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Bacterial Community Diversity and their Composition in Different Soil Ecosystems Analyzed by PCR Coupled Culture Independent Methods

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Exploration of bacterial community diversity linked with different forest and farm soil ecosystems is very essential to get information about their role in the maintenance of respective soil ecosystems. Different parameters associated with ecosystems have significant effect on inhabitance of diverse bacterial communities. Our study deals with, unraveling of bacterial community diversity and their composition in two different kinds of forest and farm soil ecosystems. This was accomplished by using molecular biology methods such as PCR-DGGE and sequencing of 16S rDNA clone library of metagenomes extracted from forest and farm soils. Study revealed that there was high level of similarity (about 77%) in bacterial community among two forest soil and more than 60% of similarity between forest and organic farm soil. However, less than 38% of similarity was seen between forest and degraded farm soil bacterial communities. Further 16S rDNA sequencing results were showed bacterial composition in each type of soil ecosystem. All soil types have shown presence of uncultured microbes with abundance of γ-proteobacterial communities. Apart from γ-proteobacteria, other bacterial communities such as Acidobacteria, Actinobacteria, γ-proteobacteria and Plactomycetes were found in organic farm soil whereas Firmicutes was present in degraded farm soil. The results of present study demonstrated that, the bacterial diversity observed in this experiment is very less irrespective of soil ecosystem. This reflects that, those may be intensively threatened by biotic/abiotic factors and human activities associated with it. Thus, these forest and farm soils are need to be protected for maintaining ecological balance.

Key words: Bacterial diversity, Soil ecosystems, culture independent method.

Soil ecosystems are rich source of microbial biodiversity. The estimated number is about 10^3 to 10^4 different bacterial ribotypes in g^{-1} of soil [1, 2]. Composite bacterial communities in diverse soil ecosystems have very potent role in maintenance of soil health, soil fertility and improvement of agro ecosystems [3, 4]. However, the understanding of bacterial diversity and their role in natural ecosystem is limited by culture dependent methods of bacterial diversity study. This limitation is overcome by culture independent molecular finger printing methods. Thus, advanced molecular approaches such as PCR based denaturing gradient gel electrophoresis (PCR-DGGE) coupled with cloning and sequencing of major finger prints are widely used to study the dynamics and structure of complex bacterial communities in diverse soil ecosystems [5]. These molecular approaches provide the prospective for significant progress in our understanding of bacterial diversity in natural ecosystems and their relation with respective soil ecosystems [2, 6, 7]. Different studies have shown that PCR coupled DGGE is effective method in studying soil bacterial diversity under the influence of different chemical,

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agronomic practices or seasonal variation [8, 9, 10, 11].

The current study was mainly focused on analysis of structural diversity of bacterial communities in forest and farm soil ecosystems based on primers specific to 16S rDNA and DGGE. Further PCR based 16S rDNA library was constructed to know the composition of bacterial communities in respective soil types.

MATERIALS AND METHOD

Soil sampling

Four soil samples from three different places of Karnataka state were collected for this study. Two forest soil (BR hills, Wildlife Sanctuary, Karnatak and D-Dandeli forest, Karnataka), one organic farm soil (PCF10- Praful Chandra farm, private farm in Shimoga district of Karnataka state) and one degraded (PCF17) which was not under cultivation from last five years (Fallow Lands other than Current Fallows) soil adjacent to organic farm soil was collected. Twenty subsamples from each place were collected from a depth of approximately 10 cm. These respective samples were then pooled to prepare master sample of each soil type. Samples were brought to the laboratory and immediately shifted at -80°C for long term storage.

DNA extraction

Triplicates of each soil sample (BR, D, PCF10 and PCF17) measuring 500 mg was taken in the respective microfuge tube. Then 200 µl of $FeCl_3$ (200 mM) was added as chemical flocculent to each tube and vortexed for 2 minutes to form a homogenous mixture prior to cell lysis [12]. These homogenous mixtures were used for soil DNA extraction by using DNA extraction protocol of Miller *et al*. (1999)[13]. Quantification of extracted soil DNA was done by using NanoDrop Spectrophotometer (ND-1000, NanoDrop Technology, Wilmington, DE, USA)

PCR amplification of 16S rDNA gene from forest and farm soil metagenomes

The extracted DNA was 20 fold diluted and used as a template for polymerase chain reaction. PCR amplification was performed by using bacteria specific DGGE primer pair PRBA968F (5'AACGCGAAGAACCTTAC3') and PRBA1406R (5'ACGGGCGGTGTGTAC3') targeting V6 and V9 variable regions of 16S rDNA gene. The GC clamp of 40 bp (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G 3') was added to the 5' end of the primer PRBA968F [14]. The reaction mixture was prepared to a final volume of 20 µl which contained 0.25 pmol of each forward and reverse primers, 0.2 mM of dNTP's, 1X *Taq*buffer B, 1.5 mM of MgCl--$_2$ and 1.5 units of *Taq* DNA polymerase (Bangalore Genei, India). The reaction was performed in automated thermal cycler (Eppendorf) with initial denaturation of 5 min at 95°C followed by denaturation at 94°C for 45 sec, annealing at 57.4°C for 45 sec and extension of 1 min 30 sec for 35 cycles followed by 10 min final extension at 72°C. The quality and size of amplicon was confirmed by agarose gel electrophoresis followed by ethidium bromide staining.

