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Genetic Diversity of *Macrophomina phaseolina* Populations, the Causal Agent of Sesame Charcoal Rot Using Inter-Simple Sequence Repeat Markers

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ABSTRACT

***Macrophomina phaseolina* is an important soil-borne pathogen causing charcoal rot in many important crop plants including sesame, in Iran. A total of 60 isolates of *M. phaseolina* were collected from the main sesame producing regions in ten provinces of Iran. The genetic diversity among *M. phaseolina* populations was estimated using Inter-Simple Sequence Repeat (ISSR), focusing particularly on geographic differentiation. Five ISSR primers generated 105 discernible DNA bands, of which 85 (77.11%) were polymorphic. The greatest value of variability (PPB: 60.00%; H: 0.185; I: 0.284) was estimated for Fars population, whereas the least variability (PPB: 9.52%; H: 0.042; I: 0.060) was estimated for Kerman population. Total gene diversity exhibited high levels of variability (H_T = 0.186). Analysis of molecular variance indicated a large proportion of genetic variability within populations.**

Keywords: *Sesamum indicum* L, ISSR, Geographic populations, Genetic differentiation.

INTRODUCTION

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

In order to design optimal breeding methods to produce sesame cultivars that can resist charcoal rot, knowledge of fungus population biology and its interaction with sesame cultivars would be quite useful. The existence of high level variations in a fungal populations increases its capability to adapt to various conditions and overcome host resistance (Trigiano *et al.,* 2008). To determine the level of variability in all organisms basic morphological traits and pathogenesis are usually used as the first character, but often these methods are time-consuming and their quantification is difficult. Molecular methods are an appropriate analysis approach for the rapid evaluation of the variability within and among species (Chakravarthi and Naravaneni, 2006).

In recent years, different DNA markers have been employed to study genetic diversity within populations of *M. phaseolina* such as Restriction Fragment

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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; Reyes-Franco *et al.,* 2006), Universal Rice Primer PCR (URP-PCR) (Jana *et al.,* 2005b), Inter Simple Sequence Repeats (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). For example, analysis of the entire fingerprint profiles indicated no clear association between DNA genotypes and geographical locations (Reyes-Franco *et al.,* 2006). Cluster analysis indicated that *M. phaseolina* isolates obtained from soybean, cotton, and chickpea hosts clearly differentiated into specific groups (Jana *et al.,* 2005b).

The advantages of ISSR over other markers are: (1) low development costs; (2) no prior information or lengthy mapping studies required, and (3) laboratory procedures can be easily applied to any plant species and fungi (Cao *et al.,* 2006). ISSR markers have been used to access taxonomic and phylogenetic comparisons and as a mapping tool in a wide range of organisms (Zietkiewicz *et al.,* 1994). In Iran, dry root occurs primarily in many economically important crops, including common bean, sesame, soybean, chickpea, sunflower and beta, among others, and leads to yield reduction. Seed infection by this pathogen is responsible for its spread in the main agricultural regions of the country. Although *M. phaseolina* has been present for decades in Iran, still information about the population biology of this pathogen in the region is not fully understood. This study was performed in order to reveal the genetic polymorphism and differentiation of *M. phaseolina* populations collected from different sesame growing regions and ten geographical locations of Iran using ISSR markers.

MATERIALS AND METHODS

Fungal Isolates

M. phaseolina isolates were collected from sesame fields showing typical symptoms and signs of charcoal rot in ten Iranian provinces, as presented in Table 1. Species-specific primer pairs (MpKFI/MpKRI) was used for identification of *M. phaseolina* isolates at the molecular level previously described by Babu *et al.* (2007). The DNA from *Sclerotinia sclerotiorum*, *Fusarium graminearum*, *Alternaria alternata*, *Trichoderma viride* and *Cytospora* Ehrenb were used as negative controls (Figure 1).

Genomic DNA Extraction

Fungal mycelia were grown on potato

Table 1. Source and number of isolates of each *M. phaseolina* population in this study.

