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EXPERIMENTAL ARTICLES

Population Structure of the Clover Rhizobia *Rhizobium leguminosarum* bv. *trifolii* upon Transition from Soil into the Nodular Niche

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Abstract—High-throughput sequencing of the amplicon gene library revealed variations in the population structure of clover rhizobia (*Rhizobium leguminosarum* bv. *trifolii*) upon transition from soil into the root nodules of the host plant (*Trifolium hybridum*). Analysis of rhizobial diversity using the *nodA* gene revealed 3258 and 1449 nucleotide sequences (allelic variants) for the soil and root nodule population, respectively. They were combined into 29 operational taxonomic units (OTU) according to the 97% identity level; 24 OTU were found in the soil population, 12 were present in the root nodule population, and 7 were common. The predominant OTE13 (77.4 and 91.5% of the soil and root nodule populations, respectively) contained 155 and 200 variants of the soil and root nodule populations, respectively, with the nucleotide diversity increasing significantly upon the "soil → root" transition. The "moving window" approach was used to reveal the sites of the *nodA* gene in which polymorphism, including that associated with increased frequency of non-synonymous substitution frequency, increased sharply upon transition from soil into root nodules. PCR analysis of the *IGS* genotypes of individual strains revealed insignificant changes in rhizobial diversity upon transition from soil into root nodules. These results indicate that acceleration of rhizobial evolution in the course of symbiosis may be associated with development of highly polymorphic virulent subpopulations subjected to directional selection in the "plant–soil" system.

Keywords: genetic structure of populations, clover rhizobia (*Rhizobium leguminosarum* bv. *trifolii*), nodulation gene *nodA,* PCR analysis, high throughput sequencing of amplicon libraries, polymorphism of DNA sequences, evolution of symbiosis, positive selection

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Symbiosis with plants or animals is an important factor of bacterial evolution. Higher genome plasticity of symbiotic bacteria due to accumulation of mobile elements (transposons), insertion and repeated DNA sequences, genomic islands and amplicons, as well as development of new types of genomic organization (multicomponent and reduced genomes in facultative and obligate symbionts, respectively) indicate substantial acceleration of bacterial evolution under symbiotic conditions [1].

Root nodule bacteria (rhizobia), the nitrogen-fixing symbionts of legumes, are convenient objects for studying the molecular and population genetic mechanisms of the evolution of symbiosis. The role of host plants in formation of the rhizobial population structure was shown to be more important than that of soil and climatic factors [2]. In the presence of hosts, the sizes of rhizobial populations increase by several orders of magnitude, up to 10^7–10^8 cells per 1 g of soil or 20–25% of the total number of culturable bacteria [3]. The high quantities of rhizobial populations under the conditions of symbiosis are usually combined with their enhanced heterogeneity [4], demonstrating the key role of the host in the evolution of microsymbionts. It has been most thoroughly studied for the strains of *Sinorhizobium meliloti* entering into symbiosis with the cultivated alfalfa, *Medicago sativa*, [5] and for the strains of *Bradyrhizobium* interacting with the wild legume *Amphicarpaea* [6]. At the same time, the absence of host plant resulted in decreased population diversity of *S. meliloti* [7] and *B. japonicum* [8, 9].

The mechanisms responsible for enhanced diversity of rhizobial populations under the conditions of symbiosis include induction of bacterial mutations in planta, as has been shown in the studies of colony morphology [10], plasmid profiles [11], and nitrogen-fixing activity [12]. Rhizobia are characterized by high intensity of gene transfer in the rhizosphere and root nodules [13–15] and therefore formation of panmictic populations [16]. However, symbiosis-specific forms of selection (frequency-dependent, inter-deme, and kin) play the key role in enhancement of genetic polymorphism of rhizobia, enabling their mutualistic interaction with plants [1].

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The difficulty of studying the microevolution of rhizobia is determined by the fact that their population dynamics has been characterized until now in restricted and unrepresentative sample groups of strains isolated by the standard microbiological methods from soil or root nodules as pure cultures. The structure of these populations was often judged by genetic markers not related to symbiosis, which restricted the study of interrelationship between the population dynamics of rhizobia and the symbiotic properties determining this dynamics.

