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Genetic diversity of bulbous barley (*Hordeum bulbosum*) accessions from Iran revealed by ISSR markers

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Summary. *Hordeum bulbosum*, a wild relative of cultivated barley (*H. vulgare*), is a valuable genetic resource because of its wide ecological adaptability across diverse regions of Iran. This study utilized Inter-Simple Sequence Repeat (ISSR) markers to assess the genetic diversity, population structure, and differentiation among *H. bulbosum* populations collected from seven ecologically and geographically distinct regions. Genomic DNA was extracted using a modified CTAB protocol, and PCR amplification was performed with seven ISSR primers, four of which produced reliable polymorphic bands. Genetic data were analyzed using PAST, STRUCTURE, PopGene, and GenAlEx software. ISSR markers were effective in detecting polymorphism (mean PIC = 0.363; D = 0.844), revealing significant genetic variation within and among populations. Analysis of molecular variance (AMOVA) attributed 74 % of the genetic variation to differences between populations, with a high genetic differentiation index ($G_{st} = 0.72$) and limited gene flow ($N_m = 0.19$). STRUCTURE analysis and the NJ dendrogram identified distinct genetic clusters, with populations 1, 2, 3, and 7 exhibiting high proportions of assignment to a single genetic cluster, while populations 4, 5, and 6 showed admixture patterns. Mantel tests and Canonical Correspondence Analysis (CCA) demonstrated that both geographic distance and environmental factors contribute to genetic structuring, indicating the combined effects of isolation by distance (IBD) and isolation by environment (IBE). These findings highlight the evolutionary importance of *H. bulbosum*, underlining its potential for use in barley improvement and stress-resistance breeding programs. Moreover, genetically unique populations were identified that warrant conservation and prioritization for inclusion in gene banks.

Генетическое разнообразие образцов *Hordeum bulbosum* из Ирана, выявленное с использованием ISSR-маркеров

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Аннотация. *Hordeum bulbosum* – дикий родственник культурного ячменя (*H. vulgare*), представляющий собой ценный источник генетических ресурсов благодаря высокой экологической адаптивности и широкому распространению в различных регионах Ирана. В настоящем исследовании ISSR-маркеры (Inter-Simple Sequence Repeat) были использованы для оценки генетического разнообразия, структуры популяций и степени генетической дифференциации между популяциями *H. bulbosum*, собранными в семи экологически и географически различных районах страны. Геномную ДНК выделяли модифицированным методом СТАВ, а ПЦР-амплификацию проводили с использованием семи ISSR-праймеров, четыре из которых обеспечили получение воспроизводимых полиморфных фрагментов. Генетические данные анализировали с применением программ PAST, STRUCTURE, PopGene и GenAlEx. ISSR-маркеры продемонстрировали высокую эффективность при выявлении полиморфизма (среднее значение PIC = 0,363; D = 0,844), что позволило установить значительный уровень генетической изменчивости как внутри исследованных популяций, так и между ними. Анализ молекулярной дисперсии (AMOVA) показал, что 74 % общей генетической изменчивости обусловлено различиями между популяциями, сопровождаемыми высоким уровнем генетической дифференциации ($G_{st} = 0,72$) и ограниченным потоком генов ($Nm = 0,19$). Анализ структуры популяций (STRUCTURE) и дендрограмма, построенная методом Neighbor-Joining, выявили несколько чётко выраженных генетических кластеров: популяции 1, 2, 3 и 7 характеризуются высокой генетической однородностью, тогда как популяции 4, 5 и 6 продемонстрировали признаки смешанного происхождения. Результаты теста Мантеля и канонического корреляционного анализа (CCA) показали, что формирование генетической структуры популяций определяется совместным влиянием географической изоляции (IBD) и различий экологических условий (IBE). Полученные результаты подчёркивают эволюционную значимость *H. bulbosum* как ценного генетического ресурса для совершенствования культурного ячменя и селекции сортов, устойчивых к неблагоприятным условиям среды. Кроме того, выявлены генетически уникальные популяции, представляющие приоритетную ценность для сохранения и включения в национальные и международные банки генетических ресурсов.

Introduction

Hordeum bulbosum L. is a perennial grass with both diploid ($2n = 14$) and tetraploid ($4n = 28$) individuals, important for agriculture and breeding. Diploid populations are mainly found in Greece and Egypt, while tetraploids are more common in the eastern regions (Morrell, Clegg, 2011). It is a significant species within the *Hordeum* genus, which includes cultivated barley (*Hordeum vulgare* L.) and is a valuable source of beneficial alleles for cereal improvement (Bothmer et al., 1995).

This species has been utilized for the genetic improvement of *H. vulgare*, including the production of doubled haploids through interspecific hybridization with *H. bulbosum* (Kasha, Kao, 1970; Maluszynski, 2003). It also contributes valuable resistance to fungal and viral pathogens in cultivated barley (Walther et al., 2000; Toubia-Rahme et al., 2003). Furthermore, its fibrous root system aids in soil conservation by enhancing shear strength and mitigating erosion through deep soil anchorage (Cammeraat et al., 2005).

Extensive research has been conducted on the evolutionary relationships and genetic structure of *Triticum* L., *Hordeum* L., and *Secale* L., and related genera like *Aegilops* L. and *Elymus* L. (Deng et al., 2016). These studies, encompassing taxonomy,

eco-geography, cytogenetics, and evolution, have provided valuable insights into the genetic structure of these grasses and have aided in understanding the ancestry of bread wheat (Feldman, Levy, 2023).

