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Studying Genetic Diversity in *Zymoseptoria tritici*, Causal Agent of Septoria Tritici Blotch, by Using ISSR and SSR Markers

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ABSTRACT

Seventy-five isolates of the fungus *Zymoseptoria tritici*, collected from seven wheat producing provinces in Iran, were studied using the molecular markers ISSR and SSR to determine its structure and genetic variations within its populations. Results indicated that the populations of the causal agent of this disease had a relatively high level of genetic diversity, with the total average genetic diversity (Ht) of 0.34 in the studied samples. When the genetic diversity was divided by the genetic diversity within and between the tested fungal populations, intra-population and inter-population genetic diversities were, respectively, 69 and 31%. Moreover, results showed that there was a strong gene flow between the studied provinces. The maximum genetic diversity among the studied provinces was observed in Ardebil and Khuzestan Provinces, and cluster analysis also revealed that the fungal populations of these two provinces had the greatest similarity with each other.

Keywords: Gene flow, Intra-population genetic diversity, Molecular markers, Wheat.

INTRODUCTION

The fungus *Zymoseptoria tritici* (Desm) (Quaedvlieg *et al.*, 2011) (teleomorph *Mycosphaerella graminicola*), which was previously named *Septoria tritici*, is the causal agent of *Septoria Tritici* Blotch (STB), which is one of the most important pathogenic agents in Dothideomycetes Class.

Knowledge of genetic diversity in various strains of pathogenic agents is of great importance in breeding wheat cultivars for producing cultivars resistant to STB (McDonald *et al*., 1995).

Numerous studies have shown that the population structure of *Zymoseptoria tritici* has high genetic diversity. Czembor and Arseniuk (1999) studied several species of *Septoria* genera and demonstrated that SSRs and ISSRs were the most sensitive markers for investigating DNA polymorphism in this subgenus. The genetic structure of *Zymoseptoria tritici* has been previously studied using markers including SCA, SRRs, RFLP, AFLP, and RAPD (Kema *et al.*, 2002; Linde *et al*., 2002; Talbot, 2015).

Schnieder *et al*. (2001) studied isolates of this fungus in Germany and showed that diversity of these isolates was related to their geographical separation. Razavi and Hoghes (2004) studied 90 isolates collected from western Canada using the RAPD marker and found that these isolates exhibited high

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molecular diversity. It was also shown in a study carried out in Kansas (United States of America) that isolates on micro-plot, macro-plot, and state-wide scales showed diversity of 98 percent (Kabbage *et al*., 2008). In a study conducted in Argentina in 2010 using ISSRs, 81 haplotype groups were identified from 126 isolates of *Mycosphaerella graminicola* collected in bread wheat from various parts of the country (Castillo *et al*., 2010).

Knowledge of genetic structure of fungi is necessary to adopt management strategies for controlling this disease. Considering the greater efficiency and speed of the ISSR and SSR markers, this research aimed to use them to study genetic diversity in this fungus.

MATERIALS AND METHODS

Collection and Purification of Isolates

During travels to various regions in Iran (in the years 2014 and 2015), wheat fields were visited and samples of leaves with symptoms of STB were collected from Ilam, Khuzestan, Hamadan, Lorestan, Markazi, Golestan, and Ardebil Provinces and transferred to the laboratory. The infected leaves were dried first to isolate the causal agent of the disease when the suitable opportunity arose. For long-term storage of these colonies, their yeast-like spores were collected and transferred to Eppendorf tubes and kept at -20˚C. In total, 75 isolates were selected for study (Table 1).

DNA Extraction

The CTAB method (Doyle and Doyle, 1990) was used with minor alterations to extract DNA from the fungal samples. About 25-50 mg of the isolated fungal spores were weighed out and poured into a sterile porcelain mortar that was cooled in freezer at -20°C beforehand, and were turned into a fine powder by adding liquid nitrogen and crushing them. Five µL of the extracted DNA were run on 0.8% agarose gel to study DNA quality. The extracted DNA was diluted to the concentration of 20 ng μL^{-1} that was further used in Polymerase Chain Reaction (PCR).

