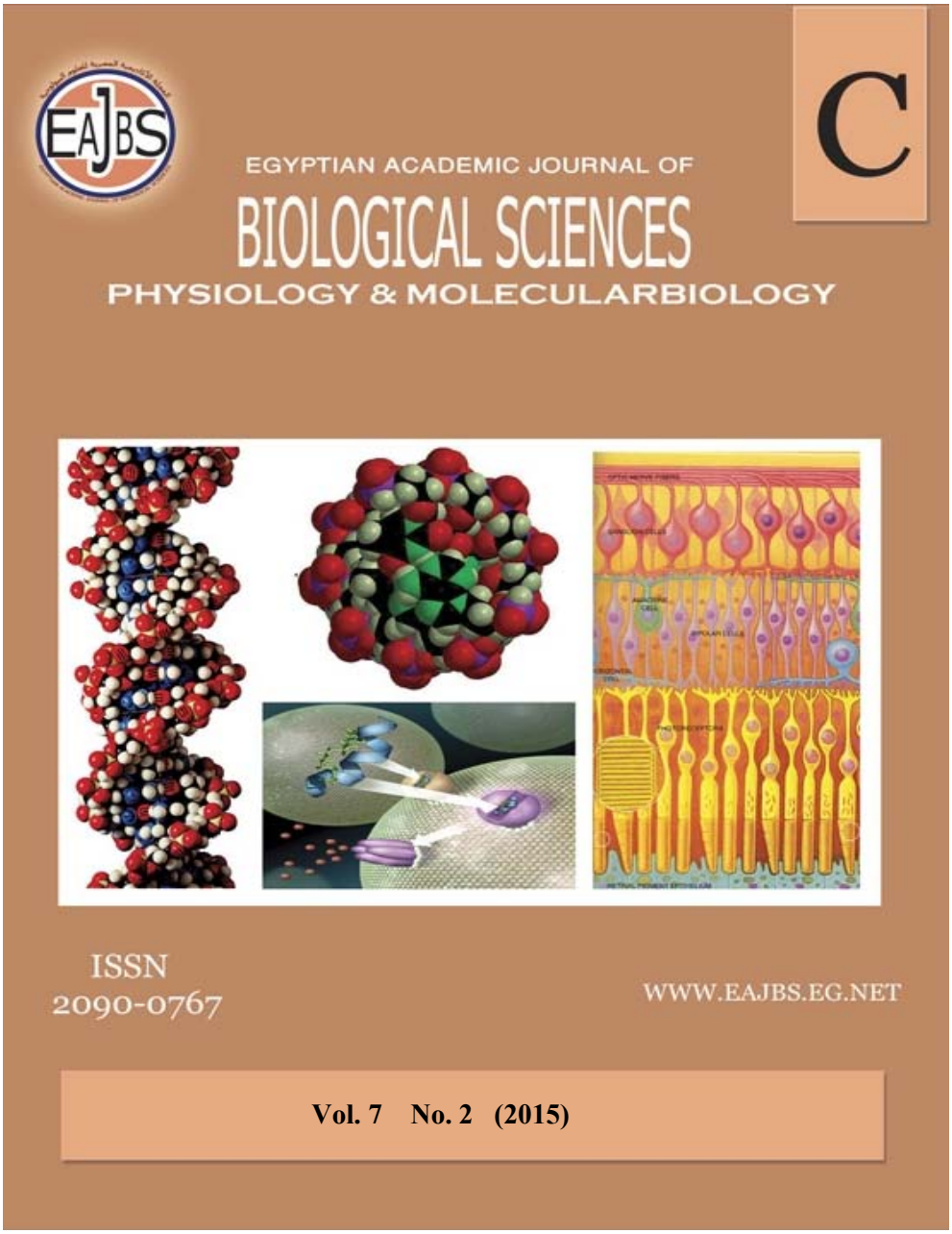

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Molecular Identification of *Aspergillus flavus* Using RAPD Markers

