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ORIGINAL RESEARCH PAPER

Analysis of virulence and genetic variability of *Alternaria alternata* associated with leaf spot disease in potato plants in Iran

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Abstract

Leaf spot disease in potato is caused by *Alternaria alternata* (Fr.) Keissler, an opportunistic pathogen that infests many agricultural crops worldwide in the field and during postharvest storage of vegetables and fruits. *Alternaria alternata* is associated with leaf spot disease in potato in Iran. Thus, there is a need to investigate the virulence and genetic variability of Iranian *A. alternata* isolates to facilitate the development of appropriate management strategies. In the present study, we analyzed a total of 28 isolates obtained from the main potato-growing regions of Iran, including the Ardebil, Hamedan, Isfahan, and Fars provinces. The pathogens were characterized based on sequence analysis of the genes encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*), plasma membrane ATPase, *Alternaria* allergen a 1 (Alt a1), calmodulin, and actin. In addition, random amplified polymorphic DNA (RAPD), intersimple sequence repeat (ISSR), and virulence studies were performed. Phylogenetic analysis of the combined dataset indicated that the five representative isolates were grouped with the subcluster comprising *A. alternata*. RAPD and ISSR analyses clustered the 28 *A. alternata* isolates into different groups with no correlation with their corresponding geographical origins. Results of the pathogenicity assay indicated that all *A. alternata* isolates were pathogenic against potato. However, the *A. alternata* isolates showed high variability in terms of virulence.

Keywords

Alternaria alternata; ISSR; RAPD; *Solanum tuberosum* L.; virulence variability

Introduction

Alternaria alternata (Fr.) Keissler has been isolated from a wide range of crops, including fruits and vegetables, nuts, and cereals [1]. *A. alternata* is a common saprobe found in many plants and other substrata worldwide and is an opportunistic pathogen that infects many agricultural crops in the field and during postharvest storage of vegetables and fruits. In potato, the *A. alternata* fungus causes brown necrotic lesions in foliage and black pit [2]. Previous reports have shown that *A. alternata* can destroy more than 20% of potato production [3,4]. In Iran, two species, namely, *A. alternata* and *A. solani* (Ellis & Martin), have been identified as the causal agents of leaf spot disease in potato, with *A. alternata* being the dominant species [5].

In recent years, multigene phylogeny has been widely employed for identification and characterization of *Alternaria* species [6,7]. Molecular approaches based on sequence analysis of gene fragments encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*), plasma membrane ATPase, *Alternaria* allergen a 1 (Alt a1), calmodulin, and actin have robustly defined the monophyly of *Alternaria* in the ascomycete family Pleosporaceae [5]. The efficacy of control strategies on the plant pathogen populations have been hampered by limited information on genetic variability [6,8,9]. The most commonly

adopted control measure is the use of fungicides and resistant cultivars. In Iran, genetic information is available based on ISSR of only 11 *A. alternata* isolates from potato plants collected in Hamedan Province. Moreover, no comprehensive study has analyzed the genetic diversity among the Iranian *A. alternata* isolates from various potato-growing regions for control planning programs [10]. Therefore, given the decline of resistance of potato plants to *A. alternata* and the lack of appropriate breeding programs, there is an urgent need to study the genetic variability within pathogen populations and should be considered one of the first steps for disease management strategy of new and aggressive isolates and evaluation of the resistance of different potato varieties [11]. Thus, considering the importance of leaf spot disease in potato and *A. alternata* in Iran [12], efforts should focus in investigating the present status of the disease.

Therefore, the present study aimed to estimate the variability among *A. alternata* isolates obtained from potato plants collected from the main potato growing regions of Iran. We aimed to obtain updated and more reliable information on the virulence and genetic variability of *A. alternata* populations, which could aid in the selection of appropriate breeding and management strategies against the *A. alternata* pathogen.

Material and methods

Fungal isolates

A total of 28 single-spore isolates [13] of *A. alternata* were obtained from the Plant Pathology Laboratory, Isfahan Research and Education Center for Agriculture and Natural Resources, Isfahan, Iran (Tab. 1). The isolates were collected in 2010 from the main potato growing regions of Iran, namely, Ardebil, Hamedan, Isfahan, and Fars provinces (Fig. 1). Population samples of *A. alternata* were collected in June to August 2010 from randomly selected fields of potato plants from 28 sites in four provinces. For each sampling site, leaves with *A. alternata* lesions were collected from the potato plants. The *A. alternata* populations originated from seven isolates from every province, namely, the Ardebil, Hamedan, Isfahan, and Fars provinces. Conidia of all isolates were produced in chains on conidiophores and presented inverted pear shapes with short beaks and dark brown color, with dimensions of 20–50 µm × 9–18 µm and two to six (four) transversal and one to four (two) longitudinal septa. Each of the 28 isolates were confirmed to be *A. alternata* based on the morphological characteristics [4].

