

Assessment of Phenotypic and Genotypic Diversity of Rhizobium Bacteria in Madhya Pradesh

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Abstract

Rhizobia are usually defined as nitrogen-fixing soil bacteria capable of inducing the formation of root stem nodules on leguminous plants in which atmospheric nitrogen is reduced to ammonia, is catalyzed by nitrogenase for the benefit of the plant. In the present investigation, fifty strains of *Rhizobium spp.* (R1.....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 (T_m). T_m of all the strains ranged between 94.3 to 95.7°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 REP-PCR, also the group differentiated with 25% similarity level into two groups in which one group with 100% similarity consists of R2 & R27 species. The least similar pattern with respect to the strains ignoring R2 and R27 maximum similarity upto 90% was seen between R21, R46, R23, and R48. On gel analysis of REP-PCR fingerprinting the bands appearing on gel also range between 100-900 bp, in the presence of 2% agarose. All restriction patterns of REP-PCR, fingerprints were coded in the binary form, and analyzed using NTSYS-pc package. 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.

1. Introduction

Biological nitrogen fixation represents the major source of nitrogen input in agricultural soil. The major nitrogen fixing systems which can play a significant role in improving the fertility and productivity of low-N soils (Zahran, 1999). Rhizobia are usually defined as nitrogen-fixing soil bacteria capable of inducing the formation of root stem nodules on leguminous plants in which atmospheric nitrogen is reduced to ammonia for the benefit of the plant. 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) and also from less explored legumes like different shrubs and herbaceous plants that have important role in certain ecosystem (Urtz et al., 1996; Laguerre et al., 1997; Khbaya et al., 1998; Jarabo-Lorenzo et al., 2000). The development of numerous molecular genetic methods has greatly contributed to these investigations. The availability of several sensitive and accurate PCR-based genotyping methods (Judd et al., 1993; Jensen et al., 1993; Selenska-Pobell et al., 1995) has enabled the differentiation among closely related bacterial strains and the detection of a higher rhizobial diversity than previous considered (Vinuesa et al., 1998; Doignon-Bourcier et al., 2000; Tan et al., 2001). Consequently, the taxonomy of root and stem nodulating bacteria has been deeply changed in the recent years. The rhizobial species are currently classified in the following genera: *Allorhizobium* (emended genus *Rhizobium*), *Mesorhizobium*, *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Bradyrhizobium* and *Methylobacterium* (van Berkum et al., 2000; Tighe et al., 2000; Young et al., 2001). DNA fingerprints can also be generated by using pairs of primers derived from repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences and the BOX element (Versalovic et al., 1994). It has been shown that REP and ERIC-like sequences are present in rhizobia and in other Gram-negative soil bacteria and that they can be used for bacterial taxonomy (Niemann et al., 1997; Di Giovanni et al., 1999; Sikora et al., 2002). The usefulness of DNA finger-printing by PCR using REP and ERIC primers for identification and classification of the members of several *Sinorhizobium* species was demonstrated (de Bruijn, 1992).

Rhizobia are taxonomically very diverse (Wolde-Meskel et al., 2004), efficient strain classification methods are needed to identify genotyping displaying, such as superior nitrogen-fixation capacity (Sikora et al., 2002). Molecular techniques have helped to develop easy and quick methods to microbial characterization including works distinguish genera, species and even strains (Schneider et al., 1996; Giongo et al., 2008). The polymerase chain reaction (PCR) and the use of primers corresponding to consensus repetitive sequences scattered in the eubacteria genome, thought as enterobacterial repetitive intergenic consensus (ERIC) and enterobacterial repetitive sequences (BOX) can create highly characteristic patterns when distinguished in

agarose gels, providing well separation on strain level (Adiguzel, A., 2006). ERIC sequences are highly protected among rhizobia genomes and to evaluate the environment effect in defined populations (Giongo et al., 2008).

