NORDIC JOURNAL OF
BOTANY

Research Article

Leaf micromorphology and genetic diversity analysis of Iranian *Hordeum* species using ISSR and SCoT markers

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Nordic Journal of Botany
2026: e05158
doi: 10.1002/njb.05158
Subject Editor: Barış Özüdoğru
Editor-in-Chief: Jalil Noroozi
Accepted 20 February 2026
Published 22 June 2026

The genus *Hordeum* (Poaceae), of the tribe Triticeae, comprises approximately 32 species with substantial potential for barley improvement. *Hordeum vulgare* is an economically important cereal widely cultivated across diverse environments, from the Arctic to desert and humid regions. In this study, we analyzed 67 individuals from 11 populations representing five Iranian *Hordeum* species using inter-simple sequence repeats (ISSR) and start codon targeted (SCoT) molecular markers. We evaluated the discriminatory power of these markers and identified diagnostic micromorphological traits of the abaxial leaf blade. Genetic variation and affinities among Iranian *Hordeum* species were assessed using molecular markers combined with micromorphology. Neighbor-net and UPGMA analyses revealed close evolutionary relationships among *Hordeum marinum*, *Hordeum bulbosum*, and *Hordeum murinum*. Micromorphological examination of the abaxial leaf surface showed significant interspecific variation in both qualitative and quantitative traits. Additionally, *H. bulbosum* exhibited close affinities with *Hordeum vulgare* subsp. *spontaneum* and, to a lesser extent, with *Hordeum brevisubulatum*. While most *Hordeum* research has focused on biological traits, salinity tolerance, and disease resistance, breeding efforts are limited by small grain size and scarce genetic information on wild taxa. Our findings provide valuable insights for the collection, conservation, and genetic management of *Hordeum* germplasm, contributing to future breeding and improvement programs. Overall, this study highlights the utility of integrating molecular and micromorphological data to guide the conservation and targeted improvement of barley and related species.

Keywords: cereal improvement, micromorphological traits, molecular markers, plant conservation, population structure, wild barley

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Introduction

Barley *Hordeum vulgare* L. is a significant global cereal crop, ranking fourth in production after wheat, rice, and maize (Marwat et al. 2012). Its importance in global agriculture is due to its wide ecological adaptability, relatively short growth cycle, and strong tolerance to abiotic stresses such as drought and salinity. In addition to being a staple food and animal feed, barley is extensively used in the malting and brewing industries, which further enhances its economic value (Naghavi et al. 2011, Marwat et al. 2012). Moreover, its rich nutritional composition – particularly its high content of dietary fiber and antioxidants – has increased its popularity in health-oriented food markets (Baik et al. 2008). In recent years, barley has also gained considerable attention as a model cereal for studying stress tolerance and resilience, supported by extensive genomic resources and its key role in sustainable agricultural systems (Jiang et al. 2025).

Iran is recognized as one of the primary centers for the origin and domestication of barley, situated on the southeastern edge of the Fertile Crescent (Khodayari et al. 2012). Archaeobotanical evidence suggests that domesticated barley, along with wheat, was cultivated in this area as early as 8500 BCE (Nesbitt et al. 1996, Zohary et al. 2000, Morrell et al. 2011). Recent population genomic studies indicate that barley domestication involved multiple wild populations across different regions of the Near East and Central Asia, suggesting a complex and regionally structured domestication process (Guo et al. 2025). The extensive history of barley domestication, together with the presence of its wild relatives in natural habitats, has resulted in a rich reservoir of genetic diversity, making them invaluable for modern crop improvement initiatives. The genus *Hordeum* (Poaceae), belonging to the Triticeae tribe, consists of approximately 32 species and 45 taxa. Many of these species are vital genetic resources for enhancing cultivated barley (Barkworth et al. 2009, Morrell et al. 2011, Dido et al. 2022). They exhibit ecological diversity, thriving in a wide variety of habitats from Arctic tundras to arid deserts and humid environments (Naghavi et al. 2011). Among the notable wild relatives are *Hordeum brevisubulatum* (Trin.) Link, *Hordeum bulbosum* L., *Hordeum marinum* Huds., and *Hordeum murinum* L., each demonstrating traits of significant agronomic interest, such as salt tolerance, winter hardiness, disease resistance, and adaptability to disturbed or marginal habitats (Morrison 1958, Von Bothmer et al. 1989, Gilpin et al. 1997, Malik et al. 2009, Wang et al. 2013, Cui et al. 2021). Recent genomic analyses of *H. bulbosum* have revealed extensive structural variation and adaptive potential, further emphasizing the value of wild *Hordeum* species as sources of beneficial alleles for barley breeding programs (Feng et al. 2025). *Hordeum bulbosum*, a perennial and primarily outcrossing species, has been integrated into breeding programs due to its contributions to disease resistance and improved winter survival (Gilpin et al. 1997). Similarly, *H. marinum* is recognized for its tolerance to salt and waterlogging, making it a valuable resource for breeding in regions prone to environmental stress (Malik et al. 2009). Wild *Hordeum* species, characterized by diverse ecological adaptations, serve as a critical genetic reservoir for breeding programs. Utilizing this diversity enables the introgression of novel traits essential for enhancing abiotic stress tolerance, resilience, and sustainable crop development (Gilpin et al. 1997, Wang et al. 2013, Cui et al. 2021, Safiyar et al. 2022).

