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MOLECULAR CHARACTERIZATION OF INDIGENOUS *RHIZOBIA* NODULATING MEDICINAL LEGUMES USING RAPD METHOD

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ABSTRACT

Rhizobia, gram-negative symbiotic bacteria, play a vital role in natural ecosystems owing to its capability to utilize dinitrogen directly with their nitrogenases. Otherwise, the plants have to get nitrogen in a chemically reduced form, which can be costly. It has been found that both the bacteria and their host plants are involved in determining host specificity. Moreover, the relation between the host plant and the rhizobial bacteria is commonly very specific. Present study deals with the Isolation and Identification of Rhizobium bacteria from root nodules of medicinal legumes, their molecular characterization and determining the genetic diversity among different strains which are isolated. The isolates were identified and characterized on the basis of colony morphology and biochemical traits via Gram Staining, Indole Production, Methyl Red, Voges-Proskauer, Citrate Utilization, Starch Hydrolysis, and Catalase Tests. Random Amplified Polymorphic DNA (RAPD), which can be used as a technique for determining genetic diversity among the bacterial strains isolated, uses short primers with an arbitrary sequence to amplify genomic DNA. In the present study, RAPD analysis was done to investigate the genetic diversity of root nodule organisms isolated from different medicinal legumes. RAPD analysis of genomic DNA of the isolates using 5 random primers such as OPA5, OPA 6, OPA 7, OPW7 and OPW8 yielded banding patterns with each primer. RAPD analysis was done with 5 random primers OPA 5, OPA 6, OPA 7, OPA 06 and OPA 07. Phylogenetic variation was determined by converting RAPD data into a frequency similarity and analysed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis to produce a phylogenetic tree. RAPD markers revealed possible relationship between host origin, mutation and genetic variation among the isolates. Thus the present study showed that isolate 2 and isolate 3 were most similar species.

KEYWORDS: **RAPD, PCR, Rhizobia, Electrophoresis**

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INTRODUCTION

Rhizobia, commonly known as nitrogen-fixing soil bacteria, are studied extensively owing to their considerable agricultural and environmental importance[1]. Over the past years, the evaluation of the rhizobial diversity among the natural populations has become significant[2]. As a result, many molecular methods including very sensitive and accurate PCR based technique have been developed to study the differentiation among closely related bacterial strains and the detection of higher rhizobial diversity than previously considered[3]. Although many studies have strived to study the exact composition and characteristics of indigenous strains isolated from different cultivated legumes using the molecular genetic methods, studies on legumes like different shrubs and herbaceous plants that have a vital role in medicine are very less[4]. In recent years, Random Amplified Polymorphic DNA (RAPD) – PCR technique has been reported as being particularly suitable for typing both within and between bacterial species. RAPD can be used as a technique for determining genetic diversity among the bacterial strains isolated. RAPD assays use short primers with an arbitrary sequence to amplify genomic DNA[5]. It has been well established that one of the vital necessity of the useful rhizobium association is the capability of the strain to compete with very diverse indigenous rhizobial strains. Thus, it is important to characterize the natural rhizobial population to improve the beneficial effects of the rhizobium association [6]. With the available evidence, the objective of the present study is to identify and characterise the genetic diversity of the root nodule organisms isolated from different medicinal legumes, including Indigo feratinctoria (true indigo), Mimosa pudica (touch me not plant), and Trigonella Foenum-graeceum (methi) using RAPD.

MATERIALS AND METHODS

Isolation of Microorganism

Our Present study is regarding isolation and molecular characterization of root nodule organisms. Root nodules occur on the roots of plants (primarily Fabaceae) that associate with symbiotic nitrogen-fixing bacteria. Under nitrogen- limiting conditions, capable plants from a symbiotic relationship with a host - specific strains of bacteria known as rhizobia.

Samples Collection

Root nodules associated Medicinal plant samples such as Indigofera tinctoria (true indigo), Mimosa pudica (touch me not plant), and Trigonella Foenum-graeceum (methi) were collected from Gandhi Krishi Vignana Kendra (GKVK), Bangalore. Root nodules were cut and separated from the root, with and washed with distilled water and crushed with sterile mortar and pestle. Serial dilution was carried out and plated on yeast extract mannitol agar media with congo red.

