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RESEARCH ARTICLE

ASSESSMENT OF MICROBIAL DIVERSITY ASSOCIATED WITH CHRONOSEQUENCE COAL MINE OVERBURDEN SPOIL USING RANDOM AMPLIFIED POLYMORPHIC DNA MARKERS

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ABSTRACT

Genetic diversity among microbial populations in six different age series coal mine overburden spoil and nearby forest soil was evaluated through culture-independent approach using RAPD markers– a PCR-based DNA fingerprinting technique. Twenty primers were used for amplification of metagenomic DNA, and banding patterns of 21 genotypes were compared to assess microbial community structure among seven different soil profiles. The Nei's genetic diversity and Shannon's information index was estimated to be 0.3558 and 0.5206 respectively. Besides, the population diversity and estimated gene flow were observed to be 0.1181 and 0.2482 respectively. Dendrogram analysis based on neighbor joining method and bootstrapping procedure revealed that 21 genotypes were well segregated into three independent clusters. Analysis of molecular variance (AMOVA) indicated 55.2% genetic variability among population was attributed to different soil profiles, where as 44.8% variability was accounted within population attributed to different habitats. The study correlated well with temporal and spatial variations among different age series coal mine overburden spoil and nearby forest soil confirming the PCR-based DNA fingerprinting technique using RAPD markers are useful means of discriminating microbial communities and estimating relatedness. Further, the analysis revealed gradual enrichment in the existing genetic composition due to reclamation over time supporting greater microbial diversity.

INTRODUCTION

Open cast coal mining activities have resulted drastic alternations in geochemical cycles and lead to land degradation with adverse changes in soil textural and structural attributes (Johnson and Skousen, 1995, Maharana and Patel, 2013). Pit scarred landscape with huge dumps of mine spoil usually presents common scenario in opencast coal mining area (Jha and Singh, 1991; Mummey *et al.*, 2002). Being deficient in plant nutrients due to the lack of biologically rich topsoil, mine spoil represents a disequilibriated geomorphic system (Agrawal *et al.*, 1993), poses problem for pedogenesis (Hearing *et al.*, 1993), revegetation (Jha and Singh, 1991) and restoration (Nath, 2004; Juwarkar *et al.*, 2004). Slow recovery of mine spoil has been reported due to the constraints in microbial growth (Whiteman *et al.*, 1998; Marcel *et al.*, 2008) and natural vegetational succession (Jha and Singh, 1991; Nath, 2004). Extremities of conditions such as nutrient deficiency and heavy metal contamination allows specific groups of microorganisms to thrive in their habitat specifically extremophiles including chemolithotrophs (Rawlings *et al.*, 1999; Tyson *et al.*, 2005), acidophiles (Kelly and Wood, 2000) and thermophiles (Kristjansson *et al.*, 2000). Besides, there is existence of some heterotrophs including gram-positive and gram-negative bacterial genera (Ellis *et al.*, 2003). Their existence of chemolithotrophs in fresh coal mine overburden spoil led to subsequent colonization of microbial population in due course of time.

Despite their abundance and importance of soil microbes for ecosystem function, it is not possible to derive a complete sense of genetic diversity and microbial community structure. Besides, it is impossible to assess the function of different groups of microbial populations, specific interactions that may exist between populations, or the independent influences of microorganisms may have on ecosystem processes. The inability to categorize soil microbial community has hampered the efforts of microbial ecologists to investigate fundamental ecological concepts such as microbial community diversity, succession redundancy or stability (Franklin *et al.*, 1999). In order to comprehend the full extent of the relationships within and between microbial community, culture-independent approach can able to evaluate attributes without relying upon the culture-based microbial growth for detection (Griffiths *et al.*, 2000; Nemergut *et al.*, 2007), which led to the development of the metagenomic approach focusing on the use of whole

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community DNA extracts (Griffiths *et al.*, 1996; Holben, 1997; Ogram and Feng, 1997; Fakruddin and Mannan, 2013).

Genetic diversity refers to the range of heritable differences of a trait or set of traits among individuals within a species. Genetic diversity is a statistical concept referring to the variations within the individual gene loci or among alleles of a gene, or gene combinations, between individual or between populations. Genetic diversity provides information about both the species diversity and ecosystem diversity. The ability of species to adapt the changing environments is directly related to the amount of genetic diversity available to natural selection (Zul *et al.*, 2007; Cardoso *et al.*, 2013). Maintenance of genetic diversity is key to the long-term survival of most species is the central paradigm in the field of conservation genetics (Smale and McBride, 1996).

The microbial diversity responds to environmental heterogeneity *via* alterations in the relative strengths of four opposing genetic forces such as mutation, migration, selection and genetic drift or gene flow, which cumulatively determine the actual level of genetic diversity at any one time. Changes in species diversity led to change in the environment, and adaptation of microbial species. The changes in genetic diversity such as loss of species lead to loss of biological diversity (Bellard *et al.*, 2012). Use of genetic diversity as a designator for environmental assessment has been suggested (Yin *et al.*, 2000; Ovreas, 2000; Balalola, 2003; Tyson *et al.*, 2004; Akbar *et al.*, 2005).

Genetic diversity can be accessed through (a) gene diversity (proportion of polymorphic loci across genom) (b) heterozygosity (fraction of individuals in a population that is heterozygous for a particular locus) and (c) alleles per locus (used to demonstrate variability). Assessment of microbial diversity can provide an insight into the ecological well being of soil habitat (Edgerton *et al.*, 1995; Moritz, 1995; White *et al.*, 1998; Findlay *et al.*, 2003; Wei *et al.*, 2006). Several workers substantiates the use of microbial diversity associated with disturbance/degradation and restoration of natural ecosystems (Volossiouk *et al.*, 1995; White *et al.*, 1998; Bickham *et al.*, 2000; Bond *et al.*, 2000, Ovreas, 2000; Yin *et al.*, 2000; Fakruddin and Mannan, 2013; Chandra, 2014).

