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Genetic Diversity Assessment of *Lotus corniculatus* L. in Khuzestan Using Molecular Markers and DNA Barcoding

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Abstract

Objective

Lotus corniculatus L. is a widely distributed, tetraploid perennial legume valued for its adaptability to diverse environments and its applications in forage production, soil improvement, and medicine. It is essential to assess its genetic diversity using molecular markers for conservation and breeding strategies. This study presents the first molecular characterization of *L. corniculatus* accessions from Khuzestan province in southwestern Iran.

Materials and methods

Twenty-one *L. corniculatus* accessions were collected from eight geographically distinct sites. Genetic variation was assessed using 12 SCoT and 6 ISSR primers. Genetic structure and relationships among accessions were analyzed using cluster analysis, principal coordinate analysis (PCoA), and the STRUCTURE software. To complement these analyses, three genetically divergent accessions were selected for sequencing of two DNA barcode regions: the internal transcribed spacer (ITS) of nuclear ribosomal DNA and the *trnH-psbA* intergenic spacer of the chloroplast genome. Barcoded sequences were analyzed using BLAST and Maximum Likelihood phylogenetic reconstruction.

Results

SCoT and ISSR markers revealed high polymorphism (96.87% and 85.8%, respectively), indicating substantial genetic diversity among the accessions. Cluster and PCoA consistently

grouped the accessions into two main clusters, with the Minoo Island accession forming a distinct and divergent group. STRUCTURE analysis supported the existence of three genetic clusters, reflecting both admixture and clear genetic differentiation. DNA barcoding revealed that all three sequenced accessions, including the genetically distinct Minoo Island accession, shared identical sequences in both the ITS and *trnH-psbA* regions, forming a single haplotype, here designated as the Iranian haplotype. The ITS sequence exhibited 99.71% identity with *L. tenuis*, while the *trnH-psbA* region showed 100% identity with *L. japonicus*. However, both barcode regions lacked sufficient resolution to distinguish intraspecific variation.

Conclusions

Both marker systems were effective in detecting genetic diversity, with SCoT markers showing higher average polymorphism and mean PIC values, and ISSR markers exhibiting stronger marker index and resolving power. The distinct clustering of the Minoo Island accession highlights the potential for local adaptation and underlines the importance of conserving regional germplasm. In contrast, the lack of variation in the barcode sequences emphasizes the limited ability of conserved regions such as ITS and *trnH-psbA* to resolve fine-scale genetic structure. Complementary tools such as chromosome counting and genome-wide markers (e.g., SNP genotyping, RAD-seq, WGS) are recommended to improve taxonomic resolution.

Keywords: Bird's-foot trefoil, Fingerprinting, Genetic differentiation, Genetic resource management, Phylogenetic relationships

Paper Type: Research Paper.

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Introduction

Lotus corniculatus L. (bird's-foot trefoil, Fabaceae) is a widely distributed perennial legume that is globally used as a high-quality forage species (Mimura et al., 2013). It thrives in acidic, nutrient-poor, and waterlogged soils and maintains relatively high productivity under abiotic stresses such as salinity, drought, and flooding. This environmental resilience, along with its value in sustainable forage systems, underscores the importance of its germplasm in future breeding and ecological applications (Chen et al., 2023). In addition to its agronomic and ecological value, this legume is also valued for its pharmacological potential, as it produces bioactive compounds like kaempferol, quercetin, and linoleamide, which exhibit antioxidant, anti-inflammatory, and antimicrobial activities (Yerlikaya et al., 2019; Abdallah et al., 2022). Its adaptability is further supported by considerable heterozygosity and phenotypic plasticity, which together enhance its ecological versatility across diverse environments. Given its broad adaptability and complex traits, recent phylogenetic studies suggest that *L. corniculatus* and its relatives originated in Central or Western Asia, with Transcaucasia proposed as a major center for genetic diversification (Chen et al., 2023). *L. corniculatus* is recognized as a tetraploid (2n = 4x = 24), probably originating from hybridization among related diploids within the *Lotus* genus. However, its precise evolutionary relationships and diploid progenitors remain unresolved. As part of the broader *L. corniculatus* complex, which comprises both diploid and tetraploid taxa, this species exemplifies the taxonomic and phylogenetic challenges associated with hybridization and gene flow among closely related members (Kramina et al., 2018).

Genetic diversity helps plant populations cope with pests, diseases, and environmental stresses, and plays a key role in their long-term survival (Salgotra & Chauhan, 2023). To support effective breeding and conservation programs, it is important to study this diversity at both within- and between-population levels. Molecular markers are widely used for this purpose in plant genetic research (Bidyananda et al., 2024). Start codon targeted (SCoT) markers are PCR-based and use conserved sequences around the ATG start codon, requiring no prior genomic information for primer design (Collard & Mackill, 2009). They have effectively revealed genetic diversity in a wide range of plant species, including those adapted to arid and saline habitats (Zabet et al., 2023; Haghighipor et al., 2024). ISSR markers amplify regions between microsatellites using a single primer, producing polymorphic bands useful for assessing genetic variation in diverse plant species (Ng & Tan, 2015). Their utility in genetic diversity and structure studies has been demonstrated in various plant taxa (Heidari & Salari, 2024; Khandanizadeh et al., 2024).

DNA barcoding provides a powerful sequence-based tool for species identification and phylogenetic studies in plants. The internal transcribed spacer (ITS) region of nuclear rDNA is favored for its high variability, broad taxonomic coverage, and reliable amplification (Letsiou et

al., 2024). Its effectiveness in resolving closely related genotypes has been demonstrated in various studies across different plant groups (Moradi Ashour et al., 2023). Among chloroplast loci, *trnH-psbA* stands out for its high resolution at the species level. It has demonstrated superior assignment accuracy in legumes compared to *matK* and *rbcLa*, making it a strong candidate for the identification of forage species (Loera-Sánchez et al., 2020).

