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MICROBIAL DIVERSITY ASSESSMENT THROUGH RAPD FINGERPRINTING OF METAGENOMES FROM DIFFERENT DRY TROPICAL ECOSYSTEMS

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ABSTRACT

The understanding of microbial diversity and their interactions with biotic and abiotic factors is considered as an integrative measure of soil quality. In order to comprehend the relationships within and between microbial communities, the metagenomic approach based on the whole community DNA extract was used for genetic profiling, which provide valuable information about the microbial community structure in different soil profiles. Genetic diversity assessment was performed between 21 microbial metagenomes isolated from seven different soil profiles using RAPD markers, which revealed higher genetic variability among microbial populations coupled with the evolution of microbial strains evident from the correlation analysis between polymorphic loci with Nei's genetic diversity. Dendrogram analysis using neighbor joining and STRUCTURE analysis indicated the segregation of 21 metagenomes into six independent clusters. AMOVA indicated the existence of genetic variability among (83%) and within (17%) the microbial populations. The study indicated gradual colonization in microbial populations supporting genetic diversity.

KEY WORDS: **Diversity index, microbial diversity, metagenomes, RAPD markers, soil quality.**

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INTRODUCTION

Soil microbes play important role in ecosystem functioning including nutrient cycling, soil structural dynamics, degradation of pollutants[1] and respond quickly to natural perturbations and environmental stress due to their short generation time. Microbes comprise a large portion of genetic diversity[2]. Besides, their intimate relationship with the ecosystem allows microbial analyses to discriminate different soil profiles, shifts in microbial population and activity, which can be used as indicators of soil quality[3,4]. The diversity studies would be grossly incomplete without the knowledge of microbial diversity as it can be extrapolated to species diversity as well as ecosystem diversity. However, the impact of soil microbes on ecosystem functioning is poorly understood[2]. Anthropogenic activities accelerate loss of soil quality that alters microclimates leading to microbial diversity. Therefore, the assessment of microbial diversity and activities in ecosystem functioning following perturbations is of crucial importance. Microbial diversity assessment associated with anthropogenic disturbances, land degradation and restoration of natural ecosystem have been advocated by several workers[5,6,7]. Genetic diversity designates gene and genotype variation within a species that composed of individuals having different genetic characteristics. Analysis of diversity is important when soil ecosystems respond to the changing environmental conditions, and such changes in microbial community structure can be crucial for the functional integrity of soil[8]. The ability of species to adapt such changing environments is directly related to genetic diversity driven by natural selection. Conservation of biodiversity includes conserving the diversity of habitats, communities, species and genetic diversity within the species for ecosystem functioning[9]. Further, the decrease in genetic diversity increases the rate of extinction due to the decline in fitness of microbial populations[10] and hence can be used as the designator for environmental assessment[11,12,13]. Genetic diversity among the microbial populations responds to the environmental heterogeneity *via* alterations in the relative strengths of four opposing genetic forces such as mutation, migration, selection, and genetic drift. The balance and cumulative history of these forces determines the actual levels of genetic diversity. Besides, the microbial interactions at the community level are more complex with individual species relying on their presence, function, and the nature of interactions among species[14]. Therefore, the quantitative and qualitative changes in microbial community structure, genetic diversity indices may serve as sensitive indicators of both short-term and long-term changes in soil health and functional integrity of terrestrial ecosystems. Molecular markers may correlate with the phenotypic expression of a genomic trait and offers numerous advantages over the conventional phenotype-based alternatives as they are stable and detectable regardless of growth and differentiation of microbes. Additionally, they are not confounded by environmental, pleiotropic and epistatic effects. An ideal molecular marker should satisfy the following attributes: (i) should be polymorphic and evenly distributed throughout ogenome; (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 small amount of DNA sample for analysis; (vi) able to link to distinct phenotypes; and (vii) require no prior information about the microbial genomic sequence. The use of metagenomic DNA for microbial phylogeny studies has been suggested by several workers [15,16,17]. With the advances in molecular biology, several highly informative DNA markers have been developed to analyze genetic polymorphisms, which can be used to determine the microbial status of ecosystem and in that sense the soil quality and sign of restoration. Random amplified polymorphic DNA (RAPD) is a PCR-based DNA fingerprinting technique to develop DNA markers of any origin or complexity using single primer with arbitrary nucleotide sequence[18]. Nucleotide variation between different sets of template DNAs resulted variation in their banding pattern associated with the presence or absence of bands because of the changes in the priming sites. RAPD fingerprinting represents dominant markers that direct amplification of discrete loci in the genome, and hence the allelism (homozygous or heterozygous) is not distinguishable in RAPD patterns. It is widely used in microbial ecology studies in order to address changes in microbial and insights into the microbial response to stress[19]. Keeping in view, the RAPD markers were used with an aim to assess the genetic variability within and between 21 metagenomes of microbial populations form seven different soil profiles (FMS, MS, DWS, GS, PTS, AS and FS), which not only pave the way of greater understanding the direction of improving soil quality, but also pre-requisite for the assessment of ecosystem functioning. Further, cluster analysis was performed based on the distance matrix in the form of a dendrogram in order to elucidate the relationship between microbial populations in seven different soil profiles with the ecosystem functioning.

