https://doi.org/10.17816/ecogen17805

PHYLOGENETIC CHARACTERISTIC OF NODUL BACTERIA ENDEMIC FOR SOUTHERN URAL SPECIES OF THE GENUS *OXYTROPIS* (FABACEAE)

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Cite this article as: Baymiev AnKh, Vladimirova AA, Akimova ES, et al. Phylogenetic characteristic of nodul bacteria endemic for Southern Ural species of the genus *Oxytropis* (Fabaceae). *Ecological genetics*. 2020;18(2):157-167. https://doi.org/10.17816/ecogen17805.

Received: *18.11.2019* Revised: *06.04.2020* Accepted: *23.06.2020*

❀ **Background.** An analysis of the spatial distribution of some taxonomically and ecologically related legumes in the Ural showed a nontrivial spatial distribution of related species of the genus *Oxytropis* DC of the Orobia Bunge section within the Uchalinsky uplands. Despite the similarities in ecology, these species practically do not grow together. Explicit spatial segregation of closely related plants over a relatively small area allows this phenomenon to be used as a convenient model for studying the effect of segregation of closely related legume species on the genetic composition of their nodule bacteria. **Materials and methods.** The genetic diversity of nodule bacteria entering into symbiosis with *O. kungurensis*, *O. baschkiriensis*, *O. approximata* and *O. gmelinii* plants was studied. In addition, the polymorphism of their symbiotic genes has also been analyzed. **Results.** Phylogenetic characteristics of nodule bacteria endemic for the Southern Ural belonging to 4 species of leguminous plants of the genus *Oxytropis* of the section Orobia: *O. kungurensis*, *O. baschkiriensis*, *O. approximata*, *O. gmelinii* which are characterized by spatial separation of the growth sites, also called plant segregation, are given. It was shown that all of them belong to the genus *Mesorhizobium* despite certain phylogenetic differences of bacteria. Analysis of the symbiotic genes of the analyzed strains revealed a lack of congruence of their phylogeny with the core part of the genome. It was found that the microsymbionts of *O. baschkiriensis* plants differ in the phylogeny of nod-genes from nodule bacteria of other plants of the *Oxytropis* genus and are close to microsymbionts of plants of the *Lupinaster* genus growing in the Southern Urals. **Conclusion.** Acquisition of the property to enter into symbiosis with nodule bacteria of plants of the genus *Lupinaster* may turn out to be an adaptive mechanism that arose as a result of segregation of *O. baschkiriensis* from other species of *Oxytropis*.

❀ **Keywords:** rhizobia; symbiosis; *Oxytropis*; phylogeny; symbiotic genes.

ФИЛОГЕНЕТИЧЕСКАЯ ХАРАКТЕРИСТИКА КЛУБЕНЬКОВЫХ БАКТЕРИЙ ЭНДЕМИЧНЫХ ДЛЯ ЮЖНОГО УРАЛА ВИДОВ РОДА *OXYTROPIS* (FABACEAE)

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Для цитирования: Баймиев Ан.Х., Владимирова А.А., Акимова Е.С., и др. Филогенетическая характеристика клубеньковых бактерий эндемичных для Южного Урала видов рода *Oxytropis* (Fabaceae) // Экологическая генетика. – 2020. – Т. 18. – № 2. – С. 157–167. https://doi.org/10.17816/ecogen17805.

Поступила: *18.11.2019* Одобрена: *06.04.2020* Принята: *23.06.2020*

❀ Проведен анализ полиморфизма и филогении клубеньковых бактерий эндемичных для Южного Урала четырех видов бобовых растений рода *Oxytropis* секции Orobia: *O. kungurensis*, *O. baschkiriensis*, *O. approximata*, *O. gmelinii*, характеризующихся пространственной разобщенностью мест произрастания, также называемой сегрегацией растений. Показано, что несмотря на определенные филогенетические различия бактерий, все они относятся к роду *Mesorhizobium*. Анализ симбиотических генов исследуемых штаммов на основании сравнительного анализа последовательностей генов *nifH* и *nodC* выявил определенные различия их филогении с коровой частью генома. Обнаружено, что микросимбионты растений *O. baschkiriensis* по филогении гена *nodC* отличаются от ризобий, полученных из клубеньков других изученных видов рода *Oxytropis* и близки к микросимбионтам растений рода *Lupinaster*, произрастающих на Южном Урале. Приобретение свойства вступать в симбиоз с клубеньковыми бактериями, характерными для растений рода *Lupinaster*, могло быть следствием сегрегации *O. baschkiriensis* от других родственных видов рода *Oxytropis*.

