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Molecular Detection and Characterization of *Fusarium sporotrichioides* based on ITS2 rDNA Polymorphism

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ABSTRACT

The genus *Fusarium* contains a number of soil-borne species with worldwide distribution. The presented PCR assays are highly selective and sensitive in detecting the *Fusarium* genus. In order to identify the eighteen *Fusarium* isolates obtained at the molecular level. PCR analysis using primer specific for the conserved ITS DNA region of *Fusarium* genus was conducted. The data indicated that all of the eighteen isolates showed a clear band corresponding to the expected molecular size of the ITS region (431bp). These results confirmed that all the tested samples belong to the genus *Fusarium.* Also, when all eighteen isolates of *Fusarium* species were analyzed by PCR for fumonisin producing ability using FUM1 gene-based primers, the expected DNA fragment of 183 bp was amplified only in *Fusarium verticillioides* (3 isolates), *Fusarium avenaceum* (3 isolates), *Fusarium semitectum* (1 isolate) and *Fusarium culmorum* (2 isolates) showed a positive result with FUM1 gene set of primers. No bands were seen in other isolates of *Fusarium* spp. and the standard (*Fusarium graminearum*). In case of zearalenone, the *PKS4* gene of *F. graminearum* has been reported to be essential for the production of zearalenone. The result indicated that the expected DNA fragment of 280 bp was amplified only in *Fusarium verticillioides* (3 isolates), *Fusarium avenaceum* (3 isolates) and *Fusarium culmorum* (2 isolates) and *Fusarium graminearum*. Microsatellite-primed PCR resembles the well-known RAPD technique but is advantageous because of the ability to generate more complex banding patterns and a high degree of reproducibility. The discriminating powers of the three MP-primers [(CTG)5, (M13) and (T3B)] used in this study were nearly the same. Cluster analyses were performed on the genomic fingerprints generated by each of the primers tested. Three dendrograms were generated with the UPGMA method. The patterns resulting from the T3B and (CTG)5 test were more distinct and T3B was the most successful primer because it always led to high polymorphic banding patterns that were suitable for interspecies comparisons. Our results indicated that there was no association between clustering in the MP-PCR dendrogram and the geographic origin and morphological identification of the tested isolates.

INTRODUCTION

The genus *Fusarium* contains a number of soil-borne species with worldwide distribution which have been known for a long time as important plant pathogens **(1).** More recently, *Fusarium* has also been reported as an emerging human pathogen in immunocompromised patients **(2).** *Fusarium* mycotoxins are among the main fungal mycotoxin contaminations in food and livestock **(3),** and some human diseases, such as Kashi neck diseases and oesophageal cancer, have been epidemiologically associated with consumption of trichothecenes **(4, 5).**

Diagnostic methods based on the polymerase chain reaction (PCR) are rapid, as there is no need to culture organisms prior to their identification. They are specific, since identification of species is made on the basis of genotypic differences, and are highly sensitive, detecting target DNA molecules in complex mixtures even when the mycelia are no longer viable. Various PCR assays have been developed for the identification of mycotoxigenic species of *Fusarium*, some of them based on single copy genes directly involved in mycotoxin biosynthesis **(6 - 10)**.

PCR-based identification has several applications such as to study the dynamics of different *Fusarium* species over time and between geographical regions in cereals or other environments or to study disease development in the field. It can also be an important tool in risk assessment of grain as a screening method to identify samples with potentially high mycotoxin content to reduce costs for chemical analyses **(11)**.

Detection of fumonisin-producing fungal species by morphological characters sometimes is not enough for accurate identification of fungal isolates at the species level. Furthermore, both morphological and mating type characterization are time-consuming and require considerable expertise in *Fusarium* taxonomy and physiology **(12, 13)**. As identification of *Fusarium* species is critical to predicting the potential mycotoxigenic risk of the isolates, there is a need for accurate and complementary tools which permit a rapid, sensitive and reliable specific diagnosis of *Fusarium* species. The last ones often amplify multicopy target sequences, such as IGS or ITS regions (intergenic spacer and internal transcribed spacer of rDNA units, respectively), which increases the sensitivity of the assay in comparison with PCR assays based on single copy sequences. The use of these PCR approaches has been already useful in epidemiological analyses **(14, 15)** and are widely used in fungal taxonomy and more recently, they have been used for the detection of fungal pathogens in plant tissues

(16). The ribosomal DNA (rDNA) regions have often been chosen for taxonomic and phylogenetic studies because sequence data are available and contain both variable and conserved regions; despite the discrimination at the genus, species, or intraspecific level. The rDNA repeat includes both highly conserved genes and more variable spacer regions **(17)**.

This study investigated the developing PCR-based techniques for identification mycotoxigenic fungi and detected the genetic heterogeneity among mycotoxigenic fungi.

MATERIALS AND METHODS

1- Fusarium species isolated:

Isolates employed in this study and their sources are listed in Table (1). *Fusarium* species were grown at 25^0C on potato dextrose agar (PDA) during 7 - 10 days. Then spores were collected with a sterile solution of 0.1% (v/v) Tween 80 and stored at – 20°C in 25% (v/v) of glycerol before use.

2- Molecular materials:

The DNA sequences of the primers which required to PCR for identification of *Fusarium spp.* are listed in Table (2).

