Genotyping of *Fusarium verticillioides* strains producing fumonisin B_1 in feed associated with animal health problems

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(Received 4 May 2010; revised version 22 Feb 2011; accepted 14 Mar 2011)

Summary

Fusarium verticillioides (*F. verticillioides*) is not only a primary pathogen of maize, but also can cause disease in other crops such as sorghum. Pathogenicity is related to mycotoxin production such as fumonisin. In the present study, 24 isolates of *F. verticillioides*, which were previously identified by phenotype based methods, were re-identified using restriction fragment length polymorphism (RFLP) analysis. Digestion of the polymerase chain reaction (PCR) products with the restriction enzyme *TasI* allowed identifying four non-*verticillioides* strains that were discarded from our study. The genetic variations among the remaining 20 strains of *F. verticillioides* were analysed by random amplified polymorphic DNA (RAPD)-PCR method with 4 primers. Of the four primers tested, two primers produced polymorphic amplification patterns. Dendrogram for each primer indicated the distance of the strains to each other. Using primers of A, B, C and D, the isolates were divided to 8, 9, 7 and 7 groups, respectively. The results of this study indicated genetic relationship among DNA polymorphic patterns with geographic regions and the severity of fumonisin B_1 (FB_1) production. It seems that RAPD analysis is a suitable technique for strain typing of *F. verticillioides*.

Key words: *Fusarium verticillioides*, Genotyping, RAPD-PCR, Maize

Introduction

Fusarium verticillioides (*F. verticillioides*) is primarily a pathogen of maize, but it can also cause disease in other crop species. Its pathogenicity is related to production of major mycotoxins such as fumonisins (Ghiasian, 2003). Mycotoxins can cause contamination of food and feedstuffs, and so, economically significant losses in both farmers and food processors (Arab Abousadi *et al*., 2007). Fumonisins have emerged as a highly visible animal health safety concern as they have been associated with many animal diseases such as leukoencephalomalacia (LEM) in horses (Marasas, 1996), pulmonary oedema syndrome (PES) in pigs (Colvin and Harrison, 1992) and hepatocarcinogenesis in rats (Gelderblom *et al*., 2001). In Iran, different *Fusarium* species, especially *F. verticillioides*, are one of the most important feed contaminators (Ghiasian, 2004).

Regarding the importance of high quantities of mycotoxin-production by this fungus and various productions in different strains of this species, it appears that more studies regarding this subject are beginning to appear. Various methods have been reported for molecular identification of *Fusarium* species such as random amplified polymorphic DNA (RAPD) - polymerase chain reaction (RAPD-PCR) (Bentley *et al*., 1995; Voiget *et al*., 1995; Yli-Mattila *et al*., 1996), PCR - restriction fragment length polymorphism (RFLP) (Liorens *et al*., 2006;

Mirhendi *et al*., 2006; Liorens, 2007) and nuclear ribosomal DNA sequence data for PCR identification of *Fusarium* genus (Abd-Elsalam *et al*., 2003). The goal of this study was to evaluate DNA polymorphism of *F. verticillioides* isolates using RAPD-PCR method with respect to the severity of fumonisin B_1 (FB_1) production and different geographical regions of the isolates in Iran.

Materials and Methods

Strains and cultivation

Twenty-four isolates of *F. verticillioides*, which were previously isolated based on morphologic and cultural characterizations, were used in this study (Table 1). These strains were confirmed in the South African Mycology Research Center (MRC). All isolates were cultured on Sabouraud dextrose agar and incubated at 27°C for 7 days. The harvested spores were transferred to Sabouraud dextrose broth and agitated at 27°C for 7 days. Cultures were filtered by vacuumed pump and the mycelial mat was washed with sterile distilled water and stored at -20°C until use (Khalil *et al*., 2003). In this study, all general chemical materials were purchased from Merck Co., Darmstadt, Germany.

