Assessment of Molecular Detecting of Anaerobic Ammonium Oxidizing (Anammox) Bacteria in Different Environmental Samples Using PCR Primers based on 16S rRNA and Functional Genes

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Abstract

Eleven published PCR primer sets for detecting genes encoding 16S rRNA, hydrazine oxidoreductase (HZO), cytochrome cd_1-containing nitrite reductase (NirS) and hydrazine synthase subunit A (HzsA) of anammox bacteria were assessed for the diversity and abundance of anammox bacteria in samples of three environments, waste water treatment plant (WW), wetland of Mai Po Nature Reserve (MP), and the South China Sea (SCS). Consistent phylogenetic results of three biomarkers (16S rRNA, *hzo*, and *hzsA*) of anammox bacteria were obtained from all samples. WW had the lowest diversity with *Candidatus* Kuenenia dominating while the SCS was dominated by *Ca*. Scalindua. MP showed the highest diversity of anammox bacteria including *Ca.* Scalindua, *Ca.* Kuenenia and *Ca.* Brocadia. Comparing different primer sets, no significant differences in specificity for 16S rRNA gene could be distinguished. Primer set CL1 showed relatively high efficiency in detecting anammox bacteria *hzo* gene from all samples, while CL2 showed greater selectivity for WW samples. The recently reported primer sets of *hzsA* gene resulted in high efficiencies in detecting anammox bacteria while *nirS* primer sets were more selective for specific samples. Results collectively indicate that the distribution of anammox bacteria is niche-specific within different ecosystems and primer specificity may cause biases on the diversity detected.

Key words: anammox bacteria; 16S rRNA; *hzo*; *nirS*; *hzsA*; diversity; nitrogen cycle

Introduction

The anaerobic ammonium-oxidizing (anammox) bacteria grow lithotrophically coupling the energy derived from oxidation of ammonium to the reduction of nitrite into dinitrogen (N_2) gas in the absence of oxygen. The existence of such microorganisms capable of ammonium oxidation with nitrite as an electron acceptor was predicted based on thermodynamics calculations in 1977 (Broda 1977). Almost 20 years later, the anammox process was first described in bioreactors of wastewater treatment plants in 1995 (Mulder et al. 1995; van de Graaf et al. 1995). As one of the latest additions to the biogeochemical nitrogen cycle, anammox bacteria have been extensively studied. Five anammox genera have been described, including the genera of *Brocadia* (Kartal et al. 2008; Oshiki et al. 2011; Park et al. 2017), *Anammoxoglobus* (Kartal et al. 2007), *Kuenenia* (Schmid et al. 2000; Strous et al. 2006), *Scalindua* (Kuypers et al. 2003; Schmid et al. 2003; Speth et al. 2017; van de Vossenberg et al. 2008) and *Jettenia* (Ali et al. 2015; Quan et al. 2008). All anammox bacteria belong to the same monophyletic branch named the *Brocadiacaea* and are related to the phylum *Planctomycetes* and have been detected in various ecosystems including extreme environments (Byrne et al. 2009; Jaeschke et al. 2009; Zhu et al. 2015), terrestrial ecosystems (Zopfi et al. 2009), freshwater and marine ecosystems (Hirsch et al. 2011; Schmid et al. 2007), mangrove (Li et al. 2011c; Meyer et al. 2005; Wang et al. 2012c), oil field (Li et al. 2010a), and also in wastewater treatment systems (Araujo et al. 2011). PCR amplification of anammox bacterial 16S rRNA and functional genes are commonly used for the detection and identification (Chouari et al. 2003; Junier et al. 2010; Li and Gu 2011; Penton et al. 2006; Quan et al. 2008; Schmid et al. 2005; Sonthiphand and Neufeld 2013; Wang et al. 2015). Fluorescence *in situ* hybridization (FISH) and stable isotope labeling also serve as useful methods to confirm the presence and activity of anammox bacteria in natural environments (Schmid et al. 2007).

The approaches using the 16S rRNA gene as a phylogenetic biomarker with universal bacterial PCR primer sets often show disadvantages due to the high sequence divergence of anammox bacteria (Schmid et al. 2005). Thus, 16S rRNA gene targeting PCR primers with

high specificity for *Planctomycetes* or different genera of anammox bacteria have been designed (Kuypers et al. 2003; Neef et al. 1998; Penton et al. 2006). As no general primer set for anammox bacterial detection has been agreed upon (Jetten et al. 2009), a combination of results obtained from different genera specific primer sets are often used to provide information about anammox bacterial abundance, diversity and distribution in natural and engineered ecosystems. Nevertheless, since the 16S rRNA gene as a molecular marker is not necessarily related to the physiology of anammox bacteria, the use of functional gene markers may provide good alternatives (Junier et al. 2010). Therefore, analysis of the functional genes encoding for enzymes involved in specific anammox biochemical reactions could significantly increase the detection efficiency and specificity (Kartal et al. 2011a). With the hypothetical metabolic pathway proposed based on this genome sequence of "*Candidatus* Kuenenia stuttgartiensis" (Strous et al. 2006) and later fully supportive experimental evidence (Kartal et al. 2011b), it is reported that first, nitrite is reduced to nitric oxide by a cytochrome cd_1-type nitric oxide/nitrite oxidoreductase (NirS), and then a hydrazine synthase (HZS) produces hydrazine from nitric oxide and ammonium, and finally hydrazine is oxidized to produce dinitrogen gas by a hydrazine oxidoreductase (HZO).

The first functional gene used for detecting anammox bacteria is HZO encoding *hzo* gene. Several different primer sets (Schmid et al. 2008) have been reported to detect anammox bacteria from different environmental samples including coastal and deep-ocean sediments (Dang et al. 2010; Hirsch et al. 2011; Li et al. 2010b), mangrove sediments (Li et al. 2011c; Wang et al. 2012c), rice paddy (Wang and Gu 2013), oilfields (Li et al. 2010a) or wastewater treatment plants (Quan et al. 2008). Results from these studies imply that the *hzo* gene is a reliable qualitative functional biomarker for the investigation of anammox bacteria in natural environments (Li et al. 2010b).

The primer set ScnirS was used to detect the cytochrome cd_1-containing nitrite reductase encoding gene (*nirS*) in anammox bacteria belonging to the *Scalindua* in Peruvian oxygen minimum zone, surface and subsurface sediments in the South China Sea (Lam et al. 2009; Li et al. 2013a; Li et al. 2013b). Another recently designed primer set, AnnirS, has also been

shown to successfully amplify *nirS* genes in other genera of anammox bacteria from coastal to deep ocean samples (Li et al. 2011b). By combining information from these two primer sets, the *nir*S gene can be considered as another functional biomarker for investigation of anammox bacteria in natural environments (Li et al. 2011b). In addition to *hzo* and *nirS*, the hydrazine synthase protein encoded by the *hzsCBA* gene cluster (kuste2859-kuste2861) has been successfully purified (Kartal et al. 2011b) and is considered to be a very good biomarker for anammox bacteria. PCR primer sets targeting the new unique functional *hzsA* were developed and were successfully tested on engineered sample and on a variety of environments indicating broad diversity (Borin et al. 2013; Harhangi et al. 2012). In addition, primer sets for *hzsB* genes have also been developed and applied to Pearl River Estuary, paddy soil, ocean sediments, coastal wetland sediments and wastewater treatment plant (WWTP) samples (Wang et al. 2012a; Wang et al. 2012b; Zhou et al. 2017), indicating a wide coverage for all known anammox genera.

