Genetic Diversity Among Some *Aspergillus flavus* Isolates by Using Inter simple sequence repeats (ISSR)

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Abstract

Aspergillus flavus isolates which are considered on conidial shape through microscopic examination and mycelial colour through cultural properties . Primary screening for the ability of *A. flavus* isolates for aflatoxin production was determined using *A.flavus parasiticus* agar medium (AFPA) as well , 7 isolates from 11 isolates give a positive result by the presence of bright yellow-orange pigments indicated the presence of aflatoxins. Molecular genetics techniques using DNA polymorphism have been increasingly used to characterize and identify genetic diversity and relationships among eleven *A. flavus* isolated from different source using the ISSR(inter simple sequence repeats) technique. Three universal primers designed at University of British Columbia (UBC 809 , UBC 810, UBC 811) were produced (18) main bands of these bands, 53 bands were polymorphic. The size of the amplified bands ranged between 100-1,500 bp. The genetic polymorphism value of each primer was ranged between 16.98-74.4%. In terms of unique banding patterns, determine the finger print for one isolate the most characteristic banding pattern was for the isolate number 5 with primer UBS primer No.809. Genetic distances ranged from 0.12599 to 0.93644 among *A.flavus* isolates. Cluster analyses were performed to construct a dendrogram placed most of the *A. flavus* was isolated from the different source at the same main group.

Keywords: Aspergillus flavus, ISSR, Aflatoxin B.

التغايرات الوراثيه بين بعض عزلات *Aspergillus flavus* بأستخدام تقنية (ISSR)

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الخلاصة

عزلات فطر الرشاشيا ت الخضراء (*A.flavus*) تم اعادة تشخيصها اعتماداً على شكل الكونيد يات مجهرياً ولون الخيوط الفطريه طبقياً. تم الكشف الاولي لقابليه الفطر على انتاج سم الافلاتوكسين(Aflatoxin B1) بواسطة الاوساط المحفزه لانتاج السم والتي تضمنت الوسط التفريقي(AFPA) ، اثبتت سبعه عزلات من ضمن احد عشر قابليتها على انتاج الافلاتوكسين وذلك بتكوين ها حلقه برتقاليه اللون . استخدمت التقنيات الجزئية للكشف عن التغايرات بين العزلات عن طريق التغاير بالدنا، حيث ان تغاير الدنا قد بيّن قابليه العزلات على انتاجها للافلاتوكسين والعلاقات الوراثيه ما بين العزلات ومن اهم هذه التقنيات (ISSR)،استخدمت ثلاث بادئات صممت من قبل جامعه (University of British Columbia) التي انتجت 18 حزمة اصلية من ضمنها 58 حزمة متعددة الاشكال و ذات حجم مابين 100−1,500 زوج من القواعد النيتروجينيه وقيمة التغايرات الوراثيه لكل بادئ وضعت بين مدى ومقداره 16.9 – 74.4 % . مصطلح الحزمه المفرده تمثل

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البصمة الوراثيه للعزل ، حزمة مفرده واحده انتجت من العزله الخامسه للبادئ رقم (UBC809) البعد الوراثي للعزلات كان بين مدى (0.12599−0.93644)، ووضحت العلاقات بين العزل على المخططات العنقوديه (الديندروكرام) الذي ميّز العزلات اعتمادا على مصدر العزل

Introduction

Mycotoxigenic filamentous fungi have the ability to contaminate a wide range of food and animal feed with one or more mycotoxins , Approximately 25 to 50 % of the crops harvested worldwide are contaminated with mycotoxins[1]. Food and feeds, especially in warm climates, are susceptible to invasion by aflatoxigenic *Aspergillus* species , *Aspergillus* species can cause deterioration of foods, including stored wheat, corn, rice, barely, flour bran, peanut and soy bean . Some species of this genus produce secondary metabolites in food as aflatoxins (AFs) which are produced mainly by *A.flavus* and *A.parasiticus* [2].

