Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Momeni and Nazari, J Plant Pathol Microbiol 2016, 7:6
DOI: 10.4172/2157-7471.1000355

Research Article Open Access

Population Genetic Structure among Iranian Isolates of *Fusarium verticillioides*

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Abstract

The genetic structure among Iranian populations of *Fusarium verticillioides* from the main corn growing areas of five provinces including Ardabil, Fars, Mazandaran, Khorasan, and Khuzestan were evaluated using VCG, RAPD and rep-PCR. Sixty-one isolates of *F. verticillioides* were placed in 14 Vegetative Compatibility Groups and 19 haplotypes. VCG3 with 14 members (23% of all isolates) was the most frequent VCG. RAPD-PCR and rep-PCR generated multiple distinct products demonstrated considerable variability among the isolates of different VCGs. Haplotype 1(HP1) had the highest frequency (0.57) in the population and was present in isolates from the majority of the locations in this study. All molecular phenotypes were distributed randomly across the various locations. Although there are some consistent between geographical origin of the isolates and their genetic similarity but VCG groups were distributed among different geographical locations and there was no correlation between geographical distribution and VCG groups. Gene diversity was 0.2909 and populations of *F. verticillioides* were placed in five distinct groups based on geographical origin. The highest genetic distance observed between Fars and Khuzestan (0.1801) and the smallest genetic distance was obtained between Ardabil and Khuzestan (0.0589). Analysis of molecular variance (AMOVA) showed a significant difference among populations of *F. verticillioides*. According to our results PhiPT was equal to 0.176 ($p <= 0.001$) and 82% of genetic variance occurred within populations and only 18% was found among populations. Moghan in Ardabil province is the main site for seed producing in Iran and the seeds that are produced there are distributed all over the country. In this study isolates of Moghan were located besides the isolates from other regions in different clusters. So it is presumed that the infected seeds from Moghan can be a major source for the spreading of the disease through all corn growing areas in Iran.

Keywords: *Fusarium verticillioides*; Corn ear rot; Rep-PCR; RAPD; Genetic diversity

Introduction

Fusarium ear rot is the most common fungal disease of corn ears that is caused by several species of Fusarium. Symptoms of the disease are a white to pink- or salmon-colored mold, beginning anywhere on the ear or scattered throughout. *Gibberella moniliformis* Wineland [anamorph *Fusarium verticillioides* (Sacc.) Nirenberg] is genetically the most intensively studied species in Fusarium section Liseola. [1]. Although yield usually is not much affected, kernel infection by Fusarium is of concern because of the loss of grain and seed quality and the potential occurrence of fumonisins and other mycotoxins [2]. *F. verticillioides* is the major species that causes ear rot on corn in Iran and is the most commonly reported fungal species associated with maize plants (*Zea mays* L.). During recent years, the disease is so severe that in some fields the entire crop has to be discarded. Host range and plant-fungus interactions are of significant interest in terms of understanding the distribution, biology, and population dynamics of this mycotoxigenic fungus [3]. The fungus can be found in plants or residues in maize fields in the United States at some time during the growing season [4].

Infection of developing corn kernels may occur through the silks, through holes and fissures in the pericarp or at points where the pericarp is torn by the emerging seedling and as a result of systemic infection of the corn plant by *F. verticillioides* [5]. *F. verticillioides* produces abundant, mostly single-celled microconidia in long chains [6].

Plant pathologist should study the population genetics of plant-pathogenic fungi, because pathogens evolve. Pathogen populations must constantly adapt to changes in their environment to survive [7]. Defining the genetic structure of populations is a logical first step in studies of fungal population genetics because the genetic structure of a population reflects its evolutionary history and its potential to evolve [7]. Knowledge of the genetic structure of pathogens is useful for developing control strategies, as the amount of genetic variation present within a population indicates how rapidly a pathogen can evolve. This information may eventually be used to predict how long control measures such as fungicides and resistant cultivars are likely to be effective [8].

Lineages that are capable of fusing (anastomosis) and forming stable and functional heterokaryons are known as sexually or vegetatively compatible, the former being frequently described as members of the same group of vegetative compatibility or vegetative compatibility group [9].

The genetic of vegetative compatibility in the entire Fusarium genus is modeled on the basic results obtained primarily with *F. verticillioides* [10]. Although Vegetative compatibility groups (VCG) is relatively a simple way to distinguish between strains that are morphologically identical [11], but as a tool for population genetic analysis in *F. verticillioides* it has not proven particularly useful, as most of the strains in a population are in different VCGs and thus the information obtained primarily is that no two strains are identical, i.e., clones in these populations are rare [12]. According to Danielsen et

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Received February 23, 2016; **Accepted** June 08, 2016; **Published** June 10, 2016

Citation: Momeni H, Nazari F (2016) Population Genetic Structure among Iranian Isolates of *Fusarium verticillioides*. J Plant Pathol Microbiol 7: 355. doi:10.4172/2157-7471.1000355

al. [13] Vegetative compatibility identified 34 vegetative compatibility groups (VCGs), of which 29 had one member and 5 had two members. Their results demonstrated that natural populations of *F. verticillioides* in Costa Rica consist of genetically diverse that represent a potential risk for disease development in corn crops.