Understanding the identification and classification of genetic variation within and among species is important for grasping evolutionary relationships, maintaining biodiversity, and ensuring effective conservation efforts (Tabaripour and Keshavarzi 2021). Recent studies on wild barley *Hordeum vulgare* subsp. *spontaneum* (K.Koch) Asch. & Graebn. have revealed pronounced genetic structure and significant marker–trait associations, highlighting the potential of these populations as valuable genetic resources for improving agronomic and physiological traits in cultivated barley (Shirvani et al. 2025). Molecular markers, including start codon targeted (SCoT) and inter-simple sequence repeat (ISSR), are valuable tools for assessing genetic diversity and elucidating relationships among closely related taxa (Collard and Mackill 2009, Khodaee et al. 2021). Although high-throughput genomic approaches are increasingly used in barley research, conventional molecular markers remain highly informative and practical for assessing genetic diversity in wild populations and regional germplasm collections (Spanner et al. 2026). A variety of molecular markers, such as RFLPs, AFLPs, STSs, and microsatellites, have been extensively used to evaluate genetic diversity within the Poaceae family. These tools have proven particularly useful for differentiating cultivars, performing genetic mapping, and clarifying phylogenetic relationships among species of *Hordeum* (Khodayari et al. 2012). For example, a study combined pedigree and phenotypic data with microsatellite markers to assess the genetic diversity of barley cultivars over a 40-year breeding period (Brbaklić et al. 2021). In addition to internal transcribed spacer (ITS) regions, some studies have indicated a close relationship between *H. marinum* and H-genome species (Blattner 2004), while others have shown a clear separation based on ITS sequences (Sun et al. 2009).

Despite extensive studies on barley and its wild relatives, gaps remain in the comprehensive assessment of Iranian *Hordeum* species combining molecular markers and micromorphological traits. This study addresses these gaps by integrating ISSR and SCoT markers with detailed leaf micromorphology, providing novel insights into population structure, interspecific relationships, and diagnostic traits that have not been previously reported for these populations.

Similar approaches have been employed in other genera within the Poaceae family apart from *Hordeum*. For instance, a study on *Triticum urartu* utilized AFLP markers (Baum and Bailey 2013), while another study on *Thinopyrum acutum* (DC.) Banfi employed microsatellites (Refoufi and Esnault 2006). Three subspecies of *Dactylis glomerata* L. have been identified using RAPD, ISSR, and AFLP markers (Costa et al. 2016). Using ISSR and SCoT markers, researchers demonstrated the ability of *Lolium* L. to distinguish

between inbreeding and outbreeding at the interspecific level (Tabaripour and Keshavarzi 2021). Similarly, ISSR markers were used in *Avena sterilis* L. and *A. fatua* L. to assess genetic diversity and population structure across different species (Aghajani et al. 2025). Additionally, *Aegilops triuncialis* L. has also shown that these markers are valuable for assessing genetic diversity within species (Khodaee et al. 2021). These examples highlight that ISSR and SCoT markers are particularly effective in intraspecific analyses and in closely related species at the interspecific level, where fragment homology is maintained.