In order to understand the microbial diversity and the factors influencing microbial metabolic functioning during reclamation of mine spoils, molecular tools are used for assessment of microbial diversity and community structure (Harris, 2003; Izquierdo *et al.*, 2005). Several studies on microbial diversity and community structure that influence reclamation have aided knowledge on ongoing process of soil microbes (Cao, 2007; Liu and Lu, 2009; Fan *et al.*, 2011; Chen *et al.*, 2012). Genetic diversity measures that support its use as ecological indicators, because (i) it is a population rather than individual measure, (ii) measure of cumulative impact of multiple stresses on microbial population, (iii) identify problems within species, before species assemblage indicators become significant, (iv) highly complementary to species assemblage and landscape indicator, (v) useful indicator of microbial population trends through temporal monitoring, (vi) quantitative measure of microbial populations and community.

There are several molecular markers developed to analyze genetic diversity, which are not confounded by environmental, pleiotropic and epistatic effects, but offer numerous advantages over conventional, phenotype-based alternatives as because they are stable and detectable regardless of their growth, differentiation, development of microbes. The ideal molecular markers should satisfy the following attributes: (i) should be polymorphic and evenly distributed throughout the genome; (ii) provide adequate resolution of genetic differences; (iii) generate multiple, independent and reliable markers to discriminate the genotypes; (iv) must be simple, quick, and inexpensive; (v) need smaller quantity of DNA sample for analysis; (vi) able to link distinct phenotypes; and (vii) require no prior information about the genome of the microorganism.

Random amplified polymorphic DNA (RAPD), an effective DNA fingerprinting technique based on polymerase chain reaction (PCR) using a single short primer with an arbitrary nucleotide sequence (Williams *et al.*, 1990) is one of the commonly used molecular techniques to develop DNA markers of any origin or complexity (Novo *et al.*, 1996). It has been widely used in microbial ecology studies in order to address the changes in microbial diversity (Novo *et al.*, 1996), and insights into the microbial response to stress (Xia *et al.*, 1995; Schimel *et al.*, 2007; Sharma *et al.*, 2013). RAPD used for genetic diversity study is an amplification based nucleic acid fingerprinting technique (concurrent detection of multiple loci without assignment of a genotype) with no prior knowledge of genome sequence that use an *in vitro* enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules (Micheli *et al.*, 1994). Due to the inexpensive and rapid analysis of polymorphisms, the RAPD markers provide a good coverage of entire genome effectively on the individuals (Nagl *et al.*, 2011).

The unprecedented rate of species extinction due to the anthropogenic disturbances have prompted extensive research on the impact of soil microbes on ecosystem functioning in coal mine overburden spoil, which may lead to the discovery of new species that bring changes in microbial/ecological properties supporting spoil reclamation over time. Besides, microbial diversity assessment can be used to determine the status of microbial ecosystem, and in that sense the quality of mine spoil and the progress of restoration. In order to link microbial diversity with soil functioning, it is pre-requisite to understand the relationship between genetic diversity and community structure, and between community structure and function. Comprehensive understanding of the relations between microbial diversity and soil function requires not only the use of molecular markers for taxonomically and functionally characterizing metagenomic DNA extracted from different age series mine spoil, but also high resolution techniques to detect inactive and active microbial cells in the mine spoil matrix. Besides, molecular approaches can be applied for better understanding of microbial diversity and ecological functions associated with the species abundance and structure. The tacit assumptions include (i) by characterizing diversity, it can be easier to manipulate the workings of ecosystems, and (ii) the ability of an ecosystem to withstand disturbances may depend in part on diversity of the system. In view of increasing mining

activities and decreasing soil fertility, it is of utmost concern to assess the genetic variation within and between the microbial populations in different age series coal mine overburden spoil in chronosequence ($OB_0 \rightarrow OB_{10}$) using RAPD markers, which not only pave the way of greater understanding the direction of improving soil fertility, but also is pre-requisite for assessing mine spoil reclamation over time.

MATERIAL AND METHODS

Study site

The present study was carried out in the Basundhara (west) open cast colliery in the Ib valley of Mahanadi Coalfields Limited (MCL), Odisha, India (Geographical location: 22º03'58" - 20º04'11" north latitude and 83º42'46" - 83º44'45" east longitude). The coal mine overburden spoil have been grouped into six different age series (fresh: OB_0, 2 yr: OB_2, 4 yr: OB_4, 6 yr: OB_6, 8 yr: OB_8 and 10 yr: OB_{10}) since inception (Figure 1). Six different age series coal mine overburden spoil in chronosequence have been selected within 10 km peripheral distance around the coal mining area. Besides, the nearby native forest soil (NF) was selected adjacent to the core coal mining area for comparison. Tropical dry deciduous forest was considered to be the natural vegetation of the site, which experiences a semi-arid climate (1300 mm rainfall y^{-1}, annual average temperature 26°C, and relative humidity 15%) with three distinct seasons i.e. summer, rainy and winter.