Population	Source	Number of isolates
B	Bushehr	8
E	Esfahan	2
F	Fars	12
G	Golestan	4
H	Hormozgan	7
K	Kerman	3
KH	Khozestan	10
KJ	Khorasan- jonoobi	4
M	Mazandaran	7
Y	Yazd	3

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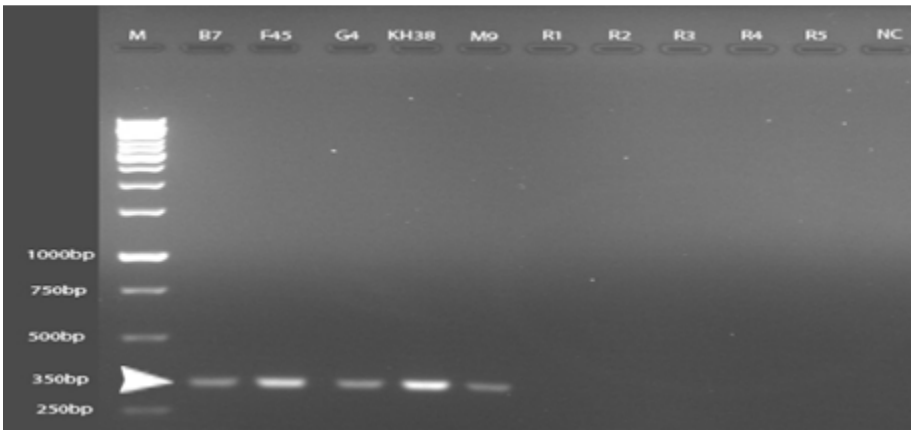

Figure 1. Template DNA was amplified using species-specific primer pair (MpKFI/MpKRI), M: 1 kb molecular ladder; B7, F45, G4, KH38 and M9: *M. phaseolina* isolates from Iran; R1: *Sclerotinia sclerotiorum*, R2: *Fusarium graminearum*, R3: *Alternaria alternata*, R4: *Trichoderma viride* and R5: *Cytospora*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

dextrose broth (PDB) for 5 days at 25 °C in darkness. Mycelium biomass was harvested by filtering through Whatman No. 1 paper, washed with sterile water and lyophilized. Samples were ground to fine powder in liquid nitrogen and stored at −80 °C. Genomic DNA was extracted from fresh mycelia according to the procedure of Safaie *et al.* (2005). The quantity and quality of DNA samples were determined by spectrophotometer (Eppendorf Biophotometer, Germany) and electrophoresis, and were stored at −20 °C until used.

Primers and Polymerase Chain Reaction (PCR)

The sequences of five ISSR primers used in this study were synthesized by CinnaGen, Tehran, and are shown in Table 2. The PCR reaction was performed in a 20-µL reaction volume containing 20-30 ng of DNA template, 1 mM primer, 200 mM each of four deoxyribonucleotide triphosphate (dNTPs), 1.5 units of Taq polymerase (CinnaGen, Tehran), 2 mM $MgCl_2$ and 2.5 mL PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0). PCR amplification was carried out with the thermocycler (Eppendorf, Mastercycler, gradient) system. The PCR conditions included initial denaturation at 95 ^{o}C for 4 min followed by 35 cycles of denaturation at 94 ^{o}C for 1 min, primer annealing according to Table 2 for 1 min, primer extension at 72 ^{o}C for 2 min, and a final primer extension at 72 ^{o}C for 7 min. The amplification product was analyzed on a 1.4% agarose gels containing 0.50 µg/mL ethidium bromide and then photographed.

Table 2. Primer names and sequences, annealing temperature, marker index (MI), effective multiplex ratio (EMR) and degree of polymorphism of amplified DNA using each of the 5 primers of ISSR.

Primer	Sequence	Annealing temperature	% polymorphism	EMR	MI
ISSR10	5'-CACCACCACCACCAC-3'	59 °C	82.2	22.92	4.56
ISSR02	5'-ACTG ACTGACTGACTG -3'	48 °C	82.2	14.45	4.24
ISSR09	5'-CCACCACCACCACCA -3'	55 °C	83.3	14.22	2.16
PcMs	5'-GTCGTCGTCGTCGTCGTCGTC-3'	55 °C	55.5	4.27	1.28
P4	5'-ATGATGATGATGATGATG -3'	51 °C	88.23	13.86	4.16

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Data Analysis

Amplified fragments were scored for the presence (1) and absence (0) of bands. Nei´s (1973) genetic diversity (h), Shannon index (Lewontin, 1972) the observed number of alleles per locus (Na), the effective number of alleles per locus (Kimura and Crow, 1964) and percentage of polymorphic bands (PPB) were calculated to estimate genetic diversity within each population.

