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Analysis of *nifH* Gene Diversity in Red Soil Amended with Manure in Jiangxi, South China

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In order to understand the community structure of diazotrophs in red soil and effects of organic manure application on the structure, four *nifH* gene libraries were constructed: the control (CK), low manure (LM), high manure (HM), and high manure adding lime (ML). Totally 150 *nifH* gene clones were screened and grouped into 21 clusters by RFLP analysis. Existence of dominant patterns was observed in all libraries, which counted for over 96% of clones in library HM and about 56~72% in other three libraries. The *nifH* sequences of the dominant patterns in all libraries were most similar to sequences of the cyanobacteria. *nifH* genes showed high diversity in red soil, dispersing throughout the *nifH* clades (*alpha-*, *beta-*, and *gamma-Proteobacteria*, *Firmicutes*, cyanobacteria, *Verrucomicrobia*, and posited group). *Bradyrhizobium* and *Burkholderia* were also important diazotrophs in low fertility soil samples. Low manure treatment increased the diversity of *nifH* genes compared with CK and high manure treatments. Manure and lime treatment led to obvious community succession. Total N to available P ratio, total carbon, and K concentrations were the main factors affecting the diversity of diazotrophs in red soil.

***Keywords*:** *nifH*, diversity, nitrogen-fixing bacteria, red soil, manure

Red soil distributes widely in tropical and subtropical area. Acidic characteristic of red soil causes deficient in many essential elements, especially N, P, K, Ca, Mg, S, Zn, B, and Cu (Wilson *et al.*, 2004). In order to improve the fertility of red soil, manures are usually employed to meliorate its quality. Organic manure added into soils can improve soil properties such as aggregation, water-holding capacity, bulk density, the degree of compaction, and fertility (Zebarth *et al.*, 1999; Franzluebbers, 2002). Lime is also a common amendment that is routinely applied to acidic agricultural soils. In tropical regions, significant increases in crop yields can be achieved with minimal applications of lime due to alleviation of Al toxicity and/or Ca deficiency (Celik and Kilic, 2004).

Microbes with the ability to fix atmospheric dinitrogen are ubiquitous in natural soil ecosystems, and biological nitrogen fixation is the most important source of N (Cleveland *et al.*, 1999). *nifH* gene coding for the Ferric protein subunit of the nitrogenase complex was reported to be conservative (Zehr and McReynolds, 1989), the outline of the *nifH* gene tree was reported to largely resemble with the 16S rRNA phylogeny (Young, 1992). Thus the *nifH* gene has been employed to identify diazotrophs with a genetic potential for N_2 fixation (Widmer *et al.*, 1999). Input of organic manure and lime may perturb the microbial community balance of red soil (Demoling *et al.*, 2008). The diversity of diazotrophs with different physiological properties might be changed. However, our knowledge of their ecological importance and their diversity in red soil remains incomplete. Description of the diversity and importance of diazotroph communities would contribute greatly to our understanding of the roles of diazotrophs in red soil N pool. In this study, we investigated the diversity of *nifH* genes containing bacteria and effects of organic manure and lime amendments on their community structure in red soil in a long-term (seven-year) fertilizer experiment.

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Materials and Methods

Field site and soil treatments

The field experiment was carried out in the Yingtan Red Soil Ecological Experimental Station (28°15'20"N, 116°55'30"E) of Chinese Academy of Sciences, Jianxi Province, China. The soil was derived from Quaternary red clay and classified as Udic Ferralsols. Under the cultivation of maize once in a year. The organic manure was fresh pig dejecta from hoggery. The total N in manure was determined to control the application rate before fertilization. The application amount of organic manure was 150 kg N ha^{-1} per year in the low manure treatment (LM), 600 kg N ha^{-1} per year in the high manure treatment (HM), and 600 kg N ha^{-1} per year plus 3000 kg $CaCO_3$ ha^{-1} every three year in the high manure and lime treatment (ML). Nothing was applied in the control (CK). Four treatments with three replicates were established in randomized blocks in the field, the plots were 2 m wide×2 m long. On July 20, 2006, soil samples were collected at a depth of 0~15 cm after the harvest of maize. For each plot, fresh soil samples were collected

from 9 points, mixed and sieved (<2 mm), with aboveground plant materials, roots and stones being removed, and then conserved for next study. Soil samples from 3 replicate plots of each treatment were mixed for DNA extraction.

