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Mangrove trees affect the community structure and distribution of anammox bacteria at an anthropogenic-polluted mangrove in the Pearl River Delta reflected by 16S rRNA and hydrazine oxidoreductase (HZO) encoding gene analyses

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Abstract Anaerobic ammonium oxidizing (anammox) bacterial community structures were investigated in surface (1–2 cm) and lower (20–21 cm) layers of mangrove sediments at sites located immediately to the mangrove trees (S0), 10 m (S1) and 1000 m (S2) away from mangrove trees in a polluted area of the Pearl River Delta. At S0, both 16S rRNA and hydrazine oxidoreductase (HZO) encoding genes of anammox bacteria showed high diversity in lower layer sediments, but they were not detectable in lower layer sediments in mangrove forest. S1 and S2 shared similar anammox bacteria communities in both surface and lower layers, which were quite different from that of S0. At all three locations, higher richness of anammox bacteria was detected in the surface layer than the lower layer; 16S rRNA genes revealed anammox bacteria were composed by four phylogenetic clusters affiliated with the "*Scalindua*" genus, and one group related to the potential anammox bacteria; while the *hzo* genes showed that in addition to sequences related to the "*Scalindua*", sequences affiliated with genera of "*Kuenenia*", "*Brocadia*", and "*Jettenia*" were also detected in mangrove sediments. Furthermore, *hzo* gene abundances decreased from 36.5×10^4 to 11.0×10^4 copies/gram dry sediment in lower layer sediments while increased from below detection limit to 31.5×10^4 copies/gram dry sediment in lower layer sediments from S0 to S2. The results indicated that anammox bacteria communities might be strongly influenced by mangrove trees. In addition, the correlation analysis showed the redox potential and the molar ratio of ammonium to nitrite in sediments might be important factors affecting the diversity and distribution of anammox bacteria in mangrove sediments.

Keywords Anammox bacteria · 16S rRNA genes · *hzo* genes · Diversity · Distribution · Abundances · Mangrove

Introduction

Anaerobic ammonium oxidation (anammox) is a microbial nitrogen transformation process that allows ammonium to be oxidized by nitrite under anoxic conditions (van de Graaf et al. 1995). The observations from both field and laboratory investigations have indicated that anammox is a key process in the global nitrogen cycle (Devol 2003; Francis et al. 2007). Anammox has been demonstrated in very diverse environments, including the suboxic zone of the Black Sea (Kuypers et al. 2003), oxygen minimum zones (OMZ) at Namibian coast and Arabian Sea (Kuypers et al. 2005; Ward et al. 2009), and a number of temperate estuarine, coastal and offshore sediments (Thamdrup and Dalsgaard 2002; Jetten et al. 2003; Risgaard-Petersen et al. 2004; Tal et al. 2005; Trimmer et al. 2005; Rich et al.

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2008), lakes (Schubert et al. 2006), freshwaters (Penton et al. 2006), polar region sediments and multiyear sea ice (Rysgaard and Glud 2004; Rysgaard et al. 2004), and deep sea hydrothermal vent (Byrne et al. 2009). Anammox microorganisms are monophyletic members of the phylum *Planctomycetes* (Strous et al. 1999; Schmid et al. 2005), including *Candidatus* "*Brocadia*", *Candidatus* "*Kuenenia*", *Candidatus* "*Anammoxoglobus*", *Candidatus* "*Scalindua*" and *Candidatus* "*Jettenia*" (Schmid et al. 2005; Kartal et al. 2007, 2008; Kuenen 2008; Quan et al. 2008).

While most available knowledge about anammox bacteria diversity was based on the 16S rRNA gene sequences, the hydrazine oxidoreductase (HZO), a key protein that dehydrogenated the unique anammox intermediate, hydrazine, to dinitrogen gas, was considered as a new biomarker for anammox bacteria recently (Schalk et al. 2000; Shimamura et al. 2007; Klotz and Stein 2008). Since *hzo* genes are definitively and specifically linked to anammox reaction, analysis of *hzo* genes diversity, abundance and expression in the environment could obviously provide a more comprehensive understanding about anammox bacteria. Several specific primers have been designed to amplify *hzo* genes from various environments, which further confirmed the *hzo* gene as a suitable target for molecular ecological studies on anammox bacteria (Schmid et al. 2008). Recently, a new PCR primer set was also used to detect *hzo* genes in various marine sediments and results indicated that anammox *hzo* genes were broadly distributed in marine environments with a high diversity related to the anthropogenic import (Li et al. 2010). However, there are only few reports using *hzo* genes as biomarkers to investigate anammox bacteria diversity (Quan et al. 2008; Schmid et al. 2008; Li et al. 2009). Thus, there is an urgent need for analysis of anammox bacteria in a wider range of natural ecosystems based on *hzo* genes, a new tool to study anammox bacteria in natural ecosystems.

