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EXPERIMENTAL ARTICLES

Genetic Structure of the Introduced and Local Populations of *Rhizobioum leguminosarum* in Plant–Soil Systems

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Abstract—Comparative study of *Rhizobium leguminosarum* populations formed under the conditions of the Srednii Island (White Sea) demonstrated the introduced clover rhizobia (*R. l.* bv. *trifolii*) to be more variable than the aboriginal vetch/vetchling rhizobia (*R. l.* bv. *viceae*) in the chromosomal *IGS* locus, while being less variable in the plasmid-located symbiotic genes *nodD* and *nifH*. The analysis of these genes revealed the most pronounced differences between the clover and vetch/vetchling rhizobia populations. These differences, together with the results of ERIC-fingerprinting, indicated that the evolution of the clover rhizobia was mainly linked with the adaptation to local soil environment, and the evolution of the vetch/vetchling rhizobia, to the adaptation to various species of the host plants. High panmixia of *R. leguminosarum* population suggests its evolution to be based on the combinatory variability associated with the transfer of *Sym*-plasmids between *R. l.* bv. *trifolii* and *R. l.* bv. *viceae*, as well as with genomic rearrangements in the resulting recombinants.

Keywords: genetic structure of a population, horizontal gene transfer, evolution of symbiosis, root nodule bacteria of clover (*Rhizobium leguminosarum* bv. *trifolii*) and vetch/vetchling (*R. l.* bv. *viceae*), genes for nodulation (*nod*) and N_2 fixation (*nif*), polymorphism of chromosomal and plasmid loci, PCR analysis, genomic fingerprinting.

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The key role in the evolution of root nodule bacteria (rhizobia), N_2-fixing symbionts of the legumes, belongs to their introduction into novel environments together with the host plants. Such introduction causes a wide range of microevolutionary processes, including the adaptation of rhizobia to the new soil environment and to the local plant species [1]. This adaptation is characterized by coevolution of the introduced strains with the aboriginal soil bacteria, based on their genetic (hybridization) and ecological (competition for survival in the *in planta* and *ex planta* ecological niches) interrelations [2]. Analysis of the role of these factors in rhizobia evolution is interesting for the study of the mechanisms affecting the formation of mutualistic microorganism–plant symbiosis and for the improvement of their practical application.

In the research of taxonomically heterogeneous rhizobial species of the genera *Rhizobium, Bradyrhizobium, Mesorhizobium*, and *Sinorhizobium*, the leading role in their evolution of the transfer of the symbiosis genes located on plasmids [3, 4] or chromosomal islands [5, 6] has been shown previously. This process promotes formation of bacterial populations with panmixia, and under specific conditions, formation of the new species of nitrogen-fixing symbionts [7, 8]. However, selective factors of rhizobial evolution are insufficiently studied. Thus, comparative analysis of the population dynamics of the introduced and local populations is necessary.

An experimental model for the analysis of genetic evolutionary processes occurring during the transition of symbiotic systems into the new environments is suggested in the current study. Dynamics of the populations formed by two biotopes of *Rhizobium leguminosarum*, the introduced (clover rhizobia *R. l.* bv. *trifolii*) and the local (vetch/vetchling rhizobia *R. l.* bv. *viceae*), was studied on the limited territory of the Srednii Island (White Sea). Therefore, the aboriginal population of *R. leguminosarum* could be considered genetically isolated. Evolution of the clover rhizobia was shown to be based on the adaptation to soil environment, while in the evolution of the vetch/vetchling rhizobia, specialization to the host plants prevailed.

MATERIALS AND METHODS

Isolation of strains and formation of the related populations of *R. leguminosarum* bv. *trifolii* and *R. l.* bv. *viceae*. For the isolation of root nodule bacterial strains, the legumes–rhizobia symbiotic systems including two *R. leguminosarum* biotypes, clover rhizobia *R. l.* bv. *trifolii* (plant hosts *Trifolium pratense,*

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T. repens), and vetch/vetchling rhizobia *R. l.* bv. *viceae* (plant hosts *Vicia cracca, V. sepium, V. silvatica, Lathyrus maritimus, L. pratense*) from the Srednii Island (White Sea, Chupa Bay estuary) were used.

