J Soils Sediments (2015) 15:1977–1986
DOI 10.1007/s11368-015-1131-5

SOILS, SEC 5 • SOIL AND LANDSCAPE ECOLOGY • RESEARCH ARTICLE

Candidatus Brocadia and *Candidatus* Kuenenia predominated in anammox bacterial community in selected Chinese paddy soils

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Received: 3 December 2014 / Accepted: 31 March 2015 / Published online: 19 April 2015

Abstract
Purpose Anammox bacteria have been widely investigated in both natural aquatic habitats and wastewater treatment reactors. However, their distribution in agricultural ecosystems which receive high reactive nitrogen input is hardly known. This study aims to examine the distribution and diversity of anammox bacteria in different Chinese paddy soils and along soil profile horizons.
Materials and methods DNA was extracted from paddy soils which were collected from ten sites and along four soil depth horizons (0–20, 20–40, 40–60, 60–80 cm). Community structure and diversity of the anammox bacteria were analyzed using cloning and sequencing methods by targeting 16S ribosomal RNA (rRNA) genes. Quantitative PCR was conducted to study the abundance of anammox *hzsB* genes.
Results and discussion Anammox bacterial 16S rRNA genes were not detected in most of the surface soil profile layers but present in all subsurface and deep horizons. Two genera, *Candidatus* Brocadia and *Candidatus* Kuenenia, were the only groups detected, and the former was predominant in tested soils. A site-specific distribution pattern of anammox bacteria and significant relationship between anammox bacteria community composition and soil pH and ammonium concentration were observed. The abundance of anammox bacteria *hzsB* gene ranged from 3.78×10^4 to 1.64×10^7 per gram of dry soil in all soils and significantly varied along the soil profile horizons.
Conclusions Anammox bacteria were widely distributed in paddy soils, especially in subsurface and profile depth horizons. *Candidatus* Brocadia and *Candidatus* Kuenenia were the dominating anammox groups in the tested soils, and the two genera showed a site-specific distribution pattern across large Chinese paddy soil areas and along soil depth profiles.

Keywords Anaerobic ammonium oxidation · Anammox bacteria · *hzs* gene · Paddy soil

Responsible editor: Yanfen Wang

Electronic supplementary material The online version of this article (doi:10.1007/s11368-015-1131-5) contains supplementary material, which is available to authorized users.

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1 Introduction

Biogeochemistry of nitrogen (N) is of great interest since N is an essential element to all lives. Microorganisms involving in nitrification and denitrification have been widely investigated due to their high activity and broad distribution in the environment. Anaerobic ammonium-oxidizing (anammox) bacteria just have been known more recently since they were firstly found being responsible for the N_2 loss from wastewater bioreactor by mediating the anaerobic oxidation of ammonia coupled nitrate reduction (Mulder et al. 1995). Later on, anammox bacteria were detected widely in natural ecosystems like marine sediments, oxygen minimum zones, and fresh water habitats (Dong et al. 2009; Lam et al. 2009; Crowe et al. 2012) and were suggested potentially responsible for the 50 % of nitrogen loss in South Atlantic midwaters (Kuypers et al. 2005). Understanding of the biodiversity and

community structure of anammox bacteria could further extend our knowledge to the mechanism of nitrogen cycle.

To date, all known anammox bacteria are the members of the phylum *Planctomycetes* and five genera have been discovered, including *Candidatus* Anammoxoglobus, *Candidatus* Brocadia, *Candidatus* Jettenia, *Candidatus* Kuenenia, and *Candidatus* Scalindua (Hirsch et al. 2011). As anammox bacteria are highly O_2-intolerant anaerobic microbes (Jetten et al. 2009), previous research mainly focused on aquatic environments like river sediments and oceans where the anoxic condition is ubiquitous (Schmid et al. 2005, 2007; Hirsch et al. 2011). A number of molecular ecology investigation based on 16S ribosomal RNA (rRNA) gene have revealed that the distributions of five known anammox genera were habitat-specific. *Candidatus* Scalindua was frequently found in the estuarine and deep-sea ecosystem, and the other four genera were mainly detected in water treatment reactors, freshwater sediments, and various types of terrestrial environments (Humbert et al. 2010). More recently, anammox bacteria have been detected in terrestrial ecosystem and showed habitat-specific distribution pattern too, as *Candidatus* Scalindua was observed predominant in the transitional zone of marine and terrestrial territories (Humbert et al. 2010; Schmid et al. 2007), and the other four genera were detected in agricultural fields, wetlands, and freshwater estuaries (Wang et al. 2012b; Long et al. 2013; Shen et al. 2013).

Paddy cultivation is one of the most important activities in agricultural production in China. Paddy soil therefore represents a large area of artificial wetlands under the frequent waterlog management and has being received high level of nitrogen fertilizer input, and the pathways of nitrogen loss from paddy soil have received great concerns due to its negative environmental influence (Zhu and Chen 2002). Although denitrification has been identified as a main soil nitrogen loss pathway under anoxic conditions (Risgaard-Petersen et al. 2005; Long et al. 2013), previous studies have indicated that the anammox process in either marine, freshwater, or terrestrial agriculture ecosystems cannot be ignored (Risgaard-Petersen et al. 2005; Crowe et al. 2012; Long et al. 2013). In a recent study in a paddy soil that received high slurry manure input, anammox was suggested to be responsible for 4–37 % of N_2 production, and *Candidatus* Brocadia was mainly detected in deep profile soils while *Candidatus* Anammoxoglobus, *Candidatus* Jettenia, and *Candidatus* Kuenenia were discovered in surface soil (Zhu et al. 2011). More recently, *Candidatus* Scalindua was identified as a dominant group in a molecular ecology survey in paddy soils in northeastern China (Wang and Gu 2013). These studies revealed the presence of anammox bacteria in agricultural soils, while the distribution and diversity of anammox bacteria in a large scale of agricultural fields are still less known.

