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RESEARCH ARTICLE

Genetic diversity of microsymbionts from legumes *Oxytropis putoranica* M. Ivanova, *Oxytropis mertensiana* Turcz., *Astragalus norvegicus* Grauer, *Astragalus tugarinovii* Basil. growing on the Putorana Plateau in Arctic Russia

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Abstract

There is a significant potential for the introduction of legumes into the Arctic regions of Russia. The ability of legumes to form a nitrogen-fixing symbiosis with nodule bacteria is one of their most important characteristics. The article studies the genetic diversity of the 24 bacterial strains isolated from root nodules from wild populations of legumes *Oxytropis putoranica* M. Ivanova, *O. mertensiana* Turcz., *Astragalus norvegicus* Grauer, and *A. tugarinovii* Basil. collected on the Putorana Plateau (Krasnoyarsk region, Arctic Russia). The microbial strains were isolated using yeast broth made with a standard method using YMA mannitol. Genomic DNA was isolated from pure cultures and the primary identification of the strains was carried out by PCR followed by sequencing of the 16S rRNA marker

gene fragment. To clarify the identity of the species, ITS sequencing of the region was performed. The isolates were assigned to six genera and to five families of the order Hyphomicrobiales: *Pararhizobium* and *Neorhizobium* (family Rhizobiaceae), *Phyllobacterium* (Phyllobacteraceae), *Microvirga* (Methylobacteriaceae), *Bosea* (Boseaceae) and *Tardiphaga* (Bradyrhizobiaceae). Isolates from *O. putoranica* nodules were identified as *Neorhizobium galegae*, *Bosea* sp., *Bosea vaviloviae*, and *Tardiphaga robiniae*. The isolated nodules of *O. mertensiana* were identified as *Pararhizobium herbae*, *Tardiphaga robiniae*, *Microvirga ossetica*, and *Microvirga* sp. Microsymbionts of *A. norvegicus* were assigned to *Bosea psychrotolerans* and to *Pararhizobium herbae* and *Tardiphaga robiniae* species, while isolates from *A. tugarinovii* were identified as *Phyllobacterium zundukense*, *Bosea* sp., and *Tardiphaga robiniae*. Symbiotic the *nod*A gene was detected in strains *P. herbae* P14/2-4 and P20/1-1, *P. zundukense* P17/1-7 and P17/3-2, while the *nod*C gene was not detected in any of the strains. The sterile test tube experiment confirmed the inability of strains *P. herbae* P14/2-4 and P20/1-1 to form nodules in host plants *O. mertensiana* and *A. norvegicus*, as well as in other wild arctic (*A. tugarinovii*, *O. putoranica*) and forage legumes (*Trifolium repens*, *Vicia cracca*, and *Lathyrus pratensis*). The results obtained expand the understanding of the taxonomic status and biodiversity of local microsymbionts of wild legumes that grow on the Putorana Plateau. The study of the symbiotic efficiency of Arctic rhizobia will allow us to identify the most promising strains for the development of effective biofertilizers for the cultivation of forage and pasture legumes under the extreme soil and climatic conditions of the Russian Arctic. In turn, the creation of highly adapted legume-rhizobial systems based on valuable genetic resources of Arctic rhizobia strains will expand the range of legume species promising for use in the creation of multi-component agrophytocenoses necessary for the sustainable development of animal husbandry in Arctic regions of Russia.

Keywords
Putorana Plateau, arctic legumes, *Astragalus*, *Oxytropis*, arctic agriculture, legume-rhizobial symbiosis, ribosomal RNA genes

Introduction

The territory of the northern regions is an important resource for the development of agriculture (Unc et al. 2021; Naydenov 2020). The total area of the Arctic sector of Russia is approximately 3 million km^2, which is 18% of the total territory of the Russian Federation, including 2.2 million km^2 of land (Pestsov 2021). In the northwestern part of the central Siberian Plateau, on an area of 250 thousand km^2, there is the Putorana mountain plateau. The plateau was formed by massive eruptions of a supervolcano about 247–252 million years ago. The solidified lava formed layers of basalt rock of varying composition. The plateau is covered by continuous permafrost. However, permafrost conditions change from west to east. In the Norilsk region, permafrost is partly discontinuous with through and interpermafrost taliks (Pospelov and Pospelova 2021). The climate is subarctic, strongly continental, but milder in some sheltered lake valleys. Winter is long and cold (average temperature -40 °C), and spring, summer, and autumn fall into three months: June, July, and August. Summer is rainy with an average temperature of +11...+13 °C. The plateau lies on the border between taiga and tundra. The vegetation is represented by larch-spruce

forests in the valley part, sparse forests, and shrub tundra on the upper slopes and on the surface of the plateau. The highest parts of the plateau, near the watershed, are dominated by rock and lichen tundra. The main part of the plateau is characterized by a uniform geomorphological structure combining plateau-like surfaces of mountain ranges and deeply incised valleys with medium and steep slopes. In terms of soil-geographic zonation, the plateau corresponds to a separate Anabar-Putorana province, which is part of the East Siberian permafrost-taiga region. The Putorana province is characterized by taiga peaty-humic, high-humic nongleyed soils with high acidity of the upper horizons, ochre podburs, tundra podburs and stony placers. Many on the soils of the Putorana plateau are specific and do not have analogues among the soils described above in other regions. The specificity of soil formation is mainly determined by the combination of mountainous relief, cold continental climate with excess moisture, and the composition of soil-forming rocks. The soils accumulate large amounts of organic matter. In general, data on the study of the soil cover of the Putorana Plateau are limited. The literature contains descriptions mainly of soils of individual mountain-tundra and forest-tundra regions (Senkov 2014; The unified state register… 2025). Lake Duluk is located in the eastern half of the plateau. The tundra in this part is shrub-sedge-moss and the surface of the plateau is a cold mountain desert. Most of the plateau is covered by the Putorana Nature Reserve, one of the largest in Russia (Pospelov, Pospelova 2021; Merganič et al. 2021). It was established to "preserve and study the natural course of natural processes and phenomena, the genetic fund of flora and fauna, individual species and communities of plants and animals, typical and unique ecological systems of the Putorana Plateau" (The statute…2025). However, many valuable ecosystems remain poorly studied due to their inaccessibility (Merganič et al. 2021).

The traditional form of economic use of the territory of the subarctic and arctic sector of Russia is reindeer husbandry. The basis for successful reindeer husbandry in the territory is, first of all, the availability of the forage base (Mizin et al. 2018). One of the most important protein-rich components of natural pastures and pastures are legumes, which are widespread in the temperate and arctic zones of the northern hemisphere. Two thirds of the species represented in the Russian Arctic belong to the tribe Galegeae (Bornn) Torr.et Gray, subtribe Astragalinae (Adans.) Benth. (Yurtsev 1986; Kamelin 2017). On the Putorana plateau, legumes are mainly represented by the genera *Oxytropis* DC., *Astragalus* L. and *Hedysarum* L., which are included in the diet of animals and birds, including reindeer, snow rams, ground squirrels, pikas, brant geese, and graylag geese (Larin 1951; Yurtsev 1986; Rosenfeld 2009; Kamelin 2017). Legume species such as *Astragalus alpinus* L. subsp. *arcticus* (Bunge) Hult., *Oxytropis adamsiana* (Trautv.) Jurtz., *Oxytropis nigrescens* (Pall.) Fisch., *Hedysarum arcticum* B. Fedtsch are ubiquitous. A significant proportion of northern oxytropes and astragals are cryophytes endemic to the Arctic or certain parts of it. For example, the rare Taimyr-Putorana endemic *Oxytropis putoranica* M. Ivanova grows mainly on open gravel surfaces, screes, and along the dry beds of running streams. *Oxytropis mertensiana* Turcz. occurs only in the eastern part of

Putorana in the highlands, in moist tundra. *Astragalus norvegicus* Grauer grows in lakeside meadows, river thickets, and damp open woodland. Astragalus tugarinovii Basil. prefers carbonate and non-turf soils. In the south-east of the Taimyr Peninsula, it is widespread on slopes and steppe meadows, common in the Golets tundra. However, on the Putorana plateau it was found only in the highlands of the dry mud tundra, at Lake Baselak and on a rocky outcrop in the western part of Lake Sobachye (Larin 1951; Pospelov and Pospelova 2021).

