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Morphological and molecular identification of Fusarium head blight isolates from wheat in north of Iran

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Abstract

Fusarium head blight (FHB) is one of the most economically important and destructive fungal diseases of wheat (*Triticum aestivum* L.). Golestan province, one of the most important zones of wheat cultivation in Iran, is also known for its high rate of digestive tract cancer, which has been attributed to many biotic factors such as fungal toxins. In order to investigate the distribution of toxicogenic *Fusarium* spp. in wheat in this region, 344 *Fusarium* isolates were collected from 7 sub-regions in Golestan province during 2010–2011. Single-spore cultures were established using routine plant pathology methods and the isolates were identified using chemotyping analysis based on morphological criteria and species-specific PCR assays. The following *Fusarium* isolates were identified: *F. graminearum, F. culmorum, F. equiseti, F. croockwellens, F. acuminatum, F. poae, F. lateritium, F. proliferatum* and *F. subglutinans*. *F. graminearum* was the most dominant species, representing 48.2% of the isolates. PCR analysis using the species-specific primers Fg161 1/2 and OPT 1/2 confirmed 96.3% isolates as *F. graminearum* and 86.4% as *F. culmorum*. Isolates morphologically identified as *F. subglutinans* or *F. proliferatum* did not show any positive reaction in species-specific PCR. The present study revealed a high occurrence of FHB in Golestan province, especially in Gonbad-Kavoos sub-regions.

Keywords: *Fusarium* species, Wheat, Head blight, specific- species primer, PCR.
Abbreviations: FHB (Fusarium head blight), DON (Deoxynivalenol), NIV (Nivalenol), DAS (Diacetoxyscirpenol), ZEA (Zearalenone).

Introduction

Bread wheat *(Triticum aestivum* L.) is a major agricultural crop and the main cereal consumed by humans in Iran. Northern parts of Iran are the main wheat cultivation areas, with an average wheat production of approximately 3.6 t/ha from 2005 to 2010 (National center of statistics in Islamic Republic of Iran, 2010). This region with hot-temperate and wet climates has favourable conditions for *Fusarium* growth at the time of kernel formation. Fusarium head blight (FHB), or scab, is one of the most economically important and destructive fungal diseases of wheat. The recent incidence reports in Asia, Canada, Europe and South America indicate that FHB is an increasing threat to the world's grain supply (Goswami and Kistler, 2004). Apart from reducing the yield, FHB damages grain quality by contamination from toxic secondary metabolites (mycotoxins), which cause a health risk to both humans and animals. The toxins produced by *Fusarium* spp. are Deoxynivalenol (DON); Diacetoxyscirpenol (DAS) (Ramdas et al., 2001), Monoacetyl-deoxynivalenol; Nivalenol (NIV); Zearalenone (ZEN) and Fusarenone-X (Bottalico and Perrone, 2002; Spanic et al., 2010). Therefore, the knowledge of *Fusarium* spp. native to a region could help plant breeders to select FHB-resistant wheat varieties The causal agents of FHB in Europe are primarily *F. graminearum* Schwabe (teleomorph *Gibberella zeae*), *F. culmorum* and *F. avenaceum* (Lemmens et al., 2004; Stepién et al., 2008) The *F. graminearum* species complex, which consists of at least 11 phylogenetically distinct species, is the predominant species causing FHB worldwide (O'Donnell et al., 2000; Ban et al., 2008), with increasing prevalence in Europe (Waalwijk et al., 2003). In northern parts of Iran, more frequently occurring species are *F. graminearum*, *F. proliferatum*, *F. acuminatum*, *F. croockwellens*, *F. equiseti* and *F. subglutinas*, but only *F. graminearum* and *F. culmorum* have shown pathogenicity to wheat (Zamani-Zadeh and Khoursandi, 1995). In Golestan province, the main species associated with FHB disease are *F. graminearum*, *F. culmorum* and *F. proliferatum* (Golzar et al., 1998), among which *F. graminearum* and *F. proliferatum* are known to produce toxins (Zamani-Zadeh and Khoursandi, 1995; Karami-Osboo et al., 2010). Classification of species within the *Fusarium* genus is very difficult because of high heterogeneity. 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 (Llorens et al., 2006). Species are also determined based on versatile differences in a single characteristic. However, these observations need some practice and are difficult for a non-specialist (Windels, 1992; Bluhm et al., 2002). Therefore, molecular approaches could support morphological diagnostics by providing a rapid and reliable assay for routine identification and classification of *Fusarium* spp.For plant pathogenic fungi, the first and most laborious step in identification is determining morphological and culture characteristics, and this is especially true in case of *Fusarium* spp. Therefore, for complete identification of *Fusarium* spp., additional molecular analysis such as species-specific PCR assays must be performed. Despite wide-range studies conducted on *F. graminearum* as the causal agent of FHB, head blight diseases of wheat and barley are prevalent

