

Genetic diversity and phylogeny of rhizobia isolated from *Caragana microphylla* growing in desert soil in Ningxia, China

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ABSTRACT. Rhizobia are soil bacteria with the capacity to induce nitrogen-fixing nodules on the roots or stems of legume plants. A total of 40 bacterial isolates from the root nodules of Caragana microphylla growing in desert soil in Ningxia, China, were analyzed for genetic diversity and phylogenetic position. These isolates were classified into 7 types of 16S ribosomal DNA (rDNA) using polymerase chain reaction-restriction fragment length polymorphism analysis. They were grouped into 4 clades, Rhizobium-Agrobacterium, Sinorhizobium, Phyllobacterium, and Bradyrhizobium, when the phylogenies of 16S rDNA, recA, and atpD genes were applied. Phylogenetic analysis showed that the tree generated from the 16S rDNA sequencing agreed with that produced from the recA and atpD genes. By analyzing phylogenetic relationship using the 3 loci, the isolates in the branches of Phyllobacterium and Sinorhizobium could be identified as *P. brassicacearum* and *S. meliloti*. The isolates in the branch of Rhizobium-Agrobacterium were the most abundant microsymbiont of C. microphylla and were designated R. leguminosarum, R. galegae, R. alamii, and A. tumefaciens. Two isolates with low sequence similarity

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to the known species of *Bradyrhizobium* might be novel species in this genus.

Key words: Phylogeny; 16S ribosomal DNA; *recA*; *atpD*; Rhizobia; *Caragana microphylla*

INTRODUCTION

Rhizobia are soil bacteria capable of forming nodules and establishing symbiosis with the roots or stems of legumes plants. During the process of symbiotic association, rhizobia reduce free N_o to nitrogen compound ammonia that is a usable nitrogen resource for the plants. The capability of rhizobia to fix nitrogen has significantly reduced the dependence of agriculture on nitrogen fertilizers. The populations and variety of rhizobia represent a valuable bioresource for the exploitation of bacterial selection in attempts to find bacterial strains with desirable traits that maximize legume crop productivity (Binde et al., 2009). Therefore, investigations of the diversity, dynamics, and characteristics of rhizobia residing in soil have practical importance in both ecology and agriculture. Such studies not only facilitate the discovery of unknown rhizobia but also support research efforts aimed at selecting effective combinations of *Rhizobium*-legume genotypes to exploit the enormous potential of increased nitrogen fixation. Owing to the ecological and economic importance of these microorganisms, their diversity and taxonomy have been extensively investigated during the last 2 decades. To date, more than 70 species of rhizobia from 11 genera in the α -Proteobacteria (including Agrobacterium, Allorhizobium, Azorhizobium, Bradyrhizobium, Devosia, Mesorhizobium, Methylobacterium, Ochrobactrum, Phyllobacterium, Rhizobium, and Sinorhizobium/Ensifer) as well as *Burkholderia*, *Cupriavidus*, and *Herbaspirillum* in the β -Proteobacteria have been reported (Chen et al., 2003; Wang et al., 2006; Willems, 2006; Chen et al., 2008; Muresu et al., 2008). More than 19,700 species of leguminous plants occur throughout the world, but the microsymbionts of only a few have been investigated (Allen, 1981). As more leguminous species in divergent geographical regions are surveyed, more novel rhizobial species may be discovered.

Caragana microphylla, a species of the subfamily Papilionoideae in Leguminosae, is a perennial leguminous shrub that is highly tolerant to drought, salt, and extreme cold in relatively poor or sandy, well-drained soil. It is often used as a windbreak to protect soil from desertification. It is also used as forage for livestock and high-energy firewood in the northern regions of China. The flowers of this plant are an excellent food source for bees, and the seeds can be used as a component of herbal medicines (Yan et al., 2007). A growing body of evidence has focused on the exploration of *Caragana* plants for pharmaceutical compounds and bioremediation potential. However, few studies have focused on the symbiotic characteristics of the plant (Tan et al., 1999; Gao et al., 2002; Yan et al., 2007).

