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PHENOTYPIC AND GENOTYPIC PROPERTIES OF BRADYRHIZOBIA NODULATING LEGUMINOUS PLANTS OF THE *GLYCINE*, *VIGNA* AND *LUPINUS* GENERA

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Objective. *To study phenotypic and genotypic features of nodule bacteria – microsymbionts of leguminous plants of the Glycine, Vigna and Lupinus genera.* ***Methods.*** *Serological affinity of 12 rhizobia strains was determined in agglutination reactions. Host specificity of strains was studied in vegetation experiments. RFLP analysis of rpoB gene was performed using MspI, HaeIII and NdeII restrictases. Sequencing of the 16S-23S rDNA intergenic spacer of rhizobia was performed on ABI 3130 Genetic Analyzer.* ***Results.*** *It was established that studied rhizobia strains differ significantly in serological properties and belong to 7 serogroups: KB11, M8, 1967, 46, B1, B2 and 367a. Microsymbionts of soybean, cowpea and mung bean form the group of cross inoculation, however, are unable to infect lupine. Alternatively, lupine rhizobia B. lupini 367a and Bradyrhizobium sp. ЛД4, enter into symbiotic interactions with lupine, however, they do not nodulate soybean. B. japonicum 631 strain is capable of symbiosis with leguminous plants of different tribes: Phaseoleae and Lupineae. Based on RFLP analysis of rpoB gene, rhizobia were grouped into four clusters with following microsymbionts: I – cowpea, II – mung bean and soybean, III – slow-growing soybean nodule bacteria, IV – lupine rhizobia and intensive-growing soybean rhizobia. 16S-23S rDNA sequencing confirmed pertinence of soybean rhizobia to the B. japonicum species (USDA 4, USDA 6, USDA 123 genetic groups). This species also included mung bean microsymbionts (USDA 4 group). Bradyrhizobium sp. B11 isolate was obtained for the first time from cowpea nodules, and it was identified as a new B. diazoefficiens species (USDA 110 group). ITS regions of B. lupini 367a and Bradyrhizobium sp. ЛД4 rhizobia were found to be identical. B. japonicum 631 strain has a 100% similarity with B. lupini 367a strain according to 16S-23S rDNA, and with high degree of probability can be included to B. lupini species.* ***Conclusions.*** *Serological heterogeneity of rhizobia from root nodules of plants of the Glycine, Vigna and Lupinus genera has been demonstrated. It was established that microsymbionts of soybean, cowpea and mung bean belong to one group of cross inoculation. 16S-23S rDNA sequencing allowed to classify the studied strains as different genetic groups and identify them as B. japonicum, B. diazoefficiens and B. lupini. The serological grouping of nodule bacteria was found to coincide with the genetic (rpoB gene and ITS region) grouping, and their host specificity was related to species affiliation.*

Keywords: Bradyrhizobium japonicum, B. diazoefficiens, B. lupini, RFLP analysis, 16S-23S rDNA, rpoB gene, soybean, cowpea, mung bean, lupine.

The *Bradyrhizobium* genus was described in the early eighties of the last century and combined the slow-growing nodule bacteria – microsymbionts of various legumes. The first described species of this genus was *B. japonicum* [1, 2]. Further isolated rhizobia were assigned to other species: *B. elkanii* [3], *B. liaoningense* [4] and *B. yuanmingense* [5]. The taxonomic status of *Bradyrhizobium* is constantly changed and renewed. Currently, 53 species of slow-growing nodule bacteria are known, in which 39 have valid names [6]. The accumulation of experimental data on the genetic heterogeneity of rhizobia in natural ecosystems and agrocenoses suggests that new *Bradyrhizobium* species still need to be identified and described [6, 7].

It was believed for a long time that *B. japonicum* bacteria can only infect soybean (*Glycine* genus). However, later it turned out that the range of their host plants could be much wider. They were isolated from the nodules of various species of cowpea (*Vigna* genus) [8], lupine (*Lupinus* genus)

and other legumes [9]. At the same time, these legumes can form a symbiosis with representatives of other types of rhizobia.

It was found that nodule bacteria with different growth rates may form symbiotic relationships with soybean, namely: slow-growing *B. japonicum* [1], *B. elkanii* [3], *B. diazoefficiens* [10], *B. liaoningense* [4], *B. yuanmingense* [4] and fast-growing *Ensifer (Sinorhizobium) fredii, S. xinjiangensis* [11] and *Mesorhizobium tianshanense* [12].

More than 20 species of rhizobia were isolated and identified from cowpea nodules [13, 14]. The roots of this plant can be simultaneously infected by nodule bacteria of several genera, in particular *Bradyrhizobium* and *Rhizobium* [14, 15].

Microsymbionts of lupine are slow-growing nodule bacteria *B. lupini*, which recently was referred to the *Rhizobium lupini* species [16]. Furthermore, it is known from the literature that the European species of lupine can be infected by *B. canariense* and *B. japonicum* nodule bacteria [6, 9], and American species – *B. japonicum* and *B. elkanii* [17]. Some reports indicate that nodules on the roots of lupine are also formed by the representatives of the *Allorhizobium, Mesorhizobium, Sinorhizobium* and *Rhizobium* genera [18, 19].

It should be noted that typical soybean microsymbionts in soils of Ukraine are slow-growing nodule bacteria of the *B. japonicum* species. Earlier among their representatives we identified strains that are characterized by increased growth rate and conventionally called "strains with intensive growth". These strains have a set of characteristic features and differ from the typical slow-growing soybean rhizobia by morphological, cultural, physiological, chemotaxonomic and genetic properties [20, 21]. It is believed that lupine microsymbionts in Ukrainian soils can be rhizobia of the *B. lupini* species. Cowpea plants can form a symbiosis with rhizobia of various genera, including *B. japonicum*.

