Assessment of genetic diversity in *Pythium aphanidermatum* isolates using ISSR and rep-PCR methods

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Abstract
Sixty isolates of *Pythium aphanidermatum* as the causal agent of sugar beet root rot were selected on the basis of their geographical origins, morphological and genetic diversity studies. Pathogenicity test using the Kruskal-Wallis analysis showed significant differences between the pathogenic potential of isolates. Based on the growth rate, isolates were categorized in two groups with low and high growth rate. The potential of ISSR and rep-PCR for fingerprinting purposes was evaluated using eight ISSR and three rep-PCR primers. A strong linear relationship was observed between resolving power of a primer, PIC, and MI. PIC value for the rep-PCR primers (0.41) was higher than the ISSR primers (0.39). Based on UPGMA method and Jaccard's coefficient, ISSR separated Khuzestan and Fars provinces isolates from other isolates at 63% similarity level, while rep-PCR separated these isolates at 61% similarity level. The PCA analysis showed that despite the similarities of both markers, rep-PCR represent a higher resolution than the ISSR. So the rep-PCR marker leads to further differentiating of the isolates. There was a relationship between genetic divergence and geographical origins of isolates. rep-PCR can replace ISSR in diversity studies because of their comparable accuracy in the interpretation of genetic diversity of isolates.

Key words: Comparative analysis, *Pythium aphanidermatum*, ISSR, Repetitive element-PCR

INTRODUCTION

Pythium root rot is one of the main causes of yield loss in crops. The most important causal agent of *Pythium* root rot and seedlings damping off in sugar beet is *Pythium aphanidermatum*. Pythium root rot caused by *P. aphanidermatum* Fitzpatrick has been reported in the states of Arizona, California, Colorado, Texas, Canada, Australia, and Iran (Von Bretzel *et al*., 1988). The first step in selecting and implementing control strategies for this pathogen is accurate identification of pathogenic species and populations within the species. In order to control the disease in plants, use of resistant cultivars is most economical and appropriate, so that an awareness of population structure is essential to develop these cultivars (Groth and Bond, 2007; Martinez and English, 1997). Due to the lack of diagnostic morphological traits, variability of morphological structures in Pythium genus and overlapping similarities, the use of morphological characteristics of the species is very difficult for identification (Martin, 2000; Levesque and de Cock, 2004). Molecular techniques can help the problems to be solved. With the advent of biochemical and molecular techniques, older methods were replaced with modern molecular tools, such as molecular markers to study fungal populations. These techniques help genetics researchers by analyzing the DNA in different fields such as, phylogeny studies determine the genetic relationships, genetic mapping, gene localization, gene transformation, and diagnosing diseases. Molecular techniques used for identification and assessment of the genetic diversity of *P. aphanidermatum* isolates are including: RFLP, ALP, AFLP, SSR, ISSR, SCAR, ITS, PCR-RFLP (Al-Sadi, 2007; Al-Sadi *et al*., 2008, 2007; Garzon *et al*., 2007, 2005; Harvey *et al*., 2000; Lee and

Moorman, 2008). Inter Simple Sequence Repeat (ISSR) assay has been used in genetic studies since 1994 (Zietkiewicz *et al.*, 1994). The ISSR primers are modified SSR primers based on sequences surrounding the microsatellite that are scattered throughout the genome. This marker showed higher polymorphism than RAPD primers because of repeated regions in the genome (Esselman *et al*., 1999). Repetitive motifs in the genomes of the *Pythium* group F isolates were shown using this marker (Vasserur *et al*., 2005).

There are inverted repeat sequences in the genome of bacteria that are highly conserved in the genomes. These sequences lead to the invention of new methods to determine the genetic variation in fungi, analysis of phylogenetic relationships and development of diagnostic methods for identification of species (de Bruijn, 1992; Rodriguez-Barradas *et al.,* 1995). First time, these sequences were identified in *Escherichia coli*, *Streptococcus pneumonia, Salmonella typhimurium* bacteria and called BOX (Martin *et al.*, 1992), REP (Stern *et al.*, 1984), PU (Gilson *et al*., 1984), IRU (Sharples *et al.*, 1990) or ERIC Elements. These regions are amplified by rep-PCR marker.

However, little attention has been given to genetic diversity within *P. aphanidermatum*. Genetic diversity studies by Garzon *et al*. (2005) and Al-sadi *et al*. (2008) reported a low level of genetic diversity within *P. aphanidermatum* isolates using AFLP marker. Despite the fact that there is information about pathogenecity and distribution of *P. aphanidermatum* in Iran (Sheikholeslami *et al*., 2002; Zamani Noor *et al*., 2004; Afzali and Ershad, 2006; Babaie- Ahary *et al*., 2004), little is known about the levels of diversity within and between populations of *P. aphanidermatum* affecting sugar beet. In the present study, we have focused on the rep-PCR and ISSR, whose high discrimination capability has been proved in the successful analysis of mycorrhizal, pathogenic, and endophytic fungi. This paper examines specific hypotheses: (i) to determine the genetic diversity of *P. aphanidermatum* populations in Iran; (ii) to test genetic differentiation among *P. aphanidermatum* populations from six provinces; (iii) to compare the analysis of genetic diversity by ISSR and rep-PCR markers and (iv) investigate the relationship between virulence power, growth rate and genetic diversity of *P. aphanidermatum.*

MATERIALS AND METHODS

Source of Pythium isolates

Sixty isolates of *P. aphanidermatum* were isolated from rhizosphere and sugar beet roots showing damping off disease from different provinces of Iran and deposited in the Sugar Beet Seed Institute of Iran (Table 1). The isolates were further purified by hyphal tip culture on 1.5% water agar (WA) and transferred to Corn Meal Agar (CMA: extract of 60 g ground maize, 15 g agar, 1 litre distilled water). In order to induce the formation of sporangia, 2-day-old cultures of *P. aphanidermatum* isolates grown on CMA amended with chloramphenicol (200mg/l) were placed in petri dishes containing sterile distilled water and a few sterilized Cannabis seeds. After 2-3 days, sexual and asexual bodies of isolates were produced and tentatively identified as *P. aphanidermatum* based on morphological characteristics and authentic keys (van-der Plaats Niterink, 1981). Finally a more authentification of isolates were done using the Pa1/ITS2 semi-specific primers.

