Genetic Biodiversity of Nitrogen-Fixing Bacteria from Agricultural Soil of Madhya Pradesh State, India

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ABSTRACT
Nitrogen fixing bacteria, especially *Bradyrhizobium* and *Rhizobium* are of great importance in sustainable agriculture. *Rhizobium* is unique because of its ability to live symbiotically with legumes and it accounts for most of the biolologically fixed nitrogen. In the present investigation, fifty strains of *Rhizobium spp.* (R1, R2, R3, R4, R5, R6.........R50) from agricultural land of Madhya Pradesh were subjected to biochemical and genetic characterization. Fifty strains confirmed as *Rhizobium* spp. were used for further microbial diversity study. Percent G+C content of all the isolated strains was calculated by determining melting temperature of DNA (Tm). Tm of all the strains ranged between 94.3 to 95.7^{0}C. However % G+C content of isolated strains ranges between 61.0 to 64.4 %. Furthermore, the pattern of genetic diversity was studied using known and established molecular biotyping methods. In the present observation isolated *Rhizobium* species were examined by RAPD-PCR genomic fingerprinting using 2% agarose. This technique helps to compare actual DNA fragments from the genome of bacteria. Genetic polymorphism is a technique most widely acceptable in presentation of genetic hierarchy. The Dendrogram show divergence at 25% similarity into two broader groups. R25 strain shows 75% dissimilarity with all the strains isolated from agricultural soil of Madhya Pradesh. RAPD analysis establishes similarity even upto 85% with R1, R39, R10 and R35 strains. The results indicated that RAPD is a very discriminative and efficient method for differentiating and studying genetic diversity of *Rhizobium* strains.
Keywords: Genetic diversity, *Rhizobium*, RAPD-PCR, Tm-value, %G+C content.

1. 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. 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). Diversity in bacterial communities is normally determined by phenotypic and methods and the culturable bacteria. It is estimated that in one gram of soil, there could be four thousand different genomic units based on DNA-DNA reassociation. There are1.9 million living species on our planet. (Stanley 2002) with almost 5000 bacterial species (Pace 1997 & 1999). The widely applied approach is to characterize of DNA from organisms in PCR with short oligonucleotide primers of arbitrary sequences to generate genetic markers. This is the basis of Random Amplified Polymorphic DNA (RAPD) method (Williams et al. 1990), arbitrarily primed polymerase chain reaction (AP-PCR) (Welsh and McClelland 1990) and DNA amplification fingerprinting (DAF) (Caetano-Anolles et al. 1991). The development of numerous molecular genetics 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). RAPD method may reveal more polymorphism if combined with the restriction digestion. RAPD analysis (Welsh and McClelland 1990; Williams et al. 1990), and can be successfully applied to identification of various bacterial strains (Mazurier et al. 1992; Dooley et al. 1993; Stephan et al. 1994).
The purpose of this study was to clarify the taxonomic position of this strain based on phylogenetic analysis of gene sequence. The results indicated that RAPD is a very discriminative and efficient method for differentiating and studying genetic diversity of *Rhizobium* strains.

2. Material and Methods

2.1. Sampling site

Soil samples (0-30cm depth) were collected from 10 agricultural sites of Neemuch, Hoshangabad, Betul- Multai, Sehore, Bhopal, Tikamgarh, Chindwara, Raisen Vidhisha- Sanchi and Ujjain districts of Madhya Pradesh (Fig. 1). These districts fall in central part of the province. Surface litter was scrapped away and soil samples stored in pre-sterilized high-density polythene (HDPE) bags (Forster 1995). Samples were passed through 2 mm sieve to have homogenous particles for further analysis. The *Rhizobium* species confirmed from soil samples were named as R1- R50.

