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Nitrite reductase genes as functional markers to investigate diversity of denitrifying bacteria during agricultural waste composting

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Abstract The purpose of this study was to investigate the diversity of denitrifier community during agricultural waste composting. The diversity and dynamics of the denitrifying genes (*nirK* and *nirS*) were determined using polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE). Relationships between physico-chemical parameters and denitrifying genes structures were simultaneously evaluated by redundancy analysis (RDA). Phylogenetic analysis indicated that *nirK* clones grouped into six clusters and *nirS* clones into two major clusters, respectively. The results showed a very high diversity of *nir* gene sequences within composting samples. RDA showed that the *nirK* and *nirS* gene structures were significantly related to pH and pile temperature ($P < 0.05$). Significant amounts of the variation (49.2 and 38.3 % for *nirK* and *nirS* genes, respectively) were explained by pH and pile temperature, suggesting that those two parameters were the most likely ones to influence, or be influenced by the denitrifiers harboring *nirK* and *nirS* genes.

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Introduction

Composting is the widely used method to stabilize agricultural organic wastes through the degradation of biodegradable components by microbial communities under controlled conditions (Maeda et al. 2010, 2011; Xie et al. 2012; Yamamoto et al. 2011; Zhang et al. 2011). During the agricultural waste composting process, organic nitrogen (N) is degraded to ammonia-N (NH_4^+–N) and lost in the subsequent ammonia volatilization or as nitrogen (N_2) and nitrous oxide (N_2O) through the nitrification–denitrification process (Chen et al. 2010; Maeda et al. 2011). Denitrification is a crucial microbial process in the N cycle in which nitrogen oxides (NO_3^-–N and NO_2^-–N) are reduced stepwise to gaseous end products (NO, N_2O, and N_2) (Zumft 1997). During agricultural waste composting, denitrification is a major cause of nitrogen loss and contributes to the production of the greenhouse gas N_2O, which is involved in global warming potential (Maeda et al. 2010). Denitrifying community are pivotal microbes involved in denitrification and responsible for nitrogen loss as well as the N_2O emission during the composting process (Maeda et al. 2011). Thus, the underlying communities of denitrifying bacteria and their responses to the composting conditions need to be deeply understood.

Denitrification is widespread among phylogenetically unrelated groups; this suggests that denitrifying bacteria are phylogenetically diverse and hence cannot to be investigated by 16S rDNA analysis methods (Philippot et al. 2007). Fortunately, some functional genes involved in the nitrite reduction, such as *nirS* and *nirK*, are often used to detect the presence of denitrifying bacteria (Tsuneda et al. 2005; Yoshida et al. 2009). This choice of the *nirS* and *nirK* as molecular markers for detecting denitrifying

communities is strengthened by the fact that: the common difference between true denitrifiers and other microorganisms with nitrate-reducing ability is that the true denitrifiers have either a copper-containing enzyme encoded by *nirK* or a cytochrome *cd_1* enzyme encoded by *nirS* (Braker et al. 2000; Zumft 1997). Furthermore, heterogeneity within the *nirS* or *nirK* allows us to examine the diversity of denitrifiers (Braker et al. 2000). Since 1998, a variety of environmental samples have been studied using these genes (Maeda et al. 2010; Tang et al. 2010; Theerachat et al. 2011). In addition, quantity of these genes can be evaluated using PCR approach (Kandeler et al. 2006). Therefore, we selected *nirS* and *nirK* as gene markers to study denitrifier community during agricultural waste composting.

Over the past few decades, denitrification and denitrifier communities have received widespread attention for their important contributions in the nitrogen cycle (Oakley et al. 2007). Some current research papers have centralized on variation and genetic analysis of the nitrite reductase genes of denitrifying bacteria during composting. Maeda et al. (2010) recently found that the use of mature compost by covering fresh cattle manure compost was related to enhancement of N_2O emission and dramatically affects the *nosZ* diversity. *NosZ*, which encodes nitrous oxide reductase, could be also used as a target for different populations of the denitrifying bacteria capable of nitrous oxide reduction (Throbäck et al. 2004). Furthermore, researchers have made great efforts to investigate the impacts of various parameters on denitrification. The main physico-chemical parameters known to influence deinitrifier community in soil are pH, pile temperature, NH_4^+–N and NO_3^-–N (Jung et al. 2011; Čuhel et al. 2010; Dang et al. 2009; Liu et al. 2003). However, little work has been undertaken to elucidate the effects of physico-chemical parameters on denitrifiers in compost. It is necessary to understand the community dynamics of denitrifiers in compost as well as their response to environmental changes.

In the present study, we investigated the denitrifier communities using the denaturing gradient gel electrophoresis (DGGE) method targeting the *nirK* and *nirS* genes during agricultural waste composting. Since the composting process consists of mesophilic, thermophilic, cooling, and the maturation stages, and the active microorganisms vary drastically with the progress of the composting process (Ishii et al. 2003), an analysis of the denitrifiers over time was performed. To achieve the deeper understanding of the denitrifier communities vary during agricultural waste composting, the effects of changes in physico-chemical parameters on the *nirK* and *nirS* genes were explored. In this paper, we try to determine the relationships between those parameters with denitrifier community. Meanwhile, it was also conducted to make sure which parameter(s) would be the critical one(s) affecting the distribution of *nirK* and *nirS* genes. It is critically important to quantify such relationships for our understanding and management of the nitrogen cycle during agricultural waste composting.

