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Genetic Diversity of *Alternaria alternata* Associated with *Citrus* spp. in Southern Iran Based on RAPD- PCR

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***Alternaria* diseases of citrus caused by *Alternaria* spp. are important diseases with worldwide distributions. Iran is a major producer of citrus. Recently, *Alternaria* diseases have been observed in many citrus orchards in southern Iran. The Random Amplified Polymorphic DNA (RAPD) technique is a cost effective approach for assessing the genetic diversity of *Alternaria alternata* isolated from citrus. In this study, 45 pathogenic isolates of *A. alternata* collected from diseased leaves of citrus in southern Iran were assessed. Genomic DNA was extracted from young mycelia and eight random decamer nucleotide primers were prepared for RAPD-PCR reactions. The primers yielded reproducible highly polymorphic bands. The highest and the lowest polymorphism information content values were recorded for primers UBC 211 and UBC 203, respectively. According to the cluster analysis, isolates were classified into 13 major clusters with a 0.68 cut-off position. Many isolates from orange cultivars, tangerine cultivars and some isolates from citron, lime, grapefruit and sweet lemons were classified in clades containing a single isolate. All of the isolates collected in the Mamasani region, many from different citrus species and cultivars in Bandar Abbas and Khuzestan, many from tangerines in Jahrom and some from oranges collected in Jahrom and Khuzestan clustered into separate clades. The results indicated a high level of genetic diversity among *A. alternata* populations associated with citrus collected in southern Iran. Some individuals in the population of *A. alternata* showed unique genetic structures. In some cases, geographical and host specialization groupings were observed among *A. alternata* isolates collected on the citrus of different regions.**

Key words: citron, geographical region, grapefruit, host specialization, lime, orange, primers, sweet lemons, tangerine, UBC 211, UBC 203.

Iran is a major producer of citrus in the world. According to FAO reports, in 2012, Iran produced 82,500 tons of citrus and ranked 15th in citrus production in the world[1]. *Alternaria* diseases of citrus caused by *Alternaria* spp. (mainly *Alternaria alternata*) are important diseases with worldwide distributions. They have been documented in the USA[2], Israel[3], South Africa[4], Turkey[5], Spain[6], Brazil and Argentina[7,8]. Recently, these diseases have been observed in many citrus orchards in southern Iran. Different diseases caused by *Alternaria* spp. on citrus species include brown spot of tangerines (*Citrus reticulata* Blanco) and their hybrids (namely the tangerine pathotype), leaf spot of rough lemon (*Citrus jambhiri* Lush) (namely the rough lemon pathotype), black rot of fruits and Mancha foliar on Mexican lime[9]. The current diseases are mainly caused by *Alternaria* fungi, which have small spores (except the causal agent of Mancha foliar on Mexican lime) and are similar in morphology but with intra-specific variations[10]. The tangerine and rough lemon pathotypes produce host-specific toxins and exhibit a narrow host range[11, 12]. *Alternaria limicola* produces symptoms on

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Mexican lime and a few other citrus varieties in western Mexico and is not known to produce host-specific toxins[13]. Thus, there is known genetic diversity among the *Alternaria* isolates associated with citrus.

Many comprehensive studies on the genetic differences in the populations of *Alternaria* spp. on citrus have been conducted[11,14-16]. In these studies, molecular techniques based on DNA markers, such as random amplified polymorphic DNA (RAPD)[17,15,18], partial sequencing of the coding regions of an endopolygalacturonase gene[10,19] and the restriction fragment length polymorphism of the rDNA IGS region[20] were applied and were cost-effective approaches for assessing the genetic diversity of *A. alternata* isolated from citrus. Because of the similar conidial morphologies and ontogenies of *A. alternata* isolates that cause different diseases in citrus species, the classification of this species remains unclear and has been challenged among researchers[21-23]. Using molecular analyses in combination with morphological studies and pathogenicity assays opens new avenues to finding evidence of host specificity for this pathogen. A few studies have been conducted to determine a clear relationship between host specificity and pathogenicity in pathogens with a broad host range based on molecular analyses[20,24]. Additionally, researchers demonstrated the existence of local pathogen populations of *A. alternata* on citrus that were restricted to geographical regions[17]. The limitation of many such studies is the limited samples collected from citrus hosts, which makes the analysis of host specificity results difficult. The aims of our study were the identification of genetic diversity in *A. alternata* populations that cause disease on different citrus species in southern Iran and the investigation of possible host specialization in restricted geographical regions based on RAPD markers.