In the present work, we used the newly developed technique of the *nodA* gene pyrosequencing for the amplicon library of clover rhizobia and showed that enhancement of bacterial diversity during the "soil → nodules" transition affected the dominant genotypic class with a high activity of nodule formation. Directional selection at one of the *nodA* gene regions suggests the possibility of using the developed system in the study of relationships between the molecular and population genetic mechanisms of symbiotic evolution.

MATERIALS AND METHODS

Collection and isolation of bacteria. The population dynamics of clover rhizobia (*Rhizobium leguminosarum* bv. *trifolii*) was analyzed in a sample taken from 100 root nodules of Alsatian clover (*Trifolium hybridum*) and the soil where it grew (collected in the Golitsky Botanical Preserve, Ternopol region, Ukraine). The meadow phytocenosis where the biological material was sampled from was characterized by high diversity of legumes: it also included other clover species (*T. montana, T. ochroleucon, T. pratense, T. repens*, and *T. rubens*), as well as members of the genera *Anthyllis, Astragalus, Coronilla, Lathyrus, Lotus, Medicago, Melilotus, Onobrychis*, and *Vicia*. The samples of Alsatian clover root nodules and soil were collected from the depth of 5–15 cm and 10–20 cm, respectively. Strains were isolated and stored by the standard methods [17].

DNA extraction. DNA was extracted from 0.2 g of frozen soil and from the combined mass of root nodules after their homogenization with glass beads for 1 min at the maximum power in FastPrep 24 (MP Medicals, United States). The material was in an extraction buffer containing 350 μL of solution A (sodium phosphate buffer, 200 mM; guanidine isothiocyanate, 240 mM; pH 7.0), 350 μL of solution B (Tris-HCl, 500 mM; SDS, 1% wt/vol; pH 7.0), and 400 μL of phenol–chloroform mixture (1 : 1). The resultant preparation was centrifuged at 13000 *g* for 5 min. The aqueous phase was collected and extracted with chloroform. DNA was precipitated by adding an equal volume of isopropyl alcohol. After centrifugation, the precipitate was washed with 70% ethanol and dissolved in water at 65°C for 5–10 min. DNA was purified by electrophoresis in 1% agarose gel and isolated by sorption on silicon oxide [18].

Analysis of nucleotide diversity of the *nodA* gene in total populations. The variability of total populations from soil and root nodules was analyzed as follows. Two pairs of universal primers flanking the *nodA* gene were constructed, based on analysis of 20 nucleotide sequences of this gene (from GenBank) belonging to the species *R. leguminosarum*. The sequences were aligned using ClustalX; degenerate primers were constructed using the Primer Premier 5 software package. Two pairs of primers (ndARL268_F DGGHYTGTAYGGAGTGC; ndARL302_F YTDGGMATCGCHCACT / ndARL591_R AGYTCSSACCCRTTT; ndARL518_R RDACGAGBACRTCTTCRGT) were used for amplification of the *nodA* gene fragment with *Taq* polymerase (Evrogen, Russia) by the scheme of two-round PCR (nested PCR). The external pair of primers (ndARL268_F and ndARL591_R) with the original DNA templates (from soil or root nodules) was used in the first round and the internal pair of primers (ndARL302_F and ndARL518_R) was used in the second round, with the amplificate from the first round used as a template (the annealing temperature in both rounds was 50°). This PCR scheme made it possible to amplify the internal *nodA* gene region (181 bp) using the soil and root nodule DNA samples as initial templates.

The fusion primers constructed for the amplicon library sequencing in a GS Junior (Roche) contained, in addition to the *nodA* sequences, multiplex identifiers providing for simultaneous sequencing of several libraries. The procedure was performed according to the Roche's protocol (unilateral reading, Lib-L library).