Growth rate and Pathogenicity test

The growth rate of isolates was measured at 26 C^{o} and 29 C^{o} temperatures, with four replications. Diameter of colonies was measured after 24 and 48 h of culture and analyzed with MVSP (MVSP 3.2) and MSTATC (MSTAT-C 2.10) software. For the pathogenicity test, Zarghan cultivar was used as the roots of mature plants. The scale of 1-9 (Buttner *et al*., 2004) was used to compare the pathogenicity of isolates. The Kruskcal Wallis test was performed to analyses variance by the SPSS software.

DNA extraction and PCR assays

Genomic DNA was extracted from the mycelia of the *P. aphanidermatum* isolates as described by Safaie *et al*. (2005). For direct detection and identification of *P. aphanidermatum* isolates, PCR reaction was performed using Pa1/ITS2 semi-specific primers (Wang *et al*., 2003). In this study, 25 ISSR primers were used for the initial screening (Table 2). Eight out of 25 ISSR primers, which showed reproducible polymorphic banding patterns in three separate experiments, were selected for diversity studies (Table 3). ISSR amplification was performed as described by Khodayari *et al*. (2009). To perform rep-PCR, the sequences of ERIC and BOX primers were adapted from Versalovic *et al.* (1994) (Table 3). Amplification was performed as described by Khodayari *et al*. (2009). The experiments were repeated three times for all primers.

Data analysis

DNA fingerprints generated by ISSR and rep-PCR markers were scored for the presence (1) or absence (0) of the bands and the cluster analysis of the resulted data was performed using Unweighed Pair-Group Method with Arithmetic Average (UPGMA), Jaccard's coefficient (Jaccard, 1908), NTSYS-pc (version 2.1; Exeter

Table1. Source of isolates of *P. aphanidermatum* and their cultural characteristics

Isolate	Origin	Growth rate (cm/day)[a]		Pathogenecity test			Date of isolation	Isolate Identity
				Rank Sum[b]	Virulence power[c]			
2D	khuzestan	5.69	A	43.90	V	ABC	2011	PK-21
3D	khuzestan	5.7	A	43.72	V	ABC	2011	PK-23
4D	khuzestan	5.72	A	39.5	V	BCDE	2011	PK-20
5D	khuzestan	5.71	A	43.80	V	ABC	2011	PK-22
6D	khuzestan	5.67	A	28.5	W	CDEFGH	2011	PK-51
10D	Lorestan	5.70	A	43.67	V	BCD	2011	PL-10
9D	Lorestan	5.61	A	22.12	W	DEFGHI	2011	PL-11
25D	Lorestan	3.00	EF	35.15	V	CDEF	2011	PL-22
13D	Kermanshah	5.45	AB	7.00	VW	FGHI	2011	K-11
34D	Kermanshah	5.5	AB	10.10	VW	FGHI	2011	K-34
36D	Kermanshah	5.36	AB	11.00	VW	FGHI	2011	K-36
54D	Markazi	5.45	AB	30	V	CDEFG	2011	PM-54
16D	Markazi	5.40	AB	33.4	V	CDEF	2011	PM-16
57D	Markazi	5.33	AB	33	V	CDEF	2011	PM-57
22D	Kermanshah	5.37	AB	31.83	W	CDEFG	2011	K-22
35D	Kermanshah	5.30	AB	32.00	W	CDEFG	2011	K-35
41D	Kermanshah	4.45	BC	29.8	W	CDEFG	2011	K-41
26A	Fars	5.25	ABC	41.02	V	BCD	2011	PS-26
42A	Fars	4.92	ABC	43.67	V	BCD	2011	PS-42
43B	Fars	5.15	ABC	40.21	V	BCD	2011	PS-43
18D	Kermanshah	4.67	BC	7.00	VW	FGHI	2011	K-18
37D	Kermanshah	4.50	BC	10.00	VW	FGHI	2011	K-37
38D	Kermanshah	4.42	BC	11.5	VW	FGHI	2011	K-38
7D	khuzestan	4.65	BC	28.83	W	CDEFGH	2011	Kh-7
19D	West Azarbayjan	4.50	BC	12.50	VW	FGHI	2011	PA-19
48D	West Azarbayjan	4.65	BC	8.50	VW	FGHI	2011	PA-48
53D	West Azarbayjan	4.40	BC	9.85	VW	FGHI	2011	PA-53
19B	Fars	4.47	BC	20.83	W	EFGHI	2011	PS-87
44A	Fars	4.44	BC	18.5	W	EFGHI	2011	PS-44
45A	Fars	4.41	BC	21.00	W	EFGHI	2011	PS-45
17D	Kermanshah	4.45	BC	47.10	V	ABC	2011	K-17
39D	Kermanshah	4.43	BC	43.83	V	ABC	2011	K-39
40D	Kermanshah	4.50	BC	45.00	V	ABC	2011	K-40
8D	West Azarbayjan	4.35	CD	54.83	HV	A	2011	PA-8
51D	West Azarbayjan	4.22	CD	52.50	HV	A	2011	PA-51
52D	West Azarbayjan	4.12	CD	50.23	HV	AB	2011	PA-52
21D	Lorestan	3.47	DE	22.17	W	DEFGHI	2011	PL-21
26D	Lorestan	3.31	DE	23.24	W	DEFGHI	2011	PL-26
33D	Lorestan	3.50	DE	24.50	W	DEFGHI	2011	PL-33
15D	Markazi	2.60	EF	27.67	W	CDEFGHI	2011	PM-15
58D	Markazi	3.00	EF	26.17	W	CDEFGHI	2011	PM-58
59D	Markazi	2.91	EF	27.51	W	CDEFGHI	2011	PM-59
23D	Lorestan	2.47	FG	26	W	DEFGHI	2011	PL-23
27D	Lorestan	2.47	FG	22.12	W	DEFGHI	2011	PL-27
32D	Lorestan	2.52	FG	24.32	W	DEFGHI	2011	PL-32
11D	Lorestan	2.45	FG	27.67	W	CDEFGHI	2011	PL-11
31D	Lorestan	2.47	FG	28.00	W	CDEFGHI	2011	PL-31
30D	Lorestan	2.50	FG	27.65	W	CDEFGHI	2011	PL-30

