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**GENETIC DIVERSITY AMONG MICROSymbionTS OF *Lathyrus*, *Vicia*, *Oxytropis* AND *Astragalus* LEGUME SPECIES FROM BAIKAL REGION****I.G. KUZNETSOVA<sup>1</sup>, A.L. SAZANOVA<sup>1</sup>, V.I. SAFRONOVA<sup>1</sup>, A.G. PINAEV<sup>1</sup>,  
A.V. VERKHOZINA<sup>2</sup>, N.Yu. TIKHOMIROVA<sup>1</sup>, Yu.S. OSLEDKIN<sup>1</sup>, A.A. BELIMOV<sup>1</sup>**<sup>1</sup>All-Russian Research Institute for Agricultural Microbiology, Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail v.safronova@rambler.ru;<sup>2</sup>Siberian Institute of Plant Physiology and Biochemistry, Federal Agency of Scientific Organizations, 132, ul. Lermontova, Irkutsk, 664033 Russia

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**Abstract**

Rhizobia are Gram-negative soil microorganisms that form intracellular nitrogen-fixing symbiosis with leguminous plants. Investigations of symbiotic systems with the participation of endemic or relict species have a particular importance for understanding of the evolution of plant-microbe interactions. The purpose of our work was to create a representative collection of microsymbionts of endemic Baikal legumes, as well as to estimate their biodiversity. The study of taxonomic positions of 69 isolates from root nodules *Lathyrus humilis*, *Vicia baicalensis*, *Astragalus mongholicus* and *Oxytropis sylvatica* was conducted. For primary identification of these isolates the methods of ITS-RFLP analysis was used that divided strains into 33 groups with identical DNA-profile. Then the taxonomy positions of isolates were determined by the 16S rRNA gene (*rrs*) and ITS region sequencing. Phylogenetic analysis revealed the considerable genetic diversity among microsymbionts of plants studied. Rhizobial isolates belonged to 5 genera: *Rhizobium* (family *Rhizobiaceae*), *Mesorhizobium* and *Phyllobacterium* (family *Phyllobacteriaceae*), *Bosea* and *Tardiphaga* (family *Bradyrhizobiaceae*). In addition, non-rhizobial isolates belonging to the genera *Herbiconiux*, *Leifsonia*, *Burkholderia* and *Stenotrophomonas* were obtained. It is known that some species of these genera may be present in the nodules of legumes, but also be inhabitants of rhizosphere or phyllosphere of different plants. The presence of atypical rhizobial microsymbionts in the studied plants was noted, which may indicate the active formation of relationships between partners in the legume-rhizobial systems of Baikal region.

Keywords: legumes of Baikal region, taxonomy of rhizobia, ribosomal genes sequencing.

Nodule bacteria (*Rhizobia*) belong to a large genetically diverse group of Gram-negative soil microorganisms that form intracellular nitrogen-fixing symbiosis with legumes. One of the urgent tasks of modern biotechnology is the study of mechanisms of interaction of legumes with rhizobia, required for evidence-based selection of highly effective plant-microbe systems [1].

Investigations of symbiotic systems with the participation of endemic or relict species have a particular importance for understanding of the evolution of plant-microbe interactions. These unique objects include Baikal legumes, such as low-growing vetchling (*Lathyrus humilis*), a Late Pleistocene relict of the South Siberian-Severouralsk area [2]; Mongolian milk vetch (*Astragalus mongholicus*), a rare medicinal plant [3, 4]; Baikal endemics Baikal pea (*Vicia baicalensis*) and forest oxytrope (*Oxytropis sylvatica*) [5].

Few data on *Astragalus* microsymbionts show a great diversity of these microorganisms belonging to different genera of order *Rhizobiales*: *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Mesorhizobium* [6-9]. Vetch-

ling and pea species are nodulated with bacteria *Rhizobium leguminosarum* bv. *viciae* [10-12], and rhizobia and mesorhizobia strains are described among oxytrope microsymbionts [7, 13]. However, microsymbionts of Baikal plants belonging to these genera have not been studied.

The purpose of this study was to create a representative collection of microsymbiont strains of endemic Baikal legumes (low-growing vetchling, Mongolian milk vetch, Baikal pea, and forest oxytrope) and to estimate their taxonomic position using the method of 16S rDNA and ITS-region sequencing.

**Technique.** A total of 69 strains isolated as described [14] from root nodules of low-growing vetchling *Lathyrus humilis*, Baikal pea *Vicia baicalensis* (two populations of each species) as well as Mongolian milk vetch *Astragalus mongholicus* and forest oxytrope *Oxytropis sylvatica* (one population of each species) which grow on the Chivyrkuy coast Bay (Baikal) were studied. Strains were grown on yeast mannitol agar (YMA) at 28 °C [7].

