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Genetic diversity among different *Crataegus azarolus* L. populations using RAPD markers in Iran

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Abstract

The genus *Crataegus azarolus* L. is well distributed in Iran as a wild plant, with numerous, inherently variable population and genotypes. In flora of Iran this species is divides into tree independent species, while Christensen believed all of them are varieties of *C. azarolus*. RAPD markers were used to study 12 hawthorn genotypes belonging to *Crataegus aronia, C. pontica, C. assadi* and *C. zarrei*. The four RAPD primers produced polymorphic bands. A phenogram based on average linkage method included four major groups. The lowest genetic variability was observed within C. azarolus var. aronia genotypes. The study demonstrated that RAPD analysis is efficient for genotyping wild-grown hawthorns.

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Introduction

Genetic markers can be defined differences at morphological, biochemical or DNA (molecular) level, which can be used to differentiate individuals or species. Morphological markers are visible plant traits controlled by Mendelian genes which congregate with genes determining the expression of the trait of interest to allow selection for suitable individuals from a population. These may include characters such as flower color, seed shape, and pigmentation. While these markers can be easily monitored across a population through visual screening, they are more prone to environmental effects. Apart from this, these markers are limited in number, sometimes appear late in plant development and may have pleiotropic gene action leading to effects on other genetic markers, or traits of interest.

Biochemical markers detect variation at the gene product level such as changes in proteins and amino acids, and include polymorphic proteins such as isozymes and allozymes. The major limitation with biochemical markers is that they are restricted in number and may be influenced by environmental factors. Apart from this, they are the translated products of genes; their expression is specific to growth stage and tissues of the plant.(Andersen and Lübberstedt,2003)

Crataegus azarolus L. is a species of hawthorn known by the common names azarole, azerole, and Mediterranean medlar. It is native to the Mediterranean and Irano-Turanian regions.It is a common plant there, growing on sites comparable to those the European common hawthorn grows on. In the Persian language is nominated to the Zalzalak. (Reidle, 1969; Khatamsaz, 1991)

C. azarolus is often divided into subspecies or varieties, for example Christensen in his monograph uses four varieties: *C. azarolus* var. *azarolus* has orange fruit.

C. azarolus var. *aronia* L., has yellowish fruit often with some red tinges, *C. azarolus* var. *chlorocarpa* (Moris) K.I.Chr. has yellowish fruit and the last one *C. azarolus* var. *pontica* (K.Koch) K.I.Chr. has yellowish or orange fruit. There is two of them(means *pontica* and *aronia* varieties) in Iran.(Christensen, 1992) Christensen believed that *C. assadi* Khatamsaz is as a variety of *C. azarolus*.(Christensen and Zielinsky,2008; Khatamsaz, 1991) In contrast Sharifnia and her colleagues using ITS marker accepted that *C. assadi* is not equal in point of view taxonomic level with other varieties of *C. azarolus* and is more separate than to other varieties. (Sharifnia *et al.* 2013) Donmez described a new species as *C. zarrei* Donmez from west of Iran. He has written in respect to pyrene number, leaf architecture and other characters, *C. zarrei* is fairly similar to *C. azarolus s. l.*(Donmez, 2009).

Due to discretion differences, twelve populations belonging to four taxa were collected from different regions of Iran. To resolve the relationships among the Iranian taxa of the *Crataegus azarolus* and to clarify phonetic position, Genetic relationships were investigated using RAPD marker.

Material and method

Preparing plant specimens

Plant material of 12 populations were collected from different localities that are desposited in herbarium of Islamic Azad University North Tehran Branch(IAUNT) shown in table 2.

DNA extraction

The total DNA was extracted from young leaves by a method proposed by Shayan Method using MBST kit (Shayan *et al.* 2007).

The MBST DNA extraction kit contains sufficient reagents for 50 µl DNA preparations and components: Lysis buffer, Binding buffer, Proteinase K, Wash buffer, Elotion buffer(Elotion buffer consists of 10 mM Tris-HCL pH 7.4 and 1 mM EDTA pH 8.0) and MBST –column.

Table 1. Localities of 12 populations of *Crataegus*.

Abbreviations:

c 1,c9,c 2=C.azarolus var, pontica, c3,c 4, c6, c7,c8 And c10=C.azarolus var. aronia, c11= C. zarrei, c12& c 5=C. assadii

Taxon	*Locality*
C1	Kerman : Bam, Mohammad abad, 1060 m , Alipour 12222 IAUNT
C2	Esfahan : Najaf abad , 1510 m , Sharifnia & Seyedipour 12223 IAUNT
C3	Azerbaijan : Ghasemloo valey , 1400 m , Sharifnia & Seyedipour 12224 IAUNT
C4	Azerbaijan : 45 Km to Ghasemloo valey Oshnavieh , 1400 m , Sharifnia & Seyedipour 12225 IAUNT
C5	Azerbaijan : Oshnavieh , Mirabad , 1600 m , Sharifnia & Seyedipour 12226 IAUNT
C6	Ghazvin : Taleghan road , Khuznan village , 1200 m , Sharifnia & Seyedipour 12227 IAUNT
C7	Kurdestan : Sardasht to Baneh , 1610 m , Sharifnia &Seyedipour 12228 IAUNT
C8	kurdestan : Sardasht to Baneh , Bariso , 1610 m , Sharifnia & Seyedipour 12229 IAUNT
C9	Azerbaijan : Piranshahr to Sardasht , 1120 m , Sharifnia & Seyedipour 12230 IAUNT
C10	Ghazvin : Taleghan road – River margin , 1200m , Sharifnia & Seyedipour 12231 IAUNT
C11	Kermanshah : 2 Km to paveh , 1100 m , Ebrahimi 12232 IAUNT
C12	Khorasan : Bojnord , Badranlo Pass , 1200 m , Behrozmand 12233 IAUNT

For extraction, briefly, the leaves were first hemogenized in 300 µl lysis buffer and the proteins were degraded with 20 µl Proteinase K for 45 min at 60°c. After addition of 580µl binding buffer and incubation for 15 min at 70°c, 440 µl ethanol (%100) was added to the solution.

