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Molecular Identification of *Aspergillus fumigatus* Using ISSR and RAPD Markers

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Abstract

The aim of this stud to isolate and identified of *A. fumigatus* from different sources and study the genetic diversity among these isolates by using RAPD and ISSR markers.Collected 20 samples from 7samples were isolated *A. fumigatus*isolates were characterized depending on its morphological, then extracted DNA from its.RAPD markersrandomly bandingwith sitesof genome more than ISSR markers where the primer OPN-07 achieved discriminative power (19.1) and 43 bands, while ISSR6 achieved discriminative power (17.1) with 32 bands.ISSR were more efficiency in specific binding then RAPD, ISSR primers has great a binding to production unique band, when 9 primers from 10 primers, ISSR9 was produce (5) unique bands, while RAPD markers was low ability to production unique bands, 3primers from 9 primers were produced unique bands.The dendrogram of RAPD was reverted than isolates number 5 and 7 had the great genetic diversity 0.33361 while the isolates number 5 and 6 had the lowest genetic similarity 0.98521 in contrast with ISSR markers was show isolates number1 and 2 greats genetic diversity 0.97826whilethe isolates number 5 and 7 had the lowest genetic similarity 0.10253.

Keywords: *Aspergillusfumigatus*, genetic diversity, ISSR, RAPD

ألتشخيص ألجزيئي لعزلات *A.fumigatus* بواسطه مؤشرات ال ISSR و الRAPD

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الخلاصه

هدفت هذا الدراسه الى عزل وتشخيص فطر *A. fumigatus* من مصادر مختلف ودراسهالتغايرات الوراثيه بين هذا العزلات بإستخدام مؤشرات التفاعل العشوائي المتعدد إلاشكال لسلسله ألدنا RAPD ومؤشرات إعاده ألسلسله ألبسيطه ISSR تم جمع 20 عينه من *A. fumigatus* من مصادر مختلفه وتم تشخيصها إعتماد على صفات المظهر الخارجي. وتم استخلاص الدنا من جميع العينات ولكن تم اختيار 7 عزلات فقط لاجراء تفاعلات ال RAPD والISSR عليها.إظهرت مؤشرات ال RAPD بأنها ذات ارتباط عشوائي بمواقع التركيب الجيني اكثر من مؤشرات الISSR, حيث ان البادئ OPN−07حقق طاقه تمييزيه بلغت 19.1 وناتج عدد حزم عشوائيه بلغ 43 مقارنه مع البادئ ISSR6 الذي حقق طاقه تمييزيه بلغت 17.1و32 حزم عشوائيه. أظهرت بادئات مؤشر ال ISSR قدره عاليه على إنتاج حزم مفرده حيث ان 7 بادئات من اصل 9 اظهرت حزمه منفرد بينما كان البادئ ISSR9 هو الاكثر إنتاج للحزم(5) حزم منفرده

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بينما بادئات مؤشر الRAPD كانت ذات قدره اقل على إنتاج الحزم المنفرده حيث انتج 3 بادئات فقط من اصل 9 اظهرت حزم منفرده وكان البادئات OPL−05, OPM−20 الاكثر إنتاج للحزم المنفرده بعدد 2 وهذا يدل على ان مؤشرات ISSR ذات كفاءه اعلى من مؤشرات RAPD في الارتباط اكثر تخصص بمواقع مميزه ،اظهرت المخططاتلشجيريه لتقنيه الRAPD اضهرت ان العزله رقم 5مع عزله رقم 7 هي الاكثر تنوع جينيه بلغت 0.33361 بينما العزله رقم 5 مع عزلة رقم 6 كانت الاقل مسافه جينيه بلغت 0.98521 اما في مايخص تقنية ISSR إضهرت أن العزلة رقم 1 وعزلة رقم 2 هي الاكبر تنوع جيني 0.97826 بينما العزلة رقم 5 و عزله رقم 7 هي الاقل تشابه جيني 0.10253.