One major component of the tropical and subtropical coastal wetlands is mangrove ecosystem, which occupies the intertidal zone of estuaries, bays, inlets and gulfs and part of the riparian zone (Alongi 2002). Mangrove ecosystems play an important role as refuge, feeding, and breeding ground for many organisms and sustain an extensive food web based on detritus (Holguin et al. 2001). In mangrove ecosystems, microbial nitrogen processes, including dinitrogen (N_2)-fixation, nitrification, denitrification, ammonification, anammox and dissimilatory nitrate reduction to ammonium, form a complex microbial nitrogen transformation (Purvaja et al. 2008). Among these processes, nitrogen fixation occurred in the rhizosphere of mangrove trees, decomposing leaves, and aerial roots and bark (Alongi 2002), while nitrification (Kristensen et al. 1998) and denitrification (Rivera-Monroy 1996) were also widely recorded in mangrove sediment since the regular tide provided an alternating aerobic and anaerobic conditions. Isotope technique had been used to detect the activity of anammox in the mangroves of Logan and Albert River system contributing 0–9% of the sediment N_2 production, which is the first evidence for the participation of anammox bacteria in nitrogen transformation in the mangrove ecosystem (Meyer et al. 2005). However, comparing to the other nitrogen microbial process, previous studies only provided limited information on the anammox process; as a result, the diversity, distribution and abundances of anammox bacteria in mangrove ecosystem are still unknown.

In the present study, we selected Mai Po Nature Reserve of Hong Kong, the largest mangrove wetland in the Southern China as our research area to investigate the anammox bacteria diversity, spatial distribution and abundance using both 16S rRNA and *hzo* genes. Environmental parameters were also analyzed to identify their influences on the anammox bacteria community structure and abundances in the mangrove sediments. The results of present study allowed us to more clearly understand the anammox bacteria in the mangrove ecosystem.

Materials and methods

Sampling and chemical analysis

Mai Po Marshes Nature Reserve, located at the northwestern corner of the New Territories of Hong Kong (22°30′ N, 114°02′ E) in the greater Pearl River Delta, is the largest remaining coastal wetland in Hong Kong. Mai Po comprises of sub-tropical mangroves, inter-tidal mudflats, as well as man-made fishponds and drainage channels. In the mangrove wetland, the dominated mangrove forests are *Kandelia obovata* (formerly known as *Kandelia candel*). Three sampling sites (S0, S1, and S2) were selected in a transect: the location of site S0 was immediately to the mangrove trees while site S1 with a distance of 10 m to the mangrove trees, and site S2 was at the intertidal mudflats about 1,000 m from S0 without any mangrove tree around. The surface layer (1–2 cm) and lower layer (20–21 cm) sediment samples were collected in triplicate at each of the three sampling sites at the same distance to mangrove trees, and all samples were immediately transferred into 4°C cooler for transport back to the laboratory for analyses (within 2 h).

Temperature, redox potential and pH of the sediment samples were measured in situ using IQ180G Bluetooth Multi-Parameter System (Hach Company, Loveland, CO) and concentration of NH_4^+-N, NO_3^--N, and NO_2^--N in pore water of sediment samples, after centrifugation and collection, were measured with an autoanalyzer

(QuickChem, Milwaukee, WI) according to standard methods by the American Public Health Association (American Public Health Association 1995). Salinity of pore water was measured using YSI 556 Multiprobe System (YSI, Yellow Springs, OH).

DNA extraction and PCR amplification

Total genomic DNA of each sediment sample was extracted using the SoilMaster DNA Extraction kit (Epicentre Biotechnologies, Madison, WI). PCR amplifications for 16S rRNA and *hzo* genes were performed according to the previous study with primer sets Brod541F-Amx820R (Schmid et al. 2000; Penton et al. 2006; Li et al. 2010) and hzocl1F11-hzoclR2 (Schmid et al. 2008), respectively. PCR products were checked by electrophoresis on 1% agarose gels and subsequent staining with ethidium bromide (0.5 μg ml^{-1}).

Cloning, sequencing and phylogenetic analysis

PCR amplified products were purified using Gel Advance-Gel Extraction System (VIOGEME, Taipei) according to the manufacturer's instructions, and cloned into the pMD 18 T-Vector (Takara, Japan). The insertion of an appropriate-sized DNA fragment was checked by PCR amplification with the primer set M13F and M13R. Different numbers of clones in each library were randomly selected for sequencing. Sequencing was performed with the Big Dye Terminate kit (Applied Sciences, Foster City, CA) and an ABI Prism 3730 DNA analyzer.

DNA sequences were examined and edited with MEGA 4.0 software (Tamura et al. 2007) and the chimera was checked using the Check Chimera program of the Ribosomal Database Project (Cole et al. 2005). For 16S rRNA gene, DNA sequences were aligned using the CLUSTAL W (Thompson et al. 1994). For *hzo* gene, DNA sequences were firstly translated into amino acids and the resulting protein sequences were aligned with referenced sequences. Phylogenetic trees were constructed by the neighbor-joining method, and bootstrap re-sampling analysis for 1,000 replicates was performed to estimate the confidence of the tree topologies.