We have not found data on infection of soybean and lupine with other types of rhizobia in Ukrainian scientific literature. Only some properties of *B. japonicum* 631, which is capable of forming nodules on soybean and lupine roots, have been studied [22]. The heterogeneity of nodule bacteria of the *Bradyrhizobium* genus requires further study using serological and molecular genetic methods. Different experimental approaches will allow to fully characterize rhizobia, establish their affinity and reveal peculiarities of formation and functioning of symbiotic systems with different legumes.

Taking into account the above, the objective of our work was to study the phenotypic and genotypic features of nodule bacteria – microsymbionts of legume plants of the *Glycine*, *Vigna* and *Lupinus* genera.

Materials and methods. The objects of study were soybean nodule bacteria strains with slow (*Bradyrhizobium japonicum* 46, *B. japonicum* КС23) and intensive (*B. japonicum* КВ11, *B. japonicum* КС19) growth rates, *B. japonicum* VKM B-1967 = USDA 6^T strain; *B. japonicum* 631 strain isolated from soybean nodules and capable to infect soybean and lupine (strain from the collection of microorganism of the All-Russian Research Institute of Agricultural Microbiology of RAAS, St. Petersburg, Russia); *B. lupini* 367a standard strain of lupine nodule bacteria; rhizobia isolated from nodules of cowpea (*Bradyrhizobium* sp. B11, *Bradyrhizobium* sp. B22), mung bean (*Bradyrhizobium* sp. КМ1, *Bradyrhizobium* sp. КМ2) and lupine (*Bradyrhizobium* sp. ЛД4); plants of soybean (*Glycine max* (L.) Merr., variety Ustia), cowpea (*Vigna unguiculata* (L.) Walp., UD0301857), mung bean (*Vigna radiata* (L.) Wilczek, variety Tadzhytskyi 1), white (*Lupinus albus* L., variety Lybid`) and yellow (*Lupinus luteus* L., variety Chernihivets) lupine. Rhizobial strains are stored in the collection of the Laboratory of Plant-Microbial Interactions and in the Collection of useful soil microorganisms at the Institute of Agricultural Microbiology and Agroindustrial Manufacture of the National Academy of Sciences of Ukraine.

In this work, immune antisera against active strains of nodule bacteria of soybean *B. japonicum* (46, М8, КВ11, 364б, 1967, OR, HR, NR), microsymbionts of cowpea *Bradyrhizobium* sp. (B1, B2) and lupine *B. lupini* (30l, 367a) were used. Pertinence of the studied strains of nodule bacteria to a certain serogroup was determined in the agglutination reaction by the Gruber-Vidal method [23].

The ability of nodule bacteria strains to enter into a symbiotic relationship with soybean, cowpea, mung bean, white and yellow lupine (seeds were provided by the NSC "Institute of Agriculture of the National Academy of Agrarian Sciences of Ukraine" and V.Ya. Yuryev National Centre for Plant Genetic Resources of NAAS of Ukraine) were studied in vegetative experiments. Cultivation of nodule bacteria was carried out in

flasks (750 ml) on liquid medium (for 72 hours), containing (g/l): K_2HPO_4 – 0.5, KH_2PO_4 – 0.5, $(NH_4)_2SO_4 \cdot 7H_2O$ – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.2, NaCl – 0.2, $CaCO_3$ (sterile) – 0.1, sucrose – 2.0, mannitol – 3.0, glucose – 10.0, broth of peas (peas seeds – 50 g per 1 litre of water) – 100.0 ml/l; pH 7.0–7.2. The titre of bacteria was $2 \cdot 10^9$ CFU/ml (colony-forming units per milliliter). Inoculation load was 200–300 thousand cells per 1 seed. Plants were grown in a 2.5 l vessel on sterile vermiculite wetted with 0.2% KH_2PO_4). The variants without inoculation were used as the controls. The repetition of the experiment was quadruple. Activity of symbiotic nitrogen fixation was measured in flowering phase by acetylene method [24].

Nodule bacteria were cultivated in TY agar medium at 28° C [25]. Total DNA was extracted from the pure cultures of nodule bacteria in exponential growth phase using a "DNA-sorb B" kit. FGPS1490-72 (5'-tgcggctggatcccctcctt-3') and FGPL132-38 (5'-ccgggtttccccatt-3') primers were used for amplification of 16S-23S rRNA intergenic spacer (ITS region) [26, 27]. The temperature-time profile of amplification: denaturation at 94°C for 30 sec, primers annealing at 55°C for 30 sec, and synthesis of complementary chain at 72°C for 1 min (30 cycles). The primers FGPS1490-72/FGPL132-38 successfully yielded PCR amplicons (835–860 bp). Sequencing was performed on ABI 3130 Genetic Analyzer. Comparative analysis of sequences obtained and sequences from GenBank database was performed using BLASTN software (version 2.9.0). Equalization of the sequences was done using the CLUSTAL W program [28]. The phylogenetic tree was plotted using Mega 6 software [29] via Neighbor-Joining algorithm [30].

RFLP analysis (*restriction fragments length polymorphism*) of rpoB gene was performed for typing of nodule bacteria strains and determination of their affinity.

RpoB83F (5′-cctcatcgaggttcagaaggc-3′) and rpoB1061R (5′-agcgtgttgcggatataggcg-3′) primers were used for amplification the rpoB gene [31]. The temperature-time profile of amplification: denaturation at 94° C for 5 min, 4 cycles, 94°C for 2 min, 58° C for 2 min, 72° C for 1 min, 31 cycles, 94° C for 30 sec, 58° C for 1 min, 72° C for 1 min, final elongation at 72° C for 7 min.

