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Identification of fast and slow growing rhizobia nodulating soybean (*Glycine max* [L.] Merr) by a multiplex PCR reaction

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Abstract

Two DNA fragments, a 730-bp and a 900-bp fragment, one homologous to host cultivar specificity genes *nolBT* of *Sinorhizobium fredii* and the other one homologous to RSα, an insertion-like sequence present in *Bradyrhizobium japonicum*, were generated by polymerase chain reaction (PCR) with two pairs of primers. The amount of each fragment generated by the multiplex PCR was proportional to the amount of template DNA present. The amplification of the 900-bp RSα fragment was more sensitive, since it was amplified from a smaller amount of template DNA than the 730-bp *nolBT* fragment. By running the multiplex reaction in the presence of template DNA isolated from different sources, we confirmed that the reaction can discriminate between *S. fredii*, *Bradyrhizobium japonicum* and *Sinorhizobium xinjiangensis*.

Keywords: Soybean; Polymerase chain reaction; *Bradyrhizobium*; *Sinorhizobium*; *Rhizobium*; Molecular marker

1. Introduction

Glycine max, the soybean, is one of the most important and oldest cultivated crops [1]. It is a summer annual herb that has never been found in the wild [2]. Soybean nodulates and fixes nitrogen in symbiosis with Gram-negative soil bacteria from the genera *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium*.

Bradyrhizobium japonicum, *Bradyrhizobium elkanii* (formerly *B. japonicum* DNA homology group II) [3] and *Bradyrhizobium liaoningense* [4] form nitrogen-fixing nodules with soybean, *B. japonicum* and *B. elkanii* have been used widely in commercial inoculants to increase soybean yields [5]. More recently, Keyser et al. [6] and Chen et al. [7,8] described the isolation of the fast growing rhizobia *Sinorhizobium fredii* [7,9], *Sinorhizobium xinjiangensis* [7,8] and *Mesorhizobium thianshanense* [8], from the soils of China. These bacteria nodulate and fix nitrogen in association with soybean as efficiently as *B. japonicum* does [10,11].

Recently, the polymerase chain reaction (PCR) has provided a variety of easy and reliable techniques for the identification of microorganisms. These include enterobacterial repetitive intergenic consensus PCR and repetitive extragenic palindromic PCR. These protocols have been used successfully to identify individual strains [12]. In addition, PCR proved to be a reliable method to identify rhizobia at the species level. Examples include *Rhizobium galegae* [13], *B. japonicum* [14], *Sinorhizobium meliloti* [15] and *S. fredii* [16].

Videira et al. [16] identified *S. fredii* and *B. japonicum* by the amplification, in two separate reactions, of a 260-bp and a 900-bp fragment, respectively. They could not amplify both DNA fragments, *nolB* and RSα, in a single reaction.

Because the identification through two independent reactions resulted in a time-consuming and costly protocol, the purpose of this work was to develop a multiplex PCR that will allow us to identify rhizobia nodulating soybean plants. Based on the *S. fredii* sequence *nolXWBTUV* and the *B. japonicum* insertion-like element RSα, we developed four primers that generated in a single reaction a 730-bp

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Table 1
Rhizobium species used in this study

Species	Strain	Geographic origin	Reference
S. fredii	SMH12	Vietnam	[17]
	SMX11	Vietnam	[17]
	SMH15	Vietnam	[17]
	HH103	China	[18]
	HH102	China	[18]
	USDA191	China	[6]
	USDA196	China	[6]
	USDA257	China	[6]
	USDA205^T	China	[6]
	S8	China	[19]
	B11	China	[19]
Sinorhizobium xinjiangense	CCBAU 110^T	China	[7]
Bradyrhizobium japonicum	E109	Argentina	INTA Argentina
Sinorhizobium meliloti	USDA1002^T	USA	[20]
	WSM419	Sardinia	[21]
Rhizobium tropici	CIAT899^T	Mexico	[22]
Rhizobium galegae	HAMBI540^T	Finland	[23]
Sinorhizobium saheli	USDA4893^T	Senegal	[24]
Sinorhizobium terangae	USDA4894^T	Senegal	[24]
Mesorhizobium ciceri	USDA3383^T	Spain	[25]
Mesorhizobium loti	USDA3015^T	New Zealand	[26]
Bradyrhizobium sp.	CB756	Africa	[27]

Stocks of all the species were kept at −70°C with 7% glycerol. Slants made on YEM were stored at 4°C.

fragment homologous to *nolBT* and/or a 900-bp fragment homologous to RSα, provided the appropriate template DNA is available. The presence or absence of these fragments allowed us to discriminate among *S. fredii*, *B. japonicum* and *S. xinjiangensis* in mixed DNA samples or in nodules.