❀ **Ключевые слова:** ризобия; симбиоз; *Oxytropis*; филогения; симбиотические гены.

INTRODUCTION

Nodule bacteria (rhizobia) represent an extensive, genetically heterogeneous group of soil Gram-negative microorganisms capable of entering into intracellular symbiosis with legumes and providing fixation of atmospheric nitrogen.

In the course of the long-term joint evolution of leguminous plants and rhizobia, a signaling system of interaction between symbionts was formed, providing specific recognition of partners and leading to their genetic integration [1]. Legumes of temperate latitudes, inhabited by the most specialized and evolutionarily young representatives of the subfamily of papilionaceous plants, are characterized by highly specific "cross-inoculation groups" interactions, in which rhizobia of a certain species or biotype enter into effective symbiosis with representatives of a certain genus or several closely related plant genera [2]. Such an increase in the specificity of partner interaction is evolutionarily accompanied by the increases in nitrogen-fixing activity [3] and dependence of leguminous plants on their microsymbionts. Therefore, the influence of soil microbiome on the distribution area of wild legumes in temperate zones, along with edaphic and climatic factors, is considered due to their close relationship with nodule bacteria and the relatively high specificity of their interaction [4, 5].

Analyzing the aspects of the spatial distribution of several taxonomically and ecologically related species of legumes in the Urals, M.S. Knyazev [5] noted a number of non-trivial cases, the explanation of which may shed light on the spatial distribution of these plants. The peculiarity of this phenomenon is most distinctly manifested in the example of the spatial distribution of closely related species of the genus *Oxytropis* DC of the section *Orobia* Bunge within the Uchalinsky hillocky area, which represents a series of low submontane, slightly sloping ranges of mountains whose foothills are covered with sparse forests, and the tops are occupied by areas of mountain steppes. Thus, the steppe species growing here, located mostly in the hilltops, are represented by a series of isolated populations. Uchalinsky hillocky area is a floristically original territory; a number of species of the Ural flora grow only or predominantly within this region, including narrow endemics of the Uchalinsky hillocky area. Here, only the habitats of five species (including one hybrid) of the genus *Oxytropis* of the Orobia section overlap; these species include *O. kungurensis Knjasev* subsp. *demidovii* (Knjasev) Knjasev (hereinafter *O. kungurensis*), *O. baschkiriensis* Knjasev subsp. *skvortsovii* Knjasev (hereinafter *O. baschkiriensis*), *O. approximata* Less., *O. gmelinii* Fisch. ex Boriss., *O. spictata* (Pall.) O. et B. Fedtach., and *O.* × *lessingiana* Knjasev. These taxa are ecological twins growing in similar communities. Despite the similarity of ecology, these species hardly grow together. This phenomenon has been designated with the term plant segregation. The distribution of the localities of two species, namely, *O. baschkiriensis* and *O. gmelinii,* in the hillocky area, framing the right bank of the Ural river along the 20 km of the valley to the north of the mouth of Mindyak river, is a typical example of segregation. Directly near the mouth of Mindyak river, on hillocks, only *O. gmelinii* grows; 2 km to the north, on Tuytube hill (575 m), under the same conditions, only *O. baschkiriensis* survived; on the hill neighboring to Tuytube, only *O. approximata* proliferated; another 2–4 km to the north, where *O. gmelinii* grew on a number of peaks of Ulutau ridge [5].

Explicit spatial segregation of closely related plants over a relatively small area enables the usage of this phenomenon as a convenient model for studying the effect of spatial separation of closely related legume species on the genetic composition of their nodule bacteria.

This work aimed to test the genetic differences in the rhizobia obtained from the nodules of closely related species of white locoweeds subject to segregation.

In this regard, we studied the genetic diversity and phylogeny of nodule bacteria entering into symbiosis with *O. kungurensis*, *O. baschkiriensis*, *O. approximata*, and *O. gmelinii* plants and analyzed the phylogeny of their symbiotic genes, namely, *nifH* (encodes the structure of nitrogenase proteins) and *nodC* (encodes the structure of the core part of the Nod factor (NF) molecule involved in signaling during nodule formation).

