Original Contribution

GENETIC DIVERSITY OF SESAME ISOLATES OF *MACROPHOMINA PHASEOLINA* USING RAPD AND ISSR MARKERS

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ABSTRACT

RAPD and ISSR were assayed to determine the genetic diversity of sesame isolates of *M. phaseolina*. A high level of polymorphism was found with both RAPD and ISSR markers and PIC values were 0.3 and 0.305 for RAPD and ISSR markers, respectively. In RAPD analyses, 96 out of 118 bands (79.81%) were polymorphic. In ISSR analyses, a total of 55 bands were detected, among which 38 bands (66.61%) were polymorphic. MR was 14.75 and 9.2 and MI was 4.42 and 2.8 for RAPD and ISSR markers, respectively. Additionally, using the MXCOPH algorithm, high cophenetic correlation between the similarity matrix and corresponding dendrogram obtained by pooled RAPD and ISSR data (0.96639), RAPD data (0.943) and ISSR data (0.91451). The results showed that both analysis were suitable for the detection of genetic polymorphism among sesame *M. phaseolina* isolates. UPGMA cluster analyses and two-dimensional plots extracted by PCO of genetic similarity matrices of pooled data of RAPD and ISSR indicated that isolates differentiated in four main groups according to geographic origin. All isolates from Isfahan with upper 70% similarity usually were clustered in one main group. Kerman isolates with upper 90% similarity were clustered together. However, Khozestan isolates clustered in two different groups.

Key words: Charcoal Rot; Iran; Molecular Markers

INTRODUCTION

Sesame (*Sesamum indicum* L.) is grown as an oilseed crop in tropical and subtropical parts of the world (1). Various diseases of sesame caused by different pathogens are described (2). The causal agent of charcoal rot of sesame, *Macrophomina phaseolina* (Tassi) Goidanich, is a important soil- and seed-borne pathogen of over 500 host plant species across 75 families (3).

Molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. It has been shown that different markers might reveal different classes of variation (Powell et al. 1996; Russell et al. 1997). It is correlated with the genome fraction surveyed by each kind of marker, their distribution throughout the genome and the extent of the DNA target that

is analyzed by each specific assay (4). Of these techniques, RAPD has several advantages, such as simplicity of use, low cost, and the use of small amount of material, etc. Inter simple sequence repeat (ISSR) markers are powerful tools which can be utilized as molecular tools to access the variation around the diverse microsatellite regions that are dispersed throughout all genomes (5). The both RAPD and ISSR markers has been successfully used to differentiate and identify many fungi (6). Molecular methods used for differentiating *M. phaseolina* populations have included Restriction Fragment Length Polymorphism (RFLP) of rDNA-ITS regions (7-10), Random Amplified Polymorphic DNA (RAPD) (7-19), Amplified Fragment Length Polymorphism (AFLP) (19-24), Universal Rice Primer PCR (URP-PCR) (25), Inter simple sequence repeats (ISSR) (26-28), and Repetitive Sequence-Based Polymerase Chain Reaction (Rep-PCR) (26).

To date, knowledge regarding the amount of genetic variation and genetic relationship at molecular marker in sesame is not available.

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The objectives of this study were to (1) reveal the genetic diversity among *M. phaseolina* isolates from sesame plant from major area of cultivation of this plant and (2) Compare RAPD and ISSR marker in assessing of genetic diversity of this fungus.

MATERIAL AND METHODS
Fungal Isolates
Isolates of *M. phaseolina* were isolated from sesame plants displaying typical symptoms and signs of infection by *M. phaseolina* from major area of cultivation of this plant in Iran (**Table 1**).

To isolate *M. phaseolina* from infected tissue, five small (0.5 cm) epidermal sections were excised from symptomatic roots or stems. Tissue sections were surface disinfested in 75% ethanol for 30 s, transferred to 2.5% sodium hypochlorite for 30 s and washed in sterile water for 1 min. Surface disinfested tissue samples were placed on potato dextrose agar (PDA) containing chloramphenicol (0.1 mg/ml). Plates were incubated at 30ºC in the dark for 5 days. Pure cultures were obtained from hyphal tips and maintained on sterile toothpicks at room temperature.

