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Random amplified polymorphic DNA (RAPD) analysis of microbial community diversity in soil affected by industrial pollutants: Reference to Mandideep industrial area

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The present study was aimed to investigate microbial diversity of industrially contaminated and uncontaminated agriculture field soil using random amplified polymorphic DNA (RAPD) analysis. Soil samples were collected from highly contaminated (industrial pollution) and uncontaminated agriculture fields away from polluted area. RAPD analysis was carried out using four ten-mer primers namely RBa-3, 4, 5 and 6 to find out the effect of industrial contamination on microbial community diversity. In total, 56 DNA fragments were generated using 4 deca-mer random primers with good reproducibility, 55 of them were polymorphic (99%) and 1 bands (loci) was monomorphic (1%). The average Jaccard's similarity coefficient based on 4 primers ranged from 0.163 (cultivated soil) to 0.462 (industrially polluted soil) in all the accessions studied, which indicates that cultivated soil supports more diverse microbes than the polluted soil. Cluster analysis was performed based on the Jaccard's similarity coefficient matrices, calculated from RAPD data. Different clustering methods including unweighed pair group method for arithmetic average (UPGMA), NJ and Bayesian tree almost produced similar results with good bootstrap and clade credibility values. In all accessions, three different clusters were obtained. These three clusters were further divided into subclusters. Samples from the agricultural field show high genetic affinity and are placed in a single cluster or clade. Results confirmed the effects of pollution on the distribution and biodiversity of soil microorganisms where most of the native beneficial microorganisms were disappeared or not cultured under these stress conditions as compared to the normal agricultural field soils, which is certainly affecting soil fertility and productivity.

Key words: Effluent contaminated soil, polymerase chain reaction-random amplified polymorphic DNA (PCR-RAPD), microbial community, lyzozyme, bacterial diversity.

INTRODUCTION

Soil biodiversity is endangered by industrial pollution (PHAs, heavy metals, carcinogenic chemicals), agricultural and manmade activities (Ahn et al., 2009; Caracciolo et al., 2011), which results in biological invasions, endangering endemic faunas and floras and changes in microbial diversity and function (Ansari and Malik, 2007). Lodge et al. (1996) has also described various threats to microbial diversity in tropical forests, which includes forest fragmentation, loss of hosts caused by logging and other anthropological activities, air pollutants, industrial

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Table 1. Different soil samples collected from the vicinity of the Mandideep Industrial area and uncontaminated soil from different distances.

Area of the study/districts	Population ID
Godrej and Hershey Ltd. (Food and Beverages Industry)	INS PS-1
HEG Ltd (Graphite producing company)	INS PS-2
Bansal Ind Ltd. (Iron product development)	INS PS-3
Crompton and greaves (AC and Refrigerator)	INS PS-4
Lupin Ltd (Pharma product development)	INS PS-5
Aristo Industries (Pharma product development)	INS PS-6
Central Mandideep uncultivated field	INS PS-7
15 km from the Industrial area (Cultivated field control)	INS PS-8
30 km from the industrial area (Cultivated field control)	INS PS-9
45 km north from the industrial area (Cultivated field control)	INS PS-10
60 km from Industrial area (Cultivated field control)	INS PS-11
40 Km south from Industrial area (Cultivated field control)	INS PS-12
Satawar plants root soil in Industrial area	INS PS-13

pollutants, etc. Soil microbial diversity is very important factor for the good cultivation of crops and plant productivity of terrestrial ecosystems. Microbes maintains the biogeochemical cycles and also improves the soil fertility by decomposition of all organic matters including persistent xenobiotics and naturally occurring polyphenolic compounds, which helps in soil fertility (Garbeva et al., 2004). Soil biodiversity is several orders of magnitude higher than that above ground (Heywood, 1995; Swift, 1999) and also a fact that fertile soils contain a greater proportion of bacteria than infertile and humans have relied directly on that to provide food (e.g. mushrooms, roots or rabbits) and medicinal products (e.g. leeches to antibiotics). The biotic elements (micro and macro-life forms) within soil interact with the soil abiotic elements (chemical and physical properties) to maintain the diverse, multi-functional value of soils (Handelsman and Wackett, 2002; Robe et al., 2003; Roose-Amslag et al., 2001; Lauber et al., 2009).

The living population inhabiting soil includes macrofauna, mesofauna, microfauna and microflora. The bacteria genus abundant in soil are: *Arthrobacter, Corynebacter, Mycobacter, Nitrobacter, Pseudomonas, Actromobacter, Radiobacter, Rhizobia,* etc and they live mainly on the surface of the soil and humus particles. 80 to 90% of the processes in soil are reactions mediated by microbes, which indicates its relationship between microbial diversity and soil functionality (Coleman and Crossley, 1996; Nannipieri et al., 2003). The soil microbial community is strongly influenced by a wide variety of factors, such as soil characteristics and field management systems.