DGGE and cluster analysis of bacterial communities

DGGE was performed with Ingenyphor U-2 system (Leiden, The Netherlands) with slight modification in earlier protocol of Muyzer *et al*. (1993) [15]. PCR amplified products of expected size from respective soils were subjected to DGGE analysis onto 8-10% (w/v) gradient of polyacrylamide gel containing 30 to 80% of chemical denaturant (7M urea and 40% formamide forms 100% denaturant). Gel was stained by the silver staining protocol of Gustavo and Peter [16]. The scoring for presence and absence of band was done manually. Similarity matrix and cluster analysis was done using UPGMA by employing NTSYS software (Version 2.02j; Applied Biostatistics) [17].

Estimation of Carrying Capacity of Forest and Farm soil ecosystems

The bacterial carrying capacity of BR, D, PCF10 and PCF17 soil ecosystems were estimated using Range weighted-richness (*Rr*) as explained by Marzorati *et al*. (2008) [18]. The *Rr* for these soil samples were determined by using formula $Rr = (N^2 \times Dg)$. Where *N* is total number of DGGE fingerprints on gel and *Dg* represents the denaturing gradient comprised between the first and last band in a lane.

Construction of PCR based 16S rDNA Library of forest and farm soil metagenomes

16S rDNA library was constructed from BR, PCF10 and PCF17 soil metagenomes only. Because, as previously reported the soil environment of these three soils is continuously getting threatened by human and human

associated activities [68, 69, 70]. The PCR reaction of 300 µl was performed for all the three soil DNA using full length universal 16S rDNA primer (27F-5'AGAGTTTGATCMTGGCTCAG3' and 1492R-5'GGTTACCTTGTTACGACTT 3') [19] as explained in the above section of polymerase chain reaction with slight change in the final extension of 45 min at 72 °C to enable T/A cloning. PCR amplified products of 1.5 kb size (Fig. 6) were gel extracted and purified using gel extraction and purification kit (Qiagen, Germany). The gel purified 16S rDNA amplicon of all three soils were used for ligation in TA cloning vector pTZ57R/T. The ligation reaction mixture was prepared as per the directions given in InsTAclone PCR Cloning Kit (Fermentas Life Sciences, EU). The ligated products were transformed into *E. coli* DH5α [20]. Finally transformed cells were selected on Luria Bertani agar plates (L.A.) containing Ampicillin in 100 µg ml^{-1} as selection pressure and X-gal, IPTG added in final concentration of 38 µg ml^{-1} and 32 µg ml^{-1} respectively.

Sequencing of 16S rDNA clones and sequence based analysis

Collectively 66 randomly selected, restriction confirmed clones from BR, PCF10 and PCF17 soils were sequenced from Ocimum Biosolution Laboratory, Hyderabad, India. Sequences were assembled by using cap contig assembly program from BioEdit software (Version 7.0.9.0) [21]. Further sequences were checked for presence of chimera using Decipher program (Version 1.8.0) [22]. Bacterial composition and taxonomic affiliation of all the sequenced clones of forest and farm was determined by using sequence match program from Ribosomal Database Project 10 (RDP10) (http:// rdp.cme.msu.edu) [23].

RESULTS

DNA extraction and PCR amplification

Our protocol used for DNA isolation resulted into extraction of high molecular weight DNA. DNA yield varied with respect to soil type (Fig. 1). It was found in between 8 to 16 µg $g^{-1}$of soil with purity $A_{260/280}$ in between 1.44 to 1.67 (Table 1). All four soil DNA templates (BR, D, PCF10 and PCF17) showed effective PCR amplification with DGGE specific primer set PRBA968F-PRBA1406R at 1:20 dilution. Single amplicons of 480 bp size were observed in each type of soil DNA on ethidium bromide stained agarose gel (Fig. 2).

DGGE and cluster analysis of bacterial communities

The DGGE fingerprints obtained using primer pair (PRBA968F- PRBA1406R) showed the presence of 28, 21, 26 and 27 operational taxonomic units (OTUs) in BR, D, PCF10 and PCF17 soil samples, respectively (Fig. 3). Structural similarity and variation among forest and farm soil bacterial communities was assessed by using similarity matrix values (Table 2). The soil bacterial community composition shared by similar ecosystem shows greater degree of similarity than that of similarities shared by different ecosystem. Similarity matrix based on DGGE fingerprints indicated that bacterial community compositions of forest soils are more similar. Both the forest soil share 77% of bacterial species whereas the organic farm soil share only 62% and 68.7% bacterial species with BR hills and Dandeli forest soil respectively. However, the organic farm soil and its adjacent degraded soil share 43.7% of the bacterial species based on DGGE profile (Fig. 4).