Table 1. *Lists of Macrophomina phaseolina isolates*

Isolate No.	Host	Province	Location
1	Sesame	Isfahan	Kashan
2	Sesame	Isfahan	Kashan
3	Sesame	Isfahan	Kashan
4	Sesame	Khozestan	Shosh
5	Sesame	Khozestan	Sabeleh
6	Sesame	Khozestan	Ahvaz
7	Sesame	Khozestan	Andimeshk
8	Sesame	Khozestan	Shahvar
9	Sesame	Khozestan	Mahinabad
10	Sesame	Khozestan	Hamidiyeh
11	Sesame	Khozestan	Shoshtar
12	Sesame	Kerman	Jiroft
13	Sesame	Kerman	Jiroft
14	Sesame	Kerman	Jiroft
15	Sesame	Kerman	Jiroft

Genomic DNA extraction
A 5mm culture plug from a 2-day-old culture of each isolate was grown in the dark at 28°C for 5 days in 250 ml glass bottles containing 50 ml potato dextrose broth (PDB). Mycelia were filtered through Whatman No. 1 filter paper and lyophilized by vacuum pump. Genomic DNA was extracted according to Lee and Taylor (29) with some modifications as described by Safaie *et al.*(30). DNA concentrations were estimated by biophotometer at 260 nm and were stored at -20°C for further use.

PCR amplification and gel electrophoresis
DNA samples of each isolate were subjected to molecular analysis by amplifying the genomic DNA in a total volume of 25 μl containing 20-30 ng of DNA. The reaction buffer consisted of 2.5 μl of 10X buffer (500 mM KCl, 15 mM MgCl$_2$, 100 mM Tris-HCl pH8.0), 0.2 μM primers

(**Table 2**), 0.2 mM of each dNTPs, and 0.5 U of Taq polymerase (Cinnagen, Iran). The PCR amplification conditions were initial denaturation at 95ºC for 3 min followed by 35-40 cycles of denaturation at 94ºC for 2 min, primer annealing according to **Table 1** for 1 min, primer extension at 72ºC for 2 min, and a final primer extension at 72ºC for 7 min. The amplification product was separated in a 1.5% agarose gel using 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA pH 8.0). The gel was stained with ethidium bromide (0.50 μg/ml) and visualized under UV to confirm DNA amplification. Amplifications and gel separation were repeated at least twice with each primer. Thirteen primers of RAPD and ISSR were screened initially. Eight primers of RAPD and six primers of ISSR were selected for final analysis based on informative banding patterns, clarity, and repeatability.

Table 2. *List of RAPD and ISSR primers, annealing temperatures, and resulting DNA polymorphisms that were employed to differentiate isolates of Macrophomina. phaseolina.*

RAPD Primer	Primer Sequence (5′–3′)	Annealing Temperature (°C)	No. of Amplified DNA fragments	No. of Polymorphic Fragments	Percent Polymorphic Fragments	PIC
OPA02	5´-TGCCGAGCTG-3´					
OPA03	5'-AGTCAGCCAC-3'	40	17	14.00	82.35	0.298
OPA04	5´-AATCGGGCTG-3´	40	16	15.00	93.75	0.316
OPA07	5´-GAAACGGGTG-3´	41	14	10.00	66.66	0.261
OPA09	5´-GGGTAACGCC-3´	42	16	15.00	93.75	0.321
OPA10	5'-GTGATCGCAG-3'	42	17	14.00	82.35	0.306
OPA11	5'-CAATCGCCGT-3'					
OPA16	5´-AGCCAGCGAA-3´					
OPA18	5'-TGAGTGGGTG-3'					
OPC18	5´-TGAGTGGGTG-3´					
OPC09	5'-CTCACCGTCC-3'	42	11	7.00	63.63	0.320
OPB04	5'-GGACTGGAGT-3'					
230	5′ – CGTCGCCCAT - 3'	39	14	10.00	71.42	0.281
232	5′ – CGGTGACATC- 3'	39	13	11.00	84.61	0.299
238	5′ – CTGTCCAGCA -3'					
Total			118	96		
Mean			14.75	12	79.815	0.300

