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Studies on *Aspergillus flavus* Link. isolated from maize in Iran

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Abstract: The *Aspergillus flavus* population structure from maize kernels was examined. During 2011, samples were collected from two main grain maize production areas in Iran (Fars and Ardebil provinces), shortly before harvest. One-hundred nine *A. flavus* isolates were recovered on Dichloran Rose Bengal Chloramphenicole (DRBC) agar and *Aspergillus flavus/parasiticus* medium (AFPA) and grouped into morphotypes and Vegetative Compatibility Groups (VCGs) based on morphological (e.g. sclerotia production), physiological (e.g. aflatoxin-producing ability) and genetic criteria (e.g. heterokaryosis). In general, morphotype and VCG composition were highly dissimilar in both provinces. In total, 43.8% and 44.3% of *A. flavus* isolates from Ardebil and Fars, respectively, produced sclerotia. Sclerotia producers were identified as *A. flavus* L and S strain morphotypes in Ardebil (66.7% and 33.3%, respectively) and Fars (29.6% and 70.4%, respectively). Furthermore, 71 isolates (65.1%) were able to produce aflatoxin (Ardebil 40.8%, Fars 59.2%). The aflatoxin values were categorized into four different classes (< 10, 10–100, 100–1,000 and > 1,000 ppb). In total, 51 aflatoxin producing isolates of *A. flavus* (Ardebil n = 22, Fars n = 29) were assigned into 26 VCGs by complementation of *nit* auxotrophs on nitrate medium. None of the *A. flavus* isolates from Ardebil complemented with any isolates from Fars. Genetic diversity of *A. flavus* isolates was 59.1% and 41.8% for Ardebil and Fars, respectively. The different geographical adaptation and genetic make-up of *A. flavus* isolates may be due to different climatic conditions, soil types and crop sequences in both maize production areas.

Key words: aflatoxin production, *Aspergillus flavus*, Iran, maize, sclerotia production, vegetative compatibility

Introduction

Maize (*Zea mays* L., Gramineae) is an economically important crop, cultivated in different regions of Iran with a production area of approximately 242,740 ha and an output of 1,642,660 tons of grains per year. Maize produced in Iran is frequently contaminated with various mycotoxins such as fumonisins, aflatoxins and ochratoxin A (Yazdanpanah *et al*. 2001a, b; Shephard *et al*. 2002; Ghiasian *et al.* 2011). Members of *Aspergillus* section *Flavi* are known for their potential to produce aflatoxins, a group of highly carcinogenic polyketides. These fungi are frequently isolated from maize which, is in particular harmful since most of the maize produced in Iran is used as animal feed (Hedayati *et al*. 2007; Abedi-Tizaki and Sabbagh 2011). In recent years, diverse health effects of aflatoxins found in dairy feed have been reported for Iran (Pirestani and Toghyani 2010; Sefidgar *et al.* 2011). Aflatoxins B are readily metabolized by mammals and excreted as aflatoxins M through the milk.

A study conducted on soil from maize fields in Iran showed that incidences of highly toxigenic *A. flavus* isolates from the Mazandaran and Semnan provinces (north and center of Iran) were approximately 30% and 20%, respectively (Razzaghi-Abyaneh *et al.* 2006).

A. flavus can be delineated into L and S strain morphotypes. *A. flavus* L strain morphotypes produce copious amounts of conidia and very few (or none) large sclerotia (average diameter above 400 μm). *A. flavus* S strain morphotypes produce very few conidia but abundant amounts of small sclerotia (average diameter less than 400 μm).

Both morphotypes can be further grouped into Vegetative Compatibility Groups (VCGs) identified by a heterokaryon incompatibility system (Papa 1986). Vegetative incompatibility limits hyphal fusion and subsequent gene flow between individuals belonging to different VCGs (Leslie 1993; Glass *et al*. 2000). Maintaining gene flow within a species is advantageous as this may lead to increased genotypic variation allowing adaptation to changing environments (Grubisha and Cotty 2010). Even within a restricted geographic area, *A. flavus* VCGs are very genetically diverse (Papa 1986; Horn and Greene 1995). They are associated with many morphological and physiological features such as aflatoxin-producing ability (Horn and Greene 1995; Horn *et al.* 1996). This vegetative compatibility system is dictated by a series of heterokaryon incompatibility (*het* or *vic*) loci whose alleles must all be identical for stable hyphal fusions to occur. VCGs have been

