

Co-occurrence of nitrite-dependent anaerobic ammonium and methane oxidation processes in subtropical acidic forest soils

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Abstract Anaerobic ammonium oxidation (anammox) and nitrite-dependent anaerobic methane oxidation (n-damo) are two new processes of recent discoveries linking the microbial nitrogen and carbon cycles. In this study, 16S ribosomal RNA (rRNA) gene of anammox bacteria and *pmoA* gene of n-damo bacteria were used to investigate their distribution and diversity in natural acidic and re-vegetated forest soils. The 16S rRNA gene sequences retrieved featured at least three species in two genera known anammox bacteria, namely *Candidatus Brocadia* anammoxidans, *Candidatus Brocadia fulgida*, and *Candidatus Kuenenia stuttgartiensis* while the *pmoA* gene amplified was affiliated with two species of known n-damo bacteria *Candidatus Methylopirabilis oxyfera* and a newly established *Candidatus Methylopirabilis* sp. According to the results, the diversity of anammox bacteria in natural forests was lower than in re-vegetated forests, but no significant difference was observed in n-damo community between them. Quantitative real-time PCR showed that both anammox and n-damo bacteria were

more abundant in the lower layer (10–20 cm) than the surface layer (0–5 cm). The abundance of anammox bacteria varied from 2.21×10^5 to 3.90×10^6 gene copies per gram dry soil, and n-damo bacteria quantities were between 1.69×10^5 and 5.07×10^6 gene copies per gram dry soil in the two different layers. Both anammox and n-damo bacteria are reported for the first time to co-occur in acidic forest soil in this study, providing a more comprehensive information on more defined microbial processes contributing to C and N cycles in the ecosystems.

Keywords Anammox bacteria · N-damo bacteria · *pmoA* gene · Forest soil · Carbon cycle · Nitrogen cycle

Introduction

Anaerobic ammonium oxidation (anammox) process, the oxidation of ammonium to dinitrogen (N₂) gas using nitrite as an electron acceptor under anoxic conditions, was first discovered in a denitrifying fluid reactor nearly 20 years ago (Mulder et al. 1995; Van de Graaf et al. 1995). The discovery changes the traditional knowledge that oxidation of ammonium occurs only under aerobic conditions. Currently, five genera, *Candidatus Kuenenia* (Chamchoi and Nitisoravut 2007; Egli et al. 2001; Schmid et al. 2000; Strous et al. 2006), *Candidatus Brocadia* (Jetten 2001; Kartal et al. 2008), *Candidatus Scalindua* (Kindaichi et al. 2007), *Candidatus Anammoxglobus* (Kartal et al. 2007), and *Candidatus Jettenia* (Quan et al. 2008) and a total of 13 species of anammox bacteria, have been established through culture independent approaches. All obtained anammox bacteria belong to the phylum of *Planctomycetes* (Strous et al. 1999).

Since 1995, anammox bacteria have been reported as distributed widely in various ecosystems, such as marine system (Dalsgaard et al. 2003; Dalsgaard et al. 2005); freshwater

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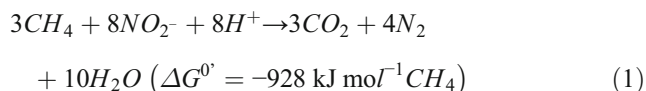
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system, including lakes (Schubert et al. 2006), rivers (Zhang et al. 2007), wetland (Li et al. 2015; Shen et al. 2015), and groundwater (Moore et al. 2011); and terrestrial system (Long et al. 2013; Wang and Gu 2013; Zhu et al. 2011); even some extreme environments like polar regions (Rysgaard et al. 2008), high-temperature hot spring (Jaeschke et al. 2009), deep-sea hydrothermal vents (Byrne et al. 2009), oil reservoirs (Li et al. 2010), guts of invertebrates (Li and Gu 2016), and fishes (Chan et al. 2016). However, though anammox process has been identified to occur in various natural as well as artificial environments, the presence of anammox bacteria in forest soils has not been clearly studied and reported previously.

Nitrite-dependent anaerobic methane oxidation (n-damo) process was first discovered in 2006 and was a novel biochemical process contributing to microbial methane oxidation (Raghoebarsing et al. 2006). It has been shown that the bacterium *Candidatus* “*Methylomirabilis oxyfera*” can couple aerobic oxidation of methane (AOM) to nitrite reduction (NIR) [1] and suggested that it is an intra-aerobic methane oxidation pathway.



Currently, the n-damo process is the only known biological process providing a linkage between two major nutrient cycles—nitrogen and carbon cycles (Raghoebarsing et al. 2006). Through enrichment culture from freshwater canal sediments and then metagenomics analysis, it has been identified that the NIR process is mediated by the bacterium named *Candidatus* “*Methylomirabilis oxyfera*,” which is affiliated with the uncultured “NC10” phylum (Ettwig et al. 2009).

Up to date, *M. oxyfera*-like bacteria have been detected in various environments such as marine sediments of the South China Sea (Chen et al. 2014), freshwater sediments (Shen et al. 2014b), paddy soils (Shen et al. 2014a; Wang et al. 2012; Zhou et al. 2014), wetlands (Chen et al. 2015; Zhu et al. 2015), and waste water treatment facilities (Luesken et al. 2011a) through 16S ribosomal RNA (rRNA) gene and/or *pmoA* gene phylogenetic analysis. However, many other environments have not been investigated for its presence to provide a more comprehensive account of the n-damo process in ecosystems.

Forests drive the dynamics of the terrestrial C cycle, and forest soils contain approximately 73 % of C in the world soils (Sedjo 1993). More than 85 % of the total N in the forest ecosystem is stored in forest soils (Reichle 1981). Moreover, forests account for more than 75 % of gross primary production (GPP) in terrestrial ecosystem (Beer et al. 2010). Forest soils also act as an important sink of both carbon and nitrogen because these nutrients are processed from leaves and litter. Obviously, forest soils have a significant role in the carbon and nitrogen cycle, but both anammox and n-damo processes

have not been studied in forest soils. Therefore, the objectives of this study were to (1) detect the presence of anammox bacteria and n-damo bacteria in acidic forest soils, (2) analyze the distribution and community structure of both natural and re-vegetated forest soils, and (3) quantify the abundance of anammox and n-damo bacteria within the different forest soils of tropical ecosystem.