The restriction analysis (RFLP) was carried out with the use of restriction endonuclease MspI, HaeIII, NdeII (Fermentas, USA) according to the manufacturer's instruction. DNA processed by restrictase was analyzed with the use of electrophoresis in 2.5% agarose gel. The DNA fragments size was calculated using Total Lab software (version 2.01).

The affinity of the resulting RFLP profiles of rpoB gene was compared using DendroUPGMA software (http://genomes.urv.cat/UPGMA/) based on unweighted pair-group method using arithmetic averages (UPGMA).

Results. Slow- and intensive-growing soybean nodule bacteria strains, as well as isolates from nodules of cowpea, mung bean and lupine were used in the work. Earlier, during study of the morphological and cultural properties of these microorganisms, it was found that they belong to the *Bradyrhizobium* genus. Soybean microsymbionts by phenotypic and genotypic features (identification of 16S rRNA gene nucleotide sequence) were classified as *B. japonicum* [20].

Serological method is a relatively simple and sufficiently informative method for investigating the diversity and affinity of nodule bacteria.

Serological properties of nodule bacteria were studied in the agglutination reaction. It has been established that the slow-growing strains of soybean rhizobia *B. japonicum* 1967, *B. japonicum* 46, *B. japonicum* KC23 are serologically different and belong to three serogroups: 1967, 46 and M8, respectively (Table 1). Intensive-growing *B. japonicum* KB11 and *B. japonicum* KC19 strains react positively with KB11 antiserum, and we have incorporated them in a separate serological group KB11. Mung bean microsymbionts *Bradyrhizobium* sp. KM1 and *Bradyrhizobium* sp. KM2 were classified as serogroup M8. The obtained results indicate their affinity with *B. japonicum* M8 strain. Isolates from cowpea nodules *Bradyrhizobium* sp. B11 and *Bradyrhizobium* sp. B22 differ in antigenic composition and belong to two serogroups: B1 and B2, respectively. The standard strain of lupine rhizobia *B. lupini* 367a and *B. japonicum* 631 strain capable to infect soybean and lupine, belong to the same serological group 367a. At the same time, *Bradyrhizobium* sp. ЛД4 isolate does not react with used antisera and its serological pertinence remains undefined. It should also be noted that the studied strains of lupine nodule bacteria show a weak positive reaction with KB11 antiserum (obtained against the intensive-growing *B. japonicum* KB11 strain) in low dilutions (up to 1:50). This fact may indicate the presence of common antigenic

determinants and serological similarity of lupine rhizobia and intensive-growing soybean rhizobia.

Despite the serological heterogeneity of investigated nodule bacteria, several serological groups (KB11, M8, and 367a) that combine strains similar in antigenic composition can be identified.

Under vegetative experiment conditions, the host specificity of the nodule bacterial strains was studied, that is, their ability to form nitrogen fixing nodules on the roots of different species of legumes: *Glycine max, Vigna unguiculata, Vigna radiata, Lupinus albus* and *Lupinus luteus* (Table 1).

It was shown that soybean microsymbionts (*B. japonicum* 46, *B. japonicum* KC23, *B. japonicum* KB11, *B. japonicum* KC19), cowpea (*Bradyrhizobium* sp. B11, *Bradyrhizobium* sp. B22) and mung bean (*Bradyrhizobium* sp. KM1, *Bradyrhizobium* sp KM2) are capable of cross-infection with these three legumes (phenotype Nod^+Fix^+ and Nod^+Fix^-), but they are not capable of forming nodules on the lupine roots (phenotype Nod^-). Standard lupine nodule bacteria strain *B. lupini* 367a and *Bradyrhizobium* sp. ЛД4 isolate, conversely, formed active symbiotic relationship with lupine (phenotype Nod^+Fix^+), but did not infect soybean (phenotype Nod^-). It should be noted that *B. japonicum* 631, isolated from soybean nodules, was able to form active nitrogen fixing nodules (phenotype Nod^+Fix^+) on the roots of plants of different tribes: soybean (*Phaseoleae* tribe) and lupine (*Lupineae* tribe).

Table 1

Host specificity and serological affinity of rhizobia strains isolated from soybean, cowpea, mung bean and lupine nodules

Strains (isolates)	Host-plants	Sero-groups	Symbiotic phenotypes				
			Phaseoleae tribe			*Lupineae* tribe	
			Glycine max	*Vigna uinguiculata*	*Vigna radiata*	*Lupinus albus*	*Lupinus luteus*
B. japonicum KB11	soybean	KB11	Nod^+Fix^+	Nod^+**Fix**$^-$	Nod^+Fix^+	**Nod**$^-$	**Nod**$^-$
B. japonicum KC19	soybean		Nod^+Fix^+	Nod^+**Fix**$^-$	Nod^+Fix^+	**Nod**$^-$	**Nod**$^-$
B. japonicum 1967	soybean	1967	Nod^+Fix^+	Nod^+Fix^+	Nod^+Fix^+	**Nod**$^-$	**Nod**$^-$
B. japonicum 46	soybean	46	Nod^+Fix^+	Nod^+Fix^+	Nod^+Fix^+	**Nod**$^-$	**Nod**$^-$
B. japonicum KC23	soybean	M8	Nod^+Fix^+	Nod^+Fix^+	Nod^+Fix^+	**Nod**$^-$	**Nod**$^-$
Bradyrhizobium sp. KM1	mung bean		Nod^+Fix^+	Nod^+Fix^+	Nod^+Fix^+	**Nod**$^-$	**Nod**$^-$
Bradyrhizobium sp. KM2	mung bean		Nod^+Fix^+	Nod^+Fix^+	Nod^+Fix^+	**Nod**$^-$	**Nod**$^-$
Bradyrhizobium sp. B11	cowpea	B1	Nod^+Fix^+	Nod^+Fix^+	Nod^+Fix^+	**Nod**$^-$	**Nod**$^-$
Bradyrhizobium sp. B22	cowpea	B2	Nod^+Fix^+	Nod^+Fix^+	Nod^+Fix^+	**Nod**$^-$	**Nod**$^-$
B. japonicum 631	soybean	367a	Nod^+Fix^+	–	–	Nod^+Fix^+	Nod^+Fix^+
B. lupini 367a	lupine		**Nod**$^-$	–	–	Nod^+Fix^+	Nod^+Fix^+
Bradyrhizobium sp. ЛД4	lupine	X	**Nod**$^-$	–	–	Nod^+Fix^+	Nod^+Fix^+