The geographical and ecological distribution of northern legumes and their role in ecosystems are largely determined by biocenotic and symbiotic relationships. Arctic soils differ from European soils in their low fertility and the presence of permafrost and seasonal frost (Larin 1951). One of the main limiting factors is the insufficient supply of nitrogen compounds readily available to plants in the soil (Beermann et al. 2015). To overcome this problem, legumes establish symbiotic relationships with nodule bacteria (rhizobia). These microorganisms are able to penetrate root hairs and form nodules in which atmospheric nitrogen is fixed. Therefore, rhizobia are important participants in the mutualistic plant-microbe symbiosis. They influence legume productivity and yield by providing additional means of survival in nitrogen deficiency and by contributing to an increase in soil fertility, which favours the introduction of new flora into local native communities (Provorov, Tikhonovich 2016). Therefore, rhizobia are important participants in mutualist plant-microbe symbiosis, influencing legume productivity and yield of legumes by providing plants with additional means of survival under nitrogen deficiency. Plants of the genera *Oxytropis* and *Astragalus* are nodulated by a wide range of rhizobial species. However, the dominant microsymbionts are members of the genus *Mesorhizobium* (Laguerre et al. 1997). In addition, members of the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Bosea*, and *Tardiphaga* can be found in *Oxytropis* and *Astragalus* nodules (Laguerre et al. 1997; Kuznetsova et al. 2015; Ampomah et al. 2017; Wdowiak and Malek 2020; Safronova et al. 2020). The low host specificity of *Oxytropis* and *Astragalus* species may indicate that they play an important role in the genetic diversification of microsymbionts, which may influence the adaptive capacity of legumes to different environmental extremes (Chen et al. 2015). There is no information in the literature on the rhizobia that inhabit the nodules of legumes that grow on the Putorana plateau. We propose that these legumes do not have an established species composition of nodule bacteria and that their nodules may contain taxonomically distinct groups of bacteria. Thus, arctic legume-rhizobial systems are good models for studying the formation of evolutionary relationships between legumes and rhizobia in harsh soil and climatic conditions of the north, which will facilitate the identification of adaptive microbial strains that can be used to obtain highly effective biofertilizers for growing legumes in various regions of the Russian Arctic.

Thus, the aim of our work was to create a collection of the wild microsymbionts of arctic legumes *O. putoranica*, *O. mertensiana*, *A. norvegicus*, and *A. tugarinovii* collected on the Putorana Plateau, to study of the genetic diversity of microorgan-

isms of the order Hyphomicrobiales by sequencing *rrs* gene sequences and the region between 16S and 23S rDNA (ITS region), to study the ability of the obtained strains to form effective symbiosis with leguminous plants by searching symbiotic *nod*A and *nod*C genes and under conditions of sterile test-tube experiment.

Materials and methods

Collection of nodules and isolation of bacterial strains

Root nodules were collected from wild populations of legumes *Oxytropis putoranica* (2 populations), *Oxytropis mertensiana*, *Astragalus norvegicus* and *Astragalus tugarinovii* growing in the vicinity of Lake Duluk (69.544246 N, 94.141467 E) during the 2022 expedition to the Putorana Plateau (Krasnoyarsk region) (Figs 1, 2).

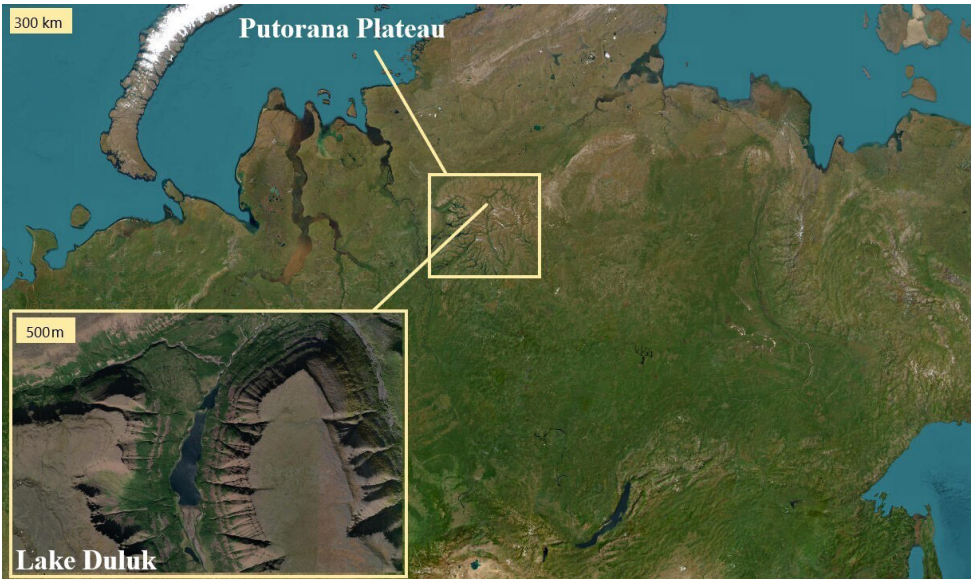

Figure 1. Putorana Plateau and Lake Duluk. Map from the site https://nakarte.me (ESRI Sputnik), 2024.

The selection of nodules was carried out from 3-5 individual plants so that the total number of nodules for each plant species was at least 20. Due to the different growing conditions and phenotypes of legumes, various tools were used to collect root nodules: garden shovels, chisels and hammers. Roots with nodules were carefully removed from the soil and placed in separate paper bags. The bags were stored in a ventilated, cool and dry place in the shade until the roots were completely dry. Five nodules of each plant species were selected in the laboratory for further work. Individual nodules were sterilized for 1 min in 96% ethanol, and strains were iso-

lated from homogenized material on yeast–mannitol agar (YMA) at 28°C using the standard technique (Novikova, Safronova 1992). To obtain pure bacterial cultures, visible colonies were picked and sequentially cloned twice in Petri dishes with YMA medium using the streak plate technique.

