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Full Length Research Paper

Genetic variation in hawthorn (*Crataegus* spp.) using RAPD markers

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In this study, we report the use of random amplified polymorphic DNA (RAPD) to determine genetic relationships in the genus *Crataegus*. Five species, including *Crataegus monogyna, Crataegus meyeri, Crataegus aronia, Crataegus pentagyna* and *Crataegus pontica* were chosen from northwest forests of Iran and analyzed. Nine RAPD primers reproducibly and strongly generated 101 discrete markers. Seventy six (76) of these were polymorphic (75.25%), with an average of 11.2 markers per primer. Jaccard's distance coefficient based on RAPD markers indicated that the relative genetic distance between species is high, ranging from a minimum of 0.575 between *C. monogyna* and *C. meyeri* to a maximum of 0.728 between *C. aronia* and *C. monogyna*. Dendrogram was constructed based on unweighted pair group method analysis (UPGMA) from RAPD data. The range in similarity coefficient indicated high genetic distance among the species of this genus. However, low variation (0.575 to 0.728) in genetic distance for *Crataegus* in northwest forests of Iran is also evident.

Key words: *Crataegus*, RAPD, genetic variation, Jaccard's coefficient.

INTRODUCTION

Hawthorn (*Crataegus* spp.) ornamentally and medically has a big name in science history. The genus *Crataegus* belongs to the family of *Rosacea* and is a mixture of trees and shrubs, which are located in America, Europe and Asia. They grow up to 5 to 15 m tall with small pomes fruit and small sharp tipped branches, typically 1 to 3 cm long. In most species leaves are either lobed or in serrate margins shape. On long shoots, leaves grow spirally and on spur shoots in clusters. The number of species in the genus varies from hundreds to thousands which is largely dependent on the Taxonomic interpretation (Phipps et al., 2003). Many *Crataegus (*Hawthorn*)* species are polyploids and can reproduce both sexually and apomictically (Lo et al., 2009). Usage spectrum of Hawthorn is very wide and is cultivated as edible fruit. The genus is of great importance in nature, and in the vulnerable ecosystems (Ferrazzini et al., 2008). It is a source of food and shelter for birds and mammals with flowers which are important for Nectar-feeding insects. Many species and hybrids are used for horticultural purposes and street trees (Phipps et al., 2003). Hawthorn has long been used as an herbal medicine to treat heart failure and cardiovascular disease all around the world (Salehi et al., 2009). They are traditionally used for their cardio-protective benefits (Peschel et al., 2008) and anti-oxidative stress potential (Chu et al., 2003). The pharmacological importance of *Crataegus* is attributed mainly to the components such as flavonoids, procyanidin, aromatic acid and cardiotonic amines (Kocyildiz et al., 2006). Lipoproteins oxidation and ischemic-reperfusion damage to the brain (Zhao, 2005) is also inhibited by flavonoid components of the Hawthorn (Zuo et al., 2006). Anti-inflammatory potential (Kao et al., 2005), lowering blood pressure (Belz et al., 2002), total cholesterol and triglyceride levels, cardiotonic, diuretic, vasorelaxant and anti-atherosclerotic properties are also recorded (Salehi et al., 2009). The classification of *Crataegus* is mainly based on traditional methods using morphological

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characteristics, thus, there are considerable differences among plant systematists concerning the existing number of this genus (Dickinson and Campbell, 1991). During the last decade several novel DNA-markers (RAPD, RFLP, SSR, ISSR, etc.) have been rapidly integrated into the tools available for genome analysis. In fact, development of molecular markers has overcome problems associated with morphology-based classification. On the other hand, these markers would be important for quality assessment in the pharmaceutical industry. One of the most widely used DNA markers are RAPDs, generally obtained by the random amplification of DNA sequences using 10- mer primers (Duran and Pérez de la vega, 2004). Among several markers available, random amplified polymorphic DNA (RAPD) (Williams et al., 1990) and inter simple sequence repeats (ISSR) markers are useful for diversity analysis. RAPD has been used extensively for classification of accessions, identification of cultivars and diversity estimation. Though, reproducibility with RAPD markers is somewhat questionable, they are quite useful due to their simplicity, low cost and throughput capabilities (Waugh and Powell, 1992). To our knowledge, no report has been published on the genetic diversity of *Crataegus* based on molecular marker in Iran. In this study, we applied RAPD markers to analyze relationship among 5 *Crataegus* species located in northwest geographical region of Iran.

