African Journal of Biotechnology Vol. 8 (12), pp. 2677-2681, 17 June, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Genetic diversity in rhizobial isolates determined by RAPDs

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Accepted 15 April, 2009

Nine soil rhizobia isolated from different field locations were subjected to RAPD analysis to study the diversity. It was found that *Rhizobium* isolates from Agricultural Research Station - Aliyar Nagar (ALN 7), Department of Agricultural Microbiology (SOB 1), isolates from TNAU Eastern block field No.36 (EB 36) and Millet Breeding station field: 9 (MBS 19) were clustered together to form cluster 1 followed by *Rhizobium* isolates Millet Breeding station field: 8 (MBS 9), isolates from the Department of Agrl. Microbiology, TNAU 14 and COG 15 belongs to cluster 2 and isolates of Cotton Breeding station field No: 9 (CBS 9) and Aliyar Nagar (ALN 2) formed the fourth cluster. Department of Agricultural Microbiology isolate, COG 15 with TNAU 14 and SOB 1 with *Rhizobium* ALN 7 were clustered together with more than 50 per cent similarity. Isolate from Cotton Breeding station field No: 12 (CBS 12) was found to have more divergence (>90 per cent) to make the separate cluster. The results indicated that RAPD is a very discriminative and efficient method for differentiating and studying genetic diversity of *Rhizobium* strains.

Key words: Rhizobium, RAPD, genetic diversity.

INTRODUCTION

Rhizobia are gram negative, rod shaped, motile bacteria belonging to the family Rhizobiaceae. They are aerobic chemoorganotrophs, grow well in the presence of oxygen and utilize relatively simple carbohydrates and amino acid (Somasegaran and Hoben, 1994). They are able to establish effective nitrogen fixing symbiosis system with legume plants, in the specialized structure called root nodules. In the symbiotic process they reduce atmospheric nitrogen to ammonia using the enzyme nitrogenase and supply this essential nutrient to the host plant. The genus *Rhizobium* is well characterized by its novel association with legumes for fixing atmospheric nitrogen. Beijerinck (1888) was the first to isolate and cultivate the microorganisms from the nodules of legumes and named it as *Bacillus radicicola* which is now member of the genus *Rhizobium* (Jordan and Allen, 1994). The present classification of root nodules bacteria as described in Bergy's Manual of Systematic Bacteriology separates the fast and slow growing microorganisms into two genera *Rhizobium* and *Bradyrhizobium*.

The fast growing rhizobia is divided into three species, *Rhizobium leguminosarum*, *Rhizobium meliloti*, and *Rhizobium loti* with *Rhizobium leguminosarum* comprising three biovars *Rhizobium leguminosarum* biovar trifolii, *Rhizobium leguminosarum* biovar phaseoli and *Rhizobium leguminosarum* biovar viciae. Rhizobial strains are currently placed in the following genera: *Allorhizobium* (recently merged with the genus *Rhizobium*), *Mesorhizobium, Rhizobium* and *Sinorhizobium*, which constitute one of the rhizobial clades; *Azorhizobium*, *Bradyrhizobium* and *Methylobacterium,* which are each found on a different and well-resolved phylogenetic branch (Sy et al., 2001; Young et al., 2001).

The availability of sensitive and accurate PCR-based genotyping among closely related bacterial strains and the detection of higher rhizobial diversity have 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 and Boonkerd, 1998). To fully

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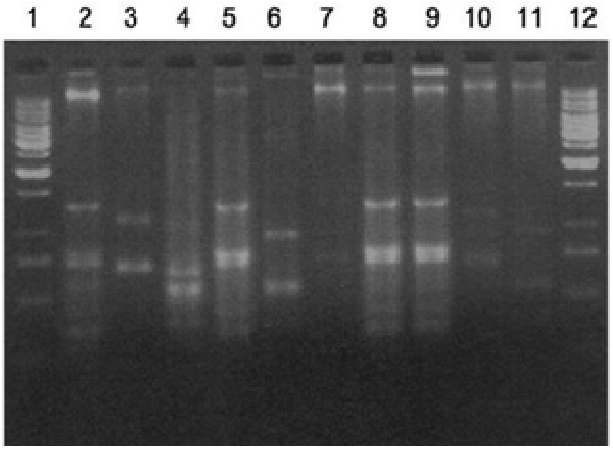

Figure 1. RAPD banding pattern of Rhizobium isolates from soil with RAPD primer (OPQ 1). Lane 1, 1 kb DNA ladder; lane 2, ALN 2; lane 3, CBS9; lane 4, CBS12; lane 5, MBS9; lane 6, MBS19; lane 7, SOB1; lane 8, TNAU14; lane 9, COG15; lane 10, ALN7; lane 11, Eb36; lane 12, 1 kb DNA ladder.

