

Research Article

## Using ISSR and URP-PCR markers in detecting genetic diversity among *Macrophomina phaseolina* isolates of sesame in Iran

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**Abstract:** To assess the variability of *Macrophomina phaseolina* (Tassi) Goid, the causal agent of charcoal rot of Sesame, sixty isolates recovered from ten geographic regions, were analyzed using inter simple sequence repeat (ISSR) and universal rice primer (URP) markers. Isolates were grouped into eight clusters at 78% genetic similarity level. Our results showed that the five ISSR primers produced 105 bands of which 77.11% were polymorphic and eight URP-PCR primers generated 135 bands of which 66.84% were polymorphic. These methods showed a considerable genetic diversity among Iranian isolates, but no correlation was found between genetic diversity and geographical origins of the isolates. Analysis of molecular variance revealed that a large proportion of genetic variability resulted from the differences among isolates within regions. The findings of this study demonstrated that the low-genetic differentiation ( $G_{ST}$ ) and high gene flow ( $N_m$ ) among populations had a significant effect on the emergence and evolutionary development of *M. phaseolina*.

**Keywords:** charcoal rot, *Sesamum indicum*, DNA fingerprinting, population genetics

### Introduction

Charcoal rot is one of the factors limiting the cultivation of Sesame *Sesamum indicum* L., an oilseed crop widely grown in different parts of the world. *Macrophomina phaseolina* (Tassi) Goid. a soil-and seed-borne fungus causes charcoal rot. The fungus can infect the root and lower stem of more than 500 plant families and has a wide geographic distribution (Dhingra and Sinclair, 1978). Charcoal rot is an important soil borne disease and is favored by hot and dry weather or when unfavorable environmental conditions impose stress on plant (Etebarian, 2006).

In order to design optimal breeding methods to produce sesame cultivars which can have resistance to charcoal rot, knowledge of population biology and its interaction with sesame cultivar would be quite useful. The use of resistant varieties is the most appropriate approach to control of *M. phaseolina* (Ahmad and Burney, 1990). The existence of high level variations in a fungal population increase its adaptation to various conditions and overcomes host resistance deployed by growers (Yeh *et al.*, 1999). To determine the level of variability in all organisms, basic morphological traits and pathogenesis as the first character are used, but usually these methods are time-consuming and their quantification is difficult. Molecular methods are an appropriate analysis approach for a rapid evaluation of the variability within and among species (Chakravarthi and Naravaneni, 2006).

Handling Editor: Vahe Minassian

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Received: 18 October 2018, Accepted: 15 June 2019  
Published online: 21 July 2019

The host correlates development of disease resistance with the accumulation of new polypeptides syntheses (Broglie *et al.*, 1986). The new protein contents depend on sensitivity to infection, host genotype and the virulence genes of the pathogens (Hlinkova and Sykora, 1996; Radwan, 2000). Pecina *et al.* (2000) reported Double-stranded RNA (dsRNA) in *M. phaseolina* with sizes ranging from 0.4kbp to 10kbp and the number of dsRNA ranging from 1 to 10.

In recent years, different DNA markers have been employed to study genetic diversity within populations of *M. phaseolina* such as Restriction Fragment Length Polymorphism (RFLP) of rDNA-ITS regions (Aghakhani and Dubey, 2009; Almeida *et al.* 2003), Random Amplified Polymorphic DNA (RAPD) (Aboshosha *et al.* 2007; Aghakhani and Dubey, 2009; Almeida *et al.* 2003), Amplified Fragment Length Polymorphism (AFLP) (Linhai *et al.* 2011; Mayek-Perez *et al.* 2001; Reyes-Franco *et al.* 2006), Universal Rice Primer PCR (URP-PCR) (Jana *et al.*, 2005b), ISSR (Jana *et al.*, 2005a), Repetitive Sequence-Based Polymerase Chain Reaction (Rep-PCR) (Purkayastha *et al.*, 2008) and Simple Sequence Repeats (SSR) (Baird *et al.*, 2010).

Among DNA markers, ISSR is a dominant molecular marker system that includes the use of one primer complementary to a target Simple Sequence Repeat (SSR) region in PCR and allows DNA amplification between SSR regions. This technique was first reported as marker to assess genetic diversity and taxonomic studies in animals, plants and later used to obtain DNA markers in fungi (Bornet and Branchard, 2001; Menzies *et al.* 2003; Zietkiewicz *et al.* 1994). The advantages of ISSR over other markers include; (1) low development costs; (2) no prior information or lengthy mapping studies are required; (3) laboratory procedures can easily be applied to any plant species and fungi (Geo *et al.*, 2006; Maleki *et al.*, 2018).

URP-PCR markers are powerful tools which can be utilized to examine genetic diversity of various organisms including Micro-organisms, animals and plants at intraspecific and interspecific levels and were designed based on

repetitive sequence of rice genome by Kang *et al.* (2002).

In Iran, dry rot occurs in many economically important crops, including common bean, sesame, soybean, chickpea, sunflower and sugarbeet, among others, and it might lead to yield reduction (Mahdizadeh *et al.*, 2011). Seed infection by this pathogen may be responsible for its spreading in the main agricultural regions of the country. Infection to this pathogen has been reported in different region of Iran (Ershad, 1995). Although *M. phaseolina* has been present for decades in Iran and a vast literature about its agronomic aspects is available, information about the population biology of this pathogen is still interesting subject.

The objective of this study was to assess genetic diversity and population structure of *M. phaseolina* pathogen of sesame in Iran, using different genetic markers, URP-PCR and ISSR and compare the discriminative power of these markers in elucidating the degree of heterogeneity in the population of *M. phaseolina*.

## Materials and Methods

### Fungal material

Sixty *M. phaseolina* isolates used in this study were obtained in a previous comprehensive sampling from different geographical regions of Iran including Khuzestan, Isfahan, Kerman, Golestan, Hormozgan, Mazandaran, Bushehr, Fars, Yazd and Khorasan-jonoobi (South Khorasan) provinces (Table 1). The isolates were confirmed using species-specific primers (MpKFI/MpKRI) previously described by Babu *et al.* (2007).

### DNA extraction

Samples were grown in 250ml glass bottles containing 50ml of Potato Dextrose Broth (PDB) for 5 days at 30 °C in the dark. Mycelia from PDB were harvested by filtering through whatman NO.1 filter paper then dried and deep frozen. Fungal DNA was extracted according to Lee and Taylor, (1990) as modified by Safaie *et al.* (2005). The Quantity and quality were determined spectrophotometrically at 260nm and electrophoretically, respectively and were stored at -20 °C for further use.