MATERIALS AND METHODS

Plant material

In this study, 5 Iranian species of *Crataegus* (*Crataegus aronia*, *Crataegus pontica*, *Crataegus meyeri*, *Crataegus monogyna* and *Crataegus pentagyna*) were selected from Northwest forests. Young leaves of *Crataegus* species were collected and stored in plastic bags containing silica gel to avoid DNA degradation. Upon arrival to the laboratory, the samples were snap frozen in liquid nitrogen and stored in -80°C until DNA extraction.

DNA extraction

DNA was extracted from fresh young leaves of five species of *Crataegus* using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990) with slight modification. The quality of genomic DNA was visualized on 0.8% agarose gel and quantified spectrophotometrically (Biophotometer Eppendorf). The purity of DNA was determined by 260/280 ratio. Samples with the ratio of 1.7-1.9 were chosen for PCR amplification.

RAPD amplification

Ten arbitrary RAPD primers were purchased from Cinnagen (Tehran, Iran). The primers are depicted in Table 1. Nine decamer primers were chosen for further analyses because they produced detectable and highly reproducible bands. The OPK-10 primer did not produce clear bands. PCR reactions were performed as follows: DNA samples of the 5 *Crataegus* species, each from 3 individual plants were adjusted to 40 ng/µl and used in the amplification reactions with a final volume of 25 µl containing 1 µl of DNA, 0.5 µl of primer (50 pmol), 12.5 µl of master mix including (dNTPS, PCR buffer, Taq DNA polymerase (5 U/µl), $MgCl_2$) and 11 µl deionized water. PCR reaction was carried out in thermocycler gradiant (Eppendorf). The steps of temperature cycling were as follows: 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 1:30 min at 43°C, 2 min at 72°C and 5 min final extension step at 72°C. PCR products were separated by electrophoresis in 1.5% agarose gel with a 0.5 X TBE buffer system at 70 V for 1 h and stained with ethidium bromide. Fragments were observed and photographed under UV light.

Band profile reproducibility

All PCR experiments were done at least twice and the best gels of the replicates were used for band scoring. Each gel was photo documented using Gel Doc 2000 system. Gene ruler 250 to 10000 bp DNA ladder was used as a reference to allow comparison among the different genotypes.

Data analysis

The gels were scored for computer analysis on the base of the presence or absence of the amplified products. If a product was present in a genotype, it was designated as 1 and if absent, it was designated as 0. This was repeated for each of the nine primers and all of the species. Estimates of genetic similarity were calculated according to Jaccard's coefficient, Sij= (NAB/ (NAB + NA + NB)), where NAB is the number of bands shared by samples, NA represents amplified fragments in sample A and NB represents amplified fragments in sample B. Jaccard's distance was estimated as Dij= 1- Sij (Jaccard, 1908). Cluster analysis was performed by the UPGMA (unweighted pair group method with arithmetic averages) method. Dendrogram was plotted using the MEGA 4 program (Tamura et al., 2007).

RESULTS AND DISCUSSION

In this study, genetic variation was investigated amongfive *Crataegus* species located in northwest of Iran. We used RAPD markers which are quick and simple to perform, enabling genetic diversity analysis in several types of plant material such as natural populations, population in breeding program and germplasm collections (Williams et al., 1990; Carvalho et al., 2004). Although, problems with reproducibility in amplification of RAPD markers and with data scoring have been reported (Penner et al., 1993; Jones et al., 1997), RAPD markers are superior when simplicity and costs were considered (Williams et al., 1990; Carvalho et al., 2004). Thus, they are widely used in genetic distance analysis of different plant species (Sharma and Mahapatra, 1995; Colombo et al., 2000). Genetic distance (D) is a measure of genetic divergence. Therefore, with no genetic divergence, D will be zero. As the taxonomic groups become more and more inclusive, the genetic distance increases. Using 9 selected RAPD primers (Table 2), a total of 101 bands

Table 1. List of the RAPD primers used in the present study.

Primer	Sequence	GC content (%)
OPA-01	5'-CAGGCCCTTC-3'	70
OPA-02	5'-TGCCGAGCTG-3'	70
OPB-10	5'-CTGCTGGGAC-3'	70
OPC-04	5'-CCGCATCTAC-3'	60
OPD-02	5'-GGACCCAACC-3'	70
OPE-08	5'-TCACCACGGT-3'	60
OPI-08	5'-TTTGCCCGGT-3'	60
OPK-10	5'-GTGCAACGTG-3'	60
OPM-17	5'-TCAGTCCGGG-3'	70
OPO-15	5'-TGGCGTCCTT-3'	60

Table 2. Primary analysis of PCR amplification products using selected primers.