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.

The aim of the present study was to investigate the suitability of the RAPD methods to distinguish *Rhizobium* strains and to detect genetic diversity in field populations among the Tamil Nadu Agricultural University (TNAU) farms.

MATERIALS AND METHODS

Soil sample collection

Each soil sample consisted of ten sub-samples, taken from 0 to 20 cm depth, which were fully homogenized. Cropping areas were chosen in each location *viz.,* continuously cropped with groundnut, legumes, non-legumes and uncultivated land. Soil analyses were performed according to Silva et al. (1999).

Genomic DNA extraction of rhizobial isolates

The total genomic DNA of rhizobial isolates was isolated by following the Ivanova et al*,* (2000) with slight modifications. The rhizobial cultures were grown in TY medium (Annexure I) at incubator shaker at 150 rpm and 30°C for 48 h. About 25 ml of actively grown rhizobial cultures were centrifuged at 6000 rpm; 5 min at 4°C to harvest the cell and suspended the cell pellets in 1 ml of TE buffer and 0.5 ml of 1-butanol and mixed well. The contents were centrifuged at 6000 rpm for 5 min at 4°C and the supernatant discarded. The cell pellets were then resuspended in 2 ml of TE buffer and centrifuged at 6000 rpm for 5 min at 4°C to remove trace of butanol. The cell pellets were added with 1 ml of TE buffer and 100 µl lysozyme (10 mg ml^{-1} freshly prepared) and incubated at room temperature for 5 min. 100 µl of 10% SDS and 25 µL of 100 µg ml^{-1} proteinase K was added, mixed well and incubated at 37°C for 1 h. After incubation, the cell lysates were added with 200 µl of 5 M NaCl; 150 µl of CTAB (10% stock) and incubated at 65°C for 10 min. The cell lysate was deproteinized with 1 ml of phenol: choloroform mixture and centrifuged at 6000 rpm for10 min at 4°C. The aqueous layer was transferred carefully to new 1.5 or 2.0 ml microcentrifuge tube, the volume was noted; added with 0.6% volume of ice-cold isopropanol and incubated at -20°C for overnight. The precipitated DNA was pelletized by centrifuge at 12000 rpm; 15 min at 4°C. After discarding the supernatant, the pellet was vacuum dried for 30 min and resuspended in 100 µl of TE buffer. One µl of DNAse free RNAse (10 mg /ml stock) was mixed by swirling, incubated at 37°C and stored at -20°C for further use.

Genetic diversity of rhizobial isolates by randomly amplified polymorphic DNA

Two RAPD primers (OPQ1 and OPM10) were used in this study. RAPD-PCR reactions were carried out as described elsewhere (Guthrie et al*.,* 1992). RAPD PCR reaction mixture (25 µl) contained 25 ng of genomic DNA, 100 µM each of dNTPs, 1 µM primer, 2.5 µM $MgCl_2$ and 1 U of Taq polymerase. PCR reactions were performed in PCR thermal cycler (Eppendoff Mastercycler, Germany) with the programme as: initial denaturation of 95°C for 3 min followed by 35 cycles of denaturation at 94°C for one min; annealing at 36°C for 45 s; extension at 75°C for one min and final extension at 72°C for 7 min. PCR products were resolved in 1.5% agarose gel, stained with ethidium bromide and documented in Alpha imager TM1200 documentation and analysis system. Strong and clear RAPD bands were scored as primer wise and similarity and clustering analysis were done using the software, NTSYS-PC 2 package (Numerical taxonomy analysis program package, Exeter software, USA). Similarity among the strains was calculated by Jaccard's coefficient (Jaccard, 1912) and the dendrogram was constructed using UPGMA method (Nei and Li, 1979).

