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SHORT COMMUNICATIONS

Phylogeny and Genetic Diversity of the *Astragalus cicer* Root Nodule Bacterial Symbionts

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The genus *Astragalus* is one of the largest among leguminous plants; it comprises up to 2000 species. Approximately 90 astragalus species forming symbioses with root nodule bacteria have been described [1]; the taxonomy and population diversity of these bacteria are, however, poorly known. Chen et al. [2] described rhizobia isolated from the Chinese species *Astragalus sinicus* as *Mesorhizobium huakuii*. The study of rhizobia isolated from *Astragalus adsurgens* root nodules in various locations in China revealed their extreme heterogeneity. They belonged to the genera *Agrobacterium*, *Rhizobium, Mesorhizobium*, and *Sinorhizobium* [3]. The suggestion was made to combine the rhizobia similar to *Rhizobium galegae* and *R. huautlense* and isolated from the root nodules of *Astragalus complanatus, A. chrysopteris* and *A. scobwerrimus* into the species *R. loessense* [4]. The index of plants of the Bashkir ASSR describes 20 astragalus species. *Astragalus cicer* (Cicer milk-vetch), widespread in Bashkortostan, is a typical representative of this genus.

We have analyzed the bacteria from root nodules of *Astragalus cicer* collected on the Chesnokovskaya mountain, Ufa raion, Republic of Bashkortostan, Russian Federation. Morphologically different nodules were collected, from small round ones (1–2 mm in diameter) on young plants to forklike and grapelike on old plants. The surface of the nodules was sterilized by 10-min exposure first to 70% ethanol then to 10% sodium hypochlorite, and then washed several times with sterile tap water. The nodule was then homogenized aseptically with tweezers in 20 μl of TY medium (yeast extract, 0.1%; bacto tryptone, 1%; $CaCl_2$, 0.1%). The suspension was centrifuged and the supernatant was inoculated on plates with TY medium. Rhizobial colonies 1–2 mm in diameter were formed after two to three days of incubation at 28°C. Well isolated colonies were then transferred to fresh TY plates. After incubation, an aliquot of the biomass was taken for analysis; the remaining biomass was resuspended in TY medium with 20% glycerol and stored at –70°C. The virulence was determined by infecting week-old, sterile *A. cicer* seedlings with the isolates. A month after inoculation, all the strains formed morphologically normal root nodules. The cross inoculation test revealed that these rhizobia did not form nodules on alfalfa, *Galega orientalis, Onobrychis arenaria*, and meadow clover. In order to determine the genetic diversity of the strains, RAPD analysis was used. Bacterial DNA was extracted by the standard procedure, with a sodium perchlorate and phenol–chloroform mixture, after treatment of cells with lysozyme and EDTA, as well as by the guanidine method with sorbents. PCR was performed on MC2 Terzik (DNK-tekhnologiya, Russia) and T1 Thermocycler (Biometra, Germany) amplifiers using standard DNA amplification kits.

The DNA polymorphism of rhizobia isolated from over 100 root nodules was analyzed with several "random" primers. Multiple coinciding bands (for some primers, up to 60% of the total number) were found in the RAPD profiles of the isolates. This observation indicated the phylogenetic relatedness of *A. cicer* root nodule bacteria. However, three arbitrary groups of these strains can be established according to the similarity between the band distribution of the amplified DNA and the 80 : 15 : 5 ratio represented in the selection. The RAPD DNA profiles of 20 bacterial isolates obtained from the same plant with the 5'-CAGGCCCTTC-3' primer are presented as an example (Fig. 1). Isolates 5 and 18 belong to group 2; isolate 17, to group 3; and the remaining ones, to group 1.

