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RESEARCH ARTICLE

Assessment of genetic diversity and population structure in alfalfa (*Medicago sativa* L.) using sequence-related amplified polymorphism (SRAP) markers

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Abstract Developing resilient cultivars with improved tolerance to environmental stresses and stronger resistance to pests and diseases requires pre-breeding efforts focused on understanding genetic diversity. In the present study, 85 alfalfa genotypes were analyzed using sequence-related amplified polymorphism (SRAP) markers. A total of 112 alleles were amplified across 10 SRAP primer combinations, with the number of alleles per primer ranging from 7 to 19. The mean polymorphism information content (PIC) was 0.295, indicating a moderate level of marker informativeness. Genetic diversity parameters, including Nei's gene diversity (He = 0.27) and Shannon's information index (I = 0.42), revealed moderate to high levels of genetic variation among the accessions. Cluster analysis based on Dice's similarity coefficients and the unweighted pair Group method with arithmetic mean (UPGMA) grouped the genotypes into two main clusters. This clustering pattern was further corroborated by principal component analysis (PCA), which showed a clear genetic differentiation among individuals. Bayesian population structure analysis conducted using STRUCTURE software identified K = 2 as the most likely number of genetic subpopulations, as determined by the Delta

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K method. Most genotypes displayed high membership coefficients ($Q \geq 0.80$), suggesting a high degree of genetic homogeneity within subpopulations. Subpopulation B exhibited greater genetic diversity ($He = 0.2612$) and lower genetic differentiation ($F_{ST} = 0.2443$), whereas subpopulation A appeared more genetically distinct ($F_{ST} = 0.3952$). These results demonstrate the effectiveness of SRAP markers in resolving genetic structure in alfalfa and underscore their utility in identifying genetically diverse and distinct individuals for breeding purposes. Moreover, the absence of clear geographic structuring among the genotypes suggests substantial gene flow across collection sites. This study emphasizes the value of molecular characterization in guiding the selection of parental lines and enhancing germplasm conservation efforts for the improvement of alfalfa cultivars.

Keywords Alfalfa · Genetic diversity · SRAP markers · Population structure

Introduction

Alfalfa (*Medicago sativa*) is one of the most extensively cultivated forage crops globally, valued for its high nutritional quality and broad adaptability to diverse climatic conditions (Annicchiarico et al. 2018). As an obligate cross-pollinated species, alfalfa exhibits substantial genetic diversity arising from a wide range of gene and allele combinations (Wang et al. 2023). This genetic variation underpins the species' capacity to adapt to heterogeneous environments and contributes significantly to the enhancement of key agronomic traits, including biomass yield, resistance to biotic stresses, and tolerance to abiotic factors such as drought (Ilhan et al. 2016). Comprehensive assessment of genetic diversity is therefore a critical component of breeding programs, facilitating effective hybridization and selection strategies. Furthermore, systematic characterization of germplasm resources is essential for the development of targeted conservation strategies and for safeguarding the long-term sustainability of alfalfa cultivation (Brummer 1999).

Local genotypes are of particular importance to breeding programs, as they constitute a valuable reservoir of adaptive traits and genetic variability (Bocianowski et al. 2024; Şakiroğlu et al. 2010). These genotypes often result from long-term natural selection and farmer-mediated selection processes under specific environmental conditions, which may include exposure to biotic and abiotic stresses such as drought, salinity, pests, and diseases (Cyplik et al. 2023; Mnafgui et al. 2023). Consequently, they frequently possess unique allelic combinations that are not commonly found in widely cultivated commercial cultivars (Mnafgui et al. 2023). Germplasm collections serve as critical repositories of this genetic diversity, offering essential resources for crop improvement through the identification and incorporation of beneficial traits into breeding pipelines (Bunjkar et al. 2024). Integrating local accessions into modern breeding strategies can significantly enhance the resilience, sustainability, and productivity of newly developed cultivars. Moreover, the genetic diversity preserved within these genotypes is vital for ensuring long-term adaptability in the context of climate change and evolving agricultural challenges (Sakiroglu and Brummer 2017). Comparative evaluation of local genotypes and registered cultivars provides insights into the genetic structure and breeding potential of alfalfa populations across varying environmental and agronomic contexts (Li and Brummer 2012). Such analyses enable the identification of genetically distinct and agronomically superior individuals, which can be prioritized for hybridization, selection, and marker-assisted breeding approaches (Annicchiarico et al. 2015). Furthermore, elucidating the genetic relationships between landraces and elite lines facilitates the strategic design of crossing schemes aimed at broadening the genetic base and reducing susceptibility to environmental fluctuations.

Germplasm diversity can be characterized using two primary approaches: morphological markers and molecular markers (Demirel et al. 2024). Although morphological markers yield valuable phenotypic information, their utility is often limited by environmental influences and developmental stage-specific variation, which can compromise their consistency and reliability (Govindaraj et al. 2015). In contrast, DNA-based molecular markers offer higher reproducibility and are not influenced by environmental conditions, making them a more robust tool for genetic analysis (Gupta and Varshney 2000). As a result, molecular markers have become the method of choice for investigating genetic diversity, population structure, and

phylogenetic relationships in alfalfa. A wide range of molecular marker systems has been employed to assess genetic variation in alfalfa, including amplified fragment length polymorphism (AFLP) (Mengoni et al. 2000), restriction fragment length polymorphism (RFLP) (Botstein et al. 1980), random amplified polymorphic DNA (RAPD) (Taški-Ajduković et al. 2014), simple sequence repeats (SSR) (Şakiroğlu et al. 2010), inter-simple sequence repeats (ISSR) (Zietkiewicz et al. 1994), and single nucleotide polymorphisms (SNPs) (Lander and Botstein 1986). Among these, sequence-related amplified polymorphism (SRAP) markers have gained prominence as a powerful and efficient tool for studying genetic diversity and population structure in alfalfa (Rhouma et al. 2017). SRAP markers are designed to preferentially amplify open reading frames (ORFs) and coding regions, thereby enabling the detection of functionally relevant polymorphisms. They exhibit high reproducibility, codominant inheritance, and broad genomic coverage, making them particularly well-suited for evaluating genetic diversity and relatedness among alfalfa genotypes (Li and Quiros 2001). In contrast to other marker systems, SRAP markers are effective in capturing variation across both coding and non-coding genomic regions, providing a comprehensive perspective on genome organization and diversity (Ferriol et al. 2003). To the best of our knowledge, no research has been conducted on Turkish alfalfa populations using SRAP markers; therefore, this is the first study to investigate the genetic diversity of natural Turkish alfalfa populations using SRAP markers.. The aim of this study is to assess the genetic diversity of 85 alfalfa (*Medicago sativa* L.) genotypes, comprising both local accessions and registered cultivars, using sequence-related amplified polymorphism (SRAP) markers. Specifically, the study seeks to compare genetic diversity and population structure between local and commercial genotypes and to identify polymorphic loci potentially associated with adaptive traits. The application of SRAP markers is expected to yield valuable insights into the genetic architecture and breeding potential of alfalfa germplasm, thereby informing future breeding strategies and contributing to the development of effective conservation programs.