Investigation of the *IGS* marker polymorphism in individual strains. Diversity of the individual strains was assessed by studying polymorphism of the *IGS* chromosome locus, an intergene region within the 16S rRNA gene cluster (primers FGPS1490 and FGPL1329) [19]. PCR was performed in a MyCyclerTM amplifier (Bio-Rad, United States) (annealing temperature, 55°C) using *Taq* polymerase and deoxynucleoside triphosphates (Helicon, Russia). Bacterial culture lysates were used as templates; each colony was resuspended in 20 μL of lysing solution (25 mM NaOH, 0.25% SDS), heated for 5 min at 95°C, brought up to 200 μL with distilled water, centrifuged, and used for the reaction in the amount of 1 μL (5–10 ng DNA).

Resultant PCR fragments were restricted with the *Msp*I enzyme (MBI Fermentas, Lithuania) and analyzed in 3% agarose gel (Helicon, Russia) with ethidium bromide (0.5 mg/L). The number of restriction fragments for each strain did not exceed 10, while their sizes varied from 50 to 700 bp.

Bioinformation and statistic analyses. The diversity of the total population was assessed at the level of the *nodA* gene sequences after their filtration with the

Table 1. Genotypic structure of *Rhizobium leguminosarum* bv. *trifolii* populations from soil and clover root nodules

Compared characteristics and population origins		Loci for genotyping the populations	
		nodA	*IGS*
Analyzed biological units		Allelic variants (DNA sequences)	Individual strains
Number of isolated units	Soil	3258	41
	Root nodules	1449	15
Revealed genotypes		Groups of allelic variants with a similarity level of at least 97% (OTU)	PCR genotypes
Number of genotypes	Total	29	8
	Soil	24	7
	Root nodules	12	6
	Common for soil and root nodules	7	5
Nei's indices	Soil	0.380	0.710
	Root nodules	0.161	0.819

GS Junior bundled software; the sequences (3600 for the soil library and 1640 for the root nodule library) were aligned using the MUSCLE algorithm (UGENE, Russia), followed by removal of the sequences with reading frame shifts, which most probably resulted from sequencing errors (the errors not resulting in a frame shift were not analyzed, since their probability did not depend on the library origin and they did not influence the results of comparison of the soil and root nodule populations). After the filtration, 3258 sequences (allele variants of the *nodA* gene) from the soil library and 1449 sequences from the root nodule library were left for further analysis (the number of different sequences in the combined metapopulation circulating in the "soil–nodules" system was 3345).

The QIIME software [20] was used for cluster analysis of the combined pool of sequences; 56 operational taxonomic units (OTUs) containing the sequences with no less than 97% similarity level were isolated. After removing the OTU with single sequences, one sequence from each of the remaining 29 OTU was taken for cluster analysis.

The levels of nucleotide polymorphism were analyzed using MEGA 5.1 [21] and DnaSP 5.10.01 [22]; reliability of the differences was analyzed using the chi-square method and Student's *t*-criterion. Genotypic polymorphism was assessed using Nei's gene diversity index: $H_N = (1 - \square F_i^2)\ [n/(n-1)]$, where F_i is the frequency of the *i*th genotype (*IGS* or OTU) and *n* is the number of revealed genotypes [23, 24].

The measure of nucleotide diversity was p-distance (the number of mismatches per nucleotide position during pairwise sequence comparison). The "sliding window" analysis of nucleotide diversity (50 bp, 5-bp pace) was performed using a p-distance analog: Pi value (the average number of mismatches per nucleotide position in the "window" for two randomly selected sequences).

The analysis of the ratio of nonsynonymic and synonymic substitutions at each codon (the *dN/dS* statistics) was performed with the HyPhy program integrated in MEGA 5.1.

RESULTS

Analysis of the *nodA* gene sequence diversity (allelic variants) at the level of operational taxonomic units (OTU) revealed (Table 1) the presence of 24 out of 29 OTU in the soil population and only 12 OTU in the root nodule population ($\chi^2 = 4.0$; $P_0 < 0.05$). At the same time, 5 and 17 OTU were strictly specific for the root nodule and soil population, respectively, and only 7 OTU were detected in both populations, which indicates their substantial differentiation coupled with the evolution of the *nodA* gene. The Nei's index for the soil population was twice the value for the root nodule one, indicating its greater diversity defined in terms of both the number of revealed genotypes (OTU) and alignment of strain distribution among the genotypes [25].