Carrying Capacity of Forest and Farm soil samples

Range weighted richness values for all forest and farm soil samples were found to be above 70 (Table 3) indicating that all the soils are habitable for bacterial microflora and carry large number of species belonging to different taxa. However, BR and PCF17 soils are more habitable than D and PCF10 soil.

Taxonomic affiliations of 16S rDNA clones

Sequence analysis of 16S rDNA clones reveal that forest and farm soils were dominated by uncultured bacteria belonging to class γ-proteobacteria. However, the BR soil showed complete dominance of different candidatus uncultured bacteria belonging to class γ-proteobacteria. On the other hand slightly different results were appeared in case of PCF10 and PCF17 soil. In spite of dominance by different uncultured bacteria belonging to γ-proteobacteria group, four of twenty six clones of organic farm soil belongs to different classes *viz;* Acidobacteria [PCF10(9)], γ-proteobacteria [PCF10(12)], Actinobacteria [PCF10(26)] and Planctomycetes [PCF10(35)]. Furthermore, one of twenty one sequenced clones of degraded farm soil evidently

visualized presence of uncultured bacteria belonging to class to Firmicutes [PCF17 (55)] (Table 4).

DISCUSSION

Soil DNA extraction and PCR amplification

The major endeavor of this study was to understand the structural diversity of bacterial communities among forest and farm soil ecosystems. Soil sampling sites selected in current metagenomic study has their own significance. Two forest soils BR and D represent largest wildlife sanctuaries in Karnataka State (India) and are major hotspots of biodiversity. Among farm soils, PCF10 was under organic farming practices from last 30 years while PCF17 was degraded (Fallow Lands other than Current Fallows) land just adjacent to organic farm soil, sharing similar edaphic and environmental conditions. Different biotic and abiotic factors linked with these soil ecosystems have strong influence on inhabitance of diverse bacterial phylotypes. To get an insight about their structural diversity, metagenomic approach was employed. Success of soil metagenomic study relies on recovery of high quality DNA from soil. Method of DNA extraction developed by Miller *et al*. (1999) [13] was used for current metagenomic study because; DNA extraction buffer used in protocol lacks EDTA. Absence of EDTA in buffer indirectly contributes toward the achieving relative purification of DNA from humic acid and its derivatives. Since, EDTA is co-extractor of humic acid from soil [24, 25, 26]. For further reduction in coextraction of humic acid along with DNA preparations was achieved by $FeCl_3$ added as chemical flocculent during sample preparation but prior to cell lyses to reduce loss of DNA [12].

Average DNA yield obtained by our protocol in respective soil type varies between 8 to 16 µg g^{-1} of soil and purity varies between 1.44 to 1.67 at A_{260}/A_{280} (Fig. 1; Table 1). The present DNA extraction method provided relatively pure DNA as compared to previously reported purity of metagenomic DNA extracted by using CTAB and PVPP (ratio of 1.25 to 1.41 at A_{260} / A_{280}) [27, 28]. The relative purity of soil DNA was demonstrated by effective PCR amplification of 16S rDNA gene at 20 fold dilution as compared to previous studies which required harsh purification and 1000 to 10000 fold dilutions for effective PCR amplification [27, 13, 29].

DGGE and diversity of soil bacterial communities

The DGGE profiles obtained using primer set PRBA968F-PRBA1406R indicated multiple bands in forest and farm soil sample. This may be

Table 1. Forest and farm soil DNA quantification

Sr. No	Soil Sample Name	*Concentration (ng / ul)	$A_{260/280}$
1	BR	408.33 (±12.04)	1.44 (±0.09)
2	D	304.66 (±10.58)	1.58 (±0.08)
3	PCF10	257.28 (±08.25)	1.63 (±0.18)
4	PCF17	202.24 (±05.63)	1.67 (±0.05)

*Average concentration of DNA taken from triplicates of each soil DNA and values in the parenthesis indicates the standard deviation

Table 2. Similarity matrix of forest and farm soil (BR, D, PCF10 AND PCF17) obtained by using DGGE profiling (968F-1406R)

ROW/COL.	BR	D	PCF10	PCF17
BR	1.000			
D	0.770	1.000		
PCF10	0.620	0.687	1.000	
PCF17	0.350	0.375	0.437	1.000

Table 3. Range-weighted richness (*Rr*) of forest and farm soils

Sr. No.	Name of Soil Ecosystem	N	N^2	Dg	$Rr = (N^2 \times Dg)$
1	BR	28	784	0.16	125.44
2	D	21	441	0.16	70.56
3	PCF10	26	676	0.12	81.12
4	PCF17	27	729	0.16	116.64

