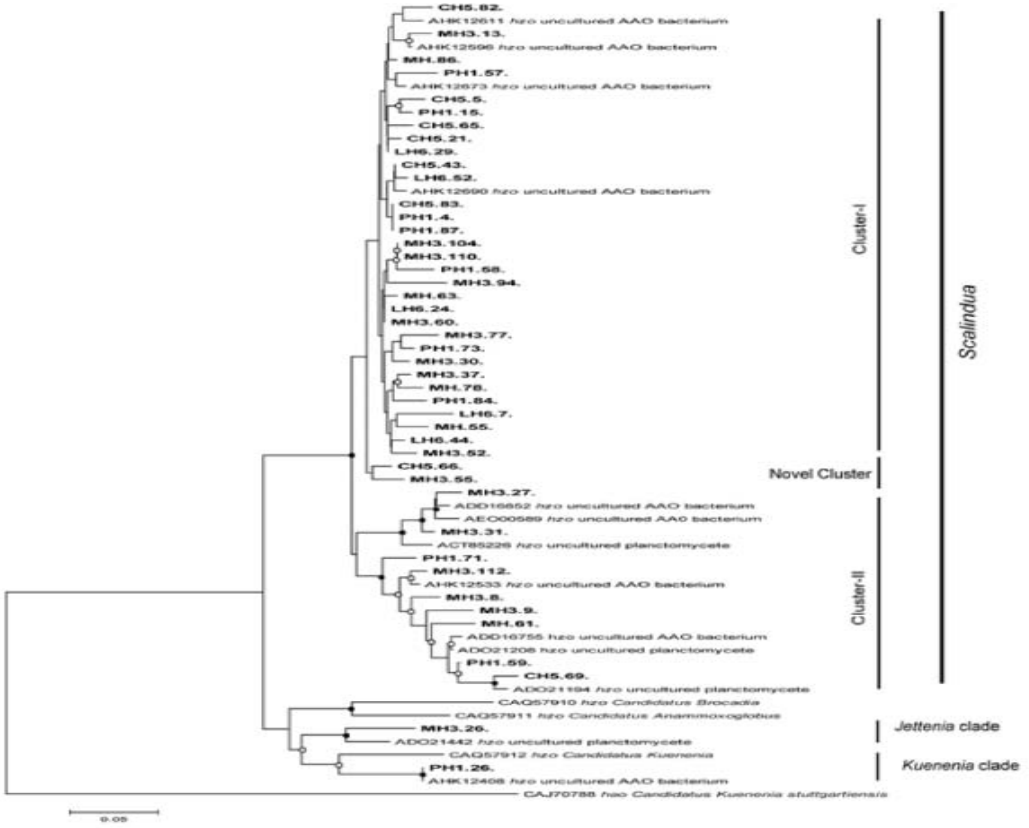

Fig. 3-Phylogenetic tree of the anammox bacterial Hzo protein sequences deduced from retrieved *hzo* gene sequences from the East China Sea and the Yellow Sea. The substitution rate of nucleotide is characterized by a distance of tree branch, and the expected number of changes per homologous position is signified by the scale bar. The open and solid circle on each node represent ≥50 but <70 and ≥70 bootstrap value respectively. The *Ca.* Kuenenia stuttgartiensis (CAJ70788) was used as out-groups.

novel genera of anammox bacteria/member of *Planctomycetes*. Due to limited data and information, these are not considered as novel genera; however, they are presumed as a novel members of *Planctomycetes*.

This clade consisted of few phylotypes showing resemblance with the sequences obtained from different marine environments, subfloor sediments of South China Sea, subfloor sediments at Good Weather Ridge and terrestrial aquatic system, and share 74.3-97.7% similarity.

Similarly, novel clade-III and IV showed their affiliation with sequences obtained from the intertidal marshes of Yangtze estuary and sub-seafloor sediments of Shimokita Peninsula with similarity level of 97.3% and 86.2-93.4%[44]. Last 3 novel clades were also recovered, discussed and classified in our previous study on Chinese marginal seas. More than 95% of the sequences detected in this study matches with the known assemblages of *Ca.* Scalindua lineage, whereas 0.2% matched with the novel *Ca. Scalindua* clade. Hence, *Ca.* Scalindua lineage was found the most dominant, prevalent and diverse throughout the target areas of the study.

