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Molecular identification and characterization of *Fusarium* spp. associated with wheat grains

Mohamed A. Mahmoud[1]* and Shereen M. Shehata[2]
[1]Plant Pathology Research Institute, Agricultural Research Center, Giza 12619, Egypt
[2]Pharmaceutical Chemistry Depart., College of Pharmacy, King Saud University, Riyadh11495
*Corresponding author: *m.a.mahmoud75@gmail.com*

Abstract

Thirty one isolates belonging to six fungal genera were found to be associated with wheat grain samples collected from three main regions in Saudi Arabia. The most common genera (average frequency) were *Aspergillus* (19.52%), *Fusarium* (32.31%), *Penicillium* (12.19%), and *Alternaria* (8.2%). In this study, different isolates of *Fusarium* spp. were isolated from wheat grains samples and identified at the molecular level by ITS-rDNA regions amplification. Twenty five isolates of *Fusarium* spp. were screened for their ability to produce Fumonisin B1 (FB1), nivalenol (NIV) and deoxynivalenol (DON) using HPLC. Eight isolates were capable of producing detectable levels of FB1, NIV and DON. Inter-simple sequence repeats (ISSR) molecular markers were used, with the aim of genetically characterizing isolates of *Fusarium* spp. and discriminate between atoxigenic and toxigenic isolates. ISSR analysis revealed a high level of genetic diversity in the *Fusarium* spp. population and useful for genetic characterization. ISSR markers were not suitable to discriminate atoxigenic and toxigenic isolates also, Clustering based on ISSR dendrograms was unrelated to geographic origin.

Keywords: wheat, *Fusarium* spp, ITS-rDNA, mycotoxins, ISSR markers.

Introduction

Wheat (*Triticum* spp) is one of the most important and strategic products in all the world, where it can provide more than 20% of the calories of daily food intake (Wiese, 1987). Wheat is grown in most parts of the world, from near-arctic to near-equatorial latitudes. In 2013, world production of wheat was 713 million tons, making it the third most-produced cereal after maize and rice (745 million tons), (FAO, 2015). The amount of wheat traded internationally exceeds that of all other grains. Furthermore, the protein and caloric content of wheat is greater than that of any other food crop. Wheat is also used as an ingredient in compound feedstuffs, starch production, and as a feed stock in ethanol production (FAO, 2009). In 2013/2014, Saudi Arabia wheat production is 660,000 Metric Ton (MT), Import 3,45 million metric tons (MMT). Several researchers have been documented wheat is attacked by several filamentous fungi, mainly *Aspergillus*, *Fusarium Penicillium*, and species responsible for common mycotoxin contaminants of many cereal grains (Al-Hazmi, 2010; Bensassi et al., 2011; Lutfullah and Hussain, 2012). Cereal plants may be contaminated by mycotoxins in two ways: field or plant pathogenic (*Fusarium* species) and storage or saprophytic (genera *Aspergillus* and *Penicillium*) (Glenn, 2007). The genus *Fusarium* has a global

distribution and many species in the genus are phytopathogenic fungi infecting a wide range of crop plants including cereals such as maize, wheat, oat and barley. Fusarium contamination is a major agricultural problem as quality and yield can be reduced, but more importantly many species in the genus produce mycotoxins responsible for serious diseases in humans and farm animals (Mankeviciene et al, 2011). Mycotoxins are secondary metabolites produced by a wide variety of fungal species that cause nutritional losses and represent a significant hazard to the food chain (Magan and Aldred, 2007).

The most important Fusarium mycotoxins are deoxynivalenol (DON), zearalenone (ZEA), T-2 toxin (T-2), fumonisins (FB), HT-2 toxin (HT-2) and nivalenol (NIV). They are common mycotoxins throughout the world, mainly associated with cereal crops, in particular corn, wheat, barley, rye, rice and oats (Omurtag et al., 2006, Severino et al., 2006).

