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RESEARCH ARTICLE

Morphological and Genetic Variation of the *Fusarium semitectum* Isolates Associated with Cereal Grains Using RAPD Markers

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ABSTRACT

Fusarium species are among the most commonly occurring and economically important member of micro-fungi. The aim of this study was to estimate the genetic diversity and relationship among 29 isolates of *F. semitectum* collected from various cereals and locations in the north of Iran by using morphological characters and RAPD-PCR polymorphisms. Based on morphological studies the isolates were divided into two distinct groups A and B. Microscopic characters (shape of macroconidia and presence of sporodochia and chlamydospore) of group A varied between isolates and lead to this group divided into two subgroups. Using eight polymorphic primers, 82 RAPD fragments were obtained with an average of 10 polymorphic bands per primer. Cluster analysis with UPGMA revealed three distinct arbitrary level of 50% similarity. Majority of isolates of group A were homogenous having similar DNA patterns. There were low levels of genetic difference among isolates of morphotype A while two of them (28 and 29) have a different banding pattern. Genetic estimates demonstrated that the isolates from morphotype A were significantly distinct from morphotype B isolates. Results of cluster analysis using RAPD data did not show any correlation with geographical distribution but the results of this analysis confirmed a genetic variation among all isolates.

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INTRODUCTION

Fusarium is a common mold in cereal fields. The infestation (superficial contamination) and infection of *Fusarium* in cereals are of great concern worldwide as plant pathogens and producers of mycotoxins. Cereal infection by *Fusarium* sp. decreased seed germination, caused production of mycotoxin and reduction of malt quality, lower seed quality (due to reduction in storage protein), cellulose and amylase (Argyris *et al.*, 2003). *Fusarium* diseases of wheat, barley and maize cause significant yield losses world-wide and are therefore of great economic importance (Sutton, 1982; Parry *et al*., 1995; Miedaner, 1997; Mesterhazy *et al*., 1999). In addition, many of these *Fusarium* species have the potential to produce a range of toxic secondary metabolites known as mycotoxins that cause a potential health risk when contaminated grain is consumed in human and animal food products (D'Mello and Macdonald, 1997; Placinta *et al*., 1999). *Fusarium semitectum* Berk. & Ravnel occur frequently among the fungal microflora associated with seedling disease. They are a major cause of seedling death in some countries (Zhang *et al*., 1996)

Awareness of genetic diversity among the isolates in the populations of plant pathogens can lead researchers to understand their genetic structure and dynamics. The latter issue plays a significant role in deducing the evolution of the pathogen and their correlation with plant hosts, which help us take any preventive measure against them.

Different molecular markers are available for the differentiation and clarification of fungal taxa. Molecular techniques based on the polymerase chain reaction (PCR) have been used a tool in genetic mapping, molecular taxonomy, evolutionary studies, and diagnosis of several fungal species (Welsh *et al.*, 1991; McDonald, 1997). Random amplified polymorphic DNA (RAPD) markers have been applied widely in the detection and genetic characterization of *Fusarium* species. Carmer *et al*. (2003) characterized genetic diversity and pathogenicity of 166 isolates of *F. oxysporium* obtained from common bean and sugar beet plants using RAPD analysis.

In the present study, 29 isolates of *F. semitectum* collected from different cereals including wheat, barley, rice and maize were identified on the basis of

morphological and cultural characteristics. In addition, the intra-species genetic variations among these isolates were studied by using RAPD-PCR method.

MATERIALS AND METHODS

Fungal isolates

Fusarium isolates were recovered from barley, wheat, rice and maize grains, collected from Golestan, Mazandaran and Gilan provinces located in north of Iran during 2011-12. Five seeds of each sample were placed onto the selective medium peptone pentachloronitro-benzene agar (PPA) (Nash & Synder, 1962) and incubated at 25°C for 7 days. The isolates purified by single spore isolation on Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany) and then grown on carnation Leaf Agar (CLA) (Fisher et al. 1982) plates at 25°C in a 12 h dark/light cycles (20W-220V) for 7 days. Morphological identification was carried out according to Nelson *et al*. (1983) and Leslie and Summerell (2006). Cultural characters were assessed by eye and by microscopic examination. Colony morphology (color, texture, sporodochia and growth rate) was recorded from cultures grown on PDA. The morphology of macroconidia, microconidia, conidiogenous cells and the chlamy-dospores was assessed from cultures grown on CLA.

