J. Agr. Sci. Tech. (2021) Vol. 23(4): 915-928

Evaluation of Genetic Diversity in Blackberry Germplasm in Iran by Using Inter Simple Sequence Repeats (ISSR) Markers

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ABSTRACT

History and background of blackberry cultivars and wild species is unclear in the south coast of the Caspian Sea as an origin of diversity center in Iran. In the present study, genetic diversity of 45 genotypes of blackberries (thorny and thornless) from the collection of Sari Agricultural Sciences and Natural Resources University (SANRU) located in south of the Caspian Sea were studied by using Inter Simple Sequence Repeats (ISSR) markers. Jaccard's similarity coefficient was used to plot the cluster diagram according to the Unweighted Pair-Group Method with Arithmetic averages (UPGMA) algorithm. Results showed that 10 ISSR primers amplified 345 fragments, of which 344 were polymorphic. The average numbers of bands were 34.5 per primers. Based on the Principal Coordinate Analysis (PCA) results, blackberry genotypes were classified in three groups. Some wild genotypes were located closed to commercial thorny cultivars. Cluster analysis divided the genotypes to six groups. Introduced genotypes that were in the same group were separated in sub-groups according to maturity time (early, mid, and late ripening cultivars). These genetic traits separated them and confirmed the morphological results, identifying them as thorny cultivars Silvan, Marion, and Tupi. The results indicated that gene pool of thornless blackberry is not limited to chimera type (as first generation of thornlessness) and it probably includes the two further steps in evolution, and even include some new and evolved types of native thornlessness.

Keywords: Genetic analysis, Principal coordinate analysis, Thornless blackberry, Wild thorny blackberry.

INTRODUCTION

Iran is one of the main areas of plant diversity in the world. It is a desirable wild and domestic genetic resource for plants, especially the Rosaceae family such as apples, pears, almonds, and cherries. Rosaceae family includes Prunoideae, Pomoideae, Rosoideae subfamilies, and also small fruits (*Rubus* and *fragaria*), of which *Rubus* belongs to the genus *Rubus*. The genus *Rubus* contains a range of 750 up to several thousand species. In this genus, there are two *Rubus* and *Idaeus* subgenus (Swanson *et al*., 2011), of which only *Rubus* or blackberry is native to Iran. The fruits of *Rubus* spp. have black color in ripening time and the receptacle is not separated from droplet, which are distinct *R. idaeus* (raspberry) fruits. The ploidy levels of *Rubus* are described from diploid to decaploid with x= 7 (Thompson, 1995). Iranian *R. sanctus* has 14 chromosomes (Kaslakheh *et al*., 2014). There are eight species and five hybrids in Iran, of which seven species grow in the south region of the Caspian Sea (Khatamsaz, 1992). Wild blackberries have a special nutritional and medicinal value in the culture of native

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[DOR: 20.1001.1.16807073.2021.23.4.5.4]

people, based on the long history of usage by native people. Blackberry leaf is rich in tannin and a significant amount of flavonoids, phenolic acids, tryptrips, mineral salts and vitamin C (Ferlemi and Lamari, 2016). Their fruit contains high levels of anthocyanins and oligothans, as well as other phenolic compounds that are involved in high antioxidant capacity (Kaume *et al*., 2012). The production of blackberries in the world dates back to 370 BC, and until the 1800s, it was taken from wildlife. Since then, due to seemingly unpleasant traits such as thorn, low yields, and high growth of wildlife plants, a breeding program has been launched on these species. In 1978, about 12,000 tons were produced commercially. In 2005, the global production of blackberries was 140,000 tons at year, which is still ongoing, and now in Mexico, the thorny Tupi cultivar is cultivated in 11,000 hectares (Strik and Stanton, 2017). In Mazandaran Province, according to the latest official statistics (Agricultural Statistics, 2018), in addition to 2,000 tons blackberry from wild used for processing and consumption inside the province, 100 tons of blackberries are sold annually from commercial orchards, indicating the development of its production. The most genetic variations of blackberry have been reported in this province (Hadadinejad *et al*., 2015; Sedighi and Rahimimalek, 2015).

Although there is no information available on domestication and import of blackberry in Iran, it appears that thorny and thornless cultivars have been introduced to the country along with strawberry in the eighteenth century. These cultivars are not known and the native and the adapted cultivars are not separated from each other because of the long-term effects of environmental conditions, the likelihood of their compatibility, and ambiguity in names. The first extensive research on blackberries in Iran was carried out by Gharaghani *et al*. (2011). They built up a collection of wild blackberry genotypes from north to south of Iran and studied their quantitative and qualitative traits. Then, they indicated four species as the main and valid species, consisting of two species *R. caesius* and *R. anatolicus* with general distribution and two species *R. hyrcanus* and *R. persicus* with limited distribution in the northern wetlands of the country. The diversity of wild blackberries in northern Iran was also investigated by Ataei *et al*. (2015). They reported a high genetic variation within the species in the population, while the genetic diversity was low among the population. In this regard, the genetic variation of *R. hyrcanus* inside-species in north of Iran was performed by Sedighi and Rahimimalek (2015) using morphological and ISSR molecular markers. Based on the results, two groups of blackberry were separated, including samples from the western and eastern parts of the southern strip of the Caspian Sea, and samples related to the center of this area. The greater diversity was observed in the central region, so, the samples from the two eastern and western regions would have likely originated from the central region samples and, therefore, the genetic basis of *R. hyrcanus* was reported fairly narrowly. They examined the diversity of the within and between populations from different regions and concluded that *R. hyrcanus* had not passed the domestication process and had not been under the pressure of selection. As a result, natural populations were preserved and a low genetic diversity was observed among populations. The evidence suggests a phenotypic differentiation (Hadadinejad *et al*., 2015), cultivar development (Effati and Hadadinejad, 2016), and major genetic variations (Hadadinejad and Moradi, 2016) in blackberries. Therefore, in addition to wildlife samples, this study investigated thorny and thornless genotypes of the old introduced, adapted varieties, and samples selected by native people as additional sources of diversity in the regional collection. Therefore, a collection of native wild blackberries of Iran, thorny and thornless plants of introduced old and adapted genotypes, was established in order to know about Iran's position and potential

