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Diversity of *nifH* Gene Reveal the Modification of the Red Paddy Soil

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Red paddy soil is one of the most important typical soils in subtropical regions of China. Appropriate fertilizer application is an important management practice to improve soil fertility and quality. Here, four paddy soil samples under long-term fertilization practices, the first was control without any treatment (CK), the second treated with only urea (N), the third treated with urea and recycled with all the rice straw (NC) and the last treated with not only urea, but also calcium magnesium phosphate and potassium chloride (NPK), were collected. Total C and N, available N, P and K and soil pH were measured. 28, 31, 27, 22 unique profiles screened by RFLP were identified from *nifH*-CK, *nifH*-N, *nifH*-NC, *nifH*-NPK libraries, respectively. The results showed that there was highest microbial population genetic diversity of N sample while NC sample and NPK sample had little change comparing with CK sample.

Key words: Diversity, *nifH*, Nitrogen-fixing bacteria, Fertilizer, Red paddy soil.

Red soil, which is considered to be the most important soil resource for the food security of China, occupies approximately 2.04 million square kilometers in tropical and subtropical region of the country land surface (Zhang, 2005). In these red soil regions, rice (*Oryza sativa* L.) is the main cereal crop, contributing 19% and 29% of the world rice area and production, respectively (Sun *et al*. 2011).

In recent years, due to the rapid population growth and a continuous decline in the amount of cultivated land area, the rate of fertilizer application keeps on rising in these regions in order to obtain high crop production in agriculture (Wang *et al*. 2010). The long-term inappropriate fertilization has caused severe degradation of the red paddy soil (Chen *et al*. 2009). Therefore, how to solve the degradation of paddy soil and maintain their sustainable development of agricultural production is an urgent problem. Many researchers suggested that application of appropriate fertilizer to soil was an effective way to modify the degraded red soil (Ladha *et al*. 2003). Biological nitrogen fixation (BNF), which could fix atmospheric nitrogen directly by nitrogen-fixing bacteria, was one of the major nitrogen inputs into the biosphere, and a high effective, low cost, low energy, no contamination process.

The nitrogenase iron protein (*nifH*) gene,

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which encoding an iron protein subunit of nitrogenase, plays an important role in regulating the molecular nitrogen (N_2) fixation process and has also been extensively used to study the diversity and function of nitrogen-fixing microorganisms (Young 1996, Zehr *et al*. 2003, Raymond *et al*. 2004, and Teng *et al*. 2009). However, our knowledge of their diversity in red paddy soil remains incomplete.

In this study, we investigated the diversity of *nifH* gene containing diazotrophs community, and effects of different fertilizer application on their community structure in red paddy soil in a long-term fertilizer experiment.

MATERIALS AND METHODS

Study sites and soil sampling

The field experiment was carried out at Yingtan Red Soil Ecological Experimental Station of Chinese Academy of Sciences, Jiangxi, China (28º152 N, 116º552 E). The local climates is of the Subtropics monsoon type, with a mean annual temperature above 17.6 °C and mean annual rainfall 1785 mm (Wu *et al*. 2008).

The soil was derived from Quaternary red clay, and was planted with rice lasted for 18 years. The experimental design consisted of nine treatments with three replications. Each plot covered an area of 30 square meters and separated with brick and cement, with good Drainage system.

Four soil samples were collected in November 2008 from the nine treatments. The first soil sample was treated with only urea (N); the second was treated with urea too, but recycled with all the rice straw (NC); the third was treated with not only urea, but also calcium magnesium phosphate and potassium chloride (NPK); the last was control without any treatment (CK). On each occasion, subsamples (approximately 50 g) were collected from the plough layer (0 ~ 15 cm) by using sterile soil probe from five points. Then, the five subsamples were mixed well and stored cool (4 °C) until processed for chemical measurements and DNA extraction.

Soil chemical characteristics

Soil organic matter, total C and N, Available N, P and K and soil pH were measured by State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, according to the methods described by Lu (Lu 2000). 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 SANplus 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. Soil pH was determined with a glass electrode.