MATERIALS AND METHODS

Study sites and Sampling

The study was carried out in the iron mining area at Noamundi maintained by TISCO located in the revenue district of West Singhbhum, Jharkhand, India (geographical location 85° 30' 33.61" east longitude and 22° 9' 49.96" north latitude). Tropical dry deciduous forest is considered to be the natural vegetation of the area. Mean annual temperature and humidity is around 19.67C and 20% respectively. Seven different sites were selected such as fresh mine spoil (FMS), 6yr iron mine spoil (MS), degraded wasteland soil (DWS), grassland soil (GS), pesticide treated soil (PTS), agricultural soil (AS) and forest soil (FS) within 10 km peripheral distance of the core iron mining area. During sampling, each site was divided into 5 blocks and from each block three soil samples were collected randomly from (0 - 15) cm soil depth. These samples were referred to as 'sub-samples' and were thoroughly mixed to form one 'composite sample'. Thus, three composite samples obtained from each site, which were sieved (2mm mesh) and stored at 4°C for analysis.

Microbial enumeration

Microbial enumeration was performed by the spread plate technique using selective media. Azotobacter population (AZB) was estimated using azotobacter mannitol agar. Arthrobacter (ARB) count was determined by arthrobacter medium[20]. Rhizobial count (RZB) was estimated by yeast extract mannitol agar with congo red dye[21]. Heterotrophic aerobic bacterial population (HAB) was enumerated using nutrient agar[22]. Enumeration of sulfur reducing bacteria (SRB) was done using sulphate reducing medium (Hi-Media). Actinomycetes count (ACT) was determined using starch-casein agar[23] with 40µl/ml streptomycin and 50µl/ml griseofulvin[24]. Yeast count (YES) was estimated using potato sucrose agar[25]. Fungal count (FUN) was determined using Rose Bengal agar with 50µl/ml streptomycin to inhibit bacterial contaminants[26].

Isolation of metagenomic DNA

The rapid direct DNA extraction from diverse soil compositions with minimal shearing was developed to address the risk of chimera formation from small template DNA during subsequent PCR by SDS-based DNA extraction method[27]. Besides, CTAB (1%) was used to reduce humic content during DNA extraction. The metagenomic DNA was extracted from soil samples by using 13.5ml of DNA extraction buffer (100mM Tris-HCl [pH 8.0], 100mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5M NaCl), 100l of proteinase K (10 mg/ml), 1.5ml of 20% SDS, 7.5M potassium acetate, chloroform:isoamyl alcohol (24:1 v/v) and RNaseA (@ 60 $g.ml^{-1}$, washed with 70% ethanol and suspended in TE buffer (10mM Tris, 0.1mM EDTA). The DNA was quantified by comparing with the known concentration of uncut DNA followed by 1.5% agarose gel electrophoresis. The DNA was diluted as 25 $ng.ml^{-1}$ for RAPD-PCR analysis.

RAPD amplification

Fifteen decamer primers (Operon Tech, USA) were used to amplify the metagenomic DNA for RAPD analysis[18]. Reaction mixture of 25µl contains 10mM Tris HCl (pH 9), 1.5mM $MgCl_2$, 50mM Tris HCl (pH 9.0), 1.5mM $MgCl_2$, 50mM KCl, 200µM of dNTPs, 0.4µM primer, 25ng DNA and 0.5U of taq polymerase (Sigma). Amplification was performed using Gene Cycler (Bio-RAD, USA) with an initial denaturation at 94^0C for 5 min, primer annealing at 37^0C for 1 min and primer extension at 72^0C for 2 min. The period of denaturation was reduced to 1 min at 92^0C in the next 42 cycles, while the primer annealing and extension time remained the same followed by the final primer extension at 72^0C for 7 min. PCR products were stored at 4^0C. About 2.5µl loading buffer (1X TAE, 50% glycerol, 0.25% xylene cyanol) was added to each reaction mixture and subjected to electrophoresis in 1.5% agarose gel containing ethidium bromide @ 0.5 $µg.ml^{-1}$ in TAE buffer (40mM Tris, 20mM sodium acetate, 20mM EDTA and glacial acetic acid, pH 7.2) for 2hrs at 50 V. The gels were documented in Gel-Doc XR 2000 (Bio-Rad, USA) and the sizes of the amplicons were determined using 100bp DNA ladder (Bangalore Genei, India). Further, the reactions were repeated twice to test the reproducibility.

Data collection and analysis

The banding patterns obtained from RAPD analysis according to their sizes were scored as present (1) or absent (0). Pairwise distance matrix was calculated using the Jaccard similarity coefficient[28] and subjected to cluster analysis by the Neighbor joining method using DARWIN (Version 5.0.158)[29]. About 1000 iteration datasets were generated with replacement of characters within the combined 1/0 data matrix. Statistically unbiased clustering of the metagenomes was performed using STRUCTURE (Version 2.3.1)[30]. POPGENE 32 software was used to calculate Nei's unbiased genetic distance among seven different soil profiles. Besides, the observed no. of alleles (Na), effective no. of alleles (Ne), Nei's gene idiversity (H), Shannon's information index (I), no. of polymorphic loci (NPL), % of polymorphic loci (PPL), population diversity (Hs) and heterozygosity (Ht) among the 21 metagenomes of microbial populations in seven soil profiles across the sites were calculated using POPGENE[31]. The resolving power (Rp) of different RAPD primers was calculated as: Rp = Σ IB where, *IB* (band informativeness) by taking the value of: 1–[2* (0.5–P)], where P being the proportion of the 21 metagenomes containing the band[32]. The polymorphic information content (PIC) was calculated based on the allele pattern[33]. Further, the RAPD data were subjected to AMOVA (Version 1.5) in order to estimate genetic variability among microbial populations[34]. The input files for AMOVA were prepared using AMOVA-PREP (Version-1.01)[35]. The non-parametric AMOVA was performed using GenAlex[34], where the genetic variability among microbial populations was partitioned among and within the populations.