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ABSTRACT

The aim of this study is to isolate and identify *A. flavus* and study the genetic diversity among these isolates by using RAPD. Eleven collected samples were characterized depending on its morphological state, then DNA was extracted from them. RAPD markers are randomly banding with sites of genome more than, markers, where the primer UBC 809achieved discriminative power (19.1) and 43 bands, while6 achieved discriminative power (17.1) with 32 bands. There were more efficiency in specific binding, then RAPD primers have great binding to produce unique band, when 9 primers from 10 primers, 9 produced (5) unique bands, while RAPD markers showed low ability to produce unique bands, 3primers from 9 primers were produced as unique bands. The dendrogram of RAPD was reverted than isolates number 5 and 7 which had the genetic diversity 0.33361, while the isolates number 5 and 6 had the lowest genetic similarity 0.98521 in contrast with markers which showed isolates number1 and 2 genetic diversity 0.97826 while the isolates number 5 and 7 had the lowest genetic similarity 0.10253.

INTRODUCTION

Aspergillus flavus is responsible for approximately 90% of invasive Aspergillosis infections for immune-compromised individuals or allergic for individuals with atopic immune system [Marr *et al*., 2002]. *A. flavus*can cause infection on inhaling of 100 spores. After inhalation, it reaches the alveoli of the lung and then reaches the whole body through the bloodstream, including kidney, liver, and brain [Segal, 2010]. The pathogen city of *A. flavus* is due to presence of virulence associated genes in the genome which can be divided into four clusters depending on phylogenomic analysis [Nireman *et al*., 2012].One of the diagnostic methods for *A. flavus* is Polymerase Chain Reaction (PCR), which can amplify some specific fragment of DNA into millions of copies[McPherson, 2001]. In recent years, different molecular typing techniques have been applied to study the genetic diversity of *Aspergillus* spp. and the possible occurrence of similarity and difference between them.

Random Amplified Polymorphic DNA (RAPD) analysis can be performed as a method for study genetic diversity with large number of different strains of microorganisms. It is inexpensive and requires less amount of DNA [Bornet *et al*., 2001]. Moreover, RAPD analysis is technically being commonly used as an indicator for determination the genetic diversity, while Inter-simple sequence repeat-technique analysis based on variation found in the regions between microsatellites has been used in genetic fingerprinting gene tagging and detection of clonal variation [McPherson, 2001]. This technique which involved amplification of DNA segment present in between two identical microsatellite repeats regions by addition the oriental in opposite direction with suitable distances. This method has been reported to produce more complex markers patterns than the RAPD markers. In addition, this method is more reproducible than RAPD method because primers are designed to anneal temperature to microsatellite sequences which are longer than RAPD primers, allowing higher annealing temperature to be used. It also because of multi locus finger printing profile obtained, it has been found to be an efficient, low cost, simple operation, and high stability [Zietkiewics *et al*., 1994]. The aim of the studyis the detection of the unique bands and polymorphism between isolates and comparative study between RAPD and markers for genetic diversity between different *A. flavus* isolates.

MATERIALS AND METHODS

Aspergillus flavus Isolates

A total of 11 *A. flavus* isolates were isolated from *Zea Mays* grains and were examined according to their microscopic features, and were sub cultured on sabouraud Dextrose Agar for using in DNA extraction. As shown in Figure 1(a) and (b).

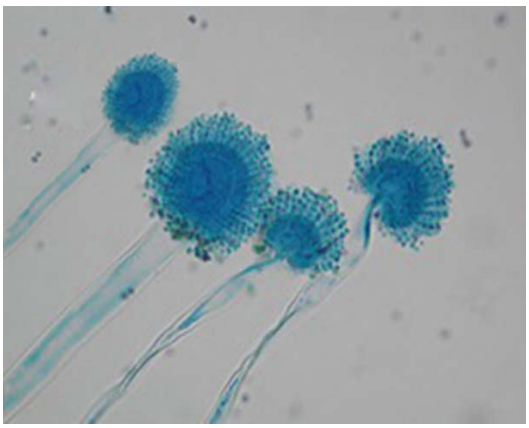

Fig. 1(a): Microscopic feature of *A. flavus* Under (40x).