14D	Lorestan	2.40	FGH	12.50	VW	FGHI	2011	PL-14
28D	Lorestan	2.46	FGH	10.50	VW	FGHI	2011	PL-28
29D	Lorestan	2.40	FGH	11.23	VW	FGHI	2011	PL-29
12D	Markazi	1.80	GHI	22.17	W	DEFGHI	2011	PM-12
55D	Markazi	1.75	GHI	21.20	W	DEFGHI	2011	PM-55
56D	Markazi	1.94	GHI	22.50	W	DEFGHI	2011	PM-56
19A	Fars	1.50	HI	34.50	V	CDEF	2011	PS-89
46B	Fars	1.65	HI	33.00	V	CDEF	2011	PS-46
47A	Fars	1.59	HI	32.4	V	CDEF		PS-72
24D	West Azarbayjan	1.45	I	50.33	HV	AB		PA-24
49D	West Azarbayjan	1.47	I	48.1	HV	AB		PA-49
50D	West Azarbayjan	1.45	I	51.5	HV	AB		PA-50
				HSD5%= 37.63				

Legend: [a] Values in growth rate column followed by different letters are significantly different according to Duncan test (P = 0.01). [b] Isolates with Rank sum equal or greater than 37.63 are significantly different according to HSD test (P = 0.05). [c] High virulence(HV), virulence(V), weak virulence(WV) and very weak virulence(VW).

Biological Software, Setauket, NY, USA) (Rohlf, 1993) and GenAlExe 6.1 software.

To calculate heterozygosity, marker index, and effective multiple ratio to identify the most effective marker in determining the genetic diversity among isolates of *P. aphanidermatum*, the presence or absence of DNA bands at each locus was considered as an allele. Then the polymorphism information content was calculated using the sum of squared allele frequencies and remove monomorph locus on the base of $PIC = 1 - \sum_{i=1}^{n} Pi2$ formula (n: The number of alleles; P_i: frequency of i allele) (Lynch and Walsh, 1998). For each primer, PIC was calculated as the average of all loci. Marker Index that expresses the degree of polymorphism, and can be an indicator for estimating the efficiency of a marker in an unknown germplasm, was calculated based on MI=PIC×EMR formula. The effective multiplex ratio that indicate the number of polymorphic loci in a germplasm was calculated by EMR= $n_p \times \beta$ formula, which n_p is the number of polymorphic loci and β is the ratio of polymorphic loci to the total number of loci (Powel *et al*., 1996).

Resolving power of a primer was calculated as Rp=∑*Ib* using the GenAlex 6.1 software. The values of *Ib* which is variable between 0-1 was calculated by Ib = 1 - (/ 0.5-p /) equation, where p is the frequency of a band (Peakall and Smouse, 2006). To evaluate the genetic distinctions among populations, PhiPT index was estimated by the GenAlex 6.1 using analyses of molecular variance (AMOVA). PhiPT index is calculated as the ratio of variance between the populations to the total variance, and shows the relationship between individuals within a population to total population.This index is similar to the Fst index (Maguire *et al*., 2002). Bila

Table 2. ISSR primers were used for initial screening

No.	ISSR primers	Sequence
1	LB-A	5'-$(GACA)_4$ TA-3'
2	LB-B	5'-$(GACA)_4$TT-3'
3	LB-C	5'-$(GACA)_4$GT-3'
4	P1	5'-$(TC)_8$C-3'
5	P4	5'-CTG$(GT)_8$-3'
6	ISSR02	5'-$(ACTG)_3$ACG-3'
7	PcMs	5'-$(GTC)_7$-3'
8	TGTC	5'-$(TGTC)_5$-3'
9	UBC 826	5'-$(AC)_8$C-3'
10	ISSR9	5'-$(AG)_8$C-3'
11	UBC 841	5'-$(GA)_8$YC-3'
12	ISSR 2	5'-$(AG)_8$T-3'
13	ISSR 4	5'-$(GA)_8$T-3'
14	LB-F	5'-$(GGAGA)_3$-3'
15	ISSR 23	5'-$(CT)_8$G-3'
16	LB-E	5'-$(GGAGA)_3$-3'
17	LB-D	5'-$(GGGTG)_3$ -3'
18	ATG	5'-$(ATG)_6$-3'
19	ISSR3	5'-$(CA)_8$A-3'
20	ISSR4	5'-$(AC)_8$YA-3'
21	ISSR 8	5'-$(AC)_8$T-3'
22	ISSR 6	5'-$(GA)_8$YG-3'
23	ISSR7	5'-$(CT)_8$T-3'
24	ISSR 5	5'-$(AC)_8$YC-3'
25	ISSR12	5'-$(TG)_8$A-3'

Table 3. Number of polymorphic band and five possible correlate of the ability of ISSR and rep-PCR primers in genetic diversity assessment of *P. aphanidermatum* isolates.

