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Full Length Research Paper

Effects of long-term fertilization on *nifH* gene diversity in agricultural black soil

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Black soil is a precious resource: it is an important grain-base commodity and a highly fertile soil. The long-term use of chemical and organic fertilisers has affected the microbial community and agricultural sustainability of black soil. We used nitrogenase reductase (*nifH)* as a biomarker of soil-borne nitrogen-fixing microbes to study the effects of long-term fertilisation on a field. Fertilisation started in 1979 and consists of the following treatments; organic, chemical, chemical and organic fertiliser combination, and no fertiliser (control treatment). The *nifH* gene pools from each fertilisation treatment were amplified and constructed into four clone libraries. Restriction fragment length polymorphisms (RFLP) analysis was then performed identifying 87 *nifH* gene types amongst the nitrogen-fixing microbial community. This analysis showed that the chemical and organic fertiliser combination resulted in a notable increase in *nifH* phylotypes and simultaneously intensified the differences among the unique phylotype quantities. The phylogenetic distribution of the clones based on *nif*H sequences from the four treatments was similar and sequences aligned with various common soils taxonomic groups, including *Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Actinobacteria, Firmicutes* and *Cyanobacteria*. The impact of mixed fertilisers on the *nifH* genotypes was complex and resulted in the selection of unique microbial flora. This study also demonstrated that the addition of organic fertiliser created better environmental conditions for the composition of the *nifH* pool. Thus, the application of organic fertilisers can greatly increase the types of nitrogen-fixing microorganisms present and is conducive to the development of a rich variety and evolution of nitrogen fixation genes.

Key words: *nifH,* long-term fertiliser, diversity.

INTRODUCTION

Black soil is a precious resource: it is an important grain-base commodity and a highly fertile soil. Therefore, using and protecting this precious soil resource is very important. Nitrogen is one of the limiting factors for crop yield and affects ecosystems and microorganism communities at both small and large scales; thus, to increase the yield, chemically synthesised nitrogen fertiliser has been widely used. However, commercial chemical fertilisers are an expensive means of supplementing the soil for growing plants. The improper or excessive use of fertilisers can also lead to nitrate pollution of groundwater. However, biological nitrogen fixation can effectively contribute to nitrogen uptake, and the identification of the major taxonomic groups of soil microorganisms is of great relevance for aiding in the

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nitrogenous fertilisation of different economically important plants.

There is no general agreement on the effects of 'long-term' mineral or organic nitrogen fertiliser use on the diazotrophic diversity in black soil. Biological nitrogen fixation is carried out by prokaryotic organisms possessing a nitrogenase enzyme that is encoded by *nif, anf* or *vnf* genes (Joerger et al., 1991; Waugh et al., 1995). The *nifH* gene encodes the iron-protein subunit of nitrogenase, and a good correlation between the *nifH* gene and 16S rRNA gene phylogeny has been found for the purposes of species identification (Young, 1992; Zehr et al, 2003). The *nifH* gene is one of the most thoroughly studied genes in the *nif* operon, and an extensive collection of sequences obtained from both cultured and uncultured microorganisms isolated from multiple environments (Deslippe and Egger, 2006; Hamelin et al, 2002; Tan et al., 2003; Zeh, 2003) is available. Using the *nifH* gene as a molecular marker in natural environments provides evidence for potential nitrogen fixation (Widmer et al., 1999). Moreover, cultivation-independent studies using clone library analyses (Ueda et al., 1995), denaturing gradient gel electrophoresis (Piceno and Lovell, 2000; Teixeira et al, 2008) and terminal or complete restriction fragment length polymorphism (Poly et al, 2001a; Mnasri et al, 2009) methods have demonstrated the great diversity of *nifH* genes in natural environments. Within agricultural soils, fertiliser applied over a period of 15 years has been shown to select for qualitatively different assemblages of bacteria and have indicated that the composition of diazotrophs is strongly influenced by the inorganic nitrogen content across a range of soil types (Poly et al., 2001b). Within the rhizosphere, however, additions of N do not significantly affect the diazotroph community structure (Tan et al, 2003; Knauth et al, 2005). It has also been reported that the addition of fertilisers only influences the bacterial populations to a minor extent, whereas the physicochemical features of the soil (e.g., the parent material, pH and prevailing management practices) are the dominating influences (Girvan et al, 2003; Marschner et al, 2003; Steenworth et al, 2003; Ulrich and Becker, 2006). In this article, we report on the biodiversity of the *nifH* gene which is involved in soil N cycling and is required for biological N_2 fixation.

A long-term fertiliser field experiment was set up in 1979 to investigate the effects of different fertiliser regimes on crop yields (Zhou and Zhang, 2005), soil phosphorus (Zhou et al., 2004), soil acidity (Zhang et al., 2008) and the transformation of bacterium soil physiology (Wang et al., 2008). Previous studies have shown that field plots that received regular applications of farmyard manure, chemical fertiliser or both (with various combinations) under long-term cultivation practices resulted in distinctly different organic carbon and nitrogen equilibria in the soil (Wang et al., 2003). In this study, we have utilised this experiment to investigate the impact of long-term fertiliser treatments on *nif*H microbial populations in agricultural black soil.

