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Gene deletion patterns in non-aflatoxigenic strains of *Aspergillus flavus*

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Abstract: Fifteen non-aflatoxigenic strains of *Aspergillus flavus*, representing a wide range of geographic regions of Iran (six provinces including Fars, Ardebil, Guilan, Golestan, Kerman and Semnan) and vegetative compatibility groups (VCGs) were collected from corn (*Zea mays* L.), peanut (*Arachis hypogaea* L.) and pistachio (*Pistachia vera* L.) soils and kernels, and were screened for the presence of aflatoxin biosynthesis genes in relation to their capability to produce aflatoxins, targeting the regulatory genes *afIR* and *aflJ*, the structural genes *aflT*, *pksA*,*ver-1*, *omtA*, *omtB*, *aflD*, *ordA*, *verA*, *norA*, *hypA*, *norB*, *cypA*, sugar utilization gene *glcA* and flanking region gene *C3* (5' end) by PCR method. This process resulted in grouping of *A. flavus* strains into twelve different amplification patterns (I-XII), characterized by 10-14 different DNA bands. Our results reveal that aflatoxin biosynthesis regulatory genes (*aflR* and *aflJ*) and the structural gene *hypA* are more important genes to detect non-aflatoxigenic strains of *A. flavus*. For non-aflatoxigenic strains of *A. flavus*, no relationship was observed between the deletion pattern and geographic origin and/or VCG; which may indicate that non-aflatoxigenic strains of *A. flavus* did not originate independently at each locality. It is concluded that the aflatoxin gene cluster variability existing in the non-aflatoxigenic populations of *A. flavus* can be useful for understanding the toxicological risk as well as the selection of biocontrol agents.

Key words: aflatoxin, gene cluster, gene defect, PCR, biosynthesis pathway.

INTRODUCTION

Aflatoxins (AFs) are highly toxic and carcinogenic in animals and humans, leading to hepatotoxicity, teratogenicity, immunotoxicity and even death (Motomura et al. 1999, Wen et al. 2005). Contamination of corn (*Zea mays* L.), peanuts (*Arachis hypogaea* L.) and pistachio (*Pistachia vera* L.) by aflatoxins is a severe economic burden for Iranian growers (Cheraghali et al. 2007, Hedayati et al. 2010, Ghiasian et al. 2011).

Aspergillus section *Flavi* includes three economically important species: *A. flavus*, *A. parasiticus* and *A. nomius*. Even though these species share numerous common features, they differ in a major attribute, their ability to produce AFs. This widely distributed group of aflatoxigenic species is considered a major problem for animal and human health, since those species are able to grow in almost any kind of crop or food.

After the discovery of a gene cluster as the regulator of AF biosynthesis (Brown et al. 1996), the biosynthetic pathway of AFs has been extensively studied, and most of the enzymes and corresponding involved genes have been identified (Ehrlich et al. 2005, Yabe & Nakajima 2004, Yu et al. 2005, Keller et al. 2005, Wen et al. 2005, Cary & Ehrlich 2006). AF biosynthesis requires at least 25 enzymes and two regulatory proteins encoded by contiguous genes in an 80-kb cluster (Scherm et al. 2005, Yu et al. 2005, Bhatnagar et al. 2006).

The functions of most of the genes required for aflatoxin production have been identified by genetic complementation (Motomura et al. 1999, Chang et al. 2000, Yu et al. 2000).

Generally, the AF biosynthesis genes of *A. flavus*, *A. parasiticus* and *A. nomius* are highly homologous, since the order of the genes within the cluster are the same (Ehrlich et al. 2005, Chang et al. 2007). A significant proportion, but not all of the non-aflatoxigenic *A. flavus* isolates have been found to contain various deletions in the AF gene cluster (Ehrlich & Cotty 2004, Chang et al. 2005, 2006)

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which are also common in some strains of *A. oryzae* (Chang et al. 2005, 2006). The loss of the ability to produce AFGs in *A. flavus* seems to result from a deletion in the terminal region of the cluster corresponding to genes *aflF* (= *norB*) and *aflU* (= *cypA*) (Ehrlich et al. 2004).

Molecular techniques have been widely applied to distinguish the aflatoxin producing and non-producing strains of *A. flavus* and related species, through the correlation of presence/absence of one or several genes involved in the AF biosynthetic pathway and the ability/inability to produce AFs. Recently, DNA–based detection systems have been introduced as powerful tools for detecting and identifying the aflatoxin producing fungi (Geisen 1996). The polymerase chain reaction (PCR) is the method of choice for this purpose (Shapira et al. 1996).

Färber et al. (1997) detected the aflatoxigenic strains of *A. flavus* in contaminated figs by performing a monomeric PCR with three sets of primers specific for three structural genes of the AF biosynthetic pathway, *aflD*, *aflM* and *aflO*. Other attempts to develop PCR-based methods for detection of aflatoxigenic fungi (*A. flavus* and *A. parasiticus*) were underway (Chen et al. 2002, Mayer et al. 2003, Scherm et al. 2005, Baird et al. 2006, Lee et al. 2006, Rahimi et al. 2008). PCR-based methods utilize primers for aflatoxin genes not necessarily unique to aflatoxigenic fungi. These methods have not been tested for reproducibility on a number of different contaminated commodities (Bhatnagar et al. 2006).

