Journal of Environmental Biology
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September 2010, 31(5) 553-556 (2010)
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Diversification of nitrogen fixing bacterial community using nifH gene as a biomarker in different geographical soils of Western Indian Himalayas

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(Received: May 13, 2009; Revised received: September 02, 2009; Accepted: September 23, 2009)

Abstract: *Six soil samples (Pantnagar, Chamoli, Almora, Ranichauri, Pithoragarh and Badrinath) belonging to different geographical locations of Western Himalayas in India, were analyzed to diversify the nitrogen fixing bacterial community using nifH gene biomarker. DNA from soil samples were isolated and amplified using nifH gene specific primers. Genomic DNA and PCR amplified products were then individually subjected to restriction digestion with tetra to octacutter enzymes (AluI, MspI, BglII, XbaI, HindIII, HaeIII, AluI, MspI and PasI. Further, restriction pattern was studied by preparing dendograms on the basis of similarity matrix and compared for the nifH community. It was observed that temperate region soils (Ranichauri and Pithoragarh) were negative for nifH marker while subalpine region (Badrinath) and tarai region soils (Pantnagar) documented similar nifH community. Moreover, the direct genomic DNA restriction analysis indicated that subalpine region soil (Badrinath) was most diversified.*

Key words: *Soil sample, Geographical regions, Diversity, RFLP, NifH*
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Introduction

The biosphere is dominated by microorganisms which are the largest unexplored reservoir on the earth. Further, the microbial diversity can be documented by both culture-dependent and culture-independent methods. However, the outcome of the uncultured microorganisms has opened new area of research by enabling unprecedented analysis of genome heterogeneity. Moreover, the potential of microbes can be explored through community fingerprinting and metagenome cloning subsequently.

Nitrogen fixation enables reduction of the atmospheric nitrogen into ammonium ion (NH_4^+) by nitrogenase enzyme. This process introduces nitrogen into the biosphere, which is responsible for its annual fixation by upto 65% of nitrogen while industrial processes represent only 25% (Newton, 1996). Further, the iron protein genes (nifH, vnfH and anfH) are highly conserved among diverse microorganism and the nifH phylogenetic tree largely resembles the 16S rRNA phylogenetic tree (Henneke *et al.,* 1985; Normand and Bousquet, 1989; Young, 1992). Therefore, the highly conserved nature of the nifH gene makes it an ideal biomarker to determine the potential nitrogen fixing community (Zehr and Capone, 1996). Moreover, Himalayas are the natural reservoirs of different microbial communities especially with nitrogen fixing abilities. In the present study, two way restriction analysis *viz.* direct soil DNA-RFLP and PCR-RFLP in-parallel to the soil nifH gene, with additional T_m analysis were conducted among soil samples collected from different geographic locations of the Western Himalayas.

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Materials and Methods

Extraction of DNA from soil samples and direct restriction digestion: Surface layer soil samples (not deeper than 15cm) were collected from different geographic location namely subalpine region (Badrinath - 79°E, 30°44'N, 3110m), temperate regions (Ranichauri - 78°30'E, 30°15'N,1950m and Pithoragarh - 80°2'E,29°47'N,1967m), subtropical regions (Almora - 28°59'W,79°2'E, 1646m and Chamoli - 30°51'N,79°4', 1300m) and tarai region (Pantnagar - 29°N, 243.8 m). Soil DNA was isolated using Power soil ™DNA isolation kit (Mobio Lab. Inc., Carlsbad, CA, USA), as described by the manufacturer and quantified by UV spectrophotometry at 260 nm.

DNA from different soil samples was digested with 10 different restriction endonucleases namely AluI, BgI-II, EcoRI, Hind III, Pas I, SmaI, xbaI, NotI and SaeII (Fermentas international Inc., Canada). A digestion reaction of 20 µl with 1µg of soil DNA and 1µl of restriction endonucleases was incubated for 16 hr at 37°C. Further, digested DNA was analyzed in 1.5% agarose gel and electrophoresed in 1X TAE at 60V. Dendograms on the basis of restricted band pattern were prepared using NTSYS software.

