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## RESEARCH ARTICLE

# GENETIC DIVERSITY OF TOXIGENIC AND NON-TOXIGENIC *ASPERGILLUS FLAVUS* STRAINS USING ISSR MARKERS

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### ABSTRACT

Aflatoxins, the most frequently studied mycotoxins, are produced by certain *Aspergillus* species/strains/isolates of fungi. The aflatoxin biosynthetic pathway studies have led to a number of discoveries. Several structural and their enzymes involved in the biosynthesis of aflatoxins have been discovered and purified. Aflatoxin production and contamination of agricultural crops are major causes of economic losses in agriculture. Thus, better methods of characterization/differentiation are required for both aflatoxigenic and non-aflatoxigenic isolates.

Polymerase Chain Reaction (PCR)-based, single sequence repeats (SSR) micro satellites 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 ranging applications in the field of genetics including kinship and population studies. These Inter simple sequence repeats (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.

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## INTRODUCTION

Aflatoxins are carcinogenic, teratogenic and immunosuppressive secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin contamination of oil seeds is one of the most important constraints to oil seed production worldwide. Aflatoxins are polyketide-derived toxic secondary metabolites produced by *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare (Bennett and Christensen 1983). Aflatoxins are hepatocarcinogenic, mutagenic and teratogenic (Moreno and Kang 1999). There are more than ten compounds named as aflatoxins (Ong 1975), the important ones being B1, B2, G1, G2, M1 and M2. Aflatoxins B1 and B2 are produced by *A. flavus*, where as B1, B2, G1 and G2 are produced by *A. parasiticus* (Ong 1975).

Lillehoy *et al.*, (1976) studied aflatoxin production in case of corn seeds due to association of *A. flavus* and *A. parasiticus*. They also stated that simply presence mould growth was not found to be an indicative for the presence of aflatoxin. While, Diener and Davis (1966) stated that all strains of *Aspergillus flavus* were not found to be aflatoxin producers as they had screened nearly 1400 strains which were isolated from different sources but only 58% strains were reported to be aflatoxigenic, Boller

and Shroeder (1966) found 94% among 284 isolates of *A. flavus* were found to be capable of aflatoxin production in rough rice. Cooke *et al.*, (1998) studied interaspecific and interspecific variation. Waals *et al.*, (2004) also studied genetic diversity among *A. solani* isolated from potato in South Africa. He further reported that isolates collect from different geographical region show relative high level of diversity amongst the isolates.

The methods for detecting and assessing genetic diversity have extended from analysis of discrete morphological traits to biochemical and molecular traits. Several DNA marker systems are now common use in diversity studies of plants. The most commonly used marker systems are restriction fragment length polymorphism (RFLP) (Soller and Beckmann, 1983), random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), inter simple sequence repeats (ISSRs) (Zietkiewicz *et al.*, 1994) and microsatellites or simple sequence repeats (SSRs). Among them to characterize DNA variation patterns within species and among closely related taxa in *Vigna* species have been RAPD (Ba *et al.*, 2004; Dikshit *et al.*, 2007), AFLP (Sivaprakash *et al.*, 2004; Fang *et al.*, 2007), RFLP (Kaga *et al.* 2000), ISSR (Ajibade *et al.*, 2000), SSRs and sequence tagged microsatellite site (STMS) (Phansak *et al.*, 2005).

Primers based on a repeat sequence and the resultant PCR reaction amplifies the sequence between two SSRs, yielding a multilocus marker system useful for

fingerprinting, diversity analysis and genome mapping. PCR products are separated on a agarose gel prior to autoradiographic visualization under ultra violet light which concludes that ISSR would be a better tool for genetic divergence and phylogenetic studies. Nagaoka and Ogihara (1997) have also reported that the ISSR primers produced several times more information than RAPD markers in wheat.

## MATERIAL AND METHODS

Different oil seeds such as groundnut (*Arachis hypogea* L.), sunflower (*Helianthus annuus* L.) and soybean (*Glycine max* L.) were collected from market places, store houses, fields from different parts of Marathwada region of Maharashtra state. These seeds were then packed in pre-sterilized polythene bags. Mycoflora associated with groundnut seeds were detected using agar plate method (ISTA, 1996). Plates were then allowed to incubate at room temperature for seven days. On seventh day of incubation the seeds were examined under stereoscopic microscope for the preliminary determination of fungal growth on them. *Aspergillus flavus* isolates were screened out and pure culture are maintained on PDA medium.

