Matching population diversity of rhizobial nodA and legume NFR5 genes in plant–microbe symbiosis

Anna A. Igolkina1,2 | Georgii A. Bazykin3,4 | Elena P. Chizhevskaya1 | Nikolai A. Provorov1 | Evgeny E. Andronov1,5,6

Abstract
We hypothesized that population diversities of partners in nitrogen-fixing rhizobium–legume symbiosis can be matched for “interplaying” genes. We tested this hypothesis using data on nucleotide polymorphism of symbiotic genes encoding two components of the plant–bacteria signaling system: (a) the rhizobial nodA acyltransferase involved in the fatty acid tail decoration of the Nod factor (signaling molecule); (b) the plant NFR5 receptor required for Nod factor binding. We collected three wild-growing legume species together with soil samples adjacent to the roots from one large 25-year fallow: Vicia sativa, Lathyrus pratensis, and Trifolium hybridum nodulated by one of the two Rhizobium leguminosarum biovars (viciae and trifolii). For each plant species, we prepared three pools for DNA extraction and further sequencing: the plant pool (30 plant indiv.), the nodule pool (90 nodules), and the soil pool (30 samples). We observed the following statistically significant conclusions: (a) a monotonic relationship between the diversity in the plant NFR5 gene pools and the nodule rhizobial nodA gene pools; (b) higher topological similarity of the NFR5 gene tree with the nodA gene tree of the nodule pool, than with the nodA gene tree of the soil pool. Both nonsynonymous diversity and Tajima’s D were increased in the nodule pools compared with the soil pools, consistent with relaxation of negative selection and/or admixture of balancing selection. We propose that the observed genetic concordance between NFR5 gene pools and nodule nodA gene pools arises from the selection of particular genotypes of the nodA gene by the host plant.

KEYWORDS
evolutionary molding, NFR5, nodA, phylogenetic congruence, rhizobium–legume symbiosis, topological diversity

1 | INTRODUCTION

One of the earliest studied types of symbiosis, the host–parasite interaction, was described by Flor’s Gene-for-Gene concept (Flor, 1971, 1942) and, in fact, the first mathematical model of coevolution was explicitly based on the assumption of a Gene-for-Gene (GFG) interaction (Mode, 1958; Thompson & Burdin, 1992). Further analyses of host–parasite interactions revealed concepts, namely matching-allele (MA) (Frank, 1994), inverse-matching-allele (IMA) (Otto & Michalakis, 1998), and inverse-gene-for-gene (IGFG) (Fenton, ...
Antonovics, & Brockhurst, 2009), which together with the GFG represent the opposite end of the same continuum of host-parasite specificity (Agrawal & Lively, 2002). These theoretical concepts, initially developed for antagonistic systems, found their reflection also in mutualistic symbiotic systems (Cregan, Sadowsky, & Keyser, 1991; Lewis-Henderson & Djordjevic, 1991; Parker, 1999; Sachs, Eisenberg, & Turcotte, 2011; Sadowsky et al., 1991). One of the possible consequences of the above-mentioned concepts is that matching between symbionts could be observed not only on the gene sequence level but also on the population structure level. We proposed that coordinated population diversity of symbionts can be a significant aspect of symbiotic interactions in a row with the difference in evolutionary rates between interacting species as considered in the Red Queen and the Red King dynamics (Bergstrom & Lachmann, 2003; Pal, Maclá, Oliver, Schachar, & Buckling, 2007; Paterson et al., 2010; Van Valen, 1973).

Previous studies have demonstrated the matching population diversities of symbionts. For example, the analysis of the symbiosis between Neorhizobium galegae and its host plant Galega indicated correspondence of population diversity levels between microsymbionts and the host Galega species (Andronov et al., 2003; Österman et al., 2011). In particular, a more genetically diverse Galega orientalis population harbors a more diverse root nodule rhizobial population, while its less diverse sympatric counterpart Galega officinalis forms symbiosis with a less diverse rhizobial population. This observation is related to the well-studied phenomenon of shaping the genetic structure of the rhizobial population through the selection of specific rhizobial genotypes by the host plant (Depret & Laguerre, 2003; Heath & Tiffin, 2007; Laguerre, Louvrier, Allard, & Noelle, 2003; Paffetti et al., 1998). Moreover, it has been shown that the topology of the nodA gene tree follows the corresponding host plant tree more strictly than the 16S rRNA-based rhizobial phylogeny (Dobert, Breil, & Triplet, 1994; Suominen, Roos, Lortet, Paulin, & Lindstro, 2001). Therefore, we expect that the interplay of symbiotic populations leads to concordance between the diversity levels in their symbiotic genes.

