Genetic Variation of Lentil Landraces Across Different Altitudes in Azerbaijan

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Abstract. Lentil is mainly grown in rainfed systems and thus plays an important role in sustainable agriculture, especially in high mountain landscapes. The aim of this study was to assess the genetic diversity of 50 lentil accessions, including landraces from high mountain regions of Azerbaijan as well as wild lentil species, using SSR markers. DNA was extracted from leaves, amplified by PCR and separated by polyacrylamide gel electrophoresis. Statistical analysis included dendrogram construction and principal coordinate analysis (PCoA). A total of 26 alleles were identified, with an average of 8.6. Primers SSR 156 and SSR 323 showed transferability between all studied species/subspecies within the genus Lens. The genetic diversity index for each primer ranged from 0.704 to 0.776, with a mean value of 0.735. Accessions from higher altitudes such as Ismayilli and Nakhchivan contributed significantly to the observed genetic variation. The dendrogram revealed four clusters in which 71.4% of the accessions were distinguishable. Lens culinary subsp. odemensis formed a distinct subcluster due to its high genetic distance. PCoA analysis revealed a dispersed sample distribution, with the first five axes accounting for 69.9% of the total variation. The study revealed significant genetic diversity among lentil accessions in Azerbaijan, including highland regions. These results highlight the need for conservation and will support future breeding and sustainable agriculture.

1 Introduction

Mountain regions are home to a wealth of biodiversity, including many legume species that are adapted to unique and challenging environments, while enhancing soil fertility through nitrogen fixation [1]. The growing issue of global protein insufficiency makes increasing grain legume yields a key task in ensuring food security also in these landscapes. Among these legumes, lentil (*Lens culinaris* subsp. *culinaris* Medik.) is possibly the most widely distributed high-protein food grain legume [2].

Lentil, one of the earliest domesticated legumes, is an annual, self-pollinating, cool-season crop with a genome size of around 4.2 Gb and a diploid chromosome number of 2n = 2x = 14. The plant provides all essential amino acids and does not accumulate harmful components like nitrates or radionuclides, making it an eco-friendly product worldwide [3-

5]. Since lentils are primarily grown in rainfed systems, they play a crucial role in sustainable agriculture in high-altitude regions where such systems are predominant.

Despite its benefits, enhancing lentil varieties is essential to maximize its use, especially in challenging environments. Enhancing lentil varieties relies on the availability of genetic diversity within both cultivated species and wild species that can interbreed with them [2]. Moreover, Liber et al. [6] found minimal gene flow between cultivated lentils and their wild ancestors, highlighting the need to preserve both genepools to maintain genetic diversity and adaptability.

Among lentil's wild relatives, *Lens culinaris* subsp. *orientalis* Boiss thrives at altitudes of 500 to 1700 meters in open or partly shaded, rocky soils with minimal competition [7]. Additionally, *Lens culinaris* subsp. *odemensis* Ladiz. was named after Mount Odem in Israel, where it was first discovered at around 1200 meters [8]. Thus, these species can provide traits such as disease resistance and environmental resilience, particularly valuable in high-altitude ecosystems. Several studies have highlighted features in wild lentil species related to their responses to biotic and abiotic stresses [9].

In Azerbaijan, lentils, along with other legumes, contribute significantly to the country's agricultural biodiversity. Local lentil landraces have been cultivated in the country for generations due to their resilience to specific environmental conditions. However, lentil production in the country falls short of meeting annual demand. In 2022, Azerbaijan produced approximately 700 tons of lentils from 544 hectares, with an average yield of 12.9 centners per hectare, according to the State Statistics Committee report.

In recent decades, many local lentil landraces in Azerbaijan and other regions have been gradually replaced by modern, high-yielding varieties. This shift, driven by commercial agriculture, has led to the erosion of valuable genetic diversity. Consequently, conserving these traditional landraces, especially those adapted to specific local environments like high-altitude regions, has become an urgent priority.