Materials and methods

Sites description

The study sites are located at Nanling National Nature Reserve in Guangdong province of China (24° 37′–24° 57′ N, 112° 30′–113° 04′ E) (Table 1), which covers an area of 58,368.4 hm² and has a typical subtropical monsoon climate. The annual average precipitation is 2108.4 mm. The maximum average temperature is 26.2 and 7.1 °C in the hottest month (July) and the coldest month (January), respectively (Gan et al. 2015). Two types of forests are present within the study sites. Forests located at an altitude above 700 m from sea level are natural, undeveloped forests, while forests located at an altitude below 700 m were once deforested and then reforested artificially through re-vegetation. Soils in those natural forests are typical yellow containing granite, metamorphic rock, and sandstone, but red soils become dominant in re-vegetated forests (Gan et al. 2015).

In this study, soils from five representative forests at different levels of altitude were selected for investigation, including three from natural forests and two from re-vegetated forests. Briefly, soil samples were collected from (i) a natural bamboo forest at the top of a mountain (altitude of 1858 m, labeled as HLS and HLD, S and D represent soil of surface and lower layer, respectively); (ii) a natural coniferous forest, predominated by *Pinus massoniana* (Lambo) (1371 m, PS and PD); (iii) a natural evergreen broad-leaf forest, dominated by *Schima superba* and *Machilus thunbergii* (1009 m, EBL S and EBLD); (iv) a man-made young *Cunninghamia lanceolata* (Lamb.) forest (615 m, CLYS and CLYD); and (v) mature *Cunninghamia lanceolata* (Lamb.) forest (529 m, CLMS and CLMD), respectively.

Field sampling and physicochemical analysis

Soil samples were taken on January 8, 2015, from five locations along elevation from 1858 to 529 m for two layers (surface—A0 layer, 0–5 cm, and lower—A1 layer, 10–20 cm) (Table 1). Three replicates were sampled randomly at each site to form a composite sample. Soils were kept in an icebox immediately with coolant and separated into two parts. One was used for physicochemical analysis and the other was brought to The University of Hong Kong for molecular analysis.

Table 1 Soil physical and chemical parameters in different forests in this study

Forest type	Sample ID	pH	Total N (g kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)	Organic matter (g kg ⁻¹)	Water content (%)	Total K (g kg ⁻¹)	Total P (g kg ⁻¹)	Hydrolyzed N (mg kg ⁻¹)	Exchangeable Al ³⁺ (cmol kg ⁻¹)	Available P (mg kg ⁻¹)
High-altitude dwarf forest	HLS	4.18 ± 0.17	5.53 ± 0.98	31.97 ± 6.65	77.92 ± 7.88	140.4 ± 25.9	39.30 ± 3.50	24.35 ± 2.03	0.212 ± 0.013	462 ± 187	6.12 ± 1.10	12.23 ± 2.77
	HLD	4.52 ± 0.02	2.56 ± 0.30	8.89 ± 2.12	34.19 ± 8.24	75.8 ± 15.4	23.08 ± 4.69	24.18 ± 3.53	0.162 ± 0.063	313 ± 79	4.79 ± 0.62	7.97 ± 2.43
Pine forest	PS	3.83 ± 0.35	6.78 ± 3.09	30.95 ± 10.74	84.05 ± 24.64	271.7 ± 133.1	44.76 ± 12.63	21.19 ± 6.81	0.254 ± 0.041	438 ± 28	10.30 ± 2.49	8.67 ± 2.59
	PD	4.47 ± 0.07	1.72 ± 0.75	8.26 ± 2.95	41.50 ± 13.91	48.5 ± 16.6	26.63 ± 4.45	25.63 ± 4.78	0.177 ± 0.034	152 ± 36	4.05 ± 0.98	3.41 ± 0.61
Evergreen broad-leaf forest	EBLS	3.80 ± 0.21	7.19 ± 3.08	33.74 ± 18.53	71.76 ± 15.61	243.3 ± 126.3	39.55 ± 7.10	15.36 ± 3.32	0.350 ± 0.061	384 ± 46	12.70 ± 3.52	8.65 ± 1.87
	EBLD	4.56 ± 0.06	1.50 ± 0.42	6.04 ± 0.40	31.78 ± 9.74	34.6 ± 1.6	21.40 ± 4.13	20.79 ± 5.26	0.185 ± 0.039	144 ± 5	6.41 ± 1.18	3.07 ± 0.72
Re-vegetated young forest	CLYS	4.45 ± 0.07	2.72 ± 0.27	20.11 ± 13.29	58.70 ± 26.85	80.47 ± 9.3	27.35 ± 1.48	12.76 ± 1.64	0.277 ± 0.008	297 ± 31	9.43 ± 0.39	3.38 ± 0.49
	CLYD	4.51 ± 0.04	1.88 ± 0.48	6.97 ± 0.28	30.39 ± 5.35	39.8 ± 9.1	27.03 ± 2.02	12.68 ± 2.68	0.232 ± 0.030	178 ± 40	8.65 ± 0.93	4.83 ± 3.08
Re-vegetated mature forest	CLMS	4.26 ± 0.09	2.87 ± 0.30	13.56 ± 1.85	42.11 ± 9.21	75.83 ± 7.1	24.07 ± 1.02	12.02 ± 0.58	0.378 ± 0.022	258 ± 70	8.01 ± 1.06	3.82 ± 1.07
	CLMD	4.31 ± 0.12	1.72 ± 0.274	5.54 ± 0.49	25.61 ± 5.22	40.9 ± 3.2	23.36 ± 1.72	11.78 ± 0.42	0.295 ± 0.005	163 ± 48	7.46 ± 0.27	3.76 ± 1.40

Physical-chemical analysis was carried out at Guangdong Institute of Eco-environment and Soil Sciences. The methods used were described elsewhere (Gan et al. 2015). Soil moisture content was measured by drying in oven at 105 °C for 24 h to constant weight.