[DOR: 20.1001.1.16807073.2021.23.4.5.4]

of blackberries and study their genetic diversity. Genetic diversity is a study that classifies a population in comparison with other populations (Abdel-Mawgood, 2012). Genetic diversity and fingerprinting studies are performed by markers (Hadadinejad *et al*. 2011). Among the Polymerase Chain Reaction Marker Techniques, the ISSR is one of the simplest and most widely used techniques. Due to the characteristics of the ISSR, it is widely used for fingerprinting, phylogeny analyzes, population structure, diversity determination, genetic mapping, and so on (Vijayan 2005). This marker has advantages such as proper repeatability, high polymorphism, and easy management (Yang *et al.*, 2016).

Dossett *et al*. (2012) used 21 polymorphic SSR markers to evaluate the genetic variation of 148 wild and domesticated black raspberries (*Rubus occidentalis* L.). Domesticated cultivars showed higher heterozygosity in comparison with wild cultivars. The results showed that wild black raspberry germplasm are pristine and suitable sources for future breeding. Debnath (2007) used ISSR markers and pedigree information to assess genetic diversity and relatedness within raspberry. This study was conducted among nine North American red raspberry cultivars (*Rubus ideatus* L.) and four Canadian breeding lines. Eighteen primers produced 306 polymorphic bands. Cluster analysis showed similarity of 24- 49% among 13 genotypes and 3-25% among 9 genotypes. Innis *et al*. (2011) examined the genetic variation of *R. phoenicolasius* compared to *R. arguta* using the ISSR marker in two habitats in the Maryland coastal forests. They considered the low genetic variation observed among the species as a short history of introducing it to farmers, high rates of self-pollination, and non-sexual propagation.

The purpose of this study was to study the genetic diversity of blackberries genotypes collected from different parts of Mazandaran Province along with some genotypes from other regions of Iran using ISSR molecular marker to identify the genetic relationships of the genotypes.

MATERIALS AND METHODS

The samples were selected from the collection of blackberries, which were 45 genotypes from different geographical regions (Table 1). The collection consists of thorny and thornless genotypes. Thorny genotypes include wild and introduced types. Wild genotypes are primocane and floricane bearing species with different habit of fruiting. Introduced genotypes consist of early-, mid- and late-ripening varieties based on morphological evaluations. Thornless blackberries include five genotypes. Genomic DNA was extracted from young leaves using the modified CTAB method (Murray and Thompson, 1980). The DNA's of samples were diluted after estimating the quality and quantity by 0.8% agarose gel and the absorption ratios of 260/280 nm by spectrophotometer (Biochrome Ltd, Cambridge, England) Ten ISSR primers were selected from 19, which produced more polymorphic bands (Table 2). PCR reactions were performed in BioRad system. The thermal cycle includes an initial denaturation step of 5 minutes at 94°C, followed by 34 cycles of 50 seconds at 94°C, 60 seconds at the annealing temperature (54-5 °C), and 80 seconds at 72°C; with a final extension step of 7 minutes at 7°C. The amplified products were separated by electrophoresis in a 1.8% (w/v) Agarose gel at 80W for 2 hours and 30 minutes in 0.5X TBE buffer, and the gel was imaged by the Gel Doc Analyzer device after staining with Ethidium bromide (Figure1). The amplified bands were scored for the presence (1) or absence (0) of the bands. Jaccard's similarity coefficient was used to plot the cluster diagram according to the unweighted Pair-Group Method with Arithmetic averages (UPGMA) algorithm. The cluster analysis and Principal Coordinate Analysis (PCA) were obtained using NTSYSpc, ver. 2.02. The Polymorphic Information Content

