



**Full Length Article**

## Mating Type Profiling and SSR-Based Genetic Diversity Analysis of Iranian *Fusarium oxysporum* f. sp. *ciceri* Causing Wilt in Chickpea

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### Abstract

Chickpea wilt disease caused by *Fusarium oxysporum* f. sp. *ciceri* (FOC) is the most important soil-borne disease of chickpea in Iran and many other chickpea growing zones worldwide. In this study, a total of 65 FOC isolates have been collected from West of Iran (Kurdistan and Kermanshah provinces) and characterized for pathogenicity, mating type alleles and genetic diversity analysis by genic- and EST-SSR molecular markers. All the FOC isolates showed virulent pattern and 86% of them were moderately virulent (21–50% of wilt damage). Mating type assay using specific PCR-based primers showed that 60% of FOC isolates possess MAT-1 allele and 40% possess MAT-2 allele. Genetic diversity of FOC isolates using 10 SSR markers revealed high genetic diversity within FOC populations with an average of 3 alleles per locus. Cluster analysis divided FOC isolates into four groups. Isolates from Kurdistan were grouped distinctly from those collected from Kermanshah province. Results of present study will be useful for breeders to design effective breeding strategies and developing necessary integrated management for Fusarium wilt in chickpea. © 2020 Friends Science Publishers

**Keywords:** Chickpea; Genetic diversity; *Fusarium* wilt; Mating type

### Introduction

Chickpea (*Cicer arietinum* L.) is an annual food legume and plays important role in human and livestock feed (Talebi *et al.* 2008). West and North-West of Iran (Kermanshah and Kurdistan provinces) are the most important chickpea produces with approximately 400 thousand hectares (Soltani *et al.* 2016). Chickpea grown in all zones in Iran and globally Iran ranked third for its cultivation area, but due to narrow genetic base of chickpea and vulnerability to biotic and abiotic stresses the average seed yield in Iran is very lower than worldwide production (Ahmadi *et al.* 2014; Ghaffari *et al.* 2014; Farahani *et al.* 2019). *Fusarium* wilt, caused by *F. oxysporum* f. sp. *ciceri* (FOC), is the main destructive soil-borne disease in chickpea worldwide and under favorable environmental conditions causing 20–100% seed yield losses (Mohamed *et al.* 2015; Nourollah and Aliaran 2017). FOC disease management is difficult due to long term survival of pathogen in soil and also in infected seeds for years without the present of host plant (Haware *et al.* 1996; Nourollah and Aliaran 2017).

Integrated disease management such as crop rotation, biological control and fungicides application are useful strategies for disease management, but in term of long survival of FOC pathogen the best and effective strategy is the using resistance sources in epidemic regions (Cook *et al.*

2012; Mengist *et al.* 2018). Sex in fungi controlled by dissimilar mating type loci named idiomorphs and plays important role of fungi germplasm diversity. Recombination during sex by dissimilar mating type loci and selection pressure imposed by fungicides enable pathogen to adopt and breakdown the resistance in commercial cultivars (Waalwijk *et al.* 2006; Aghamiri *et al.* 2015). Characterization of pathogen population structure and diversity is needed for effective integrated disease management and designing the best breeding strategies for development of resistant sources.

*F. oxysporum* f. sp. *ciceri* isolates morphology are very similar and difficult for classification and in other hand, race identification needs to differential chickpea cultivars that is time-consuming and mostly the reaction of genotypes to pathogens influenced by environmental parameters (Haware and Nene 1982; Gurjar *et al.* 2009). Therefore, rapid identification of population structure in fungi using molecular markers and specific mating-type markers are very useful and effective (Montakhabi *et al.* 2018). Different molecular markers like RAPD (Jimenez-Gasco *et al.* 2001), ISSR (Barve *et al.* 2001; Montakhabi *et al.* 2018), RFLP (Sharma *et al.* 2009), AFLP (Sivaramakrishnan *et al.* 2002) and SSR (Dubey and Singh 2008; Mohamed *et al.* 2015) have been developed and used for genetic diversity in FOC populations. Earlier workers

reported highly genetic diversity in Iranian FOC isolates by SSR and did not analyze isolates from diverse provinces and also mating type systems. Most of FOC isolates used in this study have been analyzed previously for their morphology and ISSR-based molecular diversity (Montakhabi *et al.* 2018). Therefore, the objectives of present study were (i) study the genetic diversity in Iranian FOC isolates using genic-SSR and EST-SSR markers, (ii) determining the mating type distribution in geographically collected FOC isolates, (iii) considering the possible correlation between genetic diversity, mating type and pathogenicity of FOC isolates with geographical distance.