Molecular assay:

1- Fungal growth and purification on PDA and PDB media:

Disposable polystyrene Petri dishes (4cm) were filled with 1800 µL solid medium (potato dextrose agar), on which a layer of liquid medium (1400 µL peptone yeast glucose) was added. The fungal species isolate were cultured by inoculating a small mycelial disk from stock onto the prepared Petri dishes that were subsequently incubated for 2 - 3 days at 28°C. Mycelium was lifted from the medium using sterilized inoculating loops and transferred into sterile 1.5mL microfuge tubes. For some fungal species, the mycelium mats were pelleted by centrifugation for 15 min at 4000 gin a deep well swing-bucket rotor (microcentrifuge 5804 R; Eppendorf). The mycelium pellet was washed with 600 mL TE buffer and centrifuged again for 5 min at 4000 g. Finally, the TE buffer was decanted. Isolates were purified either by single spore or hyphal tip methods and then transferred to PDA slants. Pure cultures were grown on potato dextrose broth (PDB) for 10 days at 25 – 28°C in the dark. Mycelia were

harvested by filtration through filter paper (Whatman No. 1). The harvested mycelia were either used immediately for DNA extraction or stored at –70°C until use.

2- Isolation of mycelial DNA:

A modification of the traditional sodium dodecyl sulfate (SDS) extraction procedure was adopted. Fresh fungal mats (100mg) were homogenized in 400 µL sterile salt homogenizing buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Next, 6 µL 20 mg/mL RNase A was added and mixed well. The samples were incubated at 65°C for 10 min, after which 130 µL 3 M sodium acetate, pH 5.2, was added to each sample. Samples were vortexed for 30 s at maximum speed, and incubated at -20°C for 10 min. The lysate was centrifuged at 13,000 rpm at 4°C for 15 min, and the supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample, and after mixing well, and samples were incubated at -20°C for 10 min. Samples were then centrifuged for 20 min at 4°C, at 6000 rpm.

3- Testing of DNA on gel electrophoresis:

The DNA pellets were washed twice using 700 µL washing solution (100 and 70% ethanol, respectively). DNA pellets were subsequently air dried in an oven at 40°C for at least 10 min. The resultant DNA pellet was then resuspended in 100 µL 1X TE (10 mM Tris-HCl, 1 mM EDTA) buffer, pH 8.0. Seven microliters of isolated DNA and 3 µL of 10X loading dye were loaded in a lane of 1.5% (w/v) agarose gel containing 0.05 µg/mL ethidium bromide, to check the quality of the DNA **(18)**.

4- Identification of fungal species toxins by modified microsatellite-primed PCR:

Amplifications were performed using 1 µl of DNA and the core sequence of the microsatellite M-13, 5-GAGGGTGGCGGTTCT-3, was used as a primer **(174)**. The cycling parameters were: pre-denaturation, 93°C, 3 min, followed by 45 cycles of 93°C denaturation for 1 min, 55°C annealing for 1 min, 72°C extension for 1 min and a 72°C final extension for 10 min. Amplification products were separated on 1.5% agarose gel in TAE (Tris- Acetic acid- EDTA) buffer and stained with 0.1% ethidium bromide. For quantitative measurements, a charge-coupled device camera imaging system and UVIsoft were used to capture the image and to calculate the band intensities. Only reproducible bands in repeated PCR amplification were considered for analyses. All MP-PCR patterns were analyzed with Fingerprinting

Software (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK). Bands were automatically identified, verified and edited manually. Cluster analysis was done using the unweighted pair group method based on arithmetic mean method (UPGMA) **(19)**.

5- PCR amplification for Fumonisin-producing *Fusarium* species:

Two sets of primers were used as described by Bluhm, **(176)**. One set of primer was used from the conserved ITS DNA region specific to *Fusarium* genus (ITS Forward 5 AACTCCCAAAC CCCTGTGAACATA - 3, ITS Reverse 5 - TTTAACGG CGTGGCCGC - 3) and the expected size of amplicon was 431 bp. Another set of primer specific for fumonisin production was used from 'FUM 1 gene' of *Fusarium* species (FUM1 Forward 5– CCATCAC AGTGGGACACAG–3, FUM1 Reverse 5 – CGTATCGTCAGCATGATGTAGC – 3) and expected amplicon size was 183 bp. Primers and reagents for PCR analysis were obtained from MWG, Germany.

6- PCR amplification for Zearalenone-producing *Fusarium* species:

Based on the gene sequence of polyketide synthase *PKS4* (GenBank accession number DQ019316), the forward primer F1 (5-CGTCTTCGAGAAGATGACAT-3) and the reverse primer R1(5-TGTTCTGCAAGCACTCCGA-3) were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). Sequence specificity of the primers was checked by blastn program (http://www.ncbi.nlm.nih.gov/BLAST/). For isolation of the target gene fragment of *PKS4*, a conventional PCR reaction with genomic DNA of nine zearalenone-producing *Fusarium* isolates as template was carried out using a thermocycler (Eppendorf, Hamburg, Germany). Primers for PCR amplification F1/R1 was designed by Meng, **(20)**.

RESULTS

1- Molecular Detection of toxin-producing *Fusarium* isolates:

As identification of *Fusarium* species is critical to predict the potential mycotoxigenic risk of the isolates, there is a need for accurate and complementary tools which permit a rapid, sensitive and reliable specific diagnosis of *Fusarium s*pecies. Therefore, improved and quick methods such as DNA sequencing and species-specific PCR assay for identifying fumonisin forming fungi from animal feed staff has become important especially since fumonisins are now being implicated in diseases and cancer of animals **(21, 17**).

