Genetic diversity of rhizobial strains isolated from the relict legumes *Gueldenstaedtia monophylla* and *G. verna* growing in the republics of Altai and Buryatia (Russia)

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Abstract

For the first time, bacteria were isolated and identified from the root nodules of relict legumes *Gueldenstaedtia monophylla* Fisch. and *G. verna* (Georgi) Boriss. growing in the republics of Altai and Buryatia. The taxonomic position of the 29 obtained isolates was determined by sequencing the 16S rRNA gene (*rrs*). Showing a significant biodiversity, the isolates from *G. monophylla* and *G. verna* belonged to five genera of the order *Rhizobiales*: *Mesorhizobium* and *Phyllobacterium* (family *Phyllobacteriaceae*), *Rhizobium* (family *Rhizobiaceae*), *Bosea* (family *Boseaceae*), *Bradyrhizobium* (family *Bradyrhizobiaceae*). Three isolates which belonged to the species *Bradyrhizobium valentinum* and *Rhizobium alamii* showed 100 % of *rrs*-similarity with the type strains *B. valentinum* LmjM3^T and *R. alamii* GBV016^T, respectively. Six isolates of the genera *Bosea* and *Rhizobium* had a low level of *rrs*-similarity with the closest type strains (less than 99.5 %), which indicates that they may be assigned to new species. The data obtained can be used to itemise taxonomy within the order *Rhizobiales*, as well as to reveal the mechanisms of the formation of specific plant-microbial relationships during the evolution of symbiosis by studying the intermediate link between the extinct and modern rhizobia-legume symbiotic systems.

Keywords: relict legume plants, genus *Gueldenstaedtia,* root nodule bacteria, 16S rRNA genes

Introduction

Symbiosis has been recognized as a fundamental process in the co-evolution of species, which is accompanied by an increase in the specificity of symbiotic interactions (Tikhonovich and Provorov, 2009; Provorov and Vorobyev, 2011). But the genetic basis for such specificity is still poorly understood because of the lack of adequate experimental models. Relict legume plants are promising models for studying the evolution of symbiosis between plants and rhizobia since they possess intermediate links between ancient (primitive low specific) and modern (highly specific) features of symbiotic plant-microbe systems (Safronova et al., 2018a).

Previously, from the Miocene-Pliocene relict leguminous species *Vavilovia formosa* (Caucasus, Russia); *Oxytropis triphylla*, *O. popoviana*, *O. tragacanthoides*, *Hedysarum zundukii*, *Astragalus chorinensis*, and *Glycyrrhiza uralensis* (Baikal Lake region, Russia); *Caragana jubata* (Mongolia) various bacterial strains were isolated (Safronova et al., 2014, 2015, a, b, 2017, a, b, 2019; Sazanova et al., 2019). It was shown that these isolates belonged to different rhizobial families (*Rhizobiaceae*, *Phyllobacteriaceae*, and *Bradyrhizobiaceae*), and new species *Bosea vavilo-*