Under the conditions of the Srednii Island, plant hosts of *R. l.* bv. *trifolii* are ruderal species: they grow only in the biotopes subjected to direct anthropogenic influence—along the roads and near the buildings. This is due to the fact that clover was introduced to the Srednii Island around 150 years ago, during its colonization. The host plants of *R. l.* bv. *viceae* are aboriginal: they are common in all the biotopes of the island, including forests, meadows, and the coastal zone. In ruderal biotopes, vetch and vetchling frequently grow in close contact with clover, and formation of associated micropopulations (collected from the same topographical sites) is convenient for the study of *R. l.* bv. *trifolii* and *R. l.* bv. *viceae* coevolution.

Bacteria were isolated from the root nodules and samples of rhizosphere soil collected from 90 sites where vetch/vetchling and clover grew jointly. The areas of compact (0.2–0.3 m from each other) growth of the flowering plants (distance between the sites was at least 50 m) were selected. Bacteria were isolated using the standard techniques and were maintained in the tubes with bean agar [9]; a total of 136 strains of *R. leguminosarum* were isolated. To characterize the population variability of *R. l.* bv. *viceae* from the Srednii Island, the population was used (30 strains) which was formed independently from the associated populations and represented all biotopes of vetch and vetchling.

Polymorphism of the strains in the plasmid and chromosomal loci. For evaluation of variability of the strains, polymorphism of the chromosomal *IGS* locus (FGPS1490 and FGPL1329 primers), and of the *Sym*-plasmid loci containing the *nodD* (NBA12 and NODDRL29 primers) and *nifH* (FGPD807 and FGPK4929 primers) genes were studied [10]. PCR was caried out in a MyCycler™ DNA amplifier (Bio-Rad, United States). Amplification conditions were as follows: initial denaturing at 95°C for 3 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and the final cycle at 72°C for 5 min. *Taq*-polymerase and deoxynucleoside triphosphates were used (Helicon, Russia). As a template for PCR, the lysates of bacterial cultures were used; each colony was lysed in 20 μL of a solution containing 25 mM of NaOH and 0.25% of SDS, heated at 95°C for 5 min, brought to 200 μL with distilled water, separated by centrifugation, and used in the amount of 1 μL for the reaction (5 ng of DNA).

The PCR products were restricted with *Msp*I (MBI Fermentas, Lithuania) and separated in 3% agarose gel (Helicon, Russia) in TAE buffer [11]. The gel was stained with 0.5 mg/L of ethidium bromide and photographed in UV using a Kodak EDAS 290 camera. The number of restriction fragments for each strain did not exceed 10, and their size determined using the M100 molecular weight marker (SibEnzyme) varied from 50 to 700 bp.

To characterize the polymorphism in the populations, the Nei's index was used: $H_N = (1 - \Sigma P_i^2) \cdot [n/(n-1)]$, where P_i is the frequency of the *i*th genotype [12, 13], and the Shannon index: $H_S = H_N \times \ln P$ [14]. Marker association was analyzed with the Arlequin var. 2.0 software package in order to evaluate the probability of the null hypothesis regarding the absence of loci coupling.

Rhizobia micropopulation structure. For the detailed genotyping of bacteria, genomic fingerprinting was used: PCR with the random ERIC1R primer (5'-ATGTAAGCTCCTGGGGATTCAC-3') [15]. Amplification conditions were as follows: 95°C for 30 s, 37°C for 30 s, 68°C for 3 min; 40 cycles. The PCR products were separated in 2% agarose gel, stained with ethidium bromide, and photographed in UV. Agarose gels documentation and their import to the FPQuest software was carried out using a Kodak EDAS 290 camera. Cluster analysis was carried out using Pearson's correlation for calculation of the coefficients of similarity between the fingerprints (correlation was shown in percents). Clusterization was carried out using the Ward algorithm at 1% of tolerance and 1% of fingerprints optimization; the reliability of the group distinction was assayed using the Jackknife algorithm (with mean values).

