Genetic Diversity of Rhizobial isolates revealed by PCR-RAPD fingerprinting and the data were analyzed using POPGEN

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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. The study aimed at investigating the genetic diversity of fourteen different rhizobial isolates using PCR-RAPD fingerprinting and the data were analyzed using POPGEN. Total genomic DNAs from different field isolates were amplified using five different arbitrary primers (OPA02, OPA03, OPM17, OPP07 and OPP08), the amplified DNA solution was subjected to agarose gel electrophoresis. Around 600 different bands (558 polymorphic) were produced from 14 isolates showing 93% of overall polymorphism. All the five primers produced polymorphic bands with OPA03 and OPP08 registering one hundred per cent polymorphic bands. Among these two, only OPP08 produced unique bands (2.30%). The percentage of monomorphic bands produced by OPA02, OPM17 and OPP07 were 9.65, 16.66 and 17.07 respectively. Similarly the percentages of unique bands for the above primers were 2.06, 1.19 and 2.43. Resolving power (Rp) of the primers ranged from 1.84 (OPM17) through 1.86 (OPA03, OPP07, OPP08) to 1.90 (OPA02). Summary of genetic variation statistics for all loci produced by five random primers using POPGEN version 1.31software reveal that primer OPA03 is more effective in highlighting the variation between the isolates. Nei's unbiased measures of genetic identity and genetic distance were used to construct a similarity matrix and a rectangular cladogram was constructed based on UPGMA analysis. Ewans-Watterson test for neutrality confirms the existence of genetic diversity in all the polymorphic loci indicating their neutral nature with regard to evolution of the isolates.

Keywords: - *Rhizobia, DNA profiles, RAPD, Genetic diversity, POPGEN.*

I. Introduction

Biodiversity is an important ingredient of environmental conservation and is central to agriculture production. Most microbial diversity of the soil ecosystem is confined to the rhizosphere. Rhizodeposition through plant root exudates play a major role in defining resident microflora, which differs from that in bulk soil (Lynch, 1990). Rhizobacterial diversity is influenced by both plant and soil type. Since root exudation is species-specific, it is a major factor that determines community composition within the rhizosphere (Chiarini *et al.*, 1994, Griffiths *et al.*, 1993 and Mahaffee and Kloepper, 1997). Assessment of environmental impact on microbial diversity association with human land-use practices, and application of chemical fertilizers and industrialization, are important issues in working towards sustained plant production systems (Kuske *et al.*, 2002). Among the existing practices, biological fertilizer is of utmost importance in agriculture production. Use of soil microorganisms which can fix atmospheric nitrogen, solubilize phosphorus, or stimulate plant growth through synthesis of growth-promotory substances has gained importance over the use of chemical fertilizers on account deleterious effect of the latter on soil and plant health (Glick,1995 and Thomashow *et al.*, 1997).

Rhizobia are usually defined as nitrogen fixing soil bacteria capable of forming root and stem nodules on leguminous plants, fixing atmospheric nitrogen and reducing it to ammonia for the benefit of the plant. Due to their considerable agricultural and environmental significance, these legume symbionts have been extensively studied. Knowledge of

biodiversity is essential not only for environmental conservation but also for improving agricultural production. Microbial diversity is considered as one of the most useful resources for bioprospecting. Understanding rhizobial diversity at molecular level could have a profound implication in agriculture. In the recent past, the assessment of diversity within natural populations of rhizobia in different parts of the world have received considerable attention (Madrzak *et al.*, 1995; Chen *et al.*, 2000).

However, the development and increased availability of molecular biology techniques have made it possible to obtain information regarding the genomic organization and diversity of rhizobia populations in different soils (Amarger *et al.*, 1994, Nour *et al.*, 1994, Laguerre *et al.*, 1996, Pinto *et al.*, 1998 and Oliveira *et al.*, 2000). Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between very closely related bacteria. The RAPD technique is a polymerase chain reaction (PCR) based assay that was developed to detect polymorphisms in genomic DNA (Welsh and McClelland, 1990 and Williams *et al.*, 1990). Besides being simpler and cheaper, this method is as effective as the more labor intensive RFLP for establishing genetic relationships and identifying Rhizobium strains (Laguerre *et al.*, 1996 and Selenska-Pobell *et al.*, 1996). Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) has been shown to be useful to differentiate very closely related strains (Fremont *et al.*, 1999, Mathan *et al.*, 1996, Young and Cheng *et al.*, 1998).

The characterization of rhizobial isolates from different soil samples were collected from Thanjavur district. Based on the soil type, Thanjavur district has already been classified into fourteen different soil series. From the 14 different soil series, the rhizobia would be isolated and characterized based on morphological, physiological, biochemical and molecular studies (Rajaekaran *et al.*, 2015). The present study also focuses on the diversity within the rhizobial populations isolated from different soil series of Thanjavur district.

In this study, evaluation of the genetic diversity of *rhizobium* strains from different regions of the Thanjavur district that are able to associate with *V. mungo* were performed using genomic patterns obtained by randomly amplified polymorphic DNA **(**RAPD) fingerprinting and the data were analyzed using POPGEN with the objective of identifying genetic groupings (clusters) of the strains.

II. Materials and methods

A. PCR-RAPD finger printing

a) Isolation of Genomic DNA (El-Fiki, 2006)

For total DNA isolation, culture of rhizobial isolates were grown for 4 days at 28°C in yeast mannitol medium (Vincent, 1970). Cells were pelleted by centrifugation at 3000 rpm for 10 min, washed several times and resuspended in TEN buffer (50 nM 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 dodecyle sulphate (SDS) and protinase K were then added to 1 per cent 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 precipitated in 70 per cent ethanol, dried and redissolved in 150 µl of TE buffer.

B. RAPD (Dooley et al., 1993)

A simple primer approximately 10bp in size (40-70% G+C content) is generally used in PCR fingerprinting (Dassanayake and Samaranayake, 2003). Amplifications using five different 10-mer synthetic oligonucleotide RAPD primers such as OPA02 (5'-TGCCGAGCTG-3'), OPA03 (5'-AGTCAGCCAC-3'), OPM17 (5'-TCAGTCCGGG-3'), OPP07 (5'-GTCCATGCCA-3') and OPP08 (5'-ACATCGCCCA-3') were made to perform the RAPD analysis of test isolates on an Eppendroff master cycler gradient 5331. RAPD amplification mixture consisted of 75 µl of sterile water, 10 µl of 10× assay buffer, 7.5 µl of 10 mM deoxynucleotide triphosphates, 5 µl of random primer {100 ng/µl}, 2.5 µl of *Taq* DNA polymerase, 1 µl of template DNA and 50 µl of mineral oil. The master cycler was set at 94°C for 5 min for initial denaturation cycle, followed by 40 cycles of 60 seconds duration for denaturation at 94°C, 60 seconds for annealing at 34°C and 120 seconds for extension at 72°C with a final extension for 10 min duration at 72°C. The amplified products were separated by gel electrophoresis on precast 1.2% agarose by loading 12 µl and visualized using UV transilluminator after staining with ethidium bromide (Sambrook *et al.*, 1989). The banding pattern among the 14 different test isolates is based on the PCR product obtained with 5 different 10-mer RAPD primers such as OPA02, OPA03, OPM17, OPP07 and OPP08. The sizes of amplified bands were calculated by two type of markers such as 100bp ladder and 500 bp ladder.

C. Data analysis

Differences in fingerprinting patterns between isolates were assessed visually. 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. Only sharp and clear bands were scored and Microsoft excel software was used

for this purpose. From the fingerprint data, the following parameters were calculated for each primer. Percentage of G + C content in the primer (Caetano-Anolles *et al.*, 1992). Percentage of polymorphic bands (PPB) = No of polymorphic bands / Total No. of bands × 100 (Mathan *et al.*, 1996). Percentage of monomorphic bands (PMB) = No. of monomorphic bands / Total No. of bands × 100. Percentage of unique bands (PUB) = No. of unique bands / Total No. of bands × 100. (Garg *et al.*, 2009). Resolving power (R_P) (Prevost and Wilkinson, 1999) $R_P = \Sigma Ib$, where Ib (Band informativeness) take the value of 1 – (2 x (0.5 – P)) and P = Proportion of isolates containing the band.

D. Evaluation of RAPD data using POPGENE version 3.1

POPGENE is user-friendly windows based computer package for the analysis of genetic variation among and within natural populations using Co-dominant and dominant markers and quantitative traits. It performs most types of data analysis encountered in population genetics and related fields (Yeh and Boyle, 1997; Yeh *et al.*, 1997). The following data were generated using POPGENE viz., observed number of alleles, effective number of alleles (Kimura and Crow, 1964; Hartl and Clark, 1989), Nei's gene diversity (Nei's, 1973), Shannon's information index (Shannon's and Weaver, 1949; Lewontin, 1972), Number of polymorphic loci, percentage of polymorphic loci and Ewens-Watterson Test for neutrality (Manly, 1985). POPGENE is also used to calculate Nei's unbiased measures of genetic identity (Nei's, 1972) and genetic distance (Nei, 1978) between isolates based on combined data for five primers and a similarity coefficient matrix was constructed. This matrix was subjected to the unweighed pair group method for arithmetic average analysis (UPGMA) in order to generate a rectangular cladogram of the different rhizobial isolates using TREEVIEW Software (Page, 1996).

III. Results

A. Genomic DNA Profile

Genomic DNA Profile of 14 different rhizobial isolates from 14 soil series (Table 1) was carried out by 1% agarose gel electrophoresis with ethidium bromide and visualized under UV illumination. Each isolate showed single genomic DNA in the form of bands with almost uniform intensity.

