Journal of Agricultural Technology 2011 Vol. 7(6): 1809-1822
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

The genetic diversity of Iranian isolates causing fusarium wilt of Lentil

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N. Mohammadi, E. Mohammadi Goltapeh, H. Kari Dolatabadi, A. Babaie Ahari and H. Pouralibaba (2011) The genetic diversity of Iranian isolates causing fusarium wilt of Lentil. Journal of Agricultural Technology 7(6): 1809-1822.

Fusarium wilt of lentil is one of the most important diseases which cause severe damages annually. In order to study the genetic diversity of Iranian isolates of *Fusarium oxysporum* f. sp. *lentis* (Fol), forty five isolates of the pathogen were isolated from wilted lentil plants from different lentil growing areas of Iran and two isolates were collected from International Center for Agricultural Research in the Dry Areas (ICARDA), Damascus, Syria. The analysis of banding patterns resulting from RAPD PCR amplifications showed that *Fol* isolates could be differentiated into 10 groups at 64% similarity level well in accordance with their geographic origins. Studying with ISSR technique, all six primers used indicated the diversity of the isolates and differentiated them into six groups at 74% similarity level. It could partially separate isolates based on their geographical origins. Using the MXCOPH algorithm, high cophenetic correlation values for RAPD and ISSR data (0.883 and 0.918, respectively) were obtained. Both analyses were found suitable for the evaluation of genetic polymorphisms in fungal populations. Also the correlation value (r= 0.911) between cophenetic matrix values obtained from these analyses suggested a very significant correlation between RAPD and ISSR dendrograms.

Key words: RAPD, ISSR, *Lens culinaris*, Fusarium wilt, Genetic diversity, Iran

Introduction

Fusarium wilt of lentil caused by *Fusarium oxysporum* Schlecht. emend. Snyder and Hansen f. sp. *lentis* (*Fol*) Vasudeva and Srinivasan is a major restriction to lentil production world-wide (Beniwal *et al*. 1993; Tosi and Cappelli, 2001). For the first time in 1952 the *Fusarium orthoceras* var. *lentis* was reported as a pathogen of lentil. Fusarium wilt of lentil is prevalent in

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many lentil growing countries, including the USA, India, Japan, the Netherlands, Hungary, Jordan, Nepal, Sudan, Syria and Turkey (Allen and Lenne, 1998). Fusarium wilt usually occurs near to or at the reproductive stage of crop development, and its symptoms include wilting of top leaves resembling water deficiency, stunting of plants, shrinking and curling of leaves from the lower part of the plants that progressively move up the stems of the infected plants. Plants finally become completely yellow and die. Root symptoms include reduced growth with marked brown discoloration of vascular tissues, tap root tips that are damaged, and the proliferation of secondary roots above the area of tap root injury. The discoloration of vascular tissues in the lower stem may always not be visible. Main symptoms at the seedling stage include seed rot and sudden drooping more like wilting and damping off (Khare, 1980). Determination of genetic diversity in *Fol* population is important in breeding for resistance and can facilitate investigation on its epidemiology, taxonomy and detection (Milgroom and Fry, 1997). Assessment of genetic diversity of *Fol* is needed to determine whether isolates constitute genetically distinct groups. Random Amplified Polymorphic DNA (RAPD) is a semi-quantitative method used in genetic mapping, taxonomy and phylogeny (Welsh and McClelland, 1990; Bassam *et al*. 1992). RAPD markers generated with single primers of arbitrary nucleotide sequence have been used in the detection of intraspecific polymorphisms of fungi (Assigbetse *et al.* 1994; Belabid *et al*. 2004).

In RAPD method, random primers of approximately 10 nucleotides are used in a genome-wide amplification. Several RAPD primers have been developed but for each particular species, a number of oligonucleotides are tested and those that provide the best variability among independent isolates are selected (Soll, 2000). This method has successfully been applied with *Candida albicans, Aspergillus flavus, Histoplasma capsulatum* and *Blastomyces dermatidis* (Bart-Delabesse *et al*., 1995; Buffington *et al*., 1994; Kersulyte *et al*., 1992; Yates *et al*., 1995). Microsatellites, a term for another molecular marker was first used by Litt and Luty (1989). Another technique that uses the primers designed based on the repeated simple sequences (microsatellites), ISSR makes the selective amplification of genomic DNA feasible, simultaneously provides information about many loci, and has been successfully used to identify variability within fungal species (Zeze *et al*., 1997). Molecular markers have technical differences in term of costs, speed, degrees of the polymorphism revealed, precision of genetic distance surveys and the statistical potency of tests. The purposes of this study were to identify the genetic variability in Iranian *Fol* population through RAPD and ISSR analyses, to compare the level of information provided by these markers for

estimating the genetic diversity of *Fol*, and to know which marker is appropriate to estimate genetic diversity.