Quantitative PCR assay

The copy numbers of *hzo* gene of anammox bacteria 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, which binds to double-stranded DNA during PCR amplification. Each reaction was performed in a 25 μl volume containing 2 μl of DNA template, 1 μl BSA (0.1%), 1 μl of each primer (20 μM, hzocl1F1 and hzocl1R2) and 12.5 μl of Power SYBR Green PCR Master Mix (Applied Bioshystems, Foster City, CA). The PCR cycle was started with 2 min at 50°C and 10 min at 95°C, followed by total of 48 cycles of 1 min at 95°C, 1 min at 50°C, and 1.5 min at 72°C. Standard plasmid carrying anammox *hzo* gene was generated by amplifying *hzo* gene from extracted DNA of site S1-s sediments and cloning into pMD 18 T-Vector (Takara, Japan). The copy numbers of target genes were calculated directly from the concentration of the extracted plasmid DNA. Ten-fold serial dilutions of a known copy number of the plasmid DNA were subjected to quantitative PCR assay in triplicate to generate an external standard curve. The Q-PCR amplification efficiencies ranged 0.85–0.92 (*hzo*), and correlation coefficients (r^2) were greater than 0.99.

Statistical analysis

Operational taxonomic units (OTUs) for community analysis were defined by a 2% cut-off in 16S rRNA gene nucleotide or HZO protein sequences, as determined by using the furthest neighbor algorithm in DOTUR (Schloss and Handelsman 2005). Shannon and Simpson indices of each clone library were also generated by DOTUR. To determine the significance of the difference between any of two clone libraries (e.g., X and Y), differences (ΔC) between "homologous" $C_X(D)$ and ''heterologous'' coverage curves $C_{XY}(D)$ were calculated using LIBSHUFF software version 0.96 (http://libshuff.mib.uga.edu/) according to the Singleton method (Singleton et al. 2001). The geographic distributions of anammox bacteria 16S rRNA and *hzo* genes were analyzed by the principal coordinates analysis (PCoA) and Jackknife Environment Clusters analysis as suggested previously (Lozupone et al. 2006). Meanwhile, Pearson correlation analysis between diversity and environmental variables was conducted using Microsoft Excel program. Meanwhile, Pearson correlation analysis between diversity and environmental variables was conducted using Microsoft Excel program.

Nucleotide sequence accession numbers

The GenBank accession numbers for the 16S rRNA gene sequences reported here are GQ331333 to GQ331362; and the GenBank accession numbers for the *hzo* gene sequences are GQ331363 to GQ331389.

Results

Biogeochemical characteristics of sampling sites

The biogeochemical characteristics of sampling sites, including temperature, redox, pH of sediments and nitrate,

Table 1 Sites characteristics of investigated sediments in Mai Po Nature Reserve

Sample location	Distance (m)	Depth (cm)	T (°C)	Redox (mV)	pH	NO_3^- (μM)[a]	NO_2^- (μM)[a]	NH_4^+ (μM)[a]	Salinity (‰)
S0-s	0	1–2	30.0 ± 0.1	−128.4 ± 5.0	6.48 ± 0.02	11.4 ± 0.3	0.83 ± 0.02	495.7 ± 5.0	17.1
S0-l	0	20–22	26.3 ± 0.1	−253.6 ± 6.2	5.68 ± 0.01	2.7 ± 0.2	1.25 ± 0.04	250.0 ± 1.4	17.3
S1-s	10	1–2	29.6 ± 0.1	−189.7 ± 5.6	7.04 ± 0.01	13.3 ± 0.4	2.02 ± 0.05	678.6 ± 7.9	18.6
S1-l	10	20–22	26.6 ± 0.1	−234.9 ± 6.2	5.94 ± 0.01	1.4 ± 0.1	2.71 ± 0.08	346.4 ± 2.9	18.9
S2-s	1000	1–2	29.5 ± 0.1	−148.9 ± 4.6	6.65 ± 0.02	22.8 ± 0.5	5.44 ± 0.17	355.0 ± 3.5	20.6
S2-l	1000	20–22	28.8 ± 0.1	−349.2 ± 7.2	7.24 ± 0.01	1.5 ± 0.1	1.79 ± 0.05	676.4 ± 7.1	20.6

[a] Values correspond to the concentrations in pore water

nitrite, ammonium and salinity of pore-water, are shown in Table 1. All surface layer samples for three sites showed a relatively higher redox potential, temperature and concentration of nitrate than the lower layer. However, the variations of nitrite, ammonium and pH between the surface and lower layer were quite different. In mangrove sediments (S0 and S1), surface layer samples have lower nitrite concentration but higher pH and concentration of ammonium compared to the low layer, where the concentration of ammonium in S0-l was only 250.0 μM, the lowest among all sampling sites. However, the variation trends of pH, nitrite, and ammonium concentrations were reversed in non-mangrove sediments (S2).

Phylogenetic diversity of anammox bacteria by 16S rRNA genes

All anammox-like 16S rRNA gene sequences obtained in the present study were divided into four clusters which fell deeply into *Candidatus* "*Scalindua*" clade, and the sequence similarity within each cluster was more than 97.0% (Fig. 1). In Cluster 1, 25 sequences recovered from sites S1-s and S2-s are closely related to the clones from river estuary sediments (Dale et al. 2009) and *Candidatus* "*Scalindua wagneri*" (Kuypers et al. 2003). Eight sequences from S0-s and S2-s established the Cluster 2, representing a novel cluster based on phylogenetic analysis. Cluster 3 was supported by the clones recovered from the surface sediment samples (S1-s and S0-s), and these clones were more affiliated with the 16S rRNA gene sequences of *Candidatus* "*Scalindua brodae*" and *Candidatus* "*Scalindua arabica*". Cluster 4, the most common cluster containing 96 anammox-like sequences from all sediment samples (except S0-l), was the dominant anammox bacteria, which closely related to the clone recovered from Xinyi river sediment in China. Another interesting group Cluster P contained four sequences from site S1-l and was not clustered with any known anammox bacteria, but sequences in this cluster showed a high similarity (96.6–97.4%) with those detected from hydrothermal vent, where the activity and diversity of anammox bacteria were confirmed recently (Byrne et al. 2009), thus the cluster P might be considered as a potential anammox bacteria group.