Legends: Nod^+Fix^+ – formation of nitrogen-fixing nodules; Nod^+Fix^- – formation of nodules that do not fix nitrogen, Nod^- – nodules are not formed, – – not investigated, X – undetermined serological group.

It is known that genes of the ribosomal cluster (16S rRNA and 16S-23S rRNA), as well as functionally important genes (housekeeping genes), are widely used to assess the diversity of microorganisms and determine their species specificity. We have performed a restriction analysis of one of these genes, namely the gene rpoB that encodes the β-subunit of RNA polymerase and can be used as a highly conserved genetic marker [31, 32].

When amplifying rpoB gene in the studied rhizobia, one fragment of ~900 bp was formed which was separately cleaved with restriction enzymes MspI, HaeIII and NdeII.

Under the use of MspI, nodule bacteria, regardless of species, had the same restriction patterns and were classified as MI rpoB type (Table 2). Only the typical *B. japonicum* 1967 was different from the others strains and isolates by the number of DNA fragments and formed the MII rpoB type.

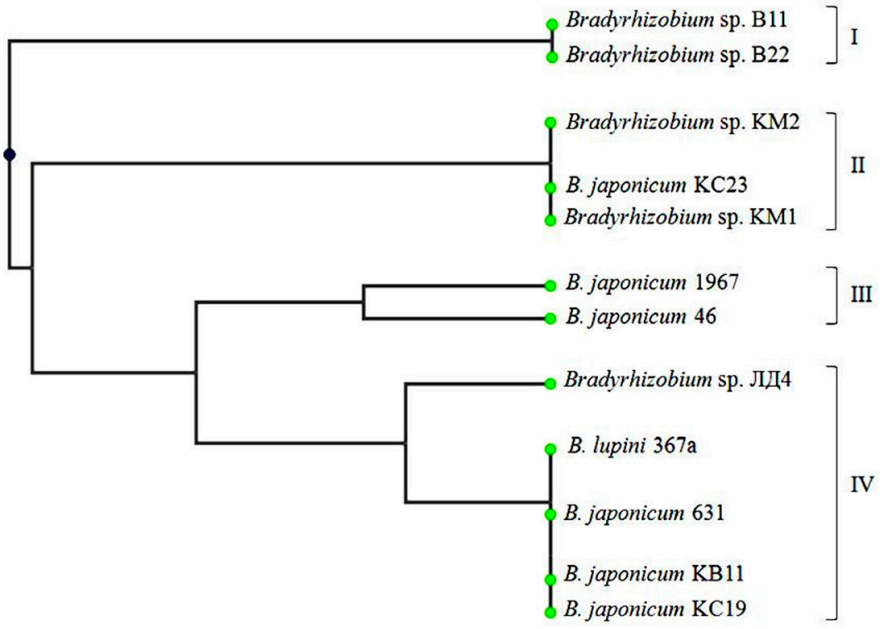
Significant differences among the nodule bacteria were detected with the use of restriction endonucleases HaeIII and NdeII.

Upon cleavage of the rpoB gene with HaeIII restriction enzyme, from three to five DNA fragments were formed, which allowed strains to be classified into five restriction types. Intensive-growing soybean rhizobia *B. japonicum* KB11, *B. japonicum* KC19, standard lupine strain *B. lupini* 367a and *B. japonicum* 631 had identical set of fragments and formed the HI rpoB type. Typical *B. japonicum* 1967 strain was characterised by the unique restriction pattern – NII rpoB type. The *B. japonicum* 46 strain and isolated from lupine nodules *Bradyrhizobium* sp. ЛД4 strain (HIII rpoB type) were similar. Microsymbionts of soybean and mung bean (*B. japonicum* KC23, *Bradyrhizobium* sp. KM1 and *Bradyrhizobium* sp. KM2) were combined into HIV rpoB type. Isolates derived from cowpea nodules *Bradyrhizobium* sp. B11 and *Bradyrhizobium* sp. B22 are classified as HV rpoB type.

Four types of DNA patterns were formed under the use of restriction endonuclease NdeII. Therefore, intensive-growing strains of soybean rhizobia *B. japonicum* KB11, *B. japonicum* KC19, lupine rhizobia *B. lupini* 367a, *Bradyrhizobium* sp. ЛД4 and *B. japonicum* 631 had identical profiles and they are combined into one rpoB type (NI). They differed from the slow-growing *B. japonicum* 1967 and *B. japonicum* 46 strains, which were classified as NII rpoB type with 4 fragments in restriction profiles. The largest number of fragments (6 units) was observed in the strains of the slow-growing *B. japonicum* KC23 and mung bean microsymbionts *Bradyrhizobium* sp. KM1, *Bradyrhizobium* sp. KM2 (NIII rpoB type). Isolates of *Bradyrhizobium* sp. B11 and *Bradyrhizobium* sp. B22 formed the NIV rpoB type.

