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Studies on Molecular characterization of Rhizobium spp. Isolates from Agricultural soil of M.P

Oraon Vinay[1*], Singh Kiran[2]

[1]Department of Microbiology, Vananchal Dental College and Hospital, Farathiya, Garhwa, Jharkhand-822114, India, Mobile No. 09955049070
[2]Department of Biological Sciences & Engineering, Maulana Azad National Institute of Technology (MANIT), Bhopal, Madhya Pradesh, India
* E-mail of the corresponding author: vinay2079@gmail.com

Abstract
Nitrogen fixation in leguminous plants by rhizobia, gram-negative symbiotic bacteria, is important in natural ecosystems and for agriculture. This is because the rhizobia are capable of utilizing dinitrogen directly with their nitrogenases, whereas the plants have to get nitrogen in a chemically reduced form, which can be costly. Both the bacteria and their host plants are involved in determining host specificity and the interaction between the host plant and the rhizobial bacteria is often highly specific. The aim of this study was to study diversity of Rhizobium spp. in agricultural lands of Madhya Pradesh. Physicochemical properties of soil were studied using standard methods while molecular methods used to study diversity within Rhizobium species. Further, population analysis of Rhizobium species in relation to genetic diversity was carried out using 16S rDNA-RFLP PCR. Rhizobium were identified and genetically by determining the %G+C content of the whole genome, followed by Restriction enzyme (Mbo I, Hap II, Taq I, Msp I, Cfo I, Nde II, Dde I, Rsa I) treatment of Polymerase Chain Reaction (PCR) amplified product of 16S rDNA segment was performed. The sequences recognized by the restriction enzymes are distributed at variable intervals in the genome of an organism and also vary in number. The separation carried out by electrophoresis (1.6-2% agarose gel) resulted in specific banding pattern differing within as well as among different species.

Key words: Rhizobium spp, NTYSY, % G+C, 16S rDNA & RFLP-PCR.

Introduction

Nitrogen is an important element to support plant growth. Plants depend on soil microorganisms for fixed nitrogen. Among the nitrogen fixing microorganisms, the role of *Rhizobium* is quite significant. DNA related values, 16S rDNA homology values and some phenotypic characteristics provide more and deeper information for the classification of these bacteria. Some species in genera *Rhizobium* and *Bradyrhizobium* were later moved into new genera based on phylogenetic analyses. At the present time, rhizobia have been classified, mainly by comparison of the sequences of the 16S rRNA genes, into 6 genera (*Mesorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Rhizobium, Sinorhizobium*) of the α-2 subclass of Proteobacteria (Wang and Martinez-Romero, 2000; Ngom et al., 2004). The 16S or small subunit ribosomal RNA gene is useful for estimating evolutionary relationships among bacteria because it is slowly evolving and the gene product is both universally essential and functionally conserved (van Berkum and Eardly, 1998). Direct sequencing of genes coding for 16S rRNA (16S rDNA) has been used to establish genetic relationships and to characterize strains at the species or higher level (Laguerre *et al.*, 1996).

PCR-RFLP is used in determining genetic relationships based upon the PCR and restriction site analysis. Specific regions of the genome are amplified and fingerprint patterns are obtained after restriction digestion of the amplification products. The banding patterns across different enzyme digests are used to estimate the genetic diversity (van Berkum and Eardly, 1998). PCR-RFLP of the 16S rDNA has been used in the analysis of legume symbionts (Laguerre *et al.*, 1994). The 16S rDNA has turned out to be a very good tool for the assessment of organismal phylogenies down to the genus level (Terefework *et al.*, 1998). PCR-RFLP analysis of 16S rDNA has been used to determine the phylogenic position of root-and stem-nodule bacteria including *Rhizobium* (Terefework *et al.*, 1998; Wang *et al.*, 1999; Diouf *et al.*, 2000), *Mesorhizobium* (Wang *et al.*, 1999). Besides PCR-RFLP of the 16S rRNA gene, some other genes have been used.

Presently the use of restriction enzymes during RFLP over amplified 16S rDNA strengthen the recognition sites occupied at different location indicating genetic variation in number. In current study 26 isolates of *Rhizobium* and characterized at molecular level on the basis of genetic study including % Guanine plus Cytosine content and 16S rDNA - RFLP, with banding pattern analysis using NTSYS software (Rohlf, 1990).