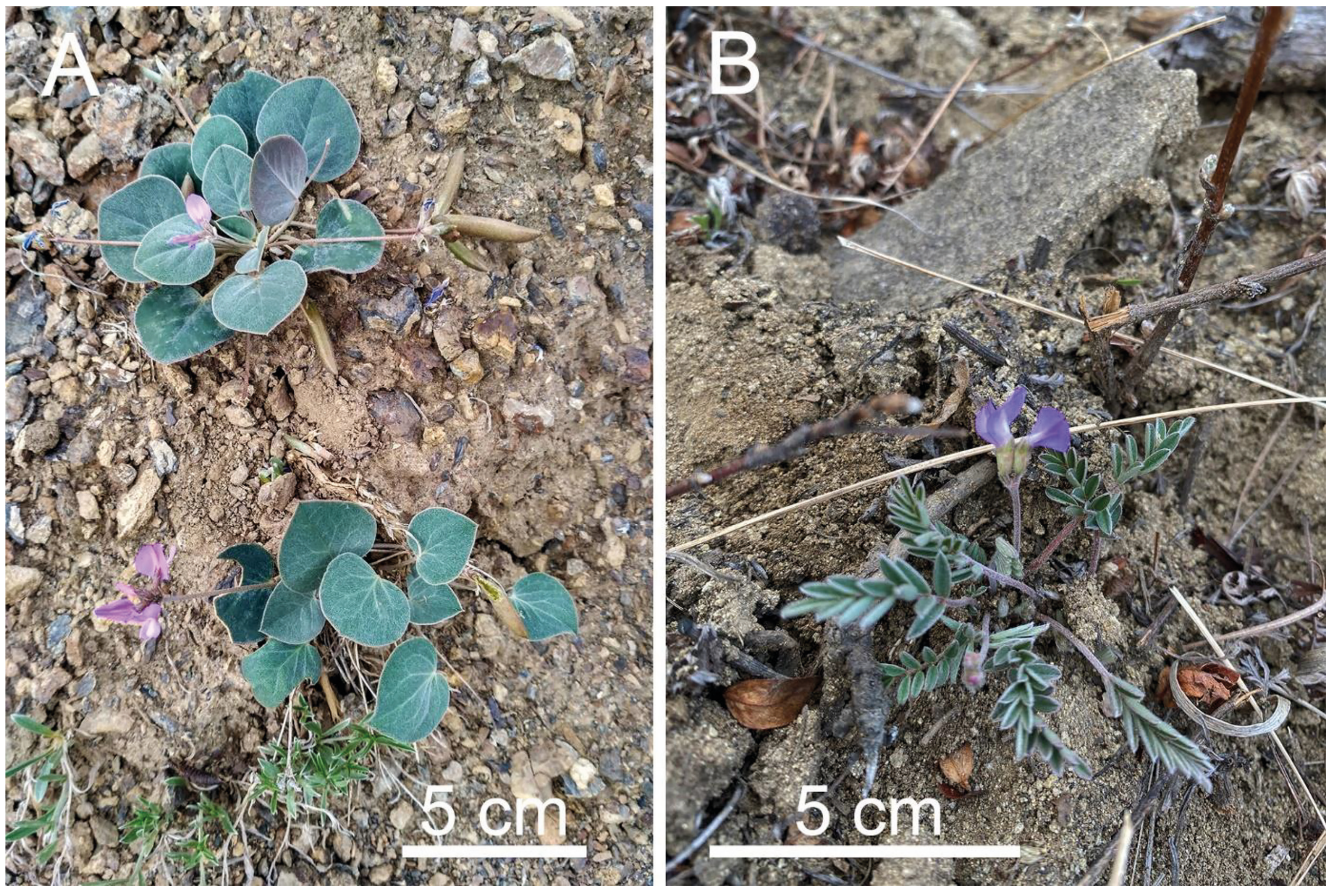

Fig. 1. Appearance of plants *Gueldenstaedtia monophyla* (A, Photo by A. A. Belimov) and *Gueldenstaedtia verna* (B, Photo by A. V. Verkhozina).

viae, *B. caraganae* and *Phyllobacterium zundukense* were described (Safronova et al., 2015a, 2018b; Sazanova et al., 2019).

Gueldenstaedtia is a small genus from the tribe *Caraganeae* (family *Fabaceae*) with unclear taxonomic status (Duan et al., 2015). This genus includes five species growing within eastern Asia (Zhu, 2004; Xie, Meng, Sun and Nie, 2016). Only two species (*G. monophylla* and *G. verna*) are represented in the flora of Russia and belong to the Miocene-Pliocene relict plants growing in the south of Siberia and the Far East (Polozhiy, 2001; Pyak, 2003; Selyutina, Konichenko and Dorogina, 2017; Stupnikova, 2018).

G. monophylla is a rare and unusual plant included in the Red Book of Russia (2008) and grows in arid habitats with stony, gravelly or sandy substrates (Selyutina, Konichenko and Karnauhova, 2014). This species is distinguished from the congeners by its solitary leaf (Fig. 1A). The species is represented by small, isolated populations and is found in the mountain-steppe belt in the Central and less often Southeast Altai, as well as in Tuva (Namzalov, 1986; Pyak, 2003; Zhu, 2004).

G. verna is a rare East Asian species with a disjunctive area, mostly located in China (Zhu, 2004). Within Russia, it is found in Western and Eastern Siberia, and in the south of the Far East (Fig. 1B). The species is listed in the Red Data Books of various regions in Russia. *G. verna* grows in xerophytic habitats: stony steppe slopes, edges of oak and pine forests, sandy sediments in river floodplains (Stupnikova, 2018).

There is no information in the literature about rhizobia inhabiting nodules of plants belonging to genus *Gueldenstaedtia*. We propose that these relict legume plants have no established species-specific composition of nodule bacteria and their nodules may contain taxonomically different bacteria. Symbiotic systems formed by relict leguminous plants will make it possible to study legume-rhizobia relationships at the early stages of evolution, as well as to identify minor microbial components that can be used for increasing symbiotic efficiency in modern crops. It was shown that microsymbionts of relict plants can help in the creation of the most effective biofertilizers (Datta, Singh, Kumar and Kumar, 2015).

The aim of this work was to create a collection of strains of relict leguminous plants *G. monophylla* and *G. verna* growing in the republics of Altai and Buryatia (Russia). Here we studied the genetic diversity of bacteria inhabiting the nodules of these plant species and belonging to the *Rhizobiales* order by sequencing the 16S rRNA gene.

Materials and methods

Isolation of pure cultures of microorganisms

Bacterial strains were isolated from the nodules of natural populations of relict legumes *G. monophylla* and *G. verna* collected in the republics of Altai (Ongudaysky district, the mouth of the Chuya River) and Buryatia (Ulan-Ude, Yuzhny settlement) in Russia. Additional strains were isolated from root nodules of *G. monophylla* obtained in the sterile test-tube experiment in laboratory (see below). The standard method was used for isolation of bacteria from surface-sterilized single nodules (Novikova and Safronova, 1992). The isolates were grown at 28 °C on the modified yeast extract mannitol agar (YMA, Vincent, 1970) supplemented with 0.5 % succinate (YMSA, Safronova et al., 2015a). All isolates were deposited in the Russian Collection of Agricultural Microorganisms (RCAM) and stored at –80 °C in the automated Tube Store (Liconic Instruments, Lichtenstein) (Safronova and Tikhonovich, 2012). Information on the isolates is available in the online RCAM database http://www.arriam.spb.ru.

Plant Nodulation Assays

Due to the insufficient number of *G. monophylla* nodules collected in natural populations in the Altai Republic, and in order to obtain additional nodules, sterile test-tube experiments were carried out. For this purpose, soil samples and seeds of *G. monophylla* were collected in the Altai Republic. Seeds of *G. monophylla* were surface sterilized by H_2SO_4 for 10 min, washed with sterile tap water and germinated on filter paper in Petri dishes at 25 °C in the dark for 4 days. Germinated seedlings were transferred to 50 mL glass test tubes (2 seedlings per test tube) which contained 10 mL of sterile agar medium of the following composition (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, $ZnSO_4$ 0.005, $MnSO_4$ 0.002, agar for micropropagation of plants (Dia-m, Russia) 5.0. Seedlings were inoculated with 1 ml soil extracts. In total, 5 soil samples, collected in different areas of growth of *G. monophylla* populations in the Altai Republic, were used. The uninoculated plants were used as negative control. The plant nodulation assay was carried out in duplicate. Plants were cultivated for 30 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).

