

Differentiated Response of Denitrifying Communities to Fertilization Regime in Paddy Soil

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Abstract The impact of fertilization regimes on sequential denitrifying communities was investigated in a rice paddy field with 17 years continuous fertilization, located in Taoyuan Agro-ecosystem Research Station (110°72" E, 28°52" N), China. The diversity, community composition, and size of denitrifying genes of *narG*, *qnorB*, and *nosZ* were determined using molecular tools including terminal restriction fragment length polymorphism, quantitative polymerase chain reaction (qPCR), cloning, and sequencing analysis. Soil samples were collected from the plots with no fertilizer (NF), urea (UR), balanced mineral fertilizers (BM), and BM

combined with rice straw (BMR). UR and BM caused marked increase in the community size of the denitrifying genes; however, BMR resulted in the highest abundance. The community size of *narG* was the most affected by the fertilization regimes, while *qnorB* was the least. Fertilization also induced some shifts in the composition of denitrifying genes, but the responses of different genes varied. However, fertilization regimes caused no significant changes to the diversity of the denitrifying genes. Potential denitrification activity (PDA) was significantly correlated with the abundance of *narG* and *nosZ* rather than *qnorB*, but there were no such correlations between PDA and the composition and diversity of denitrifying communities. Conclusively, long-term fertilization significantly affected denitrifying community size and composition, but not diversity. Among the sequential denitrifying genes, *narG* was the most, while *qnorB* was the least sensitive communities to fertilization regimes.

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Introduction

Denitrification is a complex microbial dissimilatory process in which nitrate and nitrite are reduced to gaseous NO, N₂O, and N₂ when oxygen is limited. Denitrification not only causes nitrogen loss from agricultural soil resulting in a low rate of nitrogen usage by crops but also contributes to greenhouse gas N₂O emission. This can induce destruction of the stratospheric ozone layer, and its global warming potential is about 296 times higher than that of CO₂ [1]. A wide range of taxonomic groups, including Bacteria, Archaea, and Fungi, have the capability to carry out denitrification; for instance, more than 50 genera of bacteria have been identified with denitrifying function [2]. Because of the high phylogenetic diversity among denitrifiers, 16S

rRNA gene-based approaches are not suitable to explore the denitrifying communities [2]. Instead, the use of functional genes involved in denitrification is a more direct method of detecting or identifying denitrifying bacteria [3]. Complete denitrification requires the sequential action of four enzymes, nitrate reductase (encoded by *narG* and *napA*), nitrite reductase (encoded by *nirK/nirS*), nitric oxide reductase (encoded by *nor*), and nitrous oxide reductase (encoded by *nosZ*).

In the past decades, chemical fertilization has greatly contributed to food production, and the input is increasing continuously. For example, worldwide synthetic nitrogen fertilizer consumption was about 85 Tg N in 1999 with about 56 Tg N consumed in developing countries, and global N fertilizer consumption is projected to reach 105 Tg N by 2030 [4]. The heavy input of mineral nitrogen has had serious and long-term environmental consequences [5]. Nitrogen fertilizers can promote denitrification activity in agricultural soils; subsequently, substantial amounts of applied nitrogen fertilizer are lost through the denitrification process [6–8]. Denitrification rates are influenced by soil moisture and temperature, nutrient supply as nitrate and soluble organic matter, and soil texture [9, 10]. Spatial and temporal variations of these parameters and their interactions are often linked to the high variability of soil denitrification rates [11, 12]. Although previous studies have revealed that the above soil physical and chemical conditions indirectly affect denitrification, microbial factors are of great importance for directly regulating the denitrification process. Therefore, efforts should be taken to understand nitrogen turnover processes and the functional microorganisms involved.

Literature reviews have suggested that the composition and diversity of soil denitrifier communities may be the main microbial factors affecting denitrification [13, 14]. However, Hallin et al. [15] found that the community size correlated with the denitrifying process rate. This indicates that the soil denitrifier community may differ in response to environmental conditions that indirectly control the rate of denitrification [16, 17]. Recently, we reported the long-term fertilization effects on the nitrite reductase gene (*nirK* and *nirS*) community and revealed that the *nirK* community was more sensitive to fertilization than the *nirS* community. These findings guide us to explore the complete picture of key genes involved in denitrification in paddy soil because denitrification should be considered a sequential community process. Since not all denitrifiers produce the full suite of enzymes to complete the process, it remains unclear which are the main microbial groups involved in each denitrifying process and which community's shifts are related to the potential denitrifying activity in paddy soil. However, most previous studies on soil denitrifiers have focused on a single

or a couple denitrifying genes, rather than the sequential functional genes of the entire denitrification process [18–21]. Thus, it is worth detecting and comparing the complete denitrifying communities of denitrification. In addition, most previous studies on denitrifiers have focused on upland soils; few have been conducted on paddy soil, which is important agricultural land, especially in Asian countries [22]. Unlike upland soils, frequently flooded paddy soil supplies an anaerobic condition favoring denitrification [23]. However, knowledge of the denitrifying communities in paddy soils is very limited.

The main objectives of this study were to determine the long-term effects of repeated application of mineral fertilizers and rice straw incorporation on the composition, diversity, and abundance of sequential denitrifying communities and to explore the response of each denitrifying gene community to fertilization regimes in the paddy soil.

Materials and methods

Soil samples

The same soil samples were used in this study as the previous work, and the main characteristics have been described previously [24]. Briefly, soil samples were collected from the Taoyuan Agro-ecosystem Research Station of the Chinese Academy of Sciences (110°72' E, 28°52' N), China. A long-term experiment was established in 1990, including three replicates of ten treatments. Four treatments were selected for this study as follows: NF (no fertilizer), UR (182.3 kg N ha⁻¹ a⁻¹ as urea), BM (balanced mineral fertilizer, 182.3 kg N ha⁻¹ a⁻¹, 39.3 kg P ha⁻¹ a⁻¹, 197.2 kg K ha⁻¹ a⁻¹ as urea, superphosphate, and potassium chloride, respectively) and BMR (BM combined with rice straw produced in the plot). Soil samples were collected in March 2007, 10 months after the latest fertilizer application.

Potential denitrifying activity

Potential denitrifying activity (PDA) was performed according to the C₂H₂ inhibition method [18, 25]. Briefly, 25 g fresh soil were placed in 250-ml flasks containing 25 ml substrate with 1 mM glucose and 1 mM KNO₃. Denitrifying conditions were achieved by evacuating and filling flasks with nitrogen gas three times. Acetylene was injected to a partial pressure of 10 kPa to inhibit the N₂O reductase. The soils were incubated at 25°C on a rotary shaker at 210 rpm for 6 h. Head space samples were collected every hour. Nitrous oxide was analyzed with a gas chromatograph (Hewlett 5890; Packard series II) equipped with a poraplot Q column (3 m×2 mm) and a ⁶³Ni electron capture detector.