Introduction

Aspergillusfumigatus is an opportunistic fungus, responsible for approximately 90% of invasive Aspergillosis infections for immune-compromised individuals or allergic for individuals with atopic immune system [1]. *A.fumigatus*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.[2,3]. the pathogen city of *A.fumigatus* is due to presence of virulence associated genes in the genome which can be divided into four clusters depending on phylogenomic analysis [4].One of the diagnostic methods for *A.fumigatus* is Polymerase Chain Reaction (PCR), which can amplify some specific fragment of DNA into millions of copies[5]. In recent years, different molecular typing techniques have been applied to study the genetic diversity of *Aspergillusspp.* 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 [6]. Moreover, RAPD analysis is technically being commonly used as an indicator for determination the genetic diversity, whileInter-simple sequence repeattechnique ISSR analysis based on variation found in the regions between microsatellites it has been used in genetic fingerprinting gene tagging and detection of clonal variation [7]. This technique whichinvolved amplification of DNA segment present in between two identical microsatellite repeat regions by addition the oriental in opposite direction with suitable distances ISSR method has been reported produce more complex markers patterns than the RAPD markers In addition, ISSR method are more reproducible than RAPD method Because ISSR primers designed to anneal temperature to a microsatellite sequences are long than RAPD primers, allowing higher annealing temperature to be used. It also because of multilocus fingerprinting profile obtained ISSR has been found to be an efficient, low cost, simple operation, high stability and abundance of [8].The aim of the study Detection of the unique bands and polymorphism between isolates and Comparative study between RAPD and ISSR markers for genetic diversity between different *A. fumigatus* isolates.

Materials and Methods

Aspergillusfumigatus isolates

A total of 7 *A. fumigatus* clinical and environmental isolates as shown in table-1 were examined according to their microscopic features, and were sub cultured on sabouraud Dextrose Agar, for using in DNA extraction.

Table 1- *Aspergillusfumigatus* isolates examined during this study

***A.fumigatus*isolates**	**Samples type**	**Sources**
*AFU*1	Aspergillosis(Sputum)	Clinical
*AFU*2	River Water sample	Environmental
*AFU*3	Contaminationhospital (Swap)	Clinical
*AFU*4	Soil rounded plant root	Environmental
*AFU*5	*Zea mays* grain	Environmental
*AFU*6	Otitis externa (Swap)	Clinical
*AFU*7	Air sample	Environmental

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 electrophoresiswith ethidium bromide and visualized under UV light [9]

Molecular Analysis

RAPD assay

Six of RAPDprimers were used in this study,the primers was synthase by (Bioneer-Korea) in lyophilized form and dissolved in sterile distilled water to get final conc. of (10pmol/ml)[10].The primers and their sequences are listed in table-2.

Table 2- The names and sequences of the primers used in this study

No.	Primers name	Sequences(´5 -´3)
1.	OPI - 06	AAGGCGGCAG
2.	OPE-16	GGTGACTGTT
3.	OPN-07	GAGCCCGAG
4.	OPQ-17	GAAGCCCTTG
5.	OPD-20	ACCCGGTCAC
6.	OPL-05	ACGCAGGCAC

Amplification of genomic DNA was performed with the following 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 finalextension, following table shows 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[10]. Each PCR amplification reaction was repeated twice to ensure reproducibility the products analyzed by electrophoresis in 1.5% agars gels with 0.5µl stained ethidium bromide at 5vt/cm for 3hours.

ISSR assay

Six of ISSR primers were used they were provided by (Bioneer – Korea) in lyophilized form and dissolved in sterile distilled water to get final conc. of (10pmol/ml) [10].Recommended by provider the primers which tested in this study which list in table-3.

Table 3- The names and sequences of the primers used in this study

No.	Primers Name	Sequence(´5-´3)
1.	$ISSR_2$	GACAGACAGACAGACA
2.	$ISSR_6$	AGAGAGAGAGAGAGAGAGAGC
3.	$ISSR_7$	AGAGAGAGAGAGAGAGAGAGT
4.	$ISSR_8$	CTCTCTCTCTCTCTCTCTCTA
5.	$ISSR_9$	CTCTCTCTCTCTCTCTCTCTG
6.	$ISSR_{10}$	CTCTCTCTCTCTCTCTCTCTT