The dilutions (10^{-1} to 10^{-8}) were inoculated on YEMA (Yeast Extract Mannitol Agar) plates (Subba Rao 1984) and incubated at 28° ± 2°C for 24 to 72 h. Fast growing *Rhizobium* species appeared within 24 hours and the slow growing needed cells further incubation of 72-96 h. The glistening white *Rhizobium,* like colonies were picked up and purified by continuous streaking on YEMA and CRYEMA plates (Subba Rao., 1984). The composition of media was mannitol-10g, K_2HPO_4-0.5g, $MgSO_47H_2O$-0.2g, NaCl-0.1g,yeast extracts-0.4g, agar-15.0g, distilled water-1 L and Congo red solution(10.0ml). pH of the medium was adjusted to 6.8 and sterilized at 15 psi (15 min).Yeast extract mannitol agar: (Subba Rao., 1984).had the following ingredient:mannitol-10 g, K_2HPO_4-0.5g, $MgSO_47H_2O$-0.2g, NaCl-0.1g,yeast extracts-0.4g, agar-15.0g, distilled water-1 L, pH of the medium was adjusted to 6.8 and sterilized at 15 psi (15 min).

2.2. Gram Staining: (Vincent., 1970) The isolated bacteria were subjected to microscopic examination using Gram Staining (Vincent., 1970), where the smear was prepared with a loop full of isolated bacterial culture via spreading over a clean slide in a drop of sterile normal saline and is allowed to dry in air. The smear is heat fixed by passing over the flame, brought to 25° ± 1°C and stained with crystal violet solution for one minute followed by rinsing with water and is allowed to air dry. The slide is then poured with Gram's iodine solution (Mordant) for another one minute, drained and decolorized with alcohol. Again rinsed with water and allowed to air dry. The smear thus obtained was stained with counter stain for two min., rinse with water, and allow to air dry and observed under the compound microscope. First with 40X then with 100X under oil immersion, gram-negative cell appears pink- red in color and gram positive as violet.

2.3. Biochemical analysis- Hofer's alkaline medium: (Hofer., 1935) *Agrobacterium* can grow at higher pH level than *Rhizobium* and therefore their growth in YEM broth, with high pH has been considered as a useful means to distinguishing between the two allied genera. The composition is as follows:-Mannitol-10 g, K_2HPO_4-0.5 g, $MgSO_47H_2O$-0.2 g, NaCl-0.1 g, Yeast extracts-0.4 g, Distilled water -1 L, pH of the medium was adjusted to 11.0 and sterilized at 121°C and 15 psi for 15 min. Glucose peptone agar medium: Being incapable of peptone consumption a peptone rich medium of the following composition was used to distinguish *Rhizobium*, which is a fast growing microorganism in peptone rich medium (Kleczowska et al. 1968). The composition is as follows-Glucose-10.0g,Peptone-20.0g, NaCl-5.0g, Agar-20.0g, Distilled water-1 L,pH-7.2, 1ml of 1.6% Bromocersol purple, to 1 L of the medium is added and sterilized at, 121°C and 15 psi for 15 min., for three consecutive days. *Rhizobium* showed no growth or poor growth in 24 hours as compare *Agrobacterium,* which results yellow colour of the medium due to the change of the pH from alkaline/neutral to acidic. Ketolactose test: On replacement of mannitol with lactose in YEMA medium ketolactose medium is prepared. The isolated microorganisms of 4 day old culture is then transferred to the ketolactose medium, and incubated at 28° ± 2°C for 48-52 hours followed by addition of Benedicts solution of the composition given below. The excess of solution was drained out, and samples were allowed to grow at 26° ± 2°C. The yellow colour obtained around the bacterial colonies helped in distinguishing *Rhizobium* from *Agrobacterium* producing the yellow colour. (Bernaerts and Deley 1963). Composition of reagents used is: (1) Benedict's solution: Solution A: Sodium citrate-173.0 g, Anhydrous sodium Carbonate-100.0 g, Distilled water-600.0 ml, Solution B: Copper sulphate-17.3 g, Distilled water-100.0 ml, Mixing of both the solutions A and B, and filtering them a transparent clear blue coloured solution is obtained. Liquefaction of gelatin: (Subba Rao 1979) In the nutrient agar 12% of gelatin, is added and plates were inoculated with isolated bacteria and incubated at 28° ± 2°C for three to four days and then flooded with 0.2% mercuric chloride ($HgCl_2$) in 20% HCl. The clear zone around the colonies due to the hydrolysis of gelatin appears. The composition of the medium used was as follows- Peptone-5.0 g, Beef extract-3.0 g, NaCl-5.0 g, Glucose-5.0 g, Distilled water-1L, pH -6.8.