Table 4. Taxonomic affiliations of 16S rDNA library clones of forest and farm soil PCR based library

Soil Type	Clone	Accession No.	Phylum	Sub-phylum	Genus	Species*	Similarity Score
Organic	P10(1)	HQ883944	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; EU663550*	1.000
Farm	P10(2)	HQ883945	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Escherichia coli; AJ404540*	1.000
Soil	P10(3)	JF712661	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; AY390990*	1.000
(PCF-10)	P10(4)	HQ883946	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured gamma proteobacterium; AJ005541*	0.994
	P10(5)	KJ469544	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Escherichia coli; X80731*	1.000
	P10(6)	HQ883947	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; FJ450614*	1.000
	P10(7)	HQ883948	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured Escherichia sp.; EU629739*	1.000
	P10(8)	HQ883949	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; EU663550*	1.000
	P10(9)	KJ469545	Acidobacteria	Acidobacteria Gp4	*Gp4*	*Uncultured Acidobacteriales bacterium; EU665022*	0.987
	P10(10)	HQ883950	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; EU663550*	0.989
	P10(12)	JF712662	Proteobacteria	á-proteobacteria	*Bradyrhizobium*	*Uncultured bacterium; AM749498*	0.997
	P10(15)	JF712663	Proteobacteria	ã-proteobacteria	*Salmonella*	*Uncultured bacterium; EU663550*	1.000
	P10(16)	JF712664	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; AB075097*	1.000
	P10(25)	JF712665	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured Escherichia sp.; GU229535*	0.997
	P10(26)	KJ469546	Actinobacteria	Actinobacteria	*Unclassified Actinobacterira*	*Uncultured bacterium; EU354555*	0.958
	P10(35)	KJ469547	Planctomycetes	Planctomycetes	*Unclassified Planctomycetes*	*Uncultured bacterium; EF019958*	0.969
	P10(40)	JF712666	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; DQ159121*	0.998
	P10(45)	JF712667	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured compost bacterium; AF353276*	1.000
	P10(57)	KJ469549	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Escherichia coli; X80731*	0.997
	P10(66)	JF712668	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured Escherichia sp.; EU629739*	1.000
	P10(67)	KJ469548	Proteobacteria	ã-proteobacteria	*Erwinia*	*Uncultured bacterium; FJ849509*	0.996
	P10(74)	HQ883951	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*mixed culture ruminal bacterium; AF070468*	1.000
	P10(75)	HQ883952	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*mixed culture ruminal bacterium; AF070469*	1.000
	P10(76)	HQ883953	Proteobacteria	ã-proteobacteria	*Salmonella*	*Escherichia coli; U58272*	1.000
	P10(80)	HQ883954	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; AY390990*	1.000
	P10(87)	KJ469550	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; AB075097*	1.000
Degraded	P17(8)	KJ469551	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Escherichia coli; X80731*	1.000
Farm	P17(18)	JF712669	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; AY390990*	1.000
Soil	P17(25)	HQ883955	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; EF094394*	1.000
(PCF-17)	P17(28)	HQ883956	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured Escherichia sp.; EU629739*	1.000
	P17(33)	HQ883957	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; GQ448021*	0.964
	P17(35)	JF712670	Proteobacteria	ã-proteobacteria	*Salmonella*	*Uncultured bacterium; EF094394*	1.000
	P17(45)	JF712671	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; AY537670*	0.990
	P17(48)	JF712672	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured Escherichia sp.; EU629739*	1.000
	P17(52)	JF712673	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; FJ450614*	1.000
	P17(38)	HQ883958	Proteobacteria	ã-proteobacteria	*Salmonella*	*Escherichia coli; U58272*	1.000
	P17(53)	HQ883959	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; EF440333*	0.997
	P17(55)	KJ469552	Firmicutes	Clostridia	*Acetobacterium*	*Uncultured Acetobacterium sp.; KF909223*	0.990
	P17(58)	HQ883960	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured Escherichia sp.; EU629739*	1.000
	P17(65)	HQ883961	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; EU544289*	1.000
	P17(68)	HQ883962	Proteobacteria	ã-proteobacteria	*Salmonella*	*Escherichia coli; U58272*	1.000
	P17(73)	JF712674	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; EF440333*	0.995

