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Co-occurrence and distribution of nitrite-dependent anaerobic ammonium and methane-oxidizing bacteria in a paddy soil

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Abstract

The anaerobic ammonium-oxidizing (anammox) and nitrite-dependent anaerobic methane-oxidizing (n-damo) bacteria in a paddy soil core (0–100 cm) were investigated with newly designed primers targeting the hydrazine synthase β-subunit (*hzs*B) of anammox bacteria and the recently published primers targeting the *pmo*A and 16S rRNA genes of n-damo bacteria. The *hzs*B gene was identified as a proper biomarker to explore the anammox bacterial biodiversity and abundance in soil. The anammox bacteria were present throughout the soil core with the highest abundance of 2.7×10^6 *hzsB* copies g^{-1} dry soil at 40–50 cm and were not detectable below 70 cm. Sequences related to at least three species of known anammox bacteria, 'Brocadia anammoxidans', 'Brocadia fulgida', and 'Jettenia asiatica' were detected. By combining the analysis of *pmo*A and 16S rRNA genes, the n-damo bacteria were observed to be present in 30–70 cm with abundance from 6.5×10^3 (60–70 cm) to 7.5×10^4 (30–40 cm) copies g^{-1} dry soil. The *pmo*A sequences retrieved from different depths closely related to each other and formed a unique clade. Our results showed that anammox and n-damo bacteria co-occurred in the paddy soil. Both of them were abundant in deep layers (30–60 cm) and the community structures changed along depths in the soil core.

Introduction

Ammonium (NH_4^+) and methane (CH_4) were previously assumed to be inert under anoxic conditions (Strous & Jetten, 2004; Jetten, 2008). This understanding was gradually changed by the discoveries of anaerobic ammonium oxidation (anammox) (Van de Graaf *et al.*, 1995; Strous *et al.*, 1999) and nitrite-dependent anaerobic methane oxidation (n-damo) (Raghoebarsing *et al.*, 2006; Ettwig *et al.*, 2009, 2010) in which NH_4^+ and CH_4 were oxidized anaerobically using nitrite as the electron acceptor.

With the development of molecular biomarkers (Kuypers *et al.*, 2003; Schmid *et al.*, 2005, 2008; Li *et al.*, 2010, 2011; Li & Gu, 2011), anammox bacteria have been detected in many marine ecosystems (Kuypers *et al.*, 2003; Byrne *et al.*, 2009), freshwater ecosystems (Zhang *et al.*, 2007; Zhu *et al.*, 2010), and man-made environments (Quan *et al.*, 2008; Zhu *et al.*, 2011a). Using the isotopic pairing technology, anammox has been identified as an important process in the aquatic nitrogen cycle, accounting for as much as 13% of N_2 production in freshwater Lake Tanganyika (Schubert *et al.*, 2006) and 67% in marine environments (Thamdrup & Dalsgaard, 2002). Although recently anammox bacteria were enriched from a peat soil (Hu *et al.*, 2011), relative little is known about the distribution of anammox bacteria in soil ecosystems because of the lack of suitable primers for quantitative PCR assays and high interfering background in fluorescence *in situ* hybridization (FISH) analyses by soil matrix components. Hydrazine synthase is a key enzyme in the anammox metabolism, consisting of three subunits encoded by the genes *hzs*A, *hzs*B, and *hzs*C (Strous *et al.*, 2006; Kartal *et al.*, 2011; Harhangi *et al.*, 2012), responsible for the synthesis of hydrazine from nitric oxide and ammonium (Kartal *et al.*, 2011). Previously, the *hzs*A gene was used as an anammox phylomarker (Harhangi *et al.*,

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2012). Here, we developed an additional phylomarker based on the *hzs*B gene for the biodiversity and quantitative analyses of anammox bacteria in a paddy soil.

Enrichments of n-damo bacteria, members of NC10 phylum, were started from freshwater sediments (Raghoebarsing *et al.*, 2006; Ettwig *et al.*, 2009) and wastewater treatment sludge (Luesken *et al.*, 2011a, c). In 2010, the genome of *Methylomirabilis oxyfera*, the bacterium responsible for n-damo, was assembled and analyzed (Ettwig *et al.*, 2010). The remarkable presence of genes encoding for particulate methane monooxygenase (*pmo*CAB) in this anaerobe was explained by its unusual intra aerobic metabolism. Recently published primers specifically targeting the *pmo*A subunit of n-damo bacteria were used to screen environmental samples, and n-damo bacteria were detected in a wide range of freshwater habitats (Deutzmann & Schink, 2011; Luesken *et al.*, 2011b; Kojima *et al.*, 2012).

