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RESEARCH ARTICLE

Diversity of endophytic microorganisms of endemic species legume *Oxytropis exserta* Jurtz., *O. evenorum* Jurtz. and *O. ochotensis* Bunge growing on the Kamchatka Peninsula (Russia)

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Abstract

The interaction of legume plants with nitrogen-fixing nodule bacteria (rhizobia) helps to enrich the soil with nutrients, thereby maintaining its fertility and ensuring the sustainability of pasture landscapes. In challenging soil and climatic conditions, the symbiotic relationship between legumes and rhizobia is of paramount importance. The study investigated the genetic diversity of endophytes of the order Hyphomicrobiales (Alfaproteobacteria) found in the nodules of endemic legumes *O. exserta*, *O. evenorum*, and *O. ochotensis* growing in Kamchatka (the Subarctic region of Russia). Forty strains were isolated from nodules, of which the largest number (17) were obtained from *O. ochotensis*, while 12 and 11 strains were isolated from *O. evenorum* and *O. exserta*, respectively. Microbial strains were isolated using a standard method using mannitol-yeast nutrient agar (YMA). Primary taxonomic identification of strains was performed using ribosomal protein profiling and 16S rRNA gene sequencing. The largest number of strains were *Mesorhizobium*, *Allobosea*, and *Tardiphaga*. Isolates of the genera *Rhizobium*, *Phyllobacterium*, *Arminella*, and *Pararhizobium* were also present. Incubation temperature turned out to be a key factor in the selection of cultivable bacteria. Most *Mesorhizobium* and all

Phyllobacterium were detected only at 28 °C, whereas *Arminella*, and *Pararhizobium* was found only at 15 °C. At 5 °C, no *Mesorhizobium* or *Rhizobium* isolates were obtained. A qualitative assessment of resistance to salt and hydrogen (pH) stress was carried out. A high diversity of pH stress tolerance was found among the strains studied. The widest pH range (4-10) was demonstrated by *Mesorhizobium* sp. RCAM07391, RCAM07494, *P. myrsinacearum* RCAM07525, RCAM07528, RCAM07530, and *Rhizobium* sp. RCAM07452, RCAM07474. Most strains could also grow at 3% NaCl concentration. Thus, this study expands our understanding of the biodiversity of cultivable bacteria associated with endemic legumes in subarctic regions. Rhizobial strains native to northern latitudes are of interest for further study of their symbiotic potential and adaptive properties, with a view to their potential use as microbial fertilizers for growing forage and pasture legumes in the extreme soil and climatic conditions of Russia's Arctic and subarctic regions.

Keywords
Oxytropis, endemic, Kamchatka, legume-rhizobium symbiosis, root nodule bacteria, northern agrophytocenoses, 16S rRNA gene, MALDI-TOF

Introduction

The Kamchatka Peninsula is a unique region of the Northwest Pacific with extreme climatic and soil conditions. Kamchatka's soil cover is unmatched in the entire Far East. This is explained by the fact that, in addition to the main factors, active volcanic activity influences soil formation on the peninsula (Kochneva and Cheburina 2021). Kamchatka's vegetation also has its own distinctive features, distinguished by the presence of species adapted to long, snowy winters and cool, rainy summers. Approximately 1166–1170 species and subspecies of vascular plants grow here, belonging 89 families and more than 400 genera (Yakubov 2007; Chernyagina 2004). Among the perennial plants of the legume family (Fabaceae) in Kamchatka (excluding Koryakia), there are 33 species of legumes from 8 genera: *Oxytropis* – 14 species, *Astragalus* – 7, *Trifolium* – 4, *Lathyrus* – 3, *Vicia* – 2, *Hedysarum*, *Pisum* and *Thermopsis* (1 species each). Twenty-five species (76%) are indigenous, and eight species (24%) are introduced or escaping from cultivation. The genera *Trifolium*, *Pisum*, and *Vicia* are completely adventitious (Pavlova 1989). Many legumes, including those of the genus *Oxytropis*, have a variety of beneficial properties of practical importance and are valuable sources of vegetable protein for animals (Voronkova and Kholina 2017). Some species of *Oxytropis* are pioneers in places without soil cover, in particular in areas after volcanic eruptions. As a result of their vital activity, soil properties are formed that are favorable for the settlement of other plants (Voronkova et al. 2008). The symbiosis of legumes with nodule- inducing nitrogen-fixing bacteria (rhizobia) enriches the soil with nitrogen. However, the rich potential of *Oxytropis* species is largely unexploited, and their introduction into cultivation has been poorly studied, especially for endemic species (Voronkova and Kholina 2017). For example, due to symbiosis with nodule bacteria, endemic legume species of the genus *Oxytropis*: *O. exserta* Jurtz., *O. evenorum* Jurtz., and *O. ochotensis* Bunge, can

play an important role in the formation of pasture ecosystems and maintaining soil fertility (Prevost et al. 1987). *O. exserta* is endemic to the Russian Far East, found in the south of Chukotka, the Magadan region and Kamchatka. It grows on dry river pebbles, rubble slopes and rocks, sometimes in the mountain tundra (Malyshev 2008). *O. evenorum* is endemic to Siberia and the Russian Far East (Malyshev 2008; Voronkova and Kholina 2017). A common species, it is typically associated with cryoxeromesophytic tundra, often with outcrops of small- or large-block rocks. The species is morphologically related to the widespread Arctic taxon *O. sordida* (Willd.) Pers, which is considered a valuable fattening plant for reindeer in Taimyr. *O. evenorum* is readily or satisfactorily consumed by reindeer, and its rhizomes are used in the diet of many herbivorous species, and is considered a forage plant for *Ovibos moschatus* Zimmermann (Nikolin et al. 2020). *O. ochotensis* is endemic to northeastern Russia, distributed primarily along the coasts of the Sea of Okhotsk, in the Magadan Region, Kamchatka, and Chukotka, where it grows rocky and gravelly slopes. In the tundra, it often grows in thickets, creating a backdrop to alpine and subalpine meadows during flowering. It is well adapted to the harsh conditions of high mountains and polar tundra (Malyshev 2008; Kozyrenko et al. 2020). Like other members of the genus *Oxytropis*, it can be eaten by reindeer in the tundra and highlands (Mosolov 2010).

The main nitrogen-fixing microsymbionts of *Oxytropis* are bacteria of the genus *Mesorhizobium* (family Phyllobacteriaceae), which are highly adaptable and have broad host specificity (Ampomah et al. 2017; Ashrafi et al. 2022). Among the microsymbiont of *Oxytropis*, representatives of various species have been described, such as *M. amorphae*, *M. temperatum*, *M. mediterraneum*, *M. loti*, *M. ciceri*, and *M. gobiense*; however, many isolated strains still lack species identification (Han et al. 2008; Hou et al. 2009; Safronova et al. 2020). In addition, in the nodules of various *Oxytropis* species, there are representatives of other families and genera: *Sinorhizobium* and *Rhizobium* (family Rhizobiaceae), *Phyllobacterium* (Phyllobacteriaceae), *Bradyrhizobium* and *Tardiphaga* (Bradyrhizobiaceae), *Bosea* (Boseaceae) (Laguerre et al. 1997; Ampomah et al. 2017; Safronova et al. 2020; Andrews and Andrews 2017). Since 2026, the genus *Bosea* and family Boseacea have been emended to genus *Allobosea* and family Alloboseaceae as replacement names for the illegitimate prokaryotic genus and family (Deshmukh and Oren 2026).

