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Full Length Research Paper

Genetic diversity of *Cytospora chrysosperma* isolates obtained from Iranian walnut trees using molecular markers

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To study the genetic diversity of isolates of *Cytospora chrysosperma*, the main causal agent of Cytospora canker, we collected the infected samples from the major walnut cultivation areas in 12 provinces of Iran. 58 representative isolates out of a total of 147 isolates were selected according to their geographical origins to study their genetic diversity using random amplified polymorphic deoxyribonucleic acid (RAPD) marker. Ten of 20 random primers amplified reproducible polymorphic banding patterns. Cluster analysis of the data using Centroid method and Jaccard´s similarity coefficient, divided the isolates into six groups, showing a high genetic diversity among populations of *C. chrysosperma*. Although there was no correlation between geographical origins and the resulting groups of RAPD analysis, but the amount of observed polymorphism (94%) indicated the adequacy of the molecular marker RAPD, for studying genetic diversity in *C. chrysosperma* isolates. This is the first report on the study of genetic diversity of populations of *C. chrysosperma* using RAPD marker.

Key words: Biodiversity, fungi, canker, deoxyribonucleic acid, random amplified polymorphic deoxyribonucleic acid, Iran.

INTRODUCTION

Species of *Cytospora* Ehrenb. are the causal agents of canker and dieback diseases on many woody and rarely on some herbaceous plants (Adams et al., 2006). Approximately, 560 species of *Cytospora* Ehrenb. have been recorded in the web site of the index fungorum but many of them have no known teleomorphs and also most of them are synonyms (Adams et al., 2005; http://www.indexfungorum.org). Where teleomorphs are known, they are species of *Leucostoma*, *Valsa*, *Valsella*, and *Valseutypella*, genera in the Diaporthales Ascomycota. Descriptions of *Cytospora* species are generally based on host and morphological features that might be plastic. The size, shape and arrangement of labrinthiform locules and stromatic tissues of the pycnidium are the basic characteristics used in differentiating *Cytospora* species in nature, other than conidium size (Adams et al., 2002). The earliest investigation on *Cytospora* species in Iran began with the work of Fragoso (1918), which reported species *Cytospora silene* on *Silene boryi* Boiss. Ashkan and Hedjaroude (1981, 1982) studied taxonomy and pathology of some species of *Cytospora* on fruit trees and presented a comprehensive description of the species. Ershad (2009) listed 32 species of *Cytospora* on different native and non-native plants. Fotouhifar et al., (2007) investigated taxonomy of *Cytospora* spp. In Iran

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using its rDNA (recombinant deoxyribonucleic acid) sequences and reported several new species of the genus for mycoflora of Iran. Cytospora canker is one of the most prevalent diseases of walnut trees in many areas of Iran. The disease reduces the useful lifetime of the trees and inflicts major economic losses. Unfortunately, no chemical sprays give satisfactory control of Cytospora canker. Cultural practices also do not adequately control the disease. Therefore, the most effective control would be the introduction of disease resistant cultivars. The successful management of the disease required the knowledge of identification of the pathogens and genetic diversity of the pathogens. Several species of *Cytospora* were reported on walnut trees in Iran.

