Research Article

Investigation of Genetic Variation In *Berberis Vulgaris* Using ISSR and SSR Molecular Markers

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Abstract

Barberry fruit is a medicinal plant, and it is one of the most important horticultural crops in South Khorasan province, Iran. Genetic diversity has a basic role in the successful breeding of crop varieties with durable resistance to biotic and abiotic stresses. The main objective of this study was to assess the genetic diversity of 20 ecotypes of *Berberis*, which collected from different regions of South Khorasan province, Iran, using ISSR and SSR markers. In this study, 10 ISSR primers and 5 SSR primer pairs, with the amplification of suitable polymorphic alleles were used. A total of 98 bands for ISSR markers and 43 bands for SSR markers were detected between 300 to 1300 bp and 100 to 1100 bp in size, respectively. Polymorphic ISSR-7 and CA03 primers amplified the highest number of alleles with 17 and 24 bands, respectively, while ISCS50 and CA30 primers amplified only two polymorphic alleles. The ISCS57 and GA31 primers had the highest polymorphic information content (PIC) and ISCS50 and GA04 primers had the lowest PIC. The estimated Nei's and Shannon indices for genetic diversity in ISSR markers were 0.24 and 0.35, while for SSR markers these were 0.23 and 0.34, respectively. Based on cluster analysis, five and six main groups were identified for ISSR and SSR markers, showing high genetic variations among a set of collected barberry ecotypes. Analysis of molecular variances in both ISSR and SSR markers showed that high level of total variation was due to within populations, rather. Therefore, it will be better to select within populations in breeding programs.

Keywords: *Berberis* spp, Genetic diversity, ISSR, SSR

Introduction

Barberry (*Berberis* L.) is a well-known medicinal plant, which has been used for a long time in Iran and many other ancient civilizations around the world (Heidary et al., 2009). This plant is a deciduous, evergreen and semi-evergreen shrub which grows up to 4 m high and under a wide range of ecological conditions (Bottini et al., 2000; Rezvani Moghaddam and Koocheki, 2007). Barberries are mostly diploid (2n = 2x = 28) and few are tetraploid (Cadic, 1992).

They are self-fertile and mainly autogenous (Rezaei et al., 2011). They are vegetatively and sexually propagated plants (Bottini et al., 2002). *Berberis* is the largest genus in the Berberidaceae family (Kim et al., 2004).

The genus includes about 450-500 species that grows in Asia, Europe and America. *Berberis* is a well-known medicinal plant in Iran (Sodagar et al., 2012; Shamsa et al., 1999) and five species have been reported so far, which include *B. vulgaris* L., *B. orthobotrys* Bien. ex Aitch., *B. crataegina* DC., B. *integerrima* Bunge and *B. khorasanica* Browicz and Ziel. (Alemardan et al., 2013; Rezaei and Balandary, 2015; Tavakoli et al., 2016).

Seedless barberry is one of the few unique crops cultivated only in Iran, especially in South Khorasan province (Heidary et al., 2009). In Iran, this plant occupies approximately 95% of the total cultivated area and production of this plant is located in these regions (Heidary et al., 2009).

Berberis has been reported as a tolerant plant to low temperature, drought, and wind (Varas et al., 2013). Due to salinity stress and water scarcity, most of the agricultural land in South Khorasan are not suitable for the growth of most of the other crops, hence, the seedless barberry has been introduced as a major crop, during the last 20 years. Cultivation and production of seedless barberry took place in Afin village in Ghayenat, Zirkooh district for the first time. Until 50 years ago, seedless barberry was

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mainly cultivated in Afin and Darmian villages, however, ever since seedless barberry has been cultivated in most of the villages and regions (Javadzadeh and Fallah, 2012). Nowadays, there are over 11,000 ha of cultivated areas under seedless barberry, with a production of more than 9200 tons of dried fruit per year (Alemardan et al., 2013).

The knowledge of genetic diversity is an essential tool in gene-bank management and breeding experiments. The assessment of genetic diversity and the characterization of germplasm are prerequisite to improve the chances of selecting better segregants for various characters (Dwevedi and Gaibriyal, 2009).

Determination of genetic diversity can be based on morphological, biochemical and molecular markers (Mohammadi and Prasanna, 2003; Sudre et al., 2007; Goncalves et al., 2009). However, the huge diversity, polyploidy levels, spontaneous mutations and subsequent recombinations make the identification of the species rather difficult and controversial.

The origins and relationship between seedless types have remained unclear so far. Seedless barberry has often been considered as *B. vulgaris* var. asperma in Iran as well as in Europe, but some authors described it as "*B. vulgaris* Asperma" (Hatch, 2007; Azadi, 2009).

However, recent studies indicated that Iranian seedless cultivar belongs to B. integerrima (Rezaei et al., 2011; Alemardan et al., 2013). Assessment of the relationships between seeded and seedless barberry and evaluation of genetic diversity will provide useful information to breeders.

So far, all studies have been focused on medicinal properties of barberry and little is known about the genetic diversity of seedless barberry cultivars and its wild-type relatives.