However, data on the biodiversity and properties of rhizobia associated with Kamchatka`s endemics legume remain extremely limited. Strains adapted to subarctic conditions are of particular interest as a potential basis for the development of biopreparations for northern agriculture. Therefore, the isolation and taxonomic study of endophytes *O. exserta*, *O. evenorum*, and *O. ochotensis* will enable the creation of a unique collection of bacteria and the selection of the most effective strains capable of nodulating a wide range of wild and forage legumes in a test tube and field experiments.

The aim of this study was:

(1) to isolate endophytes from nodules of endemic Kamchatka legume *O. exserta*, *O. evenorum*, and *O. ochotensis* using different temperatures;

(2) to primary taxonomically identify the isolates using mass spectrometric profiling of ribosomal proteins and 16S rRNA gene sequence analysis;

(3) to qualitatively determine tolerance of the isolates to salinity and pH.

Materials and methods

Collection of nodules and isolation of bacterial strains

Root nodules from wild-growing populations of the legumes *O. ochotensis* Bunge, *O. evenorum* Jurtzev and A.P. Khokhr, *O. exserta* Jurtz. were collected in Kamchatka (RF) during the expedition of 2016 (Fig. 1).

Figure 1. Plants *Oxytropis ochotensis*, *Oxytropis evenorum*, and *Oxytropis exserta* found during the expedition. Photo by V.V. Yakubov, 2016.

O. ochotensis nodules were collected on the southern slope of Avachinskaya Sopka volcano (53.197361, 158.771083); *O. evenorum* nodules on the northern slope of Tolbachik volcano (55.940150, 160.323383); *O. exserta* nodules on the northwestern slope of Vilyuchinskaya Sopka volcano (52.992000, 158.852483) (Fig. 2).

Figure 2. The Kamchatka Peninsula. Nodule collection points. Retrieved from https://nakarte.me (ESRI Sputnik), 2026.

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. The roots with nodules were carefully shaken off 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 totally dry. Five nodules from each plant species were selected in the laboratory for further work.

Individual nodules were surface-sterilized with 96% ethanol for 1 minute, followed by rinsing with sterile water four times (each for 1 minute). To confirm the success of a surface sterilization of root nodules, sterile nodules were rolled over the surface of the yeast mannitol agar (YMA) medium, and the Petri dishes were incubated for 7 days at 28 °C. Each sterile nodule was then homogenized in 100 µl of sterile water. Thirty microliters of bacterial suspension were transferred into three dishes with YMA medium and spread over the surface with a spatula (Novikova and Safronova 1992). The Drigalski method was used to separate colonies spatially and minimize competition during seeding: the surface of the next dish with nutrient medium was seeded using a spatula that was not burned by the flame. Cultures were

grown at 5°C, 15°C and 28°C for 30, 20 and 10 days, respectively (refrigerators HF-400-3 (POZIS, Russia), BD240 incubator (Binder, Germany). All emerging colonies were selected and recultured to obtain a pure culture. Based on MALDI-TOF-MS analysis, isolates with identical spectra (>80%) were classified as isolates with a high degree of similarity of protein profiles, and one representative from the group was selected for further work.

MALDI-TOF MS based system BactoSCREEN

Sample preparation for MALDI-TOF-MS was performed according to the manufacturer's instructions using the direct application method. Cells from a single colony were applied to a spot on a steel target and air-dried at room temperature. One ml of matrix solution was applied to each sample and air-dried (Gordeeva et al. 2020). Mass spectra were recorded on a BactoSCREEN microbiological analyzer (Litech LLC, Russia), BactoSCREEN-ID software was used for spectral analysis. Comparisons were performed using a previously created custom reference library for the analysis of spectra of bacteria of the order Hyphomicrobiales, which were not included in the main reference library. A custom library was constructed using the type strains and most common root nodule bacteria available in the Network Bioresource Collection in the Field of Genetic Technologies for Agriculture (RCAM, FSBSI ARRIAM, St. Petersburg). Sample preparation was performed as described by Ferreira et al. (Ferreira et al. 2011) except for the incubation time for slow-growing microorganisms, which was 72 h. Reagents from the 'MALDI-TOF sample kit' and a bacterial calibration standard based on ribosomal proteins of the strain *Escherichia coli* DH5α with additional proteins RNase A and myoglobin (NPF Litekh LLC, Russia) were used in the work. Measurements were then taken in accordance with the manufacturer's instructions for replenishing the custom library. Forty laser pulses (60 Hz) were used to obtain a single mass spectrum; the analyzed m/z range was 2000-20000 Da. A total of 200 individual spectra were acquired for each sample to generate a Main Spectrum (MSP) (a set of peaks with distinct masses), which was then compared against the main database. During the comparison, a matching score with the studied microorganism was calculated for each database entry: species-level identification – 0.8–1.0; genus-level identification – 0.5–0.79; no identification – < 0.5. The resulting spectral profiles of the created library were checked for self-recognition. For strains of the main symbiotic genera (*Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*), external validation was performed against the main commercial database BactoSCREEN on the genus/species level. Additional strains of different taxonomic affiliations from the RCAM collection tested to verify the accuracy and selectivity of custom library. All strains used to create the library were identified by sequencing the 16S rRNA gene, the ITS (internal transcribed spacer) region, or the complete genome.

DNA extraction and PCR protocols

For DNA extraction, pure cultures were grown in YM broth for 2–5 days at 28°C with shaking at 200 rpm (Orbital Shaker-Incubator ES20, BioSan, Latvia). DNA was isolated using a kit for isolating genomic DNA from bacterial cells (diaGene, Russia) according to manufacturer's guidance. PCR followed by sequencing of the rrs gene fragment was used for primary strain identification. PCR was performed on a T100 automated amplifier (Bio-Rad, United States). The 16S rRNA gene fragments (1268–1397 bp) were amplified using the primer pair fD1 5'-AGAGTTTGATC-CTGGCTCAG-3' and rD1 5'-AAGGAGGAGTGATCCAGCC-3' (Weisburg, Barns, Pelletier, and Lane 1997). PCR was performed in 50 µL reaction mixtures containing 150 µM dNTP (Evrogen, Russia), 5 pmol of each primer, 1 U Taq polymerase (Evrogen, Russia) and 50–100 ng purified DNA template. 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.

