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Morphological and Molecular Characterization of Iranian Wild Blackberry Species Using Multivariate Statistical Analysis and ISSR Markers

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ABSTRACT

This study was carried out to estimate the genetic diversity and relationships of 74 Iranian blackberry accessions assigned to 5 different species using inter-simple sequence repeats (ISSR) marker analysis and morphological trait characterization. Sixteen traits were analyzed, including phenological, vegetative and reproductive attributes (in 57 out of 74 accessions), and 10 ISSR primers were screened (in 74 accessions). The yield and leaf width had the highest and lowest genetic diversity, (diversity index = 62.57 and 13.74), respectively. Flowering and ripening date were recorded as traits with the strongest correlations (r = 0.98). Ten ISSR primers were selected and produced a total of 161 amplified fragments (200 to 3500 bp), of which 113 were polymorphic. The highest, lowest and average PIC values were 0.53, 0.38 and 0.44, respectively. Principle component analysis (PCA) was based on morphological traits and showed that the first six components explained 84.9% of the variations among the traits. Meanwhile, the principal coordinate analysis (PCoA) was based on ISSR data and implied the first eight principal coordinates which explained 67.06% of the total variation. Cluster analysis was based on morphological traits and ISSR data ultimately classified all accessions into two and three major groups, respectively, so that the distribution pattern of genotypes was mainly based on species and the geographic origins.

Introduction

Rubus is one of the most diverse genera in the plant kingdom. It has 12 subgenera and over 500 species (Graham and Woodhead, 2011). These species are distributed widely in temperate regions of Europe, South and North America and Asia (Hummer, 2010). Red raspberries (*R. idaeus* L., Subgenus: *Idaeobatus*), black raspberries (*R. occidentalis* L., Subgenus *Idaeobatus*) and blackberries [*Rubus* spp., Subgenus *Rubus* (formerly *Eubatus*)] are the most widely grown fruits of the commercial *Rubus* species (Finn and Clark, 2012).

Rubus species have been cultivated or harvested from wild stands, as a food source (fresh, pulp, jam, juice, etc.), and for medicinal and therapeutic properties (Clark and Finn, 2011). In every region of the world that *Rubus* is native to, local cultivation and industries have been based on local species, such as trailing raspberry (*R. parvifolius* L.) in Asia, and arctic raspberry (*R. strigosus* L.) in North America. Cultivated

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raspberries and blackberries mainly originate in North America or Europe, although Asia also has a wealth of diversity that should be useful in breeding programs for creating new cultivars with superior traits (Finn and Clark, 2012). The information about worldwide production of blackberries is limited but it has been reported that production has rapidly increased in the past decades with an estimated 140,292 tons commercially harvested from 20,035 ha in 2005 (Graham and Woodhead, 2011).

Like other crops, blackberry breeding programs have been limited by a lack of variation for important traits in germplasm (Dossett et al., 2012). Acquiring information about the genetic structure of wild populations as sources of genetic diversity can be an efficient strategy for the identification of superior and promising genotypes to create new cultivars and to improve efficiency in breeding programs (Sedighi and Rahimmalek, 2015). Various techniques have been used for evaluating plant genetic variability, including morphological trait characterization (Ahmed et al., 2014; Maro et al., 2014; Dossett and Finn, 2016) and molecular marker analysis (Amsellem et al., 1999; Sedighi and Rahimmalek, 2015; Lee et al., 2016). Dossett and Finn (2016) evaluated the genetic diversity of wild populations of *R. occidentalis* L. and reported a wide range of variation in plant vigor, morphological traits, flowering and ripening date, as well as fruit weight beyond those of existing commercial cultivars for every trait. It was confirmed that a great potential can be expected in future breeding programs. High variations in berry weight and dimensions were reported in evaluating wild populations of *R. ellipticus* Smith. (Singh et al., 2009). Innis et al. (2011) assessed the genetic diversity of *R. argutus* and *R. phoenicolasius* using ISSR markers and reported higher variations between sites, compared to differences within each site, regarding both species. In addition, Lee et al. (2016) used SSR markers to determine genetic relationships between 69 *Rubus* accessions of six *Rubus* species and showed that *Rubus* accessions were subdivided into six sub-populations.

Iran is one of the most important areas in terms of plant genetic resources. In particular, this applies to wild edible fruits like blackberry. Based on a survey by Khatamsaz (1992), *Rubus* germplasm in Iran comprises eight species assigned to two subgenera. Seven out of these 8 species belong to the subgenus *Rubus* and include *R. sanctus* Schreber (syn. *R. anatolicus* (Focke) Hausskn.), *R. caesius* L., *R. hirtus* Waldst. & Kit. (syn. *R. lanuginosus* Stev. ex Ser), *R. dolichocarpus* Juz. (syn. *R. ochtodes* Juz.), *R. discolor* Weihe and Nees (syn. *R. armeniacus* Focke), *R. persicus* Boiss (syn. *R. raddeanus* Focke) and *R. hyrcanus* Juz., whereas the only herbaceous type, *Rubus saxatilis* L., belongs to the subgenus *Cylactis*. Five inter-specific natural hybrids also exist (Khatamsaz, 1992).

While the genetic diversity of blackberry has been documented in many parts of the world, there are only a few reports on this area, and even they have covered a limited number of species or have considered a limited span of geographic distribution regarding the Iranian *Rubus* species (Gharaghani et al., 2014; Sedighi and Rahimmalek, 2015; Yazdanpour et al., 2018). In this regard, Sedighi and Rahimmalek (2015) used the ISSR marker and morphological trait characterization to assess genetic diversity in a few populations of *R. hyrcanus* in the Caspian area of Iran. They found a narrow genetic base and relatively high genetic differentiation for *R. hyrcanus* genotypes. They also concluded that the morphological analysis corresponded to those obtained through molecular analyses in most cases. Furthermore, Gharaghani et al. (2014) evaluated the variation of fruit characteristics of two populations of *R. sanctus* Schreb. as genotypes from two distinct regions in north and south of Iran. They observed high variation as well as significant differences in fruit attributes between the genotypes collected from the two different regions of Iran. Recently, Yazdanpour et al. (2018) evaluated the phenotypic variations of one *Rubus* species in Babol county of Mazandaran province, in the Caspian region. They referred to this species as black raspberry, although it seems to have been named incorrectly. Based on a photo presented in their paper, this species seems to be *R. sanctus,* which is a blackberry rather than a black raspberry. They reported high levels of variation in almost all measured traits among the phenotypic attributes, including plant and fruit characteristics. Garazhian et al. (2020) evaluated the bio-chemicals and antioxidant activity in fruits of the same set of the genotypes studied herein. They reported a vast majority of genetic diversity available for fruit bio-chemicals and antioxidant activity in the Iranian wild *Rubus* species. However, apart from the reports on taxonomic classification, mainly based on phenotypic characterization, there is insufficient information on the phenotypic and genotypic diversity as well as relationships of the available genetic resources of the wild *Rubus* species in Iran. Accordingly, the objectives of this study were to characterize phenotypic diversity along with ISSR molecular markers for evaluating the genetic diversity and relationships of 74 accessions in the Iranian gene pool of wild blackberries. Since five

species were selected, the objectives in this research also included correlation analyses among the measured traits.
This was aimed at determining the relationships between the genotypes and species via cluster analysis, according to morphological and molecular data. Ultimately, the outcomes could serve future projects for the domestication and breeding of this valuable crop.

Materials and Methods

Plant materials

Seventy-two genotypes were assigned to five Iranian wild *Rubus* species. They were selected from the first Iranian blackberry repository established in 2010 at the research field of the Department of Horticultural Science, School of Agriculture, Shiraz University, Shiraz, Iran. The geographic information of the experimental site is as follows: longitude 52° 35' 0.73"; latitude 29° 43' 43.56"; and altitude 1791.4 (m). The average long-term annual rainfall was 336 mm, the soil type was silty-loam, and plants were irrigated using drip irrigation based on the common practices of the region. The accessions of this collection were collected from 10 provinces across Iran (Fig. 1) and included five different species, *R. sanctus*, *R. hirtus*, *R. caesius*, *R. discolor* and *R. persicus* (As classified by an experienced botanist). The geographic information of the origin of studied genotypes is presented in Table1.

