# Effect of legume-cereal mixtures on the diversity of bacterial communities in the rhizosphere

**Y.J. Qiao, Z.Z. Li, X. Wang, B. Zhu, Y.G. Hu, Z.H. Zeng**

*College of Agronomy and Biotechnology, China Agricultural University, Beijing, P.R. China*

**ABSTRACT**

Aboveground plant diversity is known to influence belowground diversity and ecosystem processes. However, there is little knowledge of soil microbial succession in legume-grass mixtures. Therefore, this study was designed to determine the effect of oat and common vetch binary mixtures at three seeding rates on soil bacterial communities. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments was used to profile the structure of the bacterial community in the rhizosphere. Compared with a monoculture of common vetch and oat, the Shannon-Weaver index and species richness of the mixtures were increased. Thirteen cloned monocultures and mixtures of oat and common vetch soil 16S rDNA sequences were deposited to NCBI. Based on the sequencing results, the bands could be identified as related to *Proteobacteria*, *Bacteroidetes* and *Cyanobacteria*. Common vetch did not have some bacteria relatives to *Sphingomonas* spp. Some bacterial taxa could be detected in the ratio of 1:1 and 1:2, but not in the ratio of 1:3, e.g. *Myxococcales*. The results suggested that the belowground diversity could be promoted by mixed cropping systems.

**Keywords**: intercropping mixtures; rhizosphere microorganism; DGGE; 16S rDNA; biodiversity

The interaction between plants and surrounding soil communities has been a topic of intense research in the past decade. Plants, as producers, acquire nutrients from inorganic sources that are mostly supplied by decomposers, whereas decomposers, which are mostly soil microorganisms, obtain carbon from organic resources that are primarily supplied by producers. Previous studies investigating the effects of microbes on plants reported decreases in plant growth, increases in the rate of succession, and effects on non-native plant success (Kardol et al. 2006, Kulmatiski et al. 2008). These effects are largely due to negative plant-pathogen interactions. However, positive effects, such as increases in plant growth, and changes in plant community composition caused by mycorrhiza associations and plant-growth promoting bacteria were also reported (Compant et al. 2005). Direct effects of plants on microbial community, such as root exudates and particulate organic matter rhizodeposited by plants, can determine the nature of microbial habitats and local nutrient conditions (Puget and Drinkwater 2001, Orwin et al. 2006); these compounds can also indirectly influence bacterial metabolism and plant gene expression (Beattie and Lindow 1995). A better understanding of the role that plant species play in determining soil microbial community structure will contribute to the predictability of soil biogeochemistry. Here, we address the question of whether mixing legumes and cereals influence the diversity of bacterial communities in the rhizosphere.

Mixed cropping of certain annual legumes with cereals is extensively used for forage production all over the world (Anil et al. 1998, Papastylianou 2004). It can increase forage yield, improve forage quality, and change the seasonal distribution of forage (Droushiotis 1989, Carr et al. 1995, Jensen 1996). Mixtures have different microenvironments compared to pure stands. The effect of mixtures of

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legume-cereal is attracting increasing interest in many countries as it can provide increased yields in an environmentally sustainable manner (Park et al. 2002, Gliessman 2007).

Common vetch (*Vicia sativa* L.), an annual legume with climbing growth habit and high protein content, is usually grown in mixtures with small grain cereals for hay or forage production (Anil et al. 1998). Oat-vetch mixed cropping has been a popular cropping system for forage production and an important fodder for farm animals in the arid and semi-arid areas in China. Most of the previous studies on oat-vetch mixed cropping focused on the yield and quality, seeding ratio, interaction and competition between the two crops, and the N uptake from soil and atmosphere (Lithourgidis et al. 2006, Dhima et al. 2007). However, there is a limited understanding of the effect of oat-vetch mixed cropping on soil microbial communities. Additionally, there is a need to study the changes in the microbial community composition with different seeding ratios.

