

## **Application of the Randomly Amplified Polymorphic DNA (RAPD) Markers to Analyze the Genetic Variability in Species of the Fungus *Alternaria***

**Sahi J. Dhahi**  
*Department of Biology*  
*College of Science*  
*Mosul University*

**Akeel H. Al-Assie**  
*Department of Biology*  
*College of Science*  
*Tikrit University*

**Hadeel A. Omear**  
*Department of Biology*  
*College of Science*  
*Tikrit University*

(Received 22 / 2 / 2010 ; Accepted 20 / 9 / 2010 )

### **ABSTRACT**

The PCR-based technique of randomly amplified polymorphic DNA (RAPD) was used to fingerprint and assess the genetic relatedness among nine species of the fungus *Alternaria* isolated from various crop plants showing the leaf spot disease in Mosul, Iraq. Genomic DNA of each species was extracted at a final concentration of 300 - 400 µg / 2-3 g of wet mycelium , and at a purity of 1.6-1.8. Each DNA sample was amplified with each of 22 primers and the products were resolved electrophoretically on 1.2% agarose gel, stained with ethidium bromide and photographed under UV. One Primer failed to support amplification but the remaining 21 (95.5%) primers produced a total of 112 bands (2-10 per primer) across the nine species. Of these bands, 100 (1-10 per primer ) were polymorphic. The least efficient primer was OP-H01 (1.79%), while the most efficient one was OP-M05 (8.93 %). Primers OP-C05, OP-E20 and OP-T20 had the lowest (0.1%) discriminatory power while primer OP-M05 had the highest (10 %) power and identified all 9 species through unique patterns of banding. RAPD analysis fingerprinted eight of the nine isolates through marker bands with one or more of the 21 primers. Cluster analysis based on the genetic distances split the nine species into three distinct clusters with no obvious association between the pattern of clustering of the species and their host specificities.

**Keywords :** *Alternaria* , Genetic variability , RAPD , PCR

**(RAPD)**

***Alternaria***

PCR

RAPD

*Alternaria*

400-300

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relies on using short primers ( $\approx 10$  bases) with random sequences and less stringent demands for pairing that allows amplification of DNA polymorphisms randomly distributed all over the genome and giving varied patterns of banding on gels that can be used for distinction between species or linkage mapping (Williams *et al.*, 1990).

In the present work, an attempt was made to use the RAPD technique for checking the relatedness among nine fungal species of the genus *Alternaria* which was earlier deduced from phenetic and numerical taxonomic methods using both phenotypic and chemical characteristics (Al-Tae, 2007).

## MATERIAL AND METHODS

### Fungal isolates

A total of nine species of the genus *Alternaria* was used in the present work. The origins and other specifications of the isolates are given in Table 1. They were kindly provided by Dr. Warka S. Al-Tae, Biology Department, College of Science, Mosul University, Iraq. They were subpopulation of a larger collection of species of this genus whose details are given in Al-Tae (2007).

Table 1: Origins of the *Alternaria* species used in the present work (Al-Tae, 2007).

Numbering in the present work	Species	Corresponding No. in Al-Tae (2007)	Source of isolation (Host) (Al-Tae, 2007)
1.	<i>Alternaria radicina</i>	10	<i>Vicia faba</i>
2.	<i>A. state</i>	8	<i>Brassica oleracea</i>
3	<i>A. raphani</i>	13	<i>Solanum tuberosum</i>
4.	<i>A. tenuissima</i>	2	<i>Vigna sinensis</i>
5.	<i>A. alternata1</i>	6	<i>Beta vulgaris var cila</i>
6.	<i>A. longipes</i>	11	<i>Brassica oleracea</i>
7.	<i>A. brassicae</i>	5	<i>Raphanus sativus</i>
8.	<i>A. alternaria2</i>	15	<i>Brassica repa</i>
9.	<i>A. cheiranthi</i>	1	<i>Beta vulgaris var vulgaris</i>

### Culture media and culturing the fungal isolates

The potato sucrose agar (PSA) medium described by Dhingra and Sinclair (1983) was used. All species were subcultured on PSA plates, single spored, put on PSA slants and stored at 4°C until they were used. To grow mycelium, PS broth (PSA lacking agar) was prepared and distributed into 200ml flasks (50 ml each). To induce vigorous mycelial growth and substitute for agitation (as no shaking incubator was available at the time), 5g of yeast extract/L were included in the PS broth before distribution and autoclaving. After cooling, each flask was inoculated with a single colony grown for three days on a PSA plate. Cultures were incubated statically for 7-10 days at 24°C.

