SYMBIOGENETICS

Rhizobial isolates in active layer samples of permafrost soil of Spitsbergen, Arctic

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Abstract

Twenty-nine strains were isolated from two samples of the permafrost active layer of the Spitsbergen archipelago. The estimated number of bacteria ranged from $4.0 \cdot 10^4$ to $1.7 \cdot 10^7$ CFU·g^{-1}. As a result of sequencing of the 16S rRNA (*rrs*) genes, the isolates were assigned to 13 genera belonging to the phyla Actinobacteria, Proteobacteria (classes α, β, and γ), Bacteroidetes, and Firmicutes. Six isolates belonged to the rhizobial genus *Mesorhizobium* (order Rhizobiales). A plant nodulation assay with seedlings of legume plants *Astragalus norvegicus*, *A. frigidus*, *A. subpolaris* and *Oxytropis sordida*, originated from Khibiny (Murmansk region, Russia) and inoculated with *Mesorhizobium* isolates, showed the inability of these strains to form nodules on plant roots. Symbiotic (*sym*) genes *nodC* and *nodD* were not detected in *Mesorhizobium* strains either.

Keywords: legume plants, root nodule bacteria, 16S rRNA genes, permafrost soil, Spitsbergen

Introduction

Root nodule bacteria (rhizobia) belong to one of the most important groups of soil microorganisms that form nitrogen-fixing symbiosis with legumes, which allows them to enrich nitrogen-depleted soils, making it available to other plants. (Tikhonovich and Provorov, 2009).

Russia's northern territories will increasingly be used for agricultural land as a result of global climate warming. In the Russian Arctic zone, there has an expansion of the areas occupied by more productive plant communities, the so-called "greening" of the tundra. This is mainly associated with an increase in temperature, the growing season duration and the thawing depth change of permafrost soils. These processes activate soil microorganisms, which contributes to a more intensive accumulation of organic matter in the forming phytocenoses (Belonovskaya et al., 2016). As a result, free-living rhizobial bacteria can form highly adaptive legume–rhizobial symbioses, penetrating new landscapes, such as the Arctic tundra. Legumes are known to play an important role in pasture phytocenoses and are the main source of protein for herbivorous farm animals (Parakhin and Petrova, 2009; Pryadilshchikova, Kalabashkin and Konovalova, 2018).

From the permafrost soil of the Arctic, isolates were obtained related to the genera whose representatives can form nitrogen-fixing symbiosis with legumes. Strains of the genus *Burkholderia* were isolated from the permafrost of the Canadian Arctic (Wilhelm, Niederberger, Greer and Whyte, 2011). Isolates belonging to the genera *Bradyrhizobium*, *Rhizobium*, *Devosia*, and *Methylobacterium*, as well as the species *Mesorhizobium gobiense*, were obtained from the soils of northeastern Greenland (Ganzert, Bajerski and Wagner, 2014). It should be mentioned that the type strain *Mesorhizobium gobiense* 83330^T was isolated from the nodule of *Oxytro-*

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pis glabra growing in the desert soils of China. This strain, in addition to the host plant, is also able to form nodules on *Glycyrrhiza uralensis*, *Lotus corniculatus*, and *Robinia pseudoacacia* plants, as shown by cross-nodulating tests (Han et al., 2008). Of the Late Pleistocene (20–35 thousand years ago) permafrost soil of the Kolyma lowland, a dominant phylotype was identified, which showed 99.3 % similarity to *Bradyrhizobium canariense* (Kudryashova et al., 2013). The ability of strains of this species to form effective symbiosis with various legumes endemic from the tribe Genisteae growing in the Canary Islands has been shown (Vinuesa et al., 2005).

Isolates belonging to *Rhizobium* sp. (Singh et al., 2017) and *Burkholderia* sp. (Hansen, Herbert, Mikkelsen and Jensen, 2007) were isolated from permafrost soil of Spitsbergen. The metagenomic studies of the active permafrost layer in this archipelago revealed DNA and RNA of symbiotic nitrogen-fixing bacteria of the genera *Rhizobium* and *Bradyrhizobium* (Schostag et al., 2015). The above works were devoted to the description and study of the bacterial biodiversity, including root nodule bacteria, in the permafrost of Spitsbergen. However, their ability to form nitrogen-fixing symbioses with plants was not studied.

Currently, native species of legume plants do not grow on Spitsbergen. The presence of a randomly introduced species of *Trifolium repens* L. in the area of the village of Barentsburg (https://svalbardflora.no/index.php?id=638) and Pyramid (Svenning, Røsnes, Lund and Junttila, 2001) was noted. The clover genotypes from Spitsbergen have been shown to have a higher dry matter mass as well as greater frost resistance compared to the two varieties (Norstar and AberHerald) from central Norway (Svenning, Røsnes, Lund and Junttila, 2001). The same authors selected soil samples in various areas of Spitsbergen, including in the area of the village Pyramid. Clover plants planted in this soil formed nodules and their microsymbionts were identified as *Rhizobium leguminosarum* bv. *trifolii* (Fagerli and Svenning, 2005). Clover plants grown in other soil samples of Spitsbergen did not form nodules, which suggests the introduction of rhizobia together with clover seeds. However, attempts to use other species of leguminous plants, including Arctic ones, as a selective factor for the search for root-nodule bacteria have not been described.

It is known that the genus *Mesorhizobium* belongs to an extensive group of rhizobia, isolated from soils and root nodules of legume plants growing around the world (Helene, Dall'Agnol, Delamuta and Hungria, 2019). *Mesorhizobium* strains form nitrogen-fixing symbiosis with a wide range of host plants from the genera *Acacia, Alhagi, Amorpha, Biserrula, Caragana, Chamaecrista, Leucaena, Lotus, Phaseolus, Prosopis, Robinia* and *Sophora*, including various species of *Astragalus* (Willems, 2014). The broad distribution of *Mesorhizobium* strains, allied with the ability to establish symbiotic relationships with many genera of legumes, makes it promising for use in agriculture (Helene, Dall'Agnol, Delamuta and Hungria, 2019).

Arctic rhizobia are of interest for studying the evolutionary development of nitrogen-fixing bacteria and their adaptation to low temperatures, and also make it possible to analyze the functional relations between rhizobia and leguminous plants in isolated indigenous populations of the North (Caudry-Reznicket, Prevost and Schulman, 1986). However, the biodiversity of root nodule bacteria in the Arctic territories and their symbiotic interactions with legumes are practically not studied in Russia.