Reports on deletion patterns of non-aflatoxigenic *A. flavus* strains are different. For instance, the loss of aflatoxin production in strain *A. flavus* 649-1 is associated with large deletions in the aflatoxin gene cluster (Prieto et al. 1996). Non-aflatoxigenic *A. flavus* AF36, widely used in the management of aflatoxin contamination of cotton in Arizona, has a defect in the gene *pksA* (Ehrlich & Cotty 2004). Rodrigues et al. (2009) reported that gene expression of *aflD* in *Aspergillus* section *Flavi* was a good indicator to distinguish between toxigenic and non-toxigenic strains of *A. flavus*. Okoth et al. (2012) tested *A. flavus* and *A. parasiticus* isolates for the presence of *aflD* and *aflQ* genes.

Strains of *A. flavus* show a great variation in their ability to produce aflatoxins. There are a number of *Aspergillus* species that produce aflatoxin but are not classified as section *Flavi* (Cary & Ehrlich 2006). Greatest successes to date in biological control of aflatoxin contamination in both pre- and post-harvest crops have been achieved through application of biocompetitive non-aflatoxigenic strains of *A. flavus* and/or *A. parasiticus* (Yin et al. 2008).

In the current study, we identified genomic deletions of non-aflatoxigenic strains of *A. flavus* based on polymerase chain reaction with the aflatoxin biosynthesis gene cluster analysis.

MATERIALS AND METHODS

Fungal strains

Fifteen non-aflatoxigenic strains of *A. flavus* from a collection of 52 strains from peanut (IRG075, IRG129 and IRG517), corn (IRM074, IRM193, IRM014, IRM211, IRM031, IRM041 and IRM081) and pistachio (IRP049, IRP107, IRP082, IRP144), representing a wide range of geographic regions of Iran and different vegetative compatibility groups (IR1 to IR15; unpublished data) were used for this study (Tables 1 & 2). Morphological characterization of non-aflatoxigenic strains of *A. flavus* was based on seriation on Czapek Yeast Agar (CYA) 25. The observed characteristics on this medium were as follows: conidia ornamentation, conidia size (µm), colony color and sclerotia. The colony diameter (cm) was measured on CZ42. Conidia of *A. flavus* species have relatively thin and finely to relatively rough walls. Their shapes vary from spherical to elliptical, and when grown on Czapek-Dox (CZ), colonies of *A. flavus* are yellowish-green (Klich, 2002, Samson et al. 2004, 2006, Pildain et al. 2008) (Table 1).

Table 1. List of non-aflatoxigenic strains of *Aspergillus flavus*.

Strain	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Colony color on CYA25	AFBs on YES
IRP-049	> 400	b/u	Smooth	1.8	yellowish-green	-
IRP-107	> 400	B	Smooth	1.7	yellowish-green	-
IRP-082	-	B	Smooth	2.5	yellowish-green	-
IRP-144	> 400	B	Smooth	2	yellowish-green	-
IRG-075	> 400	b/u	Smooth	1.5	yellowish-green	-
IRG-129	-	B	Smooth	2.9	yellowish-green	-
IRM-074	> 400	b/u	Smooth	3	yellowish-green	-
IRM-193	-	B	Smooth	2	yellowish-green	-
IRM-014	> 400	n.d.	n.d.	2.8.	yellowish-green	-
IRM-211	> 400	u/b	Smooth	2.6	yellowish-green	-
IRP-179	> 400	B	Smooth	2.7	yellowish-green	-
IRG-517	-	n.d.	n.d.	1.8	yellowish-green	-
IRM-031	> 400	B	slightly rough	2.3	yellowish-green	-
IRM-041	> 400	B	smooth	2.9	yellowish-green	-
IRM-081	-	B	smooth	2.7	yellowish-green	-

-: not detected n.d.: not determined a: size, in µm: average of 15 sclerotia b: u = uniseriate; b = biseriate; u/b = predominantly uniseriate; b/u = predominantly biseriate c: average of 3 colonies, in cm.

Table 2. Non-aflatoxigenic strains of *Aspergillus flavus* used in this study.

