

UDC 579.64:631.461.52:577.2

doi: 10.15389/agrobiol.2015.5.655rus

doi: 10.15389/agrobiol.2015.5.655eng

## PHYLOGENETIC ANALYSIS OF *Rhizobium* STRAINS, ISOLATED FROM NODULES OF *Vavilovia formosa* (Stev.) Fed.

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Acknowledgements:

Supported by Russian Science Foundation (grant 14-26-00094). The equipment of ARRIAM Center for Genome Technologies, Proteomics and Cell Biology (St. Petersburg) was used

Received July 7, 2015

### Abstract

Among the 5 genera of tribe *Fabeae*, *Vavilovia* Fed. is the least studied consisting of the only species *Vavilovia formosa* (Stev.) Fed. *Vavilovia*'s area of growth is limited by the highlands of Central and Eastern Caucasus, with only several known populations on the territories of Armenia, Dagestan, North Ossetia, Azerbaijan, Iran, Iraq, Siria, Turkey, and by the environmental conditions. The only phylogenetic research of rhizobia isolated from the nodules of *vavilovia* from the North Ossetian population demonstrated significance of both slow-growing and fast-growing microsymbionts' specific and genetic diversity. It was shown that all of the fast-growing isolates, belonging to *Rhizobium leguminosarum* species, carry *nodX* gene in their genomes. Three expeditions to the regions of Armenia, Dagestan and North Ossetia succeeded in finding and collecting plants of *Vavilovia formosa* (Stev.) Fed. with its nodules, from which later rhizobia isolates were obtained. We have chosen nineteen fast-growing isolates, derived from ten plants' nodules, to identify their species affiliation, to trace geographical isolation and also to try to track down its genetic differences from rhizobia, which nodulate other plants of tribe *Fabeae*. To make this we sequenced ITS (internally transcribed spacer) fragment and *nodA* gene, and made screening of the isolates for the presence of *nodX* gene, which controls rhizobia host specificity. Obtained sequences were used to calculate genetic distances between groups of rhizobia, i.e. different regions isolates (Armenia, Dagestan, North Ossetia) and isolates from different plant hosts (*vavilovia*, pea, clover). Results of ITS sequencing showed that all strains involved in the analysis belong to *R. leguminosarum* (bv. *viciae*) species. ITS-dendrogram shows relatively high heterogeneity of isolates, but on *nodA*-dendrogram they form a very compact group. Difference in the structure of these dendrograms allows to assume that *nodA* gene, chained with the genes of host specificity, can be easily transferred within the populations of *R. leguminosarum*, providing unlimited combinations of specificity to *vavilovia* with different variants of bacterial chromosome. Comparison of genetic distances based on ITS-sequences for the isolates in this study shows tendency to geographic isolation between them. Data on *nodA*-based genetic distances along with the presence of *nodX* gene in the genomes of all *R. leguminosarum* strains in this study point out the presence of its host specificity within biovar *viciae*. It seems that correlation between strain origin and the genetic structure of *nodA* reflects presence of highly specific interactions among each group of *R. leguminosarum* strains with their plant-hosts, whereas correlations with the structure of ITS-loci reflect rhizobia adaptation to soil environment.

Keywords: *Vavilovia formosa*, *Rhizobium leguminosarum*, legume-rhizobia symbiosis.

Tribe *Fabeae* (син. *Viciae*) is among most representative ones in the family *Fabaceae* Endl. It comprises over 300 species, and, however, its taxonomy is still being revised significantly. At that, many species of the tribe are of practical use in agriculture. Most ancient cultivated plants such as pea (*Pisum sativum* L.) and tare (*Vicia sativa* L.) are the representatives of this tribe [1]. Of five genera of the tribe, namely *Lathyrus* L., *Vicia* L., *Lens* Mill., *Pisum* L. and *Vavilovia* Fed., the last one which consists of a single species *Vavilovia formosa* (Stev.) Fed. remains least studied. Its areal covers the highlands of Central and

Eastern Caucasus, and only a few populations are also known in Armenia [2], Dagestan and North Ossetia [3], Azerbaijan [4], Iran, Iraq, Syria and Turkey [5], and its vegetation is limited to conditions the environment [6], therefore, the plant itself and its microsymbionts, remained unknown for a long time.