Therefore, the main objective of this research was to study the genetic diversity of barberry ecotypes (seeded and seedless) collected from South Khorasan province using ISSR and SSR markers.

Materials and Methods

Plant Materials

In this study, 20 barberry ecotypes selected from different regions of South Khorasan province, were evaluated genetically, using ISSR and SSR molecular markers.

Among these ecotypes, 11 samples were seeded-barberry with red color berries, three samples were seeded-barberry with black color berries and six were seedless cultivated barberry (Table 1).

Young leaves of selected ecotypes were harvested in pril 2015. The collected samples were kept in ice and transferred to the laboratory snap frozen in liquid nitrogen, and then stored at -20°C until the DNA extraction.

Genomic DNA Extraction

DNA was extracted according to CTAB method (Doyle and Doyle, 1990), with minor modifications. The quantity and quality of the extracted DNA were assessed using Nano Drop 2000 spectrophotometer and agarose gel electrophoresis 0.8%. The extracted DNA was then diluted to 15 ng/µl as working concentration for PCR.

ISSR and SSR Analysis

A total of 10 ISSR primers and 5 primer pairs of SSRs developed by Roß and Durka (2006) were selected and used for PCR amplification of the DNA templates (Table 2).

PCR reactions were performed in a 20 µL volume mixture in a gradient thermal cycler (Master cycler® gradient, Eppendorf, Hamburg, Germany).

The PCR reaction mixture for ISSRs contained 1.5 µL of the 10 µ mol/l primers, 10 µL of PCR ready Master Mix (CinnaGen Co., Iran), 2 µL of template DNA (30 ng), and 6 µL of sterile water.

For SSRs, however, the reaction mixtures included 1 µL of the 10 µmol/l of each forward and reverse primers, 10 µL of PCR ready Master Mix (CinnaGen Co.), 3 µL of template DNA (45 ng), and 5 µL of sterile water.

The following PCR program was used for ISSR marker: 3 min at 94°C; followed by 35 cycles at 94°C for 30s, 43- 61°C (depending on the Tm of the primers) for 45s and 72°C for 1 min; then left at 72°C for 10 min.

For SSR marker, however, the following PCR program was set up; initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 35s, annealing for 60s (optimized temperature between 57°C and 62°C, depending on the primer pairs) and extension at 72°C for 90s with a final extension at 72°C for 10 min.

The PCR products were separated by electrophoresis on 2% agarose gel in 0.5x TBE buffer, stained with ethidium bromide, and subsequently visualized using UV lights. A DNA size marker of 100 bp (DENAzist Asia Co., Iran) was used to estimate the size of different bands amplified for each primer or primer pairs. Amplification experiments repeated twice to confirm the band amplification results.

Table 1. The list of 20 barberry ecotypes evaluated in this study with their location names and fruit and leaf characteristics

Number ecotype	Name ecotype	Location	Color and shape of fruit	Shape and Color of leaf	Fruit image	Leaf image
1	Seeded	Birjand	Round and stretched dark red	Rhombus stretched with short prickles around leaf dark green		
2	Seeded	Ghehardeh	Stretched Dark red	Elliptic without prickles around leaves Light green		
3	Seeded	Razg	Round and stretched light red	Elliptic and flattened with very small prickles around leaves Light green		
4	seedless	Birjand	Round and stretched light red	Rounded and stretched with very small prickles around leaves Light green		
5	black seeded	Behdan	Ovate Black	Elliptic stretched with prickly edge dark green		
6	Seeded	Behdan	Round and large Red	Elliptic and flattened with prickly edge dark green		
7	black seeded	Behdan	Round and stretched Black	Elliptic and flattened, Prickles around leaf to each other over long distances dark green		
8	black seeded	Behdan	Round Black	Rounded and flattened with very small prickles around leaves dark green		
9	Seeded	Behdan	Round Red	Elliptic and flattened with prickly edge Light green		
10	Seeded	Behdan	Round Pink	Elliptic and flattened with prickly edge dark green		
11	Seeded	Behdan	Round and large Dark red	Rounded, Prickles around leaf to each other over long distances dark green		
12	Seeded	Behdan	-	Rounded and flattened with Long prickles around leaves dark green, Around the red leaves	-	
13	Seeded	Behdan	Stretched light red	Elliptic stretched with edge without prickles dark green		
14	seedless	Behdan	Round and stretched light red	Elliptic and flattened with very small prickles around leaves dark green		
15	Seeded	Behdan	Round and Ovate Dark red	Rhombus and flattened with Long prickles around leaves Light green		
16	Seeded	Behdan	-	Rounded and stretched with Long prickles around leaves dark green	-	
17	seedless	Noghab	Round light red	Elliptic and stretched with edge without prickles dark green		
18	seedless	Nozad	Round and large Red	Elliptic and flattened with very small prickles around leaves Light green		
19	seedless	Dorokhsh	Round and large Red	Rhombus and flattened with prickly edge dark green		
20	seedless	Afin	Stretched light red	Elliptic and stretched flattened with prickly edge dark green		

Data Analysis

Each of the DNA fragments amplified by ISSR and SSR primers were scored 1 for presence and 0 for absence of the specific bands. The polymorphic information was calculated for all the primers (Equation 1).

