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Evaluation of Genetic Diversity of Wild Raspberry Genotypes in the Western Caspian Sea Regions by ISSR Molecular Markers

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ABSTRACT

The raspberry is an edible fruit belonging to the Rubus genus within the Rosaceae family. The species is known for its high global genetic diversity. Raspberries are highly regarded for their nutritional benefits, particularly their contributions to dietary fiber and vitamins. This species contains numerous wild genotypes, and it is essential to discover and investigate them in this experiment. Managing natural diversity in domestic cultivars and wild relatives plays a crucial role in creating targeted programs to improve plant products. The study aims to identify and examine various wild genotypes within this species. For this, 48 wild raspberry genotypes were gathered from multiple locations along the western borders of the Caspian Sea forests, specifically from Fandoghlo, Astara, Germi, and Bilesavar in Iran. The genetic diversity of these genotypes was assessed using 12 ISSR primers. The analysis of the observed bands revealed a significant range of polymorphism, varying from 33% to 100%, with an average polymorphism rate of 77%. Based on cluster analysis results, the genotypes were classified into three main clusters. The first and second cluster analysis groups were located in the west and southwest of the Caspian Sea. The findings of this research indicated that there is sufficient genetic diversity between raspberry wild genotypes, which is largely affected by the geographical regions of the genotypes. Certainly, regions that were more distantly located exhibited a higher genetic diversity between their genotypes. This phenomenon can be attributed to the role of cross-pollination facilitated by insects, as well as the influence of natural barriers.

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Introduction

Raspberry (*Rubus idaeus*), or the red raspberry, is a deciduous perennial shrub of the Rosaceae family with economically valuable soft fruit. The family contains 500 species of Rubus, which is noted for its high heterozygosity and broad range of ploidy levels (Graham and McNicol, 1995). Raspberries are plants that typically grow in the edges of forests, are native to Europe and northern Asia, and spread naturally in temperate regions (Hancock, 2008). It is asserted that the central zone of variation and scattering of raspberry species in Iran primarily occurs in the north, northwest, and northeast. It is extensively found on the slopes of the Alborz Mountain range, particularly in the Caspian provinces, such as the northern coastal provinces from Gilan to Mazandaran and Gorgan (Gharesheikhbayat and Padasht, 2020). The fruit of *R. idaeus* is a high content of healthful phytochemicals, such as anthocyanins, catechins, salicylic acid, ascorbic acid, riboflavin, and quercetin, and is very good source of nutrients (Davik *et al*., 2022), and plays a minor role in the removing of stomach and colon cancer cells (Kula *et al*., 2016). Genetic diversity research

can improve natural resource conservation strategies, provide information on genetic relations between ecotypes or populations and among species, and estimate genetic relationships to determine cultivars. This can make it easier to select parents for Hybridization, which can be beneficial (Ghanbari and Estaji, 2018). The natural diversity of domestic cultivars and crop wild genotypes is at the core of developing specifically targeted programs to enhance plant products (Naghavi *et al*., 2010).

The different methods have been established to identify genetic diversity, from morphological characteristics to various molecular markers like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), Inter-simple sequence repeat (ISSR), and simple sequence repeats or microsatellites (SSR) (Ghanbari *et al*., 2019). Morphological markers can be useful indicators but are limited in their utilization by certain determinants like low levels of polymorphism and greater sensitivity to alterations in environmental condition characteristics that molecular markers do not possess (Victoria *et al*., 2011).

Cekic and Erdem (2018) analyzed 19 wild raspberry genotypes sourced from the Black Sea region of Turkey using ISSR markers. Their findings revealed the presence of 111 bands generated from 15 UBC ISSR primers. The resulting dendrogram analysis categorized the genotypes into two primary groups: a smaller group of three genotypes and a larger group encompassing the remaining genotypes. Furthermore, ISSR markers are recognized as effective tools for genetic analysis among genotypes and commercial cultivars. This method typically utilizes DNA fragments ranging from 100 to 3000 base pairs between oriented microsatellite regions. It is characterized by its speed, simplicity, cost-effectiveness, and high reproducibility, making it capable of identifying multiple polymorphic gene loci. Consequently, ISSR markers have demonstrated their utility in genomic fingerprinting, phylogenetic research, and gene mapping (Zinodini *et al*., 2013). In this study, ISSR primers were employed to assess the genetic diversity of wild raspberries in the western regions surrounding the Caspian Sea.