MATERIALS AND METHODS

Bacterial strains and cultivation conditions

In the work, we used isolates of nodule bacteria isolated from the nodules of *O. kungurensis*,

O. baschkiriensis s. l., *O. approximata*, and *O. gmelinii*, growing in the Southern Urals in the area of Uchalinsky hillocky area.

Bacteria were isolated from nodules by obtaining punctures from the zone of bacterial generation and inoculating them on a nutrient agar YM medium (0.1% yeast extract, 1% mannitol, 0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.01% NaCl, and 1.5% agar) to grow individual colonies [6]. One pure culture of bacteria was obtained from each nodule. Preliminary testing of isolates belonging to the group of nodule bacteria was checked by polymerase chain reaction (PCR) analysis of the presence of the *nifH* gene, which is characteristic of all rhizobial species.

Isolation of total DNA

DNA was isolated from bacteria by thermocoagulation. A small amount of bacterial mass was placed in 1.5 ml tubes with 100 μL 1% Triton X100 and 1% suspension Chelex100 resin (BioRad, USA) and after suspension, incubated at 95 °C for 10 min. Cellular debris was precipitated by centrifugation at 12,000 g for 3 min. The supernatant was used as a template for PCR.

Genetic analysis of strains

The genetic diversity of the strains collected was studied using random amplified polymorphic DNA (RAPD) analysis [7] using "random" primers, namely, 1) 5'-gggcgctg-3'; 2) 5'-caggcccatc-3'; 3) 5'-gcgtccattc-3'. This analysis also enabled to reduce the number of samples by combining microorganisms with identical RAPD profiles into homogeneous groups, from which only one sample was subsequently obtained for the work.

PCR restriction fragment length polymorphism (RFLP) analysis [8] of the *16S* rRNA gene was performed using frequently cutting restriction endonucleases *Kzo91* and *HaeIII*. Universal primers fD1 5'-cccgggatccaagcttaaggaggtgatccagcc-3' and rD1 5'-ccgaattcgtcgacaacagagtttgatcctggctcag-3' were used to amplify the *16S* rRNA gene, flanking a gene fragment of approximately 1500 bp. [9]; primers RecAF 5'-ggcagttcggcaagggctcgat-3' and RecAR 5'-atctggttgatgaagatcaccat-3' were used for amplification of the *recA* genes; NifHF 5'-ttctatggaaagggcggcattggcaagct-3' and NifHR 5'-atctcgccggacatgacgatataaatttc-3' were used for amplification of the *nifH* gene; NodCF 5'-cgttt cgtcttatgcggtgctc-3' and NodCR 5'-cagctgcgtctcgtatt gat-3' were used for amplification of the *nodC* gene [10].

Nucleotide sequences were determined using an Applied Biosystems 3500 automatic sequencer made by Applied Biosystems, Inc. (USA), using Big Dye Terminator v. 3.1 kits.

Phylogenetic analysis

Phylogenetic analysis of the strains under study was performed based on multiple alignment (ClustalW) of the sequenced fragments of *16S* rRNA, *recA*, *nodC, and nifH* genes. Phylogenetic trees were constructed using the Megalign program from Lasergene software package using the neighbor-joining method (NEIGHBOR). Nucleotide sequences for comparative analysis were obtained from the GenBank database (www.ncbi.nlm.nih.gov). The statistical significance of branching (bootstrap analysis) was assessed using the corresponding function of Megalign program based on 1,000 alternative trees.

The nucleotide sequences of the *16S rRNA, recA, nodC*, and *nifH* genes of the strains under study were deposited in GenBank database under the accession numbers of MK402237-MK402258, MK511967-MK511971, MK511979, and MK511980.

Cross-inoculation experiments

The nodulation ability of the strains on the roots of the studied plant species was analyzed by presowing seed inoculation. The seeds treated with a suspension of bacteria ($3-7 \times 10^6$ CFU/ml) were planted in separate pots with sterile sand. After 30–40 days, the plant roots were analyzed visually for the presence of nodule formation. The experiments were conducted with five repetitions.