The total genetic diversity (H_T), genetic diversity within each population (H_s), and coefficient of gene differentiation (G_{st}) were also determined according to Nei (1986). Nei's unbiased genetic distance and genetic identity were calculated for each population pair using the program POPGENE version 1.31 Software (Nei, 1972). Dendrogram based on unbiased genetic distance (Nei, 1972) was generated using UPGMA of NTSYS-pc version 2.02c (Rohlf, 2000) to examine the genetic relationship among populations. The gene flow estimates (Nm) among these populations were computed as Nm ¼ (1/Gst _ 1)/4 (Nei, 1973; Slatkin and Barton, 1989). An analysis of molecular variance (AMOVA) was preformed to calculate the hierarchical apportionment of variation using GenAlEx 6.41 (Peakall and Smouse, 2006).

RESULTS

Identification of *M. phaseolina* Isolates by Species Specific Primers

The isolates were identified at the molecular level using species specific primers (MpFI/MoKRI) as designed by Babu *et al.* (2007). A fragment of 350-bp was amplified by the primer pair in tested isolates (Figure 1). The result showed that the isolates collected belonged to the species *M. phaseolina*. No fragment was detected in other fungi used as negative control.

Genetic Diversity among and within Populations

The percentage of polymorphism for each population varied from 60% (Fars) to 9.52% (Kerman), with an average of 35.90% at the population level. The observed number of alleles (Na) per locus ranged from 1.114 (Esfahan) to 1.600 (Fars) with an average of 1.350, while the effective number of alleles (Ne) ranged from 1.312 (Golestan) to 1.076 (Kerman) with an alleles average of 1.210 (Table 3).

The values of Nei's genetic distance

Table 3. Genetic diversity of ten populations of *M. phaseolina*.

Population	Na [a]	Ne [b]	H [c]	I [d]	Polymorphic loci	PPB% [e]
B	1.504(0.502)	1.298(0.364)	0.176(0.195)	0.265(0.281)	53	50.48
E	1.114(0.319)	1.114(0.319)	0.057(0.159)	0.079(0.221)	12	11.43
F	1.600(0.492)	1.307(0.357)	0.185(0.188)	0.284(0.268)	63	60.00
G	1.419(0.495)	1.312(0.390)	0.176(0.212)	0.255(0.305)	44	41.90
H	1.428(0.497)	1.266(0.354)	0.157(0.194)	0.234(0.282)	45	42.86
K	1.095(0.295)	1.076(0.236)	0.042(0.131)	0.060(0.187)	10	9.52
KH	1.542(0.500)	1.273(0.326)	0.169(0.180)	0.261(0.263)	57	54.29
KJ	1.219(0.415)	1.146(0.287)	0.086(0.166)	0.128(0.244)	23	21.90
M	1.457(0.500)	1.255(0.325)	0.157(0.184)	0.238(0.272)	48	45.71
Y	1.209(0.408)	1.167(0.327)	0.093(0.181)	0.133(0.260)	22	20.95

[a] Observed number of alleles per locus; [b] effective number of alleles per locus; [c] Nei's (1973) gene diversity: [d] Shannon's information index; [e] percentage of polymorphic bands. Values of standard deviation in are given parentheses.

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ranged from 0.0281 to 0.1721 in 10 populations. The lowest value for genetic distance was obtained between populations of Fars and Bushehr and showed that the populations from these two provinces were most similar. The highest value based on Nei's gene diversity (0.185) was obtained for Fars and the lowest for Kerman (0.042) with an average of 0.130. The Shannons index (0.284 and 0.060) was obtained for the same populations as in the Nei's gene diversity case. This result indicates that population of Fars had the highest level of variability, while the Kerman population exhibited the lowest diversity (Table 5).

Genetic Differentiation

The total gene diversity (H_t) and mean diversity within each population (H_s) were 0.186 and 0.13, respectively, for these ten populations. The mean coefficient of gene differentiation (G_{st}) among populations was 0.303, indicating 30% of differentiation between populations.

The amount of gene flow (N_m) between populations was 1.14, 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 (Table 6).

Significant correlation was found between Shannon index and the percentage of polymorphism for all populations (R=0.87, P=0.001) suggesting that ISSR markers were unevenly distributed in the populations. This result shows that ISSR divergence among populations was mainly attributed to difference of the DNA-bands frequency instead of allele fixation.

