

GENETICS
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Genetic Diversity and Phylogeny of Root Nodule Bacteria Isolated
from Nodules of Plants of the *Lupinaster* Genus
Inhabiting the Southern Urals

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Abstract—The genetic diversity and phylogeny of root nodule bacteria, microsymbionts of plants of the genus *Lupinaster* Adans. (*L. albus* Link and *L. pentaphyllus* Moench), were studied, and also their symbiotic genes were analyzed. The bacterial strains studied were shown to be phylogenetically different; however, all of them are related to the *Mezorhizobium* genus with the exception of the single strain which is related to the *Rhizobium* genus. Analysis of the symbiotic genes *nifH* and *nodC* revealed their high homology among all strains independently of strain phylogeny, as well as the phylogenetic relation of these genes to those of *Mezorhizobium*. *Mezorhizobium* bacteria are likely specific microsymbionts of these plants, while the *Rhizobium* strain acquired its symbiotic genes and became capable of nodule formation in *Lupinaster* plants through horizontal gene transfer. Thus, the genetic composition of nodule bacteria inhabiting *Lupinaster* plants represents additional support for the idea that they do not belong to the *Trifolium* genus.

Keywords: nodule bacteria, symbiotic genes, phylogeny, symbiosis, legumes

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INTRODUCTION

Legume-rhizobium symbiosis is a unique phenomenon of very significant importance. Because of the capability of assimilation of atmospheric nitrogen in symbiosis with root nodule bacteria, legumes can grow in nitrogen poor areas and enrich soils in this element. Such enrichment is, undoubtedly, very important for the rest of the biocenosis where legume plants grow.

Symbiotic interaction of legumes with nodule bacteria is highly specific; however, this specificity varies to some degree. For example, symbiosis is less specific for legumes inhabiting tropical and subtropical areas, while plants growing in a temperate climate have stricter specificity.

In this study, we analyzed the genetic heterogeneity and phylogeny of nodule bacteria which are symbionts of two legume species of the *Lupinaster* Adans. genus (*L. albus* Link and *L. pentaphyllus* Moench) inhabiting the Southern Urals. This genus together with its microsymbionts is of significant interest since its independence and systematic position have been discussed for a long time.

Although the *Lupinaster* genus was described as long ago as the middle of the 18th century, it was not always recognized as a taxonomic category, particu-

larly, by Old World taxonomists. *Lupinaster* was generally included in the *L. s. l.* (*Trifolium*) genus as a sub-generic unit, as was defined in the basic summary of *Flora of the USSR* [1]. For this reason, the generic position of species of the *Lupinaster* genus was defined for many years. However, the taxonomic distinction of *Lupinaster* was so clear that a majority of taxonomists finally recognized its independence [2, 3]. Nevertheless, even now, a number of florists still consider the *Trifolium* genus in a broader sense [4, 5]. The distinctions found concern many parameters: morphology of generative (flowers, fruits, and inflorescence) and vegetative (leaves) organs, pollen structure, karyology, and others [3, 6]. Moreover, the areas of species of the *Trifolium* and *Lupinaster* genera are also significantly different, which suggests their independent evolution. *Trifolium* species are common for European and Mediterranean countries, while *Lupinaster* species inhabit North America and only a few of them are specific to Siberia and Europe.

Currently, relations of *Lupinaster* and its position in the legume taxonomy are still arguable. This genus was and still is the most frequently ascribed to the tribe Trifolieae. However, some researchers indicate that species related to *Lupinaster* are phylogenetically closer to the predominantly American genus *Lupinus* L. from

the tribe Genisteae and that they hardly can be assigned to other subgeneric categories of *Trifolium*. A problem of definition of a new tribe Lupineae [6], which could include *Lupinaster*, *Lupinus*, and other related genera, is still to be elucidated.