Red granules of anammox bacteria were reported in a full-scale wastewater treatment plant with simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) process, and anammox bacteria in the reactor were investigated with 16S rRNA gene marker (Wang et al. 2010). The South China Sea (SCS), as an oligotrophic ecosystem and the largest semi-enclosed marginal sea basin of the West Pacific with an area of about 3.5×10^6 km^2 (Chen et al. 2001; Ning et al. 2009), has been studied extensively to examine the diversity and distribution of anammox bacteria in both surface and subsurface sediments. A high micro-diversity of anammox bacteria with *Scalindua* dominating in SCS has been demonstrated (Han and Gu 2013; Hong et al. 2011; Li et al. 2013a). The Mai Po Nature Reserve of Hong Kong, the largest area of wetland in Hong Kong, consists of intertidal mudflats, mangroves, traditionally operated shrimp ponds, fishponds, reed beds and drainage channels in between Shenzhen River and Yuen Long River (Nelson 1993). Diversity and distribution of anammox bacteria have been extensively studied spatially and seasonally (Chen and Gu 2017; Li et al. 2011a; Li et al. 2011c; Wang et al. 2012c; Wang and Gu 2012; Zhou et al. 2017).

In this study, eleven in total primer sets targeting anammox bacterial 16S rRNA (3 sets), *hzo* (4 sets), *nirS* (2 sets) and *hzsA* (2 sets) genes were selected to study the diversity of anammox bacteria in samples of three environments from a wastewater treatment plant (WW), the South China Sea (SCS) and the Mai Po Nature Reserve (MP). The objectives were to 1) compare the diversity and abundance of anammox bacteria by 16S rRNA and functional biomarkers from these three different environments, and 2) examine the efficiencies and specificities of phylogenetic and functional markers in recovering the diversity and abundance of anammox bacteria, thus providing useful information for primer selection in analysing anammox bacterial community structure in future studies. This is the first time that 16S rRNA gene and core functional gene markers have been applied in one single study for samples from three distinct environments.

Materials and Methods

Sampling and chemical analysis

Samples from three environments were selected for the current study: 1) Red granules from a long-term, simultaneous partial nitrification, anammox and denitrification (SNAD) wastewater treatment system (WW) in Taiwan (Kumar and Lin 2010; Lin et al. 2011; Ni et al. 2011; Wang et al. 2010). Red granules were collected from the influent of the wastewater treatment system in Apr. 2011. Samples were taken in triplicate and stored at -20 °C in a refrigerator after collection, 2). Mai Po Nature Reserve wetland sediment (MP) (N 22°29, E 114°01), an estuarine coastal wetland influenced by the discharge of the Pearl River, was selected based on the reported information of ammonia-oxidizing microorganisms (Li et al. 2011a; Li et al. 2011c). The Mai Po sediment samples were collected in June 2011 and kept in an ice-cooled container for transportation back to the laboratory within 2 hours for further chemical analysis and molecular processing, and 3). Deep-sea surface sediments from the South China Sea (SCS), a pristine marine ecosystem, were also chosen with the samples collected from a depth of 2350 meters at 19°38’ N, 115°31’E (Li et al. 2011a). Surface sediments (0-1 cm) were taken in triplicate and stored at -80 °C in a refrigerator after collection on board during the South China Sea Open Cruise by R/V Shiyan 3 in 2008.

Concentration of ammonia, nitrite, and nitrate in pore water of red granule and sediment samples were analyzed using the standard flow injection analysis (FIA) technique with QuickChem (Milwaukee, Wisconsin, USA) based on the protocol by Ariza (Ariza et al. 1992).

DNA extraction and PCR amplification

Thawed and homogenized granules or sediments (approximately 0.5 g, wet weight) were transferred into sterile 1.5 mL centrifuge tubes. The total genomic DNAs were extracted using the Power Soil Isolation Kit (Mo Bio, Carsbad, California) according to the manufacturer's instructions. All extracted DNAs were separately stored at -20°C for further analysis.

Primer sets used in this study and the PCR annealing temperatures are listed in Tab. 1. In a final volume of 25 µl, a PCR reaction mixture contained: 1 µL of extracted template DNA (1-10 ng), 5 µL of 5× GoTaq Flexi buffer (Promega, Hong Kong) and 2.5 µL of $MgCl_2$ (25 mM, Promega), 2 µL of dNTPs (2.5 mM, Invitrogen, Hong Kong), 0.5 µL of each forward and reverse primer (20 µM) and 0.2 µL of GoTaq Flexi polymerase (5 U ml^{-1}, Promega, Hong Kong). PCR products were analyzed by agarose gel electrophoresis in 1% agarose gels in TAE (20 mM Tris-acetate pH 8.0; 0.5 mM EDTA) at 130 V, 400 mA for 20 min (Amersham Biosciences, Electrophoresis Power Supply 301). The gels were stained by addition of GelRed nucleic acid stain (Biotium) and photographed by using a Bio-Rad® GelDoc™ station.

Cloning, sequencing and phylogenetic analysis

Clone libraries were constructed from the PCR products according to the methods described by Friedrich (Friedrich et al. 2001). Briefly, the amplified PCR products were analyzed by gel electrophoresis as described above, and then purified using a Qiaex II Gel Extraction Kit (QIAGen, Hilden, Germany). Ligation with a pMD® 18-T vector (Takara, Japan) was performed at 16°C for 10 hours in a 8 µL reaction volume consisting of 4 µL of Solution 1 (containing T4 ligase and buffer), 3.5 µL of purified PCR products and 0.5 µL of

pMD® 18-T vector. Transformation of the vectors to competent cells of *E. coli* was performed (Chung et al. 1989) afterwards. The cloned single colonies were then randomly picked for PCR amplification using the primer sets M13 (M13F: 5'-TGTAAAACGACGGCCAGT-3'; M13R: 5'-CAGGAAACAGCTATGACC-3') in a 25 μL PCR reaction volume consisting of the same components as described above. The PCR program consisted of 95°C of initial denaturation for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 80 s, followed by 10 min of final elongation at 72°C. Positive PCR products were sent for DNA sequencing performed with the Big Dye Terminator Kit (Applied Sciences, Foster City, CA) on an ABI Prism 3730 DNA analyzer.

Construction of phylogenetic trees

Clone sequences were checked on their sequence similarity with known references using the NCBI Blast nucleotide database. 16S rRNA gene sequences were inspected before alignment using the Black Box Chimera Check tool (Gontcharova et al. 2010) for the presence of chimerical sequences. Sequences were aligned and phylogenetic trees were constructed using MEGA version 5.0 (Tamura et al. 2011) and subjected to phylogenetic inference using the neighbor-joining algorithm followed by 1000 times of bootstrap.