Soil microbial DNA preparation

Soil microbial DNA was extracted according to the previous method [24]. To minimize the influence of PCR inhibitors, extracted DNA was further purified using the Agarose Gel DNA Purification Kit (Takara Bio Inc, Japan). DNA concentration and quality were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Primers for denitrifying gene fragments

Degenerate primers for *narG* and *nosZ* genes were newly designed (Table 1). In total, 14 *narG* sequences and 15 *nosZ* sequences from various families or strains derived mostly from soils (Supplementary Table S1) were retrieved from the GenBank databases and aligned using ClustalW2 software (<http://www.ebi.ac.uk/clustalw>). Conserved peptide fragments were used to design forward and reverse primers (Table 1). The primer pairs of *narG*145F/773R and *nosZ*1126F/1884R were used for terminal restriction fragment length polymorphism (T-RFLP). While the primers of *narG*571F/773R and *nosZ*1126qF/1381R were used for real-time PCR. The specificity of the newly designed primers was validated with 30 strains, which were obtained from different sources (Supplementary Table S2).

Amplification of *qnorB* gene fragments was conducted using the *qnorB* 2F/7R (T-RFLP) and 2F/5R (qPCR) [26] with a few modifications (Table 1). The qPCR for 16S rRNA was performed with 1369F/1492R primer set [27].

PCR amplification and T-RFLP detection

The PCR reaction solution consisted of about 60 ng of DNA template, 400 nM dNTP, 400 nM of each primer,

1.5 mM Mg²⁺ buffer, and 2 U Taq DNA polymerase (Tiangen, China), and dH₂O to make a final volume of 50 μL. Touchdown PCR for *narG* and *nosZ* were performed with 5 min of denaturation at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55 to 52°C, and 45 s at 72°C. The first two cycles were at 55°C, the following three cycles were at 52°C, and the last 30 cycles were at 50°C. PCR was completed with 7 min at 72°C. The program for *qnorB* was as follows: following 5 min of denaturation at 95°C, 5 cycles at the annealing temperature of 60°C, then 55°C for 35 cycles; the other program steps were the same with the *narG*.

T-RFLP analysis was carried out by Sangni Corporation (Shanghai, China) using an ABI Prism 3100 Genetic Analyzer. T-RFLP profiles for *narG* were generated with endonucleases *TaqI* and *RsaI*, for *qnorB* with *MspI* and *HhaI*, and for *nosZ* with *AluI* and *HhaI*.

Cloning and sequencing

After the PCR amplification, the expected bands of *narG*, *qnorB*, and *nosZ* genes were excised from agarose gel and purified with Wizard SV Gel and PCR clean-up Systems (Promega, Madison, USA) following manufacturer's instructions. The PCR products were cloned into a pGEM-T vector (Promega) and transformed into *Escherichia coli* strain DH-5α, followed by "blue-white screening." Plasmids with correct size inserts were screened through PCR with M13F and M13R primers. For each of the above gene, about 85 selected colonies from each treatment were sequenced by ABI Prism 3100 Genetic Analyzer (Invitrogen Biotechnology Co., Guangzhou). The detailed number of sequences is shown in supplementary material (Table S4). All *narG*, *qnorB*, and *nosZ* gene sequences determined by the BLAST program in the present study have been deposited in

Table 1 Primer sequences, peptide templates, and positions

	Primer ^a	Conserved peptide	Primer sequence ^b (5'–3')	Reference
^a The forward and reverse primers are indicated by the letters F and R, respectively. The number of each primer indicate the nucleotide position corresponding to the <i>narG</i> gene of <i>Pseudomonas aeruginosa</i> PAO1 (NC_002516), the <i>nosZ</i> gene of <i>Sinorhizobium meliloti</i> 1021 (AE007253), and 16S rRNA gene of <i>Escherichia coli</i>	<i>narG</i> 145F	THGVNCT	ACSCAYGGSGTDAACTGYAC	This study
	<i>narG</i> 571F	PIPAMSM	CCGATYCCGGCVATGTCSAT	This study
	<i>narG</i> 773R	WGS/TNV/IP	GGNACGTTNGADCCCCA	This study
	<i>nosZ</i> 1126F	GLGPLHT	GGICTBGGICCRYTGCAAYAC	This study
	<i>nosZ</i> 1126qF	GLGPLHT	GGGCTBGGGCCRTTGCA	This study
	<i>nosZ</i> 1381R	NKFSKDRF	GAAGCGRTCCTTSGARAACCTG	This study
	<i>nosZ</i> 1884R	HAL/MHL/MEM	CATYTCSAKRTGCAKGGCRTG	This study
	16S 1369F		CGGTGA ATACGTTTCYCGG	[27]
	16S 1492R		GGWTACCTTGTTACGACT	[27]
	<i>qnorB</i> 2F		GGNCAYCAGGGSTAYGA	[26]
	<i>qnorB</i> 5R		ACCCASAGRTGSACSACCCACCA	[26]
	<i>qnorB</i> 7F		GGNGGRTTDTACDGAANCC	[26]

^b S = C or G; Y = C or T; K = G or T; R = A or G; D = A, G or T; V = A, C or G; B = C, G or T; N = A, C, T or G; I = inosine

GenBank under the accession numbers FJ209539–FJ209729 (*narG*), HQ615163–HQ615366 (*qnorB*), and FJ209365–FJ209538 (*nosZ*)

Quantitative PCR

The primer pairs of 16S rRNA-1369F/1492R, *narG*-571F/773R, and *nosZ*-1126qF/1318R *qnorB*-2F/5R were employed for qPCR of 16S rRNA, *narG*, *nosZ*, and *qnorB* genes, respectively (Table 1). The real-time PCR assay was carried out in a volume of 10 μ L, and the assay mixture contained 0.25 \times SYBR green (Invitrogen, USA), 1 μ L of 10 \times reaction buffer, 0.8 μ L of 2.5 mmol L⁻¹ dNTP, 3.5 mmol L⁻¹ Mg²⁺, 0.5 U Hot Start DNA polymerase (Takara, Japan), 150 nmol L⁻¹ forward and reverse primers, and 5 ng of template DNA. Thermal cycling conditions were as follows: an initial cycle of 95°C for 3 min; 5 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s; and 30 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 20 s. qPCR was performed with an ABI Prism 7900 sequence detection system. The standard curves for 16S rRNA, *narG*, *qnorB*, and *nosZ* were created using 10-fold dilution series (10²–10⁹ copies, Supplementary Table S3 and Fig. S2) of the plasmids containing the targeted gene fragments from soil as described by Henry et al. [28]. The efficiency of the reactions was from 82% to 110% for the above genes (Supplementary Table S3). The R² values for the curves were above 0.99 (Table S3). One sharp peak was observed in each of the melt curves (Supplementary Fig. S3).

Statistical analysis

The size and relative abundance of terminal restriction fragments (T-RFs) were quantified using PeakScan version 1.0 software (Applied Biosystems, Inc.). The fragments with a signal above 1% of the sum of all peak heights were included. The peak heights of T-RFs that differed in size by ≤ 2 bp in an individual profile were summed and considered as one fragment. T-RFs with a size of more than 90 bp were used for cluster analysis. Diversity indices were calculated with PC-ORD version 5.0. Ordination techniques of correspondence analysis (CA) and canonical correspondence analysis (CCA) (CANOCO version 4.53) [29] were used for community analysis. CCA was used to assess the relationship between microbial community profiles and environmental variables. Sequence identity >95% was defined as an operational taxonomic unit (OTU) [30]. The clone sequences were aligned using ClustalX (1.83), and a neighbor-joining tree was produced from the MEGA 4.0 [31]. Bootstrap analysis was used to estimate the reliability of the phylogenetic reconstruction (1,000 replicates).