Paddy fields are characteristized by cultivation patterns including water logging, which causes anoxic soil conditions. Plant-derived organic substances additionally serve as an important carbon source for CH_4 (Lu & Conrad, 2005). In addition, application of nitrogen-rich fertilizers makes the paddy field a favorable habitat for both anammox and n-damo bacteria. In the present study, we aimed to explore the co-occurrence and vertical distributions of anammox and n-damo bacteria in a paddy soil core with our newly developed anammox primers targeting the β subunit of the hydrazine synthase (*hzs*B gene) and the primers targeting the *pmo*A and 16S rRNA genes of n-damo bacteria. Both quantitative and biodiversity analyses are reported.

Materials and methods

Site description and sampling

A paddy field with long-term manure fertilization practice in subtropical China (E120°41′50″ N30°45′50″) was selected for this study. Five soil cores (1 m distance) were collected by a stainless steel ring sampler (5 cm diameter and 100 cm depth) from the field in October 2009 at the growth season. The soil cores were placed in sterile plastic bags, sealed, and transported to the laboratory on ice. Later they were sliced at 10-cm intervals, and slices of the same depth were mixed to form one composite sample. One part was sieved through 2.0-mm sieve for the chemical analysis, and subsamples were used for DNA extraction.

To evaluate the designed primers, biomass from several anammox enrichment cultures in bioreactors from our laboratory (Nijmegen, The Netherlands) were sampled including '*Candidatus* Kuenenia stuttgartiensis', '*Candidatus* Brocadia fulgida', '*Candidatus* Brocadia anammoxidans', '*Candidatus* Scalindua sp.', and '*Candidatus* Jettenia asiatica'. The cultures were each dominated at 70–95% by single anammox species. The enrichment and cultivation profiles see the previous works (Kartal *et al.*, 2007; Quan *et al.*, 2008; Schmid *et al.*, 2008). Environmental samples from wastewater treatment plants (WWTPs) and sediment were investigated from the Rotterdam and Lichtenvoorde full-scale anammox reactors (Van der Star *et al.*, 2007) and ditches in the Ooijpolder, Olburgen, and Propionaat (The Netherlands), respectively (Hu *et al.*, 2011; Harhangi *et al.*, 2012).

Chemical and statistical analyses

Ammonium, nitrite, and nitrate were extracted from the soil with 2 M KCl and measured using a SAN++ Continuous Flow Analyzer (Skalar Analytical, The Netherlands). Total nitrogen, soil organic matter, Mn^{2+}, and Mn^{4+} were measured according to standard methods (Bao, 2000). Soil pH was determined at a soil/water ratio of 1 : 2.5. All analyses were performed in triplicate on each sample. The *in situ* measurement of oxygen concentration was achieved by OXY Meter S/N 4164 with stainless electrode sensor (Unisense, Denmark) (Gundersen *et al.*, 1998). Statistical analyses were performed using program SPSS for Windows.

DNA extraction and PCR amplification

DNA in soil and sediment samples were extracted from 0.25 g samples using the Powersoil DNA isolation kits (Mobio). DNA from enriched anammox biomass was extracted according to the method described previously (Schmid *et al.*, 2008). For the specific PCR amplification of the anammox *hzs*B gene, newly designed primer pair of *hzs*B_396F and *hzs*B_742R was applied based on our new findings in anammox molecular mechanism (Kartal *et al.*, 2011; Harhangi *et al.*, 2012). The *pmo*A gene of n-damo bacteria was amplified using a nested approach (first-step primer pair A189_b-cmo682, followed by primer pair cmo182-cmo568) according to Luesken *et al.* (2011c). The 16S rRNA gene of n-damo was amplified using a nested approach (first-step primer pair 202F-1545R, followed by primer pair qP1F-qP2R) according to Juretschko *et al.* (1998) and Ettwig *et al.* (2009). The sequences of primers and thermal profiles were shown in Table 1. PCRs were performed with the PerfeCTa SYBR Green FastMix (Quanta).

Cloning, sequencing, and phylogenetic analysis

PCR amplified fragments were cloned using the pGEM-T Easy cloning kit (Promega) according to the manufacturer's