Identification of the isolates and bioinformatic analysis

For identification of the isolates the following PCR primers were used: fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-CTTAAGGAGGTGATCCAGCC-3′) for an approximately 1400 bp segment of the 16S rRNA gene (Weisburg, Barns, Pelletier and Lane, 1991). PCR was performed in 25-μl reaction mixtures containing 150 mM dNTPs (Promega, United States), 5 pmol of each primer, 1 U of Taq polymerase (Evrogen, Russia) and 50–100 ng of purified template DNA. PCR conditions for amplification of the 16S rRNA gene were the following: 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 in 1 % agarose gel (Invitrogen, United States) in Tris-acetate-EDTA. A 100-bp GeneRuler™ and Lambda DNA/HindIII markers (Fermentas, United States) were used for sizing and approximate quantification of DNA fragments. Purification of the PCR products was usually performed by using PureLink™ Quick kit (Invitrogen, United States) according to the manufacturer's guidance. The direct sequencing of PCR products was performed by an ABI PRISM 3500xl genetic analyzer (Applied Biosystems, United States). The sequences were compared with related sequences of the type strains available in the GenBank database using BLAST analysis at NCBI. *Rrs*-dendrogram was constructed using the Maximum Likelihood method in MEGA 7.0 software package (Kumar, Stecher and Tamura, 2016). The evolutionary distances were computed using the maximum composite likelihood method. Bootstrap analysis with 1000 replicates was performed to estimate the support of clusters. The *rrs* sequences were deposited to the NCBI GenBank database under accession numbers: OL438981–OL439009.

Results and discussion

A total of 29 isolates belonging to 5 genera and 4 families from the order *Rhizobiales* (*Alphaproteobacteria*) were obtained (Table 1). Eighteen and eleven isolates were isolated from the nodules of *G. monophylla* and *G. verna,* respectively. Eleven isolates formed colonies on the 3rd day (fast-growing), seven — on the 4-5th day (meso-growing), and eleven — on the 6-7th day (slow-growing). Analysis of *rrs* gene sequences allowed assigning the obtained isolates to five genera in the order *Rhizobiales*. Thus, the isolates of *G. monophylla* belonged to the genera *Mesorhizobium* (family *Phyllobacteriaceae*) — 10 isolates; *Bosea* (*Boseaceae*) — 6 isolates; as well as to *Rhizobium* (*Rhizobiaceae*) and *Bradyrhizobium* (*Bradyrhizobiaceae*) — 1 isolate each. *G. verna* isolates were assigned to the genera *Phyllobacterium* (family *Phyllobacteriaceae*) and *Rhizobium* — 5 isolates each, and *Bosea* — 1 isolate.

It was shown that isolates belonging to the genus *Mesorhizobium*, together with the type strains *Mesorhizobium delmotii* STM4623T, *M. prunaredense* ST-

Table 1. Isolates obtained in the work and their taxonomic assignment in the order *Rhizobiales*

Host plants	Location	Genus and number of isolates				
		Mesorhizobium	*Phyllobacterium*	*Bosea*	*Rhizobium*	*Bradyrhizobium*
G. monophylla	Altai	10	–	6	1	1
G. verna	Buryatia	–	5	1	5	–

M4891^T, *M. muleiense* CCBAU 83963^T and *M. robiniae* CCNWYC 115^T, formed three clusters within a single group with a low (76 %) support level (Fig. 2). Cluster I was combined at a support level of 87 % by isolates GU2/B1-2, GU2/B2-3, GU2/M1-3, GU2/M2-3, GU5/KM1-3, and GU5/KB1-3. These isolates did not form statistically significant groups with known type strains, although they showed a rather close relationship (99.86 % similarity) with the type strains *M. delmotii* STM4623^T and *M. prunaredense* STM4891^T (Table 2; Fig. 2). Both type strains were first isolated from the root nodules of the legume plant *Anthyllis vulneraria* subsp. *carpatica,* grown on soils sampled at the border of the Avinières mine and at a non-mining site in France (Mohamad et al., 2017). It was shown that the strains *M. delmotii* STM4623^T and *M. prunaredense* STM4891^T were able to form nodules