Table 2 Copy numbers of 16S rRNA and denitrifying genes in fertilization treatments

Treatment	Gene copy numbers (copies g ⁻¹ soil)		Ratio		Ratio		Ratio		Ratio	
	<i>narG</i>	<i>nirK</i> ^b	<i>narG</i> /16S	<i>nirK</i> /16S	<i>nirS</i> ^c	<i>qnorB</i>	<i>qnorB</i> /16S	<i>nosZ</i>	<i>nosZ</i> /16S	Ratio <i>nosZ</i> /16S
NF	5.4 \times 10 ^{9Ba} (5.7 \times 10 ⁸)	2.2 \times 10 ^{8BC} (1.9 \times 10 ⁷)	5.99%	4.16%	1.4 \times 10 ^{7C} (2.4 \times 10 ⁶)	1.2 \times 10 ^{8B} (5.80 \times 10 ⁶)	0.26%	2.24%	3.1 \times 10 ^{6C} (1.1 \times 10 ⁶)	0.06%
UR	5.9 \times 10 ^{9B} (4.6 \times 10 ⁸)	3.8 \times 10 ^{8B} (7.2 \times 10 ⁷)	19.62%	6.46%	4.2 \times 10 ^{7B} (3.6 \times 10 ⁵)	1.4 \times 10 ^{8B} (3.5 \times 10 ⁶)	0.71%	2.28%	5.6 \times 10 ^{6B} (1.2 \times 10 ⁶)	0.10%
BM	6.3 \times 10 ^{9B} (4.5 \times 10 ⁸)	5.2 \times 10 ^{8B} (4.9 \times 10 ⁷)	14.48%	8.20%	3.6 \times 10 ^{7B} (3.9 \times 10 ⁶)	2.3 \times 10 ^{8A} (2.4 \times 10 ⁷)	0.57%	3.66%	8.0 \times 10 ^{6B} (8.8 \times 10 ⁵)	0.13%
BMR	1.2 \times 10 ^{10A} (8.8 \times 10 ⁸)	1.2 \times 10 ^{9A} (8.0 \times 10 ⁷)	20.85%	9.68%	1.01 \times 10 ^{8A} (3.0 \times 10 ⁶)	3.9 \times 10 ^{8A} (1.7 \times 10 ⁷)	0.82%	3.14%	1.5 \times 10 ^{7A} (2.7 \times 10 ⁶)	0.12%

^a Significant differences ($p < 0.05$) between the treatments are shown with superscripted letters A, B, or C; mean (SEM), $n = 3$ for each treatment

^{b,c} The gene numbers of *nirK* and *nirS* were cited from the reference of Chen et al. [24]

Results

Potential denitrifying activity

Long-term fertilization affected PDA. PDA was significantly higher in UR, BM, and BMR treatments (220, 161, and 394 ng N₂O–N g⁻¹ dry soil h⁻¹, respectively) than in NF (63 ng N₂O–N g⁻¹ dry soil h⁻¹). There was no significant difference between the UR and the BM treatments, and the highest PDA was observed in plots amended with rice straw (BMR).

Primer validation and denitrifying gene amplification

Both *narG* and *nosZ* gene fragments were successfully amplified from all the 12 soil samples by the primer sets of *narG*-145F/773R and *nosZ*-1126F/1884R. In total, 344 and 331 clones generated by *narG*-145F/773R and *nosZ*-1126qF/1884R, respectively, were sequenced (about 85 clones for *narG* and *nosZ*, respectively, from each treatment, details shown in Supplementary Table S4). The consequent alignment analysis by BLAST indicated that 94% of *narG* clones and 88% of *nosZ* clones were homologous to *narG* and *nosZ* genes, respectively (data not shown). These high percentages implied that the newly designed primers possess high specificity for the denitrifying genes in the soil.

Similarly, a total of 318 clones were generated by the primer set of *qnorB*-2F/7R and resulted in 97% positive proportion (307 clones) that were homologous to the *qnorB* gene fragments in the GenBank database.

The nitric oxide reductase gene, *cnorB*, was also first in our consideration because this gene is directly linked to N₂O production and is only found in denitrifiers, while the *qnorB* gene has been discovered both in denitrifiers and in some nondenitrifying bacteria [32]. Unfortunately, the *cnorB* gene failed to amplify with the published *cnorB* primers [26]. We also designed a set of *cnorB* primers and obtained the targeted size of PCR products; however, most of these products were not homogeneous to *cnorB*. Therefore, the *cnorB* gene was not used for further analysis.

Influence of fertilization on the abundance of denitrifying genes

The copy number of each denitrifying gene (*narG*, *nirK/nirS*, *qnorB*, and *nosZ*) varied similarly to the fertilization regimes (Table 2). The highest copy number was observed in the plots amended with BMR followed by the two mineral fertilization treatments (BM and UR), and the lowest occurred in NF treatment. Compared with the above denitrifying communities, interestingly, fertilization exhibited less effect on the abundance of 16S rRNA. The

mineral fertilization treatments of UR and BM did not induce significant differences compared to NF, while only BMR resulted in doubled 16S rRNA copy numbers, which was significantly higher than NF (Table 2). In contrast, the impact of fertilization on the size of denitrifying genes was more obvious because the two mineral fertilization regimes (UR and BM), especially amended with BM, caused remarkable increases in the copy numbers of the denitrifying genes. However, plots amended with BMR induced the highest copy numbers of the denitrifying genes, which was three to nine times higher compared to NF. The results implied that the abundance of the denitrifying genes of *narG*, *nirK/nirS*, *qnorB*, and *nosZ* was more sensitive to fertilization than that of 16S rRNA. However, the abundance of the denitrifying genes was not evenly influenced by the long-term fertilization, where *narG* and *nirS* communities were most sensitively responsive to the fertilizations followed by *nirK* and *nosZ*; *qnorB* was the least sensitive community.

The order of the denitrifying genes abundance was as follows: *narG* > (*nirK* + *nirS*) > *qnorB* > *nosZ*. This trend coincidentally agreed with the process order of denitrification. *narG* gene works on the first step to reduce NO₃⁻ to NO₂⁻ and possessed the highest copy number (3.2 × 10⁸ in NF), while the *nosZ* gene is involved in the last step of denitrification and possessed the lowest copy number (3.1 × 10⁶ in NF). This finding indicates clearly that the copy numbers of the key genes involved in the denitrification process in paddy soil decreased with the sequential reaction order.

Diversity variations of the denitrifying genes under fertilization regimes

The diversity variations of the denitrifying genes based on the T-RFLP results are presented in Table 3. Statistically, no significant differences in the diversity indices of *narG*, *qnorB*, and *nosZ* were detected among the fertilization treatments except that UR induced significantly lower diversity of *qnorB* than the other treatments, indicating weak effect of fertilization on the diversity of *narG*, *qnorB*, and *nosZ* gene communities after 17 years of continuous fertilization. Combined with our previous results of *nirK* and *nirS* gene diversity, it suggested that the diversity of denitrifying gene communities in paddy soil is not sensitive to fertilization.

