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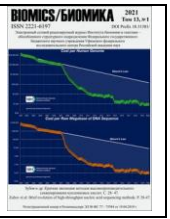

REGULARITIES OF THE GENOTYPE'S DISTRIBUTION OF PHYLOGENETICALLY HOMOGENOUS BACTERIA *RHIZOBIUM LEGUMINOSARUM* IN THE NODULES OF SEPARATE POPULATIONS OF *LATHYRUS VERNUS* (SPRING PEA) PLANTS

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Resume

The genotypes of phylogenetically homogeneous *Rhizobium leguminosarum* bacterial strains in nodules of *Lathyrus vernus* plants were studied. The degree of genetic variation between bacteria within nodules of one *L. vernus* population correlated with the distance between host plants: the greater the distance, the greater the genetic differences between their microsymbionts. This may be due to the ongoing process of exchanging genetic information between *Rhizobium* strains, with the depends on the distance between them. But in some cases, this pattern was not observed, and there were significant differences between the microsymbionts of neighboring plants. Most likely, with the exception of spatial limitations, there are some other barriers that exist to the free exchange of genetic information between nodule bacteria.

Keywords: Nodule bacteria, *Rhizobium leguminosarum*, leguminious plants, *Lathyrus vernus*, genotype

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Introduction

Rhizobia (nodule bacteria) are microorganisms capable of forming symbiotic relationships with leguminous plants by way of nodules, specialized structures on the roots, inside which nitrogen fixation takes place. Currently, this taxonomic group encompasses more than 98 species of bacteria grouped into 13 genera belonging to α-proteobacteria (*Rhizobium, Mezorhizobium, Ensifer, Bradyrhizobium, Phylobacterium, Microvirga, Azorhizobium, Ocrhobactrum, Methylobacterium, Devosia, Shinela*), and β-proteobacteria (*Burkholderia, Cupriavidus (Ralstonia)*) [Berada, Fikri-Benbrahim, 2014]. Rhizobia have two forms of existence: i) saprophytic, in which microorganisms are free-living soil microorganisms, and ii) symbiotic, in which bacteria propagate clonally in plant nodules. For successful symbiotic interaction, nodule bacteria must express symbiotic genes from their genomes, the products of which drive various elements of the relationship between bacteria and plants. *Sym*-genes encompass members of the nitrogen fixation *nif*-genes encoding the synthesis and regulation of the nitrogenase enzyme; *nod*-genes encode the synthesis of Nod factors (NF), responsible for the initiation and specificity the symbiotic relationship; and *fix*-genes, which are also necessary for nitrogen fixation, and often linked to *nif*-genes [Provorov, 1996; Franche et al., 2009].

Symbiotic genes serve no purpose to bacteria in a saprophytic state, and their loss only increases the competitiveness of bacterial cells. In contrast, for the formation of symbiosis, their presence is a prerequisite. Therefore, nodule bacteria are characterized by a constant loss/acquisition of symbiotic genes. This occurs in *Rhizobium* species, whose *sym*-genes are part of plasmids, for example, in *Rhizobium leguminosarum*. The host plant is directly involved in this process, which increases in the root zone a pool of bacteria having symbiotic genes due to their clonal propagation in nodules and further release into the soil when these structures die. In its turn, *sym*+ bacteria act both as agents capable of forming a symbiotic relationship with their host plant, and donors of *sym*-genes for *sym* bacteria. And with the loss of symbiotic genes, they can switch to a saprophytic mode of existence. The constant processes of loss and acquisition of *sym*-genes associated with "infection and release" (IR-cycles) [Provorov, Vorobyov, 2012] require from nodule bacteria high horizontal gene transfer activity. This, in turn, is one of the reasons of high polymorphism of these microorganism's populations. Indeed, heterogeneity indices for *Rhizobium* sp. are one of the highest among bacteria [Provorov, Vorobyov, 2012]. It is very likely that the high genetic diversity observed in the *Rhizobium* genus is a function of this survival strategy.

The aim of this work was to investigate the regularity of spatial distribution of strains phylogenetically homogeneous nodule bacteria *Rhizobium leguminosarum* isolated from nodules of one population of *Lathyrus vernus* plants and their comparison with microsymbionts of this plant species from another distant population.

