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Genetic diversity of *Alternaria alternata* Isolates Causing Potato Brown Leaf Spot, Using ISSR Markers in Iran

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Abstract

Sampling was carried out from different fields in Hamedan province, Iran during the years 2012 and 2013. Among the 300 obtained isolates of *Alternaria* spp. after morphological identification, it was revealed that *A. alternata* isolates from potato had the highest frequency distribution. Due to the abundance of this species in this region which is considered as a major potato producing province in the country, genetic diversity of its isolates were assessed using ISSR markers. Among *A. alternata* isolates, 11 isolates screneded from nine different regions on four potato cultivars were selected. A total of 15 ISSR primers of UBC group were used to investigate the genetic diversity of these isolates. Out of 15 primers used in this study, 5 primers had favorable results and produced a significant number of bands. Based on the results of cluster analysis using the Jaccard's coefficient, isolates were divided into two main groups and there was some correlation between the grouping of isolates regarding their geographic location, pathogenicity and potato cultivars.

Keywords: *Alternaria alternate*; Cluster analysis; Diversity analysis; ISSR markers; Potato

Introduction

Alternaria alternata (Fr.) Keissler is common saprobe found on many plants and other substrate worldwide [1-3] and can cause damage to many plants in different agro ecosystems, including potato brown leaf spot disease. It is mainly found in the soil or on decomposing plant tissues [4]. This species is also an opportunistic pathogen affecting many cultivated plants in the field and during post-harvest storage of fruit and vegetables. This fungus attacks plants including cereals, ornamental plants, magnolia, oilseeds, vegetables such as cauliflower, broccoli, eggplant, pepper, carrot, potato, tomato, bean and fruits such as citrus, apple, strawberry and peach and in some cases, is known as post-harvest pathogens [5]. In recent years, molecular markers have been used for practical studies of many organisms. In a way, that exploring the various types of molecular markers has made major progresses in genetic studies [6]. DNA is the essence of genetic differences between two specified organism and DNA fingerprinting is now one of the methods for identification of biological organisms. DNA polymorphism is the base of many genetic studies [1]. Genetic diversity is the base of evolution of species and populations [7]. The ability of a population to adapt to different environmental conditions is dependent on the level of genetic diversity [8]. Populations with higher genetic diversity are stable against changes in environmental conditions. Therefore, the first step for managing a plant pathogen is to investigate the diversity. One of the reasons for lack of success in the management of plant diseases is due to lack of information about the structure of the pathogen populations; therefore enhancing knowledge in this area will certainly be helpful in adopting effective methods of managing a pathogen. Therefore being aware of the genetic diversity of populations and the structure of populations within a species, not only checks the evolutionary processes and mechanism of that, but also provides useful information about biological conservations [9].

There are several methods for evaluating genetic diversity. The choice of molecular markers depends on their high reproducibility, simplicity of method, low cost and high reliability. In 1994, a new type of molecular markers briefly known as ISSR was introduced by Zitkovich et al. and quickly was used in different fields. These markers have high similarity to RAPD markers and are widely dispersed throughout the genome [10]. ISSR is a multilocus marker that in addition to these features does not have the restrictions of other markers such as low reproducibility in RAPD, high costs and complexity in AFLP [11]. ISSR being able to create polymorphism patterns among near-organisms and their reproducibility caused to be known as informative markers with a wide range of applications including the study of genetic diversity [12]. Recent studies show the extremely diverse nature of these markers and their potential to study the different levels of population [2]. Although for some *Alternaria* species *Lewia* is known as sexual stage but the sexual stage of *A. alternata* is unknown [3,13]. These species are likely haploid which reproduce asexually in vegetative phase and is expected to have a low genetic diversity. Guo et al. [14] used 20 ISSR primers to study genetic diversity of 112 isolates of endophytic *Alternaria alternata* isolated from pine in China. They found out tha of these, only two primers showed high levels genetic diversity of this species in pine. Their results showed no correlation between the fungal genotype and host age and endophytes of *A. alternata* had great potential for development and maintenance of genetic diversity. Zhong-hui et al. [15] also studied genetic variation in eight isolates of *A. alternata* at five region of China on Tobacco using ISSR markers. Kale et al. [16] studied genetic diversity of 20 isolates of *A. alternata* on linseed at 14 districts of India using nine ISSR primers. Their research results showed that there was a high genetic diversity among these 20 isolates and no correlation between genetic diversity, geographic region, and intensity of pathogenicity.