Soil sampling

Sampling was done in accordance with the standard microbiological procedure (Parkinson *et al.,* 1971). Sampling was done three times representing three seasons *i.e.* summer (April), rainy (July) and winter (January). Each coal mine overburden was divided into 5 blocks, and from each block five spoil samples were collected randomly from (0-15) cm soil depth by digging pits (15 x 15 x 15 cm^3) referred to as 'sub-samples'. The sub-samples collected from each block of an overburden were thoroughly mixed to form one 'composite sample'. Thus, three mine spoil samples were collected from each site, and coded according to the age of the coal mine overburden (OB_0 _S1, OB_0 _S2, and OB_0 _S3). Similar sampling strategies were followed for different coal mine overburden (OB_0, OB_2, OB_4, OB_6, OB_8 and OB_{10}) as well as nearby native forest soil (NF). The composite samples were homogenized, sieved (0.2 mm) and stored at 4°C until analyzed.

Isolation of metagenomic DNA

The metagenomic DNA from different age series coal mine overburden spoil (OB_0, OB_2, OB_4, OB_6, OB_8 and OB_{10}) as well as the nearby native forest soil (NF) was extracted by SDS method (Volossiouk *et al.,* 1995) with minor modifications. Further, the extracted DNA was purified with Proteinase K (@100 µg ml^{-1} of DNA solution), RNase A (@ 60 µg ml^{-1} of DNA solution) followed by washing with cold 70% ethanol, air dried and subsequently resuspended in TE buffer [10 mM Tris (pH 8.0) and 0.1 mM EDTA]. The quantity of DNA was estimated by comparing with known concentration of lambda uncut DNA in 1.5% agarose gel electrophoresis. Thereafter, the purified DNA was equilibrated to 25 ng ml^{-1} for RAPD analysis.

RAPD marker analysis

Twenty random decamer primers (Operon Tech, USA) OPA, OPB, OPC, OPD, OPH, OPJ, OPK, OPN, OPS, OPT and OPZ series were used for DNA amplification (Williams *et al.,* 1990). Each amplification reaction mixture of 25µl contained 30 ng template DNA, 2.5 µl of 10X assay buffer 100 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM Tris HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTPs, 0.4 µM primer, and 0.5 unit of Taq polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India). The amplification was performed in a thermal cycler (Bio-RAD, USA) programmed for initial denaturation of 94^{0}C for 5 min; 45 cycles of 2 min denaturation at 92^{0}C; 1 min annealing at 37^{0}C and 2 min extension at 72^{0}C and the final elongation at 72^{0}C for 7 min. The PCR products were stored at 4^{0}C till further analysis. The amplification products were electrophoresed in 1.5% agarose gel containing ethidium bromide @ 0.5 µg ml^{-1} in TAE buffer [1 M Tris HCl, 0.5 M EDTA and glacial acetic acid (pH 7.2)] for 2 hr at 50 Volts. A total of 2.5 µl loading buffer (Bromophenol blue 0.25%, glycerol 50%, xylene cyanol 0.25% and sterile water) was added to each reaction mixture before electrophoresis. After electrophoresis, the gels were observed under UV transilluminator, documented in Gel-Doc XR 2000 (Bio-Rad) and photographed. The size of the amplicons was determined using 250bp DNA ladder (Bangalore Genei, India) and Quantity One software. To test reproducibility, the reactions were repeated twice.

Data collection and analysis

The banding pattern of each primer was scored as present (1) or absent (0), each of which was treated as an independent character. Only the reproducible bands were observed for scoring and the light bands were omitted as they were not reproducible. The Jaccard's dissimilarity coefficient (J) was calculated, and subjected to cluster analysis by bootstrapping and neighbor-joining method using the program DARWIN (version 5.0.158) (Felsenstein, 1985). One thousand permutation data sets were generated by resampling with replacement of characters within the combined 1/0 data matrix. POPGENE software was used to calculate Nei's unbiased genetic distance among different genotypes with all markers. Data for observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage of polymorphic loci (PPL) across the 21 genotypes were analyzed (Zhao *et al.,* 2006). The population diversity (Hs) and heterozygosity (Ht) were calculated within 21 populations from seven different soil profiles (OB_0, OB_2, OB_4, OB_6, OB_8, OB_{10} and NF) by using POPGENE software (Nei, 1978).

Statistically unbiased clustering of the genotypes from six mine spoils ($OB_0 \rightarrow OB_{10}$) and nearby forest soil (NF) was performed using STRUCTURE (version 2.3.1) (Evanno *et al.,* 2005). RAPD data were subjected to hierarchical analysis of molecular variance (AMOVA) to describe genetic variability among populations *via* Gen Alex program (Excoffier *et al.,*