DNA extraction and PCR amplification

Genomic DNA was extracted from 0.25 g of soils using the PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc., USA) according to the manual. DNA concentration was measured by NanoDrop™ ND-2000 (Thermo Scientific Inc., USA). The extracted DNA was stored at -20 °C for further analysis.

Nested PCR was adopted in this study to amplify gene fragments of the targeting microbial groups. For anammox bacteria, pla46F targeting *Plantomycetes* phylum and universal primer 630R were selected as outer primer, while Amx368f–Amx820r for anammox 16S rRNA gene were selected as inner primer. For n-damo bacteria, two sets of primers, A189_b-com682r and com182f-com568r (Luesken et al. 2011b), were used to amplify the characteristic *pmoA* gene in n-damo bacteria. Detailed information of primer and corresponding thermal profiles are listed in Table 2. After amplification, PCR products were examined with gel electrophoresis in 1 % agarose gels in TAE buffer.

Cloning and sequencing

Thirty individual PCRs (triplicates for each sample) were pooled and gel-purified by using GFX™ PCR DNA and Gel Band Purification Kit by following the manufacturer’s protocol (Amersham Biosciences). Amplified gene fragments were then ligated into pMD-18 vector (Takara, Japan) and transformed into DH5α competent cells for clone library construction. After blue-white screening, positive clones were selected randomly for sequencing.

Quantitative PCR assay

Primer sets of Amx 438f–Amx 684r and qP1F–qP1R were selected to conduct real-time quantitative PCR (qPCR) to estimate the abundance of 16S rRNA gene of anammox bacteria and n-damo bacteria in soil samples, respectively (Table 2).

The qPCR assays were performed on an ABI 7000 Sequence detection system (Applied Biosystems, Foster City, CA) using SYBR green method. Standard curves for qPCR were constructed by ten serial dilutions of a known copy number of plasmid through a sequenced clone. Triplication was conducted for each sample, and each reaction was performed in a volume of 20 µl containing 1 µl of DNA template (10 ~ 100 ng DNA templates), 1 µl of each primer (10 µM), and 10 µl of SYBR Green PCR Master Mix (Applied

Table 2 PCR primers and thermal profiles used in this study

Specificity	Primers	Sequence(5'-3')	Thermal profiles	Reference
Planctomycete (16S rRNA gene)	pla46F 630R	GGATTAGGCATGCAAGTC CAKAAAGGAGGTGATCC	95 °C for 10 min followed by 35 cycles of 60 s at 95 °C, 1 min at 56 °C, 1 min at 72 °C	(Schmid et al. 2000)
Anammox (16S rRNA gene)	Amx 368f Amx 820r	TTCGCAATGCCCGAAAGG AAAACCCCTCTACTTAGTGCCC	95 °C for 10 min followed by 25 cycles of 60 s at 95 °C, 1 min at 52 °C, 1 min at 72 °C	(Schmid et al. 2000; Schmid et al. 2003)
N-damo (<i>pmoA</i> gene, nested)	A_189b Com 682R	GGN GAC TGG GAC TTY TGG AAA YCC GGC RAA GAA CGA	5 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 53–63 °C and 1 min 30 s at 72 °C; finally, 10 min at 72 °C	(Luesken et al. 2011a)
	Com 182F Com 568R	TCA CGT TGA CGC CGA TCC GCA CAT ACT CCA TCC CCA TC	5 min at 95 °C, followed by 15 cycles of 60 s at 95 °C, 60 s at 55 °C and 1 min 30 s at 72 °C; finally, 10 min at 72 °C	(Luesken et al. 2011b)
Anammox (qPCR)	Amx 438f Amx 684r	GTC RGG AGT TAD GAA ATG ACC AGA AGT TCC ACT CTC	95 °C for 5 min; 40 cycles consisting of 95 °C for 15 s, 55.5 °C for 45 s, and 72 °C for 1 min; and 95 °C for 15 s	(Schmid et al. 2003)
N-damo(qPCR)	qPIF qPIR	GGG CTT GAC ATC CCA CGA AAC CTG CGC CTT CCT CCA GCT TGA CGC	95 °C for 5 min; 40 cycles consisting of 95 °C for 15 s, 63 °C for 45 s, and 72 °C for 1 min; and 95 °C for 15 s	(Ettwig et al. 2009)

Biosystems, Foster City, CA). The reaction cycling conditions were in accordance with the PCR procedures described above. The R^2 values were 1.0, and amplification efficiency was 93.2 and 96.5 % of anammox and n-damo, respectively.

Sequences and phylogenetic analysis

Sequencing was conducted at Beijing Genomics Institute (Shenzhen, People's Republic China). Clone sequences were processed by BioEdit (Hall 1999) and aligned by Clustal X (Thompson et al. 2002). DECIPHER's Find Chimeras (Wright et al. 2012) was used to remove chimeras in anammox 16S rRNA gene sequences to ensure the reliability of sequences obtained. Processed sequences were analyzed against those in GenBank with BLAST (Altschul et al. 1997). After the identity and validity of sequences confirmed through GeneBank, sequences of *pmoA* gene were translated into amino acids for phylogeny analysis. At last, all processed sequences were imported to MEGA version 6.0 to construct phylogenetic tree, using neighbor-joining algorithm with the best model of Kimura 2-parameter for anammox and JTT method amino acid substitution model for n-damo. The reliability of branching within the tree was tested with bootstrap of 1000 replicates, and the consensus tree was presented.

Statistical analysis

To examine the distribution of anammox bacteria and *M. oxyfera*-like bacteria, the operational taxonomic units (OTUs) cutoff values of 3 % was applied to determine the diversity of anammox bacterial 16S rRNA gene sequences and sequences of *pmoA* gene using the Mothur program (Schloss et al. 2009). The Chao1 estimator, richness, and

Shannon-Wiener diversity index were also calculated with the Mothur software.