PCR-RFLP of nifH amplicon: For *nif*H detection in soil DNA, a nested PCR approach was carried out. A set of three primers were modified and used; these primers were originally developed by Widmer *et al.* (1999). However, the combination of the primer sets was followed as described by them. The first PCR was performed with the forward primer *nif*H(forA) 5'GCIATITACGGCAAAGGTGG and the reverse primer *nif*H(rev) 5'GCGTAIACGGCCATCATCTC. The second (nested) PCR was performed with the forward primer *nif*H(for B) 5' G GITGTGATCCTAAAGCCGA and the same reverse

primer that was used in first reaction . First PCR amplification was performed with primer *nif*H (for A) - 0.1 µM and *nif*H (rev) - 0.1 µM in 25 µl volume, using Taq PCR kit (New England Biolabs, Inc, Ipwich, MA, UK) and a template DNA concentration of 50ng in a thermal cycler (Bio-Rad Lab, Hercules, CA, USA) for 30 cycles (94°C for 5 min, 94°C for 45 sec. 47°C for 30 sec annealing, 72°C for 20 sec extension and 72°C for 10 min final extension). Nested PCR was performed by using first amplicon as template in 25µl volume, containing template DNA 2 µl, 0.1 µM of final concentration of primer nif (for B) and nif (rev) and Taq DNA polymerase 2 U with following programme. Initial denaturation (94°C for 5 min, 92°C for 15 sec, 50°C for 30 sec, 72°C for 10 sec) for 35 cycles, final extension (72°C) for 10 min. The PCR product were analyzed on 1.5% agarose gel (Fig. 1) and quantified by taking absorbance at 260 nm. To analyze genetic diversity of nifH community, nifH amplicon from different positive soil samples were subjected to digestion with different restriction endonucleases. A 50 µl reaction containing 1µg of amplified product and 2 µl of restriction enzyme was carried out for 1 hr at 37°C. The digested product were analysed on 4% MetaPhor gel (Biobasic Lab, Hercules, CA,USA).

In-silico Analysis: Dendograms based on similarity matrix were prepared after analyzing the RFLP gels. These dendograms were based on Euclidean coefficient also known as the Euclidean similarity coefficient. It is a statistical method used for comparing the similarity and diversity of sample sets. For the analysis of RFLP data, the character state '1' was given for a band, which could be clearly and reproducibly detected in the gel, while '0' was assigned if it was absent. The data matrix thus generated was calculated by Jaccard coefficient for each pair wise comparison. Dendogram was constructed from the similarity matrix using NTSYS software.

Tm analysis by Real time PCR (Melt Curve): Melt curve of amplified soil DNA was determined by iCycler iQTM Multicolor PCR (Bio-Rad Lab, Hercules, CA,USA) and the curve was obtained by using universal eubacterial Primer1: 5[1] CC TACGGGAGGCAGCAG, Primer 2: 5[1] ATTACCGCGGCTGCTGG (Muyzer *et al*. 1993). The product of these primer sets was about 200 bp. Real time PCR was performed in 25 µl reaction mixture using SYBR green supermix 12.5 µl, primer 1 and 2 in 0.1 µM concentration and template DNA 100 ng with PCR condition 95°C- 3 min, 94°C-1 min, 58°C-2 min. Immediately after amplification, immediately 80 melt cycles were performed by gradually increasing temperature from 55°C-90°C and continually measuring the fluorescence of each sample. The fluorescence of each signal decreases dramatically as the amplicon dissociates, causing the SYBR dye to be quenched.

Results and Discussion

The T_m analysis of the PCR amplicon is good enough to measure the comparative G+C composition among the studied samples (Kimura *et al*., 2007). The decrease in the Tm is the

