Research Article

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Giberella fujikuroi species complex isolated from maize and wheat in Iran: distribution, molecular identification and fumonisin B_1 *in vitro* biosynthesis

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Abstract

BACKGROUND: Contamination of food and agricultural crops by *Fusarium* species is a major concern of food spoilage and a potential public health hazard. In the present study, natural contamination of maize and wheat samples from main cultivation areas of Iran by *Fusarium* species belonging to the *Giberella fujikuroi* species complex was evaluated, with special attention to the ability of the isolates to produce fumonisin B_1 (FB_1).

RESULTS: A total of 55 *Fusarium* isolates were obtained from 27/32 maize samples (84.4%) and 11/15 wheat samples (73.3%). They were identified as *F. verticillioides* (47.3%), *F. proliferatum* (47.3%), *F. fujikuroi* (1.8%), *F. nygamai* (1.8%) and *F. redolens* (1.8%) by sequence analysis of translation elongation factor 1-α (TEF1-α). Twenty-two of 55 *Fusarium* isolates belonging to *F. proliferatum* (23.6%), *F. verticillioides* (14.5%) and *F. fujikuroi* (1.8%) produced FB_1 in the concentration range 230.4–9565.0 μg mL^{-1}. The dendrogram resulting from the TEF1-α profile showed that the genotypes were divided into clusters I, II and III, of which cluster III contained only *F. redolens*, its first report from Iran.

CONCLUSION: On the basis of *in vitro* FB_1 biosynthesis of the analyzed strains, the high degree of contamination of maize and wheat with *Fusarium* strains reported here should be considered as a potential public health threat, because a meaningful number of the isolates were found to produce hazardous levels of carcinogenic FB_1.

Keywords: food contamination; fumonisin B_1; *Giberella fujikuroi* species complex; TEF1-α gene; maize; wheat

INTRODUCTION

Mycotoxigenic fungi from the genera *Aspergillus*, *Fusarium* and *Penicillium* are real contaminants of food and feed, so they are potential threats to public health and agriculture throughout the world.[1] They reside in the soil and air as main reservoirs and contaminate a wide range of crops and agricultural commodities every year.[2–5]

Members of the genus *Fusarium*, especially those belonging to the *Giberella fujikuroi* species complex (recently recommended as *Fusarium fujikuroi* species complex by Geiser *et al.*[6]), are noted for their ability to cause various diseases in plants, humans and animals.[6–9] Geiser *et al.*[6] presented a phylogenetically guided circumscription identifying the genus *Fusarium* as the sole name for all *Fusarium* species of importance in plant pathology, mycotoxicology, medicine and basic research.

Fusarium species are involved in the etiology of an expanding number of plant diseases, including seed rot, seedling blight, root rot, stalk rot, ear rot, kernel rot, head blight, canker and wilt.[10] *Fusarium* diseases of wheat, barley and maize cause significant decreases in yields across the world and are therefore of major importance to the economy.[11] Fumonisins have been found as natural pollutants of maize-based foods and feedstuffs in many regions of the world. Since maize is a main food for human and animal nutrition and is quite susceptible to contamination with fumonisin B_1 (FB_1), worldwide incidence becomes a major risk factor with very serious consequences for human and animal health.[12–20] Consumption of unacceptable levels of

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FB_1-contaminated maize has been shown to be well associated with an elevated risk of esophageal cancer in humans, while it causes leukoencephalomalacia in horses, pulmonary edema syndrome in pigs and liver cancer in rats.[6,21] According to a report from the US Food and Drug Administration (FDA), fumonisin occurrence in foods and feedstuffs is regulated by an increasing number of countries all over the world, with legal limits varying from 1000 to 4000 μg kg^{-1} based on the country and the type of food or feed.[22]

Wheat and maize are two strategic agricultural crops of Iran, with annual production of 13.8 and 2.0 million tons respectively.[23] They are cultivated as major food sources in nearly all parts of Iran for direct and indirect consumption by humans and animals.[24–27] *Fusarium* species, particularly *F. verticillioides* and *F. proliferatum*, are the most important food and feed contaminants reported from maize, wheat and other agricultural crops in Iran.[28,29]