The purpose of this work was to study the biodiversity of bacterial isolates in samples of the active layer of permafrost soil of Spitsbergen (including rhizobia, capable of forming a nitrogen-fixing symbiosis with legumes), as well as to study the ability of the obtained rhizobial isolates to form root nodules with Arctic legume plants.

Materials and methods

Seeds of legumes *Astragalus norvegicus*, *A. frigidus*, *A. subpolaris* and *Oxytropis sordida* were collected in Khibiny (Kola Peninsula, Russia) in 2018. The coordinates of the collection sites are from 67°43'8.7" to 67°48'6.9" N and from 33°35'39.1" to 33°36'24.1" E.

Two samples of frozen soil were collected in 2018 by aseptic drilling of borehole 10 (56 m above sea level (a.s.l.), 77.99332° N, 14.66114° E) on the slope of pingo Fili in Grøndalen valley in the vicinity of the Russian mine Barentsburg on West Spitsbergen (Demidov et al, 2019). Frozen conditions of cores were maintained during drilling, sampling and transportation to the laboratory. Modern mean annual air temperatures equals −2.2 °C, permafrost temperature at zero amplitude depth amounts to −3.56 °C and the active layer thickness amounts to approximately 1.5 m in this part of West Spitsbergen.

The uppermost part of borehole 10 from 0 to 2.5 m below surface (b.s.) includes the modern top soil at 0 to 0.1 m b.s. with living shrub material and a buried soil formation at 0.25 to 0.4 m b.s. with decomposed similar shrub material. Both modern and buried soil were sampled and used in this study (Table 1). The minerogenic material was characterised by fine sand and loam, including gravel. The cryostructures were wavy lenticular with

Table 1. Cryolithological description of cores from borehole 10 used in this study

№ sample	Depth, m	Cryolithological description
1	0.0–0.1	Modern top soil with living shrub material, fine sand and loam with wavy lenticular cryostructures including gravel.
2	0.3–0.39	Buried soil with decomposed shrub material, fine sand and loam with wavy lenticular cryostructures including gravel.

ice lenses up to 2 cm thick. Toward 2.5 m b.s. the clay content increased as the gravel content decreased. The ion content of dry residue was about 55 mg/l, with predominantly HCO_3^- and Ca^{2+}. The pH was neutral (6.8).

Isolation of pure cultures of microorganisms

To isolate microorganisms, we used the original and 10-fold diluted modified yeast extract mannitol agar (YMA, Vincent, 1970) supplemented with 0.5 % succinate (YMSA, Safronova et al., 2015) for the cultivation of root nodule bacteria; Tryptic Soy Broth (TSB, Sigma, USA) supplemented with 20 g/L agar for heterotrophs; R2A agar medium (Oxoid, UK) for oligotrophs (Vishnivetskaya et al., 2000); meat peptone agar (GMF, NICF, Russia) for mesophilic microorganisms; Ashby's medium for nitrogen-fixing (oligonitrophils) microorganisms (Egorov, 1976). The cultivation was carried out at a temperature of 22 °C for two weeks, and results were recorded starting from the second day. To determine the number of colony-forming units (CFU), 1 g of soil was suspended in 10 ml of sterile water, followed by 10-fold dilutions and plating on solid nutrient media.

All strains were deposited in the Russian Collection of Agricultural Microorganisms (RCAM, WDCM 966) and stored at –80 °C in the automated Tube Store (Liconic Instruments, Lichtenstein) as described previously (Safronova and Tikhonovich, 2012). Information on isolates is available in the online RCAM database (http://www.arriam.spb.ru.).

Identification of the isolates and bioinformatic analysis

Identification of the isolates was determined by amplification, purification of the PCR product, and sequencing of the 16S rRNA gene, as described previously (Safronova et al., 2015, 2019). The DNA fragment was sequenced using an ABI PRISM 3500xl genetic analyzer (Applied Biosystems, USA).

The search for homologous sequences and related type strains was performed using the NCBI GenBank database (https://www.ncbi.nlm.nih.gov) and the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed using the MEGA7 program and the neighbour-joining method (Tamura et al., 2011). The evolutionary distances were computed using the maximum composite likelihood model.

The *rrs* sequences were deposited to the NCBI GenBank database under accession numbers: MT912817–MT912845.

Sterile test-tube experiment

Seeds of leguminous plants *Astragalus norvegicus*, *A. frigidus*, *A. subpolaris* and *Oxytropis sordida* were surface sterilized by treatment with H_2SO_4 for 5 min. The treated seeds were rinsed carefully with sterile water and germinated on filter paper in Petri dishes at +25 °C in the dark for 3 days. Seedlings 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, H_3BO_3 0.005, $(NH_4)_2MoO_4$ 0.005, $ZnSO_4 \times 7\ H_2O$ 0.005, $MnSO_4$ 0.002 (Novikova and Safronova, 1992). Seedlings were inoculated with *Mesorhizobium* isolates 1Y-1, 1Y-3, 1Y-5, 1Y-8, 1G-2 and 1G-4 in the approximate amount of 10^6 cells per tube as well as with two soil extracts from different horizons of the active layer of permafrost soil of borehole 10 (Spitsbergen). The uninoculated plants were used as negative control. The plant nodulation assay was carried out in duplicate. Plants were cultivated 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, 2 h), day (400 μmol m^{-2} s^{-1}, 23 °C, 12 h), evening (200 μmol m^{-2} s^{-1}, 20 °C, 2 h). Illumination was performed by L 36W/77 FLUORA lamps (Osram, Germany).

Amplification of *nodC* and *nodD* genes in rhizobial isolates

To reveal the potential ability of *Mesorhizobium* isolates to nodulate, the symbiotic *nodC* and *nodD* genes were amplified. For *nodC* genes, primers were used: nodCF (5′- AYGTHGTYGAYGACGGTTC — 3′) / nodCI (5′- CGYGACAGCCANTCKCTATTG — 3′) (Laguerre et al., 2001) and the PCR protocol as described earlier (Wei, Young and Bontemps, 2009). For nodD genes, primers were used: (5′- GCGAACGYWTTCTGACACC- 3′) / (5′- TCSGTAAATSCSGGAAG — 3′) and the PCR protocol as described earlier (Ji et al., 2015). The strains *Mesorhizobium japonicum* Opo-235 and Opo-242, as well as the strain *M. kowhaii* Ach-343 (Safronova et al., 2018; 2019) were used as reference strains.

