J. Crop Prot. 2015, 4 (4): 441-452

Research Article

Genetic diversity of *Cytospora schulzeri* isolates using RAPD-PCR and MP-PCR markers on Apples of Semirom Region of Iran

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Abstract: Genetic diversity relationships of 50 isolates of *Cytospora schulzeri* on apple from different parts of the Semirom region were analyzed using 15 polymerase chain reaction (PCR) based markers, 7 random amplified polymorphic DNAs (RAPDs) and 8 Microsatellite primed polymerase chain reaction (MP-PCR). Using 7 selected RAPD primers 113 bands were generated, of which 81 bands were polymorphic (71.7%), with an average of 11.57 polymorphic fragments per primer, and with 8 selected MP-PCR primers 107 amplified bands were observed with 78 polymorphic bands (72.3%), with an average of 9.75 polymorphic fragments per primer. In RAPD marker, number of polymorphic bands varied from 8 (241) to 15 (230, 238, OPA13) with an average of 11.57 per primer and which varied in size from 200 to 3750 bp. Percentage of polymorphism ranged from 64% (203 and 232) to a maximum of 83% (238). In MP-PCR marker, number of polymorphic bands varied from 6 (CAG) to 12 (GTG and ATG) with an average of 9.75 per primer and which varied in size from 200 to 3500 bp. Percentage of polymorphism ranged from 54% (CAG) to a maximum of 81% (ACTG). By combining markers, a total of 220 bands were detected, of which 159 bands (72%) were polymorphic and produced on an average 10.6 polymorphic bands per primer. The results showed that both markers were suitable for the detection of genetic polymorphism among apple *C. schulzeri* isolates. Estimated genetic relationship using similarity co-efficient (Jaccard's) values between different pair of accessions varied from 0.54 to 0.89 in RAPD, 0.62 to 0.89 in MP-PCR and 0.62 to 0.87 with combined markers based similarities. High cophenetic correlation between the similarity matrix and corresponding dendrogram was obtained by RAPD + MP-PCR marker (r = 0.81). Cluster analysis of the data using UPGMA based on Jaccard´s similarity coefficient, divided the isolates into six groups, showing a high genetic diversity among populations of *C. schulzeri*.

Keyword: apple, cytospora,genetic diversity, MP-PCR, RAPD

Introduction

Cytospora canker is the most important disease limiting apple *Malus pumila* Mill. production in Semirom region of Iran (Mehrabi *et al*., 2011). Ascomycetous fungi *Cytospora* Ehrenb. (Teleomorph *Valsa* Fr., *Leucostoma* (Nitschke) Höhn., *Valsella* Fuckel and *Valseutypella* Höhn. (Adams *et al*., 2005) are considered to be the most destructive pathogens for apple trees in Semirom region of Iran. Six species belonging to three genera are associated with cytospora canker disease of

Handling Editor: Naser Safaie

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Received: 05 August 2013, Accepted: 20 May 2015
Published online: 28 June 2015

apple trees in Semirom region, Iran which comprise *Cytospora cincta, C. schulzeri, C. leucostoma, C. chrysosperma, Valsa malicola* and *Leucostoma cinctum*. *C. schulzeri* (Teleomorph *Valsa malicola*) is the most dominant species on apple trees in Semirom region. High morphological diversity has been observed in the discs, ostioles, locules, size of conidia and conidiophores within the isolates of *C. schulzeri*. *Cytospora* canker is difficult to control (Mehrabi *et al.,* 2011). Once a tree is infected, the disease cannot be controlled with fungicides (Helton, 1967). Since cultural practices and chemical treatments do not adequately control the disease, the best control would be the introduction of disease resistant cultivars with acceptable horticultural characteristics (Chang *et al.,* 1991). One of the constraints in the development of resistant cultivars is the lack of knowledge regarding the identification of the pathogens and the heterogeneity of the pathogens. DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann, 1983). The polymerase chain reaction (PCR) technology has offered new marker systems for diagnosis of genetic diversity in large scale studies (Saiki *et al.,* 1988). Two methods have evinced considerable potential for the genetic diversity of isolates investigated in this research: Random Amplified Polymorphic DNA (RAPD) and Microsatellite Primed Polymerase Chain Reaction (MP-PCR). In RAPD (Random Amplified Polymorphic DNA) technique, single primers with arbitrary nucleotide sequences are employed in a polymerase chain reaction to amplify genomic DNA and the resulting polymorphisms provide a simple means to construct genetic maps and perform DNA fingerprinting (William *et al.,* 1990). Microsatellite Primed Polymerase Chain Reaction (MP-PCR) technique is a PCR based oligonucleotides complementary to microsatellites, that is, short tandem repeats or simple sequence length polymorphisms, are stretches of tandem mono-, di-, tri-, and tetranucleotide repeats of varying lengths (Bruford *et al.,* 1993; Tautz, 1989) used as single primers. If inversely repeated microsatellites are located within an amplifiable distance of one another, the inter-repeat sequences are amplified. This technique has been used successfully to amplify hypervariable repetitive DNA sequences in a wide range of animal, plant, and fungal species (Meyer *et al.,* 1993; Meyer and Mitchell, 1995; Weising *et al.,* 1995; Kahl *et al.,* 1995). Inter and intra specific genetic studies among *Cytospora* species has been demonstrated in previous studies mainly based on sequencing of the internal transcribed spacer(ITS) region of the nuclear rDNA gene (Adams *et al.*, 2006; Adams *et al*., 2005; Adams *et al*., 2002; Fotouhifar, 2007, Fotouhifar *et al.,* 2010), colony morphology and small nuclear rDNA size polymorphism(Wang *et al*., 1998), isozyme polymorphisms (Surve-Iyer *et al*., 1995), vegetative compatible groups (Adams *et al.*, 1990) and genetic diversity with RAPD and MP-PCR (Abbasi *et al*., 2011; Seifollahi *et al.,* 2013). The objectives of this study were to (1) use RAPD and MP-PCR markers to determine genetic diversity in isolates of *C. schulzeri* on apple trees in Semirom region of Iran; (2) compare genetic relationships with morphological diversity observed in this fungus.