In the present study, the following fungal species viz., *Fusarium verticillioides* (3 isolates), *Fusarium avenaceum* (3 isolates), *Fusarium semitectum* (3 isolates), *Fusarium solani* (3 isolates), *Fusarium sporotrichioides* (1 isolate), *Fusarium poae* (2 isolates), *Fusarium culmorum* (2 isolates), *Fusarium graminearum* (1 isolate) and *Fusarium graminearum* which was used as control, were subjected to PCR analysis using 'ITS' genus specific and 'FUM 1 gene' specific primers and the results are presented in Table (3). Table (4) showed mycotoxin genes specific primers used in the study. In order to identify the eighteen *Fusarium* isolates obtained at the molecular level, PCR analysis using primer specific for the conserved ITS DNA region of *Fusarium* genus was conducted. The data in Figure (1) indicated that all of the eighteen isolates showed a clear band corresponding to the expected molecular size of the ITS region (431bp). These results confirmed that all the tested samples belong to the genus *Fusarium.*

On the other hand, when all eighteen isolates of *Fusarium* species were analyzed by PCR for fumonisin producing ability using FUM 1 gene-based primers, the expected DNA fragment of 183 bp was amplified only in *Fusarium verticillioides* (3 isolates), *Fusarium avenaceum* (3 isolates), *Fusarium semitectum* (1 isolate) and *Fusarium culmorum* (2 isolates), showed a positive result with FUM1 gene set of primers. No bands were seen in other isolates of *Fusarium* and genera of standard (*Fusarium graminearum*) (Fig. 2).

In case of zearalenone, the *PKS4* gene of *F. graminearum* has been reported to be essential for the production of zearalenone **183**. By generation of *PKS4* single insertional replacement mutant, it was identified that the gene catalyzes critical steps in the synthesis zearalenone, and its product stimulates expression of another gene involved in the zearalenone synthetic pathway **177**. Therefore, *PKS4* was selected as the target gene and a primer set F1/R1 was designed in this study. The result in Fig. (3) indicated that the expected DNA fragment of 280 bp was amplified only in *Fusarium verticillioides* (3 isolates), *Fusarium avenaceum* (3 isolates) and *Fusarium culmorum* (2 isolates) and *Fusarium graminearum*. This result proved that the target sequence was specific for the zearalenone-producing *Fusarium verticillioides, Fusarium avenaceum* and *F. culmorum* as well as the previously reported *F. graminearum.*

2- **Microsatellite-primed PCR amplification of *Fusarium* species isolates:**

To check the effectiveness of primers in the detection of intraspecific polymorphisms, experiments were performed by analysing isolates of *Fusarium* isolates. Amplification of

DNA from various *Fusarium* species isolates using microsatellite repeat primers resulted in a single distinguishable marker. To determine whether the PCR fingerprint technique could be employed for species identification, each sample of genomic DNA from 19 *Fusarium* isolates was amplified separately with three primers, the microsatellite repeats (CTG)5, (M13), and a primer derived from the intergenic spacer regions (T3B). (T3B) primers gave multiple amplification products at 50°C annealing temperatures because it always led to high polymorphic banding patterns that were suitable for inter-species comparisons. The patterns resulting from the T3B and (CTG)5 test were more distinct **(22, 23, 24)**.

The discriminating powers of the three MP-primers used in this study were nearly the same. Cluster analyses were performed on the genomic fingerprints generated by each of the primers tested. Three dendrograms were generated with the UPGMA method and are shown in Figs. (4, 5 and 6). Fig. (4) showed those obtained with the dendrogram produced by T3B-based fingerprinting. Isolates belonging to the same species typically clustered together at similarity values greater than 60%. The observed intra-isolates homologies were mostly in the range of about 80 to 100%.

The highest homology (100%) were found between isolates ***F. verticillioides (F. ver.* 2**) and ***F. avenaceum (F. ave.* 1**), ***F. culmorum (F.cul. 1)*** and ***F. culmorum (F.cul. 2)***, ***F. avenaceum (F. ave. 2***) and ***F. solani (F. sol.* 3**), ***F. sporotrichioides (F. sporo.)*** and ***F. poae (F. poa.* 1*)*** and between ***F. avenaceum (F. ave. 3***), ***F. semitectum (F. semi.* 3**) and ***F. poae (F. poa. 2***). There was no clear-cut relationship between clustering in the MP-PCR dendrogram and geographic origin of tested isolates. For example, isolates ***(F.cul.* 1**) and ***(F.cul.* 2**) were isolated from USA and France, respectively, however, they showed a very high genetic homology of 100%.

Generally, it falls in the broader class of resampling methods. Bootstrap test of Phylogeny (bootstrap values of phylogenic tree) used to investigate the genetic polymorphism within and between 19 *Fusarium* isolates, MP-PCR analysis was performed. A UPGMA tree resulting from M13 cluster analysis showed 80% bootstrap value of isolates 14 and 15 whose ***Fusarium poae*** isolates (Fig. 5). The highest homology (100%) were found between isolates ***F. avenaceum* (*F. ave.* 1**) and ***F. solani* (*F. sol.* 3)**. The phylogenetic tree generated by the M13 primer did not clarify the *Fusarium* species identity.

MP-PCR based genetic similarity (GS) analysis clustered nineteen for isolates into the first main cluster (Fig. 6), the genetic similarity ranging from 32 to 90%. ***Fusarium solani*** isolates **(*F. sol. 2*)** and (***F. sol.* 3**) showed high genetic relatedness (90%). Although, these isolates were isolated from Kenya and Uzbekistan, respectively.

Also, ***Fusarium semitectum*** isolates **(*F. semi.* 2**) and **(*F. semi.* 3)** showed high homology (85%). There was no association between clustering in the MP-PCR dendrogram and the geographic origin and morphological identification of the tested isolates.

DISCUSSION

Molecular analysis:

Fusarium is one of the most heterogeneous fungal genera and classification of species within this genus is very difficult. Currently, the differentiation of *Fusarium* spp. is based on physiological and morphological characteristics such as the shape and size of the macroconidia, the presence or absence of microconidia and chlamydospores, and colony morphology **(25)**.