Materials and methods

Experimental set-up and sample collection

The typical agricultural wastes (rice straw, vegetables, and bran) were collected from the suburb of Changsha. The air-dried rice straw and several types of vegetables were passed through a cutting machine to yield a 10–20 mm particle size and used as recalcitrant and easy-degradable organic materials, respectively. Soil collected from the surface of Yuelu Mountain (Changsha, Hunan) was sieved through a 40-mesh screen to remove crude plant debris and was responsible for offering some necessary nutrients and increasing microbial population. Bran was added to adjust the initial C/N ratio of composting materials.

Three composting piles with a wet weight of 25 kg were set up indoors. Rice straw, vegetables, soil, and bran were mixed at a ratio of 11:3:8:2 to obtain an organic matter content of about 60 % (dry weight) and a C/N ratio of about 30:1 (Zhang et al. 2011). Initial moisture content of the mixture was adjusted to about 60 % and moisture content during entire composting process was also monitored and adjusted to maintain 50–60 % by the addition of sterile deionized water based on the most favorable condition for composting (Zhang et al. 2011). Then, the composting piles were packed loosely in concrete composting bins with dimension of 0.5×0.4×0.45 m (length× width×height). The experiment proceeded 50 days. The sub-samples for sample property determination were collected from different locations of the composting piles (about 0.2 m in depth) on days 1, 2, 3, 5, 8, 10, 12, 13, 15, 18, 20, 30, 40, and 50. Samples for molecular analysis were pooled, mixed, and stored immediately at −20 °C before use at each sampling occasion. Based on the pile temperature fluctuations, the mixture was turned twice a week during the first 2 weeks and once a week afterwards to provide some aeration (Zeng et al. 2009, 2010).

Physico-chemical parameter determination

Ambient and pile temperatures at the center of the composting piles were monitored by a temperature meter. The pH value was measured by a digital pH meter in water suspension, which was obtained by mechanically shaking the fresh sample in water suspension at a ratio of 1:10 (*w/v*) at 200 rpm for 40 min. NO_3^-–N and NH_4^+–N were extracted with 2 M KCl and measured using a Continuous Flow Analyzer (FIAstar 5000, FOSS, Denmark).

DNA extraction and purification

The total genomic DNA was extracted from 0.5 g of the freeze-dried compost samples according to the method described previously by Yamamoto et al. (2010). After purified using the commercial DNA purification kit (Bioteke, Beijing), the extracted DNA was dissolved in 100 μL of TE buffer

(10 Mm Tris–HCl, 1 Mm EDTA, pH=8) and stored at −20 °C before use.

PCR amplification of *nir* fragments and DGGE analysis

Primer sets F1aCu (5′-ATCATGGTSCTGCCGCG-3′):R3Cu (5′-GCCTCGATCAGR TTGTGGTT-3′) (Hallin et al. 1999) and cd3af (5′-GTSAACGTSAAGGARACSGG-3′):R3cd (5′-GASTTCGGRTGSGTCTTGA-3′) (Zhou et al. 2011) were used for the amplification of the *nirK* and *nirS* genes, respectively. The forward primers F1aCu and cd3af were attached a GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGGCGGGGGCAC GGGGGG-3′) to prevent complete separation of the DNA strands during DGGE (Muyzer et al. 1993). Each reaction mixture was prepared with 2 μL of DNA extract, 2 μL of each *nirK* or *nirS* primer (10 μM), and 25 μL of Bioteke 2× power Taq PCR MasterMix and adjusted with sterile water to a final volume of 50 μL. PCR amplification was run on a MyCycler thermal cycle (Bio-Rad, Hercules, CA, USA) using cycling conditions as follows: 95 °C for 3 min; followed by 35 cycles of 95 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min; followed by 72 °C for 10 min and end at 4 °C (Maeda et al. 2010).

DGGE analysis of the amplified *nirS* and *nirK* genes was performed using the Dcode™ Universal Mutation Detection System (Bio-Rad, USA) according to the manufacturer's instructions. The purified PCR products containing approximately equal amounts of PCR amplicons (25 μL) were loaded onto the 1-mm-thick 8 % (*w/v*) polyacrylamide gels with a denaturing gradient of 50–70 % for the *nirK* genes and 40–80 % for the *nirS* genes, respectively (Maeda et al. 2010). The electrophoresis was performed in 1× TAE buffer at 60 °C for 12 h at 120 V (Ingela et al. 2004). After stained with Du-red nucleic acid gel stain, the gels were visualized with the Gel Doc XR System (Bio-Rad, Hercules, CA, USA).

Sequencing and phylogenetic analysis

For the construction of a clone library, DNA extracts from samples collected on days 1, 12, 20, and 40 were amplified with the primer sets F1aCu:R3Cu and cd3af:R3cd without a GC-clamp for *nirK* and *nirS*, respectively. The PCR products were purified before sequencing (Majorbio Bio-Pharm Technology, Shanghai). The obtained nucleotide sequences were manually proofread and compared with available sequences in the GenBank database using the NCBI BLASTn program (http://www.ncbi.nlm.nih.gov/blast). The sequences matching well were retrieved from NCBI and aligned using the ClustalW software. Phylogenetic trees were constructed using the neighbor-joining algorithm and distance calculation by MEGA4 (Tamura et al. 2007). The clone sequences of *nirK* and *nirS* determined in this essay have been submitted to the GenBank database under the following accession numbers: *nirK*, KF011510-KF011528; *nirS*, KF011529-KF011544.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to compare the physico-chemical parameters for different sampling times and to determine whether there were any significant variation at the 95 % confidence level using the software package SPSS for Windows (version 11.5, SPSS, Chicago, IL, USA).