Materials and methods

Fungal isolates

Forty five isolates of *Fusarium oxysporum* f. sp. *lentis* were isolated from wilted lentil plants with wilt symptoms in different regions of Iran during 2008–2009 (Fig.1).

Fig 1. *F. oxysporum* f. sp. *lentis* isolates collected from diverse agro-ecological regions of Iran including (1) Ardebil, (2) East Azerbaijan, (3) West Azerbaijan, (4) North Khorasan, (5) Khorasan Razavi, (6) South Khorasan, and (7) Ilam Province.

In addition, two isolates of *Fol* were kindly provided by ICARDA. The fungus *F. oxysporum* f. sp. *lentis* was isolated from stem and crown of wilted lentils and identified according to the key for the identification of *Fusarium* species (Nelson *et al*., 1983). Each of the identified isolates were sub-cultured with a single spore and stored for a short period of time on SNA (Special Nutrient Agar), however for long period storage, the single spore cultures were transferred into the test tubes containing sterile sand and were stored at 4°C. Details on the isolates have been presented in table 1. All *F. oxysporum* f. sp. *lentis* isolates were deposited in Department of Plant Pathology Type Culture Collection, School of Agriculture, Tarbiat Modares University, Tehran, Iran.

Table1. Isolates of *Fusarium oxysporum* f. sp. *lentis* from different geographical regions of Iran

No.	Isolate	Origin	State	No.	Isolate	Origin	State
1	Fol1	Goytapeh	Ardebil	25	Fol25	Varzeghan	E. Azerbaijan
2	Fol2	Goytapeh	Ardebil	26	Fol26	Dizaj	E. Azerbaijan
3	Fol3	Goytapeh	Ardebil	27	Fol27	Kigal	E. Azerbaijan
4	Fol4	Jafarabad	Ardebil	28	Fol28	Dizaj	E. Azerbaijan
5	Fol5	Ruhkandi	Ardebil	29	Fol29	Agbolagh	E. Azerbaijan
6	Fol6	Goytapeh	Ardebil	30	Fol30	Varzeghan	E. Azerbaijan
7	Fol7	Goytapeh	Ardebil	31	Fol31	Tokhomdel	E. Azerbaijan
8	Fol8	Bilesavar	Ardebil	32	Fol32	Varzeghan	E. Azerbaijan
9	Fol9	Goytapeh	Ardebil	33	Fol33	Kigal	E. Azerbaijan
10	Fol10	Ruhkandi	Ardebil	34	Fol34	Dizaj	E. Azerbaijan
11	Fol11	Ruhkandi	Ardebil	35	Fol35	Dizaj	E. Azerbaijan
12	Fol12	Goytapeh	Ardebil	36	Fol36	Dizaj	E. Azerbaijan
13	Fol13	Ptilgan	Ardebil	37	Fol37	Ahar	E. Azerbaijan
14	Fol14	Bilesavar	Ardebil	38	Fol38	Ahar	E. Azerbaijan
15	Fol15	Goytapeh	Ardebil	39	Fol39	Ahar	E. Azerbaijan
16	Fol16	Ilam	Ilm	40	Fol40	Ahar	E. Azerbaijan
17	Fol17	Ilam	Ilam	41	Fol41	Ahar	E. Azerbaijan
18	Fol18	Ilam	Ilam	42	Fol42	Khorasan	Khorasan
19	Fol19	Ilam	Ilam	43	Fol43	Khorasan	Khorasan
20	Fol20	Ilam	Ilam	44	Fol44	Khorasan	Khorasan
21	Fol21	ICARDA	Syria	45	Fol45	Khorasan	Khorasan
22	Fol22	ICARDA	Syria	46	Fol46	Hashtrud	E. Azerbaijan
23	Fol 23	Oshnavie	W.Azerbaijan	47	Fol47	Hashtrud	E. Azerbaijan
24	Fol24	Kigal	E. Azerbaijan				

