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Genetic Diversity Analysis of Wheat Rhizobacterial Isolates from North Indian Soils Showed the Unique Occurrence of *Sporosarcina* Related Species

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Abstract:
In natural ecosystem an intimate relationship between staggering bacterial diversity and plants are observed and such a relationship can be friendly or hostile. Soil being a base of plant root – microbe interaction, serves as an excellent source of research at genetic levelowing to new techniques related to sequencing and metagenomics. The microbial diversity within rhizosphere of wheat plant from agricultural soils of north India were characterized by direct soil DNA extraction, restrictinon fragment length polymorphism (RFLP) pattern analysis of 16S gene and subsequent sequencing of V6-V9 region of 16S rRNA for clones having unique restriction patterns. Analysis of wheat rhizosphere from different regions of North India showed the presence of rich microbial diversity. Both gram positive and Gram negative bacterial communities, reported to be promoting growth activity, were found to be present in all the regions of wheat cultivation. Most of the bacteria belonged to phylum proteobacteria and firmicutes, uncultured bacterium belonging to unknown phylogeny was also found to be present. Although no regional variation was found, the occurrence of Sporosarcina related species were unique to wheat rhizosphere.

Keywords: *Rhizosphere, microbial diversity, wheat rhizosphere, PGP, 16S gene sequencing*

1. Introduction

1.1. Rhizobacteria

Association of plants with microbes is although a symbiotic relationship but it may be understood as recruitment of beneficial microbes by plants to overcome adverse conditions of pathogenecity and to boost their immunity. As seeds germinate and roots grow through the soil the loss of organic material provides the driving force for the development of active microbial populations around the root, known as the rhizosphere effect (Morgan JAW, Whipps JM.2001). Although the stimulation in microbial activity is a general phenomenon largely involving saprotrophs, specific groups of symbionts may be selectively enhanced depending upon host plant. One such group of symbionts has been termed as plant growth promoting rhizobacteria. Plant growth promoting rhizobacteria are root colonizing bacteria that are associated with many plants and are commonly present in the environment. PGPRs have gained worldwide importance and acceptance for agricultural benefits (Feguiredo et al, 2010)

1.2. Bacterial Diversity

The bacterial community diversity or structure can be used as an indicator of perturbations or disturbances in the ecosystem (agroecosystems). Disturbances caused, could be due to the presence of a plant, or changes in agronomic practices. The presence of microrganisms in the soil will depend on the number and volume of available microhabitats and bacterial activity to the amounts of available metabolic substrates found in those microhabitats (Nannipierre et al 2003). It has been known for more than a century that the abundance of microbes in soil is directly proportional to the organic matter content. Thus soils receiving large amounts of organic residues support a larger microbial population. The rhizode position corresponds to 15-60% (30% in wheat) of the total photosynthetic production of the plant and conveys an important carbon and energetic source towards the microorganisms of the rhizosphere (Darrah PR, 1996). The 16S rRNA gene present in all prokaryotes is major target area for diversity related researches as it is highly conserved between different species of bacteria and archaea. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification. Although it was originally used to identify bacteria, 16S sequencing was subsequently found to be capable of reclassifying bacteria into completely new species, or even genera. It has also been used to describe new species that have never been successfully cultured.

1.3. Wheat Agroecosystem

In view of its global significance in agriculture production and human health, wheat agroecosystem has been studied extensively from the viewpoint of bacterial diversity across various regions of the world (S. Rawat et al, 2011). Wheat is grown in a variety of soils of India. Soils with a clay loam or loam texture, good structure and moderate water holding capacity are ideal for wheat cultivation (Ministry of Agriculture, India, 2013). In roots, rhizoplane and rhizosphere of Wheat crop, fungi tend to be most abundant in the integrated system or monoculture, and bacteria in the organic system (Leszek et al, 2014). A number of bacterial species associated with the plant rhizosphere belonging to genera *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* are able to exert a beneficial effect on plant growth (Aragno, 2005). Although the soil mediated microbe plant interaction has been studied extensively but its science is still growing.

