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Genetic Characterization and Diversity Analysis of Heavy Metal-Resistant Rhizobacterial Strains Isolated from the Root Nodules of *Vigna trilobata* Determined by the R.A.P.D. Technique

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ABSTRACT

The present study characterizes twenty-one rhizobacterial strains isolated from root nodules of *Vigna trilobata* cultivars collected from various Andhra Pradesh, India districts. Rhizobacteria was isolated using selective medium Yeast Extract Mannitol Agar medium (Y.E.M.A.). All 21 strains were characterized based on colony morphology and biochemical characterization and identified up to species level by 16S rDNA sequencing analysis. Different genera of the bacteria are present in the root nodules of *Vigna trilobata.* Short random sequence primers amplify genomic D.N.A. in a process called random amplified polymorphic D.N.A. (R.A.P.D.), which were employed to assess genetic variation across isolated bacterial strains. Banding patterns were obtained via R.A.P.D. analysis of genomic D.N.A. from the isolates using five random primers, including OPA-4, OPA-9, OPA-11, OPA-12, and OPA-15. The R.A.P.D. profiles of twenty-one rhizobacterial isolates were compared separately to determine their differences by the occurrence of polymorphic D.N.A. fragments. PCR amplified of the D.N.A. isolated from twenty-one bacterial isolates yielded seventy amplified products, of which sixty-eight were polymorphic, and two were monomorphic. The investigation found that R.A.P.D. could differentiate between the strains very precisely. Characterizing and creating novel *Rhizobium* species can be aided by using molecular markers to investigate genetic diversity.

Keywords Genetic diversity, Rhizobia, Yeast Extract Mannitol Agar, Polymorphic D.N.A. fragments, R.A.P.D.

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INTRODUCTION

Nitrogen-fixing bacteria from the family Rhizobiaceae have been isolated from nodules on leguminous plants, and a symbiotic relationship has been formed. The bacteria, which are members of the genera *Rhizobium, Allorhizobium, Mesorhizobium, Bradyrhizobium,* and *Sinorhizobium* (formerly known as Ensifer), can form nodules on the host plant inside of which

they fix-nitrogen. Nitrogen-fixing soil bacteria are studied extensively owing to their big agriculture and environmental importance (Chen *et al.* 2000). The evolution of the rhizobial diversity among the natural populations has become significant (Zhang *et al.* 1999).

Many molecular methods, including very sensitive and accurate PCR-based techniques, have been developed to study the differentiation among closely related bacterial strains and the higher *Rhizobial* diversity (Selenska-Pobell *et al.*1995). In recent years, Random Amplified Polymorphic D.N.A. (R.A.P.D.)-PCR techniques have been reported as suitable for both within and between bacterial species. R.A.P.D. can be used to determine genetic diversity among isolated bacterial strains (Jarabo-Lorenzo *et al*. 2000).

Recently, R.A.P.D. analysis has been applied to identifying *Rhizobium* and the genetic study of *Bradyrhizobium,* both of which benefit from symbiotic relationships (Kosier *et al.* 1993, Kay *et al.* 1994). R.A.P.D. fingerprinting was employed to investigate the genetic variations in Rhizobium strains (Versalovic *et al.* 1994, Teaumroong and Boonkerd 1998).

DNA fingerprinting based on R.A.P.D. has efficiently distinguished closely related bacterial species. Rhizobium-associated polymorphic D.N.A. (R.A.P.D.) strain identification relies on the polymerase chain reaction (PCR) method in combination with random primers to distinguish between the genomes of various bacterial strains that are representative of various species (Teaumroong and Boonkerd 1998). Several researchers have demonstrated that R.A.P.D. is a highly discriminating and effective approach for differentiating and investigating the genetic diversity of *Rhizobium* strains. Paffetti *et al.* (1996) found substantial genetic variability among phenotypically similar *Rhizobium meliloti* strains. *R. leguminosarum* bv *Phaseoli* and *Rhizobium tropici* strains were found to have high levels of genetic variability, as demonstrated by Oliveira *et al.* (2000).