Several traits in *H. bulbosum*, one of the most important wild relatives used in barley breeding, offer potential benefits for barley improvement. These include disease resistance, pest tolerance, a perennial growth habit, and self-incompatibility. Introducing these traits into cultivated barley through breeding could enhance disease resistance, improve pest tolerance, increase genetic diversity, and potentially lead to more sustainable barley production (Johnston et al., 2009).

A groundbreaking discovery revealed that diploid *H. bulbosum* could be crossed with *H. vulgare* to produce haploid *H. vulgare* progeny, significantly impacting barley breeding programs. As a result, doubled haploid barley lines have been widely produced using *H. bulbosum* (Morrell, Clegg, 2011). Genetic diversity analyses indicate that wild barley possesses greater genetic variation than landraces and modern cultivars, suggesting that cultivated barley originates from a limited number of wild species (Naghavi et al., 2011).

Recent advances in molecular genetics and genomics have further highlighted the importance of *H. bulbosum* and other wild relatives for barley

breeding programs. For instance, molecular genomic studies have revealed novel alleles and genes in wild barley species that confer resistance to biotic and abiotic stresses, which are often absent in cultivated barley (Milner et al., 2019).

Beyond its role in elucidating the history and breeding of barley, genetic diversity is essential for breeding wild barley species. However, morphological characteristics in cultivated germplasms may exhibit limited polymorphism due to environmental influences (Naghavi et al., 2011). Since genetic markers remain unaffected by environmental factors, they provide more reliable estimates of genetic diversity within species.

Molecular techniques are crucial for identifying genetic variation in plant species, particularly as biodiversity increases and precise identification becomes more important in agriculture, ecology, and conservation (Ayaz et al., 2020). Taxonomic identification is particularly valuable in evolutionary and biodiversity research, especially in assessing interspecific genetic variability (Ayaz et al., 2020; Tabaripour, Keshavarzi, 2021; Keshavarzi et al., 2022). In this context, molecular markers serve as crucial tools for identifying and distinguishing closely related genotypes (Khodaei et al., 2021).

Several factors significantly influence plant population structure, including mutations, human and/or environmental selection, genetic drift, mating systems, and growth habits (Ben Romdhane et al., 2017; Brbaklić et al., 2021). Molecular techniques have been widely employed to identify crop cultivars and accessions, overcoming challenges associated with phenotype-based classification (Noroozisharaf et al., 2015). These techniques have been extensively applied to assess genetic variation in several species of the Poaceae family, including *Lolium* L. (Posselt et al., 2006; Hu et al., 2011; Abbaszade et al., 2013; Ghariani et al., 2015; Costa et al., 2016; Amin et al., 2017; Keshavarzi et al., 2022), *Cynodon* Rich. (Huang et al., 2012), *Triticum* (Guo et al., 2016; Abdel-Lateif, Hewedy, 2018), *Bromus* Scop. (Safari et al., 2019), *Anomochloa* Brongn. (Vieira et al., 2020), *Setaria* P. Beauv. (Palakurthi et al., 2023), and *Miscanthus* Anderss. (Dorogina et al., 2023).

Inter-Simple Sequence Repeat (ISSR) markers are dominant molecular markers that involve PCR amplification of DNA and are widely used to detect species and population genetic diversity (Keshavarzi et al., 2022). The ISSR technique can differentiate closely related genotypes and detect polymorphisms even in the absence of prior DNA sequence information for a crop (Noroozisharaf et al., 2015).

Genetically diverse populations are essential for plant breeding programs, as they enable the development of varieties with desirable traits. Studying the interaction between genes, genomes, and environments provides insights into selection processes for newly targeted varieties. Evaluating cereal crops alongside their wild relatives has always been challenging for breeders. Therefore, diverse genetic markers are required to assess genetic diversity and streamline the selection process for novel cultivars (Rani et al., 2024).

Okumus, Uzun (2007) highlighted the potential of *H. bulbosum* as a source of disease resistance for cultivated barley. They noted its resilience to harsh conditions and its possession of resistance genes against fungal and viral diseases. Furthermore, their research using RAPD markers revealed that *H. bulbosum* populations exhibit significantly higher polymorphism compared to *H. vulgare* accessions, suggesting a greater diversity of resistance genes.

While research on the genetic diversity of *H. bulbosum* using molecular markers remains relatively scarce, valuable insights can be gained from studies conducted on its close wild relative, *H. spontaneum* C. Koch. For instance, Hubner et al. (2009) demonstrated a strong correlation between the population structure of *H. spontaneum* and environmental variables such as temperature and precipitation, using 18 SSR loci across geographically distinct populations. These findings underscore the adaptive potential and genetic richness of wild barley species, which may similarly apply to *H. bulbosum* under comparable ecological pressures. Similarly, SSR markers have been used to investigate genetic variation in *H. vulgare*, revealing high polymorphism information and strong discriminating power, which highlights their effectiveness in genotype differentiation (Lahoot et al., 2016). For barley, ISSR markers have proven to be effective in analyzing genetic diversity, population structure, and phenotypic traits (Yigider et al., 2024).

This study employed ISSR molecular markers to explore the genetic diversity and population structure of *H. bulbosum* in Iran, which represents the first use of these markers for this species. The study aims to assess genetic variation within and between populations, identify genetic differentiation, and understand the species' adaptability across different Iranian regions. These findings will be crucial for informing conservation strategies, promoting resource preservation, and guiding future breeding programs.

Materials and Methods

Plant materials

Seventy-six accessions of *H. bulbosum* were collected from seven geographical populations of Iran (Table 1, Fig. 1). Voucher samples were deposited in the Herbarium of Alzahra University (ALUH).

