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AFLP, pathogenicity and mating type analysis of Iranian *Fusarium proliferatum* isolates recovered from maize, rice, sugarcane and onion

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Abstract: Assessment of eighty *Fusarium proliferatum* isolates obtained from maize, rice, sugarcane and onion using AFLP molecular marker separated the isolates into four distinct clusters according to their host's. Isolates recovered from rice clustered in a distinct group. Isolates from sugarcane grouped in two distinct groups and isolates recovered from maize and onion clustered in a unit group. As well, all studied *F. proliferatum* isolates originated from different hosts expressed pathogenicity to maize ears. However, different levels of pathogenicity were observed among and within of different host populations. Duncan's test analysis showed isolates from maize, sugarcane and onion belonged to group A and rice isolates placed in group B. Moreover, a correlation was observed between AFLP clustering and pathogenicity of the maize, sugarcane and onion isolates compare to isolates coming from rice. All of the isolates were examined for PCR based identification of mating type idiomorphs and determination of sexual fertility status. Among 142 isolates of *F. proliferatum*, 72 isolates (50.7%) were identified as *MAT–1* and 70 isolates (49.2%) were belonged to *MAT–2*. The presence of both mating type idiomorphs with favorable frequency among isolates recovered from different hosts of *F. proliferatum*, shows that there is a potential for sexual reproduction within these populations. On the other hand, female fertility examination for 40 randomly selected field isolates including 10 isolates of each host populations showed all the isolates are female–sterile. Finally, we concluded that the genetic variation within *F. proliferatum* populations in Iran is possibly a result of vegetative compatibility, parasexual cycle, various cultivars of the hosts and high amount of migration to the populations as well as sexual reproduction.

Key words: DNA fingerprinting, genetic diversity, *Fusarium* ear rot, molecular marker, VCG

INTRODUCTION

The *Gibberella fujikuroi* (Sawada) Wollenw., species complex, consists of anamorphic species of *Fusarium* section Liseola, and is composed of at least nine reproductively isolated biological species (mating populations) denoted by a base letter A through I (Leslie & Summerell 2006). *Fusarium* isolates in the *G. fujikuroi* species complex, include important fungal pathogens of agricultural crops and trees (Leslie 1995). These pathogens can parasite cultivated plants (Agrios 2005), and produce mycotoxins that pose serious hazards to human and animal health (Nelson et al. 1993).

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Gibberella intermedia (Kuhlman) Samuels, Nirenberg and Seifert is a member of *G. fujikuroi* species complex (Leslie 1991). This mating population has been assigned to anamorphic *F. proliferatum* (Matsushima) Nirenberg known as *G. fujikuroi* mating population D and is a pathogen of some economically important plants worldwide including rice, maize, citrus fruit, banana, orchids, sorghum (Leslie & Summerell 2006), onion (Galván et al. 2008) and sugarcane (Alizadeh 2010). Particularly in Iran, it has been known as the causal agent of stalk and ear rot of maize (Bujari et al. 1993, Ghiasian et al. 2004), foot rot disease of rice (Padasht–dehkayi 1993), basal and root rot disease of onion (Rabiee motlagh 2009) and knife cut disease of sugarcane (Alizadeh 2010). The species produce wide variety of mycotoxins often at high levels in seeds and crops through toxic agents including beauvericin, fumonisins, fusaproliferin, fusaric acid, fusarins and moniliformin (Leslie & Summerell 2006). Recently, the incidence and severity of these diseases have increased drastically in Iran. However, to the best of our knowledge, no comprehensive study on the Iranian *F. proliferatum* populations has been conducted. As well, little is known about genetic variability of its host in Iran. Intraspecific differences in the pathogenicity of individual isolates from different hosts have not been extensively studied either.

In the majority of plant pathogen systems much of the breakdown of plant resistance genes is due to pathogen population evolving virulence, rather than the nature of the employed resistance gene (Garcia–Arenal & McDonald 2003). Thus, to breed for durable disease resistance, a study of the population genetic and evolutionary potential of pathogen populations is in demand. The pathogens with the highest evolutionary potential pose the highest 'risk' of defeating resistance genes or counteracting other control methods such as applications of pesticides or antibiotics (McDonald & Linde 2002). Two important factors for pathogen evolution are the reproduction/ mating system and gene/genotypic flow.

Sexual fertility is an important practical parameter to understand the structure of fungal populations as an evidence of sexual cross–fertility. This is usually required when two strains are assigned to a common species (Leslie & Klein 1996). Assessing the potential for mating by toxigenic strains of *Fusarium* would increase our understanding of the genetic mechanisms that maintain intraspecific diversity as well as biological and evolutionary integrity of the species. The reproduction/mating system will affect the way that alleles are distributed in a population. Also, sexual reproduction combines favorable alleles into the same genetic background, resulting in genotypes with higher fitness or higher levels of virulence/fungicide resistance. Besides the occurrence of sexual reproduction, its frequency is also a prominent parameter to design strategies of plant pathogens. These strategies are often different for clonally and sexually reproducing organisms (McDonald & McDermott 1993).

In the previous study, vegetative compatibility grouping of the same Iranian populations of *F. proliferatum* from maize, rice, sugarcane and onion showed that natural populations of the species in Iran are genetically highly divergent and include isolates representing a potential risk for disease development (Alizadeh 2010). A correlation between VCGs grouping and host preferences also were founded. Assessing of genetic diversity based on RAPD molecular marker (Mohamadian et al. 2011) showed that maize and rice isolates of *F. proliferatum* in Iran are clearly divergent. Therefore, in order to better examination of genetic diversity, we conducted AFLP molecular marker to identify distinct *F. proliferatum* isolates from different hosts.

The objective of this research was to investigate the genetic structure of *F. proliferatum* populations from various hosts including maize, rice, sugarcane and onion in different areas of Iran using AFLP molecular markers. Specifically, we investigated the evolutionary potential of pathogen populations by determining their genetic diversity, reproduction system and the degree of gene flow among host populations. We aimed to determine whether observed patterns of genetic diversity are consistent with strict clonality, random mating, or a mixture of asexual and sexual reproduction. Furthermore, this research was conducted to determine whether host populations of the fungus are distinct populations in Iran that has evolved separately, or whether they belong to the same larger panmictic population. Thus, we studied the extent of genetic differentiation within and among populations. Also, we conducted pathogenicity test to determine whether there are differences in the ability of each of these four host populations to infect maize ears.

MATERIALS AND METHODS

Fungal isolates

A total of 142 *F. proliferatum* single–spore isolates which formerly have been collected from diseased maize, rice, sugarcane and onion from various areas of Iran were selected for population genetic analysis (Fig. 1). Among them, 79 isolates have been recovered from maize stalks and seeds obtained from nineteen locations in 10 maize producing provinces throughout of Iran (i.e. Ardabil, Isfahan, Tehran, Fars, Qazvin, Kermanshah, Golestan, Mazandaran, Hamadan and Kerman provinces) during the 2004–2005 growing season. Others were include 23 isolates from comercial rice fields in Guilan and Mazandaran provinces in north of Iran, 20 isolates from onion in Khorasan province and 20 isolates from sugarcane grown in commercial sugarcane farms in Khuzestan. These isolates were identified using the morphological characteristics of the species as described by Leslie & Summerell (2006) and Nelson et al. (1983). Isolates were stored on filter papers at –20 °C in fungal culture collection, at mycology laboratory, University College of agriculture and natural resources, university of Tehran, Karaj, Iran.