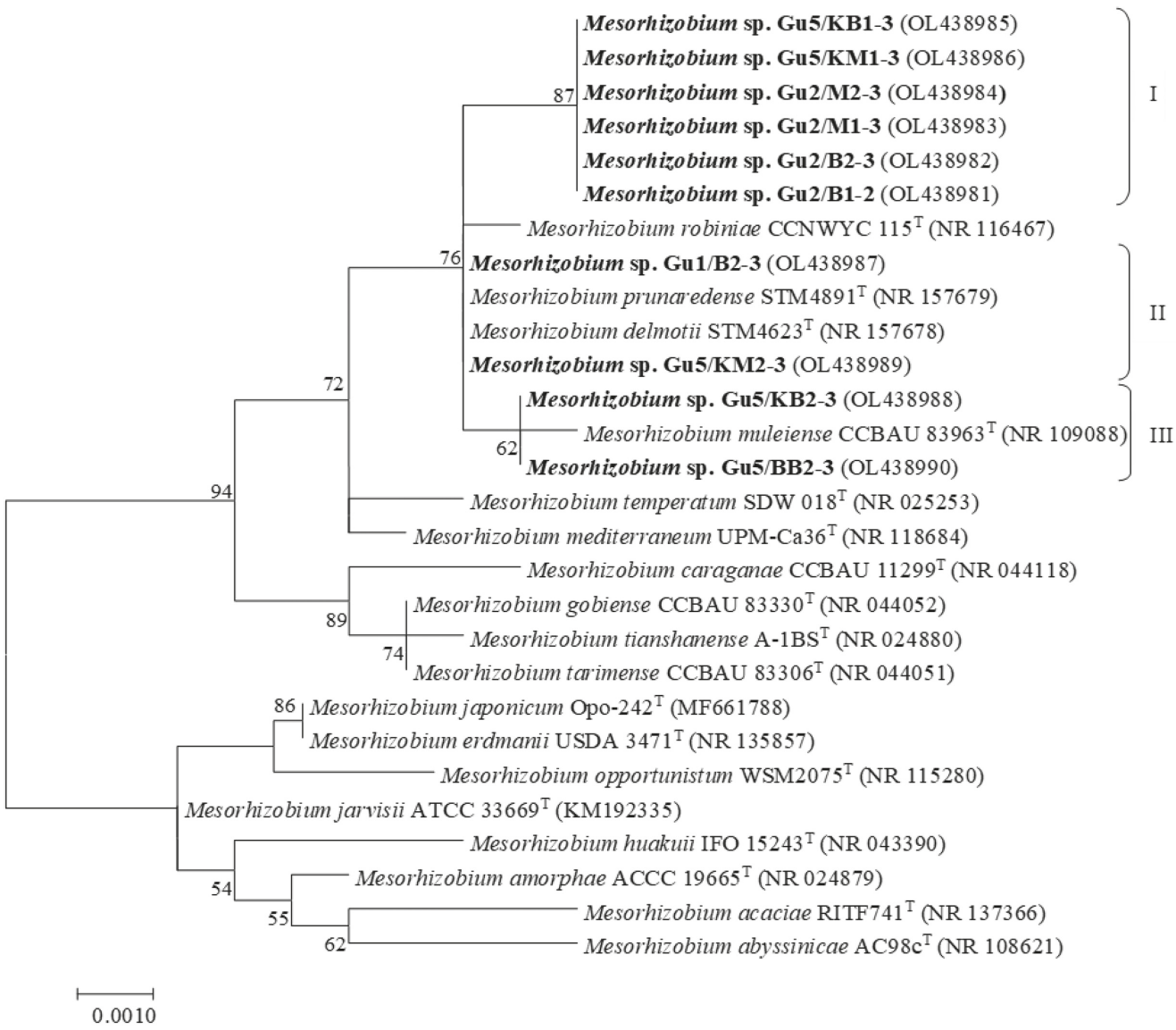

Fig. 2. Phylogenetic tree generated by the Maximum Likelihood method using 16S rRNA gene sequences of the isolated strains from *G. monophylla* nodules and representatives of closely related to *Mesorhizobium* species. The isolated strains are in bold. Type species are indicated by the letter ^{T}. I–III — clusters formed by the *Mesorhizobium* isolates obtained in the work. Bootstrap values of more than 50 % are given.

Table 2. The similarity of 16S rRNA gene between the strains isolated from *Gueldenstaedtia monophylla* nodules and the type strains of closely related *Mesorhizobium* species

Type strain	Isolates *rrs*-similarity (%)									
	Cluster I						Cluster II		Cluster III	
	GU2/ B1-2	GU2/ B2-3	GU2/ M1-3	GU2/ M2-3	GU5/ KM1-3	GU5/ KB1-3	GU1/ B2-3	GU5/ KM2-3	GU5/ KB2-3	GU5/ BB2-3
Mesorhizobium delmotii STM 4623	99.86	99.86	99.86	99.86	99.86	99.86	100	100	99.93	99.93
M. prunaredense STM 4891	99.86	99.86	99.86	99.86	99.86	99.86	100	100	99.93	99.93
M. muleiense CCBAU 83963	99.71	99.71	99.71	99.71	99.71	99.71	99.86	99.86	99.93	99.93
M. robiniae CCNWYC 115	99.70	99.70	99.70	99.70	99.70	99.70	99.85	99.85	99.78	99.78

The greatest similarity of isolate with the type strain is highlighted in gray.

and exhibit nitrogen-fixing activity with their original host plant *Anthyllis vulneraria,* which is widespread in Europe and is resistant to heavy metals (Mohamad et al., 2017). Cluster II was formed by isolates Gu1/B2-3, Gu5/KM2-3 and type strains *M. delmotii* STM4623^T and *M. prunaredense* STM4891^T, with which these isolates showed a 100 % level of *rrs*-similarity (Table 2; Fig. 2). Cluster III was formed by isolates Gu5/KB2-3, Gu5/BB2-3 and the type strain *M. muleiense* CCBAU 83963^T at a low (62 %) level of support (Fig. 2). The isolates showed the same level (99.93 %) of similarity to three type strains: *M. delmotii* STM4623^T, *M. prunaredense* STM4891^T and *M. muleiense* CCBAU 83963^T (Table 2). It should be noted that the last strain was isolated from the nodules of the legume plant *Cicer arietinum* L. growing in China (Zhang et al., 2012). It was shown that this strain formed effective nitrogen-fixing nodules with the original plant host, but no nodules were induced on other tested legumes: *Medicago truncatula*, *Trifoliium pretense*, *Pisum sativum*, *Vicia faba*, *Phaseolus vulgaris*, *Astragalus propinquus*, *Glycine max* and *Vigna aconitifolia* (Zhang et al., 2012).