MATERIALS AND METHODS

Sampling

A total of 45 isolates of *A. alternata* were obtained from the fungal collection of the Islamic Azad University of Jahrom. The isolates were sampled from leaves of different citrus species and cultivars in different citrus orchards of southern Iran in 2012 (Table 1). Pathogenicity tests were previously conducted on leaves of the same species or cultivars. Single conidial colonies were derived from each original mass hyphal sample.

DNA extraction

Genomic DNA was extracted from young mycelia of pure cultures (two-days-old) using the CTAB method. Briefly, 50 to 70 mg of pure culture mycelia was removed from the agar surface using a sterile scalpel and transferred to a mini-sized mortar. Mycelia were lyophilized using liquid nitrogen and powdered by grinding. A 2X CTAB buffer containing 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8), 2% CTAB and 1% PVP was warmed for 30 to 45 min at 65°C before using. Buffer was added to 1.5 ml Eprndorf tubes containing powdered mycelia and inverted gently for homogenizing. Tubes were incubated at 63°C for 30 min and inverted gently every 10 min. To digest proteins, 1 µl of 20 mg/ml Proteinase K was added to tubes and incubated at 37°C for 30 min. Tubes were subjected to chloroform-isoamyl alcohol (24:1) extraction and centrifuged at 4°C for 10 min in 20217 *g*. Then, the top layer was collected and transferred to new tubes. To each tube, 2 µl RNase (10 mg/ml) was added and incubated at 37°C for 30 min. An equal volume of chloroform-isoamyl alcohol was added and inverted for 5 min. Tubes were centrifuged at 4°C for 10 min in 20217 *g*. After centrifugation, the top layers were transferred to new tubes and 2/3 volume of cold-isopropanol was added. The tubes were inverted and then incubated for 15 min at –20°C. The supernatant was carefully discarded after centrifuging again. DNA pellets were washed with 500 µl of 70% ethanol and centrifuged twice. Ethanol was discarded and the DNA pellets were dried at 37°C for 30 min. The pellets were resuspended in 50 µl sterilized distilled water and placed in the refrigerator overnight at 4°C. DNA concentrations were determined by spectrophotometry using a Nanodrop. Additionally, DNA was electrophoresed in a 1% agarose gel with a subsequent ethidium bromide staining and visualized under UV light.

RAPD-PCR

Eight random decamer nucleotide primers were prepared for RAPD-PCR reactions (Table 2). PCR was performed in a total reaction volume of 25 µl containing 50 ng of template DNA, 12.5 µl of 1X reaction buffer, 200 ¼M dNTPs, 2 mM $MgCl_2$, 1.25

U of *Taq* DNA polymerase and 100 ng primers. Reactions were run in a thermocycler using the following amplification cycling conditions: an initial 5 min melt at 94°C followed by 39 cycles of 94°C for 40 sec, temperature and time for annealing were as described in Table 3, and 72°C for 2 min. The final extension step was 72°C for 10 min[25]. Amplicons were separated in 1.2% agarose gels along with a 1 kb (Fermentas; Cat. No. SM0311) size marker and then visualized by staining with ethidium bromide under UV light on a gel documentation system. At least three independent DNA extractions were made from each of the isolates to verify the repeatability of the PCR. The reproducibility of the RAPD markers was tested by performing PCR reactions with different concentrations (5–100 ng) of DNA template.