Quantitative Analysis

The abundance of anammox bacteria in the three samples was estimated by the presence of the anammox bacterial 16S rRNA, *hzo* and *hzsA* genes with the primer sets Brod, CL1 and HZSS respectively. Quantitative polymerase chain reaction (q-PCR) were performed to qualify the copy numbers of 16S rRNA, *hzo* and *hzsA* genes for anammox bacteria in triplicate using the Applied Biosystems StepOnePlus™ Real-Time PCR System. The quantification was based on the fluorescent dye SYBR-Green I. Each reaction was performed in a 20-μL volume containing 10 μL of Power SYBR Green PCR Master Mix (Applied BioSystems), 1 μL of DNA template (1-10 ng), 0.2 μL of each primer (20 μM), and 8.8 μL of autoclaved DD water. The PCR profile for the 16S rRNA and *hzsA* gene was started with 10 min at 95 °C, followed by a total of 40 cycles of 50 s at 95 °C, 50 s at 54 °C, and 50 s at

72 °C. The PCR cycle for the *hzo* gene was started 10 min at 95 °C, followed by a total of 48 cycles of 50 s at 95 °C, 1 min at 48 °C, and 1 min at 72 °C. Standard plasmids carrying the targeted gene fragments were generated by amplifying from sample DNA templates of MP and cloning into pMD 18-T Vector. The plasmid DNA concentration was determined, and the copy numbers of the target genes were calculated. Tenfold serial dilutions of a known copy number of the plasmid DNA were subjected to a Q-PCR assay in triplicate to generate an external standard curve. The correlation coefficients (r^2) were all greater than 0.99 for the three targeted genes.

Statistical analysis

All sequences associated with anammox or putative anammox bacterial gene sequences were analyzed using the DOTUR (Distance-Based OTU and Richness) program to compare their diversity and richness (Schloss and Handelsman 2005). Operational taxonomic units (OTUs) were defined at 3% variation in nucleic acid sequences for 16S rRNA genes and 5% for protein sequences. In order to examine the relationship between the distribution of these various genes among samples, all clone libraries of each target gene, namely 16S rRNA, *hzo*, *nirS* and *hzsA*, were analyzed with UniFrac (http://bmf.colorado.edu/unifrac/) using the principal coordinates analysis tool (PCoA) (Lozupone and Knight 2005). Correlations of the anammox clusters with environmental factors were also explored with canonical correspondence analysis (CCA) using the software Canoco (version 4.5; Microcomputer Power).

Nucleotide sequence accession numbers

Partial sequences of 16S rRNA, *hzo*, *nirS* and *hzsA* genes from WW, MP and SCS have been deposited to GenBank under accession numbers JQ822788-JQ823004 (WW), JQ822258-JQ822427 (MP) and JQ822536-JQ822787 (SCS), respectively.

Results

Characteristics of the samples

The physiochemical characteristics of the three types of samples from WW, MP, and SCS are shown in Tab. S1. There was no significant pH difference among samples. WW showed the highest concentration of NH_4^+, but is relatively lower in concentrations of NO_3^- and NO_2^-. MP showed a much lower concentration of NH_4^+ than WW, but higher concentrations of NO_3^- and NO_2^-. SCS shows the lowest concentrations of NH_4^+, NO_2^- and NO_3^- among all samples.

PCR amplification and sequencing

All primer sets with few exceptions resulted in positive amplification products from all three samples (Tab. 1 and S2). These exceptions included primer set CL2 worked only for sample WW., and two primer sets for *nirS* gene, ScnirS and AnnirS, worked with SCS and MP respectively. All three 16S rRNA gene primer sets demonstrated high efficiency (> 80%) in detecting anammox bacteria in all samples, except for MP-Brod (64.3%). Three *hzo* gene based primer sets (CL1, H4F and Ana) showed high efficiency (> 80%) in detecting anammox bacteria in all samples except for MP-CL1 (78.9%) and MP-Ana (44.4%). Primer CL2 only worked with sample WW with 100% efficiency. The two *nirS* gene based primer sets showed selectivity for sample SCS (ScnirS, 93.3% efficiency) and MP (AnnirS, 43.5% efficiency). For sample WW, both of *nirS* primer sets showed 100% efficiency. Both *hzsA* primer sets worked with all samples while primer set HZSL showed relatively lower efficiency at 33.0% for MP and 35.0% for the SCS.

Diversity and community structure of anammox bacteria by 16S rRNA

Retrieved 16S rRNA gene sequences confirmed to be affiliated with the anammox bacteria were aligned with known reference sequences for the phylogenetic tree construction (Fig. 1a). Three major groups are visualized: i) the *Scalindua* group, ii) a combined group including *Kuenenia*, *Brocadia*, *Jettenia* and *Anammoxoglobus*, and iii) a putative anammox bacteria group by MP sequences. The *Scalindua* group included all SCS and 87.6% MP (71/87) sequences which share at least 96% sequence similarity with the known *Scalindua* species. Sequences within the *Scalindua* group were also subdivided into six clusters (brodae,

arabica, wagneri, zhenghei-Ⅰ, zhenghei-Ⅱand zhenghei-ⅢI) as described previously (Hong et al. 2011). Most (93.5%, 86/92) SCS sequences (with primer sets AMX, BS and Brod) were clustered to the zhenghei-Ⅰcluster. The brodae cluster contained the remainder (6.5%, 6/92) of the SCS sequences (with primer sets AMX, BS and Brod) and 2.3% (2/87) of the MP sequences (with primer sets AMX and BS). Sequences from MP contributed exclusively to the other three clusters, there are 31.0% (27/87) MP sequences (with primer sets AMX, BS and Brod) included in zhenghei-Ⅱ, 2.3% (2/87) (with primer set BS) in zhenghei-Ⅱ and 46.0% (40/87) (with primer sets AMX, BS and Brod) in the wagneri cluster. No sequences obtained in this study affiliated with the arabica cluster. Only sequences from MP and WW contributed to the second group. The *Kuenenia* cluster contained 9.2% (8/87) MP sequences (with primer sets AMX and BS) and 85.7% (54/63) WW (with primer sets AMX, BS and Brod) sequences. The rest, 14.3% (9/63) WW sequences (with primer sets AMX, BS and Brod) contributed to the *Brocadia* cluster. Finally, eight sequences (with primer set AMX) from the MP grouped to a separated cluster (a new cluster).

All retrieved 16S rRNA gene sequences were analyzed with UniFrac principal coordinates analysis (PCoA) (Fig. S1a). The phylogenetic differences within the different clone libraries led to a separation into different single groups, with each accounting for a different sample type, WW, MP, and SCS. For SCS and MP clusters, the Brod libraries are relatively far from the other two (AMX and BS), indicating apparent differences of primer specificity for anammox bacteria detection in SCS and MP ecosystems.