Recently, based on phylogenetically important genes *matK*, *trnL-F* and *trnS-G*, and ITS (internally transcribed spacer) fragment, the evidence was reported that the vavilovia is taxonomically close to genera *Pisum* and *Lathyrus*, being, nevertheless, clustered separately within tribe *Fabeae* [7]. The only reported phylogenetic study of rhizobia isolates from vavilovia nodules from North Ossetian population showed a fairly broad species abundance and genetic diversity of both fast-growing and slow-growing microsymbionts [8].

In the same study it was also found that all fast-growing isolates of *R. leguminosarum* possessed the gene *nodX* peculiar to the symbionts of *Pisum sativum* cultivar Afghanistan [9]. All known plants of tribe *Fabeae* are of the same cross inoculation group and can produce nodules with the bacteria of the abovementioned species, though symbiosis formation is mostly indifferent to the presence of *nodX* gene. Nevertheless, in the *P. sativum* cv. *Afghanistan*, or Afghan pea, there is *sym2<sup>A</sup>* allele encoding receptor specific to the Nod-factor decorated with extra acetyl group bound under the *nodX* gene control [10].

At present, the studies of *nodX* gene role in individual interaction between a legume plant and rhizobia are fast developed [11]. Data summarized hereinabove, are enough to consider *Vavilovia formosa* and its microsymbiont a promising model in revealing mechanisms of specificity evolution in the legume-rhizobia symbiosis.

Due to our successful expeditions to Caucasian territories, the samples of *Vavilovia formosa* have been collected, and rhizobia from the plant nodules were isolated.

In this research, the fast-growing rhizobia isolates from *Vavilovia formosa* nodules were taxonomically attributed based on analysis of ITS region sequences, and their host specificity was studied by phylogenetic analysis of *nodA* and *nodX* genes. Additionally, the genetic distances in rhizobia isolated from different host plants of distinct locations were compared with regard to ITS and *nodA* sequences to estimate the environmentally important polymorphic parameters in studied isolates.

**Technique.** Nodulated vavilovia plants were collected in 2012-2013 in North Ossetian Reserve (North Ossetia, Alagirskii Region), Armenia and Dagestan at more than 1,500 m above sea level. Of total, 19 rhizobia strains isolated from 10 plants of three different populations were studied.

For rhizobia isolation we used common procedures [12]. From each nodule a strain was isolated and further grown on the media № 79 [13] at 28 °C. All isolated have been deposited to the ARRIAM Russian collection of agricultural microorganisms and stored at Station of Low Temperature Automated Storage of Biological Samples at -80 °C (Liconic Instruments, Lichtenstein) [14]. Data are available online at <http://www.arriam.spb.ru>.

Bacterial DNA was isolated according to standard protocol [15], and 1,000-1,300 bp ITS fragment (intergenic transcribed spacer) was amplified using primers FGPS1490-72 5'-TGCGGCTGGATCCCCCTCCTT-3' and FGPL132'-38 5'-CCGGGTTTCCCCATTCGG-3' [16, 17]. The *nodA* gene 66 bp fragment was analyzed with primers *nodA*-1 5'-GCRGTGGAARNTRN-NCTGGGAAA-3' and *nodA*-2 5'-GGNCCGTCRTCRAASGTCARGTA-3' [18]. The PCR mixture (25 µl) contained 150 µM dNTPs (Helicon, Russia), 1 U Taq-polymerase (Eurogen, Russia), 10 pM of each primers and 10-20 ng of purified DNA as matrix. Amplification was performed at a T100 amplifier (Bio-Rad, USA) as follows: ini-