**Table 1** Name and source of *Macrophomina phaseolina* isolates utilized in this study.

| Isolates | Origin    | Isolates | Origin           |
|----------|-----------|----------|------------------|
| B1       | Bushehr   | H10      | Hormozgan        |
| B4       | Bushehr   | H11      | Hormozgan        |
| B5       | Bushehr   | H12      | Hormozgan        |
| B6       | Bushehr   | K1       | Kerman           |
| B7       | Bushehr   | K2       | Kerman           |
| B8       | Bushehr   | K3       | Kerman           |
| B9       | Bushehr   | KH6      | Khuzestan        |
| B10      | Bushehr   | KH15     | Khuzestan        |
| E1       | Isfahan   | KH17     | Khuzestan        |
| E2       | Isfahan   | KH27     | Khuzestan        |
| F5       | Fars      | KH30     | Khuzestan        |
| F7       | Fars      | KH32     | Khuzestan        |
| F12      | Fars      | KH33     | Khuzestan        |
| F15      | Fars      | KH35     | Khuzestan        |
| F23      | Fars      | KH36     | Khuzestan        |
| F27      | Fars      | KH37     | Khuzestan        |
| F31      | Fars      | KJ1      | Khorasan-jonoobi |
| F33      | Fars      | KJ4      | Khorasan-jonoobi |
| F38      | Fars      | KJ7      | Khorasan-jonoobi |
| F41      | Fars      | KJ8      | Khorasan-jonoobi |
| F43      | Fars      | M2       | Mazandaran       |
| F45      | Fars      | M3       | Mazandaran       |
| G1       | Golestan  | M9       | Mazandaran       |
| G2       | Golestan  | M13      | Mazandaran       |
| G3       | Golestan  | M16      | Mazandaran       |
| G4       | Golestan  | M19      | Mazandaran       |
| H3       | Hormozgan | M20      | Mazandaran       |
| H7       | Hormozgan | Y1       | Yazd             |
| H8       | Hormozgan | Y2       | Yazd             |
| H9       | Hormozgan | Y3       | Yazd             |

### Primers and polymerase chain reaction

The sequences of five ISSR primers and 12 URP-PCR primers used for the molecular analysis are given in Table 2 and were synthesized by CinnaGen, Iran. The PCR were carried out in a thermocycler (Mastercycler® gradient, Eppendorf, Hamburg, Germany). For both methods final volume of 20µl contained 20 ng of template DNA, 1µM primer, 200mM each of four deoxyribonucleotide triphosphate (dNTP<sub>s</sub>), 1.5 units of taq polymerase (CinnaGen, Iran), 2mM MgCl<sub>2</sub> and 2.5µl PCR Buffer (100mM Tris-HCl, 500mM KCl (pH = 8.4)).

The PCR reactions were performed for both methods under following conditions: initial denaturation at 94 °C for 4min; 35 cycles of 1 min denaturation at 94 °C; 1 min annealing at a temperature specific to the primer and extension primer at 70 °C for 2min and 7min at 72 °C for the final product extension. The amplification product were separated by electrophoresis on agarose (1.4%) gel in 0.5 × TAE Buffer run for 2h at 85v and stained with ethidium bromide. A 1Kb DNA ladder (Fermentase, Lithuania) was used as standard molecular weight marker. All PCR assays were repeated at least twice to confirm the reproducibility of the results.

### Data analysis

Amplified fragments were considered as a binary character for the present (scored 1) and absent (Scored 0). Similarities based on their fingerprinting groups were estimated by Jaccard's (J) coefficient. Cluster analysis was carried out with NTSYS-pc software, UPGMA algorithm (Rohlf, 2000). Polymorphism information content (PIC) was calculated as:  $PIC = 1 - [(p) + (q)^2]$ , where p is the frequency of allele band present and q is frequency of allele band absent (Lynch and Walsh, 1998). Effective multiplex ratio (EMR) and marker index (MI) were calculated according to Powell *et al.* (1996) to determine the utility of each of the markers in *M. phaseolina* system. Percentage of Polymorphic Loci (PPL), Shannon's Information Index and Nei's gene diversity were calculated using 'POPGENE' software package ver. 1.31 (Yeh *et al.*, 1999). The total genetic diversity ( $H_T$ ), genetic diversity within each population ( $H_S$ ) coefficient of gene differentiation ( $G_{st}$ ) were also determined to characterize the gene diversity and the distribution of the variation using 'POPGENE' program. The gene flow estimates ( $N_m$ ) among these populations were computed as  $N_m = 1/(4G_{st} - 1)$  (Nei 1973; Slatkin and Barton 1989). An analysis of molecular variance (AMOVA) was performed to calculate the hierarchical apportionment of variation using GenAlEx version 6.501 (Peakall and Smouse, 2006).

**Table 2** Primer names and sequences, annealing temperature, number of generated bands, number of polymorphic bands and degree of polymorphism of amplified DNA for each primer using URP-PCR and ISSR analysis of *Macrophomina phaseolina* isolates.

| Marker  | Primer  | Sequence                    | Annealing temperature (°C) | No. bands generated | No. bands polymorphic | Polymorphism (%) |
|---------|---------|-----------------------------|----------------------------|---------------------|-----------------------|------------------|
| ISSR    | ISSR10  | 5'-CACCACCACCACCAC-3'       | 59                         | 23                  | 19                    | 82.20            |
|         | ISSR02  | 5'-ACTG ACTGACTGACTG-3'     | 48                         | 23                  | 19                    | 82.20            |
|         | ISSR09  | 5'-CCACCACCACCACCA-3'       | 55                         | 24                  | 22                    | 83.30            |
|         | PcMs    | 5'-GTCGTCGTCGTCGTCGTCGTC-3' | 55                         | 18                  | 10                    | 55.50            |
|         | P4      | 5'-ATGATGATGATGATGATG-3'    | 51                         | 17                  | 15                    | 88.23            |
| URP-PCR | URP-2F  | 5'-GTGTGCGATCAGTTGCTGGG-3'  | 47                         | 13                  | 10                    | 76.92            |
|         | URP-30F | 5'-GGACAAGAAGAGGATGTGGA-3'  | 45                         | 18                  | 11                    | 61.11            |
|         | URP-2R  | 5'-CCCAGCAACTGATCGCACAC-3'  | 45                         | 17                  | 12                    | 70.58            |
|         | URP-4R  | 5'-AGGACTCGATAACAGGCTCC-3'  | 46                         | 17                  | 11                    | 64.70            |
|         | URP-6R  | 5'-GGCAAGCTGGTGGGAGGTAC-3'  | 45                         | 17                  | 9                     | 52.94            |
|         | URP-13R | 5'-TACATCGCAAGTGACACAGG-3'  | 46                         | 18                  | 14                    | 77.77            |
|         | URP-1F  | 5'-ATCCAAGTCCGAGACAACC-3'   | 47                         | 22                  | 9                     | 69.23            |
|         | URP-17R | 5'-AATGTGGGCAAGCTGGTGGT-3'  | 55                         | 13                  | 8                     | 61.53            |
|         | URP-25F | 5'-GATGTGTTCTTGAGCCTGT-3'   | -                          | -                   | -                     | -                |
|         | URP-9F  | 5'-ATGTGTGCGATCAGTTGCTG-3'  | -                          | -                   | -                     | -                |
|         | URP-32F | 5'-TACACGTCTCGATCTACAGG-3'  | -                          | -                   | -                     | -                |
|         | URP-38F | 5'-AAGAGGCATTCTACCACCAC-3'  | -                          | -                   | -                     | -                |
|         | URP-1F  | 5'-ATCCAAGTCCGAGACAACC-3'   | -                          | -                   | -                     | -                |

## Results

### Identification of *M. phaseolina* isolates using specific primers

In the present study, sixty isolates were identified at the molecular level using species specific primers (MpFI/MoKRI) as designed by Babu *et al.* (2007). A fragment 350-bp was amplified by the primer pair in tested isolates (Fig. 1). The result showed that the isolates belong to the species *M. phaseolina*. The DNA from *Sclerotinia sclerotiorum*, *Fusarium graminearum*, *Alternaria alternata*, *Trichoderma viride* and *Cytospora* sp. were used as negative controls. No fragment was detected in the negative control.