Therefore, examining the diversity and distribution of anammox bacterial community among different soil types and soil depths is of significance to estimate the potential contribution of the anammox process to the whole nitrogen cycle in paddy soil system. To address these concerns, in this study, ten soil profiles incorporating four soil horizons (i.e., 0–20, 20–40, 40–60, and 60–80 cm with representative letters A–D, respectively) were collected from ten major rice-producing areas across the northeastern (Panjin (PJ1 and PJ2) and Shenyang (SH)), eastern (Jiaxing (JX) and Shangyu (SY)), central (Jingzhou (JZ), Xianning (XN), and Xiantao (XT)), and southern (Hengyang (HY) and Miluo (ML)) China. The diversity of anammox bacteria in these soils was investigated by cloning and sequencing 16S rRNA genes, and the abundance was estimated by quantifying *hzsB* genes.

2 Material and methods

2.1 Soil sampling site and physiochemical analysis

Soil samples were collected during the rice-growing period from ten rice plantation areas in July, 2010. The information about sampling sites is listed in Table 1. Paddy soils were sampled along soil horizons at 0–20, 20–40, 40–60, and 60–80 cm. Soil samples for DNA extraction were dried in a vacuum freeze drier, and subsamples for soil properties analysis were stored at 4 °C. Detail methods of the soil physiochemical properties were referred to a previous work (Shen et al. 2008).

2.2 DNA extraction

Approximately 0.50-g soil (dry weight) was weighted for DNA extraction using a Power Soil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) following the manufacturer's instructions. All DNA samples were then stored under −40 °C for further analysis.

2.3 PCR amplification of 16S rRNA genes

A nested PCR assay was conducted for the detection of anammox 16S rRNA genes. The first PCR amplification was performed in a 25 μl reaction with primer pair Pla46F/1037R (Schmid et al. 2011). PCR reaction contained 12.5 μl of 2× TAKARA premix (TaKaRa Bio Inc., Shiga, Japan), 0.25 μl of each primer (10 μM), 1 μl of DNA template (1–20 ng μl^{-1}), and PCR water to a final volume of 25 μl. After the initial step, PCR amplification was further performed with the primer set Amx368F/Amx820R (Schmid et al. 2000) in a 50-μl reaction system containing 10× PCR buffer (Mg^{2+} plus) 5 μl, 2.5 mM dNTPs 4 μl, 0.5 μl of Ex Taq HS polymerase (TaKaRa Bio Inc., Shiga, Japan), 1 μl of 10-μM primer each, and 2 μl of 1000× diluted templates from the first amplification.

Table 1 Sample sites and the soil properties

Location	Position	Sample ID	Soil profile (cm)	pH	OM (g kg^{-1})	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})
Hengyang, Hunan	N 27° 8′, E 112° 54′	HY-A	0–20	5.3	28.79	22.11	2.92
		HY-B	20–40	6.8	11.97	12.59	2.20
		HY-C	40–60	7.1	6.90	10.32	1.64
		HY-D	60–80	7.4	4.16	8.84	1.55
Miluo, Hunan	N 28° 44′, E 113° 2′	ML-A	0–20	4.8	30.23	23.70	3.77
		ML-B	20–40	6.4	12.53	11.74	4.82
		ML-C	40–60	7.3	8.28	9.66	3.83
		ML-D	60–80	7.1	8.90	10.85	3.48
Shangyu, Zhejiang	N 29° 57′, E 120° 55′	SY-A	0–20	5.5	32.46	32.30	4.87
		SY-B	20–40	6.8	15.12	11.31	3.20
		SY-C	40–60	7.4	8.65	9.19	4.19
		SY-D	60–80	7.4	8.21	8.62	4.03
Jiaxing, Zhejiang	N 30° 29′, E 120° ′	JX-A	0–20	6.5	24.38	35.63	1.78
		JX-B	20–40	7.5	18.04	8.22	1.16
		JX-C	40–60	7.6	10.11	7.31	0.88
		JX-D	60–80	7.5	5.28	7.20	1.27
Xianning, Hubei	N 30° 01′, E 114° 21′	XN-A	0–20	5.8	20.39	18.51	2.55
		XN-B	20–40	7.4	6.85	13.67	1.53
		XN-C	40–60	7.1	5.94	10.00	1.56
		XN-D	60–80	7.1	6.44	15.91	1.24
Jingzhou, Hubei	N 30° 21′, E 113° 03′	JZ-A	0–20	6.5	21.25	13.38	2.88
		JZ-B	20–40	7.2	7.62	5.58	2.22
		JZ-C	40–60	7.2	7.34	6.95	1.37
		JZ-D	60–80	7.3	5.28	7.74	2.54
Xiantao, Hubei	N 30° 26′, E 113° 00′	XT-A	0–20	8.2	19.85	12.80	1.81
		XT-B	20–40	8.4	7.88	8.47	1.32
		XT-C	40–60	8.5	8.99	8.04	2.30
		XT-D	60–80	8.5	9.22	9.28	5.57
Panjin, Liaoning	N 41° 13′, E 122° 00′	PJ1-A	0–20	8.7	11.61	11.87	0.11
		PJ1-B	20–40	9.2	6.23	9.53	0.25
		PJ1-C	40–60	9.2	5.18	9.51	0.42
		PJ-D	60–80	9.2	4.63	7.74	0.39
Panjin, Liaoning	N 41° 14′, E 121° 57′	PJ2-A	0–20	7.9	9.52	10.88	0.33
		PJ2-B	20–40	7.8	7.66	10.61	0.33
		PJ2-C	40–60	8.0	5.29	9.96	0.21
		PJ2-D	60–80	8.0	4.06	9.34	0.19
Shenyang, Liaoning	N 41° 31′, E 123° 24′	SH-A	0–20	6.8	15.54	32.24	3.46
		SH-B	20–40	7.2	9.71	16.85	1.37
		SH-C	40–60	7.3	7.73	16.94	1.79
		SH-D	60–80	7.4	4.50	16.31	1.18