For primary identification of strain intraspecific diversity, RFLP 16S and 23S rRNA (ITS-region) sequencing analysis was performed. Amplified DNA was digested with MspI, and the resulting DNA fragments were separated by electrophoresis in the standard mode [15]. Species affiliation of strains was determined by 16S rRNA gene (*rrs*) sequencing. The taxonomy position of *Bosea* nodule bacteria was specified using the more variable ITS-region sequencing method.

To amplify 16S rDNA (about 1500 bp), the fD1 (5'-AGAGTTTGGATCC-TGGCTCAG-3') and rD1 (5'-AAGGAGGTG-ATCCAGCC-3') primers were used, to amplify the ITS-region (800 bp), the FGPS1490-72 (5'-TGCGGC-TGGATCCCCCTCCTT-3') and FGPL-132 (5'-CCGGGTTTCCCCATTCGG-3') primers were used. The resulting PCR product was gel purified [15] for subsequent sequencing.

The search for homologous sequences was performed using the GenBank database (BLAST program). The phylogenetic tree was constructed using the MEGA v. 4.0.2 program (Neighbor-Joining method). Sequence pairs were compared using the number of different nucleotides.

**Results.** The strains used in the study are summarized in the Table.

**Taxonomic structure of microsymbiont strains isolated from root nodules in endemic Baikal legumes** (according to *rrs* and ITS-region sequencing)

Microsymbiont species	Legume microsymbiont No.			
	low-growing vetchling <i>Lathyrus humilis</i>	Mongolian milk vetch <i>Astragalus mongholicus</i>	Baikal pea <i>Vicia baicalensis</i>	Forest oxytrope <i>Oxytropis sylvatica</i>
<i>Rhizobium</i> sp.	2/5(1), 2/12M	—	—	—
<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>	1/10K, 2/10K	—	11/2, 11/3K, 11/4M, 11/7K, 11/12M, 11/19, 11/20, 11/21, 11/22, 11/23, 11/24K, 11/24M, 11/25, 11/33, 11/34K, 11/34M, 11/35, 11/36, 11/37K, 11/37M, 11/38, 11/39, 11/42K, 11/42M, 11/43K, 11/44K, 11/44M, 11/45K, 11/46, 11/47K, 11/47M, 11/48K, 11/48M, 13/3, 13/4, 13/6M, 13/7, 13/8K, 13/9M, 13/11	12/13M, 12/16K
<i>Mesorhizobium metallidurans</i>	—	3/14C1, 3/14C2	—	—
<i>Mesorhizobium ciceri</i>	2/13K	—	—	—
<i>Tardiphaga robiniae</i>	1/11M	3/6M, 3/11C, 3/21(2)	—	12/11(1)
<i>Bosea</i> sp.	—	3/5M, 3/31K	—	12/22M
<i>Bosea vaviloviae</i>	—	3/25	—	—

<i>Herbiconiux</i> sp.	1/3M, 1/5M, 1/14M, 1/15M	—	—	—
<i>Burkholderia</i> sp.	—	3/8K	13/5	—
<i>Leifsonia</i> sp.	—	3/23M, 3/27K	—	—
<i>Stenotrophomonas</i> sp.	—	3/17	11/7M	—
<i>Phyllobacterium</i> sp.	—	—	13/12M	—

Note. Dashes mean the absence of isolates.