ISSR-PCR

Ten ISSR primers supplied by the SinaClon Company (Iran) were selected according to previous data (Czembor and Arseniuk, 1999) and they used to amplify DNA fragments (Table 2).

For each PCR, 13 µL of PCR Master Mix, 1 µL of DNA, 1 µL of the primer, and 10 µL of twice-sterilized distilled water were used. The PCR master mix was supplied by either the SinaClon Company of (Iran) or the Ampliqon Company (Denmark) and contained 1.5 mM of $MgCl_2$, 0.8 mM dNTPs, and 0.4 unit of *Taq* polymerase.

Amplification was carried out in a thermocycler (Bio-Rad) and the PCR cycle was as follows: initial denaturation for 2 minutes at 94˚C, then, 40 cycles of denaturation for 2 minutes at 94˚C, annealing 40 seconds at the tested temperature of 48-53˚C and elongation for 1 minute at 72˚C. The final elongation cycle was extended to 10 minutes.

SSR-PCR

Four pairs of primers supplied by the Macrogen Inc. (South Korea) were used to amplify SSR markers (Owen *et al*., 1998) (Table 3) and PCR mixture was composed with the same ingredients as above but only master mix supplied by the Ampliqon Company (Denmark) was used for the amplification.

Amplification was carried out in the thermocycler (Bio-Rad) and was programed for initial denaturation for 2 minutes at 94˚C , 35 cycles of denaturation for 2 minutes at 94˚C, annealing for 35 seconds at the tested temperatures of 52-56˚C, and elongation for

Table 1. Codes, names, origins, and host of collection of *Zymoseptoria tritici* isolates used in this study.

No	Name of isolate	Province	Origin (Location)	Host
1	IR1	Ardebil	Moghan	Bread Wheat
2	IR2	Khuzestan	Zeydoon	Bread Wheat
3	IR3	Khuzestan	Dezful	Bread Wheat
4	IR4	Khuzestan	Dezful	Bread Wheat
5	IR5	Khuzestan	Shushtar	Bread Wheat
6	IR6	Khuzestan	Zeydoon	Bread Wheat
7	IR7	Khuzestan	Zeydoon	Bread Wheat
8	IR8	Khuzestan	Zeydoon	Bread Wheat
9	IR9	Ardebil	Moghan	Bread Wheat
10	IR10	Ardebil	Moghan	Bread Wheat
11	IR11	Khuzestan	Shushtar	Bread Wheat
12	IR12	Khuzestan	Safiabad	Bread Wheat
13	IR13	Khuzestan	Dezful	Triticale
14	IR14	Lorestan	Noorabad	Bread Wheat
15	IR15	Khuzestan	Safiabad	Durum Wheat
16	IR16	Golestan	Araghi Mahale	Bread Wheat
17	IR17	Ardebil	Moghan	Bread Wheat
18	IR18	Ardebil	Moghan	Bread Wheat
19	IR19	Lorestan	Noorabad	Bread Wheat
20	IR20	Khuzestan	Safiabad	Durum Wheat
21	IR21	Khuzestan	Safiabad	Triticale
22	IR22	Khuzestan	Andimeshk	Dry land Wheat
23	IR23	Khuzestan	Shush	Bread Wheat
24	IR24	Khuzestan	Safiabad	Durum Wheat
25	IR25	Ardebil	Moghan	Bread Wheat
26	IR26	Khuzestan	Sardasht	Dry land Wheat
27	IR27	Ardebil	Moghan	Bread Wheat
28	IR28	Markazi	Arak	Bread Wheat
29	IR29	Ardebil	Moghan	Bread Wheat
30	IR30	Khuzestan	Safiabad	Durum Wheat
31	IR31	Khuzestan	Shushtar	Bread Wheat
32	IR32	Golestan	Araghi Mahale	Bread Wheat
33	IR33	Lorestan	Noorabad	Bread Wheat
34	IR34	Khuzestan	Safiabad	Bread Wheat
35	IR35	Lorestan	Noorabad	Bread Wheat
36	IR36	Lorestan	Noorabad	Bread Wheat
37	IR37	Khuzestan	Sardasht	Dry land wheat
38	IR38	Khuzestan	Sardasht	Dry land wheat
39	IR39	Ardebil	Moghan	Bread Wheat
40	IR40	Ardebil	Moghan	Bread Wheat
41	IR41	Lorestan	Aleshtar	Bread Wheat
42	IR42	Khuzestan	Sardasht	Dry land Wheat
43	IR43	Khuzestan	Sardasht	Dry land Wheat
44	IR44	Khuzestan	Shushtar	Bread Wheat
45	IR45	Khuzestan	Zeydoon	Bread Wheat
46	IR46	Lorestan	Noorabad	Bread Wheat
47	IR47	Khuzestan	Zeydoon	Bread Wheat
48	IR48	Khuzestan	Sardasht	Dry land Wheat
49	IR49	Khuzestan	Sardasht	Bread Wheat
50	IR50	Golestan	Gorgan	Bread Wheat
51	IR51	Khuzestan	Zeydoon	Bread Wheat
52	IR52	Ilam	Ilam	Bread Wheat
53	IR53	Ilam	Ilam	Bread Wheat
54	IR54	Golestan	Araghi Mahale	Bread Wheat
55	IR55	Ilam	Mehran	Bread Wheat
56	IR56	Ilam	Mehran	Bread Wheat
57	IR57	Ilam	Mehran	Bread Wheat
58	IR58	Ilam	Mosian	Bread Wheat
59	IR59	Ilam	Mosian	Bread Wheat
60	IR60	Hamadan	Jokar	Bread Wheat
61	IR61	Hamadan	Jokar	Bread Wheat
62	IR62	Hamadan	Jokar	Bread Wheat
63	IR63	Hamadan	Jokar	Bread Wheat
64	IR64	Hamadan	Jokar	Bread Wheat
65	IR65	Hamadan	Jokar	Bread Wheat
66	IR66	Hamadan	Jokar	Bread Wheat
67	IR67	Hamadan	Jokar	Bread Wheat
68	IR68	Hamadan	Jokar	Bread Wheat
69	IR69	Hamadan	Jokar	Bread Wheat
70	IR70	Hamadan	Jokar	Bread Wheat
71	IR71	Khuzestan	Shushtar	Bread Wheat
72	IR72	Ardebil	Moghan	Bread Wheat
73	IR73	Golestan	Gorgan	Bread Wheat
74	IR74	Ardebil	Moghan	Bread Wheat
75	IR75	Khuzestan	Shushtar	Bread Wheat