Morphological Characterization

Sixteen morphological traits of 57 genotypes were studied during two growing seasons (2015 and 2016). Some of the accessions did not bear fruits so that we merely recognized genotypes with measurable morphological traits. Some of the characterizations were performed according to the guidelines provided by the International Union for the Protection of New Varieties of Plants (UPOV, 2006). Morphological traits of the cane included cane length (cm), internode length (mm), number of nodes, and spine density (No. of spine/cm of cane). Flowering and fruit ripening date were recorded on floricanes. The date of each observation was recorded for the appearance of the full bloom and the fully colored (black color) fruits. Flowering and ripening dates were determined as dates on which flowering or ripening took place. Number of fruit inflorescence/plant and number of fruits/ inflorescence were counted as they emerged on the canes. Leaf dimensions included leaf length (mm), leaf width (mm), and petiole length (mm), of mature leaves (20 leaves/accession per replicate) representing the typical characteristics of each genotype which was randomly collected from several parts of the shrubs in the summer. Fully mature fruits (black colored) were collected from different fruit clusters of the shrubs (20 fruits/accession per replicate), and immediately transferred to a refrigerator for future measurements of fruit weight (g), fruit length (mm), fruit width (mm), and number of drupelets/fruit. Characteristics such as leaf and fruit dimensions were measured by a digital caliper. The fruit weight was measured with an electronic scale with 0.01 g precision. The total yield (g/plant) was calculated by adding together the values of the repeated harvest of ripened fruits as well as the weight of fruits sampled for other measurements.

DNA Extraction

All 74 accessions were used for molecular characterization. DNA was extracted from fully expanded young leaves using the modified CTAB procedure as described by Khanuja et al. (1999). The quantity and quality of extracted DNAs were estimated with electrophoretic and spectrophotometric methods. The DNA was diluted to a working concentration of 5 ng/µl with sterile, distilled water.

ISSR Primers and PCR Reaction

Fifteen ISSR primers were screened and 11 primers produced a higher number of reproducible bands for selection (Table 4). PCR reactions were carried out in a volume of 20 µl that contained 2 µl of DNA (5 ng/µl), 3 µl of ISSR Primer (5 pg/µl), 8 µl of Master-Mix (Metabon-Germany) and 7 µl of sterile double distilled water. The optimum annealing temperature was determined for each primer (Table 4). The PCR cycling conditions for all studied primers were 3 min initial denaturation in 95 °C; followed by 35 cycles of 1 min at 95 °C, 1 min at the specific annealing temperature (52-57.5 °C), 1 min at 72 °C and a final extension step of 8 min at 72 °C. The amplified DNA fragments were separated in a 1% agarose gel at 100 W for 3 h in 1×TBE buffer (100 mM Tris–Borate, 2 mM EDTA in pH 8.0) and stained with SimplySafe (EURX company, Gdańsk, Poland).

Table 1. Geographic information of wild blackberry genotypes being used in this study

Species	Number	Genotype	Province	E (Longitude)	N (Latitude)	Altitude
R. sanctus	1	Shirgah	Mazandaran	52°55'16.42"	36°20'55.67"	361.4 m
	2	Sisangan	Mazandaran	51°47'45.31"	36°35'58.86"	-13.0
	3	Sari1(Jade Khazar)	Mazandaran	53° 1'8.24"	36°40'8.83"	544.6 m
	4	Jade Haraz	Mazandaran	52°17'17.34"	35°56'42.56"	403.5 m
	5	Chaloos	Mazandaran	51°26'4.23"	36°38'0.01"	1547.7 m
	6	Tonekabon (Nematabad)	Mazandaran	50°55'11.70"	36°45'9.33"	1821.7 m
	7	Tonekabon	Mazandaran	50°55'14.02"	36°45'10.330"	1803.5 m
	8	Namak Abrud 1	Mazandaran	51°20'47.38"	36°38'24.21"	1625.1 m
	9	Namak Abrud 3	Mazandaran	51°15'43.71"	36°39'8.39"	1376.4 m
	10	Babolsar 1	Mazandaran	52°45'29.09"	36°38'26.80"	-22.2 m
	11	Babolsar 2	Mazandaran	52°34'12.71"	36°37'42.13"	-24.6 m
	12	Sari 3	Mazandaran	53° 3'21.17"	36°34'48.79"	96.1 m
	13	Sefid Tameshk	Mazandaran	50° 38'14.81"	36° 54'57.65"	56.8 m
	14	Behshahr 2	Mazandaran	53°34'22.62"	36°40'41.79"	359.0 m
	15	Bandar Gaz	Golestan	53°56'13.67"	36°46'28.74"	409.0 m
	16	Gorgan 2	Golestan	54°25'58.79"	36°50'35.75"	501.3 m
	17	Aliabad Katul 3	Golestan	54°55'15.10"	36°52'50.12"	147.5 m
	18	Naharkhoran	Golestan	54°27'45.60"	36°47'2.595"	413.4 m
	19	Roodbar	Guilan	49°27'2.04"	36°48'10.00"	1639.5 m
	20	Kelachay	Guilan	50°22'53.58"	37° 0'22.53"	1804.1 m
	21	Roodsar	Guilan	50°16'33.97"	37° 8'24.95"	1630.3 m
	22	Kelachay (Polrood)	Guilan	50°22'29.17"	37° 4'40.88"	1680.0 m
	23	Talesh 2	Guilan	48°52'6.85"	37°48'40.60"	766.8 m
	24	Talesh 1	Guilan	48°52'6.08"	37°48'41.160	754.5 m
	25	Astara 4	Guilan	48°46'24.82"	38°25'21.13"	531.2 m
	26	Gardane Heyran 1	Guilan	46°57'4.84"	38°49'20.26"	645.8 m
	27	Gardane Heyran 4	Guilan	46°47'37.52"	38°47'13.90"	776.5m
	28	Lahijan	Guilan	49°59'5.50"	37°11'34.92"	4.0 m
	29	Anzali 3	Guilan	49°27'58.03"	37°27'32.69"	-24.2 m
	30	Jade Kandovan	Alborz	46°37'36.78"	37°59'25.16"	801.0 m
	31	Urmia 1	West Azarbayejan	44°54'20.88"	37°25'56.32"	1794.6 m
	32	Urmia 2	West Azarbayejan	44°55'30.15"	37°39'34.54"	2032.1 m
	33	Sanandaj 1	Kurdistan	47°03'40.14"	35°13'1.75"	1588.6 m
	34	Abidar	Kurdistan	50°32'40.61"	36° 2'25.56"	1890.6 m
	35	Malayer	Hamedan	48°50'41.89"	34°14'3.40"	1862.6 m
	36	Seyedan (Bag Bonyad)	Fars	52°35'3.47"	30°57'3.11"	1689.4 m
	37	Shiraz (Chamran)	Fars	52°29'41.80"	29°39'12.50"	1674.5 m
	38	Kazerun (Fathabad)	Fars	51°31'21.72"	29°43'42.59"	755.2 m
	39	Kazerun (Eslamabad)	Fars	51°34'26.50"	29°46'58.43"	797.3 m
	40	Nourabad (Bavan)	Fars	51°32'22.98"	30° 7'36.14"	1079.9 m
	41	Dasht Arzhan	Fars	51°58'46.66"	29°39'10.06"	2142.1 m
	42	Sepidan (Roodbal)	Fars	52° 2'20.75"	30° 6'0.10"	1920.2 m
	43	Beyza (Hoseinabad)	Fars	52°23'24.53"	29°58'14.28"	1637.0 m
	44	Kamfiruz	Fars	52°11'32.76"	30°19'35.13"	1783.0 m
	45	Firouzabad	Fars	52°32'21.76"	28°52'37.76"	1347.2 m
	46	Jahrom (Khafr)	Fars	53°31'31.93"	28°29'15.77"	1049.4 m
	47	Sivand	Fars	52°55'1.58"	30° 5'12.83"	1802.8 m
	48	Dena (Karyak)	Kohgiluyeh-Boyrahmad	51°25'5.97"	30°49'1.80"	2018.9 m
	49	Kakan	Kohgiluyeh-Boyrahmad	51°48'3.78"	30°37'31.62"	2013.2 m
	50	Yasuj (Naregah)	Kohgiluyeh-Boyrahmad	51°34'8.08"	30°36'55.97"	2105.2 m
R. hirtus	51	Abas Abad	Mazandaran	51° 7'24.50"	36°42'34.78"	1722.7 m
	52	Gardane Heyran 5	Guilan	46°53'1.14"	38°48'17. 6"	660.5 m
	53	Rezvanshahr	Guilan	49° 8'32.03"	37°33'33.37"	950.3 m
	54	Ashkvarat 1	Guilan	50°16'7.76"	36°48'5.24"	1835.2 m
	55	Ashkvarat 2	Guilan	50°14'7.40"	36°46'26.55"	1839.9 m
	56	Anzali 2	Guilan	49°28'44.39"	37°27'12.35"	1173.7 m
	57	Astara 1	Guilan	48°46'45.47"	38°26'56.21"	490.4 m
	58	Astara 2	Guilan	48°47'43.30"	38°22'49.72"	558.6 m
	59	Ganjname	Hamedan	48°26'6.01"	34°45'34.13"	1795.2 m
	60	Gerdbisheh	Char Mahal-Bakhtiari	50°39'37.12"	32° 8'12.91"	2062.8 m
R. caesius	61	Aliabad Katul 1	Golestan	54°54'45.18"	36°54'54.92"	150.4 m
	62	Aliabad Katul 2	Golestan	54°51'2.03"	36°53'32.20"	159.7m
	63	Gorgan 5	Golestan	54°26'21.73"	36°50'44.32"	499.8 m
	64	Gorgan 4	Golestan	54°25'11.23"	36°50'49.48"	484.2 m
	65	Naharkhoran 1	Golestan	54°28'20.19"	36°48'28.69"	360.2 m
	66	Gonbad-e Kavus	Golestan	55°10'31.47"	37°12'37.55"	43.0 m
	67	Anzali 1	Guilan	49°27'7.99"	37°27'16.12"	-29.0 m
	68	Fuman 1	Guilan	49°18'41.58"	37°12'45.17"	1325.5 m
	69	Gerdbisheh 1	Char Mahal-Bakhtiari	50°37'14.22"	32° 8'88.03"	2051.5 m
R. discolor	70	Kelachay 1	Guilan	50°22'25.3"	37° 4'41.24"	1676.0 m
R. persicus	71	Masuleh	Guilan	49° 00'2.22"	37° 9'40.34"	1231.3m
	72	Fuman	Guilan	49°17'51.93"	37° 12'32.07"	39.6 m
	73	Rasht Abad	Guilan	49° 50'25.21"	37° 15'32.05"	-4.4 m
	74	Ramsar 1	Mazandaran	50° 38'13.89"	36° 54'53.75"	95.5 m