DNA extraction

Fungal DNA isolation method was adopted as described previously by Zhu *et al*. (1993). *Fusarium* isolates were grown in 50 ml yeast extract on an orbital shaker (150 rpm) at 25°C for 5 days. Mycelia were harvested by filtration and freeze-dried. Approximately 1 g of the dried mycelia was ground in liquid nitrogen and subjected to CTAB buffer (containing 50mM Tris Buffer pH 8.0, 100mM EDTA, 150mM NaCl) and 600 *µl* of 2-mercaptoethanol. Then it was incubated at 65 °C for 20 min in an incubator shaker at 60 rpm. DNA was extracted by adding an equal volume of Chloroform/Isoamyl Alcohol (24:1 V/V) mixture, vortexed for 3-4 s, and centrifuged at 10000 rpm for 12 min. Aqueous viscous supernatant was removed to a fresh tube. DNA was precipitated with ice-cold isopropanol. After centrifuged at 4000 rpm for 2 min, it was washed in 70% ethanol, air-dried and suspended in 100 µl of deionized water.

RAPD amplification analysis

Polymerase chain reactions were performed in 25 *µl* reactions containing 0.5 *µl* dNTPs mix (10mM), 1 *µl* primer, 20 *ng* template DNA, 1.2 *µl* MgCl2 (50mM), 1.5 *µl* reaction buffer (10X), 1.5 U *Taq* DNA polymerase and deionized water 18.5 *µl*. Amplification were carried out in a thermo cycler (Bio Rad, USA) programmed as follows: 2 min denaturation at 94°C, 35 cycles of 94°C 20 sec, 36°C 30 sec, 72°C 1 min and followed by an extension step of 5 min 72°C. Eight primers (SinaClon, Iran) were evaluated for their ability to produce polymorphic bands on a subset of isolates (Table 1).

Amplifications were performed twice to confirm consistency of the method. Negative control (without template DNA) was maintained for each set of experiment to test for the presence of non-specific banding.

The PCR products were separated electrophoretically at 85 V on 1.2% agarose gels in 1xTAE (40 mm Tris-acetate and 1 mm EDTA) buffer. The gels were stained with red safe and visualised in a UV-transilluminator and the gel were photographed using Gel Doc system.

Data analysis

DNA fingerprints were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix. Genetic similarity (GS) among all the isolates was created with Jaccard's coefficient of similarity. The similarity matrix was then subjected to the unweighted pair group method with arithmetical mean (UPGMA) (SneathandSokal, 1973) cluster analysis to generate a dendrogram using NTSYS pc-2.02e program (Rohlf, 1998). Genetic diversity parameters were calculated using PopGene software v. 1.32 (Yeh *et al*., 1999).

RESULTS

All single-spored isolates of *Fusarium* that were successfully isolated were initially identified based on the cultural characteristics and microscopic characteristics. For species determination, the descriptions by Nelson *et al*. (1983) and Leslie and Summerell (2006) were adopted. In this study 29 isolates were identified as *F. semitectum* based on the morphological characters. The most distinctive character is the abundant production of straight, spindle-shaped mesoconoidia from polyphialides in the aerial mycelia. These mesoconidia are easily observed microscopically *in situ* and often have the appearance of rabbit ears. Culture usually grow rapidly (3-7 cm in 5 days at 24^0C) and produce abundant dense aerial mycelia that initially is off white and becomes brown with age and brown color pigmentation on reverse of the colony was also observed. Some isolates of *F. semitectum* produced chlamydospore and light sporodochia. The results indicate that all isolates of *F. semeitectum* can be divided into 2 different groups assigned as morphotypes A and B. Isolates belongigng to morphotype A produce abundant brown floccose aerial mycelia with growth rate between 3-5 cm in 5 days while morphotype B isolates have abundant white uniform mycelia with 5-7 cm growth rate. Among the 29 isolates, 24 isolates were grouped as morphotype A and 5 isolates were grouped as morphotype B. Table 2 shows Different factors like morphological characters, type of host and location of isolates was taken into account regarding classifying of isolates.