### Screening of *Aspergillus flavus* isolates for Aflatoxin producing ability

Isolates of *Aspergillus flavus* obtained were screened for their Aflatoxin-producing potentials in SMKY liquid media (Sucrose- 200gm, Magnesium sulphate- 0.5gm, Potassium nitrate- 3gm, yeast extract- 7dm and distilled water 1000ml). *Aspergillus flavus* isolated from different oil seeds were tested for their toxicity by ammonia vapor test (Fente *et al.*, 2001). Ammonia vapors treatment method is inexpensive, less time consuming and ideal for pre screening of large numbers of *Aspergillus flavus* isolates to identify Aflatoxin producing isolates turned into Pink/Red, from creamy or yellow colour after the treatment of Ammonia vapours. But no colour change occurred in the non toxic isolates. Various degrees of colour development (i.e pink, moderate, red and dark red) were observed within 10-20 min. of treatment and as a result the strains were categorized as highly, moderate and mildly toxigenic.

### DNA extraction

12 different strains of *Aspergillus flavus* which were isolates from different oil seed were grown separately in glucose nitrate medium contained in 100 ml flasks and grown at 25 °C for 7 days in an orbital shaker (120 rpm). Mycelium was harvested by filtration through Whatman filter paper No. 3 and immediately frozen in liquid nitrogen. The frozen mycelium was pulverized, freeze-dried and ground to a fine powder using a sterile mortar and pestle. The mycelia powder was stored at 20°C.

### DNA Amplification condition and gel electrophoresis

DNA was isolated from ground fine powder of mycelium using D. Neasy Plant mini kit (Quigen, USA) by following the protocol modified by Liu *et al.* (2000).

### PCR

PCR technique has promoted the development of a range of molecular assay systems which detect polymorphism at molecular level. In this study we used the most widely adopted PCR based ISSR marker technology for characterizing *Aspergillus flavus*.

The PCR amplification was carried out in 200 µl reaction mixture tube containing 10×PCR buffer (2.0 µl),

20 ng of genomic DNA (3 µl), 2 µl of UBC ISSR primer (3 pmol/µl), 10 mM dNTPs (2.0 µl), 3U/µl *Taq* DNA polymerase (0.4 µl), Tween 20 (0.2 µl). The final volume was made to 20 µl by adding SMQ. PCR reactions were carried out in a Finnzyme make thermal cycler.

PCR reactions were carried out in a Finnzyme make thermal cycler using ISSR primers. Three ISSR primer sets were ordered from University of British Columbia (UBC). The primer sequences are as follows.  
Primer 809 - AGA GAG AGA GAG AGA GG  
Primer 810 - GAG AGA GAG AGA GAG AT  
Primer 811 - GAG AGA GAG AGA GAG AC

PCR amplified product separated on 1% agarose gel. Gel stained using ethidium bromide and visualized under the UV Transilluminator. For each sample, each fragment / band that was amplified using ISSR primers was treated as a unit rearrangement in genome. The primers which were given scorable and consistently reproducible amplicons were considered. The gel pictures were taken and documented to computer by using Alpha Imager gel documentation system and size of each amplicon was measured by using Alpha Imager Software with respect to standard molecular weight DNA ladder and molecular weight of each of the potential specific bands was calculated using the software program Alpha Imager (Naik and Taware, 2009).

### Data Analysis

Marker index for ISSR markers was calculated in order to characterize the capacity of each primer to detect polymorphic loci among the genotypes. It is the sum total of the polymorphism information content (PIC) values of all the bands produced by a particular primer. The data obtained by scoring the ISSR profiles with different primers individually as well as collectively were subjected to the construction of similarity matrix using Neighbor-joining tree construction method of Nei and Li/Dice. The similarity values were used for cluster analysis. Data analysis was done using Free Tree and Tree View bioinformatics software.