ISSR Polymorphism

The five ISSR primers produced 105 scorable bands, of which 85 (77.11%) were polymorphic (Figure 2). In terms of investigating the discriminatory feature of ISSR primers, effective multiplex ratio and marker index were calculated for all the primers. The EMR of the five primers ranged from 22.92 (ISSR10) to 4.27 (PcMs) with an average of 13.94 and marker index values were between 1.28 (PcMs) and 4.56 (ISSR10) (Table 2).

Genetic Relationship

Genetic identities among populations ranged from 0.9722 to 0.8419 with an average of 0.9292. The greatest genetic identity was obtained among Hormozgan, Fars, and Bushehr populations and indicated that the populations from these three regions were most similar.

In this study, the relationship among populations was examined based on Nei's genetic distance. Cluster analysis demonstrated that clusters were not related to the geographic distance between populations. The UPGMA analysis showed that the populations could be separated into four clusters. Cluster I consisted of seven populations from Bushehr, Fars, Hormozgan, Khozestan, Mazandaran, Khorasan-jonoobi and Yazd, while clusters II, III, and IV was comprised of Esfahan, Golestan, and Kerman provinces, respectively (Figure 3). In cluster I, populations of Bushehr and Fars showed 100% similarity coefficient. Both Fars and Bushehr are located in south and share similar climates. The co-phenetic correlation was r= 0.76.

Analysis of Molecular Variance

AMOVA analysis showed that, from the total genetic variability, 9% ($p<0.01$) could be attributed by the differences among populations while 91% ($p<0.01$) could be

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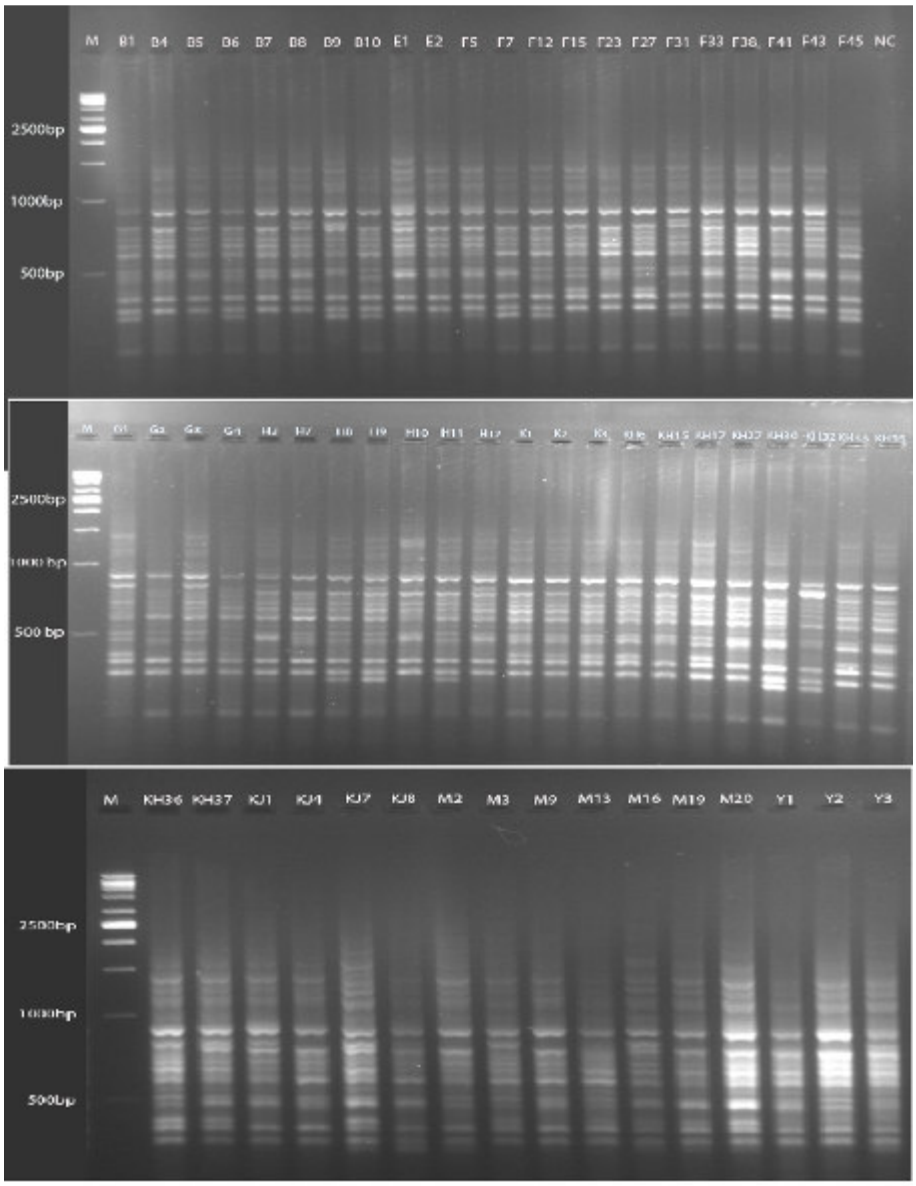