Diversity and community structure of anammox bacteria by *hzo* gene

Deduced amino acid sequences obtained from all *hzo* gene clone libraries were aligned with known reference protein sequences of anammox bacteria to build a phylogenetic tree, in which three separated groups were indicated (Fig. 1b). Five (5.4%, 5/93) SCS sequences (with primer set CL1) form their own cluster, which shared only 77% to 81% similarity with sequences retrieved from an anammox reactor (Li et al. 2009), and thus suggesting a new phylogenetic group. Cluster 1a consists of the rest (94.6%, 88/93) of the SCS sequences (with primer sets CL1, H4F and Ana) and half (12/24) of the MP sequences (with primer sets CL1,

H4F and Ana). Cluster 1b was contributed to by 33.3% (8/24) of the MP sequences (with primer set CL1). Cluster 1a and cluster 1b were assembled as the *Scalindua* group. In the second group, cluster 2a contained 16.7% (4/24) MP sequences (with primer sets H4F and Ana). Cluster 2b was contributed to by 5.7% (4/70) of the WW sequences (with primer sets CL1), cluster 2c was formed by 80% (56/70) WW sequences (with primer sets CL1, H4F and Ana) and cluster 3 by 14.3% (10/70) WW sequences (with primer set CL2). Cluster 3 is an independent cluster, consisting of 10 WW sequences sharing 99% of the amino acid sequence similarity with *Kuenenia stuttgartiensis.* In addition, cluster 3 is relatively distant from the above clusters, indicating an apparent specificity in the *Kuenenia*.

The phylogenetic differences among clone libraries formed three clusters for the three sample types (Fig. S1b). Within each of the clusters, clone libraries constructed with the ANA and the H4F are relatively closer to one another than to CL1 libraries. The WW-CL2 is located far away from all other three libraries, indicating a greater diversity of HZO proteins.

Diversity and community structure of anammox bacteria by *nirS* gene

Deduced amino acid sequences obtained were aligned with known reference sequences of *nir*S genes of both anammox and denitrifying bacteria to build a phylogenetic tree (Fig. 1c). Five clusters form anammox's *nirS* group which is separated from the denitrifier's *nirS* group. Sequences obtained from each of the samples formed a different cluster: cluster 1b (SCS with primer set ScnirS), cluster 1c (MP with primer set AnnirS) and cluster 2 (WW with primer sets ScnirS and AnnirS). In addition, more than half (56.5%, 13/23) of the MP sequences (with primer set AnnirS) were affiliated with denitrifiers. Retrieved *nirS* gene sequences were analyzed with PCoA (Fig. S1c), resulting in separated libraries in terms of sample type, consistent with the phylogenetic analysis.

Diversity and community structure of anammox bacteria by *hzs*A gene

Deduced amino acid sequences of all six *hzsA* gene clone libraries were aligned with the known reference sequences of anammox bacteria to build two phylogenetic trees (Figs. 1d and 1e). Since products amplified with primer HZSL and HZSS were not from the same

region of the *hzsA* gene, two trees were constructed separately. Sequences obtained with primer HZSL and reported sequences could be divided into 5 clusters within two large groups. For sequences obtained with primer HZSS, SCS sequences contributed to cluster 1a (11.8%, 2/17), cluster 1b (64.7%, 11/17) and cluster 1c (23.6%, 4/17). MP sequences contributed to cluster 1c (33.3%, 5/15), cluster 1d (20%, 3/15) and cluster 2a (46.7%, 7/15). All WW sequences were included in cluster 2a and were closely related to hydrazine synthase subunit A from *Ca.* Kuenenia stuttgartiensis. Two independent PCoA analyses show similar community structures for anammox bacteria except MP, which is more diverse, and distant, from *Scalindua* in the HZSS clone library (Fig. S1e).

Abundance of Anammox Bacteria

The abundance of anammox bacteria was estimated by the copy numbers of the 16S rRNA, *hzo* and *hzsA* genes (Tab. 2). Results showed that 16S rRNA gene abundance was 1.69×10^6 to 2.51×10^6 copies per gram of dry sediment and the *hzo* gene and *hzsA* gene were 1.21×10^5 to 1.11×10^6 and 1.67×10^5 to 1.26×10^6 copies per gram of dry sediment, respectively. In addition, the ratio of copies of 16S rRNA gene to *hzo* gene ranged from 2.26 (WW) to 13.9 (MP), the ratio of copies of 16S rRNA gene to *hzsA* gene ranged from 1.60 (SCS) to 10.12 (MP).

The relationship of environmental factors and anammox communities

Correlations of the anammox diversity based on 16S rRNA phylogenetic analysis with environmental parameters were analyzed via CCA (Fig. 2). The environmental variables in the first two CCA dimensions (CCA1 and CCA2) explained 97.4% of the total variance in the anammox species composition and 82.3% of the cumulative variance of the anammox environment relationship. Correlations of the anammox bacterial assemblages with environmental parameters analyzed by CCA indicated that ammonia has a strong relationship with the amount of *Kuenenia* and *Brocadia* in certain environmental samples, that means much of the distribution of *Kuenenia* and Brocadia in some samples can be explained by ammonia concentration. In addition, the concentration of nitrite and nitrate could also contribute to the distribution of clusters zhenghei-Ⅰ, zhenghei-Ⅱ and wagneri, the nitrite to

nitrite plus nitrate ratio significantly affected brodae and zhenghei-Ⅲ clusters.

Discussion

A closer look at the samples reveals that WW was by far the easiest sample to amplify anammox bacterial sequences with all primer sets. Based on the phylogenetic analysis with 16S rRNA, *hzo* and *hzsA* gene clone libraries, WW was dominated by *Kuenenia* while *Brocadia* was also detected, representing agreement with previous studies (Wang et al. 2010). Mai Po showed the highest diversity of anammox bacteria among all three samples. All primer sets, except for CL2 and ScnirS, were able to amplify anammox bacterial gene sequences, showing several different clusters within the *Scalindua, Kuenenia*, and *Brocadia*, as well as some sequences of putative anammox bacteria, representing agreement with previous studies (Li et al. 2011a). The SCS showed micro-diversity with two clusters (zhenghei-Ⅰ and Brodae), all belonging to *Scalindua* based on phylogenetic analyses of the 16S rRNA gene sequences obtained. The SCS-CL1 new cluster based on the phylogenetic analyses of HZO proteins also indicated the possibility of hidden diversity of anammox bacteria in the marine environment. Most sequences obtained with all primer pairs used showed a similarity of 97-100% to *Scalindua* type anammox bacteria from previous studies of marine environment (Hong et al. 2011; Woebken et al. 2008).

The simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) process has been frequently applied in nitrogen removal and in bioreactors (Chen et al. 2009; Daverey et al. 2012; Lin et al. 2011; Wang et al. 2010). Previous studies have revealed the existence and dominance of anammox bacteria in SNAD bioreactors (Lin et al. 2011; Wang et al. 2010). Phylogenetic analysis based on 16S rRNA and functional genes in this study support this finding. However, it should be noted that many questions remain on how the conditions and processes shape the anammox bacterial community structure, and how the interactions among anammox bacteria, nitrifiers and denitrifier's regulate removal of organic carbon and nitrogen waste.