Fotouhifar et al., (2007) reported three species of *Cytospora* from walnut trees including *Valsa sordida* (anamorph; *C. chrysosperma*), *Cytospora cincta* and *Cytospora leucosperma.* Moreover, Javadi (2008) reported species of *Cytospora chrysosperma*, *C. leucostoma* and *Cytospora rubescens* (in most cases synonymized with *C*. *cincta*) from the walnut trees in Iran. Recently, another species, *Cytospora schulzeri* was reported from walnut trees in Iran (Abbasi et al., 2010). *C*. *chrysosperma* was determined as the prevalent species of *Cytospora* on walnut trees (Javadi, 2008; Abbasi, 2010). Abbasi et al. (2010) indicated that the higher frequency of *C. chrysosperma* on walnut trees in Iran could be due to its high virulence. *V. sordida* and its anamorph, *C. chrysosperma* is the causal agent of canker on different species of plant genera; *Populus* and *Salix* and more rarely on other angiosperms in all over the world. Wounded trees and trees injured by insects or attacked by other pathogens are more susceptible to infection (Hayova and Minter, 1998). Fotouhifar and Hedjaroude, (2010) isolated anamorphic and teleomorphic states of the fungus on many new host plant genera in Iran, including: *Armeniaca vulgaris*, *Juglans regia*, *Crataegus azarolus*, *Fraxinus excelsior*, *Morus alba*, *Ligustrum latifolium*, *Persica vulgaris*, *Platanus orientalis*, *Robinia pseudo-acacia*, *Tamarix* sp., *Thuja orientalis*. Different isolates of the *C*. *chrysosperma* obtained from various plant hosts in Iran had different pathogenicity capabilities (Aghapour, 2010). This study was aimed to estimate the extent of genetic diversity in *C. chrysosperma* isolates collected from different agro-climatic walnut tree growing regions of Iran using RAPD markers.

MATERIALS AND METHODS

Fungal isolates

During 2008 to 2009, 242 samples of infected twigs and branches of walnut trees (*J. regia*) with symptoms of Cytospora canker having fungal fruiting bodies were collected from different areas in 12 provinces of Iran including: Hamedan, Kurdistan, Kermanshah, Ilam, West Azarbaijan, Zanjan, Markazi, Lorestan, Chaharmahal va Bakhtiari, Isfahan, Kohgiloye va Boyerahmad and Fars. The fungal isolates were identified based on morphological characteristics of asexual fruiting bodies on the bark, including: presence or absence of conceptacle, shape, size and number of ostiole, shape and color of disk, arrangement of locules, color of tendrils (spore masses), size of conidia, conidiophores and conidiogenous cells and branching type of conidiophores. Pure colonies of *C. chrysosperma* isolates were produced as described by Fotouhifar et al., (2007). Small pieces of infected bark with fruiting bodies were cut and surface sterilized by 70% ethanol. The specimens were then placed in a Petri dish containing moist filter paper. After oozing of the gelatinous matrix (conidial masses), little amount of the spore mass from an individual fruiting body was streaked on 2% water agar medium by sterile needle and plates were incubated for 24 h at 25°C and single germinated conidium was transferred to a new plate containing potato dextrose agar (PDA). Colonies were grown at 25°C for 7 to 10 days.

Finally, 147 fungal isolates belonging to *C*. *chrysosperma* were obtained from infected plant tissues. Fungal isolates were grown on PDA slants at 25°C for 7 to 10 days and then stored at 4°C for future use. Of these, 58 representative isolates were selected according to their geographical distribution and origin for assessment of their genetic diversity (Table 1).

DNA extraction

Pure culture of selected isolates of *C. chrysosperma* was first grown on PDA (pathological demand avoidance). After four days, three mycelial plugs with 6 ml diameter taken from the edge of the growing colony of each isolate were transferred aseptically to a 250 ml Erlenmeyer flask containing 100 ml potato-dextrose broth (PDB). After inoculation, flasks were put on rotary shaker incubator with 140 rpm rotation speed for four to seven days at 25°C. Then mycelial mats were harvested by vacuum filtration, washed three times with sterile deionized water, dried and stored at –20°C. Total genomic DNA was extracted from bulked mycelium following the procedure described by Safaei et al. (2005). The extracted genomic DNA was diluted in 50 µl distilled water and checked in terms of quality and quantity by means of both 0.8% agarose gel electrophoresis and spectrophotometery technique using absorption at 260 nm. Finally, the extracted DNA was stored in -20°C for future use.