DNA extraction

A total genomic DNA was extracted and purified, using glass bead disruption according to the Mirhendi *et al*. (2006) method. Briefly, an aliquot of mycelial mat of each isolate was added to 300 µl of lysis buffer (100 mM Tris, 10 mM EDTA pH = 8, 1% SDS, 100 mM NaCl, 2% Triton X-100), 300 µl of phenol-chloroform (1:1) and 300 mg of glass beads (0.5 mm in diameter). The samples were strongly shaken for 5 min to disrupt the cells perfectly. Fungal debris was separated by centrifugation at 5000 rpm for 5 min and the supernatant was separated and an equal volume of chloroform was added. The suspension was centrifuged at 5000 rpm for 5 min again and the supernatant was

Table 1: Characterizations of 24 under study isolates of *Fusarium verticillioides* based on geographical regions and severity of toxicity in 3 groups including Low (L), Moderate (M) and High (H)

Code No.	MRC[a]	FB_1 µg/g[b]	Severity of toxicity[c]	Geographic region
1[d]	8288	1735	M	Intermediate
2	8289	2371	M	Intermediate
3	8290	411	L	Intermediate
4[d]	8291	6895	H	Intermediate
5	8292	3653	M	Intermediate
6	8293	1854	M	Intermediate
7	8296	258	L	Intermediate
8	8297	309	L	Intermediate
9	8299	666	L	Intermediate
10	8300	418	L	Intermediate
12	8302	9661	H	Tropical
13	8304	404	L	Tropical
14[d]	8305	2798	M	Tropical
16	8307	8220	H	Tropical
17	8316	5675	H	Cold
21	8320	3563	M	Cold
23	8328	396	L	Humid
24	8329	720	L	Humid
28	8308	5795	H	Tropical
29	8310	788	L	Tropical
30	8311	233	L	Tropical
31	8312	323	L	Tropical
32	8314	849	L	Tropical
33[d]	8315	4149	M	Tropical

[a] MRC: Mycological Research Center of South Africa, [b] FB_1: Fumonisin B_1, [c] Low toxicity (L): ≤1000 µg/g, Moderate toxicity (M): >1000 to 5000 µg/g, High toxicity (H): >5000 µg/g, and [d] Identified as non-*Fusarium verticillioides*

separated by adding 0.1 volume of 3 M sodium acetate and an equal volume with supernatant of 2-isopropanol to precipitate total DNA. Precipitant was washed with 70% ethanol, air-dried, resuspended in 50 µl of deionized distilled water and stored at -20°C until use.

PCR conditions

PCR amplification was carried out in a final volume of 50 µl. PCR reaction mixture contained 1 µl of template DNA (100 pg), 0.5 µl of each forward (ITS1, 5'-TTC GTA GAA CCTGCG G-3') and reverse (ITS4, 5'-TCC TCCGCT TAT TGA TATGC-3') primer (0.5 mM), 0.5 µl of each deoxynucleoside triphosphate (dNTP, 200 mM), 5 µl of 10X PCR buffer and 0.5 µl of Taq DNA polymerase. The PCR profile was entreated at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min and extension step of 72°C for 6 min. Amplified products were visualized by 1.5% (W/V) agarose gel electrophoresis in TBE buffer (0.09 M Tris, 0.09 M boric acid and 2 mM EDTA, pH = 8.3), stained with ethidium bromide (0.5 µg/ml) and then photographed.

RFLP analysis

TasI was selected as a restriction enzyme based on species-specific length patterns data. RFLP reaction mixture contained 11 µl of PCR product, 0.5 µl of enzyme *TasI* (*Fermentase*, *Litvani*), 1.5 µl of enzyme buffer and 2 µl of deionized distilled water (total volume, 15 µl). Digestion was performed by incubation at 65°C for 2 h. Restriction fragments were separated by 2% agarose gel electrophoresis in TBE buffer for 45 min at 120 v and visualized by staining with ethidium bromide and photography was performed.