Strain isolate	VCGs	Geographical origin	Source	Sclerotium production
IRP-049	IR1	Rafsanjan	pistachio soil	+/L
IRP-107	IR2	Rafsanjan	pistachio soil	+/L
IRP-082	IR3	Damghan	pistachio soil	-
IRP-144	IR4	Damghan	pistachio soil	+/L
IRG-075	IR5	Minoodasht	Peanut soil	+/L
IRG-129	IR6	Astaneh-e Ashrafieh	Peanut soil	-
IRM-074	IR7	Darab	Maize soil	+/L
IRM-193	IR8	Fasa	Maize soil	-
IRM-014	IR9	Parsabad	Maize soil	+/L
IRM-211	IR10	Parsabad	Maize soil	+/L
IRP-179	IR11	Rafsanjan	pistachio kernel	+/L
IRG-517	IR12	Astaneh-e Ashrafieh	Peanut kernel	-
IRM-031	IR13	Parsabad	Maize soil	+/L
IRM-041	IR14	Darab	Maize kernel	+/L
IRM-081	IR15	Darab	Maize kernel	-

+: Sclerotia producer -: Sclerotia non-producer L: Sclerotia >400 µm on CYA25

Aflatoxin analysis

All of the isolates were initially screened for aflatoxigenic ability on yeast extract sucrose agar medium (YES: Yeast Extract 20 g/L, Saccharose 150 g/L, Agar 15 g/L) amended with 0.3% (w/v) methylated β-cyclodextrin (mß-CD) (Fente et al. 2001). All of the non-aflatoxigenic strains of *A. flavus* were re-tested for confirmation of AFs production in AF-inducing YES. Strains were inoculated on Petri dishes containing YES and incubated at 25-27 °C for 7 days in the dark. A Method based on HPLC with fluorescence detection has been suggested to measure aflatoxins B in *A. flavus* isolates (Bragulat et al. 2001). Briefly, extraction of aflatoxin using MeOH/H2O (80:20, v/v) and purification by an immunoaffinity column cleanup were carried out. HPLC system was equipped with an autosampler; a pump (Sykam 2100) and a reverse phase C18 column (Genesis ODS2, 250×4.6 mm, 5 µm), fitted with a precolumn with the same stationary phase and a fluorescence detector (RF-10AXL).

The injection volume was 100 µL. The fluorescence detection was carried out at excitation and emission wavelengths of 365 nm and 435 nm, respectively (detection limit = 0.5 ng/g). A standard solution of AFB1 and AFB2 (Sigma Co.) was used. HPLC grade solvents (methanol and acetonitrile) were used to prepare the AF standards in the sample extraction, and also to prepare the mobile phase. The *A. flavus* aflatoxin producing strain SRKC-G1907 which released only aflatoxins of group B was used as the reference strain.

Isolation of genomic DNA

Total DNA was extracted from mycelia of fungal isolates obtained from 7-day-old cultures grown in YES liquid media according to Prabha et al. (2013) with minor modifications.

The DNA concentration was measured with a UV-Vis spectrophotometer (NanoDrop ND-1000, Wilmington, Delaware USA).

Molecular detection of *A. flavus* isolates

All the non-aflatoxigenic strains of *A. flavus* were detected based on sequence analysis of ITS2 rDNA by using specific primer pair FVAVIQ1: 5'-GTC-GTC-CCC-TCT-CCG-G-3´ and FLAQ2: 5´-CTG-GAA-AAA-GAT-TGA-TTT-GCG-3', as described by Sardiñas et al. (2011). The primer pairs were based on three multicopy ITS2 rDNA target sequences. The primers FLAVIQ1/FLAQ2 yielded an amplicon of 100 bp in *A. flavus*.

Analysis of aflatoxin biosynthesis gene cluster PCR primers

To design oligonucleotide primers, known sequences for *aflT*, *pksA*, *afIR*, *aflJ*, *ver-1*, *omtA*, *omtB*, *aflD*, *ordA*, *verA*, *norA*, *hypA*,*norB-cypA* intergenic region (*norB* and *cypA* genes), *glcA*(sugar utilization gene) and *C3* (flanking region gene, 5'end) were derived from the aflatoxin biosynthetic pathway genes of *A. flavus* AF36 (AY 510455), AF70 (AY 510453), AF13 (AY510451), BN008 (AY 510451.1) and *A. parasiticus* AY371490 were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). Primer pairs designed using OLIGO (version 5.0; National Biosciences) are depicted in Table 3. Some primer sets were based on Chang et al. (2005) and Ehrlich et al. (2005). The housekeeping gene *tub1* coding for ß-tubulin (primer pair tub1-F/tub1-R) was chosen as a system control for PCR (internal amplification control). The PCR products were analyzed by electrophoresis in 1.5% agarose gels, which were stained with DNA green viewer dye (green fluorescent stain, 10 mg/ml).

PCR analysis

The fifteen non-aflatoxigenic strains of *A. flavus* were tested for presence of 14 genes and one intergenic region of aflatoxin biosynthesis gene cluster by PCR amplification in a 25 µl reaction mixture in a Biometra Thermal Cycler (T1 thermocycler; Biometra, Göttingen, Germany).

Table 3. Primers used in this study, target gene, sequence and expected PCR product size.

