MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *FUSARIUM* ISOLATED FROM MAIZE EARS IN IRAN

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SUMMARY

A total of 191 *Fusarium* isolates were recovered from maize ears collected from 11 different geographic regions in Iran during 2004 and 2005. *Fusarium* isolates were identified on morphological characters and using species-specific PCR assays. DNA sequence-based identification of some unknown isolates was achieved using the translation elongation factor 1-α (TEF) gene region. *Fusarium verticillioides* was the most prevalent species representing 69.6% of the isolates. PCR analysis using the species-specific primers VER1/2 and PRO1/2 confirmed 98.5% and 85.1% of the morphological identifications of *F. verticillioides* and *Fusarium proliferatum* respectively. Based on sequence analysis five isolates were identified as either *Fusarium oxysporum*, *Fusarium* cf. *bullatum* or *Fusarium thapsinum*. Two isolates appeared to represent a new unnamed *Fusarium* species. This is the first report of *F.* cf. *bullatum* and *F. thapsinum* on maize kernels in Iran.

Key words: DNA sequencing, *Gibberella fujikuroi*, PCR, TEF, survey.

INTRODUCTION

Maize (*Zea mays*) is one of the major crops grown in Iran with production of approximately two million tons per year (Anonymous, 2005). Maize plants are attacked by several *Fusarium* species responsible for diseases such as root rot, stalk rot, seedling blight and ear rot. Fusarium ear rot is the most important disease of maize in Iran (Zamani, 1998). Several species of *Fusarium* belonging to section *Liseola* can cause Fusarium ear rot of maize, but *F. verticillioides* (Sacc.) Nirenberg has been reported as the most prevalent species on maize worldwide (Leslie, 1991; Chulze *et al.,* 2000; Danielsen *et al.*, 1998). Based on the taxonomic system of Gerlach and Nirenberg (1982) the teleomorph of *F. verticillioides* is *Gibberella moniliformis* Wineland, which corresponds to mating population A (MPA) of the *G. fujikuroi* species complex (Leslie, 1991; Danielsen *et al.*, 1998). Limited investigations on the prevalence of *Fusarium* species causing Fusarium ear rot of maize in Iran indicate that *F. verticillioides* is the dominant species. Other *Fusarium* species, especially *F. proliferatum* (Matsushima) Nirenberg (teleomorph: *G. intermedia*, *G. fujikuroi* mating population D), were reported also as pathogenic to maize kernels (Zamani, 1998; Ghiasian *et al.*, 2004).

Morphological identification of plant pathogenic fungi is the first and the most difficult step in the identification process. This is especially true for *Fusarium* species. Although morphological observations may not suffice for complete identification, a great deal of information is usually obtained on the culture at this stage. However, for species that cannot be reliably identified in this way, especially for members of the *G. fujikuroi* complex, additional analysis such as DNA sequencing and species-specific PCR assays must be conducted.

The translation elongation factor 1-α (TEF) gene appears to occur consistently as single-copy in *Fusarium*, and shows a high level of sequence polymorphism among closely related species, even when compared with the intron-rich portions of protein-coding genes such as calmodulin, β-tubulin and histone H3. Thus, TEF has become the marker of choice as a single-locus identification tool in *Fusarium* (Geiser *et al.*, 2004).

Geiser *et al.* (2004) created the first generation of FUSARIUM- ID v.1.0, a publicly available database currently consisting of 441 sequences representing a phylogenetically diverse selection of TEF sequences from the genus and placed it on a local BLAST server, which can be accessed at http://fusarium.cbio.psu.edu (Geiser *et al.*, 2004).

Some species-specific PCR primers have been developed, but in most cases they have yet to be more widely tested, and their reliability for analyses of strains from various crops and/or geographic locations is unproven. Some researchers have used species-specific PCR assay to identify some *Fusarium* species in the *G. fujikuroi* species complex. (Murillo *et al.*, 1998; Moeller *et al.,* 1999). For example, Mulé *et al.* (2004) used species-specific primers to identify *F. subglutinans*, *F. proliferatum*

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and *F. verticillioides* from maize. These primer pairs were based on partial calmodulin gene sequences.