RAPD-PCR

Among various random primers, in total 4 different 10-mer primers were selected for RAPD analysis as follows: A:5'-GGGGGTTAGG-3', B:5'-GCTGTAGTGT-3', C:5'-GTATTGCCCT-3', D:5'-GGTTCT GGCA-3' (Abd-Elsalam *et al.*, 2003). The reactions were carried out in a final volume of 50 µl, containing 2 µl of template DNA, each primer (A, B, C and D) at 0.5 mM, 1.5 mM of $MgCl_2$, 1.25 units of 10X PCR buffer, 0.5 mM of dNTP and 0.5 µl of Taq DNA polymerase. The RAPD-PCR profile was entreated at 35°C for 6 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 32°C for 1 min and extension at 72°C for 1.5 min, with a final extension step of 72°C for 10 min. Amplified products were visualized by 1.3% (W/V) agarose gel electrophoresis in TBE buffer and stained with ethidium bromide.

Statistical analysis for RAPD

The pattern of RAPD products was separately obtained for each primer. Amplified DNA fragments reproducible in 2 to 3 reactions were scored as 0 (fragment absent) and 1 (fragment present) in a data matrix.

A phenogram was constructed by the unweighted pair group method with arithmetic mean (UPGMA) after determining the association coefficients by the simple matching method.

Results

Fungus-specific universal primer pairs (ITS1, ITS4) were successfully able to amplify the ITS region of the isolates of *F. verticillioides* tested, providing a single PCR product of 540 bp.

To ensure all of the isolates were *F. verticillioides*, restriction digestion of PCR products was performed with the enzyme *TasI* (Fig. 1). Based on the restriction pattern of standard strain (MCR 8559), 4 isolates were differentiated from the others, which were eliminated as the non-*verticillioides* strains. RAPD-PCR method was used to analyse the genetic variations and obtained differences among the isolates of *F. verticillioides*. In spite of similar bands, remarkable differences were seen among them. These findings represented more confident and logical results by UPGMA method (Fig. 2a-d). Then, drawing the dendrogram for each primer and all primers together indicated the distance of the strains from each other (Fig. 3). Using primer A: the isolates were divided to 8 groups, primer B: 9 groups, primer C: 7 groups and primer

D: 7 groups. Based on this grouping, primers A and D had the most similarities (Tables 2 and 3).

In spite of the similarity of the molecular patterns and the severity of toxicity, significant differences were observed between the isolates under study ($P<0.05$). Also, the molecular similarities among isolates in each region showed significant correlations.

In this study, with respect to DNA polymorphic patterns, the clear-cut relationship among *F. verticillioides* isolates and geographic regions with the severity of FB_1 production were observed.

Discussion

F. verticillioides is genetically the most intensively studied species in *Fusarium* section *Liseola*. The most prominent toxins produced by this fungus are the fumonisins, in particular FB_1 (Bentley *et al*., 1995). FB_1, either in purified form or in naturally contaminated maize or maize based feeds has been reported to cause the diseases LEM

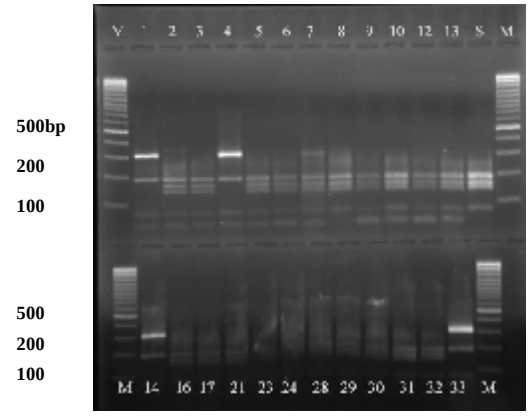

Fig. 1: Restriction digestion of PCR products of 24 under study isolates with the enzyme *TasI* and (MRC 8559) strain with molecular weight ladder 100 bp (1-33: Number of under study isolates, S: Standard strain, and M: Marker)