Although morphological criteria are in the first line of identification of members of the *G. fujikuroi* species complex, molecular approaches are essential for confirmation of identity owing to the huge inter- and intra-species similarities. The translation elongation factor 1-α (TEF1-α) gene encodes the essential part of protein translation, which provides a lot of information on *Fusarium* species. This gene has a single copy in the genus *Fusarium* and shows high levels of polymorphic sequences among species that are closely related to each other, in comparison with the intron-rich parts of protein-coding genes such as calmodulin, β-tubulin and histone. The first generation of FUSARIUM-ID v.1.0 as a publicly available database consisting of 600–700 sequences represented a phylogenetically diverse selection of TEF sequences from the genus and is placed on a local BLAST server accessible at http://fusarium.cbio.psu.edu.30 This makes the TEF1-α gene a valuable factor in the differentiation and definitive identification of *Fusarium* species. The aim of this study was (i) to determine the extent of contamination of maize and wheat by *Fusarium* species belonging to the *G. fujikuroi* species complex, (ii) to assess the distribution and identification of *Fusarium* species using DNA sequence analysis with the TEF1-α gene and (iii) to evaluate the fumonisin-producing ability of *Fusarium* isolates distributed in five different species.

MATERIALS AND METHODS

Sampling sites and identification of *Fusarium* species

A total of 32 maize and 15 wheat fields located in 25 main growing regions of ten provinces of Iran were sampled during the growing seasons of 2009 (Fig. 1). Each field was arbitrarily divided into five parts, and four samples were randomly taken from each part. Samples from each field were pooled and used for the isolation of *Fusarium* species. Each sample (20 g) was surface-disinfected for 2 min with 10 g L^{-1} sodium hypochlorite solution and rinsed three times with sterile distilled water. *Fusarium* species were isolated by the flotation method on malachite green agar and the freeze blotter technique according to Kachuei *et al.*[26] Suspected cottony fungal colonies were transferred to potato dextrose agar (PDA) slants, and single spores were obtained by the agar block method on 20 g L^{-1} aqueous agar containing 0.05 g L^{-1} chloramphenicol. Pure cultures on PDA were morphologically identified using the keys of Nelson *et al.*[31] and Leslie and Summerell.[32]

Molecular characterization of *Fusarium* isolates by TEF1-α partial gene sequencing

DNA extraction

Fungal genomic DNA of all *Fusarium* isolates was extracted according to Stewart and Via.[33] Each isolate was cultured on potato dextrose broth (Scharlau, Barcelona, Spain) for 4 days at 25 °C. The mycelium mat was collected and homogenized with liquid nitrogen. A 650 μL aliquot of lysis buffer (20 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 100 mmol L^{-1} Tris–HCl, 1.4 mol L^{-1} NaCl, 20 g L^{-1} cetyltrimethylammonium bromide, 10 g L^{-1} $Na_2S_2O_5$, 10 g L^{-1} sodium dodecyl sulfate) was added to 50–100 mg of ground mycelium, mixed thoroughly and incubated for 45 min at 65 °C. The same volume of chloroform/isoamyl alcohol (24:1 v/v) was then added and the mixture was shaken vigorously for 10 min at room temperature and centrifuged for 10 min at 9000 × *g*. The aqueous upper phase was separated and the total DNA was precipitated with 0.7 volume of isopropanol for 20 min at −20 °C. The pellet was centrifuged for 5 min at 9000 × *g*. The DNA was washed twice with 500 μL of ethanol (700 mL L^{-1}) and centrifuged for 5 min at 9000 × *g*. The pellet was air-dried and dissolved in 50 μL of TE buffer (10 mmol L^{-1} Tris, 1 mmol L^{-1} EDTA, pH 8.0). Nucleic acid concentrations were determined with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and the integrity of each DNA sample was tested on 12 g L^{-1} agarose gel.

TEF1-α gene sequencing

Polymerase chain reaction (PCR) of the TEF1-α gene was performed with the primers EF-1 (5′-GTTAAGAGGCGCGGTGTCGGTGT G-3′) and EF-2 (5′-GGAAGTACCAGTGATCATGTT-3′)[30] in a 25 μL reaction mixture containing 12.5 μL of PCR Master Mix buffer, 1 μmol L^{-1} of each primer and 20 ng of fungal genomic DNA. The reaction was carried out in a PTC-100 DNA Engine™ Peltier Thermal Cycler (MJ Research; Bio-Rad Laboratories, Inc., Hercules, CA, USA) by an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 35 s, annealing at 53 °C for 35 s and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. Identification of PCR products was performed in Tris/Borate/EDTA (TBE) buffer on 10 g L^{-1} agarose gel. The size of the band was estimated by comparison with a 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania). PCR products were purified using a GeneElute PCR Clean-up Kit (Sigma, St Louis, MO, USA) and directly sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit, Version 3.1 (Applied Biosystems, Waltham, MA, USA). Reaction products were analyzed on an ABI Prism 3700 automated DNA analyzer (Applied Biosystems). The obtained sequences were edited using Lasergene 2001, Version 5 software (DNASTAR Inc., Madison, WI, USA), and the nucleotide sequences obtained were examined using a local BLAST server accessible at http://fusarium.cbio.psu.edu. DNA sequences were arranged with the profile of ClustalW, and an evolutionary distance matrix was produced with the Kimura 2 parameter substitution model. The evolutionary tree for the data was made with the neighbor-joining algorithm, and the confidence of the tree was assessed by bootstrap analysis based on 5000 bootstrap replications using MEGA 4 software (Miami, FL, USA). *Aspergillus fumigatus* Af 293 was inserted in the analysis as an outgroup.