Chemical properties of soils

Soil pH was determined with a glass electrode. Soil total C and total N were determined by dichromate oxidation and Kjeldahl digestion method, respectively. NH_4^+-N and NO_3^--N in the extracts were determined by an automated procedure (Skalar SAN^{plus} Segmented Flow Analyzer). Available P in soil was determined using the molybdenum-blue method. Available K in soil was extracted by ammonium acetate and determined by flame photometry. All methods were described by Bao (1999). The data were subjected to analysis of variance, and the means and standard deviations for three replicates were calculated. Significant differences of means in all treatments were judged by LSD multiple comparison tests.

Extraction of DNA from red soil

The total community DNA was extracted by the direct lysis method described by Zhou *et al.* (1996). Five-gram soil samples were mixed with 2 g of sterile sand and transferred to a baked mortar. The samples were frozen in liquid nitrogen and ground until thawed. The process of freezing in liquid nitrogen and grinding until thawed was repeated twice. Ground samples were transferred to 50 ml polypropylene centrifuge tubes, and treated with SDS for cell lysis. All crude DNA was purified using the dialysis method.

PCR amplification of *nifH* and restriction fragment length polymorphism (RFLP) analysis

The primer pair *nifH*-F (forward) and *nifH*-R (reverse) was used for the amplification of the nitrogenase gene *nifH* (Rösch *et al.*, 2002). PCR amplification was performed in a PTC-200^{TM} thermocycler (BIO-RAD Laboratories, USA). The thermal profile for amplification was as follows: 4 min at 94°C; 30 cycles of 40 sec at 94°C, 40 sec at 52°C, and 1 min at 72°C; 5 min at 72°C, 10 min at 10°C. The PCR products were electrophoresed on 1.0% agarose to ascertain their size and quality. To avoid potential biases and to obtain enough PCR products for cloning, three replicate amplifications were carried out for each DNA sample, and the *nifH* amplificons were then pooled. The amplification products were purified with a Qiaquick gel extraction kit (BioFlus, China) and cloned into the pMD18-T Vector (TaKaRa, China) according to the protocols of the manufactures. The plasmids were transformed into competent *E. coli* DH10B. Clones containing a correct insert were re-amplified with the primers (*nifH*-F and *nifH*-R). Unique clones were digested by two tetrametric enzymes (*Hha*I and *Rsa*I). Each phylotype was defined as a group of amplifications that had indistinguishable *Hha*I and *Rsa*I restriction patterns. The digested products were electrophoresed in 8% (w/v) polyacrylamide (acrylamide-bisacrylamide [29:1]) gels and stained with $AgNO_3$. The RFLP patterns were analyzed and clustered by using the unweighted pair group method with arithmetic averages and the Jaccard algorithm (Braker *et al.*, 2000). The resulting clusters were validated visually by comparing the clusters with gel images.

Sequencing and phylogenetic analysis

nifH representatives for each RFLP patterns were sequenced. The nucleotide sequence of all the sequenced clones was queried against GenBank (http://www.ncbi.nlm.nih.gov) using BLAST search to get the most closely related bacteria in the database. The nucleotide sequences determined in this study or obtained from the GenBank database were aligned by CLUSTAL, and translations and phylogenetic analysis of sequences were performed with MEGA version 4.0 (http://www.megasoftware.net). The unrooted Neighbour-Joining phylogenetic tree was constructed by using the amino acid sequence distance measurement Poisson correction model (MEGA 4.0 software). Bootstrapping was used to estimate reliability of phylogenetic trees with 1,000 replicate trees.

The sequences generated in this study have been deposited in the GenBank database under accession numbers: EU544201-EU544223, EU586055-EU586056, and FJ466691-FJ466700.

Statistics

In order to reveal relationships between the distribution of bacterial groups and environmental variables from the red soil samples, a redundancy analysis (RDA) was used with the software CANOCO 4.53 (Biometris-Plant Research International). The environmental factors that best described the composition of bacteria were identified by forward selection. This procedure gives information about the importance of individual variables (ter Braak and Verdonschot, 1995). Explanatory variables were added until addition of further variables failed to improve the model's explanatory power significantly ($P<0.05$). This was assessed in permutation tests with 499 unrestricted Monte Carlo permutations. The environmental variables tested were pH, total N, total C, NH_4^+-N, NO_3^--N, P, and K. All of the group's data and environmental data except pH data were transformed as log (1+x).