Realizing the full potential of *L. corniculatus* requires a thorough understanding of its genetic diversity, which influences adaptability, productivity, and breeding prospects. Previous studies have assessed genetic relationships in *L. corniculatus* using RAPD (Steiner and García de los Santos, 2001), AFLP and SSR (Savo Sardaro et al., 2008), and ISSR markers (Kramina et al., 2012, Kramina, 2013; Abraham et al., 2015; Merkouropoulos et al., 2016). Sequence-based markers, including the internal transcribed spacer (ITS) and plastid intergenic spacer *trnH-psbA*, have provided valuable insights into the population structure and phylogenetic relationships within the genus *Lotus* (Degtjareva et al., 2008, 2025; Kramina et al., 2012, 2016), while markers such as *trnL-trnF* have specifically contributed to understanding the *L. corniculatus* complex (Kramina et al., 2018). In this study, we used SCoT and ISSR markers, along with nuclear (ITS) and chloroplast (*trnH-psbA*) DNA barcodes, to assess the genetic diversity of *L. corniculatus* accessions collected from Khuzestan province in southwestern Iran. SCoT and ISSR were selected for their cost-effectiveness, reproducibility, and ability to detect polymorphisms without prior genomic data, while DNA barcodes were applied for their high sequence resolution and usefulness in species-level identification. Khuzestan, an ecologically vulnerable region with high salinity, arid climate, and anthropogenic stress, was selected for this study. This is the first molecular investigation of *L. corniculatus* in this region and provides baseline data for breeding and conservation under arid and saline conditions.

Materials and methods

Plant material and DNA extraction: Twenty-one accessions representing eight distinct geographic sites in Khuzestan province were selected from a larger set of 126 genotypes previously assessed for morphological diversity (Mousavi & Nasernakhaei, 2025). Despite the limited sample size, the accessions covered the observed phenotypic variation. The geographic distribution of accessions and their corresponding codes and site details are provided (Figure 1, Table 1). Genomic DNA was extracted from young fresh leaves using the Anacell Kit, which was chosen after preliminary comparisons with alternative protocols demonstrated superior yield and purity for this species. DNA quality was checked by 0.8% agarose gel electrophoresis, and concentration was measured using a NanoDrop spectrophotometer.

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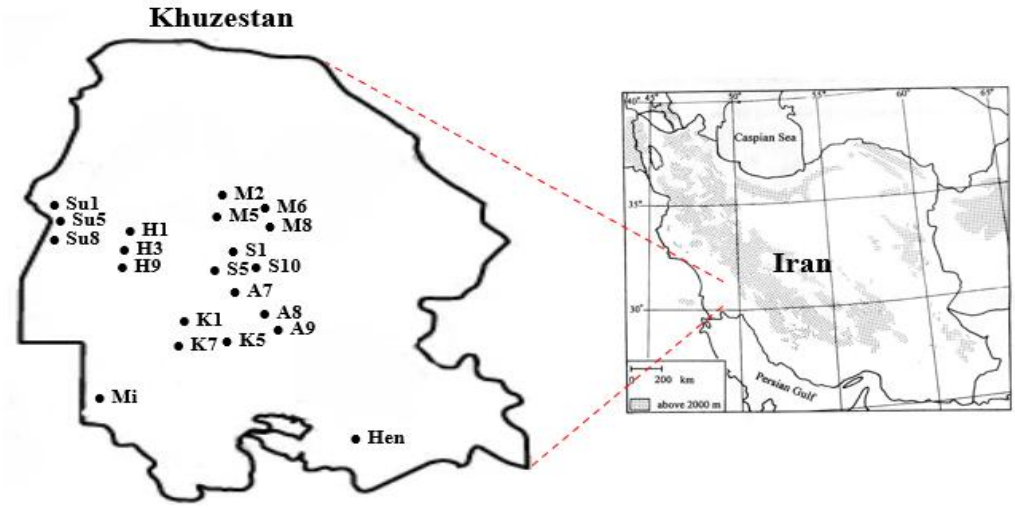

Figure 1. Geographic distribution map of the 21 *L. corniculatus* accessions collected from Khuzestan province. Each collecting-site is marked with its corresponding accession code, as detailed in Table 1.

Table 1. Information of sampling location

Collecting-sites	Accession code	Latitude (N)	Longitude (E)
Ahvaz	A7, A8, A9	31º21 2430′	48º44 0620′
Sheyban	S1, S5, S10	31º23 4920′	48º47 2480′
Mollasani	M2, M5, M6, M8	31º34 0520′	48º52 9760′
Hamidiye	H1, H3, H9	31º28 6420′	48º26 3900′
Susangerd	Su1, Su5, Su8	31º32 7810′	48º11 9570′
Karun	K1, K5, K7	31º13 2230′	48º38 8310′
Minoo Island	Mi	30º20 3150′	48º12 1320′
Hendijan	Hen	30º13 6530′	49º43 5970′

SCoT and ISSR PCR amplification: Twelve SCoT primers were initially selected following Collard & Mackill (2009), but one (SCoT 11) was excluded due to lack of reproducibility, yielding 11 reliable primers. These, alongside six ISSR primers, were used to assess genetic variability (Table 2). PCR reactions for SCoT were performed in a 15 µL volume containing 7.5 µL of 2X PCR master mix (Ampliqon, Denmark), 5.4 µL double-distilled water (ddH_2O), 1.5 µL of genomic DNA (35-45 ng) and 0.6 µL of primer (10 pmol/µL) (Table 2). ISSR amplification were conducted in a 10 µL reaction volume containing of 5 µL of 2X PCR master mix, 0.7 µL of DNA (35-45 ng), 0.4 µL of primer (Table 2) and 3.9 µL ddH_2O. PCR amplifications were carried out using a Bio-Rad T100 thermal cycler. For ISSR markers, amplification conditions were optimized in preliminary experiments using gradient PCR to determine the best annealing temperature and extension time for each primer. The final ISSR protocol included an initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, annealing at

primer-specific temperatures (mostly 51 °C, but 46.4 °C for ISSR 4 and ISSR 9) for 45 s, and extension at 72 °C for 2 min (90 s for ISSR 4 and ISSR 9), with a final extension at 72 °C for 5-7 min depending on the primer. In contrast, SCoT amplifications were performed using uniform conditions: an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 5 min. PCR products were separated on 1.5% agarose gels stained with YTA Safe Stain (Yekta Tajhiz, Iran) and visualized under UV light.