2. Materials and Methods

2.1. Sample Collection

Soil samples under wheat cultivation were collected in the months of Jan-May from the upper layer (10-15 cm) of Wheat rhizospheric zones respectively during pre-harvest (Jan-March) and post-harvest (May) period from seven different North Indian locales namely Bijvasan, New Delhi (28.38°N ,77.13°E), Aligarh, U.P (27.88°N, 78.08°E), Kanpur, U.P (26.47°N, 80.35°E), Sonepat, Haryana (28.98°N, 77.02°E), Rohtak, Haryana (28. 54°N, 76. 34°E), Karnal, Haryana (29.68°N, 76.98°E) and Gaya, Bihar (24.78°N, 85.0°E). Soil from at least ten locations from each site was sampled, collected composited and homogenized by sieving and stored at 4°C until further processing.

All soil samples were subjected to physical and chemical analysis according to the standard procedures described by Olson (1984) and Soil Survey Staff (1993).

2.2. Direct Soil DNA Extraction

DNA extraction from soil samples was extracted using method ASM modified Zhou's method (Shagufta et al, 2012). 5g of soil sample and 13.5 ml of DNA extraction buffer (100mM TRIS–HCl, pH- 8.0; 1.5 M NaCl) were mixed. 100 µl of Proteinase K (10mg per ml) was added and the samples were incubated at 37º C for half an hour.1.5 % $CaCl_2$ was added and sample were vortexed for 30 seconds. 1.5 ml of 20% SDS was added and Samples were incubated at 70ºC for 15-20 min and centrifuged at 10,000 g for 10 min. The supernatant thus obtained was subjected to isopropanol precipitation and quantified by Ultraviolet (UV) spectrophotometry at 260 nm. Assessment of purity and quality of DNA was done spectrophotometrically by calculating A260/A280 and A260/230 ratios respectively.

2.3. Amplification of 16S gene

PCR procedure was performed in 50µl of reaction mixture containing 5µl of 10X reaction buffer (10mM Tris (pH 9.0 at 25˚C), 1.5 mM $MgCl_2$, 50mM KCl, and 0.01% gelatin) and 1U of Taq Polymerase (Banglore genei, India), 200µM of each dNTPs (Banglore genei, India), 10 pmole of 16 S gene specific universal primer set 1 (27F, 1525R; G Biosciences, India) and 10µl of tenfold diluted template concentration (50 ng) in a pTC thermal block (MyGenei32). Details of Primers and pTC conditions are given in table below (Table 1 & 2).

S.NO	Programme	Primer Set I	Primer Set II	Primer Set III
1.	Hot start	95˚ C for 3 min	94˚ C for 5 min	94˚ C for 5 min
2.	Denaturation	94˚C for 30 secs	94˚C for 1 min	95˚C for 1 min
3.	Annealing	50˚C for 30 secs	55˚C for 1 min	50˚C-55˚C for 30 secs
4.	Extension	72˚ C for 1 min	72˚ C for 2 min	72˚ C for 2 min
5.	Final extension	72˚ C for 7 min	72˚C for 10 min	72˚C for 10 min
6.	Total	30 cycles	35 cycles	35 cycles

Table 1: PCR cycling parameters

Primer	16S rDNA target (basenumber)	Primer sequence	References
PR338F	Bacteria V3 region (338-358) Set 3	5'AC TCC TAC GGG AGG CAG CAG 3'	(Lane, 1991)
PR518R	Universal V3 region (534-518) Set 3	5'ATT ACC GCG GCT GCT GG 3'	(Muyzer et al., 1993)
PR968F	Bacteria V6 region (968-983) Set 2	5'AA CGC GAA GAA CCT TAC 3'	(Nübel et al., 1996)
PR1406R	Bacteria V9 region (1406-1392) Set 2	5'ACG GGC GGT GTG TAC 3'	(Lane et al., 1988)
PR27F	Bacteria 16S rDNA (8-25) Set 1	5'AGA GTT TGA TCM TGG CTC AG 3'	(Gutleret al., 1996)
PR1525R	Bacteria 16S rDNA (1525-1507) Set 1	5'T/CGG GTC TCG CTC GTT G/ACC 3'	(Gutler et al., 1996)

Table 2: Oligonucleotide primers used for amplification of bacterial 16S rRNA gene.

