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Original Research Article

RAPD analysis of microbial population from coir retting area, Colachel, Kanyakumari District, India

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A B S T R A C T

Random amplified polymorphic DNA (RAPD) analysis was performed on 5 bacterial, 3 fungal and 2 actinomycetes isolates isolated from coir retting area near Colachel. Bacterial species of *Enterobacter, Klebsiella, Micrococcus, Salmonella* and *Pseudomonas* were isolated and identified. The isolates produced reproducible amplification products which were sufficiently polymorphic to allow differentiation of the strains. Three primers such as OPA01, OPA17, and OPAD08 were used for the RAPD analysis. DNA banding patterns generated by RAPD were scored for the presence (1) or for absence (0) of each amplified band. For genetic distance analysis, using NTSYS software Cluster analysis was based on similarity matrices using the Unweighted Pair Group Method Analysis (UPGMA) program in the software package. The Jaccard coefficient was used for dendrogram construction. A dendrogram based on these results showed a high level of genetic similarity between different bacterial isolates, moderate level of genetic similarity between fungal and actinomycetes isolates and genetic differences were expressed in clusters. The genetic difference between the overall populations was low and showed similarity with each population.

Introduction

Random amplified polymorphic DNA (RAPD) is a convenient and sensitive method of species identification that is finding increasing application in such fields as epidemiology, molecular genetics, microbial ecology, and molecular evolution and taxonomy (Berg *et al.*, 1994). Following purification of genomic DNA, PCR amplification is performed using a short primer of arbitrary sequence. Amplification of a particular genomic sequence depends on the fortuitous nearby occurrence, on opposite DNA strands, of a pair of sequences complementary to the primer. These fragments are resolved by gel electrophoresis, and comparison of the resulting patterns of bands provides information about the relatedness of the

organisms in question. The chief drawback of the RAPD method is its sensitivity to experimental conditions and the consequent variability from run to run, and from lab to lab.

Molecular diversity by PCR based RAPD analysis allows identification and placement of the strains in genetically distinct and related groups (Lee and Henry, 2001). Randomly amplified polymorphic DNA (RAPD) involves the use of random primers in PCR reactions (Williams *et al.,* 1990). It has been used increasingly to distinguish closely related organisms (Bassam *et al.,* 1992; Hadrys *et al.,* 1992) based on polymorphisms in the RAPD product patterns. RAPD technology is very useful, fast, and informative and is unique in that no information concerning specific sequence is needed as random primers are used. Further, the technique gives the opportunity to get information about the biodiversity in a group of isolates (Hansen *et al.,* 1998).

In this context, a study was conducted on the isolation of DNA from the microbial population in the coir retting area near Colachel. Also diversity analysis was determined by PCR based RAPD analysis to understand the genetic variation among organisms.

Materials and Methods

Microbial isolates: A total of 5 bacterial, 3 fungal and 2 actinomycetes isolates were isolated from coir retting yards near Colachel, Kanyakumari district by serial dilution agar plating method.

Identification of bacteria

The identification of bacteria was performed by macroscopic, microscopic and biochemical methods described by Cappucino and Sherman (1998).

DNA preparation

The genomic DNA isolation from microbes was described by the method Murray and Thompson (1980) and Sillhavy *et al.* (1984). CTAB method was the most commonly used protocol for the preparation of genomic DNA. 1.5ml of microbial culture was taken in a micro centrifuge tube and centrifuged for 2 minutes and the supernatant was discarded. The pellet was resuspended in 567µl of TE by repeated pipetting. Then 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase-k was added, mixed thoroughly and incubated at 37^{o} C for 1 hour. 100 µl of 5M NaCl was added and thoroughly mixed. And then 80 µl CTAB/NaCl solutions was added mixed thoroughly and incubated for 10 minutes at 65^{o}C. Equal volume of chloroform was added, mixed thoroughly and spinned for 4 to 5 minutes in a microcentrifuge. This extraction removes CTAB protein/polysaccharide complexes. A white interface should be visible after centrifugation. The aqueous phase with phenol/chloroform was extracted and spinned in a microcentrifuge for 5 minutes. The supernatant was transferred to a fresh tube. 0.6 volume of isopropanol was added for the nucleic acid precipitation. The precipitate was transferred to a fresh tube containing 70% ethanol. The precipitate was centrifuged at 10,000 rpm for 5 minutes at room temperature. The pellet was redissolved in 100 µl TE buffer.

