



# Diversity of endophytic bacteria in *Caragana microphylla* grown in the desert grassland of the Ningxia Hui Autonomous Region of China

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**ABSTRACT.** The diversity of endophytic bacteria in the sand-fixation plant *Caragana microphylla* was investigated by amplified rDNA restriction analysis and by sequence and phylogenetic comparisons of the 16S rRNA genes. A total of 24, 19, and 17 operational taxonomic units were identified from 16S rDNA libraries of the plant roots, stems and leaves, respectively. Homology analysis revealed a 92-100% identity of bacterial 16S rDNA sequences compared with those in the GenBank database. The bacteria identified by sequence homology fell into the following groups:  $\alpha$ -Proteobacteria,  $\beta$ -Proteobacteria,  $\gamma$ -Proteobacteria, bacilli, and uncultured bacterium. Sequence analysis demonstrated that the roots were colonized predominantly by Bradyrhizobiaceae, while bacteria from Burkholderiaceae and Sphingomonadaceae were predominant in the stems and leaves, respectively. Additionally, the endophytic bacterial community in leaves was more diverse than those in the roots and stems.

Overall, the most abundant bacteria in all three tissues analyzed were from the Sphingomonadaceae and Burkholderiaceae families, although many bacterial populations were found in only a single tissue. These results suggest that the bacterial population of *C. microphylla* is diverse.

**Key words:** Diversity; 16S rDNA; Endophytic bacteria; *Caragana microphylla*

## INTRODUCTION

Endophytic bacteria are defined as those bacteria that can colonize the internal tissue of a living plant for all or part of their life cycle, causing asymptomatic infections entirely within plant tissues (Wilson, 1995). It has been proposed that endophytic bacteria originated in the rhizosphere (Germida et al., 2006). Members of this microbial community have been suggested to play diverse positive roles in the development and growth of host plants, since they are capable of producing phytohormones and siderophores, increasing the hosts' resistance to pathogens and parasites (Burd et al., 1998; Compant et al., 2005; Berg et al., 2005; Feng et al., 2006), as well as promoting biological nitrogen fixation (Han et al., 2005). Additionally, many endophytes can produce bioactive natural compounds that are a potential source of novel products for use in medicine, agriculture, and industry. These natural products are highly effective, with low toxicity and minor environmental impact, and thus are resources for discovering and developing novel antibiotics (Strobel et al., 2004), chemotherapeutic agents (Strobel and Daisy, 2003), and agrochemicals (Brooks et al., 1994; Ryan et al., 2008). To date, endophytic bacteria have been found in a variety of plants, including soybean, potato, rice, wheat, and sugarcane (Coombs and Franco, 2003; Kuklinsky-Sobral et al., 2004; Sun et al., 2008). Understanding the diversity of bacterial endophytes in plants is therefore important for increasing crop production, conserving biodiversity and sustaining agro-ecosystems, as well as for the discovery of novel drugs for the treatment of diseases in humans, plants, and animals (Ryan et al., 2008).

*Caragana microphylla*, a species of the genus *Caragana*, a subfamily of Papilionoideae in the Leguminosae, is a perennial leguminous shrub that is highly tolerant of drought, salt, and extreme cold in relatively poor or sandy, well-drained soils. In the northern regions of China, it has been widely used as a windbreak to protect soils from desertification, as well as forage for livestock, and high-energy firewood (Yan et al., 2007). Furthermore, the roots, flowers, and seeds of *C. microphylla* are used in herbal medicine for their antineuralgic, antirheumatic, and antiarthritic properties. However, to date, relatively little information is available regarding the bacterial community in *C. microphylla*. The aim of this study was to investigate the diversity of the endophytic population in the roots, stems, and leaves of *C. microphylla* by 16S rDNA analysis.