Fumonisin B_1 extraction and analysis

Fusarium isolates were cultured in 100 mL Erlenmeyer flasks containing 25 mL of conductive broth medium (20 g L^{-1} fructose, 0.5 g L^{-1} malt extract, 1 g L^{-1} yeast extract, 1 g L^{-1} peptone,

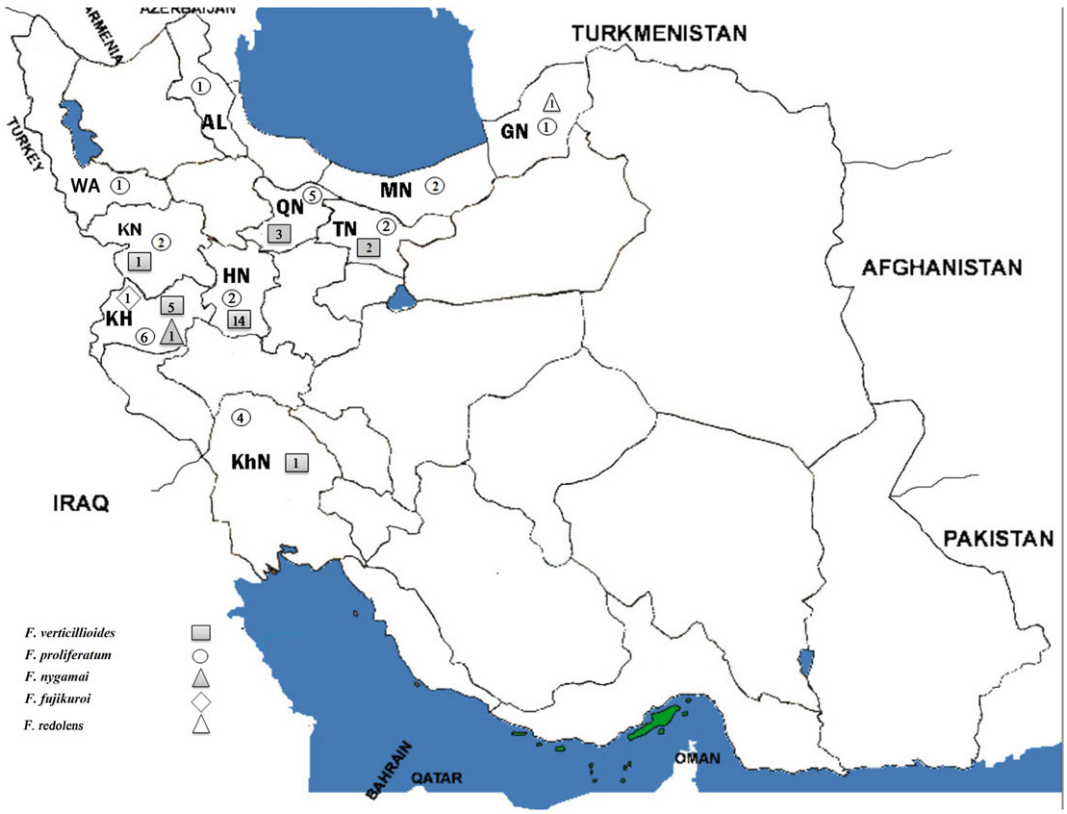

Figure 1. Map of Iran showing sampling sites and distribution patterns of *Fusarium* isolates within *Giberella fujikuroi* species complex isolated from maize and wheat in ten provinces during year 2009. Capital letters on the map indicate province names: Ardabil (AL), Golestan (GN), Hamedan (HN), Kerman (KN), Kermanshah (KH), Khuzestan (KhN), Mazandaran (MN), Qazvin (QN), Tehran (TN) and West Azerbaijan (WA).