In this study, we focused on two symbiotic genes that can be considered interacting as both encode the essential components of the rhizobium–legume signaling system; these are associated with each other through a lipochito-oligosaccharide called Nod factor (NF) (Figure 1). The first component is the rhizobial nodA gene which encodes an acyltransferase enzyme essential in NF biosynthesis, specifically in the attachment of the long-chain fatty acid tail to the oligosaccharide backbone (Déharié, Debellé, & Promé, 1996; Esseling & Emons, 2004; Oldroyd, 2013). The second component is one of the plant symbiotic receptor genes, NFR5, which is a homologue of LjNFR5, MtNFP, PsSym10 genes. Its product recognizes NFs (signaling molecules) by three extracellular LysM domains and triggers the formation of root nodule primordia giving the green light to the process of bacterial infection (Oldroyd, 2013). The NFs are major determinants of host specificity: rhizobia produce NFs with different structures, and host plants perceive only those NFs that have a certain composition (Mergaert & Montagu, 1997). The variation of NFs structure is observed not only between rhizobia species but also at the intra-species level (Spank, 2000); one rhizobia species produces a mixture of NFs that vary in the fatty tail modifications. As proposed, the nodA product can vary in its fatty acid specificity, thus contributing to the bacterial host range (Déharié et al., 1996; Moulin, Béna, & Stepkowski, 2004; Ritsema, Wijfjes, Lugtenberg, & Spank, 1996; Roche et al., 1996). It is logical to assume that the nodA gene diversity in a rhizobial population can reflect the structural variation of NFs produced by this population. Indeed, it has been shown that minor differences in the structure of fatty acids tail can affect intra-species host specificity (Li et al., 2011). On the host plant side, NFs are recognized by high-affinity legume receptors (Broghammer et al., 2012; Moulin et al., 2004). Studies on the model legumes revealed NFR5 as one of the major receptors to perceive NFs (Radutoiu et al., 2007). Mutant analysis showed that single amino acid differences in one domain of the NFR5 receptor change recognition of NF variants (Broghammer et al., 2012; Radutoiu et al., 2007). Such mediation of NFs between rhizobial nodA and legume NFR5 genes make them good candidates for testing the hypothesis that population diversities of partners in nitrogen-fixing rhizobium–legume symbiosis are matched.

We tested the hypothesis on symbiotic systems of three wild-growing legume species (Vicia sativa, Lathyrus pratensis and Trifolium hybridum) with their rhizobial microsymbionts. Sampling of the experimental material was performed uniformly on the one large natural fallow (more than 25 years) field in order to avoid the influence of ecological factors. We collected 30 plant individuals for each of three species and, for each individual, collected (a) leaves, (b) nodules, and (c) soil samples. After the pooling, we obtained nine samples (3 species × 3 types of materials) each containing aggregated information of 30 samples (Figure S1). Vicia and Lathyrus species represent the

**FIGURE 1** A part of the signal transduction system that governs the rhizobium–legume symbiosis. The rhizobial nodA gene encodes the acyltransferase that participates in the attachment of the hydrophobic long-chain fatty acid tail to the Nod factor core. Plant NFR5 gene encodes the symbiotic receptor recognizing the rhizobial Nod factor followed by symbiosis formation.
same cross-inoculation group nodulated with *Rhizobium leguminosarum* bv. *viciae* strains, while *Trifolium* belongs to a separate group nodulated with *R. leguminosarum* bv. * trifolii*. One of the important traits of the rhizobium-plant symbiosis is the annual cycle of rhizobia, consisting of nodule formation with consequent amplification of rhizobia inside of the nodule, followed by release of the nodule rhizobia back into the soil after nodule degradation leading to an increase of the frequency of this rhizobial genotype in the soil (Spaïnk, Kondorosi, & Hooykaas, 1998). Therefore, we analyzed both soil and nodule populations of rhizobia, which affect each other.

Testing the hypothesis of matching population diversities required comparison of structural (topological) characteristics of plant and rhizobia populations. Traditionally, the topological similarity between two populations is estimated as the congruence of two respective labeled trees (Leigh, Lapointe, Lopez, & Baptiste, 2011). Here, we propose a novel method to compare topologies of two gene trees with unlabeled leaves. The method is based on the gCEED approach (Choi & Gomez, 2009) that translates each population to the Gaussian mixture model in a K-dimensional space. This method can be classified as a kind of beta-diversity metric, which, by analogy with taxonomic (Jost, Chao, & Chazdon, 2011) and phylogenetic methods (e.g., UniFrac, Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011), could be denoted as “topological beta-diversity.” We apply it to show that the tree structures are concordant between the two symbiont species.

### 2 | MATERIALS AND METHODS

#### 2.1 | Sampling

Three wild-growing legume species (30 samples per species) together with rhizosphere soil—the common vetch *V. sativa*, the meadow vetchling *L. pratensis*, and the alsike clover *T. hybridum*—were uniformly collected from the large natural fallow (more than 25 years) field near the town Vyritsa (Gatchinskii region of Leningradskaya oblast, Russia, 59°24’7.74″N; 30°15’28.74″E). All sampled plants had formed nitrogen-fixing symbiotic root nodules that were selected and thoroughly washed. Soil samples were collected from close proximity to the plant roots (1-5 cm). Despite the fact that a host plant has an influence on the rhizobial population within these soil samples, these samples are referred to as soil samples. Three nodules from each plant sample were picked from the main or the closest to the main lateral roots. For each legume species, we prepared three pools for DNA extraction: the plant pool (30 leaf pieces, 0.1 g each), the nodule pool (90 nodules, 3 nodules per plant individual), and the soil pool (30 soil samples, 0.2 g each).