ISSR and SCoT markers are widely utilized for their simplicity, reproducibility, and high polymorphic potential across various taxonomic levels. Their effectiveness in delineating species has been demonstrated in several genera, including *Epilobium* Dill. ex L. (Rahimi et al. 2022), *Lallemantia* Fisch. & C.A.Mey. (Koohdar et al. 2021), and *Crocus* L. (Sheidai et al. 2018). While these markers are particularly robust for identifying intraspecific and interspecific variations – as shown in *Plantago* L., *Nepeta* L., and oat varieties (Mohsenzadeh et al. 2020, Talebi et al. 2022) – their informativeness typically decreases with greater taxonomic divergence due to fragment homology decay. Nevertheless, combining these marker systems can provide comprehensive insights into complex genetic structures, a strategy employed in the present study to evaluate interspecific relationships.

In the genus *Hordeum*, polyploidization is essential for speciation (Brassac and Blattner 2015). A study utilized next-generation sequencing of multiple nuclear and chloroplast loci to reconstruct a comprehensive multilocus phylogeny of all diploid and polyploid *Hordeum* species. This research revealed detailed progenitor-derivative relationships and the evolutionary history of the genus, providing a crucial framework for understanding its genetic diversity and speciation processes.

Understanding genetic diversity and structure is crucial for breeding programs and germplasm conservation in *Hordeum* (Safiyar et al. 2022). In this study, we combined ISSR and SCoT markers with detailed leaf micromorphology to investigate Iranian *Hordeum* species.

The aims of this research are as follows: 1) to enhance understanding of the genetic diversity and population structure of *Hordeum* species in Iran, 2) to employ ISSR and SCoT molecular markers to assess genetic variation and infer relationships among *Hordeum* taxa, with a focus on the structure and differentiation of populations within Iran, and 3) to identify diagnostic micromorphological traits of *Hordeum* leaves to complement the molecular data.

Material and methods

Plant samples

For ISSR and SCoT studies, 67 individuals (from 11 populations) of five species of the genus *Hordeum* were used in this research (Table 1, Fig. 1). Voucher specimens of the studied *Hordeum* taxa were collected from multiple populations across Iran and deposited in the Herbaria of Alzahra University (ALUH) and Ferdowsi University of Mashhad (FUMH). Sampling sites were selected to represent the

Table 1. Locality information for taxa of the genus *Hordeum* for ISSR and SCoT studies. *ALUH, Alzahra University Herbarium; FUMH, Ferdowsi University of Mashhad.

Taxa	Locality, herbarium no* and acronym	Number of specimens sampled	Samples analyzed for micromorphology	Ploidy level
H. vulgare subsp. *spontaneum* (K. Koch) Thell.	Razavi Khorasan: southeast of Mashhad, 1370 m a.s.l., Basiri 1026 (ALUH) Razavi Khorasan: southeast of Mashhad, Green belt road, 1051 m a.s.l., Basiri 1027 (ALUH)	8,2	+	2x
H. vulgare subsp. *vulgare* L.	Razavi Khorasan: southeast of Mashhad, Green belt road, 1051 m a.s.l., Basiri 1028 (ALUH)	10	+	2x
H. bulbosum L.	Razavi Khorasan: Toroq, 998 m a.s.l., Basiri 1029 (ALUH)	10	+	2x–4x
H. brevisubulatum (Trin.) Link	Gilan: pastures of Gadook, 2230 m a.s.l., Pouramini 1030 (ALUH) Gilan: Kiare, 2240 m a.s.l., Pouramini 1031 (ALUH) Gilan: between Majareh village and Gardaneh-ye Tūnī, 2250 m a.s.l., Pouramini, 1032 (ALUH) Gilan: Asalem to Khalkhal, Kormon Yaylak, 2055 m a.s.l., Pouramini 1033 (ALUH)	2, 2, 5, 1	+	2x–4x–6x
H. murinum subsp. *glaucum* (Steud.) Tzvelev	Gilan: Talish-Asalem, 40 m a.s.l., Pouramini 1015 (ALUH)	10	+	2x
H. murinum subsp. *leporinum* (Link) Arcang.	Khuzestan: city entrance of Ramhormoz, 161 m a.s.l., Farasat 1034 (ALUH)	10	+	4x–6x
H. marinum subsp. *gussoneanum* (Parl.) Thell.	Razavi Khorasan: 25 km north of Kashmar to Neyshabur, 1600 m a.s.l., Faghihnia and Zanguei 20735 (FUMH)	7	+	2x–4x