RESULTS

Polymorphism of population in plasmid and chromosomal markers. Analysis of the related populations of two rhizobia biotopes demonstrated (Table 1, Fig. 1) that their structure in the plasmid (symbiosis) loci differed significantly: the ratio of *R. l.* bv. *trifolii* and *R. l.* bv. *viceae* strains depended on certain *nodD* or *nifH* genotypes. For both loci in the dominating genotype, the maximal frequency of the clover rhizobia was observed; in the next frequently occurring genotype, the maximal frequency of the vetch/vetchling rhizobia. According to the chromosomal loci, the differences between the clover and vetch/vetchling rhizobia were not found. Diversification of these biotypes was probably limited by a small (20000–30000 bp [16]) locus which is located on *R. leguminosarum Sym* plasmid and contains *nod* and *nif* genes playing the main role in symbiosis [17].

At the same time, the variability of the clover and vetch/vetchling rhizobia for the above-mentioned loci was different. Polymorphism of the strains for the *IGS* locus was maximal for *R. l.* bv. *trifolii*, and polymorphism for the symbiotic loci, for *R. l.* bv. *viceae* (Table 1). The *nodD* and *nifH* alleles located on the *Sym* plasmid were randomly associated with both the alleles of the *IGS* chromosomal locus and with each other: the probability of the null hypothesis regarding the absence of three loci coupling (after two by two

Table 1. Analysis of *R. leguminosarum* population structure (bv. *trifolii*, bv. *viceae*) for the chromosomal (*IGS*) and *Sym* plasmid (*nodD*, *nifH*) markers

Locus (genotypes revealed)	Comparison parameters	Biotype *trifolii*	Biotype *viceae*
nodD (4)	Number of strains studied	53	41
	Number of genotypes revealed	4	3
	Number of genotypes common for two biotypes	3 (75%)	
	Nei's index	0.33	0.64
	Shannon index	0.46	0.71
	$\chi^2(P_0)$*	29.57 ($P_0 < 0.001$)	
nifH (10)	Number of strains studied	77	52
	Number of genotypes revealed	8	8
	Number of genotypes common for two biotypes	6 (60%)	
	Nei's index	0.63	0.82
	Shannon index	1.31	1.71
	$\chi^2(P_0)$*	36.93 ($P_0 < 0.001$)	
IGS (12)	Number of strains studied	65	43
	Number of genotypes revealed	10	5
	Number of genotypes common for two biotypes	3 (25%)	
	Nei's index	0.64	0.58 (0.91)**
	Shannon index	1.47	0.93 (2.40)**
	$\chi^2(P_0)$*	1.45 ($P_0 > 0.05$)	

Notes: * Values of χ^2 (corresponding to the probabilities of the null hypothesis) are given for the comparison of the population structure of different biovars, distribution of the strains among the genotypes revealed for a certain locus.

** Diversity indices revealed by analysis of the *R. l.* bv. *viceae* population collected over the Srednii Island territory are shown in brackets.

comparison) was 0.20–0.40. It could be suggested that in *R. leguminosarum* population an active transfer of the *Sym* plasmids takes place (both between various biotopes and within them) with subsequent recombination of the transferred and resident plasmids resulting in the formation of novel genotypes.

Among 12 chromosomal (*IGS*) genotypes found in *R. leguminosarum* populations, only three genotypes (*a, b*, and *c*) common for the clover and vetch/vetchling rhizobia and dominating in their populations have been revealed (Table 1, Fig. 1). These genotypes probably appeared in *R. l.* bv. *trifolii* by the transfer of the *Sym* plasmids determining the specificity to clover to *R. l.* bv. *viceae* strains which lost their own plasmids [18]. The following diversification of the newly obtained recombinants occurred mainly in the chromosomal genes and led to the appearance of new genotypes, not characteristic for vetch/vetchling rhizobia; this caused high variability of the clover rhizobia in the chromosomal *IGS* locus.

Analysis of the *nodD* and *nifH* loci showed that most of the genotypes found were common for two biotypes of *R. leguminosarum*. This suggests the possibility of combination of the same *nodD* and *nifH* alleles, universal for most rhizobia species [16, 19], with the genes determining differences in the host specificity.

Therefore, in ruderal biotopes including *R. l.* bv. *trifolii* and *R. l.* bv. *viceae* populations, the maximal competition occurs for the soil, their common ecological niche. Adaptation to soil is very important for the introduced *R. l.* bv. *trifolii* biotype, resulting in the diversification of its chromosomal *IGS* locus. This diversification resulted in formation of a set of 7 minor *IGS* genotypes which were not found in *R. l.* bv. *viceae*. Due to the absence of competition for the intranodule niches, a number of common symbiotic genotypes of *R. l.* bv. *trifolii* and *R. l.* bv. *viceae* exists.

