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GENETIC DIVERSITY OF PLANT PATHOGEN *Valsa sordida* USING MICROSATELLITE MARKERS

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The genetic diversity of *Valsa sordida* isolates from different geographical regions and hosts was investigated using MP-PCR markers. PCR amplifications were done using eight primers. Of them, only four primers [$(ATC)_7$, $(ACTG)_4$, $(CGA)_5$, and $(AAC)_8$] produced polymorphic bands. At least 88.5% polymorphism was revealed by four primers and the maximum polymorphism (97%) was generated by $(ACTG)_4$ primer and three diagnosable groups (1, 2 and 3) were resolved in the resulting dendrogram constructed by the UPGMA algorithm. The results showed high polymorphism among the isolates and confirmed the merit and accuracy of the MP-PCR markers for studying the genetic variability of *V. sordida* isolates at the intra-species level. We have not found any correlations between observed genetic diversity and the geographical region or host plant of the isolates, unless in limited cases. The abundant formation of the sexual state of the fungus in the infected parts of trees, as well as possible asexual recombination during asexual reproduction, are suggested as influencing factors of high genetic variability among the individuals.

Key words: Cytospora chrysosperma, canker disease, tree hosts, molecular marker, MP-PCR

INTRODUCTION

Cytospora canker, sometimes called Valsa canker, Leucostoma canker, or perennial canker, is a serious and spreading disease on angiosperm and gymnosperm woody plants throughout the world (ADAMS *et al*., 2005). The genus *Cytospora* was first described by EHRENBERG (1818), and its species are plant pathogens or saprobes with a worldwide distribution and are among the most important pathogenic taxa of the Fungi, causing canker and dieback diseases on the twigs of a wide range of host plants (NORPHANPHOUN *et al*., 2018). The first

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report on species of the genus *Cytospora* in Iran was published by FRAGOZO (1918) introducing *Cytospora silenes* Gonz. Frag. on *Silene boryi* Boiss. FOTOUHIFAR *et al*. (2010) studied the taxonomy of *Cytospora* species in Iran using the ITS nuclear rDNA sequences analysis. Parsimony analysis established five distinct major clades and 12 sub-clades, which were correspond to the accepted species and genera. *Valsa sordida* Nitscke and its anamorphic state, *Cytospora chrysosperma* (Pers.) Fr., are the causal agents of widespread canker disease on trees such as species of *Populus* and *Salix* and numerous other host plants (FOTOUHIFAR, 2007). Many *Cytospora* species were listed as causal agents of Cytospora canker worldwide. However, species identification in this group of fungi is controversial, because, *Cytospora* fruiting bodies and vegetative structures, as well as spore size, vary considerably even in the isolates of the same species (SPIELMAN, 1985).

Microsatellite loci are short tandemly repeated motifs of 1-6 bases, also known as simple sequence repeats (SSR), and are widely used as optimal genetic markers, because they have a considerably polymorphic nature within the population, highly abundant, and also dispersed throughout the eukaryotic genomes, inherited in a co-dominant fashion, and fast and easy for fingerprinting (DUTECH *et al.*, 2007; WEISING *et al.*, 1995). Moreover, they allow inferences of population genetic parameters such as gene flow, effective population size, or the reproductive system, to be made with high accuracy (BREUILLIN *et al.*, 2006). The technique of microsatellite-primed PCR (MP-PCR) was originally introduced by MEYER *et al.* (1993). Several studies have shown the importance of using microsatellites to understand epidemiological processes in plant pathogenic fungi (BREUILLIN *et al.*, 2006).