## MATERIAL AND METHODS

### Sampling of plants and DNA extraction

Plant materials were obtained from Baijitan National Nature Reserves of Ningxia Hui Autonomous Region, China. This is a typical desert-type region, located on the south rim of the Maowusu sandland (106°20'-106°37' E, 37°49'-38°20' N). In May 2011, the roots, stems, and leaves of *C. microphylla* were collected randomly from plants separated by at least 100 m

in a plantation that was more than 10 years old. The tissues collected were washed with sterile distilled water and their surfaces disinfected by immersion in 70% ethanol and then in fresh sodium hypochlorite solution (2.5% Cl<sup>-</sup> concentration) for 3 min each, followed by extensive rinsing several times with sterile distilled water. To confirm that the sterilization process was successful, water from the final rinse was tested for bacterial growth on LB medium plates. Uncontaminated tissues were then used for DNA extraction by the CTAB procedure, as described previously (Marquez-Santacruz et al., 2010).

### PCR amplification and cloning of bacterial 16S rDNA

The 16S rDNA of endophytic bacteria was amplified using the universal primers 799f: 5'-AACAGGATTAGATACCCTG-3' and 1492r: 5'-GGTTACCTTGTTACGACTT-3'. The 50- $\mu$ L PCR mixture contained 50 ng plant tissue DNA, 250 pmol each primer, 5  $\mu$ L 10X PCR buffer, 2.5 U Taq DNA polymerase (Takara, Dalian, China), and 100  $\mu$ M dNTP mixture. The PCR temperature cycling program consisted of an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min; followed by a final extension at 72°C for 7 min. The PCR products were examined by running them on a 1% agarose gel, and the desired PCR product was purified using the PCR Purification Kit (Promega, USA) as per the manufacturer instructions. The purified PCR fragments were then cloned into the pGM-T Easy Vector (TIANGEN, Beijing, China), and positive clones with DNA insertions were identified as white colonies on LB medium containing 80  $\mu$ g/mL X-Gal and 0.5 mM IPTG.

### Amplified ribosomal DNA restriction analysis (ARDRA), sequencing, and phylogenetic analyses

One hundred positive clones bearing the 16 rDNA PCR product amplified from DNA extracted from roots, stems, or leaves were randomly picked, and plasmid DNAs isolated from these clones were then digested with *Hae*III or *Alu*I restriction endonuclease (Fermentas, USA). The restriction fragments were separated on 2% agarose gels. Clones that had identical ARDRA patterns in digests with each of the two restriction endonucleases were grouped into an operational taxonomic unit (OTU). Plasmids of three representative clones from each OTU from the roots, stems, and leaves of the plants were sequenced on an ABI 3730 DNA sequencer at Sangon Biotech Co. Ltd. (Shanghai, China). The 16S rDNA sequences acquired were further analyzed for the presence of possible chimeric sequences by using the CHIMERA-CHECK program and then analyzed using BLAST in the GenBank database (<http://www.ncbi.nlm.nih.gov/>) to explore the identity by sequence alignment and the CLUSTAL X1.8 software (BioWareDB.org) was also used. Phylogenetic trees were constructed by the neighbor-joining method using the MEGA 4.0 software. The statistical significance levels of the interior nodes were determined by bootstrap analysis with 1000 replications of each sequence.

## RESULTS

### ARDRA analysis of bacterial communities

16S rDNA clone libraries were constructed from the endophytic bacteria of *C. micro-*

*phylla* and analyzed based on their ARDRA restriction patterns. The analysis identified 24, 19, and 17 OTUs in libraries from *C. microphylla* roots, stems, and leaves, respectively. Homology analysis of OTU representatives revealed that the bacterial 16S rDNA sequences were 92-100% identical to those in the GenBank database. The sequences obtained in this study can be accessed in GenBank (accession Nos. JQ795016-JQ795086).

The sequence analysis showed that all the isolated bacterial clones were from  $\alpha$ -Proteobacteria,  $\beta$ -Proteobacteria,  $\gamma$ -Proteobacteria, bacilli, and uncultured bacterium, with a majority from the Proteobacterial phyla (Tables 1-3).

