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Triphasic approach to assessment of bacterial population in different soil systems

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Using more than one approach to the analysis of soil systems has always been a better option than a single approach due to the presence of complex genomes in metagenomic DNA samples. A combined triphasic approach using qPCR, DGGE and SSU ribosomal gene sequencing was applied to analyze the bacterial load on different soil systems (subalpine, temperate, subtropical and tarai) of the Western Indian Himalayas. Temperate soil (Pithoragarh, 80°2' E, 29°47' N, 1967 m) showed the maximum copy number (3.97×10^{10}/µl of DNA) or concentration (8.56 ± 1.06 ng/µl of DNA) of 16S rRNA gene. DGGE data and cloning of 16S rRNA gene has shown that this soil is most diverse among all the soils analysed in the present study. The lowest ribosomal gene copy number (8.85×10^{9}/µl of DNA) was observed in subalpine soil (a glacier, 30.44° N, 79° E, 3 133 m). It was concluded from this study that Pithoragarh (a temperate region) soil has a better bacterial load and diversity (among all the soil samples tested) and would be a good option for agricultural practices.

Key words: 16S rRNA, DGGE, qPCR, soil community, the Western Himalayas

INTRODUCTION

Microbes are the key members of soil ecosystems (Garbeva et al., 2004) and take part in increasing soil productivity by getting involved in various processes such as nitrogen fixation, *P* solubilization and bioremediation. Hence, it is important to know the bacterial load on soil being used for agricultural purposes. There are various biotechnological tools which are commonly used for analyzing the community structure of an environment like SSU RNA gene sequencing (Stephen et al., 1996; Yeates et al., 1998), RFLP (Poly et al., 2001), ARDRA (Zhang et al., 2008), TGGE (Muyzer, 1999), DGGE (Wartiainen et al., 2008) and Real Time PCR (Hermansson, Lindgren, 2001; Kimura et al., 1999). Most of these tools are based on the conserved 16S rRNA gene sequences. The use of any of these tools alone provides key information, but a combination of two or more approaches gives a complete picture. Thus, this offers a more powerful methodology for selecting an efficient soil system for agricultural purposes.

In this study, three approaches *viz.* qPCR, DGGE and ribosomal gene sequencing, were used to assess soils in different environments of the Western Himalayas (below alpine or tree line). The study area lies in the Western Himalayas region which comprises tarai (<1 200 m), subtropical (1 200 to 1 800 m), temperate (1 800 to 2 800 m) and subalpine (2 800 to 3 800 m) zones. These regions are also characterized by seasonal changes in physical and biochemical properties due to cold winters with snowfall for a long time, good rainfall in the monsoon, and mild summers. Due to this climatic shift, it possesses diverse flora and fauna. Further, it is known to harbour a variety of useful bacterial communities which are highly adapted to the varying extremities of weather (Pandey, Palni, 1998). Therefore, it is imperative to study the dominant bacterial traits and their load in the particular soil systems of this region.

METHODS

Soil sample collection and soil DNA extraction

Surface layer soil samples (not deeper than 15 cm) were collected during winter from different geographic locations of the Western Himalayas in India, namely subalpine (Badrinath and Mana Glacier), temperate (Ranichauri, Pithoragarh), subtropical (Chamoli) and Tarai (Pantnagar). Details of the sample collection sites are given in Table 1. All samples were kept at 4 °C till further use. Soil DNA was extracted using a Power soil™ DNA isolation kit (Mobio Lab.

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Inc., Carlsbad, CA, USA). The purity of the isolated DNA was checked with a UV spectrophotometer ((Perkin Elmer 35-lambda UV-vis spectrophotometer, Shelton, CT, USA) at 260 / 280 nm.

DGGE analysis

PCR for DGGE analysis was performed using the protocol and the primers (EUB f933 5'-GC-clamp-GCACAAGCGGTGGAGCATGTGG-3', EUB r1387 5'-GCCCGGGAACGTATTCAC

CG-3', GC-clamp 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) by Mako et al. (2002). DGGE was performed on a Dcode system (Bio-Rad Lab, Hercules, CA, USA), with 1 mm thick acrylamide–bisacrylamide (37.5 : 1) gel run in 0.5x TAE at 60 °C and 90 V. Gel solutions were made according to the manufacturer's instructions. The products were separated on a 6% (w/v) acrylamide–bisacrylamide gel with a 40–70% denaturing gradient for 6 h. The gels were stained for 30 min in SybrGreen gel stainer in 1x TAE (Invitrogen, Paisley, UK) and visualized with a Gel Documentation system (Bio-Rad Lab, Hercules, CA, USA). The phylogenetic analysis of the banding pattern was performed using Quantity One software provided with a gel documentation system (Bio-Rad) using the neighbor-joining method.