RESULTS AND DISCUSSION

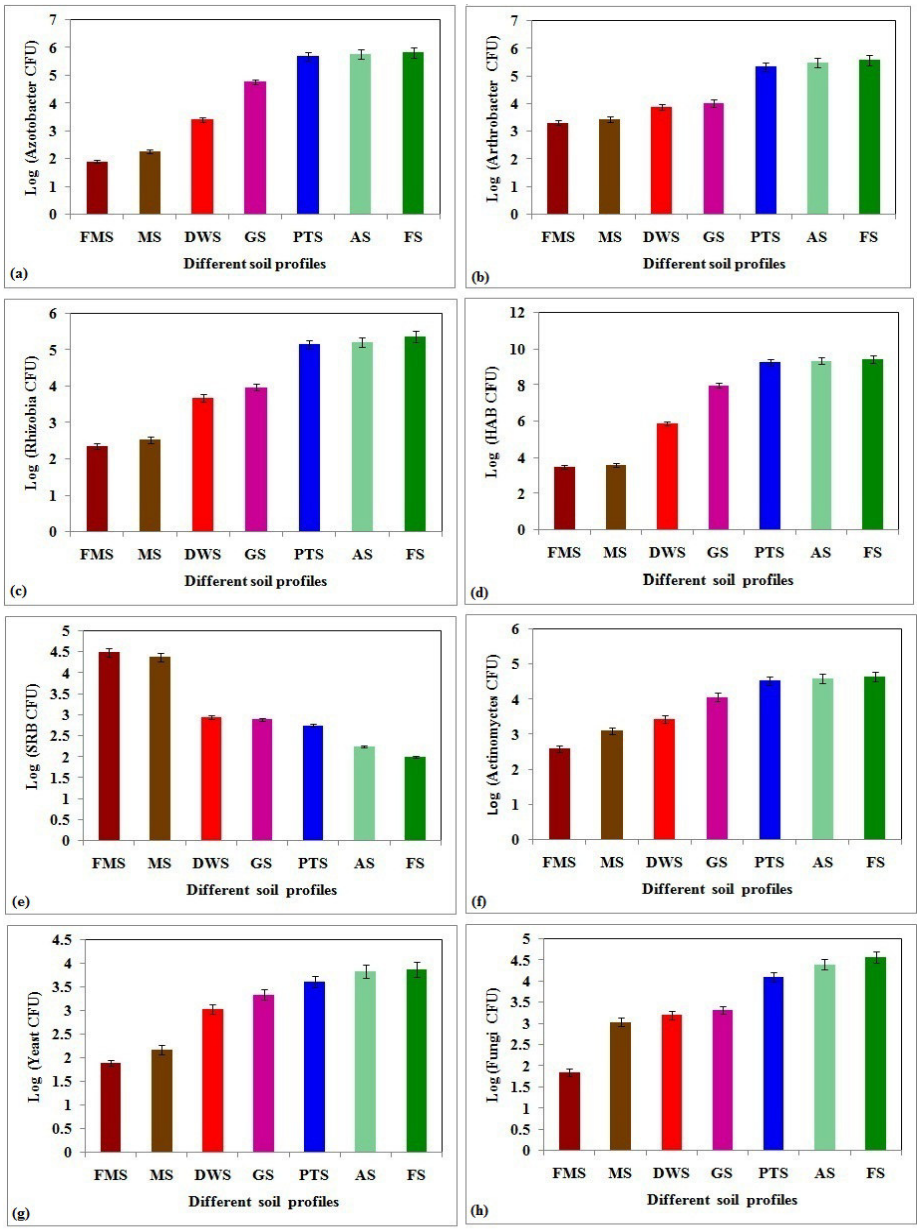
Microbial enumeration

The variation in their relative abundance among seven different soil profiles were presented in terms of log_{10} transformed of CFU/g soil (Figure 1). The AZB count exhibited an increasing trend from FMS ($8x10^{-1}$) to FS ($68x10^{-4}$) across the sites (Figure 1a). Relatively higher AZB abundance was estimated in AS ($59.2x10^{-4}$) than PTS ($48.9x10^{-4}$) and GS ($59x10^{-3}$). ARB count revealed similar trend with minimum in FMS ($21x10^{-2}$) and maximum in FS ($38.9x10^{-4}$) (Figure 1b). ARB population was found to be relatively higher in AS ($31.5x10^{-4}$) than PTS ($22.5x10^{-4}$) and GS ($10.8x10^{-3}$). The RZB count varied from $23x10^{-1}$ (FMS) to $23.8x10^{-4}$ (FS) across the sites (Figure 1c). Progressive increases in HAB count from $32x10^{-2}$ (FMS) to $28.2x10^{-8}$ (FS) (Figure 1d) and AMC population from $4x10^{-2}$ (FMS) to $45x10^{-3}$ (FS) were observed among seven different soil profiles (Figure 1f). However, the SRB count exhibited a reverse trend *i.e.* maximum in FMS ($31.2x10^{-3}$) and the minimum in FS ($10x10^{-1}$) (Figure 1e). The SRB count in MS ($24x10^{-3}$) was found to be relatively higher than DWS ($8.9x10^{-2}$) and GS ($79x10^{-1}$). Further, the relative distribution of YES varies from $8x10^{-1}$ (FMS) to $7.5x10^{-3}$ (FS) (Figure 1g). The MS ($15x10^{-1}$) exhibited relatively higher YES count than FMS. The YES count in GS ($22x10^{-2}$) was relatively higher than DWS ($11x10^{-2}$). Comparatively higher YES count was exhibited by AS ($69x10^{-2}$) than PTS ($41x10^{-2}$). Similar trend with respect