B. PCR-RAPD finger printing

After Amplification, 12 µl of PCR products from 14 different rhizobial isolates were loaded on 1.2% agarose gel with ethidium bromide and viewed under UV-light. The electrophoretic pattern for the 14 different test isolates is shown in Figure 1-5.

C. Scoring and Data analysis

The amplified bands were in the range of 300 bp to 4750 bp (0.3 to 4.75kg. bp). Considering all the primers and rhizobial isolates, a total of 600 bands were produced from 14 strains, out of which 558 were polymorphic showing 93% of overall polymorphism. Number of bands produced per genotype (i.e., isolate) ranged from 30 to 53 with an average of 43 bands per genotype. Among the isolates, maximum number of bands were produced by Pdg3 (53; 50 polymorphic) while minimum by Alg10 (30; 27 polymorphic) (Table 2-4). The number of bands produced by various primers were in the range of 82 (OPP07) to 159 (OPA03) with an average of 120 bands per primer. The total number of monomorphic bands (NMB) produced by 5 random primer was 42 (7%) with a mean of 8.4 per primer. Out of 5, only 3 viz., OPA02, OPM17 and OPP07 produced monomorphic bands of equal number. However, the percentage of monomorphic bands (PMB) produced by 3 different primers showed variations such as 9.65% (OPA02), 16.66% (OPM17) and 17.07% (OPP07). The total number of unique bands produced by 5 random primers was 9 (1.5%) with an average of 1.8 per primer. The number of unique bands (NUB) produced per primer was 0 (OPA03) to 3 (OPA02 and OPP08). The percentage of unique bands (PUB) per primer ranged from 0 to 2.43%. The primers used in the study had G+C content ranging from 60 (OPA03, OPP07, OPP08) to 70 (OPA02 and OPM17). The resolving power of the primers varied from 1.84 (OPM17), 1.86 (OPA03, OPP07 and OPP08) to 1.90 (OPA02) (Table 5; Fig.6-7).

D. Evaluation on RAPD data by POPGENE Version 1.31 Software

The banding pattern of the gels for the rhizobial isolates were scored manually and transferred to the present/absent scale (1 or 0 for each allele and genotype). These data were subjected to analysis using POPGENE version 1.31software. Summary of genetic variation statistics for all loci as revealed by the five different primers are presented in table 19. Random primer OPA03 was able to reveal a higher percentage of polymorphic loci indicating higher genetic diversity among the rhizobial isolates. The observed number of alleles (na) produced by 5 different primers range from 1.4074 ± 0.5007 (OPM17) to 1.8519 ± 0.3620 (OPA03). The effective number of alleles (ne) produced by 5 different primers range from 1.2019 ± 0.3242 (OPM17) to 1.5963 ± 0.3218 (OPA03). The gene diversity (h) range from 0.1224 ± 0.1776 (OPM17) to 0.3428 ± 0.1624 (OPA03) and the Shannon's information

Index (I) range from 0.1891 ± 0.2585 (OPM17) to 0.5021 ± 0.2274 (OPA03) (Table 6).

The total number of polymorphic loci produced by 5 different primers was 91 (67.40%) with a mean of 18.2 per primer. The range of polymorphic loci (NPL) produced per primer was 11 (OPM17) to 23 (OPA03). The percentage of polymorphic loci (PPL) in the 14 rhizobial isolates as revealed by the various primers were in the range of 40.74 (OPM17) to 85.19% (OPA03) (Table 6). Similarity coefficient matrix for Nei's unbiased measures of genetic identity and genetic distance between isolates calculated based on combined data for five primers is presented in table 7. Genetic identity between the isolates ranged from 0.8998 (Pvr9 and Alg10) to 1.0365 (Mdk1 and Adn5). The genetic distance among the isolates varied from -0.0008 (Mdk1 and Klt2) to 0.0819 (Alt7 and Pvr9). Further UPGMA of similarity matrix used to generate a cladogram grouped the rhizobial isolates as shown in fig.8. From the cladogram the following groups could be recognized.

Group I: Mdk1, Klt2 and Mud8

Group II: Alt7, Bdl11 and Klv13

Group III: Pdg3, Pkt4 and Adn5

Group IV: Mlk12, RF14 and Alg10

Isolates Vlm6 and Pvr9 did not fall under any group.

E. Ewens-Watterson test for neutrality

Ewens-Watterson neutrality test was performed to test the neutrality of all loci produced by all the primers. The statistics for the test were calculated using 1000 simulated samples. The result suggested that the F value (sum of square of allelic frequency) for entire test loci lie within the lower and upper limit of 95% confidence region in all the primers indicating that all the loci were neutral to selection. The results of Ewens-Watterson test for neutrality elucidated using POPGENE version 1.31 software, revealed that in all the polymorphic loci, the observed sum of the squared allelic frequency for the various polymorphic loci varied between 0.5000 to 0.7551, which confirms the existence of genetic diversity among the rhizobial isolates of Thanjavur district. Neutrality test results have evolutionary significance (Table 8 - 12).

IV. Discussion

Knowledge of biodiversity is essential not only for environmental conservation but also for improving agricultural production. Rhizobia are relatively unique among the majority of soil microorganisms, in that they have an extensive soil phase as free living saprophytic, heterotrophic microorganisms, yet in conjunction with leguminous plants they have the ability to form species specific N_2 fixing symbiotic associations. In the present study, diversity of the rhizobia present in soil of Thanjavur district has been discussed in terms of their morphological, biochemical, molecular characteristics besides, their population structure and agronomic influence.

A. PCR-RAPD analysis

Recently, studies have aimed to uncover the nature of rhizobial symbionts in their native environments as it has been recognized that one of the major problems in the application of BNF technology is the establishment of inoculant strains. Several factors are responsible for this problem, including competition that occurs between the inoculated strains and those already present in the soil, which generally have low effectiveness. Competitiveness studies that have been conducted to select bacterial genotypes have been limited due to problems in identifying strains that nodulate legumes. In those organisms, similar antigenic structures (cross-reactivity) are common as seen through serologic tests or classic identification methods (Schmidt *et al.*, 1968). However, the development and increased availability of molecular biology techniques have made it possible to obtain information regarding diversity and population structure of bacteria. The 16S rRNA gene sequences are an indispensable parameter in *Rhizobium* taxonomy and methods based on differences in ribosomal RNA genes have been frequently applied to species identification (Laguerre *et al.*, 1994; Ganesh kumar, 2008). Nevertheless, the conservative nature of 16S rRNA genes limits its use for discrimination at the strain level. The intergenic spacer between 16S and 23S rRNA genes was described to be more variable (Massol-Deya *et al.*, 1995). The development of PCR led to new fingerprinting methods RFLP of PCR amplified IGS was used for characterization of *Rhizobium* strains (Nour *et al.*, 1994; Selenska-Pobell *et al.*, 1996; Sessitsch *et al.*, 1997). Arbitrary oligonucleotide PCR primers of random sequences (RAPD) have been used to generate strain specific fingerprints of *Rhizobium* (Selenska-Pobell *et al.*, 1995; Mathan *et al.*, 1996; Paffetti *et al.*, 1996; Oliveira *et al.*, 2000; Pinto *et al.*, 2004; El-Fiki, 2006; Rajasundari *et al.*, 2009). PCR analysis identified adequate primers for strain identification and this was evident from the studies of Olivera *et al.* (2000) and Pinto *et al.* (2004). The RAPD technique is a PCR based assay that was developed to detect polymorphisms in genomic DNA (Welsh and McClelland, 1990; Williams *et al.*, 1990). Besides being simpler and cheaper, this method is as effective as the more labour intensive RFLP for establishing genetic relationships and identifying *Rhizobium* strains (Laguerre *et al.*, 1996; Selenska-Pobell *et al.*, 1996).

PCR fingerprinting of DNA enables the amplification of characteristic fingerprints of DNA bands from bacterial genomes. The precision and reproducibility of these fingerprints allow perfect discrimination between different strains of the same species of *Rhizobium*. These patterns are known to be stable and conserved even in clonal descendants of the same strain that had been propagated independently for some years (Versalovic *et al*., 1994; Teaumroong and Boonkerd, 1998). Therefore, patterns of DNA (fingerprints) are excellent tools for verification and identification of strains. In the present study also RAPD profiles were generated for the rhizobial isolates from different sites representing fourteen soil series of Thanjavur district, since the traditional methods viz., morphological, physiological, biochemical could not distinguish the isolates from various sites. At least 3 isolates from each site were analyzed for their RAPD profile.

For generating RAPD profile, Dassanayake and Samaranayake (2003) reported that a single primer of approximately 10 bp size having 40-70% G+C content is used. The primer used in the study were all decamer primers (i.e., 10 bp size) having a G+C content ranging from 60-70%. Madthan *et al.* (1996) investigated the root nodulating strains from *Arachis hypogaea* isolated from eight farm sites for their phenotypic features and RAPD profiles of the genomic DNA. The intrasite RAPD profile did not show any variation with respect to the number and position of amplification products. However, between the sites, when the RAPD profiles were generated for 11 random decamer primers, the percentage of polymorphic products ranged from 76.5 to 100. No primer showed a uniform profile for all the genotypes analysed and most of the genotypes could be identified using one or two primers. Pinto *et al.* (2004) characterized Rhizobia that nodulate *Arachis pinati* by RAPD analysis. In their study also, the RAPD patterns generated were not uniform and the percentage of polymorphic products ranged from 67 to 100 depending on the primer used.

