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Community Genomics Involving Culture Independent Approach for Assessing the Phylogenetic Diversity of Mangrove Sediment

KEYWORDS: Metagenomics, 16Sr DNA, ARDRA, Phylogeny

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ABSTRACT *Soil community genomics or metagenomics is employed in this study to analyze the evolutionary relatedness of mangrove microbial community. The metagenomic DNA was isolated from mangrove sediment and 16SrDNA was amplified using universal primers. The amplicons were ligated into pTZ57R/T cloning vector and transformed onto E. coli JM109 host cells. The recombinant plasmids were isolated from positive clones and the insert was confirmed by its reamplification. The amplicons were subjected to Amplified Ribosomal DNA Restriction Analysis (ARDRA) using three different tetra cutter restriction enzymes namely Sau3A1, Hha1 and HpaII. The 16SrDNA insert were sequenced and their identity was determined. The sequences were submitted to NCBI database and accession numbers obtained. The phylogenetic tree was constructed based on Neighbor-Joining technique. Clones belonged to two major phyla of the bacterial domain, namely Firmicutes and Proteobacteria, with members of Firmicutes predominating. The microbial diversity of the mangrove sediment was explored in this manner.*

INTRODUCTION

Mangroves are highly productive ecosystems with immense ecological values. The microbial communities in the mangrove sediment represent some of the most complex microbial habitats on the Earth. There may be several thousand species of bacteria in 1g of soil (Torsvik, 1990). Since only a small percentile (<1%) of existing bacteria are cultivable by standard microbiological methods, the others remain largely unexplored. Hence to assess the diversity of an environmental sample, modern molecular tools based on the PCR amplification of 16S rRNA gene, the phylogenetic anchor, are employed (Handlesman, 2004). In metagenomic approach, 16S rDNAs are amplified by PCR from nucleic acids extracted from environmental samples, whereafter the PCR products are cloned and sequenced. Amplified Ribosomal DNA restriction analysis (ARDRA) which is based on DNA sequence variations present in PCR-amplified 16S rRNA genes(Smit, 1997) also serve as an effective tool in phylogenetic analysis.

In the present study, the 16S rDNA clone library was constructed with the metagenomic DNA isolated from mangrove sediment. Clones were clustered according to the dendrograms of ARDRA results and sequenced. The partial 16S rRNA gene sequences were compared with NCBI data base and taxonomic hierarchy was assigned.

MATERIALS AND METHODS

Isolation of metagenomic DNA

The benthic sediment samples collected from mangroves of Kochi, Kerala, India (09°52'43.3"N 76°15'50.6"E), were transported to the laboratory under refrigeration. The metagenomic or total soil DNA was isolated from sediment sample according to Zhou et al (1996) and purified using UltraClean Soil DNA isolation kit (MoBio, CA, USA). The isolated DNA was electrophoresed on 1% agarose gel at 80V for 1 hour, stained using ethidium bromide (Sambrook, 2000) and visualized under ultraviolet illumination and gel pictures were captured using Gel documentation system (Syngene, CA, USA). The DNA was quantified using a UV-Visible spectrophotometer (Shimadzu, Kyoto, Japan).

PCR amplification of 16SrDNA sequences of metagenomic DNA

The 16SrDNA sequences of the metagenomic DNA was amplified using universal 16SrDNA forward (5'AGAGTTTGATCCTGGCTCAG 3') and reverse primer (5' ACGGCTACCTTGTTACGACTT 3') (Shivaji, 2000). 50ng template DNA was used in a 20µL reaction with an initial denaturation for 2 min at 94 °C, 34 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec and extension at 72 °C for 2 min and with a final extension for 10 min at 72 °C. PCR was performed in a thermal cycler (Biorad, CA, USA). The amplicon was then electrophoresed and visualized using gel documentation system (Syngene). The PCR product was purified using Gel and PCR Clean-up kit (Macherey-Nagel, Duren, Germany) and the purified PCR product was used for cloning.

Construction of 16SrDNA library

The 16SrDNA amplicons were ligated into TA cloning vector pTZ57R/T and transformed onto *E. coli* JM109 host cells using InsTAclone PCR cloning kit (Thermo Scientific, MA, USA). The transformed colonies were screened for α-complementation by using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D thiogalactoside). All positive clones were confirmed by reamplification of 16S rDNA inserts from the recombinant plasmids, isolated according to alkali lysis method, and were stored on LB agar (HiMedia, Mumbai, India) plates containing ampicillin.