Objectives of this study were: (i) identify the species belonging to *Fusarium* section *Liseola* isolated from maize ears in Iran; (ii) determine the dominant *Fusarium* species infecting maize in different geographic areas; and (iii) use species-specific PCR and DNA sequence analysis to confirm the morphological identifications and to identify unknown isolates.

MATERIALS AND METHODS

Fungal isolates. A total of 90 maize fields in 23 regions of 11 provinces were sampled during the growing seasons of 2004 and 2005 (Fig. 1). The fields were located in the main maize growing area in Iran with different climates. Each field was arbitrarily divided into five circular plots approximately 100 m in diameter and two to four samples were randomly taken from each plot. Samples were pooled in each field and two infected ears from each field were selected and used for *Fusarium* species isolation. A total of 191 *Fusarium* isolates were recovered (Fig. 1) from 180 maize ear samples collected from different geographic regions.

Identification of *Fusarium* species. Two maize seeds of each sample were surface-sterilized for 1 min with a 1% sodium hypochlorite solution, rinsed twice in sterile distilled water and dried in a laminar flow cabinet. Two growth media, Potato Dextrose Agar (PDA) and Peptone PCNB Agar (PPA), were used for fungal isolations. The plates were incubated at 25ºC in the dark for 5-7 days. All *Fusarium* isolates were subcultured on PDA, Spezieller Nahrstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA), using a single spore technique (Leslie and Summerell, 2006). PDA cultures were incubated at 25ºC and CLA and SNA cultures were incubated at 25ºC under near UV light for two to four weeks.

Cultural characters were assessed by eye and by microscopic examination. Colony morphology was recorded from cultures grown on PDA. The morphology of macroconidia, microconidia, conidiogenous cells and the chlamydospores was assessed from cultures grown on SNA and CLA. Morphological identifications of isolates were made using the criteria of Gerlach and Nirenberg (1982) and Leslie and Summerell (2006).

DNA extraction. *Fusarium* isolates were grown on PDA plates for 7 days and mycelia were harvested and ground in liquid nitrogen. Total DNA was extracted from ground mycelium of each isolate (~100 mg wet weight) using a DNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's instructions.

Species-specific PCR. The primer pairs VER1/2, PRO1/2 and SUB1/2 (Table 1) were used in species-specific PCR assays to identify *F. verticillioides, F. proliferatum* and *F. subglutinans,* respectively (Mulé *et al.*, 2004). The reaction mixtures were prepared in a total volume of 25 µl with a final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM of each dNTP and 1.5 mM $MgCl_2$. For each reaction 0.6 U of Ampli Taq polymerase (Applied Biosystems, USA), 15 pmol of each primer and approximately 25 ng of fungal template DNA were used. Reactions were performed in a GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems, USA) using the following PCR conditions: denaturation at 95ºC for 5 min, 35 cycles of denaturation at 94ºC for 50 sec, annealing at 56ºC for 50 sec, extension at 72°C for 1 min, final extension at 72°C for 7 min, followed by cooling at 4°C until recovery of the samples. Amplification products were visualized in 1.2% agarose gels stained with ethidium bromide (Mulé *et al.*, 2004).

DNA sequencing. A standard PCR reaction was used to amplify the TEF gene region of 26 samples (Table 2). The primer pair ef1 and ef2 (O'Donnell *et al.*, 1998) (Table 1) was used in a PCR reaction with an annealing temperature of 53ºC (Geiser *et al.*, 2004). After amplification, products were purified by using a Gene Elute PCR clean-up kit (Sigma, USA). and were directly sequenced using an ABI PRISM® BigDye Terminator Cycle Se-

Table 1. Sequences of primers used in the experiments.