Based on RFLP analysis of rpoB gene, using three restrictases, we have plotted UPGMA dendrogram of relationships of 12 strains and isolates of nodule bacteria – microsymbionts of different legumes (Fig. 1).

As can be seen from the diagram, the studied microorganisms were divided into four clusters (I–IV). A separate group was formed by intensive-growing strains of soybean rhizobia *B. japonicum* KB11, *B. japonicum* KC19, strains of lupine nodule bacteria *B. lupini* 367a, *Bradyrhizobium* sp. ЛД4 and *B. japonicum* 631,



F i g. 1. UPGMA dendrogram, showing clustering of strains and isolates of soybean, cowpea, mung bean and lupine nodule bacteria based on RFLP analysis of rpoB gene for the use of restriction enzymes MspI, HaeIII and NdeII.

Table 2

RFLP types and restriction fragments determined by PCR-RFLP analysis of rpoB gene of soybean, cowpea, mung bean and lupine microsymbionts

Strains (isolates)	Restriction endonuclease					
	MspI		HaeIII		NdeII	
	Restricted fragments size (bp)	rpoB type	Restricted fragments size (bp)	rpoB type	Restricted fragments size (bp)	rpoB type
B. japonicum КВ11	260, 170, 140	MI	370, 160, 130, 90, 65	HI	500, 170, 120	NI
B. japonicum КС19	260, 170, 140	MI	370, 160, 130, 90, 65	HI	500, 170, 120	NI
B. japonicum 1967	260, 170, 160, 140	MII	525, 160, 100, 90, 65	HII	300, 200, 170, 120	NII
B. japonicum 46	260, 170, 140	MI	525, 160, 130, 90, 65	HIII	300, 200, 170, 120	NII
B. japonicum КС23	260, 170, 140	MI	525, 200, 160, 80	HIV	300, 200, 170, 130, 120, 100	NIII
Bradyrhizobium sp. КМ1	260, 170, 140	MI	525, 200, 160, 80	HIV	300, 200, 170, 130, 120, 100	NIII
Bradyrhizobium sp. КМ2	260, 170, 140	MI	525, 200, 160, 80	HIV	300, 200, 170, 130, 120, 100	NIII
Bradyrhizobium sp. В11	260, 170, 140	MI	630, 160, 90	HV	380, 150, 120, 100	NIV
Bradyrhizobium sp. В22	260, 170, 140	MI	630, 160, 90	HV	380, 150, 120, 100	NIV
B. japonicum 631	260, 170, 140	MI	370, 160, 130, 90, 65	HI	500, 170, 120	NI
B. lupini 367a	260, 170, 140	MI	370, 160, 130, 90, 65	HI	500, 170, 120	NI
Bradyrhizobium sp. ЛД4	260, 170, 140	MI	525, 160, 130, 90, 65	HIII	500, 170, 120	NI

indicating their close phylogenetic affinity. At the same time, the slow-growing strains of soybean nodule bacteria *B. japonicum* 1967, *B. japonicum* 46 and *B. japonicum* KC23 turned out to be phylogenetically distant from lupine microsymbionts, and they entered into two different clusters. One cluster combined similar strains of *B. japonicum* 1967 and *B. japonicum* 46, while another cluster included mung bean microsymbionts *Bradyrhizobium* sp. KM1 and *Bradyrhizobium* sp. KM2 along with *B. japonicum* KC23. The genetic similarity on the dendrogram has been demonstrated by cowpea microsymbionts *Bradyrhizobium* sp. B11 and *Bradyrhizobium* sp. B22.

We have performed 16S-23S rDNA sequencing (ITS region) to identify isolates of nodule bacteria and to specify the species affiliation of strains. The resolution of this genetic marker is quite high and allows to find differences between microorganisms at the interspecies level.

The analysis of 16S-23S rDNA nucleotide sequences and their comparison with the sequence of *B. japonicum* USDA 6^T and *B. japonicum* strains from GenBank confirmed the pertinence of soybean microsymbionts to *B. japonicum* species (Table 3). As can be seen from the filogram (Fig. 1), the studied strains form three separate genetic groups by 99.0-100.0% of the support level: USDA 6 (*B. japonicum* 1967, *B. japonicum* 46), USDA 4 (*B. japonicum* KC23), USDA 123 (*B. japonicum* KB11 and *B. japonicum* KC19).

