CIBTech Journal of Microbiology ISSN: 2319-3867 (Online)
An Online International Journal Available at http://www.cibtech.org/cjm.htm
2016 Vol. 5 (4) October-December, pp.17-24/Patel and Bhatt
Research Article

DIVERSITY OF FREE LIVING NITROGEN FIXING BACTERIA FROM DIFFERENT SOILS OF NORTH GUJARAT, INDIA

***P. Patel[1] and S. Bhatt[2]**
[1]*Department of Biotechnology, Mehsana Urban Institute of Sciences, Mehsana-Gozariya Highway, Ganpat University-384012, Gujarat, India,*
[1]*Department of Life Sciences, Hemchandracharya North Gujarat University, Patan-384265, Gujarat, India*
**Author for Correspondence*

ABSTRACT
The North Gujarat region is geographically having varied terrestrial ecosystem which is comprised of semiarid to arid and highly arid saline soil types. It also has tropical dry deciduous Forest patches. Thus, considering these ecoclimatic conditions, it is thought to have different diazotrophic populations which might be explored for potential biofertilizer to improve crop productivity in the region. In this context, the long-lasting challenges in the development of effective methods to know the types of microorganisms present in soils, and to determine functions which the microbes perform in situ. Total 68 free living nitrogen fixing bacteria having ability to grow on N_2 free media were categorized in different group and identified at species level by ARDRA analysis and 16S r-RNA sequences. One isolate is closely related to uncultured bacteria based on complete sequence analysis. Our result indicated that higher diversity of free-living nitrogen-fixers was mainly affected by the physico-chemical properties of the soils and plant species, especially the changes of nutrient.

Keywords: *Free Living Nitrogen Fixing Bacteria, 16S r-RNA Sequencing, Diversity*

INTRODUCTION
Symbiotic systems represent the most significant biological N-source for many terrestrial ecosystems (Kennedy and Islam, 2001). However, free-living nitrogen-fixing microorganisms inhabiting soils can significantly contribute to the N budgets of a number of ecosystems (Kahindi *et al.*, 1997; Deslippe *et al.*, 2005; Unkovich and Baldock, 2008; Hsu and Buckley, 2009). Free-living aerobic N_2-fixing bacterial genera found in soil include *Azotobacter*, *Beijerinckia* and *Derxia*, but the majority are Microaerophilic (e.g. *Azospirillum*, *Herbaspirillum*) or facultative and obligate anaerobes (e.g. *Klebsiella*, *Clostridium*, *Erwinia*) (Giller and Day, 1985).
The ability of free-living nitrogen fixing bacteria to actually fix N_2 in the field is strongly influenced by the prevailing environmental condition. Although the tropics contain some of most productive environments in the word, they also contain their fair share of hostile environments. The main environmental stresses which occur in the tropics can be divided in to predominantly physical factor (temperature, moisture) and into chemical factors which include both toxic effects like acidity and nutrient deficiencies (McGrath *et al.*, 1995; Alexander and Zuberer, 1989; Tan *et al.*, 2003; Zhang *et al.*, 2006).
The molecular-phylogenetic perspective is a reference framework within which microbial diversity is described; the sequences of genes can be used to identify organisms (Amann *et al.*, 1995). A number of approaches have been developed to study molecular microbial diversity.
These include DNA re-association, DNA– DNA and mRNA-DNA hybridization, DNA cloning and sequencing and other PCR-based methods such as Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Ribosomal Intergenic Spacer Analysis (RISA) and Automated Ribosomal Intergenic Spacer Analysis (ARISA), Ribosomal DNA Restriction Analysis (ARDRA) (Kirk *et al.*, 2004).
In this study, we have monitored the diversity of free-living nitrogen-fixing bacteria from rhizosphere and non-rhizosphere region of cultivable and non-cultivable plants by using ARDRA and 16S-rRNA analysis.

The objectives of the research were (1) to compare the diversity of free-living nitrogen fixing bacteria in different soils of North Gujarat Region; and (2) to explore the main factors resulting in the changes of free-living nitrogen-fixing microbial community structure in this region.

