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STUDY OF THE GENETIC DIVERSITY OF MICROSYMBIONTS ISOLATED FROM *Hedysarum gmelinii* subsp. *setigerum*, GROWING IN THE BAIKAL LAKE REGION

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Abstract

One of the urgent problems of modern microbiology and biotechnology is the study of the mechanisms of interaction between leguminous plants and root nodule bacteria (rhizobia), which are an extensive group of microorganisms capable to form nitrogen-fixing symbiosis with a host plant. Knowledge of these mechanisms is necessary for carrying out scientifically based selection of highly effective rhizobia-legume symbiotic systems. To understand the evolution of the specificity of plant-microbe interactions, symbiotic systems with the participation of relic leguminous plants, which are an intermediate link between the extinct and modern species, are of particular importance. One of these unique objects is the pleistocene relict *Hedysarum gmelinii* Ledeb. subsp. *setigerum* (Turcz. ex Fischer et Mey.) Kurbatsky. The aim of this study was to isolate and identify the world's first collection of microsymbionts of this plant species growing in the Lake Baikal region. The study of taxonomic positions of 19 isolates from root nodules of *H. gmelini* subsp. *setigerum* plants was conducted by the methods of ITS-RFLP and 16S rRNA gene (*rrs*) sequencing. Phylogenetic analysis revealed the considerable genetic diversity among microsymbionts of the plant species studied. Fourteen rhizobial isolates belonged to 3 genera: *Rhizobium* (family *Rhizobiaceae*), *Phyllobacterium* (family *Phyllobacteriaceae*) and *Bosea* (family *Bradyrhizobiaceae*). It was noted the presence in the root nodules of non-symbiotic rhizobial species that are not able to form symbiosis with leguminous plants (*Phyllobacterium endophyticum*, *Ph. loti* and *Bosea* sp.). In addition, five non-rhizobial isolates belonging to the genera *Acinetobacter, Stenotrophomonas, Sphingomonas* and *Agromyces* were obtained. The obtained data may indicate that the relic rhizobia-legume symbioses, formed in particular by the *H. gmelini* subsp. *setigerum* plants, are prototypes of modern symbiotic systems and reflect the evolutionary pathways in the direction of recruiting symbiotic genes of different microorganisms and increasing the specificity of plant-microbe interactions. It is possible that strains of non-symbiotic rhizobial species are present in nodules as a source of genes that do not participate directly in the formation of symbiosis, but affect its activity. Such strains, after appropriate genetic and phenotypic study, can be used for the production of biopreparations with increased efficacy.

Keywords: leguminous plants of the Baikal region, *Hedysarum gmelinii* subsp. *setigerum*, ribosomal RNA genes sequences, *Rhizobiaceae* taxonomy

Rhizobia, a large genetically diverse group of Gram-negative soil microorganisms, are capable of establishing intracellular symbiosis with leguminous plants and performing fixation of atmospheric nitrogen by forming symbiotic nodules on the roots of host plants, due to which these microorganisms are also known as nodule bacteria. Scientific selection of highly effective plant-microbial systems necessitates understanding the molecular mechanisms that ensure the interaction of leguminous plants with rhizobia, so these studies are among most

urgent in modern microbiology and biotechnology [1]. Symbiotic systems of relict leguminous plants, which are an intermediate link between extinct and existing species, are of particular importance for understanding evolution of specific plant-microbial interactions. These unique objects include the sweetvetch *Hedysarum gmelinii* Ledeb. subsp. *setigerum* (Turcz. ex Fischer et Mey.) Kurbatsky which grows in the Baikal region [2]. Botanical and geographical analysis showed the belonging of this species to Pleistocene petrophytic steppe relics of South Siberian origin [3, 4].

The evidence was reported that bacteria *Pantoea agglomerans, Enterobacter kobei*, *Enterobacter cloacae*, *Leclercia adecarboxylata*, *Escherichia vulneris* and *Pseudomonas* sp. (class *Gammaproteobacteria*) were isolated from the nodules of *Hedysarum spinosissimum* subsp. *capitatum* and some other species of this genus (*H. pallidum, H. carnosum*) growing in the Mediterranean region [5]. *H. coronarium* is nodulated by *Rhizobium sullae* strains [6]. According to Chinese researchers [7], *Rhizobium* strains were isolated from the nodules of *H. scoparium* and *H. polybotrys* which grow in the Chinese northwest. In 2011, it was shown that the plants of the alpine sweet vetch *H. alpinum* are nodulated by representatives of *Mesorhizobium* genus [8].