Table 1. Chemical properties of the red soils

Treatment	pH	Total C g/kg	Total N g/kg	C/N	NH_4^+-N mg/kg	NO_3-N mg/kg	Available P mg/kg	Available K mg/kg	tN/P
CK	4.8(0.4)^a	3.57(0.26)^a	0.48(0.03)^a	7.4	2.8(0.1)^a	4.3(0.2)^a	1.8(1.1)^a	90.0(8.0)^a	267
LM	5.0(0.1)^a	5.98(0.27)^b	0.74(0.04)^b	8.0	6.2(0.9)^a	26.3(10.9)^b	31.9(7.4)^b	125.8(2.2)^b	23
HM	5.3(0.0)^a	8.25(0.14)^c	1.01(0.03)^c	8.1	15.2(1.6)^b	53.6(7.8)^c	205.9(7.4)^c	204.2(3.0)^c	4.9
ML	7.1(0.2)^b	8.28(0.24)^c	1.04(0.00)^c	8.0	37.6(1.1)^c	83.6(8.8)^d	196.8(9.8)^c	205.0(6.6)^c	5.2

Note: C/N = Total C/Total N, tN/P = Total N/available P. SDs are shown in parentheses. Values within the same column not followed by the same letter differ significantly (N=3, $P<0.05$)

Table 2. Diversity indices based on *Rsa*I-*Hha*I RFLP phylotypes in *nifH* gene clone libraries from four soil samples

Library	Coverage (C)[a]	H'[b]	D[b]	E[b]	D_M[b]	Numbers of clones
CK	94.29%	1.36	0.46	0.26	1.13	35
LM	88.24%	1.99	0.62	0.35	1.82	51
HM	96.42%	0.22	0.07	0.05	0.30	28
ML	97.22%	1.49	0.58	0.29	0.84	36

[a] Coverage (C) was calculated as described by Good (1953), [b] H', D_M, E, D were described by Hill *et al*. (2003) H' was Shannon index, D_M was Margalef index, E was Shannon evenness, and D was Simpson's index.

Results

Treatment effects on soils

The properties of soil samples are given in Table 1. Red soil was seriously deficient in available nutrients, especially phosphorus. Manure amendments significantly increased the fertility of the red soil. The concentrations of available nitrogen and phosphorus in LM and HM treatment plots were 4.6 and 17.7, 9.7 and 114 times higher than those in control plot, respectively. Although pig manure had a higher C/N ratio (about 13), the soil C/N ratio kept nearly the same in all treatments. tN/P ratio in each treatment varied greatly from 267 to 5, approximately. Manure amendments increased the pH value of the acidic red soil slightly. This was consistent with the observation of Noble *et al.* (1996). Addition of lime further increased the pH of soil to 7.1 and increase in NH_4^+-N and NO_3^--N was also observed in ML.

RFLP analysis of the *nifH* clone libraries

Four *nifH* gene libraries were constructed by PCR amplification with the DNA from CK, LM, HM, and ML soil samples. Different diversity patterns were observed for these *nifH* clones as indicated by RFLP analysis. 150 clones were grouped into 21 clusters according to the RFLP profiles, abbreviated as CK-I to V, LM-I to X, HM-I and II, and ML-I to IV hereafter. The clones were subjected to percent coverage analysis to check the sufficiency of the libraries (Good, 1953). Coverage for clone libraries showed that all four clone libraries were significantly near satu-

