

Genetic Diversity in Rhizobia Determined by Random Amplified Polymorphic DNA Analysis

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ABSTRACT

Rhizobia are soil bacteria which specifically nodulate legume roots thus forming a nitrogen fixing root nodule symbiosis, which has a great importance to agriculture in nitrogen deficient environments. RAPD fingerprinting was used for strain identification and the assessment of genetic diversity within a field population of *Rhizobium* (*Bradyrhizobium archus*, *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *Trifolii*). Total genomic DNAs from different field isolates were amplified using two different arbitrary primers. Different band patterns were obtained for all strains. Cluster analysis showed the relationship of *R. leguminosarum* bv. *Trifolii* with *B. archus* (69%) and *B. japonicum* (63%). The results indicated that RAPD is a very discriminative and efficient method for differentiating and studying genetic diversity of *Rhizobium* strains.

Key Words: Genetic diversity; Plasmid profiles; RAPD; Rhizobia

INTRODUCTION

Rhizobia are usually defined as nitrogen fixing soil bacteria capable of forming root or stem nodules on leguminous plants fixing atmospheric nitrogen and reducing to ammonia for the benefit of the plant. Due to their considerable agricultural and environmental significance, these legume symbionts have been extensively studied. During the last years, the assessment of diversity within rhizobial natural populations in various regions of the world has received considerable attention (Madrzak *et al.*, 1995; Chen *et al.*, 2000). Many attempts have been made to determine the actual composition and characteristics of indigenous strains isolated from different cultivated legumes (Laguerre *et al.*, 1996; Carelli *et al.*, 2000). Although most studies were conducted on crop plants, less explored legumes like different shrubs and herbaceous plants have important roles in certain ecosystems (Jarabo-Lorenzo *et al.*, 2000). The development of numerous molecular genetic methods has greatly contributed to these investigations. The availability of sensitive and accurate PCR-based genotyping among closely related bacterial strains and the detection of higher rhizobial diversity has been greatly considered (Vinuesa *et al.*, 1998; Doignon-Bourcier *et al.*, 2000; Tan *et al.*, 2001).

The *Rhizobium* strains were studied by using randomly amplified polymorphic DNA (RAPD) fingerprinting, as the technique is frequently used recently for exploring genetic polymorphisms (Versalovic *et al.*, 1994; Teaumroong & Boonkerd, 1998). In order to fully exploit RAPD results for studies of the genetic structures of populations, it is necessary to utilize a method of data analysis that permits identification of variations within a

population and permits correlation of genetic variations with environmental effects.

The aim of the present study was to investigate the suitability of the RAPD method to distinguish *Rhizobium* strains and to detect genetic diversity in field populations.

MATERIALS AND METHODS

Bacterial strains. Strains of *Rhizobia* were isolated from root nodules harvested from young seedlings of soybean (*B. archus* & *B. japonicum*) and clover (*Rhizobium leguminosarum* bv. *trifolii*) inoculated with 1 mL of soil suspension. These soil samples were collected from various regions of Egypt and were screened for the presence of *Rhizobia* as described by De Lajudie *et al.* (1994). *Rhizobia* strains were maintained on yeast extract mannitol medium Vincent (1970).

Genomic DNA preparation. For total DNA isolation, culture of *Rhizobia* strains were grown for 4 days at 28°C in yeast mannitol medium (Vincent, 1970). Cells were pelleted several times by centrifugation at 3000 rpm for 10 min. washed and resuspended in TEN buffer (50 mM Tris-HCl, 20 mM disodium EDTA, 50 mM NaCl, pH 8.0). Lysozyme was added to a final concentration of 0.5 mg mL, and cell suspensions were incubated at 37°C for 1 h. Sodium dodecyl sulphate (SDS) and proteinase K were then added to 1% and 200 µg mL final concentrations, respectively. After 3 h incubation at 37°C the lysate was extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1 v/v) and once with phenol: chloroform (24: 1 v/v). DNA was ethanol precipitated in 70% ethanol, dried and redissolved in 150 µl of TE buffer. The concentration and the purity of DNA were estimated

spectrophotometrically at 260-280 nm.

Oligonucleotide primers and PCR amplification conditions. Two different primers were chosen arbitrarily. The primers used in this work were 20 and 15 nucleotides in length. Primer sequences (5' to 3') were as follows: P1- GTG TGT GTG TGT GTG TGT GT, P2- GAC AGA CAG ACA GACA. Amplification reactions were performed in a 25 μ L volume, containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 200 μ M each of dNTPs, 1 μ M primer, 30 ng of genomic DNA and 1.5 U of *Taq* DNA polymerase. The reaction mixture was overlaid with two drops of mineral oil, incubated for 5 min. at 95°C for initial denaturation, and then amplified for 35 cycles consisting of 30 s at 94°C, 30 s at 36°C and 60 s at 72 s followed by a 7 min. incubation at 72°C. Amplification product were separated by gel electrophoresis on precast 0.8% Agarose and visualized under UV illumination after staining with ethidium bromide.