The data of physical-chemical parameters and abundance of anammox bacteria were imported to R to conduct independent sample *t* test and analysis of variance (ANOVA) to investigate any significant differences between samples from different layers or forests (Table S1). To investigate relationships between diversity of anammox communities, n-damo communities and soil physical-chemical parameters, the Pearson moment correlation was carried out using PAleontological STatistics version 3.0 (Hammer 2012). Detrended correspondence analysis (DCA) was conducted for anammox and n-damo analysis using Canoco for Windows 4.5 (Ter Braak and Smilauer 2012). A linear model was applied, and the constrained ordination method was redundancy analysis (RDA) using Canoco.

Finally, UniFrac function under Mothur software was used to perform the principal coordinate analysis (PCoA). Results generated by Mothur were imported into OriginPro8 (OriginLab, USA) to construct illustrations.

Nucleotide sequences

The anammox bacteria n-damo *pmoA* gene sequences were deposited in NCBI under accession number KU894711-KU894735 and KU894736-KU894777, respectively.

Results

Physical-chemical parameters

A range of soil properties were investigated including pH, organic matter, total N, total P, total K, NH_4^+ -N, NO_3^- -N,

hydrolyzed nitrogen, available phosphorus, exchangeable Al^{3+} , and water content for a total of these 11 parameters of 10 samples in triplicate. The pH values of soil samples ranged from 3.66 to 4.59, which were classified as extremely acidic to very strongly acidic (USDA 1999). The details of chemical properties of soil are listed in Table 1. According to the results of ANOVA (Table S1), the surface layer of soil was more acidic than the lower layer on average. Moreover, surface layer contained significantly higher concentration of organic matter, total N, NH_4^+ -N, NO_3^- -N, hydrolyzed N, and exchangeable Al^{3+} than lower layer. Total P and available P were higher in surface soil than the deep in natural forest, but this trend did not persist in re-vegetated forest. There was no significant difference of total K content between these two layers.

Comparison of soil properties between different forests was also made using ANOVA. The five forests were divided into natural and re-vegetated forests. Based on results obtained, soil samples from natural forest contained significantly higher organic matter, total N, NH_4^+ -N, NO_3^- -N, total K, and available P than those of re-vegetated ones. The re-vegetated forest soils yielded higher total P than natural forest. There was no significant difference in pH values and exchangeable Al^{3+} between the two soil types. ANOVA was also conducted to compare the differences between the two layers of an individual forest. Regardless of the forest type, majority of physical-chemical parameters did not show a significant difference between the two forests. Significant differences were only observed for total K content in natural forest, and pH value and total P content in re-vegetated forest.

Phylogenetic analysis of anammox bacteria and n-damo bacteria

The coverage of clone library for anammox and n-damo bacteria reached 96 to 100 % (Table 3), indicating nearly complete obtainment of information of two communities. The

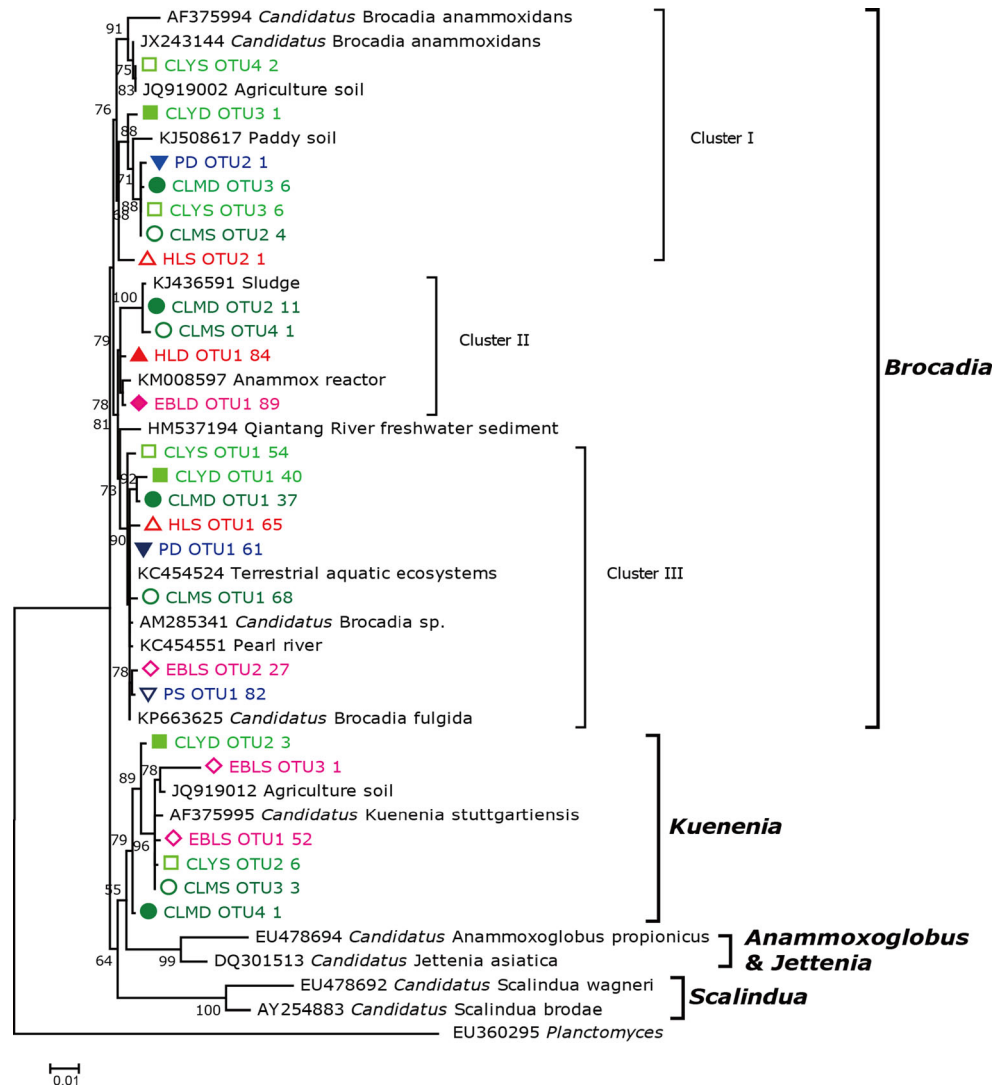
phylogenetic tree constructed by 16S rRNA gene indicates that two known anammox bacterial genera, namely *Brocadia* and *Kuenenia*, were detected in the forest soils (Fig. 1). Majority of sequences (636 out of 702) were closely related to *Candidatus Brocadia*, and could be further divided into three subclusters according to the different environments that sequences affiliated with. Cluster I comprised mainly sequences from re-vegetated forest (20 out of 21). This clade was most closely related to *Candidatus Brocadia* anammoxidans and sequences from agriculture soil. Cluster II consisted of sequences from samples HLS, EBLs, and CLYS and were closely affiliated with uncultured anaerobic ammonium-oxidizing bacteria clones from sludge and reactor. In cluster III, all of the sequences in this cluster showed 98–99 % identity to the sequences of *Candidatus Brocadia fulgida* and grouped with references retrieved from freshwater ecosystem. The remaining 66 anammox 16S rRNA gene sequences were closely related to *Candidatus Kuenenia stuttgartiensis* 16S rRNA gene (AF375995) in GenBank (identities up to 97–99 % for nucleotide). But these sequences were only detected from sample EBLs and samples from re-vegetated forests. Overall, sequences from re-vegetated forest were more diverse and scattered, which could be detected in every cluster of *Brocadia* and *Kuenenia*. In natural forest, majority of sequences obtained were distributed in *Brocadia* in a more centralized manner, except sequences from EBLs.

