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Molecular diversity within and between Ajowan (*Carum copticum* L.) populations based on inter simple sequence repeat (ISSR) markers

M. Modareskia[1], R. Darvishzadeh[2,3,*], A. Hassani[1] and M. Kholghi[2]

1. Department of Horticulture, Urmia University, Urmia, Iran.
2. Department of Agronomy and Plant Breeding, Urmia University, Urmia, Iran. [3]Institue of Biotechnology, Urmia University, Urmia, Iran.
*Corresponding author email: r.darvishzadeh@urmia.ac.ir

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Abstract

Study of genetic relationships is a prerequisite for plant breeding activities as well as for conservation of genetic resources. In the present study, genetic diversity among and within 15 Iranian native Ajowan (*Carum copticum* L.) populations were determined using inter simple sequence repeat (ISSR) markers. Twelve selected primers produced 153 discernible bands, with 93 (60.78%) being polymorphic, indicating considerable genetic diversity at the population level. Number of polymorphic bands per primer ranged from 4 to 11 with an average of 7.75. The largest percentage of polymorphic loci was 78.57% for primer UBC818. Based on an un-weighted pair-group method using arithmetic average (UPGMA) clustering algorithm, four distinct groups were established. Results of the principal coordinate analysis (PCoA) corresponded to those obtained through cluster analysis. Analysis of molecular variance (AMOVA) showed that the genetic variation was found mainly within populations (61%), but variance among populations was only 39%. This study has detected significant genetic differentiation among *C. copticum* populations. The current study confirmed the importance of molecular studies in detecting genetic variation among genotypes in selecting diverse parents to carry out crossing program successfully.

Key words: *Carum copticum*, cluster analysis, genetic diversity, molecular markers.

Introduction

Ajowan (*Carum copticum* L.) with 2n=18 chromosomes is a medicinal plant belonging to family Apiaceae. It is an annual and cross-pollinated plant which grows in the east of India, Iran, Pakistan and Egypt with white flowers and small brownish fruits (Zargary 1991). Ajowan is one of the aromatic seed spices, which is generally used as a digestive stimulant or to treat liver disorders. The phytochemical studies on Ajowan seeds have revealed the presence of multiple constituents such as steroptin, cumene, thymene, amino acids like lysine and threonine, calcium, iron, starch, tannins and dietary fiber (Uma et al. 1993). Seeds also contain essential oil (2–3%), which has thymol (40–50%), γ-terpinene, p-cymene, α-pinene, β-pinene and carvacrol (Ballba et al. 1973). Thymol as a major phenolic compound present in Ajowan has been reported to be a germicide, antispasmodic and antifungal

agent (Nagalakshmi and Shankaracharya 2000). Thymol is also used in toothpaste and perfume industries (Krishnamoorthy and Madalageri 1999; Joshi 2000).

Genetic diversity studies are fundamental for plant breeding programs and are commonly measured by genetic distances or similarities (Weir 1990). Morphological and biochemical markers tend to be restricted to relatively few traits, display a low degree of polymorphism, are often environmentally variable in their manifestation and depend on the expression of several unlinked genes (Melchinger et al. 1991). In contrast, molecular marker-based genetic diversity analysis has potential for assessing changes in genetic diversity over time and space (Duwick 1984). Molecular markers have been extensively used in characterization of plant genetic resources. DNA-based molecular markers can help breeders to improve medicinal plant species. These markers can be used for assessing genetic diversity, authenticating plant material used for drugs and for marker-assisted breeding (Joshi et al. 2004; Canter et al. 2005).

Understanding genetic variation within and between populations is essential for the establishment of effective and efficient conservation practices for plant species. Several aspects of conservation biology, such as loss of genetic diversity in conservation programs and restoration of threatened populations, can only be addressed by detailed population genetic studies (Hamrick and Godt 1996). Molecular fingerprinting allows non-coding DNA sequences to be examined, thereby providing more penetrating insights into population genetic structures. Among DNA fingerprinting techniques, inter simple sequence repeats (ISSR) representing non-coding sequences between simple sequence repeats, provide an effective way to assess genetic diversity. ISSR was first published by Zietkiewicz et al. (1994) and it does not require previous knowledge of the sequence of the genome being tested. ISSR amplification utilizes anchored SSR primers that are complementary to genomic microsatellites and target numerous and highly variable loci (Zietkiewicz et al. 1994; Gilbert et al. 1999; Prevost and Wilkinson 1999; Joshi et al. 2000). As a consequence, ISSR amplification reveals a much larger number of polymorphic fragments per primer than RAPD does (Qian et al. 2001). This method uses single primers of 15–20-nucleotides with a 3′ or 5′ anchor sequence. ISSR markers due to repeatability are being more popular and easier to use than other markers such as RAPD and AFLP (Chennaout-Kourda et al. 2007). ISSR studies of *Aegiceras corniculatum* and *Viola pubescens* natural populations demonstrated a hyper variable nature of these DNA fingerprints and their potential use for population level studies (Ge and Sun 1999; Culley and Wolfe 2001). The ISSR technique has been successfully applied to study genetic diversity in many plants species such as *Citrus* [*Poncirus trifoliata* (L.) Raf., Fang et al. 1997], hop (*Humulus lupulus* L., Patzak 2001), chickpea (genus *Cicer*, Iruela et al. 2002), *Changium smyrnioides* (Qiu et al. 2004), blackgram (Souframanien and Gopalakrishna 2004), *Phyllanthus amarus* (Palaniappan et al. 2008), *Tribulus terrestris* (Sarwat et al. 2008), *Salvia miltiorrhiza* (Song et al. 2010) and Rhubarb (Wang 2010) as well as quality control of the medicinal plant *Armillaria mellea* (Zheng et al., 2009), authentication of medicinal plant *Swertia chirayita* (Tamhankar et al. 2009) and to identify zygotic plantlets in *Citrus* species (Tusa et al. 2002). It has been shown to be useful in population genetic studies of *Oryza granulate* (Qian et al. 2001), *Hordeum spontaneum* (K. Koch) synonym of *Hordeum vulgare* L. subsp. *spontaneum* (K. Koch) Thell (Tanyolac 2003) and *Phaseolus vulgaris* (Gonzalez et al. 2005). ISSR technique has also been used to study genetic diversity in wild populations of endangered medicinal plant *Emmenopterys henryi* (Li and Jin 2008) and a perennial herb *Glycyrrhiza uralensis* (Yao et al. 2008).