Virulence assay

Pathogenicity tests were conducted in a greenhouse using all 28 *A. alternata* isolates. The experiment was carried out based on a completely randomized design with 10 replicates (pots) for infected plants and control plants following the methods described by Mamgain et al. [8]. Potato (*Solanum tuberosum*) 'Agria' seed tubers were planted in plastic pots (30 cm diameter) containing soil and perlite under greenhouse conditions (18–25°C) at the Plant Protection Research Department, Isfahan Agricultural and Natural Resources Research and Education Center, (AREEO), Isfahan, Iran. Potato tuber seeds consisted of small whole tubers weighing approximately 50 g each. One tuber seed piece was planted in each pot. Tubers were treated with thiabendazole (tekto WP60%, Golsam Gorgan Company, Tehran) before planting. Plants were irrigated once per day or as needed. Each replication consisted of ten leaves of each plant. Conidial suspension spray (103 spores/mL) was used to inoculate 1-month-old potato 'Agria' plants after emergence. After pathogen inoculation, the plants were covered for 48 h with plastic bags in the greenhouse with 14 h of light and 10 h of darkness [14]. For estimation of the proportion of the leaf surfaces infected with *A. alternata*, disease rating was conducted at 7 days after inoculation based on the following 5-point scoring scale: 0 = no disease symptoms, lesions as pinpoints and nonmeasurable; 1 = <10% of the leaves have brown necrotic lesions; 2 = 10% to 25%; 3 = more than 25% to 50%; 4 = more than 50% to 75% of the leaves have brown necrotic lesions; and 5 = more than 75% to 100% of the leaves have brown necrotic lesions or appeared completely brown

Tab. 1 Origins and virulence variability of *Alternaria alternata* isolates used in this study.

No.[1]	Isolate name	Location	State	Percent disease severity (PDS)[2]
1	Aa-H1	Asad Abad	Hamedan	23.83 [ab]
2	Aa-H2	Shirin Su	Hamedan	48.00 [ab]
3	Aa-H3	Kabodar Ahang	Hamedan	49.50 [ab]
4	Aa-H4	Bahar and Saleh Abad	Hamedan	45.33 [ab]
5	Aa-H5	Hamedan	Hamedan	67.50 [ab]
6	Aa-H6	Qabaq Tappeh	Hamedan	12.50 [b]
7	Aa-H7	Famenin	Hamedan	31.67 [ab]
8	Aa-A1	Agha Bagher village	Ardebil	16.50 [b]
9	Aa-A2	Khalifeh Lu village	Ardebil	28.83 [ab]
10	Aa-A3	Yunjalu village	Ardebil	52.33 [ab]
11	Aa-A4	Tupraghlu village	Ardebil	17.67 [b]
12	Aa-A5	Samian village	Ardebil	44.67 [ab]
13	Aa-A6	Soltan Abad	Ardebil	42.83 [ab]
14	Aa-A7	Ardebil	Ardebil	37.67 [ab]
15	Aa-F1	Kushk Mola village	Fars	48.88 [ab]
16	Aa-F2	Boroj village	Fars	30.67 [ab]
17	Aa-F3	Dariun village	Fars	36.50 [ab]
18	Aa-F4	Deh Bid	Fars	69.17 [a]
19	Aa-F5	Shirin Abad village	Fars	50.00 [ab]
20	Aa-F6	Hasan Abad village	Fars	38.00 [ab]
21	Aa-F7	Bovanat village	Fars	36.00 [ab]
22	Aa-I1	Chadegan	Isfahan	46.00 [ab]
23	Aa-I2	Daran	Isfahan	52.50 [ab]
24	Aa-I3	Rozveh	Isfahan	55.00 [ab]
25	Aa-I4	Semirom	Isfahan	27.17 [ab]
26	Aa-I5	Golpayegan	Isfahan	43.33 [ab]
27	Aa-I6	Mehdi Abad	Isfahan	38.00 [ab]
28	Aa-I7	Nisian	Isfahan	49.33 [ab]

[1] *Alternaria alternata* isolates causing leaf spot of potato.
[2] Values followed by the same letter in the column did not differ significantly (0.05 level) in Duncan's multiple range test.