ISSR primers	**Sequence**	NSB	NPB	PIC	B	EMR	MI	Rp
LB-A	5'-$(GACA)_4$ TA-3'	17	17	0.32	1.00	17.00	5.44	6.94
LB-B	5'-$(GACA)_4$TT-3'	22	22	0.26	1.00	22.00	5.72	5.36
LB-C	5'-$(GACA)_4$GT-3'	20	20	0.69	1.00	20.00	13.8	6.42
P1	5'-$(TC)_8$C-3'	13	10	0.46	0.77	7.70	3.54	5.36
P4	5'-CTG$(GT)_8$-3'	18	15	0.35	0.83	12.45	4.35	3.68
ISSR02	5'-$(ACTG)_3$ACG-3'	18	17	0.38	0.94	16.00	6.8	4.94
PcMs	5'-$(GTC)_7$-3'	14	13	0.37	0.93	12.10	4.47	3.47
TGTC	5'-$(TGTC)_5$-3'	14	12	0.36	0.86	10.32	3.71	4.25
Total		136	126					
Average				0.39	0.91	14.70	5.97	
Rep-PCR primers	**Sequence**							
ERIC1R	5'-ATGTAAGCTCCTGGGGATTCAC-3'	16	15	0.35	0.94	14.10	4.93	5.36
ERIC2I	5'-AAGTAAGTGACTGGGGTGAGCG-3'	20	19	0.51	0.95	18.05	9.2	5.78
BOX	5'-CTACGGCAAGGCGACGCTGACG-3'	12	12	0.37	1.00	12.00	4.44	3.58
Total		48	46					
Average				0.41	0.96	14.72	6.19	
Total markers		184	172					

NSB: Number of scored band; NPB: Number of polymorphic band; PIC: Polymorphism information contents; β: np/(np+nnp); EMR: Effective Multiplex Ratio; MI: Marker index; R_P: Resolving power.

teral matrix was establish-ed based on Jaccard's similarity coefficient using NTSYS 2.02 software. For each dendrogram, the co-phenetic coefficients between the matrix of genetic similarities and the matrix of co-phenetic values were computed using appropriate routines of the NTSYS-pc 2.02 package and mantel test (Mantel, 1967). This test was also used to check the alignment and comparison of the similarity matrix of rep-PCR and ISSR data. To confirm the results of cluster analysis, principal coordinates analysis (PCA) was conducted using NTSYS software and plot of the relationship between the samples was drawn.

RESULTS AND DISCUSSION

Morphology, growth rate, and pathogenicity test

Based on morphological characters of sexual and asexual organs and colony morphology, the isolates were identified as *P. aphanidermatum* (Table 1). Isolates produced colonies with cottony aerial mycelium on CMA with terminal and stuck straight oogonial and sporangia consisting of a terminal complex of swollen hyphal branches of varying length (Figure 1). Based on the of growth rate, isolates were divided into two groups with high rate growth (58% of isolates) and low rate growth (42% of isolates). The isolates induced typical symptoms of rot on roots. Kruskal Wallis analysis indicated that isolates had significant differences and by using MSTAT-C and UPGMA analysis, the isolates were classified into four distinct groups based on their virulence (P = 0.05); nevertheless, no correlation was observed between pathogenicity and genetic diversity (Table 1).

Molecular identification of *P. aphanidermatum*

A single band of 210 bp was amplified in all tested isolates using the Pa1/ITS2 semi-specific primers and isolates were confirmed as a *P. aphanidermatum*. Such a band was not amplified in *P. oliganderum* and *Phytophtora* (Figure 2a).

ISSR and rep-PCR assays and polymorphism information contents

In this study, rep-PCR and ISSR markers were compared for genetic diversity of 60 isolates of *P. aphanidermatum*. All eight ISSR primers and three rep-PCR primers used in this study showed polymorphism (Figure 2b). One hundred and thirty six bands for ISSR marker and 48 bands for rep-PCR marker were scored. In ISSR marker, the largest number of loci was ampli-

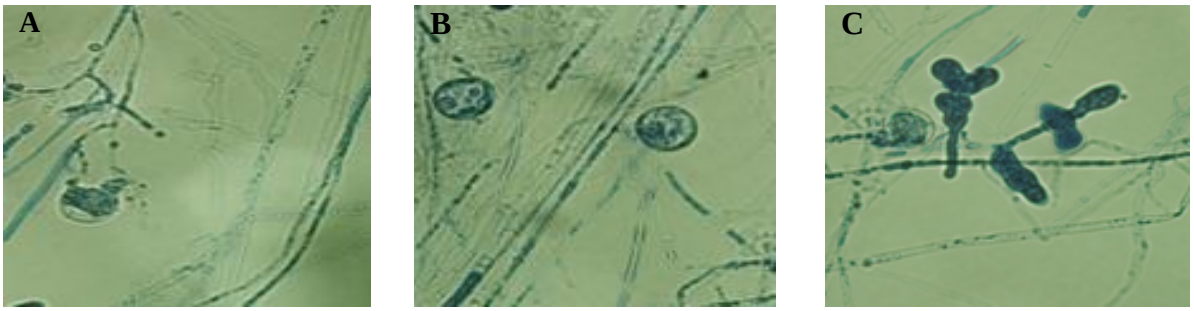


Figure 1. Sexual and asexual organs of *P. aphanidermatum* isolated from six provinces of Iran; **A:** oogonium and antheridium; **B:** oospore; **C:** sporangium.