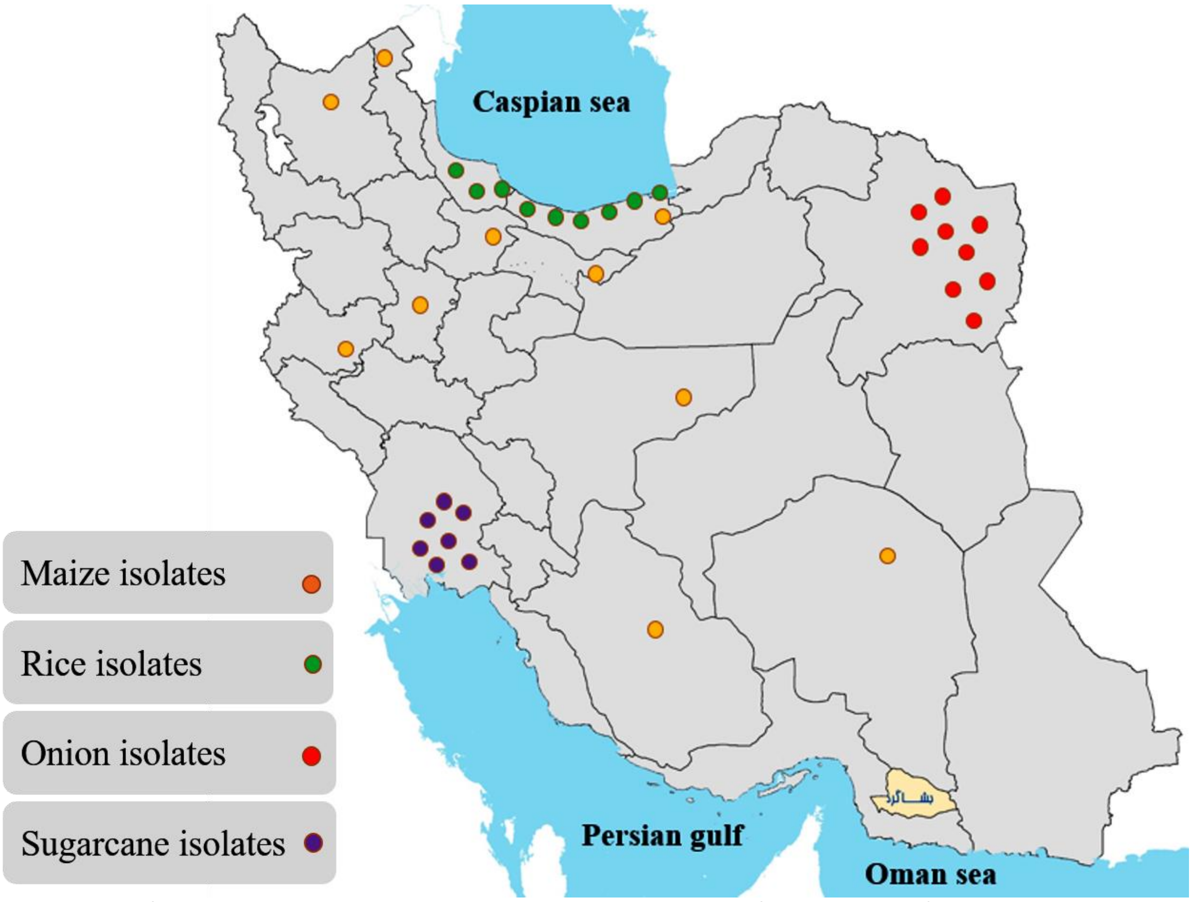

Fig. 1. Map of Iran showing sampling regions and data related to *F. proliferatum* isolated from maize ears in 10 Iranian provinces (i.e. Ardabil, Isfahan, Tehran, Fars, Qazvin, Kermanshah, Golestan, Mazandaran, Hamadan and Kerman provinces) during 2004 and 2005. Rice isolates obtained from commercial rice fields in Guilan and Mazandaran provinces in north of Iran. Onion isolates recovered from in Khorasan province and sugarcane isolates recovered from commercial sugarcane farms in Khuzestan.

The isolates are summarized (including their host plant species, year of isolation, geographical origin, mating type and VCG s groups) in Table 1. For the Maize and onion *F. proliferatum* isolates used in this study, morphological identification was confirmed by PCR assay using specie–specific primers, conducted by Rahjoo et al. (2008) and Rabiee Motlagh (2009), previously.

Amplified fragment length polymorphism (AFLP) Analysis

For AFLP analysis, fungal isolates were cultured in 100 ml flasks containing 50 ml of potato dextrose broth (PDB), and grown for seven to 10 days in dark, at 25°C. Fungal mycelium was harvested, dried by vacuum filtration through a filter paper, and lyophilized overnight.

Total fungal DNA was extracted from 20–40 mg of lyophilized mycelia grounded in 2 ml tubes using a Core–one$^{\text{tm}}$ Plant Genomic DNA isolation Kit (Corebio, Korea) according to the manufacturer’s instructions.

Four hundred nano–grams of DNA suspension was used for AFLP reactions. AFLP fingerprinting was done as described by Vos et al. (1995) using combination of two restriction enzymes: *Eco*RI–*Mse*I. After digestion and ligation, pre–amplification was performed in a volume of 20 µl using *Eco*RI0: 5'–CTCGTAGACTGCGTACCAATTC–3' and *Mse*I0: 5'–CACGATGAGTCCTGAGTAA–3' primers without extra–nucleotides. Subsequently, six primer pair combinations were used for selective amplification (Table 2). Reactions were performed in 25 µl containing 5 µl aliquot of the pre–amplification template (v/v 1/15), 50 ng primer, 0.2 mM of all four dNTPs, and 0.2 U Taq polymerase (Smart Taq, Fermentase, Germany) in PCR buffer (PCR buffer, Sinagen co., Iran). The amplification was done in a termocycler CG1–96 (Corbett Research, Australia), and consisted of an initial denaturation for 2 min at 94 °C followed by 10 cycles of denaturation at 95 °C for 30 s; annealing at 63 °C for 30 s and extension at 72 °C for 2 min, followed by 25 cycles of denaturation at 95 °C for 30 s; annealing at 54 °C for 30 s and extension at 72 °C for 2 min, with a final extension for 5 min at 72 °C. From each sample, 5 µl of AFLP products was loaded on a 6% denaturing polyacryl–amide gel (Merk, Germany), and gel electrophoresis was performed in a Biorad gel electrophoresis system (Biorad, USA).

Table 1. *Fusarium proliferatum* isolates; host plant species, year of isolation, geographical origin, mating type and VCG groups.

Isolates	Host – tissue	Year	Location (Province)	Mating type	VCGs
BTa1	Maize– ear	2005	Kerman	*MAT–1*	1
GNn1	Maize	2005	Golestan	*MAT–2*	–
QLf4	Maize	2005	Mazandaran	*MAT–2*	–
QLg3	Maize	2005	Mazandaran	*MAT–2*	–
QLg3	Maize	2005	Mazandaran	*MAT–2*	–
QLc9	Maize	2005	Mazandaran	*MAT–1*	–
ADa1–1	Maize	2005	Hamadan	*MAT–1*	–
HNa2–1	Maize	2005	Kermanshah	*MAT–1*	–
PHa11–1	Maize	2005	Kermanshah	*MAT–2*	–
PHa11–2	Maize	2005	Kermanshah	*MAT–2*	–
PHa11–3	Maize	2005	Kermanshah	*MAT–2*	–
EDd1–1	Maize	2005	Kermanshah	*MAT–1*	–
EDd1–3	Maize	2005	Kermanshah	*MAT–1*	4
EDb4–1	Maize	2005	Kermanshah	*MAT–1*	–
EDb4–2	Maize	2005	Kermanshah	*MAT–1*	–
EDb4–3	Maize	2005	Kermanshah	*MAT–1*	–
EDd1–2	Maize	2005	Kermanshah	*MAT–1*	–
QNd10	Maize	2005	Qazvin	*MAT–2*	–
QNh3	Maize	2005	Qazvin	*MAT–1*	–
QNi1	Maize	2005	Qazvin	*MAT–1*	–
QNi2	Maize	2005	Qazvin	*MAT–1*	–
QNj1	Maize	2005	Qazvin	*MAT–1*	–
ZNa1	Maize	2005	Fars	*MAT–1*	–
ZNe1	Maize	2005	Fars	*MAT–2*	–
MTa1	Maize	2005	Fars	*MAT–1*	–
MTaf4–2	Maize	2005	Fars	*MAT–1*	1
MTf4–1	Maize	2005	Fars	*MAT–1*	–
MTf4–3	Maize	2005	Fars	*MAT–1*	–
MTf8	Maize	2005	Fars	*MAT–2*	–
ENf2	Maize	2005	Isfahan	*MAT–2*	3
MNa3	Maize	2005	Ardabil	*MAT–2*	–
MNf4	Maize	2005	Ardabil	*MAT–1*	8
MNF6–1	Maize	2005	Ardabil	*MAT–1*	–
MNd1	Maize	2005	Ardabil	*MAT–2*	1
Mnb4–1	Maize	2005	Ardabil	*MAT–1*	–
Mnb1	Maize	2005	Ardabil	*MAT–2*	1
Mne8	Maize	2005	Ardabil	*MAT–1*	–
Mnc8	Maize	2005	Ardabil	*MAT–1*	–
Mnf7	Maize	2005	Ardabil	*MAT–2*	–
Mne3	Maize	2005	Ardabil	*MAT–2*	–
Mni1	Maize	2005	Ardabil	*MAT–1*	–
Mnh5	Maize	2005	Ardabil	*MAT–1*	–
KHc1–2	Maize	2005	Kermanshah	*MAT–1*	–
KHc1–3	Maize	2005	Kermanshah	*MAT–1*	–
ADa3–3	Maize	2005	Hamadan	*MAT2*	–
QB19	Maize	2005	Qazvin	*MAT–1*	–
QB20	Maize	2005	Qazvin	*MAT–1*	–
MQ30	Maize	2005	Mazandaran	*MAT–2*	11
QA34	Maize	2005	Mazandaran	*MAT–1*	12
FM35	Maize	2005	Fras	*MAT–2*	–
FM36	Maize	2005	Fras	*MAT–1*	–
FS39	Maize	2005	Fras	*MAT–2*	–
AM41	Maize	2005	Ardabil	*MAT–1*	–
AM45	Maize	2005	Ardabil	*MAT1*	–
AM46	Maize	2005	Ardabil	*MAT2*	–
AM47	Maize	2005	Ardabil	*MAT–2*	–
AM51	Maize	2005	Ardabil	*MAT–1*	–
AM52	Maize	2005	Ardabil	*MAT–2*	–
AM53	Maize	2005	Ardabil	*MAT–1*	–
QQ56	Maize	2005	Qazvin	*MAT–1*	–
QQ57	Maize	2005	Qazvin	*MAT–1*	–
AZ58	Maize	2005	East Azerbaijan	*MAT–1*	9
TK76	Maize	2005	Tehran	*MAT–1*	13
KE89	Maize	2005	Kermanshah	*MAT–2*	–

