U.P. Shrivastava (2017) Int. J. Appl. Sci. Biotechnol. Vol 5(1): 72-80
DOI: 10.3126/ijasbt.v5i1.17029

Research Article

Molecular Diversity Assessment of Plant Growth Promoting Rhizobacteria Using Denaturing Gradient Gel Electrophoresis (DGGE) of 16s rRNA Gene

Umesh Prasad Shrivastava*

Department of Botany, tribhuvan University, Thakur Ram Multiple Campus, Birgunj, Nepal

*Email: upshrivastava@gmail.com

Abstract

The rhizobacteria were isolated from rhizosphere of rice plant of different fields of 4 districts of Nepal and 5 districts of Bihar and Uttar Pradesh, adjoining states of India with Nepal. The DGGE analysis was performed for diversity analysis. For the construction of dendrogram, 16S *rRNA* gene was amplified by two different sets of primers. The DGGE ladder consisting of PCR amplified products of nine pure bacterial cultures were obtained. The first DGGE ladder was prepared by 400 bp fragment of 16S rDNA with GC clamp and the second DGGE ladder was prepared with 200 bp fragment of 16S rDNA with GC clamp. The perpendicular DGGE of these amplicons based on their melting behavior clearly demonstrated separation of different isolates. The 16S rDNA fragment amplified with primer set of V2-V3 regions with GC clamp showed separation between 40-60% of denaturant. The DGGE profile based on primer set F352T and 519r for various bacteria present in soil samples of 5 districts of India and 4 districts of Nepal revealed that the number of bands which might be specific for diazotrophic isolates varied from 2 to 11. The dendrogram constructed based on DGGE profile of various samples of 5 districts of India and 4 districts of Nepal showed that all the samples could be clustered in nine groups with 58-96% similarity to each other. Among all these 37 samples, only Var-4 and Var-5 showed 100% similarity, no other samples from any site showed 100% similarity.

Keywords: DGGE; Molecular Diversity; Rhizobacteria; PGPR; Rice plant

Introduction

The fraction of soil adhering and surrounding to plant roots is defined as the rhizosphere. It is believed that rhizosphere plays import role in plant health and soil fertility. Root exudates selectively influence the growth of microbial populations by altering the presence of substrates in soil near roots (Jaeger et al., 1999; Yang and Crowley, 2000). The structural and functional diversity of rhizospheric microbial populations is supposed to be influenced by alterations in root exudation and rhizodeposition in different root zones, and in relation to soil type, plant species, growth stage, cultural practices, such as tillage and crop rotation and other environmental factors (De Leij et al., 1994; Latour et al., 1996; Westover et al., 1997; Grayston et al., 1998; Horwath et al., 1998; Lupwayi et al., 1998; Shrivastava, 2013b). Microorganisms present in rhizosphere in turn exert strong effects on plant growth and health by nutrient solubilisation (Shrivastava, 2015), N_2 fixation, or by the production of plant hormones (Höflich et al., 1994; van Loon et al., 1998; Shrivastava, 2013a) or by siderophore production (Shrivastava and Kumar, 2011) or by 1-Aminocyclopropane-1-Carboxylate Deaminase (ACCD) activity (Shrivastava and Kumar, 2013). Increased plant productivity also results from the suppression of deleterious microorganisms by antagonistic bacteria while soil-borne pathogens can greatly reduce plant growth. It has been evident influence on bacterial and fungal communities as well as on the plant (Watkinson, 1998).

A trusted technique for deciding the diversity of organisms in the environment and environmental samples has for quite some time been looked for by microbiologists. In recent days, denaturing inclination gel electrophoresis (DGGE) analysis of *16S rRNA* gene has been proved a better tool for complex microbial diversity study (Muyzer et al., 1993) and analyse the phylogenetic relationship among group individuals (Muyzer and de Waal, 1994; Muyzer et al., 1995). GC region rich PCR product of *16S rRNA gene* is utilized for diversity study (Wawer and Muyzer. 1995). One of the PCR primers includes a GC-rich sequence (GC clamp) on its 59 end that imparts melting stability to the PCR products in a denaturing gradient gel. As a result, all a similar size products are separated into distinct similar

bands during electrophoresis through an acrylamide gel that contains an increasing linear gradient of denaturants. Individual double-stranded DNA molecules denature along their length adjacent to the GC clamp according to their melting characteristics (i.e., sequences). This partial denaturation causes their migration to essentially halt at unique positions, forming discrete bands in the gel. Individual bands can be probed by Southern blotting (Abrams and Stanton. 1992; Muyzer et al., 1993), or they can be excised and the nucleotide sequences can be determined (Muyzer and de Waal, 1994; Muyzer et al., 1995). Useful information can also be obtained through analyses of banding patterns alone, since each band may represent a different microbial population.

The present study is an attempt to examine molecular diversity of rhizobacteria associated with the rice plant of Indo-Nepal border using the technique of DGGE.

Materials and Methods

Study Sites and Sample Collection

The rhizobacteria were isolated from rhizosphere of rice plant of different fields of Nepal and Bihar and Uttar Pradesh, only two states of India adjoining to the border area of Nepal.

