Closely Related Strains Of *Bradyrhizobium* Contained In Commercial Inoculates Of Soybean Are Identified By A Set Of PCR Reactions

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Abstract

The aim of this work was to identify closely related rhizobia, used to formulate commercial inoculants, using polymerase chain reaction (PCR). Repetitive extra-genic palindromic (REP) and BOX fingerprints hardly discriminate among a set of commercial strains. PCR targeted at repetitive RSα successfully allow discriminating within representatives of Bradyrhizobium. These fingerprints clustered isolates at a higher level of similarity and proved to be an important tool to complement the information provided by the other markers. The results suggest that mutants occur along the bacterial culture, during inoculant production. However, independently of the number of amplification reactions used to characterize and identify organisms, mispriming always generates artifactual diversity. In addition to this, it seems that combining reactions such as BOX or REP fingerprinting with reactions targeted at the RSα sequence, generates a more reliable identification tool to characterize closely related bradyrhizobia.

Keywords: *Inoculant; molecular markers; rhizobia.*

1. Introduction

Among legumes, soybean (*Glycine max* L. Merr) is one of the most important crops worldwide. Three different species of *Bradyrhizobium* nodulate soybean. These include *Bradyrhizobium japonicum* [1], *Bradyrhizobium elkanii* [2], and the extra slow growing *Bradyrhizobium liaoningense* [3]. All these species of *Bradyrhizobium* nodulating soybean belong to the biovar glycinearum [4]. Furthermore, soybean is also nodulated by the bacterium *Sinorhizobium fredii* in a cultivar-specific manner [5].

In Argentina, Brazil, and Uruguay, soybean is an exotic species. In these soils, rhizobia had been introduced through inoculation, as it was done in Australia [6]. Today, at least in Argentina and Brazil, most of the soils contain naturalized bradyrhizobia, whose origin might have been commercial inoculants [7]. The soybean production area in Argentina occupies approximately 19 million ha and most of these soils are inoculated every year [8]. The scenario in Brazil and Uruguay is most likely similar. In each of these countries, a different set of strains are used as a source of commercial inoculants. While in Brazil and Uruguay, inoculants are made of a mixture either of *B. elkanii* SEMIA5019 or SEMIA587 and *B. japonicum* strain SEMIA5079 or 5080. In Argentina, soils were inoculated mostly with the strain E109, which is a derivative of USDA138 [9].

Several reasons justify the development of precise and accurate identification techniques for bacteria used as inoculants. One of the reasons is that the factories might have a permanent control of the different batches of organisms produced by fingerprinting their genomes [10]. Another reason is that the frequency of mutations per generation in bacteria is high, 1 in 1×10^6 [11], which might contribute to the growth of mutants when they are multiplied. In addition, rhizobia might present colony dimorphisms like those reported by Mac Innes *et al.* [12], or may change morphologically as a result of the conditions of growth or storage [13]. Furthermore, some strains like SEMIA5079 and SEMIA5080 are closely related and cannot be identified easily (Hungría personal communication). Therefore, the availability of fast and reliable methods for the identification of strains is crucial, considering that the quality of an inoculant resides not only in the nitrogen-fixing ability of the selected strain but also in its purity and cell number.

Polymerase chain reaction (PCR) has provided a variety of reliable techniques for the identification and typing of microorganism, either by widely distributed sequences among bacteria [14–16] or by species-specific

primers, such as those described for *Rhizobium galegae* [17], *B. japonicum* [18], *Sinorhizobium melilotii* [19] and *S. fredii* [20, 21]. However, PCR procedures developed to generate fingerprints for the identification of strains, particularly closely related ones are lacking.

B. japonicum and *B. elkanii* contain in their genomes repetitive sequences known as RSα and RSβ [22, 23]. These sequences occur at different frequencies within the genome but they are stable [24, 25], so they can be used to identify *Bradyrhizobium* strains [26, 27]. Therefore, the purpose of this work was to develop a fast and accurate method for the precise identification of closely related rhizobium strains from commercial inoculants.

2. Methods

2.1. Bacterial strains

The strains included in the study SEMIA587, SEMIA5019, SEMIA5079, SEMIA5080 (Strains that belong to the Culture Collection of EMBRAPA) and E109 (Culture Collection of INTA, Argentina) were grown on yeast extract–mannitol (YEM) medium at 28°C either on solid (medium supplemented with agar) or in liquid medium, at 150 rev/min [20]. Stocks were prepared by supplementing 10% glycerol to a 500 ml aliquot of a 1 x 10^9 cell culture and were kept in the freezer at −70°C. Tubes containing cultures of the strains on YEM agar were kept at 5°C and used as starter cultures.