ISSR Primers and PCR Reaction

Fifteen ISSR primers were screened and 11 primers produced a higher number of reproducible bands for selection (Table 4). PCR reactions were carried out in a volume of 20 µl that contained 2 µl of DNA (5 ng/µl), 3 µl of ISSR Primer (5 pg/µl), 8 µl of Master-Mix (Metabon-Germany) and 7 µl of sterile double distilled water. The optimum annealing temperature was determined for each primer (Table 4). The PCR cycling conditions for all studied primers were 3 min initial denaturation in 95 °C; followed by 35 cycles of 1 min at 95 °C, 1 min at the specific annealing temperature (52-57.5 °C), 1 min at 72 °C and a final extension step of 8 min at 72 °C. The amplified DNA fragments were separated in a 1% agarose gel at 100 W for 3 h in 1×TBE buffer (100 mM Tris–Borate, 2 mM EDTA in pH 8.0) and stained with SimplySafe (EURX company, Gdańsk, Poland).

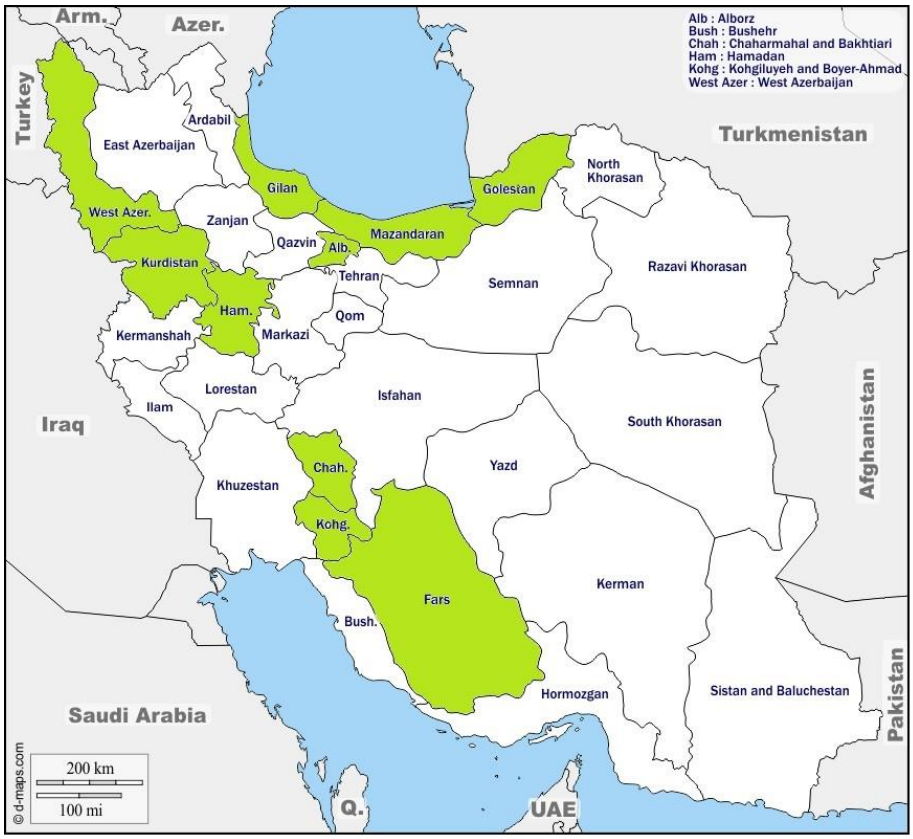

Fig. 1. Approximate distribution of Iranian blackberry genotypes (the green colored provinces). The original map is obtained from d-map (https://d-maps.com/carte.php?num_car=5496&lang=en) and are modified (colored) using paint software of Microsoft Windows 10.

Table 4. List of the selected ISSR primers and the degree of polymorphism

Primer	Motif	Annealing temperature	Number of bands (n)	Number of polymorphic bands (np)	Polymorphisms ratio (%)	PIC
P1	5'-(CA)8 G-3'	52.5	21	14	77.57	0.46
P2	5'-(AC)8 G-3'	52.0	18	13	81.25	0.44
P3	5'-(CA)7 CTA-3'	54.0	12	10	84.61	0.42
P5	5'-(AG)8 C-3'	53.5	15	11	71.66	0.53
P6	5'-(GA)8 C-3'	52.5	17	11	73.63	0.38
P7	5'-(AC)8 C-3'	52.5	25	17	74.41	0.48
P9	5'-(CA)8 CCCT-3'	53.5	11	9	90.20	0.44
P10	5'-(CA)8 GGGT-3'	53.5	13	10	82.90	0.45
P11	5'-T(AG)7-3'	55.5	14	9	69.23	0.46
P13	5'-G(TC)7-3'	57.5	15	9	81.81	0.39
Total	-	-	161	113	-	-
Average	-	-	16.1	11.3	78.72	0.44

Statistical Analysis

Morphological data pertaining to two years were compared to determine the significance of each year. Therefore, average data of two years were used because the effect of the year and year×genotype were not significant at $p < 0.05$, for almost all of the measured traits. The range and estimated diversity index (DI) were used as indicators of variability (Diversity Index = (Std. dev./Mean)×100) (Sorkheh et al., 2009). The simple correlation coefficient was calculated to determine the relationships between the studied morphological variables using the Pearson correlation coefficient by Minitab, version 16 (Minitab, Inc., 1998). Relationships among genotypes were investigated by principal component analysis (PCA) using Minitab statistics software. The morphological similarity coefficients according to Euclidian distance matrix using Ward method were calculated using Minitab, version 16 and the dendrogram was constructed. A scatter plot was drawn according to the PC1 and PC2 using PAST statistics software (Hammer et al., 2001). The analysis of polymorphic ISSR bands in each gel were scored as absent (0) or present (1). The cluster analysis and principal coordinate analysis (PCoA) were conducted by the NTSYs software Version 2.01 (Rohlf, 2000). Polymorphic information content (PIC) was calculated using the formula; PICi=2f (1−f), where PICi is the polymorphic information content of marker i, fi is the frequency of the present fragments and (1−fi) is the frequency of the absent fragments (Powell et al., 1996). Genetic similarity among all genotypes was calculated according to Simple Matching (SM) similarity index, using the similarity of qualitative data (Simqual) routine. The dendrogram was constructed using the unweighted pair group method average (UPGMA) clustering procedure. Also, cophenetic correlation coefficients were calculated by COPH and MXCOMP procedures for each combination.

Results

Morphological diversity analysis and variations of morphological traits

Based on a combined analysis of variance (ANOVA), there was no significant difference among the studied traits in the two years, but significant variations were observed in most of the measured traits among genotypes. Year × genotype interaction was also not significant (data not shown). Considering cane characteristics, cane length varied from 73 cm (Jade Kandovan; *R. sanctus*) to 197 cm (Masule; *R. persicus*). The number of nodes were between 15.5 (Jade Kandovan; *R. sanctus*) to 48.5 (Kazerun-Eslamabad; *R. sanctus*) and internode length ranged from 30.0 mm (Chaloos; *R. sanctus*) to 85.6 mm (Masule; *R. persicus*). Variations in spine density were recorded between 0.4 (Gardane Heyran 5; *R. hirtus*) and 4.45 per centimeter of cane length (Aliabad Katul 1; *R. caesius*) (Table 2, A and B).

