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Assessment of the genetic diversity and population structure of *Vaccaria hispanica* (Mill.) Rauschert germplasms of Türkiye based on SRAP and SSR markers

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Abstract: *Vaccaria hispanica* (Mill.) Rauschert has been studied for its potential economic value. Its seeds contain valuable starch granules, triterpenic saponins, and cyclopeptides. In the present study, sequence-related amplified polymorphism (SRAP) and microsatellite simple sequence repeat (SSR) markers were used to assess the genetic diversity and population structure of *V. hispanica* genotypes. The plant material belonged to 66 genotypes collected from different parts (27 squares, with at least two locations in each square) of Türkiye. The application of 49 SRAP primer pairs produced 476 bands, of which 197 (41.38%) were polymorphic. The polymorphic information content (PIC) ranged from 0.12 to 0.46 with a mean of 0.30. The genetic distance coefficients among the accessions ranged from 0.52 to 0.91, with an average of 0.70. The 17 SSR primers previously developed for related species produced a total of 69 alleles and 42 polymorphic alleles, and the number of polymorphic alleles produced in each locus ranged from 1 to 6, with an average of 2.47 alleles per locus. The PIC ranged from 0.21 to 0.49 with a mean of 0.40. The unweighted pair group method of arithmetic averages (UPGMA) dendrogram obtained based on the markers revealed that the genotypes were genetically different from each other and were partially geographically grouped. Bayesian Structure analysis carried out using the marker data divided the genotypes into two subpopulations. It showed a general agreement with the UPGMA dendrogram on the genetic relationships among the accessions and clustering. Thus, it was determined that genetically distinct genotypes exist, and subpopulations for breeding can be established using the characterized genotypes.

Key words: Cow cockle, genetic relationship, genetic variation, molecular markers, wild accessions

1. Introduction

Vaccaria hispanica (Mill) Rauschert (synonyms *V. pyramidata*, *V. segetalis*, *Saponaria vaccaria*), belonging to the family Caryophyllaceae, is an annual herb widely distributed in Asia, Europe, and other parts of the world (Sang et al., 2003). *V. hispanica* is also known as 'cow cockle' in English and 'ekinebesi' in Turkish. It has been used in traditional medicine in China for centuries and is cultivated in Japan as a garden plant to a limited extent (Sang et al., 2003; Meesapyodsuk et al., 2007).

Different parts of *V. hispanica*, including the roots, stems, leaves, flowers, and seeds, have been reported to have medicinal usage (Balsevich et al. 2006). According to Duddu et al. (2015), because of its medicinal and industrially valuable seed constituents, *V. hispanica* is being considered for commercial production. Regarding medicine, it includes several quillaic acids, gypsogenin bisdesmosides, flavonoids, cyclopeptides, and significant amounts of mono- and bisdesmosidic triterpene saponins (Jia et al., 2002) and has promising beneficial effects, including prolactin-like, estrogen-like, antitumor, antiangiogenesis, and antioxidant activity (Tian et al., 2021). In addition, as an industrially important compound, *V. hispanica* seeds have a high amount of small (0.5–1.6 µm in diameter) starch grains (Balsevich, 2008). All of these and other compounds (i.e. cyclopeptides, antioxidant phenolics, ribosome-inactivating proteins, and oils) have potential use in the medical, vaccine (as an adjuvant), cosmetic, herbal, food, nutraceutical, feed (as an additive), and veterinary industries (Balsevich, 2008). Consequently, its valuable seed components and advantageous agricultural characteristics have recently made *V. hispanica* an important candidate as a new alternative medical-industrial product for Canada (Willenborg and Johnson, 2013).

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However, *V. hispanica* has been recognized as a significant weed species all over the world for a long time and has been subjected to extensive herbicide treatment. As a result, there have been reports of declining populations of *V. hispanica* species worldwide in recent years. For example, the frequency and distribution of the species has dramatically decreased in Canada (Harker et al., 2009). Likewise, *V. hispanica* species have been declining in Europe (Torzewski et al., 2018). It was considered extinct in the Czech Republic (Kropáč, 2006) and Poland (Węgrzynek, 2014; EX category, Kaźmierczakowa et al., 2016; category RE), and endangered in Austria (Niklfeld and Schratt-Ehrendorfer, 1999) and Croatia (Hulina, 2005). As for Türkiye, although it is reported that the *V. hispanica* species grows naturally and is spread widely in almost every part of the country with different climatic and geographical conditions, the species is also under various pressures. Thus, it is necessary to characterize the available germplasms for conservation as well as better utilization of the species. In a previous study, the 66 natural Turkish *V. hispanica* genotypes, the same genotypes used in this study that represent the genetic pool of the country, were characterized for 21 morphological and six agronomic traits (Arı et al., 2022). However, genetic characterization is needed to better evaluate the breeding utility of these genotypes.

Genetically distinct genotypes that adapt to different climatic and soil conditions have a valuable potential as a genetic resource. Establishing gene pools representing the available genetic variation will allow for the inclusion of genotypes with desired characteristics in breeding new cultivars. Knowledge about the geographic origin and genetic diversity of the species will contribute significantly to the collection and conservation of genetic resources. Analyses of the genetic variation and population structure of different genotypes are important for their effective conservation and the utilization of valuable genetic resources.

The evaluation of genetic diversity can be enhanced using DNA markers. Although high-throughput genotyping technologies can provide many markers, the traditional markers can still be useful for some studies, such as genetic diversity analysis, and sequencing of all of the genomes may not be necessary (Zhang et al. 2014; Giordani et al., 2019). Sequence-related amplified polymorphism (SRAP) targets open reading frames (ORFs) in the genome and is more reproducible, stable, and less complicated than other marker systems (Agyenim-Boateng et al., 2019; Li and Quiros, 2001). SRAP has been employed successfully for the construction of genetic maps (Levi et al., 2011; Guo et al., 2012; Wang et al., 2018), identification of quantitative trait loci (QTL) (Chen et al., 2007; Zhang et al., 2009; Li et al., 2015), and analysis of genetic diversity in different plant species (Zheng et al., 2017; Ma et al., 2021; Zhou et al., 2021).

Microsatellites, also known as SSRs are highly polymorphic, distributed throughout the genome abundantly (Miah et al., 2013), and have been utilized for the development of genetic maps, identification of QTL (Wang et al., 2017; Bhagya et al., 2020; Ma et al., 2020), and analysis of genetic diversity (Ali et al., 2019; Adjebeng-Danquah et al., 2020) in many plant species. Although SSR markers are developed as species-specific, they can be transferred to related species. SSRs are more transferable between species of the same genus (Hendre et al., 2008; Mnejja et al., 2010).

However, to our knowledge, the genetic relationship of *Vaccaria* genotypes has not been studied in Türkiye or in the world. Therefore, the present study aimed to analyze the population structure and genetic diversity of *V. hispanica* genotypes collected from different parts of Türkiye using SRAP and SSR molecular markers.

2. Materials and methods

All 66 germplasm accessions of *V. hispanica* (Mill.) Rauschert used in the present study were collected from 61 different provinces and districts (27 squares, with at least two locations in each square) of Türkiye based on the Turkish Grid System (Davis, 1970). The samples were collected with the official permission of the Republic of Türkiye Ministry of Agriculture and Forestry. The collected seeds were germinated in seedling containers and transferred to the greenhouse at Akdeniz University, Antalya, Türkiye. The fresh leaves were collected from each genotype and stored at –20 °C until DNA extraction. Detailed information about the samples and localities is presented in Table 1 and Figure 1. Genomic DNA was extracted from ~120 mg of frozen leaf tissue using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). The quality of the DNA was estimated through electrophoresis using 1% (w/v) agarose gel with ethidium bromide staining. The DNA concentration was assessed using a NanoDrop Spectrophotometer (ND-1000; NanoDrop Technologies, Inc, Wilmington, DE, USA). The DNA was diluted to 50 ng using sterile DNase-free water.