The objective of the present study was to evaluate the effect of oat and common vetch monocultures as well as mixed cropping in three seeding ratios, 1:1, 1:2, and 1:3, on the microbial community structure in rhizosphere soil. The community structures were assessed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

## MATERIAL AND METHODS

**Greenhouse experiment and soil sample collection**. The experiment was conducted in a greenhouse from Sep 2009 to Jan 2010 using sandy loam soil (pH 6.8, organic matter content 1.15%, soil moisture 17.8%, previous crop was corn). Soil was sampled in Yanqing County, Beijing P.R. China in a dry summer day. After sieving and homogenizing, 5 kg wind-dried soil was filled into greenhouse pots (39 × 15 × 27 cm). There were five treatments with three replications: H1 was common vetch (333/A) monoculture, H2 was oat (Baiyan7) monoculture and H3, H4, and H5 were common vetch and oat intercropped at 1:1, 1:2, and 1:3 seeding ratios, respectively. Each pot was planted with 24 plants in three rows, with 6.5 cm row spacing, and 4 cm plant spacing.

Soil samples were collected in the rhizosphere of common vetch and oat, intercropped soil samples were mixed with both of them. They were taken at the jointing stage of oat with three replicate cores from each pot, and analysed separately. Soil samples were totally homogenized, removed roots and stored at −20°C until further processing.

**DNA extraction, PCR and DGGE analysis**. Total soil DNA was extracted from approximately 0.3 g soil using a soil DNAout kit (TIANDZ, Beijing, China) following the manufacturer's instructions. The variable (V3) region of 16S rDNA was amplified by PCR using the primers V357F-GC clamp and V517R (Muyzer et al. 1993).

DGGE analysis of microbial community structure was performed using the D-Code system (Bio-Rad Laboratories, Hercules, USA) by loading 13 μL PCR products (approx.300 ng/μL, and 7 μL loading buffer) onto 6–12% (w/v) polyacrylamide gels in a 0.5 × TAE electrophoresis buffer. The denaturing gradient was established with 30–50% denaturant. Electrophoresis was performed at a constant voltage 200 V for 5 h at 61°C. The gel was stained by SYBRs Green I (Molecular Probes, Eugene, USA) for 30 min, and scanned using the Alpha Imager 2200 Imaging System (Alpha Innotech, CA, USA).

**Sequencing analysis**. Treatment-specific and dominant bands were excised from the DGGE gel and eluted overnight in diffusion buffer for re-amplification. The amplified 16S rDNA segments were inserted into pEASY-T3 vectors (TransGen Biotech, Beijing, China) and transformed into *E. coli* DH5α (Li et al. 2007). Subsequently, positive recombinants were submitted for sequencing using an ABI3730 DNA Sequencer (CA, USA) with T3 primer at SunBiotech Co., Ltd. The closest match to each sequence was obtained using the NCBI basic local alignment search tool (http://www.ncbi.nlm.gov/blast/). Alignment of 16S rDNA gene sequences were performed using ClustalX 1.83 (Thompson et al. 1997) and a neighbor-joining phylogenetic tree was constructed based on evolutionary distances that were calculated with the Kimura 2-parameter model using Mega 5 with bootstrap confidence values obtained from 500 replications.

**Statistical analyses**. Diversity analysis of DGGE patterns was performed using Quantity One 4.2.3 (Bio-Rad Laboratories, Hercules, USA) by the unweighted pair-group method with arithmetic averages (UPGMA). Soil microbial community diversity was assessed by Shannon-Weaver index (H), richness (S) and evenness (E). The Shannon-Weaver diversity index was calculated from the number of bands and their intensities in each lane (Shannon and Weaver 1949). The formulas are as follows:

$$pi = n_i/N$$

$$H = -\sum_{i=1}^{s}(pi)(Lnpi)$$

$$E = H/H_{max} = H/LnS$$

Where: $n_i$ – intensity of the $i^{th}$ band; N – total intensity of the bands; *pi* – relative intensity of the $i^{th}$ band. Significance