Hamedan province in Iran, through having good and adapted

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weather for the cultivation of potato has dedicated a large percentage of potato production in the country to itself, With cultivated area equal to 25,000 hectares and annual production of 900 thousand tons potato tubers has gained an special position in production of potato in the country.

Since *A. alternata* is one of the most important pathogens causing potato brown leaf spot in this region, the aim of this study was determined to investigate the genetic diversity of isolates of this species on potato using ISSR markers in Hamedan province, Iran.

Materials and Methods

Sampling, isolation and identification

Sampling was done during the spring, summer and autumn of 2012 and 2013 from potato fields, in different locations of Hamedan province, Iran. The collected samples were placed separately in paper bags and plant names, place and date of sample collection was recorded. In order to conduct further studies plant tissues were transported to the laboratory and stored in a refrigerator at 4°C. Plant organs with suspected symptoms of contamination were washed under running water for 5 minutes. Sections possess symptoms were cut from healthy sections and segmented into parts of 0.5 to 1 cm. These segments were surface sterilized with 10 percent sodium hypochlorite for 2 minutes and immediately were washed twice with sterile distilled water. The pieces on the dewatering filter paper were transferred to the petri dishes containing potato dextrose agar (PDA) medium. Petri dishes were kept in an incubator at 23-25 °C. Five to seven days after incubation, isolates were purified using single spore method and then were transferred to the tubes containing potato carrot agar (PCA) medium for storage and subsequent studies. Microscopic identification at the genus level was achieved using Lica microscope according to the imperfect fungi key [17].

Evaluation of morphological features

To study the morphological characteristics of purified isolates at the species levels, subcultures were transferred to the petri dishes containing PCA medium. These petri dishes were kept at 23 to 25°C under fluorescent light with light cycle of 8 hours light and 16 hours dark and were studied after five to seven days. For white light, two 40 W white fluorescent lamps were used at the distance of 40 cm from the surface of petri dishes. Microscopic identification was achieved using Lica microscope according to descripion of *Alternaria* species [18] After morphological identifications, *Alternaria* isolates with higher frequency were selected for further studies such as study of thier pathogenicity and genetic diversiy.

Pathogenicity test

To conduct the pathogenicity test, healthy potato tubers were cultured in sterilized pots containing pasteurized soil. Potato seedlings with 5-6 leaves, were sprayed separately with *A. alternata* spore suspension (10^6 spores per ml) of each isolate. 48 h before pathogen inoculation, plants were covered with plastic bags to keep the relative humidity at 100%. To avoid air trapped small holes was created at the top of the bags. After pathogen inoculation, the plants covered for 48 hours with plastic bags and were kept in greenhouse conditions with 14 hours light and 10 hours of darkness.

Molecular studies

Among 165 obtained isolates of *A. Alternata* from potato in differrent reagions of Hamedan province, screening was achived according to the main reagiones of potato production in this province and kind of potato cultivatrs that isolates were isolated from those, therefore due to the great similarity among isolates from each reagion and considering to potato cultivare 11 isolates as representative were screened for study of gentics diversity.

DNA extraction

Mycelia of *A. alternata* isolates grown in potato dextrose broth (PDB) medium were collected, washed and were kept at -20˚c. DNA was extracted using modified method of Sharma et al. [19]. Frozen mycelia were poured in a porcelain mortar which was pre-chilled in the freezer and were powdered in liquid nitrogen. 200 mg of powdered mycelia were transferred to each 1.5 ml tubes and 750 µl of extracted buffer stored in 60˚c (2% PVP-40, pH 8.0 EDTA 20 mM, CTAB 5% (W/V) Tris-HCl pH 8.0 100 mM, NaCl 1.4 M, 2.0% mercaptoethanol) was added to each sample, mixed and for 35 minutes was kept in 60˚C hot water bath and at this time the contents of the tubes were shaken gently several times. Equivalent to the volume of the tube, the mixture of chloroform – isoamyl alcohol (1:24) was added to each tube containing the sample and was mixed gently for one minute.