## Materials and Methods

### FOC isolation and pathogenicity test

Infected chickpea plants were collected from geographically distant chickpea fields from North-West of Iran in two provinces; Kurdistan and Kermanshah. Infected stems that showing vascular discoloration symptoms were washed with distilled water and sterilized with 1% hypochlorite sodium for 2 min.

Sixty-five samples (Table 1) were cut into small pieces and plated on potato dextrose agar (PDA) medium and incubated at 24°C (Mohamed *et al.* 2015; Montakhabi *et al.* 2018). After 10 days single-spore FOC purified and plated on potato dextrose broth (PDB) (potato 200 g, dextrose 20 g, agar 18 g and 1 L water) plates for 10 days. For pathogenicity test, mycelium were harvested and dissolved in sterilized distilled water. Inoculum suspension was adjusted to  $5 \times 10^6$  conidia/ml. For pathogenicity test, a susceptible chickpea cv. Bivanij were grown in perlite in greenhouse. 14-days old seedlings were inoculated using root-inoculated method as described by Pande *et al.* (2007). Two-week-old seedling plants were inoculated using root-inoculated method as described by Pande *et al.* (2007).

Twenty eight days after inoculation, seedlings were scored based on percentage of death plants for each isolates and FOC isolates categorized into four group based on their pathogenicity; (I) avirulent FOC isolates (showed 0% wilt), (II) less virulent (showed 1–20% wilt), (III) moderately virulent (showed 21–50% wilt) and (IV) highly virulent (showed >51% wilt) (Kashyap *et al.* 2016).

### DNA extraction, mating types and SSR markers analysis

FOC isolates were cultured on PDB medium and after 8 days, fungal mycelium harvested and used for DNA extraction using CTAB method as described by Kumar *et al.* (2013). DNA sample were concentration were diluted to 20–30 ng  $\mu\text{L}^{-1}$  for further molecular analysis using SSRs and mating type specific primers.

Mating type of FOC isolates were analyzed by specific PCR-based primers, MAT1-F (5'-GTCGTCGATGGTGATGAAAGAAA-3'), MAT1-R (5'-

CCGCACTGGAGCTCAAATGGT-3'), MAT2-F (5'-GTTGCATCTCCGTCTGCGCCA-3') and MAT2-R (5'-GGCTG CAAGGATGACTGGCAT-3') that have been developed previously by Kashyap *et al.* (2015). PCR amplification was performed in 20  $\mu\text{L}$  reaction containing 1 $\times$  PCR buffer, 20 ng DNA, 4  $\mu\text{M}$  primers, 250  $\mu\text{M}$  of each dNTP, 2 mM  $\text{MgCl}_2$  and 1 unit of Taq DNA polymerase. PCR amplifications were done as follows: initial 95°C for 3 min, followed by 35 cycles of 94°C for 1 min, annealing at 57.5/60.8°C (MAT1/MAT2) for 1 min, extension at 68°C for 1 min and 68 °C for 10 min as final extension.

For molecular diversity a set of ten SSR markers (6 genic-SSR and 4 EST-SSR) were used. These markers have been reported previously for their highly allelic divergence in different FOC populations (Bogale *et al.* 2005; Kumar *et al.* 2013) (Table 2).

The PCR was performed in 20  $\mu\text{L}$  reaction volume containing 1 $\times$  PCR buffer, 15 ng sample DNA, 4  $\mu\text{M}$  primer, 250  $\mu\text{M}$  of each dNTP, 2 mM  $\text{MgCl}_2$  and 1 unit of Taq DNA polymerase. PCR Cycles for SSR were conducted as following: 3 min at 95°C; 32 cycles of 1 min at 94°C, 1 min of annealing temperature, 2 min at 72°C and finally 7 min at 72°C. PCR products of mating type specific primers were resolved on 1.5% agarose gel and SSR primers were resolved on 2.5% metaphoragarose gel.