Prasad (2014) demonstrated the evolutionary connection between four *Rhizobial* species collected from different plant nodules. In contrast, Abo-Aba *et al.* (2015) reported the differences between three native *Rhizobium leguminosarum bv. trifolii* isolates from the *Trifolium alexandrinum* plant. Wekesa *et al.* (2017) investigated the genetic characterization of rhizosphere bacteria found in common bean nodules in soils in Western Kenya. *Delfitia, Rhizobia, Providencia, Acinetobacter, Enterobacter, Pseudomonas,* and *Klebsiella* were among the 24 rhizobacterial strains recovered from common bean nodules. Most of the isolates within each genus were genetically varied except for a few individuals. Kuklinsky-Sobral *et al.* (2005) reported the isolation of nodule endophytes from the genera *Rhodopseudomonas, Phyllobacterium, Mycobacterium, Pseudomonas, Sphingomonas,* and *Bacillus* from soybean nodules.

In the family Leguminoceae, Vigna is one of the most important nodulating genera. Over 100–150 species of perennial or annual legumes make up the genus Vigna. In a recent study by, Mortuza and group (2020), multiphase characterization of wild Vigna-associated root nodule bacteria from Japanese subtropical islands unveiled the novel high-temperature resistant *Bradyrhizobium* strains having high symbiotic compatibility with soybean and mungbean in India, *Vigna trilobata,* also known as *Pilli pesara,* was mostly grown as a temporary Fodder and farmyard manure crop. Nodulation studies were described, and the distribution of root Nodules of *Vigna trilobata* varied from cultivar to cultivar (Kumar and Silpa 2023). The symbiont in root nodules has been described as a rhizobial strain, although it has not been fully defined as of yet. Understanding their variety was aided by the emergence of several molecular genetics techniques. To identify variations among rhizobacterial strains and investigate their genetic relationships. More people are using PCR for genotyping. This approach is equally efficient as the time-consuming RFLP at identifying rhizobial isolates, yet it is also much easier and less expensive. Therefore, the current study, R.A.P.D. analysis, was conducted to evaluate the rhizobacterial diversity among isolates collected from root nodules of *Vigna trilobata* plants that grow in soils from several districts of Andhra Pradesh.

MATERIALS AND METHODS

Isolation of rhizobacterial strains

Rhizobial strains were identified from the nodules of

V. trilobata plants grown in soils from the agricultural soils near the arsenic-contaminated industrial effluents of Andhra Pradesh (Deepika *et al,* 2016). After 21 days from seeding, the *V. trilobata* plants were carefully uprooted, surface cleansed with 0.1% Mercuric chloride, and rinsed multiple times with sterile distilled water to collect the pink, healthy root nodules for isolation. A bacterial suspension was created by crushing these nodules with a sterile glass rod in sterile distilled water. After incubating the suspension at room temperature for three days, we spotted it on media plates using a selective medium (Y.E.M.A.) with 0.1% Congo red. After incubation, pure cultures were preserved by repeatedly subculturing the same medium and isolating the white, translucent, convex colonies that produced a lot of mucilage. Twenty-one isolates were tested for their ability to nodulate on homologous hosts and their identity as Rhizobium using standard biochemical techniques (Somasegaran and Hoben 1994). Twenty-one strains were sequenced for their 16S rDNA (Macrogen, South Korea), and the resulting sequence data was submitted to the GenBank for future study.

Isolation of genomic D.N.A. and R.A.P.D. analysis

Bacterial genomic D.N.A. was isolated using a modified protocol reported in (Sa *et al.*1993). This was followed by R.A.P.D. analysis. The bacterial D.N.A. was amplified using a Perkin-Elmer thermocycler. The PCR amplification reaction mixture consisted of 40 cycles, each consisting of 15s of denaturation at 94°C, 30s of annealing at 35°C, and one minute of polymerization at 72°C for 7 min; an extra cycle of an extension was performed. Each reaction mixture contained 25µL and contained the following ingredients: 2.5 mM $MgCl_2$, 10 mM Tris KCl, and 0.01 mM dNTPs (dATP, dCTP, dGTP, dTTP) 25 ng of D.N.A., 1 U of Taq D.N.A. polymerase, 0.4 mM of primer, and 11 µL of H_2O. Five different primer sets (OPA-04, OPA-09, OPA-11, OPA-12, and OPA-15) from the Operon Kit (Operon Technologies Inc.) were evaluated.

Primer design and selection

For the preliminary primer screening, 20 primers from the O.P.A. series (Operon technology) were used to analyze molecular variation. The diversity of the strains was analyzed using primers that allowed for repeatable and measurable amplification.

Scoring of the DNA fragments and data analysis

All amplification products were documented as potential RAPD markers. All DNA fragments and characteristics found were documented in a data entry matrix. Each isolate's RAPD pattern was analyzed by assigning character state "1" to all DNA fragments capable of being reliably spotted in the gel and "O" to those that could not.