2.4 Construction of clone libraries, sequencing, and phylogenetic analysis

The obtained PCR products were purified using Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) and ligated into a pMD 18-T vector (TaKaRa Bio Inc., Shiga, Japan) at 4 °C overnight. The ligation was then transformed into *E. coli* competent cells (JM109, TaKaRa Bio Inc., Shiga, Japan). Approximately 30 positive colonies were randomly selected and then sequenced

by automatic sequencer ABI3730 (Applied Biosystems, USA).

2.5 Quantitative PCR of *hzsB* genes

The abundance of anammox bacteria was estimated by quantifying *hzsB* gene with primer pair hzsB_396F (5′-ARGG HTGGGGHAGYTGGAAG-3′) and hzsB_742R (5′-GTYC CHACRTCATGVGTCTG-3′) (Wang et al. 2012a). Quantitative PCR reactions were conducted in 25-μl volume containing 12.5 μl of SYBR Green premix (TaKaRa Bio Inc., Shiga, Japan), 0.6 μl of each primer (10 μM), and 1 μl of DNA template (1–20 ng/μl). The touchdown PCR cycle was started with a 5-min denaturation at 95 °C, followed by five cycles of 95 °C for 1 min, 64 °C (decrease 1 °C each cycle) for 1 min, and 72 °C for 45 s. Then, it was continued with 35 cycles of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 45 s, and a final 72 °C for 5 min. PCR product amplified with the same primer pair as above from the DNA template was cloned into a pMD 18-T vector (Takara); then, the positive clones were sequenced, and the plasmid DNA carrying the *hzsB* genes was extracted and purified. The concentration of the plasmid was then determined by NanoDrop, and the copy number of *hzsB* gene per microliter was calculated. The tenfold serial dilutions of the plasmid were utilized as standard for quantitative PCR.

2.6 Statistical analysis and phylogenetic analysis

By utilizing SPSS (version 19.0, IBM), Pearson correlation analysis was used to test the relationship between environmental factors and *hzsB* gene abundance, and Duncan test of one-way ANOVA was conducted to test the differences of hzsB abundance between soil profiles; $P<0.05$ level was considered significant. The obtained clone sequences were analyzed manually using BLAST program in GenBank database. The sequences sharing more than 97 % identity were defined as one operation taxonomic unit (OTU), and then, one sequence for each anammox bacteria OTU and its closest clone from GenBank was chosen to construct the phylogenetic tree using the neighbor-joining method in MEGA 5 software with 1000 bootstraps (Kumar et al. 2008). The definition of OTUs and calculation of diversity indices were carried out using DOTUR program (Schloss and Handelsman 2005). The percentage of coverage of cloning libraries was calculated based on the equation $C = [1 - (N_s/N)] \times 100\ \%$, where C represents percentage of coverage, N denotes the amount of positive clones in one library, and N_s is the number of clones that presents only once in the library (Table 2). CANOCO software (version 4.5, Microcomputer Power) was used for principal components analysis (PCA) and canonical correspondence analysis (CCA). Percentages of each OTUs occupied in all cloning library were used as the "species" dataset, and the measured soil physiochemical properties were utilized as environmental variables.

3 Results and discussion

3.1 Detection of anammox bacteria in paddy soils based on 16S rRNA genes

In total, 40 samples including 10 surface and 30 profile samples were used in this study. The target fragment was successfully amplified from 32 samples, while eight of ten layer A (0–10 cm) samples failed to get amplification except JZ-A and SH-A (Table 2). In total, 913 sequences were obtained from 32 cloning libraries and 789 sequences in 29 OTUs at 97 % sequence identity level were identified as anammox bacteria genera *Candidatus* Brocadia and *Candidatus* Kuenenia after BLASTing in GenBank and phylogenetic analysis (Fig. 1). OTU18 falling within Brocadia cluster was the most frequently detected which represented 141 sequences which was detected from eight sites, while 15 OTUs were only detected in one site (Fig. 1). The rest 124 sequences formed a new phylogenetic cluster far from the known anammox clusters within the phylum *Planctomycetes* in the phylogenetic tree (Fig. 1), and most of them showed 88–96 % identities to uncultured planctomycete clones in GenBank. These non-anammox bacteria sequences were mainly retrieved from two sites including ML-B, ML-C, SH-A, and SH-B but showed high diversity and represented 80 OTUs at 97 % sequence identity level. The non-specific amplification of anammox 16S rRNA gene implied the absence of known anammox bacteria in these samples, whether these planctomycete-related clones are potential anammox bacteria is of interest in future studies. Furthermore, cloning libraries dominated by known anammox bacteria presented coverage more than 90 %, and Shannon index ranged from 0 to 1.59, Simpson index 0 to 0.93, and Chao1 index from 1 to 5.5. In contrast, cloning libraries dominated with unknown bacteria showed much lower coverage and higher diversity (Table 2).