Materials and methods

Plant material

In this study, a total of 85 *Medicago sativa* L. genotypes were analyzed, comprising 56 wild genotypes collected from six provinces of Türkiye (Ankara, Erzurum, Eskişehir, Kirikkale, Konya and Sivas) and 29 commercially used alfalfa cultivars (Table 1 and Fig. 1).

DNA ekstraksiyonu

Genomic DNA was extracted following the protocol described by Zeinalzadehtabrizi et al. (2015). For each genotype, DNA was extracted from a single individual plant. The quality and integrity of the DNA extracted were assessed by electrophoresis on 0.8% agarose gels. DNA concentration and purity were measured using a NanoDrop® ND-1000 UV/Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For SRAP analysis, DNA samples were diluted to a working concentration of 50 µg/µL. The diluted samples were stored at − 20 °C until further use in SRAP-PCR assays (Turkoglu 2023).

SRAP analysis

SRAP primers were synthesized by Shanghai Sangon Biotech Co. A total of 50 primer combinations were generated from 15 forward to 17 reverse primers previously described by Li and Quiros (2001). Of these, 10 combinations exhibiting the highest levels of polymorphism were selected for subsequent analyses (Table 2). PCR amplification was performed in a 20 µL reaction mixture containing 1 × PCR buffer, 2 µM (20 pmol) of each primer, 0.25 mM of each dNTP, 2 mM $MgCl_2$, 0.5 U of Taq DNA polymerase, and 50 ng/µL of template DNA. Amplification was carried out in a 96-well thermal cycler (SensoQuest Labcycler, Göttingen, Germany) using the protocol described by Türkoğlu et al. (2022). The thermal cycling program consisted of an initial denaturation at 95 °C for 3 min, followed by 37 cycles of denaturation at 95 °C for 60 s, annealing at 50–58 °C for 60 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 5 min. PCR products were separated on 3% agarose gels in 1× SB buffer at 150 V/cm for 120 min.

Table 1 Geographic and selected characteristics of wild and cultivated *Medicago sativa* L. genotypes used in the study

No	Province	District	Trait	No	Province	District	Trait
G1	Ankara	Şereflikoçhisar	Wild	G44	Konya	Ereğli	Wild
G2	Ankara	Şereflikoçhisar	Wild	G45	Konya	Ereğli	Wild
G3	Ankara	Şereflikoçhisar	Wild	G46	Konya	Cihanbeyli	Wild
G4	Ankara	Şereflikoçhisar	Wild	G47	Konya	Cihanbeyli	Wild
G5	Ankara	Haymana	Wild	G48	Konya	Cihanbeyli	Wild
G6	Ankara	Haymana	Wild	G49	Sivas	Hafik	Wild
G7	Ankara	Haymana	Wild	G50	Sivas	Hafik	Wild
G8	Ankara	Haymana	Wild	G51	Sivas	Hafik	Wild
G9	Ankara	Haymana	Wild	G52	Sivas	Hafik	Wild
G10	Ankara	Haymana	Wild	G53	Sivas	Hafik	Wild
G11	Ankara	Çubuk	Wild	G54	Sivas	Suşehri	Wild
G12	Ankara	Çubuk	Wild	G55	Sivas	Suşehri	Wild
G13	Erzurum	Pasinler	Wild	G56	Sivas	Suşehri	Wild
G14	Erzurum	Pasinler	Wild	G57	Speeda	Mas Seeds Seed Company Co. Ltd	
G15	Erzurum	Pasinler	Wild	G58	Nimet	Eastern Mediterranean Agricultural Research Institute/Adana	
G16	Erzurum	Hınıs	Wild	G59	Prosementi	Tasaco Agriculture Industry and Trade Inc	
G17	Erzurum	Hınıs	Wild	G60	Gea	Maro Agriculture Construction Trade and Industry Inc	
G18	Erzurum	Hınıs	Wild	G61	Melissa	May-Agro Seed Industry and Trade Inc	
G19	Erzurum	Hınıs	Wild	G62	Magna-601	Biotek Seed Agriculture Products Industry and Trade Inc	
G20	Erzurum	Aşkale	Wild	G63	Toros	N.E.O.B.I. Seed Inc	
G21	Erzurum	Aşkale	Wild	G64	Maryo	Maro Agriculture Construction Trade and Industry Inc	
G22	Erzurum	Aşkale	Wild	G65	Özpınar	Aegean Agricultural Research Institute	
G23	Eskişehir	Sivrihisar	Wild	G66	Planet	Maro Agriculture Construction Trade and Industry Inc	
G24	Eskişehir	Sivrihisar	Wild	G67	Marina	Palmiye Seeds	
G25	Eskişehir	Mahmudiye	Wild	G68	Plato	Kazak Agriculture Construction Contracting Transport Industry and Trade Inc	
G26	Eskişehir	Mahmudiye	Wild	G69	Magna-905	Biotek Seed Agriculture Products Industry and Trade Inc	
G27	Eskişehir	Mahmudiye	Wild	G70	Queen	N.E.O.B.I. Seed Inc	
G28	Eskişehir	Mahmudiye	Wild	G71	Bilensoy-80	Field Crops Central Research Institute	
G29	Kirikkale	Bahşılı	Wild	G72	Savaş	Eastern Anatolia Agricultural Research Institute	
G30	Kirikkale	Bahşılı	Wild	G73	Hüseyin Aga	Aegean Agricultural Research Institute	
G31	Kirikkale	Bahşılı	Wild	G74	Zenith	Ulusoy Seed Agriculture Industry and Trade Co. Ltd	
G32	Kirikkale	Bahşılı	Wild	G75	Sünter	Mutlu Seed Industry and Trade Co. Ltd	
G33	Kirikkale	Bahşılı	Wild	G76	Kayseri	Local genotype	
G34	Kirikkale	Bahşılı	Wild	G77	La Torre	Maro Agriculture Construction Trade and Industry Inc	
G35	Kirikkale	Bahşılı	Wild	G78	Kalender	N.E.O.B.I. Seed Inc	
G36	Kirikkale	Bahşılı	Wild	G79	İside	May-Agro Seed Industry and Trade Inc	
G37	Kirikkale	Karakeçili	Wild	G80	La Bella	Samen-unternehmung Cristoph Böhrer (Austrian Company)	
G38	Kirikkale	Karakeçili	Wild	G81	Magnum 7	Biotek Seed Agriculture Products Industry and Trade Inc	
G39	Kirikkale	Karakeçili	Wild	G82	Elçi	Ankara University Faculty of Agriculture	
G40	Kirikkale	Karakeçili	Wild	G83	Giulia	Mutlu Seed Industry and Trade Co. Ltd	
G41	Konya	Ereğli	Wild	G84	Emiliana	Palmiye Seed Agriculture Industry and Trade Co. Ltd	
G42	Konya	Ereğli	Wild	G85	Ezzelina	Alfa Seed Agriculture Food Construction Livestock Marketing Industry and Trade Co. Ltd	
G43	Konya	Ereğli	Wild				