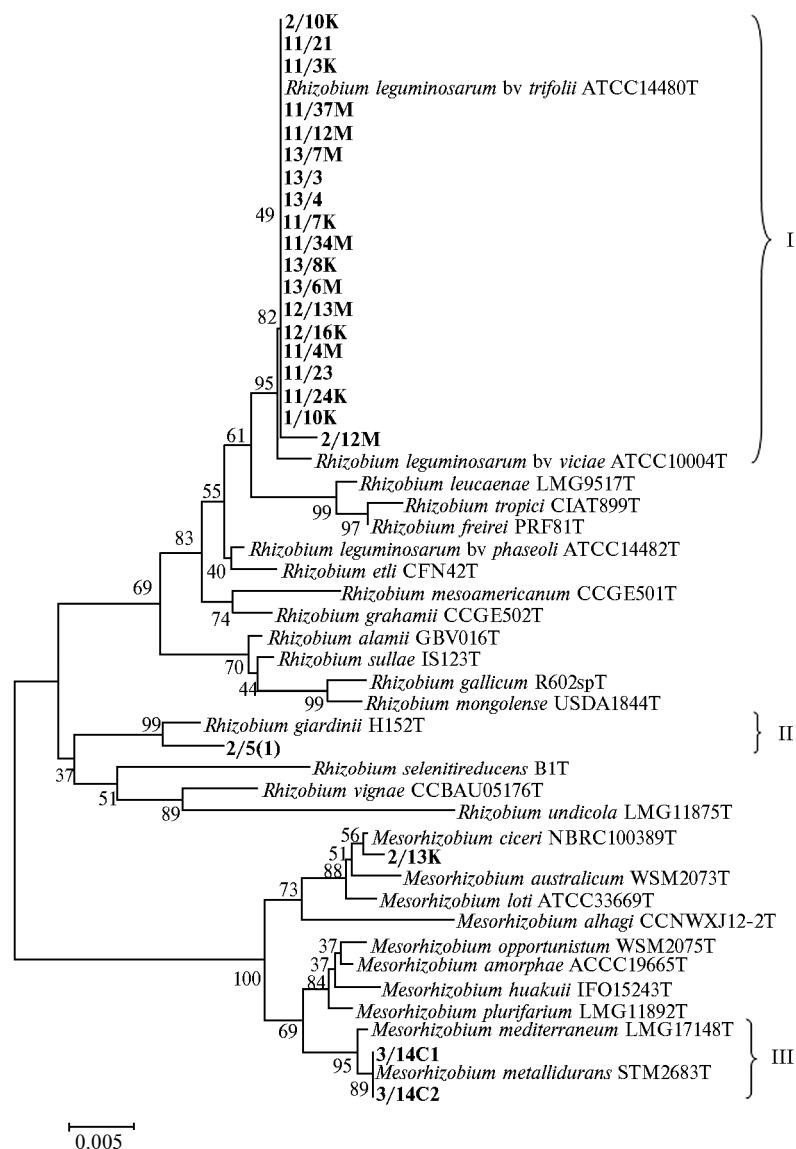
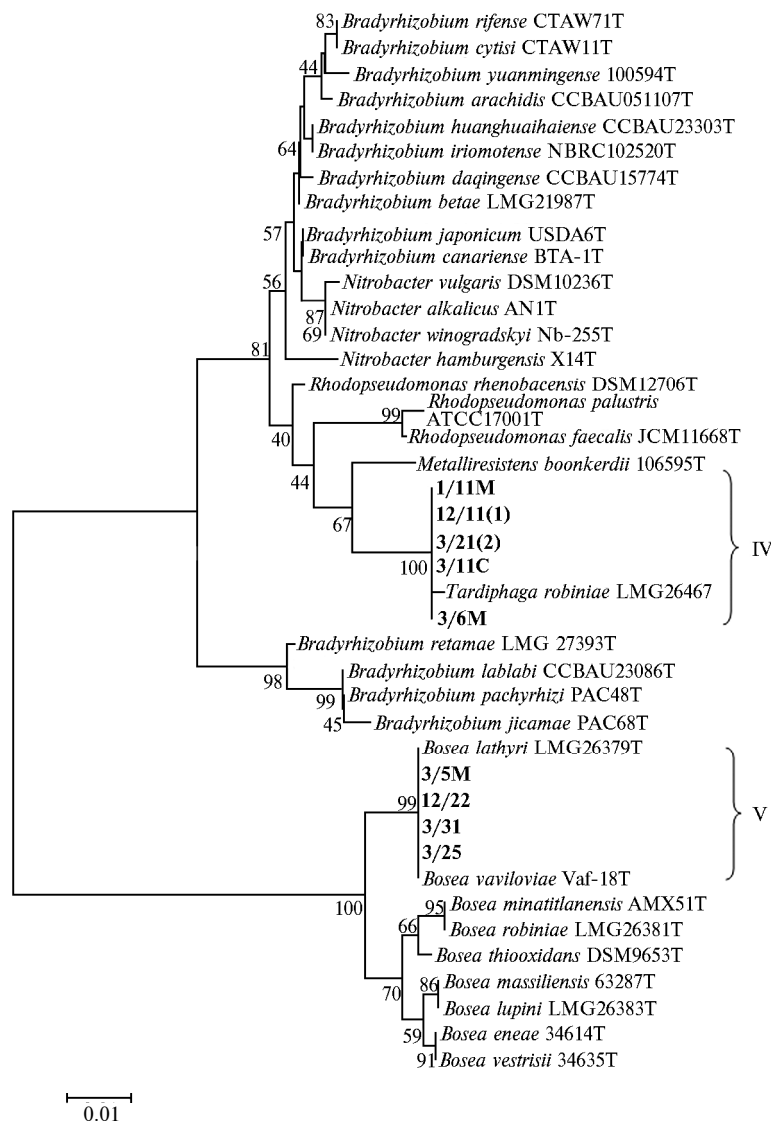


Fig. 1. *Rrs*-phylogram demonstrating the taxonomic position of genera *Rhizobium* and *Mesorhizobium* isolates. The tested strains are marked in bold. Letter «T» marks typical strains; I-III — significantly different clusters.