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widely used to study genetic relationships in anamorphic populations of fungi. VCGs are typically identified by complementation of nitrate nonutilizing (*nit*) auxotrophs, and members of the same VCG are considered to be clonal (Grubisha and Cotty 2010). The Ardebil (Pars-Abad) and Fars (Darab and Fasa) are two major agricultural areas in Iran with long grain maize cropping histories but different climatic conditions (e.g. average temperature and rainfall, soil composition, hours of sunshine per day) and agricultural practices (e.g. crop sequence history and management practices, pest infestation). The current study provides an assessment of the *A. flavus* population structure found on maize in two different agro-ecosystems of Iran. Information about the fungal population structure in different maize growing areas in Iran assist in developing effective aflatoxin management strategies. In this research, *A. flavus* isolates were grouped into morphotypes and VCGs based on morphological (e.g. sclerotia production), physiological (e.g. aflatoxin-producing ability) and genetic criteria (e.g. heterokaryosis).

Materials and Methods

Sampling sites

All maize samples were collected from the major grain maize production areas of the Ardebil (Northwestern Iran, Pars-Abad region) and Fars (southern Iran, Fasa and Darab regions) provinces. Fasa and Darab lie on the geographical coordinates of 53°41′E, 28°56′N and 54°17′E, 28°47′N, respectively. Latitude and longitude of Pars-Abad is 39°65′N, 47°92′E. Climatic conditions of Fars and Ardebil vary from one place to another in accordance with the geographical features. The climate of Pars-Abad (Ardebil province) is moderate, semi-arid with an average annual precipitation of 271.2 mm. While, Fasa and Darab have an average annual rainfall of 300 mm and < 350 mm, respectively. The climates of the Darab and Fasa (Fars province) can be described as having warm dry summers and fairly moderate and rainy winters. Throughout the year, there is virtually no rainfall in Darab. In Fasa and Darab, the mean annual temperature ranges from 7.9°C to 15.2°C and for Pars-Abad, from 18.5°C to 22°C.

Sample collection

In September 2011, 160 maize ears were randomly sampled from each of eight grain maize fields (0.5–2 ha) in the Fars and/or Ardebil provinces (20 ears from each of field) (Fig. 1), 40 days after tasselling (all the husk leaves were dry). In each field, five maize ears (combined samples) were randomly selected and shelled immediately afterwards. The extracted seeds were artificially dried to a 14.0% moisture content, at 45°C in a Thermax batch type seed drier.

Fungal isolation and identification

Methods used for isolation, identification, and characterization of *Aspergillus* section *Flavi* isolates have been described previously (Cotty 1997). Briefly, maize ears were shelled and dried. Maize seeds were collected and mixed uniformly. After being mixed, 100 seeds were randomly selected and surface sterilized in a 2.5% NaOCl solution for 1 min followed by washing three times in sterile distilled water. The sterilized maize seeds were placed on Dichloran Rose Bengal Chloramphenicole (DRBC) agar and/or *A. flavus/parasiticus* (AFPA) medium amended with 50 mg chloramphenicole per liter (Sigma-Aldrich Chemie GmbH/ Switzerland). Plates were incubated for 48–72 h at 30°C. A plug of mycelium was aseptically removed from the orange medium below each infected seed and sub-cultured on Czapek-Yeast Extract Agar (CYA) at 25°C for 5 days. At the end of the incubation period, species and strains were identified based on macroscopic and microscopic characteristics. The colonies of *A. flavus* S and L strain isolates were transferred to plants of Czapek-Dox Agar (CDA) and incubated for 14 days at 25°C in the dark.