Many Molecular techniques based on DNA analysis are being widely used to identify and discriminate among isolates within a species and to develop rapid, sensitive, and accurate detection methods (Mule et al., 2005; Nicolaisen et al., 2005). Furthermore specific PCR helps in accurate discrimination of fumonisin (FB) producing and nonproducing *Fusarium* isolates occurring on cereal grain (Dawidziuk et al., 2014). Researchers use genes from the FUM cluster as a good additional marker for phylogenetic and taxonomic studies of the fumonisin-producing *Fusarium* species (Baird et al. 2008; Stepien et al. 2011). FB produced by *F. culmorum*, *F. graminearum*, *F. verticillioides* and *F. proliferatum* (Dawidziuk et al., 2014). Based on chemical structures and position of acetyl groups, in type B trichothecenes identified three chemotypes Deoxynivalenol (DON), Nivalenol (NIV) and derivatives. The ability to produce a certain type of mycotoxin could be established both through biochemical and molecular techniques. The molecular techniques are based on detection of different gene clusters, by using specific primers. As target for amplification could be used primers designed from the various gene such as *Tri3*, *Tri7*, *Tri12* and *Tri13* (Cornea et al., 2013).

The present study was aimed at (1) morphological and molecular identification of *Fusarium* spp. associated with wheat grains (2) assessment of the ability of *Fusarium* spp. to produce FB1, NIV and DON and (3) study of the and genetic diversity of *Fusarium* spp. using inters simple sequence repeat (ISSR) markers.

Materials and Methods

Collection of samples

Fifteen samples (1000 g each) of each grain type were collected from different markets located in Riyadh, Dammam, and Abha, in the Kingdom of Saudi Arabia, during May 2013 for this experimental study. The samples were stored at 2°C until being used (Czerwiecki et al., 2002).

The samples were hard wheat and their moisture around 13%, while the percentage of protein about 12.5%.

Identification *Fusarium* isolated

Samples were surface sterilized with 5% sodium hypochlorite solution for 1 min, before they were rinsed three times with sterile distilled water. Ten grains were placed randomly on potato dextrose agar (PDA) on three 9-cm Petri dishes. The Petri dishes were incubated at 25±2°Cand observed daily for emergence of colonies for 5 days in darkness. The replication was carried out three times separately.

The developing fungal colonies were sub-cultured onto PDA and identified based on their macro and microscopic features (Raper and Fennell, 1965; Domsch et al., 1993). Fusarium isolates were purified by single spore method and then cultured on synthetic nutrient agar (SNA) medium in 25±2°C in 12 hours light and 12 hours darkness condition for 7 to 14 days. *Fusarium* isolates were identified by both morphology and characteristic of colonies based on Leslie et al., 2006, Nirenberg, et al., 1998 Nelson et al., 1983 descriptions. Identification of fungal isolates was carried out on the basis of morphological and microscopic characteristics at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

DNA extraction and PCR amplification of partial sequence of ITS-rDNA

Genomic DNA was extracted from all *Fusarium* spp. inoculated into double-layer (one solid and one liquid) media in 50-mm Petri dishes. The base solid medium was a film of PDA, and the top medium was liquid PYG. DNA was extracted according to the method reported by Mahmoud (2015). Samples were stored at −80 °C until further use.

Molecular identification of *Fusarium* spp.

ITS-rDNA regions of 6 *Fusarium* species (*F. chlamydosporum*, *F. culmorum*, *F. gramineaum*, *F. oxysporum*, *F. proliferatum*, *F. verticillioides*) were amplified by PCR using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') synthesized from e-oligos brand products manufactured by Gene Link, USA.

PCR amplifications were run using mixtures with a final volume of 25 µL according to (Gaikwad et al., 2013). PCR was carried out on T Personal Thermo Cycler (Biometra, Germany). The amplified PCR products were sequenced using an automated ABI-Prism 377 DNA Sequencer (Applied Biosystems Inc., Foster City, CA, USA). The sequences were aligned using the MEGA 7.01 software against nucleotide National Center for Biotechnology Information (NCBI) for final identification. A phylogenetic tree was constructed using the neighbour-joining method (Saitou et al., 1987). Identification at species-level was based on per cent similarity between ITS sequences (Higgins et al., 2007).