Methods and Materials

Soil samples (0-30cm depth) were collected from 10 agricultural sites of Neemuch, Hoshangabad, Betul- Multai, Sehore, Bhopal, Tikamgarh, Chindwara, Raisen Vidhisha- Sanchi and Ujjain districts of Madhya Pradesh. These districts fall in central part of the province. Surface litter was scrapped away and soil samples stored in pre-sterilized high-density polythene (HDPE) bags (Forster J, 1995). Samples were passed through 2 mm sieve to have homogenous particles for further analysis. The *Rhizobium* species confirmed from soil samples were named as R1- R26.

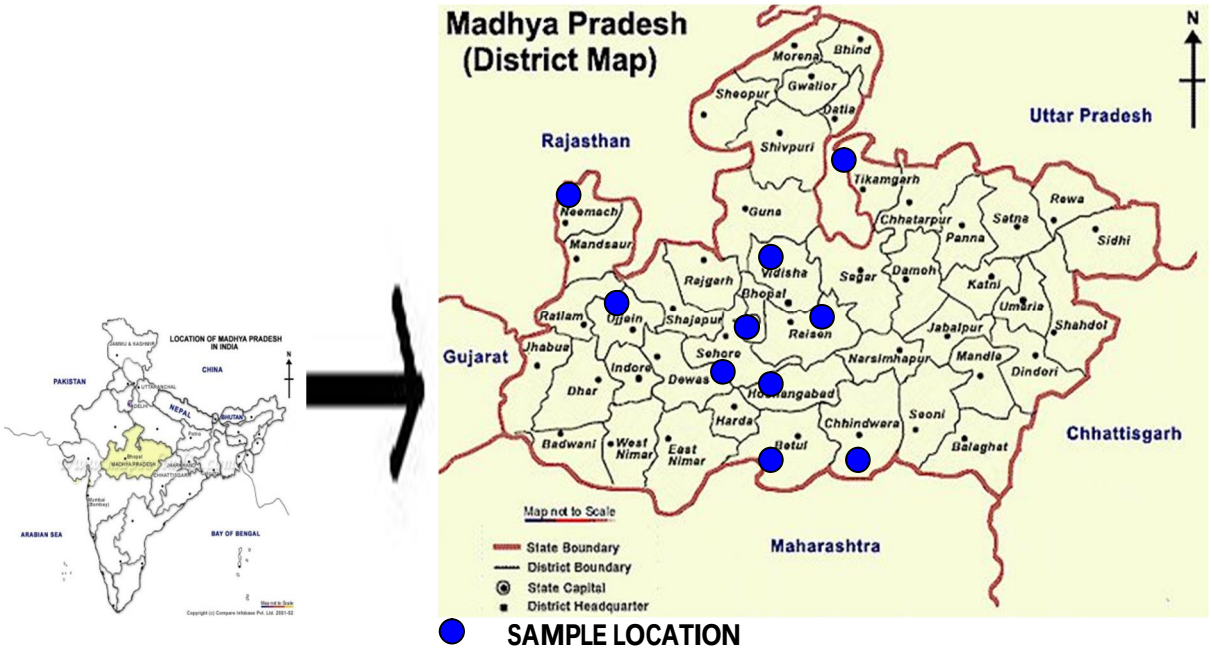


Fig. 1 Map showing sampling locations

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

DNA isolation

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

Determination of Tm value

The Tm of each DNA sample was determined as described by Mandel and Marmur (Mandel et al., 1968; Marmur J, 1961). The % G+C content of the samples was determined by using the equation %G+C = 2.44 (Tm – 69.4) as suggested by De Ley, J. (1970).

PCR-RFLP of amplicon

Polymerase chain reaction (PCR) is most useful widely used genetic tool in study of molecular biology of organisms. It is widely applied on cloning, sequencing and phylogenetic study. The efficiency of PCR technique is based on "master mix'' preparation consisting buffer, dNTP's mix (2mM), Primer 1, Primer 2, Taq polymerase, sterile water except the DNA template. The reproducibility and reliability of results depended upon proper pipetting of all the components of "master mix" and their further distribution. After addition of template DNA, it was exposed to temperature cycles in a thermal cycler (PTC-1148, MJ Mini Thermal cycler, BIO-RAD, USA). T he conserved sequence in DNA i.e., the 16S rDNA was amplified using the reverse primer (5' A CGGCTACCTTCTTAGCACTT-3') and the forward primer (5' AGAGTTTGATCCTGGCTCA G-3') at 55°C (annealing) for 30 cycles in PCR unit (PTC-1148, MJ Mini Thermal cycler, BIO-RAD, USA). Amplified DNA was subjected to RFLP analysis using restriction enzymes (*Mbo I, Hap II, Taq I, Msp I, Cfo I, Nde II, Dde I, Rsa I*) after amplification. The amplified 16S rDNA