CZEMBOR and ARSENIUK (1999) studied the genetic variability of the *Stagonospora* ssp. and *Septoria tritici* isolates using MP-PCR. The molecular marker revealed low similarities, resulting from the high mutation rate of the microsatellites and transposons activity. MP-PCR has also successfully been used in species and sub-species differentiation in many pathogenic fungal genera, including *Colletotrichum*. Genetic analyses based on 52 MP-PCR markers revealed a highly significant differentiation between two populations of *Colletotrichum gloeosporioides* Penz. from yam plants (ABANG *et al.*, 2005). Also, this molecular marker could be used to identify the mycelial compatibility groups of *Valsa malicola* (SEIFOLLAHI *et al.*, 2014). Application of MP-PCR to study the genetic diversity of *Cytospora schulzeri*, and *Cytospora cincta* showed high genetic variability among the isolates obtained from different geographical places and different parts of apple trees. But the identified groups did not correlate with the geographical places or origin of the isolates on the sampled trees (MERHRABI *et al.*, 2015).

AGHAPOUR (2010) has also investigated the diversity and genetic structure of *Valsa sordida* populations from Golestan province, using the rep-PCR molecular marker. For this, 30 isolates of *C. chrysosperma*, 10 isolates of *Cytospora* sp.1, one isolate of *Valsa* sp.1, and one isolate of *Cytospora* sp.2 were evaluated using the ERIC and BOX primers. Results showed two separate groups of strains in *V. sordida*. As a result, rep-PCR was suggested as an appropriate molecular marker for species delimitation in this fungus.

ABBASI (2010) and ABBASI *et al.* (2011) have investigated the genetic diversity of *C. chrysosperma* populations using RAPD and ISSR molecular markers, and the results showed high genetic variability among the populations. They have not observed any correlation between

geographical origins and the identified groups in RAPD and ISSR analyses, but the amount of the observed polymorphism, 94%, and 95%, respectively, indicated the adequacy of molecular markers for studying the genetic diversity of *C. chrysosperma* isolates.

In addition, MEHRABI *et al.* (2015) have evaluated the genetic diversity of isolates of two species, *Cytospora schulzeri,* and *Cytospora cincta*, recovered from Iran, using RAPD and MP-PCR molecular markers. However, they also have not found any correlation between the geographical origins of the isolates and their host tree, but the amount of observed polymorphism was very considerable, and the results indicated the eligibility of using molecular markers to study the genetic variability in *C. schulzeri* and *C. cincta* isolates.

In order to complete the previous studies on the genetic diversity of this species, the study of intra-species genetic variability using collected isolates from different hosts and geographical regions with an appropriate molecular marker is necessary. Because of the economic importance of diseases caused by *Valsa sordida* on a wide range of host plants, the study of its genetic diversity in Iran could be very valuable. On the other hand, due to the high accuracy of the MP-PCR marker in detecting the high level of genetic diversity among the isolates of a given species and grouping of isolates in different populations, and even in identified mycelial compatibility groups, the selection of this marker will be useful for the study of genetic variability. So, the main aim of the present study was the investigation of the genetic diversity of *Valsa sordida* isolates obtained from different hosts and geographical areas in Iran using the MP-PCR molecular marker.

MATERIALS AND METHODS

Fungal isolates and DNA extraction

Ten isolates of *Valsa sordida* have been recovered from infected twigs and barks of apple (*Malus pumila*), willow (*Salix babylonica* and *Salix excelsa*), poplar (*Populus deltoides*), walnut (*Juglans regia*) and oleaster (*Elaeagnus angustifolia*) trees that were collected in November 2010 from different regions of Isfahan province, Iran. Pure colonies of the fungal isolates were obtained using the method described by FOTOUHIFAR (2007) based on a blotter test. Other isolates (30 isolates) were obtained from the fungal collection of the University of Tehran, Department of Plant Protection that were deposited previously by FOTOUHIFAR (2007), AGHAPOUR (2010), and BOZORGMANESH (2012). Overall, 40 isolates of *Cytospora chrysosperma* that were used in this study were recovered from infected tree samples obtained from different geographical areas and host plants in Iran (Table 1).