RESULTS AND DISCUSSION

Nodules were collected from the roots of the studied plants growing in the Southern Urals, from which pure cultures of rhizobia were isolated, to study the genetic diversity of microsymbionts. In total, 32 pure bacterial cultures were obtained from the nodules of 19 *O. approximata* plants, 56 from the nodules of 28 *O. baschkiriensis* plants, 16 from the nodules of 8 *O. kungurensis* plants, and 6 from the nodules of 4 *O. gmelinii* plants. The ratios of the numbers of pure

cultures and plants were due to the limited number of nodules (no more than 2–3) on the roots of each plant. Subsequently, one pure culture of bacteria was obtained from each nodule. The study of the genetic diversity of the obtained isolates by RAPD analysis revealed a certain polymorphism in the DNA of samples under study, which formed 27 genetically homogeneous groups. Thus, the isolates obtained from the nodules of *O. approximata* belonged to 7 genetically homogeneous groups, those from the nodules of *O. baschkiriensis* s. l. belonged to 13 groups, those from *O. kungurensis* s. l. belonged to 6 groups, and those from nodules of *O. gmelinii* belonged to 1 group (Fig. 1).

Preliminary phylogenetic analysis conducted with the use of 16S-RFLP revealed that the strains formed eight monophyletic groups. Accordingly, the strains of rhizobia from the nodules of *O. kungurensis* formed 2 groups, those of *O. baschkiriensis* s. l., 3 groups; those of *O. approximate,* 2 groups, and those of *O. gmelinii,* 1 monophyletic group.

The sequencing of conserved genes (*16S* rRNA and *recA*) and their comparative analysis with other similar genes deposited in GenBank were performed to determine the phylogenetic affiliation of the representatives of the identified groups of microorganisms. The results showed that the studied strains of rhizobia, despite exhibiting certain phylogenetic differences, all belong to the genus *Mesorhizobium*. For the *16S* rRNA gene, the similarity of the strains ranged from 98.4% to 99.8%, and for the *recA* gene, the value ranged from 89.7% to 96.7%. The degree of phylogenetic relationship of the strains was independent of whether they were symbionts of the same or different plant species, given that in plants of the same species, nodules contained bacteria with greater phylogenetic differences than those isolated from nodules of different *Oxytropis* species (Figs. 2 and 3). Thus, the spatial separation and the absence of joint growth of plants under study cannot be explained by the influence of species composition and phylogenetic differences of their rhizobia.

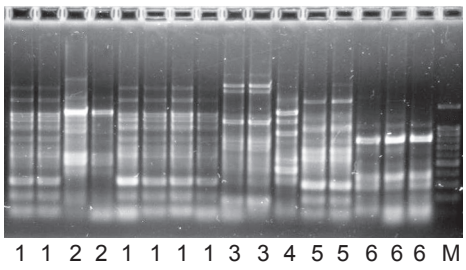

Fig. 1. Foregram of RAPD analysis of DNA of rhizobia isolated from nodules of *O. kungurensis*. The digits indicate the numbers of genetically homogeneous groups. M: 100 bp marker

Products of specialized *sym*-genes caused the interaction with macrosymbionts in nodule bacteria. These products include *nif*-genes, which are responsible for nitrogen fixation and encode the synthesis and regulation of nitrogenase enzyme; *nod*-genes encoding the synthesis of NFs, which are responsible for the initiation and specificity of the symbiosis formed; *fix*-genes are necessary for nitrogen fixation and often linked to *nif*-genes but are not homologous to them [11, 12].

To date, a large number of studies have indicated the high mobility of *sym*-genes and susceptibility of their horizontal gene transfer (HGT) [13–18]. Such process is an integral part of the evolution of legume–rhizobial relationships [19–22] and often leads to the appearance of strains with altered host specificity or inclusion of new types of microorganisms to the group of nodule bacteria [23]. The participation of HGT in the evolution of rhizobia is confirmed by the localization of *sym*-genes on mobile genetic elements (plasmids or chromosomal islets bounded by IS-like elements) and by their characteristic panmictic population structure [24]. The wide expansion of *sym*-genes in plant-associated bacterial communities by means of HGT is considered to be the most probable method for the formation of the modern diversity of rhizobia and manifests itself in different phylogenies of symbiotic genes and constitutive genes [14, 25]. Therefore, the analysis of symbiotic genes is an integral part of research on the diversity of nodule bacteria.