The phylogeny of the investigated strains was determined by 16S rRNA gene sequencing. The nucleotide sequences were determined with an ABI PRISM 310 automatic sequencer (Applied Biosystems, Inc., United States) using the Big Dye Terminator v.3.0 kit. The search for related sequences in the NCBI Megablast database (www.ncbi.nlm.nih.gov) revealed that the 872 bp sequenced fragment, which was common for all three groups of *A. cicer* bacteria, was completely identical to the *Agrobacterium tumefaciens* IAM 14 (D13294) 16S r RNA gene described by Yanagi and Yamasato [5]. Multiple alignment of the isolated gene sequences and of those obtained from EMBL/GenBank/DDBJ international databases was performed with the MegAlign program from the Lasergene soft-

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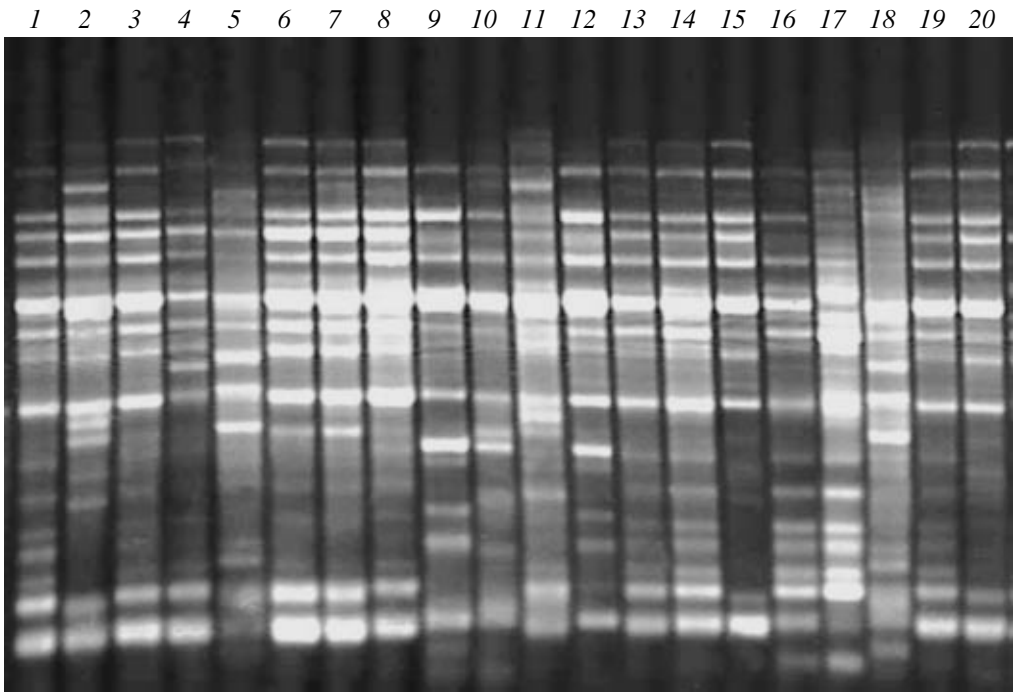

Fig. 1. Electrophoregram of RAPD analysis of the amplified DNA of *Astragalus cicer* root nodule bacteria (*1–20*) with the random oligonucleotide primer 5'-CAGGCCCTTC-3'.