Protection of soil is therefore, of high priority and a thorough understanding of ecosystem processes, and is critical factor in assuring soil health (Wilhjelm committee, 2001). Microbial activity in soil depends on moisture content, temperature, soil enzymes, dissolution of soil minerals and breakdown of toxic chemicals. Variety of molecular methods based on direct isolation and analysis of nucleic acids, proteins and lipids from environmental samples have been discovered and revealed structural and functional information about microbial communities. Molecular approaches such as genetic fingerprinting, metagenomics, metaproteomics, metatranscriptomics and proteogenomics are vital for discovering and characterizing the vast microbial diversity and understanding their interactions with biotic and abiotic environmental factors.

The present study was focused on the effect of industrial pollution on soil microbial diversity in highly contaminated and stressed local environment (Industrial Area, Mandideep, India). The study was performed not only because of the significant reduction in land fertility and productivity, but also the public health risk resulted from the widespread distribution of chemicals, PHAs and heavy metals.

MATERIALS AND METHODS

Collection of soil samples

Thirteen soil-collecting points were specified in the industrial area Mandideep situated in Madhya Pradesh, India (Table 1 and Figure 1). Each soil samples were collected from the periphery of the different industries, contaminated with industrial effluent drained from the individual industry and also from the agricultural field as control sample. For each soil sampling site, three 10 cm depth cores were taken, tightly sealed in plastic bag and transferred to the laboratory to be processed and samples were kept at -20°C until the experiment began.

Extraction of DNA

Triplicate of each soil samples were used for DNA extraction. Three soil cores from each sampling point were mixed and sieved through a 2 mm wire mesh to remove plant debris. The soil samples were cultured in the nutrient broth for 48 h at 37°C and then centrifuged to pellet the soil. The DNA extraction was then preceded with the enzymatic lysis of samples (Steffan and Atlas, 1988). Briefly samples

Table 2. Polymorphism pattern demonstration by RAPD analysis in different populations of microbes in soil samples.

Primer	Accessions	Total no. of bands (*a*)	Total no. of polymorphic bands (*b*)	Total no. of monomorphic bands (*b*)	Polymorphism (*bla* × 100) (%)
RBa-3	AM 773772	16	16	0	100
RBa-4	AM911679	12	12	0	100
RBa-5	AM 911680	18	18	0	100
RBa-6	AM773778	10	9	1	90
Total		56	55	1	98.21

Figure 1. Soil sample collection sites of Mandideep industrial area (India).

were suspended in 2 ml of a lyzozyme soln (150 mM NaCl, 100 mM EDTA, 5 mg/ml lyzozyme). The tubes were then inverted several times to mix the contents and placed in a 37°C water bath for 2 h. Subsequently, 500 µL Protienase K (2.5mg/ml) was added, and the content of the tube was gently mixed and placed in a 55°C water bath for 15 min. An equivalent volume of phenol : chloroform : isoamyl alcohol (25:24:1) was added and the mixture was gently vertexed for 1 min. The mixture was centrifuged at 14000 g at 4°C for 10 min and the supernatant was collected. To precipitate DNA, 0.7 vol. cooled isoproponol and 1/10 vol. 3 M sodium acetate were added to the supernatant. The mixture was gently mixed (5-10 times) and kept at -20°C overnight. The samples were centrifuged at 14000 g for 10 min, and pellet was washed three times with cold 70% ethanol and then resuspended in 100 microlitre TE Buffer (10 mM Tris-HCl, 0.1mM EDTA, pH-8.0).

Yield and purity of DNA

The yield and purity of DNA was determined by calculating the ration of absorbance at 260 and 280 nm using a Nano Drop UV

