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Bacterial community analysis using temporal temperature gradient gel electrophoresis (TTGE) of 16S rDNA PCR products of soil metagenome

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Separation of amplified 16S rDNA products by temporal temperature gradient gel electrophoresis (TTGE) was carried out for studying bacterial community composition in bulk soil samples in the different tracts (subalpine, temperate and subtropical) of the Western Himalayas (India). Bacterial universal primers were used to amplify the 16S rDNA product. Characteristic profiles of the communities were obtained by TTGE. Bands resolved by TTGE suggested the presence of a moderate diversity among the dominating soil bacterial species of this region. Further, it was observed that the diversity of the bacterial population varies within the same climatic conditions. The study has also revealed that the method is efficient for screening multiple samples.

Key words: TTGE, metagenomics, microbial diversity, the Western Himalayas

The Western Himalayas which comprise different – subtropical (1 200 to 1 800 m), temperate (1 800 to 2 800 m) and subalpine (2 800 to 3 800 m) zones – are known for their rich biodiversity. This region is also characterized by seasonal changes in physical and biochemical properties due to cold winters with snowfall for an extended period, abundant rainfall in the monsoon, and mild summers. Due to this climatic shift, its flora and fauna is diversified and is known to harbour a variety of useful bacterial communities which are highly adapted to the varying extremities of weather (Pandey, Palni, 1997). Earlier studies on bacterial communities from subalpine, temperate and subtropical soils of the Western Himalayas (Pandey, Palni, 2007; Selvakumar et al., 2008) gave an idea about the microbial diversity in this region, but the comparative bacterial load still needs further exploration and authentic documentation.

After introduction of temporal temperature gradient gel electrophoresis (TTGE) (Yoshino et al., 1991) and Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993), the face of microbial ecological research has been changed. These two tools are regularly helping in exploration of microbial communities of different environments (Campbell et al., 2009; Kaartokallio et al., 2008; Wei et al., 2007; Zeng et al., 2009). TTGE exploits the principle on which DGGE is based, without requiring a chemical denaturing gradient. An amplified product of soil DNA is loaded onto a polyacrylamide gel with a constant concentration of urea. During the run time, the temperature is increased gradually and uniformly. The result is a linear temperature gradient over the length of the electrophoresis run. Finally, DNA fragments of different nucleotide sequences are separated on the gel. The use of DGGE for community analysis is now common (Cherif et al., 2008; Chong et al., 2009; Lai et al., 2007; Nakatsu et al., 2000; Ning et al., 2009; Wang et al., 2008; Yoshida et al., 2008), but TTGE has not been much explored. The objective of this study was to determine whether the separation of 16S rRNA amplified sequences from soil DNA using TTGE could provide qualitative and quantitative information about soil microbial community composition without further analysis, wherein the method was tested on soils of different agroclimatic regions (from subalpine to subtropical) of the Western Indian Himalayas.

MATERIALS AND METHODS

Collection of soil samples and isolation of soil DNA

Surface layer soil samples (not deeper than 15 cm) were collected during winter from different geographical locations of the Western Himalayas viz. subalpine (Badrinath, Mana Glacier), temperate (Almora, Ranikhet, Ranichauri, Champawat and Pithoragarh), and subtropical (Rishikesh, Dehradun, Chamoli). Details of the sample collection sites are given in

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Table 1. **Geographical details of soil sample collection sites**

Sample site	Longitude / longitude	Elevation m	Climate
Rishikesh	30.12° N, 78.32°E	372	Subtropical
Dehradun	30.20° N, 78.03°E	635	Subtropical
Chamoli	30.51° N, 79.4°E	1300	Subtropical
Champawat	29.3333° N, 80.1E	1615	Subtropical
Almora	29.37° N, 79.40°E	1651	Subtropical
Ranikhet	29.39° N, 79.25°E	1869	Temperate
Ranichauri	30.15° N, 78.30°E	1950	Temperate
Pithoragarh	29.47° N, 80.2°E	1967	Temperate
Badrinath	30.44° N, 79°E	3110	Subalpine
Glacier	30.44° N, 79°E	3133	Subalpine

Table 1. All samples were kept at 4 °C till further use. Soil DNA was extracted using the Power soil™ DNA isolation kit (Mobio Lab. 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.