The objective of this study was to isolate and characterize the rhizobial populations naturally associated agriculturally land originating from different ecological areas by a polyphasic approach including the evaluation of phenotypic properties as well as genotypic characteristics.

2. Material and Methods

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. 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 $28^{\circ} \pm 2^{\circ}\text{C}$ 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, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -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, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -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).

2.1. 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 $25^{\circ} \pm 1^{\circ}\text{C}$ 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.

2.2 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, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -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 $28^{\circ} \pm 2^{\circ}\text{C}$ 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 $26^{\circ} \pm 2^{\circ}\text{C}$. 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 $28^{\circ} \pm 2^{\circ}\text{C}$ 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.

2.3 DNA preparation

Total genomic DNAs from all strains were isolated using standard phenol-chloroform-isoamyl extraction and ethanol precipitation in the presence of sodium acetate (0.3 mol/L). The pellets were washed with 70 % ethanol, dried and redissolved in 150 μ L of TE buffer. The concentration and purity of DNA were estimated spectrophotometrically at 260 and 280 nm, respectively.

2.4 Determination of T_m value- The T_m 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 (T_m – 69.4) as suggested by (De Ley, J., 1970).

2.5 REP-PCR genomic fingerprinting

The 18 nucleotides in the REP primer make it specific primer for genomic DNA analysis. Both reverse primer; REP 1R (5' IIIICgIcATCIGgC-3') (Versalovic et Al., 1991) and forward primer REP 2I (5'-ICgICTTATCIGg CCTAC-3') (Versalovic et Al., 1991) amplifies the complementary sequence found in the genomic DNA varying in size were latter analyzed by gel electrophoresis. The master mix was prepared for all the 50 samples as mentioned below-Master Mix (25 μ L-1x) 5X Gitsschier buffer (5.0 μ L), BSA-20 mg/ml (0.2 μ L), DMSO-100% (2.5 μ L), Mix of dNTP's-2mM (1.25 μ L), Primer1 (1 μ L), Primer2 (1 μ L), Taq DNA polymerase-5U (0.4 μ L), ddH₂O (9.65 μ L), DNA template (4.0 μ L). 25 μ L of master mix is distributed PCR tubes and subject to the following temperature cycle for amplification (35 cycles). Initial denaturation (95° C for 7min), Denaturation (94° C for 1min), Annealing (40° C for 1min), Extension (72° C for 1min), Final Extension (72° C for 16 min), Final hold (4° C for 0 Sec).

2.6 Electrophoresis of REP PCR product

Agarose of 2% (W/V) was used for the PCR product separation at 60mV at 25° \pm 1°C for 6 hours. The amplified product is then analyzed under gel documentation unit (Vilber Lourmat, FRANCE, CN-UV/WL), after electrophoresis at 2% agarose gel (Fig.1).

2.7 Data analysis

All restriction patterns as well as REP-PCR, ERIC-PCR, BOX-PCR fingerprints were coded in the binary form, and analyzed using NTSYS-pc package (Rohlf, F J., 1990). 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.

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. 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 glistening 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 (T_m). 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 T_m ranged between 94.3 to 95.6°C. However % G+C content of isolated strains range between 60.7 to 63.9%. All the bacteria tested and examined for T_m 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 patterns of the resulting PCR products were found to be highly specific for each strain, suggesting that the REP and ERIC-PCR method is useful for the identification and classification of bacterial strains since they allow the fingerprinting of individual genera, species and strains and help to determine phylogenetic relationships.

All DNA fingerprinting methods used in this study revealed high level of genetic diversity among inoculants strains and field isolates. Genotyping of REP-PCR also the group differentiated with 25% similarity level into two groups in which one group with 100% similarity consists of R2 & R27 species. The least similar pattern with respect to the strains ignoring R2 and R27 maximum similarity upto 90% was seen between R21, R46, R23, R48 (Fig. 2). On gel analysis of REP-PCR fingerprinting the bands appearing on gel also range between 100-900 bp, in the presence of 2% agarose.