To harvest mycelia for DNA extraction, fungal isolates were grown in the hand-made potato dextrose broth (PDB) containing extract of 200 g boiled potato and 20 g dextrose mixed in 1000 ml double distilled water, for 7-10 days at 25-28 °C. Mycelial biomasses were filtered and washed using filter paper, Buchner funnel, and a vacuum pump. DNA was extracted from the lyophilized mycelia using the mini-preparation method described by LIU *et al*. (2000). The extracted genomic DNA was diluted in 50 µl distilled water and checked in terms of quality and quantity using 1% agarose gel electrophoresis. The gel image was recorded using a gel documentation system (B&L system, Netherlands IMAGO). Finally, the extracted DNA was stored at -20°C for future use.

Polymerase chain reaction

The total number of eight MP-PCR primers were screened using the genomic DNA extracted from some isolates (Table 2). Four primers that generated polymorphic and reproducible bands were selected and used for evaluation of all 40 isolates. PCR amplifications were performed using a thermocycler with a total reaction volume of 20 µl. The PCR was performed as follows: about 10 ng of DNA were added to a reaction mixture containing 2.5 µl 10× PCR buffer containing [500 mM KCl and Tris-HCl (pH 8.4)] (CinnaGen Co., Iran), 2.5 mM $MgCl_2$, 0.5 mM dNTP mix, 1 unit of *Taq* DNA polymerase (CinnaGen Co., Iran) and 1 µM of each single primer. Amplification reactions were carried out using the following cycle profile: initial denaturation at 95 °C for 5 min followed by 40 cycles; denaturation at 94 °C for 30 s, annealing at 40-50 °C (according to the used primer) for 1 min, extension at 72 °C for 35 s and a final extension at 72 °C for 7 min. PCR products were separated using a 1.4% agarose gel electrophoresis technique for 3 h at 85 V in 0.1×TBE buffer, stained with ethidium bromide, and photographed by the Gel Documentation system.

Statistical analysis

The relationships of 40 isolates were estimated by means of scorable DNA bands amplified from different microsatellite loci. Each band was considered as a binary character of present (scored as 1) and absent (scored as 0). Cluster analyses were performed by the unweighted pair group method with an arithmetic average (UPGMA) algorithm using NTSYS-pc software (ver. 2.01). The resulting dendrogram was used to reveal the relationships among the different isolates.

RESULTS

All recovered isolates of *V. sordida* were used in this study. Eight microsatellite primers were used to study the genetic diversity of *V. sordida* isolates. The obtained isolates were deposited in the culture collection of the Department of Plant Protection, University of Tehran. Primer $(CT)_8$ with dinucleotide motif, has not produced any banding pattern, so, this primer was omitted from the analysis. Primers $(TATG)_4$, $(CTA)_5$, and $(TGTC)_4$ have not produced enough polymorphic loci and these primers were also omitted from further analysis. Finally, four of eight tested microsatellite primers including $(ACTG)_4$, $(CGA)_5$, $(ATC)_7$ (Figure 1), and $(AAC)_8$ were selected based on generated results and their reproducibility and the numbers of produced polymorphic bands.

Cluster analysis of the resulting MP-PCR fingerprinting patterns was done with the UPGMA method using the Jaccard's coefficient. All selected primers generated polymorphic loci with various frequencies. The highest percentages of produced polymorphic DNA fragments, 93%, 97%, 93%, and 88.5%, were produced by $(ATC)_7$, $(ACTG)_4$, $(CGA)_5$, and $(AAC)_8$ primers, respectively. Different levels of polymorphism and different numbers of loci were obtained by using these four primers. Generated loci ranged from 225 to 2500 bp for each tested isolate. The largest number of polymorphic DNA fragments was generated by microsatellite primer $(ACTG)_4$ in PCR amplification. The constructed dendrogram, based on the results generated by all primers, revealed different levels of genetic similarity among the isolates that were obtained

from different hosts and geographical regions. In the resulting UPGMA dendrogram (Figure 2), three distinct groups were identified in the investigated isolates. The biggest group (group 1) includes 16 isolates, with considerable spatial distance, that all were collected from 13 different hosts (*Salix babylonica*, *Populus deltoides*, *Salix excelsa*, *Salix alba*, *Salix triandra*, *Juglans regia*, *Populus nigra*, *Morus alba*, *Malus pumila*, *Robinia pseudoacacia*, *Armeniaca vulgaris*, *Hibiscus syriacus*, and *Fraxinus excelsior*) and eight different provinces (Isfahan, Golestan, Tehran, Razavi Khorasan, Hamadan, Lorestan, Alborz, and Markazi) of Iran.