Microscopic characters including present or absent of sporodochia, shape of macroconidia and produce of chlamydospore varied between isolates of morphotype A since could be divided this morphotype into two subgroups assigned as A-I and A-II. Subgroup A-I produce light sporodochia and the basal shape of macroconidia was foot shaped while sub-group A-II do not produce sporodochia and the basal shape was notched shaped. On the other hand, isolates of morphotype B are similar to each other and all of them have light sporodochia and no chlamydospore on CLA culture.

Of the 10 arbitrary primers tested, 8 primers generating reproducible banding patterns were selected for RAPD-PCR. The size of RAPDs ranged from 500 to

Table 1: Primers used for RAPD-PCR amplification of *Fusarium semitectum* isolates

Primer name	Primer sequence 5-3	Number of fragment	Primer name	Primer sequence 5-3	Number of fragment
OPC- 5	GATGACCGCC	8	OPD- 2	GGACCCAACC	12
OPC- 7	GTCCCGACGA	10	OPD- 3	GTCGCCGTCA	9
OPC- 9	CTCACCGTCC	no	OPD- 5	TGAGCGGACA	13
OPC- 10	TGTCTGGGTG	12	OPD- 8	GTGTGCCCCA	4
OPC- 18	TGAGTGGGTG	11	OPD- 9	CTCTGGAGAC	no

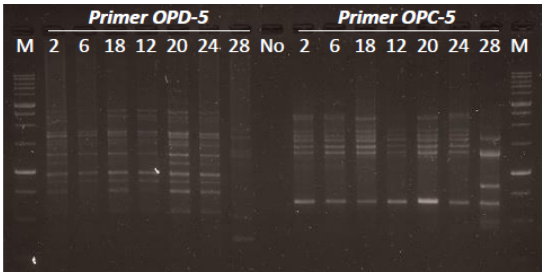

Fig. 1: RAPD band profile generated using OPD 5 and OPC 5 primers visualized 1.2% (v/v) agarose. Lane M: 1kbp DNA ladder

2500 bp (Figure 1). Using eight polymorphic primers, 79 RAPD fragments were obtained with an average of 10 polymorphic bands per primer. Primer number OPD-5 produced the maximum number of fragments (13 band position) and number OPD-8 produced the minimum number (4 band position) of fragments (Table 1). RAPD analysis of genomic DNA from the isolates revealed the presence of three lineages at the arbitrary level of 50% similarity (Figure 2). Lineage A was the largest cluster occupying 82.75% of total samples that included almost all isolates representing morphotype A except isolates 28 and 29. Lineage B consisted of two isolates, 23 and 11 from morphotype B. lineages C included one isolate 12, 25 and 26. The cophenetic correlation coefficient between Jaccard´s similarity matrices and the dendrogram of RAPD data was 0.99, showing very good fit.

The polymorphic loci in morphotype A (44.29%) was lower than morphotype B (62.86%). The Nei,s gene diversity calculated for morphotypes A and B were 0.078 and 0.262, respectively. Also, the values of Shannon information index were 0.138 for morphotype A and 0.381 for other morphotype.