**Table 1.** Diversity of the community of endophytic bacteria in the roots of *Caragana microphylla*.

Class	Family (No. of OTUs)	NCBI alignment (No. of clones)	Max identity %
$\alpha$ -Proteobacteria	Bradyrhizobiaceae (6)	<i>Bradyrhizobium</i> sp FR753090 (2)	97%
		<i>Bradyrhizobium</i> sp HQ233230 (4)	99%
		<i>Bradyrhizobium</i> sp JN377665 (5)	99%
		<i>Bradyrhizobium japonicum</i> FJ436370 (8)	96%
		Uncultured <i>Bradyrhizobium</i> sp AB512187 (6)	100%
		Uncultured <i>Bradyrhizobium</i> sp GQ129971 (4)	99%
	Sphingomonadaceae (1)	<i>Sphingomonas</i> sp HQ530510 (8)	95%
		Uncultured Erythrobacteraceae bacterium clone FJ516791 (4)	92%
		Erythrobacteraceae (1)	
		Caulobacteraceae (1)	<i>Phenylobacterium lituiforme</i> NR_029117 (1)
	Rhizobiaceae (1)	<i>Rhizobium</i> sp EF638788 (6)	97%
		-	Alpha proteobacterium FJ711201 (4)
-		Uncultured Burkholderiales bacterium clone JF277911 (2)	98%
$\beta$ -Proteobacteria	Burkholderiaceae (2)	<i>Cupriavidus respiraculi</i> EU221394 (6)	99%
		<i>Lysobacter yangpyeongensis</i> NR_043625 (5)	96%
$\gamma$ -Proteobacteria	Xanthomonadaceae (6)	<i>Lysobacter</i> sp GU385868 (2)	96%
		Uncultured Xanthomonadaceae bacterium clone JN082695 (3)	96%
		Uncultured Xanthomonadaceae bacterium clone EU305594 (1)	96%
		Uncultured Xanthomonadaceae bacterium clone DQ230964 (1)	99%
		Uncultured gamma proteobacterium clone AY622241 (1)	97%
		-	<i>Paenibacillus elgii</i> AY090110 (4)
Bacilli	Paenibacillaceae (1)	<i>Bacillus</i> sp AY556409 (1)	99%
	Bacillaceae (2)	<i>Bacillus</i> sp JN604334 (4)	99%
-	-	Uncultured bacterium clone HQ190331 (8)	99%
-	-	Uncultured bacterium clone FJ612147 (10)	99%

**Table 2.** Diversity of the community of endophytic bacteria in the stems of *Caragana microphylla*.

Class	Family (No. of OTUs)	NCBI alignment (No. of clones)	Max identity %
$\alpha$ -Proteobacteria	Sphingomonadaceae (1)	<i>Sphingomonas</i> sp AB265150 (6)	97%
	Phyllobacteriaceae (1)	<i>Mesorhizobium</i> sp AB604651 (3)	97%
	Bradyrhizobiaceae (1)	Uncultured <i>Bradyrhizobium</i> sp clone AB512187 (5)	100%
$\beta$ -Proteobacteria	Comamonadaceae (1)	<i>Delftia</i> sp EU304256 (8)	100%
	Burkholderiaceae (4)	<i>Burkholderia</i> sp FJ025138 (4)	93%
		<i>Burkholderia caledonica</i> NR_025057 (1)	94%
		<i>Ralstonia</i> sp FJ984446 (23)	97%
		<i>Ralstonia solanacearum</i> EF016361 (2)	93%
$\gamma$ -Proteobacteria	Pseudomonadaceae (1)	<i>Pseudomonas</i> sp AY247063 (4)	100%
	Pasteurellaceae (1)	<i>Haemophilus</i> sp AM420172 (4)	95%
	Enterobacteriaceae (3)	<i>Serratia</i> sp HM245061 (2)	96%
		<i>Pantoea</i> sp HM159968 (11)	95%
		<i>Enterobacter</i> sp GQ478257 (7)	97%
Bacilli	Streptococcaceae (2)	<i>Streptococcus</i> sp EU071520 (12)	99%
		<i>Streptococcus</i> sp EU341245 (2)	97%
-	Staphylococcaceae (1)	<i>Staphylococcus</i> sp FJ957767 (1)	99%
-	-	Uncultured bacterium clone AY218754 (2)	95%
-	-	Uncultured bacterium clone EF397376 (2)	92%
-	-	Uncultured bacterium clone FJ983108 (1)	93%