ARDRA profiling of metagenomic clones

The 16SrDNA amplicons were digested using 3 different tetrameric restriction enzymes Sau3A1, Hha1 and HpaII (Thermo Scientific) (Gurtler, 1991) with specific restriction sites, ('GATC, GCG'C and C'CGG respectively). Five units of each enzyme was mixed with 5µL of PCR amplicon in separate reaction mixtures and incubated for 4 hours at 37°C. The digested fragments were analyzed on 2% agarose gel, and visualized using gel documentation system (Syngene). Dendrograms were constructed from the distance matrix by using the Unweighted Pair Group Method with Arithmetic means (UPGMA) (Sneath, 1973). The plasmids with discrete ARDRA banding pattern for the 16SrDNA insert were sequenced.

Sequencing of 16SrDNA amplicons

The 16SrDNA was sequenced from the recombinant plasmids by Sanger's Dideoxy method using ABI 3730 Excel (Applied Biosystems, CA, USA). The identity of the sequences was determined by comparing with the sequences in the NCBI database using BLAST software (Altschul, 1990). The sequences were compiled and aligned using ClustalX program (Thompson, 1997) using BioEdit software (Hall, 1999).

Volume : 3 | Issue : 10 | Oct 2013 | ISSN - 2249-555X

The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou, 1987) with 1000 resampling bootstrap using MEGA software (Tamura, 2007).

RESULTS

Construction of 16SrDNA library

The total community or metagenomic DNA isolated from the mangrove sediment with a concentration of 1.56µg/µL was used as template for 16SrDNA amplification. Cloning of purified 16SrDNA PCR product yielded 20 phylogenetic clones after α-complementation and reamplification. The recombinant plasmids isolated from the positive clones showed distinct band shift when compared with control plasmid (Fig.1).

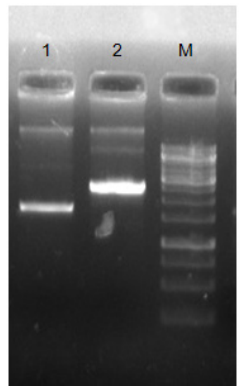

Fig.1: Agarose gel showing band shift of recombinant plasmid

Lane1 – control plasmid pTZ57R/T

Lane2 – recombinant plasmid

Lane M– 1kb marker (Thermo Scientific)

ARDRA profiling of metagenomic clones

The ARDRA profile of 16SrDNA clones obtained with the enzyme Sau3A1 showed distinct patterns on 2% agarose gel (Fig. 2).