The mixture was centrifuged for 15 min at 7,000 rpm, then supernatant was taken and poured into a new sterile tube. Sodium acetate of 3 M, 5.2 PH and 0.6 volume of cold isopropanol solution was added to one to thirty of volume and the solution in the tubes was gently mixed several times, at this stage, the DNA strands were formed which were easily visible. The tubes containing DNA strands were centrifuged for 10 min at 7000 rpm at 4˚C and the supernatant was emptied gently so that the DNA remained intact inside the tube. Then 500 ml 70% ethanol was added to the tubes containing DNA, and centrifuged at 13,000 rpm at 4˚C for 5 min. The upper phase was discarded and tubes in the air upside down were placed on absorbent paper so that the deposition driedand finally 50 µl of sterile double- distilled water was added to each tube. The samples were stored overnight in the refrigerator until the mass of DNA distilled in water. To detect the extracted DNA, 1.2% Agarose gel in TBE buffer was used and then 5 µl of DNA with double amount of loading buffer was mixed and electrophorzed at a constant voltage of 80 volts for 1.5 hours. Quantity and quality of extracted DNA was determined by spectrophotometry and agarose gel electrophoresis.

Regulation of polymerase chain reaction (PCR)

To confirm the morphological identification, one of the isolates was selected as representative for molecular analysis and protein encoding gene of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplyfied and sequenced. To assess the genetic diversity of isolates of this species, ISSR marker was selected and 15 UBC primers were used for conducting the test in polymerase chain reaction. The names and gpd_1, gpd_2 sequences of primers and specific UBC primer are listed in Table 1.

At the time of conducting tests, to prevent the time error, taking very small amounts of materials and to practice more easily and quickly, master mix of PCR reactions was prepared as shown in Table 2. Master mix includes all materials needed for the PCR reaction except DNA sample and primer.

Firstly, 2 µl of DNA of each sample was poured into PCR specific micro-tubes, then 2 µl primers (with concentration of 10 pmol/µl) was added and kept in the refrigerator. Immediately, 21 µl of the master mix was added to the each micro-tube containing DNA and primer. Thermocycler device of Techne model TC-512 was used

The name of primer	Sequence
UBC 807	5'-AGAGAGAGAGAGAGAGT-3'
UBC 808	5'-AGAGAGAGAGAGAGAGC-3'
UBC 809	5'-AGAGAGAGAGAGAGAGG-3'
UBC 818	5'-CACACACACACACACAG-3'
UBC 822	5'-TCTCTCTCTCTCTCTCA-3'
UBC 834	5'-AGAGAGAGAGAGAGAGCT-3'
UBC 835	5'-AGAGAGAGAGAGAGAGCC-3'
UBC 840	5'-GAGAGAGAGAGAGAGATT-3'
UBC 841	5'-GAGAGAGAGAGAGAGACC-3'
UBC 842	5'-GAGAGAGAGAGAGAGATG-3'
UBC 846	5'-CACACACACACACACAAT-3'
UBC 849	5'-GTGTGTGTGTGTGTGTCA-3'
UBC 850	5'-GTGTGTGTGTGTGTGTCA-3'
UBC 856	5'-ACACACACACACACACCA-3'
gpd1	5'-CAACGGCTTCGGTCGCATTG-3'
gpd2	5'-GCCAAGCAGTTGGTTGTGC-3'

Table 1: Names and sequences of primers used in this study.

Per one reaction	Final concentration	Basal concentration	Material
15.2 µl	-	-	Sterile double distilled water
2.5 µl	1X	10X	PCR buffer
1.6 µl	3.2 Mm	50 Mm	$MgCl_2$
1.5 µl	0.6 Mm	10 Mm	dNTPS
0.2 µl	1 Unit	5 Unit/µl	Taq DNA polymerase enzyme
21 µl	-	-	*Total volume of master mix

Table 2: Ingredients and amounts for the preparation of master mix used in the PCR reaction. *Final volume of PCR reaction: (2 µl DNA+2 µl Primer+21 µl master mix)=25 µl.