[15]. The experiments were repeated twice, and reisolation of the inoculated fungi was performed to fulfill Koch's postulate. The following formula was used to calculate percent disease severity (PDS) in each replication: PDS = $(\Sigma RT \times 100)/(SN)$, where T represents the total number of leaves in each category, R is the disease severity scale, N is the total number of leaves tested, and S is the highest number in the scale.

Data were transformed to arcsine square root and analyzed by conducting analysis of variance procedure (ANOVA, $p < 0.05$). Means were compared by performing Duncan's multiple range test using SAS software version 9.2.

DNA extraction, PCR amplification

Total genomic DNA was extracted from all isolates using the CTAB method [16]. A NanoDrop ND-1000 spectrophotometer (LMS Co., Ltd, Tokio, Japan) was used to verify the quality and concentration of genomic DNA extracts. To characterize the isolates, the following genes were PCR-amplified using the indicated primers: a region of the *gpd*

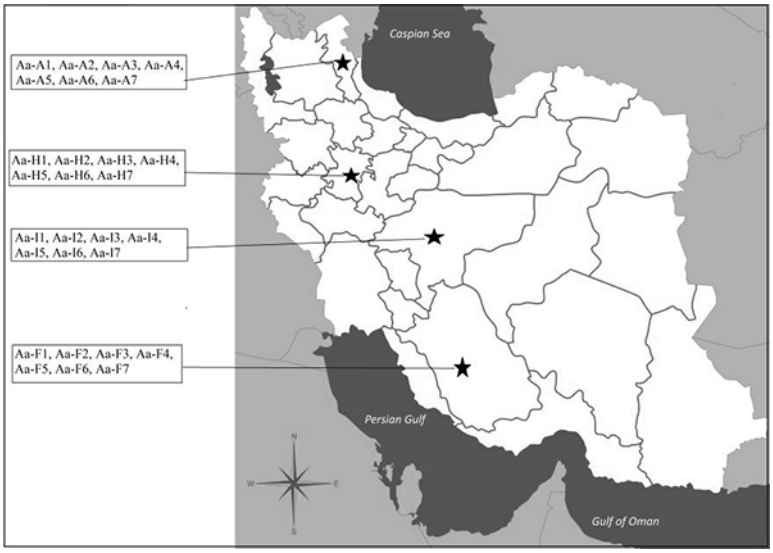

Fig. 1 Map of Iran showing the locations of potato plantations where the 28 *Alternaria alternata* isolates were collected. The names of the isolates are based on the names indicated in Tab. 1.

gene using the primers gpd1 and gpd2 [17], Alt a1 using primers Alt-a1-for and Alt-a1-rev [18], calmodulin using primers CALDF1 and CALDR1 [5], actin using primers ACTDF1 and ACTDR1 [5], and plasma membrane ATPase using primers ATPDF1 and ATPDR1 [5]. PCR amplification of the gene sequences was performed as described by Lawrence et al. [5]. PCR products were then purified using the Gene JETTM commercial PCR Purification Kit (Fermentas, Axon Scientific, Malaysia) and sequenced using the same forward and reverse primers by a commercial sequencing service provider (Iraizol Old Extraction DNA Kits, Rona Bio-Fanavaran Co., Iran).

Tab. 2 RAPD and ISSR primers utilized to identify and assess interspecific genetic diversity among *Alternaria alternata* isolates collected from potato plants.

RAPD primer[1]	Sequence (5′–3′)	ISSR primer[2]	Sequence (5′–3′)
OPA-03	AGTCAGCCAC	UBC-807*	AGAGAGAGAGAGAGAGT
OPA-04	AATCGGGCTG	UBC-808*	AGAGAGAGAGAGAGAGC
OPA-13	CAGCACCCAC	UBC-809*	AGAGAGAGAGAGAGAGG
OPA-16*	AGCCAGCGAA	UBC-818	CACACACACACACACAG
OPB-17	AGGGAACGAG	UBC-834*	AGAGAGAGAGAGAGAGYT
OPB-18	CCACAGCAGT	UBC-835*	AGAGAGAGAGAGAGAGYC
OPC-06*	GAACGGACTC	UBC-840*	GAGAGAGAGAGAGAGAYT
OPC-08*	TGGACCGGTG	UBC-841*	GAGAGAGAGAGAGAGAYC
OPE-01	CCCAAGGTCC	UBC-842	GAGAGAGAGAGAGAGAYG
OPP-16*	CCAAGCTGCC	UBC-846	CACACACACACACACART
OPP-17	TGACCCGCCT	UBC-849*	GTGTGTGTGTGTGTGTYA
OPP-18	GGCTTGGCCT	UBC-856*	ACACACACACACACACYA
OPP-19*	GGGAAGGACA		
OPX-12*	TCGCCAGCCA		
OPX-14	ACAGGTGCTG		