	P17(75)	JF712675	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; EU357412*	0.997
	P17(78)	HQ883963	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; EU663550*	0.997
	P17(85)	HQ883964	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; EU663550*	1.000
	P17(88)	HQ883965	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured Escherichia sp.; GU229535*	0.997
	P17(98)	HQ883966	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Escherichia coli; GU415745*	1.000
BR	b1	HQ883928	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Escherichia coli; AB496849*	1.000
Hill	b10	HQ883929	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured compost bacterium; AF353276*	1.000
Forest	b17	KJ469542	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Shigella boydii; EU438863*	0.984
Soil	b19	HQ883930	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; AB205998*	1.000
(BR)	b23	HQ883931	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured bacterium; AY574446*	1.000
	b24	HQ883932	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; EU215260*	0.996
	b25	HQ883933	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured Escherichia sp.; EU629359*	1.000
	b30	HQ883934	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Escherichia coli; AB496851*	0.996
	b32	HQ883935	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Escherichia coli; AB496849*	0.996
	b34	HQ883936	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Escherichia coli; AB496850*	0.996
	b38	HQ883937	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured Escherichia sp.; AY325737*	0.997
	b44	HQ883938	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured Escherichia sp.; EU629359*	1.000
	b49	KJ469543	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; AJ487022*	0.992
	b53	HQ883939	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Escherichia coli; AB496849*	1.000
	b56	HQ883940	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured Photorhabdus sp.; AM084246*	0.996
	b59	HQ883941	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured bacterium; DQ159121*	0.998
	b60	JF712676	Proteobacteria	ã-proteobacteria	*Unclassified Enterobacteriaceae*	*Uncultured compost bacterium; AF353276*	1.000
	b64	HQ883942	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured Escherichia sp.; EU629360*	1.000
	b68	HQ883943	Proteobacteria	ã-proteobacteria	*Escherichia/Shigella*	*Uncultured Escherichia sp.; EU629359*	0.997

* The numbers followed by species name indicates the accession number of respective nucleotide sequence

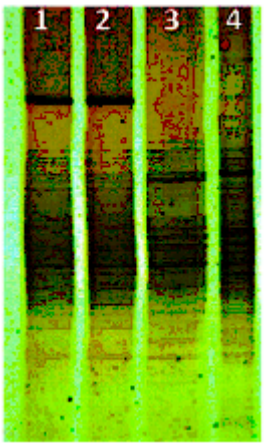
Lane 1: λ Hind III digest ladder; Lane 2: BR soil DNA; Lane 3: D soil DNA; Lane 5: PCF10 soil DNA; Lane5. PCF17 soil DNA

Fig. 1. Genomic DNA of forest and farm soil

Lane 1: BR soil; Lane 2: D soil; Lane 3: PCF10 soil; Lane 4: PCF17 soil; Lane 5: 100 bp ladder

Fig. 2. PCR amplification of 16S rRNA gene from forest and farm soil using primer 968F and 1406R

due to the efficient amplification of different bacterial 16S rDNA nucleotide sequences from all four soil types. Also, the amplicons obtained using this primer pair covers V6 to V9 variable regions which contributes toward the discrimination among fingerprints on gel. It is interesting to note that, another possible reason may be the coverage rate of Primer set PRBA 968F - PRBA 1406R. Fascinatingly, coverage range for the primer set estimated by using probe match program from RDP database showed that primer pair PRBA968F and PRBA1406R targets 35 different bacterial phyla with variable coverage rate in each phylum when two mismatches were allowed. The per cent coverage of this primer pair for five major bacterial phyla appearing in soils were shown in figure 5 [30]. These result concise to results of Nakatsu *et al*. (2000) [14]. Each band on the DGGE gel is considered as one bacterial genus/species or defines one OTU [31, 32]. Thus, DGGE fingerprinting pattern obtained using this primer (PRBA968F-PRBA1406R) showed existence of 28, 21, 26 and 27 OTUs in BR, D, PCF10 and PCF17 soil samples, respectively. Variable abundance of soil bacterial communities was indicated by differential pattern of DGGE fingerprints and their intensity [33].



Lane 1: BR soil DNA; Lane 2: D soil DNA; Lane 3: PCF10 soil DNA; Lane 4:PCF17 soil DNA

Fig. 3. Denaturing gradient gel electrophoresis finger prints of forest and farm soil obtained using primer pair 968F-1406R

Similarity matrix values based on DGGE fingerprint with PRBA968F-PRBA1406R primer pair indicated that microflora of similar soil ecosystem shared maximum similarity. Both forest soil bacterial communities showed closer similarity with the bacterial microflora of organic farm soil but lesser extent with degraded farm soil bacterial microflora. This may be due to the variability in soil health conditions, natural environmental factors and biotic/abiotic factors or organic farming practices associated with respective soil ecosystem. These listed factors one or the other way may leads to increase/decrease or static nature of soil microflora. Several studies in the last decade have provided strong evidences for such changes in bacterial