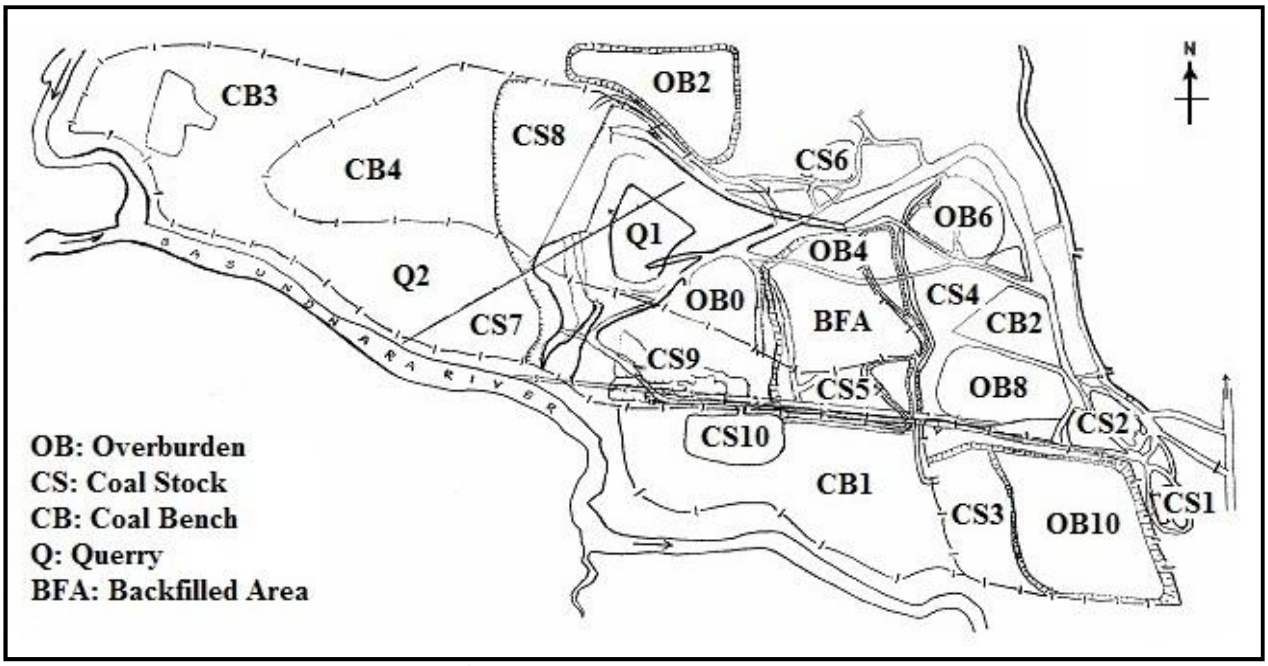

Figure 1 Site map of Basundhara (west) open cast colliery, Sundargarh, Odisha.

1992), where the variation component was partitioned among microbial population (seven soil profiles) and within microbial population (three samples from each soil profile). The input files for AMOVA were prepared using AMOVA-PREP version 1.01 (Miller, 1988). Resolving power (Rp) of twenty RAPD primers was calculated (Prevost and Wilkinson, 1999) as: $Rp = \Sigma\ IB$ where, *IB* (band informativeness) by taking the value of: 1–[2* (0.5–P)], where P being the proportion of the 21 genotypes containing the band. Further, the frequency of polymorphism obtained in the genotypes was calculated on the basis of presence (1) and absence (0) of the bands amplified. The polymorphic information content (PIC) was calculated according to the procedure described by Anderson *et al.* (1993).

RESULTS AND DISCUSSION

The uneven distribution of soil microorganisms in terrestrial ecosystems suggested intense variability in microbial community structure at small spatial scales depending upon the heterogeneities in different age series mine overburden spoil matrix and localized nutrient availability. Exceptionally heterogeneous existence of microbial community structure as well as the complexity of coal mine spoil matrix requires robust technique to assess genetic diversity and changes in microbial community structure through time and space. In the present study, microbial diversity in different age series coal mine overburden spoil in chronosequence was assessed through the metagenomic approach using RAPD markers. The implementation of RAPD profiling for small sample size as a means to study microbial diversity provides an opportunity to examine the spatial scale of variance in community structure in different age series coal mine overburden spoil. Appropriate number of replicates must be taken for efficient RAPD analysis necessary for statistical significance, which most of the other techniques exhibit practical limitations. RAPD fingerprinting technique allows the microbial ecologists to elucidate microbial community structure towards precise understanding of the overall interactions among/between microbial populations with the ecosystems (Franklin *et al.*, 1999). Further, genetic diversity assessment is an important index of monitoring changes that occur within different age series coal mine overburden spoil and further brings about mine spoil genesis supplementing reclamation in due course of time (Daniel, 2004).

DNA content

The mean DNA content in six different age series coal mine overburden spoil in chronosequence revealed an increasing trend, which varied from 0.058 mg/g spoil to 0.185 mg/g spoil with minimum in OB_0 and maximum in OB_{10}. Besides, the mean DNA content in nearby forest soil (1.168 mg/g soil) was found to be relatively higher as compared to different age series mine overburden spoil across the sites. The analysis revealed wide genetic variation among microbial populations with consistent increase in DNA content from OB_0 to OB_{10} suggested gradual increase in microbial colonization, which might be due to the coupling of genetic composition with the increase in age of mine overburden spoil, influence of environmental variables including soil textural composition, gradual accumulation of soil nutrients such as organic C, total N, and available P, establishment of vegetation cover *etc* over time (Maharana and Patel, 2013). Corresponding to the consistent increase in DNA content with respect to the age of coal mine spoil, higher extent of genetic variation between populations was also evident from differential banding patterns and appearance of high number of polymorphic RAPD markers.

RAPD marker analysis

Molecular fingerprinting of 21 genotypes using 20 primers was used to regenerate RAPD markers that responded amplification with good coverage of genome, and provide substantial polymorphism with multiple band profiles varying from 9 to

Table 1 RAPD primers, their nucleotide sequence, (G+C)%, number of bands amplified, nature of bands, resolving power (Rp) and polymorphic information content (PIC) of 20 different primers used for RAPD fingerprinting of 21 genotypes collected from six different age series coal mine overburden spoil ($OB_0 \rightarrow OB_{10}$) in chronosequence and nearby NF soil across the sites.