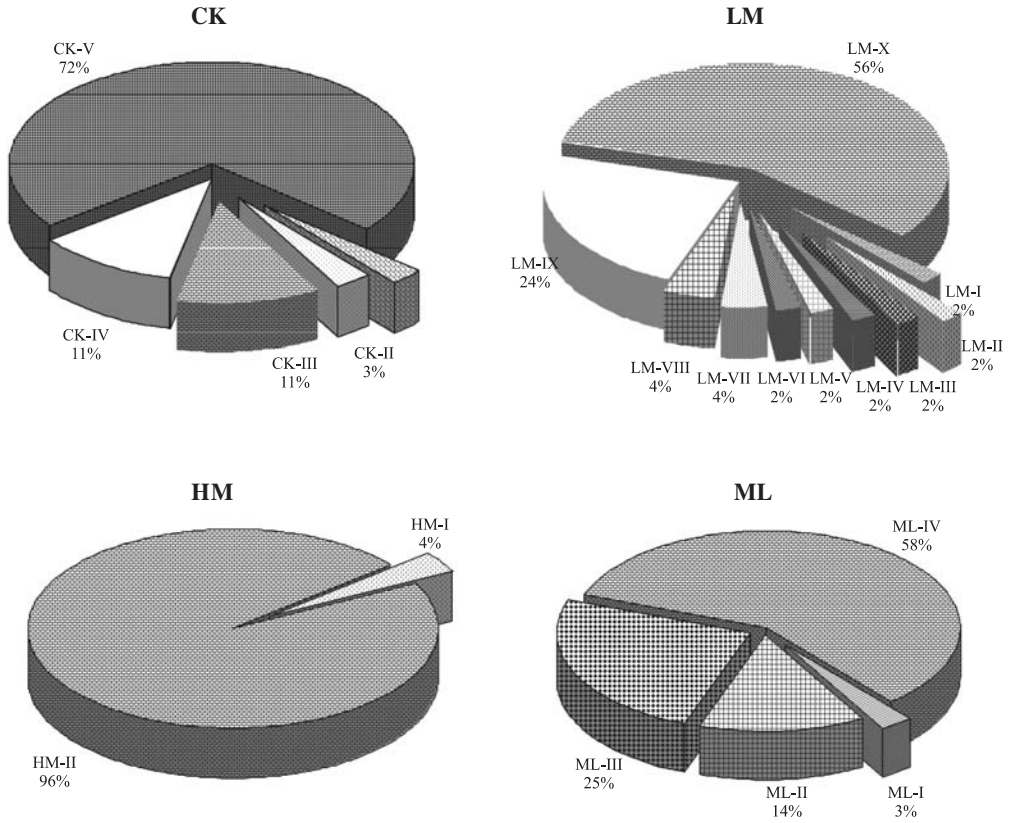

Fig. 1. Frequencies of bacterial phylogenetic lineages detected in *nifH* gene clone libraries from all samples. Calculations were made based on the total number of clones associated with phylotypes from which the representative clones had been sequenced. The same clusters in different libraries are represented by same pattern. Abbreviation: CK, the control; LM, the low manure sample; HM, the high manure sample; ML, the high manure and lime sample.

ration (Table 2), indicating that all clone libraries could represent the four environmental samples, although the number of the clones in each clone library was less than that of previous research (Zhang *et al.*, 2006; Rodrigues Coelho *et al.*, 2008). The proportions of each *nifH* clusters were shown in Fig. 1.

Biological richness index was used to describe the phylotype richness of the four libraries. As shown in Table 2, biological richness indexes indicated that application of organic manure affected the community of diazotrophs. Addition of low-level organic manure to red soil could increase the diversity of diazotrophs and the diazotrophic community in LM became more even than in others. However, under the condition of high organic manure in red soil, the diversity of diazotrophs in HM was changed obviously. The result suggested that the applications of proper amount of organic manure in red soil had increased the richness of diazotrophs in red soil ecosystems.