Primers	Gene(s)	Primer sequence (5' → 3')	PCR product Size (bp)	Reference
aflT-F	*aflT*	tgcggacatctaacgaccat	750	Chang et al.(2005)
aflT-R		aggtcacttcgttcgtgaagg		
pksA-II-F	*pksA*	cagttgctcccaaggagtggt	518	This study
pksA-II-R		gctgggrttctgcatgggtt		
aflR-II-F	*aflR*	aaccgcatccacaatctcat	794	This study
aflR-II-R		gcagttcrctcagaacragctg		
aflJ-II-F	*aflJ*	cttcaacaacgaccmaaggtt	788	This study
aflJ-II-R		tcggttgtcatcgttatcca		
aflM-II-F	*ver-1*	agccaaagtcgtggtkaact	786	This study
aflM-II-R		ccatccaccmcaatgatct		
aflP-II-F	*omtA*	ctcctcwaccagyggcttcg	593	This study
aflP-II-R		caggatatcattgtggaygg		
aflO-III-F	*omtB*	acttggcattcygaataggc	643	This study
aflO-III-R		aacccasaataggtcgcatc		
aflD-II-F	*nor1*	accgctacgccggcrctctcggcac	400	This study
aflD-II-R		gttggccgccagcttcgacactccg		
aflQ-II-F	*ordA*	ttaaggcagcggaatacaag	719	This study
aflQ-II-R		gacgsccaaagccraacacaaa		
aflN-II-F	*verA*	ccgcaacaccacmaagtagca	424	This study
aflN-II-R		aaacgctctccaggcmcctt		
aflE-F	*norA*	gtgttcgtgtgtcgcccttа	770	Chang et al. (2005)
aflE-R		gtcggtgcttctcatcctga		
aflY-F	*hypA*	gcatgtccgtcgtcctgata	654	Ehrlich et al. (2005).
aflY-R		cccattgatcaatctcggat		
C3-F	*C3*	tctggagtcggaggttaggtt	544	Chang et al. (2005)
C3-R		gagcaacacgatcattgcat		
glcA-F	*glcA*	aagacacagtcatcgcctgtt	745	Chang et al.(2005)
glcA-R		acgcctttatcgagccaata		
norB-cypA-F	*norB, cypA*	gtgcccagcatcttggtcca	300,800,1800	Ehrlich et al.(2004)
norB-cypA-F		aggacttgatgattcctcgtc		
Tub1-F	*tub1*	gtccggtgctggtaacaact	902	Chang et al.(2005)
Tub1-R		ggaggtggagtttccaatga		

DNA amplification conditions

Fourteen aflatoxin clustered genes and the intergenic region *norb-cypA* were amplified using the primers at a concentration of 10 pmol/µl, 50 ng template DNA, 50 µmol of each of the four dNTPs and 5 units of Taq polymerase (BioNeer Inc., Korea) in a total volume of 25 µl containing 10× assay buffer (1× contains 10 mmol/l Tris-HCl, pH 8.8 at 25˚C, 50 mmol/l KCl, 1.5 mmol/l MgCl2). PCR conditions were as follows: denaturation at 95˚C for 2 min; 30 cycles of 94˚C for 60 sec, primer-specific annealing temperature at 45˚C for 60 sec, extension at 72˚C for 90 sec and a final extension at 72˚C for 5 min. The reaction was carried out in the Biometra Thermal Cycler.

Gel electrophoresis

The PCR products were resolved by electrophoresis in a 1.5% agarose gel in 0.5X TBE buffer. The amplified products were visualized under UV transilluminator (UV solo TS gel documentation system, Biometra Co.) and compared with a standard DNA size marker (Thermo Fisher Scientific Inc., Fermentas, Germany).

RESULTS

Aflatoxin analysis

In our study, absence of fluorescence on mß-CD was correlated with no AFs production (determined by HPLC). This medium did not yield any false-negatives. In the other words, all the *A. flavus* strains had already been characterized for their aflatoxigenic ability, after mycelium collection YES broth were analyzed by reverse-phase HPLC to confirm the AF production. Since AF production is extremely dependent on growth conditions, it was important to determine aflatoxigenic ability under the current test conditions.

Molecular detection of *A. flavus* isolates

All the fifteen non-aflatoxigenic strains of *A. flavus* had the species specific gene, and were therefore confirmed as *A. flavus* (Fig 1).

Deletions in the aflatoxin gene cluster

Oligonucleotide primer sets (Table 3) targeted PCR products of 0.3–1.8 kb (300-1800 bp). In the present study, additional set of primers specific for the *aflR*, *pksA*, *aflJ*, *ver1*, *omtA*, *omtB* and *aflD* genes were also used. The PCR results are summarized in Table 4. On the basis of PCR assay, we grouped the fifteen non-aflatoxigenic strains of *A. flavus* into deletion patterns. The genomic deletions were identified in all the fifteen *A. flavus* strains examined, resulting in loss of parts of genes from the aflatoxin gene cluster (Table 2). Based on the banding patterns, twelve deletion patterns (I-XII), designated as A to L were detected among the non-aflatoxigenic strains of *A. flavus*. The deletion patterns of examined non-aflatoxigenic strains of *A. flavus* are shown in Table 5. The non-aflatoxigenic strains of *A. flavus* were placed into five groups based on their number of

amplified genes (Table 6). Two strains IRP049 and IRP082 from pistachio have identical deletion patterns (C and I, respectively, Table 6). Deletion patterns were mainly in the left side of aflatoxin gene cluster (*hypA*, about 10-15 kb) and regulatory genes *aflR* and *aflJ* (approximately 35 kb).