4. Conclusion

Research techniques described above are instances of the methods which have been extensively used in estimating phenotypic and genotypic diversity of soil bacteria. These techniques provide a different level of perspective to interpret the phenotypic and genotypic variations amongst legume symbionts. Most studies employed the combination of several methodologies to characterize and to examine genetic relationships of these specific groups of bacteria. The ranges of discriminating power and respective levels of resolution and limitations have been evaluated.

All *Rhizobium* strains used in this study will be further characterized for their symbiotic properties in order to select the highest quality and the most suitable strains for soybean inoculation under particular agroecological conditions.

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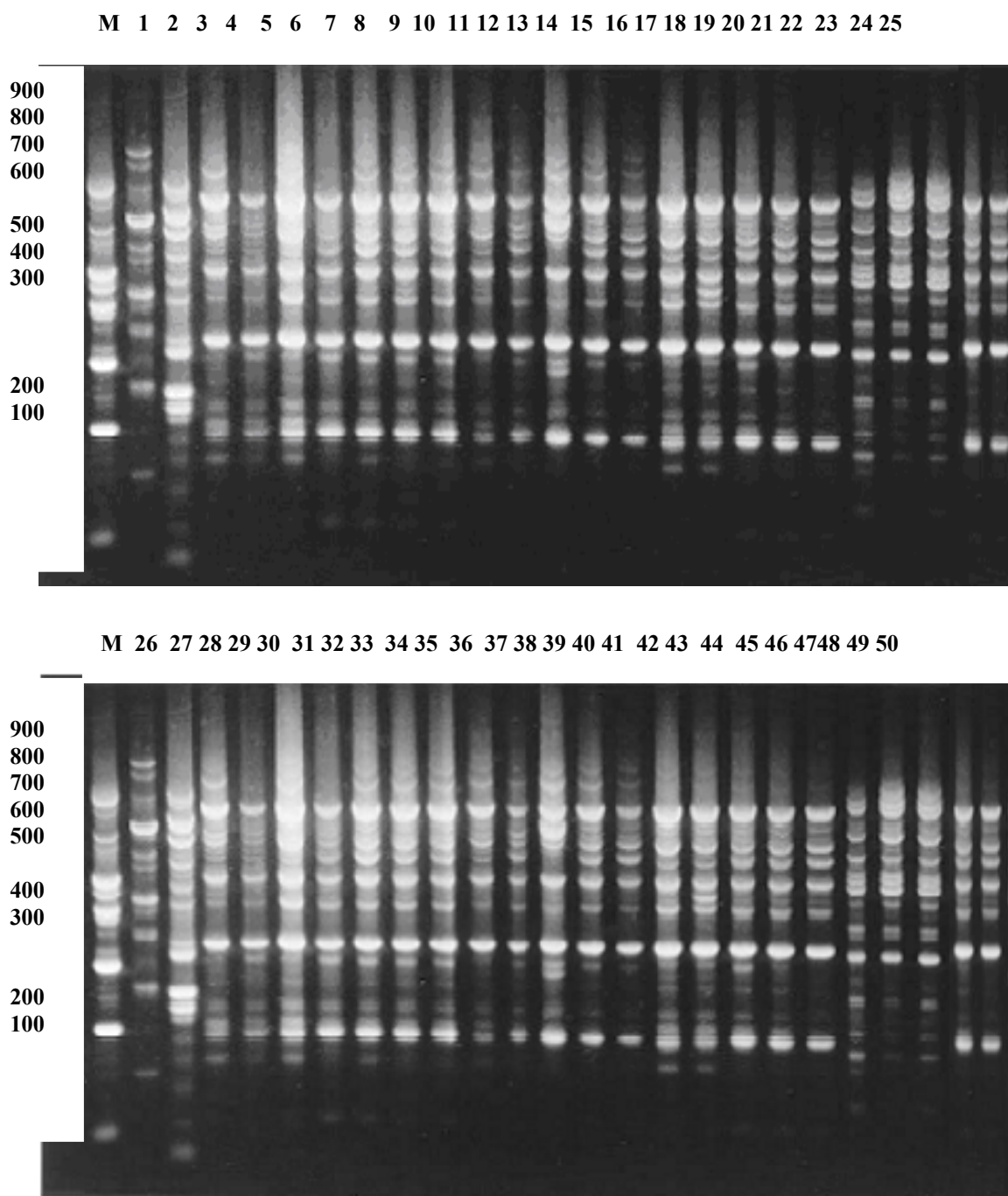