16S rRNA gene amplification, cloning screening and sequencing

The 16S rRNA genes were amplified from isolated soil DNA, using universal eubacterial primers (primer 1- 5' CCTACGGGAGGCAGCAG 3' and primer 2- 5' ATTACCGCGGCTGCTGG 3') (Muyzer et al., 1993). Undiluted soil DNA (1 µl) was added to 25 µl (final volume) of a mixture containing 0.1 µM of each primer, 250 µM of each dNTP (New England Biolab, MA, USA), 2.5 µl of 10x buffer (New England Biolab) and 1 U *Taq* polymerase (New England Biolab). PCR was run at 94 °C for 3 min (initial denaturation), followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min and 72 °C for 10 min (final extension). The amplified 16SrRNA gene amplicons were cloned using a Qiagen PCR cloning plus (Qiagen, Qiagen Institute, Valenica, CA) kit. Transformants were checked on Luria Agar medium plates (with 100 μgl^{-1} ampicillin). The selected clones were then sequenced at South Campus, Delhi University (India). These clones were sequenced with an ABI-PRISM DNA sequencer (Model 3730, version 3.0) using T3 and T7 sequencing primers.

All the sequences were submitted to NCBI GenBank with accession numbers EU647765 to EU647790, EU689116 and FJ785820 to FJ785825 and FJ792808 to FJ792812.

Quantitative PCR

The qPCR was set in an iCycler iQ™ Multicolor (Bio-Rad Lab, Hercules, CA, USA) instrument using SYBER green chemistry. The PCR mixture included 3 µl of purified soil DNA, 12.5 µl of 2x SYBR supermix and 2.5 µl (0.1 µM) of each universal primers (primer 1- 5' CCTACGGGAGGCAGCAG 3' and primer 2- 5' ATTACCGCGGCTGCTGG 3'). PCR cycles consisted of 5 min of initial denaturation at 95 °C, followed by 35 cycles of 1 min at 95 °C and 2 min at 58 °C. A standard curve was prepared using genomic DNA from *Pseudomonas putida* (DQ205427) taken from the departmental culture collection. The extracted bacterial DNA was subjected to a conventional PCR in a volume of 25 µl containing 0.1 µM of each primer, 250 µM of each dNTP (New England Biolab, MA, USA), 2.5 µl of 10x buffer (New England Biolab) and 1 U *Taq* polymerase (New England Biolab). The PCR was run at 94 °C for 3 min (initial denaturation) followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min and 72 °C for 10 min (final extension). The PCR product was then analyzed by gel electrophoresis and purified with Montage™ PCR Centrifugal Filter Devices (Millipore Corporation, Bedford, MA, USA). The purified product was quantified by spectroscopy at 260 nm. Two-fold serial dilution was done (starting from 10 ng) and was used as an external standard for quantification by real time PCR. With the help of a standard curve, the amount of DNA was quantified by the software provided by the manufacturer (Bio-Rad). The copy number estimation was done according to the formula: the number of copies = (amount $\times 6.022 \times 10^{23}$ / (length $\times 10^9 \times 650$), assuming one copy of ribosomal gene per genome (Kabir et al., 2003).

Phylogenetic analysis

All the sequences were compared to the GENBANK database using BLAST*n* (http://blast.ncbi.nlm.nih.gov/Blast.cgi. Bethesda, MD, USA). Homologous sequences were retrieved from NCBI database and aligned with clone sequences using a ClustalX multiple sequence alignment tool (version 1.81). Further, the phylogenetic tree was prepared using the neighbor joining method (Saitou, Nei, 1987), in MEGA 4.1 software. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) in the units of the number of base substitutions per site. Moreover, all positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analysis was conducted in MEGA4 (Tamura et al., 2007). Further, for generic homology, the clone sequences were also aligned with 1519 available assembled eubacterial genome sequences at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

RESULTS AND DISCUSSION

qPCR and DGGE analysis

The real-time PCR was performed using a similar primer set as the one used for 16S rRNA gene cloning. Quantification of the ribosomal DNA from all sources was done using the standard curve drawn on the basis of the known concentration of standard DNA. The quantification showed a linear correlation ($R^2 = 0.997$) between the log values of bacterial

