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RAPD based Molecular Diversity Analysis of Different *Alternaria alternata* (Fr.) Keissler Isolates of Chilli Fruit Rot

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Genetic diversity in chilli fruit rot pathogen (*Alternaria alternata*) was analyzed using eight isolates collected from major chilli growing regions of Gujarat. The genomic DNA extracted from each isolates of *Alternaria alternata* was subjected to polymerase chain reaction using 65 random decamer primers from OPA, OPD and OPF series. Only 10 of the 65 RAPD primers were selected based on repeatability. The 10 earmarked RAPD primers selected from these series amplified 214 DNA fragments with size ranging from 119.93 to 3236.45 bp. Out of these, 98 were polymorphic giving 98.98 per cent polymorphism. The total number of amplified fragments varied from 19 in OPA-3 to 25 in OPA-13. The average polymorphic bands per primer were 9.8 and percent polymorphism ranged from 85.71 in OPF-1 to 100 in rest of the primers. The PIC value varied from 0.746 in OPF-1 to 0.910 in OPA-16. Dendrogram generated by pooled molecular data of 10 RAPD primers formed two clusters namely 'A' and 'B'. The cluster 'A' included Aa-1 and Aa-2 isolates, while the cluster 'B' included Aa-3, Aa-4, Aa-5, Aa-6, Aa-7 and Aa-8 isolates. Thus, the molecular characterization of eight isolates of *A. alternata* by RAPD revealed existence of variations.

Key words: Random Amplification of Polymorphic DNA, Molecular diversity, *Alternaria alternata*, Chilli fruit rot.

Chilli (*Capsicum annum* L.) belongs to family: Solanaceae popularly known as 'Mirchi' is an important spice cum vegetable crop. The crop is vulnerable to many diseases and pests due to its extreme delicacy and succulence. Diseases caused by fungi, bacteria and viruses are major constrains to chilli production. Among the fungal diseases, fruit rot is a major constraint in chilli causing several losses in terms of quality and quantity (Sreekantiah *et al*., 1973). In India, the first report of *Alternaria* sp. was made from Delhi by Dutt in 1937. Narain *et al*. (2000) reported *Alternaria alternata* causing fruit rot of chilli on fruits, as initially small blackish brown, circular to elongated water soaked depressed lesions are formed on the pericarp of fruits, which leads to rotting of fruits in later stage. The characteristic lesions observed at semi ripe stage of chilli fruits. The present study carried out to ascertain molecular polymorphism in different isolates of *Alternaria alternata*.

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MATERIALS AND METHODS

The present investigation entitled RAPD based molecular diversity analysis of different *Alternaria alternata* (Fr.) Keissler isolates of chilli fruit rot was carried out at the Department of Plant Pathology, B. A. College of Agriculture, Anand Agricultural University, Anand.

***Alternaria alternata* isolates**

The experimental materials comprising eight *Alternaria alternata* isolates *viz.*, Anand (Aa-1), Kheda (Aa-2), Bharuch (Aa-3), Navsari (Aa-4), Vadodara (Aa-5), Surat (Aa-6), Ahmedabad (Aa-7) and Rajkot (Aa-8) were collected from different chilli growing area of Gujarat.

Isolation of *Alternaria alternata*

Total eight isolates of *Alternaria alternata* were collected from different parts of Gujarat. The pathogen was isolated by following standard tissue isolation procedure (Tuite, 1969).

DNA isolation

The DNA was extracted from eight isolates of *Alternaria alternata*. Potato dextrose agar medium was used to culture the isolates and mycelia were harvested after 7 days of incubation as described by Coddington and Gould (1992). DNA extraction of the isolates of *A. alternata* and their further molecular characterization were done by Cetyl trimethyl ammonium bromide (CTAB) method with slight modification (Niu *et al.*, 2008), and DNA was amplified with RAPD-PCR technique using random primers.

Genomic DNA extraction from mycelia

Solutions used for DNA isolation

1) DNA extraction buffer
   a) 10% CTAB
   b) 1 M Tris-HCl (pH 8.0)
   c) 0.5 M EDTA (pH 8.0)
   d) 5 M NaCl
   e) Double distilled water
   f) β- mercaptoethanol
2) Chloroform and iso-amyl alcohol (C: I) solution in the ratio of 24:1
3) DNase-free RNase A (10 mg ml^{-1})
4) 70 % Ethanol (Chilled, Absolute)
5) TE buffer (1M Tris-HCl (pH 8.0) and 0.5M EDTA (pH 8.0).