The microsymbionts of the *H. gmelinii* Ledeb. subsp. *setigerum* have never been isolated before. In the present work, we have created and described the world's first collection of rhizobial microsymbionts of this relict leguminous plant growing in the Baikal region.

The purpose of our study was to isolate the *Hedysarum gmelinii* subsp. *setigerum* microsymbionts and determine the taxonomic position of the strains using the ITS-RFLP method and 16S rDNA sequencing.

Techniques. The objects of the study were 19 strains isolated using the traditional method [9] from the root nodules of *H. gmelinii* subsp. *setigerum* growing on the Zunduk Cape (mainland coast of the Baikal strait Maloye More, coordinates 53.383333, 107.41666753°23′00″ N. 107°25′00″ E). Microorganisms were grown on modified mannitol yeast agar YMSA with addition of 0.5 % succinic acid [10] at 28 °C. All isolates are deposited in the Departmental Collection of Useful Agricultural Microorganisms and placed at the Station for Low-Temperature Automated Storage of Biological Samples (Liconic Instruments, Liechtenstein) [11]. The information on the strains is available in the Internet database of the Russian Collection of Agricultural Microorganisms [12].

In the initial assessment of the intra-species diversity of strains, RFLP (restriction fragment length polymorphism) analysis of sequences between the genes 16S and 23S rRNA (ITS-RFLP method) was performed. For this, the amplified DNA fragment was cleaved with MspI restriction endonuclease (Promega, USA) and the restricted DNA fragments were separated electrophoretically in the standard mode [13]. For species identification of the strains, the nucleotide sequence of the 16S rRNA (*rrs*) gene was used.

For amplification of 800 bp ITS region, the primers FGPS1490-72 (5′-TGCGGCTGGATCCCCTCCTT-3′) and FGPL-132 (5′-CCGGGTTTCCCC-ATTCGG-3′) were used, for amplification of 16S rDNA of about 1500 bp, the primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAG-GTGATCCAGCC-3′) were used. The resultant PCR product was isolated from the gel and purified [14] for RFLP analysis or sequencing on a genetic analyzer ABI PRISM 3500xl (Applied Biosystems, USA). Detection of homologous sequences was performed using the NCBI GenBank database and the BLAST program [15]. A neighbor-joining tree was constructed by using the program MEGA5 [16] were used. Differences in the number of nucleotides which differed between the obtained sequences were revealed by a pairwise com-

parison. To assess the levels of clusters support, a bootstrap analysis was performed based on 1000 replicates. The resulting sequences were deposited in the GenBank database (accession numbers KY290459-KY290467, KY290469, KY290470 and KY290472-KY290474).

Results. Isolates of nodule bacteria are presented in Tables 1 and 2.

1. 16S rRNA gene sequence homology (%) in fast-growing isolates from nodules of sweetvetch *Hedysarum gmelinii* subsp. *setigerum* (Baikal region) and type strains of *Phyllobacterium* and *Rhizobium*

Type strain	Isolate										
	Rhizobium sp.	*Ph. loti*			*Ph. endophyticum*		*Phyllobacterium* sp.				
	Hse-26	Hse-9	Hse-19	Hse-30	Hse-10	Hse-24	Hse-13	Hse-14	Hse-17	Hse-20	Hse-29
1	95.7	98.3	98.3	98.2	97.4	97.1	99.9	96.9	98.6	98.3	98.7
2	94.5	99.6	99.6	99.5	97.9	97.7	99.6	97.4	98.9	99.7	98.9
3	94.9	99.1	99.1	99.0	97.1	96.8	99.9	96.6	98.1	99.2	98.1
4	94.6	98.6	98.6	98.6	96.6	97.1	99.2	96.1	97.7	98.6	97.7
5	94.3	98.6	98.1	98.6	99.9	99.6	99.4	99.3	98.8	98.6	98.9
6	95.1	98.5	98.6	98.4	98.5	98.2	99.6	98.0	99.3	98.6	99.3
7	95.1	99.8	99.8	99.8	98.5	98.3	99.6	98.0	99.0	99.8	99.1
8	98.9	94.4	94.4	94.3	93.5	93.3	95.0	93.1	94.8	94.4	94.8
9	96.4	93.5	93.5	93.5	92.5	92.3	93.4	92.2	93.8	93.6	93.8

Note. 1 — *Ph. myrsinacearum* STM 948T, 2 — *Ph. trifolii* PETP02T, 3 — *Ph. ifriqiyense* STM 370T, 4 — *Ph. catacumbae* CSC19T, 5 — *Ph. endophyticum* PEPV15T, 6 — *Ph. brassicacearum* STM 196T, 7 — *Ph. loti* S658T, 8 — *Rh. giardinii* NBRC 107135, 9 — *Rh. alamii* GBV016T.