Quantification and identification of *Fusarium* species have traditionally relied on culture methods and morphological classification that require specific expertise and experience. Culturing methods take time and are dependent on living propagules, which may not be related to toxin levels. In recent years, several PCR-based techniques have been developed to overcome this problem **(26)**.

PCR-based identification has several applications such as to study the dynamics of different *Fusarium* species over time and between geographical regions in cereals or other environments or to study disease development in the field. It can also be an important tool in risk assessment of grain as a screening method to identify samples with potentially high mycotoxin content to reduce costs for chemical analyses **(11)**.

Diagnostic methods based on the polymerase chain reaction (PCR) are rapid, as there is no need to culture organisms prior to their identification. They are specific, since identification of species is made on the basis of genotypic differences, and are highly sensitive, detecting target DNA molecules in complex mixtures even when the mycelia are no longer viable. Various PCR assays have been developed for the identification of mycotoxigenic species of

Fusarium, some of them based on single copy genes directly involved in mycotoxin biosynthesis **(6 - 10, 27).**

Amplification of target DNA through PCR with sequence specific primers is potentially more sensitive and rapid than microbiologic techniques, as a number of constraints are removed. Taxon-selective ITS amplification has already been used for detection of fungal pathogens such as *Fusarium* **(252)** and *Verticillium* spp. The data in Table (3) and Figure (1) indicated that all of the eighteen isolates showed a clear band corresponding to the expected molecular size of the ITS region (431bp). The rRNA genes, commonly used in identification and taxonomic studies, were confirmed in the present study to be particularly appropriate for the purpose of providing target sequences for molecular detection **(28)**.

In case of zearalenone, the *PKS4* gene of *F. graminearum* has been reported to be essential for the production of zearalenone (Table 4 and Figure 3). Polyketides (derived from polyketone) are a class of secondary metabolites produced by most organisms, but they have been most extensively examined in bacteria and fungi. In fungi, the polyketide synthases (*PKS*) are an important enzyme class involved in the production of secondary metabolites of fungi and were include a range of compounds such as the mycotoxins aurofusarin, aflatoxin **(29)**, and zearalenone and spore pigments **(30)**. Now, the selective cloning of genes encoding polyketide synthases (*PKSs*) can precede identification of a product and contribute to the overall analysis of polyketide diversity and function in an organism. As genomic sequences of fungi from a broad group of lifestyles become available, comparisons among studies of *PKS* genes will further our understanding of the evolution and ecological significance of this diverse group of compounds **(31)**.

Detection of fumonisin-producing *Fusarium* species based on the molecular methods and this method results are specific since identification of species is made on the basis of genotypic differences and are highly sensitive, detecting the target DNA molecules in complex mixtures, even when the mycelia are no longer viable **(32)**. The data in Figure (2) showed that the expected DNA fragment of 183 bp was amplified only in *Fusarium verticillioides* (3 isolates), *Fusarium avenaceum* (3 isolates), *Fusarium semitectum* (1 isolate) and *Fusarium culmorum* (2 isolates). So, targeting the mycotoxigenic genes is the best way especially where a particular mycotoxin can be produced by a number of species. Regions of homology within mycotoxin biosynthetic gene from the different species can be then used to develop specific primers allowing the detection of these relevant mycotoxigenic species **(33)**.

Microsatellite-primed PCR resembles the well-known RAPD technique **(34)** but is advantageous because of the ability to generate more complex banding patterns and a high degree of reproducibility **(22, 35 - 36)**.

In this study, we have focused on microsatellite-based methods supplying patterns specific for *Fusarium* isolates. Based on the specific PCR fingerprints and the high interspecies variation of these banding patterns, a clear distinction between all species was possible. The ability of selected primers to produce species-specific fingerprints was apparent. Isolates that were deemed to represent different species according to conventional morphological as well as molecular phylogenetic criteria gave rise to distinct PCR fingerprints, whereas isolates of the same species had similar banding patterns.

T3B was the most successful primer because it always led to high polymorphic banding patterns that were suitable for interspecies comparisons. Complex fingerprints are generated, allowing detection of polymorphisms at inter- and intraspecific levels and subsequent identification **(24)**. The high discriminatory power **(37, 38)** of this technique explains why it revealed a great heterogeneity among isolates.

In conclusion, it was observed that as the extract concentration increased the inhibitory effect increased. In other words, the inhibitory effect of the extract is proportional to its concentration and clove (*Syzygium aromaticum*) proved to be very effective oil and gave the most promising antifungal effect against *Fusarium* species tested.

The results presented here indicate that a microsatellite technique provides an efficient tool for the identification of poly and monomorphic loci that can be used to monitor the genetic differences between phytopathogenic fungi.

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Molecular identification of *Macrophomina phaseolina* by microsatellite-based fingerprint

Table (1) List of *Fusarium* isolates used in the study.

Serial No.	Isolate code	Fungi	Crop	Origin country
1	*F. ver.* 1	*F. verticillioides*	Yellow corn	Canada
2	*F. ver.* 2	*F. verticillioides*	Wheat	Germany
3	*F. ver.* 3	*F. verticillioides*	Wheat	Uzbekistan
4	*F. ave.* 1	*F. avenaceum*	Wheat	Canada
5	*F. ave.* 2	*F. avenaceum*	Wheat	USA
6	*F. ave.* 3	*F. avenaceum*	Wheat	USA
7	*F. semi.* 1	*F. semitectum*	Barely	Sudan
8	*F. semi.* 2	*F. semitectum*	Wheat	France
9	*F. semi.* 3	*F. semitectum*	Wheat	Australia
10	*F. sol.* 1	*F. solani*	Corn	Nigeria