Rarefaction analysis indicated that site S0-s had the greatest anammox-16S rRNA genes diversity while site S2-l (except S0-l) was the lowest. At the same time, the rarefaction analysis also showed the surface layer samples had higher diversity of OTUs than that of lower layer samples at each sampling site, consistent with the values of Simpson and Shannon indices (Fig. S-1A; Table 2). It was interesting that no anammox-like bacterial 16S rRNA gene sequences were detected in a total of 29 clones from site S0-l, where the low layer was the only one in the mangrove forest (Table 2).

Phylogenetic diversity of anammox bacteria by *hzo* genes

All *hzo* gene sequences recovered in the present study showed high similarity (>84% in protein sequences) with those published in previous studies. Phylogenetic analysis showed that recovered HZO sequences were grouped into five distinct sub-clusters, designated as Cluster 1-1, 1-2, 1-3, 1-4, and 1-5 shown in Fig. 2, within the recently proposed *hzo* Cluster 1 (Schmid et al. 2008). Sequences in Cluster 1-1 were closely related to HZO proteins from Uncultured planctomycete (ACF95877), the enrichment culture *Candidatus* "*Kuenenia* sp." (CAQ57912), *Candidatus* "*Brocadia* sp." enrichment culture (CAQ57910), and the two octaheme cytochrome *c* gene product present in the genome of *Candidatus* "*Kuenenia stuttgartiensis*" (CAJ72085 and CAJ71439). However, sequences in Clusters 1-2 were more closely related to the uncultured planctomycete HZO proteins CAQ57913 and CAQ57914 than ACF95877, CAQ57912, and CAQ57910. In addition, the deduced HZO protein sequences in Clusters 1-3, 1-4 and 1-5 were more closely affiliated to CAQ57913 and CAQ57914, as well as

Fig. 1 Consensus phylogenetic tree constructed after subjecting to an alignment of anammox bacterial 16S rRNA gene sequences from this study and those retrieved from database to neighbor-joining analysis. Numbers in parenthesis refer to the clone numbers retrieved by amplification with the primer set (Brod541F–Amx820R) and assigned to an individual phylotype. The numbers at the nodes are percentages that indicate the levels of bootstrap support based on 1,000 resampled data sets (only values greater than 50% are shown). Branch lengths correspond to sequence differences as indicated by the *scale bar*