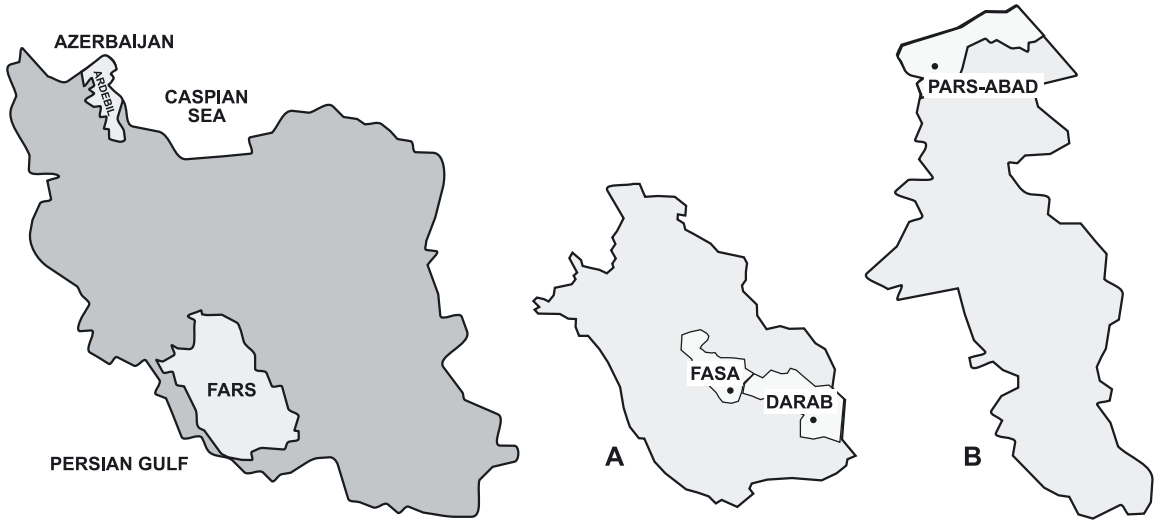

Fig. 1. Map of the Iran Islamic Republic showing the Ardebil and Fars provinces from which the *A. flavus* maize kernel isolates were obtained: (A) Darab and Fasa, Fars province; (B) Pars-Abad, Ardebil province

Sclerotia production

Formation of sclerotia as well as sclerotial sizes was recorded for single-spored *A. flavus* isolates incubated on CDA containing 3% $NaNO_3$ for 21 days at 30°C (three replications for each isolate). Sclerotia larger than 400 µm were classified as large (L strain morphotype) and sclerotia smaller than 400 µm were classified as small (S strain morphotype) (Abbas *et al.* 2005; Giorni *et al*. 2007; Atehnkeng *et al*. 2008).

Aflatoxin analysis

Isolates were placed on Yeast Extract Sucrose (YES) agar containing 0.3% methyl *β*-cyclodextrin, and incubated for 2–3 days at 28°C in the dark (Fente *et al.* 2001). At the end of the incubation period, fluorescence was examined under UV light (365 nm). The production of aflatoxin B1 (AFB1) was confirmed with thin-layer chromatography (TLC). In brief, chlorophormic extracts of culture broth were spotted on silica gel plate (Silica gel G60, 20 × 20 cm, 0.25 mm thick, Merck 5721, Germany), and developed with a chloroform-acetone mixture (90 : 10). AFB1 was visualized under 365-nm UV light and photographed and quantified with a TLC scanner equipped by densitometer (CAMAG Reprostar 3, CAMAG, Switzerland). In all the aflatoxin analyses, two aflatoxigenic (SRKC-G1907) and non-aflatoxigenic (F3W4) *A. flavus* strains (Courtesy Dr. Schmidt-Heydt, Institute Max Rubner, Germany) were used as the positive and negative controls.

VCG analysis

Nitrate non-utilizing mutants (*nit* mutants) of each wild type isolate were generated on CDA (sole nitrogen source – NO_3) supplemented with potassium chlorate (25 g/l) and incubated for a minimum of two weeks or until a *nit* mutant appeared at 30°C in the dark (Cove 1976). The mutants were distinguished by the production of fine hyaline mycelia, with no conidial production. The growing tip of the mutant was transferred to CDA to confirm that a *nit* mutant was produced (Papa 1986). *Nit* mutants were characterized by the type of nitrogen the mutants were able to utilize. The sodium nitrate in CDA was replaced with hypoxanthine (0.2 g/l), ammonium tartrate (1 g/l) or sodium nitrite (0.5 g/l) (Papa 1986). Mutants were scored by the absence or presence of growth and classified into one of three mutant types: *cnx* (molybdenum cofactor gene mutants), *niaD* (apoenzyme gene mutants) or *nirA* (nitrate and nitrite reductase system mutants). An agar plug of each wild-type served as a control in each test. Compatibility tests based on complementation of *nit* mutants were conducted by cutting a mycelial plug (5 mm in diameter) from the edge of *cnx* and *nirA* mutants (if not *niaD* and *nirA* mutants or *niaD* and *cnx*) of different isolates and placing a pair of agar plugs 4 cm apart on a 9-cm-diameter plate of CDA. Pairings were made between two different *nit* mutants in all possible combinations, and also between the same isolate (self-fusion), which were included for each test run on the same plate as a negative control. The plates were incubated for three weeks at 28°C in the dark. A complementary reaction was determined by the development of dense heterokaryotic aerial mycelial growth at the zone of hyphal contact. If one or more mutants from a given isolate formed a heterokaryon with one or more mutants from another isolate, the isolates were placed in the same VCG. All *cnx* and *nirA* mutants that did not fall into a VCG were also paired with the remaining *niaD* mutants and cultivated for three weeks. Diversity of VCGs of the *A. flavus* isolates was calculated as the number of groups divided by the total number of isolates (Horn and Greene 1995).