2.4. DNA isolation-Isolation of DNA was done by Marmur's method (*1961; 1963*), with slight modifications. The *Rhizobium* inoculum (2.0 O.D) was added to 50ml YEM broth and put at 28° ± 2°C (24 h) in a shaking incubator, the cell pellet obtained by centrifugation (10,000 rpm). Cells were suspended in 25ml saline EDTA solution in Erlenmeyer flasks. Lysis of cells as done by addition of 1ml of lysozyme (37°C, 30 min) followed by 25% of 2ml SDS at 60°C (10min). The suspension was allowed to cool at room temperature, and 5ml of 3M sodium acetate was gently mixed in 50ml of 24:1 Chloroform-isoamyl alcohol followed by centrifugation (10,000 rpm, 30min). Out of the three layers obtained following centrifugation, the uppermost one bearing nucleic acid was pipetted out in 100 ml beaker and two volumes of chilled ethanol added (Helms et al 1985; Tracy 1981 and Maniatis et al. 1982). The white fibrous precipitate at the interface was gently pooled out with the help of glass rod.

2.5. Determination of Tm value- The Tm of each DNA sample was determined as described by (Mandel and Marmur, 1968). The % G+C content of the samples was determined by using the equation %G+C = 2.44 (Tm –

69.4) as suggested by (De Ley J. 1970).

2.6. RAPD-PCR of amplicon-Polymerase chain reaction (PCR) is most useful widely used genetic tool in study of molecular biology of organisms. It is widely applied on cloning, sequencing and phylogenetic study. The efficiency of PCR technique is based on "master mix'' preparation consisting buffer, dNTP's mix (2mM), Primer 1, Primer 2, Taq polymerase, sterile water except the DNA template. The reproducibility and reliability of results depended upon proper pipetting of all the components of "master mix" and their further distribution. After addition of template DNA, it was exposed to temperature cycles in a thermal cycler (PTC-1148, MJ Mini Thermal cycler, BIO-RAD, USA). The conserved sequence in DNA i.e., the 16S rDNA was amplified using the reverse primer (5' ACGGCTACCTTCTTAGCACTT-3') and the forward primer (5' AG AGTTTGATCCTGGCTCAG-3') at 55°C (annealing) for 30 cycles in PCR unit (PTC-1148, MJ Mini Thermal cycler, BIO-RAD, USA). Amplified DNA was subjected to RAPD analysis using restriction enzymes (OPK-13, OPK-11, OPK-15, OPK-20) after amplification. The master mix was prepared for 50 DNA samples and then equally distributed in PCR tubes for 30 cycles at the RAPD temperature cycle programme. The composition of master mix was as follows-**Reaction mix (25µl-1x),** 10x buffer-3.0µl, *Taq* Polymerase (1.5U)-0.5µl, dNTP's (2mM)-5.0µl, Primer (50pM)-5.0 µl, H_2O-7.5µl, DNA template-4.0µl. The master mix was prepared under aseptic conditions under the laminar air flow hood, and distributed to 50 different dilution tubes, and then subject to following temperature cycles (30 times). Initial denaturation-94°C (7 min), Denaturation-94°C (30 Sec), Annealing-55°C (40 Sec), Extension-72°C (1min), Final Extension-72°C (10 min), Final hold-4°C (0 Sec). Once the PCR reaction was over, 25µl of the product was separated on 1.6% agarose gel by electrophoresis (Walker 1998). Agarose gel (1.6%) was used for the separation on RAPD products and electrophoresis done at 45-50mV (4 h 25° ± 1°C). Enzyme activity was stopped by low temperature (4°C) and by adding 2µl of 6x loading buffer. Further, the enzyme digested PCR product along with 1kb DNA ladder (Bangalore Genie, India) in a separate well was estimated by electrophoresis (Walker 1998) at 55mV on (2% agarose, 2h).The Bio-Rad Gel Doc^{TM} XR and ChemiDoc^{TM} XRS gel documentation system are easy-to-use, high-performance systems. They use a CCD camera to capture image in real time, which allows you to more accurately position and focus the image (Molecular Imager Gel Doc XR System 170-8170, 170-8171, BIO-RAD, USA).