Primer	Number of band	Number of polymorphic band	Polymorphism (%)	Molecular weight range(bp)
OPA-01	9	6	66.66	300-1800
OPA-02	11	10	90.90	400-1500
OPB-10	14	12	85.57	300-1500
OPC-04	16	10	62.5	200-1200
OPD-02	9	8	88.88	300-1400
OPE-08	8	6	75	300-2000
OPI-08	6	4	66.66	300-1500
OPM-17	15	11	73.33	200-1500
OPO-15	13	9	69.23	300-1500

were generated (an average of 11.2 bands per primer) of which 76 bands were polymorphic (75.25%). Number of bands varied from 6 (OPI-08) to 16 (OPC-04). The size of the amplified fragments varied from 200 to 2000 bp. Percentage of polymorphism ranged from 62.5 to 90.9% (Table 2). Estimates of genetic relationship were obtained from Jaccard's distance coefficient data. Pair wise comparison indicated that the relative genetic distance between species is high, ranging from a minimum 0.575 between *C. monogyna* and *C. meyeri* to a maximum 0.728 between *C. aronia* and *C. monogyna*. Figure 1, is the representative of the polymorphism extent in 5 *Crataegus* species revealed by primers OPB- 10, OPM-17, OPO-15 and OPE-08. The genetic distance derived from RAPD data are presented in Table 3. The phylo-genetic tree comprising a total of 5 Hawthorn species was constructed as shown in Figure 2. The 5 species of *Crataegus* could be grouped into two clusters. Cluster I comprised two species *C. pentagyna* and *C. monogyna* and *C. meyeri*. *C. aronia* and *C. pontica* are grouped as cluster II. The reliability of RAPD markers in genetic diversity of Hawthorn had recently been shown (Dai et al., 2009). All species of *Crataegus* possess the base chromosome number of 17 and the classification of the genus is mainly based on morphological characteristics (Campbell et al., 1991, 1995; Evans and Campbell, 2002). Therefore, the systematic study of the *Crataegus* is still in question, due to the fact that hybridization, introgression, polyploidy and apomixes may occur in this genus (Dickinson and Campbell, 1991). In principle, the genetic structure of natural populations is strongly affected by both intrinsic factors, such as migratory capabilities and mating systems and extrinsic factors including the ecological characteristics of their habitats and historical events (Xiao et al., 2004). Studies on genetic variability of *European C. monogyna* and *C. laevigata* showed low level of diversity based on chloroplast DNA markers, although, the *Crataegus* plants were sampled from seven European countries including, UK, Sweden, Spain, France, Germany, Greek and Italy (Fineschi et al., 2005). Studies on genetic variability of *C. monogyna* population located in northern Italy based on RAPD showed high level of diversity (Ferrazini et al., 2008). Our results exhibited low variance in genetic dis-