Results

Fungal isolation and identification

In the present study, a total of 226 *Aspergillus* section *Flavi* isolates were recovered from maize seeds collected in 2011. When placed on AFPA, 134 showed yellow-orange reverse coloration; from which 109 were identified as *A. flavus* (48 Ardebil, 61 Fars).

Sclerotia production

In total, 43.8% and 44.3% of *A. flavus* isolates from Ardebil and Fars, respectively, produced sclerotia. In Ardebil, 66.7% of sclerotia producing isolates belonged to the *A. flavus* L strain morphotype and 33.3% were *A. flavus* S strain morphotypes. In Fars, the majority of sclerotia producing isolates were *A. flavus* S strain morphotypes (70.4%), the minority (29.6%) were identified as L strain morphotypes.

Aflatoxin production

Seventy-one (65.1%) of the obtained *A. flavus* isolates produced aflatoxins. The frequencies of aflatoxigenic isolates of *A. flavus* in maize seeds from the Fars and Ardabil provinces were 59.2% and 40.8%, respectively. The aflatoxin values were categorized into four different classes, low AFB1 greater than three and less than or equal to 10 ppb AFB1, medium AFB1 greater than 10 ppb and less than or equal to 100 ppb AFB1, high AFB1 greater than 100 ppb and less than or equal to 1000 and very high AFB1 greater than 1,000 ppb AFB1 (Table 1).

Nit mutant production

Nit mutants were successfully obtained for 51 (58.6%, 22 Ardebil, 29 Fars) of the wild-type isolates. In total, 473 (84.3%) and 285 (79.8%) *nit* mutants were generated from 561 and 357 chlorate resistant sectors of *A. flavus* isolates from the Fars and Ardebil provinces, respectively. Therefore, the results of crosses among *nit* mutants of each wild type *A. flavus* isolate, revealed that 52 and 26 *nit* mutants from the Fars and Ardebil did not complement each other and were excluded from further analysis, respectively.

Recovery rates of *nit* mutant phenotypes were highly variable among *A. flavus* isolates. In Ardebil, 59.5% were *niaD*, 32.4% *nirA* and 8.1% *cnx*. In Fars, 67.2% of *nit* mutants were *niaD*, 23.3% *nirA* and 9.5% *cnx*.

Table 1. Mean comparison of aflatoxin B1 (AFB1) production of *A. flavus* strains isolated from maize kernels in the Pars-Abad (Ardabil province), Darab and Fasa (Fars province)

Sampling region	AFB1 production [ppb]			
	A. flavus strain L		*A. flavus* strain S	
	range	mean ±SD	range	mean ±SD
Fasa	3–91	53.51±11.18 b	95–1697	813.81±49.42 b
Darab	5–172	77.31±16.43 ab	84–1893	937.33±53.27 ab
Pars-Abad	3–218	126.53±28.33 a	137–2042	1129.73±71.16 a

Average data in each column with dissimilar letters are significantly different (Tukey's test, 5% level)

Table 2. Production of sclerotia, type of strain, number of *nit* mutants and vegetative compatibility groups of *A. flavus* isolates from maize in the Fars province, Iran

Isolate	Sclerotia production/strain isolate	Mutant phenotype			Vegetative compatibility groups (VCG)[d]
		niaD	*nirA*	*cnx*	
dar104	+/L[b]	1	–	–	A
dar111	+/L	1	1	–	A
dar120	+/L	1	–	–	A
dar123	+/L	1	–	–	A
fas150	+/L	1	–	–	A
fas152	+/L	1	1	1	B
fas161	+/L	1	1	–	B
dar117	+/L	1	–	–	B
fas144	none[a]	1	1	1	C
dar132	none	1	–	–	C
dar136	none	1	1	–	C
dar137	none	1	–	–	C
dar113	+/S[c]	1	1	–	D
fas151	+/S	1	–	–	D
fas157	+/S	1	–	–	D
fas139	+/S	1	–	–	D
dar114	+/S	1	–	–	D
dar126	none	1	1	–	E
fas147	none	1	–	–	E
fas163	none	1	1	1	E
dar112	none	1	1	1	F
dar118	none	1	1	–	G
fas143	none	1	–	–	H
dar127	+/L	1	–	–	I
fas156	none	1	–	–	J
dar128	none	1	–	–	K
dar131	none	1	–	–	L
fas160	+/L	1	–	–	M
fas166	none		1	–	N