The Jaccard similarity coefficient for each pairwise comparison was then determined using the resulting data matrix. The coefficients were determined *in Silico* using the following formula, which is based on work by Jaccard (1908):

Similarity co-efficient= a/n

Where,

a = Number of matching band for each pair of comparisons
n= Total number of DNA fragments observed in two samples

A U.P.G.M.A. (unweighted pair-group technique on arithmetic average) cluster analysis was performed using the similarity coefficient to classify the isolates by their shared characteristics. Cluster analysis and dendrogram construction were performed using tools from SPSS 10.0.

RESULTS

i) Molecular diversity and cluster analysis of rhizobacterial strains

Evaluation using RAPD-PCR All rhizobial strains have their genomic D.N.A. extracted and separated using agarose gel electrophoresis. Primers were utilized to amplify whole genomic D.N.A. from 21 rhizobial strains, and the GenBank accession numbers were used as references (Table 1). The R.A.P.D. profiles of twenty-one bacterial strains were compared

Table 1. Species names along with strain number and GenBank accession number.

Sl. No.	Species name	Strain number	GeneBank accession no.
1	*Agrobacterium tumifaciens*	MRR 108	KC415690
2	*Mycobacterium wolinskyi*	MRR 120	KF621019
3	*Ensifer terangae*	MRR 121	KC503883
4	*Ensifer* sp.	MRR 126	KC503886
5	*Ensifer* sp.	MRR 101	KC428651
6	*A.tumifaciens*	MRR 102	KC428652
7	*Rhizobium* sp.	MRR 103	JX576499
8	*Bacillus altitudinis*	MRR 122	KF621021
9	*Rhizobium* sp.	MRR 123	KC503884
10	*Ensifer kostisense*	MRR 104	KC428653
11	*Ensifer xinjiangense*	MRR 110	KC415691
12	*Paenibacillus* sp.	MRR124	KF621017
13	*A. tumifaciens*	MRR 111	KC415692
14	*Rhizobium* sp.	MRR 112	KF621018
15	*Rhizobium* sp.	MRR 106	KC428655
16	*Ensifer* sp.	MRR 114	KC503887
17	*A.tumifaciens*	MRR 105	KC428654
18	*A.tumifaciens*	MRR 115	KC415693
19	*Ensifer* sp.	MRR 125	KC503885
20	*Enterobacter cloaceae*	MRR 127	KF621020
21	*Ensifer kostisense*	MRR 117	KC415695

individually for each primer and separately to determine their differences by the presence of polymorphic D.N.A. fragments. Four primers O.P.A.- 4, O.P.A. 9, O.P.A. 11 and O.P.A. 12 produced 100% discrimination power (Plates I-V) (Fig. 1).

ii) Genetic relationship of twenty-one rhizobacterial strains as determined by R.A.P.D. analysis

The dendrogram obtained by statistical gel data analysis was categorized into two major clusters (Fig. 2). Twelve strains were grouped into one cluster, and the remaining nine were included in the second cluster. The first cluster was further divided into four groups. The first group of the first cluster consisted of six strains, including *Rhizobium* sp. MRR 123, *Ensifer* sp. MRR 125, *E. kostisense* MRR 117, *Enterobacter cloaceae* MRR 127, *A. tumifaciens* MRR 115 and *Ensifer* sp. MRR 101. The second group consisted of *A. tumifaciens* MRR 111, *Rhizobium* sp. MRR 112 and *E. xinjiangense* MRR 110. *Ensifer* sp. MRR 114 was individually grouped into a third group. The fourth group consisted of *A. tumifaciens* MRR 105 and *Rhizobium* sp. MRR 106. The second cluster is further divided into two groups. The first group of the second cluster consisted of *A. tumifaciens* MRR 108 and *Ensifer* sp. MRR 109. Whereas *E. terange* MRR 121, *Paenibacillus* sp. MRR 124, *Mycobacterium wolinskyi* MRR 120, *Bacillus altitudinis* 178 MRR 122,