It has been proved that anammox bacteria are highly intolerant to oxygen and could hardly survive when the O_2 concentration is higher than a certain level (Jetten et al. 2009). The concentration of O_2 could decrease intensively along the paddy soil depth profile and steadily maintain the gradient over time. As a consequence, the distribution pattern of microbes that are sensitive to oxygen could be influenced by this gradient in paddy sediments (DeAngelis et al. 2010). It was observed that the relative contribution of anammox to total N_2 production and the relative abundance of anammox bacteria varied greatly along the sediment profiles (Rooks et al. 2012). All these may explain the unsuccessful amplification of anammox 16S rRNA gene in most of the surface horizon of profiles, and the results suggested that there is a strong soil-oxygen gradient

Table 2 Cloning libraries and the diversity indices of anammox bacteria based on 16S rRNA genes by 3 % cutoff

Sample ID	No. of sequences	Coverage (%)	OTUs	Shannon	Simpson	Chao1
HY-A[a]	N/A	N/A	N/A	N/A	N/A	N/A
HY-B	29	97	3	0.40	0.80	3
HY-C	30	97	2	0.15	0.93	2
HY-D	30	100	2	0.68	0.49	2
ML-A[a]	N/A	N/A	N/A	N/A	N/A	N/A
ML-B[b]	28	64	15	2.53	0.07	25
ML-C[b]	29	62	14	2.63	0.06	30.75
ML-D	30	97	3	0.53	0.70	3
SY-A[a]	N/A	N/A	N/A	N/A	N/A	N/A
SY-B	30	100	1	0.50	0.67	2
SY-C	29	100	1	0.74	0.56	3
SY-D	28	96	3	0.66	0.67	4
JX-A[a]	N/A	N/A	N/A	N/A	N/A	N/A
JX-B	22	91	5	1.72	0.18	7.5
JX-C	29	97	3	0.92	0.50	4
JX-D	29	93	5	1.09	0.42	6
XN-A[a]	N/A	N/A	N/A	N/A	N/A	N/A
XN-B	29	100	2	1.19	0.38	5
XN-C	29	93	5	1.12	0.37	5.5
XN-D	28	93	4	0.94	0.52	5.5
JZ-A	29	93	3	0.30	0.86	4
JZ-B	29	97	3	1.01	0.43	7
JZ-C	28	100	2	0.68	0.49	2
JZ-D	29	100	2	0.74	0.56	3
XT-A[a]	N/A	N/A	N/A	N/A	N/A	N/A
XT-B	29	90	6	1.78	0.18	9.5
XT-C	30	100	3	1.59	0.21	6
XT-D	29	97	4	1.59	0.21	6
PJ1-A[a]	N/A	N/A	N/A	N/A	N/A	N/A
PJ1-B	26	100	1	0.16	0.92	1
PJ1-C	30	100	1	0.24	1.00	2
PJ-D	26	100	2	0.75	0.57	3
PJ2-A[a]	N/A	N/A	N/A	N/A	N/A	N/A
PJ2-B	26	96	3	0.94	0.47	4
PJ2-C	28	96	3	0.82	0.45	3
PJ2-D	29	100	1	0.00	0.00	1
SH-A[b]	29	34	21	3.27	0.02	127
SH-B[b]	27	7	25	3.24	0.00	176
SH-C	30	100	2	0.50	0.67	2
SH-D	30	100	2	0.61	0.57	2

[a] Samples that no PCR product was retrieved

[b] Means that only non-anammox clones were detected in the cloning libraries. These samples were excluded from the correlation analysis between diversity indices and environmental factors

effect on the succession of vertical distribution of anammox bacteria in paddy soil horizons.

Among the 29 anammox OTUs, 18 OTUs were classified into *Candidatus* Brocadia and 11 OTUs into *Candidatus* Kuenenia, which accounted for 57.9 and 42.1 % of retrieved anammox bacteria sequences, respectively. No sequence was identified as *Candidatus* Scalindua, *Candidatus* Jettenia, and *Candidatus* Anammoxoglobus. *Candidatus* Brocadia was detected from nine out of ten sampling sites except in site PJ2. Though no any known anammox sequence was retrieved from

horizons A, B, and C of site ML and horizons A and B of site SH, *Candidatus* Brocadia was largely discovered in the deeper horizons (layers C and D) from these two sites. *Candidatus* Kuenenia was found in six out of ten sites and was largely detected in SY, XN, PJ1, and PJ2. It was also the only anammox bacterial group detected in PJ2. The results were consistent with the observation of Humbert et al. showing that *Candidatus* Brocadia and *Candidatus* Kuenenia were more dominant than other groups in various terrestrial ecosystems (Humbert et al. 2010). Furthermore, although *Candidatus* Jettenia and *Candidatus* Anammoxoglobus were detected from the five and three out of 32 Chinese agricultural fields in the work of Shen and his colleagues (2013), *Candidatus* Brocadia and *Candidatus* Kuenenia were predominant groups, occupying 92 % of their detected sequences. The wide distribution of *Candidatus* Brocadia could be explained by their versatile metabolism system which favors their adaptation to various environments (Gori et al. 2011).

Some sequences affiliating into *Candidatus* Brocadia showed high identity to *Candidatus Brocadia fulgida* and *Candidatus Brocadia anammoxidans*, while more sequences were closely related to the uncultured clones from river and freshwater sediments. Especially, the BLAST analysis against GenBank reveals that 68.7 % of *Candidatus* Brocadia OTUs detected in this study had a great identity to the clones from Yangtze River marsh sediments (Hou et al. 2013) and freshwater wetlands (Zhu et al. 2011a). Consequently, these results indicated that this linkage of the genus *Candidatus* Brocadia may be preferable to freshwater ecosystems.