Materials and Methods

The experiments described in this study were carried out on nodule bacteria of *L. vernus* (spring pea) plants. Nodules were collected from the roots of spring pea plants (before flowering) belonging to two populations growing in two different districts of the Republic of Bashkortostan (Russia): I – Tatyshlinsky (coordinates: 56°17′N 55°51′E) (Fig. 1(I)) and II – Beloretsky (coordinates: 53°50′N 58°36′E) (Fig. 1(II)). The selected populations were characterized by a uniform distribution of plants over the entire area of their growth.

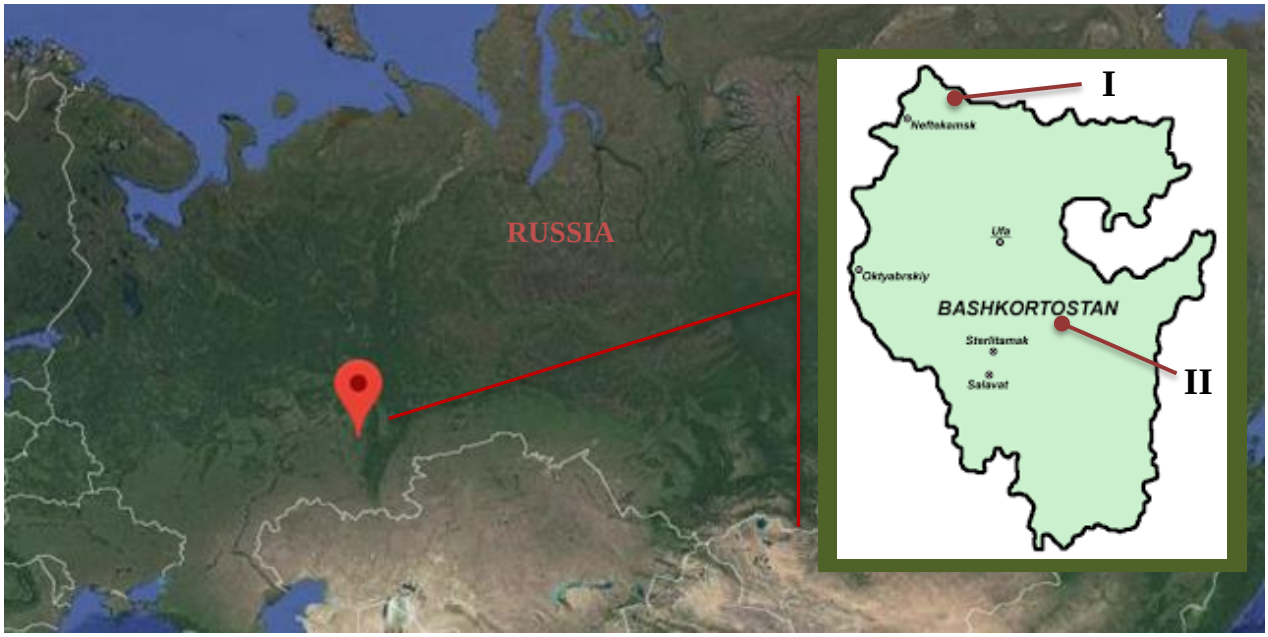

Fig. 1. The map demonstrating coordinates where *Lathyrus vernus* roots' nodules collected in Republic of Bashkortostan, Russia. **I** - Tatyshlinsky district (56°17′N 55°51′E) and **II** - Beloretsky district (53°50′N 58°36′E).

In the Tatyshlinsky district of the RB (Fig.1(I)) nodules were collected from 15 plants of the same population, separated from each other with a distance of 10 m according to the scheme shown in Fig. 2., from which pure cultures of nodule bacteria were subsequently isolated. 5 to 15 nodules were selected from each plant.

