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Full Length Research Paper

Bacterial community structure in the *Cerasus sachalinensis* Kom. rhizosphere based on the polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE) method

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The bacterial community structures of the *Cerasus sachalinensis* Kom. rhizosphere in wild and cultivated soil were studied and the community changes in different growth stages were analyzed by the PCR-denaturing gradient gel electrophoresis (PCR-DGGE) method. The results showed that the bacterial community diversity in the cultivated *C. sachalinensis* rhizosphere was always higher than the wild, while the evenness and dominance indices followed a different pattern as compared to band richness in the wild and cultivated conditions. The plant growth stages also had an influence on the bacterial community structures. The richness and diversity of the bacteria both corresponded to: bud-breaking phase > growing phase > defoliation phase. Cluster analysis based on DGGE banding patterns showed that the bacterial community structures were affected by growth conditions and agricultural management practices. Accordingly, the dendrogram was divided into Clusters I and II, which respectively contained the cultivated and wild soil samples. Additionally, specific bacterial species were found in the wild and cultivated *C. sachalinensis* rhizosphere. 13 dominant DGGE bands were excised, sequenced, and divided into eight groups, in which 3 bands were identified as *Actinobacteria*, 2 as *Sphingobacteria*, 2 as *α-Proteobacteria*, 2 as *Firmicutes*, and the remaining 4 as *γ-Proteobacteria*, *Acidobacteria*, *Gemmatimonadetes* and unclassified bacteria, respectively. Uncultivable bacteria were predominant in the total bacterial groups.

Key words: *Cerasus sachalinensis* Kom., bacteria, community structure, denaturing gradient gel electrophoresis (DGGE), diversity.

INTRODUCTION

As the most important components of soil, microbes especially bacteria play a pivotal role in regulating and influencing processes such as nutrient transformation, litter decomposition, soil structure, fertility maintenance, and plant health (Kennedy, 1999; Zak et al., 2003). Understanding the complex bacterial community has proven to be a challenging task for developing the best management practices for agro-ecosystems (Garbeva et al., 2004).

Cerasus sachalinensis Kom. is native to the north-eastern mountains of China and is the most important

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Table 1. Mean soil nutrient and pH values of the four samples.

Soil sample	pH	Available N (mg·kg^{-1})	Available P (mg·kg^{-1})	Available K (mg·kg^{-1})	Organic matter (mg·kg^{-1})
W_{CK}[a)]	6.01±0.03	42.00±3.50	6.94±0.09	65.13±4.25	19.34±0.60
W_{Cs}	6.83±0.05	32.67±4.25	6.74±0.13	61.62±1.34	13.96±0.13
C_{CK}	7.03±0.10	159.8±10.69	227.3±0.06	183.3±5.56	27.84±0.51
C_{Cs}	7.43±0.04	86.33±7.29	154.6±3.08	112.4±6.10	25.87±0.13

a): $W_{CK:}$ the control in wild condition; $W_{Cs:}$ rhizospheric soil of *C. sachalinensis* in the wild condition; $C_{CK:}$ the control in cultivated condition; $C_{Cs:}$ rhizospheric soil of *C. sachalinensis* in the cultivated condition.

rootstock of cherries in cold areas. However, in the cultivated soil, it shows poor resistance to crown gall disease, limiting its application in production (Lü et al., 2008). But during the two-year's field investigation, we found the *C. sachalinensis* plants were healthy and hardly with root diseases when grown in wild. What is more, the bacterial community structures changed firstly when rhizospheric environment was not adaptable. So, it could be speculated that the different growing condition of cultivated and wild soil was one of the most important factors which affected the bacterial community structures. Understanding the bacterial community structural shifts of the *C. sachalinensis* rhizosphere in wild growth conditions may help to determine specific rhizospheric characteristics and develop effective management practices. However, research on bacterial community diversity in the *C. sachalinensis* rhizosphere is at an early stage (Yu et al., 2007; Lü et al., 2008). Unfortunately, reports about the rhizospheric characteristics of wild *C. sachalinensis* and the different environmental factors between wild and cultivated soils in the *C. sachalinensis* rhizosphere have not been found. The objective of this study was to reveal distinctive features of bacterial communities in the respective habitats of wild and cultivated soil using PCR-DGGE.