The diversity and community structure of anammox bacteria have been studied

seasonally (Li et al. 2011a; Wang et al. 2012c), spatially (Li et al. 2010b) and also on a basis of soil types (Wang et al. 2012c) from the mangrove sediments and mudflats in the area of the Mai Po marsh. Since the Mai Po marsh is a typical wetland with a mixture of freshwater, soil, seawater, wetlands and mangrove trees, it is not unexpected that a higher diversity of anammox bacteria was retrieved with almost all described genera. In contrast, the dominance of *Scalindua* in the South China Sea samples may be a function of substrate limitation contributing to the competitive fitness of this bacterium in the marine realm. Recent work based on metagenomic studies suggests that *Scalindua* may be well adapted to the marine environments (van de Vossenberg et al. 2013), but further studies are clearly warranted. Recent findings concerning the micro-diversity and niche-specific distribution of anammox bacteria in the South China Sea (Han and Gu 2013; Hong et al. 2011) may inspire the need of more detailed physiological studies.

The consistency of phylogenetic analysis of 16S rRNA, *hzo* and *hzsA* genes from all three samples suggests that these three biomarkers could be successfully applied in detecting anammox bacteria (Fig. 1). Nevertheless, functional gene markers may reflect more physiological information: 1) distinctive WW-CL2 sequences indicate *Kuenenia*-specific HZO proteins; 2) the SCS-CL1 new cluster may indicate higher diversity of *Scalindua*-specific HZO proteins; 3) the large differences in the ratios of 16S rRNA gene copies to functional genes copies among the three samples suggest even more complicated metabolic mechanisms involved in anaerobic ammonia oxidation. To date, genomic information is available for *Kuenenia* (Speth et al. 2012; Strous et al. 2006), *Jettenia* (Hira et al. 2012; Hu et al. 2012), *Scalindua* (Speth et al. 2017; van de Vossenberg et al. 2013) and *Brocadia* (Park et al. 2017) and the core metabolic mechanisms have been described. However, the presence of eight highly divergent *hzo* genes in the genome of *Ca.* Kuenenia stuttgartiensis (Strous et al. 2006), and the identification of copper-containing nitrite reductase NirK in *Ca.* Jettenia asiatica (Hu et al. 2012) and organism KSU1(Hira et al. 2012) instead of cytochrome cd_1-type nitrite reductase NirS identified in *Ca.* Kuenenia stuttgartiensis and *Ca.* Scalindua profunda, indicate variable mechanisms involved in

403 metabolism of anammox bacteria in terms of nitrite reduction to NO, and further complicate
404 interpretation of the retrieved sequence data.

405 All three 16S rRNA gene based primer sets showed high efficiency in recovering
406 anammox bacterial amplicons with high specificity (Tab. S2). In contrast to former studies
407 using these primer sets (Kuypers et al. 2003; Penton et al. 2006; Schmid et al. 2000), no
408 significant differences in specificity on the genus level. PCoA suggested that AMX and BS
409 clone libraries of each sample, and Brod except in the MP sample, are clustered very closely
410 to each other. The formerly reported *Scalindua* specific Brod541F (Penton et al. 2006) was
411 combined with the *Brocadia* and *Kuenenia* specific Amx820R primer (Schmid et al. 2000)
412 and described as the Brod primer pair in this study. This combination could also amplify most
413 of the different genera of known anammox bacteria, but the *Kuenenia* type clade detected
414 with the other two 16S rRNA gene primer sets from MP could not be recovered with the
415 Brod primer pair. In general, primer set BS showed the best coverage in detecting all
416 recovered clusters (Fig.1a).

417 All three *hzo* gene based primer sets showed their ability in detecting taxa closely related
418 to anammox bacteria from the three samples used in this study (Fig. 1b). There are also
419 primer-specific clusters obtained, namely the new SCS cluster, MP cluster 1b and WW
420 cluster 2b with primer set CL1, and WW cluster 3 with primer set CL2. In addition, cluster 2a
421 is a CL1 and CL2 exclusive cluster with only sequences obtained by primer set Ana and H4F.
422 In particular, the new cluster of marine anammox bacterial *hzo* genes (SCS new cluster)
423 shares only up to 81% sequence identity to the known anammox organisms (Dalsgaard et al.
424 2003; Kuypers et al. 2003; Ward et al. 2009), indicating hidden diversity of anammox
425 bacteria in marine ecosystems and the relatively higher efficiency of primer set CL1. The
426 CL2 primer set amplified solely a specific sequence of the open reading frame kustc1061
427 encoding one of the *hzo* genes of *K. stuttgartiensis* from the WW. In general, primer CL1 has
428 a higher efficiency in recovering most of the anammox genera from different samples,
429 indicating similar patterns found by 16S rRNA gene diversity analyses. CL2 only worked
430 with sample WW, indicating the existence of the *hzo* gene in the *K. stuttgartiensis* genome is

more diverse and needs further study.

The specificity of the ScnirS primer set for *Scalindua* (Lam and Kuypers 2011) has also been observed in this study, as demonstrated by SCS sequences which all fall into a *Scalindua* cluster 1b (Fig..1c). However, MP sequences determined with the AnnirS primer set did contribute to another *Scalindua* cluster 1c. Cluster 2 contained exclusively sequences from sample WW, which was dominated by *Kuenenia.* Compared to ScnirS, the AnnirS primer set performed better in detecting anammox bacteria from sample MP, while a large portion (56.5%, 13/23, Tab. S2) of denitrifier *nirS* sequence was also obtained due to the lower specificity. In general, both of the *nirS* primer sets showed their selectivity, however, there is a need to improve the efficiency and specificity of both ScnirS and AnnirS in detecting anammox bacteria.

Two sets of primers for *hzsA* gene amplification share the same reverse primer, while the length of the HZSL product is much longer than HZSS (Tab. 1). Both of the primer sets have been used recently (Harhangi et al. 2012) and were compared in this study. Based on the efficiency results and phylogenetic analyses, HZSS was more effective in detecting anammox bacteria than HZSL. Even though the length of PCR products with HZSS is much shorter than that with HZSL, sequences obtained with HZSS contained sufficient meaningful phylogenetic information. However, neither of the *hzsA* primer sets recovered genes affiliated with *Brocadia* from the WW and MP samples, as shown in phylogenetic analyses based on 16S rRNA gene sequences and HZO protein sequences, indicating the limitation of this functional gene marker.

In conclusion, diversity and community structure of anammox bacteria were studied with 16S rRNA and three functional gene markers from samples of three environments. Results showed agreement with previous studies while new functional gene clusters indicated the diverse metabolic mechanisms among different genera of anammox bacteria. Primers were also assessed and most are of high efficiencies and specificities in detecting anammox bacteria while some showed selectivity and lower specificities. In general, 16S rRNA gene

458 targeting primer set BS, *hzo* gene targeting primer set CL1 and *hzsA* gene targeting primer set
459 HZSS are highly recommended.