MATERIALS AND METHODS

Soil sampling and processing

A long-term fertiliser field experiment site and scientific observation field (45°40′ N/126° 35′ W, 151 m elevation, black soil, smooth topography and a temperate zone continental monsoon climate) was built in 1979 at Harbin farm, Heilongjiang Province, China under a wheat–soybean–corn crop rotation. The total experiment area was 8060 m^2; the plot area was 168 m^2 with 8 ridges in each plot area. The ridges were 30 m long and 70 cm apart. We obtained samples for this study in 2008, which was the 29th growing season. Soils from four different fertiliser treatments were sampled: plot "M" included approximately 1240 kg $acre^{-1}$ horse manure added as the organic fertiliser for the corn crop at each rotation cycle time; plot "NPK" received NH_4NO_3 10 kg $acre^{-1}$, P_2O_5 5 kg $acre^{-1}$ and K_2O 5 kg $acre^{-1}$ during the wheat and corn seasons and NH_4NO_3 5 kg $acre^{-1}$, P_2O_5 10 kg $acre^{-1}$ and K_2O 5 kg $acre^{-1}$ during the soybean season; plot "MNPK" received the same amount of manure as the "M" plots received plus the same amount of chemical fertiliser as the "NPK" plots received. Neither organic nor chemical fertiliser was applied in the control plot, "CK". The four treatments, with three replicates, were arranged in a randomised block design. On May 1, 2008 soil samples were collected from 16 points at a depth of 0 to 20 cm. The soil samples were collected, mixed and sieved (<2 mm) to remove plant materials, roots and stones and were stored at -70°C until further study. The soil samples from the 3 replicate plots of each treatment were pooled for subsequent molecular and physico-chemical analyses.

Soil DNA extraction and amplification of *nifH* genes

Total soil DNA was directly extracted from soil samples using the Soil Master™ DNA Extraction Kit (EPICENTRE, U.S.A.) and subsequently purified with the Soil DNA Purification Kit (GENMED SCIENTIFICS INC. U.S.A). The DNA quality and integrity was checked by electrophoresis on a 0.8% agarose gel. The *nifH* gene fragments were amplified by PCR with the primer pair, nifH-F and nifH-R (Röch et al., 2002). The 20 µl PCR mixtures included a 0.2 mM final concentration of each dNTP, 1×PCR buffer, 100 ng of the template DNA, 2.0 mM $MgCl_2$, 0.5 µM of each primer, 2U of Taq DNA polymerase (TAKARA, Japan) and 1% dimethyl sulphoxide (DMSO). The amplification was performed as follows: denaturation for 5 min at 95°C, 30 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C; the final elongation was for 5 min at 72°C. The PCR products were analysed by electrophoresis on a 2.0% agarose gel with ethidium bromide (EtBr) and were visualised with UV light to ascertain their size and quality.

nifH gene clone libraries and restriction fragment length polymorphism (RFLP) analysis

The *nifH* gene pools from the four soil samples were cloned into the pMD18-T vector (TAKARA BIO. GROUP) and transformed into *Escherichia coli* DH5α competent cells following the manufacturer's instructions. A total of 250 clones were picked, and the presence of inserts of the correct size was verified by PCR using M13f (5'-GTAAAACGACGGCCAG-3') and M13r (5'-CAGGAAACAGCTATGAC-3') primers under the following conditions: 25 cycles of 94°C for 30 s, 46°C for 30 s, and 72°C for 50 s.

A 2 µl sample of each amplification product was digested

Table 1. Chemical properties of the black soils.

Treatment	pH	Organic matter (%)	Total N (g/kg)	Total P (g/kg)	Available P (mg/kg)	Available K (mg/kg)
CK	6.82(0.10)[a]	2.66(0.06)[a]	1.23(0.06)[a]	0.7(0.10)[a]	13.3(0.05)[a]	133(1.15)[a]
M	6.87(0.17)[a]	2.96(0.13)[b]	1.34(0.04)[b]	0.81(0.03)[a]	24.2(3.19)[b]	150(12.53)[a]
NPK	6.07(0.23)[b]	3.09(0.42)[ab]	1.33(0.01)[b]	1.11(0.19)[ab]	83.1(11.00)[c]	178(6.03)[b]
MNPK	6.20(0.12)[b]	3.03(0.10)[b]	1.42(0.07)[b]	1.23(0.05)[b]	89.7(34.92)[c]	213(9.61)[c]

Values within the same column not followed by the same letter differ significantly (N=3, $P<0.05$). Methods for the determination of soil characteristics were described by Bear (1955). Values in parentheses are standard errors of the estimates.

overnight with *Hae I* and *Rsa* I for restriction fragment length polymorphism (RFLP) analysis. This procedure was repeated at least two times for each sample to verify the consistency of the patterns. The DNA fragment sizes were estimated by comparing them to molecular weight standards (500-bp DNA ladder, Promega), and clones were grouped into phylotypes according to their banding patterns. Representative *nifH* fragments for each RFLP pattern were sequenced by SHANGHAI SANGON.