2.2. Genomic DNA extraction and PCR amplification

Rhizobial strains were cultured in liquid YEM medium at 150 rev/min at 28°C for 5–7 days. Total genomic DNA from each isolate was obtained as described before [28]. The repetitive extra-genic palindromic (REP), BOX, and RSα primers used for the PCR were previously described by de Bruijn [14], Versalovic *et al.* [29], and Minamisawa *et al*. [27], respectively.

The BOX–PCR reaction contained 50–100 ng of DNA template, 50 pmol of primer, 2.0 mM of dNTP, 1× PCR buffer, and 0.6 U *Taq*, DNA polymerase in a total volume of 15 µl. Reactions were performed in a Thermocycler M&J Research. The PCR conditions were set for an initial step at 94°C for 7 min, followed by 35 cycles at 94°C for 1 min, 53°C for 1 min and a final step at 65°C for 8 min and a final cycle at 65°C for 16 min. The PCR fragments were separated on 1.5% agarose gels, stained with ethidium bromide. Each REP–PCR contained 50 ng of DNA template, 12.5 pM of each primer, 2.5 mM of dNTP, 1× PCR buffer and 2.5 U *Taq* DNA polymerase in a total volume of 50 µl. The thermocycler M&J Research PTC 1152 was programmed as follows: one cycle of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 at 50°C, 1 min at 72°C and the final cycle was set as 1 cycle of 10 min at 72°C.

The amplification of the RSα sequence was performed by a set of two reactions, one with primers SAR and SAL followed by another one with primers 17 and 18, homologous to the 5′ and 3′-ends of the previously amplified DNA [18, 30]. The reactions were carried out in a 15 µl reaction mixture that contained 10–20 ng of PCR-amplified DNA fragment; 1 U of *Taq* Polymerase; 2,0 mM of dNTP; 1× PCR buffer and 400 nM of each primer. The thermocycler was programmed as follows: 1 cycle of 240 s at 95°C, 60 s at 55°C, and 60 s at 72°C; 30 cycles of 60 s at 94°C, 60 s at 55°C, and 60 s at 72°C; and 1 cycle of 300 s at 72°C.

2.3. Identification of the strains in the batch cultures

Two samples of commercial inoculants C1 and C2 were kindly provided by Ing. Agr. Gustavo Gonzalez Anta. (Rhizobacter Argentina, S.A.). The inoculants contained 1 x 10^9 cell/ml of a mixture of *B. japonicum* SEMIA5079 and SEMIA5080 (C1) or of *B. elkanii* SEMIA587 and SEMIA5019 (C2). Aliquots of the batches were serially diluted 1/10; 1/100; 1/1000; 1/10,000; 1/100,000; 1/1,000,000 and several 50 µl samples, the last three dilutions were plated on Congo Red YEM [31]. We counted and transferred individually the colonies using a sterile toothpick to tubes containing 5 ml of YEM liquid media and were incubated in an orbital shaker at 150 rev/min at 28°C for 96 h. At the end of the incubation period, a 50 µl aliquot of each tube was used as a source of template DNA to run PCRs as described before.

The sizes of the fragments in each analysis were normalized according to the MW of the DNA markers (1000–100 bp INBIO-Highway, Tandil, Argentina). The fingerprints obtained by the REP, BOX, and RSα PCR were

analyzed via PCGene software (Intelli-Genetics, Mountain View, CA, USA). Phenograms were built using PCGene Software, using the unweighted pair-grouping method with arithmetic mean (UPGMA) algorithm with the coefficient of DICE ($p < 0.05$).

By means of the PCGene software, we combined in a single analysis; the fingerprints generated by the BOX, REP and RSα primers and generated a single phenogram. This was performed with the fingerprints that were generated either using clean DNA or a bacterial suspension culture as template. All the PCRs were repeated at least thrice, but because they were identical, for simplicity only one picture of each is presented.