Isolates were divided into two groups by their growth rate. In 9 strains, visible colonies were formed from the day 5 to 6 of growth on YMA medium, other strains formed colonies from the day 3 to 4. Based on the results of ITS-region RFLP-analysis, studied isolates were divided into 33 groups with identical DNA profile. One species of each group was selected for *rrs* sequencing. *Rrs* sequencing analysis showed that 23 strains belonged to genera *Rhizo-*

*bium* and *Mesorhizobium* and formed three significantly different clusters with the level of support of more than 95 % (Fig. 1).



**Fig. 2. Rrs-phylogram demonstrating the taxonomic position of isolates within the *Bradyrhizobiaceae* family.** Tested strains are marked in bold. Letter «T» marks typical strains; IV, V — significantly different clusters.

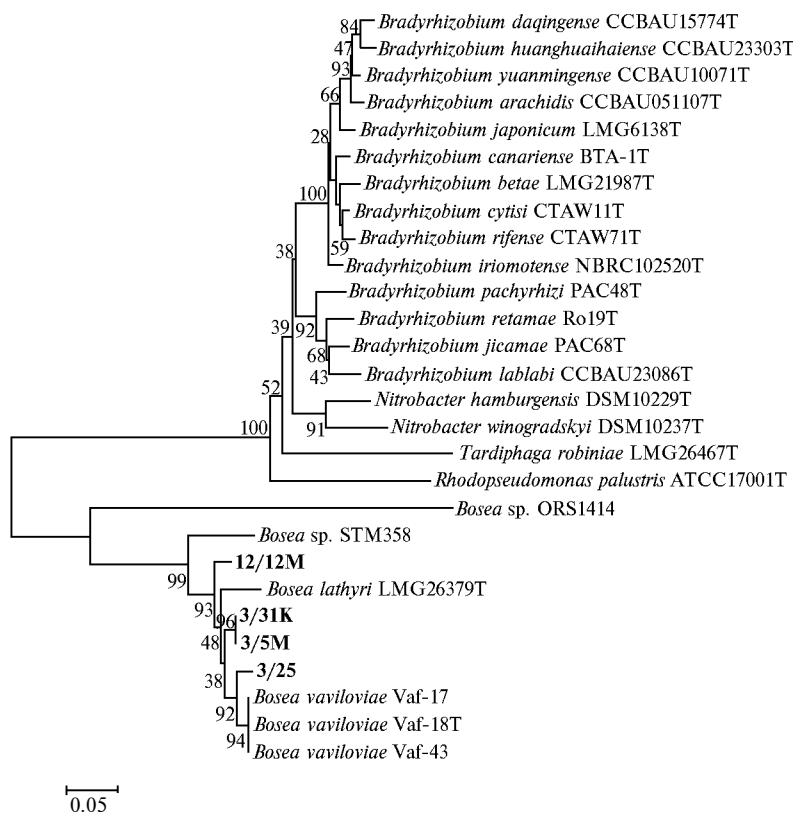
Cluster I included 19 strains isolated from low-growing vetchling, baikal pea and forest oxytrope (3, 14 and 2 strains, respectively) and two typical strains *R. leguminosarum* bv. *trifolii* ATCC14480T and *R. leguminosarum* bv. *viciae* ATCC10004T. Based on the results of *rrs* sequencing (see Table), isolates 1/10K, 2/10K, 11/3K, 11/4M, 11/7K, 11/12M, 11/21, 11/23, 11/24K, 11/34M, 11/37M, 12/13M, 12/16K, 13/3, 13/4, 13/6M, 13/7M, and 13/8K were identified as *R. leguminosarum* bv. *trifolii* (index of similarity to typical strain ATCC14480T was 99.7-99.9 %).

Cluster II was formed by strain 2/5(1) isolated from low-growing vetchling and typical strain H152T *Rhizobium giardinii* which has been described as microsymbiont strain [16]. However, since the similarity between these strains in gene *rrs* was 99.0 % only, strain 2/5(1) was identified as

*Rhizobium* sp. (see Table).

