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RESEARCH ARTICLE

Assessment of genetic diversity among Crataegus genotypes by Application of ISSR markers in Ardabil province

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ABSTRACT: The genus *Crataegus* spp. (Hawthorn) is belongs to Rosaceae family. The hawthorn can be utilized as a rootstock as well as an ornamental plant. The fruits are sources of many essential nutrients and have beneficial effects on human health. The Crataegus genus has been founded in different areas such as Iran, especially in the Ardabil region. The collection and conservation of genetic material is important for future breeding programs. In this purpose, thirty hawthorn genotypes collected from several regions (Fandoghlo, Namin, Khalkhal, Aladizgeh, Germi) of Ardebil province and their genetic variations investigated with 15 ISSR markers. According to the results, all 15 primers amplified and produced total 128 bands (average = 8.53), as well as showed high polymorphism information content (average = %38.06). The results showed that there is a high level of genetic diversity and polymorphism ratio among the wild genotypes of hawthorn in the Ardabil region. Based on WARD technique, genotypes classified into three main clusters with several sub-clusters that exhibited high genetic diversity. The Germi genotypes completely isolated from the rest of the genotypes, which according to the structure analysis results, were similar to the Khalkhal genotypes.

KEYWORDS: Hawthorn, ISSR marker, Cluster analysis, Structure analysis

INTRODUCTION

Hawthorn belongs to the genus Crataegus and Rosaceae family. This genus is a complex group of small shrubs and tree, mostly growing to 5–15 m tall, with small pome fruit and usually thorny branches. The fruits are berry-like but structurally such as a pome. The *Crataegus* species distributed in Asia, Europe and North America [16]. The extracts of Hawthorn fruit exhibited antioxidant and radical scavenging activities, moreover heart failure and cardiovascular disease treatment capability is cited in several researches [21]. Hawthorn also has numerous health effects including hypolipidaemic, antiatherosclerotic, hypotensive, cardioprotective and blood vessel relaxing activities [16].

The wild hawthorn plants naturally grow in the Northwest (such as Ardabil province) and west of Iran. *Crataegus pontica* which naturally grows in these locations has morphological genetic diversity in flower, leaf, fruit and leaf characteristics [13]. This genetic variation is because that, most birds and mammals eat the fruits and distribute the seed [25]. In Europe, the main species are *Crataegus laevigata*, *Crataegus monogyna*, *Crataegus pentagyna*, *Crataegus nigra*, and *Crataegus azarolus* [6].

There are many species of this genus in northern and western apart of Iran. Hawthorn is a popular and important tree fruit from aspects of medicinal and rootstock. Because of the high diversity of this plant, primary studies are necessary to breeding programs. The molecular method is one of the applicable methods which can be used to selecting and conserving plants. Among the marker-based polymerase chain reaction methods, inter-

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simple sequence repeats (ISSR) is one of the simple markers that is widely used for genetic diversity investigation [24]. The ISSR marker involves amplification of DNA segments located between two identical microsatellite repeated regions, oriented in opposite direction. The ISSR technique uses microsatellites sequences, usually 16–25 bp long. The ability of molecular tools for evolutionary studies is due to the insensitivity of the genetic markers to environmental factors [12]. In a study by Erfani-Moghadam et al. (2015) investigated both morphological characteristics and molecular data of some hawthorn species in Iran. This study clearly indicated that a wide biodiversity occurred among hawthorn germplasms found in Iran. Also in other studies, Torkashvand et al. (2017) and Beigmohamadi and Rahmani, (2011) evaluated genetic variation in hawthorn by SSR and RAPD markers, respectively. However, there aren't any studies on genetic variation of hawthorn in Ardabil area. Therefore, the aim of this study is to evaluate 23 hawthorn genotypes using inter-simple sequence repeat (ISSR) markers.

MATERIALS AND METHODS

plant material

This study was conducted on 23 genotypes of hawthorn from several regions of Ardabil (Fandoghlo, Namin, Khalkhal, Aladizgeh, Germi), Iran in 2017 (Table 1). The Ardabil province has a temperate climate, with almost warm and moist summers and, wet and cold winters.