In the present study, the percentage of polymorphic products ranged from 82 to 100 and none of the primers showed a uniform profile for all the genotypes (isolates) tested and most of the genotypes (isolates) could be identified using one or two primers. Thus the present study also produced results with regard to variation among the rhizobial isolates, in conformity with the trend reported earlier by Mathan *et al.* (1996), Oliveira *et al.* (2000), Pinto *et al.* (2004) and Rajasundari *et al.*, (2009). Out of the 5 primers used in the present investigation four (OPA02, OPM17, OPP07 and OPP08) of them revealed unique bands. These unique bands may be useful in detecting mixes between isolates as was reported by Fernandez *et al.* (2002) for detecting mixes between cultivars.

Molecular genetic markers (RAPD, SSR, RFLP, AFLP) have become increasingly available in a variety of plant species and will likely continue to do so (Westman and Kresovich, 1997). They can be used to examine a group of individuals or populations to estimate various diversity measures and genetic distances, infer population structure and clustering patterns, test polymorphic loci for evidence of selective neutrality. Studying variation at marker loci allows genetic classification of populations and can lend insight into their history and inherited changes during their improvement (Labate, 2000). Many software programmes for molecular population genetic studies have been developed for personal computers such as Tools for Population Genetic Analyses (TFPGA). Arleguin, GDA, GENEPOP, Genestrut, POPGENE etc. They have the advantage of accommodating a variety of molecular marker types and perform many different types of analyses.

As already mentioned, RAPD marker has been used in the present investigation to study the variation among the rhizobial isolates of Thanjavur district. From the RAPD profile, data were generated and analysed using POPGENE program. The data generated include summary of genetic variation statistics for all loci produced by five different primers, neutrality test of the various loci, Nei's genetic identity and genetic distance. Genetic variation statistics includes data regarding observed number of alleles, effective number of alleles, gene diversity, Shannon's Information index regarding genotypic diversity, percentage of polymorphic loci etc. Knowing species genetic diversity helps in understanding their evolution. Presence of higher percentage of polymorphic bands among the test isolates was suggestive of high genetic variability and this was evident from the work of Gouveia *et al.* (2005) on genetic diversity of *Hemileia vastatrix* based on RAPD markers. Polymorphisms can be detected by using various random primers. The primer that produces higher percentage of polymorphism with better resolving power can be used to discriminate the isolates. Accordingly, OPA03 is the primer that can be used to distinguish the rhizobial isolates in the present study. The gene diversity and Shannon's Information index value for the various loci observed with this primer are also maximum thus justifying its usefulness.

Compared to gene diversity, the Shannon's Index values observed were higher in all the primers tested (Table 6). This suggests that the genetic diversity is distributed mainly among clonal lineages and this view gains support from the work of Gouveia *et al.* (2005). Accordingly, it may be inferred that all the rhizobial isolates in the present study could be descendents of clonal lineage. However this view needs to be strengthened with actual field study.

Judging from the cladogram (Fig.8) generated using similarly matrix data, the isolates Vlm6 and Pvr9 could be considered as genetically unique. Isolates in groups I, II and III may be considered as descendants of the same clone. In group IV, the isolates may represent different clonality. However, this inference needs to be substantiated. Gouveia *et al.* (2005) while carrying out genetic diversity studies on *Hemileia vastatrix* isolates based on RAPD markers, reported that high genetic similarity could be noticed between isolates from different geographical regions. In the present study also the isolates from different geographical areas of Thanjavur district showed high genetic similarity indicating that the sites did not have any direct influence on the grouping of strains. However, there are also reports indicating the direct influence of the site on the grouping of strains (Mathan *et al.*, 1996; Handley *et al.*, 1998; Young and Chang, 1998; Fremont *et al.*, 1999).

Population genetics is the study of allele frequency distribution and change under the influence of four main evolutionary processes: natural selection, genetic drift, mutation and gene flow. A population is a group of organisms of the same species living within a prescribed geographical area. Geographically widespread species are often subdivided into subpopulations. The complete set of genetic information contained within the individuals in a population is called gene pool, which includes all genetic loci and all the alleles for each locus present in the population. The gene pool of a population can be described and characterized by certain statistical properties such as allele frequency (gene frequency), polymorphism etc. Polymorphism refers to the proportion of all genetic loci that exist in more than one allelic forms. It can be measured as a measure of genetic variation within populations.

Applying the above concepts to the present study, an attempt has been made to understand the process of evolution among the rhizobial isolates of Thanjavur district. One definition of evolution is "Allele frequency change over time". Polymorphism can be used as a measure of genetic variation within populations. The percentage of polymorphic loci that could be detected among rhizobial isolates in the present study ranged from 40.74 to 85.197 with different primers. One of the factors responsible for genetic variation is mutation. Mutation can alter the allele frequency in a locus. The impact of the change in the allele frequency of rhizobial population was tested using Ewens-Watterson test for Neutrality which has evolutionary significance. The results suggested that all the loci were neutral to selection as the value of allelic frequency lied within the lower and upper limit of 95% confidence region in all the primers (Table 8 - 12).

Genetic variation at the molecular level commonly is associated with neutral changes in gene sequences. According to Neutral theory of evolution proposed by Motoo Kimura, the genetic variation (re-polymorphism) observed in natural populations is due to the accumulation of neutral mutations. Neutral mutations do not affect the phenotype of the organism. As neutral alleles are not acted on by natural selection, the change in the genetic makeup caused by forces such as mutation did not have any effect leading to the evolution of isolates in the population to the next stage. That is why all the isolates of the present study from the different soil series of Thanjavur district are treated under a single genus viz., *Rhizobium*. From the foregoing discussion it is clear that rhizobial isolates representing various soil series exhibit diversity in many respects such as morphological, physiological, metabolic, ecological, genetic and molecular characteristics. Among them the isolate Pdg3 is found to be more efficient compared to other isolates.

One of the most fascinating and attractive aspects of the world is its extraordinary diversity. It seems that almost every possible experiment in shape, size, physiology and life-style has been tried (Prescott, 2005). The present study which focused on the diversity of rhizobial population of Thanjavur district stands as a testimony to the above statement. *Rhizobium* is a prokaryotic bacterial species. A prokaryotic species is a collection of strains that share many stable properties and differ significantly from other groups of strains. A strain is a population of organisms that is distinguishable from atleast some other populations within a particular taxonomic category. It is considered to have descended from a single organism or pure culture isolate (Prescott, 2005).

All the genes in a population make up the gene pool. In this regard, each member of the population is viewed as receiving its genes from the gene pool. Population geneticists study the genetic variation within the gene pool and the causes of observed patterns of genetic variation within and among populations. In so doing, it seeks to explain the underlying genetic basis for evolutionary change. The genetic structure of a population is described by the number and frequencies of genotypes at each locus. The genetic structure of a species can vary both geographically and temporally. Mutation, genetic drift, migration and natural selection are processes that can alter allele frequencies of a population.

The larger a population, the more potential there is for a novel mutation to arise, but mutation by itself change allele frequencies only a little. Genetic drift, a chance change in allele frequency caused by sampling error can have important evolutionary and survival implications for small populations. Genetic

drift leads to loss of genetic variation within populations, genetic divergence among populations, and random fluctuation in the allele frequencies of a population over time. Genetic drift can also explain how molecules in different species accumulate differences in a seemingly regular basis, forming the basis for the neutral theory of molecular evolution.

Genetic variation at the molecular level commonly is associated with neutral changes in gene sequences. In 1968, Motoo Kimura from Japan proposed the neutral theory of molecular evolution. According to this theory, a new mutation that is not selectively disadvantageous but neutral with respect to natural selection initially will be at a low frequency. Therefore, it most likely will be lost from the population as a result of genetic drift. Occasionally, however, by chance alone, the new mutation will drift to fixation and the gene will have evolved. Such neutral mutations do not affect the phenotype of the organism and the neutral alleles are not acted on by natural selection (Peter and Russel, 2006).

The genetic structure of rhizobial isolates in the present investigation revealed the existence of polymorphism to a considerable extent which could be the result of altered allele frequencies. Ewans neutrality test clearly indicates that the altered allele frequencies are neutral in nature without affecting the phenotype of the organism and hence all the isolates of *Rhizobium* from the various soil series could be considered as descendants from a single organism. Though the variation might not have been evolutionarily significant, it gives an insight into the effectiveness of a particular isolate so that it can be exploited by inoculant industry.

CONCLUSION

Rhizobial isolates representing the various soil series of Thanjavur district exhibit diversity in many respects including the genetic structure. The existence of polymorphism in the rhizobial population in the present study could be attributed to altered allele frequencies and the same is evident from the evaluation of RAPD fingerprinting data using POPGENE software. However, the altered allele frequencies are neutral in nature as per the results of Ewans neutrality test and this has not resulted in any change in the phenotype of the organism. Hence, all the isolates could be considered as descendants from a single organism through genetic variation which gets exemplified in the form of structural and functional diversity. Though the variation might not have been evolutionarily significant, it gives an insight into the effectiveness of a particular isolate that can be exploited by inoculant industry.