Data analysis

The phylotypes shared between the samples were analysed. The sequences were aligned using Clustal X (Thompson et al., 1997) and imported into DNADIST in PHYLIP version 3.6 (Felsenstein, 2004) to generate distance matrices using the Juke-Cantor correction for multiple substitutions. OTUs were assigned by DOTUR (Schloss and Handelsman., 2005). To estimate species richness, the nonparametric Chao1 estimate was calculated with log-linear transformed confidence intervals at 95%. Coverage (C) was used as a measurement of captured diversity (Chelius et al., 2001), and the number of unique phylotypes (phylotype richness, S) were used for the creation of rarefaction curves (Simberloff 1978).

The Simpson's index = $1-\Sigma\left(\frac{n_i}{n}\right)^2$, was used to measure the 'evenness' of the community from 0 to 1.

Where ni is the number of individuals of taxon *i*.

The Shannon index = $H=-\Sigma\frac{n_i}{n}\ln\left(\frac{n_i}{n}\right)$, was also calculated to account for the number of individuals as well as number of taxa.

This varies from 0, for communities with only a single taxon, to high values, for communities with many taxa, each with few individuals.

Evenness, = e^H / S

Where S is number of taxa. In Menhinick's richness index, the ratio of the number of taxa to the square root of sample size.

The significance of the between-plot differences in the community profile diversity was calculated using a one-way analysis of similarities (ANOSIM) test by the distance measure of Bray-Curtis. ANOSIM R values of 1 indicated that replicates within the plots were more similar to each other than to any samples from the other plots, whereas an R-value of 0 indicated that there was as much variation within the plots as between the plots. In deciding whether the plots were different, both the R-value and significance level were considered. All statistical analyses were carried out using PAST statistical software (http://folk.uio.no/ohammer/past).

The *nif*H sequences, as determined by highest sequence similarity during BLAST analysis, were subsequently aligned using Clustal X version 1.81 alongside cultured and environmental nitrogen-fixing bacteria. Phylogenetic trees were calculated by the Poisson correction model and the neighbour-joining algorithm using 1,000 bootstraps to assign confidence levels by MEGA 4.0 (http://www.megasoftware.net) to draw the phyloenetic tree. The sequences generated in this study were deposited in NCBI GenBank under accession numbers GU111776-GU111862.

RESULTS AND DISCUSSION

The influence of fertilisation on soils characteristics

The properties of the soil samples listed in Table 1 showed that the long-term chemical fertiliser application (NPK) and application of chemical fertiliser with the organic manure (MNPK) reduced soil pH. However, the field under the application of manure (M) was of the same pH as the non-fertilised control field (CK). The organic and/or chemical fertiliser treatments notably influenced the organic matter, total N, P and K when compared to the non-fertilised soil. The soil organic matter was significantly increased after the application of organic manure (with or without the addition of chemical fertilisers), whereas there was only a slight effect when the chemical fertiliser was applied alone. Compared to the control the total N and available P and K increased significantly in response to all fertiliser additions. Similar results have been reported by Rasmussen et al. (1998), Wang et al. (2003), Sun (2004) and Bai et al. (2008).

nifH clone libraries

Four *nifH* gene libraries were constructed from the CK-, M-, NPK- and MNPK-managed soil samples. Rarefaction curves (Figure 1) and the percent library coverage (Table 2) indicated that the *nif*H genes identified from the community DNA extracted from the soil samples were adequately sampled to cover their diversity. The *Hae*III and *Rsa*I RFLP analysis revealed 87 identical phylotypes. Among those, 15% were shared among the fourlibraries. The characteristic phylotype of the M treatment accounted for 16.1%, MNPK for 8.0%, CK for 8.0% and NPK for 4.6% of the total phylotypes. The M soil contained the highest percentage of characteristic phylotypes, which suggests that the organic fertiliser increased diversity. In contrast, the chemical fertilisers decreased