Protocol for isolation of fungal genomic DNA

The fungal tissue was harvested exponentially from liquid medium (Potato dextrose broth) by shaking, for 5-7 days at 26°C. Mycelia were collected by filtration through sterilized filter paper lining a Buchner funnel and then extensively washed with water. The mycelia were blotted dry between the layers of tissue and immediately frozen in liquid nitrogen in foil packets. The tissue was gently broken into fine pieces by crushing the foil envelops with a pestle and mortar. Care was taken not to allow the tissue to thaw as this causes lyses and the release of endogenous nuclease.

Protocol for DNA extraction

1. Pestle and mortar were pre-cooled at 4°C, and then 300 mg of mycelium was grounded to a fine powder in liquid nitrogen and transferred to a plastic sterilized tube by ensuring that the tissue did not thaw.
2. Ten ml of pre-thawed isolation buffer was added to it, and then incubated for 60 minutes with occasional stirring.
3. Extraction was carried out for 10 minutes with equal volume of chloroform: isoamylalcohal (24:1).
4. Centrifugation was carried out at 10,000 rpm for 20 minutes at room temperature (24°C).
5. Aqueous phase was separated and transferred to a fresh tube.
6. To this aqueous phase, 0.6 ml of ice-cold isopropanol and 0.1 ml of sodium acetate were added, and then incubated at 20° C for 30 minutes.
7. Centrifugation was carried out at 10,000 rpm for 10 minutes at 4°C, and then the supernatant was collected, whereas the aqueous phase was discarded.
8. Again, wash was given and the DNA pellet was obtained with 70 per cent ethanol (5 ml).
9. Centrifugation was carried out at 10,000 rpm for 10 minutes at 4°C and then aqueous phase was discarded.
10. DNA pellet was dried, and then dissolved in 200 µl of TE buffer.
11. Then, RNase treatment was given to the samples by adding 6 µl of RNase, and then samples were kept at 37^0C in hot water bath for 1 h and latter at 65^0C for 10 minutes.

Spectrophotometric analysis (Nanodrop)

To check the quality and quantity of isolated genomic DNA in relation to protein and RNA spectroscopic analysis on nanodrop was carried out. The data were analyzed by using the software N.D. (V.3.3.0). One µl DNA was loaded into the well of nanodrop spectrophotometer and the concentration of DNA and absorbance at 260 nm /280 nm was measured. Quantification and purity of DNA was checked on 0.8% agarose gel at voltage of 60V/cm by using 1X TBE buffer and ethidium bromide (4 µl/70 ml) staining. On

completion of run, gel was viewed under UV light and observations were recorded on Alpha EaseFC4.0.0 gel documentation system.

Random Amplification of Polymorphic DNA analysis

A total of 65-decamer primer belonging to OPA, OPD and OPF series (Operon Technologies, USA) were screened for RAPD analysis. Of these, 10 primers were selected based on repeatability. The list of RAPD primers were used for the analysis of random amplification of polymorphic DNA (Table 1) to study the polymorphism present in the isolates of *A. alternata*.

PCR Protocol

Preparation of Reaction Mixture

The reaction mixture for RAPD analysis was prepared as under:

Millipore sterilized water	10.45 µl
Taq Buffer A (10X)	1.5 µl
dNTPs (2.5mM each) mix	0.4 µl
Taq polymerase (3U/µl)	0.15 µl
Primer (10 pmoles/µl)	1.0 µl
Template DNA (40 ng/µl)	1.5 µl
Total	15 µl

The master mix was prepared by adding initially millipore sterilized water followed by Taq buffer A 1.5 µl, 0.4 µl dNTPs, 0.15 µl Taq polymerase, 1 µl primer and finally 1.5 µl template DNA was added. The reagents were mixed thoroughly by slight vortex followed by spinning in microcentrifuge. The tubes were then placed in thermal cycler for cyclic amplification. The conditions for amplification were as follows:

Programming for PCR

Step 1 : 94°C for 7 min. (Initial denaturation)
Step 2 : 94°C for 1 min. (Denaturation after every cycle)
Step 3 : 38°C for 1 min. (Primer annealing)
Step 4 : 72°C for 1.20 min. (Extension of annealed primer)
Step 5 : 72°C for 7 min. (Final Extension) Step 2, 3 and 4 comprised of one cycle and the total reaction was carried out for 40 cycles.