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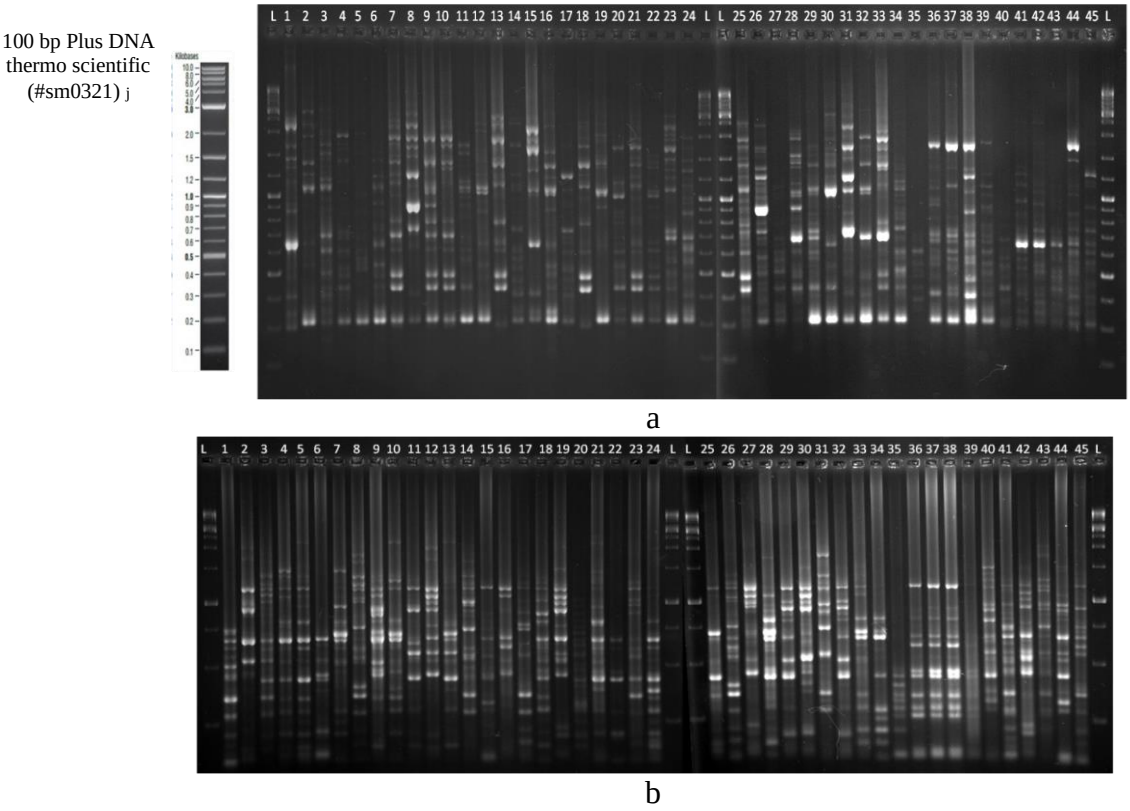

Figure 1. The band pattern of blackberry genotypes created by ISSR 15 (A) and ISSR 1 (B).

Table 1. The origin of blackberry genotypes used in evaluation of molecular marker ISSR**.**

No	Genotype name	Place of origin	Property	No	Genotype name	Place of origin	Property
1	D12	Qaemshahr	Wild thorny	23	B3	Kermanshah	Wild thorny
2	D7	Qaemshahr	Wild thorny	24	C22	Noor	Wild thorny
3	A5	Gilan	Wild thorny	25	D4	Sari	Wild thorny
4	A7	Noor	Wild thorny	26	D1	Sari	Wild thorny
5	C27	Savadkouh	Wild thorny	27	C19	Amol	Wild thorny
6	D11	Sari	Wild thorny	28	B22	Ghaemshahr	Wild thorny
7	D3	Sari	Wild thorny	29	A11	Gorgan	Wild thorny
8	D2	Sari	Wild thorny	30	C4	Sari	Wild thorny
9	B17	Amol	Wild thorny	31	B16	Damavand	Wild thorny
10	C7	Gilan	Wild thorny	32	A12	Gorgan	Wild thorny
11	B20	Noor	Wild thorny	33	B4	Fars	Wild thorny
12	B8	Amol	Wild thorny	34	A9	Kohgiluyeh	Wild thorny
13	C29	Sari	Wild thorny	35	A24	Babol	Introduced thornless
14	B9	Gilan	Wild thorny	36	A25	Amol	Introduced thornless
15	C8	Lahijan	Wild thorny	37	C2	Sari	Introduced thornless
16	B15	Savadkouh	Wild thorny	38	C18	Qaemshahr	Introduced thornless
17	C17	Noor	Wild thorny	39	D6	Babol	Introduced thornless
18	B12	Babolsar	Wild thorny	40	D20	Sari	Thorny
19	D10	Sari	Wild thorny	41	A1	Karaj	Thorny cultivar
20	B14	Sari	Wild thorny	42	A22	Babol	Thorny cultivar
21	B13	Babolsar	Wild thorny	43	A4	Sari	Thorny cultivar
22	B11	Babolsar	Wild thorny	44	C26	Sari	Thorny cultivar
				45	A	Sari	Thorny cultivar

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Table 2. ISSR primers information used for evaluation of blackberry genotypes genetic diversity.

Primer no.	Primer sequence	Annealing temperature (°C)
ISSR 1	*5′ GAGAGAGAGAGAGAGAGAA*	55
ISSR 2	*5′ GAGAGAGAGAGAGAGAGAC*	55
ISSR 7	*5′CGAGAGAGAGAGAGAGA*	54
ISSR 8	*5′ CTCTCTCTCTCTCTCTG*	55
ISSR 10	*5′GAGAGAGAGAGAGAGAG*	55
ISSR 11	*5′ GAGAGAGAGAGAGAGAGAC*	54
ISSR 14	*5′ TCTCTCTCTCTCTCTCG*	55
ISSR 15	*5′ACACACACACACACACG*	55
ISSR 18	*5′ATCATCATCATCATCATCT*	55
ISSR 19	*5′ATCATCATCATCATCATCC*	55

(PIC) was calculated with formula for dominant markers: PICi= 2pi (1–pi), in which pi is the frequency of amplified allele. Marker Index (MI), showing the efficiency of marker, for each primer was calculated by PIC×the percentage of polymorphism. Shannon's index (I), showing the diversity in each primer, expected and observed heterozygosity, the number of alleles, and the number of effective alleles were determined by GeneAlex ver. 6.5.