DNA extraction and PCR amplification of *nifH* gene

DNA was extracted separately from the 4 soil samples according to the method of Esther (Esther *et al*. 2003). Extracted DNA were visualized on 0.75% (w/v) agarose gels to assess their integrity and purified by AxyPrpp^{TW} DNA Gel Extraction kit. Purified DNA was diluted 50-folds, which can reduce the influence of the PCR inhibitors such as humic acids, and used for PCR targeting *nifH*. To amplify *nifH* gene, primers *nifH*-1F: 5'-AAAGGYGGWATCGGYAARTCCACCA-3' and *nif*H-2R: 5'-TTGTTSGCSGCRTACATS GCCATCAT-3' were used (Rosch *et al*. 2002). Amplification conditions and program were as described by Zhang (Zhang *et al*. 2005)Singh et al., 2006 B.K. Singh, L. Nazaries, S. Munro, I.C. Anderson and C.D. Campbell, Use of multiplex terminal restriction fragment length polymorphism for rapid and simultaneous analysis of different components of the soil microbial community, *Applied and Environmental Microbiology* 72 (2006), pp. 7278–7285. Full Text via CrossRef | View Record in Scopus | Cited By in Scopus (20) eo. Each 50µl reaction containing 2.5µl 10×Buffer, 2.5µl Mg^{2+} (25mmol/L), 2.5µl dNTP (2.5 mmol/L each), 1.0µl each primer (10pmol/µl), 0.3µl Tag DNA polymerase (5U/µl), and 1 µl template DNA. DNA amplification was performed by PCR with the following parameters: an initial cycle of 5 min at 95°C; 10 cycles of 40s at 95°C, 30s at 55°C and declined by 0.5°C per cycle and 1min at 72°C; then 30 cycles of 40s at 95 °C, 30s at 50°C and 1min at 72°C; and a final cycle of 5 min at 72°C. Amplification products were analyzed on 2% (w/v) agarose gel stained with ethidium bromide, and photographed with a UVP system, then purified with a AxyPrpp^{TW} DNA Gel Extraction kit and stored

at -20°C.

Construction of clonal libraries and RFLP analysis

The purified *nifH* gene fragments were subsequently cloned to pMD18-T Vector (TaKaRa, Dalian, China), and the ligation products were used to transform into competent *E.coli* DH5± cells. Transformants were then plated on LB-agar containing 100 µg/ml ampicillin and incubated 12 ~ 16 hours at 37 °C. Colonies of transformed cells were picked at random and then four clonal libraries of the four soil sample were constructed, separately named *nifH*-N library, *nifH*-NClibrary, *nifH*-NPK library, *nifH*-CK library.

Colonies of transformed cells were suspended in PCR reaction mixtures with primers M13-47 and RV-M. PCR products of *nifH* fragments were digested for two hours at 37 °C using the tandem tetrameric restriction endonucleases *Hha*I and *Rsa*I, separately. The restriction products were run for 2.5 h 300 V on a 8% (w/v) polyacrylamide gels and stained with $AgNO_3$. Restriction fragment length polymorphism (RFLP) patterns were compared, and OTUs (Operational Taxonomic Unit) were defined (Hughes *et al.* 2002), by using Tannon Digital Image analysis software.

Sequencing and phylogenetic analysis

Representative clones from RFLP patterns were chosen for sequencing. The *nifH* sequences obtained were identified by BLAST algorithm using the NCBI GenBank database.

RESULTS

Soil analysis

The properties of soil samples are given in Table 1. So far, there was no obvious difference on soil pH with different fertilization treatments. The treatment applied N fertilizer and straw had the highest content of organic matter, NPK treatment followed, while the N treatment had no obvious difference with control. The trends of total carbon content in different treatments were same with organic matter. The total nitrogen content of N treatment was not significant different with the control, however, NC treatment and NPK treatment were significantly higher than the control. The NPK treatment had the highest content of available phosphorus, while the other treatments were not significantly different with control. The control had the highest content of available K, NC treatment was followed, then N and NPK, and this

Table 1. Chemical properties of the red soils

Treatment	pH(1 mol L^{-1}KCl)	OM	Total C	Total N	Total C/ Total N	Available N	Available P	Aavailable K
		($g.kg^{-1}$)				($mg.kg^{-1}$)		
CK	4.86 b	13.1 c	7.38 b	0.78b	9.46b	63.4c	0.57a	13.52f
N	4.95 bc	13.9 bc	8.22 b	0.75b	10.96b	65.2bc	1.04ab	9.45cd
NC	5.02 bc	16.9 a	9.74 c	1.11c	8.77ab	88.2abc	1.67ab	11.53e
NPK	5.15 c	15.2 abc	8.83 bc	1.12c	7.88a	91.9ab	6.64d	8.42c

Note: Different letters in the same columnmean significant difference at $p < 0.05$. Values affixed with the same letter are not significantly different between treatments

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

Library	Clone	OTUs	C	H'	D	d	E
nifH-CK	70	28	80%	2.989	0.935	6.355	0.897
NifH-N	58	31	65.5%	3.105	0.945	7.388	0.904
NifH-NC	64	27	75%	2.838	0.904	6.252	0.861
NifH-NPK	48	22	72.9%	2.673	0.900	5.425	0.865

Note: C was Coverage, H2 was Shannon index, D was Margalef index, E was Shannon evenness, and d was Simpson2 s index.

phenomenon may be related to the uptake of rice.