11	*F. sol.* 2	*F. solani*	Yellow corn	Kenya
12	*F. sol.* 3	*F. solani*	Wheat	Uzbekistan
13	*F. sporo.*	*F. sporotrichioides*	Yellow corn	Kenya
14	*F. poa.* 1	*F. poae*	Soy	USA
15	*F. poa.* 2	*F. poae*	Soy	Canada
16	*F.cul.* 1	*F. culmorum*	Corn	USA
17	*F.cul.* 2	*F. culmorum*	Wheat	France
18	*F. gram.*	*F. graminearum*	Wheat	Germany
19	*F. annu.* 1	*F. annulatum*	Barely	Sudan
20	*F. annu.* 2	*F. annulatum*	Sesame	Kenya

21	*F. tric.* 1	*F. tricinctum*	Wheat	Australia
22	*F. tric.* 2	*F. tricinctum*	Wheat	Australia
23	*F. brev.*	*F. brevicatenulatum*	Sorghum	Nigeria
24	*F. fuji.* 1	*F. fujikuroi*	Wheat	Germany
25	*F. fuji.* 2	*F. fujikuroi*	Wheat	Australia
26	*F. floc.*	*F. flocciferum*	Wheat	Germany
27	*F. heter.*	*F. heterosporum*	Wheat	Uzbekistan
28	*F. equi.*	*F. equiseti*	Barely	Sudan
29	*F. dece.*	*F. decemcellulara*	Barely	Sudan
30	*F. oxy.*	*F. oxysporum*	Wheat	Australia
31	*F. cili.*	*F. ciliatum*	Yellow corn	Canada
32	*F. acum.*	*F. acuminatum*	Wheat	France

Table (2): DNA sequences of the primers used in the present study and size of the corresponding PCR products

Primer code	Primers sequence	Amplicon size	Reference
ITS	ITS F– 5-AACTCCCAAACCCCTGTGAACATA-3 ITS R– 5-TTTAACGG CGTGGCCGC-3	500 bp	Whit, *et al* 1990.
F1	5-CGTCTTCGAGAAGATGACAT-3	280 bp	Meng, K. *et al*, 2010.
R1	5-TGTTCTGCAAGCACTCCGA-3		
FUM1 F	5-CCATC ACAGTG GGACACAGT-3	430 bp	Bluhm, *et al* 2004.
FUM1 R	5-CGTATCGTCAGCATGATGTA GC-3		
M13	5-GAGGGTGGCGGTTCT-3	200 - 2200 bp	Kuhls, *et al.* 1995
T3B	5-AGG TCG CGGGTT CGA ATC C-3	160 - 2100 bp	Godoy, *et al.* 2004
(CTG)5	5-CTG CTG CTG CTG CTG-3	100 - 2000 bp	Abd-Elsalam, *et al* 2011

Table (3): The number of fungi tested and number of fungi showing positive for ITS and FUM1 gene as analysis by PCR.

Fungi	Number of Isolates	Positive for ITS region	Positive for FUM I gene
Fusarium verticillioides	3	3	3
Fusarium avenaceum	3	3	3
Fusarium semitectum	3	3	1
Fusarium solani	3	3	0
Fusarium sporotrichioides	1	1	0
Fusarium poae	2	2	0
Fusarium culmorum	2	2	2
Fusarium graminearum	1	1	0
Fusarium graminearum	standard	1	0

Table (4): *Fusarium* species screened and scored for presence and absence of mycotoxin genes using specific primers

Serial	Isolate code	*Fusarium* species	Mycotoxin gene specific-primers		
			Zea	FUM	ITS
1.	*F. ver. 1*	*F. verticillioides*	+	+	+
2.	*F. ver. 2*	*F. verticillioides*	+	+	+
3.	*F. ver. 3*	*F. verticillioides*	+	+	+
4.	*F. ave. 1*	*F. avenaceum*	+	+	+
5.	*F. ave. 2*	*F. avenaceum*	+	+	+
6.	*F. ave. 3*	*F. avenaceum*	+	+	+
7.	*F. semi. 1*	*F. semitectum*	-	+	+
8.	*F. semi. 2*	*F. semitectum*	-	-	+
9.	*F. semi. 3*	*F. semitectum*	-	-	+
10.	*F. sol. 1*	*F. solani*	-	-	+
11.	*F. sol. 2*	*F. solani*	-	-	+
12.	*F. sol. 3*	*F. solani*	-	-	+
13.	*F. sporo.*	*F. sporotrichioides*	-	-	+
14.	*F. poa. 1*	*F. poae*	-	-	+
15.	*F. poa. 2*	*F. poae*	-	-	+
16.	*F.cul. 1*	*F. culmorum*	+	+	+
17.	*F.cul. 2*	*F. culmorum*	+	+	+
18.	*F. gram.*	*F. graminearum*	+	-	+
19.	Standard	*F. graminearum*	+	-	+