Primer name	Primer sequence (5' → 3')	Species-specificity
SUB1	CTGTCGCTAACCTCTTTATCCA	*F. subglutinans*[a]
SUB2	CAGTATGGACGTTGGTATTATATCTAA	
PRO1	CTTTCCGCCAAGTTTCTTC	*F. proliferatum*[a]
PRO2	TGTCAGTAACTCGACGTTGTTG	
VER1	CTTCCTGCGATGTTTCTCC	*F. verticillioides*[a]
VER2	AATTGGCCATTGGTATTATATATCTA	
EF1	ATGGGTAAGGA(A/G)GACAAGAC	All *Fusarium* species[b]
EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT	
EF22	AGGAACCCTTACCGAGCTC	

[a]Mulé *et al.*, 2004; [b]O'Donnell *et al.*, 1998; Geiser *et al.*, 2004

quencing. Ready Reaction Kit v.1.1 (Applied Biosystems, USA). The internal reverse ef22 primer (Table 1) was used in the sequencing reactions which generated ~ 450 bp of sequence data (Geiser *et al.,* 2004). All sequencing products were precipitated by ethanol according to the manufacturer's protocol and were analyzed on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA). DNA sequences were edited with SeqMan™II 4.03 (Lasergene Sequence Analysis Software, DNASTAR, USA) then used as a query to search for similarities using the BLAST network services at the National Centre for Biotechnology Information (NCBI) (Altschul *et al.*, 1997) and the FUSARIUM-ID v.1.0 database (http://fusarium.cbio.psu.edu) (Geiser *et al.*, 2004).

DNA cloning. DNA from 18 isolates gave amplified fragments with primers VER 1/2 and PRO1/2. Therefore, the TEF gene region of 10 representative isolates was amplified with primers ef1/ef2 (O'Donnell *et al.*, 1998) and cloned into chemically competent TOP10 One Shot *Escherichia coli* using a TOPO TA Cloning Kit (Invitrogen, USA) according to manufacturer's instructions. From each isolate nine white colonies were selected for PCR amplification using the primers PUC1 (5'-TATCTGCGCCTCTGCTGAA-3') and PUC2 (5'-CCAAATACTGTTCTTCTAGTG-3'). The amplicons from six clones were randomly chosen for purification and determination of the DNA sequence as described above.

RESULTS

Morphological identification. Of the 191 *Fusarium* isolates, 140 were identified as *F. verticillioides*, 47 as *F. proliferatum* and one as *F. oxysporum*, based on morphological criteria. Three further isolates could not be identified to species level.

PCR amplification. All isolates were tested with the species-specific primers VER 1/2 and PRO 1/2. Of the 140 isolates morphologically identified as *F. verticillioides,* 131 were identified using the VER 1/2 primers, whereas of the 47 isolates morphologically identified as *F. proliferatum*, 40 were identified using the PRO 1/2 primers. A small number of isolates morphologically identified as either *F. verticillioides* or *F. proliferatum* did not show any positive signal in the species-specific PCR reactions. Eighteen isolates yielded PCR products

Table 2. Identification based on sequencing of the translation elongation factor 1-α gene region.

Isolate	Location	Morphological identification	Sequence based identification	Sequence with best match	Identity (%)
15	Golestan	*F. verticillioides*	*F. verticillioides*	NRRL22172	99
36	Fars	*F. proliferatum*	*F. proliferatum* [1]	MUCL31970	99
37	Kerman	*F. verticillioides*	*F. verticillioides* [1]	NRRL22172	99
39	Fars	*F. proliferatum*	*F. proliferatum* [1]	NRRL31071	100
40	Qazvin	*F. oxysporum*	*F. oxysporum*	NRRL	99
53	Ardabil	*F. proliferatum*	*F. proliferatum* [1]	NRRL31071	99
57	Qazvin	*F. proliferatum*	*F. proliferatum* [1]	NRRL31071	100
58	Azerbaijan	*F. proliferatum*	*F. proliferatum*	NRRL31071	99
93	Kurdistan	*F. verticillioides*	*F. verticillioides*	isolate FvMM2-4	100
96	Fars	*F. verticillioides*	*F. verticillioides* [1]	NRRL22172	99
134	Fars	*F. proliferatum*	*F. proliferatum*	NRRL31071	99
139	Mazandaran	*F. proliferatum*	*F. proliferatum* [1]	MUCL31970	99
140	Hamedan	*F. proliferatum*	*F. proliferatum* [1]	MUCL31970	99
144	Khuzestan	*F. verticillioides*	*F. verticillioides* [1]	NRRL22172	99
149	Ardabil	*F. proliferatum*	*F. proliferatum*	NRRL31071	99
152	Tehran	*Fusarium* sp.	*F.* cf. *bullatum*	NRRL 31005	98
153	Hamedan	*Fusarium* sp.	*F.* cf. *bullatum*	NRRL 31005	98
156	Isfahan	*F. proliferatum*	*F. proliferatum*	NRRL31071	99
163	Ardabil	*F. verticillioides*	*F. verticillioides* [1]	NRRL22172	99
165	Kermanshah	*F. proliferatum*	*F. proliferatum*	NRRL31071	99
167	Ardabil	*F. proliferatum*	*F. proliferatum*	NRRL31071	99
176	Qazvin	*Fusarium* sp.	*F.* cf. *bullatum*	NRRL 31005	98
184	Golestan	*F. verticillioides*	*Fusarium* sp.	NRRL 26793	94
187	Khuzestan	*F. verticillioides*	*Fusarium* sp.	NRRL 26793	94
192	Fars	*F. proliferatum*	*F. proliferatum*	NRRL31071	99
196	Tehran	*F. verticillioides*	*F. thapsinum*	strain NRRL22045	98