DNA extraction and PCR protocols

DNA was isolated using KIT (Thermo Scientific, EU) according to the manufacturer's instructions. The strains were identified by sequencing of 16S rDNA and ITS region. Primary identification of the strains was performed by PCR amplification of 16S marker fragments of the rRNA gene (*rrs*) fragments (1341–1404 bp) with subsequent sequencing of the amplicons. Amplification was carried out with the primers fD1 5′-AGAGTTTGATCCTGGCTCAG-3′ and rD1 5′-AAGGAGGTGATCCAGCC-3′. PCR was performed on a T100 automated amplifier (Bio-Rad, United States). PCR was carried out in 50 μL reaction mixtures containing 150 μM dNTP (Promega, United States), 5 pmol of each primer, 1 U Taq polymerase (Helicon, Russia) and 50–100 ng purified DNA template. The PCR conditions for 16S rDNA amplification were as follows: 95 °C 3.5 min; 94 °C, 1 min 10 s; 56°C, 40 s; 72°C, 2 min 10 s and final elongation 72 °C, 6 min 10 s. To amplify the ITS-region we used primers FGPS FGPS1490-72 5'-TGCGGCTGGGGATCCCCTCCTT-3' and FGPL132'-38 5'-CCGGGGTTTCCCCATTCGG-3'. The PCR condition for ITS region amplification: 95°C, 3.5 min; 94°C, 1 min; 50°C, 1 min; and 72 °C, 2 min with final elongation 72°C, 6 min 10 s (Normand et al. 1996).

Amplification of the *nod*C and *nod*A genes in isolates

To determine the nodulation capacity, a 666 bp fragment of the *nod*A gene was sequenced using the primers *nod*A-1 5′-GCRGTGGAARNTRNNCTGGGAAA-3 and *nod*A-2 5′-GNCCGTCRTCRAASGTCARGTA-3′ (Haukka et al. 1998); and a 930 bp fragment of the *nod*C gene using the primers nodCF 5′-AYGTHGTYGAYGACGGTTC-3′ and *nod*CR 5′-CGYGACAGCCANTCKCTATTG-3′ (Laguerre et al. 2021). PCR fragments amplified:95°C, 2 min; 94°C, 30 s; 50°C (primer pair *nod*A-1, *nod*A-2) or 53°C (primer pair *nod*CF, *nod*CR) for 1 min and 72°C, 1 min with final elongation 72°C, 3 min. The strain *Rhizobium leguminosarum* bv. *trifolii* RCAM1365 was used as a positive control.

Visualization and purification of the PCR product

Electrophoresis was performed on 1% agarose gel (Invitrogen, United States) in 0.5% TAE. The GeneRuler 1 Kb Plus DNA Ladder TM and Lambda DNA/HindIII marker (Fermentas, Lithuania) were used for the determination of the size and approximate quantification of the DNA fragments. The PCR product was purified using a Cleanup S-Cap Kit (Eurogen, Russia) according to the manufacturer's instructions.

Figure 2. Plants *Oxytropis putoranica*, *Oxytropis mertensiana*, *Astragalus norvegicus* and *Astragalus tugarinovii* found during the expedition.

Sequencing and data processing

The sequencing of the prepared PCR products was performed on the ABI PRISM 3500×l genetic analyzer (Life Technologies, United State) in the Core Centrum "Genomic Technologies, Proteomics and Cell Biology', All-Russia Research Institute for Agricultural Microbiology. The DNA sequences obtained were analyzed using the ChromasLite 2.6.4 program. Sequences of closely related strains were searched for in the GenBank database (https://www.ncbi.nlm.nih.gov) and the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed using the MEGA -X, XI software package (Tamura et al. 2011). Evolutionary distances were computed using the maximum composite likelihood method. Bootstrap analysis with 1000 replications was performed to estimate the support of clusters.

The nucleotide sequences were deposited in the GenBank database with accession numbers PQ870313 – PQ870336 for the *rrs* gene, PQ871498 – PQ871503 for the ITS region.

Storage of strains

Pure microsymbiont cultures (after sequential double cloning) were placed in the UNU 'Station for low-temperature automated storage of biological samples' at -80 °C (Liconic Instruments, Liechtenstein) for long-term storage. Information on the strains is available in the RCAM Internet database (https://arriam.ru/kollekciya-kul-tur1/).

Sterile test tube experiment

The seeds of wild arctic leguminous plants *A. norvegicus*, *A. tugarinovii*, *O. putoranica*, *O. mertensiana* and the forage legumes *Trifolium repens*, *Lathyrus pratensis*, *Vicia cracca* were surface sterilized by treatment with 98% H_2SO_4 for 5–10 min (depending on the size of the seeds). The treated seeds were carefully rinsed with sterile water and germinated on filter paper in Petri dishes at +25 °C in the dark for 3–5 days. The seeds were transferred in 50 ml glass tubes (2 seedlings per tube) containing 3 g of sterile vermiculite. Each glass tube was supplemented with 6 ml of the nutrient solution (g/l): K_2HPO_4 – 1.0, KH_2PO_4 – 0.25, $MgSO_4$ – 1.0, $Ca_3(PO_4)_2$ – 0.2, $FeSO_4$ – 0.02, as well as microelements according to M.V. Fedorov in a volume of 1 ml of the following composition: H_3BO_3 – 0.005, $(NH_4)_2MoO_4$ – 0.005, $ZnSO_4 \times 7\ H_2O$ – 0.005, $MnSO_4$ – 0.002 g/l. The seedlings were inoculated with *P. herbae* P14/2-4 and P20/1-1 in an approximate amount of 10^6 cells per tube. Uninoculated plants were used as negative control. The plant nodulation assay was carried out in duplicate. The plants were grown for 35 days in the growth chamber with 50 % relative humidity and four levels of illumination and temperature: night (dark, 18 °C, 8 h), morning (200 μmol $m^{-2}s^{-1}$, 20 °C, 2h), day (400 μmol $m^{-2}s^{-1}$, 23 °C, 12h), evening (200 μmol $m^{-2}s^{-1}$, 20 °C, 2h). Illumination was performed with L36W / 77 FLUORA lamps (Osram, Germany).

Results

Molecular phylogenetic identification of strains

As a result of this work, 24 bacterial isolates were obtained from nodules collected from the roots of *O. putoranica*, *O. mertensiana*, *A. norvegicus*, and *A. tugarinovii*. Four of them were fast-growing and formed colonies on the third day, while the rest appeared on the fourth or fifth day. BLAST analysis of the *rrs* gene sequences allowed all the isolates obtained to be assigned to six genera of the order Hyphomi-

crobiales (formerly Rhizobiales): *Pararhizobium* and *Neorhizobium* (family Rhizobiaceae), *Phyllobacterium* (Phyllobacteriaceae), *Microvirga* (Methylobacteriaceae), *Bosea* (Boseaceae), and *Tardiphaga* (Bradyrhizobiaceae) (Table 1).

