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Diversity of endophytic bacteria associated with nodules of two indigenous legumes at different altitudes of the Qilian Mountains in China

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ABSTRACT

A total of 201 endophytic root nodule-associated bacteria collected from two legumes indigenous to different Qilian Mountain altitudes (Hexi Corridor) were characterized through 16S rDNA polymerase chain reaction (PCR)-restriction fragment length polymorphism, 16S rRNA gene sequence analysis, and enterobacterial repetitive intergenic consensus-PCR clustering. The isolates phylogenetically belonged to 35 species in the *Phyllobacterium*, *Ensifer*, *Rhizobium*, *Microvirga*, *Sphingomonas*, *Paracoccus*, *Mycobacterium*, *Paenibacillus*, *Cohnella*, *Sporosarcina*, *Bacillus*, *Staphylococcus*, *Brevibacterium*, *Xenophilus*, *Erwinia*, *Leclercia*, *Acinetobacter*, and *Pseudomonas* genera. Phylogenetic *nodA* sequence analysis showed higher similarity to *Sinorhizobium meliloti* with strains related to the *Rhizobium*, *Sinorhizobium*, and *Acinetobacter* genera. Sequence analysis of the *nifH* gene revealed that the strains belonging to *Xenophilus*, *Acinetobacter*, *Phyllobacterium*, and *Rhizobium* had genes similar to those of *Mesorhizobium* and *Sinorhizobium*. The results indicated that horizontal gene transfer could have occurred between rhizobia and non-rhizobial endophytes. Canonical correspondence analysis revealed that altitude and host plant species contributed more to the bacterial endosymbiont separation than other ecological factors. This study provided valuable information on the interactions between symbiotic bacteria, non-symbiotic bacteria and their habitats, and thus provided knowledge on their genetic diversity and ecology.

Introduction

The Qilian Mountains (93.5° E–103° E, 36.5° N–39.5° N) are located on the northeastern margin of the Tibetan Plateau, a transitional area between the Asian Summer Monsoon and the westerlies [25]. Their northern part is the Hexi Corridor of Gansu Province, whereas the southern part includes the Qaidam basin and Qinghai Lake of Qinghai Province [22]. The ecosystems in the area are responsive to climate change. The Qilian Mountains provide 95% of the water consumed by the people and crops in the oases in the Hexi Corridor [18,30]. Thus, the mountains are a large ecological barrier to northwest China, especially to the Hexi Corridor. The plants on the mountains are distributed according to elevation zones. *Caragana jubata* and *Oxytropis ochrocephala* are the dominant leguminous species at an altitude of 3000 m above and below the mountains, respectively.

Caragana, which belongs to subfamily *Papilionoideae* of the family *Leguminosae*, is diverse in the southwestern and Sino-Himalayan regions of Asia. Approximately 100 *Caragana* species are recorded. *Caragana* is a perennial leguminous shrub that is highly tolerant to drought, a salty environment, and extreme cold, and it grows in relatively poor or sandy well-drained soil. Over 70 species within this genus have been recorded [47]. *Caragana* spp. are good livestock feed and high-energy firewood sources, as well as being used as windbreaks in the northern regions of China to protect soil from desertification. *Caragana* spp. flowers are good food sources for bees, and their seeds are used as herbal medicines [30,42]. *Oxytropis* is also a perennial wild plant belonging to subfamily *Faboideae* of the family *Leguminosae*. This plant is distributed in some regions of Gansu, Qinghai, and Sichuan Provinces and is often used as foliage or green manure, as well as in preventing soil erosion. The entire plant is used in Chinese herbal medicine for clearing heat,

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detoxification, invigorating blood circulation, and dissolving stasis [22]. Rhizobia isolated from root nodules of the plants in these two genera have been studied previously [5,6,9,11,23,31,45], and they revealed that distinct rhizobia may nodulate with *Caragana* grown in different Chinese regions. However, few studies demonstrate clear correlations between *Caragana* rhizobia and their geographic origins through statistical analysis. In addition, little has been reported on endophytic bacterial diversity within *Caragana* and *Oxytropis* nodules at different elevations on the regional scale.

An increasing number of endophytic bacteria have been isolated recently from the sterilized nodule surfaces of several legumes. These bacteria do not symbiotically induce nodules or fix nitrogen but their coexistence or assistant nodulation ability has been proven by some earlier studies [3,20,21,24]. It has been proposed that the bacteria form an ecological niche for survival [27,29]. Endophytes isolated from *C. jubata* and *O. ochrocephala* of the Qilian Mountains were collected and characterized in this study to investigate the rhizobial endophyte resource in virginal areas of these mountains, and to elucidate the relationship between the endophyte population and the biogeography of this special environment.