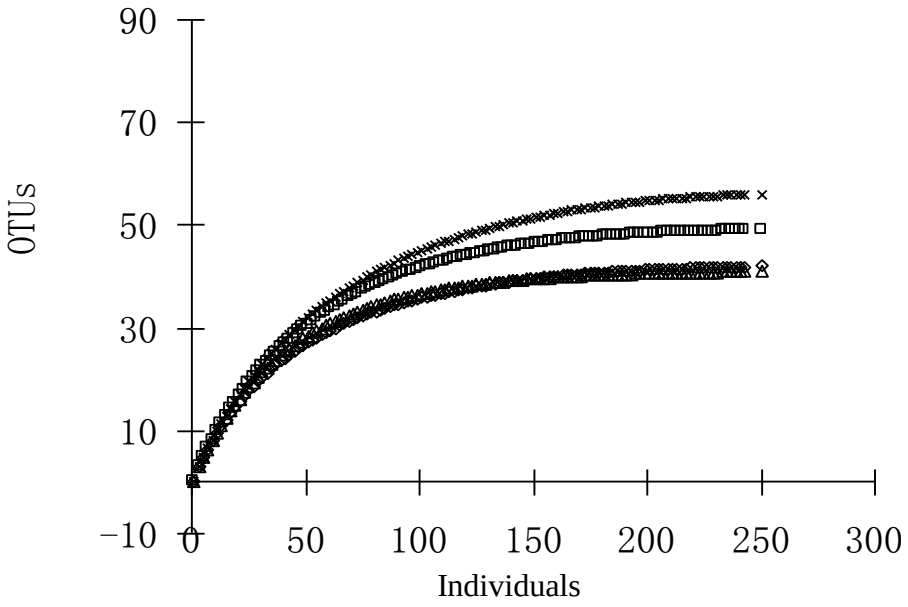

Figure 1. Phylotype richness curves for clone libraries. Sampling curves were calculated by rarefaction. Symbols: ◇ — CK, □ — M, △ — NPK, × — MNPK.

Table 2. *nifH* gene diversity measures from clone libraries constructed from soils subjected to differing levels of fertilization (CK = control, M = Manure, NPK = chemical fertilizer, MNPK = chemical fertilizer and manure).

Sample	No. of phylotypes	Total No. of clones	Coverage %	Shannon	Simpson	Margalef	Equitability	Chao 1 Estimate
CK	42	250	99.2	3.478	0.964	7.063	0.9427	45.88
M	49	250	99.6	3.707	0.972	8.331	0.9628	60.46
NPK	41	250	99.6	3.471	0.966	6.339	0.9687	69.33
MNPK	56	250	99.2	3.795	0.974	9.599	0.9514	74.82

the biodiversity of *nif*H microorganisms. With regard to the samples that were treated with both organic and chemical fertilisers, the latter treatment appeared to reduce significantly the impact of organic fertiliser on phylotype diversity. The diversity index, including the Shannon-Wiener index and Simpson's diversity index, indicated that the *nifH* patterns were the most diverse in the MNPK soil and that the addition of organic manure could increase the diversity of *nifH* (that is, MNPK>M>NPK>CK). In addition, the equitability index showed that the number of individual phylotypes in the NPK clone library had a more uniform distribution (that is, NPK>M>MNPK>CK), whereas the margalef index indicated that the MNPK treatment increased the number of individual phylotypes in the MNPK clone library (that is, MNPK>M>CK>NPK). Our data showed that the diversity and evenness indices of the four soil samples did not agree. This indicated that the high uniformity of a certain *nifH* from the soil sample pool compensated for the reduction in its abundance (for example, NPK), whereas the MNPK treatment impacted the *nifH* library relatively more positively and increased the *nifH* phylotypes. However, an imbalance remained, and this simultaneously intensified the degree of the difference among the unique phylotype abundance. Hence, the gene phylotypes increased caused aggravated competition, which induced an increase in the abundance index.

The ANOSIM test further indicated that similarities in the community structure existed between plots (Table 3). We assigned the CK sample as the frame of reference and then examined the R-value of the M, NPK and MNPK samples. The results indicated that using the M or NPK treatment alone had little effect on the diversity of the *nifH* gens, compared to the treatment which combined both together (MNPK). The R-values obtained from the NPK-CK and MNPK-CK and NPK-M and MNPK-M treatment comparisons revealed that, the addition of organic fertiliser to the inorganic fertiliser resulted in greater differences in *nif*H gene diversity between the clone libraries.

We speculate that different types of fertilisation would have some selectivity on the soil *nifH* community; in our

Table 3. A one-way analysis of similarities (ANOSIM) test to examine RFLP community profile differences in response to 4 fertiliser treatments.

Fertiliser	CK	M	NPK	MNPK
CK	-	0.6127	0.6741	0.2942
M	0.6127	-	0.3958	0.1653
NPK	0.6741	0.3958	-	0.6036
MNPK	0.2942	0.1653	0.6036	-

study, organic fertiliser and mineral fertiliser had the opposite effect on *nifH* selectivity. Thus, given sufficient time and evolutionary gene variation, it would be possible to form two completely different *nifH* libraries. Our results indicated that the use of organic and chemical fertilisers in combination effectively neutralised the influence of each other. This also suggests that organic fertiliser could create better environmental conditions for the expansion of the *nifH* composition. Considering a certain impact of the control of pests, this indicates that the cultivation of the crops had also influenced the *nifH* composition.