#### 2.2 | nodA amplification and sequencing

DNA was isolated from soil and nodule pools by bead beating homogenization (Precellys 24) and purification (PowerSoil DNA Isolation Kit; MoBio). Two pairs of nested degenerate oligonucleotide primers were designed for *nodA* gene of *R. leguminosarum* bv. *viciae* and bv. *trifolii*. The first round of nested PCR with external primers—forward (5′-DDGHTYGTAYGAGTGC-3′) and reverse (5′-AGYTCSSACCCRTTT-3′)—produces a 324 bp amplicon product; the second round with inner primers—forward (5′-YTDGGMATCGCCHACT-3′) and reverse (5′-RDACGAGBACRTTCCRT-3′)—produces a 217 bp amplicon product. The reaction conditions in the first and the second round of the PCR consisted of the initial denaturation step at 94°C for 3 min followed by 35 cycles with denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s, and extension at 72°C for 1 min. The bar-coded PCR products from six *nodA* libraries (soil and nodule libraries from three plant species) were sequenced with a Roche 454 GS Junior (following the manufacturer’s protocols) generating an average of 3,000–4,000 reads per library. All obtained sequences were subjected to filtration by quality (quality score higher than 25), length (longer than 170 bp), and separating into libraries according to barcodes in QiIME. We introduced the term “pool” to designate a set of *nodA* gene sequences from nodule or soil rhizobia population. We performed the rarefaction analysis of the α nucleotide diversity within rhizobial pools to demonstrate whether the number of resultant sequences was sufficient to estimate the diversity (Figure S2).

The sequencing data were deposited at the NCBI short read archive under the bioproject number PRJNA297503. The multiple alignments of the remaining sequences within each pool were performed with ClustalW algorithm as implemented in MEGA (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Sequences with frame-shift errors were removed. The resultant multiple alignment for each pool did not contain gaps.

#### 2.3 | NFR5 amplification and sequencing

DNA from plant leaf pools was isolated by AxioPrep kit (Axigen) and was used as the template DNA for PCR amplifications. Approximately 0.9 kb DNA fragments encoding all three LysM domains of the plant receptor gene, NFR5, were amplified with the following pairs of primers: forward “NFR5-for4” (5′-AGCTTGTTGTTTCTTGGCC-3′) and reverse “NFR5-Grev3” (5′-CAACATGGAAAGTACTTATTACGCA-3′) for *V. sativa*; forward “NFR5-for4” and reverse “NFR5-Grev3” (5′-TGCTCTAGCTTGGCTACTTAC-3′) for *L. pratensis*; forward “NFR5-for4” and reverse “NFR5-Grev6” (5′-CATACATTGGGTGCTTAC-3′) for *T. hybridum*. The standard PCR protocol was used: initial denaturation at 95°C for 3 min, 30 cycles with denaturation at 94°C for 30 s, primer annealing at 48°C for 30 s, extension at 72°C for 1 min, and final extension for 4 min. PCR fragments were extracted from agarose gel (Onishchuk, Chizhevskaya, Kurchak, Andronov, & Simarov, 2015) and cloned into the plasmid pTZ57R/T (Thermo Scientific). For each plant species, 100 randomly selected cloned fragments of NFR5 genes were sequenced by Sanger method in an automated ABI 3500XL sequencer (Applied Biosystems) using standard M13 (−20) and (−26) primers. Sequences were deposited in the GenBank database under the Pool accession number 1041522217. The multiple alignment of 100
sequences was performed with ClustalW as implemented in MEGA6 (Tamura et al., 2013).

2.4 | Gene trees
The total nodA gene sequences from nodule and soil pools aligned with ClustalW were clustered into operational taxonomic units (OTUs) at 95% nucleotide sequence identity threshold using the UCLUST algorithm implemented in QIIME 1.9. A neighbor-joining (NJ) dendrogram based on the numbers of differences between representatives of each OTU was constructed in MEGA6 and rooted using the outgroup nodA gene sequence of Sinorhizobium melloti (GenBank ID AZNW01000092.1).

2.5 | Diversity analysis
To compare the levels of nucleotide diversity \( \pi \) between plant and nodule rhizobia pools, we constructed the distributions of \( \pi \) statistics for each plant population subsampling with replacement of 70 sequences in 2,000 trials from each plant NFR5 pool that initially contained 100 sequences. Subsequently, we randomly formed pairs of \( \pi \) diversity values from the obtained distributions for the plant pools and the respective nodule pools. The total set of 6,000 pairs (2,000 per plant sp.) was taken as a paired sample data. The relationship between host plants (NFR5 gene) and nodule rhizobial (nodA gene) population diversity was assessed as the value of Spearman’s rank correlation coefficient for the paired sample data. The values higher than 0.8 were taken to imply monotonic relationship in \( \pi \) between plant and nodule rhizobial pools.

All calculations were implemented in MATLAB. The link to the GitHub repository containing MATLAB scripts for the diversity analysis is provided in the Supporting information.