3. Result and Discussion

The soil samples analyzed at various study areas e.g., A1 to A10 reveal slight variations in mechanical properties as (%) that varies from 14.1 to 15.4 at A5 and A2, respectively and % silt content fell between 24.3 to 25.5 at A9 and A5, respectively. However clay content showed a range of (57.6 to 59.1 %) at A6 and A1, respectively. In contrast all the sampling stations (from A1 to A10) showed the range pH between 7.4 to 8.2 as most of the soils found were alkaline. The soil alkalinity as observed, were more suited as far as the availability of ions was concerned. Water holding capacity was in the narrow range (49.2 to 51.12 %) at side A3 and A1, respectively. The observations capacity at 10 sampling stations, showed somewhat water holding. Nitrogen availability monitored (in terms of nitrate), it ranged between 2.1 to 3.02 mg/gm at A1 and A6, respectively. The N content varied between 78 to 80 ppm at all the sampling sites. Similarly, potassium content also appeared to be sufficient in the range of 200-210 ppm (Table 1). Soil sample for all the sites were further examined under laboratory conditions on pre-sterilized synthetic bacterial growth medium.

Present study, however, concentrated on the nitrogen fixing strains. Hofer's alkaline broth test conducted is based on the fact that *Agrobacterium* grows at higher pH levels and not rhizobia. The isolates strains failed to utilize peptone when were grown on glucose peptone agar medium. *Rhizobium* respond negatively ketolactose test. Microscopic observations on pure culture cells confirmed the gram negative nature. Also, gelatin was not liquefied by cells grow on gelatin medium. Bacterial cells once inoculated on pre-sterilized yeast extract Mannitol Agar (YEMA) produced white, translucent glistering colonies with entire margin soil samples from Neemuch, Hoshangabad, Betul-Multai, Ujjain, Sehore, Bhopal, Tikamgarh, Chindwara, Sanchi-Vidisha, Raisen etc were subjected to the abovementioned biochemical parameters.

The composition of DNA in bacterial genome is similar as it shows presence of all the four defined bases. The helix of DNA with double stranded structure shows pairing between A+T and G+C, thus (A+T)/(G+C) ratio or (G+C) content reflects compatibility of microbial strain in relation to evolutionary stress. The G+C content is examined in the form of temperature of melting (Tm). The bases are joined by hydrogen bonds and show regular pairing. It is obvious that the DNA with higher G+C content will stand higher melting temperature as more energy is needed to separate the double stranded DNA. The melting temperature thus is calculated by observing mid point of the rising curve. The optical density of DNA shows further use in the presence of greater amount of G+C content. The composition of G+C content with slight variation shows similar base sequences thus giving emphasis on relatedness among species in contrast to dissimilarity as observed in PCR-based observations. Samples analyzed presently, amongst 50 strains i.e., R1 to R50, the Tm ranged between 94.3 to 95.6°C. However % G+C content of isolated strains range between 60.7 to 63.9% (Table1). All

the bacteria tested and examined for Tm values and G+C content, were similar with narrow range of difference with respect to % G+C.