tial denaturation at 95 °C for 2 minutes; 35 cycles comprising denaturation at 94 °C for 30 seconds, annealing at 50 °C with FGPS1490-72/FGPL132'-38 primers or at 49 °C with nodA-1/nodA-2 primers for 30 seconds, elongation at 72 °C for 1 minute; final elongation at 72 °C for 3 minutes.. PCR fragments were separated electroforetically in 1 % agarose gel (Amresco, USA) with 0,5× TAE, with DNA molecular weigh marker 100 bp + 1,5 Kb + 3 Kb (Sibenzyme, Russia). PCR products were purified as described [19]. ITS region and *nodA* gene were sequenced on a ABI PRISM 3500xl genetic analyzer (Applied Biosystems, USA), with UGENE program (Unipro, Russia) for primary data processing [20]. The similarity was found by BLAST analysis using GeneBank database (<http://www.ncbi.nlm.nih.gov/>). The obtained data were used to design two dendrograms by joint neighbor method (MEGA v. 5.0) [21]. Evolution distances were evaluated by p-distance technique. A bootstrap analysis for 1,000 random samples was used for reliable clustering.

The *nodX* gene in isolated was detected in PCR with primers oMP199-F 5'-CCATGGGACCATCCAATGAAC-3' and oMP196-R 5'-TTAAGCGACG-GAAAGCCTTC-3' [22]. The PCR mix composition and protocol was the same as described hereinabove except annealing at 53 °C. *R. leguminosarum* bv. *viciae* A1 strain [22] producing nodules with Afghan pea plants was a positive control.

Genetic distances were calculated for ITS fragments and *nodA* genes for each taxonomic and geographically distant group of rhizobia the same as between these groups. A genetic distance as estimated on ITS region and *nodA* gene sequences was compared in two distinct groups of microorganisma. The first one comprised *R. leguminosarum* bv. *viciae* (pea rhizobia), *R. leguminosarum* bv. *trifolii* (clover rhizobia) and tested isolated with correlation to host specificity, and the second one consisted of vavilovia isolated as correlated to geografic origin. We compared genetic distances both within and between the groups. The p-distance parameter estimated using MEGA v. 5.0 [21].

**Results.** ITS fragments were amplified and sequenced, partially or completely, in all tested isolated, and in Dagestanian isolates there were at least two different copies of ribosomal operon with ITS fragments of different size. In the dendrogram based on ITS sequencing (Fig. 1) these sequences are marker as l (long) and s (short).

In the Table 1 we indicated the size of amplified fragments and their homology to ITS sequence of typical strains of *R. leguminosarum* species of which TOM strain possesses *nodX* gene. Genomes of these strains are sequenced and available online in the NCBI database. Note, with regard to ITS, strains isolated from vavilovia nodules are similar to *viciae* biovar rather than to *trifolii* biovar, so far as an average similarity coefficient was 83±0.9 % in strain 3841, 89±0.8 % in strain TOM and only 80±1.0 % in strain WSM2304.

### 1. Origin of strains isolated from vavilovia *Vavilovia formosa* (Stev.) Fed. nodules and similarity of their ITS regions to referent strains sequences deposited in GenBank

Strain	Plant №	ITS frag- ment size, np	Similarity, %		
			<i>Rhizobium legumino- sarum</i> bv. <i>viciae</i> 3841	<i>Rhizobium legumino- sarum</i> bv. <i>viciae</i> TOM	<i>Rhizobium leguminosa- rum</i> bv. <i>trifolii</i> WSM2304
North Ossetia					
Vaf-01	1	565	96	86	90
Vaf-09		613	75	90	75
Vaf-10	3	1206	83	95	81
Vaf-12		1184	85	89	88
Vaf-23	4	565	99	88	100
Vaf-25		649	80	99	72
Vaf-26	5	1113	91	94	91