2.6 | Statistical methods for detecting selection
The \( D^N/D^S \) ratio is a widely used measure to quantify the selection pressure acting on a set of homologous protein-coding gene regions, where \( D^N \) and \( D^S \) are two measures of divergence between species, with \( D^N \) corresponding to the number of nonsynonymous substitutions per nonsynonymous site and \( D^S \) to the number of synonymous substitutions per synonymous site. The \( pN/pS \) statistic is analogous to the \( D^N \) and \( D^S \) statistics but are used for levels of polymorphism within a population rather than divergence (Kryazhimskiy & Plotkin, 2008). As we analyzed the gene regions that are smaller than the probable length of linkage disequilibrium blocks, we assumed that \( pN \) and \( pS \) value within this gene region can be nonindependent. Under the assumption of the nonindependence of \( pN \) and \( pS \), the \( pS \) statistic does not reflect neutral mutations, and the \( pN/pS \) ratio became inconsistent. Thus, we analyzed \( pN \), \( pS \), and \( pN/pS \) separately, mostly focusing on \( pN \) as natural selection acts on nonsynonymous changes. Comparing the nodule and soil pools, we assumed that the increase of \( pN \) in one of them indicates the potential presence of stronger positive selection in it or the presence of stronger negative selection in the other pool. We hypothesized that the \( pN \) value in a soil rhizobial pool is greater than or equal to the \( pN \) value in the respective nodule pool. We tested this hypothesis for each legume species separately, performing Welch’s t test considering 0.01 level of significance as described above.

Tajima’s \( D \) statistic represents the difference between the observed and the theoretically expected nucleotide diversity (Tajima, 1989). If the mutations are neutral and the population adheres to the Wright–Fisher assumptions (Hartl & Clark, 2007), Tajima’s \( D \) equals zero. Values significantly higher than zero indicate the deficit of rare alleles (e.g., due to a recent decrease in population size or balancing selection), and values significantly lower than zero indicate the excess of rare alleles (e.g., due to a recent population size expansion or purifying selection).

In order to identify the selection type underlying the transformation of a soil pool to the respective nodule pool, we compared the values of Tajima’s \( D \) between the pools. We considered the hypothesis that the Tajima’s \( D \) in the soil pool is higher or equal to that in nodule pool. We tested this hypothesis using Welch’s t test at 0.01 level of significance.

The calculations were performed using MATLAB PGE Toolbox (Population Genetics Evolution Toolbox). The link to the GitHub repository containing MATLAB scripts for the analysis of selection is provided in the Supporting information.

2.7 | Topological organization of diversity in plant and rhizobia pools
Let two populations of different sizes be represented by a set of aligned sequences. Let \( p \) be a population index, \( p \in \{1, 2\} \). At the first step, the method identifies the unique haplotypes and their frequencies in each population, denoted as \( \{h_i^p, f_i^p\} \). Let \( n_p \) be the number of unique haplotypes in the population \( p \). Let \( D_{i,j}^p \) be the symmetric distance matrix between each pair of haplotypes in a population \( p \). At the second step, the hierarchical agglomerative clustering method merges haplotypes into clusters until the number of clusters equals to a predefined number \( m \). We fix a population \( p \) and omit this index. The clustering algorithm starts with placing each haplotype in its own cluster that is described by two parameters: frequency \( f_i \) and mean difference \( a_i \) (initialized to zero). Then, the following procedure is repeated until exactly \( m \) clusters occur. Two clusters, the \( i \)-th and \( j \)-th with the smallest pairwise distance \( D_{i,j}^p = \min_{i,j} D_{i,j} \), are merged into one new cluster. A distance from this cluster to a \( k \)-th cluster is calculated as follows:

\[
D_{i,j} = \frac{\sum_{k=1}^{m} \left( (h_k^i f_k^i + D_{i}^p (h_k^j f_k^j) / h_k^i f_k^j) - (h_k^i f_k^i + D_{j}^p (h_k^j f_k^j) / h_k^i f_k^j) \right)}{h_k^i f_k^j + D_{j}^p (h_k^j f_k^j) / h_k^i f_k^j}
\]

The frequency of the new cluster is \( f_i + f_j \) and the mean difference of the new cluster is \( a_i + a_j \).

The described hierarchical clustering is applied to each population and yields the \( m \) clusters of haplotypes with the reduced distance matrix between them \( D_{i,j}^p \), the frequencies \( f_i^p \), and the mean differences within clusters \( a_i^p \), \( i,j = \{1, m\} \). In order to normalize the \( D_{i,j}^p \) and \( a_i^p \) values between two populations, we divided these values by the median across \( D_{i,j}^p \) and \( a_i^p \) values. If a cluster contains only one haplotype and its \( a_i^p \) is equal to zero, we set \( a_i^p = \min_{i,j} a_i^p \), \( f_i^p = 1 \).
At the third step, the set of clusters for each population was translated into $K$-dimensional Euclidean space (in the current project, we worked with 3D space) by Metric multidimensional scaling (Metric MDS) that transforms the distance matrix $(D_{ij})_{i,j=1,...,m}$ into a set of coordinates $(x^p_i)_{i=1,...,m}$. Then, the Gaussian mixture model (GMM) is introduced for the population $p$ as follows:

$$G_p(x) = \sum_{m=1}^{m} f_i N(x|x^p_i, \sigma_i),$$

The adjustment of two GMMs is carried out by Procrustes superimposition: the minimization of the dissimilarity between mixtures ($\Delta G$) via translating, rotating, and mirror reflection. The lower the $\Delta G$ value is, the more similar two GMMs are and, consequently, the more similar the topologies of two population structures are. The exact formula to compute $\Delta G$ is presented in Choi and Gomez (2009; equation 12).