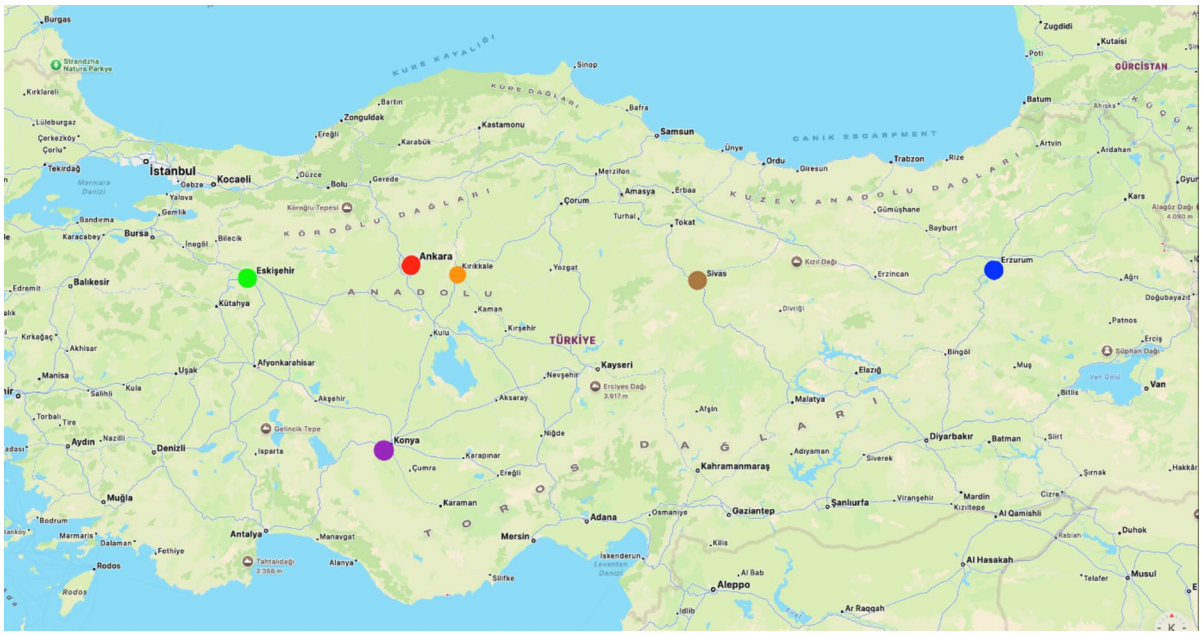

Fig. 1 Geographic distribution of the sampling sites for alfalfa (*Medicago sativa* L.) germplasm in Türkiye. Provinces highlighted include Ankara, Eskişehir, Kırıkkale, Konya, Sivas, and Erzurum, representing the collection sites of wild accessions, while cultivars were obtained from national breeding programs

Bands were visualized using ethidium bromide (EtBr) staining (Sigma-Aldrich GmbH, Darmstadt, Germany), and fragment sizes were estimated using 50 bp and 1000 bp DNA ladders (Vivantis, product no. NM2421), as described by Türkoğlu et al. (2023).

Data analysis

Amplified bands from each sample were scored as either present (1) or absent (0), and the resulting data were used to construct a binary matrix using TotalLab TL120 software (TotalLab Ltd., Gosforth, Newcastle upon Tyne, UK). Genetic similarity among genotypes was assessed using the Dice similarity (Dice 1945), and cluster analysis was performed with the NTSYSpc software (version 2.0). The unweighted pair group method with arithmetic mean (UPGMA) (Rohlf 2000) and principal component analysis (PCA) were employed to visualize genetic relationships among genotypes. The discriminatory power of each SRAP primer was evaluated based on the polymorphism information content (PIC), calculated using the formula:

$$PIC_i = 1 - \sum_{j=1}^{n} p_{ij}^2,$$

where p_{ij} represents the frequency of the j-th pattern amplified by the i-th primer (Wolko et al. 2014). Genetic diversity parameters—including the effective number of alleles (Ne), Nei's genetic diversity index (He), and Shannon's information index (I) were computed using POPGENE version 1.32 (Talebi et al. 2011). The population genetic structure was examined through model-based Bayesian clustering implemented in STRUCTURE v2.2 software (Pritchard et al. 2000). Simulations were performed for K values ranging from 2 to 10, with each K scenario replicated across 10 independent runs. Each cluster was defined by a distinct set of allele frequencies at each locus, and individuals were probabilistically assigned to clusters. The analysis employed a Markov Chain Monte Carlo (MCMC) approach, with a burn-in period of 10,000 iterations followed by 100,000 iterations under the admixture model with correlated allele frequencies. The optimal number of clusters (K) was determined using the ΔK method proposed by Evanno et al. (2005), and results were visualized