Aspergillus flavus is the main producer of the well known carcinogenic aflatoxins, The presence of this fungus and aflatoxins is of huge concern in terms of food safety because its the most potent naturally occurring toxic and hepatocarcinogenic compounds [3,4].Growth of *Aspergillus* species, spore and/or toxins production is affected by temperature, pH, water activity (aw) availability of air, and nutritional factors [2].

Although aflatoxins are not automatically produced whenever grain becomes moldy, *A. flavus* genome has revealed numerous genes and gene clusters that are potentially involved in the formation of aflatoxin and other secondary metabolites[5]. Lin *et al*., (1998) used conventional methods to distinguish between toxigenic and non-toxigenic isolates in the *A. flavus* group by culturing on suitable inducing media[6].

Polymerase Chain Reaction (PCR)-based, inter single sequence repeats (ISSR) analysis has been used successfully in the analysis of DNA relatedness of species of fungi, bacteria, plants and animals. SSR micro satellites analysis present in nuclear and organelles DNA can be used as molecular markers and has wide rang applications in the field of genetics including kinship and population studies, (ISSR) represent genome region between micro satellite loci. Sequences amplified by ISSR-PCR can be used for delimiting species. Dendograms which evaluate the likeness between different isolates has also been used[7]. Because of the harmful activity of *A.flavus* necessary to document genetic diversity among some local Iraqi isolates through determination the ability of *A.flavus* isolates for aflatoxin B1 production by using conventional method and investigation of genetic diversity , attempt to find the DNA fingerprint of isolates , and studying the genetic distance between *A. flavus* isolates using ISSR-PCR technique.

Materials and Methods

A.flavus isolates

The isolates of *A.flavus* (1,2) isolated from Rice , *A.flavus* isolates (6,7,8,9,10 and 11) isolated from maize ,were obtained from Department of Biology, Collage of sciences, University of Baghdad *A. flavus* (3,4 and5) ,isolated from Maize were obtained from Department of Biotechnology, University of Baghdad .All fungal isolates were cultivated at 28 ˚C on potato dextrose agar (PDA) and stored at 4˚C.

Rapid detection of aflatoxigenic *Aspergillus flavus* using (AFPA):

Aspergillus flavus and *parasiticus* agar (AFPA) is a selective medium for detecting *A. flavus* aflatoxigenic isolates from the other contaminant moulds. AFPA preparation: peptone 10g, YE 20g, ammonium ferric citrate 0.5g, chloramphenicol 0.2g, agar 15g, dichloran 1 ml .All materials were mixed in (500 mL) Erlenmayer flasks. After adjustment of pH, they were sterilized by autoclaving at 121˚C for 15 min under15 Psi [8] .

Genomic DNA Isolation

Total genomic DNA of all the studied isolates were obtained by using EZ-10 Spin Column Fungal Genomic DNA Mini –Preps Kit.to produce a rapid extraction and high quality extracted DNA. Purity and concentration of DNA was measured by spectrophotometer [9]. Genomic DNA integrity was detected by running on 1% agarose gel electrophoresis followed by staining with ethidium bromide and visualized under UV light [10].

Primer selection and ISSR assay

Three decamers of oligonucleotides primers sequence were used in a lyophilized form and were dissolved in sterile deionizer distilled water to give a final concentration of (10pmol/µl). Three ISSR primer sets were ordered from University of British Columbia (UBC). The primers and their sequences As : UBC 809 AGAGAGAGAGAGAGAGG , UBC 810 GAG AGA GAG AGA GAG AT , UBC 811 GAG AGA GAG AGA GAG AC.