Multi-substrate testing

The enzymatic activities of the isolates were studied using the GENIII MicroPlate microassay system (BioLog, USA), which analyzes the ability of bacteria to metabolize 71 carbon sources and resistance to 23 chemicals. For the test analysis, isolates belonging to the bacterial genera presented in the GEN III database were selected. The analysis was performed according to the manufacturer's recommendations, with the exception of the extended incubation period for strains 2G-2 and 2R-3 (2 days).

Results and discussion

Isolation of pure cultures of microorganisms

The number of microorganisms varied within $1.4 \cdot 10^6$–$1.7 \cdot 10^7$ CFU·g^{-1} (sample 1) and $4.0 \cdot 10^4$–$1.1 \cdot 10^5$ CFU·g^{-1} (sample 2) depending on the medium and the dilution rate. The data obtained are comparable with the results of previous studies on the number of microorganisms in the active layer of the permafrost soil of Spitsbergen (Singh et al., 2017; Trubitsyn et al., 2019). For sample 1, the maximum number of CFU/g was detected on YMSA, 1/10 R2A and 1/10 TSA; for sample 2 — on YMSA medium.

Due to the use of various nutrient media, it was possible to expand the range of isolated bacteria. Representatives of four bacterial taxa (*Streptomyces fildesensis*, *Arthrobacter humicola*, *Mesorhizobium* sp. and *Pseudomonas* sp.) were isolated on two or more media. The other strains were isolated on one medium: on YMSA — representatives of the genera *Rhodococcus*, *Kocuria*, *Citricoccus*, *Sphingomonas*, *Mucilaginibacter* and *Planococcus*; on R2A — *Streptomyces*, *Streptacidiphilus* and *Arthrobacter*; on GMF — *Arthrobacter*; on TSA — *Luteibacter*; on Ashby — *Caballeronia*.

Phylogenetic analysis

A total of 29 bacterial isolates were obtained, grouped into 13 genera and 16 species (Table 2). Sequence analysis of the *rrs* gene of the obtained isolates showed that they belong to the following bacterial phyla: *Actinobacteria*, *Proteobacteria* (classes α, β and γ), *Bacteroidetes* and *Firmicutes*. The most numerous (both in the number of isolated strains and in the number of genera) was the phylum *Actinobacteria*, represented by the genera *Arthrobacter* (6 strains), *Streptomyces* (5 strains), as well as *Streptacidiphilus*, *Rhodococcus*, *Kocuria*, and *Citricoccus* (1 strain each). The phylum *Proteobacteria* was represented by the genera *Mesorhizobium* and

Table 2. The similarity between the isolates obtained in the work and the closest type strains based on the 16S rRNA gene sequencing

Isolate number*	Closest type strain	Isolation source	Similarity, %	Phylogenetic group	Accession number
1R-6, 1G-1, 1A-1, 1T-1	*Streptomyces fildesensis* GW25-^{5}T	Soil of Antarctica	99.36–99.63	*Actinobacteria*	DQ408297
1Y-4	*Rhodococcus qingshengii* JCM 15477^T	Carbendazim-contaminated soil	99.36		DQ090961
1R-3	*Streptomyces paucisporeus* 1413^T	Forest soil	98.10		AY876943
1R-4	*Streptacidiphilus durhamensis* FSCA6^{7}T	Forest soil	98.13		NR_125637
2A-2, 2G-1, 2R-2, 2Y-4	*Arthrobacter humicola* KV-653^T	Soil	99.93–100		AB279890
2G-2	*Arthrobacter humicola* KV-653^T	Soil	98.27		AB279890
2R-3	*Arthrobacter oryzae* KV-651^T	Soil	98.56		AB279889
2Y-1	*Kocuria rosea* DSM 20447^T	Soil	100		X87756
2Y-2	*Citricoccus nitrophenolicus* PNP1^T *Citricoccus alkalitolerans* YIM 7001^{0}T	Sludge from a wastewater treatment plant Desert soil	100 100		GU797177 AY376164
1Y-1, 1Y-3, 1Y-5, 1Y-8, 1G-2, 1G-4	*Mesorhizobium* sp. CCANP61 *Mesorhizobium qingshengii* CCBAU 33460^T *Mesorhizobium shangrilense* CCBAU 6532^{7}T	Nodules *Cicer canariense* Nodules *Astragalus sinicus* Nodules *Caragana bicolor*	100 99.92 99.85	*Alphaproteobacteria*	HF931055 NR_109565 NR_116163
1Y-7	*Sphingomonas panacis* DCY99^T	Rhizosphere *Panax ginseng*	99.05		NR_146850
1A-2, 1A-4	*Caballeronia udeis* Hg2^T *Caballeronia sordidicola* S5-B^T	PAH** — contaminated soil fungus *Phanerochaete sordida*	98.81 98.60	*Beta proteobacteria*	NR_125500 NR_104563
1T-2	*Luteibacter anthropi* CCUG 2503^{6}T	Human blood	99.28	*Gammaproteobacteria*	NR_116911
2A-1, 2R-1	*Pseudomonas frederiksbergensis* JAJ28^T	Soil	99.02		AJ249382
1Y-6	*Mucilaginibacter dorajii* DR-f4^T	Rhizosphere *Platycodon grandiflorus*	98.15	*Bacteroidetes*	GU139697
2Y-3	*Planomicrobium soli* XN1^{3}T	Soil	99.47	*Firmicutes*	NR_134133