Effect of fertilization on denitrifying community composition

The community compositions of *narG*, *qnorB*, and *nosZ* genes were determined by T-RFLP. The T-RF profiles of the three genes from triplicate samples of each treatment were similar, indicating that the results were reproducible and

Table 3 Diversity parameters from the T-RFLP profile analysis of *narG* and *nosZ* gene fragments amplified from different fertilization practices

	<i>narG</i>		<i>qnorB</i>		<i>nosZ</i>	
	<i>TaqI</i>	<i>RsaI</i>	<i>MspI</i>	<i>HhaI</i>	<i>AluI</i>	<i>HhaI</i>
NF ^a	2.09 (0.08)A ^b	2.24 (0.09)A	2.09 (0.08)A	2.29 (0.04)A	1.91 (0.18)A	1.73 (0.18)A
UR	1.99 (0.11)A	2.09 (0.18)A	1.82 (0.04)B	2.01 (0.03)B	1.97 (0.27)A	2.01 (0.16)A
BM	2.11 (0.06)A	2.15 (0.11)A	2.10 (0.04)A	2.22 (0.01)A	2.02 (0.07)A	1.85 (0.11)A
BMR	2.18 (0.04)A	2.27 (0.05)A	2.06 (0.05)A	2.31 (0.07)A	1.88 (0.12)A	2.02 (0.12)A

^a $n=3$ for the treatment

^b Diversity = Shannon's diversity index $H' = -\sum (p_i) (\ln p_i)$, where p_i = T-RF abundance divided by total abundance in a profile. The uppercase letter A in the column means no significant difference within $p>0.05$ level

representative for the denitrifier communities in the soils (Supplementary Fig. S1). Therefore, the average relative abundances of the T-RFs for *narG* (*TaqI*), *qnorB* (*HhaI*) and *nosZ* (*AluI*) are presented in Fig. 1. It is obvious that the community composition of *narG* varied significantly under both mineral fertilizations (UR and BM) and rice straw incorporation (BMR), while the community composition of *nosZ* was only affected by the rice straw incorporation (BMR), which induced shifts of some dominant T-RFs. Comparatively, the community composition of *qnorB* was less affected by the fertilization regimes.

The relative abundance of *narG* T-RFs of 251 and 263 bp declined markedly in the plots amended with UR, BM, and BMR compared to the NF (Fig. 1a). However, the *narG* T-RFs of 275 and 437 bp were nearly undetectable in NF but dominant in UR, BM, and BMR. Obvious

variations were also detected on *narG* T-RFs of 205 and 254 bp. The T-RFLP patterns of *narG* in BM and BMR were similar, although the relative abundance of some less-abundant fragments showed a little difference. Fewer variations in the relative abundance of *qnorB* and *nosZ* T-RFs were observed among the treatments (Fig. 1b, c) in comparison with *narG*. For the profiles of *qnorB*, no significant differences were detected on the relative abundance of several dominant T-RFs (93, 107, 187, and 242 bp) and less-dominant T-RFs (165, 442, and 463 bp), indicating that the *qnorB* patterns of the four treatments were similar. For the profiles of *nosZ*, it was obvious that NF, UR, and BM treatments shared similar T-RFs patterns characterized by a predominant T-RF of 273 bp. Application of BMR resulted in lower proportion of T-RF of 273 bp and higher proportion of T-RF of 141 bp compared to other

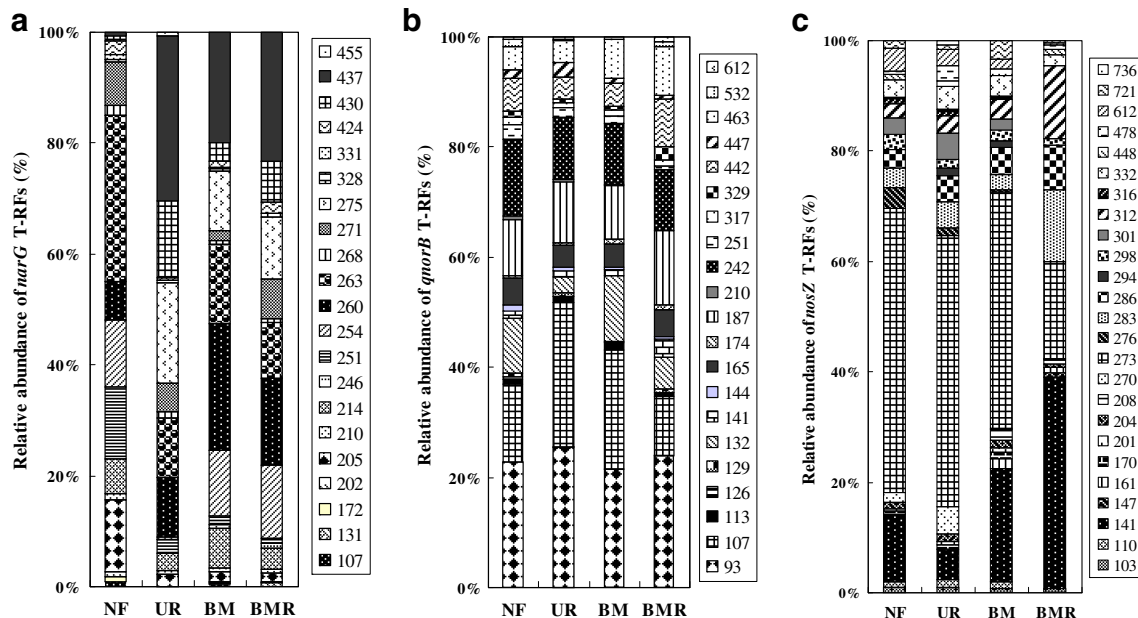


Figure 1 Average relative abundances of *narG* (a) T-RFs with endonuclease *TaqI*, *qnorB* (b) with *HhaI*, and *nosZ* (c) with *AluI* from long-term fertilization treatments. The relative abundance of T-RFs is

given as a percentage of the total peak height. Fragment sizes within the graph indicate the sizes (bp) of the experimental T-RFs by T-RFLP

treatments. In addition, BMR treatment also induced three dominant groups of T-RFs of 283, 286, and 316 bp, which were minority groups in NF, UR, and BM treatments.

Correspondence analysis (CA) was employed to determine the fertilization effects on the *narG*-, *qnorB*-, and *nosZ*-defined communities (Fig. 2). The *narG* community composition formed three distinct groups (Fig. 2a) among the four treatments with three replicates, in which BM and BMR grouped together while NF and UR were grouped independently, indicating that mineral fertilizations obviously altered *narG* communities rather than rice straw incorporation. *qnorB* and *nosZ* denitrifier communities were not separated like *narG*. The *qnorB* samples from the four treatments scattered around the center of the ordination plot, suggesting that fertilization had a weak effect on *qnorB*-denitrifier community composition (Fig. 2b). For *nosZ*, only the replicates of BMR grouped independently, and the rest of the treatments grouped together, implying rice straw incorporation caused obvious alteration of *nosZ* community composition, while mineral fertilizers did not result in severe changes (Fig. 2c).

The relationship between PDA and composition and abundance of denitrifying community

PDA was not coupled to the denitrifier community composition, as determined by CCA analysis. However, statistical calculation showed that PDA was significantly correlated with the copy numbers of *narG*, *nirK*, *nirS*, and *nosZ* gene ($R^2=0.792, 0.811, 0.855, \text{ and } 0.732$ respectively, $P<0.01$ by Pearson correlation test). No such correlation existed between PDA and *qnorB* copy number ($R^2=0.352, P>0.05$).