Table 3

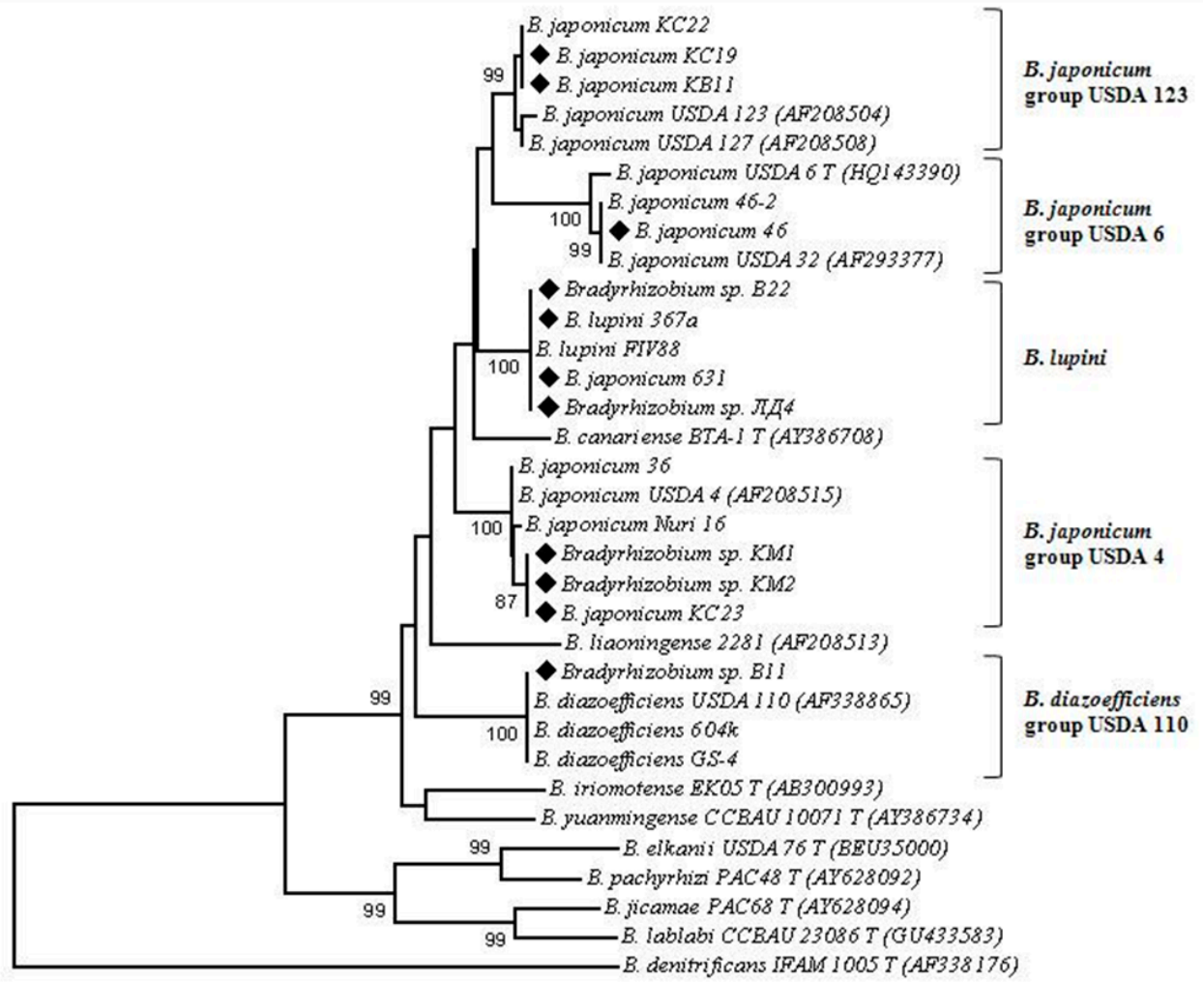
Identity of nucleotide sequences 16S-23S rDNA of microsymbionts of soybean, cowpea, mung bean and lupine with ITS sequences of nodule bacteria of the *Bradyrhizobium* genus

Strains (isolates)	Reference strains (GenBank accession numbers)	Similarity by ITS, %
B. japonicum KB11	*B. japonicum* USDA 127 (AF208508)	99.8
	B. japonicum USDA 123 (AF208504)	99.5
B. japonicum KC19	*B. japonicum* USDA 127(AF208508)	99.8
	B. japonicum USDA 123 (AF208504)	99.5
B. japonicum 1967 (USDA 6^T)	*B. japonicum* USDA 6^T (HQ143390)	100.0
B. japonicum 46	*B. japonicum* USDA 6^T (HQ143390)	98.7
B. japonicum KC23	*B. japonicum* USDA 4 (AF208515)	99.1
Bradyrhizobium sp. KM1	*B. japonicum* USDA 4 (AF208515)	99.1
	B. japonicum KC23	100.0
Bradyrhizobium sp. KM2	*B. japonicum* USDA 4 (AF208515)	99.1
	B. japonicum KC23	100.0
Bradyrhizobium sp. B11	*B. diazoefficiens* USDA 110^T (AF338865)	100.0
	B. diazoefficiens XF7 (CP029603)	100.0
Bradyrhizobium sp. B22	*B. lupini* 367a	100.0
	B. japonicum USDA 6^T (HQ143390)	93.1
	B. diazoefficiens USDA 110^T (AF338865)	93.7
B. japonicum 631	*B. lupini* 367a	100.0
	B. japonicum USDA 6^T (HQ143390)	93.1
	B. japonicum USDA 123 (AF208504)	96.6
B. lupini 367a	*B. lupini* FIV88	100.0
	B. japonicum USDA 123 (AF208504)	96.6
Bradyrhizobium sp. ЛД4	*B. lupini* 367a	100.0
	B. japonicum USDA 123 (AF208504)	96.6

According to the structure of the ITS region, isolates from mung bean nodule *Bradyrhizobium* sp. KM1 and *Bradyrhizobium* sp. KM2 is 99.1% similar to the *B. japonicum* USDA 4 strain deposited in GenBank (Table 3). The highest level of homology (100.0%) of these strains was noted with *B. japonicum* KC23 strain selected by us, which also belongs to USDA 4 genetic group (Fig. 2) and serologic group M8 (Table 1). Based on the sequencing results of 16S-23S rRNA intergenic spacer, the study of the structure of the rpoB gene, as well as the analysis of serological affinity and symbiotic properties, mung bean microsymbionts were identified as *B. japonicum.*

Cowpea microsymbionts entered into two different statistically reliable clusters (Fig. 2) on the phylogenetic tree. ITS sequence of *Bradyrhizobium* sp. B11 isolate was found to be 100.0% similar to the sequences of soybean strains of *B. diazoefficiens* USDA 110^T and *B. diazoefficiens* XF7 from GenBank (genetic group USDA 110). Considering the high degree of homology in the structure of 16S-23S rRNA intergenic region, this isolate can be classified as *B. diazoefficiens*. It should be noted that a relatively recent a group of strains of soybean and other legumes (*B. japonicum* group Ia), were reclassified to this species, and they closely interconnected but differ from the nodule bacteria *B. japonicum* by the phenotypic and genotypic features [10].