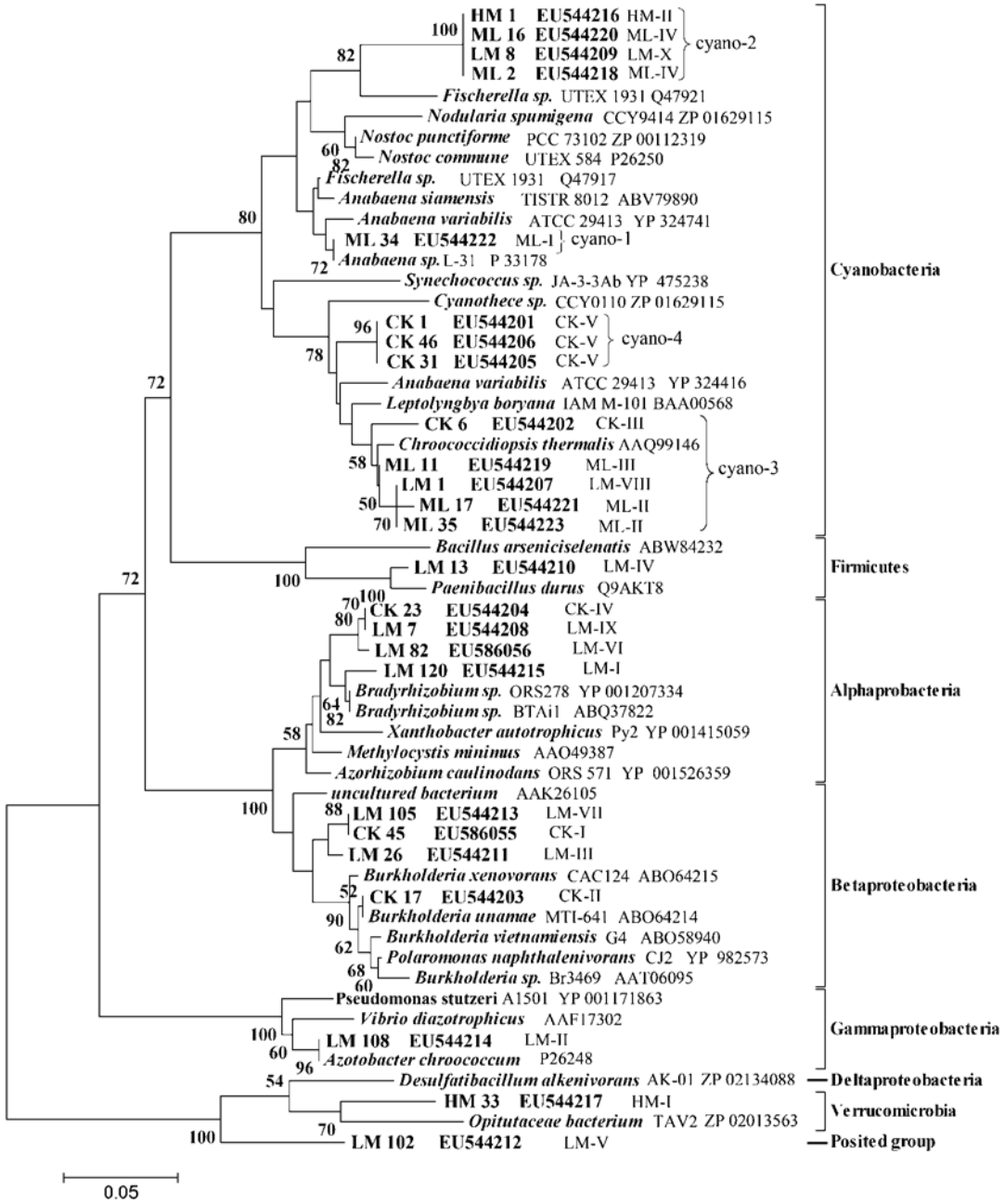

Fig. 2. Unrooted Neighbour-Joining phylogenetic tree based on partial *nifH* sequences (152 amino acids) retrieved from all libraries in this study and GenBank of NCBI. The accession numbers and the group names for each sequence are attached. One thousand bootstrap analyses were conducted, and percentages greater than 50% are indicated at the notes. The scale bar indicates five changes per 100 amino acid positions.

Phylogenetic analysis of the *nifH* sequences

Phylogenic analysis of the *nifH* genes from red soil indicated that there was a rich diversity of diazotrophs in red soil. 38 representative clones of all clusters from the four libraries were sequenced. The similarity of sequenced *nifH* genes ranged from 73% to 100% between each other. From the sequences, a phylogenetic tree was constructed and analyzed (Fig. 2). Phylogenetic analysis of the *nifH* amino acid sequences clustered them into 7 groups, including α, *β*, and *γ* subclasses of the *Proteobacteria*, *Firmicutes*, *Verrucomicrobia*, cyanobacteria and a posited group.