Table 1. BLAST results of 16S rRNA gene fragments comparison for strains of nodules of *O. putoranica*, *O. mertensiana*, *A. norvegicus*, and *A. tugarinovii* plants collected near Lake Duluk (Putorana Plateau, Krasnoyarsk region)

Strain ID	Species	Source of isolation	Closely related type strain(s) identified by BLAST	Similarity, %
P13/1-1	*Tardiphaga robiniae*	*O. putoranica*	*Tardiphaga robiniae* R-45977	99.71
P13/1-2	*B. vaviloviae*		*Bosea vaviloviae* Vaf-18	100.00
P13/2-2	*B. vaviloviae*		*Bosea vaviloviae* Vaf-18	100.00
P13/2-3	*Tardiphaga robiniae*		*Tardiphaga robiniae* R-45977	99.78
P18/1-2	*Tardiphaga robiniae*		*Tardiphaga robiniae* R-45977	99.93
P18/3-2	*Neorhizobium galegae*		*Neorhizobium galegae* NBRC 14965, *Neorhizobium vignae* CCBAU 05176	99.48, 99.77
P18/3-6	*Tardiphaga robiniae*		*Tardiphaga robiniae* R-45977	99.85
P18/5-1	*Neorhizobium galegae*		*Neorhizobium galegae* NBRC 14965, *Neorhizobium vignae* CCBAU 05176	99.41, 99.66
P18/5-2	*Bosea* sp.		*B. lathyri* R-46060, *B. caraganae* RCAM04680, *B. psychrotolerans* 1131	99.14
P14/2-2	*Bosea psychrotolerans*	*A. norvegicus*	*Bosea psychrotolerans* 1131	99.93
P14/2-3	*Tardiphaga robiniae*		*Tardiphaga robiniae* R-45977	99.86
P14/2-4	*Pararhizobium herbae*		*Pararhizobium herbae* CCBAU 83011	100.00
P14/3-3	*Bosea psychrotolerans*		*Bosea psychrotolerans* 1131	99.48
P14/5-1	*Bosea psychrotolerans*		*Bosea psychrotolerans* 1131	99.33
P20/1-1	*Pararhizobium herbae*	*O. mertensiana*	*Pararhizobium herbae* CCBAU 83011	100.00
P20/1-2	*Tardiphaga robiniae*		*Tardiphaga robiniae* R-45977	99.86
P20/2-1	*Tardiphaga robiniae*		*Tardiphaga robiniae* R-45977	99.86
P20/4-1	*Microvirga ossetica*		*Microvirga ossetica* V5/3M	99.86
P20/5-1	*Microvirga* sp.		*Microvirga ossetica* V5/3M	99.18
P17/1-6	*Bosea* sp.	*A. tugarinovii*	*Bosea caraganae* RCAM04680	99.34
P17/1-7	*P. zundukense*		*Phyllobacterium zundukense* Tri-48	99.93
P17/2-3	*Tardiphaga robiniae*		*Tardiphaga robiniae* R-45977	99.93
P17/3-2	*P. zundukense*		*Phyllobacterium zundukense* Tri-48	99.93
P17/3-3	*Tardiphaga robiniae*		*Tardiphaga robiniae* R-45977	99.93

Four fast growing isolates P18/3-2 and P18/5-1 from *O. putoranica* nodules, P14/2-4 from *A. norvegicus* nodule and P20/1-1 from *O. mertensiana* nodule were assigned to the family Rhizobiaceae based on the results of the sequencing of the *rrs* gene fragment. These isolates were grouped into two groups on the phylogenetic tree (Fig. 3).

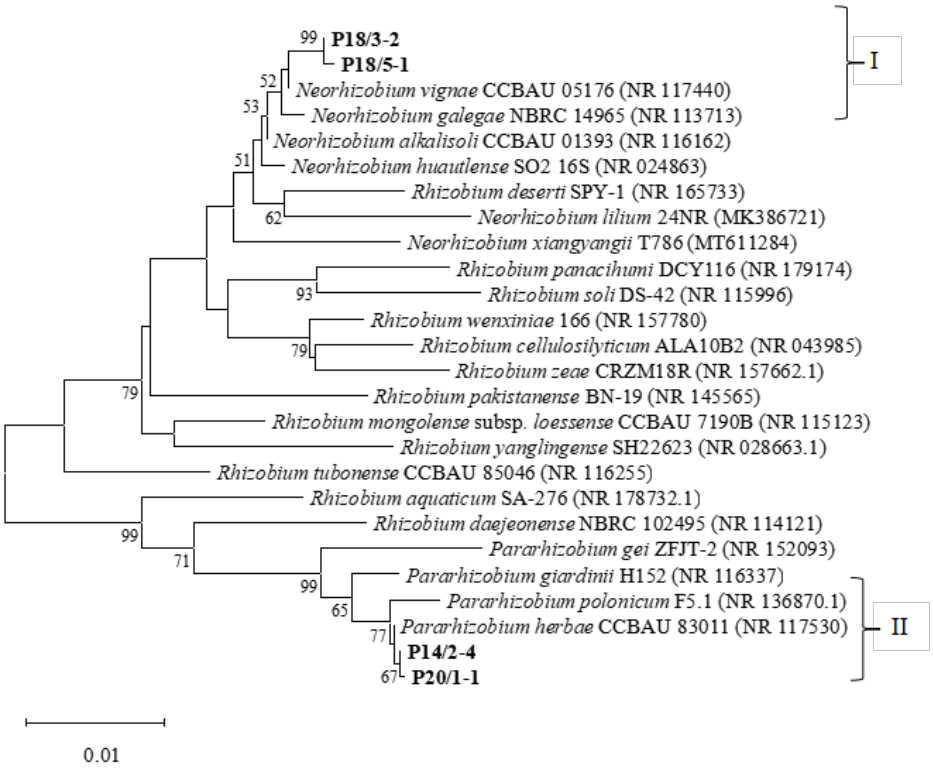

Figure 3. Unrooted phylogenetic tree constructed on the basis of a comparative analysis of the nucleotide sequences of 16S rRNA gene fragment of strains obtained from nodules of *O. putoranica*, *O. mertensiana*, *A. norvegicus* and representatives of the related species *Rhizobium*, *Neorhizobium*, and *Pararhizobium*. Isolates are shown in bold. Clusters I–II are statistically different clusters. Support levels above 50% are indicated.

Cluster I included isolates P18/3-2, P18/5-1 and type strains *Neorhizobium vignae* CCBAU 05176 and *Neorhizobium galegae* NBRC 14965 with a low level of support (Fig. 3). The level of similarity of *rrs* between isolates P18/3-2, P18/5-1 and the type strain *N. vignae* CCBAU 05176 was 99.77 and 99.66%, respectively, whereas the level of *rrs* similarity between isolates P18/3-2, P18/5-1 and the type strain of *N. galegae* NBRC 14965 was 99.48 and 99.41%, respectively (Table 1).

Cluster II was formed by isolates P14/2-4, P20/1-1 and the type strains *Pararhizobium polonicum* F5.1 and *Pararhizobium herbae* CCBAU 83011 with a relatively low (77%) level of support. The isolates had a 100% *rrs* similarity to the type strain *P. herbae* CCBAU 83011, while the level of *rrs*- similarity between isolates and the type strain *P. polonicum* F5.1 was 99.61% (Table1).

For isolates P18/3-2, P18/5-1, P14/2-4 and P20/1-1, the ITS fragment was sequenced and a phylogenetic tree constructed. These isolates were grouped into two groups (Fig. 4).