Table 2. SCoT, ISSR, ITS, and *trnH-psbA* primers information

Primer No.	Primer	5′→3′ sequence	Tm (°C)	GC%
P1	SCoT 1	CAACAATGGCTACCACCA	48	50
P2	SCoT 3	CAACAATGGCTACCACCG	50.3	55.6
P3	SCoT 5	CAACAATGGCTACCACGA	48	50
P4	SCoT 11	AAGCAATGGCTACCACCA	48	50
P5	SCoT 13	ACGACATGGCGACCATCG	52.6	61.1
P6	SCoT 14	ACGACATGGCGACCACGC	54.9	66.7
P7	SCoT 19	ACCATGGCTACCACCGGC	54.6	66.7
P8	SCoT 20	ACCATGGCTACCACCGCG	54.9	66.7
P9	SCoT 21	ACGACATGGCGACCCACA	52.6	61.1
P10	SCoT 26	ACCATGGCTACCACCGTC	52.6	61.1
P11	SCoT 34	ACCATGGCTACCACCGCA	52.6	61.1
P12	SCoT 35	CATGGCTACCACCGGCCC	57.1	72.2
P13	ISSR 4	AGAGAGAGAGAGAGAGT	44.7	47.1
P14	ISSR 5	AGAGAGAGAGAGAGAGC	47	52.9
P15	ISSR 7	ACACACACACACACACG	47	52.9
P16	ISSR 9	GGAGAGGAGAGGAGA	44.7	60
P17	ISSR 10	GAGAGAGAGAGAGAGAGT	48	50
P18	ISSR 11	GAGAGAGAGAGAGAGATC	48	50
P19	ITS 1-F	AGAAGTCGTAACAAGGTTTCCGTAGG	61.7	46.2
	ITS 4-R	TCCTCCGCTTATTGATATGC	54.1	45
P20	*trnH-psbA*-F	CGCGCATGGTGGATTCACAATCC	64	56.5
	trnH-psbA-R	GTTATGCATGAACGTAATGCTC	55.3	40.9

DNA barcode amplification and sequencing: To ensure representative sampling for DNA barcoding, three accessions (A7, Hen, and Mi) were selected based on their high genetic divergence as revealed by SCoT and ISSR analyses. These accessions were chosen to capture the widest possible range of genetic variation for subsequent molecular characterization. PCR amplification of the ITS (White et al., 1990) and *trnH-psbA* (Sang et al., 1997; Tate & Simpson, 2003) regions was performed in a 25 µL reaction mixture containing 11.6 µL of ddH_2O, 12.5 µL of 2X PCR master mix, 0.4 µL (30 ng) of genomic DNA, and 0.3 µL of each primer (Table 2). Thermal cycling conditions are listed in Table 3. Amplification success was confirmed by the presence of specific bands on 1.5% agarose gel stained with YTA Safe Stain. The PCR products

were subsequently purified and sequenced bidirectionally using the same primers employed for amplification. Sequencing was performed by GenFanAvaran (Tehran, Iran), enabling accurate downstream phylogenetic analysis.

Table 3. PCR amplification program for ITS and *trnH-psbA*

Step	Temperature ℃	Time	Number of cycles
Initial denaturation	95	3 min	1x
Denaturation	95	30 s	10x
Annealing	62 (ITS) / 64 (*trnH-psbA*)	30 s	
Extension	72	55 s (ITS) / 90 s (*trnH-psbA*)	
Denaturation	95	30 s	22x (ITS) 25x (*trnH-psbA*)
Annealing	52 (ITS) / 54 (*trnH-psbA*)	30 s	
Extension	72	55 s (ITS) / 90 s (*trnH-psbA*)	
Final extension	72	10 min	1x
Hold	4	∞	-

Data analysis-SCoT and ISSR: Amplification bands were scored as present (1) or absent (0) to generate a binary matrix using CLIQS 1D ver. 1.5 software (Total Lab). The total number of bands (N), polymorphic bands (np), and percentage of polymorphism (P%) were calculated. Genetic diversity parameters, including Nei's gene diversity (h) and Shannon's information index (I) were computed using POPGENE ver. 1.32**.** (Yeh et al., 1999). The discriminatory power of SCoT and ISSR markers was assessed by calculating resolving power (Rp) and polymorphism information content (PIC)**,** where PIC was calculated using the formula PIC = 2fi (1-fi), with fi representing the frequency of the ith allele (Serrote et al., 2020). To further assess the efficiency of the marker system, the effective multiplex ratio (EMR) and marker index (MI) were calculated, following the method described by Chesnokov & Artemyeva (2015). A similarity matrix was generated and used to construct a dendrogram based on the unweighted pair group method with arithmetic mean (UPGMA) and Dice coefficient. Furthermore, principal coordinate analysis (PCoA) was performed using PAST ver. 4.16c (Hammer et al., 2001) based on the same similarity coefficients (Dice). Genetic structure was analyzed using the Bayesian approach implemented in STRUCTURE software ver. 2.3.4. (Pritchard et al., 2000). The program was executed with a burn-in period of 5000 generations, followed by 50,000 Markov Chain Monte Carlo (MCMC) iterations. The number of hypothetical genetic clusters (K) was tested from 1 to 10. Each K value was evaluated in ten independent runs. The optimal K was determined using the method of

Evanno et al. (2005), which is based on changes in log probability (ΔK). The best K value was identified using StructureSelector (Li & Liu, 2018).

ITS and *trnH-psbA*: The initial quality of the sequences was assessed using ChromasPro ver. 1.41 (Technelysium Pty. Ltd.). Subsequently, sequences were manually edited and aligned in BioEdit ver. 7.1.10 (Hall, 1999) to ensure high accuracy. The aligned sequences were registered in the National Center for Biotechnology Information (NCBI) database. To identify molecular haplotypes, BLAST searches were conducted against the NCBI database, and sequences with the highest percent identity were selected for further analysis. To evaluate genetic relationships among the identified molecular variants, cluster analysis was performed. Genetic clustering was inferred using the Maximum Likelihood method, with 1000 bootstrap replicates and the Tamura 3-parameter model for both ITS and *trnH-psbA* sequences. All phylogenetic analyses were conducted using MEGA ver. 12.0.11 software (Kumar et al., 2024).