to FUN distribution was observed, which ranges from $7x10^{-1}$ (FMS) to $38x10^{-3}$ (FS) (Figure 1h). Relatively higher FUN population was observed in AS ($25x10^{-3}$) as compared to PTS ($12.6x10^{-3}$) and GS ($21x10^{-2}$). Similarly, higher FUN dominance was observed in DWS ($16x10^{-2}$) than MS ($11x10^{-2}$) and FMS ($7x10^{-1}$). Comparative assessment with respect to relative distribution of microbial populations among seven different soil profiles suggested that the enriched soil usually harbours relatively higher microbial community than the nutrient deficient degraded soil (Figure 1).

Figure 1

(a-h). Relative distribution of (a) Azotobacter (AZB), (b) Arthrobacter (ARB), (c) Rhizobia (RZB), (d) Heterotrophic aerobic bacteria (HAB), (e) Sulphur-reducing bacteria (SRB), (f) Actinomycetes (ACT), (g) Yeast (YES) and (h) Fungal (FUN) populations in seven different soil profiles.



DNA content

The analysis indicated wide variation in the metagenomic DNA content among the seven soil profiles across the sites. The MS (0.078 mg/g soil) exhibited relatively higher DNA content than FMS (0.063 mg/g soil). Relatively higher DNA content was estimated in GS (0.207 mg/g soil) as compared to DWS (0.108 mg/g soil). The DNA content ranges from 0.063 mg/g soil to 1.174 mg/g soil with minimum in FMS and maximum in FS, which may be due to the gradual improvement in microbial colonization. The study revealed relatively higher DNA content in FS as compared to AS and DWS, which was substantiated by other findings[36]. Corresponding to the consistent increase in DNA content associated with higher genetic variability among the microbial population was also evident from their CFU counts in seven different soil profiles[37]. The correlation between the metagenomic DNA content and log (CFU) of different soil microbial populations was found to significant (Table 1).

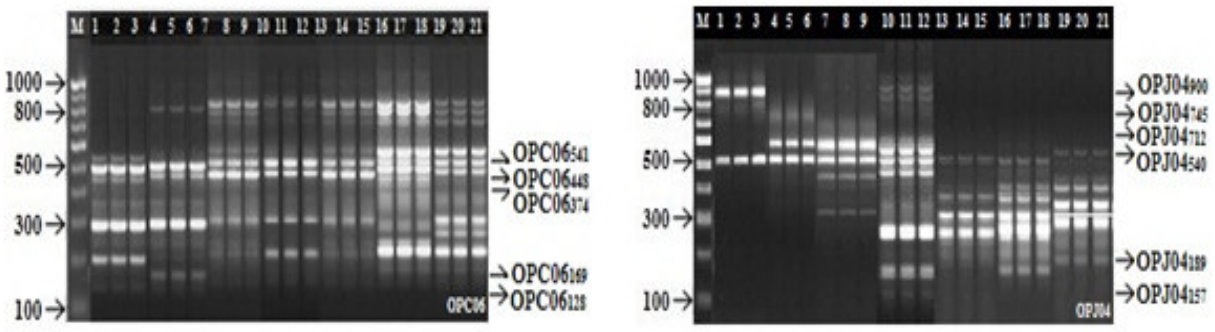
Table 1
Correlation between the metagenomic DNA content and log (CFU) of different microbial populations in seven different soil profiles.

Parameters		Equation(s)	Coefficient of determinant (R^2)	Correlation coefficient (r)	Level of significance
x	y				
CFU count (AZB)	DNA content (mg/g soil)	y = 2.7403x + 2.7295	R^2 = 0.7806	r = 0.883	$p < 0.01$
CFU count (ARB)		y = 1.8012x + 3.4534	R^2 = 0.9675	r = 0.983	$p < 0.001$
CFU count (RZB)		y = 2.1361x + 2.8703	R^2 = 0.8476	r = 0.920	$p < 0.001$
CFU count (HAB)		y = 4.2232x + 4.6837	R^2 = 0.7534	r = 0.867	$p < 0.01$
CFU count (SRB)		y = -1.401x + 3.8739	R^2 = 0.6172	r = 0.785	$p < 0.05$
CFU count (AMC)		y = 1.3187x + 3.1305	R^2 = 0.7830	r = 0.884	$p < 0.01$
CFU count (YES)		y = 1.1990x + 2.4528	R^2 = 0.6861	r = 0.828	$p < 0.01$
CFU count (FUN)		y = 1.5234x + 2.6601	R^2 = 0.7704	r = 0.877	$p < 0.001$

RAPD marker size and patterns

PCR amplification of 21 metagenomes of microbial populations revealed that the total number of amplified bands varied from 71 (FMS) to 159 (FS). Higher genetic variability among the microbial populations was evident from the increasing trend in total number of bands from 221 (FMS) to 474 (FS). The molecular fingerprinting revealed several amplified fragments produced per primer with varying sizes (Figure 2a-b), which was analytically appropriate[13,17,38].