DNA extraction

Sterile potato dextrose broth (PDB) medium was inoculated with a bit of the fungus *Fol* growth from a fresh culture. The inoculated broth media were incubated on a shaker (120 rpm) at room temperature (25± 1˚C) for 4 days. Then they were placed under florescent light at 25±2˚C with alternation of 12 / 12 hours light / darkness for 10 days. DNA was extracted by the cetyl trimethyle ammonium bromide (CTAB) method (Doyle and Doyle, 1990) with a minor modification. DNA was amplified using a thermocycler (Mastercycler, Eppendorf AG, Germany). The quality of the extracted DNA was visually checked on 1.5% agarose gels.

RAPD-PCR

The protocol of Balmas *et al*. (2005) was employed. PCR reaction was performed in a thermocycler (Mastercycler, Eppendorf AG, Germany). RAPD-PCR was performed in the total volume of 25 µl of reaction containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.32 µM primer, 1.5 mM $MgCl_2$, 0.125 mM dNTPs, and 0.6 U Taq polymerase (MBI, Fermantas, Germany). The mixtures were subjected to the following conditions: initial denaturation step (95ºC, 1 min), followed by 40 cycles of denaturing (94ºC, 1 min), annealing (37ºC, 38ºC, 39ºC, and 40ºC; 1 min) for different primers (Table 2), extension (72˚C, 2 min) and a final elongation step (72ºC, 5 min).

Table 2. List of RAPD primers, annealing temperatures and the resulted DNA polymorphisms used to differentiate isolates of *Fusarium oxysporum* f. sp. *lentis*

Primer	Sequence	Anneling temperature°C	Polymorphism (%)	No. Amplified Fragments
238	5′– CTGTCCAGCA -3'	39	94	17
241	5′– GCCCGACGCG -3'	38	95	20
230	5′– CGTCGCCCAT - 3'	40	94	17
OPA13	5′– CAGCACCCAC -3'	37	96	23
OPA02	5′–TGCCGAGCTG-3'	39	86	14
RCO09	5′–GATAACGCAC-3'	38	100	17
OPA04	5'–AATCGGGCTG-3'	39	96	27
ROTH20	5'–GTTGCGATCC-3'	38	90	21

ISSR

The protocol (Meyer *et al*. 1993) was applied. PCR reactions were performed using an Eppendorf Gradient (Germany) thermocycler. ISSR-PCR was performed in a total volume of 25 µl of the reaction containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.5 mM MgCl2, 0.24 µM primer, 0.2 mM dNTPs, and 1 U Taq polymerase (MBI, Fermantas, Germany). Amplification was performed as follow: initial denaturation (95ºC, 5 min) followed by 40 cycles of denaturing (94ºC, 30s), annealing (60ºC, 59ºC, 58ºC , 54ºC, 52ºC and 57ºC) for different primers (Table 3), 1 min, extension (72ºC, 30s) and a final extension step (72ºC, 6 min). The products of PCR amplification were separated by horizontal gel electrophoresis in 1.5% agarose gel prepared with 1x TBE buffer (0.1 M tris, 0.05M boric acid and 0.001M ethylene diamine tetra acetate (EDTA)) and run with the electric potential difference of 80 V for 180 min and subsequently stained with ethidium bromide solution 0.5mg·ml^{-} and photographed with Gel Doc under UV translaminator. Lourmat T-5×20-2A. A

1kb ladder (Gene Ruler^{TM}, Fermantas, France) was used as a molecular size standard ruler. All PCR assays were repeated at least twice.