MATERIALS AND METHODS

Study Area

Research locations are spread within the four districts of North Gujarat viz., Sabarkantha, Mehsana, Banaskantha and Patan. Total five towns were selected from each district and from each town place five different sampling sites were selected.

These sites were selected for the studies, based on general observation for the topographic situations, soil conditions, and vegetation cover. Mean annual rainfall in North Gujarat according to the records of the Indian Meteorological Department is 650 mm, of which more than 95% is received during the monsoon season.

The rains are highly erratic in both intensity and duration. Sometimes, the intervals between the two successive rains may extend more than 20 days.

Total numbers of rainy days are thirty to thirty five. The post monsoon period is generally hot and air temperature is generally higher during this period. Winter is mild with mean maximum temperature of 25℃ and mean minimum of 15 ℃. Summer is hot and dry.

During the month of May, the mean maximum temperature goes up to 43 ℃ and mean minimum temperature reaches 28 ℃.

There is no moisture surplus throughout the year and there is consistent moisture deficiency except in the months of July and August. The underlying rock at Sabarkantha and Banaskantha districts in North Gujarat is basalt. In situ weathering leads to fragmentation of rocks by spheroid weathering of concentric rings.

The soil derived from the rocks can be divided in to three basic types; viz; (1) ‘Morrrum’ on hill tops and exposed plateau, (2) Thin sandy clayey loam intermixed with pebbles on slopes and (3) Clayey black cotton soil in the low lying areas and plains which are highly sticky and form characteristic cracks upon drying.

At the research site the soil varies in thickness from 15 cm to 1 m. At several places, rocks are exposed. Uppermost layer of the rock is amygdaloidal with vesicular cavities, filled with secondary deposits of quartzite, calcite, zeolite, agate etc. Sometimes, the metamorphosed instrugens may be 10 cm in diameter. The most prevalent color of the rock is grayish green.

Soil Samples Collection and Isolation of Free Living Nitrogen Fixers

The soil samples were collected from four districts of North Gujarat region viz., Sabarkantha, Mehsana, Banaskantha and Patan. Soil samples were collected from rhizosphere and non-rhizosphere regions of various plants.

Samples were also collected from uncultivated area, undisturbed forest during year 2011-2012 and from the areas where intensive agricultural production using high rate of pesticides and fertilizers is carried out. Soil samples were collected during post monsoon and winter season as during that period more plant diversity is seen.

Microclimatic data such as temperature, moisture and soil physicochemical characteristics during different seasons were recorded. Total 68 strains of free living nitrogen fixing bacteria were isolated from the semi-arid soils of North Gujarat. The purity of the cultures was verified by repeated streaking of single colony of isolates on Nitrogen free agar medium (Ashby’s Mannitol medium). Isolates, which were found to be pure and confirmed, were only used for further study.

Physical and Chemical Analysis of Soil

The soil samples were dried at room temperature (30.5 + 2 °C) then passed through 1 mm sieve prior to oven dry at 105 °C. These soil samples were used for chemical analysis as follows:

Soil pH was estimated by making soil paste in distilled water in ratio of 1:5 and measured on a Digital pH meter using glass electrode (Systronic make, model- 335).

Soil organic carbon was estimated by wet oxidation method (Modified Walkely Black method) as per Makeague (1978).

The organic matter was calculated by multiplying organic carbon by the factor 1.72 as per Richards (1968). Total soil nitrogen was determined by micro-kjedahl method (Peach and Tracey, 1956).

Soil phosphorous was estimated by phosphomolybdate blue color method (Jackson, 1973). Exchangeable Na, K, Ca, and Mg of the soil samples were estimated on atomic absorption spectrophotometer (Varian Techtron make, AA-6D).

Genetic Characterization of the Isolates

DNA Isolation

Chromosomal DNA was isolated using the genomic DNA extraction kit (Chromus Biotech). The isolates were freshly grown on N2 free agar slopes and were re-suspended into 10ml of N2 free broth, before being spun down and resuspended in 1ml of N2 free broth. Genomic DNA was isolated as per the protocol provided with the kit.