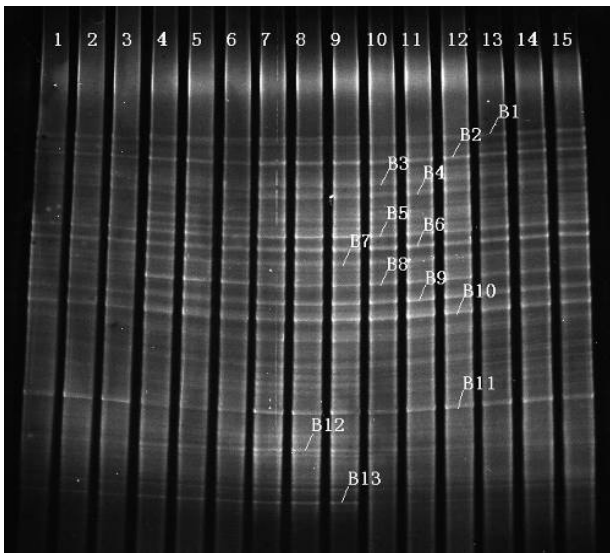


Figure 1. PCR-denaturing gradient gel electrophoresis patterns of 16S rDNA fragments obtained from soil DNA of oat and common vetch monocultures as well as their mixture: 1–3 – oat monoculture; 4–6 – common vetch monoculture; 7–9, 10–12, and 13–15 – the mixtures of oat and common vetch at the ratio of 1:1, 1:2, and 1:3, respectively

of differences in the indices was tested by the Duncan's multiple range test in SAS V8.0, $P < 0.05$.

## RESULTS

Based on the PCR-DGGE banding patterns (Figure 1), Shannon-Weaver diversity index and corresponding evenness and richness were calculated. The results from the monoculture and the mixed cropping are presented in Table 1. Compared with the monocultures, the Shannon-Weaver index and richness of the soil bacteria were higher in the mixed cropping oat and common vetch. H1 had the lowest Shannon-Weaver index and significantly differed from mixtures H, and H5. H3 had the highest Shannon-Weaver index and significantly differed from H1, H2, and H4. H4 had the highest richness and significantly differed from others.

Table 1. Shannon-Weaver diversity index, evenness and richness calculated from the PCR-DGGE patterns obtained from the soil samples

| Treatment | Shannon-Weaver (H)' | Richness (S) | Evenness (E) |
|---|---|---|---|
| H1 | $2.86^{cd}$ | $22.3^{d}$ | $0.920^{a}$ |
| H2 | $2.93^{bc}$ | $24.0^{c}$ | $0.923^{a}$ |
| H3 | $3.06^{a}$ | $27.0^{b}$ | $0.927^{a}$ |
| H4 | $2.94^{bc}$ | $28.3^{a}$ | $0.883^{b}$ |
| H5 | $2.98^{ab}$ | $27.0^{b}$ | $0.903^{ab}$ |

*Different letters within a row indicate significant differences between the means (Duncan's multiple range test, $P < 0.05$; $n = 3$)

PCR-DGGE analysis with bacterial primers was performed to analyze the total bacterial communities in the soil of common vetch and oat monocultures as well as mixtures of them. The effects of the monocultures and mixtures were clearly visible from the UPGMA clustering of the PCR-DGGE gels (Figure 2). Based on the monoculture and the mixture of common vetch and oat, the DGGE patterns obtained from soil can be grouped into three separate clusters. Cluster 1 included all the common vetch monocultures, Cluster 2 included all the oat monocultures, and Cluster 3 included all the mixtures of the two crops at different ratios. In addition, Cluster 3 had two branches; one included the mixtures of oat and common vetch at the ratio of 1:1 while the other bigger branch included the other two ratios.