DGGE band profiles for *nirK* and *nirS* genes were digitized after average background subtraction for the entire gel. Band position and intensity data for each sample were calculated and exported to an excel spreadsheet prior to further statistical analysis. Band numbers and relative intensity (within lane) were quantified with the QuantityOne software (version 2.0, Bio-Rad, Hercules, CA, USA) as described previously (Zhang et al. 2011). The Shannon diversity index (*H*) was calculated as:

$$H = \sum (N_i/N)\ln(N_i/N)$$

where N_i is the relative intensity of a specific band *i*, *i* is the number of bands in each DGGE profile, and *N* is the sum of all intensities of all bands in a given DGGE profile. Bands with relative intensity below 1 % were discarded before analysis. Canoco (version 4.5, Centre for Biometry, Wageningen, The Netherlands) was used for the further determination of multivariate relationships between structures of *nirK* and *nirS* genes and the physico-chemical parameters. Detrended correspondence analysis (DCA) was performed first to decide between linear or unimodal response model for these genes data. The length of the first DCA ordination axis is 1.890 for *nirK* gene and 2.658 for *nirS* gene data, respectively, which did not indicate clear unimodal species responses (Lepš and Šmilauer 2003; Zhang et al. 2011, 2013). Therefore, redundancy analysis was carried out to ordinate the temporal compositions of the *nirS* and *nirK* genes to the measured composting parameters (Lepš and Šmilauer 2003). Forward selections were performed to test which parameter(s) had significant influence on the *nirK* and *nirS* genes community composition, respectively. The selection procedures were stopped when the parameters to be added failed to be significant (*P*<0.05). Beyond that, by following the forward selection, variation partitioning analysis was conducted to discriminate the influence of each significant parameter after elimination of the possible effects using partial RDA (Borcard et al. 1992; Lepš and Šmilauer 2003; Zhang et al. 2011, 2013). The significant parameter was treated as constraining variable, while the other significant ones were regarded as covariables, which enabled us to evaluate the proportion of variation solely explained by each of them separately. Monte Carlo reduced model tests with 499 unrestricted permutations were used to statistically estimate the significance of all the canonical axes. Statistical significance was maintained at *P*<0.05.

Results

Physico-chemical parameters

The changes of pH and pile temperature during the composting process are shown in Fig. 1a. According to the tendency of pile temperature, the composting process could be divided into four stages: mesophilic stage (days 1–2), thermophilic stage (days 3–12), cooling stage (days 13–25), and the maturation stage (days 26–50). The maximum temperature increased significantly to 62 °C on day 10. The pH gradually increased from 7.15 to 9.45 during the first 2 weeks and decreased to 9.09 at the later stage.

Figure 1b shows the profiles of the NH_4^+–N and NO_3^-–N. As a result of mineralization of organic nitrogen compounds and increase of pH at the beginning of thermophilic stage, the

NH_4^+–N accumulated rapidly and reached peak values on day 5. Afterwards, its content decreased to low level by the NH_3 volatilization and the immobilization by microorganisms. The NO_3^-–N showed a significant increase in the first 3 days. Then, the content decreased sharply to 974.60 mg kg^{-1} (dry weight) on the 12th day (Fig. 1b). Afterwards, it gradually increased to 1401 mg kg^{-1} (dry weight) during the later thermophilic stage and cooling stage.

NirK and nirS genes DGGE profiles

The fingerprint of the microbial community structure was obtained in the resulting DNA band pattern, in which each band represents a group of denitrifying species harboring the nirK or nirS genes (Figs. 2 and 3). The denitrifiers harboring nirS gene were very dynamic during the composting process,

Fig. 1 Changes of physico-chemical parameters: **a** pile temperature and pH and **b** NO_3^-–N and NH_4^+–N during composting process. The *bars* represent the standard deviations of the mean values (*n*=3)
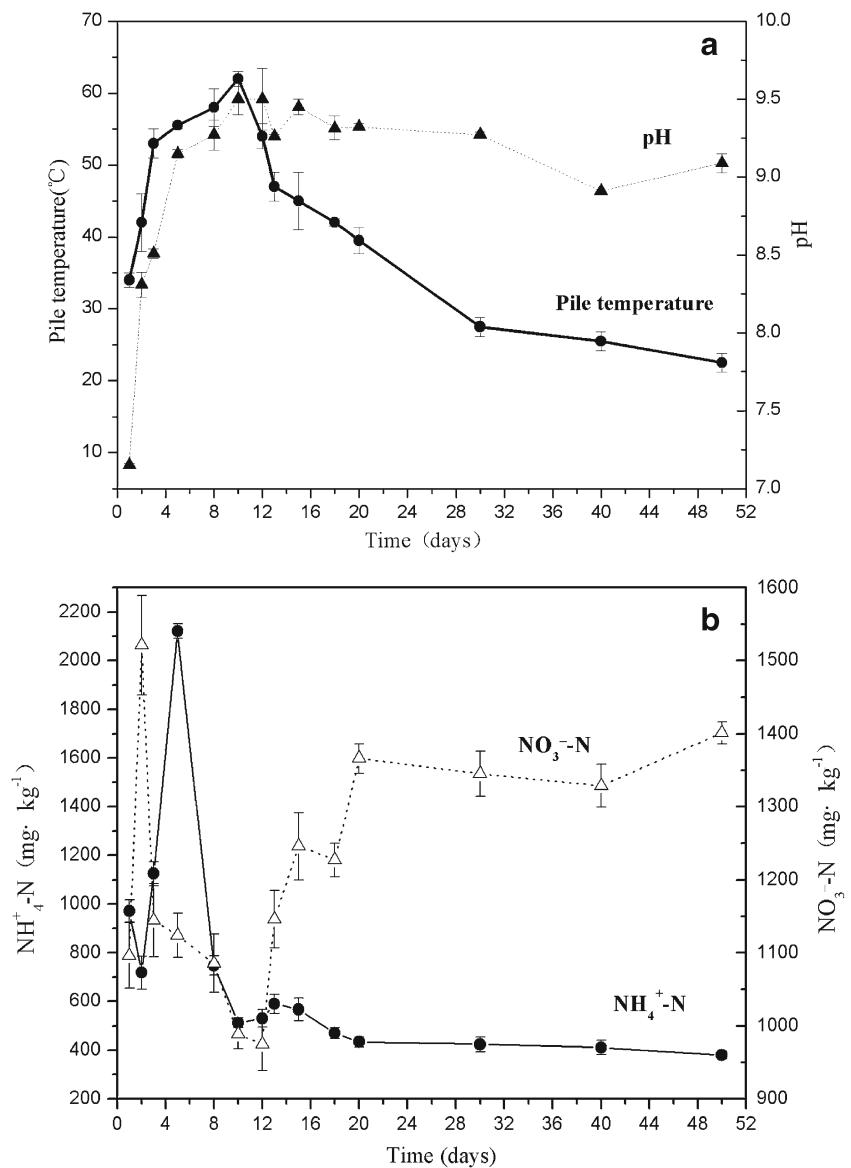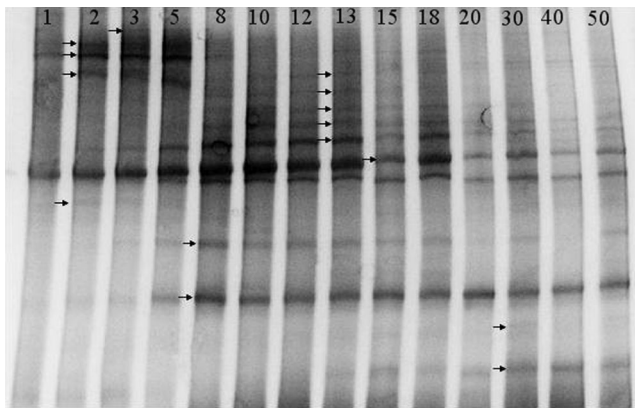
Fig. 2 DGGE profile of amplified *nirK* (nitrite reductase) gene fragments from the compost samples. The *numbers above the lanes* refer to the sampling days. The *arrows* indicate the *nirK* DGGE bands