MATERIALS AND METHODS

Experimental points

The wild *C. sachalinensis* rhizospheric soil samples were collected from the Pristine mountain area, a successional uncultivated field since 1970 in Benxi city (41°24′ N, 124°17′ E), Liaoning province of China. The altitude reached 500 m where *C. sachalinensis* grown intensively. *C. sachalinensis* plants were grown naturally and randomly all over the mountain without cultivation. Around the *C. sachalinensis* plants, there were many other trees such as oak wood (*Quercus mongolica*), larch (*Larix gmelinii (Ruprecht)* Kuzeneva), and hawthorn (*Crataegus pinnatifida* Bge.) etc. The cultivated soil was collected from the fruit tree experimental base of ShenYang Agricultural University in Shenyang city (41°48′ N, 123°25′ E), Liaoning province of China, which is conventionally tilled.

Soil sampling

The roots of *C. sachalinensis* were mainly distributed at a depth of 20-40 cm. The rhizospheric soil that adhered tightly to the roots within 0.1 cm in 30 cm depth was collected at different growth stages: bud-breaking phase (2008-4-20), growing phase (2008-8-8), and defoliation phase (2008-10-20), respectively. Fallow soil of the same depth by the side of *C. sachalinensis* was used as a control (CK). Replicates were taken randomly from six trees at each study site. For each tree, we collected the soil samples respectively from the 4 directions, cut 20 fine roots and shake off the rhizospheric soil into sterile bags. Finally, insured the total weight of soil samples were not less than 100 g. The soil samples for DNA extraction, PCR and DGGE analysis were mixtures of the replicates. The soil samples were marked, mixed thoroughly, sieved through a 2 mm screen, and stored at -70°C until DNA extraction.

Soil chemical analysis

Soil pH was measured by a combination glass electrode (soil: water, 1: 2.5). Total Organic C was determined by dichromate oxidation, as well as the available phosphorus, inorganic N and available potassium analysis were undertaken by the method used by Shi and Bao (1988). The characteristics for all soil samples are presented in Table 1.

DNA extraction from soil

DNA extraction was performed with a modified direct lysis method, as described by Zhou et al. (1996). Briefly, 0.5 g soil was mixed with 1 ml DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, and 1% CTAB) in a microcentrifuge tube, vortexed for 5 min, and incubated in a 65°C water bath for 10 min. Subsequently, the samples were subjected to three freeze-thaw cycles (-70 to 65°C) for complete cell lysis. The supernatant was collected by centrifugation at 12,000×*g* for 10 min at room temperature and mixed with an equal volume of chloroform: isoamylalcohol (24:1, *v/v*). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. Crude nucleic acid extracts were washed with cold 70% ethanol and re-suspended in TE buffer to a final volume of 50 µl. The crude extracts were further purified with the Mini-DNA fragment Rapid Purification Kit (BioDev, China), according to the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification

Primers GC (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCG), 341f (5'-CCTACGGGAGGCAGCAG-3'), and 758r (5'-CTACCAGGGTATCTAATCC-3') were used for direct amplification of 16S rDNA sequences from purified DNA (Tresse et al., 2004; Bottos et al., 2008). All PCR amplifications were

carried out in 50 µl reactions containing 5 µl 10×PCR reaction buffer (TakaRa, Japan), 20 ng DNA template, 20 pmol of each primer, 200 µmol dNTP mixture, and 2 U of Ex *Taq* DNA Polymerase (TakaRa, Japan). The PCR reaction conditions used for primers 341f and 758r were: 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis to determine yield and purity.

Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed with the DCcode mutation detection system (Bio-Rad, USA). The conditions for separation were as follows: 40 µl PCR products/lane, running at 180 V for 6 h in 1× TAE buffer at 60°C; and 6% polyacrylamide gel with a denaturing gradient from 30 to 60%.

After electrophoresis, gels were stained with 10 µl GeneFinder™ DNA dye (Beijing BIO-V Gentech Co., Ltd., Beijing, China) in 100 ml 1× TAE buffer for 30 min. Gel images were digitally captured by UV transillumination and documented with a GelDoc system (Bio-Rad, USA). Cluster analysis and dendrograms were calculated with UPGMA using Quantity One 4.1 gel analysis software (Bio-Rad, USA).