DISCUSSION

Fusarium semitectum commonly is isolated from soil (Burgess *et al*., 1988; Leslie *et al*., 1990) and from diver aerial plant parts in tropical and subtropical areas (Wallbridge, 1981). Although there are many reports of *F. semitectum* being implicated in various disease it often is not regarded as an important plant pathogen. It has been reported to cause a reduced seed germination and seedling growth of sorghum (Gopinath *et al*., 1985), pod and seed rot of beans (Dhingra and Muchovej, 1979), storage rot problems of mushrooms (Seth and Shandilya, 1978) and is one of the dominant fungi on the grain of pearl millet (Wilson, 2002).

In this study, the isolates were characterized primarily based on culture characteristics. Single spore isolates of *F. semitectum* showed a considerable variation in culture characteristics and 29 isolates were classified into 2 morphotypes. Abd-Elsalam *et al*. (2003) reported that isolates of *F. semitectum* associated with a seedling disease of cotton in Egypt also produced floccose and powdery aerial mycelium with whitish, buff, ochreous and peachy color aerial mycelium. Similar study by Hava *et al*. (2010) also indicated that a vast difference in the culture characteristics of isolates of *F. semitectum* were observed which can be divided into 2 different morphological types, the fast growing with peach mycelia and the slow growing with beige to brown mycelia.

Two varieties of *F. semitectum*, *F. s.* var. *semitectum* and *F. s.* var. *majus* are illustrated by Gerlach & Nirenberg (1982). *F. semitectum* var. *semitectum* produced 1-5 septate while var. *majus* produced 1-7 septate macroconidia (Booth, 1971; Gerlach & Nirenberg, 1982). In our study, the isolates produce shorter macroconidia (varied between 3 and 5) which fulfilled the criteria of *F. s.* var. *semitectum*.

Molecular analysis at the species level will lead to the re-evaluation of phenotypic characters for fungi species delimitation, and will determine DNA polymorphism among populations of phytopathogenic fungi (Schnider *et al*., 1998). RAPD analysis has been used with increasing frequency as a method for the molecular typing and genetic characterization of various *Fusarium* spp. (Donaldson *et al.*, 1995). RAPD analysis revealed similar polymorphic patterns among most of the isolates from different both provinces and hosts. Majority isolates of morphotype A placed in one lineage indicating that this group has a homogenous population structure across the north of Iran. For example, isolates 3, 12 and 19 were collected from different both province and host included in the same lineage. Also results shows that the present or absent of sporodochia and chlamydospore had have any significant roles in polymorphic patterns of isolates. The shape of apical basal cells of macroconidia (that produce in sporodochia) is one of the best and significant characters for identification of *Fusarium* spp. (Lesslile *et al*., 2006). There are two different shape of macroconidia in isolates of morphotype A. however, these isolates with varied macroconidia placed in same group fingerprint of RAPD analysis as showed in figures 1 and 2. Comparison of genetic differentiation estimates revealed low levels of genetic differentiation among morphotypeA isolates.

In this study, two isolates of morphotype A (28 and 29) were placed in the separate fingerprinting group by random amplified polymorphic DNA (RAPD) analysis. These isolates recovered from maize of Golestan province. Li *et al*. (2012) used inter-simple sequence repeats (ISSR) markers to assess genetic diversity among 21 *Fusarium* strains of section Martiella. They showed that within the same species collected from the same regions and same host plants, there existed definite genetic difference among the strains of the same species.

Table 2: Characteristics of isolates *F. semitectum* sampled from different cereals in the North of Iran during 2011-12

