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Genetic diversity and distribution of rhizobia associated with the medicinal legumes *Astragalus* spp. and *Hedysarum polybotrys* in agricultural soils

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ABSTRACT

With the increasing cultivation of medicinal legumes in agricultural fields, the rhizobia associated with these plants are facing new stresses, mainly from fertilization and irrigation. In this study, investigations on the nodulation of three cultivated medicinal legumes, *Astragalus mongholicus*, *Astragalus membranaceus* and *Hedysarum polybotrys* were performed. Bacterial isolates from root nodules of these legumes were subjected to genetic diversity and multilocus sequence analyses. In addition, the distribution of nodule bacteria related to soil factors and host plants was studied. A total 367 bacterial isolates were obtained and 13 genospecies were identified. The predominant microsymbionts were identified as *Mesorhizobium septentrionale*, *Mesorhizobium temperatum*, *Mesorhizobium tianshanense*, *Mesorhizobium ciceri* and *Mesorhizobium muleiense*. *M. septentrionale* was found in most root nodules especially from legumes grown in the barren soils (with low available nitrogen and low organic carbon contents), while *M. temperatum* was predominant in nodules where the plants were grown in the nitrogen-rich fields. *A. mongholicus* tended to be associated with *M. septentrionale*, *M. temperatum* and *M. ciceri* in different soils, while *A. membranaceus* and *H. polybotrys* tended to be associated with *M. tianshanense* and *M. septentrionale*, respectively. This study showed that soil fertility may be the main determinant for the distribution of rhizobia associated with these cultured legume plants.

Introduction

Compared with natural environments, agricultural cultivation systems provide strong selection stress for both the plants themselves and for the microflora associated with them. Cultivation systems generally provide sufficient light, plentiful water resources, nutrient supplementation in the form of fertilizer and lower competition with other plants. It could be hypothesized that agricultural practices have led to shifts in the associated microbiota, especially the rhizobia because the diversity and composition of rhizobial communities are strongly affected by environmental factors, as evidenced by studies on rhizobia of soybean [11,50], chickpea [47] and common bean [41]. Rhizobia associated with most cultivated crops, like soybean (*Glycine max*), alfalfa (*Medicago sativa*), pea (*Pisum sativum*), broad bean (*Vicia faba*) and common bean (*Phaseolus vulgaris*), have no natural distribution in natural ecosystems. Therefore, comparative studies on the rhizobial communities associated with the same legume species in natural environments and in agricultural fields are almost absent. Previous studies have been conducted on rhizobia of cultivated species and their wild relative species, such as *G. max* and *Glycine soya* [46], or wild *Vicia* species and cultivated broad bean [23]. Armas-Capote et al. reported that nine *Mesorhizobium* genospecies could nodulate with wild *Cicer canariense* in a natural habitat [1], while *Mesorhizobium ciceri*, *Mesorhizobium mediterraneum* and *Mesorhizobium muleiense* were the main microsymbionts of chickpea (*Cicer arietinum*) in farmland [19,47]. However, these studies could not differentiate the rhizobial differences from the same plant species grown in the cultured or wild conditions.

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Astragalus mongholicus Bunge and *Astragalus membranaceus* Bunge are nodulating legumes of medicinal importance, which have been used as health-promoting herbs for more than 2000 years in China [22,34]. With increasing demand for their production as medicines, such as those to treat influenza and to increase immunity, the wild resources of these *Astragalus* species are rather limited. In addition, large scale digging of the roots of these species destroys the vegetation of the ecosystems. Therefore, cultivation of these medicinal legumes has been rapidly and widely developed, especially in the provinces in the northern regions of China. Some rhizobial isolates originating from these two species grown in the wild have been characterized [32,45,51]; the major genera isolated were *Mesorhizobium* and *Rhizobium*, with only a few strains belonging to *Sinorhizobium* (now *Ensifer*) and *Bradyrhizobium* [3,7,9,10,18,21,49,51]. The biogeographic patterns of rhizobial symbionts of *Astragalus* have not been clearly described and the nodulation and diversity of rhizobia associated with cultivated *Astragalus* have not been fully investigated.

The aim of the present study was to investigate the nodulation, diversity and geographic distribution of rhizobia associated with cultured medicinal *Astragalus* species. A small number of rhizobia nodulating with the medicinal legume *Hedysarum polybotrys* were also included because this plant is medicinally similar to *A. membranaceus* [4]. Our current study will provide candidate rhizobial inoculants for these medicinal legumes grown in the agricultural areas.