Statistical analysis

DGGE gels were analyzed using Quantity One 4.1 gel analysis software (Bio-Rad, USA); bands with intensity <0.05 were excluded from the analysis. The Shannon-Wiener index of diversity (*H*) (Shannon and Weaver, 1963) was used to determine the diversity of the bacterial community. This index was calculated with the formula: $H = -\sum pi \ln(pi)$; where, *pi* is the proportion of the bands in the track (it was calculated as follows: $pi = ni/N$; where *ni* is the intensity of band *i* in the densitometric curve, and *N* is the sum of the intensities of all bands). The PieLou index of evenness (*J*) was calculated using the formula: $J = H / \ln(R)$; where *R* is the total number of different DGGE bands (richness). The Simpson index of dominance, *S* (Gafan et al., 2005), was calculated with the formula: $S = \sum pi^2$. EXCEL software were used in calculation and data analysis.

Isolation and sequencing of DGGE bands

Prominent DGGE bands were excised from the UV-illuminated acrylamide gels, and the DNA was eluted from the excised gel by incubation in 30 µl TE at 4°C overnight. Eluted DNA was used for PCR reaction, as described above, and the products were again analyzed by DGGE. PCR products with single bands on the second or third DGGE were purified for analysis using a PCR purification kit (Mini-DNA fragment Rapid Purification Kit, BioDev, Beijing) with a final elution volume of 50 µl. Sequencing was performed by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.

The PCR product was direct sequenced with the same primer sets. Sequences were searched in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/). On the basis of the BLASTN results, highly similar GenBank sequences were added to the data set for CLUSTAL W multiple sequence alignments (Larkin et al., 2007). Phylogenetic distance trees were constructed by MEGA 3.1 (method of Neighbor-Joining). DNA sequences were submitted to GenBank with accession numbers GQ981474-GQ981486.

RESULTS

Analyses of PCR products by denaturing gradient gel electrophoresis (DGGE)

The DGGE banding patterns of 16S rDNA genes from the soil samples are shown in Figure 1. The DGGE band number, position, and density were all different between wild and cultivated *C. sachalinensis* soils and also varied with different growth stages. The detected band numbers showed that the bacteria community richness in cultivated soil (21, 20, 20) was higher than that in soil of wild condition (20, 18, 13) (Table 2), respectively; higher by 5.00, 11.11 and 53.85%, which was in agreement with the Shannon-Weiner index (*H*). However, the PieLou index (*J*) and the Simpson index (*S*) were reversed, indicating that the richness of soil bacteria in the wild was higher than the cultivated (Table 2). Additionally, specific bands were detected in both the wild and cultivated soils. In Figure 1, bands 10-13 were detected only in the soil of wild condition, and 1-7 only in the cultivated soil. In addition, there were only two mutual bands (8 and 9) between soil of the wild and cultivated condition. Thus, differences of the bacterial communities between wild and cultivated growth conditions were clear in this study.

The bacterial community structures were also influenced by plant growth stages. The band numbers (R) and Shannon-Weiner indices (*H*) of the wild and cultivated conditions (except wild CK soil, CK meant the control) all followed bud-breaking phase > growing phase > defoliation phase, but the Simpson index (*S*) showed different patterns between the rhizospheric and control soils. The Simpson index (*S*) in rhizospheric soil had the same dynamic change as band numbers (R) and Shannon-Weiner indices, but the control had not obviously ordered. The PieLou index (*J*) was highest at the defoliation phase in both land-use conditions, except cultivated rhizospheric soils (Figure 1 and Table 2). On the whole, the band numbers (R) and Shannon-Weiner index (*H*) of the rhizospheric soil from wild and cultivated *C. sachalinensis* in the bud-breaking phase were both higher than the control, whereas the PieLou index (*J*) and Simpson index (*S*) were reversed. However, each index of the wild and cultivated soil at the growing and defoliation phases performed differently. When the band numbers (R) and Shannon-Weiner index (*H*) of the rhizospheric soil were higher than the control in the cultivated condition and the control was higher than the rhizospheric soil in the wild condition, the PieLou index (*J*) and Simpson index (*S*) were reversed.