There are not any reports concerning to genetic diversity studies on Iranian Ajowan population by using molecular markers. The main aims of present study were to (1) assess levels of genetic diversity in Iranian native Ajowan populations; (2) partitioning the genetic variations to among

and within population diversity; and (3) provide basic information for conservation and breeding programs of this medicinal plant species.

Materials and Methods

Plant materials

Fifteen Ajowan populations collected from different geographical regions of Iran (Table 1) were investigated in the present study. The seeds of each population were collected from several individuals. The populations' seeds of present study have been kindly provided by Research Institute of Forests and Range and Pakan Seed Company. Four plants per population were randomly chosen, and genomic DNA was extracted from the leaves of 4-weeks-old seedlings following the CTAB-based modified method originally described by Doyle and Doyle (1987). The concentration of genomic DNA samples was determined spectrophotometrically at 260 nm using the BioPhotometer 6131 (Eppendorf, Germany). DNA quality was checked by running 1µl DNA in 0.8% (w/v) agarose gels in 0.5× TBE buffer (45mM Tris base, 45mM boric acid, 1mM EDTA pH 8.0). DNA samples that gave a smear in the gel were discarded.

ISSR analysis

Twelve ISSR primers (from University of British Columbia, Vancouver, Canada) (Table 2) were used for DNA fingerprinting. Polymerase chain reactions (PCR) were performed in 25µl containing 12.5µl of PCR master mix [200mM Tris–HCl pH 8.55, 160mM $(NH_4)_2SO_4$ 0.1% (v/v), 3.0mM $MgCl_2$, 0.4mM of dNTPs, 1.0 Unit of Taq DNA polymerase] (Cinna Gen Inc., Tehran, Iran), 2µM ISSR primer (Cinna Gen Inc., Tehran, Iran), 50ng of genomic DNA and bidistillated water. DNA amplifications were performed using a 96-well Eppendorf Mastercycler Gradient (Type 5331, Eppendorf AG, Hamburg, Germany) programmed for a preliminary step of 4 min at 94ºC, followed by 35 cycles of 94ºC for 40s, 35-60ºC (depend on primers sequence) for 40s and 72 ºC for 2 min. A final extension was 5 min at 72 ºC. The reaction products were then mixed with an equal volume of formamide dye [98% (v/v) formamide, 10mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol] and resolved in a 1.8% (w/v) agarose gel in 0.5× TBE and visualized with ethidium bromide (1.0 µg ml^{-1}). The electrophoretic patterns of the PCR products were photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 PRO, USA).

Data analysis

The PCR amplification products were scored for the presence (1) or absence (0) of each band across the 60 individuals and a binary matrix was constructed. Number of bands, percentage of polymorphic bands, number of bands with a frequency higher or equal to 5%, number of private bands, number of less common band with frequency lower or equal to 25% and 50%, mean heterozygosity (Lynch and Milligan, 1994), and standard error of mean heterozygosity were calculated for each population using GenAlEx 6 software (Peakall and Smouse 2006). Jaccard (Jaccard 1908), Dice (Nei and Li 1979) and Simple Matching (Sneath and Sokal 1973) similarity coefficients were used to define the genetic relationship among individuals. UPGMA, complete linkage and single linkage were used for clustering individuals. Efficiency of clustering algorithms and their goodness of fit were determined based on Cophenetic correlation coefficients. Significance of observed Cophenetic correlation was tested using the Mantel matrix correspondence test (Mantel 1967). In addition to cluster analysis, principal co-ordinates analysis (PCoA) (Kovach 1999) was used to confirm the results of cluster analysis. Data analyses were performed using NTSYS-pc version 2.11 software (Rolf 1998). For studying diversity between populations, Nei genetic distance (Nei 1972) was calculated. Visualization of the relationship among population was carried out by UPGMA dendrograms and also by principal coordinate analysis. To know partition of total genetic variation within and among populations, analysis of molecular variance (AMOVA) was carried out by using GenAlEx 6 software (Peakall and Smouse 2006).