[1,2] RAPD and ISSR Primers with an asterisk (*) were utilized to identify and assess interspecific genetic diversity among *Alternaria alternata* isolates. These primers with high polymorphism and reproductive profiles were chosen among all primers based on the results of initial screening against a set of representative isolates.

Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analyses

Based on the results of initial screening against a set of representative isolates (Tab. 2), we selected six out of 15 primers, namely, OPA-16, OPC-06, OPC-08, OPP-16, OPP-19, and OPX-12 (Operon Technologies Inc., Alameda, CA, USA) for RAPD analysis of the *A. alternata* isolates. The above-mentioned primers amplify regions with high polymorphism and reproductive profiles. For the ISSR analysis, nine primers, namely, UBC-807, UBC-808, UBC-809, UBC-834, UBC-835, UBC-840, UBC-841, UBC-849, and UBC-856, were selected among 12 primers based on the results of initial screening against a set of representative isolates (Tab. 2). RAPD and ISSR analyses were carried out as previously described by Nasehi et al. [6].

RAPD and ISSR analyses

All PCR runs were performed in triplicate to confirm the consistency of amplification. The bands were considered as binary characters and were scored as either 1 (presence of DNA band) or 0 (absence of DNA band). The scores were then entered into a matrix and analyzed based on the numerical taxonomy and multivariate analysis using the program NTSYS-pc 1.8 (Applied Biostatistics Inc., Setauket, NY, USA) [19]. The similarity matrix was calculated using Jaccard's similarity coefficient. Clustering was performed using the unweighted pair group method using arithmetic averages (UPGMA) to generate the dendrogram.

Sequence alignment and phylogenetic analysis

For molecular identification, five representative isolates, namely, Aa-A5, Aa-A6, Aa-H2, Aa-H3, and Aa-I1, were selected from the 28 isolates. One isolate was selected from each of the five groups based on the RAPD and ISSR grouping. Consensus sequences were generated from the forward and reverse DNA strands using BioEdit Sequence Alignment Editor [20]. To analyze the phylogenetic relationships between the *A. alternata* isolates and other *Alternaria* species, the sequences of the five representative isolates from this study and sequences of all the reference of the species-groups of *Alternaria* species [5] were aligned using the Clustal W multiple alignment program [21]. *Stemphylium callistephi* was used as the out-group taxon. We performed phylogenetic analysis of the combined dataset comprising the *gpd*, plasma membrane ATPase, Alt a1, calmodulin, and actin genes using maximum likelihood method with the Kimura two-parameter model in MEGA 7.0 (GenBank accession, Fig. 2) [22]. Branch support of the trees obtained from maximum likelihood analysis was assessed by bootstrapping with 1,000 replications to estimate the reliability of inferred monophyletic groups. MEGA7: Molecular Evolutionary Genetics Analysis version as used 7.0 for larger datasets [22].

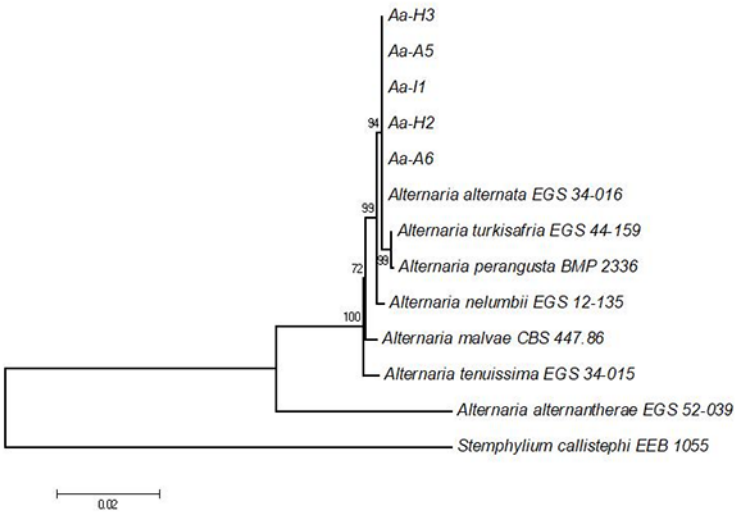

Fig. 2 Phylogenetic tree constructed based on the combined dataset (five target genes) using five representative isolates from this study and other *Alternaria* species retrieved from GenBank. *Stemphylium callistephi* was used as the out-group taxon. The bar indicates the number of nucleotide substitutions per site. Numbers of bootstrap support values ≥50% based on 1,000 replicates.