was then digested separately with 8 different restriction enzymes, by incubating overnight at 37°C. Enzyme activity was stopped by low temperature (4°C) and by adding 2µl of 6x loading buffer. Further, the enzyme digested PCR product along with 1kb DNA ladder (Bangalore Genie, India) in a separate well was estimated by electrophoresis (Walker et al., 1998) at 55mV on (2% agarose, 2h). The Bio-Rad Gel Doc^{TM} XR and ChemiDoc^{TM} XRS gel documentation system are easy-to-use, high-performance systems. They use a CCD camera to capture image in real time, which allows you to more accurately position and focus the image (Molecular Imager Gel Doc XR System 170-8170, 170-8171, BIO-RAD, USA).

Data analysis:

All restriction patterns were coded in binary form and analysed using NTSYS software (Rohlf, 1990). A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

Result and Discussion

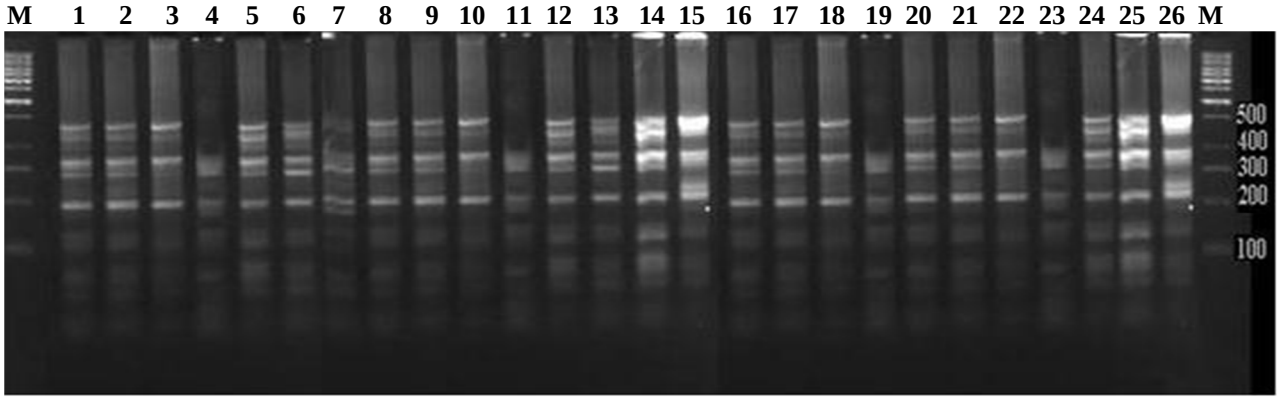
Present study, however, concentrated on the nitrogen fixing strains. Hofer's alkaline broth test conducted is because *Agrobacterium* grows at higher pH levels and not rhizobia. The isolates strains failed to utilize peptone when were grown on glucose peptone agar medium. *Rhizobium* respond negatively ketolactose test (Table 1). Microscopic observations on pure culture cells confirmed the gram-negative nature. In addition, gelatin was not liquefied by cells grow on gelatin medium. Bacterial cells once inoculated on pre-sterilized yeast extract Mannitol Agar (YEMA) produced white, translucent glistering colonies with entire margin soil samples from Neemuch, Hoshangabad, Betul-Multai, Ujjain, Sehore, Bhopal, Tikamgarh, Chindwara, Sanchi-Vidisha, Raisen etc were subjected to the above mentioned biochemical parameters.

