Taxonomically Different Co-Microsymbionts of a Relict Legume, *Oxytropis popoviana*, Have Complementary Sets of Symbiotic Genes and Together Increase the Efficiency of Plant Nodulation

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Ten rhizobial strains were isolated from root nodules of a relict legume *Oxytropis popoviana* Peschkova. For identification of the isolates, sequencing of *rrs*, the internal transcribed spacer region, and housekeeping genes *recA*, *glnII*, and *rpoB* was used. Nine fast-growing isolates were *Mesorhizobium*-related; eight strains were identified as *M. japonicum* and one isolate belonged to *M. kowhaii*. The only slow-growing isolate was identified as a *Bradyrhizobium* sp. Two strains, *M. japonicum* Opo-242 and *Bradyrhizobium* sp. strain Opo-243, were isolated from the same nodule. Symbiotic genes of these isolates were searched throughout the whole-genome sequences. The common *nodABC* genes and other symbiotic genes required for plant nodulation and nitrogen fixation were present in the isolate Opo-242. Strain Opo-243 did not contain the principal *nod*, *nif*, and *fix* genes; however, five genes (*nodP*, *nodQ*, *nifL*, *nolK*, and *noeL*) affecting the specificity of plant-rhizobia interactions but absent in isolate Opo-242 were detected. Strain Opo-243 could not induce nodules but significantly accelerated the root nodule formation after coinoculation with isolate Opo-242. Thus, we demonstrated that taxonomically different strains of the archaic symbiotic system can be co-microsymbionts infecting the same nodule and promoting the nodulation process due to complementary sets of symbiotic genes.

Symbiosis has been recognized as a fundamental process in the coevolution of species that is accompanied by an increase in the specificity of symbiotic interactions (Provorov and Vorobyev 2011; Tikhonovich and Provorov 2009). But the genetic basis for such specificity is still poorly understood because of the lack of adequate experimental models. In this respect, the promising models for studying the evolution of plant-microbial relationships are symbiotic systems of relict legume species.

Information about strains isolated from root nodules of the relict legume *Vavilovia formosa*, which is the closest relative of the extinct ancestor of the tribe *Fabeae* (Kenicer et al. 2009; Sinjushin et al. 2009), was recently published (Safronova et al. 2014). It was shown that *V. formosa* had a wide range of nodulating strains belonging to different families, such as *Rhizobiaceae*, *Phyllobacteriaceae*, and *Bradyrhizobiaceae* (Safronova et al. 2014; 2015b) and a new species, *Bosea vaviloviae*, was described (Safronova et al. 2015a). Then, a collection of taxonomically different microsymbionts of legumes *Oxytropis triphylla*, *O. popoviana*, *O. tragacanthoides*, *Hedysarum zundukii*, *Astragalus chorinensis*, and *Glycyrrhiza uralensis* belonging to the Miocene-Pliocene relics (Malyshev 2006; Turuta et al. 2015) and originated from the Baikal Lake region was created (Safronova et al. 2017a).

The impact of natural rhizobial diversity and resulting multipartite interactions between symbionts on plant growth is currently poorly understood. The influence of inoculation with single- and multi-strains on the productivity of two Australian *Acacia* species (*A. salicina* and *A. stenophylla*) interacting with highly diverse communities of rhizobia was recently estimated (Barrett et al. 2015). It was shown that the multi-strain treatments had a negative effect on plant growth and were not able to enhance plant productivity through mechanisms of either complementarity or selection. As a result, the ‘antagonism hypothesis’ was supported, according to which the presence of multiple rhizobial strains in the rhizosphere leads to strong competition between strains with different levels of nitrogen-fixing activity, the formation of low- or ineffective symbiosis, and decrease in plant productivity. However, the data describing multipartite interactions between the plant and different rhizobial co-microsymbionts that jointly participate in the formation of the same nodule have not yet been published.