Table 2. ISSR markers used in this study.

No.	ISSR marker	Marker sequence 5′→3′	Annealing temperature (°C)	Number of polymorphic bands	*PIC*
1	(GACA)4	*GACAGACAGACAGACA*	48	6	0.36
2	(AAC)7	*AACAACAACAACAACAACAAC*	52	7	0.41
3	(ATC)7	*ATCATCATCATCATCATCATC*	52	8	0.43
4	(AC)9	*ACACACACACACACACAC*	53	8	0.48
5	(AAG)7	*AAGAAGAAGAAGAAGAAGAAG*	52	11	0.41
6	(AG)9	*AGAGAGAGAGAGAGAGAG*	53	9	0.46
7	(AGC)5	*AGCAGCAGCAGCAGC*	52	8	0.47
8	(CAG)5	*CAGCAGCAGCAGCAG*	52	8	0.48
9	(GTG)5	*GTGGTGGTGGTGGTG*	52	12	0.48
10	(GACAC)3	*GACACGACACGACAC*	48	11	0.47

Table 3. SSR markers used in this study.

No	SSR marker	Primer sequence 5′→3′ (forward)	Primer sequence 3′→5′ (reverse)	Annealing temperature (°C)
1	ST1E7	*GATCTCGAGCAGGGCGGAAGT*	*TCACACGCTGGTCTGTGAATC*	54
2	ST2E4	*GAAGATCAACAGCAT GGGCGG*	*CTCCAGAGGGATCACAAAGGC*	56
3	ST1D7	*ATCCTCCATTCACTACTGCAT*	*TGTGGAACAGGAATAGGCTTG*	56
4	ST1G7	*ATGCTGAGAAGTTCGGTGAGG*	*CGTTCTTCCACCTCCTCCAACACT*	52

1 minute at 72˚C.The final elongation cycle was extended to 10 minutes.