Symbiotic association between rhizobia and legumes plants might be affected by environmental factors. A recent study has shown that rhizobia species are abundant in drought areas in northwest China (Wei et al., 2008). Ningxia is a geographic region with typical features of an arid, semi-arid, and low rainfall area with very high evaporation potential. The soils in this area are poor in organic matter with high salinity and alkalinity. Considering the ecological importance of *C. microphylla* and the lack of information about its microsymbionts, systematic study of the relationship of rhizobia and legumes plants is essential. To this end, the genetic diversity and phylogenetic relationship of rhizobia associated with *C. microphylla*

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grown in the desertification grassland of the Ningxia Hui Autonomous Region were analyzed in this study using methods of 16S ribosomal DNA (rDNA) restriction fragment length polymorphism (RFLP) and sequencing analysis of 16S rDNA, *atpD*, and *recA* genes.

MATERIAL AND METHODS

Bacterial isolation from root nodules of C. microphylla

Fresh root nodules were collected from *C. microphylla* growing in the Baijitan National Nature Reserve of Ningxia. This region is a typical desert in the south rim of the Maowusu sandland (106°20'-106°37' E, 37°49'-38°20' N). The surfaces of collected nodules were first sterilized with 75% ethanol and 0.1% HgCl₂ for 3 min each, then extensively rinsed several times with sterile distilled water before being crushed. The exudate of the crushed nodules was cultured on yeast-mannitol agar (YMA) medium at 28°C for 3-5 days, and a single colony was selected for further culture (Vincent, 1970). To confirm that the sterilization process was successful, we tested the water from the final rinse for bacterial growth in YMA medium. The purity of the culture was validated by repeated streaking on YMA medium and microscopic examination and identification of cellular morphology. All isolates were incubated on YMA slants at 28°C and maintained at 4°C for temporary storage or in 20% glycerol at -70°C for long-term storage. A total of 40 individual bacterial isolates from 120 nodules of 40 plants were analyzed in this study (Table 1).

DNA extraction and 16S rDNA PCR-RFLP

Isolated bacteria were cultured in YMA liquid medium at 28°C for 48-72 h, and the cells were collected by centrifugation at 10,000 rpm at 4°C for 10 min. Total genomic DNA of each strain was extracted as described elsewhere (Menna et al., 2006). Universal primers corresponding to *Escherichia coli* 16S rDNA positions 8-27 and 1492-1509 (Weisburg et al., 1991) were used to amplify 16S rDNA using PCR (Table 2). The PCR products were digested with restriction endonucleases *MspI*, *Hae*III, and *Hin*fI (Takara Biotech, Japan) as recommended elsewhere (Laguerre et al., 1994). The resulting fragments were resolved with electrophoresis on 2.5% (w/v) agarose gels. The RFLP patterns obtained from the digestion were combined and used in cluster analysis with the Gelcompar II software package (Vauterin and Vauterin, 1992). Isolates that had identical RFLP patterns were regarded as the same 16S rDNA type. The PCR products of 16S rDNA were confirmed through sequencing.

Sequencing and phylogenetic analyses of 16S rDNA, recA, and atpD genes

The *atpD* and *recA* genes of representative strains from the seven 16S rDNA types were amplified according to a previously described method (Vinuesa et al., 2005). The parameters and primers for the amplification of *recA* and *atpD* are listed in Table 1. The PCR products were further sequenced on an ABI 3730 DNA sequencer at Sangon Biotech Co., Ltd. (Shanghai, China). The acquired 16S rDNA, *recA*, and *atpD* gene sequences were then blasted in the GenBank database (http://www.ncbi.nlm.nih.gov/) to find similar sequences and were further aligned with CLUSTAL X1.8 (Thompson et al., 1997). Phylogenetic trees were constructed with the neighbor-joining method using Molecular Evolutionary Genetics Analysis 4.0 (Tamura et al., 2007). Statistical significance levels of interior nodes were determined via

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bootstrap with 1000 replications of each sequence.