Cluster dendrogram analysis of DGGE profiles

Cluster analysis (UPGMA) of DGGE band patterns of wild and cultivated *C. sachalinensis* rhizospheric soil showed

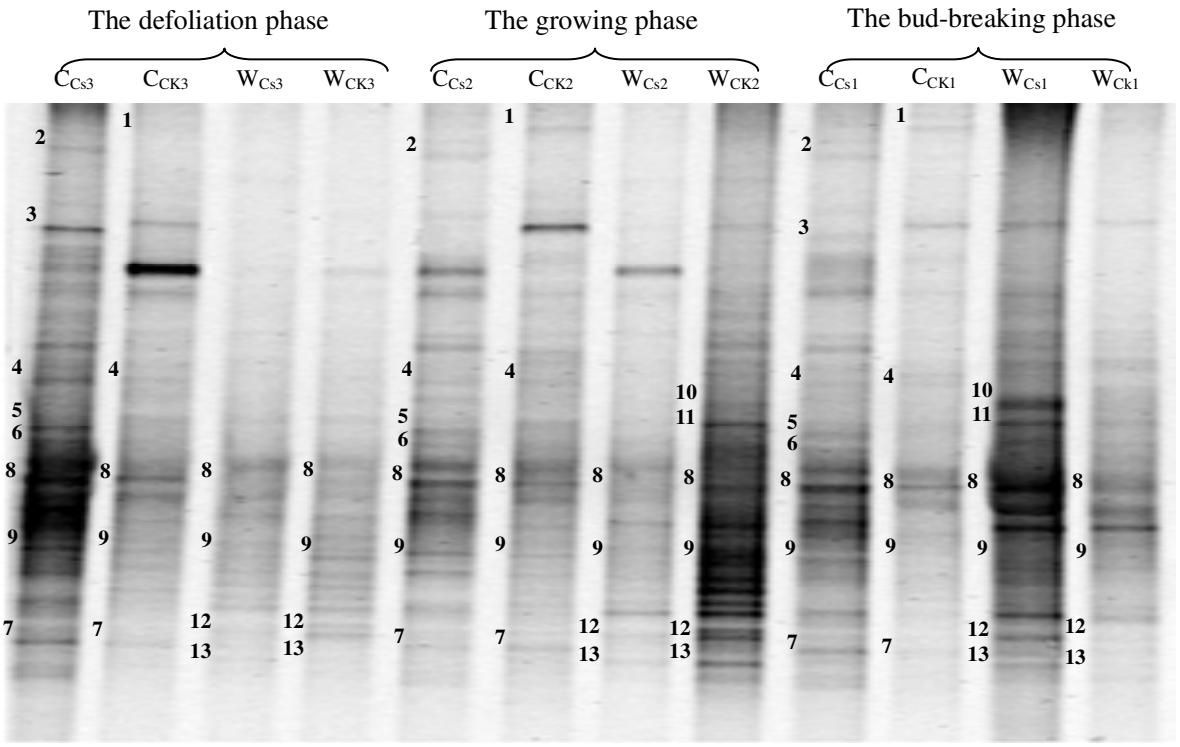

Figure 1. DGGE profiles of bacterial communities in wild and cultivated *C. sachalinensis* rhizospheres at different growth stages. W_{CK1}, W_{Cs1}, C_{CK1} and C_{Cs1} represent the Table 1 soil samples in the germinating phase; W_{CK2}, W_{Cs2}, C_{CK2} and C_{Cs2} represent the Table 1 soil samples in the growing phase; W_{CK3}, W_{Cs3}, C_{CK3} and C_{Cs3} represent the Table 1 soil samples in the defoliation phase. The same nomenclature scheme is the same as that of Figure 2.

Table 2. Detected band numbers, Shannon-Weiner, PieLou and Simpson indices in DGGE profiles of wild and cultivated *C. sachalinensis* rhizospheric bacteria.

Soil sample	Detected band number (R)	Shannon-Weiner index (*H*)	PieLou index (*J*)	Simpson index (*S*)
W_{CK1}	13	2.563	0.9991	0.0773
W_{Cs1}	20	2.988	0.9975	0.0507
C_{CK1}	13	2.564	0.9995	0.0771
C_{Cs1}	21	3.041	0.9987	0.0480
W_{CK2}	21	3.034	0.9966	0.0486
W_{Cs2}	18	2.889	0.9997	0.0557
C_{CK2}	17	2.832	0.9994	0.0590
C_{Cs2}	20	2.993	0.9989	0.0503
W_{CK3}	17	2.832	0.9997	0.0589
W_{Cs3}	13	2.564	0.9997	0.0771
C_{CK3}	19	2.943	0.9995	0.0528
C_{Cs3}	20	2.984	0.9961	0.0511