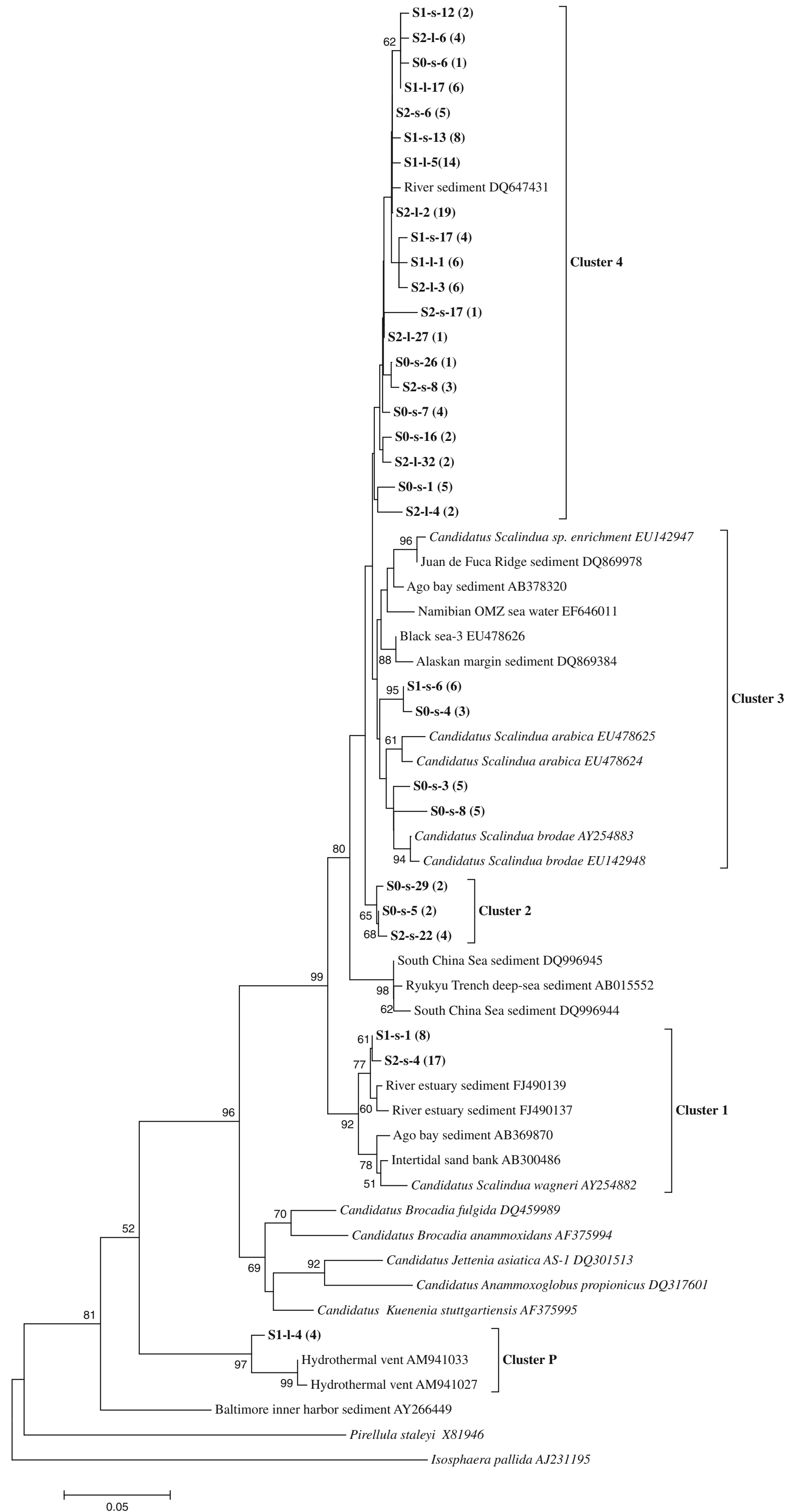

Table 2 Diversity characteristics of 16S rRNA gene and the deduced HZO amino acid sequences recovered from each of the three sampling sites at two depths

Biomarker	Sites	No. of screened clone	OTUs	Shannon Index	Simpson Index
16S rRNA	S0-s	30	7	1.85	0.14
	S0-l	29	0	ND	ND
	S1-s	28	6	1.4	0.27
	S1-l	30	6	0.95	0.54
	S2-s	30	6	1.25	0.36
	S2-l	34	3	0.49	0.73
HZO	S0-s	45	13	1.68	0.24
	S0-l	ND	ND	ND	ND
	S1-s	59	10	1.63	0.25
	S1-l	36	9	1.69	0.23
	S2-s	43	9	1.52	0.31
	S2-l	33	2	0.42	0.73

ND No PCR product was amplified successfully

one sequence obtained from *Candidatus* "*Scalindua* sp." (CAQ57909). The deduced HZO protein sequences in Cluster 1-3, Cluster 1-4, and Cluster 1-5 constituted sister clades with Cluster 1-2 which contained only sequences unique to our investigated area (Fig. 2).

Rarefaction analysis showed the greatest *hzo* genes diversity occurred at site S0-s, and the lowest richness at site S2-l (except site S0-l, where no PCR amplicon could be obtained), consistent with the results of 16S rRNA gene (Fig. S-1B). Both Simpson and Shannon indices indicated a higher diversity in surface layer samples at each site, except the Shannon index in site S1 (Table 2).

Abundance of *hzo* gene of anammox bacteria in mangrove sediments

Except no *hzo* genes were detected at site S0-l, the obtained anammox *hzo* gene abundances ranged from 7.5×10^4 to 36.5×10^4 copies per gram of sediments (dry weight) (Fig. 3). The *hzo* gene determined at sites S1 and S0 showed a significant higher copy number in surface layer samples than lower layer samples ($p < 0.05$, $n = 3$). However, in site S2, the surface layer sample had a lower *hzo* gene copy number than the lower layer samples ($p < 0.05$, $n = 3$). Interestingly, *hzo* abundances decreased in surface layer from S0 to S2 along with the distance between sampling sites and mangrove trees, while increased in lower layer along with the same transect, showing a significant positive correlation ($r = 0.98$, $n = 3$) (Fig. 3).

Anammox bacterial community structure comparison and relationships with environmental factors

Based on the *P*-values calculated using LIBSHUFF software, there was little significant compositional overlap of anammox bacteria community structure in the five sediment samples (Table S-1). The 16S rRNA gene sequences showed no significant difference between S1-s and S2-s, or S1-l and S2-l, while statistical difference in the other two sites. For *hzo* gene, only S1-s vs. S2-s and S2-s vs. S2-l showed similar clone libraries composition. It was needed to point out that both 16S rRNA and *hzo* gene clone libraries at site S0-s were significantly different from the other sampling sites, indicating a special anammox bacterial community structure. As for the clone library comparison, PCoA and Jackknife Environment Clusters analyses of 16S rRNA and *hzo* genes showed that sites S1-s and S2-s shared quite similar anammox bacteria community structures; and lower layer samples (S1-l and S2-l) also shared similar community structures based on 16S rRNA gene though *hzo* genes at the two sites were not closely related to each other (Fig. 4). It was very interesting that the anammox bacterial community structure in S0-s, the only site in mangrove forest, formed a clearly distinct biogeographic cluster, separating from all other sites for the anammox bacterial structures. Furthermore, Pearson moment correlation analysis showed that the OTUs and Shannon index of 16S rRNA and *hzo* genes significantly correlated with redox potential of the sediments, and *hzo* gene abundances strongly correlated with the molar ratio of ammonium to nitrite, but no significant correlations were detected for the anammox diversity and abundances with other biogeochemical parameters (Table 3).