Material and methods

Sampling sites and isolation of the nodule bacteria

Root nodules were collected from the cultured plants of *A. mongholicus*, *A. membranaceus* and *H. polybotrys* grown in 14 sampling sites in the north regions of China (Supplementary Fig. S1). Sites S1 and S2 were in Shanxi Province, N1 through N7 corresponded to Ningxia Province and G1 through G5 were located in Gansu Province. These three provinces were the main cultivation area for the two *Astragalus* species. *A. mongholicus* was abundantly cultured in the three provinces, while *A. membranaceus* and *H. polybotrys* were scatteredly planted in Ningxia and Gansu, respectively. No rhizobial inoculation history was recorded in these sites during the cultivation of these legumes. Specially, site S1 located on a mountain with sandy soil and no fertilizer was ever applied there, while the other sampling sites were in the farming areas with chemical fertilizer used annually. *A. mongholicus* were intercropped with maize only in site S2, while in other sites *A. mongholicus* were grown in a monoculture system.

Root nodules were collected and put into plastic tubes filled with silica gel and were brought to laboratory for further treatments. The shriveled dry nodules were waterlogged to original shape and were surface sterilized using 3% NaClO for 5 min followed by washing 7 times with sterilized water. The bacteria were isolated and purified on yeast mannitol agar (YMA) medium using the standard method [42]. Rhizobial isolates were cultured at 28 °C and were storage at 4 °C for a short term or in YM broth supplemented with 20% (w/v) glycerol at −80 °C for long term reservation.

Characterization of the soil samples

The sample soils were collected from the root zone. After air dried and grinded into powder, the soil samples were sieved through 1.00 mm and 0.15 mm mesh screen successively. Soil samples were delivered to Beijing Academy of Agriculture and Forestry Science for analyses of the pH, total nitrogen (TN), organic carbon (OC), available nitrogen (AN), available phosphorus (AP), available potassium (AK) and electrical conductivity (EC), with the standard methods [6,13,25,35].

PCR-restriction fragment length polymorphism (RFLP) analysis

Total genomic DNA was extracted from each of the nodule isolates and the reference strains for *Bradyrhizobium*, *Mesorhizobium*, *Ensifer* and *Rhizobium* species using the guanidinium thiocyanate chloride (GuTC) method [40]. Amplification of 16S rRNA genes was carried out using the primers P1 and P6 and the protocol of Tan et al. [39]. PCR products (10 μL) were digested separately with each (5U) of the four restriction endonucleases, *Msp*I, *Hinf*I, *Hae*III and *Alu*I at 37 °C for 4 h. Digested products were separated by electrophoresis in 2.5% (w/v) agarose gel and stained with 0.5 $\mu g\,mL^{-1}$ ethidium bromide. The DNA bands were visualized under UV light and their sizes were estimated by comparing with a 100-bp DNA ladder. As reported previously [44], similarity of the electrophoretic profiles was estimated by using the Dice coefficient and cluster analysis was performed by using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm and GelCompar II software (version 4.5) (Applied Maths, Sint-Martens-Latem, Belgium). Isolates with identical restriction patterns were classified into the same 16S rRNA type.

The 16S-23S rRNA gene intergenic spacer (ITS) sequences were amplified using the primers FGPL132 and FGPS1490 [24] with the PCR program of Laguerre et al. [16]. PCR products were digested with each of the restriction endonucleases *Hae*III, *Msp*I, *Hinf*I and *Hha*I under the same conditions used for the 16S rRNA gene. Separation of the restriction products and analysis of the electrophoretic profiles were as described above. In addition, the restriction fragment length polymorphism (RFLP) patterns of the 16S rRNA gene and ITS (2:1 weight) sequences were combined in a clustering analysis to obtain a dendrogram for differentiating the genomic species [8].

Multilocus sequence analysis (MLSA) of different genes

According to the result of the combined RFLP analysis, representative isolates were selected to represent each genomic species to further MLSA of different genes. The following genes were amplified: 16S rRNA gene with the primers P1 and P6 [39]; the housekeeping genes *recA*, *glnII* and *atpD* according to the protocols of Vinuesa et al. [43] with the primer pairs recA41F/recA640R, glnII12F/glnII689R and atpD255F/atpD782R, respectively; symbiotic gene *nodC* (about 700 bp) using the primer pair nodCF540/nodCR1160 and the protocol of Sarita et al. [33]; *nifH* gene (784 bp) with the primer pair nifHF/nifHR and the procedure of Laguerre et al. [17]. The PCR products were sequenced bi-directionally using the corresponding primers in Beijing Genomics Institute. Together with the related sequences recruited form GenBank database, the obtained sequences in this study were aligned using ClustalW program integrated in MEGA 5.0 [38] and the phylogenetic trees with 1000 bootstrap replications were constructed using the fitted model and the Maximum Likelihood (ML). Sequences of the three housekeeping genes *recA*, *glnII* and *atpD* were concatenated and a ML phylogenetic tree was constructed. Genospecies were further confirmed by the phylogenetic relationships and by >95% intraspecies sequence similarity value [15,26].

Nodulation tests

Representative isolates for different genospecies were cultured separately in 5 mL of YM broth with shaking up to the late exponential phase (about 48 h, $OD_{600} \approx 1.5$) and were inoculated to *A. mongholicus* and *A. membranaceus*, respectively, for testing the