Genotypic variability (Nei's indices) was much higher at the *IGS* locus, functionally unrelated to symbiosis, than at the *nodA*, since the dominant genotype (OTU13) by the *nodA* locus was detected in both populations, while no dominant PCR genotype by the *IGS* locus was found. Genotypic diversity by the *IGS* locus in the root nodule population was insignificantly higher than in the soil population, while the diversity by the *nodA* locus was much higher in the soil popula-

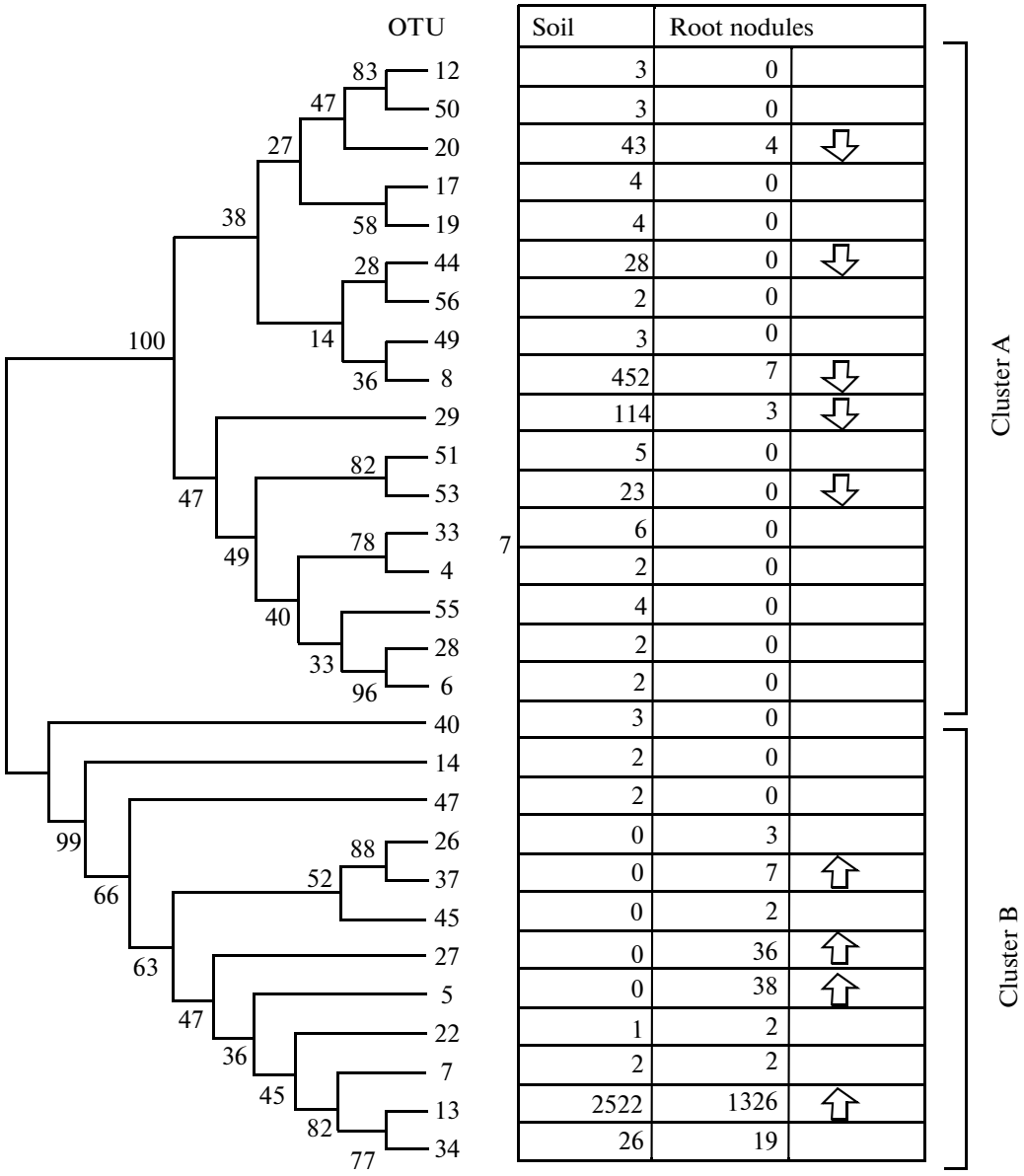

Fig. 1. Phylogenetic relationships between the operational taxonomic units (OTU) revealed by analysis of rhizobial polymorphism according to the *nodA* gene. The upward or downward arrows indicate significant ($P_0 < 0.01$) increase or decrease in the OTU frequencies during the "soil → nodules" transition, respectively. For the sequences from the dominant OTU13, the frequency is 91.5 ± 0.73% (1326/1449) in the root nodule population and 77.4 ± 0.72% (2522/3258) in the soil population.

tion. Only 7 out of 29 OTU (24%) and most of the 8 *IGS* genotypes were common for the root nodule and soil populations (5 genotypes or 63%; the difference was significant at $P_0 < 0.05$) (Table 1).

Phylogenetic data analysis showed the combined population of the *nodA* gene sequences to be distributed among two clusters (A and B) with bootstrap support of more than 99% (Fig. 1). The OTU found only in soil but absent in the root nodules predominated in cluster A (14 out of 17, or 82 ± 9.3%) but were in the minority in cluster B (3 out of 12, or 25 ± 12.5%; the difference was significant at $P_0 < 0.01$). Nine OTU demonstrated highly significant ($P_0 < 0.01$) differences in