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Characteristics of Natural Selection in Populations of Nodule Bacteria (*Rhizobium leguminosarum*) Interacting with Different Host Plants

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Abstract—Using high throughput sequencing of the *nod*A gene, we studied the population dynamics of *Rhizobium leguminosarum* (bv. *viciae*, bv. *trifolii*) in rhizospheric and nodular subpopulations associated with the leguminous plants representing different cross-inoculation groups (*Vicia sativa, Lathyrus pratensis* of the vetch/vetchling/pea group and *Trifolium hybridum* of the clover group). The “rhizosphere → nodules” transitions result in either an increase or decrease in the frequencies of 10 of the 23 operational taxonomic units (OTUs) (which were identified with 95% similarity) depending on the symbiotic specificity and phylogenetic positions of OTUs. Statistical and bioinformatical analysis of the population structures suggest that the type of natural selection responsible for these changes may be diversifying at the whole-population level and frequency-dependent at the OTU-specific level, ensuring the divergent evolution of rhizobia interacting with different host species.

Keywords: natural (diversifying, frequency-dependent) selection, divergent evolution, nodule bacteria (*Rhizobium leguminosarum*), legume-rhizobia symbiosis, common vetch (*Vicia sativa*), meadow vetchling (*Lathyrus pratensis*), alsike clover (*Trifolium hybridum*), high throughput sequencing, *nod*A gene.

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INTRODUCTION

The formation and functioning of legume-rhizobial symbiosis is accompanied by severe transformations of bacterial populations that occur during rhizobial transitions from the soil and rhizosphere into nodule niches provided by plant hosts [1]. These transitions are related to signal interactions between the partners: lipochitooligosaccharide Nod-factors synthesized by rhizobia under the control of *nod*-genes affect plant receptors encoded by *NFR*-genes and activating nodules development [2]. The colonization of intra- and intercellular compartments in rhizobia activated by these signals is accompanied by the induction of symbiosis-specific selection forms, which directs its evolution toward an increase in N_2-fixation intensity and the ecological adaptability of partners [3].

However, experimental data allowing the study of the direction, form, and intensity of the selection occurring in the rhizobial population during symbiosis with other plants are rather limited, possibly because the analysis of population rhizobial dynamics was performed on a small sample of strains isolated from the soil or nodules by traditional microbiological methods and therefore represents an insignificant part of rhizobial genepool.

Using the method of high-throughput sequencing of *nod*A gene (encoding for Nod-factors acylation [2]), we previously reported [4] rapid modifications in the gene structure in *Rhizobium leguminosarum* bv. *trifolii* populations as a result of their migration from the soil to the nodules of clover *Trifolium hybridum*. In the present study we characterized the form and direction of a natural selection causing these changes in a more diverse *R. leguminosarum* population consisting of two biotypes—bv. *trifolii* (clover rhizobia) and bv. *viciae* (vetch/vetchling/pea rhizobia)—interacting with symbiotic host plants. Our findings show that the selection in the rhizobial population during the migration from rhizosphere to nodule niches demonstrates a diversifying and frequency-dependent character that causes divergent rhizobial evolution via interaction with different cross-inoculation groups (CIG) of host plants.

MATERIALS AND METHODS

Experimental material collected on a long-fallow field near Vyritsa (Gatchinskii region of Leningradskaya oblast, 59°24′7.74″ N; 30°15′28.74″ E) was used in the study. Thirty nodule-containing plants, as well as rhizospheric soil probes for three leguminous species were selected: the common vetch *Vicia sativa*, the meadow vetchling *Lathyrus pratensis* (both species belong to vetch/vetchling/pea CIG and symbiotically interact with *Rhizobium leguminosarum* bv. *viciae*), and the alsike clover *Trifolium hybridum* (from clover CIG symbiotically interacting with *R. leguminosarum* bv. *trifolii*). The nodules collected from these plants were combined into three pools, and three soil pools obtained from the combination of samples selected from vetch, vetchling, and clover rhizosphere were also formed.