Extraction of DNA and PCR Amplification

The young leaves were dried and ground into a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. The samples were then incubated at 65 °C for 1 h in Cetyltrimethylammonium bromide (CTAB) extraction buffer. Genomic DNA was extracted following the protocol described by Wang (2009). To evaluate DNA quality, the extracted

Table 1. Geographical information and voucher numbers of sampled populations of *Hordeum bulbosum*

Population code	Locality addresses	Geographic coordinates			Voucher no.	Individual quantity
		Altitude a. s. l.	latitude	longitude		
1	Razavi Khorasan province: Southeast of Mashhad, Balendar village, Basiri	1370 m	36.0769°	59.3044°	1035 (ALUH)	14
2	Mazandaran province: Kelardasht, Pouramini	1140 m	36.5347°	51.1784°	1036 (ALUH)	10
3	Gilan province: Asalem to Khalkhal road, after Charsou Yeylak, Pouramini	2020 m	37.6056°	48.6939°	1037 (ALUH)	10
4	Razavi Khorasan province: Southeast of Mashhad, between Orfi village and Balendar village, Basiri	1450 m	36.0899°	59.2882°	1038 (ALUH)	12
5	Golestan province: near the Golestan National Park, Rajani	982 m	37.3247°	56.0192°	1039 (ALUH)	10
6	Golestan province: Olang-Ramian to Shahrud, after Malacharan village, Rajani	1780 m	36.8486°	55.2411°	1040 (ALUH)	10
7	Khuzestan province: Izeh-Eshkaft-e Salman, Farasat	850 m	36.8166°	49.8477°	1041 (ALUH)	10

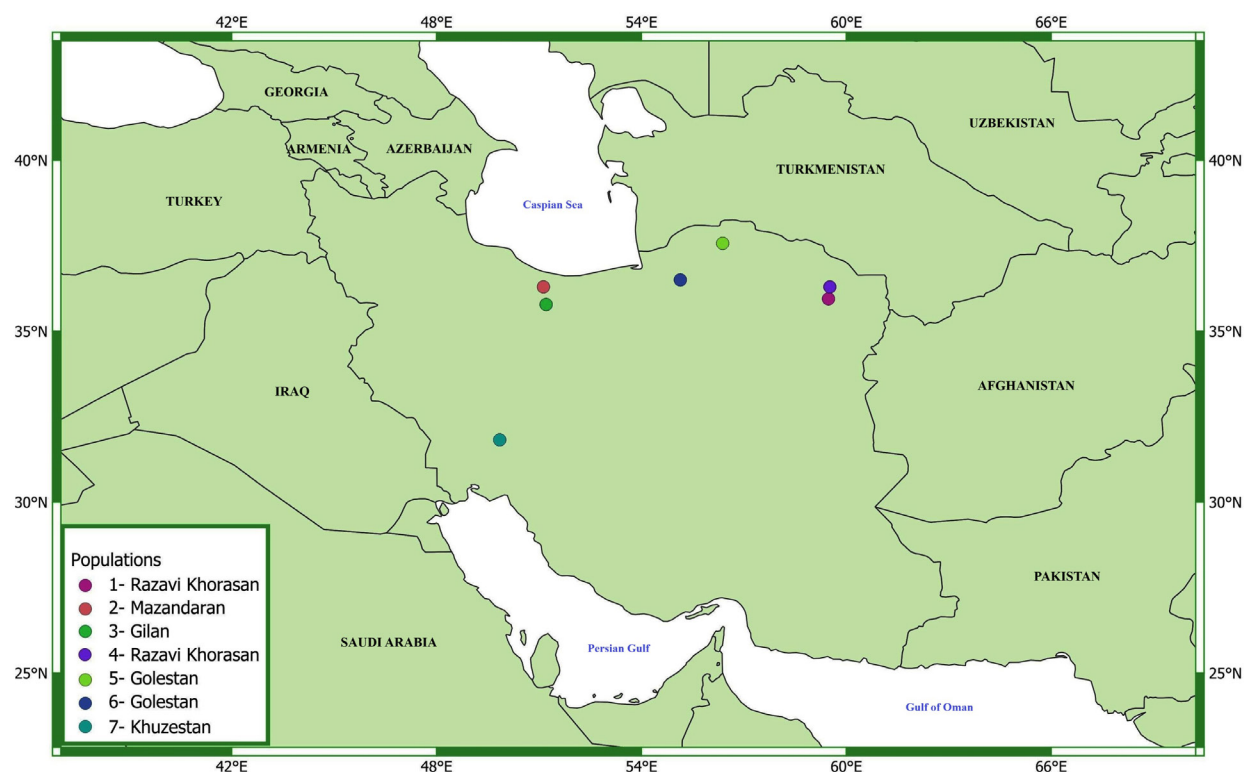


Fig. 1. Geographical distribution map of *Hordeum bulbosum* studied populations.

DNA was electrophoresed on a 0.8 % agarose gel. A total of four ISSR primers were used in the PCR reactions (Table 2).

Table 2. Properties of the ISSR primers used (commercialized by the University of British Columbia)

Primer	Sequences (5'–3')
UBC807	AGAGAGAGAGAGAGAGT
UBC812	GAGAGAGAGAGAGAGAA
UBC817	CACACACACACACAA
(AGC)5GA	AGCAGCAGCAGCAGCGA

To perform PCR amplification, we used a 25 µl containing: 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 0.2 µM of each dNTP, 3 U (units) of Taq DNA polymerase, and 0.2 mM of a single primer.