RAPD analysis

RAPD bands were scored from gel photographs as 1 (present) or 0 (absent) using the individual primers in the study. Each band was regarded as a locus. A phenogram was generated among 45 isolates by the unweighted pair group method with arithmetic means (UPGMA) based on a distance matrix (Jaccard coefficient) using the NTSYC-pc software package. Results were used to construct individual and final dendrograms. Polymorphic information content (PIC) values were calculated for each RAPD primer according to the formula:

$$PIC = 1-\Sigma(P_{ij})^2$$

where P_{ij} is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers[26].

RESULTS

All of the eight primers produced highly polymorphic reproducible bands among the 45 isolates used in this study (Fig. 1). A total of 217 bands ranging from 0.4 to 4.0 kb were detected, revealing different patterns among isolates. For the polymorphic bands, higher and lower numbers of bands were found in an analysis with primers OPH-12 and UBC 203, respectively. The average PIC was 0.93, ranging from 0.89 to 0.99. The highest and the lowest PIC values were recorded for primers UBC 211 and UBC 203, respectively (Table 4).

According to a cluster analysis of RAPD data, isolates were classified in different groups based on the primers used and the cut-off line position (Table 5). As shown in this Table 5 some isolates grouped into single clades. For the collection regions, many isolates from orange

Table 1. Description of *Alternaria alternata* isolates used in the present study and their origins

Strain	Host of isolation	Isolation place
Alt1	Orange (Blood CV.)	Khuzestan
Alt2	Tangerine (Minneola CV.)	Khuzestan
Alt3	Sour orange	Khuzestan
Alt4	Tangerine (unknown CV.)	Khuzestan
Alt5	Tangerine (Tangelo CV.)	Khuzestan
Alt6	Orange (Lisbon CV.)	Khuzestan
Alt7	Citron	Khuzestan
Alt8	Tangerine (Kinnow CV.)	Khuzestan
Alt9	Orange (Parson Brown CV.)	Khuzestan
Alt10	Orange (Marrs CV.)	Khuzestan
Alt11	Orange (Frost Valencia CV.)	Jahrom
Alt12	Orange (Campbell Valencia CV.)	Jahrom
Alt13	Orange (Parson Brown CV.)	Jahrom
Alt14	Orange (Salustiana CV.)	Jahrom
Alt15	Orange (Pineapple CV.)	Jahrom
Alt16	Orange (Thomson Navel CV.)	Jahrom
Alt17	Orange (Hamlin CV.)	Jahrom
Alt18	Orange (Marrs CV.)	Jahrom
Alt19	Orange (Tarocco CV.)	Jahrom
Alt20	Orange (Navel CV.)	Jahrom
Alt21	Tangerine (Kinnow CV.)	Bandar Abbas
Alt22	Lime	Bandar Abbas
Alt23	Trifoliate orange	Bandar Abbas
Alt24	Orange	Bandar Abbas
Alt25	Orange	Bandar Abbas
Alt26	Grapefruit (White CV.)	Bandar Abbas
Alt27	Grapefruit (Pink CV.)	Bandar Abbas
Alt28	Sweet lime	Bandar Abbas
Alt29	Orange (Valencia CV.)	Bandar Abbas
Alt30	Orange (Washigton Navel CV.)	Bandar Abbas
Alt31	Orange (Washington Navel CV.)	Bandar Abbas
Alt32	Tangerine (Peache CV.)	Jahrom
Alt33	Tangerine (Ponkan CV.)	Jahrom
Alt34	Tangerine (Lee CV.)	Jahrom
Alt35	Tangerine (Klemantine CV.)	Jahrom
Alt36	Tangerine (Kinnow CV.)	Jahrom
Alt37	Tangerine (Oseola CV.)	Jahrom
Alt38	Tangerine (Fortune CV.)	Jahrom
Alt39	Tangerine (Local CV.)	Jahrom
Alt40	Orange (Navel CV.)	Mamasani
Alt41	Lime	Mamasani
Alt42	Orange	Mamasani
Alt43	Sweet lime (Wekiwa)	Mamasani
Alt44	Sweet lime	Mamasani
Alt45	Orange (Valencia CV.)	Mamasani