Genomic DNA Isolation

5 mL of exponentially grown culture was harvested by centrifugation at 8000 rpm for 5 min. The pellet was washed with 1 mL STE (Sodium chloride-Tris-EDTA buffer) and resuspended in 567 µL TE buffer. 30 µL SDS (10%) and 3 µL proteinase K (20 mg/mL) were added and then the samples were properly mixed by vortexing and incubated for 1 h for complete lysis of cells. Thereafter, 100 µL 5 M NaCl was added followed by the addition of 80 µL CTAB/NaCl and the tube was incubated at 65ºC for 10 min. Equal volume of phenol/chloroform/ isoamyl alcohol (25:24:1) was added to the suspension, mixed and spun at 12000 rpm for 10 min at 4ºC. Upper aqueous phase was collected carefully in fresh centrifuge tube (1.5 mL) and again equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed and spun as mentioned above. Again upper clear aqueous phase was collected in fresh centrifuge tube and then 2 µL of DNase inactivated RNase (US Biologicals, Massachusetts, USA) from 10 mg/mL stock was added followed by incubation at 37ºC for 1 h. DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of isopropyl alcohol. The precipitated DNA was washed twice with 70% ethanol, and the DNA pellet was air-dried. Finally, DNA was suspended in minimal volume of TE. Electrophoresis of genomic DNA was performed in a horizontal slab gel of 0.8 % agarose in a LKB 2012 Maxiphor Electrophoresis Unit attached with LKB 2301 Macro Drive Power Supply (Hoefer Scientific Instruments, San Fransisco, CA, USA). Ethidium bromide was present in the gel at a final concentration of 0.5µg/mL. 1 X TAE buffer was used for electrophoresis. DNA samples were prepared in 1 X gel loading buffer and the samples were run at 50 V for 3 h. The genomic DNA was monitored under long wave UV-light by a trans-illuminator (Bio-Rad Laboratories, USA).

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

The DGGE analysis was performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). PCR-DGGE amplicons were loaded in polyacrylamide gels (8% wt/vol acrylamide in 0.5· TAE buffer) with denaturant gradients of 40–70% for 16S rDNA-DGGE. The gels were electrophoresed at a constant voltage of 20 V for 15 min followed by 5 h 30 min at 200 V. After electrophoresis, gels were stained with ethidium bromide for 5 min and destained in distilled water for 20 min with agitation, after which their photograph was taken with gel documentation unit (Bio-Rad Laboratories, USA). Construction of dendrogram was performed with the help of NTSYSp_c software, version 2.11a (Exeter Software, Setauket, NY). Similarities between each pair of isolates were estimated from the proportion of shared restriction fragments/amplicons identified in all the strains examined. Dendrograms were constructed from the similarity matrix by the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) method.

Reagents for DGGE and CDGE

40% Acrylamide/Bis (37.5:1)

40% Acrylamide/Bis (37.5:1) was prepared using 38.93 g Acrylamide & 1.07 g Bis-acrylamide to make it 100.0 ml by adding DDW. Prepared solution was Filter through a 0.45 µm filter and store at 4 °C.

10% (w/v) Ammonium persulfate

10% (w/v) Ammonium persulfate was generally freshly prepared. Sometimes stored at -20 ºC for about a week, if necessary.

50X TAE Buffer

50X TAE Buffer was prepared by 242.0 g of Tris base (2M), 57.1 mL of glacial acetic acid (1M), 100.0 mL of 0.5 M EDTA, pH 8.0 (50 mM) to make 1,000.0 mL after adding DDW. After mixing, it was autoclaved for 20–30 min and stored at room temperature.

1X TAE Running Buffer

140 mL of 50X TAE buffer was added in 6,860 mL of DDW to make 7,000 mL of 1X TAE Running Buffer

Working reagents required for DGGE/ CDGE gel preparation is shown in Table 1 and 2X Gel loading dye is shown in Table-1

.

Table 1: Working reagents required for DGGE/ CDGE gel preparation

Reagents	Gel %	Denaturing Percentage							
		20%	45%	50%	53%	55%	60%	80%	100%
	6%	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
40% Acrylamide (mL)	7%	4.375	4.375	4.375	4.375	4.375	4.375	4.375	4.375
	8%	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
50X TAE buffer (µL)		500.0	500.0	500.0	500.0	500.0	500.0	500.0	500.0
Formamide (mL)		2.0	4.5	5.0	5.3	5.5	6.0	8.0	10.0
Urea (g)		2.1	4.725	5.25	5.565	5.775	6.3	8.4	10.5
TEMED (µL)		30	30	30	30	30	30	30	30
10% APS ((µL)		90	90	90	90	90	90	90	90
Total volume with DDW (mL)		25	25	25	25	25	25	25	25

10% APS and TEMED were added quickly, mixed properly and cast the gel immediately

Table 2: 2X Gel loading dye

Reagent	**Amount (**mL)	**Final Concentration**
2% Bromophenol blue	0.25	0.05%
2% Xylene cyanol	0.25	0.05%
100% Glycerol	7.0	70%
DDW	2.5	
Total volume	10.0	

Stored at room temperature.

Amplification of 16S rRNA gene with GC clamp for DGGE and CDGE

16S rRNA gene (400 bp with GC clamp) was amplified with the forward primer containing GC clamp at 5' end (Forward:5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TGG CGG ACG GGT GAG TAA-3' and Reverse: 5'-CGT ATT ACC GCG GCT GCT GG-3'). Amplification of 16S rRNA gene was done with thermal profile set at; initial denaturation at 95ºC for 3 min, 35 cycles of denaturation at 94ºC for 1 min and annealing at 70ºC for 20 s without any extension and storage at 4ºC. 50 µL of reaction mixture contained 36.6 µL of MQ water, 1X *Taq* DNA polymerase assay buffer, 5 mM of each dNTPs, 25 pmol of each primer, 1.5 U of *Taq* DNA polymerase and 250 ng of each DNA template. To examine the effect of annealing temperature on the amplification of desired band and DGGE profile, various annealing temperatures (45-72ºC) were tested, the other steps being identical to those of the initial PCR program. Amplification of desired gene was found in each annealing temperature used but non specific bands also appeared, the desired result without non specific band was obtained at 70ºC.