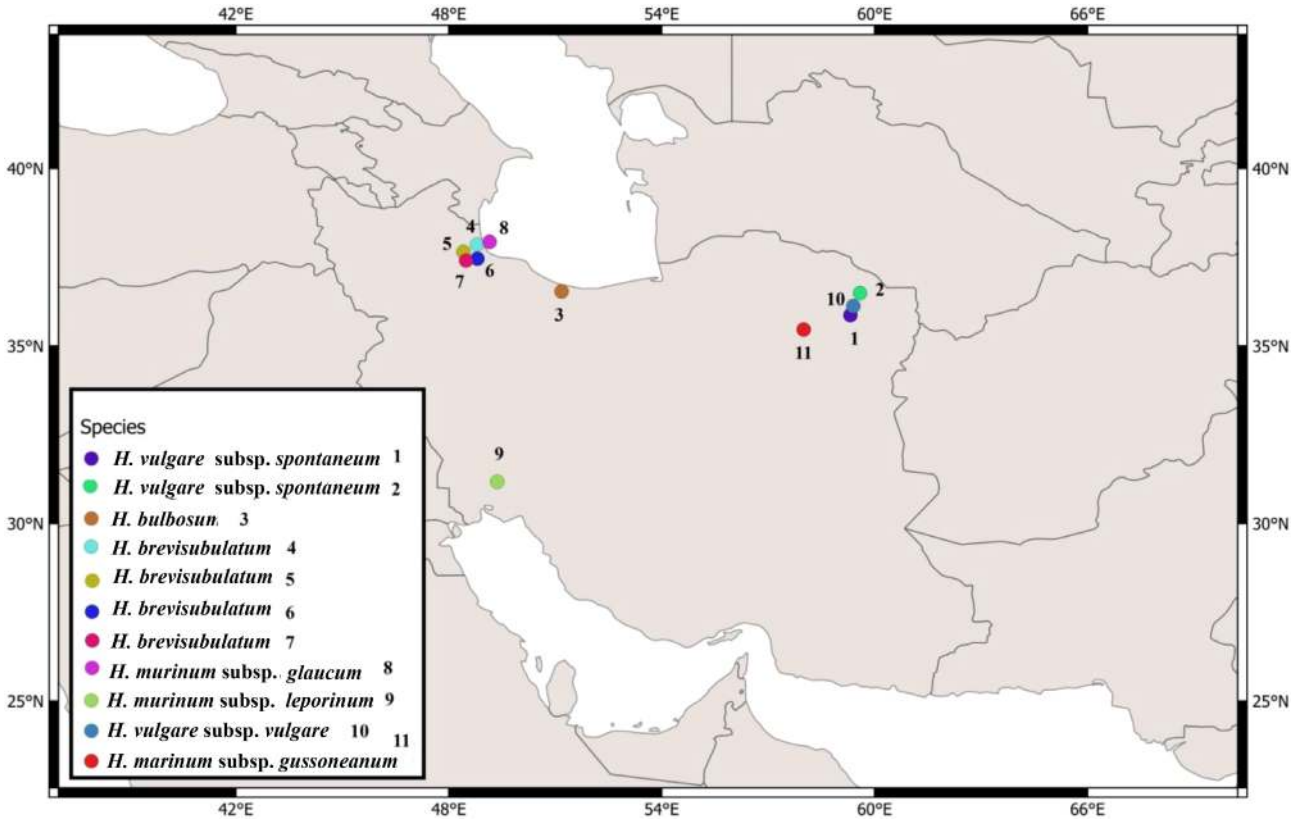


Figure 1. Distribution map of the studied *Hordeum* populations in Iran.

geographical distribution and ecological variability of each species, ensuring that populations from different environmental conditions and centers of diversity were included. The corresponding voucher numbers can be found in Table 1. Species identification was performed by RT, and the specimens are available for public reference at ALUH. The names were checked based on Plants of the World Online (POWO; www.plantsoftheworldonline.org/). Accordingly, *Hordeum gussoneanum* (Parl.) Thell. is treated here as a subspecies of *H. marinum*, following the current classification in POWO, while alternative treatments considering it as a separate species (Brassac and Blattner 2015) were also noted.