1 g L^{-1} KH_2PO_4, 0.3 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.3 g L^{-1} KCl, 0.05 g L^{-1} $ZnSO_4 \cdot 7H_2O$ and 0.01 g L^{-1} AQOK? Do not use '%' here. $CuSO_4 \cdot 5H_2O$ in 1 L of distilled water).[34] Flasks were inoculated with 10^6 spores from 7-day-old fungal cultures in PDA and incubated in a static condition for 21 days at 20 °C. Culture media were separated from fungal mycelia by filtration through Whatman No. 1 paper and extracted for FB_1 using ethyl acetate. The ethyl acetate extracts were concentrated to near dryness in a rotary evaporator (N-1000, EYELA, Tokyo, Japan). The residues were re-suspended in 0.5 mL of acetonitrile/water (1:1 v/v). Standard FB_1 (CAS 116355-83-0, Sigma-Aldrich, St Louis, MO, USA) was dissolved in acetonitrile/water (1:1 v/v) to a final concentration of 1 mg mL^{-1}. Extraction was performed according to the method of Jimenez *et al.*[34] with slight variations.

FB_1 was measured in the culture filtrates by high-performance liquid chromatography (HPLC) according to Sydenham *et al.*[35] Aliquots (50 µL) of acetonitrile extracts of the culture filtrates were derivatized with 250 µL of *o*-phthaldialdehyde (OPA) (40 mg of OPA in 1 mL of methanol, diluted with 5 mL of 0.1 mol L^{-1} sodium tetraborate and 50 µL of 2-mercaptoethanol). A 20 µL aliquot of each FB_1 OPA derivative was injected into a reverse phase HPLC column (TSKgel ODS-80TS, 4.6 mm i.d. × 150 mm; Tosoh Bioscience, Tokyo, Japan) at excitation and emission wavelengths of 335 and 430 nm respectively. Samples were eluted at a flow rate of 1 mL min^{-1} with 0.1 mol L^{-1} sodium dihydrogen phosphate/methanol (20:80 v/v, adjusted to pH 3.35 with *o*-phosphoric acid) as mobile phase. The elution time of samples was compared with that of pure FB_1, and samples were quantified on the basis of the ratio of the peak area of the sample to that of the standard. For this purpose, a standard curve of FB_1 was plotted using various concentrations of FB_1 standard (1, 5 and 1 mg mL^{-1} stock solution), and the amounts of FB_1 in unknown samples were calculated by comparing the under-curve area of the sample with that of authentic FB_1. The percentage efficiency of the method was evaluated using known concentrations of FB_1 standard. About 91% of FB_1 was recovered after extraction with ethyl acetate.

Statistical analysis

Data of FB_1 amounts were subjected to one-way analysis of variance (ANOVA) and Tukey's test using SPSS Statistics Version 16.0 for Windows (http://www.spss.com/). Differences with $P < 0.05$ were considered significant.

RESULTS

Isolation and identification of *Fusarium* species

A total of 55 *Fusarium* isolates were obtained from 27/32 maize samples (84.4%) and 11/15 wheat samples (73.3%). *Fusarium verticillioides* and *F. proliferatum* were the most prevalent species (each in 26 isolates); the other three identified species were *F. fujikuroi*, *F. redolens* and *F. nygamai* (Table 1). Isolates of *F. verticillioides* and *F. proliferatum* were distributed in both wheat and maize samples, while *F. fujikuroi* and *F. nygamai* were recovered from maize only. *Fusarium redolens* was isolated from wheat in Gorgan province (Table 1). All isolates have been deposited in the Culture Collection of the Department of Mycology, Pasteur Institute of Iran (http://fa.pasteur.ac.ir/MBankResult.aspx).

Table 1. Geographical distribution, FB_1 production and TEF1-α gene sequence-based identification of *Fusarium* species isolated from maize and wheat