2.1. SRAP analysis

To screen the 66 *V. hispanica* accessions, 208 SRAP combinations were used, including 13 forward and 16 reverse SRAP primers (Li and Quiros, 2001). The SRAP primer combinations were previously evaluated in bulk DNA of *V. hispanica*, including equal amounts of DNA of 10 randomly selected genotypes, and 49 SRAP primers that produced a high level of polymorphism and clear banding pattern were selected (Table 2).

Table 1. Geographical descriptions for the collection areas of the *V. hispanica* genotypes in Türkiye.

Gen. no.	Square no.	Collection location	Altitude (m)	Gen. no.	Square no.	Collection location	Altitude (m)
1	A1-1	Çanakkale – Lapseki	225	34	B8-2	Bingöl – Yedisu	1.440
2	A1-2	Çanakkale – Lapseki	681	35	B9-1	Bitlis – Tatvan	1.795
3	A2-1	Bursa – İnegöl	918	36	B9-2	Van – Erciş	1.654
4	A2-2	Bursa	758	37	C1-1	Muğla – Bencik	585
5	A3-1	Bolu – Mudurnu	979	38	C1-2	Aydın – Didim	92
6	A3-2	Ankara – Nallıhan	793	39	C2-1	Antalya – Elmalı	1.070
7	A4-1	Çankırı – Ilgaz	873	40	C2-2	Denizli – Honaz	1.110
8	A4-2	Kırıkkale – Kalecik	642	41	C3-1	Antalya – Korkuteli	915
9	A5-1	Sinop – Boyabat	411	42	C3-2	Isparta – Burdur	1.346
10	A5-2	Tokat – Zile	1.148	43	C4-1	Konya – Çumra	1.058
11	A6-1	Tokat – Niksar	566	44	C4-2	Karaman	1.038
12	A6-2	Sivas – Suşehri	818	45	C5-1	Konya – Ereğli	1.046
13	A7-1	Giresun – Şebinkarahisar	1.399	46	C5-2	Adana – Saimbeyli	1.180
14	A7-2	Giresun – Kelkit	1.411	47	C6-1	Kahramanmaraş	1.106
15	A8-1	Erzurum – Bayburt	1.543	48	C6-2	Adıyaman – Gölbaşı	907
16	A8-2	Erzurum – İspir	1.269	49	C7-1	Adıyaman – Kahta	670
17	A9-2	Erzurum – Horasan	1.556	50	C7-2	Urfa – Siverek	748
18	A9-2	Iğdır – Kağızman	1.044	51	C8-1	Mardin	570
19	B1-1	İzmir – Sabunbeli	593	52	C8-2	Batman – Gercüş	901
20	B1-2	Balıkesir	192	53	C9-1	Siirt – Eruh	965
21	B2-1	Manisa – Gördes	731	54	C9-2	Şırnak	1.577
22	B2-2	Kütahya – Çavdarhisar	1.011	55	A10	Iğdır	847
23	B3-1	Afyon – Dinar	1.004	56	A6-3	Sivas – Suşehri	1006
24	B3-2	Eskişehir – Sivrihisar	1.191	57	A7-3	Gümüşhane – Salyazı	1.674
25	B4-1	Konya – Kulu	1.101	58	A7-4	Bayburt – Pazaryolu	1.398
26	B4-2	Konya – Altınekin	973	59	B1-3	Manisa – Kırkağaç	240
27	B5-1	Kayseri – Develi	1.092	60	B8-3	Erzurum – Çat	1.894
28	B5-2	Yozgat – Sarıkaya	1.438	61	C2-3	Antalya – Kaş	859
29	B6-1	Kayseri – Sarız	1.581	62	C2-4	Denizli – Honaz	1.137
30	B6-2	Sivas- Ulaş	1.338	63	C2-5	Antalya – Elmalı	1.070
31	B7-1	Malatya	1.066	64	C2-6	Antalya – Elmalı	1.033
32	B7-2	Erzincan – Alpköy	1.085	65	C2-6	Antalya – Elmalı	1.033
33	B8-1	Muş	1.280	66	C2-6	Antalya – Elmalı	1.033

Polymerase chain reaction (PCR) amplification was performed with a total reaction volume of 15 µL using a Bio-Rad T100 thermal cycler (Hercules, CA, USA). The reaction mix consisted of 1.5 µL of 10X Taq polymerase buffer, 1.5 µL of 25 mM $MgCl_2$, 1.5 µL of 2 mM dNTPs, 0.8 U of Taq DNA polymerase (5 U/µL) (Thermo Fisher Scientific Inc., Waltham, MA, USA), 1 µL of 10 µM primer/s, 1 µL of template DNA (~50 ng), and 6.7 µL of sterilized distilled water. SRAP-PCR analysis was carried out according to the procedure described by Li and Quiros (2001) and the PCR cycling profile involved 1 cycle for 1 min at 94 °C, 5 cycles of 1 min at 94 °C, 1 min at 35 °C, 1 min at 72 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C, and a final extension for 5 min at 72 °C. The PCR products were separated on 2% agarose (Amresco; VWR International, LLC., Radnor, PA, USA) gel electrophoresis in 1× Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer.

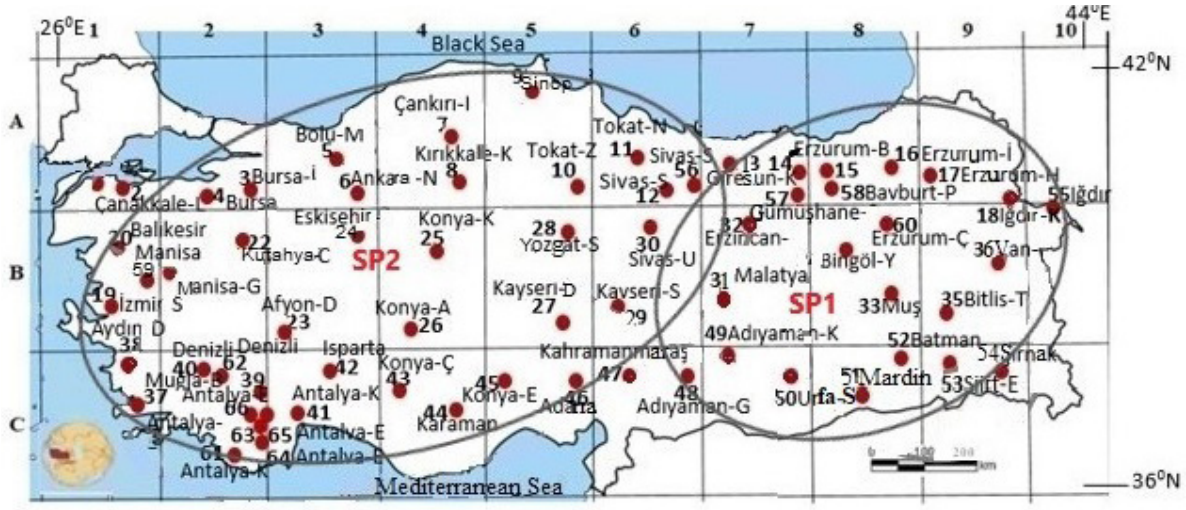

Figure 1. Map of the area where the *V. hispanica* samples were collected. Representation of the two subpopulations (SP1 and SP2) determined as a result of the Structure analysis.

Table 2. List of the SRAP primers used to detect the polymorphisms, number of amplified products, polymorphism percentage, and polymorphism information content.