Agarose Gel Electrophoresis, Staining and Extraction

DNA was separated on 1% agarose gels run at ~100V in Tris Acetate (40mM) EDTA (1mM) buffer. DNA samples were loaded on to each well with a solution of 30% glycerol, 0.25% bromophenol blue used as a loading buffer (x6 concentration).

A 1kb ladder was used as a size marker. Gels were stained in ethidium bromide solution (0.8µg/ml) before the DNA was visualised under UV light. DNA was extracted from gels as required using the Gel Extraction Kit (Medox) following the Gel Extraction Kit micro centrifuge protocol as supplied with the kit.

DNA Purification

DNA was purified using phenol extraction or ethanol precipitation. Phenol extraction was carried out by adding equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) to the DNA sample, which was vortexed before centrifuging at 13,000 rpm for 5 minutes.

The aqueous layer was then removed and kept in refrigerator. Ethanol precipitation was carried out by adding 10% volume of 3 M sodium acetate, then equal volume of 100% ethanol to the DNA sample. Samples were then centrifuged at 13,000 rpm for 20 minutes. The samples were then washed twice in 70% ethanol before air-dry.

ARDRA (Amplified Ribosomal DNA Restriction Analysis)

In order to choose the restriction enzymes to be used for ARDRA, the 16S rRNA sequences of different nitrogen fixing bacteria retrieved from the GenBank were virtually restricted using the serial cloner software (Version 2.6).

Crude DNA was isolated from bacterial culture using Chromous Bacterial genomic DNA Isolation Kit RKN. The 16S r-RNA gene was amplified by means of universal 16S r-DNA primers and the reactions were run for 35 cycles. The product obtained (1.5kb) was digested with TaqI, HpaII and RsaI (4 base cutters).

Five units of each enzyme were added to 12.5 µl of the amplification product and incubated for 5 h at 37 °C in a total volume of 25 µl.

The digests were resolved by electrophoresis on a 2% agarose gel along with 100bp and 250bp DNA ladder.

Gels were stained in ethidium bromide for 15 min and rinsed for 5 min in distilled water. Gel electronic images were visualized to check the ARDRA pattern.

The ARDRA pattern of all the isolates was compared with the ARDRA profile of different nitrogen fixing known reference strain of bacteria.

The comparison of amplified DNA profiles was performed on the basis of the presence (1) or absence (0) of fragments was generated by using PyElph 1.4 software. The similarity matrix was generated with jaccard coefficient and the distance matrix was used for constructing the dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages).The dandrogram was generated in Newick format are shown in figure 1.