cultivars (Alt 1, Alt 9 to Alt 14, Alt 17 to Alt 20, and Alt 40), tangerine cultivars (Alt 2, Alt 4, Alt 5, Alt 32, Alt 33, Alt 35, Alt 36 and Alt 38) and some isolates from citron (Alt 7), lime (Alt 22), grapefruit (Alt 32) and sweet lemons (Alt 43 and Alt 44) were classified in clades containing single isolates, representing unique genetic characteristics of these isolates. UBC 17 and UBC 211 primers created different band patterns for an isolate from lime (Alt 22) and UBC 17 and P 27 primers for isolates from sweet lime, Alt 44 and Alt 43, respectively.

The similarity coefficients among *A. alternata* isolates ranged from 0.99 between Alt 29 and Alt 28 (isolates from sweet lime and orange in Bandar Abbas) to 0.13 between Alt 31 and Alt 13 (isolates from orange in Bandar Abbas and Jahrom) (Table 6).

Based on the RAPD fingerprinting of all eight primers, the *A. alternata* isolates were classified into 13 major clusters with a 0.68 cut-off position, which was the mean of the similarity matrix data, on dendrogram (Figure 2). Isolates Alt 1, Alt 2, Alt 13, Alt 14, Alt 18, Alt 26 and Alt 38 were in single clusters, representing different genetic characteristics compared with the other isolates. Most of the isolates grouped into cluster 8, which, with some exceptions, included isolates from different citrus species and cultivars mainly

Table 2. Primers used in RAPD-PCR

No	Primer	Sequence
1	UBC 30	5'-GATTCCGGCC-3'
2	UBC 17	5'-CTCCGGGTCC-3'
3	UBC 203	5'-CACGGCGAGT-3'
4	UBC 208	5'-ACGGCCGACC-3'
5	UBC 211	5'-GAAGCGCGAT-3'
6	UBC 285	5'-GATCAGCGGGG-3'
7	OPH-12	5'-ACGCGCATGT-3'
8	P 27	5'-CCGTGCAGTT-3'

Table 3. Annealing temperatures and times for primers in RAPD-PCR

Primer	Annealing temperatures (°C)	Time (Sec)
UBC 17	33	40
UBC 30	31	40
UBC 203	32	40
UBC 208	34	40
UBC 211	30	40
UBC 285	32	40
OPH-12	38	40
P 27	30	40