For *hzo* gene sequences, AB1F/R primers were used initially to amplify a product around 1500bp.

In addition, the product obtained was used further with AB4F/R primers to amplify a final product of 600bp (Fig 1S–Electronic Supplementary Material). The cloned 475 Hzo amino acid sequences were grouped into 47 distinct OTUs with 3% cutoff value of amino acid variation and five gene clone libraries were generated. Variable numbers of OTUs (6-9) with distinct diversities were also estimated with Hzo protein sequences out of 5 gene clone libraries established (Table 2). Based on the number of OTUs and other diversity indices, the highest diversity of anammox bacteria was observed at ME3 site of the ECS, and lowest diversity was detected at the L06 site of YS (Table 2 and Fig. 4b).

When distributions of OTUs were probed along the whole gene libraries generated with deduce amino acid sequences, it was observed that six OTUs were ubiquitous in all the gene libraries, including (MH3-61, L6-44-52, CH5-43-66 & PH1-4) which accounted for 21%, 14.5% each, 7.3%, 5.6%, 7.5% and 5.9% of the total Hzo amino acid sequences used in all the gene libraries constructed. Therefore, it can be assumed that all these groups of anammox bacteria are found as potentially most predominant in the sediments of ECS and YS.

The phylogenetic tree constructed with Hzo protein sequences indicated that nearly all the sequences were closely related to the known *Ca.* Scalindua lineage except for one novel clade (Fig 3). Similarly, two other known genera of anammox bacteria, including *Ca.* Jettenia and *Ca.* Kuenenia were also recovered from distinct locations of ECS.

Approximately, 86.4% sequences retrieved, belonged to known *Ca.* Scalindua lineage, whereas nearly 13% represented the novel *Ca. Scalindua* clade. All the sequences used in the phylogenetic consensus tree had an affiliation with sequences recovered from different environmental samples, including surface sediments of the Pearl estuary, samples from various aquatic ecosystems, hypernutrified Jiaozhou Bay and deep-sea subsurface sediments of the South China Sea [21, 22, 43 & 45].

The most prevailing clade-I resembled with sequences recovered from the surface sediment of Pearl estuary with similarity level of 94.3-98.6% and 93.6-97.9% [43]. The unique position of the novel clade-I in the consensus tree and distant similarity level with all the known *Ca.* Scalindua clades indicated its exceptional identity and hence proposed to be the novel clade in *Ca.* Scalindua group.

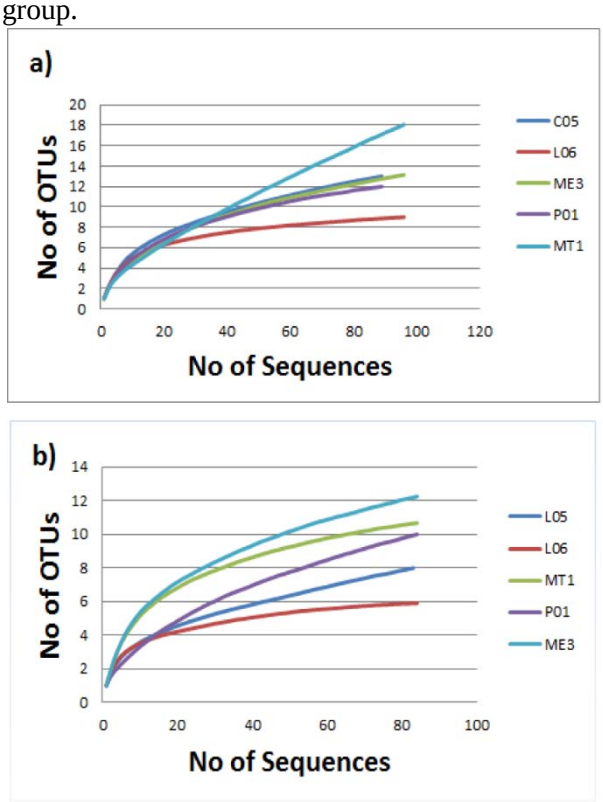

Fig. 4-Rarefaction analysis of anammox bacterial community with a) 16S rRNA gene sequences and b) deduced Hzo protein Sequences retrieved from the East China Sea and the Yellow Sea by using DOTUR program with a 3 % cutoff value.

