Genetic diversity of *Valsa malicola* isolates assessed by microsatellite-primed PCR (MP-PCR)

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(Received 12 November 2013; accepted 12 November 2013)

Genetic diversity of 40 isolates of *Valsa malicola* in Iran was investigated by MP-PCR. Isolates were obtained from different host plants in diverse regions during 2004–2010. Of the six microsatellite primers tested, only four primers; (ACTG)$_4$, (GACA)$_4$, (AC)$_8$ and (CGA)$_5$, were able to amplify DNA fragments. With these four primers, 120 loci were identified; of which eight were monomorphic (6.7%) and 112 were polymorphic (93.3%). Approximate size of amplified DNA fragments ranged from 0.2 to 3 kb. Observed high genetic diversity in isolates of *V. malicola* indicates the eligibility of the marker to investigate ranks below the species level. The results of cluster analysis indicated that isolates are related to each other with 45.63% similarity and four groups (1, 2, 3 and 4) were identified in the dendrogram. Group 3 included 37 isolates that were obtained from different hosts and geographical regions and each of groups 1, 2 and 4 only had one isolate. Some correlations between identified groups and host origins or geographic distributions of the isolates were found.

**Keywords:** Cytospora schulzeri; Apple canker; fungus; genome; polymorphism

Introduction

*Valsa* canker or *Cytospora* canker is an important disease on apple trees and it causes severe economic losses (Wang et al. 2007). The pathogens infect the inner bark that is also referred to as the bark periderm. Also, they discolor adjacent sapwood in hardwood plants (Adams et al. 2006). *Valsa malicola* and its anamorph, *Cytospora schulzeri*, the causal agents of canker diseases, have been found as new taxa for mycoflora of Iran, and have been recovered from new host plants, such as *Cerasus vulgaris*, *Colutea* sp. and species of *Malus*, *Cerataegus* and *Thuja*, that had been collected and identified from all over Iran by Fotouhifar (2007). These taxa predominantly infect members of Rosaceae family (mainly apple trees) throughout the world (Fotouhifar et al. 2010) and other hosts are listed by Gvritishvili (1982) and Hayova and Minter (1998). Fotouhifar et al. (2010) showed that *V. malicola* isolates have diverse nucleotide sequence in the ITS regions and based on nucleotide sequence of ITS regions, isolates of *V. malicola* were placed in a not well-resolved clade.

DNA fingerprinting technique is used to study the population structure, epidemiology and systematics of phytopathogenic fungi (Abd-Elsalam et al. 2009) and can provide valuable information about genetic diversity, taxonomy and resistance of fungal pathogens.

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Microsatellite loci are short tandem motifs of 1–6 bases (Hanlon et al. 2000; Dutech et al. 2007) and also known as simple sequence repeats (SSRs) (Dutech et al. 2007) that exist at multiple sites (up to 10^5) in eukaryotic genomes (Geistlinger et al. 1997) and have been utilised widely as genetic markers because of their ubiquity, ease of scoring, reproducibility, assumed neutrality and high level of polymorphism (Dutech et al. 2007). They are highly polymorphic and can be used to discriminate closely related taxa even at the level of intra-species (Weising et al. 1995; Hanlon et al. 2000) or individual isolates in a solitary species (Abd-Elsalam et al. 2009). The use of SSR as primers for PCR is called microsatellite-primed PCR, random amplification of microsatellites, direct amplification of microsatellite-region DNA polymerase chain reaction (DAMD-PCR) and SSR-PCR (Jana et al. 2005). Purkayastha et al. (2008) pointed out that the major advantages of MP-PCR technique are simplicity, universality of primers and tolerance of wide range of DNA concentrations to produce repeatable results. MP-PCR uses single primer to produce DNA fingerprint profiles (Bahkali et al. 2012).