Table1. Geographical coordinates of the studied areas

Region	Latitude	Longitude	Height (m)	Genotype
Fandoghlo	38° 30' N	47° 59' E	1620	1-5
Namin	38° 27' N	48° 34' E	1536	6-10
Khalkhal	38° 19' N	48° 35' E	1850	11-14
Aladizgeh	37° 35' N	48° 32' E	1337	15-19
Germi	38° 57' N	48° 10' E	1062	20-23

Table 2. Selected primers for ISSR analyses in *Crataegus* spp.

ISSR Markers	Repeat motif	Tm (˚C)	Reference
ISSR 10	5′(GACA)4 3′	48.2	[3]
ISSR 11	5′(GAC)3 ′	51.6	[3]
ISSR 12	5′(GA)6 CC 3′	44	[3]
ISSR 13	5′(CT)8 AC 3′	54	[20]
ISSR 14	5′(ATG)6 3′	47	[20]
ISSR 15	5′(ACTC)4 3′	56	[3]
ISSR 17	5′(CA)8 AG 3′	54	[3]
UBC807	5′(AG)8 T 3′	50	[2]
UBC809	5′(AG)8 G 3′	48	[3]
UBC810	5′ (GA)8 T 3′	50	[3]
UBC814	5′(CT)8 A 3′	50	[3]
UBC817	5′(CA)8 A 3′	50	[19]
UBC823	5′(TC)8 C 3′	52	[2]
UBC825	5′(AC)8 T 3′	51	[3]
UBC860	5′ (TG)8 A3′	50	[3]

DNA extraction

Genomic DNA was extracted from 100 mg of fresh mature leaves from each of the 23 genotypes. The modified CTAB method was used for extraction [8]. The leaves were thoroughly powdered with liquid nitrogen in a pre-sterilized mortar and pestle. After the addition of 1 ml of extraction buffer [100 mM Tris-HCl, 2 M NaCl, 20 mM EDTA, 2% (w/v) PVP, pH:8], the mixture was homogenized and incubated at 65˚C water bath for 60 min and mix thoroughly by vortexing. The tubes were cooled in room temperature, add an equal volume of 24:1 (v/v) mix of chloroform: isoamyl alcohol and shaken vigorously to form an emulsion. After centrifugation at 11000g for 20 min, the supernatant was separated and transferred to a new tube and mixed with 0.7 (v/v) volumes of cold isopropanol (-20˚C). After that, tubes were centrifuged at 10,000 g for 5 min and the upper, aqueous phase transferred to fresh tubes. The precipitated DNA was washed in 70% (v/v) ethanol and then pellets were dried and dissolved in 0.2 ml of Double-distilled water. Finally, DNA quality was evaluated with 0.8 % Agarose gel electrophoresis.

ISSR fingerprinting

Fifteen ISSR primers were chosen for amplifying the genetic materials of Crataegus (Table 2). Amplified DNA fragments were selected based on the degree of polymorphism, clearness and reproducibility. DNA amplification was carried out in 10 µl reactions. The 10 µl volume PCR reactions contained 20 ng of genomic DNA, 5 µl of a PCR kit (Sigma, St. Louis, MO, USA), 1.1 µl of primer and 2.5 µl of double distilled water. The amplification was performed by using Q-cycler thermocycler (HainLifescience, UK), programmed for 5 min denaturation step at 94 ℃, then 35 cycles, of 45 s at

94 ℃, 30 s at 44-52 ℃ (depending on the primers) and 1 min at 72˚C, followed by 10 min 72˚C. Amplification products were separated in 1.5% (w/v) agarose gel containing 10% (v/v) fluorescent dye in 1×TBE buffer at 85 V for 60 min. The ISSR bands observed under a UV light and photographed with a digital camera. A 100 bp DNA molecular weight marker (New England Bio Labs, USA, CAS number: 15628-02-9) was used for standard sizes.

Data analysis

The observed bands were evaluated based on the present (coded1) or absent (coded 0) for each entry. Very faint and non-reproducible bands were excluded from scoring. The genetic parameters such as; Number of scored band (NSB), Number of polymorphic (NPB), observed number of alleles (Na), effective number of alleles (Ne) [18], Nei's (1987) gene diversity (H), and Shannon's Information index (I) = [14] were calculated by PopGene program version 1.31 [22]. Cluster tree was performed by WARD (minimum spherical cluster) dissimilarity index using Windows (DARwin5) software [19]. For the population structure, a model analysis was fulfilled to infer the genetic structure and to clarify the number of sub-populations using the software Structure (version 2.3.4) [26]. This clustering method is based on an algorithm that confers genotypes to analogous groups, given a number of clusters (K). The number of supposed populations (K) was set from one to ten and most plausible number (K) of subpopulations was identified following Evanno et al. (2005). The run with the maximum likelihood was employed to set genotypes into subpopulations.