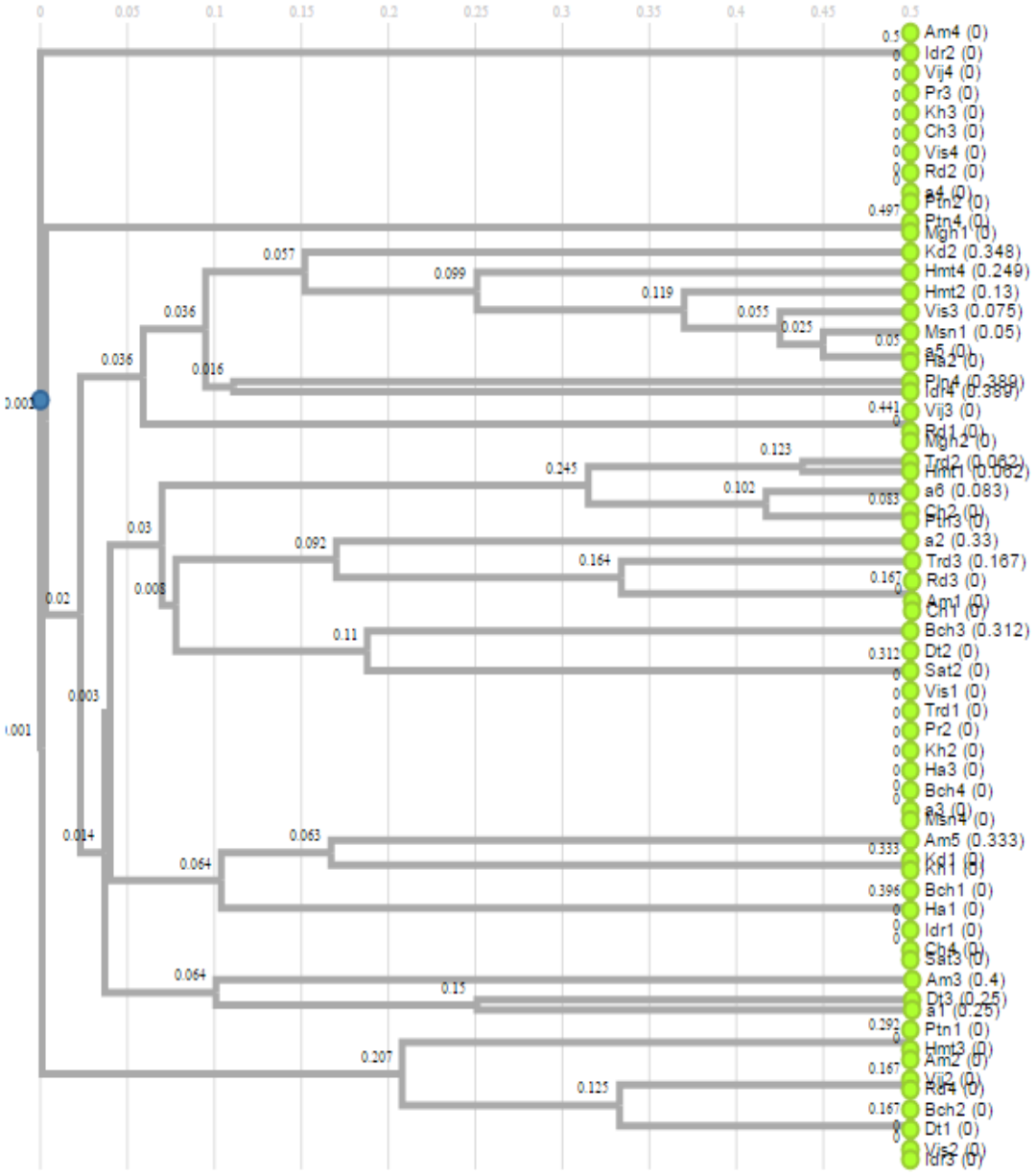

Figure 1: UPGMA Dendrogram of all the Isolates Taken under Study Including Five Reference Strains Digested with Hpa II

PCR-Sequencing

After purification of the PCR product it was sent for sequencing to Chromus Biotech pvt. Ltd., India. The sequencing of the purified products were done as follows. The sequencing mix composition and PCR conditions used is described later. Sequencing Machine used was ABI 3500 XL Genetic Analyzer and the chemicals for chemistry used were: Big Dye Terminator version 3.1 ” Cycle sequencing kit. For Polymer & Capillary Array: POP_7 polymer was used for 50 cm Capillary Array. The results were analyzed by using protocol BDTv3-KB-Denovo_v 5.2. Data analysis was carried out by Seq Scape_ v 5.2 Software and the reaction plates used in the sequencing were Applied Biosystem Micro Amp Optical 96- Well Reaction plates. The partial rRNA gene sequences of the isolates were preliminarily compared with those

present in the Basic BLAST search (Altschul *et al.,* 1997), and then aligned using the ClustalW program (Higgins *et al.,* 1992) available at the European Bioinformatic Institute website (http://www.ebi.ac. uk/clustalw/) with the sequences retrieved from the GenBank database (Benson *et al.,* 2003) available at the NCBI website (http://www.ncbi.nlm.nih. gov/) and then phylogenetic tree of all the isolates were generated.