2. 16S rRNA gene sequence homology (%) in slow-growing isolates from nodules of sweetvetch *Hedysarum gmelinii* subsp. *setigerum* (Baikal region) and type *Bosea* strains

Type strain	Isolate *Bosea sp.*		
	Hse-21	Hse-22	Hse-32
B. vaviloviae Vaf-18T	98,5	98,0	98,5
B. massiliensis 63287T	98,4	97,5	98,4
B. eneae 34614T	98,6	97,7	98,6
B. vestrisii 34635T	98,6	97,7	98,6

All the studied isolates were divided into two groups based on the growth rate: three strains formed colonies on YMSA on days 4-5, and in the remaining strains, visible growth occurred on day 3. Since each isolate generated a unique ITS-RFLP pattern (data not shown), all the strains studied were identified by *rrs* gene sequencing.

Sequence analysis showed that 11 fast-growing strains belong to the genera *Phyllobacterium* and *Rhizobium* and form 3 statistically reliably different clusters with a support level of 100 % (Fig. 1). The first cluster included the strains Hse-14, Hse-24 and Hse-10, and also the type strain *Ph. endophyticum* PEPV15T. Hse-24 and Hse-10 strains which were identified as *Ph. endophyticum* showed high homology of *rrs* gene with that of the type strain PEPV15T (99.6 % and 99.9 %, respectively). The Hse-14 strain has been identified as *Phyllobacterium* sp. (Table 1). The second cluster combined the strains Hse-29, Hse-17, Hse-20, Hse-30, Hse-19, Hse-9, Hse-13 and the type strains *Ph. sophorae* CCBAU03422T, *Ph. bourgognense* STM201T, *Ph. brassicacearum* STM 196T, *Ph. loti* S658T, *Ph. trifolii* PETP02T, *Ph. catacumbae* CSC19T, *Ph. myrsinacearum* STM 948T and *Ph. ifriqiyense* STM 370T (Fig. 1). The Hse-20 strain was identified as *Phyllobacterium* sp., since being simultaneously the closest to two strains, *Ph. trifolii* PETP02T and *Ph. loti* S658T, by the *rrs* gene (the homology was 99.7 % and 99.8 %, respectively). Similarity of *rrs* gene in the strains Hse-9, Hse-19 and Hse-30 and the type strain *Ph. loti* S658T reached 99.8 %. Hence, these strains were assigned to *Ph. loti* (see Table 1). The closest to the isolates *Phyllobacterium* sp. Hse-17 and Hse-29 was the type strain *Ph. brassicacearum* STM 196T (99.3 % homology of *rrs* gene).