2.4. AFLP Analysis and Sequencing of 16S gene

The amplicons generated from primer set 1 were gel purified and band extracted as per manufacturer's instruction (Gel extraction kit, Banglore Genei, India) and ligated into T-cloning vector using PCR cloning kit (Promega Corporation, USA) using the recommended ratio of the insert DNA and the vectors from 1:3 to 3:1. Recombinant colonies were selected for colony PCR using primer set 1. Re-

amplified products were restricted by 1U each of the three tetra cutter restriction enzymes, Hae *III,* Msp *I* and Sau *3AI* (New England Bio labs, Beverly Mass) in a final volume of 20µl, for 3 h at 37°C *in* a common reaction. The resultant products of digestion were separated on 2% agarose gel and banding pattern was visualized by ethidium bromide staining and UV-Vis illumination (Perkin Elmer UV- Vis illuminator, Shelton CT, USA).

RFLP pattern were analyzed visually and representative clone of each pattern were selected for amplification of variable region (V3 and V6 –V9) in a 50µl volumes containing 5µl of 10X reaction buffer (10mM Tris (pH 9.0 at 25˚C), 1.5 mM $MgCl_2$, 50mM KCl, and 0.01% gelatin) and 1U of Taq Polymerase (Banglore genei, India), 200µM of each dNTPs (Banglore genei, India), 10 pmole of each primer (G Biosciences, India) and 10 ng of template. The purified amplicon extracted using PCR purification kit (Mo bio) were then sequenced using 8µl big dye terminator ABI 3130 (4 capillary) or 3730Xl (96 capillary) electrophoresis instruments (applied Biosystems, Thermofisher Scientific, India). All the sequences were evaluated using CHECK-CHIMERA program and were compared to GeneBank database using BLASTn (http://blast.ncbi.nlm.nih.gov. Bethesda, MD, USA). Homologous sequences were retrieved from database and aligned with clone sequences using multiple sequence alignment tool clustalX version 2.1 and a newick tree was constructed by N-J methodology with 1000 bootstrap value using Seaview4 and viewed by MEGA version5.1. The 16S partial sequences reported in this paper have been deposited in the GenBank database under accession numbers KU659072-KU659082 (SMC1-5, SMM1, 4, 9, 12& 15) and KU664324-KU664325 (SMC6 & SMM17)

3. Results and Discussion

The enormous range of complexity in soil microbial communities has made it an incredibly challenging ecosystem to study (Torsvik et al, 2002). Direct isolation of DNA from environmental samples and their molecular analysis leads to the exploitation of microflora to a greater extent and overcomes the need to culture a single organism. Often, culture conditions not exactly mimic the environmental conditions of bacterial growth. Amplification of Soil DNA with primer set 1 resulted in the presence of the expected band size of 1500 bp in almost all the samples. In the present study clones were generated for SSU rRNA using set of universal primer directed at the conserved regions at the ends of the 16S gene and subjected to digestion using three tetracutter restriction enzymes namely *Hae* III, *Msp* I and *Sau* 3AI. According to Menachem Y, 2009, Profiles are found to be unique to genera for two or more REs, even at a high error level of 20% in the sizing of the restriction fragments (as might occur in agarose gels). However, at least three enzymes were necessary to differentiate species. In this study 12 unique restriction patterns (Patterns 1-10 from pre Harvest samples and patterns11-12 from post-harvest sample clones) with 2-6 bands (summation equaling approximately 1550 bp) were obtained from RFLP study carried on a total of 80 clones picked at random from positive transformants. (Fig 1)and a single representative of each restriction pattern was selected for sequencing.

Out of 80 clones under study for wheat rhizosphere seven clones had pattern 1, five clones had pattern 2. There were six representatives each of pattern 3, 9, 10 and 11.Fifteen clones had a clear and reproducible pattern 8, three clones showed the presence of pattern 12, four had pattern 4, two clones each had clear and reproducible AFLP pattern (pattern 5 and 6 respectively) and 18 clones showed pattern 7. The fragments with 600 bp were common in clones (SMC4 & SMM17). DNA bands of size 500 bp were present in SMC1, SMC3, SMM1, SMM12 and SMC6. In SMC2 and SMM5, band corresponding to 300 bp were common. SMC5 & SMM14 showed similar banding pattern except the presence of 50 bp.