For PCR, the following program was used: 94 °C for 5 min; 40 cycles of 94 °C for 1 min; annealing at 53 °C for 45 s and extension at 72 °C for 90 s; final extension at 72 °C for 7 min. By running the products on gels containing 1 % agarose and staining them with Power Load, the products were visualized. To detect the size of bands, we used a molecular size ladder of 100 bp.

Molecular analyses

For analyses of genetic diversity and polymorphism parameters, ISSR bands were coded as absence = 0 and presence = 1. GenAlEx ver. 6.4 was used to estimate parameters of genetic differentiation, such as different bands number with frequencies of ≥ 5 , ≤ 25 , and ≤ 50 % (no. LComm bands of ≥ 5 , no. LComm bands of ≤ 25 and no. LComm bands of ≤ 50 %), number of unique bands in a single population (no. private bands), different alleles number (Na), effective alleles number (Ne), Nei's unbiased genetic distance, unbiased expected heterozygosity (uHe), polymorphic loci percentage (P), expected heterozygosity (He), and Shannon's Information Index (I). Moreover, AMOVA was performed with 1000 permutations to determine genetic differentiation between the studied populations (Peakall, Smouse, 2006).

To assess the discriminating power of ISSRs loci, POPGENE v. 1.31 (Yeh, 1999) performs GST (genetic diversity residing among species) analysis. Using the online marker efficiency calculator (iMEC), the heterozygosity index (H), polymorphism information content (PIC), effective multiplex ratio (E), arithmetic mean of H (H.av),

marker indexes (MI) and effective multiplex ratios (EMR) are calculated for each primer (Amiryousefi et al., 2018). This program can be found at <https://irscope.shinyapps.io/iMEC/>.

Based on the following formula, these indices were calculated: $H = 1 - \sum p_i^2$, combined allele frequencies for all i-th alleles are summed, where p_i is the frequency for the i-th allele; $PIC = 1 - \sum p_i^2 - \sum \sum p_i^2 p_j^2$, Population frequency p_i and p_j are the frequencies of i-th and j-th alleles. Initially, the summation represents the total number of alleles, while the two subsequent summations represent i and j where $i \neq j$; $E = n\beta$, defining $\beta = np / (np + nnp)$, where p and np indicate the polymorphic and nonpolymorphic fraction of the markers, so np and nnp represent their respective counting numbers; $H_{avp} = \sum H_n / np$. In this case, H_n represents the heterozygosity of the polymorphic fraction of markers, and the summation is over all the polymorphic loci np; $MI = E \times H_{avp}$, where H_{avp} is the expected average heterozygosity of polymorphic markers. It is equal to $\sum H_p / np$, where the summation is over all polymorphic sites with H_p and np defined as above; $D = 1 - C$, where C is the confusion probability; $R = \sum I_\beta$, where I_β or band informativeness is represented on a scale of 0–1 and is defined as $I_\beta = 1 - (2 \times |0.5 - p|)$, where p is the portion of the samples containing the observed band (Amiryousefi et al., 2018).

PAST ver. 4.10 was applied to classify the populations based on a neighbor-joining (NJ) tree and to examine the relationship between genetic and geographical distances between accessions (Podani, 2000).

Using DARwin software version 6 (Perrier, Jacquemoud-Collet, 2006), reticulation analysis (Legendre et al., 2002) is investigated potential genetic connections among populations. A supporting phylogenetic tree using NJ is built. A reticulation branch at each stage of the algorithm minimizes the least squares (Legendre et al., 2002; Makarenkov, Lapointe, 2004).

The STRUCTURE analysis uses Bayesian clustering and Markov Chain Monte Carlo estimation. A total of 10 000 burns were performed during the period, and 10000 MCMC repeats were performed after the burn-in (Porras-Hurtado et al., 2013). The Evanno test was utilized to determine the most probable value for K (genetic clusters) based on K statistics (Evanno et al., 2005). To determine the best K-value, Pseudo-F and Bayesian Information Criteria were applied to STRUCTURE version 2.3.4 (Meirmans, Van Tienderen, 2004).

Results

The examined genetic diversity and polymorphism parameters were summarized in Table 3.

A total of 179 bands were detected, with population 1 exhibiting the highest number of loci (36) and the highest polymorphism percentage (54.90 %), whereas population 2 displayed the lowest values of loci (4) and polymorphism percentage (7.84 %).

The average frequency of bands with a prevalence equal to or greater than 5 % was 25.57. Locally common bands with frequencies less than or equal to 25 % and 50 % were observed at 0 and 4.86, respectively. Among the studied populations, Population 1 showed the highest values for these parameters, while Population 7 had the lowest. This pattern was also reflected in other genetic diversity indices, such as the Shannon Information Index, expected heterozygosity (H_e), and observed heterozygosity (H_o). The mean number of alleles across populations was 0.731, with a maximum of 1.255 in Population 1 and a minimum of 0.412 in Population 7.

Additionally, the average marker index (MI), which represents marker efficiency, was calculated to be 0.002. All primers demonstrated high

polymorphic information content (PIC) values (~ 0.363). Among them, UBC807 exhibited the highest PIC value (0.381), while UBC812 and UBC817 had the lowest (0.350). The resolving power (R_p) of the primers varied from 3.923 (UBC807) to 5.718 (UBC812), with a mean of 4.974. The diversity index (D) of the primers ranged between 0.795 (UBC817) and 0.903 (UBC807), averaging 0.844. A summary of these calculations is presented in Table 4.

The AMOVA results indicate a significant level of genetic differentiation among the populations ($\Phi_{PT} = 0.740$, $P = 0.001$), with the majority of genetic variation (74 %) attributed to differences between populations rather than within them (26 %). Pairwise Φ_{PT} values ranged from 0.387 (between Populations 5 and 6) to 0.922 (between Populations 2 and 5), suggesting a broad range of genetic divergence across populations.