[1]Sequences obtained after DNA cloning

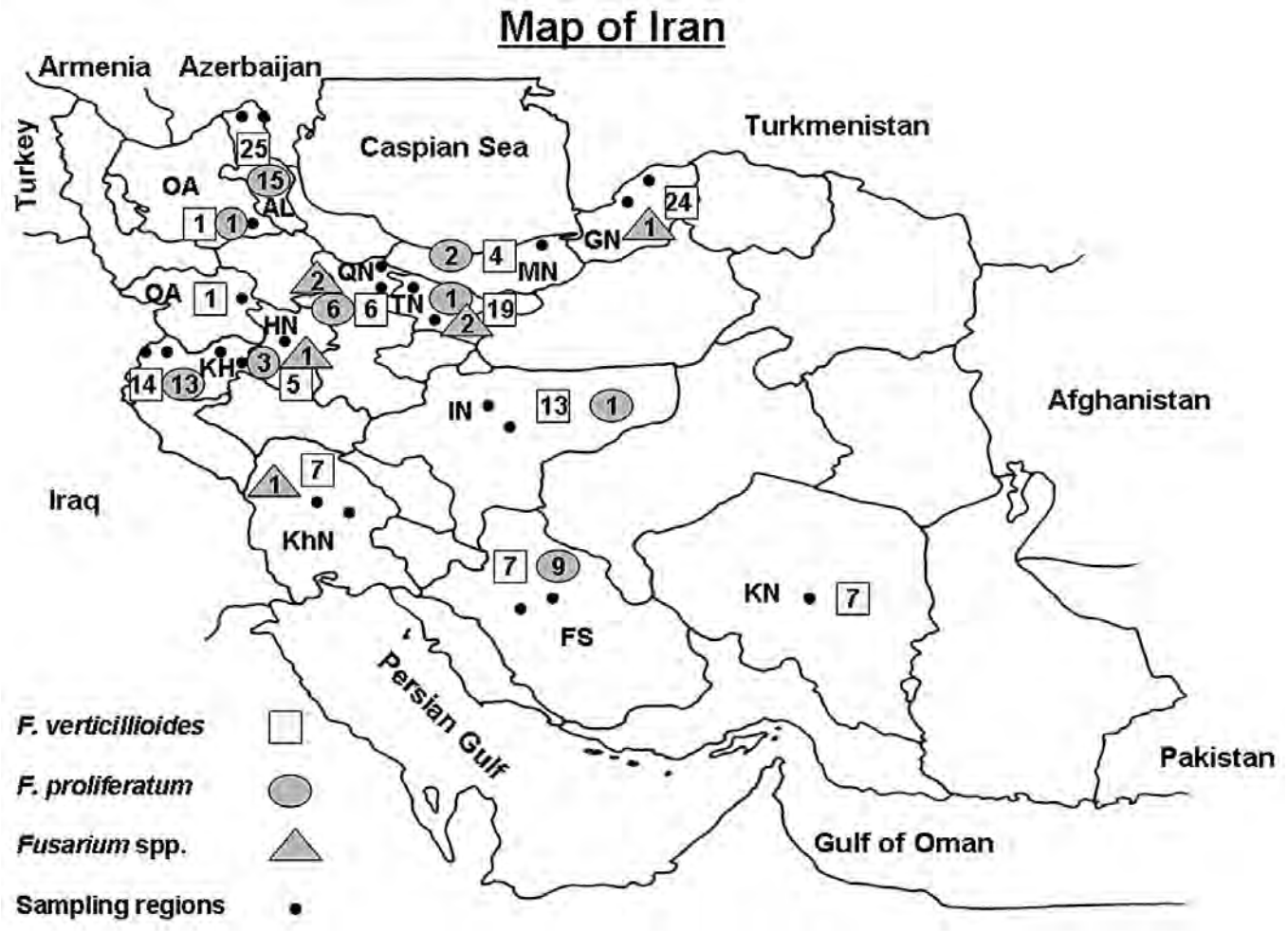

Fig. 1. Map of Iran showing sampling regions and data related to *Fusarium* isolated from maize ears in 11 Iranian provinces during 2004 and 2005. Capital letters in the map indicate province names: AL=Ardabil; FS=Fars; GN=Golestan; HN=Hamedan; KN=Kerman; KH=Kermanshah; KhN=Khuzestan; IN=Isfahan; MN=Mazandaran; QN=Qazvin; TN=Tehran and OA=Other Areas.

using primer pairs VER 1/2 and PRO 1/2. Of the three isolates morphologically identified only as *Fusarium* sp., no amplification fragments were observed in any of the species-specific PCR assays.

No isolate was identified as *F. subglutinans* with morphological analysis, but since species-specific primers for *F. subglutinans* were available (SUB 1/2), all unknown isolates and those that had not given any amplified PCR product using the *F. verticillioides*- and *F. proliferatum*-specific PCR primers, were tested with SUB 1/2. As expected, no positive signal was obtained with these primers.

DNA cloning and sequencing. The TEF region of 16 unknown isolates or isolates which showed no positive signal in any of the species-specific PCR assays were selected to be sequenced. As 18 DNA samples gave an amplified fragment with both the VER 1/2 and PRO1/2 primer pairs, extracted DNA from 10 of these isolates were randomly chosen for further amplification of the TEF region followed by cloning and sequence analysis. Six randomly selected clones from each isolate showed identical DNA sequence for each of the clones originating from the same isolate. These isolates were identified with a BLAST search for similarities present in the NCBI database as well as by comparing their sequences against the FUSARIUM-ID database.