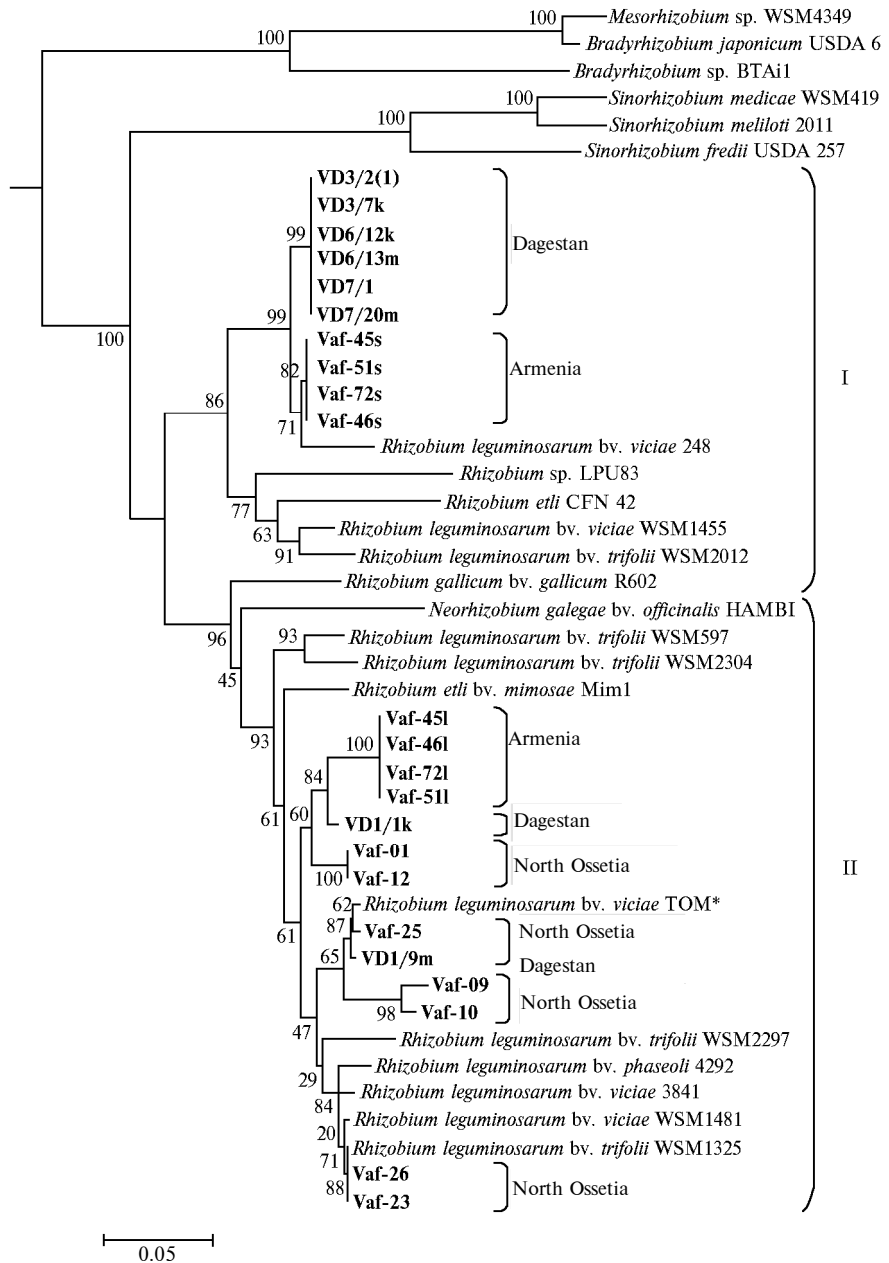
		Armenia			
Vaf-45	7	1199	82	93	80
		1010	78	90	79
Vaf-72		1199	82	93	80
		1010	78	90	79
Vaf-46	8	1199	82	93	80
		1010	78	90	79
Vaf-51		1199	82	93	80
		1010	78	90	79
		Dagestan			
VD1/1k	1D	1105	92	86	87
VD1/9m		1287	86	94	75
VD3/2(1)	3D	1032	82	83	77
VD3/7k		1032	82	83	77
VD6/12k	6D	1032	82	83	77
VD6/13m		1032	82	83	77
VD7/1	7D	1032	82	83	77
VD7/20m		1032	82	83	77

ITS dendrogram showed that all tested strains was attributed to family *Rhizobiaceae* and clustered into two groups of bacterial species with different types of ribosomal operon (the clusters I and II). Moreover, strains were grouped with no regard to taxonomic attribution or geographical prevalence. *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, and *R. etli*, the same as different vavilovia isolates from Armenia and Dagestan were representatives of both groups. It probably could be due to only few chromosome types providing adaptation to environmental conditions and viability in bacteria.