RAPD analyses

Three primers designated OPA01, OPA17 and OPAD08 (Operon Biotechnology) were used (Table 1). RAPD analysis was carried out essentially as described by Brons and Von (2008). A 25µl PCR reaction mixture contained 10X PCR buffer- 1x, 25mM MgCl- 2.5mM, 10mM dNTP mix- 800µM, 25Pico moles Primer- 5 Pico moles, Taq

DNA polymerase- 0.75U, Template DNA- 45ng, Milli Q water- Variable for the PCR amplification of Bacteria. In the PCR amplification of fungi, 25µl PCR reaction mixture contained 10X PCR buffer- 1x, 25mM MgCl- 2.5mM, 10mM dNTP mix- 800µM, 25Pico moles Primer- 0.5 Pico moles, Taq DNA polymerase- 0.5U, Template DNA- 50ng, Milli Q water- Variable. The reaction mixture differ for actinomycetes and it consists of 10X PCR buffer- 1x, 25mM Mgcl- 1.5mM, 10mM dNTP mix- 600µM, 25Pico moles Primer- 2 Pico moles, Taq DNA polymerase- 0.5U, Template DNA- 50ng, Milli Q water- Variable.

After the solutions were mixed, the tubes containing the mixtures were placed in a PTC-100 programmable thermal controller (MJ Research, Inc.). The thermal cycler was programmed for 1 cycle of initial denaturation for 3 minutes at 94°C. This was followed by 40 cycles programmed for denaturation at 94°C for 50 seconds, annealing at 37°C for 1 minute, and extension at 72°C for 1.30 minutes. An additional cycle of at 7 minutes at 72°C was used for final extension followed by 40 cycles of 94°C for 1 min, 30°C for 2 min, and 72°C for 2 min and a final extension period of 72°C for 7 min. Randomly amplified products were analyzed by electrophoresis on a 1.2% agarose gel in Tris-borate-EDTA buffer (0.5MTris, 0.5Mboric acid, 10 mM EDTA [pH 8.0]) and visualized by ethidium bromide staining. The molecular size standards used were ranges from 100bp to 1kb.

Analysis of data from RAPD

DNA banding patterns generated by RAPD were scored for the presence (1) or for absence (0) of each amplified band. All RAPD assays were repeated twice and only the reproducible bands were scored. For considering a marker as polymorphic, the absence of an amplified product in at least one species was used as a criterion. For genetic distance analysis, using NTSYS software Cluster analysis was based on similarity matrices using the unweighted pair group method analysis (UPGMA) program in the software package. The Jaccard coefficient was used for dendrogram construction.

Results and Discussion

Microbial isolates

The microbial isolates were isolated from coir retting area by serial dilution agar plating method. After the incubation, serially diluted agar plated samples showed five different types of bacterial isolates (B1, B2, B3, B4, B5), 3 fungal isolates (F1, F2, F3) and 2 actinomycetes isolates (A1, A2). After the characterization of bacteria, the isolates were identified as *Enterobacter* sp, *Klebsiella* sp, *Micrococcus* sp, *Salmonella* sp and *Pseudomonas* sp based upon its macroscopic, microscopic and biochemical properties on comparison with Bergey's Manual of Determinative Bacteriology (Bergey *et al.*, 1994).

RAPD Analysis in bacteria

RAPD analysis was carried out in five bacterial isolates using 3 random primers for the genomic DNA. High level of genetic similarity was observed between organisms. Three random primers, each with 10 bases generated a total of 15 polymorphic bands out of 41 total bands. In addition to the morphological variations a significant level of (36.58%) polymorphism was observed. The results were listed in Table 2 and Figure 1.

RAPD data analysis of bacteria

Cluster analysis was carried out based on UPGMA Jaccard coefficient. Dendrogram divided into two clusters. Cluster I consist of *Enterobacter sp* (B1), *Pseudomonas sp* (B5) and *Micrococcus sp* (B3). Among this, *Micrococcus sp* (B3) and *Pseudomonas sp* (B5) showed close relationships while *Enterobacter sp* (B1) was related with the above two. The second cluster contains *Klebsiella sp* (B2) and *Salmonella sp* (B4) that showed similarity. The *Klebsiella sp* (B2) differs from the other group. The genetic difference between the overall populations showed low level. Dendrogram revealed that these populations showed close relationship. The result revealed low level of polymorphism among this species. The phylogenetic tree for bacteria was displayed in Figure 2.

RAPD analysis in fungi

RAPD analysis was carried out in fungal isolates F1, F2 and F5 using 3 random primers for the genomic DNA. Moderate level of genetic similarity was observed between organisms. Three random primers, each with 10 bases generated a total of 6 polymorphic bands out of 19 total bands. In addition to the morphological variations a significant level of (31.57%) polymorphism was observed. The results were displayed in Table 3.