The sequences from the Paddy Soil, subsurface sediment of the Pearl estuary, hypernutrified Jiaozhou Bay and deep-sea subsurface sediments of South China Sea and various aquatic ecosystems showed an affiliation with the phylotypes of clade-II, with resemblance of 90-97.1%, 93.6-99.3%, 94.4-99.3% and 94.3-97.9%[21, 22, 43, 45 & 46]. Similarly, some of the obtained phylotypes used in the study showed identity with *Jettenia* clade retrieved from the Black River sediments and those with *Kuenenia* clade was recovered from enrichment culture and subsurface sediment of the Pearl estuary[22, 43 & 47]. The deduced amino acid sequences shared 82.3-97.5% sequence identity with sequences retrieved in this analysis and 95-100% similarity with top-hit GenBank sequences in the NCBI.

The topographical distribution of anammox bacterial community from the selected sites was analyzed with both 16S rRNA gene sequences and Hzo protein sequences, and the impact of environmental variables were determined by using CCA. CCA analysis, after using 16S rRNA gene sequences and environmental parameters, indicated that NH_4^+ had a positive correlation with addition, SiO_3^{2-} and TOC also played a reasonable role to shape the pattern of anammox communities from sites ME3 and P01 (Fig. 5b).

Similarly, when 16S rRNA sequences were used for environmental clustering with the help of Weighted UniFrac by Jackknife method, the result revealed few clusters of anammox bacterial assemblages (Fig. 6Sa). A similar result was displayed by PCoA (P1 and P2) (Fig. 5Sa), which describe 75.26% of the total anammox bacterial community variability between all the stations together.

When environmental clustering was carried out with the help of Weighted UniFrac by Jackknife method, the results indicated the identity and segregation of few clusters of anammox bacterial assemblages with Hzo protein sequences (Fig. 6Sb). The site ME3 was evidently different from other sites; this classification was further established by PCoA using Weighted UniFrac (Fig 5Sb). The site ME3 could be distinguished from rest of the sites along the first principal coordinate (PCoA), which explain 62.83% of total anammox bacterial variability.

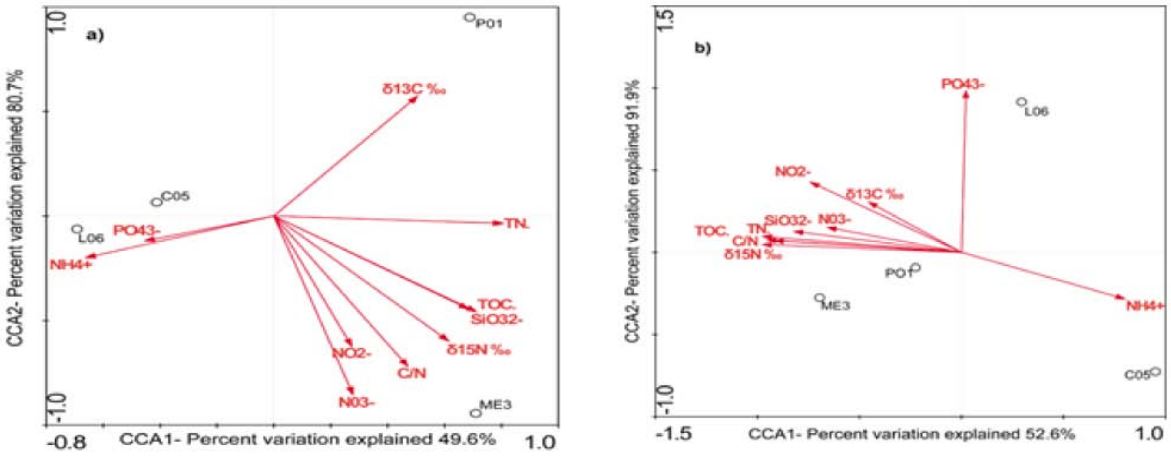

Fig. 5-CCA ordination plots for the first two dimensions to display the correlations to display the correlation between the anammox bacterial diversity with ecological factors studied by using b) 16S rRNA gene and a) deduced Hzo protein sequences retrieved from sediments of five different sites of the East China Sea and the Yellow Sea. The length and angle of the arrows represent the relationships among ecological factors and CCA axis.