RESULTS AND DISCUSSION

All the ten primers had a favorable polymorphism on the population. A total of 345 bands were created, of which 344 bands were polymorphic in the whole genotypes. The number of bands varied from 54 bands for primer number 1, to 13 bands for primer number 11. Both primers having the same nucleotide sequences and differing only in 3' anchor. The average number of bands for primers were 34.5. The average Number of observed alleles (Na), effective alleles (Ne), expected Heterozygosity (He) and observed Heterozygosity (Ho) and Shannon (I) information index for all primers were 1.99, 1.28, 0, 0.21 and 0.33, respectively. The average of information content of Polymorphic Information (PIC) and Marker Index (MI) for all primers was 29.2 and 29.22, respectively. Primer number 7 obtained the highest amount of Shannon (I), observed alleles (Ne) and observed Heterozygosity (Ho) (0.45, 1.43, and 29.2, respectively). Primer number 8 had the lowest PIC, MI, I, Ne and Ho with the rate of 0.22, 22.39, 0.26, 1.7 and 0.14, respectively. Expected heterozygosity is critical for determining genetic variation (Jakše *et al*., 2001). In some primers, the observed heterozygosity was more than the expected heterozygosity. It shows an increase in genetic variation. The observed and expected heterozygosity had an average of 0.21 and 0.19, respectively. Wright stabilization index, which indicates the reduction or increase in the observed impurity to expected impurity for each primer based on the comparison of observed and expected heterozygosity, was negative, which shows increase in heterozygotes (Boudchicha *et al*., 2018). It is probably due to maintaining high level of heterozygosity by non-sexual propagation and increase in heterozygosity rate by high mutations during generations (Radosavljević *et al*., 2015). According to these results, the selected population has been genetically diversified. However, Ataei *et al*. (2015) reported that there was little genetic differentiation among the wild blackberry population. The self-complementarity among ISSR primers including TA nucleotide sequences makes the proliferation difficult in proper and specific loci (Fang and Roose, 1997). Therefore, in this study, ISSR primers with repetitive sequences AG, CT, TC and ATC were used. These primers, with two or three nucleotides, repeat sequences and 54 and 55 degrees Celsius were successfully optimized. This conclusion suggests that

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complementary sequences of this type of primers are abundant throughout the genome of blackberries in this study. The Polymorphic Information Content (PIC) range for dominant markers, such as the ISSR, is 0 to 0.5, and for co-dominant markers, this range is between zero and one. The average PIC is 0.29 and indicates adequate polymorphism. Primer 7 obtained the highest PIC (0.39). The rate of PIC has important role in differentiation. The more PIC rate has the more polymorphism in primer loci. Therefore, high-PIC primers are useful for differentiating closely related genotypes (Thimmappaiah *et al*., 2008). MI (Marker Index) is a general criterion for determining the efficiency of a primer in polymorphism estimation and higher MI primers have been more effective in determining the variation between genotypes. Primer number 7 followed by primer number 2 (with 39.17 and 35.05, respectively) had the highest MI rate, and indicated the high efficiency and the better detection ability of determining the genetic distance. These primers also had the highest levels of Shannon index, effective allele and heterozygosity for these two primers with 0.45, 0.42, 1.43 and 1.41, 0.29 and 0.27, respectively, which could better justify the genetic diversity within the population and are introduced as the preferred primer for this study (Table 3). According to the results, it can be stated that repeated GA sequences in this study are more efficient than other sequences such as CT, according to Mandak *et al*. (2014) on the genetic diversity of the *Thymus vulgaris* population. Therefore, in future studies on blackberries, it could be recommended to use GA nucleotide repeat sequences.

Principal Component Analysis (PCA)

In this study, the first three components explained 33% of total variation, and showed an appropriate distribution of ISSR markers in the whole genome. According to the principal components' analysis, the genotypes can be categorized in three groups consisting of: (1) Old and adapted thornless introduced genotypes, (2) Wild thorny genotypes, and (3) Wild thorny genotypes and old adapted introduced thorny genotypes.

A new part of the blackberry variety is observed in Iran in the first group, which has not been reported by researchers. This group of genotypes (old and adapted introduced thornless varieties), which is in the upper of the 2D plot (Figure 2) was completely separate from other genotypes, and there is

Table 3. The result of statistical analysis for each primer.

Primer	Total band	Polymorphic band	Polymorphic percent	Na[a]	Ne[b]	I[c]	Ho[d]	He[e]	PIC[f]	MI[g]	F[h]
ISSR 1	54	54	100	2	1.23	0.29	0.17	0.16	0.26	26.4	-0.009
ISSR 2	20	20	100	2	1.41	0.42	0.27	0.26	0.35	35.03	-0.011
ISSR 7	20	20	100	2	1.43	0.45	0.29	0.28	0.39	39.71	-0.012
ISSR 8	31	31	100	1.97	1.17	0.26	0.14	0.14	0.22	22.39	-0.011
ISSR 10	44	44	100	2	1.31	0.35	0.22	0.21	0.30	30.67	-0.011
ISSR 11	13	13	100	2	1.35	0.38	0.23	0.23	0.30	30.37	-0.011
ISSR 14	33	33	100	2	1.27	0.33	0.20	0.19	0.29	29.83	-0.011
ISSR 15	53	53	100	2	1.22	0.28	0.16	0.16	0.25	25.48	-0.012
ISSR 18	34	34	100	2	1.37	0.36	0.23	0.23	0.29	29.08	-0.011
ISSR 19	43	42	97.67	2	1.22	0.28	0.16	0.15	0.23	23.27	-0.011
Mean	34.5	34.4	99.76	1.99	1.28	0.33	0.21	0.19	0.29	29.22	-0.011

[a] Number of different alleles; [b] Number of effective alleles; [c] Shannon's information index; [d] Observed Heterozygosity; [e] Expected Heterozygosity; [f] Polymorphic Information Contents; [g] Marker Index, and [h] Wright's Fixation index.