To effectively conserve lentil biodiversity, evaluating and characterizing the genetic diversity of local landraces and their wild relatives is essential. Molecular markers are powerful tools for this purpose, allowing for precise identification and differentiation of accessions and providing insights into genetic relationships and variation critical for conservation and breeding efforts.

In Azerbaijan, molecular markers have been used to assess genetic diversity in various legume species, including local and introduced accessions of chickpea, grass pea, and common bean [10, 11]. Studies using DNA markers on these crops have highlighted the genetic variation within these important species, demonstrating the value of molecular markers in biodiversity assessments.

Among molecular markers, simple sequence repeats (SSRs) are particularly useful for analyzing genetic diversity due to their co-dominant inheritance, high polymorphism, transferability among close species, locus specificity and reproducibility [12]. Over the past two decades, SSRs have been the most commonly utilized markers for plant genotyping. Mutations that escape the DNA mismatch repair system generate new alleles at SSR loci, allowing multiple alleles to exist at the same location [13]. This makes SSRs more informative than other markers like SNPs. New SSR markers are continuously being developed [14].

The main objective of this study is to evaluate the genetic diversity of local lentil landraces from different altitudes of Azerbaijan, along with several wild lentil species, using SSR markers. By identifying diverse and resilient accessions this evaluation can support sustainable agriculture, protecting both the agricultural biodiversity and the wider ecosystem.

2 Material and methods

The research material comprised 50 lentil accessions, including 44 landraces collected from various regions of Azerbaijan, 2 improved varieties, 1 cultivated, and 3 wild accessions (*L. culinaris* subsp. *orientalis* Boiss, *L. culinaris* subsp. *odemensis* Ladiz., and *L. ervoides* (Brign.) Grande) obtained from the Ukrainian gene bank (Table 1). The local landraces included accessions from high-altitude regions like Ismayilli (1140 m) and Nakhchivan (860 m), as well as moderate-altitude areas (450-500 m).

Table 1. Lentil accessions used in the study.

№	Cat. №	№	Cat. №	№	Cat. №
1	134461	18	134458	35	134448
2	134464	19	134459	36	134460
3	134465*	20	123599	37	134469
4	134466*	21	134467	38	70172*
5	123682	22	70167	39	73957
6	123683	23	123773	40	123774
7	123684	24	132681*	41	123775
8	123603	25	134451	42	123798
9	123618	26	134453	43	123800
10	134440	27	123681	44	134452
11	134444*	28	123685	45	Jasmin
12	123679	29	123620	46	Arzu
13	134446	30	134443*	47	*L. culinaris*
14	134447	31	123676	48	*L. odemensis*
15	134450	32	123678	49	*L. ervoides*
16	134456	33	123680	50	*L. orientalis*
17	134457	34	134445*		

*- accessions collected from moderate to high altitudes

Genomic DNA was isolated from fresh leaf tissue using a modified CTAB protocol based on Doyle and Doyle [15]. For SSR markers, 8 primers were initially screened in a 2.5% agarose gel, and 3 primers that produced clear, consistent bands with the least number of missing alleles were chosen for further analysis (Table 2) [16].

The amplification reactions were set up in a total volume of 20 μL, including: 2 μL of 10x PCR buffer; 0.4 μL of a 10 mM dNTP mixture; 0.6 μL of 50 mM $MgCl_2$; 1 μL of each forward and reverse primer (10 μM); 0.1 μL of Taq polymerase (5 U/μL); and 2 μL of template DNA (50 ng/μL). PCR amplifications were carried out in a 2720 thermal cycler (Applied Biosystems) under the following conditions: an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 15 seconds at 94°C, 15 seconds at the primer-specific annealing temperature, and 30 seconds at 72°C. The resulting PCR products were resolved on an 8% polyacrylamide gel, with fragment sizes compared against 100-bp ladder.