Each of the nodule probes was frozen in liquid nitrogen, homogenized, and used for DNA isolation, together with soil samples using PowerSoil® DNA Isolation Kit (MoBio, United States). Purified DNA was used in two-round PCR with two primer pairs. For the first round we used degenerate primers for *nod*A gene ndARL268_F DGG HYTGTAYGGAGTGC and ndARL591_R AGYTCSSACCCRTTT. The second round of PCR was performed with primers ndARL302_F AYTDGGMATCGCHCACT and ndARL518_R RDACGAGBACRTCTTCRGT. These primers allow the amplification of *nod*A gene alleles in clover, vetch, and vetchling rhizobia with the same efficacy. Two-round PCR allows the amplification of PCR product from soil DNA, in which the *nod*A gene content is extremely low. Secondary sequences, including adapters and multiplex identifiers necessary for pyrosequencing, were added to the primers used in the second round of PCR according to Roche protocol. All PCR included high-fidelity Encyclo polymerase (Evrogen, Russia) under the following temperature conditions: 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s (35 cycles).

The libraries were sequenced with GS Junior Analyzer (Roche) in the Genomic Technologies and Cell Biology collective use center of the All-Russia Research Institute for Agricultural Microbiology. For each library three to four thousand sequences 200 bp in length were obtained and analyzed with the QIIME package. The analysis included a rejection of short sequences, as well as sequences containing inserts and deletions with a shifted reading frame. The remaining sequences were sorted by multiplex identifiers into six libraries. The sequences were aligned with efficient algorithms (pyNAST), while cluster analysis and division into operational taxonomic units (OTU selection) was conducted with the UCLUST algorithm at 95% similarity. After the deletion of minor OTUs represented by one to two sequences, 23 OTUs remained, and the selection of a representative sequence for cluster analysis was performed for each of them.

We used Nei's diversity index to assess genotypic polymorphism: $H_N = \left(1 - \sum F_i^2\right)[n/(n-1)]$, where F_i is the frequency of ith genotype (OTU) and n is the number of detected OTUs [5, 6]. The Student's t-test was used for statistical data analysis [7]. A previously developed model [8] of multistrain competition for nodule formation was used to characterize the selection affecting rhizobia.

RESULTS

Analysis of the data revealed that the frequencies of 23 detected OTUs significantly varied (ranging from 0 to 96.99%) depending on the DNA library origin in six rhizobial subpopulation isolated from rhizospheric soil and nodules of three plant species (Table 1, Fig. 1). For the ten most numerous OTUs, we observed significant differences in the frequencies of at least one plant in the rhizospheric and nodule libraries, which is evidence of a positive or negative selection affecting this OTU during the rhizobial transition into the symbiotic phase of the life cycle. It is necessary to mention that all OTUs lacking frequency differences during the rhizosphere-to-nodule migration are minor (with a frequency of less than 0.3%), and, hence, the selection related to plant inoculation appears to be a significant condition for the maintenance of increased strain quantity in populations.

The previously proposed indices of nodule niche inoculations (n_i) characterize the dependence between the frequencies of the ith strains in the inoculum (f_i) and in nodules $\left(g_i^{ni}\right)$. To analyze the studied system, inoculation indices were calculated by modified formula:

$$n_i = \sqrt[3]{\frac{\ln(g_i) + \ln(N_N/N_S)}{\ln(f_i)}},$$

where n_i is the inoculation index of the nodule niche of a distinct host plant of the ith OTU, N_N and N_S are the summarized (for all OTUs) numbers of the sequences in the nodule and rhizospheric subpopulations, f_i is the frequency of the ith OTU in the rhizospheric subpopulation, and g_i is the frequency in the nodule subpopulation.

Data analysis demonstrated (Table 2) that the selection acting on the ith OTU is positive (its frequency significantly increases with "rhizosphere → nodule" transition) under $n_i \leq 0.90$ and is negative (its frequency decreases) under $n_i \geq 1.14$, while selection is absent (the OTU frequencies in rhizospheric and nodule niches have no significant differences) in the case of intermediate n_i values.