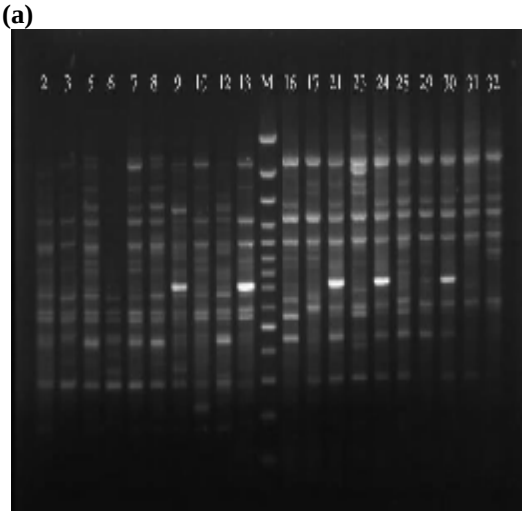

Fig. 2: RAPD-PCR products of 20 isolates of *F. verticillioides* with primers A (1), B (2), C (3) and D (4) and 100 bp marker (M: Marker)

and PES (Colvin and Harrison, 1992; Marasas, 1996). It also causes liver toxicity and liver cancer in rats, atherosclerosis in monkeys and immunosuppression in poultry (Henry *et al*., 2000). Many taxonomic problems related to the morphological and physiological variation within the species have recently been shown by using biological and molecular approaches (Leslie *et al*., 2001). Variation obtained by PCR amplification of the IGS region combined with RFLP analysis have been frequently applied to test intraspecific phenotypic-based taxonomic hypothesis in the genus *Fusarium* (Apple and Gordon, 1995; Carter *et al*., 2000) and was therefore the choice for the present study.

In this study, 24 isolates of *F. verticillioides*, which were previously identified by phenotype-based methods, were re-identified and confirmed using RFLP. All *F. verticillioides* strains in our study produced FB_1, which was previously reported by Ghiasian (2004). These results were consistent with studies in other Asian and European countries (Lee *et al*., 1994;

Table 2: The number of bands observed through RAPD-PCR technique with primers A, B, C and D in each of 20 isolates of *F. verticillioides*

Isolate No.	Primer			
	A	B	C	D
2	9	5	8	9
3	8	5	7	7
5	12	7	8	12
6	4	4	5	4
7	9	7	9	9
8	11	9	9	11
9	10	7	10	10
10	10	5	10	10
12	9	6	10	10
13	9	8	9	9
9	9	7	16	7
16	7	9	9	7
17	8	9	10	8
21	9	9	9	9
23	9	10	11	9
24	8	10	9	8
28	9	9	10	9
29	7	7	10	7
30	9	9	10	8
31	8	8	10	7
32	5	7	10	5