Table 1. Continued

Isolates	**Host – tissue**	**Year**	**Location (Province)**	**Mating type**	**VCGs**
KK90	Maize	2005	Kermanshah	*MAT–2*	–
KK92	Maize	2005	Kermanshah	*MAT–2*	–
AM116	Maize	2005	Ardabil	*MAT–1*	–
AM120	Maize	2005	Ardabil	*MAT–2*	–
MQ139	Maize	2005	Mazandaran	*MAT–1*	–
HA140	Maize	2005	Hamadan	*MAT–1*	–
AM149	Maize	2005	Ardabil	*MAT–2*	1
AM151	Maize	2005	Ardabil	*MAT–1*	–
EE156	Maize	2005	Isfahan	*MAT2*	10
KE158	Maize	2005	Kermanshah	*MAT–1*	–
AM162	Maize	2005	Ardabil	*MAT–1*	–
AM167	Maize	2005	Ardabil	*MAT–2*	3
AM169	Maize	2005	Ardabil	*MAT–2*	–
AM171	Maize	2005	Ardabil	*MAT–2*	–
QQ177	Maize	2005	Qazvin	*MAT–2*	–
GRP3	Rice	2004	Guilan	*MAT–1*	14
GRP7	Rice	2004	Guilan	*MAT–1*	15
GRP9	Rice	2004	Guilan	*MAT–1*	–
GRP17	Rice	2004	Guilan	*MAT–2*	7
GRP19	Rice	2004	Guilan	*MAT–2*	16
GRP23	Rice	2004	Guilan	*MAT–1*	2
GRP29	Rice	2004	Guilan	*MAT–1*	2
GRP109	Rice	2004	Guilan	*MAT–2*	17
MRP6	Rice	2004	Mazandaran	*MAT–1*	6
MRP9	Rice	2004	Mazandaran	*MAT–1*	18
MRP17	Rice	2004	Mazandaran	*MAT–2*	–
MRP23	Rice	2004	Mazandaran	*MAT–2*	2
MRP25	Rice	2004	Mazandaran	*MAT–1*	–
MRP28	Rice	2004	Mazandaran	*MAT–2*	5
MRP29	Rice	2004	Mazandaran	*MAT–2*	2
MRP32	Rice	2004	Mazandaran	*MAT–1*	–
MRP36	Rice	2004	Mazandaran	*MAT–1*	–
MRP46	Rice	2004	Mazandaran	*MAT–1*	–
MRP1	Rice	2004	Mazandaran	*MAT–1*	–
MRP12	Rice	2004	Mazandaran	*MAT–2*	19
MRP21	Rice	2004	Mazandaran	*MAT–1*	21
MRP24	Rice	2004	Mazandaran	*MAT–2*	24
Khs1	Sugarcane	2001	Khuzestan	*MAT–1*	25
Khs2	Sugarcane	2002	Khuzestan	*MAT–2*	1
Khs3	Sugarcane	2002	Khuzestan	*MAT–1*	–
Khs4	Sugarcane	2002	Khuzestan	*MAT–1*	1
Khs5	Sugarcane	2003	Khuzestan	*MAT–1*	–
Khs6	Sugarcane	1997	Khuzestan	*MAT–1*	–
Khs7	Sugarcane	1997	Khuzestan	*MAT–1*	–
Khs8	Sugarcane	2002	Khuzestan	*MAT–1*	–
Khs9	Sugarcane	2002	Khuzestan	*MAT–1*	–
Khs10	Sugarcane	2002	Khuzestan	*MAT–2*	–
Khs11	Sugarcane	2002	Khuzestan	*MAT–2*	–
Khs12	Sugarcane	2000	Khuzestan	*MAT–1*	–
Khs13	Sugarcane	2002	Khuzestan	*MAT–1*	–
Khs14	Sugarcane	2002	Khuzestan	*MAT–2*	–
Khs15	Sugarcane	2002	Khuzestan	*MAT–2*	–
Khs16	Sugarcane	2002	Khuzestan	*MAT–1*	28
Khs17	Sugarcane	2000	Khuzestan	*MAT–1*	–
Khs18	Sugarcane	2001	Khuzestan	*MAT–2*	29
Khs19	Sugarcane	2001	Khuzestan	*MAT–1*	30
Khs20	Sugarcane	2001	Khuzestan	*MAT–2*	–
E7'	Onion – root	2006	North Khorasan	*MAT–2*	1
E10'	Onion – root	2006	North Khorasan	*MAT–2*	–
E6'	Onion – root	2006	North Khorasan	*MAT–1*	–
R2	Onion – root	2006	Razavi Khorasan	*MAT–2*	–
E1'	Onion – seed	2006	North Khorasan	*MAT–2*	–
Eh7	Onion – root	2006	North Khorasan	*MAT–2*	–
Eg7	Onion – root	2006	North Khorasan	*MAT–2*	–
Mk1	Onion – basal	2006	Razavi Khorasan	*MAT–2*	1

Table 1. Continued

Isolates	Host – tissue	Year	Location (Province)	Mating type	VCGs
Mta'8	Onion – seed	2006	Razavi Khorasan	*MAT–1*	–
Eo9	Onion – root	2006	North Khorasan	*MAT–2*	1
E2	Onion – seed	2006	North Khorasan	*MAT–2*	–
Ns'9	Onion – seed	2006	North Khorasan	*MAT–2*	–
Ep4	Onion – scale	2006	North Khorasan	*MAT–2*	1
Td2	Onion – basal	2006	Razavi Khorasan	*MAT–1*	–
Eo10	Onion – root	2006	North Khorasan	*MAT–2*	1
Eg5	Onion – scale	2006	North Khorasan	*MAT–1*	–
Eh6	Onion – root	2006	North Khorasan	*MAT–2*	–
R3	Onion – root	2006	Razavi Khorasan	*MAT–2*	–
Td3	Onion – basal	2006	Razavi Khorasan	*MAT–1*	–
Ns'11	Onion – root	2006	North Khorasan	*MAT–2*	–

Table 2. Six primer pair combinations were used for selective amplification in AFLP analysis.