Table 1. The primers and thermal profiles used in this study

Primer	Sequence (5′-3′)	Specificity	Position	Thermal profiles	References
*hzs*B_396F ((q) PCR)	ARGGHTGGGGHA GYTGGAAG	Anammox *hzs*B		10 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 59 °C and 45 sat 72 °C (PCR) 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 59 °C and 30 sat 72 °C (qPCR)	This study
*hzs*B_742R ((q) PCR)	GTYCCHACRTCAT GVGTCTG	Anammox *hzs*B			
202F (PCR)	GACCAAAGGGGG CGAGCG	NC10 phylum 16S	193	10 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 55–64 °C and 60 s at 72 °C	Juretschko *et al.* (1998)
1545R (PCR)	CAKAAAGGAGGT GATCC	Bacteria 16S	1529–1545		
qP1F ((q)PCR)	GGGCTTGACATCCC ACGAACCTG	n-damo 16S	1001	10 min at 95 °C, followed by 30 cycles of 60 s at 95 °C, 60 s at 63 °C and 45 sat 72 °C (PCR, qP1F – qP2R)	Ettwig *et al.* (2009)
qP1R (qPCR)	CGCCTTCCTCCAGC TTGACGC	n-damo 16S	1201		
qP2R (PCR)	CTCAGCGACTTCGA GTACAG	n-damo 16S	1481–1500	3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 63 °C and 30 sat 72 °C (qPCR, qP1F – qP1R)	
A189_b (PCR)	GGNGACTGGGACT TYTGG	n-damo *pmo*A		10 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 55–64 °C and 60 sat 72 °C	Luesken *et al.* (2011a, b, c)
cmo682 (PCR)	AAAYCCGGCRAAG AACGA	n-damo *pmo*A			
cmo182 (PCR)	TCACGTTGACGCC GATCC	n-damo *pmo*A		10 min at 95 °C, followed by 30 cycles of 60 s at 95 °C, 60 s at 59 °C and 60 sat 72 °C	
cmo568 (PCR)	GCACATACCCATC CCCATC	n-damo *pmo*A			
616F (qPCR)	AGAGTTTGATYMTG GCTCAG	All bacteria	8–27	3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 sat 68 °C (qPCR)	Juretschko *et al.* (1998)
Eub338-I R (qPCR)	GCTGCCTCCCGTA GGAGT	Most bacteria	338		Amann *et al.* (1990)

instructions. Plasmid DNA was isolated with the GeneJET Plasmid Miniprep kit (Fermentas, Lithuania). Plasmids were digested with EcoRI enzyme, and the digestion products were examined for an insert with expected size by agarose (1%) gel electrophoresis. Selected clones were sequenced using primer of M13f targeting vector sequences adjacent to the multiple cloning sites. Phylogenetic analysis was performed using MEGA 5.0 software (Tamura *et al.*, 2011) by neighbor-joining (NJ) with the Jukes-Cantor correction. Diversity indices, including Chaol, Shannon, and Simpson, were generated by DOTUR for each clone library (Schloss & Handelsman, 2005).

Quantitative PCR analysis

Quantitative PCR was performed on a Bio-Rad iQ5 real-time PCR instrument (Bio-Rad) with a SYBR Green qPCR kit (Quanta). The quantitative PCR of anammox *hzs*B gene was performed with the same primer pair used for the PCR cloning. The quantitative PCR of n-damo 16S rRNA gene was performed with specific primers qP1F-qP1R described previously (Ettwig *et al.*, 2009). Total bacterial numbers were quantified with the primer pair 616F-Eub338-IR specific for the 16S rRNA gene (Amann *et al.*, 1990; Juretschko *et al.*, 1998). Standard curves were obtained with serial dilutions of plasmid DNA containing the target genes.

Nucleic acid sequence accession numbers

The sequences reported in this study have been deposited in the GenBank database under accession numbers JN704402–JN704415 (n-damo *pmo*A), JN704416–JN704466 (n-damo 16S rRNA), and JN704467–JN704568 (anammox *hzs*B).

Results and discussion

Biogeochemistry of the soil core

Owing to the long-term fertilizations, the concentrations of nitrogen compounds (NH_4^+-N, NO_x^--N and total nitrogen) and total organic matter (TOM) in soil were

very high (Supporting Information, Fig. S1). Most of the highest values were observed in the upper 10-cm layers except for NH_4^+-N which was peaked at 10–20 cm (up to 158.8 mg kg^{-1} dry soil). For NO_x^--N, the common electron acceptor for anammox and n-damo bacteria, the highest concentration (53.8 mg kg^{-1} dry soil) was present at 0–10 cm. After a rapid decrease at 10–30 cm (11.6 ± 0.3 mg kg^{-1} dry soil), a slight increase in NO_x^--N was observed at 30–50 cm of 12.5 ± 0.3 mg kg^{-1} dry soil, providing a potentially suitable condition for the growth of anammox and n-damo bacteria.