Materials and Methods

Sample collection and identification

During spring and autumn seasons of 2007-2008, 50 isolates of *C. schulzeri* were collected from apple orchards with dieback and canker symptoms in Semirom region of Iran. Identification of species were based on study of morphological features of the sexual and asexual fruiting bodies produced on infected plant tissues. Isolation and morphological characteristics of species were described by Mehrabi *et al.* (2011). Reference number and location of isolates are listed in Table 1.

DNA extraction

Single spore cultures were isolated and subcultured on potato-dextrose agar at 25 ºC. Each isolate was grown in a standard culture medium (10 gr glucose, 10 gr yeast extract, 2.5 gr. malt and 2.5 gr peptone in 1 lit distilled water) broth for 7-14 days at 25 ºC on a shaker at 120 rpm. Mycelia from cultures on standard broth medium were vacuum filtered, lyophilized, and subsequently stored at -20 (Fotouhifar, 2007). Total DNA was isolated from lyophilized mycelia following the CTAB method as described by Doyle and Doyle (1990) with slight modification. Approximately 25mg of lyophilized mycelia were ground to fine powder in liquid nitrogen using mortar and pestle. It was then transferred to pre-warmed extraction buffer and incubated at 65 °C for 1 h. The mixture was extracted twice with chloroform and centrifuged to remove solids. The water-soluble fraction was precipitated with two volumes of absolute ethanol and centrifugation, followed by a rinse with 70% ethanol and a second centrifugation. The precipitate was air-dried then resuspended in 50 µl water. The DNA extracted was quantified spectrophotometrically at 260 nm and homogeneity was checked on 0.8% agarose gel by electrophoresis. For PCR, DNA samples were diluted to 50 ng/µl.

Microsatellite-primed PCR

Microsatellite motifs used in MP-PCR assay as primers were chosen according to literature data (Meyer *et al.*, 1993; Longato and Bonfante, 1997; Stepansky *et al.*, 1999; Amicucci *et al*., 2002; Vasseur *et al.,* 2005; Sharma *et al.,* 2005). Primer sequences used for each of the PCR techniques were synthesized by CinaGen Incorporation in Tehran. The annealing temperature for each primer in MP-PCR assay was established experimentally. PCR was performed in 25 µl reaction volumes containing 2.5 µl PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0), 1 mM $MgCl_2$, 0.5 mM dNTP; 1 µM primer (CinaGen Inc.); 0.3 units of Tag DNA polymerase (CinaGen Inc.) and 50 ng of template DNA.

Table 1 Isolates of *Cytospora schulzeri* used in this study with reference number and location.