Results and discussion

SCoT and ISSR markers analysis and genetic diversity: Among the 12 initially tested SCoT primers, SCoT 11 was excluded due to poor reproducibility, resulting in 11 reliable primers for analysis. These primers generated a total of 879 scorable fragments (Table 4), with SCoT 21 producing the highest number of bands (147) and SCoT 5 the lowest (10). The average percentage of polymorphism across all SCoT primers was 96.87%, with most primers showing 100% polymorphism, while SCoT 14 exhibited the lowest polymorphism (65.57%). The resolving power (Rp) ranged from 0.95 (SCoT 5) to 11.71 (SCoT 1), with an average of 7.61. The polymorphism information content (PIC) values varied between 0.22 (SCoT 14) and 0.42 (SCoT 21), averaging 0.32 per primer. Similarly, SCoT 21 showed the highest values of effective multiplex ratio (EMR = 147) and marker index (MI = 61.83), reflecting its high efficiency and informativeness. This level of genetic diversity is essential for guiding breeding strategies aimed at improving stress tolerance and for ensuring the long-term conservation of valuable *L. corniculatus* genetic resources. For ISSR analysis, six primers generated 1004 amplified fragments. ISSR 9 produced the highest number of bands (214), while ISSR 7 yielded the lowest (123 bands). The mean percentage of polymorphism was 85.8%. Three primers (ISSR 7, ISSR 9, and ISSR 11) showed 100% polymorphism, while ISSR 10 had the lowest level (60.37%). The average resolving power was 15.94, ranging from 11.71 (ISSR 7) to 20.38 (ISSR 9). PIC values ranged from 0.19 (ISSR 10) to 0.35 (ISSR 7), with a mean of 0.27. ISSR 9 also exhibited the highest values of effective multiplex ratio (EMR = 214) and marker index (MI = 58.02). Further details on the primers are given in Table 4. These findings are consistent with earlier studies on

L. corniculatus, which have also reported variable levels of polymorphism using ISSR markers. For instance, Kramina et al. (2012) reported 86% polymorphic loci; and in a subsequent study, Kramina (2013) observed approximately 95% polymorphism in *L. corniculatus*, confirming similar patterns. Abraham et al. (2015) observed 78.26% polymorphism using seven ISSR primers and Merkouropoulos et al. (2016) reported 100% polymorphism for ISSR 4 and ISSR 5. The variability in ISSR marker efficiency observed in our study aligns with these previous findings, further emphasizing the utility of ISSR markers in assessing genetic diversity. These results collectively highlight the robustness of ISSR markers for genetic differentiation in *L. corniculatus*, and underscore the importance of careful primer selection to maximize polymorphism detection and analytical resolution. In terms of classical diversity indices calculated using POPGENE software ver. 1.32., SCoT markers revealed a mean Nei's gene diversity (h) of 0.25 and Shannon's index (I) of 0.40. In contrast, ISSR markers showed slightly higher genetic diversity values with h = 0.29 and I = 0.44. Although SCoT primers showed higher average polymorphism (96.87%) and mean PIC (0.32) values, indicating their effectiveness in detecting variation within coding regions, ISSR primers outperformed in terms of resolving power (Rp), effective multiplex ratio (EMR), and marker index (MI). These differences reflect the broader genomic scope and strong discriminatory power of ISSR markers. This complementarity highlights the advantage of integrating both marker systems to obtain a more balanced and comprehensive picture of genetic diversity in *L. corniculatus*. While SCoT markers target gene-rich regions flanking the ATG start codon, ISSR markers amplify more variable intergenic sequences enriched in microsatellites. The high polymorphism observed, particularly with SCoT (P% > 96%), likely reflects the high adaptability of *L. corniculatus* to harsh conditions in Khuzestan, as well as its outcrossing breeding system, which enhances genotypic variability. The observed marker performance differences, rooted in their genomic targets, justify their combined use in future studies aiming to inform breeding, conservation, and ecological adaptation strategies for this resilient legume.

Relationships and genetic structure analysis of *L. corniculatus* based on SCoT and ISSR markers: Dendrograms were constructed for 21 accessions using the UPGMA method based on SCoT, ISSR, and combined SCoT+ISSR datasets. The dendrograms generated from all three datasets exhibited a similar clustering pattern. The number of clusters was determined based on a cutoff point on the dendrogram, selected at the position where the largest genetic distance between groups was clearly observed, resulting in the division of accessions into two major clusters. Cluster I was subdivided into two sub-clusters, one containing the accession from Hendijan and the other comprising the remaining accessions. Cluster II exclusively contained accession from Minoo Island demonstrating clear genetic differentiation (Figure 2). This genetic separation likely

reflects ecological isolation and site-specific selection pressures such as salinity gradients, waterlogging, microclimatic factors, and proximity to the Zohreh River in Hendijan. Similar findings have been reported in other studies, where geographical and ecological factors significantly influenced genetic variation in *L. corniculatus* (Steiner & García de los Santos, 2001; Merkouropoulos et al., 2016).

Principal Coordinate Analysis (PCoA) confirmed the clustering results and explained 55.76% of total genetic variation in the first three principal coordinates based on the combined dataset. Notably, SCoT markers exhibited higher eigenvalues than ISSR, indicating broader genome coverage. This finding supports the complementary nature of the markers, with SCoT targeting gene-rich regions and ISSR amplifying intergenic sequences. The clear separation of the Minoo Island accession and partial divergence of Hendijan accession supports the existence of genetically unique ecotypes. This spatial genetic structure highlights potentially valuable gene pools for targeted conservation and breeding programs focused on stress tolerance.