Genetic diversity among *F. verticillioides* isolates was analyzed using VCG and RAPD [14]. According to their results RAPD could differentiate VCGs except in two cases. Genetic diversity among Iranian isolates of *F. verticillioides* was analyzed using VCGs [15]. Their results demonstrated that natural populations of *F. verticillioides* in Iran are genetically highly divergent and include isolates representing a potential risk for disease development. Genetic diversity of *F. verticillioides* was investigated by Mohammadi et al. [16] using VCGs in Iran among isolates that were recovered from Seed samples had been collected from the major producing area in Khuzestan and Ardabil provinces. Specific relation was not observed between VCGs and geographic origin of the isolates in their study and genetic diversity among population of *F. verticillioides* was very high. Isolates of *F. verticillioides* were recovered from diseased sugarcanes in Iran [17]. According to their results Forty-eight VCGs of *F. verticillioides* were isolated and none of the VCGs was common.

A simple procedure that can be used to detect infection by *F. verticillioides* from infected plant tissues has been developed [18]. A Polymerase Chain Reaction–Based assays was used for species-specific detection of Fusarium [14,19]. This technique has been successfully used to assess genetic variability within many plant pathogenic fungi, including Fusarium section *Liseola* [4,20-22]. There are two detailed genetic maps of Fusarium species available, one for *F. verticillioides* [1] and the other for *F. graminearum* [23].

Genetic diversity among 41 isolate of *F. verticillioides* collected from rice in Iran was determined using vegetative compatibility groups and RAPD. High level of genetic diversity was observed among *F. verticillioides* isolates [24].

Edel et al. [25] used ERIC and REP primers as molecular methods along with RFLP and PCR-amplified IGS for characterization of *Fusarium oxysporum* strains. Good correlation was found between the groupings obtained by the three methods. According to their results discrimination of closely related strains within IGS genotypes could be achieved by ERIC- or REP-PCR fingerprinting, which is the most efficient procedure in terms of simplicity and rapidity.

Karimi Dehkordi et al. [26] used rep-PCR to determine genetic diversity of 55 isolates of *F. verticillioides* from infected ears and stems of *Zea maize* and *Oryzae sativa* from different corn and rice producing areas of Iran. Their results suggested that *F. verticillioides* isolates from rice and corn are genetically different and that rep-PCR is a convenient and rapid method for analysis of genetic diversity and strain differentiation in *F. verticillioides*.

McDonald et al. [27] investigated the potential of repetitive-sequence-based polymerase chain reaction (rep-PCR) fingerprinting of fungal genomic DNA as a rapid and simple alternative to random amplified polymorphic DNA (RAPD) analysis in the study of phylogenetic relationships, and also as a diagnostic method among some species of Tilletia.

Jedryczka et al. [28] used REP, ERIC and BOX primers for rep-PCR genomic fingerprinting to assess the ability of rep -PCR genomic fingerprinting methods to characterize a collection of 90 isolates of *Leptosphaeria maculans* from Poland.

The present survey was undertaken in order to obtain a current picture of genetic structure among Iranian populations of *F. verticillioides* collected from the main corn producing areas. A second analysis was conducted to determine how many molecular phenotypes (haplotypes) were present in the population.

Materials and Methods

Sample collection and fungal isolation

A survey of corn fields was conducted in 2012-2013. Ears with symptoms resembling fusarium ear rot were collected from fields (each field ≥ 1 ha) in the 5 main corn producing provinces including 14 locations (Figure 1). A total of 5 infected ears were collected per field.

Seed samples were surface disinfected in 5.25% solution of Naocl for 1 min, rinsed three times in sterilized water and air dried on sterile paper towel. Sterilized corn seeds were cut in half and plated cut side down onto Nash and Snyder selective medium [29,30] that allows formation of easily recognizable colonies [31]. Fusarium colonies were transferred to Carnation-Leaf Agar (CLA), Potato Dextrose Agar (PDA) and Spezieller Nahrstoffarmer Agar (SNA), and were incubated for 7 days at 25°C [32]. Sixty-one Single spore isolates of *F. verticillioides* were identified based on the morphological criteria of Leslie and Summerell [10].

Recovered isolates were grouped into 5 populations based on geographical distances. Each population represented one province.

Pathogenicity test

Pathogenicity test was carried out according to Danielsen et al. [13] using SC301 corn seeds. The inoculums of isolates were prepared by transferring plugs of PDA containing 7-day old *F. verticillioides* to vials containing autoclaved toothpicks and PDB medium. After 7 week, 3 plants (stalks) per isolates were inoculated by insertion infected toothpicks about 5 cm above soil level and the inoculated site were sealed with parafilm. The control was the stalks that were inoculated with sterile toothpicks. The length of the necrotic region at the insertion point was measured and compared with the control.