Following master amplification reaction

Materials	Final concentration	Volume for 1tube
PCR Premix	1x	5µI
Deionised D.W.	—	17 µI
Primer(10pmol/ml)	10pmol/ml	2µI
DNA template	100ng	1µI

Final concentration was performed in a volume of 25µl. PCR program for ISSR assay using the following program: No. of cycles= 40 cycles between initial denaturation and final extension, the following table shows the ISSR program:

Steps	Temperature(°C)	Time (min.)
Initial denaturation	94	5
Denaturation	94	1
Annealing	50	1
Extension	72	1
Final extension	72	10

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

Data analysis

Estimation of molecular weight

Computer softwarePhoto-Capture M.W. program was used to determine molecular weight based on comparing the RAPD-PCR and ISSR-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 ISSR markers result bands were a score for each bands on the molecular size (1 for present, 0 for absence) the commercial soft word [11]. Only major bands consistently amplified were scored. Polymorphism of each primer was calculated based on the following formula:

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

Where Np= the number of polymorphic bands of random primer, Nt= the total number of bands of the same primer. Efficiency and discriminatory power of each primer 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

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 six RAPD primers were used for study the genetic differences between seven *A.fumigatus* isolates, amplified341 bands,126 bands were polymorphic, with average of (3-43) polymorphic bands, that OPD-20 produce 3 polymorphic bands only ,were OPN-07 can be produce 43 polymorphic bands with average range size (100-2000)bp,(figure -1). Some isolates could be distinguished from all other isolates with selection of these primers, for instance OPN-07 primers can be produce higher discrimination power 19.1 bands only, while OPL-05 gave 2 unique bands patterns table -4.