Phylogenetic analysis

Of the total 322 *narG* clone sequences, 143 OTUs were identified from all the treatments and formed the phyloge-

netic tree together with a selected published *narG* reference sequences (Fig. 3). The tree can be divided into six main clusters and some subclusters based on the location of NarG from cultivated microorganisms. Cluster I contained 46% of the total clones, which were closely related to the *narG* of Alphaproteobacteria (~92% identical at the protein level), and the proportion of the clones from each treatment was similar (43–50%). Unlike cluster I, clusters II and V were related to Betaproteobacteria, and the clones were unevenly distributed in these two clusters in relation to the fertilization regimes. For example, in cluster V, the clones from BM accounted for 25% of the total, whereas it was only about 3% for NF. Clusters III and IV were related to Gammaproteobacteria, and only a few clones (2%) were from BMR in cluster IV, while no obvious differences in clone distribution were observed in cluster III. Interestingly, about 2% of the *narG* clones were similar to Gram-positive bacteria (*Mycobacterium*), which were located in cluster VI. These results indicate that paddy soil contains highly diversified *narG*-containing denitrifiers, and fertilization regimes intensively influenced *narG* phylogeny.

nosZ phylogenetic tree containing 103 OTUs mainly grouped into seven clusters. Most of the *nosZ* OTUs from paddy soil were related to Alpha-, Beta- and Gammaproteobacteria, the first two groups accounting for 96% of the total OTUs (Fig. 4). Forty-five *nosZ* Alphaproteobacterial OTUs were distributed in clusters I and III, closely related to the *nosZ* gene of *Bradyrhizobium* (83–95% identical at the protein level). *nosZ*-containing Betaproteobacteria accounted for 54 OTUs and allocated in clusters II, V, and VI; among them, cluster V was unique in which not a single clone was isolated from BMR treatment. The *nosZ*-containing Gammaproteobacteria comprised a very small group of clones, which were mainly isolated from UR and BMR treatments (cluster IV) and related to *Pseudomonas stutzeri* strain A1501.

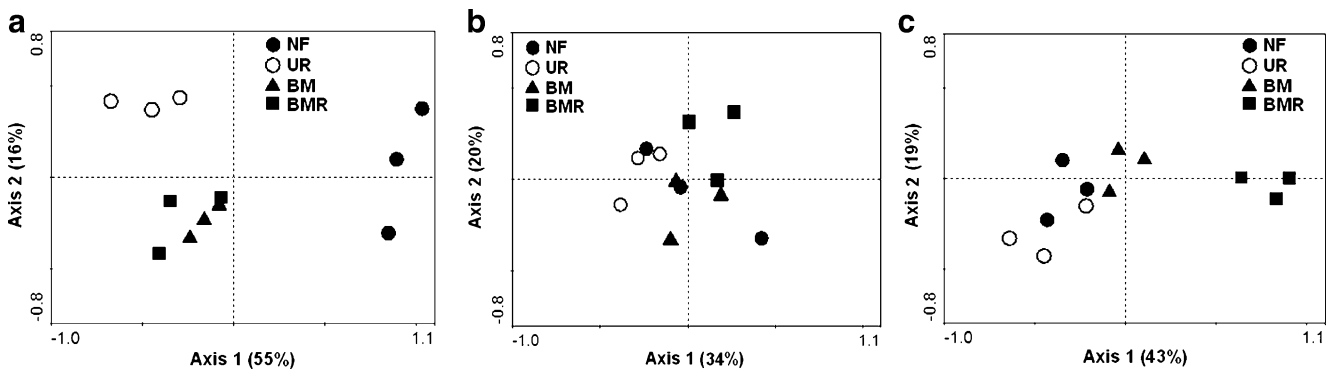
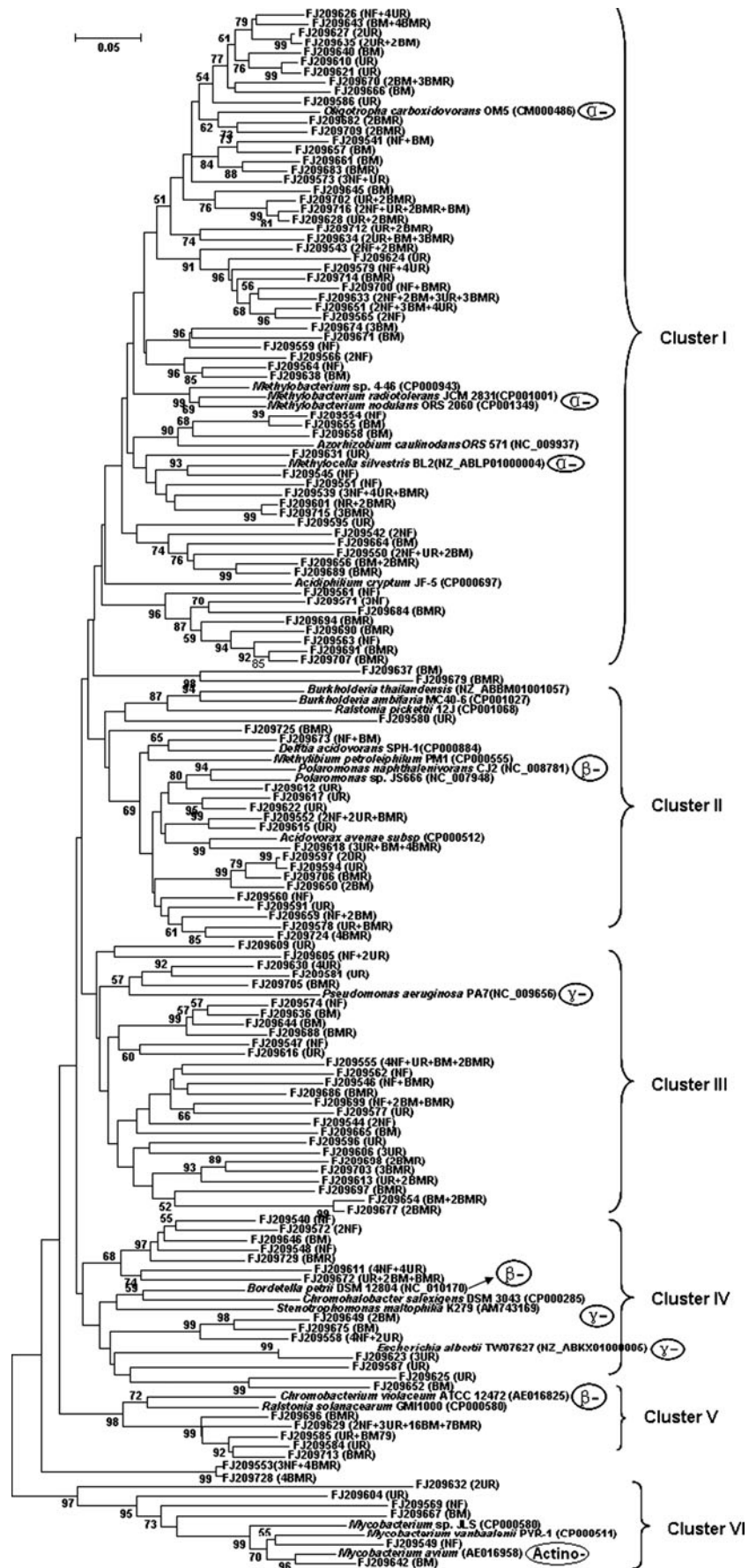