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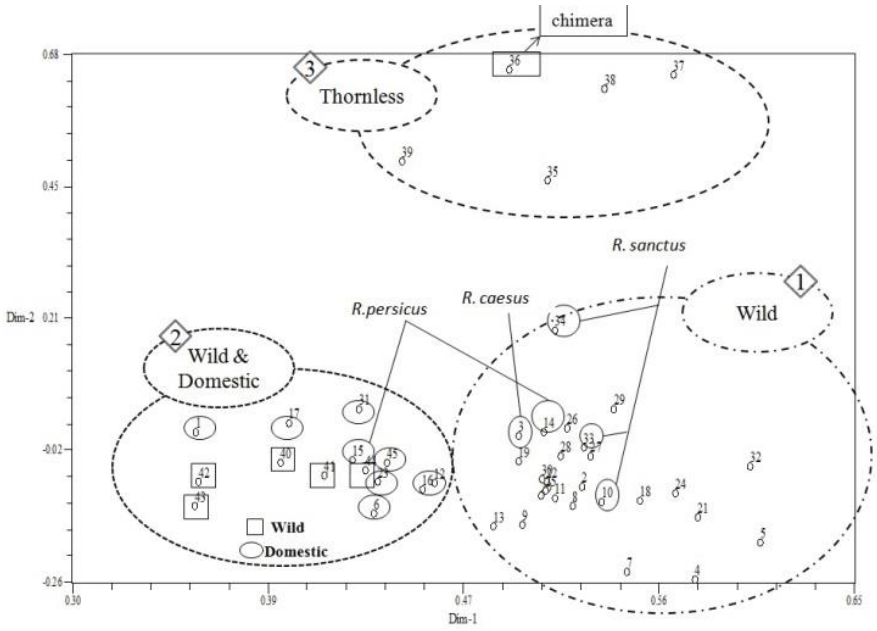

Figure 2. Di plot of the principal coordinates analysis based on Jaccard's similarity matrix in blackberry genotypes.

no exact or well documented history of their entrance to Iran, but are fully adapted to the conditions of the region and variation among them may occur during adaptation.

The second group belonged to thorny genotypes. There are some certificated genotypes according to the result of Gharaghani *et al*. (2011), so, some unknown genotypes in this group can be identified by their remoteness and proximity to these specific species. Genotypes 27 (WildTA), 29 (WildTGor), 2 (WildTQ), which are adjacent to the *R. sanctus* genotype, are categorized as *R. sanctus* and genotype 26 (WildTS) belongs to *R. persicus*. The rest of the interspecific genotypes of these identifiable species, and those that are in far distance can be attributed to the cross pollination and hybridization in wild populations of blackberries. An important point is that the distribution of similar species (*R. sanctus*= 10, 33 and 34, and *R. persicus*= 14 and 15) was observed in different regions, which can be explained by interspecies diversity of the samples studied. Sediqi and Rahimmalek (2015) and Ataei *et al*. (2015) reported, respectively, the diversity of inter and within the species in native blackberry of Iran; and they explained it due to the difference in the conditions of the ecological niche of these species and the possibility of inter and within specific crossing in addition to non-sexual reproduction.

In the third group, some genotypes from different wild species are located with old introduced thorny cultivars, which are adapted to the south region of the Caspian Sea. The emphasis of one of the Hypotheses of this research was that the distribution of genotypes in the last group, which included the important wild genotypes of number 1 from Ghaemshahr and number 17 of Noor, were along with the thorny introduced cultivar, and whose names has been revealed as Marion cv. and Tupi cv, via seed characteristics evaluation (Salehi *et al*., 2018). According to this distribution, based on the experience and the ancient background, native people of Mazandaran have identified and selected the superior genotypes, and may have taken a step in the

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domestication of wild genotypes, which is consistent with the goals and priorities of blackberry breeder in the 20th century to produce superior cultivars.

Cluster Analysis

The separation of the three main groups of blackberries was observed in this study. According to the results of Jaccard's similarity matrix, the highest similarity was observed with Sari (cvTLS= 37) and Ghaemshahr (cvTLQ= 38) thornless blackberry genotypes with a value of 73%. They were in the same groups based on morphological characteristics such as plant growth habit, leaflet length and width, leaf shape, flower size, flowering time, and fruit arrival (data not shown). The least similarity was found between two thorny genotypes Ghaemshahr (WildTQ= 1) from northern Iran and Kermanshah (WildTKER= 23) in the highlands of western Iran with a value of 0.07. It is related to the geographical distance and high altitude difference between their origins. They were also different in some botanical properties. For example, the average number of thorns at the distance between the two internodes for the WildTQ and wildTKER genotypes is 4 with a size of 41.3 mm and 13 with a size of 48.2 mm, respectively. Ruiz *et al*. (2007) showed that the presence of plants in the highlands with longer periods of cold weather led to the formation of a higher need for them. It seems this difference between these genotypes distinguished them from each other effectively.