Table 2 Some parameters of 10 sequence-related amplified polymorphism (SRAP) markers of 85 alfalfa genotypes

Primer	Primer	Type	Sequence (5′–3′)	Allele number	PIC[a]	Ne	h	I
SRAP1	F-me 1	Forward	TGAGTCCAAACCGGATA	11	0.27	1.42	0.27	0.43
	R-em 1	Reverse	GACTGCGTACGAATTAAT					
SRAP2	F-me 3	Forward	TGAGTCCAAACCGGAAT	8	0.35	1.37	0.23	0.36
	R-em 1	Reverse	GACTGCGTACGAATTAAT					
SRAP3	F-me 4	Forward	TGAGTCCAAACCGGACC	7	0.39	1.68	0.39	0.57
	R-em 1	Reverse	GACTGCGTACGAATTAAT					
SRAP4	F-me 5	Forward	TGAGTCCAAACCGGAAG	13	0.18	1.26	0.17	0.29
	R-em 1	Reverse	GACTGCGTACGAATTAAT					
SRAP5	F-me 7	Forward	TGAGTCCAAACCGGTCC	14	0.33	1.55	0.33	0.50
	R-em 1	Reverse	GACTGCGTACGAATTAAT					
SRAP6	F-me 3	Forward	TGAGTCCAAACCGGAAT	18	0.20	1.26	0.19	0.34
	R-em 3	Reverse	GACTGCGTACGAATTGAC					
SRAP7	F-me 5	Forward	TGAGTCCAAACCGGAAG	11	0.28	1.46	0.27	0.43
	R-em 5	Reverse	GACTGCGTACGAATTAAC					
SRAP8	F-me 7	Forward	TGAGTCCAAACCGGTCC	19	0.36	1.58	0.35	0.53
	R-em 7	Reverse	GACTGCGTACGAATTCAA					
SRAP9	F-me 8	Forward	TGAGTCCAAACCGGTGC	14	0.13	1.20	0.12	0.20
	R-em 1	Reverse	GACTGCGTACGAATTAAT					
SRAP10	F-me 9	Forward	GACTGCGTACGAATTCGA	9	0.46	1.87	0.46	0.65
	R-em 1	Reverse	GACTGCGTACGAATTAAT					
Mean				12.4	0.295	1.44	0.27	0.42

[a]PIC: Polymorphism information content; ne: the effective number of alleles; h: Nei's gene diversity and I: shannon's Information Index

using the STRUCTURE HARVESTER tool (Kopelman et al. 2015). Population differentiation was further quantified using the fixation index (F_{ST}), which estimates the proportion of genetic variance attributable to population subdivision. An F_{ST} value of 0 indicates no genetic differentiation, whereas a value of 1 indicates complete genetic divergence (Bird et al. 2017).

Results

DNA polymorphism and genetic diversity

A total of 112 alleles were amplified using 10 SRAP markers across 85 *Medicago sativa* genotypes. The number of alleles per marker ranged from 7 (SRAP3) to 19 (SRAP8), with an average of 12.4 alleles, indicating moderate to high locus-specific allelic richness within the studied population. The polymorphism information content (PIC), which quantifies the informativeness and discriminatory capacity of each marker, ranged from 0.13 (SRAP9) to 0.46 (SRAP10), with a mean PIC value of 0.295 (Table 2).

The effective number of alleles (Ne) ranged from 1.20 to 1.87, with a mean value of 1.44, reflecting moderate allelic diversity per locus. Nei's gene diversity index (He) varied from 0.12 (SRAP9) to 0.46 (SRAP10), with an average of 0.27, while Shannon's Information Index (I), which accounts for both allele richness and evenness, ranged from 0.20 (SRAP9) to 0.65 (SRAP10), with a mean value of 0.42. These indices collectively suggest substantial genetic variation among the analyzed genotypes.

Clustering analysis of alfalfa genotypes based on SRAP markers

The genetic similarity matrices matrix for alfalfa genotypes collected from different provinces of Türkiye is presented in Supplementary Table 1. The genetic similarity between genotypes ranged from 0.00% (G1

and G75, G2 and G75, G22 and G68, G31 and G80, G34 and G85, G46 and G85, G47 and G85 and G50 and G85) to 80.0% (G70 and G72). The UPGMA dendrogram constructed based on Dice's similarity coefficients derived from SRAP markers revealed a distinct genetic structure among the 85 analyzed *Medicago sativa* genotypes. Hierarchical clustering divided the genotypes into two principal groups: Cluster I and cluster II. Cluster I encompassed a relatively small subset of genotypes, suggesting the presence of a genetically distinct lineage. In contrast, cluster II included the majority of genotypes and exhibited greater internal branching, indicative of higher intragroup genetic variability. The relatively long branch lengths separating cluster I from cluster II highlight considerable genetic divergence between the two groups, potentially reflecting differences in geographic origin, domestication history, or breeding practices. However, the formation of only two major clusters may suggest limited resolution or the amplification of more conserved genomic regions by the SRAP markers. Examination of the dendrogram also revealed that some genotypes collected from the same geographic region clustered closely together, reflecting potential genetic relatedness due to shared ancestry or localized gene flow. For instance, genotypes G35 and G39 were closely grouped, suggesting strong genetic similarity, which is likely attributable to their common collection site. Similarly, the commercial cultivars 'Sunter' (G75) and 'Kayseri' (G76) clustered within cluster II, albeit on separate branches. This pattern suggests that while these cultivars may have originated from distinct breeding lines, they retain a level of genetic similarity, possibly due to shared ancestral germplasm or parallel selection pressures during domestication. Notably, both cultivars are widely cultivated across Turkey and were developed from diverse Anatolian landraces during variety improvement programs. Conversely, some genotypes originating from the same geographic location were distributed across different clusters, indicating a lack of strict geographic structuring. For example, G1 and G85, although collected from the same region, were assigned to cluster I and cluster II, respectively. This observation underscores the presence of substantial genetic heterogeneity within local populations, likely driven by the highly outcrossing reproductive nature of alfalfa and extensive gene flow occurring over generations (Fig. 2).