Earlier, four pairs of strains with different growth rates were isolated in approximately equal proportion from root nodules of *O. triphylla* (Safronova et al. 2017b). Identification of these

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All sequences have been deposited to the National Center for Biotechnology Information GenBank database, the whole-genome sequences under accession numbers MZXX000000000, for isolate *M. japonicum* Opo-242, and MZXW000000000, for the isolate *Bradyrhizobium* sp. strain Opo-243 and *rrs* sequences under accession numbers MF661781 to MF661790.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures are published online.

strains by 16S ribosomal RNA (rRNA) gene sequencing showed that one strain of each pair belonged to the fast-growing bacteria of genus *Phyllobacterium* but the other strain belonged to the slow-growing bacteria of genus *Bosea* (Safronova et al. 2017b). Further study of these strains was postponed because of the difficulties of cultivating *O. triphylla* plants that grow in an extremely arid region, in the gnotobiotic conditions used.

The aim of this work was to study rhizobial strains isolated from the root nodules of another relict legume, *Oxytropis popoviana*, originating from the restricted area of Buryatia (the Baikal Lake region). Special attention was paid to the presence of different strains in the same nodule and analysis of symbiotic gene complementation by the comparison of whole genome sequences and nodulation assay.

RESULTS AND DISCUSSION

Isolation of rhizobial strains in a pot experiment.

Soil analysis showed that samples used for a pot experiment belonged to podzols type, in accordance with the World Reference Base for Soil Resources, and were characterized by low values of available P (5.5 mg $P_2O_5/100$ g), total C (1.9%), and total N (0.19%). Such poor soils should favor formation of rhizobia-legume symbiosis (Tikhonovich and Provorov 2007).

Figures 1A and 1B show *O. popoviana* plant and root nodules obtained at the end of the pot experiment. A total of 35 nodules were collected. Most nodules were pink, suggesting the presence of leghemoglobin and nitrogen-fixing activity. Their shape corresponded to the elongate-indeterminate type of nodules (Sprent et al. 2013), usually with one branch or, rarely, two branches (Fig. 1B). Nodule size varied from 1 to 5 mm. Nine fast-growing rhizobial strains (Opo-225, Opo-228, Opo-235, Opo-239, Opo-240, Opo-242, Opo-245, Opo-249, and Opo-324) as well as one slow-growing strain, Opo-243, were isolated from eight nodules. Colonies of fast- and slow-growing strains appeared on yeast extract mannitol agar (Vincent 1970) supplemented with succinate at 5 and 7 days, respectively. Two strains, Opo-242 and Opo-243, differing in growth rate, were isolated from the same nodule and presented approximately in equal proportion in the amount of 10^4 CFU.

Identification of isolates by the analysis of 16S rDNA, internal transcribed spacer (ITS) region, and housekeeping genes *recA*, *glnII*, and *rpoB*.

The preliminary identification of the isolates by sequencing of the 16S rRNA gene (Table 1; Fig. 2) showed that all fast-growing strains belonged to genus *Mesorhizobium*. The isolates

Fig. 1. *Oxytropis popoviana* plant and root nodules **A** and **B**, in the pot experiment and **C** through **H**, in the gnotobiotic nodulation assay after 2, 4, and 6 weeks of plant vegetation. **C**, **E**, and **G**, Inoculation with the isolate Opo-242 and **D**, **F**, and **H**, coinoculation with the isolates Opo-242 and Opo-243, after 2 (**C** and **D**), 4 (**E** and **F**), and 6 weeks (**G** and **H**). The single inoculation with the strain Opo-243 did not produce nodules.

Opo-225, Opo-228, Opo-235, Opo-239, Opo-240, Opo-242, Opo-245, and Opo-249 formed a separate cluster at 91% bootstrap support with the type strains *M. japonicum* MAFF 303099^T and *M. erdmanii* USDA 3471^T (Fig. 2) and had 99.61 to 100% and 99.68 to 100% *rrs* similarity with these type strains, respectively (Table 1). However, the *rrs* sequence of type strain

M. erdmanii (accession number NR135857.1) was characterized by the 35 nucleotides deletion 5'-GGCAGCGAGACCGCGAGGTCGAGCTAATCTCCAAA-3' from the 1190 position. Thus, we identified the fast-growing isolates mentioned above as a *M. japonicum* species, which was recently described for two *Lotus*-nodulating strains (Martínez-Hidalgo et al.