* Strains isolated on media: R — R2A; G — GMF; A — Ashby; T — TSA; Y — YMSA

**PAH — polycyclic aromatic hydrocarbon

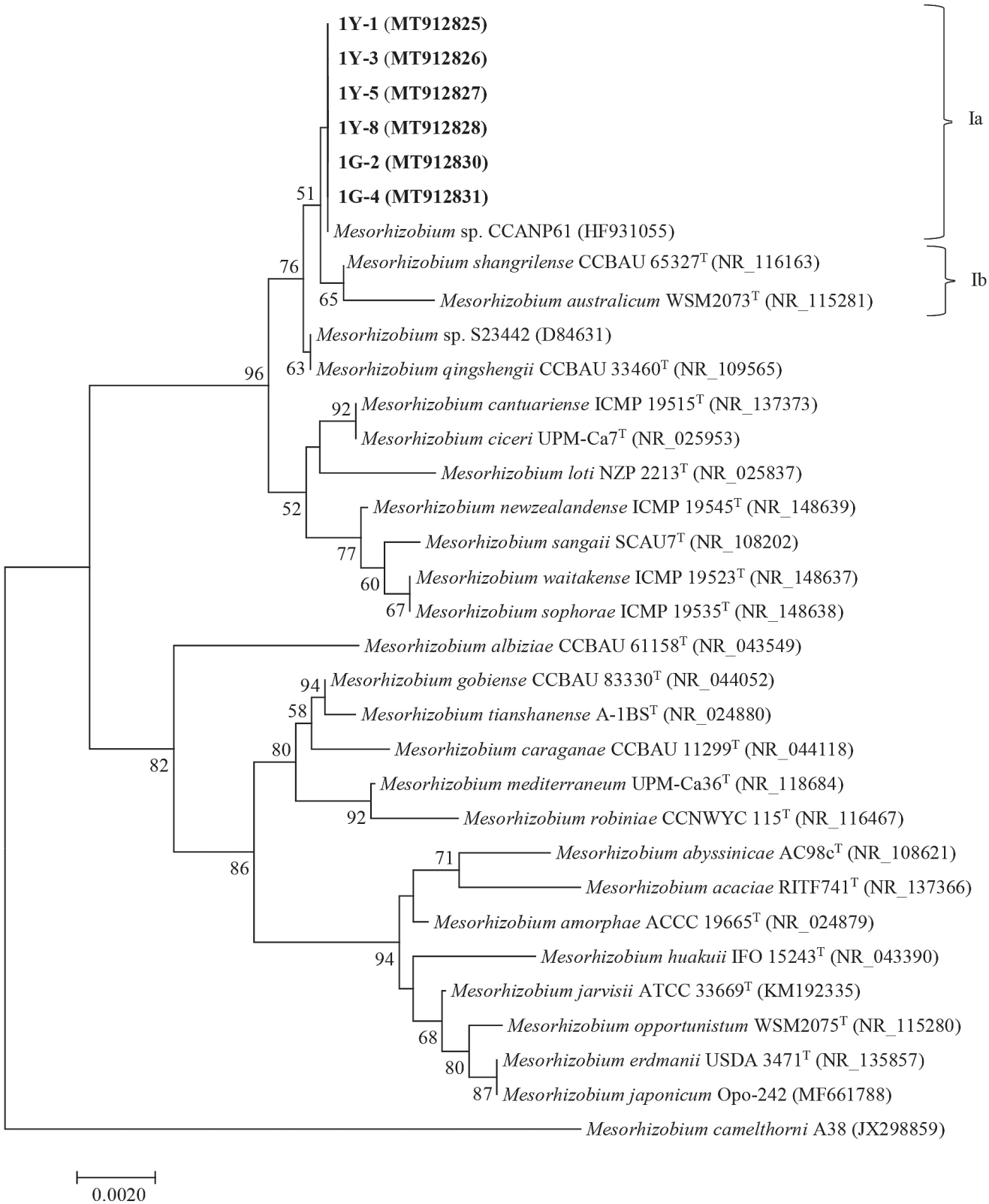

Fig. 1. Phylogenetic tree generated by the neighbour-joining method using partial 16S rRNA gene sequences of the strains isolated from active layer of permafrost soil and representatives of closely related *Mesorhizobium* species. The isolated strains are in bold. Type strains are indicated by the letter T. Subclusters Ia and Ib are formed by *Mesorhizobium* isolates obtained in this work and representatives of closely related species. Bootstrap values of more than 50 % are given.

Table 3. Phenotypic properties of some strains obtained in the study

Phenotypic property	1Y-4	2A-2	2G-2	2R-3	2A-1	2R-1	2Y-1
Growth at:							
pH 6	+	+	+	+	+	+	–
pH 5	–	–	–	–	–	+	–
1 % NaCl	+	+	–	+	+	+	+
4 % NaCl	–	–	–	–	–	–	–
8 % NaCl	–	–	–	–	–	–	–
Utilization of:							
D-Maltose	+	+	–	–	–	–	–
D-Trehalose	+	+	–	–	–	–	–
D-Cellobiose	+	+	–	–	–	–	–
Sucrose	+	+	+	–	+	+	–
D-Turanose	+	+	–	+	–	–	–
Stachyose	+	+	+	–	–	–	–
D-Raffinose	+	+	–	+	–	–	–
α-D-Lactose	–	+	+	–	–	–	–
β-Methyl-D-Glucoside	–	–	+	–	–	–	–
N-Acetyl-D-Glucosamine	–	–	+	–	–	–	–
N-Acetyl-D-Galactosamine	–	–	+	–	+	–	–
N-Acetyl Neuraminic Acid	–	+	+	–	–	–	–
α-D-Glucose	–	+	–	–	+	+	–
D-Mannose	–	+	+	+	–	–	–
D-Fructose	–	–	+	+	+	+	–
D-Galactose	–	+	–	–	+	–	–
3-Methyl Glucose	+	+	–	–	–	–	–
D-Fucose	–	+	–	+	–	–	–
L-Fucose	–	+	+	–	–	–	–
L-Rhamnose	–	+	–	–	–	–	–
1 % Sodium Lactate	+	+	–	–	+	+	–
Fusidic Acid	–	–	–	–	–	–	–
D-Serine	+	–	–	–	–	–	–
D-Sorbitol	+	+	–	+	+	+	–
D-Mannitol	–	+	–	+	+	–	–
D-Arabitol	–	–	+	–	+	+	–
myo-Inositol	–	+	+	–	+	+	–
Glycerol	–	–	+	+	+	+	–

Phenotypic property	1Y-4	2A-2	2G-2	2R-3	2A-1	2R-1	2Y-1
Utilization of:							
D-Fructose-6-phosphate	–	+	–	–	–	–	–
D-Aspartic Acid	+	+	–	–	+	+	–
Glycyl-L-Proline	–	–	+	–	–	–	–
L-Alanine	–	+	–	–	+	+	–
L-Arginine	+	+	+	–	+	+	–
L-Aspartic Acid	+	+	+	–	+	+	–
L-Glutamic Acid	+	+	+	–	+	+	–
L-Histidine	–	–	–	–	+	+	–
L-Pyroglutamic Acid	+	–	+	–	+	+	–
L-Serine	+	–	+	–	+	+	–
Pectin	–	+	–	–	+	+	–
D-Galacturonic Acid	–	+	–	–	+	+	–
L-Galactonic Acid Lactone	–	–	–	–	+	+	–
D-Gluconic Acid	+	+	+	+	+	+	–
D-Glucuronic Acid	+	+	–	–	+	+	–
Glucuronamide	+	–	–	–	+	+	–
Mucic Acid	+	+	–	–	+	+	–
Quinic Acid	+	–	+	–	+	+	–
D-Saccharic Acid	+	+	–	–	+	+	–
p-Hydroxy-Phenylacetic Acid	–	–	–	–	+	+	–
D-Lactic Acid Methyl Ester	–	–	+	–	–	–	–
D-Lactic Acid	–	+	+	–	+	+	–
Citric Acid	+	+	–	–	+	+	–
α-Keto-Glutaric Acid	–	+	+	–	+	+	–
D-Malic Acid	–	+	–	–	+	+	–
L-Malic Acid	+	–	+	–	+	+	–
Bromo-Succinic Acid	–	–	+	–	–	–	–
Tween 40	+	+	+	+	–	–	+
γ-Amino-Butryric Acid	+	+	–	–	+	+	–
α-Hydroxy-Butyric Acid	+	+	–	–	–	–	–
β-Hydroxy-D, L-Butyric Acid	+	+	–	–	–	–	–