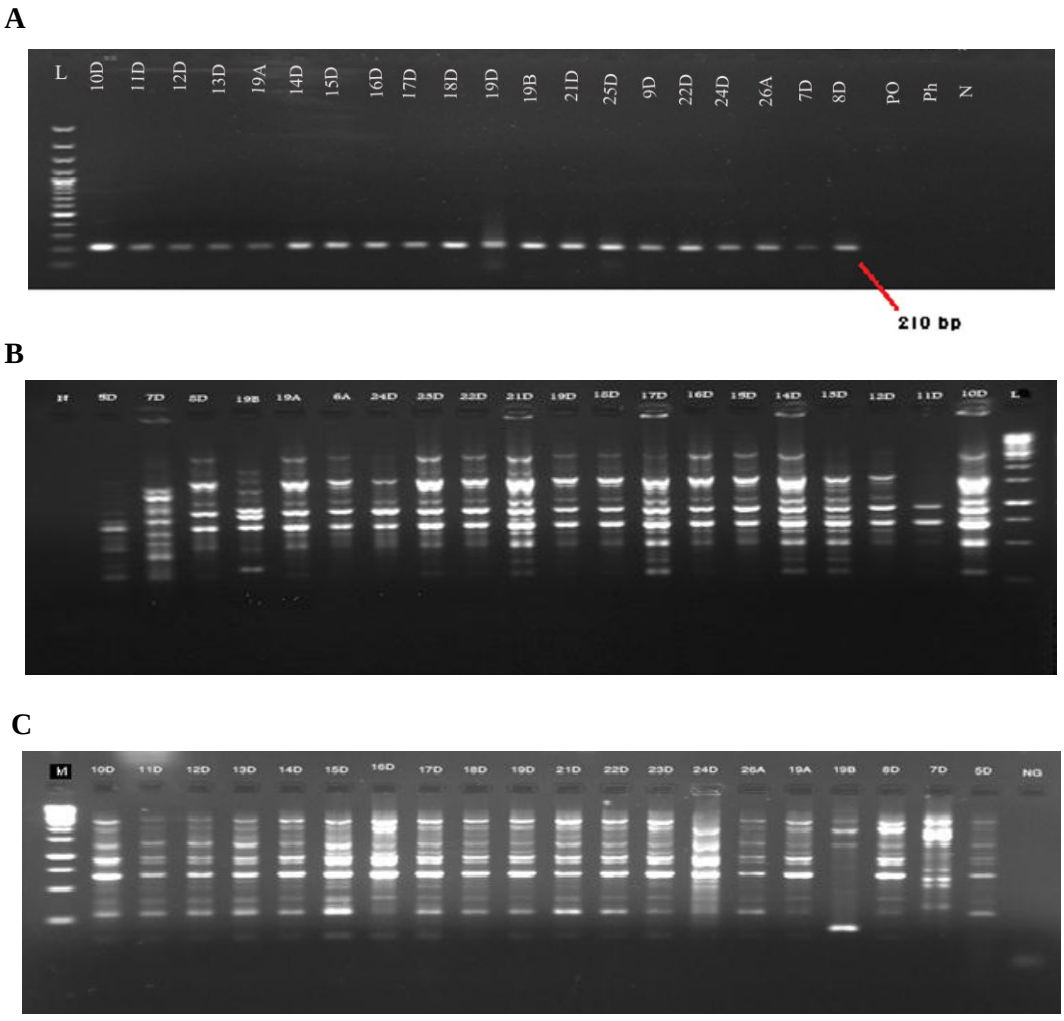


Figure 2. A: DNA fingerprint profiles of 20 selected isolates of *P. aphanidermatum*, one isolate of *P. oliganderum* (PO) and Phytophtora (Ph) using Pa1/ITS2 semi-specific primers (L; 100bp ladder; N; negative control) . DNA fingerprint profiles of selected 20 isolates of *P. aphanidermatum* obtained with **B:** ISSR02 primer and **C:** ERIC 2I; L – Size marker (1 Kb); N – Negative control.

fied by the LB-B primer and the lowest number of loci was amplified by the P1 primer (Table 3). An average16 loci were obtained for rep-PCR marker (2I with the highest and BOX with the lowest number of loci).

For each primer, PIC, MI and EMR indices were calculated separately using the allelic frequency. The average PIC value of all ISSR primers (0.39) and rep-PCR assay (0.41) were similar. LB-C primer of ISSR and ERIC 2I primer of rep-PCR with the highest PIC values of0.69 and 0.51, respectively, were able to identify the genetic distance better than the other primers. Resolving power (Rp) is a parameter that indicates resolving ability of primers. Average resolving power of the eight ISSR primers and three rep-PCR primers were 5.05 and 4.9, respectively. LB-A of ISSR and Eric 2I from rep-PCR possessed high Rp values (6.9 and 5.78, respectively) and were able to distinct all isolates.

Comparison of cluster analysis in rep-PCR and ISSR assay

Comparison of cluster analysis of two markers indicated that ISSR separated Khuzestan and Fars provinces isolates from other isolates at 63% similarity level, while rep-PCR marker separated Khuzestan and Fars provinces isolates (except 26A and 45A) from other isolates at 61% similarity level. It is possible that these two isolates (26A and 45A) belong to other geographical regions that distributed in the Fars province. rep-PCR could separate these two isolates. Analysis of 184 alleles from the combined data of ISSR and rep-PCR assays, based on UPGMA method and Jaccard's coefficient, separated Khuzestan and Fars provinces isolates (except 26A and 45A) from other isolates at 54% similarity level (Figure3). Cumulative arrangement of 60 isolates studied with principal coordinates analysis (PCA) based on Jaccard's similarity matrix, has been shown in Figure 3. The distribution of isolates in this plot was compatible with clustering of isolates in the dendrogram.