the distribution of anammox bacteria on sampling site L06 and C05, phosphate (PO_4^{3-}) contributed moderately; whereas, the rest of variables showed no significance on the distribution of anammox bacterial community across the studied sites (Fig. 5a). Likewise, the CCA analysis with Hzo protein sequences also displayed congruent outcomes as determined by 16S rRNA gene sequences. In

Discussion

The views of the scientist about the nitrogen cycling pathways has been changed after few decades of discovery of anammox process. Therefore, currently anaerobic nitrogen production is considered to be an imperative pathway in a marine environment[3, 12 & 15].

Relatively high diversity and abundance of

anammox bacteria have been reported previously from anoxic or hypoxic environments[16 & 19]. Our analysis reveals that *Ca.* Scalindua genus of anammox bacteria found predominantly in low-temperature marine environments, including surface and subsurface marine sediments and sea water column, these findings are in agreement with studies performed previously[40 & 48]. In this study, 16S rRNA and *hzo* gene primers were used to evaluate anammox bacterial diversity and their distribution in sediment samples from five different sites of ECS and YS. However, *Ca.* Scalindua clade was the most pervasive and predominant anammox bacterial group with both the gene markers. Similar findings were previously documented from the sediments of Bohai Sea in the former study[37]. The existence of *Ca*. Scalindua lineage with both the gene biomarker in the sediments of ECS and YS not only validated the taxonomy of this group with 16S rRNA gene, it also exposed the potential role of *Ca*. Scalindua in the nitrogen sink of studied ecosystem with *hzo* gene sequences. Previous studies, based on 16S rRNA gene, indicated that overall diversity of anammox bacteria in marine samples was quite low except for *Ca.* Scalindua lineage[40, 49-51]. Results retrieved from the diversity indices showed that increased diversity was estimated with 16S rRNA sequences as compared to the data obtained by deduced Hzo amino acid sequences (Table 2) from the sediments of ECS and YS. The highest diversity of anammox bacteria was observed with 16S rRNA gene sequences from the sediments of MT1 site located in ECS near the Cheju Island, whereas the lowest diversity was detected from sediments of L06 site in the YS (Fig. 1 and Table 2). Rich diversity of anammox bacteria from sediments of the site MT1 might be due to the close proximity with the Cheju Island. Cheju Island, because of its tourism and agricultural activities and other factors like the high load of sewage water, may contribute substantially to shift the diverse types of bacterial communities and associated nutrients through runoff water.