Table 1. *Rrs* sequence similarities between *Mesorhizobium*-related isolates from *Oxytropis popoviana* and the reference type strains

Isolate	<i>rrs</i> sequence similarities (%) ^a				
	<i>M. erdmanii</i> USDA 3471 ^T			<i>M. opportunistum</i> WSM2075 ^T	<i>M. kowhaii</i> ICMP 19512 ^T
	<i>M. japonicum</i> MAFF 303099 ^T	Without deletion	With deletion		
Opo-225	99.86	99.85	97.35	99.64	97.85
Opo-228	100	100	97.10	99.79	98.00
Opo-235	100	100	97.49	99.78	98.00
Opo-239	99.61	99.68	97.21	99.38	97.59
Opo-240	99.85	99.85	97.31	99.64	97.91
Opo-242	100	100	97.17	99.80	98.00
Opo-245	99.71	99.71	97.48	99.50	97.77
Opo-249	100	100	97.30	99.77	97.91
Opo-324	98.11	98.06	95.56	98.18	100

^a The nucleotide deletion is 5'-GGCAGCGAGACCGCGAGGTCGAGCTAATCTCCAAA-3'.

Fig. 2. Phylogenetic tree generated by the neighbor-joining method, using partial 16S rRNA gene sequences (1,400 nt) of the *Mesorhizobium*-related isolates from *Oxytropis popoviana* (in bold) and closely related species. Bootstrap values more than 50% are given. Type strains are indicated by the letter T.

2016). The isolate Opo-324 had 100% *rrs* similarity with the type strain *M. kowhaii* ICMP 19512^T (Table 1) isolated from the root nodule of the *Sophora microphylla* plant in New Zealand (De Meyer et al. 2016).

A *Rrs* phylogenetic tree including the only slow-growing isolate, Opo-243 (Fig. 3), showed that this isolate belonged to genus *Bradyrhizobium* and was most closely related to the type strain *B. diazoefficiens* USDA 110^T, which was isolated from *Glycine max* and initially described as *B. japonicum* (Delamuta et al. 2013). However, the low bootstrap value (52%) indicated that these two strains were quite distinct. *Rrs* similarity between isolate Opo-243 and the type strain of *B. diazoefficiens* was 99.48% (Table 2). To specify the taxonomic position of isolate Opo-243, the ITS region as well as the housekeeping genes *recA*, *glnII*, and *rpoB* obtained from the whole-genome sequence were analyzed (Figs. 4 and 5; Supplementary Figs. S1, S2, and S3). Unlike the *rrs* dendrogram, isolate Opo-243 formed the separate ITS cluster at 98% bootstrap support with type strains *B. japonicum* USDA 6^T, *B. canariense* BTA-1^T, *B. betae* LMG

21987^T, *B. rifense* CTAW71^T, and *B. cytisi* CTAW11^T (Fig. 4). The concatenated *recA*, *glnII*, and *rpoB* gene tree showed that the isolate Opo-243 was most closely related to the type strains *B. canariense* BTA-1^T, *B. rifense* CTAW71^T, and *B. cytisi* CTAW11^T, yet distinct from the type strains of species *B. diazoefficiens*, *B. japonicum*, and *B. betae* (Fig. 5). ITS, *recA*, *glnII*, and *rpoB* gene similarities between the isolate Opo-243 and all type strains mentioned above are presented in Table 2. Based on the obtained data, we identified isolate Opo-243 as '*Bradyrhizobium* sp.' because of the difficulty in assigning its species.

Analysis of symbiotic genes of the isolates *M. japonicum* Opo-242 and *Bradyrhizobium* sp. strain Opo-243 by whole-genome sequencing.

Symbiotic genes of the isolates Opo-242 and Opo-243 that were together present in the same nodule of *O. popoviana* were searched throughout the whole-genome sequences obtained. The common *nodABC* genes necessary for legume nodulation (Wais et al. 2002) and some other *nod* and *noe* genes participating

Fig. 3. Phylogenetic tree generated by the neighbor-joining method, using partial 16S rRNA gene sequences (1,400 nt) of the *Bradyrhizobium*-related isolate Opo-243 from *Oxytropis popoviana* (in bold) and closely related species. Bootstrap values more than 50% are given. Type strains are indicated by the letter T.