Diversity of *nifH*

A total of 70 *nifH* clones from *nifH*-CK library were screened by RFLP and 28 unique profiles were identified; 58 *nifH* clones from *nifH*-N library were screened by RFLP and 31 unique profiles were identified; 64 *nifH* clones from *nifH*-NC library were screened by RFLP and 27 unique profiles were identified; while 48 *nifH* clones from *nifH*-NPK library were screened by RFLP and 22 unique profiles were identified(Table 2). A phenotype rarefaction curve was made to check the sufficiency of the libraries (Figure 1), Figure 1 showed that all the four libraries' phenotypes rarefaction curve tend to plateau, indicating that the four clone libraries could represent environmental samples. Otherwise, the Coverage C value also supports such a result, the lowest Coverage C of the four libraries have reached to 65.5%. Generally, we used diversity index to describe the diversity of microbial community. Shannon-Wienner index, Simpson index, species richness and evenness of N sample were 2.989, 0.935, 6.355 and 0.897, separately (Good 1953, Hill *et al* 2003). All the indices have provided the evidence for highest microbial population genetic diversity of N sample, while NC sample and NPK sample had little change comparing with CK sample.

Figure 2 showed there were one dominant micro-flora, accounting for 14%, and two sub-dominant micro-floras in CK sample; two dominant micro-floras in N sample, accounting for 12%, separately; one dominant micro-flora, accounting for 25%, and one sub-dominant micro-flora in NC sample; while one dominant micro-flora, accounting for 25%, and two sub-dominant micro-floras in NPK sample. The proportion of RFLP pattern which contained one clone and two clones of the four soil samples were 34%, 56%, 43%, 49%, respectively; moreover, the proportion of RFLP pattern which contained three and three or more clones were 66%, 44%, 57%, 51%, respectively. There was same dominant micro-flora in the four soil samples by comparing the RFLP pattern. The dominant micro-flora of CK sample were same with N sample, while it was sub-dominant micro-flora of NPK sample; similarly, NC sample, NPK sample and N sample have same dominant micro-flora, and this dominant micro-flora was the sub-dominant micro-flora of CK sample.