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2 \quad (1)$$

In which, *pi* is the *i*th allele frequency for co-dominant markers and *i*th band frequency for dominant markers (Anderson et al., 1993; Powel et al., 1996).

These similarity coefficients were estimated according to Jacard (J) index (Nei and Li, 1979). Cluster analysis was performed based on the unweighted pair group method with arithmetic averages (UPGMA) algorithm and employing the sequential, agglomerative, hierarchical, and nested clustering (SAHN) using NTSYS PC2.02 program (Rohlf, 1998).

To determine the genetic relationships between the studied ecotypes and to categorize them, a principal coordinate analysis (PCoA) as a complementary method for cluster analysis (Huff *et al*., 1993) was also performed using the same program.

Barberry ecotypes collected were grouped According to the geographical collection site in three populations named as Birjand (including seedless, seeded and black seeded barberry Birjand, Ghehardeh, Razg and Behdan), Ghayenat (including seedless barberry Dorokhsh and Afin) and Darmian (seedless barberry Noghab and Nozad).

Analysis of molecular variance (AMOVA) to determine the amount of genetic variation within and between populations was performed using GenAlEx version 6.1 (Huff et al., 1993; Excoffier et al., 1992). Genetic coefficients and indices such as number of polymorphic loci, percentage of polymorphic loci, observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (*h*), Shannon's Information index (I), heterozygosity within populations (Hs), total heterozygosity between populations (Ht), Gst Factor and Nm of gene flow were analyzed using the POPGENE program version 1.32 (Yeh *et al*., 1997). Differences among populations were quantified using Wright's inbreeding coefficient (Fst).

Fst = 1-HS / HT

Table 2. ISSR and SSR primers used in the present study

Name marker	Primer seq. (5'- 3')	Annealing temperature (°C)
ISCS1	5' - (TCC) $_6$ C - 3'	61
ISCS7	5' - (TC) $_9$C - 3'	48
ISCS50	5' -(CA) $_8$ RC - 3'	47
ISCS57	5' -(GA) $_8$ YG - 3'	39
ISCS80	5' -(TG) $_8$ C - 3'	51
ISSR2	5' -(AG) $_8$ YT -3'	46
ISSR3	5' -(AG) $_8$ T - 3'	45
ISSR4	5' -(GA) $_8$ T - 3'	45
ISSR6	5' -DBD (AC) $_7$ - 3'	43
ISSR7	5' -(AG) $_8$ RC - 3'	49
GA33	F: GAT CAG GTC CAT AAT ATC AAA GTT C (25mer) R: CAG ACA AGG AGA GTG CTT GTA CC (23 mer)	62
CA03	F: GGG GTG TGA CCG TTT TTA TG (20 mer) R: CAA TGC CCG AAA GTT ACG TC (20 mer)	58
CA30	F: TGC ATT TTC GAC CCA TCT AC (20 mer) R: TCT CCT CAC ATG CAA CAA AAG (21 mer)	57
GA04	F: ACC CAT TGG AGC TCT CTC AG (20 mer) R: TTG ATT TTG AAG CCG AGA TG (20 mer)	57
GA31	F: TCA CAA TAG TTT ATT TGA GTT TAT TTG (27 mer) R: CAC TGT CTG GCT CAA TTT TGT C (22 mer)	57

Results

Genetic Parameters Based on ISSR Markers

In this study, 55 ISSR primers were initially screened for DNA amplification of tested barberry ecotypes using a gradient PCR. Among the tested ISSR primers, only 10 primers produced reproducible bands across 20 barberry ecotypes (Figure 1).

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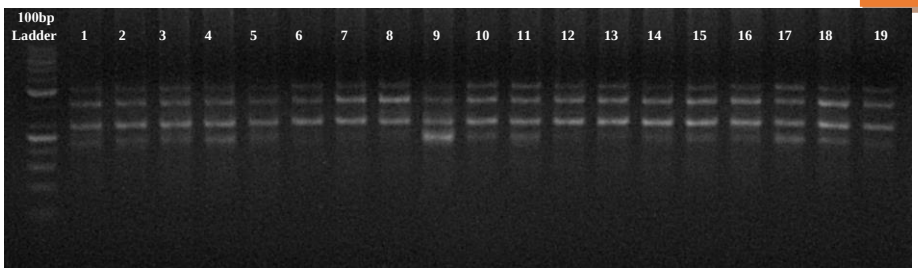

Figure 1. Amplified fragments of ISCS80 for 19 ecotypes

A total of 98 bands were detected using these 10 ISSR markers, out of which 12 were monomorphic and 86 were scorable polymorphic bands (Table 3). The number of amplified fragments varied from 3 (ISCS 50) to 18 (ISSR 4), with an average of 9.8 bands per primer. The maximum number of polymorphic bands (18 bands) were observed for ISSR 4; the average number of polymorphic bands was 8.6 per primer. The size of ISSR bands obtained varied from 30 to 1500 bps (Table 3).