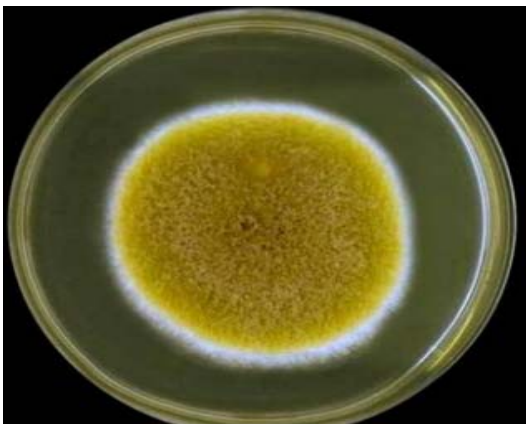

Fig. 1(b): Top view of *A. flavus* under on SDA at 25-27°C after 7 days of

Genomic DNA Extraction

The DNA was extracted by small-scale method commercial kit (Bionner-Korea). DNA Purity was measured depending on optical density by spectrophotometer. DNA quality was visualized by agars gel electrophoresis with ethidium bromide and visualized under UV light [Sambrook *et al*., 2001]

Molecular Analysis

RAPD Assay

Three of RAPD primers were used in this study, the primers was synthesd by (Bioneer-Korea) in lyophilized form and dissolved in sterile distilled water to get

final conc. of (10pmol/ml) [Hatti *et al*., 2010]. The primers and their sequences are listed in Table1.

Amplification of genomic DNA was performed with the following master amplification reaction (Table 2).

Table 1: The names and sequences of the primers used in this study.

No.	Primers name	Sequences (´5 -´3)
1.	OPE_16	GATGACCGCC
2.	OPD_20	ACCCGGTCAC
3.	OPc_16	GGTGACTGTT

Table 2: Master amplification reaction.

Materials	Final concentration	Volume for 1 tube
PCR pre mix	1x	5µI
Deionised D.W	——	11µI
Primer(10pmol/	10pmol /µl	2µI
DNA template	100ng	2µI

RAPD-PCR premix (final reaction volume = 20 µI).No. of cycles = 40 cycles between initial denaturation and final extension, the following table shows the RAPD program (Table 3).

Table 3: The RAPD program.

Steps	Temperature (°C)	Time (min.)
Initial denaturation	94	5
Denaturaion	94	1
Annealing	36	1
Extension	72	2
Final extension	72	10

Followed by a hold at 4°C [Hatti *et al*., 2010], each PCR amplification reaction was repeated twice to ensure reproducibility of the products analyzed by electrophoresis in a 1.5% agars gels with 0.5µl stained ethidium bromide at 7vt/cm for 3hours.

Each PCR amplification reaction was repeated twice to ensure reproducibility of the products analyzed by electrophoresis in a 1.5% agars gels with stained ethidium bromide 0.5µl at 5vt/cm for 2hour.

Data Analysis

Estimation of Molecular Weight

Computer software Photo-Capture M.W. program was used to determine molecular weight based on comparing the RAPD-PCR and -PCR products depending on molecular weight of bands and number bands of a 2000bp DNA ladder Bioneer (which consist of 13 bands from 100 to 2000 bp.).

Estimation of Polymorphism, Efficiency, and Discriminatory Power

Data generated for molecular weight RAPD and markers result bands were a score for each bands on the molecular size (1 for present, 0 for absence) the commercial soft word [Bibi *et al*., 2010]. Only major bands consistently amplified were scored. Polymorphism of each primer was calculated based on the following formula:

$$\textbf{Polymorphism \%} = (Np / Nt) \times 100$$

Where Np = the number of polymorphic bands of random primer and Nt = the total number of bands of the same primer. Efficiency and discriminatory power of each primer were calculated according to the formula below:

- Efficiency=number of polymorphic bands to each primer / total number of bands to the same primer.
- Discriminating power= number of polymorphic band to each primer / total number of polymorphic band to all primer X100 %.