Sequences and phylogenic analysis showed that cyanobacteria were the dominant diazotrophs in red soil. Cyanobacteria-like *nifH* covered 87% of the *nifH* genes cloned from the soils (130/150). The significant dominant cluster CK-V was 96% similar to the *nifH*1 gene of *Anabaena variabilis.* *Fischerella* UTEX1931 related *nifH* clones dominated LM (representative clone LM8, the same below), HM (clone HM1), and ML (clone ML2) libraries with a similarity of 92%. Though cluster CK-III (clone CK6) shared the same RFLP pattern as *Fischerella* UTEX1931 related *nifH* clones, their nucleotides sequences differed significantly and separated them into different subgroups on phylogenic tree.

Bradyrhizobium was also important nitrogen fixer in red soil of low fertility, it only appeared in CK and LM libraries. Appropriate manure application promoted the growth of *Bradyrhizobium*-like diazotrophs, the appearance percentage increased from 11% in CK to 24% in LM. However, *Bradyrhizobium*-like *nifH* gene could not be detected in HM and ML libraries. Appropriate manure application also increased the diversity of other diazotrophs, *nifH* genes belonging to *β-Proteobacteria*, *γ-proteobacteria*, *Firmicutes*, and an unknown type were detected in low manure treatment. *Burkholderia*-like *nifH* genes were also frequently detected in CK and LM libraries and showed high diversity level (5 clones into 4 clusters).

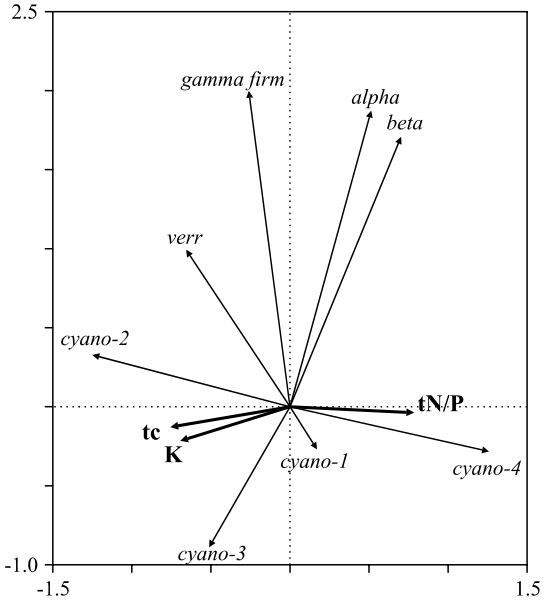

Fig. 3. RDA biplots. Different bacterial groups in relation to the strongest environmental variables. *alpha*, *Alphaproteobacteria*; *beta*, *Betaproteobacteria*; *gamma*, *Gammaproteobacteria*; cyano, Cyanobacteria and it was divided into four subgroups: cyano-1, -2, -3, and -4 (as shown in Fig. 2); *verr, Verrucomicrobia.*

RDA results

RFLP patterns clustered together on phylogenetic tree were designated as a group. Cyanobacteria were divided into 4 subgroups, i.e. cyano1, cyano2, cyano3, and cyano4. The group data set was analyzed by RDA to clarify the influence of the environmental factors of interest on the composition of the bacterial communities. In the RDA model total carbon content (tC), total nitrogen to available P ratio (tN/P), and available K (K) were found to be the environmental variables that statistically best explained the variations in distribution of bacterial groups among all samples ($P<0.05$) (Fig. 3). The RDA model statistically explained 79.1% of the variation of the data. The first and second axis explained 48.8% and 30.3% of the variation, respectively. Cyanobacterial subgroups 2, 3, and Verr were correlated with high tC and K conditions in the RDA, whereas subgroups 1, 4, α and β groups of diazotrophs were correlated with high tN/P condition.

Discussion

Biological nitrogen fixation plays important role in the supplying of nitrogen to the ecosystems. Numerous researches had been carried out to investigate the structure and function of diazotrophs in terrestrial and aquatic ecosystems (Zehr *et al.*, 2003; Wood *et al.*, 2008). Diazotrophs represent a physiologically and phylogenetically highly diverse functional group including *Proteobacteria*, green sulfur bacteria, cyanobacteria, *Firmicutes*, *Spirochaetes*, and *Archaea*. Results based on *nifH* as the molecular marker showed that heterotrophic nitrogen fixation microorganisms are the most commonly found diazotrophs in terrestrial ecosystems they include *α*, *β*, *γ*, *δ-Proteobacteria*, *Firmicutes*, and *Archaes* (Zehr *et al*., 2003).