Table 3. ISSR and SSR primers used in the study including number of total bands (NT), size range of amplified fragments (SR), number of polymorphic bands (NP), percentage of polymorphic fragment (PP) and polymorphic information content (PICavg) of 10 ISSR primers and 5 SSR primer pairs.

Name Primer	NT	SR	NP	PP (%)	PIC_{avg}
ISCS50	3	500 - 800	2	66.66	0.56
ISCS57	5	500 - 750	4	80	0.72
ISCS7	11	400 - 900	11	100	0.68
ISCS80	4	500- 800	1	25	0.68
ISCS1	8	400 - 700	8	100	0.71
ISSR2	6	400 - 1000	4	66/66	0.61
ISSR3	13	300 - 1200	10	76.92	0.65
ISSR4	18	350 - 1000	18	100	0.61
ISSR6	13	350 - 1150	11	84.61	0.69
ISSR7	17	400 - 1300	17	100	0.68
Mean	9.8	-	8.6	79.98	0.65
GA33	12	500 – 900	12	100	0.35
CA03	24	300 - 1100	24	100	0.38
CA30	2	100 - 220	2	100	0.4
GA04	3	100 - 300	3	100	0.33
GA31	2	100 - 300	2	100	0.46
Mean	8.6	-	8.6	100	0.38

The average of total heterozygosity between populations, heterozygosity within populations, diversity among populations (Gst), the fixation Index (Fst) and gene flow (Nm) were 0.30, 0.15, 0.96, 0.47 and 5.65, respectively (Table 4). The highest (1.22) and lowest (0.23) total of heterozygosity were observed for ISCS1 and ISCS7 primers, respectively. ISCS50 and ISSR6 primers had the highest (0.21) and lowest (0.07) heterozygosity within populations.

The primer ISCS50 also showed the highest coefficient of variation between populations with 6.34, while the lowest value for coefficient of variation between populations was observed for ISCS1 with 0.1. Gene flow and Fst ranging from 37.69 (ISCS1) to 0.98 (ISCS80) and 0.62 (ISSR6) to 0.13 (ISCS1), respectively.

When individuals in a population are quite similar in terms of allelic frequency, Fst values is equal to 0, when they possess different alleles, this value would be equal to one (Holsinger & Weir, 2009).

One of the important indicators for genetic diversity is the number of alleles detected at each position for the studied individuals (Nevo, 1978). The average number of alleles (Na) and the number of effective alleles (Ne) were 1.37 and 1.19 respectively (Table 4).

The Na value ranged from 1.08 (ISCS80) to 1.62 (ISCS1) and the value for Ne ranged from 1.08 (ISCS80) to 1.32 (ISCS1). The mean Nei's genetic diversity index (H) and Shannon index (I) were 0.24 and 0.35, respectively. The highest Nei's genetic diversity index and Shannon index were observed for ISCS1 primer with the value of 0.19 and 0.30, respectively whereas the lowest Nei's genetic diversity index and Shannon index were observed for ISCS80 primer, with 0.04 and 0.05, respectively.

Genetic parameters based on SSR markers

All five SSR markers showed reproducible bands with three to five amplified fragments in all 20 barberry ecotypes. In total, 43 alleles were identified by five primer pairs (Table 3).

Table 4. Genetic diversity data and differentiation parameters from ISSR and SSR molecular markers for three natural populations of barberry in South Khorasan province. Number of total heterozygosity between populations (Ht), heterozygosity within populations (Hs), Diversity among populations (Gst), Fixation Index (Fst), Gene flow (Nm), observed alleles (Na), Number of effective alleles (Ne), Nei's gene diversity (H), Shannon's information index (I).

Primer	Ht	Hs	Gst	Fst	Nm	Na	Ne	H	I
Mean (ISSR)	0.30	0.15	0.96	0.47	5.65	1.37	1.19	0.24	0.35
Mean (SSR)	0.29	0.23	0.18	0.22	25.15	1.61	1.4	0.23	0.34

Number of alleles ranged from two (CA30 and GA31) to 24 (CA03), with an average of 8.6 alleles per locus. The overall size of amplified products ranged from 100 bp (CA30, GA04 and GA31) to 1100 bp (CA03). The size difference between the smallest and largest allele at a given SSR locus varied from 120 (CA30) to 800 (CA03). Multiple alleles were observed at a rate of 100% in all ecotypes for the SSR markers. PIC values ranged from 0.33 to 0.46, with an average value of 0.38 per locus. The most informative markers were ISCS57 and ISCS1 with PIC values of 0.72 and 0.71, respectively (Table 3).

For SSR primers, the mean of heterozygosity, heterozygosity within populations, coefficient of variation between populations, the Fst and gene flow were 0.29, 0.23, 0.18, 0.22 and 25.15, respectively (Table 4). The highest and lowest total of heterozygosity were observed for GA31 primer with 0.46 and CA03 with 0.2, respectively. GA31 and GA33 primers showed the highest (0.36) and lowest (0.13) heterozygosity within populations, respectively.