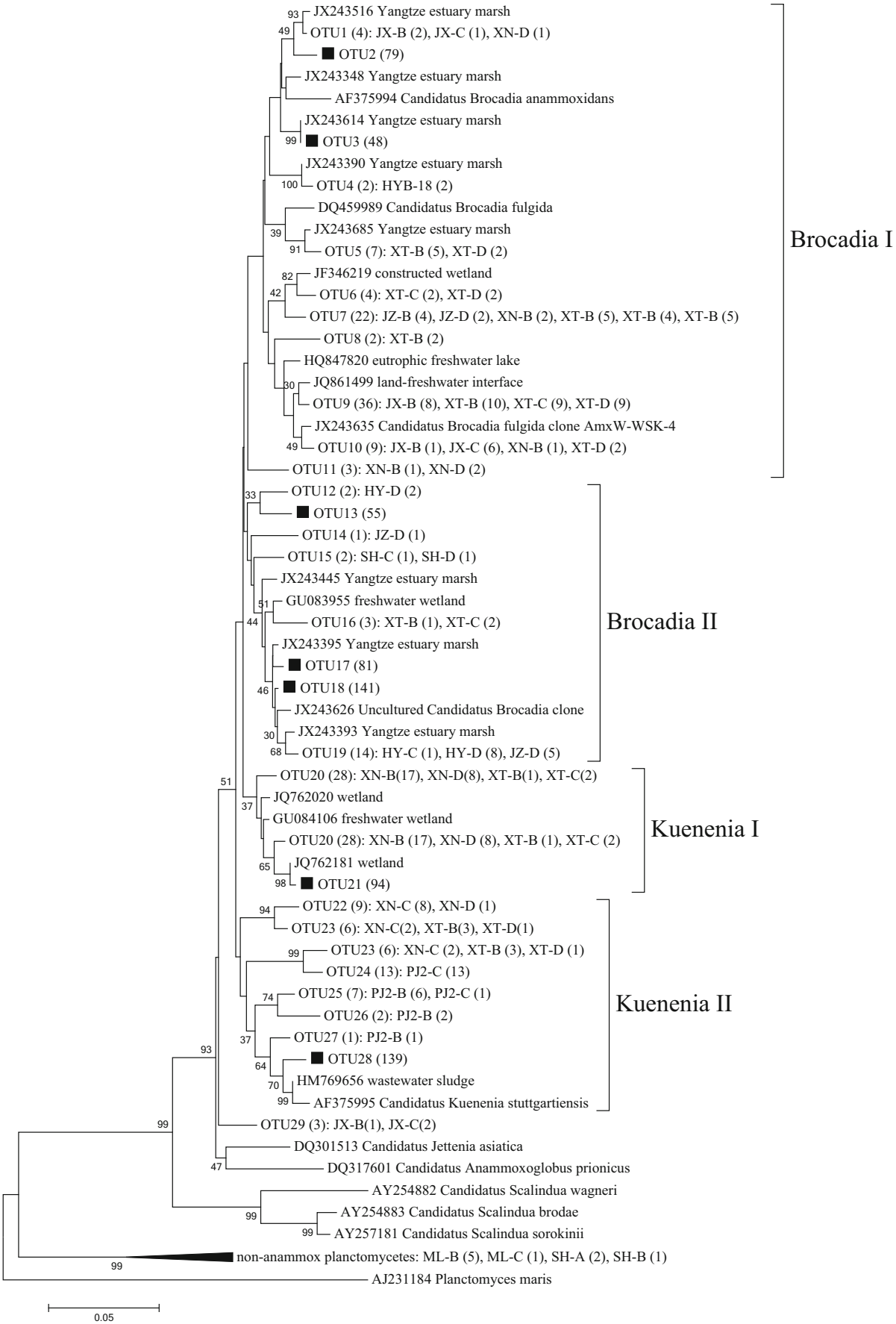
None of our sequences was identified as either *Candidatus* Scalindua or Scalindua-like in the present study. Similarly, this genus neither detected in the paddy soil received high N input from Zhejiang Province of China where other four genera were discovered (Zhu et al. 2011). *Candidatus* Scalindua has been reported as a niche-specific group that thrives in the marine ecosystems (Schmid et al. 2007; Humbert et al. 2010; Han and Gu 2013) but is considered as relatively less abundant in terrestrial ecosystems (Humbert et al. 2010). However, it is interesting that another investigation based on 16S rRNA genes with primers Amx368F/Amx820R has found that *Candidatus* Scalindua is the most dominant group in northeastern Chinese paddy soils (Han and Gu 2013), while we only observed *Candidatus* Brocadia- or *Candidatus* Kuenenia-dominated anammox community in three soil profiles (PJ1, PJ2, and SY) from this area. Such inconsistency implies a possible variation of anammox bacteria distribution within relatively small areas and potential bias of the existing primer sets.

3.2 Community structure of anammox bacteria and environmental factors

Except to eight surface samples failing to produce PCR products and four samples predominated by non-anammox bacteria, *Candidatus* Brocadia and *Candidatus* Kuenenia were dominant in the rest 28 samples (Fig. 2). PCA based on all retrieved OTUs including non-anammox sequences showed that sampling sites with different planctomycete groups were separated from each other in the first two PCA dimensions (Fig. S1, Electronic Supplementary Material), indicating a site-specific distribution pattern. *Candidatus* Kuenenia predominated all layer B, C, and D samples from Panjin (PJ1 and PJ2) with a proportion more than 96.2 %, while *Candidatus* Brocadia exclusively dominated samples from Jingzhou (JZ-B, JZ-C, and JZ-D), Hengyang (HY-B, HY-C, HY-D), and Shenyang (SH-C and SH-D) and take overwhelming proportions in samples from Xiantao (XT-B, XT-C, XT-D), Jiaxing (JX-B, JX-C, JX-D), and Miluo (ML-D) (Fig. 2). Only samples from XN (XN-B, XN-C, and XN-D) and Shangyu (SY-B, SY-C, and SY-D) were inhabited by two genera (Figs. 2 and 3). The result indicated a strong site effect on shaping the community structure of anammox bacteria (Figs. 2 and S1, Electronic Supplementary Material). Although environmental conditions that influence anammox bacteria community have not been well established, spatial variation in their distribution due to local soil types has also been reported in the study of Shen (2013).