Isolate no.	Location	Date of collection
S1	Padena (Sheibani)	2007/01/01
S7	Padena (Sheibani)	2007/01/03
S121	Padena (Sheibani)	2007/09/10
S140	Padena (Sheibani)	2007/09/12
S149	Padena (Sheibani)	2007/09/12
S125	Padena (Sheibani)	2007/09/12
S134	Padena (Sheibani)	2007/09/12
S75	Padena (Sheibani)	2007/09/08
S84	Padena (Sheibani)	2007/09/08
S100	Padena (Sheibani)	2007/09/10
S68	Padena (Sheibani)	2007/09/08
S78	Padena (Sheibani)	2007/09/08
S161	Padena (Sheibani)	2007/09/15
S8	Padena (Sheibani)	2007/01/03
S23	Padena (Kahangan)	2007/01/03
S132	Padena (Kahangan)	2007/09/12
S116	Padena (Kahangan)	2007/09/10
S118	Padena (Kahangan)	2007/09/10
S71	Padena(Kahangan)	2007/01/08
S82	Padena (Kahangan)	2007/01/08
S95	Padena (barand)	2007/01/08
S97	Padena (barand)	2007/01/08
S25	Padena (barand)	2007/01/03
S103	Padena (Bideh)	2007/09/10
S150	Padena (Bideh)	2007/09/15
S152	Padena (Bideh)	2007/09/15
S160	Padena (Bideh)	2007/09/15
S148	Padena (Bideh)	2007/09/15
S93	Padena (Bideh)	2007/09/08
S77	Padena (Bideh)	2007/09/08
S69	Padena (Bideh)	2007/09/08
S87	Hanna	2007/09/08
S123	Hanna	2007/09/12
S155	Hanna	2007/09/15
S138	Hanna	2007/09/12
S117	Hanna	2007/09/10
S83	Komeh	2007/09/08
S135	Bardekan	2007/09/12
S111	Mehrgerd	2007/09/10
S146	Mehrgerd	2007/09/15
S127	Mehrgerd	2007/09/10
S114	Semirom	2007/09/10
S89	Semirom	2007/09/08
S151	Semirom	2007/09/15
S136	Semirom	2007/09/12
S122	Semirom	2007/09/12
S101	Semirom	2007/09/10
S88	Semirom	2007/01/08
S86	Semirom	2007/01/08
S76	Semirom	2007/01/08

Amplification was performed in a Mastercycler gradient PCR machine (Eppendorf AG, Germany).

The temperature regime suggested by a slightly modified method of Meyer *et al* (1993) was followed: an initial denaturation step at 5 min at 94 ºC, 40 cycles of 20 s at 94 ºC, an annealing step of 1 min at various temperatures depending on the experiment, and 72 ºC for 20s and then a final extension at 72 ºC for 6 min. the amplification products were separated by electrophoresis in 1.4 % agarose gel ((1 X TBE buffer (89 mM Tris, 89 mM boric acid, and 2mM EDTA), 80 V, 3h)) stained with ethidium bromide (0.1 µg µl-1) and amplimers were observed over a UV light source. Gel images were acquired with a Gel Doc (Vilbert, Lourmat, Marne La Vallee, France). For each of the primers used at least two independent PCR assay with negative control (H_2O instead of DNA template) were conducted.

RAPD PCR

RAPD PCR was carried out in 25 µl of reaction mixtures as described for microsatellite-primed PCR. Seven 10-mer oligonucleotides were chosen randomly according to Chen *et al*., (1999) and tested as primer sequences (Table 2). Amplification was performed by method of Balmas *et al.* (2005) and run in a Mastercycler gradient PCR machine (Eppendorf AG, Germany) programmed for one cycle of 1 min at 95 °C, 40 cycles of 1 min at 94 °C, 1 min at 39 °C, 2 min at 72 °C, one final cycle of 10 min at 72 °C. RAPD analysis was repeated at least twice for each isolate. The same amplification conditions were used as detailed for microsatellite-primed PCR in same experiments. Samples were separated on agarose gels as described above.

Table 2 PCR primers used in this study.

Primer code	Primer sequence 5' → 3'	Annealing temperature (ºC)
	MP-PCR assay	
$(GTG)_5$	GTGGTGGTGGTGGTG	64
$(ATG)_6$	ATGATGATGATGATGATG	57
$(GAC)_5$	GACGACGACGACGAC	58
$(CAG)_5$	CAGCAGCAGCAGCAG	57
$(CCA)_5$	CCACCACCACCACCA	56
$(CAC)_5$	CACCACCACCACCAC	58
$(ACTG)_4$	ACTGACTGACTGACTG	58
$(TGTC)_4$	TGTCTGTCTGTCTGTC	46
	RAPD-PCR assay	
203	CACGGCGAGT	39
211	GAAGCGCGAT	39
230	CGTCGCCCAT	39
232	CGGTGACATC	39
238	CTGTCCAGCA	39
241	GCCCGACGCG	39
OPA13	CAGCACCCAC	39

Cluster analysis of RAPD and Microsatellite-primed PCR data

The relatedness of DNA samples was assessed by comparing RAPD and microsatellite-primed PCR fragments of DNA separated according to their sizes and the presence/absence of shared fragments. The banding patterns obtained from RAPD and microsatellite-primed PCR were used to compare relatedness of the isolates. For each isolate, a data record was constructed in which each band of a particular molecular weight, as generated by each primer, was represented into a binary data matrix ("1" for the presence and "0" for the absence of a band at each particular locus). Polymorphic information content (PIC) and the marker index values for each RAPD and MP-PCR primer was calculated according to the formula:

$PIC = 1-\sum(Pij)^2$,

marker index (MI) = PIC × no. of polymorphic bands.

