Original Article:
Study of Genetic Diversity Among *Crataegus* Species (Hawthorn) Using ISSR Markers in Northwestern of Iran

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Article info:
Received: 04 Mar 2020
Accepted: 09 Nov 2020

Keywords:
Genetic variation, Hawthorn, Medicinal plants, Pharmaceutical activity, Polymorphism

ABSTRACT

Background: *Crataegus* spp. (Hawthorn) are used for horticultural and medicinal purposes.

Objectives: In the present study, the genetic relationships among the five Iranian *Crataegus* species were evaluated by Inter Simple Sequence Repeats (ISSR) molecular marker.

Methods: PCR reactions were performed using six primers (811, 827, 834, 845, 856, and 868) and genetic similarity was calculated based on Jacquard's similarity coefficient.

Results: Six primers generated 79 products in total, of which 71 were polymorphic (89.9%), with an average of 13.1 bands per primer. The percentage of polymorphic bands ranged from 77 to 100. Primer 856 produced the highest number of bands, while the lowest was generated by primer 845. The Jacquard's similarity coefficient, derived from ISSR marker analysis, ranged from 0.164 to 0.337, indicating high genetic variation among *Crataegus* species in Iran.

Conclusion: This study provides important data for identifying species relationships and helps develop plant breeding strategies to improve the medicinal properties of this genus in the future.

Introduction

The research on natural plant compounds has demonstrated their pharmaceutical properties of [1]. Hawthorn (*Crataegus* spp.) belongs to the Maloideae subfamily and is one of the largest genera in the predominantly woody Rosacea [2, 3]. The species is one of the most important edible and popular medicinal plants, with approximately 280 species present in Europe, North Africa, West Asia, and North America [4].

Hawthorn has a long history in treating various ailments such as cardiovascular disorders, immune system, eyes, central nervous system, reproductive system, liver, and kidney. The species exhibits biological and pharmaceutical activities in the wide range of cytotoxic, gastroprotective, anti-inflammatory, anti-HIV, antimicrobial, antitumor, antispasmodic, anti-atherosclerotic, anti-atherosclerotic and anti-atheromatous, anti-atheromatous. It has cardiotonic, coronarodilatating, and diuretic properties and used to treat cancer, diabetes, cough, flu, asthma, hypotensive, stomach ache, rheumatic pain, nephritis, and hemorrhoids [4-9].

Citation Beigmohamadi M, Rahmani F, Mirzaei L. Study of Genetic Diversity Among Crataegus Species (Hawthorn) Using ISSR Markers in Northwestern of Iran. Pharmaceutical and Biomedical Research. 2021; 7(1):55-62. http://dx.doi.org/10.18502/pbr.v7i1.7357

doi: http://dx.doi.org/10.18502/pbr.v7i1.7357

The bioflavonoids (oligomeric procyanidins, vitexin, quercetin, and hyperoside), flavonoid, phenolic compounds, polyphenols, polysaccharides, triterpenes, cyanogenetics, glycosides, catecholamines, saponins, antioxidants, phytosterols, vitamins, tannins, fats, and fixed oils have been reported as the main active phytochemicals of the *Crataegus* species [5, 10-13].

Leaves, flowers, and fruits of hawthorn could be an excellent source of antioxidants, namely, hyperoside, isoquercetin, epicatechin, chlorogenic acid, quercetin, rutin, and protocatechuic acid [14-20]. Antioxidant activity has already been reported for *Crataegus* species (*C. pentagyna*, *C. aronia*, *C. monogyna*, *C. meyeri*, *C. pontica*) [21]. However, few studies have been carried out on the genetic diversity of this genus in Iran.

DNA markers are the best estimate to evaluate genetic variation due to their independent effects on environmental factors [22]. Molecular markers have different principles, characteristics, critical points during assay procedure, advantages, and disadvantages [23]. In the eukaryotic genome, the target sequences of ISSR molecular markers are abundant, revealing many polymorphic loci compared to other dominant markers. ISSR markers are composed of di-, tri-, tetra-, or pentanucleotide repetitions, with or without a one-to-three nucleotide anchor targeting the microsatellite region of the genome. The marker is attractive because it does not require previous genomic sequencing [24].