CCA based on OTU matrix belonging to anammox sequences showed that *x*- and *y*-axes accounted for 32.1 and 25.4 % variance in the relationship between species and environmental factors, respectively (Fig. 3). Monte Carlo test (999 permutation) further showed that the community composition of anammox bacteria significantly correlated with soil pH and NH_4^+, accounting for 22.2 and 27.8 % of observed variance, respectively (Fig. 3). CCA based on OTUs and environmental parameters showed that OTU9, OTU13, and OTU28 responded to pH increase more sensitively while OTU2 tended to distribute in high ammonium condition (Fig. S2,

Fig. 1 Phylogenetic tree of anammox bacteria 16S rRNA gene sequences retrieved with primer sets Pla46F/1037R and Amx368F/Amx820R from the paddy soils. The obtained sequences and their related sequences and standard species from GenBank were combined with neighbor-joining method. The clone library name and *number in parenthesis* after the OTU names denoted the number of clones in which the OTU represented in each library. The sequences obtained in this study are available in GenBank under accession numbers KJ512714 to KJ512741. Details of OTUs labeled with *solid squares* are as follows: OTU2: HY-C (1), JX-C (1), JZ-A (26), JZ-B (3), JZ-C (6), ML-D (4), SH-C (24), SH-D (6), XN-B (6), XN-D (1), and XT-D (1); OTU3: JX-B (3), JX-C (16), JX-D (6), JZ-B (3), JZ-C (6), JZ-D (6), SH-D (3), XT-B (1), XT-C (1), and XT-D (3); OTU13: HY-B (1), HY-D (15), JX-B (1), JZ-B (1), SY-B (2), SY-C (1), SY-D (3), and XT-B (30); OTU17: JX-B (3), JX-C (3), JX-D (18), JZ-B (18), JZ-C (13), JZ-D (16), ML-D (1), XT-B (2), XT-C (3), and XT-D (4); OTU18: HY-B (26), HY-C (28), HY-D (5), JX-B (1), JX-C (4), ML-D (24), PJ1-D (3), SH-C (5), SH-D (20), SY-B (6), SY-C (7), SY-D (2), XN-B (2), XN-C (1), XN-D (4), and XT-C (3); OTU21: JX-B (1), JX-D(3), SY-B (21), SY-C (21), SY-D (20), XN-B (1), XN-B (15), and XN-D (12); and OTU28: PJ1-B (26), PJ1-C (30), PJ1-D (24), PJ2-B (16), PJ2-C (14), and PJ2-D (29)


JX243516 Yangtze estuary marsh
OTU1 (4): JX-B (2), JX-C (1), XN-D (1)
OTU2 (79)
JX243348 Yangtze estuary marsh
AF375994 Candidatus Brocadia anammoxidans
JX243614 Yangtze estuary marsh
OTU3 (48)
JX243390 Yangtze estuary marsh
OTU4 (2): HYB-18 (2)
DQ459989 Candidatus Brocadia fulgida
JX243685 Yangtze estuary marsh
OTU5 (7): XT-B (5), XT-D (2)
JF346219 constructed wetland
OTU6 (4): XT-C (2), XT-D (2)
OTU7 (22): JZ-B (4), JZ-D (2), XN-B (2), XT-B (5), XT-B (4), XT-B (5)
OTU8 (2): XT-B (2)
HQ847820 eutrophic freshwater lake
JQ861499 land-freshwater interface
OTU9 (36): JX-B (8), XT-B (10), XT-C (9), XT-D (9)
JX243635 Candidatus Brocadia fulgida clone AmxW-WSK-4
OTU10 (9): JX-B (1), JX-C (6), XN-B (1), XT-D (2)
OTU11 (3): XN-B (1), XN-D (2)
Brocadia I
OTU12 (2): HY-D (2)
OTU13 (55)
OTU14 (1): JZ-D (1)
OTU15 (2): SH-C (1), SH-D (1)
JX243445 Yangtze estuary marsh
GU083955 freshwater wetland
OTU16 (3): XT-B (1), XT-C (2)
JX243395 Yangtze estuary marsh
OTU17 (81)
OTU18 (141)
JX243626 Uncultured Candidatus Brocadia clone
JX243393 Yangtze estuary marsh
OTU19 (14): HY-C (1), HY-D (8), JZ-D (5)
Brocadia II
OTU20 (28): XN-B(17), XN-D(8), XT-B(1), XT-C(2)
JQ762020 wetland
GU084106 freshwater wetland
OTU20 (28): XN-B (17), XN-D (8), XT-B (1), XT-C (2)
JQ762181 wetland
OTU21 (94)
Kuenenia I
OTU22 (9): XN-C (8), XN-D (1)
OTU23 (6): XN-C(2), XT-B(3), XT-D(1)
OTU23 (6): XN-C (2), XT-B (3), XT-D (1)
OTU24 (13): PJ2-C (13)
OTU25 (7): PJ2-B (6), PJ2-C (1)
OTU26 (2): PJ2-B (2)
OTU27 (1): PJ2-B (1)
OTU28 (139)
HM769656 wastewater sludge
AF375995 Candidatus Kuenenia stuttgartiensis
Kuenenia II
OTU29 (3): JX-B(1), JX-C(2)
DQ301513 Candidatus Jettenia asiatica
DQ317601 Candidatus Anammoxoglobus prionicus
AY254882 Candidatus Scalindua wagneri
AY254883 Candidatus Scalindua brodae
AY257181 Candidatus Scalindua sorokinii
non-anammox planctomycetes: ML-B (5), ML-C (1), SH-A (2), SH-B (1)
AJ231184 Planctomyces maris
0.05