To complement macromorphological observations and taxonomic identification, a detailed micromorphological analysis was performed. Both light microscopy (LM) and scanning electron microscopy (SEM) were employed to examine the abaxial leaf surfaces of the studied species. For this purpose, 3–5 individuals per population were selected from the 67 individuals used in the molecular (ISSR and SCoT) analyses, ensuring direct comparability between the molecular and micromorphological datasets.

Locality information for taxa of the genus *Hordeum*, including herbarium vouchers for specimens used for ISSR and SCoT analyses, is mentioned in Table 1.

Ploidy levels of the studied taxa were also considered based on available literature. *Hordeum vulgare* subspecies (*spontaneum* and *vulgare*) are diploid (2×, 2n = 14). *Hordeum bulbosum* and *H. marinum* subsp. *gussoneanum* are typically tetraploid (4×). In contrast, *H. brevisubulatum* and *H. murinum* subspecies exhibit intraspecific ploidy variation ranging from diploid (2×) to hexaploid (6×), as documented in cytogenetic and genome size studies (Jakob et al. 2004, Brassac and Blattner 2015).

Leaf micromorphology

Light microscopy and scanning electron microscopy

A total of seven taxa from five species were used to study the micromorphology of the leaf abaxial epidermis (Table 1). The micromorphology of the leaf abaxial epidermis in the studied species was examined using tissue removal method and staining with Basic Toluidine Blue O (BTO). Examination of the prepared tissues was conducted with the aid of an Olympus optical microscope and Dino-Lite AM 4023 digital eyepiece cameras (Table 2).

Leaf sections from the central part of the blade were excised and mounted directly on aluminum stubs using double-sided carbon tape, then sputter-coated with a layer of gold for SEM observation. To prepare the specimens for SEM, mature leaves were first cleaned and, to prevent structural collapse during imaging, dehydrated through a graded ethanol series of 25, 50, 75, and 95%, followed by three exchanges in 100% anhydrous ethanol. Critical point drying was then performed to replace ethanol with liquid CO_2 and dry the

Table 2. The abaxial foliar epidermal characters evaluated in *Hordeum* taxa.

No.	Char.	Char. state
1	prickle in costal zone	1 = absent, 2 = present
2	prickle in intercostal zone	1 = absent, 2 = present
3	macro hair	1 = absent, 2 = present
4	silica bodies in costal zone	1 = absent, 2 = present
5	silica bodies in intercostal zone	1 = absent, 2 = present
6	wax shape	1 = membranous platelets and granules, 2 = membranous platelets
7	short cell in intercostal zone	1 = absent, 2 = present
8	stomata cell wall shape	1 = parallel, 2 = low domed, 3 = quadrate
9	stomata row number	1 = two, 2 = four
10	cell wall shape between long cells	1 = diagonal, 2 = straight-diagonal, 3 = straight
11	long cell shape	1 = fusiform, 2 = rectangular

specimens under supercritical conditions, minimizing surface tension artifacts, Micromorphological features were examined using a VEGA3 TESCAN scanning electron microscope (TESCAN, Brno, Czech Republic) at an accelerating voltage of 15 kV. Specimens were sputter-coated with a 20–30 Å layer of a 60:40 gold–palladium (Au/Pd) alloy to enhance surface conductivity and reduce charging and thermal damage during imaging. SEM micrographs were captured at magnifications of 35× and 250× to analyze epidermal traits. The traits analyzed for the leaf blades are presented in Table 2, and terminology followed Ellis (1979) and Barthlott et al. (1998). Quantitative measurements were performed using Digimizer software (ver. 5.3.4) (Schoonjans 2012).