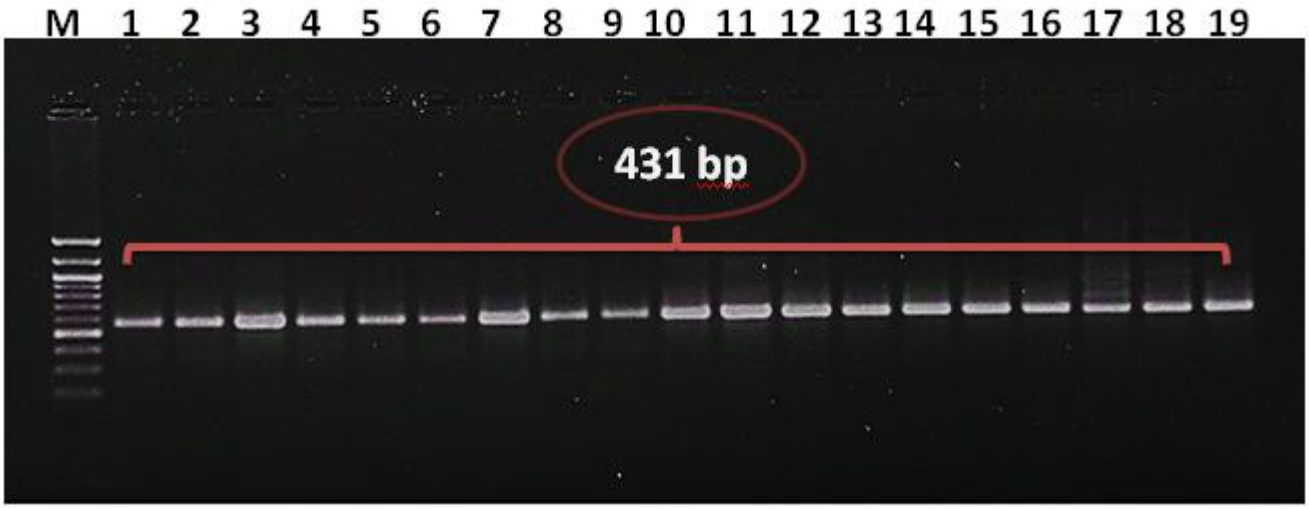

Fig. (1): Full-internal-transcribed spacer (ITS) PCR products amplified from different *Fusarium* species isolates using ITS1/ITS4 primers.

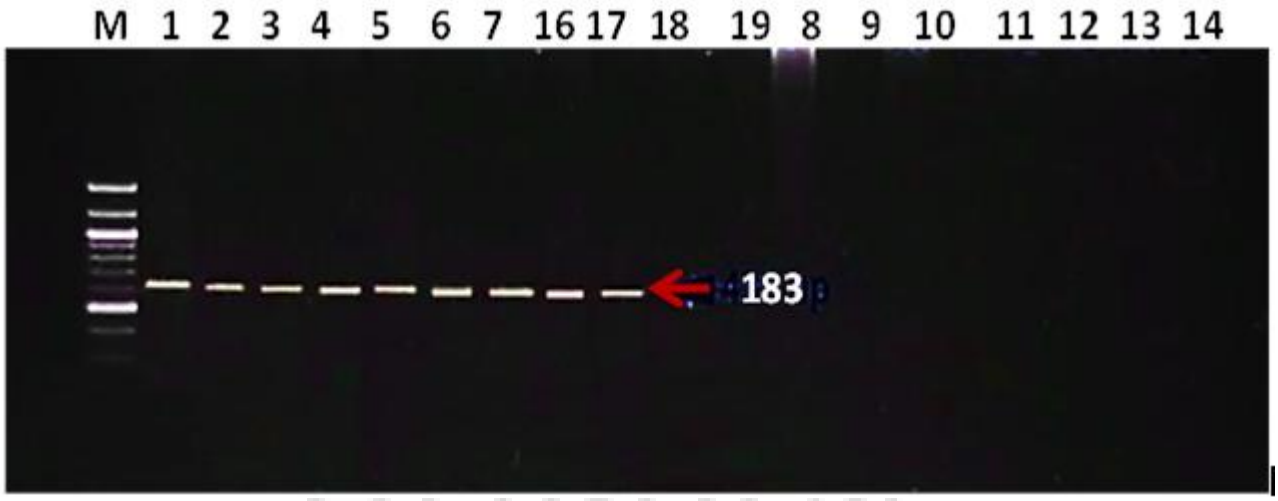

Fig. (2): PCR analysis for Fumanisin-producing *Fusarium* isolates genomic DNA using two different primers FUM1-F and FUM1-R with the M 100-bp used as molecular marker.

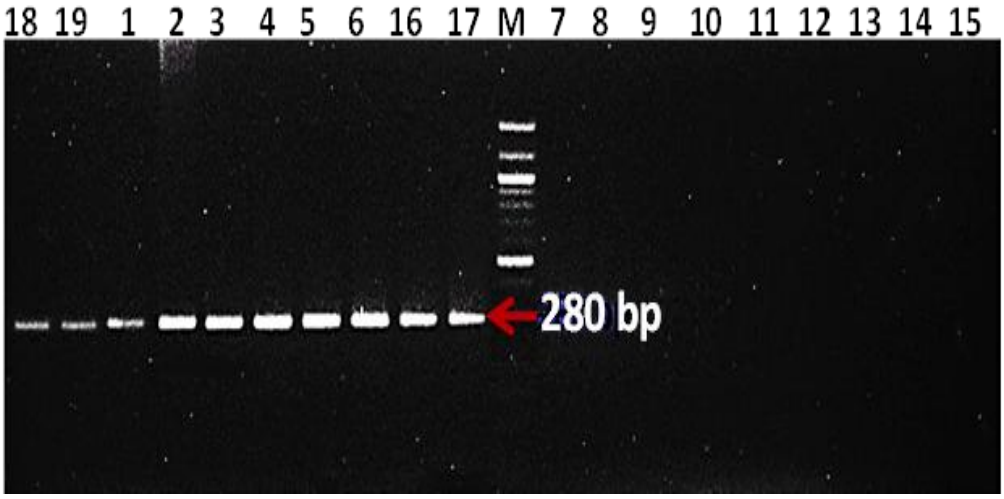

Fig. (3): PCR analysis for the Zearalenone-producing *Fusarium* isolates genomic DNA using two different primers F1 and R1 with the M 100-bp used as molecular marker.