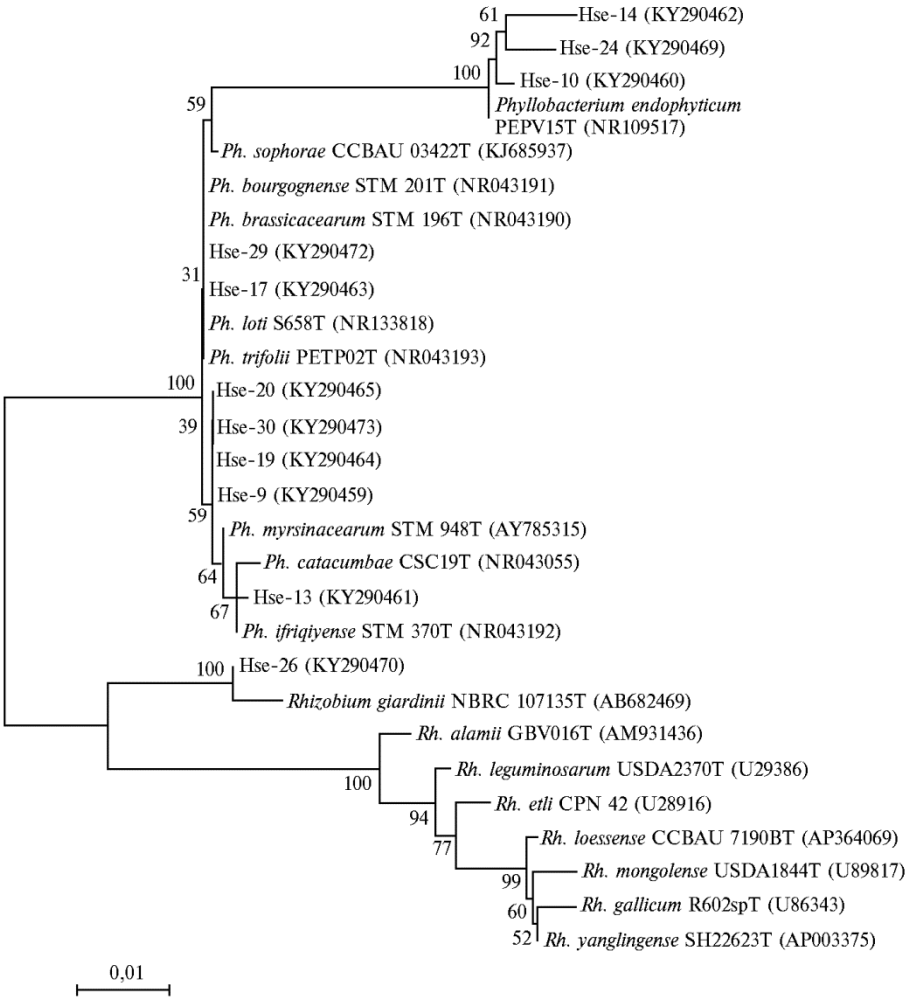

Fig. 1. *rrs*-Phylogram of the fast-growing strains isolated from nodules of sweetvetch *Hedysarum gmelinii* subsp. *setigerum* in Baikal region and the representatives of related species *Phyllobacterium* and *Rhizobium*. The obtained isolates are designated as Hse, type strains are marked with the letter "T".

The isolate Hse-13 and two type strains, *Ph. myrsinacearum* STM 948T and *Ph. ifriqiyense* STM 370T, showed 99.9 % similarity; therefore, Hse-13 was identified only to the genus *Phyllobacterium* sp. (see Table 1). The third cluster was formed by the strain Hse-26 and the type strain *Rhizobium giardinii* NBRC 107135. Based on *rrs* gene sequencing (see Table 1), the Hse-26 isolate was identified as *Rhizobium* sp. with 98.9 % similarity to the type strain *Rhizobium giardinii* NBRC 107135.

Fig. 2 shows the *rrs*-dendrogram reflecting the taxonomic position of three slow-growing rhizobial isolates within the *Bradyrhizobiaceae* genera. The strains Hse-21 and Hse-32 showed 98.5 % *rrs* gene homology with the type strain *Bosea vaviloviae* Vaf-18T and 98.6 % homology with the type strains *B. eneae* 34614T and *B. vestrisii* 34635T (see Table 2). The similarity of *rrs* genes in the Hse-22 isolate and the closest type strain *vaviloviae* Vaf-18T was 98.0 % (see Table 2). On this basis, the strains Hse-21, Hse-22 and Hse-32 were identified as *Bosea* sp. It should be noted that *B. vaviloviae* was described quite recently, in 2015, when three microsymbiont strains were discovered in the relict legume plant *Vavilovia formosa* which grows in North Ossetia [10]. In addition, the microorganisms of the *Bosea* and *Phyllobacterium* genera were not isolated