Three isolates were classified as *Mesorhizobium* (see Fig. 1, Table). Strains 3/14C1 and 3/14C2 (cluster III) isolated from Mongolian milk vetch were identified as *Mesorhizobium metallidurans* and *M. ciceri* (*rrs* homology to typical strains STM2683T and NBRC100389T was 100 and 99.6 %, respectively).

Figure 2 shows the *rrs*-phylogram demonstrating the taxonomic position of 9 slow-growing *Rhizobium* isolates within the *Bradyrhizobiaceae* family. Strains 1/11M, 3/6M, 3/11C, 3/21(2), and 12/11(1) isolated from various plants formed cluster IV with typical strain *Tardiphaga robiniae* LMG26467T and were assigned to this species (*rrs* index of similarity of 99.8-99.9 %). Cluster V with the level of statistical support of 99.0 % was formed by four strains 3/5M, 3/31K, 12/22M, and 3/25 and two typical *Bosea* (*B. lathyri* and *B. vaviloviae*) strains. The *rrs* similarity of isolates to typical strains *B. lathyri* LMG26379T and *B. vaviloviae* Vaf-18T was 98.4-99.4 and 99.4-100 %, respectively. It should be noted that species *B. vaviloviae* was described as recently as 2015 for three micro-symbiont strains of a relic legume *Vavilovia formosa* which grows in North Ossetia [17]. Therefore, Baikal isolates belonging to genus *Bosea* and having a high index of similarity to species *B. vaviloviae* are of great interest for further study.



**Fig. 3.** ITS-phylogram demonstrating the taxonomic position of genera *Bosea* isolates isolated from Mongolian milk vetch (*Astragalus mongholicus*) and forest oxytrope (*Oxytropis sylvatica*) nodules. Tested strains are marked in bold. Letter «T» marks typical strains.

ITS-region sequencing was performed in strains 3/5M, 3/31K, 12/22M, and 3/25 as this method provides higher resolution in the identification of closely related strains than *rrs* sequencing [9]. The dendrogram shown in Figure 3 demonstrates the correlation between the results of *rrs* and ITS-region sequencing. All isolates could be clustered with typical strains *B. lathyri* LMG26379T and *B. va-*

*viloviae* Vaf-18T under the statistical support level of 99.0%. However, the maximum levels of ITS-region homology between these isolates and typical strains *B. lathyri* and *B. vaviloviae* were low (of 84.9 and 89.4 %, respectively). Isolate 3/25 was an exception, its ITS-region similarity to typical strain *B. vaviloviae* Vaf-18T was 97.8 %. Based on the results of *rrs* ITS-region sequencing, it was assigned to species *B. vaviloviae*. The taxonomic position of the other *Bosea* isolates is unclear as it is considered that nodule bacteria strains belonging to one species can not be less than 95 % homologous in ITS-region [9]. Analysis of *rrs* sequencing in other Baikal isolates revealed their belonging to genera *Herbiconiux* (4 strains), *Leifsonia* (2 strains), *Burkholderia* (2 strains), *Stenotrophomonas* (2 strains), and *Phyllobacterium* (1 strain). Reported in the literature, some species of these genera may be present in legume nodules [18, 19] and be inhabitants of rhizosphere or phyllosphere of different plants.

Thus, rhizobial strains belonging to species *Rhizobium* spp., *Mesorhizobium ciceri*, *Tardiphaga robiniae*, and species of genus *Herbiconiux* (family *Microbacteriaceae*) can be found among the low-growing vetchling microsymbionts. Species of genera *Mesorhizobium*, *Bosea*, *Tardiphaga*, *Leifsonia*, *Burkholderia* and *Stenotrophomonas* were found among Mongolian milk vetch symbionts. Isolates of species *R. leguminosarum* bv. *trifolii*, *T. robiniae* and *Bosea* sp. were obtained from forest oxytrope nodules. A total of 40 strains belonging to species *R. leguminosarum* bv. *trifolii* were isolated from Baikal pea nodules, as well as one *Phyllobacterium* and one *Burkholderia* species. We would like to note that the presence of atypical rhizobial microsymbionts in legume nodules (*M. ciceri* and *T. robiniae* in vetchling; *Bosea* spp. and *T. robiniae* in milk vetch and oxytrope; *Phyllobacterium* sp. in pea) may indicate the active formation of relationships between partners in the legume-rhizobial systems of Baikal region.

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