No.	Primer code	NTB*	NPB**	Polymorphism (%)	PIC	No.	Primer code	NTB	NPB	Polymorphism (%)	PIC
1	Me1Em4	11	4	36	0.26	26	Me7Em5	10	3	30	0.34
2	Me1Em5	11	2	18	0.30	27	Me8Em2	12	9	75	0.38
3	Me1Em10	15	5	33	0.18	28	Me8Em6	10	5	50	0.36
4	Me1Em12	11	6	54	0.26	29	Me8Em11	11	2	18	0.24
5	Me1Em15	7	3	42	0.42	30	Me9Em2	11	8	72.7	0.34
6	Me2Em6	12	4	33	0.36	31	Me9Em5	8	4	50	0.22
7	Me2Em7	6	3	50	0.40	32	Me9Em7	8	3	37.5	0.45
8	Me2Em8	8	1	12.5	0.14	33	Me9Em11	3	2	66.6	0.17
9	Me2Em15	10	3	30	0.39	34	Me10Em1	12	2	16.6	0.37
10	Me3Em8	5	1	20	0.27	35	Me10Em2	11	7	63.6	0.25
11	Me3Em10	10	4	40	0.31	36	Me10Em4	17	6	35.2	0.41
12	Me3Em11	6	2	33.3	0.34	37	Me10Em6	6	4	66.6	0.12
13	Me3Em12	7	4	57.1	0.24	38	Me10Em7	16	9	56.25	0.30
14	Me3Em14	9	3	33.3	0.41	39	Me10Em8	11	4	36.3	0.39
15	Me4Em3	12	4	33.3	0.23	40	Me10Em9	8	3	37.5	0.27
16	Me5Em2	12	4	33.3	0.33	41	Me11Em2	14	14	100	0.27
17	Me5Em3	12	5	41.6	0.35	42	Me11Em9	12	3	25	0.24
18	Me5Em5	6	3	50	0.18	43	Me11Em12	6	2	33.3	0.26
19	Me5Em6	8	3	37.5	0.24	44	Me12Em11	7	4	57.14	0.24
20	Me5Em7	8	4	50	0.36	45	Me13Em1	5	1	20	0.29
21	Me5Em8	11	2	18.8	0.46	46	Me13Em3	14	7	50	0.35
22	Me5Em9	8	4	50	0.22	47	Me13Em4	9	4	44.4	0.42
23	Me6Em1	10	5	50	0.39	48	Me13Em5	5	3	60	0.23
24	Me6Em2	9	4	44.4	0.23	49	Me13Em6	16	3	18.75	0.26
25	Me7Em3	10	2	**20**	0.26	T		476	197		
						M		9.71	4.02		

*NTB: Number of total bands,
**NPB: number of polymorphic bands.

2.2. SSR analysis

A total of 17 SSR primer pairs, comprising AGA1-150, AGA5-123, and AG9-49 developed for *Arenaria grandiflora* L. (Zavodna et al., 2009); CB011a, CB020a, CB041a, CB047a, and CB060a developed for Carnation (*Dianthus caryophyllus*) (Kimura et al., 2009); SA06, SA30, SA32, SA04, SA09, SA16, and SA36 developed for *Schiedea adamantis* (Culley et al., 2008); and SpI_c2 and SpI_d3 developed for *Spergularia media* (L.) C. Presl. (Prinz et al., 2009) and previously tested for amplification and polymorphism performance in *V. hispanica* (İkten et al., 2016) were employed to estimate genetic diversity among 66 *V. hispanica* genotypes. The information about the SSR primers employed for the SSR-PCR reactions is presented in Table 3.

PCR was performed in 15-mL reaction volumes containing 1.5 µL of 10X Taq polymerase buffer, 1 µL of 25 mM $MgCl_2$, 1 µL of 2 mM dNTPs, 0.5 U of Taq DNA polymerase (5 U/µL) (Thermo Fisher Scientific), 1 µL of 10 µM primer/s, 1 µL of template DNA (~50 ng), and 8 µL of sterilized distilled water. The amplification was carried out using the following PCR conditions: 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 30 s at 53–60 °C (the annealing temperature varied depending on the primer used), and 45 s at 72 °C, with a final 3 min incubation at 72 °C. The PCR products were separated on 2.5% superfine resolution agarose (Amresco) gel electrophoresis in 1× TAE buffer.

2.3. Data analysis

The amplified products for the SRAP and SSR markers were scored visually as 1 for the presence and 0 for the absence of a band to generate a binary data matrix. First, a similarity matrix was created and then cluster analysis was performed to construct a dendrogram based on the similarity matrix data obtained by unweighted pair group method using arithmetic averages (UPGMA) using the SimQual mode of the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc version 2.1; Exeter Software, Setauket, NY, USA) (Rohlf, 1993). Polymorphism information content (PIC) values were calculated for each SRAP and SSR primer according to the formula of Roldan-Ruiz et al. (2000): PICi = 2fi(1 - fi), where PICi is the polymorphism information content of allele I and fi is the frequency of occurrence of allele I (fragment present) in the 66 individuals. The Mantel test (Mantel, 1967) (MXcomp in NTSYS-pc version 2.1) was used to estimate the level of correlation between the SRAP and SSR similarity matrices data.

Table 3. List of the simple SSR primers used for the genotyping of 66 *V. hispanica* accessions, allele size, number of polymorphic alleles, total bands amplified, polymorphism percentage, and PIC value.

Locus primer ID	Allele size (bp)	Number of alleles (number of polymorphic bands)	Number of total bands	Polymorphism (%)	PIC
AGA1-150	350	1	3	33.3	0.39
AGA5-123	300, 200, 180, 90	4	5	80	0.38
AG9-49	450, 300, 350	3	6	50	0.32
CB011a	150	1	1	100	0.49
CB020a	250, 240, 230, 520, 510, 490	6	6	100	0.21
CB041a	400, 300, 280	3	4	75	0.39
CB047a	350	1	2	50	0.46
CB060a	280	1	3	33.3	0.49
SA06	200	1	2	50	0.49
SA30	300, 280	2	4	50	0.38
SA32	230, 240, 250, 500, 530, 540	6	6	100	0.34
SA04	320, 300	2	5	40	0.48
SA09	300	1	4	25	0.49
SA16	260, 250, 480	3	5	60	0.38
SA36	350, 400	2	5	40	0.37
SpI_c2	600, 280	2	4	50	0.49
SpI_d3	500, 270, 250	3	4	75	0.25
	Total	42	69		
	Mean	2.47	4.05	59.5	0.40

Principal components analysis (PCA) was performed using the Eigen module in PAST 2.17 software (Hammer et al., 2001) for the 66 *V. hispanica* genotypes. Bayesian model-based clustering with assumed K populations was employed to analyze the structure of the population. A burn-in period of 10,000 replications and 100,000 Markov chain Monte Carlo replications, along with the admixture model, were used in Structure 2.3.4 (Pritchard et al., 2000). A total of 10 independent runs were performed for each value of K (from 1 to 10) (Evanno et al., 2005). The results were input into Structure Harvester (Earl and VonHoldt, 2012) to determine the best K (number of clusters) value. The number of subpopulations determined with Structure was used for an analysis of molecular variance (AMOVA). Distribution of the genetic variation of the populations was analyzed using AMOVA with 1000 permutations and pairwise F_{ST} was performed in Arlequin software 3.1 (Excoffier et al., 2005) based on the SRAP and SSR markers.

3. Results

3.1. SRAP analysis

Initially, the *V. hispanica* genotypes were analyzed with bulk DNA containing equal amounts of DNA of 10 randomly selected genotypes using 208 SRAP primer combinations, of which 49 produced reproducible polymorphic banding patterns. The 49 selected primers were used for PCR reactions with the 66 genotypes. A total of 476 bands were scored, of which 197 (41%) were polymorphic. The size of the amplified products varied from 100 to 1500 bp. The number of total bands generated per primer varied from 3 (Me9Em11) to 17 (Me10Em4), with a mean of 9.71 and the polymorphic bands varied from 1 (Me2Em8, Me3Em8, and Me13Em1) to 14 (Me11Em2), with a mean of 4.02. Representative banding patterns generated using primer Me10Em7 are shown in Figure 2. The mean PIC calculated from the frequency of the polymorphic bands across all of the genotypes was 0.30 (Table 2). Primer Me5Em8 provided the highest PIC value of 0.46, followed by primer Me9Em7 (0.45), and primer Me10Em6 provided the lowest PIC value of 0.12. In the genetic similarity analysis of the SRAP data, the similarity coefficient ranged from 0.48 to 0.91. The similarity was the lowest between accessions originating from Konya and Antalya, Sivas, and Bursa, and the highest between the ones from Bolu and Çanakkale. The genetic similarity values between genotypes 3-A2 (Bursa) and 5-A3 (Bolu) (0.89) and 3-A2 (Bursa) and 2-A1 (Çanakkale) (0.88) collected from proximal provinces of the northwest part of Türkiye (A-1, A-2, and A-3) were also high. UPGMA cluster analysis illustrated the overall genetic relationship among the genotypes (Figure 3). The genotypes were divided into 6 major clusters with a mean similarity value of 0.70. First, the 66 accessions were clustered into 2 at a 0.59 genetic similarity value. Cluster A contained a single genetically distinct accession (C5-45) from Konya. Cluster B contained the remaining 65 genotypes and was