Primers	Primer sequence (5' ï 3')	(G+C)%	Max. No. of bands	Polymorphic bands	Monomorphic bands	Specific bands	Rp	PIC
OPA03	5'AGTCAGCCAC3'	60	18	12	0	6	16.00	0.916
OPA07	5'GAAACGGGTG3'	60	16	13	1	2	14.66	0.904
OPA09	5'GGGTAACGCC3'	70	12	8	3	1	16.09	0.895
OPB05	5'TGCGCCCTTC3'	70	15	13	1	1	15.90	0.923
OPC02	5'GTGAGGCGTC3'	70	14	7	2	5	15.90	0.901
OPC05	5'GATGACCGCC3'	70	18	14	0	4	20.19	0.921
OPD03	5'GTCGCCGTCA3'	70	09	5	2	2	12.19	0.846
OPD07	5'TTGGCACGGG3'	70	14	10	1	3	17.14	0.911
OPD18	5'GAGAGCCAAC3'	60	12	9	2	1	14.19	0.870
OPH11	5'CTTCCGCAGT3'	60	16	12	1	3	18.19	0.905
OPJ04	5'CCGAACACGG3'	70	16	13	2	1	19.23	0.922
OPJ06	5'TCGTTCCGCA3'	60	13	11	0	2	14.57	0.891
OPK20	5'GTGTCGCGAG3'	70	16	10	2	4	16.38	0.898
OPN10	5'ACAACTGGGG3'	60	11	6	1	4	10.95	0.869
OPN12	5'CACAGACACC3'	60	17	16	1	0	20.38	0.928
OPS01	5'CTACTGCGCT3'	60	12	11	1	0	12.57	0.890
OPS06	5'GATACCTCGG3'	60	13	9	1	3	12.19	0.877
OPT08	5'AACGGCGACA3'	60	09	4	2	3	9.619	0.827
OPZ04	5'AGGCTGTGCT3'	60	15	10	4	1	18.38	0.908
OPZ07	5'CCAGGAGGAC3'	70	12	6	3	3	12.76	0.890
	Total	--	278	199	30	49	--	--

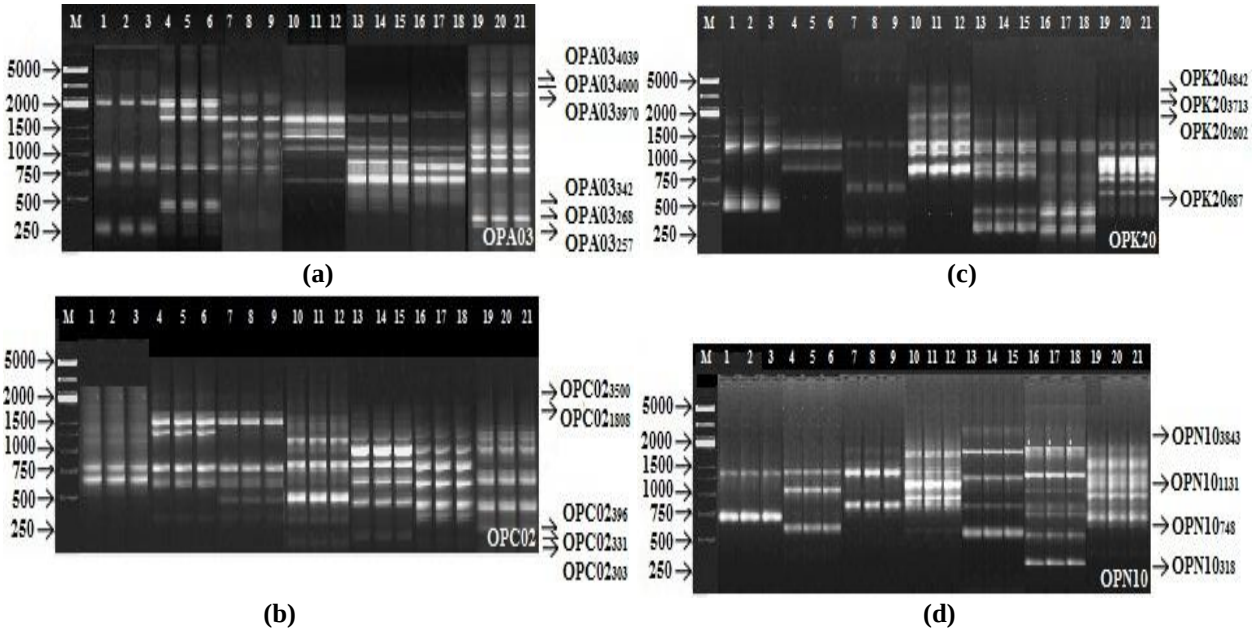

Figure 2 Pattern of PCR amplified DNA fragments of metagenomes sampled from six different age series coal mine overburden spoil and nearby NF soil using primers (a) OPA03, (b) OPC02, (c) OPK20, and (d) OPN10. Lane 1: Marker DNA (250bp ladder); Lanes 1-3: Fresh coal mine spoil (OB_0); Lanes 4-6: 2yr coal mine spoil (OB_2); Lanes 7-9: 4yr coal mine spoil (OB_4); Lanes 10-12: 6yr coal mine spoil (OB_6); Lanes 13-15: 8yr coal mine spoil (OB_8); Lanes 16-18: 10yr coal mine spoil (OB_{10}); and Lanes 19-21: nearby forest soil (NF).