Results

Virulence assay

Symptoms of the leaf spot disease were observed in all the inoculated potato plants at 7 days after inoculation. Foliar lesions were initially visible as pinpoints that were irregular to circular in shape. Brown spots were usually observed first on the lower leaves and subsequently spread as circular spots throughout the leaves. Control plants did not exhibit any symptoms of the disease. *Alternaria alternata* isolates were reisolated from inoculated plants and were found to be identical to the original isolates used. These results reconfirmed the *A. alternata* isolates as the causal agents of leaf spot disease in the potato plants. However, we observed high variability in virulence among the four isolates (Tab. 1). The Aa-F4 (PDS of 69.17) isolate was found to be the most virulent isolate, while the three isolates, namely, Aa-H6 (PDS of 12.50), Aa-A1 (PDS of 16.50), and Aa-A4 (PDS of 17.67), were the least virulent isolates. Other isolates showed moderate virulence towards potato 'Agria' plants with no significant differences in virulence.

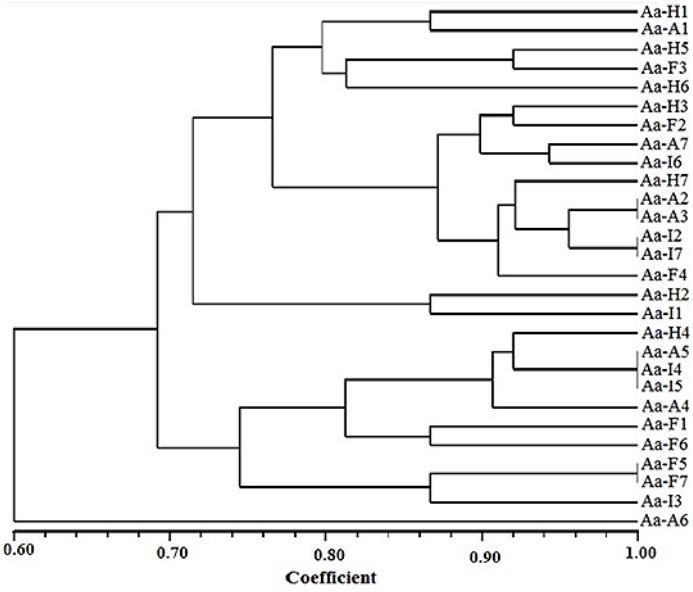

Fig. 3 Dendrogram generated via the UPGMA method showing the genetic variability of the 28 *Alternaria alternata* isolates based on six RAPD primers (OPA-16, OPC-06, OPC-08, OPP-16, OPP-19, and OPX-12). The names of the isolates are based on the names indicated in Tab. 1.

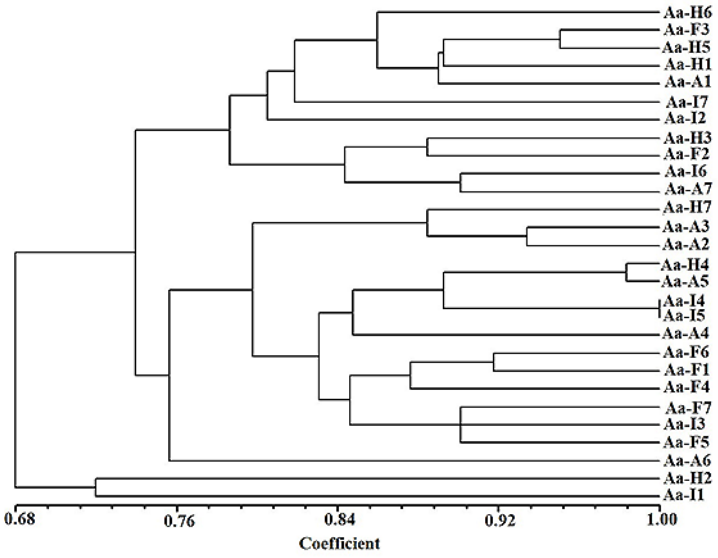

Fig. 4 Dendrogram generated via the UPGMA method showing the genetic variability of 28 *Alternaria alternata* isolates derived from a combination of nine ISSR primers (UBC-807, UBC-808, UBC-809, UBC-834, UBC-835, UBC-840, UBC-841, UBC-849, and UBC-856). The names of the isolates are based on the names indicated in Tab. 1.