Discussions

The phylogenetic diversity of anammox bacteria in the environments is studied based on the retrieved 16S rRNA gene sequences; however, specific 16S rRNA gene sequences belonging to the anammox group have historically been difficult to recover from environmental samples (Schmid et al. 2005; Penton et al. 2006). According to our

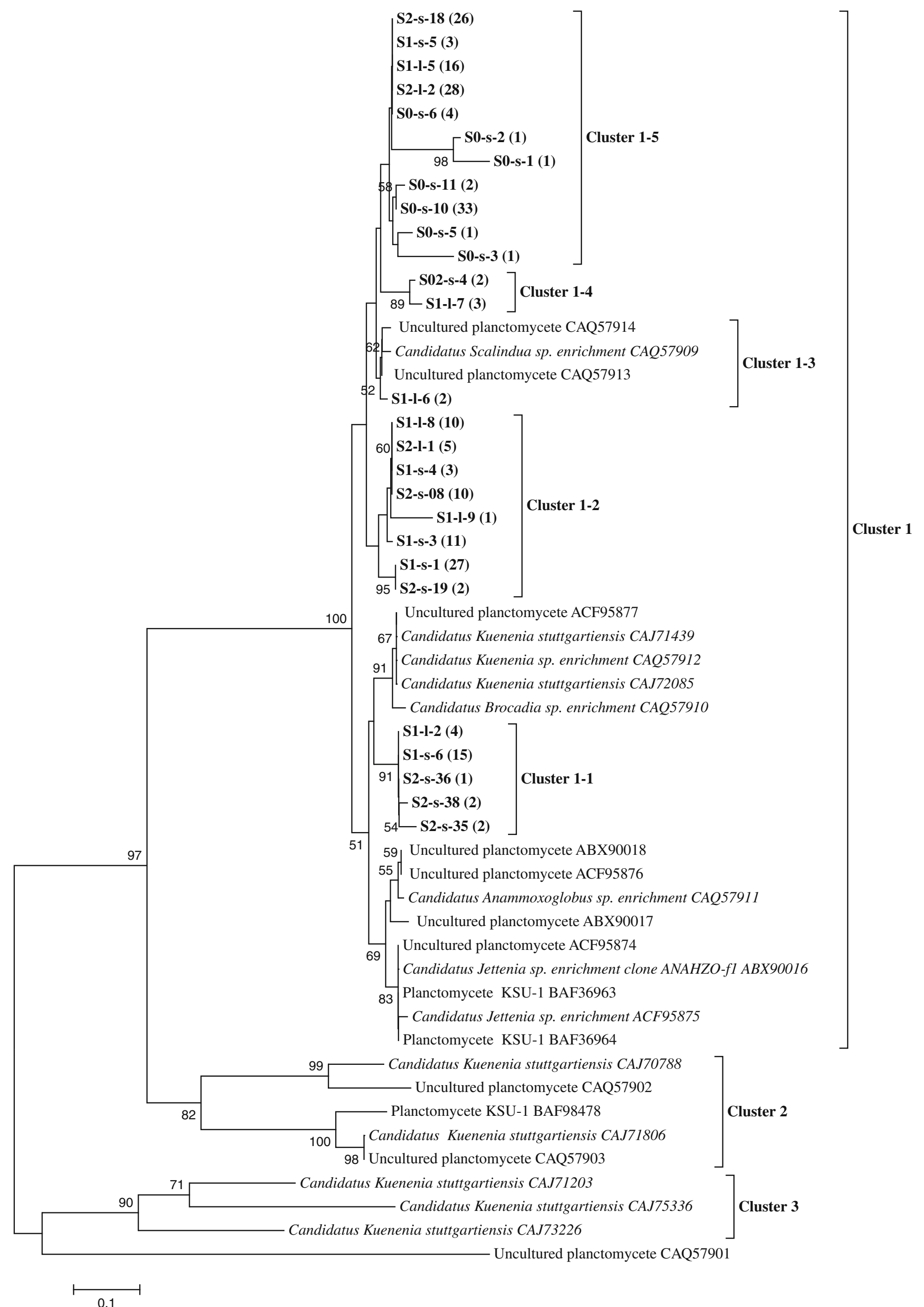

Fig. 2 Consensus phylogenetic tree constructed after subjecting to an alignment of deduced HZO protein sequences from this study and those retrieved from database to neighbor-joining analysis. Numbers in parenthesis refer to *hzo* gene clone numbers retrieved using the primer set (hzocl1F1-hzocl1R2) and assigned to an individual phylotype. The numbers at the nodes are percentages that indicate the levels of bootstrap support based on 1,000 resampled data sets (only values greater than 50% are shown). Branch lengths correspond to sequence differences as indicated by the scale bar. Major clades were named following a proposal of Schmid et al. (2008)