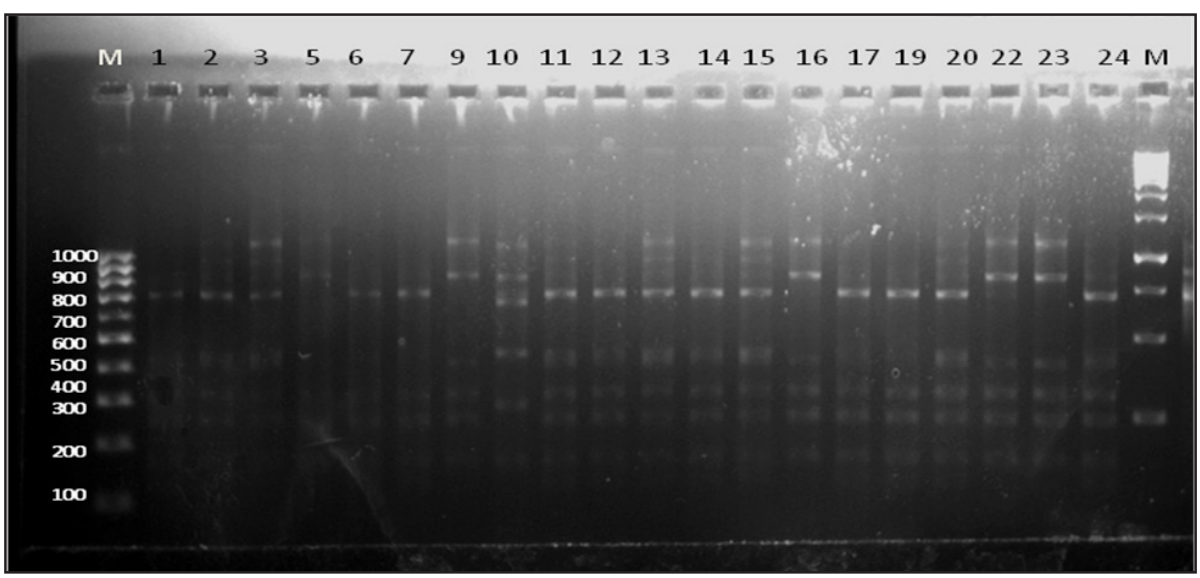

Fig.2: ARDRA profile of 16SrDNA clones with Sau3A1

Lane numbers represent the clone numbers
M represent 1kb DNA marker
Clustering of the clones based on the ARDRA banding pattern using the enzyme Sau3A1 is as depicted in Fig. 3

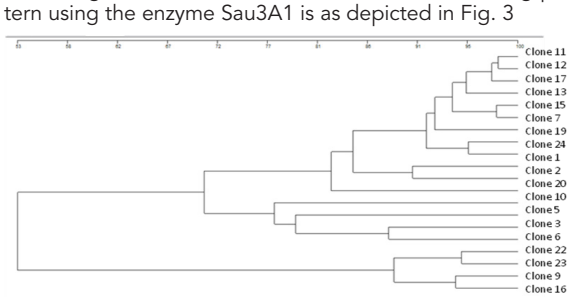

Fig.3. Clustering based on ARDRA pattern using Sau3A1

Most of the clones showed discrete banding pattern with a few exceptions. Clones 11, 12 and 17 showed similar banding pattern and they clustered together. Similarly, clones 22,23,9 and 16 showed similar pattern and they formed a separate cluster.

The ARDRA profile of 16SrDNA clones obtained with the enzyme Hha1 showed similar patterns on 2% agarose gel for most of the clones indicating that the number of Hha1 restriction sites is same in most of the clones. However clone 10 showed a variation in the banding pattern. (Fig. 4)

Volume : 3 | Issue : 10 | Oct 2013 | ISSN - 2249-555X

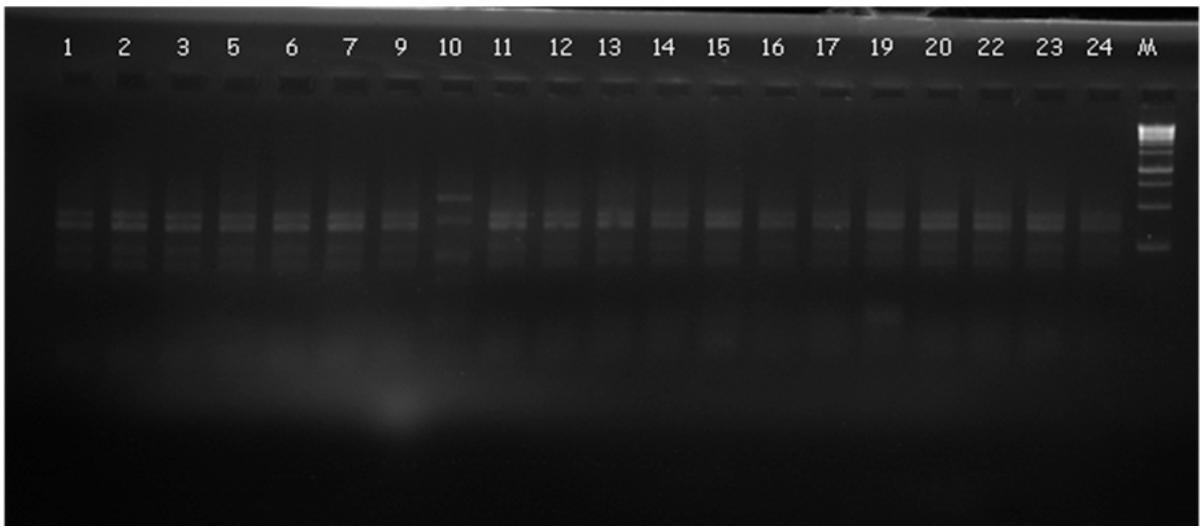

Fig.4: ARDRA profile of 16SrDNA clones with Hha1

Lane numbers represent the clone numbers

M represent DNA marker (Lambda DNA/EcoR1/HindIII double digest)