Table 2. Sequence similarities between *Bradyrhizobium* sp. strain Opo-243 isolated from *Oxytropis popoviana* and the type strains of most closely related species

Locus	Sequence similarities (%)					
	<i>B. diazoefficiens</i> USDA 110 ^T	<i>B. japonicum</i> USDA 6 ^T	<i>B. canariense</i> BTA-1 ^T	<i>B. betae</i> LMG 21987 ^T	<i>B. rifense</i> CTAW71 ^T	<i>B. cytisi</i> CTAW11 ^T
<i>rrs</i>	99.48	99.15	99.34	99.75	99.53	99.43
ITS	91.96	91.14	91.75	95.17	95.69	94.19
<i>recA</i>	95.14	94.56	94.52	94.69	94.00	94.84
<i>glnII</i>	95.32	96.23	94.11	94.19	95.91	95.91
<i>rpoB</i>	95.83	95.69	93.51	95.83	94.72	96.38

in biosynthesis, modification, and transport of rhizobial Nod factors (NFs) (Aoki et al. 2013; Lopez-Lara et al. 2001a and b; Perret et al. 2000) were present in isolate Opo-242 (Table 3). Isolate Opo-242 also contained *nif* and *fix* genes required for nitrogen fixation, *nifHDK* and *nifENB* genes encoding structural and catalytic components of the nitrogenase complex (Dos Santos et al. 2012), as well as *fixABCX*, *fixNOPQ*, and *fixGHIS* genes participating in electron transfer to nitrogenase

and symbiotically essential oxidase complex (Edgren and Nordlund 2004; Fischer 1994). Strain Opo-243 did not contain the principal *nod*, *nif*, and *fix* genes, however, some other symbiotic genes were detected: *nodNTGPQ*, *nifLS*, *fixNOPQGHISJKL*, *nolK*, and *noeL* (Table 3). All symbiotic genes of isolate Opo-242 were most similar to different representatives of the genus *Mesorhizobium* (*M. japonicum*, *M. loti*, *M. amorpha*, *M. huakuii*, *M. ciceri*), while symbiotic genes

Fig. 4. Phylogenetic tree generated by the neighbor-joining method, using internal transcribed spacer region sequences of the isolate Opo-243 (in bold) and closely related species. Bootstrap values more than 50% are given. Type strains are indicated by T.

Fig. 5. Phylogenetic tree of the isolate Opo-243 (in bold) and phylogenetically related species, based on concatenated *recA*, *glnII*, and *rpoB* gene sequences generated by the neighbor-joining method. Bootstrap values more than 50% are given. Type species are indicated by T.

of isolate Opo-243 were closest to *B. japonicum* and *B. diazoefficiens* strains. Levels of similarity between the symbiotic genes revealed and homolog sequences presented in the GenBank database ranged from 80 to 100% (data not shown). It was noted that isolate Opo-243 contained at least five genes (*nodP*, *nodQ*, *nifL*, *nolK*, and *noeL*) that were absent in isolate Opo-242 (Table 3). Four of them affect the specificity of plant-rhizobium interactions by NF modification; *nolK* and *noeL* genes are responsible for NF fucosylation (Kaneko et al. 2011; Mergaert et al. 1997) and *nodP* and *nodQ* genes are involved in their sulfation (Roche et al. 1991). It was shown that mutations in these four genes lead to an inability of rhizobia to trigger host responses or significantly decrease competitiveness to nodulate legume plants (Lamrabet et al. 1999; Snoeck et al. 2003; Wais et al. 2002). For example, the rate of nodule formation on *Lotus japonicus* roots exhibited by the *nodP*, *nodQ* mutant of strain *M. loti* NZP-2235 was decreased by approximately 50% compared with the wild type (Townsend et al. 2006).

Symbiotic phenotype of the isolates *M. japonicum* Opo-242 and *Bradyrhizobium* sp. strain Opo-243.