BIOCHEMICAL CHARACTERIZATION

Indole Production Test

Tryptone broth was prepared and autoclaved, followed by inoculation of culture and incubation at 37° C for 24 hours. Development of cherry red colour on addition of Kovac's reagent would indicate a positive result.

Methyl Red Test

MR-VP broth was prepared and autoclaved & Glucose solution was sterilized by filtration and 0.25ml was added to each tube that will give a final concentration of 0.5%. Test culture was inoculated in the MR broth and incubated at 37^0C for 48 hours. After incubation 5-6 drops of methyl red solution were added. A bright red color indicating a pH of 4.2 or less is a positive test.

Voges – Proskauer Test

MR-VP broth was prepared and autoclaved and Glucose solution was sterilized by filtration and 0.25ml was added to each tube that will give a final concentration of 0.5%. The test culture was inoculated in the VP broth inoculated in the VP broth incubated at 37^0C for 48 hours. After incubation, 1ml of 40% potassium hydroxide (plus creatinine) and 3ml of a 5% solution of alpha- naphthol were added in absolute ethanol. A positive reaction is indicated by the development of a pink or crimson colour

Citrate Utilization Test

Test tubes containing Simon's citrate agar were autoclaved and followed by inoculation of culture and incubation at 37° C for 24 hours. Change of color of the slant form green to blue would indicate a positive result.

Starch Hydrolysis

Starch agar plates were prepared and a single line of streak of the organisms was made across the centre of the starch agar plate. The plates were incubated at 37^0C for 24 hours for sufficient growth. After incubation the plates was flooded with iodine solution. Hydrolysis is indicated by clear zone formation around the growth and unchanged starch gives as blue colour. The medium should preferably contain no glucose as this may diminish starch hydrolysis. The iodine normally used for Gram's stain.

Catalase Test

Nutrient agar plates were prepared and streaked with test cultures and the plates were incubated at 37^0 C for 24 hours. After incubation, 1ml of 3% hydrogen peroxide was added to the plates after 5 minutes the plates were examined immediately for the evolution of bubbles, which indicates a positive test.

Casein Hydrolysis

The cultures were inoculated on skim milk agar plates and incubated at 37^0C for 24 to 48 hours. It is used to observe for clearing around colonies of casein hydrolyzing organism. To detect false clearing, pair 10% solution of $MgCl_2$ in 20% HCL over this medium. The disappearance of cleaned area indicates false hydrolysis.

DNA Fingerprinting by RAPD

PCR is *in-vitro* nucleic acid synthesizing reaction, it amplifies the template DNA flanked by primers. Thermo cycler controls the temperature at various stages of the replication cycle. Five primers were screened to amplify the DNA isolated as given in the table 1.

Primers Used

Table 1

Primers were used for DNA Fingerprinting by RAPD analysis

NAME	SEQUENCE (5'-3')
OPA 05	5' AGGGGTCTTG 3'
OPA 06	5' GGTCCTGAC 3'
OPA 07	5' GAAACGGGTG 3'
OPW 07	5' CTGGACGTCA 3'
OPW 08	5' GACTGCCTCT 3'

Molecular Marker Analysis

The amplified products were resolved by electrophoresis in 1.5% of agarose gel electrophoresis using 1X TAE buffer (appendix 5) at 50 volts for 2.5 hours. A 100 base pair ladder was included as molecular size marker. Gels were visualized by staining with Ethidium bromide (1µl/10ml) and banding patterns were photographed over UV light. Phylogenetic variation was determined by converting RAPD data into a frequency similarity and analysed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis to produce a phylogenetic tree.

RESULTS

In order to observe biochemical activities of microorganisms which were specific to individual genus and species various kinds of specially prepared media were inoculated with pure cultures of microorganisms. Many distinctive enzyme activities be can demonstrated by observing for the products resulting from the action of enzymes on specific substrates within the specially prepared media. Different mediums were used for the biochemical characterization of the isolated and selected bacteria for their identification according to Bergey's Manual of Determinative Bacteriology as mentioned in the table 2.