16S rDNA (200 bp with GC clamp) was amplified with the forward primer containing GC clamp at 5' end (F352T:5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C- 3' and 519r: 5'-ACC GCG GCT GCT GGC AC- 3') in 25 µl of reaction mixture containing 1×PCR buffer, 2.5mM $MgCl_2$(Bangalore Genei, India), 100 ng of the template DNA, 25.0 pmol each of the forward and reverse primers,

250 µM each of dNTPs, and 1 U of *Taq* DNA polymerase (Bangalore Genei, India). The touchdown PCR program was performed which consisted of an initial denaturation at 95°C for 5 min, followed by 6 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min, in which the annealing temperature was reduced by 0.5°C/cycle from the preceding cycle, and then 24 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 30 s; and a final extension at 72°C for 5 min.

Perpendicular DGGE

Perpendicular DGGE was performed with a "The Decode Universal Mutation Detection System" (Bio-Rad Laboratories, USA). A uniform gradient gel of 0% to 100% denaturant was prepared which was changed several times so as to optimize suitable concentration and finally 20 to 60% denaturant was found optimal for the best result. Gels of 6% polyacrylamide (37:1 acrylamide-bisacrylamide) were prepared at every time. The gel was kept for polymerization for 1-2 h, and then it was run at 100V for 3 h at constant temperature of 60°C in 1X TAE buffer. After electrophoresis, the gels were stained in 1 X TAE buffer containing ethidium bromide (0.1 mg/L) and photograph was taken with gel documentation unit (Bio-Rad Laboratories, USA).

Construction and Validation of the DGGE Ladder

The DGGE ladder consisting of PCR amplified products of nine pure bacterial cultures were obtained by using two different sets of primers. The first DGGE ladder was prepared by 400 bp fragment of 16S rDNA with GC clamp and the second DGGE ladder was prepared with 200 bp fragment of 16S rDNA with GC clamp (see Fig. 2 A, B). Gels of 8% acrylamide (37:1 acrylamide-bisacrylamide) were prepared with 40 and 60% denaturant for 400 bp primer and between 40 and 70% denaturant for 200 bp primer where 100% denaturant defined as 7M urea and 40% (vol/vol) formamide. The gels polymerized in about 2 h and were run at 100 V for 3 h and thereafter changed to 120V for 5 h and 50 minutes, the temperature was kept at 60°C constantly in 1 X TAE buffer. After electrophoresis, the gels were stained in 1 X TAE buffer containing ethidium bromide (0.1 mg/L) and photograph of gel was taken in gel documentation unit (Bio-Rad Laboratories, USA).

Results and Discussions

16S rRNA Gene Amplification and DGGE

For performing DGGE and its optimization, two primers V_2-V_3 regions with GC clamp (400 bp amplicon) and F352T-GC clamp and 519r (200 bp amplicon) were selected which showed amplification of desired amplicon in all the isolates. The perpendicular DGGE of these amplicons based on their melting behavior clearly demonstrated separation of different isolates. The 16S rDNA fragment amplified with primer set of V_2-V_3 regions with GC clamp showed separation between 40-60% of denaturant (Fig. 1) whereas amplicon amplified with F352T and 519r primer set was separated between 40-70% of denaturants.

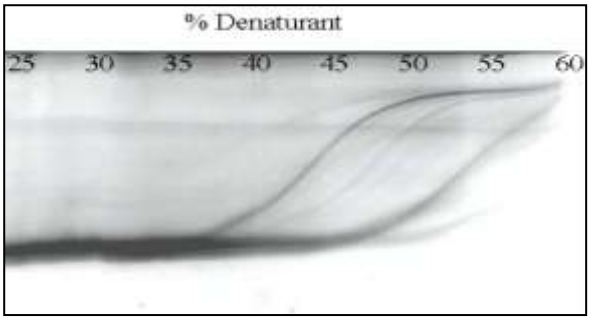

Fig. 1: Negative image of ethidium bromide stained perpendicular DGGE showing pattern of PCR amplified 16S rDNA fragments (~ 400 bp) of selected isolates.

Validation of the DGGE Identification Ladder

Nine identified strains of this study and one reference strain of *E. coli* JM109 were subjected to DGGE so as to obtain a standard profile of various fragments which could be used for the identification of unknown isolates. Accordingly, two sets of primers were used; i) the 16S *rRNA* gene primer of V_2-V_3 region with GC clamp of 400 bp in forward primer; and ii) 16S *rRNA* gene primer set namely, F352T and 519r. The pattern of DGGE profile for the first primer is shown in Fig. 2A and the profile of other primer is shown in Fig. 2B. It is evident with banding pattern of Fig. 2A that two strains of *Agrobacterium* sp. (AF-1D and BN-2A) showed band of equal size. Similarly, *Pseudomonas* sp. (AF-4B and PN-4D) showed homology in size of band which is evident from lane numbers 4 and 7. Other isolates such as *Serratia*, *Klebsiella* and *Microbacterium* showed distinct pattern and thereby could be easily separated and used as ladder for identification purpose. However it is evident that the first pair of primer could not show diversity on strain level. To resolve this problem the second primer was used. It is evident from Fig. 2B that the primer set F352T and 519r produced a reproducible and different banding pattern for each strain. Once, it became clear that the primer set F352T and 519r could produce a different size of band in different strains, we employed this primer set for diversity analysis of unknown samples.