Table 3. List of ISSR primers, annealing temperatures and resulting DNA polymorphisms applied to differentiate isolates of *Fusarium oxysporum* f. sp. *lentis*

Primer	Sequence	TA(°C)	Polymorphism (%)	No. Amplified Fragments
$(CAG)_5$	5' - CAGCAGCAGCAGCAG - 3'	58	93	15
$(GAC)_5$	5' - GACGACGACGACGAC - 3'	60	90	10
$(GTG)_5$	5'- GTGGTGGTGGTGGTG - 3	64	88	17
PCMS	5'-GTCGTCGTCGTCGTCGTCGTC-3'	57	86	7
ISSR02	5'-ACTGACTGACTGACTG-3'	52	50	6
ISSR10	5'-CACCACCACCACCAC-3'	54	93	15

Data analysis

With RAPD or ISSR studies, each band of a determined molecular weight was scored with code for the presence of a given band (1) or another code for the absence of a given band (0). A binary matrix combined all the data records for all isolates. The numerical taxonomic software package NTSYS-pc, version 2.0 (Rohlf, 2000) was used to order the isolates by un-weighed paired group method with arithmetic averages (UPGMA) using the Sequential Agglomerative Hierarchical Nested (SAHN) cluster analysis module (Rohlf, 2000) based on Dice's coefficient of similarity. The Mantel test (Mantel, 1967) was used to analyze the correlations between the similarity matrices based on different methods. This test compares the elements of two matrices and estimates the degree of the correlation between them by means of a product-moment correlation (r). Mantel tests were made using by the NTSYS-pc package (Rohlf, 2000). The information content of each marker system was estimated for each marker and locus using the Polymorphism Information Content (PIC) (Lynch and Walsh, 1998) which provides an estimate of the distinguishing power of a *locus* by taking into accounts not only the number of the alleles expressed and their relative frequencies. Calculations were carried out following the formula below:

$$\text{PIC} = 1 - \sum_{i=1}^{n} (P_i)^2$$

Where P_i is the frequency of the i^{th} allele (Lynche and Walsh, 1998), effective multiplex ratio (EMR) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay. And marker index (MI) is defined as the product of average diversity index for polymorphic

bands in any assay and the EMR for that assay. MI was calculated according to Powell *et al*. (1996).

Results

RAPD-PCR Analysis

To assess the genetic diversity in *F. oxysporum* f. sp. *lentis*, 47 isolates were analyzed (Table 1). PCR amplification with eight selected primers resulted in the production of 156 different RAPD fragments as revealed through UPGMA analysis. The number of fragments was variable ranging from 14 to 23 fragments per profile. The size of fragments ranged from 100 to 4000 bp dependant on the primers used and the isolate (Fig. 2). The analysis showed that *F. oxysporum* f.sp. *lentis* isolates could be differentiated into 10 groups at 64 % similarity according to geographic origins (Fig. 3). Group I included 15 isolates from Ardebil; Cluster II consisted three isolates from East Azerbaijan (E. Az); cluster III contained all isolates from Ilam; cluster IV entailed one isolate from West Azerbaijan and two isolate from E. Az (Zolbin); Cluster V included all isolates from E. Az (Ahar); Cluster VI contained four isolates from E. Az; cluster VII included four isolates from E. Az; Cluster VII also entailed two isolates from E. Az (Dizaj village); Cluster IX included four isolates from Khorasan; Cluster X consisted two isolates from ICARDA.

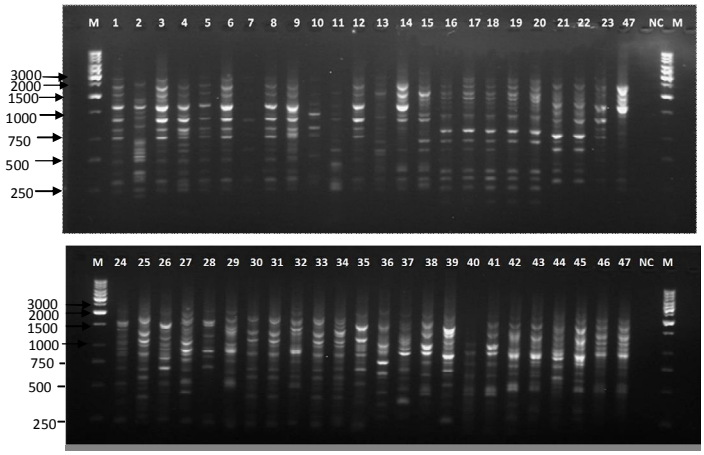

Fig2. PCR fingerprintig patterns from genomic DNA of *F. oxysporum* f.sp. *lentis* from different geograhic regions of Iran with primer OPA-04.