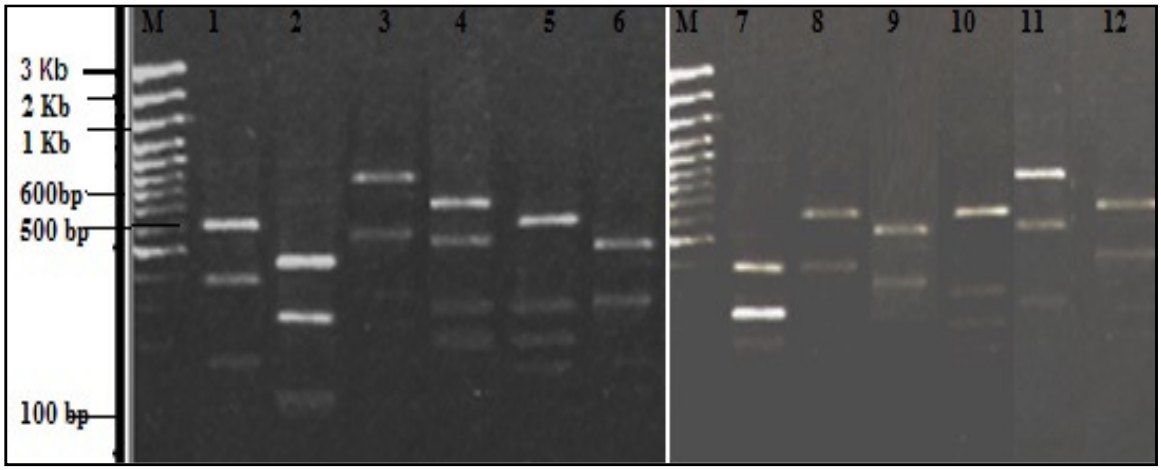

Figure 1: Gel image showing restriction patterns of 16S rDNA clones. Lane M; 3 Kb Marker, Lanes 1-5; Clones SMC 1-5, lanes 6-10; SMM1, 5,9,12 &14. Lanes 11 and 12 (post-harvest samples); Clones SMC6 and SMM17.

Re-amplification of clones corresponding to unique restriction pattern revealed clear and sharp bands of approximate size 450 bp with primer set II (fig 2), however, no bands were observed for bacterial V3 region (180bp) for any of the clones, the reason may be its small sized PCR fragments (150 bp) which can be often missed on long running gels. Variable region, V6-V9, have been widely used in diversity analysis according to the literature. The V6–V9 region was found to accurately classify 96.43% of the phyla, 94.54% of the classes, 88.64% of the orders, 86.32% of the families, and 72.65% of the genera (Claudia Vilo and Qunfeng Dong, 2012).

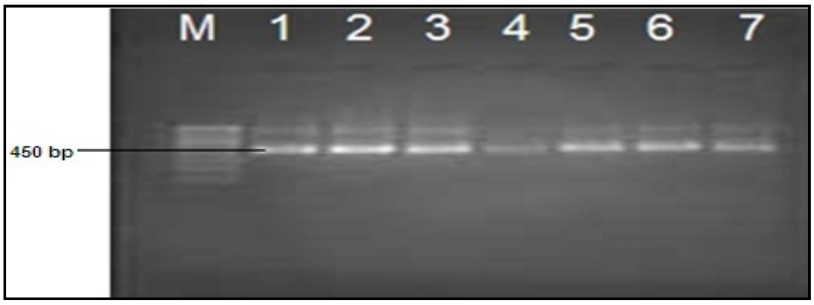

Figure 2: Gel image showing amplified V6-V9 region. Lane M; 100 bp marker, Lane 1-7 PCR amplified products

Phylogenetic analysis done so far revealed that all of the observed sequences had a bacterial origin (fig 2). Sequencing of V6-V9 region of bacterial 16S gene for ten (10/12) representative clones obtained by RFLP screening from pre harvest rhizospheric samples showed the presence of three phyla in wheat rhizosphere of North India namely proteobacteria, firmicutes, and actinobacteria out of which phylum proteobateria accounted for 50% followed by phylum firmicutes which accounting 30% of total sequences analyzed. A total of 10% each of analyzed sequences of wheat rhizospheric isolates belonged to phylum actinobacteria and novel bacterial group as it couldn't be assigned to any phyla or lineages. All of the clones from pre harvest soil samples showed a similarity percentage of >80% with 16S sequences available at NCBI.