Statistical data analysis makes it possible to distinguish two clusters, A and B, containing 12 and 11 OTUs, respectively (Fig. 1). The negative selection during the migration from rhizosphere to *V. sativa* and *L. pratensis* nodules acts on the OTUs comprising cluster A, while the positive selection acts during migration in *T. hybridum* nodules (Table 3). The frequencies of the *nod*A sequences of cluster A were 4.7–4.8 times higher in samples of rhizospheric soil from clover than in samples from vetch and vetchling, while in the nodules they differed by more than 40 times.

Table 1. Frequencies (%) of OTUs detected as a result of the analysis of *nod*A gene sequences in rhizospheric (R) and nodule (N) populations of *Rhizobium leguminosarum* interacting with three leguminous species

OTU (Fig. 1)	Origin of *nod*A sequences					
	Vicia sativa		*Lathyrus pratensis*		*Trifolium hybridum*	
	R	N	R	N	R	N
1	0	0	0	0	0.03 ± 0.03	0.11 ± 0.05
3	0	0.07 ± 0.04	0	0	0.27 ± 0.07	0.11 ± 0.05
10	0	0.02 ± 0.02	0.02 ± 0.02	0	0.73 ± 0.14	0.05 ± 0.04↓
11	0	0	0.02 ± 0.02	0	0.27 ± 0.07	0.22 ± 0.06
17	0.08 ± 0.05	0.02 ± 0.02	0.17 ± 0.06	0.03 ± 0.03	0.03 ± 0.03	0
12	0	0	0.02 ± 0.02	0	0.05 ± 0.04	0
4	0.03 ± 0.03	0	0.02 ± 0.02	0	0	0
23	15.71 ± 0.59	1.89 ± 0.21↓	16.42 ± 0.58	0.25 ± 0.08↓	75.73 ± 0.61	82.89 ± 0.52↑
20	1.01 ± 0.16	0.10 ± 0.05↓	0.15 ± 0.06	0.11 ± 0.05	2.91 ± 0.19	15.07 ± 0.58↑
7	0.05 ± 0.03	0	0.02 ± 0.02	0	0.14 ± 0.05	0.09 ± 0.05
21	0.69 ± 0.14	0.02 ± 0.02↓	0.10 ± 0.05	0.08 ± 0.05	1.64 ± 0.20	1.19 ± 0.16
19	0.05 ± 0.03	0	0	0	0.03 ± 0.03	0.16 ± 0.05
14	0.03 ± 0.03	0	0	0	0	0
5	0.05 ± 0.03	0	0	0.03 ± 0.03	0.03 ± 0.03	0
9	0	0	0.10 ± 0.05	0	0	0
2	0.03 ± 0.03	0.05 ± 0.04	0	0.03 ± 0.03	0	0
16	1.07 ± 0.17	1.30 ± 0.18	0.07 ± 0.04	0.63 ± 0.13↑	0	0
13	0	0	0	0.05 ± 0.03	0	0
18	1.15 ± 0.17	5.38 ± 0.36↑	1.05 ± 0.16	1.01 ± 0.16	0	0
0	0	0.39 ± 0.10↑	0.05 ± 0.03	0.16 ± 0.07	0	0
22	78.85 ± 0.67	83.53 ± 0.58↑	81.76 ± 0.02	96.99 ± 0.28↑	18.15 ± 0.22	0.11 ± 0.04↓
15	1.20 ± 0.18	6.87 ± 0.40↑	0.02 ± 0.02	0.63 ± 0.13↑	0	0
8	0	0.34 ± 0.09↑	0	0	0	0
N	3749	4073	4111	3650	3713	4472