Table 1. Code number and accession name of the 15 Iranian Ajowan populations used to evaluate genetic diversity by using inter simple sequence repeat (ISSR) markers

Code	Accession name	Altitude (m)	Latitude (E)	Longitude (N)
P1	Rafsanjan I	1529	56°36′30″	30°29′20″
P2	Ardabil	1347	48°17′58.30″	38°14′49.40″
P3	Rafsanjan II	1529	56°25′25″	30°26′30″
P4	Fars	1830	53°02′45.21″	29°06′15.77″
P5	Damyal	1347	48°15′10″	38°20′02″
P6	Isfahan	1575	51°40′45.09″	32°39′05″
P7	Hamadan I	1832	48°26′34.65″	34°32′47.54″
P8	Hamadan II	1791	48°30′58.49″	34°48′23.40″
P9	Ghazvin	1407	50°25′	36°29′
P10	Gorgan	138	45°28′48″	36°50′11.81″
P11	Kerman	1253	57°04′01.27″	30°16′48.97″
P12	Bam	1063	58°21′14.22″	29°06′37.94″
P13	Marand	1425	45°46′10.69″	38°25′30.42″
P14	Sardasht	1435	45°28′48″	36°09′17″
P15	Khorasan	29	54°00′20.25″	26°32′25.44″

Table 2. Primers, amplification conditions and polymorphism of ISSR markers used on 15 Iranian Ajowan populations.

Primer name	Sequence(5'→3')	Annealing Temperature(°C)	Total number of bands	Polymorphic bands	Percentage of polymorphic bands	Band size (bp)
UBC818	$(CA)_8G$	45	14	11	78.57	300–2,900
UBC812	$(GA)_8A$	45	15	4	26.67	350–2,000
A7	$(AG)_{10}T$	50	10	7	70.00	200–1,500
A13	$(GT)_6CC$	48	11	7	63.64	250–2,000
UBC857	$(AC)_8YG$	40	12	5	41.67	100–2,000
UBC840	$(GA)_8YT$	43	8	4	50.00	200–1,500
UBC849	$(GT)_8CG$	56	14	10	71.43	300–2,000
CAG5	$(CAg)_5$	60	13	8	61.54	500–2,000
UBC825	$(AC)_8T$	60	14	10	71.42	250–1,000
UBC848	$(CA)_8RG$	57	17	10	58.82	100–1,000
UBC807	$(AG)_8T$	46	13	9	69.23	250–1,500
UBC810	$(GA)_8T$	48	12	8	66.66	300–1,500
Total	-	-	153	93	60.78	
Mean	-	-	12.75	7.75	60.80	

Results and Discussion

Genetic diversity among and within 15 Iranian native Ajowan populations was investigated by using 12 ISSR marker loci. Primer sequence, annealing temperature, total number of bands, and polymorphic bands are shown in table 2. ISSR primers produced a total of 153 reproducible bands, 93 were polymorphic. Number of total bands per primer ranged from 8 [primer $(GA)_8YT$] to 17 [primer $(CA)_8$ RG], with an average of 12.75 (Table 2). Number of polymorphic bands per primer ranged from 4 to 11 with an average of 7.75 (Table 2). The size of the amplified fragments ranged from 100 to 2900 bp. ISSR markers revealed high levels of polymorphism in Ajowan natural populations. ISSR markers have been also utilized to study genetic diversity in several crops and they are known as highly polymorphic and suitable for DNA fingerprinting (Bornet and Branchard 2001; Reddy et al. 2002; Pharmawati et al. 2005, Aghaei et al. 2012).

Features of amplified bands were reported in Table 3. Private band was only detected in Kerman population (P11). That is, the ISSR divergence among populations was mainly attributed to differences of the DNA fragment frequency rather than allele fixation. The region-specific band detected in this study can be used for the authentication of this medicinal herb. The region-specific bands were also reported by Sarwat et al. (2008) in the medicinal plant *Tribulus terrestris* by using ISSR and RAPD markers. Mean of heterozygosity varied from 0.043 (P10: Gorgan) to 0.107 (P7: Hamadan I), averaging 0.077 (Table 3). These values indicate P7 (Hamadan I) would be the population in which more genetic diversity and alleles would be expected.

Use of different similarity coefficients and algorithms for grouping the studied Ajowan individuals and calculation of co-phenetic correlation coefficient for each one, revealed that Jaccard's similarity coefficient with UPGMA clustering algorithm resulted in the highest correlation coefficient ($r=0.759$; $P\leq0.05$) (Table 4). The UPGMA clustering dendrogram representing relationships among individuals are shown in figure 1. The UPGMA dendrogram shows the trend to group individuals according to the population which they belong, resembling the different populations. Results of PCoA corresponded to those obtained through cluster analysis and classified Ajowan individuals mostly by resembling the populations (Figure.2).

Analysis of molecular variance (AMOVA) revealed significant genetic variation among populations, however it was lower (39%) than within population (61%) (Table 5). Barbosa et al.(2010) studied the medicinal plant *Palicourea coriacea* (Rubiaceae) with molecular markers and reported 23% of genetic variability was found among populations and 77% of genetic variability within populations. The genetic structure of plant populations reflects the interaction of various factors, including long-term evolutionary history of the species, genetic drift, mating system, and gene flow (Hogbin and Peakall 1999). The out crossing system of *C. copticum* probably seems to account for high levels of genetic variation within populations, but, population differentiation could indicate certain independent evolution.