as strong shifts in the DGGE profiles were observed between the different sampling times (Fig. 3). A total of 15 different *nirK* bands and 35 *nirS* bands were detected in the DGGE patterns. Most of those bands appeared to be ubiquitous but varied in abundance for different composting stages. There also appeared to be some dominant denitrifying communities containing *nirK* or *nirS* genes.

Phylogenetic diversity of the *nir* (*nirK* and *nirS*) PCR products

Diversity of the denitrifying community was evaluated by Shannon diversity index. The diversity indices of the two genes were various in the different composting sample time. Higher diversity indices appeared during the thermophilic stage, and the diversity index reached the lowest value on day 15 (Table 1).
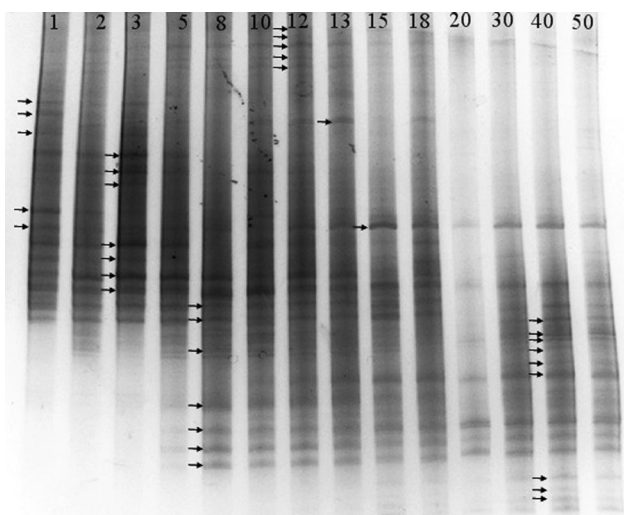

Fig. 3 DGGE profile of amplified *nirS* (nitrite reductase) gene fragments from the compost samples. The *numbers above the lanes* refer to the sampling days. The *arrows* indicate the *nirS* DGGE bands

The PCR products were cloned and randomly chosen and sequenced. A total of 120 sequences were obtained. Using BLAST, comparison of the obtained sequences with the NCBI database exhibited that all the sequences shown similarities ranging between 54 and 99 % with the closest known *nirK* and *nirS* sequences. Phylogenetic trees based on the *nirK* and *nirS* nucleic acid sequences (480 and 371–386 bp) are shown in Figs. 4 and 5, respectively. The clones of the two genes obtained in this study spread throughout the trees and affiliated to *Proteobacteria*. The phylogenetic tree of the *nirK* gene was divided into six major clusters (clusters I–VI). The clones belonging to cluster I were more closely related to *Alcaligenaceae* than affiliated to *Betaproteobacteria*. The clones belonging to clusters II, III, IV, V, and VI were closely related to *Phyllobacteriaceae*, *Bradyrhizobiaceae*, *Rhizobiaceae*, *Rhodobacteraceae*, and *Brucellaceae*, respectively, affiliated to *Alphaproteobacteria*.

The *nirS* tree was divided into two major clusters. The *nirS* clones in cluster I were primarily related to *Pseudomonadaceae*, affiliated to *Gammaproteobacteria*. While cluster II were relatively related to *Rhodocyclaceae*, affiliated to *Betaproteobacteria*.