Phylogenetic analysis by 16S rRNA gene sequences displayed that the novel clades identified in this study are predominantly composed of phylotypes and sequences retrieved from MT1 site adjacent to the Cheju Island. This validated the inimitable diverse nature of the targeted site with enriched environment for anammox bacterial community and potentially distinct environmental features. However, most of the environmental parameters used throughout the study could not be detected at this site, giving an impression that several unidentified ecological parameters might contribute to a high diversity of anammox bacteria. The phylogenetic analysis reveals that *Ca.* Scalindua was the most prevalent genera of the anammox bacteria identified from all the studies sites. In addition, detection of novel phylotype related to *Ca.* Scalindua authenticated the diversity and richness of this genus throughout the targeted areas. Similarly, identification of novel phylotypes of *Planctomycetes* indicated that various other novel members of anammox bacteria can be discovered by improving the PCR strategies and by using more targeted primers. Several studies have previously reported various *Ca.* Scalindua species from different ecosystems[4, 13, 37, 40, 43 & 45]. Novel clades identified in the study placed a discrete clustering in the consensus tree. In addition, the distance-based pair wise similarity level and high bootstrap values after phylogenetic analysis with other known genera/species of anammox bacterial lineages were quite distant as well. Thus the hypothesis of this novel clade to be announced as discrete specie of *Ca. Scalindua* is adopted from a previous study performed on the sediments of Bohai Sea and Pearl estuary[37 & 43]. The novel clade clustering in *Ca.* Scalindua group showed less than 95% of similarity with top-hit sequences in the GenBank databases; high bootstrap values between the clusters after phylogenetic analysis also confirmed their novelty. This phylotype was therefore considered to be the novel *Ca.* Scalindua specie, and was provisionally named as *Ca.* Scalindua flavia (Figs. 2S, 3S, 4S–Electronic Supplementary Material) [37]. In the current study, after investigation with 16r rRNA gene, none of the documented genera of anammox bacteria could be determined except *Ca.* Scalindua besides some presumably novel member of anammox/*Planctomycetes* bacteria. The low coverage, non-specificity of 16r rRNA gene and inappropriate PCR techniques might be the possible reasons responsible for a lower detection limit of anammox bacterial communities[29 & 52]. Previous studies have proven that 16S rRNA gene-targeted primers lack the ability to cover all the anammox bacterial lineages; therefore, functional gene markers such as *hzo* gene targeting primers are the ideal options to get the desirable results[23].

When *hzo* gene sequences were analyzed, the diversity indices and richness estimators indicated that a highest diversity of anammox bacteria from the ME3 sampling site of the ECS situated near

the Zhejiang River. However, like previous results with 16S rRNA gene primers, the lowest diversity was reported at the site L06 (Fig. 1). The ME3 site is relatively close to the coastal areas of Zhejiang province, which is densely populated area and possibly anthropogenic disturbances could be the possible reason to affect the studied site. The Changjiang River contributes significantly to shape the community structure due to huge penetration of fresh water and associated nutrients into the ECS. In addition, the ECS receives a huge pressure of anthropogenic interferences from the coastal areas and drainage sink of Changjiang River[27]. It has also been observed that different concentrations of nitrite, silicate and especially ammonium and phosphates might have a significant impact on the diversity of anammox bacterial community in the area mentioned above. Hzo protein, as a member of cytochrome C oxidoreductase protein family, played a key role in anammox process to dehydrogenate hydrazine to N_2; the protein has already been isolated from most of the anammox bacteria, including new genera[7 & 53]. It has been recommended that *hzo* gene could be used effectively for phylogenetic analysis as the functional marker gene[20 & 47].

Phylogenetic analysis with *hzo* gene primers revealed a diverse community composition of anammox bacteria. With two clades of *Ca.* Scalindua, one novel *Ca.* Scalindua clade and two other known genera of anammox bacteria were also detected. Identification of *Kuenenia* and *Jettenia* clade along with novel *Ca. Scalindua* clade indicated a high coverage potential of *hzo* gene primers together with higher specificity for the anammox bacteriallineages[7 & 37]. The *Ca.* Brocadia and *Ca.* Kuenenia were usually found in waste-water treatment plants or reactors. Therefore, it could be assumed that the P01 site might have been slightly affected by anthropogenic activities via nearby coastal areas of Zhejiang province or Changjiang River[46].In congruence to previous studies, with Hzo protein sequences, *Ca*. Scalindua was found to be the most prevailing group identified from all the gene libraries[40]. The distribution and niche specificity of four other genera of anammox was further evident when *Ca.* Scalindua was found prevalent throughout the study area[46].

The anammox bacterial classification and community structure showed the uniqueness of the site ME3 as compared to the rest of four studied sites when Jackknife method and PCoA analysis was done by using 16S rRNA gene sequences (Fig. 5Sa and 6Sa–Electronic Supplementary Material). Likewise, UniFrac environmental clustering and PCoA analysis with Hzo protein sequences further validated the distinctiveness of anammox bacterial community from the above mentioned site (Fig. 5Sband 6Sb–Electronic Supplementary Material).