RAPD amplification

The total number of 20 RAPD primers were screened using genomic DNA extracted from ten isolates. Ten primers that gave reproducible results were selected and used in the analyses of all the 58 isolates. PCR amplifications were performed using a thermocycler (Corbett Research, Australia) with total reaction volume of 25 µl containing 10 mM Tris-HCl (pH~8), 50 mM KCl, 3.2 mM $MgCl_2$, 1 µM of each single primer, 0.2 mM dNTP, 1 unit of *Taq* DNA polymerase and 30 ng of template DNA. Amplification reactions were carried out using the following cycle profile: initial denaturation at 94°C for 4 min followed by 40 cycles; denaturation at 94°C for 30s, annealing at 37 to 40°C (according to the primer used) for 40s, extension at 72°C for 90 s and a final extension at 72°C for 5 min. PCR products were separated using 1.2% agarose gel electrophoresis technique with 50X TAE buffer (EDTA, trise-base, acid acetic, pH~8), stained with ethidium bromide and finally visualized under ultraviolet illuminator. The gel image was recorded using gel documentation system (UVP, UK).

Data analyses

The produced RAPD bands were scored based on their presence/absence in each isolate, which then were transformed

Table 1. Used *Cytospora chrysosperma* isolates.

Isolate	Origin	Date of sampling
A.kah.2	West Azarbaijan	2008/10/28
A.kah.6	West Azarbaijan	2008/10/28
A.koh.2	West Azarbaijan	2008/10/28
A.sal.1	West Azarbaijan	2008/10/28
A.sal.4	West Azarbaijan	2008/10/28
CH.ave.2	Chaharmahal va Bakhtiari	2008/8/2
CH.far.1	Chaharmahal va Bakhtiari	2008/8/2
CH.far.2	Chaharmahal va Bakhtiari	2008/8/2
CH.sam.1	Chaharmahal va Bakhtiari	2008/8/2
CH.sam.4	Chaharmahal va Bakhtiari	2008/8/2
CH.sh.1	Chaharmahal va Bakhtiari	2008/8/2
F.saf.4	Fars	2008/8/31
H.nah.1	Hamedan	2008/8/2
H.nah.3	Hamedan	2008/8/2
H.nah.6	Hamedan	2008/8/9
H.nah.8	Hamedan	2008/8/9
H.nah.18	Hamedan	2008/8/9
H.ser.1	Hamedan	2008/10/10
H.toi.1	Hamedan	2008/10/10
H.toi.2	Hamedan	2008/8/9
H.toi.4	Hamedan	2008/10/10
H.toi.8	Hamedan	2008/10/10
IL.il.2	Ilam	2008/8/31
IS.dar.1	Isfahan	2008/8/2
IS.rez.3	Isfahan	2008/8/2
IS.rez.4	Isfahan	2008/8/2
IS.rez.5	Isfahan	2008/8/2
KE.gah.1	Kermanshah	2008/10/10
KE.kan.2	Kermanshah	2008/10/10
KE.sah.6	Kermanshah	2008/10/16
KE.sah.13	Kermanshah	2008/10/16
KE.son.2	Kermanshah	2008/7/28
KE.sah.5	Kermanshah	2008/10/16
KE.son.7	Kermanshah	2008/7/28
KO.sagh.1	Kurdistan	2008/10/21
KO.san.8	Kurdistan	2008/10/18
KO.san.5	Kurdistan	2008/10/18
KO.san.7	Kurdistan	2008/10/18
KO.san.17	Kurdistan	2008/10/18
KO.san.19	Kurdistan	2008/10/18
KO.san.26	Kurdistan	2008/10/18
L.ali.1	Lorestan	2008/8/2
L.azn.1	Lorestan	2008/10/23
L.bor.1	Lorestan	2008/8/2
L.dor.1	Lorestan	2008/8/2
L.dor.2	Lorestan	2008/8/2
L.osh.1	Lorestan	2008/8/2
L.osh.2	Lorestan	2008/8/2
M.ara.1	Markazi	2008/7/3
M.ara.2	Markazi	2008/7/3
M.ara.3	Markazi	2008/7/3

Table 1. Continue.