Where, Pij is the frequency of the ith pattern revealed by the jth primer summed across all patterns revealed by the primers (Botstein *et al*., 1980). Similarity matrices were obtained by the unweighted pair group method using arithmetic averages (UPGMA) and Jaccard's coefficient. Clustering analysis was performed on data generated from each primer separately and on the combination data gained from each primer with

the NTSYS-pc version 2 program (Rohlf, 2000). The co-phenetic correlation coefficient was calculated and Mantel test (Mantel, 1967) was performed to check the goodness of fit of a cluster analysis to the matrix. For determination of the best point of dendrogram cut, analysis of molecular variance (AMOVA) within and between groups and calculation of F index using the software GenAlEx version 6.1 were carried out. These points had the highest F values (Peakall and Smouse, 2006).

Result

MP-PCR analysis

Fifty isolates of *Cytospora schulzeri* obtained from Semirom region (Semirom-Hanna-Sheibani-Barand- Kahangan- Mehrgerd-Komme-Bideh) of Iran were amplified using the 8 oligonucleotides as primers (Table 2). Amplification patterns obtained by Microsatellite-primed PCR with primer (ACTG) are shown in figure 1. Using 8 selected MP-PCR primers, a total of 107 bands were generated of which 78 bands were polymorphic (72.3%). The number of polymorphic bands varied from 6 (CAG) to 12 (GTG –ATG) with an average number of bands per primer and average number of polymorphic bands per primer being 13.37 and 9.75, respectively (Table 3). The size of the amplified fragments varied from 200 to 3500 bp. The PIC varied from 0.295(ACTG) to 0.434 (CAG) with an average of 0.347 and the Marker Index from 2.086 (TGTC) to 4.452 (GTG) with an average of 3.373. Among the MP-PCR primers used ACTG, GTG, CCA and CAC were highly informative.

RAPD analysis

Seven different oligonucleotide primers (Table 2) were tested to amplify polymorphic DNA fragments among *C. schulzeri* isolates obtained in Semirom region. Amplification patterns obtained with primer 238 in 50 isolates of *C. schulzeri* are shown in figure 2. Using 7 selected RAPD primers, a total of 113 bands were generated of which 81 bands were polymorphic (71.7%). The number of polymorphic bands varied from 8 (241) to 15 (230, 238, OPA13) with an average of 11.57 per primer (Table 3). The size of the amplified fragments varied from 200 to 3750 bp. The polymorphic information content (PIC) varied from 0.289 (238) to 0.413 (211) with an average PIC of 0.347 and the marker index (MI) varied from 2.844 (203) to 5.04 (OPA13) with an average of 3.867. The most informative primers were 238 and 230.