With regard to all genotypes, the leaf length ranged from 30.2-94.2 mm, leaf width varied from 28.2 to 59.4 mm, and petiole length ranged from 14.5-52.6 mm (Table 2). The highest values of all leaf parameters were measured in Masule (*R. persicus*), while the lowest values of leaf length and width were measured in Kamfiruz (*R. sanctus*). Meanwhile, Jadeh Kandovan (*R. sanctus*) had the lowest petiole length.

Phenological traits also showed high variations among genotypes. The flowering time varied from May 14th (Gerdbisheh, *R. hirtus*) to August 1st (Masule; *R. persicus*), while genotypes were harvested from May 30th (Gerdbisheh, *R. hirtus*) to August 24th (Anzali 2, *R. hirtus*) (Table 2).

The number of inflorescences/plant varied from 17.5-75 (Kamfiruz and Sanandaj; *R. sanctus*), while the number of fruits/inflorescence ranged from 3-16 (in Babolsar 2 and Astara 4; *R. sanctus*) among all studied genotypes. The range of fruit length was 6.5–16.2 mm, while the fruit width was between 05.3-15.5 mm (Table 2). Collections from Ganjname and Abas Abad (both of which belong to the *R. hirtus*) were recorded as genotypes having the highest and lowest fruit dimensions, respectively. The highest value for fruit weight was 1.38 g (Sepidan-Roodbal; *R. sanctus*), while the lowest value for fruit weight was 0.14 g (Babolsar 2; *R. sanctus*).

The number of drupelets/fruit showed a high level of variation ranging from 5.5-36.0 drupelets per fruit. Gardane Heyran 1 and Babolsar 2 (both of which belong to the *R. sanctus*) were the genotypes having the highest and lowest number of druplets/fruit, respectively (Table 2). The highest (944.7 g/plant) and lowest (16.8 g/plant) yield were harvested from Sanandaj 1 and Babolsar 2 genotypes, both of which are accessions of *R. sanctus*. Considering the estimated diversity index (DI), the studied vegetative, phenological and pomological attributes were categorized into three groups, i.e. high diversity (DI ≥ 40), medium diversity (DI from 20-40) and low diversity (DI ≤ 20). Based on this grouping, yield (DI=62.57) and spine density (DI=53.54) were recorded as having the highest diversity, while ripening date, leaf width, fruit width, flowering date, fruit length, leaf length, cane length and internode length were categorized as traits having low diversity.

Table 2(A). Descriptive Statistics of morphological traits of studied blackberry genotypes from Iran

Code	Traits	Mean	Std. error	Range	Max	Min	Std. dev.	Diversity Index
1	Cane length (cm)	123.71	1.35	124.00	197.00	73.00	20.78	16.79
2	Leaf length (mm)	50.00	0.50	64.00	94.20	30.20	8.10	16.11
3	Leaf width (mm)	44.50	0.40	31.20	59.40	28.20	6.10	13.74
4	Petiole length (mm)	23.10	0.40	38.10	52.60	14.50	5.70	28.84
5	Internode's length (mm)	42.70	0.50	55.60	85.60	30.00	7.90	18.49
6	Number of nodes	29.88	0.47	33.00	48.50	15.50	7.23	24.19
7	Spine density (No. in 1 cm of cane)	1.21	0.04	4.05	4.45	0.40	6.49	53.54
8	Flowering date	107.96	1.22	79.00	August 1th	May14th	15.91	14.74
9	Number of inflorescences	36.50	1.01	57.50	75.00	17.50	13.21	36.18
10	Fruits/ inflorescences	7.49	0.19	13.00	16.00	3.00	2.44	32.51
11	Ripening date	127.75	1.32	86.00	August 24th	May 30th	17.20	13.47
12	Number of drupelets/Fruit	20.05	0.51	30.50	36.00	5.50	6.68	33.32
13	Fruit width (mm)	11.70	0.10	10.20	15.50	5.30	1.60	14.06
14	Fruit length (mm)	11.90	0.10	9.70	16.20	6.50	1.80	14.80
15	Fruit weight (g)	0.82	0.02	1.19	1.33	0.14	0.25	30.25
16	Yield (g)	233.90	11.20	927.90	944.70	16.80	146.30	62.57

Table 2(B). Leven's statistic

Variable	Leven's statistic	P-value
Number of nodes	3.02	0.074
Internodes length	0.25	0.614
Cane length	3.08	0.080
Spine density	0.11	0.739
Leaf length	0.00	0.951
Leaf width	0.01	0.924
Petiole length	0.64	0.424
Flowering date	0.03	0.862
Ripening date	0.04	0.847
Fruit length	0.04	0.844
Fruit width	0.07	0.786
Number of drupelets/Fruit	0.14	0.710
Number of inflorescences	2.37	0.124
Fruits/inflorescences	0.02	0.901
Fruit weight	0.46	0.496
Yield	3.31	0.095

Other groups of evaluated characteristics were, namely, the number of nodes, petiole length, fruit weight, number of fruits/inflorescence, number of drupelets/fruit and number of inflorescences/plant, which were found to have medium diversity (Table 2).

Correlations among traits, principle component analysis (PCA) and cluster analysis

The analyses of correlation among traits are presented in Table 5. As expected, the cane lengths showed a significant positive correlation ($r = 0.76$) with number of nodes and, interestingly, a significant but negative correlation ($r = -0.32$) occurred with spine density. Leaf length, leaf width, and petiole length were also highly correlated to each other (ranging from $r = 0.71$ to $r = 0.79$). Leaf length and petiole length also had positive correlation with internode length ($r = 0.32$ and $r = 0.37$, respectively). Flowering and ripening dates were highly correlated to each other ($r = 0.98$). Not surprisingly, yield positively correlated to its components including number of inflorescences ($r = 0.60$), number of fruits/inflorescence ($r = 0.69$), fruit weight ($r = 0.54$) and number of drupelets/fruit ($r = 0.48$). Interestingly, yield and many of its components were negatively correlated to spine density (ranging from $r = -0.25$ in number of fruits/inflorescence to $r = -0.50$ in fruit weight). Fruit weight had positive correlations with fruit length ($r = 0.42$), fruit width ($r = 0.40$), and number of drupelets/fruit ($r = 0.42$).

The results of principle component analysis (PCA) showed that the first four components explained 67% of the variations of traits in the studied genotypes (Table 3). The first component, which accounted for 25.3% of total variations, featured mainly reproductive attributes including fruit weight, yield, number of drupelets, fruit length and leaf length. The second component, however, explained 15.8% of total variations (i.e. flowering date, ripening date and number of nodes) (Table 5).

Table 5. Correlations coefficients among studied morphological traits.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1-Cane length	1.00	0.257 *	0.142 ns	0.144 ns	-0.035 ns	0.766**	-0.320*	0.054 ns	-0.031 ns	-0.055 ns	0.043 ns	0.138 ns	0.011 ns	-0.033 ns	0.189 ns	0.098 ns
2-Leaf length		1.00	0.792**	0.714**	0.326*	0.009 ns	0.178ns	0.059 ns	-0.043 ns	-0.220 *	0.026 ns	-0.290*	-0.212 *	-0.209*	-0.040 ns	-0.220 ns
3-Leaf width			1.00	0.561**	0.096 ns	0.088 ns	0.277*	0.003 ns	-0.035 ns	-0.225 *	-0.026 ns	-0.247 *	-0.145 ns	-0.130 ns	-0.097 ns	-0.245 ns
4-Petiole length				1.00	0.373**	-0.131 ns	0.221*	0.131 ns	0.200 *	-0.130 ns	0.094 ns	-0.300*	-0.159 ns	-0.128 ns	-0.240 *	-0.120 ns
5-Internodes length					1.00	-0.658**	0.058 ns	-0.014 ns	-0.241 *	0.211 *	-0.046 ns	-0.071 ns	0.023 ns	-0.045 ns	-0.101 ns	-0.097 ns
6-Number of nodes						1.00	-0.282*	0.073 ns	0.121 ns	-0.151 ns	0.084 ns	0.150 ns	-0.004 ns	-0.006 ns	0.224*	0.146 ns
7-Spine density							1.00	-0.257 *	0.120*	-0.270*	-0.254 *	-0.380**	0.016 ns	0.057 ns	-0.500**	-0.337**
8-Flowering date								1.00	-0.074 ns	0.119 ns	0.980**	0.139 ns	-0.130 ns	-0.100 ns	0.050 ns	0.030 ns
9-Number of inflorescences									1.00	0.103 ns	-0.030 ns	0.016 ns	-0.028ns	0.129 ns	-0.025 ns	0.602**
10-Fruits/ inflorescences										1.00	0.13 ns	0.427**	0.125 ns	0.154 ns	0.330**	0.693**
11-Ripening date											1.00	0.150 ns	-0.140 ns	-0.090 ns	0.080 ns	0.080 ns
12-Number of drupelets												1.00	0.439**	0.444**	0.421**	0.485**
13-Fruit width													1.00	0.890**	0.406**	0.228**
14-Fruit length														1.00	0.421**	0.325**
15-Fruit weight															1.00	0.542**
16-Yield																1.00

*and **: significant different at 5% and 1% probability levels respectively; ns: non-significant.