Visualization and purification of the PCR product

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

Sequencing and data processing

Sequencing of the prepared PCR products was performed on the ABI PRISM 3500×l genetic analyzer (Life Technologies, United State) at 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 type strains were searched for in the GenBank database (https://www.ncbi.nlm.nih.gov) and BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Evolutionary analyses were conducted in MEGA12 utilizing up to 3 parallel computing threads (Kumar 2024). The phylogeny was inferred using the Maximum Likelihood method and Kimura 2-parameter model with 1000 bootstrap replications. This analysis involved 29 (for *Mesorhizobium* and *Phyllobacterium*) and 30 (for *Rhizobium* and *Pararhizobium*) partial 16S ribosomal RNA gene nucleotide sequences. There were a total of 1285 and 1321 positions in the final datasets, respectively. The nucleotide sequences of the strains of the order Hyphomicrobiales were deposited in the GenBank database as: PZ142538– PZ142562, for rrs gene.

Primary screening of isolates for abiotic stress tolerance

The ability of isolates to grow in salinity was tested by each isolate into an YMA medium containing 0.5, 1, 2, 3, 4, 5, 6, 7, and 8% (weight/volume) NaCl. The tolerance of the isolates to pH was examined in YMA medium with pH adjusted between 4 (1 N HCl) and 10 (1 N NaOH), at an increment of 1 pH unit. The pH of the medium was adjusted before autoclaving, taking into account possible shifts during sterilization, and checked after sterilization. Bacterial strains were inoculated on the surface of the nutrient medium in three replicates and incubated for 5-10 days at a temperature of 28 °C. *Rhizobium ruizurguezonis* RCAM0626, isolated from a *Vicia sativa* L. root nodule growing in the Leningrad Region, and *Mesorhizobium* sp. RCAM2923, isolated from an *Onobrychis arenaria* (Kit.) DC. root nodule growing in the Altai Mountains, were used as reference strains. The strains were provided by the Network Bioresource Collection in the Field of Genetic Technologies for Agriculture (FSBSI ARRIAM, St. Petersburg).

Storage of strains

Pure cultures of endophytess (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/).

Results

As a result of this work, nodule endophytes of endemic legumes of the genus *Oxytropis* growing in different places of the Kamchatka were studied. Fourty bacterial isolates were obtained from 15 nodules (5 for each plant species) collected from the roots of *O. ochotensis*, *O. evenorum* and *O. exserta*. Nodule suspensions were grown on YMA medium at temperatures of 5 °C, 15 °C and 28 °C.

The isolated strains were identified using two methods: rapid identification by MALDI-TOF MS on a BactoSCREEN analyzer (Litech, Russia) using our own protein spectra database, and 16S rRNA gene sequencing for isolates belonging to the genera *Mesorhizobium*, *Rhizobium*, *Pararhizobium*, *Arminella*, and *Phyllobacterium* (typical symbionts of legumes) and some *Tardiphaga* and *Allobosea* isolates to refine the initial identification (Tables 1, 2).

Mesorhizobium strains were isolated from *O. ochotensis* and *O. exserta* nodules. *Phyllobacterium* were found only in *O. exserta* nodules. *Rhizobium* strains were isolated from *O. ochotensis* and *O. evenorum* nodules. *Pararhizobium* and *Arminella* strains were also found in an *O. evenorum* nodules. *Allobosea* were described for all *Oxytropis* species, while *Tardiphaga* were found only in *O. ochotensis* and *O. evenorum*.

Note that most *Mesorhizobium*, *Phyllobacterium*, and *Rhizobium* strains nodulated a wide range of plants from the genera *Oxytropis*, *Hedysarum* L., *Astragalus* L., *Onobrychis* Mill, *Caragana* Fabr., and *Robinia* L., but did not nodulate *Vicia* L., *Trifolium* L., and *Lotus* L. under conditions of sterile test-tube experiments (data not shown). Based on the results of rrs gene fragment sequencing, thirteen strains isolated from nodules of *O. ochotensis*, and *O. exserta* were divided into two groups on the phylogenetic tree (Fig. 3).

Table 1. Results of BLAST analysis of 16S rRNA gene fragments for strains isolated from nodules of *O. ochotensis*, *O. evenorum*, and *O. exserta* plants growing in Kamchatka

Strain ID	Name of the strain when isolated	Species	T °C*	Closely related type strain(s) identified by BLAST	Similarity, %	Sequence query coverage, %
			O. ochotensis			
RCAM07452	K6.1.3_28	*Rhizobium* sp.	28	*Rhizobium rhizogenes* NBRC 13257 *R. lusitanum* P1-7	99.86 99.86	100.00
RCAM07392	K6.3.1_28	*Rhizobium* sp.	28	*R. rhizogenes* NBRC 13257 *R. lusitanum* P1-7	99.78 99.63	100.00
RCAM07474	K6.3.3_15	*Rhizobium* sp.	15			
RCAM07390	K6.1.4_28	*Mesorhizobium* sp.	28	*Mesorhizobium jarvisii* ATCC 33669 *M. amorphae* NBRC 102496 *M. huakuii* NBRC 15243	99.93 99.85 99.85	100.00
RCAM07391	K6.2.1_28	*Mesorhizobium* sp.	28			
RCAM07470	K6.1.2_15	*Mesorhizobium* sp.	15	*M. jarvisii* ATCC 33669 *M. amorphae* NBRC 102496 *M. huakuii* NBRC 15243	99.93 99.71 99.71	100.00
RCAM07494	K6.4.7_15	*Mesorhizobium* sp.	15			
RCAM07455	K6.3.2_28	*Tardiphaga robiniae*	28	*Tardiphaga robiniae* R-45977	99.64	100.00
RCAM07430	K6.3.5_28	*Allobosea vaviloviae*	28	*Allobosea vaviloviae* Vaf-18	99.62	100.00
RCAM07429	K6.4.5_28	*Allobosea vaviloviae*	28	*A. vaviloviae* Vaf-18	99.85	100.00
RCAM07469	K6.6.4_28	*Allobosea vaviloviae*	28	*A. vaviloviae* Vaf-18	99.85	100.00
			O. evenorum			
RCAM07506	K16.6.2_28	*Allobosea vaviloviae*	28	*A. vaviloviae* Vaf-18	99.77	100.00
RCAM07577	K16.1.2_15	*Arminella tubonensis*	15	*Ar. tubonensis* CCBAU 85046	100.00	100.00
RCAM07509	K16.1.4_15	*Rhizobium* sp.	15	*Rhizobium beringeri* SM51 *Rhizobium binxianense* BJ04	100.00	100.00