In the case where plants belong to the *Trifolium* genus, they are highly likely to have nitrogen-fixing symbiosis with nodule bacteria of *Rhizobium leguminosarum* bv. *trifolii* [7]. Otherwise, if there is a separate genus in the Trifolieae tribe, symbionts are likely represented by *Sinorhizobium* spp. since plants of the other genera (*Trigonella* L., *Melilotus* Mill., and *Medicago* L.) have symbiotic association with these microbial species [8–10]. The only exceptions to this symbiotic specificity are *Medicago ruthenica* (L.) Trautv., which is associated with *R. mongolense* [11], and the *Ononis* L. genus, whose representatives interact with bacteria from a broader range of strains [12, 13]. In this connection, a study on the genetic diversity and phylogeny of nodule bacteria associated with species of the *Lupinaster* genus may shed light on its taxonomic position and phylogenetic relations.

MATERIALS AND METHODS

Strains and culture growth. Root nodule bacteria were isolated from nodules of *L. albus* and *L. pentaphyllus* plants from Uchalinskii Hummocks located in the Southern Urals.

Bacteria were isolated from their propagation areas and streaked on an JM agar medium (0.1% yeast extract, 1% mannitol, 0.05% K₂HPO₄, 0.05% MgSO₄, 0.01% NaCl, 1.5% agar) to obtain single colonies [14]. Each isolate corresponded to a separate nodule.

Isolation of total DNA. DNA was isolated by cell lysis in 1% Triton X100 and 1% Chelex100 suspension. A small amount of bacterial mass was placed in a 1.5 mL tube with 100 µL 1% Triton X100 and 1% Chelex100 suspension. Cells were vortexed and incubated at 95°C for 10 min. Cell debris was pelleted at 12000 g for 3 min. Supernatants were used as PCR templates.

Genetic analysis of strains. RAPD analysis was used to study the strain genetic diversity [15]. The “random” primers were 5'-GGGCGCTG-3', 5'-CAGGCCATC-3', and 5'-GCGTCCATTC-3'.

PCR-RFLP analysis [16] of the *16S* rRNA genes was carried out with rare-cutting restriction endonucleases *Kzo91* and *HaeIII*. DNA fragments of these genes (1500 bp) were amplified with the following universal primers: fD1 5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3' and rD1 5'-CCGAATTCGTGACAACAGAGTTTGATCCTGGCTCAG-3' [17]. The *recA*, *nifH*, and *nodC* gene sequences were amplified with the following primer pairs, respectively [18]: RecAF 5'-GGCAGTTCGGCAAGGGCTC-GAT-3' and RecAR 5'-ATCTGGTTGATGAAGAT-CACCAT-3'; NifHF 5'-TTCTATGGAAAGGGCG-

GCATTGGCAAGCT-3' and NifHR 5'-ATCTCGC-CGGACATGACGATATAAATTC-3'; and NodCF 5'-CGTTTCGTCTTATGCGGTGCTC-3' and NodCR 5'-CAGCTGCGTCTCGTATTGAT.

For sequencing, an automated Applied Biosystems 3500 system (Applied Biosystems Inc., United States) and a Big Dye Terminator v. 3.1 kit were used.

The nucleotide sequences were analyzed with the Lasergene program package (DNASTAR Inc., United States). Nucleotide sequences for comparison were from the GenBank database (www.ncbi.nlm.nih.gov).

Phylogenetic analysis. Phylogenetic analysis of the strains was based on the multiple alignment (ClustalW) of the sequenced *16S* rRNA, *recA*, *nodC*, and *nifH* genes. Phylogenetic trees were constructed by a neighbor-joining method with the use of the Megalign program of the Lasergene package. Branching significance (bootstrap analysis) was estimated with the use of the corresponding function of the Megalign program based on 1000 alternative trees.

The nucleotide sequences of *16S* rRNA, *recA*, *nodC*, and *nifH* were submitted to GenBank and their accession numbers are ku725684–89, ku702936–47, and ku672512–17.

RESULTS AND DISCUSSION

The *L. albus* and *L. pentaphyllus* plants inhabiting the Southern Urals were a source of nodules from which 23 authentic cultures of nodule bacteria were isolated. The RAPD analysis was used to study polymorphism in these strains. The results obtained demonstrated relatively high heterogeneity among the strains which composed 11 groups of genetically homogeneous isolates (strains). Preliminary phylogenetic analysis based on 16S RFLP demonstrated that all strains were organized in six monophyletic groups. Some groups included microsymbionts of both *L. albus* and *L. pentaphyllus*. These plants seem to be hosts of the same species of nodule bacteria. Strains predominantly found in *L. albus* or in *L. pentaphyllus* nodules were designated as Lal and Lpe strains, respectively.