the frequency of occurrence in soil and root nodule populations, including 4 cases of significant ($P_0 < 0.01$) increase in OTU frequency during the "soil → nodules" transition (all of them within cluster B) and 5 cases of decrease in this frequency (all within cluster A). It was shown that these clusters were formed by the highly diverged allelic variants of the *nodA* gene: the average nucleotide diversity (p-distance) was 0.055 ± 0.009 and 0.073 ± 0.011 within clusters A and B and 0.277 ± 0.025 between the clusters.

Cluster B was shown to contain the dominant OTU13 with a significantly higher frequency of occurrence in the root nodule population compared to the

Table 2. Comparison of the *nodA* gene polymorphism (partial sequence, 181 bp) from soil and root nodule populations (for the dominant operational taxonomic unit OTU13)

Compared parameters	Populations		Statistical comparisons*
	soil	root nodule	
Number of allelic variants (out of 1100 variants studied for each population)	155	200	$\chi^2 = 5.70$ ($P_0 < 0.01$)
Diversity of allelic variants (Nei's index)	0.451 ± 0.019	0.762 ± 0.001	$t_{St} = 16.32$ ($P_0 < 0.001$)
Number of nucleotide positions with the highest polymorphism**	60	96	$\chi^2 = 8.31$ ($P_0 < 0.01$)
Number of "sliding windows" (50 bp) with the highest polymorphism***	8	19	$\chi^2 = 4.48$ ($P_0 < 0.05$)
p-Distances (calculated for random samples out of 50 and 100 allelic variants)	0.030 ± 0.004 and 0.044 ± 0.006	0.070 ± 0.007 and 0.080 ± 0.005	$t_{St} = 4.91$ and 4.62 ($P_0 < 0.001$)

* χ^2 values were calculated to compare experimental data to the 1 : 1 ratio anticipated in the absence of differences between the soil and root nodule populations.