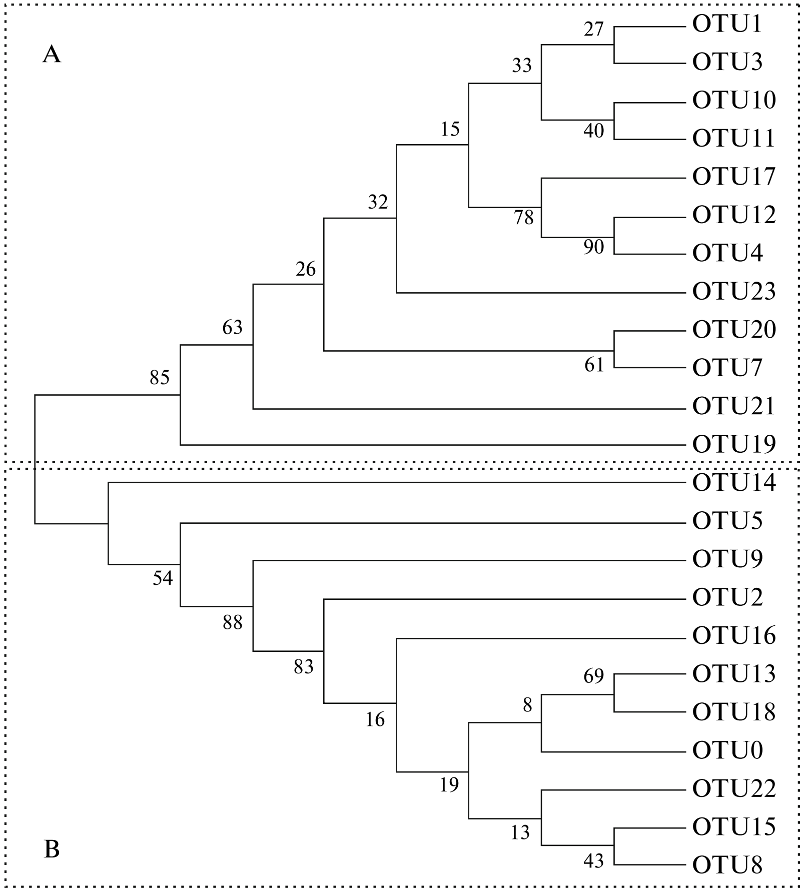
OTU frequencies with standard errors are shown. The arrows directed up or down stand for a significant ($P_0 < 0.01$) increase or decrease in the frequencies during rhizobial migration from rhizosphere into nodule niche; the gray background indicates combinations of OTU frequencies as evidence in the selection (*N* is the total number of sequences).

OTU23 represents the most numerous OTU detected in this cluster specific to *T. hybridum* (Table 1).

Other results were obtained for *nod*A sequences from cluster B (Fig. 1, Table 1): a positive selection during the migration from rhizosphere to *V. sativa* and *L. pratensis* nodules affects the included OTUs; however, the migration to *T. hybridum* nodules results in negative selection. The frequencies of cluster B *nod*A sequences were 4.5 times lower in the samples of rhizospheric soil from clover than in the samples from vetch and vetchling, while there was a 900-fold difference in the nodules (Table 3). OTU22 appears to be the most common OTU detected in cluster B specific for *V. sativa* and *L. pratensis* (Table 1).

The number of OTUs detected in the rhizospheric rhizobial population in all three leguminous species appeared to be higher than in the nodule population (Table 4). All nodule OTUs are presented in rhizospheric soil in the clover-associated rhizobia, while three OTUs were detected in vetch- and vetchling-associated rhizobia present in the nodules rather than in rhizosphere. For the vetch and vetchling rhizobia, the quantity of OTUs detected only in rhizospheric subpopulations was higher than in clover rhizobia (Table 4).

It should be noted that four of the ten OTUs detected in clover nodules were absent in vetch and vetchling nodules (they all belong to cluster A). Eight of 15 OTUs detected in vetch and vetchling nodules were absent in clover nodules (seven of them belong to cluster B). All OTUs associated only with one CIG are