Data analysis. The sizes of RAPD fragments were estimated by comparison with the marker. RAPD fingerprints were recorded in the binary form, i.e., 1 = presence of a band and 0 = absence of a band. All data were scored twice by two independent scorings. A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram by using NTSYS-pc package (Rohlf, 1990).

Plasmid DNA isolation. The plasmid DNA was isolated as described by Brinboin and Doly (1979).

RESULTS AND DISCUSSION

Total genomic DNA from five field isolates of, *B. aruches*, *B. japonicum* and *R. leguminosarum* bv. *trifolli* were used as templates for RAPD fingerprinting. In order to obtain sufficient number of polymorphic bands that permitted reliable comparison, two different arbitrary primers were used in amplification reaction. All the isolates were further genotypically characterized by RAPD fingerprinting. The primers produced multiple DNA products ranging in size from 0.546 to 3.010 kb. A total of 11 bands were scored, with an average of 3 bands per score (Fig. 1). The number of scorable bands generated by primer ranged from as low as 1 to as many as 4. One diagnostic marker in *B. japonicum* and *B. aruches* with molecular weight 1.241 kb and two diagnostic marker were identified each in *R. leguminosarum* bv. *Trifolli* with molecular weight of 3.010 and 1.915 kb. This and various other studies show showed that RAPD analysis can effectively distinguish different *Rhizobia*, because it is a fast and simple method once the primers revealing the polymorphism are identified, and PCR conditions optimized. Slight differences in primer sequences can cause significantly different RAPD patterns that enable an easy visual discrimination among strains. In addition this method has various advantages like faster, less labor intensive, eliminates the need for pure DNA and only

Fig. 1. Representative RAPD profiles of *Rhizobia* strains. Amplification of the genomic DNA was performed using primers GT1 and GACA. Lanes: 3 and 4, *B. japonicum*; lane: 1, *B. archus*; lanes: 2 and 5, *R.leguminosarum* and Lane M, lambda DAN Hind III marker

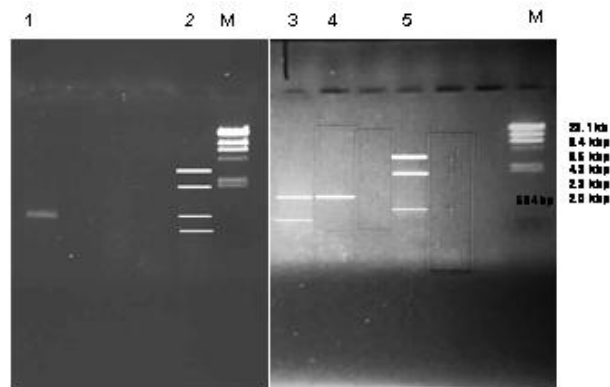
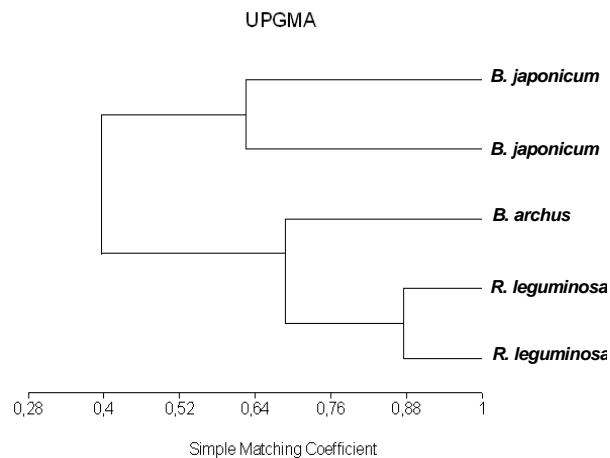


Fig. 2. Dendrogram of *Rhizobia* strains derived from RAPD fingerprints generated by using two different primers



a small amount of template is required for the amplification reaction method gives better discrimination among bacterial strains (Bostock *et al.*, 1993; Wang *et al.*, 1993). Sikora *et al.* (1997) demonstrates that *B. japonicum* strains could be easily differentiated by RAPD fingerprinting, thus supporting the validity of this fast and accurate technique in studying diversity of *B. japonicum* field population. Further investigations are needed for the assessment of the effects of releasing *B. japonicum* strains in the environment and for a better understanding of the relationships among field populations.