Phenotypic property	1Y-4	2A-2	2G-2	2R-3	2A-1	2R-1	2Y-1
Utilization of:							
α-Keto-Butyric Acid	+	+	–	+	–	–	–
Acetoacetic Acid	+	–	–	+	+	–	+
Propionic Acid	+	+	–	–	+	–	+
Acetic Acid	+	+	+	+	+	+	+
Formic Acid	+	–	–	–	–	–	–
Resistance to:							
Troleandomycin	–	–	–	–	+	+	–
Rifamycin SV	–	–	–	–	+	+	–
Minocycline	–	–	–	–	–	–	–
Lincomycin	–	–	–	–	+	+	–
Guanidine HCl	–	–	–	–	–	–	–
Niaproof4	–	–	–	–	+	+	–
Vancomycin	–	–	–	–	+	+	–
Nalidixic Acid	+	+	+	+	–	–	+
Aztreonam	+	+	+	–	–	–	–
Lithium Chloride	+	–	–	–	–	–	–
Potassium Tellurite	+	–	–	+	+	+	–
Sodium Butyrate	+	–	–	–	–	–	+
Tetrazollium Violet	–	–	–	–	+	+	–
Tetrazollium Blue	–	–	–	–	+	+	–

Sphingomonas (6 and 1 strain, respectively) related to class α-*Proteobacteria*, by the genera *Caballeronia* and *Luteibacter* (2 and 1 strain, respectively) related to class β-*Proteobacteria*, as well as by the genus *Pseudomonas* (2 strains) related to class γ-*Proteobacteria*. The phyla *Bacteroidetes* and *Firmicutes* were represented by the genera *Mucilaginibacter* and *Planomicrobium*, respectively (1 strain each).

From sample 1, 18 strains were isolated, while 11 strains were isolated from sample 2 (Table 2). There were no coincidences at the genus level between the isolates of both samples. In sample 1, the greatest number of strains belonged to the genera *Mesorhizobium* and *Streptomyces* (6 and 5 strains, respectively), while in sample 2 most of the strains were affiliated to the genus *Arthrobacter* (6 strains).

Isolates 1Y-1, 1Y-3, 1Y-5, 1Y-8, 1G-2, and 1G-4 showed 100 % similarity of the *rrs* gene to the strain *Mesorhizobium* sp. CCANP61 (family *Phyllobacteriaceae*) isolated from the root nodule of the legume plant *Cicer canariense*, an endemic to the Canary Islands (Armas-Capote et al., 2014). The closest type strains to this group were *Mesorhizobium qingshengii* CCBAU 33460^T and *Mesorhizobium shangrilense* CCBAU 65327^T, which showed a high rate of *rrs*-similarity to the obtained isolates (99.92 and 99.85 %, respectively). On the phylogenetic tree (Fig. 1) isolates 1Y-1, 1Y-3, 1Y-5, 1Y-8, 1G-2, 1G-4 and strain *Mesorhizobium* sp. CCANP61 formed the single subcluster Ia; *Mesorhizobium shangrilense* CCBAU 65327^T and *Mesorhizobium australicum* WSM2073^T formed subcluster Ib. The levels of support of these subclusters were low (45 and 65 %, respectively). Both subclusters were grouped together with a 51 % support level. The type strain *Mesorhizobium qingshengii* CCBAU 33460^T previously isolated from the root nodule of the legume plant *Astragalus sinicus* did not form a cluster with the obtained isolates. It was shown that this strain contains the symbiotic genes *nifH* and *nodC* and effectively nodulates the host plant, which is traditionally used as a green manure in wintry fallow paddy fields (Zheng et al., 2013). The strain *Mesorhizobium shangrilense* CCBAU 65327^T was isolated from the root nodule of *Caragana bicolor* growing in China. Strain CCBAU 65327^T was able to nodulate a wide range of plant species of genera *Caragana*, *Glycyrrhiza*, *Astragalus*, *Vigna* and *Phaseolus* (Lu et al., 2009).

Isolates 2G-1, 2A-2, 2R-2, 2Y-4 showed a high level of *rrs*-similarity (99.93–100 %) with the type strain *Arthrobacter humicola* KV-653^T, isolated from a paddy soil sample in Saitama prefecture in Japan (Kageyama, Morisaki, Ōmura and Takahashi, 2008), while the isolate 2Y-1 was identified as *Kocuria rosea* (100 % *rrs*-similarity with the type strain DSM 20447^T) (Stackebrandt, Koch, Gvozdiak and Schumann, 1995).

Isolate 2Y-2 showed a 100 % similarity with the type strains *Citricoccus nitrophenolicus* PNP1^T and *Citricoccus alkalitolerans* YIM 70010^T. The strain PNP1^T was isolated from sludge from a wastewater treatment plant at a chemical factory producing pesticides in Denmark. It has been shown that this strain can utilize the xenobiotic *para*-nitrophenol (pNP), which is a product of human activity and a common pollutant in the environment (Nielsen, Kjeldsen and Ingvorsen, 2011). The strain *Citricoccus alkalitolerans* YIM 70010^T was isolated from desert soil in Egypt (Li et al., 2005). The rest of the isolates showed a low level of *rrs*-similarity (98.1–99.47 %) to the closest type strains.