Amplification reactions were performed in a volume 20 µl (ISSR-PCR PreMix(Bioneer),(final reaction volume = 20 µl). Amplification was carried out using a thermocycler (Eppendorf-Germany), using the following program:- cycle of 5 min at 94°C for initial strand separation, followed by 40 cycles of 1min at 94˚C for denaturation and 1min at 50˚C for anneling and 1min 72˚C for primer extension. Finally, 1 cycle of 10 min at 72˚C was used for the final extension, and this program resulted by optimization.

Approximately, 12µl of PCR amplified products were separated by electrophoresis in 1.2% agarose gels (2 hrs, 5V/cm, 1X Tris-borate buffer). Gels were stained with ethidium bromide, PCR products were visualized by U.V transilluminator and then were imaged by gel documentation system. The amplified products usually consist of 1-10 discrete bands and may reach to 19 bands**,** the size of ISSR-PCR products estimated by comparing with the marker DNA ladder (100-1500) bp (promega).

Data Analysis

Molecular Weight Estimation :Molecular weight was estimated by using a computer software M.W. Detection program, Photo-Capture M.W. program from Consort, based on comparing the PCR products with the known size of DNA fragments of a100bp DNA ladder (consists of 11 bands from 100 to 1,500 bp) for ISSR-PCR products.

Estimation of Genetic Distance

Data generated from the detection of polymorphic fragments were analyzed. The amplification profile of all the used varieties for any given primer was scored as "1" with the presence of each band and was scored as "0" with the absence of the same band of the same size in other isolates [11]. Estimates of genetic distance (G.D) were calculated between all pairs of the varieties based on the data matrix based on the following formula:

$G.D = 1-\{2Nab/(Na + Nb)\}$

Where Na = the total number of fragments detected in individual 'a'; Nb = the total number of fragments shown by individual 'b' and Nab = the number of fragments shared by individuals 'a' and 'b [12]. Polymorphism of each primer was calculated based on the following formula:Polymorphism $\%=(Np/Nt)\times100$,where –(Np)= the number of polymorphic bands of random primer.-(Nt)= the total number of bands of the same primer.Estimation of efficiency of primers and the discriminatory : Efficient discriminatory power of each primer calculated according to the formula :Primer efficiency of each primer was calculated as the number of polymorphic bands to the total number of bands to the primer, Primer efficiency ranged between (0-1).Discrimination power of each primer was calculated as the percentage of the number of polymorphic bands to each primer to the total number of polymorphic bands to all primers[13] .Cluster analysis was performed to construct genetic relationship tree diagrams among studied barley varieties using an Unweighted Pair-Group Method with Arithmetic Average (UPGMA)[14] .All computations were carried out using the Numerical Taxonomy and Multivariate Analysis System Version 1.80 package[15].

Results and Discussion

Rapid detection of aflatoxigenic isolates

Seven isolates of *A. flavus* able to produce aflatoxin from 11 *A.flavus* isolates that detected through examined under UV light after three days of incubation. Isolates No.(1,2)were isolated from Rice and isolates No.(9,6) isolated from Maize shows un ability of these isolate to produce aflatoxin. Isolates No.(3,4,5,7,8,10,11)isolated from maize Shows the ability of isolates to produce bright yellow-orange pigments by their growth on mentioned media after 3 days and this revealed the ability of aflatoxin production[16]. As shown in figure (1).