Primer combinations	No. of polymorphic bands	Percent of polymorphic bands
*Mse*I + TG/*Eco*RI + AT	26	69
*Mse*I + GC/*Eco*RI + TA	59	83
*Mse*I + AT/*Eco*RI + TG	51	84
*Mse*I + TC/*Eco*RI + TG	64	90
*Mse*I + TA/*Eco*RI + CA	52	97
*Mse*I + AT/*Eco*RI + CA	49	98

Polyacrylamide gels were stained by silver nitrate and then photographed. Images were scored manually for the presence or absence of bands and bands between 200 and 700 bp in length were scored manually. Analysis was performed based on 248 polymorphic AFLP bands. The AFLP fingerprint patterns obtained were converted into binary data matrices containing arrays of 0 and 1.

The presence of a band was scored as 1 and its absence as 0. Only bands that amplified conspicuously over several DNA extractions and PCR experiments with the isolates were considered as reproducible and used for analysis.

The binary matrices was analyzed with NTSYS–pc ver. 2.20 (Rohlf 2000) software using the band–based DICE similarity coefficient and the clustering of fingerprints was performed with the unweighted pair group (UPGMA) method by using average linkages (Nei & Li 1979). DNA samples from three isolates were submitted to the AFLP procedure repeated in triplicate. In order to compare isolates from different hosts, polymorphic bands were analyzed by POPGENE 32 ver. 1.31, GenALEX version 6.1 and Nei coefficient. Nei's unbiased measure of gene diversity, *H* (Nei 1978) and Shannon's Information index, *I* (Lewontin 1972) for all four host populations were estimated in POPGENE 32 ver. 1.31 (Yeh et al. 1999). To estimate the distribution of genetic variation among and within host populations, a hierarchical analysis of molecular variance (AMOVA) and the significance levels of genetic variations were calculated using a permutation test with 1000 permutations using GENALEX ver. 6.1 (Peakall & Smouse 2006). Nei's unbiased genetic distance was estimated for all four host populations in POPGENE 32 ver. 1.31 (Yeh et al. 1999). The extent of gene flow between populations was estimated from GST (Slatkin & Barton 1989) in POPGENE 32 ver. 1.31 (Yeh et al. 1999).

Sexual crossing and female fertility

Crosses to confirm mating population, investigation of sexual fertility status and identifying of mating types were made in carrot agar using the protocol developed by Klittich and Leslie (1988a). Standard tester strains 8549 (*MATD–1*) and 8550 (*MATD–2*) as the female parent and the uncharacterized field isolates as male parent were used for crosses. In all crosses, a positive and negative control was included. The female parent was inoculated by transferring a plug of mycelia onto a 60–mm carrot agar plate, and simultaneously the male parent was inoculated onto a PDA slant (8 ml medium in a 25 × 150 mm culture tube). After 7 days, the male conidia were suspended in 3–5 ml of a 2.5% tween 60 solution, and 1–1.5 ml of the suspension were gently spread over the surface of the female parent colony with a glass rod by thoroughly wetting the mycelium. Plates were incubated in the dark at 25°C for 2–3 days in order to provide mycelia contact. Finally, cultures were transferred to an incubator where temperature was maintained at 22–23°C with high humidity for about a month. A mixture of cool white fluorescence and near–UV was used for providing the desired light intensity to cultures. After the incubation period, plates were examined daily for the growth of fertile perithecia under stereomicroscope and crosses recorded positive when ascospore–oozing perithecia were observed. For each cross, fertility was confirmed by the observation of cirrhus on top of the perithecia. Female fertility examination for 40 randomly selected field isolates including 10 isolates of each host populations was conducted in crosses using the protocol described above (Klittich & Leslie 1988a) in which the field isolates used as female parents and the standard testers were the male parents. After 4–6 week, plates were examined daily for the growth of the fertile perithecia.

Multiplex PCR for *MAT–1* and *MAT–2* idiomorphs

Fusarium isolates were grown on PDA plates for 7 days and mycelia were harvested and grounded in liquid nitrogen. Total DNA was extracted from grounded mycelia of each isolate (~200 mg wet weight) using a Core–one^{tm} Plant Genomic DNA isolation Kit (Corebio, Korea) according to the manufacturer's instructions.

Multiplex PCR was conducted using the two pairs of degenerate PCR primers as introduced by Ker΄enyi et al. (2004) to amplify highly conserved alpha box and HMG box domains found in the *MAT1* and *MAT2* portions, respectively. The sequences of the primers used were as follow: FusALPHA forward: 5′–CGCCC TCT(GT)AA(CT)G(GC)CTTCATG–3′, FusALPHA reverse: 5′–GGA(AG)TA(AG)AC (CT)TTAGCAAT (CT)AGGGC–3′, FusHMG forward: 5′–CGACCTC CCAA(CT)GC(CT) TACAT–3′ and FusHMG reverse: 5′–TGGGCGGTA CTGGTA(AG)TC (AG) GG–3′. The oligonucleotides were synthesized by Eurofins MWG operon (Germany). Each PCR mixture (20 µl) contained 12.5 µl of sterilized distilled water, 2 µl of 10 × PCR buffer, 0.8 µl (2 mM) $MgCl_2$, 1 µl (0.5 mM) of dNTPs, 1.2 µl (6 µM) primer, 0.5 µl *Smar Taq* DNA Polymerase (2.5 U/ µl; CinnaGen Co., Iran) and 2 µl (~10 ng) DNA template. The PCR amplification was performed using the following programs: an initial denaturation at 94 °C for 2 min followed by 35 cycles of 30 s at 94 °C, 45 s at 55 °C and 60 s at 72 °C with a final extension of 10 min at 72 °C in a CG1–96 thermocycler (Corbett Research, Australia). Amplification products were separated by electrophoresis on a 1% (wt/vol) agarose gel for 90 min at 100 V in 1X TBE buffer (0.09 M Tris, 0.09 M boric acid and 0.002 M EDTA, pH 8.0). To visualize the amplified DNA, gels were stained with 1% ethidium bromide and then photographed by trans illumination using a Gel–Documentation (IMAGO, B & L System, the Netherland).

Pathogenicity

A total of 32 isolates (8 isolates from each host: maize, rice, sugarcane and onion) were selected for pathogenicity test on maize ear. A susceptible maize genotype K74/1 line would be a likely good candidate for *Fusarium* colonization and was selected as the experimental genotype for pathogenicity test. Experiments were conducted during the growing season of 2012 in irrigated sites at the Seed and Plant Improvement Institute (SPII), Karaj, Iran. All soils were fertilized according to regional recommenda–tion. Field inoculations were performed according to Yates and Sparks (2008) at post–pollination during the brown silk stage (Fig. 2a). Each of ears was inoculated with a 5 ml of a suspension of 1×10^6 *F. proliferatum* conidia with a 400–gauge needle. The needle was inserted into the ear until resistance from the cob was felt and then was withdrawn gently while the conidial suspension was slowly dispensed. Pathogenicity test performed in a randomized complete block design (RCBD), 3 blocks and 33 lines in each block assigned for the pathogenicity test. Five maize ears in each line of each block inoculated by one *F. proliferatum* isolate. Also, one line in each block specified as control randomly and inoculated with 5 ml double deionized sterile water. Thus, the total ears analyzed were 2400 based on 32 (isolates) × 5 (replicate) × 3 (plot). Ears were harvested at kernel maturity during mid–October of year. Disease severity was scaled according to scale proposed by Reid & Zhu (2002) (Fig. 2b).