Higher polymorphism of the symbiotic loci indicated for *R. l.* bv. *trifolii* (if compared with *R. l.* bv. *viceae*) is probably determined by higher specificity of the former biotype to various plant species. For the clover rhizobia, the qualitative variability in the specificity of symbiotic features is known to be atypical [20]: quantitative changes in the indices of root nodule formation or nitrogen fixation after interaction of the strains with various *Trifolium* species prevails. Indeed, the study of *R. l.* bv. *trifolii* population did not reveal any differences in the three analyzed loci (χ^2 =

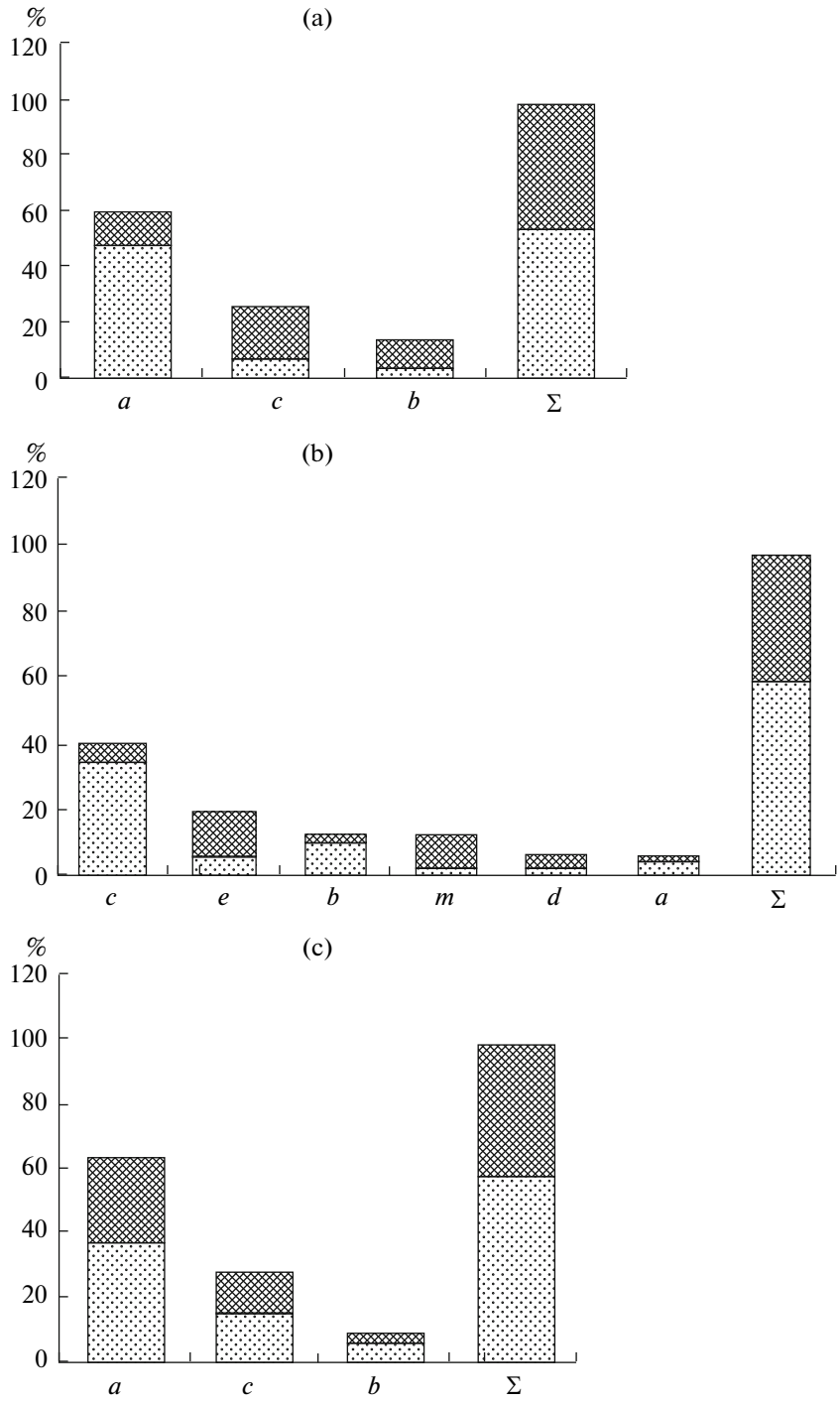

Fig. 1. Genetic structure of *R. leguminosarum* population for the *nodD* (a), *nifH* (b), and *IGS* (c) loci. The data on the frequency (% by the vertical axis) of the genotypes (marked by Latin characters under the horizontal axis), which are common for two biotypes of *R. l.* bv. *trifolii* (thatched) and *R. l.* bv. *viceae* (cross-thatched) are shown for each locus. The total number of strains for each locus is taken for 100% (shown as Σ). Distribution of *R. l.* bv. *trifolii* and *R. l.* bv. *viceae* strains by genotypes reliably differ for the *nodD* and *nifH* loci but not for the *IGS* locus (Table 1).

Table 2. Analysis of *R. leguminosarum* bv. *viceae* strains specificity to various plant species revealed in the study of population polymorphism

Plant species	*nifH*-locus		*nodD*-locus		*IGS*-locus	
	m-genotype	Other genotypes	*b*-genotype	Other genotypes	*a*-genotype	Other genotypes
Vicia cracca	9	6	8	5	7	3
Other species*	1	21	3	18	11	12
χ^2	12.50 ($P_0 < 0.01$)		6.17 ($P_0 < 0.05$)		0.63 ($P_0 > 0.05$)	

* *V. sepium, V. silvatica*, *L. pratense*.

0.01–1.78; $P_0 > 0.05$) between the strains isolated from two clover species.

R. l. bv. *viceae* strains often demonstrated both quantitative and qualitative variability of the symbiosis specificity with various species of *Vicia, Lathyrus*, and *Pisum* [3, 21, 22]. A new approach was used for statistical analysis of the relationship between the populational genetic structure and host-specificity of