Inter-populations genetic distance, represented by the UPGMA algorithm in figure 3 and principal coordinate analysis in figure 4, ranged from 0.031 to 0.205 (Table 6). The least genetic distance resulted between populations from "Sardasht" and "Khorasan" (0.031). Samples from "Ardabil" and "Damyal" resulted in a high genetic distance to samples of "Kerman" (0.205) (Table 6), which is related to a high geographical distance. It indicates geographic distance could be the cause of a substantial barrier to gene exchange among these populations promoting the appearance of genetic differentiation between both regions. However, it is not true for all the populations, for example populations from Rafsajan (geographically closer to Kerman than to Ardabil or Damyal) resulted more similar according to ISSR pattern to Ardabil than to Kerman.

Genetic drift caused by small population size and or reduced gene flow or dispersal among patchy populations may also shape the current genetic structure of *C. copticum* populations. Murthy and Arunachalam (1966) showed genetic drift and

Table 3. Characteristics of amplified ISSR loci on studied Iranian Ajowan populations.

Population	No. Bands[a]	No. Bands Freq. >= 5%[b]	No. Private Bands[c]	No. LComm Bands (<=25%)[d]	No. LComm Bands (<=50%)[e]	Mean He[f]	Standard error of Mean He
P1	100	100	0	0	5	0.068	0.014
P2	99	99	0	0	2	0.084	0.016
P3	99	99	0	1	2	0.086	0.017
P4	96	96	0	1	5	0.091	0.016
P5	100	100	0	0	2	0.088	0.017
P6	96	96	0	0	2	0.055	0.013
P7	97	97	0	1	4	0.107	0.018
P8	96	96	0	0	5	0.076	0.016
P9	91	91	0	0	2	0.078	0.016
P10	87	87	0	0	0	0.043	0.011
P11	101	101	1	0	6	0.093	0.017
P12	101	101	0	0	5	0.09	0.016
P13	97	97	0	0	5	0.078	0.016
P14	98	98	0	0	5	0.073	0.015
P15	91	91	0	0	4	0.054	0.014

[a]No. Bands = Number of different bands; [b]No. Bands Freq. >= 5% = Number of different bands with a frequency >= 5%; [c]No. Private Bands = Number of bands unique to a single population; [d]No. LComm Bands (<=25%) = Number of locally common bands (Freq. >= 5%) found in 25% or fewer populations; [e]No. LComm Bands (<=50%) = Number of locally common bands (Freq. >= 5%) found in 50% or fewer populations; [f]He = Expected heterozygosity = $2 \times p \times q$.

Table 4. Comparison of different methods for constructing similarity matrices and dendrograms

Similarity matrices	Algorithm	Co-phenetic coefficient
Jaccard	UPGMA	r = 0.759
	Complete linkage	r = 0.634
	Single linkage	r = 0.707
Dice (Nei and Li)	UPGMA	r = 0.746
	Complete linkage	r = 0.619
	Single linkage	r = 0.694
Simple Matching	UPGMA	r = 0.745
	Complete linkage	r = 0.699
	Single linkage	r = 0.677

Dic (Nei and Li, 1979); Jaccard (Jaccard, 1908); Simple Matching (Sneath and Sokal, 1973). UPGMA: un-weighted pair-group method using arithmetic average.

Table 5. Analysis of Molecular Variance (AMOVA) for Iranian Ajowan populations.

Source	df	SS	MS	Est. Var.	%	Stat	Value	P(rand >= data)
Among Pops	14	455.767	32.555	5.822	39%	PhiPT	0.386	0.001
Within Pops	45	417.000	9.267	9.267	61%			
Total	59	872.767		15.089	100%			

df = degrees of freedom; SS: sum of square; MS = mean of squares.

Table 6. Nei genetic distance among 15 Iranian Ajowan populations.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
P1	0.000														
P2	0.076	0.000													
P3	0.069	0.106	0.000												
P4	0.126	0.118	0.091	0.000											
P5	0.086	0.081	0.098	0.108	0.000										
P6	0.114	0.119	0.126	0.138	0.105	0.000									
P7	0.118	0.099	0.131	0.122	0.116	0.057	0.000								
P8	0.166	0.174	0.157	0.154	0.183	0.112	0.099	0.000							
P9	0.130	0.119	0.128	0.161	0.119	0.130	0.114	0.193	0.000						
P100	0.143	0.150	0.155	0.177	0.156	0.181	0.150	0.168	0.101	0.000					
P11	0.203	0.205	0.155	0.175	0.205	0.166	0.187	0.148	0.165	0.165	0.000				
P12	0.172	0.169	0.136	0.148	0.154	0.134	0.148	0.153	0.129	0.142	0.057	0.000			
P13	0.161	0.176	0.152	0.157	0.164	0.149	0.166	0.148	0.132	0.126	0.068	0.049	0.000		
P14	0.149	0.151	0.161	0.164	0.169	0.130	0.143	0.141	0.136	0.110	0.060	0.065	0.064	0.000	
P15	0.155	0.142	0.163	0.160	0.182	0.137	0.140	0.137	0.140	0.113	0.071	0.071	0.063	0.031	0.000

selection in different environments can cause greater diversity among genotypes than geographic distance cause. Therefore, selection of parental material in breeding programs simply based on geographic diversity may not be rewarding. One possible reason for the genetic similarity among germplasm from different regions is that the materials might have originally been introduced from the same region. Crossing individual from clusters with maximum inter-clusters distance may be result in high heterosis. It is well documented that crosses between unrelated, and consequently genetically distant parents, show greater hybrid vigor than crosses between closely related genotypes (Reif et al. 2007; Solomon et al. 2007).