### Data analysis

FOC isolates mating type determined based on amplification of a 320 bp and 650 bp for MAT-1 and MAT-2 locus, respectively.

PCR products of SSR primers for each FOC were scored as 0 and 1 for absence and present of bands, respectively. Binary matrix of ten SSR markers was used for cluster analysis using UPGMA algorithm by DARwin program package (Perrier and Jacquemoud-Collet 2006). Polymorphic information content (PIC) value of SSR marker and also Analysis of molecular variance (AMOVA) were performed in GenAlex ver.6.5 software

## Results

### Pathogenicity and mating types of the *F. oxysporum* f. sp. *ciceri* Isolates

The virulence and pathogenicity of FOC isolates were tested on susceptible chickpea cultivar cv. Bivanij. FOC isolates showed high variability for their pathogenicity. Out of 65 isolates, only four isolates (FOC9, FOC14, FOC34 and FOC36) were less virulent (1–20% wilt damage) (Table 1). All of these isolates belonged to Kurdistan province. Five isolates (FOC2, FOC3, FOC18, FOC20 and FOC33) showed highly virulent pattern (>51% wilt damage) were from Kermanshah province. Remaining 56 FOC isolates belonged to moderately virulent group (21–50% wilt damage) (Table 1).

**Table 1:** Description of 65 Iranian isolates of *F. oxysporum* f. sp. *ciceri* for their collection sites, pathogenicity level and mating type alleles

FOC ID	Province	Pathogenicity level	Mating type	FOC ID	Province	Pathogenicity level	Mating type
FOC1	Kermanshah	moderately virulent	MAT-2	FOC36	Kurdistan	less virulent	MAT-1
FOC2	Kermanshah	highly virulent	MAT-1	FOC37	Kurdistan	moderately virulent	MAT-1
FOC3	Kermanshah	highly virulent	MAT-2	FOC38	Kurdistan	moderately virulent	MAT-1
FOC4	Kermanshah	moderately virulent	MAT-1	FOC39	Kurdistan	moderately virulent	MAT-1
FOC5	Kermanshah	moderately virulent	MAT-2	FOC40	Kurdistan	moderately virulent	MAT-1
FOC6	Kermanshah	moderately virulent	MAT-2	FOC41	Kurdistan	moderately virulent	MAT-1
FOC7	Kermanshah	moderately virulent	MAT-2	FOC42	Kurdistan	moderately virulent	MAT-2
FOC8	Kurdistan	moderately virulent	MAT-1	FOC43	Kurdistan	moderately virulent	MAT-1
FOC9	Kurdistan	less virulent	MAT-1	FOC44	Kurdistan	moderately virulent	MAT-1
FOC10	Kurdistan	moderately virulent	MAT-1	FOC45	Kurdistan	moderately virulent	MAT-1
FOC11	Kurdistan	moderately virulent	MAT-1	FOC46	Kermanshah	moderately virulent	MAT-2
FOC12	Kurdistan	moderately virulent	MAT-2	FOC47	Kermanshah	moderately virulent	MAT-1
FOC13	Kurdistan	moderately virulent	MAT-1	FOC48	Kermanshah	moderately virulent	MAT-2
FOC14	Kurdistan	less virulent	MAT-1	FOC49	Kermanshah	moderately virulent	MAT-2
FOC15	Kurdistan	moderately virulent	MAT-2	FOC50	Kermanshah	moderately virulent	MAT-1
FOC16	Kurdistan	moderately virulent	MAT-1	FOC51	Kermanshah	moderately virulent	MAT-1
FOC17	Kermanshah	moderately virulent	MAT-1	FOC52	Kermanshah	moderately virulent	MAT-1
FOC18	Kermanshah	highly virulent	MAT-1	FOC53	Kermanshah	moderately virulent	MAT-2
FOC19	Kermanshah	moderately virulent	MAT-1	FOC54	Kermanshah	moderately virulent	MAT-2
FOC20	Kermanshah	highly virulent	MAT-2	FOC55	Kermanshah	moderately virulent	MAT-1
FOC21	Kermanshah	moderately virulent	MAT-1	FOC56	Kermanshah	moderately virulent	MAT-1
FOC22	Kermanshah	moderately virulent	MAT-2	FOC57	Kermanshah	moderately virulent	MAT-1
FOC23	Kermanshah	moderately virulent	MAT-1	FOC58	Kermanshah	moderately virulent	MAT-2
FOC24	Kermanshah	moderately virulent	MAT-1	FOC59	Kermanshah	moderately virulent	MAT-2
FOC25	Kermanshah	moderately virulent	MAT-2	FOC60	Kermanshah	moderately virulent	MAT-1
FOC26	Kermanshah	moderately virulent	MAT-1	FOC61	Kermanshah	moderately virulent	MAT-2
FOC27	Kermanshah	moderately virulent	MAT-2	FOC62	Kermanshah	moderately virulent	MAT-1
FOC28	Kermanshah	moderately virulent	MAT-1	FOC63	Kermanshah	moderately virulent	MAT-2
FOC29	Kermanshah	moderately virulent	MAT-1	FOC64	Kermanshah	moderately virulent	MAT-2
FOC30	Kermanshah	moderately virulent	MAT-2	FOC65	Kermanshah	moderately virulent	MAT-1
FOC31	Kermanshah	moderately virulent	MAT-2				
FOC32	Kermanshah	moderately virulent	MAT-1				
FOC33	Kermanshah	highly virulent	MAT-2				
FOC34	Kurdistan	less virulent	MAT-1				
FOC35	Kurdistan	moderately virulent	MAT-2				