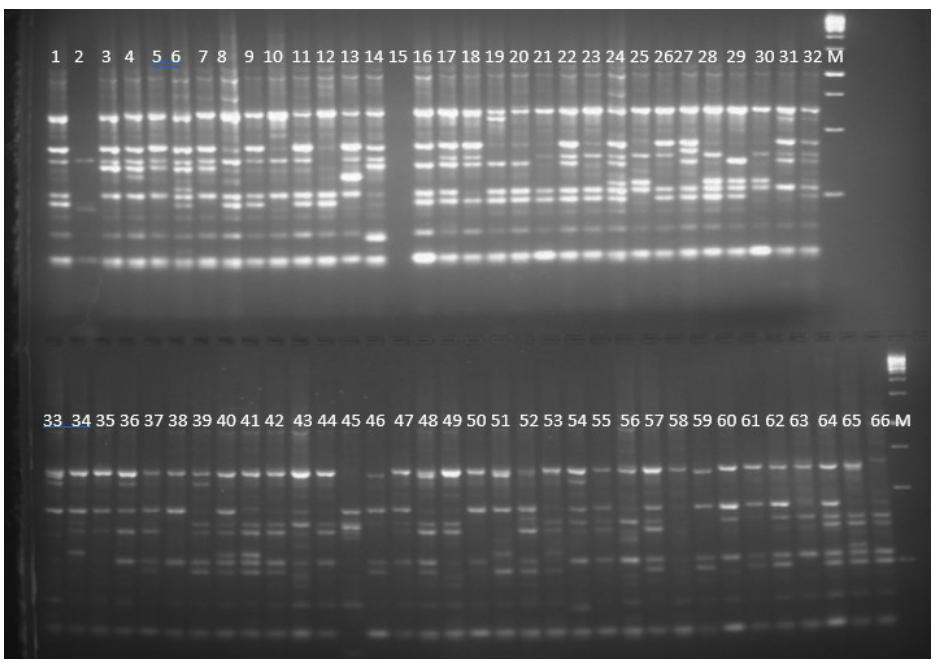

Figure 2. DNA amplification patterns of the 66 *V hispanica* accessions screened using the Me10Em7 SRAP primer. The marker (1 kb) is loaded in the last well of each row.

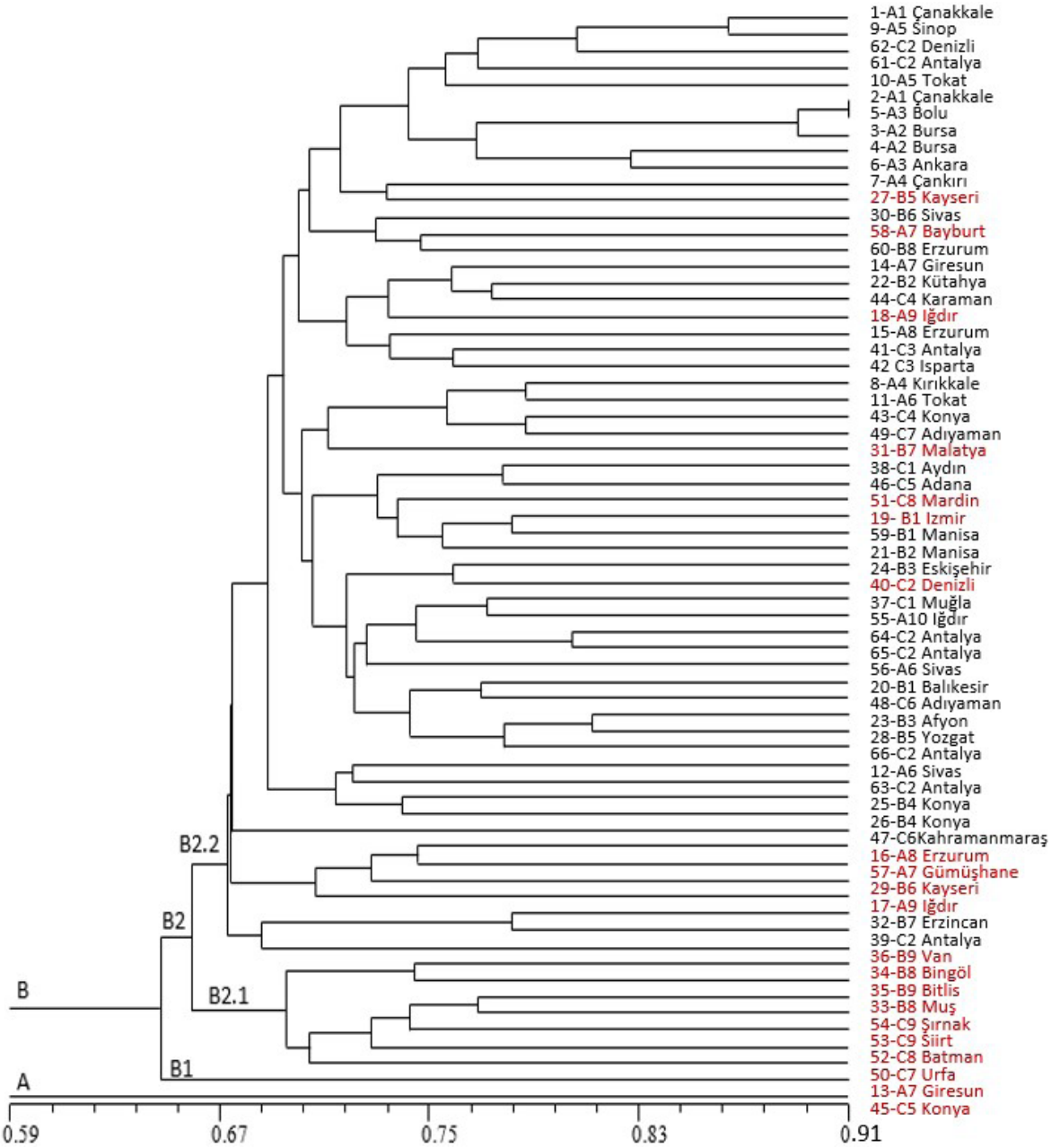

Figure 3. Dendrogram of the 66 *V hispanica* accessions derived from the UPGMA cluster analysis based on the SRAP markers. Genotypes marked in red are those included in SP1.

divided into 2 subclusters (Clusters B1 and B2). Cluster B1 included a single genotype from Giresun. The other 64 genotypes were placed in Cluster B2 and divided into 2 subclusters (B2.1 and B2.2). Subcluster B2.1 comprised 7 genotypes from Bingöl, Bitlis, Muş, Şırnak, Siirt, Batman, and Urfa, which are geographically proximal locations with similar climates. Subcluster B2.2 comprised the majority of the genotypes (45 genotypes) further divided into two subclusters at a similarity coefficient of 0.69. Genotypes 2-A1 from Çanakkale and 5-A3 from Bolu were genetically similar (0.91). The genotypes were generally not strongly clustered based on their geographic origins. However, the SRAP markers partially grouped some of the genotypes based on their geographic location, such as 3-A2 and 4-A2 from Bursa were placed close to 2-A1 from Çanakkale and 5-A3 from Bolu. Similarly, genotypes 58-A7 (Bayburt), 60-B8 (Erzurum), A7-14 (Giresun), 17-A9 and 15A8 (Erzurum), 59-B1 and 21-B2 (Manisa), 64-C2 and 65-

C2 (Antalya-Elmalı), and 25-B4 and 26-B4 (Konya) were also closely clustered within their respective groups in the dendrogram.