For anammox activity, the equal presence of ammonium and nitrite were accessible, either between aerobic and anaerobic interfaces or nearby sediments[48]. The anammox bacterial distribution was substantially influenced by NH_4^+, particularly at sites L06 and C05 that showed a reasonable effect posed by this parameter. Similarly, PO_4^{3-} also contributed equitably to shape the distributional pattern of anammox community of both these sites. Moreover, SiO_3^{2-} and TOC also contributed marginally significant to shape the anammox bacterial community distribution from station ME3 and P01. It was also observed in the study that various ecological parameters in different magnitude augmented different anammox bacterial communities in the environment[37].

Conclusions

The current study reveals that both the neighboring seas had an immense diversity of anammox community in the sediments when both the 16SrRNA and *hzo* gene sequences were used. Based on data related to the diversity of anammox bacteria (Table 2), with both the gene markers, a relatively rich diversity was observed with 16S rRNA gene sequences as compared to *hzo* gene sequences across all the studied sites. Like previous studies, *Ca.* Scalindua was found to be the most ubiquitous and predominant group of anammox bacteria in the study area. It was also revealed that some of the environmental factors like ammonium, phosphate, silicone and total organic carbon played some reasonable role in shaping the distribution and community structure of resident anammox bacteria. Due to the detection of one novel specie of *Ca.* Scalindua and some unknown members of *Planctomycetes,* it was also confirmed that the study area had a remarkably diverse nature for anammox community. In order to understand the exact reasons for structuring the resident anammox community, numerous ecological parameters need to be investigated.

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Supplementary Material

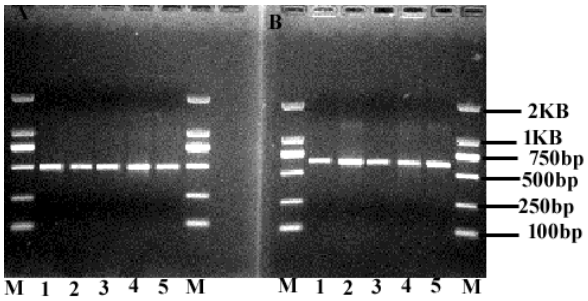

Fig. S1-Gel electrophoresis results of a) 16S rRNA and b) *hzo* gene band with 477 & 600bp sequences respectively. Each number represent sampling site of sediment i.e. (**1=PO1, 2=MT1, 3=ME3, 4=LO6 & 5=CO5**) with 2kb marker used.

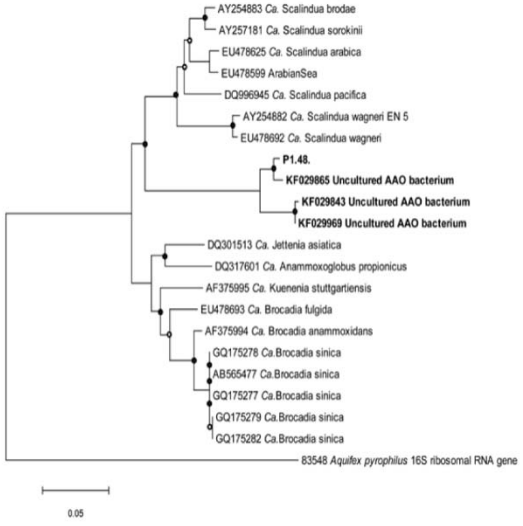

Fig. S2-Neighbor joining phylogenetic tree of the anammox bacterial 16S rRNA genes retrieved in the study with nearly full-length well-defined known anammox species. The open and solid circles on each node represent ≥50 but <70, and ≥70 bootstrap value respectively. The substitution rate of nucleotide is characterized by distance of tree branch, and the expected number of changes per homologous position is signified by scale bar. The *Aquifex pyrophilus* (M83548) was used as out-groups.