Figure 2
Banding pattern of amplified DNA fragments of 21 metagenomes from seven soil profiles using primers (a) OPC06 and (b) OPJ04. Lane 1: Marker DNA (100bp ladder); Lanes 1-3: FMS; Lanes 4-6: MS; Lanes 7-9: DWS; Lanes 10-12: GS; Lanes 13-15: PTS; Lanes 16-18: AS and Lanes 19-21: FS.



DNA amplification studies revealed the distribution of RAPD markers with variable sizes and produced multiple band profiles varying from 8 to 19 (Table 2). Out of 208 bands, 143 (68.75%) were polymorphic, 28 (13.46%) monomorphic and 37 (17.78%) specific bands (Table 2). The study indicated that 14 primers (except OPA03) revealed wide polymorphisms among soil profiles. Rp values of 15 primers ranged from 11.04 (OPN03) to 18.95 (OPC06) (Table 2). Primers such as OPC06, OPS04, OPD03, OPA09, OPH07 and OPC02 exhibited higher Rp values, which can able to distinguish 21 metagenomes of microbial populations in seven different soil profiles. Polymorphic information content (PIC) defines the ability of the marker to detect polymorphism within the population or set of metagenomes by taking into account not only the number of alleles that are expressed, but also the relative frequencies of alleles per locus. The OPD03 showed highest level of polymorphism with PIC value 0.932, whereas PIC value of other RAPD markers were with in the range of 0.860-0.926 (Table 2).

Table 2

RAPD primers and their nucleotide sequence, (GC) content (%), total and maximum number of bands amplified, nature of bands, resolving power and polymorphic information content (PIC) of 15 different primers used for RAPD fingerprinting of 21 metagenomes of microbial populations from seven different soil profiles across the sites

Primer	Primer sequence (5' 3')	GC content (%)	Total no. of bands amplified*	Maximum no. of bands	Polymorphic bands	Monomorphic bands	Specific bands	Resolving power	PIC**
OPA03	5'AGTCAGCCAC3'	60	123	08	06	2	0	11.71	0.860
OPA09	5'GGGTAACGCC3'	70	177	15	10	2	3	16.85	0.918
OPC01	5'TTCGAGCCAG3'	60	135	12	10	0	2	12.85	0.898
OPC02	5'GTGAGGCGTC3'	70	174	14	11	2	1	16.57	0.917
OPC06	5'GAACGGACTC3'	60	199	17	09	3	5	18.95	0.919
OPD03	5'GTCGCCGTCA3'	70	191	19	14	1	4	18.19	0.932
OPD08	5'GTGTGCCCCA3'	70	159	14	10	1	3	15.14	0.910
OPH07	5'CTGCATCGTG3'	60	176	14	08	2	4	16.76	0.908
OPJ04	5'CCGAACACGG3'	70	162	18	11	1	6	15.42	0.925
OPJ05	5'CTCCATGGGG3'	70	167	11	04	6	1	15.90	0.891
OPN03	5'GGTACTCCCC3'	70	116	09	07	1	1	11.04	0.870
OPN12	5'CACAGACACC3'	60	159	13	10	2	1	15.14	0.909
OPS01	5'CTACTGCGCT3'	60	163	13	11	1	1	15.52	0.907
OPS04	5'CACCCCCTTG3'	70	197	16	12	3	1	18.76	0.926
OPZ04	5'AGGCTGTGCT3'	60	159	15	10	1	4	15.14	0.913
Total	--	--	**2457**	**208**	**143**	**28**	**37**	--	--