The use of MP-PCR for different purposes in different fungi has shown some adequacies. In a study on genetic variability of monopycnidial and monopycnidiospore isolates of Stagonospora spp. and Septoria tritici, microsatellite primers produced polymorphic bands within pycnidial sets with various frequencies, and MP-PCR was considered as the most sensitive technique for detecting DNA polymorphism among three different techniques used (RAPD, MP-PCR and rep-PCR) (Czembor & Arseniuk 1999). Meinhardt et al. (2002) reported that MP-PCR was able to show genetic variability within MCGs of Sclerotinia sclerotiorum. This molecular marker revealed genetic variation of Mycosphaerella fijiensis isolates which were obtained from the same lesion as well as isolates from different location and also indicated that different isolates can be grouped according to their plant host origin (Muller et al. 1995). Braganca et al. (2011), using MP-PCR, identified different species of Cryphonectria that were obtained from different host and geographical origins. Jana et al. (2005), using MP-PCR, clustered isolates of Macrophomina phaseolina into groups according to their hosts and geographical regions, and the produced polymorphism showed that the microsatellite loci was useful in population studies. Purkayastha et al. (2008) found that chlorate-resistant and chlorate-sensitive isolates of M. phaseolina were separated by MP-PCR. Using of MP-PCR has shown high level of polymorphism in intra-species level among Iranian isolates of Cytospora cincta (Mehrabi 2009), Cytospora chrysosperma (Abbasi 2009) and C. schulzeri (Mehrab 2009) for the study of genetic diversity.

V. malicola is an important plant pathogen in Iran. The main aim of the study was the evaluation of genetic diversity of V. malicola that were collected from different host plants and geographical regions of Iran using MP-PCR molecular marker.

Materials and methods

Fungal isolates

In this study, 40 isolates of V. malicola were used. For obtaining some fungal isolates (25 isolates), infected twigs and barks of apple trees from different regions of Seminrom city in Isfahan province of Iran were collected in November of 2010. Pure colonies of fungal isolates were obtained from infected plant materials using the method provided by Fotouhiifar (2007). Some other isolates (15 isolates) were obtained from mycology collections of University of Tehran and University of Tarbiat Modares that were found by Fotouhiifar (2007) and Mehrabi (2009), respectively. The geographic and host origins of the isolates are shown in Table 1. All studied specimens are kept in the herbarium of
Department of Plant Pathology, University of Tehran, Karaj, Iran, and will be kept in the herbarium of Iran (Iranian Research Institute of Plant Protection, Tehran, Iran). For long-term storage, the pure fungal isolates were grown on the sterile filter paper in the Petri-dishes containing PDA culture medium and then the filter papers were dried and cut in to pieces and were put in 1.5 ml tubes and kept at −20 °C.

**DNA extraction**

For genomic DNA extraction, isolate were grown in 100 ml flasks containing 50 ml potato dextrose broth on a rotary shaker with a speed of 150 rpm for 7–10 days at room temperature. Then, mycelia were harvested by vacuum filtration on the sterile filter paper, lyophilised and kept at −20 °C. Genomic DNA was extracted from lyophilised mycelia by mini-prep method provided by Liu et al. (2000). Extracted DNA was eluted in 30 μl of distilled water and DNA samples were kept at −20 °C for future use. Quantity and quality of extracted DNA were evaluated by electrophoresis method using 1% agarose gel (1X TBE buffer, 100 V, 1 h) stained with 5% Ethidium bromide and then visualised in IMAGO gel documentation device (B and L System, Netherlands) under the UV light.

**PCR amplification**

Polymerase chain reaction was performed by MP-PCR method using single microsatellite primers (Table 2) including (ACTG)₄, (GACA)₄, (AC)₈, (AAC)₈, (TGTC)₄ and (CGA)₅ (Generay Biotech, Shanghai, China). PCR reactions had 10.5 μl deionised water, 2.5 μl PCR buffer 10X (Sinagen, Iran), 2.5 mM of MgCl₂, 0.5 mM of dNTP mix, 1 μM of Primer, 1 U of Taq DNA Polymeras (Sinagen, Iran) and approximately 10 ng of template DNA in the final volume of 20 μl. PCR amplifications were done in an Eppendorf thermal Cycler (Mastercycler, ep gradient) with the following programme; an initial DNA denaturation step at 95 °C for 5 min, 40 cycles of 30 s at 94 °C, 45 s at 45–50 °C (depending on the type of used primers, Table 1), 35 s at 72 °C, followed by a final extension step at 72 °C for 7 min (Meyer et al. 1993). PCR products of each used primers were separated by electrophoresis in 1.4% agarose gel (Meyer et al. 1993) at 90 V for 2 h and 45 min and using 1X TBE buffer. Size of PCR products was estimated using VC 100 bp Plus DNA Ladder (Vivantis technologies, Malaysia). Agarose gel was stained with 5% Ethidium bromide and then photographed with IMAGO gel documentation device under the UV light.