The genetic distance among populations is a valuable parameter for germplasm conservation and for plant breeding programs. Hybridization/crossing between any distantly related populations is expected to yield more heterosis and vigorous plants constituting much of the different traits contained in the two parental lines. Therefore, hybridization or crossing between genetically distant populations of the present study could be an appropriate strategy for inter-populations landrace improvement programs.

The perhaps most striking result of this investigation is the high level of differentiation between populations from different habitats, as demonstrated by analysis of molecular variance, dendrogram based on Nei´s distance, and principal coordinate analysis. However, it can not be explained only by geographical distance,

other aspect such as genetic drift could explain totally the results. The usual idea that, the higher spatial distance between populations increases, the higher genetic differentiation is usually expected (Slatkin 1993) is not totally supported by the present investigation.

Conclusion

The present study is the first report on molecular relationships of Ajowan populations and can be considered as an initial point for further research on the genetic relationships and evolution as well as marker-assisted selection in Ajowan breeding programs. Our study demonstrated that ISSR technique was very effective in determining genetic diversity of the *C. copticum* populations. Cluster analysis based on ISSR profiles clearly classified Ajowan populations and provided a molecular diagnosis tool for the authentication of valuable medicinal plant populations. The great genetic diversity found among Ajowan

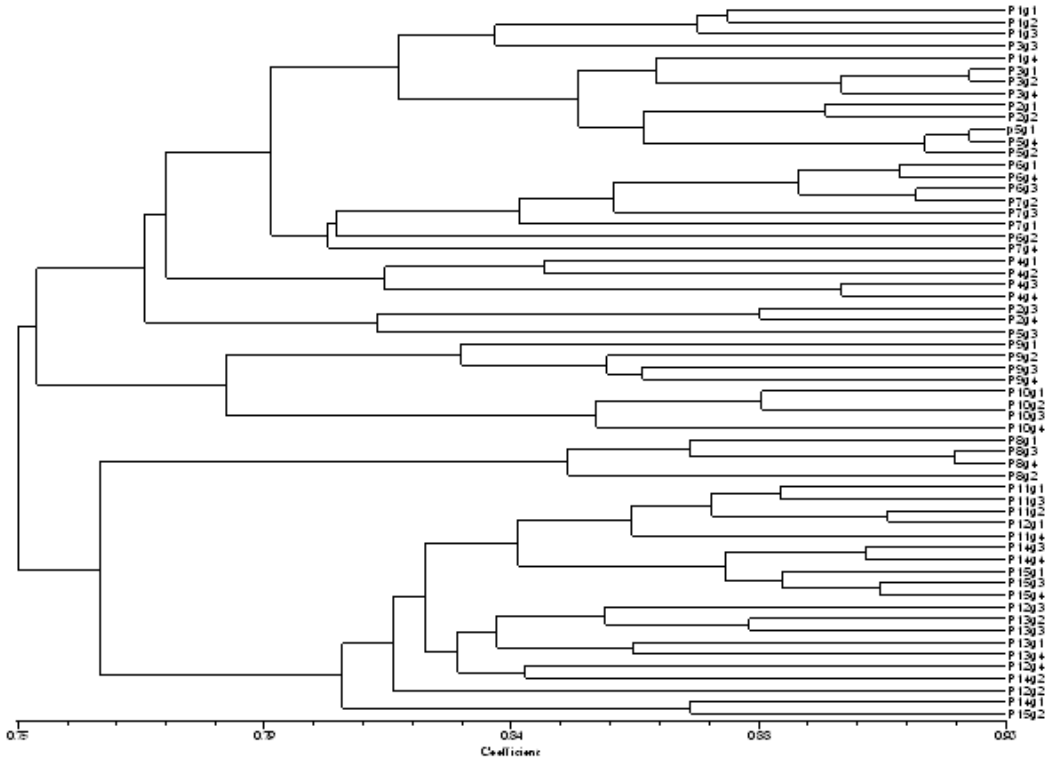

Figure 1. UPGMA clustering of 60 Iranian Ajowan genotypes based on Jaccard's similarity coefficient.