PCR products were run on electrophoresis with known molecular marker (mol wt. 100 bp) in 1.5% Agarose gel.

Data scoring

Data was scored on the basis of presence (1) or absence (0) for analysis. If a product was present in an isolate, it was considered as '1' and if absent, was considered as '0'. The data were maintained in the MS-Excel sheet format for further analysis. The polymorphism percentage was calculated as per the method suggested by Blair *et al.* (1999).

$$\text{Polymorphism}\,(\%) = \frac{\text{Total number of bands - Number of monomorphic bands}}{\text{Total number of bands}} \times 100$$

The molecular weights of the PCR products were estimated by Alpha Ease FC (V.4.0.0) software (Alpha Innotech Corporation, USA) for each primer to analyzed alleles range.

Data analysis

The data generated by RAPD were analyzed with the software NTSYS (Numerical Taxonomy and Multivariate Analysis System). Diploid data analysis for dominant marker was performed with the assumption of Hardy-Weinberg equilibrium. Multiple populations were used for the estimation of effective allele number, polymorphic loci, Jaccard's similarity coefficient measures of genetic identity and genetic distance as well as for dendrogram construction.

Dendrogram

A dendrogram drawn, based on Jaccard's similarity coefficient (1908) by UPGMA (Unweighted Pair Group Method of Arithmetic averages) method. This program is an adoption of program NEIGHBOR of PHYLIP version 3.5c by Joe F elsenstein. The drawing was executed for multiple populations.

RESULTS AND DISCUSSION

Assay of DNA from the isolates of *A. alternata* obtained through nanodrop technique

The DNA isolated from eight isolates of *A. alternata* was analyzed by Nanodrop Spectrophotometry. The results of absorbance ratio of DNA at 260/280 nm wavelength and concentration of DNA (ng/µl) are presented in Table 2.

Random Amplification of Polymorphic DNA study

A total of 65-decamer primer belonging to OPA, OPD and OPF series were screened for RAPD analysis. From these, 10 primers were selected based on repeatability. The fragment sizes were detected by comparing the amplicons with a 100 bp DNA Ladder. A common dendrogram was generated using the NTSYS-pc software version

2.2 (Exeter Software, New York, USA). Result is collectively discussed for all the10 primers.

Pooled RAPD

Dendrogram (Fig.1) based on Jaccard's similarity coefficient (1908) (Table 3) by "UPGMA method" formed two clusters namely 'A' and 'B'. The cluster 'A' consisted Aa-1 and Aa-2 isolates. The cluster 'B' consisted Aa-3, Aa-4, Aa-5, Aa-6, Aa-7 and Aa-8 isolates. Cluster 'A' was further subdivided into two clusters *i.e.* A_1 and A_2 that includes the isolates Anand (Aa-1) and Kheda (Aa-2), respectively. The cluster 'B' was subdivided into two clusters *viz.*, B_1 and B_2. The cluster B_1 was further subdivided into two sub-clusters namely B_1a and B_1b. The cluster B_1a was further subdivided into two sub clusters B_1aa and B_1ba, which included Bharuch (Aa-3) and Surat (Aa-6) isolates, respectively. Simultaneously, the cluster B_1b was further subdivided into two sub-clusters namely B_1ab, which included Navsari isolate (Aa-4) and B_1bb that was further subdivided into two sub clusters namely B_1abb and B_1bbb that included Ahmedabad (Aa-7) and Rajkot (Aa-8) isolates, respectively. The cluster B_2 included Vadodara isolate (Aa-5).