M.kho.1	Markazi	2008/9/12
M.kho.2	Markazi	2008/9/12
M.kho.3	Markazi	2008/9/12
Z.abh.1	Zanjan	2008/8/10
Z.zan.1	Zanjan	2008/8/1
Z.zan.2	Zanjan	2008/8/1
Z.zan.8	Zanjan	2008/8/1

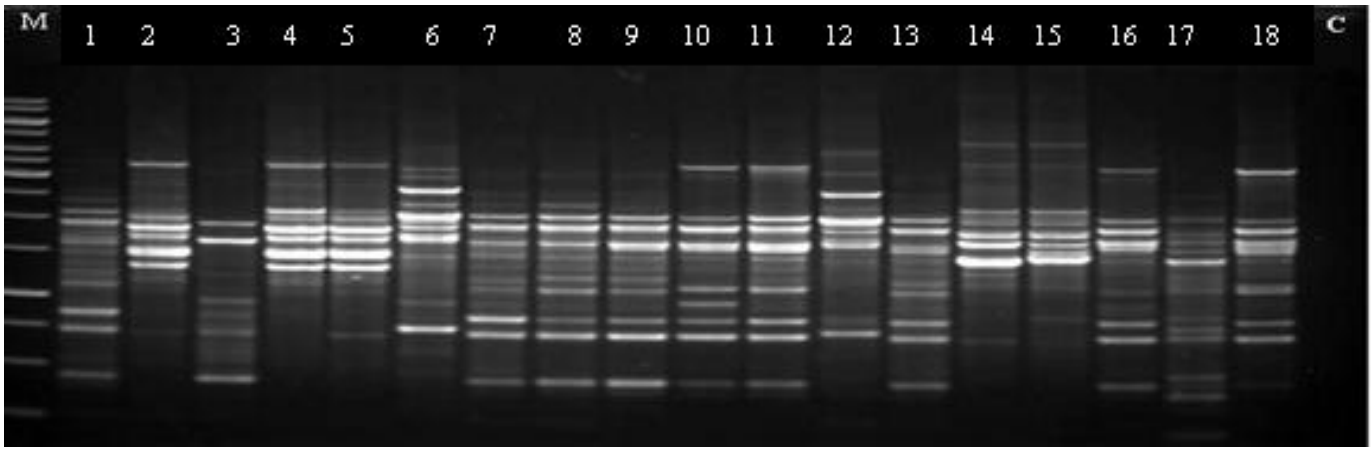

Figure 1. The results of PCR amplification using primer U17 detected by means of agarose gel electrophoresis technique. First lane, DNA ladder (1 Kb) and the last lane negative control.

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) values for each RAPD primer was calculated according to the formula:

PIC = 1 - Σ(Pij)2,

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). Pair-wise genetic similarity (GS) between individuals for RAPD marker was estimated using the Jaccard's similarity coefficient (Jaccard, 1908). Cluster analysis of data was performed with MVSP version 3.131 software (Kovach Computing Services, Anglesey, Wales) and Centroid method. Principal coordinate analysis (PoCA) was performed using a batch mode of NTSYS-pc version 2.11 V software (Exeter software, NY, USA) (Rohlf, 2000). 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).

RESULTS

Out of 20 primers that were primarily used, only ten primers produced scorable and polymorphic bands (Figure 1). A total of 94 loci were found using these ten primers, out of which 89 loci were polymorphic. Primers E17, U17 and UBC1 had the lowest percentage of polymorphic bands (85%) (Table 2). The highest number of common fragments was 15 bands using the primer OPC07. The genetic relationships among *C. chrysosperma*isolates were presented in a dendrogram (Figure 2). Results of the analysis via cluster analysis and PCoA (Figure 3) were slightly different. According to the analysis of molecular variance (AMOVA) and PCoA of the combined fingerprinting data using Centroid method, the isolates were classified into six groups.