Principal component analysis (PCA) of alfalfa genotypes

The genetic relationships among the 85 *Medicago sativa* genotypes were further examined through principal component analysis (PCA) based on SRAP marker data. The two-dimensional scatter plot, derived from the genetic distance matrix, revealed a clear separation of genotypes into two major clusters along the first two principal coordinates (Fig. 2). This spatial distribution corroborates the clustering pattern identified by the UPGMA dendrogram, thereby reinforcing the reliability of the inferred population structure. Genotypes forming the green cluster, predominantly located in the upper-left quadrant of the PCA plot, correspond to individuals grouped within Cluster I of the UPGMA analysis. Conversely, the orange cluster, occupying the lower-right quadrant, includes genotypes aligned with Cluster II, which comprises the majority of the analyzed accessions. The concordance between PCA and hierarchical clustering highlights the consistency and robustness of the genetic differentiation captured by SRAP markers.

Population structure analysis based on bayesian clustering

To determine the most probable number of genetic clusters (K) among the 85 alfalfa genotypes, the Evanno method (Evanno et al. 2005) was employed using STRUCTURE Harvester software. STRUCTURE simulations across K = 2–10 (Fig. 2) indicated that the most likely number of clusters was K = 2, as supported by the ΔK method (Evanno et al. 2005). Moreover, the results are consistent with those obtained from UPGMA and principal component analysis (PCA), both of which support the existence of two genetically distinct groups within the studied population. Based on membership coefficients, the genotypes were grouped into two distinct subpopulations: subpopulation A (red) and subpopulation B (green). Specifically, genotypes G1 through G50 were assigned to subpopulation A, while genotypes G51 through G85 were assigned to subpopulation B. The membership probabilities of the 85 alfalfa genotypes were estimated using STRUCTURE software, with individuals assigned to subpopulations based on their Q-values (Supplementary Table 2). The analysis revealed that 78 genotypes (91.76%) were classified

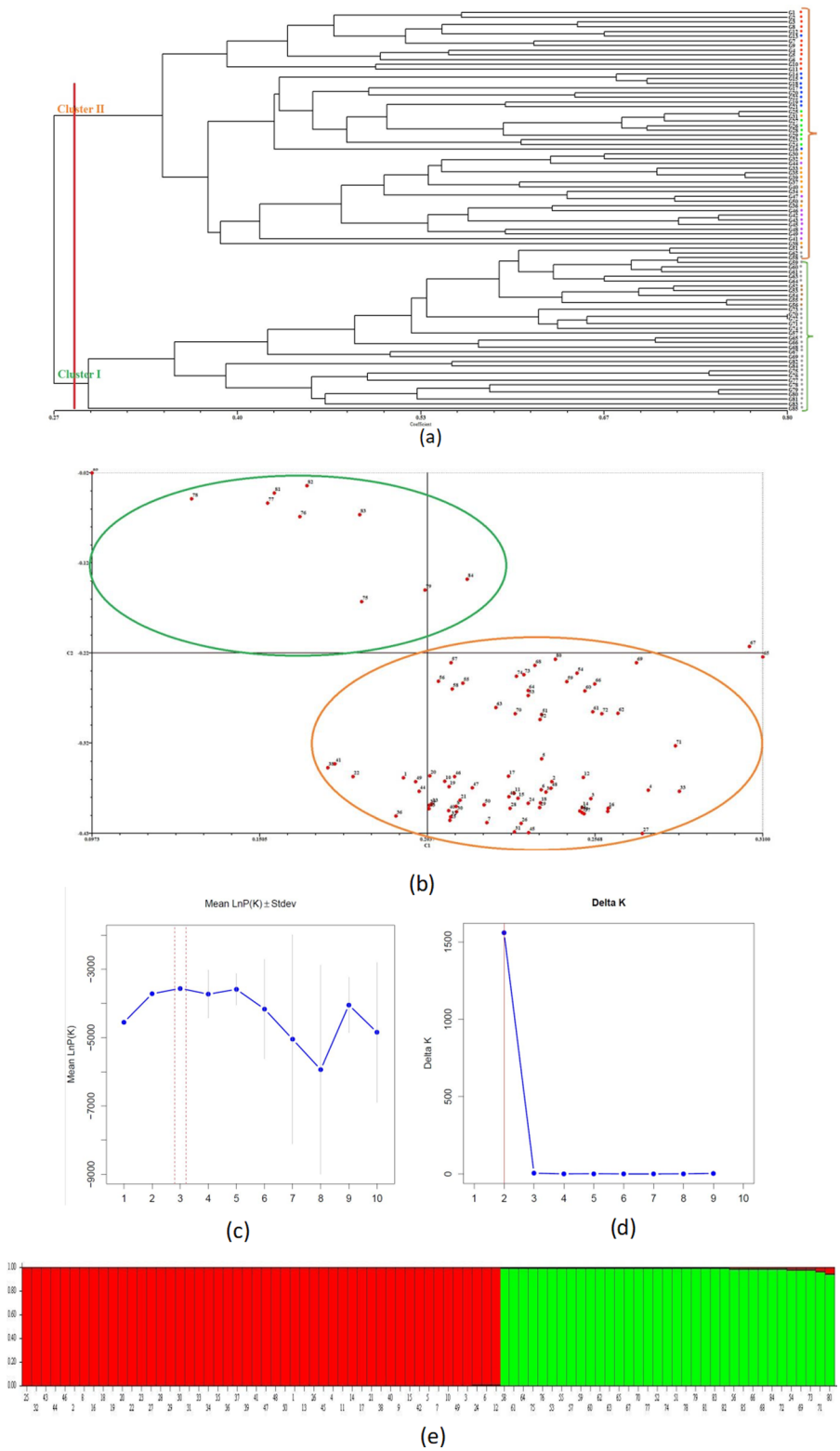

Fig. 2 Genetic structure of 85 alfalfa (*Medicago sativa* L.) genotypes revealed by Sequence-related amplified polymorphism (SRAP) markers. **a** Unweighted pair group method with arithmetic mean (UPGMA) dendrogram constructed using Dice similarity coefficients, with branches color-coded by geographic origin: Ankara (red), Erzurum (blue), Eskişehir (green), Kırıkkale (orange), Konya (purple), Sivas (brown), and cultivars (gray). Two main clusters were identified: Cluster I and Cluster II. **b** Principal component analysis (PCA) showing differentiation of wild and cultivated germplasm into two main groups. **c** Mean LnP(K) values, **d** ΔK statistics from STRUCTURE analysis, indicating K = 2 as the most likely number of clusters. **e** Population structure bar plot illustrating two distinct genetic subpopulations, consistent with dendrogram and PCA results