Primers targeting *pmoA* gene of n-damo bacteria were successfully applied on all samples in this study. Deduced amino acid sequences were grouped into six clusters with the *pmoA* sequence of *M. oxyfera* retrieved to show the phylogeny (Fig. 2). Cluster I, which contained 714 sequences in total, included sequences from all soil samples. Five hundred eighty-two sequences were 100 % identical to the sequence from sediment of Panjin swamp on nucleotide level; 93 sequences were closely affiliated with the sequences from freshwater sediments; interestingly, 11 sequences in this cluster having 95%

Table 3 Alpha diversity of anammox bacteria and n-damo bacteria in forest samples (based on percentage sequence identity of 97% similarity)

Sample ID	Anammox 16S rRNA gene					<i>pmoA</i> gene				
	No. of sequences	OTUs	Chao1	Coverage (%)	Shannon	No. of sequences	OTUs	Chao1	Coverage (%)	Shannon
HLS	66	2	2	98.48	0.08	80	4	7	96.25	0.2
HLD	86	3	4	97.67	0.13	85	4	7	96.47	0.19
PS	82	1	1	100	0	71	6	6.5	97.18	1.29
PD	62	2	2	98.39	0.08	82	3	3	100	1.1
EBLS	80	3	3	98.75	0.7	77	3	4	97.4	0.14
EBLD	89	1	1	100	0	92	5	6	97.83	0.47
CLYS	68	4	4	100	0.72	88	1	1	100	0
CLYD	44	3	3	97.73	0.36	82	5	5	98.78	1.05
CLMS	76	4	4	98.68	0.44	89	5	5	98.88	0.65
CLMD	55	4	4	98.18	0.9	85	6	12	95.29	0.41

Fig. 1 Phylogenetic tree constructed by neighbor-joining method after an alignment of anammox bacterial 16S rRNA gene sequences along with some known species from GenBank. Numbers beside the sample ID refer to the number of clones in each clone library. The numbers at the nodes are bootstrap calculation obtained based on 1000 times replicates



identity to KC341442 (sediment of Panjin swamp) did not affiliate to any known cultured species. Cluster II was composed of 60 sequences, mainly from samples PS and PD, which were closely related to n-damo sequences from Qiantang River sediment. Cluster III contained only sequences from PS and PD and these sequences were most similar to *M. oxyfera* (FP565575) among all sequences retrieved. There were only two sequences in cluster IV, which were closely related to the sequence retrieved from aquifer environment. Cluster V contained four sequences closely related to samples from coastal wetland sediment with 93–96 % identity to the sequence of *Candidatus Methyloirabilis* sp. Rs1 (KT443986), which was a novel denitrifying methanotroph of NC10 phylum enriched (unpublished data). Cluster VI included nine sequences showing 100 % identity to the *Candidatus Methyloirabilis* sp. Rs1 in amino acid level (ALL28944). In short, clusters I to III were more related to known specie of *M. oxyfera* while clusters IV to VI were closely affiliated to novel specie *Candidatus Methyloirabilis* sp. Rs1.

Based on phylogenetic tree constructed, principal coordinate analysis (PCoA) was conducted. According to the results of PCoA, the first and second principles in total contributed about 66% of structure variations for anammox bacteria (Fig. 3). The community of samples retrieved from re-vegetated forests was more centralized while anammox community in natural forests was more scattered comparatively. For n-damo community structure, the first and second principles combined to contribute about 58% of variations in PCoA. Samples could be grouped together according to their corresponding forest types.

Genetic diversity analyzed of anammox bacteria and n-damo bacteria

A total of 708 sequences of anammox bacteria and 831 sequences of *pmoA* gene were analyzed. The diversity of anammox and n-damo bacteria was investigated via number of OTUs, Chao1 estimators and Shannon index calculations.