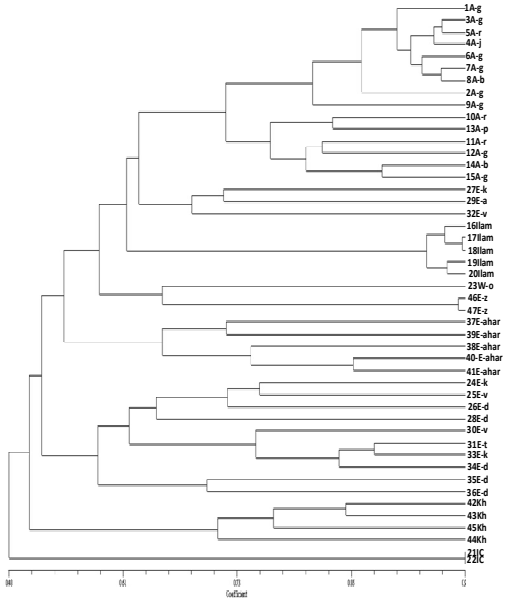

Fig 3. Dendrogram derived from cluster analysis (UPGMA) showing relationship among the 47 *Fusarium oxysporum* f. sp. *lentis* isolates listed in Table 1. Genetic similarity was obtained based on RAPD markers and Dice's similarity coefficient.

ISSR analysis

PCR amplification using six selected primers resulted in the production of 70 different ISSR fragments. The number of fragments was variable, ranging from 6 to 17 in each profile. The size of fragments ranged from 150 to 3500 bp dependant combination of primer- isolates under study (Fig. 4). The analysis showed that *F. oxysporum* f.sp. *lentis* isolates could be differentiated into six groups at 74% similarity (Fig. 5). Cluster I that included 32 isolates from Ardebil (15), one isolate from West Azerbaijan, all isolates from Ilam, Khorasan, and seven isolates from different regions of East Azerbaijan. As the greatest group of isolates, this cluster included 68.08% of all isolates; Cluster II contained three isolates from East Azerbaijan (Ahar) except from two isolates of Ahar grouped in Cluster 1; Cluster III entailed two isolates from ICARDA; cluster IV consisted five isolates from East Azerbaijan; Cluster V included five isolates from E. Az., and cluster VI contained two isolates from East Azerbaijan (Dizaj village).

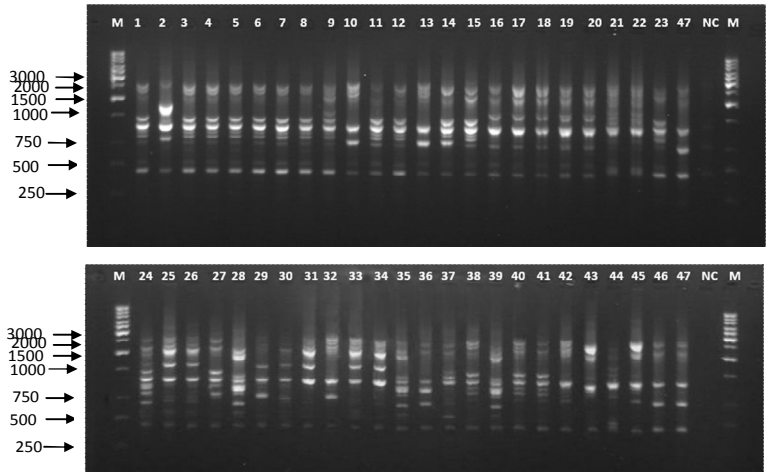

Fig 4. PCR fingerprints of the genomic DNA of *F. oxysporum* f.sp. *lentis* isolates from different geographic regions of Iran with primer PCMS.