**Cluster analysis**

In order to investigate the genetic diversity of the *V. malicola* isolates, produced banding pattern in each used primers in MP-PCR was evaluated based on the presence (1) or absence (0) of each bands in all studied fungal isolates and results were generated in all used primers and were prepared as a table in Microsoft Excel 2003 software (Microsoft, USA). Similarity, matrix were produced by Jaccard’s coefficient of similarity (Jaccard 1908) and cluster analysis was done using unweighted pair group method with an arithmetic average (UPGMA) algorithm in PAST software version 2.17c. To determine the support for each clade, bootstrap analysis was performed with 1000 replicates by a computer programme cluster analysis PAST version 2.17c. Only the bootstraps values more than 40% were shown in the dendrogram (Figure 1). The resulting dendrogram
Table 1. Examined isolates of *V. malicola*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Host</th>
<th>Geographic origin</th>
<th>Collector</th>
<th>Year</th>
</tr>
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<tr>
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<td>S1</td>
<td><em>Malus pumila</em></td>
<td>Isfahan-Deh Nesa Sofia</td>
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<td>2010</td>
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<td>8</td>
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<td>Isfahan-Sheibani</td>
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<td>2007</td>
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(Continued)
was saved and visualised for evaluation of genetic diversity of used fungal isolates (Figure 1).

Result

In MP-PCR assay, among the examined primers, only (ACTG)$_4$, (GACA)$_4$, (AC)$_8$ and (CGA)$_5$ primers were able to amplify fragments from genomic DNA of fungal isolates and produced appreciable banding patterns on agarose gel. (ACTG)$_4$, (GACA)$_4$, (AC)$_8$ and (CGA)$_5$ primers revealed high level of polymorphism among the isolates of *V. malicola* and the highest level of polymorphism was produced by (ACTG)$_4$ primer with 100% polymorphic bands and the lowest level of polymorphism was generated by (CGA)$_5$ with 86.96% polymorphic bands. Two primers (GACA)$_4$ and (AC)$_8$ produced 93.3 and 89.29% polymorphic bands, respectively. Based on the banding patterns
resulted from four used primers, 120 loci including 8 monomorphic (6.7%) and 112 polymorphic (93.3%) were identified. Approximate size of visible bands ranged from 0.2 to 3 kb. According to the results, MP-PCR molecular marker was eligible for study of genetic diversity of *V. malicola* isolates and it can be used for the investigation of ranks below the species level. The banding patterns of four used primers were

Figure 1. The final dendrogram showing four groups in 40 isolates of *V. malicola* produced with combination of the results obtained from four single primers (ACTG)$_4$, (GACA)$_3$, (AC)$_8$ and (CGA)$_5$ based on Jaccard’s coefficient and UPGMA method in PAST software ver. 2.17c. Numbers on the branches of the dendrogram represent the results of bootstrap analysis with 1000 replicates.
considered and a final dendrogram was produced (Figure 1). The aggregate result generated by four primers indicates that the total similarity of the isolates is 45.6% and four main groups (1, 2, 3 and 4) can be identified based on all characteristics including similarity, host and geographic origins. Groups were named in order from top to bottom in resulting dendrogram (Figure 1). Obtained groups and their members were the same with groups resulted from mycelial (vegetative) compatibility test on PDA culture medium, but were different from groups obtained in mycelial (vegetative) compatibility test on OMA culture medium (Seifollahi 2012).