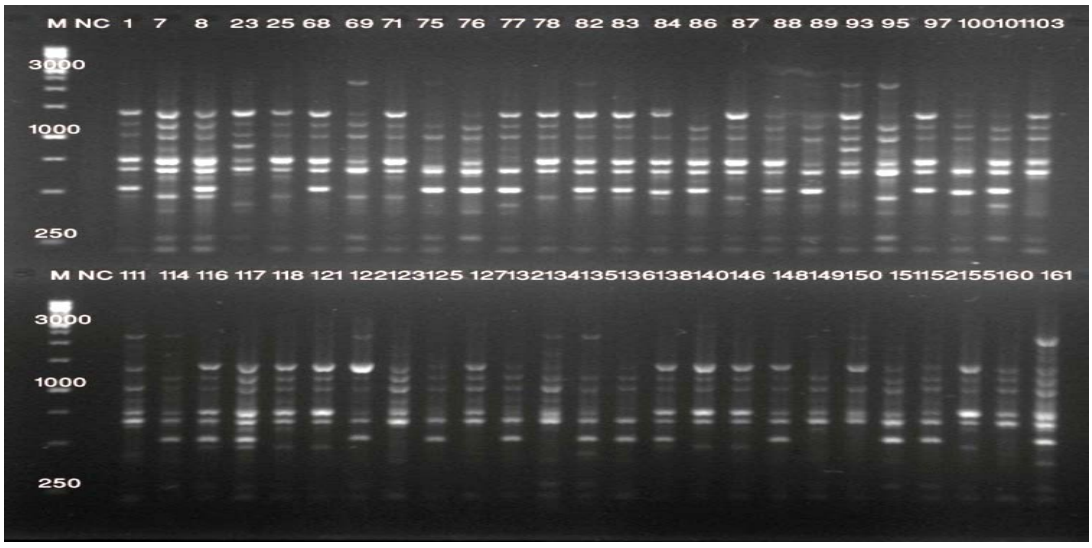

Figure 1 Amplification patterns obtained by Microsatellite-primed PCR with primer (ACTG) in 50 isolates of *Cytospora schulzeri*. M: molecular weight marker (1 kb DNA ladder) NC: negative control.

Table 3 Polymorphism detected with 7 RAPD and 8 MP-PCR primers in 50 isolates of *Cytospora schulzeri*.

Primer no.	No. of total bands	No. of monomorphic bands	Polymorphism		Total no. of bands amplified	PIC*	MI*	Amplicons band size (bp)
			No. of bands	%				
MP-PCR								
$(GTG)_5$	15	3	12	80	541	0.371	4.452	400-1700
$(GAC)_5$	16	6	10	62.5	608	0.338	3.38	300-3200
$(CAG)_5$	11	5	6	54	425	0.434	2.604	350-2500
$(CCA)_5$	14	3	11	78	435	0.323	3.553	400-3000
$(CAC)_5$	14	3	11	78	380	0.394	4.334	650-3500
$(TGTC)_4$	10	3	7	70	292	0.298	2.086	350-2000
$(ATG)_6$	16	4	12	75	568	0.327	3.924	250-1800
$(ACTG)_4$	11	2	9	81	323	0.295	2.655	200-2300
Total/mean	107	29	78	72.3	3572	0.347	3.373	
RAPD								
203	14	5	9	64	409	0.316	2.844	400-2500
211	14	4	10	69	332	0.413	3.717	300-1800
230	20	5	15	75	713	0.303	4.545	350-3750
232	14	5	9	64	393	0.402	3.618	250-3000
238	18	3	15	83	510	0.289	4.335	250-3000
241	12	4	8	66.6	431	0.372	2.976	250-2000
OPA13	21	6	15	71	707	0.336	5.04	200-3000
Total/mean	113	32	81	71.7	3495	0.347	3.867	

*PIC: polymorphic information content: MI: marker index.

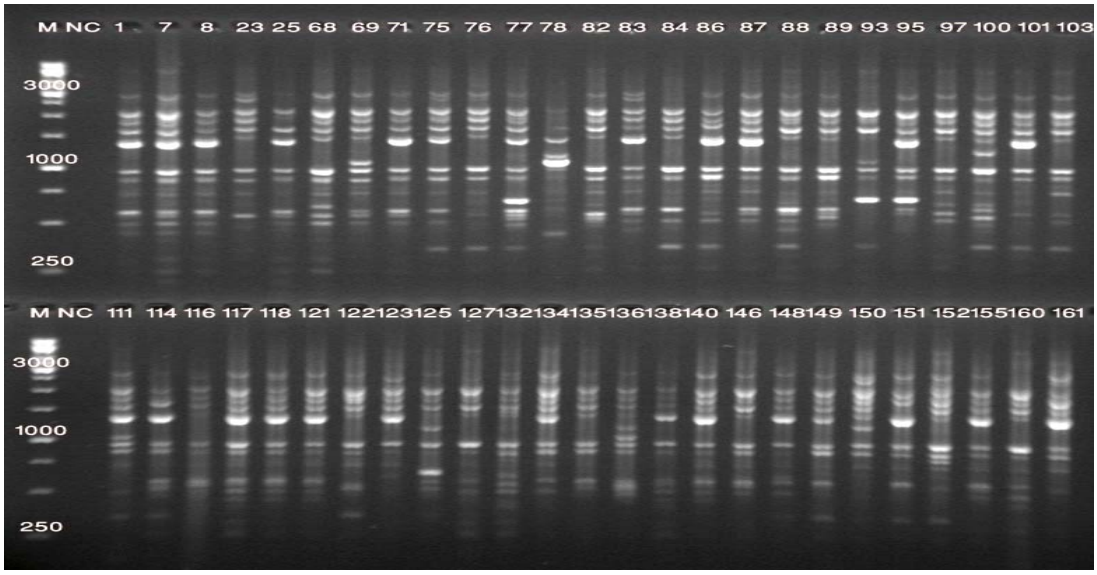

Figure 2 Amplification patterns obtained by random amplified polymorphic DNA with primer 238 in 50 isolates of *Cytospora schulzeri*. (M: molecular weight marker (1 kb DNA ladder) NC: negative control).