In this work, the phylogeny of symbiotic genes of the strains was studied based on a comparative analysis of the sequences of *nodC* and *nifH* genes with similar sequences of other nodule bacteria obtained from GenBank database (Figs. 4 and 5, respectively).

The study of the phylogeny of the *nifH* gene of all the bacteria analyzed showed their similarity to analogous genes that are mainly found in bacteria of genus *Mesorhizobium*. At the same time, the differences in *nifH* nucleotide sequences of all the bacteria analyzed were insignificant. The greatest difference

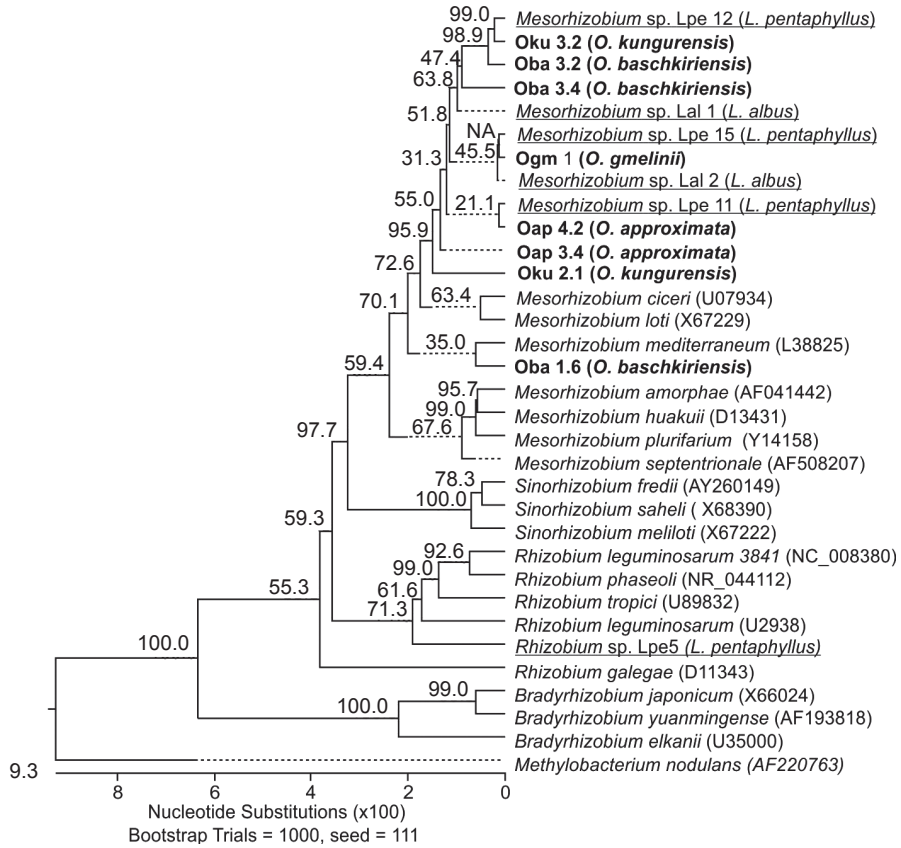

Fig. 2. Phylogenetic tree of nodule bacteria constructed on the basis of the comparative analysis of *16S* rRNA gene sequences. The strains of microorganisms studied in this work are marked bold; the strains isolated from the nodules of *L. pentaphyllus* and *L. Albus* are underlined