Redundancy analysis and variation partitioning

In order to determine to what extent the four parameters (pile temperature, pH, NH_4^+–N and NO_3^-–N) affected the *nir* genes diversity, both *nirK* and *nirS* DGGE fingerprints were analyzed by redundancy analysis. These results are shown in Table 2. Monte Carlo tests for the first and all canonical axes were highly significant ($P<0.05$), suggesting that these composting parameters may play important roles in explaining the genes diversity. The first two canonical axes for *nirK* gene DGGE fingerprints explained 70.0 and 8.50 % of the variation in the species data, respectively. For *nirS* species data, 59.8 and 6.5 % of the variation were explained by the first two canonical axes, respectively.

Forward selection was performed to help us to identify the parameters that best described the most influential gradients. Explanatory variables were added until addition of further parameters failed to improve the model explanatory power significantly ($P<0.05$). In this procedure, pile temperature and pH were found to statistically explain the variation ($P<0.05$) in the distribution of the denitrification gene (*nirK* and *nirS*) species data. NH_4^+–N and NO_3^-–N cannot statistically explain the variation ($P>0.05$), so we are unable to know exactly how much NH_4^+–N and NO_3^-–N contributes to the variation by statistical analysis. For *nirK* and *nirS* genes, the RDA model (pile temperature and pH) statistically explained up to 49.2 % of the variation ($P=0.002$) and 38.3 % of the variation ($P=0.002$), respectively (Table 3). These results implied that those parameters (pH and pile temperature) as well as the interactions among them had prominent impacts on

**Table 1** The *nir* gene band number and Shannon diversity index (*H*) of DGGE profiles for each compost sample

Index		Days of composting													
		1	2	3	5	8	10	12	13	15	18	20	30	40	50
nirK gene DGGE	Band number	2	4	5	8	9	10	11	10	10	9	5	7	7	8
	Shannon diversity index (*H*)	0.499	1.290	1.407	1.916	1.753	1.835	1.865	1.827	1.929	1.943	1.267	1.826	1.768	1.972
nirS gene DGGE	Band number	12	12	13	12	15	15	16	15	10	11	4	11	13	9
	Shannon diversity index (*H*)	2.431	2.391	2.459	2.372	2.602	2.533	2.609	2.484	2.284	2.140	1.240	2.313	2.465	2.169

denitrifying community composition. However, it did not imply that NH_4^+–N and NO_3^-–N were of no importance in determining *nirK* and *nirS* genes structure. It can be only concluded that NH_4^+–N and NO_3^-–N were not significantly related to temporal changes in the compositions of the *nirK* and *nirS* genes in this research. Some variations remained unexplained (18.9 and 29.8 % for *nirK* and *nirS* genes species data, respectively). Perhaps, part of the unexplained temporal variations could relate to other microbial species (e.g., nitrifier) and physico-chemical parameters (e.g., WSC and moisture), which were not measured here.

Variation partitioning analysis was performed to extract the variation in the denitrifying community composition explained by each of the two significant parameters without the effects of the other. Percentages of variation explained by each of the significant parameters in Table 3 are those without the shared variation. Pile temperature solely explained 14.4 % (*P*=0.002) of the variation in the *nirK* gene DGGE profiles, whereas pH