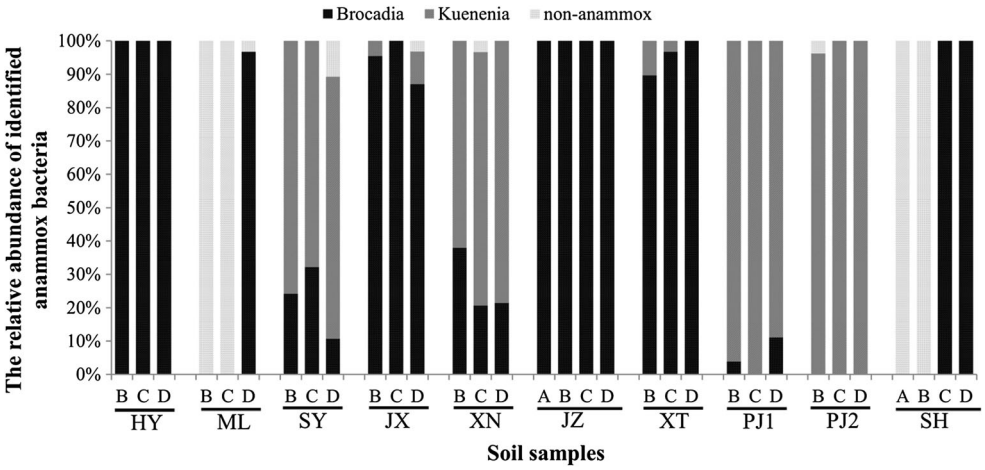


Fig. 2 Community composition of anammox bacteria among paddy soil profile horizons *A* (0–20 cm), *B* (20–40 cm), *C* (40–60 cm), and *D* (60–80 cm) detected by primer sets Pla46F/1037R and Amx368F/Amx820R

Electronic Supplementary Material). Ammonium concentration in agricultural soils is greatly influenced by nitrogen fertilization, and the results thus imply an anthropogenic effect on the distribution of anammox bacteria. A similar result that ammonium significantly influenced the community structure of anammox bacteria has been reported in mangrove sediments, in which the change of ammonium concentration could strongly alter community composition of anammox bacteria (Li and Gu 2013). Previous studies have also revealed that neutral or weakly alkaline environments are favorable to anammox bacteria (Yamamoto et al. 2008; Chen et al. 2009). Furthermore, a recent report has indicated that anammox bacteria are sensitive to the change of pH and their diversity increased in response to both acid and alkaline conditions (Wang and Gu 2014). Coincidently, pH was identified as a significant factor correlated to the community composition of anammox bacteria. These results consistently demonstrated that soil pH is a potential factor influencing the community structure of anammox bacteria' various ecosystems. As ammonium is one of the essential substrates in anammox process, it is not surprising that pH plays an essential role in shaping anammox bacterial communities, as it generally affects the availability of ammonium.

3.3 Abundance of anammox functional genes

The abundance of anammox bacteria was estimated further by quantifying *hzsB* genes (Fig. 4). Although anammox bacterial 16S rRNA genes were hardly amplified in the surface paddy soils, *hzsB* genes could be detected from all the soil layers within the range of 3.78×10^4 to 1.64×10^7 per gram of dry soil. The numbers are comparable to previous investigations on paddy soils based on *hzsA* and *hzsB* genes (Humbert et al. 2010; Zhu et al. 2011; Wang et al. 2012a).

Significant difference of *hzsB* gene abundance could be observed between soil horizons in each sampling site except site HY. Zhu et al. have found that *hzsB* gene copy numbers were the highest at the depth of 40–50 cm within the same soil

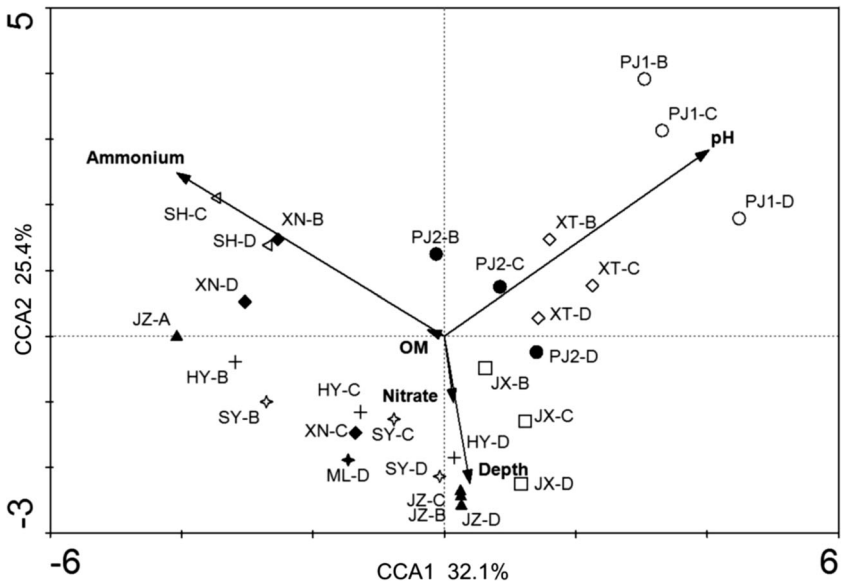


Fig. 3 Canonical correlation analysis (CCA) between soil properties and anammox bacteria communities based on 97 % cutoff OTUs excluding non-anammox OTUs