DGGE Analysis of the Diazotrophic Rhizospheric Bacteria

The DGGE profile based on primer set F352T and 519r for various bacteria present in soil samples of 5 districts of India and 4 districts of Nepal revealed that the number of bands which might be specific for diazotrophic isolates varied from 2 to 11. Analysis of bands showed that the highest number (11) of isolates was present in BN-3 location of Bara district of Nepal and the lowest number (2) of isolates in MGI-2 location of India and RN-3 of Nepal. Further analysis revealed appreciable diversity in diazotrophic populations of all the districts of India and Nepal (Fig. 3).

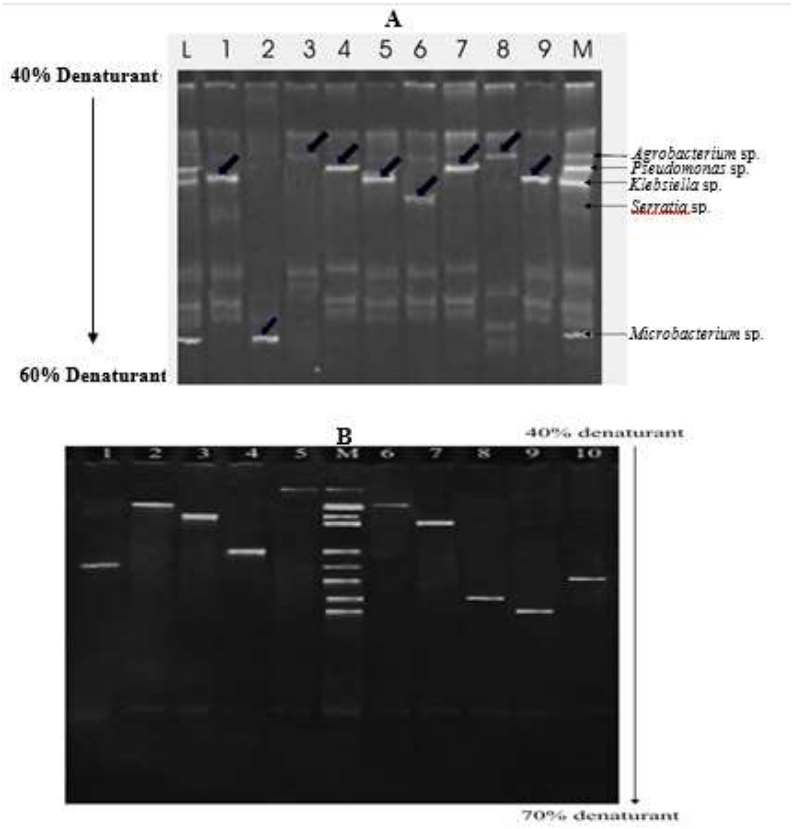

Fig. 2: DGGE of 16S rDNA amplicon for the selection of the DGGE identification marker ladder.

[A. Based on 400 bp fragment with GC clamp of 16S rDNA: Lane L, amplicon of mixed DNA templates of 1 to 9; Lane1-*Klebsiella* sp. ECI-10A; 2-*Microbacterium* sp. ECI-12*A*; 3-*Agrobacterium* sp. AF-1D; 4-*Pseudomonas* sp. AF-4*B*; 5-*Klebsiella* sp. AF-4C; 6-*Serratisia* sp. AF-5A; 7-*Pseudomonas* sp. PN-4D; 8-*Agrobacterium* sp. BN-2A; 9-*Klebsiella* sp. BN-4A and Lane M, PCR mixed amplicons of isolates 1 to 9.

B. Based on 200 bp fragment with GC clamp of 16S rDNA: Lane1-*Klebsiella* sp. ECI-10A; 2-*Microbacterium* sp. ECI-12A; 3-*Pseudomonas* sp. AF-4B; 4-*Klebsiella* sp. AF-4C; 5-*Serratisia* sp. AF-5A; M- PCR mixed amplicons of isolates 1 to 10; 6-*Pseudomonas* sp.PN-4D; 7-*Agrobacterium* sp.BN-2A;8-*Klebsiella* sp. BN-4A; 9-*E. coli*. strain JM109, and 10-*Agrobacterium* sp.AF-1D]

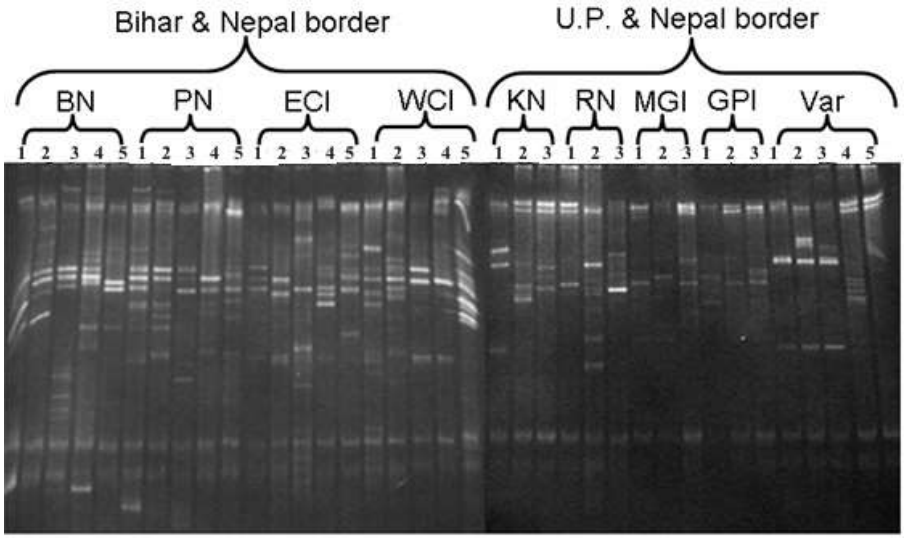

Fig. 3: Ethidium bromide stained gel showing DGGE analysis of 16S rDNA amplicons (200 bp with GC clamp) generated by PCR from diazotrophic bacteria recovered from rhizosphere region of rice plants collected from certain districts of border area of India and Nepal.