Fumonisin B1 (FB1), NIV and DON quantification

For mycotoxin production assays, the 25 *Fusarium* isolates were cultured in 250 mL Erlenmeyer flasks containing 50 mL of rice culture medium (50 g of rice flour per 500 mL deionized water). The top of each flask was covered with aluminum foil, autoclaved and was allowed to cool down in a hood for about 40 min.

Mycelia from PDA plates were gently scrapped from the agar using sterile scalpels. Then, each flask was inoculated by adding the mycelia from the Fusarium isolates. The flasks were incubated in a regulated room at 24 °C for about 28 days. Mycelia were harvested by filtration through Whatman no. 1 paper. The dry mycelium was ground to fine powder using a coffee blender with ethanol cleaning between samples. The ground samples were stored at 0 °C until use.

Determination of Fumonisin B1 (FB1), NIV and DON quantification by HPLC

High-performance liquid chromatography (HPLC) was used to detect the following mycotoxins: FB1, NIV and DON. Standards of these toxins were purchased from Sigma Chemical Company (St. Louis, MO, USA) and stored at 4 °C in darkness. The procedure was performed as described by (Kushiro, 2013).

ISSR analysis

10 ISSR primers were synthesized by e-oligos brand products manufactured by Gene Link, USA. (Table 1) and used for the amplification of microsatellite loci. To select primers that produced a higher number of polymorphic bands for the characterization of *Fusarium* spp., 10 ISSR primers with di-or trinucleotide repeats were screened using DNA samples from representative isolates. PCR was performed using a thermalcycler (T personal, Biometra, Germany). PCR amplification was carried out in a 20 µL total volume containing 2 µL of 10× Taq buffer, 0.2 mmol L−1 dNTPs (2.5 mmol L−1 each), 1.5 mmol L−1 MgCl2, 10 pmol L−1 of each primer, 1 U of Taq DNA polymerase and 50 ng of genomic DNA. PCR was performed by initial denaturation at 94 ◦C for 4min, followed by 40 cycles of denaturation at 94 ◦C, annealing for 1min (the appropriate annealing temperature was used for each primer; Table 1). The thermal cycles were terminated by an extension for 2min at 72 ◦C and a final extension for 10 min at 72 ◦C. Amplified products were separated on 12 g L−1 agarose gel at 60 V cm−1 using 1× TBE buffer.

Table (1). Features of ISSR markers

No.	Primer sequence	T_a (°C)	Total bands	Polymorphic bands	Polymorphism(%)
1	CTCTCTCTCTCTCTCTAC	40	8	7	87.5
2	CACACACACACAAC	43	12	7	58.3
3	GTGTGTGTGTGTGG	40	11	5	45.4
4	GAGAGAGAGAGACC	40	10	6	60
5	AGAGAGAGAGAGAGC	41	13	7	53.8
6	GAGAGAGAGAGAGAGAT	46	9	7	77.7
7	CTCTCTCTCTCTCTCTGC	43	12	8	66.6
8	CTCCTCCTCGC	43	9	6	66.6
9	CCACCACCACCACCACCA	68	9	7	77.7
10	ATGATGATGATGATGATG	50	9	5	55.5

T*a*, annealing temperature

Results

Thirty one isolates belonging to six fungal genera were obtained from the test samples in the Riyadh region (Table 2). The frequency (42%) of *Fusarium* genus was the most predominant isolates. Thirteen isolates for *Fusarium* spp. such as *F. chlamydosporum* (1), *F. culmorum* (3), *F. gramineaum* (2), *F. oxysporum* (1), *F. proliferatum* (2) and *F. verticillioides* (4).

Table (2): Occurrence and frequency seed borne fungi isolated from wheat grains in different Saudi regions on PDA media at 25°C for 7 days.

