

Distinctive Features of Rhizobia Species *Sinorhizobium fredii* and *Bradyrhizobium japonicum* Inhabiting Soils of the Russian Far East

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Abstract—In the framework of the comprehensive research of Far Eastern natural populations of soybean nodule bacteria, laboratory experiments have been conducted at the All-Russia Scientific Research Institute of Soybean (Blagoveshchensk) with the purpose to identify distinctive features of rhizobia species *Sinorhizobium fredii* (Scholla and Elkan, 1984) and *Bradyrhizobium japonicum* (Jordan, 1982) whose pure cultures were isolated from soils of Far Eastern regions practicing soybean cultivation. It is established that *B. japonicum* strains start growing in Petri dishes on the seventh to tenth and even on the 20th day after the inoculation, assimilate a limited number of carbon nutrition sources, release mostly alkaline metabolic products, and feature a relatively low osmotic resistance. Representatives of this species are susceptible to extreme environmental conditions; their growth sharply slows down on acidic and alkaline nutrient media and stops at high (37–42°C) temperatures. However, under the optimal conditions, this rhizobia species dominates in the nodulation of soybean plants due to its high and persistent virulence. The restriction analysis of the studied *B. japonicum* strains confirmed their identity. *S. fredii* strains start growing in Petri dishes on the second to fourth day after the inoculation, assimilate well a broad spectrum of carbon nutrition sources, and release acidic metabolic products. Most strains of this species feature high osmotic resistance. Cultures retaining universal growth capacity under extreme environmental conditions (high temperatures and low and high pH values) have been identified in the group of *S. fredii* strains. This rhizobia species can predominate in the formation of symbiotic mechanisms in years featuring extreme weather conditions. Enzymatic fermentation of gene 16S rRNA in the studied *S. fredii* strains was performed using restriction enzyme *HaeIII*; the analysis of the fermentation results confirmed the identity of these strains. The RAPD-PCR analysis has demonstrated the intraspecific specificity of the studied *B. japonicum* and *S. fredii* strains: these species feature high degrees of polymorphism reflecting their population heterogeneity.

Keywords: soybean rhizobia, *Bradyrhizobium japonicum*, *Sinorhizobium fredii*, strains, virulence, extreme conditions, metabolic products, restriction analysis, RAPD-PCR analysis

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INTRODUCTION

A distinctive feature of the Russian Far East is the presence of natural soybean rhizobia populations in its soils. Their high activity makes it possible to perform selective breeding of nitrifying microorganisms for subsequent economic use. In the 1970s, large-scale works involving the selection of nutrient media and development and introduction of analytical selection techniques for soybean nodule bacteria have been carried out at the All-Russia Scientific Research Institute of Soybean on the basis of concepts proposed by the leading researchers of microbial nitrogen fixation: E.M. Mishustin, V.K. Shilnikova, and L.M. Dorosinsky [1, 2]. Pure cultures of 11–95 soybean rhizobia strains were isolated on an annual basis; in total, over 2000 forms of soybean nodule bacteria have been isolated over the course of the study period [3]. Up until the 1980s, it was believed that only slow-growing rhi-

zobia (e.g. *Rhizobium japonicum*) can form nodules on soybean plants [4, 5]. Therefore, studies of rhizobia carried out in the Far Eastern region were focused up until recently only on this species, although the first rare forms of fast-growing nodule bacteria had been identified at the All-Russia Scientific Research Institute of Soybean as early as in the 1970s [6]. Large amounts of data collected using modern genetic relationship identification techniques made it possible to distinguish from the genus *Rhizobium* (Frank, 1889) two independent genera: *Bradyrhizobium* (Jordan, 1982) and *Sinorhizobium* (Chen et al., 1988) [7, 8]. This discovery, in turn, made it possible to identify fast-growing pure rhizobia cultures isolated from natural Far Eastern populations as the species *Sinorhizobium fredii*, while slow-growing ones as the species *Bradyrhizobium japonicum* [9, 10]. Currently, the collection of pure cultures of soybean nodule bacteria

maintained at the All-Russia Scientific Research Institute of Soybean includes 289 strains [11].

The purposes of this study were to identify distinctive features of rhizobia strains belonging to the species *Bradyrhizobium japonicum* and *Sinorhizobium fredii* isolated from Far Eastern soils and estimate the population variation of aboriginal rhizobia using the restriction and RAPD-PCR analyses with the purpose to assess the species diversity of Far Eastern natural soybean nodule-forming rhizobia populations.