For each plant species, we performed two comparisons: "plant population versus nodule pool" and "plant population versus soil pool." We used joint rhizobial nodule and soil populations before clustering and MDS, and separated them before the Procrustes analysis. This manipulation ensured that the same haplotypes in both comparisons were taken into account in the same way. We tested the null hypothesis that the $\Delta G$ value in the first comparison is greater than or equal to the $\Delta G$ value in the second comparison. In other words, the similarity between nodule rhizobia and plant pool topologies is not higher than the similarity between soil rhizobia and plant pool topologies. For each of the two comparisons, we obtained the set of $\Delta G$ values bootstrapping sequences in rhizobial pools. To test the hypothesis, we compared two obtained sets of $\Delta G$ values by one-sided Mann–Whitney U test with 0.01 level of significance.

For visual comparison of plant and rhizobial populations, we constructed tanglegrams based on adjusted GMMs after Procrustes superimposition. A tanglegram is a diagram with a pair of two binary trees with matching leaves connected by edges. To construct it, we built two NJ gene trees for $m$ plant NFR5 clusters and $m$ rhizobium $nodA$ clusters based on the between-cluster distance matrices and plotted two trees face to face. A pair of leaves from two trees was connected by an edge if a 3D point corresponding to one leaf was located within the five closest points to a point of another leaf and vice versa.

The link to the GitHub repository containing MATLAB scripts for topological beta-diversity analysis is provided in the Supporting information.

### 3 | RESULTS

#### 3.1 $nodA$ gene: OTUs and cluster analysis

$nodA$ gene libraries (for root nodules and for soil samples) were sequenced by NGS technology. Sequencing and filtration of $nodA$ gene libraries produced a total of 22,463 sequences, for an average of 3,750 sequences per pool. Clustering by 95% sequence identity produced 15 OTUs, which frequencies differed between the pools. The NJ tree constructed for OTU representatives showed that the rhizobium population consisted of two clusters (orange and blue in Figure 2). Almost all sequences from the first cluster (9 OTUs) belonged to Vicia and Lathyrus pools, while sequences from the second cluster (6 OTUs) were mostly detected in Trifolium pools. The converse was also true: most of the Vicia-Lathyrus pool belonged to the first cluster (90% average), while most of the Trifolium pool belonged to the second cluster. This distribution of OTUs was in agreement with the "cross-inoculation groups" concept, which refers to the fact that separate groups of legume species can be successfully inoculated by only the specific groups of rhizobia. We attributed the first cluster to $R.\ leguminosarum$ bv. $viciae$ and the second to $R.\ leguminosarum$ bv. $trifolii$. For further analysis, we kept in Vicia-Lathyrus pool only sequences from the first cluster, and in Trifolium pools, only sequences from the second cluster. A small fraction of sequences in nodule pools ($<5\%$) was from the improper cluster likely
corresponding to a minor admixture of soil rhizobia adhered to the nodule surface.

### 3.2 Relationship between the bacteria and host plant diversities

We calculated the diversity levels \( \pi \) in each plant population and found that the ranking of the three species was the same as the ranking based on the \( \pi \) nucleotide diversity values in corresponding nodule pools. By bootstrapping the plant and rhizobial nodule pools, we estimated the Spearman correlation between nucleotide diversities in these pools. The 0.89 value, which was higher than the predefined threshold, indicated that the monotonic relationship between diversities in pools of plant NFR5 gene sequences and in bacterial nodA node pools is statistically significant (Figure 3).

### 3.3 Concordance of gene trees

A visual comparison of topologies of plant NFR5 trees with those of corresponding rhizobial nodA trees for nodule and soil pools revealed that the topology of clades in the plant trees was more similar to the topology of clades in the nodule trees than in the soil trees (Figure S4, Appendix S1). Based on this observation, we proposed that the gene tree of the nodule rhizobia is more similar to that of the host plant than the gene tree of the soil rhizobia.

To formally test this, we developed a method for comparing structures (topologies) between two pools. We tested the null hypothesis that the topological similarity between a nodule rhizobia pool and a plant pool is not higher than the topological similarity between the soil rhizobia pool and the plant pool. This hypothesis was rejected at the 0.01 level of significance. We constructed tanglegrams that also illustrated a higher similarity of the topology of the nodule rhizobial nodA gene trees (Figure 4, left tanglegram) than the soil rhizobial nodA gene trees (Figure 4, right tanglegram) to that of plant NFR5 gene tree. The statistics \( \Delta G \), which reflects the topological difference between structures of plant and bacteria populations, can be referred to as "topological beta-diversity."

The obtained tanglegrams demonstrated that nodule pools contained clades of genotypes that were not detected in the corresponding soil pools (Figure 4). The presence of these clades can be a result of host plant selection of rare soil genotypes and is responsible for the increased topological similarity between NFR5 gene pools with nodule nodA gene pools.

### 3.4 Analysis of selection

Analysis of population structures revealed the significant linkage between synonymous and nonsynonymous polymorphic sites, suggesting that the \( pN \) and \( pS \) statistics were not independent (see Appendix S2). Further comparison of \( pN/pS \) values between nodule and soil pools did not establish the significant difference (Figure S3). Despite the inconsistency of \( pN/pS \) in our case, we observed that the values of both statistics, \( pN \) and \( pS \), were significantly increased (Welch's t test, \( p \)-values < .01) in the nodule pools, indicating that both nonsynonymous and synonymous diversity were elevated there (Table 1). The values of Tajima's \( D \) were significantly lower than 0 in all of the rhizobial nodA pools, indicating the presence of negative selection (Table 1). However, within each plant, they were significantly higher in nodule pools than in the soil pools (\( p \)-values < .01), suggestive of relaxation of negative selection or admixture of balancing selection in the former. *Trifolium* pools displayed the highest values of this statistic, consistently with the strongest admixture of balancing selection, while the *Lathyrus* pools were characterized by the lowest values.