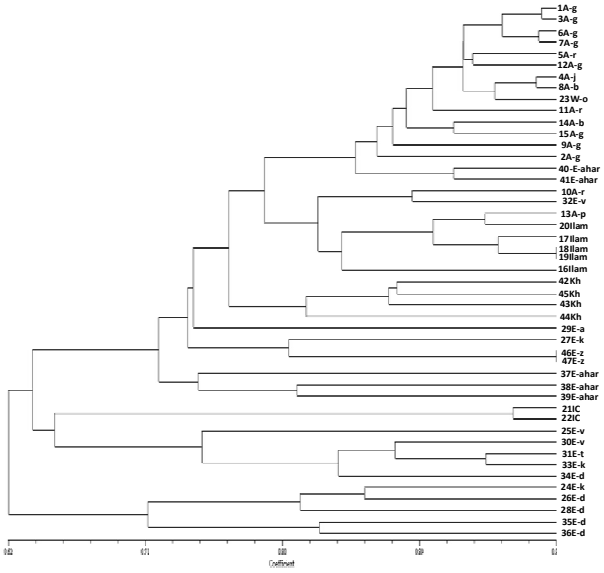

Fig 5. Dendrogram derived from cluster analysis (UPGMA) showing relationship among the 47 *Fusarium oxysporum* f. sp. *lentis* isolates listed in Table 1. Genetic similarity was obtained by ISSR marker based on Dice's similarity coefficient.

Joint analysis of ISSR and RAPD fingerprints

The dendrogram obtained through joint analysis of ISSR and RAPD fingerprints indicated *F. oxysporum* f. sp. *lentis* isolates could be differentiated

into eight groups at 66% similarity according to their geographic origins (Fig. 6). Cluster I included 24 isolates, from Ardebil (15) and West Azerbaijan (1), Ilam (5) and East Azerbaijan (3); Cluster II contained two isolates from East Azerbaijan (Zolbin) with different climate conditions; cluster III contained five isolates from Ahar; cluster IV consisted four isolates of Khorasan); Cluster V included four isolates from E. Az; cluster VI contained two isolates from E. Az. (dizaj); Cluster VII included four isolates from E. Az., and cluster VIII intailed two isolates from ICARDA.

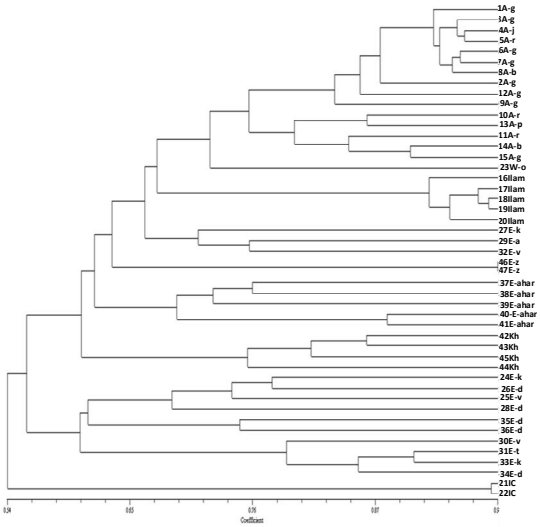

Fig 6. Dendrogram derived from cluster analysis (UPGMA) showing the relationships among 47 *Fusarium oxysporum* f. sp. *lentis* isolates listed in Table 1. Genetic similarity was obtained by ISSR and RAPD markers using Dice's similarity coefficient

Comparison of RAPD and ISSR markers

Both of the PCR techniques differed in the degree of polymorphism detected. Moreover, the polymorphic bands varied for each of the marker systems including 6 primers for ISSR, and 8 primers of RAPD. As a result, the total number of bands scored for the each marker varied. RAPD primers produced a total of 156 amplification products (19 bands/ primer) of which 147 were polymorphic (94%) and 9 remaining bands were monomorphic. With ISSR primers, 70 bands were revealed out of them 61 bands (83%) were polymorphic (Table 6). Marker index (MI) was used as criterion for the assessment of the usefulness of each marker. As MI is the product of the

polymorphism information content (PIC) where *pi* is the frequency of allele and EMR is an effective multiplex ratio. PIC-based degrees of polymorphism were calculated for RAPD (0.226) as well as ISSR (0.234). The average EMR values for RAPD (17.34) and ISSR (9.01) were determined. The MI values were calculated for RAPD and ISSR markers as 3.79 and 2.37, respectively (table 4).

Table 4. Estimates of key statistics for evaluating the performance of ISSR and markers in 47 isolates of *Fusarium oxysporum* f. sp. *Lentis*

Component	ISSR	RAPD
Mean PIC	0.234	0.226
Mean EMR	9.01	17.34
Mean MI	2.37	3.79
Total number of assay	6	8
Total polymorphic Products	61	147
Total polymorphic of assay	70	156
Mean number of Products per assay	11.66	19.5
Percentage of polymorphism percentage	0.83	0.94

Correlations between ISSR and RAPD

Mantel test was performed and the dendorograms resulted from RAPD and ISSR markers and their combination had cophentic correlation efficient of 0.883, 0.918, 0. 911 respectively (Table 5).