Fig. 4 Phylogram for *nirK* based on partial gene fragments. Sequences with the names start with AB1K. *NirS* sequences of the reference known denitrifying bacteria are indicated by each accession number in *parentheses*. Bootstrap values (%) were generated from 1,000 replicates, and the values>50 % are shown. The scale bar represents 0.1 nucleotide substitutions per site
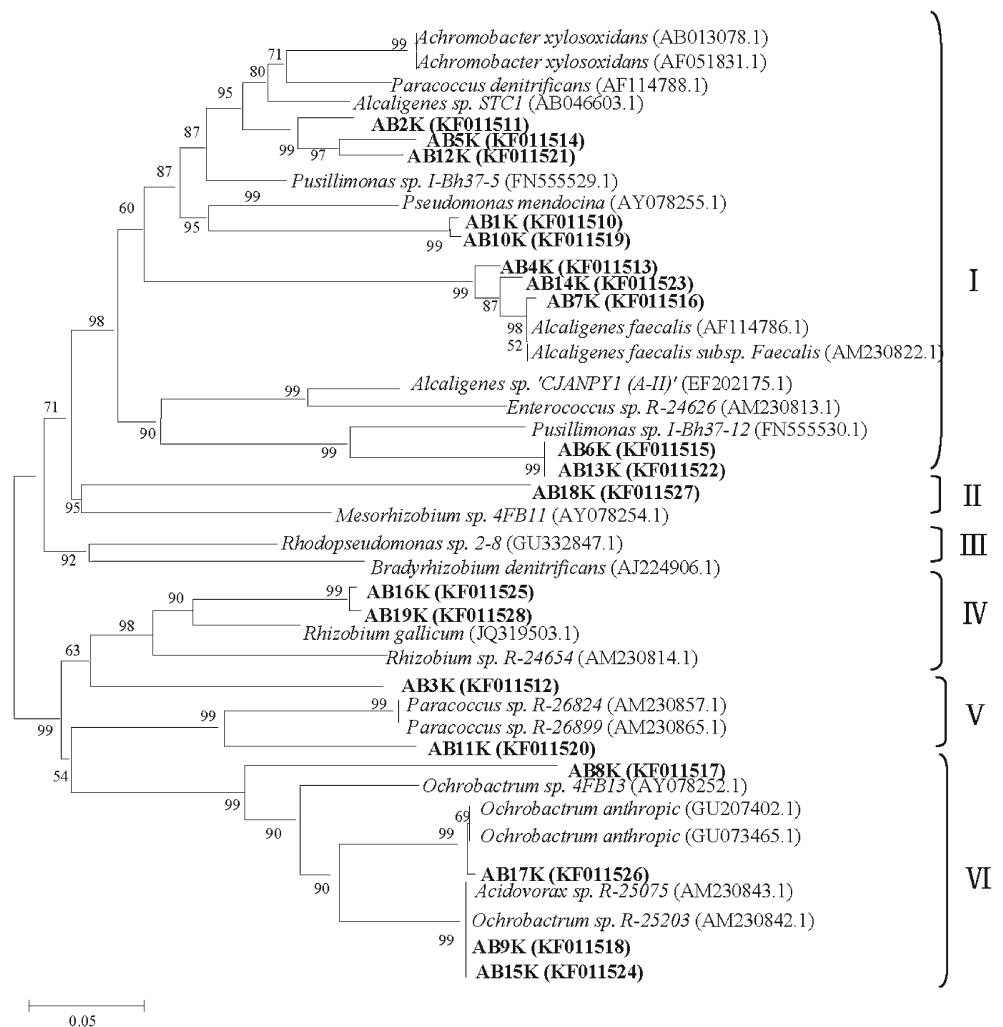
Fig. 5 Phylogram for *nirS* based on partial gene fragments. Sequences with the names start with CD1S. *NirS* sequences of the reference known denitrifying bacteria are indicated by each accession number in parentheses. Bootstrap values (%) were generated from 1,000 replicates, and the values >50 % are shown. The scale bar represents 0.1 nucleotide substitutions per site
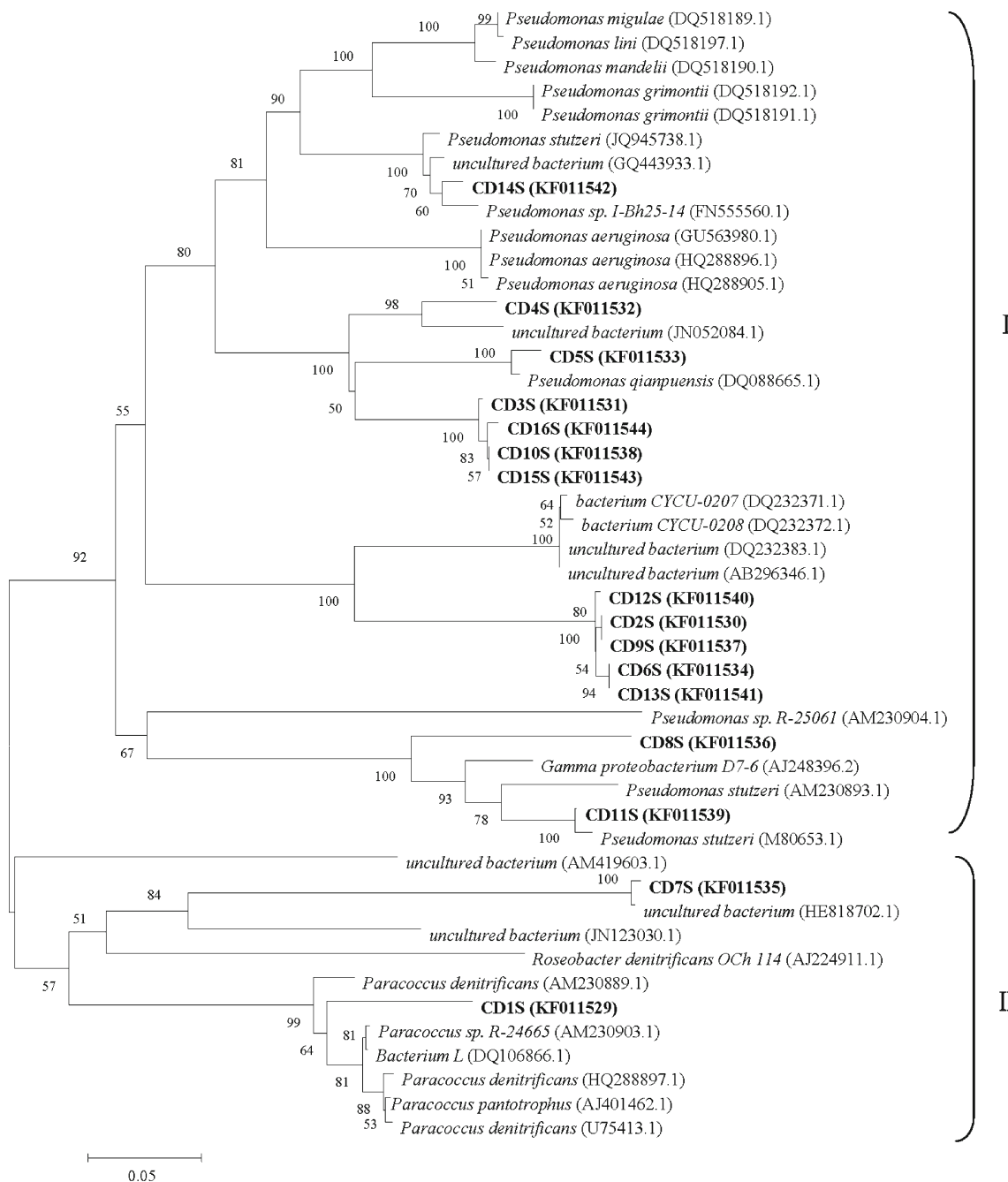
explained 19.6 % (P=0.004). The variation shared by pile temperature, pH was 15.2 %. For the *nirS* gene, pile temperature explained 16.4 % (P=0.002) of the variation, and pH explained 7.9 % (P=0.04). The shared variation was 14.0 %. The positions of each sampling days in regard to the first two environmental axes are shown in Fig. 6a for *nirK* gene species data and Fig. 6b for *nirS* gene species data. The composting condition of each sampling time can be visualized in these biplots.