Materials and methods

Collection of root nodules

Root nodules were collected in July 2009 and 2010 from *C. jubata* and *O. ochrocephala* nodules grown at ten Qilian Mountain altitudes (Table 1). The vertical distance between the sampling zones was at least 50 m, and the distance between the individual plants was at least 10 m. The geographical locations of the sampling sites were determined using GPS. The plants were photographed, with the foliage and flowers being preserved as specimens for further identification. Root-attached soils were also collected and stored at 4 °C for subsequent characterization. The physicochemical characteristics of the soil samples, such as organic C content, total N content, C/N, total K content, total P content, and pH, were analyzed (Table S1).

Strain isolation

The tested strains were isolated from nodules using the standard method [39]. Nodules were surface sterilized with 95% alcohol for 30 s and 0.1% $HgCl_2$ for 2 min. Subsequently, the nodules were rinsed five times with sterile distilled water to thoroughly eliminate $HgCl_2$, and then they were crushed using aseptic forceps and streaked onto yeast-mannitol agar (YMA) plates. The bacteria isolated from the nodules were purified by repeatedly streaking on YMA medium, and the purity of each isolate was checked by observing the colony and its cellular morphology. Authenticated cultures were stored on YMA slants at 4 °C for temporary storage and 30% (v/v) glycerol at −80 °C for long-term storage.

Amplification of 16S rRNA, nifH, and nodA genes

The total genomic DNA of each strain was extracted as described by Moulin et al. [26].

The purities and concentrations of the DNA extracts were spectrophotometrically determined at 260 and 280 nm. The polymerase chain reaction (PCR) protocol of 16S rRNA was performed as described by Terefework et al. [35]. Primers P1 (5′-AGAGTTTGATCCTGGCTCAGAACGAACGCT-3′) and P6 (5′-TACGGCTACCTTGTTACGACTTCACCCC-3′), corresponding to *Escherichia coli* 16S rRNA gene positions 8–37 and 1479–506, respectively, were used. *nodA* (acyltransferase) and *nifH* (nitrogenase reductase) genes were amplified in order to confirm the existence of the symbiotic genes. The sequences of the two genes were amplified using the primers *nifH*F/*nifH*I (*nifH*F: 5′-AAGTGCGTGGAGTCCGGTGG-3′; *nifH*I: 5′-GTTCGGCAAGCATCTGCTCG-3′) and *nodA*R/*nodA*F (*nodA*R: TGCRGTGGARDCTRYGCTGGGAAA; *nodA*F: GGNCCGTCRTCRAASGTCARGTA) with a touchdown PCR program, which was performed as described by Xu et al. [43].

PCR-based RFLP fingerprinting of rhizobial isolates

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of genomic genes is useful in rhizobial taxonomy [1,7,17,37]. Restriction endonucleases *Hea*III, *Hinf*I, and *Msp*I were used to separately and directly digest 8 μL of each PCR product, as recommended by Laguerre et al. [16]. Restriction fragments were separated through electrophoresis in 2% (w/v) agarose gels at 80 mV for approximately 3 h. Strains sharing the same RFLP pattern were assigned to one type for RFLP pattern cluster analysis. The representative PCR products were sequenced in both directions with the primers used for PCR amplification, and the sequences were manually corrected using the Vector NTI software (Invitrogen). The different restriction (RFLP) patterns obtained from the three endonucleases were combined and analyzed. Strains with identical combined RFLP patterns were designated as a genotype.

ERIC-PCR fingerprint analysis

Repetitive sequences based on the PCR are widely used to differentiate closely related rhizobia and can be accurate to the strain level [15,24]. Primers ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were used for ERIC-PCR. The amplified ERIC and patterns were screened with electrophoresis in 1.5% (w/v) agarose gel, stained with ethidium bromide, visualized under UV radiation, and photographed.

Phylogenetic analyses

Phylogenetic trees were constructed using the sequence alignments of the *rrs* and symbiotic genes. The sequences were aligned using the CLUSTAL W software [36]. The phylo_win software [8] was used to build neighbor-joining trees with the Jukes–Cantor parameter correction method. The acquired nucleotide sequences were deposited in the GenBank database.

Statistical analysis

The statistical analysis of the ERIC-PCR DNA fingerprints was performed using UPGMA in the NTSYS pc2.1 software package [40]. The computer-assisted analysis of the fingerprints was performed using GELCOMPAR II (version 1.5; Applied Maths, Kortrijk, Belgium).

Statistical and clustering analysis

The endophyte genospecies and the geographic origin correlations were examined through canonical correspondence analysis (CCA) by using the Biodiversity R package of the R environment (R Development Core Team, 2007). The geographic origin and endophyte genospecies (RFLP and ERIC) were treated as two variables. In addition, ten levels, corresponding to the sampling sites, were included in the geographic variable origin in the analysis. The 35 combined RFLPs and 82 ERIC types (clusters) defined in the endophytes were treated as levels in the bacterial species variable. The results of the correspondence analysis are presented in a two-dimensional figure, in which the different levels of the variables were grouped.