Slow-growing isolates belonging to the genus *Bosea* formed two clusters on the phylogenetic tree (Fig. 3). Cluster I was formed by the type strains *B. minatitlanensis* AMX51^T, *B. robiniae* R-46070^T and the isolate A3/3-2 with a low (53 %) support level. The isolate showed 100 and 99.78 % similarity with the first and second type strains, respectively, and most likely belongs to the species *B. minatitlanensis*. The type strain AMX 51^T of this species is a strictly aerobic bacterium, although it was isolated as a transient microorganism from anaerobic digester sludge in Mexico (Ouattara et al., 2003). The type strain *B. robiniae* R-46070^T was isolated from root nodules of legume *Robinia pseudoacacia* in Flanders (Belgium) (De Meyer and Willems, 2012). Cluster II was formed by the type strains *B. lathyri* R-46060^T, *B. vaviloviae* Vaf18^T, *B. psychrotolerans* 1131^T and the isolates A3/1-3M, A3/4-2, A3/3-1, A5/1-1, A5/1-2 at a rather high (80 %) support level (Fig. 3). Within this cluster the isolates A3/4-2, A3/3-1, A5/1-1 and A5/1-2 formed a statistically significant (98 %) group. The isolates A3/3-1, A5/1-1 and A5/1-2 showed a low level of similarity (99.06 %) with the closest type strain *B. lathyri* R-46060^T, while the isolate A3/4-2 was the most similar (99.40 %) with the closest type strain *B. vaviloviae* Vaf18^T (Table 3). Isolate A3/1-3M showed a very low level of similarity (98.61 %) with the closest type strain *B. lathyri* R-46060^T (Table 3). It can be assumed that the isolates A3/1-3M, A3/4-2, A3/3-1, A5/1-1 and A5/1-2 may represent new *Bosea* species. Currently, genus *Bosea* consists of eleven species, of which five species (*B. lupini*, *B. lathyri*, *B. robiniae*, *B. vaviloviae* and *B. caraganae*) were isolated from nodules of the legume genera *Lupinus*, *Lathyrus*, *Robinia*, *Vavilovia* and *Caragana*, respectively. However, the capacity of the strains to independently form symbioses has not yet been shown (De Meyer and Willems, 2012; Safronova et al., 2015a; Sazanova et al., 2019). The strain B17/10-m isolated from the *G. verna* nodule did not form any cluster with other isolates or known type strains (Fig. 3), although it showed a rather close relationship at the levels 99.86 and 99.79 % with the type strains *B. vestrisii* LMG 26222^T and *B. eneae* LMG 26220^T, respectively (Table 3). Both type strains were isolated from hospital water supplies in France (La Scola, Mallet, Grimont and Raoult, 2003).

The slow growing isolate A3/3-3 had a 100 % *rrs*-similarity with the type strain *Bradyrhizobium valentinum* LmjM3^T and formed a single cluster with it at a high (94 %) level of support (Fig. 3). Strain LmjM3^T was isolated from a nitrogen-fixing nodule of *Lupinus mariae-josephae*, an endemic of basic-lime soils in Eastern Spain (Duran et al., 2014). The strain LmjM3^T established a nitrogen-fixing symbiosis with *L. mariae-josephae* but not with the other lupine species tested (*L. angustifolius*, *L. cosentinii*, *L. luteus* and *L. micranthus*). The strain was also able to efficiently nodulate and fix nitrogen with legumes *Retama raetam* and *R. sphaerocarpa* growing in North Africa, the Levant and some parts of southern Europe (Duran et al., 2014). Based on the data

Table 3. The similarity of 16S rRNA gene between the strains isolated from *G. monophylla* and *G. verna* nodules and the type strains of closely related *Bosea* species

Type strain	Isolates *rrs*-similarity (%)						
	Cluster I	Unclustered	Cluster II				
	A3/3-2	B17/10-m*	A3/1-3M	A3/4-2	A3/3-1	A5/1-1	A5/1-2
Bosea lathyri R-46060	98.88	98.15	98.61	99.30	99.06	99.06	99.06
B. vaviloviae Vaf18	98.66	98.58	98.46	99.40	98.76	98.84	98.84
B. psychrotolerans 1131	98.53	98.37	98.52	99.28	98.89	98.88	98.88
B. vestrisii 34635	99.55	99.86	98.54	98.29	98.17	98.18	98.18
B. robiniae R-46070	99.78	98.86	97.36	97.48	97.36	97.46	97.46
B. minatitlanensis AMX51	100	98.43	97.07	97.68	97.29	97.31	97.31
B. eneae 34614	99.33	99.79	98.54	98.29	98.17	98.18	98.18

The greatest similarity of an isolate with the type strain is highlighted in gray.
* — strain isolated from *G. verna*.

obtained, the isolate A3/3-3 was identified as *Bradyrhizobium valentinum*.

Fast-growing isolates from the genus *Rhizobium* formed four clusters on the phylogenetic tree (Fig. 4). All isolates, except for GU1/B2-2, were isolated from *G. verna* nodules. Cluster I was formed by isolates B17/10-cm, B24/10-m, as well as two type strains *R. lusitanum* P1-7^T and *R. rhizogenes* ATCC11325^T at a support level of 76 %. Both isolates showed 99.93 % similarity with these type strains (Table 4). *R. lusitanum* P1-7^T was isolated from *Phaseolus vulgaris* growing in Portugal, on the roots of which it formed effective nodules (Valverde et al., 2006). The strain *R. rhizogenes* ATCC 11325^T was isolated from apple and was able to induce hairy roots in plants and also to nodulate *Phaseolus vulgaris* (Riker et al., 1930; Velázquez et al., 2005). Cluster II was formed by the type strains *R. giardinii* H152^T, *R. herbae* CCBAU83011^T and the isolate B17/6-k at a 100 % support level (Fig. 4). However, this isolate showed a very low level of *rrs*-similarity (99.22 %) with the closest type strain *R. giardinii* H152^T (Table 4), therefore it is possible that it is a representative of a new *Rhizobium* species. Cluster III was formed with 99 % support by the type strains *R. leguminosarum* LMG 14904^T, *R. sophorae* CCBAU 03386^T, *R. anhuiense* CCBAU 23252^T, *R. laguerreae* FB206^T and the isolate GU1/B2-2, which showed 99.93 % similarity with all these type strains (Fig. 4; Table 4). To clarify the species identity of this isolate, an additional analysis of the housekeeping genes (*dna*K, *atp*D, *rpo*B, *gyr*B, *rec*A) is required. Cluster IV was formed at a high level of support (93 %) by isolates B18/3-k, B18/3-m and the type strain *R. alamii* GBV016^T (Fig. 4).