the investigated vetch/vetchling rhizobia strains, since host plant species were very diverse and the selection of the strains isolated from each species was scarce. For *Vicia cracca* (major part of the isolated strains), the most numerous genotype was identified for each locus (*nodD, nifH*, and *IGS*) and the table for χ^2 value calculation was constructed based on the number of strains: (a) isolated from *V. cracca* and representing the "maximal" genotype; (b) from *V. cracca* incoming into the other genotypes; (c) from other species of *Vicia* and *Lathyrus* demonstrating the "maximal" genotype for *V. cracca*; (d) from other plant species with other genotypes.

The use of this approach showed (Table 2) that the specificity of the isolates of *R. l.* bv. *viceae* from *V. cracca* and the isolates from other species of *Vicia* and *Lathyrus* for the *nifH* and *nodD* loci was statistically reliable. Specific for *V. cracca* were the *nifH m* genotype and the *nodD b* genotype; more than 80% of the strains isolated from this plant species had the *nifH(m)nodD(b)* genotype. In contrast, for the *IGS* locus such specificity did not exist. In the evolution of *R. l.* bv. *viceae* specialization to the host plants based on polymorphism of the symbiotic loci plays apparently a more important role than in the evolution of *R. l.* bv. *trifolii*.

Analysis of the structure of micropopulations by genomic fingerprinting. For the detailed characterization of the population structure of two *R. leguminosarum* biotypes, the genotyping of strains by fingerprinting with the nonspecific ERIC1R primer was carried out (Fig. 2). The number of DNA fragments revealed after fingerprint analysis was 20–30 per strain, the size of the fragments varied from 100 to 2000 bp. This method made it possible to reveal high variability of the population (among 136 strains, 131 ERIC-genotypes were indicated, the Nei's diversity index was 0.995), thus providing detailed characterization of the micropopulations within various biotypes (*R. l.* bv. *trifolii*, *R. l.* bv. *viceae*), genotypes (*nodD*, *nifH, IGS*), and topographical sites.