The symbiotic phenotype of isolates Opo-242 and Opo-243 was studied in a gnotobiotic plant nodulation assay with *O. popoviana* plants, using treatments of mono- and coinoculation. After 42 days of plant cultivation, the negative (uninoculated) control and plants inoculated with Opo-243 did not show any nodules, which was expected due to the absence of common *nodABC* genes in this strain. A simultaneous inoculation with strains Opo-242 and Opo-243 accelerated root nodule formation as compared with inoculation with a single strain, Opo-242. The first nodules appeared after 2 weeks, while isolate Opo-242 alone induced nodule formation only after 3 weeks (Fig. 6). The number of nodules significantly increased in the course of our experiment and stabilized after 4 weeks, in the case of coinoculation, and after 5 weeks when plants were inoculated with single Opo-242. It can be assumed that the presence of *nolK*, *noeL*, *nodP* and *nodQ* genes in strain Opo-243 were involved in the modification of NFs and triggered the nodulation process. Apparently, strain Opo-242 synthesizes and excretes NFs, which are then modified outside the cells by another strain.

Pictures of nodules obtained in the gnotobiotic assay 2, 4, and 6 weeks after inoculation are presented in the Figure 1C through H. The shape of nodules was round in the early stages (Fig. 1D and E) but, later on, they were more elongated with predominantly one branch (Fig. 1F through H). An obvious difference in the size of nodules was found. Nodules formed by strain Opo-242 were about half as large as the nodules that appeared after inoculation with a mixture of strains Opo-242 and Opo-243. This phenomenon can be associated with an earlier formation of nodules in the coinoculation treatment.

Dry shoot and root biomass as well as the acetylene reduction activity measurements are given in Table 4. Compared with the uninoculated control and inoculation with single non-nodulating strain Opo-243, strain Opo-242 significantly increased root biomass and acetylene reduction activity, while, after coinoculation with strains Opo-242 and Opo-243, all three

parameters were statistically greater. There was also a tendency for an increase in dry shoot and root biomass (by 14 and 20%, respectively) of plants inoculated with the strain mixture as compared with the strain Opo-242, however the differences were insignificant because of variation of the data. Even though the acetylene reduction measured at the end of experiments was similar in both variants with nodules (Opo-242 and Opo-242 + Opo-243), earlier nodule formation in the coinoculation treatment had some positive effect on plant growth.

The strains re-isolated from nodules, as estimated by 16S rDNA sequencing, corresponded to those used for inoculation. The ratio of strain Opo-242 to Opo-243 in nodules was approximately 20 to 1. It is yet unknown how isolate Opo-243 penetrated into nodules. However, it was shown that some *Bradyrhizobium*-related strains having no common *nodABC* genes infected tropical legume plants *Aeschynomene* spp. through epidermal fissures via the ‘crack-entry’ process (Giraud et al. 2007; Mornico et al. 2012; Okubo et al. 2013).

Thus, we demonstrated for the first time that the strains isolated from the same root nodule of the relict legume *O. popoviana* are co-microsymbionts having complementary sets of symbiotic genes and their simultaneous presence on roots increases the efficiency of host plant nodulation. The results obtained here are in agreement with previous reports (Safronova et al. 2014; 2015a and b; 2017b), suggesting that symbiotic systems of relict plants are archaic and can be formed with different rhizobial strains belonging to various taxonomic groups. It may be proposed that the phenomenon of low host specificity in the relict rhizobia-legume symbiotic systems allows infection with various bacteria related to plant-microbe symbiotic interactions and creates prerequisites for an effective exchange of genetic material between microsymbionts jointly inhabiting the root nodules. This hypothesis helps to explain evolution of rhizobia through the recruitment in one strain of a complete set of genes that are mandatory or promising for symbiosis but distributed over different bacterial taxa.

MATERIALS AND METHODS

Isolation of rhizobial strains.

Soil samples and seeds of *O. popoviana* were collected in the Baikal Lake region (51°22'37.67"N, 106°28'24.06"E, Buryatia). The content of available P was determined in 0.2 N HCl soil extract (Watanabe and Olsen 1965), using a Smart Spec Plus spectrophotometer (Bio-Rad). The content of total N was determined by the Kjeldahl method, using a Kjeltac Auto 1030 analyzer (Tecator) according to the manufacturer's instructions. The total C was determined by the Tjuri's oxidation method (Arinushkina 1970). Seeds were surface-sterilized and scarified by treatment with 0.1% HgCl₂ for 10 min and, then, 5% NaOCl for 8 min, were rinsed carefully with sterile tap water, and were germinated on filter paper in petri dishes at 25°C in the dark for 4 days. Seedlings were transferred to three sterile plastic pots (one pot with five seeds) containing 250 g of soil. Plants were cultivated for 60 days in the growth chamber, with 50% relative humidity and four-level illumination/temperatures mode: night (dark, 18°C, 8 h), morning (200 μmol m⁻² s⁻¹, 20°C, 2 h), day