Table 3 Average expected heterozygosity and fixation index (F_{ST}) for alfalfa subpopulations

Subpopulation (K)	Average distances (expected heterozygosity)	Mean value of Fst
Subpopulation A	0.2026	0.3952
Subpopulation B	0.2612	0.2443

Table 4 Pairwise nei's genetic distance between alfalfa subpopulations

Subpopulation (K)	A	B
Subpopulation A	–	0.1088
Subpopulation B	0.1088	–

as genetically pure, exhibiting high membership probabilities to either Subpopulation A or subpopulation B. subpopulation A comprised the majority of genotypes (G1 to G49), each displaying Q-values greater than 0.98, indicating strong genetic assignment and a well-differentiated cluster. Similarly, Subpopulation B included genotypes G50 to G85, most of which also exhibited high membership coefficients ($Q > 0.98$), further supporting the presence of a distinct genetic grouping. Only a few genotypes (e.g., G54, G68, G71, G72, and G84) exhibited evidence of admixture, with Q-values ranging from 0.947 to 0.985, suggesting a limited degree of gene flow between the two subpopulations. The most admixed individual was genotype G80, with a Q-value of 0.947 for Subpopulation B, indicating potential hybrid ancestry or recent gene exchange. Genetic diversity parameters derived from the STRUCTURE analysis indicated marked differentiation between the two subpopulations (Tables 3, 4). The expected heterozygosity (He), representing the average genetic variation within each subpopulation, was estimated at 0.2026 for Subpopulation A and 0.2612 for Subpopulation B, suggesting that the latter possesses relatively greater genetic variability. The fixation index (F_{ST}), which quantifies genetic differentiation between populations, was calculated as 0.3952 for Subpopulation A and 0.2443 for Subpopulation B. These values indicate a moderate to high level of genetic differentiation, with Subpopulation A exhibiting a more genetically isolated structure, as reflected by its higher F_{ST} value. In contrast, the lower F_{ST} observed in Subpopulation B suggests a comparatively more admixed or genetically diverse composition.

Discussion

Understanding the genetic diversity and population structure of *Medicago sativa* L. is essential for the development of effective conservation strategies and for guiding breeding programs aimed at enhancing agronomic traits and adaptive potential (Demirel et al. 2024; Eren et al. 2023). Sequence-Related Amplified Polymorphism (SRAP) markers, developed by Li and Quiros (2001), constitute a molecular marker system specifically designed to target open reading frames (ORFs) in plant genomes. This system generates polymorphic bands through the use of primers that anneal to the initiation regions of selected ORFs. Although structurally similar to other single-primer-pair techniques such as Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) markers, the SRAP system offers enhanced reproducibility and greater discriminatory power due to its proximity to coding regions (Chen et al. 2004; Li and Quiros 2001). Numerous investigations have demonstrated the efficacy of SRAP markers in detecting both intraspecific and interspecific genetic diversity, particularly in cultivated crops and natural populations (Aakanksha et al. 2023). Owing to their high percentage of polymorphism and superior reproducibility compared to other dominant marker systems, SRAP markers have been widely employed in applications such as genetic mapping, population structure analysis, and cultivar identification (Robarts and Wolfe 2014). Beyond their application in diversity research, SRAP markers have been successfully employed in genetic mapping and gene tagging, particularly because of their preferential amplification of ORF-associated regions (Li and Quiros 2001; Ferriol et al. 2003). For example, Talebi et al. (2011) and Rhouma et al. (2017) demonstrated the integration of SRAP markers in mapping research in alfalfa populations. Although their dominant inheritance limits their resolution compared to SNPs and SSRs, SRAPs remain valuable in constructing preliminary linkage maps, detecting QTLs, and complementing high-throughput markers in advanced mapping efforts. Although SRAP

markers are not typically used for direct marker-assisted selection of agronomic traits, their ability to reveal diversity in coding regions makes them valuable for identifying genetically distinct parental lines (Li and Quiros 2001; Robarts and Wolfe 2014). Breeders can exploit these markers to strategically select divergent genotypes that maximize heterosis and enhance adaptive potential, while trait-specific selection should ideally be combined with SSR, SNP, or GWAS-based markers (Aakanksha et al. 2023).

In the present study, a total of 112 alleles were amplified using 10 based on SRAP primer combinations across 85 alfalfa genotypes, indicating a moderate to high level of genetic polymorphism. The average polymorphism information content (PIC) value of 0.295 observed here exceeds the 0.14 PIC reported in a previous iPBS-based study by Eren et al. (2023), suggesting that SRAP markers may offer greater resolution for detecting genetic variation in alfalfa populations. Similarly, Talebi et al. (2011) reported that 95 of 193 fragments generated by 14 SRAP primer combinations were polymorphic, with an average of 6.78 polymorphic fragments per primer and a PIC value of 0.343. Furthermore, Rhouma et al. (2017), in their analysis of 110 alfalfa individuals from diverse cultivars, landraces, and natural populations, found that 10 SRAP primer combinations yielded 137 fully polymorphic bands, with PIC values ranging from 0.81 to 0.94 (mean = 0.90), thereby confirming the high discriminatory capacity and reliability of SRAP markers in capturing genetic variation across diverse geographic regions. In comparison with previous SRAP-based research, our mean PIC value (0.295) was lower than the range reported by Rhouma et al. (2017) (0.81–0.94) but similar to the values obtained by Talebi et al. (2011) (0.343). These differences can be attributed to the specific primer sets used, the number of loci analyzed, and the genetic background of the populations examined. While Rhouma et al. evaluated highly diverse germplasm from multiple continents, our analysis focused primarily on Turkish accessions, which may harbor more localized variation. Thus, although absolute PIC values differ, the overall conclusion remains consistent: SRAP markers are effective tools for resolving genetic diversity and population structure in alfalfa. Collectively, these findings underscore the utility of SRAP markers as highly effective tools for assessing genetic diversity within alfalfa germplasm and demonstrate their consistent informativeness across a range of investigations and populations.