[BN- Bara Nepal, PN- Parsa Nepal, ECI- East Champaran India, WCI- West Champaran India, KN- Kaski Nepal, RN- Rupandehi Nepal, MGI- Maharajganj India, GPI- Gorakhpur India, Var- Varanasi India.]

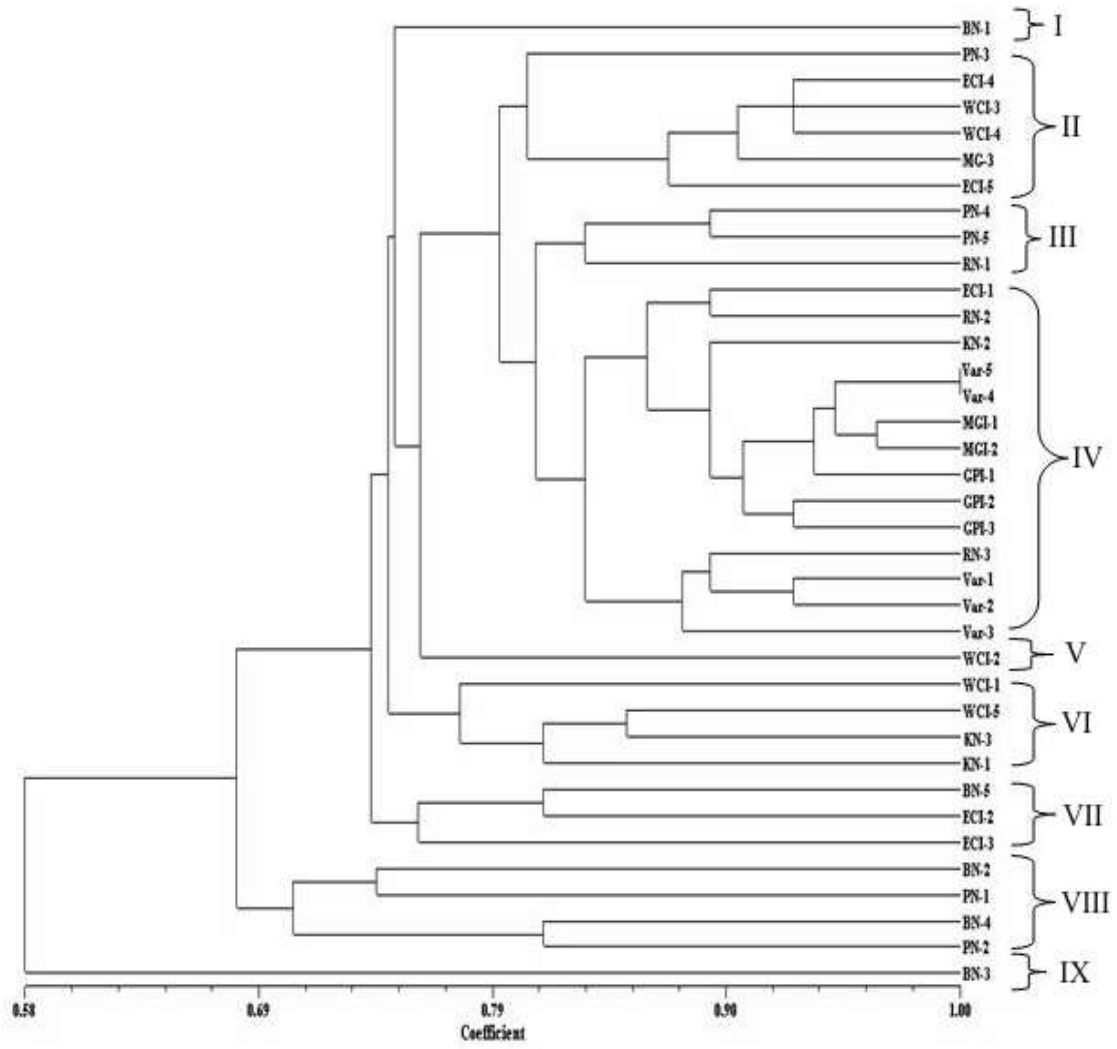

Fig. 4: Dendrogram showing diversity based on DGGE profile of 16S rRNA gene of diazotrophic bacterial population of the rice rhizosphere of Indo-Nepal border.

Dendrogram Based on DGGE Profile

The dendrogram constructed on the basis of DGGE profile of various samples of 5 districts of India and 4 districts of Nepal is shown in Fig. 4. It is evident from the results that all the samples could be clustered in nine groups with 58-96% similarity to each other. Among all these 37 samples, only Var-4 and Var-5 showed 100% similarity, no other samples from any site showed 100% similarity.

Discussion

In general, genetic information on natural bacterial communities and correlation with their specific environmental conditions are limited and such study has not been made in Indo-Nepal border region until now. With the advent of molecular techniques, elucidation of microbial community structure, physiological functions of each member of the community, and genetic interaction in nature have become easier. In the present study we have employed DNA fingerprinting techniques to decipher phylogenetic relationship among diazotrophic bacterial populations obtained from the rhizosphere of rice plant, specifically from Indo-Nepal border, after enrichment in modified JNFb$^-$ medium (Döbereiner, 1995; Shrivastava, 2013a, b, c). Accordingly we selected 16S rDNA to establish phylogenetic relationships among various isolates because this gene is universally present and there are many variable regions in alternate fashion between conserved regions which makes easier to compare the bacterial populations. The variable region between any two conserved regions provides easy way to amplify short fragment of sequence, which helps for sequence-based reliable phylogenetic analysis (Maidak *et al*., 2001; Shrivastava, 2016).