Table 3. Presence of symbiotic genes in the isolates *Mesorhizobium japonicum* Opo-242 and *Bradyrhizobium* sp. strain Opo-243^a

Isolate	Genes				
	<i>nod</i>	<i>nif</i>	<i>fix</i>	<i>nol</i>	<i>noe</i>
Opo-242	ABCDIJFEGNLMZT	HDKENABQTZXWSU	ABCXNOPQGHISJKL	–	K
Opo-243	NTG[PQ]	[LJS]	NOPQGHISJKL	[K]	[L]

^a Genes present in isolate Opo-243 and complementary to symbiotic genes of isolate Opo-242 appear in brackets.

(400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 23°C, 12 h), evening (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 20°C, 2 h). Illumination was performed by L 36W/77 FLUORA lamps (Osram). Then, roots of individual plants were removed from soil and were washed with tap water. Pictures of plants and nodules were performed by the stereo microscope Stemi 508 (Zeiss) and the microscope color camera AxioCam ERc 5 s (Zeiss). Strains of nodule bacteria were isolated from the obtained nodules by the standard method described by Novikova and Safronova (1992), using modified yeast extract mannitol agar (Vincent 1970) supplemented with 0.5% succinate (Safronova et al. 2015a). All strains were deposited in the Russian Collection of Agricultural Microorganisms (WDCM 966) and were stored at -80°C in the automated Tube Store (Liconic Instruments) as described previously (Safronova and Tikhonovich 2012).

Whole-genome sequencing of isolates Opo-242 and Opo-243.

Genomic DNA was extracted using the Genomic DNA purification kit (Thermo Scientific) according to the recommendation of the manufacturer. DNA was fragmented by the focused ultrasonicator Covaris S2 (Covaris). Fragment DNA libraries were prepared with NEBNext DNA library kit (NEB), and their quality was estimated with a High Sensitivity DNA kit on Bioanalyzer 2100 (Agilent). The DNA amount was estimated with a dsDNA high sensitivity kit on Qubit 1.0 (Invitrogen). Genome sequencing was performed on a MiSeq genomic sequencer (Illumina) by standard protocol with a MiSeq reagent kit, 600 cycles (Illumina) at the Siberian Branch of The Russian Academy of Sciences Genomics Core Facility (Institute of Chemical Biology and Fundamental Medicine). Genome was assembled de novo, using SPAdes 3.5.0 software (Bankevich et al. 2012). Quality control was performed by QUAST 3.0 (Gurevich et al. 2013). Search for genes in the assembled contigs was performed using the RAST annotation service (Overbeek et al. 2014). Search for homologs of the 16S rRNA gene, ITS region, housekeeping genes *recA*, *glnII*, and *rpoB*, as well as symbiotic genes in annotated genomes was performed, with CLC Genomics Workbench 7.5.1 software, using local BLASTn and tBLASTx.

Identification of isolates by analysis of the 16S rDNA, ITS region, and housekeeping genes *recA*, *glnII*, and *rpoB*.

For identification of all isolates, the following PCR primers were used: fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-CTTAAGGAGGTGATCCAGCC-3'), for an approximately 1,400-bp segment of the 16S rRNA gene (Weisburg et al. 1991). PCR was performed in 25- μl reaction mixtures containing 150 μM dNTPs (Promega), 5 pmol of each primer, 1 U of *Taq* polymerase (Helicon), and 50 to 100 ng of purified template DNA. PCR conditions for 16S rDNA amplification were as follows: initial denaturation at 95°C for 3 min 30 s, 35 cycles of denaturation at 94°C for 1 min 10 s, annealing at 56°C for 40 s, and extension at 72°C for 2 min 10 s, final extension at 72°C for 6 min 10 s. Electrophoresis was carried out with 1% agarose gel (Invitrogen) in Tris-acetate-EDTA. A 100-bp GeneRuler and Lambda DNA/*HindIII* markers (Fermentas) were used for sizing and approximate quantification of DNA fragments. Purification of the PCR products was usually performed by using PureLink Quick kit (Invitrogen) according to the manufacturer's guidance. The direct sequencing of PCR products was performed on an ABI PRISM 3500xl genetic analyzer (Applied Biosystems) in the Core Centrum 'Genomic Technologies, Proteomics and Cell Biology' at the All-Russia Research Institute for Agricultural Microbiology.