Furthermore, Nei's genetic distance analysis revealed values ranging from 0.114 (between Populations 5 and 6) to 0.541 (among most other population pairs), confirming the presence of considerable genetic differentiation (Table 5).

Based on ISSR data, the NJ tree and the MDS plot (Figs. 2, 3) showed broadly similar patterns of population arrangement. Population 3 was clearly separated from the other populations, indicating

Table 3. The detected parameters of genetic diversity and polymorphism of the evaluated population of *Hordeum bulbosum* (populations coding as in Table 1)

Parameters/populations	Population1	Population2	Population3	Population4	Population5	Population6	Population7	Total/Mean
Total band no.	36	21	34	21	25	26	16	179
Polymorphic loci no.	28	4	12	8	5	20	5	82
Polymorphic loci percentage	54.90	7.84	23.53	15.69	9.80	39.22	9.80	22.97
No. Bands Freq. $\geq 5\%$	36	21	34	21	25	26	16	25.57
No. Private Bands	5	1	8	0	0	0	0	2
No. LComm Bands ($\leq 25\%$)	0	0	0	0	0	0	0	0
No. LComm Bands ($\leq 50\%$)	9	5	7	0	4	7	2	4.86
Mean of N_a	1.255	0.490	0.902	0.569	0.588	0.902	0.412	0.731
Standard deviation of N_a	0.125	0.090	0.106	0.106	0.094	0.132	0.094	0.043
Mean of N_e	1.285	1.034	1.150	1.081	1.019	1.262	1.045	1.125
Standard deviation of N_e	0.054	0.022	0.044	0.036	0.011	0.053	0.025	0.015
Mean of I	0.250	0.032	0.126	0.067	0.026	0.217	0.043	0.109
Standard deviation of I	0.039	0.018	0.034	0.026	0.013	0.041	0.020	0.012
Mean of H_e	0.165	0.020	0.085	0.044	0.014	0.148	0.027	0.072
Standard deviation of H_e	0.028	0.012	0.024	0.018	0.008	0.029	0.014	0.008
Mean of uH_e	0.171	0.021	0.089	0.046	0.015	0.155	0.029	0.075
Standard deviation of uH_e	0.029	0.013	0.025	0.019	0.008	0.030	0.014	0.009

Note: No. Bands Freq. $\geq 5\%$ = No. of Different Bands with a Frequency $\geq 5\%$. No. Private Bands = No. of Bands Unique to a Single Population; No. LComm Bands ($\leq 25\%$) = No. of Locally Common Bands (Freq. $\geq 5\%$) Found in 25 % or Fewer Populations; No. LComm Bands ($\leq 50\%$) = No. of Locally Common Bands (Freq. $\geq 5\%$) Found in 50 % or Fewer Populations; N_a : mean number of alleles, N_e : number of effective alleles, I : Shannon's information index, H_e : expected heterozygosity, uH_e : unbiased expected heterozygosity.

Table 4. Marker parameters of genetic variation calculated for ISSR primers of *Hordeum bulbosum*

ISSR primers	H	PIC	EMR	H.av	MI	D	Rp
UBC807	0.429	0.381	4.371	0.0004	0.002	0.903	3.923
UBC812	0.495	0.350	5.859	0.0005	0.003	0.797	5.718
UBC817	0.495	0.350	4.526	0.0006	0.003	0.795	5.359
(AGC)5GA	0.451	0.371	4.808	0.0004	0.002	0.882	4.897
Mean	0.467	0.363	4.891	0.0005	0.002	0.844	4.974

Note: Expected heterozygosity (H), an average of polymorphic information content (PIC), effective multiplex ratio (EMR), mean heterozygosity (H.av), marker index (MI), discriminating power (D) and resolving power (Rp).

Table 5. Nei genetic distance between *Hordeum bulbosum* studied populations

Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7
0.000						Population1
0.187	0.000					Pop2
0.366	0.335	0.000				Pop3
0.193	0.230	0.367	0.000			Pop4
0.193	0.362	0.440	0.146	0.000		Pop5
0.197	0.325	0.401	0.099	0.114	0.000	Pop6
0.306	0.341	0.541	0.202	0.250	0.149	0.000 Pop7