18 (Table 1). Out of the total 278 RAPD bands, 199 (71.58%) were found to be polymorphic, 30 (10.79%) monomorphic, and 49 (17.62%) specific bands respectively. The study revealed that 18 primers (except OPN12 and OPS01) showed specific band profiles exhibiting wide polymorphisms among different soil profiles. Size of amplified bands varied from 258bp to 4842bp (Figure 2). Percentage of polymorphic bands (PPB) amplified by 20 primers varied from 44.44% (OPT08) to 91.66% (OPS01). Resolving power (Rp) is the characteristic of a primer, which reflects overall suitability of a marker system for the purpose of identification, as it is related to the number of accessions distinguished by that primer. Rp value of 20 primers ranged from 9.61 (OPT08) to 20.38 (OPN12). Five primers (OPN12, OPC05, OPJ04, OPZ04 and OPH11) with higher Rp values 20.38, 20.19, 19.23, 18.38 and 18.19 respectively can able to distinguish 21 genotypes associated with microbial populations from seven different soil profiles across the sites (Table 1). The polymorphism information content (PIC) revealed that 'OPN12' (0.928) showed the highest level of polymorphism, whereas the rest of the RAPD markers were within the range of 0.827-0.928 (Table 1).

PIC refers to the value of a marker for detecting polymorphism within a population or set of genotypes by taking into account not only the number of alleles that are expressed, but also the relative frequencies of alleles per locus. The representative gels revealed heterogeneous banding patterns among 21 genotypes across the sites (Figure 2a-d).

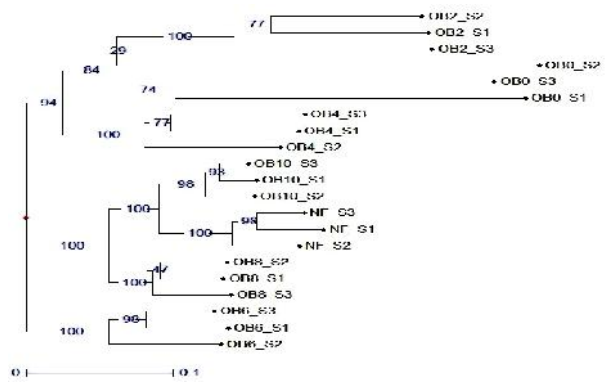

Figure 3 Neighbor joining tree representing clustering (calculated from 278 RAPD bands produced by 20 primers) of 21 genotypes of microbial populations associated with six age series coal mine overburden spoil and nearby NF soil supported by bootstrap values based on RAPD profiling.

with other findings (Novo *et al.*, 1996; Waltenbury *et al.*, 2005; Akbar *et al.*, 2005; Zul *et al.*, 2007).

Genetic diversity assessment

The distribution of different marker size and banding patters in six mine overburden spoil ($OB_0 \rightarrow OB_{10}$) and nearby NF soil were utilized for the estimation of Nei's genetic diversity indices. For the purpose, the RAPD marker data were arranged in binary matrix. The genetic similarity between microbial populations in seven different soil profiles was estimated, which varied from 0.4101 (between OB_0_S1 and NF_S2) to 0.9353 (between OB_{10}_S1 and OB_{10}_S3). Besides, the corresponding figures of genetic distance were calculated, which varied from the maximum of 0.8914 (between OB_0_S1 and NF_S2) to the minimum of 0.0669 (between OB_{10}_S3 and

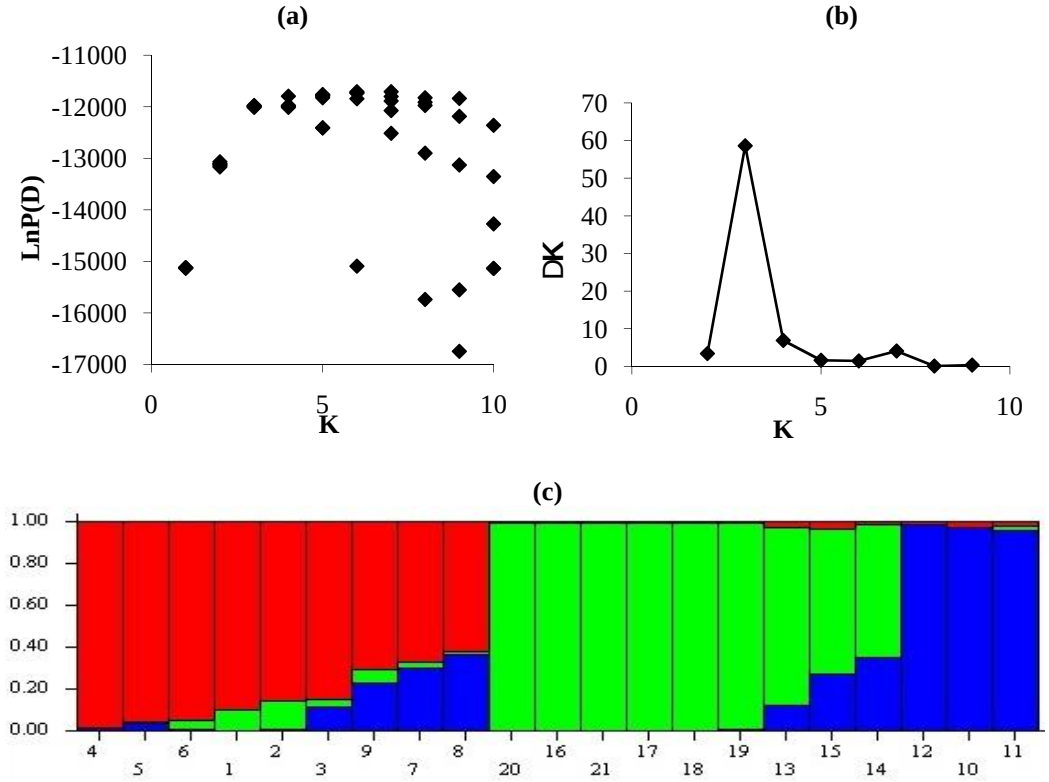

Figure 4 (a) Relationship between number of clusters (K) and the estimated likelihood of data (LnP(D)); (b) Relationship between K and ΔK based on STRUCTURE analysis; (c) STRUCTURE analysis of 21 genotypes based on RAPD data showing grouping of genotypes, when K = 3.