Strain Name	Incubation at 28°± 1°C for period in Hours	YEMA/ CRYMA	Slow(S)/ Moderate(M)/ Fast(F) growing sp.	Hofer's Alkaline media	Glucose peptone agar	Ketolactose test	Gram staining	Liquefaction of Gelatin	Action on Milk
R-1	96	+/+	S	-	-	-	-ve	-ve	+
R-2	96	+/+	S	-	-	-	-ve	-ve	+
R-3	96	+/+	S	-	-	-	-ve	-ve	+
R-4	72	+/+	S	-	-	-	-ve	-ve	+
R-5	72	+/+	S	-	-	-	-ve	-ve	+
R-6	96	+/+	S	-	-	-	-ve	-ve	+
R-7	96	+/+	M	-	-	-	-ve	-ve	+
R-8	72	+/+	M	-	-	-	-ve	-ve	+
R-9	72	+/+	M	-	-	-	-ve	-ve	+
R-10	72	+/+	M	-	-	-	-ve	-ve	+
R-11	72	+/+	M	-	-	-	-ve	-ve	+
R-12	96	+/+	M	-	-	-	-ve	-ve	+
R-13	96	+/+	M	-	-	-	-ve	-ve	+
R-14	96	+/+	M	-	-	-	-ve	-ve	+
R-15	96	+/+	M	-	-	-	-ve	-ve	+
R-16	96	+/+	M	-	-	-	-ve	-ve	+
R-17	96	+/+	M	-	-	-	-ve	-ve	+
R-18	96	+/+	M	-	-	-	-ve	-ve	+
R-19	96	+/+	M	-	-	-	-ve	-ve	+
R-20	96	+/+	S	-	-	-	-ve	-ve	+
R-21	72	+/+	S	-	-	-	-ve	-ve	+

R-22	72	+/+	S	-	-	-	-ve	-ve	+
R-23	72	+/+	S	-	-	-	-ve	-ve	+
R-24	72	+/+	S	-	-	-	-ve	-ve	+
R-25	72	+/+	S	-	-	-	-ve	-ve	+
R-26	72	+/+	S	-	-	-	-ve	-ve	+

Table 1. Showing bacterial isolates and Biochemical characterization of *Rhizobium spp*

The composition of %G+C content with slight variation shows similar base sequences thus giving emphasis on relatedness among species in contrast to dissimilarity as observed in PCR-based observations. Samples analyzed presently, amongst 26 strains i.e., R1 to R26, the Tm ranged between 94.3 to 95.6°C. However, % G+C content of isolated strains ranges between 60.7 to 63.9%. All the bacteria tested and examined for Tm values and G+C content, were similar with narrow range of difference with respect to % G+C. Thus present observation as listed in provides most similarity amongst microbial isolates in relation to DNA as a parameter. Furthermore, the pattern of genetic diversity was studied using known and established molecular biotyping methods. Genotyping of the isolates was done by using molecular methods. *Taq I* restriction enzyme when used to have 16S rRNA digestion the group of strains showed variation in pattern on DNA profiling studies with 2.0 % agarose (Fig. 1& 2). In the presence of *Taq I* the digestion of 16S rDNA showed bands between 100 bp and 500bp. DNA profile once put on computation data on NTSYS using unweigheted pair group method with arithmetic averaging (UPGMA) over *Taq I* digested rDNA, *Rhizobium* isolates. The dendrogram shows divergence at 100% similarity into three broader groups and S19 strain shows 80% dissimilarity with all the strains isolated from agricultural soil of Madhya Pradesh. The significance of nodulation by *Rhizobium* also throws several genetic factor related to establishment of symbiotic relationship. Therefore, the study of genetic variation with existing molecular techniques along with biochemical analysis helped in specifying and determining phylogeny amongst isolated *Rhizobium* cultures.



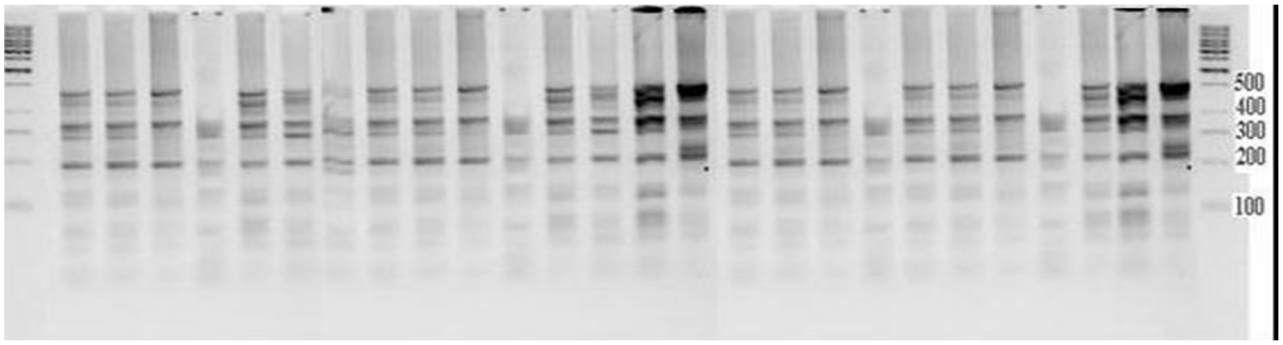


Figure 1: Restriction enzyme Taq I treated 16s rDNA segments of isolated strains of *Rhizobium spp.* on 2% agarose gel