[a] sclerotia were not produced under cultural conditions; [b] strain produce sclerotia > 400 μm in diameter; [c] strain produce sclerotia < 400 μm in diameter (Cotty 1989); [d] designation correlative with Novas and Cabral (2002)

VCG analysis

Fifty-one aflatoxigenic isolates of *A. flavus* were successfully placed into 26 VCGs (14 Fars, 12 Ardebil) by complementation of *nit* auxotrophs. A total of nine multi-member VCGs were detected. Five multi-member VCGs (containing three to five members each) and nine single-member VCGs were detected in the Fars province (Table 2). Furthermore, four multi-member VCGs (containing three to four members each) and eight single-member VCGs

Table 3. Production of sclerotia, type of strain, number of nit-mutants and vegetative compatibility groups of *A. flavus* isolates from maize in the Ardebil province, Iran

Isolate	Sclerotia production/strain isolate	Mutant phenotype			Vegetative compatibility groups (VCG)[d]
		niaD	*nirA*	*cnx*	
prs104	+/L[b]	1	1	–	O
prs125	+/L	1	–	–	O
prs129	+/L	1	–	–	O
prs105	none[a]	1	1	1	P
prs113	none	1	1	1	P
prs114	none	1	1	–	P
prs124	none	1	–	–	P
prs132	none	1	–	–	Q
prs108	none	1	–	–	Q
prs111	none	1	–	–	Q
prs112	none	1	1	–	Q
prs117	none	1	1	1	R
prs119	none	1	1	–	R
prs126	none	1	–	–	R
prs128	+/S[c]	1	1	–	S
prs101	+/S	1	–	–	T
prs110	none	1	1	–	U
prs109	none	1	–	–	V
prs118	+/L	1	1	–	W
prs122	none	1	1	–	X
prs127	none	1	1	–	Y
prs139	none	1	–	–	Z

[a] sclerotia were not produced under cultural conditions; [b] strain produce sclerotia > 400 μm in diameter; [c] strain produce sclerotia < 400 μm in diameter; [d] designation correlative with Novas and Cabral (2002)

were identified in the Ardebil province (Table 3). Results revealed that none of the *A. flavus* isolates from Ardebil complemented with any isolates from Fars. The sclerotia producers within each VCG were either L or S strain, not both. Diversity indices for the Ardebil and Fars VCGs, expressed as the number of VCGs divided by the total numbers of isolates (Horn and Greene 1995) resulted in 59.1% and 41.8%, (62.5% Darab and 69.2% Fasa) respectively.

Discussion

Maize (*Zea mays* L.) has been cultivated in Iran for centuries. Although *A. flavus* is mostly considered a storage fungus, it may cause a very high percentage of maize grain infection under specific agroecological field conditions.

Previous studies showed that *A. flavus* was widely distributed throughout the world and that there were predominant species isolated from maize kernels (Marín *et al*. 2012; Muthomi *et al.* 2012). There are not enough data available on the characteristics and toxicological potentials of *A. flavus* populations in the Ardebil and Fars provinces. The *A. flavus* population structures observed by us, varied within and between two major grain maize growing areas of Iran: the Ardebil and Fars provinces.

Physiological (sclerotia and aflatoxin production) and genetic (VCG compositions) characteristics of 109 and 71 *A. flavus* isolates were compared, respectively. Sclerotia production was greater than previously reported for *A. flavus* populations found in maize fields of Iran (Mazandaran and Semnan provinces) (Razzaghi-Abyaneh *et al.* 2006). Sclerotia producing isolates were found for both *A. flavus* L and S strain morphotypes. Strain L is very common throughout the world, while strain S has been established in the China (Gao *et al*. 2007) and Kenya (Probst *et al*. 2007).