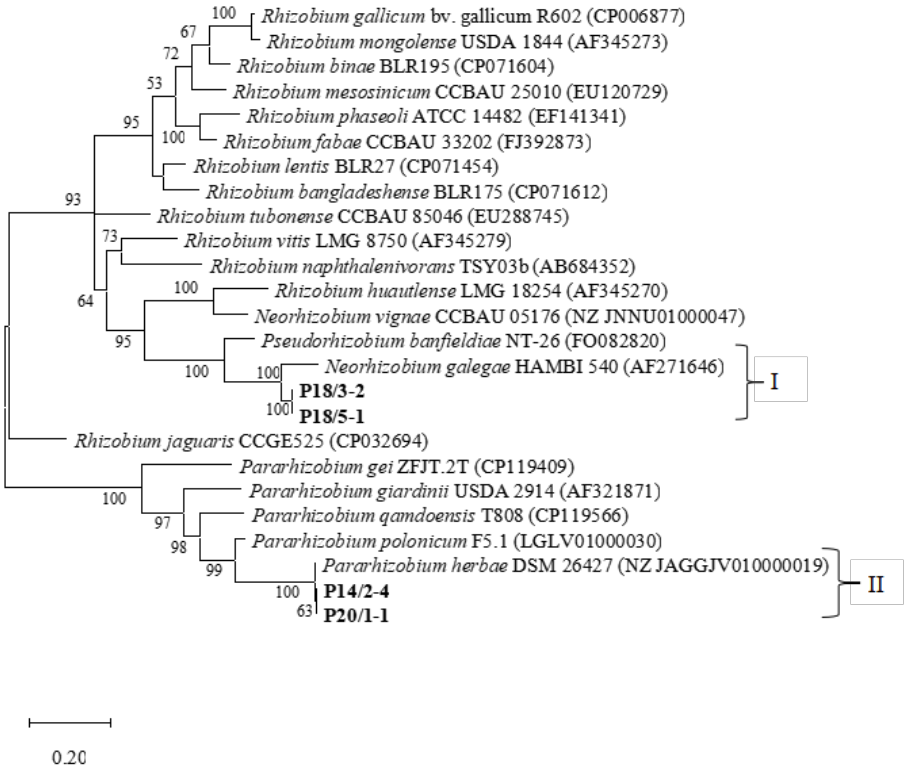

Figure 4. Unrooted phylogenetic tree constructed on the basis of a comparative analysis of the nucleotide sequences of the fragment of the ITS region of four strains, as well as representatives of related species of *Rhizobium*, *Neorhizobium*, *Pseudorhizobium* and *Pararhizobium*. Isolates are shown in bold. Clusters I–II are statistically different clusters. Support levels above 50% are indicated.

Cluster I included isolates P18/3-2, P18/5-1 and the type strain *N. galegae* HAMBI 540 at 100% support level (Fig. 4). The level of ITS region similarity between isolates P18/3-2, P18/5-1 and this type strain was 96.16 and 95.97%, respectively (Table 2).

Table 2. Similarity levels (%) of the ITS region of strains P18/3-2, P18/5-1, P14/2-4, and P20/1-1, isolated from root nodules of *O. putoranica*, *O. mertensiana*, *A. norvegicus* and closely related type strains, belonging to the genera *Neorhizobium*, *Pararhizobium*, *Pseudorhizobium* and *Rhizobium*

Closely related type strains	Strain ID			
	P18/3-2	**P18/5-1**	**P14/2-4**	**P20/1-1**
N. galegae HAMBI 540	**96.16**	**95.97**	-	-
N. vignae CCBAU 05176	86.36	86.06	-	-
P. banfieldiae NT-26	83.01	82.24	-	-
R. lentis BLR27	79.04	77.89	-	-
P. gei ZFJT.2T	-	-	87.77	87.80
P. giardinii USDA 2914	-	-	84.74	84.93
P. herbae DSM26427	-	-	**98.91**	**99.00**
P. polonicum F5.1	-	-	89.64	89.64

Cluster II included isolates P14/2-4, P20/1-1, and the type strain *P. herbae* DSM26427 at 100% support level (Fig. 4). The ITS regions of isolates P14/2-4 and P20/1-1 showed 98.91 and 99.00% similarity to this type strain, respectively (Table 2). In order for a strain to be classified as a specific species, there must be at least 95.5% similarity in the ITS sequence between them (Ferraz et al. 2022). The results of the ITS sequencing confirmed that the isolates P14/2-4 and P20 / 1-1 were related to the species *P. herbae*, while P18/3-2 and P18/5-1 to the species *N. galegae*.

Phylogenetic analysis of the sequences of the fragments of the *rrs* gene of isolates P17/1-7 and P17/3-2 from *A. tugarinovii* nodules showed that they formed a cluster I with the type strain *Phyllobacterium zundukense* Tri-48 at a support level (Fig. 5) and had 99.93% similarity to it (Table 2).

On the ITS tree, strains P17/1-7 and P17/3-2 formed a group I (97% support level) with the type strain *P. zundukense* Tri-48 (Fig. 6) and had 98.20 and 98.23% similarity in the ITS gene, respectively. Therefore, isolates P17/1-7 and P17/3-2 were identified as *P. zundukense*.

The isolates P13/1-2, P13/2-2, and P18/5-2 from the *O. putoranica* nodules, P14/2-2, P14/3-3, and P14/5-1 from the *A. norvegicus* nodules and the P17/1-6 from the *A. tugarinovii* nodule were assigned to the *Bosea* genus according to the results of *rrs* gene fragment (Table 1, 3).

These isolates were grouped into four groups on the phylogenetic tree (Fig. 7).

Cluster I was formed by isolates P14/2-3, P14/3-3, P14/5-1 and the type strain *Bosea psychrotolerans* 1131 with a rather high support level (Fig. 7). The isolate P14/2-2 showed 99.93 and 99.85%, the isolate P14/3-3 showed 99.48 and 99.14%, the isolate P14/5-1 showed 99.21 and 99.14% *rrs* similarity to the closest type strain *B. psychrotolerans* 1131 and *B. vaviloviae* Vaf-18, respectively (Table 3). Thus, these strains were assigned to the species *B. psychrotolerans*.

Table 3. Similarity levels (%) of the 16S rRNA gene fragments comparison for strains from nodules of *O. putoranica*, *A. norvegicus*, *A. tugarinovii*, and closely related type strains, belonging to genera *Bosea*

Strain ID	Closely related type strains				
	B. caraganae **RCAM04680**	***B. lathyri*** **R-46060**	***B. psychrotolerans*** **1131**	***B. vaviloviae*** **Vaf-18**	***B. massiliensis*** **63287**
P13/1-2	98.71	99.07	99.78	**100**	98.35
P13/2-2	98.68	99.05	99.78	**100**	98.32
P14/2-2	98.67	99.19	**99.93**	99.85	98.16
P14/3-3	98.00	98.50	**99.48**	99.14	97.57
P14/5-1	98.00	98.50	**99.21**	99.14	97.56
P17/1-6	**99.34**	98.98	98.83	98.91	98.98
P18/5-2	**99.14**	**99.14**	**99.14**	99.07	97.92