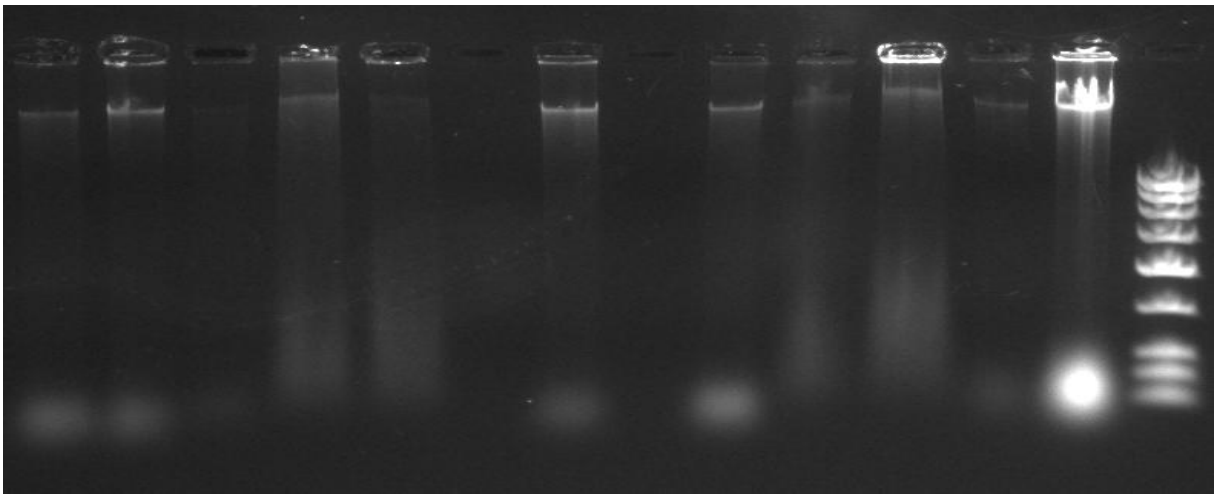

Figure 2. Integrity of isolated bacterial DNA from the soil samples obtained from Mandideep industrial area.

Spectrophotometer (ND-1000). The DNA was diluted to final concentration of 50 ng/µl using TE buffer and used as template DNA for RAPD analysis. DNA concentration and purity was also determined by running the sample on 1% agarose gel based on the intensities of band when compared with the DNA marker.

RAPD-PCR analysis

RAPD profiles were generated using DNA extracted from 13 soil samples. Four decamer random oligonucleotide primers (Bangalore Genei, India) were used for amplification in polymerase chain reaction (PCR) following the standard protocol according to the RAPD amplification kit (Bangalore Genei). RAPD Primer accessions are shown in Table 2. Each amplification reaction mixture of 50 µl volume contained about 1 µl of genomic template DNA (50 ng), 25 µl of 2x red dye, 2 µl of primer (Bangalore Genei Pvt. Ltd., Bangalore, India) and 22 µl de-ionised water (RNAse, DNAse free). The mixture was centrifuged briefly to mix well. The reactions were carried out in Gradient Automatic Thermal Cycler (PCR), (eppendorf) in the following temperature cycles: holding at 94°C for 5 min at start, followed by 8 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 1.5 min and 35 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 1 min and a final additional extension at 72°C for 10 min and a holding temperature of 4°C. Amplified PCR-products were stored at 4°C. All the experiments were repeated thrice to ensure reproducibility.

Agarose gel electrophoresis

Amplified PCR products were electrophoreticaly separated in 1.2% (w/v) agarose gel, in 1×TAE buffer for 3 to 4 h at 60 V and visualized under UV light by ethidium bromide staining and photographed using Gel documentation system (Alpha Innotech). To determine the size of the polymorphic fragments, 100 bp DNA ladder (Quarta Bio), was used as size standard molecular marker. Raw gel images were recorded through Alpha View Software. All the experiments were repeated thrice to ensure reproducibility. The best gels of the replicates were used for band scoring.

RAPD data analysis and scoring

Bands on RAPD gels were scored as (1) when present or (0) when absent. Evaluation of fragment patterns was carried out by similarity index. The similarity index (SI) values between the RAPD profile of any two individual were calculated using the Nei's genetic similarity index (Nei and Li, 1979), on the basis of the equation:

$$SI = 2Nij / (Ni + Nj),$$

Where Nij is the number of common bands shared between 2 samples i and j, Ni and Nj are the total number of DNA bands for genotypes i and j, respectively.

Using dice coefficients, a similarity matrix involving 13 samples was generated with PAST (paleontological statistics) software (Hammer et al., 2001). The similarity matrix data was subjected to unweighed pair group method for arithmetic average (UPGMA) cluster analysis to generate a dendrogram using average linkage procedure.

RESULTS AND DISCUSSION

Soil is under considerable threat from over-exploitation, pollution from industrial waste, human anthropological activities and its misuse. Soil contaminants can have significant deleterious consequences on the ecosystems including chemistry changes, alteration in the metabolism of the endemic microorganisms, arthropods and plants in a given soil environment, biomagnifications of contaminants through the food chain, reducing crop yields, soil erosion and the formation of derivative chemicals from the decay of primary soil contaminants (Stegmann, 2001; Negraa et al., 2005). As we know that the soil microorganism plays very important role in maintaining the soil health and early warning indicators of environmental changes, it became very essential to study its genetic diversity.