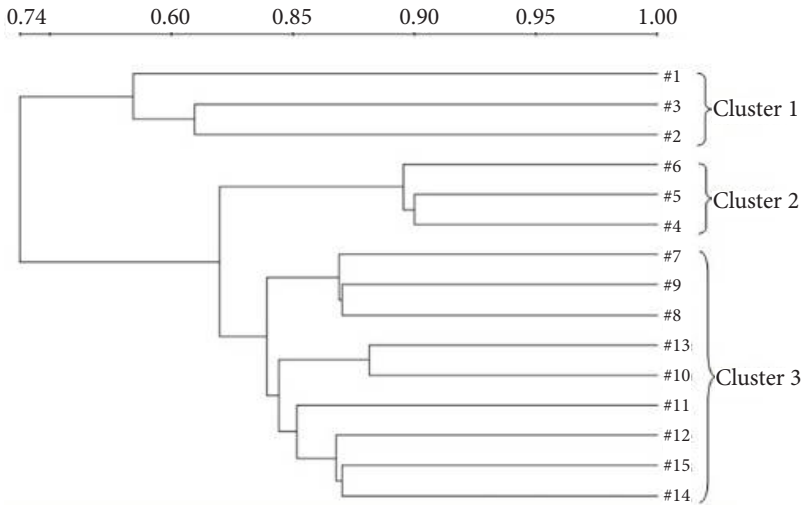


Figure 2. Dendrogram representing the similarity of PCR-DGGE profiles generated from the soil DNA of oat and common vetch monocultures as well as their mixture: 1–15 marked as Figure 1

Table 2. Sequencing results of 16S rDNA DGGE fragment

| DGGE bands | Sequences | Bacteria with the highest identity and accession No. | The highest identity (%) |
|---|---|---|---|
| B1 | CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGC CTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTG TAAAGCT CTTTTACCCGGGATGATAATGACAGTACCGGGAGAA TAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT | *Sphingomonas* sp. (GQ484890.1) | 100 |
| B2 | ATTACCGCGGCTGCTGGCACGGAATTATCCGGGGCTTGCTCAA GCGGGACCGTCATATTCCGCGCGAGTAAAAAACGTCTTCTGGT TCAAAGCCTCTATCACTCACGAAGCATAGGTCCATCATGCTTT CCCCCATTGTGCCTAATTCCCCGCCGCTGCCTCCGGTAAG | *uncultured Synechococcus* sp. (AY664055.1) | 97 |
| B3 | CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAA AGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGG TTGTAAAGCTCTTTTACCAGGGATGATAATGACAGTACCTGGAG AATAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA | *Erythrobacter* sp. (AB526331.1) | 99 |
| B4 | ATTACCGCGGCTGCTGGCACGGGGATATTACCGCGGCT GCTGGCACGGGGATATTACCGCGGCTGCTGGCACGGAGTTAGC CGGTGCTTATTCATATAGTACCGTCAGTTCCTCTCGCAAGAGTG TTTTCTTCCTATATAAAAGCAGTTTACAACCCAGAAGGCCTTCT TCCTGCACGCGGCATGGCTGGGTCAGACTTCCGTCCATTGCCCA ATATTCCCTACTGCTGCCTCCCGTAGGA | *Adhaeribacter aquaticus* (AJ626894.2) | 97 |
| B5 | CCTACCGGAGGCGCCGGTGGGGAATTTTGCGGAATGGGCGCA AGCCTGACCCAGCAATGCCTCGTGAGTGAAGAAGGCCTTCGG GTTGTAAAGCTCTTTCACTCGGGACGAATTTGAGGGTGATGC TTTCACCCGTTGTCGAAAATTCCCCGCTACTGCCTCCCGTAAT | *uncultured Nitrosomonas* sp. (FM997796.1) | 92 |
| B6 | GGCTGCTGGCACGGGGATATTACCGCGGCTGCTGGCACGGG GATATTACCGCGGCTGCTGGCACGGAGTTAGCCGATCCTTAT TCTTACAGTACCGTCAAGCTGGTTCACGAACCAGTGTTTCTTC CTGTACAAAAGCAGTTTACAATCCATAGGACCGTCATCCTGCAC GCGGCATGGCTGGTTCAGGCTTGCGCCCATTGACCAATATTCC TCACTGCTGCCTCCCGTAGG | *Flavobacterium* sp. (AM934649.1) | 100 |
| B7 | CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAA GCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGT TGTAAACCTCTTTTACCCGGGATGATAATGCCTCTACCGGGAG AAAAAGCCCCGGCTAACTACGTGCCGGCTACCTCGGTAAT | *Sphingomonas* sp. (EU855783.1) | 93 |
| B8 | CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAA GCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGT TGTAAAGCTCTTTTACCCGGGATGATAATGACAGTACCGGGAGA ATAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAGCA GCCGCCAGCAGCCGCGGTAAT | *Sphingomonas kaistensis* (GQ505344.1) | 100 |
| B9 | CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAA ACCCTGACCCAGCGACGCCGCGTGAGTGATGAAGGCCTTATG GTTGGAAAGCTCTGTTACCCCGCAAGATAATGACCGTACCGG GAGAATAATGTCCGGCTAACTCCGTGCCAGCACCCGCGGCAAT | *Sphingomonas* sp. (FJ434127.2) | 91 |
| B10 | ATTACGGCAGGCAGTGGCGGGGACTATTGGACAATGGGCGAAA CCTGATCCTCAATGCCGCAGCTGGCACGGAGTTCTGCGGAG CTTATTCTCCTTTTACTGTCATTATCATCCCTGGAAAAAAAAC TCCGCAACCTCAAGGCCTTCATCCGCGACGCGGCATTGCTGG ATCAGGCTTTCGGGAATTACTGAATATAAACCACGCGT | *Hydrogenophaga pseudoflav*a (DQ133432.1) | 89 |
| B11 | CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCG AAGGCCTGATCCAGCAATGCCGCGTGGGTGATGAAGGCCT TAGGGTTGTAAAGCTCTTTTACCCGGGATGATAATGACAGT ACCGGGAGAATAAGCCCCGGCTAACTCCGTGCCAGCAGC CGCGGTAATACGGGTGCCAGCAGCCGCGGTAATATCCCCG TGCCAGCAGCCGCGGTAAT | *Sphingomonadaceae bacterium* (FJ263045.1) | 98 |
| B12 | CCTACGGGAGGCAGCAGTAGGGAATATTGGTCAATGGATG CAAGTCTGAACCAGCCATGCCGCGTGCAGGAAGAAGGCCT TCTGGGTTGTAAACTGCTTTTGCCGGGGGATAAAATTCCCA TGCGTGGGACATTGAAGGTACCCGGTGAATAAGCCACGG CTAACTACGTGCCAGCAGCCGCGGTAAT | *uncultured Sphingobacteriales bacterium* (AM935101.1) | 100 |
| B13 | CCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCG AAAGCCTGACGCAGCGACGCCGCGTGGGTGATGAAGGCC TTCGGGTTGTAAAGCCCTGTGGGGAGGGACGAACAAGCT ACGACCTAATACGTCGTGGCCCTGACGGTACCTCCTTAGC AAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAAT | *Myxococcales bacterium* (AB245340.1) | 95 |