Group 1 included only the isolate 150 (2.5% of total isolates) and was obtained from Plum tree (*Prunus domestica*) that has been collected from Kohgiluyeh Boyer Ahmad province. Isolate 150 was related to isolates of groups 2 and 3 with 50.16% similarity and also placed in the single membered MCG on PDA (Seifollahi 2012). Group 2 only had isolate 143 (2.5% of total isolates) that was obtained from *Colutea* sp. collected from Fars province and was related to isolates of group 3 with 50.4% similarity. Isolate 143 placed in the single membered MCG on PDA (Seifollahi 2012). There were 37 isolates in group 3, including: S40, S12, 7, 212, S20, S22, S29, S15, S16, S19, S32, 82, 167, S1, S2, S33, S37, 57, S30, 138, S7, S4, S9, S14, S41, 122, 8, 233-2, 256-1, 167-1, S10, S11, S24, 132, S38, S42 and S44 that included 92.5% of the total isolates. The members of group 3 were related to each other with 52.8% similarity and were collected from different geographical regions in Iran. Most of the members of group 3 were obtained from apple trees (*Malus pumila*) except isolates 167-1 and 167 that both were recovered from Hawthorn (*Crataegus pseudoheterophylla*). All isolates of group 3 placed in the same mycelial compatibility group (MCG) on PDA culture medium (Seifollahi 2012). Group 4 also included only the isolate 270-2 (2.5% of total isolates) that was obtained from Quince (*Cydonia oblonga*) that has been collected from Kurdistan province. The isolate 270-2 was related to isolates of groups 1, 2 and 3 with 45.6% similarity and also placed in the single membered MCG on PDA (Seifollahi 2012). According to the dendrogram, the maximum similarity between the isolates was observed in group 3 between isolates S10 and S11 that were related to each other with 82.56% similarity and both have been collected from the same host (*M. pumila*) but from different geographical regions (isolates S10 and S11 were collected from Isfahan-Fath Abad and Isfahan-Mehr Gerd respectively) with close distance (about 5–6 km).

**Discussion**

“Knowledge on the population genetic structure of the pathogen may offer insight into the best breeding strategy for durable resistance” (McDonald & Linde 2002) and it is required to perform effective control strategies (McDonald 1997).

Based on the results of DNA fingerprinting of *V. malicola* isolates using four microsatellite primers, 93.3% polymorphic bands were observed and the cluster analysis indicated that the isolates are related to each other with 45.6% similarity and four groups (1, 2, 3 and 4) can be identified at this similarity level. The high level of polymorphism produced by MP-PCR in the isolates of *V. malicola* reflects the adequacy of the marker for determining genetic diversity at the intra-specific level. In some cases MP-PCR did not reveal high genetic diversity at intra-specific level in some fungi such as Morels (Buscot et al. 1996) and plant pathogenic fungus, *Ascochyta rabiei* (Geistlinger et al. 1997). High level of genetic diversity in *V. malicola* isolates could be the result of sexual recombination of the fungus in the nature. Sexual form of *V. malicola* sometimes is formed in orchards in Iran. Despite of sexual reproduction,
asexual reproduction of the fungus occurs abundantly in the nature and in most cases
the fungus overwinter as conidial masses in the conidiomata (it was observed during
sampling), so the factors that cause genetic variation during asexual reproduction are
not to be missed. The factors such as activity of transposons, high mutation rates of
microsatellites, location of microsatellite sequences in the genome, hyphal anastomosis
between fungal individuals that are genetically different and asexual recombination
possibly can be the reasons of high genetic diversity. Also, in *Stagonospora* spp. and
*Septoria tritici*, the produced polymorphism by different microsatellite primers varied
between 6 and 89% and activity of transposons, high mutation rates of microsatellites
and hyphal anastomosis between fungal individuals that are genetically different were
mentioned as the reasons for genetic diversity during asexual reproduction (Czembor &
Arseniuk 1999).