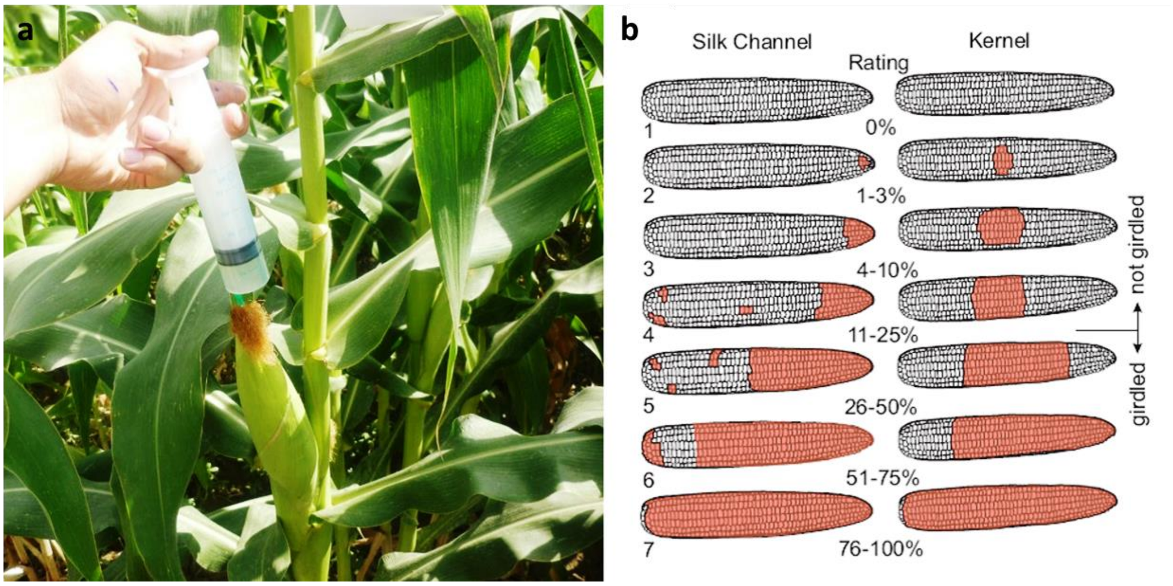

Fig 2. a. Ears inoculation at brown silk development stage through the silk channel. **b.** The scales for the estimation of pathogenicity of *F. proliferatum* isolates on maize ear proposed by Reid & Zhu (2002).

RESULTS

A total of 142 field isolates were crossed with standard tester isolates. Among those, 134 isolates were found fertile and produced ascospore–oozing perithecia 4 to 6 weeks after crossing. Furthermore, morphological identification of the isolates as *F. proliferatum* was confirmed by sexual crosses (Fig. 3). Only, eight isolates did not produce perithecium in the crosses. All of the 40 isolates were used for determination of male–female fertility in current study were female–sterile or male–fertile.

Identification of mating type idiomorphs

The applicability of the diagnostic PCR method for mating type identification was tested on 142 *F. proliferatum* isolates obtained from maize, rice, sugarcane and onion plants. Both *MAT–1* and *MAT–2* individuals were identified among these isolates (Fig. 4). The *MAT–1–* and *MAT–2–*specific fragments of 200 and 260 bp, respectively, were amplified in different isolates of *Fusarium*. Based on PCR amplification, among 142 isolates, 72 isolates (50.7%) including 40 isolates (50.6%) from maize, 14 isolates (60.8%) from rice, 5 isolates (25%) from onion and 13 isolates (65%) from sugarcane were identified as *MAT–1* and 70 isolates (49.2%) including 39 isolates (49.3%) from maize, 9 isolates (39.1%) from rice, 15 isolates (75%) from onion and 7 isolates (35%) from sugarcane belonged to *MAT–2*.

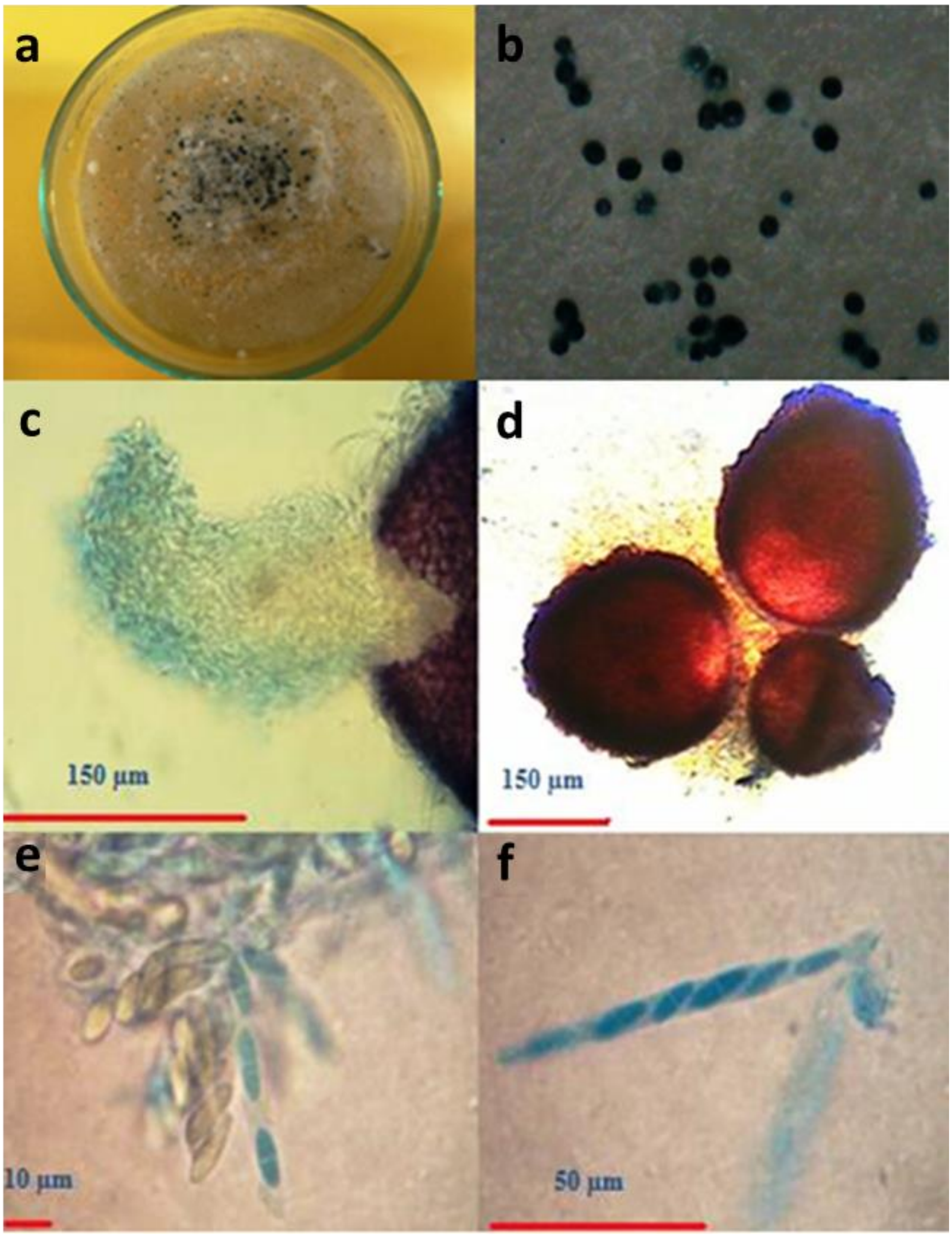

Fig 3. *Fusarium Proliferatum*. **a–c.** Perithecia of formed from crossing between field isolates as the male parents and standard tester isolates as the female parents on carrot agar medium after 2–4 weeks. **d.** Oozing ascospores in the cirrhus of the perithecia. **e–f.** asci containing mature two celled ascospores.

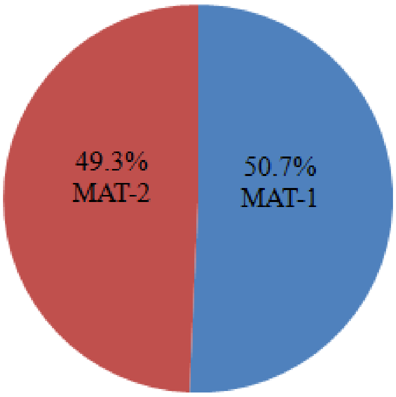

Fig 4. *MAT–1* and *MAT–2* frequency in 142 *F. proliferatum* isolates obtained from maize, rice, onion and sugarcane.