Vegetative compatibility groups

VCGs assignment was based on complementation of nitrate non-utilizing (*nit*) mutants. Heterokaryon formation was demonstrated by pairing mutants that were unable to reduce nitrate [33]. Generation of mutants from 61 *F. verticillioides* isolates carried out according to Correll et al. [10] on PDA, malt agar (MA), corn meal agar (CMA) and minimal medium (MM) amended with 3 to 4.5 % potassium Cholorate [34]. Hyphal tips from Fast-growing, Chlorate-resistant sectors were transferred to minimal medium. Colonies with an expanding thin mycelium were considered *nit* mutants. All *nit* mutants showed wild-type growth on PDA.

The *nit* mutants were assigned phenotypically as *nit*1, *nit*3 or NitM based on differential growth on Minimal Medium (MM) amended with NaNo3, NaNo2, hypoxanthine, ammonium tartrate and Uric acid as sole nitrogen sources [35].

Complementation tests were carried out between NitM (or *nit3*) and *nit1* mutants on minimal medium [36]. After 10 days in 25°C, Pairs of isolates that exhibited robust growth at the line of contact between the two colonies were determined as vegetatively compatible, otherwise were grouped in different VCGs [34,37]. All pairing were performed twice.

Figure 1: Provinces and locations where the isolates of *Fusarium verticillioides* were collected in Iran. 1-Mashhad 2-Neyshabur 3-Chenaran 4-Dashtenaz 5-Gharakheil 6-Moghan 7-Shushtar 8-Shush 9-Dezful 10-Andimeshk 11-Marvdasht 12-Zarghan 13-Fasa 14-Darab.

Genomic DNA extraction

Single spore colonies of all isolates were established and grown on PDA Medium. An inoculum disk was taken from each colony and used to inoculate 50 ml of liquid PDB (Potato Dextrose Broth) medium [38]. The cultures were incubated for 7 days, at 25°C, after which the mycelium was harvested, washed and used for extraction.

Total genomic DNA was isolated from mycelium by a microextraction protocol according to Möller et al. [39]. DNA was quantified by comparison with known amounts of genomic DNA on a 1.5% agarose. Appropriate dilution of the samples ensured a DNA sample of 10 ng genomic DNA for PCR reactions.

RAPD–PCR

DNA from individual single spore colonies was taken and used for each PCR reaction. Three previously identified primers by the name OPR11, OPR14, OPR16 [14] and four new primers including UBC682, UBC648, UBC199 and UBC196 (metabion international AG, Martinsried/Deutschland) were selected for PCR reactions. These primers were initially tested on three isolates and reactions repeated two times to insure of production of reproducible bands.

Reactions were performed with a BioRad thermocycler (Icycler model) in a 25 µl total volume containing 50 ng genomic DNA, 1.25 X PCR buffer (Fermentaz, Germany), 0.2 mM of each of the four dNTPs, 2.5 mM MgCl2, 12.5 pmol of each primers and 1 U *Taq* DNA Polymerase (Fermentaz, Germany). Reaction conditions consisted of an initial denaturation step at 95°C for 2 min, followed by 35 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, and then a final extension step at 72°C for 7 min. The fragment analysis was performed on 2% agarose gels in 1XTBE buffer.

Rep-PCR reactions

Rep-PCR reactions was carried out using BOX (5′- CTA CGG CAA GGC GAC GCT GAC G-3′), ERIC (ERIC1: 5′-ATG TAA GCT CCT GGG GAT TCA C-3′ ERIC2: 5′-AAG TAA GTG ACT GGG GTG AGC G-3′) and REP (REP1: 5′-IIII CGI CGI CAT CIG GC-3′ REP2: 5′-ICG ICI TAT CIG GCC TAC-3′) primers [40].

Reaction conditions was the same as RAPD-PCR but PCR components was different including 30 ng genomic DNA, 1X PCR buffer (Fermentaz, Germany), 0.1 mM of each of the four dNTPs, 1.5 mM MgCl2, 5 pmol of each forward and reverse primers, and 1 U *Taq* DNA Polymerase (Fermentaz, Germany) in a 25 µl total volume.

Data analysis

Haplotype: Determination of molecular phenotype (Haplotype) was carried out according to the DNA banding patterns of all seven primers that were used in RAPD based on Kolmer et al. [41]. So each isolates of *F. verticillioides* was given a seven-digit number that shows the haplotype of that isolates.