W_{CK}: the control in wild condition; W_{Cs}: rhizospheric soil of C. *sachalinensis* in the wild condition; C_{CK}: the control in cultivated condition; C_{Cs}: rhizospheric soil of C. *sachalinensis* in the cultivated condition. 1, 2, 3 represent the defoliation phase, the growing phase and the bud-breaking phase respectively.

that the dendrogram was divided into two groups, named Clusters I and II. Cluster I consisted of all soil samples obtained from the cultivated condition, and Cluster II contained the soil samples collected in the wild condition (Figure 2). The similarity reached 55 and 61%, respectively. However, the similarity between Clusters I

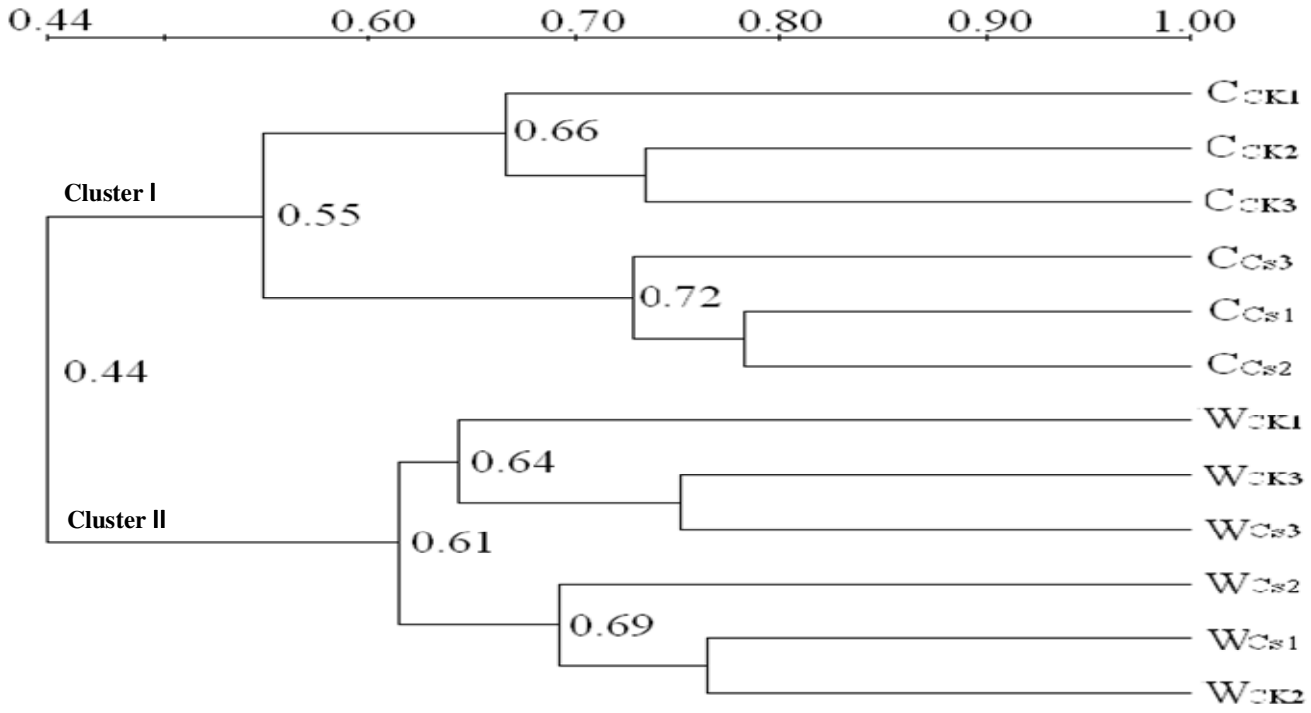

Figure 2. Cluster dendrogram analysis of DGGE profiles of bacterial communities in wild and cultivated *C. sachalinensis* Kom. rhizospheres at different growth stages.

and II is only 44%. This result clearly showed a distinction of the bacterial community between wild and cultivated soil. Higher similarities were found among the similar growth conditions and divergent relationships were observed in the different conditions, demonstrating that the growth conditions and agricultural management practices greatly influenced the soil bacterial community structures.