Results from AMOVA indicated that 6% of the genetic variation is attributable to differences among accessions groups while 94% of the genetic variation is attributable to between accessions within accessions groups. Detailed results from AMOVA are given in Table 3. Jaccard's coefficient of genetic similarity (GS) matrix was computed. The maximum similarity was detected between isolates 24 and 43 (GS 0.90) and the minimum was found between isolates 35 and 39 (GS 0.19). The average PIC was 0.38, ranging from 0.30 to 0.45. The lowest and the highest PIC values were recorded for primers AB1 and E16, respectively.

Although there was no correlation between geographical origins and the resulting groups of RAPD analysis, but the amount of observed polymorphism (94%) indicated the adequacy of the molecular marker, RAPD

Table 2. RAPD primers and their produced number and type of fragments.

Primer	Primer sequence 5′-3′	Annealing temperature (°C)	Number of scored band	Number of Polymorphic bands	Percentage of polymorphic band (%)	*PIX	**MI
OPC07	GTCCCGACGA	40	15	15	100	0.41	4.05
E16	GGTGACTGTG	40	10	9	90	0.45	3.66
OPC15	GACGGATCAG	40	6	6	100	0.4	2.38
A7	GAAACGGGTG	40	10	10	100	0.37	3.72
AB1	CCGTCGGTAG	40	8	8	100	0.3	2.11
E17	CTACTGCCGT	40	7	6	85.71	0.4	2.05
U17	ACCTGGGGAG	37	7	6	85.71	0.35	1.82
U11	AGACCCAGAG	40	9	9	100	0.38	3.45
OPC13	AAGCCTCGTC	40	15	14	93.33	0.37	4.78
UBC1	CCTGGGCTTC	37	7	6	85.71	0.36	1.86

*PIC, polymorphic information content.;** MI, marker index.