**Table 3.** Diversity of the community of endophytic bacteria in the leaves of *Caragana microphylla*.

Class	Family (No. of OTUs)	NCBI alignment (No. of clones)	Max identity %
$\alpha$ -Proteobacteria	Sphingomonadaceae (1)	<i>Novosphingobium</i> sp FJ889321 (30)	97%
	Rhodobacteraceae (1)	<i>Paracoccus</i> sp JN561152 (1)	98%
	Acetobacteraceae (1)	<i>Acidisphaera</i> sp AB512155 (1)	97%
$\beta$ -Proteobacteria	-	Uncultured alpha proteobacterium FJ517710 (2)	93%
	Comamonadaceae (2)	<i>Pelomonas puraquae</i> AM501441 (5)	97%
	-	<i>Delftia</i> sp GU560163 (12)	99%
	Alcaligenaceae (1)	<i>Achromobacter</i> sp GU056301 (14)	99%
	Burkholderiaceae (2)	<i>Burkholderia</i> sp EU071528 (7)	99%
$\gamma$ -Proteobacteria	-	<i>Burkholderia</i> sp GQ181152 (1)	95%
	Pseudomonadaceae (2)	<i>Pseudomonas</i> sp AM913900 (1)	97%
	-	<i>Pseudomonas</i> sp AY288072 (3)	97%
	Moraxellaceae (1)	<i>Acinetobacter</i> sp DQ421391 (1)	99%
	Xanthomonadaceae (1)	<i>Stenotrophomonas</i> sp GU179638 (1)	99%
Bacilli	Enterobacteriaceae (1)	<i>Enterobacter</i> sp FN433019 (12)	99%
	Staphylococcaceae (1)	<i>Staphylococcus hominis</i> JN644561 (1)	99%
	Bacillaceae (1)	<i>Bacillus</i> sp JN604334 (2)	99%
	Enterococcaceae (1)	<i>Enterococcus</i> sp JF346884 (6)	99%

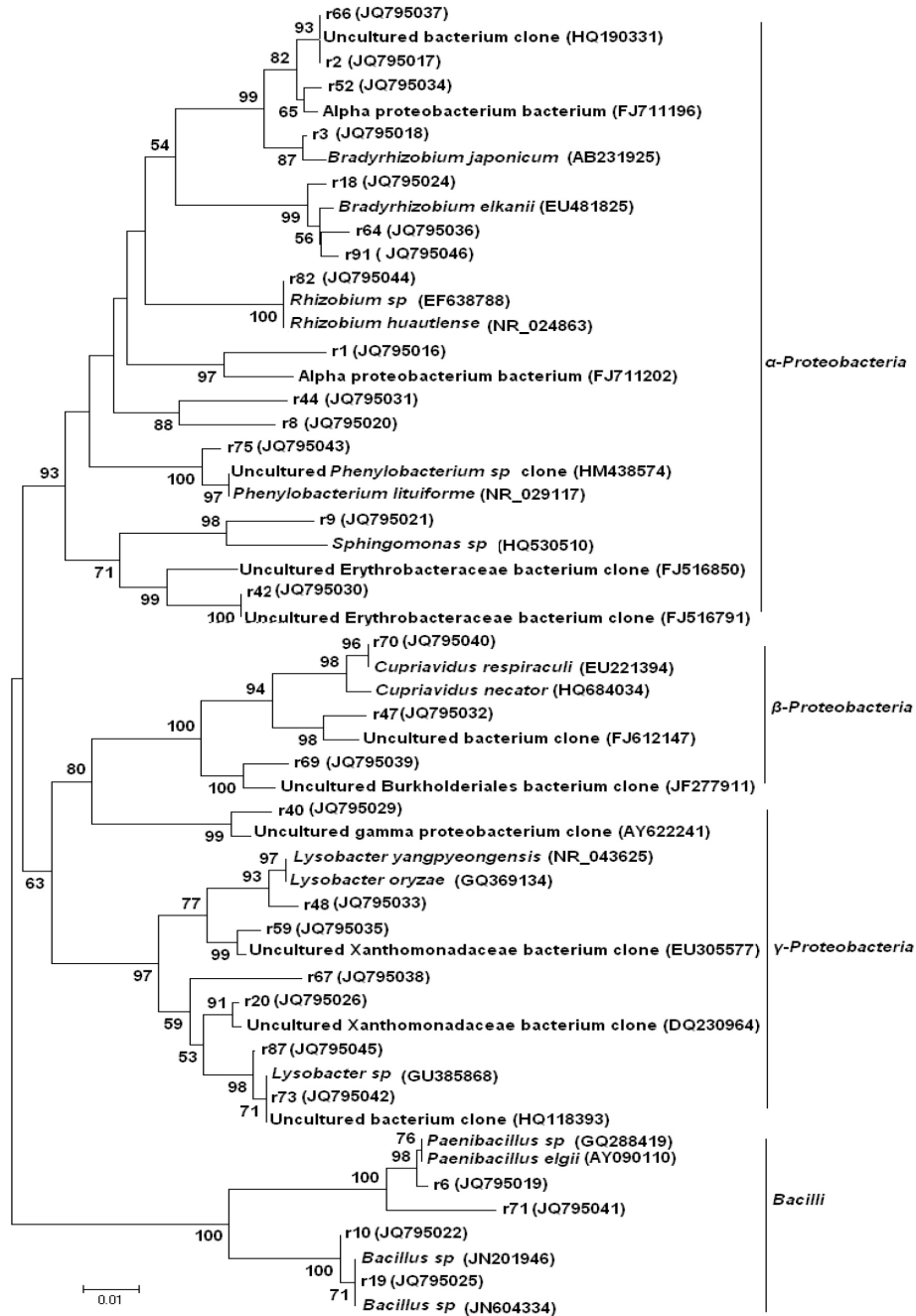
### Phylogenetic analysis of the bacterial community in different plant tissues