RESULTS

PCR-RFLP and sequence analysis of 16S rDNA

All isolates were amplified an 1500 bp of single band using a 16S rDNA primer set. and the size of the amplicon was in agreement with the expected size of the 16S rDNA among bacteria as reported elsewhere (Weisburg et al., 1991). Seven rDNA types were identified among the 40 tested strains based on the results of the 16S rDNA PCR-RFLP patterns (see Table 1). Types 2 and 5 were the major groups, representing 30 and 22.5% of the isolates, respectively. To elucidate the phylogenetic relationships of the isolates further, 1 isolate from each rDNA type was selected for additional analysis by sequencing the entire 16S rDNA sequence (7 total isolates were selected). The phylogenetic tree is presented in Figure 1. The result indicated that all of the isolates could be grouped into 4 clades corresponding to *Rhizobium-Agrobacterium*, Sinorhizobium, Phyllobacterium, and Bradyrhizobium. Types 1 and 3 rDNA formed the Phyllobacterium clade, and the representative isolate CNXE1 shared 100, 97, and 97% rDNA similarity to P. brassicacearum, P. myrsinacearum, and P. bourgognense, respectively. Types 2-5 rDNA consisted of the *Rhizobium-Agrobacterium* clades, which contained 30 isolates. Their representative strains had identical 16S rDNA sequences and shared 100, 100, 99, and 100% rDNA similarity to R. leguminosarum, R. galegae, R. alamii, and A. tumefaciens, respectively. Type 6 rDNA represented the Bradyrhizobium clade; isolate CNX211 was identical to Bradyrhizobium sp and they shared 99% similarity of 16S rDNA sequences. However, CNX211 shared only 92% sequence similarity to *B. japonicum* and *B. yuanmingense*. The isolates in rDNA type 7 belonged to the Sinorhizobium clade, which contained 5 isolates. The isolate CNXC3 shared 100% sequence similarity to S. meliloti and 99% to S. medicae, S. fredii, and S. xingjiangensis.

Isolates	rDNA type	NCBI closest match (Sequence similarity %)			
		16S rDNA	recA	atpD	
CNXE1 [†] CNXF1, CNXF2	1	P. brassicacearum (100%)	Phyllobacterium sp (89%)	P. brassicacearum (90%)	
CNXLW24 [†] CNXE2, CNXE3, CNXE4, CNXE5, CNXE7, CNXF5, CNXF6, CNXF7, CNXF8, CNXLW14, CNXLW20	2	R. leguminosarum (100%)	R. leguminosarum (99%)	R. leguminosarum (97%)	
CNXE6 [†] CNX053, CNX054	3	<i>R. galegae</i> (100%)	R. galegae (97%)	R galegae (99%)	
CNXF3 [†] CNXF4, CNXLW17, CNXC7, CNXC8, CNXC10	4	R. alamii (100%)	R. alamii (97%)	R. alamii (95%)	
CNX404 [†] CNX401, CNX402, CNX403, CNX411, CNX412, CNX413, CNX501, CNX502	5	A. tumefaciens (100%)	A. tumefaciens (99%)	A. tumefaciens (99%)	
CNX211 [†] CNX333	6	Bradyrhizobium sp (99%)	Bradyrhizobium sp (93%)	Bradyrhizobium sp (93%)	
CNXC3 [†] CNXC1, CNXC2, CNXC4, CNXC9	7	S. meliloti (100%)	S. meliloti (98%)	S. meliloti (98%)	

†Representative isolates used for sequencing and phylogenetic analysis.