Multi-substrate testing of isolates

Seven isolates were tested for the ability to utilize the major classes of carbon sources and for resistance to different inhibiting chemicals (Table 3). Each strain had a unique metabolic profile. All strains grew at 1 % (w/v)

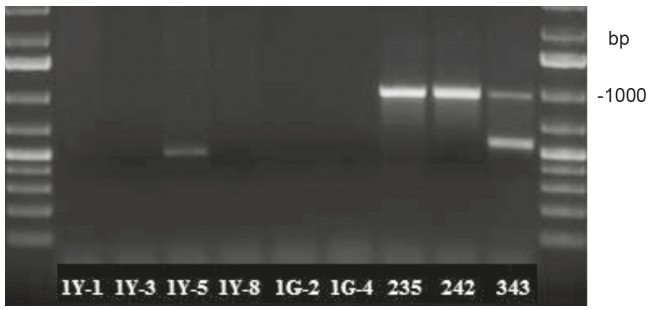

Fig. 2. Agarose gel electrophoresis of nodC PCR-products from rhizobial isolates *Mesorhizobium* and reference strains. 235 — *M. japonicum* Opo-235; 242 — *M. japonicum* Opo-242; 343 — *M. kowhaii* Ach-343

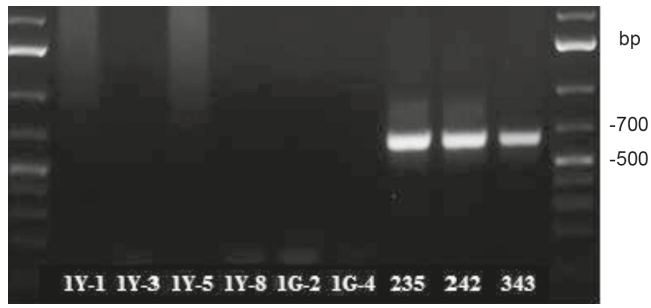

Fig. 3. Agarose gel electrophoresis of *nodD* PCR-products from rhizobial isolates *Mesorhizobium* and reference strains. 235 — *M. japonicum* Opo-235; 242 — *M. japonicum* Opo-242; 343 — *M. kowhaii* Ach-343

NaCl and pH 6 with the exception of 2G-2 and 2Y-1. Growth at pH 5 was only in strain 2R-1. Isolate *Arthrobacter* sp. 2G-2 was able to utilize β-methyl-D-glucoside, N-acetyl-D-glucosamine, glycyl-L-proline, D-lactic acid methyl ester and bromo-succinic acid; isolate of *Arthrobacter* sp. 2A-2 utilized L-rhamnose and D-fructose-6-phosphate and isolate *Rhodococcus* sp. 1Y-4 utilized fusidic and formic acids.

Strains *Pseudomonas* sp. 2A-1 and 2R-1 had nearly identical phenotypic profiles (Table 3).

The common assimilated substrates for isolates *Arthrobacter* spp. 2A-2, 2G-2 and 2R-3 were D-mannose, D-gluconic acid and acetic acid. All strains grew at pH 6 and in the presence of Tween 40, and also were sensitive to nalidixic acid.

Isolates *Pseudomonas* sp. 2A-1 and *Arthrobacter humicola* 2A-2 had the largest range of utilized substrates (42 and 38 substrates, respectively), isolate *Kocuria rosea* 2Y-1 revealed the smallest spectrum (3 substrates). Isolate 2Y-1 assimilated acetoacetic acid, propionic acid and acetic acid.

Sterile test-tube experiment and the search for *nodC* and *nodD* genes in *Mesorhizobium* isolates

To study the nodulating activity of *Mesorhizobium* isolates 1Y-1, 1Y-3, 1Y-5, 1Y-8, 1G-2 and 1G-4, a sterile test-tube experiment with legume plants growing in the Murmansk region (*Astragalus norvegicus, A. frigidus, A. subpolaris u Oxytropis sordida)* was performed. The obtained result showed the lack of ability of isolates to form nodules on the roots of these plant species. The presence in *Mesorhizobium*-related isolates of symbiotic genes *nodC* and *nodD*, which belong to the common nodulation genes, was not revealed (Figs. 2 and 3).

Conclusions

In total, 29 isolates were isolated from samples of the active layer of Spitsbergen permafrost due to the use of a wide range of nutrient media. The isolates belonged to the phyla *Actinobacteria*, *Proteobacteria* (classes α, β and γ), *Bacteroidetes* and *Firmicutes*, and represented 13 genera and 16 species. Most of the isolated strains had a low level of *rrs*-similarity (less than 99.5 %) with the closest type strains, which indicates they may possibly belong to new species of microorganisms. A number of strains belong to the practically valuable groups of microorganisms that promote plant growth (genera *Arthrobacter* and *Caballeronia*) (Chung et al., 2010; Palaniappan et al., 2010) or biodegradation of xenobiotics (genus *Citricoccus*) (Nielsen, Kjeldsen and Ingvorsen, 2011). The multi-substrate analysis of the isolates revealed a significant variety of their phenotypic profiles. At the same time, some strains had a number of features (growth in the presence of antibiotics and heavy metal salts, acid and salt tolerance) that can be of practical use. Despite the fact that most of the isolates belong to non-symbiotic bacteria, six isolates belonging to root nodule bacteria *Mesorhizobium* were isolated. To clarify the species affiliation of the *Mesorhizobium* isolates, additional analysis of the "housekeeping" genes is required. Although representatives of this genus are known as symbiotic microorganisms, nodulating a wide range of legumes, the isolated *Mesorhizobium* strains did not form nodules on the roots of the Arctic species *Astragalus norvegicus, A. frigidus, A. subpolaris* or *Oxytropis sordida*. The presence of the common nodulation genes *nodC* and *nodD* was also not detected in the obtained isolates. The negative results of the plant nodulation assay and amplification of *nod*-genes in these isolates could be associated with either the actual absence of these genes or their different structure. For further study of the nodulation capacity of rhizobial isolates, it is necessary to obtain their whole genome sequences and analyze symbiotic genes. The data obtained make it possible to consider the collection of strains isolated from the active layer of permafrost soil of Spitsbergen as a promising object for further research.

The selection of frost-resistant and effective strains of nodule bacteria and soil microorganisms, and analysis of the whole genomic sequences of the obtained isolates, will allow us to evaluate their agricultural potential

for the formation of new pasture phytocenoses and for the restoration of previously disturbed lands in the Arctic regions of Russia.