The endophytic bacterial community of *C. microphylla* consisted of 20 families. Phylogenetic analysis showed that the community composition varied in different plant tissues. The bacteria identified from the roots, stems, and leaves fell into 9, 10, and 13 families, respectively. Among them, bacteria representing Paenibacillaceae, Erythrobacteraceae, Caulobacteraceae, and Rhizobiaceae were detected only in the roots; members of Streptococcaceae, Pasteurellaceae, and Phyllobacteriaceae were found exclusively in the stems; and bacteria from Rhodobacteraceae, Enterococcaceae, Acetobacteraceae, Moraxellaceae, and Alcaligenaceae were identified only in leaf tissues. The most common bacteria were members of the Sphingomonadaceae and Burkholderiaceae families, which were detected in all three tissues (Tables 1-3).

The results of phylogenetic analysis indicated that the bacterial clones from the roots of *C. microphylla* consisted of five groups, with a majority (52%) from the  $\alpha$ -Proteobacteria, with *Bradyrhizobium* the predominant genus, making up 29% of the total (Figure 1). The  $\gamma$ -Proteobacteria were assigned to the family Xanthomonadaceae, which accounted for 12% of root clones and had 96-99% sequence identity with *Lysobacter* and uncultured bacterium.  $\beta$ -Proteobacteria were represented by a single genus, *Cupriavidus*. Bacilli consisted of Paenibacillaceae and Bacillaceae, and showed 99% 16S rDNA sequence identity with *Paenibacillus* and *Bacillus*.