Blackberry genotypes were divided into 6 groups at a similarity level of 0.21 (Figure 3). The first group included wild thorny genotypes. All introduced thorny cultivars (old and adapted cultivars) were in the second group. Twenty-four blackberry genotypes were placed in the third group, all of which were wild thorny. Thornless genotypes were separately in the fourth group. The fifth group included nine other genotypes of wild thorny blackberries. The sixth group belonged to the Lahijan wild blackberry genotype alone.

The first group consisted of genotypes number 1 (wildTQ) and six (wildTS), respectively, belonging to the wild thorny blackberries of Qaemshahr and Sari. Qaemshahr and Sari are considered as the old and prosperous cities of the southern strip of the Caspian Sea, and also according to local information, the area of collection of genotype number 1 has been taken attention by native people. Therefore, it seems that the presence of these two wild-type genotypes along with Group 2, which includes introduced breeding varieties (Figure 2 the wild and domestic samples), suggests the selection of these two genotypes by native people.

The second group consisted of introduced thorny cultivars that were divided into three subgroups with a 0.32 similarity level, in which two introduced thorny blackberry were collected from Sari (cvTS= 44) and (cvTS= 40) were placed in a separate subgroup and thorny genotypes from the University of Tehran, Babol, and Sari (41-43, respectively) were placed in another subgroup. The genotypes of this group can be completely separated from each other based on the maturity time, including early ripening (SANRUizadyar), mid-ripening (41 and 42), and late ripening (40 and 44). They are old and adapted cultivars, their specific name was recently noted as Silvan, Marion and Tupy, respectively (Salehi *et al*., 2018), according to observed genetic diversity in them. Early ripening and flowering in blackberry are a genetic attribute, and despite the similarity of the two genotypes 44 and 41 in morphological traits, they are completely different genetically. They separated from each other in 44 similarity values. Salehi *et al*. (2018) indicated the name of 41 and 44 as Marion (Chehalem×Olallie) and Silvan (Osc742×Marion), which shows that their similarity is due to their relatedness in pedigree (Wada, 2010; Fin *et al*., 2005). The similarity in the sample gathered from Karaj with the Babol sample indicates the

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traveling of the plant material between these two important and well-known cities located in high-traffic areas of the country.

The results of morphological studies performed on wild genotypes (Hadadinejad and Moradi, 2016) showed that WildTQ and WildTS genotypes collected from Qaemshahr and Sari were placed in the same group with about 90 percent similarity based on some traits such as number of lateral branches, lateral branch, leaf shape, end leaflet width and length. The same traits were similar in WildTNur, WildTSav, WildTS, and WildTA genotypes of Noor, Savadkouh, Sari and Amol, respectively, which were grouped in the same study.

The third group, the wild blackberry genotypes, were divided into two subgroups in the similarity level of about 24. The first subgroup included wild blackberries of one Qaemshahr genotype, three Sari genotypes, two Amol genotypes and two Gorgan genotypes. The second subgroup consisted of two genotypes of Noor and Savadkooh, three Sari genotypes, two genotypes of Amol and Gilan, three Bahnamir genotypes, and two Shiraz genotypes.

Due to the reported identification of genotypes (Gharaghani *et al.*, 2011), they belonged to *R. sanctus*, and adjacent genotypes in this grouping like WildTA= 9 genotype could be identified in this species according to its botanical characteristics. Also, in this division, primocane bearing genotypes, with a similarity of about 34, are in the same subgroup, which are one of the most important genotypes in the collection due to the formation of fruit in the end part of the primocane. Released cultivars of these species without chilling requirement will bear on primocane at the end of the growth season and are suitable for planting in areas with severe frosts to delete freezing injury to floricanes. The new thorny and thornless varieties are introduced from them (Clark and Salgado, 2016).

Samples number 10, 33, and 34 all belong to *R. sanctus*, however, they are placed such that numbers 33 and 10 had a similarity of 16%, numbers 34 and 10 with similarity of 22%, and numbers 33 and 34 with similarity of 40% in the cluster. This level of similarity may indicate an interspecies diversity among wild populations. KhatamSaz (1992) has reported that crossing between wild species is possible and a new variety is produced. According to studies by Kollmann *et al*. (2000) on European *Rubus*, as well as Ataei *et al*. (2015) on *Rubus* in northern Iran, it has been suggested that cross pollination affects seed retention and fruit quality, while increasing the polyploidy levels and the affiliation taxonomy of species. This phenomenon can also be found in the wild blackberries of this study and justify the diversity of the interspecies observed in this group, which is the largest species from the east (29 and 32) to the west (11) of the southern strip of the Caspian Sea. Sedighi and Rahimimalek (2015) reported a small interspecies variation on *R. hirtus* blackberries collected from the east to the west of the southern strip of the Caspian Sea. However, the most diverse samples were collected from the central region and the genetic diversity of this area was more than other areas. They indicated that samples from the two eastern and western regions of the southern strip of the Caspian Sea also originated from the central region. Blackberry collection of SANRU is located in the central region; it has succeeded to collect an interspecies variation. Internal hybridization changes in species typically do not appear in morphology of genotypes, so, morphological markers alone cannot be a reliable tool for determining the species background. Nuclear markers such as microsatellites are affected by internal hybridization changes in species and will not have the expected results. Therefore, along with nuclear markers such as the ISSR, it is possible to propose organ markers (DNA mitochondria and chloroplasts) to determine the species borders for the study of blackberry genus in Iran (Ataei *et al*., 2015).