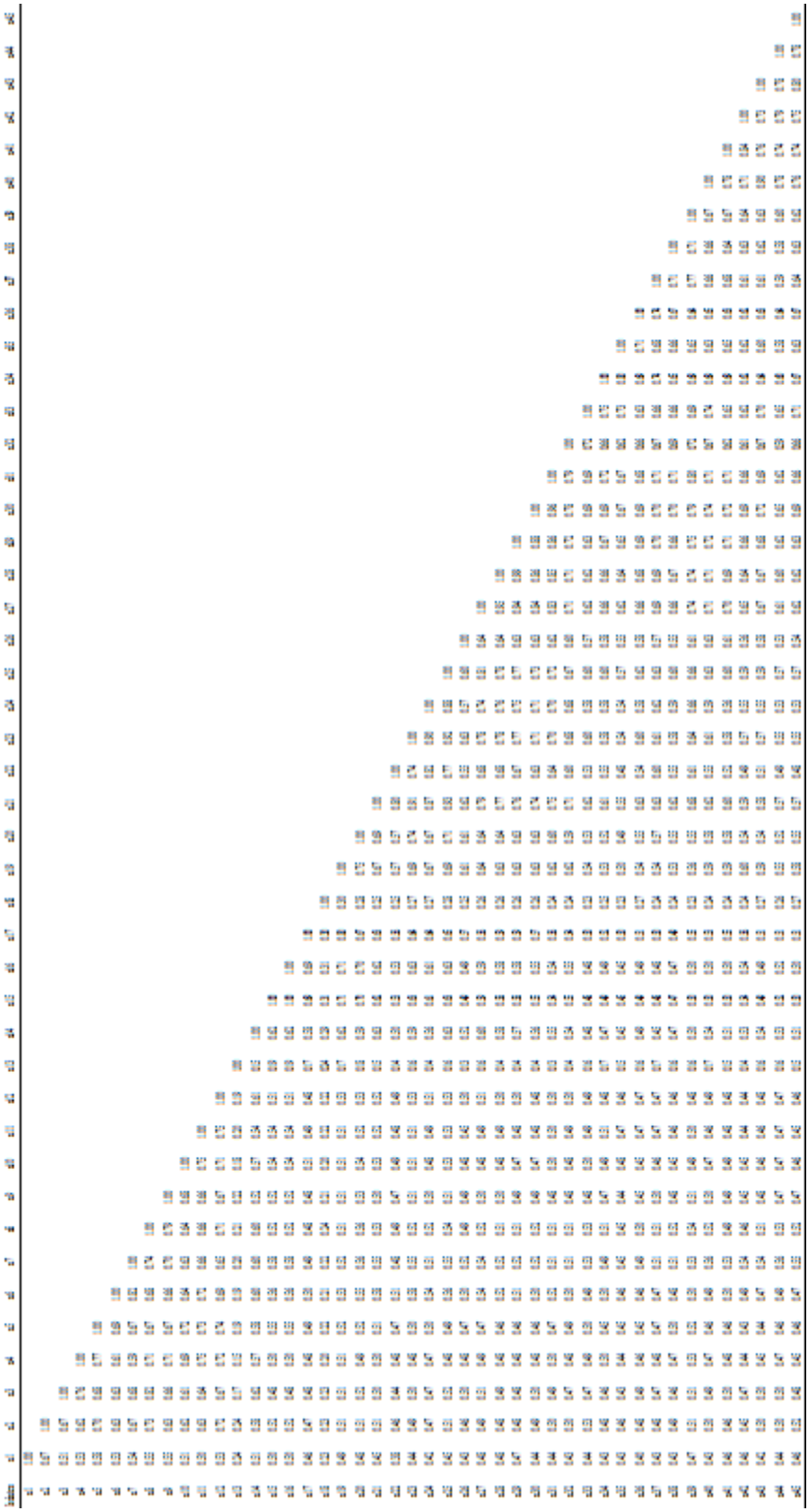
Table 4. Amplified bands and polymorphism information content (PIC) values for the RAPD analysis of *Alternaria alternata* isolates from citrus in southern Iran

PIC	Amplified bands (no.)	Primer
UBC 17	29	0.90
UBC 30	31	0.91
UBC 203	19	0.89
UBC 208	27	0.94
UBC 211	24	0.99
UBC 285	25	0.93
OPH-12	36	0.94
P 27	26	0.91
Total	217	7.41
Mean	27.125	0.926

Table 5. Groupings of *Alternaria alternata* isolates from citrus in southern Iran based on cluster analyses of different primers

Primer	Clade (No.)	Cut off line levels	Isolates in single clade
UBC 17	13	0.85	Alt 6, Alt 13, Alt 18, Alt 26, Alt 35, Alt 44
UBC 30	13	0.70	Alt 13, Alt 18, Alt 19, Alt 20, Alt 22, Alt 33, Alt 38
UBC 203	7	0.75	Alt 1, Alt 13, Alt 18
UBC 208	18	0.80	Alt 13, Alt 14, Alt 17, Alt 18, Alt 26, Alt 32
UBC 211	15	0.72	Alt 4, Alt 5, Alt 6, Alt 10, Alt 11, Alt 13, Alt 14, Alt 18, Alt 22, Alt 26
UBC 285	19	0.75	Alt 1, Alt 5, Alt 9, Alt 13, Alt 14, Alt 17, Alt 18, Alt 36
OPH-12	14	0.72	Alt 1, Alt 2, Alt 9, Alt 13, Alt 14, Alt 18, Alt 20, Alt 26
P 27	18	0.70	Alt 4, Alt 6, Alt 7, Alt 13, Alt 18, Alt 40, Alt 43