**Table 2:** SSR markers used for the genetic diversity study of *F. oxysporum* f. sp. *ciceri* isolates

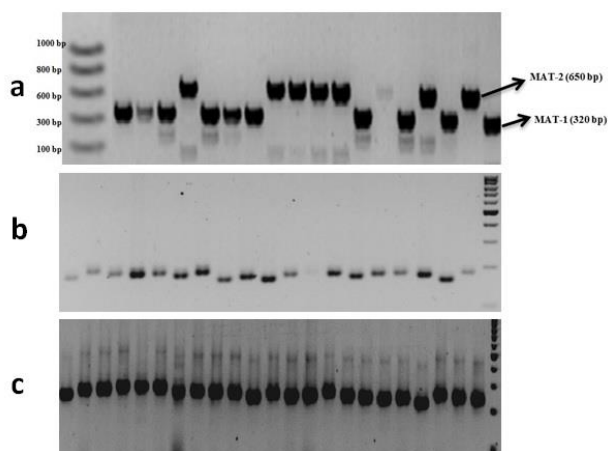
	Locus	Primer sequence (5'-3')	Tm (°C)	No. of Alleles	PIC	MI
Genic-SSR	MB2	F: TGCTGTGTATGGATGGATGG	57	4	0.43	1.72
		R: CATGGTCGATAGCTTGTCTCAG				
	MB5	F: ACTTGGAGGAAATGGGCTTC	54	3	0.35	1.05
		R: GGATGGCGTTTAATAAATCTGG				
	MB11	F: GTGGACGAACACCTGCATC	60	3	0.33	0.99
		R: AGATCCTCCACCTCCACCTC				
	MB14	F: CGTCTCTGAACCACCTTCATC	60	3	0.37	1.11
		R: TTCCTCCGTCCATCCTGAC				
MB17	F: ACTGATTCACCGATCCTTGG	60	4	0.51	2.04	
	R: GCTGGCCTGACTTGTATCG					
MB18	F: GGTAGGAAATGACGAAGCTGAC	55	4	0.64	2.56	
	R: TGAGCACTCTAGCACTCCAAAC					
EST-SSR	FOL2	F: CTCGCATACTACTACCGCACAG	58	2	0.31	0.62
		R: GCAGATAAGGGAGATGCAAAAC				
	FOL4	F: CCAGTCAATCCAACCCTACTT	56	2	0.29	0.58
		R: AGGCTTATCTGCGTCAGTTTCT				
	FOL5	F: ACCTAACTCTTGGAGGACGAT	57	2	0.34	1.29
R: CTGC ATAGCCTTGGTTGTGTGA						
FOL7	F: CAAGTC AGC AACCAACT	58	3	0.43		
		R: GTCCTCCATTCTTCTACCACC				