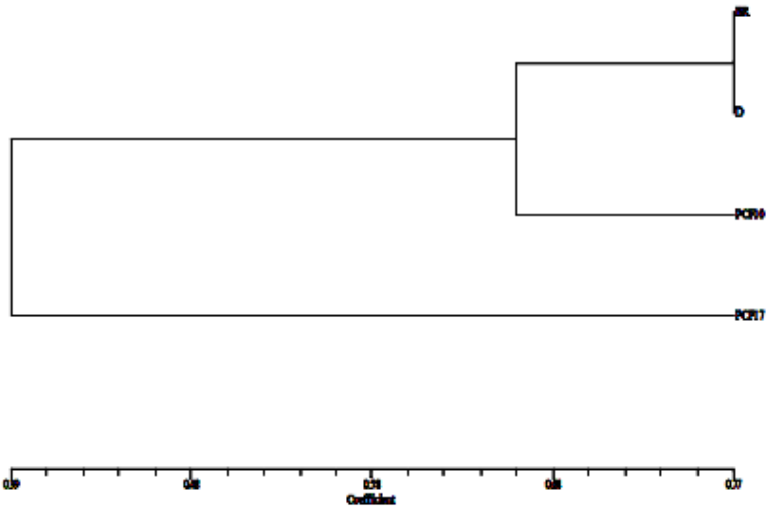
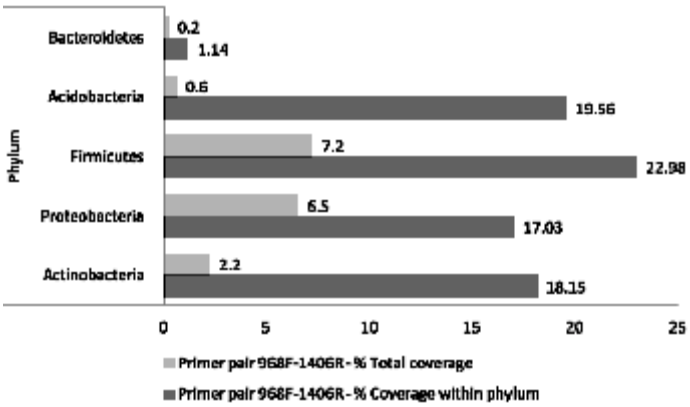


Fig. 4. Phylogenetic tree of forest and farm soil constructed using NTSYS software (Version 2.02j; Applied Biostatistics) based on manual scoring of presence and absence of bands

community diversity with respect to different factors associated with soil [34, 35, 36, 37, 7, 38, 39, 11, 40, 41].

Carrying capacity of forest and farm soil samples

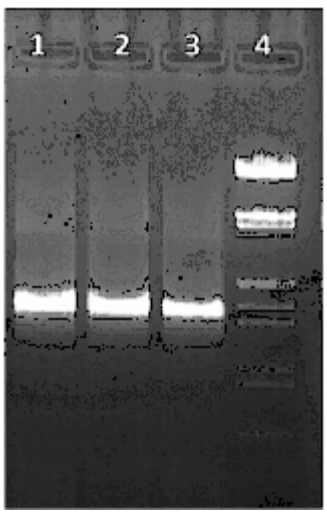
We addressed that, irrespective to soil type the bacterial carrying capacity for all selected forest and farm soil samples were above 70. This clearly indicates the richness of bacterial community diversity in respective soil ecosystems. However, in case of PCF17 soil the *Rr* value was 116.64. But, this is not necessary that, degraded farm soil should show less number of DGGE finger prints of bacterial communities. Because, adverse environmental conditions may leads to depletion of the one or more bacterial communities in soil but at the same time one or several other kinds of bacterial communities may increase in number to cope up with the surrounding conditions [7, 42, 43, 44]. Head *et al.* (1998) [45] showed that number and intensity of DGGE finger prints on gel not necessarily represents the accurate picture of



[Total % coverage = (No. sequence matches found for given phyla / Total no. sequences of 5 major phyla in database) ×100]

[% coverage within the phylum is = (No. Of sequence matches found for given phyla / total number of sequences of given phylum in database) × 100]

Fig. 5. Per cent coverage rates of primer pair 968F-1406R for five major soil bacterial phyla



Lane 1: BR soil DNA; Lane 2: PCF10 soil DNA; Lane 3: PCF17 soil DNA; Lane 4: λ EcoRI , Hind III double digest ladder

Fig. 6. PCR amplification of full length 16S rRNA gene from forest and farm soil using primer pair 27F-1492R