Three independent deletions as type I, type II and type III were found in the *norB-cypA* region (Table 4). Only in *A. flavus* strain IRG75 from peanut the *norB* and *cypA* (*norB-cypA* intergenic region, deletion pattern type III) were not amplified. In addition, in twelve strains of *A. flavus* from pistachio and maize and two peanut strains (IRG129, IRG517), the fragments 0.8 and 0.3 kbp were amplified by primer pair norB-cypA-F/norB–cypA-R. Individual VCGs contain isolates from different deletion patterns (Table 6).

DISCUSSION

In this study, 15 of the 52 non-aflatoxigenic strains of *A. flavus* collected from soil and kernel of peanut, maize and pistachio belonging to various geographical regions and distinct VCGs were analyzed for aflatoxin gene cluster deletion patterns. The 15 non-aflatoxigenic strains of *A. flavus* were selected in this study, because they had proved to produce polymorphic DNA fragments based on microsatellite primed-PCR (MP-PCR) marker and different VCGs in previous studies (Houshyarfard et al. 2014).

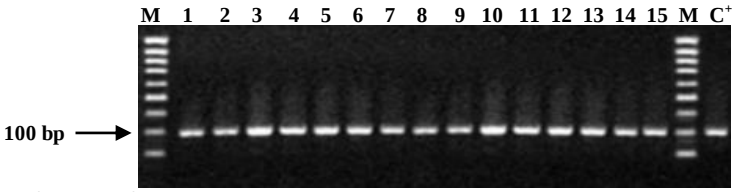

Fig.1. Specific amplification of internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) of extracted genomic DNA electrophoresed in 1.5% (w/v) agarose gel from fifteen non-aflatoxigenic strains of *Aspergillus flavus*. C^+ = positive control, M = Molecular marker (50 bp DNA Ladder, Fermentas).

Table 4. Prevalence of aflatoxin-associated genes among fifteen Iranian non-aflatoxigenic strains of *A. flavus.*

		Genes**														
Strain	Source*	*aflT*	*pksA*	*aflR*	*aflJ*	*ver1*	*omtA*	*omtB*	*aflD*	*ordA*	*verA*	*norA*	*hypA*	*C3*	*glcA*	*norB-cypA*
IRP-049	S/P	+	-	+	+	+	+	+	+	+	+	+	+	+	+	II
IRP-107	S/P	-	+	-	+	+	+	+	+	+	+	+	+	+	+	II
IRP-082	S/P	+	+	-	-	+	+	-	+	+	-	+	-	+	+	II
IRP-144	S/P	-	+	-	-	+	+	+	+	+	+	+	-	+	+	II
IRG-075	S/G	+	+	-	+	+	+	+	+	+	+	+	-	+	+	III
IRG-129	S/G	+	+	-	-	+	+	+	+	+	+	+	-	+	+	I
IRM-074	S/M	+	+	-	+	+	+	+	+	+	+	+	-	+	+	II
IRM-193	S/M	+	+	-	+	+	+	+	+	+	+	+	-	+	+	II
IRM-014	S/M	+	+	-	+	+	+	+	+	+	+	+	-	+	+	II
IRM-211	S/M	+	+	-	-	+	+	+	+	+	+	+	-	+	+	II
IRP-179	K/P	+	-	-	+	+	+	+	+	+	+	-	-	+	+	II
IRG-517	K/G	+	+	+	+	+	+	+	+	+	+	+	-	-	+	I
IRM-031	S/M	+	+	-	-	+	+	+	+	+	-	+	-	-	+	II
IRM-041	K/M	+	+	-	+	+	+	+	+	+	+	-	-	+	+	II
IRM-081	K/M	+	+	-	-	+	+	+	+	+	+	+	-	+	+	II
SRKC-G1907	Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*: S : Soil K: Kernel P: Pistachio G: Peanut M: Maize +: present -: absent Isolate name in bold indicates the aflatoxin producer.

Table 5. Deletion patterns (A-L) in the aflatoxin gene cluster of non-aflatoxigenic strains of *A. flavus*.