Table 1. **Samples profiling details in relation to selected clones**

Sampling site	Longitude	Elevation, m	Concentration of amplified 16SrRNA gene(ng / µl of DNA)	Copy no. of ribosomal gene[a]	Selected clones
Tarai-plane (TP) – Pantnagar	29.00° N	243.8	6.07	2.81×10^{10}	PE2, PE3, PE5, PE6
Subtropical (ST) – Chamoli	30.51° N, 79.4° E	1300	5.23	2.42×10^{10}	CE2, CE11, CE13, CE9, CE8, CE10, CE12
Temperate sample 1 (TS1) – Ranichauri	78°30′ E, 30°15′ N	1950	2.83	1.31×10^{10}	RE1, RE8, RE10, RE11, RE12, RE13
Temperate sample 2 (TS2) – Pithoragarh	80°2′ E, 29°47′ N	1967	8.56	3.97×10^{10}	PT3, PT5, EP1, EP2, EP3, EP5, EP6, EP7, EP8, EP9, EP13
Subalpine sample 1 (SA 1) – Badrinath	30.44° N, 79° E	3110	4.89	2.27×10^{10}	BE1, BE3, BE10
Subalpine sample 2 (SA 2) – glacier	30.44° N, 79° E	3133	1.91	8.85×10^{9}	GE2, GE3, GE6, GE7, EG1, EG4

[a]The formula used for calculating the number of copies is: number of copies = (amount · N / (length · 1×10^9 · m), where m is the average weight of a base pair (bp) is 650 Daltons, N is the Avogadro's number (6.022×10^{23} molecules / mole).

genomic DNA and real-time PCR threshold cycles, over the range of examined DNA concentrations (Fig. 1). Quantification of the above-mentioned soil samples showed that Pithoragarh soil had the highest 16S rDNA copy number / 10 mg soil (3.97×10^{10}/ µl of DNA), followed by Pantnagar (2.81×10^{10}/ µl of DNA), Chamoli (2.42×10^{10}/ µl of DNA) and Badrinath (2.27×10^{10}/ µl of DNA). However, the Ranichauri (1.31×10^{10}/ µl of DNA) and glacier (8.85×10^{9}/ µl of DNA) soil samples showed the least presence of 16S rDNA (Table 1).

Further, DGGE results revealed a significant diversity of 16S rRNA gene among all the soil samples. The phylogenetic tree prepared from the individual banding pattern, reported three clusters in which temperate soil sample 2 (Pithoragarh soil) was separated from the other samples, indicating its diversified nature. This was further supported by the sequences of clones as most of the clones were subsequently recovered from this soil sample (Table 2).

PCR amplification, cloning and sequencing

After extraction of soil DNA, 16S rRNA genes were amplified, cloned and sequenced for individual soil samples. The identical clone sequences were removed, and the total of 38 clones were selected for phylogenetic analysis (Table 1).

The phylogenetic tree was constructed on the basis of Blast*n* homology results (Fig. 2). Blast*n* results revealed that out of the total selected clones, 19.5% had the homology with purely culturable bacteria, while the remaining 80.5% showed a homology (more than 95% with the score >200) with unculturable bacterial sequences available in the NCBI database. However, these clones were also aligned with the 1 519 assembled bacterial genome database (NCBI) where more than 70% homology was considered for generic confirmation. The majority of clones (50%) belonged to the proteobacteria group, while the rest were from actinobacteria (22%) and firmicutes (14%). One clone, RE13, was found to have no significant similarity with the available culturable genome