## RESULTS AND DISCUSSION

### Screening of Aflatoxin production

The oil seed collected from different locality were classified into four different abnormal category (Shrunken, Under sized, Discolored, cracked). Five different *Aspergillus* species were isolated from these abnormal oil seed category i.e. *Aspergillus flavus*, *A. niger*, *A. ustus*, *A. glaucus* and *A. terreus*, out of which *Aspergillus flavus* and *A. niger* were found to be more dominant.

From Table 1 it is clearly observed that 12 *Aspergillus flavus* strains were isolated from different oil seed, out of which four isolate were found to nontoxic (GnAF4, SoAF1, SoAF2 and SoAF3) and eight isolates were found to be toxic. From these eight toxic isolates, GnAF2 and SuAF3 are found to be highly toxic, GnAF3 and SoAF4 are found to be moderate toxic, were as isolates GnAF1, SuAf1, SuAf2 and A F4 are mildly toxic.

During the present studies, total 03 primers produced the amplification in terms of repeatability and scorability. These 03 primers produced total 28 bands and all 28 bands were polymorphic.



Fig. 1. Toxicity by ammonia vapor test

M 1 2 3 4 5 6 7 8 9 10 11 12

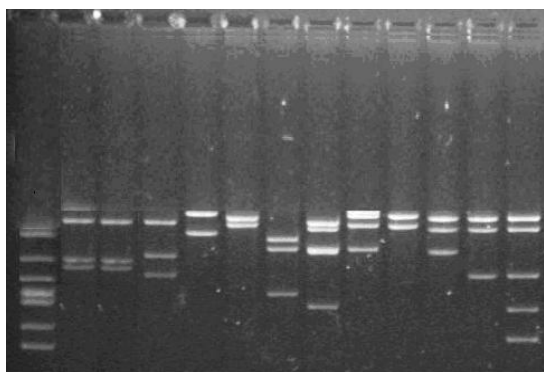


Fig. 2. UBC Primer No. 809

M 1 2 3 4 5 6 7 8 9 10 11 12

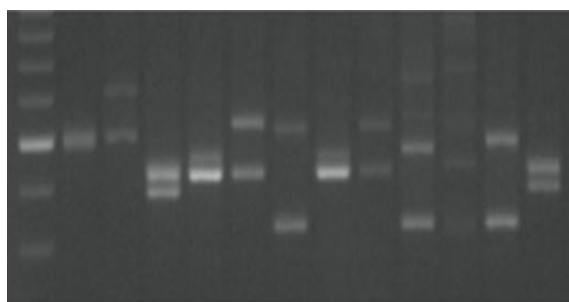


Fig. 2. UBC Primer 810

M 1 2 3 4 5 6 7 8 9 10 11 12

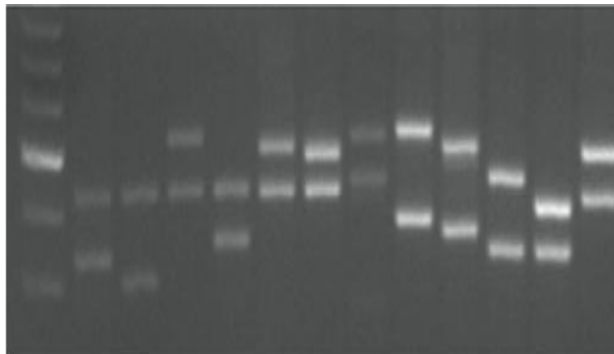


Fig. 3. UBC Primer No. 811

Table 1. Aflatoxin detection by Rapid technique

Isolates	Category	Aflatoxin
<b>Groundnut</b>		
Gn. A F1	Us	+
Gn. A F2	Dc	+++
Gn. A F3	Dc	++
Gn. A F4	Sh	-
<b>Sunflower</b>		
Su.A F1	Sh	+
Su.A F2	Us	+
Su.A F3	Cr	+++
Su.A F4	Dc	+
<b>Soybean</b>		
So.A F1	Sh	-
So.A F2	Us	-
So.A F3	Cr	-
So.A F4	Dc	++

Non toxic = -, Moderate toxic = +, Mildly toxic = ++, Highly toxic = +++  
 Sh. = Shrunken, Us.= Under sized, Dc.= Discolored, Cr.= cracked