Number of cycles	Conducted Steps	Time	Temperature (°C)
1 Cycle	Initial denaturation	5 min	94
35 Cycles	Denaturation	1 min	94
	Annealing	75 second	Depending on the type of primer used
	Extension	2 min	72
1 cycle	Final extension	10 min	72

Table 3: Time and temperature required for conducting different stages of PCR for primers gpd_1, gpd_2 and specific UBC primers.

for amplification. According to the program given to the machine, polymerase chain reaction (PCR) was conducted using primers based on the conditions shown in Table 3.

Electrophoresis of PCR products

For electrophoresis of each sample, 5 µl of the initial PCR product was removed and after mixing with 2 µl of loading buffer, electrophorzed on the 1.2% agarose gel in TBE buffer with constant voltage of 80 kV for 2.5 hours. The samples were run on a solution of ethidium bromide for 30 minutes (5.0 mg per µl). After washing the gel with distilled water, the gel was subjected to image analysis with a gel document device (DIGI.DOC H101 model).

Sequenced regions of *gpd*

To determine the DNA sequence of a representative isolate, regions of *gpd* were amplified and sequenced. Obtained sequences were blasted with the sequences of these regions related to the isolates of this species in the GenBank.

Analysis of data obtained from UBC primers

The resulting band patterns of each isolate were scored for the presence or absence of amplified products. Cluster analysis of the data was done using UPGMA, by the use of Jaccard's similarity coefficient in the NTSYS-PC software (version 2) and a dendrogram was constructed for each primers and total primers. Decomposition to main coordinates on similarity matrix was obtained through Jaccard's similarity coefficient. To determine the optimal number of clusters in which the highest distinction between the groups is achieved, analysis of molecular variance (AMOVA) was used.

Results and Discussion

In this study, 300 isolates of *Alternaria* were obtained from different hosts and regions in Hamedan province and were identified at species level, based on morphological characteristics. Among identified isolates, 165 isolates were belonged to the *A. alternata* species isolated from potato crop (Table 4). Since isolates of *A. alternata* from potato were the most frequent spcies, genetic diversity of these isolates was studied by using ISSR marker. Among collected isolates from potato crops in this study, 11 isolates were selected from nine different regions and four different potato cultivars in Hamedan province (Table 5).

Item	Name of isolates	Host	Number of isolates
1	*Alternaria alternata*	*Solanum tuberosum*	165
2	*Alternaria alternata*	*Medicago sativa*	2
3	*Alternaria alternata*	*Prunus persica*	2
4	*Alternaria alternata*	*Cucumis sativus*	3
5	*Alternaria alternata*	*Salix* sp.	2
6	*Alternaria alternata*	*Rosa* sp.	2
7	*Alternaria alternata*	*Rumex alpinus*	3
8	*Alternaria alternata*	*Solanum lycopersicum*	3
9	*Alternaria alternata*	*Lactuca sativa*	2
10	*Alternaria alternata*	*Amarantus albus*	1
11	*Alternaria alternata*	*Acroptilon repens*	2
12	*Alternaria alternata*	*Prunus domestica*	3
13	*Alternaria alternata*	*Juglans regia*	4
14	*Alternaria alternata*	*Althaea officinalis*	2
15	*Alternaria alternata*	*Lepidium draba*	2
16	*Alternaria alternata*	*Fraxinus excelsior*	3
17	*Alternaria alternata*	*Triticum aestivum*	3
18	*Alternaria alternata*	*Diospyros* sp.	3
19	*Alternaria arborescens*	*Solanum tuberosum*	8
20	*Alternaria arborescens*	*Juglans regia*	3
21	*Alternaria arborescens*	*Lactuca sativa*	3
22	*Alternaria arborescens*	*Solanum lycopersicum*	6
23	*Alternaria arborescens*	*Lepidium draba*	2
24	*Alternaria arborescens*	Triticum aestivum	2
25	*Alternaria arborescens*	*Carex* sp.	2
26	*Alternaria arborescens*	*Althaea officinalis*	2
27	*Alternaria dumosa*	*Solanum tuberosum*	8
28	*Alternaria dumosa*	*Althaea officinalis*	2
29	*Alternaria infectoria*	*Solanum tuberosum*	3
30	*Alternaria infectoria*	*Althaea officinalis*	1
31	*Alternaria rosae*	*Alisma plantago*	2
32	*Alternaria solani*	*Solanum tuberosum*	15
33	*Alternaria tenuissima*	*Malus domestica*	2
34	*Alternaria tenuissima*	*Medicago sativa*	3
35	*Alternaria tenuissima*	*Fragaria ananassa*	24
36	*Alternaria tenuissima*	*Solanum tuberosum*	5