2. Materials and methods

2.1. Bacterial strains

Bacterial species and strains used in this work are presented in Table 1. Stock cultures were kept at −70°C in 7% glycerol [28]. Cultures were initiated by inoculating a loop of bacteria taken from agar slants maintained at 4°C. Bacteria were grown in yeast extract–mannitol (YEM) medium [29] in an orbital shaker at 150 rpm at 28°C. *S. fredii* strains were kindly provided by Dr. Steven G. Pueppke (University of Missouri, Columbia, MO, USA) and Jose Ruiz-Sainz (Universidad de Sevilla, Seville, Spain). *S. meliloti*, *Rhizobium tropici*, *Mesorhizobium loti* and *Rhizobium leguminosarum* bv. *trifolii* strains were provided by Dr. Peter van Berkum, USDA-ARS Soybean and Alfalfa Research Laboratory, Beltsville, MD, USA.

2.2. DNA Isolation and PCR

DNAs were isolated from two sources, pure cultures and nodules. The procedure used to isolate DNA from

Table 2
Sequences of the 20-mer primers that specifically amplify the *nolXWBTUV* locus of *S. fredii* and the RSα repetitive sequence of *B. japonicum*

Primer	5′-3′ sequence	Fragment size (bp)	%GC	T_m (°C)
nolXWBTUV locus				
Forward primer nolBT	tgggcaagcgacgctgccgg		75	68.0[a]
				65.5[b]
Reverse primer nolBT	cgctcttgcaactgggtata	730	50	57.8
				55.0
RSα				
Forward SAL1	agcgggcgcggatagttctgttg		61	65.5
				69.0
Reverse SAR1	ggctcggctctgtcgttgtatgc	950	61	65.5
				69.0

Forward primer nolBT [17] corresponds to position 2530–2550 bp of the *nolXWBTUV* locus (GenBank accession number L12251). Reverse primer nolBT corresponds to position 3259–3239 bp of the *nolXWBTUV* locus. SAL1 and SAR1 of RSα were described by Hartmann et al. [15].
[a] T_m calculated based on the following formula: $T_m = 63.3 + 0.41 \times GC\% - 500/length$.
[b] T_m calculated based on the following formula: $T_m = (A+T) + 4 \times (C+G) - 5$.

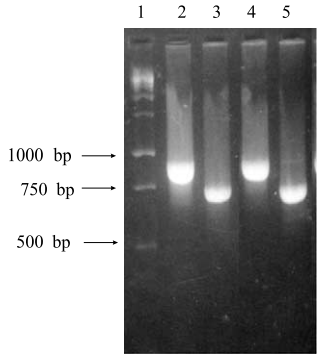

Fig. 1. PCR of RSα and *nolBT* from template DNA derived from *B. japonicum* and *S. fredii*. Lane 1, 1-kb ladder range 250–10 000 (Promega); lane 2, reaction with template DNA from *B. japonicum* and RSα primers; lane 3, reaction with template DNA from *S. fredii* and primers nolBT; lane 4, reaction with template DNA from *B. japonicum* in the presence of primers RSα and nolBT; lane 5, reaction with template DNA from *S. fredii* in the presence of primers RSα and nolBT. Arrows indicate the sizes of the 1000-, 750- and 500-bp bands generated by the marker.

cultured strains was described by Meinhardt et al. [30]. DNA from nodules was extracted by crushing surface-sterilized nodules in Eppendorf tubes filled with 200 μl of distilled water. Then the tubes were heated at 95°C for 20 min and centrifuged for 10 min. An aliquot of the supernatant solution was used as template for the PCRs.

PCRs were performed in a 15-μl volume with a PTC-100 M&J Research Thermocycler programmed as follows: an initial step at 94°C for 3 min; then 29 cycles of 45 s at 95°C and 1 min at 65°C. There was a final extension of 4 min at 72°C.

PCR was performed using as template DNA either intact rhizobium cells or nodule extracts or pure DNA. The reactions were performed with 10–20 ng of DNA or 4 μl of nodule extracts or whole bacterial cells from liquid cultures or water extract of rhizobia (a loop of bacteria was resuspended in water and heated at 99°C for 5 min). Reactions contained 1.5 mM $MgCl_2$, 0.125 mM of primers nolBT1 and nolBT2, 0.375 mM primers SAR and SAL, 0.200 mM dNTPs, 1 U of T-Plus DNA polymerase (Highway Molecular Biology-INBIO-UNICEN) in 1×reaction buffer containing 50 mM KCl; 10 mM Tris–HCl, pH 9.0 at 25°C; 1% Triton® X-100. The PCR products were visualized by electrophoresis in 2% agarose gels stained with ethidium bromide and were exposed to UV light with a transilluminator. Gels were photographed with a Polaroid PS34 camera. Pictures were scanned, analyzed and printed.