Table 1. Primers used for the RAPD analysis

S. No.	Primer	Sequence (5'-3')	Base pair
1	OPA-3	AGT CAG CCA C	10
2	OPA-4	AAT CGG GCT G	10
3	OPA-13	CAG CAC CCA C	10
4	OPA-16	AGC CAG CGA A	10
5	OPA-20	GTT GCG ATC C	10
6	OPD-2	GGA CCC AAC C	10
7	OPD-3	GTC GCC GTC A	10
8	OPF-1	ACG GAT CCT G	10
9	OPF-3	CCT GAT CAC C	10
10	OPF-14	TGC TGC AGG T	10
Total			1000

The RAPD analysis of the eight isolates of *A. alternata* collected from major chilli growing regions of Gujarat and analyzed by 10 random primers produced 99 loci. Out of which, 98 loci were polymorphic. The selected random primers produced as much as 211 bands. On an average, 98.98 per cent polymorphism was observed. However, average numbers of bands and per primer were 21.1. Moreover, average numbers of polymorphic loci obtained per primer (Assay

Table 2. Assay of DNA from the *A. alternata* isolates obtained through nanodrop technique

S. No.	Designation of Isolates	Location of isolates	District	Wavelength Ratio (260/280) nm	Concentration (ng/µl)
1	Aa-1	Anand	Anand	1.96	861.9
2	Aa-2	Kheda	Kheda	1.95	617.3
3	Aa-3	Bharuch	Bharuch	1.72	149.4
4	Aa-4	Navsari	Navsari	2.00	305.0
5	Aa-5	Vadodara	Vadodara	2.11	831.2
6	Aa-6	Surat	Surat	1.95	669.3
7	Aa-7	Ahmedabad	Ahmedabad	2.06	370.8
8	Aa-8	Rajkot	Rajkot	1.91	196.3

Table 3. Genetic similarity matrix of pooled RAPD data based on Jaccard's similarity coefficient

Isolates	Aa-1	Aa-2	Aa-3	Aa-4	Aa-5	Aa-6	Aa-7	Aa-8
Aa-1	1.0000							
Aa-2	0.3143	1.0000						
Aa-3	0.2000	0.1364	1.0000					
Aa-4	0.1556	0.1163	0.1667	1.0000				
Aa-5	0.1064	0.1707	0.1200	0.1489	1.0000			
Aa-6	0.0833	0.1163	0.3023	0.1489	0.1739	1.0000		
Aa-7	0.1277	0.0652	0.1875	0.1702	0.1458	0.2500	1.0000	
Aa-8	0.0612	0.0909	0.1200	0.2273	0.1250	0.1489	0.2791	1.0000

Efficiency Index) were 9.8. As much as nine primers out of the selected 10 primers *viz.*, OPA 3, OPA 4, OPA 13, OPA 16, OPA 20, OPD 2, OPD 3, OPF 3 and OPF 14 showed cent percent polymorphism. The lowest polymorphism (85.71%) was found in the primer OPF1. The molecular weight of the amplicons ranged from 119.93 to 3236.45 bp (Table 4).

The maximum similarity index value of 0.3143 was observed between the isolates Kheda (Aa-2) and Anand (Aa-1), both of which are from Middle Gujarat region. The least similarity index value of 0.0612 was found between the isolates Rajkot (Aa-8) and Anand (Aa-1). The average similarity coefficient among isolates was 0.346 (Table 3).

The efficacy of the RAPD primers selected in distinguishing the isolates of *A. alternata* was apparent by the fact that out of 10 primers selected as many as nine generated amplicons with 100% polymorphism (Table 4). Further, the representative RAPD profiles presented in Plate 1-10 indicated the extent of genetic differences prevailing among the isolates of *A. alternata*.

In the present study a total of 65 primers comprising of OPA, OPD and OPF series were screened against isolates of *A. alternata.* Out of these, ten primers were found useful for amplification of DNA of *A. alternata.* Among the 10 primers, all primer gave 100% polymorphism of the DNA, which helped to ascertain variability except, OPF-1 primer having 85.71% polymorphism. Therefore, these primers would be very useful for ascertaining variability among the population of other species of *Alternaria*.

In consonance to the present findings, Varma *et al*. (2007) and Kumar *et al*. (2008) have also studied on RAPD marker against *A. solani* by using different primers. In the present study, a