The complete volume was transferred to the MBST-column. The MBST-column was first centrifuged and washed twice with 500 µl Wash buffer. Finally, DNA was eluted from the carrier with Elution buffer. The quality of DNA was determined using agarose gel (1%) electrophoresis.

RAPD amplification

Amplification of RAPD fragments was performed according to Jayoti method.

A total of 4 different random 10-mer primers were used for RAPD analysis. The sequences of the selected primers are presented in Table 2. Polymerase chain reaction (PCR) was conducted with 50 µl reactions containing 6 µl genomic DNA, 10 µl primer, 9 µl H2O and 25 µl Ampliqon Master Mix (including Tris-HCL pH 8.5, (NH4)2SO4, 2 mM MgCl2, 0.2% tween 20, 0.4 mM dNTPs, 0.2 units/µl Ampliqon Tag DNA polymerase). The PCR reactions was carried out in a thermo cycler (Perkin Elmer, Massachusetts, USA) with following conditions, i.e. denaturation at 97°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 2 min. A final extension step was carried at 72 °C for 4 min. (Jayoti *et al*, 2010).

Table 2. The sequence of four primers

RAPD Perimers:		GC content(%)
OPB-10	5'-CTGCTGGGAC-3'	70
OPE-08	5'-TCACCACGGT-3'	60
OPM-17	5'-TCAGTCCGGG-3'	70
OPO-15	5'-TGGCGTCCTT-3'	60

Detection of PCR products

The amplified products were detected using agarose gel electrophoresis (1% gel in 1X TAE buffer), stained with ethidium bromide and visualized with UV transilluminator and photographed using gel documentation system.

Data analysis

The RAPD bands were scored as "1" for presence and "0" for absence across all *Crataegus* populations for each primer. The data was analyzed for genetic diversity with the help software SPSS version 20 and genetic distances were calculated on the basis of average linkage method Coefficient (Fig. 1)

Results

Random amplified polymorphic DNA (RAPD) technology via the polymerase chain reaction (PCR) has fast become a means of investigating genetic diversity within and between populations and has been applied to many plant species. The genetic structure of *Crataegus azarolus* populations reflects the interactions of different processes including long-term evolutionary history of the species.

Analysis of RAPD data was shown in Fig. 1. In taxonomic distance of twenty five, the phenogram was divided into two main clusters. In one of them, c12 12 and c5 5 were located. They are actually *C. assadii* and placed in a separate group, which is in accordance with Sharfnia and her colleagues'research. They found that the formers are in higher taxonomic level compared with *C.azarolus* and it may constitute independent group and in contrast of Christensen and Zielinsky believed this species is a variety of *Crataegus azarolus*. (sharifnia *et al.*2013; Christensen and Zielinsky, 2008). While c11 11 is exactly *C. zarrei* and along with *C. azarolus var. pontica* and this taxon can be at maximum as a variety of *C. azarolus*. In phylogenetic analysis of hawthorn species using Internal Transcribed Spacer (ITS), all of them(means *Crataegus azarolus*var. *pontica*, *Crataegus azarolus* var. *aronia* and *Crataegus zarrei*)are located as a polytomy in cladograms which is made using maximum likelyhood and Baysian methods. (Sharifnia *et al* 2013) polytomy of *C.azarolus* var. *aroni* and *C. azarolus* var. *pontica* is not resolved. c2 2, c9 9 and c 1 1 are morphologically shrubs without thorn and belong to *C.azarolus* group. Species called *C.zarrei* by Donmez is without thorn and its habitat is in conformity with *C. azarolus var. pontica*. Finally, c 3 3, c4 4, c10 10, c6 6, and c8 8 are morphologically shrubs with thorn and in a phenogram, they are in same cluster with *C. azarolus var. aronia*.

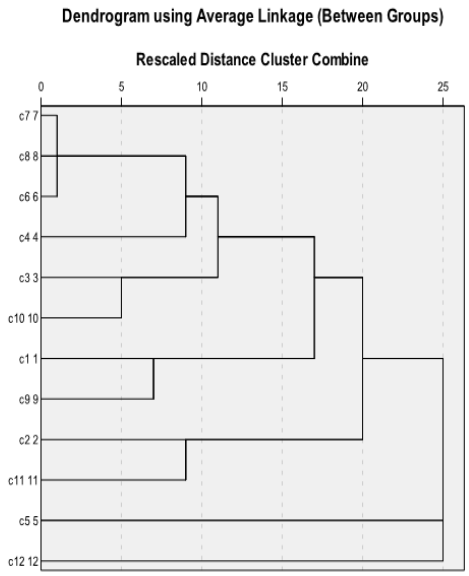

Fig. 1. phenogram of Crataegus populations using RAPD bands.

Abbreviations:c1 1,c9 9,c2 2=C.azarolus var, pontica, c3 3,c4 4, c6 6, c7 7,c8 8

And c 10 10=C.azarolus var. aronia, c11 11= C. zarrei, c12 12& c5 5=C. assadii

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