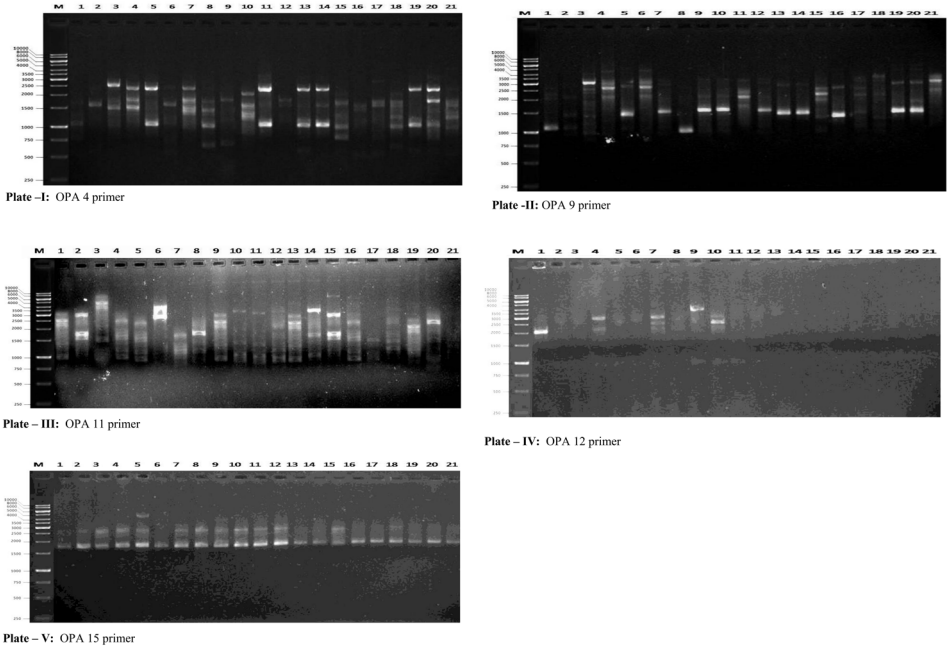

Fig. 1. (Plates I-V) RAPD-PCR analysis for evaluating the genetic relationship of twenty-one rhizobacterial strains.

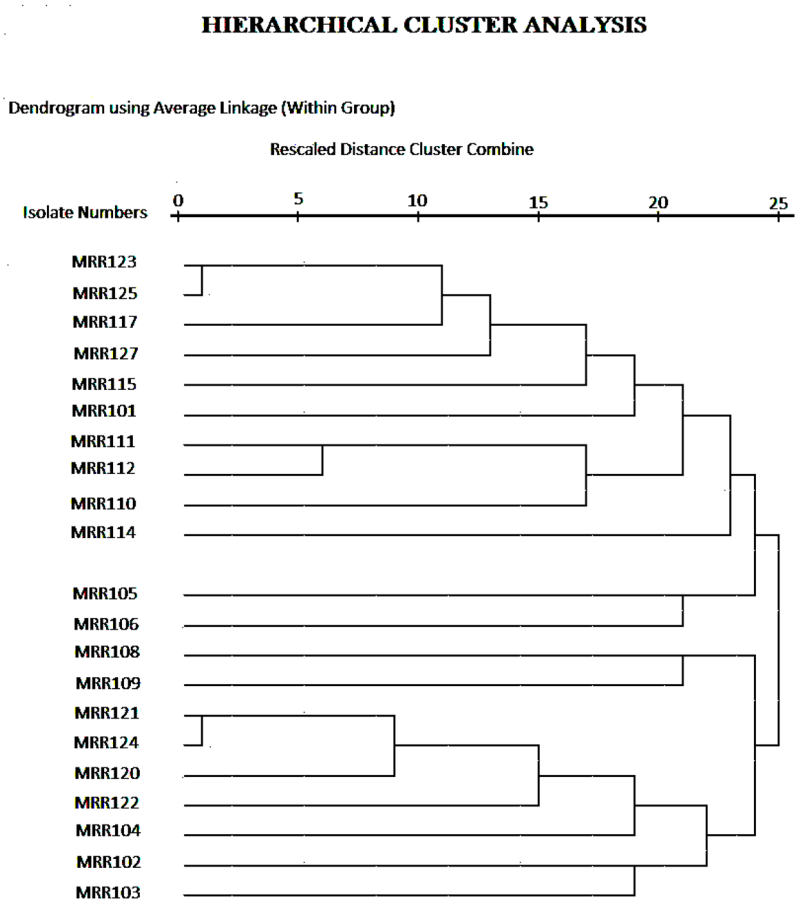

Fig. 2. Dendrogram for evaluating the evolutionary relationship of twenty-one rhizobacterial strains using hierarchical cluster analysis.