Amplification of 16S rRNA gene

Amplification of 16S rRNA gene for TTGE analysis was performed using the protocol and primers (EUB f933 5'-GC-clamp-GCACAAGCGGTGGAGCATGTGG-3',EUB r1387 5'-GCCCGGGAACGTATTCACCG-3',GC-clamp5'CGCCCGCCGCGCGCGGC GGGCGGGGCGGGGGCACGGGGGG) described by Mako et al. (2002). This primer set amplifies the V_6–V_8 region of the bacterial 16S rRNA gene. The polymerase chain reaction (50 µl) mixture contained 0.1 µM of each primer, 250 µM of dNTPs (New England Biolab, MA, USA), 5 µl of 10X buffer (New England Biolab), 2 U *Taq* polymerase (New England Biolab) and 1 µl of undiluted soil DNA. The total volume of the reaction mixture was maintained with sterilized triple distilled water. A touchdown PCR was performed in a BioRad iQ™5, multicolor real time PCR detection system and was carried out as follows: a single denaturation step at 95 °C for 7 min, followed by a 20 cycle programme which included denaturation at 94 °C for 1 min, annealing at a temperature between 66–56 °C (decrease of 0.5 °C in every cycle) for 1 min and extension at 72 °C for 3 min. This was followed by an additional 20 cycle programme which involved a denaturation step at 94 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 3 min and a final extension at 72 °C for 10 min.

TTGE analysis

TTGE was performed on a Dcode system (Bio-Rad Lab, Hercules, CA, USA). The products were separated on a 8% (w/v) acrylamide–bisacrylamide gel containing 7 M of urea as a denaturant, 1.25x TAE, 0.1% TEMED, 1 gL^{-1} ammonium persulfate and acrylamide–bisacrylamide solution (from 40% stock) following the manufacturer's recommendation (BioRad). The temperature ranged within 58–70 °C, while the running time ranged between 5 to 6 h. Electrophoresis was carried out at 120 V. The gel was stained for 30 min in a SybrGreen gel stainer in 1× TAE (Invitrogen, Paisley, UK) and visualized by Gel Documentation system (Bio-Rad Lab, Hercules, CA, USA).

Statistical analysis

The phylogenetic tree was drawn on the basis of the banding pattern by Quantity One software (Bio-Rad, CA, USA) using the neighbor-joining method. Relatedness of microbial communities was also determined using similarity coefficients of bands among different samples. First, the total number of different bands was determined for samples being compared, and each sample was scored on the basis of the presence or absence of each band in its profile when compared with the profile of each of the other samples. Sørensen's similarity coefficient [$Cs = 2j / (a + b)$] was used for a pairwise calculation of band sharing between samples (Sørensen, 1948). In this equation, a is the number of bands in sample A, b is the number of bands in sample B, and j is the number of bands common to both A and B samples.

RESULTS

The TTGE profile using the above set of primers documented the separation of different bands along with a little smear due to the presence of the 16S rRNA gene fragments which may have a minor difference in their sequences. Smears of the bands from bulk soils were similar, but not identical in all samples (Lerner et al., 2006). Further, the number of intense bands obtained on the gel was less than ten. These intense bands illustrate the dominating soil bacterial species, but the presence of smear in the amplified product suggests the presence of diverse bacterial populations which were in a lower amount. The least numbers of bands were observed in Rishikesh soil.