Table 4. DNA banding profile of 10 primers in eight isolates of *A. alternata*

S. No.	Primer	Molecular weight range (bp)	Total bands	No. of loci	No. of polymorphic loci	Polymor-phism (%)	PIC* value
1	OPA 3	165.87-1615.57	19	9	9	100	0.853
2	OPA 4	282.40-1995.40	20	7	7	100	0.840
3	OPA 13	129.82-2950.19	25	10	10	100	0.883
4	OPA 16	119.93-3236.45	20	12	12	100	0.910
5	OPA 20	135.24-2055.56	23	12	12	100	0.903
6	OPD 2	336.10-2718.50	20	11	11	100	0.855
7	OPD 3	228.18-2979.18	23	12	12	100	0.877
8	OPF 1	337.77-2636.75	21	7	6	85.71	0.746
9	OPF 3	327.47-3049.89	21	11	11	100	0.888
10	OPF 14	161.65-1846.09	22	8	8	100	0.834
Total	-	-	214	99	98	-	-
Average	-	-	21.4	9.9	9.8	98.98	0.859

*PIC= Polymorphic Information Content

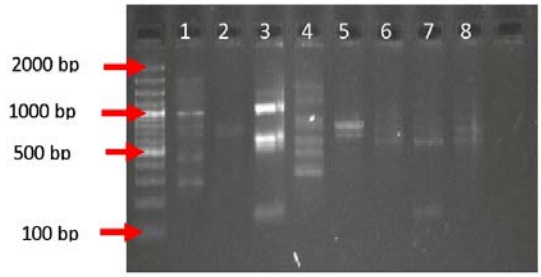


Plate 1. RAPD profile of OPA 3

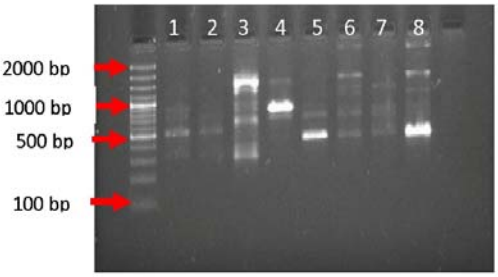


Plate 2. RAPD profile of OPA 4

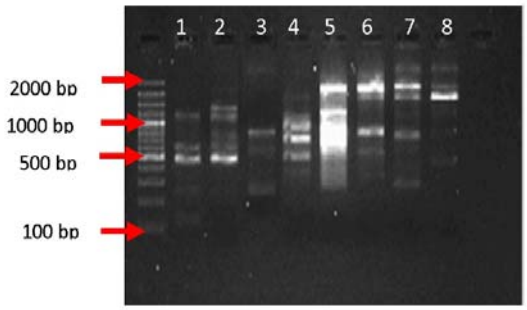


Plate 3. RAPD profile of OPA 13

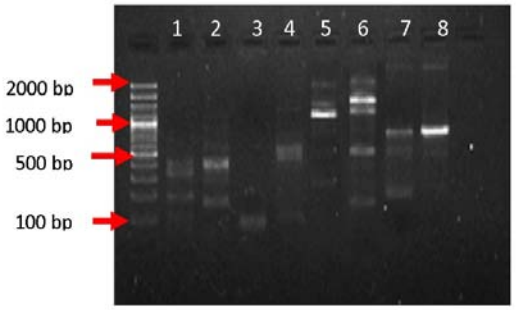
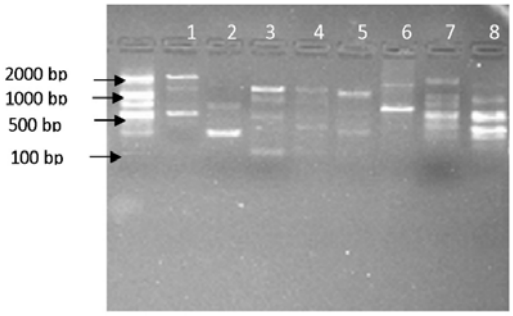


Plate 4. RAPD profile of OPA 16



M= 100 + 800 kbp DNa ladder; 1= Aa-1; 2= Aa-2; 3= Aa-3; 4= Aa-4; 8= Aa-8; 6= Aa-6; 7= Aa-7; 8=Aa-8 isolate