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Figure 1. Phylogenetic trees generated with the 16S rDNA gene sequence. Dendrogram shows the relationships of rhizobial isolates from *Caragana microphylia* root nodules to reference nodule bacteria. The clading pattern was produced by the neighbor-joining method. Numbers at nodes indicate levels of bootstrap support and were based on neighbor-joining analysis of 1000 resampled datasets. Bootstrap confidence levels greater than or equal to 60% are indicated at internodes. Scale bar indicates number of substitutions per site.

Phylogenetic analyses of the *recA* and *atpD* genes

The *recA* gene encodes some of the proteins of the DNA recombination and repair system (Lloyd and Sharp, 1993; Cox, 2003), and numerous phylogenies of *recA* in the bacteria are consistent with the corresponding 16S rDNA phylogenies (Gaunt et al., 2001; Ghosh and Roy, 2006; Martens et al., 2007). The *atpD* gene is a ubiquitous and conserved gene encoding the beta subunit of membrane ATP synthase, which is essential for energy production (Gaunt et al., 2001). The phylogenies of the *recA* and *atpD* genes were very similar to that of 16S rDNA, with well-supported clades for *Rhizobium-Agrobacterium*, *Sinorhizobium*, *Phyllobacterium*, and *Bradyrhizobium* (Figures 2 and 3). The sequence similarities of the representative isolates are listed in Table 2. Isolate CNXE1 and the reference strains consisted of the *Phyllo*-

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bacterium clade, but they shared only 89% similarity of the *recA* gene sequence to *Phyllobacterium* sp and shared 90% *atpD* gene sequence similarity to *P. brassicacearum*. The sequences similarities of the *recA* and *atpD* genes between CNXE1 and other known species of the genus *Phyllobacterium* were less than 88%. The representative strains of 16S rDNA types 2-5 CNXLW24, CNXE6, CNXF3, and CNX404 composing the *Rhizobium-Agrobacterium* clade shared 99, 97, 97, and 99% *recA* gene sequence similarity to *R. leguminosarum*, *R. galegae*, *R. alamii*, and *A. tumefaciens*, respectively. The sequence similarities of the *atpD* gene in these representative strains to the above reference strains were 97, 99, 95, and 99%, respectively. The CNXC3 isolate had 98 and 91% *recA* and *atpD* gene sequence similarity to *S. meliloti* and *S. meldicae*, respectively. Isolate CNX211 shared 93% *recA* and *atpD* gene sequence similarity to *B. liaoningense*, and *B. japonicum* were 85-88%.



Figure 2. Phylogenetic trees generated with the *recA* gene sequence. Dendrogram shows the relationships of rhizobial isolates from *Caragana microphylia* root nodules to reference nodule bacteria. The clading pattern was produced by the neighbor-joining method. Numbers at nodes indicate levels of bootstrap support and were based on neighbor-joining analysis of 1000 resampled datasets. Bootstrap confidence levels greater than or equal to 60% are indicated at internodes. Scale bar indicates number of substitutions per site.

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Figure 3. Phylogenetic trees generated with the *atpD* gene sequence. Dendrogram shows the relationships of rhizobial isolates from *Caragana microphylia* root nodules to reference nodule bacteria. The clading pattern was produced by the neighbor-joining method. Numbers at nodes indicate levels of bootstrap support and were based on neighbor-joining analysis of 1000 resampled datasets. Bootstrap confidence levels greater than or equal to 60% are indicated at internodes. Scale bar indicates number of substitutions per site.

Table 2. The parameters and primers of PCR.						
Primer	Sequence (5'-3')	PCR parameters ^a	Amplicon size (bp)	GenBank accession No.		
16s rDNA	Forward: 5'-AGAGTTTGATCCTGGCTCAG-3'	94°C 60 s	1500	AF041447		
	Reverse: 5'-TACGGYTACCTTGTTACGACTT-3'	60°C 45 s				
		72°C 60 s				
recA Forv Rev	Forward: 5'-TTCGGCAAGGGMTCGRTSATG-3'	94°C 60 s	500	AJ294376		
	Reverse: 5'-ACATSACRCCGATCTTCATGC-3'	58°C 40 s				
		72°C 60 s				
atpD For Rev	Forward: 5'-ATCGGCGAGCCGGTCGACGA-3'	94°C 60 s	470	AJ294405		
	Reverse: 5'-GCCGACACTTCCGAACCNGCCTG-3'	58°C 40 s				
		72°C 60 s				

^aThe reactions were incubated at 94°C for 4 min prior to amplification using the listed parameters for 30 thermal cycles.