throughout Iran(Safaie and Alizadeh, 2001; Safaie et al., 2005; Sanjarian et al., 2005), molecular identification studies using specific primers to confirm morphological diagnostics have not yet been conducted. Different *Fusarium* spp. have been reported to infect wheat crops in northern Iran (Zamani-Zadeh and Khoursandi, 1995), but no instances of molecular confirmation have been reported on the FHB in Golestan province. PCR can be used for identification of *Fusarium* spp., either as an alternative or as a complement to morphological identification methods (Spanic et al., 2010). It has proven to be very useful and more suitable than whole-seed plating for identification of *Fusarium* spp. (Yli-Mattila et al., 2004; Demeke et al., 2005; Rahjoo et al., 2008) PCR can also be used for routine detection and identification of *Fusarium* spp., without the need for isolation and morphological investigation (Koncz et al., 2008). Species-specific primers have been developed and used for PCR detection as well as screening of *Fusarium* spp. (Spanic et al., 2010). The main objectives of the current study were threefold: (1) To identify the *Fusarium* spp. affecting wheat in one of the most important wheat cultivation regions of Iran; (2) To determine the dominant *Fusarium* spp. infecting wheat ear and its infection percentage in different areas; (3) To use species-specific PCR for confirming morphological identification.

Results

Morphological identification

In total, 344 *Fusarium* isolates were collected during the wheat-growing season of 2010–2011. All isolates were classified into 9 species based on morphological criteria using various keys for *Fusarium* spp. identification. Of the 344 *Fusarium* isolates, 166 were identified as *F. graminearum*. *F. graminearum* was also the most prevalent species in the sampled regions. Other species were identified as *F. culmorum* (17.1%), *F. acuminatum* (10.1%), *F. equiseti* (8.1%) and *F. poae* (5.5%) (Table 2).

Species-specific PCR assay

Molecular identification of the selected isolates was further confirmed using species-specific primers of the 5 species with high frequency. Species-specific PCR assay was performed on the genomic DNA of the selected isolates. Of the 166 isolates morphologically identified as *F. graminearum*, 160 were confirmed by using Fg16F/Fg16R primers. Similarly, of the 59 isolates morphologically identified as *F. culmorum*, 51 were confirmed by using OPT18F/OPT18R primers, and of the 35 isolates morphologically identified as *F. acuminatum*, 30 were confirmed by using FAC-F/FAC-R primers. A total of 28 *F. equiseti* isolates and 19 *F. poae* isolates showed expected results in PCR amplification performed using their specific primers. A few isolates, morphologically identified as either *F. subglutinans* or *F. proliferatum*, did not show any positive reaction in species-specific PCR.

Distribution of isolates

FHB was observed in all 162 sampling fields in different regions of Golestan province (Gorgan, Kordkuy, Bandargaz, Gonbadkaboos, Minoodasht, Kalaleh and Azadshar) The Gonbad-Kavoos region showed the highest percentage of infected farms for surface (80%) and ear (45.9%) infections. It is possible that cultures of the sensitive cultivar Tajan and crop rotation with corn and sorghum are the causes of FHB prevalence in this region.