Comparison of 2-dimensional plot of the PCA in the two markers showed that despite the similarities of both markers, rep-PCR represent a higher resolution than the ISSR. Co-phenetic coefficient and correlation between the similarity matrices obtained by rep-PCR and ISSR data together and separately were determined using the Mantel test.I In all cases there was a significant positive correlation between the two markers(Table4).

The genetic similarity between isolates from six geographic regions was analyzed using the GenAlExe 6.1 software. The genetic similarity between isolates of different areas ranged from 0.96 to 0.757 (Table 5). The lowest similarity coefficient was observed between isolates of Kermanshah and Khuzestan Province and the highest similarity coefficient was observed between isolates of Kermanshah and Lorestan provinces.

Analysis of molecular variance (AMOVA)

The analysis of molecular variance (AMOVA) was done using the GenAlEx 6.1 software. This software is able to calculate the variance components. Analysis of molecular variance showed that both markers represent the low PhiPT value (0.249 for ISSR and 0.26 for rep-PCR). Approximately 75% of the total variance was related to within-population variation and 25% of it was due to the variation among populations (Table 6). Thus, the high genetic diversity within populations was observed due to the low levels of PhiPT.

Comparison of these molecular markers in this study and a previous study (Khodayari *et al*., 2009) showed that the highest number of scored DNA bands and a higher percentage of polymorphism were produced by the ERIC primers. Therefore, the rep-PCR was more efficient than ISSR PCR. Polymorphism information content (PIC) is one of the most important indicators for comparing different markers and provides sufficient discriminating ability to eval uate the genetic diversity among genomes (Barth *et al*., 2002). Botstein *et al*. (1980) reported that PIC> 0.5 represents a very efficient marker, 0.5> PIC> 0.25 represents a useful marker and PIC <0.25 represents a poor performance of a marker. Also the obtained PIC value for the rep-PCR marker (0.41) was more than ISSR (0.39), if a higher number of rep-PCR primers had been a higher PIC value might have been obtained. Moreover, rep-PCR had more MI and β than ISSR. Therefore, rep-PCR marker was considered as a more effective marker for the genetic diversity. In order to determine the efficiency of markers, polymorphism content, marker index and EMR were calculated. Because of the high number of repetitive sequences and using large number of primers, these indicators provide more information on the ISSR marker. It should be noted that according to the genome of the fungal isolates, EMR index varies by changing the number and type of primers. Prevost and Wilkinson (1999) introduced the Rp parameters as an indicator to measure the ability of differentiation of markers. High alignment of the Rp values with the other indices was indicated that primer LB-C is more efficient than other primers.

The ISSR and rep-PCR clusters were defined according to the principle coordinate analysis (PCoA) results. In a molecular genetic variation study, it is better that the markers have a homogenous distribution

Table 4. Mantel test results for two ISSR and rep-PCR markers.

	ISSR	Rep-PCR	ISSR + repPCR
Correlation between the similarity matrices and Co-phenetic coefficient	r= 0.98 p= 0.002	r= 0.93 p= 0.002	r= 0.98 p= 0.002

Table 5. The genetic similarity matrix of 60 isolates of *P. aphanidermatum* from six geographical regions based on combined data analysis of rep-PCR and ISSR.

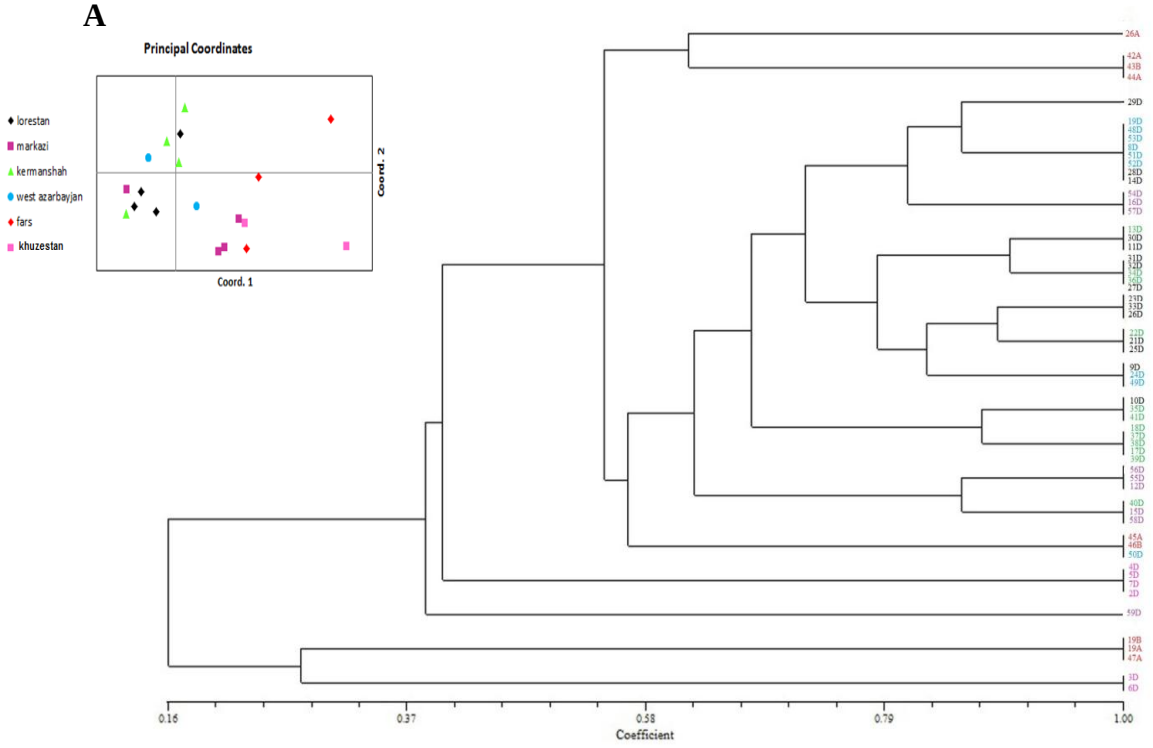
west	west azarbayjan	fars	Kermanshah	Lorestan	Markazi	Khuzestan
azarbayjan	1.000					
Fars	0.853	1.000				
kermanshah	0.925	0.871	1.000			
lorestan	0.944	0.860	0.965	1.000		
markazi	0.924	0.864	0.943	0.930	1.000	
Khuzestan	0.764	0.794	0.761	0.771	0.757	1.000