RAPD markers has been successfully used for genetic diversity studies and found suitable for use with 21 genotypes of microbial populations across the sites, because of its ability to generate reproducible polymorphic markers. The microbial diversity analysis suggested that the total number of polymorphic bands exhibited an increasing trend from OB_0 to OB_{10}, which can be considered as the reflection of the increase in genetic variability among six different mine overburden spoil in chronosequence over time (Harry *et al.*, 2001; Zul *et al.*, 2007).

There have been usual agreements that the microbial population in disturbed habitat showed a reduced diversity in comparison to undisturbed habitat (Yin *et al.*, 2000; Zhou *et al.*, 2002; Findlay *et al.*, 2003; Tyson *et al.*, 2004; Zul *et al.*, 2007). Further, the number of amplified fragments produced per primer and their sizes was analytically appropriate confirming

OB_{10}_S1). The study suggested minimal genetic distance between OB_{10}_S3 and OB_{10}_S1.

The similarity coefficient values were subjected to dendrogram analysis based on bootstrapping and neighbor joining (NJ) method, which indicated that the microbial populations from six different mine overburden spoil and nearby NF soil grouped 21 genotypes into three distinct clusters, which are further extensively divided into mini clusters: cluster-I (9 genotypes), cluster-II (9 genotypes), and cluster-III (3 genotypes) (Figure 3). Since all the three clusters showing the bootstrap value above 50%, the tree likeness of the original (unrandomized) tree was statistically well resolved.

An unbiased clustering of genotypes based on STRUCTURE program without prior knowledge about the microbial populations collected from six mine overburden spoil and

nearby NF soil clustered 21 genotypes into three major groups. Under the admixed model, STRUCTURE calculated that the estimate of likelihood of the data (LnP(D)) was greatest when K = 3 (Figure 4a). For K > 3, LnP(D) increased slightly but more or less plateued, *i.e.* ΔK reached its maximum value when K = 3 (Figure 4b). RAPD data suggested that 21 genotypes representing different microbial populations across seven different soil profiles fell into one of the 3 clusters albeit minor interference. The dendrogram analysis revealed the sequence of clusters as follows: $OB_0 \rightarrow OB_2 \rightarrow OB_4 \rightarrow OB_6 \rightarrow OB_8 \rightarrow OB_{10} \rightarrow$ NF. The variation in microbial community structure across the sites may be the possible reason for such shifting in position of different clusters. These results were observed to be almost similar to the splitting in the neighbor joining tree. Overall the cluster analysis based on the neighbor joining method as well as STRUCTURE analysis strongly suggested that 21 genotypes associated with microbial population in seven different soil profiles were well segregated (with high likelihood probability) into three independent clusters (Figure 4c).

Table 2 Summary of analysis of molecular variance (AMOVA) based on RAPD profiling using 21 genotypes from six different age series coal mine overburden spoil ($OB_0 \rightarrow OB_{10}$) in chronosequence and nearby NF soil across the sites.

Source of variance	d.f.	S.S.D.	M.S.	Estimated variance (%)	*P*-value
Among populations	6	694.000	115.667	55.2	$p < 0.05$
Within population	14	344.667	24.619	44.8	$p < 0.05$
Total	20	1038.667	140.286	100	--

(Level of significance is based on 1000 iteration steps; df: degree of freedom; SSD: sum of square deviation; MS: mean square deviation; P-value: probability of null distribution)

Further, the observed number of alleles (Na) was found to be minimal in OB_{10} (1.1295) and maximal in OB_0 (1.5683), where as the effective number of alleles (Ne) was observed to be minimal in OB_{10} (1.1036) and maximal in OB_0 (1.4547) among the microbial populations in six different age series coal mine overburden spoils across the sites. The Nei's genetic diversity (H) computed among 21 genotypes showed a range of 0.0576 (OB_{10}) to 0.2526 (OB_0). Similar trend of heterogeneity was descended through Shannon's information index (I), which varied from 0.0824 (OB_{10}) to 0.3618 (OB_0). Besides, the total genetic heterozygosity (Ht) varied from 0.0576 (OB_{10}) to 0.2526 (OB_0). The number of polymorphic loci varied from 36 (OB_{10}) to 158 (OB_0) having average polymorphic loci estimated to be 26.565%. Incidence of increasing polymorphic loci is considered to be a reflection of higher degree of genetic variability between microbial populations in seven different soil profiles (Bickham *et al.*, 2000; Bond *et al.*, 2000; Yin *et al.*, 2000; Simpson *et al.*, 2002; Balalola, 2003; Tyson *et al.*, 2004; Akbar *et al.*, 2005; Waltenbury *et al.*, 2005; Volossiouk *et al.*, 1995; Zul *et al.*, 2007). Nevertheless, the microbial diversity assessment among 21 genotypes with respect to seven different soil profiles in terms of observed number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (H), Shannon's information index (I), heterozygosity (Ht), number of polymorphic loci (NPL), and percentage of polymorphic loci (PPL) revealed higher genetic variability across the sites.