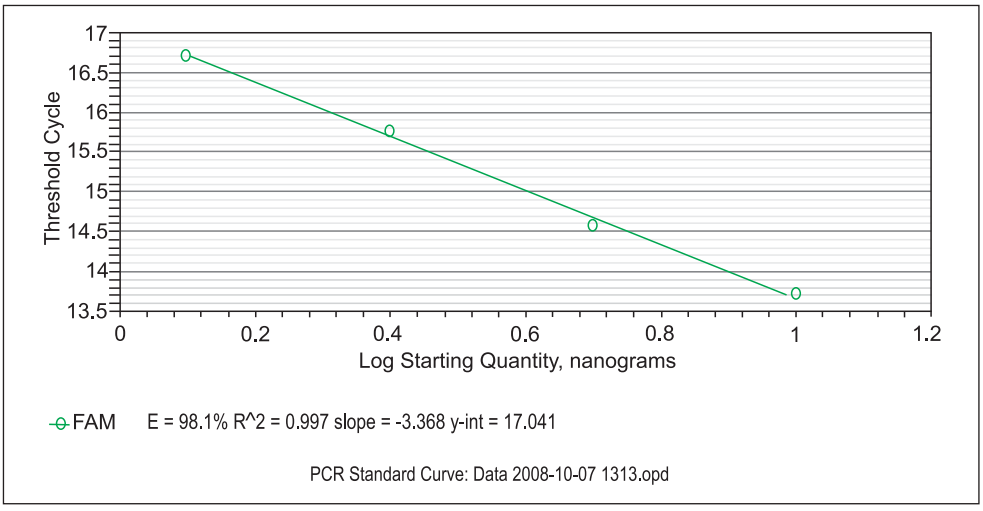

Fig. 1. Standard curve obtained by plotting log starting concentration of 2-fold dilution (starting with 10 ng) genomic DNA of *Pseudomonas putida* strain KNP9 (DQ205427) versus the cycle number required to elevate the fluorescence signal above the threshold

Table 2. **Blast homology (assembled genomes) among 16S rDNA clones of the Western Indian Himalayan soil**

Climate	Clone	Genus	Homology (%)
Subalpine	BE1	*Bacillus*	99
	BE3	*Exiguobacterium*	95
	BE10	*Stigmatella*	84
	GE2	Pseudomonas	100
	GE3	Mesorhizobium	99
	GE6	Janthinobacterium	96
	GE7	Bacillus	97
	GE1	Mycobacterium	83
	GE4	Clostridium	94
Temperate	EP1	*Acidithiobacillus*	83
	EP2	*Arthrobacter*	98
	EP3	*Xanthomonas*	94
	EP5	*Sphingopyxis*	96
	EP6	*Sphingopyxis*	98
	EP7	*Microscilla*	87
	Ep8	*Flavobacterium*	89
	EP9	*Syntrophus*	85
	EP13	*Sphingopyxis*	97
	PT3	*Exiguobacterium*	90
	PT5	*Rhodococcus*	97
	RE1	*Acidobacteria*	93
	RE8	*Arthrobacter*	99
	RE10	*Arthrobacter*	99
	RE11	*Geobacter*	83
	RE12	*Janibacter*	98
	RE13	No significant homology	–
Subtropical	CE2	*Parvibaculum*	92
	CE8	*Geobacter*	81
	CE9	Flavobacterium	87
	CE10	*Azorcus*	82
	CE11	*Mesorhisobium*	94
	CE12	*Mycobacterium*	90
	CE13	*Rubrobacter*	86
	CE14	*Azorhizobium*	91
Tarai	PE2	*Sphingomonas*	98
	PE3	*Coxiella*	90
	PE5	*Nostoc*	90
	PE6	*Sphingopyxis*	99

database (assembled). However, among proteobacteria, the dominating subdivision was the alpha group.

Several studies on bacterial communities from subalpine, temperate and subtropical soils of the Western Himalayas (Selvakumar, 2008; Pandey, Palni, 2007, 1998; Zhang et al., 2006) have already been documented. However, the comparative bacterial load in this region needs to be analyzed. Previous culture-dependent studies by our and other groups confirmed that Western Indian Himalayan soil has the bacterial community with a tremendous potential of biodegradation (Soni et al., 2008; Satlewal et al., 2008; Goel et al., 2008) and plant growth promotion properties (Pandey et al., 2006). Moreover, the cultivation-based analysis has its limitations due to the fact that only 1–3% of bacteria are culturable. Quantitative (Filion et al., 2003; Shilpi et al., 2007) and sequence-based metagenomic analyses (Lozupone, Knight, 2007) of microbial biomass from any environmental samples provide a complete idea about the potentiality of soil and its productivity.

Further, temperate soil 2 (TS2) was found to have the highest bacterial DNA load (3.97×10^{10} copies/µl of DNA). These data were supported by DGGE analysis in which this soil came in a separate cluster and cloning of 16S rRNA gene, with the maximum numbers of diverse clones. However, DNA concentration decreased (from 6.07 ± 0.37 ng/µl to 2.83 ± 0.29 ng/µl of DNA) with the altitude (240 m to 1950 m). Thereafter, it suddenly increased in temperate soil, followed by a decline in subalpine soil samples (4.89 ± 0.95 ng/µl and 1.91 ± 0.53 ng/µl of DNA), respectively). These data suggest that the bacterial biomass is affected not only by the altitude, but also by some other factors such as soil pH (Pandey, Palni, 1997) and local flora and fauna (Pandey, Palni, 2007). Nonetheless, seasonal variations such as temperature and humidity fluctuation also affect the microbial community structure (Schmidt, Lipson, 2004).