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Diversity of rhizobia isolates associated with C. microphylla

Among the analyzed bacteria, 3 isolates were identified as *Phyllobacterium* and shared 100 and 90% 16S rDNA and *atpD* sequence similarity, respectively, to *P. brassicacearum*. Systematic analysis of the phylogenetic relationship at these 3 loci further identified *Phyllobacterium* as *P. brassicacearum*. Most of the isolates belonged to *Rhizobium*, which were 16S rDNA types 2-4 (containing the 21 isolates listed in Table 2). These isolates had a high sequence identity at all 3 analyzed loci and shared the highest sequence similarity to *R. leguminosarum*, *R. galegae*, and *R. alamii*. Together, the sequencing results of 16S rDNA and *recA* and *atpD* genes indicated they should be designated as *R. leguminosarum*, *R. galegae*, and *R. alamii*. Nine isolates of 16S rDNA type 5 belonged to *Agrobacterium*, and their sequences were identical to that of *A. tumefaciens*. Five isolates from 16S rDNA type 7 were grouped in *Sinorhizobium*. These strains were identified as *S. meliloti* based on their similarities to this bacterium in all analyzed categories.

DISCUSSION

In the present study, PCR-RFLP analysis of 16S rDNA and phylogeny based on 16S rDNA, *recA*, and *atpD* genes were used to analyze the genetic diversity of rhizobia isolates associated with *C. microphylla* growing in desert soil in Ningxia. The results demonstrated the genetic diversity and phylogeny of rhizobia in this region.

Studies of the diversity of rhizobia rely mainly on the analysis of 16S rDNA sequences for species-level identification. However, several studies have shown that 16S rDNA genes are capable of undergoing recombination and horizontal transfer that might result in sequence mosaicism (Eardly et al., 1996; Vinuesa et al., 2005). Another disadvantage of bacterial identification based on 16S rDNA analysis is the difficulty in discriminating between closely related species owing to high levels of sequence conservation between these species (Acinas et al., 2004; Martens et al., 2007). Thus, a phylogenetic tree generated from a single locus may inadequately reflect the phylogenetic relationship of bacteria. The use of additional gene loci with great sequence divergence as well as sufficient conserved sequences for phylogenetic analysis may be an alternative approach that overcomes the drawbacks of 16S rDNA analysis alone. For example, the use of a housekeeping protein encoding as a supplementary genetic marker has been introduced to identify rhizobia (Martens et al., 2007, 2008; Chen et al., 2008). Additional housekeeping genes including atpD, glnI, glnII, recA, and dnaK have recently been used in taxonomic and phylogenetic studies of rhizobia (Gaunt et al., 2001; Naser et al., 2005; Vinuesa et al., 2005; Ghosh and Roy, 2006; Wang et al., 2007). Martens et al. (2008) have demonstrated that the sequencing of housekeeping genes is superior to DNA-DNA hybridization for the assessment of genetic relationships between species of the genus *Ensifer*.

A number of *Agrobacterium* strains isolated from root nodules of various legumes have been reported to lose nodulating capability with their host plants (Tan et al., 1999; Gao et al., 2004). One reasonable explanation is that they were never symbiotic but could invade nodules as endophytic bacteria (Wang et al., 2006). The group defined as *S. meliloti* has often been considered a rhizobial species with a host range limited to some species in the genera *Medicago, Melilotus,* and *Trigonella* (Szeto et al., 1984). However, a novel biovar of *S. meliloti* effectively nodulating *Phaseolus vulgaris* has been recently isolated in Africa (Mnasri et al., 2007), which might indicate that the 5 *S. meliloti* isolates identified as originating from *C*.