3.2. SSR analysis

The total number of alleles amplified by the 17 polymorphic SSR primers was 42, with an average of 2.47 alleles per locus. The number of amplified bands varied from 1 (AGA1-150, CB011a, CB047a, CB060a, SA06, and SA09) to 6 (CB020a, SA32). The PIC values ranged from 0.21 (CB020) to 0.49 (SA06, SA09, CB11a, and CB060) with an average of 0.40 (Table 3). Representative banding patterns generated using primer SpI_c2 are shown in Figure 4. The results of the similarity matrix revealed a high genetic diversity among the genotypes. The lowest genetic similarity (0.42) was observed between the genotypes from Antalya-Kaş (61-C2) and Adıyaman (49-C7), followed by genotypes 53-C9 (Siirt) and 19-B1 (İzmir). The highest genetic similarity (0.84) was observed between genotypes 25-B4 (Konya) and 10-A5 (Tokat), 28-B5 (Yozgat), and 32-B7 (Erzincan) (data not shown).

The dendrogram was constructed using UPGMA cluster analysis divided into 2 main clusters (A and B) at a similarity coefficient of 0.57 (Figure 5). In cluster A, there were 3 genotypes, 18-A9 (Iğdır), 61-C2 (Antalya), and 14-A7 (Giresun), which originated from distinct geographical locations. Cluster B consisted of the rest of the genotypes and was divided into subclusters B1 and B2. There were 7 genotypes in subcluster B2 (1-A1, 2-A1,5-A3, 3-A2, 13A-7, 17-A9, and 45-C5). The remaining 59 genotypes were placed in subcluster B1, which was further divided into 2 branches (B1.1 and B1.2) with a similarity coefficient value of 0.60. Branch B1.1 included only 2 genotypes, 65-C2 and 49-C7, and Branch B1.2 was divided into 2 subcluster again (B1.2.1 and B1.2.2). Subcluster B1.2.1 contained 10 genotypes (53-C9, 36-B9, 52-C8, 54-C9, 50-C7, 55-A10, 27-B5, 46-B5, 35-B9, and 34-B8) that were all collected from neighboring provinces located in the southeast region of Türkiye. Some accessions originating from the same provinces were placed in the same subgroup in the dendrogram. Genotype 1A-1 and 2A-1 from Çanakkale, 25-B4 and 43-C4 from Konya, and 63-C2 and 39-C2 from Antalya-Elmalı were placed in the same group and in very close proximity in the dendrogram.

3.3. Combined data analysis

The highest genetic similarity (0.90) was achieved between 2-A1 (Çanakkale) and 5-A3 (Bolu), while the lowest (0.49) was obtained between 45-C5 and 39-C2 in the genetic similarity matrix (data not shown).

The genetic similarity coefficients ranged from 0.58 to 0.90 in the dendrogram, and the genotypes were grouped into 2 clusters (A and B) by the UPGMA clustering analysis (Figure 6). There was a partial relationship between the genetic proximity of the germplasms collected from the same location. In the dendrogram generated with the combined data of the SRAP and SSR, 9 genotypes (1-A1, 2-A1, 5-A3, 3-A2, 4-A2, and 6-A3) collected from the square A-1, A-2, and A-3 were in the same group at the top of the dendrogram and were more closely related than the other genotypes. Cluster A included only 1 genotype (45-C5) collected from Konya, and cluster B, which included the remaining 65 genotypes, was divided into 2 subclusters (B1 and B2) with a genetic similarity value of 0.67. Subcluster B1 included 9 genotypes, 8 of which (35-B9, 34-B8, 52-C8, 50-C7, 54-C9, 33-B8, 36-B9, 31-B7, and 53-C9) were collected from Bitlis, Bingöl, Batman, Urfa, Şırnak, Muş, Van, Malatya, and Siirt, all located in the southeast region of Türkiye (Figure 6). Genotypes 2-A1 (Çanakkale) and 5-A3 (Bolu) were genetically similar (0.90) and placed in the same cluster along with genotypes 1-A1 from Çanakkale and 3-A2 from Bursa. The PCA

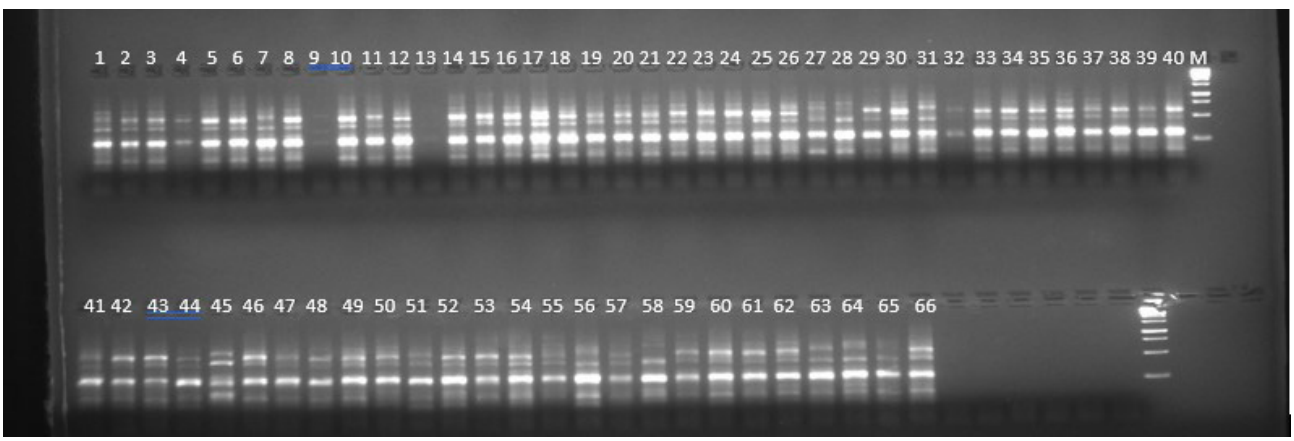

Figure 4. DNA amplification patterns of the 66 *V. hispanica* accessions screened using the SpI_c2 SSR primer. The marker (1 kb) is loaded in the last well of each row.