Table 4. The similarity of 16S rRNA gene between the strains isolated from *G. verna* and *G. monophylla* nodules and the type strains of closely related *Rhizobium* species

Type strain	Isolates *rrs*-similarity (%)					
	Cluster I		Cluster II	Cluster III	Cluster IV	
	B17/10-cm	B24/10-m	B17/6-k	GU1/B2-2*	B18/3-k	B18/3-m
Rhizobium giardinii H152	96.00	96.07	99.22	95.79	95.37	95.31
R. herbae CCBAU 83011	95.76	95.76	99.09	95.46	95.16	95.16
R. rhizogenes NBRC 13257	99.93	99.93	96.48	98.20	96.62	96.59
R. lusitanum P1-7	99.93	99.93	96.36	98.14	96.50	96.45
R. alamii GBV016	96.48	96.50	95.65	97.96	100	100
R. sophorae LMG 27901; *R. anhuiense* CCBAU23252; *R. laguerreae* FB206; *R. leguminosarum* LMG14904	98.18	98.21	96.23	99.93	98.07	98.04

The greatest similarity of an isolate with the type strain is highlighted in gray.
* Strain isolated from *G. monophylla*.

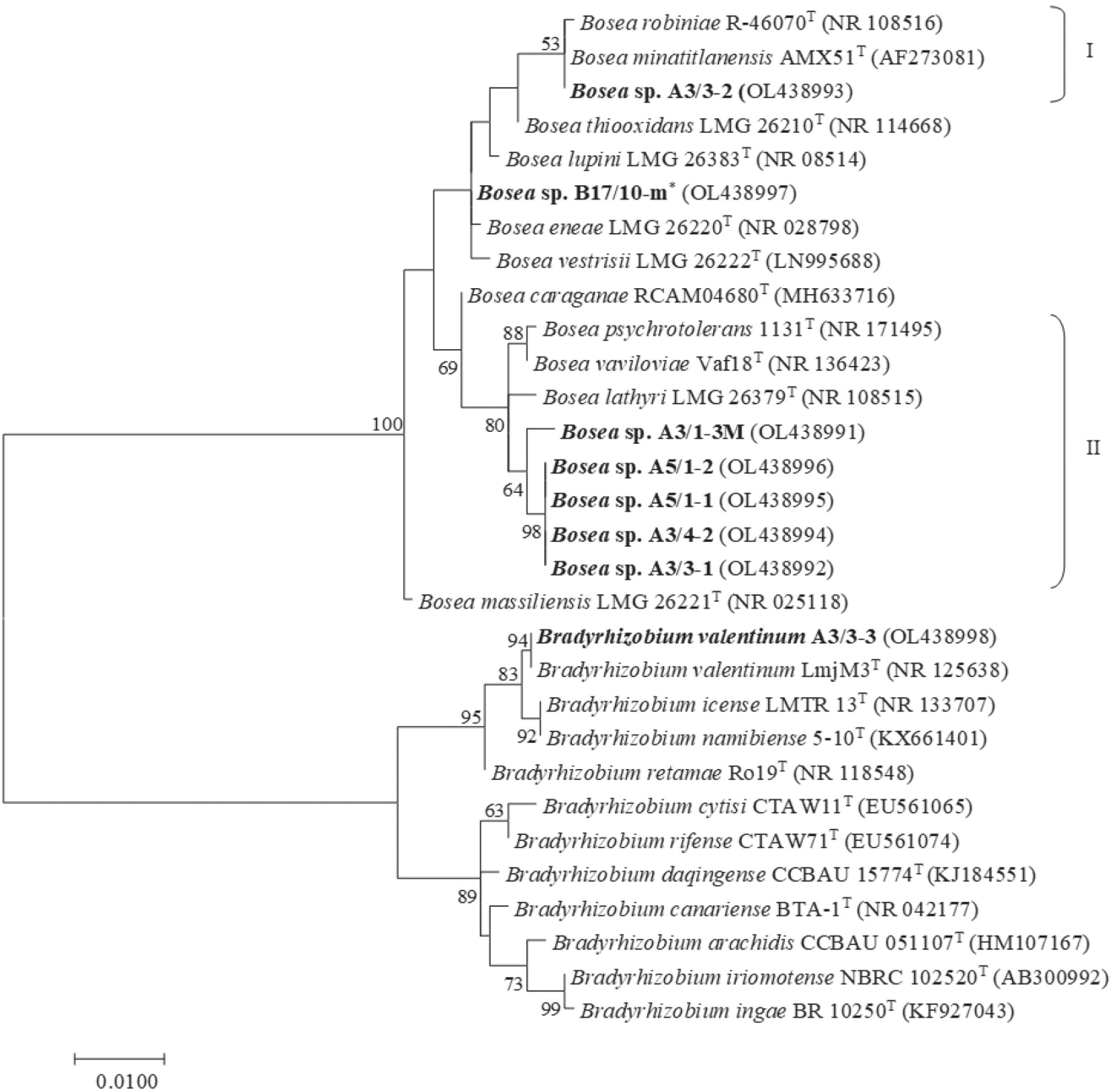

Fig. 3. Phylogenetic tree generated by the Maximum Likelihood method using 16S rRNA gene sequences of the isolated strains from *G. monophylla* and *G. verna* nodules and representatives of species closely related to *Bosea* and *Bradyrhizobium*. Isolate B17/10-m* was isolated from *G. verna*. The isolated strains are in bold. Type species are indicated by the letter T. I and II — clusters formed by *Bosea* and the isolates obtained in the work. Bootstrap values more than 50 % are given.