RESULTS AND DISCUSSION

The Soils

The recorded soil temperature showed inverse relationship with the soil depth. Soil moisture was found maximum during the monsoon months and it gradually decreased in the subsequent months, however, it was little more at 30 cm soil depth. There was some marked difference in soil texture and colour in the three sites.

Site of Patan was having pH in range of 6.5 to 7.46, while sites of Banaskantha and Sabarkantha were having pH in range of 7.11 to 7.67 and the sites of Mehsana were having pH in range of 7.07 to 7.62. Soil chemical characters show that there was not much variations in their characters, however, soils of Patan district contained little more organic carbon and total nitrogen than the soils at the other districts. Seasonal variation in total versus diazotropic bacterial counts from rhizospheric and non-rhizospheric region are shows that the counts of bacteria are higher in post monsoon season (Data not shown).

ARDRA Profile and Diversity of Free Living Nitrogen Fixing Bacteria

Stringent PCR conditions allowed amplification of a single 16S r-DNA fragment. All isolates yielded a band of ~1.5kb in size after amplification with the universal eubacterial primer. ARDRA (Amplified restriction digestion analysis) of extracted DNA of each bacteria were digested with TaqI, HpaII and RsaI (4 base cutters) resulted in the number of pattern type. The isolates were grouped into different 18 ARDRA types. From 18 ARDRA group, organisms belonging to 08 groups were identified using similarity with reference strains. ARDRA groups which were not identified by ARDRA were identified by 16S r-RNA sequencing.

Phylogenetic Analysis

A total of 68 Free living nitrogen fixing bacterial DNA band positions could be differentiated in ARDRA analysis. The ARDRA pattern of all the isolates was compared with the ARDRA profile of different nitrogen fixing known reference strain of bacteria. The comparison of amplified DNA profiles was performed on the basis of the presence (1) or absence (0) of fragments was generated by using PyElph 1.4 software. The similarity matrix was generated with jaccard coefficient and the distance matrix was used for constructing the dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages). The dandrogram was generated in Newick formats are shown in figure 1. All the bands were also retrieved, followed by PCR, cloning and sequencing. As a result, total 18 different types of free living bacterial species were identified up to species level. The list of identified isolates with their accession number is shown in table 1.

Discussion

Successful isolation of the free living diazotrophs from different region of North Gujarat are able to grow on N_2 free medium, suggested that all the 68 isolates of bacteria are free living Nitrogen fixing bacteria. In the North Gujarat region the soil types where more vegetation is found, recorded more of free living diazotrophs few of which have been found to be potential N_2 fixers and those can be further tested to be used as Biofertilizer in the soils of same districts as well as others. The diversity of free living diazotrophs is studied by various methods. The morphological and cultural characteristics found that out of 68 free-living nitrogen fixing bacterial isolates, majority of the strains are gram negative short rods, some of the strains are gram positive rods and filamentous and some are gram positive cocci (Data not shown). The result was agreed with Chocnhahirum (1986) who found that 259 bacterial cultures isolated from acid soil of Thailand were gram negative rod shape. Another study on the physiological diversity of rhizoshere *Spartina alternitlona* (smoot cord grass) in salt marsh in North America was done by Bagwell *et al.,*

(1998) and they found that all 339 strains are gram negative. The majority of strains were motile rods which were confirmed gram negative of free-living nitrogen fixing bacteria.

Table 1: The Recognized Strains of the Free–Living Diazotrophs Isolated from North Gujarat