RESULTS

Genetic diversity of ground nut *Rhizobium* isolates

The DNA extracted from different *Rhizobium* isolates was resolved in 0.8% agarose gel. In total 10 groundnuts *Rhizobium* isolates were studied for their polymorphism at molecular level. The electrophoretic profile of groundnut *Rhizobium* isolates generated by RAPD-PCR by using the primer OPQ 1 and OPM 10 are presented in Figures 1 and 2, respectively. All the isolates were further genotypically characterized by RAPD fingerprinting. From the electrophoretic banding pattern, cluster analysis was carried out using Jaccard similarity co-efficient. From the result, it was found that *Rhizobium* isolates ALN 7, SOB 1, EB 36 and MBS 19 were clustered together to form cluster 1 followed by *Rhizobium* isolates MBS 9, TNAU 14, COG 15 were belong to cluster 2 and CBS 9 and ALN 2 formed the fourth cluster. Isolate CBS 12 was found to have more divergence (>90%) to make the separate cluster. COG 15 with TNAU 14 and SOB 1 with *Rhizobium* ALN 7 were clustered together with more than 50% similarity (Figure 3). The results of this study indicated that RAPD provided a high degree of discrimination between the strains.

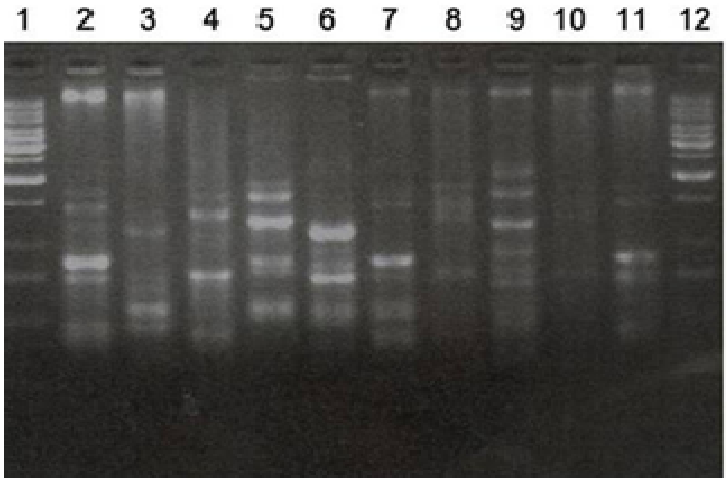

Figure 2. RAPD banding pattern of Rhizobium isolates from soil with RAPD primer (OPQ 1). Lane 1, 1 kb DNA ladder; lane 2, ALN 2; lane 3, CBS9; lane 4, CBS12; lane 5, MBS9; lane 6, MBS19; lane 7, SOB1; lane 8, TNAU14; lane 9, COG15; lane 10, ALN7; lane 11, Eb36; lane 12, 1 kb DNA ladder.