The present study aimed to differentiate and determine molecular relationships among five *Crataegus* species employing ISSR markers. The selected species have common medicinal properties and accessibility in the Northwest of Iran. They are *C. monogyna*, *C. meyeri*, *C. pentagyna*, *C. pontica*, and *C. aronia*. This paper reports on developing a fingerprint key for *Crataegus*.

Materials and Methods

Plant material

Young leaves of five Iranian *Crataegus* species were randomly collected from the Northwest of Iran and stored in zip-lock plastic bags with silica gel. Upon arrival to the laboratory, species were morphologically identified by a systematic specialist at the Herbarium laboratory of Urmia University as *C. monogyna*, *C. meyeri*, *C. aronia*, *C. pentagyna*, and *C. pontica*. Leaf tissues were transferred to liquid nitrogen and then stored at -80°C until DNA isolation.

Genomic DNA extraction

Genomic DNA was extracted from fresh leaves tissue according to the Cetyltrimethylammonium Bromide (CTAB) method [25]. The quality of DNA was assessed using 0.8% agarose gel electrophoresis. The extracted DNA was quantified spectrophotometrically by BioPhotometer (Eppendorf, Germany), and samples yielding good quality (A_{260}/A_{280} ratio 1.7–1.9) were chosen for subsequent PCR reactions.

Table 1. List of ISSR primers used in this research

Primer Name	Sequence (5'-3')	GC Content (%)
811	$(GA)_8C$	52
814	$(CT)_8A$	47
827	$(AC)_8G$	52
834	$(AG)_8CTT$	47
845	$(CT)_8AGG$	52
846	$(CA)_8AGT$	47
848	$(CA)_8AGC$	52
856	$(AC)_8CTA$	47
858	$(TG)_8AGT$	47
868	$CGTAGTCGT(CA)_7$	52

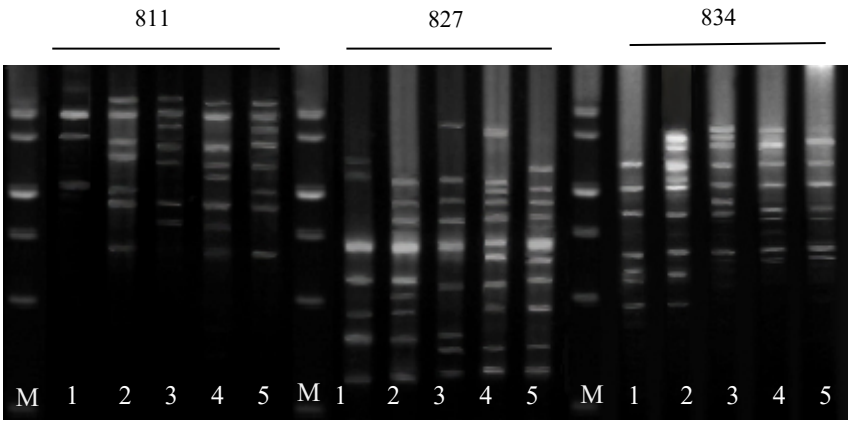

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Figure 1. ISSR DNA profile using 827 and 834 primers. 1: *C. aronia*, 2: *C. pontica*, 3: *C. meyeri*, 4: *C. monogyna*, 5: *C. pentagyna* M; DNA ladder

ISSR analysis

Ten arbitrary ISSR primers (Cinnagen, Tehran) were tested (Table 1). After optimizing different concentrations of genomic DNA and primers, six primers (811, 827,834, 845, 856, and 868) were finally chosen for further analyses due to the generation of detectable and highly reproducible bands (Table 2). PCR reactions were carried out in total volume of 25 µL containing 1 µL of DNA (25 ng/µL), 0.4 µL of primer (100 µM), 12.5 µL of Master Mix (Cinnagen, Tehran), including dNTPs, PCR buffer, $MgCl_2$, *Taq* DNA polymerase (5 U/µL) and 11.1 µL deionized water. DNA amplification was performed in Mastercycler Gradient (Eppendorf, Germany) using the cycling parameters. The amplification conditions for ISSR started with the initial step of 3 min at 94°C followed by 35 cycles of 45 s denaturation at 94°C, 2 min annealing at 50°C (Table 1), 2 min extension at 72°C, and a final extension at 72°C for 10 min. PCR products were separated on 1.5% agarose gel in 0.5 ×TBE buffer running at 70 V for 1 h. After separation, the gel was viewed under a UV transilluminator and photographed with the help of a gel documentation system (Gel Doc 2000).