Fungal isolates	Riyadh		Dammam		Abha	
Fusarium isolate	No. isolates	Frequency	No. isolates	Frequency	No. isolates	Frequency
F. chlamydosporum	1	3.22	1	4.76	0	0.00
F. culmorum	3	9.67	1	4.76	1	4.34
F. gramineaum	2	6.45	0	0.00	1	4.34
F. oxysporum	1	3.22	2	9.52	1	4.34
F. proliferatum	2	6.45	2	9.52	2	8.69
F. verticillioides	4	12.90	1	4.76	0	0.00
Aspergillus isolate						
A. flavus	3	9.67	2	9.52	1	4.34
A. parasiticus	2	6.45	0	0.00	1	4.34
A. niger	2	6.45	1	4.76	3	13.04
Other Fungi						
Alternaria alternata	1	3.22	1	4.76	3	13.04
Penicillium spp.	3	9.67	2	9.52	4	17.39
Mucor spp.	4	12.90	3	14.28	3	13.04
Rhizopus spp.	3	9.67	5	23.80	3	13.04

In Dammam, *Fusarium* genus was the dominant fungi from wheat grains, with a frequency 33.3% comparing *Aspergillus* and *Penicillium* 14.3% and 9.5%, respectively. Seven isolates for *Fusarium* spp. such as *F. chlamydosporum* (1), *F. culmorum* (1), *F. oxysporum* (2), *F. proliferatum* (2) and *F. verticillioides* (1). In Abha, *Fusarium* and *Aspergillus* genera were the dominant fungi from wheat grains, with a frequency 21.7% comparing *Aspergillus* and *Penicillium* 14.3% and 9.5%, respectively.

Molecular identification of Fusarium spp. and phylogenetic analysis

Fusarium spp. were identified morphologically based three types of spores, i.e., microconidia, macroconidia and chlamydospores. The identification of individual species was done accurately by sequencing the ITS-rDNA (Fig. 1) from each of the isolates comparison with available sequences in the data base (NCBI) using online BLAST analysis program.

The neighbour-joining tree constructed using the ITS-rDNA sequences of test isolates clustered the isolates of the same species in distinct groups. The results revealed that Clade I contained eight isolates (32%) were *F. verticillioides*, Clade II contained five (20%) were *F. proliferatum.* Clade III and VI contained two (8%) for both *F. oxysporum* and *F. chlamydosporum*. Clade IV and V contained four (16%) for both *F. culmorum* and *F. gramineaum*.