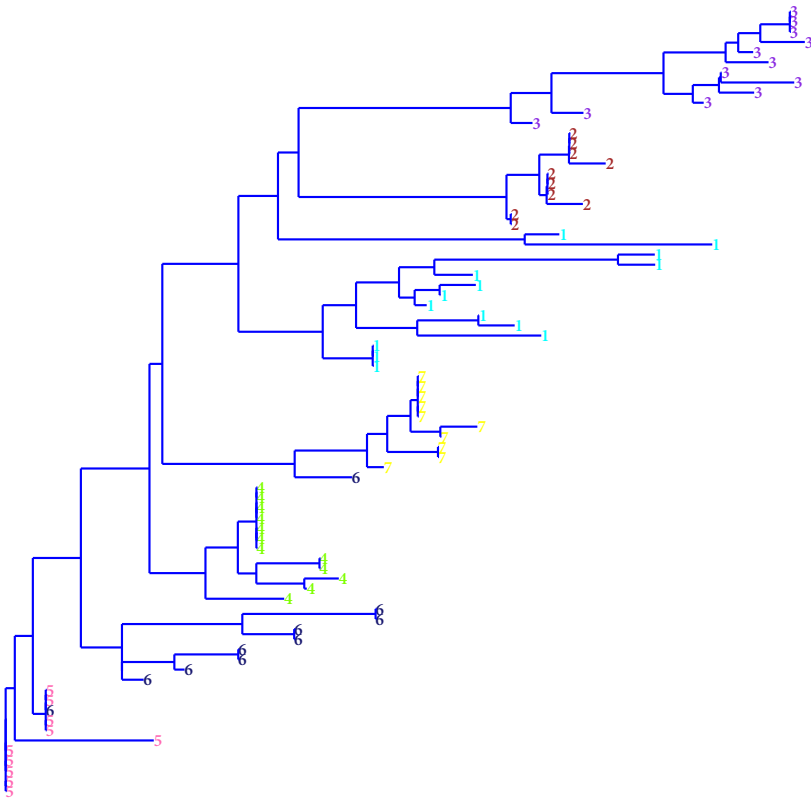


Fig. 2. NJ tree of the studied populations based on ISSR data (the population code is according to Table 1).

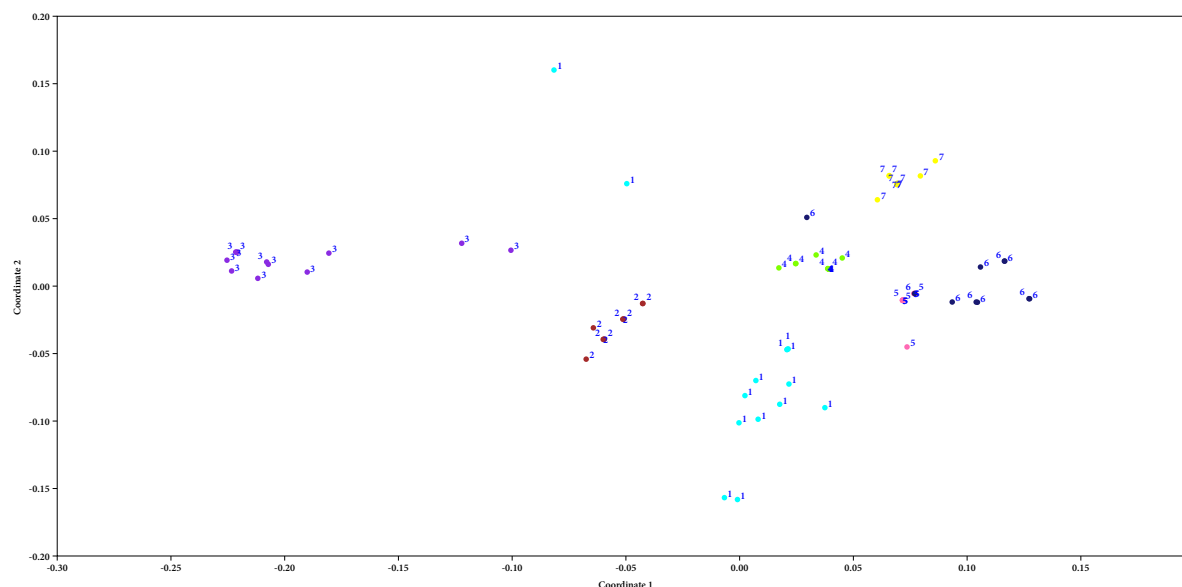


Fig. 3. MDS plot of ISSR data in studied populations (the population code is according to Table 1).

marked genetic divergence. Population 2 was positioned near population 3, but it remained distinct, whereas population 1 also formed a relatively separate group with greater dispersion among its individuals. By contrast, populations 4, 6, and 7 were located closer to each other, suggesting relatively greater genetic affinity. Population 5 occupied a more isolated position than most of the other populations, reflecting its distinct genetic background. Taken together, these findings are consistent with the AMOVA results and support the existence of clear genetic structuring among the studied populations.

STRUCTURE analysis provided valuable insights into the genetic makeup and diversity of *H. bulbosum* populations by revealing distinct genetic clusters and their proportions within the overall population (Fig. 4). Populations 4–6 displayed greater genetic similarity due to shared

ancestral alleles, while populations 2 and 7 exhibited distinct allele compositions, as evidenced by their unique color segments. This grouping pattern aligns with the results from the Neighbor-Joining (NJ) tree, indicating a strong correlation between the genetic structure and genetic distance.

The study found that total genetic diversity ($H_T = 0.26$) was significantly greater than within-population genetic diversity ($H_s = 0.07$), with a genetic differentiation index (G_{ST}) of 0.72, indicating high genetic differentiation between populations. Limited gene flow ($N_m = 0.19$) may have contributed to the high level of genetic differentiation observed among populations.

A reticulogram analysis, by visually depicting complex evolutionary relationships that involve genetic mixing through events like recombination, hybridization, and horizontal gene transfer, provides a more comprehensive understanding of population