Strain ID	Name of the strain when isolated	Species	T °C*	Closely related type strain(s) identified by BLAST	Similarity, %	Sequence query coverage, %
RCAM07600	K16.4.1_15	*Pararhizobium* sp.	15	*P. herbae* CCBAU 83011 *P. polonicum* F5.1	99.70 99.30	97.00 95.00
			O. exserta			
RCAM07525	K9.1.1_28	*P. myrsinacearum*	28	*Phyllobacterium myrsinacearum* NBRC 100019	100.00	100.00
RCAM07528	K9.2.1_28	*P. myrsinacearum*	28			
RCAM07530	K9.3.1_28	*P. myrsinacearum*	28			
RCAM07529	K9.2.2_28	*Mesorhizobium* sp.	28			
RCAM07531	K9.3.2_28	*Mesorhizobium* sp.	28			
RCAM07535	K9.4.3_28	*Mesorhizobium* sp.	28	*M. jarvisii* ATCC 33669 *M. huakuii* NBRC 15243	99.93 99.71	100.00
RCAM07538	K9.5.3_28	*Mesorhizobium* sp.	28			
RCAM07542	K9.6.4_28	*Mesorhizobium* sp.	28			
RCAM07546	K9.2.3_15	*Mesorhizobium* sp.	15			
RCAM07539	K9.6.1_28	*Allobosea* sp.	28	*A. psychrotolerans* 1131 *A. vaviloviae* Vaf-18	99.93 99.86	96.00 100.00

Note: *– Incubation temperature for strain isolation.

Table 2. Results of express identification (BactoSCREEN, MALDI-TOF MS) of endophytic strains isolated from nodules of *O. ochotensis*, *O. evenorum*, and *O. exserta* plants growing in Kamchatka

Strain ID	Name of the strain when isolated	Species	T °C*	Identification by BactoScreen	Reliability coefficient
		O. ochotensis			
RCAM07439	K6.2.2_28	*Tardiphaga* sp.	28	*Tardiphaga robiniae*	0.80
RCAM07441	K6.5.6_28	*Tardiphaga* sp.	28	*Tardiphaga robiniae*	0.91
RCAM07480	K6.1.2_5	*Tardiphaga* sp.	5	*Tardiphaga robiniae*	0.83
RCAM07482	K6.2.6_5	*Tardiphaga* sp.	5	*Tardiphaga robiniae*	0.80
RCAM07486	K6.4.2_5	*Tardiphaga* sp.	5	*Tardiphaga robiniae*	0.85
RCAM07493	K6.3.6_15	*Tardiphaga* sp.	15	*Tardiphaga robiniae*	0.84
		O. evenorum			
RCAM07497	K16.1.1_28	*Allobosea* sp.	28	*A. vaviloviae*	0.84
RCAM07499	K16.1.5_28	*Tardiphaga* sp.	28	*Tardiphaga robiniae*	0.87
RCAM07500	K16.4.1_28	*Allobosea* sp.	28	*A. vaviloviae*	0.84
RCAM07503	K16.5.2_28	*Allobosea* sp.	28	*A. vaviloviae*	0.83
RCAM07504	K16.5.3_28	*Tardiphaga* sp.	28	*Tardiphaga robiniae*	0.89
RCAM07505	K16.6.1_28	*Allobosea* sp.	28	*A. vaviloviae*	0.80
RCAM07514	K16.6.2_15	*Allobosea* sp.	15	*A. vaviloviae*	0.86
RCAM07601	K16.6.5_15	*Tardiphaga* sp.	15	*Tardiphaga robiniae*	0.90

Strain ID	Name of the strain when isolated	Species	T °C*	Identification by BactoScreen	Reliability coefficient
		O. exserta			
RCAM07533	K9.4.1_28	*Allobosea* sp.	28	*A. vaviloviae*	0.82

Note: *– Incubation temperature for strain isolation.

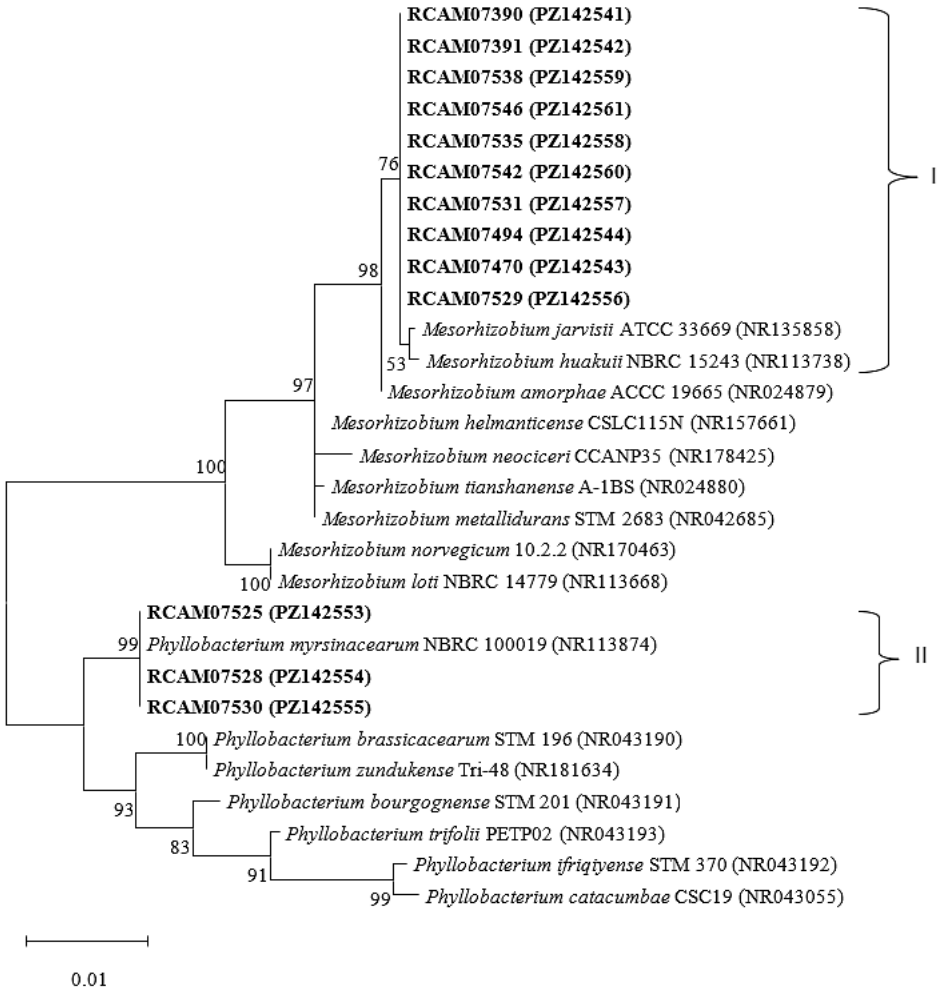

Figure 3. Phylogenetic tree constructed on the basis of a comparative analysis of the nucleotide sequences of 16S rRNA gene fragment of strains obtained from nodules of *O. ochotensis*, *O. exserta*, and representatives of the related type species *Mesorhizobium* and *Phyllobacterium*. The isolates are shown in bold. Tentative group (I) and cluster (II) are indicated by curly brackets. Support levels above 50% are indicated.