Specific PCR-based primers were used for determining mating types (MAT-1 and MAT-2) in 65 FOC isolates. Both mating types specific gene amplified in collected isolates. A 320 bp amplicon from MAT-1 gene was obtained in 39 isolates. Similarly, a 650 bp amplicon from MAT-1 gene

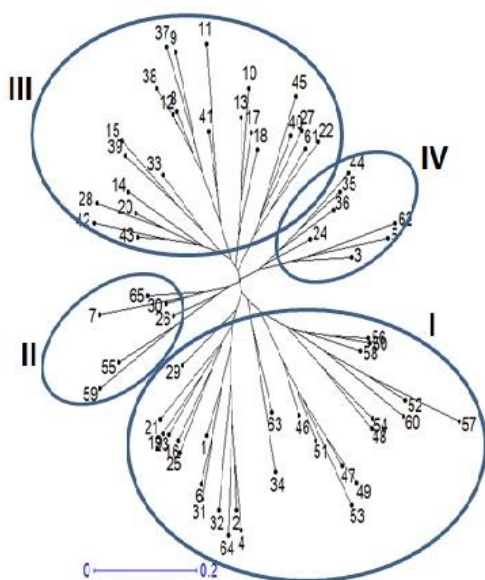
observed in 26 FOC isolates (Fig. 1). Interestingly, among 21 FOC isolates collected from Kurdistan province, only 4 isolate showed MAT-2 gene, while FOC isolates from Kermanshah province showed both MAT genes, although the frequency of MAT-1 was higher than MAT-2 (Table 1).

**Table 3:** Analysis of molecular variance (AMOVA) within and between FOC populations based on SSR markers

SOV	df	SS	MS	% of variation	P value
Among populations	1	42.17	42.17**	6.5	0.01
Within populations	63	183.18	2.90	93.5	
Total	64	225.35	3.52	100	



**Fig. 1:** Amplification profile obtained with mating type specific marker (a), genic-SSR (b), EST-SSR (c) markers in *F. oxysporum* f. sp. *ciceri* isolates



**Fig. 2:** Neighbor joining (NJ) phylogenetic tree using SSR molecular data in 65 *F. oxysporum* f. sp. *ciceri* isolates

**Microsatellite markers analysis and diversity pattern of FOC isolates**

Ten SSR markers comprised six genic-SSRs and four EST-SSRs were used for genetic diversity analysis in 65 FOC isolates. A total of 30 alleles (21 by genic-SSRs and 9 by EST-SSRs) were amplified across all FOC isolates with

average of 3 alleles per locus (Table 2). Polymorphism information content (PIC) of SSR markers ranged from 0.29 (FOL4) to 0.64 (MB18) with an average value of 0.40. Marker index ranged from 0.58 to 2.56, with an average value of 1.26. Cluster analysis based on SSR markers, grouped 65 FOC isolates into 4 groups (Fig. 2). Cluster I comprised 28 FOC isolates, which all of them collected from Kermanshah province. Cluster II contained 6 FOC isolates that all from Kermanshah. Cluster III comprised 24 isolates, which divided into two sub-clusters. Sub-cluster I comprised FOC isolates collected from Kurdistan province and another sub-cluster contained four FOC isolates from Kurdistan and 6 isolates from Kermanshah province. Cluster IV, contained 7 FOC isolates from both Kermanshah and Kurdistan provinces (Fig. 2).

The AMOVA analysis based on SSR markers data showed 6.5% of the variation among populations and 93.5% between populations (Table 3). In general, our results showed relatively clear pattern of diversity between isolates according to their geographical collection site, that suggest the impact of environmental conditions on population genetics on FOC isolates.

**Discussion**

FOC is known as highly variable fungi in morphology, virulence ability that consists of different races and pathotypes (Jendoubi *et al.* 2017). Study of genetic diversity of pathogens is critical for effective management of disease, selection of resistant chickpea sources and development of resistant cultivars, especially if isolates are collected from various agro-climatic zones (Gurjar *et al.* 2009). In this context, pathogenicity and molecular markers viz. genic-SSR, EST-SSR and mating type locus were employed for genetic diversity in 65 Iranian FOC isolates that collected from West of Iran (Kermanshah and Kurdistan provinces). All FOC isolates in this study showed virulent pattern on susceptible chickpea cv. Bivanij. Four and five isolates showed less and highly virulence, respectively. Remaining isolates showed moderately virulent (21–50% wilt damage). Previously, most of isolates used in this study, has been characterized for their morphological characteristics like as colony color and clamydospore position (Montakhabi *et al.* 2018) and based on present results there is no significant correlation between geographically distribution of these isolates with virulence pattern and morphological attributes.