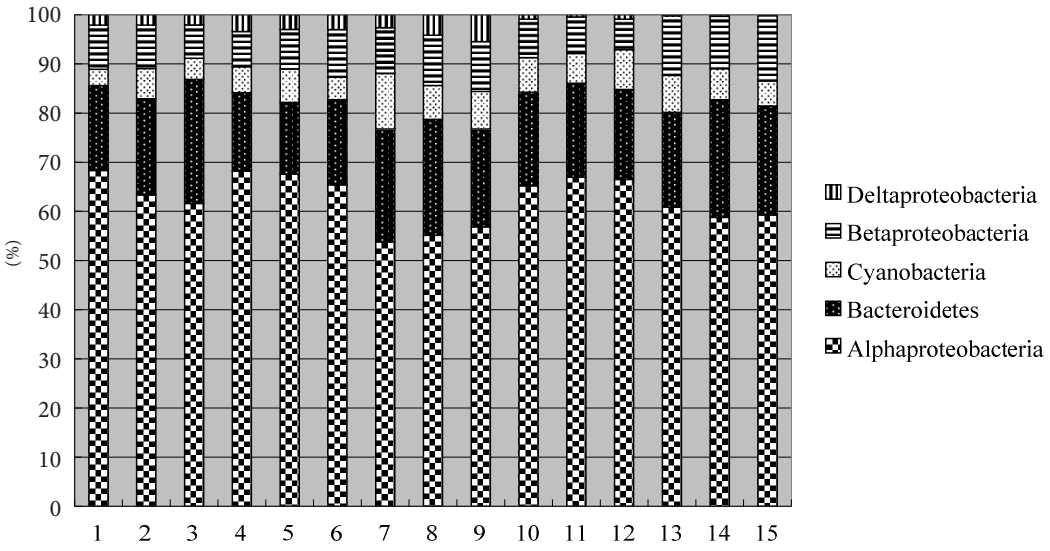


Figure 3. Comparison of the bacterial community among H1-H5. Percentage was calculated according to the total number of DGGE bands representing different bacteria