Anammox-specific primer design and verification

In addition to the previous work exploiting the *hzs*A gene (Harhangi *et al.*, 2012), we focused on the *hzs*B gene in this study. A data set with hydrazine synthase β-subunit DNA and protein sequences from the known anammox bacteria of *Candidatus* genera 'Jettenia', 'Brocadia', 'Scalindua', 'Kuenenia', and Planctomycete KSU-1 available from metagenome sequencing projects and GenBank were aligned. Conserved regions of the aligned sequences were identified and used as the targets for designing degenerate primers (Fig. S2). Six forward and five reverse degenerate primers were designed based on the alignment. The sequences and positions on the gene were shown in Table S1 and Fig. S3. Different combinations of the designed primers were tested and evaluated with template DNA extracted from anammox enrichment cultures. High intensities of specific band (*c.* 365 bp) were observed (Figs S4–S7) using the primer pair of *hzs*B_396F and *hzs*B_742R (at annealing temperature 59 °C and with 2–2.5 mM $MgCl_2$) by single-step amplification instead of nested PCR which was previously required for soil samples (Humbert *et al.*, 2010; Hu *et al.*, 2011; Zhu *et al.*, 2011b). The PCR products were cloned and sequenced, and a phylogenetic tree of the retrieved *hzs*B sequences from anammox enrichment cultures was constructed (Fig. S8a). The phylogeny of *hzs*B was consistent with that of the 16S rRNA gene (Fig. S8b) (Schmid *et al.*, 2008) and the *hzs*A gene (Harhangi *et al.*, 2012).

For the molecular detection of anammox bacteria in soil, the 16S rRNA gene was the most common used biomarker (Humbert *et al.*, 2010; Hu *et al.*, 2011; Zhu *et al.*, 2011b). However, the amplified PCR products must be verified with subsequent cloning and sequence analyses because nonanammox bacterial 16S rRNA genes were also amplified with the current PCR protocols (Song & Tobias, 2011). In this study, the specificity was promising when using functional *hzs*B gene as the biomarker that the retrieved sequences were all closely related to the known anammox bacteria. Four catalytic proteins (nitrite and nitrate reductases, hydrazine synthase, and hydrazine dehydrogenase) were possibly used as the biomarkers for the molecular detection of anammox bacteria in present study (Strous *et al.*, 2006; Kartal *et al.*, 2011). The hydrazine synthase was the most unique one (no multiple copies present) (Harhangi *et al.*, 2012) compare with the other functional genes that were present in both anammox and nitrifying or denitrifying bacteria (Song & Tobias, 2011). The application of *hzs*B gene would avoid the ambiguous differentiation between the anammox and nitrifiers or denitrifiers sequences.

Community structures of anammox bacteria

The community structures of anammox from four representative depths (0–10, 20–30, 40–50, and 60–70 cm) of the soil core were analyzed by amplifying their *hzs*B gene. Ninety-two anammox *hzs*B clone sequences were retrieved and shown to be closely related to the 'Kuenenia stuttgartiensis' *hzs*B gene (AB365070) present in GenBank (identities up to 82–85% for nucleotide and 90–93% for protein sequence). Phylogenetic analysis showed that the clone sequences were related to the anammox bacterial genera *Candidatus* 'Brocadia' and 'Jettenia' (Fig. 1). Most of the sequences (79/92) were closely related to *Candidatus* genus 'Brocadia' which comprises the '*Candidatus* Brocadia fulgida' of group 1 (44/79) and '*Candidatus* Brocadia anammoxidans' of group 2 (35/79). It confirmed the previous conclusion that representatives of the *Candidatus* genus 'Brocadia' were the most frequently detected anammox genus in soils (Humbert *et al.*, 2010). Group 3 with 13 sequences was clustering in between the *Candidatus* genera 'Brocadia' and 'Jettenia'. It was in agreement with a recent study revealing unknown anammox species distantly related to *Candidatus* 'Brocadia' and *Candidatus* 'Jettenia' in soil (Hu *et al.*, 2011). However, this result must be interpreted with caution because of the absence of *Candidatus* genera 'Anammoxoglobus propionicus' as a reference in the phylogenetic analysis. It is noted that all the 16 sequences retrieved from the surface soil (0–10 cm) were identical (difference up to 98–100% nucleotide identity) and most closely related to the '*Candidatus* Brocadia anammoxidans' group. In contrast, sequences from other depths were very divergent. These results confirmed that the community composition of anammox bacteria in soil changed with depth (Zhu *et al.*, 2011b).

The biodiversity and coverage analysis of the clone library targeting the *hzs*B gene were conducted and compared with the 16S rRNA gene at the same location of our previous study (Zhu *et al.*, 2011b). The rarefaction curve built from the 16S rRNA gene reached the saturation