RAPD data analysis in fungi

Cluster analysis was carried out based on UPGMA Jaccard coefficient. Dendrogram divided into II clusters. Cluster I contain F1 and F5 showed close relationships and cluster II contains F2. The fungal isolate F2 is distinct from the other two clusters. The genetic difference between the overall populations was low. Dendrogram revealed that these populations showed distinct relationship. The result revealed that low level of polymorphism among this species. The phylogenetic tree for fungi was displayed in Figure 3.

RAPD analysis in actinomycetes

RAPD analysis was carried out in actinomycetes isolates A1 and A2, using 3 random primers for the genomic DNA. Moderate level of genetic similarity was observed between organisms. Three random primers, each with 10 bases generated a total of 3 polymorphic bands out of 7 total bands. In addition to the morphological variations a significant level of (42.58%) polymorphism was observed. The results were listed in Table 4.

RAPD data analysis in actinomycetes

Cluster analysis was carried out based on UPGMA Jaccard coefficient. In this Dendrogram the actinomycetes isolates A1 and A2 showed the close relationship. The genetic difference between the overall populations was moderate level. Dendrogram revealed that these populations showed close relationship. The result revealed that moderate level of polymorphism among this species. The phylogenetic tree for actinomycetes and fungi was displayed in Figure 4.

RAPD analysis was carried out to find distribution of genetic variation between organisms present in coir retting area. The RAPD technique provides a high number of marker loci and high levels of polymorphisms and allows detection of genotypes in a population. Here RAPD analysis was performed in five isolated bacteria, 3 fungal and 2 actinomycetes isolates using three different primers and phylogenetic tree was constructed. The same

type of result was showed in studies of diversity in different organisms (12 strains of *E coli* and four other enteric bacterial species) using RAPD, produced complex of patterns of bands from which inferences about strain and species relatedness can be made. They used 25 Pico mole RAPD analysis primers for RAPD (Berg *et al.*, 1994). RAPD analysis is the most technically simple and often detects variation between isolates that are invariant with RFLP analysis (Pfaller, 1992; Rath, 2001). Although it is advisable that RAPD analysis be made with several primers, the use, only, of one of them with high discriminatory power, may be enough to manage the outbreak, avoiding more assays money and time consuming (Anderson *et al.,* 1996). In conclusion, RAPD analysis is a truly rapid and reliable tool in DNA fingerprinting. Patterns may be easier to repeat and interpret when drastically prolonged ramp times between annealing and extension are used.

Table.1 Oligonucleotides used for the RAPD reactions

Sl. No	Primer	Sequences (5' – 3')	GC content (%)
1	OPA01	CAGGCCCTTC	70.00%
2	OPA 17	GACCGCTTGT	60.00%
3	OPAD08	GGCAGGCAAG	70.00%

Table.2 Genetic diversity analysis using RAPD markers in bacteria

Sl. No	Name of primer (Operon Tech)	Sequence of the primer 5' – 3'	Amplicons size range (bp)	Number of amplified bands	Number of polymorphic bands	Percentage of polymorphism
1	OPA01	CAGGCCCTTC	500-600bp	10	1	10
2	OPA 17	GACCGCTTGT	500-1200bp	16	10	62.5
3	OPAD08	GGCAGGCAAG	500-900bp	15	4	27
			Total	41	15	36.58

Table.3 Genetic diversity analysis using RAPD markers in fungi

Sl. No	Name of primer (Operon Tech)	Sequence of the primer 5' – 3'	Amplicons size range (bp)	Number of amplified bands	Number of polymorphic bands	Percentage of polymorphism
1	OPA01	CAGGCCCTTC	500-630bp	5	1	20
2	OPA 17	GACCGCTTGT	500-900bp	6	3	50
3	OPAD08	GGCAGGCAAG	500-900bp	8	2	25
			Total	19	6	31.57

Table.4 Genetic diversity analysis using RAPD markers in actinomycetes

Sl. No	Name of primer (Operon Tech)	Sequence of the primer 5' – 3'	Amplicons size range(bp)	Number of amplified bands	Number of polymorphic bands	Percentage of polymorphism
1	OPA01	CAGGCCCTTC	550-600bp	2	0	0
2	OPA 17	GACCGCTTGT	450-500bp	2	0	0
3	OPAD08	GGCAGGCAAG	500-900bp	3	3	100
			Total	7	3	42.58