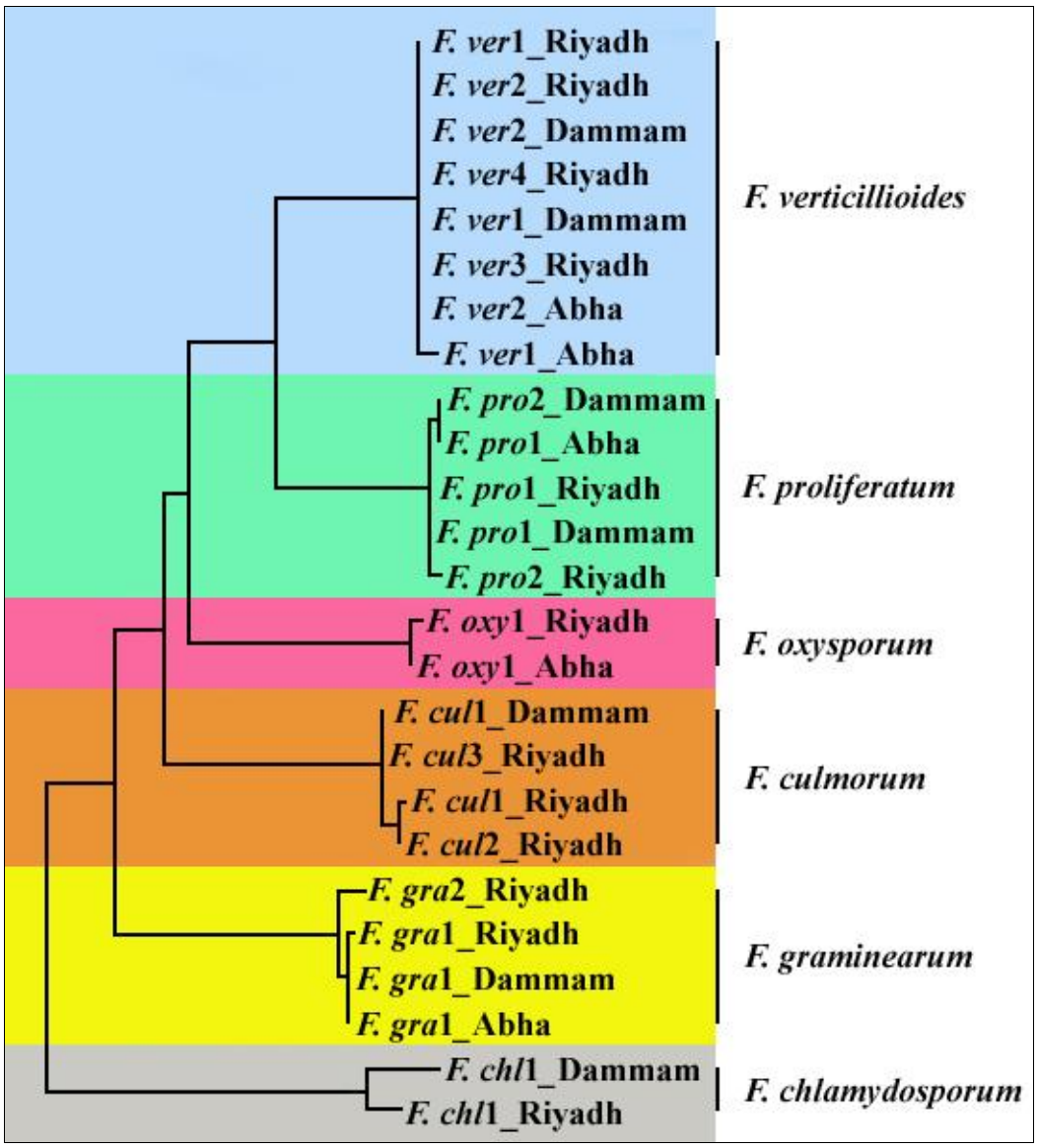

Fig. 1. Neighbour-joining consensus tree for ITS-rDNA sequences of *Fusarium* spp.

FB1, NIV and DON toxin production

Eight isolates were capable of producing detectable levels of FB1, NIV and DON, although seventeen isolates failed to produce any detectable amount (Table 3). The highest level (1.844 mg/mL) of FB1 toxin was obtained from isolate *F. culmorum* 1 from Dammam region while lowest level (1.177 mg/mL) obtained from isolate *F. verticillioides*2 from Abha region. For DON toxin, the highest amount (0.736 mg/mL) was obtained from isolate *F. proliferatum*2 from Dammam region, while lowest level (0.389 mg/mL) obtained from isolate *F. proliferatum*2 from Riyadh region. The highest production for NIV toxin was reached to 1.391 mg/mL for *F. culmorum*1 from Dammam region, while lowest production was decreased to 1.106 mg/mL F. gramineaum1 from Riyadh region. All isolates of *F. chlamydosporum* and *F. oxysporum* failure to producing any detectable amount of different toxins.

Table (3). Aflatoxin production by *Fusarium* spp. isolated from wheat grains collected from the Riyadh, Dammam, and Abha regions.