**** Total number of bands amplified for all 21 soil sample.***

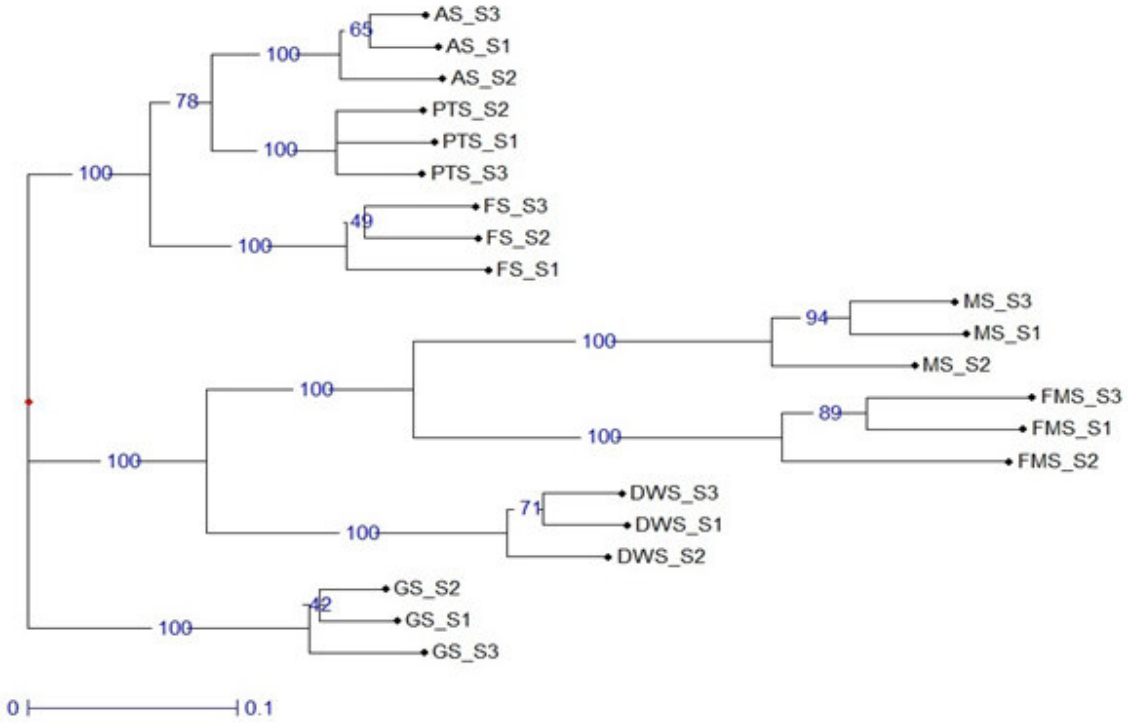
***** Polymorphic information content.***

Genetic diversity assessment

To estimate the genetic variability among microbial populations, similarity coefficient between 21 metagenomes were analyzed. The similarity coefficient values were subjected to cluster analysis using NJ method, which revealed the convergence of 21 metagenomes of microbial populations from seven different soil profiles into six distinct clusters supported by bootstrap values: cluster- I (3 metagenomes representing FMS), cluster- II (3 metagenomes representing MS), cluster- III (3 metagenomes representing DWS), cluster-IV (3 metagenomes representing GS), cluster-V (6 metagenomes representing PTS and AS) and cluster-VI (3 metagenomes representing FS) (Figure 3). The association of different clusters revealed appropriate chronosequence (FMS → MS → DWS → GS → PTS → AS → FS) based on the similarity matrix among microbial populations collected from seven different soil profiles. Since all the six clusters showing bootstrap values above 50%, the tree likeness of the original (unrandomized) tree was statistically well resolved (Figure 3).

Figure 3

RAPD profiling based on Neighbor joining method represent six independent clusters of 21 metagenomes of microbial populations from seven different soil profiles supported by the bootstrap values



An unbiased clustering of 21 metagenomes from seven different soil profiles based on STRUCTURE analysis without prior knowledge about the microbial populations across the sites clustered into six major groups. Under the admixed model, STRUCTURE calculated that the estimate of the likelihood of the data (LnP(D)) was greatest when K = 6. For K > 6, LnP(D) increased slightly but more or less plateued (Figure 4a) *i.e.* δK reached its maximum value when K = 6 (Figure 4b). The analysis suggested that the 21 metagenomes representing microbial populations from different soil profiles fell into one of the 6 clusters albeit minor interference (Figure 4c). These results are almost similar to the splitting in the neighbor joining tree (Figure 3). Overall the cluster analysis based on NJ method as well as STRUCTURE analysis strongly suggested that the 21 metagenomes of microbial populations from seven different soil profiles across the sites were well segregated (with high likelihood probability) into six independent clusters.

Table 3
Summary of the analysis of molecular variance (AMOVA) based on RAPD fingerprinting of 21 metagenomes of microbial populations from seven soil profiles

Source of variance	*df*	*SSD*	*MS*	*Estimated variance (%)*	*p-value*
Among populations	6	635.619	105.937	83	$p < 0.001$
Within population	14	95.333	6.81	17	$p < 0.001$
Total	20	730.952	112.747	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, wide genetic variation among the 21 metagenomes of microbial populations from seven different soil profiles was evident from high number of polymorphic markers. Higher number of polymorphic loci (180) suggested profound polymorphism (86.54%). The observed number of alleles (Na) and an effective number of alleles (Ne) was found to be 1.8654 and 1.5907 respectively (Table 4). Genetic diversity (H) and Shannon's information index (I) was found to be 0.3347 and 0.4916 respectively. Similarly, the heterozygosity (Ht) among the microbial populations in seven different soil profiles was estimated to be 0.3347 and within populations (Hs) was found to be 0.0437. Gene differentiation (Gst) among the 21 metagenomes of microbial populations was estimated to be 0.8696. Moreover, the estimated gene flow (Nm) was estimated to be 0.0750 among the microbial populations from seven different soil profiles across the sites (Table 4).