Primer efficiency ranged between (0-1). Discrimination power of each primer

RESULTS AND DISCUSSION

RAPD-PCR Analysis

Tables 4, 5, 6 &7 and Figs. 2, 3, 4 & 5 summarize all information obtained from RAPD assay, and based on RAPD assay, the data developed from the PCR analysis demonstrated that some primers generate several bands, while other generates only a few bands. A total of three RAPD primers were used for studying the genetic differences between eleven *A. flavus* isolates, amplified 341 bands,126 bands were polymorphic, with average of (3-43) polymorphic bands, that OPD-20 produce 3 polymorphic bands only, were OPE_16 can be produce 43 polymorphic bands with average range size (100-2000)bp. (Fig. 2). Some isolates could be distinguished from all other isolates with selection of these primers, for instance OPE_16 primers can produce higher discrimination power 19.1 bands only, while OPL-05 gave 2 unique bands patterns.

Table 4:The polymorphic, monomorphic and unique bands with their molecular weight for primer OPE_16.

No.	Band M. wt.	1	2	3	4	5	6	7	8	9	10	11
1	1000	-	-	1	-	-	-	1	1	1	1	-
2	700	1	0	1	1	1	-	-	-	-	-	-
3	500	-	-	-	-	-	-	1	1	1	1	-
4	200	-	1	-	1	1	-	-	-	-	1	-

Table 5:The polymorphic, monomorphic and unique bands with their molecular weight .

2	700	-	1	-	-	-	-	-	-	-	-	-
3	600	1	-	1	1	1	0	1	1	1	1	1
4	200	-	1	-	-	-	-	-	-	-	-	-
5	100	1	-	1	1	-	-	-	-	-	-	-

Table 6:The polymorphic, monomorphic and unique bands with their molecular weight for primer OPc_16.

No.	Band M. wt.	1	2	3	4	5	6	7	8	9	10	11
1	1000	1	1	1	-	-	-	-	-	-	-	1
2	800	1	1	1	-	-	-	-	-	-	-	-
3	600	-	-	1	-	-	1	1	1	1	1	1
4	500	-	-	-	1	-	-	-	-	-	-	-
5	300	-	-	-	0	1	1	1	1	1	1	1

Table 7: Distinct characteristics of , primers including in the study: primers name, total number of bands, number of polymorphic bands, number of unique bands, percentage of polymorphism, primer efficiency and discrimination value.

No.	Name of primers	Total number of main bands	Number unique bands	Number polymorphic bands	Polymorphism %	Primer efficiency	Discrimination power
1	P1	20	2	20	2.9	1	100
2	P2	16	-	16	3.635	1	100
3	P3	22	1	22	2.636	1	100

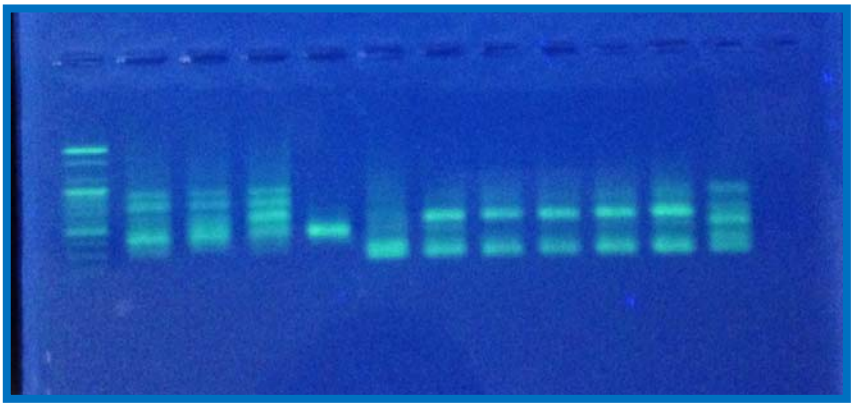

Fig. 2: PCR produced RAPD primerOPE_16 on 1.5% agarose gel electrophoresis with ethidium bromide, M=1000 bp., N=negative control, Lines=*A. flavus* isolates (AFU1, AFU2, AFU3, AFU4, AFU5, AFU6, AFU7), (1X TBE, 5Volt, 2hr, 0.5 romide).