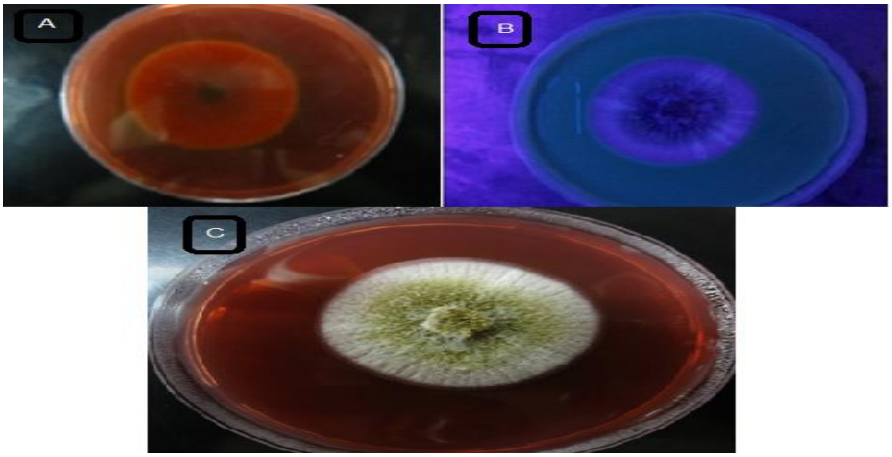

Figure 1- Ability of *A. flavus* to produce aflatoxin in (AFPA) medium incubated at 37 c for 3 days . (A,B):*A. flavus* produce aflatoxin (present of a bright yellow-orange pigments); (C):*A.flavus* non produce of aflatoxin).

Genomic DNA Extraction

The DNA was extracted efficiently by using EZ-10 Spin Column Fungal Genomic DNA Mini –Preps Kit . Purity and concentration measured using the standard method [9]. The yield of the extracted DNA was in range of (490-570) µg per ml of fungal growth with purity of (1.7-1.9).

ISSR-PCR Analysis

In this study, ISSR-PCR technique was used to reveal the genetic diversity between different studied *A. flavus* isolates, in order to search the polymorphisum that could be used as a DNA marker for diagnosis of *A. flavus* isolates and study the differences that come from environment . ISSR markers was calculated in order to characterize the capacity of each primer to detect polymorphic loci among the genotypes. Three primers used to detect this diversity were ordered from University of British Columbia (UBC), [7]. Figure (2) was showed PCR product with UBC primer No.809,810,811.

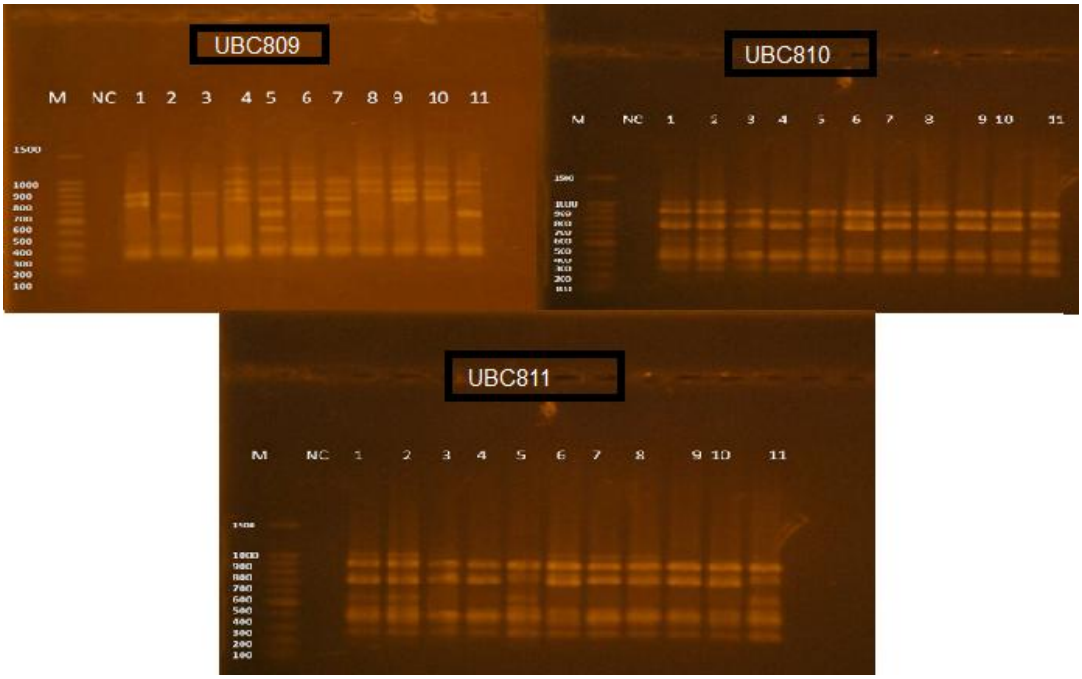

Figure 2- PCR product with UBC primer No.810 on 1.2% agarose gel bromide. M: 100 bp DNA ladder. N: negative control. . Isolates(1,2 from rice),(3,4,5,6,7,8,9,10,11)from maize.