	Vic_nod	Vic_soil	Lath_nod	Lath_soil	Trif_nod	Trif_soil
OTU1	0	0	0	0	5	1
OTU3	3	0	0	0	5	10
OTU10	1	0	0	1	2	27
OTU11	0	0	0	1	10	10
OTU17	1	3	1	7	0	1
OTU12	0	0	0	1	0	2
OTU4	0	1	0	1	0	0
OTU23	77	589	9	675	3707	2812
OTU20	4	38	4	6	674	108
OTU7	0	2	0	1	4	5
OTU21	1	26	3	4	53	61
OTU19	0	2	0	0	7	1
OTU14	0	1	0	0	0	0
OTU5	0	2	1	0	0	1
OTU9	0	0	0	4	0	0
OTU2	2	1	1	0	0	0
OTU16	53	40	23	3	0	0
OTU13	0	0	2	0	0	0
OTU18	219	43	37	43	0	0
OTU0	16	0	6	2	0	0
OTU22	3402	2956	3540	3361	5	674
OTU15	280	45	23	1	0	0
OTU8	14	0	0	0	0	0

Fig. 1. Cluster analysis of *nod*A gene sequence distributions by OTUs detected as a result of analysis of libraries from rhizospheric soil (soil) and *Vicia, Trifolium*, and *Lathyrus* nodules (nod).

Table 2. Inoculation indices (n_i) of nodule subniches of three plant species during rhizobial migration from rhizospheric subniches into them (for OTUs represented in less than four of six *Rhizobium leguminosarum* populations)

Clusters (Fig. 1)	OTU	*Vicia sativa*	*Lathyrus pratensis*	*Trifolium hybridum*
A	OTU23	1.28↓	1.50↓	0.22↑
	OTU20	1.14↓	1.02	0.78↑
	OTU21	1.18↓	1.01	1.01
	OTU17	1.05	1.09	N.d.
B	OTU22	0.74↑	0.90↑	1.57↓
	OTU15	0.84↑	0.85↑	N.d.
	OTU16	0.98	0.90↑	N.d.
	OTU18	0.86↑	1.01	N.d.

Arrows indicate a significant ($P_0 < 0.01$) increase or decrease in OTU frequency during rhizobial migration from the rhizosphere into the nodule niche (n.d. indicates no data).

Table 3. Summarized frequencies (%) of *Nod*A gene sequences in the clusters combining OTUs detected in rhizospheric (R) and nodule (N) *Rhizobium leguminosarum* subpopulations

Cluster (OTU quantity)	Origin of *nod*A sequences					
	Vicia sativa		*Lathyrus pratensis*		*Trifolium hybridum*	
	R	N	R	N	R	N
A (12)	17.6 ± 0.62	2.1 ± 0.22↓	17.0 ± 0.59↓	0.5 ± 0.11↓	81.8 ± 0.63	99.9 ± 0.05↑
B (11)	82.4 ± 0.62	97.9 ± 0.22↑	83.0 ± 0.59↑	99.5 ± 0.11↑	18.2 ± 0.63	0.1 ± 0.05↓

See Table 1 for Note.

Table 4. Distribution of OTUs in rhizospheric (R) and nodule (N) niches of various plant species

The number of detected OTUs	*Vicia sativa*		*Lathyrus pratensis*		*Trifolium hybridum*	
	R	N	R	N	R	N
In total	14	13	15	12	13	10
Only in this niche	5	3	6	3	3	0
Common for two niches	8		9		10	

minor, while the dominant OTU22 and OTU23 are present in all subpopulations (Table 1).

Cluster analysis of the OTU frequency distributions in the rhizospheric and nodule *nod*A libraries demonstrated a high similarity between *R. leguminosarum* subpopulations associated with *V. sativa* and *L. pratensis*, as well as their differences from subpopulations associated with *T. hybridum* (Fig. 2). The minimal values of genetic distances (D) in the multiscale space of data concerning the frequencies of different OTUs were observed during a comparison of subpopulations from the vetch and vetchling rhizosphere and nodules ($D = 0.051$ and 0.085), while the maximal values were detected during a comparison of the subpopulation from clover nodules with populations from vetch and vetchling nodules ($D = 0.445$ and 0.470). Comparison of the rhizospheric populations from leguminous representing different CIG resulted in an intermediate value of genetic distances ($D = 0.229$), which indicates the divergence of subpopulations as being in the periapical plant zones (rhizospheric effect). Pair-wise comparisons of leguminous species revealed that the divergence was higher for nodule rhizobial subpopulations ($D = 0.085$–0.470) than for rhizospheric subpopulations ($D = 0.051$–0.299). This divergence might result in increased Nei's indices, which characterize subpopulations variability during "rhizosphere → nodules" transitions (Table 5). Comparison of legumes from different CIGs demonstrated