Recently, denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene has been used to profile complex microbial communities (Mako et al., 2002; Ferris, Ward, 1997). A similar approach has been used, wherein DGGE was tested

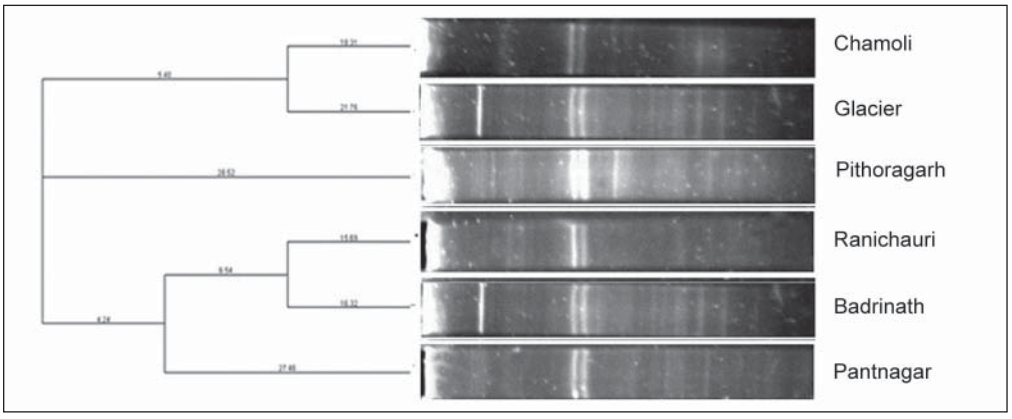

Fig. 2. Phylogenetic analysis of 16S rRNA gene sequences amplified from different soils of the Western Himalayan soil metagenome. The tree was constructed using the neighbor-joining method. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees

as a means to study microbial community composition in bulk soil samples (Nakatsu et al., 2000). Our results from qPCR supported by the DGGE phylogenetic tree pattern, in which the subtropical sample (ST) and the subalpine sample (SA 2) are in same cluster, both have approximately the same 16S rRNA gene concentration (5.23 ± 0.13 ng and 4.89 ± 0.95 ng, respectively). Moreover, the temperate soil sample (Ranichauri, TS 1) and the subalpine sample (Glacier (SA2)) containing approximately equal concentrations of the ribosomal gene (2.83 ng ± 0.29 µl of DNA ng and 1.91 ng ± 0.53 µl of DNA) are placed in a similar cluster also (Fig. 2).

Further, the 16S rRNA gene sequence-based analysis has indicated that subalpine, temperate, subtropical and lower (tarai) regions of Himalayan soils have a diversified bacterial community. The point of interest here is that Blastn alignment data showed the majority of unculturable bacteria (80.5%) in the Western Indian Himalayan soil. Further, results from the Blastn (Basic Blast, http://blast.ncbi.nlm.nih.gov/Blast.cgi) in combination with the results of BLAST assembled genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) analysis provide a satisfactory scenario regarding the existence of a dominant phyla in a respective soil sample. This study has revealed that all the soil samples exhibited the dominance of phylum proteobacteria, especially those of nitrogen-fixing genera *viz*. the species *Azoarcus, Mesorhizobium, Azorhizobium, Mycobacterium, Nostoc* and *Clostridium*. There were also several uncommon soil bacterial genera, such as *Parvibaculum*, *Exiguobacterium, Stigmatella* and *Sphingopyxis.* One clone (RE13) did not show a significant homology with the existing bacterial genomes (Table 2).

The present study is a sincere effort to explore the microbial community of the Indian Himalayan region, which needs to be documented properly. Here, the temperate soil of Pithoragarh was the most diverse soil with an efficient content of bacterial load, and it would be a good agricultural soil. Overall, it implies that the phylogenetic tree with clones gives an idea about the unculturable diversity of the soil, but the diversity among the soils was determined with the help of DGGE and real-time PCR. This combined approach thus widely explains the community structure in general. Further, it also signifies the implication of culture-independent approaches to community structure analysis.

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