Among the three decamer oligonucleotide primers, primers varied greatly in their ability to resolve variability among *A. flavus*. Some primers generated several bands, while others

generated a few bands. Table-1 showed that three primers (UBC809,UBC810,UBC811) produced total of (18) useful main bands across 11 isolates, from these (18) PCR products generated (7) were monomorphic main band across all isolates , one unique main band ,The remaining (10) main bands of the total products scored were polymorphic among the studied isolates.The UBC primer No.809 gave the highest number of polymorphic bands (35), while the minimum number of polymorphic bands (9) by using UBC primer No. 811.[7] used same primers and agree with present study. Polymorphism of each primer was calculated as the percentage of polymorphic bands to the number of total bands produced by the designated primer[17]. Polymorphism ranged between (16.98-74.45%), primer UBC primer No.809 produced the highest percent of polymorphism compared with primer UBC primer primer efficiency represented the ability of primer to appear polymorphic between isolates and not means gave high number of amplified bands. Primer efficiency of each primer ranged between (0.16-0.76) UBC primer No. 811 gave the minimum efficiency (0.16) this primer from the primers that produced monomorphic bands therefore not have higher efficiency, while the UBS primers No.809 gave the highest efficiency (0.74) these primers appeared height polymorphic between the isolates in our study. The value of discrimination power represented the ability to descry and diagnosis of the isolates, The UBC primer No.809 gave the highest percentage of Discrimination power (64.8%) while the UBC primer No.811 gave the lowest percentage (16.6%).Table (1) shows number of isolates, total number of bands, polymorphic bands , percentages of polymorphism among isolates, primer efficiency and Discrimination power to each primer.

Table 1- Distinct characteristic of ISSR primers included in the study: primer's name, total number of bands,number of polymorphic bands, percentage of polymorphism , Primer efficiency and Discrimination value.

No.	Name	Total number of main bands	Number of polymorphic bands	Polymorphism %	Primer efficiency	Discrimination power
1	**UBC primer No.809**	**7**	**35**	**74.4%**	**0.74**	**64.8%**
2	**UBC primer No.810**	**5**	**10**	**31.25%**	**0.31**	**18.5%**
3	**UBC primer No.811**	**6**	**9**	**16.98%**	**0.16**	**16.6%**

The total number of bands generated by each primer varied, UBC primer No.811 generated maximum number of bands (53) while UBC primer No. 810 amplified minimum number of bands (32), The size of the amplified fragments had ranged from (200 bp to 1,200 bp).

Analysis of Genetic Distance

The genetic distance among *A. flavus* isolates was calculated using genetic ~~prog~~ram (Numerical Taxonomy and Multivariate Analysis System Version 1.80 package) depending on shared bands between each variety when increasing bands number that lead to decreasing of genetic distance and vice versa. The values of genetic distance of *A.flavus* isolates studied and determined the genetic distance between 11 *A.flavus* isolates this occurs through intersection between each of the two isolates. The highest value of genetic distance occurs between isolates (2,9) :0.93644 and the lowest value between (10,6):0.12599.

Cluster analysis

Dendrogram was constructed genetic distance using UPGMA cluster analysis and depicted genetic relationships among 11 isolates of *A.flavus*[12], Figure (3) showed the four major clusters.