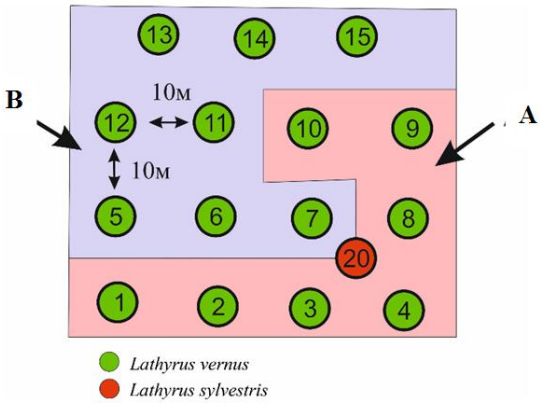

Fig. 2. Scheme of *L. vernus* plants (from the nodules of which rhizobia were isolated) locations in Tatyshlinsky district (Republic of Bashkortostan, Russia). Plant numbers are marked in the circles. **A** - plants, whose rhizobia form cluster A in cluster analysis; **B** - plants, whose rhizobia form cluster B in cluster analysis.

Bacteria were isolated from the nodules of plants. Nodule surfaces were sterilized for 2 min in 70% ethyl alcohol solution followed by 2 min in 10% sodium hypochlorite solution. Then the nodules were repeatedly washed with sterile water. Using sterile needles from 5 ml disposable syringes, the peel was removed from the distal part of the nodule. Then, with a different sterile needle, the contents of the nodule were scraped and plated on a solid TY nutrient medium (0.1% yeast extract, 1% bacto-tryptone, 0.1% $CaCl_2$, 1.5% agar).

DNA from bacteria was extracted by cell lysis in 1% Triton X-100 and 1% Chelex 100 suspension. For this, a small amount of the bacterial mass was placed into test tubes containing 100 µl of 1% Triton X-100 and 1% Chelex 100 suspension, incubated at 95°C for 10 min, then centrifuged at 12 000 g for 3 min. The supernatant was taken as a matrix for PCR. PCR was performed on amplifiers "T1 Thermocycler" ("Biometra", Germany) using DNA amplification kits "PCR-Kit" ("Syntol", Russia). The genetic diversity of the isolated strains was studied by the method of RAPD-analysis (Random Amplified Polymorphic DNA) [Williams et al., 1990] using the following "random" primers: LMBD8 5'-gggcgctg-3', Opa1 5'-caggcccatc-3', AFK3 5'-gcgtccattc-3'. To standardize the amplification conditions, a common PCR mixture was used. The DNA concentration of all samples was determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific) and 0.1 µg of DNA was taken for each reaction.

Amplification was performed using the following program: denaturation 95°C - 30 sec., annealing 33°C - 30 sec., elongation 72°C - 30 sec. To determine the size of the electrophoresis bands and their alignment in the course of analysis, 100 bp DNA "ladder" (NEB, USA) was used.

Cluster analysis of RAPD profiles was carried out using GelComparII (Applied Maths) using the method of unweighted pairwise mean - Unweighted Pair-Group Method Using Arithmetic Averages (UPGMA and similarity percentage calculated using a Dice coefficient with 2.5% tolerance. Phylogenetic homogeneity of bacteria was tested by RFLP (restriction fragment length polymorphism) [Laguerre et al. 1996] on the 16S rRNA gene using restriction endonucleases Kzo91 and HaeIII. Primers Y1 (5'-tggctcagaacgaacgctggcggc-3') and Y3 (5'-taccttgttacgacttcaccccagtc-3') were used to amplify a 1400 bp fragment of the 16S rRNA gene [Young et al., 1991; Cruz et al., 2001]. The bacteria in our sample set were confirmed to be *R. leguminosarum* by phylogenetic analysis of 16S rRNA and recA sequences. Sequences were generated on the ABI 3500 platform ("Applied Biosystems", USA) using sets of "Big Dye Terminator V.3.1".

Results and discussions

Of the nodules collected from 15 plants of one population growing in the Tatyshlinsky district of the Republic of Bashkortostan (Fig. 1(I)), spaced from each other at a distance of 10 m according to the scheme shown in Fig. 2, pure cultures of nodule bacteria were isolated. 5 to 15 nodules were selected from each plant. RAPD analysis of isolated strains' DNA was carried out, the results of which after were analyzed using cluster analysis. For comparison to the cluster analysis RAPD profiles were also generated for strains isolated from nodules of another plant population growing in the Beloretsk region (Republic of Bashortostan, Russia) and from strains isolated from nodules of *Lathyrus sylvestris* growing together with *Lathyrus vernus* the Tatyshlinsky region (Fig. 3). All strains analyzed by the 16S rRNA gene sequence were phylogenetically homogeneous and related to *R. leguminosarum* (> 99.5% similarity).