Additionally, for identification of slow-growing isolate Opo-243, the ITS region as well as housekeeping genes *recA*, *glnII*,

and *rpoB*, obtained from the whole-genome sequence, were analyzed. The sequences were compared with related sequences of the type strains available in the GenBank database, using BLAST analysis (Basic logical alignment search tool) at the National Center for Biotechnology Information. The *Rrs* dendrogram and the phylogenetic tree based on the concatenated *recA*, *glnII*, and *rpoB* sequences were constructed, using the neighbor-joining method, in the MEGA 5.0 software package (Tamura et al. 2011). The evolutionary distances were computed using the maximum composite likelihood method. Bootstrap analysis with 1,000 replicates was performed to estimate the support of clusters.

Plant nodulation assay.

Seeds were surface-sterilized, scarified, and germinated as described above. The uniformly germinated seedlings were transferred to polypropylene pots (Duchefa) containing 20 g of vermiculite (15 pots with two seeds per each treatment). Each pot was supplemented with 40 ml of the nutrient solution (grams per liter): K_2HPO_4 1.0, KH_2PO_4 0.25, MgSO_4 1.0, $\text{Ca}_3(\text{PO}_4)_2$ 0.2, FeSO_4 0.02, H_3BO_3 0.005, $(\text{NH}_4)_2\text{MoO}_4$ 0.005, $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.005, MnSO_4 0.002. Pots were inoculated with isolates either Opo-242, Opo-243, or their combination in the amount of 10^6 cells per pot. The uninoculated plants were used as negative control. Plants were cultivated for 42 days in the growth chamber, as described above. The number of nodules on six plants per treatment was counted 2, 3, 4, 5, and 6 weeks after planting. Nodules were photographed by the stereo microscope Stemi 508 (Zeiss). At the end of the experiments, the plants were dried at room temperature and the biomass of shoots and

Fig. 6. Nodule formation on *Oxytropis popoviana* roots after mono- and coinoculation with isolates Opo-242 and Opo-243 in the gnotobiotic plant nodulation assays. Vertical bars represent standard error. Asterisks indicate probability at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) (Student's *t*-test). The data of two experiments with six replications each is shown.

Table 4. Effects of mono- and coinoculation of *Oxytropis popoviana* plants with isolates *Mesorhizobium japonicum* Opo-242 and *Bradyrhizobium* sp. strain Opo-243 in the gnotobiotic plant nodulation assays^a

Treatment	Shoot biomass (mg DW plant ⁻¹)	Root biomass (mg DW plant ⁻¹)	Acetylene reduction activity (nmol C ₂ H ₄ plant ⁻¹ h ⁻¹)
Uninoculated control	2.2 ± 0.3 a	0.7 ± 0.2 a	0.1 ± 0.02 a
Opo-243	2.3 ± 0.2 a	0.6 ± 0.1 a	0.1 ± 0.01 a
Opo-242	2.8 ± 0.2 ab	1.0 ± 0.1 b	2.1 ± 0.4 b
Opo-242 + Opo-243	3.2 ± 0.3 b	1.2 ± 0.2 b	2.3 ± 0.3 b

^a The strain Opo-243 did not produce nodules. The data is shown as mean ± standard error of one representative experiment ($n = 12$). Different letters show significant differences between treatments (least significant difference test, $P < 0.05$). DW = dry weight.

roots was weighed. Nitrogen fixation of nodules was measured by the acetylene-reduction method (Turner and Gibson 1980), using gas chromatograph GC-2014 (Shimadzu), at the end of the experiment. The experiment on nodulation assay was performed twice. Three nodules from each treatment were used for re-isolation of microsymbionts and their identification was performed by 16S rDNA sequencing as described above. The data were processed by the standard method of variance analysis, using the software STATISTICA version 10 (StatSoft Inc.). Fisher's least significant differences test and Student's *t*-test were used to evaluate differences between means.

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