There are several approaches which are frequently used in phylogenetic studies specifically based on the rDNA sequences; electrophoretic pattern obtained after restriction digestion of the 16S rDNA amplicon is one of the simple and convenient methods for deriving phylogenetic relatedness. This technique, popularly known as Amplified Ribosomal DNA Restriction Analysis (ARDRA) has been successfully used to analyze phylogenetic relatedness in the

bacterial communities (Heyndrickz *et al*., 1996). Taking into account the above approach, 16S rDNA was amplified in the selected 33 isolates with a pair of primer designed from the conserved region of the gene (Weisburg, 1991) and digested with two tetra-cutting restriction endonucleases in order to reveal the restriction fragment length polymorphism of 16S rDNA. On the basis of shared DNA fragments, the isolates were found to be phylogenetically different among each other. Similar to our study, PCR-RFLP analysis of small subunit of rDNA has also been used for grouping large number of rhizobia nodulating Australian *Acacia* sp. (Lafay and Burdon, 2001). This method has also been applied in biodiversity study of an *Acinetobacter* population isolated from activated sludge (Barbeiro and Fani, 1998). The phylogenetic distances among the isolates are deciphered by a dendrogram, which is built after calculating the phylogenetic distances on the basis of number of shared DNA fragments. In the present investigation level of similarity varied from 71% to 98% in different isolates suggesting significant genetic relatedness.

ARDRA data showed genetic diversity among all the 33 isolates but for further confirmation another approach which shows multilocus analysis, ERIC-PCR was preferred and used for rapid study of molecular polymorphism among bacterial isolates. Any one PCR-based assay is not sufficient for molecular diversity analysis or taxonomic assignment therefore it is always useful to apply a number of molecular tests. In general the distribution of repetitive sequences (BOX and ERIC) has been employed in elucidating the genomic diversity in a number of bacteria (Selenska-Pobel *et al.*, 1995). In our study, the bacterial isolates were analyzed by ERIC-PCR where 2-7 bands were generated and 76-97% similarity was observed. Reproducible result of the ERIC profile was evident by the fact that identical banding pattern was obtained when replicate samples were run together. ERIC-PCR- generated fingerprints, allowed us to grouping the isolates into nine clusters. Similar to our approach other workers have also applied ERIC- and BOX-PCR for studying the bacterial diversity of rice-associated diazotrophic bacteria (Mirza *et al.*, 2000) and putative endophytic bacteria isolated from seedlings of rice (Stoltzfus *et al.*, 1997).

The sequencing of 16S rDNA and phylogenetic analysis approach for identification and similarity studies have been used in this study. The sequences extracted from amplified DNA represented mainly three bacterial groups (α-proteobacteria, γ-proteobacteria and actinobacteria). The isolate AF-5A, an isolate from Varanasi showed similarity with *Serratiamarcescen* which is consistent with the previous report (Grimont *et al.*, 1981).*S. marcescens* isolated from soil or plant materials and non-clinical *S. marcescens* are regarded as beneficial rhizobacterium due to its chitinase activityand its ability to induce systematic resistance in plants (Kalbe *et al.*, 1996). In another report*S. marcescens* strain IRBG 500 capableof N_2fixing, was isolated from the roots of rice, and had a growth-promoting effect on rice seedlings (Gyaneshwar *et al.*, 2001;Tan *et al.*, 2001).Our three isolates (ECI-10A, AF-4C and BN-4A) belong to *Klebsiella* sp, a finding similar to the report of Bagwell *et al.*, 1998; Lovell *et al.*, 2000. In a recent report, various strains of *Pseudomonas* sp. and *Agrobacterium* sp. showing plant growth promoting potentials have been isolated from the rhizosphere of Chinese cabbage (Yim *et al.*,2009).Our findings showed that isolates PN-4D and AF-4B show 99.0% and 100.0% similarity with *Pseudomonas* sp., thereby can be easily placed in this genus. AF-1D and BN-2A showed 100.0% similarity with *Agrobacterium* sp. and resembles to the isolate reported by Yim *et al*. (2009).

In addition to DNA fingerprinting, protein profile of selected eight isolates was also analyzed to establish phylogenetic relationship. Clustering of isolates on the basis of SDS-PAGE pattern clearly demonstrated that they were clustered in 4 groups. Electrophoretic pattern showed subtle differences among all the isolates suggesting that they are not closely related species or strain of any individual bacterium. Results of DNA finger printing and protein profile allow us to conclude that molecular diversity does exist among all these rhizospheric diazotrophs.

At present denaturing gradient gel electrophoresis (DGGE) of 16S rDNA gene has become more common for a direct comparison of structural diversity among different microbial communities. This molecular method has been used by many researchers to detect shifts of environmental changes such as heavy metals contaminations, agricultural practices, seasonal fluctuations, fluctuations due to temperature and soil particle size fractions on the relative frequency of dominant phylotypes of the whole community (Cherif *et. al.*, 2008). In the present study, DGGE profiles from 5 districts of India and 4 districts of Nepal showedcomplex banding patterns, which indicate the high level of diversity of bacteria. Comparison between DGGE patterns, based on the comparison of the dominant species colonizing each rice rhizosphere sample showed close similarities between isolates of India and Nepal a fact similar to previous report (Cherif *et. al.*, 2008). Predominance of gram-negative bacteria has been reported in the rice straw (Sugano *et al.*, 2005) and rice straw compost (Tanahashi *et al*., 2005) added into the flooded paddy field soil. Furthermore members of α-, γ- and δ-proteobacteria, Chloroflexi, Bacteroidetes, Acidobacteria, Verrucomicrobia and Spirochaetes were found to be close relatives of the predominant bacteria present in rice straw (Sugano *et al*., 2005) and/or in rice straw compost (Tanahashi *et al.*, 2005). In the present study sequencing of some of the isolates showed predominance of gram-negative bacteria belonging to α- and γ- proteobacteria and matched with the previous report (Tanahashi *et al.*, 2005). Altogether DGGE profile of rice rhizosphere of Indo-Nepal

border showed high diversity, however detailed study is required to resolve the total bacterial population structure and range of diversity prevailing therein.