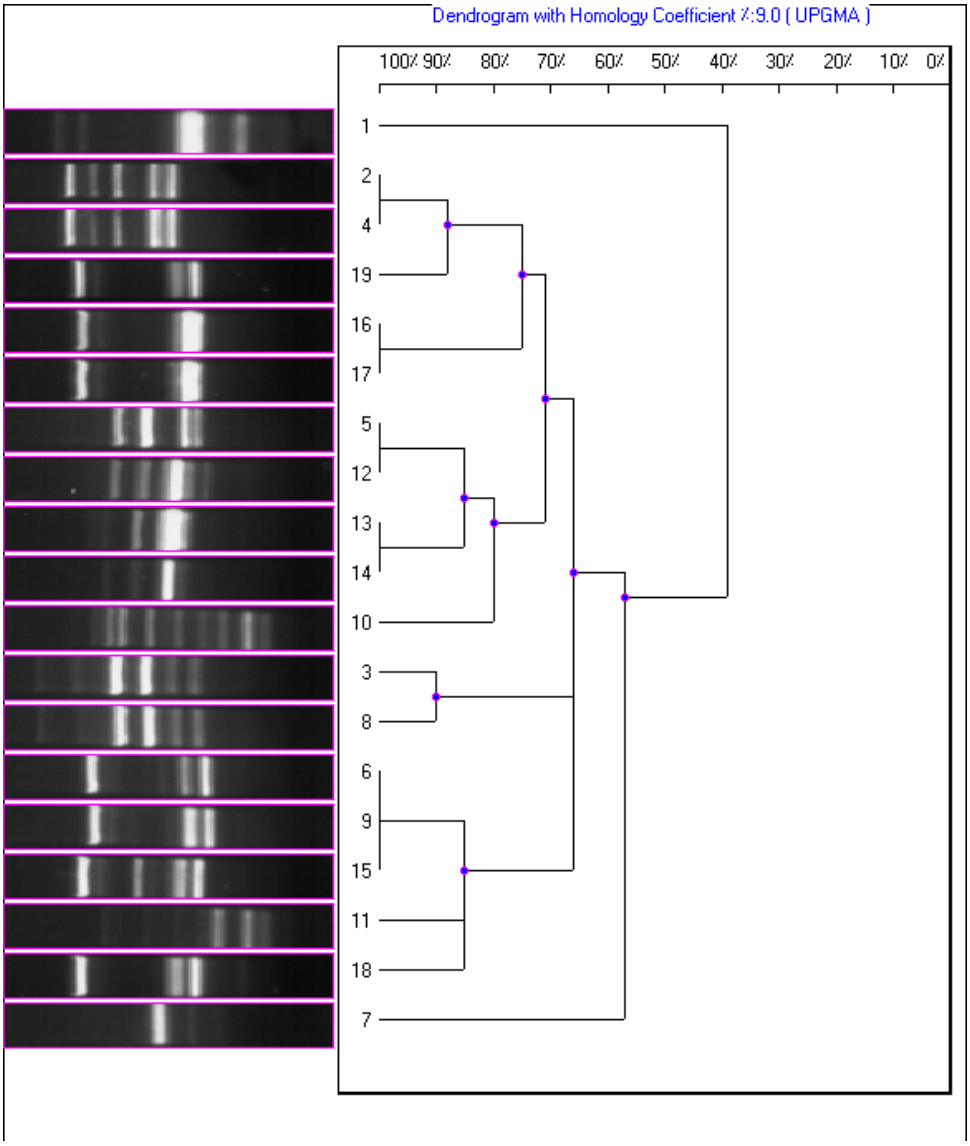

Fig. (4): Cluster analysis (unweighted pair-group method using arithmetic averages, UPGMA) of *Fusarium* species isolates based on DNA fingerprinting using microsatellite-primed PCR (T3B primer).