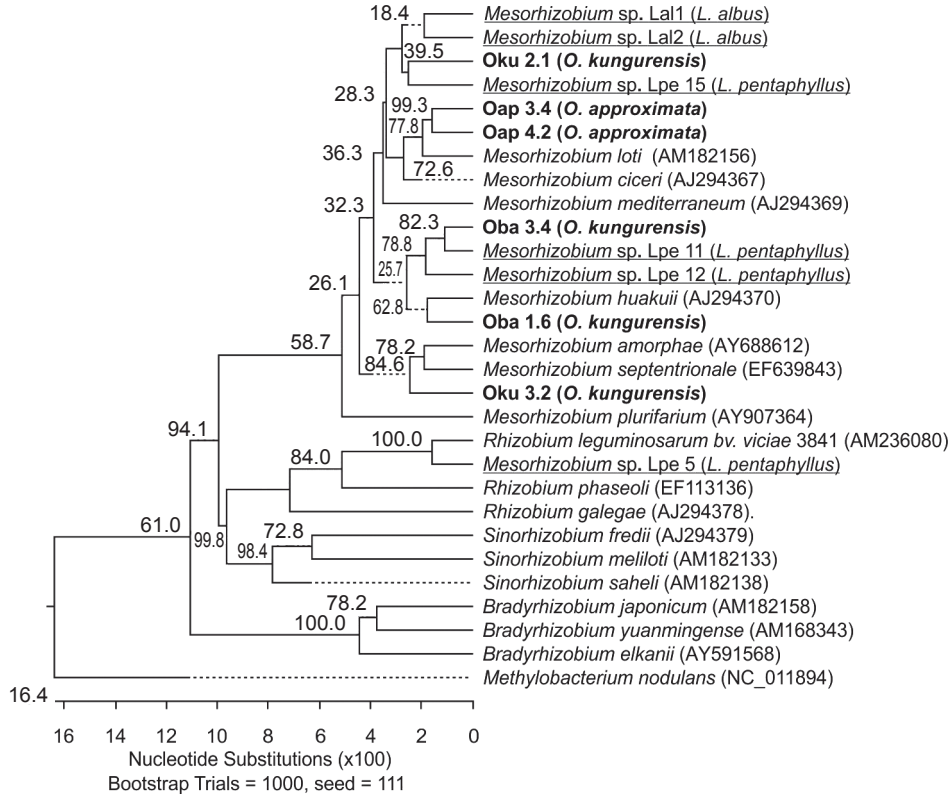

Fig. 3. Phylogenetic tree of nodule bacteria constructed on the basis of the comparative analysis of *recA* gene sequences. The strains of microorganisms studied in this work are marked bold; the strains isolated from the nodules of *L. pentaphyllus* and *L. Albus* are underlined

was revealed between the sequences of the *nifH* gene of two strains, namely, Oku 3.2 and Oku 2.1, which were symbiosis with *O. kungurensis* plants. Nevertheless, the similarity between them was 92%, which indicates the conservation of the nitrogenase genes of these bacteria.

Investigation of *nod*-gene sequences revealed interesting patterns. PCR-RFLP analysis of the *nodC* gene of the representatives of all homogeneous groups of microorganisms under study revealed the division of bacteria into two groups based on the similarity of bands on the foregram. Group 1 consisted of microorganisms isolated from the nodules of *O. baschkiriensis,* whereas Group 2 included microorganisms isolated from the nodules of other studied plants (data not presented). When analyzing the phylogeny of the *nodC* gene based on the comparative analysis of nucleotide sequences, the nodule bacteria of *O. baschkiriensis* plants for the *nodC* gene differed significantly from the rhizobia of other representatives of *Oxytropis* growing in Southern Urals (similarity 74.5%–78%). At the same time, they exhibited 99% or more similarity with microsymbionts of plants, which were studied earlier [26], of the genus *Lupinaster* Fabr. (*L. pentaphyllus* и *L. albus*), which grows together with *Oxytropis* species (Fig. 4). At present, the systematic position of plants of the genus *Lupinaster* is disputable, and a consensus on this issue has not been formed. Earlier, we discovered that these plants enter into symbiosis with the bacteria of genus *Mesorhizobium*, which is not typical for the plants of tribe Trifolieae, to which they are attributed [26]. However, despite the controversial situation with the systematic position of genus *Lupinaster*, these plants are not related to the plants of genus *Oxytropis*. The presence of *nodC* gene in the genome of the nodule bacteria *O. baschkiriensis*, which is almost identical to the analogous genes of rhizobia of *Lupinaster* plants, indicates several adaptive evolutionary processes. Studies should still determine whether the preference of *O. baschkiriensis* to enter into symbiosis with nodule bacteria with *nodC* genes, which is not typical for other species of white locoweed plants growing in Southern Urals, causes segregation or is a consequence of the spatial separation of this species from other species of white locoweed. A certain pattern of differences in the composition of nodule bacteria of segregating plant species has been revealed, which may help to determine the endemicity of several species of leguminous plants. Notably, not a single case of deviation from the segregation rule has been revealed for *O. baschkiriensis* on the Uchalinsky hillocky area, whereas for other white locoweed plants, isolated cases have been revealed (for example, the joint growth of O. kungurensis s. l. and *O. approximata*). *O. baschkiriensis* was isolated relatively recently [27] from the widespread species *O. ambigua* (Pall.) DC. s. l. (Eastern Europe up to the Vologda Region in the west, Western and Eastern Siberia, Mongolia) and differed in terms of nonessential traits. Perhaps, such a vast habitat of *O. ambigua* s. l. (including *O. baschkiriensis* s. str.), which is uncharacteristic of the species of the *Orobia* section, is associated with the genetic similarity of *Lupinaster pentaphyllus* s. l. rhizobia, which are also characterized by wide distribution (from Eastern Europe to Mongolia and the Far East) [28, 29]