** Equal polymorphism was revealed for 25 positions in the populations.

*** Equal polymorphism was revealed for 1 "window" in the populations. Reliably ($t_{St} = 3.79$; $P_0 < 0.001$) higher Pi value in the root nodule population compared to the soil one was revealed for the set of 28 windows.

soil population (91.51 ± 0.73 and $77.4 \pm 0.72\%$; $P_0 < 0.001$). This OTU was used to analyze the *nodA* gene polymorphism at individual nucleotide positions (a 181-bp gene fragment was analyzed). This analysis (Table 2) showed that the root nodule population considerably surpassed the soil population in the number of allelic variants of the *nodA* gene revealed in OTU13 (200 and 155, respectively) and in the diversity of both individual nucleotide positions (Nei's indices: 0.762 and 0.451, respectively) and the groups of 50 adjacent positions revealed by the "sliding window" method (Table 2, Fig. 2). At the same time, the studied gene fragment was shown to contain two compact regions with higher frequencies of nucleotide substitutions in the root nodule population compared to the soil population, and a region between them with an insignificant predominance of substitutions in the soil population.

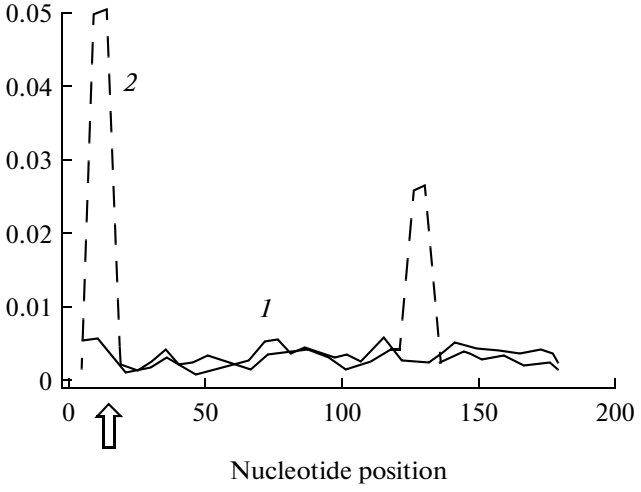

Fig. 2. Comparison of nucleotide polymorphism of the *nodA* gene fragment (181 bp) in soil (*1*, solid line, Pi1) and root nodule (*2*, dotted line, Pi2) populations within OTU13 analyzed by the "sliding window" method (window size, 50 bp; step, 5 bp; the Y-axis shows Pi values). The arrow indicates the nonsynonymous nucleotide substitution in the CTT→TTT codon (enhanced frequency of its occurrence shows the action of dynamic selection during transition of the population from soil into root nodules).

It should be noted that local enhancement of nucleotide diversity in the former region was determined mainly by substitution in the triplet CTT→TTT (positions 298–300 in the full-sized *nodA* gene, 12–15 in the fragment shown on Fig. 2) nearly in the half of "root nodule" sequences. This nucleotide substitution is not synonymous and leads to an amino acid substitution (Leu→Phe) in the NodA protein. Statistical analysis revealed that the frequency of this substitution in the root nodule population was significantly higher than the frequency anticipated during the neutral evolution ($dS = 1.755$; $dN = 13.596$; $p = 0.0069$), which was an evidence of the directional character of selection occurring at this codon during the transition of rhizobial population from soil into root nodules.

DISCUSSION

Acceleration of bacterial evolution during the interactions with plant or animal hosts is typical for various forms of symbiosis, including mutualistic and antagonistic interrelationships. This acceleration has been documented both at the level of the overall bacterial genome structure and in organization of individual "symbiotic" genes [1]; however, its population mechanisms are poorly studied. The experimental

Table 3. Changes in the structural diversity of the *nodA* gene during the "soil → nodules" transition revealed at different levels of population analysis

Levels of population structure analysis	Total population	Predominant genotype
Compared genetic units	Operational taxonomic units (OTU)	Allelic variants in the dominant OTU13
Number of revealed units	24 → 12	155 → 200
Nei's indices	0.380 → 0.161	0.451 → 0.762
Frequencies (%) of allelic variants	97.3 → 43.3	77.4 → 91.5
Nucleotide diversity (p-distance)	0.034 → 0.009	0.003 → 0.007

data used to assess the population dynamics of symbiotic bacteria are still extremely insufficient, because they have been obtained from the analysis of restricted and unrepresentative strain samples, which, even in case of the most thoroughly studied rhizobia, represent less than 1% of their actual genetic diversity.