Table 4. SCoT and ISSR amplification results

Primers	N	Np	P%	Rp	PIC	EMR	MI
SCoT 1	123	123	100	11.71	0.26	123	31.98
SCoT 3	102	102	100	9.71	0.38	102	39.03
SCoT 5	10	10	100	0.95	0.36	10	3.63
SCoT 13	94	94	100	8.95	0.33	94	30.83
SCoT 14	61	40	65.57	5.81	0.22	26.22	5.85
SCoT 19	70	70	100	6.67	0.33	70	22.9
SCoT 20	59	59	100	5.62	0.26	59	15.59
SCoT 21	147	147	100	14	0.42	147	61.83
SCoT 26	54	54	100	5.14	0.29	54	15.6
SCoT 34	53	53	100	5.05	0.36	53	19.05
SCoT 35	106	106	100	10.10	0.33	106	34.61
Average			96.87	7.61	0.32	76.74	25.54
ISSR 4	180	138	76.66	17.14	0.23	105.8	24.07
ISSR 5	189	147	77.77	18	0.26	114.33	30.16
ISSR 7	123	123	100	11.71	0.35	123	43.19
ISSR 9	214	214	100	20.38	0.27	214	58.02
ISSR 10	159	96	60.37	15.14	0.19	57.96	10.83
ISSR 11	139	139	100	13.24	0.34	139	47.73
Average			85.8	15.94	0.27	125.68	35.67

Total number of bands (N), polymorphic bands (np), percentage of polymorphism (P%), resolving power (Rp), polymorphism information content (PIC), effective multiplex ratio (EMR), marker index (MI)

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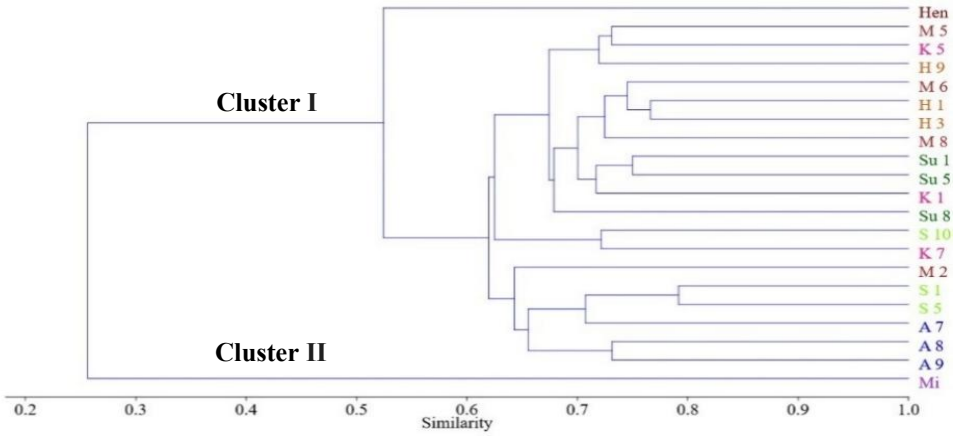

Figure 2. Cluster analysis of *L. corniculatus* based on SCoT+ISSR data (Each branch is labeled with the accession code, corresponding to its respective collecting-sites as detailed in Table 1)

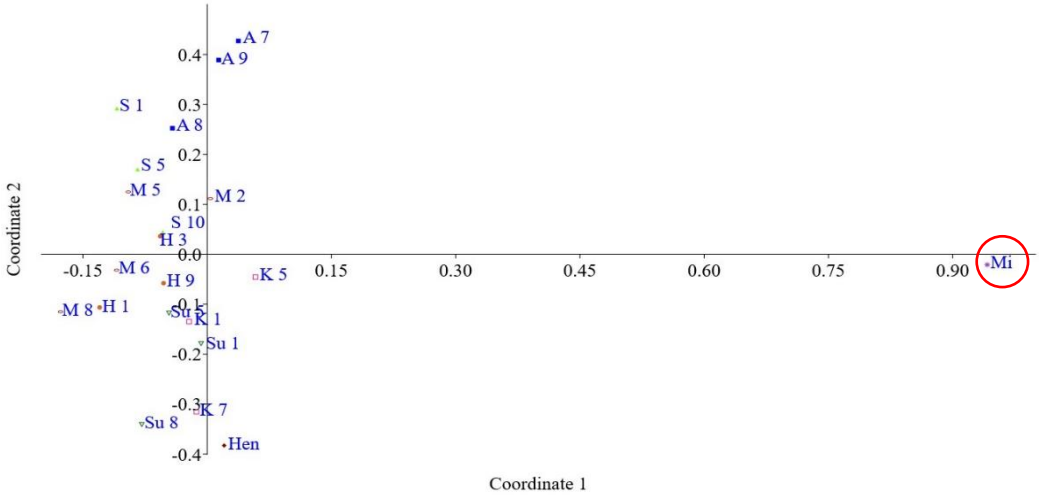

Figure 3. PCoA of 21 accessions of *L. corniculatus* based on combined SCoT+ISSR data (The accession codes are detailed alongside their respective collecting-sites in Table 1)

STRUCTURE analysis, based on SCoT, ISSR, and combined datasets, was performed to assess the genetic structure of the studied accessions. Using the ΔK method proposed by Evanno et al. (2005), K = 3 was identified as the most likely number of genetic clusters (Figure 4). The results revealed clear structuring, with both admixture and distinct groupings among accessions, suggesting the presence of at least three gene pools. This pattern is likely considering the outcrossing reproductive system of *L. corniculatus*, which facilitates widespread gene flow among accessions. STRUCTURE-based clustering thus provides a valuable framework for identifying conservation units and selecting genetically diverse individuals for breeding programs.