3. Results and Discussion

Rhizobia have been successfully identified by several fingerprinting techniques that have been used to screen diversity within populations and/or as identification tools, which is particularly important in ecological studies and also to assess the quality of inoculants [14, 25, 10]. We fingerprinted the genomes of the five most important strains used in commercial inoculants in Brazil, Uruguay, and Argentina with BOX and REP primers (Figure 1A and 1B) and the fingerprints were similar whether template DNA was obtained by the lysis method, cleaned with phenol:chloroform or a bacterial suspension culture [32]. Both fingerprints grouped *B. japonicum* and *B. elkanii* in different clusters at different levels of similarity, which was more evident within the *B. japonicum* isolates (Figure 1). Similarity within *B. elkanii* and *B. japonicum* clusters was 80% (BOX), 69% (REP), and 52% (BOX), 49% (REP), respectively (Figure 1). Judd *et al.* [26] found that although amplification reactions like ERIC and REP-PCR effectively grouped strains, they might have overestimated their relative genetic similarity. Besides, protocols such as REP, ERIC, and BOXA1R generate amplification profiles that are prone to variation, which was suggested to be due to potential mispriming [10]. In addition, the fingerprint patterns generated with these protocols have a considerable number of poorly resolved bands that are not always consistent between PCRs. Repetitive sequences like RSα, have been found to be distributed within the genome and to be stable, though still prone to variation. Therefore, they might be useful tools to differentiate isolates of *Bradyrhizobium*. By means of oligonucleotides homologous to the RSα sequence, we generated fingerprints of the strains and found two clusters. One corresponded to *B. elkanii* strains and the other one corresponded to *B. japonicum*. Each was clustered at a level of similarity of 94% and 58% respectively, which were higher than those obtained by means of REP and BOX primers. Furthermore, the fingerprints generated with the RSα primers showed, as expected, a closer relationship between Brazilian strains SEMIA5079 and 5080 (75% similarity) than with the Argentinian strain E109.

The analysis of the REP, BOX, and RSα fingerprints together clustered strains in two main groups (data not shown), one that included *B. elkanii* strains SEMIA587 and 5019 and another one that included *B. japonicum* strains. Within the latter group, Brazilian strains SEMIA5079 and 5080 appeared more closely related than with E109, than the commercial strain of Argentina.

The combined use of the three different fingerprints was a more reliable tool for the precise identification of strains, including mutants that might develop along the bacterial culture. The fingerprints including the bands generated by the different primers are shown in Figure 2. It can be seen that the similarity indexes found between control strains were similar to those found when only control strains were analyzed (Supplementary Information). It can also be seen that the complexity of the analysis was such, that even though the colonies were clones of the same strain they all had a unique genotype, most probably this was due to small errors of the software or some rather unstable amplified fragments or mispriming. However, within the C2 sample, which contained a mixture of *B. elkanii* strains, control strain 5019 and colonies C2-2 and C2-3 had a 97% similarity. Other colonies such as C2-1, C2-4, C2-5, and C2-8 were 90% similar to control strain 587. The C1 batch, as can be seen in Figure 2, contained *Bradyrhizobium* strains 5079 and 5080. Like with the other clusters, each colony presented a unique fingerprint. Again, the CFU C1-1, C1-5, and C1-2 were 90% similar to *B. japonicum* 5080. Other CFU were also 90% similar to control strain SEMIA5079 and both clusters were well differentiated from E109. These results suggest that, independently of the number of amplification reactions used to characterize and identify rhizobia, mispriming might always generate artifactual diversity. Therefore, the combination of PCRs such as BOX or REP fingerprinting with reactions targeted at the RSα sequences, generate a more reliable identification of closely related bradyrhizobia.

Abbreviations

PCR: Polymerase Chain Reaction; REP: Repetitive Extra-genic Palindromic; YEM: Yeast Extract Mannitol; UPGMA: Unweighted Pair-Grouping Method with Arithmetic Mean.

Competing Interests

The authors are researchers working at the Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, and their field of expertise is nitrogen fixation of soybean. Particularly they work on selection and evaluation of newly isolated rhizobia and in the development of identification tools of microorganisms.

Authors' Contributions

SMYL did the amplification reactions, the analysis and wrote part of the paper; PAB helped in the analysis of the data and in writing and editing of the manuscript.

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Figure 1.