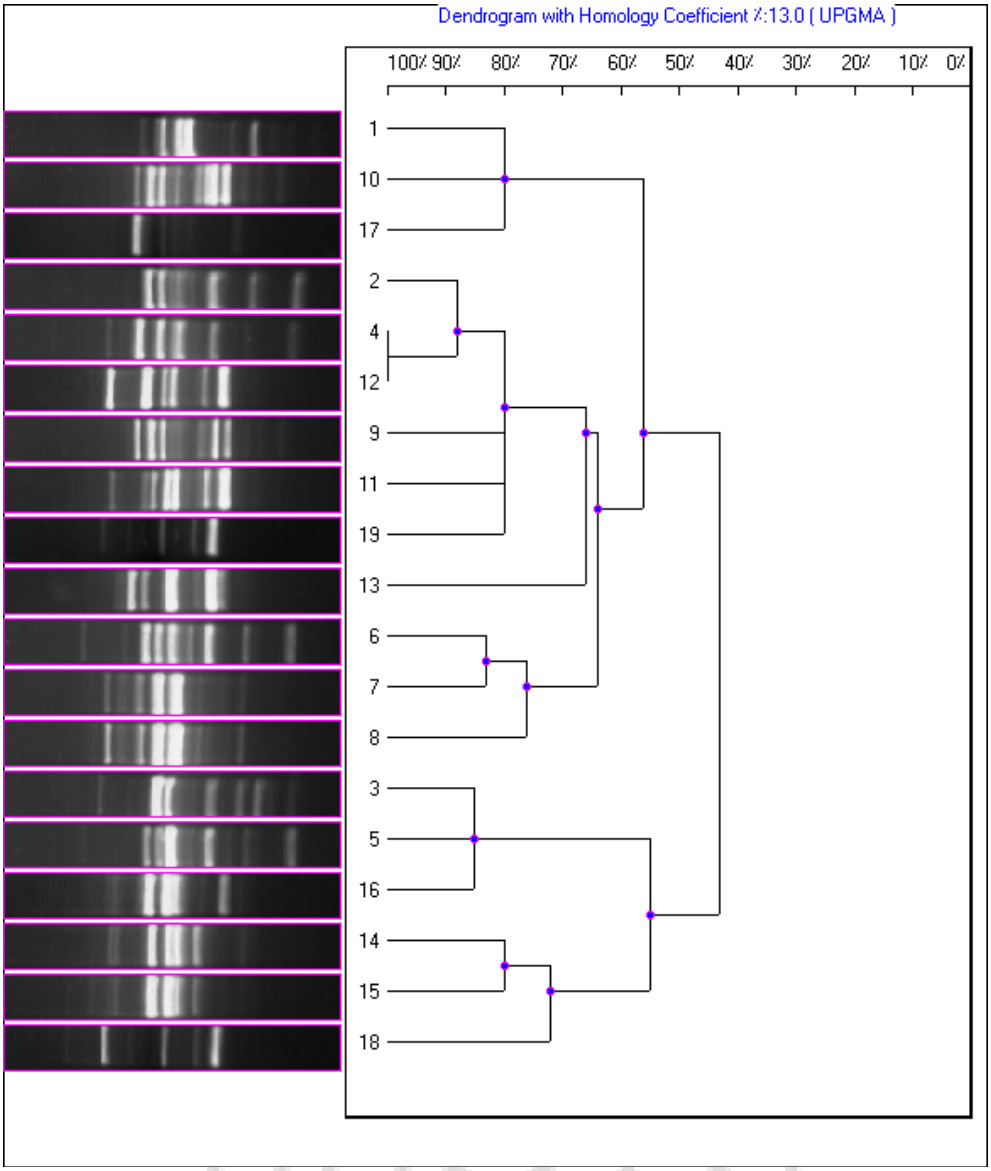

Fig. (5): Cluster analysis (unweighted pair-group method using arithmetic averages, UPGMA) of *Fusarium* species isolates based on DNA fingerprinting using microsatellite-primed PCR (M13 primer).

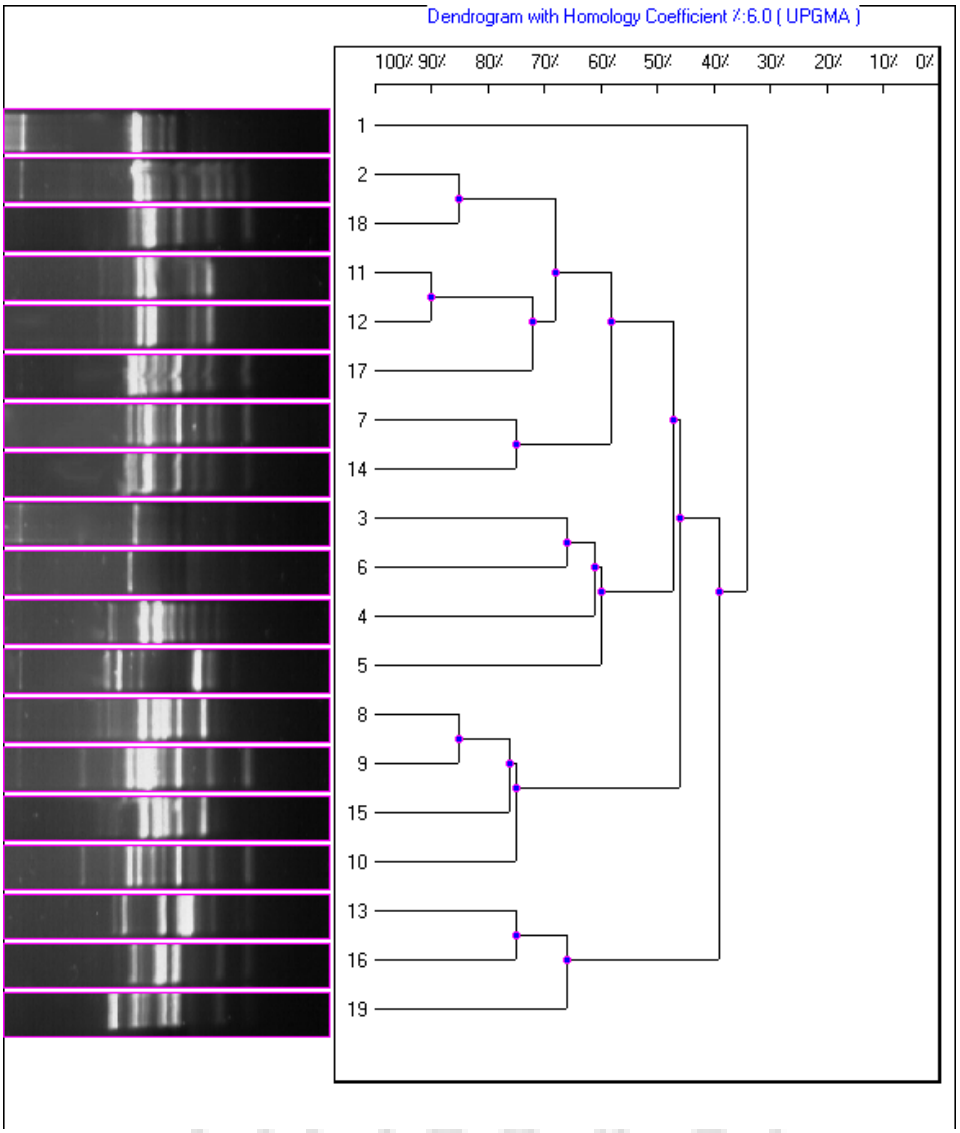

Fig. (6): Cluster analysis (unweighted pair-group method using arithmetic averages, UPGMA) of *Fusarium* species isolates based on DNA fingerprinting using microsatellite-primed PCR (CTG)5 primer.