Discussion

Fusarium spp. are causal agents of ear rot in cereals cultured in hot and humid areas worldwide. The toxins produced by different species of this genus decrease the baking quality of wheat and are a health risk for both humans and animals. FHB of wheat and barley are two important diseases in Golestan province, the main wheat cultivation area in Iran (Golzar et al., 1998). *F. graminearum* is proposed as the most dominant species in several zones in northern Iran. This species was reported for the first time in Mazandaran province (Ershad, 1995). It has also been isolated from barley crops in central Iran.(Babadost, 1996; Golzar et al., 1998). Our morphological observation is in accordance with that in previous studies in other parts of northern Iran (Zamani-Zadeh and Khoursandi, 1995). Gonbad-Kavoos fields showed a higher frequency of isolated species causing head blight on the surface of and inside wheat kernels. In this study, other species of *Fusarium* with less frequent prevalence were isolated from infected wheat ears. These species were morphologically identified as *F. culmorum, F. equiseti, F. poae, F. croockwellens, F. lateritium, F. proliferatum* and *F. subglutinans.* These species have been reported to cause grain and stem rot of maize in regions with moderate to temperate climate (Rahjoo et al., 2008). Among these species, *F. subglutinans* was reported for the first time in these regions. Classification and identification of *Fusarium* spp. using morphological characteristics is difficult even for specialists because of the number, varieties and forms of species and also because of the large morphological variation of isolates within a single species (Klittich et al., 1997; Leslie et al., 2006). Hence, species-specific PCR assays are usually needed for accurate identification. Accordingly, we used species-specific PCR to confirm our morphological identifications. In Iran, most of the identification of these fungi has been made based on morphological criteria, which is time consuming and requires considerable expertise in taxonomy and physiology. As identification of *Fusarium* spp. is critical to predict the potential mycotoxigenic risk of the isolates, there is a need for accurate and complementary tools that permit rapid, sensitive, reliable and specific diagnosis of *Fusarium* spp. (Sampietrod et al., 2009). The principal objective of this study was precise identification of *Fusarium* isolates collected from infected wheat ears showing FHB symptoms to chemotype determination analysis in further studies. Some authors have reported wheat FHB in northern Iran (Ershad, 1995; Zare and Ershad, 1997; Golzar et al., 1998), but no molecular technique has been used for species confirmation. For closely related *Fusarium* spp., molecular techniques such as species-specific primer assay could be used for routine detection and identification of *Fusarium* spp., without the need for isolation and morphological investigation (Koncz et al., 2008).Morphological assessment showed that 166 of 344 isolates were *F. graminearum.* Of the 166 isolates morphologically identified as *F. graminearum*, 160 (96.3%) were confirmed by using Fg16F/Fg16R primers . Similarly, of the 59 isolates morphologically identified as *F. culmorum*, 51 (86.4%) were confirmed by using OPT18F/OPT18R primers. All primers used in this study amplified the expected product size of species tested in PCR. None of the isolates showed any amplification with the VER 1/2 PCR primers specific for *F. proliferatum*. This is the first study where species-specific primers were used for the identification *Fusarium* spp. causing FHB in this region.

Table 1. Sequences of primers used in the experiments.

Species-specificity	Primer name*	Primer Sequence (5'→ 3')	Amplification (size bp)	Reference
F. graminearum	Fg16F	CTCCGGATATGTTGCGTCAA	420-520	Nicholson et al. (1998)
	Fg16R	GGTAGGTATCCGACATGGCAA		
F. culmorum	OPT18F	F-GAT GCC AGA CCA AGA CGA R-AG	470	Schilling et al. (1996)
	OPT18R	GAT GCC AGA CGC ACT AAG AT		
F. equiseti	FEF	CAT ACC TAT ACG TTG CCT CG	400	Mishra et al. (2003)
	FER	TTA CCA GTA ACG AGG TGT ATG		
F. acuminatum	FAC-F	GGG ATA TCG GGC CTC A	600	Williams et al. (2002)
	FAC-R	GGG ATA TCG GCA AGA TCG		
F. poae	Fp 82F	CAAGCAAACAGGCTCTTCACC	220-bp	Parry and Nicholson (1996)
	Fp 82R	TGTTCCACCTCAGTGACAGGT		
F. subglutinans	SUBF	CTGTCGCTAACCTCTTTATCCA	631bp	Mulè et al.(2004)
	SUBR	CAGTATGGACGTTGGTATTATATCT		
F. proliferatum	VERF	TGTCAGTAACTCGACGTTGTTG	420 bp	Mulé et al. (2004)
	VERR	CTTCCTGCGATGTTTCTCC		