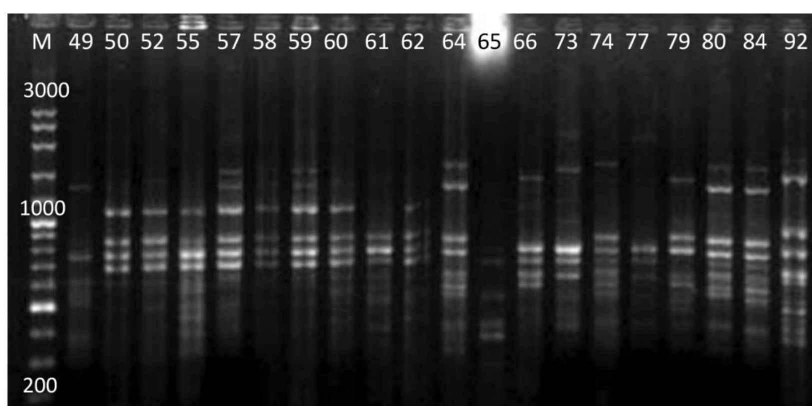

Figure 1. The DNA fingerprinting profile of some *Valsa sordida* isolates generated by $(ATC)_7$ primer. M: Molecular size marker (Gene Ruler™ 100bp DNA, Vivantis technologies, Malaysia); Lanes 49-92: different isolates of *Valsa sordida* including: G81, G87, G89, G104, G108, G109, G110, G95, 331, K-1, 334, 335, 316, 300, 304, 225, 216-1, 233-1, 284-1 and K-2. PCR products were resolved in 1.4% agarose gel using TBE (1x) buffer.

The second group (group 2) consists of nine isolates that all were collected from six different hosts (*Ficus carica*, *Populus deltoides*, *Salix excelsa*, *Elaeagnus angustifolia*, *Juglans regia*, and *Malus pumila*) and two different provinces (Isfahan and Fars) of Iran. The third group (group 3) consists of 15 isolates that all were recovered from 11 different hosts (*Salix babylonica*, *Populus deltoides*, *Salix excelsa*, *Salix* sp., *Prunus domestica*, *Thuja orientalis*, *Populus nigra*, *Parthenocissus tricuspidata*, *Malus pumila*, *Olea sativa*, and *Eucalyptus* sp.) and eight different provinces (Fars, Golestan, Mazandaran, Tehran, Kohkiluyeh and Buyer Ahmad, Lorestan, Alborz and Kurdistan) of Iran. Also, there is considerable spatial distance among the isolates in these three groups. In this study, high genetic diversity was observed among the studied fungal isolates, inside the identified groups, and also among the groups. However, clear relationships between estimated genetic diversity and geographical distribution or host preference of the isolates, except in some cases, were difficult to achieve.