Results of the identification of the 16 unknown isolates as determined by the partial DNA sequence of their TEF region (Table 2), showed that of the five isolates initially identified as *F. verticillioides* by morphological characterization two, which could not be identified using the VER 1/2 primer pair, were found to be *F. verticillioides*, one was *F. thapsinum*, a species morphologically very similar to *F. verticillioides*, and the last two belonged to a putative new unnamed *Fusarium* species. Similarly, the seven isolates identified as *F. proliferatum* based on morphological characters, which had not yielded amplicons with PRO 1/2 primers, proved to be *F. proliferatum*. Three isolates that could not be identi-

fied to the species level based on morphology, most closely matched *F.* cf. *bullatum* isolate NRRL 31005 in the FUSARIUM-ID database. One isolate was identified as *F. oxysporum* morphologically and by TEF.

Results of sequence analysis of the cloned genomic DNA fragments of 10 representative isolates confirmed their morphological identification (Table 2).

DISCUSSION

Distinguishing species within the *G. fujikuroi* species complex using morphological characters is difficult even for specialists (Summerell *et al.*, 2003; Leslie and Summerell, 2006), so DNA sequence-based identifications and species-specific PCR assays are usually needed to accurately identify species within the complex. Thus, we used species-specific PCR and sequence analyses in some cases to confirm our morphological identifications and to identify unknown isolates.

Based on morphology, 140 of 191 isolates were identified as *F. verticillioides*. Molecular identification assays confirmed 133 of the 140 isolates to be *F. verticillioides* and showed that seven of 140 isolates belonged to other *Fusarium* species. i.e. *F. thapsinum* (one isolate), *F. proliferatum* (four isolates) and an unidentified *Fusarium* sp. (two isolates).