* F: forward primer, R: reverse primer

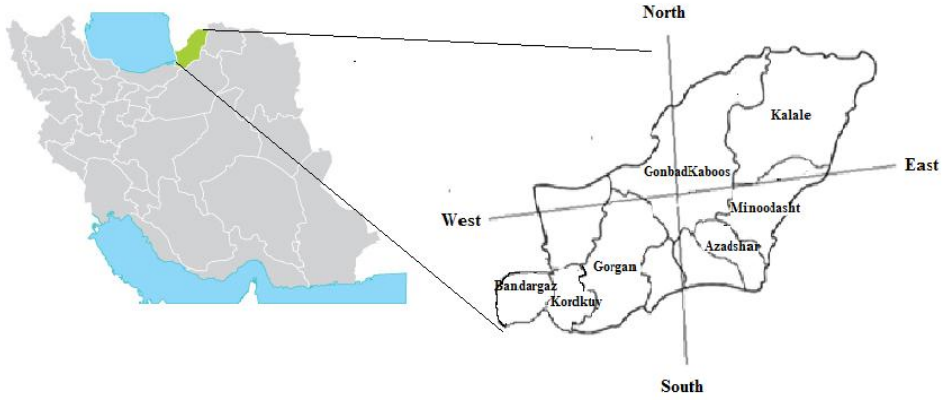

Fig 1. Map of Golestan province showing sampling regions during 2010 -2011.

Results of the amplified fragments of 9–18 representative isolates in PCR using species-specific primers revealed that the DNA in these samples was from pure strains corresponding to a single species rather than to a mixture of species (Fig. 2; a-e). These data confirmed our morphological observations. Species-specific primer studies conducted to identify *Fusarium* isolates from maize ears revealed that species-specific PCR primers are not specific for all *F. proliferatum* and *F. verticillioides* species (Rahjoo et al., 2008), but these results cannot ignore the role of specific primers in accurate identification of *Fusarium spp*..Of the 344 *Fusarium* isolates collected in the study area, *F. graminearum* was the most prevalent with a frequency of 48.2% (166 of 344), followed by *F. culmorum* with a frequency of 17.1% (59 of 344). The dominance of *F. graminearum* affecting the wheat cultured in Golestan province is in accordance with previous reports from other provinces in northern Iran; this is not true for *F. culmorum* (Zare and Ershad, 1997). *F. graminearum* is known to produce strong mycotoxins such as deoxynivalenol and zearalenone, which are detrimental to humans and domestic animals (Mc Mullen et al., 1997). These toxins are suspected to lead to digestive tract cancers in humans and are implicated in a number of animal diseases (Desjardins, 2008). Therefore, accurate and rapid identification techniques for early detection and identification of these pathogens are needed.

Materials and methods

Sampling

A total of 162 wheat fields in 7 zones of Golestan province were sampled during the two growing seasons of 2010–2011 (Fig. 1). Each field was arbitrarily divided into 5 circular plots approximately 100 m in diameter, and 3–5 samples (ears with visible infection symptoms) were randomly taken from each plot. Samples were pooled in each field and 2 infected ears from each field were selected and used for *Fusarium* spp. isolation. The most popular wheat varieties were Tajan, Zagros and Koohdasht. In these regions, crop rotation was practised with corn and sorghum.

Identification of Fusarium species

A total of 350 kernels collected were surface-sterilized in 3% (w/v) chloramine T (Sigma, saint–Quentin, France) in water plus a drop of Tween 80 for 15 min, and then rinsed at least 4 times with sterile distilled water. The kernels were then treated with Vitavax 200 (thiram+carboxin) at 200 g/100 kg for protection against seed-borne diseases.

Table 2. *Fusarium* spp. collected from wheat ears in Golestan province during 2010–2011.

Fusarium species	Number	Percent%
F. graminearum	166	48.2
F. culmorum	59	17.1
F. acuminatum	35	10.1
F. equiseti	28	8.1
F. poae	19	5.5
F. croockwellens	13	3.7
F. lateritium	10	2.9
F. proliferatum	9	2.6
F. subglutinans	5	1.4
Total isolates	344	