The comparative analysis of nucleotide sequences revealed significant differences between the symbiotic *nod*-genes of nodule bacteria of *O. baschkiriensis* and *Lupinaster* plants from all known *nod*-genes previously described in the bacteria of genus *Mesorhizobium*. Thus, these plants formed a separate clade on the phylogenetic tree (Fig. 4). At the same time, the symbionts of *O. baschkiriensis, L. pentaphyllus,* and *L. albus* plants have 99% or more similarities with each other for this gene, regardless of the bacterial phylogeny. This finding suggests that these plants should have a high and unique specificity with their microsymbionts. Most likely, these plants belong to different groups of cross-inoculation with other plants entering into symbiosis with the bacteria of *Mesorhizobium*.

Our research confirmed this assumption. Experiments on the cross inoculation of *O. baschkiriensis* and *O. approximata* with nodule bacteria isolated from the nodules of these plants showed that numerous pink (active) nodules formed only in the case of plant interaction with strains isolated from the nodules of the same species; when plants were inoculated with the rhizobia of another species, nodules were not formed, or small white nodules were formed, which indicate the low functionality of these nodules. At the same time, the cross inoculation of *O. baschkiriensis, L. pentaphyllus*, and *L. albus*

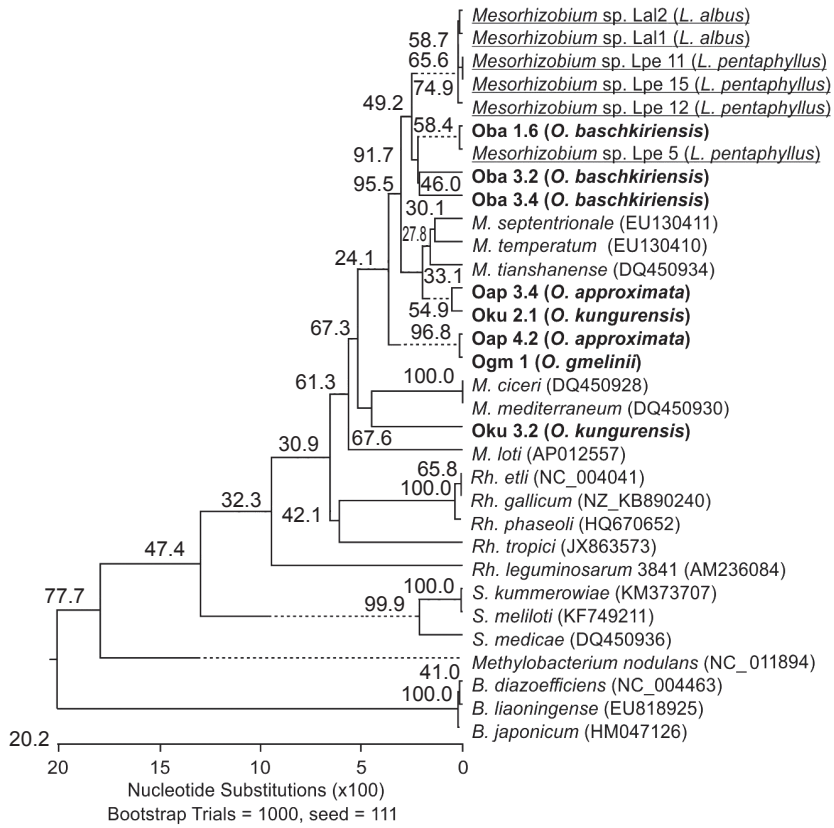

Fig. 4. Phylogenetic tree of nodule bacteria constructed on the basis of the comparative analysis of *nifH* gene sequences. The strains of microorganisms studied in this work are marked bold; the strains isolated from the nodules of *L. pentaphyllus* and *L. Albus* are underlined