Rhizobia isolated from vavilovia nodules did not form an individual cluster, and separated into groups demonstrating tendency to geographical isolation in the tested microorganisms. All Armenian strains, the same as 6 of 8 strains from Dagestan, were clustered at high statistical level ( $> 82\%$ ) in group I. ITS fragments of vavilovia strains from North Ossetia was found only in group II and clustered along with strains from the same territory (Vaf-01 and Vaf-12, Vaf-09 and Vaf-10, Vaf-23 and Vaf-26). Vaf-25 strain was the only exception and grouped at 87 % bootstrap level to strains VD1/9m from Dagestan and *R. leguminosarum* bv. *viciae* TOM with *nodX* gene. Similar clusterization pattern we observed earlier in North Ossetinian isolates [8]. Therefore, it was reasonable to study whether there was *nodX* gene in the isolates from Armenia and Dagestan (Fig. 2), and it was found it all tested isolates.

Importantly, the ITS sequence, being the core part of the genome, allows us to understand only the taxonomic attribution of the stains whereas host specificity can be understood only from studying genes involved in formation of legume-rhizobia symbiosis. These are the genes of *nod* operons [23]. So we studied *nodA* gene. Though *nodA* gene does not control the symbiotic specificity directly, it is linked to genes encoding factors which provide host specificity. This gene is convenient to study due to a single copy in the rhizobia genome [18]. ITS and *nodA* based dendrograms differed sufficiently (Fig. 1, 3). In the *nodA* dendrogram, all isolates clustered with *R. leguminosarum* bv. *viciae* strains at 100 % statistical level. Thus, these isolates may be considered the rhizobia of the pea cross inoculation group. Based on *nodA* gene analysis, the isolates of this cluster were divided into three groups which did not coincide with ITS groups. In this, the isolates from North Ossetia and Dagestan clustered together into groups I and III, while Armenian isolates were found only in group II (see Fig. 3). It should be noted that in the *nodA* dendrogram the strain *R. leguminosarum* bv. *viciae* TOM was most closely related to the studied isolated. The strains from other taxones were not found in all three clusters comprising vavilovia isolates, that may be

consider an additional evidence toward isolation of these strains.

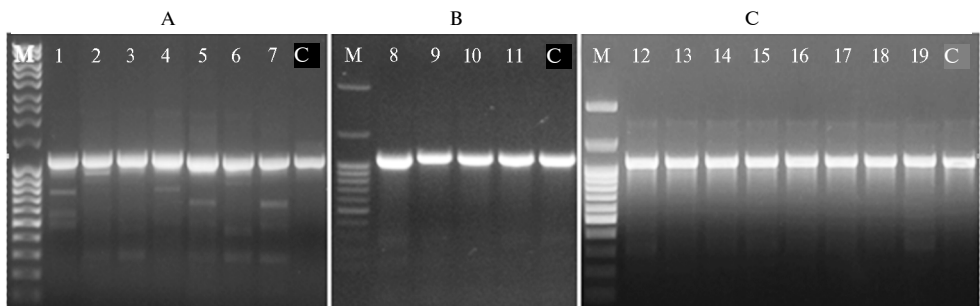


**Fig. 1. Dendrogram based on ITS (758 bp) sequences in rhizobium strains isolated from *Vavilovia formosa* (Stev.) Fed. plants depending on geographical origin.** Strains are grouped by the «neighbor-joining» method. Phylogenetic tree of the isolates reflects their taxonomy. Bootstrap value > 45 % for 1,000 random samples are show. I and II are statistically reliable clusters. Strain with *nodX* gene is marked (\*); s (short) and l (long) are Armenian strains with different copies of ITS fragments. Studied strains are mark bold.