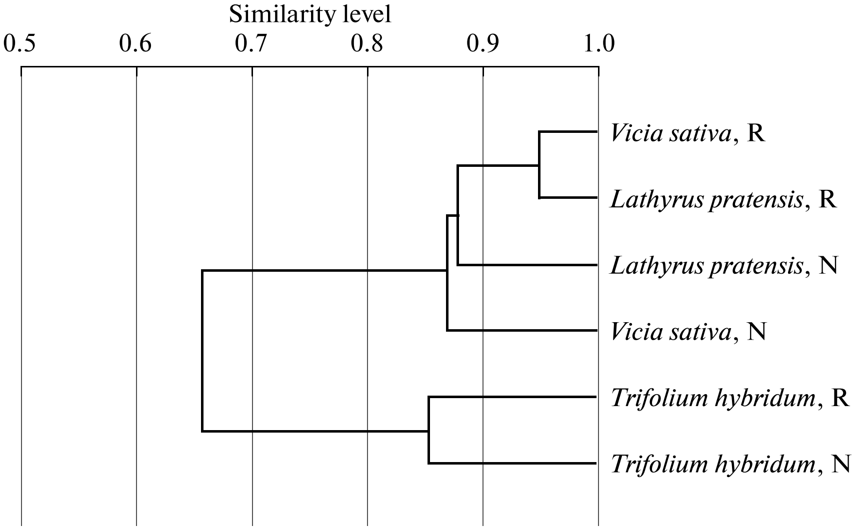


Fig. 2. Dendrogram of similarity between the frequencies of various *nod*A gene sequences in rhizospheric (R) and nodule (N) niches of three leguminous species. Dendrogram was constructed based on the genetic distances between OTUs calculated according to Table 1 data with the formula:

$$D_{mn} = \frac{2}{\pi}\arcsin\left(\frac{1}{2}\sqrt{\sum_{i=1}^{K}\left(\sqrt{p_{\mathrm{ni}}} - \sqrt{p_{\mathrm{mi}}}\right)^2}\right),$$

where p_{mi}, p_{ni} is the frequencies of the *m*th and *n*th OTU.

greater differences between rhizospheric and nodule subpopulations than between plants from the one CIG ($D = 0.346 \pm 0.008$ and 0.122 ± 0.004; $P_0 < 0.001$).

DISCUSSION

The main evolutionary direction of legume-rhizobial symbiosis includes an increase in its structural-functional organization (the formation of a system of subcellular compartments for rhizobial arrangement, as well as the interorganism pathways of C/N metabolism) and efficacy (increased ecological adaptability of partners), which is determined by symbiosis-specific selection forms [1, 9]. To study it, we analyzed the dynamics of the *nod*A gene via high-throughput sequencing in *Rhizobium leguminosarum* populations interacting with three leguminous plants: the vetch *Vicia sativa*, the meadow vetchling *Lathyrus pratensis* (from vetch, vetchling, pea CIG possessing the common symbionts *R. leguminosarum* bv. *viciae*), and the alsike clover *Trifolium hybridum* (from clover CIG symbiotically interacting with *R. leguminosarum* bv. *trifolii*, differing from bv. *viciae* in the structure of a small OTU 20 kb and the region of the *Sym*-plasmid containing the genes of host specificity [10]).