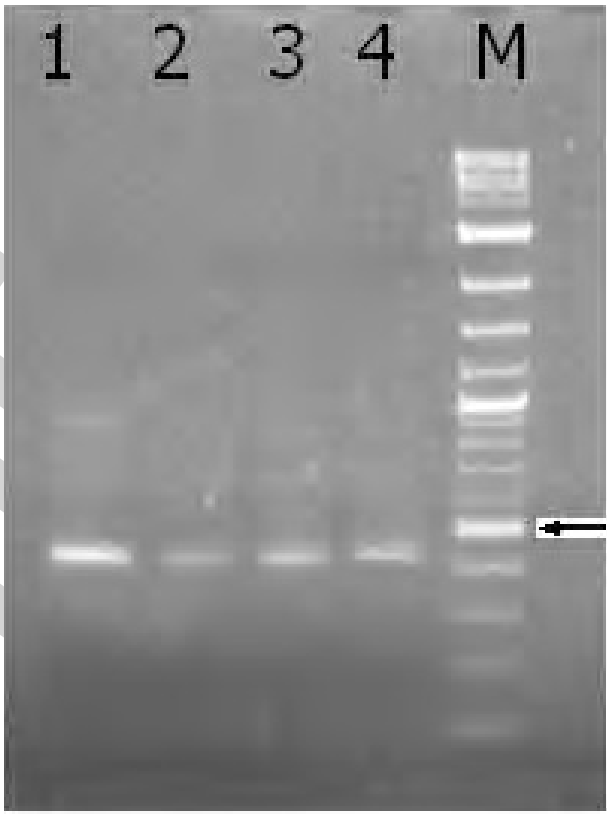

Fig. 1: Agarose (1.5%) gel containing 5-ml portions of the nested amplification products obtained from Tarai soil - Pantnagar (lane 1), subtropical soil - Chamoli (lane 2), subalpine soil - Badrinath (lane 3), and subtropical soil - Almora (lane 4) soil metagenome. Lane M shows 2 Log Ladder (Bio-Rad Lab,CA, USA)

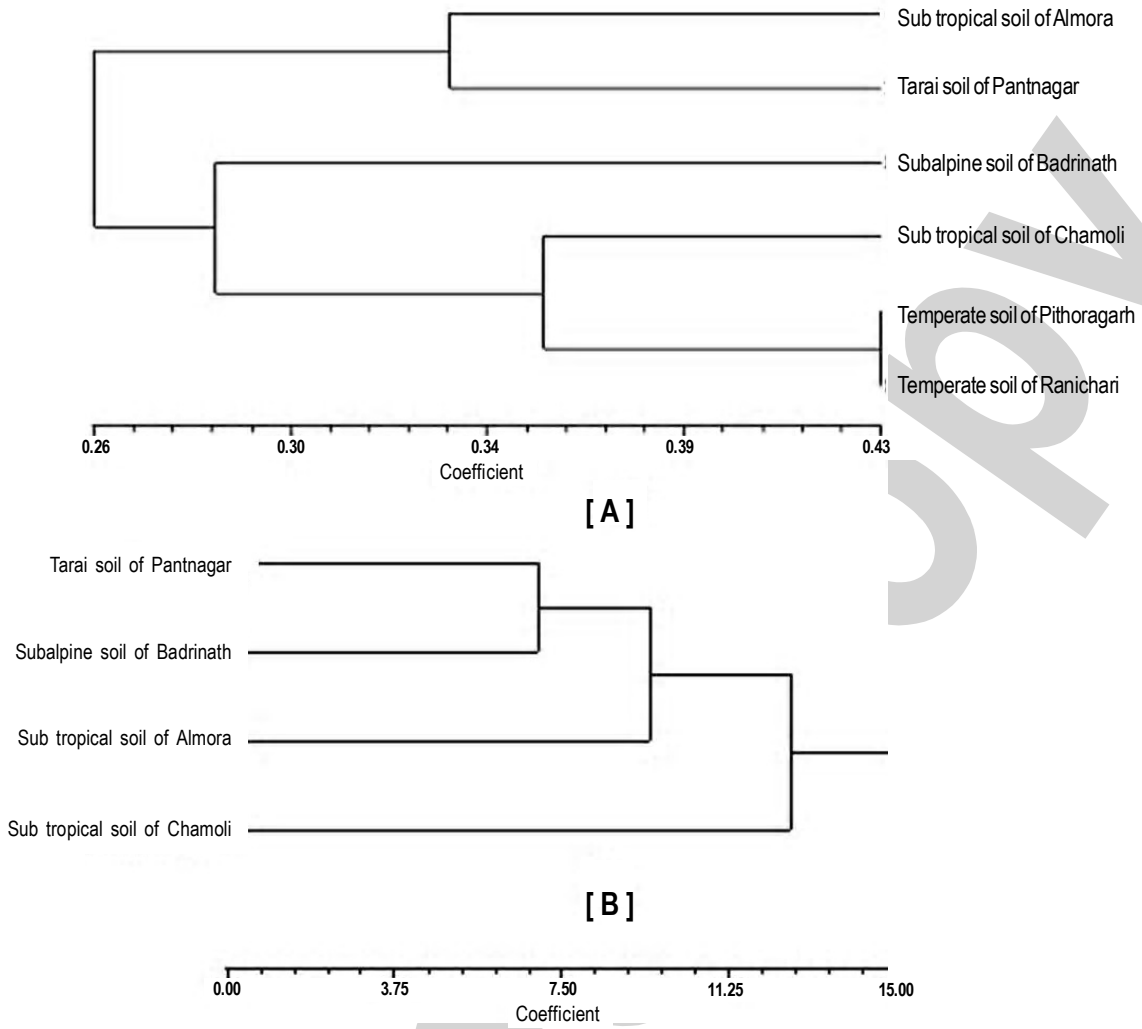

Fig. 2: Dendograms showing the relatedness (A) among different soils in similarity coefficient of 34. Each soil DNA was restricted digested with AluI, MspI, BglII, XbaI and HindIII and (B) among the nifH community of different soils DNA at similarity coefficient of 15. In each case amplified soil DNA was restricted with HaeIII, AluI, MspI and PasI enzymes. Dendograms based on similarity matrix prepared after analyzing the RFLP gels using NTSYS software. For the analysis of RFLP data, the character state '1' was given for a band, which could be clearly and reproducibly detected in the gel and '0' was assigned if it was absent