3. Results and discussion

Soybean nodulates and fixes nitrogen in association with different rhizobial species [3,4,6–12] and so evaluation of the efficiency of field inoculations and the survival and competitive ability of rhizobia introduced by means of inoculants is quite dependent upon the availability of reliable techniques of identification.

Videira et al. [16] identified rhizobia nodulating soybean by means of two DNA sequences amplified in two independent reactions. However, the authors failed to amplify both fragments in a single PCR reaction, most probably due to the differences in the melting temperature of both pairs of primers and/or the different length of the DNA fragments amplified by each pair of primers.

In order to overcome this shortcoming, a larger fragment of DNA, the *nolBT* region, was amplified by using primers nolBT1 and nolBT2 (Table 2), which were derived from the previously published sequence of *S. fredii* USDA257 [30]. The PCR yielded a 730-bp band from genomic DNA of *S. fredii*. The same PCR program with the SAR/SAL pair of primers generated a 900-bp fragment, RSα, from *B. japonicum* template DNA (Fig. 1) [14]. The two fragments were amplified even though there were two pairs of primers in the reaction tube. Furthermore, both fragments were generated from a mixture of template DNA (Fig. 3). This finding suggests that both reactions are specific and that neither the mixture of primers nor the mixture of template DNAs inhibited the amplification of the specific fragments.

Table 3
Template DNA isolated from different species and strains of rhizobia and their ability to act as templates of the multiplex PCR reaction

	730-bp *nolBT*	900-bp RSα
Bradyrhizobium japonicum	–	+
Sinorhizobium fredii		
USDA205^T	+	–
USDA191	+	–
USDA196	+	–
USDA197	+	–
USDA257	+	–
HH103	+	–
S8	+	–
B11	–	–
SMH10	+	–
SMX11	+	–
SMH13	+	–
SMH15	+	–
Rhizobium tropici		
CIAT 899^T	–	–
Sinorhizobium meliloti		
USDA1002^T	–	–
WSM419	–	–
Sinorhizobium saheli		
USDA4893^T	–	–
Rhizobium galegae		
HAMBI 540^T	–	–
Sinorhizobium terangae		
USDA 4894^T	–	–
Sinorhizobium xinjiangensis		
CCBAU110^T	–	–
Bradyrhizobium sp.		
CB756	–	–
Mesorhizobium loti		
USDA3015^T	–	–

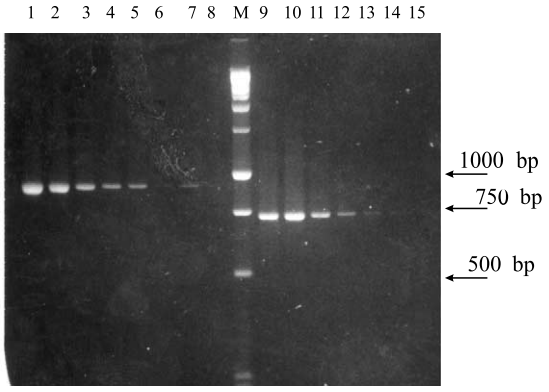

Fig. 2. Amplification of RSα and *nolBT* in the presence of decreasing amounts of template DNA from *B. japonicum* and *S. fredii*, respectively. Lanes 1–8, *B. japonicum* template DNA; lanes 10–15, *S. fredii* template DNA. Lanes 1 and 9, 820 pg; lanes 2 and 10, 410 pg; lanes 3 and 11, 200 pg; lanes 4 and 12, 100 pg; lanes 5 and 13, 50 pg; lanes 6 and 14, 25 pg; lanes 7 and 15, 12 pg of DNA; lane 8, no template DNA. Lane M, 1-kb ladder range 250–10 000 (Promega).

Whether the multiplex reaction is a useful tool depends upon the specificity of the reaction. Several multiplex amplifications were performed in the presence of template DNA isolated from related and unrelated rhizobia (Table 3). The specificity of the reaction was evaluated among 12 *S. fredii* strains, and we found that genomic DNA from all the strains, except for B11, generated the 730-bp fragment specific to *S. fredii*. The *nolXWBTUV* locus is conserved among *S. fredii* strains [16,30]. Furthermore, it has also been found in the closely related strain *Rhizobium* sp. NGR234 [12]. Strain B11 has been recently isolated from the soils of China [19] and although it was isolated from nodulated soybean cv. Jing Dou19, it failed to nodulate other soybean cultivars like Williams, Asgrow 4404 and Heinong 33. Furthermore, strain B11 showed different plasmid, lipopolysaccharide and RAPD profiles compared to other strains isolated from nodulated soybean plants cv. Jing Dou19. These findings suggest that strain B11 might not be *S. fredii*.