In different groups of rhizobia no relationship with host specificity was traced based on ITS fragment sequencing analysis, since genetic distance within the groups was statistically indistinguishable from that between the groups (Table 2). Thus, more reasonably, there is a tendency to geographical isolation of the vavilovia microsymbionts involved in our study.

## 2. Genetic distance on ITS and *nodA* gene sequences in rhizobia isolated from different host plants depending on geographical origin

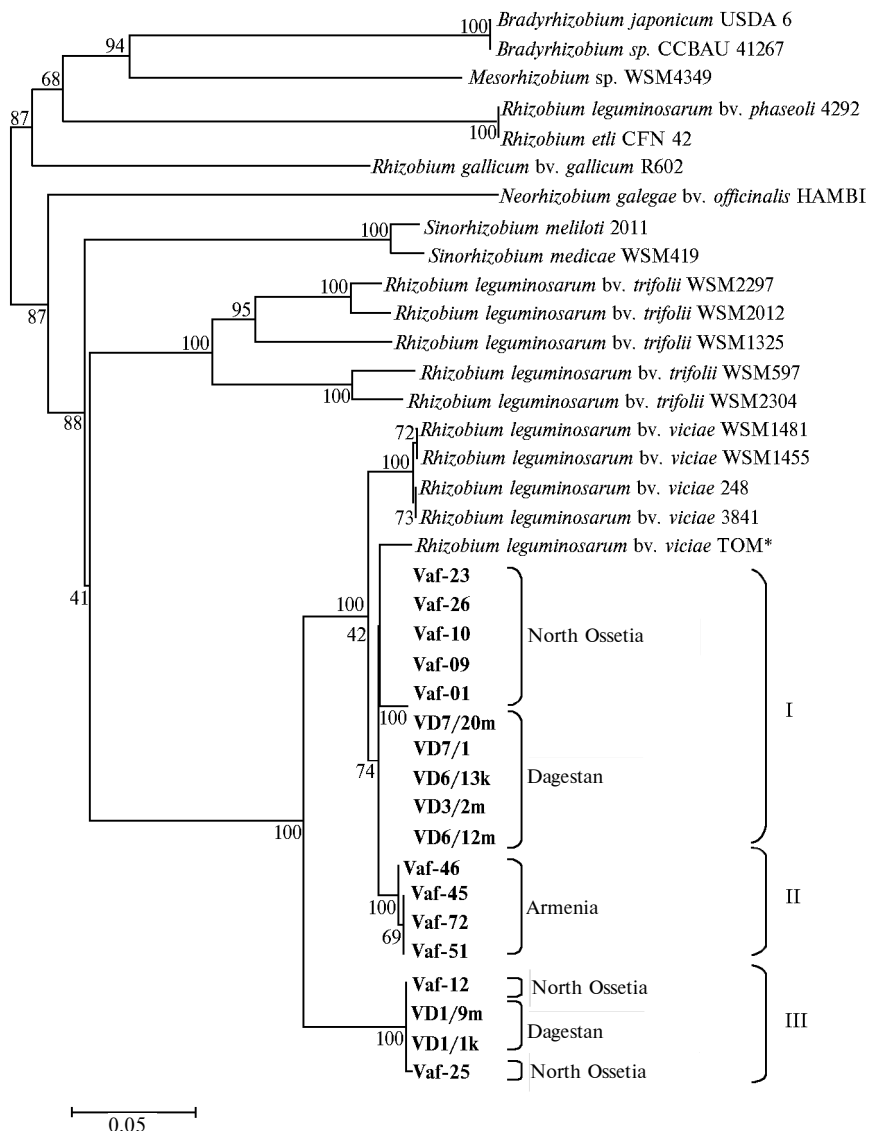
Strain, origin	Genetic distance	
	ITS	<i>nodA</i>
Correlation to host plant		
As compared within group:		
from vavilovia ( <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> )	0.082±0.008	0.036±0.005
from pea ( <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> )	0.115±0.010	0.016±0.003
from clover ( <i>R. leguminosarum</i> bv. <i>trifolii</i> )	0.089±0.008	0.119±0.010
As compared between groups:		
vavilovia—pea	0.096±0.008	0.045±0.006
vavilovia —clover	0.099±0.008	0.252±0.015
pea—clover	0.100±0.008	0.254±0.016
Correlation to geographical origin		
As compared within group:		
Armenia	0.000±0.000	0.001±0.001
Armenia	0.054±0.007	0.040±0.005
North Ossetia	0.050±0.006	0.040±0.005
As compared between groups:		
Armenia — Armenia	0.116±0.012	0.039±0.006
Armenia — North Ossetia	0.060±0.007	0.039±0.006
Armenia — North Ossetia	0.111±0.011	0.034±0.005