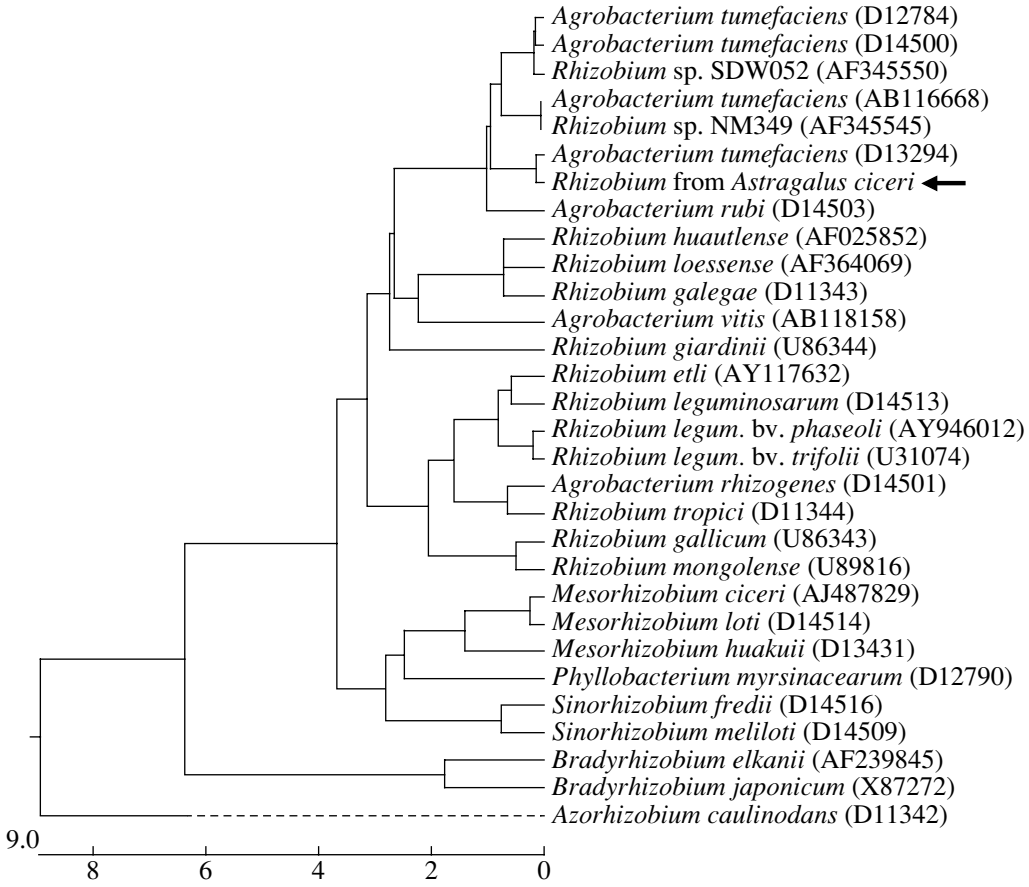

Fig. 2. Phylogenetic tree of 16S rRNA gene sequences of bacteria of the family *Rhizobiaceae* constructed with the MegAlign Lasergene (DNASTAR, United States) software package. X axis, the weight of alignments in the number of nucleotide substitutions. The phylogenetic position of the strains under investigation is indicated by the arrow.

ware package (DNASTAR Inc., United States). The phylogenetic tree of *Rhizobiaceae* was built (Fig. 2).

Chen and coworkers have already mentioned the similarity between the 16S rRNA genes of agrobacteria and *Astragalus* root nodule bacteria [3, 4]. They have revealed high phylogenetic diversity of the microsymbionts of various *Astragalus* species (*A. adsurgens, A. complanatus, A. chrysopterus*, and *A. scobwerrimus*). Several isolates were described as *R. loessense.* This species forms a monophyletic branch together with *Galega* symbiont *R. galegae* and *Sesbania herbaceae* symbiont *R. huautlense.* These two bacterial species have been previously combined together with *Agrobacterium vitis*; transfer of these rhizobia to the genus *Agrobacterium* and even into a separate genus were discussed [6]. These same authors, however, have described *Rhizobium* sp. strains SDW052 and NM349. Although they were not assigned the species status, they formed symbioses with *Astragalus adsurgens*; their 16S rRNA gene sequences were identical to those of *Agrobacterium tumefaciens* (Fig. 2). It is important to note that the genus *Agrobacterium* is included in the family *Rhizobiaceae*, in spite of the ecological differences. Moreover, Ti plasmid-free *Agrobacterium tumefaciens* strains gained the capability to infect leguminous plants and even to form "efficient" root nodules after the introduction of rhizobial symbiotic plasmids [7]. Thus, saprotrophic soil agrobacteria deprived of Ti plasmids (*Agrobacterium radiobacter*) can receive the symbiotic plasmids and in theory are capable of "becoming rhizobia." It becomes clear that the initial criterion for rhizobia, i.e., the ability to form symbioses with leguminous plants, is related mostly to the set of symbiotic plasmids, rather then to phylogeny.

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