RAPD and MP-PCR analysis

Combining the markers of both primers, a total of 220 markers were detected, out of which 159 bands (72) were polymorphic with an average of 10.6 polymorphic markers per primer (Table 4).

However, RAPD markers detected greater number of polymorphic bands (11.57 bands/primer) than MP-PCR (9.75 bands/primer). Thus RAPD marker was more efficient marker system than MP-PCR for detecting polymorphism and to determine diversity. The genetic relationships among *C. schulzeri* isolates were presented in a dendrogram (Fig. 3). According to the analysis of molecular variance (AMOVA) of the combined fingerprinting data (RAPD + MP-PCR), the isolates were classified into six groups at 65.5% similarity level. Groups A, B, C, D and E included 64%, 26%, 4% and 4% of Padena and Semirom isolates, respectively. Group F cosisted of one isolate. Results from AMOVA indicated that 17% of the genetic variation is attributable to differences among accessions groups while 83% of the genetic variation is attributable to between accessions within accessions groups. Detailed results are presented in Table 5 (AMOVA).

Table 4 A comparative list showing different markers details (RAPD, MP-PCR and RAPD + MP-PCR).

primer	RAPD	MP-PCR	RAPD+MP-PCR
Number of primers used	7	8	15
Total number of bands	113	107	220
Total number of polymorphic bands	81	78	159
Total number of monomorphic bands	32	29	61
Total number of bands amplified	3495	3572	7067
Percentage polymorphism (%)	71.7	72.3	72
Average number of bands/primer	16	13.37	14.6
Average number of polymorphic bands/ primer	11.57	9.75	10.6
Maximum genetic similarity(%)	0.89	0.89	0.87
Minimum genetic similarity	0.54	0.62	0.62
Polymorphic information content (PIC)	0.347	0.347	0.347
Marker index (MI)	3.867	3.373	3.62
Cophentic correlation (r)	0.81	0.9	0.81

Estimated genetic relationship. was obtained from the markers data using similarity coefficient ((Jaccard's (Table 4)). Pair-wise comparison of accessions indicated relative genetic similarity between accessions ranging from a maximum of 0.89 to a minimum of 0.54 in RAPD and from a maximum of 0.89 to a minimum of 0.62 in MP-PCR. By combining markers data (RAPD + MP-PCR), the similarity coefficient varied from a maximum of 0.87 to a minimum of 0.62. Maximum similarity based on combined markers (RAPD + MP-PCR) was found between isolates S71 and S155. The cophenetic correlation (r) was 0.81 indicating a good fit of the cluster analysis to the similarity data.

Discussion

RAPD and MP-PCR molecular markers were used to determine the genetic diversity among *C. schulzeri* isolates from apples tree in Semirom region of Iran. Diversity in plant-pathogenic fungi can be generated by factors such as recombination, mutation and gene flow (Burdon and Silk, 1997). Recombination in plant pathogens occurs either through sexual reproduction or through a process of somatic hybridization, in which nuclear and cytoplasmic material may be exchanged (Burdon and Silk, 1997). The observation of high genetic diversity among *C. schulzeri* isolates in our study could be due to sexual or asexual reproduction because in used isolates abundant mitotic spore was generated in conidiomata and also (*Valsa malicola* the telomorphic state of *C. schulzeri*) was observed. In MP-PCR marker. percentage of polymorphism ranged from 54% (CAG) to a maximum of 81% (ACTG), with an average of 72.3% polymorphism. Our results confirm and extend previous observations that PCR primed by microsatellites can detect genetic polymorphisms in eukaryotic genome (Meyer *et al.*, 1993; Longato and Bonfante, 1997; Stepansky *et al,*. 1999; Amicucci *et al,*. 2002; Vasseur *et al.,* 2005; Sharma *et al.,* 2005). Distinct, polymorphic banding patterns were produced by most of the primers consisting of tetranucleotide or GC-rich trinucleotide repeats

and some trinucleotide repeats, generated a smear with a few superimposed bands, probably as a consequence of the high number of putative primer target sites which is in accordance with those obtained in the study reported by (Weising *et al.,* 1995). Also in this case a high polymorphism was observed using GTG, CCA, CAC and ACTG primers. These microsatellites in *C. schulzeri* isolates were reproducible and the use of microsatellite primed PCR allowed us to study intra-specific variability in *C. schulzeri* isolates, in particular for the GTG and ACTG regions.