The *nod*A gene was chosen as a model for our study of selection. It encodes the stage of signal Nod-factor synthesis, which is universal for all rhizobia. The addition of fatty-acid residue to oligochitin chain was also included [2]. An earlier analysis of modifications in the *R. leguminosarum* bv. *trifolii* population structure, which occurred as a result of rhizobial migration from the soil into *T. hybridum* nodules, demonstrated that the direction of selection in them depended on the phylogenetic position of OTUs comprised of *nod*A gene sequences. The present study revealed that the selection form, direction, and intensity depended on the host plant. Analysis of the findings made it possible

Table 5. Changes in Nei's index of genetic diversity in *Rhizobium leguminosarum* during "rhizosphere → nodules" migration

Cluster comprising OTUs (Fig. 1)	*Vicia sativa*	*Lathyrus pratensis*	*Trifolium hybridum*
A	0.202 → 0.214	0.061 → 0.670	0.142 → 0.288
B	0.082 → 0.263	0.028 → 0.051	No data

to divide the 23 detected OTUs into two clusters characterized by multidirectional modifications in the frequencies during the interaction with plants from two CIGs that contrast in their symbiotic specificity. Hence, at a whole-population level, *R. leguminosarum* selection has a diversifying character. This results in the divergence of a subpopulation specialized to different leguminous species that select strains with contrasting host-specificity from the whole population.

The fact that the most frequent OTUs, OTU22 and OTU23, are isolated from the rhizosphere and nodule niches in all three plants is related to the involvement of *nod*A in the synthesis of the core part of Nod-factor rather than in its modifications determining host specificity (the level of saturation with acyl residue or the addition of various chemical radicals to oligochitin chain [2, 10]). At the same time, several minor OTUs appeared to be related to the plants of the one CIG. It might be caused by severe linkage of the *nod*A gene with the genes of host specificity [10], resulting in nonrandom associations of *nod*A alleles of different rhizobial strains and host plant species.

The action of diversifying selection was detected earlier in phytopathogenic bacteria *Pseudomonas syringae* in an analysis of *hrp*-genes, which control the formation of type 3 secretion systems (which are responsible for signal proteins transfer into the plant cells) [11]. However, this action was established in a small sample of strains (22) that were analyzed for the relation of synonymous and nonsynonymous modifications in *hrp*A, *hrp*B, *hrp*D, *hrp*E, and *hrp*Z genes, and the cladograms of various *hrp* genes were compared by indirect selection parameters.

In the present study, a diversifying selection by *nod*A was shown by a direct method—a shift analysis of population structures during rhizobial migration from rhizospheric soil into the nodules. These shifts were detected by our group by an analysis of rhizospheric and nodule DNA libraries, which were characterized by the presence of about 20000 *nod*A sequences comprising 23 OTUs (95% similarity) and two clusters (containing 12 and 11 OTUs). Statistical analysis revealed that the positive selection affected the OTUs comprising cluster B during “rhizosphere → nodules” transition in the case of symbiosis with vetchling and vetch, while a negative selection was observed in the case of symbiosis with clover. At the same time, a positive selection with clover symbiosis was observed in cluster A, and the negative selection was observed in symbiosis with vetch and vetchling. Since the studied plants representing various CIG grow symbiotically, it might be supposed that the induced diversifying selection might cause sympatric species formation in the united *R. leguminosarum* population. In contrast to previously studied higher organisms [12], these bacteria might be responsible for diversifying evolution without the enhancement of reproductive isolation, since, as was shown previously [13], an intensive transfer of “symbiotic” genes occurred between *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* biotypes symbiotically interacting with plants.

A diversifying selection is known to be induced in the population when rare genotypes obtain selective advantages after frequency-dependent selection [14, 15]. To analyze the possibility of its action in the studied rhizobial-plant scheme, we compared the levels of *nod*A sequence polymorphisms in conjugated (associated with the same host) rhizospheric and nodule populations using Nei’s index, which characterizes the uniformity of distribution of the studied genotypes (*nod*A gene sequences) by classes (OTU). The values of this index appeared to increase in clusters A and B during rhizobial interaction with three plant species (Table 5). Previous mathematical modeling of symbiosis evolution suggests [1, 16] that such a modification in the population structure might be caused by a negative frequency-dependent selection, leading to the propagation of rare rhizobial genotypes in endosymbiotic niches.