However, our research showed that cyanobacteria were the dominant diazotrophs in red soil of south China. 22 clones of the 38 sequenced *nifH* genes belonged to the class of cyanobacteria (Fig. 2). Cyanobacteria have been reported as dominant diazotrophs in aquatic environment (Zehr *et al*., 2003). They are also main nitrogen fixers in biological soil crust in arid area (Belnap, 2002; Warren-Rhodes *et al.*, 2007; Yeager *et al.*, 2007) and arctic soils (Eckford *et al.*, 2002). Cyanobacteria were detected in rice field of Uruguay by culture-dependent method (Irisarri *et al.*, 2001) and south China by 16S rDNA PCR-DGGE (Song *et al.*, 2005). But they were not detected by PCR amplification of *nifH* genes in slit loam soil of south China (Wartiainen *et al.*, 2008) which shared similar climate condition with our research site except the soil type. The soil water content was rather low at sampling time (data not shown), though the average annual rainfall in the experimental station is quite high (1795 mm per year). The ability to endure repeated desiccation and hydration (Cavacini, 2001) might help cyanobacteria to survive and dominate in red soil. This phenomenon was also observed in the Dry Valleys of Eastern Antarctica,

Table 3. Similarity matrix for phylotype composition for clone libraries

Library	Similarity[a]			
	CK	LM	HM	ML
CK		53.26%	49.60%	41.32%
LM			58.64%	56.72%
HM				60.00%

[a] Pairwise similarity values were calculated as follows: [Sorensen index : 2c/(a+b)×100%], a, b was the number of clones only occurring in library a and b, c was the number of clones occurring in both library (Goodall, 1973).

where the liquid water content of soil increased because the global warming promotes the survival of aquatic cyanobacteria in dry soil (Wood *et al*., 2008). Although we did not measure the nitrogenase activity in red soil, field investigation with dark chamber indicated that cyanobacteria played important roles in biological nitrogen fixation in Spanish rice field soils (Quesada *et al.*, 1997).

Many environmental parameters affect the activity and diversity of soil bacteria. In red soil, the lack of nitrogen and phosphorus was the most common condition, which limited the development and yield of crops. This lack of nutrition also affected the diazotrophic diversity in red soil. The Shannon-Weiner index of control was significantly lower than that of low manure treatment (LM). Application of organic manure to the red soil efficiently improved the fertility of red soil (Table 1), which led to obvious diazotrophic community succession in this research site. Diazotrophs community in CK had a low similarity (<50%) with HM and ML treatments, while those of LM, HM, and ML treatment had a higher similarity of nearly 60% (Table 3). Increase of nutrients such as N, P, and K might stimulate the growth of diazotrophs. High nitrogen content in soil inhibited the nitrogen fixation rate and phosphorus fertilization stimulated the nitrogen fixation activity (Liengen, 1999). However, some research showed that N to P ratio was a key environmental factor controlling the biological nitrogen fixation in soil (Smith, 1992). Under high manure treatment, diversity of diazotrophs decreased to very low level (H'=0.22). Cyanobacteria were detected as significant dominant group under such condition.

The soil surface in the flooded paddy field is characterized by the presence of algae and cyanobacteria. A number of researches on the function of cyanobacteria on the fertility of paddy soils had been carried out (Valiente *et al.*, 2000; Chunleuchanon *et al.*, 2003; Pandey *et al.*, 2005; Jha and Prasad, 2006; Irisarri *et al.*, 2007; Prasanna and Nayak, 2007). Cyanobacteria play important roles in supplying of nitrogen in rice production. The estimated amount of the nitrogen fixed in places where no cyanobacterial presence was visually apparent ranged from 0.23 to 75.5 kg N ha^{-1} $year^{-1}$ in a Spanish rice field, when cyanobacterial blooms were present, it reached 2 kg N ha^{-1} day^{-1} (Quesada *et al*., 1997). In this research, the red soil was managed under unflooded format. Results showed that cyanobacteria were significantly dominant diazotrophs in this research site. The ecological meaning of the appearance of cyanobacteria and their contribution to soil nitrogen pool deserve profound research.

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