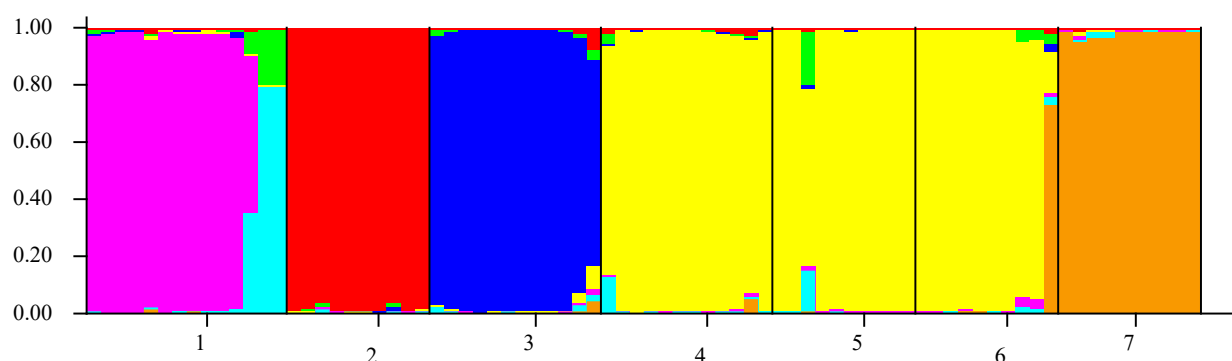
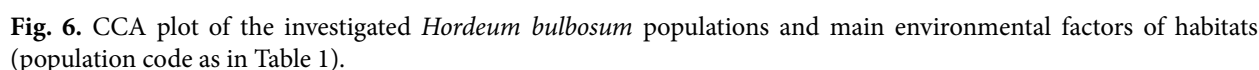
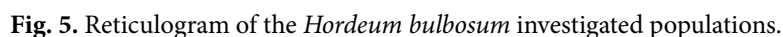


Fig. 4. STRUCTURE plot of the studied *Hordeum bulbosum* populations (the population code is according to Table 1).

and others (like green and blue colors) displaying greater internal variation.

Canonical Correspondence Analysis (CCA) of environmental factors revealed that different *H. bulbosum* populations are associated with distinct ecological conditions (Fig. 6). Specifically, populations 1, 4, and 6 were found at higher latitudes compared to others, and population 3 was separated from the rest based on latitude and elevation.

These findings highlight the significant role of environmental variables in shaping the genetic



diversity and structure of *H. bulbosum* populations. Additionally, significant correlation between genetic diversity and geographic distance ($r = 0.49$, $P = 0.0002$) suggests isolation by distance.

Discussion

A variety of molecular markers has been utilized to investigate genetic diversity and genotype associations (Yigider et al., 2024). Genetic diversity is a fundamental component of plant evolutionary potential and adaptive capacity, reflecting the differences in the genetic composition of populations or individuals within populations. It serves as a valuable reference in studies on the origin, evolution, adaptation, and utilization of species, especially under changing environmental conditions (Hu et al., 2024). ISSR markers are commonly used to evaluate genetic diversity because they are polymorphic, reproducible, cost-effective, and do not require prior sequence information (Bornet et al., 2002).

The AMOVA results revealed a high level of genetic differentiation among the studied populations ($\Phi_{PT} = 0.740$, $P = 0.001$), with 74 % of the total genetic variation attributed to differences among populations. This considerable divergence may be associated with restricted gene flow, habitat fragmentation, or geographical barriers, which can promote population isolation and divergence over time (Hamrick, Godt, 1996; Nybom, 2004). The pairwise Φ_{PT} values ranged from 0.387 between Populations 5 and 6 to 0.922 between Populations 2 and 5, indicating a wide range of genetic structuring. The relatively low genetic differentiation between Populations 5 and 6 may suggest recent common ancestry or ongoing gene flow, whereas the high differentiation between Populations 2 and 5 suggests stronger genetic isolation or divergent evolutionary trajectories. In line with these observations, Nei's genetic distances ranged from 0.114 to 0.541, further supporting the presence of substantial genetic variation. These findings highlight the importance of both geographical distance and ecological factors in shaping the genetic structure of natural populations (Frankham et al., 2010).

The results of the STRUCTURE analysis revealed clear patterns of genetic composition among the studied populations. Populations 1, 2, 3, and 7 were characterized by high proportions of assignment to a single genetic cluster, indicating limited gene flow with other populations. In contrast, Populations 4, 5, and 6 exhibited notable levels of admixture, suggesting higher interpopulation gene flow,

potentially due to geographic proximity or shared ecological conditions.

High proportions of assignment to a single genetic cluster in populations is often associated with historical isolation or restricted gene exchange, whereas admixture can reflect genetic connectivity among populations. Similar patterns have been reported in various plant species. For instance, studies on *Zea mays* ssp. *parviglumis* H. H. Iltis et Doebley have shown that geographically isolated populations maintain high genetic integrity, whereas those in more continuous landscapes experience greater gene exchange (van Heerwaarden et al., 2011), whereas *Centaurea diffusa* Lam. showed substantial genetic admixture associated with multiple introductions and long-range dispersal during range expansion (Marrs et al., 2008). These findings align with the results of AMOVA and clustering analyses, reinforcing the existence of distinct genetic structures among some populations and greater genetic connectivity among others. The combined evidence supports a scenario where both historical isolation and geographic connectivity may have contributed to the genetic structure observed of the studied populations. In this study, *H. bulbosum* populations 4, 5, and 6 showed relatively high levels of admixture, indicating a more complex genetic composition and a relatively recent shared ancestry compared to the other populations. This pattern may be associated with geographic proximity, which could facilitate genetic exchange among populations through pollen or seed dispersal. Similar trends have been observed in other Poaceae members. For example, recent genomic research on *H. bulbosum* has revealed multiple independent origins of autotetraploid cytotypes, followed by gene flow between diploid and tetraploid populations (Feng et al., 2025). Moreover, *H. spontaneum*, a closely related wild barley species, exhibits evidence of admixture among neighboring populations due to extensive pollen-mediated gene flow (Civán et al., 2021). These findings support the observed genetic structure in our study and highlight the role of gene flow in shaping diversity within wild *Hordeum* taxa. The presence of admixture in some individuals also points to occasional hybridization events, which can be important for introducing novel genetic combinations (Pritchard et al., 2000).