Table 4
Overall genetic variability among the 21metagenomes of microbial populations with respect to their distribution in seven different soil profiles 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)	Heterozygosity (Ht)	Population diversity (Hs)	Number of polymorphic loci	Percentage of polymorphic loci
21	1.8654 (0.3421)	1.5907 (0.3504)	0.3347 (0.1702)	0.4916 (0.2307)	0.3347 (0.0290)	0.0437 (0.0039)	180	86.54

Microbial populations in their natural habitats are usually subjected to horizontal gene transfer[13,17,43]. However, there exists limitation for the microbial colonization in hostile fresh iron mine overburden spoil (FMS). Subsequently, the microbial colonization may be due to the gradual establishment of vegetation leading to the development of heterogeneous microbial groups that acted as the stock for horizontal gene transfer resulting molecular variation. The process of gene flow among the microbial populations can be well assessed through the estimation of a projected gene flow[44,45,46]. In the present study, the estimate of the gene flow *i.e.* 0.0750 limits the operation of gene transfer between the microbial populations in seven soil profiles. The positive correlation between CFU and DNA content revealed higher genetic variability, which may be due to the evolution of microbial strains across the sites. Besides, the variation in microclimatic conditions may cause such variation among the microbial populations. Further, the microbiological study indicated 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[7]. There has been increasing efforts for the assessment of genetic diversity of soil microbial populations using different molecular techniques[13,17,41,44]. The molecular genetic variability among the microbial populations has been studied by several workers[17,44]. Efficiency of such molecular approaches used for the microbial ecology studies lies with the fact that this can unravel the relative distribution and abundance of different microbial populations in their natural habitat and can explain their functional role for sustainability of the soil subsystem[7,47,48]. The conclusive outcome of the investigations is to provide a comprehensive study of RAPD markers that can supplement molecular analysis useful for studying the microbial diversity. The study correlated well with temporal and spatial variations among seven different soil profiles across the sites confirming the PCR based DNA fingerprinting

technique using RAPD markers through metagenomic approaches were useful means of discriminating the soil microbial communities and useful for estimating microbial community relatedness. The study indicated relatively higher genetic variability among the microbial metagenomes from 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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REFERENCES

1. Bloem J, de Ruiter P and Bouwman LA, Soil food webs and nutrient cycling in agroecosystems. In: J.D. van Elsas, J.T. Trevors, and H.M.E. Wellington (eds.), *Modern Soil Microbiology,* Marcel Dekker, New York, 1994. p. 245 – 278.
2. Marcel GA, Van de H, Bardgett RD and Nico MS, The unseen majority soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol. Letter, 2008; 11: 296 - 310.
3. Kennedy P and Smith KL, Soil microbial diversity and sustainability of agricultural soils. Plant Soil, 1995; 170: 75 - 86.
4. Pankhurst CE, Hawke BG, MacDonald HJ, Kirkby CA, Buckerfield JC, Michelsen P, O'brien KA, Gupta VVSR and Doube BM, Evaluation of soil biological properties as potential bioindicators of soil health. Aust. J. Exp. Agri, 1995; 35: 1015 - 1028.
5. Bickham JW, Sandhu S, Hebert PDN, Chikhi L and Athwal R, Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutation Research, 2000; 463: 33 - 51.
6. Ovreas L, Population and community level approaches for analyzing microbial diversity in natural environments. Ecol. Letter, 2000; 3: 236 - 251.
7. Yin B, Crowley D, Sparovek G, De Melo WJ and Borneman J, Bacterial functional redundancy along a soil reclamation gradient. Appl. Environ Microbiol., 2000; 66: 4361 – 4365.
8. Insam H, Developments in soil microbiology since the mid 1960s. Geoderma, 2001; 100: 389 - 402.
9. Emmerling C, Schloter M, Hartmann A and Kandeler E, Functional diversity of soil organism a review of recent research activities in Germany. Plant Nutri. Soil Sci., 2002; 165: 408 - 420.
10. Steffan-Dewenter I and Tscharntke T, Insect communities and biotic interactions on fragmented calcareous grasslands. a mini review. Biol. Conser., 2002; 104: 275 - 284.
11. Babalola OO, Molecular techniques: An overview of methods for the detection of bacteria. African J. Biotech., 2003; 2(12): 710 - 713.
12. Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM, Solovyev VV, Rubin EM, Rokhsar DS and Banfield JF, Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature, 2004; 428: 37 - 43.
13. Akbar T, Akhtar K, Ghauri MA, Anwar MA, Rehman M, Rehman M, Zafar Y and Khalid AM, Relationship among acidophilic bacteria from diverse environments as determined by randomly amplified polymorphic DNA analysis (RAPD). World J. Microbiol. Biotech., 2005; 21: 645 - 648.
14. Hill GT, Mitkowski NA, Aldrich-Wolfe L, Emele LR, Jurkonie DD, Ficke A, Maldonado- Ramirez S, Lynch ST and Nelson EB, Methods for assessing the composition and diversity of soil microbial communities. Appl. Soil Ecol., 2000; 15: 25 - 36.
15. Volossiouk T, Robb EJ and Nazar RN, Direct DNA Extraction for PCR mediated assays of soil organisms. Appl. Environ. Microbiol., 2005; 3972 - 3976.
16. Baker BJ and Banfield JF, Microbial communities in acid mine drainage. FEMS Microbiol. Ecol., 2003; 44: 139 - 152.
17. Waltenbury DR, Leduc LG and Ferroni GD, The use of RAPD genomic fingerprinting to study relatedness in strains of *Acidithiobacillus ferrooxidans*. Tropical Ecol., 2005; 62: 103 - 112.
18. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic acids Res., 1990; 18: 6531 - 6535.
19. Sharma N, Singh G and Sudarsan Y, Assessment of microbial diversity under arid plants by culture dependent and culture independent approaches. African J. Biotech., 2013; 12(40): 5860 - 5868.
20. Hagedorn C and Holt JG, Ecology of soil arthrobacters in Clarion-Webster top sequences of Iowa. Appl. Microbol., 1975; 29: 11-218.
21. Vincent JM. A manual for the practical study of root-nodule bacteria. IBP Handbook of methods. No.15.Blackwell Scientific Publication, Oxford, 1970.
22. Gray TRG. Soil Bacteria. Soil Biology Guide. John Wiley and Sons (ed.). New York, 1990.