Figure 2 CA analysis of T-RFLP profiles examining the effect of different fertilization regimes on the composition of *narG* (a), *qnorB* (b), and *nosZ* (c) denitrifier communities in long-term fertilization

plots. The *nraG*, *qnorB*, and *nosZ* distributions were based on the relative abundance of T-RFs after restriction using *TaqI*, *HhaI* and *AluI* enzymes, respectively

Figure 3 Phylogenetic tree of *narG* sequences cloned from long-term fertilization plots. Bootstrap values (>50%) are indicated at the branch points. GenBank accession number followed by treatment abbreviation in parentheses, and the numbers before each treatment abbreviation represent the respective number of clones. The scale bar represents a 5% estimated sequence divergence



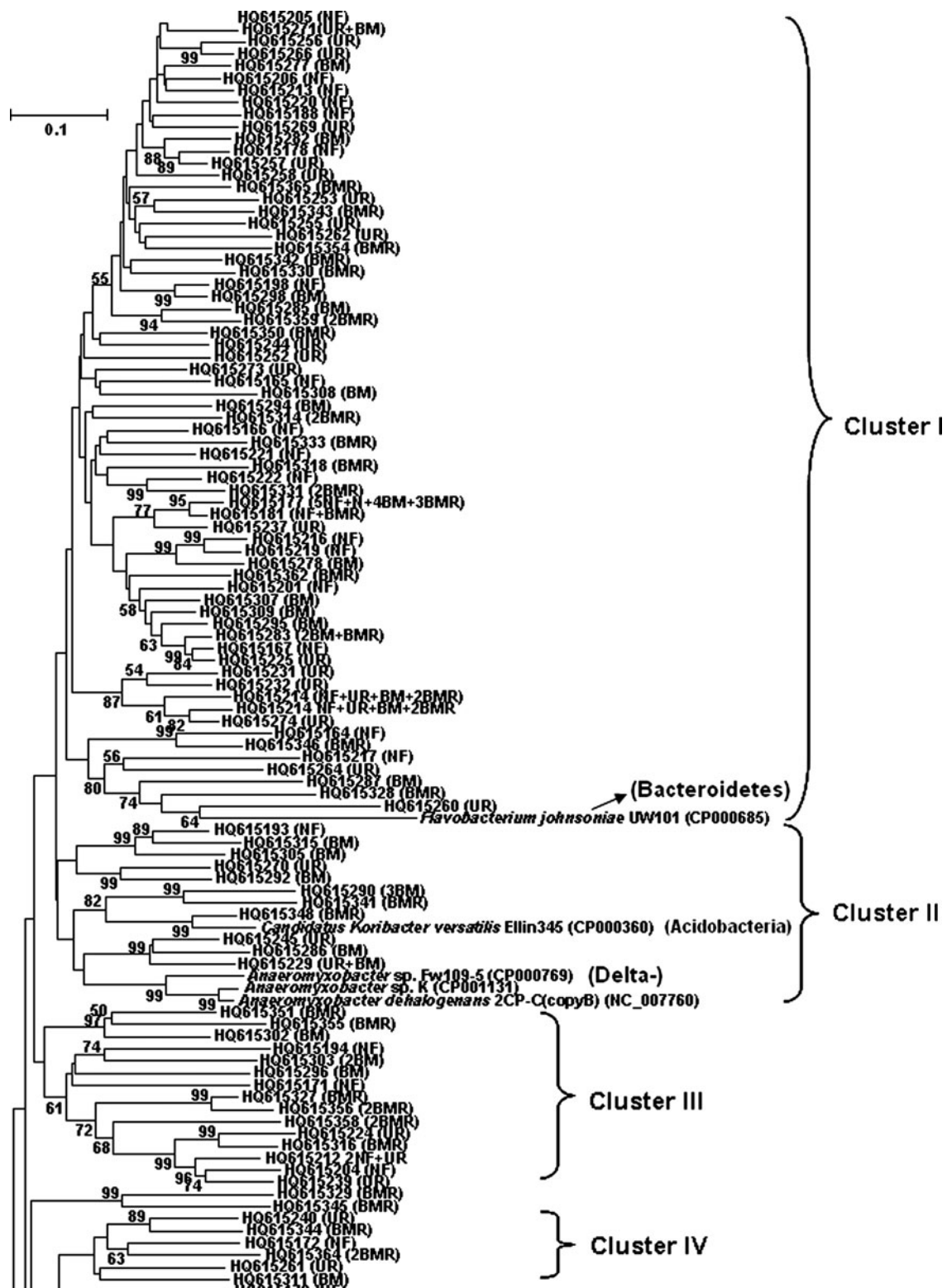


Figure 4 Phylogenetic tree of *qnorB* sequences from a paddy field in long-term fertilization. Bootstrap values (>50%) are indicated at the branch points. GenBank accession number followed by treatment

abbreviation in parentheses, and the numbers before each treatment abbreviation represent the respective number of clones. The scale bar represents a 10% estimated sequence divergence

Unlike the features of *narG* and *nosZ*, *qnorB* phylogeny is incongruent with the 16S rRNA phylogeny at the family

or genus level (Fig. 5). For example, cluster VII contained most Betaproteobacteria, but was intermingled with the