AFLP analysis of *F. proliferatum* isolates

The AFLP analysis of the fungal collection using six primer combinations yielded a total of 301 bands. Table 2 shows the number and percentage of polymorphic bands reproduced by each primer combinations. Finally, analysis was performed based on 248 polymorphic markers. Figure 5 shows the phenetic relationships among *F. proliferatum* isolates from four different hosts including: maize, rice, sugarcane and onion for the combined data set, including all six primer combinations. Based on denderogram, the isolates of *F. proliferatum* were separated according to the host plants. In general, *F. proliferatum* isolates were separated into four distinct groups. The isolates from sugarcane were grouped into two distinct groups.

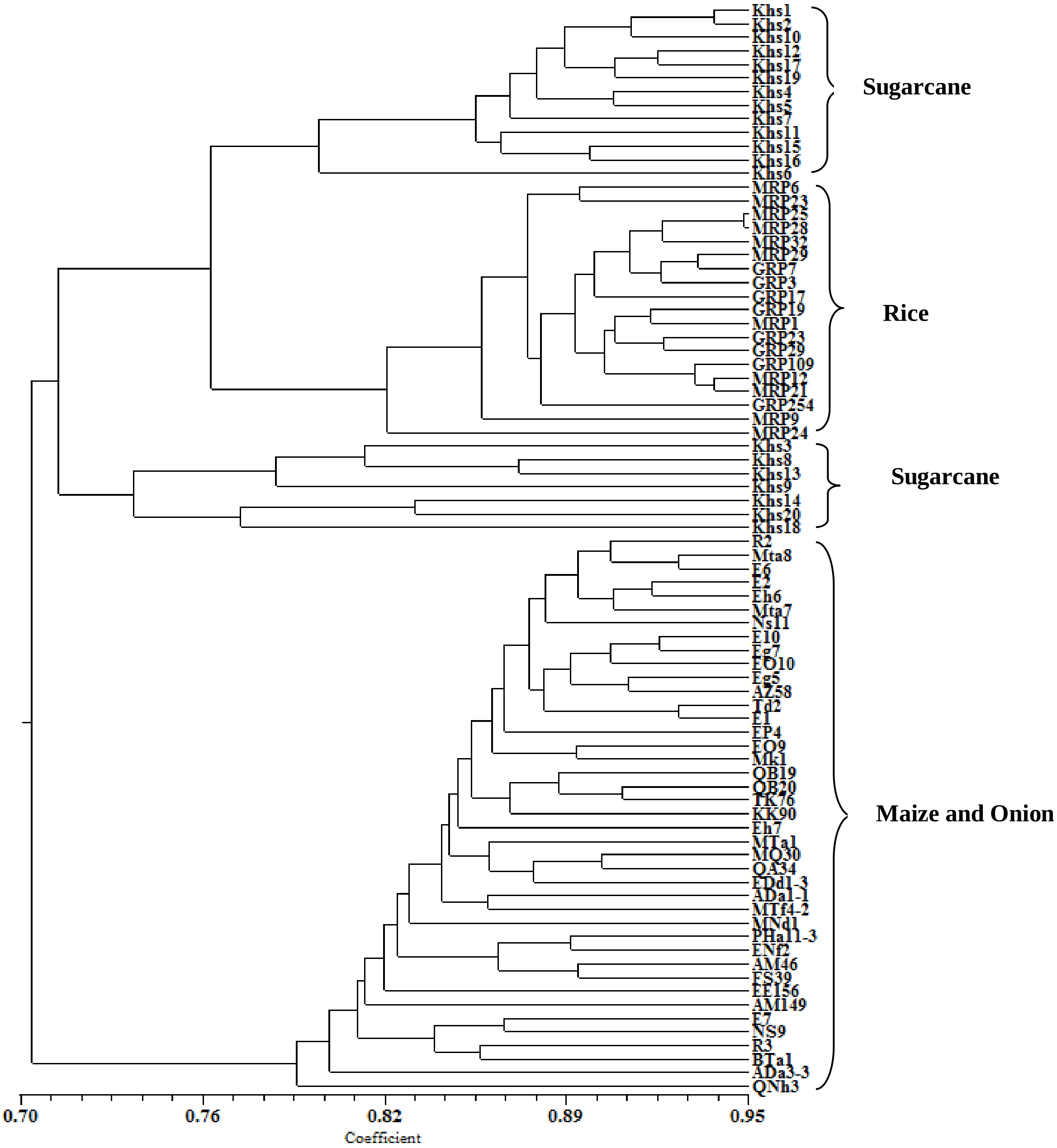

Fig. 5. Dendrogram generated from UPGMA cluster analysis of 80 *Fusarium proliferatum* isolates using DICE similarity coefficient, showing four groups according to the hosts.

The isolates from rice were grouped in a unique distinct group. However, maize and onion isolates were grouped in a unit fingerprinting group. Genetic similarity between four mentioned host populations was more than 70%.

Population genetics analysis

Nei's unbiased gene diversity (*H*) for four populations ranged from 0.1518 to 0.3928. Largest gene diversity values were for sugarcane and maize populations and smallest values were for rice and onion populations, respectively (Table 3).

Also, Shanon`s information index (*I*) ranged from 0.2307 to 0.3295. Sugarcane, maize, onion and rice host populations have largest to smallest account of *I* (Table 3).

In total, from 248 polymorphic AFLP bands, the number of polymorphic bands in each of the populations was from 48% for onion populations to 75% for sugarcane populations (Table 3).

Likewise, gene flow (Nm) values among populations comparisons was 0.86.

Nei's unbiased genetic distance (Nei 1978) between pairwise comparisons of host populations ranged from 0.0346 to 0.3048. The smallest genetic distance in pairwise comparisons was seen between maize and onion populations and the largest were for rice and onion populations (Fig. 6).

The results of AMOVA showed that 60% of the total genetic variation was attributable to the differences among populations, whereas 40% was due to the variation within populations.

Pathogenicity

Pathogenicity tests compared the effects of the maize, rice, onion and sugarcane isolates on maize ears. Typical rotting symptoms developed and the fungal mycelia grew over the ears (Fig. 7b). Evaluation of virulence of isolates was done on the basis of disease severity (%DS) index at physiological stage. The results of the analysis using of variance of data by Duncan's test showed that difference between treatments were statistically significant (α = 1%). The data, summarized in Table 4, showed that all studied *F. proliferatum* isolates originated from maize, rice, onion and sugarcane were pathogenic to maize ears and there were a considerable differences among the isolates for their virulence ability. So that different levels of pathogenicity were observed among the isolates. No disease symptoms were found in the sterile water control treatments (Fig. 7a). Among all of the isolates the maize isolates had greatest (35%) and the rice isolates showed smallest (6%) degree of pathogenicity in maize ears. Also, sugarcane and onion isolates showed 32% and 24% of pathogenicity, respectively. Totally, MTF4–3 isolate originating from maize showed the highest level of virulence (64%) and isolates MRP29 recovered from rice showed the lowest levels of virulence (2.2%) among all isolates.

Table 3. Accounts of *H*, *I*, number of polymorphic bands and percentage of polymorphic bands in the populations of *Fusarium proloferatum.*

Host population	H	I	No. of polymorphic loci	Percentage of polymorphic loci
Maize	0.2138	0.3295	177	71
Rice	0.1518	0.2307	119	48
Sugarcane	0.2615	0.3928	186	75
Onion	0.1705	0.2589	129	52
Total	0.3149	0.4739	246	100

H = Nei gene diversity
I = Shanon` s information index [Lewontin (1972)]

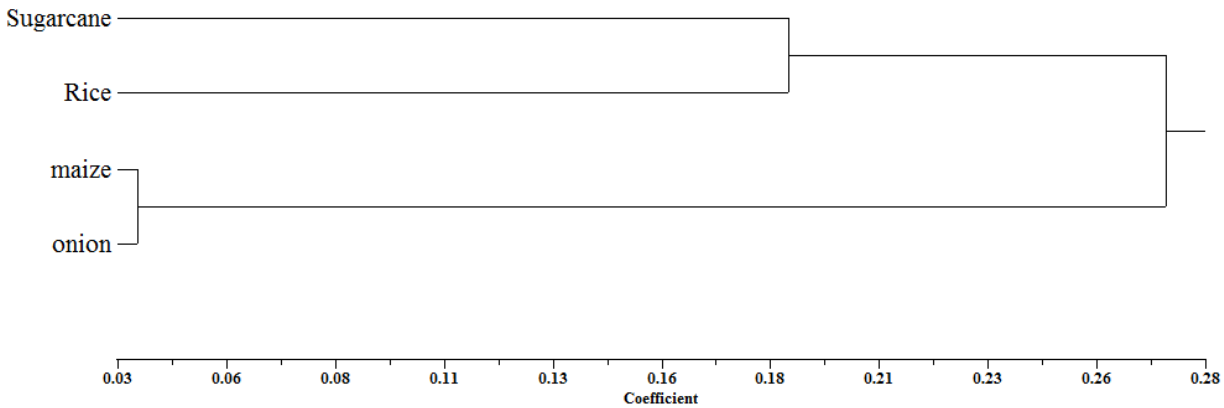

Fig. 6. Nei's unbiased genetic distance dendrogram between pairwise comparisons of host populations of *Fusarium proliferatum*.