The results of the cluster analysis show that the strains entering into symbiosis with one plant schematically are combined predominantly in separate clades with more than 85% support. The similarity between these bacteria is most likely caused by the numerical predominance of a certain strain of nodule bacteria in the rhizosphere, which occurs due to the reproduction of virulent clones in nodules, where only part of the bacterial cells, are differentiated into nitrogen-fixing bacteroids. Significant number of bacteria (up to 10^6 – 10^7 per nodule) retains the properties of free-living cells and after the decomposition of nodules are released into the soil, providing their numerical superiority over other strains and, accordingly, increasing the likelihood of nodule formation by bacteria of this strain. This is especially true for perennial plant species including *Lathyrus vernus* [Hirsh, 1996].

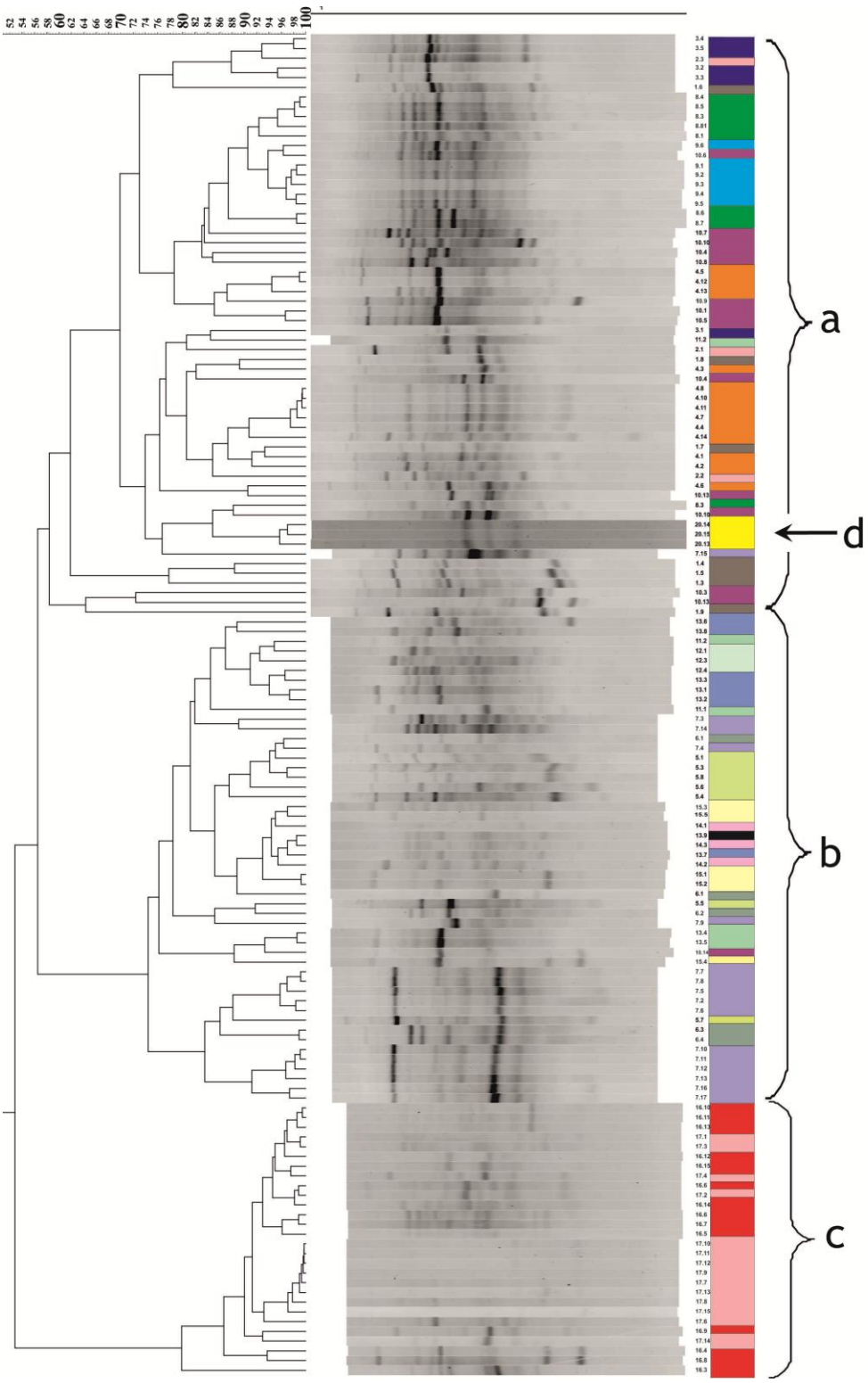

Fig. 3. Scheme of *L. vernus* plants (from the nodules of which rhizobia were isolated) locations in Tatyshlinsky district (Republic of Bashkortostan, Russia). Plant numbers are marked in the circles. A - plants, whose rhizobia form cluster A in cluster analysis; B - plants, whose rhizobia form cluster B in cluster analysis.

The similarity of RAPD-DNA profiles of bacteria reflect the influence of distance between locations from which samples were collected. Thus, plant microsymbionts growing in neighbouring areas exhibit a higher percentage of similarity in their RAPD profiles, compared to those collected from locations more distant to each other. But extraordinary is the fact that on the dendrogram all strains isolated from plant nodules from the Tatyshlinsky district are divided into two separate clades, designated as (A) and (B), having a similarity level of 56%, while the strains inside these clades, with some exceptions, have a similarity level of 70% and above. So, clade (A) is formed by strains from plant nodules 1, 2, 3, 4, 8, 9, 10, and clade (B) is formed by strains of plant nodules 5, 6, 7, 11, 12, 13, 14, 15 (Fig. 3). RAPD-profiles of strains isolated from the nodules of plants growing in the Beloretsky region, which were taken for comparison, form a separate clade (C), and this clade has 52% of similarity level with the clades (A) and (B). At the same time, RAPD-profiles of strains isolated from the nodules of the *Lathyrus sylvestris*, growing together with the *Lathyrus vernus* in the Tatyshlinsky district, are referred to the clade (A), and even have a significant similarity (84%) with RAPD-profiles of strains isolated from the nodules of neighbouring *L. vernus* plants.