Clustering of the clones based on the ARDRA banding pattern using Hha1 is depicted in Fig. 5, which clearly showed that while most others clustered together, Clone 10 remained separate from all the others

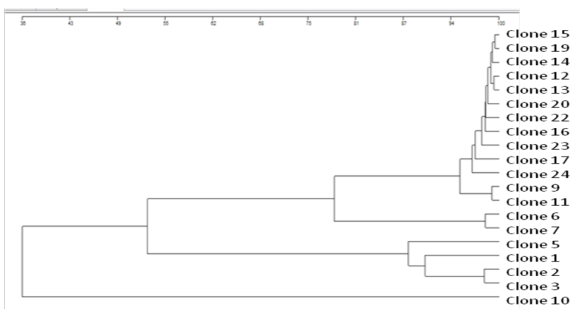

Fig.5. Clustering based on ARDRA pattern using Hha1

The ARDRA profile of 16SrDNA clones obtained with the enzyme HpaII showed varied patterns on 2% agarose gel for most of the clones (Fig. 6).

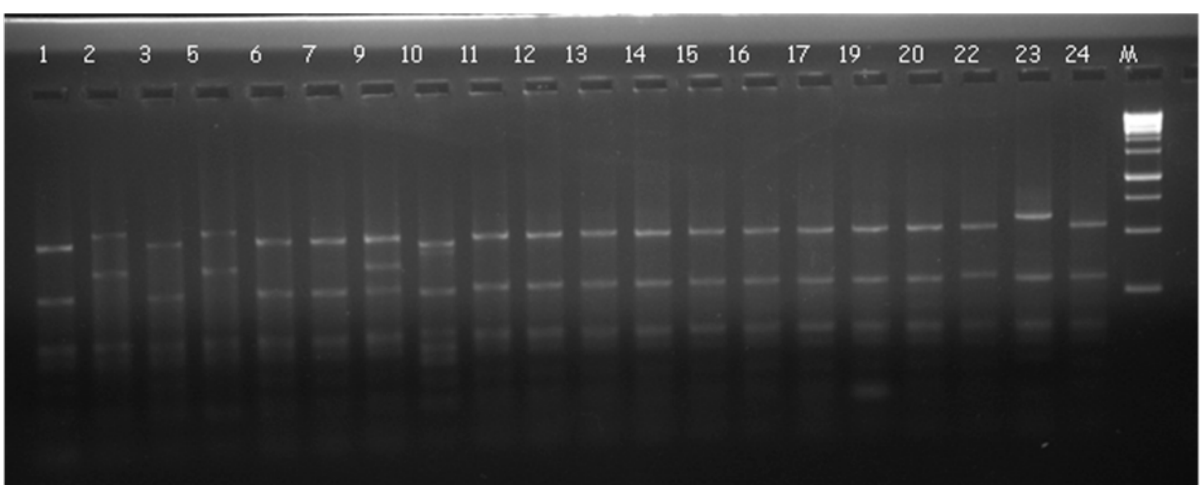

Fig.6: ARDRA profile of 16SrDNA clones with HpaII

Lane numbers represent the clone numbers

M represent DNA marker (Lambda DNA/EcoR1/HindIII double digest)

Clustering of the clones based on the ARDRA banding pattern using the enzyme HpaII is depicted in Fig. 7

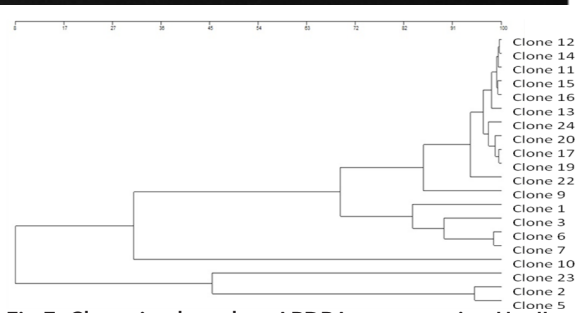

Fig.7. Clustering based on ARDRA pattern using HpaII

Volume : 3 | Issue : 10 | Oct 2013 | ISSN - 2249-555X

Clones 11, 12, 13, 14, 15 and 16 illustrated a particular banding pattern and therefore claded together while clones 2 and 5 showing a different banding pattern formed another cluster.