Scoring and data analysis

PCR reactions for each ISSR primer were performed twice to make sure reproducibility. After observing and comparing the bands across two PCR amplification replicates for each primer, the bands were regarded as reproducible and scorable and used in the subsequent analyses. Clear and intense bands were scored. The band profiles of each primer were scored in a binary pattern (presence [1]/absence [0]) of co-migrating fragments for all species.

The genetic similarity between the two samples is calculated according to the following formula; $Sij= N_{AB}/(N_{AB} + N_A + N_B)$, where N_{AB} is the number of bands present in both samples (A and B), N_A represents amplified fragments in sample A, and N_B represents fragments in

Table 2. Primary analyses of PCR amplification products

Primer	Scorable Bands	Polymorphic Bands	Polymorphism (%)	Product Size Range (bp)
811	10	8	80	700-3000
827	16	16	100	100-1750
834	14	11	78.5	100-1800
845	9	7	77	250-700
856	17	17	100	200-2000
868	13	12	92	200-1250
Total	79	71	89.9	

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Table 3. Jacquard's genetic similarity values based on ISSR data among 5 Crataegus species

Species	*C. aronia*	*C. pontica*	*C. meyeri*	*C. monogyna*	*C. pentagyna*
C. aronia	1.000				
C. pontica	0.337	1.000			
C. meyeri	0.181	0.232	1.000		
C. monogyna	0.166	0.258	0.323	1.000	
C. pentagyna	0.164	0.250	0.301	0.334	1.000

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sample B [26]. The cluster analysis was performed based on the Jacquard coefficient and the UPGMA (Un-weighted Pair Group Method with Arithmetic Average) method and then plotted in dendrogram form. Data analysis was carried using NTSYS-pc version 2.11 software [27].

Results

The ISSR oligonucleotides were used for amplification of all 5 *Crataegus* species. Six out of ten primers (811, 827, 834, 845, 856, and 868) gave rise to reproducible amplified products, while the four others (814, 846, 848, and 858) did not produce any fragment. All of the primers were 3′-Anchored. Six primers produced 79 bands across 5 species on average, of which 71 bands were polymorphic. The number of bands varied from 9 (845) to 17 (856). The scored fragments size ranged from 100 to 3000 bp, and polymorphism percentage varied from 77% to 100%. Figure 1 shows the extent of polymorphism in five *Crataegus* species using 811, 827, and 834 primers. The most informative primer was 856, which amplified 17 polymorphic products (Table 2). The lowest genetic similarity (0.164) was obtained between *C. aronia* and *C. pentagyna*, and the highest value (0.337) was achieved between *C. aronia* and *C. pontica* (Table 3). The phylogenetic tree of 5 Hawthorn species was shown in Figure 2. Dendrogram indicated that the five species could be grouped into two clusters. The first cluster included *C. aronia* and *C. pontica*, and the second cluster consisted of *C. monogyna*, *C. pentagyna*, and *C. meyeri*.

Discussion

The classification of the *Crataegus* genus using morphological traits is still in question by systematic specialists due to the hybridization, introgression, polyploidy, and apomixes [28], especially because *Crataegus* species