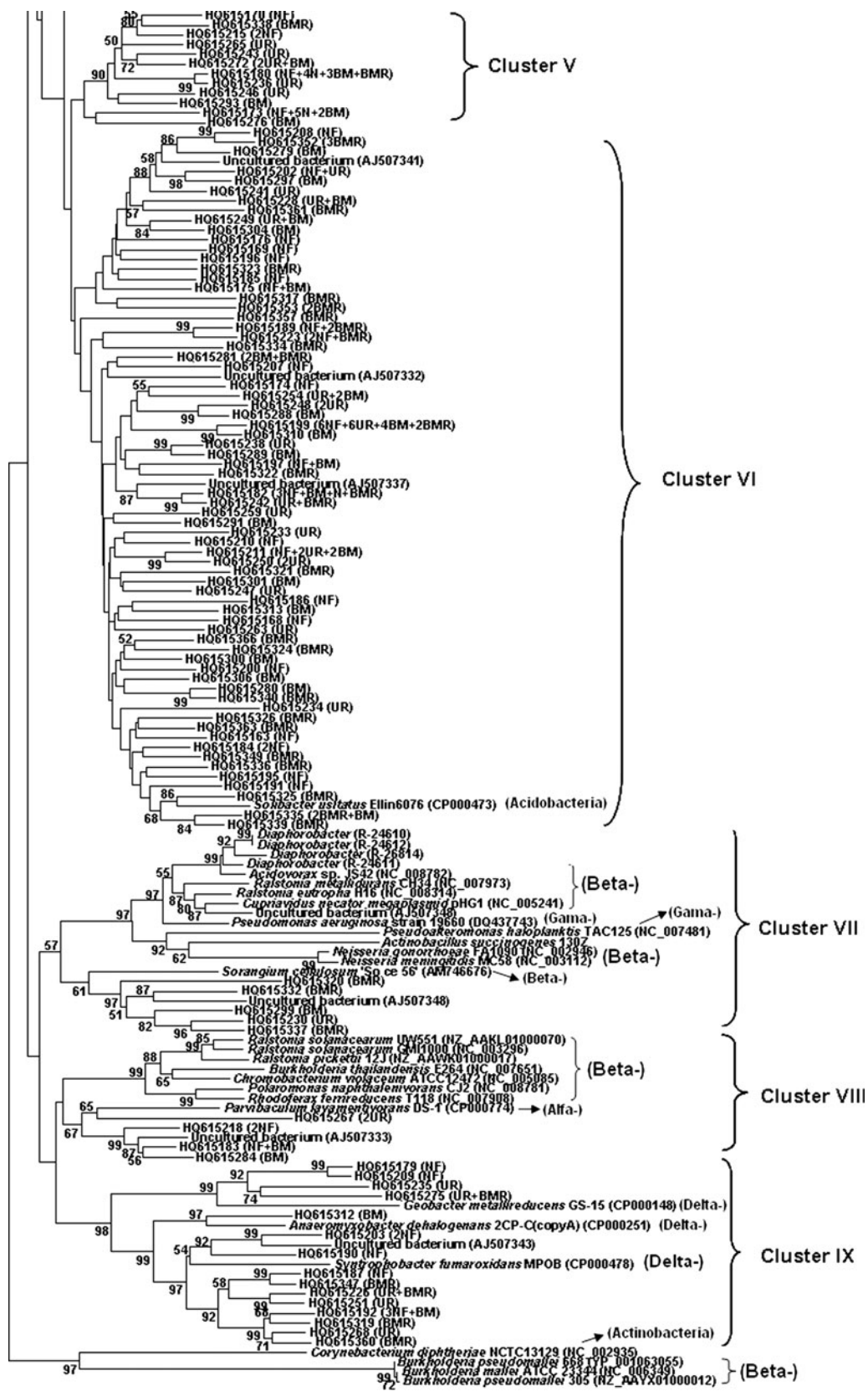
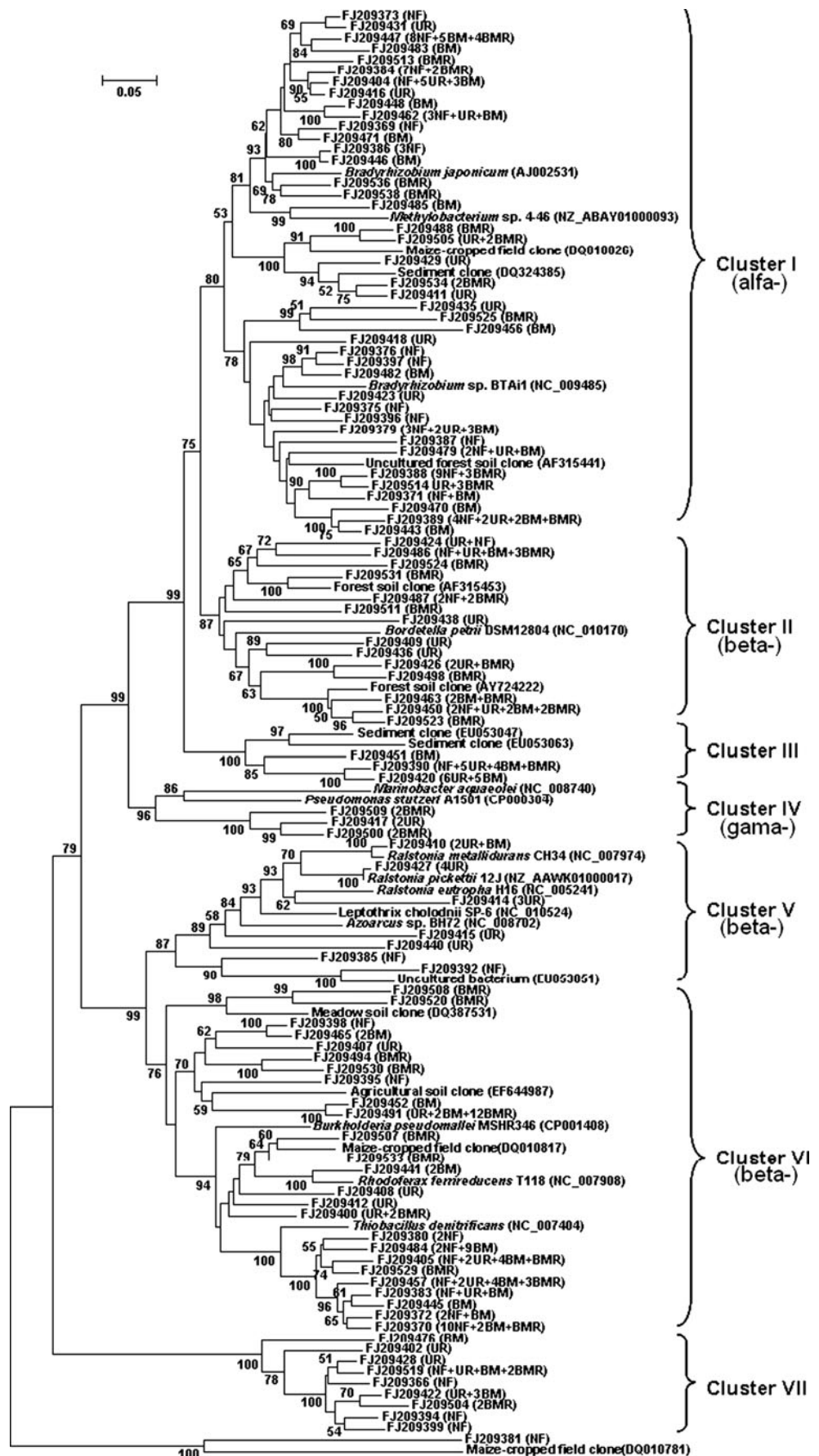


Figure 4 (continued)

Figure 5 Phylogenetic tree of *nosZ* sequences from a paddy field in long-term fertilization. Bootstrap values (>50%) are indicated at the branch points. GenBank accession number followed by treatment abbreviation in parentheses, and the numbers before each treatment abbreviation represent the respective number of clones. The scale bar represents a 5% estimated sequence divergence



sequences from Gammaproteobacteria; the species of *Ralstonia* genus branched in different clusters (clusters VII and VIII) and grouped with bacteria of other genus. Another feature of the *qnorB* phylogenetic tree was that more than half of the *qnorB* clones (56%) assembled with non-cultured species in clusters III and VI. The effect of fertilization on *qnorB* phylogeny was in congruence with the T-RFLP analysis, indicating that the fertilization impact was not obvious. For example, in the two major clusters (I and VI), the proportions of the clones obtained from the fertilization treatments were quite evenly distributed (27–30% in cluster I and 35–40% in cluster VI).