Furthermore, the pattern of genetic diversity was studied using known and established molecular biotyping methods. Genotyping of the isolates was done by using molecular methods (RAPD-PCR, REP-PCR, 16SrDNA-RFLP PCR). The isolated strains expressed pattern of banding between 400 to 900 bp on 2% agarose gel using RAPD-PCR fingerprint with OPK 13 primer (Fig. 2). The construction of tree is based upon distance data and is performed using computer software, by the unweigheted pair group method with arithmetic averaging (UPGMA) using PHYLIP (Phylogenetic Interference package) or NTSYS software. The dendrograms shows divergence at 25% similarity into two broader groups. R25 strain shows 75% dissimilarity with all the strains isolated from agricultural soil of Madhya Pradesh. RAPD analysis establishes similarity even upto 85% with R1, R39, R10 and R35 strains (Fig. 3).

Conclusion

Diversity in soil bacteria has been revealed by many studies and almost all of the data reported previously indicate that there is a high level of genetic diversity in these bacteria. An assessment of the genetic diversity and genetic relationships among strains could provide valuable information about bacterial genotypes that are well adapted to a certain environment. The *Rhizobium* strains were studied by using randomly amplified polymorphic DNA (RAPD) fingerprinting, as the technique is frequently used recently for exploring genetic polymorphisms. 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 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.

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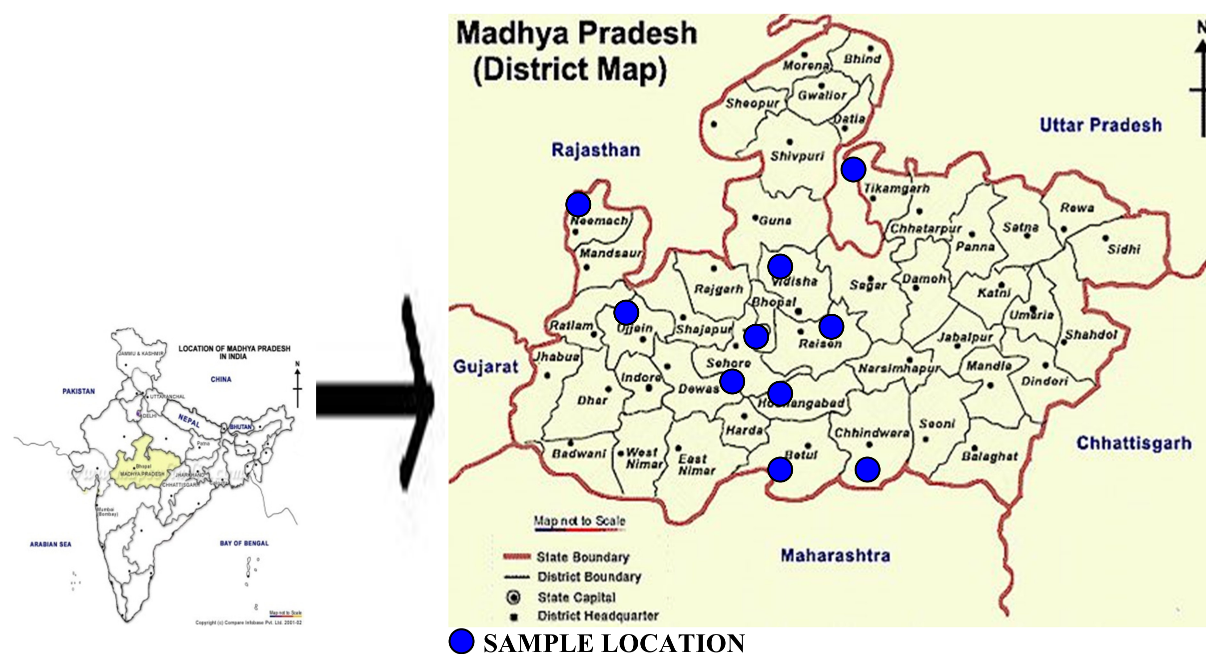
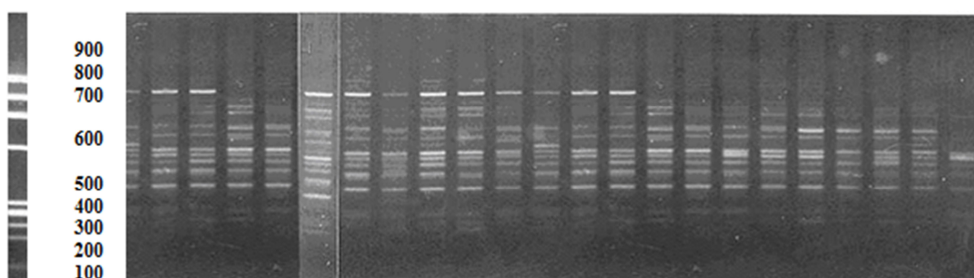