RAPD analysis

Amplification using six RAPD primers generated a total of 47 consistently amplified fragments (100 to 3,000 bp), of which 100% were polymorphic. The average number of bands per primer was 7.83. The dendrogram produced using UPGMA analysis based on Jaccard's coefficient indicated relatively high variability among the 28 *A. alternata* isolates. The Jaccard's similarity coefficient index ranged from 100% to 60%, with the mean value of 0.80 (Fig. 3). The genetic diversity showed no association with the geographical origin of the isolates.

ISSR analysis

The nine ISSR primers generated a total of 61 consistently amplified DNA bands, of which 83.61% were polymorphic. The average number of bands per primer was 6.8. The bands ranged in size from approximately 100 to 3,000 bp. The dendrogram also indicated high variability among the 28 *A. alternata* isolates and grouped the isolates into different groups, with no correlation with the geographical regions of the isolates (Fig. 4). The Jaccard's similarity coefficient ranged from 100% to 68% with the mean value of 0.84.

Phylogenetic analysis

PCR amplification of the genes corresponding to *gpd*, plasma membrane ATPase, Alt a1, calmodulin, and actin in the five representative isolates produced fragments with sizes 797, 1,194, 467, 780, and 939 bp, respectively. Phylogenetic analysis of the combined dataset indicated that the five representative isolates were clustered in a subdistinct cluster, which included *A. alternata* with a high bootstrap value of 94% (Fig. 2). The five isolates were clearly distinguished from other selected *Alternaria* species, which were found to be highly similar to the *A. alternata* isolates in the present study. *Stemphylium callistephi* was phylogenetically distant to *Alternaria* and clustered as its sister taxon.

Discussion

Results of the virulence assays indicated that all the *A. alternata* isolates were pathogenic against potato 'Agria' plants, and disease symptoms were observed in the leaves of all potato 'Agria' plants inoculated with *A. alternata*. Reisolation of *A. alternata* from all the inoculated samples confirmed Koch's postulates. Therefore, all *A. alternata* isolates were verified to be the causal agents of the leaf spot disease in the potato 'Agria' plants.

Our findings also revealed high variability in virulence among the *A. alternata* isolates. In addition, the grouping of the isolates based on virulence was not found to be correlated with the results of RAPD and ISSR analyses and the geographical origins of the isolates. Therefore, further detailed studies must be conducted using a larger number of *A. alternata* isolates. Our current findings were consistent with those of previous studies on *A. alternata* isolates obtained from citrus hybrids [23], potato plants [10,24], and tomato plants [24,25] throughout Iran.

The RAPD and ISSR techniques are extremely powerful tools for distinguishing among individuals that exhibit intraspecific and interspecific variability [26]. In the present study, 100% and 84% of the isolates were found to be polymorphic with respect to the RAPD and ISSR markers, respectively. The similarity indices were 84% and 80% for the RAPD and ISSR markers, respectively. RAPD and ISSR markers exhibit lower polymorphism rates than those of AFLP and other molecular markers. However, the AFLP method is more laborious, time-consuming and costly and involves the use of radioactive reagents. Therefore, the RAPD and ISSR techniques could serve as reliable alternatives for molecular characterization because they are easier to implement and are less costly [9,24]. The RAPD and ISSR methods have been employed to generate comprehensive information regarding the genetic variability of *A. alternata* populations in citrus [23], linseed [27], pistachio [28], potato [10,24], tangerine [13], and tomato [24,25]. These methods have also been used to investigate the genetic variability of other fungal species, such as *Stemphylium lycopersici*, which is associated with leaf spot disease in vegetable crops [26]. In the present study, the results of RAPD and ISSR analyses were very similar and showed that the markers were 84% to 100% polymorphic, respectively. Therefore, the 28 *A. alternata* isolates exhibited relatively high diversity in the main potato-growing regions of Iran. The RAPD and ISSR markers showed no correlation with the geographical origins of the isolates, suggesting that the isolates are widely dispersed across Iran. The above results were consistent with those of a previous study, in which ISSR markers were used to assess the genetic diversity of 11 *A. alternata* isolates that caused brown leaf spot disease in potato plants throughout Iran [10]. Furthermore, our findings indicated that both RAPD and ISSR markers can be used to reliably evaluate the diversity of *A. alternata* isolates.