E. kostisense MRR 104, *A. tumifaciens* MRR 102 and *Rhizobium* sp. MRR 103 strains fell into the second group. Data of R.A.P.D. markers scanned from twenty-one bacterial strains with five reproducible primers were used to generate a similarly coefficient. A maximum similarity value of 100% was observed between three bacterial strains of *Rhizobium* sp. MRR 123, *Paenibacillus* sp. MRR 124 and *Ensifer* sp. MRR 125 followed by *A. tumifaciens* MRR 111 and *Rhizobium* sp. MRR 112 (83.3%), whereas 9.1% was found to be genetically most diverse *A. tumifaciens* MRR 108 and *A. tumifaciens* MRR 102. From this, it is evident that most of the strains nodulating the root nodules of *V. trilobata* are genetically diversified. From the molecular characterization studies, it can be concluded that the genetic diversity among rhizobacterial strains of *V. trilobata* depends on the strains' physiological characteristics and the geographical location from which they were isolated. This variation among the strains may be since fast-growing rhizobacteria exhibit much more diversity than slow-growing ones.

Discrimination power of the primers

The discrimination power (%) values calculated for each primer are presented in (Table 2). Four primers O.P.A.- 4, O.P.A. 9, O.P.A. 11 and O.P.A. 12 produced 100% discrimination power, whereas Primer O.P.A. 15 produced 33% discrimination power, respectively.

Table 2. List of primers, no. of DNA fragments and polymorphism in each primer.

Sl. No.	Primers	Primer sequences	Total no. of polymorphic DNA fragments	Total no. of DNA fragments	Percentage of DNA polymorphism
1	O.P.A. 15	5¹TTCCGAACCC3¹	1	3	33.00%
2	O.P.A. 4	5¹AGTCAGCCAC3¹	3	3	100.00%
3	O.P.A. 9	5¹GGGTAACGCC3¹	15	15	100.00%
4	O.P.A. 11	5¹CAATCGCCGT3¹	28	28	100.00%
5	O.P.A. 12	5¹TCGGCGATAG3¹	21	21	100.00%
			68	70	99.1%

DISCUSSION

The selected strains were recovered from *Vigna radiata's* root nodules, based on viscous EPS production and arsenic tolerant capacity, as identified by 16 S rDNA sequencing as evidenced in a previously published paper (Deepika *et al,* 2016). R.A.P.D. analysis reveals the genetic diversity among rhizobacterial strains. Numerous studies have shown R.A.P.D. analysis to be an efficient tool for identifying and classifying Rhizobia. Once the primers exposing the polymorphism have been identified and the PCR conditions have been tuned, even a small variation in primer sequence can result in dramatically different R.A.P.D. patterns, allowing for straightforward strain classification. This approach is advantageous since it only needs a tiny amount of template for amplification, and that template doesn't even have to be pure D.N.A. (Bostock *et al.* 1993, Wang *et al.* 1993). A preliminary round of PCR- R.A.P.D. was performed to discover genomic diversity between strains using five specific primers. The R.A.P.D. patterns of the strains were found to differ. The number and size of D.N.A. fragments amplified by different primers. The present work demonstrated that *Rhizobium* diversity exists and that PCR-based R.A.P.D. analysis is more effective than RFLP (Fig. 1). The 16S rDNA sequence analysis identified 21 strains, including Five *Agrobacterium* sp., Eight *Ensifer* sp, four *Rhizobium* sp. and four other rhizobacterial strains. However, by R.A.P.D. analysis, they were grouped into 2 major clusters (Fig. 2).

First major cluster (with 12 strains) is further divided into four groups: The 1st group includes 6 strains, the 2nd group includes 3 strains, the 3rd with one strain and the 4th with two strains. The second large cluster contained two subgroups inside the second large cluster, which contained the remaining nine strains. The high degree of resemblance between these groups results from the fact that they share similar genotypes. The RAPD analysis shows the strains with high similarity of 100% viz., *Rhizobium* sp. MRR 123, *Paenibacillus* sp. MRR 124 and *Ensifer* sp. MRR 125 was isolated from soils of different locations. Even the strains with very low similarity values of 9.1%, viz., Agrobacterium tumifaciens MRR 108, and Agrobacterium tumifaciens MRR102, were isolated from soils of different locations. This clearly shows that the diversity among rhizobacterial strains isolated from *V.trilobata* was independent of the location from which they were isolated. However, Pinto *et al.* (2004) reported a direct influence of the isolation site in the grouping of *Rhizobium* strains from *Arachis pintoi.* Hassan *et al.* (2015) from their studies also proved that PCR-RAPD fingerprinting is a useful technique for conducting ongoing and competitive studies in *Rhizobia.* Finding that R.A.P.D. attributes might detect genetic variation among the same species of *Rhizobium* was also discovered by Valverde *et al.* (2011). In their studies, Hassan *et al.* (2015) isolated 8 Rhizobium sp. from *Vicia faba* nodules from different geographical areas. The average number of fragments per primer in R.A.P.D. investigations was 13.83, the average number of polymorphic fragments was 10.5, and the average percentage of polymorphic fragments was 75.12. The diameters of the fragments varied from 50 bp to 1520 bp. The eight strains were divided into two groups in the resulting dendrogram.