In order to overcome this restriction, we analyzed a representative amplicon library of rhizobial populations using the *nodA* gene, which is a specific component of the symbiotic system. This gene belongs to the group of "common *nod* genes" involved in the synthesis of the core part of signaling Nod factors, which activate the development of root nodules colonized by rhizobia [26]. The *nodA* gene is structurally homologous and functionally interchangeable in taxonomically distant groups of rhizobia, which is due to its conservative function (attachment of the acyl residue to the oligochitin chain of the Nod factor). The *nodA* gene is absent in proteobacteria related to rhizobia; it is likely that rhizobia have acquired it via horizontal gene transfer from gram-positive soil bacteria or mycorrhizal fungi [27]. Since most of the *sym* genes have been "recruited" to perform symbiotic functions from native rhizobial genomes and have close relatives in the related free-living bacteria [1], the *nodA* gene can be used to characterize not only the general population structure of rhizobia, but also the polymorphism of the evolutionally plastic symbiotic system.

Our data show (Table 3) that the number of genotypes (OTUs) revealed by the *nodA* gene, as well as their diversity indices, drastically decreased during the transition of clover rhizobia from soil into the root nodule niche of the host plant. This decrease may be due to the fact that only the virulent genotypes showing high competitiveness under field conditions penetrate the root nodules.

At the same time, the *nodA* gene diversity revealed by analysis of the sequences (allelic variants) belonging to the dominant OTU13 drastically increased during the "soil → nodules" transition. These data confirm the effect of enhancement of rhizobial polymorphism during the transition into the root nodule niche as a result of frequency-dependent selection that has been predicted previously based on the analysis of mathematical models of symbiotic evolution [1].

In order to demonstrate the interrelationship between the changes in population structure revealed during the "soil → nodules" transition and the symbiotic functions of bacteria, we compared the parameters of population polymorphism according to the symbiotic *nodA* gene and the *IGS* locus, which is not functionally related to symbiosis and localized in the chromosomal part of the genome (the *nodA* gene in clover rhizobia is extrachromosomal). It was shown that the genotypic compositions of the soil and root nodule populations of rhizobia at the *IGS* and *nodA* loci were substantially different (Table 1). Differentiation of these populations is probably associated with divergence of the nodulation genes: it may be supposed that not all of the *nodA* alleles provide for efficient infection of plants under field conditions. The genotypic structure of the population at the *IGS* locus, which is not associated with symbiosis, insignificantly changed during the "soil → nodules" transition (Table 1), demonstrating that the revealed changes in rhizobial population were indeed determined by development of the symbiosis.

Data analysis suggests that enhanced diversity of rhizobia observed during their interaction with plants influences mostly the symbiotically active part of the population, which continuously circulates between the soil and root nodule niches. This part of the population undergoes the most significant selection, which may be revealed by analyzing the structure of the *nodA* gene responsible for the synthesis of the signaling Nod factor determining infection of the host.

It should be noted that the frequency ratios of nucleotide substitutions in the root nodule and soil populations varied for different regions of the *nodA* gene (Fig. 2): the region characterized by the highest frequency of substitutions in the root nodule population was shown to contain a codon, in which nonsynonymous nucleotide substitutions dominated over synonymous substitutions, demonstrating the effect of dynamic selection. Hence, it may be suggested that the NodA protein contains functionally different domains that may be influenced by differently directed selection pressures induced during the plant–bacteria interaction (disruptive selection). The extremely low frequency of occurrence of the allele of the *nodA* gene with the TTT codon at positions 298–300 in the soil population is of particular interest because, according

to the available data [26, 27], this gene is inactive in free-living rhizobia and, consequently, is not to be influenced by selection.

Elucidation of the forms and quantitative characterization of symbiosis-specific forms of selection is of great interest and will be an object of our further research. However, the available data show that the interaction between rhizobia and plant hosts is accompanied by substantial enhancement of the tempos of evolution, which is associated with the changes in the genes controlling the transition of bacteria from soil into endosymbiotic (root nodule) niche.

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