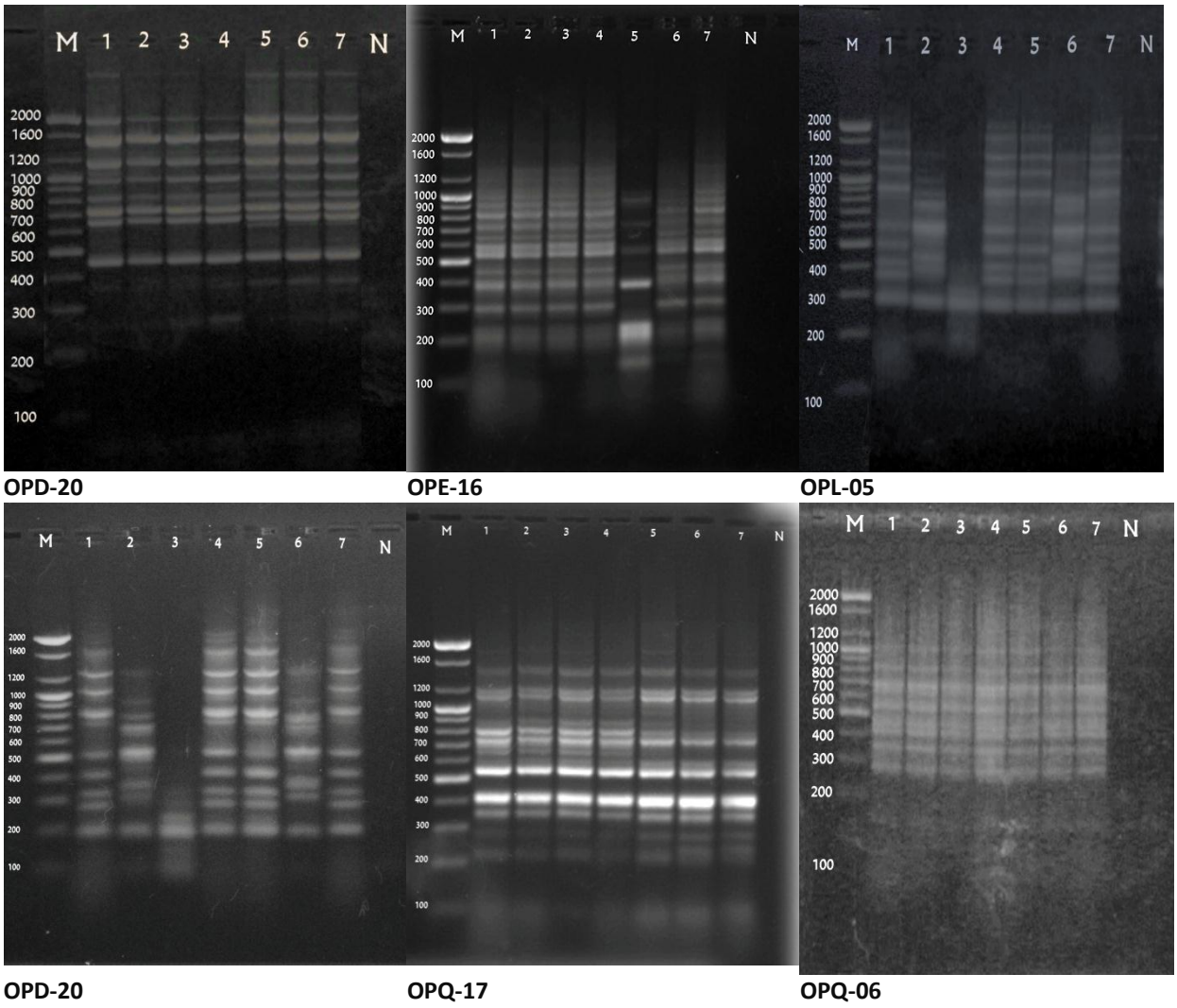

Figure 1- PCR producedRAPD primer on 1% agars gel electrophoresis with ethidium bromide,M=1000bp.,N= negative control, Lines= *A.fumigatus* isolates (AFU1,AFU2, AFU3, AFU4, AFU5, AFU6, AFU7), (1X TBE,7Volt,2hr,0.5ethidium bromide)

Table 4- Distinct characteristics of RAPD 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 primer	Total number of main bands	Number of unique bands	Numberof polymorphic bands	Polymorphism (%)	Primer efficiency	Discrimination Power (%)
1	OPI-06	7	0	18	39.1	0.39	8
2	OPE-16	12	1	35	50	0.5	15.5
3	OPQ-17	9	0	5	8.4	0.08	2.22
4	OPD-20	10	0	3	4.5	0.04	1.33
5	OPL-05	12	2	42	85.7	0.85	18.6
6	OPN-07	12	0	43	84.3	0.84	19.1

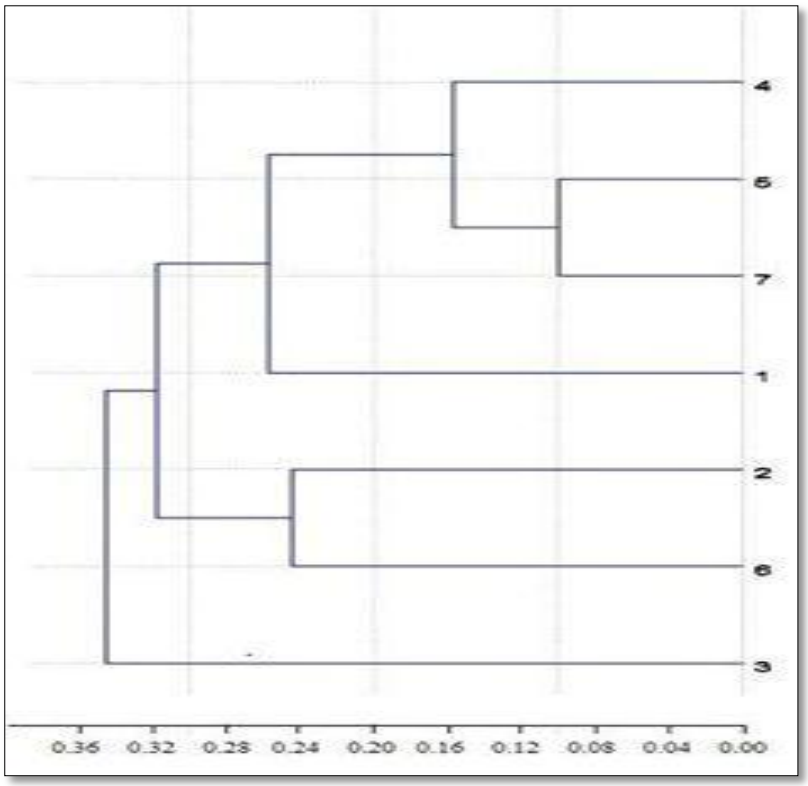

Figure 2- Dendrogram illustrated genetic fingerprint and relationship between *A.fumigatus* isolates developed from RAPD data.