Both isolates showed a 100 % level of similarity with this type strain (Table 4). Strain GBV016^T was isolated from rhizosphere soil of *Arabidopsis thaliana* (Berge et al., 2009). It was shown that *R. alamii* GBV016^T is capable of producing gel-forming exopolysaccharides (EPS) that play an active role in plant development in a non-symbiotic context (Alami, Achouak, Marol and Heulin, 2000; Berge et al., 2009). Based on the data obtained, the isolates B18/3-k and B18/3-m were assigned to the species *R. alamii*.

Five meso-growing isolates isolated from *G. verna* nodules were assigned to the genus *Phyllobacterium* (Table 5). Isolate B17/8-m, most likely, belongs to the *P. loti* species, since it has 100 % *rrs*-similarity with the type strain *P. loti* S658^T isolated from a *Lotus corniculatus* nodule in a soil sample obtained in Uruguay (Sanchez et al., 2014). However, the ability of strain S658^T to form nodules was not confirmed. Isolate B24/4-2 had a 100 % *rrs*-similarity with the type strains *P. brassicacearum* STM 196^T and *P. sophorae* CCBAU 03422^T.

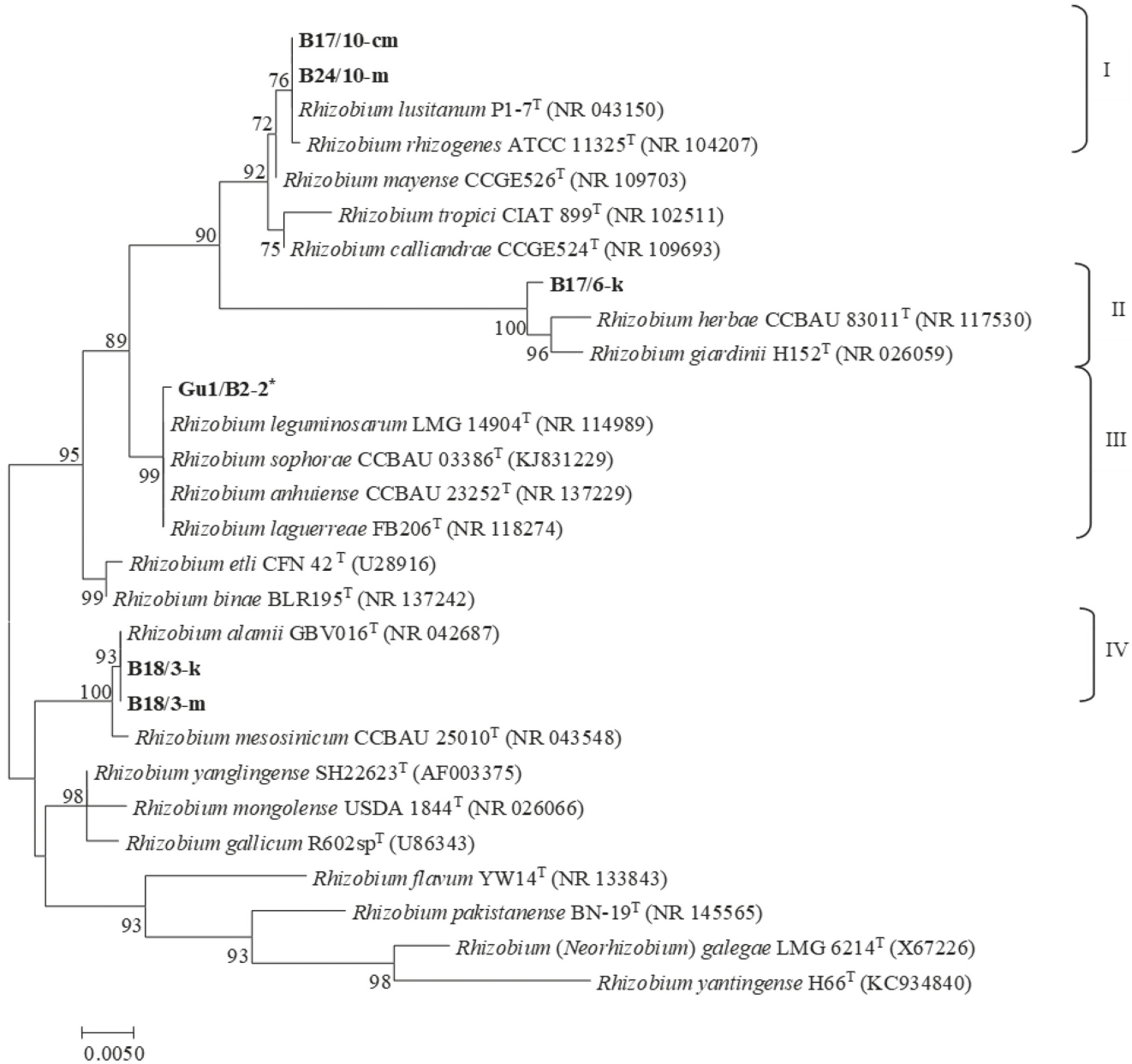

Fig. 4. Phylogenetic tree generated by the Maximum Likelihood method using 16S rRNA gene sequences of the isolated strains from *G. verna* and *G. monophylla* nodules and representatives of species closely related to *Rhizobium*. Strain Gu1/B2-2* was isolated from *G. monophylla*. The isolated strains are in bold. Type species are indicated by the letter T. I–IV — clusters formed by the *Rhizobium* isolates obtained in the work. Bootstrap values more than 50 % are given.