After the amplification, PCR products were electrophoresed on 1.5% agarose gel at 90 volts for 1 hour (although it is probably insensitive to ±10% variation in fragment size). After electrophoresis, gels were photographed on a UV transilluminator.

Analysis of Molecular Data

The gel images were evaluated for presence (score 1) or absence (score 0) of specific amplicons. The PopGene version 1.32 (Yeh *et al*., 1999) and GenAlex version 6.501 (Peakall and Smouse, 2006) software were applied to calculate the number and percentage of polymorphic markers, Polymorphism Information Content (PIC) of each primer, Nei's genetic diversity (h), Shannon's Index (I), Nei's genetic Distance (D), total genetic diversity (Ht), inter-population diversity (Gst), and intra-population diversity (Hst), (Yeh *et al*., 1999). The NTSYSpc version 2.02e software was applied for analysis of the genetic relationship among isolates according to Dice's similarity coefficient and the Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Rohlf, 2010).

RESULTS AND DISCUSSION

All of the 10 ISSR primers produced polymorphic bands that could be scored, and the lengths of the amplified DNA fragments varied from 100 to 3,000 bp, but only bands 100 to 1,500 bp long were used in analyses and weak bands were removed. Only two of the SSR primers (ST1D7 and ST1G7) produced distinct band pattern. In total, 91 polymorphic bands were considered in further analyses. The *PIC* index, varied from 0.36 to 0.48 (Table 3).

Results of Cluster Analysis

Based on molecular data, the diversity of isolates was distributed in a completely homogeneous manner within and between the sampled populations (Figure 1). Based on this dendrogram, the 75 studied samples were put within their distinctly related populations.

Results of the cluster analysis indicated that two clusters had the maximum number of isolates. One of them included most of the isolates from Lorestan, Hamadan, Markazi, and Ilam Provinces, and the other most of the isolates from Khuzestan and Ardebil Provinces.

Genetic Diversity of the Populations

Statistical analysis of genetic diversity within and between populations of *Zymoseptoria tritici* in Iran indicated that the maximum number of polymorphic loci belonged to the isolates from Khuzestan Province, with eighty-nine loci (97.8%). This was followed by the isolates from Ardebil Province with eighty-seven loci (95.6%) and the minimum to the isolates