Tentative group I was formed by isolates RCAM07390, RCAM07391, RCAM07470, RCAM07494, RCAM07529, RCAM07531, RCAM07535, RCAM07538, RCAM07542, RCAM07546 and type strains *M. jarvisii* ATCC 33669 and *M. huakuii* NBRC 15243 with a relatively high support level of 76%. (Fig. 3). The all isolates had a 99.93% rrs-similarity to the type strain *M. jarvisii* ATCC 33669, and a 99.85% rrs-similarity to the type strain *M. huakuii* NBRC 15243. Thus, these isolates were left without species affiliation.

Cluster II was formed by isolates RCAM07525, RCAM07528, and RCAM07530 from *O. exserta*, and the type strain *Phyllobacterium myrsinacearum* NBRC 100019 at 99% level of support. The isolates had a 100% rrs-similarity to the closest type strain *P. myrsinacearum* NBRC 100019, as well as 99.42% similarity in the rrs gene with other close strains *P. bourgognense* STM 201 and *P. brassicacearum* STM 196. Thus, isolates RCAM07525, RCAM07528, and RCAM07530 were tentatively assigned to *P. myrsinacearum*.

On the phylogenetic tree, isolates RCAM07452, RCAM07392, RCAM07474, RCAM07509, RCAM07577, and RCAM07600 were divided into four groups (Fig. 4).

Tentative group I was formed by the isolate RCAM07509 from *O. evenorum*, and type strains *R. beringeri* SM51, and *R. binxianense* BJ04, at 75% level of support. The levels of rrs-similarity of isolate RCAM07509 and these type strains were 100%. The levels of rrs-similarity with type strains from the "*Rhizobium leguminosarum*" complex (*R. leguminosarum* LMG 14904, *R. sophorae* LMG 27901, *R. ruizarguesonis* UPM1133, *R. laguerreae* FB206, *R. indicum* JKLM 12A2, *R. anhuiense* CCBAU 23252, *R. brockwellii* CC275e) was 99.2%. The isolate RCAM07509 was assigned to the *Rhizobium* sp.

Tentative group II was formed by isolates RCAM07452, RCAM07392, and RCAM07474 from *O. ochotensis*, and the type strains *R. lusitanum* P1-7, and *R. rhizogenes* NBRC 13257 at 72% level of support. The isolates had a 99.6-99.9% rrs-similarity to the closest type strain *R. lusitanum* P1-7 and *Rhizobium rhizogenes* NBRC 13257. The isolates RCAM07452, RCAM07392, and RCAM07474 were assigned to the *Rhizobium* sp.

Cluster III with a support level of 99% was formed by the isolate RCAM07577 from *O. evenorum* and the type strain *Arminella tubonensis* CCBAU 85046 (previously *R. tubenense* CCBAU 85046). The genus *Arminella* was separated from the genus *Rhizobium* in 2026 (Naranjo-Robayo et al. 2026). The rrs similarity of the isolate RCAM07577 to the type strain *A. tubonensis* CCBAU 85046 was 100%. Meanwhile, with other closely related type strains, *Neorhizobium huautlense* SO2 and *Arminella tumorigenes* 1078, the similarity was 97.8%, which is below the species similarity threshold of 98.65% (Kim et al. 2014). Thus, the isolate RCAM07577 was tentatively assigned to the species *A. tubonensis*.

Cluster IV was formed by the isolate RCAM07600 from *O. evenorum*, and type strains *Pararhizobium herbae* CCBAU 83011, and *Pararhizobium polonicum* F5.1, with a relatively high level of support of 83%. The rrs similarity of the isolate

RCAM07600 to the closest type strain *P. herbae* CCBAU 83011 was 99.7% (query cover 97%). At the time as the rrs similarity of the isolate RCAM07600 to the type strain *P. polonicum* F5.1 was 99.3% (query cover 95%). Thus, the isolate RCAM07600 was assigned to the *Pararhizobium* sp.

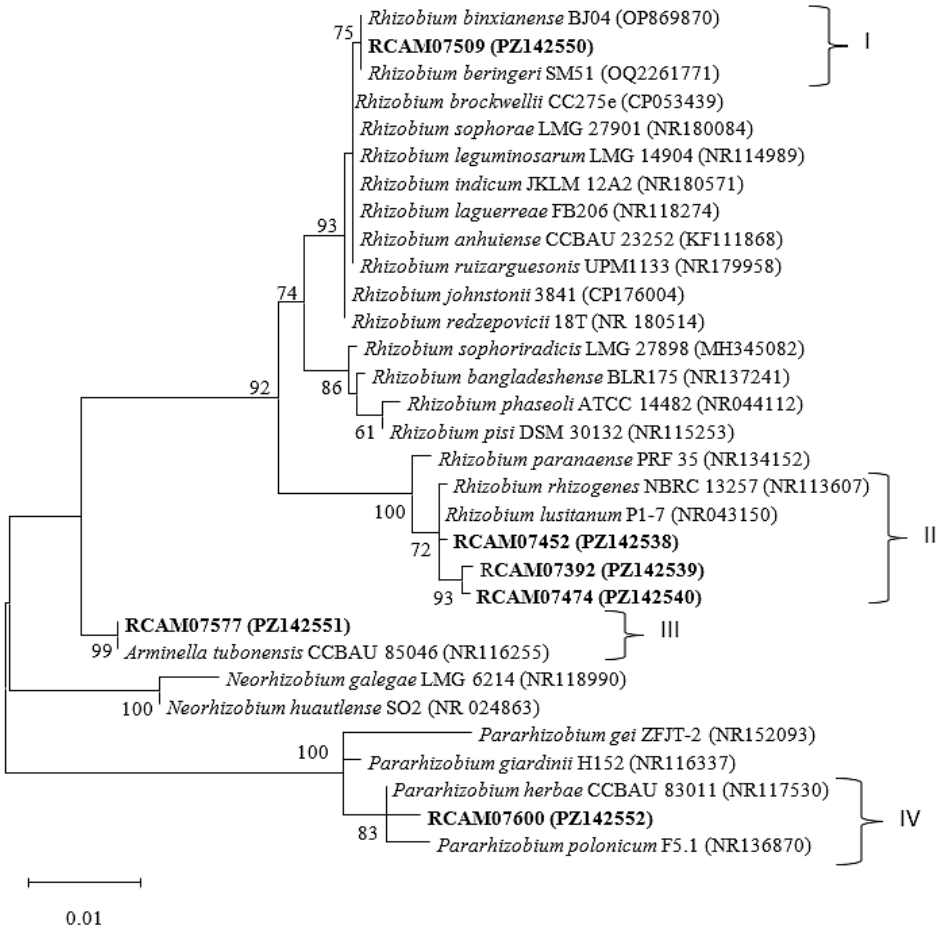

Figure 4. Phylogenetic tree constructed on the basis of a comparative analysis of the nucleotide sequences of 16S rRNA gene fragment of strains obtained from nodules of *O. ochotensis*, *O. evenorum*, and representatives of the related type species *Rhizobium* and *Pararhizobium*. The isolates are shown in bold. Tentative groups (I, II) and clusters (III, IV) are indicated by curly brackets. Support levels above 50% are indicated.