Recently, sequencing of different DNA regions has been used as an alternative to morphology-based identification of *Alternaria* species [5,7,29] and other fungal species. Consequently, different DNA regions have been used for the characterization and identification of various *Alternaria* species [5]. Studies have indicated that the combination of various DNA genes, including *gpd*, plasma membrane ATPase, Alt a1, calmodulin, and actin, can successfully determine the monophyly of *Alternaria* species. The above-mentioned genes showed higher polymorphism than those of the translation elongation factor 1-alpha (*TEF*) and beta-tubulin genes. In the present study, phylogenetic analysis of the combined dataset comprising the *gpd*, plasma membrane ATPase, Alt a1, calmodulin, and actin genes confirmed that all five representative isolates, namely, Aa-A5, Aa-A6, Aa-H2, Aa-H3, and Aa-I1, were *A. alternata* species. Furthermore, we detected no significant differences in the sequences of the five target genes among the representative isolates.

Conclusions

In conclusion, RAPD and ISSR markers were successfully used to genetically distinguish all the *A. alternata* isolates. The results of the present study revealed the extant diversity in pathogenicity and genetics of *A. alternata* isolates. Therefore, new and aggressive isolates should be screened to evaluate the potential resistance of potato varieties, which would be useful for the selection of appropriate breeding strategies against the potato pathogen *A. alternata*. To our knowledge, no comprehensive study has investigated the genetic diversity of *A. alternata* isolates that cause leaf spot disease in potato plants from various geographical regions in Iran. RAPD and ISSR markers could also be used to investigate a broader range of *A. alternata* isolates from other regions of the world and can also be extended to other host plants. Given that temperature, wind speed, potato cultivar, and other factors can affect the population structure of fungi,

future studies should investigate the population structure of *A. alternata* under various growing conditions. In addition, further studies are required to determine pathogen specialization of different cultivars.

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References

1. Andersen B, Hansen ME, Smedsgaard J. Automated and unbiased image analyses as tools in phenotypic classification of small-spored *Alternaria* spp. Phytopathology. 2005;95:1021–1029. https://doi.org/10.1094/PHYTO-95-1021
2. Droby S, Dinoor A, Prusky D, Barkai-Golan R. Pathogenicity of *Alternaria alternata* on potato in Israel. Phytopathology. 1984;74:537–542. https://doi.org/10.1094/Phyto-74-537
3. Rotem J. The genus *Alternaria*; biology, epidemiology, and pathogenicity. Saint Paul, MN: American Phytopathological Society Press; 1998.
4. van der Waals JE, Pitsi BE, Marais C, Wairuri CK. First report of *Alternaria alternata* causing leaf blight of potatoes in South Africa. Plant Dis. 2011;95(3):363–363. https://doi.org/10.1094/PDIS-11-10-0820
5. Lawrence DP, Gannibal PB, Peever TL, Pryor BM. The sections of *Alternaria*: formalizing species-groups concepts. Mycologia. 2013;105:530–546. https://doi.org/10.3852/12-249
6. Nasehi A, Kadir JB, Ashtiani FA, Nasr-Esfahani M, Wong MY, Rambe SK, et al. *Alternaria capsicicola* sp. nov., a new species causing leaf spot of pepper (*Capsicum annuum*) in Malaysia. Mycol Prog. 2014;13(4):991. https://doi.org/10.1007/s11557-014-0991-1
7. Woudenberg JHC, Groenewald JZ, Binder M, Crous PW. *Alternaria* redefined. Stud Mycol. 2013;75:171–212. https://doi.org/10.3114/sim0015
8. Mamgain A, Chowdhury R, Tah J. *Alternaria* pathogenicity and its strategic controls. Research Journal of Biology. 2013;1:1–9.
9. Vandecasteele M, Landschoot S, Carrette J, Verwaeren J, Höfte M, Audenaert K, et al. Species prevalence and disease progression studies demonstrate a seasonal shift in the *Alternaria* population composition on potato. Plant Pathol. 2018;67:327–336. https://doi.org/10.1111/ppa.12734
10. Bagherabadi S, Zafari D, Soleimani MJ. Genetic diversity of *Alternaria alternata* isolates causing potato brown leaf spot, using ISSR markers in Iran. J Plant Pathol Microbiol. 2015;6:286. https://doi.org/10.4172/2157-7471.1000286
11. McDonald BA, Linde C. Pathogen population genetics, evolutionary potential, and durable resistance. Annu Rev Phytopathol. 2002;40:349–379. https://doi.org/10.1146/annurev.phyto.40.120501.101443
12. Nasr Esfahani M. Identification of *Ulocladium atrum* causing potato leaf blight in Iran. Phytopathol Mediterr. 2018;57(1):112–114. https://doi.org/10.14601/Phytopathol_Mediterr-22282
13. Demartelaere ACF, Nascimento LC, Marinho CO, Nunes MC, Abraão PC, Gomes RSS, et al. Diversity among isolates of the tangerine pathotype of *Alternaria alternata*. American Journal of Plant Sciences. 2018;9:81987. https://doi.org/10.4236/ajps.2018.92017
14. Shahbazi H, Aminian H, Sahebani N. Halterman D. Effect of *Alternaria solani* exudates on resistant and susceptible potato cultivars from two different pathogen isolates. Plant Pathol J. 2011;27(1):14–19. https://doi.org/10.5423/PPJ.2011.27.1.014
15. National Institute of Agricultural Botany. Disease assessment manual for crop variety trials. Cambridge: National Institute of Agricultural Botany; 1985.
16. Talbot N, editor. Molecular and cellular biology of filamentous fungi. New York, NY: Oxford University Press; 2001. (Practical Approach Series).
17. Berbee ML, Pirseyedi M, Hubbard S. *Cochliobolus* phylogenetics and the origin