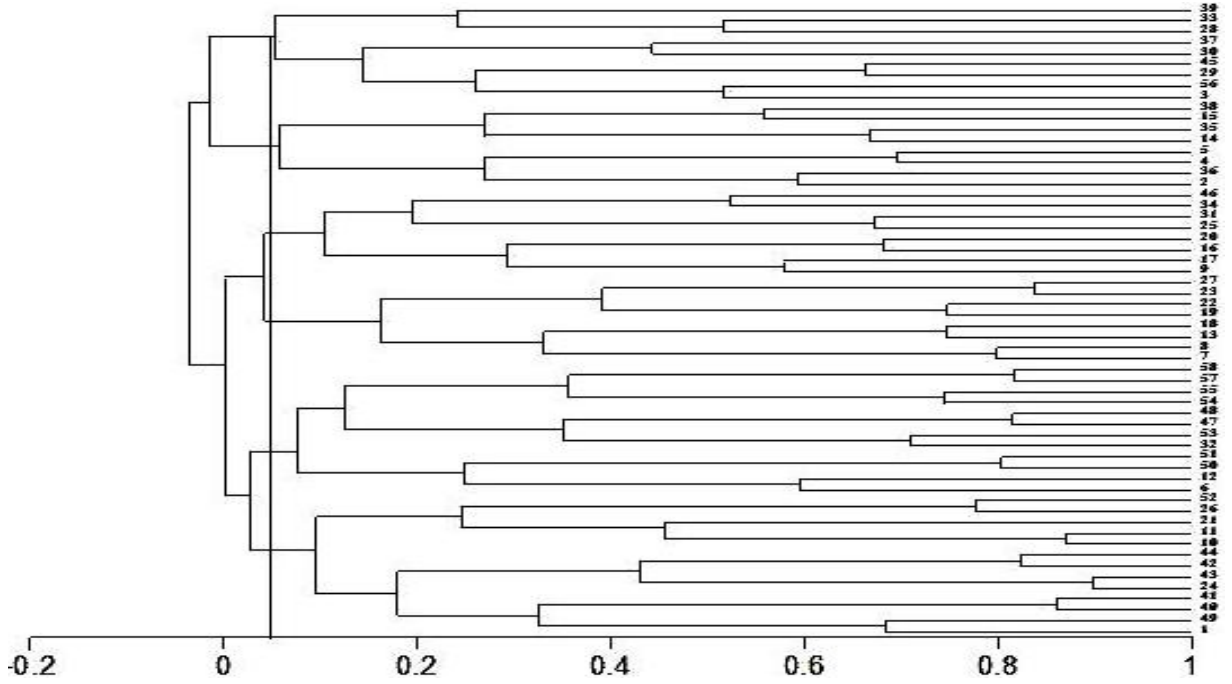

Figure 2. Dendrogram depicting genetic relationship among the 58 isolates of *Cytospora chrysosperma,* produced based on RAPD data, using Centroid method and Jaccard´s similarity coefficient.

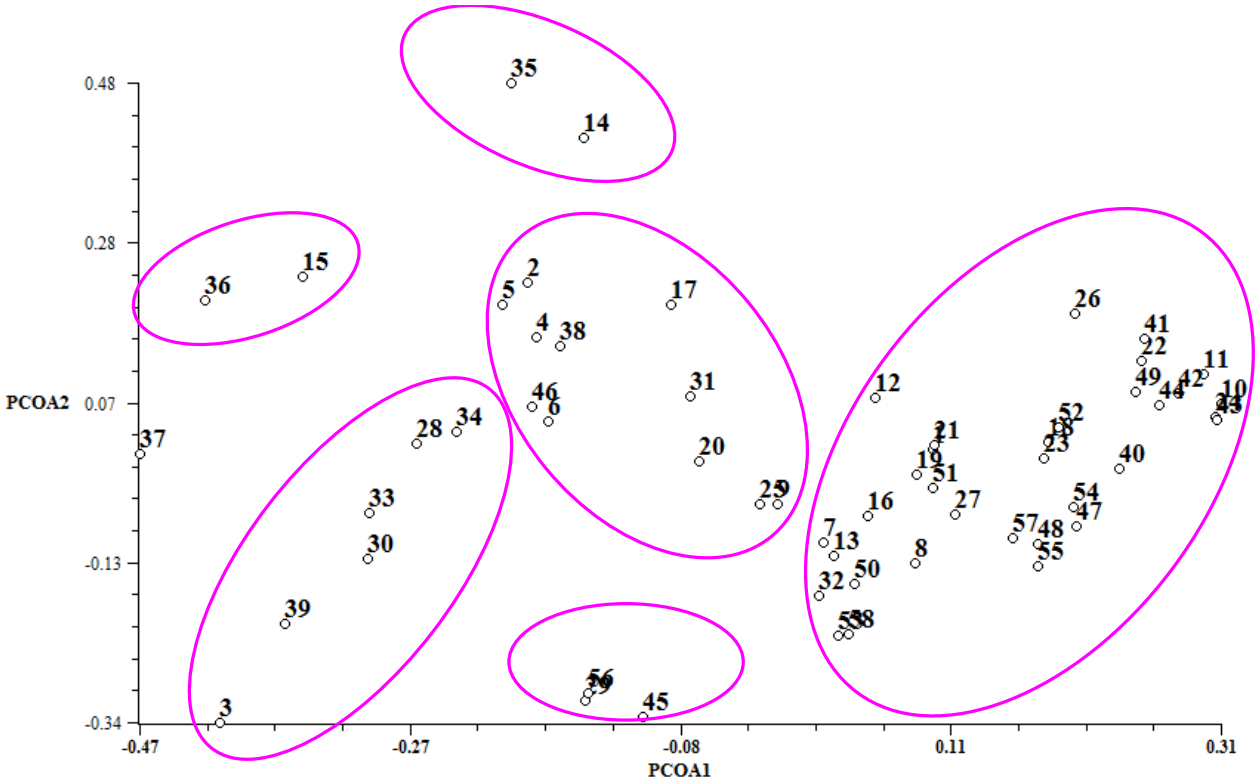

Figure 3. Distribution of the 58 isolates of *Cytospora chrysosperma* in two-dimensional PCoA in NTSYS-pc ver. 2.11 V software using data produced by 10 RAPD primers.