The effective number of alleles (ne), Nei's gene diversity (h), and Shannon's Information Index (I) further underscored the efficiency of SRAP markers in capturing the extent of genetic variation within the studied alfalfa genotypes. Comparable findings were reported by Talebi et al. (2011), who observed ne values ranging from 1.50 to 1.57, h values between 0.29 and 0.33, and I value from 0.44 to 0.49 in Iranian alfalfa populations, reinforcing the capacity of SRAP markers to reveal moderate to high levels of intra-population genetic variation. Similarly, Rhouma et al. (2017), in an analysis of 110 individuals representing 13 alfalfa cultivars, landraces, and natural populations from Tunisia, Australia, Serbia, and Kazakhstan, reported ne values between 1.57 and 1.70, Nei's gene diversity (h) values from 0.21 to 0.26, and shannon's information index (I) values from 0.32 to 0.38. These findings collectively validate the utility of SRAP markers in effectively capturing within-population genetic diversity across broad geographic ranges. Such results are consistent with previous research demonstrating the robustness of based on markers in assessing intra-specific diversity, particularly in species with complex, outcrossing breeding systems such as alfalfa (Yu et al. 2008). In the present study, the efficiency of SRAP markers in detecting genetic variation among the 85 alfalfa genotypes suggests that they may offer a more refined resolution of genetic structure than iPBS markers. The higher values of PIC, h, and I observed in this study indicate superior marker informativeness, highlighting their potential utility in future genetic mapping, diversity assessments, and breeding program applications. This finding is consistent with previous research employing iPBS markers in alfalfa, such as Eren et al. (2023), who reported lower PIC values (0.14) compared to those obtained with SRAPs in the present study.

The UPGMA dendrogram constructed using Dice similarity coefficients revealed two major genetic clusters, indicating clear genetic structuring among the analyzed genotypes. Notably, while some genotypes from the same geographic origin clustered together, others did not, reflecting the high level of within-population genetic diversity characteristic of outcrossing species like alfalfa (Julier et al. 2003). This finding is partially consistent with the

four-cluster genetic structure reported by Eren et al. (2023) using iPBS markers; however, discrepancies may be attributed to differences in marker type and their respective genomic targets. The hierarchical clustering tree also revealed that genotypes originating from the same collection sites were not consistently grouped together, suggesting an absence of strict geographic structuring. This pattern is likely a consequence of alfalfa's allogamous (outcrossing) reproductive system, combined with the potential for seed and pollen exchange among populations. Rhouma et al. (2017) similarly observed that alfalfa genotypes, when analyzed using a neighbor-joining dendrogram based on a genetic distance matrix, clustered into three primary groups. To further elucidate population structure, a model-based Bayesian clustering analysis was conducted; in that study, the second-highest Delta K peak occurred at K = 2, while the highest peak was observed at K = 7, suggesting a more complex substructure.

Likewise, Talebi et al. (2011) evaluated 48 alfalfa genotypes using SRAP markers and found that a UPGMA dendrogram constructed with Jaccard's similarity coefficient successfully differentiated all genotypes, with a cophenetic correlation coefficient of r = 0.66. However, no strong correlation with geographic origin was detected. In addition, Principal Component Analysis (PCA) revealed that the first three principal components accounted for only 32.88% of the total genetic variation, indicating that a substantial proportion of the underlying genetic structure remains unexplained in low-dimensional space. These collective findings reinforce the conclusion that genetic structuring in alfalfa is predominantly shaped by allelic variation and gene flow, rather than by strict geographic patterns consistent with the biological characteristics of this highly recombining, outcrossing species.

Although a formal Mantel test was not performed, the observed concordance between the genetic similarity matrices and the dendrogram topology suggests a moderate to strong correlation, thereby supporting the reliability of the clustering patterns obtained (Eren et al. 2023). This structured genetic diversity is of considerable significance for germplasm conservation and hybrid breeding programs, where the strategic selection of genetically divergent parental lines can enhance heterosis and improve adaptive potential (Li and Brummer 2012). Comparable patterns of structured diversity accompanied by substantial intra-population variation were reported by Talebi et al. (2011), where UPGMA dendrograms constructed using SRAP markers delineated distinct genotype clusters despite pronounced within-population heterogeneity.

Further insights were obtained through STRUCTURE analysis, which clearly partitioned the 85 alfalfa genotypes into two genetically distinct subpopulations (K = 2), a result strongly supported by the Delta K method of Evanno et al. (2005). The bipartite grouping identified by STRUCTURE corresponds broadly to the improvement status of the germplasm, with wild accessions (G1–G50) clustering in Subpopulation A and cultivars (G51–G85) in Subpopulation B. This separation reflects both the selective pressures applied during cultivar development and the maintenance of greater genetic differentiation in wild populations. Geographic origin also contributed to stratification: Central Anatolian genotypes (Ankara, Konya, Kırıkkale) were more closely related, while Eastern Anatolian accessions (Sivas, Erzurum) showed greater divergence, suggesting local adaptation and restricted gene flow. PCA plots displayed a more continuous distribution compared to STRUCTURE, which is expected given that PCA partitions variance, whereas STRUCTURE models allele frequency–based ancestry. Together, these complementary analyses highlight the strong differentiation between wild and cultivated germplasm as well as the contribution of geographic origin to population structure. The ordered clustering pattern, where genotypes G1–G50 grouped into Subpopulation A and G51–G85 into Subpopulation B, can be attributed to the sensitivity of SRAP markers in detecting ORF-based polymorphisms. Because the first group largely comprised wild accessions and the second group cultivars, SRAPs effectively captured their distinct genetic backgrounds, producing a clear separation between the two subpopulations. This bipartite clustering contrasts with the four-subpopulation model identified in iPBS-based research (Eren et al. 2023), likely reflecting inherent differences in the genomic targets of the marker systems. While iPBS markers are designed to amplify sequences flanking insertions distributed across the genome, SRAP markers preferentially target open reading frame (ORF)-associated regions, potentially revealing distinct layers of genetic structure (Boban et al. 2022). Because SRAP