### **DNA extraction**

DNA was extracted from the mycelia using the cetyltrimethyl ammonium bromide (CTAB) method described by Weigand *et al.*, (1993). The concentration and purity of the extracted genomic DNA were determined spectrophotometrically as described by Sambrook *et al.*, (1989). Readings were taken at 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of DNA in the sample. An OD of 1 corresponds to approximately 50 µg/ml for double-stranded DNA. Therefore, if a 100 fold diluted sample of the extract was used the concentration of the original sample will be (µg/ml) = OD at 260 nm × dilution factor (100) × 50 (µg/ml).

The ratio between the readings at 260 nm and 280 nm (OD 260 nm / OD 280 nm) provides an estimate for the purity of the DNA. Pure preparations of DNA have OD 260 nm / OD 280 nm of 1.8.

### **Determination of the molecular size of the DNA fragments (bands)**

The molecular size the genomic DNA samples was determined by running them on a 1% agarose gel and comparing with undigested λ DNA sample (Promega, USA) run in a separate lane on the same gel. The molecular sizes of the DNA fragments amplified by the PCR with each primer were determined by calibration to a curve made for a λ DNA sample simultaneously digested with *EcoRI* and *HindIII* restriction enzymes (Promega, USA) (Sambrook *et al.*, 1989). The digest contained fragments with known molecular sizes and was run in a separate lane on a 1.2 % agarose gel having a control lane and 9 other lanes for the 9 PCR products of the fungal species with each primer. The λ DNA digest produced 12 DNA size markers. They were, from well downwards; 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 974, 831, and 564 bp.

### **The RAPD amplification conditions**

The RAPD reactions were done for the nine fungal genomic DNA samples using 22 primers whose numbers and sequences are shown in Table 2. These are a random set of a larger standard set of 520 random primers (OP-A01 – OP-Z20) commercially available (Operon Technology, USA). All of these primers are 10-mers oligonucleotides with 60-70 percent GC content and are used to all types of organisms irrespective of the GC content of the DNA amplified. As these primers are randomly designed and not complementary to specific sequences in the amplifies genome, the high GC content is essential for the non-stringent initial annealing of the primers to their target genomic DNA (Newton and Graham, 1997; Rafalski, 1997). If the RAPD markers are used to detect polymorphism and genetic relationships, as in the present work, a large number of the primers is recommended as many primers could produce monomorphic bands across the species tested (Rafalski, 1997; Tahir, 2008).

The reaction conditions were as described by Williams *et al.*, (1990). The concentration of the genomic DNA in all samples was adjusted with TE buffer to be 25-50 ng/µl. Amplification reactions were performed in volumes of 25 µl containing the components shown in Table 3.

Table 2: Numbers and sequences of the RAPD primers used

Primer	Sequence
OP-C05	5'GATGACCGCC <sup>3'</sup>
OP-E20	5'AACGGTGACC <sup>3'</sup>
OP-H01	5'GGTCGGAGAA <sup>3'</sup>
OP-I02	5'GGAGGAGAGG <sup>3'</sup>
OP-K01	5'CATTGAGGCC <sup>3'</sup>
OP-L05	5'ACGCAGGCAC <sup>3'</sup>
OP-L20	5'TGGTGGACCA <sup>3'</sup>
OP-M05	5'GGGAACGTGT <sup>3'</sup>
OP-M06	5'CTGGGCAACT <sup>3'</sup>
OP-M14	5'AGGGTCGTTT <sup>3'</sup>
OP-M20	5'AGGTCTTGGG <sup>3'</sup>
OP-N16	5'AAGCGACCTG <sup>3'</sup>
Op-P04	5'GTGTCTCAGG <sup>3'</sup>
OP-R06	5'GTCTACGGCA <sup>3'</sup>
OP-R12	5'ACAGGTGCGT <sup>3'</sup>
OP-R14	5'CAGGATTCCC <sup>3'</sup>
OP-T19	5'GTCCGTATGG <sup>3'</sup>
OP-T20	5'GACCAATGCC <sup>3'</sup>
OP-V02	5'AGTCACTCCC <sup>3'</sup>
OP-V09	5'TGTACCCGTC <sup>3'</sup>
OP-V14	5'AGATCCCGCC <sup>3'</sup>
OP-V19	5'GGGTGTGCAG <sup>3'</sup>

Table 3: Compositions of the PCR reaction master mix.