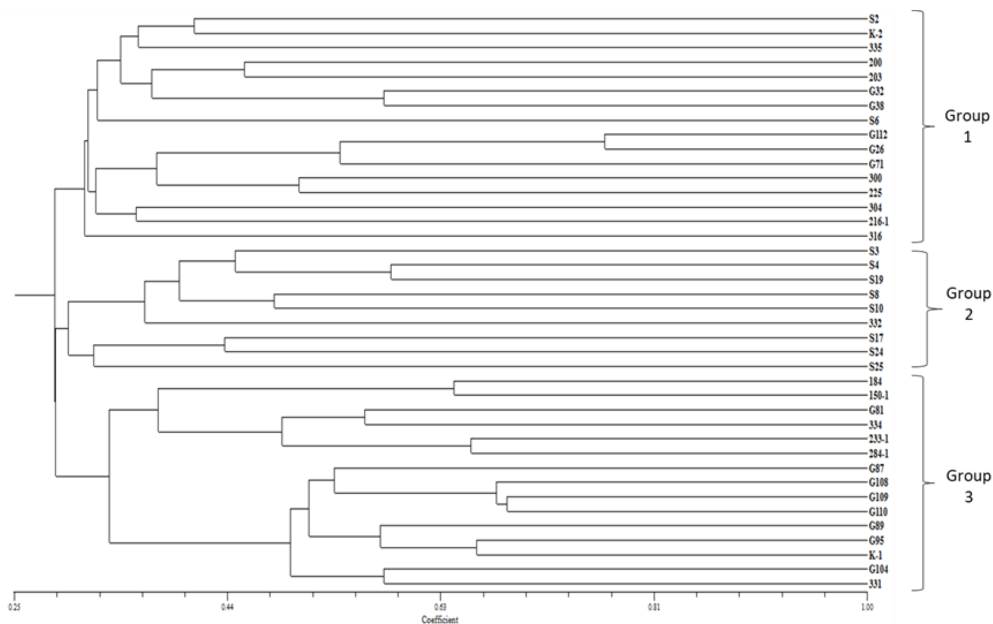

Figure 2. Dendrogram of genetic distance among 40 isolates of *Valsa sordida* was obtained by combining the results of four MP-PCR primers [$(ATC)_7$, $(ACTG)_4$, $(CGA)_5$ and $(AAC)_8$] based on the unweighted pair-group method using arithmetic averages (UPGMA) in NTSYS-pc software (ver. 2.01).

DISCUSSION

Valsa sordida is one of the important and destructive species in the Valsaceae family, causing canker disease in tree hosts. In this study, *V. sordida* isolates were recovered from 20 different host tree species that were growing in 12 different provinces of Iran. The fungus has a very broad host range, including fruit trees such as apple, walnut, fig, olive, and even wild or ornamental trees such as *Populus* spp., *Salix* spp., and eucalyptus. Originally, *V. sordida* was reported confining to the species of *Populus* and *Salix* (HAYOVA and MINTER, 1998). The results of this study are indicating that this species is increasing its host range in Iran. This could be the result of growing many different tree hosts in sympatric and even in allopatric situations with dry climates that predispose the host trees that are infected by *V. sordida*.

Investigation of genetic diversity within each population of a pathogen that is responsible for disease epidemics is essential for the development of effective control strategies (ABANG *et al.*, 2005). Because of high polymorphism, ease of score, and co-dominance, microsatellite loci have been proved to be invaluable in many fields of biology, such as population genetics, paternity testing, and genome mapping in forensics. Furthermore, they can be developed as

powerful tools for inferring the evolutionary and demographic parameters of a given organism (DUTECH *et al.*, 2007). DUTECH *et al.* (2007) claimed the high polymorphism of trial microsatellites in fungi and also microsatellites of fungi are harder to isolate and exhibit lower polymorphism in comparison to other organisms.

Most of the microsatellites used in this research were also successfully applied in studies of the genetic diversity of other fungal species (GROPPE *et al*., 1995; SEIFOLLAHI *et al*., 2014; MEHRABI *et al*., 2015). Evaluating primers with di, tri, and tetranucleotide motifs indicated that the primers with dinucleotide motifs do not produce considerable banding patterns for comparison. The results of this research showed that the clear polymorphic banding patterns were produced mostly by tri- and tetranucleotide primers, and all dinucleotide primers produced smears, which is consistent with prior studies (WEISING *et al*., 1995). Based on the obtained results, the high polymorphism was produced by $(ACTG)_4$ primer. Most of the identified loci for all primers were polymorphic. Similarly, the highest level of polymorphism with $(ACTG)_4$ primer was observed in *C. schulzrei*, another devastating species of *Cytospora* in Iran that infects mainly the apple trees (MEHRABI *et al.* 2015). The MP-PCR assay with $(CTA)_5$, and $(TGTC)_4$ primers did not produce any detectable bands. One logical explanation is that it is probably the annealing sites for these primers missed from the genome of the studied fungi (CZEMBOR and ARSENIUK, 1999). In contrast to our results, in the assessment of genetic diversity of *Fusarium oxysporum* f. sp. *radicis-lycopersici* and f. sp. *lycopersici* isolates, it was proved that microsatellite-primers such as $(TGTC)_4$ produced 100% polymorphism in two populations of these fungi (BALMAS *et al.*, 2005).