The first main group:

Included three subgroup, the first subgroup contains the isolate number 3 , the second sub group contains two sub group the first one contains isolates number '4 and 10' and the second one contain the isolate number،6,9; he third subgroup contains two sub sub group first one contains isolate number 7 and the second one contain isolate number 11,8.

The second main group:

This group included the isolate number 5.

The third maingroup:

Included two subgroups, the first subgroup contains the isolates number 2 while the second subgroup contains the isolates number 1.

Figure-3 was explained the convergence and divergence between (11 isolates) isolated from different source , isolate number 1 and 2 isolated from Rice so these isolates are near from each others, remaining isolates was isolated from maize that occured away from the first two. Convergence among *A. flavus* isolates came from many different reason like source of Isolation , similarity in morphological characteristic , Genetic characteristic and other character.

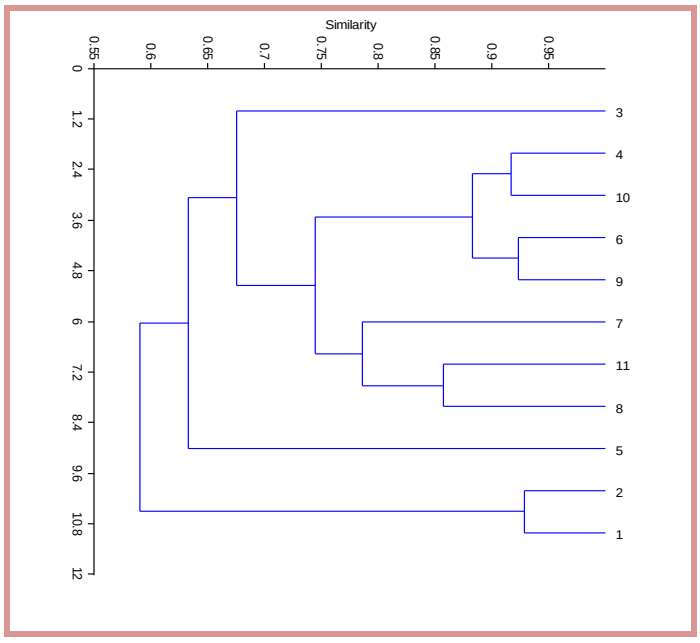

Figure 3-Dendrogram illustrated genetic fingerprint and relationships between *A. flavus* isolates developed from ISSR data

RAPD technique with the same programming of cluster analysis and genetic distance was used to make Investigation on genetic diversity, relationships and attempt to find the DNA fingerprint of isolates, in order to identify the various isolates and studying genetic distance between *A. fumigatus* isolates [18].There are many studies indicated that ISSR – PCR is depended on the sensitive reaction condition , optimization of PCR condition is necessary to get the highest specificity and product yield[19,20], Optimization of PCR condition , including temperature , number cycle , Reagent and other parameters all these are very necessary to get successful ISSR-PCR reaction[13].The analysis of ISSR-PCR and variation between eleven isolates including the absence or presence of bands, differences in molecular weight and intensity of amplified bands[21]. variation in the number of bands amplified by different primers influenced by variable factors such as primer structure and the annealing sites in the genome[22] , so in the present study was used three different structural primer for reveal the genetic diversity between (11) *A.flavus* isolates and appears high rate variation between them, but the intensity of the bands was not taken into account because of the DNA concentration was not controlling exactly at 50 ng for each sample, which means the

number of copies of DNA template was not the same for each sample, that may affected the intensity of the resulted bands, In this study a unique band , polymorphic band were generated, unique bands means that presence of band in specific variety and absence of the same band of the same size in all other varieties , They further suggested the application of unique markers in DNA typing and variety identification, presence of numerous unique markers in scented rice germplasm and Egyptian rice varieties important in variety identification[23,24] .High rate of polymorphic band led to Polymorphism percentage due to the polymorphism of each primer was calculated as the percentage of polymorphic bands to the number of total bands produced by the designated primer[17] .

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