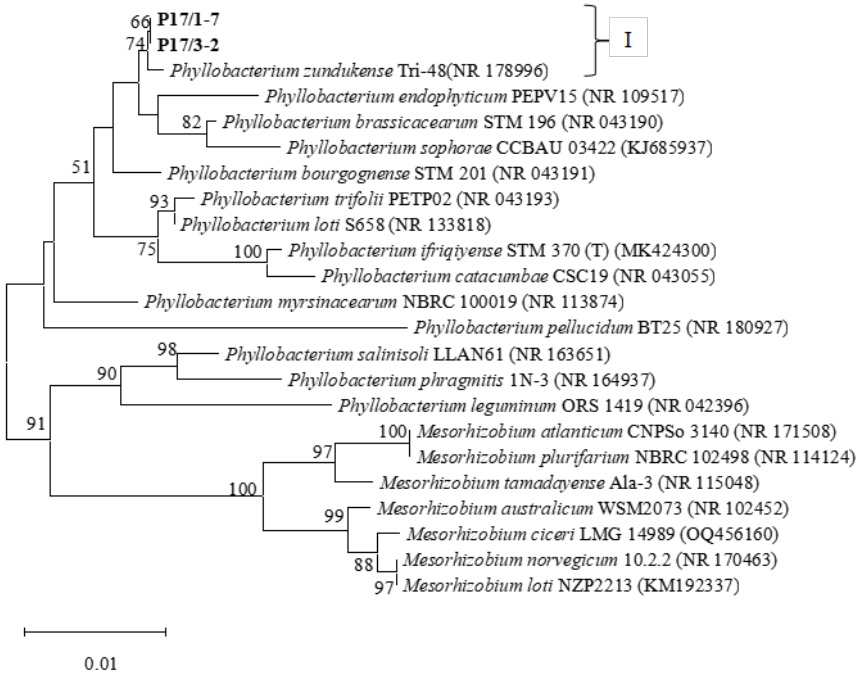

Figure 5. Unrooted phylogenetic tree constructed on the basis of a comparative analysis of the nucleotide sequences of the 16S rRNA gene fragment of strains obtained from nodules of *A. tugarinovii* and representatives of the related species *Phyllobacterium* and *Mesorhizobium*. Isolates are shown in bold. Cluster I is a statistically different cluster. Support levels above 50% are indicated.

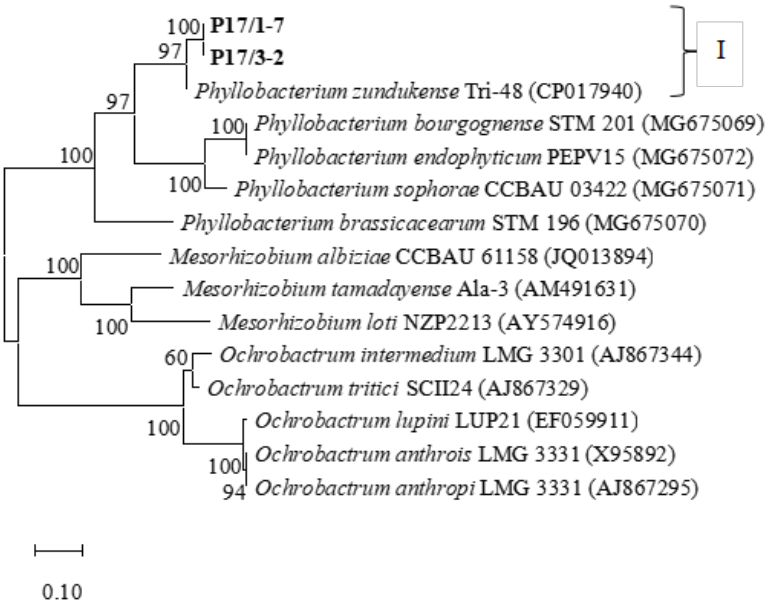

Figure 6. Unrooted phylogenetic tree constructed on the basis of a comparative analysis of the nucleotide sequences of the fragment of the ITS region of two strains, as well as representatives of related species of *Phyllobacterium* and *Mesorhizobium*. Isolates are shown in bold. Cluster I is a statistically different cluster. Support levels above 50% are indicated.

Cluster II included isolates P13/1-2, P13/2-2 and the type strain *Bosea vaviloviae* Vaf-18 at a support level (Fig. 7). The 16S rRNA sequence analysis showed that isolates P13/1-2 and P13/2-2 were 100 and 99.78% similar to the closest type strain *B. vaviloviae* Vaf-18 and *B. psychrotolerans* 1131, respectively (Table 3). Thus, isolates P13/1-2 and P13/2-2 were assigned to species *B. vaviloviae*.

Cluster III included the isolate P18/5-2 and the type strain *Bosea lathyri* R-46060 with a low level of support (50%, Fig. 7). The similarity between the isolate and the type strains *B. lathyri* R-46060, *B. caraganae* RCAM04680, *B. psychrotolerans* 1131 was 99.14% (Table 3). Therefore, isolate P18/5-2 was identified as *Bosea* sp.

Cluster IV was formed by isolate P17/1-6 and the type strain *Bosea caraganae* RCAM04680 with a low support level (Fig. 7). The level of *rrs* similarity between the isolate and the type strain *B. caraganae* RCAM04680 was 99.34% (Table 3). The isolate P17 / 1-6 was identified as Bosea sp. To clarify the species identity of isolates P18/5-2 and P17/1-6, sequencing and phylogenetic analysis of the ITS gene should be performed.

Slow growing strains P20/4-1 and P20/5-1 were isolated from root nodules of *O. mertensiana*. Based on the results of the fragment of the sequencing of the *rrs* gene, the isolates were assigned to the genus *Microvirga* (Table 1). On the phyloge-

netic tree, they were grouped into cluster V with the type strain *Microvirga ossetica* V5/3M at a high support level (Fig. 7). The *rrs* similarity between isolates P20/4-1, P20/5-1 and the closest type strain of *M. ossetica* V5/3M was 99.86 and 99.17%, respectively. The *rrs*-similarity between isolates P20/4-1, P20 / 5-1, and the type strain of *Microvirga zambiensis* WSM3693 was 98.63 and 97.91%, respectively. The strain P20 / 4-1 was identified as *M. ossetica*, while strain P20/5-1 was identified as *Microvirga* sp. More molecular phylogenetic analysis is needed to clarify the species identity of strain P20/5-1.