previous results, we optimized a new primer combination (Brod541F-Amx820R) to detect anammox bacteria from various marine sediments, which showed higher specificity and efficiency than other primer sets (Li et al. 2010). Thus, using primer set Brod541F-Amx820R in the present study, results indicated multiple ecotypes of "*Scalindua*" genus anammox bacteria in mangrove sediments. However, from the phylogeny results of *hzo* gene, five sub-clusters of HZO sequences were found in mangrove sediment samples, which related not only to enrichment cultures of *Candidatus* "*Scalindua* sp.", but also sequences related to *Candidatus* "*Kuenenia* sp.", and *Candidatus* "*Brocadia* sp." (Fig. 2). Although the total number of clones screened for anammox bacterial 16S rRNA and *hzo* genes was not very large when considering the sample sizes in this study, our further studies with much more 16S rRNA and *hzo* gene sequences provide a similar community structure of anammox bacteria at this research area, supporting the obtained results in the present study (Li et al. 2011b). The sediment samples of the present study were directly collected from inside or outside of the mangrove forest, which has been strongly affected by anthropogenic input including wastewater and urbanization development (Wang et al. 2006). According to the results from a long-term ecological

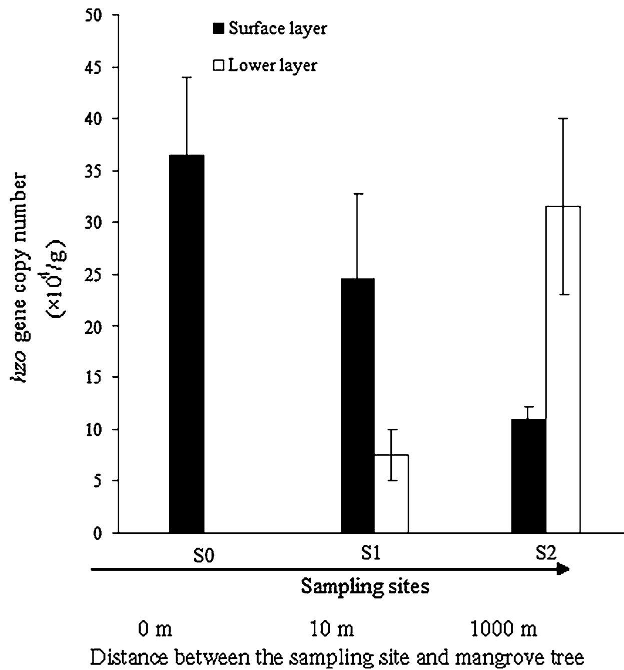

Fig. 3 Abundance of the *hzo* gene of anammox bacteria (gene abundance/g dry sediment weight) at the Mai Po mangrove sediments with different distances from the mangrove forest

monitoring project, large quantity of wastewater with different types of pollutants, including heavy metals, excessive nutrients and organic substances, is directly discharged into this area, which seriously threatens the biodiversity in this ecosystem (Mai Po Inner Deep Bay Ramsar Site Monitoring Programme reports from 2003 to 2008, "unpublished data"). Mangrove serves a conjunct region of terrestrial and marine environments at this area, where the anammox bacteria such as "*Kuenenia*" and "*Brocadia*" usually found in freshwater and terrestrial ecosystems delivered by the adjacent river would mix with the dominant marine anammox bacteria "*Scalindua*" mixed by regular tides. As a result, not only "*Scalindua*" related *hzo* gene sequences but also some related to the "*Kuenenia*" and "*Brocadia*" anammox bacteria are also detected. These *Kuenenia*-like or *Brocadia*-like anammox bacteria cannot be detected by 16S rRNA gene possibly due to the discrimination of the highly specific 16S rRNA gene targeting PCR primers, but indicated that there are still many non-described marine anammox bacteria in mangrove sediments.

One of the most interesting findings from the present study is the spatial distribution of anammox bacteria in mangrove sediments. Due to the river discharge and tidal movement carrying available nutrients in water and also anoxic condition in mangrove sediments, it is not surprising to find the existence of anammox bacteria with high diversity in all surface sediment samples. However, in the lower layer sediment samples collected closely to the mangrove trees, anammox bacteria have low diversity and abundance, even could not be detected in some location (S0-l), regardless of 16S rRNA or *hzo* genes used as biomarkers (Figs. 1, 2). Furthermore, anammox bacteria at