The genetic diversity index (GDI) was computed following the method outlined by Weir [17]. A dissimilarity matrix was generated using binary data with the Jaccard coefficient, and DARwin version 6 software [18] was employed to construct an unweighted neighbor-joining (UNJ) dendrogram and perform Principal Coordinate Analysis (PCoA).

3 Results and discussions

Understanding the genetic diversity and relationships among lentil landraces is crucial for the effective utilization of germplasm resources in breeding, as well as for conserving biodiversity. Previous studies have employed SSR markers to assess genetic variation in cultivated lentils [19-22].

In this study, the genetic diversity of 50 lentil accessions, including 44 local landraces was evaluated using three SSR markers selected from an initial set of eight primers. These markers revealed a total of 26 alleles, with an average of 8.6. The number of alleles per primer ranged from 8 to 9 (Table 2). For comparison, Chowdhury et al. [23] identified 33 alleles with an average of 8.3, using 7 primers for 20 lentil genotypes, while Tomar et al. [24] analyzed 37 lentil genotypes with 10 SSR primers, finding an average of 3.6 alleles on agarose gel. The higher number of alleles identified in our study, despite using fewer primers, can be attributed to the use of high-resolution polyacrylamide gel, as well as the diverse genetic background of the accessions analyzed, which included landraces from various regions with different elevations and soil-climatic conditions.

Using SSR 156 primer, eight alleles were detected, and unique genetic profiles were observed for accessions ILL 134459, ILL 123620, and ILL 134448. Notably, among the wild accessions, *L. orientalis*, widely recognized as the wild ancestor of cultivated lentils, exhibited a distinct profile with SSR 156 primer. The specie can thrive in rocky, mountainous environments, such as the high-altitude Mount Hermon and the medium-altitude Mount Tabor in Israel, which highlights its natural resilience to harsh environmental conditions [8]. These traits make *L. orientalis* a valuable genetic resource for improving sustainability in lentil breeding programs.

The SSR 323 primer pair synthesized 9 alleles across the lentil collection, with a unique allele identified for the wild *L. ervoides* accession. Using the SSR 167 primer pair, a fragment of the expected length was amplified in most accessions, with unique bands and patterns observed in several cases. Notably, *L. odemensis* (No. 48) and one cultivated accession (No. 47) from the Ukrainian gene bank exhibited distinct profiles at this locus. The unique alleles identified in the wild species *L. ervoides* and *L. odemensis* underscore the importance of preserving these species for their potential contributions to crop improvement.

Table 2. The genetic diversity parameters determined in a lentil collection with SSR primers.

Primer name	Primer sequences	Number of alleles	Genetic diversity index	Expected allele size, bp
SSR 156	F: GTACATTGAACAGCATCATC R: CAAATGGGCATGAAAGGAG	8	0.724	176
SSR 167	F: CACATATGAAGATTGGTCAC R: CATTTATGTCTCACACACAC	9	0.704	160
SSR 323	F: AGTGACAACAAAATGTGAGT R: GTACCTAGTTTCATCATTG	9	0.776	250
Total		**26**		
Mean		**8.6**	**0.735**	

To summarize, SSR markers demonstrated a high level of effectiveness in distinguishing wild species, emphasizing their utility in genetic diversity studies. SSR 156 and SSR 323 primers demonstrated transferability across all studied species or subspecies, while SSR 167 primers showed transferability in at least 3 species or subspecies within the genus *Lens*. The observed transferability of these SSR markers is influenced by the

conserved nature of both the SSR loci and the flanking regions, as noted by Tuler et al. [25]. Our results highlight the interspecies conservation of the microsatellite loci used in this study.