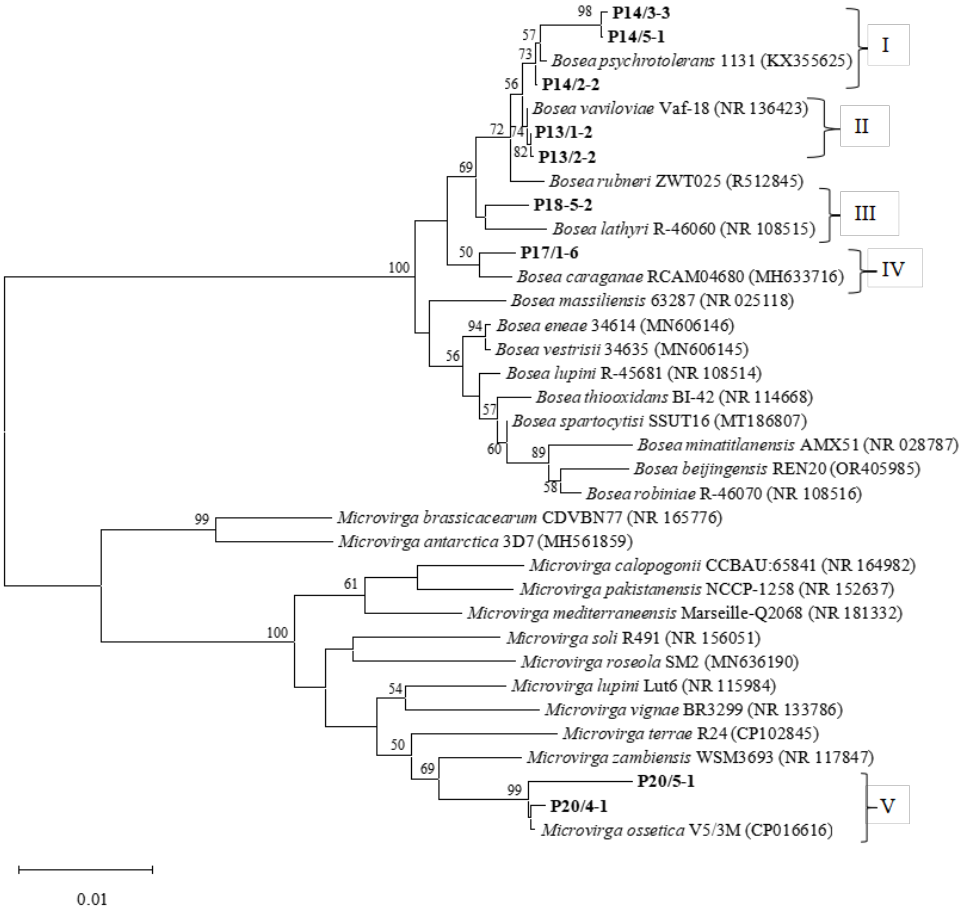

Figure 7. Unrooted phylogenetic tree constructed on the basis of a comparative analysis of the nucleotide sequences of the 16S rRNA gene fragment of strains obtained from nodules of *O. putoranica*, *O. mertensiana*, *A. norvegicus*, and *A. tugarinovii* and representatives of the related species *Bosea* and *Microvirga*. Isolates are shown in bold. Clusters I-V are statistically different clusters. Support levels above 50% are indicated.

Slow growing strains P13/1-1, P13/2-3, P18/1-2, and P18/3-6 were isolated from *O. putoranica* nodules, P14/2-3 from the *A. norvegicus* nodule, P20/2 and P20/2-1 from *O. mertensiana* nodules, P17/2-3 and P17/3-3 from the *A. tugarinovii* nodules. The similarity *rrs* between the isolates and the closest type strain of *Tardiphaga robiniae* R-45977 and *Tardiphaga zeae* SS122 varied from 99.71 to 99.93% and from 99.34 to 99.56%, respectively. All of these isolates were classified as *T. robiniae*.

Analyzing the Genetic Diversity of Symbiotic Genes

The search for symbiotic (symbiotic) *nod*A and *nod*C genes was carried out in strains of *N. galegae* P18/3-2 and P18/5-1 of *O. putoranica* nodules, *P. herbae* P14/2-4 of *A. norvegicus* nodule, *P. herbae* P20/1-1, *M. ossetica* P20/4-1 and *Microvirga* sp. P20/5-1 strains from *O. mertensiana* nodules, *Phyllobacterium zundukense* P17/1-7, and P17/3-2 strains from *A. tugarinovii* nodules. The nodA gene was detected in strains *P. herbae* P14/2-4 and P20/1-1, *P. zundukense* P17/1-7 and P17/3-2, while the *nod*C gene was not detected in any of the strains. Both symbiotic genes were present in the positive control *Rhizobium leguminosarum* bv. *trifolii* RCAM1365.

Sterile test tube experiment

To study the nodulating activity of strains *P. herbae* P14/2-4 and P20/1-1, a sterile test tube experiment with legume plants *A. norvegicus*, *A. tugarinovii*, *O. putoranica*, *O. mertensiana*, *T. repens*, *L. pratensis*, and *V. cracca* was performed. The result obtained showed the lack of ability of isolates to form nodules on the roots of these plant species.

Discussion

As a result, nodules of the wild arctic legumes *O. putoranica*, *O. mertensiana*, *A. norvegicus*, and *A. tugarinovii* that grow on the shore of Lake Duluk (Putorana plateau, Krasnoyarsk region) were collected. Twenty-four bacterial isolates belonging to six genera of the order Hyphomicrobiales: *Pararhizobium* and *Neorhizobium* (Rhizobiaceae), *Phyllobacterium* (Phyllobacteraceae), *Microvirga* (Methylobacteriaceae), *Bosea* (Boseaceae) and *Tardiphaga* (Bradyrhizobiaceae) were isolated and studied. Species affiliation was established for 18 isolates.

The microsymbionts of *O. putoranica* belonged to *N. galegae*, *B. vaviloviae*, *Bosea* sp., and *T. robiniae*, the microsymbionts of *A. norvegicus* were represented by *P. herbae*, *Bosea* sp. and *T. robiniae*. The microsymbionts of *O. mertensiana* were associated with *P. herbae*, *T. robiniae*, *M. ossetica*, and *Microvirga* sp. The microsymbionts of *A. tugarinovii* were related to *P. zundukense*, *Bosea* sp., and *T. robiniae*.

The genus *Neorhizobium* was segregated from the genus *Rhizobium* based on multilocus sequence analysis (MLSA) of six housekeeping genes. The type strain

N. galegae HAMBI 540 was shown to be able to form nitrogen-fixing root nodules on *Galega orientalis* Lam. (Lindström 1989; Mousavi et al. 2014). The four species *N. galegae*, *N. vignae*, *N. huautlense*, and *N. alkalisoli* are members of the "*R. galegae* complex" and are closely related phylogenetically. Some strains were described as nodule microsymbionts of various legumes (Wang et al. 1998; Ren, Chen et al. 2011). Other strains were found in the soil (Zhang et al. 2012; Soenens et al. 2019; Pan et al. 2022).

The genus *Pararhizobium* was separated from the genus *Rhizobium* based on MLSA analysis of housekeeping genes (Mousavi et al. 2015). The strain *R. herbae* CCBAU 83011 was first isolated from Nodules of *Astragalus membranaceus* (Fisch.) The bundle was growing in Xinjiang (China) and was renamed in 2015 *Pararhizobium herbae* (Ren, Wang et al. 2011; Mousavi et al. 2015). Representatives of the species were also found in the nodules of various leguminous plants of the genera *Astragalus*, *Oxytropis*, *Vicia* and *Lathyrus* (Kuznetsova et al. 2023), while other species of *Pararhizobium* were isolated, for example, from Antarctic water samples (Naqvi et al. 2017) and Alpine soils (Zhang et al. 2017).

The genus *Phyllobacterium* was first described by Knoesel in 1962 (Knösel 1962) for bacteria developing in leaf nodules of tropical ornamental plants. *Phyllobacterium* strains were isolated from root nodules of various legumes, including *Astragalus* (Mantelin et al. 2006) and *Oxytropis* (Safronova et al. 2018), from the *Brassica* rhizoplane (Mantelin et al. 2006) or from leaf nodules of *Ardisia* (Mergaert et al. 2002). It is known that strains *P. sophorae* CCBAU 03422 and *P. trifolii* PETP02 possessed the core nodulation nodACD and nifH genes, encoding Fe protein nitrogenase, and were capable of forming an effective symbiosis with host plants (Valverde et al. 2005; Jiao et al. 2015). The genomes of *P. zundukense* strains of *O. triphylla* nodules lacked the common *nod*ABC genes required for legume nodulation, although some other symbiotic *nod* and fix genes were detected (Safronova et al. 2018). Thus, *Neorhizobium*, *Pararhizobium*, and *Phyllobacterium* species can be both free-living and plant-associated bacteria, indicating their high ability to adapt to the environment, accompanied by the complexity of microbial genomes and deep specialization to host plants.

The genus *Microvirga* was first described in 2003 (Kanso, Patel 2003). Most species of *Microvirga* were described as isolates from soil, air, and water samples. However, strains of several species were isolated from root nodules of legumes and can form effective symbioses with host plants (Ardley et al. 2012; Radl et al. 2014). The type strain *Microvirga ossetica* V5/3M was isolated from root nodules of *Vicia alpestris* Steven growing in the north Ossetia region (Caucasus). However, it was unable to form nodules or swellings on the roots of *V. alpestris* and *V. formosa* plants in pot experiments (Safronova et al. 2017).