### 4 DISCUSSION

Symbiotic interactions represent a special case of ecological interactions when one of the partners provides an "environment" for another. In *On the Origin of Species* (Darwin, 1872), Charles Darwin proposed that "the life of each species depends in a more important manner on the presence of other already defined organic forms, than on climate." This is particularly true for organisms in deeply integrated symbiotic systems.

Here, we traced the coordination in levels of population diversities between partners within the essential components of the rhizobium–legume signaling system: plant symbiotic receptor gene *NFR5* and *Rhizobium* symbiotic gene nodA involved in the synthesis of signaling molecules Nod factors (NFs), ensuring the first stage of partner recognition (Oldroyd, 2013). The matching was detected in two phenomena. The first is the monotonic relationship between the diversity of plant gene pools and the diversity of nodule rhizobial gene pools (Spearman correlation = 0.89). The second is the higher topological similarity between plant gene pool with nodule gene pool than with soil gene pool. The last phenomena were demonstrated using the developed method to compute "topological beta-diversity"—the difference in topological structures.
of two population sets (plant and rhizobia) of gene sequences. The observed results allowed us to accept the hypothesis that population diversities of partners in nitrogen-fixing rhizobium–legume symbiosis are matched.

We characterized the most pronounced characteristics of the mechanism underlying the matching population diversities. First, the analysis of the selection imposed by plants revealed significantly increased nonsynonymous diversity (pN) and Tajima's D values in the nodule gene pool (Table 1). This may be indicative of weaker negative selection in a nodule gene pool in a comparison with the respective soil gene pool, but is also consistent with a contribution of balancing selection or presence of stronger population structure in the former. In the previous study, it was shown that in symbiotic systems, besides the above-mentioned types of selection, negative frequency-dependent selection in favor of rare genotypes during the competition of rhizospheric bacteria for root nodulation (Amarger & Lobreau, 1982) may also play an important role (Andronov, Igolkina, Kimeklis, Vorobyov, & Provorov, 2015; Provorov & Vorobyov, 2000, 2006). Second, trees of nodule gene pools contained unique clades of genotypes, which were not detected in soil pools likely due to the low frequency of the genotypes in soil. These genotypes are probably responsible for increased topological similarity between plant and nodule rhizobia gene pools, so that a nodule population is selected by the host plant to supply some needs of the latter. In other words, our results demonstrated the transformation of the initial soil nodA pool by the template of the host plant receptor pool, and this conclusion is in line with the numerous works studying the interplay between diversities of host plant and rhizobia (Andronov et al., 2003; Bailly, Olivier, Mita, Cleyt-Marel, & Bena, 2006; Barrett, Zee, Bever, Miller, & Thrall, 2016; Bena, Lyet, Huguet, & Olivier,
study, we modeled the 3D sandwich-like structures of the NFR5-K1 heterodimeric receptor with its ligand Nod factor and observed the mutually polymorphic areas in the contact zone between NFR5 and K1 that were overlapped with known structural variation of Nod factor (in the fatty acid part) produced by \textit{R. leguminosarum} nodA receptors to variations in NF structure likely resulting in matching by \textit{a faster evolving partner, rhizobia in our case. We proposed that according to the effect described, the relationship in population diversity between rhizobia and host plant may be observed not only within the pair of nodA-NFR5 genes (which are related through the NF) but also within any pair of interplaying genes from plant and bacterial sides, and that genome-wide scanning for “matching” genes can be an extension to the traditional methods of functional analysis of genes.

ACKNOWLEDGMENTS

This study is supported by Russian Science Foundation (RSF) grant 19-16-00081.

CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

NodA gene sequencing data were deposited at the NCBI short read archive under the bioproject number PRJNA297503. NFR5 gene sequences were deposited in the GenBank database under the Pool accession number 104152217.

ORCID

Anna A. Igolkina https://orcid.org/0000-0001-8851-9621

REFERENCES

Agrawal, A., & Lively, C. M. (2002). Infection genetics: Gen-for-gene versus matching alleles models and all points in between. \textit{Evolutionary Ecology Research}, 4, 79–90.

Amarger, N., & Lobreau, J. P. (1982). Quantitative study of nodulation competitiveness in rhizobium strains. \textit{Applied and Environmental Microbiology}, 44(3), 583–588.