Isolate number	Source	Geographical location	Molecular identification	FB_1 (production on TLC)	FB_1 (HPLC analysis) ($\mu g\ mL^{-1}$)
6	Maize	Tehran	*F. verticillioides*	–	ND
63	Wheat	Kurdistan	*F. verticillioides*	–	ND
94	Maize	Hamedan	*F. verticillioides*	–	ND
95	Maize	Qazvin	*F. verticillioides*	–	ND
96	Maize	Hamedan	*F. verticillioides*	–	ND
97	Maize	Hamedan	*F. verticillioides*	–	ND
103	Maize	Hamedan	*F. verticillioides*	+	5103.4 ± 550.2
104	Maize	Hamedan	*F. verticillioides*	+	1930.0 ± 202.4
106	Maize	Hamedan	*F. verticillioides*	+	9565.0 ± 989.8
107	Maize	Kermanshah	*F. verticillioides*	–	ND
108	Maize	Kermanshah	*F. verticillioides*	+	7335.0 ± 894.3
109	Maize	Hamedan	*F. verticillioides*	–	ND
116	Maize	Hamedan	*F. verticillioides*	+	6853.8 ± 736.3
117	Maize	Kermanshah	*F. verticillioides*	+	6508.2 ± 685.9
118	Maize	Hamedan	*F. verticillioides*	–	ND
120	Maize	Qazvin	*F. verticillioides*	–	ND
121	Maize	Hamedan	*F. verticillioides*	–	ND
123	Maize	Hamedan	*F. verticillioides*	–	ND
124	Maize	Qazvin	*F. verticillioides*	–	ND
131	Maize	Kermanshah	*F. verticillioides*	–	ND
132	Maize	Hamedan	*F. verticillioides*	–	ND
134	Maize	Khuzestan	*F. verticillioides*	+	1880.8 ± 195.9
138	Maize	Kermanshah	*F. verticillioides*	–	ND
140	Maize	Hamedan	*F. verticillioides*	+	1560.1 ± 161.6
153	Wheat	Tehran	*F. verticillioides*	–	ND
185	Maize	Hamedan	*F. verticillioides*	–	ND
5	Maize	Gorgan	*F. proliferatum*	–	ND
7	Maize	Khuzestan	*F. proliferatum*	+	1332.7 ± 138.8
8	Wheat	Tehran	*F. proliferatum*	–	ND
9	Maize	Khuzestan	*F. proliferatum*	+	1334.4 ± 147.9
55	Wheat	Ardabil	*F. proliferatum*	+	1808.8 ± 175.0
64	Wheat	Hamedan	*F. proliferatum*	+	1709.3 ± 195.5
69	Wheat	Mazandaran	*F. proliferatum*	+	1725.0 ± 183.1
70	Wheat	West Azerbaijan	*F. proliferatum*	–	ND
81	Maize	Khuzestan	*F. proliferatum*	+	1681.8 ± 175.3
83	Wheat	Kermanshah	*F. proliferatum*	–	ND
91	Maize	Qazvin	*F. proliferatum*	+	2250.6 ± 226.0
99	Maize	Kermanshah	*F. proliferatum*	–	ND
101	Maize	Qazvin	*F. proliferatum*	+	2200.3 ± 221.3
113	Maize	Qazvin	*F. proliferatum*	–	ND
114	Maize	Kermanshah	*F. proliferatum*	–	ND
122	Maize	Qazvin	*F. proliferatum*	+	2253.0 ± 225.1
125	Maize	Qazvin	*F. proliferatum*	–	ND
126	Maize	Kermanshah	*F. proliferatum*	–	ND
127	Maize	Hamedan	*F. proliferatum*	+	1751.6 ± 178.3
130	Maize	Kermanshah	*F. proliferatum*	+	2335.8 ± 245.5
135	Maize	Kermanshah	*F. proliferatum*	–	ND
139	Maize	Khuzestan	*F. proliferatum*	–	ND
142	Wheat	Mazandaran	*F. proliferatum*	–	ND
149	Wheat	Kurdistan	*F. proliferatum*	+	3782.5 ± 391.1
163	Wheat	Tehran	*F. proliferatum*	–	ND
190	Wheat	Kurdistan	*F. proliferatum*	+	5230.5 ± 543.7
1	Maize	Kermanshah	*F. fujikuroi*	+	230.4 ± 24.4
2	Wheat	Gorgan	*F. redolens*	–	ND
4	Maize	Kermanshah	*F. nygamai*	–	ND

ND, not detected.

DNA sequencing and phylogenetic analysis

Figure 2 illustrates the dendrogram based on sequence analysis of the TEF1-α gene of isolated *Fusarium* species. The genotypes were grouped into three main clusters I, II and III. Cluster I comprised all 26 isolates of *F. verticillioides* in group A_1 and only one isolate of *F. nygamai* in group A_2, which were distributed as FB_1 producers and FB_1 non-producers. Cluster II was divided into two main groups B_1 and B_2. Group B_1 comprised only *F. fujikuroi* (one isolate) and group B_2 contained *F. proliferatum* (26 isolates), which were distributed as FB_1 producers and FB_1 non-producers. Cluster III contained only one species, *F. redolens*, which is reported from Iran for the first time. This species was not able to produce FB_1. Clusters I and II contained both FB_1 producers and non-producers, with a random distribution among the species *F. proliferatum*, *F. verticillioides* and *F. fujikuroi*, while the single isolates of *F. redolens* and *F. nygamai* were not able to produce FB_1. Based on the clusters observed in the dendrogram, *F. verticillioides* isolates were clearly distinguished from *F. proliferatum* isolates as the two main species found in this study. *Aspergillus fumigatus* Af 293 resided in a separate line as an outgroup.