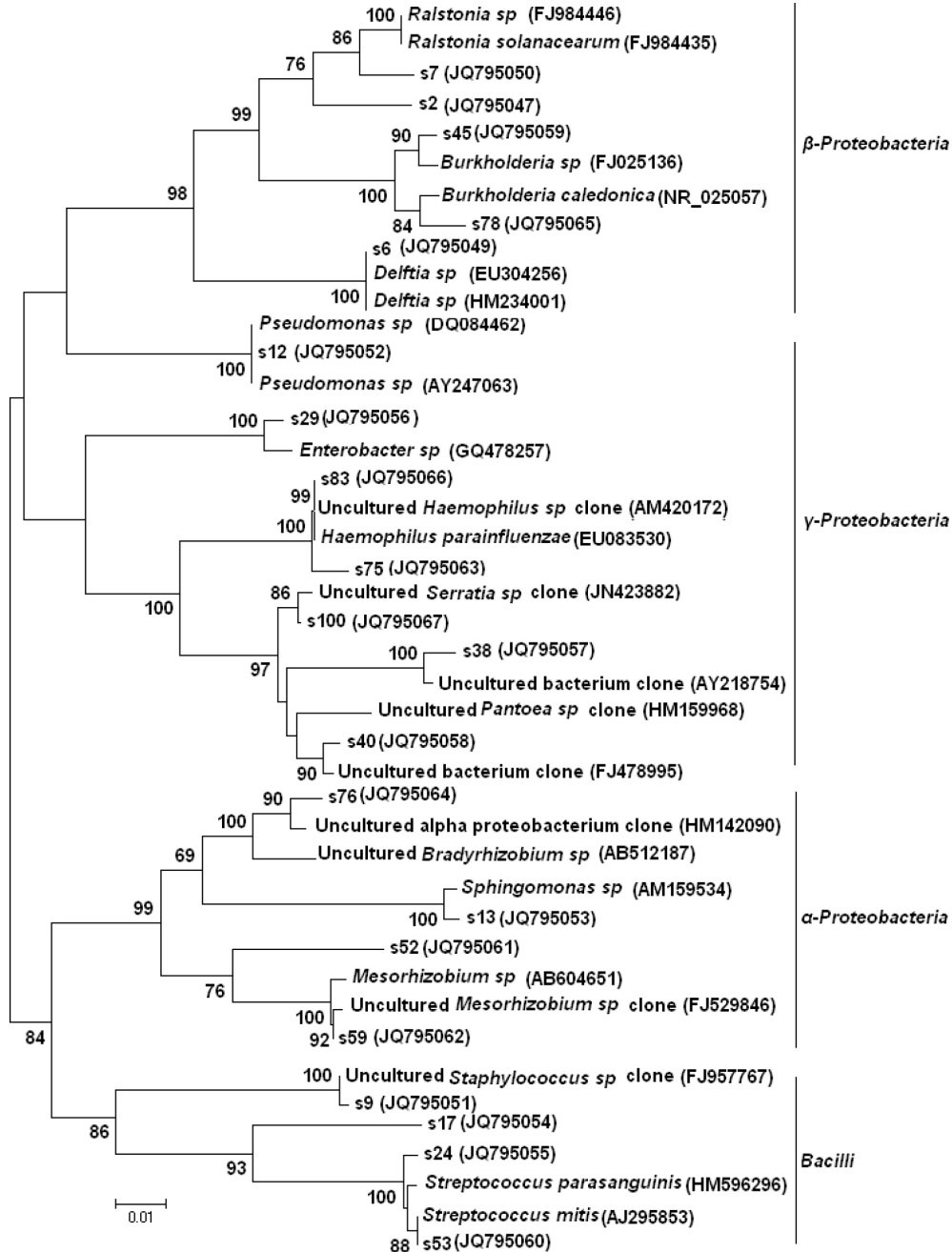
Bacterial clones from the stems also fell into five groups:  $\alpha$ -Proteobacteria (14%),  $\beta$ -Proteobacteria (38%),  $\gamma$ -Proteobacteria (28%), bacilli (15%), and uncultured bacterium (5%) (Figure 2). The predominant clones were from the Burkholderiaceae family (30%). The bacterial clones isolated from the stem were assigned to 13 genera, among which *Ralstonia*, *Pantoea*, and *Streptococcus* were the most common. Eleven of the 13 genera detected in the stems were not found in the root library. Only *Bradyrhizobium* and *Sphingomonas* occurred in both libraries.

The bacterial clones detected in leaf tissues fell into four classes, the most common being  $\alpha$ -Proteobacteria (34%) and  $\beta$ -Proteobacteria (39%), with  $\gamma$ -Proteobacteria and Bacilli accounting for 18% and 9%, respectively (Figure 3). The predominant clones were from the Sphingomonadaceae family, of which *Novosphingobium*, *Delftia*, and *Achromobacter* were the major genera. The diversity of endophytic bacteria in the leaves of this plant was greater than that in the roots and stems, comprising 13 families. Fourteen genera could be assigned, among which eight were detected only in the leaves.