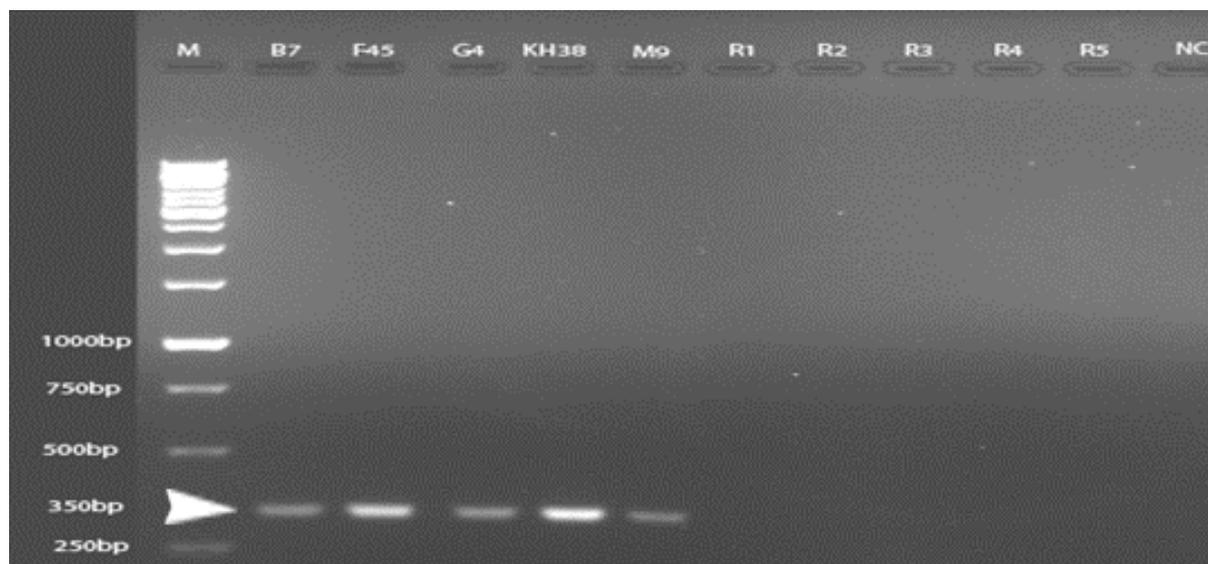
### ISSR analysis

Five ISSR primers used to assess genetic diversity of 60 *M. phaseolina* isolates yielded reproducible and polymorphic amplification patterns. A total of 105 bands were produced of which 85 (80.95%) bands were polymorphic. The number of ISSR bands varied from 24 (for the ISSR10) to 17 (for the P4 primers), ranging

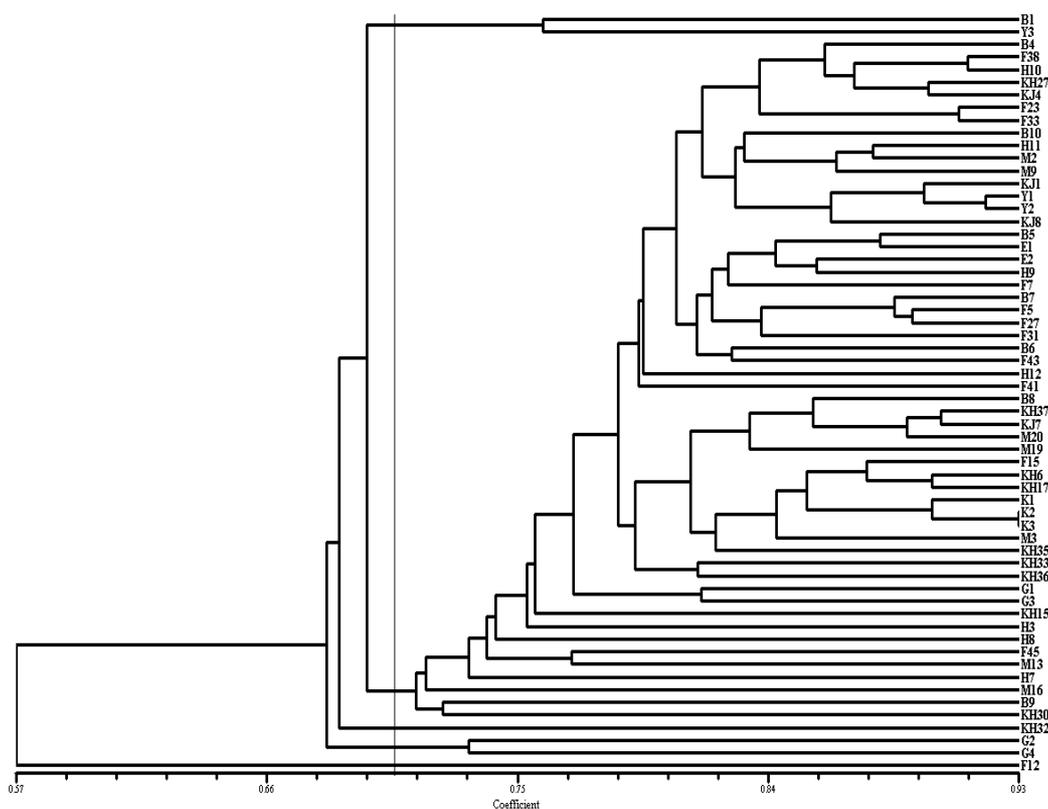
in size from 250 to 3000 bp. Genetic similarity coefficient varied from 0.51 to 0.91.

The dendrogram obtained by UPGMA analysis indicated that the isolates were grouped into five clusters at 70% similarity coefficient level (Fig. 2). Cluster I was comprised of two isolates from Bushehr and Yazd. Cluster II contained 54 isolates, consisting of seven isolates from Bushehr, 11 isolates from Fars, nine isolates from Khuzestan, two isolates from Golestan, two isolates Yazd, all isolates from Khorasan-jonoobi, Kerman, Mazandaran, Hormozgan and Isfahan provinces. Clusters III, IV and V contained one, two and one isolates from Khuzestan, Golestan and Fars provinces, respectively. Fifty four isolates grouped in one cluster indicating there is high similarity among isolates from different regions.