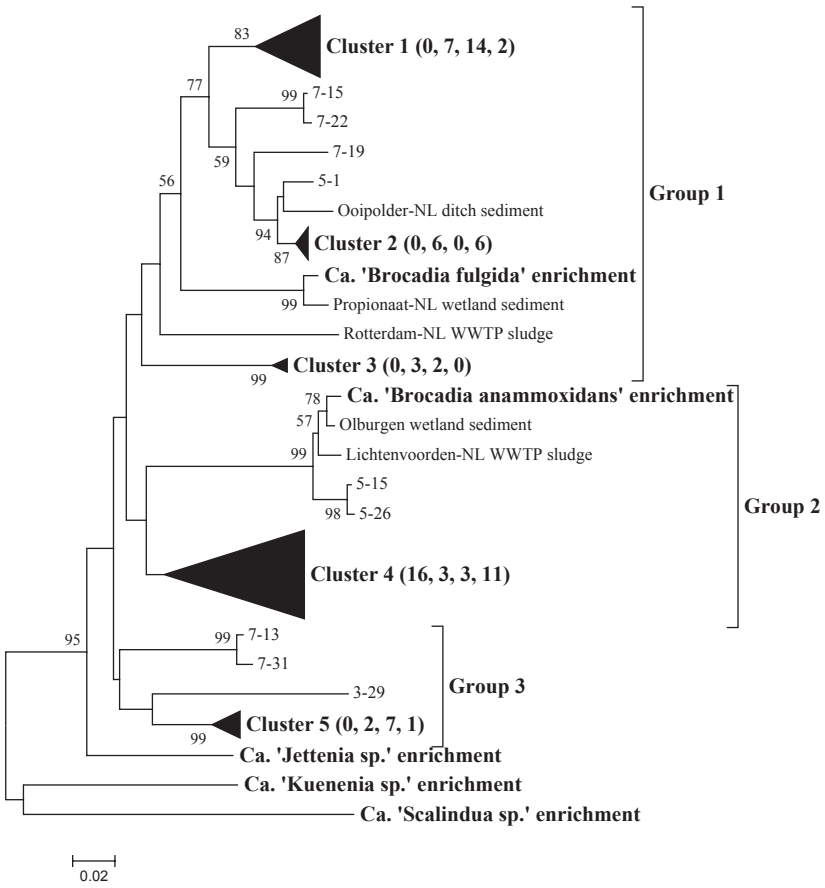

Fig. 1. Phylogenetic tree of the deduced anammox bacterial *hzs*B gene sequences. The reference sequences were amplified from the genomic DNA of highly enriched anammox bacteria. The numbers of clones in four depths (10, 30, 50 and 70 cm) of each cluster are indicated in the parentheses. The evolutionary distances were computed using the Jukes-Cantor method. Branches corresponding to partitions reproduced in < 50% bootstrap replicates were collapsed. The bar represents 2% sequence divergence.

easily, whereas that of the *hzs*B gene suggested that higher diversity could be observed if more clones were sequenced (Fig. S9). It is further confirmed by the coverage estimators of Chao1, which showed a high value of the *hzs*B clone library than that of the 16S rRNA gene (16.9 vs. 5). The Shannon (2.2 vs. 1.35) and Simpson (0.14 vs. 0.27) indices also implied a higher diversity of anammox bacteria by amplifying the *hzs*B gene. Compared with primers targeting the *hzs*A subunits, similarly high specificities were observed that no false positives were detected in 92 (*hzs*B) and 46 (*hzs*A) clones.

Abundance of anammox bacteria

The primer pair of *hzs*B_396F and *hzs*B_742R was applied for the quantification of anammox bacterial abundance in the soil core. The copy number measured in the surface sample (0–10 cm) was $7.0 \pm 0.3 \times 10^5$ copies g^{-1} dry soil and decreased slightly to $2.0 \pm 0.9 \times 10^5$ copies g^{-1} dry soil at 20–30 cm depth as shown in Fig. 2a. Below this depth, *hzs*B gene copy numbers increased and peaked at 40–50 cm depth of $2.7 \pm 1.3 \times 10^6$ copies g^{-1} dry soil, which accounts for about 2.3% of total bacterial cells (Fig. 2c) assuming that the anammox bacteria contained one copy of the *hzs*CBA gene cluster (Strous *et al.*, 2006; Kartal *et al.*, 2011) and 3.8 copies of the 16S rRNA gene for all bacteria (Fogel *et al.*, 1999). For the samples below 60 cm, the copy numbers decreased below the detection limit of the qPCR assay.

The variety in anammox bacterial abundance in the soil core was more or less similar to the result based on 16S rRNA gene from the same site (Zhu *et al.*, 2011b). Little significant correlation was observed between the abundance of anammox bacteria and environmental factors (Table 2). Similar to the anammox in stratified water columns and sediments where active anammox was restricted to specific layers (Dalsgaard *et al.*, 2003, 2005), anammox bacteria seemed to prefer selective niches at particular depths in soil (Humbert *et al.*, 2010). Owing to the high interfering background in soil samples, only the primers targeting the 16S rRNA gene were capable for the *in situ* quantification of soil sample until now (Hamersley *et al.*, 2007; Hu *et al.*, 2011; Zhu *et al.*, 2011b). As the specificity and sensitivity of 16S rRNA gene detection are highly dependent on the abundance of anammox bacteria in environmental samples (Song & Tobias, 2011), the *hzs*B gene would be a more precise biomarker for the quantification of anammox in soil.