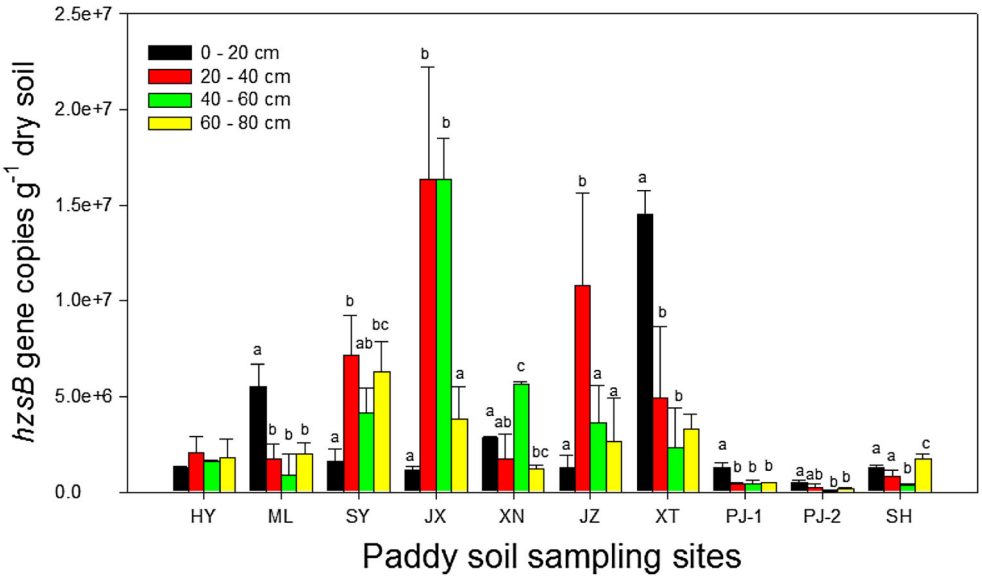


Fig. 4 The abundance of anammox bacteria in four soil horizons from ten sampling sites as indicated by *hzsB* gene copy numbers (the labels "*a*, *b*, *c*" *above the bars* indicate significant differences of *hzsB* gene abundance within each sampling site by one-way ANOVA test)

type (Zhu et al. 2011), while no similar trend was observed in this study, and the highest gene abundance could be found in any of the soil horizon, implying a heterogeneous distribution of anammox bacteria abundance. The result is similar to the observation in the work of Shen and colleagues (2013) in which they found that abundance of anammox bacteria changed largely among samples from different locations.

Generally, *hzsB* gene abundance was the lowest in PJ1 and PJ2 from Liaoning, in which organic carbon and nitrate were relatively low and the detected 16S rRNA genes were mostly composed of *Candidatus* Kuenenia. However, *hzsB* gene abundance showed no significant correlation with any tested physiochemical parameters ($P>0.05$). Similarly, Wang et al. (2012a) found that *hzsB* gene abundance showed no significant correlation with ammonium, nitrate, and other environmental factors in paddy soils, while Han and Gu (2013) discovered that nitrate concentration in wastewater treatment plants and marine and wetland sediments significantly influenced the abundance of anammox bacteria based on the quantification of 16S rRNA genes, and Shen et al. (2013) considered ammonium content to be the most influential factor in agricultural soils based on quantitative analysis of *hzsA* genes. All these suggested that different gene markers may produce different conclusions and more research is needed to get a comprehensive understanding on the distribution of anammox bacteria.

Moreover, 16S rRNA genes of anammox bacteria were absent in the most surface soil in this study, while *hzsB* gene could be detected in all the samples and was abundant in horizon A of sites PJ1, PJ2, ML, and XT. This inconsistency between 16S rRNA gene and *hzs* genes may be caused by the primer bias. Although the functional genes are generally more efficient in the study of microbial abundance since their direct relations to the physiology of target organisms perform ecological function, the primers designed specifically for *hzs* genes are still under test and may be amended further. The difference in detection efficiency on anammox bacteria between 16S rRNA genes and *hzsA* genes has been reported in the work of Hu and colleagues (Hu et al. 2013), in which they found that the *hzsA* primer pairs recovered a much broader diversity of organisms in an enrichment of anammox bacteria community from paddy soils. Wang and colleagues also found that the copy numbers of anammox 16S rRNA gene were approximately 10^2-fold lower than those of *hzsB* genes in estuary sediments (Wang et al. 2012b). The coverage and specificity of the two primer sets need to be compared in future work.

4 Conclusions

Diversity and abundance of anammox bacteria in ten Chinese paddy soil profiles were investigated in the present study. Anammox bacterial 16S rRNA genes were not detected in most of the surface soil profile layers but present in all subsurface and deep horizons. Cloning and sequencing analysis showed that *Candidatus* Brocadia and *Candidatus* Kuenenia were the dominating anammox groups in the tested soils and *Candidatus* Brocadia had a broader distribution. Meanwhile, two genera showed a clear site-specific distribution pattern among different sampling sites and soil pH and ammonium concentration significantly correlated to community structure of anammox bacteria. It is suggested that other essential factors in anammox process such as O_2 concentration and soil oxidation-reduction potential could be taken into consideration in future studies.

Acknowledgments This work was financially supported by the National Science Foundation of China (41090281, 41025004, 41322007). We would like to thank Prof. Wenxue Wei and Ms. Yijun Zhu for their assistance in soil sampling and Drs. Peng Cao and Yu Dai for their help in soil physiochemical analysis.

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