Table 6. Coefficient of similarity values for *Alternaria alternata* isolates from citrus in southern Iran based on proportion of shared fragments generated by using simple matching algorithm of the NTSYS program



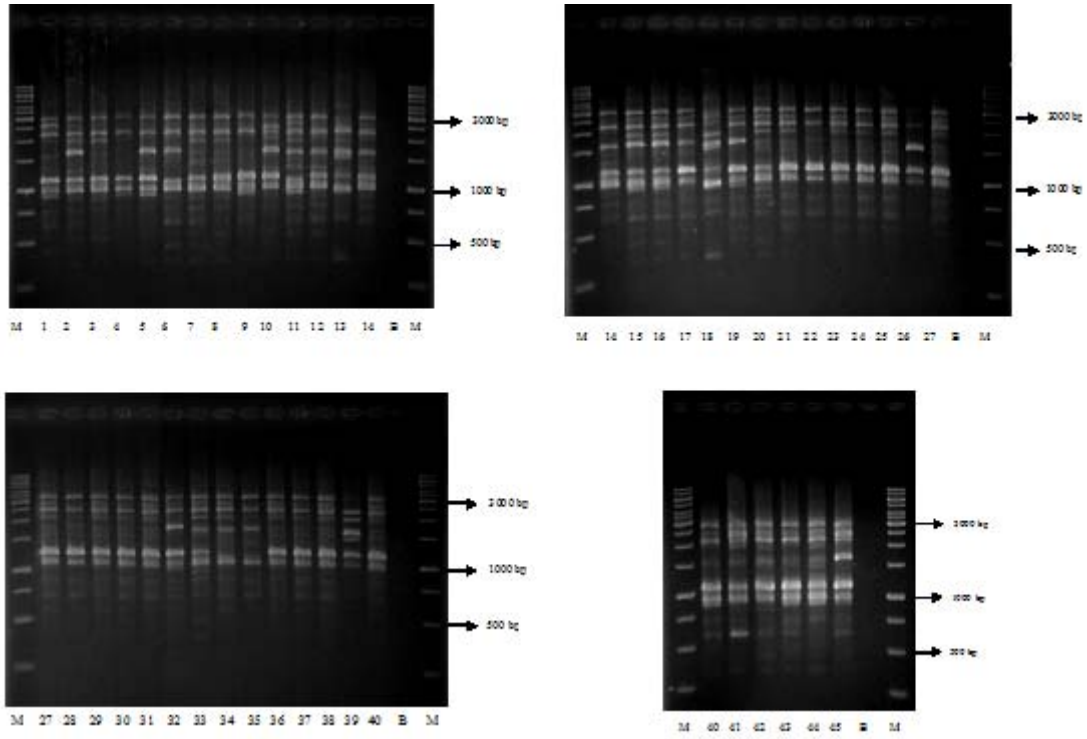


Fig. 1. RAPD band profiles generated by the operon primer UBC211 for the *Alternaria alternata* isolates from citrus in southern Iran included in this study. The isolate numbers correspond to those in Table 1. The molecular weight marker (M), a 1 kb ladder, is shown at the right. Empty lanes (B)