Table 3. Eigenvectors of the eight principal component axes from PCA analysis of morphological variables in studied Iranian blackberry accessions

Traits	Component					
	1	2	3	4	5	6
Cane length	0.056	0.384	0.067	0.369	-0.129	-0.228
Leaf length	-0.284	0.279	0.406	0.064	-0.046	-0.07
Leaf width	-0.265	0.218	0.355	0.147	-0.035	0.075
Petiole length	-0.266	0.217	0.407	-0.059	0.155	0.067
Internodes length	-0.127	-0.077	0.349	-0.380	-0.161	-0.348
Number of nodes	0.123	0.353	-0.161	0.509	0.003	0.051
Spine density	-0.256	-0.280	0.139	0.055	0.127	0.381
Flowering date	0.046	0.429	-0.122	-0.388	-0.079	0.356
inflorescences Number of	0.080	0.015	0.115	0.115	0.705	0.206
Fruits/inflorescences	0.284	0.018	0.154	-0.300	0.225	-0.289
Ripening date	0.065	0.425	-0.136	-0.384	-0.045	0.361
Number of drupelets	0.398	0.069	0.131	-0.048	-0.130	-0.026
Fruit width	0.276	-0.203	0.316	0.103	-0.298	0.314
Fruit length	0.290	-0.189	0.333	0.096	-0.165	0.391
Fruit Weight	0.362	0.135	0.197	0.055	-0.160	-0.133
Yield	0.367	0.079	0.185	-0.039	0.446	-0.105
Proportion of total variance	0.253	0.157	0.132	0.128	0.097	0.082
Cumulative % of total variance	25.3	41.0	54.2	67.0	76.7	84.9

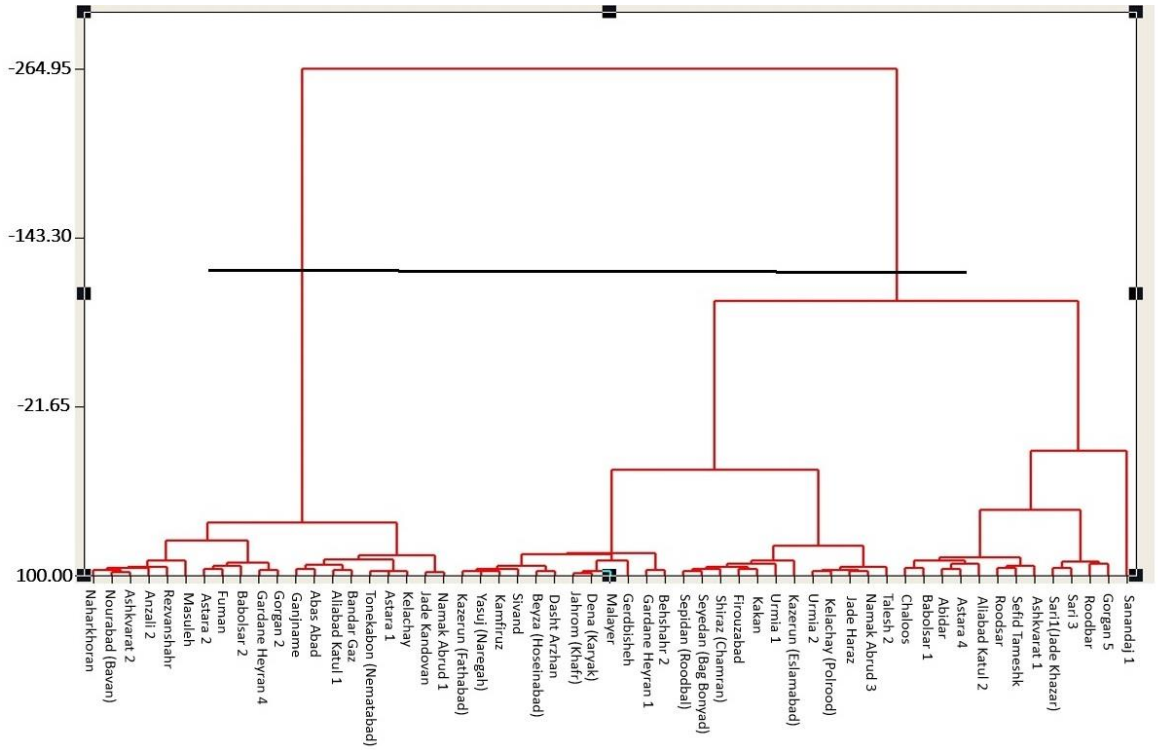

Fig. 2. Projection of genotypes on the first (PC1) and second (PC2) principal components based on bi-plot. Discrimination vector of different morphological traits based on loading plot.

The bi-plot of PCA ultimately grouped the samples into their phenotypic resemblance and morphological characteristics. The results showed that all genotypes were divided into two main categories based on the first two components according to the loading plot (Fig. 2). The cluster analysis of accessions was planned on the basis of morphological characteristics using Ward's method (Fig. 3) which divided them into two main clusters. The first cluster, which contains the majority of *R. sanctus* accessions, was divided into 2 sub-clusters. In the first sub-cluster, despite the Abidar and Sanandaj1 (from Kurdistan Province in west of Iran), other genotypes were from the Caspian Sea region in the north of Iran. Most of the genotypes originated from west and south of the country and a few genotypes from the north of Iran. These included Talesh 2, Namak Abrud 3, Jadeh Haraz and Kelachay-Polrood which were placed in the second sub-cluster (Fig. 3). The second cluster contained 20 genotypes including 6 out of 8 genotypes of *R. hirtus* (except Gerdbisheh and Ashkvarat 1), 2 genotype of *R. persicus*, 2 out of 4 genotypes of *R. caesius* (except Gorgan 5 and Aliabad Katul 2) and 10 genotypes of *R. sanctus* species. Except for Nourabad which is from Fars province, other *R. sanctus* genotypes in this cluster were from the Caspian Sea region in Golestan, Mazandaran and Guilan provinces.

ISSR Analysis, primer amplification and diversity indices

The selected primers produced a total of 161 amplified fragments in which 113 were polymorphic. The sizes of the amplified products ranged from 200 to 3500 bp. Considering all genotypes, the number of amplified bands per primer varied from 11 (P9) to 25 (P7), while the number of polymorphic bands per primer ranged from 9 (in P9, P11 and P13) to 17 (in P7) with an average of 11.3 polymorphic fragments per primer. The rate of polymorphism ranged from 69.23% (in P11) to 90.2% (P9) with an average of 78.72% (Table 4). The highest and lowest PIC values were observed in P5 (0.53) and P6 (0.38), respectively, and the average PIC was 0.44 (Table4).

Genetic diversity, cluster analysis and PCo analyses

A dendrogram was generated by the Jaccard similarity matrix. The UPGMA method revealed genetic relationships among genotypes of different species (Fig. 4). Jaccard similarity coefficients ranged from 0.33 to 0.90. The highest genetic similarity (90%) was observed between Astara 1 and Astara 2 genotypes (both belong to *R. hirtus* species). Meanwhile, genotypes of R. persicus had the highest genetic distance (67%) from the rest of the genotypes. According to the generated cluster, based on the similarity matrix, genotypes were placed in three major groups according to the species. All genotypes of R. sanctus which originated from different regions of the country were located in the first large cluster. The second cluster contained 20 genotypes, including 10 genotypes of *R. hirtus*, 9 genotypes of *R. caesius*, and one genotype of R. discolor. Cluster 3 consisted of four genotypes and belonged to *R. persicus*. The PCo analysis implied that the first eight principal coordinates totally explained 67.06% of the total variation (54.20% in the first three principal coordinates). In most cases, PCo analysis showed that the result corresponded to those obtained through cluster analysis and classified genotypes into four groups as follows: group 1 comprised all *R. sanctus* genotypes, group 2 comprised all *R. hirtus* genotypes, group 3 comprised all *R. caesius* genotypes along with the only genotype of *R. discolor*, and group 4 comprised all *R. persicus* genotypes. However, the majority of *R. hirtus*, *R. caesius* and *R. discolor* genotypes were classified in one group, according to the morphological cluster analysis (Fig. 3).