460

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697 **Table 1** PCR primer sets used for amplification of the four anammox bacterial marker genes
698 chosen in this study
699

Target gene	Primer pair	Primer name	Sequence 5'-3'	Product size (bp)	Annealing temperature	References
16S rRNA	AMX	AMX368F	CCTTTCGGGCATTGCGAA	452	56°C	(Kuypers et al. 2003)
		AMX820R	AAAACCCCTCTACTTAGTGCCC			(Schmid et al. 2000)
	BS	AMX368F	CCTTTCGGGCATTGCGAA	452	56°C	(Kuypers et al. 2003)
		BS820R	TAATTCCCTCTACTTAGTGCCC			(Kuypers et al. 2003)
	Brod	Brod541F	GAGCACGTAGGTGGGTTTGT	279	56°C	(Penton et al. 2006)
		AMX820R	AAAACCCCTCTACTTAGTGCCC			(Schmid et al. 2000)
hzo	CL1	hzocl1F1	TGYAAGACYTGYCAYTGG	470	48°C	(Schmid et al. 2008)
		hzocl1R2	ACTCCAGATRTGCTGACC			
	H4F	hzoF1	TGTGCATGGTCAATTGAAAG	1000	53°C	(Li et al. 2010b)
		hzoR1	CAACCTCTTCWGCAGGTGCATG			
	Ana	Ana-hzo1F	TGTGCATGGTCAATTGAAAG	1000	53°C	(Quan et al. 2008)
		Ana-hzo2R	ACCTCTTCWGCAGGTGCAT			
	CL2	hzocl2aF1	GGTTGYCACACAAGGC	525	48°C	(Schmid et al. 2008)
		hzocl2aR2	ATATTCACCATGYTTCCAG			
*nir*S	ScnirS	ScnirS372F	TGTAGCCAGCATTGTAGCGT	473	59°C	(Lam et al. 2009)
		ScnirS845R	TCAAGCCAGACCCATTTGCT			
	AnnirS	AnnirS379F	TCTATCGTTGCATCGCATTT	442	51°C	(Li et al. 2011b)
		AnnirS821R	GGATGGGTCTTGATAAACA			
*hzs*A	HZSL	hzsA_526F	TAYTTTGAAGGDGACTGG	1331	54°C	(Harhangi et al. 2012)
		hzsA_1857R	AAABGGYGAATCATARTGGC			
	HZSS	hzsA_1597F	WTYGGKTATCARTATGTAG	260	54°C	
		hzsA_1857R	AAABGGYGAATCATARTGGC			

700

701 **Table 2** Copy numbers of 16S rRNA, *hzo* and *hzsA* genes

Sample	Gene copy no. per gram (dry weight)		
	16S rRNA gene	*hzo* gene (Ratio*)	*hzsA* gene (Ratio*)
WW	2.51×10^{6}	1.11×10^{6} (2.26)	1.14×10^{6} (2.20)
MP	1.69×10^{6}	1.21×10^{5} (13.97)	1.67×10^{5} (10.12)
SCS	2.02×10^{6}	2.29×10^{5} (8.82)	1.26×10^{6} (1.60)

702 Ratio*: compared by 16S rRNA gene copy numbers

Figure Captions

Figure 1 Phylogenetic tree constructed with distance and neighbor-joining method from an alignment of PCR amplified (a)16S rRNA gene sequences, (b) HZO protein sequences, (c) NirS protein sequences, (d) HzsA protein sequences (with primer set HZSL) and (e) HzsA protein sequences (with primer set HZSS) along with their closely related sequences from GenBank. WW (Green): wastewater treatment plant; SCS (red): the South China Sea; MP (blue): Mai Po Nature Reserve. Primer sets used were placed behind the sample name and numbers refer to how many clones retrieved. The numbers at the nodes are percentages that indicate the levels of bootstrap support based on 1000 resampled data sets (only values greater than 50% are shown). Branch lengths correspond to sequence differences as indicated by the scale bar.

Figure 2 CCA ordination plots for the first two principal dimensions of the relationship between the distribution of anammox species and clusters with environmental parameters of WW, MP and SCS used in this study. Correlations between environmental variables and CCA axes are represented by the length and angle of arrows (environmental factor vectors). (Abbreviations are the same as in Figure 1)

Table S1 Physiochemical characteristics of samples used in this study

Table S2 Diversity and richness indices from the three samples based on sequences of anammox 16S rRNA gene, HZO, NirS and HzsA protein

Figure S1 PCoA analyses with 100 replicates Jackknife supporting test of anammox (a) 16S rRNA gene sequences, (b) HZO protein sequences, (c) NirS protein sequences and (d, e) HzsA protein sequences in WW, MP and the SCS on the basis of the online software UniFrac.