There were some correlations between four identified groups (group 1, 2, 3 and 4)
in the dendrogram and the host and geographical origins of the fungal isolates.
Members of group 3 were obtained mainly from apple trees (Rosaceae family) except
two isolates, 167 and 167-1 that were recovered from Hawthorn (Rosaceae family), and
all host plants are considered as pome fruit trees. Group 2 consisted of a single isolate
143 that was recovered from *Colutea* sp. (Fabaceae family). A single isolate 150 was
placed in group 1 that was obtained from stone fruit tree, Plum (Rosaceae family) and
group 4 contained a single isolate 270-2 that was recovered from pome fruit tree,
Quince (Rosaceae family). Also, it can be mentioned that distribution of the isolates
obtained from different hosts or regions in the same group is due to the lack of enforce-
ment of quarantine principles between different areas of Iran, handling of agricultural
materials, lack of sanitation during pruning and use of contaminated agricultural equip-
ments. The same results also were obtained by Mehrabi (2009) and Mehrabi et al.
(2009) during the investigation of genetic diversity of Iranian isolates of *C. schulzeri*
and *C. cincta* by MP-PCR. Investigation of genetic diversity of Iranian isolates of *C.
chrysosperma* obtained from walnut trees grown in different provinces by ISSR revealed
the high level of genetic diversity (95.8% polymorphism) among the isolates that could
be due to the sexual reproduction of the fungus and the result of cluster analysis was
not compatible much with geographical distribution of the isolates. It was mentioned
that grouping of the isolates with different geographical origins in the same cluster
suggests that these isolates may have migrated between locations by infected plant
materials and they did not evolve independently from each other (Abbasi 2009). Study
on genetic diversity of 40 isolates of *M. phaseolina* collected from cotton and soybean
plants in America and India using the MP-PCR put them into three major groups
corresponding to their hosts and geographical distribution and it revealed that the
isolates obtained from soybean and cotton were genetically distinct (Jana et al. 2005).
According to the final dendrogram, the maximum similarity was observed between
isolates S10 and S11 that both were collected from the same host but form different
geographical areas with close distance (about 5–6 km), most likely that these isolates
have been cloned from the same ancestor. Isolates 82 and 167 were placed in the same
clade and are related to each other with 78.72% similarity which was collected from
different hosts and geographical regions. It can be mentioned that probably these
isolates have been cloned from the same ancestor and disseminated to different climatic
conditions by infected plant materials.

Seifollahi (2012) used PDA and OMA culture media to investigate the mycelial
compatibility, and mycelial compatibility and incompatibility reactions were
recognisable on both culture media. In mycelial compatibility tests, PDA culture
medium was preferred because of more mycelial compatibility between isolates and required shorter time to get the results. In some species such as *Leucocytospora kunzei* (Proffer & Hart 1988), PDA has been used for mycelial compatibility test, but OMA has been used in some others such as *V. ceratosperma* (Suzaki 2008) and *Leucostoma persoonii* (Adams et al. 1990). In compatibility reactions, merging colonies of two isolates form no barrage zone at the line of contact regions (Adams et al. 1990) and colonies of two isolates merge together uniformly (Proffer & Hart 1988). In incompatibility reactions, merging colonies of two isolates form barrage zone as a dark coloured line (Proffer & Hart 1988; Adams et al. 1990) or lack hyphal growth between two colonies (Poloni et al. 2009). On PDA culture medium, four MCGs including three single member groups and a 58 member group were identified. Also, eight groups were obtained on OMA culture medium, including a group containing 50 members, a group containing six members and six groups having a single isolate.

It was determined that the results of grouping based on cluster analysis of total results of four primers were compatible with results of grouping of isolates based on mycelial (vegetative) compatibility test on PDA and the number of obtained groups and their members were identical in both methods (Seifollahi 2012). Therefore some physiological and biological characteristics of the *V. malicola* isolates can be predicted based on molecular data as well as amount of genetic diversity that can be predicted by mycelial compatibility tests and revealing the complexity of population genetic structure, indicating that MCG test and molecular methods like MP-PCR are correlative for investigation of genetic diversity.

Based on results of MP-PCR analysis, isolates 143, 270-2 and 150 are placed in the single membered groups 2, 4 and 1, respectively, and also placed in the single membered groups MCG1, MCG2 and MCG3, respectively, in mycelial compatibility grouping, indicating that these isolates are different from other isolates not only in host and geographic origins but in the type of vic or het loci, as well as type and number of microsatellite loci. The high genetic diversity was observed among the members of group 3 in dendrogram (Figure 1) and also the members of this group placed in the same mycelial compatibility group (MCG4), so it can be mentioned that microsatellite primers are capable to show genetic variation within a MCG, if the members of a MCG belong to different races, maybe it can be separated by use of MP-PCR marker.

It was determined that MP-PCR molecular marker is able to detect genetic variability among the isolates of *V. malicola* in the intra-specific level and it is a robust marker to study the population genetics of this species and provides valuable data for investigation of ranks below the species level for special aims such as taxonomy or production of resistant plant varieties.

**Acknowledgement**

This research was supported by a grant [03/6/73145563] from University of Tehran, Tehran, Iran, so we are grateful for the financial support of University of Tehran.

**Abbreviations**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MP-PCR</td>
<td>Microsatellite primed PCR</td>
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<td>MCG</td>
<td>Mycelial compatibility group</td>
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</table>
References


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