Table-5 summarized the in formations which can be obtained from RAPD analysis, and from genetic distance the ration genetic diversity among the *A.fumigatus* isolates 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). [12].

Table 5- Values of genetic distance among*A.fumigatus* Isolates calculated according to Nei and Lei, 1979).

	1	2	3	4	5	6	7
1	0.00000						
2	0.69226	0.00000					
3	0.85701	0.69857	0.00000				
4	0.64981	0.97773	0.97398	0.00000			
5	0.10389	0.62219	0.95737	0.45770	0.00000		
6	0.64708	0.48562	0.56583	0.87972	0.98521	0.00000	
7	0.74012	0.69881	0.92887	0.67972	0.33367	0.58919	0.00000

Cluster analysisillustrated genetic relationship among seven of *A.fumigatus* isolates showing two major clusters figure-2, 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 introduced from environmental and clinical source, 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, present result appeared multiple difference in isolates of *A. fumigatus*came from two factors including genetic factor and environment factor, also the results indicate that the clinical isolate have greater genetic variability than the environment isolates during gene distance and dendrogram, genetically different may be 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

ability isolates that infected patients to reactive and general more variability in relation to the original strain [13]. Geneticdiversity may be attributed to mutation or recombination that occurs in fungal cell into resistance to anti-mycotic treatment or under environmental stress [14]. Environmental and clinical isolates of *A.fumigatus* may be different in genotype consisted of gene involved in transport, regulation of transcription, metabolism of molecular with 1-3 carbon and paroxysm all proteins [15,16].

ISSR-PCR analysis

In this study, ISSR-PCR technique was used to reveal the genetic diversity among different studied*A.fumigatus*isolates in order to search the genetic diversity between *A.fumigatus* isolates and study the differences that come from environment.A total of 178 use full bands were scored from the amplifiedproducts with the seven Inter Simple Sequence Repeat (ISSR), 120 bands were polymorphic, with average of (6) polymorphic bands ISSR10,and ISSR6 produce 32 polymorphic bands with average range size (100-2000)bp. figure-3. ISSR9 primers can be produce high unique bands can be produce 5 unique bands, table-6.