Fig. 7. Pathogenicity test of the maize, rice, onion and sugarcane isolates on maize ears. **a.** Control treatments. **b.** Typical rotting symptoms developed over the ears by *Fusarium proliferatum* isolates.

Result of pathogenicity test showed that there were two statistical groups among four host populations. Based on Duncan's test analysis, isolates from maize, sugarcane and onion belonged to group A and rice isolates placed in group B (Table 4). Likewise, isolates of each host plant showed significant variation based on disease severity. Maize isolates placed in four (A–D) statistically groups (Table 5). Rice and sugarcane isolates placed in two statistically groups (Table 6 and 7, respectively) and finally onion groups belonged to one statistically group (Table 8).

Table 4. Evaluation of virulence of *Fusarium proliferatum* isolates obtained from different hosts on the basis of disease severity (DS) index estimated by Duncan's test.

Host	**DS (%)**	**N**	**Duncan's grouping**
Maize	34.450	24	A
Sugarcane	32.167	24	A
Onion	24.758	24	A
Rice	6.217	24	B

Table 5. Evaluation of virulence of *Fusarium proliferatum* isolates obtained from maize on the basis of disease severity (DS) index estimated by Duncan's test.

Isolates	**DS (%)**	**N**	**Duncan's grouping**	
MTF43	64.333	3	A	
Mnb41	46.667	3	B	
KE158	40.667	3	B	C
QB20	29.667	3	D	C
BTa1	28.867	3	D	C
QNd10	27.000	3	D	C
ADa11	22.533	3	D	

DISCUSSION

Fusarium proliferatum has not often been studied in depth from a population genetics perspective in Iran. Understanding of the genetic structure of a population reflects its evolutionary history and its potential to evolve.

Table 6. Evaluation of virulence of *Fusarium proliferatum* isolates obtained from rice on the basis of disease severity (DS) index estimated by Duncan's test.

Isolates	**DS (%)**	**N**	**Duncan's grouping**	
MRP12	17.333	3	A	
GRP17	8.800	3	B	
GRP29	6.067	3	B	C
GRP23	5.200	3	B	C
GRP109	3.733	3	B	C
MRP23	3.667	3	B	C
MRP17	2.733	3	B	C
MRP29	2.200	3		C

Table 7. Evaluation of virulence of *Fusarium proliferatum* isolates obtained from sugarcane on the basis of disease severity (%DS) index estimated by Duncan's test.

Isolates	**DS (%)**	**N**	**Duncan's grouping**		
Khs12	46.533	3	A		
Khs201	43.333	3	A	B	
Khs17	34.667	3	A	B	C
Khs11	32.267	3	A	B	C
Khs20	26.867	3		B	C
Khs5	26.667	3		B	C
Khs15	24.533	3			C
Khs4	22.467	3			C

Table 8. Evaluation of virulence of *Fusarium proliferatum* isolates obtained from onion on the basis of disease severity (%DS) index estimated by Duncan's test.

Isolates	**DS (%)**	**N**	**Duncan's grouping**
EO10	32.733	3	A
NS9	32.733	3	A
EH7	32.067	3	A
MTA8	24.267	3	A
E7	22.867	3	A
R2	19.067	3	A
TD2	18.133	3	A
E1	16.200	3	A

Therefore, knowledge of the genetic structure of the *F. proliferatum* populations might be useful in order to establish effective strategies for controlling the disease (McDonald 2004). The main goal of this study was to study on genetic diversity of the Iranian *F. proliferatum* isolates recovered from maize, rice, sugarcane and onion in agricultural fields using AFLP molecular marker. The results of previous studies on *F. proliferatum* isolates showed there is high genetic diversity within Iranian populations of *F. proliferatum* in Iran. Alian (2005) reported that, diversity for VCGs is very high in *F. proliferatum* isolates from rice recovered from different regions of Mazandaran province in Iran, where 23 different VCGs were found among 29 isolates. In the another study which conducted by Mohammadian et al. (2011), assessment of the *F. proliferatum* isolates from rice and corn using RAPD–PCR showed these isolates separated into two distinct clusters at 69% similarity level according to the hosts. Also, these data proved there is a high genetic variation among the isolates of *F. proliferatum* from rice and corn. In the previous study (Alizadeh et al. 2010), assessment of vegetative compatibility grouping of Iranian *F. proliferatum* isolates from different hosts, showed there is a high genetic diversity within Iranian populations of *F. proliferatum* from different host plants and a correlation between VCGs grouping and host preferences were founded. Therefore, we concluded that natural populations of *F. proliferatum* in Iran are probably genetically divergent and include isolates representing a potential risk for disease development. Thus, in the present study in order to improve understanding of the structure of populations, additional isolates from other hosts growing regions were tested and AFLP molecular marker used to examine if the *F. proliferatum* isolates from different hosts are distinct populations.

In this study, assessment of the *F. proliferatum* isolates from different hosts using AFLP molecular marker showed this technique is able to separate the isolates of this species into four distinct clusters at 64 % similarity level according to their hosts. Isolates recovered from rice clustered in a distinct group. Isolates of sugarcane grouped in two groups at 70% similarity level. Isolates recovered from maize and onion grouped in a unit group. The results of AMOVA analysis confirmed segregation of the four host populations and revealed 40% of the total genetic variation was attributable to the differences among populations, whereas 60% was due to the variation within populations. Low amount of gene flow that achieved in population genetics analysis, can explain segregation of the isolates of the fungus from different host populations. These results are in agreement with results from previous studies on *F. proliferatum*, demonstrating that the host populations of this fungus are genotypically highly divergent in Iran (Mohammadian 2011, Alizadeh et al. 2010).

Correlation between AFLP and VCGs and other traits is reported in numerous studies such as *F. oxysporum* (Baayen et al. 2000), *F. sterilihyphosum* in *G. fujikuroi* species complex (Lima et al. 2009) and *Colletotrichum* (Heilmann et al. 2006). Patino et al. (2004) investigated thirty–three isolates of *F. verticillioides*, isolated from diverse origins and hosts. Analysis of the IGS region by PCR–RFLP discriminated two groups of isolates based on fumonisin production and host preferences. Moretti et al. (2004) reported that, isolates of *F. verticillioides* from banana showed different traits such as pathogenicity, toxin profile, fertility and AFLP fingerprint than members of the same fungus from maize. In the present study, a correlation was observed between VCG and AFLP clustering. Vegetative compatibility grouping of the same *F. proliferatum* isolates identified 30 VCGs among 57 isolates. Of these, only 23 groups had one member and the remaining 34 isolates belonged to seven multimembers VCGs. VCG1 was the largest and the most frequent group in Iran and consisted of 20 isolates, VCG2 included four isolates whereas the other five multimember VCGs each had two members. Isolates belonging to VCG1 were collected from eight provinces, indicating that genetic variation across geographic locations occurs in Iran, confirming results obtained in previous studies (Desjardins 2003, Bargen et al. 2009). VCG1 included isolates from maize, onion and sugarcane plants and VCG2 included isolates from maize and rice. Each of VCG 3, VCG 4 and VCG 5 included two isolates from maize and each of VCG 6 and VCG 7 included two isolates from rice. Additionally, none of the isolates from rice complemented with any other isolates from onion and sugarcane. Also, non–complementation occurred between onion and sugarcane isolates. In VCG1 only one complementation occurred between one isolate from maize and sugarcane isolates. Also, in VCG2 only one isolate from maize complemented with rice isolates. Therefore except some cases, host preferences observed in complementation tests and assignment of isolates to VCG groups. In VCGs test, various complementations were occurred between maize and onion isolates. Also, AFLP clustering showed that, maize and onion isolates grouped in a unit cluster together. Similarly, Nei's unbiased genetic distance (Nei 1978) estimated by POPGENE32 software showed the Maize and onion isolates present smallest genetic distance. Finally, more interestingly, VCGs results were in consistent with AFLP results.