Fumonisin B_1 production

As shown in Table 1, 22/55 *Fusarium* isolates (40%) produced FB_1 in the concentration range 230.4–9565.0 $\mu g\ mL^{-1}$ ($P < 0.05$). They belonged to *F. verticillioides* (eight isolates, 14.5%), *F. proliferatum* (13 isolates, 23.7%) and *F. fujikuroi* (one isolate, 1.8%). The single isolates of the other two identified species *F. nygamai* and *F. redolens* did not produce detectable amounts of FB_1. Fumonisin B_1 was produced by toxigenic *Fusarium* isolates in the concentration range 1560.1–9565.0 $\mu g\ mL^{-1}$ for 8/26 *F. verticillioides* isolates, 1332.7–5230.5 $\mu g\ mL^{-1}$ for 13/26 *F. proliferatum* isolates and 230.4 $\mu g\ mL^{-1}$ for 1/1 *F. fujikuroi* isolate (Table 1). Mean levels of FB_1 produced by *Fusarium* species are shown in Table 2. Isolates belonging to *F. verticillioides* and *F. proliferatum* produced FB_1 in concentrations ranging from 1560.1 to 9565.0 $\mu g\ mL^{-1}$ (mean 5090.5 $\mu g\ mL^{-1}$) and from 1332.7 to 5230.5 $\mu g\ mL^{-1}$ (mean 2261.3 $\mu g\ mL^{-1}$) respectively. *Fusarium verticillioides* isolates recovered from Kermanshah province had the highest mean level of FB_1, followed by the isolates from Hamedan and Kurdistan provinces. For *F. proliferatum*, the highest level of toxin production was found for the isolates obtained from Kurdistan province.

DISCUSSION

Contamination of food, feed and agricultural crops with *Fusarium* species belonging to the *G. fujikuroi* species complex is a real public health hazard owing to the production of carcinogenic fumonisins. Thus there is an urgent necessity to survey the incidence and distribution pattern of these fungi in various crops all over the world. DNA-based diagnostic methods such as TEF 1-α gene sequence analysis can be used to confirm and complement morphological identification of the genus *Fusarium* and species within the *G. fujikuroi* species complex.[36,37]

In the present study, 55 *Fusarium* isolates belonging to five species were isolated from maize (42 isolates) and wheat (13 isolates) and their identity was confirmed by a combination of morphological and molecular analyses. *Fusarium verticillioides* and *F. proliferatum* were the most prevalent species, followed by *F. fujikuroi*, *F. nygamai* and *F. redolens*. A considerable number of the isolates were found to produce FB_1 in various amounts.

Investigation of the geographical distribution of *Fusarium* species has shown various results regarding the type of species and their toxigenicity in different parts of the world.[27,28,38–44] Rahjoo *et al.*[27] reported *F. verticillioides* and *F. proliferatum* in maize samples from different geographical regions of Iran at frequencies of 69.6 and 26.7% respectively. In a report by Chehri *et al.*,[28] a contamination rate of 58% by *Fusarium* species was reported for wheat samples, and *F. graminearum*, *F. verticillioides* and *F. proliferatum* were isolated as the main species. Fandohan *et al.*[38] reported the contamination of maize samples with *F. verticillioides* and *F. proliferatum* at frequencies of 68 and 31% respectively. An analysis of Colombian feedstuffs by Acuna *et al.*[39] showed a contamination rate of 100 and 40% respectively for maize and wheat samples by *Fusarium* species. The predominant species reported by these authors were *F. verticillioides* (70.8%) and *F. proliferatum* (25%). It seems that weather patterns, agronomic practices and/or postharvest handling and storage practices could significantly affect the geographical distribution and toxigenicity of *Fusarium* species.[38] However, in most cases, the latter two species were always predominant in all studied geographical regions and climates, especially in tropical and subtropical zones of the world, indicating that these species may able to occupy ecological niches in the environment, since they produce a larger number of airborne conidia in comparison with other species and have similar optimal growth conditions. In addition, they may have additional powerful tools to adapt to unusual environmental conditions during various seasons, especially hard winters, which needs to be further studied.