Abbasi *et al.* (2011) investigated genetic diversity of *Cytospora chrysosperma*_isolates obtained from Iranian walnut trees using RAPD marker. They obtained polymorphism of 94% in this species. Our results showed that in RAPD marker percentage of polymorphism ranged from 64% (203 and 232) to a maximum of 83% (238), with an average of 71.7% polymorphism that indicated the adequacy of RAPD marker for studying genetic diversity in *C. schulzeri* isolates which is in agreement with results obtained by Abbasi *et al*, (2011). Also, Seifollahi *et al.* (2013) studied genetic diversity of 40 isolates of *Valsa malicola* from different host plants in Iran by MP-PCR. Their study determined 93.3% polymorphism in this species. Less polymorphism in this study (71.7 in RAPD, 72.3 in MP-PCR) is perhaps due to the small size of the region as is discussed. High cophenetic correlation between the similarity matrix and corresponding dendrogram obtained by RAPD (0.81), MP-PCR (0.9) and RAPD + MP-PCR marker (r = 0.81) indicates a good fit of the cluster analysis to the similarity data. High percentage of polymorphism (> 70%) observed with RAPD and MP-PCR markers indicated high level of genetic variation existing among population of *C. schulzeri*. Both markers are equally important for genetic diversity analysis in *C. schulzeri.* With regards to polymorphism detection, RAPD detected 71.7% as compared to 72.3% for MP-PCR markers. The higher Marker index (MI) calculated for RAPD (3.867) in comparison to MP-PCR (3.373) in this study proves the efficiency of RAPD.

High morphological diversity was observed in the discs, ostioles, locules, size of conidia and conidiophores within the isolates of *C. schulzeri* (Mehrabi *et al*., 2001). For example isolates 77 and 136 had spores with size <5.5 µm and isolates 93; 75; 76; 151; 134; 101 and 121 had spore sizes >5.5. Dendrogram obtained from RAPD and MP-PCR (data not shown) revealed a significant correlation between size of spores and the resulting groupings by markers analysis. But dendrogram of combining the results of eight RAPD and MP-PCR showed that these isolates are placed in the same group. So it seems that the size of the spores of this fungus differentiate the populations. This study could not show a significant relationship between the number of ostioles and size of conidiophore. Fotouhifar (2007) identified 26 species belonging to three genera *Cytospora*, *Valsa* and *Leucostoma* from different plant hosts in Iran, also phylogenetic relationships of these species has been studied based on nucleotide sequences of ITS1-5.8S-ITS2 region of the nuclear rDNA. Fotouhifar (2007) and Fotouhifar *et al.* (2010) concluded that the isolates belonging to the species *Valsa malicola* (anamorph: *C. schulzer*) no separate within specified group and overlaps clade 11 based on sequences of the ITS regions. He concluded that the nucleotide sequences of ITS1-5.8S-ITS2 region of the nuclear rDNA of this species is of high diversity which is in accordance with the results of this study. The dendrogram obtained from these markers did not reveal a significant correlation between geographical origins and the resulting groups of markers analysis. This is the first study of genetic diversity of *C. schulzeri* using RAPD molecular marker, as well as the first study that compared two PCR-markers to define genetic relationships among apple isolates of *C. schulzeri.* Our studies could demonstrate that the molecular analyses of RAPD and MP-PCR genomic fingerprinting were very useful for studying intra-specific genetic relationships between *C. schulzeri* isolates. The results of this study can help future research on the population and evolutionary genetic diversity in *Cytospora schulzeri.*