The second isolate from nodules of cowpea *Bradyrhizobium* sp. B22 showed the maximum degree of ITS homology (100.0%) with the standard *B. lupini* 367a strain and almost 100.0%



F i g. 2. Phylogenetic tree designed based on the comparative analysis of sequences of intergenic region 16S–23S rRNA of nodule bacteria with the use of the Neighbor-Joining algorithm. The scale corresponds with 2 substitutions to 100 founding pair (evolutionary distances). The figures show the statistical reliability of branching order (in %), which is estimated with the help of "bootstrap" – the analysis of 1000 alternative tree.

support level entered into a statistically significant cluster of *B. lupini* species bacteria. Considering that this strain is not able to infect yellow and white lupine, but forms nitrogen fixing nodules on soybean, cowpea and mung bean roots (Table 1), further molecular genetic studies are needed to clarify its systematic position.

All lupine microsymbionts entered into separate cluster on the ITS dendrogram with a 100.0%. support level. The standard *B. lupini* 367a strain showed 100.0% similarity to *B. lupini* FIV88, and ITS region of *Bradyrhizobium* sp. ЛД4 isolate was 100.0% identical to ITS region of *B. lupini* 367a strain (Table 3). The fact that *B. japonicum* 631, isolated from soybean nodules, had 100.0% homology with *B. lupini* 367a strain is of particular importance. At the same time, the similarity of 16S-23S rDNA of this strain and the typical strain of soybean rhizobia *B. japonicum* USDA 6^T was only 93.1%, which is considered low for such a high-conservative locus. Strains of the same species cannot have less than 95.0% homology in ITS region [33, 34]. Based on the obtained genetic data and the ability of the studied strain to nodulate not only soybean, but also lupine, it can be classified as *B. lupini* with high degree of probability.

Discussion. It is known that the intense cultivation of legumes promotes the increase of the biological diversity of their microsymbionts. New genotypes of nodule bacteria can occur as a result of mutations, recombinations, horizontal transfer of genes between strains of one species and another rhizobial microbiota [33].

In the modern literature, considerable attention is paid to the research of genetic resources and diversity of microsymbionts of both traditional and rare legumes. However, in Ukraine these issues remain virtually unexplored. Information on phenotypic and genotypic features of rhizobia can be useful in studying their population genetics, ecology, and also for agricultural practice [33, 35].

In this work, we have studied 12 strains and isolates of nodule bacteria – soybean, cowpea, mung bean and lupine microsymbionts. Their serological and genetic features, as well as the ability to form a symbiotic relationship with leguminous plants of different tribes: *Phaseoleae* (genus *Glycine* and *Vigna*) and *Lupineae* (genus *Lupinus*), have been studied.

We have established a significant genetic diversity of studied nodule bacteria. RFLP analysis of the rpoB gene allowed the grouping of nodule bacteria into four statistically reliable clusters. As a result of 16S-23S rDNA nucleotide sequences determination and comparing them with the standard and typical (from GenBank) strains, the rhizobia were classified into five genetic groups. In particular, three genetic groups were formed by representatives of *B. japonicum* species – soybean and mung bean microsymbionts. Rhizobia of *B. diazoefficiens* and *B. lupini* species were classified into two separate groups. It should be noted that we were the first who discovered *B. diazoefficiens* nodule bacteria in Ukrainian soils. They can form nodules on soybeans, cowpea and mung beans roots. The data that we obtained are consistent with the reports of scientists who studied the diversity of soybean nodule bacteria, cowpea and lupine in the soils of European, Asian, and African countries. The studies of Pudełko K. [19], Sikora S. [36], Appunu C. [37], Chidebe I. [38] et al. demonstrated a high degree of local rhizobia polymorphism according to the ITS region and housekeeping genes, which allowed to classify the strains into different genetic groups. Moreover, as in our studies, the number of formed groups depended on used genetic marker and restriction endonuclease [19, 36–39].

The serologic typing of *Bradyrhizobium* strains showed that they differ significantly in serological properties and belong to seven serogroups. Four serogroups (KB11, 1967, 46, M8) are microsymbionts of soybean and mung bean. And bacteria from root nodules of cowpea and lupine were included in three serogroups (B1, B2, 367a). A significant serological diversity of soybean and cowpea microsymbionts was revealed in the works of other scientists [40, 41].

Comparing the data obtained by us, a certain relationship was found between the grouping of microsymbionts by genetic markers and serological composition. For example, serologically related strains belonging to the serogroup KB11 formed a separate group according to the nucleotide sequences of 16S-23S rDNA and restriction profiles of rpoB gene. Similarly, the representatives of the serogroup M8 – microsymbionts of mung bean and *B. japonicum* KC23 strain were combined. It is noteworthy that nodule bacteria of various species of *B. japonicum* (intensive-growing strains) and *B. lupini* have similar antigenic determinants and entered into one cluster by RFLP profiles of rpoB gene. The obtained results may indicate their serological and genetic affinity.