Acknowledgement

Financial supports by ICCR, Government of India is acknowledged. The author is also grateful to A. Kumar, Professor, School of Biotechnology, Banaras Hindu University, Varanasi, India for guidance and all facilities.

References

Abrams ES and Stanton VP (1992) Use of denaturing gradient gel electrophoresis to study conformational transitions in nucleic acids. *Methods Enzymol* **212**: 71–104. DOI: 10.1016/0076-6879(92)12006-C

Bagwell CE, Piceno YM, Ashburne-Lucas A, Lovell CR (1998) Physiological diversity of the rhizosphere diazotroph assemblages of selected salt marsh grasses. *Appl Environ Microbiol* **64:** 4276-4282.

Cherif H, Ouzari H, Marzorati M, Brusetti L, Jedidi N, Hassen A, Daffonchio D (2008) Bacterial community diversity assessment in municipal solid waste compost amended soil using DGGE and ARISA fingerprinting methods. *World J Microbiol Biotechnol* **24**:1159-1167. DOI: 10.1007/s11274-007-9588-z

De Leij FAAM, Whipps JM and Lynch JM (1994) The use of colony development for the characterization of bacterial communities in soil and on roots. *Microb Ecol* **27**: 81–97. DOI: 10.1007/BF00170116

Döbereiner J (1995) Isolation and identification of aerobic nitrogen-fixing bacteria from soil and plants. In: Alef K, Nannipieri P (eds) *Methods in applied soil Microbiol and biochemistry*, Academic Press, London, pp 134-141.

Grayston SJ, Wang S, Campbell CD and Edwards AC (1998) Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol Biochem* **30**: 369–378. DOI: 10.1016/S0038-0717(97)00124-7

Grimont PAD, Grimont F, Starr MP (1981) *Serratia* species isolated from plants. *Curr Microbiol* **5:** 317-322. DOI: 10.1007/BF01567926

Gyaneshwar P, James EK, Mathan N, Reddy PM, Reinhold-Hurek B, Ladha JK (2001) Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. *J Bacteriol* **183:** 2634-2645. DOI: 0.1128/JB.183.8.2634-2645.2001

Heyndrickz M, Vautenin L, Vandamme P, Kersters K, de Vos P (1996) Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *J Microbiol Methods* **26**: 247-259. DOI: 10.1016/0167-7012(96)00916-5

Höflich G, Wiehe W and Kühn G (1994) Plant growth stimulation by inoculation with symbiotic and associative rhizosphere microorganisms. *Cellular and Molecular Life Sciences* **50**(10): 897-905. DOI: 10.1007/BF01923476

Horwath WR, Elliott LF and Lynch JM (1998) Influence of soil quality on the function of inhibitory rhizobacteria. *Lett Appl Microbiol* **26**: 87–92. DOI: 10.1046/j.1472-765X.1998.00278.x

Jaeger CH III, Lindow SE, Miller W, Clark E and Firestone MK (1999) Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. *Appl Environ Microbiol* **65**: 2685–2690.

Kalbe C, Marten P, Berg G (1996) Strains of the genus Serratia as beneficial rhizobacteria of oilseed rape with antifungal properties. *Microbiol Res* **151**: 433-439. DOI: 10.1016/S0944-5013(96)80014-0

Lafay B and Burdon JJ (2001) Small-subunit rRNA genotyping of Rhizobia nodulating Australian Acacia spp. *Appl Environ Microbiol* **67**: 396-402. DOI: 10.1128/AEM.67.1.396-402.2001

Latour X, Philippot L, Corberand T and Lemanceau P (1996) The establishment of an introduced community of fluorescent pseudomonads in the soil and in the rhizosphere is affected by the soil type. *FEMS Microbiol Ecol* **30**: 163–170. DOI: 10.1111/j.1574-6941.1999.tb00645.x

Lovell CR, Piceno YM, Quattro JM, Bagwell CE (2000) Molecular analysis of diazotroph diversity in the rhizosphere of the smooth cordgrass, Spartina alterniflora. *Appl Environ Microbiol* **66**: 3814-3822. DOI: 10.1128/AEM.66.9.3814-3822.2000

Lupwayi NZ, Rice WA and Clayton GW (1998) Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. *Soil Biol Biochem* **30**: 1733–1741. DOI: 10.1016/S0038-0717(98)00025-X

Maidak BL, Cole JR, Lilburn TG, Parker CT, Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM, Tiedje JM (2001) The RDP-II (ribosomal database project). *Nucleic Acids Res* **29**: 173-174. DOI: 10.1093/nar/29.1.173

Mirza MS, Rasul G, Mehnaz S, Ladha JK, So RB, Ali S, Malik KA (2000) Beneficial effects of inoculated nitrogen-fixing bacteria on rice. In: JK Ladha and PM Reddy (eds) The quest for nitrogen fixation in rice. International Rice Research Institute, Manila, Philippines, pp 191-204.