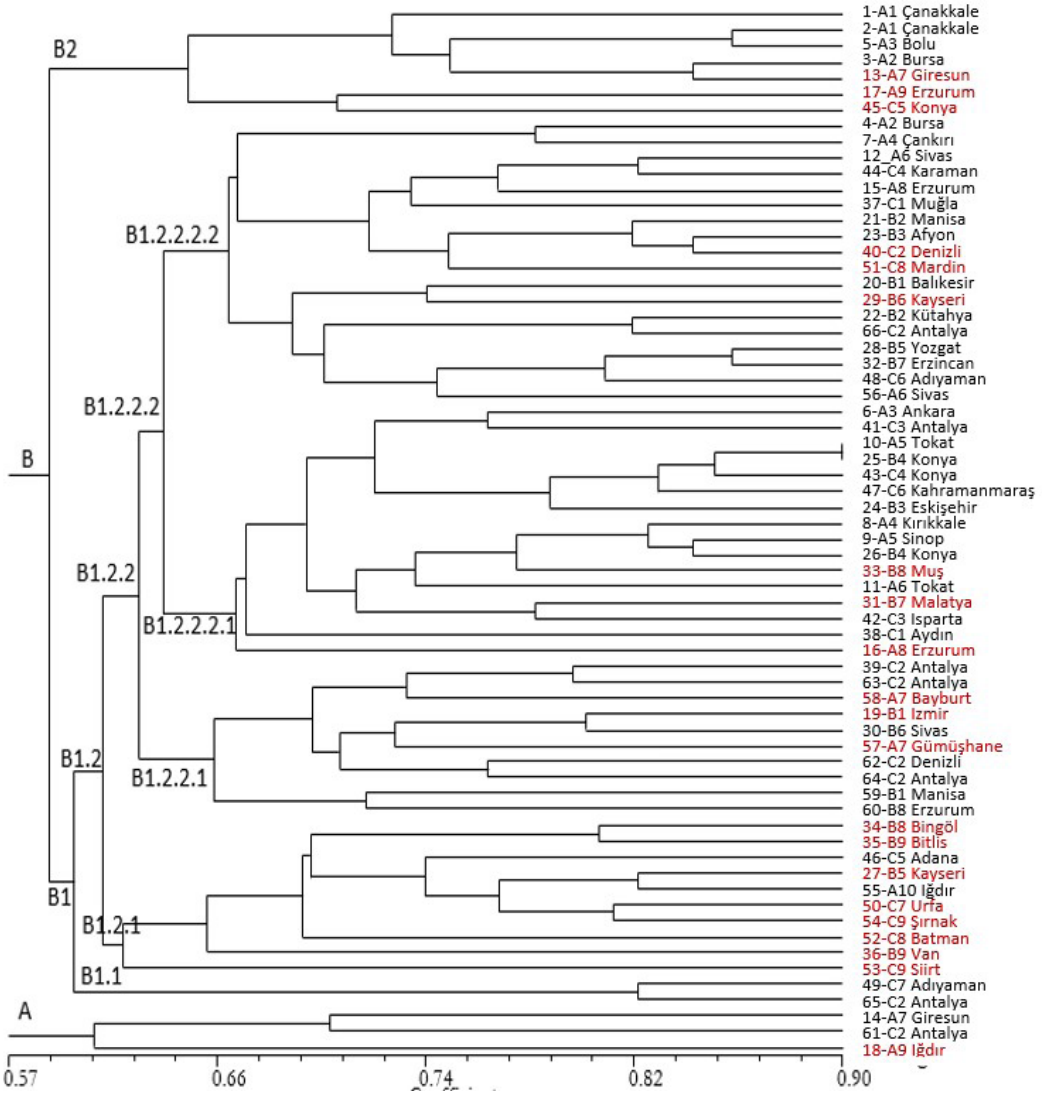

Figure 5. Dendrogram of the 66 *V. hispanica* accessions derived from the UPGMA cluster analysis based on the SSR markers. Genotypes marked in red are those included in SP1.

analysis of these genotypes showed a similar result (Figure 7). Geographic proximity tended to cluster genotypes on the dendrogram, such as 43-C4 and 25-B4 from Konya, 64-C2 and 65-C2 from Antalya-Elmalı, 21-B2 and 59-B1 from Manisa were placed closely in the dendrogram. The genotypes (16-A8, 57-A7, 17-A9, 12-A6, and 15-A8) collected from neighboring provinces (Gümüşhane, Erzurum, and Sivas) were also placed closely in the same cluster (B-2-1).

The Mantel test was performed to test the correlation between 2 matrices and a positive low correlation (r = 0.17) was found between the SRAP and SSR markers.

3.4. Population structure

The molecular marker data generated using the 66 *V. hispanica* genotypes and employing the 49 SRAP and 17 SSR primers were subjected to an admixture model-based analysis by varying K from 1 to 10. The results of the combined data of the SRAP and SSR marker analysis demonstrated that the maximum delta K value was obtained at K = 2 (Figure 8), where the 66 accessions were best grouped into 2 subpopulations. The Bayesian bar plots were constructed using the Bayesian method with the admixture model (Figure 9). Furthermore, based on the inferred ancestry of the individuals, considering the membership fractions, accessions with membership probabilities greater than 0.50 were considered to belong to the same group. Of the accessions, 32% (21) were assigned to subpopulation 1 (SP1) collected from geographically adjacent provinces located in the southeast region, such as Şırnak, Batman, Muş, Bitlis, and Siirt, and

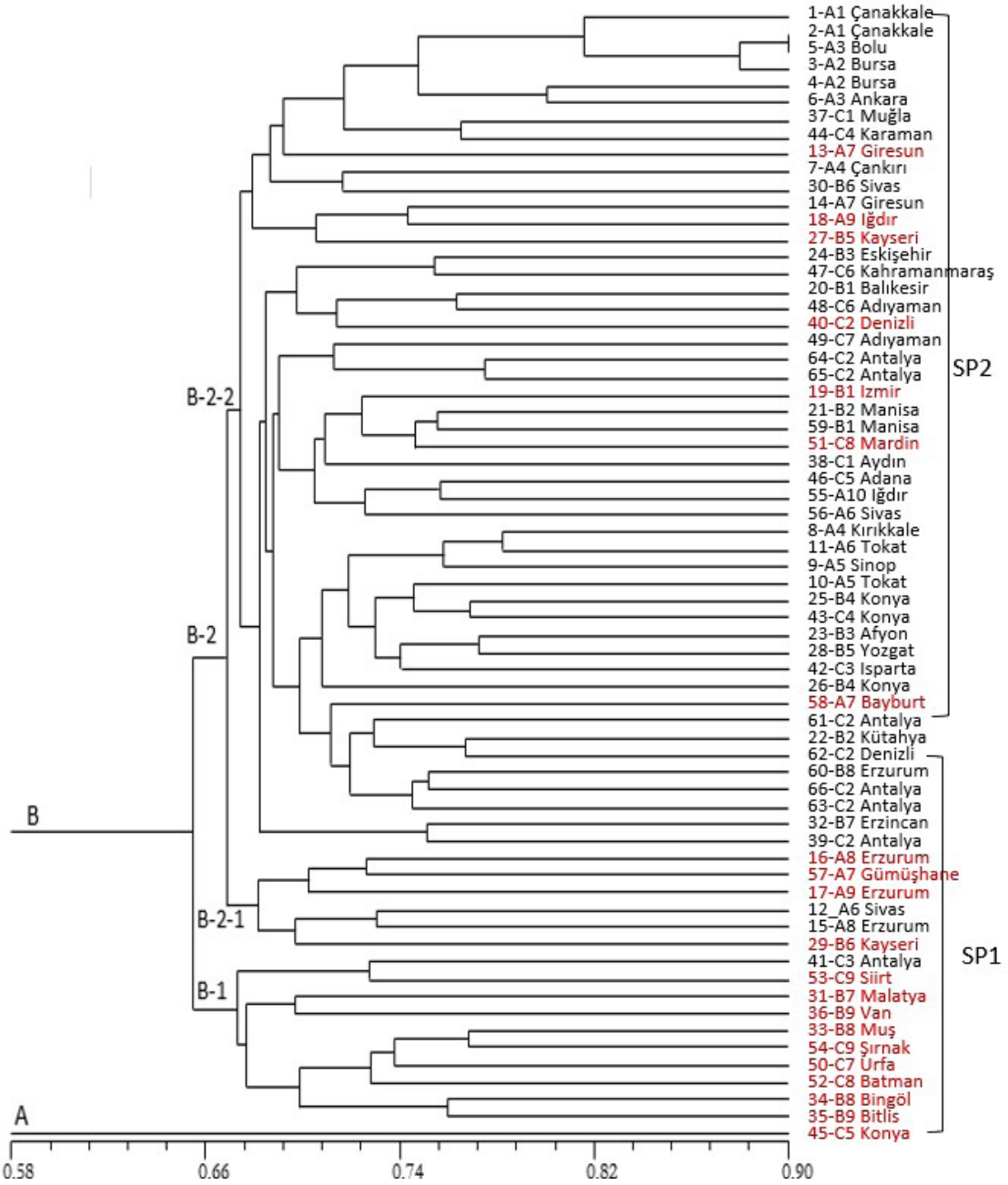

Figure 6. Dendrogram of the 66 *V. hispanica* accessions derived from the UPGMA cluster analysis based on the combined data of the SRAP and SSR markers. Genotypes marked in red are those included in SP1

Bingöl, Mardin, Van, Malatya, Gümüşhane, and Bayburt but extended to some northeast Anatolian (Erzurum and Giresun) and western parts of Türkiye (Denizli). Subpopulation 2 (SP2) represented the remaining 68% of the accessions (45) and generally covered the provinces in central Anatolian region (Sivas, Kayseri, and Yozgat), but extended toward western, Kütahya, Afyon, Aydın, Muğla, and Denizli), southern (Adana, Kahramanmaraş, Mardin, and Antalya), and northern Anatolian regions (Çankırı, Tokat, Sinop, Giresun, Erzurum, Erzincan, and Iğdır), as well as the provinces located in the northwest part of Türkiye, including Çanakkale, Bursa, and Ankara (the Structure results estimated the fixation index (F_{st}) for each of the subpopulations). The F_{st} value is a measure of population differentiation based on genetic structure (Luo et al. 2019). F_{st} values of 0.0048 and 0.2271 were obtained