In the present study, the average number of D.N.A. fragments per primer was 14; out of 70 amplified PCR products, 68 are highly polymorphic, and

only 2 are monomorphic. The dendrogram generated separated the 21 strains into two major clusters—four primers OPA-4, 9, 11 and 12 produced polymorphism with 100% discrimination power. However, Pinto *et al.* (2004) reported that O.P.A. 10, 04, and O.P.C. 10, 03 primers showed the highest polymorphism in *Rhizobium* isolated from *Arachis pintoi*, ranging from 67%-100%. As El-Fiki *et al.* (2006) showed, R.A.P.D. fingerprinting provides a fast and accurate method for distinguishing between *Bradyrhizobium* strains.

Similarly, Abo-Aba *et al.* (2015) for *Rhizobium* strains from *Trifolium alexandrinum;* Hassan *et al.* (2015) from *Rhizobium* of *Vicia faba,* Rajasundari *et al.* (2009) for *Rhizobium* isolates from different locations in Tamilnadu; Berrada *et al.* (2012) for *Rhizobium* isolates from various legumes, also reported the suitability of R.A.P.D. fingerprinting analysis in studying the variations in between *Rhizobium* isolates. R.A.P.D. investigated rhizobial strains from Brazilian soils, and the results showed that there was between 6% and 46% genetic variation between the strains (Oliveira *et al*. 2000). In comparison to *Bradyrhizobium* and *Azorhizobium,* which grow slowly, fast-growing nodulating bacteria tend to be more taxonomically varied and ecologically adaptive. The *nif* genes, like those in slow-growing strains, are found on chromosomal D.N.A. in fast-growing strains, they are on plasmid D.N.A. These features promote hybridization among the several soil *Rhizobium* spp. Amplification of genomic D.N.A. of the most popular 24 Indian mungbean cultivars with these R.A.P.D. primers yielded 249 fragments that could be scored, of which 224 were polymorphic, with an average of 7.0 polymorphic fragments per primer (Datta *et al.* 2012). All R.A.P.D. primers were found to recognize polymorphic D.N.A. fragments, as reported, and RAPD proved to be a highly discriminatory and productive technique for analyzing genetic variation.

In the present study, all the rhizobacterial strains are fast-growing, hence this may be the possible reason for the diversity. It can also be concluded that genetic diversity among rhizobacteria of *Vigna trilobata* is mainly dependent on physiological characteristics and is independent of the geographical locations from which they were isolated.

CONCLUSION

This study was the first to document genetic variation among the 21 rhizobial strains isolated from *Vigna trilobata* root nodules. Diverse genetic characteristics with varying nitrogen-fixing abilities require a high level of genetic diversity. Newer *Rhizobium* strains with strong nitrogen-fixing capability may be discovered through diversity analysis. Strikingly, the 16S rRNA sequencing studies of 21 strains were identified as species belonging to 7 different genera viz., *Rhizobium, Agrobacterium, Ensifer, Mycobacterium, Bacillus, Paenibacillus* and *Enterobacter.* RAPD analysis reveals that these strains were divided into two main clusters; which 1st cluster includes nine strains and the 2nd cluster in 12 strains. Genetic diversity among rhizobacterial strains depends on their physiological characteristics, independent of the locality from which they were isolated. Intriguingly, RAPD was used for genomic DNA fingerprinting to distinguish between bacterial species closely related to rhizobial strains. Twenty-one rhizobial isolates were chosen to reflect the various morphological classes and geographical regions from which soil samples were obtained. Characterizing and generating novel species of *Rhizobium* can be aided by studying their genetic diversity using molecular markers.

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