Table 2
Assay of biochemical activities of microorganisms

S.No	BIOCHEMICAL TEST	Sample 1	Sample 2	Sample 3	Sample 4
1.	Indole Production Test	Negative	Negative	Negative	Negative
2.	Methyl Red Test	Negative	Negative	Negative	Negative
3.	Voges Proskauer Test	Negative	Negative	Negative	Negative
4.	Citrate Utilization Test	Negative	Positive	Negative	Negative
5.	Catalase Test	Positive	Positive	Positive	Positive
6.	Starch Hydrolysis Test	Negative	Negative	Negative	Negative
7.	Casein Hydrolysis Test	Negative	Negative	Negative	Negative

Genetic Diversity by RAPD Analysis

The genetic diversity among six species was determined by RAPD technique using random primers. The PCR products obtained were analysed by using 1.5% agarose gel electrophoresis with Ethidium Bromide stain and visualized over UV light as given in the figure 1, 2 and 3.

DNA samples amplified with OPW 07

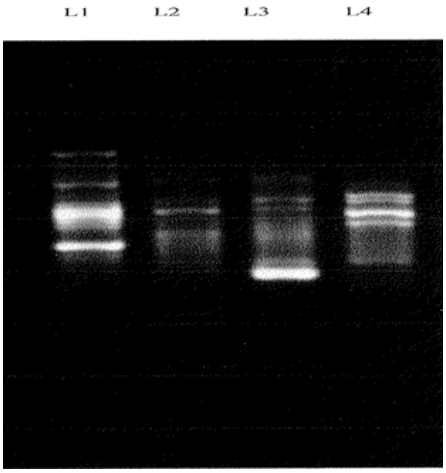


Figure 1
L1 - isolated from methi, L2 -isolated from methi, L3 -isolated From indigo, L4 -isolated from mimosa.

DNA samples amplified with OPA5, OPA6, OPA7

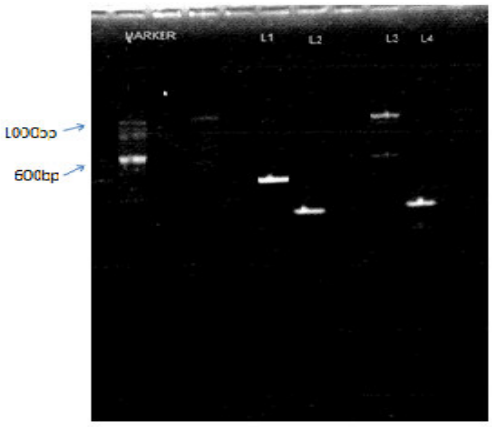


Figure 2
L1 -isolated from methi, L2- isolated from methi, L3-isolated from indigo, L4- isolated from mimosa.