ISSR Primer	Primer Sequence (5′–3′)	Annealing Temperature (°C)	No. of Amplified DNA fragments	No. of Polymorphic Fragments	Percent Polymorphic Fragments	PIC
ISSR2	5'-ACTGACTGACTGACTG-3'	48	10	6	40.00	0.284
ISSR5	5'-GACACGACACGACACGACAC-3'	50				
ISSR09	5'-CCACCACCACCACCA-3'	47	9	8	88.88	0.360
ISSR10	5'-CACCACCACCACCAC-3'	52				
PcMs	5'-GTCGTCGTCGTCGTCGTCGTC-3	57	11	7	63.63	0.409
P4	5'-ATGATGATGATGATGATG-3'					
P5	5'-ACACACACACACACACYC-3'		-	-	-	
P6	5'-GAGAGAGAGAGAGAGAYG-3'	46	12	8.00	66.67	0.290
LMB-A	5'-GACAGACAGACAGACATA-3'					
LMB-B	5'-GACAGACAGACAGACATT-3					
LMB-C	5'-GACAGACAGACAGACAGT-3'	50	7	4.00	57.14	0.218
Stag 1	5'-ACAACAACAACAACA-3'					
Stag 3	5'-CAGCAGCAGCAGCAG-3'	46	6	5.00	83.33	0.267
Total			55	38		
Mean			9.17	6.33	66.61	0.305

Statistical analysis

RAPD and ISSR reproducible fragments were scored as present (1) or absent (0), and bands were entered in a computer file as a binary matrix, one for each molecular marker. The binary matrix was analyzed using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) package, version 2.0 (31) to calculate the similarity values. After obtaining the similarity matrix, clustering was performed by sequential agglomerative hierarchical nested clustering (SAHN). The graphical representation of the cluster was obtained by using the unweighted pair group method of mathematical averages (UPGMA). Bootstrap analysis of the data was performed with 1000 replications using the WINBOOT program (32) to estimate structural stability of clusters. Polymorphic information content (PIC) or average heterozygosity was calculated as per the formula of Roldán-Ruiz *et al.*(33). Average heterozygosity (H_{av}) is obtained by taking the average of PIC values obtained for all the markers. Multiplex ratio (MR) for each assay was estimated by dividing the total number of bands (monomorphic and polymorphic) amplified by the total number of assays (34, 35). Marker index (MI) was obtained by multiplying the average heterozygosity (H_{av}) with MR (Powell et al. 1996). A principal coordinate analysis (PCO) was performed EIGEN procedure in the NTSYSpc in order to highlight the

resolving power of the ordination. To estimate the congruence among dendrograms, cophenetic matrices for each marker type were computed and compared using the Mantel matrix correspondence test. This test was performed using the MXCOMP procedure in the NTSYSpc

RESULTS
Fungal Isolates
Fifteen isolates of *M. phaseolina* was isolated from three main regions of growing of sesame in Iran. **(Table 1)**. Khozestan is the largest producer of sesame in Iran as major of isolates were from this province.