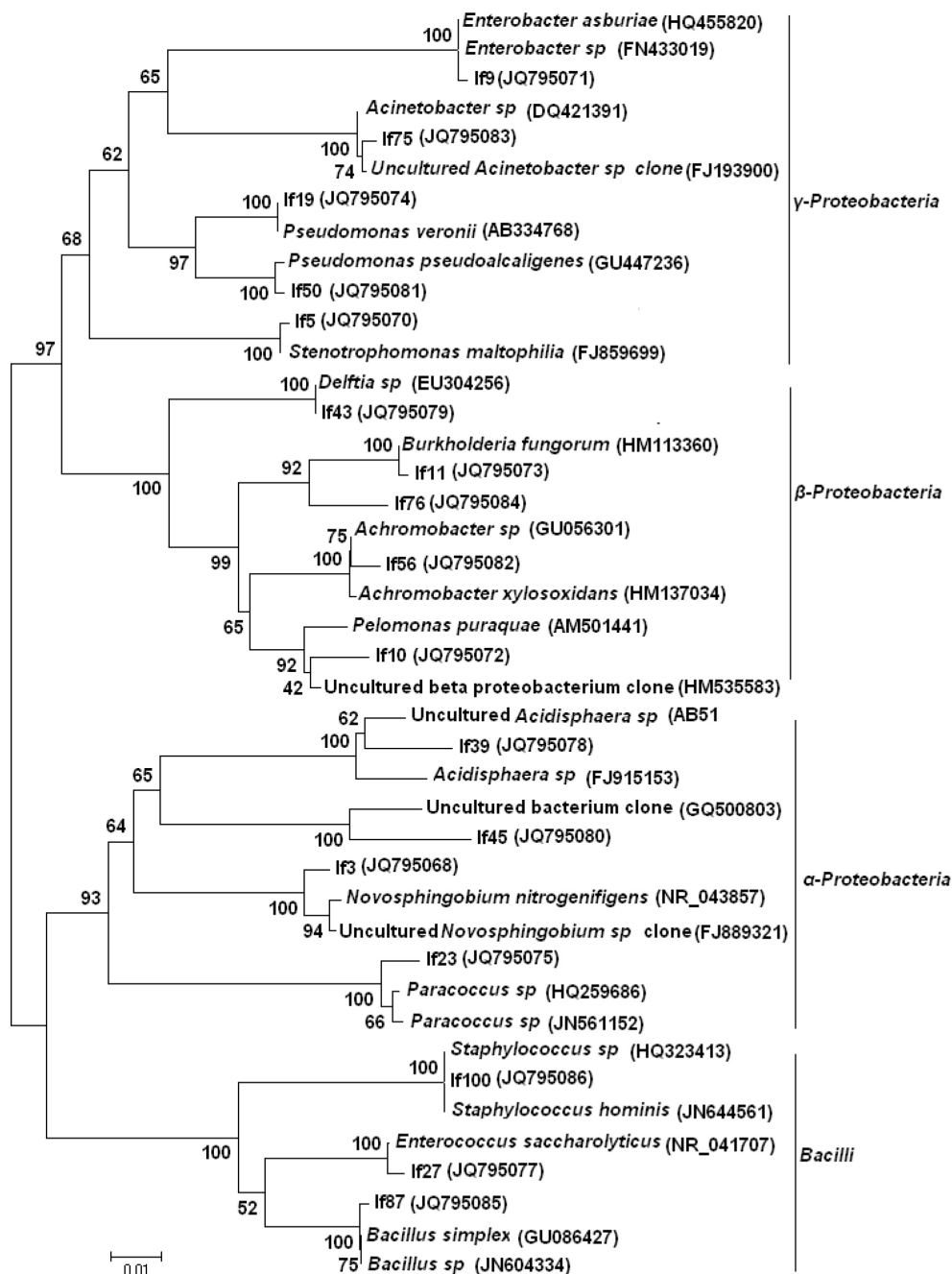


**Figure 1.** Phylogenetic tree based on the 16S rDNA sequences of endophytic bacteria clones from the roots of *Caragana microphylla*. The dendrogram was constructed with the neighbor-joining method. Bootstrap values greater than 50% (based on 1000 bootstrap resamplings) were labeled at the nodes. Scale bar represents 1% sequence divergence.