Reticulogram analysis revealed insights into gene pool structure by showing clusters representing independent gene pools and reticulation patterns indicating gene flow between populations. This means some groups are relatively isolated while

others share genetic material. This supports the hypothesis that *H. bulbosum* in Iran comprises at least 5–6 distinct gene pools, although confirmation of their exact number requires integration with ecogeographic data, such as climate and elevation populations exhibited distinct genetic structures that were associated with differences in their spatial and ecological contexts. For example, Population 1 (Blander village), located in an arid, high-temperature region with fluctuating climates, displayed high internal diversity and broader genetic dispersion, likely resulting from adaptation to harsh environmental conditions. In contrast, Population 3 (Asalem–Khalkhal road), which resides in a humid, stable mountain environment, exhibited a more uniform genetic composition, consistent with low intra-population differentiation and high environmental stability.

These patterns are shaped by ecological pressures, reproductive isolation, and environmental gradients. These results suggest that isolation by environment (IBE) acts as a strong force in creation genetic differentiation among these populations. IBE occurs when ecological divergence reduces gene flow, change in flowering time and pollination period, leading to distinct genetic patterns in populations living in different environments (Wang, Bradburd, 2014).

Nei's genetic distance values revealed substantial variation in genetic relationships among the studied populations, with some closely located populations showing relatively low genetic distances. Populations 4 and 5, as well as 5 and 6, showed the closest genetic relationships (0.099 and 0.114, respectively), suggesting that their physical proximity may have facilitated more frequent interbreeding. Conversely, Populations 3 and 7 (0.541) and 3 and 6 (0.401) exhibited the greatest genetic distances, may be associated with geographic separation and ecological divergence driven by environmental variables such as altitude and precipitation. These findings underscore the importance of both geographic and ecological factors in shaping population structure, with isolation by distance acting as a significant driver.

This pattern has been similarly observed in other Poaceae species. For instance, in a study of *Aegilops tauschii* Coss. Wang et al. (2013) found that populations located at higher altitudes and greater geographic distances exhibited significantly greater genetic divergence, influenced by reduced gene flow and environmental heterogeneity. These results parallel our findings in *H. bulbosum*, supporting the role of geographic separation and environmental heterogeneity in structuring genetic variation.

The high polymorphism observed in this study, with a mean PIC value of 0.363 and D values ranging from 0.795 to 0.903 (mean 0.844), demonstrates the effectiveness of ISSR markers in assessing genetic diversity. Comparable levels of polymorphism have been reported in other plant species, including lentil (Fikiru et al., 2007), yarrow (Farajpour et al., 2012), safflower (Ali et al., 2020), and ryegrass (Keshavarzi et al., 2022). Dominant markers with PIC values near 0.5 are considered highly informative for population genetics studies (Roldán-Ruiz et al., 2000).

Genetic diversity was further estimated through expected heterozygosity (H_e), which ranged from 0.014 (Population 5) to 0.165 (Population 1), with a mean of 0.072. These values are consistent with low gene flow and genetic isolation. For comparison, Yigider et al. (2024) reported a higher H_e (0.306) for *H. vulgare*, emphasizing the differentiation between cultivated and wild barley.

From a conservation and breeding perspective, these results have important practical implications. Populations with relatively low genetic distances, such as Populations 4 and 5, are suitable for breeding and hybridization transfer of potentially valuable traits. In contrast, highly distinct populations like Population 3 should be prioritized for *in situ* and *ex situ* conservation, as they represent unique genetic reservoirs. These findings can inform the establishment of gene banks and native seed reserves for the long-term conservation and utilization of valuable genetic resources (Salgotra, Chauhan, 2023).

Integrating genetic structure data with ecological parameters such as soil type, rainfall, altitude, and temperature provides a more comprehensive understanding of environmental adaptation in *H. bulbosum*. This study is among the first to integrate molecular diversity data with ecological variables in this species. The ability of *H. bulbosum* to adapt to a wide range of environmental conditions underlines its potential as a genetic resource for cultivated barley (*H. vulgare*), particularly for traits related to drought, cold, pest, and disease resistance.

The research highlights how genetic diversity, restricted genetic connectivity, and local adaptation have significantly influenced the genetic structure of *H. bulbosum* in Iran. The study utilizes various molecular techniques to analyze this, offering insights into evolutionary processes and providing valuable data for conservation and research related to *Triticeae* species.

The study revealed pronounced genetic differentiation and a complex population structure among the Iranian *H. bulbosum* populations,

suggesting distinct gene pools and high levels of genetic differentiation. Restricted gene flow, local adaptation to different environments, and geographical isolation, including isolation by distance and isolation by environment, have all contributed to the observed genetic variation. The findings also demonstrated that ISSR markers were effective for evaluating genetic diversity in *H. bulbosum*, a wild relative of barley, and providing useful molecular information for future conservation and breeding efforts. The study emphasizes the importance of preserving *H. bulbosum* genetic resources to enhance barley's resilience and sustainability, as well as to protect global biodiversity.

Conclusion

This study showed that Iranian populations of *H. bulbosum* possess substantial genetic differentiation and a clear population structure. ISSR

markers were effective in detecting polymorphism and revealing patterns of genetic differentiation among populations. The results suggest that limited gene flow, geographic isolation, and environmental differences have all contributed to the observed genetic structure. Some populations were genetically more distinct, whereas others showed signs of admixture, may reflect the combined influence of historical separation and ecological connectivity. These findings highlight the importance of *H. bulbosum* as a valuable wild genetic resource and provide useful information for its conservation and for future barley breeding programs, especially those aimed at improving stress tolerance.

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