The dendogram based on the banding pattern showed three major clusters. The subtropical soil (Rishikesh) inhabited a separate cluster. The second cluster was formed by the samples of the Ranikhet, Ranichauri and Chamoli regions,

Table 2. **Similarity coefficient (Cs*) of bacterial populations in Western Indian Himalayan soils**

	Sørensen coefficient (Cs*)									
Lanes compared	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1									
2	0.50	1								
3	0.86	0.43	1							
4	0.80	0.40	0.70	1						
5	0.92	0.31	0.80	0.75	1					
6	0.77	0.31	0.53	0.50	0.57	1				
7	0.71	0.57	0.62	0.70	0.80	0.93	1			
8	0.57	0.42	0.50	0.59	0.66	0.67	0.62	1		
9	0.86	0.42	0.62	0.70	0.80	0.80	0.75	0.75	1	
10	0.86	0.71	0.62	0.82	0.80	0.67	0.87	0.75	0.87	1

Lanes 1–10: Ranikhet, Rishikesh, Ranichuari, Pithoragarh, Champawat, Glacier, Dehradun, Chamoli, Badrinath, Almora, respectively.

Cs* = 2 (No. of bands shared) / total No. of bands. Lanes correspond to TTGE profile of PCR amplified product of soil DNA as illustrated in Fig. 1

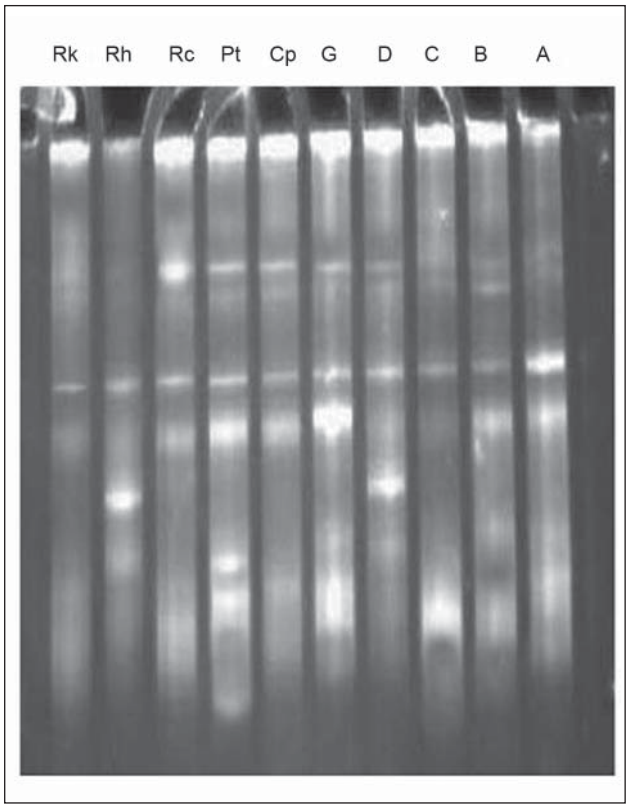

Fig. 1. Temporal temperature gel electrophoresis (TTGE) of different regions of the Indian Western Himalayan soil (temperature range 58–70 °C, running time 5–6 h). Electrophoresis was carried out at 120 V. The gel was stained for 30 min in SybrGreen gel stainer in 1× TAE).

Rk – Ranikhet, Rh – Rishikesh, Rc – Ranichuari, Pt – Pithoragarh, Cp – Champawat, G – Glacier, D – Dehradun, C – Chamoli, B – Badrinath, A – Almora, respectively

while the third cluster contained the rest of the samples. The subalpine soil of the Badrinath region was found to more closely resemble the subtropical soil of Almora (temperate) than its neighbour subalpine soil of Glacier. Another interesting observation was that diversity was present within similar climatic regions as the temperate soils of Ranikhet and Pithoragarh were found to be more different from each other. Similarly, all subtropical soils were also in a separate cluster.