Genetic diversity: Sixty-one isolates of *F. verticillioides* from different geographical regions along with an isolate of *Fusarium proliferatum* were analyzed for genetic diversity based on pooled data that was obtained from RAPD and rep-PCR reactions. Genetic diversity within each population and for the entire isolates (Ht) computed by the program POPGENE Version 1.31. [42]. Genetic diversity was calculated

as H= (1-$\sum pi^2$), where i is the frequency of allele i at the locus [43]. Gene flow that is shown with *Nm* (Number of migrants) is calculated as $Nm=0.5(1-G_{ST})/ G_{ST}$, and G_{ST} is the Coefficient of gene differentiation [43]. Genetic distances were calculated between different populations based on Nei [43,44] and a dendrogram was generated using the unweighted pair group method with arithmetic averaging (UPGMA). The consensus tree was displayed using TREEVIEW v. 1.6.6 [45]. Shannon's information index (I) as a measure of gene diversity was estimated as well.

Analysis of molecular variance (AMOVA): Distribution of genetic variation and genetic structure was evaluated using analysis of molecular variance (AMOVA) that computed by GenAlEx6 [46]. We used AMOVA to estimate the partitioning of the total genetic diversity among and within the 5 studied populations including Fars, Khuzestan, Ardabil, Mazandaran and Khorasan. PhiPT is a measure facilitating AMOVA is calculated with the formulae of *PhiPT = AP / (WP + AP)*, where AP is estimated variance Among Populations and WP is estimated variance Within Populations. Genetic Distance (GD) matrix was obtained between all isolates of *F. verticillioides*.

Cluster analysis: Cluster analysis was computed by the help of NTSYSpc-2.02e. The input file was an Excel with binary data including 1 for the presence and 0 for the absence of each amplified band. The SimQual program was used to calculate the Dice similarity coefficients [47]. The resulting similarity matrix was used for unweighted pair group method with arithmetic averages (UPGMA) based dendrogram [48] using the sequential agglomerative hierarchical nested cluster analysis (SAHN) module of NTSYSpc. An isolate of *F. proliferatum* was used as outlier.

Results

Identification of VCGs

Based on mycological characteristics, 61 isolates were identified as *F. verticillioides* [10]. Pathogenecity test demonstrated that all isolates are pathogenic and inoculated corn stalks showed discoloration. Nitrate non-utilizing (*nit*) mutants were recovered from all 61 isolates of *F. verticillioides* and used in complementation tests and each isolate was assigned to a unique VCG group (Table 1).

Ninty-four percent of the sectors recovered were unable to utilize nitrate as the sole nitrogen source. A total number of 434 mutants of *F. verticillioides* were obtained with 49% of *nit*1, 29% of *nit*3 and 22% of NitM. Isolates of *F. verticillioides* were grouped into 14 VCGs based on complementation tests between NitM of one isolates with *nit1* or *nit3* of other isolates. NitM of isolates Fv1, Fv8, Fv14, Fv28, Fv31, Fv39, Fv41, Fv44, Fv47, Fv49, Fv53 and Fv58 were assigned as testers for 12 VCG groups and Two VCG groups including VCG13 and VCG14 that have only one member, no tester were considered. VCG3 with 14 members (23% of all isolates) was the largest and the most frequent VCG.

Determination of molecular phenotype based on RAPD data

According to the patterns of all seven random decamer primers (Table 2), 19 molecular phenotypes (haplotypes) were determined among 61 isolates of *F. verticillioides* (Table 1).

Haplotype 1(HP1) had the highest frequency (0.57) in the population and was present in isolates from the majority of the locations in this study. Fourteen molecular phenotypes occurred only once in the population. All molecular phenotypes were distributed randomly across the various locations. We didn't found a clear consistent between haplotype of the isolates and their VCGs.