However, spatial separation cannot be the sole explanation of differences in RAPD-profiles of nodule bacteria isolated from the nodules of *L. vernus* in the Tatyshlinsky district, forming two separate clade (A) and (B). The observed genetic differences are likely driven by biological barriers that restrict recombination between these strains. Limiting horizontal gene transfer between bacteria, forming clade A and bacteria forming clade B indicates about more significant differences between these bacteria and about the existence of a “friend - foe” identification mechanism in them. The exchange of genetic information, which occurs mainly within a certain group of rhizobia, allows them to prevent their losses by diluting them in the total mass of bacteria identified as relevant for the survival of plasmids or individual genes [Smith et al., 1993]. Probably, rhizobia form a certain genetic cluster, within which the frequency of horizontal gene transfer between cells is very significant and is limited between the representatives of other clusters. Thus, there is the formation of local bacteria groups with a specific genetic profile in addition to their own genome of the whole species, which by analogy with Internet technologies can be called "cloud" genome, access to which is open due to the possibility of genetic information exchange, and the level of access to it is likely to correlate with phylogenetic proximity of bacteria.

The similarity of RAPD-profiles of nodule bacteria from the nodules of *Lathyrus sylvestris* (D) with RAPD-profiles of nodule bacteria from *L. vernus* nodules in the joint growth of these species demonstrates that plants referred to the same group of cross-inoculation do not differ in the preference of certain bacteria genotypes. High recombination activity is peculiar for many types of soil bacteria and represents the mechanism for their adaptation to changing environmental conditions. *R. leguminosarum* is not an exception in this sense. Besides, the generation of a local groups of bacteria with a high frequency of genetic metabolism gives them additional opportunities for survival, because it results in to increase of the amount of accessible "cloud" genetic material.

Conclusions

Overall, the results of our work on the example of the distribution of nodule bacteria *R. leguminosarum* strains in *L. vernus* nodules indicated that the initial levels of the organization of the pangenome of bacteria, where due to different genetic integration between different groups of strains local pangenomes are formed, which together probably form a higher level of pangenome.

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