Analysis of the similarity coefficients obtained by ERIC genotyping demonstrated that the level of variability of the strains between and within *R. l.* bv. *trifolii* and *R. l.* bv. *viceae* biotypes did not differ significantly (Table 3). This could be due to the high genetic plasticity of the populations, resulting both from panmixia and genomic instability of certain strains (mainly recombinant ones). The differences in host plant specificity for *R. l.* bv. *trifolii* and *R. l.* bv. *viceae* are determined by the allele condition of a small number of *nod* genes coupled with *nodD* and *nifH* [16]. The random character of distribution of the ERIC genotypes for *R. l.* bv. *trifolii* and *R. l.* bv. *viceae* biotypes confirmed free combination of the *Sym* plasmids and the chromosome in *R. leguminosarum* population, which was shown previously by the analysis of *IGS, nodD*, and *nifH* loci.

For the strains from the dominant *IGS* genotypes *a* and *c*, the similiarity coefficients were at the level of intragroup comparison (Table 3). However, we revealed the group of 4 minor *IGS* genotypes (*p, r, n*, and *s*) of *R. l.* bv. *trifolii* within which the similarity coefficient was much higher (75.0%) than when this group was compared with the dominant *a* and *c* genotypes (4.5–12.5%). Apparently, this group of *IGS* genotypes had common origin and appeared as a result of adaptation of one of the introduced *R. l.* bv. *trifolii* genotypes to the conditions of the Srednii Island.

The distribution of the ERIC genotypes among the symbiotic genotypes was also not random; as for the *nodD* genotype *b* and the *nifH* genotype *m*, the intragroup similarity coefficients were significantly higher than the intergroup coefficients (Table 3). Notably, these two genotypes were specific for the isolates from *V. cracca* (Table 2). Therefore, genomic fingerprinting confirmed the leading role of specialization for the host plants in the evolution of *R. l.* bv. *viceae*.

To assess the impact of the local adaptive processes on the evolution of *R. leguminosarum*, similarity coefficients were determined in 7 micropopulations, each of which was isolated from a certain topographical