VCG group	Haplotype group	Haplotype (primer OPR11-OPR14- OPR16-UBC196-UBC199-UBC648-UBC682)	Locations	Province (population)	Isolates
VCG1	HP1	1111111	Fasa	Fars	Fv1
VCG3	HP1	1111111	Fasa	Fars	Fv18
VCG3	HP1	1111111	Fasa	Fars	Fv19
VCG3	HP1	1111111	Fasa	Fars	Fv20
VCG3	HP1	1111111	Darab	Fars	Fv14
VCG3	HP1	1111111	Darab	Fars	Fv15
VCG3	HP1	1111111	Darab	Fars	Fv16
VCG3	HP1	1111111	Darab	Fars	Fv17
VCG8	HP14	1112212	Darab	Fars	Fv46
VCG3	HP1	1111111	Marvdasht	Fars	Fv21
VCG3	HP1	1111111	Marvdasht	Fars	Fv22
VCG3	HP1	1111111	Zarghan	Fars	Fv23
VCG3	HP1	1111111	Zarghan	Fars	Fv24
VCG3	HP1	1111111	Zarghan	Fars	Fv25
VCG1	HP1	1111111	Dashte naz	Mazandaran	Fv2
VCG3	HP1	1111111	Dashte naz	Mazandaran	Fv26
VCG3	HP1	1111111	Dashte naz	Mazandaran	Fv27
VCG12	HP19	1121111	Dashte naz	Mazandaran	Fv59
VCG1	HP2	1114131	Gharakheil	Mazandaran	Fv3
VCG1	HP1	1111111	Gharakheil	Mazandaran	Fv4
VCG9	HP1	1111111	Gharakheil	Mazandaran	Fv47
VCG9	HP15	1111112	Gharakheil	Mazandaran	Fv48
VCG1	HP1	1111111	Neyshabur	Khorasan Razavi	Fv5
VCG2	HP2	1114131	Neyshabur	Khorasan Razavi	Fv8
VCG5	HP12	1111211	Mashhad	Khorasan Razavi	Fv35
VCG5	HP12	1111211	Mashhad	Khorasan Razavi	Fv36
VCG5	HP12	1111211	Chenaran	Khorasan Razavi	Fv37
VCG5	HP1	1111111	Chenaran	Khorasan Razavi	Fv38
VCG1	HP1	1111111	Moghan	Ardabil	Fv6
VCG2	HP4	2111411	Moghan	Ardabil	Fv9
VCG5	HP1	1111111	Moghan	Ardabil	Fv31
VCG5	HP9	1141141	Moghan	Ardabil	Fv32
VCG1	HP3	1124131	Moghan	Ardabil	Fv7
VCG2	HP5	2111111	Moghan	Ardabil	Fv10
VCG2	HP1	1111111	Moghan	Ardabil	Fv11
VCG2	HP6	1421111	Moghan	Ardabil	Fv12
VCG2	HP1	1111111	Moghan	Ardabil	Fv13
VCG7	HP1	1111111	Moghan	Ardabil	Fv41
VCG7	HP1	1111111	Moghan	Ardabil	Fv42
VCG7	HP1	1111111	Moghan	Ardabil	Fv43
VCG5	HP10	1112331	Moghan	Ardabil	Fv33
VCG5	HP11	1111131	Moghan	Ardabil	Fv34
VCG11	HP13	1111121	Moghan	Ardabil	Fv52
VCG11	HP1	1111111	Moghan	Ardabil	Fv53
VCG11	HP1	1111111	Moghan	Ardabil	Fv54
VCG11	HP15	1111112	Moghan	Ardabil	Fv55
VCG11	HP18	1111113	Moghan	Ardabil	Fv56
VCG11	HP1	1111111	Moghan	Ardabil	Fv57
VCG4	HP1	1111111	Dezful	Khuzestan	Fv28
VCG6	HP1	1111111	Dezful	Khuzestan	Fv39
VCG6	HP1	1111111	Dezful	Khuzestan	Fv40
VCG10	HP13	1111121	Dezful	Khuzestan	Fv49
VCG4	HP7	1111141	Andimeshk	Khuzestan	Fv29

VCG4	HP8	1111411	Andimeshk	Khuzestan	Fv30
VCG13	HP1	1111111	Andimeshk	Khuzestan	Fv60
VCG14	HP13	1111121	Andimeshk	Khuzestan	Fv61
VCG8	HP1	1111111	Shush	Khuzestan	Fv44
VCG10	HP16	1112231	Shush	Khuzestan	Fv50
VCG10	HP17	1112111	Shush	Khuzestan	Fv51
VCG8	HP13	1111121	Shushtar	Khuzestan	Fv45
VCG12	HP13	1111121	Shushtar	Khuzestan	Fv58

Table 1: Populations, Locations, Haplotypes and VCG groups of the isolates of *Fusarium verticillioides* that were used in this study.

Size range of scorable bands	Polymorphic bands	G+C (%)	5'-Sequence-3'	Primer
300-2500	16	60	5´-CTG CGA CGG T-3´	UBC682
150-2500	19	70	5´-GCA CGC GAG A-3´	UBC648
200-1800	14	80	5´-GCT CCC CCA C -3´	UBC199
200-2000	17	80	5´-CTC CTC CCC C -3´	UBC196
300-3000	14	60	5´- GTA GCC GTC T -3´	OPR11
300-2500	16	60	5´- CAG GAT TCC C -3´	OPR14
150-2000	20	70	5´- CTC TGC GCG T -3´	OPR16

Table 2: Characteristics of RAPD primers that were used in PCR reactions with the isolates of *Fusarium verticillioides* in this study.

Population genetic diversity and differentiation

The evaluation was carried out based on pooled data derived from rep-PCR and RAPD. Nei's analysis [44] of gene diversity estimated gene flow (*Nm*) 1.7624 and *Gst* equal to 0.2210. Genetic diversity for the entire collection (Ht) was 0.2909. A dendrogram based on Nei's [44] genetic distance with the method UPGMA and modified from NEIGHBOR procedure of PHYLIP Version 3.5 was obtained and populations were placed in 5 distinct groups based on geographical origin (Figure 2).