Materials and Methods

Plant material

This research was carried out in 2021, focusing on 48 genotypes of wild raspberry. The genotypes were sourced from various locations along the western edges of the Caspian Sea forests, specifically from Fandoghlo, Astara, Germi, and Bilesavar in Iran (Fig. 1). This area is characterized by a temperate climate, featuring warm, humid summers and cold, wet winters. (Table 1).

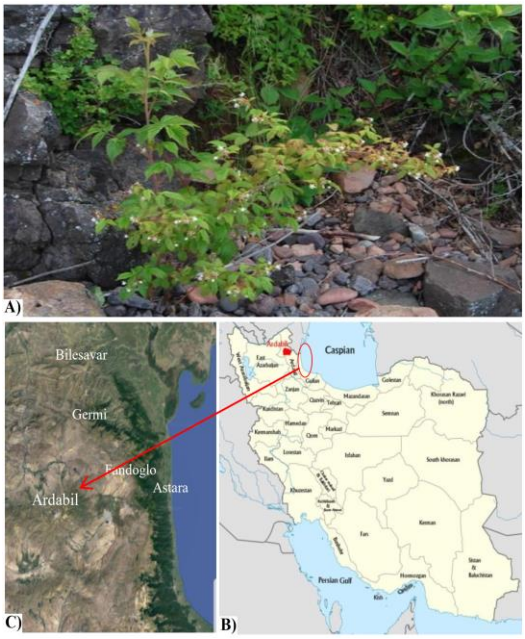

Fig. 1. Place of collection of raspberries in the western edges of Caspian Sea forests: A) A raspberry genotype in the region of the Astara; B and C) Collection areas of raspberry genotypes.

DNA Extraction

Genomic DNA was extracted from 100 mg of fresh mature leaves from each of the 48 genotypes using a modified CTAB method (Ghanbari and Estaji, 2018). The leaves were finely ground in a pre-sterilized mortar and pestle with liquid nitrogen. Following this, 1 ml of extraction buffer, composed of 100 mM, Tris-HCl; 2 M, NaCl; 20 mM, $EDTA_{Na2}$; and 2% (w/v), Polyvinylpyrrolidone at pH 8.0, was added to the powdered leaves. The mixture was blended and incubated in a water bath at 65˚C for 45 minutes, followed by thorough vortexing. Once the tubes reached room temperature, an equal volume of a 24:1 (v/v) chloroform:

isoamyl alcohol mixture was added, and the solution was shaken vigorously to create an emulsion. After centrifugation at 11000×g for 20 minutes, the supernatant was carefully collected and transferred to a new tube, where it was mixed with 0.8 (v/v) volumes of cold isopropanol (-20˚C). This mixture was then centrifuged for 5 minutes at 10000× g, allowing for the separation of the upper aqueous phase. The resulting DNA precipitate washed with 70% (v/v) ethanol, after which the pellets were dried and resolved in 20 µl of sterile double-distilled water. The quality of the extracted DNA was evaluated using 0.8% agarose gel electrophoresis.

Table 1. Geographical coordinates of the studied areas.

Regions	latitude	Longitude	Height	Number of plants studied
Fandoghlo	38º 30' N	47º 59' E	1620	1-10
Astara	38º 27' N	48º 34' E	1536	11-31
Bilesavar	38º 19' N	48º 35' E	1850	32-40
Germi	38˚ 57' N	48º 10' E	1062	40-48

ISSR fingerprinting

Twelve ISSR primers were chosen to amplify genetic material from raspberry, as shown in Table 2. The selection criteria for amplified DNA fragments were polymorphism, clarity, and reproducibility. DNA amplification was conducted in reactions of 10 µl. Each 10 µl PCR reaction contained 20 ng of genomic DNA, 5 µl of a PCR kit (Sigma, St. Louis, MO, USA), 1.1 µl of primer, and 3 µl of double-distilled water. The amplification was generated using a Q-cycler thermocycler (Hain Lifescience, UK), programmed for a 5 min denaturation step at 94˚C, then 35 cycles, of 45 s at 94˚C, 30 s at 44-52˚C (depending on the primers), and 1 min at 72˚C, followed by 7 min at 72˚C. Amplification products were distinguished in 1.5% (w/v) agarose gels containing 10% (v/v) fluorescent dye (GelRed Ampliqon) in 1×TBE buffer at 85 V for 60 min. The ISSR bands were visualized under UV light and photographed using a digital camera. A 100-base pair DNA molecular weight marker (New England Bio Labs, USA) was used for standard sizes.