Fig: 1 REP- PCR fingerprint of *Rhizobium* strains isolated from agricultural field of M.P.

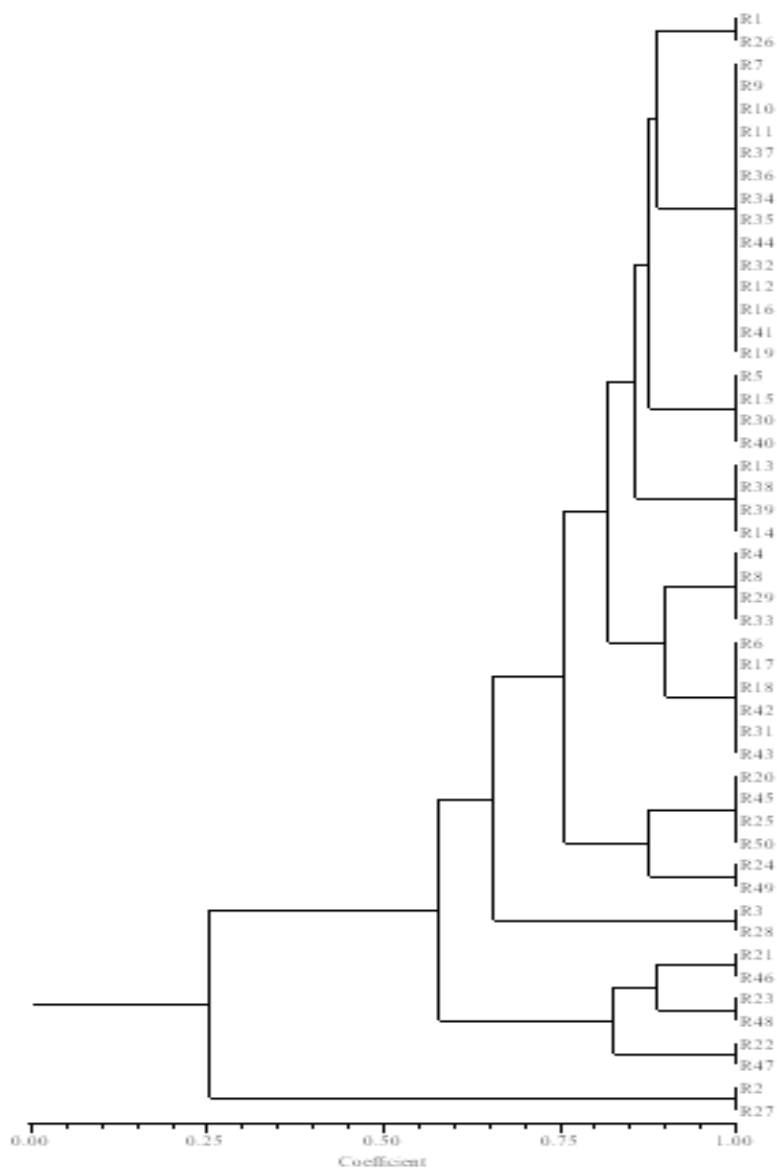


Fig: 2 Dendrogram based on REP PCR of *Rhizobium* isolates showing genetic relatedness using Jaccard's similarity coefficient and UPGMA cluster method

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