MATERIALS AND METHODS

The subjects of this study were pure rhizobia cultures belonging to the species *Bradyrhizobium japonicum* and *Sinorhizobium fredii* isolated from natural populations inhabiting the Russian Far East. A reference strain for the species *B. japonicum*, V-1967, was received in 2014 from the All-Russia Collection of Microorganisms, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino); a reference strain for the species *S. fredii*, KNRb, was received in 1990 from the Chinese collection.

The laboratory microbiological experiments were performed in accordance with the commonly accepted methodologies [12–14]. The mineral–plant nutrient medium (MPM) was used. Deep inoculation was used to determine the appearance timing and colony sizes of soybean rhizobia. The susceptibility of rhizobia to the salt concentration in the nutrient medium was assessed by their ability to grow on the minimum agar medium at various concentrations of sodium chloride. To determine their ability to assimilate various carbon nutrition sources, pure cultures of rhizobia were grown on the MPM containing, aside from mannitol, other carbonaceous compounds. To measure changes in the pH reaction, bromthymol blue was added to the MPM (5 mL of 0.4% alcohol solution/L of medium). The virulence of the collection strains was determined by growing bacterized seeds in test tubes with the nutrient medium for plants [15].

The chromosomal DNA was extracted and refined using the phenol method [16, 17]. The polymerase chain reaction (PCR) was carried out using a Gene-Amp PCR System 2700 thermal cycler (Applied Biosystems, United States). The following eubacterial primers were used for amplification of genes 16S rRNA: 5'-AGAGTTTGATCCTGGCTCAG-3' (27f, an upstream primer) and 5'-TACGGYTACCTTGT-TACGACTT-3' (1492r, a downstream primer) [18, 19]. The PCR products were separated in 1% agarose gel with the addition of ethidium bromide in an electrophoresis chamber in 0.5-fold TBE buffer using the standard methodology [20]. The isolated strains were grouped based on the restriction analysis of genes 16S rRNA [21]. The reaction products were separated using the electrophoresis method in a Helicon electro-

phoresis chamber in 1.3% agarose gel in TBE buffer under the voltage of 90 V. The electrophoresis results were processed using ViTran image processing system (Biokom, Moscow).

The RAPD-PCR analysis was performed with primer M13 (5'-GAGGGTGGCGGTTCT-3') (22). The PCR was carried out in 25 µL of the mixture containing the following components: 2.5 µL of tenfold PCR buffer (Fermentas, Lithuania), 1.5 µL of 25 mM MgCl₂, 2.5 µL of the mixture of 4 deoxynucleoside triphosphates (2.5 mM each), 10 pM of the primer, 0.1–0.5 µg DNA, and 1U of Taq polymerase (Fermentas, Lithuania). After the preliminary denaturation (94°C, 2 min), 30 amplification cycles were carried out under the following conditions: denaturation (94°C, 60 s), annealing (40°C, 30 s), and synthesis (72°C, 120 s). The PCR products were separated in 1.4% agarose gel with the addition of ethidium bromide in an electrophoresis chamber in the 0.5-fold TBE buffer under the voltage of 90 V. The rows have been visualized in an UV transilluminator.

RESULTS AND DISCUSSION

B. japonicum strains of the Amur selection start growing in Petri dishes on the seventh to tenth and even on the 20th day after the inoculation, assimilate a limited number of carbon nutrition sources, release mostly alkaline metabolic products, and feature a relatively low osmotic resistance (Table 1). Bacteria of this species are susceptible to extreme environmental conditions; their growth sharply slows down on acidic and alkaline nutrient media and stops at high (37–42°C) temperatures. However, under optimal conditions, this rhizobia species dominates in the nodulation of soybean plants due to its high and persistent virulence.

S. fredii strains start growing in Petri dishes on the second to fourth day after the inoculation, assimilate well a broad spectrum of carbon nutrition sources, and release acidic metabolic products. Most strains of this species feature high osmotic resistance. Cultures retaining universal growth capacity under extreme environmental conditions (high temperatures and low and high pH values) have been identified in the group of *S. fredii* strains. Strains of this species feature a lower virulence in comparison with *B. japonicum* and may lose virulence in the course of repeated reinoculations. Overall, *S. fredii* can predominate in the formation of symbiotic mechanism in years featuring extreme weather conditions.

Restriction and RAPD-PCR analyses have been applied to 35 collection strains of *B. japonicum* and *S. fredii* bred at the All-Russia Scientific Research Institute of Soybean to estimate the population variation of aboriginal rhizobia. The following reference cultures were used as the control: strain V-1967 for *B. japonicum* and strain KNRb for *S. fredii*.