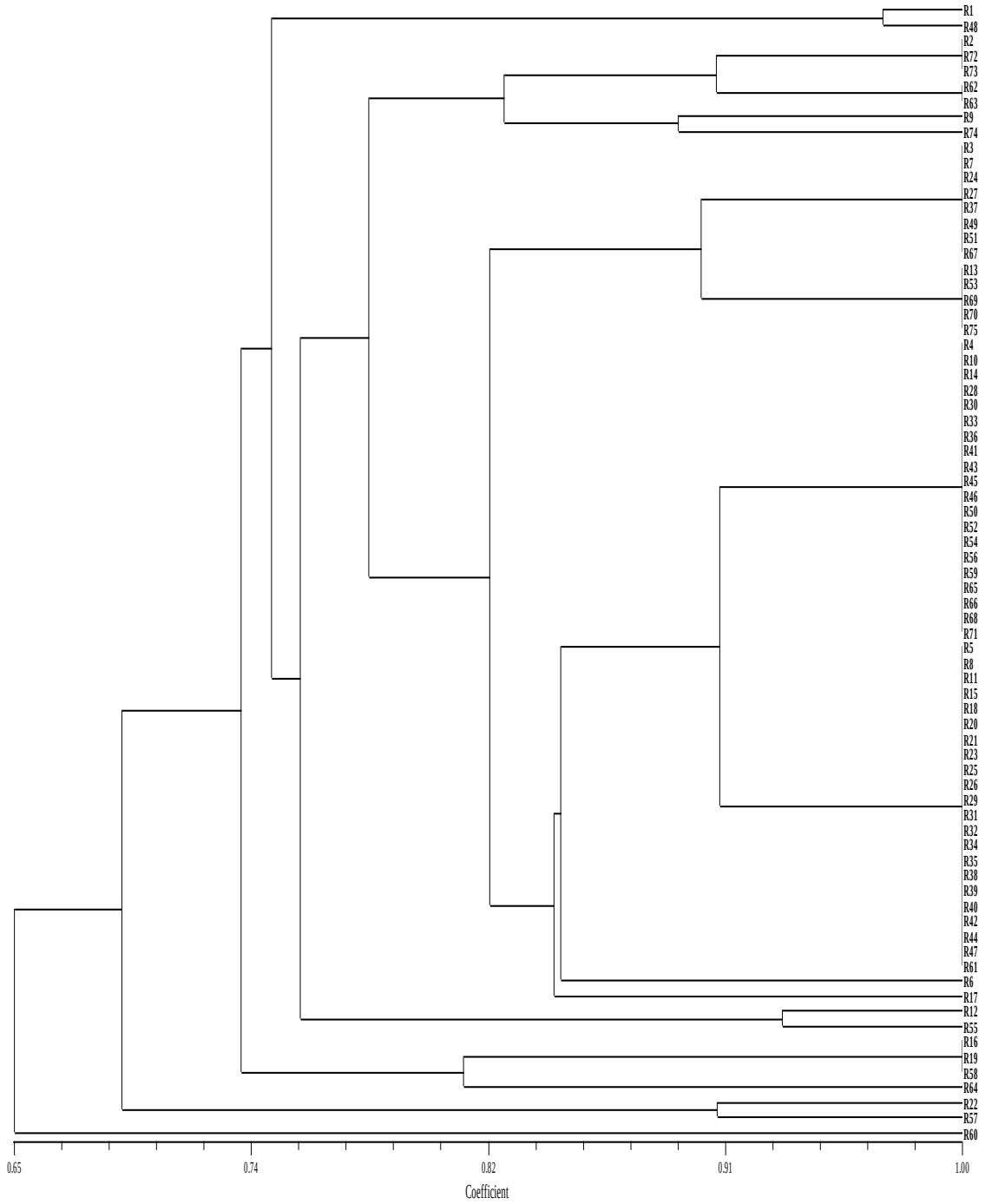

Figure 1. Dendrogram for 75 isolates of *Zymoseptoria tritici*, based on DNA polymorphism revealed by ISSR and SSR molecular markers using Unweighted Pair Group Method of Arithmetic average (UPGMA) from pairwise comparisons employing the Dice genetic similarity coefficient.

from Ilam Province with thirty-nine loci (42.86%) (Table 4).

The largest genetic diversity based on Nei's genetic diversity (h) was that of Ardebil Province (0.3382) and the smallest that of Ilam Province (0.166).

Results of this research showed that the fungus causing STB in Iran had relatively high genetic diversity (Table 5). The average total genetic diversity was 0.34, of which 0.23 (equivalent to 69%) was that of intra-population diversity and the rest (31%) was that of inter-population diversity. Therefore, most of the genetic diversity was contributed by intra-population diversity.

Analysis of Molecular Variance

Analysis of Molecular Variance (AMOVA) showed significant variance within and between populations and was highly related (91%) to variation within populations (Table 6).

Grouping of Populations Based on Genetic Diversity

Genetic grouping of populations based on Nei's genetic diversity showed that the maximum similarity existed between the populations collected in Khuzestan and Ardebil Provinces (Table 7 and Figure 2).

The word population cannot be an accurate definition for pathogens because, allelically, pathogens in various regions are basically combinations of various isolates that can behave as a population (Boeger *et al*., 1993). Nevertheless, study of genetic diversity in pathogenic agents attracts the interest of plant pathologists in many ways. Therefore, researchers search for causes of and influential factors in pathogenicity of

Table 4. Genetic diversity of the studied population *Zymoseptoria tritici* using Nei's genetic diversity index and Shannon index.

Population	Nei's genetic diversity index	Shannon index	Observed alleles means	Effective alleles means	Number of polymorphic loci	Polymorphic percentage
Khuzestan	0.3376	0.5081	1.9780	1.5678	89	97.8%
Lorestan	0.2741	0.4082	1.7473	1.4686	68	74.73%
Ilam	0.1660	0.2438	1.4286	1.2913	39	42.86%
Markazi	0	0	1	1	0	0
Ardebil	0.3382	0.5060	1.9560	1.5791	87	95.60%
Golestan	0.2777	0.4067	1.7033	1.4913	64	70.33%
Hamedan	0.2383	0.3595	1.7253	1.4061	44	72.53%
Means	0.3517	0.5258	2	1.6030		

Table 5. The genetic diversity indices of all populations *Zymoseptoria tritici*.