RESULTS AND DISCUSSION

The only bright and non-ambiguous amplified products were scored as present (1) or absent (0). In this study, a total of 128 reproducible amplified products were generated (an average of 8.53 bands per primer) of which 113 were polymorphic among the samples (Table 3). The size of DNA bands shown in amplification products ranged from 100-1500 bp. The number of DNA amplified fragment revealed by the ISSR varied from 6 to 14, with a mean value of 7.52 per primer. The results showed that there is a high level of genetic diversity with high polymorphism ratio among the wild hawthorn germplasm in the Ardebil region. Marker UBC823 yielded the largest number of scored bands (15), while UBC809, ISSR 13 and ISSR 14 produced the smallest number [8]. These ISSR primers gave a high Polymorphism information content (PIC) value of 47 for primer ISSR 13 (Fig 1) and low PIC value of 33 for primer UBC814, with an average PIC value of 38.06 per primer. This value is a reflection of allele diversity and frequency among the varieties. There is some investigation on genetic diversity especially ISSR molecular markers, such as; Dai et al. (2008) studied eight species of Crataegus and reported PIC values between 0.08 and 0.94. Erfani-Moghadam et al. (2015) investigated genetic diversity among some hawthorn species in Iran by RAPD marker and published PIC between 0.64 and 0.79. In the present study the mean percent of polymorphism was 60%. The parameters such as PIC have been used for assessing the informative potential of ISSR markers [11].

The PIC value will be zero if there isn't any allelic variation (monomorphic) and it can reach to maximum (or 1.0) if there is an individual new allele, which is a rare phenomenon. The mean value of observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H) and Shannon index (I) were 8.53, 7.52, 0.41 and 0.58 respectively (Table 3).

Cluster analysis

A WARD algorithm, produced by Dice coefficients, revealed the relationships among the all genotypes showed in Fig 2. The dendrogram was grouped into three main clusters. The first cluster 11 genotypes (15, 6, 11, 10, 14, 12, 13, 2, 1, 3, and 4) subdivided into two subgroups. According to Fig 2, at the first group most of the genotypes were belong to Namin, Fandoghlo and Khalkhal regions which were close together, but in long distance from the rest of the genotypes. The group 2 was included three genotypes from Aladizgeh and Namin. The most genotypes of the Aladizgeh region and some genotypes of Namin region were in same cluster.

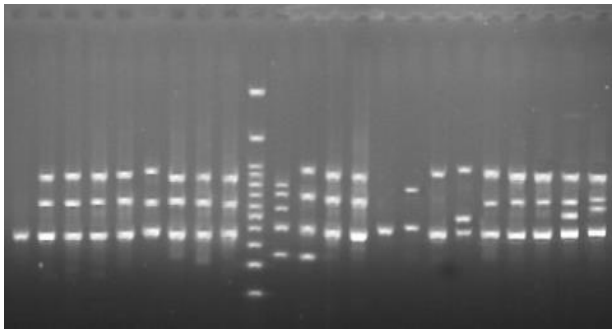

Figure 1. ISSR banding pattern of *Crataegus* genotypes amplified by ISSR.

Table 3. Comparison of informativeness obtained with ISSR markers in *Crataegus* spp.

ISSR Markers	Na	Ne	H	I	PIC
UBC807	8	7.1	0.41	0.6	38
UBC810	8	6.29	0.31	0.47	34
UBC814	10	9.3	0.45	0.64	33
UBC823	**14**	**13.42**	0.46	0.66	36
UBC860	8	7.57	0.46	0.66	39
UBC817	8	7.32	0.44	0.64	41
UBC809	6	4.4	0.29	0.45	41
UBC825	10	9.09	0.44	0.63	35
ISSR 10	8	6.62	0.37	0.55	37
ISSR 11	10	8.47	0.4	0.59	33
ISSR 12	10	8.7	0.4	0.59	35
ISSR 13	6	4.97	**0.63**	0.58	**47**
ISSR 14	6	4.38	0.28	0.45	42
ISSR 15	8	7.42	0.45	0.65	41
ISSR 17	8	7.86	0.48	0.68	39
Mean	8.53	7.52	0.41	0.58	38.06

Na = observed number of alleles, Ne =effective number of alleles, H = gene diversity, I = Shannon's Information index, PIC= polymorphic information content.