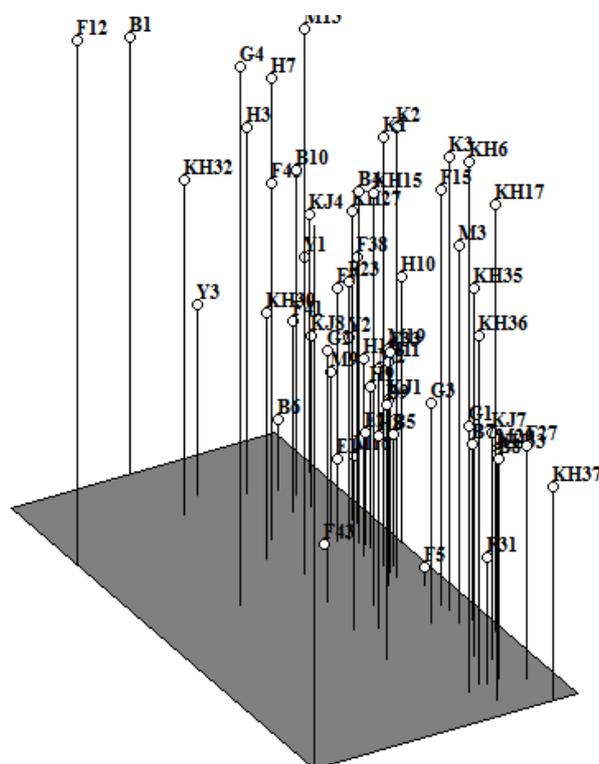
The first three most informative principal components of ISSR data explained 80.17% of the total genetic variation observed among isolates. The result of three-dimensional plot of principal component analysis confirmed the cluster analysis results and it indicated that F12 isolate from Fars is the most genetically distant from the other isolates (Fig. 3).



**Figure 1** Template DNA was amplified using species-specific primers (MpKFI/MpKRI), M: 1 kb molecular ladder; B7, F45, G4, KH38 and M9 *M. phaseolina* isolates from Iran. R1: *Sclerotinia sclerotiorum*, R2: *Fusarium graminearum*, R3: *Alternaria alternata*, R4 *Trichoderma viride* and R5: *Cytospora ehrenb* are negative controls; NC: Negative control.



**Figure 2** Dendrogram derived (constructed) from 60 isolates *Macrophomina phaseolina* by UPGMA clustering based on ISSR.



**Figure 3** Genetic relationships of 60 *Macrophomina phaseolina* isolates based on principal component analysis.

#### UPR-PCR analysis

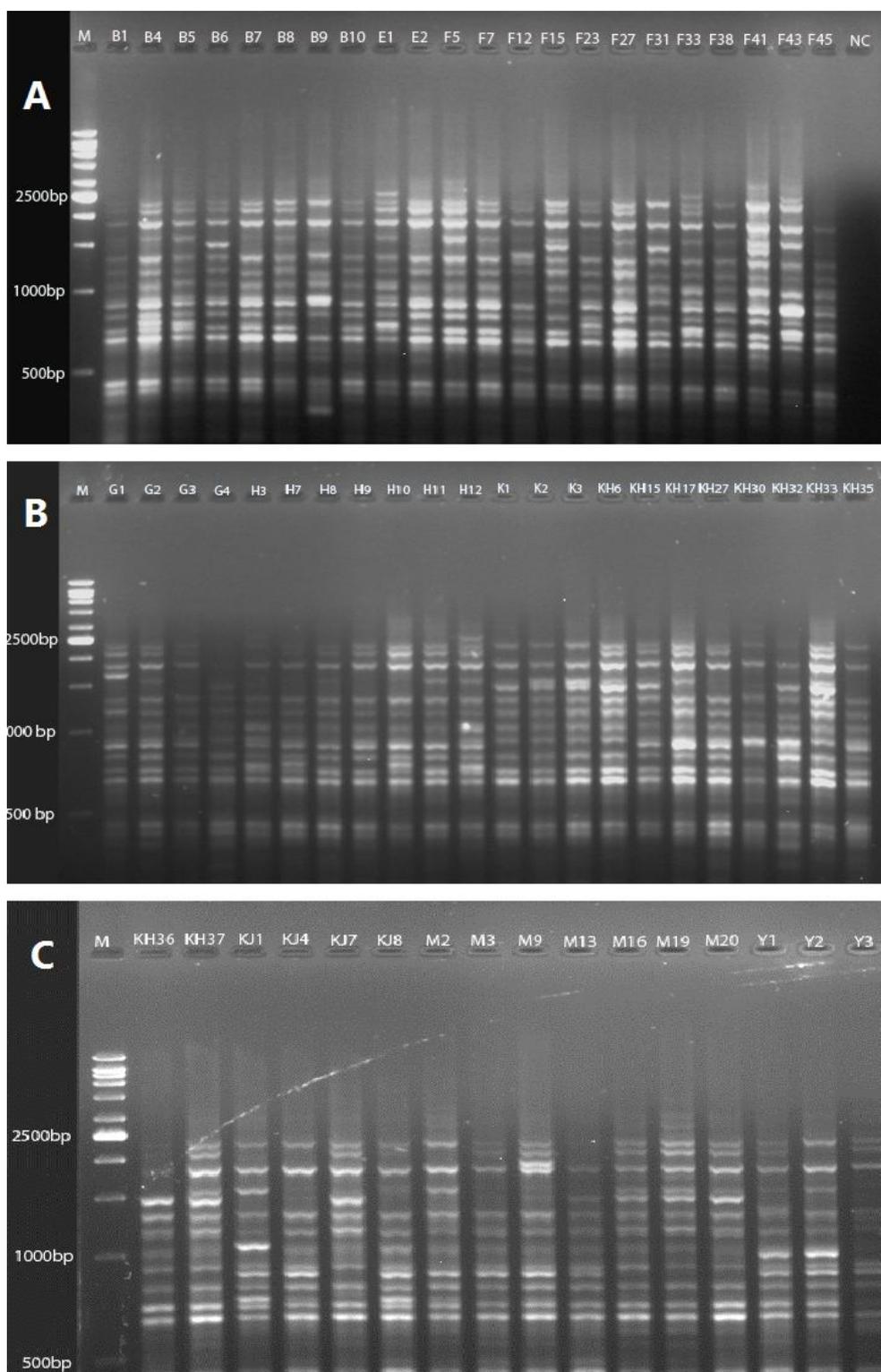
Out of 12 URP-PCR primers tested for amplification, 8 primers generated reproducible and well-defined fragments (Fig. 4). A total of 135 fragments were generated, 84 (62.22%) of them were polymorphic. Amplified fragments were from 200bp to 2500bp. The number of polymorphic fragments ranged from 8 to 15, with an average of 10.5 fragments. Genetic similarity coefficient was varied from 0.61 to 0.93. Based on URP-PCR analysis, the isolates were grouped into six clusters by UPGMA at the 77% similarity coefficient level (Fig. 5). Cluster I contained 53 isolates, consisting of 2 sub-clusters; the first sub-cluster contained six isolates from Bushehr, seven isolates from Fars, three from Khorasan-jonoobi, six from Hormozgan, two from Mazandaran, two from Khuzestan, one from Golestan and all isolates from both Isfahan and Yazd provinces. The second sub-cluster included six isolates from Khuzestan, four from Fars, four from Mazandaran, all isolates from Kerman and a

single isolate from Bushehr, Khorasan-jonoobi, Hormozgan and Golestan provinces. Cluster III consisted of only two isolates from Fars and Mazandaran and cluster IV contained two isolates from Khuzestan. Clusters II, V and VI are single isolate, namely G1, F45 and M13 which were collected from Golestan, Fars and Mazandaran, respectively.