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TABLE 1. Rhizobia isolated from different soil series

S. No.	Soil series	Place of collection	Code Name of isolates
1.	Madukkur	Kattuthottam	Mdk1
2.	Kalathur	Thittai	Klt2
3.	Padugai	Mathur	Pdg3
4.	Pattukkottai	Kudikadu	Pkt4
5.	Adonur	Thandangorai	Adn5
6.	Vallam	Achampatti	Vlm6
7.	Alathur	Sorakudipatti	Alt7
8.	Mudukulam	Vilar	Mud8
9.	Peravurani	Puthu Parapanar Vayal	Pvr9
10.	Alangudi	Annappanpettai	Alg10
11.	Budalur	Budalur	Bdl11
12.	Melkadu	Parakalakottai	Mlk12
13.	Kollivayal	Nadiyam	Klv13
14.	Reserved Forest	Adirampattinam	RF14

TABLE 2. Details of amplified genomic DNA profile

S.No.	Details	
1.	Number of random primers used	5
2.	GC %	60 to 70
3.	Total number of bands	600
4.	Size range (bp)	300 bp to 4750 bp
5.	Total number of polymorphic bands	558
6.	Total number of monomorphic bands	42
7.	Total number of unique bands	9
8.	Percentage of polymorphic bands	93
9.	Percentage of monomorphic bands	7
10.	Percentage of unique bands	1.5
11.	Average number of bands per primer	120
12.	Average number of polymorphic bands per primer	111.6
13.	Average number of monomorphic bands per primer	8.4
14.	Average number of unique bands per primer	1.8
15.	Total resolving power	9.32
16.	Total number of loci	135
17.	Total number of polymorphic loci	91
18.	Percentage of polymorphic loci	67.40
19.	Average number of polymorphic loci	18.2

TABLE 3. Total number of bands detected from rhizobial isolates by five different random primers

S. No.	Random primers	Sequence of the random primers	Rhizobial isolates code name														
			Mdk1	Klt2	Pdg3	Pkt4	Adn5	Vlm6	Alt7	Mud8	Pvr9	Alg10	Bdl11	Mlk12	Klv13	RF14	Total
1.	OPA02	5′-TGCCGAGCTG-3′	12	6	12	10	12	11	12	10	9	9	10	10	10	12	145
2.	OPA03	5′-AGTCAGCCAC-3′	15	12	15	10	12	10	11	14	12	8	9	9	10	12	159
3.	OPM17	5′-TCAGTCCGGG-3′	7	6	6	8	8	8	4	4	8	5	5	4	5	6	84
4.	OPP07	5′-GTCCATGCCA-3′	7	7	10	10	7	7	6	5	8	2	4	3	3	3	82
5.	OPP08	5′-ACATCGCCCA-3′	10	7	10	13	13	11	7	8	9	6	10	7	10	9	130
		Total	51	38	53	51	52	47	40	41	46	30	38	33	38	42	600

TABLE 4. Total number of polymorphic bands detected from rhizobial isolates by five different random primers

S. No.	Random primers	Sequence of the random primers	Rhizobial isolates													
			Mdk1	Klt2	Pdg3	Pkt4	Adn5	Vlm6	Alt7	Mud8	Pvr9	Alg10	Bdl11	Mlk12	Klv13	RF14	Total
1.	OPA02	5′-TGCCGAGCTG-3′	11	5	11	9	11	10	11	9	8	8	9	9	9	11	131
2.	OPA03	5′-AGTCAGCCAC-3′	15	12	15	10	12	10	11	14	12	8	9	9	10	12	159
3.	OPM17	5′-TCAGTCCGGG-3′	6	5	5	7	7	7	3	3	7	4	4	3	4	5	70
4.	OPP07	5′-GTCCATGCCA-3′	6	6	9	9	6	6	5	4	7	1	3	2	2	2	68
5.	OPP08	5′-ACATCGCCCA-3′	10	7	10	13	13	11	7	8	9	6	10	7	10	9	130
		Total	48	35	50	48	49	44	37	38	43	27	35	30	35	39	558

TABLE 5. Fingerprint details of rhizobial isolates as revealed by five different random primers

S. No.	Random primers	Sequence of the random primers	GC %	NB	NB Size range (bp)	NPB	NMB	NMB Size range (bp)	NUB	NUB Size rnage (bp)	PPB	PMB	PUB	Rp
1.	OPA02	5′-TGCCGAGCTG-3′	70	145	600-4250	131	14	3000	3	850, 1250, 3750	90.34	9.65	2.06	1.90
2.	OPA03	5′-AGTCAGCCAC-3′	60	159	500-4750	159	0	-	0	-	100	-	-	1.86
3.	OPM17	5′-TCAGTCCGGG-3′	70	84	900-4500	70	14	1500	1	4000	83.33	16.66	1.19	1.84
4.	OPP07	5′-GTCCATGCCA-3′	60	82	500-4000	68	14	500	2	3250, 3500	82.92	17.07	2.43	1.86
5.	OPP08	5′-ACATCGCCCA-3′	60	130	300-4250	130	0	-	3	300, 400, 4000	100	-	2.30	1.86

GC% : Guanine and Cytosine percentage, NB: Number of bands, NPB: Number of polymorphic bands, NMB: Number of monomorphic bands, NUB: Number of unique bands, PPB: Percentage of polymorphic bands, PMB: Percentage of monomorphic bands, PUB: Percentage of unique bands, Rp: Resolving power.

TABLE 6. Summary of genetic variation statistics for all loci produced by five different random primers using POPGENE Version 1.31

S.No.	Random primers	Sequence of the Random primers	TNL	na* !(M ± Sd) (n = 27)	ne* !(M ± Sd) (n = 27)	h* !(M ± Sd) (n = 27)	I* !(M ± Sd) (n = 27)	NPL	PPL
1.	OPA02	5'-TGCCGAGCTG-3'	27	1.7407 ± 0.4466	1.3784 ± 0.3143	0.2370 ± 0.1737	0.3647 ± 0.2496	20	74.07%
2.	OPA03	5'-AGTCAGCCAC-3'	27	1.8519 ± 0.3620	1.5963 ± 0.3218	0.3428 ± 0.1624	0.5021 ± 0.2274	23	85.19%
3.	OPM17	5'-TCAGTCCGGG-3'	27	1.4074 ± 0.5007	1.2019 ± 0.3242	0.1224 ± 0.1776	0.1891 ± 0.2585	11	40.74%
4.	OPP07	5'-GTCCATGCCA-3'	27	1.5926 ± 0.5007	1.3090 ± 0.3745	0.1829 ± 0.1955	0.2799 ± 0.2766	16	59.26%
5.	OPP08	5'-ACATCGCCCA-3'	27	1.7778 ± 0.4237	1.4803 ± 0.3761	0.2782 ± 0.1922	0.4145 ± 0.2659	21	77.78%

*na = Observed number of alleles, *ne = Effective number of alleles (Kimura and Crow, 1964), *h = Nei's (1973) Gene diversity, *I = Shannon's Information index (Lewontin, 1972), TNL = Total number of loci, NPL = Number of polymorphic loci, PPL = Percentage of polymorphic loci.
!The result are expressed by mean ± standard deviation.
n = number of loci

TABLE 7. Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) of rhizobial isolates produced by POPGENE version 1.31

pop ID	Mdk1	Klt2	Pdg3	Pkt4	Adn5	Vlm6	Alt7	Mud8	Pvr9	Alg10	Bdl11	Mlk12	Klv13	RF14
Mdk1	****	1.0008	1.0275	0.9793	1.0365	1.0085	1.0014	1.0041	0.9730	0.9636	0.9794	0.9658	0.9928	1.0017
Klt2	-0.0008	****	0.9911	0.9775	0.9703	0.9731	0.9893	1.0152	0.9477	0.9962	1.0031	0.9808	1.0186	0.9710
Pdg3	-0.0271	0.0090	****	1.0037	1.0115	1.0037	0.9730	0.9898	0.9636	0.9636	0.9840	0.9567	0.9928	0.9969
Pkt4	0.0209	0.0228	-0.0036	****	1.0173	0.9667	0.9829	0.9809	0.9597	0.9681	0.9793	0.9704	1.0122	1.0112
Adn5	-0.0359	0.0301	-0.0114	-0.0172	****	0.9887	0.9959	0.9841	0.9863	0.9401	0.9697	0.9513	0.9869	0.9961
Vlm6	-0.0084	0.0273	-0.0037	0.0339	0.0114	****	0.9552	0.9577	0.9642	0.9192	0.9603	0.9429	0.9699	0.9645
Alt7	-0.0014	0.0108	0.0273	0.0172	0.0041	0.0458	****	1.0144	0.9214	0.9818	1.0101	0.9841	1.042	0.9804
Mud8	-0.0040	-0.0151	0.0102	0.0193	0.0160	0.0432	-0.0143	****	0.9419	1.0019	1.0174	0.9913	1.0115	0.9922
Pvr9	0.0273	0.0537	0.0371	0.0411	0.0138	0.0365	0.0819	0.0599	****	0.8998	0.9278	0.9408	0.9813	0.9442
Alg10	0.0371	0-.0038	0.0371	0.0324	0.0618	0.0843	0.0184	-0.0019	0.1056	****	0.9841	0.9924	1.0056	0.9910
Bdl11	0.0208	-0.0030	0.0161	0.0209	0.0308	0.0405	-0.0101	-0.0173	0.0750	0.0160	****	0.9699	1.0036	0.9629
Mlk12	0.0348	0.0194	0.0443	0.0301	0.0499	0.0588	0.0160	0.0088	0.0611	0.0076	0.0306	****	1.0169	1.0022
Klv13	0.0072	-0.0184	0.0072	-0.0121	0.0132	0.0306	-0.0042	-0.0115	0.0189	-0.0056	-0.0036	-0.0167	****	1.0092
RF14	-0.0017	0.0294	0.0031	-0.0112	0.0039	0.0361	0.0198	0.0079	0.0575	0.0090	0.0378	-0.0022	-0.0092	****