Reagent*	Final concentration	Amount per one reaction ( $\mu\text{l}$ )	Master mix (x10) ( $\mu\text{l}$ )
PCR buffer (x10)	1x	2.5	25
dNTPs	200 $\mu\text{m}$	2.5	25
Primer	10 picomole	2	20
dH <sub>2</sub> O	—	15.8	158
<i>Taq</i> polymerase (5U/ $\mu\text{l}$ )	1 U.	0.2	2
Total		23	230
An aliquot of 23 $\mu\text{l}$ from the master mix was pipetted into each of 9 thin-walled eppendorf tubes			
Genomic DNA sample	25-50 ng/ml	2	
Final volume	25		

\* The reagents should be added in the order listed in the table.

Due to the small amounts of reagents used per individual reactions and to ensure accuracy, a master reaction mixture enough for 10 reactions (the nine genomic DNAs plus one spare reaction) was prepared as in Table 3. Amplification was performed in a Hybrid (USA) DNA Thermal Cycler programmed for a period of 4 minutes at 94°C for initial strand separation followed by 40 cycles of 30 sec at 94°C, 45 sec at 36°C to allow primer annealing, 45 sec at 72°C for primer extension and a final period of 7 minutes at 72°C for extension completion. Amplification products were analyzed by electrophoresis in 1.2 % agarose, detected by staining with ethidium bromide, and photographed under UV.

### **Analysis of the amplified DNA polymorphisms**

The banding patterns on the gels were transformed into tables of binary characters where the appearance of a band was given the number one (1) while the absence of the band was denoted by zero (0). The tables were used to (i) determine the total number of bands together with their molecular weights that were produced by a primer across all isolates starting from the lowest weight (bottom of the gel) to the highest weight (top of the gel) (Cao *et al.*, 1999) (ii) to determine the monomorphic or common bands (appearing in all isolates) and polymorphic bands (appearing in some isolates only) amplified by a primer (iii) to determine the % efficiency of a primer. This was estimated as a percentage of the total number of bands amplified by the primer out of the total number of bands amplified by all primers across all species. This represents the availability of sequences complementary to the primer in the genome (iv) to determine the discriminatory power of the primer (Grundman *et al.*, 1995). This is a percentage of the polymorphic bands amplified by a primer out of the total number of polymorphic bands given by all primers in all isolates (v) to determine the number of patterns of banding produced by a primer across all isolates amplified by this primer. This will be used to identify an isolate : (a) Through the presence of a unique pattern of banding (number of bands and their molecular weights) that distinguishes it from other isolates amplified by the same primer (Zaid *et al.*, 1999). (b) To fingerprint it through the absence of a common band that is present in all other isolates amplified by the primer. Or, through a unique or specific band (s) or marker (s) present in this isolate and not in other isolates when amplified by the same primer. This specific marker or band can be useful in two ways ; first it can be scraped from the gel and re-amplified with the same primer but in the presence of a labeled dNTP to produce labeled probes that can be used for hybridization and detection of any Restriction Fragment Length Polymorphism (RFLP) that can be produced by any restriction enzyme within the original specific RAPD fragment (band) (Williams *et al.*, 1990). Second, the specific RAPD bands or markers can be sequenced and used to prepare primers that are specific for this marker that can be used to specifically identify the isolate producing these specific markers (Al-aaraji, 2003). (vi) The tables of binary characters can be used to determine the genetic distances between the various species. To this end the tables of binary character were fed into the computer program Similarity for Qualitative Data (SIMQUAL).

Calculation of the genetic distance (GD) between isolate (i) and isolate (j) is based on the equation of Nei and Li (1979) where

$$GD = 1 - [2 * N_{ij} / (N_i + N_j)]$$

Where

$N_{ij}$  = number of bands common to both the i and the j isolates .

$N_i$  = Total number of bands appearing in i .

$N_j$  = Total number of bands appearing in j .

The resulting matrix of the genetic distances is used to draw the clustering of the isolates within a dendrogram depending on the genetic closeness of members of each cluster. This was achieved by feeding the data of the matrix in to the Sequential Agglomerative Hierarchical and Nested Clustering (Sneath and Sokal, 1973) which depends on the Unweighted Pair-Group Method with Arithmetic Averages (UPGMA).

All these were with the statistical package : Numerical Taxonomy and Multivariate Analysis system NTSYS-PC version 1.80 (Rohlf, 1993).