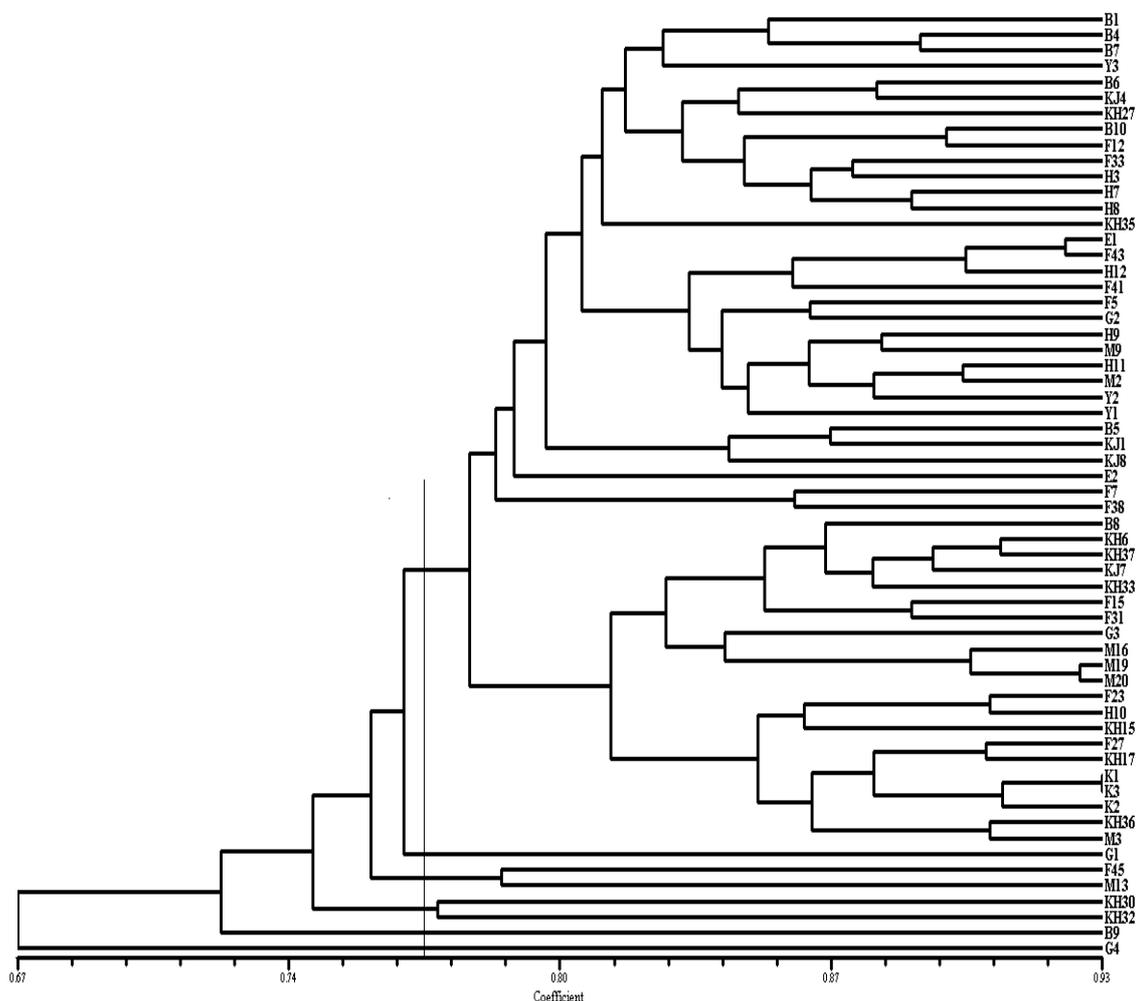
The first three principal components of URP-PCR data explained 83.34% of the total variation observed among isolates. The grouping of isolates by three first principal components was in agreement with results in clustering. It indicated that G4 isolate from eastern Golestan is the most genetically distant isolate from the other isolates.

#### Combination of ISSR and URP-PCR analysis

The UPGMA dendrogram obtained from combination of URP-PCR and ISSR fingerprints indicated that *M. phaseolina* isolates could be differentiated into eight clusters at 78% similarity coefficient level (Fig. 6).



**Figure 4** URP-PCR profile generated using URP-13R; B = Bushehr; E = Isfahan; F = Fars; G = Golestan; H = Hormozgan; K = Kerman; KH = Khuzestan; KJ = Khorasan-jonoobi; M = Mazandaran; Y = Yazd; M = molecular weight marker GeneRuler™ 1 kb DNA ladder (Fermentase, Lithuania); NC = Negative control.

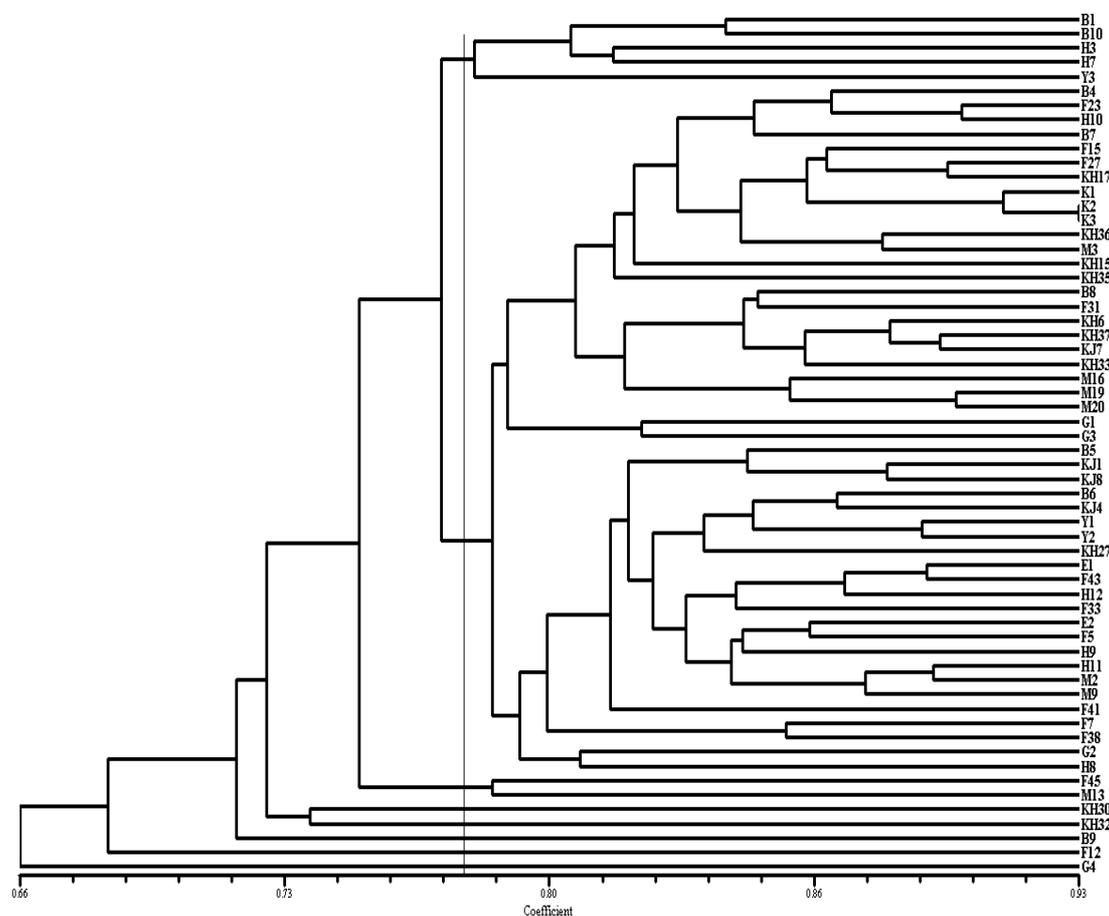


**Figure 5** Dendrogram derived (constructed) from 60 isolates *Macrophomina phaseolina* by UPGMA clustering based on URP-PCR.