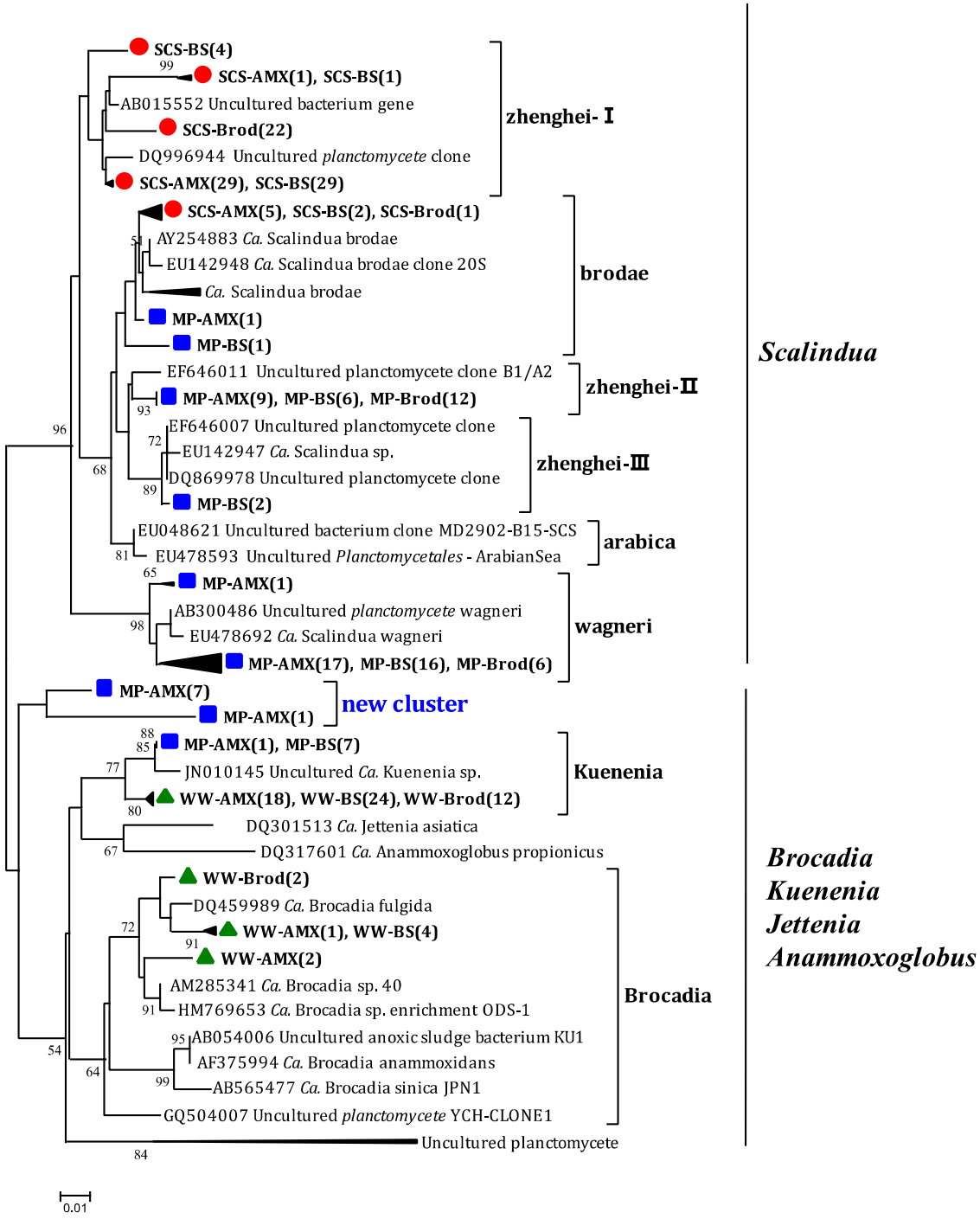
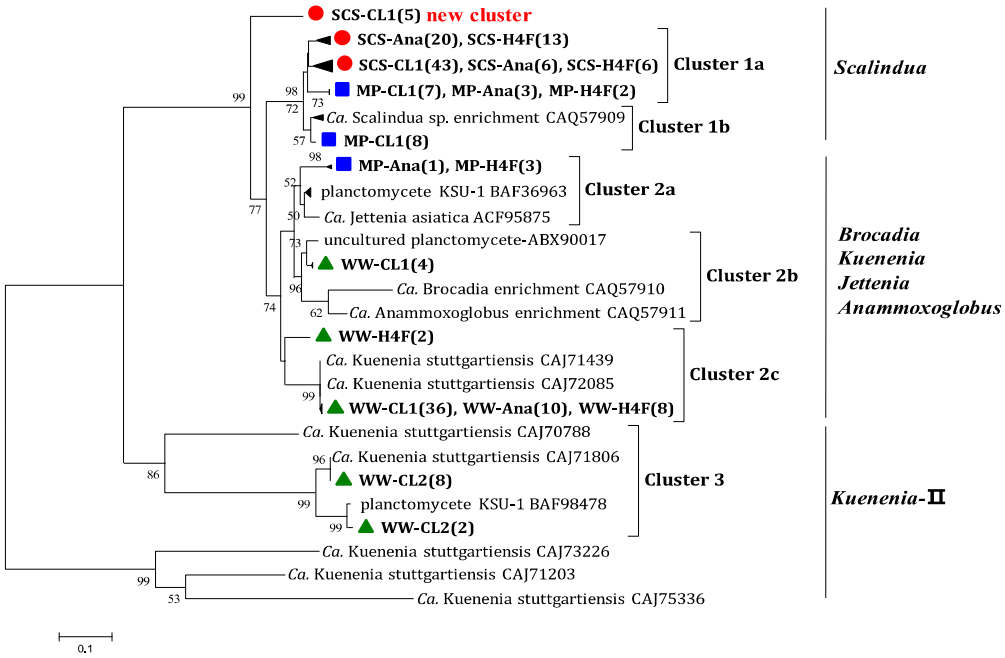

SCS-BS(4)
99
SCS-AMX(1), SCS-BS(1)
AB015552 Uncultured bacterium gene
SCS-Brod(22)
DQ996944 Uncultured planctomycete clone
SCS-AMX(29), SCS-BS(29)
zhenghei- Ⅰ
SCS-AMX(5), SCS-BS(2), SCS-Brod(1)
AY254883 Ca. Scalindua brodae
EU142948 Ca. Scalindua brodae clone 20S
Ca. Scalindua brodae
MP-AMX(1)
MP-BS(1)
brodae
EF646011 Uncultured planctomycete clone B1/A2
MP-AMX(9), MP-BS(6), MP-Brod(12)
zhenghei-Ⅱ
96
72
EF646007 Uncultured planctomycete clone
EU142947 Ca. Scalindua sp.
68
89
DQ869978 Uncultured planctomycete clone
MP-BS(2)
zhenghei-Ⅲ
EU048621 Uncultured bacterium clone MD2902-B15-SCS
81
EU478593 Uncultured Planctomycetales - ArabianSea
arabica
65
MP-AMX(1)
AB300486 Uncultured planctomycete wagneri
98
EU478692 Ca. Scalindua wagneri
MP-AMX(17), MP-BS(16), MP-Brod(6)
wagneri
Scalindua
MP-AMX(7)
MP-AMX(1)
new cluster
88
85
MP-AMX(1), MP-BS(7)
77
JN010145 Uncultured Ca. Kuenenia sp.
80
WW-AMX(18), WW-BS(24), WW-Brod(12)
Kuenenia
DQ301513 Ca. Jettenia asiatica
67
DQ317601 Ca. Anammoxoglobus propionicus
WW-Brod(2)
DQ459989 Ca. Brocadia fulgida
72
WW-AMX(1), WW-BS(4)
91
WW-AMX(2)
AM285341 Ca. Brocadia sp. 40
91
HM769653 Ca. Brocadia sp. enrichment ODS-1
95
AB054006 Uncultured anoxic sludge bacterium KU1
54
AF375994 Ca. Brocadia anammoxidans
64
99
AB565477 Ca. Brocadia sinica JPN1
GQ504007 Uncultured planctomycete YCH-CLONE1
Brocadia
Uncultured planctomycete
84
Brocadia
Kuenenia
Jettenia
Anammoxoglobus
0.01