Genetic diversity
RAPD analysis
DNA fragments that resulted from amplification with eight RAPD primers ranged in size from 300 to 3,500 bp **(Fig. 1)**. A total of 118 bands were detected, among which 96 bands (79.81%) were polymorphic with the mean of 12 per primer **(Table 2)**. For each primer, the number of bands ranged from 11 to 17, with an average of 14.75. The average polymorphic information content (PIC) was 0.300, ranging from 0.261 to 0.321. The lowest and the highest PIC values were recorded for primer OPA07 (0.261) and OPA09 (0.321), respectively.
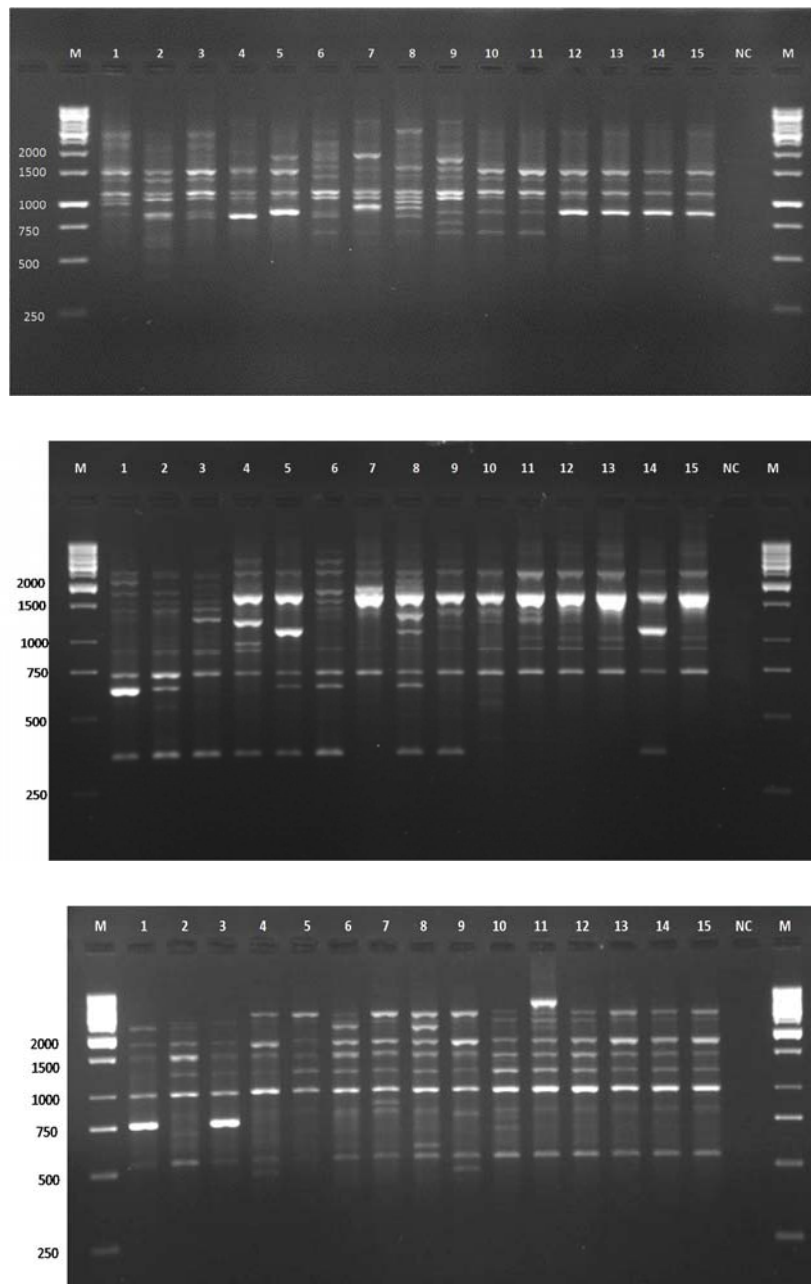

Fig. 1. RAPD banding pattern of *Macrophomina phaseolina* isolates generated by primer OPA04 (A), OPA10, and OPA07 (B). M: standard 1kb molecular ladder, NC: no-DNA control.

The relationships within and between groups were estimated by a UPGMA cluster analysis of GS matrices **(Fig 2).** Based on the resulting dendrogram, in level of 57% similarity, isolates clustered in four main groups. In group I, only one isolate from Khozestan-shosh was placed. The group II included three isolates from Khozestan. In group III, isolates from Kerman and Khozestan and in group IV, isolates from Isfahan were placed.

ISSR analysis
DNA fragments that resulted from amplification with six ISSR primers ranged in size from 250 to 3,300 bp **(Fig. 3).** A total of 55 bands were observed, with 9.17 bands per primer **(Table 3).** Thirty-eight out of 55 bands

(66.61%) were polymorphic, among which 4 to 8 polymorphic bands were detected by each primer. The average PIC was 0.305, and the lowest and highest PIC values were 0.218 (LMB-C) and 0.409 (PcMs), respectively.