Fig. 2 Phylogenetic tree of *pmoA* nucleotide sequences including the sequences as out group from GenBank. The evolutionary distances were computed using the Tamura-Nei method. The numbers at the nodes are bootstrap calculation obtained based on 1000 times replicates



The operational taxonomic unit (OTU) cutoff was examined by the 97 % sequence similarity to reflect phylogeny diversity in each sample (Table 3). A total of one to four and one to six OTUs of anammox 16S rRNA and *pmoA* genes were

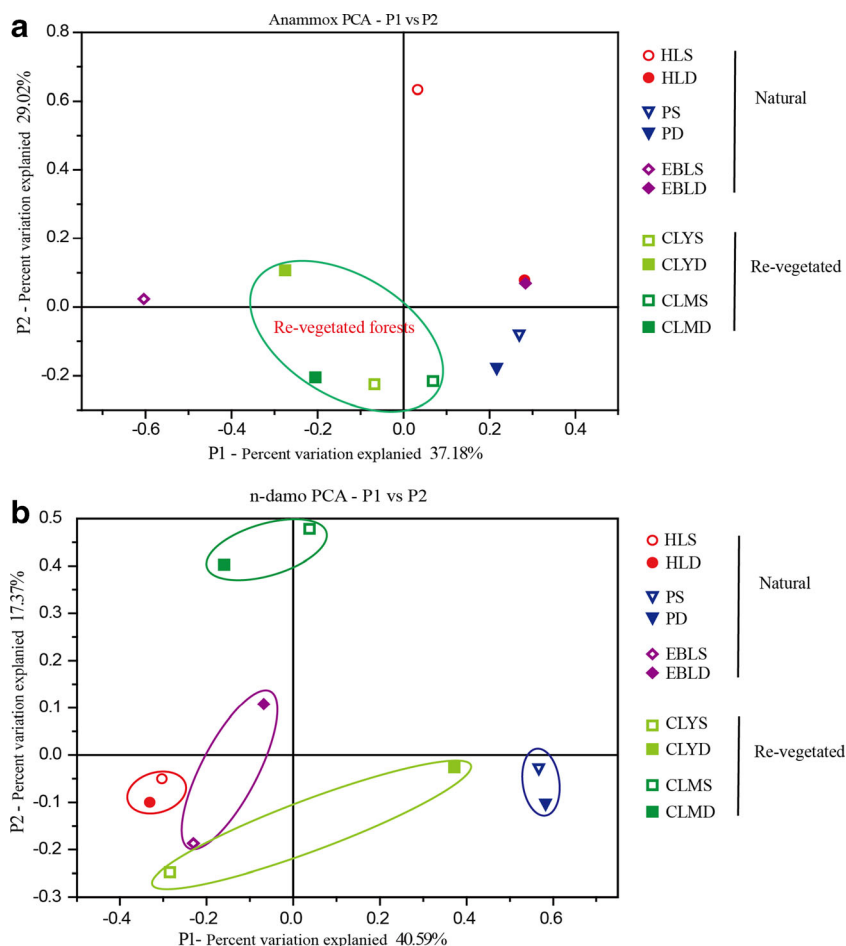
observed, respectively. The diversity of anammox bacteria in natural forest was lower than that in re-vegetated forest while no significant difference was detected in n-damo diversity between two types forests (Table 2). Compared to studies in

other ecosystems, anammox bacteria showed a notably lower diversity while no difference was observed in n-damo community in this acidic forest ecosystem.

Abundance of anammox bacteria and n-damo bacteria

The primer pair of Amx438f and Amx684r was applied for the quantification of anammox bacterial abundance in the soil samples. Standard curves were generated by eight orders of magnitude of the standard plasmid containing cloned 16S rRNA gene fragment. A significantly linear relationship (R^2 , 1.0) was obtained and an amplification efficiency of 93.2 % was yielded. The melting curve analyses revealed only one peak appearing at 84.5 °C, confirming that fluorescent signals were derived from specific PCR products in the process of the qPCR quantification, and thereby reliable results. The gene copy number measured in the surface sample (0–5 cm) was 2.21×10^5 – 3.91×10^5 gene copies per gram dry soil in natural forest and ranged from 4.44×10^5 to 1.20×10^6 gene copies per gram dry soil in the lower layer (Fig. 4a). Compared to natural forest, the abundances of anammox bacteria in re-vegetated forest were higher, ranging from 6.68×10^5 to 3.90×10^6 gene copies per gram dry soil.

Fig. 3 Principal coordinates analysis (PCoA) of anammox bacteria (a) and n-damo bacteria (b) in five forest soils. *Red*, high-altitude forest; *blue*, pine forest; *purple*, evergreen broad-leaf forest; *shallow green*, re-vegetated young forest; *dark green*, re-vegetated mature forest



The abundance of n-damo bacteria was estimated through qualifying 16S rRNA gene by qP1F and qP1R primers. With respect to its R^2 value (R^2 , 1.0) and amplification efficiency (96.5%), the result was suggested to be reliable. The gene copy numbers ranged from 1.69×10^5 to 5.07×10^6 gene copies per gram dry soil. In natural forest, the abundance of surface samples was two to ten times lower than the deep layer samples, and the highest gene abundance was retrieved from pine forest sample (Fig. 4b), while samples from young forest resulted in fewer gene copies than matured forest under re-vegetation. The quantities of gene copies in the lower layer were higher than in the surface layers in two re-vegetated forests.

Discussion

Distribution and diversity of anammox bacteria and n-damo bacteria

In this study, anammox and n-damo bacteria were reported for the first time to be detected in acidic forest soils in tropic

region. This contributes to better understanding on the global distribution of anammox and n-damo bacteria to fully account for C and N cycling. Two genera of anammox bacteria—*Candidatus Brocadia* and *Candidatus Kuenenia*—were found in this study. Both genera of anammox bacteria were reported as dominant types in terrestrial ecosystem (Humbert et al. 2010). *Brocadia* and *Kuenenia* were reported to be able to use ferrous iron and a variety of organic compounds, such like formate, acetate, propionate, and methylamines, as electron donors, and the versatile metabolism of *Brocadia* and *Kuenenia* organisms may result in better adaptations in various terrestrial environments (Kartal et al. 2008; Strous et al. 2006). The detection of these two genera was in agreement with our current understanding and information based on environmental conditions of the sampling sites.