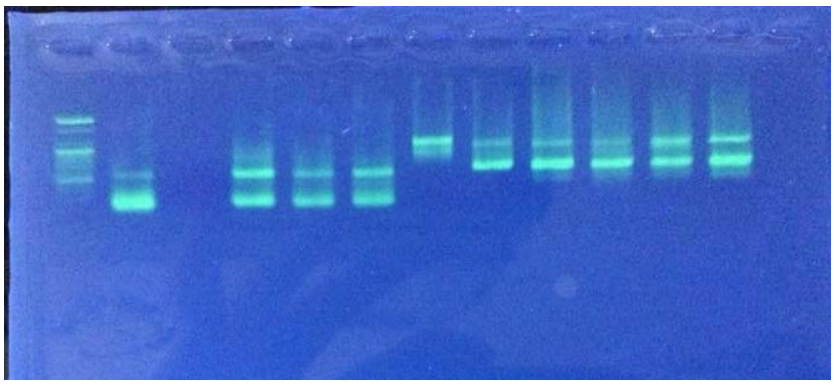

Fig. 3: PCR produced RAPD primerOPD_20on 1.5% agarose gel electrophoresis with ethidium bromide, M=1000 bp. , N=negative control, Lines=*A. flavus* isolates (1X TBE, 5Volt, 2hr, 0.5 romide).

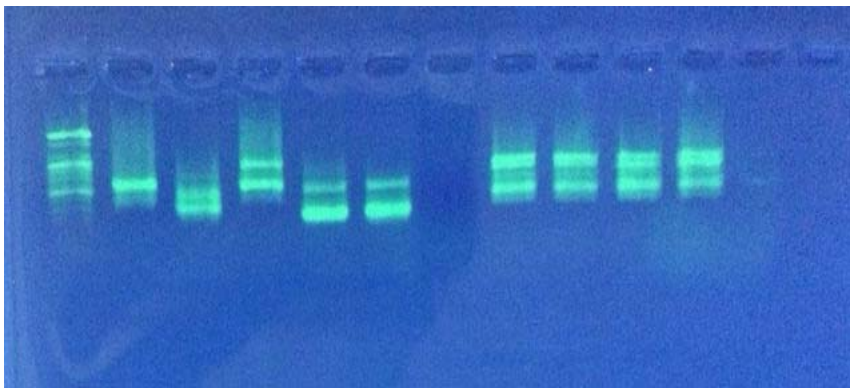

Fig. 4: PCR produced RAPD primerOPc_16 on 1.5% agarose gel electrophoresis with ethidium bromide, M=1000 bp. , N=negative control, Lines=*A. flavus* isolates (1X TBE, 5Volt, 2hr, 0.5 romide).

Table 7 summarized the information which can be obtained from RAPD analysis, and from genetic distance, the ration genetic diversity among the *A. flavus* isolates is from 0.9852 to 0.3336. The highest similarity 0.9852 (98.5%) was obtained between isolates numbers (5 and 6) while 0.48562 (48.5%) similarity between isolates numbers (2 and 6), the lowest level of similarity 0.3336 (33.3%) was obtained between isolate number (5 and 7). [Nei and lei,1979].

Cluster analysis illustrated genetic relationship among seven of *A. flavus* isolates showing two major clusters (Figure5 and Table 6), the first cluster contained two main groups, first group, 5 and 7 isolated in one sub group cluster with low genetic distance 0.3336. These were introduced from environmental sources and isolated number 1 formed separated line due to different in isolate source, while isolate number 2 and 6 formed another sub clusters with genetic distance 0.48652. These isolates were introduced from environmental and clinical sources. Second group contained isolate number 3 only, during clusters analysis showing the levels of genetic relatedness also dendrogram indicates difference between isolates based on source of the isolates.