Sequence analysis and phylogenetic assignment

Following the sequence analysis of each phylotype, we were able to examine the the influence of the applications of organic manure and chemical fertiliser on the composition of the *nif*H. The clones from the four treatments clustered into various taxonomic groups, including *Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Actinobacteria, Firmicutes* and *Cyanobacteria* (Figure 2). The characteristic phylotypes of the M sample had a broader distribution in the phylogenetic tree, whereas the distribution of the unique phylotypes of the CK, NPK and MNPK samples was relatively narrow (mainly in the middle area of the phylogenetic tree). Additionally, the common taxa in the four libraries had little distribution in the middle of the phylogenetic tree (they were mainly grouped at both ends of the tree) and had no *Firmicutes* or *Betaproteobacteria* classification unit, although it did have the majority of the *Alphaproteobacteria* classification unit. This indicated that the characteristic genotypes of the CK, NPK and MNPK samples were closely related, with an identity of 83%. These data showed that the organic manure wasmore widely selective for the *nifH* pool. The common genotypes of the four libraries reflected the intrinsic link among the four kinds of processing but also may reflect the roles of other factors in addition to the four fertilisation treatments on the *nifH* structure. *nif*H clones belonging to *Azorhizobium* sp., *Azospirillum* sp., *Beijerinckia* sp., *Bradyrhizobium* sp., *Burkholderia* sp., *Nostoc* sp., *Pectobacterium* sp., *Pseudomonas* sp., *Rhodopseudomonas* sp. and *Verrucomicrobiae* sp. were found in the four libraries. The presence of *nifH* genes from *Rhizobium* and *Bradyrhizobium* spp. (also found in all of the libraries) could be explained by the practice of crop rotation (wheat–soybean–corn) with a leguminous plant (soybean) in the sites where the soil samples were collected. Moreover, *Rhizobium* spp. is well known to colonise the roots of a broad range of non-legumes, such as wheat (Marcia et al., 2008). Clones affiliated with *Paenibacillus durus* were found in the M, NPK and MNPK libraries. In addition to fixing nitrogen, *Paenibacillus* species can influence plant growth and health via phytohormones, chitinases, proteases, antimicrobial compounds and the solubilisation of phosphate (Coelho et al., 2007), which may be one of the reasons that the M, NPK and MNPK treatment improved both some properties of the soil and plant production (Zhou and Zhang, 2005). However, there were some quantitative differences in the frequencies of individual taxonomic units in the different libraries (Figure 3). *Alphaproteobacteria* was abundant in all of the libraries and was the most represented taxonomic unit in all of the four clone libraries (Figure 3). There were variations in the percentage of genotypes of each taxonomic unit in the total group of genotypes subjected to different treatments. The chemical fertiliser increased the percentage of *Alphaproteobacteria*, *Gammaproteobacteria* and unknown bacteria, whereas the organic fertiliser increased the percentage of *Actinobacteria* and *Archaea*. Within the control soil *Betaproteobacteria* and *Cyanobacteria* were abundant, whilst in the mixed fertilisation plots an increased abundance of *Firmicutes* were present. In some taxonomic groups, a large genotype percentage also had a larger proportion of the number of clones. However, the genotype percentages of *Deltaproteobacteria* and *Cyanobacteria* treated with organic fertiliser were not the highest; the proportion of the number of clones was the largest. We found that *Deltaproteobacteria* and *Cyanobacteria* became the dominant phylotypes as a result of the treatment with organic manure. Both the organic fertiliser and the organic-chemical fertiliser mix had a greater contribution to the *nifH* genotypes and the number of clones in the taxonomic units of *Actinobacteria.* This revealed that the organic matter had a strong selection for nitrogen-fixing *Actinobacteria.* From the perspective of the phylotypes, mixing of the fertilizers did not result in a simple