	Fusarium mycotoxins (mg/mL)		
Fusarium isolate code	FB1	DON	NIV
Riyadh region			
*F. chl*1	nd	nd	nd
*F. cul*1	1.158	0.539	1.218
*F. cul*2	nd	nd	nd
*F. cul*3	nd	nd	nd
*F. gra*1	1.614	0.425	1.106
*F. gra*2	1.127	nd	nd
*F. oxy*1	nd	nd	nd
*F. pro*1	nd	nd	nd
*F. pro*2	1.322	0.389	1.148
*F. ver*1	nd	nd	nd
*F. ver*2	nd	nd	nd
*F. ver*3	1.5.78	nd	nd
*F. ver*4	nd	nd	nd
Dammam region			
*F. chl*1	nd	nd	nd
*F. cul*1	1.844	0.658	1.391
*F. gra*1	nd	nd	nd
*F. pro*1	nd	nd	nd
*F. pro*2	1.687	0.736	1.252
*F. ver*1	nd	nd	nd
*F. ver*2	nd	nd	nd
Abha region			
*F. gra*1	nd	nd	nd
*F. oxy*1	nd	nd	nd
*F. pro*1	nd	nd	nd
*F. ver*1	nd	nd	nd
*F. ver*2	1.117	0.419	1.295

ISSR analysis

To investigate the degree of genetic diversity among and within different *Fusarium* species, the 25 isolates representing three different species were analysed with 10 primers. The ISSR primers generated very distinct amplification products, showing considerable variability among the isolates belonging to different species of *Fusarium*. The dendrogram of ISSR analyses grouped the 25 isolates into two clusters. Main cluster I contained thirteen isolates and main cluster II contained twelve isolates of *Fusarium* spp. (Fig. 2). Both main cluster I and II had two groups. The first main cluster contained two groups (I A and I B) with 13 isolates with genetic similarity (GS) of 19.58%. In main cluster I, the first group (I A) contained nine isolates with GS of 22.69% based on the banding pattern. Group (I A) included *F. Cul* 1, 3, *F. ver* 1, 2, 3, *F. pro*1 and *F. Chl* 1 from Riyadh region also *F. ver* 1 *and F. chl* 1 from Dammam region. In the second group (I B), four isolate with GS of 38.29% two isolates from Riyadh and two from Abha region. Main cluster I included nine atoxigenic isolates and four toxigenic isolates belonging to different species of *Fusarium*. Main cluster I included nine isolates from Riyadh region, two isolates for both Dammam and Abha regions.