The fourth group consisted of old, introduced and adapted thornless including Amol, Sari, Qaemshahr genotypes and two genotypes of Babol. They were divided into

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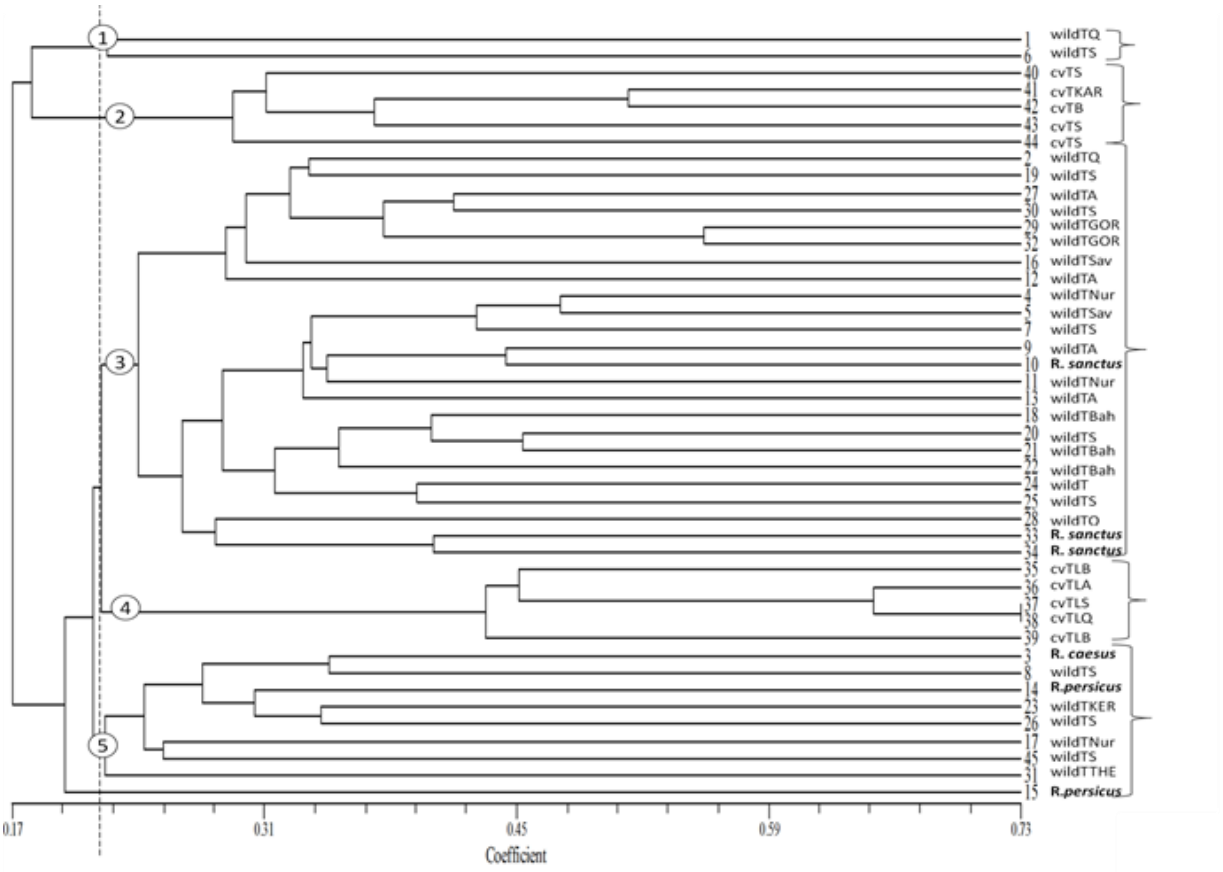

Figure 3. Cluster analysis of 45 blackberry genotypes based on Jaccard's similarity coefficient and UPGMA algorithm. In the cluster analysis, the summery consists of three parts: The first part- such as wild, CV: Introduced varieties from domestication or hybridization and selection, R and W respectively mean wild known (*Rubus*) or unknown (Wild) genotypes, The second part- the Thorny (T) or Thornless (TL), The third part- the first letter of the collection site of Noor: Nur, Savadkouh: Sav, Sari: S, Ghaemshahr: Q, Karaj: KAR, Kermanshah: KER, Gorgan: Gor, Bahnamir: Bah, Babol: B, Amol: A.

two subgroups with the similarity of about 0.45, and one of Babol genotypes in subgroup separated from the others. Genotype number 36 (cvTLA), one of the four genotypes located in a subgroup, produces thorny vines by propagating via root cutting, which indicates its belonging to the chimera variety (Thornless Evergreen). "Evergreen" was the parent of the first thornless cultivar generation, and was from the wild germplasm (*R. Laciniantus*). In this thorny cultivar, a mutation occurred and the name 'Thornless Evergreen' became the source of the development of thornless cultivars (Swanson *et al*., 2011). This group, which included 5 blackberry genotypes, was morphologically similar in some of the traits like plant growth habit, branch cross section, and number of leaflets. The results of cluster analysis (Figure 3) showed that the most similarity was found to be 70% between two samples from Qaemshahr and Sari. They probably are the same according to high genetic similarity, low morphological difference, and close geographical location. However, the observed differences can be further investigated. Thornless Evergreen is similar with these two samples to about 60%, based on evidence of their genetic and recessive thornlessness (Effati and Hadadinejad, 2016). It seems that these two samples are derived from early cultivar

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(Everthornless), which originated from tissue culture of chimera layer (McPheeters and Skirvin, 1983, 1995, 2000), and in some ways are part of the second generation of the thornless cultivars' evolution; and for this reason, they have been distinguished from the chimer genotype despite the similarity. In research by Coyner *et al*. (2008), cultivars, which had a distance less than 50 percent of the cultivars with the chimera sample, classified in the same thornless group and related them to chimera sample. A sample of the Amirkola-Babol Region (in north of Babol and at low altitude) was placed with 40% similarity in this category, and another example of the west Bandpey Area in the Village of Babol Sangrudpey separated (located near the highlands of the Alborz Mountains) with more than 60 percent difference. These samples are related to subsequent generations of thornless, such as Merton, or are the result of compatibility of previous samples with the diverse conditions of Mazandaran Province. Therefore, it can be concluded that the Iranian blackberry germplasm is not limited to the first and chimera type. Rather, it is likely to include samples of the next two steps of evolution, and may even include some of their new and evolving ones.