markers target ORF-associated regions, they are considered non-neutral and may reveal polymorphisms influenced by selection. This characteristic enhances their utility in detecting adaptive differentiation and functional diversity, making them valuable for identifying divergent parental lines and understanding adaptive divergence among populations. However, this also means that SRAP-based phylogenies may not exclusively reflect neutral evolutionary processes but may instead be biased toward genomic regions under selection. Consequently, while SRAPs provide powerful insights into population structure and adaptive diversity, their use in phylogenetic reconstruction should ideally be complemented with neutral marker systems such as SSRs or SNPs to obtain a more balanced and comprehensive view of genetic relationships (Li and Quiros 2001). A similar observation was made by Rhouma et al. (2017), who reported that SRAP-based STRUCTURE analysis grouped 110 alfalfa genotypes into four main genetic clusters (K = 7), further confirming the effectiveness of SRAP markers in detecting fine-scale population differentiation and highlighting the genetic divergence among genotypes of diverse geographic origin. Genetic differentiation analysis revealed moderate to high levels of population structure, with fixation index (F_{ST}) values of 0.3952 for Subpopulation A and 0.2443 for Subpopulation B, indicating restricted gene flow and historical genetic isolation between the two subpopulations. STRUCTURE analysis revealed that most individuals exhibited high Q-values (> 0.947), which indicates strong assignment to a single subpopulation and therefore low levels of admixture. Only a limited number of genotypes displayed intermediate Q-values, suggesting restricted but detectable gene flow between Subpopulation A and Subpopulation B.

Although SRAP markers are not directly linked to specific agronomic traits, they proved effective in distinguishing divergent subpopulations. Individuals from Subpopulation A, with higher genetic differentiation (FST = 0.3952), and those from Subpopulation B, with greater internal variability (He = 0.2612), represent valuable sources of parental material. Crossing genetically distinct representatives from both groups may broaden the genetic base and enhance heterosis in alfalfa breeding programs. These F_{ST} values are notably higher than those reported in previous alfalfa research, which typically ranged from 0.18 to 0.30 (Eren et al. 2023), suggesting that the genetic distinctiveness of the subpopulations examined here has been well preserved, potentially due to geographic isolation, ecological divergence, or targeted breeding practices. In contrast, Talebi et al. (2011) reported relatively low yet statistically significant genetic differentiation ($F_{ST} = 0.068$) among Iranian alfalfa populations, highlighting the influence of the species obligate outcrossing nature and localized adaptation on gene flow patterns.

Analysis of membership coefficients further supported the presence of well-defined subpopulations, with 91.76% of genotypes exhibiting high assignment probabilities (Q > 0.98), indicative of genetically pure groups with minimal admixture. Only a small subset of individuals, such as G80, displayed intermediate Q-values, suggesting historical hybridization or incomplete lineage sorting. The overall low admixture observed contrasts with findings in other alfalfa populations and underscores the robustness of SRAP markers for inferring population structure (Pritchard et al. 2000). Similarly, Rhouma et al. (2017) reported that 95.45% of genotypes were confidently assigned to one of seven genetic clusters based on SRAP analysis, reinforcing the utility of this marker system in resolving fine-scale genetic stratification across geographically and genetically diverse germplasm. Collectively, these findings affirm that SRAP markers are effective and informative tools for assessing genetic diversity and population structure in Medicago sativa. As a complement to other marker systems such as iPBS and SSR, SRAPs offer practical advantages for applications in germplasm conservation, association mapping, and the identification of genetically divergent parental lines in breeding programs. Unlike SNP or SSR markers, SRAPs are scored as dominant bands and are not associated with predefined genomic coordinates. Their primers preferentially target ORF-associated regions, which makes them useful for detecting functional polymorphisms (Li and Quiros 2001). Determining the exact chromosomal positions of these fragments would require sequencing and alignment to the alfalfa reference genome, a step that was beyond the scope of the present study but remains a promising avenue for future research.

Future research integrating SRAP data with genomic, epigenomic, and phenotypic datasets holds promise for enhancing the resolution of population structure and for uncovering functionally relevant alleles associated with agronomic traits. Both

Talebi et al. (2011) and Rhouma et al. (2017) have emphasized the utility of SRAP markers in characterizing genetic variation across diverse alfalfa populations, supporting their continued application in the design of informed breeding and conservation strategies. Overall, the present study demonstrates that SRAP markers provide a reliable and high-resolution method for elucidating genetic diversity and structure within alfalfa germplasm. The clear delineation of two genetically distinct subpopulations, alongside the presence of individuals with high heterozygosity, highlights the potential of this material as a valuable resource for future breeding efforts aimed at exploiting genetic divergence and enhancing heterosis.

Conclusion

In conclusion, this study demonstrated that Sequence-Related Amplified Polymorphism (SRAP) markers are highly informative and reliable molecular tools for evaluating genetic diversity and population structure in Medicago sativa L. The observed moderate-to-high levels of polymorphism (mean PIC = 0.295; He = 0.27) and the clear genetic stratification of the 85 genotypes into two primary clusters (K = 2) underscore the effectiveness of SRAP markers in capturing genetic variability within alfalfa germplasm. Consistent results across STRUCTURE, UPGMA, and PCA (Principal Component Analysis) confirmed the presence of two well-defined subpopulations, with the majority of genotypes exhibiting high membership coefficients, indicative of genetic homogeneity within each cluster. Subpopulation B displayed greater internal genetic diversity, while Subpopulation A was more genetically distinct and isolated, as reflected by its higher fixation index (F_{ST}) value. These findings offer valuable insights into the genetic architecture of both local and commercial alfalfa genotypes, emphasizing the utility of SRAP markers in germplasm characterization, conservation, and breeding initiatives. The identification of genetically divergent and highly pure genotypes provides a robust foundation for parental selection in hybrid breeding programs aimed at maximizing heterosis and improving traits such as abiotic stress tolerance, yield performance, and forage quality.

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Declarations

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