Figure 2. DNA fingerprint profiles of 60 isolates of *M. phaseolina* obtained with PcMs: NC Negative control; B: Bushehr; E: Esfahan; F: Fars; G Golestan; H: Hormozgan; K: Kerman; KH: Khozestan; KJ: Khorasan-jonobi; M: Mazandaran; Y: Yazd; M: molecular marker GeneRuler™ 1 kb DNA Ladder.

Table 4. Nested AMOVA for *M. phaseolina* populations.

Source	Df	Sum of squares	Mean squares	Est. Var.	%
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%

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Table 5. Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) between populations of *M. phaseolina*.

Population	B [a]	E [b]	F [c]	G [d]	H [e]	K [f]	KH [g]	KJ [h]	M [i]	Y [j]
B	0	0.928	0.9722	0.9263	0.971	0.9239	0.9582	0.9519	0.9694	0.9393
E	0.0747	0	0.9425	0.8834	0.937	0.8419	0.9029	0.9341	0.9126	0.8995
F	0.0281	0.0592	0	0.9207	0.9722	0.9154	0.9594	0.9435	0.9539	0.918
G	0.0766	0.124	0.0827	0	0.9412	0.8882	0.932	0.8922	0.9184	0.8613
H	0.0294	0.065	0.0282	0.0606	0	0.9318	0.9598	0.9487	0.9504	0.928
K	0.0791	0.1721	0.0883	0.1186	0.0707	0	0.9308	0.8988	0.9252	0.888
KH	0.0427	0.1022	0.0414	0.0705	0.0411	0.0718	0	0.9465	0.9552	0.9184
KJ	0.0493	0.0682	0.0582	0.1141	0.0527	0.1067	0.055	0	0.9464	0.9463
M	0.0311	0.0914	0.0472	0.0852	0.0508	0.0777	0.0458	0.0551	0	0.9254
Y	0.0626	0.1059	0.0856	0.1493	0.0748	0.1188	0.0851	0.0552	0.0776	0

[a] Bushehr, [b] Esfahan, [c] Fars, [d] Golestan, [e] Hormozgan, [f] Kerman, [g] Khozestan, [h] Khorasan- jonoobi, [i] Mazandaran, [j] Yazd.

Table 6. Genetic structure estimated by Nei's genetic parameters for ten populations of *M. phaseolina* from ten Iranian provinces

Eatimative	H_T [a]	H_S [b]	G_{ST} [c]	N_m [d]
Mean	0.186	0.13	0. 303	1.146
St. Dev	0.328	0.015		

[a] Total genetic diversity in the pooled populations, [b] Mean diversity within each population, [c] The mean coefficient of gene differentiation (Gst), [d] Estimate of gene flow from Gst, Nm = 0.5(1 - Gst)/Gst.

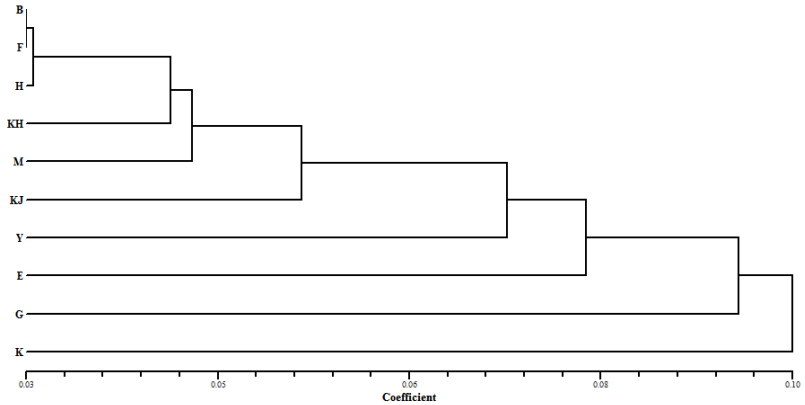

Figure3. UPGMA dendrogram for populations of *M. phaseolina* based on Nei's genetic distance.

attributed by the differences within populations (Table 4).