We found a high level of polymorphism in the investigated isolates of *V. sordida*. However, we were unable to find any correlation between estimated genetic diversity and the geographical or host origins of the studied isolates. High level of polymorphism and lack of correlation between genetic diversity and geographic or host origins were also encountered in the assessment of genetic diversity in *C. schulzeri*, *C. cincta* (SEIFOLLAHI *et al*., 2014; MEHRABI *et al*., 2015), *Stagonospora* ssp. and *Septoria tritici* (CZEMBOR and ARSENIUK, 1999) using MP-PCR and *C. chrysoperma* (ABBASI, 2010; ABBASI *et al*., 2011) using ISSR and RAPD markers. So, the eligibility and accuracy of the MP-PCR markers to investigate the genetic diversity of *V. sordida* and related species is proved. The high level of genetic diversity within the *V. sordida* population could be the result of the sexual recombination during sexual reproduction and/or asexual recombination during the asexual reproduction of the fungus in nature. Sexual fruiting bodies of *V. sordida* can always be observed in diseased parts of the different host species in Iran. FOTOUHIFAR *et al*. (2010) have observed the sexual state of this species at least in three different plant species (*Salix aegyptiaca*, *Juglans regia*, and *Populus alba*). The asexual stage of the fungus is more abundant in cankered areas of trees throughout the year, although it was thought to be rare in the orchards. This means that a huge number of conidia are produced from each conidioma. Mitotic production of abundant conidia in even one canker could result in mitotic recombination, which is a source of genetic differentiation. Also, hyphal anastomosis between individuals could occur as shown by BOZORGMANESH *et al*. (2015) through the investigation of the mycelial compatibility of isolates in this species. Similar results were also obtained in a survey of the genetic diversity of *C. chrysosperma* isolates that were recovered from walnut trees in 12 provinces of Iran using the molecular markers RAPD and ISSR. No clear

correlations between observed genetic diversity and the geographical origins of isolates were found in these studies (ABBASI, 2010; ABBASI *et al.*, 2011). Cluster analysis of DNA fingerprinting using MP-PCR primers in isolates of *C. schulzrei* and *C. cincta*, which has been done by MEHRABI (2009), also revealed the high genetic diversity within the isolates of these two species. However, no correlations between geographical origins of the isolates, and cluster analysis of DNA fingerprints were found. CZEMBOR and ARSENIUK (1999) studied the genetic variability among the isolates of *Stagonospora* ssp. and *Septoria tritici* using three molecular markers, RAPD-PCR, MP-PCR, and rep-PCR. In this study, the MP-PCR assay was found to be the most sensitive technique for the detection of DNA polymorphism in studied taxa.