The genetic diversity index for each primer in this study ranged from 0.704 to 0.776, with an average of 0.735. This indicates that the lentil landraces from various regions of Azerbaijan are highly genetically diverse. Notably, accessions from higher altitudes, such as those from Ismayilli (1140 m) and Nakhchivan (860 m), contributed significantly to the genetic variation observed, highlighting the importance of conserving biodiversity in mountainous regions. High genetic diversity was also noted in Ethiopian lentils by Mekonnen et al. [26]. Similar levels of genetic diversity have been reported by Tomar et al. [24], who observed a range of 0.5 to 0.79 in genetic diversity among 37 advanced lentil breeding lines in India. Lentil landraces, well-adapted to specific regions and rainfed conditions, are particularly valuable for cultivation in mountainous and high-elevation landscapes. High diversity among landraces helps identify resilient varieties and supports sustainable agriculture in these challenging environments.

In our study, the *L. orientalis* Boiss samples from the wild exhibited successful amplification with only two primers (SSR 156 and SSR 323), while no amplification was observed with other primers. Consequently, the analysis was focused on 49 accessions. Using binary data for these 49 accessions, the Jaccard similarity coefficient was calculated.

Evaluating the degree of differentiation among accessions is essential for choosing effective parents in breeding programs [27]. Thus, to provide a visual representation of the genetic relationships within the lentil collection, a dendrogram was constructed using the DARwin software, as shown in Fig. 1. The UNJ dendrogram revealed four main clusters, distinguishing 71.4% of the accessions from each other. The genetic distance index ranged from 0 to 1, with both maximum and minimum values observed among various genotypes. Notably, there was no clear correlation between the clustering of genotypes and traits such as seed size, color, or pattern of the seed coat. This finding aligns with Chowdhury et al. [23], who analyzed 20 lentil genotypes using 7 SSR markers. Their study grouped the samples into four clusters; however, these clusters did not correspond with the dendrogram based on yield indicators.

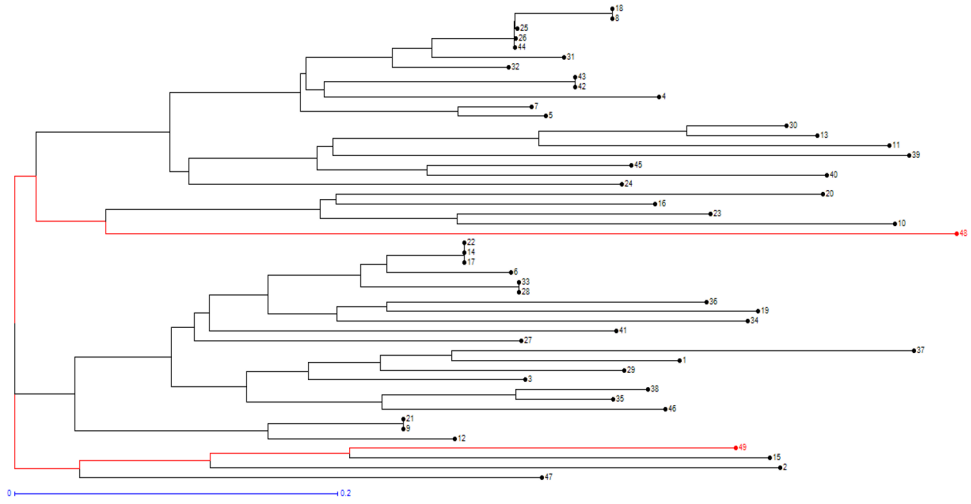

Fig. 1. UNJ dendrogram showing genetic relationship among studied lentil accessions. Wild samples are indicated in red.

Cluster I, comprising 19 accessions, included 7 samples from the Lankaran region. Within this cluster, 5 accessions exhibited similarity across all three loci. Additionally, 2 of the 4 Nakhchivan accessions collected from 860 m.a.s.l. were grouped together, with a genetic distance of 0.43 between them (ILL 134443 and ILL 134444). The accession ILL 132681 from the high-altitude Ismayilli district (1150 m) formed a distinct subgroup within this cluster, showing a genetic distance ranging from 0.38 to 0.89 from other genotypes. The accession ILL 134466 from the medium-altitude Jabrayil area (550 m) demonstrated genetic similarity with ILL 123798 and ILL 123800.