Recently, different molecular techniques were successfully employed based on genetic diversity such as RNA-based stable isotope probing (Hatamoto et al., 2008),16S rRNA gene sequencing (Betancourt et al., 2008; Norton et al., 2008; Sallam and Steinbu¨chel, 2008), RAPD, microsatellites, minisatellites, restriction fragment length polymorphisms and DNA sequence data

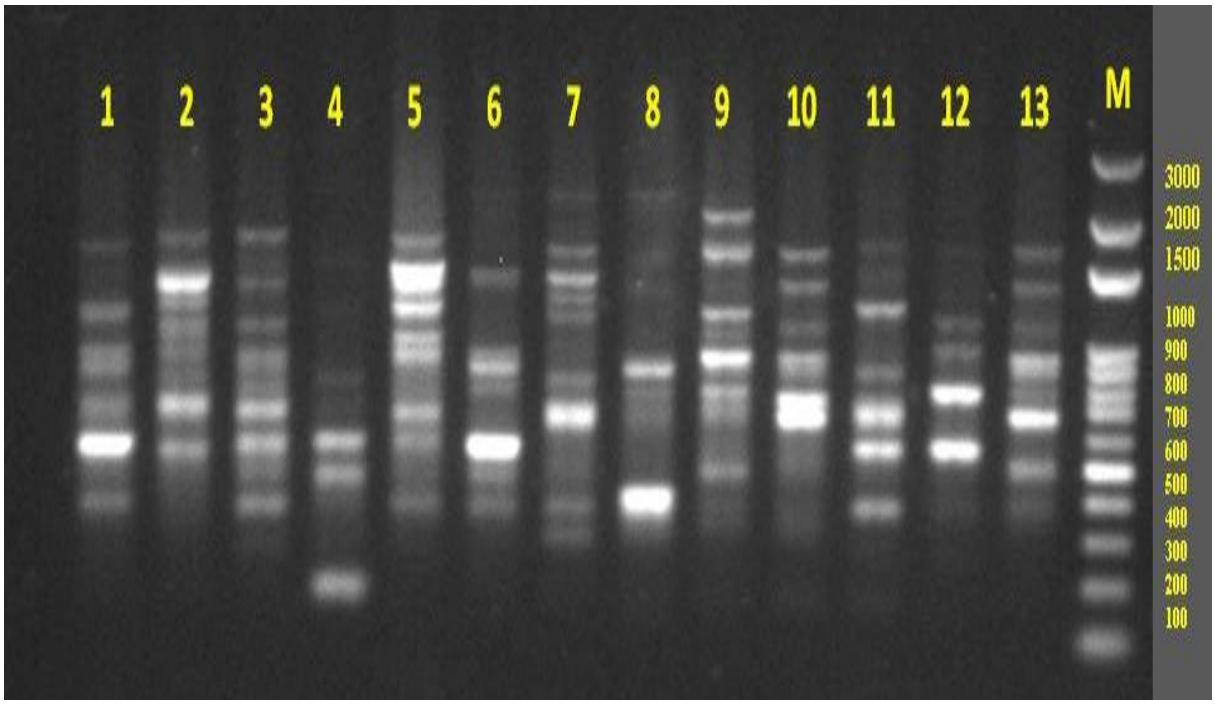

Figure 3. Electrophoretic pattern of PCR amplified polymorphic soil bacterial DNA fragments generated using RBa-3 RAPD primer (Bangalore Genei).

(Allan and Max, 2010). They were manipulated either for defining biodiversity of certain environment as general or more specifically for searching of indicator or biodegrading microorganisms.

In the present study for analysis of microbial communities in the environment sample particularly from soil, an efficient, culture based enzymatic lysis (lysozyme) DNA extraction method was used. There are different methods used for the DNA extraction from soil such as chemical lysis, physical lysis and enzymatic lysis methods which are also described by many scientists. Analysis of DNA extraction by enzymatic lysis of the cells proved to be very efficient in providing sufficient amount of DNA for amplification. The purity obtained at A260/280 nm ranges from 1.61 to 2.08 and the concentration obtained was from 500 to 1200 µg/µl, which is quite enough for the amplification The quality of DNA was also observed using the agarose gel (Figure 2), which indicates that the banding pattern not very clear but even though it has produced good amplification.

PCR amplifications were tested with 4 random decamer oligonucleotide primers purchased from Bangalore Genei (RBa-3 to RBa-6) with thirteen different accessions obtained from nearby vicinity of different industries (Table 2 and Figure 1). All the four primers showed (RBa-3, RBa-4, RBa-5 and RBa-6) satisfactory, scorable and reproducible amplifications (Figures 3, 4, 5 and 6) and generated total of 56 bands, 55 of them being polymorphic (99%) and 1 bands (loci) being monomorphic with mean percentage of 1%, in all the accessions studied (Table 2). Primers RBa-5 produced the highest number of bands (18 bands) followed by RBa-3, RBa-4 (16, 12 bands), while, primer RBa-6 produced the lowest number of bands (10 bands). Some bands were specific for some of the accessions, which may be used in the accessions discrimination.

Among the accessions studied, the similarity coefficient based on 4 primers ranged from 0.163 to 0.462. INS PS-1 and INS PS-9, showed the lowest value of similarity index, while the highest value of similarity index occurred between the INS PS-2 and INS PS-3 (Table 3) since these industries are very close to each other.