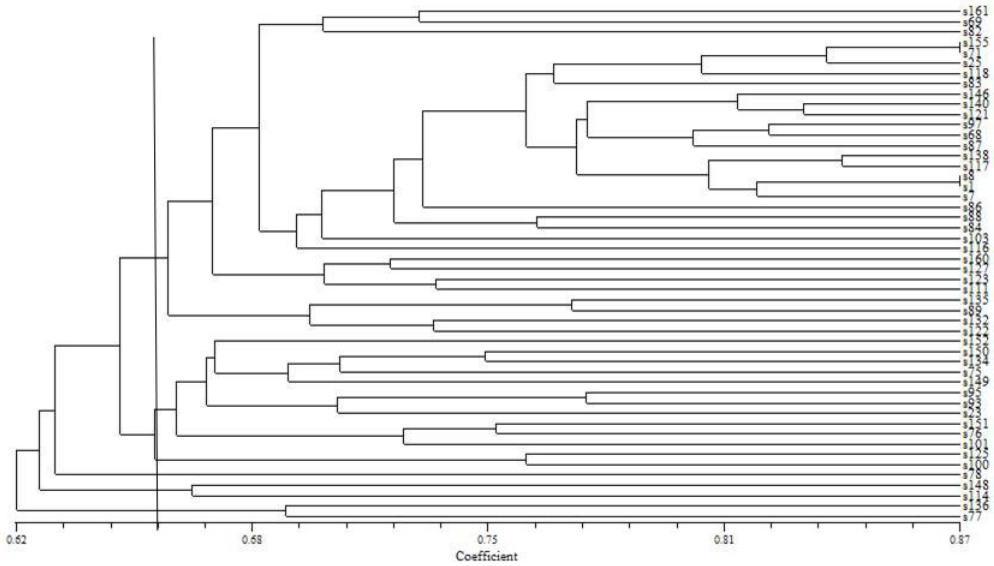

Figure 3 Dendrograms generated using unweighted pair of group method with arithmetic average analysis, showing relationships between *Cytospora schulzeri* isolates, using RAPD+MP-PCR data.

Table 5 Analysis of molecular variance (AMOVA) based on RAPD+MP-PCR marker.

source	df	SS	EV	(%)
Among groups	5	279.207	5.171	17*
Within groups	43	1068.916	24.859	83*
Total	48	1348.122	30.029	100

df: degrees of freedom, SS: sum of squares, EV: estimated variance, %: percentage of total variation.
*Significant at $P < 0.01$ level.

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تنوع ژنتیکی جدایه‌های *Cytospora schulzeri* با استفاده از نشانگرهای RAPD و MP-PCR روی درختان سیب منطقه سمیرم ایران

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دریافت: ۱۴ مرداد ۱۳۹۲؛ پذیرش: ۳۰ اردیبهشت ۱۳۹۴

چکیده: روابط تنوع ژنتیکی ۵۰ جدایه از قارچ *Cytospora schulzeri* روی درختان سیب از نقاط مختلف منطقه سمیرم با استفاده از ۱۵ آغازگر (۷ آغازگر RAPD و ۸ آغازگر MP-PCR) تجزیه و تحلیل گردید. با استفاده از ۷ آغازگر RAPD، ۱۱۳ باند ایجاد گردید که ۸۱ باند پلی‌مورفیک بودند (۷۱/۷ درصد) که به‌طور میانگین ۱۱/۵۷ قطعه پلی‌مورفیک برای هر آغازگر محاسبه می‌گردد و با ۸ آغازگر MP-PCR، ۱۰۷ باند مشاهده گردید که ۷۸ باند پلی‌مورفیک بودند (۷۲/۳ درصد) که به‌طور میانگین ۹/۷۵ قطعه پلی‌مورفیک برای هر آغازگر محاسبه گردید. در نشانگر RAPD، تعداد باندهای پلی‌مورفیک از ۸ (در آغازگر ۲۴۱) تا ۱۵ عدد (در آغازگرهای ۲۳۰، ۲۳۸، OPA13) و اندازه باندها از ۲۰۰ تا ۳۷۵۰ جفت باز متغیر بود درصد پلی‌مورفیسم در این نشانگر از ۶۴ درصد (در آغازگرهای ۲۰۳ و ۲۳۲) تا ۸۳ درصد (در آغازگر ۲۳۸) متغیر بود. در نشانگر MP-PCR، تعداد باندهای پلی‌مورفیک از ۶ (در آغازگر CAG) تا ۱۲ عدد (در آغازگرهای GTG و ATG) و اندازه باندها از ۲۰۰ تا ۳۵۰۰ جفت باز متغیر بود درصد پلی‌مورفیسم در این نشانگر از ۵۴ درصد (در آغازگر CAG) تا ۸۱ درصد (در آغازگر ACTG) متغیر بود. با ترکیب اطلاعات دو نشانگر، در مجموع ۲۲۰ باند حاصل گردید که ۱۵۹ باند (۷۲ درصد) پلی مورفیک بودند. نتایج این تحقیق نشان داد که هر دو نشانگر برای بررسی تنوع ژنتیکی در جدایه‌های *C. schulzeri* مناسب می‌باشند. تخمین روابط ژنتیکی با استفاده از ضریب تشابه جاکارد از ۰/۵۴ تا ۰/۸۹ در نشانگر RAPD و ۰/۶۲ تا ۰/۸۹ در نشانگر MP-PCR متغیر بود. ضریب همبستگی کوفنتیکی بالایی بین ماتریس شباهت و دندروگرام به‌دست آمده از ترکیب دو نشانگر حاصل گردید. آنالیز داده‌ها با استفاده از روش UPGMA براساس ضریب تشابه جاکارد جدایه‌ها را به شش گروه تقسیم کرد که نشان‌دهنده تنوع ژنتیکی بالا در میان جمعیت‌های *C. schulzeri* می‌باشد.

واژگان کلیدی: سیب، سیتوسپورا، تنوع ژنتیکی، MP-PCR، RAPD