Discussion

Diversity of *nir* (*nirK* and *nirS*)

The Shannon diversity index (H) of *nirK* varied in different composting time. Indices in samples collected during the thermophilic stage were higher than other stages (Table 1). The organic compounds are relative abundant at the beginning

Table 2 Redundancy analysis results of *nirK* and *nirS* DGGE profiles

Axis		Eigen value	Species–environment correlation	Cumulative % variation of species	Cumulative % variation of species-environment	Sum of all canonical eigenvalues
nirK	Axis 1	0.700	0.965	70.0	86.4	0.811
	Axis 2	0.085	0.888	78.5	96.8	
	Axis 3	0.023	0.461	80.8	99.6	
	Axis 4	0.003	0.578	81.1	100.0	
nirS	Axis 1	0.598	0.927	59.8	85.2	0.702
	Axis 2	0.065	0.753	66.3	94.4	
	Axis 3	0.030	0.640	69.3	98.7	
	Axis 4	0.009	0.508	70.2	100.0	

Monte Carlo significance tests for *nirK* gene data: sum of all eigenvalues, 1.000; significance of first canonical axis, F value=21.048, P=0.002; significance of all canonical axes, F value=9.627, P=0.002. Monte Carlo significance tests for *nirS* gene data: sum of all eigenvalues, 1.000; significance of first canonical axis, F value=13.384, P=0.002; significance of all canonical axes: F value=5.304, P=0.004. F and P values were estimated using Monte Carlo permutations

of the composting, so the denitrifiers were found that rose with the time prolonged. Thus, the diversity of *nirK*-harboring denitrifiers tend to increase after the mesophilic stage and reach the peak value on the thermophilic stage. The diversity indices in this study were lower than those reported previously in rice paddy field soil (Yoshida et al. 2009). Soil is the best natural medium for microbes. Temperature, pH, oxygen, and moisture and other environmental conditions in soil can better meet the needs of microbial growth (Guo et al. 2011). It indicates that the anaerobic environment in rice paddy field soil may be more suitable for denitrifier community than that of the compost.

Based on the *nirK* clone library analysis, *nirK* clones were widespread in the phylogenetic tree, indicating that the *nirK* diversity was large in the composting. The majority of clones from clusters II, III, IV, V, to VI were closely related to *Rhizobiales* (*Phyllobacteriaceae*, *Bradyrhizobiaceae*,

Table 3 Eigenvalues, *F* values, and *P* values obtained from the partial RDAs testing the influence of the significant parameters on the *nirK* and *nirS* diversity, respectively

Parameters included in the model		Eigen value	% variation explains solely	*F* value	*P* value
nirK	Pile temperature	0.144	14.4	10.434	0.002
	pH	0.196	19.6	11.929	0.004
	All the above together	0.492	49.2	27.103	0.002
nirS	Pile temperature	0.164	16.4	9.907	0.002
	pH	0.079	7.9	3.631	0.04
	All the above together	0.383	38.3	10.321	0.002

Partial RDAs based on Monte Carlo permutation (*n*=499) maintained only the significant parameters in the models. For each partial model, the other significant parameter was used as covariable. *F* and *P* values were estimated using Monte Carlo permutations. For both partial RDAs, sum of all eigenvalues were 1.000

Rhizobiaceae, *Rhodobacteraceae*, and *Brucellaceae*), and few clones in cluster I were closely related to *Burkholderiales* (*Alcaligenaceae*), similar to the previous study (Bremer et al. 2007). However, this does not necessarily mean that these *nirK*-harboring denitrifier communities belonged to *Rhizobiales* or *Burkholderiales*, since *nirK* phylogeny was incompatible with the 16S rDNA phylogeny (Guo et al. 2011; Yoshida et al. 2009).

Similar to *nirK* gene, the higher diversity indices of *nirS* were also appeared on earlier thermophilic stage. The result indicates that denitrifier communities harboring *nirS* or *nirK* were vibrant on thermophilic stage. This phenomenon was similar with the result of Fukumoto et al. (2003), who found that the denitrifiers were more active on the thermophilic stage of the composting process. It also confirmed the reasons for the decline in nitrate on thermophilic stage (Fig. 1b). Meanwhile, we found the diversity indices of the two genes were both relatively constant among samples except the one collected on day 20. At the beginning of the compost, easily available organic compounds were relatively abundant, the microorganism were extraordinarily active and grew fast. Thus, the diversity indices of the two genes increased smoothly. As the compost entering the continuous high temperature phase and the depletion of easily degradable substances, the species that were less sensitive to environmental changing tend to survive. Meanwhile, the species that have poor tolerances to circumstance alteration tend to be reduced or even be eliminated (Zhou et al. 2011). Thus, the diversity indices of the two genes were reduced promptly on day 20. As the composting process proceeds, microbial populations gradually adapt to the changes of environment and recover their activities. Hence, we observed that the diversity indices of the *nirK* and *nirS* increased.