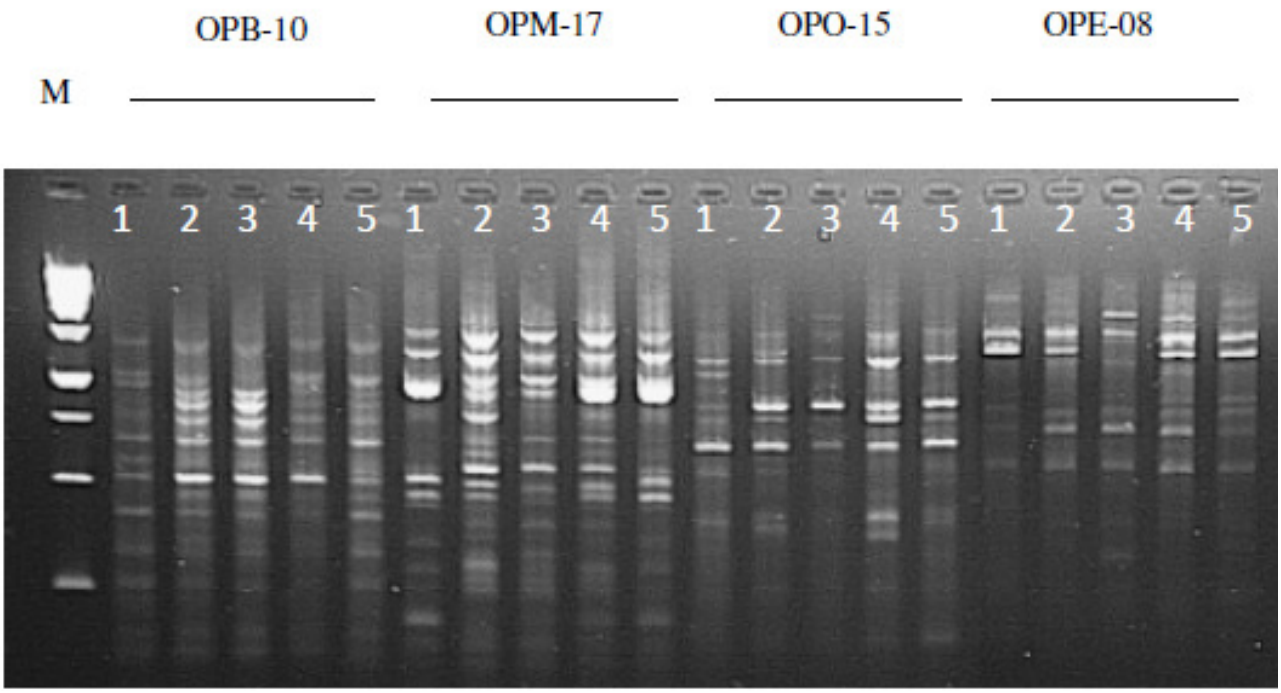

Figure 1. RAPD DNA profile among five *Crataegus* species using OPB-10, OPM-17, OPO-15 and OPE-08 primers.1-5, Species: *C. aronia, C. pontica*, *C. meyeri, C. monogyna, C. pentagyna*. M; DNA ladder.

Table 3. Distance matrix values on RAPD data between 5 *Crataegus* species.

Parameter	*C. aronia*	*C. pontica*	*C. meyeri*	*C. monogyna*	*C. pentagyna*
C. aronia	0.000				
C. pontica	0.630	0.000			
C. meyeri	0.720	0.643	0.000		
C. monogyna	0.728	0.651	0.575	0.000	
C. pentagyna	0.700	0.681	0.607	0.599	0.000

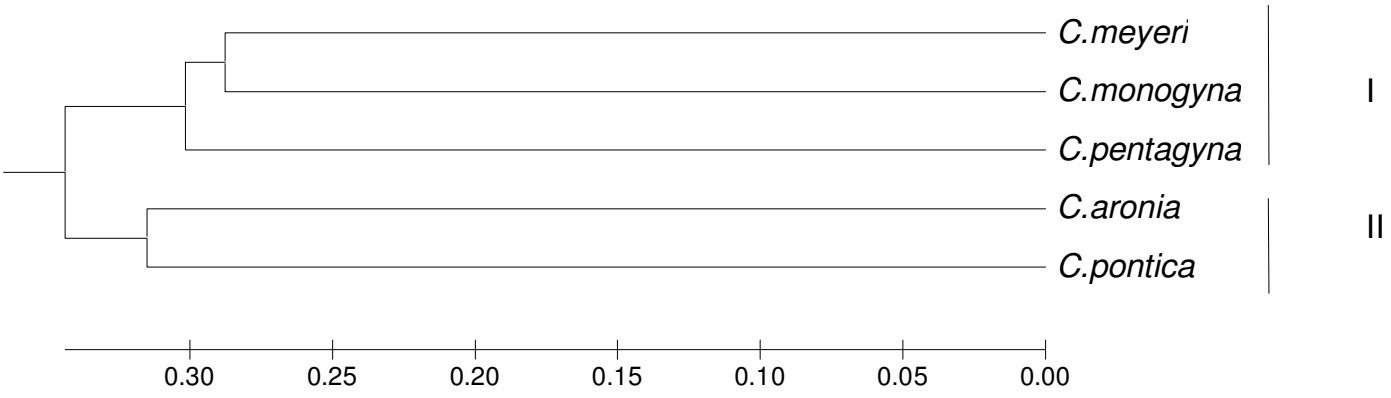

Figure 2. Dendrogram derived from UPGMA cluster analysis using Jaccard's coefficient of RAPD markers.

tance among *Crataegus* species of northwest Iran (0.575 to 0.728). The high value for genetic distance reflects high level of polymorphism at the DNA level in those species. Since no report has been recorded on *Crataegus* in Iran, we hope the result of this study can provide information about the genetic variability of *Crataegus* in northwest forests of Iran. This study will also be a starting point for future investigation aimed at defining the level of intra- and inter-specific genetic diversity, distinguishing hybrids among species and to preserve the diversity of the species.

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