## RESULT AND DISCUSSION

The method of Weigand *et al.*, (1995) for extracting DNA from the fungal mycelia proved sufficient in the present work where genomic DNA was obtained at a concentration of 300-500  $\mu\text{g}/\mu\text{l}$  for each 2-3 g of mycelium sample. The purity of the DNA preparations (as determined by spectrophotometer reading at 260nm and 280nm) ranged between 1.8-2.0. The average size of the genomic DNA as deduced from electrophoresis on 1.2% agarose gel was about 50kb as judged from comparison with an undigested  $\lambda$  DNA sample run on the same gel (Fig. 1.a).

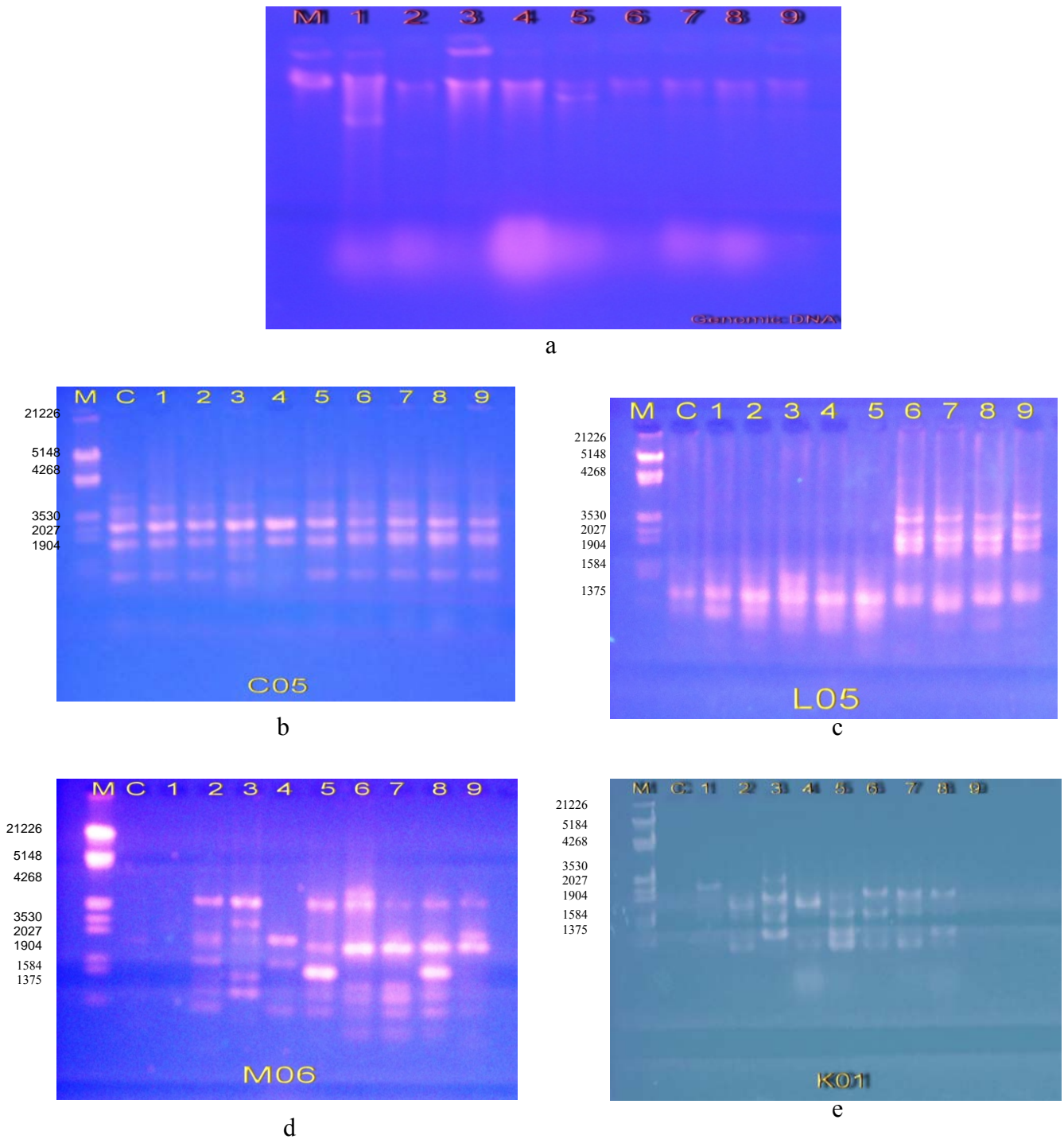


Fig. 1: Electrophoresis gels( 1% agarose) of the genomic DNA (a) and (1.2% agarose) of its PCR products with four representative RAPD (OP-C05, OP-L05,OP-M06 and OP-K01) primers (b-e). Lane M (marker) contains intact  $\lambda$  DNA in (a) and  $\lambda$  DNA digested with *EcoRI* and *HindIII* (fragment sizes in bp are shown) in b-e. Lane C (control) contains the master mix without genomic DNA. Other lanes contain genomic (a) and the PCR products (b-e) with genomic DNA from 1-*A.radicina*, 2-*A.state*, 3-*A.raphani*, 4-*A.tenuissima* , 5-*A.alternata 1*, 6-*A.longipes* , 7-*A.brassicae*, 8-*A.alternata 2*, 9-*A.cheiranthi* ; monomorphic bands with OP-C05 ; two monomorphic groups with OP-L05 and polymorphic bands with OP-M06 and OP-K01.



The concentration of genomic DNA was 25-50 ng/reaction. The condition for RAPD reaction set by Williams *et al.*, (1993) were quite satisfactory and all but one primer gave amplified fragments in the present work. The single primer that gave no amplified fragments, despite several repeats, was OP-V02. This might be attributed to lack of complementary sequences to this primer among the genomic DNA from the 9 fungal species (Devos and Gale, 1992).

The remaining 21 (95.5%) primers all gave amplified bands (representative of these PCR products are shown in Fig1). Primers OP-C05 (Fig1.b), OP-E20 and OP-T20 all gave monomorphic bands except one polymorphic band per each primer (Table 4). Primers OP-L05 (Fig1.c) and OP-N16, on the other hand, each split the PCR products of the nine fungal species in to two