Table 1. Distinctive features of collection soybean rhizobia strains belonging to the species *B. japonicum* and *S. fredii* isolated from soils of the Russian Far East

#	Feature	Soybean rhizobia species	
		<i>B. japonicum</i>	<i>S. fredii</i>
1	Time between inoculation and appearance of colonies in Petri dishes, days	7–20	2–4
2	Changes in the nutrient media pH reaction in the course of the growth	Alkalization (up to 90% of strains)	Acidification (100%)
3	Assimilation of carbonaceous compounds	Mannitol and glucose	Monosaccharides, oligosaccharides, polyatomic alcohols, etc
4	Osmotic resistance	Low	High
5	Growth on acidic and alkaline nutrient media;	Stops or slows down	Remains the same or slows down
6	Growth at high (37–42°C) temperatures	Stops	Bacteria demonstrate growth
7	Virulence and soybean nodule formation intensity	High (up to 100%)	Decreased (10–100%)
8	Loss of the nodule formation capacity (virulence)	Never lose	May lose

A comparative analysis of the restriction fragments of genes 16S rRNA was performed to differentiate the isolates. As is known, the size of gene 16S rRNA amplified using universal eubacterial primers is some 1450 base pairs (bp) for all bacteria. However, various bacterial species feature different nucleotide sequences in this gene region. Using restriction endonucleases, it is possible to distinguish between bacteria belonging to various species.

The gel electrophoresis of the restriction products of *B. japonicum* strains is shown in Fig. 1. The comparative analysis of the obtained fragments made it possible to identify restriction enzyme profiles of the amplified genes 16S rRNA with the length of 150, 250, 300, and 600 bp. Therefore, the analysis of the results of enzymatic fermentation of gene 16S rRNA in the studied *B. japonicum* strains performed using restriction enzyme *Hae*III confirms their identity.

The gel electrophoresis of the restriction products of *S. fredii* strains is shown in Fig. 2. The comparative analysis of the obtained fragments made it possible to

identify restriction enzyme profiles of the amplified genes 16S rRNA with the length of 100, 150, 200, 300, and 600 bp. Therefore, the analysis of the results of enzymatic fermentation of gene 16S rRNA in the studied *S. fredii* strains performed using restriction enzyme *Hae*III confirms their identity.

The restriction analysis alone may be unable to reveal polymorphism in the course of differentiation of closely related organisms. The restriction enzyme profiles of genes 16S rRNA were identical for all studied strains of *B. japonicum* and *S. fredii*; therefore, a more sensitive method, RAPD-PCR analysis, was used to estimate their population variation. All the amplification products had the length of 100–2000 bp; six to ten products were obtained per strain.

Based on the fingerprint analysis results, the studied *B. japonicum* strains have been divided into five groups (Fig. 3). Identical RAPD DNA profiles have been obtained for nine strains subsumed under group I: V-1967, BuD-63, TS-196, 648a, TM-437, MS-63, MM-117, TA-125, and TA-40. Four strains have been

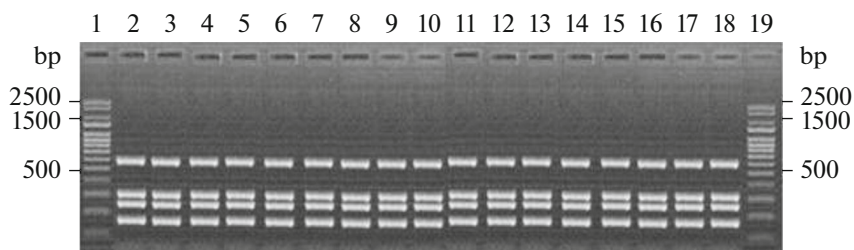


Fig. 1. Restriction analysis of amplified genes 16S rRNA in the studied *B. japonicum* strains performed with endonuclease *Hae*III. Rows 2–18 represent restriction enzyme profiles of the following strains: (2) V-1967, (3) SM-42k, (4) SM-46, (5) MM-121, (6) BM-91, (7) BuD-63, (8) TS-196, (9) 648a, (10) TM-437, (11) MC-63, (12) AS-17, (13) MM-117, (14) TA-125, (15) TA-40, (16) MM-125, (17) MM-124, and (18) BM-58. Rows 1 and 19 represent molecular mass markers in base pairs (bp) (Fermentas, Lithuania).