DNA samples amplified with OPW 08

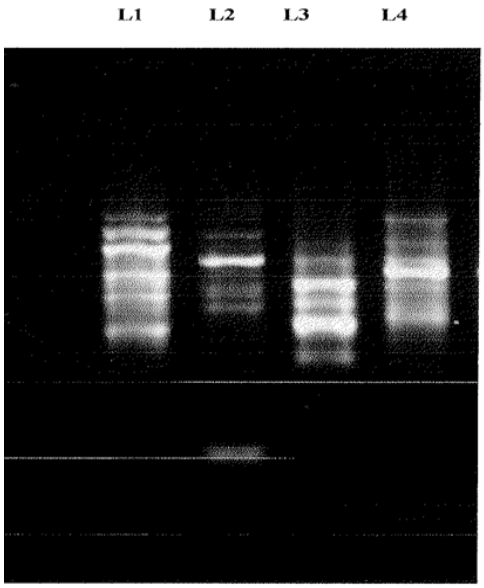
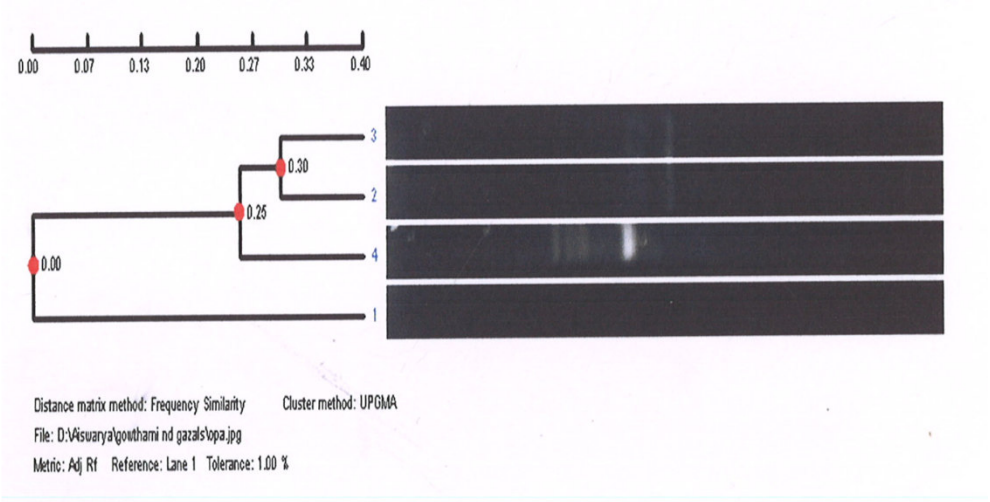


Figure 3
L1- isolated from methi, L2- isolated from methi,
L3-isolated from indigo, L4 - isolated from mimosa

Phylogenetic analysis using Dendrogram - OPA 05

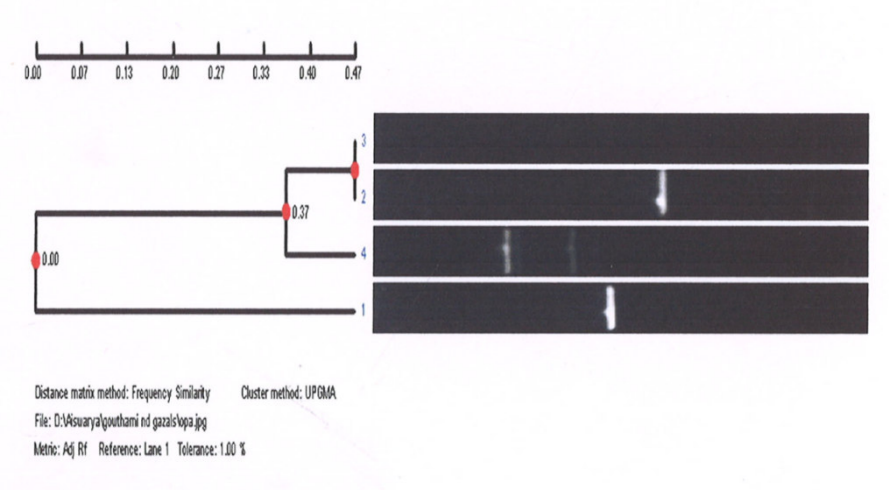


1.	100.00	20.00	0.00	40.00
2.	20.00	100.00	80.00	80.00
3.	00.00	80.00	100.00	60.00
4.	40.00	80.00	60.00	100.00

Figure 4
Phylogenetic analysis using Dendrogram of OPA 05

As shown in the figure 4, the phylogenetic tree produced from the UPGMA average cluster using Primer OPA 05 Matrix analysis shows that, the isolate 1 and 3 showed 6 bands and isolate 2 showed 9 bands and isolate 4 showed 8 bands.