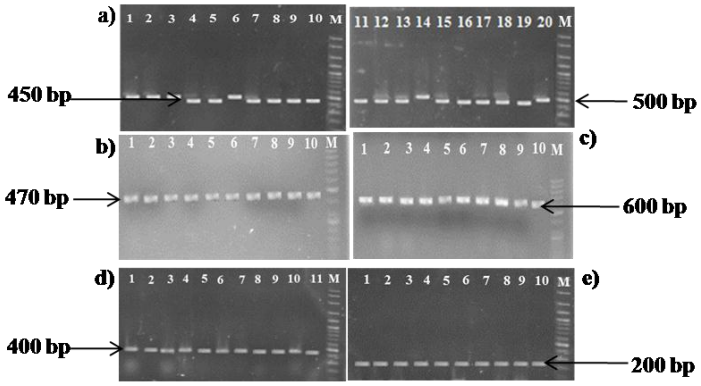

Fig 2. Species-specific PCR assays. (a) *F. graminearum* with Fg16F/Fg16R primers (Lane 1–18) and standard *F. graminearum* isolate (Lane 19–20); DNA marker (M). (b) *F. culmorum* with OPT18F/OPT18R primers (Lane 1–8) and standard *F. culmorum* isolate (Lane 9–10); DNA marker (M). (c) *F. acuminatum* with FAC-F/FAC-R primers (Lane 1–9) and standard *F. acuminatum* isolate (Lane 10); DNA marker (M). (d) *F. equiseti* with FEF1/ FER1 primers (Lane 1–10) and standard *F. equiseti* isolate (Lane 11); DNA marker (M). (e) *F. poae* with Fp 82F/Fp 82R primers (Lane 1–9) and standard *F. poae* isolate. (Lane 10); DNA marker (M).

Table 3. Percentage of wheat ears infected with Fusarium head blight in sampled farms.

Region of sampling	Number of field	Surface(ha)	Infected field with *Fusarium* (%)	Average of ear infected (%)
Gorgan	22	29	19	40.1
Kordkuy	19	24	11	29.7
Bandargaz	26	27	14	34.1
GonbadKaboos	30	34	24	45.9
Minoodasht	21	22	13	37.5
Kalaleh	24	25	16	36.8
Azadshahar	20	21	13	34.3
Total	162	182		

All disinfected grains were air dried in a laminar flow cabinet, and then incubated in synthetic nutrient agar (SNA) with 2 antibiotics (chlortetracycline, 0.01 g; streptomycin sulphate, 0.05 g) (Nirenberg, 1981) in a controlled environment (dark cycle, temperature 24 ± 3°C and humidity 80%) for 5–7 days. All *Fusarium* isolates were sub-cultured on Potato Dextrose Agar PDA (Merk, Germany) and Spezieller Nahrstoffarmer Agar using a single-spore technique (Leslie et al., 2006). Culture characteristics were assessed by eye and microscopic examination. The morphology of macroconidia, microconidia, conidiogenous cells and chlamydospores was assessed from cultures grown on SNA and CLA (Carnation Leaf Agar). Morphological identifications of isolates were carried out using the criteria of Leslie and Summerell (2006).

Determination of disease severity

Ear contamination by FHB was monitored in 2010–2011. Five fields from each plot were chosen for sampling, from seed production until ear formation. Two hundred samples from each field were randomly selected for disease severity assay. Average ear infection severity in the 7 regions studied was calculated using the following formula:

$\Sigma i^n = 1(Di \times Si)/\Sigma i^n = 1\ Si$

where l = number of farms, Di = percentage of infected plants in the farm and Si = surface area of the farm in hectares.

DNA extraction from fungi isolates

The *Fusarium* isolates were grown in liquid potato dextrose broth medium (Merck, Germany), and incubated on a rotaryshaker at 120 rpm for 5 days at 25°C. For DNA extraction of fungal cultures, the mycelium were harvested and ground to fine powder in liquid nitrogen. Total genomic DNA was extracted using the CTAB method (Nicholson et al., 1997).

PCR assay

Species- specific PCR assay with specific primers (Table 1) was used to identify *F. graminearum*, *F. culmorum*, *F.*

acuminatum, F. equiseti and F. poae species. 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, 1.5U of Taq polymerase (Fermentase, Sinagen, Iran), 15 pmol of each primer and approximately 25 ng of fungal template DNA were used. Reactions were performed in a thermal cycler (Eppendrof, Germany) 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) and photographed under UV light in the Bio-Imaging system (Bio-RAD, USA).

Conclusion

In the present work, a PCR assay was used to confirm the morphological identification of *F. graminearum, F. culmorum, F. acuminatum, F. equiseti* and *F. poae. F. graminearum* was the most frequently isolated species from infected wheat ears in all sampled areas. We conclude that molecular identification using species-specific primers of all collected isolates was almost in accordance with morphological identification. The results presented here demonstrate that the 5 *Fusarium* species mentioned above could be differentiated from each other on the basis of a single PCR amplification with high confidence and precision. Due to the predominance of *F. graminearum*, we advocate the use of this species for screening in wheat cultivation areas in northern Iran, one of the main areas of wheat production. For identification of similar *Fusarium* spp. and elucidation of their taxonomic relationships, DNA sequence analyses could be applied.

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