**Fig. 2.** PCR analysis of rhizobia isolated from *Vavilovia formosa* (Stev.) Fed. in North Ossetia (A), Armenia (B) and Dagestan (C) with primers omp196/omp199: 1 — Vaf-01, 2 — Vaf-09, 3 — Vaf-10, 4 — Vaf-12, 5 — Vaf-23, 6 — Vaf-25, 7 — Vaf-26, 8 — Vaf-45, 9 — Vaf-72, 10 — Vaf-46, 11 — Vaf-51, 12 — VD1/1k, 13 — VD1/9m, 14 — VD3/2(1), 15 — VD3/7k, 16 — VD6/12k, 17 — VD6/13m, 18 — VD7/1, 19 — VD7/20m. DNA of *Rhizobium leguminosarum* bv. *viciae* A1 was used as control (C). Molecular weight marker (M) is MassRuler (Thermo Scientific, USA) (A) и 100 bp + 1.5 Kb + 3Kb (Sibenzyme, Russia) (B, C).

Nevertheless, in the genetic distance assessment based on the *nodA* gene sequence homology, clear correlation to the host specificity was disclosed though no relationship to the geographical origin of vavilovia plants was found. The *nodA* gene determines binding fatty acid residue to oligochitin chain which is common in the Nod-factor synthesis for all rhizobia, therefore, the observed correlation is probably due to *nodA* linkage to the host specificity genes. In *R. leguminosarum* these genes are grouped as compact clusters of about 20,000 bp in size on the *Sym*-plasmids. The revealed correlation seems to reflect the fact of high specific interactions between each group of *R. leguminosarum* strains and the corresponding host plants.

Because of no similarity in *nodA* and ITS based dendrogram patterns, it is reasonably to assume an easy exchange of the plasmid-located *nod*-genes in *R. leguminosarum* populations which provides free combination of the bacterial genes of host specificity to vavilovia and different types of bacterial chromosomes in biovar *viciae* and, in some cases, in biovar *trifolii*. This is evidenced by 100 % homology of ITS region in one vavilovia rhizobial isolate of those involved in our investigation and in a representative strain WSM2304, being in line with earlier reported probability of active *Sym*-plasmids transfer between mentioned biovars in nature.



**Fig. 3.** Dendrogram based on *nodA* (531 bp) sequence in rhizobia strains isolated from *Vavilovia formosa* (Stev.) Fed. plants depending on geographical origin. Strains are grouped by the «neighbor-joining» method. Phylogenetic tree of the isolates reflects their taxonomy. Bootstrap value > 45 % for 1,000 random samples are show. I, II and II are statistically reliable clusters. Strain with *nodX* gene is marked (\*). Studied strains are marked bold.

Thus, from ITS sequencing analysis, all rhizobia strains isolated from *Vavilovia formosa* (Stev.) Fed. nodules and involved in our investigation were taxonomically attributed to *Rhizobium leguminosarum*. Moreover, taking into account the phylogenetic analysis of both ITS region and *nodA* gene sequences, it is possible to attribute these strains to the boivar *viciae*. According to the calculated genetic distances, all these isolates form a distinct statistically separated group in which a tendency to geographical isolation and strict host specificity is traced. Grouping *R. leguminosarum* bv. *viciae* strains into clusters separately from other strains, and disclosing Afghan pea nodulation gene *nodX* in all isolates from *Vavilovia formosa* can be basic for further study of these rhizobia group in the view to its possible attribution as an individual taxonomic group of *R. leguminosarum* species.

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