	Total genetic diversity(Ht)	Inter populations genetic diversity (Hs)	Gst	Nm
Total Populations indices	0.3377	0.2331	0.3087	1.1197

Table 6. Analysis of Molecular Variance (AMOVA) for 75 *Zymoseptoria tritici* isolates.

Source	*Df*	*SS*	*MS*	*Est Var*	%	*Pr> F*
Among Pops	5	212.911	42.582	2.047	9%	<0.0001**
Within Pops	68	1366.562	20.096	20.096	91%	<0.0001**
Total	73	1579.473		22.144	100%	

** Significant at *P*= 0.01.

Table 7. Pairwise population matrix of mean pop binary genetic distance.[a]

khuzestan	lorestan	Ilam	Ardebil	Golestan	Hamadan	
****	0.9378	0.8309	0.7404	0.9663	0.9063	khuzestan
0.0643	****	0.8158	0.6997	0.9204	0.8915	lorestan
0.1853	0.2036	****	0.643	0.8072	0.8506	Ilam
0.3006	0.3571	0.4417	****	0.7483	0.7203	Ardebil
0.0343	0.0829	0.2142	0.2900	****	0.9092	Golestan
0.0983	0.1149	0.1619	0.3280	0.0952	****	Hamadan

[a] Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

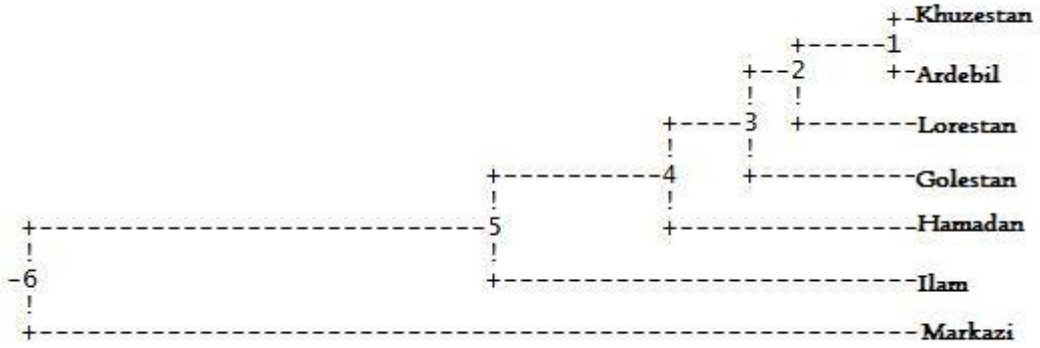

Figure 2. Dendrogram (UPGMA) of *Zymoseptoria tritici* isolates based on Nei's (1978) Genetic distance.

pathogens (Pryor, 2000). Various researchers have previously studied diversity in pathogenicity of *Z. tritici* isolates (Gurung *et al*., 2011).

Relatively similar results have been obtained in research carried out on this subject. For example, in the United States, genetic diversity reported from various states varied from 0.31 to 0.56 (Gurung *et al*., 2013). Furthermore, Razavi and Hughes (2004) reported that genetic diversity in 90 isolates collected in Canada was 0.44, of which 88% was related to intra-population and 12% to inter-population diversity. In another study conducted by Linde *et al*. (2002) on samples from various countries including Switzerland and the United States, it was found that genetic diversity varied from 0.44 to 0.50, of which 77% was related to intra-population and 23% to inter-population diversity.

Although the diversity obtained in the present research was lower compared to some countries, yet it was relatively high. In the mentioned studies, it was mentioned that gene flow was the reason for the higher share of intra-population diversity compared to inter-population diversity. In addition, in the present research, the higher intra-population diversity compared to inter-population diversity, as well as the high Nm index (1.1197), suggested the occurrence of a strong gene flow between the studied provinces. This result is in contrast to a previous study conducted in Iran. Abrinbana *et al*. (2010) found an Nm of 0.78 and noted the considerable contrast with other regional (Schneider *et al*., 2001) and global (Linde *et al*., 2002) scales. Of course, populations of these two studies are not the same and are different in terms of the host type and the locations of the isolate collection.