monomorphic groups. It's to be noticed in (Fig1.a and b) that there are spillages, during loading, of the PCR products from lane 1 to the control (lane c) as can be judged from the identical patterns of banding in lane 1 and lane c in both cases. The remaining 16 primers gave variable patterns of polymorphic and monomorphic bandings across the nine spices (e.g. Fig. 1.d and e).

All in all, the 21 primers gave amplified fragments totalling at 112 and ranging between 2 (in OP-H01) and 10 (in OP-M05) across the 9 fungal species with a corresponding primer efficiency of amplification ranging between 1.79% (OP-H01) and 8.93% (OP-M05) (Table 4). High efficiency of a primer is indicative of a large area of the genome that complements and allows base pairing between the primer and the genomic DNA (Karp and Edwards, 1997). Moreover it has been suggested (Fristch *et al.*, 1993) that high efficiency positively correlates with the high GC content of the primer. This suggestion, however, is not supported by results of the present work as primer OP-C05(with70% GC) and primer OP-E20 ( with 60% GC ) have equivalent efficiencies (3.57% each ) (Table 4).

Table 4: Fragments amplified by the 21 primers in the 9 fungal species and the % efficiency of amplification and discriminatory power of each primer.

primer	Sequence	No. of bands amplified in all 9 species		Primer efficiency (%)	Primer discriminatory power (%)
		Total	polymorphic		
OP-C05	5'GATGACCGCC3'	4	1	3.57	1.0
OP-E20	5'AACGGTGACC3'	4	1	3.57	1.0
OP-H01	5'GGTCGGAGAA3'	2	2	1.79	2.0
OP-I02	5'GGAGGAGAGG3'	6	6	5.36	6.0
OP-K01	5'CATTTCGAGCC3'	8	8	7.14	8.0
OP-L05	5'ACGCAGGCAC3'	7	6	6.25	6.0
OP-L20	5'TGGTGGACCA3'	9	9	8.04	9.0
OP-M05	5'GGGAACGTGT3'	10	10	8.93	10.0
OP-M06	5'CTGGGCAACT3'	9	9	8.04	9.0
OP-M14	5'AGGGTCGTTC3'	7	7	6.25	7.0
OP-M20	5'AGGTCTTGGG3'	3	3	2.68	3.0
OP-N16	5'AAGCGACCTG3'	6	6	5.36	6.0
Op-P04	5'GTGTCTCAGG3'	5	5	4.46	5.0
OP-R06	5'GTCTACGGCA3'	3	3	2.68	3.0
OP-R12	5'ACAGGTGCGT3'	5	5	4.46	5.0
OP-R14	5'CAGGATCCCC3'	4	3	3.57	3.0
OP-T19	5'GTCCGTATGG3'	4	4	3.57	4.0
OP-T20	5'GACCAATGCC3'	3	1	2.68	1.0
OP-V09	5'TGTACCCGTC3'	3	3	2.68	3.0
OP-V14	5'AGATCCCGCC3'	4	3	3.57	3.0
OP-V19	5'GGGTGTGCAG3'	6	5	5.36	5.0
<b>Total</b>		112	100	-	-