The highest genetic distance observed between Fars and Khuzestan (0.1801) and the smallest genetic distance was obtained between Ardabil and Khuzestan (0.0589) (Table 3).

Results from analysis of molecular variance (AMOVA) showed a significant difference among 5 populations of *F. verticillioides* (Table 4). According to our results PhiPT was equal to 0.176 (p<= 0.001) and 82% of genetic variance occurred within populations and only 18% was found among populations.

Cluster analysis

When subjected to NTSYS-pc version 2/02e with UPGMA cluster analysis and Dice coefficient four clusters (I, II, III and IV) were found on the level of genetic similarity of 41% (Figure 3).

As expected the isolate of *F. proliferatum* was placed separately in the dendrogram. Subdividing of cluster I lead to 6 further groups (A to F). All of the isolates in group A have been originated from the Province Fars only. With one exception all isolates in group B are from Ardabil province. Isolates in group C are from Mazandaran (4 isolates) and Khorasan Razavi (3 isolates) and one isolates has been originated from Khuzestan. Except the isolate Fv20 from Fars other three isolates in group D are from Ardabil. Group E has only one member from Ardabil. Isolates in group F are from Ardabil and Khuzestan that shows the highest genetic identity in Figure 2. Cluster II has isolates from three provinces including Fars, Khuzestan and Ardabil. Isolates in Cluster III compromised isolates from Khorasan and Mazandaran, two populations that shows more genetic identity comparing with other populations. All isolates in Cluster IV are from Ardabil.

We didn't found a clear separation of Haplotype and VCG distribution among populations that evaluated in this study although there are some consistent between geographical origin of the isolates and their genetic similarity. Isolates Fv1 and Fv14 showed the highest similarity (87%) both of them from Fars province but from different location, Fasa and Darab.

Discussion

The results presented here demonstrated that the selected *F. verticillioides* populations from Iran consist of highly genetically diverse isolates indicated by the high level of VCG and Haplotype polymorphism. These results are in agreement with results from other studies on *F. verticillioides*, demonstrating that this fungus is genotypically highly diverse [13,15,36,49].

This may important because pathogen populations with high

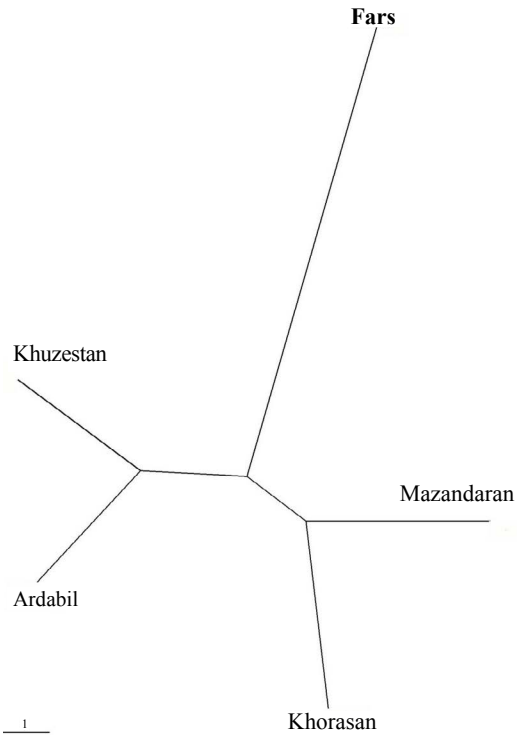

Figure 2: Dendrogram Based on Nei's [44] Genetic distance with the use of UPGMA method among five populations of *Fusarium verticillioides*.

POP ID	Fars	Mazandaran	Khorasan	Ardabil	Khuzestan
Fars	****	0.8783	0.8526	0.9141	0.8352
Mazandaran	0.1298	****	0.9305	0.9251	0.8982
Khorasan	0.1595	0.0721	****	0.9179	0.8769
Ardabil	0.0898	0.0778	0.0857	****	0.9428
Khuzestan	0.1801	0.1074	0.1314	0.0589	****

Table 3: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) between five populations of *Fusarium verticillioides* in this study.

Source	df	SS	MS	Est. Var.	Variation (%)
Among Pops	4	355.704	88.926	5.423	18%
Within Pops	56	1425.148	25.449	25.449	82%
Total	60	1780.852		30.872	100%

*P value=0.001 and number of permutations is 999.

Table 4: AMOVA results for five populations of *Fusarium verticillioides*.