The highest coefficient of variation between populations and Fst were observed for GA33 primer with 0.19 and 0.30, respectively and the lowest coefficient of variation between populations and Fst were observed for GA04 primer with 0.02 and 0.03, respectively. The highest gene flow was recorded for GA04 primer with a value 87.98, while the lowest value for this parameter was observed for CA30 primer with a value of 2.34.

The average number of alleles (Na), number of effective alleles (Ne), Nei's genetic diversity index (H) and Shannon index (I) were 1.61, 1.4, 0.23 and 0.34, respectively (Table 4). The highest number of alleles, the number of effective alleles, Nei's genetic diversity index and Shannon index were observed for GA31 primer with 1.83, 1.65, 0.36 and 0.52, respectively, while the lowest number of alleles, the number of effective alleles, Nei's genetic diversity index and Shannon index were detected for GA33 primer with 1.47, 1.22, 0.13 and 0.21, respectively.

Analysis of molecular variance and cluster analysis based on ISSR and SSR markers

Analysis of molecular variance using ISSR and SSR markers showed that, the greatest variation was related to within populations. Variations within populations were 20 and 11 for ISSRs and SSR markers, while the variation between populations were 80 and 89, respectively (Figure 2).

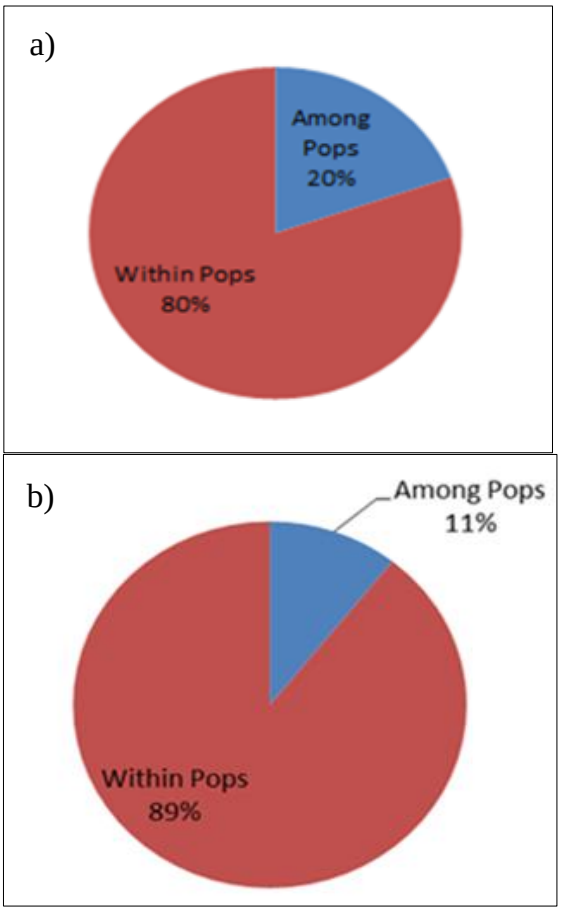

Figure 2. The percentage of molecular variance between and within populations of 20 barberry ecotypes investigated using a) ISSR primers and b) SSR markers

The similarity matrix for ISSR and SSR primers based on the Jacard coefficient for 20 barberry ecotypes were shown in Tables 5 and 6.

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Table 5. Similarity matrix for the 20 barberry ecotypes investigated using ISSR markers

Name genotype	w	W	w	c	wb1	w1	wb2	wb3	w2	w3	w4	w5	w6	C	w7	w8	c	c	c	c
1- w	1																			
2- w	0.56	1																		
3- w	0.62	0.61	1																	
4- c	0.38	0.31	0.46	1																
5- wb1	0.36	0.32	0.39	0.36	1															
6- w1	0.54	0.42	0.41	0.41	0.46	1														
7- wb2	0.60	0.53	0.58	0.40	0.44	0.53	1													
8- wb3	0.45	0.43	0.37	0.29	0.38	0.43	0.56	1												
9- w2	0.62	0.49	0.54	0.43	0.44	0.53	0.69	0.51	1											
10- w3	0.44	0.45	0.56	0.54	0.38	0.37	0.49	0.53	0.56	1										
11- w4	0.40	0.41	0.46	0.44	0.28	0.38	0.40	0.40	0.35	0.51	1									
12- w5	0.52	0.52	0.48	0.35	0.30	0.40	0.50	0.44	0.42	0.47	0.46	1								
13- w6	0.51	0.51	0.56	0.36	0.36	0.39	0.35	0.43	0.35	0.55	0.54	0.43	1							
14- c	0.44	0.42	0.47	0.51	0.37	0.48	0.38	0.40	0.38	0.43	0.51	0.38	0.55	1						
15- w7	0.36	0.35	0.47	0.32	0.43	0.32	0.33	0.33	0.38	0.35	0.35	0.38	0.40	0.28	1					
16- w8	0.37	0.33	0.34	0.24	0.31	0.33	0.42	0.39	0.36	0.33	0.40	0.36	0.31	0.36	0.32	1				
17- c	0.56	0.50	0.64	0.47	0.37	0.39	0.48	0.48	0.49	0.63	0.53	0.52	0.61	0.48	0.42	0.30	1			
18- c	0.40	0.41	0.53	0.46	0.36	0.35	0.41	0.41	0.42	0.44	0.33	0.32	0.41	0.38	0.47	0.20	0.61	1		
19- c	0.28	0.20	0.31	0.19	0.24	0.23	0.30	0.27	0.28	0.32	0.28	0.19	0.26	0.32	0.18	0.31	0.32	0.24	1	
20- c	0.37	0.29	0.33	0.15	0.19	0.21	0.35	0.25	0.25	0.20	0.25	0.34	0.19	0.24	0.25	0.34	0.30	0.17	0.37	1