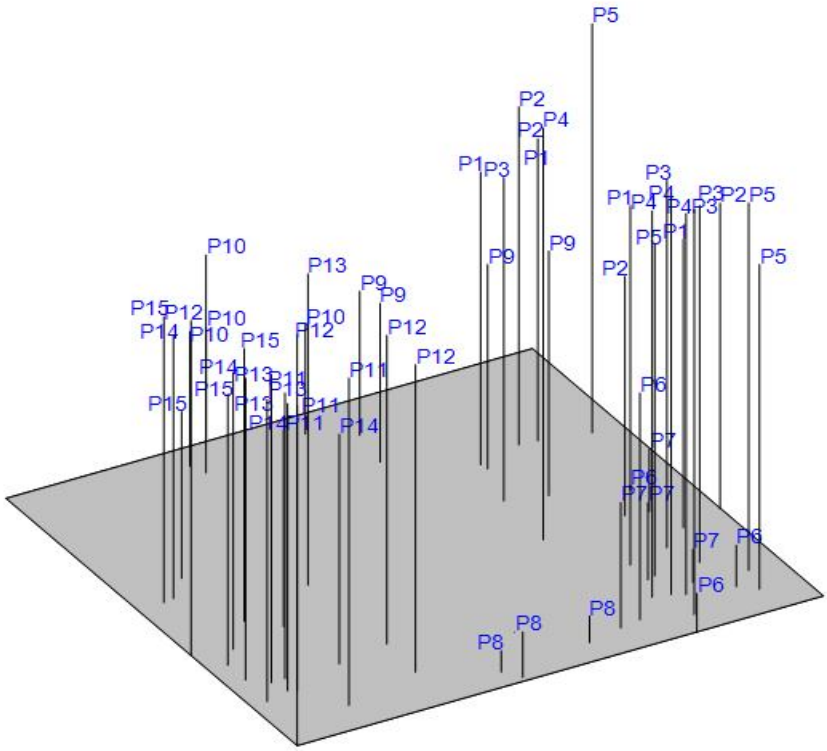

Figure 2. Three-dimensional graph from the principal coordinate analysis of 60 Iranian Ajowan genotypes (15 populations and 4 plants per population) by using12 ISSR markers.

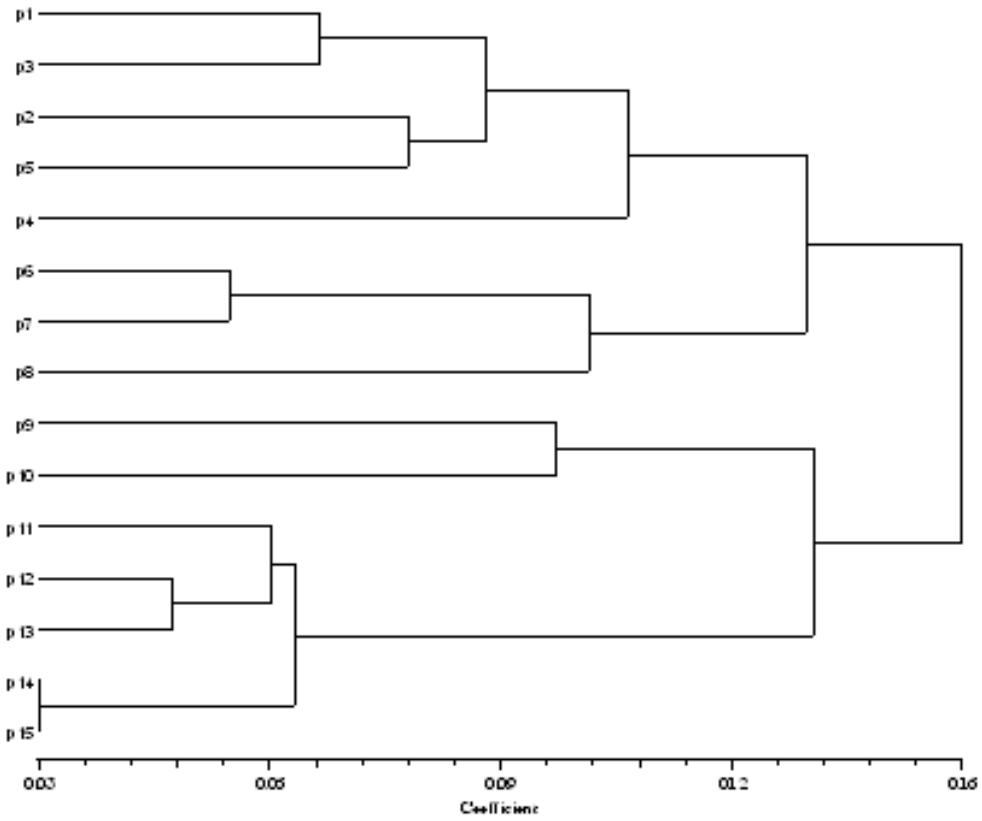

Figure 3. UPGMA clustering of 15 Iranian Ajowan populations based on Nei genetic distance.

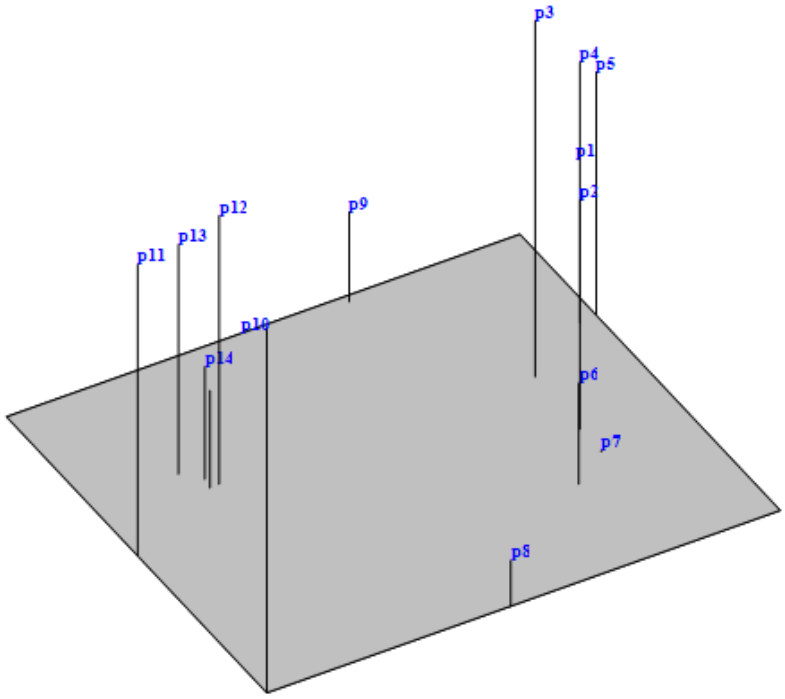

Figure 4. Three-dimensional graph from the principal coordinate analysis of 15 Iranian Ajowan populations by using 12 ISSR markers.

assists parental selection in current and future breeding programs.