Sr. No.	Isolate Name	Isolate Identified as	Accession Number
1	Hmt 4	*Azotobacter salinestris*	JX437935
2	Vis 2	*Azotobacter tropicalis*	JN591767
3	Msn 4	*Azotobacter vinelandii*	JX564632
4	Bch 3	*Nocardioides nitrophenolicus*	JX564633
5	Ch 4	*Streptomyces thermocarboxydovorans*	JN580892
6	Idr 2	*Agrobacterium tumifaciens*	JX564634
7	Am 5	Isolate similar with Uncultured bacteriumsp.	--
8	Ptn 4	*Variovorax soil strain*	JX564635
9	Am 3	*Mycobacterium cosmeticum*	KF418747
10	Rd 1	*Enterobacter ludwigi*	KF418748
11	Pln 4	*Rhodococcus* corynebacterioides	KF418749
12	Vij 2	*Sinorhizobium saheli*	KF418750
13	Trd 3	*Bacillus niabensis*	KF535156
14	Idr4	*Planococcus sp.*	KJ538560
15	Kd 2	*Microbacterium sp.*	KJ538561
16	Dt 3	*Arthrobacter sp.*	KJ538562

ARDRA is commonly utilized as an alternative to more laborious and expensive methods for the identification of eubacteria, being the analysis of the rRNA cistron a good criterion for microbial classification at both genus and species level (Grimont and Grimont, 1986; Massol-Deya *et al.,* 1995). However, this molecular tool has not been utilized on Azotobacteraceae and other nitrogen fixing bacteria whose identification, traditionally based on conventional biochemical tests, is often tentative. Therefore, the development of a simple identification method yielding reliable and unambiguous results appears to be useful.

Nitrogen-fixing microorganisms are highly adapted to different environmental conditions and considered to be important for the nitrogen input to soil, they are rarely dominant in terrestrial ecosystems (Wartiainen *et al.,* 2008; Coelho *et al.,* 2009) and susceptible to environmental condition. In this study, the diversity of nitrogen-fixing microbial community in different soils of North Gujarat varied with the physico-chemical properties of the soils, plant species, was influenced by plant rhizosphere. In the cultivated area of the North Gujarat at which dense vegetation are available with high nutrient contents and less toxicity of heavy metals, the nitrogen-fixers in rhizosphere and non-rhizosphere samples displayed higher diversity than that in non cultivated area. Tan *et al.,* (2003) suggested that the environmental parameters, especially, N content had a strong influence on the community of root-colonizing diazotrophs, rather than the plant species. Sato *et al.,* (2009) reported that the diversity and phylogenetic composition of nifH genes depended on soil properties. Previous work also confirmed the key effect of C and N content on the nitrogen-fixing bacterial community (Poly *et al.,* 2001; Zhang *et al.,* 2006; Coelho *et al.,* 2008, 2009).

In this study, significant difference in the free-living nitrogen fixing microbial community can be seen due to the type of plant community. These results suggested the significant effect of plant species on the diversity and structure of nitrogen-fixers communities in the tailings. It has been already shown that composition, number and distribution of nitrogen-fixers were associated with plant species (Tan *et al.*, 2003; Diallo *et al.*, 2004; Duc *et al.*, 2009). According to these results, the physico-chemical properties of North Gujarat and plant species were proved to be the dominant factors determining the structure of free-living nitrogen-fixing microbial community in this region.

ACKNOWLEDGEMENT
The authors are thankful to the Department of Biotechnology, Mehsana Urban Institute of Sciences, Ganpat University for providing the facilities for this research.