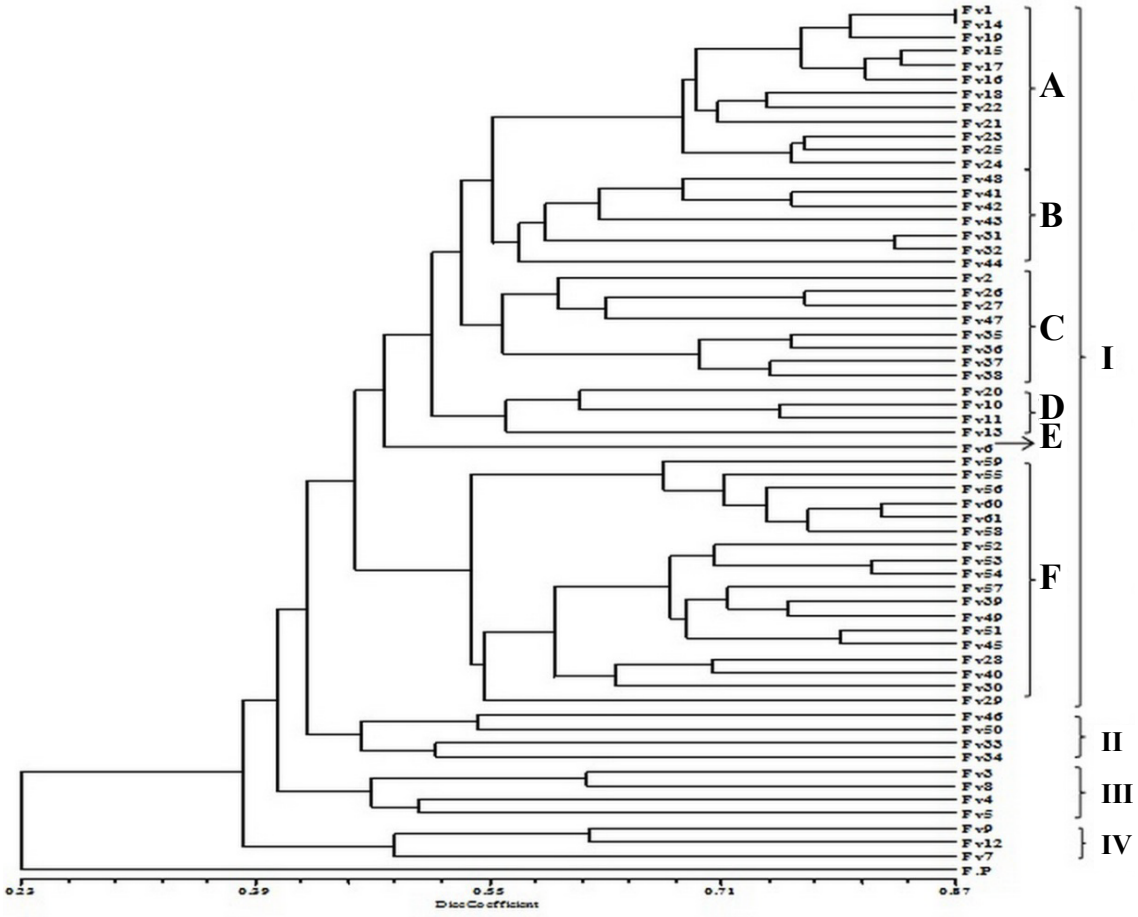

Figure 3: Cluster analysis of *Fusarium verticillioides* isolates based on the data derived from RAPD and rep-PCR reactions using UPGMA methods and Dice coefficient. **I**: Isolates from Fars, Ardabile, Mazandaran, Khorasan Razavi and Khuzestan provinces **II**: Isolates from Fars, Khuzestan and Ardabile provinces **III**: Isolates from Khorasan and Mazandaran provinces **IV**: isolates from Ardabile province.

genetic variation are potentially capable of rapidly evolving responses to changing environmental conditions [50]. A possible explanation for the high levels of genetic diversity found among isolates of *F. verticillioides* could be natural chance mutations, combined with the fact that the fungus can produce abundant numbers of spores in a relatively short period of time. However high levels of genetic variation are usually due to recombination, which occurs sexually through mating or asexually through the parasexual cycle [14]. Since parasexuality is dependent on the formation of a heterokaryon, parasexual recombination occurs only between members of the same VCG [9]. The high degree of VCG polymorphism in this study supports the assumption that the genotypic diversity of *F. verticillioides* is primarily caused by recombination during the sexual state. In spite of sexual state that maintains high level of diversity in the population, asexual reproduction decrease diversity because of selection and genetic drift.

Farrokhi-Nejad and Leslie [51] also found high degree of diversity among isolates of *F. verticillioides* in USA. In a similar investigation extensive variation was detected among isolates of *F. verticillioides* by Huang et al. [14]. Comparing with their results we saw some consistence between geographical separation and genetic clustering. Although this correlation was not perfect and several exceptions were observed. Isolates of Moghan in Ardabil province were scattered among isolates of other populations indicating that the initial inoculums of *F. verticillioides* may be distributed through country with seeds that are mainly produced in Moghan. Seed-borne inoculums might be important for long distance gene flow, but compared to the large fungal population in the soil, its effect on population diversity is probably small [14]. Moghan in Ardabil province is the main site for seed producing in Iran and the seeds that are produced there are distributed over the country. So infected seeds in Moghan are very important for the spreading of fungus through the maize producing areas. In this study isolates of Moghan are seen besides other isolates in different clusters (Figure 3). Spores of this pathogen are distributed by wind between corn fields [52] but long distance distribution is made up mainly by seed infection.