Cluster II, with 5 accessions, included samples from Lankaran, Salyan, and Shamakhi, representing plain areas. The *L. odemensis* accession (No. 48) was included in this cluster but formed a distinct subcluster due to a high genetic distance from other samples. The specie exhibited the smallest genetic distance of 0.75 between the Arzu variety, which was largely influenced by the SSR 156 primer. The high genetic distance values, ranging from 0.75 to 1, further underscore the distinct genetic profile of *L. odemensis*. This species was first discovered at approximately 1200 meters above sea level in the Odem Mountains of Israel [8], where it has adapted to high-altitude conditions. *L. odemensis* possesses valuable genetic traits such as disease resistance and environmental resilience. These traits are crucial for enhancing cultivated lentil varieties and ensuring food security in the face of climate change.

Cluster III, which contained the majority of samples, included genotypes from diverse regions. Accessions from high altitudes of Nakhchivan region (ILL 134445, ILL 70172) were grouped with Lankaran accessions, showing a genetic distance of 0.14 to 0.5. Within this cluster, 2 accessions from Lankaran, 2 from Salyan, and 1 from Jabrayil shared identical alleles at all three loci.

The final cluster comprised four genetically distinct genotypes, including the wild *L. ervoides*. Two accessions from Lankaran and one from the Ukrainian gene bank also fell into this cluster, with genetic distances between the *L. ervoides* accession and the other genotypes ranging from 0.5 to 0.67.

Overall, there was no direct correlation between the clustering of accessions and their geographical origin, consistent with findings by Toklu et al. [28]. This lack of geographical pattern could be attributed to the limited number of loci or the adaptation of landraces to specific environmental conditions, as well as potential movement of landraces across locations [6]. In modern plant breeding, selecting diverse and suitable parents for hybridization is crucial for maximizing genetic gains. Thus cross-breeding among samples from different clusters can further enhance the genetic diversity and broaden the range of recombinants, contributing to the rich biodiversity of lentils.

Breeding varieties obtained through single plant selection were placed in distinct clusters. The Jasmin variety showed closer genetic similarity to ILL 123774 (GD = 0.38), while the Arzu variety was more similar to ILL 70172 and ILL 134448 from Nakhchivan and Lankaran (GD = 0.29 to 0.38). The genetic distance between wild accessions was 0.67, underscoring the importance of conserving and utilizing diverse genetic resources for future breeding programs.

The Principal Coordinates Analysis (PCoA) aligns with the cluster analysis by providing a more detailed view of the sample distribution (Fig. 2). The plot reveals that samples are dispersed rather than forming distinct groups. The first axis explained only 23% of the variation, while the first three axes accounted for 54.1% and the first five axes captured 69.9% of the variation. This limited explanatory power is primarily due to the small number of markers used. Notably, genotypes such as ILL 73957, ILL 123774, and ILL 134446 emerged as relatively distinct.