Discussion

Morphological diversity analysis, variations of morphological traits

Like many fruit crops, commercial cultivars of blackberries have a narrow genetic background that makes them not only vulnerable to new biotic and abiotic stresses, but also limits breeding programs with a lack of variation (Dossett et al., 2012). Acquiring comprehensive information about the genetic diversity and population structure of wild species is a good strategy for identifying superior genotypes to be introduced as new cultivars, or else to introduce promising parents in future breeding programs (Sedighi and Rahimmalek, 2015). The results of this study revealed a high level of genetic variation among and within Iranian wild blackberry species, regarding vegetative, phenological and reproductive traits (Table 2). The vegetative and reproductive traits, as well as the genetic factor, greatly depended on the environment. Usually, species or accessions from regions of greater annual precipitation almost always have greater leaf dimensions (Sorkheh et al., 2009). Ryabova (2007) showed a high level of morphological, phenological and genetic variations of wild raspberry and concluded that these variations -

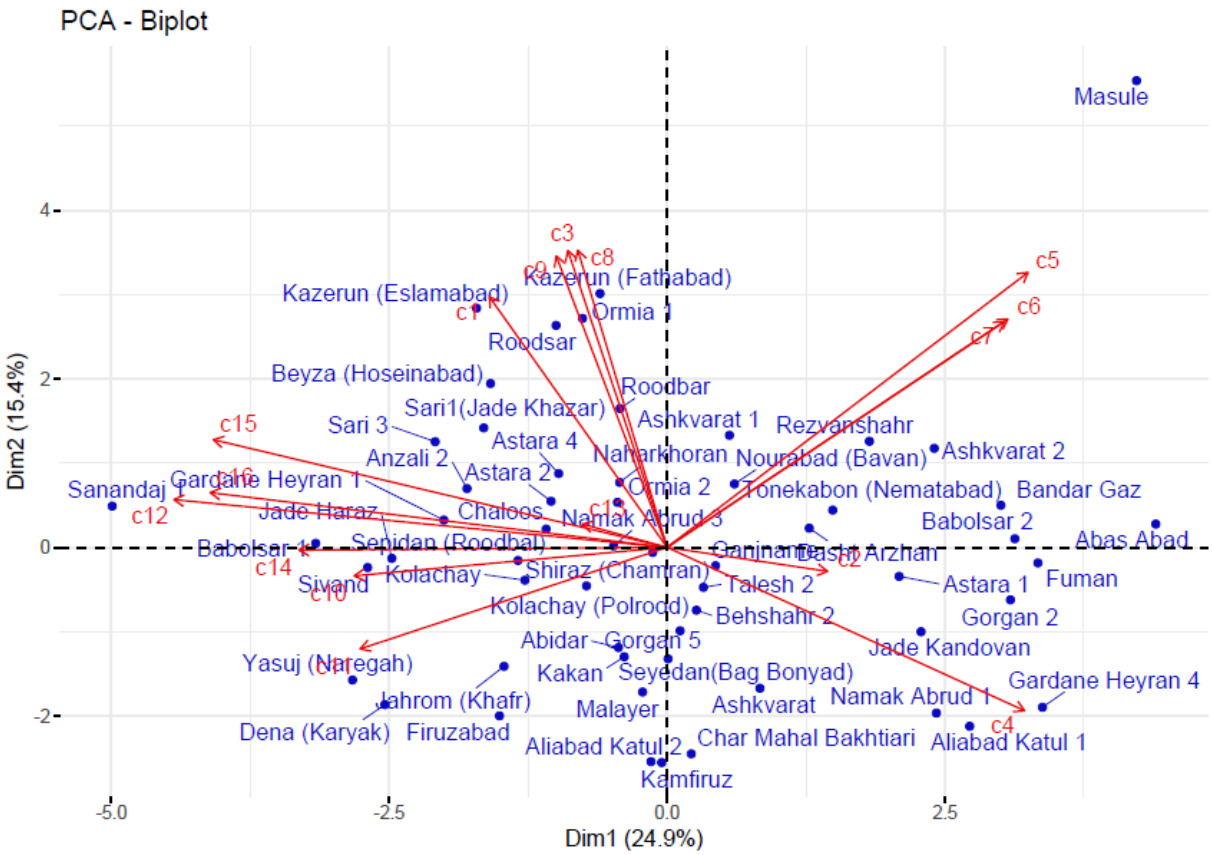

Fig. 3. Dendrogram of blackberry genotypes based on cluster analysis of morphological traits.

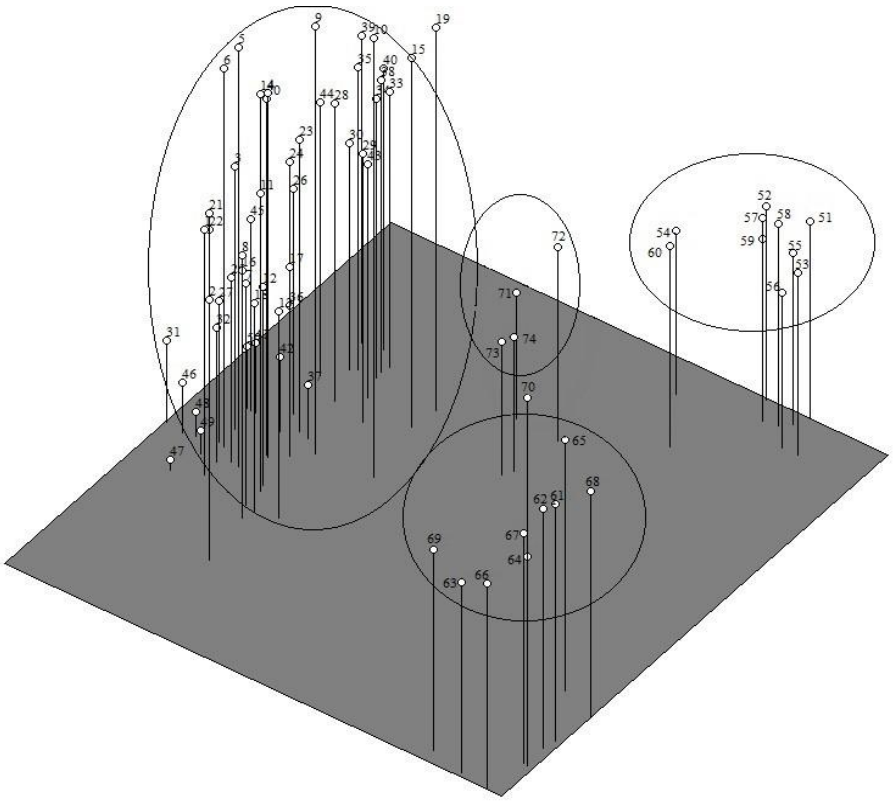

Fig. 4. Patterns of relationships among blackberry genotypes being used in this study based on ISSR data that revealed by PCoA

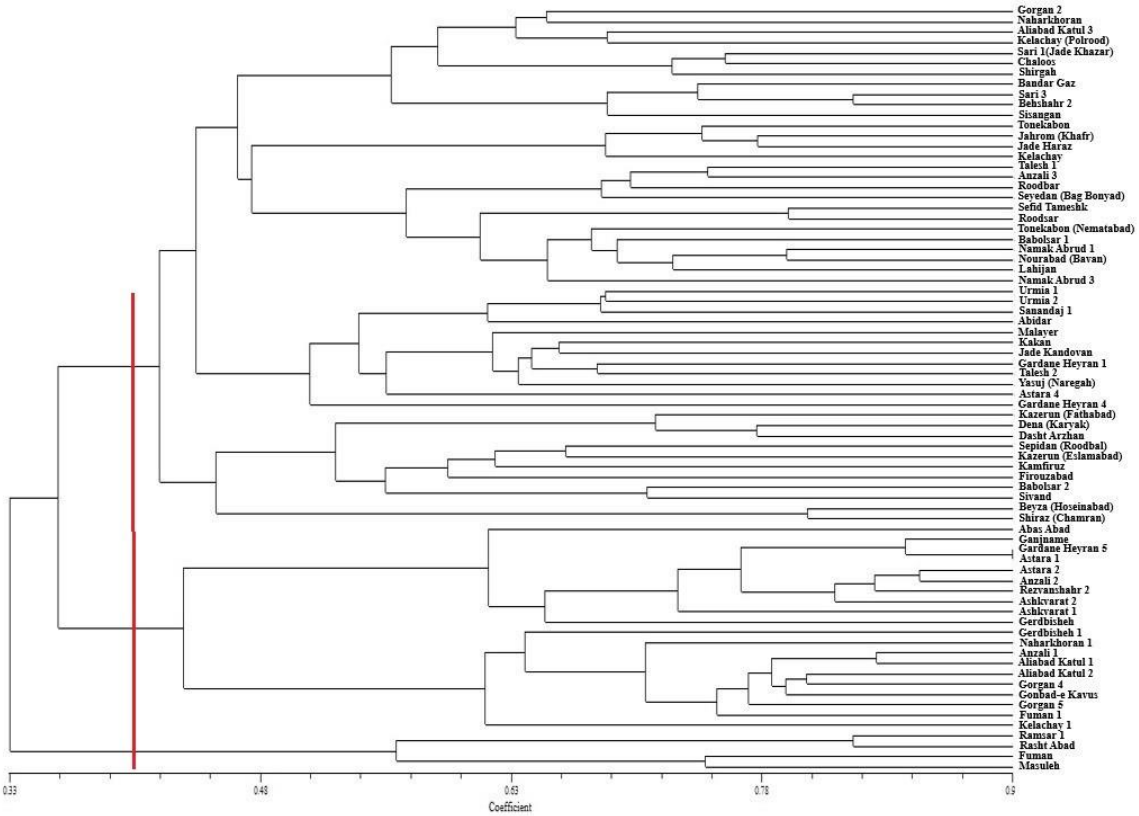

Fig. 5. UPGMA dendrogram generated based on ISSR data using Jaccard similarity coefficient for blackberry genotypes being used in this study.

might be affected by soil properties and climatic - conditions, as well as the topography of the location. Furthermore, vegetative traits such as the number of nodes, shoot length, and plant size have depended on light which the plant receives (Glisic et al., 2009). Also, hybridization between close species and changes in ploidy level could be one reason for the wide range of diversity in vegetative trait (Amsellem et al., 2001).