Based on the presence or absence of the bands in each sample, Sørenson's similarity coefficients (Cs) were determined for the TTGE profile (Table 2). A value of 1.0 indicates that all bands between the samples analysed are common, whereas 0.0 indicates no bands in common. Analysis of the Cs can also be used for determining the similarity between species within the same climatic region. When the temperate soils were compared on the basis of Cs, it was observed that the similarity between Ranikhet and Ranichauri soil microbial species was higher (Cs = 0.86) than for the Ranikhet and Pithoragarh soil samples (Cs = 0.80). Among the subtropical soils, Cs was highest between Almora and Dehradun whereas the subalpine soils had Cs = 0.67. Furthermore, the results have shown that the TTGE approach for diversity analysis of various ecosystems seems as effective as DGGE. A combination of PCR of 16S rDNA and TTGE can be used to differentiate the microbial community composition in different soils (Figs. 1 and 2). The results suggest that a bacterial community can vary also within the same climatic conditions. The technique enables to distinguish diversity among various environments, but there are limitations to quantifying the extent of differences among highly diverse communities. The results of the present study have revealed that the Western Himalayan climatic tracts have a highly diverse bacterial community. Earlier, culture-dependent studies have also documented that the bacterial community of Western Himalayan soil has a tremendous potential of biodegradation (Goel et al., 2008; Satlewal, 2008; Soni et al., 2008) and PGPR properties (Pandey, Palni, 2007; Pandey et al., 2006). However, more efforts are needed to explore the microbial diversity of this biotic zone. In future, the advanced molecular biology tools like TTGE and DGGE would play a key role in the systematic documentation of microbial population.

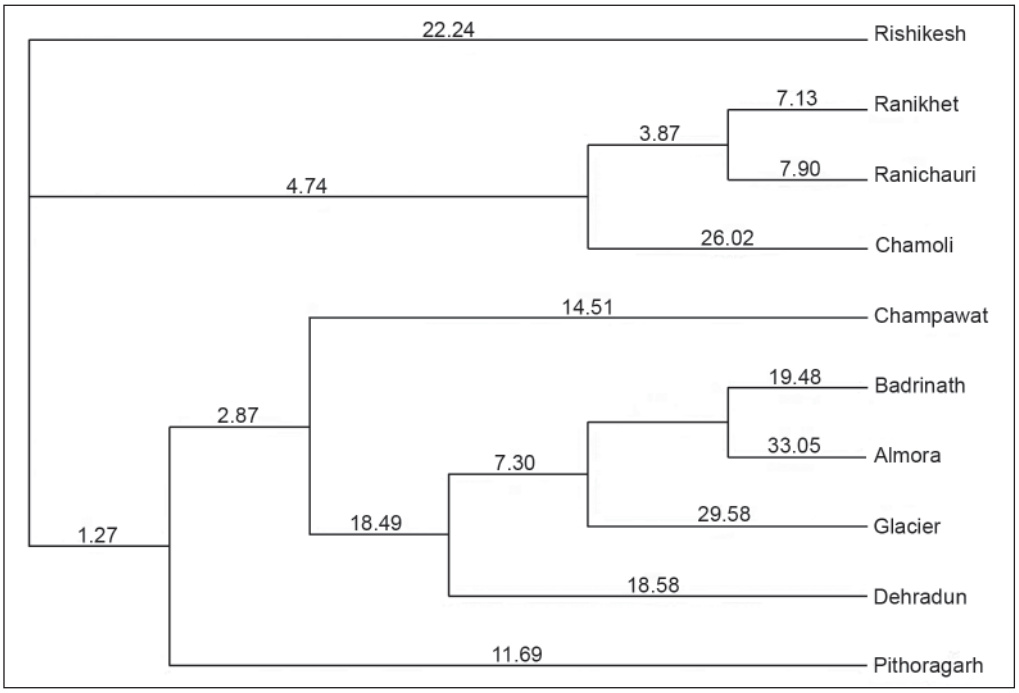

Fig. 2. Phylogenetic tree on the basis of individual soil banding pattern by quantity one software (Biorad) using the neighbor-joining method

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