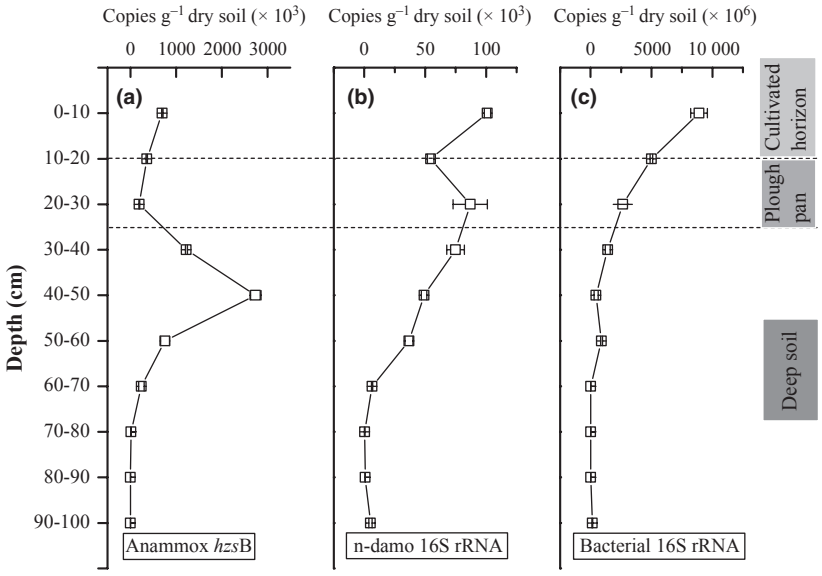

Fig. 2. Abundance of anammox bacteria targeting the *hzs*B gene (a), n-damo bacteria targeting their 16S rRNA gene (b) and total bacteria using broad specificity primers targeting the 16S rRNA gene (c) in every 10 cm. Error bars indicate standard deviation and were calculated from three replicates per site.

Table 2. Correlation analysis of main chemical profiles and the abundance of anammox, n-damo, and all bacteria (n = 10)

Item	n-damo	n-damo/bacteria	Anammox	Anammox/bacteria	Bacteria
pH	− **0.928***	0.367	− 0.284	0.252	− **0.808***
NH_4^+-N	**0.717**†	− 0.433	− 0.097	− 0.373	**0.842***
NO_x^--N	**0.710**†	− 0.262	0.190	− 0.163	**0.879***
NH_4^+/NO_x^-	0.395	− 0.339	− 0.180	− 0.318	0.440
TN	**0.813***	− 0.405	0.050	− 0.322	**0.977***
TOM	**0.917***	− 0.409	0.183	− 0.309	**0.914***
DO	0.553	− 0.241	0.031	− 0.186	**0.843***
Mn^{2+}	− 0.568	− 0.085	− 0.398	− 0.279	− 0.386
Mn^{4+}	**0.748**†	− 0.129	0.272	− 0.021	**0.812***
Mn^{2+}/Mn^{4+}	− 0.514	− 0.114	− 0.372	− 0.260	− 0.336
General bacteria abundance	**0.759**†	− 0.417	0.001	− 0.341	–

*Correlation is significant at 0.01 level (2-tailed).
†Correlation is significant at 0.05 level (2-tailed).

Community structures of n-damo bacteria

To analyze the community structure of n-damo bacteria on a functional level, primers targeting the *pmo*A gene were used in samples from representative depths (0–10, 20–30, 40–50, and 60–70 cm). The n-damo-specific *pmo*A primer A189_b was combined with the widely applied cmo682 primer (Holmes *et al.*, 1995; Luesken *et al.*, 2011c). Following by a nested PCR approach (cmo182-cmo568) (Luesken *et al.*, 2011c), sequences clustering with the *pmo*A sequence present in the genome of *M. oxyfera* were retrieved in the samples except the surface soil (0–10 cm), which did not result in a positive PCR product at the second amplification. The absence of *pmo*A sequence in surface soil suggested a preferred habitat in deep soil for n-damo bacteria. The 14 sequences retrieved from the other three depths together with the published *pmo*A, *pxm*A and *amo*A nucleic acid sequences were phylogenetically analyzed (Fig. 3). Most of the sequences in this study showed high identity to each other and were closely related (difference up to 90–92% nucleotide and up to 94–95% protein identity) to the *pmo*A gene of *M. oxyfera* (FP565575 or CBE69519). The sequences obtained from the paddy soil formed a unique clade in the tree along with other *pmo*A sequences from ditch, aquifer environments, and WWTPs reported previously (Luesken *et al.*, 2011a, c). The low diversity of *pmo*A sequences obtained from the paddy soil was consistent with previous studies (Deutzmann & Schink, 2011; Luesken *et al.*, 2011c; Kojima *et al.*, 2012). The fact that the sequences obtained were not highly divergent from each other was