Cluster analysis was performed based on the Jaccard's similarity coefficient matrices, calculated from RAPD data. Different clustering methods including unweighed pair group method for arithmetic average (UPGMA) and Neigbour Joining almost produced similar results with good bootstrap and clade credibility values (Figures 7 and 8). In all accessions, three different clusters were obtained. These three clusters were further divided into subclusters. Samples from the agricultural field show high genetic affinity and are placed in a single cluster or clade due to less distance. The samples obtained from the industrially polluted soil showed genetic similarity and form a single cluster. All the three clusters are placed far from each other and join together with greater distance indicating their genetic difference with the others. The results of this study indicated that RAPD provide high

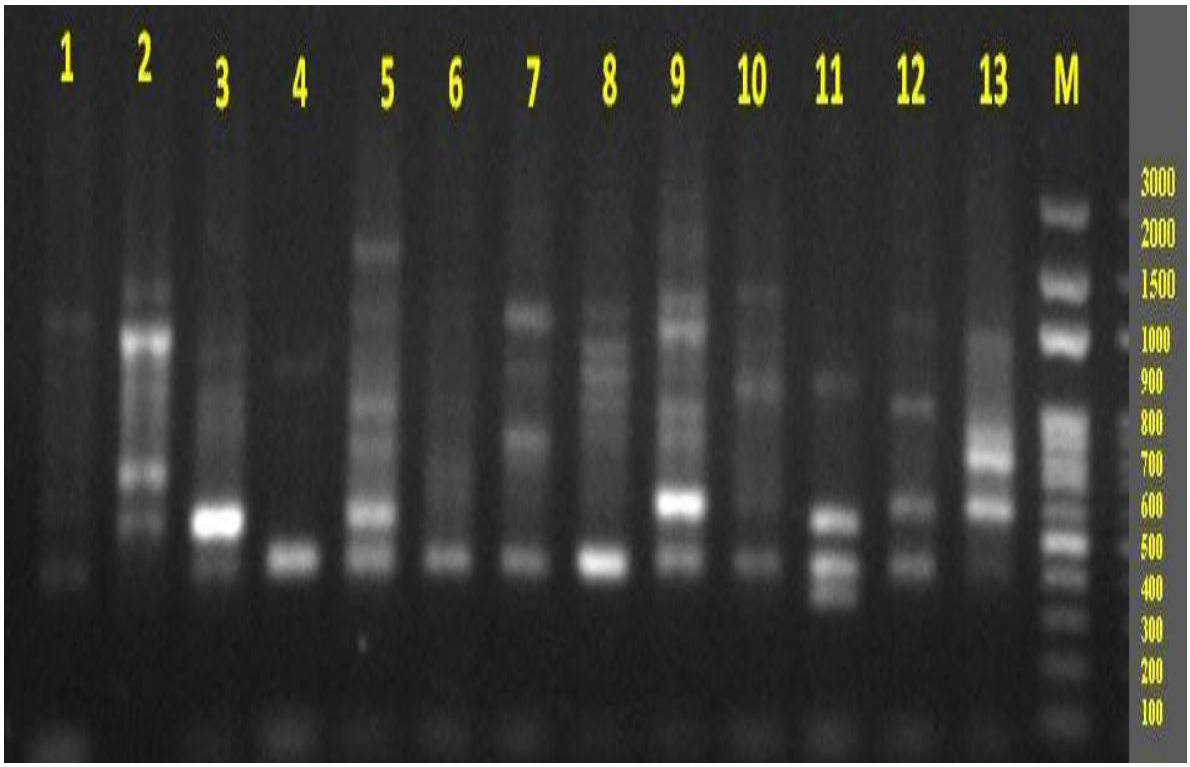

Figure 4. Electrophoretic pattern of PCR amplified polymorphic soil bacterial DNA fragments generated using RBa-4 RAPD primer (Bangalore Genei).