Cluster I consisted of two isolates from Bushehr, two from Hormozgan and one from Yazd. Cluster II had two sub-cluster, the first sub-cluster was comprised of three isolates from Bushehr, four from Fars, seven from Khuzestan, four from Mazandaran, all isolates from Kerman, two from Golestan and single isolates from Hormozgan and Khorasan-jonoobi provinces and the second sub-cluster was composed of two isolates from Bushehr, three from Khorasan-jonoobi, two from Yazd, six from Fars, four from Hormozgan, two from Mazandaran, all isolates from Isfahan and single one each from Khuzestan and Golestan provinces. Cluster III contained two isolates

from Fars and Mazandaran provinces. Clusters IV, V, VI, VII and VIII that each contained one isolate from Khuzestan, Khuzestan, Bushehr, Fars and Golestan, respectively. Genetic similarity coefficient was varied from 0.61 to 0.90.

The first four principal components from combination of ISSR and URP-PCR data explained 82.74% of the total variation observed among isolates. The result of three-dimensional plot of principle component analysis confirmed the cluster analysis results and it indicated that G4 isolate from Golestan is the most genetically distant from the others.



**Figure 6** Dendrogram derived (constructed) from 60 isolates *Macrophomina phaseolina* by UPGMA clustering based on URP-PCR and ISSR.

### Comparison of ISSR and URP-PCR

EMR, MI and the product of the polymorphism information content (PIC) were used as criterion for the assessment the usefulness of each marker. For ISSR marker, MI, EMR and PIC were 3.28, 13.94 and 0.246, respectively, while for URP-PCR these indices were 1.56, 5.54 and 0.33, respectively.

The cophenetic correlation ( $r$ ) value for similarity matrices of URP-PCR and ISSR were 0.76 and 0.86, respectively, demonstrated a good fit of the cluster analysis to the similarity data. The cophenetic correlation with combination of URP-PCR and ISSR fingerprints was  $r = 0.83$ , which according to Mantel, (1967) is significant.

The Mantel test showed that the correlation coefficient between the two similarity matrices

obtained by ISSR and URP-PCR was significant ( $r = 0.44$ ,  $P = 0.002$ ), suggesting existence of positive correlation between two molecular markers.

### Genetic differentiation between populations

The higher values, Shannon's Information Index ( $0.258 \pm 0.269$ ) and PPL (53.33%) were in Fars populations and Nei's gene diversity ( $0.17 \pm 0.2096$ ) in Golestan populations. Percentage of Polymorphic Loci (PPL) (9.58%), Shannon's Information Index ( $0.061 \pm 0.1878$ ) and Nei's gene diversity ( $0.042 \pm 0.1311$ ) had the lowest values in the populations of Kerman (Table 3).

The AMOVA conducted using URP-PCR + ISSR data showed that from the total genetic variability, 10% ( $p < 0.01$ ) could be

attributed by the differences between populations while 90% ( $p < 0.01$ ) could be attributed by the differences within populations. The within and among population variability or the URP-PCR and ISSR data were 88-12% and 91-9%, respectively (Table 4).

**Table 3** Genetic analysis among *Macrophomina phaseolina* populations obtained from URP-PCR and ISSR data.

| PGP              | N  | H             | I             | p     |
|------------------|----|---------------|---------------|-------|
| Bushehr          | 8  | 0.157 ± 0.191 | 0.235 ± 0.278 | 45.00 |
| Isfahan          | 2  | 0.068 ± 0.172 | 0.095 ± 0.293 | 13.75 |
| Fars             | 12 | 0.168 ± 0.187 | 0.258 ± 0.269 | 53.33 |
| Golestan         | 4  | 0.170 ± 0.209 | 0.248 ± 0.302 | 40.83 |
| Hormozgan        | 7  | 0.131 ± 0.188 | 0.195 ± 0.274 | 35.42 |
| Kerman           | 3  | 0.042 ± 0.131 | 0.061 ± 0.187 | 9.58  |
| Khuzestan        | 10 | 0.164 ± 0.188 | 0.250 ± 0.273 | 49.58 |
| Khorasan-jonoobi | 4  | 0.112 ± 0.185 | 0.164 ± 0.269 | 27.50 |
| Mazandaran       | 7  | 0.142 ± 0.188 | 0.213 ± 0.275 | 39.58 |
| Yazd             | 3  | 0.085 ± 0.175 | 0.122 ± 0.251 | 19.17 |

Population genetic parameters (PGP).

Samples size (N).

Nei's (1973) gene diversity (H).

Shannon Information Index (I).

% of polymorphic loci (P).

**Table 4** Nested analysis of molecular variances (AMOVA) for *Macrophomina phaseolina* isolates from 10 regions of Iran.

| Source         | df | Sum of squares | Mean squares | Est. Var. | %   |
|----------------|----|----------------|--------------|-----------|-----|
| ISSR           |    |                |              |           |     |
| Among Pops     | 9  | 133.655        | 14.851       | 0.912     | 9   |
| Within Pops    | 50 | 477.495        | 9.550        | 9.550     | 91  |
| Total          | 59 | 611.150        |              | 10.461    | 100 |
| URP-PCR        |    |                |              |           |     |
| Among Pops     | 9  | 172.770        | 19.197       | 1.439     | 12  |
| Within Pops    | 50 | 541.430        | 10.829       | 10.829    | 88  |
| Total          | 59 | 714.200        |              | 12.268    | 100 |
| ISSR + URP-PCR |    |                |              |           |     |
| Among Pops     | 9  | 306.425        | 34.047       | 2.351     | 10  |
| Within Pops    | 50 | 1018.925       | 20.379       | 20.379    | 90  |
| Total          | 59 | 1325.350       |              | 22.729    | 100 |

The total gene diversity ( $H_t$ ) and mean diversity within each population ( $H_s$ ) belonging to URP-PCR + ISSR data were obtained for the populations which were 0.181 and 0.124, respectively, and the mean coefficient of gene differentiation ( $G_{st}$ ) among populations was 0.313, indicating 31% of differentiation between populations (Table 5). Similarly, almost relevant results were obtained for relative differentiation ( $G_{st}$ ), total Heterozygosity ( $H_t$ ), intra-population heterozygosity ( $H_s$ ) indices over populations for either individual datasets of each marker analyses.

**Table 5** Genetic structure estimated by Nei's genetic parameters among *Macrophomina phaseolina* populations obtained from URP-PCR + ISSR data.

| Population genetic parameters         | Obtained values |
|---------------------------------------|-----------------|
| Intra-population ( $H_s$ )            | 0.124 ± 0.016   |
| Total heterozygosity ( $H_t$ )        | 0.181 ± 0.034   |
| Relative differentiation ( $G_{st}$ ) | 0.313           |
| Estimate gene flow ( $N_m$ )          | 1.09            |

The amount of gene flow ( $N_m$ ) from URP-PCR + ISSR data between populations was 1.09, where  $N_m$  is the average number of migrants among the population. According to Wright, (1951), the  $N_m > 1$  shows the existence of little differentiation among populations. No specific fragments were found in either population. This result shows that ISSR and URP-PCR divergence among populations was mainly ascribed to difference of the DNA-bands frequency instead of allele fixation.