The nucleotide sequences of the conservative genes *16S* rRNA and *recA* were compared with their homologs from GenBank to study the phylogeny of the group representatives. According to the sequences of both genes, the majority of bacteria were closely related to the *Mesorhizobium* genus (Figs. 1 and 2) with the exception of one strain which was more related to the *Rhizobium* genus.

The genes *nodC* and *nifH* were compared with their homologs of other nodule bacteria from the GenBank database to analyze the phylogeny of symbiotic genes (Figs. 3 and 4).

This analysis revealed that all strains of the nodule bacteria studied have symbiotic genes predominantly related to those of the *Mesorhizobium* genus, including

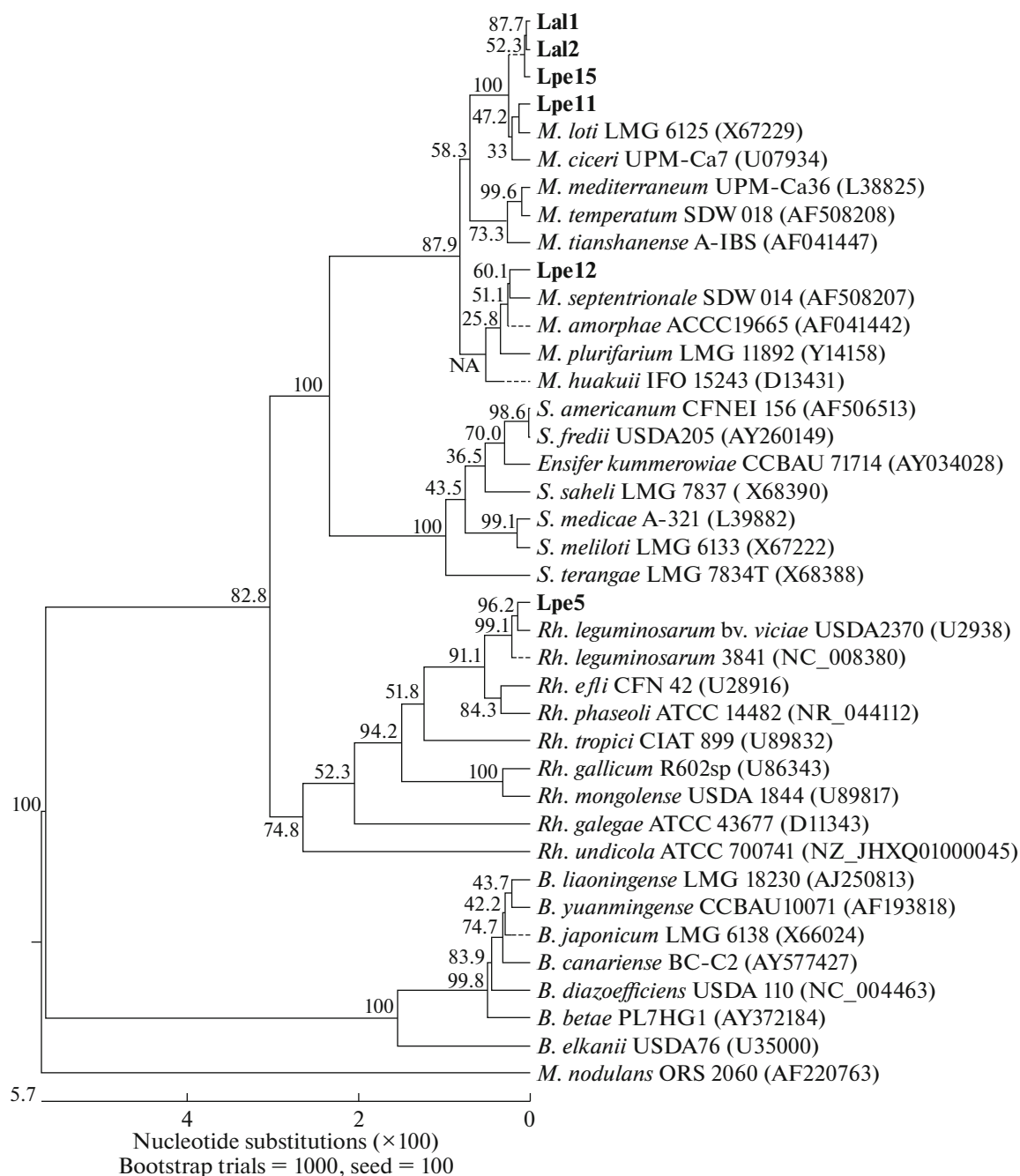


Fig. 1. Phylogenetic tree of nodule bacteria based on comparison of 16S rRNA gene sequences. Strains of microorganisms studied by us are shown in bold.

the strain which is more related to the *Rhizobium* genus according to its housekeeping genes. It is possible that this chimeric *Rhizobium* sp. strain acquired symbiotic genes of the *Mesorhizobium* genus through horizontal gene transfer (HGT).

One of the distinguishing traits of legumes is their capability of nitrogen-fixing symbiosis with nodule bacteria. This interaction of plant and bacteria is characterized by high specificity in which each legume spe-

cies is associated with a definite microsymbiont range and each strain of nodule bacteria has its own host range [19]. According to a theory of cross-inoculation groups developed by Fred et al. [20], legume plants consist of species which can be easily infected by rhizobia within a group and have a common pool of microsymbionts [21]. Although 16 cross-inoculation groups previously were proposed, more studies in this field resulted in only four such groups. One of them includes the *Trifolium* genus and its *Rhizobium legumi-*