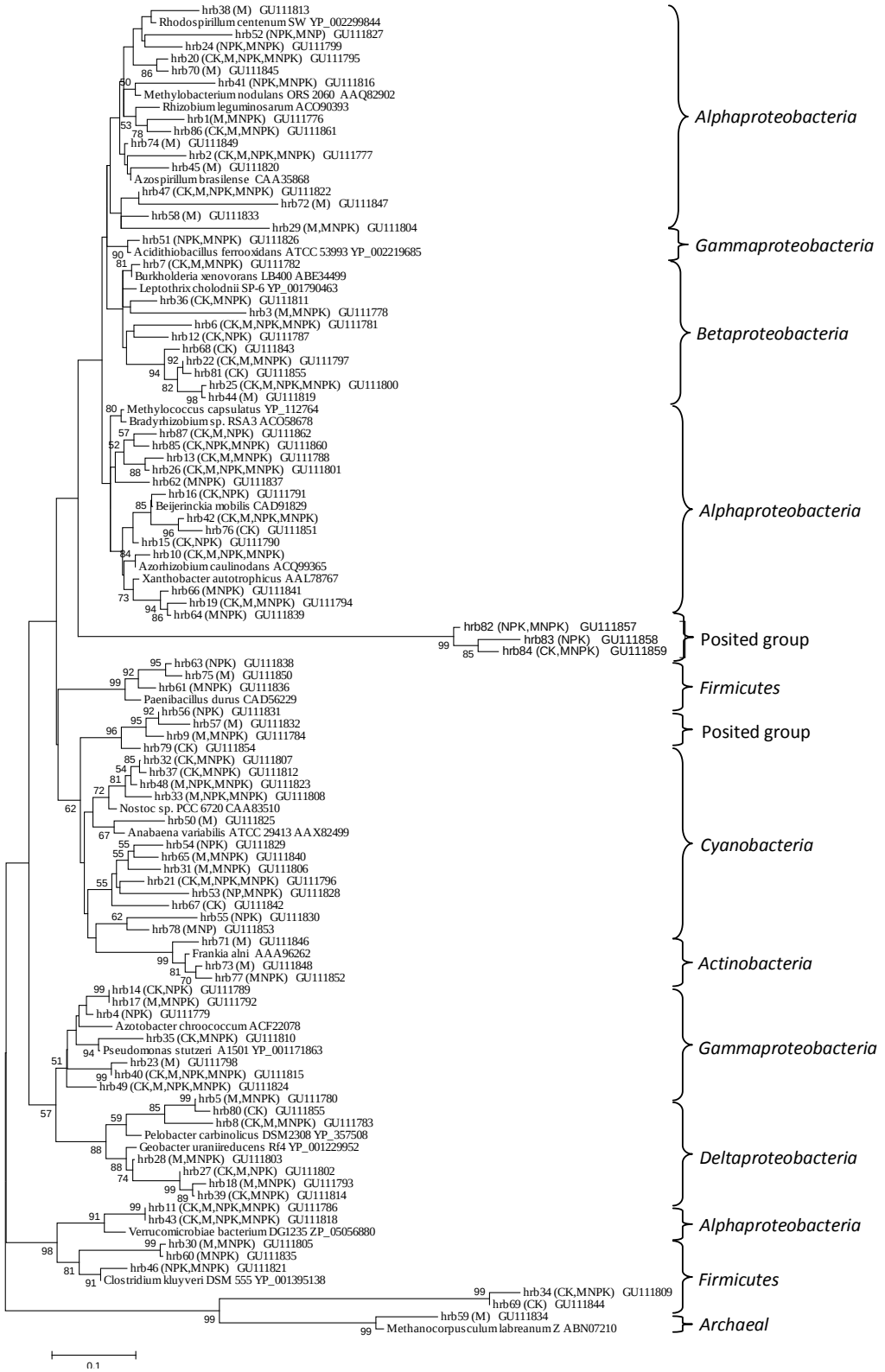

Figure 2. Phylogeny of *nif*H amino sequences using 22 partial sequences from the GenBank database and all sequences obtained from the fourfertiliser treatments. GenBank database accession numbers are indicated next to the bacterial names. The tree was constructed by the neighbor-joining method, and bootstrap values above 50 from 1,000 resamplings are shown for each node. Scale bars represent amino acid (NifH) substitutions per site. Origins of the phylogeny are given in parentheses, and accession numbers are behind.