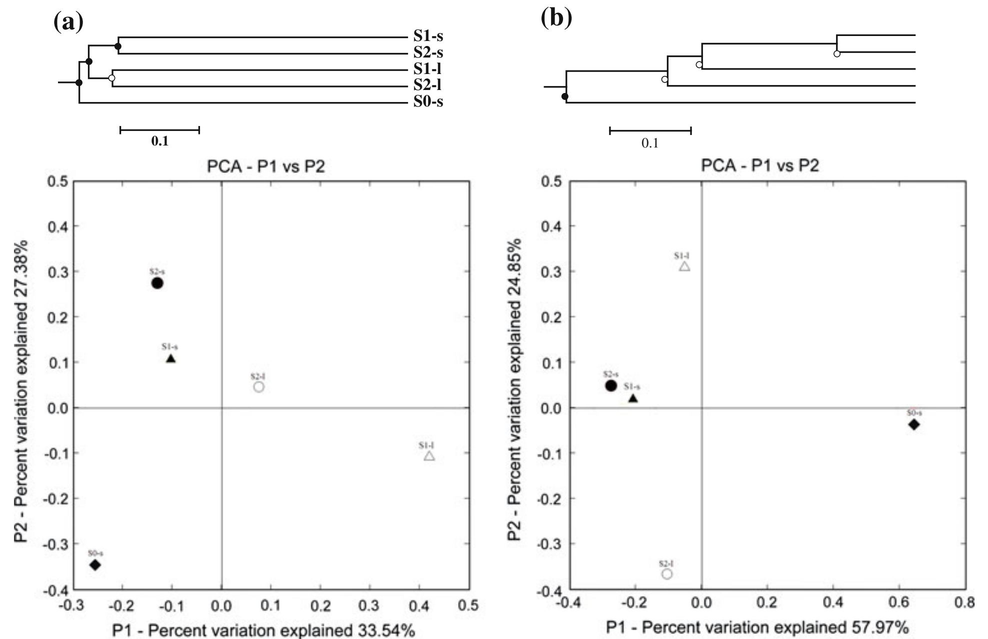

Fig. 4 Dendrogram and PCoA based on the UniFrac metric of 16S rRNA gene (**a**) and deduced HZO protein (**b**) sequences on diversity of anammox bacteria, based on sequence abundance data. *Circles* in dendrogram trees represent Jackknife support for the monophyly at the node. *Solid circles* 90–99.9%; *open circles* 50–90%

Table 3 Statistical analyses of physicochemical parameters and anammox bacteria diversity and abundance

Parameter	Pearson moment correlation[a]						
	OTUs		Shannon Index		Simpson Index		Abundance
	16S rRNA	*hzo*	16S rRNA	*hzo*	16S rRNA	*hzo*	*hzo* gene
Temperature	0.45	0.26	0.74	−0.01	0.80	−0.02	0.64
Redox	0.98*	0.93*	0.88*	0.87*	0.86	0.85	−0.09
pH	−0.38	−0.53	−0.04	−0.67	0.07	−0.67	0.56
NH_4^+	−0.35	−0.40	−0.03	−0.54	0.05	−0.53	0.71
NO_3^-	0.66	0.47	0.65	0.44	0.71	0.42	−0.15
NO_2-	0.04	−0.07	−0.16	0.14	−0.11	0.12	−0.77
$NO_3^- + NO_2^-$	0.27	0.12	0.13	0.27	0.19	0.24	−0.61
NH_4^+/NO_2^-	0.18	0.20	0.48	−0.14	0.44	−0.13	0.95*
$NH_4^+/(NO_2^- + NO_3^-)$	−0.14	0.20	0.17	−0.35	0.23	−0.34	0.78
Salinity	−0.68	−0.79	−0.68	−0.63	−0.60	−0.64	−0.40

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

* 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: $t = \times\sqrt{\frac{n-2}{(1-r^2)}}$. The number of samples is given by $n = 5$

S0-s, the site immediately to the mangrove trees, are significantly different from the other sampling sites, forming a unique community structure (Fig. 4). In addition, *hzo* abundances were decreasing at surface layer while increasing at lower layer along with distances to mangrove trees, and mangrove surface layer sediment samples have higher *hzo* gene abundances than the lower layer samples, while the sample (S2) located far away from mangrove has the reversed trend (Fig. 3). Previous studies have proposed a close microbe-nutrient-plant relationship that functions as a mechanism to recycle and conserve nutrients, such as nitrogen, in the mangrove ecosystem (Holguin et al. 2001). The diverse microbial community with high activities continuously transforms nutrients from dead mangrove vegetation into sources of nitrogen, phosphorus, and other nutrients that can be used by the mangrove trees again. In turn, plant-roots transport O_2 into sediments and their exudates serve as substrates for the microorganisms (Holguin et al. 2001). Since anammox bacteria is strictly anaerobic bacteria, which require nitrite and ammonium as substrates for growth, the existence of mangrove trees would have competitive interaction with anammox bacteria for available nitrogen or some other terms. Meanwhile, different nitrogen-utilizing bacteria, such as the ammonium-oxidizers, nitrite-oxidizers, and denitrifiers in mangrove sediments might also have a complex coupling with anammox bacteria due to the same nitrogen substrates or products in these nitrogen microbial processes (Flores-Mireles et al. 2007). A complex interaction between anammox bacteria and other nitrogen-utilizing ones for consumption or production of ammonium or nitrite in the Black Sea water column and Peruvian oxygen minimum zone are revealed (Lam et al. 2007, 2009). Thus, the special distribution of anammox bacteria in mangrove sediment spatially might also due to the interactions between anammox bacteria and other nitrogen-utilizing microorganisms, such as ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). In our previous study, we reported that AOA diversity and abundances were significantly correlated with *hzo* gene abundance at the same research sites, which provides further evidence on the interactions between AOA and anammox bacteria (Li et al. 2011a).

On the other hand, correlation analysis further shows that 16S rRNA and *hzo* gene diversities have strong positive correlations with sediment redox potential, and *hzo* gene abundance has a significant correlation with the molar ratio of ammonium to nitrite, which are reasonable as oxygen and nitrogen in sediments are important factors affecting the diversity and abundances of anammox bacteria.

In conclusion, the phylogenetic diversity, distribution and abundances of anammox bacteria are investigated in mangrove sediment transect with different depths. The phylogeny of functional biomarker HZO protein shows a more complex anammox bacteria community structure than the phylogeny of 16S rRNA gene in these research areas.

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