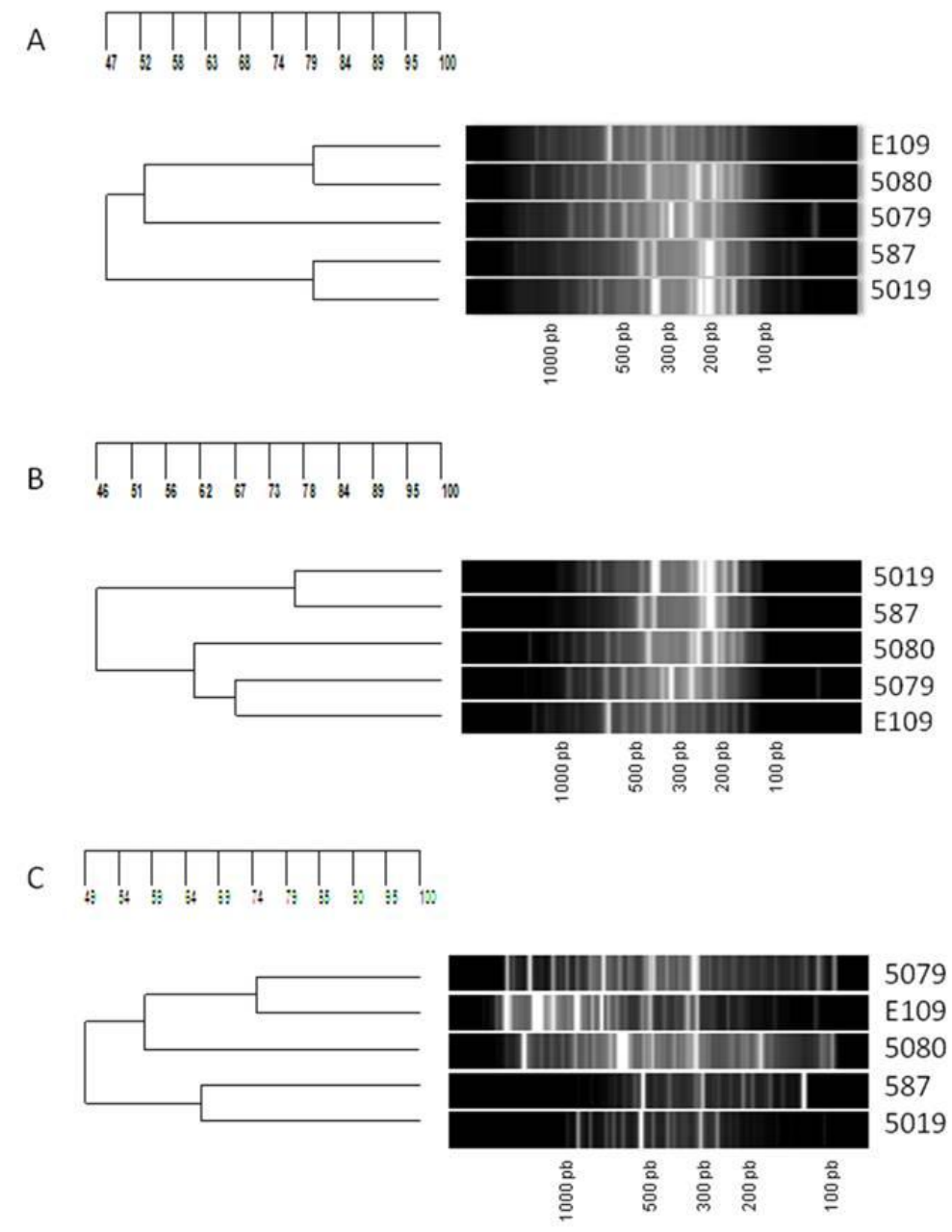


Figure 1: Fingerprints of *B. japonicum* strains SEMIA5079, SEMIA5080 and E109 and *B. elkanii* SEMIA587 and SEMIA5019. A) BOX primers B) REP primers C) RSα (2 pairs of primers). Reactions were repeated at least three times using either liquid cultures or colonies or DNA as templates but they were similar for simplicity only one picture of each is presented. Phenograms were built by means of the PCGene Software developed by Syngene. The sizes of the bands at the side of the gels corresponded to the 1000-100 bp molecular marker (INBIO, Highway Tandil, Argentina).