Data analysis

The bands observed were scored according to the presence being coded 1, or absence being coded 0, of each entry. Very faint and non-reproducible bands were excluded from scoring. The genetic parameters, such as number of scored band (NSB), number of polymorphic (NPB), observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H), and Shannon's Information index (I), was calculated by PopGene program version 1.31 (Jamali *et al*., 2019). Cluster tree was performed by WARD (Minimum spherical cluster) dissimilarity index using Windows (DARwin5) software (Perrier *et al*., 2006).

Results and discussion

In the present study, twelve ISSR primers were used to examine the 48 genotypes of raspberry. The 99 bands were obtained, of which 80 were polymorphic (Table 3). The number of polymorphic bands ranged from 12 (ISSR12) to 6 (ISSR5 and ISSR8); the average number of polymorphic bands was determined to be 8.2 per primer. The rate of polymorphism varied extensively, from 33% to 100%, and averaged 77% polymorphism. PIC values varied from 0.47 for ISSR5 to 0.64 for ISSR12, and averaged 0.57 for all primers. The observed and effective number of alleles varied from 24 to 19.87 for ISSR 12, and 4 to 3.07 for ISSR8 (Fig. 3), with all means summing to 11.46. The values of gene diversity for Nei varied between 0.48 for ISSR12 and 0.33 for ISSR8, with a mean of 0.40 (Table 3).

Cluster analysis

Based on the cluster analysis results (Fig. 2), genotypes were divided into three groups. The first group comprised genotypes 1-15, 17-28, 30-32, and 47 (30 genotypes). The second group comprised genotypes 33-37, 39-41, 46-43, and 48 (15 genotypes), and the third group comprised genotypes 38, 16, and 42 (three genotypes), which are shown in Figure 2. The genotypes in the first group belonged to the

western Caspian Sea regions, specifically the eastern parts of Pars-Abad and Bileh-Savar, which had minor variation in terms of geographical distance and were grouped under the same locality. This region is characterized by a temperate climate with dry summers. The average winter temperature is 3°C. The second group genotypes were southwest of the Caspian Sea (East Nemin and Garmi forests). The exception was the three genotypes from different regions that were placed in one category.

Table 2. Characteristics of primers used to investigate the raspberry genotype.

ISSR markers	Sequences (5′→3′)	Tm (ºC)	References
ISSR1	5′-DBD $(CA)_7$ -3′	52	Bahmani *et al.*, 2015
ISSR2	5′- $(GA)_8$C-3′	54	Brake *et al.*, 2014
ISSR3	5′- $(AG)_8$YA- 3′	53	Brake *et al.*, 2014
ISSR4	5′- $(CA)_8$ G- 3′	54	Bahmani *et al.*, 2015
ISSR5	5′- $(CA)_8$ A- 3′	50	Brake *et al.*, 2014
ISSR6	5′- $(AG)_8$T- 3′	50	Pivorienė and Pašakinskienė, 2008
ISSR7	5′- $(GGG\ T)_4$- 3′	58	Brake *et al.*, 2014
ISSR8	5′- $(CT)_8$CRC- 3′	55	Brake *et al.*, 2014
ISSR9	5′- $(GA)_8$T- 3′	50	Brake *et al.*, 2014
ISS10	5′- $(GA)_6$ CC- 3′	44	Brake *et al.*, 2014
ISSR11	5′- $(ATG)_6$- 3′	47	Brake *et al.*, 2014
ISSR12	5′- $(CT)_8$ TT- 3′	52	Pivorienė and Pašakinskienė, 2008

D= A, G or T; B= C, G or T; V= A, C, or G; Y= C or T; R= G or A

Table 3. List of primers and genetic variation characteristics between 48 Rubus genotypes.