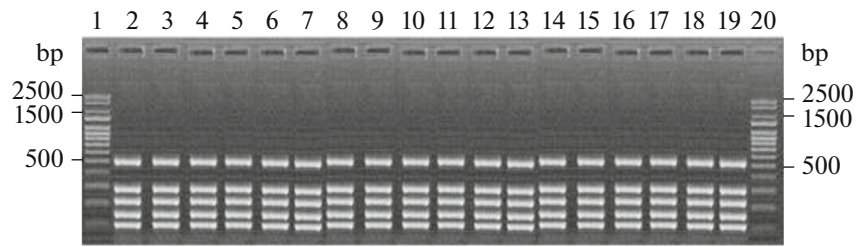


Fig. 2. Restriction analysis of amplified genes 16S rRNA in the studied *S. fredii* strains performed with endonuclease *Hae*III. Rows 2–19 represent restriction enzyme profiles of the following strains: (2) KNRb, (3) MB-85k, (4) BB-87k, (5) SB-51k, (6) KB11, (7) TB-491, (8) SB-43k, (9) TB-524k, (10) BB-55k, (11) OB-42, (12) TB-522, (13) TB-365, (14) TB-398, (15) SB-38, (16) TB-508, (17) TB-587, (18) TB-467k, and (19) BB-90k. Rows 1 and 20 represent molecular mass markers in base pairs (bp) (Fermentas, Lithuania).

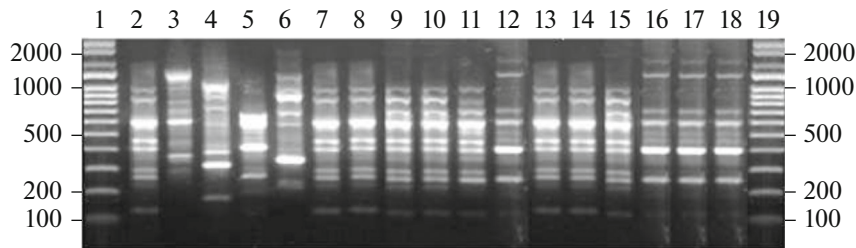


Fig. 3. RAPD-PCR DNA profiling of the studied *B. japonicum* strains with primer M13. Rows 2–18 represent strain profiles (see the Fig. 1 caption). Rows 1 and 19 represent molecular mass markers in base pairs (bp) (Fermentas, Lithuania).

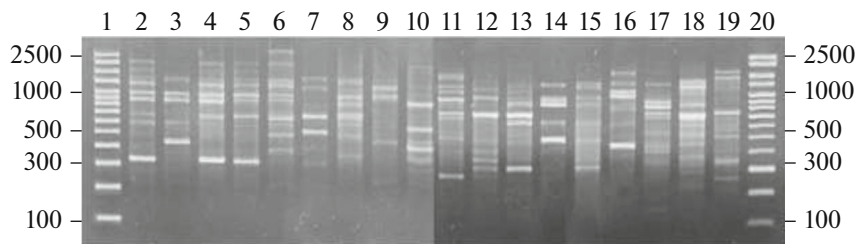


Fig. 4. RAPD-PCR DNA profiling of the studied *S. fredii* strains with primer M13. Rows 2–19 represent strain profiles (see the Fig. 2 caption). Rows 1 and 20 represent molecular mass markers in base pairs (bp) (Fermentas, Lithuania).

subsumed under group II: MM-125, MM-124, BM-58, and AS-17. Groups III, IV, and V consist of only one strain each: SM-42k, SM-46, and MM-121, respectively.

The performed RAPD analysis has identified significant differences between the studied *S. fredii* strains (Fig. 4). All the amplification products had the length of 100–2000 bp; five to 11 products were obtained per strain. Identical RAPD DNA profiles have been obtained for three strains subsumed under group I: KNRb, SB-43k, and TB-491. Strains TB-522 and SB-38 have been subsumed under group II. The RAPD DNA profiles of the remaining 13 *S. fredii* strains were not completely identical. The RAPD-PCR analysis has demonstrated the intraspecific specificity of the studied *B. japonicum* and *S. fredii* strains, which indicates that these species feature high degrees of polymorphism.

CONCLUSIONS

The studied *B. japonicum* and *S. fredii* strains of the Far Eastern selection have significant interspecific differences in their cultural and biochemical properties. Strains of the fast-growing species *S. fredii* feature higher resistance to extreme environmental conditions than strains of the slow-growing species *B. japonicum*. However, under the optimal conditions, *B. japonicum* strains never lose their high virulence.

The restriction analysis of amplified genes 16S rRNA in the studied *B. japonicum* and *S. fredii* strains confirmed their identity. A more sensitive method, RAPD-PCR DNA profiling, made it possible to divide the *B. japonicum* strains into five groups. The *S. fredii* strains are more diverse, which indicates the population heterogeneity of these species. Overall, Far Eastern natural populations turned out to be more diverse by their species composition than previously thought.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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