The fifth group consisted of wild blackberry genotypes, which were divided at a similarity level of about 0.24 into two subgroups. The first subgroup included Gilan, Kermanshah, and two Sari genotypes. The second subgroup included Noor and Sari genotypes. Gilan genotype number 3 belonged to *R. caesus* species. The nearest genotype to it is Sari wild thorny genotype number 8, and was placed in the same subgroup with similarity to about 0.34. Genotype number 14 (Gilan) of this group belongs to *R. persicus*, and indicated the diversity of species among the studied species. Ataei *et al*. (2015) reported the genetic variation in blackberries is determined by the plant propagation system and that there is cross pollination among polyploidy blackberry species. This phenomenon can also be found in the wild blackberries of this study and justifies the similarity of the observed species among the different species belonging to a subgroup.

The sixth group was related to Lahijan genotype belonging to *R. persicus* species based on overlap by botanical systematic method by Gharghani *et al*. (2011).

CONCLUSIONS

Blackberry genotypes of this study included the primary gene pool including adapted thorny and thornless cultivars, and have secondary gene pool including wild thorny blackberry genotypes. Since different species of blackberries have different ploidy levels, the ISSR marker performance for this plant is desirable. However, the lack of co-dominance of this marker requires more accuracy in comparison to the SSR marker. The wild species of this study were categorized in a separate group with a similarity of about 21%, so, there are variation among species in the wild populations. Also, the relatively high distance in the *R. sanctus* species, as the largest and most diverse species, showed the diversity within the samples. Interestingly, this study found that wild blackberries samples used by the native people in areas close to the major centers of human activities were close to the old introduced cultivars. Among these old varieties, also observed variation that confirmed their genetic difference and differentiate synonyms. However, in thornless blackberries, this diversity was seen differently and, consistent with the global approach to the development of thornless blackberries, samples of various generations include Thornless Evergreen, thornless from tissue culture (Ever thornless), and even Merton, or a variety of blackberries that are adapted to the climate and environment.

ACKNOWLEDGEMENTS

This research work was sponsored financially by grants from Genetics and Agricultural Biotechnology Institute of

[DOR: 20.1001.1.16807073.2021.23.4.5.4]

Tabarestan (GABIT) and is greatly appreciated.

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[DOR: 20.1001.1.16807073.2021.23.4.5.4]

بررسی تنوع ژنتیکی ژنوتیپ‌های تمشک‌های سیاه در ایران به وسیله نشانگر مولکولی ISSR

ن. عبدی، ح. مرادی، و م. حدادی نژاد

چکیده

پیشینه ارقام و گونه‌های وحشی تمشک سیاه سواحل جنوبی دریای مازندران به عنوان یک محل تنوع، نامشخص است. این پژوهش به منظور بررسی تنوع ژنتیکی ۴۵ ژنوتیپ تمشک سیاه (وحشی و وارداتی) جمع‌آوری شده از نقاط مختلف ایران با استفاده از نشانگر مولکولی ISSR صورت گرفت. از ضریب تشابه جاکارد برای ترسیم نمودار خوشه‌ای بر اساس الگوریتم UPGMA، برای گروه‌بندی ژنوتیپ‌ها استفاده شد. نایج نشان داد ۱۰ آغازگر در مجموع ۳۴۵ باند ایجاد کردند که ۳۴۴ باند چندشکل بودند و میانگین تولید تعداد باند برای هر پرایمر ۳۴/۵ بود. براساس نتایج PCA، ژنوتیپ ها در سه گروه طبقه بندی شدند. برخی از ژنوتیپ‌های وحشی در نزدیکی ارقام تجاری خاردار قرار گرفتند. نتایج تجزیه خوشه‌ای ژنوتیپ‌ها را به شش گروه تفکیک نمود. ژنوتیپ‌های وارداتی در این تقسیم‌بندی در یک گروه اما در زیرگروه مجزا قرار گرفتند که از لحاظ زمان رسیدن کاملا از یکدیگر قابل تفکیک بودند و شامل ارقام زودرس، میانرس و دیررس می‌باشند. این صفات ژنتیکی آنها را از هم جدا کرده و نتیجه مورفولوژی را تأیید و آنها را به عنوان ارقام خاردار Silvan، Marion و Tupi معرفی نمود. علاوه بر این نتایج نشان داد خزانه‌ژنی تمشک سیاه بی‌خار ایران فقط محدود به نوع اولیه و شیمر آن نمی‌باشد. بلکه احتمالا نمونه‌هایی از دو گام تکامل بعدی آن را در بر داشته و حتی ممکن است شامل برخی نمونه‌های جدید و تکامل یافته‌تر آنها نیز باشد.

[DOR: 20.1001.1.16807073.2021.23.4.5.4]