Deletion pattern	*C3*	norB-cypA	*aflT*	*pksA*	*aflD* (*nor1*)	*aflR*	*aflJ* (*afls*)	*aflE* (*norA*)	*aflM* (*ver-1*)	*aflN* (*verA*)	*aflO* (*omtB*)	*aflP* (omtA)	*aflQ* (ordA)	*aflY* (*hypA*)	*glcA*
A	○	I	●	●	●	●	●	●	●	●	●	●	●	○	●
B	●	I	●	●	●	○	○	●	●	●	●	●	●	○	●
C	●	II	●	○	●	●	●	●	●	●	●	●	●	●	●
D	●	II	○	●	●	○	●	●	●	●	●	●	●	●	●
E	●	II	●	●	●	○	●	●	●	●	●	●	●	○	●
F	●	II	●	●	●	○	●	○	●	●	●	●	●	○	●
G	●	II	●	●	●	○	○	●	●	●	●	●	●	○	●
H	●	II	●	○	●	○	●	○	●	●	●	●	●	○	●
I	●	II	●	●	●	○	○	●	●	○	○	●	●	○	●
J	○	II	●	●	●	○	○	●	●	○	●	●	●	○	●
K	●	II	○	●	●	○	○	●	●	●	●	●	●	○	●
L	●	III	●	●	●	○	●	●	●	●	●	●	●	○	●

Filled and empty circles indicate positive and negative PCR products in Iranian strains, respectively. Original gene names are above and new names are below (Yu et al. 2004). Types I, II and III deletions in the *norB-cypA* region indicate 0.3 kb, 0.8 kb and no product, respectively. *C3* and *glcA* genes are in the flanking and sugar utilization regions of the aflatoxin gene cluster.

Table 6. Grouping of non-aflatoxigenic strains of *A. flavus* based on the number of amplified genes and one intergenic region.

Group	No. of amplicons	Strains	Deletion pattern
1	14	IRP049	C
2	13	IRP107, IRM193, IRM074, IRM014, IRG517	A,D,E
3	12	IRM041,IRM081,IRM211,IRG129,IRG075	B,F,G,L
4	11	IRP179,IRP144	H,K
5	10	IRM031, IRP082	I,J

It is to be emphasized that the PCR detection of *A. flavus* is no guarantee of aflatoxin production, since genes other than those involved in the biosynthesis of aflatoxins are not targeted for amplification. Bearing in mind that our molecular studies were applied to a limited number of non-aflatoxigenic strains of *A. flavus* and that it was not our goal to study barcoding sequences, our results strengthen the hypothesis that the ITS region is suitable for the identification of *A. flavus* isolates.

Analysis of deletions within the aflatoxin biosynthesis gene cluster for the fifteen Iranian non-aflatoxigenic strains of *A. flavus* revealed that *A. flavus* strains had different deletions in the aflatoxin gene cluster. The detection of mycotoxigenic fungi has relied, for the most part, on the time consuming isolation and culturing techniques that require taxonomical expertise (Bhatnagar et al. 2006). The non-aflatoxigenic strains of *A. flavus* lacked 3-5 different genes (the aflatoxigenic strain of *A. flavus* used as a positive control had all of the genes). The deletion patterns observed in this study have not been previously reported. Some researchers reported that the analysis of deletion patterns in aflatoxin gene cluster was a useful marker for the identification of non-aflatoxigenic strains (Kusumoto et al. 2000, Ehrlich et al. 2005).

The variable amplicons of genes from aflatoxin biosynthesis gene cluster were produced by the non-aflatoxigenic strains of *A. flavus* from pistachio (10, 11, 13 & 14), maize (10, 12 & 13) and peanut (12 & 13), soil and kernels. The *pksA*, *aflT* and *omtB* amplicons were not detected in pistachio non-aflatoxigenic strains of *A. flavus*. The only product amplified in the fifteen strains of *A. flavus* was *C3* which belonged to pistachio strains. The *C3* is the sugar utilization genes in the flanking region of the aflatoxin gene cluster. Donner et al. (2010) suggested that remnants of the aflatoxin gene cluster are not necessary for the isolates to exclude aflatoxin producers during the host infection, effectively. The genes *ver-1*, *nor-1*, *ordA*, *omtA* and *glcA* were present in all of the non-aflatoxigenic strains. The *glcA* is a gene in the sugar utilization cluster adjacent to the 3´end of the aflatoxin cluster.

Scherm et al. (2005) studied 13 strains of *A. parasiticus* and/or *A. flavus* and found consistency of *aflD*, *aflO* and *aflP* genes in detecting AF production ability, further indicating them as potential markers. The *aflD* gene encodes an enzyme that catalyzes the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid to averantin in *A. flavus* (Papa 1982), while the *aflQ* gene is involved in the conversion of *O*-methylsterigmatocystin (omst) to aflatoxin B_1 (AFB1) and aflatoxin G_1 (AFG2), and also dihydro-*O*-methylsterigmatocystin (dmdhst) to aflatoxin B_2 (AFB2) and aflatoxin G_2 (AFG2) in *A. parasiticus* (Cleavland 1989) and *A. flavus* (Yu et al. 1998). It was assumed that the lack of amplicons revealed an evidence of the genetic variability for non-aflatoxigenic strains of *A. flavus*. It should be noted that aflatoxin biosynthesis pathway is highly complex, and just the key genes directly related to aflatoxin biosynthesis are useful for analyses of aflatoxin gene cluster. Therefore, the genes encoding the key enzymes necessary for aflatoxin production are used in this study.