Figure 2

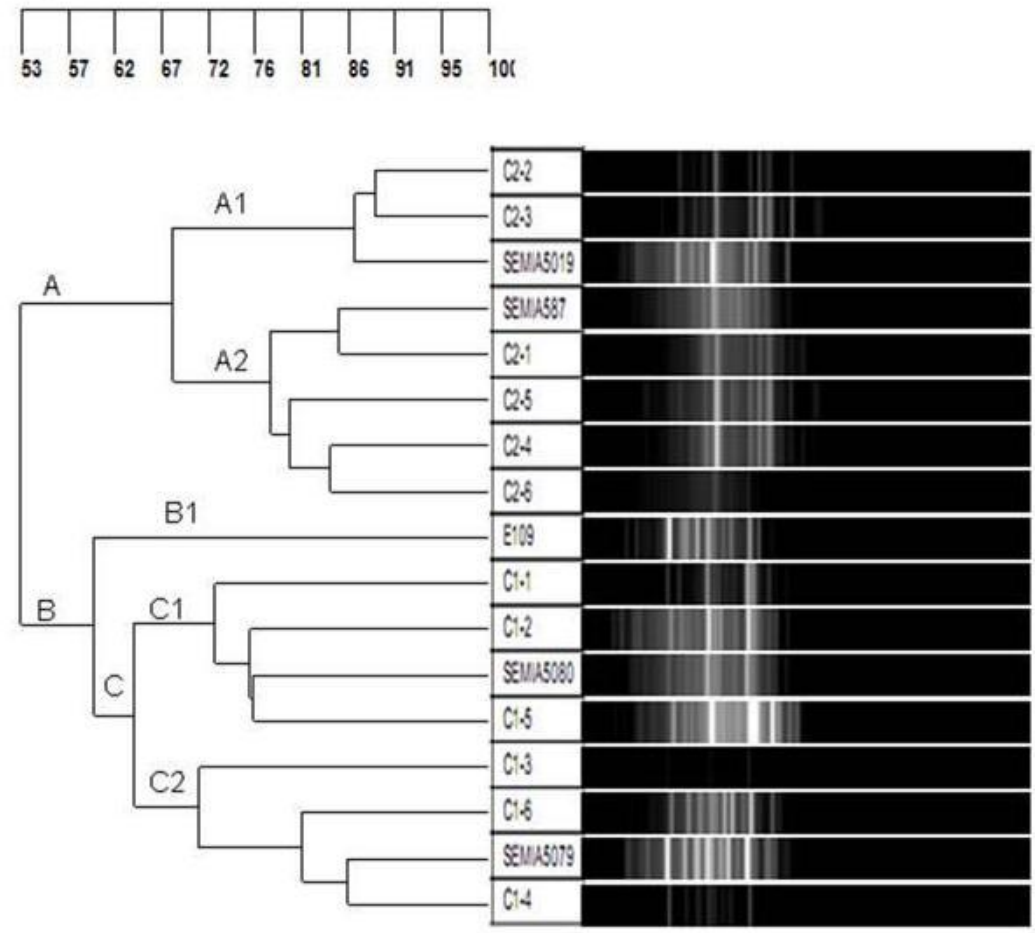
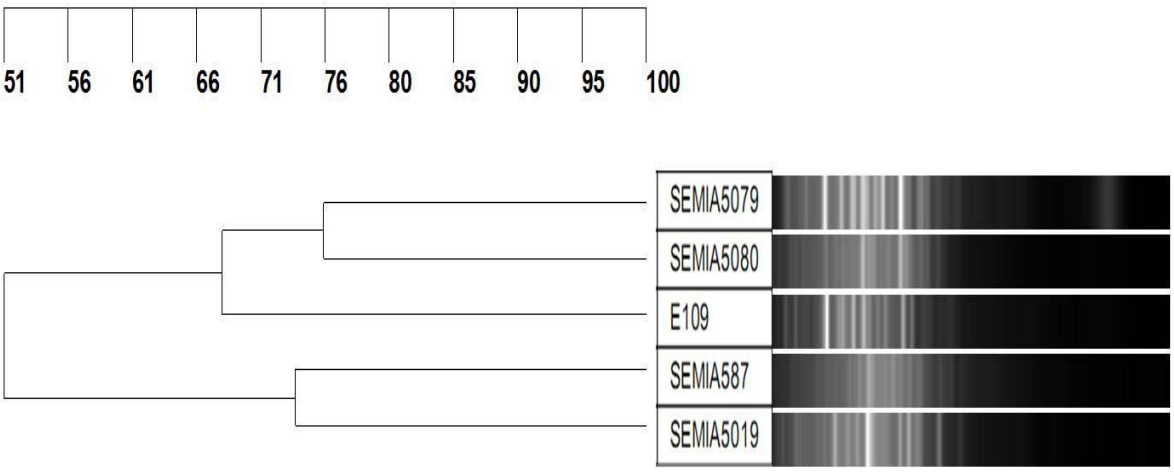


Figure 2: Fingerprints of control strains and colonies obtained from a serial dilution series of the two batch cultures analyzed C1 and C2. Fingerprints were generated by means of the BOX, REP and RSα (2 pairs of primers). The fingerprints were superimposed and analyzed by means of PCGene Tools Syngene. Gels were normalized considering the position of the bands of a 1000-100 bp marker allowing a 1% variance. Letters identify the clusters formed.



Supplementary Information

Fingerprints of control strain colonies obtained from pure cultures of a serial dilution series of the two batch cultures analyzed C1 and C2 of SEMIA5080, SEMIA5079, SEMIA587, and SEMIA5019. Fingerprints were generated by means of the BOX, REP, and RSα (2 pairs of primers). The fingerprints were superimposed and analyzed by means of PCGene Tools Syngene. Gels were normalized considering the position of the bands of a 1000–100 bp marker allowing a 1% variance.