Ahmed et al. (2014) studied the genetic diversity of wild raspberry and reported leaf lengths and leaf widths between 91-124 mm and 94-122 mm, respectively, which were higher than those obtained in this study. Sedighi and Rahimmalek (2015) studied the morphological traits of wild *R. hyrcanus* and reported that the leaf length, leaf width, and petiole length were between 53 to 73 mm, 21.3 to 48.1 mm and 10.9 to 18.7 mm, respectively, which were in line but much lower than the values recorded in the current study. These differences could be mainly due to the higher number of species included herein, and larger geographical areas covered in this study, compared to the previous study that considered one species in a limited habitat.

Graham et al. (2003) showed that wild *R. ideaus* populations had a flowering time of 21 days, and a fruit ripening time that spanned 65 days, which were much lower than those recorded in this study (79 and 86 days on average, respectively). Also, Graham et al. (2009) evaluated fruit ripening time in different populations of raspberry during 4 years in different locations. They reported that the ripening time was from 21 April to 24 July in 2003, 18 May to 7 July in 2004, 10 May to 23 July in 2005 and 19 May to 6 July in 2006. They suggested that this variation was affected by longitude and altitude. In the higher altitude, flowering and ripening dates were later than in other areas. Also, the cultivated raspberries tend to have completed flowering and fruit set before any other wild population (Graham et al., 2003). In addition, geographic conditions and pollen grain also can affect the flowering and ripening time (Graham et al., 2009).

Some reproductive traits such as fruit size and the number of drupelets (as a yield component) have been a primary objective in all blackberry breeding programs (Clark and Finn, 2011). Unlike vegetative traits, fruit traits appear to be less influenced by rainfall or elevation. Yilmaz et al. (2009) reported that the fruit length and width in wild populations of *R. fruticosus* L. ranged from 7.8 to 11.4 mm and from 9.4 to 13.0 mm, respectively. Furthermore, they showed fruit weights varied from 0.4 to 1.2 g in wild genotypes.

These results are generally in agreement with the findings of the current research and the occurrence of minor differences may be due to different species and a varied number of genotypes in the two studies. Maro et al. (2014) evaluated genetic diversity in Brazilian raspberry cultivars and showed that fruit length in mountainous regions and in plains ranged from 13.5 to 23.8 mm and from 10.5 to 23.5 mm, respectively. Moreover, they reported that the fruit diameter ranged from 16.5 to 21.6 mm and from 13.8 to 21.5 mm in mountainous regions and in plains, respectively. These differences could be due to higher temperatures in the plains (Maro et al., 2014). The number of drupelets had a direct effect on fruit size and yield and ranged from 50 to 80 drupelets/fruit in commercial cultivars (Maro et al., 2014) which meant twice as much as that of the best genotype studied herein. Various factors can affect the number of druplets in wild genotypes, including pollen source (weakness of pollen grain in wild genotype), pollination, pollinator incompatibility and ploidy level (Graham et al., 2009).

Maro et al. (2014) studied genetic diversity in raspberry and reported the diversity index of fruit length, fruit width, fruit mass and number of drupelets which measured 9.63, 7.78, 20.29% and 19.37%, respectively. Singh et al. (2009) reviewed the genetic diversity of wild genotypes in the north-western Himalayan region and reported phenotypic coefficients of variation (PCV) in berry weight, berry length, and berry width which were 24.11, 13.3, and 11.5, respectively. They concluded that climatic conditions like temperature, sunlight, rainfall and snowfall can affect these traits. Blackberry yield varied substantially among cultivars, in response to different management practices and locations of production. In floricane blackberry, the yield depends on cane number, fruiting laterals per cane, and fruit weight. In primocane types, however, yield depends on cane number and amount of branching (Clark and Finn, 2011; Finn and Clark, 2012). Moreover, Weber et al. (2005) reported that blackberry yield ranged from 6.8 to 2.6 t/ha which was much more than the values recorded in the wild genotypes of the current study. They explained that the differences among yields resulted from genetic factors and environmental conditions which could be affected also by susceptibility to disease, pests and the production system (Weber et al., 2005).

Correlation among traits, principle component analysis (PCA) and cluster analysis

Associations among traits indicated whether the selection of one trait had an effect on another. Strong correlations among traits can assist breeders select important traits indirectly. This can accelerate and facilitate breeding programs. Established relationships between desirable traits can also help breeders with parental partner selection in breeding programs (Garazhian et al., 2020).

According to the results, a significant positive correlation was observed between cane lengths and number of nodes. Interestingly, a significant but negative association was proved between cane length and spine density. Almost all leaf attributes, including leaf length, leaf width, and petiole length correlated highly to each other as well as to internode length. The high positive correlations between the leaf characteristics and traits related to plant growth indicated that more leaf expansion can lead to stronger shrub vegetative growth (Khadivi-Khub & Anjam, 2014).

Flowering and ripening dates were highly correlated to each other. Yazdanpour et al. (2018) also reported a high and positive correlation between flowering and fruit ripening times ($r = 0.62$), which is in accordance but weaker than that reported in this study ($r = 0.98$). These differences can arise from the fact that the current study evaluated all genotypes in a single site, thereby eliminating the environmental effects. Although later-flowering accessions tended to have later fruit, the fruit growth and development was slightly accelerated, perhaps due to increased temperatures later in spring and summer (Lewers et al., 2010).

Not surprisingly, yield was positively correlated to its components including the number of inflorescences, number of fruits/inflorescence, fruit weight and number of drupelets/fruit. Interestingly, yield and many of its component were negatively correlated to spine density. Sonsteby et al. (2009) reported that yield was associated with cane height and the number of lateral buds in 'Glen Ample' raspberry. Perasovic (2013) reported that yield correlated strongly with the number of lateral shoots per cane. However, in the current study, no correlation was observed between cane length and yield. Also, they showed that raspberry yield was highly and positively correlated to fruit size ($r = 0.497$) which was consistent with our findings ($r = 0.54$). Negative correlation between spine density and some vegetative and reproductive traits could be explained since the production of spines can consume some of the potential carbohydrate sources in plants to produce protective organs against probable biotic and abiotic hazards.

Fruit weight had positive correlations with fruit

dimensions and number of drupelets/fruit. Gharaghani et al. (2014) and Yazdanpour et al. (2018) reported a positive correlation among fruit weight and fruit dimensions in Rubus species from Iran. Detecting useful and highly significant correlations between traits can help breeders with indirect selection of traits (easily or even cheaper) in germplasm, parental materials and seedling population's characterization. It also can help to improve the selection efficiency by using the proper combination of the traits.

Considering the results of principle component analysis (PCA), the first component (25.3% of total variations) featured mainly reproductive attributes, whereas the second component (15.8% of total variations) included phenological traits (i.e. flowering and ripening date) and number of nodes (Table 5). A high number of PCAs with small eigen values meant that the studied traits have wide distributions between genomic groups (maybe different chromosomes), and this will lead to a low resolution of clustering when using bi-plot analysis. The bi-plot of PCA showed that all genotypes were divided into two main categories based on the first two components according to the loading plot (Fig. 2). The cosine of the angles between vectors shows the extent of correlation among traits which is in accordance to the correlation analysis in almost all of the traits. The acute angles (<90°) represented positive correlations, whereas wide obtuse angles (>90°) showed negative correlations. The length of the vectors connecting traits to the origin showed the extent of variability. The PCA and bi-plot are in line with those reported by Yazdanpour et al. (2018).