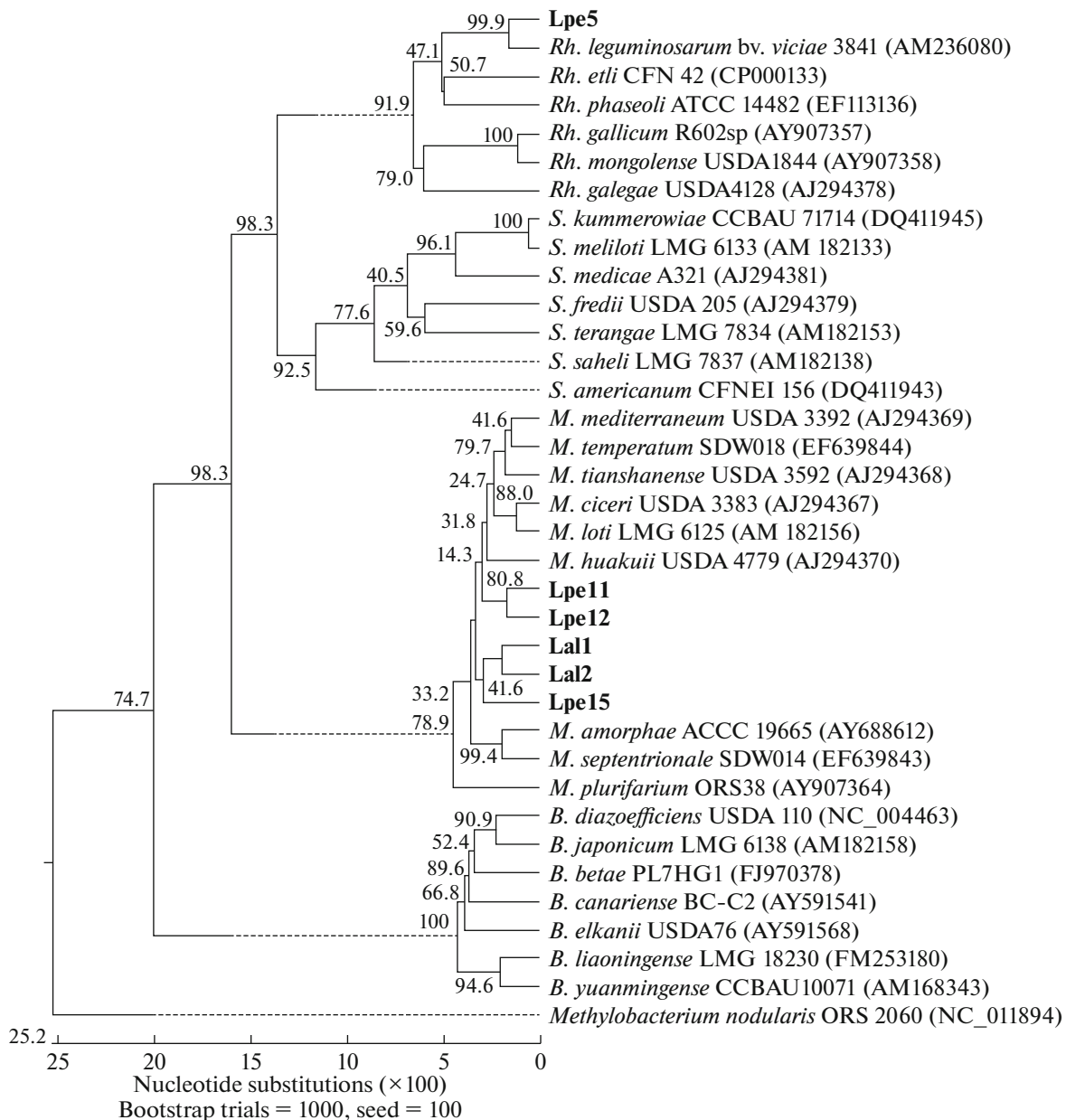


Fig. 2. Phylogenetic tree of nodule bacteria based on comparison of *recA* sequences. The microbial strains studied are shown in bold.

nosarum symbionts which previously were categorized as the biovar *trifolii*. This strictly specific interaction of plants with a definite group of bacteria represents an evolutionary phenomenon and, therefore, has to be considered in the phylogeny and systematics of legumes. Our data demonstrate that the *Lupinaster* species studied are not symbiotically associated with the *Rhizobium* representatives which are natural for *Trifolium* plants. Conversely, the *Lupinaster* plants are associated with bacteria of the *Mesorhizobium* genus. This genus is more specific to other legume groups, for example, the tribe Galegeae [22].

Rhizobial symbiotic genes represented by the virulence genes *nod*, *nol*, and *noe* are responsible for plant host specificity. These genes along with the other symbiotic genes, *nif* and *fix*, are clustered and localized on large plasmids or on chromosomes in regions confined by IS-like elements, which makes possible HGT [23]. This, in turn, leads to strains of rhizobia with foreign symbiotic genes and changed specificity. To exclude the HGT effect on the pool of the symbiotic bacteria derived from the plants studied, phylogenetic analysis of several symbiotic genes was performed. These results demonstrated that the bacterial strains carry *sym* genes, a characteristic of the *Mesorhizobium*