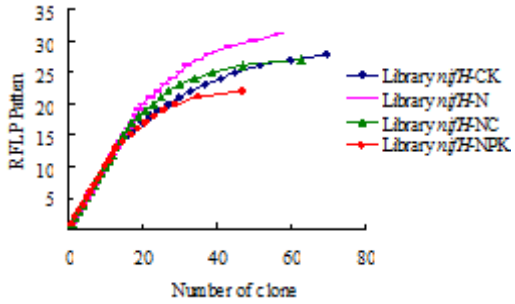

Fig. 1. The phytotypes rarefaction curve of four *nifH* gene clone libraries

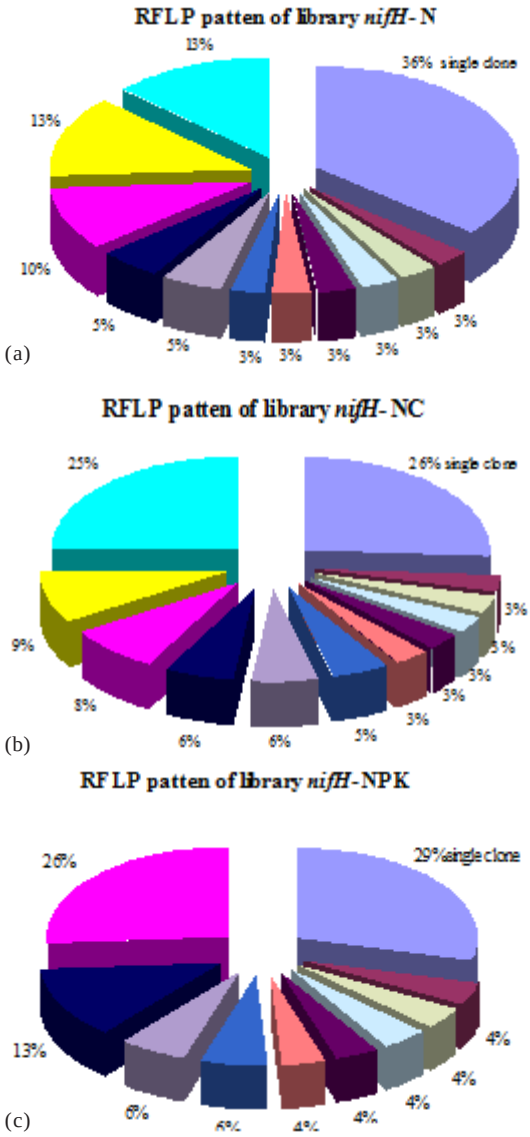

Fig. 2. Proportion of each clone in the four libraries. A, B, C, for, N, NC, NPK

Phylogenetic analysis

A total of 22 nucleotide sequences of *nifH* clones were sequenced, obtained nucleotide sequences had the identity of 80.56% with the estimations of DNAMAN software. All sequences obtained in this study have been submitted to the

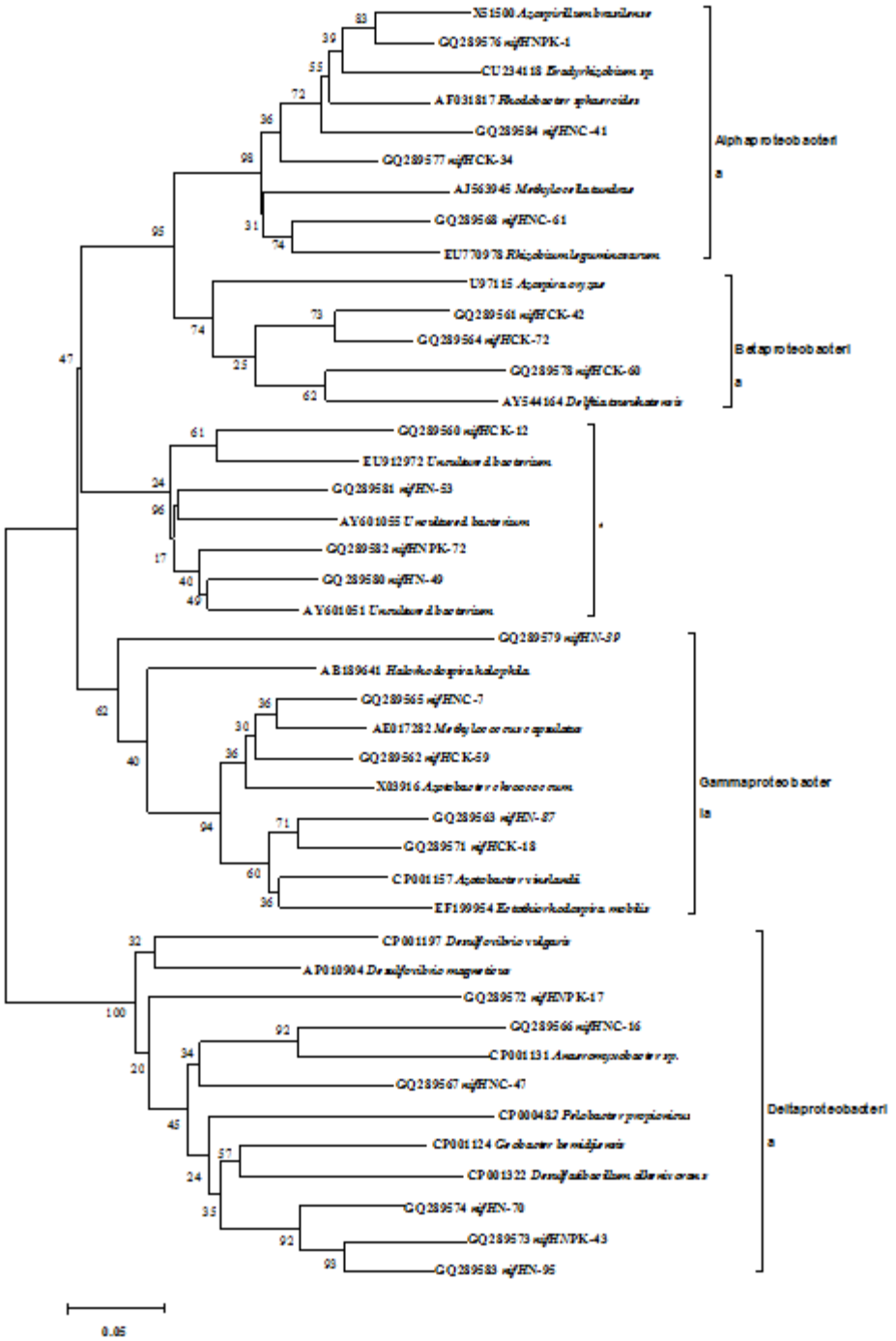

Fig. 3. Unrooted Neighbour-Joining phylogenetic tree based on partial *nifH* sequences retrieved from all libraries in this study and GenBank of NCBI

GenBank database under accession numbers GQ289560 to GQ289584. Sequence data were compared to public database entries using the BLAST search program of the National Center for Biotechnology Information (NCBI) website. A phylogenetic tree of *nifH* was constructed by 1000-fold bootstrap analysis using the neighbor-joining method with the ClustalW program and Mega software (Figure 3).