It was assumed that non-aflatoxigenicity of our *A. flavus* strains might be associated with no DNA amplification. In the other words, the lack of aflatoxin production in the strains of *A. flavus* may be due to the lack of genes in their genome. Deletion of portions of the aflatoxin biosynthesis gene cluster within atoxigenic *A. flavus* strain is not rare (Chang et al. 2005). The loss of aflatoxin production by Iranian non-aflatoxigenic strains of *A. flavus* is not well understood. It should be considered that the lack of AFB production in non-aflatoxigenic strains of *A. flavus* does not clearly specify that their inability is only due to the partial loss of aflatoxin gene cluster (small deletions). Our findings should be demonstrated by other molecular methods as well as positive-negative PCR. For example, although *A. oryzae* strains have the aflatoxin biosynthesis gene cluster, it is not functional (Tominaga et al. 2006). Several reports have demonstrated that the risk of loss of gene, DNA recombinations, DNA inversions, partial deletions, translocations and other genomic disorders in the aflatoxin gene cluster are associated with proximity to the telomere region of fungal chromosome (Carbone et al. 2007). The aflatoxin biosynthesis genes in *A. oryzae* contain deletions, frame-shift mutations and base pair substitutions that explain the lack of aflatoxin production (Tominaga et al. 2006). Until recently, there have been very few verifiable reports of deletion patterns from aflatoxin biosynthesis gene cluster in the Iranian non-aflatoxigenic strains of *A. flavus*. Chang et al. (2005) reported eight deletion patterns (A to H) in the

aflatoxin genes of 38 non-aflatoxigenic strains of *A. flavus*. They supported the hypothesis that deal with the relationship between the inability to produce aflatoxin and a partial or entire gene deletion or mutations in the aflatoxin gene cluster of non-aflatoxigenic strains of *A. flavus*. Criseo et al. (2001) reported that 85 of 134 non-aflatoxigenic strains of *A. flavus* had numerous deletions in aflatoxin gene cluster. Yin et al. (2009) showed that 24 of 35 isolates containing no detectable aflatoxins had the entire aflatoxin gene cluster. Eleven non-aflatoxigenic isolates had five different deletion patterns in the cluster. Mauro et al. (2013) detected six deletion patterns in the aflatoxin biosynthesis gene cluster of Italian non-aflatoxigenic strains of *A. flavus*. No deletions in the cluster were detected for twelve non-aflatoxigenic isolates and ten had the entire cluster deleted.

The presence of two strategic genes of the AF biosynthetic pathway, *aflR* and *aflJ* was associated with the aflatoxigenic ability of our isolates. Our findings revealed that aflatoxin biosynthesis regulatory genes (*aflR* and *aflJ*) and the structural gene *hypA* are more important genes to detect and identify Iranian non-aflatoxigenic strains of *A. flavus*.

The *aflR* and *aflJ* genes play an important role in the aflatoxin biosynthetic pathway by regulating the activity of other structural genes such as *omt-A, ver-1* and *nor-1* (Bennett & Klich 2003, Yu et al. 2005).

The *aflJ* gene which is adjacent to *aflR* is necessary for expression of other genes in the aflatoxin cluster (Chang 2003). However, these findings are not according to some reports. These reports indicated that *aflR* gene could not distinguish and differentiate between aflatoxigenic and non-aflatoxigenic strains of *A. flavus* (Rodrigues et al. 2009). Gallo et al. (2012) studied the two regulatory genes (*aflR* and *aflJ*) and five structural genes (*aflD, aflM, aflO, aflP* and *aflQ*) of non-aflatoxigenic strains of *A. flavus* from maize, and reported four different groups (I-IV) based on the gene amplification patterns. Erami et al. (2007) differentiated the aflatoxigenic strains among fourteen *A. flavus* strains using three structural genes *ver-1*, *nor*-1, *omt-1* and one aflatoxin biosynthesis regulatory gene (*aflR*). Other reports suggest that DNA banding patterns of non-aflatoxigenic strains of *A. flavus* are variable based on lack of the genes *aflP, aflM, aflD* and *aflR* (Criseo et al. 2008). Kale et al. (2007) showed that the regulation of aflatoxin biosynthesis in some strains of *A. parasiticus* was not due to lack of defects in three aflatoxin regulatory genes *aflR, aflJ* and *laeA*. It is likely that secondary metabolic pathways in these types of *A. parasiticus* strains were not associated with *aflR* and *aflJ* genes and were independent of other aflatoxin positive regulators. So far, the molecular mechanisms responsible for the lack of aflatoxin production in some strains of *A. flavus* have not been known (Schmidt-Heydt et al. 2009).