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microphylla in the present study enlarged the host range of *S. meliloti*. Despite 2 isolates having been previously identified as *Bradyrhizobium*, a common microsymbiont of many tropical legumes (Qian et al., 2003), their 16S rDNA similarities to all known species of the genus *Bradyrhizobium* were less than a "threshold" for species definition: 97% (Stackebrandt and Goebel, 1994). Moreover, the sequence similarities of *recA* and *atpD* genes to *B. yuanmingense*, *B. liaoningense*, and *B. japonicum* were very low (85-88%) as well. Therefore, these 2 isolates might represent novel species.

In this study, 40 rhizobia isolates originating from nodules of *C. microphylla* growing in desert soil in Ningxia, China, were classified into seven 16S rDNA types using PCR-RFLP and grouped into 4 clades-*Rhizobium-Agrobacterium*, *Sinorhizobium*, *Phyllobacterium*, and *Bradyrhizobium*-based on the phylogenies of 16S rDNA, *recA*, and *atpD* genes. Most of these isolates (21; 52%) belonged to the genus *Rhizobium*, and another 22.5% (9 isolates) were classified in the genus *Agrobacterium*. The other isolates were assigned to the genus *Sinorhizobium* (7 isolates), *Phyllobacterium* (7 isolates), and *Bradyrhizobium* (2 isolates).

These results differed from those of a previous report, in which agrobacteria-related strains were found to be the most abundant bacteria in the root nodules of *Caragana interme-dia* growing in the Maowusu sandland (Inner Mongolia, China) (Gao et al., 2002), as well as a report that *Mesorhizobium* spp are the main microsymbionts of *Caragana* spp growing in the Liaoning Province of China (Yan et al., 2007). *Caragana* sp have the capability for stringent selection of rhizobia symbiotic genotype rather than genomic background, even though lateral transfer of symbiotic genes has been observed in *Caragana* rhizobia and their biogeography exhibit a clear correlation between rhizobial genospecies and eco-regions (Lu et al., 2009). Although the symbiotic association between the partners, it might be also affected by environmental factors. This hypothesis is supported by the fact that legume plants can nodulate with specific rhizobia in distinctive geographic regions, whereas various legume species in the same region might nodulate with the same rhizobial species-such as *B. japonicum* and *Mesorhizobium plurifarium* in tropical regions.

Furthermore, the effects of soil pH on the interaction between rhizobia and their legume hosts have been demonstrated (Yang et al., 2001), as have the effects of abiotic stress on the expression of *nod* genes (Duzan et al., 2004). Our results further confirmed the diversity of rhizobial populations or communities in a distinctive geographic region, suggesting that the symbiotic association between legumes and rhizobia is a result of interactions among rhizobia, legumes, and biogeographic and environmental factors.

The neighbor-joining tree generated in this study using 16S rDNA was consistent with that generated with *recA* and *atpD* genes for the majority of the strains, although the precise branching patterns of various genes in some isolates were not in tight agreement with sequence trees produced with the 16S rDNA gene. For instance, the position of *Phyllobacterium* was ambiguous for each of the 3 genes. This result suggested that phylogenetic position can be influenced by the genes used for analysis, which is consistent with a finding that discordant phylogenies within different loci of rhizobia can result in different phylogenetic tree topologies for rhizobia species (van Berkum et al., 2003).

In conclusion, the results in this study demonstrated that *C. microphylla* growing in desert soil of Ningxia nodulated with diverse rhizobia species, and the phylogenetic lineage *Rhizobium-Agrobacterium* was the most abundant microsymbiont. These data offer new in-

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formation about the *Caragana*-rhizobia association in general and the genetic diversity and phylogeny of symbiotic legume rhizobia in desert soil in particular. Further studies of the correlation of rhizobia, host plant, and environmental factors are required.

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