The highest frequency of *F. verticillioides* was found in Hamadan province (14/26 isolates), while *F. proliferatum* was mainly isolated from Kermanshah province (5/21 isolates). The dominance of *F. verticillioides* and *F. proliferatum* in Iranian maize kernels is in accordance with previous reports from all over the world.[29,38,39] Of the three *Fusarium* isolates identified as species other than *F. verticillioides* and *F. proliferatum* (Fig. 1), one was a maize isolate of *F. fujikuroi* recovered from Kermanshah province, one was a maize isolate of *F. nygamai* obtained from Kermanshah province and one was a wheat isolate of *F. redolens* isolated from Gorgan province. This is the first report of *F. redolens* from Iran. This species reported from various legumes such as oat, wheat, barley and also from agricultural soils is a root and crown pathogen.[40,41]

Production of fumonisins has been reported for several *Fusarium* species.[42–44] In the present study, a total of 22 out of 55 *Fusarium* isolates produced FB_1 at various levels. They belonged to *F. verticillioides*, *F. proliferatum* and *F. fujikuroi*. Other species, i.e. *F. nygamai* and *F. redolens*, were not capable of producing FB_1. Levels of FB_1 production by our isolates are comparable with previous results from Iran and other countries.[28,29,45,46] In a study by Ghiasian *et al.*,[29] all *F. verticillioides* and *F. proliferatum* isolates recovered from maize were reported to produce FB_1 at concentrations of 233–9661 and 48–1725 $\mu g\ mL^{-1}$ respectively. In a study of 52 corn samples from main corn production areas of Iran, Ghiasian *et al.*[47] reported 21.4–59.0% contamination of samples by *F. verticillioides*, with a mean level of fumonisins (FB_1, FB_2, FB_3 and 3-epi-FB_3) of 10 647 $\mu g\ kg^{-1}$. In separate studies, about 66% of *F. verticillioides* strains isolated from Taiwan and 20% of *F. verticillioides* strains from maize in Italy were reported to produce FB_1.[13,45] In a report from Colombia, 97% of *F. verticillioides* and 91% of *F. proliferatum* isolates were able to produce FB_1 in the concentration range 5.6–25 846.5 $\mu g\ mL^{-1}$.[39] Ross *et al.*[48] reported that 75% of *F. moniliforme* and *F. proliferatum* isolates associated with equine leukoencephalomalacia and pulmonary edema syndrome in swine in the USA produced fumonisins in various amounts.