Phylogenetic analysis using Dendrogram - OPA 06



1.	100.00	0.00	0.00	20.00
2.	0.00	100.00	100.00	80.00
3.	00.00	100.00	100.00	80.00
4.	20.00	80.00	80.00	100.00

Figure 5
Phylogenetic analysis using Dendrogram of OPA 06

1.	100.00	0.00	0.00	33.33
2.	0.00	100.00	100.00	66.67
3.	00.00	100.00	100.00	66.67
4.	33.33	66.67	66.67	100.00

As shown in the figure 5, the phylogenetic tree produced from the UPGMA average cluster using Primer OPA 06 Matrix analysis shows that, the isolate 2 and 3 are 100 % similar that means identical and isolate 2 is 80% similar to both isolate 4 &3.

Phylogenetic analysis using Dendrogram - OPA 7

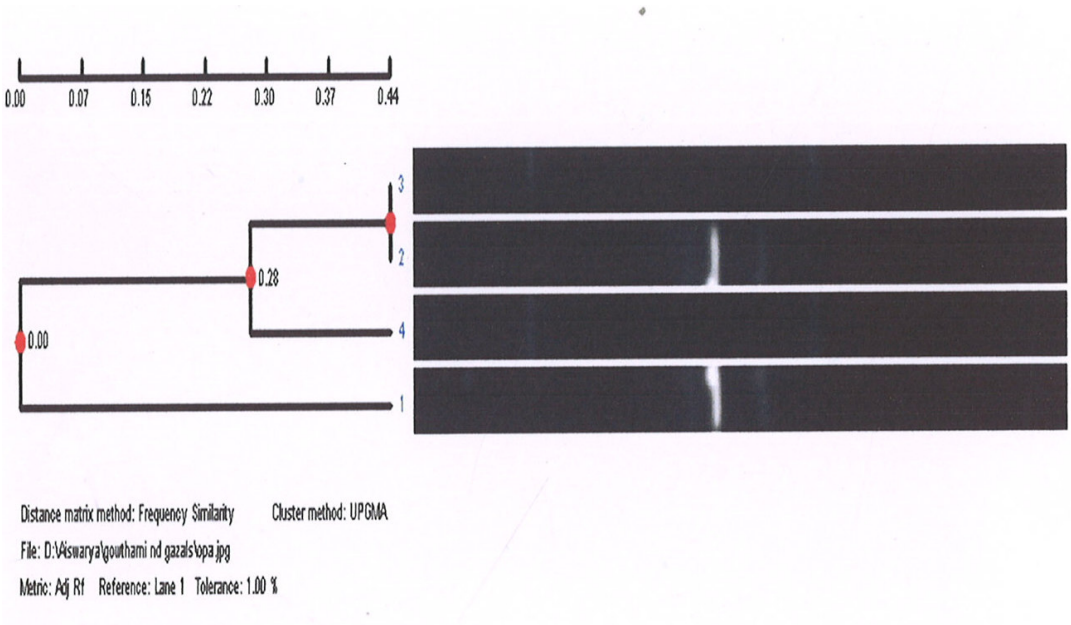
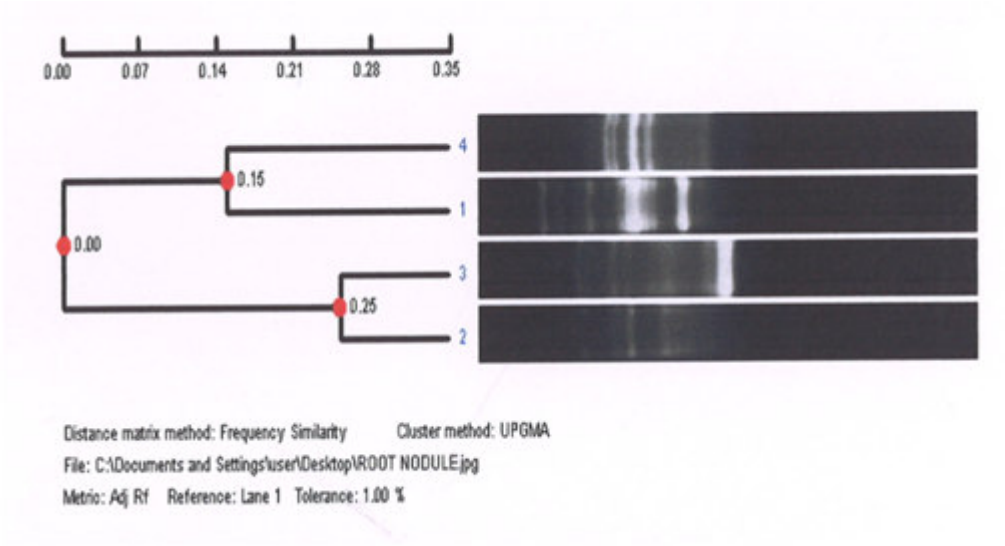