Andronov, E. E., Igolkina, A. A., Kimeklius, A. K., Vorobyov, N. I., & Provorov, N. A. (2015). Characteristics of natural selection in populations of nodule bacteria (\textit{Rhizobium leguminosarum}) interacting with different host plants. \textit{Genetika}, 51(10), 949–956. https://doi.org/10.1134/S1022779515100026

Andronov, E. E., Terefework, Z., Roumiantseva, M. L., Dzyubenko, N. I., Onichtchouk, O. P., Kurchak, O. N., ... Lindstro, K. (2003). Symbiotic and genetic diversity of \textit{Rhizobium galegae} isolates collected from the \textit{Galega orientalis} gene center in the Caucasus. \textit{Applied and Environmental Microbiology}, 69(2), 1067–1074. https://doi.org/10.1128/AEM.69.2.1067

Bailly, X., Olivieri, I., De Mita, S., Cleyt-Marel, J.-C., & Benà, G. (2006). Recombination and selection shape the molecular diversity pattern of nitrogen-fixing \textit{Sinorhizobium} sp. associated to \textit{Medicago}. \textit{Molecular Ecology}, 15, 2719–2734. https://doi.org/10.1111/j.1365-294X.2006.02969.x

Barrett, L. G., Zee, P. C., Bever, J. D., Miller, J. T., & Thrall, P. H. (2016). Evolutionary history shapes patterns of mutualistic benefit in \textit{Acacia}–rhizobial interactions. \textit{Evolution}, 70(7), 1473–1485. https://doi.org/10.1111/evo.12966

Bena, G., Lyet, A., Huguet, T., & Olivieri, I. (2005). \textit{Medicago} – \textit{sinorhizobium} symbiotic specificity evolution and the geographic expansion of \textit{Medicago}. \textit{European Society for Evolutionary Biology}, 18, 1547–1558. https://doi.org/10.1111/j.1420-9101.2005.00952.x

Bergstrom, C. T., & Lachmann, M. (2003). The red king effect: When the slowest runner wins the coevolutionary race. \textit{Proceedings of the National Academy of Sciences of the USA}, 100(2), 593–598.

Broghammer, A., Krusell, L., Blaise, M., Sauer, J., Sullivan, J. T., Maolanon, N., ... Stougard, J. (2012). Legume receptors perceive the rhizobial lipochitooligosaccharide signal molecules by direct binding. \textit{Proceedings of the National Academy of Sciences of the USA}, 109(34), 13859–13864. https://doi.org/10.1073/pnas.1205171109

Choi, K., & Gomez, S. M. (2009). Comparison of phylogenetic trees through alignment of embedded evolutionary distances. \textit{BMC Bioinformatics}, 15, 1–15. https://doi.org/10.1186/1471-2105-10-423

Cregan, P. B., Sadowsky, M. J., & Keyser, H. H. (1991). Gene-for-gene interaction in the legume-Rhizobium symbiosis. In D. L. Keister, & P. B. Cregan (Eds.), \textit{The rhizosphere and plant growth}, Vol. 2 (pp. 163–171). Dordrecht, the Netherlands: Springer Netherlands. https://doi.org/10.1007/978-94-011-3364-4_32

Darwin, C. (1872). On the origin of species by means of natural selection, or, the preservation of favoured races in the struggle for life. London, UK: J. Murray.

Dénarié, J., Debelle, F., & Promé, J.-C. (1996). \textit{Rhizobium} lipo-chitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. \textit{Annual Review of Biochemistry}, 65(1), 503–535. https://doi.org/10.1146/annurev.bi.65.070196.002443
Depret, G., & Laguerre, G. (2008). Plant phenology and genetic variability in root and nodule development strongly influence genetic structuring of Rhizobium leguminosarum biovar viciae populations nodulating pea. New Phytologist, 179, 224–235. https://doi.org/10.1111/j.1469-8137.2008.02430.x

Dobert, R. C., Breil, B. T., & Triplett, E. W. (1994). DNA sequence of the common nodulation genes of Bradyrhizobium elkanii and their phylogenetic relationship to those of other nodulating bacteria. Molecular Plant-Microbe Interactions, 7(5), 564–572.

Esseling, J. J., & Emons, A. M. C. (2004). Dissection of nod factor signalling in legumes: Cell biology, mutants and pharmacological approaches. Journal of Microscopy, 214, 104–113.

Fenton, A., Antonovic, J., & Brockhurst, M. A. (2009). Inverse-gene-for-gene infection genetics and coevolutionary dynamics. The American Naturalist, 174(6), E230–E242. https://doi.org/10.1086/645087

Flor, H. H. (1942). Inheritance of pathogenicity in Melampsora lini. Phytopathology, 32, 653–669.

Flor, H. H. (1971). Current status of the gene-fob-gene concept. Annual Review of Phytopathology, 9, 275–296.

Frank, S. A. (1994). Recognition and polymorphism in host-parasite genetics. Philosophical Transactions of the Royal Society B: Biological Sciences, 346(1317), 283–293. https://doi.org/10.1098/rstb.1994.0145

Hartl, D. L., & Clark, A. G. (2007). Principles of population genetics, 4th ed. Sunderland, MA: Sinauer Associates.

Heath, K. D., & Tiffin, P. (2007). Context dependence in the coevolution of plant and rhizobial mutualists. Proceedings of the Royal Society B, 274(May), 1905–1912. https://doi.org/10.1098/rspb.2007.0495

Igolkina, A. A., Porozov, Y. B., Chizhevskaya, E. P., & Andronov, E. E. (2018). Structural insight into the role of mutual polymorphism and conservatism in the contact zone of the NFR5–K1 heterodimer with the nod factor. Frontiers in Plant Science, 9, 344. https://doi.org/10.3389/fpls.2018.00344

Jost, L., Chao, A., & Chazdon, R. L. (2011). Compositional similarity and b (Beta) diversity. In A. E. Magurran & B. J. McGill (Eds.), Biological diversity – Frontiers in measurement and assessment (pp. 66–84), Oxford, England: Oxford University Press.