TABLE 8. The Ewens-Watterson test for Neutrality of 27 loci produced by OPA02 primer using POPGENE version 1.31

S.No.	Locus bp	n	k	Obs.F	Min F	Max F	Mean*	SE*	L95*	U95*
1.	300	14	1	1.0000	1.0000	1.0000	****	****	****	****
2.	400	14	1	1.0000	1.0000	1.0000	****	****	****	****
3.	500	14	1	1.0000	1.0000	1.0000	****	****	****	****
4.	600	14.	2	0.6633	0.5000	0.8673	0.7096	0.0185	0.5000	0.8673
5.	650	14	2	0.7551	0.5000	0.8673	0.7123	0.0183	0.5000	0.8673
6.	700	14	2	0.6633	0.5000	0.8673	0.7066	0.0190	0.5000	0.8673
7.	750	14	1	1.0000	1.0000	1.0000	****	****	****	****
8.	800	14	2	0.5918	0.5000	0.8673	0.7089	0.0191	0.5000	0.8673
9.	850	14	2	0.8673	0.5000	0.8673	0.7131	0.0190	0.5000	0.8673
10.	900	14	2	0.7551	0.5000	0.8673	0.7075	0.0194	0.5000	0.8673
11.	950	14	2	0.5918	0.5000	0.8673	0.7056	0.0189	0.5000	0.8673
12.	1000	14	2	0.5918	0.5000	0.8673	0.7043	0.0193	0.5000	0.8673
13.	1250	14.	2	0.8673	0.5000	0.8673	0.7086	0.0199	0.5000	0.8673
14.	1500	14	2	0.7551	0.5000	0.8673	0.6995	0.0195	0.5000	0.8673
15.	1750	14	2	0.5918	0.5000	0.8673	0.7056	0.0184	0.5000	0.8673
16.	2000	14	2	0.7551	0.5000	0.8673	0.7076	0.0195	0.5000	0.8673
17.	2250	14	2	0.5408	0.5000	0.8673	0.7038	0.0190	0.5000	0.8673
18.	2500	14	2	0.5918	0.5000	0.8673	0.7126	0.0185	0.5000	0.8673
19.	2750	14	2	0.7551	0.5000	0.8673	0.7065	0.0186	0.5000	0.8673
20.	3000	14	1	1.0000	1.0000	1.0000	****	****	****	****
21.	3250	14	2	0.5408	0.5000	0.8673	0.7072	0.0194	0.5000	0.8673
22.	3500	14.	2	0.5102	0.5000	0.8673	0.7115	0.0185	0.5000	0.8673
23.	3750	14	2	0.8673	0.5000	0.8673	0.7131	0.0181	0.5000	0.8673
24.	4000	14	2	0.7551	0.5000	0.8673	0.7093	0.0197	0.5000	0.8673
25.	4250	14	2	0.5918	0.5000	0.8673	0.7065	0.0182	0.5000	0.8673
26.	4500	14	1	1.0000	1.0000	1.0000	****	****	****	****
27.	4750	14	1	1.0000	1.0000	1.0000	****	****	****	****

* These statistics were calculated using 1000 simulated samples. k: No. of alleles; Obs. F: Observed sum of the squared of allelic frequency; L95, U95: The 95% confidence interval upper and lower limit; SE: Standard error for observed F were calculated using 1000 simulated sample; *: F-Value that outside the limit (lower and upper) of 95 per cent confidence region.

TABLE 9. The Ewens-Watterson test for Neutrality of 27 loci produced by OPA03 primer using POPGENE version 1.31

S. No.	Locus bp	n	k	Obs. F	Min F	Max F	Mean*	SE*	L95*	U95*
1.	300	14	1	1.0000	1.0000	1.0000	****	****	****	****
2.	400	14	1	1.0000	1.0000	1.0000	****	****	****	****
3.	500	14	2	0.6633	0.5000	0.8673	0.7038	0.0193	0.5000	0.8673
4.	600	14	2	0.6633	0.5000	0.8673	0.7086	0.0187	0.5000	0.8673
5.	650	14	2	0.5000	0.5000	0.8673	0.7054	0.0193	0.5000	0.8673
6.	700	14	2	0.5408	0.5000	0.8673	0.7096	0.0190	0.5000	0.8673
7.	750	14	2	0.7551	0.5000	0.8673	0.7018	0.0184	0.5000	0.8673
8.	800	14	2	0.5102	0.5000	0.8673	0.7106	0.0194	0.5000	0.8673
9.	850	14	1	1.0000	1.0000	1.0000	****	****	****	****
10.	900	14	2	0.5918	0.5000	0.8673	0.7090	0.0194	0.5000	0.8673
11.	9500	14	2	0.5102	0.5000	0.8673	0.7056	0.0191	0.5000	0.8673
12.	1000	14	2	0.6633	0.5000	0.8673	0.7103	0.0190	0.5000	0.8673
13.	1250	14	2	0.5918	0.5000	0.8673	0.7080	0.0190	0.5000	0.8673
14.	1500	14	2	0.5408	0.5000	0.8673	0.7073	0.0195	0.5000	0.8673
15.	1750	14	2	0.6633	0.5000	0.8673	0.7048	0.0190	0.5000	0.8673
16.	2000	14	2	0.5000	0.5000	0.8673	0.7079	0.0194	0.5000	0.8673
17.	2500	14	2	0.5918	0.5000	0.8673	0.7065	0.0186	0.5000	0.8673
18.	2500	14	2	0.5918	0.5000	0.8673	0.7124	0.0184	0.5000	0.8673
19.	2750	14	2	0.7551	0.5000	0.8673	0.6991	0.0191	0.5000	0.8673
20.	3000	14	2	0.5000	0.5000	0.8673	0.7127	0.0186	0.5000	0.8673
21.	3250	14	2	0.5918	0.5000	0.8673	0.7093	0.0187	0.5000	0.8673
22.	3500	14	2	0.5102	0.5000	0.8673	0.7062	0.0189	0.5000	0.8673
23.	3750	14	1	1.0000	1.0000	1.0000	****	****	****	****
24.	4000	14	2	0.5918	0.5000	0.8673	0.7123	0.0187	0.5000	0.8673
25.	4250	14	2	0.6633	0.5000	0.8673	0.7053	0.0193	0.5000	0.8673
26.	4500	14	2	0.5918	0.5000	0.8673	0.7103	0.0198	0.5000	0.8673
27.	4750	14	2	0.6633	0.5000	0.8673	0.7021	0.0190	0.5000	0.8673

* These statistics were calculated using 1000 simulated samples. k: No. of alleles; Obs. F: Observed sum of the squared of allelic frequency; L95, U95: The 95 per cent confidence interval upper and lower limit; SE: Standard error for observed F were calculated using 1000 simulated sample; *: F-Value that outside the limit (lower and upper) of 95 per cent confidence region.

TABLE 10. The Ewens-Watterson test for Neutrality of 27 loci produced by OPM17 primer using POPGENE version 1.31

S.No.	Locus bp	n	k	Obs.F	Min F	Max F	Mean*	SE*	L95*	U95*
1.	300	14	1	1.0000	1.0000	1.0000	****	****	****	****
2.	400	14	1	1.0000	1.0000	1.0000	****	****	****	****
3.	500	14	1	1.0000	1.0000	1.0000	****	****	****	****
4.	600	14	1	1.0000	1.0000	1.0000	****	****	****	****
5.	650	14	1	1.0000	1.0000	1.0000	****	****	****	****
6.	700	14	1	1.0000	1.0000	1.0000	****	****	****	****
7.	750	14	1	1.0000	1.0000	1.0000	****	****	****	****
8.	800	14	1	1.0000	1.0000	1.0000	****	****	****	****
9.	850	14	1	1.0000	1.0000	1.0000	****	****	****	****
10.	900	14	2	0.5000	0.5000	0.8673	0.7038	0.0193	0.5000	0.8673
11.	950	14	2	0.5408	0.5000	0.8673	0.7086	0.0187	0.5000	0.8673
12.	1000	14	2	0.8673	0.5000	0.8673	0.7054	0.0193	0.5000	0.8673
13.	1250	14.	1	1.0000	1.0000	1.0000	****	****	****	****
14.	1500	14	1	1.0000	1.0000	1.0000	****	****	****	****
15.	1750	14	2	0.7551	0.5000	0.8673	0.7096	0.0190	0.5000	0.8673
16.	2000	14	2	0.6633	0.5000	0.8673	0.7018	0.0184	0.5000	0.8673
17.	2250	14	2	0.8673	0.5000	0.8673	0.7106	0.0194	0.5000	0.8673
18.	2500	14	2	0.8673	0.5000	0.8673	0.7090	0.0194	0.5000	0.8673
19.	2750	14	1	1.0000	1.0000	1.0000	****	****	****	****
20.	3000	14	1	1.0000	1.0000	1.0000	****	****	****	****
21.	3250	14	1	1.0000	1.0000	1.0000	****	****	****	****
22.	3500	14.	2	0.5102	0.5000	0.8673	0.7056	0.0191	0.5000	0.8673
23.	3750	14	1	1.0000	1.0000	1.0000	****	****	****	****
24.	4000	14	2	0.8673	0.5000	0.8673	0.7103	0.0190	0.5000	0.8673
25.	4250	14	2	0.5918	0.5000	0.8673	0.7080	0.0190	0.5000	0.8673
26.	4500	14	2	0.6633	0.5000	0.8673	0.7073	0.0195	0.5000	0.8673
27.	4750	14	1	1.0000	1.0000	1.0000	****	****	****	****

* These statistics were calculated using 1000 simulated samples. k: No. of alleles; Obs. F: Observed sum of the squared of allelic frequency; L95, U95: The 95 per cent confidence interval upper and lower limit; SE: Standard error for observed F were calculated using 1000 simulated sample; *: F-Value that outside the limit (lower and upper) of 95 per cent confidence region.