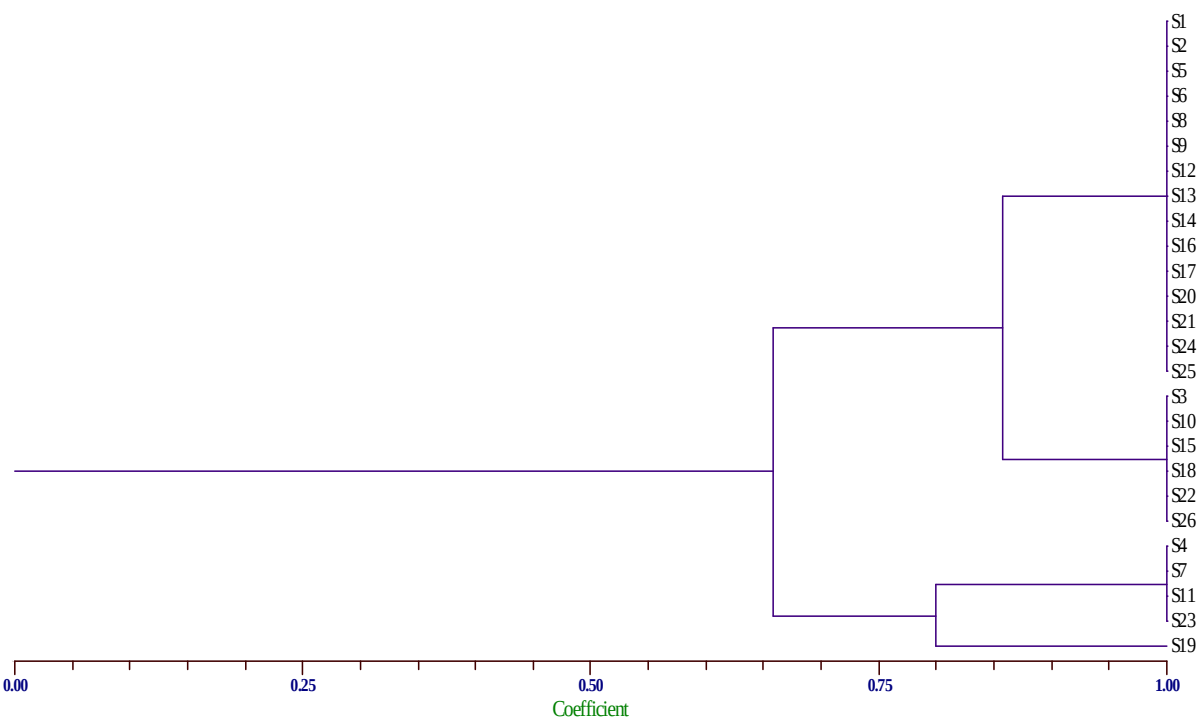


Fig.: 2. Dendrogram based on analysis of 16SrDNA segment with restriction enzymes Taq I of R*hizobium* using Jaccord's Coefficient and UPGMA cluster analysis method

Conclusions

Diversity in soil bacteria has been revealed by many studies and almost all of the data reported previously indicate that there is a high level of genetic diversity in these bacteria. An assessment of the genetic diversity and genetic relationships among strains could provide valuable information about bacterial genotypes that are well adapted to a certain environment (Niemann et al., 1997). On the basis of different physiological and biochemical parameter studied, it seems all the strain of *Rhizobium* sp. are same. Findings on the isolated *Rhizobium* strains from agricultural soils of M.P. with regard to the level of gene sequences will help establish the improved strains as biofertilizers.

The utility of present observation falls with relative similarity between patterns of genome i.e., alignment of bases of DNA, although preference was given to the pattern of base-sequences in NTSYS based genomic analysis and give an insight on molecular orientation of *Rhizobium* species occurring naturally in the agricultural soils of Madhya Pradesh.

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A Brief Author Biography

1st Author Name – Dr. Vinay Oraon
Designation: Lecturer
Qualification: M.Sc. Ph.D.
Research Area: Bacteriology, Bioinformatics, Molecular Biology and Phylogenetics
e-mail: vinay2079@gmail.com

2nd Author Name – Dr. Kiran Singh
Designation: Professor & HOD
Qualification: M.Sc. Ph.D. from BHU
Research Area: Meta genomics, Molecular Biology and Phylogenetics
e-mail: kiransingh@manit.ac.in