According to the cluster analysis, the genotypes being studied were divided into two main clusters (Fig. 3). These results showed relatively good differentiation of genotypes based on species and to some extent based on their origin. Since there was a possibility of plant exchange among the studied regions, genotypes of different geographic origin may group together whereas genotypes of similar geographic origin may not have been placed necessarily in the same cluster. However, it should be mentioned that this analysis is based on a limited number of morphological traits and their corresponding data. Therefore, it may not be as reliable as larger numbers of morpho-chemical traits or molecular data. On the other hand, morphological markers are greatly affected by environmental conditions, so that using molecular markers (which were generated in the current study) can help to complete and refine the results (Clark and Finn, 2011). Ryu et al. (2014) evaluated the genetic diversity among fifty-six blackberries (*R. fructicosus*) using morphological characteristics. Based on their report, the studied blackberry germplasm was grouped into six clusters and two independent groups indicated an unclear pattern of division among the groups. Amsellem et al. (2001) studied genetic diversity among local and introduced genetic resources of *R. alceifolius* by cluster analysis and showed that the germplasm could be clustered into two main groups of native and exotic genotypes. Also Gharaghani et al. (2014) studied genetic diversity among *R. sanctus* genotypes grown in two distinct climatic conditions of Iran and found that 16 blackberry genotypes were grouped into two main clusters regardless of their origins, meaning that their results are largely consistent with the results of the current study. Yazdanpour et al. (2018) also used hierarchical clustering based on the dissimilarity of the genotypes to group *Rubus* germplasm of Babol County in the north of Iran and showed that the clustering pattern is mainly based on the location of genotypes. Garazhian et al. (2020) evaluated fruit bio-chemicals and antioxidant activity in a same set of genotypes that were studied herein, and reported differentiation of genotypes based on species and to some extent based on their origin. Graham et al. (2003) analyzed genetic similarity among wild accessions and cultivars of *Rubus ideaus* from different sites and showed that the clustering pattern of wild plant materials was largely according to their origin and the cultivars were genetically distinct from the wild populations.

ISSR Analysis, primer amplification and diversity indices

The ISSR markers analysis also provided a comprehensive insight of the genetic diversity among Iranian wild species. Considering all genotypes, the average polymorphic fragments per primer was of 11.3 with an average polymorphism of 78.72% (Table 4). These results are in accordance with previous results in similar studies on Rosaceae using ISSR markers (Hong et al., 2003), especially in the case of Rubus. In this regard, Sedighi and Rahimmalek (2015) used ISSR primer to assess the genetic diversity in several populations of *R. hyrcanus* from various geographical regions of the Caspian Sea in Iran. They reported an average polymorphism of 77% that was similar to the results of this study (78.72%). They reported relatively low levels of genetic diversity within populations but high variation among populations of this species collected from different geographical regions. Meanwhile, the results of the current research indicated a high genetic diversity among Iranian wild blackberry species. These differences can be because several species were evaluated in the

current study, compared to only one species (R. hyrcanus) in their research. Innis et al. (2011) surveyed genetic diversity in *R. phoenicolasius* and *R. argutus* using ISSR markers and concluded that a lack of genetic diversity in these clonal invasive species was due to fewer introductions into their invaded habitat or frequent self-fertilization and clonal reproduction. Lee et al. (2016) also estimated the genetic relationships among 69 *Rubus accessions* and reported that the average polymorphism was 0.76, which was largely in accordance with the findings of this research.

The highest and lowest PIC values were observed in P5 (0.53) and P6 (0.38), respectively, and the average PIC was 0.44 (Table 4). The average PIC of 0.55 and 0.49 were reported in red raspberry (Castillo et al., 2010), wild and cultivated black raspberry (Dossett et al., 2012), respectively, which was largely in agreement with the results of the current study.

Genetic diversity, cluster analysis and principal coordinate analyses (PCoA)

A dendrogram was generated by the Jaccard similarity matrix and by the UPGMA method, thereby revealing genetic relationships among genotypes of different species (Fig. 4). A high cophenetic correlation coefficient (0.897) was observed, indicating a good fit between the dendrogram clusters and the similarity matrices, which was higher than that (0.80) reported by Sedighi and Rahimmalek (2015) using the same molecular marker (ISSR). Jaccard similarity coefficients ranged from 0.33 to 0.90. The highest genetic similarity (90%) was observed between Astara 1 and Astara 2 genotypes (both belong to R. hirtus species). Meanwhile, genotypes of *R. persicus* had the highest genetic distance (67%) from the rest of the genotypes. The high molecular variation among wild Iranian *Rubus* species could be explained by the fact that they have not undergone any domestication process (suffering less selection pressure). Also, plant propagation systems and cross-pollination can affect variations among species (Sedighi and Rahimmalek, 2015; Amsellem et al., 2001).

According to the generated cluster based on the similarity matrix, genotypes were placed in three major groups based on their species. The separation of genotypes within this cluster was based on the species. With a few exceptions, the pattern of grouping within the main clusters was also based on the species and origin of the accessions, showing that clustering was based on the molecular data which not only differentiated among species but also assigned genotypes with relatively similar origin in identical groups within the main clusters. For instance, all genotypes of *R. sanctus* originated from different regions of the country and were located in the first large cluster. Within this large clad, the majority of *R. sanctus* genotypes from southern (Fars and Kohgiluyeh-Boyrahmad), western (Hamadan, Kurdistan and West Azarbayejan) and northern (Guilan, Mazandaran and Golestan) Iran were located in separate sub-clusters. However, some genotypes were clustered irrespective of their origin (for instance, the grouping of Jahrom-Khafr and Seyedan (Bag Bonyad) from Fars province in the south among genotypes of northern Iran) which could be due to germplasm transmission and exchange between regions (Gharaghani et al., 2014). The second cluster contained 20 genotypes including 10 genotypes of R. hirtus, 9 genotypes of *R. caesius* and one genotype of *R. discolor*. Furthermore, our finding suggested that the only genotype belonged to R. discolor and was placed in the sub-cluster of *R. caesius* genotypes, but quite distantly (Fig. 4 and 5).

The PCo analysis implied that the first eight principal coordinates totally explained 67.06% of the total variation (54.20% in the first three principal coordinates). In most cases, PCo analysis showed that the result corresponded to those obtained through cluster analysis and, thus, classified genotypes into four groups as follows: group1; all *R. sanctus* genotypes, group 2; all *R. hirtus* genotypes, group 3; all *R. caesius* genotypes and the only genotype of *R. discolor*, group 4; all *R. persicus* genotypes. This is while the majority of *R. hirtus*, *R. caesius* and *R. discolor* genotypes were classified in one group based on morphological cluster analysis (Fig. 3). The results of PCoA confirmed the results of cluster analysis based on similarity matrix (Fig. 4). A comparison between the clusters was obtained from morphological and molecular data. It showed higher and stronger discrimination ability of molecular markers in separating the genotypes. The results of morphological analysis were in general agreement with those obtained through molecular analyses, but had a lower resolution.

Lee et al. (2016) estimated the genetic relationships among sixty-nine *Rubus* accessions and found that all 69 *Rubus* accessions belonged to six species, which were classified into three groups, thereby concluding that an undertrained diversity of species and artificial groups of the *Rubus* genus have brought confusions with respect to the correct classification of the species at commercial and scientific levels.

Sedighi and Rahimmalek (2015) showed a relatively high agreement between the morphological and molecular data in *R. hyrcanus*

and concluded that this may be due to low environmental variation in the sampling regions. Also, Amsellem et al. (2001) illustrated a strong congruence in taxonomy and genetic diversity based on morphological and molecular markers on wild populations of *R. alceifolius*. In contrast, Ryu et al. (2014) reported a low correlation between the cluster analysis of morphological and molecular markers. A low correlation occurred between molecular and morphological markers, which might have been caused by primers of the intron regions of the genome. Also, it is specified that biological characteristics in plants and habitat fragmentation could affect the performance of morphological traits and this might therefore play an important role in weak associations between the results obtained by molecular and morphological markers (Sedighi and Rahimmalek, 2015).

Conclusion

The results of this study revealed a high level of genetic variation among and within Iranian wild blackberry species, with respect to vegetative, phenological and reproductive traits, which could be utilized in future breeding programs. Also, the results implied some positive/negative correlations between the traits which could be very useful in an efficient screening of germplasm and in breeding populations. An analysis of morphologic data with PCA showed that the genotypes were divided into two main categories which reflected known differentiation among species and among their origin. The ISSR marker analysis also provided a comprehensive insight of the genetic diversity among Iranian wild species. The results of morphological cluster analysis were in general agreement, but had lower resolution compared to those obtained through molecular analyses. The current study provided basic information for phylogeny, taxonomy and breeding programs in some Iranian Rubus species. However, it seems that more research on genetic diversity can encompass more genotypes as well as commercial cultivars in order to categorize, clarify and classify more precisely, confirm and approve the current results.

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Data availability statement

All necessary data are presented in the manuscript and no other data are publicly available.

Author contributions

AG and SE design of the work. MG and AT carried out the analysis. AG and MG carried out the interpretation of data. MG, AG, and SE have drafted the work.

Supplementary material

No supplementary data is presented.

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Conflict of interest

The authors declare that they have no conflict of interest.

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