Plate 5. RAPD profile of OPA 20

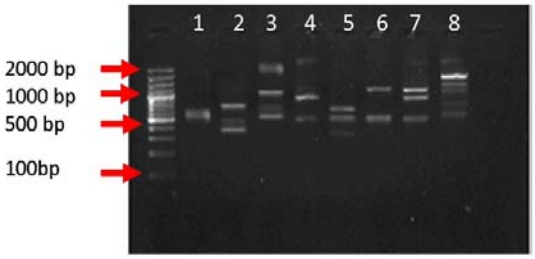


Plate 6. RAPD profile of OPD 2

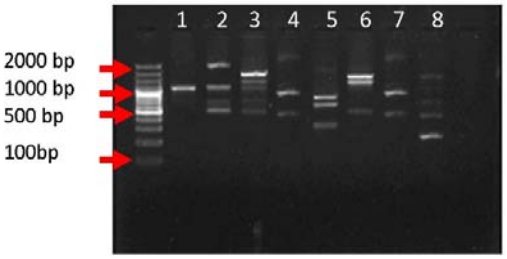


Plate 7. RAPD profile of OPD 3

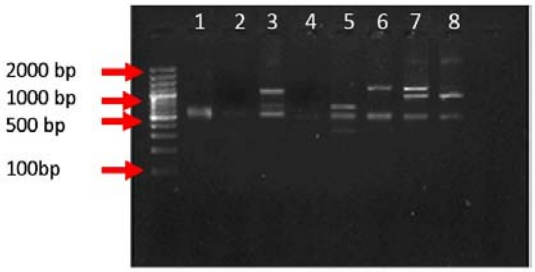


Plate 8. RAPD profile of OPF 1

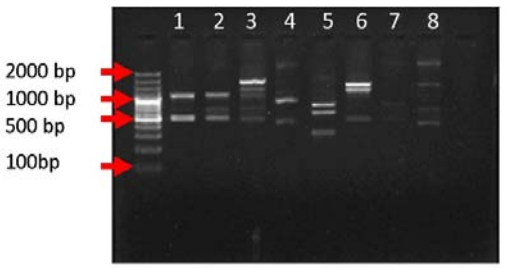
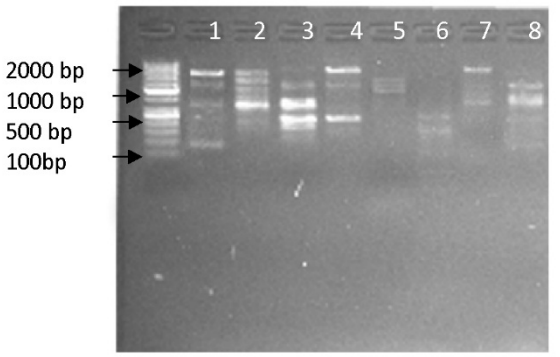


Plate 9. RAPD profile of OPF 3



M= 100 + 800 kbp DNa ladder; 1= Aa-1; 2= Aa-2; 3= Aa-3; 4= Aa-4; 8= Aa-8; 6= Aa-6; 7= Aa-7; 8=Aa-8 isolate

Plate 10. RAPD profile of OPF 14

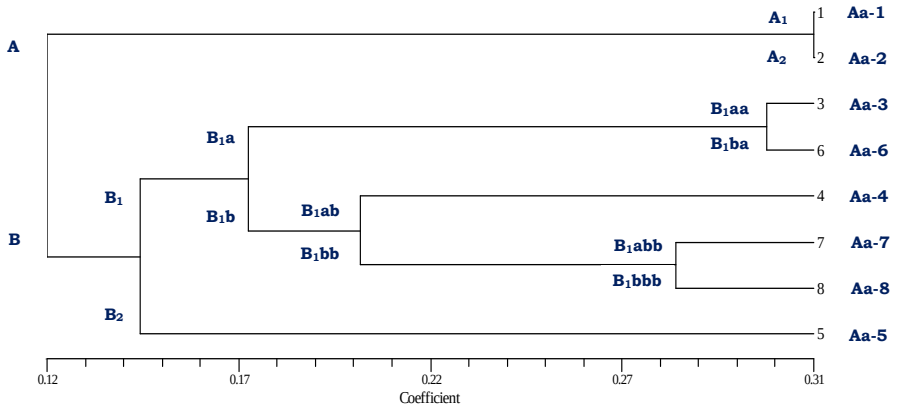


Fig. 1. Dendrogram of eight isolates of *A. alternata* Jaccard's similarity coefficient (1908) using UPGMA as the clustering method for RAPD

sizeable number of OPA, OPD and OPF primers were screened to find out the variability. The present results are in agreement with above workers.

The primers identified here might be useful in distinguish the isolates of *A. alternata* and in their characterization and such molecular profiles may prove to be ideal for identification of the virulent strains when a large-scale study is undertaken.

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