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References

Aghaei, M., Darvishzadeh, R., Hassani, A. 2012. Molecular characterization and similarity relationships among Iranian basil (*Ocimum basilicum* L.) accessions using inter simple sequence repeat markers. Rev Ciênc Agron, 43: 312-320.

Ballba, S.I., Hilal, S.H., Haggag, M.Y., 1973. The volatile oil from the herb and fruits of *Carum copticum* at different stages of growth. Planta Med, 23: 312–320.

Barbosa, T.C.S., Sibov, S.T., Telles, M.P.C. and Soares, T.N. 2010. Genetic characterization of natural populations of the medicinal plant *Palicourea coriacea* (Rubiaceae) with molecular markers. Genet Mol Res, 9: 695–704.

Bornet, B. and Branchard, M. 2001. Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. Plant Mol Biol Rep, 19: 209–215.

Canter, P.H., Thomas, H. and Ernst, E. 2005. Bringing medicinal plant species into cultivation: opportunities and challenges for biotechnology. Trends Biotech, 23: 180–185.

Chennaoui-Kourda, H., Marghali, S., Marrkchi, M. and Trifi-Farah, N. 2007. Genetic diversity of Sulla genus (Hedysarea) and related species using Inter-simple Sequence Repeat (ISSR) markers. Biochem System Ecol, 20: 1–7.

Culley, T.M. and Wolfe, A.D. 2001. Population genetic structure of the cleitogamous plant species *Viola pubescens* Aiton (Violaceae), as indicated by allozyme and ISSR molecular markers. Heredity, 86: 545–556.

Doyle, J. and Doyle, J. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull, 19: 11–15.

Duwick, D.N. 1984. Genetic diversity in major farm crops on the farm and reserve. Econ Bot, 32: 161–178.

Fang, D.Q., Roose, M.L., Krueger, R.R. and Federici, C.T. 1997. Fingerprinting trifoliate orange germplasm accessions with isozymes, RFLPs, and inter-simple sequences repeat markers. Theor Appl Genet, 95: 211–219.

Ge, X.J. and Sun, M. 1999. Reproductive biology and genetic diversity of a cryptoviviparous mangrove *Aegiceras corniculatum* (Myrsinaceae) using allozyme and inter simple sequence repeat (ISSR) analysis. Mol Ecol, 8: 2061–2069.

Gilbert, J.E., Lewis, R.V., Wilkinson, M.J. and Caligari, P.D.S. 1999. Developing an appropriate strategy to assess genetic variability in plant germplasm collections. Theor Appl Genet, 98: 1125–1131.

Gonzalez, A., Wong, A., Delgado-Salinas, A., Papa, R. and Gepts, P. 2005. Assessment of inter simple sequence repeat markers to differentiate sympatric wild and domesticated populations of common bean. Crop Sci, 45: 606–615.

Hamrick, J.L. and Godt, M.J.W. 1996. Conservation genetics of endemic plant species. *In*: Avise JC, Hamrick JL, eds. Conservation genetics: case histories from nature. New York, NY: Chapman and Hall, pp. 281–304.

Hogbin, P.M. and Peakall, R, 1999. Evaluation of the contribution of genetic research to the management of the endangered plant *Zieria prostrata*. Conserv Biol, 13: 514–522.

Iruela, M., Rubio, J., Cubero, J.I., Gil, J. and Millan, T. 2002. Phylogenetic analysis in the genus *Cicer* and cultivated chickpea using RAPD and ISSR markers. Theor Appl Genet, 104: 643–651.

Jaccard, P. 1908. Nouvelles recherches sur la distribution florale. Bull Soc Vaud Sci. Natur, 44: 223-270.

Joshi, K., Chavan, P., Warude, D. and Patwardhan, B. 2004. Molecular markers in herbal drug technology. Curr Sci, 87: 159–165.

Joshi, S.G. 2000. Medicinal Plants. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, India, 362.

Joshi, S.P., Gupta, V.S., Aggarwal, R.K., Ranjekar, P.K. and Brar, D.S. 2000. Genetic diversity and phylogenetic relationships as revealed by inter simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. Theor Appl Genet, 100: 1311–1320.

Kovach, W. 1999. MVSP-A Multivariate Statistical Package for Windows, ver. 3.1. Kovach Computing Services, Pentraeth, Wales, UK, 133 pp.

Krishnamoorthy, V. and Madalageri, M.B. 1999. Bishop weed (*Trachyspermum ammi*): an essential crop for north Karnatka. J Med Aromat Plant Sci, 21: 996–998.

Li, J.M. and Jin, Z.X. 2008. Genetic structure of endangered *Emmenopterys henryi* Oliv. based on ISSR polymorphism and implications for its conservation. Genet,133, 227–234.

Lynch, M. and Milligan, B. 1994. Analysis of population-genetic structure using RAPD markers. Mol Ecol, 3: 91-99.

Mantel, N.A. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res, 27: 209–220.

Melchinger, A.E., Messmer, M., Lee, M., Woodman, W.L. and Lamkey, K.R. 1991. Diversity and relationships among U.S. maize inbreds revealed by restriction fragment length polymorphisms. Crop Sci, 31: 669–678.