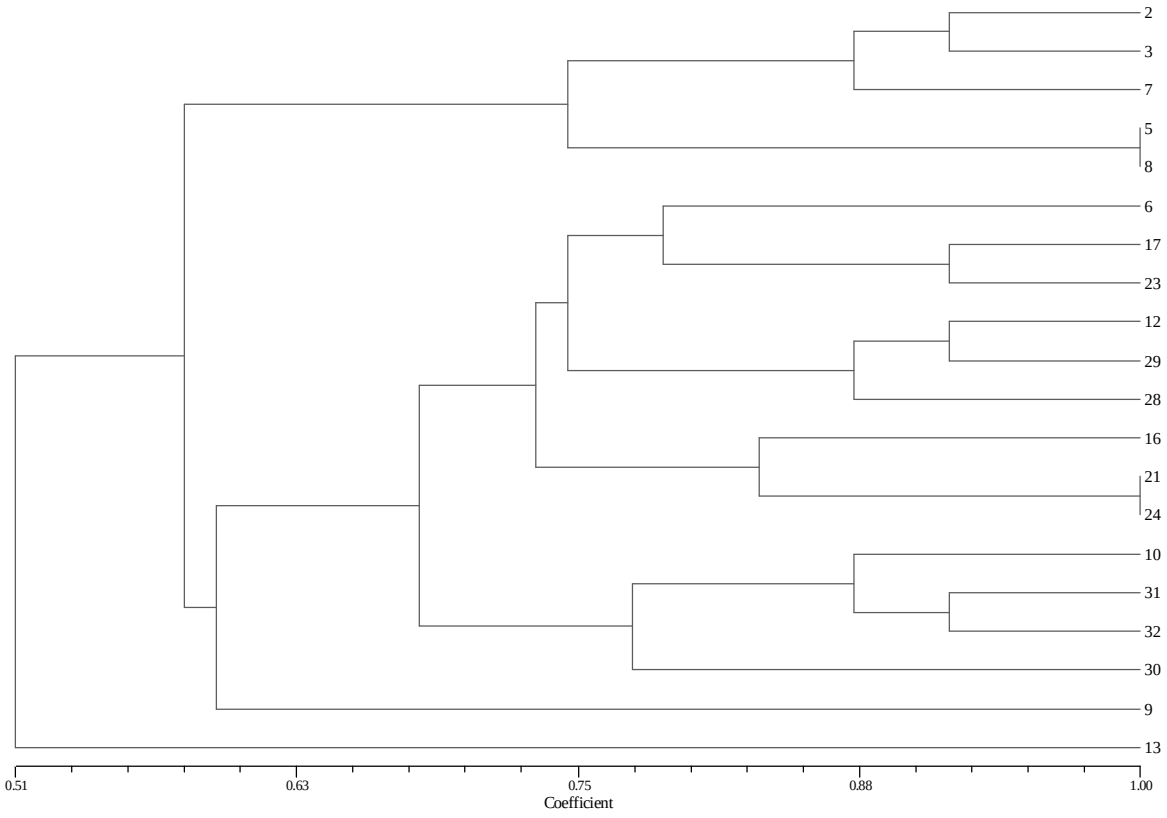

Fig. 3: Phenogram depicting relationships among twenty operating taxonomic units of *F. verticillioides* fungus using 57 characters (from all RAPD-PCR reactions) and obtained by UPGMA

Table 3: Similarity indexes among 20 genotypes of *F. verticillioides* means 2, 3, 5, … , 31, 31 and 32 (G = genotype)

32	31	30	29	28	24	23	21	17	16	13	12	10	9	8	7	6	5	3	2	G
																			1	2
																		1	0.92	3
																	1	0.75	0.67	5
																1	0.5	0.75	0.67	6
															1	0.67	0.83	0.92	0.83	7
														1	0.83	0.5	1	0.75	0.67	8
													1	0.58	0.42	0.58	0.58	0.5	0.58	9
												1	0.5	0.58	0.58	0.58	0.58	0.5	0.58	10
											1	0.67	0.67	0.75	0.58	0.75	0.75	0.67	0.58	12
										1	0.58	0.75	0.58	0.5	0.33	0.33	0.5	0.25	0.33	13
									1	0.58	0.67	0.67	0.5	0.58	0.58	0.75	0.58	0.5	0.42	16
								1	0.67	0.42	0.67	0.67	0.83	0.58	0.58	0.75	0.58	0.67	0.75	17
							1	0.67	0.83	0.58	0.67	0.5	0.67	0.42	0.42	0.75	0.42	0.5	0.42	21
						1	0.75	0.92	0.75	0.33	0.75	0.58	0.75	0.67	0.67	0.83	0.67	0.75	0.67	23
					1	0.75	1	0.67	0.83	0.58	0.67	0.5	0.67	0.42	0.42	0.75	0.42	0.5	0.42	24
				1	0.83	0.75	0.83	0.67	0.83	0.58	0.83	0.67	0.5	0.58	0.58	0.75	0.58	0.67	0.58	28
			1	0.92	0.75	0.83	0.75	0.75	0.75	0.5	0.92	0.75	0.58	0.67	0.67	0.83	0.67	0.75	0.67	29
		1	0.83	0.75	0.75	0.67	0.75	0.58	0.75	0.67	0.75	0.75	0.58	0.67	0.67	0.67	0.67	0.58	0.5	30
	1	0.83	0.83	0.75	0.58	0.67	0.58	0.58	0.75	0.67	0.75	0.92	0.42	0.67	0.67	0.67	0.67	0.58	0.5	31
1	0.92	0.75	0.75	0.67	0.67	0.75	0.67	0.67	0.83	0.58	0.67	0.83	0.5	0.58	0.58	0.75	0.58	0.5	0.42	32