Based on the number of OTU identified and diversity indices calculated, the results of phylogenetic study revealed that the diversity in these study sites were generally lower, compared to results reported in other ecosystems, such as rice paddy field or wetlands (Shen et al. 2015; Zhu et al. 2011). The lower diversity of anammox bacteria might be attributed

to several factors. First, soil pH may be a critical one. According to a reported study on metabolism of anammox bacteria, and the performance of anammox bacteria in bioreactors, anammox bacteria grow within the pH range of 6.7 to 8.3, being optimum at slightly alkaline conditions (Kartal et al. 2011) whereas even slightly acidic condition would weaken the tolerance of resting anammox cells to NO_2^- toxicity (Altschul et al. 1997). As mentioned previously, the pH of soil samples in this study ranged from 3.66 to 4.59. This highly acidic nature of soil may impede growth of anammox bacteria and lead to the result of low diversity.

Limited nitrogen sources may be another possible explanation for the low diversity observed. As anammox bacteria make use of ammonium and nitrite for metabolism, reactive nitrogen level would be an important factor for anammox bacteria to establish. Compared to environments in which anammox bacteria were frequently reported, such as rice paddy field (Zhu et al. 2011) and wastewater treatment facilities (Wu et al. 2015), forest soils are relatively infertile in terms of nitrogen. At the study sites, shed leave litter would probably be the sole nitrogen source to soil. On the other hand, agricultural soil and domestic wastewater generally contain a huge quantity of nitrogen available to microbes due to addition of fertilizers and decomposition of nitrogen-containing compounds, respectively. Thus, abundant nitrogen sources could support a more diverse, complex community of anammox bacteria in these environments.

Vegetation may also exert an adverse effect on the diversity of anammox bacteria. As the sampling sites were covered by extensive, various kinds of trees and herbaceous plants, a strong association between plants and soil properties could be expected in the rhizosphere. Plants release organic acids to the rhizosphere via the roots (Haoliang et al. 2007), and organic acids are generally unfavorable to anammox bacteria (Van de Graaf et al. 1996). Therefore, the growth and in turn diversity of anammox bacteria may be hindered by the extensive local vegetation.

Although anammox and n-damo bacteria seem to be competitive for nitrite which serves as the electron acceptor for each process (Haroon et al. 2013), co-occurrence was reported in paddy soil (Wang et al. 2012), saline lake (Yang et al. 2012), and wetlands (Shen et al. 2015) recently as well as in this study. According to the phylogeny analysis of n-damo bacteria (Fig. 2), all clones affiliated with the sequences from wetland and freshwater ecosystem, especially the cluster I accounting for 85.9% of all *pmoA* sequences closely related to the sediment of Panjin Swamp which is one of the best preserved wetlands in the world (Zhu et al. 2015).

This was indeed out of expectations because environmental conditions of forest soils were very different from that of wetland sediment. To explain this, in subtropical forest, moss and litter in surface layer may retain moisture for the soil from rainfall and, under anaerobic environments, litters would

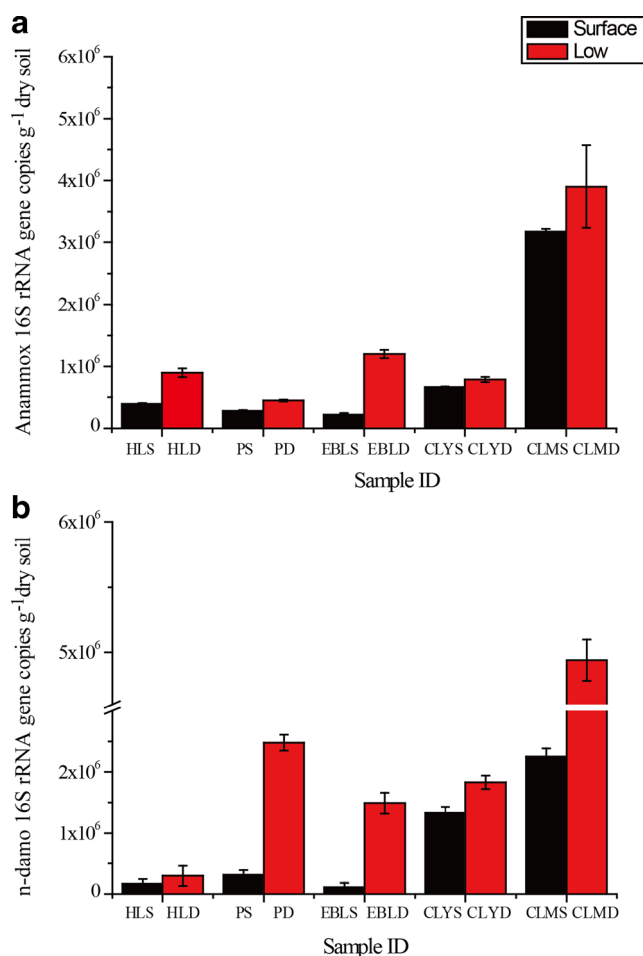


Fig. 4 The number of copies of anammox bacterial 16S rRNA gene (a) and n-damo bacterial 16S rRNA gene (b) in five forests soils

Table 4 Statistical analyses of physical-chemical parameters of anammox and n-damo bacterial diversity

Parameters	Abundance		OTUs		S_{ACE}		Shannon	
	Anammox	n-damo	Anammox	n-damo	Anammox	n-damo	Anammox	n-damo
pH	0.60277	0.60585	0.10909	-0.15433	0.16959	-0.04802	-0.18574	-0.11901
Organic matter (g kg ⁻¹)	-0.74566*	-0.74435*	-0.32091	0.026471	-0.43511	-0.2088	-0.079903	0.07443
Total N (g kg ⁻¹)	-0.74422*	-0.77852**	-0.26909	-0.023021	-0.42476	-0.26862	-0.054159	-0.052838
Total P (g kg ⁻¹)	0.019449	0.13219	0.5758	0.029998	0.60834	0.23085	0.68943*	-0.15159
Total K (g kg ⁻¹)	-0.54456	-0.38973	-0.7263*	-0.050231	-0.84941**	-0.4527	-0.82167**	0.14872
Hydrolyzed N (mg kg ⁻¹)	-0.73074*	-0.83062**	-0.17736	-0.1206	-0.38342	-0.38304	-0.14957	-0.21755
Available P (mg kg ⁻¹)	-0.59574	-0.9054**	-0.30965	0.054436	-0.53883	-0.32151	-0.29467	-0.17298
NO ₃ ⁻ -N (mg kg ⁻¹)	-0.7826**	-0.7124*	-0.20791	-0.22234	-0.37982	-0.37553	-0.019598	-0.17394
NH ₄ ⁺ -N (mg kg ⁻¹)	-0.83765**	-0.67247*	-0.33446	-0.21758	-0.43825	-0.40087	-0.1664	-0.03387

Based on Pearson moment correlation; numbers in bold are regarded as significant, * $p < 0.05$, ** $p < 0.01$

degrade to release substrates for n-damo bacteria. Degradation of organic carbon may provide similar conditions for n-damo bacteria in wetlands to establish and grow.