Sequences of DGGE bands

The bands in the DGGE profiles represent the dominant microbial populations (Muyzer et al., 1993). To gain insight into the major bacterial populations in the wild and cultivated *C. sachalinensis* rhizosphere, thirteen prominent DGGE bands were excised for sequencing (Figure 1). A neighbor-joining tree based on the sequences of the 13 bands, together with reference sequences obtained by BLAST searches from the DNA database is shown in Figure 3. It demonstrates that the dominant populations could be divided into eight groups: *Sphingobacteria*, *γ-Proteobacteria*, *α-Proteobacteria*, *Acidobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Actinobacteria*, and unclassified bacteria. Sequence information is shown in Table 3.

Among the 13 sequenced bands, the *Actinobacteria* and *Proteobacteria* both covered 23.08% (3/13) and thus, represented the most dominant bacteria groups in the *C. sachalinensis* rhizosphere. The *Firmicutes* and *Sphingobacteria* followed at 15.38% (2/13) of the bacterial community in the soil samples. Additionally, *Acidobacteria*, *Gemmatimonadetes*, and unclassified bacteria each represented a smaller proportion of 7.69% (1/13). Interestingly, *Sphingobacteria*, *Acinetobacter* (*γ-Proteobacteria*), *Arthrobacter* (*Actinobacteria*), and unclassified bacteria were detected only in the cultivated soil, while *Firmicutes*, *Gemmatimonadetes*, and unidentified *eubacterium* (*Actinobacteria*) were only in the soil of wild condition. However, *α-Proteobacteria* and *Acidobacteria* were common among both growth conditions of *C. sachalinensis*.

DISCUSSION

This study investigates the shifts of bacterial community structures in the *C. sachalinensis* rhizosphere due to growth stages and growth conditions. Generally, the complex microbial community in the soil environment had been proven to be related to multiple factors (Kennedy et al., 2005; Ausec et al., 2009). However, microbial-based indicators of soil quality are believed to be more dynamic than those based on physical and chemical properties, with the potential to serve as early signals of soil degradation or improvement (Salinas-García et al., 2002).