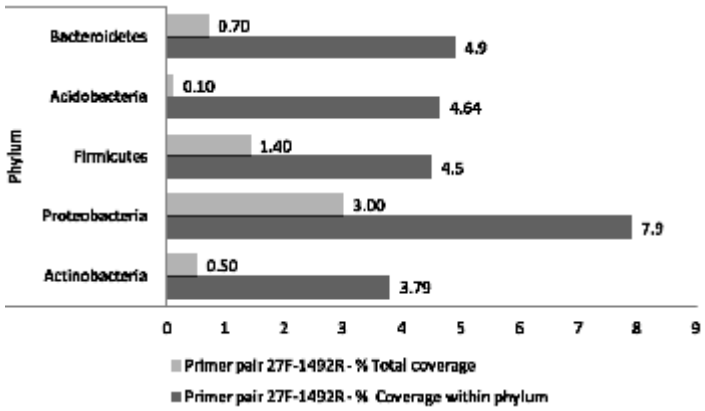
bacterial communities as one organism may produce more than one band as result of multiple copies and heterogeneous nature of rRNA operons. There are several other possible reasons for getting less or more DGGE profiles on gel irrespective to nature of soil samples such as, concentration of 16S rDNA targets in sample under investigation, in case of partial 16S rRNA gene sequences one band may represent more than one bacterial species and also PCR artifacts occurred during PCR amplification process [18]. The range weighted richness is based on number of bands (OTU's) observed in a range of denaturant. Increase in either the number of bands or the denaturant range or both will increase in the range weighted richness. In this study, the difference in the number of OTU's observed between the samples is not much, but the range of denaturant in which the OTU's got separated leads to large carrying capacity in case of degraded soil. Irrespective of taxonomical

correlation, several studies in the last decade have shown that per cent GC content can be used to study diversity and dynamics of microbial communities [1, 46, 47]. Even different environmental factor seems to influence the GC content of complex microbial communities. Thus, GC content of bacterial communities may make them to adopt the adverse conditions in soil like degraded farm soil [48].

Soil bacterial communities and their interaction with soil ecosystems

Sequencing of 16S rDNA clones of forest and farm soil gave a partial insight about nature of bacterial communities in respective soil types. Irrespective of soil type, γ-proteobacteria group appeared as dominant bacterial population. This may be due to preferential PCR amplification of 16S rDNA from bacteria belonging to γ-proteobacteria group by primers pairs used. Hence, this indicates the primer bias. Hong *et al*. (2009) [49] used same primer pair for the study of marine tidal flat bacterial communities. They found that 25 % clones sequenced from library I and 23 % clones sequenced from library II belongs to sub-phylum γ-proteobacteria. There are several other studies that have reported similar kind of results due to primer bias [50, 51, 52].

Also, we have determined the coverage



[Total % coverage = (No. sequence matches found for given phyla / Total no. sequences of 5 major phyla in database) ×100]

[% coverage within the phylum is = (No. of sequence matches found for given phyla / total number of sequences of given phylum in database) ×100]

Fig. 7. Per cent coverage rates of primer pair 27F-1492R for five major soil bacterial phyla

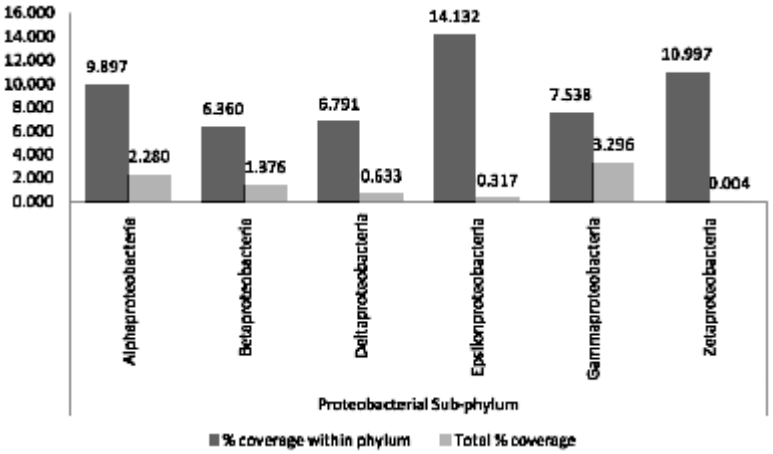


Fig. 8. Per cent coverage rates of primer pair 27F-1492R for different Proteobacterial Sub-phyla

rates of primer used for construction of 16S rDNA library in RDP database. The primer pair 27F and 1492R covers about 35 different bacterial phyla with variable number of sequence match in each phylum when two mismatches were allowed. The per cent coverage rate of primer pair 27F - 1492R for five major soil bacterial phyla were shown in figure 7 [30]. This primer pair has shown maximum overall per cent sequence coverage in proteobacterial phylum [more specifically in sub-phylum γ-proteobacteria compared to other Proteobacterial sub-phyla (Fig. 8) as compared to sequence match in other bacterial phyla (Fig. 7) [53].

Janssen PH. (2006) [54] reviewed that, major dominant phyla in the in metagenomic 16S rDNA libraries (Including forest and agricultural farm soil) are Proteobacteria, Acidobacteria, Actinobacteria, Verruco-microbia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmati- monadetes, and Firmicutes. He summarized in his survey that, on an average 92% of 16S rDNA sequences in soil libraries were contributed by members of these nine phyla. Among these phyla only Proteobacterial phyla covers about average 39% of total 16S rDNA sequences in soil libraries. While among Proteobacterial phyla, sub-phylum γ-proteobacteria shown third highest abundance of 16S rDNA clones. Surprisingly this is strong evidence for the sequencing results of BR soil (forest) which have shown the presence of complete domninance of bacteira belonging to sub-phylum γ-proteobacteria.