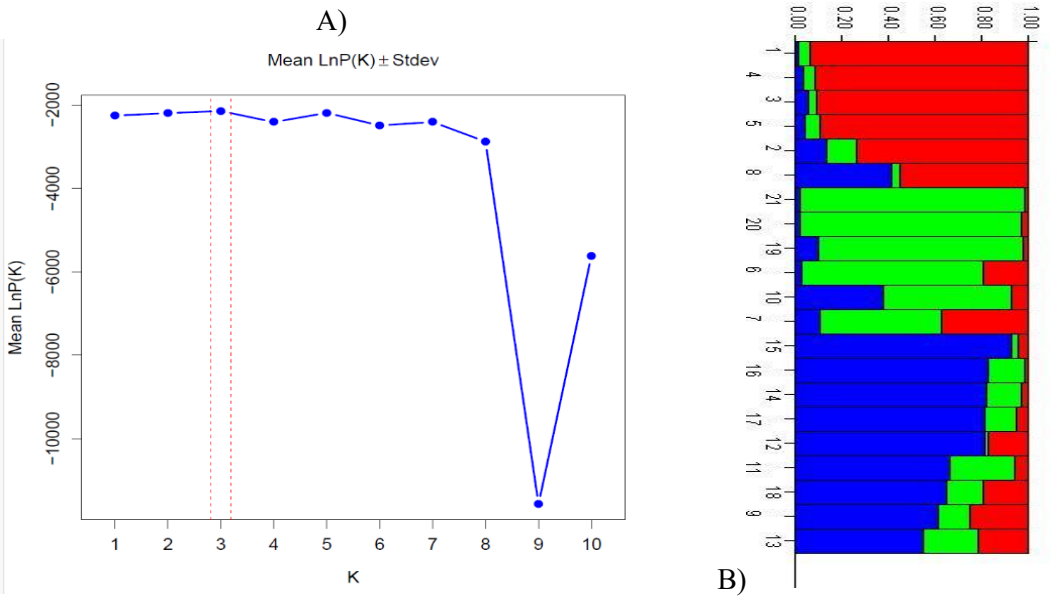

Figure 4. Genetic structure of *L. corniculatus* accessions using STRUCTURE, A) ΔK values for different numbers of clusters; B) bar plot representation for K = 3

Genetic variation assessment of *L. corniculatus* using ITS and *trnH-psbA* barcode markers: Analysis of the ITS region in three genetically divergent accessions (A7: PQ459600, Hen: PQ459601, and Mi: PQ459602), selected based on SCoT and ISSR markers, yielded sequences ranging from 667 to 692 bp. All ITS sequences were identical, indicating a single, highly conserved sequence type, here designated the Iranian haplotype. This represents the first report of ITS sequence data for *L. corniculatus* from Iran and, to our knowledge, globally. BLAST comparisons of the ITS sequences revealed 99.71% identity with *L. tenuis*, indicating close similarity. In the same accessions (A7: PQ616113, Hen: PQ616114, Mi: PQ616115), sequencing of the *trnH-psbA* intergenic spacer produced fragments between 349 and 417 bp. All sequences were identical and defined as the Iranian *trnH-psbA* haplotype. BLAST analysis showed 100% identity and coverage with *L. japonicus*, although no prior records exist for this plastid region in Iranian *L. corniculatus* accessions. The lack of intraspecific variation observed in both ITS and *trnH-psbA* sharply contrasts with the high levels of polymorphism detected using SCoT and ISSR markers. This discrepancy indicates the limited resolution of these conserved barcode regions for detecting fine-scale genetic structure among the studied accessions of *L. corniculatus*. It is also possible that the limited number of sequenced accessions restricted the detection of rare or localized haplotypes. Similar findings have been reported in previous studies. Gauthier et al. (1998) documented low cpDNA haplotype variability across *Lotus* species. Likewise, Kramina et al. (2016) found that although some variation exists among ITS sequences within the *L.*

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corniculatus complex (a taxonomic group comprising both diploid and tetraploid species such as *L. tenuis*, *L. japonicus*, *L. frondosus*, *L. stepposus*, and *L. corniculatus*), individuals of the same species do not always form well-resolved phylogenetic clusters. Consistent with this, our analysis revealed identical ITS and *trnH-psbA* sequences across morphologically and molecularly divergent accessions, underscoring the limited capacity of these barcode regions to detect intraspecific genetic structure. This observation supports the idea that limited sequence divergence and evolutionary processes such as hybridization, introgression, and incomplete lineage sorting may blur species boundaries within this genus (Kramina et al., 2018).

Phylogenetic analysis of *L. corniculatus* based on ITS and *trnH-psbA* data: Maximum Likelihood phylogenetic analysis using the Tamura 3-parameter model showed that the Iranian *L. corniculatus* haplotype clustered with *L. tenuis* (China) and *L. frondosus* (Russia), both diploid species with broad Eurasian distributions, with strong bootstrap support (100%) (Figure 5A). This grouping may reflect a shared ancestral signal or result from the limited resolution of the ITS region in distinguishing closely related taxa, as previously noted (Kramina et al., 2016, 2018). In contrast, several *L. corniculatus* accessions from geographically distant regions, including the United States, New Zealand, the United Kingdom, and Russia, formed a distinct clade. This pattern likely reflects the global dissemination of a common genetic lineage, shaped by historical forage introductions and human-mediated germplasm movement. By comparison, phylogenetic analysis of the *trnH-psbA* haplotype revealed a polytomous topology, in which the Iranian *L. corniculatus* haplotype clustered within an unresolved clade alongside *L. japonicus*, *L. ucrainicus*, *L. stepposus*, and *L. corniculatus* accessions from Italy, the UK, the USA, and Russia (Figure 5B). This unresolved branching pattern reflects the low resolution and conserved nature of the plastid marker, which hinders differentiation among closely related species within the *L. corniculatus* complex. Such patterns may also result from recent divergence, low mutation rates in chloroplast DNA, or historical germplasm exchange. The discordance between nuclear (ITS) and plastid (*trnH-psbA*) markers aligns with previous reports of phylogenetic complexity in *Lotus*, driven by hybridization, differential inheritance, and incomplete lineage sorting (Bailey et al., 2003; Kramina et al., 2016; Degtjareva et al., 2025).