The values of Nei's genetic distance ranged from 0.027 to 0.123 for 10 populations. The lowest value of genetic distance was obtained between Fars and Hormozgan populations and showed that the populations from these two regions were most similar (Table 6).

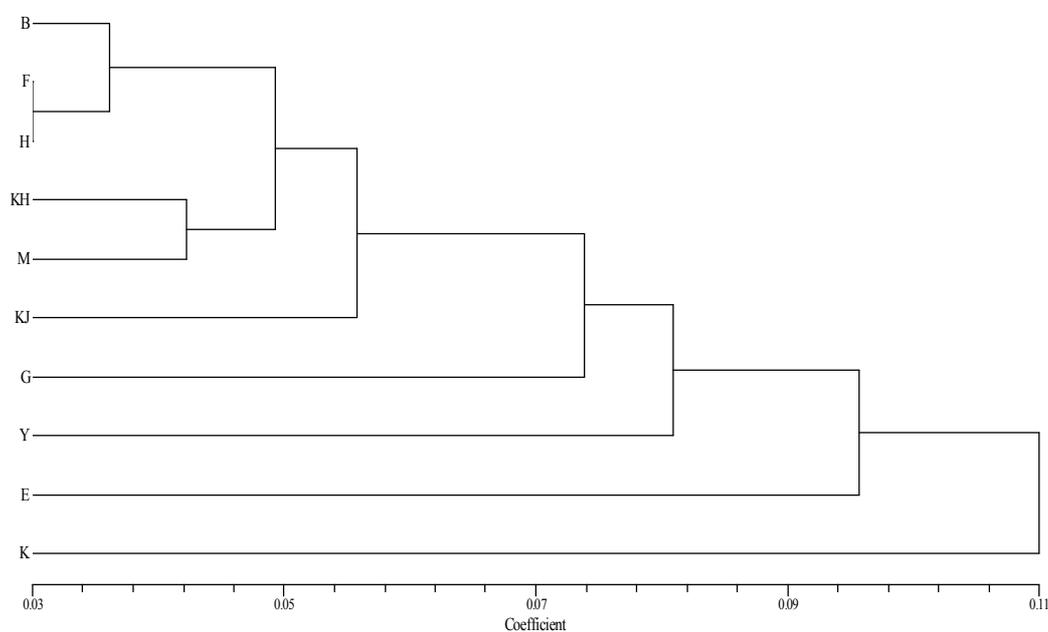
The relationship among populations was estimated based on Nei's genetic distance. The UPGMA dendrogram revealed that clusters were not related to the geographic distance

between populations. Cluster analysis (UPGMA) demonstrated that the populations could be differentiated into six clusters. Cluster I consisted of five populations from Bushehr, Fars, Hormozgan, Khuzestan and Mazandaran, clusters II, III, IV, V and VI were comprised of Khorasan-jonoobi, Golestan, Yazd, Isfahan, and

Kerman provinces, respectively (Fig. 7). In cluster I populations of Hormozgan and Fars showed 100% similarity coefficient. Geographically, both Fars and Hormozgan populations have grown at low latitude and almost in similar climates, which result in high genetic similarity between them.

**Table 6** Nei's genetic distance measured among ten populations of *Macrophomina phaseolina*.

| Population | B     | E     | F     | G     | H     | K     | KH    | KJ    | M     | Y     |
|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| B          | 0.000 |       |       |       |       |       |       |       |       |       |
| E          | 0.084 | 0.000 |       |       |       |       |       |       |       |       |
| F          | 0.028 | 0.064 | 0.000 |       |       |       |       |       |       |       |
| G          | 0.067 | 0.116 | 0.066 | 0.000 |       |       |       |       |       |       |
| H          | 0.038 | 0.070 | 0.027 | 0.062 | 0.000 |       |       |       |       |       |
| K          | 0.095 | 0.184 | 0.087 | 0.120 | 0.094 | 0.000 |       |       |       |       |
| KH         | 0.046 | 0.106 | 0.042 | 0.063 | 0.045 | 0.064 | 0.000 |       |       |       |
| KJ         | 0.050 | 0.094 | 0.055 | 0.092 | 0.050 | 0.109 | 0.051 | 0.000 |       |       |
| M          | 0.048 | 0.093 | 0.045 | 0.071 | 0.050 | 0.075 | 0.039 | 0.056 | 0.000 |       |
| Y          | 0.072 | 0.106 | 0.077 | 0.114 | 0.067 | 0.123 | 0.081 | 0.063 | 0.065 | 0.000 |



**Figure 7** UPGMA cluster using Nei's genetic distance among ten populations of *Macrophomina phaseolina*. B = Bushehr, E = Isfahan, F = Fars, G = Golestan, H = Hormozgan, K = Kerman, Kh = Khuzestan, KJ = Khorasan-jonoobi, M = Mazandaran, Y = Yazd.

## Discussion

*Macrophomina phaseolina* the causal agent of charcoal rot of sesame, is an important fungal pathogen causing economically important crop losses in oil seeds in Iran. Because of the high level of heterogeneity, the use of resistant cultivars was proposed as the most effective and practical method to control charcoal rot of sesame (Reyes-Franco *et al.*, 2006). Understanding genetic diversity within and between populations is essential for the improvement of disease management systems and sesame breeding program against charcoal rot. In this study, the distribution of genetic diversity among 60 *M. phaseolina* isolates from different sesame growing regions in Iran was investigated and URP-PCR and ISSR genomic fingerprinting revealed different levels of heterogeneity in populations of this fungus.

The dendrogram obtained with combination of URP-PCR and ISSR fingerprints showed that isolates of *M. phaseolina* were separated into eight groups. The significant correlation ( $r = 0.83$ ) between cophenetic matrices value generated from ISSR and URP-PCR datasets demonstrated that both molecular markers are powerful tools for analyzing the intraspecific variability among *M. phaseolina* isolates.

The existence of genetic diversity among isolates of *M. phaseolina* in Iran could be due to variations in the climatic conditions and cropping patterns in different parts of the country, also transportation of the pathogen (Aghakhani *et al.*, 2009; Baird *et al.* 2010)

Cluster analysis indicated that *M. phaseolina* isolates obtained from soybean, cotton, and chickpea hosts clearly differentiated into specific groups (Jana *et al.*, 2005b).

In the present study URP-PCR marker revealed a high genetic diversity within and between groups.

Different molecular approaches have been used in the analysis of genetic variation among *M. phaseolina* from various plant hosts and geographic locations.

AFLP assessment of the polymorphism of *M. phaseolina* isolates from sesame in China has shown a high level variations of 73.9% among the isolates (Linhai *et al.*, 2011). ISSR evaluation of *M. phaseolina* populations from soybean and cotton grown in India and USA has shown highly significant genetic heterogeneity among and within populations and isolates could be grouped into three major clusters according to their hosts and geographical region (Jana *et al.*, 2005a). Genetic variation of *M. phaseolina* isolates in India using RAPD marker has grouped the isolates into six categories at 40% genetic similarity (Aghakhani *et al.*, 2009). Using AFLP analysis, Reyes-Franco *et al.* (2006) reported that genetic variation of isolates was not strongly correlated to geographic origin for Mexico and other countries.