Discussion

PCR primers with high specificity and universality are a prerequisite for molecular analysis of microbial communities, since they ultimately determine what is detected in the environmental sample. In this study, the *narG* and *nosZ* primers were designed in our laboratory based on the sequences isolated mainly from soil samples, to facilitate the specific soil denitrifier community. The specificity and universality of the newly designed *narG* primers was verified by amplifying *narG* denitrifiers representing a spectrum of Proteobacteria and Gram-positive bacteria. The tested *nosZ* primers could successfully amplify the majority of the Proteobacteria except for Gram-positive bacteria and Firmicutes, indicating the specificity for *nosZ* of Gram-negative bacteria, which coincided with previous studies [33–35]. However, the primer set *nosZ*-1126F/1884R showed the advantage of amplifying *nosZ*-containing Alpha- and Betaproteobacteria even compared to some previous *nosZ* primers with bias toward Alpha- or Betaproteobacteria [20, 34, 36]. Furthermore, the newly designed *narG*-145F/773 and *nosZ*-1126F/1884R primers obtained 92% and 87% of positive clones, respectively, which covered more than ten genera of Alpha-, Beta-, and Gammaproteobacteria and a large number of uncultured species, showing that amplification with the new primers could detect a wide range of the corresponding gene communities.

Denitrification is an important stepwise process in soil nitrogen turnover, which is closely related to fertilization. Soil microbial denitrifiers, the major organisms in driving denitrification, can be affected by fertilizers [15]. However, little is known about how fertilization regimes affect stepwise denitrifying communities. As the influence of fertilization on denitrifiers would be an accumulative consequence after continuous application of fertilizers to soil, short-term experiment might not reflect the shifts of the functional microorganisms. Miller et al. [37] found that neither crop residues nor nitrogen fertilizer alone or in combinations induced a measurable change in the abundance

of the *nosZ* gene in the short term. A long-term paddy field experiment was employed in this study to investigate the impact of fertilization regimes on denitrifying communities. We found that the indices of the diversity of *narG*, *qnorB*, and *nosZ* genes were little affected by the fertilization treatments, although some shifts of denitrifier composition occurred. It might mean that paddy soil possesses a strong capacity in maintaining a stable divergence of denitrifiers under applying fertilizer application, including mineral fertilizers and rice straw incorporation [38, 39]. In contrast, fertilization regimes induced a significant increase in the community size of all the denitrifying genes. Furthermore, we found that this influence was more obvious on denitrifying communities than on 16S rRNA because the increase in the copy numbers of each denitrifying gene (*narG*, *nirK/nirS*, *qnorB*, and *nosZ*) in the plots amended with fertilizers was about ten times higher than that of 16S rRNA. It might suggest that denitrifying communities were sensitive groups to fertilization practices within bacteria society. This might be due to the input of nitrogen substrates stimulating nitrogen turnover bacteria, and other nutrients supply, including C, P, and K, might accelerate the growth and reproduction of these functional microorganisms [40].

We found that the abundance of the denitrifying genes were quite different, *narG* possessed the highest copy number followed by *nirK/nirS* and *qnorB*, and *nosZ* had the lowest copy numbers, and the abundance of *narG* was about 100 times higher than *nosZ*. Coincidentally, this abundance order of the genes is consistent with the step order of denitrification. The mechanism of such a big difference in abundance existing among the denitrifying genes is not clear. This phenomenon might be related to the gene copies within the genomes and the highly distinct physiologies between *narG*- and *nosZ*-containing organisms. It has been reported that *nirK*, *qnorB*, and *nosZ* only have one copy per genome, but *narG* could have up to three copies [41]. Substrate concentration might also play an important role in the different abundance. Nitrate is the first substrate for denitrification under anaerobic condition, and it is the most abundant compared to nitrite, NO, and N₂O. Considering denitrification, plant uptake, leaching, and gas emission, the concentrations of the denitrifying intermediates in soil decrease significantly followed by the reduction order [42]. These substrate differences could be closely related to the abundance of the corresponding genes. Besides, the nitrate reductase encoded by *narG* gene is also involved in other dissimilatory pathway for nitrate reduction [43], and thus, more *narG*-containing bacteria might be required. Furthermore, the *nosZ* gene has been found absent in nearly one third of denitrifiers that can harbor *nir* and *nor* genes [44], and the reduction of N₂O to N₂ catalyzed by *nosZ* contributes relatively little to the overall bioenergetic needs of denitrifying organisms [45]. In addition, it is known that

the results of gene abundance obtained in PCR-based studies are influenced by the initial choice of primer set [34]. Thus, the coverage of denitrifier communities to be amplified might be different for each PCR primer.

Fertilization regimes exhibited various effects on the abundance of the denitrifiers. Among them, BMR induced the highest copy numbers of all the denitrifying genes, which were 1.7- to 2.9-fold higher than BM. Rice straw incorporation might be an important contributor to the high copy numbers of the genes due to the supplementation of carbon and other nutrients for the microbial communities, and the majority of denitrifiers are heterotrophs, which rely on organic matter [40, 46]. Kandeler et al. [47] also found that the highest correlation existed between the quantity of soil organic carbon and the number of denitrifying genes in a receding glacier foreland. However, applying organic fertilizer alone without chemical fertilizers did not significantly increase the copy number of denitrifying genes (*narG*, *nir*, and *nosZ*) [15]. In the present study, the balanced mineral fertilization (BM) also produced significant increases in the abundance of the denitrifying genes compared to NF. The BM treatment obviously improved rice growth and yield, and as a result, more plant residues (roots) and secretions left in the soil could stimulate the reproduction of denitrifiers. However, it is poorly understood whether applying nitrogen alone also significantly increases the abundance of *narG*, *nirS*, and *nosZ* in comparison with the NF treatment, since plant growth was similar between the two treatments. It could be hypothesized that some strains favoring N might flourish under continuous input of nitrogen fertilizer. However, high abundance of denitrifiers in the plots amended with fertilizers might link to high denitrification rate, causing more nitrogen loss through N_2O and N_2 . This speculation can be supported by the fact that soil potential denitrification activity was significantly correlated with the copy numbers of denitrifying genes, which was found both in this study and other work [15, 24].

We also detected that different denitrifying community compositions responded differentially to fertilization regimes. Mineral fertilization regimes induced obvious shifts of *narG* gene community composition; however, the influence of rice straw incorporation was very limited. In contrast, only rice straw resulted in notable variations of *nosZ* community structure, whereas the composition of *qnorB* was little affected by the fertilization regimes. Since these functional genes mostly belonged to different bacterial strains, it might be deduced that *narG* community composition was more sensitive to mineral fertilizers rather than rice straw incorporation, while *nosZ* composition was more susceptible to the later. Previous work indicated that *nirK/nirS* communities differentially responded to fertilization [24, 48]. Therefore, it is obvious that different denitrifying

gene communities possess differential responses to fertilization practice. Although we do not know much about how significant these differential changes can influence nitrogen cycling, it might be an important factor in determining nitrogen turnover rate.

In conclusion, this study demonstrates that the sequential denitrifying gene communities responded differently to fertilization regimes. Long-term fertilization significantly affected the denitrifying community size and the denitrifying community composition. However, fertilization regimes did not result in significant variations of the diversity of denitrifiers. Functional communities involved in nitrogen cycling provide good models in microbial ecology for studying the role of the size and structure of microbial communities in corresponding process rates and ecosystem functioning.

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