Furthermore, abundance of proteobacteria (γ-proteobacteria) is probably due to biotic factors such as interaction among soil and other animals like birds, grazing animals and insects or organic manures added in soil [55, 56, 57]. However, presences of few clones (*Unclassified Actinobacteria, Uncultured Acidobactria, Uncultured Acetobacteria, Uncultured Bradyrhizobium, Uncultured Erwinia,* and *Unclassified Planctomycetes*) other than γ-proteobacteria are excellent indicator of their contribution towards respective soil health and soil environment. Our results are supported by the results of Zhang *et al.* (2013) [58]. Their finding emphasize that, Proteobacteria were highest in percentage irrespective to the treatment of organic matter in soil. Further they found that, most of Proteobacteria are gram negative and have potential role in maintenance of soil environment such as nitrogen fixation. Feris *et al.* (2003) [59] suggested that contaminated soils have abundance of Proteobacteria and a wide variety of pathogens such as *Agrobacterium, Escherichia, Salmonella* and *Helicobacter* are also Proteobacteria. While Urich *et al.* (2008) [60] suggested that environmentally protected soils (Free from human interventions) have lesser Proteobacterial load.

However, the results of Smit *et al.* (2001) [6] suggested that healthy nutritive soil with a readily available nutrients favors positive selection for á- and γ-proteobacteria. The ratio between the number of Proteobacteria and Acidobacteria is a good indicator of nutritional status of soils. Their findings showed that, this ratio was found to be high in agricultural soil with high organic content, medium in agricultural soil with low organic input and low in oligotrophic soil. These statements are found to be true for our sequencing results of forest and farm soil which are dominated by bacteria belonging to sub-phylum γ-proteobacteria. This indicates that all soil types are nutritionally healthy with high organic content.

Microbial community dynamics during the composting of organic waste in an aerobic industrial composting process examined by culture independent survey through 16S rDNA sequence analysis demonstrated the predominance of Enterobacteriaceae family including *Escherichia* during initial stages of compost digestion (Alfreider *et al*., 2002) [61]. So there may be possibility that coincidently the organic matter present in forest and farm soil samples were in initial stages of decomposition which might have caused dominance of bacterial community from Enterobacteriaceae family. Compared to artificial process of decomposition natural process of organic matter decomposition is slow because of which *Escherichia* may survive for long time in soil.

The sequencing results of 16S rDNA clone library of forest and farm soil ecosystems have revealed dominance of γ-proteobacterial species belonging to family Enterobacteriaceae mainly classified into genus *Escherichia*. However *Escherichia* genus is not native soil bacterial community, but the sequencing of 16S rDNA showed their dominance in forest and farm soil. It

is mostly likely that it might have added into soil as result of amendment of organic matter (bovine or dairy manure based compost) through natural means or by artificial means or a source of contamination [62, 63, 64]. Since all the soil types including forest soil used this research investigation have strong link with organic matter. The birds, animals, wild animals or polluted water or soil source may be the reason for getting the *Escherichia* genus in forest soil (Wildlife sanctuary).

The reports from Government of Karnataka (GoI 2003b and 2003c) [65,66] which states that, 40% of Karnataka state has soil degradation problem and around 30% forest in Karnataka state are degraded due to problems like mining, industrialization, quarrying, livestock grazing, fuel wood collection, fragmentation of habitats, unsustainable management practices etc. The climate change is another threat to Indian forests which effects into altered forest productivity, resilience and biodiversity (GoI 2007a). India's forests are exceedingly pressurized from population growth, commercial need for forest products, grazing livestock and fires [67]. Strangely, only few species belonging to particular phyla identified by sequencing of 16S rDNA from forest soil confirms these reports.

Thus we conclude that, the DGGE is capable of discriminating bacterial community diversity among forest and farm soil ecosystems. However, further sequencing of dominant DGGE profiles will give more information about the relation between different factors associated with forest and farm soil ecosystems and inhabitance of diverse bacterial communities. Cloning and sequencing of 16S rDNA clones has given partial information about the composition and role of bacterial communities in respective forest and farm soil ecosystems. The sequencing results have shown alarming situation that forest and farm soil used for current study may be intensively threatened by climate change and human activities. So it suggests that care must be taken in order to conserve natural biodiversity and maintain environmental/ecological balance. Further sequencing of more number of 16S rDNA clones will give us fine idea about diverse bacterial communities in forest and farm soil ecosystems. However, the determination of exact composition of bacterial communities by culture independent method will be challenging task due to enormous diversity of microorganisms. But the methodological and technical refinement in experimentation will reduce the bias and help to give solid inferences about the experiments.

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