DISCUSSION

ISSR genomic fingerprinting revealed a high level of heterogeneity among M. *phaseoina* populations. 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 asexuall propagules and polyphagous nature of this fungus.

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 genotypes of different hosts, high selection pressure, and breeding system have been used to explain high levels of genetic diversity fungus (Aghakhani and Dubey, 2009; Almeida *et al.,* 2008; Purkayastha *et al.,* 2008).

AFLP assessment of the polymorphism of *M. phaseolina* isolates from sesame in China has shown high level of variations (73.9%) among the isolates (Linhai *et al.,* 2011). ISSR evaluation of *M. phaseolina* populations originated from soybean and cotton grown in India and the 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 isolated into six categories with 40% genetic similarity (Aghakhani and Dubey, 2009). The results of the present study are in corroboration with the above findings that there are a high genotypic diversity levels among *M. phaseolina* isolates. The results showed that populations originating from Fars and Golestan provinces have the highest heterozygosity (18.5%) among the ten tested populations. This might be caused by diversity of sesame varieties in these two regions.

The high similarity observed among populations from Fars, Bushehr, and Hormozgan provinces is a powerful finding, suggesting that the isolates used in this study did not evolve independently from each other and that these populations might originate from the same ancestor (Almeida *et al.,* 2003). Also, the observed similarity among isolates from different geographic areas confirmed genetic exchange and free genetic information flux in the genus, reducing the probability of formation of delimited groups (Mihail and Taylor, 1995).

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In this study, both genetic differentiation and AMOVA indicated that most of the genetic diversity existed within the populations. The existence of high levels of variations within populations, the expression of disease resistance to *M. phaseolina* might be due to genetic diversity of the pathogen. One reason for high genetic diversity within populations might be parasexual recombination among isolates (Carlile, 1986).

The level of gene flow (N_m) was measured at 1.14, 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 the means of vegetative stage and sclerotia or may occur 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.

These results confirmed 9% and 19.42% of the genetic variation existing between populations, as measured by AMOVA analysis and Shannon's diversity index, respectively. In conclusion, because of high levels of variations among the isolates of *M. phaseolina* in Iran, to control charcoal rot, different disease management systems should be integrated by combining chemical, cultural, and biological methods.

Our study provided preliminary information about the population genetics of *M. phaseolina* from different geographical regions in Iran. The results of the present research indicated that ISSR are appropriate markers to determine genetic diversity among populations of *M. phaseolina.*

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بررسی ساختار ژنتیکی جمعیت های *Macrophomina phaseolina* عامل ایجاد کننده پوسیدگی ذغالی کنجد با استفاده از نشانگر ISSR

ر. صلاحلو، ن. صفایی، و م. شمس بخش

چکیده

قارچ *M. phaseolina* یکی از مهمترین بیمارگرهای خاکبرد است که باعث بیماری پوسیدگی ذغالی در بسیاری از گیاهان زراعی مهم، از جمله کنجد، در ایران می‌شود. تعداد ۶۰ جدایه *M. phaseolina* از مناطق اصلی تولید کننده کنجد کشور شامل ۱۰ استان جمع آوری گردید. تنوع ژنتیکی میان جمعیت‌های *M. phaseolina* با استفاده از نشانگر ISSR با تاکید بر تمایز جغرافیایی بررسی گردید. پنج آغازگر ISSR در مجموع ۱۰۵ نوار قابل امتیاز دهی ایجاد شد که در این بین ۸۵(۷۷.۱۱٪) قطعه چند شکلی بودند. بیشترین مقدار تنوع (PPB=۶۰٪ ، H=۰/۱۸۵، I= ۰/ ۲۸۴)برای جمعیت فارس محاسبه شد. در حالی که کمترین مقدار تنوع (PPB=۹/۵۲٪ ، H=۰/۰۴۲، I= ۰/ ۰۶۰). برای جمعیت کرمان دیده شد. شاخص تنوع ژنی کل میزان بالایی از تنوع را نشان داد (H_T ۰/۱۸۶ =). نتایج تجزیه واریانس مولکولی نشان داد که سهم بالایی از تنوع ژنتیکی مربوط به تنوع درون جمعیت ها می باشد.

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