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References

Armas-Capote, N., Pérez-Yépez, J., Martínez-Hidalgo, P., Garzón-Machado, V., del Arco-Aguilar, M., Velázquez, E., and León-Barrios, M. 2014. Core and symbiotic genes reveal nine *Mesorhizobium* genospecies and three symbiotic lineages among the rhizobia nodulating *Cicer canariense* in its natural habitat (La Palma, Canary Islands). *Systematic and Applied Microbiology* 37(2):140–148. https://doi.org/10.1016/j.syapm.2013.08.004

Belonovskaya, E. A., Tishkov, A. A., Vaisfel'd, M. A., Glazov, P. M., Krenke-ml., A. N., Morozova, O. V., Pokrovskaya, I. V., Tsarevskaya, N. G., and Tertitskii, G. M. 2016. "Greening" of the Russian Arctic and the modern trends of transformation of its biota. *Izvestiya Rossiiskoi Akademii Nauk. Seriya Geograficheskaya* 3:28–39. https://doi.org/10.15356/0373-2444-2016-3-28-39 (In Russian)

Caudry-Reznick, S., Prevost, D., and Schulman, H. M. 1986. Some properties of arctic rhizobia. *Archives of Microbiology* 146(1):12–18. https://doi.org/10.1007/BF00690151

Chung, E. J., Park, J. H., Park, T. S., Ahn, J. W., and Chung, Y. R. 2010. Production of a phytotoxic compound, 3-phenylpropionic acid by a bacterial endophyte, *Arthrobacter humicola* YC6002 isolated from the root of *Zoysia japonica*. *The Plant Pathology Journal* 26:245–252. https://doi.org/10.5423/PPJ. 2010.26.3.245

Demidov, N., Wetterich, S., Verkulich, S., Ekaykin, A., Meyer, H., Anisimov, M., Schirrmeister, L., Demidov, V., and Hodson, A. J. 2019. Geochemical signatures of pingo ice and its origin in Grøndalen, West Spitsbergen. *The Cryosphere* 13:3155–3169. https://doi.org/10.5194/tc-13-3155-2019

Egorov, N. S. 1976. Workshop on Microbiology. Izdatelstvo Moskovskogo Universiteta, Moscow. 307 p. (In Russian)

Fagerli, I. L. and Svenning, M. M. 2005. Arctic and subarctic soil populations of *Rhizobium leguminosarum* biovar *trifolii* nodulating three different clover species: characterisation by diversity at chromosomal and symbiosis loci. *Plant and Soil* 275(1–2):371–381. https://doi.org/10.1007/s11104-005-3103-9

Ganzert, L., Bajerski, F., and Wagner, D. 2014. Bacterial community composition and diversity of five different permafrost-affected soils of Northeast Greenland. *FEMS Microbiology Ecology* 89(2):426–441. https://doi.org/10.1111/1574-6941.12352

Han, T. X., Han, L. L., Wu, L. J., Chen, W. F., Sui, X. H., Gu, J. G., Wang, E. T., and Chen, W. X. 2008. *Mesorhizobium gobiense* sp. nov. and *Mesorhizobium tarimense* sp. nov., isolated from wild legumes growing in desert soils of Xinjiang, China. *International Journal of Systematic and Evolutionary Microbiology* 58(11):2610–2618. https://doi.org/10.1099/ijs.0.2008/000125-0

Hansen, A. A., Herbert, R. A., Mikkelsen, K., and Jensen, L. L. 2007. Viability, diversity and composition of the bacterial community in a high Arctic permafrost soil from Spitsbergen, Northern Norway. *Environmental Microbiology* 9:2870–2884. https://doi.org/10.1111/j.1462-2920.2007.01403.x

Helene, L. C. F., Dall'Agnol, R. F., Delamuta, J. R. M., and Hungria, M. 2019. *Mesorhizobium atlanticum* sp. nov., a new nitrogen-fixing species from soils of the Brazilian Atlantic Forest biome. *International Journal of Systematic and Evolutionary Microbiology* 69(6):1800–1806. https://doi.org/10.1099/ijsem.0.003397

Ji, Z., Yan, H., Cui, Q., Wang, E., Chen, W., and Chen, W. 2015. Genetic divergence and gene flow among *Mesorhizobium* strains nodulating the shrub legume *Caragana*. *Systematic and Applied Microbiology* 38(3):176–183. https://doi.org/10.1016/j.syapm.2015.02.007

Kageyama, A., Morisaki, K., Ōmura, S., and Takahashi, Y. 2008. *Arthrobacter oryzae* sp. nov. and *Arthrobacter humicola* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 58(1):53–56. https://doi.org/10.1099/ijs.0.64875-0

Kudryashova, E. B., Chernousova, E. Y., Suzina, N. E., Ariskina, E. V., and Gilichinsky, D. A. 2013. Microbial diversity of Late Pleistocene Siberian permafrost samples. *Microbiology* 82(3):341–351. https://doi.org/10.1134/S0026261713020082

Laguerre, G., Nour, S. M., Macheret, V., Sanjuan, J., Drouin, P., and Amarger, N. 2001. Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Microbiology* 147(4):981–993. https://doi.org/10.1099/00221287-147-4-981

Li, W. J., Chen, H. H., Zhang, Y. Q., Kim, C. J., Park, D. J., Lee, J. C., Xu, L.-H., and Jiang, C. L. 2005. *Citricoccus alkalitolerans* sp. nov., a novel actinobacterium isolated from a desert soil in Egypt. *International Journal of Systematic and Evolutionary Microbiology* 55(1):87–90. https://doi.org/10.1099/ijs.0.63237-0

Lu, Y. L., Chen, W. F., Wang, E. T., Han, L. L., Zhang, X. X., Chen, W. X., and Han, S. Z. 2009. *Mesorhizobium shangrilense* sp. nov., isolated from root nodules of *Caragana* species. *International Journal of Systematic and Evolutionary Microbiology* 59(12):3012–3018. https://doi.org/10.1099/ijs.0.007393-0

Nielsen, M. B., Kjeldsen, K. U., and Ingvorsen, K. 2011. Description of *Citricoccus nitrophenolicus* sp. nov., a para-nitrophenol degrading actinobacterium isolated from a wastewater treatment plant and emended description of the genus *Citricoccus* Altenburger et al. 2002. *Antonie van Leeuwenhoek* 99(3):489–499. https://doi.org/10.1007/s10482-010-9513-6

Novikova, N. and Safronova, V. 1992. Transconjugants of *Agrobacterium radiobacter* harbouring sym genes of *Rhizobium galegae* can form an effective symbiosis with *Medicago sativa*. *FEMS Microbiology Letters* 93:261–268. https://doi.org/10.1111/j.1574-6968.1992.tb05107.x

Palaniappan, P., Chauhan, P. S., Saravanan, V. S., Anandham, R., and Sa, T. 2010. Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodule of *Lespedeza* sp. *Biology and Fertility of Soils* 46(8):807–816. https://doi.org/10.1007/s00374-010-0485-5

Parahin, N. V. and Petrova, S. N. 2009. Symbiotically fixed nitrogen in agroecosystems. *Bulletin of Agrarian Science* 18(3):41–45. (In Russian)