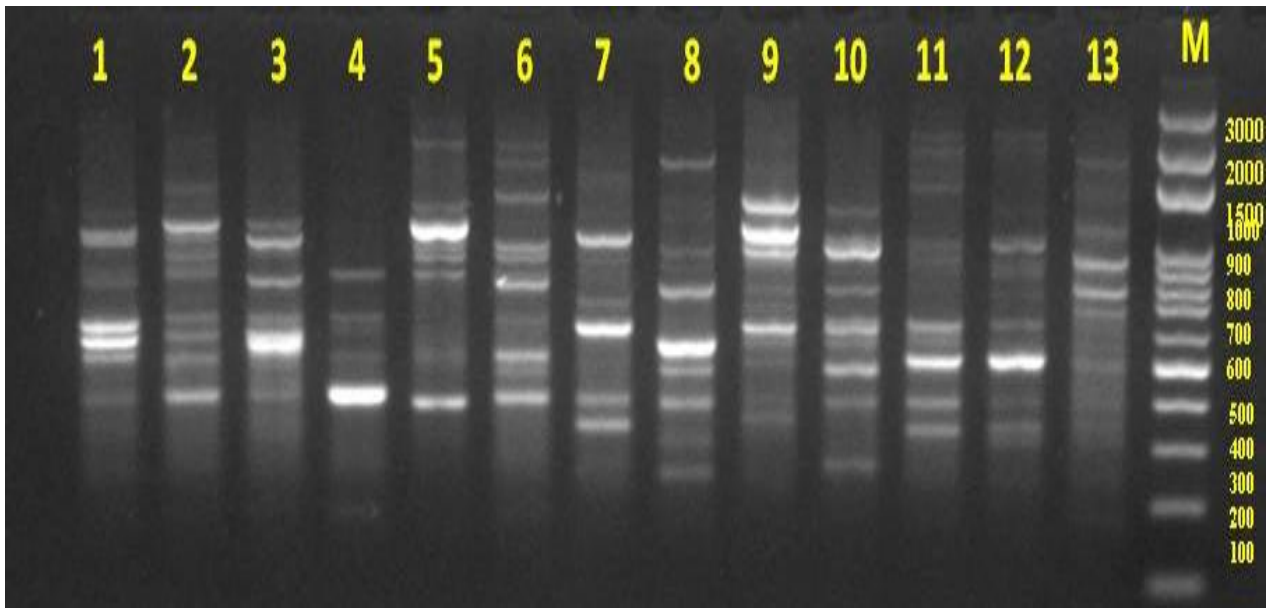

Figure 5. Electrophoretic pattern of PCR amplified polymorphic soil bacterial DNA fragments generated using RBa-5 RAPD primer (Bangalore Genei).

degree of discrimination between the bacterial communities. This and various other studies on RAPD analysis showed that it is a simple, fast and can effectively distinguish different microbial communities. Once the primer

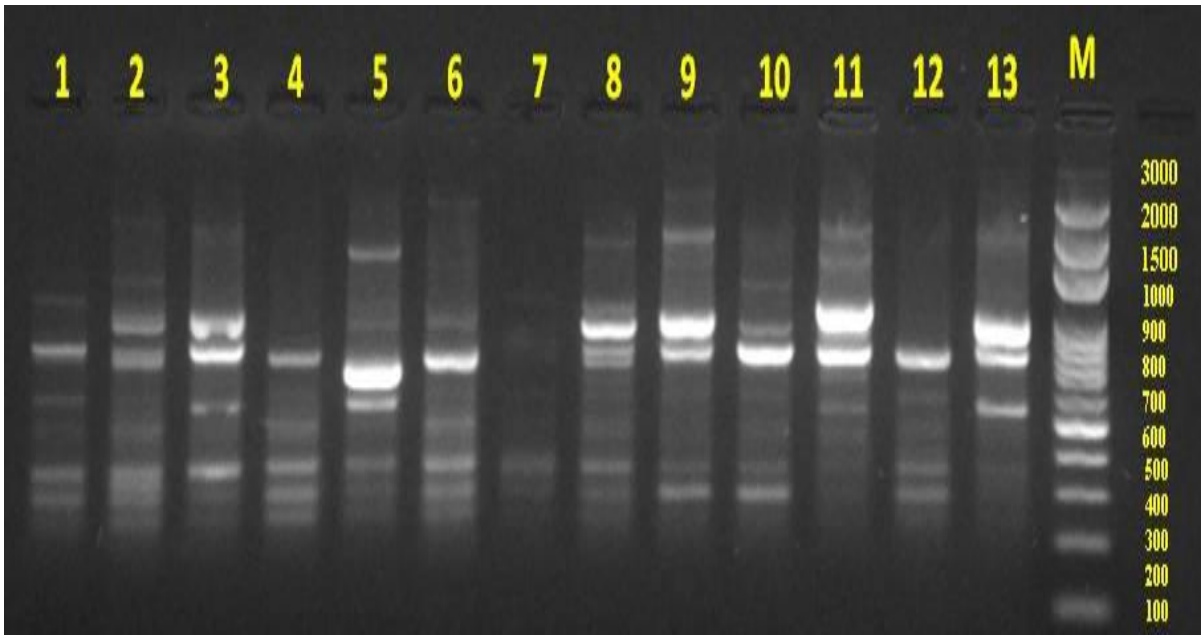

Figure 6. Electrophoretic pattern of PCR amplified polymorphic soil bacterial DNA fragments generated using RBa-6 RAPD primer (Bangalore Genei). Lane 1: Godrej Hershey Ltd., Lane 2: HEG Ltd., Lane 3: Bansal Iron industries Ltd., Lane 4:Crompton and Greaves Ltd., Lane 5: Lupin Ltd., Lane 6: Aristo Ltd., Lane 7 Central Mandideep Industrial area, Lane 8: Cultivated field 15 Km (north), Lane 9: Cultivated field 30 Km (north), Lane 10: Cultivated field 45 Km (north), Lane 11: Cultivated field 60 Km (north), Lane 12: Ratibarh cultivated field 50 km (south), Lane 13: Satawar plant roots soil near industrial area, Lane M: molecular size of a DNA marker.