The relationships within and between groups were estimated by a UPGMA cluster analysis of GS matrices **(Fig 4)**. Based on the resulting dendrogram, in level of 71% similarity, isolates clustered in four main groups. The group I contained four isolates from Khozestan. The group II, included only one isolates from Khozestan. In group III, four isolates from Kerman and three isolates from Khozestan were placed. The group IV separated all isolates from Isfahan.
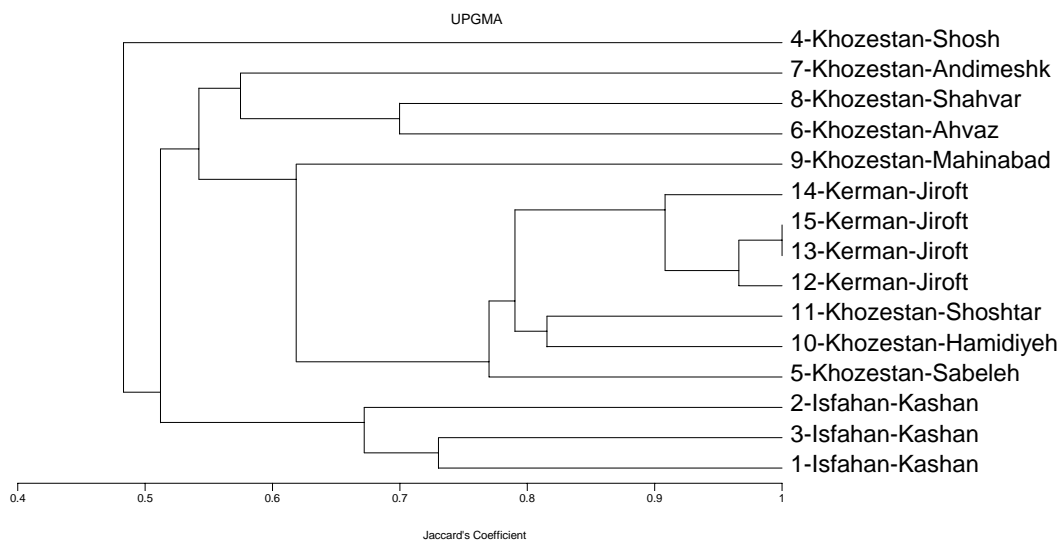

Fig. 2. Dendrogram of isolates of *Macrophomina phaseolina* derived from RAPD analysis with total primers by UPGMA.

Table 3. *Effectiveness of RAPD and ISSR Markers in detecting polymorphism of Macrophomina phaseolina*

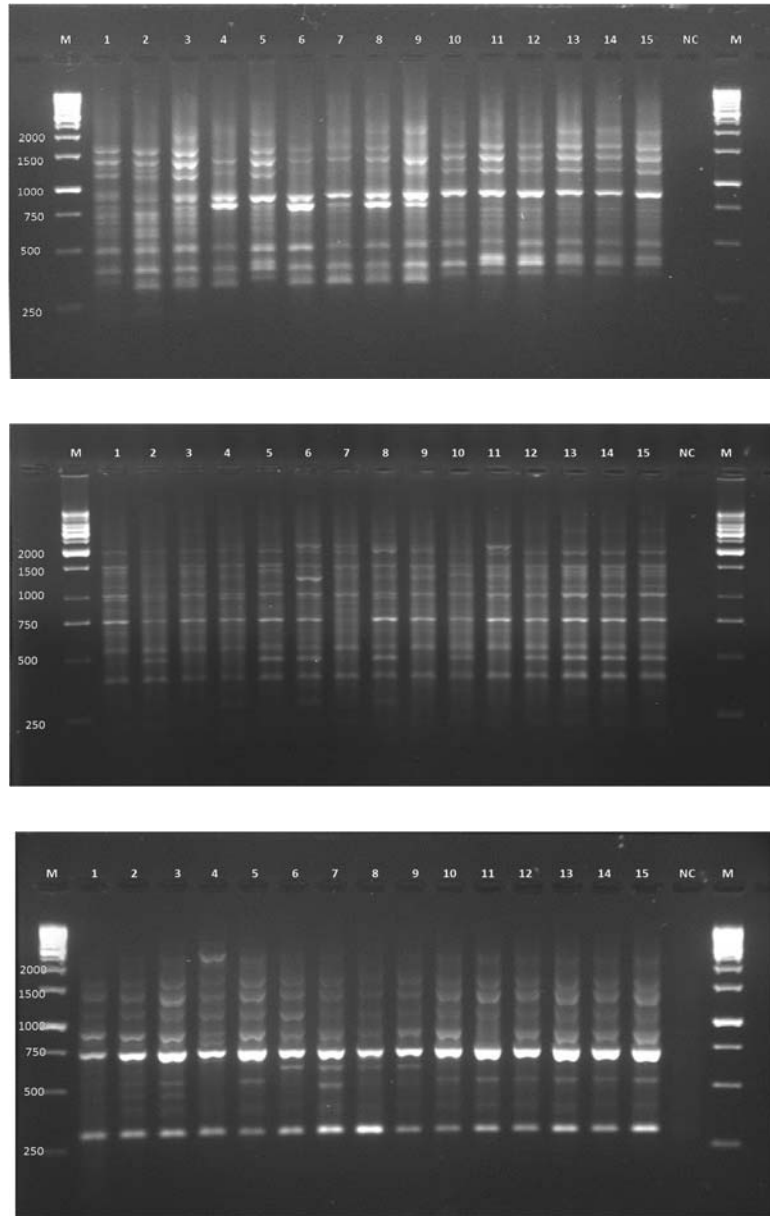| INDEX	RAPD	ISSR
No. of Primers	8	6
No. of Markers	118	55
No. of Polymorphic Markers	96	38
Minimum Polymorphism Scored per Primer	63.63	40.00
Maximum Polymorphism Scored per Primer	93.75	88.88
Average Polymorphism Scored per Primer	79.81	66.61
Average Heterozygosity	0.300	0.305
Multiplex Ratio	14.75	9.2
Marker index	4.425	2.80