SAMPLE LOCATION

Fig. 1 Map showing sampling locations

Table 1 Physiochemical features of soil from different sites.

S.No	Soil parameter	Agriculture fields									
1.	Mechanical properties	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
	(i) Sand (%)	15.2	15.4	14.7	14.3	14.1	14.4	14.9	1.4.6	15.1	14.9
	(ii) Silt (%)	25	24.4	24.7	25.1	25.5	24.6	24.9	24.8	24.3	24.8
	(iii) Clay (%)	59.1	58.6	58.8	58.2	57.9	57.6	58.6	58.5	59.1	58.3
2.	Physio-Chemical properties										
	(i) (pH)	8.1	7.4	7.9	8.0	8.2	7.9	7.5	7.8	8.1	7.9
	(ii)Electrical conductivity (mhos/cm)	0.217	0.214	0.218	0.215	0.218	0.216	0.217	0.216	0.218	0.215
	(iii) Water holding capacity (%)	50.12	50.05	49.22	51.50	50.11	50.89	50.99	51.02	51.06	49.68
	(iv) Nitrate (ppm)	2.121	2.214	2.311	2.105	2.314	3.029	2.245	2.299	2.798	2.325
	(v) Nitrogen content (ppm)	80.0	78.0	80.9	80.4	79.2	78.9	79.9	80.6	80.4	80.7
	(vi) Phosphorus (ppm)	1.065	1.023	1.089	1.054	1.022	1.014	1.099	0.982	0.921	1.041
	(vii) Potassium content (ppm)	210	201	205	204	208	205	204	200	202	207
	(viii) Organic content (mg/g)	0.128	0.122	0.142	0.126	0.129	0.124	0.123	0.123	0.124	0.125



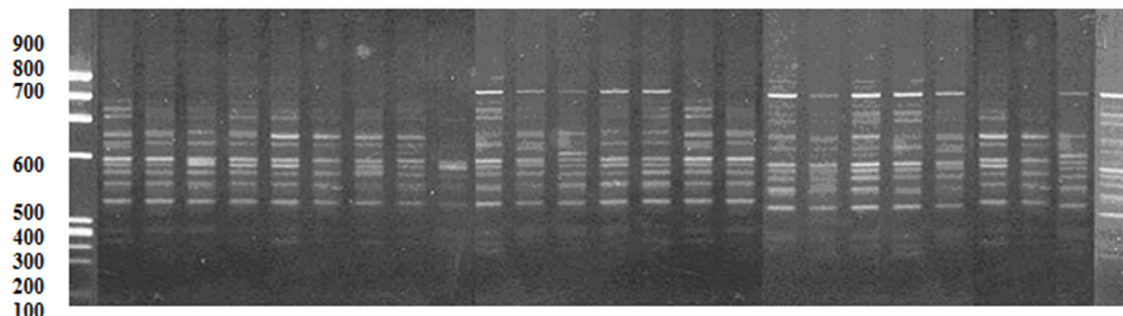


Fig. 2 RAPD- PCR fingerprint of Rhizobium strains from agricultural fields using OPK 13 primer (2 % agarose gel).