TABLE 11. The Ewens-Watterson test for Neutrality of 27 loci produced by OPP07 primer using POPGENE version 1.31

S.No.	Locus bp	n	k	Obs.F	Min F	Max F	Mean*	SE*	L95*	U95*
1.	300	14	1	1.0000	1.0000	1.0000	****	****	****	****
2.	400	14	1	1.0000	1.0000	1.0000	****	****	****	****
3.	500	14	1	1.0000	1.0000	1.0000	****	****	****	****
4.	600	14	2	0.8673	0.5000	0.8673	0.7048	0.0190	0.5000	0.8673
5.	650	14	2	0.6633	0.5000	0.8673	0.7079	0.0194	0.5000	0.8673
6.	700	14	2	0.6633	0.5000	0.8673	0.7065	0.0186	0.5000	0.8673
7.	750	14	1	1.0000	1.0000	1.0000	****	****	****	****
8.	800	14	2	0.7551	0.5000	0.8673	0.7124	0.0184	0.5000	0.8673
9.	850	14	2	0.5102	0.5000	0.8673	0.6991	0.0191	0.5000	0.8673
10.	900	14	1	1.0000	1.0000	1.0000	****	****	****	****
11.	950	14	1	1.0000	1.0000	1.0000	****	****	****	****
12.	1000	14	2	0.5408	0.5000	0.8673	0.7127	0.0186	0.5000	0.8673
13.	1250	14.	2	0.5000	0.5000	0.8673	0.7093	0.0187	0.5000	0.8673
14.	1500	14	2	0.8673	0.5000	0.8673	0.7062	0.0189	0.5000	0.8673
15.	1750	14	2	0.8673	0.5000	0.8673	0.7123	0.0187	0.5000	0.8673
16.	2000	14	1	1.0000	1.0000	1.0000	****	****	****	****
17.	2250	14	1	1.0000	1.0000	1.0000	****	****	****	****
18.	2500	14	2	0.5408	0.5000	0.8673	0.7053	0.0193	0.5000	0.8673
19.	2750	14	2	0.5102	0.5000	0.8673	0.7103	0.0198	0.5000	0.8673
20.	3000	14	2	0.8673	0.5000	0.8673	0.7021	0.0190	0.5000	0.8673
21.	3250	14	2	0.8673	0.5000	0.8673	0.7125	0.0188	0.5000	0.8673
22.	3500	14.	2	0.8673	0.5000	0.8673	0.7084	0.0182	0.5000	0.8673
23.	3750	14	2	0.6633	0.5000	0.8673	0.7066	0.0187	0.5000	0.8673
24.	4000	14	2	0.5102	0.5000	0.8673	0.7120	0.0191	0.5000	0.8673
25.	4250	14	1	1.0000	1.0000	1.0000	****	****	****	****
26.	4500	14	1	1.0000	1.0000	1.0000	****	****	****	****
27.	4750	14	1	1.0000	1.0000	1.0000	****	****	****	****

* These statistics were calculated using 1000 simulated samples. k: No. of alleles; Obs. F: Observed sum of the squared of allelic frequency; L95, U95: The 95 per cent confidence interval upper and lower limit; SE: Standard error for observed F were calculated using 1000 simulated sample; *: F-Value that outside the limit (lower and upper) of 95 per cent confidence region.

TABLE 12. The Ewens-Watterson test for Neutrality of 27 loci produced by OPP08 primer using POPGENE version 1.31

S.No.	Locus bp	n	k	Obs.F	Min F	Max F	Mean*	SE*	L95*	U95*
1.	300	14	2	0.7551	0.5000	0.8673	0.7154	0.0199	0.5000	0.8673
2.	400	14	2	0.8673	0.5000	0.8673	0.7064	0.0190	0.5000	0.8673
3.	500	14	2	0.5408	0.5000	0.8673	0.7060	0.0187	0.5000	0.8673
4.	600	14	2	0.8673	0.5000	0.8673	0.7030	0.0187	0.5000	0.8673
5.	650	14	2	0.5000	0.5000	0.8673	0.7014	0.0196	0.5000	0.8673
6.	700	14	2	0.5408	0.5000	0.8673	0.7156	0.0188	0.5000	0.8673
7.	750	14	1	1.0000	1.0000	1.0000	****	****	****	****
8.	800	14	2	0.8673	0.5000	0.8673	0.7057	0.0193	0.5000	0.8673
9.	850	14	2	0.6633	0.5000	0.8673	0.7090	0.0190	0.5000	0.8673
10.	900	14	1	1.0000	1.0000	1.0000	****	****	****	****
11.	950	14	1	1.0000	1.0000	1.0000	****	****	****	****
12.	1000	14	2	0.5000	0.5000	0.8673	0.7104	0.0191	0.5000	0.8673
13.	1250	14.	2	0.6633	0.5000	0.8673	0.7047	0.0190	0.5000	0.8673
14.	1500	14	2	0.5918	0.5000	0.8673	0.6997	0.0195	0.5000	0.8673
15.	1750	14	2	0.5102	0.5000	0.8673	0.7052	0.0191	0.5000	0.8673
16.	2000	14	2	0.7551	0.5000	1.0000	0.7058	0.0188	0.5000	0.8673
17.	2250	14	2	0.5102	0.5000	1.0000	0.7093	0.0196	0.5000	0.8673
18.	2500	14	2	0.5918	0.5000	0.8673	0.7120	0.0186	0.5000	0.8673
19.	2750	14	2	0.5408	0.5000	0.8673	0.7055	0.0194	0.5000	0.8673
20.	3000	14	2	0.6633	0.5000	0.8673	0.7022	0.0184	0.5000	0.8673
21.	3250	14	1	1.0000	1.0000	1.0000	****	****	****	****
22.	3500	14.	2	0.5918	0.5000	0.8673	0.7077	0.0189	0.5000	0.8673
23.	3750	14	2	0.5918	0.5000	0.8673	0.7130	0.0197	0.5000	0.8673
24.	4000	14	2	0.8673	0.5000	0.8673	0.7027	0.0194	0.5000	0.8673
25.	4250	14	2	0.5102	0.5000	0.8673	0.7123	0.0191	0.5000	0.8673
26.	4500	14	1	1.0000	1.0000	1.0000	****	****	****	****
27.	4750	14	1	1.0000	1.0000	1.0000	****	****	****	****

* These statistics were calculated using 1000 simulated samples. k: No. of alleles; Obs. F: Observed sum of the squared of allelic frequency; L95, U95: The 95 per cent confidence interval upper and lower limit; SE: Standard error for observed F were calculated using 1000 simulated sample; *: F-Value that outside the limit (lower and upper) of 95 per cent confidence region.