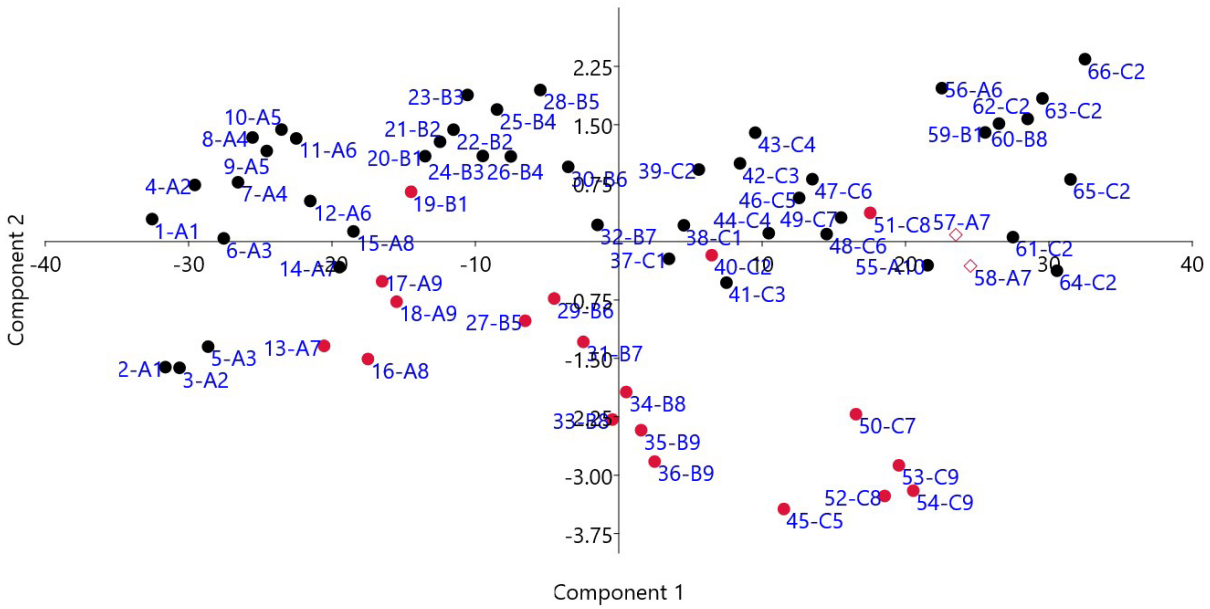

Figure 7. PCoA plot based on the combined data of the SRAP and SSR markers for the 66 *V. hispanica* accessions. Red dots represent SP1.

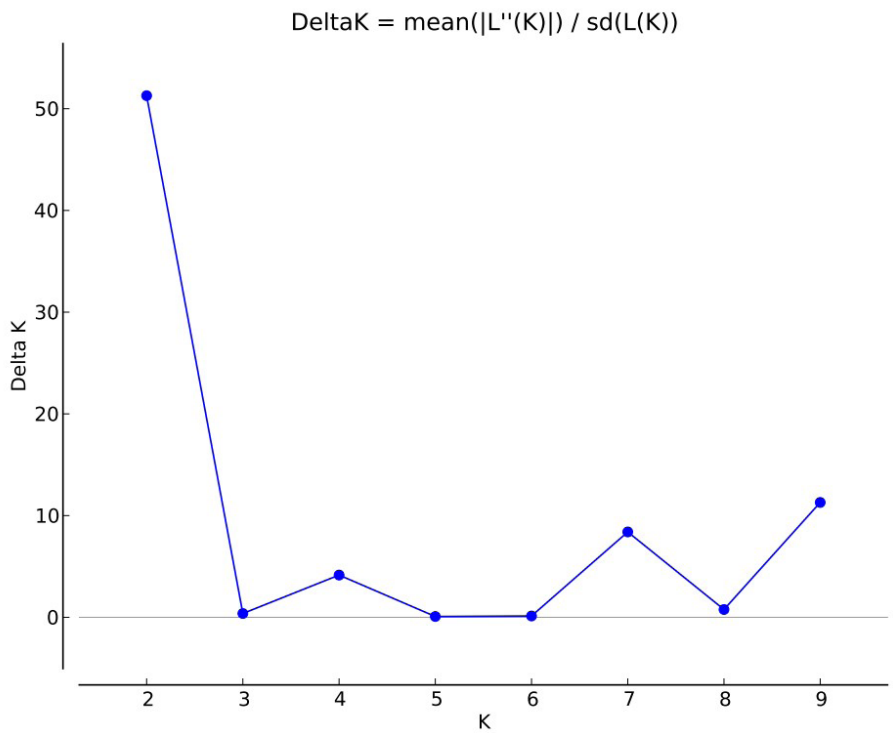

Figure 8. Structure estimation of the number of subgroups for the K values ranging from 1 to 10, based on the delta K (ΔK) values.

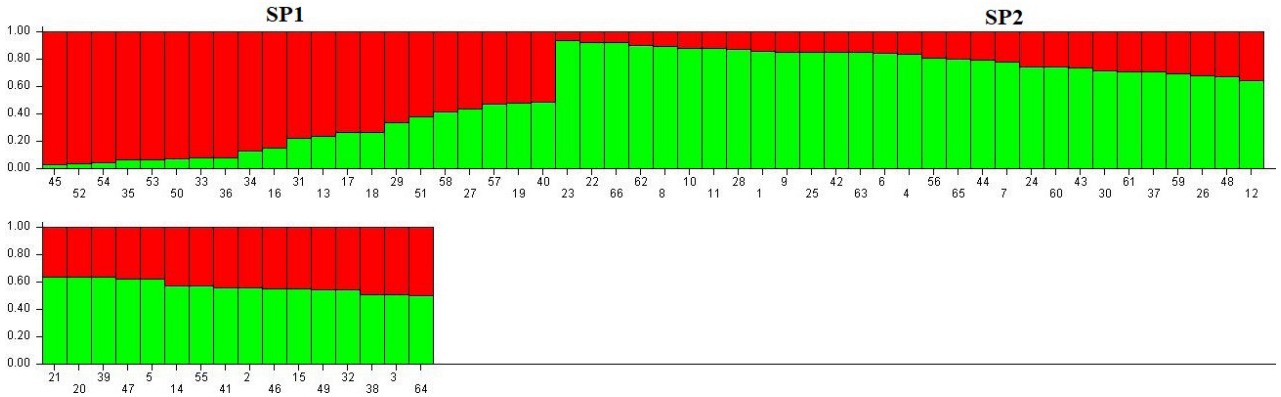

Figure 9. Genetic structures of the 66 *V. hispanica* accessions, derived from the Structure analysis using the combined data of the SRAP and SSR markers based on an admixture model with K = 2. The identified SPs are indicated in different colors. The accession numbers refer to Table 1.

for SP1 and SP2, respectively. Pairwise F_{st} comparisons evaluated the divergence between the subgroups given by the Structure results and a low F_{st} value was obtained between SP1 and SP2 (F_{st} = 0.0245), indicating a low genetic differentiation between these subpopulations. The average distances (expected heterozygosity) between individuals in the same subpopulation were 0.3562 and 0.2891 for SP1 and SP2, respectively (Table S1). In accordance with NTSYS analysis, the Structure and PCA analysis graph showed that genotypes collected from close geographical locations tended to be placed together. AMOVA revealed that 93% of the total genetic variations were contributed by differences within the *V. hispanica* population, which was significantly higher than that among populations (6%), as summarized in Table S2.