Table 4: List of hosts of *Alternaria* spp. isolates.

Item	Number of isolates	Name of fungus	Host	Cultivar	Region
1	16	*Alternaria alternata*	Potato	Esprit	Asadabad
2	218	*A. alternata*	Potato	Sante	Asadabad
3	15	*A. alternata*	Potato	Marfona	Hamedan
4	226	*A. alternata*	Potato	Sante	Hamedan
5	223	*A. alternata*	Potato	Agria	Nahavand
6	215	*A. alternata*	Potato	Agria	Qorveh
7	214	*A. alternata*	Potato	Agria	Gahavand
8	120	*A. alternata*	Potato	Agria	Bahar
9	91	*A. alternata*	Potato	Agria	Gheidar
10	269	*A. alternata*	Potato	Agria	Kabodrahng
11	286	*A. alternata*	Potato	Agria	Razan

Table 5: List of the used isolates to determine the genetic diversity of along with the host, cultivars and sample locations.

Pathogenicity test

After three weeks the symptoms of the disease caused by the 214, 269 and 286 isolates were observed on tested plants. Samples were taken from diseased plant and cultured on PDA and again after ten days of incubation, the pathogen resembles to those of the original isolates, *A. alternata* was identified. However, no symptoms were observed in other tested isolates.

Results of molecular studies

Blast search from the *gpd* gene sequences showed 99% similarity between representative and *A. alternata* in the gene bank (Accesion number: KP057228) that confirmed the morphological identification results. The results of molecular studies to determine the genetic diversity of *A. alternata* isolates using ISSR markers showed that theses markers are excellent choices for fingerprinting genomes of these fungi. Among 15 primers used in this study that they have been selected accroding thier good resultes in previous studies by reseachers, 10 primers did not have favorable results for genotyping in this study, therefore remaining 5 primers were used to study the genetic relationships of this species. Based on the results obtained from these tests, the binding temperature of the primer in most cases, were close to their melting temperature (Table 6).

In this study, ISSR primers, fragments with sizes of 100 to 3000 bp were amplified. Totally, ISSR primers showed 540 bands that 408 bands were polymorphic. The results showed that UBC807 primer with 157 bands had the highest number of bands and UBC809 primer with 77 bands had the lowest bands. According to the (Figures 1 and 2), UBC809 primer had the highest polymorphism (92%) and UBC807 primer had the lowest levels of polymorphism (75%). Finally, 78 bands loci were amplified by ISSR primers that 63 loci of them were polymorphic. In this study we have examined the same ISSR primers which Kale et al. [16] were used in their conducted research, in order to study the genetic diversity of *A. alternata* isolates in linseed. In the study of Kale et al. [16] mean percentage of polymorphic was 98%, whereas in this study, ISSR markers showed 83.3% polymorphic. The details of used primers in this study are listed in Tables 7 and 8.

Cluster analysis based on ISSR markers

Comparative analysis of molecular variance showed that the highest difference between the two groups at the cutting point of two groups was obtained at similarity level of 55%, indicating the genetic diversity among *A. alternata* isolates. Isolates regarding to their collected regions were placed in two groups. Isolates collected from Asadabad (Esprit *cv*), Hamadan (Marfona *cv*), Asadabad (Sante *cv*), Hamadan (Sante *cv*), Qorveh (Agria *cv*), Gheidar (Agria *cv*) and Nahavand (Agria *cv*) in a separate group and isolates collected from Ghahavand (Agria), Razan (Agria) and Kabodrahng (Agria) were placed in another group.