The results of a screening study of the pH range and NaCl concentration at which growth of isolates and commercial strains of *Rhizobium ruizurguezonis* RCAM0626 and *Mesorhizobium* sp. RCAM2923 was observed are presented in Fig. 5.

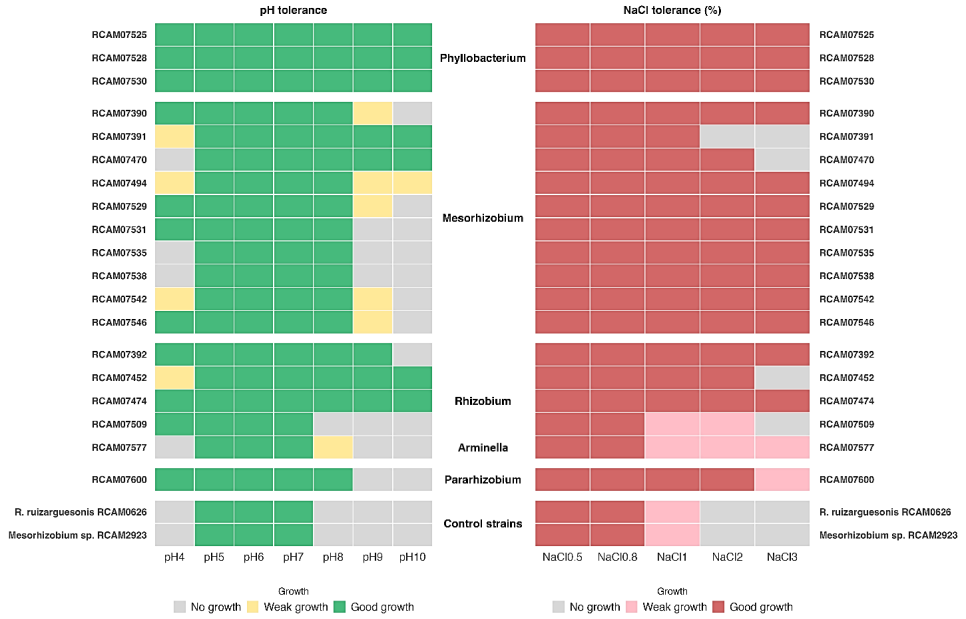

Figure 5. Results of the determination of the pH range and NaCl concentration for growth of isolates *Mesorhizobium*, *Phyllobacterium*, *Rhizobium*, *Arminella*, *Pararhizobium* and commercial strains *Rhizobium ruizurguezonis* RCAM0626 and *Mesorhizobium* sp. RCAM2923 from the RCAM collection.

The pH range for isolates *Mesorhizobium* sp. RCAM07391, RCAM07494, *P. myrsinacearum* RCAM07525, RCAM07528, RCAM07530, and *Rhizobium* sp. RCAM07452, RCAM07474 was from 4 to 10, whereas for the remaining strains of *Mesorhizobium*, *Rhizobium* and *Pararhizobium* the pH range was from 4-5 to 8-9. It should be note that commercial strains *Rhizobium* sp. RCAM0626 and *Mesorhizobium* sp. RCAM2923 grew in the pH range from 5 to 7.

For most strains of *Mesorhizobium*, *Rhizobium* and all strains related to *P. myrsinacearum,* and *Pararhizobium*, the maximum NaCl concentration for growth was 3%, whereas for commercial strains *Rhizobium* sp. RCAM0626 and *Mesorhizobium* sp. RCAM2923, the maximum NaCl concentration was 1%. Strains *Mesorhizobium* sp. RCAM07470, *Rhizobium* RCAM07452 and RCAM07509 grew at a salt concentration of 2%, for the strain *Mesorhizobium* sp. RCAM07391 the maximum NaCl concentration for growth was 1%.

Discussion

As a result of this study, a collection of endophytes from the nodules of three endemic *Oxytropis* species from the Kamchatka Peninsula was isolated and characterized for the first time. Using the YMA medium culture method, 40 strains belonging

to seven genera of the order *Hyphomicrobiales* were obtained: *Mesorhizobium*, *Phyllobacterium*, *Rhizobium*, *Pararhizobium*, *Arminella*, *Allobosea*, *Tardiphaga*.

Based on the analysis of the 16S rRNA gene, it was established that the majority of isolates from the nodules of *O. ochotensis* and *O. exserta* belonged to the genus *Mesorhizobium*. The latter also contained strains related to *Phyllobacterium myrsinacearum*, belonging to the Phyllobacteriaceae family. *P. myrsinacearum* was first discovered in leaf nodules of tropical ornamental plants (species of *Myrsinaceae* and *Rubiaceae*) and on the phylloplane and rhizoplane of other plants (Mergaert, Cnockaert, and Swings 2002). *Rhizobium* species were present in *O. ochotensis* and *O. evenorum* nodules. One isolate *Arminella tubonensis* RCAM07577 from *O. evenorum* showed 100% similarity to the type strain of *A. tubonensis* CCBAU 85046 (*R. tubenense* CCBAU 85046), which was isolated from nodules of *O. glabra* (Lam.) DC. in Tibet (Zhang et al. 2011). The genus *Arminella* was separated from the genus *Rhizobium* in 2026 and includes the species *A. rhododendri*, *A. tumorigenes* and *A. tubonensis* (Naranjo-Robayo et al. 2026). Strain RCAM07509 of *O. evenorum* was most closely related to the type strain *R. beringeri* SM51 and *R. binxianense* BJ04 which were isolated, respectively, from a root nodule of legume *T. repens* growing together with cereal *Lolium perenne* L. at the breeding site in Denmark: Zealand (Young et al. 2023), and *Phaseolus vulgaris* L. growing in Heilongjiang Province of China (Liu et al. 2025). Isolates RCAM07392, RCAM07452, and RCAM07474 from *O. ochotensis* on the dendrogram formed a separate lineage, phylogenetically close to *R. rhizogenes* and *R. lusitanum*. A type strain *R. rhizogenes* NBRC 13257 (previously *Agrobacterium rhizogenes*), related to our strain, was isolated from an infectious lesion of the root hairs of apple trees. It is known that species *R. rhizogenes* includes pathogenic or nonpathogenic strains, depending on the presence of Ti or Ri plasmids (Young et al. 2001). Various strains of *R. rhizogenes* are used as transformative tools in plant biotechnology. It has been established that a wide range of plant species, including angiosperms (both dicotyledonous and monocotyledonous plants), gymnosperms, and even moss, are susceptible to successful infection by *R. rhizogenes*. Strains of this species are used as effective rooting inducers for difficult-to-root plants (Ying et al. 2023). The type strain *R. lusitanum* P1-7 was isolated from a nodule of *Phaseolus vulgaris* L. in Portugal. This strain does not carry virulence gene virA present in the type strain of *R. rhizogenes*, ATCC 11325, and form effective nodules effective in *P. vulgaris*, *Macroptilium atropurpureum* (DC.) Urb. and *L. leucocephala* (Lam.) de Wi and ineffective nodules in *Medicago sativa* L. (Valverde et al. 2006). Thus, the results of our study showed significant species diversity of the studied isolates of the genus *Rhizobium*, while closely related type strains of these isolates were isolated from a wide range of legume species growing in different geographic regions of the Earth.