The genus *Bosea* is currently represented by 14 species. Members of the genus *Bosea* may be present in nodules of various legumes of the genera *Lupinus*, *Lathyrus*, *Robinia*, *Vavilovia*, *Caragana*, *Spartocytisus*, *Vicia*, *Astragalus*, *Oxytropis*, and *Hedysarum* (De Meyer and Willems 2012; Sazanova et al. 2019; Pulido-Suárez et

al. 2022; Kuznetsova et al. 2024). Strains of *B. vaviloviae* were detected in the nodules of *Vavilovia formosa* (Steven) Fed (North Caucasus), *A. schelichowii* (Norilsk) Turcz. and narrowly endemic Kamchatka species of *Oxytropis* (Safronova et al. 2020; Kuznetsova et al. 2023). *B. psychrotolerans*, which is phylo-genetically related to *B. vaviloviae*, was described in 2019 as a psychrotrophic alphaproteobacterial species isolated from Lake Michigan water (Albert et al. 2019). The *nodule* genes involved in the formation of nodules on legume roots were found to be present in the genomes of several *Bosea* species. However, the ability of these species to form nodules independently was not demonstrated (Sazanova et al. 2019).

The genus *Tardiphaga* is currently represented by two species, *T. robiniae* and *T. alba*. The first *T. robiniae* was isolated from *Robinia pseudoacacia* L., growing in Flanders (Belgium) (De Meyer et al. 2012). *T. robiniae* strains have also been found in nodules of *V. formosa*, *A. schelichowii*, and in narrow local endemics of the genus *Oxytropis* (Safronova et al. 2020, Kuznetsova et al. 2023). Some symbiotic (*sym*) genes responsible for nodulation and nitrogen fixation may be present in the genome of *T. robiniae* strains. However, the ability of *Tardiphaga* representatives to form nodules independently was not identified (Safronova et al. 2020). The type strain *T. alba* SK50-23 was isolated from Thallium-loaded garden soil in Japan (Bao et al. 2024). Thus, despite the lack of the ability of *Bosea* and *Tardiphaga* strains to form symbiotic nodules, the frequency of their occurrence and the presence of individual *sym* genes probably indicate their ability to influence the efficiency of legume-rhizobium symbiosis.

A study of the genetic diversity of symbiotic genes was carried out to investigate the ability of strains of the genus *Neorhizobium*, *Pararhizobium*, *Phyllobacterium*, and *Microvirga* to form nodules. The strains *Pararhizobium* sp. P14 / -2, P20 / 1-1, and *P. zundukense* P17/1-7, P17/3-2 possessed the main symbiotic (symbiotic) gene *nod*A, but lacked the *nod*C gene, without which nodule formation is impossible, while other strains lacked both genes. The *nod*A and *nod*C genes are common genes and, together with the *nod*B gene, are responsible for the synthesis of the cortical part of the *Nod* factors. *Nod*ABC genes are located in plasmids in fast-growing rhizobia, are involved in horizontal transfer genes processes and can be easily lost by bacterial cells (Provorov, Tikhonovich 2016). The negative results of the amplification of the *nod*A and *nod*C gene in the studied isolates may be due to the absence of target genes or their peculiar structure. The absence of nodules in a sterile test tube experiment using strains *P. herbae* P14/2-4 and P20/1-1 and legumes *A. norvegicus*, *O. mertensiana*, *A. tugarinovii*, *O. putoranica*, *T. repens*, *L. pratensis*, and *V. cracca* confirmed the negative results of amplification of the *nod*C gene in these strains. Thus, based on the results of the nod-gene search and the test tube experiment, no strains capable of forming nodules on host plants were identified, which may be due to both the mediocre quality of the collected nodules and the suboptimal conditions for culturing Arctic bacteria. In the future, it is proposed to use additional nutrient medium and different temperature cultivation modes to identify nodulating strains.

It is important to note the presence of two or three strains belonging to different families of the order Hyphomicrobiales in one nodule. Thus, strains *N. galegae* P18/3-2 and P18/5-1 (Rhizobiaceae), *T. robiniae* P18/3-6 (Bradyrhizobiaceae), and *Bosea* sp. P18/5-2 (Boseaceae) were detected in nodules of *O. putoranica*. Strains *P. herbae* P14/2-4 (Rhizobiaceae), *B. psychrotolerans* P14 / 2-2 (Boseaceae), and *T. robiniae* P14/2-3 (Bradyrhizobiaceae) were isolated from a nodule of *A. norvegicus*. The strains *P. herbae* P20/1-1 (Rhizobiaceae) and *T. robiniae* P20/1-2 (Bradyrhizobiaceae) were isolated from a nodule of *O. mertensiana*. Strains *P. zundukense* P17/1-7 and P17/3-2 (Phyllobacteriaceae), *Bosea* P17/1-6 (Boseaceae), and *T. robiniae* P17/3-3 (Bradyrhizobiaceae) were isolated from the nodules of *A. tugarinovii*. This confirms the hypothesis that legume symbiotic systems can be multicomponent and include rhizobial strains from different taxonomic groups. This creates conditions for effective exchange of genetic material between microsymbionts cohabiting in root nodules (Safronova et al. 2023).

Conclusions

This paper presents the first data on the genetic diversity of native arctic bacteria associated with nodules of the wild legumes *O. putoranica*, *O. mertensiana*, *A. norvegicus*, and *A. tugarinovii* growing in the vicinity of Lake Duluk (Putorana Plateau, Krasnoyarsk Krai). A total of 24 isolates belonging to six genera of the order Hyphomicrobiales were obtained: *Pararhizobium* and *Neorhizobium*, *Phyllobacterium*, *Microvirga*, *Bosea*, and *Tardiphaga*. The species *N. galegae* (fam. Rhizobiaceae), *T. robiniae* (fam. Bradyrhizobiaceae), *B. vaviloviae* and *Bosea* sp. (fam. Boseaceae) were described as microsymbionts of *O. putoranica*. The species *P. herbae* (fam. Rhizobiaceae), *T. robiniae*, *M. ossetica*, and *Microvirga* sp. (fam. Methylobacteriaceae) were described as microsymbionts of *O. mertensiana*. Representatives of *P. herbae*, *B. psychrotolerans*, and *T. robiniae* were isolated from the nodules of *A. norvegicus*, and representatives of *P. zundukense* (fam. Phyllobacteriaceae), *Bosea* sp. and *T. robiniae* were found in the nodules of *A. tugarinovii*. To clarify the species assignment of the strains, *Microvirga* sp. P20/5-1, *Bosea* sp. P17/1-6 and P18/5-2 require the sequencing and analysis of additional marker genes. In addition, strains of different taxonomic groups of the order of Hyphomicrobiales order are present in the nodules of all legume species, which can affect the increase in the potential of symbiotic complementarity for increasing the productivity of plant-microbe interactions due to the possibility of joint localization of taxonomically different co-microsymbionts in one nodule. Such symbiotic systems are promising models for studying the formation of the specificity of legume-rhizobial interaction and its effect on the productivity of symbiosis in extreme Arctic conditions. Therefore, the creation of plant-microbial systems based on the genetic resources of Arctic rhizobia will allow us to expand the range of leguminous plant species that are promising as a high-protein component in the creation of highly productive agrophytocenoses for the ustainable development of northern livestock

Arctic landscapes.

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