Muyzer G and de Waal EC (1994) Determination of the genetic diversity of microbial communities using DGGE analysis of PCR-amplified 16S rDNA. In: Stal LJ and Caumette P (eds) *Microbial mats: structure, development and environmental significance.* Springer-Verlag, Heidelberg, Germany, 207–214. DOI: 10.1007/978-3-642-78991-5_21

Muyzer G, De Waal EC and Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**: 695–700.

Muyzer G, Teske A, Wirsen CO, and Jannasch HW (1995) Phylogenetic relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**:165–172. DOI: 10.1007/BF02529967

Selenska-Pobell S, Evguenieva Hackenberg E, Schwickerath O (1995) Random and repetitive primer amplified polymorphic DNA analysis of five soil and two clinical isolates of Rahnella aquatilis. *Syst Appl Microbiol* **18**:425-438. DOI: 10.1016/S0723-2020(11)80435-2

Shrivastava UP (2013a) Characterization of Diazotrophic Rhizobacteria under Various Conditions. Int. J. Appl. Sci. Biotechnol. 1(3): 110-117. DOI: 10.3126/ijasbt.v1i3.8607

Shrivastava UP (2013b) Isolation and initial characterization of diazotrophic plant growth-promoting rhizobacteria (PGPR) from rice rhizosphere of Parsa and Bara district of Nepal. *Int. J. Pharma. Life Sci.* **4**: 2481-2488.

Shrivastava UP (2013c) Isolation and Characterization of Plant Growth Promoting Rhizobacteria (PGPR) from Rice Rhizosphere of Varanasi and East Champaran District of India. Academic Voices 3: 81-90 DOI: 10.3126/av.v3i1.9542

Shrivastava UP (2015) Isolation and Characterization of Phosphate Solubilizing Diazotrophic Bacteria from Rhizosphere of Rice Plant of Indo-Nepal Border. Academic Voices 5: 79-87. DOI: 10.3126/av.v5i0.15856

Shrivastava UP (2016) Diversity of Plant Growth Promoting Rhizobacteria (PGPR). Int. J. Grad. Res. Rev. 2(3): 56-64.

Shrivastava UP and Kumar A (2011) Biochemical Characterization of Siderophore Producing Plant Growth Promoting Rhizobacteria (PGPR) Isolated from Rice Rhizosphere. Nep. J. Integrated Sci. 1: 31-37

Shrivastava UP and Kumar A (2013) Characterization and optimization of 1-Aminocyclopropane-1-Carboxylate Deaminase (ACCD) activity in different rhizospheric PFPR along with Microbacterium sp. strain ECI-12A. Int. J. Appl. Sci. Biotechnol. 1(1): 11-15. DOI: http://dx.doi.org/10.3126/ijasbt.v1i1.7921

Stoltzfus JR, de Bruijn J (2000) Evaluating diazotrophy, diversity, and endophytic colonization ability of bacteria isolated from surface-sterilized rice. In: The quest for nitrogen fixation. Ladha JK and Reddy PM, (eds.), International Rice Research Institute, Manila, Philippines, pp 63-91.

Sugano A, Tsuchimoto H, Tun CC, Asakawa S, Kimura M (2005) Succession and phylogenetic profile of eubacterial communities in rice straw incorporated into a rice field: Estimation by PCR-DGGE analysis. *Soil Sci Plant Nutri* **51**: 51-60. DOI: 10.1111/j.1747-0765.2005.tb00006.x

Tan Z, Hurek T, Vinuesa P, Muller P, Ladha JK, Reinhold-Hurek B (2001) Specific detection of Bradyrhizobium and Rhizobium strains colonizing rice (Oryza sativa) roots by 16S-23S Ribosomal DNA Intergenic Spacer-Targeted PCR. *Appl Environ Microbiol* **67**: 3655-3664. DOI: 10.1128/AEM.67.8.3655-3664.2001.

Tanahashi T, Murase J, Matsuya K, Hayashi M, Kimura M, Asakawa S (2005) Bacterial communities responsible for the decomposition of rice straw compost in a Japanese rice paddy field estimated by DGGE analysis of amplified 16S rDNA and 16S rRNA fragments. *Soil Sci Plant Nutri* **51**: 351-360. DOI: 10.1111/j.1747-0765.2005.tb00040.x

Van Loon LC, Bakker PAHM and Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol* 36: 453–483. DOI: 10.1146/annurev.phyto.36.1.453

Watkinson AR (1998) The role of the soil community in plant population dynamics. *Trends in ecology & evolution* **13**: 171–172. DOI: 10.1016/S0169-5347(98)01329-9

Wawer C, and Muyzer G (1995) Genetic diversity of Desulfovibrio spp. In environmental samples analyzed by denaturing gradient gel electrophoresis of (NiFe) hydrogenase gene fragments. *Appl Environ Microbiol* **61**: 2203–2210.

Weisburg WG, Barns SM, Pelletier DA, Lane DL (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697-703. DOI: 10.1128/jb.173.2.697-703.1991

Westover KM, Kennedy AC and Kelley SE (1997) Patterns of rhizosphere microbial community structure associated with co-occurring plant species. *J Ecol* **85**: 863–873. DOI: 10.2307/2960607

Yang C-H and Crowley D E 2000 Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl Environ Microbiol* **66**: 345–351. DOI: 10.1128/AEM.66.1.345-351.2000

Yim W, Poonguzhali S, Madhaiyan M, Palaniappan P, Siddikee M, Sa T (2009) Characterization of plant-growth promoting diazotrophic bacteria isolated from field grown Chinese cabbage under different fertilization conditions. *J Microbiol* **47**: 147-155. DOI: 10.1007/s12275-008-0201-4