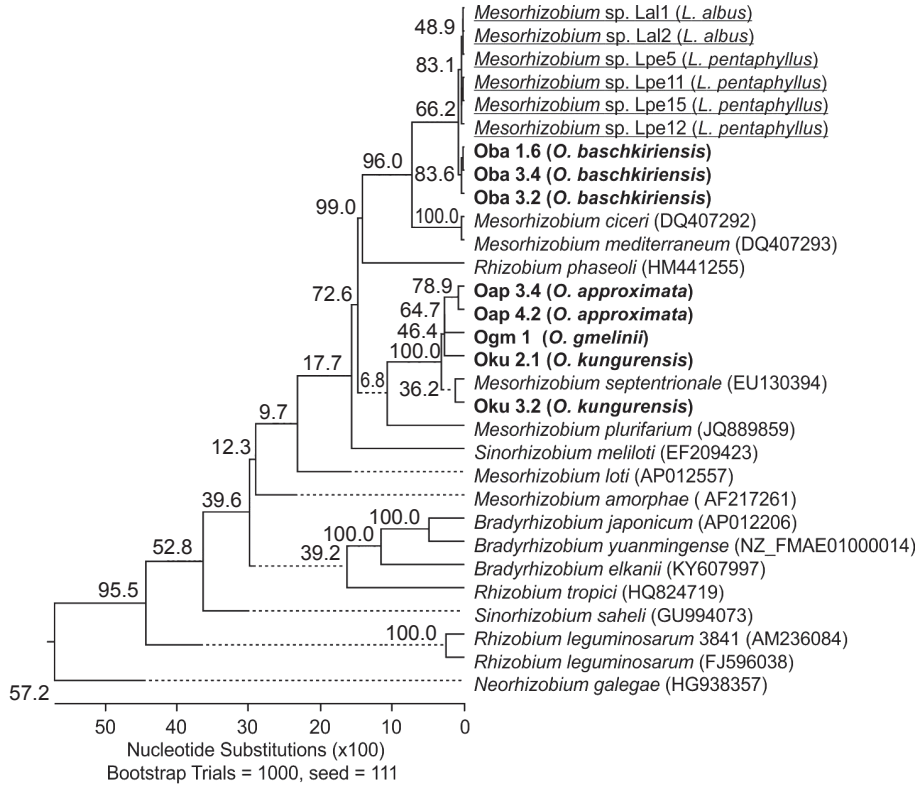

Fig. 5. Phylogenetic tree of nodule bacteria constructed on the basis of the comparative analysis of the *nodC* gene sequences. The strains of microorganisms studied in this work are marked bold; the strains isolated from the nodules of *L. pentaphyllus* and *L. Albus* are underlined

plants with their microsymbionts led to the formation of active nodules in all combinations, which indicates that the above species belong to the same group of cross inoculation.

The differences in the phylogeny of *nod*- and *nif*-genes of the strains analyzed were observed probably because the polymorphism of *nod*-genes is more correlated with the taxonomy of host plants than with the core elements of its genome. Furthermore, the genes responsible for nitrogen fixation, given the conservative function of the proteins that they encode, are less variable, and their polymorphism often has a strong correlation with the divergence of the core part of the bacterial genome. Both groups of genes can only jointly impart the properties of symbiotic nitrogen fixation to bacteria; in the genome, they form islets of symbiosis in *Mesorhizobium* bacteria and are therefore also transmitted together during HGT. This condition affects their evolution and leads to incomplete coincidence of the phylogeny of *nif*-genes and the core part of the genome [30, 31].

We have shown that the segregation of closely related leguminous plants can lead, in certain cases, to changes in the genetic composition of their nodule bacteria, which render their cross-inoculation impossible. For *O. baschkiriensis* plants, the acquisition of the ability to enter into symbiosis with native strains of the nodule *Mesorhizobium* bacteria containing unique *nod*-genes, which are detected nowadays only in the nodule bacteria of legumes of Southern Urals, can become an adaptive mechanism that can contribute to the fixation of *O. baschkiriensis* in new areas.

This work was financially supported by the Russian Foundation for Basic Research grant No. 17-44-020201.

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