As the primer efficiency depends on the total number of bands amplified by the primer and this could include a number of common (monomorphic) ones (representing conserved sequences among various species, efficiency would not be very informative in identifying species. A more informative parameter in this respect is the primer discriminatory power (Grrundman *et al.*, 1995). Discriminatory value of a primer depends only on the number of polymorphic bands produced by the primer relative to the total number of polymorphic bands produced by all primers. Polymorphic bands are essential in identification in two ways; first in generating patterns of banding that are unique to individual species and second in exhibiting or lacking unique band (s) (marker bands) that distinguish an individual from the rest of the population. Therefore discriminatory power of a primer is more important than its efficiency in determining its DNA fingerprint and two primers with similar efficiencies (e.g. OP-L05 and OP-M14 both with total number of bands of 7 and an efficiency of 6.25 for both of them) gave different powers, 6.0 for OP-L05 to identify two isolates (1,3) only, and 7 for OP-M06 to identify seven out of the nine isolates tested (Tables 4 and 5). However, primers with equal discriminatory values may have different capacities in identifying species (e.g. OP-I02 and OP-L05 both with discriminatory power of 6 but the first primer gave 9 unique patterns of banding and identified all 9 species while the second gave 4 patterns and identified only 2 species (1,3) (Tables 4 and 5)). Obviously, primer OP-I05 was the best primer in identification as, with just 6 polymorphic bands, it

distinguished all nine species whether by unique patterns of banding or by the marker bands. Primer OP-M05 did similarly but only with 9 polymorphic bands (Table 5). There were two primers (OP-E20 and OP-T20) that could not distinguish any of the nine species whether by patterns or by marker bands. The remaining markers managed to differentiate various numbers of the species whether by patterns, by marker bands or by both (Table 5).

Table 5: Species identified by the unique patterns of banding and/or by the marker bands of the RAPD data.

Primer	No. of bands	No. of polymorphic bands	No. of unique patterns of banding	Species identified by	
				Unique patterns	Marker bands: Fingerprinted
OP-C05	4	1	2	4*	4 <sup>*</sup> ,(-1.48)
OP-E20	4	1	2	None	None
OP-H01	2	2	4	5	None
OP-I02	6	6	9	1,2,3,4,5,6,7,8,9	1,(1.05);2,(-1.12)
OP-K01	8	8	7	1,3,5,8,9	1,(2.1);3,(2.25,1.5);9,(All bands lacking)
OP-L05	7	6	4	1,3	3,(1.66)
OP-L20	9	9	8	1,2,3,4,5,7,9	1,(1.52)
OP-M05	10	10	9	1,2,3,4,5,6,7,8,9	1,(1.6,-1.52,1.32);6(1.75)
OP-M06	9	9	8	1,2,3,4,6,7,9	1,(All bands lacking); 3,(1.9); 6,(2.4)
OP-M14	7	7	8	1,4,5,6,7,8,9	1,(1.23); 9,(1.32)
OP-M20	3	3	4	1	1,(-1.82)
OP-N16	6	6	5	1,9	1,(1.91)
OP-P04	5	5	8	1,2,3,4,5,6,7	None
OP-R06	3	3	4	1	1,(-1.48)
OP-R12	5	5	8	1,2,3,4,5,6,8	None
OP-R14	4	3	4	1,5,8	1,(3.16); 5,(2.24); 8,(-1.86)
OP-T19	4	4	5	1,2,3,4	None
OP-T20	3	1	2	None	None
OP-V09	3	3	5	1,3,9	None
OP-V14	4	3	6	2,6,9	6,(2.29)
OP-V19	6	5	8	1,3,5,6,7,8,9	3,(1.82); 8,(1.29)

- \*, Numbers in these columns refer to the fungal species numbers (Table 1) .
- Bracketed values are sizes (kbp) of marker bands .
- -, indicates absence of a common band.