The DGGE bands obtained from the patterns generated from the sampling were excised and sequenced to analyze the dominant bacterial populations in the monocultures and the mixtures. Thirteen cloned monocultures and mixtures of oat and common vetch soil 16S rDNA sequences (about 170 bp) were deposited at the NCBI database. Based on the sequencing results, six bands (B1, B3, B7, B8, B9, B11) could be identified as related to *Alphaproteobacteria*, three bands (B4, B6, B12) related to *Bacteroidetes*, B5 and B10 related to *Betaproteobacteria*, of the remaining two bands, B2 related to *Cyanobacteria*, and B13 related to *Deltaproteobacteria* (Table 2). The intensity of each band was different between treatments, e.g. H1 did not have B7 and B8; B13 was not detected either in H1 or H5. According to Figure 3, *Alphaproteobacteria* were dominant in all the bacterial communities, accounting for 53–68%; *Deltaproteobacteria* were the less abundant among the sequenced bands. Figure 4 shows the phylogenetic relationships of the 16S rDNA sequences representing the respective excised 13 DGGE band. They were grouped into 2 big clusters.

## DISCUSSION

The clear distinction in bacterial community structure between the pure oat, pure common vetch and the mixtures of them is probably mainly due to variation in the amount and chemical composition of the rhizodeposits. Our findings were consistent with those of previous reports that plant species was the most important factor in determining bacterial community composition (Westover et al. 1997, Marschner et al. 2001, Garbeva et al. 2008). Apart from the differences in soil bacterial community structures between the monocultures of oat and common vetch (common vetch did not have some bacteria relatives to *Sphingomonas* spp.), differences among the seeding ratios were also examined. Some bacterial taxa could be detected in the ratio of 1:1 and 1:2, but not in the ratio of 1:3, e.g. *Myxococcales*. This may be explained by the different proportion of oat and common vetch in the mixtures releasing different qualities and quantities of root cell components and root exudates that enter the soil, which in turn modifies the microbial community structure and activity. Furthermore, some new soil bacterial species could be detected in the mixtures soil, but not in the monocultures of oat or common vetch (Figure 1). In addition to the aforementioned process occurring in mixtures, some new chemicals produced by the mixture could explain this result.

In our study, higher plant diversity increased the soil microbial diversity, which was consistent with the findings of previous studies that plant species richness had a positive effect on the diversity of soil microbes (Spehn et al. 2000). Carney and Matson (2005) found that plant diversity had a significant effect on microbial community composition by altering microbial abundance rather than community composition. Other studies reported that aboveground net primary productivity could increase soil carbon input by enhancing the turno-