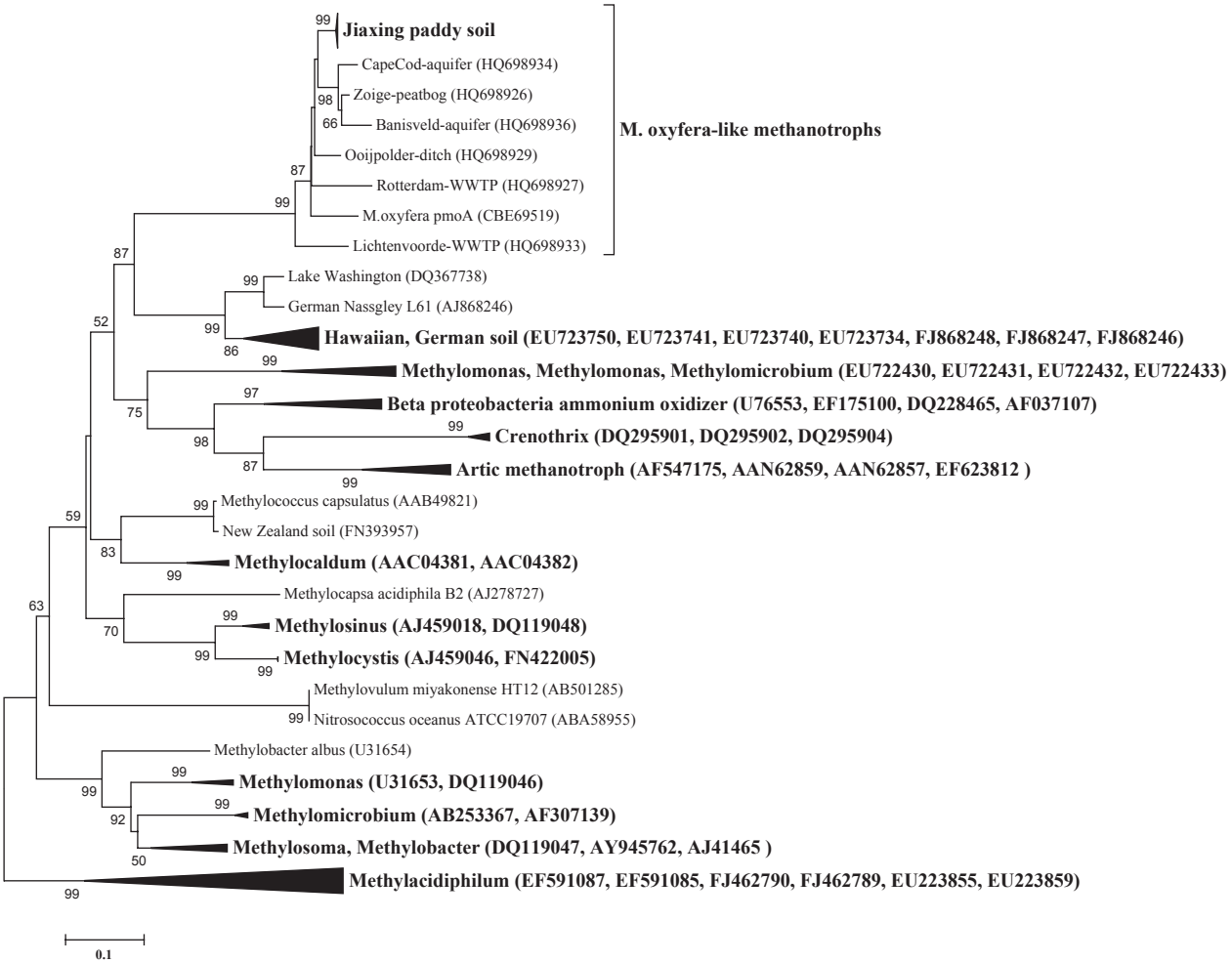

Fig. 3. Phylogenetic tree of *amo*A, *pmo*A and *pxm*A nucleotide sequences including the sequences obtained in paddy soil with *Methylacidphilum* species as the out group. The evolutionary distances were computed using the Jukes-Cantor method. Branches corresponding to partitions reproduced in < 50% bootstrap replicates were collapsed. The bar represents 10% sequence divergence.

probably caused by the functional conservation of *pmo*A gene reflected by the unique oxygenic pathway of n-damo bacteria (Luesken *et al.*, 2011c). In addition, the primers used in this study were designed based on the limited references available. It cannot be ruled out that they were too narrow to cover all the *pmo*A gene of the n-damo bacteria (Deutzmann & Schink, 2011). Therefore, further improvement in specific primers was needed to analyze the diversity of the n-damo at a functional level (Kojima *et al.*, 2012).