Primer name	Primer Binding Temperature (Ta)	Primer Melting Temperature (Tm)
UBC807	45	43
UBC809	44	44
UBC834	48	47
UBC835	48	48
UBC842	44	44
gpd1, gpd2	55	58

Table 6: The name of primer, melting temperature and binding temperature of effective specific primers.

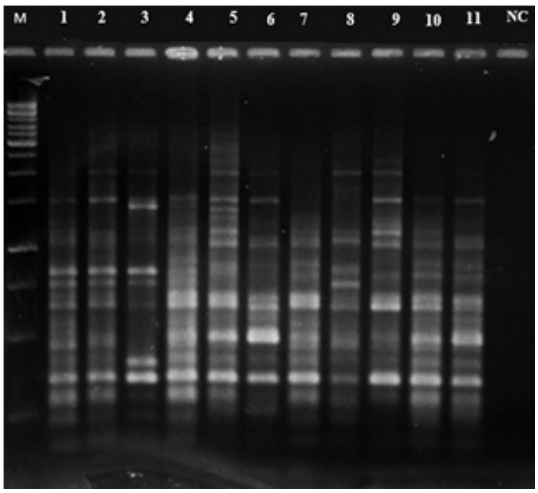

Figure 1: The banding pattern of UBC807 primer application. Molecular marker 1 Kb. 1: Isolates No. 16, 2: Isolates No. 218, 3: Isolates No. 159, 4: Isolates No. 226, 5: Isolates No.223, 6: Isolates No. 215, 7: Isolates No. 214. 8: Isolates No. 120, 9: Isolates No. 91, 10: Isolates No.269, 11: Isolates No. 286, NC: Negative Control.

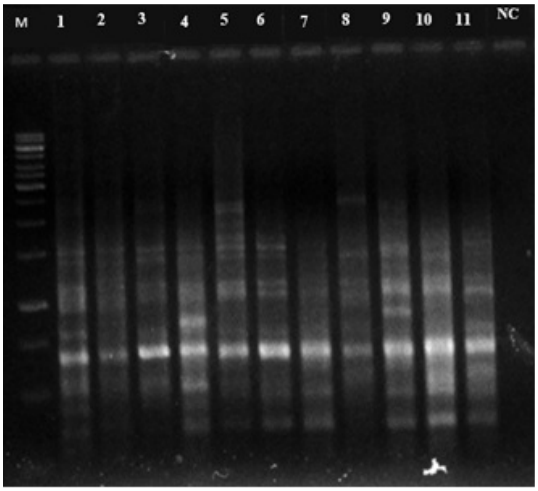

Figure 2: The banding pattern of UBC809 primer application. Molecular marker 1 Kb. 1: Isolates No. 16, 2: Isolates No. 218, 3: Isolates No. 159, 4: Isolates No. 226, 5: Isolates No.223, 6: Isolates No. 215, 7: Isolates No. 214. 8: Isolates No. 120, 9: Isolates No. 91, 10: Isolates No.269, 11: Isolates No. 286, NC: Negative Control.

The results also showed that isolates of *A. alternata* on potato had interaspecific variation which indicates that this marker could demonstrate the genetic diversity of this species. The results of cluster analysis showed that in the first group isolates with different cultivars and geographically dispersed areas and non-pathogenic were placed and in second group same cultivar and geographic region close to each other and pathogenic were placed, thus it can be said that there is some correlation between the genetic diversity of this species, geographical distribution, pathogenicity of potato variation. So this might be concluded that isolates from the same locations showed a tendency to group together compared to geographically farther ones. As can be seen in the dendrogram (Figure 3) isolates obtained from Razan, Kabodrahng and Ghahavand with Agria cultivar and pathogenicity in the north part of Hamedan Province placed in one group and the remaining isolates on different cultivars and non-pathogenic obtained from other areas of the Hamadan province placed in another group.

Primer name	Number of band loci	Number of polymorphic loci	Total number of loci	Number of polymorphic bands
UBC807	20	15	157	102
UBC809	13	12	77	66
UBC834	17	14	120	87
UBC835	15	13	104	82
UBC842	13	11	82	60

Table 7: Data on locations of bands, polymorphic loci, total bands and polymorphic bands per each primer.