The multiplex PCR for identification of rhizobia should be sensitive enough to detect small amounts of DNA. The amount of the amplified sequences by PCR was found to be proportional to the concentration of template DNA, whether its source was fast growing *S. fredii* strains or slow growing *B. japonicum* (Fig. 2). The sensitivity of RSα was 10 times higher compared to the *nolBT* fragment, based on the amount of template DNA from *S. fredii* or from *B. japonicum* (Fig. 2) required for a successful amplification.

The minimal concentration of DNA needed to amplify the 900-bp fragment was insufficient for the amplification of the 730-bp fragment homologous to *nolBT* (Fig. 2). This was confirmed with reactions performed with cell suspensions of rhizobia grown on solid or liquid media. The amplification of the 730-bp fragment showed a threshold detection limit of 1×10^5 cells (data not shown) while RSα had a much lower detection limit, which was found to be between 1.6 and 3.4×10^3 [14]. Whenever the multiplex reaction was performed with whole cells, its sensitivity was reduced, most probably due to the inhibitory effect of cell constituents that remain in the reaction mixture.

There are two possible explanations for the difference in sensitivity between the *S. fredii* 730-bp and the *B. japonicum* 900-bp fragments. One is that the number of copies of the target sequence is different. The number of copies of RSα varies between species of *B. japonicum* and *B. elkanii* [14], while *nolBT* is a single copy locus of *S. fredii* [30]. In order to increase the sensitivity of the reaction for *S. fredii* strains we increased the number of cycles of the reaction from 30 to 40 and 50 cycles and found that although the addition of PCR cycles improved the sensitivity of the reaction, RSα was still much more sensitive than *nolBT*. The other explanation of the higher sensitivity of the RSα fragment is that unlike the nolBT primers, the SAR/SAL primers have identical melting temperatures and GC content, which should contribute to achieve a higher level of specificity.

A single nodule may be occupied by more than one strain or species of rhizobia [16]. In order to rapidly determine if a nodule is cohabited by *B. japonicum* and *S. fredii* strains, it is better to amplify both fragments at the same time. We thus performed multiplex PCRs in the presence of increasing concentrations of genomic DNA from *S. fredii* mixed with decreasing amounts of genomic *B. japonicum* DNA (Fig. 3). Although the amplification reactions were quite dependent upon the DNA concentration, both DNA fragments were amplified concomitantly, even if the template DNA was supplied in a concentration of 1 ng in the case of *B. japonicum* and 3 ng in the case of *S. fredii* (Fig. 3).

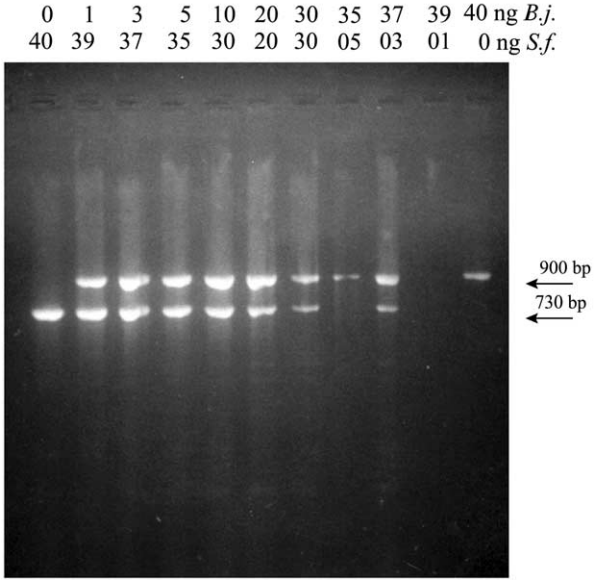

Fig. 3. RSα and *nolBT* amplification from a mixture of template DNA derived from *B. japonicum* and *S. fredii*. Reactions were performed in the presence of increasing concentrations of template DNA from *B. japonicum* and decreasing concentrations of *S. fredii* template DNA.

The 900-bp fragment homologous to RSα specifically identified *B. japonicum* as described by Hartmann et al. [14] and Videira et al. [16]. Therefore, the PCR multiplex reaction allows us to discriminate in a single reaction whether a bacterium nodulating soybean is a fast growing or a slow growing *B. japonicum*. No other species of rhizobia other than *S. fredii* and *B. japonicum* provided template DNA that allowed the amplification of any of the two identification bands (Table 3), confirming the specificity of the reaction. However, *S. xinjiangense* could not be identified except by the absence of the two fragments, which still is not a confirmation of the bacterial identity, since soybean can also be nodulated by *B. liaoningense*. Future work should be aimed at developing a multiplex reaction that can also amplify DNA bands that will specifically identify and/or discriminate *B. liaoningense* and *S. xinjiangensis*.

The availability of this multiplex PCR will allow us to determine in a short time the identity of bacteria occupying nodules, enabling a study of the competitive ability of fast and slow growing rhizobia and their ability to nodulate soybeans.

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