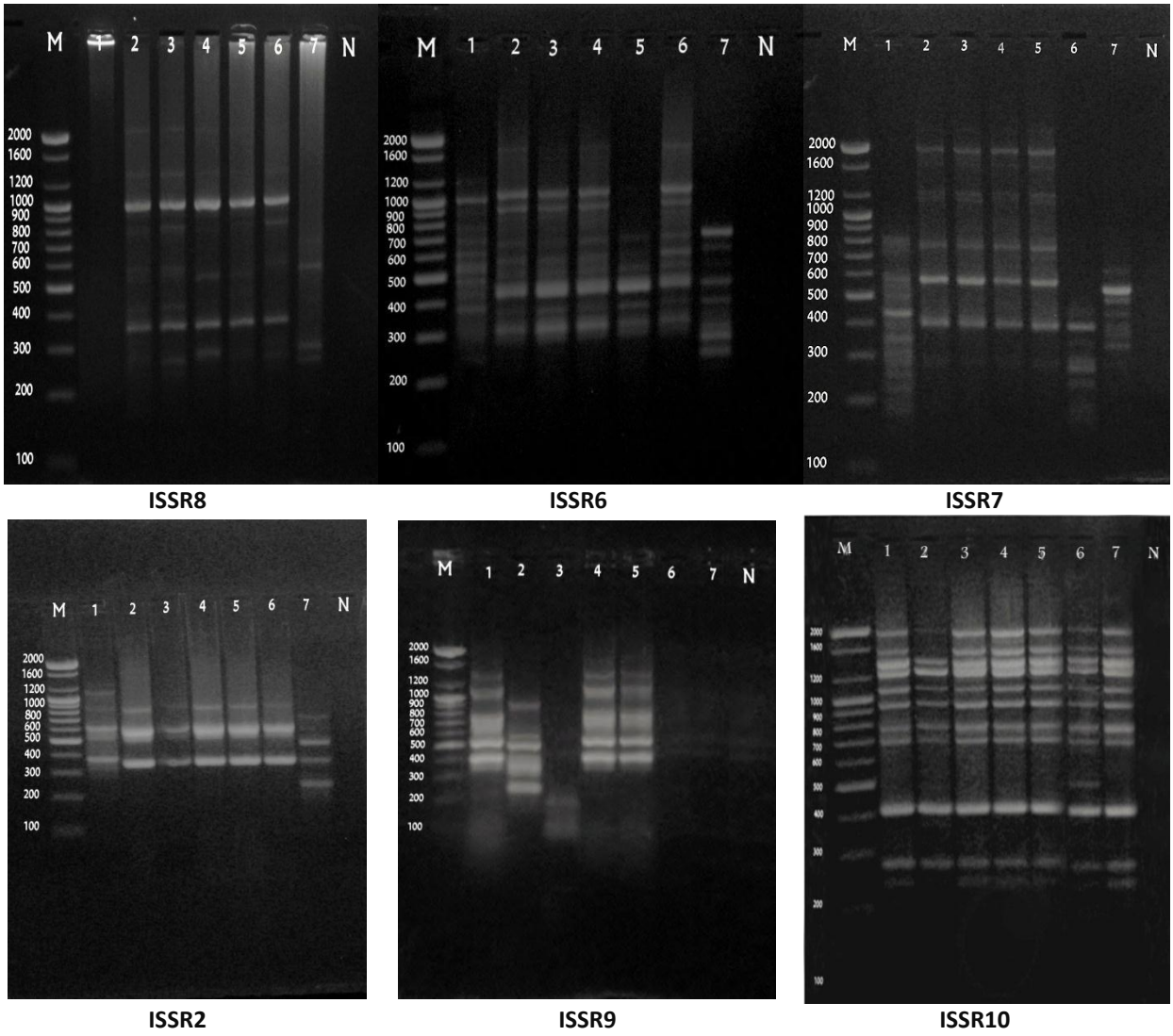

Figure 3- PCR produced ISSR primer on 1.5% agars gel electrophoresis with ethidium bromide,M=1000bp., N=negative control, Lines =*A.fumigatus* isolates (AFU1, AFU2, AFU3, AFU4, AFU5, AFU6, AFU7), (1X TBE,5Volt,3hr,0.5ethidumbbromide).

Table 6- Distinct characteristics of ISSR 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 primer	Total number of main bands	Number of unique bands	Numberof polymorphic bands	Polymorphism (%)	Primer efficiency	Discrimination Power (%)
1	ISSR2	7	4	12	52.1	0.52	6.41
2	ISSR6	10	3	32	91.4	0.91	17.1
3	ISSR7	10	4	25	83.3	0.83	13.3
4	ISSR8	11	2	30	93.7	0.39	16
5	ISSR9	10	5	15	75	0.57	8.02
6	ISSR10	11	1	6	8.5	0.85	3.2