Based on the *nirS* clone library analysis, the overwhelming majority of clones (cluster I) were related to *Pseudomonadaceae*, and only few clones in cluster II were related to *Rhodocyclacea*. Similar to this study, Guo et al. (2011) reported that majority of
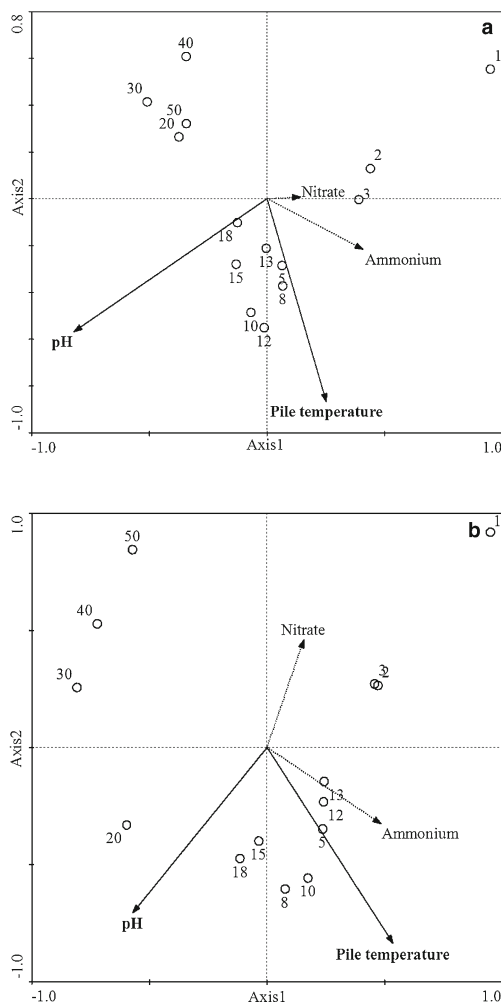
Fig. 6 DGGE band data redundancy analysis for **a** *nirK* and **b** *nirS* gene. Significant composting parameters are indicated by *solid lines* with *filled arrows*, while supplementary parameters are shown using *gray dotted lines* with *unfilled arrows*. Samples are represented by *open circles*, and sample numbers refer to the sampling days

the *nirS* clones were similar to the *nirS* from *Pseudomonadaceae* and *Rhodocyclaceae* bacteria in an agricultural soil.

Interrelations between physico-chemical parameters and *nir* (*nirK* and *nirS*)

Variation partitioning analysis suggested that pile temperature and pH have a predominant effect on denitrifier community composition. The interactions between denitrifier community with physico-chemical parameters (pH, pile temperature, NH_4^+–N and NO_3^-–N) have attracted considerable attention of biologists. The importance of pile temperature variation for the nitrifier community and the denitrifier community has been highlighted by our previous study (Zhang et al. 2011). In addition, Saad et al. (1993) also pointed out that pile temperature seemed to be the main factor determining the nitric oxide-producing nitrate-reducing bacterial populations in soil. As another important parameter, pH is one of the

important factors to affect microbial population structure. The variation of the pH is closely related to the ammonification and mineralization of organic nitrogen in the composting process (Sundberg et al. 2004). Several studies have reported that pH could dramatically affect the denitrifying community in different environments (Čuhel et al. 2010). The significant correlation between pH and denitrifier community composition was also obtained in this study. However, there were some other researchers who suggested that pH had no significant effect on the denitrifying genes in glacier foreland and long-term fertilization soils (Enwall K et al. 2005; Kandeler E et al. 2006). Earlier studies reported that the denitrification rate is greatly affected by soil pH (Šimek et al. 2002). In addition to affecting the denitrification rate, pH also substantially influences the two denitrification end products, N_2O and N_2 (Šimek and Cooper 2002). Under acidic pH, the activity of N_2O reductase is lowered and the synthesis of N_2O is inhibited. Oppositely, a higher nitrification rate was found in the alkaline environment (De et al. 2001; Nugroho et al. 2007). Thus, the acidic environment suggests a decrease in nitrifying activity, and no significant relation was found between pH and the denitrifying genes. The pH of the agricultural waste composting in this study is basic. Therefore, the strong correlations between pH and denitrifying community activity were observed in our work and Čuhel et al. (2010). While in an acidic environment, it has been shown that pH may had no significant effect on the denitrifying genes (Enwall et al. 2005; Kandeler et al. 2006). As an electron acceptor in the process of denitrification, the influence of NO_3^-–N on denitrifying communities has been proved (Liu et al. 2003). But in this study, there is no significant correlation between the denitrification gene and NO_3^-–N. Smith et al. (2007) also found the *nirK*, *nirS* and *nosZ* genes has been less effected by the nitrate concentration in the estuarine sediments. NH_4^+–N, which can be oxidized to nitrate during the composting process, also has no significant connection with the two genes in this study. Kong et al. (2010) has pointed out that changes in ammonia-oxidizing bacterial and deinitrifier communities in soil microenvironment did not relate to changes in NH_4^+–N or NO_3^-–N concentration. Notwithstanding, no significant correlation with neither *nirK* nor *nirS* gene community was found in this study, it did not imply that those parameters were of no importance in determining *nirK* and *nirS* genes community compositions. It can be only concluded by statistical analysis in this research. This result might not be necessarily applied in other fields and more research efforts should be conducted to explain the denitrification mechanism during the composting process.

In contrast to the previous report in the compost (Maeda et al. 2010), one of the notable findings in this study was that both of the *nirK*- and *nirS*-harboring denitrifiers were well detected. Our results clearly demonstrated the diversity in both *nirK*- and *nirS*-harboring denitrifiers, indicating that the nitrite

reducers with copper-containing and cytochrome cd_1 nitrite reductase are both important in the nitrite reduction step in the agricultural composting in this study. In the future, the relations of those functional genes involved in denitrification should be an important aspect.

In conclusion, this study has investigated the diversity of genes (nirK and nirS) encoding nitrite reductase in agricultural waste composting. The results showed that the diversity indices of the two genes were various in the different composting sample time, and the higher diversity indices appeared during the thermophilic stage. We also indicated that nirK clones grouped into six clusters and nirS clones into two major clusters. In addition, pile temperature and pH explained the most variation in distribution of nirK and nirS genes species data. Variance partition analysis indicated a predominant effect of the pile temperature and pH on the nirK and nirS genes structure, respectively.

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