Our findings indicate the differential adaptation of *A. flavus* L and S morphotypes to different agro-ecological maize growing areas in Iran. Ardebil (Pars-Abad) and Fars (Darab and Fasa) are two major agricultural areas in Iran with long maize cropping histories but different climatic conditions (e.g. average temperature and rainfall, soil composition, hours of sunshine per day), and agricultural practices (e.g. crop sequence history and management practices, pest infestation). Precipitation factors as indicators of climate conditions in the two studied areas show that conditions were arid and semi-arid, respectively. In Fars, the very low precipitation factor or arid conditions that favoured proliferation of *A. flavus* morphotypes had also been described in Kenya (Muthomi *et al.* 2012) and the USA (Abbas *et al*. 2006). Considerably more *A. flavus*

S strain morphotypes were detected in the Fars province. Bock *et al.* (2004) speculated that S strain morphotypes may be better adapted to arid and semi-arid climates. The opposite was observed for Ardebil. Our study found that *A. flavus* S strain morphotypes favored the hotter and drier Fars province. The *A. flavus* S strain is suspected to be an important causal agent of aflatoxin contamination in several areas worldwide, including USA (Cotty *et al*. 2001) and Argentina (Novas and Cabral 2002).

It was reported that increased elevation has been associated with reduced S-strain incidence within agricultural areas in Arizona (Bigelow *et al.* 2001), however, this is not a factor in the Fars province. Fars (Darab – 1180 m, Fasa – 1382 m) and Ardebil (Pars Abad – 20–40 m) provinces are two areas with dissimilar altitudes which differed in incidence of % S from very low (Ardebil province) to very high incidences (Fars province).

In general, S strain isolates of *A. flavus* produced more B aflatoxins (range: 84–2042 ppb, mean: 960.29±57.95 ppb) than the L strain isolates (range: 3–218 ppb, mean: 85.78±18.64 ppb).

Because L strain isolates of *A. flavus* do not produce AFB1, or if they do its concentrations are low, those isolates are known as atoxigenic isolates. Characterization of the *A. flavus* strain isolates is an important initial step for development of management procedures (Probst *et al*. 2007).

In the Fars and Ardebil, five (VCGs A, B, D, I and M) out of 14 VCGs, and five out of 12 VCGs produced sclerotia, respectively. Some isolates within a VCG that produced sclerotia, which is consistent with previous reports (Horn *et al.* 1996). We evaluated possible relationships between VCGs and sclerotia production, sclerotia size and geographical origin. It has been shown that isolates within the same VCGs share common features such as ability to produce aflatoxins (Grubisha and Cotty 2010). Furthermore, AFB1 production within a multi-member VCG was variable. All L strain isolates of *A. flavus* (VCGs A, B, I, M, O and W) produced medium (10–100 ppb) to high levels of AFB1 (100–1,000 ppb). While, AFB1 produced by S strain isolates (VCGs D, S and T) was greater than 1,000 ppb. In general, the higher the incidence of S strain morphotypes, the greater the severity of aflatoxin contamination (Probst *et al.* 2007). All Iranian non-producing sclerotia isolates of *A. flavus* from maize (VCGs C, E–H, J–L, N–R, U–V, X–Z) were classified in toxin group 1 and/or 2 (AFB1 ≤ 10 ppb and 10–100 ppb AFB1). It is assumed that the morphotypes and vegetative compatibility heavily influence the average aflatoxin production. The relationship of *A. flavus* VCG and aflatoxin production in the Fars and Ardebil populations still has to be investigated. There were associations between VCG and sclerotium production in *A. flavus* isolates from the Fars and Ardebil. Besides maize, both agricultural areas also cultivate cotton. Aerial spore dispersal of *A. flavus* from infected maize and cotton in each area, could affect the variability of *A. flavus* isolates. The genetic variability of an *A. flavus* population, usually expressed as the number of VCGs found in specific area, is an important criterion when developing aflatoxin management strategies (Dorner and Cole 2002). Both areas may be inhabited by several genetically distinct individuals. Furthermore, no genetic agreement was found in the population structures of *A. flavus* strain isolates from maize in two studied areas, dissimilar in size and climate, suggesting that the identified VCGs are locally adapted. None of the Ardebil L strain VCGs were found in the Fars suggesting selective pressure in *A. flavus* populations in response to a multitude of environmental parameters as had been described by Horn *et al*. (1996). The *A. flavus* population from Ardebil was also more genetically diverse (a diversity value of 50.1%) than in Fars (41.8%), and encompassed four multimember VCGs and eight single member VCGs indicating that *A. flavus* has not been well adapted to the area.

The current study provided a first insight into the genetic and structural compositions of *A. flavus* populations endemic in maize cultivating areas in Iran. A foundation for future investigations of *A. flavus* populations in other agricultural areas has been laid. Further studies are required to confirm the relationships among VCGs, genotypes, and phenotypes, such as sclerotia formation (size and number of sclerotia) and aflatoxin production, using more *A. flavus* isolates collected from diverse geographical area in Iran.

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