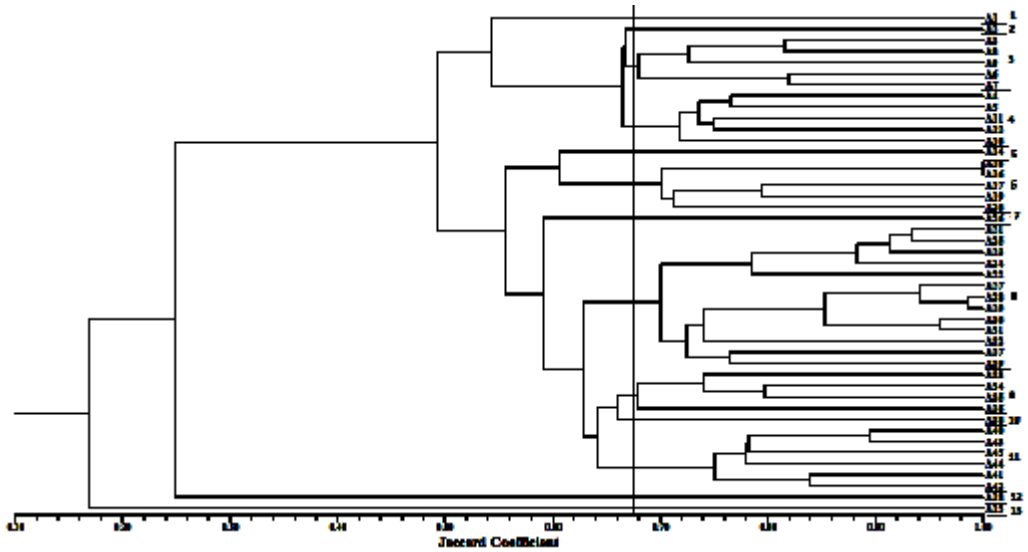


Fig. 2. An UPGMA dendrogram based on the similarity index among 45 *Alternaria alternata* isolates from citrus in southern Iran using eight RAPD primers

collected from Bandar Abbas. All of the isolates collected from Mamasani region were classified into cluster 11. Many isolates from tangerine collected in Jahrom grouped in cluster 9. Most of the isolates from orange collected in Jahrom and Khuzestan clustered into two separate clusters, 4 and 6. Cluster 3 included isolates from different citrus species collected in the Khuzestan region.

DISCUSSION

Knowledge of the genetic structures of plant pathogen populations is an important factor in plant disease management[27]. Genetic population structures of plant pathogens vary over the time, and many plant pathogens adapt themselves to environmental changes, such as host resistance, climatic changes and applied fungicides, by altering their genetic structure. Thus, studies on genetic variations in plant pathogens can be applied to selecting appropriate control strategies[16]. This study was conducted to evaluate the genetic variation among *A. alternata* isolates associated with citrus from different regions of southern Iran using the RAPD-PCR technique. In this study, primers UBC 211 and UBC 203 showed the highest and the lowest levels of polymorphism, respectively. This shows the importance of primer selection s in population genetic variation studies of *A. alternata*. The obtained results were in accordance with the results of Kakvan *et al.* (2012).

In our study some single isolates represented clades. These isolates had unique genetic structures compared with other isolates, which signifies the high level of genetic diversity among the populations of *A. alternata*. Kakvan [et al]. (2012) reported a high level of genetic variation among *A. alternata* isolates associated with citrus collected from northern Iran. Individual isolates from populations of *A. alternata* have different genetic structures and may be specialized for developing on special species or cultivars of citrus hosts.

Some tangerine, lime, citron, grapefruit, sweet lemon and orange isolates were separated into groups based on RAPD band patterns. These results indicated that these isolates are genetically distinct from other isolates. Previously, the terms tangerine pathotype and lemon pathotype have been applied to denote the unique biological attributes of appropriate isolates[28]. Our results revealed that, in addition to isolates from tangerine and lemon, some other isolates, such as those from lime, citron, grape fruit, sweet lemon and orange, were genetically distinct, which may represent specialization to the hosts. We speculate that these populations are in the early stages of adaptation to, and possible speciation on, these hosts; although, more research is needed to confirm this hypothesis. Research showed that an *A. alternata* population that was active on lime was genetically distinct from other citrus isolates[29]. Additionally, there was an *A. alternata* isolated specifically from lime, termed in the old nomenclature as *Alternaria citri*[30-33]. Research conducted in the USA indicates populations of *Alternaria* spp. sampled from grapefruit were highly differentiated from populations sampled from tangerine×grapefruit by RAPD markers[18].