Fig. 3. ISSR banding pattern of *Macrophomina phaseolina* isolates generated by primer PcMs (A), P6, and ISSR2 (B). M: standard 1kb molecular ladder, NC: no-DNA control.
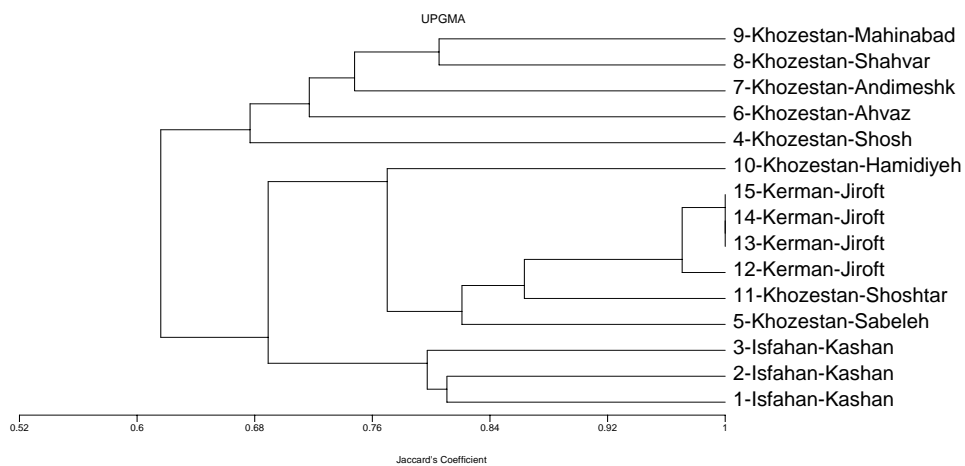

Fig. 4. Dendrogram of isolates of *Macrophomina phaseolina* derived from ISSR analysis with total primers by UPGMA.

RAPD and ISSR analysis

The relationships within and between groups were estimated by a UPGMA cluster analysis of GS matrices (Fig 5). Resulting dendrogram was similar to dendrogram of RAPD as in level of 61% isolates clustered in four main groups. In group I, major isolates from Khozestan were placed. The group II included only one isolate from Khozestan. In group III, isolates from Kerman and Khozestan were placed. The group IV contained all isolates from Isfahan.

Both RAPD and ISSR markers varied in number of primers, number of marker,

Multiplex ratio (MR), number of polymorphic markers, average polymorphism, polymorphic information content (average heterozygosity) and marker index **(Table 3)**.