To demonstrate the relationships between the isolates with different fingerprint profiles, the summarized

Table I. Similarity matrix for five *Rhizobia* strains

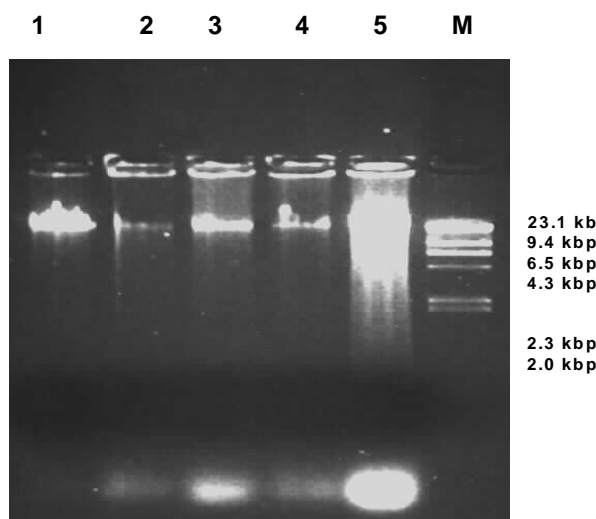
	R. leguminosarum	R. Leguminosarum	B. archus	B. japonicum	B. japonicum
<i>R. leguminosarum</i>	1,000				
<i>R. leguminosarum</i>	0,875	1,000			
<i>B. archus</i>	0,750	0,625	1,000		
<i>B. japonicum</i>	0,500	0,375	0,500	1,000	
<i>B. japonicum</i>	0,375	250	0,375	0,625	1,000

information gained by the banding patterns was analyzed statistically. Analysis of banding patterns by peak comparison of the the isolates into two main cluster revealed that one cluster consisted of *B. japonicum* strains and the other one that of *R. leguminosarum* bv. *Trifolli* strains (Fig. 2). Cluster analysis of the genetic diversity values was performed to generate a dendrogram illustrating the overall genetic relationships between *Rhizobia* studied individually and within genera. Within the first cluster, which included *B. japonicum*, the isolates together and the similarity between them was 0.63. The scnd cluster, which include *R. leguminosarum* bv. *Trifolli*, the isolates closely linked with them and the similarity was 0.88. However, the similarity between *B. japonicum* and *B. aruches* was 0.63. While, the similarity between *B. aruches* and *R. leguminosarum* bv. *Trifolli* was 0.69. Data from the similarity matrix indicate that, the relationships between Rhizobial strains are different (Table I). Whereas, the relationships within *R. leguminosarum* bv. *Trifolli* was 88%, while these relationships with-in *B. japonicum* were 63%. The arbitrary nucleotide sequences, RAPD fingerprinting, is frequently used technique for investigating genetic polymorphisms (Versalovic *et al.*, 1994; Teaumroong & Boonkerd, 1998). The results of this study indicated that RAPD provied a high degree of discrimination between *B. japonicum*, *B. aruches*

and *R. leguminosarum* bv. *Trifolli*. RAPD profiles were reproducibly obtained for all *B. japonicum*, *B. aruches* and *R. leguminosarum* bv. *Trifolli*. By the RAPD analysis clear differences were observed between *B. japonicum*, *B. aruches* and *R. leguminosarum* bv. *Trifolli*. These results suggest the presence of adapted indigenous strains *B. japonicum*, *B. aruches* and *R. leguminosarum* bv. *Trifolli*, probably with higher competitive ability. This study indicated that the *Rhizoba* isolates can be divided into two main groups, as of at least two diverent groups within *B. japonicum* and *R. leguminosarum* bv. *Trifolli* exist (Sikora *et al.*, 1997; Josic *et al.*, 2002). These findings are further agree to the report of Madrzak *et al.* (1995), Ando and Yokoyama (1999), Niemann *et al.* (1997) who studied diversity among natural rhizobial populations.

The isolated plasmids from the *Rhizobia* strains were the same in molecular weight (21, 148 kb), being one in all strains (Fig. 3). Characterization of the Rhizobium genome at the molecular level is the most discriminating method to evaluate the diversity among bacteria. Most species and genes essential for symbiosis and in particular genes that determine the plant host specificity are carried on a symbiotic plasmid. *Rhizobium* strains may additionally contain other phenotypically cryptic plasmids. Plasmid DNA represented an important part of the genome. The number and sizes of plasmid have helped in differentiating *Rhizbium* strains and variability among them (Shishido & Pepper, 1990).

Fig. 3. Plasmid number and size in *Rhizobia* strains. Lanes: 1 and 2, *B. japonicum*; lane: 3, *B. archus*; lanes: 4 and 5, *R.leguminosarum* and Lane M, lambda DNA Hind III marker



CONCLUSION

The results in this study showed that, one diagnostic marker was identified each in *B. japonicum* and *B. aruches* and two diagnostic markers in *R. leguminosarum* bv. *Trifolli*. RAPD considered fast and simple method gives discrimination among rhizobial strains.

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