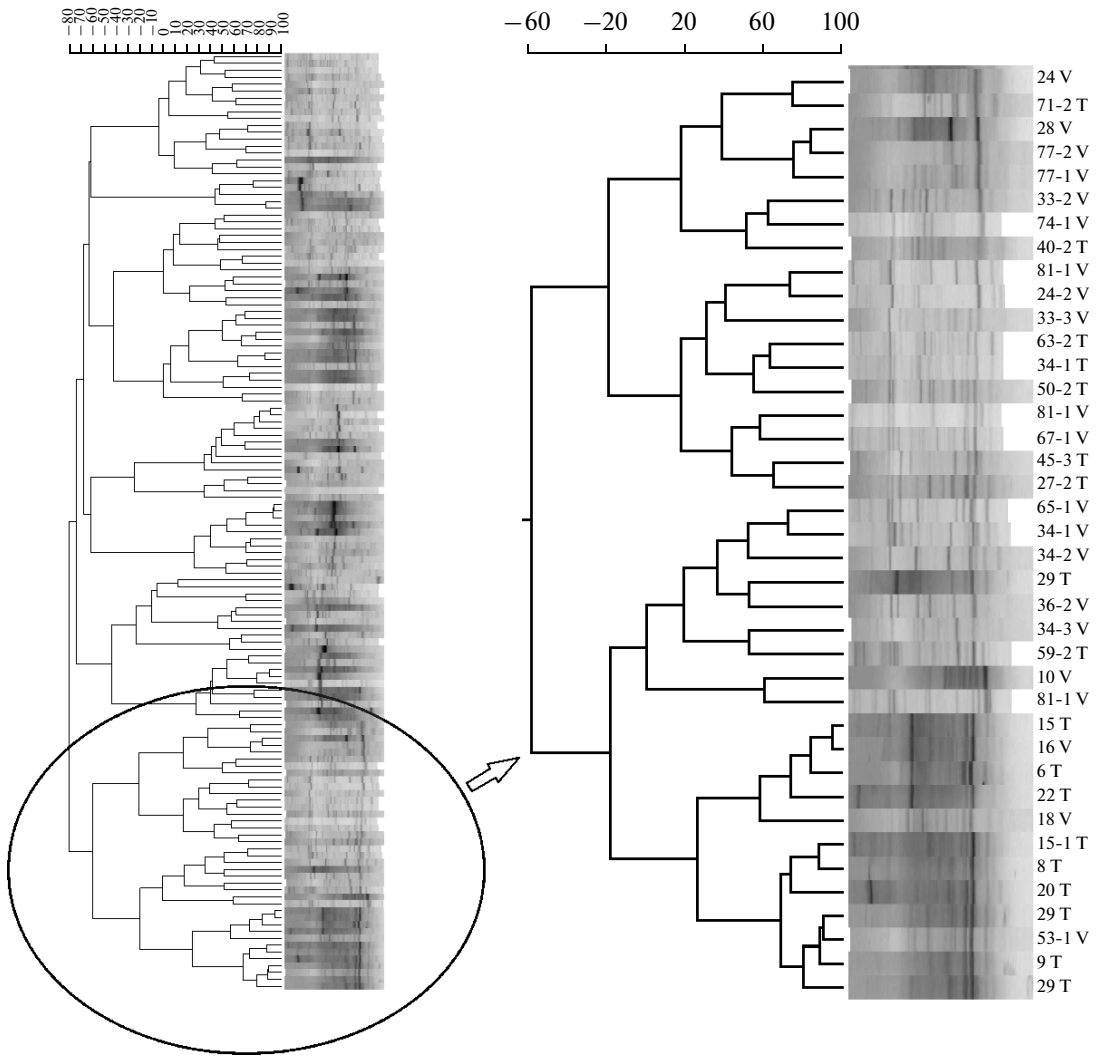

Fig. 2. Cluster analysis of genomic ERIC fingerprints of the native *R. leguminosarum* isolates. The Cluster demonstrating the highest polymorphism is shown separately, as well as the absence of correlation with the *viceae* (V) and *trifolii* (T) biotype of the strains.

site, and compared with the intragroup coefficients calculated after comparison of the micropopulations from various sites. All 7 populations were genetically diverse in the *nodD, nifH* or *IGS* loci, and 3 of them contained the strains from different biotypes (Table 4). The intragroup similarity coefficients calculated using the data of genomic fingerprinting were 50–100% (in average 72.7%), while the intergroup coefficients were 0–33% (in average 4.5%). These data suggest that diversification that occurs in local micropopulations during adaptation to the soil environments and host plants plays the key role in the evolution of the studied bacteria.

DISCUSSION

Comparative analysis of the introduced and local populations of rhizobia made it possible to study the genetic principles of their adaptive evolution. This process is most intensive when rhizobia together with legumes move to new ecological zones, and new forms of symbionts are formed [1, 2]. To study this evolution, we used the populations of two *R. leguminosarum* biotypes, symbionts of clover (*R. l.* bv. *trifolii*) and of vetch/vetchling (*R. l.* bv. *viceae*). Accessibility of *R. leguminosarum* for molecular genetic analysis (including its complete genome sequences [23]) and diversity of its host plants makes it possible to study the effect of a wide range of microevolutionary factors and to evaluate their role in the formation of microbial populations during their migration to new habitats together with their host plants.

Analysis of the genetic structure of the island (Srednii Island, the White Sea) *R. leguminosarum* population was carried out using the primers specific for the chromosomal (*IGS*) and plasmid (*nodD, nifH*)

Table 3. Similarity coefficients calculated for various biotypes and genotype classes of *R. leguminosarum* on the basis of genomic fingerprinting results

Criteria for distinction of the strains into groups	Dominant biotypes or genotypes	Similarity coefficients	
		Intragroup	Intergroup: min–max (average)
Host specificity	bv. *trifolii*	50.65	38.98–49.35
	bv. *viceae*	61.02	(44.21)
IGS-Genotyping	*a*	44.78	50.0–55.22
	c	50.0	(52.60)
nodD-Genotyping	*a*	15.52	0.0–51.72
	b	89.47*	(21.04)
	c	68.97	
nifH-Genotyping	*b*	28.57	5.26–50.0
	c	12.0	(19.23)
	e	50.0	
	m	78.95*	

* Similarity coefficients of the strains included into these genotypes are significantly higher than in the inter-group comparative studies (evaluated using the Student criteria).