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REFERENCES

1. Provorov, N.A. and Vorob’ev, N.I., *Geneticheskie osnovy evolyutsii rastitel’no-mikrobnogo simbioza* (Evolutionary Genetics of Plant–Microbe Symbiosis), Tikhonovich, I.A., Ed., St. Petersburg: Inform-Navigator, 2012.
2. Wang, D., Yang, S., Tang, F., and Zhu, H., Symbiosis specificity in the legume-rhizobial mutualism, *Cell. Microbiol.*, 2012, vol. 14, no. 3, pp. 334–342.
3. Provorov, N.A., Tsyganova, A.V., Brewin, N.J., et al., Evolution of symbiotic bacteria within the extra- and intra-cellular plant compartments: experimental evidence and mathematical simulation (mini-review), *Symbiosis*, 2012, vol. 58, nos. 1–3, pp. 39–50.
4. Andronov, E.E., Onishchuk, O.P., Kurchak, O.N., and Provorov, N.A., Population structure of the clover rhizobia *Rhizobium leguminosarum* bv. *trifolii* upon transition from soil into the nodular niche, *Microbiology* (Moscow), 2014, vol. 83, no. 4, pp. 422–429.
5. Nei, M., Estimation of average heterozygosity and genetic distance from a small number of individuals, *Genetics*, 1978, vol. 89, no. 4, pp. 583–590.
6. Selander, R.K., Caugant, D.A., Ochman, H., et al., Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics, *Appl. Environ. Microbiol.*, 1986, vol. 51, no. 5, pp. 873–884.
7. Lakin, G.F., *Biometriya* (Biometry), Moscow: Vysshaya Shkola, 1990.
8. Vorobyov, N.I. and Provorov, N.A., Simulation of legume–*Rhizobium* symbiosis evolution under multistrain competition among bacteria for inoculation of symbiotic niches, *Ekol. Genet.*, 2008, vol. 6, no. 4, pp. 3–11.

9. Provorov, N.A. and Tikhonovich, I.A., Genetic and molecular bases of symbiotic adaptations, *Usp. Sovrem. Biol.*, 2014, vol. 134, no. 3, pp. 211–226.
10. Shlaman, Kh., Fillips, D., and Kondoroshi, E., Genetic organization and transcriptional regulation of rhizobia genes controlling nodulation, in *Rhizobiaceae: molekulyarnaya biologiya bakterii, vzaimodeistvuyushchikh s rasteniyami* (Rhizobiaceae: Molecular Biology of Bacteria Interacting with Plants), Spaink, G., Kondoroshi, A., and Khukas, P., Eds., St. Petersburg: Biont, 2002, pp. 389–416.
11. Guttman, D.S., Gropp, S.J., Morgan, R.L., and Wang, P.W., Diversifying selection drives the evolution of the type III secretion system pilus of *Pseudomonas syringae*, *Mol. Biol. Evol.*, 2006, vol. 23, no. 12, pp. 2342–2354.
12. Timofeev-Resovskii, N.V., Vorontsov, N.N., and Yablokov, A.V., *Kratkii ocherk teorii evolyutsii* (Short Essay on the Theory of Evolution), Moscow: Nauka, 1977.
13. Provorov, N.A., Andronov, E.E., Onishchuk, O.P., et al., Genetic structure of the introduced and local populations of *Rhizobioum leguminosarum* in plant–soil systems, *Microbiology* (Moscow), 2012, vol. 81, no. 2, pp. 224–232.
14. Trotter, M.V. and Spencer, H.G., Frequency-dependent selection and the maintenance of genetic variation: exploring the parameter space of the multiallelic pairwise interaction model, *Genetics*, 2007, vol. 176, no. 3, pp. 1729–1740.
15. Friesen, M.L., Saxer, G., Travisano, M., and Doeveli, M., Experimental evidence for sympartric ecological diversification due to frequency-dependent competition in *Escherichia coli*, *Evolution*, 2004, vol. 58, no. 2, pp. 245–260.
16. Provorov, N.A. and Vorob'ev, N.I., Microevolution of nodule bacteria upon generation of mutants with altered survival in the plant–soil system, *Russ. J. Genet.*, 2003, vol. 39, no. 12, pp. 1349–1359.

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