of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. Mycologia. 1999;91:964–977. https://doi.org/10.2307/3761627

18. Hong SG, Cramerb RA, Lawrence CB, Pryora BM. Alt a 1 allergen homologs from *Alternaria* and related taxa: analysis of phylogenetic content and secondary structure. Fungal Genet Biol. 2005;42:119–129. https://doi.org/10.1016/j.fgb.2004.10.009
19. Rohlf EJ. NTSYS-pc: numerical taxonomy and multivariate analysis system, version 1.80. Setauket, NY: Applied Biostatistics, Inc.; 1993.
20. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser (Oxf). 1999;41:95–98.
21. Thompson JD, Higgins DG, Gibson TJ. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–4680. https://doi.org/10.1093/nar/22.22.4673
22. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870–1874. https://doi.org/10.1093/molbev/msw054
23. Kakvan N, Zamanizadeh H, Morid B, Taheri H, Hajmansor S. Study on pathogenic and genetic diversity of *Alternaria alternata* isolated from citrus hybrids of Iran, based on RAPD-PCR technique. Eur J Exp Biol. 2012;2(3):570–576.
24. Weir TL, Huff DR, Christ BJ, Romaine CP. RAPD-PCR analysis of genetic variation among isolates of *Alternaria solani* and *Alternaria alternata* from potato and tomato. Mycologia. 1998;90(5):813–821. https://doi.org/10.2307/3761323
25. Morris PF, Connolly MS, St Clair DA. Genetic diversity of *Alternaria alternata* isolated from tomato in California assessed using RAPDs. Mycol Res. 2000;104(03):286–292. https://doi.org/10.1017/S0953756299008758
26. Nasehi A, Kadir JB, Nasr Esfahani M, Abed Ashtiani F, Wong MY, Rambe SK, et al. Analysis of genetic and virulence variability of *Stemphylium lycopersici* associated with leaf spot of vegetable crops. Eur J Plant Pathol. 2014;140(2):261–273. https://doi.org/10.1007/s10658-014-0460-3
27. Kale SM, Pardeshi VC, Gurjar GS, Gupta VS, Gohokar RT, Ghorpade PB, et al. Inter-simple sequence repeat markers reveal high genetic diversity among isolates of Indian origin. J Mycol Plant Pathol. 2012;42(2):194.
28. Pryor BM, Michailides TJ. Morphological, pathogenic, and molecular characterization of *Alternaria* isolates associated with *Alternaria* late blight of pistachio. Phytopathology. 2002;92(4):406–416. https://doi.org/10.1094/PHYTO.2002.92.4.406
29. Woudenberg JHC, Seidl MF, Groenewald JZ, de Vries M, Stielow JB, Thomma BPHJ, et al. *Alternaria* section *Alternaria*: species, formae speciales or pathotypes? Stud Mycol. 2015;82:1–21. https://doi.org/10.1016/j.simyco.2015.07.001