Kryazhimskiy, S., & Plotkin, J. B. (2008). The population genetics of dN/dS. PloS Genetics, 4(12), e1000304. https://doi.org/10.1371/journal.pgen.1000304

Laguerre, G., Louvrier, P., Allard, M. R., & Noelle, A. (2003). Compatibility of rhizobial genotypes within natural populations of Rhizobium leguminosarum biovar viciae for nodulation of host legumes. Applied and Environmental Microbiology, 69(4), 2276–2283. https://doi.org/10.1128/AEM.69.4.2276

Laguerre, G., Haradjid, L. M., & Noelle, A. (2006). Interplay of Darwinian and freelinking processes in the evolution of symbiosis. Trends in Plant Science, 11(1), 39–46. https://doi.org/10.1016/j.tplants.2005.10.002

Leigh, J. W., Lopinto, F. J., Lopez, P., & Baptiste, E. (2011). Evaluating phylogenetic congruence in the post-genomic era. Genome Biology and Evolution, 3(1), 571–587. https://doi.org/10.1093/gbe/evr050

Lewis-Henderson, W. R., & Djordjevic, M. A. (1991). A cultivar-specific interaction between Rhizobium leguminosarum Bv. Trifolii and subterranean clover is controlled by NodM, other bacterial cultivar specificity genes, and a single recessive host gene. Journal of Bacteriology, 173(9), 2791–2799. https://doi.org/10.1128/ JB.173.9.2791-2799.1991

Li, R., Knox, M. R., Edwards, A., Bridget Hogg, T. H., Ellis, N., Wei, G., ... Downtie, J. A. (2011). Natural variation in host-specific nodulation of pea is associated with a haplotype of the SYM37 LysM-type receptor-like kinase. Molecular Plant-Microbe Interactions, 24(11), 1396–1403. https://doi.org/10.1094/MPMI-01-11-0004

Lizupone, C., Lladser, M. E., Knights, D., Stombaugh, J., & Knight, R. (2011). UniFrac: An effective distance metric for microbial community comparison. The ISME Journal, 5(2), 169–172. https://doi.org/10.1038/ismej.2010.133

Mergaert, P., Van Montagu, M., & Hols, M. (1997). Molecular mechanisms of nod factor diversity. Molecular Microbiology, 25(5), 811–817. https://doi.org/10.1111/j.1365-2958.1997.mmi526.x
are host-range determinants. *Proceedings of the National Academy of Sciences of the USA*, 93(December), 15305–15310. https://doi.org/10.1073/pnas.93.26.15305

Sachs, J. L., Essenberg, C. J., & Turcotte, M. M. (2011). New paradigms for the evolution of beneficial infections. *Trends in Ecology and Evolution*, 26(4), 202–209. https://doi.org/10.1016/j.tree.2011.01.010

Sadowsky, M. J., Cregan, P. B., Gottfert, M., Sharma, A., Gerhold, D., Rodriguez-Quinones, F., ... Stacey, G. (1991). The Bradyrhizobium japonicum NolA Gene and Its Involvement in the Genotype-Specific Nodulation of Soybeans. *Proceedings of the National Academy of Sciences of the USA*, 88(2), 637–641. https://doi.org/10.1073/pnas.88.2.637

Spaink, H. P. (2000). Root nodulation and infection factors produced by rhizobial bacteria. *Annual Review of Microbiology*, 2000(54), 257–288. https://doi.org/10.1146/annurev.micro.54.1.257

Spaink, H. P., Kondorosi, A., & Hooykaas, P. J. J. (1998). Soil biology of the Rhizobiaceae. In H.P. Spaink, A. Kondorosi, & P.J.J. Hooykaas (Eds.), *The Rhizobiaceae: Molecular biology of model plant-associated bacteria* (pp. 155–172). Dordrecht, the Netherlands: Springer Science & Business Media. https://doi.org/10.1007/978-94-011-5060-6

Suominen, L., Roos, C., Lortet, G., Paulin, L., & Lindstro, K. (2001). Identification and structure of the *Rhizobium galegae* common nodulation genes: Evidence for horizontal gene transfer. *Molecular Biology and Evolution*, 18(6), 907–916.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123, 585–595.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

Thompson, J. N., & Burdin, J. J. (1992). Gene-for-gene coevolution between plants and parasites. *Nature*, 360, 121–125. https://doi.org/10.1038/360121a0

Van Valen, L. (1973). A new evolutionary law. *Evolutionary Theory*, 1, 1–30.

Vuong, H. B., Thrall, P. H., & Barrett, L. G. (2016). Host species and environmental variation can influence rhizobial community composition. *Journal of Ecology*, 105(2), 540–548. https://doi.org/10.1111/1365-2745.12687

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Igolkina AA, Bazykin GA, Chizhevskaya EP, Provorov NA, Andronov EE. Matching population diversity of rhizobial nodA and legume NFR5 genes in plant–microbe symbiosis. *Ecol Evol*. 2019;9:10377–10386. https://doi.org/10.1002/ece3.5556