Pryadilshchikova, E. N., Kalabashkin, P. N., and Konovalova, S. S. 2018. Formation of pasture phytocenoses on the basis of new varieties of leguminous grasses under conditions of the European North of Russia. *Vladimir Agricolist* 1(83):32–35. https://doi.org/10.24411/2225-2584-2018-00008 (In Russian)

Safronova, V. and Tikhonovich, I. 2012. Automated cryobank of microorganisms: Unique possibilities for long-term authorized depositing of commercial mi-

crobial strains; pp. 331–334 in Microbes in applied research: current advances and challenges. https://doi.org/10.1142/9789814405041_0066

Safronova, V. I., Kuznetsova, I. G., Sazanova, A. L., Kimeklis, A. K., Belimov, A. A., Andronov, E. E., Pinaev, A. G., Chizhevskaya, E. P., Pukhaev, A. R., Popov, K. P., Willems, A., and Tikhonovich, I. A. 2015. *Bosea vaviloviae* sp. nov., a new species of slow-growing rhizobia isolated from nodules of the relict species *Vavilovia formosa* (Stev.) Fed. *Antonie van Leeuwenhoek* 107(4):911–920. https://doi.org/10.1007/s10482-015-0383-9

Safronova, V. I., Belimov, A. A., Sazanova, A. L., Chirak, E. R., Verkhozina, A. V., Kuznetsova, I. G., Andronov, E. E., Puhalsky, J. V., and Tikhonovich, I. A. 2018. Taxonomically different co-microsymbionts of a relict legume, *Oxytropis popoviana*, have complementary sets of symbiotic genes and together increase the efficiency of plant nodulation. *Molecular Plant-Microbe Interactions* 31(8):833–841. https://doi.org/10.1094/MPMI-01-18-0011-R

Safronova, V., Belimov, A., Sazanova, A., Chirak, E., Kuznetsova, I., Andronov, E., Pinaev, A., Tsyganova, A., Seliverstova, E., Kitaeva, A., Tsyganov, V., and Tikhonovich, I. 2019. Two broad host range rhizobial strains isolated from relict legumes have various complementary effects on symbiotic parameters of co-inoculated plants. *Frontiers in Microbiology* 10:514. https://doi.org/10.3389/fmicb.2019.00514

Schostag, M., Stibal, M., Jacobsen, C. S., Bælum, J., Taş, N., Elberling, B., Jansson, J. K., Semenchuk, P., and Priemé, A. 2015. Distinct summer and winter bacterial communities in the active layer of Svalbard permafrost revealed by DNA-and RNA-based analyses. *Frontiers in Microbiology* 6:399. https://doi.org/10.3389/fmicb.2015.00399

Singh, P., Singh, S. M., Singh, R. N., Naik, S., Roy, U., Srivastava, A., and Bölter, M. 2017. Bacterial communities in ancient permafrost profiles of Svalbard, Arctic. *Journal of Basic Microbiology* 57(12):1018–1036. https://doi.org/10.1002/jobm.201700061

Stackebrandt, E., Koch, C., Gvozdiak, O., and Schumann, P. 1995. Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. *International Journal of Systematic and Evolutionary Microbiology* 45(4):682–692. https://doi.org/10.1099/00207713-45-4-682

Svenning, M. M., Røsnes, K., Lund, L., and Junttila, O. 2001. Vegetative growth and freezing tolerance of white clover (*Trifolium repens* L.) genotypes from Svalbard. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science* 51(1):10–16. https://doi.org/10.1080/090647101317187843

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28(10):2731–2739. https://doi.org/10.1093/molbev/msr121

Tikhonovich, I. A. and Provorov, N. A. 2009. *Symbioses of plants and microorganisms: molecular genetics of agrosystems of the future*. St. Petersburg University Press, Saint Petersburg. 210 p. (In Russian)

Trubitsyn, V. E., Rhyzhmanova, Y. V., Zaharuk, A. G., Oshurkova, V. I., Laurinavichius, K. S., Spirina, E. V., Shcherbakova, V. A., and Rivkina, E. M. 2019. Permafrost sediments, West Spitsbergen, microbial communities, psychrophilic microorganisms, anaerobic prokaryotes. *Earth* 23(6):31–38. https://doi.org/10.21782/EC2541-9994-2019-6(31-38)

Vincent, J. M. 1970. A manual for the practical study of root nodule bacteria; pp. 73–97 in IBP Handbook. Oxford and Edinburgh: Blackwell Scientific Publications.

Vinuesa, P., León-Barrios, M., Silva, C., Willems, A., Jarabo-Lorenzo, A., Pérez-Galdona, R., Werner, D., and Martínez-Romero, E. 2005. *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (*Papilionoideae*: *Genisteae*) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta. *International Journal of Systematic and Evolutionary Microbiology* 55(2):569–575. https://doi.org/10.1099/ijs.0.63292-0

Vishnivetskaya, T., Kathariou, S., McGrath, J., Gilichinsky, D., and Tiedje, J. M. 2000. Low-temperature recovery strategies for the isolation of bacteria from ancient permafrost sediments. *Extremophiles* 4(3):165–173. https://doi.org/10.1007/s007920070031

Wei, G., Chen, W., Young, J. P. W., and Bontemps, C. 2009. A new clade of *Mesorhizobium* nodulating *Alhagi sparsifolia*. *Systematic and Applied Microbiology* 32(1):8–16. https://doi.org/10.1016/j.syapm.2008.11.003

Wilhelm, R. C., Niederberger, T. D., Greer, C., and Whyte, L. G. 2011. Microbial diversity of active layer and permafrost in an acidic wetland from the Canadian High Arctic. *Canadian Journal of Microbiology* 57(4):303–315. https://doi.org/10.1139/w11-004

Willems, A. 2014. The family Phyllobacteriaceae; pp. 355–418 in Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., Thompson, F. (Eds). The Prokaryotes: Alphaproteobacteria and Betaproteobacteria 4th edn. Springer: Berlin, Germany. https://doi.org/10.1007/978-3-642-30197-1_298

Zheng, W. T., Li Jr, Y., Wang, R., Sui, X. H., Zhang, X. X., Zhang, J. J., Wang, E. T., and Chen, W. X. 2013. *Mesorhizobium qingshengii* sp. nov., isolated from effective nodules of *Astragalus sinicus*. *International Journal of Systematic and Evolutionary Microbiology* 63(6):2002–2007. https://doi.org/10.1099/ijs.0.044362-0