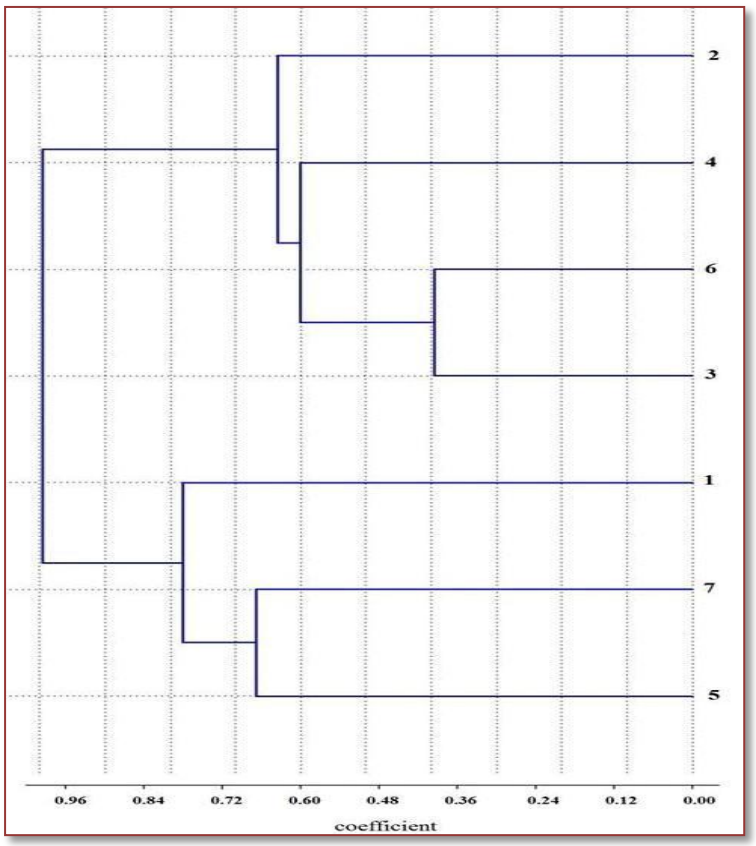

Figure 4- Dendrogram illustrated genetic fingerprint and relationship among *A.fumigatus* isolates developed from ISSR data.

From genetic distance, the ratio of genetic similarity among the seven *A.fumigatus* isolates from 0.97868 to 0.1025,showing table-7.The highest similarity 0.97868 (99.8%) was obtained between isolates number (1 and 2)while 0.21556 (21.5%) genetic similarity between isolate number 3 and 6, but lowest level of similarity 0.1025 (10.2%) was appearsbetween isolate number (5 and 7).

Table 7- Values of genetic distance among*A.fumigatus* Isolates calculated according to Nei and Lei, 1979).

	1	2	3	4	5	6	7
1	0.00000						
2	0.99826	0.00000					
3	0.81082	0.68246	0.00000				
4	0.86553	0.4055	0.7	0.0000			
5	0.54189	0.94553	0.94855	0.86744	0.0000		
6	0.78038	0.74189	0.21556	0.55687	0.70305	0.0000	
7	0.55263	0.8465	0.98682	0.87868	0.10251	0.7613	0.0000

During dendrogramwere constructed based on Nei and Lei (1979).Genetic distance using UPGMA cluster analysis and depicted genetic relationship among seven *A.fumigatus* isolates showing two major cluster,first cluster contained two maindistance, these were introduced from patients sources and isolate number 2 formed separated line that came from environmental sources, and second cluster contained isolate number (7,5) with lowest genetic similarity 0.1025.These isolates introduced from patients and environment sources, figure-4 group, first group contain isolates number (1,2) with higher genetic similarity 0.9782

In this study, each of genetic distance based on ISSR and on RAPD markers don't show geographic profiling between isolates.It has been reported that the dendrogram generated by ISSR better with genealogy and the know pedigree of the ISSR 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 ISSR data that based on number of chromosomes, ISSR markers are highly polymorphic and are useful in studies on genetic diversity [17]. Numbers of analysis studies used both ISSR and RAPD technique were found that ISSR produce more information with fewer number primerthan number RAPD primer, during among study found a number polymorphic bands was still higher with less number [18,19]. ISSR less primers means less time, less DNA, less supplied and less samples, RAPD markers don't have the specific target comparing to ISSR markers.In fact ISSR 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 according with the dendrogram based on ISSR markers, thus, both dendrogram are in agreement with the groups of geographic origin, but RAPD markers greatly agree with these group than ISSR markers, the differences in clustering pattern of genotypes using RAPD and ISSR markers also may be attributed to markers sampling error and the level of polymorphic detected [20].

Conclusion

ISSR markers produced high rate from polymorphism depending on polymorphic rate, the ISSR technique can be produce high level from unique bands a comparative with another markers, ISSR lessefficiency in dendrogramresults.

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