This indicates that these genotypes probably transmitted over the years by human. The Group 3 was involved six genotypes followed by 21, 19, 15, 18, 22 and 23. The most of these genotypes belonged to the Germi region, which is distinguished by climate condition (it has a warmer weather) and geography from other regions.

The high differentiation among populations was caused by factors, such as isolation of populations, hybridization, introgression, polyploidy, seed and pollen dispersal systems [4]. Ferrazini et al. (2008) studies on genetic variability using RAPD marker on *C. monogyna* population, which showed high level of diversity. Beigmohamadi et al. (2011) evaluated five species of genus Crataegus using RAPD marker. Dai et al. (2008) used RAPD and ISSR markers to study the genetic diversity of 28 hawthorn accessions. Moreover, Albarouki and Peterson (2007) evaluated the genetic diversity of Cratagus accessions by using plastid DNA (cpDNA) sequences (trnL-trnF, psbAtrnH) and morphological data. In all these studies, molecular markers are appropriate methods to study the genetic relationships among Crataegus species and genotypes. In fact, we used only native genotypes, which are subjected to diverse environmental condition from different parts of the Ardabil region therefore; this high separation ratio would be expected. Finally, according to our data, three clear genetic pools distinguished in Ardabil regions.

Structure analysis

Structural genetic analysis was carried out on 23 hawthorn genotypes by Structure 2.3.4 and Structure Harvester programs on the same data set in order to better detection of population substructures. The results indicated that the Crataegus genotypes had two separate speciation events (The highest Delta K value occurred at DK = 2) (Fig. 3). According to DK = 2, the 1–8 as well as 13-20 genotypes were present in the same group and 9, 10, 11, 12, 21, 22 and 23 were in second group. Depends on the structure analysis, Germi and Khalkhal genotypes had different genetic structures rest of genotypes (Fig. 4). It can be, because of geographical distance of these regions. The distance of Germi (located in the north of Ardabil) to Khalkhal (located in the south of Ardabil) and Ardabil is 110 and 120 km respectively. However, the genetic similarity of the Germi and Khalkhal regions with a distance of 230km is slightly questionable. According to studies Taeb, 1995, origin of this plant is from the Hyrcanian region (north of Iran), so that, it can be argued that the Khalkhal area was first origin and then transferred to Germi region. The genotypes of the Namin, Fandoghlo and Aladizgeh regions, which are adjacent to each other, have similar genetic structure that may be associated with vicinity and cross pollination.

In this work, we examined some of ISSR markers to studies of population Structure, genetic diversity in Hawthorn. This information can be used for pre-breeding

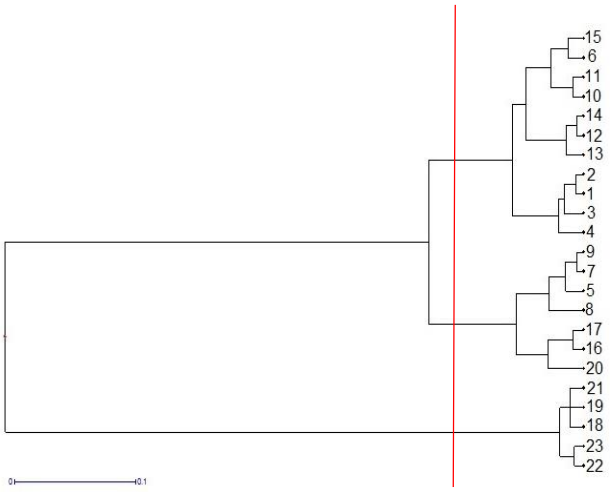

Figure 2. Dendrogram of *Crataegus* spp. genotypes based on Dice's similarity coefficient and WARD method.