REFERENCES

Alexander DB and Zuberer DA (1989). 15N 2 fixation by bacteria associated with maize roots at a low partial O2 pressure. *Applied and Environmental Microbiology* **55** 1748-1753.
Altschul SF, Madden TL, Schaffer AA, Zhang Z, Miller W and Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25** 3389–3402.
Amann RI, Ludwig W and Schleifer KH (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**(l) 143-69.
Bagwell CE, Piceno YM, Ashburne-lucas A and Lovell CR (1998). Physiological diversity of rhizosphere diazotroph assemblages of selected salt marsh grasses. *Applied and Environmental Microbiology* **64** 4276-4262.
Chocnhahirum A (1986). Chemotaxonomy of some nitrogen-fixing bacteria isolated from the rhizosphere of rice (*Oryza sativa* L.) grown in Thailand. Master's Thesis, Chulalongkorn University.
Coelho MRR, Marriel IE, Jenkins SN, Lanyon CV and O'Donnell AG (2009). Molecular detection and quantification of nifH gene sequences in the rhizosphere of sorghum (Sorghum bicolor) sown with two levels of nitrogen fertilizer. *Applied Soil Ecology* **42** 48–53.
Deslippe JR, Egger KN and Henry GHR (2005). Impacts of warming and fertilization on nitrogen fixing microbial communities in the Canadian High Arctic. *FEMS Microbiology Ecology* **53** 41–50.
Duc L, Noll M, Meier E, Burgmann H and Zeyer J (2009). High diversity of diazotrophs in the forefield of a receding alpine glacier. *Microbial Ecology* **57** 179–90.
Giller KE and Wilson KJ (1991). *Nitrogen Fixation in Tropical Cropping System*, (CAB International, Wallingford, UK) 313.
Grimont F and Grimont PAD (1986). Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Annales de l'Institut Pasteur / Microbiologie* **137** 165–175.
Higgins D, Blasby A, Fuchs R and Clustal V (1992). Improved software for multiple sequence analysis. *Computer Applications in the Biosciences* **8** 88– 192.
Hsu SF and Buckley DH (2009). Evidence for the functional significance of diazotroph community structure in soil. *International Society for Microbial Ecology* **3** 124–36.
Jackson ML (1973). *Soil Chemical Analysis*, **347**, (Prentice Hall of India Pvt. Ltd., New Delhi, India).
Kahindi JHP, Woomer P, George T, de Souza Moreira FM, Karanja NK and Giller KE (1997). Agricultural intensification, soil biodiversity and ecosystem function in the tropics: the role of nitrogen-fixing bacteria. *Applied Soil Ecology* **6** 55–76.
Kennedy IR and Islam N (2001). The current and potential contribution of asymbiotic nitrogen fixation to nitrogen requirements on farms: a review. *Australian Journal of Experimental Agriculture* **41** 447-457.
Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H and Trevors JT (2004). Methods of studying soil microbial diversity. *Journal of Microbiological Methods* **58** 169–188.
Makeague JA (1978). *Manual of Soil Sampling and Method of Analysis*, 101, (Canadian Society of Soil Science, Canada, USA).

Massol-Deya AA, Odelson DAM, Hickey F and Tiedje JM (1995). Bacterial community fingerprinting of amplified 16S and 16 – 23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). *Microbial Ecology* **3.3.2** 1 – 8.

McGrath SP, Chaudr AM and Giller KE (1995). Long-term effects of metals in sewage sludge on soils, microorganisms and plants. *Journal of Industrial Microbiology* **14** 94–104.

Peach K and Tracey MV (1956). *Modern Methods of Plant Analysis*, (Springer and Verlag, New York, USA).

Poly F, Ranjard L, Nazaret S, Gourbière F and Monrozier LJ (2001). Comparison of nif Hgene pools in soils and soil microenvironments with contrasting properties. *Applied and Environmental Microbiology* **67** 2255–62.

Sato A, Watanabe T, Unno Y, Purnomo E, Osaki M and Shinano T (2009). Analysis of diversity of diazotrophic bacteria associated with the rhizosphere of a tropical arbor, Melastoma malabathricum L. *Microbes and Environments* **24**(2) 81–7.

Tan Z, Hurek T and Reinhold-Hurek B (2003). Effect of N-fertilization, plant genotype and environmental conditions on nifH gene pools in roots of rice. *Environmental Microbiology* **5** 1009–15.

Unkovich M and Baldock J (2008). Measurement of asymbiotic N2 fixation in Australian agriculture. *Soil Biology and Biochemistry* **40** 2915–21.

Wartiainen I, Eriksson T, Zhang W and Rasmussen U (2008). Variation in the active diazotrophic community in rice paddy – nifH PCR-DGGE analysis of rhizosphere and bulk soil. *Applied Soil Ecology* **39** 65–75.

Zhang Y, Li D, Wang H, Xiao HM and Liu XD (2006). Molecular diversity of nitrogen-fixing bacteria from the Tibetan Plateau, China. *FEMS Microbiology Letters* **260** 134–42.