Mahdizadeh *et al.* (2011) reported that isolates from 24 host plant species did not clearly differentiate to the specific group according to the host or geographical origins, however, usually the isolates from the same host or the same geographic origin tend to group nearer. The results of this study correspond with above findings that there exists a high genotypic diversity level among *M. phaseolina* isolates.

In populations of *M. phaseolina*, diversity could be attributed to parasexual recombination between nuclear genes or fusion of vegetative cells favoring heterokaryons (Carlile 1986). Factors such as immigration, climatic conditions, different patterns of culture, use of various different host genotypes, high selection pressure and breeding system might explain high levels of genetic diversity of the fungus (Aghakhani *et al.* 2009; Almeida *et al.* 2008; Purkayastha *et al.* 2008). Miao *et al.* (1991) reported that unstable B chromosome may be one of the mechanisms for generating variation in fungi.

According to our results, no clear relationship was found between genetic diversity and the geographical origin of the isolates using URP-PCR and ISSR molecular markers. Previous studies showed that

application of AFLP (Mayek-Perez *et al.*, 2001), ISSR (Jana *et al.*, 2005a) markers could beneficially be used to group isolates based on their geographical areas but there are some reports that they could not clearly be grouped according to geographical areas (Aghakhani *et al.* 2009; Baird *et al.* 2010; Khan *et al.* 2017; Linhai *et al.* 2011; Mahdizadeh *et al.* 2011). Sanchez *et al.* (2017) using SSR markers reported a clear tendency in isolates to cluster by origin, and making evident a genetic distance between Chilean and Spanish strawberry isolates.

The results of the present findings revealed considerable genetic similarities among the isolates from different geographic areas. Hamer *et al.* (1989) provided evidence at the DNA sequence level for pathogen genotype selection by a host species.

We suggest that these isolates may evolve from the same ancestral population. This result is in agreement with the results obtained by Almeida *et al.* (2003) and Linhai *et al.* (2011) on isolates from the hosts as Sorghum, Soybean, Chickpea, Corn and Sesame. In addition, the similarity among isolates of remote origin confirmed free genetic information flux and genetic exchange in the genus, reducing the probability of delimited groups formation (Mihail and Taylor, 1995).

The results showed that the Fars and Golestan populations have the highest heterozygosity among the ten populations. This might be caused by diversity of sesame varieties in the two regions.

Comparison of two molecular marker systems used in this study demonstrated that the average marker index was between 3.28 (ISSR) to 1.56 (URP-PCR). Compared with URP-PCR, ISSR markers are much more informative and powerful tools for assessing variability in *M. phaseolina*.

The low level of observed genetic differentiation could be related to level of gene flow among populations. In addition, the observation of low levels of differentiation in populations could be explained by asexual

propagules and polyphagy of this fungus (Salahlou *et al.*, 2018).

In this study, both genetic differentiation and AMOVA indicated that most of the genetic diversity is within populations. One reason for high genetic diversity within populations might be parasexual recombination among isolates (Carlile, 1986). The high level of genetic variation within a fungal population could be due to the uniformity of its substrate and previous environmental experience (Ijaz *et al.*, 2013). Kendrick, (1992) reported that degree of sexual reproduction within a population is closely related to genetic diversity.

The level of gene flow ( $N_m$ ) was measured at 1.09, demonstrating a very high migration rate between populations. This gene flow might be due to movement of *M. phaseolina* through infected seeds and soil with planting materials by means of vegetative stage and sclerotia and may be occurring through natural dispersal mechanisms involving ascospores (Kohn, 1995). Gene flow induces replacement of new alleles or genotypes in new areas via introduction, reproduction and survival of the introduced organism. The  $G_{st}$  and  $N_m$  indices indicated high gene flow and low genetic differentiation among populations. Gene flow and Genetic differentiation amount generated from both molecular marker systems separately were similar to those generated with URP-PCR and ISSR data. Our survey suggests that frequent gene flow and recombination between populations of *M. phaseolina* have significantly impressed the evolution and development of this fungus in Iran. Gordon and Martyn, (1997) reported that high level of pathogenic and genetic diversity in asexual fungus is also observed with accumulation of genetic mutation over time.

Investigation of genetic variability induced by parasexual reproduction of fungi has two crucial factors including activity of transposons and a high mutation rate of microsatellites (Purkayastha *et al.*, 2008).

In conclusion, we discovered a high level of intraspecific variation among *M. phaseolina* pathogens using URP-PCR and ISSR. Both methods are shown to be useful for population

analysis of *M. phaseolina* isolates. Comparison of the two molecular marker systems used in this study showed that ISSR markers were highly informative to study the polymorphisms among *M. phaseolina* populations causing charcoal rot of Sesame. However, to evaluate genetic diversity among races of *M. phaseolina* the utilization of these molecular marker systems, especially ISSR markers should be considered as one of the priority aims of studies in future. Our study provided primary information on the population genetics of *M. phaseolina* from different geographical regions in Iran, which is essential for sesame breeding program against the charcoal rot.

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## استفاده از نشانگرهای ISSR و URP-PCR در بررسی تنوع ژنتیکی جدایه‌های *Macrophomina phaseolina* روی کنجد در ایران

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دریافت: ۲۶ مهر ۱۳۹۷؛ پذیرش: ۲۵ خرداد ۱۳۹۸

**چکیده:** به منظور بررسی تنوع *Macrophomina phaseolina* عامل پوسیدگی ذغالی کنجد، شصت جدایه از ده منطقه جغرافیایی جمع‌آوری شده، با استفاده از نشانگرهای مولکولی ISSR و URP-PCR مورد تجزیه و تحلیل قرار گرفتند. جدایه‌ها به هشت گروه در سطح تشابه ژنتیکی 78 درصد تقسیم شدند. این نتایج نشان داد که از پنج آغازگر ISSR مورد مطالعه در مجموع ۱۰۵ لوکوس که ۷۷.۱۱ درصد چند شکلی بودند و هشت آغازگر URP-PCR تولید ۱۳۵ لوکوس که ۶۶.۸۴ درصد چند شکلی بودند. این روش‌ها تنوع ژنتیکی قابل توجهی را در بین جدایه‌های ایران نشان داد اما هیچ‌گونه هم‌بستگی بین تنوع ژنتیکی و مناطق جغرافیایی جدایه‌ها مشاهده نشد. تجزیه و تحلیل واریانس مولکولی نشان داد که تنوع ژنتیکی نسبتاً زیاد، ناشی از تفاوت جدایه‌ها در داخل مناطق جغرافیایی است. نتایج یافته‌های حاضر نشان داد که تمایز ژنتیکی کم ( $G_{ST}$ ) و جریان ژن بالا ( $N_m$ ) در میان جمعیت‌ها تأثیر قابل توجهی بر ظهور و سیر تکاملی *M. phaseolina* داشته است.

**واژگان کلیدی:** پوسیدگی ذغالی، *Sesamum indicum*، اثر انگشت دی ان آ، ژنتیک جمعیت