Another observation on n-damo bacteria was that the majority of sequences from different forests were highly concentrated in cluster I, except a sequence obtained from pine forest soil. Regarding sequences from samples PS and PD, an even distribution pattern was found in clusters I, II, and III, illustrating a more diverse community of n-damo bacteria in the soil samples concerned. Clear explanations and mechanisms of such difference are not available. It could be the consequences of complex interactions among physical-chemical parameters of soil, local vegetation, and microbial communities. Furthermore, less or minimum anthropogenic interruption may also contribute to the characteristic community of n-damo bacteria at this site for the pine forest is located at a steeper slope at relatively higher elevation.

Abundance of anammox bacteria and n-damo bacteria

The PCR primer Amx438f and Amx684r could recover a more refined community structure of anammox bacteria from a wider environmental samples (Han and Gu 2013; Humbert et al. 2012), they were used to detect the abundance of anammox bacteria in this study. Overall, the abundance of anammox bacteria observed in this study ranged from 2.21×10^5 to 3.90×10^6 gene copies g⁻¹ dry soil, which is lower than that reported in paddy soil (10^5 – 10^7 copies g⁻¹ dry soil) (Wang et al. 2012; Zhu et al. 2011). In fact, the surface layer of forest soil (A0 layer) is not exposed to air directly. Instead, it is generally covered by shed and moist leaves, in turn limiting the flux of air and producing a partially anaerobic environment at the surface soil. Thus, this may explain the existence of anammox bacteria in the surface layer of forest soil. However, growth and metabolism of anammox bacteria are highly sensitive and greatly inhibited by O₂ (Jetten et al.

2009). In addition, unlike wetland sediment, forest soil in this study was porous in nature, and the anaerobic condition generated by shed leaves was not always guaranteed, e.g., drying season. Therefore, certain amount of oxygen may still frequently be present in the surface layer of soil and hamper the growth of anammox. This may explain the low detection frequency and lower abundance of anammox bacteria in the surface layer.

The abundance of n-damo bacteria (1.68×10^5 – 4.94×10^6) observed in this study was similar to those reported in flooded paddy field (10^5 – 10^6 copies per gram dry soil) (Shen et al. 2014a), freshwater lakes (10^4 – 10^6 copies per gram dry sediment) (Kojima et al. 2012; Liu et al. 2015), and wetland sediment (10^5 copies per gram dry sediment with qP1F-P2R) (Chen et al. 2015). Compared to anammox bacteria, n-damo bacteria yielded higher abundance and seemed to be more adaptive in acidic forest soil. However, as n-damo has been discovered only for a short period of time, current designed primers are not optimized and specific to n-damo bacteria. Instead, non-specific amplification of some other bacteria, such as *Acidobacteria*, has been detected in this acidic forest soil after sequencing. Thus, the value of abundance of n-damo bacteria obtained would be likely over-estimated, and it is hard to evaluate the real ratio of anammox and n-damo with the current available techniques.

Significant correlations were observed between the abundance of anammox bacteria and environmental factors (Table 4). Abundances of anammox and n-damo bacteria were observed to be negatively correlated to parameters including hydrolyzed N, NH₄⁺-N, and NO₃⁻-N and organic matter. Organic matter had a negative correlation with the abundance of anammox bacteria and this agrees with the general thought that organic matter (e.g., organic carbon) has an adverse effect on the growth of anammox bacteria because co-occurrence of ammonium, nitrite, and organic matter would encourage

growth of heterotrophic denitrifiers which compete nitrite ions with anammox bacteria (Tang et al. 2010). Anammox bacteria, as a result, are less competitive because of their lithotrophic nature and slow growth (Tang et al. 2010). Also, organic alcohol, especially methanol, shows inhibitory effect on activities of anammox bacteria (Güven et al. 2005; Tang et al. 2010; Van de Graaf et al. 1996). Thus, negative correlation between organic matter and abundance of anammox bacteria was expected in forest soil.

An unusual phenomenon observed is that abundances of two bacteria gave negative correlation to nitrogen in soil, including ammonium, nitrite, or total nitrogen content. Since anammox bacteria utilize NH_4^+ and NO_2^- , while n-damo bacteria utilize NO_2^- as energy source, a positive correlation is expected between nitrogen content and abundance of these two bacteria (Fig. S1). However, the abundance of bacteria should be considered as the final outcome of all environmental conditions in an open and dynamic ecosystem. Indeed, both bacteria prefer anaerobic condition and oxygen level would be lower at deep layer and, meanwhile, the nitrogen content in soil usually decreases with depth. In case oxygen concentration is a determining factor than other parameters, the abundance of these two bacteria would be higher at lower layer than the surface. In other words, the negative correlation observed in our study would probably be a coincidence than a causation effect.

Anammox and n-damo bacteria co-occurred in the acidic forest soil of tropical forest ecosystem and they were more abundant in lower layers than the surface. Differences were observed between re-vegetated and natural forests on the diversity and abundance of them. Lower diversity of anammox community in the forest ecosystem than other ecosystems reported and n-damo community did not show much difference. These observations may be the results of interactions among various physical-chemical parameters, vegetation, and microbial communities, which warrants further investigations to reveal mechanisms involved in forest ecosystem to fully account the C and N cycling.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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