According to the results of similarity matrices based on ISSR primer, seeded ecotypes of Behdan (w2) and black of Behdan (wb3) had the highest similarity (0.69), but seedless ecotypes of Birjand and Afin showed the lowest (0.15).

The similarity matrices based on SSR primers have also shown that seeded ecotypes of Behdan (w2) and black of Behdan (wb3) had the highest similarity (0.56), while seeded ecotypes of Behdan (w4) and seedless of Afin represented the lowest similarity (0.04) (Tables 5, 6).

Cluster analysis of the studied ecotypes based on UPGMA method for ISSRs, grouped all ecotypes into five clusters (Figure 3). The first cluster (A), included two subgroups. The first subgroup (A1) consisted five seeded ecotypes; including one from Birjand, two seeded ecotypes of Behdan (w1, w2) and two black seeded ecotypes of Behdan (wb1, wb2). The second subgroup (A2) comprised seven ecotypes, including two seeded ecotypes of Chehardeh and Razg, four seeded ecotypes of Behdan (w5, w3, w4, w6) and one seedless ecotype of Noghab.

The second cluster (B) contained only two seedless ecotypes of Birjand and Behdan. In the third cluster (C), one seeded and one black seeded ecotypes from Behdan (w7, wb1) and one seedless ecotype from Nozad were located (Figure 3).

Table 6. Similarity matrix for the 20 barberry ecotypes investigated using SSR markers

Name genotype	w	W	w	c	wb1	w1	wb2	wb3	w2	w3	w4	w5	w6	C	w7	w8	c	c	c	c
1- w	1																			
2- w	0.28	1																		
3- w	0.38	0.48	1																	
4- c	0.25	0.40	0.34	1																
5- wb1	0.15	0.40	0.25	0.44	1															
6- w1	0.25	0.17	0.25	0.34	0.24	1														
7- wb2	0.14	0.25	0.23	0.40	0.35	0.32	1													
8- wb3	0.15	0.31	0.29	0.44	0.38	0.29	0.46	1												
9- w2	0.25	0.26	0.25	0.38	0.38	0.34	0.40	0.56	1											
10- w3	0.27	0.25	0.26	0.41	0.25	0.38	0.44	0.47	0.54	1										
11- w4	0.16	0.27	0.41	0.29	0.25	0.25	0.48	0.52	0.45	0.50	1									
12- w5	0.23	0.25	0.29	0.28	0.18	0.50	0.30	0.39	0.39	0.42	0.34	1								
13- w6	0.21	0.29	0.21	0.26	0.16	0.26	0.40	0.20	0.31	0.42	0.33	0.25	1							
14- c	0.20	0.14	0.16	0.30	0.36	0.47	0.33	0.25	0.30	0.21	0.20	0.44	0.09	1						
15- w7	0.22	0.29	0.33	0.22	0.32	0.16	0.29	0.22	0.26	0.19	0.33	0.31	0.08	0.35	1					
16- w8	0.22	0.25	0.35	0.16	0.12	0.35	0.20	0.07	0.12	0.18	0.17	0.33	0.23	0.22	0.13	1				
17- c	0.26	0.14	0.11	0.11	0.15	0.13	0.14	0.15	0.11	0.16	0.07	0.13	0.09	0.09	0.12	0.15	1			
18- c	0.18	0.21	0.19	0.18	0.33	0.28	0.36	0.23	0.33	0.36	0.29	0.27	0.25	0.23	0.20	0.26	0.23	1		
19- c	0.21	0.14	0.16	0.11	0.16	0.26	0.14	0.16	0.20	0.22	0.07	0.25	0.10	0.21	0.08	0.31	0.53	0.38	1	
20- c	0.28	0.18	0.15	0.14	0.09	0.18	0.08	0.09	0.14	0.15	0.04	0.17	0.06	0.20	0.16	0.23	0.20	0.25	0.21	1

In the cluster analysis based on SSRs, 20 ecotypes were grouped into six clusters (Figure 4). The first cluster (A), included four seeded ecotypes of Birjand, Chehardeh, Razg and Behdan (W7).

The second cluster (B), included two subgroups. The first subgroup (B1) consisted of three black seeded ecotypes from Behdan (Wb1, Wb2, Wb3), three seeded ecotypes from Behdan (W2, W3, W4) and one seedless ecotype from Birjand.