In order to a better understanding of relationships between *F. proliferatum* host populations, we conducted particular population genetic analysis for four host populations (maize, rice, sugarcane and onion) of *F. proliferatum*. Nei's unbiased gene diversity (*H*), Shanon` s information index (*I*) and number and percentage of polymorphic bands in each populations were estimated for different host populations. Sugarcane, maize, onion and rice host populations had largest to smallest amounts of H, I, number and percentage of polymorphic bands, respectively.

The smallest genetic distance in pairwise comparisons was estimated between maize and onion populations and the largest observed for rice and onion populations. These data showed that the maize and onion populations are the most similar populations, however, onion and rice host populations are the most different populations. These data are in consistence with VCG results, whereas none of rice isolates complemented with any isolates from onion. Various complementations were also occurred between maize and onion isolates.

Moreover, we decided to assess the variability of *F. proliferatum* isolates coming from different host species to understand if there is pathogenicity variation between Iranian *F. proliferatum* isolates. Therefore, all studied *F. proliferatum* isolates originated from different hosts expressed pathogenicity to maize ears and demonstrated different levels of pathogenicity among isolates from different host populations. The most interesting finding of pathogenicity test was the differences in disease severity ratings between different *F. proliferatum* host populations. Duncan's test analysis revealed isolates from maize, sugarcane and onion belonging to group A and rice isolates placed in group B. The highest severities were found for maize isolates, while, lowest severities were record for rice isolates.

Finally, in this study, a good correlation was observed between AFLP clustering, VCGs and pathogenicity of maize, sugarcane and onion isolates compare to isolates coming from rice.

Among 142 field isolates used for crossing tests with standard tester isolates, 134 isolates were fertile and produced perithecia that exuded a cirrhus of ascosporous two weeks after fertilization. Thus, in these isolates the morphological identification results as *G. intermedia* was confirmed by sexual crosses. In addition, eight isolates obtained from maize and sugarcane were sterile and did not produce any perithecium in the crosses. However, PCR amplification revealed they carried the *MAT* idiomorphs. Moreover, our findings clearly showed that conserved *MAT* specific sequences were present and *MAT* alleles were expressed in those eight isolates. This could be ascribed to the fact that besides the existence of *MAT* idiomorphs, there are also a number of different factors that can affect the ability of a strain to complete the sexual portion of the life cycle (Leslie & Summerell 2006).

In this study, the multiplex PCR reaction was used for scoring mating types within the Iranian populations of *F. proliferatum* in order to analyze the natural populations of this fungus swiftly. In total, the *MAT–1* and *MAT–2* segregated in ratio of 72:70, respectively, for isolates of *F. proliferatum* recovered from different hosts from various areas of Iran. This ratio was similar to those of Leslie & Klein (1996) and Schulz et al. (2000). Furthermore, PCR based identification of mating type idiomorphs confirmed the crossing results.

Besides the mating type alleles and their ratio, the proportion of female–fertile isolates (hermaphrodites) in a population is an indicator of the frequency at which sexual reproduction occurs. For a sexual cross to occur, both strains must be in the same biological species. One strain must carry the *MAT–1* allele and the other the *MAT–2* allele, although it does not matter which parent (male or female) carries the mating type allele. Moreover, at least one of the strains must be female fertile (Leslie & Summerell 2006). The existence of both opposite mating type alleles with favorable frequency is important for the genetic diversity in the populations of fungi to occur. In the present study, the presence of both mating type idiomorphs with favorable frequency among isolates recovered from different hosts of *F. proliferatum*, shows that there is a potential for sexual reproduction within these populations and, thus, an expansion of the genetic diversity by recombination. On the other hand, female fertility examination for 40 randomly selected field isolates including 10 isolates of each host populations showed all the isolates are female–sterile. This was in agreement with results of Mohammadian et al. (2011) and suggested that sexual reproduction did not occur frequently in the *F. proliferatum* populations. However, Abbaszadeh et al. (2004) reported that Iranian *F. proliferatum* isolates can reproduce both sexually and asexually, and the alternating frequency of both sexual and asexual reproduction can affect the structure of the populations. These data propose that the high level of genetic diversity within *F. proliferatum* populations in Iran is possibly a result of parasexual cycle, vegetative compatibility, various cultivars of the hosts and high amount of migration to populations as well as sexual reproduction.

Finally, the outcome of this research could be used to assess disease control strategies that prevent or limit genotypic variation and rearrangement within the pathogen populations. McDonald & McDermott (1993) reported that high levels of race–specific resistance can be developed in plant cultivars against clonally reproducing organisms, whereas horizontal resistance could be more effective against pathogens comprising genetically diverse populations as a result of mating and meiotic recombination. In addition, the results of this study could reflect important differences in ecology and natural history of the populations from maize, sugarcane, rice and onion and should encourage further studies into the investigation of other traits of the fungus such as variation in toxin production.

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چکیده: ارزیابی تنوع ژنتیکی ۸۰ جدایه *Fusarium proliferatum* به دست آمده از گیاهان ذرت، برنج، نیشکر و پیاز با استفاده از نشانگر مولکولی AFLP، جدایه‌ها را در چهار گروه قرار داد. جدایه‌های بدست آمده از برنج در یک گروه مجزا و جدایه‌های نیشکر در دو گروه جداگانه قرار گرفتند. جدایه‌های بدست آمده از ذرت و پیاز نیز در یک گروه در کنار همدیگر قرار گرفتند. آزمون بیماری‌زایی جدایه‌های بدست آمده از میزبان‌های مختلف بیماری‌زایی همه این جدایه‌ها روی خوشه گیاه ذرت را نشان داد. با این حال، درجات مختلفی از بیماری‌زایی بر اساس جمعیت‌های میزبانی مختلف قارچ مشاهده شد. تجزیه و تحلیل بیماری‌زایی به روش آزمون دانکن، جدایه‌های ذرت، نیشکر و پیاز را در گروه A و جدایه‌های برنج را در گروه B قرار داد. همچنین ارتباط مستقیمی بین نتایج حاصل از انگشت نگاری DNA به روش AFLP و بیماری‌زایی جدایه‌های ذرت، نیشکر و پیاز در مقایسه با جدایه‌های برنج مشاهده شد. ارزیابی سازگاری جنسی و ردیابی ایدیومورف‌های تیپ آمیزشی به روش PCR برای ۱۴۲ جدایه از جمعیت‌های میزبانی مختلف انجام شد که ۷۲ جدایه به عنوان تیپ آمیزشی *MAT–1* و ۷۰ جدایه به عنوان تیپ آمیزشی *MAT–2* شناخته شدند. حضور هر دو تیپ آمیزشی با فراوانی نزدیک در جدایه‌های بدست آمده از میزبان‌های مختلف نشان می‌دهد پتانسیل وقوع تولیدمثل جنسی در جمعیت های *F. proliferatum* در ایران وجود دارد. با این وجود، آزمون تعیین ماده بارور– نر باور بودن ۴۰ جدایه انتخاب شده بطور تصادفی شامل ۱۰ جدایه از هر میزبان نشان داد همه جدایه‌های بررسی شده نر بارور می‌باشند. در نهایت، می‌توان نتیجه گرفت علاوه بر تولید مثل جنسی سایر عوامل نظیر چرخه شبه جنسی، سازگاری رویشی، تنوع در ارقام گیاهان میزبان و جریان ژنی از طریق مهاجرت می‌توانند در بروز تنوع ژنتیکی جمعیت‌های *F. proliferatum* در ایران موثر باشند.

کلمات کلیدی: انگشت نگاری DNA، تنوع ژنتیکی، پوسیدگی فوزاریومی خوشه، نشانگر مولکولی، VCG

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