Murthy, B.R. and Arunachalam, V. 1966. The nature of genetic divergence in relation to breeding system in crop plants. Indian J Genet Plant Breed, 26: 188–189.

Nagalakshmi, S. and Shankaracharya, N. 2000. Studies on chemical and technological aspects of Ajowan. J Food Sci, Technol. 37: 277–281.

Nei, M. 1972. Genetic distance between populations. Am Natur, 106, 283 292.

Nei, N.M. and Li, W. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. National Acad Sci, 76: 5269-5273.

Palaniappan, M. and Marappa, M. 2008. RAPD and ISSR analyses reveal low levels of genetic diversity in *Phyllanthus amarus*. Acta Hort, 765: 179–187.

Patzak, J. 2001. Comparison of RAPD, STS, ISSR and AFLP molecular methods used for assessment of genetic diversity in hop (*Humulus lupulus* L.). Euphytica, 121: 9–18.

Peakall, R. and Smouse, P.E. 2006. GENALEX 6: Genetic Analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes, 6: 288–295.

Pharmawati, M., Yan, G. and Finnegan, P.M. 2005. Molecular variation and fingerprinting of *Leucadendron* cultivars (Proteaceae) by ISSR markers. Ann Bot, 95: 1163–1170.

Prevost, A. and Wilkinson, M. 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor Appl Genet, 98: 107–112.

Qian, W., Ge, S. and Hong, D.Y. 2001**.** Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers. Theor Appl Genet, 102: 440–449.

Qiu, Y.X., Hong, D.Y., Fu, C.X. and Cameron, K.M. 2004. Genetic variation in the endangered and endemic species *Changium smyrnioides* (Apiaceae). Biochem System Ecol, 32: 583–596.

Reddy, M.P., Sarla, N. and Siddiq, E.A. 2002. Inter Simple Sequence Repeat (ISSR) polymorphism and its application in plant breeding. Euphytica, 128: 9–17.

Reif, J.C., Gumpert, F., Fischer, S. and Melchiger, A.E. 2007. Impact of genetic divergence on additive and dominance variance in hybrid populations. Genet, 176: 1931–1934.

Rolf, F.J. 1998. NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.02. Exter Software, Setauket, New York.

Sarwat, M., Das, S. and Srivastava, P.S. 2008. Analysis of genetic diversity through AFLP, SAMPL, ISSR and RAPD markers in *Tribulus terrestris*, a medicinal herb. Plant Cell Rep, 27: 519–528.

Slatkin, M. 1993. Isolation by distance in equilibrium and non-equilibrium populations. Evol, 47: 264–279.

Sneath, P.H.A. and Sokal, R.R. 1973. Numerical Taxonomy. Freeman WH, San Francisco, USA.

Solomon, K.F., Labuschagne, M.T. and Viljoen, C.D. 2007. Estimates of heterosis and association of genetic distance with heterosis in durum wheat under different moisture regimes. J Agric Sci, 145: 239–248.

Song, Z., Li, X., Wang, H. and Wang, J. 2010. Genetic diversity and population structure of *Salvia miltiorrhiza* Bge in China revealed by ISSR and SRAP. Genet, 138: 241–249.

Souframanien, J. and Gopalakrishna, T. 2004. A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers. Theor Appl Genet, 109: 1687–1693.

Tamhankar, S., Ghate, V., Raut, A. and Rajput, B. 2009. Molecular profling of "Chirayat" complex using inter simple sequence repeat (ISSR) markers. Planta Med, 75, 1266–1270.

Tanyolac, B. 2003. Inter-simple sequence repeats (ISSR) and RAPD variation among wild barley (*Hordeumvulgare* subsp. spontaneum) populations from west Turkey. Genet Res Crop Evol, 50: 611–614.

Tusa, N., Abbate, L., Ferrante, S., Lucretti, S. and Scarano, M.T. 2002. Identification of zygotic and nucellar seedlings in Citrus interploid crosses by means of isozymes, fow cytometry and ISSR-PCR. Cell Mol Biol Lett, 7: 703–708.

Uma, P.K., Geervani, P. and Eggum, B.O. 1993. Common Indian spices: nutrient composition, consumption and contribution of dietary value. Plant Foods Human Nut, 44: 137–148.

Wang, X.M. 2010. Optimization of DNA isolation, ISSR-PCR system and primers screening of genuine species of rhubarb, an important herbal medicine in China. J Med Plants Res, 4: 904–908.

Weir, B.S. 1990. Genetic Data Analysis: Methods for Discrete Population Genetic Data. Sinauer Associates, Sunderland, MA.

Yao, H., Zhao, Y., Chen, D.F., Chen, J.K. and Zhou, T.S. 2008. ISSR primer screening and preliminary evaluation of genetic diversity in wild populations of *Glycyrrhiza uralensis*. Biol Plant, 52: 117–120.

Zargary, A. 1991. Medicinal plants, Tehran University Press, Tehran Vol. 2. pp 942 (In Persian).

Zheng, P., Meng, X., Yu, Z., Wu, Y., Bao, Y., Yu, C. and Li, Y. 2009. Genetic and phytochemical analysis of *Armillaria mellea* by RAPD, ISSR, and HPLC. Anal Lett, 42: 1479–1494.

Zietkiewicz, E., Rafalske, A. and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genome, 20: 178–183.