Sequencing of 16SrDNA amplicons of the clones
The 16SrDNA insert of 15 recombinant plasmids with discrete ARDRA profile were sequenced and the sequences were submitted to NCBI database and accession numbers were assigned (JQ868595 - JQ868601, JQ898300 - JQ898308, JQ805720 - JQ805723).

The phylogenetic tree constructed based on the 16S rDNA sequences of these 15 clones are as shown in figure 8.

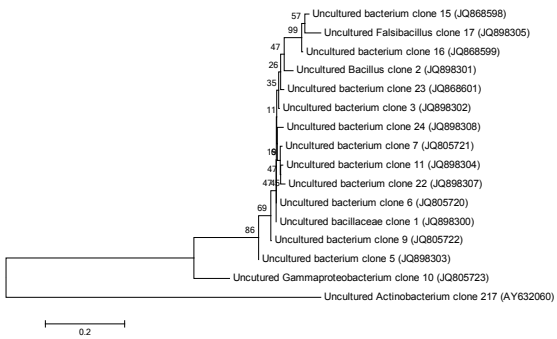

Figure 8: Phylogentic relationship based on partial 16SrDNA sequences of 15 selected clones. *Actinobacterium* clone (accession number AY632060) was used as outgroup. Accession numbers are given in parentheses. The number at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on a Neighbour-Joining analysis of 1,000 resampled data sets.

The phylogenetic tree in figure 8 is based on the16S rRNA sequences of the 15 clones. Clone 10 showed similarity to *Gamma Proteobacteria*, while most others showed similarity to uncultured bacteria. Clone 1 was most similar to unclassified *Bacillaceae*, while Clone 2 to uncultured *Bacillus*. Clone 17 showed identity to uncultured *Falsibacillus*. They all belong to phylum *Firmicutes* indicating that the predominant group of microbes in the mangrove sediment analyzed belonged to phylum *Firmicutes*.

DISCUSSION

In the present study, 20 different 16SrDNA clones of mangrove sediment microbial community generated using metagenomic approach were analysed to study their phylogenetic diversity. Sequence analysis indicated that they belonged to two major phyla of bacterial domain, namely, *Firmicutes* and *Proteobacteria*. The predominant group was phylum *Firmicutes*. The *Proteobacteria*, especially the *Gammaproteobacteria* were found to be abundant in the Sundarban mangrove(Ghosh, 2010). Many other diverse phylotypes like *Flexibacteria, Actinobacteria, Acidobacteria, Chloroflexi*, *Planctomycetes* and *Gammatimonadates* were also reported previously from different environmental samples like marine sediments(Gray, 1996) and Oklahoma prairie soil(Anne, 2009), etc. Urakawa et al. (1999) reported five major lineages of the domain *Bacteria*: the gamma, delta and epsilon *Proteobacteria*, Gram-positive bacteria and the division *Verrucomicrobia* from Sagami Bay and Tokyo Bay. It can be assumed that the dominant phylum *Firmicutes*, especially the class *Bacillaceae*, may have been carried to the mangroves from the land by rain as they are mainly terrestrial inhabitants.

The restriction enzymes, Sau3A1 and HpaII produced clear ARDRA patterns capable of distinguishing the clones from each other. The restriction pattern formed by Hha1 was not significant. Guan *et al*. (2003) reported the identification of *Lactobacillus* in crops of broilers using ARDRA pattern generated by enzymes, HaeIII and MseI. Wu *et al* (2006) reported the identification of *Bacillus* sp. based on the ARDRA pattern generated using six different enzymes namely, AluI, TaqI, HhaI, MboI, RsaI and MspI.

The sequence analysis as well as the ARDRA pattern analysis indicated the phylogenetic diversity of the mangrove microbial community.

The present study confirms that the mangrove microbial community is highly diverse. Since culture dependent studies are highly limiting, culture independent analysis or metagenomics serves as a valuable tool for assessing the phylogenetic diversity of environmental samples.

CONCLUSION

The present study analyzed the phylogenetic diversity of mangrove sediment microbial community using metagenomic approaches. The analysis revealed that the community comprised of two major phylum of bacterial domain namely, *Proteobacteria* and *Firmicutes* with phylum *Firmicutes* predominating. The study needs to be extended for the understanding of many other different phylogroups present in this unique exosystem.

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