UBC Primer No. 809 produced 10 polymorphic bands, UBC primer No. 810 produced 09 polymorphic bands and UBC primer no. 811 produced 09 polymorphic bands. An average 9.33 polymorphic bands per primer was produced. The dendrogram analysis divided the total 12 samples into five different clusters. Cluster I comprises of Sample 4, Sample 11, Sample 10 and Sample 9; while cluster II contains Sample 7 and Sample 8 only. Further cluster III possesses Sample 6, Sample 1 and Sample 2. The IVth cluster have Sample 6, Sample 1 and Sample 2. Sample 5 grouped into cluster V showed its separate identity in comparison with other samples.

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**Table. UBC Primer No. 809**

Sample Name	1	2	3	4	5	6	7	8	9	10	11	12
Lane	1	2	3	4	5	6	7	8	9	10	11	12
Fragment size In bp	-	-	-	1100	-	-	-	1100	1100	1100	1100	1100
	1050	1050	1050	-	1050	-	1050	-	-	-	-	-
	-	-	-	1000	1000	-	1000	1000	1000	1000	1000	1000
	-	-	-	-	-	960	-	-	-	-	-	-
	-	-	950	-	-	-	-	-	-	950	-	-
	920	920	-	-	-	920	920	920	-	-	-	-
	830	830	830	-	-	-	-	-	-	-	830	830
	-	-	-	-	-	-	-	630	-	-	-	-
	-	-	-	-	-	-	580	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	380

L Lane M (Marker) –100 bp DNA Marker

**Table. UBC Primer 810**

Sample Name	1	2	3	4	5	6	7	8	9	10	11	12
Lane	1	2	3	4	5	6	7	8	9	10	11	12
Fragment size In bp	-	-	-	-	-	-	-	-	780	780	-	-
	-	700	-	-	-	-	-	-	700	700	-	-
	-	-	-	-	630	630	-	630	-	-	-	-
	600	600	-	-	-	-	-	-	-	-	600	-
	-	-	-	-	-	-	-	-	590	-	-	-
	-	-	-	560	-	-	560	-	-	-	-	-
	-	-	550	550	550	-	550	550	-	550	-	550
	-	-	500	-	-	-	-	-	-	-	-	500
	-	-	-	-	-	460	-	-	460	460	460	-

Lane M (Marker) – 100 bp DNA Marker

**Table. UBC Primer No. 811**

Sample Name	1	2	3	4	5	6	7	8	9	10	11	12
Lane	1	2	3	4	5	6	7	8	9	10	11	12
Fragment size In bp	-	-	-	-	-	-	690	690	-	-	-	-
	-	-	670	-	670	670	-	-	670	-	-	670
	-	-	-	-	-	-	-	-	-	600	-	-
	-	-	-	-	-	-	580	-	-	-	-	-
	550	550	550	550	550	550	-	-	-	-	550	550
	-	-	-	-	-	-	-	500	-	-	-	-
	-	-	-	480	-	-	-	-	480	480	480	-
	460	-	-	-	-	-	-	-	-	-	-	-
	-	410	-	-	-	-	-	-	-	-	-	-