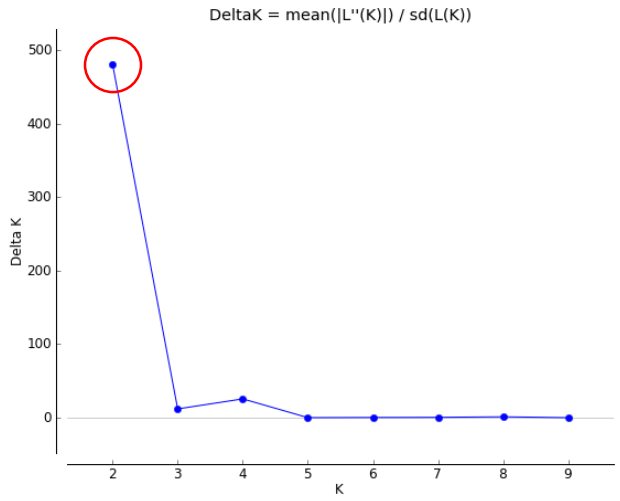

Figure 3. The value of DK estimated for the structure analysis of *Crataegus* spp. genotypes (DK = 2).

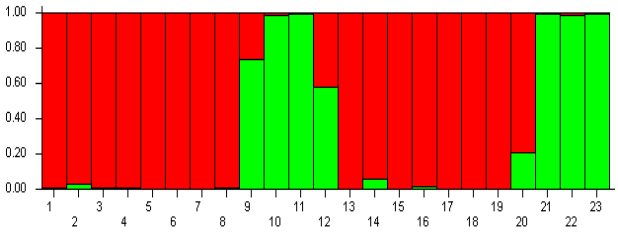

Figure 4. Results of the BAPS analysis of *Crataegus* spp. genotypes, each vertical bar represent one individual genotype.

and breeding programs. The results associated with bands analysis indicate high level of genetic diversity as well as high polymorphism ratio among the wild hawthorn germplasm. According to results of cluster and structure analysis, Germi genotypes were completely isolated from the rest of genotypes due to the climatic conditions and the distance from the other regions. However, according to the results of the Structure, and the initial origin of the species of hawthorn (Hyrcanian region) has revealed, the genotypes have been transmitted from the Khalkhal to Germi, although due to the spatial separation over time,there have been differences in the genetic variation. These genetic differences are especially important in cross-species interactions.

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بررسی تنوع ژنتیکی ژنوتیپ‌های زالزالک در استان اردبیل با نشانگرهای ISSR

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چکیده

جنس *Crataegus* (زالزالک) از خانواده روزاسه می‌باشد. زالزالک می‌تواند به عنوان پایه و یا به عنوان یک گیاه زینتی مورد استفاده قرار گیرد. میوه‌های آن منبعی از مواد مغذی بوده که برای سلامتی انسان مفید می‌باشد. گونه‌های این جنس در مناطق مختلف ایران و بویژه در اردبیل شناسایی شده است. جمع‌آوری و محافظت از مواد گیاهی برای برنامه‌های اصلاحی آینده حائز اهمیت است. به این منظور، ۳۰ ژنوتیپ زالزالک از چندین منطقه (فندقلو، نمین، خلخال، آلادیزگه و گرمی) استان اردبیل جمع‌آوری و تنوع ژنتیکی آنها با ۱۵ نشانگر ISSR بررسی شد. براساس نتایج، همه آغازگرها باند داشتند که در مجموع ۱۲۸ باند (میانگین ۸/۵۳) تولید شد، و همچنین میزان اطلاعات چندشکلی بالایی (میانگین ۳۸/۰۶٪) را نشان دادند. نتایج نشان داد که تنوع ژنتیکی و میزان چندشکلی مطلوبی در بین ژنوتیپ‌های وحشی زالزالک در استان اردبیل وجود دارد. براساس تکنیک WARD، ژنوتیپ‌ها در سه خوشه اصلی با زیرخوشه‌ها قرار گرفتند که نشان از تنوع ژنتیکی مطلوب در این مطالعه داشت. همچنین ژنوتیپ‌های گرمی بطور کامل از بقیه ژنوتیپ‌ها جدا شدند که براساس آنالیز ساختار جمعیت مشابه با ژنوتیپ‌های خلخال بودند.

کلمات کلیدی: زالزالک، نشانگر ISSR، آنالیز خوشه‌ای، آنالیز ساختار