**Figure 2.** Phylogenetic tree based on the 16S rDNA sequences of endophytic bacteria clones from the stems of *Caragana microphylla*. The dendrogram was constructed with the neighbour-joining method. Bootstrap values greater than 50% (based on 1000 bootstrap resamplings) were labeled at the nodes. Scale bar represents 1% sequence divergence.



**Figure 3.** Phylogenetic tree based on the 16S rDNA sequences of endophytic bacteria clones from the leaves of *Caragana microphylla*. The dendrogram was constructed with the neighbour-joining method. Bootstrap values greater than 50% (based on 1000 bootstrap resamplings) were labeled at the nodes. Scale bar represents 1% sequence divergence.



## DISCUSSION

Microbial endophytes establish communities by colonizing plant tissues. They are capable of developing interactions, not only among themselves, but also with their host plants, by which they may influence plant development and growth. Thus, understanding the diversity of endophytic bacteria is important for both ecological and biotechnological studies. Here, we show the diversity of endophytic bacteria in the root, stem, and leaf tissues of *C. microphylla* growing in the desert of Ningxia, China.

The results reported in this study suggest a tissue-dependent endophytic population in *C. microphylla*. Bradyrhizobiaceae were the predominant microbial population in the roots, while Burkholderiaceae and Sphingomonadaceae were the predominant bacteria in the stem and leaf tissues of the plant, respectively. Importantly, many clones identified in this study were found in only one of the three plant tissues. For example, members of *Rhizobium*, *Paenibacillus*, *Phenylobacterium*, *Cupriavidus*, and *Lysobacter* were detected only in the roots; bacteria from *Mesorhizobium*, *Haemophilus*, *Pantoea*, *Serratia*, and *Streptococcus* were found only in the stems; and bacterial members of *Novosphingobium*, *Paracoccus*, *Acidisphaera*, *Pelomonas*, *Achromobacter*, *Acinetobacter*, *Stenotrophomona*, and *Enterococcus* were identified only in leaf tissue. The endophytic bacterial community in leaves was more diverse than in the roots and stems: there was a larger number of different genera, falling into four groups, possibly a result of the selective pressure plants exert on their associated bacterial populations. Endophytic bacteria of plants colonize specific ecological niches in different tissues, which might be a reason for the diversity observed within the bacterial community in this study. This finding is consistent with those from other studies (Hallmann et al., 1997; Ulrich et al., 2008).

The most abundant bacteria detected in this study were Sphingomonadaceae and Burkholderiaceae. With their ability to fix nitrogen and their potential to promote plant development and growth, plant-associated Burkholderiaceae species have recently received increased attention. For example, endophytic Burkholderiaceae species have been detected in several crops (Sun et al., 2008). Similarly, members of the Sphingomonadaceae family are widespread in different types of soils, sediments, and pelagic aquatic environments, and are known for their ability to utilize a wide variety of carbon sources; several are in fact renowned degraders of recalcitrant (xenobiotic) molecules (Leys et al., 2004). Additionally, some sphingomonads were also found to play important roles in the mycorrhizosphere (Boersma et al., 2009).

Other 16S rDNA sequences identified in this study also showed a high degree of identity with common genera within the Bradyrhizobiaceae, Bacillaceae, Enterobacteriaceae, and Pseudomonadaceae families, such as *Bradyrhizobium*, *Bacillus*, *Enterobacter*, and *Pseudomonas*. Members of these genera have been widely studied as endophytes because of their potential use for promoting plant growth and plant health by means of their nitrogen fixation, antifungal, and antiviral activities.

In summary, the data presented in this study add to our knowledge of the diversity of endophytic bacteria associated with *C. microphylla*, a major sand-fixation plant species inhabiting desert and semi-desert areas of China. The culture-independent approach was a limitation of this study. Further studies using plants collected from different geographic location and a culture-dependent approach are required.

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