The variation in genetic relatedness among the microbial populations in six different age series coal mine overburden spoil ($OB_0 \rightarrow OB_{10}$) in chronosequence as well as nearby NF soil, and within the microbial population were analyzed to find out the molecular variance using AMOVA (Table 2). The study revealed that out of total variation in the studied populations, 55.2% could be accounted for differences among the microbial populations in seven different soil profiles (OB_0, OB_2, OB_4, OB_6, OB_8, OB_{10} and NF) with a further 44.8% being accounted for the variation within population (3 samples from each soil profile), which was statistically significant ($p < 0.05$). The analysis of molecular variance (AMOVA) based on RAPD patterns further substantiated higher genetic variation among seven soil profiles, which might be due to the intra-population interaction, extreme spatial heterogeneity, multi-phase environmental interactions, and the shift in complex physico-chemical and microbiological properties of coal mine overburden spoil over time (Daniel, 2005). Incidence in observed and expected allelic variation among six age series coal mine overburden spoil and nearby NF soil reflects variability in microbial community structure.

Keeping the overall genetic variability on genotype basis across 21 metagenomes with respect to six mine overburden spoil ($OB_0 \rightarrow OB_{10}$) and nearby NF soil into consideration, the observed number of alleles (Na) and the effective number of alleles (Ne) was found to be 1.8921, and 1.6304 per locus respectively (Table 3). Besides, the genetic diversity (H) and Shannon's information index (I) was found to be 0.3558 and 0.5206 respectively. Similarly, the total genetic heterozygosity (Ht) among the microbial populations in 21 genotypes across the sites was estimated to be 0.3558 and within populations (Hs) was found to be 0.1181 (Table 3). Further, gene differentiation (Gst) among the 21 microbial populations was found to be 0.6682. The process of gene flow among the microbial populations can be well assessed through the estimation of a projected gene flow (Kelly and Wood, 2000; Findlay *et al.*, 2003; Wulf-Durand, 1997; Pobell *et al.*, 1997; Waltenbury *et al.*, 2005). The estimated gene flow (Nm) using Gst value was found to be 0.2482, which limits the operation of gene transfer among microbial populations in six mine overburden spoil ($OB_0 \rightarrow OB_{10}$) and nearby NF soil across the sites. Besides, the variation in ecological conditions, the variation in physico-chemical properties can also be explained

Table 3 Overall genetic variability between the microbial populations in 21 genotypes with respect to their distribution among six different age series coal mine overburden spoils ($OB_0 \rightarrow OB_{10}$) as well as nearby NF soil across the sites

Sample size	Observed No. of alleles (Na)	Effective No. of alleles (Ne)	Nei's gene diversity (H)	Shannon's information index (I)	Hetero zygosity (Ht)	Population diversity (Hs)	No. of polymorphic loci	% of Polymorphic loci
21	1.8921 (0.3108)	1.6304 (0.3308)	0.3558 (0.1558)	0.5206 (0.2096)	0.3558 (0.0243)	0.1181 (0.0116)	248	89.21

to cause such variation among microbial populations. Further, the microbiological study substantiate the fact that the restoration of disturbed to original undisturbed natural habitat is often associated with the increase in microbial functional redundancy due to the gene flow among the existing microbial groups (Yin *et al.*, 2000).

Microbial colonization in fresh mine spoil (OB_0) was found to be limited due to hostile environment with extremities in soil pH and temperature along with multiple stresses. Subsequent colonization of different heterotrophic microbial populations may be possible due to the gradual establishment of vegetation that acted as stock for the horizontal gene transfer among the microbial populations resulting molecular variation (Novo *et al.*, 1996; Akbar *et al.*, 2005, Patel and Behera, 2011).

The total genetic diversity among six different age series coal mine overburden spoil ($OB_0 \rightarrow OB_{10}$) as well as nearby NF soil was estimated to be 0.3558, and within population was found to be 0.1181. The study indicated that the restoration of disturbed mine spoil to undisturbed natural forest habitat is often associated with the increase in genome-genome and genome-environment interactions of existing microbial communities (Yin *et al.*, 2000), which may be due to stress induced genetic variation and adaptation to existing abiotic stress (Zhou *et al.*, 2002).

There has been increasing efforts for the assessment of genetic diversity of soil microbial populations using different molecular techniques (Kelly and Wood, 2000; Nowrouzian *et al.*, 2001; Findlay *et al.*, 2003; Akbar *et al.*, 2005; Waltenbury *et al.*, 2005). Molecular genetic variability among microbial populations has been studied by several workers (Kelly and Wood, 2000; Waltenbury *et al.*, 2005; Akbar *et al.*, 2005). Efficiency of metagenomic approaches used for microbial ecology studies lies with the fact that this can unravel the relative distribution of different microbial populations in their natural habitat and can explain the functional role for sustainability of soil subsystem (Jastrow *et al.*, 1998; Malik and Scullion, 1998; Yin *et al.*, 2000).

CONCLUSION

The comprehensive study of RAPD markers correlated well with temporal and spatial variations among seven different soil profiles confirming the PCR-based DNA fingerprinting technique was useful means of discriminating microbial communities and estimating community relatedness.

The dendrogram analysis based on neighbor joining method as well as STRUCTURE analysis segregate the microbial populations into three distinct clusters, which indicated higher genetic variability among six different age series coal mine overburden spoil ($OB_0 \rightarrow OB_{10}$) and nearby NF soil across the sites.

The analysis of molecular variance based on RAPD patterns further substantiated higher genetic variability among seven different soil profiles, which appears to be a need to maintain sufficiently large microbial populations that can be used as scalable indices for the assessment of restoration process.

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