In this study, populations of *A. alternata* from different citrus species, cultivars and locations, with some exceptions, were strongly differentiated. Populations of the pathogen sampled from Bandar Abbas, Mamasani and many isolates from Khuzestan grouped into three separate clusters. These results suggest significant genetic and geographical distinctions among isolates from those locations. The homogeneity of *A. alternata* populations on citrus in those regions may be due to the limited movement of nursery materials among the regions at the time of grove establishment. RAPD markers could differentiate *A. alternata* isolates sampled from the citrus of southern and northern Iran[17]. No geographical groupings were observed among *A. alternata* isolates collected from the citrus of different regions of California, USA[34].

Most of the isolates from tangerine cultivars in Jahrom, and some isolates from orange in Jahrom and Kuzestan, grouped into different clusters. Brown spot has never been reported on orange leaves in northern of Iran. The current results suggest that *A. alternata* exists as discrete, independently evolving lineages on those hosts. One hypothesis is that the differentiation of tangerine and orange isolates is the result of selection by their hosts. Many researchers believe in the high level of specialization of *A. alternata* isolates on tangerine and its hybrids[12,35]. In Florida, *A. alternata* isolates from tangerine were

pathogenic only on tangerine and could not cause disease on lime[35]. Genetic differentiation among isolates from orange and tangerine with RAPD markers were previously reported by Kakvan et al. (2012). They analyzed isolates from tangerine and orange collected in the citrus orchards of southern Iran and observed that they grouped into separate clusters. Despite genetic differentiation with RAPD markers among grapefruit and other tangerine×grapefruit hybrid isolates, no pathogenic specialization was detected between grapefruit and 'Nova' isolates[18].

The results showed that the tested isolates from tangerines in different regions and of different cultivars did not group into a single clade. Therefore, we concluded that, in addition to the host specification of isolates from tangerine, there are significant genetic differences among *A. alternata* populations on tangerines of different cultivars and regions. Isolates from tangerine of the 'Mineola' cultivar sampled in different regions of southern Iran grouped into two separate clades based on RAPD band patterns[17]. Using RAPD markers, Peever et al. (2000) reported that *A. alternata* isolates from 'Mineola', 'Sunburst', 'Robinson' and 'Orlando' grouped into different clades. Studies using RAPD patterns of *A. alternata* isolates collected worldwide on the 'Mineola' cultivar of tangerine revealed that isolates from Colombia and Florida, USA were genetically different than the other isolates[10].

In our study some clusters containing isolates from different citrus species and cultivars, such as sour orange, orange (Lisbon and Parson Brown CV.), tangerine (Kinnow CV.) and citron (Figure 2, clade no. 3). These isolates are likely variants of *A. alternata* that gradually acquired the ability to cause disease in different citrus cultivars and species. Further studies are needed to confirm this hypothesis.

It seems that the *A. alternata* active on citrus in southern Iran consists of a main population that can cause disease on many citrus species and cultivars in different geographical regions and environmental conditions, and specialized populations that originated from the main population that can cause diseases on specific citrus species and cultivars. Nishimura and Kohmoto (1983) raised the hypothesis that pathogenic *A. alternata* populations evolved from local and non-pathogenic populations that can produce toxins to cause disease. Although more molecular studies are needed to determine the relationship between toxin production, pathogenicity and molecular markers in our isolates, the present results support the hypothesis of Nishimura and Kohmoto. Additionally, this hypothesis may explain the reason for genetic differences among pathogenic isolates collected from a particular region.

We should consider that several factors are involved in genetic relationships among isolates. These include the primers used, targeted genes, the distribution of the markers in the genome of the analyzed samples, the nature of the genetic evolutionary mechanisms underlying the variation measured and the number of individuals in the studied population[36]. Thus, additional primers, markers or techniques may lead to new results and the answers to more questions in this field.

In conclusion, the results of this study showed a high level of genetic diversity among citrus isolates and the revealed primers that clearly differentiated isolates based on host species, cultivars and geographical regions. Results corroborated previous studies regarding genetic diversity among *A. alternata* populations of citrus[14]. Studies on genetic pathogen variability are the first step aimed at obtaining more information on the disease etiology, which will affect disease control measures. All this information will serve as the basis for future studies on the management of *A. alternata* diseases in the citrus orchards of southern Iran.

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