We identified three distinct groups in the generated dendrogram. The genetic similarity between groups was very low (about 28.8%), which indicates the high genetic diversity between the identified groups. Also, the genetic similarity between isolates of the same group was considerably low (30.6% for group 1, 28.8% for group 2, and 33.5% for group 3). These results indicate that the genetic diversity between the groups and among the isolates of the same group is relatively high. In some cases, there was a higher genetic similarity between isolates, within the groups, with the same host plant of the same geographical origins. For example, in group 1, isolates 200, and 203 (from the ash tree in Markazi province) and G112 and G26 (from the willow tree in Golestan province), in group 2, isolates S8 and S10 (from the poplar tree in Isfahan province) were closely related to each other. In these cases, we assume that genetically close isolates are infecting the same host plant in a sympatric area. In contrast, in group 1, isolates 300 and 225 (isolated from the mulberry and black locust trees, respectively, in Hamadan province), in group 2, isolates S4 and S19 (collected from the poplar and the apple trees, respectively, in Isfahan province) and in group 3, isolates G109 and G160 (isolated from the poplar and willow trees, respectively, in Golestan province) were closely related to each other. In these cases, it is obvious that under selection pressure, the pathogen has extended its host range in the sympatric area. Also, in group 1, isolates S2 and K-2 (from the willow tree in Isfahan province and *Hibiscus syriacus* in Alborz province, respectively) and in group 3, isolates 184 and 150-1 (from the willow tree in Fars province and plum tree in Kohkiluyeh and Buyer Ahmad province, respectively), isolates 233-1 and 184-1 (from the apple tree in Lorestan province and *Thuja orientalis* in Kurdistan province, respectively), isolates G95 and K-1 (from the eucalyptus in Golestan province and from the Boston ivy in Alborz province, respectively) and isolates G104 and 331 (from the willow tree in Golestan province and from the olive tree in Mazandaran province, respectively) were closely related to each other. In these examples, plant materials are easily transported between provinces in huge amounts, even with considerable distance between them, and could expand the host range and the geographical distribution of the pathogen.

The use of MP-PCR molecular markers, allowed us to study the intra-specific genetic variability of *V. sordida* isolates, and these markers revealed high polymorphism among the investigated isolates, which can be used to select infra-specific markers for further genetic evaluation of the pathogen. In conclusion, this study has demonstrated the potential of microsatellite loci for the characterization of the genetic diversity and genetic structure of *V. sordida* populations. Obviously, the results of this study can be used in management strategies to control the spread of canker disease in many shrubs and trees, especially fruit trees, in Iran.

CONCLUSION

Valsa sordida and its anamorphic state, *Cytospora chrysosperma*, are among the most devastating plant pathogens that cause canker and dieback diseases in many woody plants all over the world. The resulting cankers can be seen throughout the year on the infected parts of the trees, producing the asexual fruiting bodies and huge amounts of conidia. In the present study, the genetic diversity of *Valsa sordida* isolates from different geographical regions and hosts was investigated using four microsatellite primers [$(ATC)_7$, $(ACTG)_4$, $(CGA)_5$, and $(AAC)_8$] in MP-PCR. The results revealed high polymorphism (at least 88.5%) among the investigated isolates of *V. sordida*. The results of the present study can be considered in the management strategies for the control of resulting canker disease in many shrubs and trees, especially in fruit trees, in Iran.

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GENETIČKI DIVERZITET BILJNOG PATOGENA *Valsa sordida* KORIŠĆENJEM MIKROSATELITSKIH MARKERA

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Izvod

Genetički diverzitet izolata *Valsa sordida* iz različitih geografskih regionai domaćina je ispitan sa MP-PCR markerima. PCR amplifikacija je urađena sa osam prajmera. Od njih, samo četiri [$(ATC)_7$, $(ACTG)_4$, $(CGA)_5$, i $(AAC)_8$] su dali polimorfne trake. Bar 88.5% polimorfizma je otkriveno sa četiri prajmera i max polimorfizam (97%) je dobijen sa $(ACTG)_4$ prajmerom I tri grupe (1, 2 i 3) se razlikuju na dendogramu sa UPGMA algorimom. Rezultati pokazuju visok polimorfizam između izolata i potvrđuju pouzdanost MP-PCR markera za ispitivanje genetičke varijabilnosti izolata *V. sordida* na intra specijskom nivou. Nije nađena korelacija između genetičkog diverziteta i geografskog regiona ili domaćina. Obilno formiranje polnog stanja gljive u zaraženim delovima drveća, kao i moguća aseksualna rekombinacija tokom aseksualne reprodukcije, sugerišu se kao uticajni faktori visoke genetske varijabilnosti među jedinkama.

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