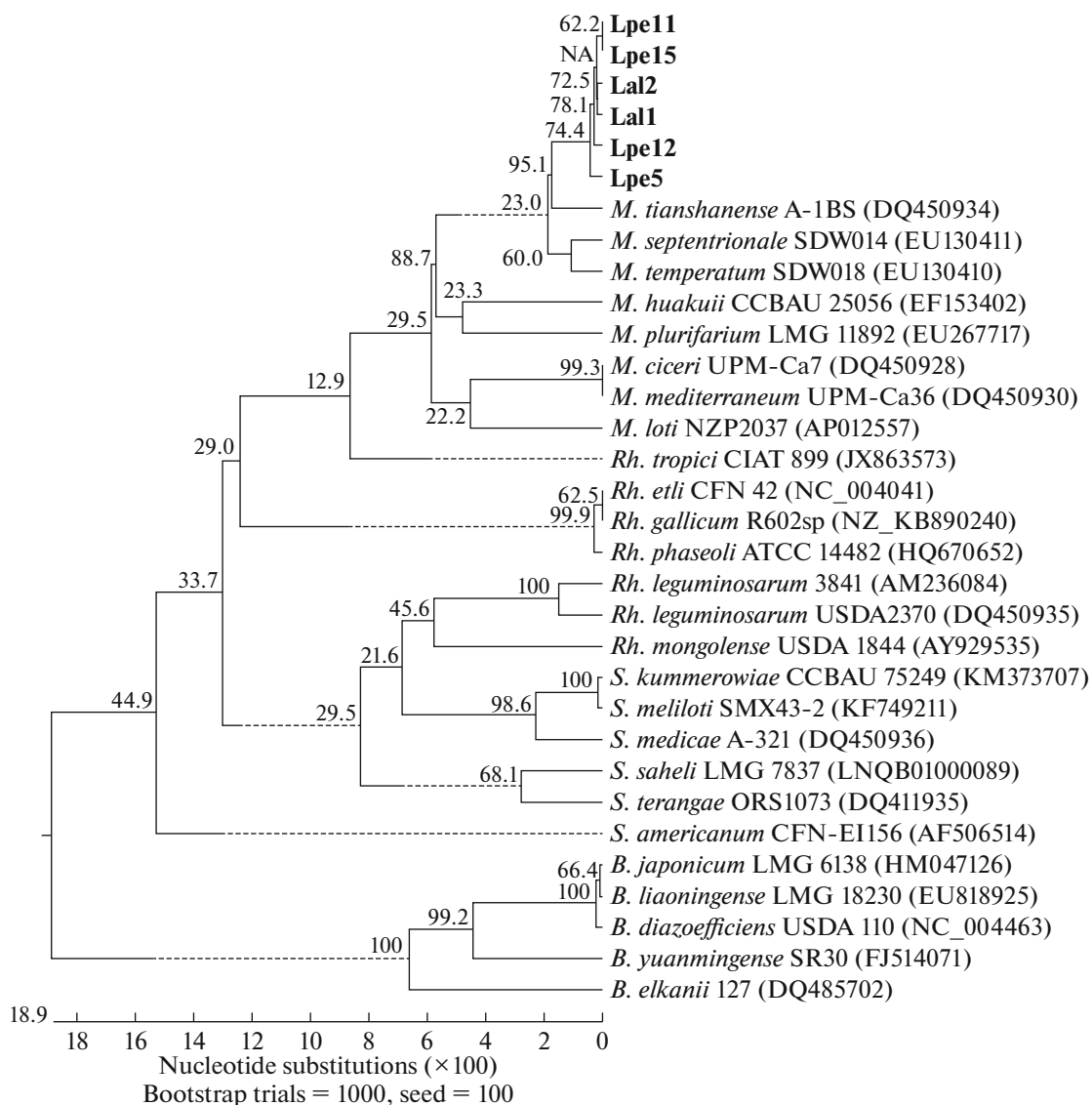


Fig. 3. Phylogenetic tree of nodule bacteria based on comparison of *nifH* sequences. The microbial strains studied are shown in bold.

genus. Bacteria of this genus are likely common microsymbionts of *Lupinaster* plants, which suggests their changed specificity as the result of HGT. For example, symbiotic genes of *R. leguminosarum* bv. *trifolii*, whose partners are *Trifolium* plants, might be transferred to *Lupinaster* species. The fact that the single strain identified as *R. leguminosarum* carries the symbiotic genes found in the representatives of the *Mesorhizobium* genus also suggests the specific plant–microbial interaction. These symbiotic genes, possibly, were horizontally transferred, which allowed the symbiotic interaction with *Lupinaster* plants. Although chimeric strains of nodule bacteria carrying foreign symbiotic genes are not stable and efficient, their presence in nodules of wild type legume species is not exceptional. The genetic pool of the nodule bacteria

from the *Lupinaster* species studied is additional evidence to support a hypothesis that these plants are not members of the *Trifolium* genus.