Phylogenetic analysis placed the *nifH* genes cloned in the present study into five clusters, the majority of the sequences were closely related to *proteobacteria*, especially, almost all the dominant micro-floras of the four samples grouped in *³-proteobacteria* and *´-proteobacteria*. A number of clones were affiliated with known diazotrophs, *nifH* NC-47 representing the dominant micro-floras of the four samples had similarity of 86%082 with *Desulfovibrio magneticus*0 *Desulfovibrio vulgaris*, separately; the other dominant micro-flora *nifH* N-87 also had high similarity with *Azotobacter vinelandii* of *³-Proteobacteria*; *nifH* N-70 stand in *´-Proteobacteria* cluster had the similarity of 88% with *Geobacter bemidjiensis*; *Methylococcus capsulatus* which belonging to *³-Proteobacteria* had high similarity of 90%088% with *nifH* NC-70*nifH* CK-59, separately; *nifH* CK-72 was also affiliated with *Azospira oryzae*, with the similarity of 87%.

Some sequences from the phylogenetic tree were only distantly related to the sequences of cultivated diazotrophs, reversely, they were closely related to *nifH* genes of uncultured bacteria. These sequences contained 29 clones, having a proportion of 12% of the total clones, and almost of these clones were the same clones in the four samples. The date indicated that novel lineages of *nifH* genes were detected in the course of this study.

DISCUSSION

Our primary objective was to examine the restoration of degraded red soil in the perspective of diversity of diazotrophs community. The second objective was to suggest a series of advice to control the quality of red soil.

Our results were different from Qihui Teng's, which had more cyanobacteria of nitrogen-fixing bacteria, and lower diversity of *nifH* (Teng *et al* 2008). In our study, we had more diazotrophs in *Proteobacteria*, which was consistent with Zhang (Zhang *et al* 2005). *Desulfovibrio.sp* and *Azotobacter.sp* were the primary diazotrophs in the four soil samples. Crops in the soil which was abundant in *Azotobacter.sp* had good state. Xi *et al* found out that the oats would have increased shoot length, higher chlorophyll content, higher aboveground dry weight and belowground dry weight, and higher crude protein (2007). Tao found that the effect of two bio-products on the growth and yield had showed that the *Azotobacter.sp* treatments had positive effect of promoting growth, increasing yield, and fertilizing soil (Tao *et al* 2005). The moisture content of paddy soil was the most important factor that affected the population and activity of *desulfovibrio.sp*, which was microbe and like the environment of high moisture content and poor ventilation in reddish paddy soil (Chen *et al* 2004).

There was close relationship in soil microbial community and quality, and fertilization was the most effective way to affect the soil quality. We had undertaken a great deal of research on the effect of long-term fertilization on soil microbial community, but still have no unified conclusion (Huo *et al* 2008). From the results of RFLP analysis and sequencing, we found that the main bands and dominant micro-floras of the four soil samples were same, which meant that the dominant microorganisms in the soil sample was relatively stable and free from the impact of fertilizer. Compared with sample CK, the proportion of dominant bacteria in the fertilizer treated soil all had increased. Fertilizer could promote the dominant bacteria in soil. After analysis of the diversity index, we found the N fertilizer treated soil had the highest diversity of diazotrophs, NPK treatment had the lowest diversity of diazotrophs, while the NC sample had little difference with sample CK. Petra Marschner in the study of structure and function of the soil microbial community in a long-term fertilizer experiment pointed out, C/N ratio is the most critical factors affecting soil microbial (Petra *et al* 2003). In our study, the C/N ratio of four soil samples and the rich index of *nifH* gene had a high degree of correlation, with the correlation coefficient of 0.984. Therefore, we could not analyze the effect of fertilizers on soil microbial community in one-sided

perspective. The advisable choice was to evaluate the impact of fertilizers on soil quality from soil chemical properties and biological properties, considering the own fertility of red soil.

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