Polymorphic bands or fragments were also useful in deciding the genetic similarity between species and estimating the genetic distances between each pair of species (Nei and Li, 1979). The longest distance was 0.7879 separating between the two species *A.radicina* and *A.raphani* , while the closest species to each other were *A.state* and *A.tenuissima* with a genetic distance of only 0.1854 (Table 6) the remaining genetic distances were between

these two limits (Table 6). These distances were used to produce the clustering dendrogram of the nine species (Fig. 2).

In addition to identify the isolates through the production of unique patterns of banding in the various species, the primers also succeeded in fingerprinting most (8 of 9) of the isolates. Fingerprinting of an isolate is indicated when it shows a specific band or bands (marker bands) which is absent in other isolates when amplified by the same primer. Or vice versa, it is manifested by the absence from the isolate of a band which is common to all isolates treated with same primer. In this respect, all strains but one (strain 7) were fingerprinted by one primer (strains 2,4,5,8), two primers (strain 9), three primers (strain 6), four primer (strain 3) or ten primers (strain 1) (Table 5). Obviously, strain 1 (*A. radicina*) has the most variable genome among the nine species tested as it showed 12 specific RAPD alleles (specific bands) (Table 5).

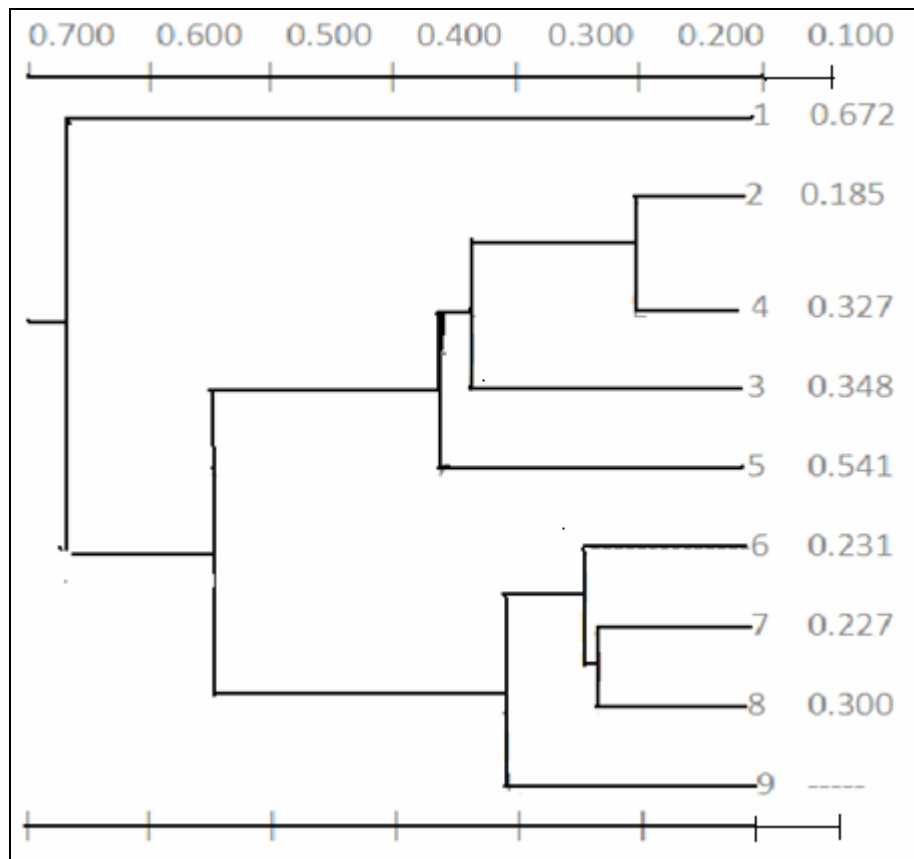
Table 6: Matrix of genetic distance between the nine *Alternaria* species as deduced from their RAPD markers.

	1	2	3	4	5	6	7	8	9
1	0								
2	0.6063	0							
3	<b>0.7897</b>	0.2801	0						
4	0.7146	<b>0.1854</b>	0.3747	0					
5	0.5097	0.3097	0.3534	0.3823	0				
6	0.6861	0.5886	0.5672	0.7305	0.4207	0			
7	0.6779	0.4934	0.5050	0.6132	0.4126	0.2272	0		
8	0.7666	0.5773	0.5534	0.7277	0.4028	0.2349	0.2268	0	
9	0.6276	0.4611	0.5566	0.5951	0.4498	0.3406	0.2601	0.2986	0

1-*A.rancidina* 2- *A.state*, 3- *A.raphani*, 4-*A.tenuissima* ,5- *A.alternata*1,6-*A.longipes*,7-*A.brassicae*,8-*A.alternata*2,9-*A.cheiranthi*.