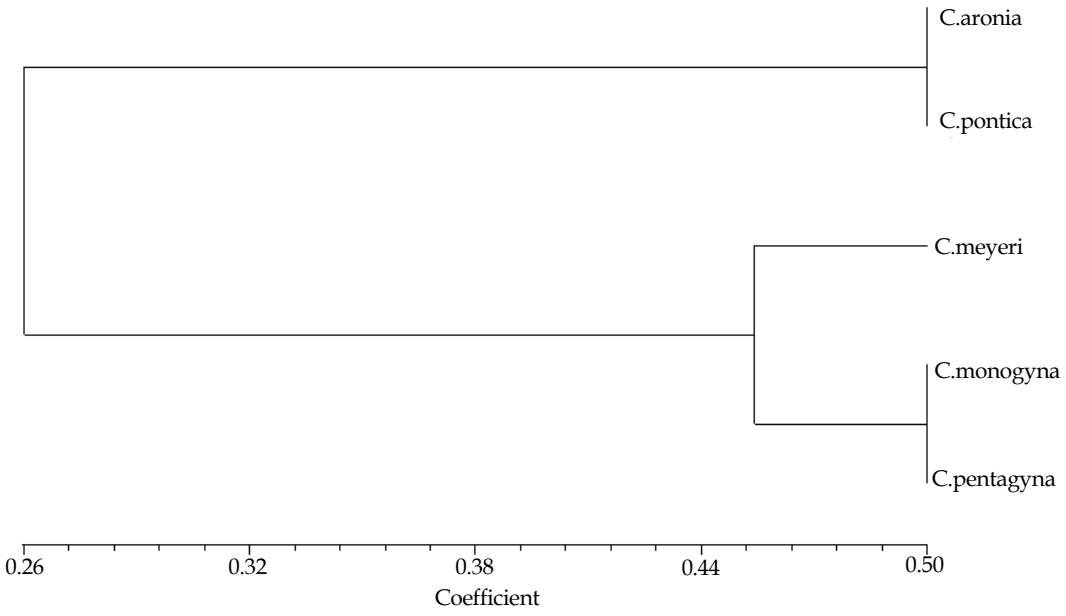

Figure 2. Dendrogram obtained from cluster analysis of five Crataegus species based on ISSR data

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possess the base chromosome number of n=17 [29]. It is essential to investigate genetic diversity, and the relationship between *Crataegus* species [30, 31] since genetic variation studies provide essential preliminary information for identifying relationships between genotypes and help developing plant breeding strategies in the future.

DNA markers are ideal for genetic relationship investigation because they are not affected by environmental factors or plant developmental stages [32]. ISSR markers are reliable, reproducible, polymorphic, inexpensive, easy to perform, and do not require prior DNA sequence knowledge. They need little DNA and have been used to evaluate genetic variation among closely related populations [33, 34]. Considering these advantages, this molecular marker system was employed to study genetic distance among *Crataegus* species. Although it is challenging to identify *Crataegus* species morphologically, our results demonstrated that ISSR primers could distinguish the *Crataegus* species.

The existence of 0.164 to 0.337 genetic similarity represents a very high genetic distance among this species in Iran. Interestingly, our present data support our previous clustering results based on Random Amplified Polymorphic DNA (RAPD) analysis and indicates the capability of both markers in identifying the genetic structure of *Crataegus* [35].

Few investigations have so far been conducted on the molecular characterization of *Crataegus* species. Investigations on genetic variability of the *C. monogyna* population revealed a high level of diversity in northern Italy using RAPD [36]. On the other hand, Fineschi et al. detected a low level of genetic diversity for *C. monogyna* and *C. laevigata* based on chloroplast DNA markers [37]. Beigmohamadi et al. [35] and Sheng et al. [38] reported 93.37% and 98.35% polymorphism among *Crataegus pontica* and *Crataegus songarica* genotypes, respectively, showing a high genetic diversity [38]. The presence of 0.38 to 1.00 genetic similarity was observed in the characterization of 91 hawthorn accessions using SSR primers by Güney et al. [39].

Our results show a low variance (0.663 to 0.836) in the genetic distance among *Crataegus* species of Northwest in Iran. However, the amount of polymorphism (89.9%) indicates the efficiency of the ISSR marker to investigate genetic variation in this species. Furthermore, the high value of genetic distance indicates a high level of polymorphism at the DNA level.

Conclusion

The result of the present study can be used to improve the medicinal properties of this genus.

Ethical Considerations

Compliance with ethical guidelines

There are no ethical considerations to be considered in this research.

Funding

The paper was extracted from the MSc. thesis of the first author at the Department of Biology, Faculty of Sciences, Urmia University.

Authors' contributions

Conceptualization, supervision, and methodology: Fatemeh Rahmani; Investigation, writing – original draft: Mina Beimohamadi; Review & editing: Fatemeh Rahmani; Data collection and data analysis: Mina Beigmohamadi and Leila Mirzaei.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

The authors would like to thank Dr. Mohammad Ali Rajamand for the identification of the specimens. The authors are also grateful to the Head of the Institute of Biotechnology, Urmia University, for providing laboratory facilities.

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