Table 5. Results of Mantel test for different marker assays of 47 isolates of *Fusarium oxysporum* f. sp. *Lentis*

	ISSR	RAPD	ISSR+RAPD
R	0.918	0.883	0. 911
P	1	1	1

Discussion

RAPD and ISSR analyses of genomic DNA of 47 isolates of *Fusarium oxysporum* f. sp. *lentis* revealed the presence of high level of genetic diversity. RAPD analysis potentially provides information about entire genome, while ISSR analysis is only capable to reveal variation within special regions of genome (Arabi and Jawhar, 2007; Aghakhani, 2009). Using RAPD analysis, researchers have grouped the fungal populations from different regions (Singh *et al*., 2006). In previous studies, RAPD analysis has successfully been used to form groups within form species of *F. oxysporum.* One group was characterized within *F. oxysporum* f. sp. *albedinis* (Tantaoui *et al*., 1996), two groups within

f. sp. *dianthi* (Manulis *et al*., 1994), two to three groups within f. sp. *pisi* (Grajal-Martin *et al.,* 1993), and eight groups within *Fusarium oxysporum* f. sp. *fragariae* using RAPD and rDNA RFLP analyses (Nagarajan *et al*., 2004). A strong correlation was found between the geographic origins of the studied isolates and the groups they were belonged to them based on RAPD PCR analysis. This was in agreement with the report on *F. oxysporum* f. sp. *vasinfectum* in where a close correlation had been declared between RAPD pattern and geographic area (Assigbetse *et al*., 1994). Also, a similar correlation between RAPD pattern and geographic region has been reported for *F.oxysporum* f. sp. *pisi* (Pomazi *et al*., 1994). It was reported before that RAPD analysis could also reliably differentiate between non pathogenic and pathogenic isolates of *F. oxysporum* (Tantaoui *et al.,* 1996). No clear relationship was found between RAPD based dendrogram clusters and geographic origin of tested isolates. ISSR analyses were very found useful in the assessment of the diversity of *F. oxysporum* (Bayraktar and Dolar, 2008), however compared to RAPD markers, ISSR markers could only slightly separate isolates based on their geographical origins and were unable to show the correlation between geographical origin and isolates. Mantel test was performed using the original similarity matrix to form the cophenetic value matrix using the matrix comparison model (MXCOPH algorithm) in NTSYS-pc. The cophenetic correlation values for RAPD and ISSR were 0.883 and 0.918, respectively. Based on the high cophenetic correlation values we concluded that both molecular markers were powerful tools for the analysis of the genetic diversity in *F.oxysporum* f. sp. *lentis*. The correlation value (r= 0.911) between cophenetic matrix values obtained from these analyses suggested a very high correlation between RAPD and ISSR dendrograms. This was in contrast to the reported very low correlation between these two markers (Bayraktar and Dolar, 2008). Also comparison of their dendrograms revealed remarkable similarities in genetic relationships among *F.oxysporum* f. sp. *lentis* isolates. Both methods provided almost similar resolution, although there were differences in the distribution of the isolates and the ratio of genetic similarity in the dendrograms (Bayraktar and Dolar, 2008). Despite of some accomplishments using RAPD method, it is difficult to reproduce results not only among laboratories but also within the same laboratory due to the methodological aspects of PCR associated with the concentration of reaction components, DNA purity, thermal cycler and the source of *Taq* polymerase that affect amplification (Ellsworth *et al*., 1993). Therefore to avoid these problems, RAPD marker was used together with another marker, ISSR marker that led to better, reliable and reproducible data.

This study showed that these two markers applied together could well indicate genetic diversity of *F.oxysporum* f. sp. *lentis* isolates. Such results would be of potential applications in future breeding programs as well as in the production of resistant cultivars and other genetic programs.

Acknowledgments

We dedicate our spiritual thanks to the Department of Plant Protection, Mashhad University, Mashhad, Iran for their kind provision of some fungal isolates and Department of Plant Pathology, School of Agriculture, Terabit Modares University for their financial aid and facilities.

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(Received 15 May 2011; accepted 1 October 2011)