Table 6. Measured variation among and within populations of Pythium aphanidermatum using analysis of molecular variance.

Source	df	Sum of squares	Mean of squares	Variance component	Variation (%)	F_{ST}^{z}
ISSR						
Among Population	5	44.100	8.820	0.686	25	0.249
Within Population	54	111.600	2.067	2.067	75	…
Rep-PCR						
Among Population	5	40.35	8.07	0.644	25	0.259
Within Population	54	100.35	1.85	1.858	75	…
Combined ISSR and rep-PCR						
Among Population	5	84.45	16.89	1.318	25	0.251
Within Population	54	211.95	3.925	3.925	75	…

in the genome to be able to cover the whole genome. T-herefore, if marker is selected from different parts of the genome, the correlation between them will be small and as a result, a greater number of markers need to justify the changes (Ahkami *et al*., 2007). The lower changes by two basic markers indicates the proper distribution of markers used in the genome. The rep-PCR marker separated Khuzestan and Fars provinces isolates (except 26A and 45A) from other isolates at 61% similarity level, while ISSR separated at 63% similarity level. The rep-PCR marker leads to further differentiation of isolates. According to these markers, the maximum similarity was observed between isolates of Kermanshah and Lorestan while the minimum similarity was observed between Markazi and Khuzestan provinces. As a result, there was a relationship between genetic divergence and geographical origin. Although most of the isolates of close geographical regions located in the same groups, but some isolates with different geographical regions located in a group. Therefore, the position of the isolates with different geographical origin together in the same group showed that common

A


Principal Coordinates
lorestan
markazi
kermanshah
west azarbayjan
fars
khuzestan
Coord. 2
Coord. 1
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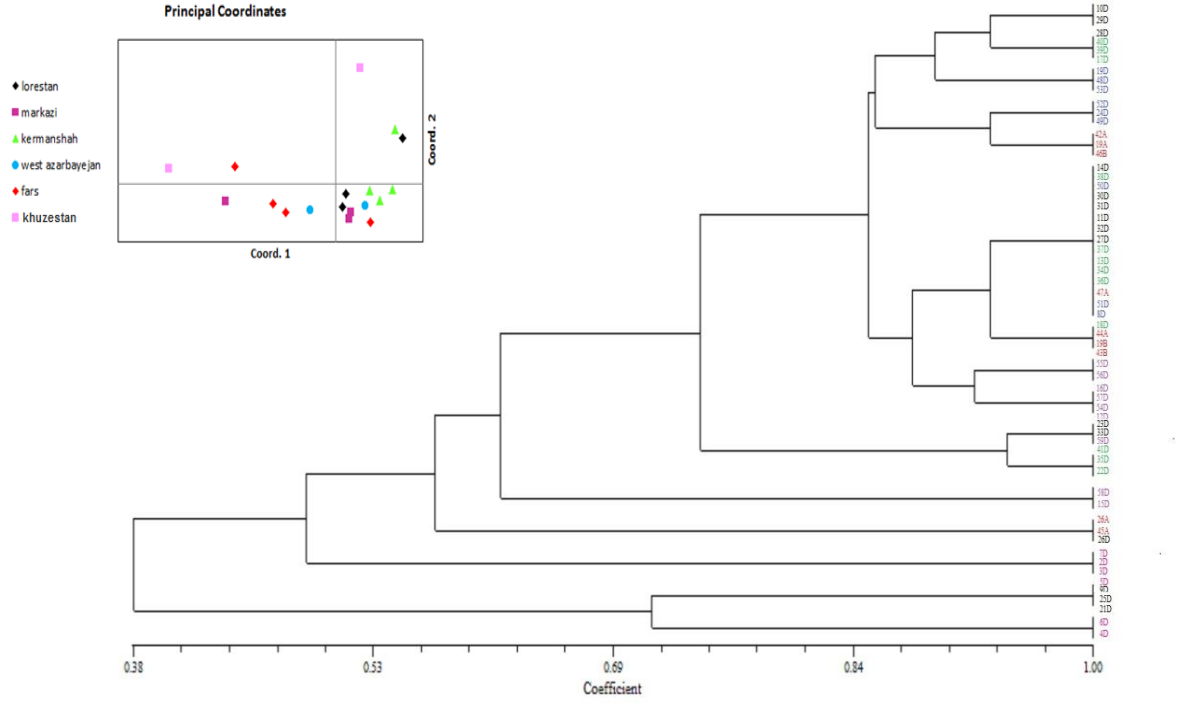
0.37
0.58
0.79
1.00
Coefficient

B


Principal Coordinates
lorestan
markazi
kermanshah
west azarbayejan
fars
khuzestan
Coord. 2
Coord. 1
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0.53
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0.84
1.00
Coefficient