There are few reports in the literature that link the serology of nodule bacteria with molecular

genetic studies. For example, van Berkum et al. investigated the evolutionary relationships between 52 strains of soybean nodule bacteria, which belong to 17 serological groups. As a result, it was found that the strains within each serogroup had little difference in the nucleotide sequence of the 16S rDNA and ITS region. Correspondence of serological and genetic groups was confirmed by AFLP (amplified fragment length polymorphism) analysis. At the same time, *B. liaoningense* 2281 and *B. japonicum* USDA 135 strains cross-reacted in a serological reaction. We believe that the use of various experimental approaches and involvement of the higher number of strains is necessary for better understanding the relationship between serological and genetic organization of strains.

It should also be noted that the host specificity of the studied rhizobia strains corresponds to their genetic organization (ITS region). However, the relationship between plants and some strains does not fit into the scheme of their classification into species for the ability to infect one or other legumes. For example, we have obtained an isolate from cowpea nodules *Bradyrhizobium* sp. B22, which is identified as *B. lupini* by 100.0% homology in ITS region, but it is not capable to form nodules on white and yellow lupine roots. *B. japonicum* 631 strain, isolated from soybean nodules, is interesting. This strain is able to nodulate soybean and lupine, and according to the nucleotide sequence of ITS region can be classified as *B. lupini*. That is, for a more reliable determination of the boundaries of the species, complex polyphasic studies of *Bradyrhizobium* strains are required, as many researchers point out [33, 35].

Thus, we have shown the serological heterogeneity of rhizobia from root nodules of the *Glycine, Vigna* and *Lupinus* genera. It has been established that soybean, cowpea and mung bean microsymbionts belong to the same group of cross-inoculation. By the results of sequencing of ITS region, the studied strains examined were classified in different genetic groups and identified as *B. japonicum, B. diazoefficiens* and *B. lupini*. The serological grouping of nodule bacteria was found to coincide with the genetic (rpoB gene and ITS region) grouping, and their host specificity was related to species affiliation.

Further study of nodule bacteria – microsymbionts of different legumes will allow to establish more deeply the feature of their biodiversity formation in soils of Ukraine.

ФЕНОТИПОВІ ТА ГЕНОТИПОВІ ВЛАСТИВОСТІ БРАДІРИЗОБІЙ, ЗДАТНИХ НОДУЛЮВАТИ БОБОВІ РОСЛИНИ РОДІВ *GLYCINE, VIGNA* ТА *LUPINUS*

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Резюме

Мета. Вивчити фенотипові та генотипові ознаки бульбочкових бактерій – мікросимбіонтів бобових рослин родів *Glycine*, *Vigna* та *Lupinus*. **Методи.** Серологічну спорідненість 12 штамів ризобій визначали у реакції аглютинації. Хазяйську специфічність штамів досліджували у вегетаційних дослідах. RFLP аналіз гену rpoB проводили з використанням рестриктаз *Msp*I, *Hae*III та *Nde*II. Секвенування 16S-23S рДНК здійснювали на автоматичному ДНК-секвенаторі ABI 3130 Genetic Analyser. **Результати.** Встановлено, що досліджувані ризобії істотно розрізняються за серологічними властивостями і належать до 7 серогруп: КВ11, М8, 1967, 46, В1, В2 та 367а. Мікросимбіонти сої, вигни та машу утворюють групу перехресної інокуляції, проте не спроможні інфікувати люпин. Ризобії люпину *B. lupini* 367а та *Bradyrhizobium* sp. ЛД4, навпаки, вступають у симбіотичні взаємовідносини з люпином, але не нодулюють сою. Штам *B. japonicum* 631 здатний до симбіозу з бобовими рослинами різних триб: *Phaseoleae* та *Lupineae*. На основі RFLP аналізу rpoB гену мікросимбіонти згрупувались у чотири кластери: I – ризобії вигни, II – бульбочкові бактерії машу та сої, III – повільнорослі бульбочкові бактерії сої, IV – ризобії люпину та інтенсивнорослі ризобії сої. Секвенування 16S-23S рДНК підтвердило належність бульбочкових бактерій сої до виду *B. japonicum* (генетичні групи USDA 4, USDA 6, USDA 123). До цього ж виду віднесені мікросимбіонти машу (група USDA 4). Вперше з бульбочок вигни отримано ізолят *Bradyrhizobium* sp. В11, який ідентифіковано як новий вид *B. diazoefficiens* (група USDA 110). Ідентичними виявились ITS-регіони ризобій *B. lupini* 367а та *Bradyrhizobium* sp. ЛД4. Штам *B. japonicum* 631 на 100% подібний зі штамом *B. lupini* 367а за 16S-23S рДНК і з високою долею ймовірності може бути віднесений

до виду *B. lupini*. **Висновки.** Продемонстровано серологічну різнорідність ризобій з кореневих бульбочок рослин родів *Glycine*, *Vigna* та *Lupinus*. Встановлено, що мікросимбіонти сої, вигни та машу належать до однієї групи перехресної інокуляції. Секвенування 16S-23S рДНК дозволило віднести досліджувані штами до різних генетичних груп та ідентифікувати їх як *B. japonicum*, *B. diazoefficiens* та *B. lupini*. Виявлено, що серологічне групування бульбочкових бактерій співпадає з генетичним (rpoB ген і ITS регіон) групуванням, а їх хазяйська специфічність пов'язана з видовою належністю.

Ключові слова: Bradyrhizobium japonicum, B. diazoefficiens, B. lupini, RFLP аналіз, 16S-23S рДНК, rpoB ген, соя, вигна, маш, люпин.

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