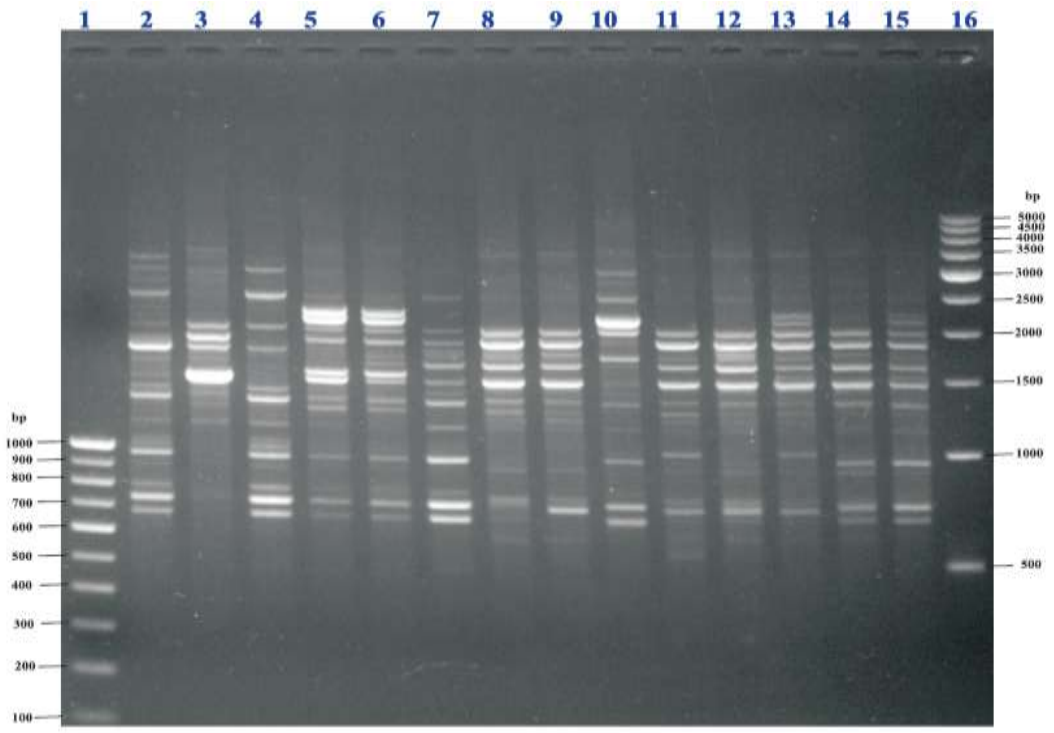

Fig.1. RAPD banding pattern of rhizobial isolates generated using OPA02 primer

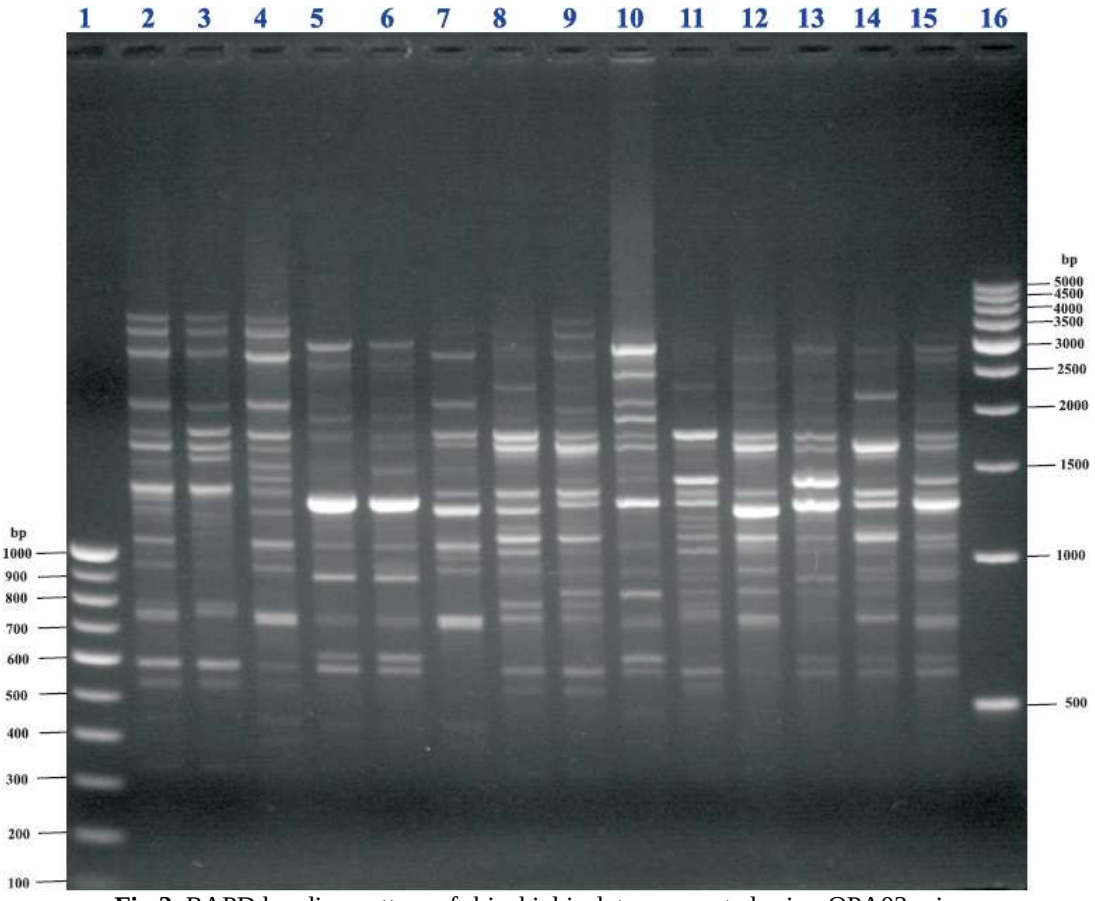

Fig.2. RAPD banding pattern of rhizobial isolates generated using OPA03 primer

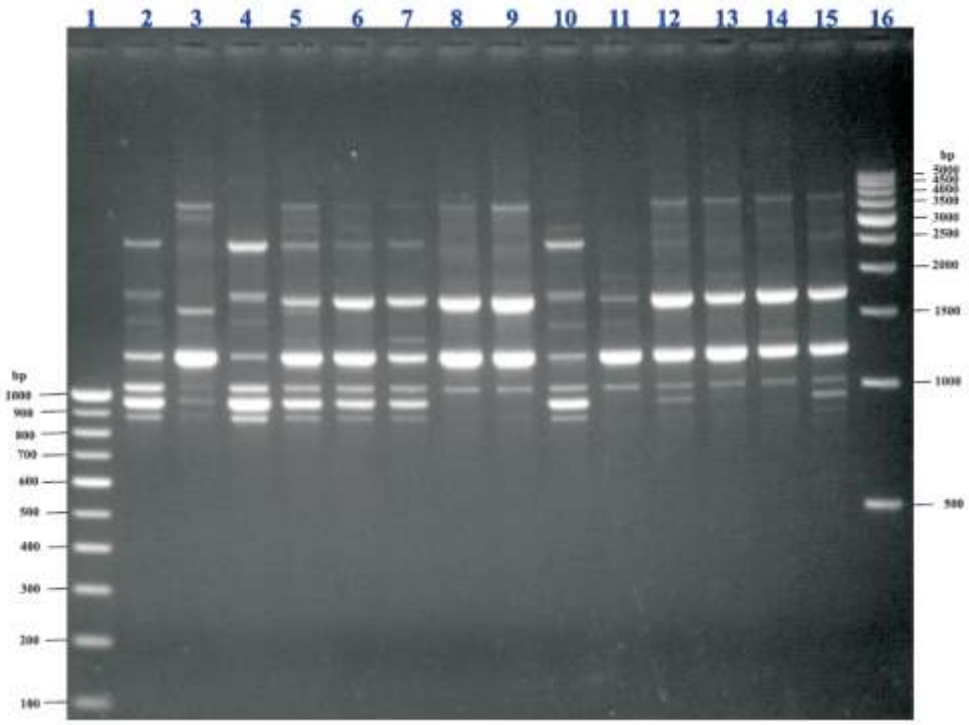

Fig.3. RAPD banding pattern of rhizobial isolates generated using OPM17 primer

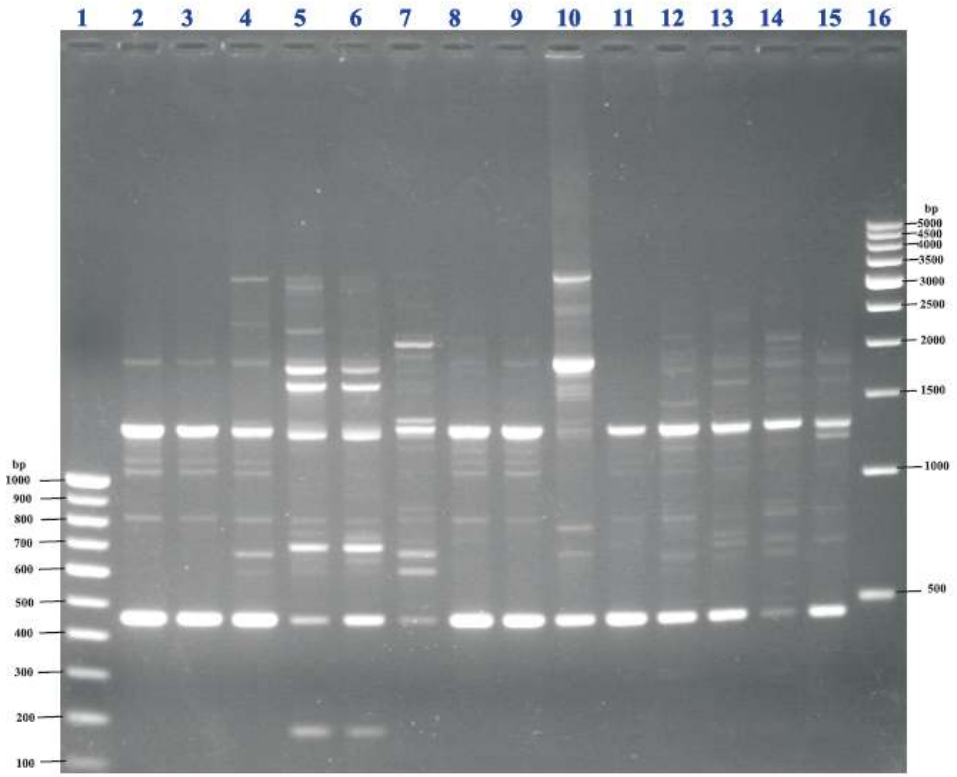

Fig.4. RAPD banding pattern of rhizobial isolates generated using OPP07 primer

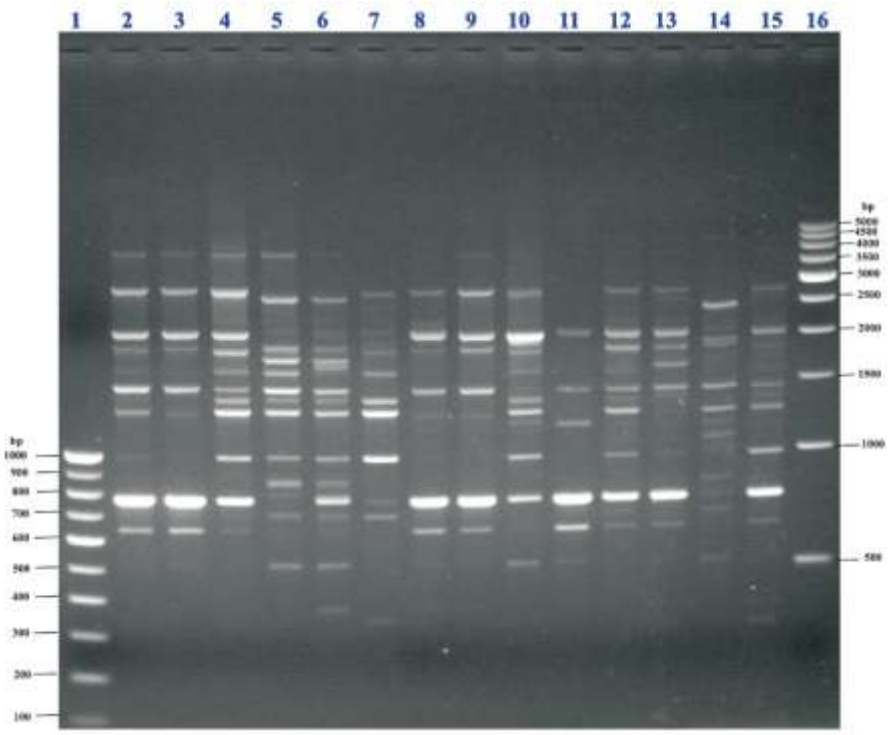

Fig.5. RAPD banding pattern of rhizobial isolates generated using OPP08 primer