ISSR markers	Total fragments	Polymorphic bands	%Polymorphic	Na	Ne	H	I	PIC
ISSR 1	7	7	100	14	11.71	0.38	0.56	56
ISSR 2	**9**	9	100	**18**	**15.31**	0.39	0.58	57
ISSR 3	7	5	71	10	8.10	0.36	0.54	54
ISSR 4	**11**	9	81	**18**	**15.74**	0.41	0.60	61
ISSR 5	6	3	50	6	5.80	0.42	0.67	47
ISSR 6	7	5	71	10	9.18	0.44	0.63	63
ISSR 7	8	7	87	14	12.70	0.44	0.63	63
ISSR 8	6	2	33	4	3.07	0.33	0.51	50
ISSR 9	9	7	77	14	12.68	0.44	0.63	63
ISSR 10	9	8	88	16	13.86	0.41	0.60	59
ISSR 11	8	6	75	12	8.50	0.35	0.53	53
ISSR 12	**12**	12	100	**24**	**19.87**	**0.48**	0.53	62
mean	99	80	77.75	11.46	11.46	0.40	0.58	57.33

Na= Observed number of alleles; Ne= Effective number of alleles; H= Nei's gene diversity; I= Shannon's information index; PIC= Polymorphism information content.

Genetic distance was estimated by the Dice coefficient, on a scale of 0 to 1. The genetic distance among the wild genotypes of raspberry varied from 0.09 to 0.59. In addition, the calculated mean genetic distance was 0.29. A small genetic distance was observed between genotypes 8 and 25 (0.09), and between genotype 25 and genotypes 6, 9, 10, 15, and 24 (0.12). These genotypes were all grouped in the first group and were found in the same geographical region. The maximum genetic distance between genotypes 30 and 42 was 0.59; These genotypes, which were grouped into the first and second clusters, were located in the western and southwestern regions adjacent to the Caspian Sea. This genetic distance measure reveals a high level of genetic diversity among the raspberry genotypes investigated (da Silva Angelo *et al*., 2014).

Genetic diversity is a fundamental requirement for enhancing plant breeding genetics, while heterosis relies upon the level of genetic divergence among the parental lines (Linos *et al*., 2014). In addition, genetic distance among various genotypes is also associated with their respective geographical positions (Atapour *et al*., 2022). Graham and McNicol (1995) explained that populations of wild raspberries comprise isolated clusters, which are usually spatially separated and exhibit local adaptation. The

results of cluster analysis and genetic distance suggest that the genetic differences between the raspberry populations of the western and southwestern Caspian Sea can be caused by environmental factors, such as pollen, seed disruption, and geographical distance, which may affect gene flow between populations.