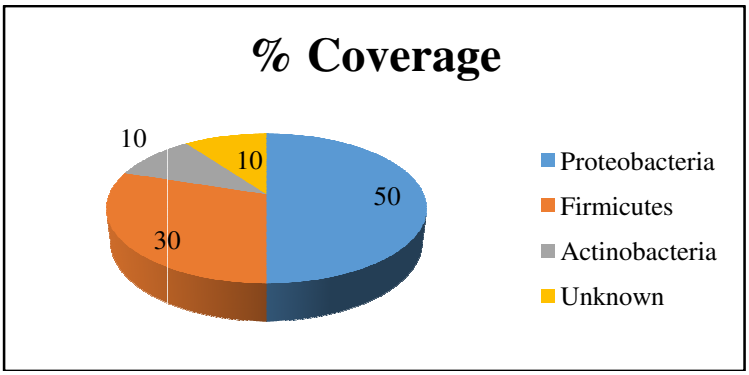

Figure 3: Chart showing percentage coverage of each phylum

The Proteobacteria encompass an enormous diversity, and are of great importance to global carbon, nitrogen and sulfur cycling (Kresters et al, 2006 and Spain AM et al 2009). Proteobacteria is the most diverse phylum of bacteria and is divided into three very different classes: Purple bacteria, Chemoautotrophic proteobacteria, and Chemoheterotrophic proteobacteria. In this study, phylum proteobacteria was represented by genera *Stenotrophomonas and Pseudomonas* from pre harvest soils samplesand Genus *Escherechia* and *Gamma proteobacteria* from pst harvest samples. As retrieved from table (2) and fig (3) Clones SMM1, 5, 9 and 12 showed similarity with *Pseudomonas chlororaphis, Pseudomonas stutzeri, Pseudomonas fluoroscens* and *Pseudomonas putida* strains respectively. Sequence SMC-2 showed relatedness to *Stenotrophomonas sp.*

Sequence no.	Clone Name	Closest match	% similarity	Phylogenetic group
1.	SMC1	*Bacillus subtilis*	98	Firmicutes
2.	SMC2	*Stenotrophomonas sp.*	99	Proteobacteria
3.	SMC3	*Bacillus sp.*	93	Firmicutes
4.	SMC4	*Uncultured bacterium clone*	92	Unknown
5.	SMC5	*Arthrobacter sp*	88	Actinobacteria
6.	SMM1	*Pseudomonas chlororaphis*	94	Proteobacteria
7.	SMM5	*Pseudomonas stutzeri*	97	Proteobacteria
8.	SMM9	*Pseudomonas fluorescens*	84	Proteobacteria
9.	SMM12	*Pseudomonas putida strain DNSL06*	100	Proteobacteria
10.	SMM14	*Sporosarcina sp*	99	Firmicutes
11.	SMC6	*Escherichia coli*	98	Proteobacteria
12.	SMM17	Uncultured gamma proteobacterium	91	Proteobacteria

Table 2: Similarity values of observed bacterial 16S rDNA sequences retrieved from rhizospheric soil

Seq1-10 (Clones generated from Wheat rhizosphere before harvesting of the crop), Seq11-12 (Clones generated from Wheat rhizosphere after harvesting of the crop).

Phylum firmicutes mainly comprised of genus *Bacillus* (SMC1, SMC3) and *Sporosarcina* (SMM14). Studies by M Koberl, 2011 revealed the enrichment of this Gram-positive group in the rhizosphere as well as endorrhiza of medicinal plants. They are well-characterised plant-associated genera with antagonistic properties towards fungal plant pathogens (Janssen P.H, 2006). Actinobacteria are a distinct group of bacteria that are widely distributed in nature and comprise of eight groups with 48 genera. In nature, Actinobacteria play an important role in the cycling of organic compounds and have been associated with soil organic matter production, including production of the black pigments called melanin, which are related to soil humic acid (Gomes et al 2006 and Monique Suela Silva et al, 2011). The phylum in our study is represented by single genus, Arthrobacter (SMC5). An extensive research work done on PGPR worldwide for over last five decades has classified the members of above mentioned genera to be highly efficient in promoting plant growth by their high antiphytophathogenic activity and root colonising activity. Clone SMC4 showed resemblance with uncultured bacterium clone of undefined phylogeny and henceforth can be characterised as novel Bacteria.

However, the post-harvest soil samples from same site showed least diversity (2/12) and bacterial count with the only dominance of phylum proteobacteria represented by genera *Eschereshia* (SMC6)and *unknown gamma proteobacteria* (SMM17), that can be attributed to absence of wheat plant and its exudates influencing bacterial growth owing to sudden change in growth environment. Released root exudates of plants can stimulate, or inhibit the growth of soil organisms (Watt and Passioura, 2006).