Table 3. Analysis of molecular variance (AMOVA). Statistics includes degrees of freedom (df), sum of squares, estimated variance (EV) and percentage of total variation (%).

Source	df	Sum of squares	EV	Percentage (%)
Among groups	5	139.418	1.114	6**
Within groups	52	895.203	17.215	94**
Total	57	1034.621	18.329	100

**Significant at P < 0.01 level.

for the study of genetic diversity in *C. chrysosperma* isolates. This is the first report on the study of genetic diversity of populations of *C. chrysosperma* using RAPD marker.

DISCUSSION

Knowledge of the genetic structure of pathogen populations has direct applications to agricultural ecosystems. It is evident that whatever genetic diversity in pathogen population is high; the efficiency of management practices such as the use of fungicides will be reduced and the possibility of occurrence of more virulent strains will be increased (Mcdonald and Martin, 1990). In this study, a high level of polymorphism was observed among the isolates. This can be attributed to sexual reproduction in *C. chrysosperma*. Although there was no correlation between geographical origins and the resulting groups of RAPD analysis, isolates from the same locations showed a tendency to classify together. Since, walnut is a commercial product that is grown in diverse climatic regions of Iran, grouping of isolates with different geographical origin in a same cluster suggests that these isolates may have migrated between locations through infected saplings and they did not evolve independently from each other. The lack of correlation between geographical origins and resulting groups of molecular analysis of some plant pathogenic fungi have been reported in the other studies (Khalil et al., 2003; Almeida

et al., 2003; Lachquer and Sedra, 2002). This study reveals that PCR based fingerprinting technique (RAPD), is informative for estimating the extent of genetic diversity among *C. chrysosperma* isolates. Choice of appropriate distance or similarity measure and clustering algorithm is a critical step in the analysis of genetic diversity and determination of true genetic relationship between individuals using molecular marker data. Although, various types of distances or similarity mea-sures are available, there are no acceptable universal approaches to assess the distance or similarity between individuals with molecular markers. We used different measures including those based on binary matrix and those using allele frequencies to assess the relationships between isolates. Chaining effect in the dendrogram constructed using unweighted pair group method with arithmetic mean (UPGMA) algorithm prevented clear grouping pattern, therefore, grouping based on Centroid method algorithm with better pattern is used to describe the relationships among entries (Mohammadi et al., 2008).

REFERENCES

Abbasi K (2010). Study on genetic diversity of *Cytospora chrysosperma* isolates obtained from walnut trees using molecular markers. M.Sc thesis, University of Kurdistan, Sanandaj, Iran, p. 113.

Abbasi K, Abbasi S, Fotouhifar KB (2010). First report of *Cytospora schulzeri* on the walnut trees from Iran. 19th Iranian Plant Protection Congress, Tehran, Iran, p. 127.

Abbasi S, Abbasi K, Bahraminejad S, Khaledi N (2010). Comparative Virulence of Four *Cytospora* speicies Isolated from Walnut Trees in Iran. 16th Asian Agricultural Symposium and 1st International Symposium on Agricultural Technology. Bangkok, Thailand.

Adams GC, Roux J, Wingfield MJ (2006). *Cytospora* species (Ascomycota, Diaporthales, Valsaceae): introduced and native pathogens of tree in South Africa. Australas. Plant Pathol. 35: 521-548.

Adams GC, Wingfield MJ, Common R, Roux J (2005). Phylogenetic relationships and morphology of *Cytospora* species and related teleomorphs (Ascomycota, Diaporthales, Valsaceae) form Eucalyptus. Stud. Mycol. 52: 11-44.

Adams, GC, Surve-Iyer RS, Iezzoni A (2002). Ribosomal DNA sequence divergence and group I introns within *Leucostoma* species, *L. cinctum*, *L. persoonii* and *L. parapersoonii* sp. nov., ascomycetes that cause Cytospora canker of fruit trees. Mycologia, 94: 947-967.