Abundance of n-damo bacteria

Because there was no suitable primer pair targeting the *pmo*A gene for qPCR so far, the abundance of n-damo bacteria was estimated by quantifying their 16S rRNA gene. The copy numbers ranged from $1.0 \pm 0.1 \times 10^5$ (0–10 cm) to $7.5 \pm 0.4 \times 10^4$ copies g^{-1} dry soil (30–40 cm; Fig. 2b). Below 40 cm depth, the abundance decreased gradually from $4.9 \pm 0.1 \times 10^4$ (40–50 cm) to $6.5 \pm 0.4 \times 10^3$ (60–70 cm) copies g^{-1} dry soil. Below 70 cm depth, the abundance decreased beyond the limit of detection. As the primers used were designed based on enrichment samples and have not been previously applied on environmental samples. Therefore, the clones of 16S rRNA gene were also sequenced for a comparison with the known n-damo bacteria (Fig. S10). The phylogenetic analysis showed that sequences from 40 to 50 and 60 to 70 cm depths clustered within group *a*, which comprises sequences closely related to the enrichment n-damo bacteria (DQ369742) (Ettwig *et al.*, 2009), whereas sequences from 0 to 10 and 20 to 30 cm depths were distantly related to the known n-damo bacteria. This means the quantification based on the 16S rRNA gene

probably overestimated the abundance in the upper soils because of the less specificity of the primer set. Taken together, n-damo bacteria were most abundant at 30–70 cm depth below the plough pan layer, which was in agreement with the previous study that the n-damo bacteria was only present in profundal sediment (Deutzmann & Schink, 2011). Correlation analysis showed that chemical profiles like pH and TOM correlated well with the abundance of n-damo as shown in Table 2. But in consideration of the flaws in specificity of the primers used, it was hard to find connections between the abundance of n-damo and chemical profiles. There was not a clear interpretation for the vertical distribution of n-damo bacteria in natural ecosystem so far. However, recent enrichment study of n-damo has identified that the addition of oxygen resulted in an instant decrease in methane and nitrite conversion rates (Luesken *et al.*, 2012). Therefore, the absence of n-damo bacteria in surface soil might be caused by the possible penetration of oxygen into the surface soil that negatively affects these anaerobes.

On the whole, the results in this study showed that the anammox and n-damo bacteria co-occurred in the paddy soil. The *hzs*B gene was identified as a novel biomarker for the molecular detection of anammox bacteria. The quantitative PCR and clone library analyses performed in this study indicated both of anammox and n-damo bacteria were abundant in deep layers (30–60 cm). Further studies are required to explore the function and relation of anammox and n-damo bacteria in paddy soil.

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Supporting Information

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

Fig. S1. Vertical profiles of NH_4^+-N, NO_x^--N, pH, total nitrogen (TN), total organic matter (TOM), disolved oxygen (DO) and Mn (II–IV) in the paddy soil.

Fig. S2. Sequence alignment of *hzs* gene β subunit and primers design.

Fig. S3. Primers designed in this study and positions indicated refer to the 'Ca. Kuenenia stuttgartiensis' *hzs*B gene (kuste2860).

Fig. S4. PCR test result of primer combinations on enriched Kuenenia gDNA (annealing temperature 55 °C).

Fig. S5. PCR test result of primer combinations on enriched Brocadia gDNA (annealing temperature 55 °C).

Fig. S6. PCR test result of selected primer combinations on different enriched gDNA (annealing temperature 55 °C).

Fig. S7. PCR test result of selected primer combinations on enriched Brocadia gDNA in a gradient PCR with the annealing temperature ranging from 53.5 to 58.4 °C.

Fig. S8. (a) Phylogenetic analysis of *hzsB* gene sequences from anammox enrichment cultures with designed primer set hzsB_396F and hzsB_742R. The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in < 50% bootstrap replicates were collapsed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are shown as numbers of base substitutions per site. (b) For comparison, a 16S rRNA gene-based phylogenetic tree was shown [adapted from reference (Schmid *et al.*, 2008)]

Fig. S9. Rarefaction and diversity analysis of anammox (*hzs*B and 16S rRNA genes) bacteria.

Fig. S10. Phylogenetic tree of the deduced n-damo and NC10 phylum bacterial 16S rRNA gene sequences (shown in bold) from paddy soil.

Table S1. Sequences of designed hydrazine synthase primers targeting the hzsB subunit of anammox bacteria.

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