Pathogenicity and fitness of fungi may be influenced by mating types (Arie *et al.* 2000). In FOC fungi the mating types are controlled by two alleles in a locus (MAT-1 and MAT-2) related to alpha box domain and HMG box domain, respectively (Cepni *et al.* 2013). Our results for mating type characterization using specific PCR-based primers showed relatively equal distribution of both alleles (60% MAT-1 and 40% MAT-2) in FOC isolates without positive correlation with collection origins, which is agreement with previous reports for distribution of mating

types in different *Fusarium* species from different countries (Irzykowska *et al.* 2013; Kashyap *et al.* 2015, 2016). None of Isolates showed both MAT alleles, which can be concluded that FOC has a heterothallic origin and previous studies supported the hypothesis for sporadic and cryptic sexual cycle in *Fusarium* species (Taylor *et al.* 1999; Kashyap *et al.* 2016). Similar results have been reported for un-equal distribution of MAT alleles for *F. oxysporum* isolates in different crop species like as common bean (Karimian *et al.* 2010). Although the maximum effective reproductive occurred when 1:1 ration observed in mating type idiomorphes, but it seems that MAT-1 is a predominant mating type in the FOC population in Iran, even a larger population from diverse geographical regions needed to be analyzed before making a final decision.

Ten SSR markers used in this study showed relatively high allelic variation in FOC isolates. The polymorphism of SSR markers showed 2 to 4 alleles with an average 3 alleles per locus. The average PIC value for SSR markers ranged from 0.29 to 0.64 with an average value of 0.40. Highly PIC value of SSR marker types revealed in this study showed the diverse nature of collected FOC isolates and was comparable with previous studies using SSR markers in different *Fusarium* species (Mahfooz *et al.* 2012; Kumar *et al.* 2012, 2013).

Cluster analysis based on SSR markers, grouped 65 FOC isolates into 4 groups. In general, FOC isolates from Kurdistan provinces grouped distinctly from those collected from Kermanshah. Our results showed that over 93.5% of genetic diversity was distributed within populations and isolates from close geographical distance. This highly genetic similarity of FOC isolates may be concluded by gene flow due to movement of conidia by contaminated seeds or infected plant debris from short distances geographical regions.

This is in agreement with previous reports stated that FOC is a monophyletic group which is derived from small size population or single individual and somatic recombination occurred through parasexuality (Jimenez-Gasco *et al.* 2001). Although, high degree of pathogenic and genetic diversity in FOC as a asexual fungi may occur by accumulation of mutations over time (Jendoubi *et al.* 2017; Nourollah and Aliaran 2017).

In this study, pathogenicity test for identification of FOC races are not used, because it can be influenced by environmental factors and inoculum density of pathogens (Navas-Cortes *et al.* 2007; Kashyap *et al.* 2016). Therefore, characterization of FOC isolates using both SSR markers and mating type specific markers enable us to effectively detect the level of genetic diversity in collected FOC isolates. This information may provide insights into the evolutionary processes of FOC isolates caused by migration, gene flow between populations and epidemiology of pathogen. The results are also very useful for developing integrated strategies for disease management and drawing effective breeding programs for stable and effective

resistance against FOC in growing chickpea zones from West of Iran.

## Conclusion

The present study showed high genetic diversity in Iranian FOC populations. Moreover, to the best of our knowledge, the present study provides first report regarding to genetic diversity of FOC isolates from West of Iran using EST-SSR and mating type specific primers. Diversity analyses carried out using genic- and EST-SSR markers grouped the isolates into three clusters. High proportion of diversity was among isolates and high similarity was observed between populations from distinct geographical regions, which are valuable information for FOC pathogen population. Therefore, this high similarity between populations can be concluded that gene flow may occur across long distances by distribution of infected seed. These results will help breeder to choose strategies for regional breeding programs for developing FOC resistance chickpea cultivars and prevent to introduction of more diverse isolates into these populations and prevent transmission any isolates from this area to other regions of the country.

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