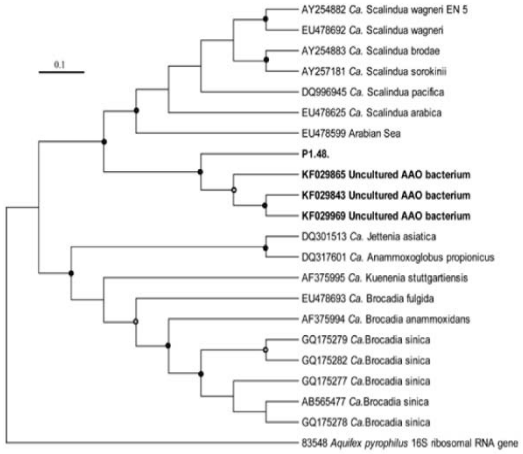

Fig. S3-Parsimony phylogenetic tree of the anammox bacterial 16S rRNA genes retrieved in the study with nearly full-length well-defined known anammox species. Bootstrap values (n=1,000 replicates) higher than 70% are shown with solid circle symbols, and those less than 70% but greater than or equal to 50% are shown with open circle symbols on the corresponding nodes. The substitution rate of nucleotide is characterized by distance of tree branch, and the expected number of changes per homologous position is signified by scale bar. The *Aquifex pyrophilus* (M83548) was used as out-groups.

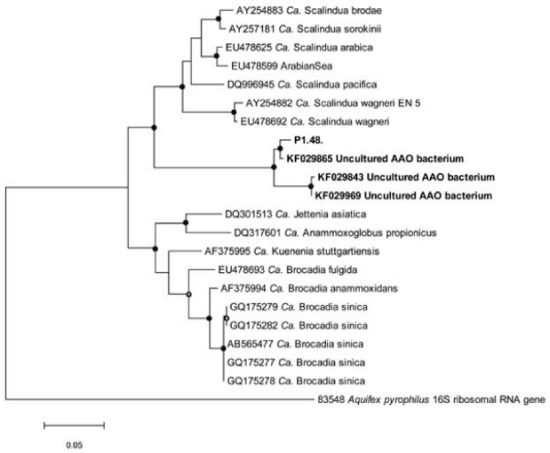

Fig. S4-Maximum likelihood phylogenetic tree of the anammox bacterial 16S rRNA genes retrieved in the study with nearly full-length well-defined known anammox species. The open and solid circles on each node represent ≥50 but <70, and ≥70 bootstrap value respectively. The substitution rate of nucleotide is characterized by distance of tree branch, and the expected number of changes per homologous position is signified by scale bar. The *Aquifex pyrophilus* (M83548) was used as out-groups.

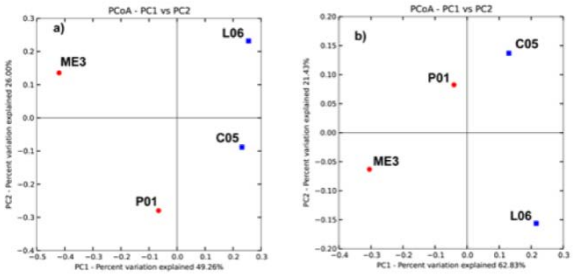

Fig. S5-Principal Coordinate plot by Weighted UniFrac analysis of 16S rRNA gene sequences (a) and Hzo protein sequences (b) recovered from sediments of Yellow Sea and East China Sea.

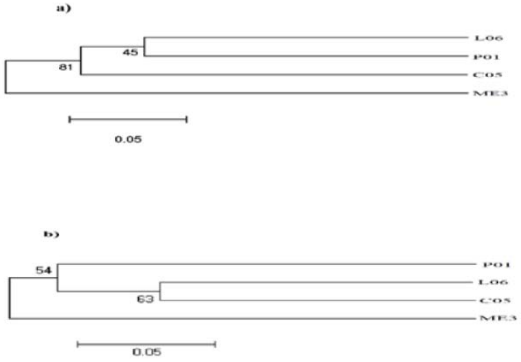

Fig. S6-Dendrograms of the hierarchical clustering analysis of 16S rRNA sequences (a) and *hzo* gene sequences (b) from the sediment of Y.S and E.C.S. Both the clustering dendrograms were obtained by using weight jackknife environment clusters statistical method by Weight UniFrac Fast.