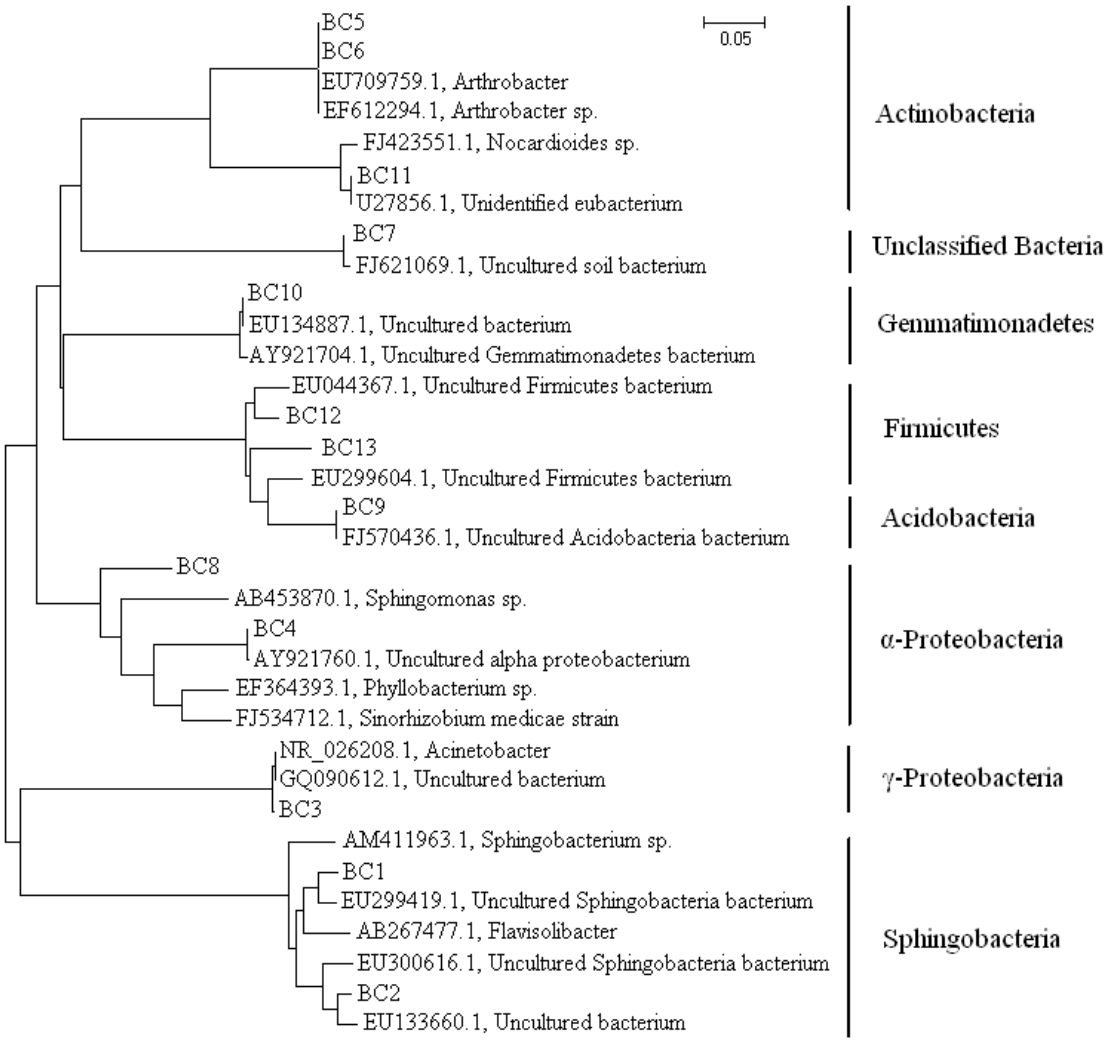

Figure 3. Phylogenetic tree including the thirteen excised DGGE bands and their related sequences. The scale bar indicates 0.05 estimated changes per nucleotide, and the numbers indicate bootstrap values representing percent confidence of 500 replicate analyses.

The importance of edaphic factors in shaping microbial communities has been established by a number of studies (Bååth and Anderson, 2003; Singh et al., 2006). Buckley et al. (2006) found that both soil management history and compost amendment had significant effects on the *Planctomycetes* diversity, and variations in soil organic matter, Ca^{2+} content, pH and spatial heterogeneity of nitrate were associated with variations in the *Planctomycetes* community composition. In the two soil types in this study, conventionally tilled (cultivated soil) and uncultivated (soil of wild condition), the pH values and nutrient contents were higher in the former than the later, which played critical roles in bacterial diversity (Tables 1 and 2). In accordance with Gelsomino et al. (1999), our results confirm the hypothesis that similar land-use conditions tend to contain similar bacterial community structures (example, dominating bacterial types) as revealed by the DGGE profiles. Similarly, Lauber et al. (2008) demonstrated that the composition of bacterial and fungal communities was most strongly correlated with specific soil properties, especially pH. A recent study investigating several sites in the North Carolina pocosin bogs and Florida Everglades showed that bacterial community composition and diversity responded strongly to soil pH, with an increase in the abundance of *Acidobacteria* at lower pH (Hartman et al., 2008). Our data clearly indicate that bacterial community structures are not only correlated with the soil pH but also nutrient status. Soil management practices can directly change the rhizosphere environment of plants. Extensive studies demonstrated perturbbations of microbial community equilibrium populations

Table 3. Informations of the sequenced bands of wild and cultivated *C. sachalinensis* rhizospheric bacteria.

Band	Number of base	GenBank accession number	Related GenBank sequence	Similarity identity (%)	Putative species
BC1	405	GQ981474	EU299419.1 AB267477.1	95 94	*Sphingobacteria*
BC2	405	GQ981475	AM411963.1 EU133660.1 EU300616.1	94 96 94	
BC3	420	GQ981476	NR_026208.1 GQ090612.1	99 99	*γ-Proteobacteria*
BC4	391	GQ981477	AY921760.1 EF364393.1 FJ534712.1	99 92 92	*α-Proteobacteria*
BC5	413	GQ981478	EU709759.1	99	*Actinobacteria*
BC6	415	GQ981479	EF612294.1	99	
BC7	418	GQ981480	FJ621069.1	98	*unclassified bacteria*
BC8	396	GQ981481	AB453870.1	91	*α-Proteobacteria*
BC9	412	GQ981482	FJ570436.1	99	*Acidobacteria*
BC10	425	GQ981483	EU134887.1 AY921704.1	98 98	*Gemmatimonadetes*
BC11	411	GQ981484	FJ423551.1 U27856.1	98 98	*Actinobacteria*
BC12	399	GQ981485	EU044367.1	97	*Firmicutes*
BC13	412	GQ981486	EU299604.1	94	