Evolutionary context of the *L. corniculatus* complex: To contextualize these findings, it is essential to consider the complex evolutionary history of *L. corniculatus*. The *L. corniculatus* complex includes both diploid (2n = 2x = 12) and tetraploid (2n = 4x = 24) species. Cytogenetic and plastid data recognize *L. krylovii*, *L. frondosus*, *L. tenuis*, and *L. stepposus* as diploids, while *L. corniculatus* and *L. ucrainicus* are tetraploid members of the complex (Kramina et al., 2018). Despite apparent monophyly, evolutionary relationships among these taxa remain unresolved due to frequent interspecific gene flow. Grant & Small (1996) proposed a model in which *L.*

corniculatus originated from hybridization between diploid ancestors, notably *L. uliginosus* and *L. tenuis*. Their hypothesis is supported by shared morphological, biochemical, and cytogenetic traits. Additional maternal contributions may have come from species such as *L. alpinus* and *L. japonicus*, underscoring the complexity of reticulate evolution in this complex. These complexities are further compounded by morphological similarities between diploid and tetraploid taxa within the *L. corniculatus* complex, which may hinder accurate species identification. As Roberts (1984) suggested, chromosome counting remains a valuable complementary tool for distinguishing the tetraploid *L. corniculatus* from its diploid progenitors. Taken together, our findings emphasize the limitations of barcode-based approaches in resolving intraspecific variation within *L. corniculatus* and support the integration of additional methodologies such as cytogenetics, broader geographic sampling, and advanced genome-wide tools including SNP genotyping, RAD-seq, and whole-genome sequencing (WGS) to enhance phylogenetic clarity and detect cryptic diversity in this ecologically and agriculturally valuable species.

Conclusions

This study provides the first comprehensive molecular assessment of genetic diversity in *Lotus corniculatus* from Khuzestan, an ecologically vulnerable region in southwestern Iran. Using SCoT and ISSR markers, we detected high levels of polymorphism, with SCoT markers showing slightly greater discriminatory power due to higher polymorphism and mean PIC values. The clear genetic distinctiveness of the Minoo Island accession and the partial separation of Hendijan accession suggest the presence of unique ecotypes shaped by local environmental factors such as salinity, hydrological regime, and microclimate. These findings highlight the importance of conserving genetically distinct accessions, particularly those from isolated or stressed habitats, to maintain adaptive potential in future breeding or restoration programs. STRUCTURE analysis revealed at least three genetic clusters, supporting the existence of differentiated gene pools within the studied accessions. This pattern is likely driven by the outcrossing reproductive system of *L. corniculatus*, which promotes gene flow and admixture. In contrast, DNA barcoding using ITS and *trnH-psbA* regions failed to detect intraspecific variation among the sampled accessions. This result underscores the limitations of conserved barcode loci for fine-scale genetic studies in *L. corniculatus*, likely due to both marker conservation and the small number of sequenced accessions. Given the tetraploid nature of *L. corniculatus* and the history of hybridization in the genus *Lotus*, we recommend the integration of cytogenetic methods (e.g., chromosome counting) and high-resolution molecular tools (e.g., SNP genotyping) to complement barcoding approaches.

Overall, this study establishes a foundational understanding of genetic structure in *L. corniculatus* from a key region and offers practical guidance for the conservation and utilization of its germplasm. Future research should expand the geographic and genomic scope to better resolve ecotypic variation and guide sustainable management of this valuable forage species.