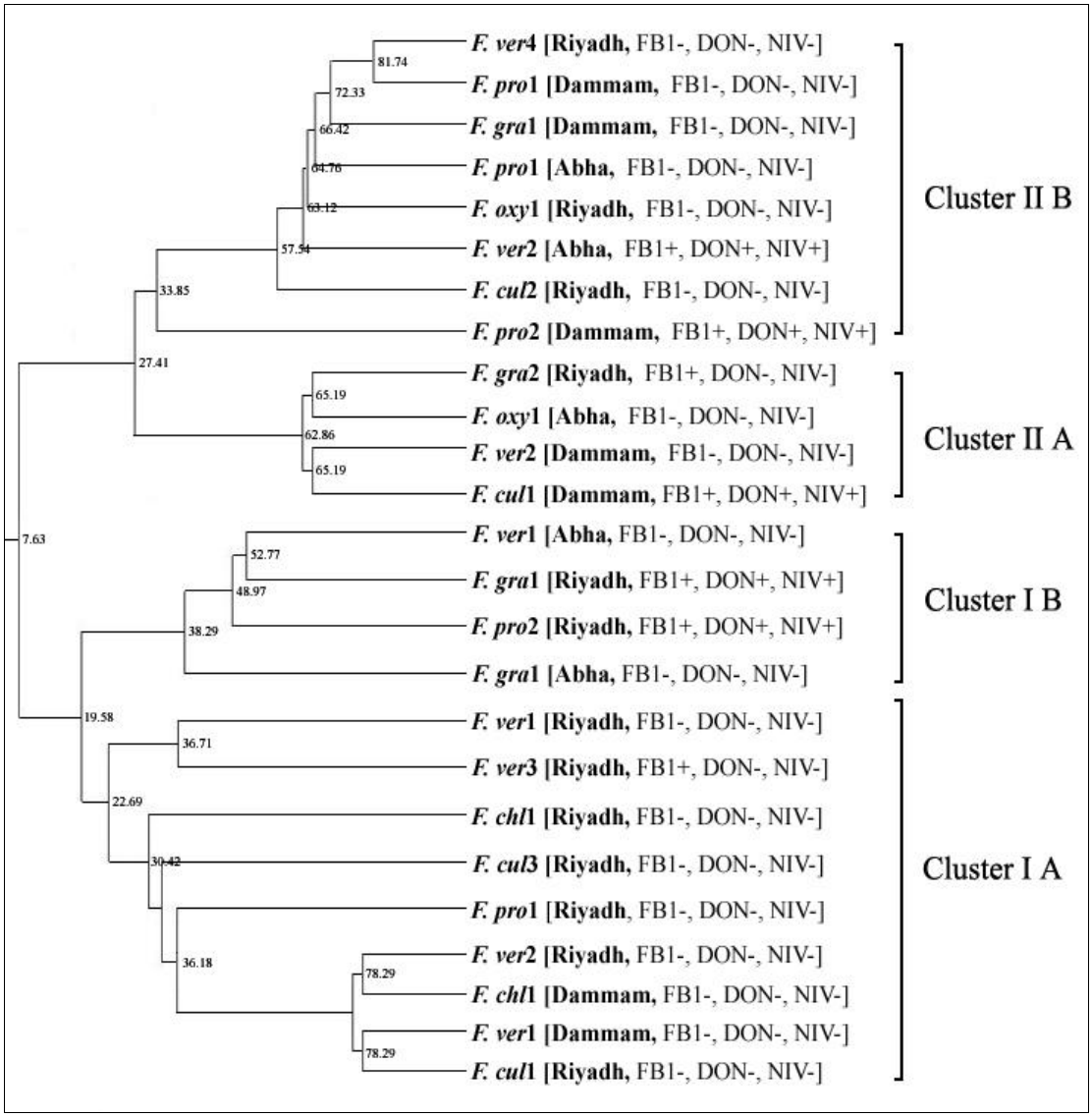

Fig. 2. Dendrogram based on ISSR fingerprints of 25 isolates of *Fusarium* spp. from wheat grains by UPGMA cluster analysis.

The second main cluster II contained two groups (II A and II B) with 12 isolates at 27.41% GS. The first group (II A) was divided into two subgroups. The first subgroup of (II A) contained two isolates at 65.19% GS from Dammam. The second subgroup of (II A) contained two isolates at 65.19% GS one from Abha and other from Riyadh. The second group (II B) included overlapping of eight isolates. Main cluster II included eight atoxigenic isolates and four toxigenic isolates belonging to different species of *Fusarium*. Main cluster II included four isolates from Riyadh region, five isolates form Dammam, three isolates Abha region.

FB1, NIV and DON-producing isolates resided in both main clusters with a random distribution and there was no obvious correlation between toxigenicity and cluster patterns of the isolates. On the basis of the dendrogram data, an exact association was not established between ISSR genotypes and the ability to produce FB1, NIV and DON. There was no clear-cut relationship between clustering in the ISSR dendrogram and geographic origin of the isolates tested.