Visconti and Doko, 1994), but in contrast to a Taiwanese study, in which only 66% of the *F. verticillioides* strains produced FB_1 (Tseng *et al*., 1995).

Our finding indicated no relationship between RAPD profiles and severity of toxicity and the conclusions were in consistent with those obtained by Liorens *et al*. (2006). The results of that study showed qualitative and quantitative intraspecific variation in the secondary metabolite profiles and toxicity with PCR-RFLP analysis and Mycotoxin-producing capacity (Liorens *et al*., 2006; Liorens, 2007; Qu *et al*., 2008).

In our study, according to RAPD-PCR reactions, the phenon line, which represented the mean similarities, was at 62%. At this level four groups were characterized as follows: The first one formed by 2, 3, 7, 5, and 8 isolates, the second one formed by 6, 17, 23, 12, 29, 28, 16, 21, 24, 10, 31, 32, 30 isolates, the third one formed by 9 isolates, the forth one formed by 13 isolates. The similarity index for distinct isolates (9 and 13) was 50%, staying below the phenon line. These indexes showed that RAPD profile did not enable the precise study of polymorphism between the understudy isolates. Digestion of the PCR products with one restriction enzyme (*TasI*), in comparison with a standard strain (MRC 8559), differentiated 4 non-*verticillioides* strains. This finding confirmed the accuracy and simplicity of molecular biological techniques in comparison with phenotype-based methods. This finding was consistent with the observation by Kosiak *et al*. (2005) who reported the correlation of morphological and molecular variations among 27 isolates of *F. equiseti* from Norwegian cereals. In the current study, RAPD-PCR was carried out with 4 primers for analysing the genetic variation among this species. Of the 4 primers tested, 2 primers produced more polymorphic amplification patterns by using dendrograms. The polymorphisms observed for RAPD markers revealed a high degree of genetic diversity in strains of *F. verticillioides* isolated from different regions of Iran. Our findings were consistent with other reports that many strains of *F. verticillioides* and other *Fusarium* species isolated from different geographical regions have genetic diversities among the strains (Pamphila and Azevedo, 2002; Mishra *et al*., 2005; Qu *et al*., 2008). In addition, Apple and Gordon (1995) reported considerable intraspecific variation in the IGS region among isolates of *F. oxysporum* from different hosts and geographical origins. In contrast, in a study conducted by Khalil *et al*. (2003), no correlation was observed between genetic affinity of *Fusarium* species such as *F. solani*, *F. moniliforme*, *F. oxysporum*, *F. avenaceum* and *F. chlamydosporum* and the origin of the fungi tested. In another study by Abdel-Satar *et al*. (2003), no correlations were observed among different *Fusarium* species including *F. oxysporum*, *F. solani*, *F. chlamydosporum*, *F. moniligorme* and *F. avenaceum* and their geographic regions using AFLP analysis. The contradictory observations may be explained by

differences in growth conditions used in culturing the fungi, genotyping in species level and sensitivity of the detection methods. The present study showed that RAPD-PCR is a rapid and suitable technique that allows genetic polymorphism among intraspecific species and the genetic relationships within the isolates, geographic origins and toxicity levels.

Acknowledgements

This work was supported by the Research Council of the University of Tehran. The author would like to thank Dr. M. R. Khorramisadeh for statistical software analysis.

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