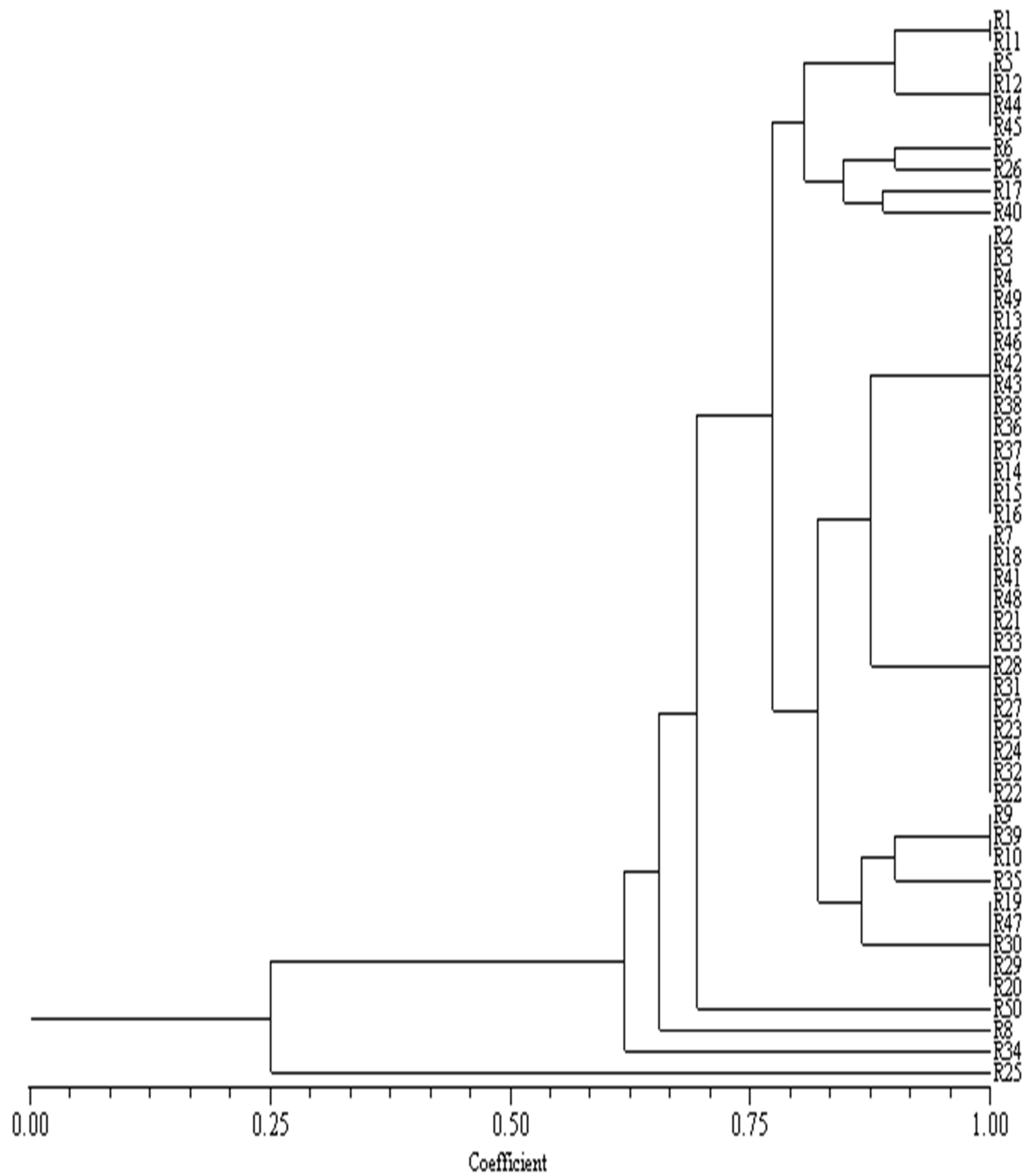


Fig. 3 Dendogram based on RAPD PCR of *Rhizobium* isolates showing genetic relatedness (based on Jaccord's similarity coefficient and UPGMA cluster method).

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