Aghapour B (2010). Study on morphology and genetic diversity of isolates of *Valsa sordida*, the causal agent of tree canker, in Golestan province using molecular marker, rep-PCR. M.Sc thesis, University of Tehran, Karaj, Iran.

Almeida AMR, Abdelnoor RV, Arias CAA, Carvalho VP, Jacoud-Filho DS, Marin SRR, Benato LC, Pinto MC, Carvalho CGP (2003). Genotypic diversity among Brazilian isolates of Macrophomina phaseolina revealed by RAPD. Fitopatol. Brasileira, 28: 279-285.

Ashkan M, Hedjaroude G (1981). Taxonomic and pathologic studies of form genus *Cytospora* on fruit trees in Iran, I. Taxonomy. Iran. J. Plant Pathol. 17(1-4): 21-68.

Ashkan M, Hedjaroude G (1982). Taxonomic and pathologic studies of form genus *Cytospora* on fruit trees in Iran, II. Pathology. Iran. J. Plant Pathol. 18(1-4): 20-67.

Botstein D, White RL, Skolnick M, Davis RW (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32: 314-331.

Ershad D (2009). Fungi of Iran. 3nd edition, Agricultural Research, Education and Extension Organization, Tehran, Iran, p. 531.

Fotouhifar KB, Hedjaroude GA (2010). ITS rDNA phylogeny of Iranian strains of Cytospora and associated teleomorphs. Mycologia, 102(6): 1369-1382.

Fotouhifar Kh B, Hedjaroude G, Ershad, D, Moussavi SM, Okhovvat SM, Javan-Nikkhah M (2007). New information on the form-genus *Cytospora* in Iran (I). Rostaniha 8(2): 129-149 (in Farsi with English summary).

Fragoso RG (1918). *Pugillus secundus* mycetorum Persiae (Lecti a ferd. Martinez de la Escalera). Boletín de la Real Sociedad Española de Historia Natural, 18: 78-85.

Hayova VP, Minter DW (1998). *Valsa sordida*. IMI Descriptions of Fungi and Bacteria. Set 137, NO. 1370, CAB International, UK.

Jaccard P (1908). *Nouvelles recherches* sur la distribution florale. Bul. Soc. Vaud. Sci. Nat. 44: 223–270.

Javadi EAR (2008). Introduction of *Cytospora* species on walnut trees in Iran. 18th Iranian Plant Protection Congress, Bu-Ali Sina University, Hamadan, Iran, p. 639.

Khalil MS, Abdel-Sattar MA, Aly IN, Abd-Elsalam KA, Verreet JA (2003). Genetic affinities of Fusarim spp. and their correlation with origin and pathogenicity. Afr. J. Biotechnol. 2(5): 109-113

Lachquer K, Sedra MH (2002). Characterisation of *Verticillium dahliae* Kleb. isolates from *Olea europea* using RAPD markers. Phytopathol. Mediterr. 41: 170–178.

McDonald BA Martines JP (1990). DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolates and host cultivars. Phytopathology, 86: 200-212.

Mohammadi SA, Khodarahmi M, Jamalirad S, Jalal Kamali, MR (2008). Genetic diversity in a collection of old and new bread wheat cultivars from Iran as revealed by simple sequence repeat-based analysis. Ann. Appl. Biol. 154: 67–76.

Peakall R, Smouse PE (2006). Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes, 6(1): 288-295.

Rohlf FJ (2000). NTSYS-pc: Numerical Taxanomy and Multivariate Analysis System, Version 1.8. Exeter Software: Setauket, New York, USA.

Safaei N, Alizadeh A, Rahimian H, Adam G (2005). Molecular characterization and evaluation of genetic variation in Iranian populations of causal agent of spike blight of wheat. Iran. J. Plant Pathol. 41: 171-189.