The cophenetic correlation between the similarity matrix and corresponding dendrogram obtained by RAPD was greater than ISSR. The cophenetic correlation between the similarity matrix and corresponding dendrogram obtained by pooled RAPD and ISSR data was greater than each one of them **(Table 4)**.
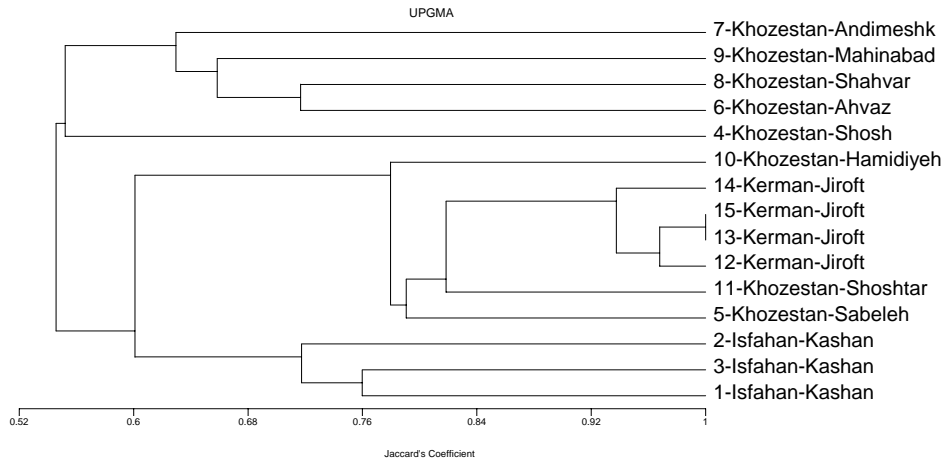

Fig. 5. Dendrogram of isolates of *Macrophomina phaseolina* derived from pooled RAPD and ISSR data analysis by UPGMA.

Table 4. *Result of Mantel test for different marker assay of sesame isolates of Macrophomina phaseolina*

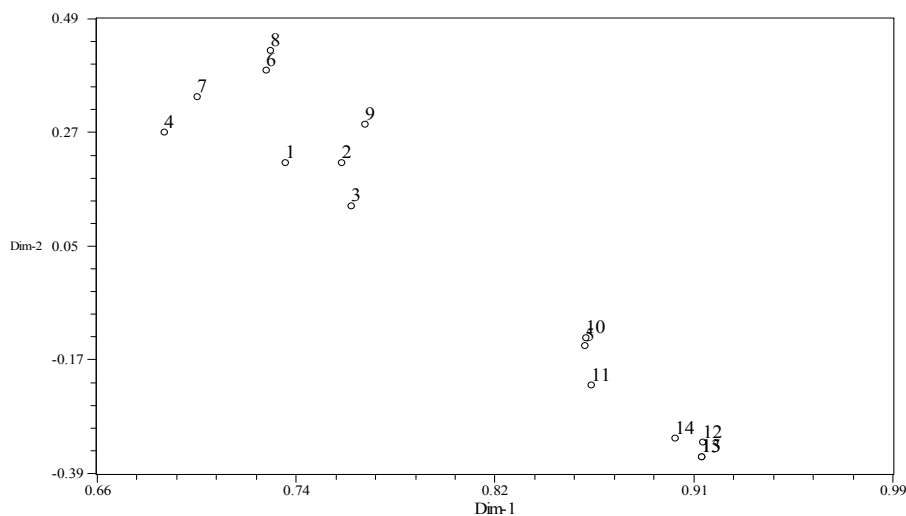| Marker	RAPD	ISSR	RAPD + ISSR
r	0.94349	0.91451	0.96639
p	1.0000	1.0000	1.0000

Fig 6. Two-dimensional plots extracted by principal coordinate analysis of pooled RAPD and ISSR amplification products from 15 isolates of *Macrophomina phaseolina.* The letters plotted represent individual isolates listed in **Table 1**.