Table 5. The similarity of 16S rRNA gene between the strains isolated from *G. verna* nodules and the type strains of closely related *Phyllobacterium* species

Type strain	Isolates *rrs*-similarity (%)				
	B17/8-m	B24/1-m	B24/4-1	B24/4-2	B24/7-2
Phyllobacterium loti S658	100	100	100	97.38	100
P. trifolii PETP02	99.93	100	100	98.61	100
P. bourgognense STM201	99.14	100	100	98.90	100
P. brassicacearum STM 196	98.71	100	100	100	100
P. sophorae CCBAU 03422	98.03	100	100	100	100
P. zundukense Tri 48	99.00	100	100	99.42	100

The greatest similarity of an isolate with the type strain is highlighted in gray.

The strain STM 196^T was isolated from the rhizoplane of *Brassica napus* in France (Mantelin et al., 2006a) and was recognized as a plant-growth-promoting bacterium in plant culture of *Brassica napus* (Bertrand, Nalin, Bally and Cleyet-Marel, 2001; Larcher et al., 2003) and *Arabidopsis thaliana* (Mantelin et al., 2006b). The strain CCBAU 03422^T was isolated from root nodules of *Sophora flavescens*, a legume traditionally used as a herbal medicine in China (Jiao et al., 2015). Nodulation tests have demonstrated that the strain CCBAU 03422^T was able to form nitrogen-fixing nodules on *S. flavescens*. Isolates B24/1-m, B24/4-1 and B24/7-2 showed a 100 % *rrs*-similarity with six type strains at once (Table 5). To clarify the species identity of these isolates, additional phenotypic and genetic analyses are required. Currently, genus *Phyllobacterium* is represented by only 13 species, most of which are isolated from the root nodules of leguminous plants (Valverde et al., 2005; Mantelin et al., 2006a; Flores-Felix et al., 2013; Sanchez et al., 2014; Jiao et al., 2015; Safronova et al., 2018b). It was shown that only *P. trifolii* and *P. sophorae* strains are able to form nodules on host plants (Valverde et al., 2005; Jiao et al., 2015).

Conclusions

For the first time, a significant diversity of bacteria isolated from the root nodules of relict legumes *G. monophylla* and *G. verna*, was revealed. A total of 29 isolates were isolated belonging to 5 genera from order *Rhizobiales*: *Mesorhizobium* and *Phyllobacterium*, *Rhizobium*, *Bosea* and *Bradyrhizobium*. It is known that representatives of many species of *Mesorhizobium* and *Rhizobium* form nitrogen-fixing symbioses with a wide range of legume host plants. At the same time, such ability was shown only for two *Phyllobacterium* species (*P. trifolii* and *P. sophorae*) (Valverde et al., 2005; Jiao et al., 2015), while the formation of nodules by *Bosea* strains has not yet been described. In order to study the ability of the obtained isolates to form an effective symbiosis with *G. monophylla* and *G. verna*, as well as to assess the spectrum of host plants, additional sterile test-tube experiments should be carried out.

Eighteen and eleven strains were isolated from *G. monophylla* and *G. verna* nodules, respectively. Most of the strains isolated from *G. monophylla* nodules belonged to the genera *Mesorhizobium* and *Bosea*, while the strains isolated from *G. verna* nodules mainly belonged to *Phyllobacterium* and *Rhizobium*. We assume that the difference in the spectrum of *G. monophylla* and *G. verna* microsymbionts may be associated with both the host plant specificity and the differences in soil characteristics and microbiomes. In order to draw a certain conclusion, the cross-nodulation experiments using the isolated strains, as well as soil samples from the cites where the studied plants were collected, are required.

Five strains belonging to *Bosea* and one *Rhizobium* strain isolated from *G. monophylla* nodules 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. Only 3 isolates of the genus *Bradyrhizobium* and *Rhizobium* were identified at the species level: the isolate A3/3-3 was assigned to the species *Bradyrhizobium valentinum* and the isolates B18/3-k and B18/3-m — to the species *R. alamii*. Isolates A3/3-2 and B17/8-m most likely belong to the species *B. minatitlanensis* and *P. loti*, respectively, since these isolates have 100 % *rrs*-similarity with the corresponding type strains AMX51^T and S658^T. To clarify the taxonomy position of isolates with an uncertain species affiliation, additional phenotypic and genetic analyses such as housekeeping genes sequencing and multisubstrate analysis MicroPlate GENIII BioLog are required.

The data gathered can be used to improve the taxonomy of nodule bacteria of the order *Rhizobiales*, as well as to reveal the mechanisms of the formation of specific plant-microbial relationships during the evolution of symbiosis by studying the intermediate links between the extinct relict and the modern legume-rhizobia symbiotic relationships. The results obtained can be of great practical value, since legumes are important agricultural objects. At the same time, in the process of plant cultivation, the potential of plant-microbial interactions is steadily decreasing, since the use of organic and mineral fertilizers does not support the evolutionary selection of effective symbiotic microorganisms. Therefore, relict legume-rhizobia systems having ancestral microbial gene pool show promise for the development of biopreparations which will be highly efficient for modern cultivated plants.

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