revealing the polymorphism are identified and PCR condition optimised, the slight differences in primer sequence can cause significantly different RAPD patterns that enable an easy visual discrimination among strains. The method has other various advantages like it is less labour intensive, eliminates the need for pure DNA and only a small amount of template DNA is required for amplification which gives better discrimination of popu-lation (Bostock et al., 1993; Wang et al., 1993). The use of these random primers overcame the limitation of prior sequence knowledge for PCR analysis and is applicable to all organisms, facilitated the development of genetic markers for a variety of purposes. The major drawback of this method is that the profiling is dependent on reaction conditions which can vary between laboratories; even a difference of a degree in temperature is sufficient to produce different patterns. Additionally, as several discrete loci are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (Bardakci, 2001). Arbitrarily primed polymerase chain reaction (AP-PCR) and DNA amplification fingerprinting (DAF) are independently developed methodologies, which are variants of RAPD. For AP-PCR (Welsh and McClelland, 1990), a single primer, 10–15 nucleotides long, is used and involves amplification for initial two PCR cycles at low stringency. Thereafter, the remaining cycles are carried out at higher stringency by increasing the annealing temperatures. According to the RAPD analysis, the high genetic diversity between the samples was observed, which is an expected pattern due to the high pollution status. These results confirmed the hypothesis that, most of the endemic micro flora (considered invasive) in the investigated soil are resistant and can cope with such type and levels of soil contamination by degradation (organic: hydrocarbons) or accumulation (inorganic: heavy metals) but certainly they adversely affect the soil fertility, especially with the absence of the native beneficial ones (Bater, 1996). Due to the high toxic effect of pollutants, the distribution and diversity of the microbial communities was disturbed as also studied by different authors (Frey et al., 2006; Joynt et al., 2006; Sa´nchez-Moreno et al., 2006; Tobor-Kaplon et al., 2006; Sun et al., 2007; Yin et al., 2008). This definitely resulted in a shift in the species composition of the native population towards more resistant species.

Genetic diversity can identify individual organisms from some unique part of their DNA or RNA providing definitive information on soil biodiversity (Ward et al., 1990; Zhao et al., 2006; Betancourt et al., 2008; Hatamoto et al., 2008; Mocali et al., 2008; Norton et al., 2008; Sallam and Steinbu¨chel, 2008; Wang et al., 2008) and has also been recognized for its economic potential through bioprospecting. Diverse plant species also supported the effect of plants on the microbial population. The clustering of samples from contaminated and uncontaminated soil, shown to be highly diverse, joining the group at a higher level of diversity, supports the fact that the practice of industrial effluents treatment adopted by the industry is

Table 3. Jaccard's similarity coefficient among different Microbial population through RAPD analysis.

Sample ID	IND PS-1	IND PS-2	IND PS-3	IND PS-4	IND PS-5	IND PS-6	IND PS-7	IND PS-8	IND PS-9	IND PS-10	IND PS-11	IND PS-12	IND PS-13
IND PS-1	1												
IND PS-2	0.384	1											
IND PS-3	0.387	0.462	1										
IND PS-4	0.313	0.2	0.25	1									
IND PS-5	0.25	0.298	0.381	0.225	1								
IND PS-6	0.3	0.349	0.375	0.314	0.311	1							
IND PS-7	0.2	0.2	0.244	0.273	0.375	0.209	1						
IND PS-8	0.302	0.292	0.311	0.191	0.370	0.395	0.217	1					
IND PS-9	0.163	0.340	0.333	0.186	0.391	0.298	0.267	0.3	1				
IND PS-10	0.205	0.283	0.302	0.306	0.25	0.296	0.233	0.356	0.319	1			
IND PS-11	0.310	0.356	0.415	0.290	0.409	0.405	0.342	0.313	0.306	0.25	1		
IND PS-12	0.256	0.310	0.3	0.194	0.273	0.359	0.256	0.296	0.261	0.286	0.302	1	
IND PS-13	0.186	0.239	0.286	0.184	0.381	0.25	0.275	0.283	0.429	0.273	0.318	0.3	1