23. Hunter-Cevera JC and Eveleigh DE. Actinomycetes Soil Biology Guide, John Wiley and Sons. New York, 1990.
24. Alharbi SA, Arunachalam C, Murugan AM and Wainwright M. Antibacterial activity of actinomycetes isolated from terrestrial soil of Saudi Arabia. J. Food Agril. Environ, 2012; 10 (2): 1093-1097.
25. Krishna H, Carpenter A. and Potter F. Effect of washing additives on the incidence of rots and an enumeration of surface microbes in stored squash. New Zealand Plant Protection. 2001; 54: 76-79.
26. Alef K and Nannipieri P. Methods in applied soil microbiology and biochemistry. Accademic Press, London, 1995.
27. Zhou J, Bruns M and Tiedje JM, DNA Recovery from soils of diverse composition. Appl. Environ. Microbiol., 1996; 62(2): 316 – 322.
28. Sneath PHA, Sokal RR. Numerical taxonomy: The principles and practice of numerical classification. W.H. Freeman & Co., San Francisco, 1973. p. 573.
29. Felsenstein J, Confidence limits on phylogenics: An approach using the bootstrap. Evolution, 1985; 39: 783 - 791.
30. Evanno G, Regnaut S and Goudet J, Detecting the number of clusters of individuals using software structure: a simulation study. Molecular Ecol., 2005; 14: 2611 - 2620.
31. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individual. Genetics, 1978; 89: 583 - 590.
32. Prevost A and Wilkinson MJ, A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor. Appl. Genet., 1999; 98: 107 - 112.
33. Anderson JA, Churchill GA, Autrique JE, Tanksley SD and Sorrells ME, Optimizing parental selection for genetic linkage maps. Genome, 1993; 36: 181 - 186.
34. Excoffier L, Smouse PE and Quattro JM, Analysis of molecular variance inferred from metric distance among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 1992; 131: 479 - 491.
35. Miller MP, AMOVA-PREP 1.01: A Program for the Preparation of the AMOVA input files from dominant-marker raw data. Dept. of Biological Sciences, Northern Arizona University, Flagstaff, AZ, 1998.
36. Grantina L, Seile E, Kenigsvalde K, Kasparinskis R, Tabors G, Nikolajeva V, Jungerius P and Muiznieks I, The influence of the land use on abundance and diversity of soil fungi: comparison of conventional and molecular methods of analysis. Environ. Experimental Biology, 2011; 9: 9 - 21.
37. Kujur M and Patel AK, Microbial soil respiration and its dependency on soil microbial communities, organic carbon and moisture in different dry tropical ecosystems. Int J. Pharm. Bio Sciences, 2014; 5(1): B21 - 36.
38. Zul D, Denzel S, Kotz A and Overmann J, Effects of plant biomass, plant diversity and water content on bacterial communities in soil Lysimeters: Implications for the determinants of bacterial Diversity. Appl. Environ. Microbiol., 2007; 6916 - 6929.
39. Simpson JM, Santo-Domingo JW and Reasoner DJ, Microbial source tracking: state of the science. Environ. Sci. Technol., 2002; 36: 5279 - 5288.
40. Zhou JB, Xia DS, Treves LY, Wu TL and Marsh RV, Spatial and resource factors influencing high microbial diversity in soil. Appl. Environ. Microbiol., 2002; 68: 326 - 334.
41. Findlay SEG, Sinsabaugh RL, Sobczak WV and Hoostal M, Metabolic and structural response of hyporheic microbial communities to variations in supply of dissolved organic matter. Limnol. Oceanography, 2003; 48(4): 1608 - 1617.
42. Bond PL, Smriga SP and Banfield JF, Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. Appl. Environ. Microbiol, 2000; 66: 3842 - 3849.
43. Novo MTM, De Souza AP, Garcia Jr. O and Ottoboni LMM, RAPD genomic fingerprinting differentiates *Thiobacillus ferrooxidans* strains. Syst. Appl. Microbiol., 1996; 19: 91 - 95.
44. Kelly DP and Wood AP, Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. Int. J. Syst. Evol. Microbiol., 2000; 50: 511 - 516.
45. Wulf-Durand P, Bryant LJ and Sly LI, PCR-mediated detection of acidophilic, bioleaching associated bacteria. Appl. Environ. Microbiol., 1997; 63: 2944 – 2948.
46. Pobell S, Otto A and Kutschke S, Identification and discrimination of *Thiobacilli* using ARDREA, RAPD and rep-APD. J. Appl. Microbiol., 1997; 84: 1085 - 1091.
47. Jastrow JD, Miller RM and Lussenhop J, Contribution of interacting biological mechanisms to soil aggregate stabilization in restored prairie. Soil Biol. Biochem., 1998; 30: 905 - 916.
48. Malik A, Scullion J, Soil development on restored opencast coal sites with particular reference to organic matter and aggregate stability. Soil Use Manag., 1998; 14: 234 - 239.