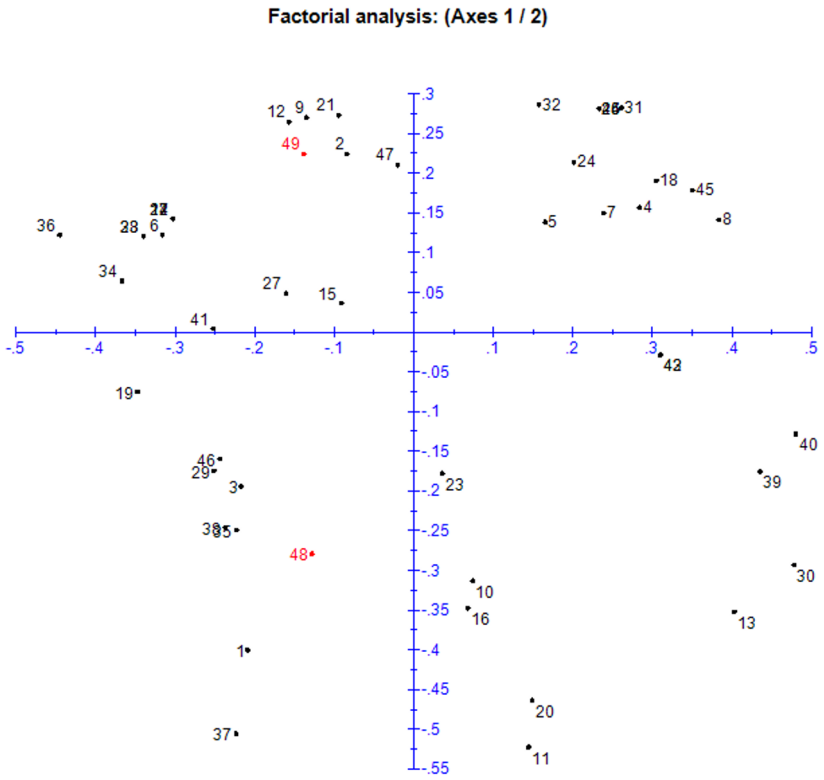

Fig. 2. Principle coordinate analysis for lentil genotypes based on SSR data. Wild samples are indicated in red.

In the PCoA analysis, wild genotypes were located in separate quadrants, consistent with the cluster analysis. The genetic affinity of *L. odemensis* to the Arzu variety (No. 46) and *L. ervoides* to the cultivated accession from the Ukrainian gene bank (No. 47) is more apparent here. *L. ervoides* is considered a sister species to *L. odemensis* [6]. The observed genetic distance between these samples can be attributed to the fact that only one representative from each species was analyzed.

Although *L. orientalis* is the primary wild progenitor of cultivated lentils (*L. culinaris*) and other wild species contribute only minimally to the domesticated gene pool, these species still have potential for broadening the genetic base of cultivated lentils through crossing, which varies among species. For instance, successful crossings with *L. odemensis* may differ depending on the parent combinations [29], whereas crossings between *L. culinaris* and *L. ervoides* have been achieved using embryo rescue techniques [30], illustrating practical methods for integrating diverse genetic resources.

In conclusion, this study demonstrates that the lentil accessions collected from various regions of Azerbaijan, including its high-altitude areas, exhibit substantial genetic diversity. This rich biodiversity highlights the unique genetic resources present and underscores the necessity of conserving these landraces. The findings on genetic diversity distribution will aid in the effective preservation, characterization, and future breeding programs, ensuring that this biodiversity supports sustainable agricultural practices.

4 Conclusions

This study provides valuable information on the genetic diversity of lentil landraces, highlighting the importance of conserving these resources for future breeding programs. The use of SSR markers revealed high levels of genetic variability among 50 lentil accessions, including 44 landraces, with an average of 8.6 alleles per primer. The observed genetic diversity was particularly pronounced in high-altitude accessions, which contribute significantly to the total variability, highlighting the importance of conserving biodiversity in mountainous regions. In addition, the wild species L. orientalis, L. ervoides, and L. odemensis exhibited distinct genetic profiles, highlighting their potential for improving cultivated lentil varieties, especially in terms of disease resistance and environmental tolerance. Despite the lack of clear correlations between geographical origin and genetic clustering, the results indicate that lentil landraces have adapted to different environmental conditions, which may have affected their genetic diversity. Cluster and PCoA analyses further confirmed significant genetic differentiation among the accessions, confirming the potential for crossbreeding between different groups to increase genetic diversity. Ultimately, this study highlights the value of integrating wild species and landraces into breeding programs to promote genetic improvement and sustainability in lentil cultivation.

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