Figure 6
Phylogenetic analysis using Dendrogram of OPA 07

As shown in the figure 6, the phylogenetic tree produced from the UPGMA average cluster using Primer OPA 07 Matrix analysis shows that, the isolate 2 and 3 are 100 % similar that means identical and isolate 1 is 33% similar to isolate 4 and no similarity is seen between isolate1 and isolate 3 and isolate 2. 66.67% similarity is obtained between isolate 3 and 4

Phylogenetic analysis using Dendrogram - OPW 07

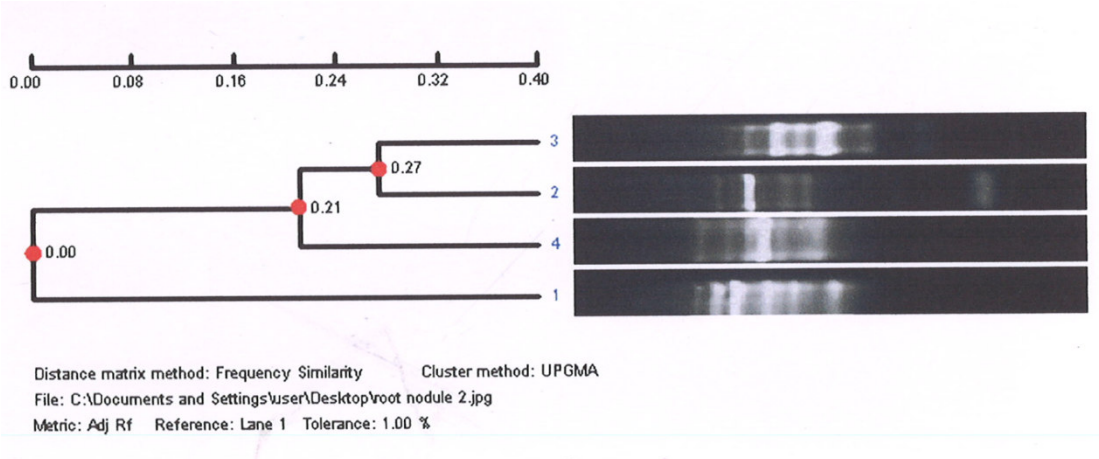


1.	100.00	20.00	0.00	60.00
2.	20.00	100.00	80.00	60.00
3.	00.00	80.00	100.00	40.00
4.	60.00	60.00	40.00	100.00

Figure 7
Phylogenetic analysis using Dendrogram of OPW 07

As shown in the figure 7, the phylogenetic tree produced from the UPGMA average cluster using Primer OPW 07 Matrix analysis shows that, the isolate 2 and 1 are 20 % similar and isolate 1 is 60% similar to isolate 4 and 60% similarity between isolate 2 and isolate 4 .40% similarity was observed between isolate 3 and isolate 4.and 80% similarity between isolate 2 and 3.

Phylogenetic analysis using Dendrogram - OPW 08



1.	100.00	25.00	25.00	12.50
2.	25.00	100.00	75.00	62.50
3.	25.00	75.00	100.00	62.50
4.	12.50	62.50	62.50	100.00

Figure 8
Phylogenetic analysis using Dendrogram of OPW 08

As shown in the figure 8, the phylogenetic tree produced from the UPGMA average cluster using Primer OPW 08 Matrix analysis shows that, the isolate 2 and 1 are 25 % similar and isolate 1 is 25% similar to isolate 3 and 12.5% similarity between isolate 1 and isolate 4 .75% similarity was observed between isolate 3 and isolate 2 and 62% similarity between isolate2 and 4 and also isolate 3 &4.Thus indicating isolate 2 and 3 are similar species.