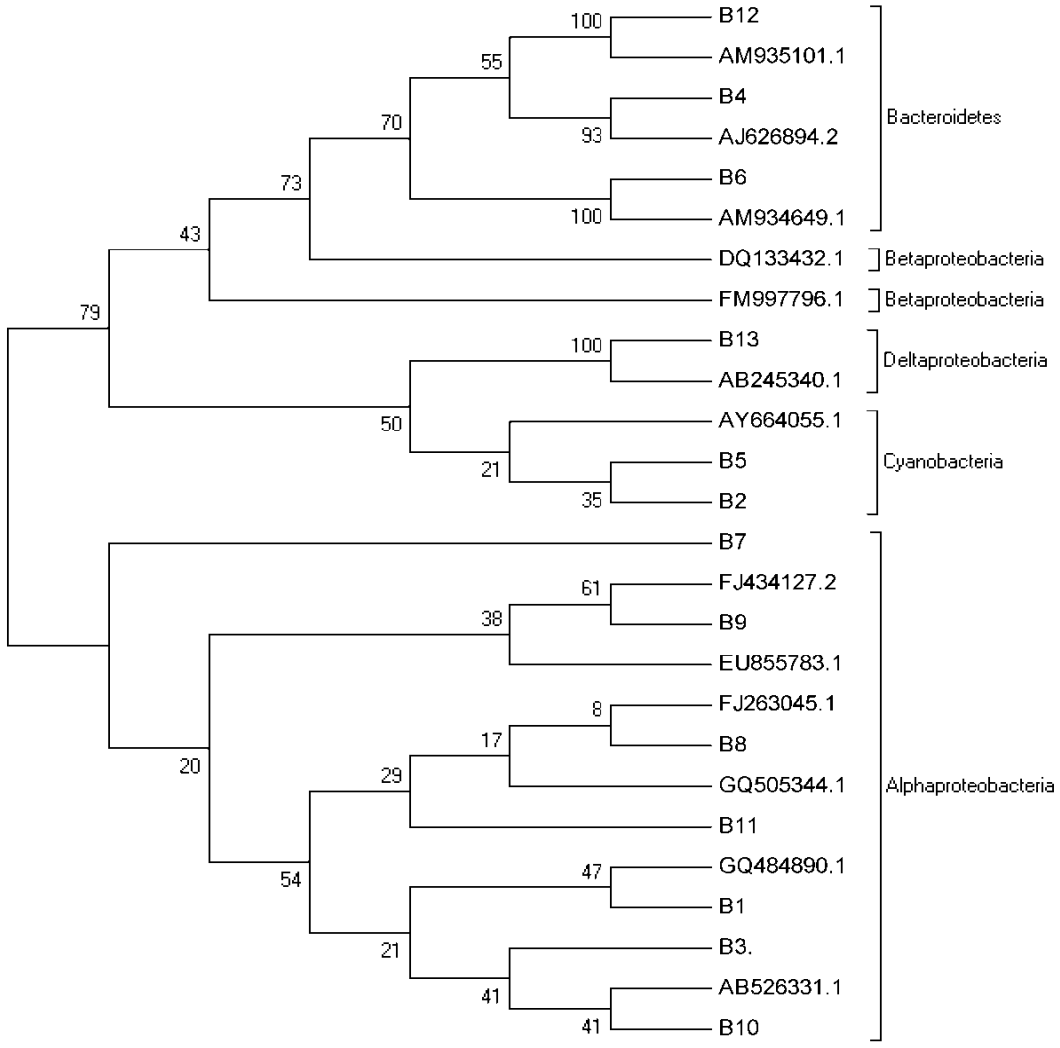


Figure 4. Phylogenetic relationships of 16S rDNA sequences retrieved using denaturing gradient gel electrophoresis of soil DNA of the oat and common vetch monocultures as well as their mixture

ver of plant biomass and root exudation, which may therefore influence carbon-limited microbial communities in the soil (Ebersberger et al. 2003).

A few points should be noted when interpreting the DGGE patterns. Part of the DNA extracted may derive from dead cells in the soil and therefore the band pattern does not only reflect the current community composition but also previous ones. Since the bands are separated out by their denaturation characteristics and GC content, a band may contain more than one species, which means species number may be underestimated. On the other hand, some species contain several copies of the amplified section and can generate several bands leading to overestimation of species number. Usually band intensity differences between species cannot be used as an indicator of species abundance (Muyzer and Smalla 1998, Gelsomino et al. 1999). However, if the intensity of a given band increases or decreases between different samples, this could indicate a relative increase or decrease in abundance of this species.

Although our study provided some insight into the effects of common vetch-oat mixtures on microbial communities in the rhizosphere, we still do not know the exact mechanisms through which mixtures affect the composition of bacterial populations. Moreover, there is still much research to be done to examine the dynamics of soil microbial diversity during the period of plant growth. Subsequent research could focus on the influence of the quality and quantity of root exudates that the mixtures release into the soil on the diversity of soil microorganisms. Differences in the influence of mixtures on soil microbial diversity between two and more plant species, interaction between the diversity of bacterial communities and the mixtures productivity in the field, as well as the role of soil characteristics and field managements, should be explored in future research.

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*Corresponding author:*

Dr. Zhaohai Zeng, China Agricultural University, College of Agronomy and Biotechnology, Beijing, P.R. China
phone: + 86 10 6273 4884, fax: + 86 10 6273 2441, e-mail: zengzhaohai@cau.edu.cn