The second subgroup (B2), however, included two ecotypes from Behdan (W1, W5). Other two clusters (C and D) each consisted only one seeded ecotype from Behdan (W6 and W8), respectively.

The fifth cluster (E) further divided into two subgroups. Two seedless ecotypes of Noghab and Dorokhsh were located in the one subgroup (E1), while, the other subgroup (E2) consisted only one seedless ecotype of Nozad. The sixth group (F) had only one seedless ecotype of Afin (Figure 4).

The results of principal coordinate analysis, as a complementary method for cluster analysis, for ISSR and SSR markers were consistent with the results of cluster analysis.In this method, those ecotypes that are located in the same area of two- and three-dimensional plot supposed to have higher genetic similarity.

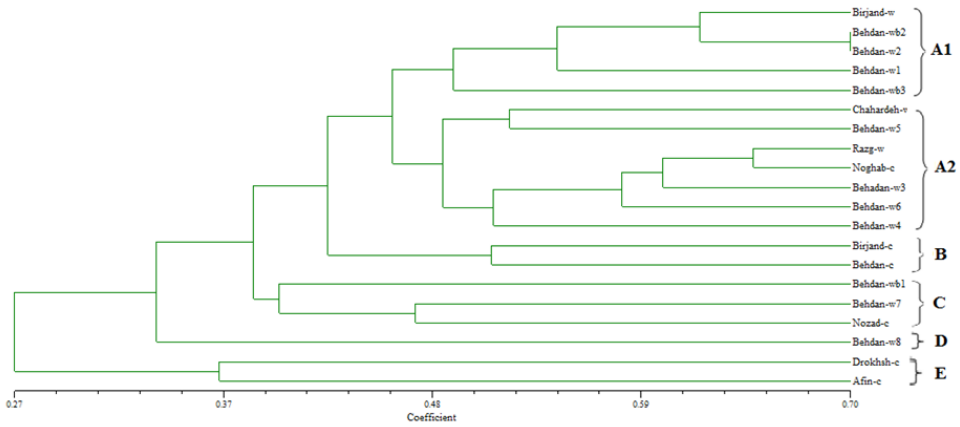

Figure 3. UPGMA clustering of barberry ecotypes based on Jaccard similarity coefficient calculated from ISSR markers

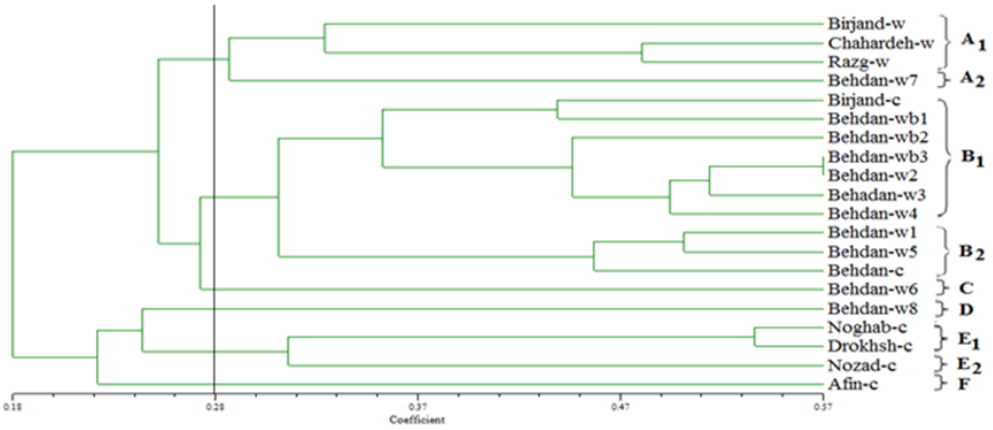

Figure 4. UPGMA clustering of barberry ecotypes based on Jaccard similarity coefficient calculated from SSR markers

Results of principal coordinate analysis based on ISSR and SSR markers were consistent with the cluster analysis result, in which those ecotypes hat were located in a group, came together in two- and three-dimensional distribution diagrams (Figures 5, 6).

Discussion

In this study, the genetic diversity of 20 barberry ecotypes from different regions of South Khorasan province, Iran was evaluated via SSR and ISSR markers. High levels of polymorphism obtained from ISSR and SSR markers representing a great diversity of the studied ecotypes as well as high power of the markers in the molecular diversity analysis.

The average PIC for ISSR marker systems was 0.65 representing the efficiency of this marker in differentiation of barberry populations. Analysis of the molecular variance showed that genetic diversity within populations was higher than between populations. According to the cluster analysis, classification of ecotypes did not follow the geographical diversity. With the closer look at the groups, most of ecotypes which located in a cluster had a higher similarity coefficient. In some cases, however, some ecotypes were grouped together despite the low level of similarity coefficient and high geographical distance, which it can be caused by differences in the quality of DNA affecting banding patterns, the lack of precision in scoring bands, non-uniform distribution of marker in the genome.