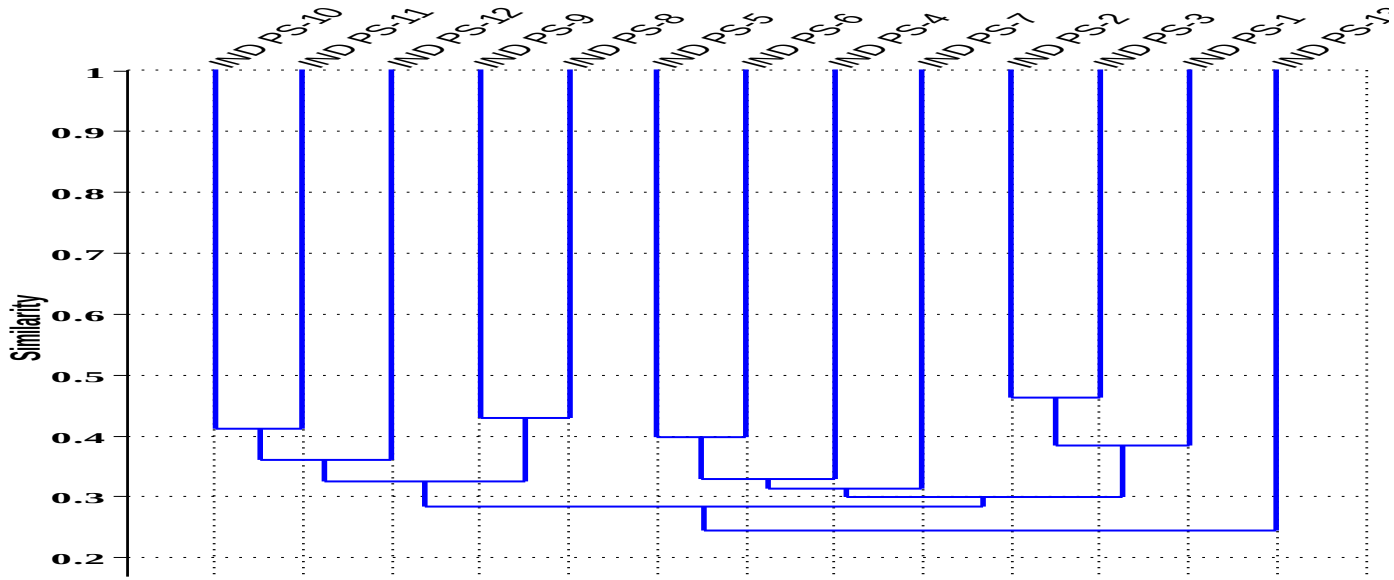

Figure 7. Paired Group Jaccard coefficient matrix of microbial population from industrially contaminated and uncontaminated soil samples based on RAPD marker.

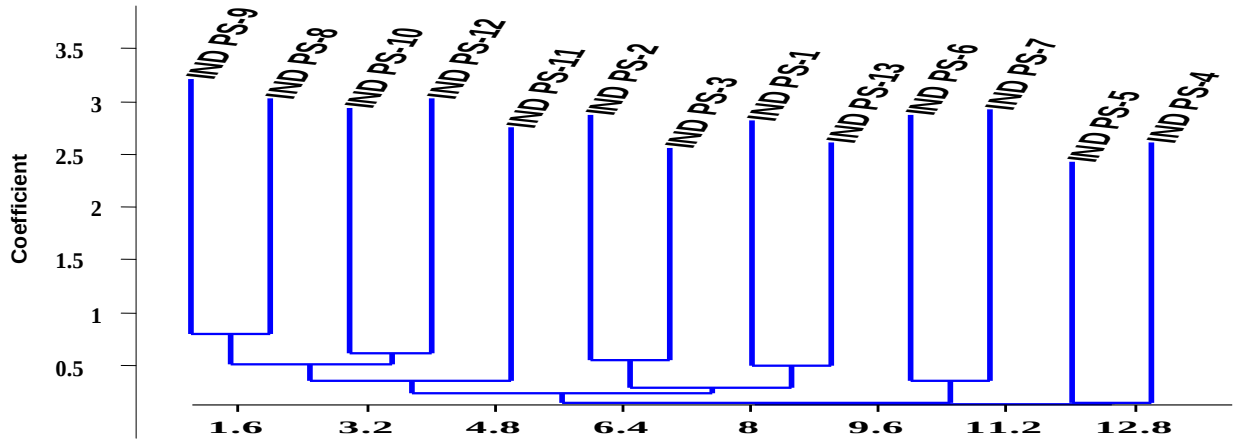

Figure 8. Euclidean matrix (Neighbor Joining) using similarity index (SI) of *O. sanctum* accessions based on RAPD marker.

to be modified. The difference was observed between cultivated and uncultivated area. This indicates that effect of the effluents draining practices, change the microbial diversity pattern in the soil.

Conclusion

RAPD is a PCR-based technique, using arbitrary primers to detect changes in the DNA sequence at sites in the genome. This approach is used for molecular epidemiological typing, biodiversity study and conservation concerns, as it is relatively fast and easy. The polymorphism within the set of DNA fragments generated has been used in discriminating micro-organisms both at the interspecies and intraspecies level. In the present study, the use of RAPD technique for assessing the soil microbial diversity showed good discrimination, with reproducible, scorable banding pattern. Hence this can be concluded that the RAPD-PCR is simple, easy and rapid tool for assessing the biodiversity of complexed microorganisms and can be used for the protection of our ecosystems and in turn human health.

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