loci, and the non-specific primers for genome RFLP fingerprinting. This analysis showed that the differences between the two biotypes (clover rhizobia *R. l.* bv. *trifolii* and vetch/vetchling rhizobia *R. l.* bv. *viceae*) were more pronounced in the case of the symbiotic loci. The absence of coupling of the analyzed markers is in accord with the data obtained previously for various biotypes of *R. leguminosarum* [10, 24] and demonstrates the main source of genetic variability for adaptive evolution to be recombination processes associated with the horizontal transfer of the *Sym* plasmids and with genomic changes induced in the recombinants. High panmixia of *R. leguminosarum* populations found in ruderal biotopes may be connected with anthropogenic effects, namely with xenobiotic pollution or mechanical impacts. These factors could affect the demonstrated enhanced panmixia of rhizobia from the cultured soils in contrast with the rhizobia from the fallow soils [25].

Analysis of the data showed that the evolution of a genetically separated population of *R. leguminosarum* from the Srednii Island includes several interconnected processes. One of them is the adaptation of bacteria to the new edaphic factors, which is based on the survival selection in the soil niches. This evolutionary direction is typical of the introduced clover rhizobia (*R. l.* bv. *trifolii*) for which the highest variability was revealed in the chromosomal markers regulating the saprophytic phase of its life cycle. We have previously shown enhanced adaptation of rhizobia to local soil, climatic and anthropogenic factors after their introduction to agrocenoses in a comparative study of *R. l.* bv. *viceae* from the areas of wide species diversity of vetch (Middle Asia) and of intensive culturing of its adapted forms (Ukraine) [26]. Under the conditions of the Srednii Island where the biotype *R. l.* bv. *viceae* is aboriginal, its adaptive evolution is associated mainly with bacterial specialization to the host plants (high diversity in the symbiosis genes) (Table 1).

It is important to note that the adaptation of rhizobia to symbiotic and soil niches takes place in the local micropopulations (Table 4) where the main selective processes stem from differentiated proliferation of the symbiotically active strains in the rhizosphere and root nodule niches, and after the symbiosis is completed, from their differentiated decline in soil [27]. These processes determine the enhanced (if compared with

Table 4. Genetic structures of *R. leguminosarum* populations isolated from various sites of the host plant growth

Sites (similarity coefficients within micropopulations)	Biotypes (host plant species)*	Genotypes revealed in strains after various loci analysis		
		IGS	*nifH*	*nodD*
63 (50.0)	*trifolii* (Tp)	*a*	*d*	*c*
	trifolii (Tp)	*a*	*d*	*c*
	trifolii (Tp)	*a*	*c*	*a*
	trifolii (Tp)	*a*	*c*	*a*
28 (66.7)	*trifolii* (Tr)	*a*	*c*	ND**
	trifolii (Tr)	*a*	*c*	*a*
	viceae (Vs)	*a*	*d*	*c*
55 (100)	*viceae* (Lp)	*a*	*e*	*c*
	viceae (Lp)	*a*	*e*	*c*
	viceae (Lp)	*a*	*g*	*c*
47 (100)	*viceae* (Lp)	*d*	*a*	ND**
	viceae (Lp)	*p*	*e*	*c*
	viceae (Lp)	*b*	*a*	*a*
	viceae (Lp)	*b*	*a*	*a*
68 (66.7)	*trifolii* (Tp)	*a*	*e*	*c*
	trifolii (Tp)	*a*	*e*	*c*
	trifolii (Tp)	*a*	*c*	*a*
29 (75.0)	*viceae* (Vc)	*a*	*c*	*a*
	viceae (Vs)	*c*	*e*	*b*
	trifolii (Tr)	*c*	*m*	*b*
	trifolii (Tp)	*a*	*c*	*a*
34 (50.0)	*viceae* (Vc)	*a*	*m*	*b*
	viceae (Vc)	*a*	*m*	*c*
	trifolii (Tr)	*a*	*m*	*b*
	viceae (Vc)	*a*	*m*	*b*

Notes: * Tp—*Trifolium pratense*, Tr—*T. repens*, Lp—*Lathyrus pratense*, Vc—*Vicia cracca*, Vs—*V. sepium*.

** ND stands for no data. Mean values of the similarity coefficients during pairwise comparison of the strains were 72.7 ± 7.8 within the micropopulations and 4.5 ± 1.6 between the populations.

free-living bacteria) evolution rate of symbiotic bacteria in the host–environment systems.

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