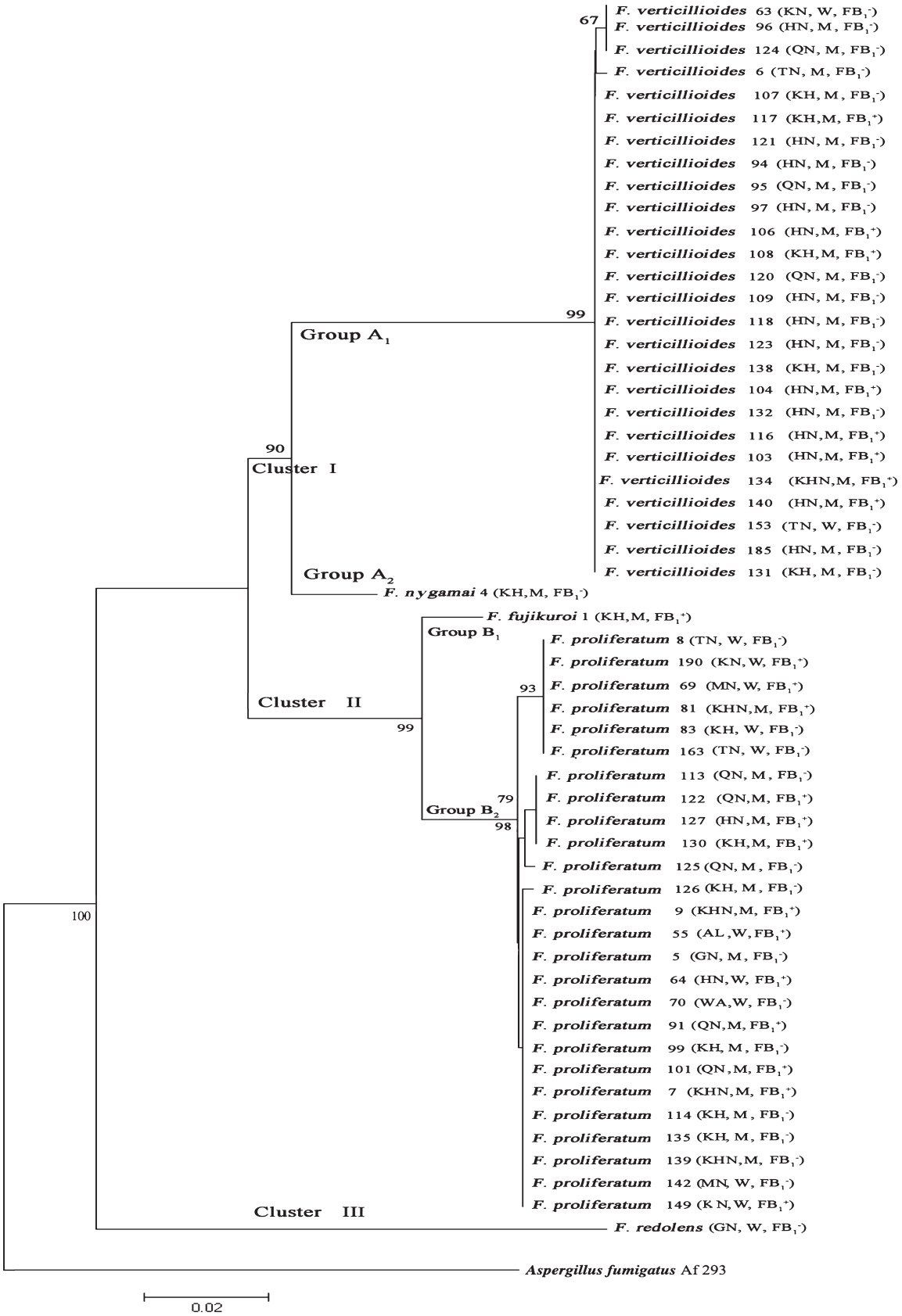

Figure 2. Dendrogram obtained from analysis of TEF1-α profiles showing genetic relationship between *Fusarium* isolates within *Giberella fujikuroi* species complex classified into three major clusters: *F. verticillioides* and *F. nygamai* (cluster I), *F. proliferatum* and *F. fujikuroi* (cluster II) and *F. redolens* (cluster III). The dendrogram shows the general characteristics of isolation source (wheat or maize), isolation location and FB_1-producing ability. Numbers at branch nodes are bootstrap values; only values above 50% are indicated. *Aspergillus fumigatus* Af 293 resided in a separate line as an outgroup.

Table 2. Mean level of FB_1 produced by toxigenic *Fusarium* species from various provinces in Iran

Province	*Fusarium* species	Number of FB_1-producing isolates	FB_1 ($\mu g\ mL^{-1}$)
Hamedan	*F. verticillioides*	5	5081.6
	F. proliferatum	2	1730.45
Kermanshah	*F. verticillioides*	2	6917.5
	F. proliferatum	1	2335.8
	F. fujikuroi	1	230.3
Kurdistan	*F. proliferatum*	3	4505.5
Khuzestan	*F. proliferatum*	3	1449.6
	F. verticillioides	1	1880.8
Qazvin	*F. proliferatum*	3	2234.6
Mazandaran	*F. proliferatum*	1	1725
Ardabil	*F. proliferatum*	1	1808.8

In this study, the highest levels of FB_1 production were shown by *F. verticillioides* isolates recovered from Hamedan province, ranging from 1560.1 to 9565 $\mu g\ mL^{-1}$ with a mean of 5081.6 $\mu g\ mL^{-1}$. These results are comparable to FB_1 amounts produced by *F. verticillioides* strains isolated from Benin, China and Argentina, which varied between 8160 and 17 900 $mg\ kg^{-1}$.[38] It has been shown that isolates of the *G. fujikuroi* species complex can be divided into low and high producers according to the amount of FB_1 produced.[48] By this definition, all our *Fusarium* isolates except one were classified in the group of high producers.

Genetic relatedness of *Fusarium* species was evaluated by comparing TEF1-α sequences among the isolates. Figure 2 shows the dendrogram originating from the TEF1-α profile of the *Fusarium* isolates, which resided in three main clusters I, II and III. All isolates belonging to *F. verticillioides* resided in cluster I, while the isolates of the other species except *F. nygamai* (in cluster I) and *F. redolens* (in cluster III) were classified in cluster II. Isolates of *F. verticillioides* and *F. nygamai* were grouped in the African clade, which shows some similarities between these two species. The African clade consists of a number of other pathogens of the *G. fujikuroi* complex that are important to farming.[19,49–51] Isolates of *F. proliferatum* and *F. fujikuroi* were grouped in the Asian clade; these two species have morphological and biological similarities, a common ancestor and cause bakanae disease.[49,50] In the present work, there was no association between phylogeny and FB_1-producing ability among the isolates. This is in accordance with the results of other researchers and means that toxigenic and non-toxigenic isolates of different species are randomly distributed in the phylogenetically identified clusters.

Taken together, the results of this study indicate a high degree of contamination of wheat and maize samples with FB_1-producing and non-producing isolates of *Fusarium* species belonging to the *G. fujikuroi* species complex, particularly *F. verticillioides* and *F. proliferatum*. This contamination must be considered as a potential public health threat not only with regard to food spoilage but also because carcinogenic FB_1 was found to be produced at high levels by a meaningful number of the isolates.

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