The species clustered in three major groups. The first one included the single species *A.radicina* (Fig. 2). This result corresponded well with the result obtained by AL-Tae (2007) who used phenotypic characteristics and put this species in a distinct group. Therefore, phenetic features do reflect real genetic specificities as detected by the RAPD analysis . the second cluster included four species; *A.state* , *A. tenuissima* , *A. raphani* and *A. alternata*1. However, phenotypic classification of AL-Tae, (2007) put *A. state* and *A. tenuissima* closely together in a single group while the two species *A. raphani* and *A. alternata*1 were put into a separate group. Comparing sequences of the internal transcribed spacer (ITS) of the ribosomal (rDNA) and the mitochondrial small subunit (mtSSU)

ribosomal DNA of various *Alternaria* and related fungal species, Pryor and Gilbertson, (2000) were able to separate the two species *A. state* and *A. tenuissima* into a group distinct from the rest of *Alternaria* species. Result from the RAPD analysis reported in the present work put *A. state* and *A. tenuissima* into a separate group with the least genetic distance (0.1854) between any other pair of the nine *Alternaria* species tested (Table 6), but still within the same major group comprising the four species *A. state*, *A. tenuissima*, *A. raphani*, and *A. alternata1* (Fig. 2). Putting these four species, by the RAPD analysis, into a single major cluster might indicate a common sequences into a noncoding DNA in the species and hence phenotypic clustering and RAPD clustering might not correspond.



1-*A.rancidina*, 2- *A.state*, 3- *A.raphani*, 4-*A.tenuissima*, 5- *A.alternata1*, 6-*A.longipes*, 7-*A.brassicae*, 8-*A.alternata2*, 9-*A. cheiranthi*.

Fig.2. The genetic dendrogram of the nine *Alternaria* species revealed by their RAPD markers.

The third major cluster comprised four species ; *A. alternata2*, *A. cheiranthi*, *A. brassicae* and *A. longipes* (Fig. 2) with genetic distances ranging between 0.2272 (between *A. longipes* and *A. brassicae*) and 0.3406 (between *A. longipes* and *A. cheiranthi*) (Table 6). Phenotypic classification (AL-Tae, 2007), however, separated these four species into two distinct groups (*A. longipes* and *A. brassicae* in one group, and *A. alternata2* and *cheiranthi* in another group). Again, this RAPD grouping of the four species into a single group as opposed to two groups established by phenotypic criteria might reflect the presence of common sequences of no coding function in the four species, so that they

could exhibit banding similarity but phenotypic diversity. Furthermore, the RAPD data did not reveal a clear association between the clustering patterns of the species (Fig. 2) and their host distribution (Table 1). The four species of the second cluster had been isolated from four different hosts and similarly the four members of the third cluster had also been isolated from four different crop plants. Moreover, *A. state* of the second cluster and *A. longipes* of the third cluster shared the same host, *Brassica oleracea* (Fig. 2 and Table 1). Association between phenotypic characteristics of organisms and patterns resulting from typing these organisms with DNA molecular (RAPD, AFLP) markers seems to vary. Ozbey *et al.*, (2005) found no association between RAPD fingerprints and serotypes in the bacteria *Ornithobacterium rhinotracheale* isolated from chicken, for they found different patterns within the same serotype (A) and similar patterns within two (A and B) serotypes. Lack of association between RAPD pattern and source of isolation have also been reported for the fungus *Fusarium sambucinum* (Sabir, 2006). Also, cluster analysis did not reveal association between pathotypes and AFLP (Amplified fragment length polymorphism) markers in the fungus *Cochliobolus sativum* (Zhong and Steffenson, 2001). However cluster analysis did reveal close association between RAPD patterns and virulence and geographic origins of the fungus *Fusarium oxysporum* f. sp. *vasinfectum* (Assigbetse *et al.*, 1994).

### ACKNOWLEDGEMENTS

The authors are deeply indebted to Dr. Warka S. Q. AL-Tae, for providing the species of *Alternaria* used in the present analysis.

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