Lane M (Marker) – 100 bp DNA Marker

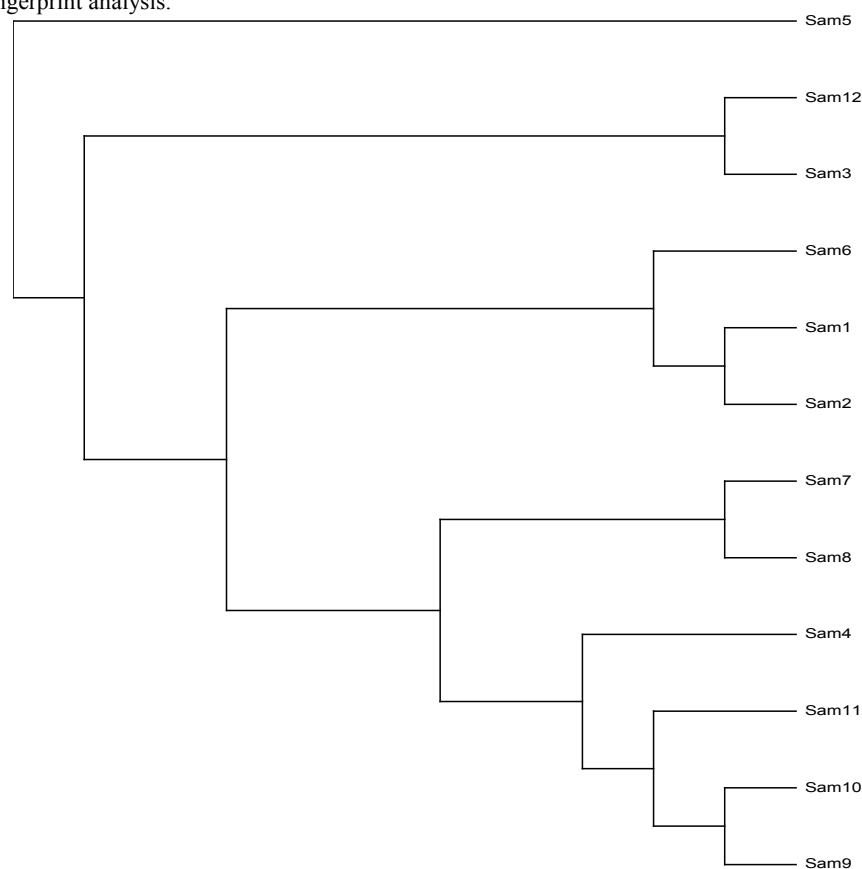
**Distance Similarity Matrix:**

Distance Similarity Matrix was calculated by using Neighbor-joining tree construction method of Nei and Li/Dice.

	Sam1	Sam2	Sam3	Sam4	Sam5	Sam6	Sam7	Sam8	Sam9	Sam10	Sam11	Sam12
Sam1		0.23077	0.57143	0.81818	0.66667	0.66667	0.66667	0.85714	1.00000	1.00000	0.53846	0.69231
Sam2	0.23077		0.60000	0.83333	0.69231	0.69231	0.75000	0.86667	0.86667	0.87500	0.57143	0.71429
Sam3	0.57143	0.60000		0.69231	0.42857	0.71429	0.64706	0.75000	0.75000	0.64706	0.60000	0.33333
Sam4	0.81818	0.83333	0.69231		0.45455	0.81818	0.57143	0.69231	0.69231	0.57143	0.50000	0.50000
Sam5	0.66667	0.69231	0.42857	0.45455		0.50000	0.60000	0.57143	0.71429	0.73333	0.69231	0.38462
Sam6	0.66667	0.69231	0.71429	0.81818	0.50000		0.86667	0.71429	0.71429	0.86667	0.69231	0.69231
Sam7	0.73333	0.75000	0.64706	0.57143	0.60000	0.86667		0.41176	0.76471	0.66667	0.75000	0.75000
Sam8	0.85714	0.86667	0.75000	0.69231	0.57143	0.71429	0.41176		0.75000	0.64706	0.73333	0.73333
Sam9	1.00000	0.86667	0.75000	0.69231	0.71429	0.71429	0.76471	0.75000		0.29412	0.46667	0.73333
Sam10	1.00000	0.87500	0.64706	0.57143	0.73333	0.86667	0.66667	0.64706	0.29412		0.50000	0.75000
Sam11	0.53846	0.57143	0.60000	0.50000	0.69231	0.69231	0.75000	0.73333	0.46667	0.50000		0.57143
Sam12	0.69231	0.71429	0.33333	0.50000	0.38462	0.69231	0.75000	0.73333	0.73333	0.75000	0.57143	

## Dendrogram

The Dendrogram analysis was carried out by using bioinformatics phylogeny tool Free Tree and Tree View of DNA fingerprint analysis.



(Sample1=GnAf1, Sample2= GnAf2, Sample3 =GnAf3, Sample4= GnAf4, Sample5=SuAF1, Sample6= SuAF2, Sample7= SuAF3, Sample8= SuAF4, Sample9=SoAf1, Sample10= SoAf2, Sample11= SoAf3 and Sample12= SoAf4)

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