DISCUSSION

Molecular markers are useful tools for assessing variation rapidly within and among species (36). In this study, we have compared two different molecular marker systems, RAPD and ISSR, to define genetic relationships among sesame isolates of M. phaseolina, and to investigate which marker system can be more effectively used. Using RAPD, eight primer combinations were sufficient to generate 96 polymorphic markers. A total of 55 bands were obtained from the 6 SSR primers amplified and 36 bands were polymorphic across all the isolates studied. In this study, RAPD marker was found to be more efficient in estimation of molecular diversity of different Isolates of M. phaseolina than ISSR marker as evident from large values of polymorphic loci and average number of polymorphic bands per primer. However, comparison of PIC values for two marker systems indicated that the PIC values for RAPD primers was 0.300, while of ISSR it was 0.305. Two ISSR primers PCMS and ISSR09 had largest amount of PIC, so average heterozygosity of ISSR marker have been larger than RAPD. Average heterozygosity corresponds to a probability that two alleles taken at random from a population can be distinguished using the marker in question (35).

The higher level of polymorphism detected by RAPD markers than with ISSR highlights the discriminating capacity of both markers. Polymorphism in a given population is often due to the existence of genetic variants represented by the number of alleles at a locus and their frequency of distribution in a population (35). A possible explanation for the difference in resolution of RAPDs and ISSRs is that the two-marker techniques target different portions of the genome (37). A comparison of the overall efficiency of the two marker systems was provided by the marker index (MI). Almost two-fold higher MI calculated for RAPD (4.425) in comparison to ISSR (2.80) reveals more efficiency of RAPD in this study. The distinctive value of MI for RAPD data is related to the multiplex ratio of RAPD (14.75) than ISSR (9.2). In other words, it depends more on the high number of polymorphic bands obtained per experiment than on the allelic heterozygosity found among isolates (38).

The cophenetic correlation between the similarity matrix and corresponding dendrogram obtained by pooled RAPD and ISSR analysis (r = 0.96639), RAPD analysis (r = 0.94349) and ISSR analysis (r = 0.91451) revealed a high degree of fit (r = 0.96). It is probably due to a large number of pair-wise genetic similarity coefficients with high values, which allow a number of similar variants for dendrogram branching (39).

Nevertheless, both marker techniques revealed a high degree of similarity in dendrogram topologies, because the three main clusters in the dendrograms were consistent for both marker systems. According to dendrogram obtained from pooled RAPD and ISSR data, all three isolates from Isfahan with upper 70% similarity usually were clustered in one main group. Kerman isolates with upper 90% similarity were clustered together. However, Khozestan isolates clustered in different groups. Tree isolate of Khozestan include 5, 10 and 11 clustered in group of Kerman isolates as this results suggest that there is possibly of same origin of isolates from these provinces. Isolate "4" from Khozestan were always placed in distinct group. Other isolates from Khozestan include 6, 7, 8, and 9 usually were grouped together with upper 60% similarity. These results showed that there is a diverse population of M. phaseolina in largest area of production of sesame in the country. The differences found among the dendrogram generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analyzed (118 for RAPDs and 55 for ISSRs) reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among M. phaseolina.

It was the first study that investigated genetic relationships among sesame isolates of M. phaseolina, as well as the first study that compared two PCR-markers to define genetic relationships among sesame isolates of M. phaseolina. Only, Purkayastha et al. (26) compared two different markers rep-PCR and MSP-PCR to investigate genetic relationships among isolates of M. phaseolina from different hosts.

CONCLUSION

RAPD and ISSR profiling techniques may provide useful information on the level of polymorphism and diversity in sesame isolates of M. phaseolina. In addition, other studies using RAPD (7-9, 11-15, 17, 18, 40) and ISSR (26-28) were shown the both markers are useful for studying molecular characterization of M. phaseolina in other plant hosts. Both marker systems have comparable accuracy in grouping isolates of this species according to their geographic origin. RAPD and ISSR techniques generated numerous polymorphic markers for use in genetic variation studies of M. Phaseolina. The study suggests that well-chosen primers could result in the quick estimate of genetic

diversity and geographical distribution studies of M. phaseolina. Based on the high percentage polymorphism, PIC, MI, similarity values and cost involved per polymorphic marker, it is concluded that RAPD was some powerful than ISSR. Our result revealed that similar to Macrophomina populations in other countries, the Iranian population is highly genetically diverse based on genotypic data. High levels of genotypic variability are most likely due in part to the exposure of the pathogen to diverse environments and a wide host range within the country.

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