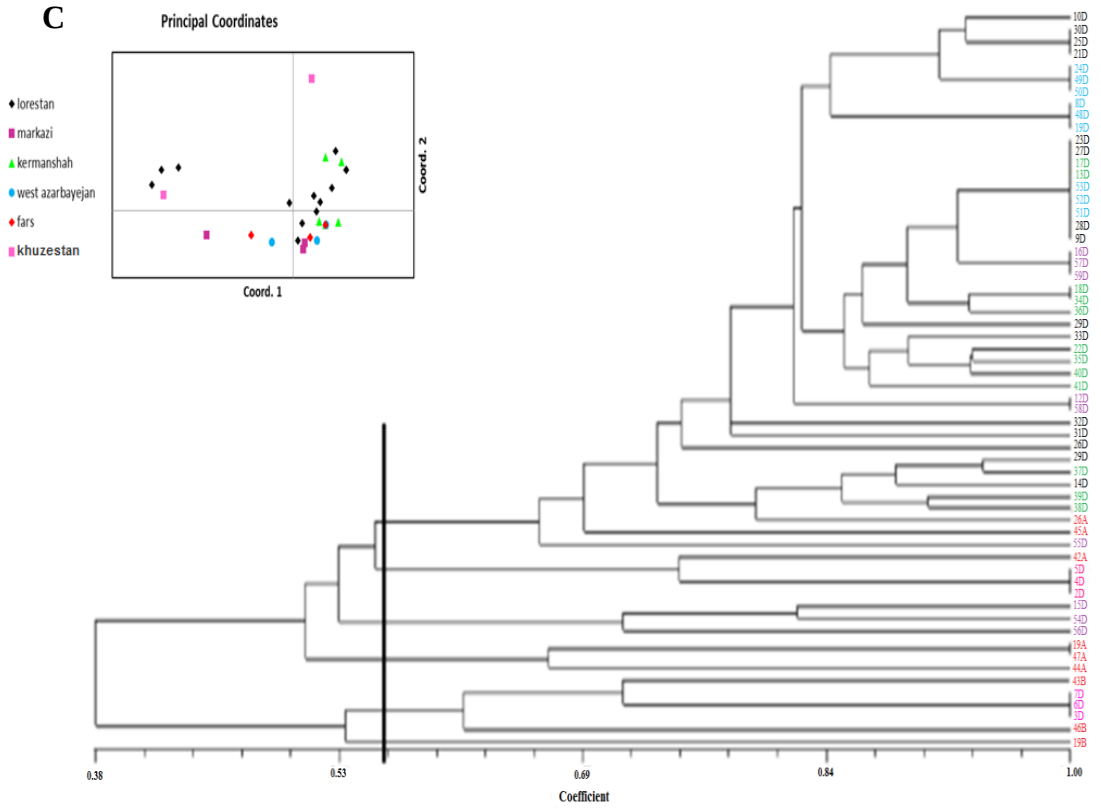

Figure 3. A: Dendrogram and tow dimension plot of PCA for ISSR data; **B:** Dendrogram and tow dimension plot of PCA for for rep-PCR data **C:** Dendrogram and tow dimension plot of PCA for ISSR and rep-PCR combined data.

alleles exist between them. Mismatching between molecular data and geographical areas may be because of the phenomenon of gene flow between populations, similar climatic conditions, or physical exchange of materials between areas. Also the relationship between genetic diversity of the isolates with virulence and growth rate of isolates was assayed. But, no relationship was observed between genetic divergence based on molecular markers, virulence power, and their growth rate. Comparing the co-phenetic with Jaccard matrix of each markers using the Mantel test, showed a high correlation between this two matrices indicating a high compatibility with the UPGMA dendrograms and similarity matrices. In general, because in both markers the absence of band measure will not enter in metrics, thus there was little difference between the correlations. Nevertheless, the concordance among *P. aphanidermatum* isolates grouping after cluster analysis was high (r = 0.98), indicating that rep-PCR and ISSR-based diversity assessments in this germplasm array were generally consistent.

Pythium aphanidermatum is one of the most prevalent and invasive species in sugar beet fields in Iran. Although, the *P. aphanidermatum* complex is considered as an important limiting factor in agriculture in Iran, the genetic diversity of Iranian *P. aphanidermatum* population is poorly understood. Therefore, we investigated the pathogenicity and morphological characteristics of 60 *P. aphanidermatum* isolates as well as their genetic variations, using ISSR and rep-PCR markers. In our studies, the morphological characters like terminal and stuck straight oogonial and sporangia were in consonance with van-der Plaats Niterink (1981). The growth rate analysis showed that isolates could be categorized into two distinct groups with low (2.31 cm/day) and high (4.93 cm/day) growth rates. Fast and accurate identification of *P. aphanidermatum* isolates using the Pa1/ITS2 semi-specific primers, which produced a single band of 210 bp, showed that PCR-based technology is a convenient molecular tool for an easy and rapid detection. The isolates were classified into four distinct groups based on their virulence. Comparison of two

molecular marker systems used in this study showed that despite some common characteristics such as dominance, simplicity, speed in both markers and similar distinguish resolution by each marker, rep-PCR marker in comparison with ISSR marker was shown to be more powerful for the interpretation of genetic diversity of isolates. Because of long primers and high annealing temperature, markers generated by rep-PCR are more robust, reproducible and specific than markers generated by ISSR PCR (Kang *et al*., 2002). The results provided an optimized method for evaluating the genetic diversity of *P. aphanidermatum* using rep-PCR marker which was useful for further investigation. Rep-PCR markers can be successfully employed to assess the level of polymorphism and diversity in *P. aphanidermatum*.

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