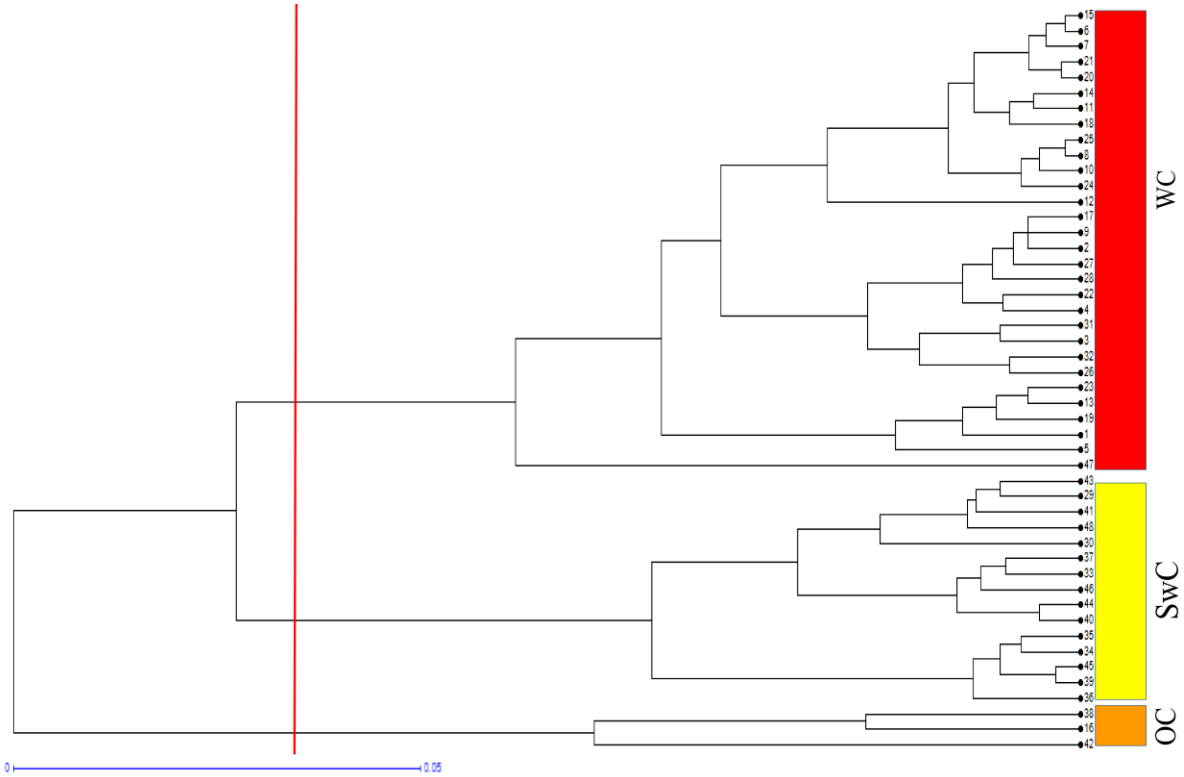

Fig. 2. Cluster analysis of wild raspberry ecotype by WARD method: WC= Western Caspian Sea regions; SwC= Southwest regions of the Caspian Sea; OC= Outspread genotypes.

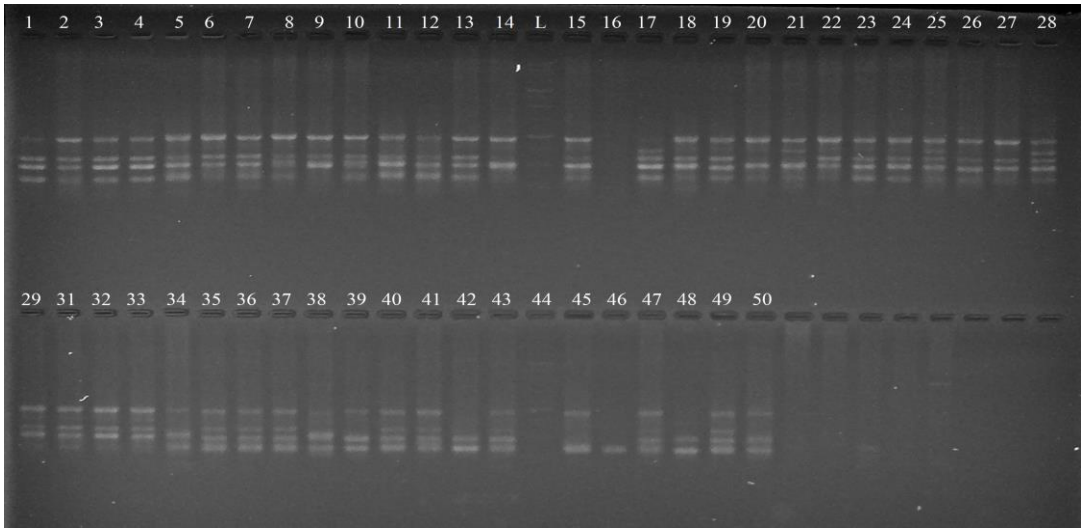

Fig. 3. Patterns of bands of Raspberry genotypes amplified by ISSR8. The DNA ladder of 100 bp (New England Bio Labs, USA) placed between lines 14 and 15, as well as 42 and 43.

The genetic diversity observed among individuals of the same agroclimatic regions was low. Several scientists have already discussed distinctions among populations of various plant

species determined by ecological conditions (Patamsytė *et al*., 2010). Numerous environmental factors have been found to cause isolation and influence genetic exchange in plant populations.

Conclusion

Raspberry is a forest and forest-edge species. The plant species is properly distributed in Iran, and, as it is important, its cultivation regions and genetic variation should be studied. Thus, genetic diversity of raspberry was studied; the findings revealed that 48 genotypes studied with ISSR markers possessed high genetic diversity in the southwestern and western regions of the Caspian Sea and they were divided into three main groups that were genetically far from each other with high genetic distance and this genetic diversity was associated with the studied geographical regions.

Conflict of interests

The authors declare no conflict of interest.

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