DISCUSSION

The present study deals with the isolation of root nodule organism from different medicinal plants and its molecular characterization organisms were isolated in yeast extract mannitol agar and *Rhizobium* were identified as mucoid red colonies further gram staining was done and all the isolates were observed as gram negative rods. 7 biochemical tests also performed for the further confirmation of the microorganisms. Genomic DNA was extracted according to Santos et al 2008 phenol chloroform method and quantified in nano drop. The quality of the DNA was accessed in 1% agarose gel[7]. Randomly Amplified Polymorphic DNA (RAPD) assays use short primers with an arbitrary sequence to amplify genomic DNA. In this study, the use of RAPD analysis to investigate the genetic diversity root nodule organisms isolates from different medicinal legumesis described. In recent years, the RAPD-PCR technique has been reported as being particularly suitable for typing both within and between bacterial species[5]. RAPD analysis of genomic DNA of the isolates using 5 random primers such as OPA5, OPA6, OPA7, OPW7, OPW8 yielding banding patterns with each primer. Amplification of DNA with arbitrary primers that involves the whole genome analysis is a powerful approach for the study of DNA polymorphism. AP-PCR is widely used for the comparison of genomes from eukaryotes or bacteria [8]. Total 122 bands were amplified with 5 different primers. Isolate 1 showed 6 bands, isolate 2 showed 9 bands, isolate 3 showed 6 and isolate 4 showed 8 bands respectively, with OPA5.Phylogenetic tree was created by the un-weighted pair-group method arithmetic (UPGMA) average cluster analysis[9,10]. RAPD data were recorded in to similarity matrix and UPGMA to produce a Phylogenetic tree. Similarity matrix with OPA6 showed 100% similarity between isolates 2 and isolate 3 and 20% similarity between isolates 1 and isolate 4. No similarity was observed between isolates 1 and isolate 3, and isolate 2. 80% similarity was observed between, isolate 3 and isolate 4. Similarity matrix with OPA7 showed 100% similarity between isolates 2 and isolates 3 and 33% similarity between isolates 1 and isolates 4. No similarity was observed between isolates 1 and isolate 3, and isolate 2. 66.67% similarity was observed between, isolate 3 and isolate 4. Similarity matrix with OPW 7 showed 20% similarity between isolates 1 and isolate 2 and 60% similarity between isolates 1 and isolate 4, 60% similarity between isolates 2 and isolate 4. 40% similarity was observed between, isolate 3 and isolate 4. 80% similarity between isolate 2 and isolate 3 was observed. No similarity was observed between isolate 1 and isolate 3.Similarity matrix with OPW 8 showed 25% similarity between isolate 1 and isolate 2 and 25% similarity between isolate 1 and isolate 3. 12.5% similarity between isolate 1 and isolate 4. 75% similarity was observed between, isolate 2 and isolate 3. 62% similarity was observed between, isolates 2 and isolates 4 and also between isolate 3 and isolate 4.RAPD markers revealed the possible relationship between host origin, mutation and genetic variation among the isolates, and this demonstrated its fingerprinting and diagnostic potential. Thus the present study showed that isolate 2 and isolate 3 were most similar species.

CONCLUSION

Prevailing evidence suggest that the study of genetic diversity and genetic association between the bacterial strains might provide important data about the bacterial genotypes which are well tailored to the natural environment. Consequently, the present study, which isolated the rhizobia of the medicinal legumes to characterize the molecular and genetic diversity of the bacterial strains using RAPD, has found 100% similarity between isolates 2 and isolate 3 and 20% similarity between isolates 1 and isolate 4. However, no similarity was observed between isolates 1 and isolate 3, and isolate 2. Moreover, 80% similarity was observed among isolates 3 and isolate 4. These strains which are diverse in species and symbiotic association remain a source of genetic substance which can be used to produce superior inoculants bacterial strains.

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