VCGs assignment based on complementation of nitrate non-utilizing (*nit*) mutants results in grouping isolates into 14 VCGs in this study accounting for a genetic diversity (number of VCGs/number of isolates) of 0.23 that was lower comparing with the diversity that was obtained with some other studies [14,51,53]. Most of the isolates in this study have been collected from Moghan where is the hot spot of the disease and is the main corn seed production site in Iran. This can cause low number of VCG groups and haplotypes. Long distance gene flow with seedborne inoculums from Moghan to other provinces/populations can have an effect on low diversity comparing with other studies. Isolates of Ardabil have been distributed through 11 of 19 haplotypes that were achieved in this study. The frequency of *nit1* mutants was higher than the frequency of the other types of *nit* mutants

that is in agreement with the results of Bowden and Leslie [54] and Puhalla [33]. Sectoring frequency of *F. verticilioides* has been shown to be heritable and to vary among isolates [34]. The wide range of sectoring frequency in plant pathogenic fungi on different concentrations of chlorate has also been suggested as a selective advantage for rapid adaptation to environmental stresses such as fungicides and host resistance [35].

VCG groups were distributed among different geographical locations and there was no correlation between geographical distribution, VCG groups and genetic similarity of the isolates. Of 14 VCG groups 6 contain isolates from more than one population (province).

Unlike the VCG assays that are based on only one marker, RAPD uses a numerous markers. Of 19 haplotypes that were obtained among 61 isolates of *F. verticiliioides* (0.31 haplotype per isolate) in our study 15 of them belong to only one VCG indicating that RAPD shows more diversity than VCG assignment.

In consistence with our results, Zamani et al. [55] and Bahmani et al. [24] didn't find a close relationship between VCGs and RAPDs in Iran, but unlike our results Huang et al. [14] found a clear relationship between VCGs and RAPDs data and they found 0.66 haplotype per isolate which is more than the diversity we obtained in this study.

According to Bodker et al. [56], in a population with high VCG diversity, it usually is not possible to correlate VCG to another trait. In contrast, in populations those consist of only a few VCGs, these VCGs can sometimes be correlated to other trait.

Evidence for the usefulness of the RAPD technique for evaluation genetic diversity among isolates of *F. verticillioides* was provided by many researchers [14,57]. Use of rep-PCR with three primers including BOX, ERIC and REP as a molecular marker beside RAPD, increased the reproducibility, specificity and credibility of the results.

Cluster analysis of combined data derived from rep-PCR and RAPD resolved the isolates into four main clusters and six more groups within the first cluster and that all of them were distant from *F. proliferatum* which is included as outlier. Cluster analysis also showed that the maximum similarity among isolates of *F. verticillioides* was approximately 87% and that no identical isolates were detected, indicating that every isolate was a unique genotype. The highest genetic distance observed between Fars and Khuzestan (0.1801) and the smallest genetic distance was obtained between Ardabil and Khuzestan (0.0589) (Table 3).

There are some consistent between geographical origin of the isolates and their genetic similarity, although we didn't found any correlation between haplotypes and geographical distribution of isolates. Furthermore there is no clear clustering of isolates according to VCG groups.

Some of previous studies on *F. verticillioides* have shown no geographic clustering of isolates [14,16,17]. Their results indicated that the various geographical populations are not genetically isolated, which may be due to dissemination of propagules by biotic and abiotic factors. We consider the distribution of corn seeds and vegetative material over a wide geographical area to be another cause of genetic variation of *F. verticillioides* in Iran.

Nineteen haplotypes were identified among the 61 isolates tested. Many researchers have used random amplified polymorphic DNA as a marker for assessing genetic diversity in *F. verticillioides* and some other fungal species [58,59]. Khalil et al. used RAPD analysis combination with pathogenicity assays to study the taxonomy of five *Fusarium* species [38]. Kini et al. used RAPD for determination of genetic variation among *F. verticillioides* isolates that were collected from seeds of different host species [57]. MacDonald and Chapman [21] divided isolates of *F. verticillioides* from Kenya into two subgroups based on RAPDs, with some correlation to the tissue-origin of isolates. Results of this study indicated that isolates of *F. verticillioides* in Iran are genetically diverse populations. One of the strategies for the control of the disease can be limitations of seed movement from the main producing seed production site in Moghan, Ardabil to other corn producing areas and also seed treatment with suitable fungicides.

Acknowledgement

Financial support by Iranian Research Institute of Plant Protection (IRIPP) (to H. M.) is gratefully acknowledged.

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