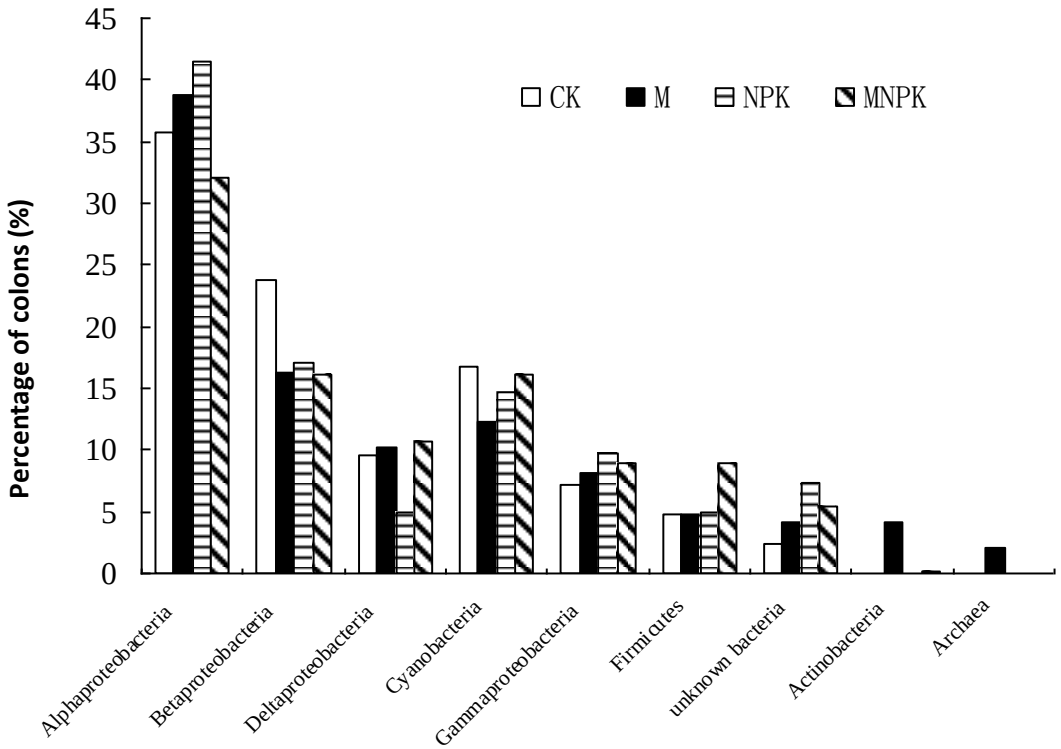

Figure 3. Phylogenetic diversity identified in four *nifH* clone libraries. It also shows the effects of different fertilizer treatments upon soil microbial community composition.

additive effect of the genotypes found in the M and NPK treatments, because a different environment was provided, and thus, a different set of microbial flora existed. We suggest that the application of organic fertiliser can greatly increase the types of nitrogen-fixing microorganisms and is conducive to the richness and evolution of nitrogen fixation genes. Many other authors have described sequences corresponding to diverse unidentified diazotrophs (Ueda, 1995; Widmer et al, 1999; Piceno and Lovell, 2000; Poly, 2001b; Zehr et al, 2003). These non-cultivated diazotrophs may be the dominant nitrogen-fixing organisms in soil systems (Widmer et al, 1999; Poly, 2001b; Tan et al, 2003) and could represent a new and unexplored group that may have an important role in the nitrogen cycle and nitrogen input into the soil.

Conclusion

The present biodiversity study of *nifH*-containing microbial communities from a long-term fertilisation field experiment enriched our knowledge about the diazotrophic community present in black soil. We used *nif*H as a biomarker of the functional soil microbial communities, and it proved to be a successful marker to examine how different fertiliser treatments affect the nitrogen-fixing microbial community. The application of organic fertilisers can greatly increase the types of nitrogen-fixing microorganisms present and is conducive to the development of a rich variety and evolution of nitrogen fixation genes. However, as the expression of the *nif*H genes were analysed merely by a DNA-based PCR approach, future studies should focus on the abundance of the *nifH* gene using Q-PCR to quantify the contribution of active nitrogenases of diazotrophic organisms to nitrogen uptake and crop yield.

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REFERENCES

Bai Z, Zhang M, Song DY (2008). Effect of long-term fertilization on microbial community in a Chinese arable Mollisol. J. Grad. School of the Chinese Acad. Sci, 25: 479-486

Chelius MK, Triplett EW (2001). The diversity of archaea and bacteria in association with the roots of *Zea mays* L. Microb. Ecol., 41: 252–263.

Coelho MRR, Fabio FDM, Newton PC, Ivanildo EM (2007). Diversity of *Paenibacillus* spp. in the Rhizosphere of Four Sorghum (*Sorghum bicolor*) Cultivars Sown with Two Contrasting Levels of Nitrogen Fertilizer Assessed by rpoB-Based PCR-DGGE and Sequencing Analysis. J. Microbiol. Biotechnol., 17(5): 753–760.

Deslippe J, Egger K (2006). Molecular diversity of *nifH* genes from bacteria associated with high arctic dwarf shrubs. Microbial. Ecol., 51: 516–525.

Felsenstein J (2004). PHYLIP (Phylogeny Inference Package) version 3.6. Department of Genome Sciences, University of Washington, Seattle.

Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS (2003). Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl. Environ. Microbiol., 69: 1800-1809.

Hamelin J, Nathalie F, Sonia T, Sylvie TC, Michel A (2002). *nifH* gene diversity in the bacterial community associated with the rhizosphere of *Molinia coerulea*, an oligonitrophilic perennial grass. Environ. Microbio., l4: 477-481.

Knauth S, Hurek T, Brar D, Reinhold-Hurek B (2005). Influence of different oryza cultivars on expression of *nifH* gene pools in roots of rice. Environ. Microbio., l7: 1725-1733.

Joerger RD, Wolfinger ED, Bishop PE (1991). The gene encoding dinitrogenase reductase is required for expression of the second alternative nitrogenase from *Azotobacter vinelandii*. J. Bacteriol., 173: 4440-4446.