Strain RCAM07600 was isolated from *O. evenorum* nodule, which formed a common cluster with *Pararhizobium polonicum* F5.1T and *Pararhizobium herbae* DSM 26427.The type strain *P. polonicum* F5.1T was isolated from gall on *Prunus avium* L. rootstock in Poland (Pulawska et al. 2016), whereas the type strain *P.*

herbae DSM 26427 is a mesophilic prokaryote that was isolated from root nodule of herb legumes *Astragalus membranaceus* grown in Xinjiang, China (Ren et al. 2011). Strain *P. herbae* DSM 26427 was able to form nitrogen-fixing and ineffective nodules on the host plant and *Albizia julibrissin* Durazz, respectively, whereas no nodules were observed on the cultivated legumes *Phaseolus vulgaris*, *Medicago sativa* L., *Pisum sativum* Lam., and *Trifolium pratense* L. (Mousavi et al. 2014). *P. herbae* also found in nodules *O. mertensiana* Turcz., *Astragalus norvegicus* Grauer, *Vicia cracca* L., and *Lathyrus pratensis* L., growing in the Arctic region of the Russian Federation (Kuznetsova et al. 2024, 2025). It should be emphasized that the genus *Pararhizobium* was separated from the larger *Rhizobium* group relatively recently). Many of these species exhibit the ability to form symbiotic relationships with a variety of wild and cultivated legumes (Mousavi et al. 2014).

However, further molecular genetic studies are needed to determine the species affiliation of the isolated *Mesorhizobium* strains and most *Rhizobium* strains, since many species are closely related to each other and have similar homology of the 16S rRNA gene.

The results of rapid strain identification using MALDI-TOF MS and 16S rRNA gene analysis revealed that some bacterial isolates also belonged to the order Hyphomicrobiales, but belonged to genera incapable of independently forming nodules. These genera included *Allobosea* (previously, *Bosea*) from family Alloboseaceae (previously, family Boseaceae) and *Tardiphaga* (family Bradyrhizobiaceae). Individual symbiotic genes involved in the formation of root nodules in legumes were described in some *Allobosea* and *Tardiphaga* species (Sazanova et al. 2019, 2020). *Allobosea* were found in all three *Oxytropis* species, with particular abundance in *O. evenorum* and *O. ochotensis*. Taxonomic analysis revealed that strains were most closely related to *Allobosea vaviloviae*, which was first isolated from the relict legume *Vavilovia formosa* (Safronova et al. 2015), and was also found in nodules of narrowly endemic species of legumes *Oxytropis erecta* Kom., *O. anadyrensis* Vass., *O. kamtschatica* Hulten, and *O. pumilio* (Pall.) Ledeb. (Kamchatka Peninsula), and in nodules of *O. putoranica* M. Ivanova (Putorana Plateau, Arctic Russia) (Safronova et al. 2020; Kuznetsova et al. 2025). Strain RCAM07539 from *O. exserta* according to the rrs gene sequence, was closest not only to *A. vaviloviae*, but also to *A. psychrotolerans*, which was described as a psychrotrophic species of alphaproteobacteria isolated from Lake Michigan water in 2019 (Albert et al. 2019).

Ten strains were assigned to the genus *Tardiphaga*, isolated primarily from nodules of *O. ochotensis*. All strains assigned to the genus *Tardiphaga* were most closely related to the species *Tardiphaga robiniae*. The first *T. robiniae* was isolated from nodules of *Robinia pseudoacacia* L., growing in Flanders (Belgium) (De Meyer et al. 2012). *T. robiniae* strains have also been found in nodules of the legumes *V. formosa*, *O. erecta*, *O. anadyrensis*, *O. kamtschatica*, and *O. pumilio*, growing on the Kamchatka Peninsula (Safronova et al. 2020).

Mass spectrometric analysis using MALDI-TOF demonstrated a high level of strain identification, allowing us to classify the strains as belonging to a specific spe-

cies (the confidence coefficient exceeded 0.79). However, due to the lack of genetic confirmation, we retained the strain description at the genus level.

When culturing suspensions from nodules on YMA medium at temperatures of 5°C, 15°C and 28°C, differences in the spectrum of isolated bacteria were observed. The greatest taxonomic diversity was observed at 28 °C, with 27 strains belonging to five genera isolated. Ten *Allobosea* strains were isolated from nodules of all three plants. Additionally, 7 isolates of the genus *Mesorhizobium* (from *O. ochotensis* and *O. exserta*), 2 isolates of *Rhizobium* (from *O. ochotensis*), 5 isolates of *Tardiphaga* (from *O. ochotensis* and *O. evenorum*) and 3 isolates of *Phyllobacterium* (from *O. exserta*) were obtained.

At a temperature of 15 °C, 10 strains were isolated, of which representatives of the classical genera of nodule bacteria predominated: 3 strains of *Mesorhizobium*, 2 strains of *Rhizobium*, and 1 strain each of *Pararhizobium* and *Arminella*. One strain of *Allobosea* and two strains of *Tardiphaga* were also isolated from nodules of *O. evenorum* and *O. ochotensis*. The most limited spectrum of bacteria species was observed during incubation at 5 °C: only three strains assigned to the genus *Tardiphaga* were isolated exclusively from *O. ochotensis* nodules. Isolates belonging to typical microsymbionts of legume plants (*Mesorhizobium* and *Rhizobium*) were not obtained at this temperature. Thus, a decrease in cultivation temperature was accompanied by a decrease in both the total number of isolates and the taxonomic richness of the cultivated community. The composition of cultivable bacteria shifted from a high relative abundance of classical rhizobia genera at 15°C to their complete absence and a predominance of *Tardiphaga* at 5 °C.