Discussion

Wheat is one of the world's most important food crops. Foods made from wheat and its derivatives are a major part of a diet for over a world's people and as well animal feed (FAO, 2015). The contamination of wheat grains by fungi is a worldwide problem, where there are several reports documenting the presence of these fungi in samples from the United States (Cleveland et al., 2003), Argentina (Roige et al., 2009), Spain (Sardiñas et al., 2011), and Algeria (Riba et al., 2010). The results of isolation trials refer that, many of fungal genera and species associated with wheat grains which collected from 3 different regions as Riyadh, Dammam and Abha. The differentiation of occurrence may be return to location and reflects different agronomic practices and climatic conditions, which dictate the fungi that are present in a farming system Bryden (2012). In the present study, all 25 isolates of *Fusarium* spp. were morphologically identified and species determination was confirmed by PCR with primers specific for the ITS‑rDNA region sequencing. Nucleotide sequences were subsequently deposited with the NCBI database. The results showed that, out of the 25 isolates, eight were *F. verticillioides*, five were *F. proliferatum*, four for both *F. culmorum* and *F. gramineaum* and finally two for both *F. oxysporum* and *F. chlamydosporum* associated with Fusarium spp. are difficult to study, because strains with similar morphology represent different biological groups that include endophytes (Bacon and Hinton, 1996), saprophytes (Fracchia et al, 2000) and plant pathogens (Chandra et al., 2009). That is why it is difficult to establish systems of taxonomy for Fusarium spp., and therefore the importance of molecular methods to differentiate taxa is emphasized. The sequences most commonly used to distinguish Fusarium spp. are portions of genomic sequences encoding translation elongation factor-1α (Divakara et al, 2014), β-tubulin (O'Donnell et al., 1998), calmodulin (O'Donnell et al., 2000), internal transcribed spacer regions in the ribosomal repeat region (ITS1 and ITS2) Gaikwad et al., 2013) and the intergenic spacer region (IGS) (Yli-Mattila et al., 2010). Eight isolates were capable of producing detectable levels of FB1, NIV and DON, although seventeen isolates failed to produce any detectable amount. The amount of toxin produced will depend on physical factors (moisture, relative humidity, temperature and mechanical damage), chemical factors (carbon dioxide, oxygen, composition of substrate, pesticide and fungicides), and biological factors (plant variety, stress, insects, spore load) (Wicklow, 1995). Moisture and temperature have a major influence on mould growth and mycotoxin production (Pitt and Hocking, 1997). Analytical methods for mycotoxins in cereals and cereal-based products require three major steps, including extraction, clean-up, and detection/determination of the toxin. Different chromatographic methods are commonly used for quantitative determination of mycotoxins in cereals, including gas-chromatography, HPLC, and liquid chromatography-tandem mass spectrometry. HPLC affords sensitive and accurate determination of mycotoxins in unprocessed cereals and cereal-based products (Pascale, 2009).

In the present study, 10 ISSR markers are very useful for investigating the genetic characterization, diversity and population structure. No correlation was shown between FB1, NIV and DON producers and non producer with ISSR dendrogram. In view of the geographic isolation, the dendrogram produced using the ISSR marker showed no clear-cut relationship between clustering in the ISSR dendrogram and geographic origin. The ISSR method is a robust PCR-based technique that produces dominant molecular markers by DNA amplification of putative microsatellite regions (Zietkiewicz et al., 1994). ISSR markers showed a higher level of polymorphism than RAPD markers (Esselman et al., 1999) and have been used extensively in fungal population analysis (Tooley et al., 2000). Three ISSR markers were used to characterize 19 *F. verticillioides* isolates in terms of their genetic variability and their mycotoxigenic profiles. The intraspecific genetic similarity between *F. verticillioides* indicated a high level of genetic variation. Additionally, clustering of ISSR genotype was not related to toxigenic profiles of isolates (Mahmoud and Abd-El-Aziz Abeer, 2016). 19 ISSR primers produced 209 bands with a number of polymorphic bands and showed wide genetic diversity among the Fusarium spp. isolates of India. Similar observations have been made by many researchers on *Fusarium* spp. of other major food crops (Divakara et al., 2014). Four ISSR primers yielding highly polymorphic markers proved to be authentic and reliable for inferring the genetic relationships within and between *Fusarium* spp (Mishra et al., 2003). Phylogenetic analysis based on the fingerprints obtained through ISSR analysis indicated the presence of wide genetic diversity among *F. oxysporum* f.sp. *cubense* isolates of India (Thangavelu etal., 2012).

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