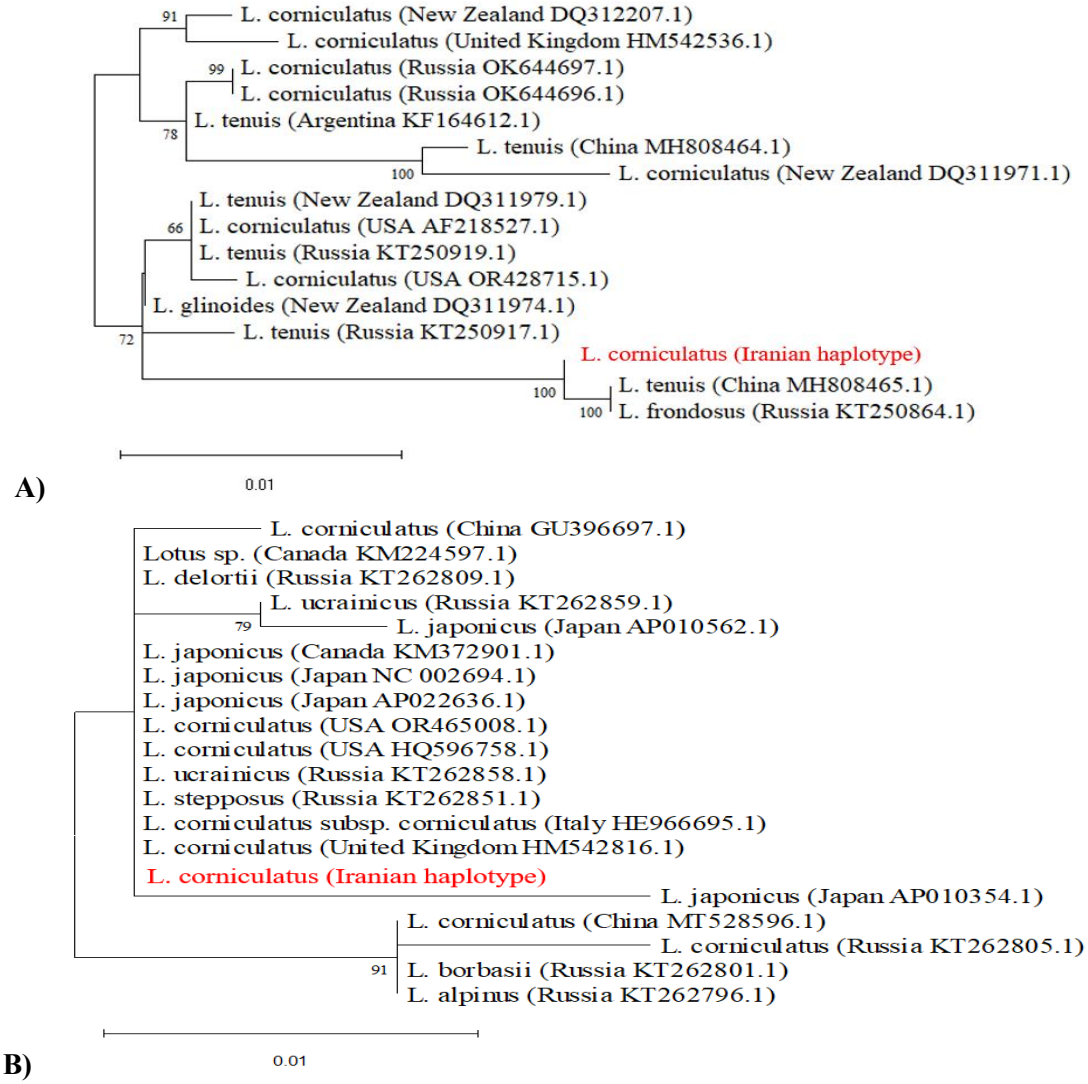

Figure 5. Phylogenetic trees reconstructed using the Maximum Likelihood method with the Tamura 3-parameter model: (A) ITS region and (B) *trnH-psbA* intergenic spacer. Each tree illustrates the evolutionary relationships between sequences from *L. corniculatus* in this study (highlighted in red) and reference sequences obtained from the NCBI GenBank database (black). Bootstrap values (based on 1,000 replicates) are shown at the branch nodes. The scale bar represents 0.01 substitutions per nucleotide site. GenBank sequences are labeled with species names, countries of origin, and accession numbers.

Author contributions

Zahra Sadat Mousavi: Formal analysis, Investigation, Resources, Writing-original draft, Writing-review & editing. Fatemeh Nasernakhaei: Conceptualization, Supervision, Writing-original draft, Writing-review & editing.

Data availability statement

The data can be provided by the corresponding author on reasonable request.

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Ethical considerations

The authors avoided data fabrication, falsification, plagiarism, and misconduct.

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Conflict of interest

The authors declare no conflict of interest.

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ارزیابی تنوع ژنتیکی *Lotus corniculatus* L. در خوزستان با استفاده از نشانگرهای مولکولی و DNA بارکد

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چکیده

هدف: گونه *Lotus corniculatus* L. یک لگوم چندساله، تتراپلوئید و با پراکنش گسترده است که به‌دلیل سازگاری با شرایط متنوع محیطی و کاربردهای آن در تولید علوفه، بهبود خاک و مصارف دارویی، ارزش بالایی دارد. ارزیابی تنوع ژنتیکی این گونه با استفاده از نشانگرهای مولکولی، نقش کلیدی در تدوین راهبردهای اصلاح نژاد و حفاظت ژرم‌پلاسم ایفا می‌کند. این مطالعه، نخستین بررسی مولکولی این گونه را در استان خوزستان، جنوب‌غرب ایران، ارائه می‌دهد.

مواد و روش‌ها: در این مطالعه، ۲۱ اکسشن *L. corniculatus* از هشت منطقه جغرافیایی متفاوت در خوزستان جمع‌آوری شد. برای بررسی تنوع ژنتیکی، از ۱۲ آغازگر SCoT و ۶ آغازگر ISSR استفاده گردید. روابط و ساختار ژنتیکی با بهره‌گیری از تحلیل خوشه‌ای، تحلیل مختصات اصلی (PCoA) و نرم‌افزار STRUCTURE بررسی گردید. به منظور تکمیل تحلیل مبتنی بر نشانگر، سه اکسشن دارای بیش‌ترین فاصله ژنتیکی برای توالی‌یابی دو ناحیه بارکدی ITS و *trnH-psbA* انتخاب شدند. جایگاه فیلوژنتیکی توالی‌های به‌دست‌آمده با BLAST و تحلیل Maximum Likelihood بررسی شد.

نتایج: نشانگرهای SCoT و ISSR به ترتیب ۹۶/۸۷ درصد و ۸۵/۸ درصد چندشکلی را نشان دادند که بیانگر تنوع ژنتیکی قابل توجه در میان اکسشن‌ها بود. تحلیل خوشه‌ای و PCoA دو گروه ژنتیکی اصلی را شناسایی کردند به طوری‌که اکسشن جزیره مینو یک خوشه متمایز را تشکیل داد. تحلیل STRUCTURE وجود سه گروه ژنتیکی را تأیید کرد که بیانگر هم‌آمیختگی ژنتیکی و

گروه‌بندی‌های مجزا در میان اکسشن‌ها بود. توالی‌یابی بارکدهای DNA نشان داد که هر سه اکشن منتخب (شامل اکسشن جزیره مینو) دارای توالی یکسان در نواحی ITS و *trnH-psbA* بودند که به‌عنوان هاپلوتیپ ایرانی تعریف شد. توالی ITS شباهت ۹۹/۷۱ درصدی با *L. tenuis* و ناحیه *trnH-psbA* تطابق کامل (۱۰۰ درصد) با *L. japonicus* را نشان داد. با این حال، این نواحی حفاظت شده توان تفکیک مناسبی در سطح درون‌گونه‌ای نداشتند.

نتیجه‌گیری: هر دو نشانگر عملکرد مناسبی داشتند؛ نشانگرهای SCoT مقادیر بالاتری از درصد چندشکلی و میانگین محتوای اطلاعات چندشکلی را نشان دادند؛ در حالی که نشانگرهای ISSR قدرت تفکیک و شاخص نشانگر بیش‌تری داشتند. تمایز ژنتیکی آشکار اکسشن جزیره مینو نشان‌دهنده‌ی سازگاری محلی و ضرورت حفاظت منطقه‌ای است. در مقابل، عدم تنوع در توالی‌های بارکدی، محدودیت نواحی محافظت‌شده مانند ITS و *trnH-psbA* را در شناسایی ساختار ژنتیکی ظریف نشان می‌دهد. استفاده از ابزارهای مکمل مانند شمارش کروموزومی و نشانگرهای ژنومی (تعیین ژنوتیپ SNP، توالی‌یابی RAD و توالی‌یابی کل ژنوم) برای بهبود تفکیک تاکسونومیکی پیشنهاد می‌شود.

کلمات کلیدی: انگشت‌نگاری ژنتیکی، تمایز ژنتیکی، روابط فیلوژنتیکی، مدیریت منابع ژنتیکی، یونجه پاکلاغی

نوع مقاله: پژوهشی.

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