Fig. 1-5. Lane Description: - Lane 1: 100 bp DNA Ladder, Lane 2: Mdk1, Lane 3: Klt2, Lane 4: Pdg3, Lane 5: Pkt4, Lane 6: Adn5, Lane 6: Vlm6, Lane 7: Alt7, Lane 8: Alt7, Lane 9: Mud8, Lane 10: Pvr9, Lane 11: Alg10, Lane 12: Bdl11, Lane 13: Mlk12, Lane 14: Klv13, Lane 15: RF14, Lane 16: 500 bp DNA Ladder

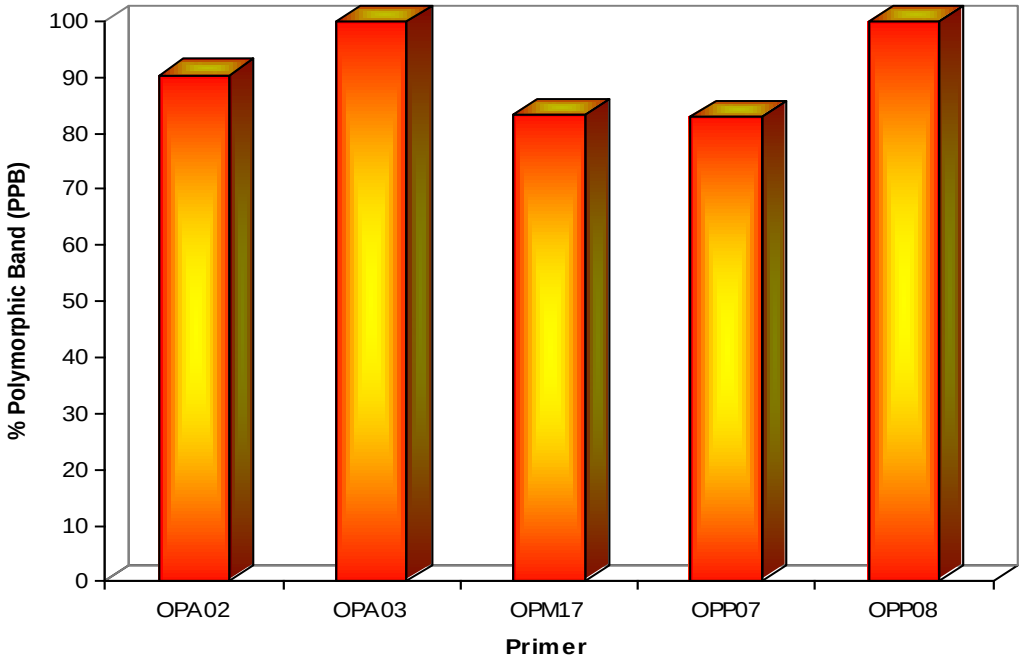

Fig.6. Percentage of polymorphic bands produced by rhizobial isolates using five different random primers

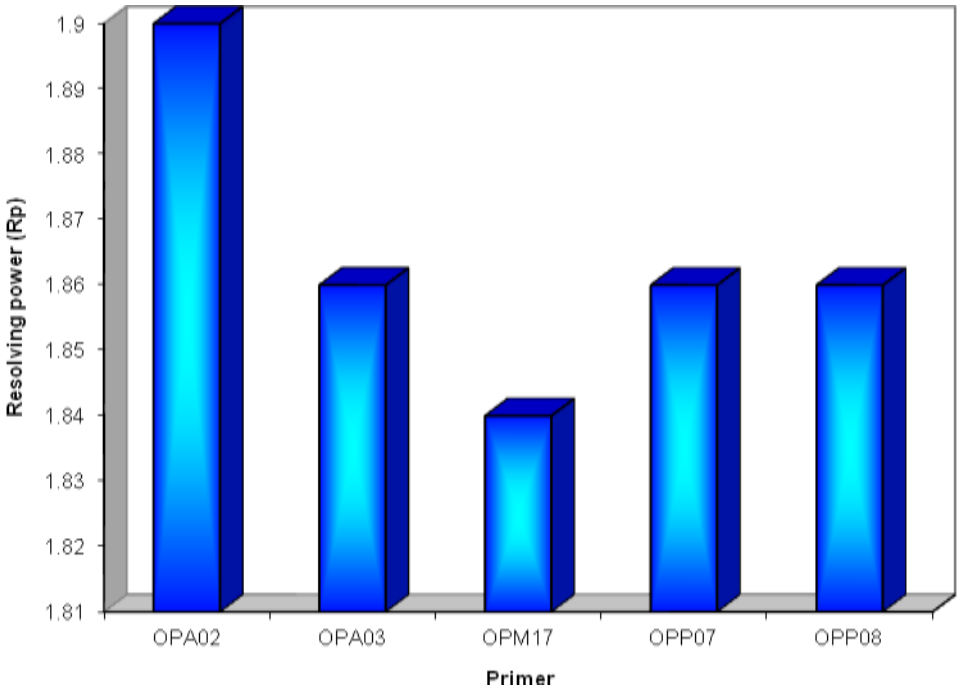

Fig.7. Resolving power of five different random primers

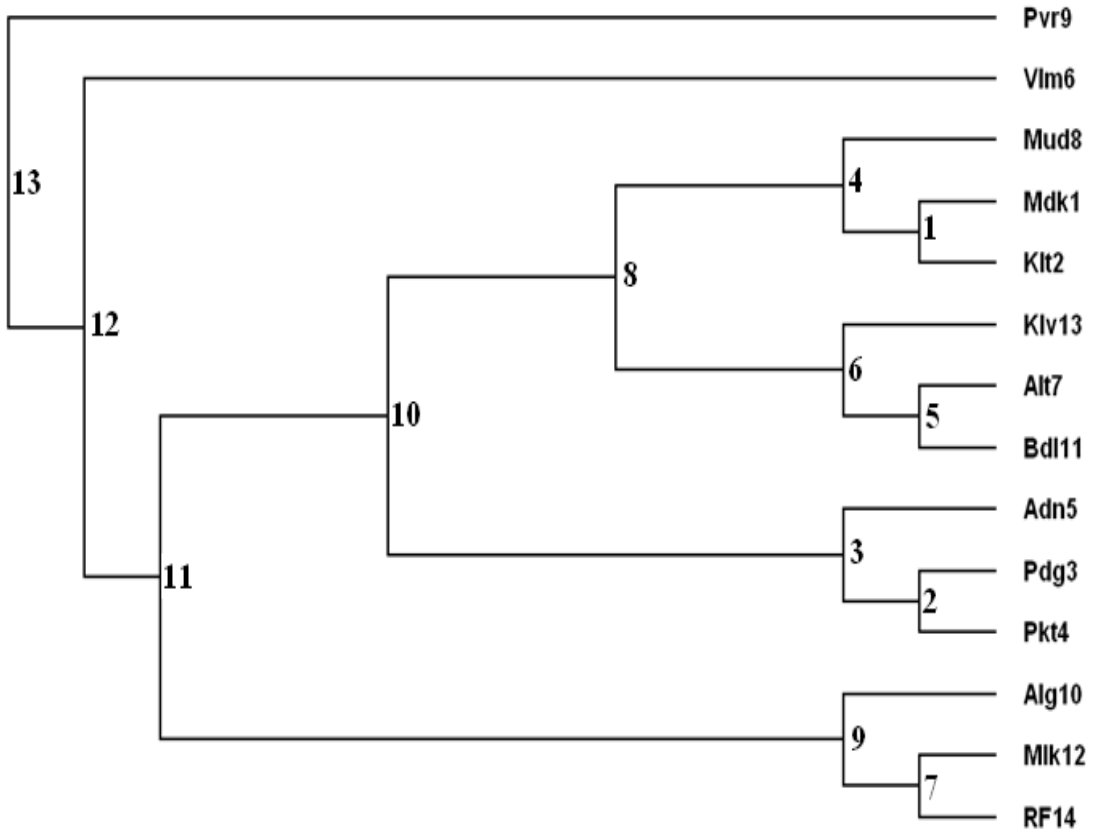

Fig.8. Rectangular cladogram of rhizobial isolates using neighbour-joining cluster analysis method produced from Nei's (1978) unbiased measures of genetic identity and genetic distance