In general, there are three ways in which gene flow happens in *Z. tritici*: formation of a sexual reproduction cycle, presence of intermediate hosts such as some grasses that can play the role of a green bridge, and

movement of infected seeds and straw from one place to another by people.

Formation of the sexual reproduction cycle and ascospore production have not been reported in Iran, but existence of a strong gene flow in this fungus and of its sexual and asexual reproduction that were previously reported in Iran (Abrinbana *et al*., 2010) raise the probability of the formation of the sexual reproduction cycle. This requires further research.

In general, we cannot ignore the role played by humans in the transmission of pathogenic agents to remote places through transportation of infected straw and seeds. Although it has not been proven that infected seeds could give rise to infected seedlings (Schnieder *et al*., 2001), we assumed that infected grains might have contributed to such a gene flow (Consolo *et al*. 2009).

In grouping the various populations through genetic diversity, it was found that the greatest similarity existed between populations collected from Khuzestan and Ardebil Provinces (Figure 3). Similar results were obtained by Abrinbana *et al*. (2010) on genetic diversity of this fungus through using mating types, so that the fungal population in Khuzestan Province had the greatest similarity to that of Fars Province, followed by that of Ardebil Province.

Study of percentages of polymorphism indicated that the maximum polymorphism was also observed in these provinces. In general, in this research, the higher the degree of similarity, the greater was the degree of relatedness between the populations. One reason for this phenomenon can be the fact that fungal populations that have higher degrees of polymorphism naturally possess a greater number of genes to exchange. Moreover, geographical conditions, especially relative humidity in the air, and agricultural practices such as irrigation of most areas under cultivation in places including Khuzestan Province and the Dasht-e Moghan area allow the annual incidence of the disease. This, by itself, can be effective in expanding, displacing, and creating diversity in the causal agent of the disease and can influence diversity in pathogenicity.

The importance that the effect of diversity in pathogenicity has on genetic diversity must not be ignored either, because many of these loci are associated with pathogenicity genes, and any factor that is involved in selection of pathogenicity genes, including the type of the involved host, also influences genetic diversity. In general, STB is a sporadic disease in most Iranian provinces, and is strongly dependent on environmental conditions, especially relative humidity and farming practices. Therefore, in many provinces, this disease appears once every few years. This, in itself, strongly influences the increases in the volume and diversity of the population and allows permanent exchange with other populations. Moreover, gene drift must not be ignored when interpreting small populations that are different from other populations.

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بررسی تنوع ژنتیکی قارچ *Zymoseptoria tritici* عامل بیماری سپتوریوز برگی گندم با استفاده از نشانگرهای ISSR وSSR

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چکیده

برای بررسی ساختار و ژنتیک جمعیت قارچ *Zymoseptoria tritici* عامل بیماری سپتوریوز برگی گندم ۷۵ ایزوله این قارچ که از 7استان گندم خیز ایران جمع آوری شده بودند توسط نشانگرهای مولکولی ISSR و SSRمورد مطالعه قرار گرفتند .نتایج این بررسی نشان داد جمعیت قارچ عامل بیماری از تنوع ژنتیکی نسبتاً بالایی برخوردار است بطوریکه میانگین تنوع ژنتیکی کل (Ht) در نمونه هی مورد بررسی ۰/۳۳ بود و وقتی تنوع ژنتیکی به تنوع ژنتیکی داخل و بین جمعیت‌های قارچ مورد آزمایش تقسیم شد مشخص شد ۶۹٪ تنوع در داخل جمعیت‌ها و ۳۱٪ تنوع در بین جمعیت‌ها وجود دارد .همچنین نتایج این تحقیق نشان از وقوع جریان ژنی قوی در بین استان‌های مورد بررسی داشت.در بین استانهای مورد بررسی بیشترین تنوع ژنتیکی دراستانهای اردبیل و خوزستان مشاهده گردید که تجزیه خوشه ای نیز نشان داد جمعیتهای این دو استان بیشترین شباهت را با هم دارند.