4. Discussion

In the current study with *V. hispanica*, the highest number of total bands and polymorphic bands obtained with SRAP primer was 17 with Me10Em4 and 14 with Me11Em2, respectively. The percentage of polymorphic markers for each primer pair ranged from 12.5% (Me2Em8) to 100% (Me11Em2). The high level of polymorphism detected by the SRAP markers in this study was comparable with that of previous studies of other perennial species. Fu et al. (2008) evaluated the genetic relationship of annual ornamental plants belonging to the family Caryophyllaceae, Chinese pink (*Dianthus chinensis* L.), *D. barbatus*, and *D. superbus* with SRAP primers, where the number of total bands generated ranged from 7 (Em2Me8) to 21 (Em01Me04). The percentage of their polymorphic markers produced by each primer pair ranged from 85.71% to 100%. In another study, Szabo et al. (2021) obtained the highest number of total bands (16 bands) with primer combinations Me6Em1 in *Astragalus exscapus* subsp. *transsilvanicushe*. The highest number of total bands was recorded as 10 in the current study. In another study conducted by Mokhtari et al. (2013) in *Carthamus* species, Me4-Em1 and Me5-Em2 primers were found to be the most informative SRAP markers based on the PIC values (0.34), where they distinguished all of the studied *Carthamus* species. In the current study, Me5Em2 produced 12 bands, 4 of which were polymorphic, with a PIC value of 0.33. Szabo et al (2021) reported PIC values of 0.25, 0.37, and 0.36 for primers Me5Em6, Me6Em1, and Me8Em2, respectively, which were very similar to the values reported herein (0.24, 0.39, and 0.38) with these primers in *V. hispanica*. Hodaei et al. (2019) reported average polymorphic bands and PIC values of 21.3 and 0.46, respectively, with the highest PIC value of 0.496 (Me2-Em3) and lowest of 0.433 (Me5-Em2). Talebi et al. (2015) obtained the highest PIC value of 0.333 (Me4Em3) with an average of 0.284 in *Thymus daenensis.* The PIC value was obtained to calculate the effectiveness of polymorphic loci in assessing the genetic relationship among the genotypes. In the current study, the mean PIC value was 0.30, and the highest PIC value was obtained with primer Me5Em8 (0.46), followed by Me9Em7 (0.45), Me13Em4 (0.42), and Me10Em4 (0.41), indicating that the SRAP primers had the potential to yield highly informative markers. Thus, SRAP markers can be employed in genetic diversity or mapping studies of *V. hispanica*. Comparing the results of the SSR primers in previous studies, primer AFA9-49 produced 9 alleles in *Arenaria grandiflora* L. (Zavodna et al., 2009) and 3 alleles in the present study. Primer SA32 produced 2 alleles in *S. adamantis* (Culley et al, 2008) and 6 in *V. hispanica*. Most of the primers developed for *S. adamantis* (SA06, SA30, SA09, AGA5-123, and SA36) in a previous study by Culley et al. (2008), gave similar results to those for *V. hispanica*. Similarly, in another study testing the transferability of SSR markers developed for wheat and rye to triticale, Kuleung et al. (2004) reported the banding pattern and size of the SSR markers in triticale were usually similar to

those of the donor. In the present study, the average number of alleles per locus for primers AGA1-150, AGA5-123, and AG9-49 was lower (4.6) than those obtained for *Arenaria grandiflora* (5.6) by Zavodna et al. (2009), in contrast, the number of alleles per locus (6) observed in the present study with primers SA32 and CB020a was greater than that of the 2 alleles per locus (SA32) obtained by Culley et al. (2008) in *S. adamantis* (Caryophyllaceae) and the 1 alle per locus (CB020a) obtained by Kimura et al. (2009) in carnation (*D. caryophyllus*). A low level of correlation was found between the SSR and SRAP markers (r = 0.17). Similar results were reported in pepper (r = –0.033) (Du et al., 2010), buffalograss (r = 0.10) (Budak et al., 2004), tea (r = 0.01) (Zhang et al., 2018), and wild soybean (r = 0.2) (Sun et al., 2013). This may be because both marker systems amplify different targets in the genome. SRAP are mostly attached to ORFs. Core sequence of the forward primers contain the CCGG and usually target exons, while reverse primers contain the TTAA sequence and target introns. In contrast, SSR markers amplify repetitive regions, which are mostly found in intron and untranslated regions. The use of multiple marker systems that amplify different regions in the genome allows for better prediction of genomic differences.

In the Structure analysis, the genotypes collected from the east Anatolian region of Türkiye (Urfa (50-C7), Mardin (51-C8), Şırnak (54-C9), Batman (52-C8), Muş (33-B8), Bitlis (35-B9), Siirt (53-C9), Bingöl (34-B8), Van (36-B9), Malatya (31-B7), Gümüşhane (57-A7), Bayburt (58-A7), Erzurum (16-A8), and Giresun (13-A7) were clustered in SP1. The UPGMA clustering and PCA graph were in agreement with the results obtained by the Structure analysis. The genetic variation within SP1 (F_{st} = 0.0048) was lower than that among the populations (F_{st} = 0.0245), whereas the genetic variation within SP2 (0.227) was higher than that among populations. One of the reasons for this may be that the gene flow has been restricted due to geographical isolation within SP1 for some time. Genetic variation can be preserved in large populations over wide geographical distances. SP2 inhabits a much larger area than SP1, with a larger representation (45 genotypes) (Figure 1). It is conceivable that SP2 individuals with different genetic structure or with a particular genotype will reproduce and preserve genetic variation, which, in turn, may result in higher genetic diversity. In the present study, the AMOVA results showed that 94% of the total variation resided within the populations, while only 6% was among populations. The results represent a low genetic variation between 2 subpopulations similar to those of Luo et al. (2019) in which 4% variance among the subpopulations and 96% variance within the populations was reported. In another outcrossing species, a similar result was reported by Li and Wei (2022).

The SSR and SRAP markers selected in this study yielded reproducible polymorphic bands in the *V. hispanica* accessions and showed that they provide a powerful and reliable molecular tool for analyzing genetic diversity and relationships among *V. hispanica* genotypes. The results in the current study illustrate the combined effectiveness of the SSR and SRAP techniques in discriminating the *V. hispanica* genotypes analyzed.

These findings will be useful in conserving and utilizing the genetic resources of *V. hispanica*, selecting the desired parent plants in hybrid breeding programs, and developing superior genotypes with a high content ratio. Genetic diversity has a valuable contribution to creating new cultivars. There have been no reports to date on the genetic diversity of *V. hispanica* using molecular markers. Balsevich et al. (2006) analyzed the profiles of seed extracts from 3 different plant sources of *Saponaria vaccaria* L. and reported all 3 contained the same type of saponin in different concentrations. Cam et al. (2018) analyzed the physical and chemical properties of the seeds of the 66 *V. hispanica* genotypes used in the current study, and the starch, segetalin, and saponin contents were highly variable, indicating the agronomic value of the accessions. In order to use genetic resources effectively in breeding programs, some important characters need to be tagged or mapped. Further studies on *V. hispanica* could be based on employing the existing genetic diversity and contributing genotypes that are superior in terms of important characteristics in breeding programs.

5. Conclusion

In this study, the genetic variations among 66 genotypes of *V. hispanica* were revealed. The results showed the potential of SRAP and SSR markers for characterizing the genetic diversity of *V. hispanica*. The diversity found among the genotypes could be of great importance for improving *V. hispanica*. To the best of our knowledge, this is the first study reporting the genetic diversity of *V. hispanica* genotypes based on molecular markers. Molecular analyses and morphological data can be used effectively in further studies for mapping important characters such as the saponin content or serve as genetic materials for breeding programs. The results in this study will also help in future studies on mapping genes associated with important characters or developing markers for some important characters in *V. hispanica*.

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Supplementary Tables

Table S1. Structure results of the 66 *V. hispanica* accessions for the fixation index (F_{st}), average distances (expected heterozygosity/He), and number of genotypes assigned to each subpopulation.

	Inferred clusters	Mean F_{st}	Expected He	Number of genotypes
SP1	0.433	0.0048	0.3562	21
SP2	0.567	0.2271	0.2891	45

Table S2. Analysis of the molecular variance (AMOVA) for the 66 individuals in the 2 SPs of *V. hispanica.*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	1	99.172	1.14215 Va	6.05
Among individuals within the population	64	2271.252	17.74416 Vb	93.95
Within individuals	66	0.000	0.00000 Vc	0.00
Total	131	2370.424	18.88631	