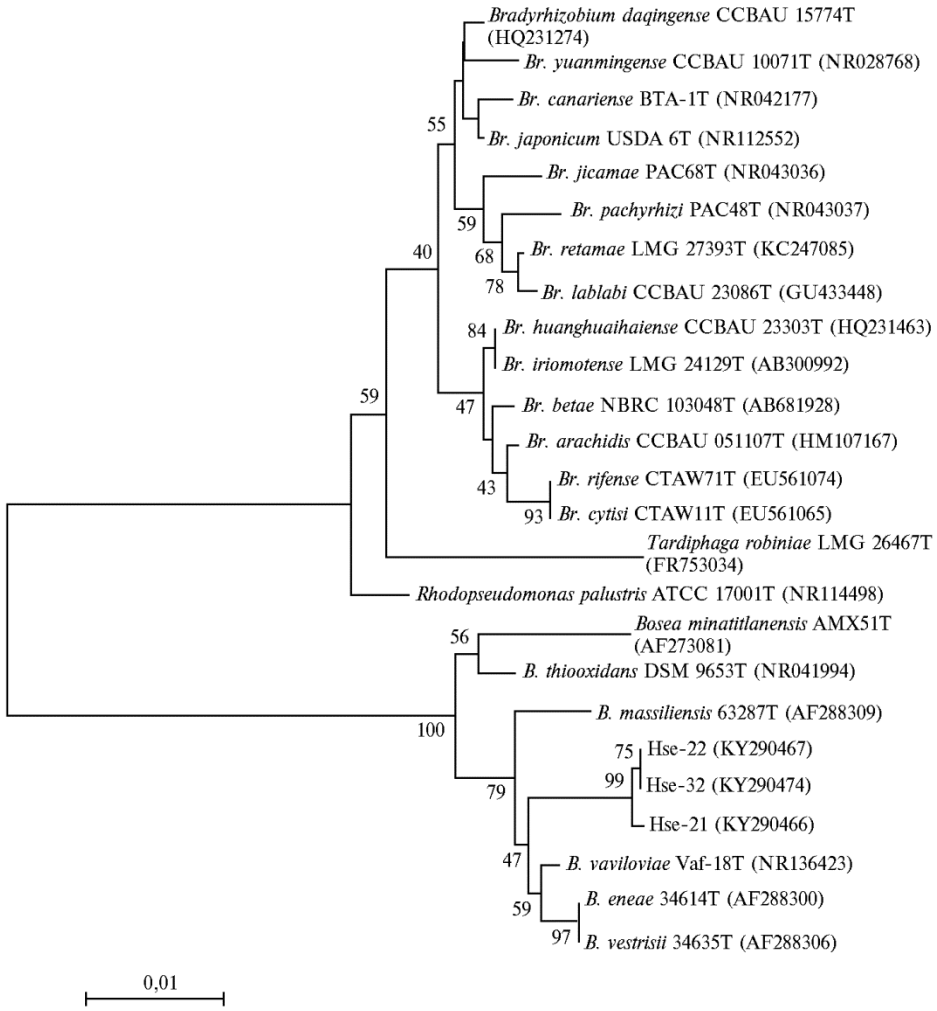

Fig. 2. *rrs*-Phylogram of the slow-growing strains isolated from nodules of sweetvetch *Hedysarum gmelinii* subsp. *setigerum* in Baikal region and the representatives of related species *Bosea*. The obtained isolates are designated as Hse, type strains are marked with the letter "T".

from the nodules of other sweetvetch species that grow in the Mediterranean, northwestern regions of China and central Russia [5-8].

The ability of any members of *Bosea* genera to form a nitrogen-fixing symbiosis has not been confirmed before, although strains of four species, *B. lupini*, *B. lathyri, B. robiniae* and *B. vaviloviae*, were isolated from the nodules of *Lupinus*, *Lathyrus, Robinia* and *Vavilovia* leguminous plants, respectively [10, 17]. However, at least two *Phyllobacterium* species, *Ph. sophorae* and *Ph. trifolii*, were described as effective microsymbionts of *Sophora flavescens* [18], *Trifolium repens* and *Lupinus albus* [19]. Therefore Baikal isolates of *Bosea* genera, which show high similarity to *B. vaviloviae* species, and *Phyllobacterium* bacteria are of great interest for further study.

Based on *rrs* gene sequences, five non-rhizobial isolates from sweetvetch *Hedysarum gmelinii* subsp. *setigerum* nodules were attributed to genera *Acinetobacter*, *Stenotrophomonas*, *Sphingomonas* and *Agromyces* (data not shown). Bacteria of *Stenotrophomonas* genera were isolated from other leguminous plants of the Baikal region [20]. According to data reported, the members of these genera can inhabit the nodules of legumes and also the rhizosphere and phyllosphere of various plant species [21-25].

Thus, in the present paper, for the first time, we have obtained a collection of strains isolated from the relict leguminous plant, the sweetvetch *Hedysarum gmelinii* Ledeb. subsp. *setigerum*, which grows in the Baikal region. Our study showed that among the microsymbionts of this plant there are strains of symbiotic species of nodule bacteria (*Rhizobium* sp.), as well as atypical species, which representatives do not form symbiosis (*Phyllobacterium endophyticum, Ph. loti* and *Bosea* sp.). Strains of non-symbiotic rhizobial species may be present in nodules as carriers of genes that do not directly participate in the formation of symbiosis, but can affect its effectiveness. Further phenotypic and genetic study of isolated microorganisms can make a significant contribution to understanding the ways of evolution and development of plant-microbial interactions in the legume-rhizobial system.

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