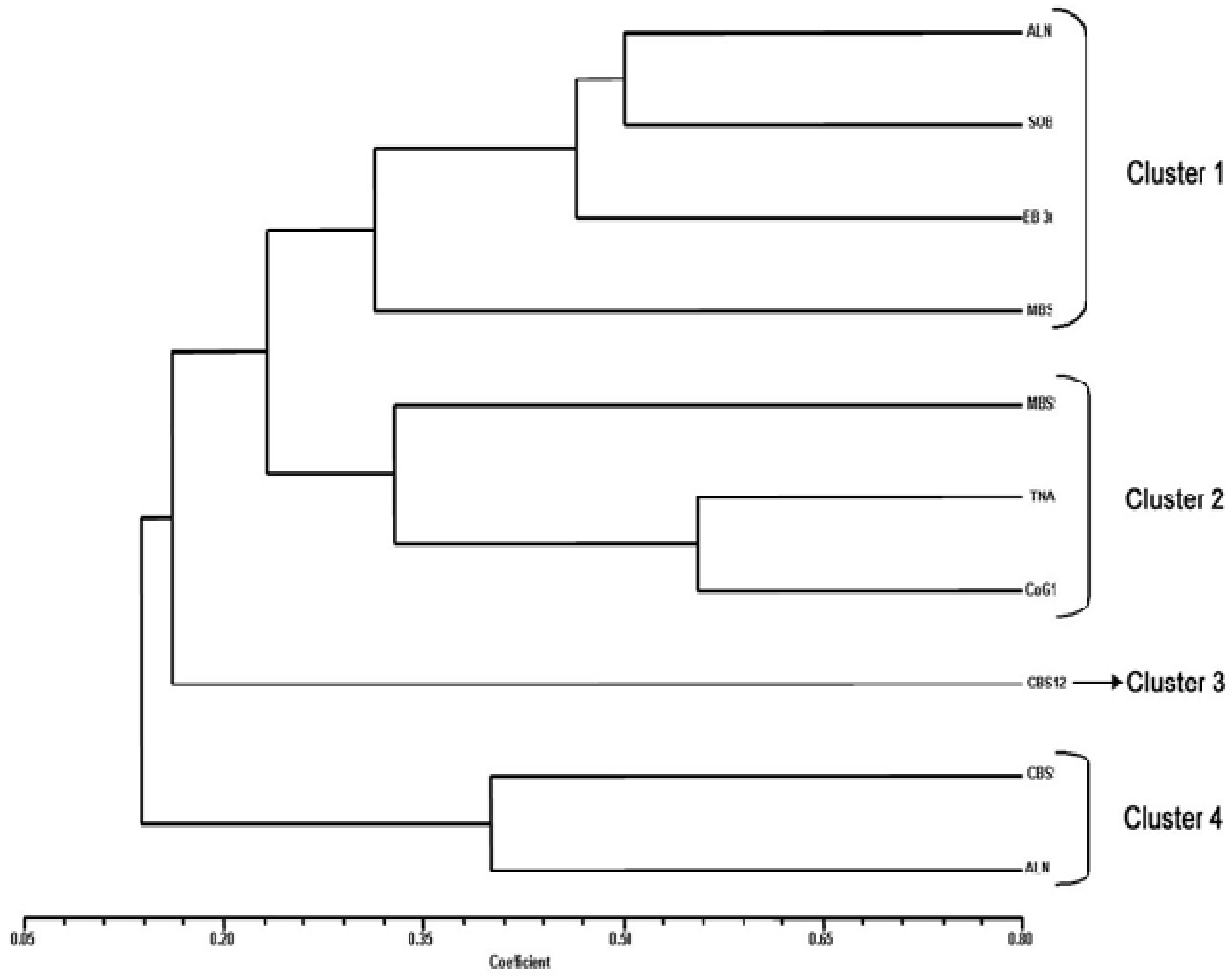

Figure 3. Phylogenetic relationship between the rhizobial isolates by RAPD analysis.

DISCUSSION

An increasing interest has emerged with respect to the importance of microbial diversity in soil habitats. The extent of the diversity of microorganisms in soil seems to be critical for the maintenance of soil habitat and quality, as a wide range of microorganisms are involved in important soil function. For successful functioning of introduced microbial bioinoculants and their influence on soil health, exhaustive efforts have been made to explore soil microbial diversity of indigenous community, their distribution and behaviour in soil habitat (Hill, 2000). Diversity is important for genetic characterization with different nitrogen fixing capacities. Using diversity analysis, novelties of new *Rhizobium* sp. with high nitrogen fixing potential could be revealed which have not been identified ever before. Genomic DNA finger printing using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between closely related bacteria. Ten selected rhizobial isolates, representing both the different morphological groups and the areas where soil samples have been collected, were analyzed by RAPD.

As a rapid method, this technology has been used for grouping and identifying rhizobia. The grouping results of PCR-RFLP patterns of 16S rRNA genes agree well with those of multilocus enzyme electrophoresis, numerical taxonomy and sequencing of 16S rRNA genes (Laguerre et al., 1994; Tan et al., 1999; Wang et al., 1998).

Randomly amplified polymorphic DNA (RAPD) profiles have provided new tools for investigating genetic polymorphism. This method was used by Van Rossum et al. (1995) for genetic analysis of *Bradyrhizobium* strains nodulating *Arachis hypogea* and nodule isolates of *Arachis* sp. (Khbaya et al., 1998). Using diversity analysis, novelties of new *Rhizobium* sp. with high nitrogen fixing potential could be revealed which have not been identified before. Genomic DNA finger printing using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between closely related bacteria. Ten selected rhizobial isolates, representing both the different morphological groups and the areas where soil samples have been collected, were analyzed by RAPD. Rhizobial isolates of cluster 1(ALN 7, SOB 1, EB 36, MBS 19) is genetically distant from the cluster IV (CBS 9 and *Rhizobium* ALN 2). Isolate CBS 12 was found to have more divergence (>90%) to make the separate cluster. This result implies that *Rhizobium* isolates belonging to cluster 1 and those from cluster 4 are significantly diverse.

The results indicate that RAPD is discriminative and efficient method for differentiating and studying genomic diversity of *Rhizobium* strains. RAPD finger printing was used for identification and the assessment of genetic diversity within the field population of *B. japonicum* by Sikora et al. (1997). From the analysis it is clear that most of the genetic diversity was found within the population of soil isolates which were isolated from different farm location and research station of Tamil Nadu Agricultural University.

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