indication of the increment in the G+C composition (Wallenstein and Vilgalys, 2005). Melting temperature of ribosomal gene product suggests moderate variability which ranges from 85 to 87.5°C. The results show that the subalpine soil (Badrinath) has lowest Tm value *i.e.* 85.54°C, whereas tarai (Pantnagar) soil (86°C), subtropical soils (Chamoli - 87.43°C and Almora - 86.93°C) and temperate soils (Ranichauri - 87.34°C and Pithoragarh - 87.50°C) documented variable Tm values respectively, therefore, confirming diversified nature of these samples.

Further, soil samples from tarai region, subtropical region and subalpine region were positive for nifH amplification and give an amplicon of ~ 460bp. Six restriction enzymes (AluI, MspI, BglII, XbaI and HindIII) out of ten used in this study evidently showed good restriction pattern with soil DNA, whereas, in the case of nifH PCR-RFLP only four enzymes (HaeIII, AluI, MspI and PasI) documented the significant pattern. RFLP for diversity analysis has been commonly being used in the past by different labs (Noda *et al.*, 1999; Shaffer *et al.,* 2000; Poly *et al.,* 2001a). However, PCR-RFLP has been used to analyse the nifH gene pool composition in various environments (Poly *et al.,* 2001b; Chelius *et al.,* 1999; Ohkuma *et al.,* 1999). Moreover, the nifH gene is preferably being studied in environment samples for fertility indicator (Bardgett *et al.,* 1999; Roger and Ladha, 1992; Ladha and Reddy, 2003). The presence of nifH gene community in the soil indicates the presence of N_2 fixing population. The direct restriction analysis of the soil samples suggests that other than temperate region soils, soils from different geographic locations have diverse N_2 fixing bacterial community in terms of nifH gene biomarker. Further, temperate soil is also negative for *nif*H gene amplification (Fig. 1).

The *in silico* analysis of restricted fragments from different soil DNA samples significantly emphasizes the available bacterial diversity in studied soils. Here the dendogram clearly shows that at 34% similarity coefficient, different soil samples are grouped into four different clusters. At this similarity coefficient Temperate soils collected from two different regions are related forming a same cluster showing that not much of diversity in terms of bacterial community is present. But, the subtropical soils collected from two different locations illustrated diverse nature, with the former (Chamoli) showing more resemblance with cluster of temperate region while latter (Almora) showing resemblance with tarai region soil (Fig. 2A) sample. Further, subalpine region soil formed a separate cluster altogether and resembled more with the soils of temperate regions.

On the other side, dendogram prepared from soil restriction analysis documents that tarai region soil and subalpine region soil are in the same cluster with respect to nifH gene community. It shows that both soils have similar nitrogen fixing gene community (clustered in 6.75% similarity coefficient). However, soil from subtropical regions of Almora and Chamoli lies in two separate clusters irrespective of their same geographic location which depicts the diversity among *nif*H gene community (Fig. 2B).

Considering the two dendograms, it is clear that bacterial diversity exists among the same geographic location soils. Moreover, temperate soils have similar bacterial diversity but when analyzed with *nif*H gene marker, they show negative results. Similarly, tarai soil marks similarity with the subtropical soil in terms of whole bacterial population, but in *nif*H-PCR, they forms separate cluster and tarai soil is showing more similarity with subalpine soil.

The present study is a non sequential approach for studying the comparative diversity of microflora among different soils. It was illustrated that when soils having diverse bacterial community were analyzed for N_2 fixing population using *nif*H gene as a biomarker, bacterial diversity was found to exist even within same geographic soils. This suggests that knowing the microbial diversity or load of particular soil sample is not enough for deciding its fertility and productivity standards, but the consideration of particular functional gene alongwith diversity gives a better explanation.

Acknowledgments

This work is being funded by National Bureau of Agriculturally Important Microorganisms (NBAIM)/ Indian Council of Agricultural Research (ICAR), grant to RG. We thank to Dr. B. Saini, Professor of English for revising the manuscript.

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