Figure.1 Pattern of bands using primer OPA01, OPA17 AND OPAD08

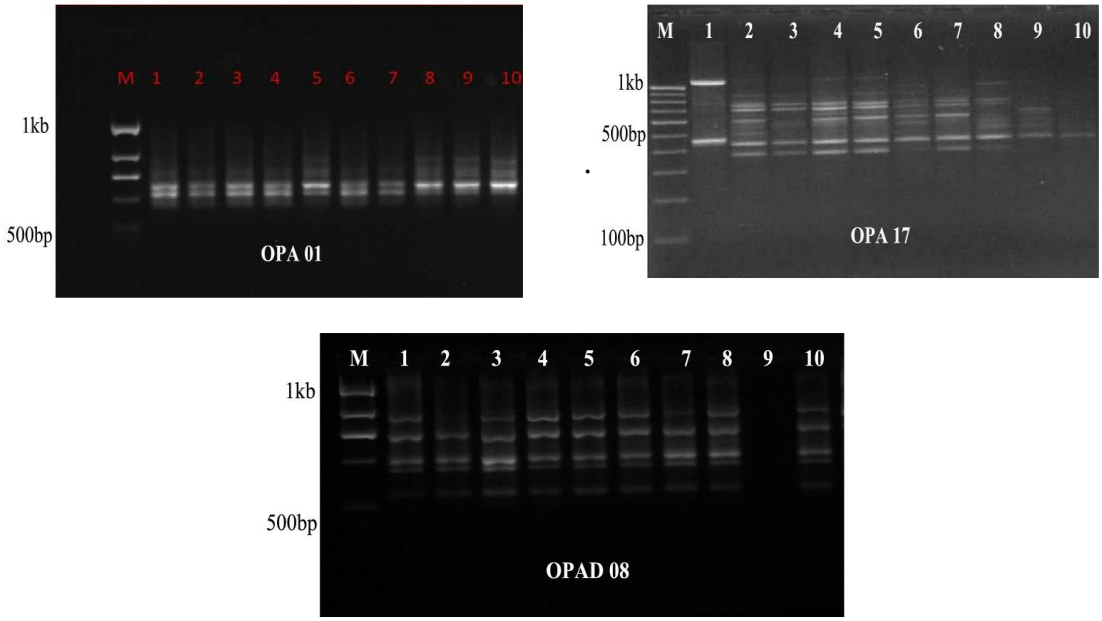
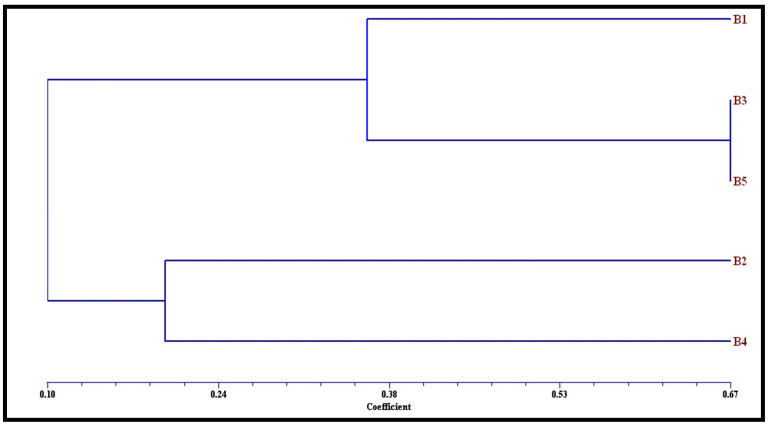
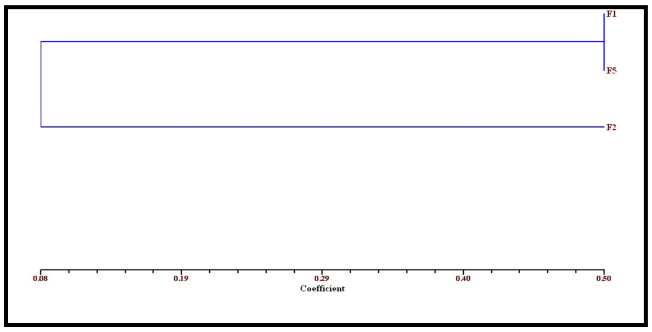


Figure.2 Phylogenetic tree for bacteria



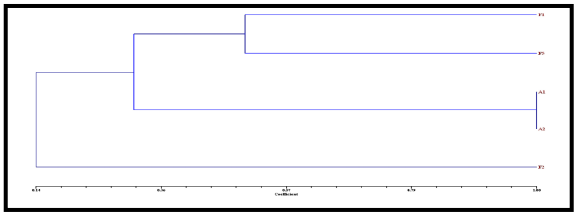
In Figure 2, B1 indicates the *Enterobacter sp*, B2 indicates *Klebsiella sp*, B3 indicates *Micrococcus sp*, B4 indicates *Salmonella sp*, B5 indicates *Pseudomonas sp.*

Figure.3 Phylogenetic tree for fungi



In Figure 3, F1, F2 and F5 indicate the fungal isolates.

Figure.4 Phylogenetic tree for fungi and actinomycetes



In Figure 4, F1, F2, F5 indicate the fungal isolates and A1, A2 indicate the actinomycetes isolates.

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