Also the results of the similarity matrix, PCoA analysis and cluster analysis showed barberry samples collected from the villages of Afin and Dorokhsh, had the low similarity and high genetic distance with other samples. The lowest percentage of similarity was observed between seedless barberry of Afin with seedless barberry of Birjand. Hybridization between populations with high genetic distance using genetic engineering techniques, protoplast fusion and in vitro fertilization can be an appropriate strategy for

improvement programs between populations of seedless barberry.

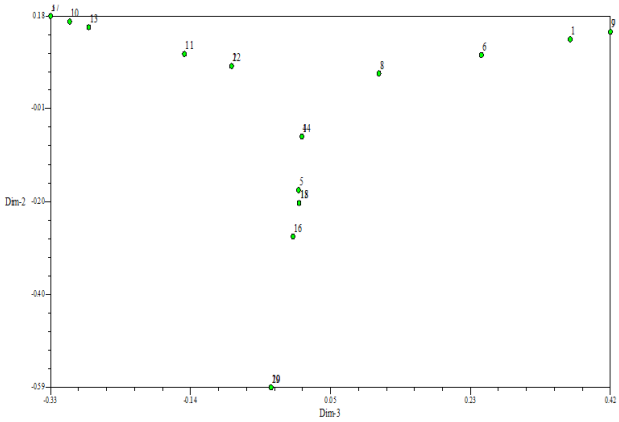

Figure 5. Distribution of the 20 barberry ecotypes revealed by PCoA analysis based on genetic similarity estimates calculated from ISSR data

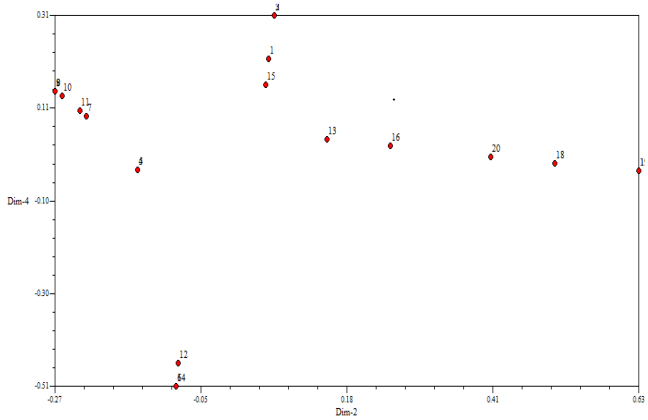

Figure 6. Distribution of the 20 barberry ecotypes revealed by PCoA analysis based on genetic similarity estimates calculated from SSR data

In a study by Bottini et al. (1999; 2000) on the genus Berberis native to Argentina, high morphological variation among Berberis species was observed and great differences in genomic content and ploidy levels were also reported.

Using the AFLP technique, Bottini et al. (2002) evaluated genetic variation of 13 Berberis species and the relationship within diploid and polyploid populations growing in southern Argentina and Chile. In general, results of the cluster analysis showed that populations of the same species were closely related in a group with high coefficients of similarity. In this study, collected ecotypes from South Khorasan province showed a high genetic diversity. All cultivated seedless barberry and wild barberry ecotypes collected from South Khorasan belong to B. integerrima (Rezaei et al., 2011). The seedless cultivars as B. integerrima were classified by both SSR and ISSR data within the wild type barberries. The high percentage of molecular variance within population indicating the genetic diversity between individuals of the same population. Rezaei et al (2011) assumed that self-incompatibility systems, which resulted from the occurrence of spontaneous mutations in B. integerrima, is responsible for the incidence of seedless barberry cultivars. They evaluated the genetic diversity of 45 barberry samples collected from Khorasan region using microsatellite markers. The results of cluster analysis and principal coordinate analysis showed that seedless barberry and species B. khorasanica had high affinity with B. integerrima species.

In agreement with the results of this study, Heidary et al. (2009) showed that the individuals of the seedless barberry cultivar were inserted between the population of B. integerrima based on AFLP markers. They studied the genetic diversity of 33 barberry ecotypes belonging to the 11 regions of Khorasan and two other species of ornamental barberry and one species of Mahonia aquifolium by AFLP marker and they concluded that barberry and Mahonia were located in two separate groups. At 17% similarity level, B. thunbergii was also located in a separate group from other species of the genus Berberis such as species of B. gagnepaini, B. vulgaris and B. integerrima which showed high level of similarities. Based on the results of this study, despite the geographical distances of samples of seedless barberry, they were in close genetic distance to each other.

Conclusion

Results from this study indicate that ISSR and SSR markers used in this study, showed high level of polymorphisms in this the genome studied ecotypes. High levels of polymorphism obtained from microsatellite markers representing a great diversity of the studied ecotypes as well as high power of this markers in molecular diversity analysis. High levels of PIC for ISSR57, ISSR1 and GA31 markers representing its high performance in the differentiation of used genotypes that we suggest them for similar studies. Characterization of the barberry germpalsms and understanding of genetic identity of cultivated and wild species can be used to improve quantity and quality of Berberis crops.

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