The *F. verticillioides*-specific primers VER 1/2 confirmed 98.5% (131 of 133 isolates) of the identifications as *F. verticillioides.* The PRO 1/2 primers confirmed 85.1% (40 of 47 isolates) of the morphological identifications as *F. proliferatum*. None of the isolates showed any amplification with the SUB 1/2 PCR primers specific for *F. subglutinans*. This is the first study where species-specific primers were used for the identification of members of the *G. fujikuroi* species complex in Iran.

When the DNA sequence was determined of the TEF regions of nine isolates (seven *F. proliferatum* and two *F. verticillioides* isolates) that were not amplified by VER 1/2 or PRO 1/2 primers, morphological identifications were confirmed. It is noteworthy that 18 isolates showed positive signals using the VER 1/2 and PRO 1/2 primers.

Results of sequence analysis of the cloned genomic DNA fragments of 10 representative isolates revealed that these DNA samples were from pure strains corresponding to a single species rather than to a mixture of species, since only a single DNA sequence was obtained from all clones from the same amplicon. An explanation of the VER 1/2 and PRO 1/2 PCR results may be that high genetic diversity exists among Iranian *Fusarium* isolates and that the so-called species-specific PCR primers are not specific for all strains of *F. proliferatum* and *F. verticillioides*.

Detailed studies on more isolates originating from different geographic regions of the world are required to answer this question. As these primers were designed on partial sequences of the calmodulin gene of isolates originating from Europe and the US, it may be that the Iranian populations of these species are genetically more diverse than those from Europe and the US. Since TEF alleles within *F. proliferatum* may vary by as much as 1.5% (Geiser *et al.*, 2004) and probably less so in *F. verticillioides*, the failure of the species-specific primers to work on some isolates of both fungi can be expected.

All TEF sequences of unknown isolates were compared with sequences in the NCBI GenBank database and in the FUSARIUM-ID database, but due to the lack of quality control on the correct identification of sequences in the GenBank database, we used the FUSARIUM-ID database (Geiser *et al.*, 2004) for species determination. In this way three isolates were identified as a *Fusarium* species very closely related to *F. bullatum* (99% nucleotide identity). Their morphological characters, especially the absence of microconidia, the shape and size of macroconidia and conidiophores, the colour and growth rate of colonies indicated that this fungus was quite similar to *F. equiseti* var. *bullatum*, synonymous with *F. bullatum* (Gerlach and Nirenberg, 1982).

Of the 191 *Fusarium* isolates collected in Iran, *F. verticillioides* was the most prevalent with a frequency of 69.6% (133 of 191), followed by *F. proliferatum* with a frequency of 26.7% (51 of 191). However, in Fars province *F. proliferatum* was the most common, whereas in Qazvin province, *F. proliferatum* and *F. verticillioides* occurred with an equal frequency (Fig. 1). The lowest and highest incidence of *F. verticillioides* were observed in Qazvin (42.9%) and Kerman province (100%), respectively. The dominance of *F. verticillioides* in Iranian maize kernels is in accordance with previous reports from Iran (Zamani, 1998; Ghiasian *et al.*, 2004) and other countries (Leslie, 1991; Chulze *et al.*, 2000; Marasas, 2001).

Of the seven isolates (Table 2) identified as species other than *F. proliferatum* and *F. verticillioides*, one was a *F. oxysporum* strain isolated from the Qazvin province, three, coming from three different geographic regions (Qazvin, Tehran and Hamedan provinces), belonged to a species most closely related to *F.* cf. *bullatum* (isolate NRRL 31005), one was a *F. thapsinum* strain isolated from Tehran province and two were *Fusarium* sp. strains isolated from the Golestan and Khuzestan provinces. This is the first report of *F. thapsinum* and *Fusarium* sp. (*F.* cf. *bullatum*) on maize in Iran.

F. verticillioides is known as a strong mycotoxins producer, especially fumonisins, which can also be produced by *F. proliferatum* (Leslie *et al.*, 1992). These toxins are suspected to be carcinogenic to humans and are implicated in a number of animal diseases (Danielsen *et al.*, 1998). Therefore, the use of accurate and rapid identification techniques for early detection and identification of these pathogens is needed. Although morphological characteristics and species-specific PCR as-

says are useful for the identification of *Fusarium* species, especially members of the *G. fujikuroi* species complex, additional species-specific PCR assays and DNA sequence analyses are desirable to facilitate accurate identifications of all fusaria.

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