Marcia RRC, Marjon V, Newton PC, Ivanildo EM, Edilson P, Lucy S (2008). Diversityof *nifH* gene pools in the rhizosphere of two cultivars of sorghum (*Sorghum bicolor*) treatedwith contrasting levels of nitrogenfertilizer. FEMS. Microbiol. Lett., 279: 15–22.

Marschner P, Kandeler E, Marschner B (2003). Structure and function of the soil microbial community in a long-term fertilizer experiment. Soil Biol. Biochem. 35: 453-461.

Mnasri B, Badri Y, Saïdi S, de Lajudie P, Mhamdi R (2009). Symbiotic diversity of *Ensifer meliloti* strains recovered from various legume species in Tunisia. Syst. Appl. Microbiol., 32(8): 583-92**.**

Piceno YM, Lovell CR (2000). Stability in natural bacterial communities: I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. Microb. Ecol., 39: 32-40.

Poly F, Monrozier LJ, Bally R (2001a). Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Res. Microbiol., 152: 95-103.

Poly F, Ranjard L, Nazaret S, Gourbiere F, Monrozier LJ (2001b). Comparison of *nifH* gene pools in soils and soil microenvironments with contrasting properties. Appl. Environ. Microbiol., 67: 2255-2262

Rasmussen PE, Goulding KWT, Brown JR, Grace PR, Körschens M (1998). Long-term agro-ecosystem experiments: assessing agricultural sustainability and global change. Science, 282: 893-896

Röch C, Mergel A, Bothe H (2002). Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl. Environ. Microbiol., 68: 3818-3829.

Schloss PD, Handelsman J (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol., 71: 1501–1506

Simberloff D (1978). Use of rarefaction and related methods in ecology. In: Dickson KL, Cairns JJ, Livingston RJ (eds) Biological data in water pollution assessment: quantitative and statistical analyses. American Society for Testing and Materials, West Conshohocken, pp. 150–165.

Steenworth KL, Jackson LE, Calderó FJ, Stromberg MR, Scow KM (2003). Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. Soil Biol. Biochem., 35: 489-500

Sun RL (2004). Effects of long-term fertilization on soil microorganism and its role in adjusting and controlling soil fertility. Chin. J. Appl. Ecol., 15: 1907-1910.

Tan Z, Hurek T, Reinhold-Hurek B (2003). Effect of N-fertilization, plant genotype and environmental conditions on *nifH* gene pools in root of rice. Environ. Microbiol., 5: 1009-1015.

Teixeira RL, von der Weid I, Seldin L, Rosado AS (2008). Differential expression of *nifH* and *anfH* genes in *Paenibacillus durus* analysed by reverse transcriptase-PCR and denaturing gradient gel electrophoresis. Lett. Appl. Microbiol., 46(3): 344-9.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res., 25: 4876–4882.

Ueda T, Suga Y, Yahiro N, Matsuguchi T (1995). Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. J. Bacteriol., 177: 1414-1417.

Ulrich A, Becker R (2006). Soil parent material is a key determinant of the bacterial community structure in arable soils. FEMS Microbiol. Ecol., 56: 430-443.

Wang Y, Wang S, Wei D, Zhou BK, Wang YF (2008). Effects of long-term located fertilization on soil physiology transformation bacterium. Ecol. Environ. 17: 2418-2420

Wang GL, Duan JN, Li XL (2003). Change of soil organic matter contents under a long term experiment. Chinese J. Soil Sci., 34: 589-591.

Waugh SI, Paulsen DM, Mylona PV, Maynard RH, Premakumar R, Bishop PE (1995). The genes encoding the delta subunits of dinitrogenases and are required for Mo-independent diazotrophic growth by *Azotobacter vinelandii*. J. Bacteriol., 177: 1505-1510.

Widmer FB, Shaffer T, Porteous LA, Seidler RJ (1999). Analysis of *nifH* gene pool complexity in soil and litter at a douglas fir forest site in the oregon cascade mountain range. Appl. Environ. Microbiol., 65: 374-380.

Young JP (1992). Phylogenetic classification of nitrogen-fixing organisms. Biological Nitrogen Fixation (Stacey G, Burris RH & Evens HJ, eds), Chapman, Hall, New York, pp. 43–87.

Zehr JP, Jenkins BD, Shortand SM, Steward GF (2003). Nitrogenase gene diversity and microbial community structure: a crosssystem comparison. Environ. Microbiol., 5: 539-554.

Zhang XL, Zhou BK, Sun L, Gao DC (2008). Black soil acidity as affected by applying fertilizer and manure. Chinese J. Soil Sci., 39: 1221-1223.

Zhou BK, Zhang XL (2005). Effect of long-term fertilization on crop yield in black soil. Syst. Sci. Compr. Stud. Agric., 21: 37-39

Zhou BK, Zhang XL, Li SL, Cong XB, Zhao RG (2004). Study on soil phosphorus accumulation and its availability through long - term fertilization to balck soil. Heilongjiang Agric. Sci., 4: 5-8.