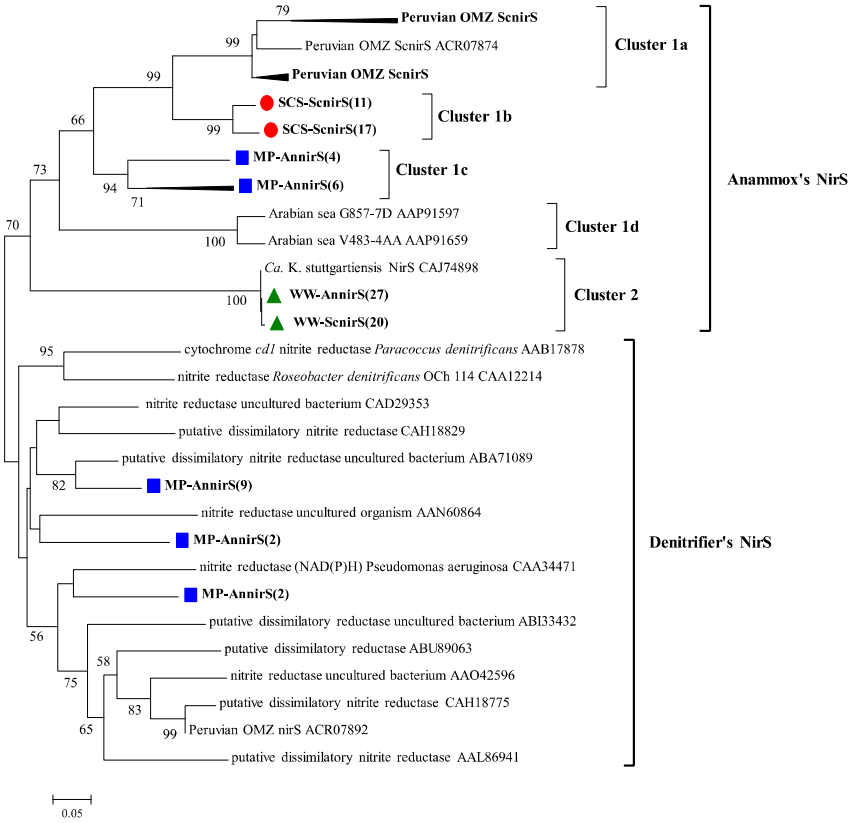
Figure 1a



736

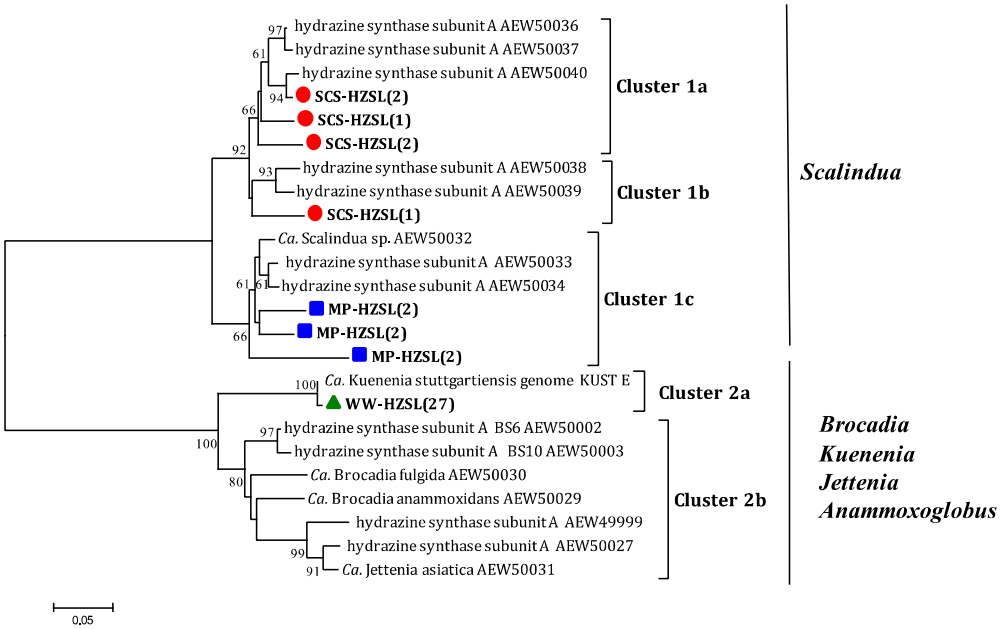
737 **Figure 1b**

738



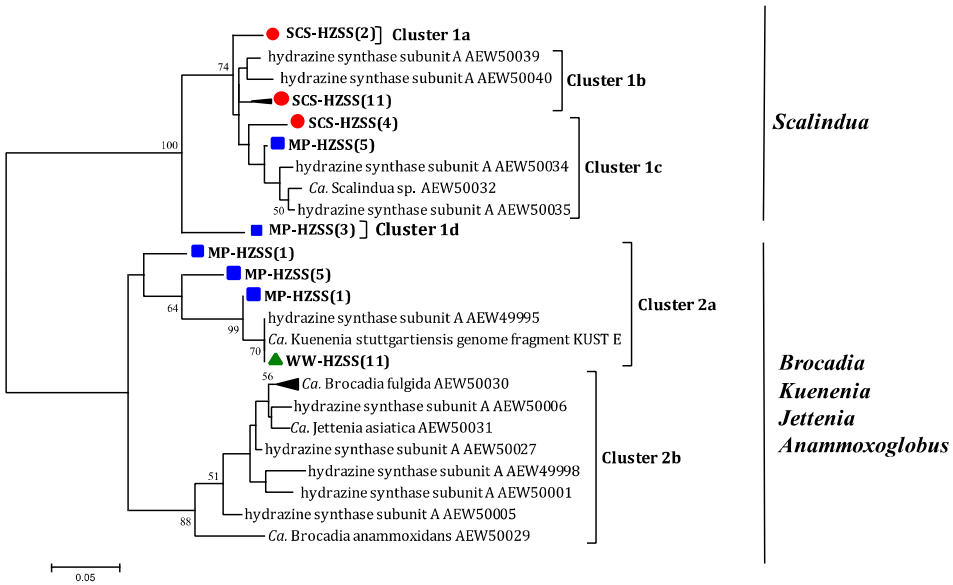
739

740 **Figure 1c**



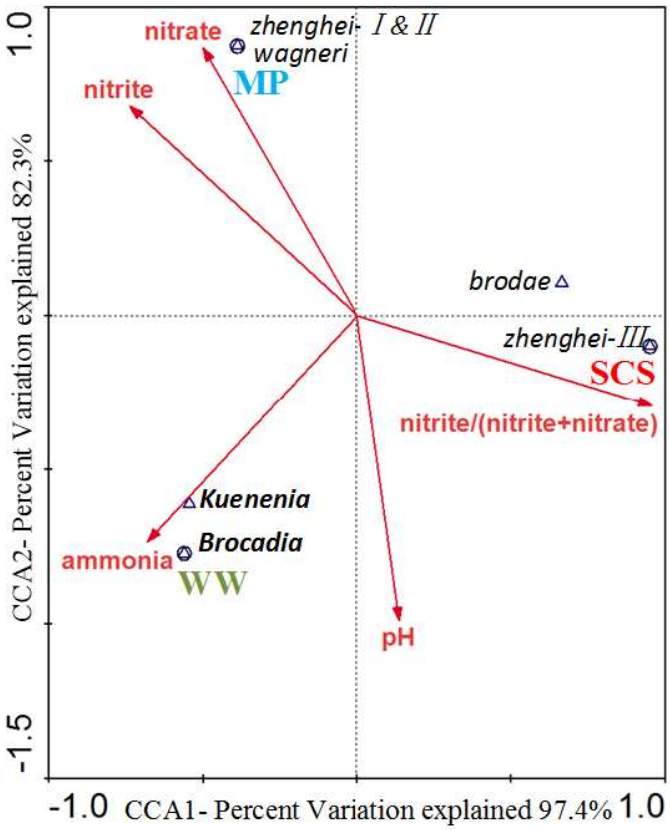
741

742 **Figure 1d**



743

744 **Figure 1e**



745

746 **Figure 2**

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748