Factors influencing the growth and distribution of rhizobia species include not only temperature, but also pH, salinity, and the distribution of suitable hosts (Dludlu et al. 2018; Laranjo and Oliveira 2011). The results of the screening study showed that pH affected Kamchatka strains of the genera *Mesorhizobium* and *Rhizobium* differently. Some of them grew in a fairly wide pH range (4-10), while others had narrower growth pH ranges (4-7 or 5-8). It is known that rhizobia generally prefer neutral or slightly acidic conditions (pH 6.0-7.5) but exhibit remarkable adaptability: some strains are capable of growing in extremely acidic (pH 4.0-4.6) or even alkaline (up to pH 10) soils, reflecting adaptation to a variety of environmental conditions (Shah et al. 2022). Some *Mesorhizobium* species can tolerate a wide range of pH values (3-10) (Brigido et al. 2007). For example, *Mesorhizobium* was found to be the dominant symbiont of *Cicer arietinum* L. plants growing on alkaline soils in China (Zhang et al. 2012). On the other hand, a study of *Mesorhizobium* strains nodulating *Cicer* plants in Portuguese soils showed that some strains were able to tolerate acidic conditions down to a minimum pH of 3 (Brigido et al. 2007). This suggests that *Mesorhizobium* have a wide tolerance to different pH values and our results confirms this.

Salt can influence symbiosis at different stages: growth and survival of rhizobia in soil, root colonization, infection and nodule development processes, and nodule functioning (Kulkarni and Nautiyal 2000; Laranjo and Oliveira 2011). In

general, the effect of NaCl is a good indicator of the response of rhizobia to different salinity conditions (Abdelmoumen et al. 1999). According to the literature, *Mesorhizobium* can tolerate a maximum of 1-2% NaCl, depending on the species (Laranjo and Oliveira 2011). However, described *Mesorizobium* strains from *Cicer arietinum* L. (Haryana, India) that grow at 8% NaCl (Sehrawat et al. 2018). For most *Phyllobacterium*, optimal growth occurs at levels up to 1% NaCl (Willems 2014). But, for example, *Phyllobacterium salinisoli* LLAN61, isolated from a root nodule of *Lotus lancerottensis* Webb and Berthel. in saline soil on the island of Lanzarote, could grow at 3.5% NaCl, and the type strain *P. myrsinacearum* IAM 13584 at 3% (Leon-Barrios et. al. 2018). The acceptable NaCl limits for different *Rhizobium* species vary greatly. For example, in a study examining the survival limits of *Rhizobium* under extreme conditions, strains of *Rhizobium* sp. NBRI0102 and *Rhizobium* sp. NBRI2505 from nodules of *Sesbania aculeata* (Willd.) Pers. were described, which tolerated at 10% and 28% salt (NaCl, wt/vol) for 18 hours of incubation at 30 °C (Kulkarni et al. 2000). In our study, most *Mesorhizobium* and *Rhizobium* strains, as well as all *Phyllobacterium*, could grow at a concentration of 3% NaCl, unlike commercial strains, for which the maximum was 1% NaCl. To study the physiological and biochemical properties and determine the optimal growth parameters (temperature, salt concentration and pH), the strains will be incubated in a liquid medium under various stress factors and taking into account the number of microbial cells at different points in time. The search for and selection of stress-resistant rhizobia strains is essential for increasing the productivity of legumes and their resilience to the challenging soil and climatic conditions of Russia's regions. Highly effective stress-resistant rhizobia strains with broad adaptive capabilities also hold promise for use in soil bioremediation under conditions of abrupt climate change. Rhizobia are known to possess the biochemical and ecological capacity to degrade organic pollutants, making them useful for the remediation of contaminated soils. Furthermore, various rhizobia species can be used for phytoremediation, stimulating plant growth through nitrogen fixation, reducing the phytotoxicity of metals, and influencing their transport and accumulation in plants (Johnson et al. 2004; Teng et al. 2015). The presence of rhizobia can also directly or indirectly influence the functioning of soil microbial communities, there by promoting the restoration of disturbed lands (Li, Liang et al. 2013; Teng et al. 2015). For example, the effective use of the microbial-plant association *Rhizobium galegae-Galega orientalis* Lam. in extreme conditions is determined by the presence of a complex of physiological and biochemical properties of the *R. galegae* microsymbiont (synthesis of exopolysaccharides, β-IAA, tolerance to petroleum hydrocarbons and their use as a carbon source, and salt tolerance), which ensures their active functioning in extreme conditions, the destruction of pollutants, and the stability of the plant component (Kartyzhova and Kondratskaya 2022). Thus, the selection of naturally resistant rhizobia strains is a challenging, but at the same time more practical and less expensive biotechnological task, than creating genetically modified resistant strains.

Conclusion

This paper presents the first data on the genetic diversity of endophytic microorganisms of endemic legumes *O. exserta*, *O. evenorum* and *O. ochotensis* growing in Kamchatka. Forty strains were isolated from nodules, most of which belonged to the genus *Mesorhizobium* (10 strains, isolated from *O. ochotensis* and *O. exserta*), *Allobosea* (11 strains, isolated from all legume species), and *Tardiphaga* (10 strains, isolated from *O. ochotensis* and *O. evenorum*). Isolates of the genera *Rhizobium* (4 strains, isolated from *O. ochotensis* and *O. evenorum*), *Phyllobacterium* (3 strains, isolated from *O. exserta*), and *Arminella* and *Pararhizobium* (1 strain each, isolated from *O. evenorum*) were also present. The obtained data are consistent with the literature data on the dominant role of *Mesorhizobium* in symbiosis with *Oxytropis* spp. Incubation temperature was found to be one of the key factors in the selection of cultured rhizobia. Most *Mesorhizobium* and all *Phyllobacterium* were detected only at 28 °C, whereas *Pararhizobium* was found only at 15 °C. Note, that no isolates from these taxonomic groups were obtained at a temperature of 5 °C, whereas only representatives of the endophytic genus *Tardiphaga* were isolated under these conditions. For *O. exserta*, a specific microbial composition was noted, represented only by the families Phyllobacteriaceae (*Mesorhizobium* and *Phyllobacterium*) and Alloboseaceae (*Allobosea*), whereas the nodules of other species contained strains from the families Rhizobiaceae (*Rhizobium*, *Pararhizobium*, and *Arminella* for *O. evenorum*), Phyllobacteriaceae (*Mesorhizobium* for *O. ochotensis*), Alloboseaceae (*Allobosea*) and Bradyrhizobiaceae (*Tardiphaga*).

The study revealed that the studied microbial strains exhibit significant variability in their tolerance to acidic and alkaline conditions. So, strains *Mesorhizobium* sp. RCAM07391, RCAM07494; *P. myrsinacearum* RCAM07525, RCAM07528, RCAM07530; and *Rhizobium* sp. RCAM07452 and RCAM07474 demonstrated the ability to grow in the widest range of pH values from 4 to 10, whereas the remaining strains were able to grow in a narrower range of pH values. Most strains also demonstrated significant salt tolerance and could grow at 3% NaCl.

Thus, the study expands our understanding of the biodiversity of cultured bacteria associated with endemic legumes in subarctic regions. Rhizobial strains selected under conditions close to natural ones are of interest for further study of their symbiotic potential and adaptive properties, with a view to their possible application in the greening of agriculture and the sustainable use of natural resources across regions of Russia which are characterized by contrasting soil and climatic conditions.

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