In addition, the *norB* and *cypA* (*norB-cypA* intergenic region) amplicons were not detected in peanut strains of *A. flavus*. Yin et al. (2009) showed that fragments 0.3 and 0.8 kbp were amplified in fifteen *A. flavus* strains by the primer pair norB-cypA-F/norB–cypA-R.

Products from *verA* and *norA* were amplified from peanut strains of *A. flavus*. The *hypA* amplicon was detected in almost all the non-aflatoxigenic strains of *A. flavus* (except for pistachio strains IRP049 and IRP107).The *hypA* is a gene adjacent to sugar utilization genes in the flanking regions and adjacent to the 3′end of the aflatoxin gene cluster. Ehrlich et al. (2005) showed that the sugar utilization gene cluster on the right side of the aflatoxin gene cluster is well-conserved.

In the current study, some isolates belonging to different VCGs had identical deletion patterns and were closely related. Most of the non-aflatoxigenic strains of the examined VCGs of *A. flavus* produced amplicons for each of the aflatoxin biosynthesis genes examined. In nature, VCGs largely behave as clonal lineages (Ehrlich et al. 2007).

As a result of this study, the basis is provided for initial selection of endemic non-aflatoxigenic strains of *A. flavus* for biological control of aflatoxin contamination in Iran.

CONCLUSION

An attempt has been made in this study to highlight that PCR-based methods have provided a rapid and effective method for identification of genes potentially involved in aflatoxin formation and detection of non-aflatoxigenic fungus *A. flavus* for researchers. But, AF biosynthesis is based on a highly complex pathway. It is thus not surprising that genetic protocols that able to fully differentiate between AF producers and non-producers have not yet been successfully established.

The use of conventional PCR, utilizing primers targeting the *aflR, aflJ* and *hypA* genes appears to offer some promise in detecting Iranian non-aflatoxigenic strains of *A. flavus*, particularly with respect to the ability to distinguish characteristic DNA banding patterns derived from amplicons of appropriate size.

Since the *A. flavus* strains used in this study were all non-aflatoxigenic, we consider that more strains of the species *A. flavus*, which is extremely variable in terms of AF production need to be tested in order to guarantee the ability of *aflR, aflJ* and *hypA* to be used as a molecular marker for this characteristic. Considering the potential role that the aflatoxin biosynthesis gene cluster has in the aflatoxigenic strains of *A. flavus*, studying the genes in aflatoxin non-producing strains of *A. flavus* collected from different regions and substrates (soil and kernel) may provide insights into the significance of these genes for the aflatoxigenicity of the *A. flavus* isolates.

The present work is far from being a finished business, and a lot of windows have been left open.

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الگوهای حذف ژنی در استرین های غیر مولد افلاتوکسین *Aspergillus flavus*

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چکیده: پانزده استرین غیر مولد افلاتوکسین *Aspergillus flavus* به عنوان نماینده طیف وسیعی از مناطق جغرافیایی (شش استان فارس، اردبیل، گیلان، گلستان، کرمان و سمنان) و گروه های سازگار رویشی از خاک و میوه ذرت (*Zea mays*)، بادام زمینی (*Arachis hypogaea*) و پسته (*Pistachia vera*) جمع آوری و با استفاده از PCR از نظر وجود ژن های تنظیم کننده *afIR* و *aflJ* و ساختمانی خوشه ژنی بیوسنتز افلاتوکسین شامل *aflT*، *pksA*، *ver-1*، *omtA*، *omtB*، *aflD*، *ordA*، *verA*، *norA*، *hypA*، *norB* و *cypA* و دو ژن *glcA* و *C3* واقع در دو ناحیه مجاور خوشه ژنی افلاتوکسین (کلاستر قند و انتهای ´۵) مرتبط با توکسین زایی، مطالعه شدند. بر اساس نتایج تکثیر ژن ها، استرین های *A. flavus* با تولید ۱۴-۱۰ باند DNA مختلف در دوازده الگوی مختلف (I-XII) گروه بندی شدند. نتایج نشان داد که، ژن های تنظیم کننده بیوسنتز افلاتوکسین (*aflR* و *aflJ*) و ژن ساختمانی *hypA* در تشخیص استرین های غیر مولد افلاتوکسین *A. flavus* از اهمیت بیشتری برخوردار می باشند. هیچگونه رابطه ایی بین الگوی حذف و منشاء جغرافیایی و یا گروه سازگار رویشی جدایه های غیر مولد افلاتوکسین *A. flavus* مشاهده نگردید. این موضوع بیانگر آن است که، استرین های غیر مولد افلاتوکسین مورد مطالعه در هر منطقه، منشاء مستقلی ندارند. نتیجه گیری کلی این است که، وجود تنوع در جمعیت های غیر مولد افلاتوکسین *A. flavus* برای فهم خطر سمیت ناشی از افلاتوکسین و نیز گزینش عوامل کنترل بیولوژیک سودمند می باشد.

کلمات کلیدی: افلاتوکسین،خوشه ژنی، نقص ژنی، PCR، مسیر بیوسنتز

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