by changes in environmental conditions and soil management practices (Peacock et al., 2001; Smit et al., 2001). Results from this study also suggest that cultivation significantly affects bacterial community structures, distinct dominant groups exist in conventionally tilled soil and uncultivated soil (Figure 1 and Table 2). Buckley and Schmidt (2003) have shown that particular microbial groups are affected by the local environment, patterns of community structures are related to field management, and the effects of cultivation on these communities are long lasting.

Temporal changes in microbial community composition are observed to occur at scales that are relevant to seasonal (or perhaps even meteorological) events. Our data show that the diversities of the bacterial community differs with different growth stages (Figure 1 and Table 2), consistent with most reports (Burke et al., 2003; Mocali et al., 2003). Baudoin et al. (2002) has shown that the influence of the maize rhizosphere environment on bacterial metabolic potentialities is mainly based on the developmental state of the plant. Thus, temporal variability in the composition of soil microbial communities may complicate the interpretation of spatial patterns of microbial abundance in relation to the field characteristics. Mocali et al. (2003) believe that seasonal population dynamics are the result of physiological responses to temperature increases that often accompany plant growth under natural conditions, but Burke et al. (2003) consider this a response to increasing resources from plant growth itself. Different C and N flux throughout the plant growth stages in soil because of plant uptake is a key factor (Tscherko et al., 2004).

DGGE analysis shows different band patterns of bacterial community structures and the richness and evenness of species in the agro-ecosystem (Sun et al., 2004). Some of the discrete DGGE bands were further

purified and sequenced to reveal specific differences in the bacterial community. Our data show that *Proteobacteria* and *Actinobacteria* are the most dominant, followed by the *Firmicutes* and *Sphingobacteria*, and finally *Acidobacteria*, *Gemmatimonadetes* and unclassified bacteria. It is known that *Proteobacteria* are commonly found in agricultural ecosystems (Smit et al., 2001; Sun et al., 2004). Smit et al. (2001) showed that *Proteobacteria* comprised 35% of the bacterial community in a wheat field in the Netherlands. In this study, 23.08% of sequenced DGGE bands were classified as *Proteobacteria*; bands 4 and 8 represent *α-Proteobacteria*, and band 3 represents *γ-Proteobacteria* (Figure 3 and Table 3). Interestingly, bands 3 and 4 were only derived from cultivated soil.

The *Actinobacteria* are a group of Gram-positive bacteria, mostly found in the soil which play an important role in the decomposition of organic materials such as sugars, amino acids, cellulose and chitin (Aislabie et al., 2006). Bands 5, 6 and 11 represented *Actinobacteria* in this study (Figure 3 and Table 3), which also covered 23.08% of sequenced DGGE bands. Bands 5 and 6 were specific to cultivated soil, and band 11 was only detected in the soil of wild condition. Band 9 was common, representing *Acidobacteria* (Figure 3 and Table 3). As other specific components in the soil of wild condition, bands 12 and 13 represented *Firmicutes*, and band 10 represented *Gemmatimonadetes*. The dominance of *Firmicutes* in agro-ecosystems has been reported in Californian soils (Ibekwe et al., 2001; Valinsky et al., 2002). *Gemmatimonas aurantiaca* firstly, was isolated by Zhang et al. (2003) and had the ability to accumulate polyphosphate. However, Wang et al. (2009) reported that *G. aurantiaca* may have important roles in phosphorus removal. Bands 1, 2 and 7 were also specific to cultured soil. Bands 1 and 2 were classified as *Sphingobacteria*, and Band 7 was unclassified. The presence of this major group of bacteria in soil from a clover-grass pasture was also reported (Calheiros et al., 2009). Moreover, It should be noted that the sequences of 13 bands were similar to 16S rDNA sequences reported for unculturable bacteria (except bands 5, 6 and 8), which indicated that the majority of dominating bacteria in the *C. sachalinensis* Kom. rhizosphere were unculturable.

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