Interestingly, the phylogeny of the symbiotic genes and that of the housekeeping genes are definitely different. The dendrogram based on the *rrs* and *recA* sequences demonstrates that the bacteria studied belong to different clades within the *Mesorhizobium* genus and even to the entirely different *Rhizobium* genus. Conversely, according to the dendrogram based on the symbiotic genes, all the samples are included in a single compact clade. This means that microsymbionts of *Lupinaster* plants have homologous symbiotic genes, although they are phylogenetically distinct. This also suggests that the microbial phenotype determined by distinct genetic variants of symbiotic genes is

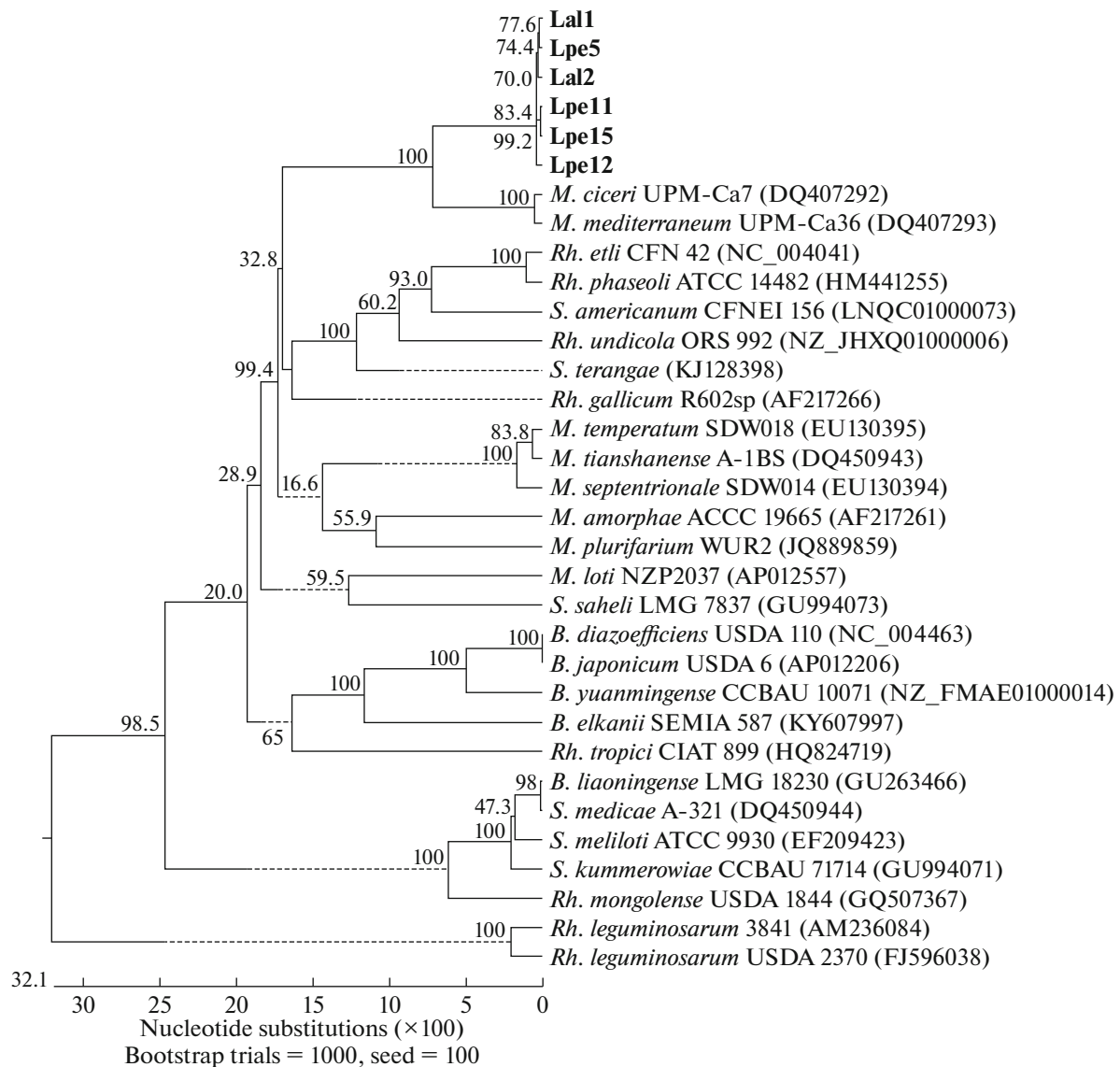


Fig. 4. Phylogenetic tree of nodule bacteria based on comparison of *nodC* sequences. The microbial strains studied are shown in bold.

more important in a choice of a microsymbiont by a plant host than a microbial species to which this microsymbiont belongs.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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