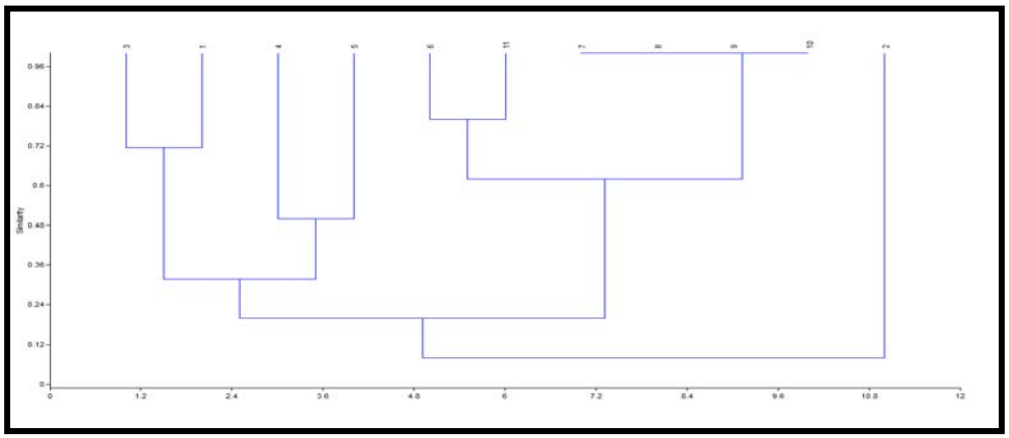

Fig. 5: Dendrogram illustrated genetic fingerprint and relationship between *A. flavus* isolates developed from RAPD data.

Table 8: values of genetic distance between *A. flavus* Isolates calculated according to Nei and Lei, 1979.

Similarity and distance indices

	1	2	3	4	5	6	7	8	9	10	11
1	0										
2	0.42857	0									
3	0.14286	0.57143	0								
4	0.28571	0.57143	0.42857	0							
5	0.35714	0.5	0.5	0.21429	0						
6	0.5	0.64286	0.5	0.5	0.28571	0					
7	0.64286	0.78571	0.5	0.64286	0.42857	0.14286	0				
8	0.64286	0.78571	0.5	0.64286	0.42857	0.14286	0	0			
9	0.64286	0.78571	0.5	0.64286	0.42857	0.14286	0	0	0		
10	0.64286	0.78571	0.5	0.64286	0.42857	0.14286	0	0	0	0	
11	0.42857	0.57143	0.42857	0.57143	0.35714	0.071429	0.21429	0.21429	0.21429	0.21429	0

Present result showed multiple differences in isolates of *A. flavus* which came from two factors including genetic factor and environment factor, also the results indicate that the clinical isolate has greater genetic variability than the

environment isolates during gene distance and dendrogram. Genetic difference may come from clinical ones, on the other hand the clinical isolates of patients constitute one group, according to genetic characteristic, with the environmental isolates. Genetic difference observed in this study come from adept fungi to grow and isolates that infected patients to reactive and generally more variability in relation to the original strain [Latge,2010]. Genetic diversity may be attributed to mutation or recombination that occurs in fungal cell into resistance to anti-mycotic treatment or under environmental stress [Tramutoli,2005]. Environmental and clinical isolates of *A. flavus* may be different in genotype consisted of gene involved in transport, regulation of transcription, and metabolism of molecular with 1-3 carbon and paroxysm all proteins [Gercia *et al*., 2011; Hynes *et al*., 2006].

In this study, each of genetic distance based on RAPD markers doesn't show geographic profiling between isolates. It has been reported that the dendrogram generated by markers is better with genealogy and the pedigree of the markers than RAPD results. On the another hand, it has been found that the data on RAPD genetic distance have more relationship with the geographic distribution in comparative with markers data that based on number of chromosomes. Markers are highly polymorphic and are useful in studies on genetic diversity [K. S. Wu *et al*.,1994]. Numbers of analysis studies used both markers and RAPD technique and found that markers produce more information with fewer numberof primer than the number of RAPD primers. During this study it was found that a number of polymorphic bands were still higher [Lanham and Brennan, 2000; Nagoaka, 1979]. Less primers means less time, less DNA, less supplied, and less samples. RAPD markers don't have the specific target comparing to markers. In fact, markers are known to be more sensitive than RAPD markers. In this study, it was obvious that the dendrogram based on RAPD markers was not in accordance with the dendrogram based on markers, thus, both dendrogram are in agreement with the groups of geographic origin, but RAPD markers greatly agree with these groups than markers. The differences in clustering pattern of genotypes using RAPD and markers also may be attributed to markers sampling error and the level of polymorphic detected [M. E. Ferandez *et al*., 2012].

CONCLUSION

Markers produced high rate from polymorphism depending on polymorphic rate. The technique can produce high level from unique bands a comparative with another markers that are less efficient in dendrogram results.

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