Studied Parameters	Results
Total number of bands	540
amplified polymorphic bands	408
number of amplified loci	78
The number of amplified loci that were polymorphic	65
The number of amplified loci that were not polymorphic	13
Percentage of polymorphic	%83/3
The highest percentage of polymorphic	%92 (UBC809)
The lowest percentage of polymorphic	%75 (UBC807)
The highest number of bands	UBC807))157
The lowest number of bands	UBC809))77
The range of band	100-3000 bp

Table 8: The summery of the results obtained for primer used in this study.

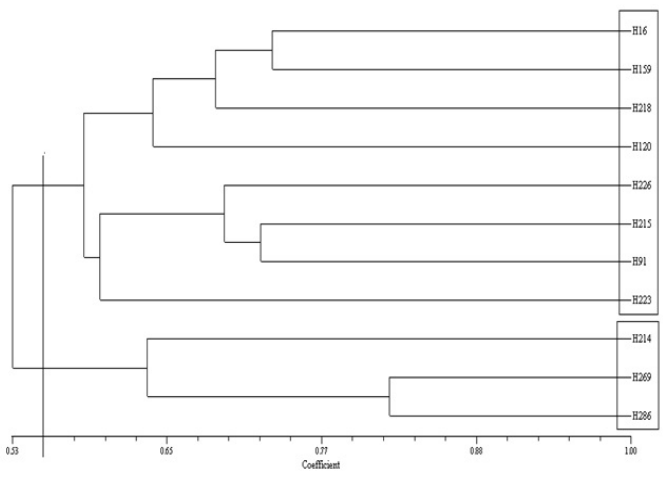

Figure 3: Dendrogram of total ISSR primers used in this study based on Jaccard's similarity coefficient and UPGMA cluster analysis.

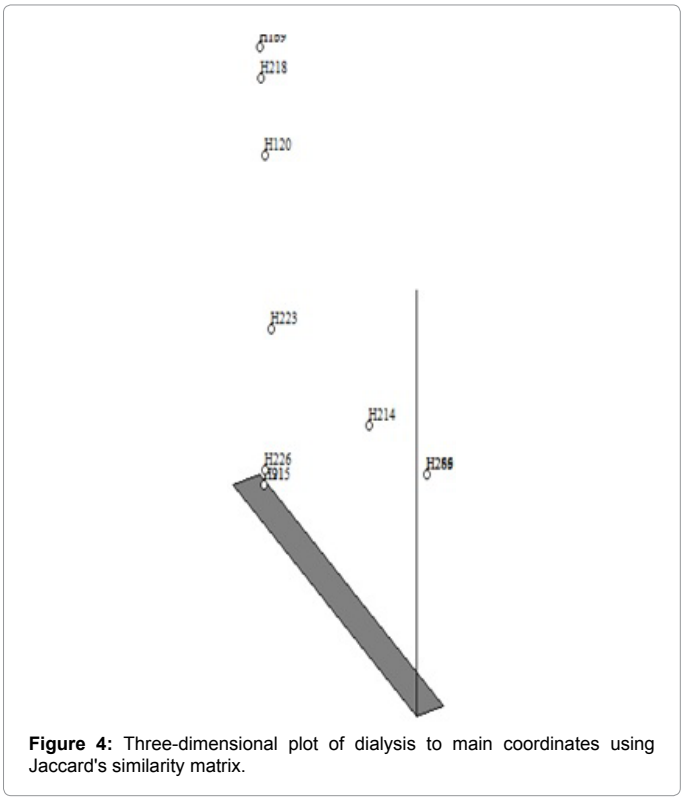

Figure 4: Three-dimensional plot of dialysis to main coordinates using Jaccard's similarity matrix.

Dialysis to main components partially confirmed the cluster analysis results. The first two components justified 70% of the changes that indicates favorable sampling of these remarks from the whole genome. Thus, each of the markers used in different parts of the genome had less correlation. This method also segregated some isolates from each other in different geographical areas (Figure 4).

This study demonstrated the existence of a narrow range of genetic diversity among potato isolates of *A. alternata* from Hamedan province, Iran. The isolates had mixed response for cultivars and pathogenicity characteri.

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