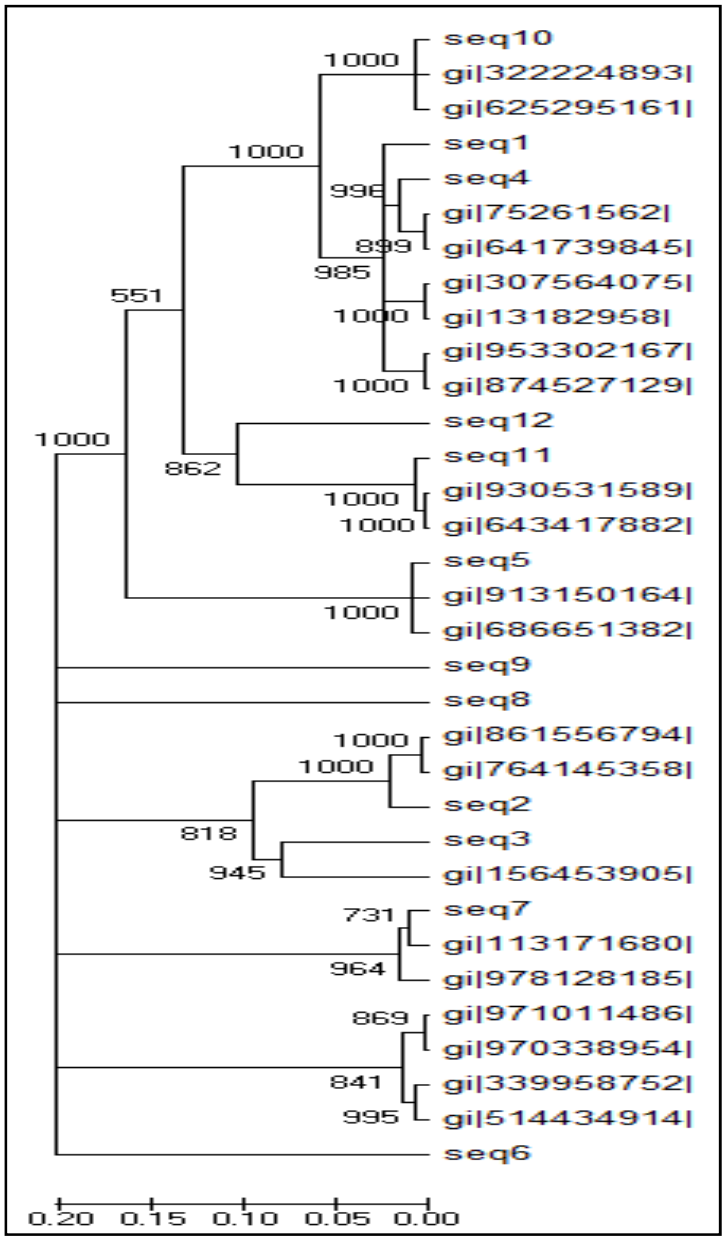

Figure 2: Phylogenetic tree showing relationship among Bacterial 16S rRNA gene sequences from wheat rhizosphere, North India with reference sequences obtained through BLAST analysis. Sequences obtained in this study are represented by seq.numbers (1-12). The tree includes 12 clone sequences with 30 reference sequences represented by their gi numbers. The tree was constructed by the neighbor joining method (Saitou and Nei, 1987) with NJ plot (Perrière and Gouy, 1996). The scale bar denotes 0.05 % of sequence distance.

4. Conclusion

Our findings revealed thatrich bacterial diversity is present in wheat rhizosphere comprising three phyla in the months of Jan-March owing to the growing stage of plant. *Pseudomonas sp* was found to be dominating in wheat rhizosphere of North India followed by *Bacillus.* No significant regional variation was observed in species composition in rhizospheric soil although the presence of genus *Sporosarcina* were unique to North Indian soils as they are not common inhabitants of wheat rhizosphere (reported in the thesis work of Ms. Shagufta Nahid, 2013 for the first time). Uncultured bacterium belonging to unclassified phylum was also found in this study, Sharp decrease in rhizobacteria was seen after harvesting of wheat crop from the same fields. The findings of this study can be exploited for increasing wheat production for future consumption in developing countries and the role of uncultured microorganisms in supporting wheat growth, obtained in this study can be enumerated by functional analysis of presence and absence of growth supporting attribute like production of antibiotics, growth hormones, siderophores etc.

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