Isolate Number	Host	Location	Colony color	*Sporodochia*	*Chlamydospore*	*Septa*	*Morphotype*
1	wheat	Mazandaran	brown	no	cluster	3	A-I
2	wheat	Mazandaran	brown	yes	cluster	3(4)	A-II
3	wheat	Mazandaran	brown	yes	cluster	3(4)	A-II
4	wheat	Mazandaran	brown	yes	cluster	3	A-II
5	wheat	Mazandaran	brown	yes	no	3(4)	A-II
6	wheat	Golestan	brown	no	cluster	3	A-I
7	wheat	Golestan	brown	no	cluster	3	A-I
8	wheat	Golestan	brown	yes	cluster	3(4)	A-II
9	wheat	Golestan	brown	yes	no	3(4)	A-II
10	wheat	Golestan	white	yes	no	3(4)	B
11	wheat	Golestan	white	yes	no	3(4)	B
12	barley	Mazandaran	brown	no	cluster	3(5)	A-I
13	barley	Mazandaran	brown	yes	cluster	3(4)	A-II
14	barley	Mazandaran	brown	yes	cluster	3(4)	A-II
15	barley	Golestan	brown	no	cluster	3	A-I
16	barley	Golestan	brown	no	cluster	3	A-I
17	barley	Golestan	brown	yes	no	3	A-II
18	barley	Golestan	brown	yes	cluster	3(4)	A-II
19	rice	Gilan	brown	yes	cluster	3-4	A-II
20	rice	Gilan	brown	no	cluster	3-4	A-I
21	rice	Mazandaran	brown	no	cluster	3-4	A-I
22	rice	Mazandaran	brown	no	cluster	3-4	A-I
23	rice	Mazandaran	white	yes	no	3(4)	B
24	rice	Mazandaran	brown	yes	cluster	3-4	A-II
25	rice	Mazandaran	white	yes	no	3(4)	B
26	rice	Mazandaran	white	yes	no	3(4)	B
27	rice	Golestan	brown	yes	cluster	3-4	A-II
28	maize	Golestan	brown	yes	cluster	3	A-II
29	maize	Golestan	brown	yes	cluster	3	A-II

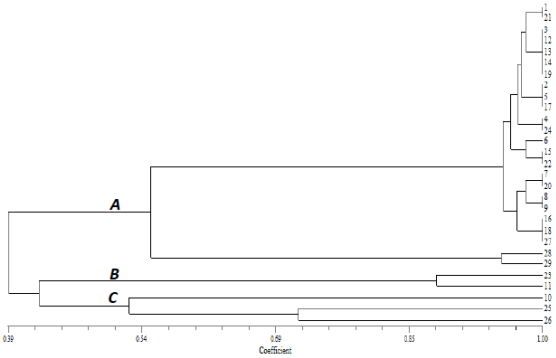

Fig. 2: UPGMA dendrogram constructed for 29 isolates of *Fusariumsemitectum* sampled from north of Iran based on Jaccard´s coefficient of similarity

Genetic variation of *F. semitectum* isolates of morphotype B was also observed using RAPD analysis. A study by Edel *et al*. (2001) of *F. oxysporium* isolated from seedling different soil samples in France revealed intraspecific variation. In their study, a good correlation was also obtained between genomic groups of RAPD and AFLP and culture characteristics. Gupta *et al*. (2009) reported the genetic polymorphism and diversity in isolates of *F. solani* isolated from wilt disease of Guava in India. Similar results was also obtained by Zaccardelli *et al.* (2006) in which the clustering of *F. semitectum* from alfalfa (*Medicago sativa*) were divide into 2 distinct AFLP clusters and the clustering corresponds to the morphological characteristics.

No correlation was found between the geographical origin and clustering patterns of the isolates. Similar results were obtained by Galvan *et al*. (2008) who classified 43 isolates of *F. oxysporum* F. sp. *cepae* representing various geographical regions into two groups based on AFLP markers from three primer combinations. They observed high genetic similarity among the isolates representing different countries, but not detected correlation between AFLP groups and the origins of isolates. However, in RAPD-PCR analysis of *F. oxysporium* isolates, a correlation has been found between genetic similarity and geographic region (Assigbetse *et al*., 1994).

Conclusions

In conclusion, results of present study demonstrated that two morphotypes are recognized in the species *F. semitectum*. There was no correlation between molecular markers used and geographic origin of the isolates. Morphological variation and genetic polymorphism within *F. semitectum* isolates suggested that *F. semitectum* as a spesies complex. However, further analysis with a large number of isolates and data from coding regions such as alpha-elongation, beta-tubulin would be useful for study of *F. semitectum* diversity and phylogeny.

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