Molecular studies

DNA extraction, amplification, and ISSR and SCoT assay

Young dried leaves were ground into a fine powder in liquid nitrogen using a pestle. After that, they were transferred to cetyl trimethyl-ammonium bromide (CTAB) extraction buffer and incubated at 65°C for 1 h. The protocol in Wang (2009) was used to extract genomic DNA. The quality of the extracted DNA was evaluated by running on a 0.8% agarose gel. For the analysis of several DNA samples, we used four inter simple sequence repeat (ISSR) primers – UBC-807, UBC-812, UBC-817, and $(AGC)_5GA$ (AGCAGCAGCAGCAGCGA) – commercially available from the University of British Columbia (Zietkiewicz et al. 1994). In addition, four start codon targeted (SCoT) primers – SCoT-2, SCoT-22, SCoT-36, and SCoT-44, also sourced from the University of British Columbia – were employed (Collard and Mackill 2009) (Table 3).

PCR reactions were performed in a total volume of 25 µl containing 10 mM Tris-HCl buffer (pH = 8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 20 ng of genomic DNA, and 3U of Taq DNA polymerase. All reagents were obtained from Bioron, Germany. The following program was used to amplify ISSR markers in a Techne thermocycler (Germany): 94°C for 5 min; 40 cycles of 94°C for 1 min; annealing at 53.4°C for 45 s and extension at 72°C for 90 s; final extension at 72°C for 7 min. On the other hand, for the SCoT marker, the program was 94°C for 5 min; 35 cycles of 94°C for 1 min; annealing at 50–53°C for 1 min and extension at 72°C for 90 s; final extension at 72°C for 7 min. Products were visualized by running them on 1% agarose gels and staining them with Power Load. A 100 bp molecular size ladder (Fermentas, Germany) is evaluated for fragment size.

Data analyses

Molecular studies

To provide a robust assessment of genetic diversity and evolutionary relationships, a multi-faceted analytical approach was employed. We combined distance-based clustering, character-based Bayesian analysis, and network-based methods to capture different aspects of genetic structure, including potential hybridization and reticulate evolution.

ISSR and SCoT bands were scored as binary characters (1 = presence, 0 = absence), and various genetic diversity parameters – including Nei's gene diversity (He), Shannon information index (I), and percentage of polymorphism – were evaluated using GenAlEx 6.4 (Weising et al. 2005, Peakall and Smouse 2006). G_{st} (genetic diversity residing among species) analysis was performed using POPGENE ver. 1.31 (Yeh et al. 1999) to survey the discriminating power of ISSR and SCoT loci. Heterozygosity index (H), Polymorphism information content (PIC), Effective

Table 3. The primer used for the analysis of *Hordeum* species.

ISSR primers		SCoT primers	
primer code	sequence (5′–3′)	primer code	sequence (5′–3′)
UBC807	AGAGAGAGAGAGAGAGT	SC_2	CAACAATGGCTACCACCT
UBC812	GAGAGAGAGAGAGAGAA	SC_{22}	AACCATGGCTACCACCAC
UBC817	CACACACACACACACAA	SC_{36}	GCAACAATGGCTACCAC
(AGC)5GA	AGCAGCAGCAGCAGCGA	SC_{44}	CAATGGCTACCATTAGCC

multiplex ratio (E), Arithmetic mean of H (H.av), Marker index (MI), Effective multiplex ratio (EMR), Discriminating power (D), and Resolving power (R) for each primer were calculated by the program online marker efficiency calculator (iMEC) (Amiryousefi et al. 2018) (https://irscope.shinyapps.io/iMEC/).

These indices were calculated using the following formulas:

$H = 1 - \Sigma\ pi^2$, $E = n(n/(np - nnp))$, $H_avp = \Sigma\ Hn/np$, $MI = E \times H_avp$, $D = 1 - C$, $R = \Sigma\ I_b$, and $PIC = 1 - \Sigma\ pi^2 - \Sigma\ \Sigma\ pi^2\ pj^2$, where pi is the allele frequency for the i-th allele, pj is the population frequency of the j-th allele, p and np are the polymorphic and non-polymorphic fractions of the markers, C is the confusion probability, and H_n is the heterozygosity of the polymorphic fraction of markers, calculated across all polymorphic loci (n_p). Band informativeness (I_b) is measured on a scale from 0 to 1 (Amiryousefi et al. 2018).

To visualize the genetic relationships among taxa, species were grouped using neighbor-joining (NJ) trees in PAST software (ver. 4.10). Considering that strictly bifurcating trees may not accurately represent evolutionary histories involving hybridization, NeighborNet networks were constructed using SplitsTree 4.13 with 100 bootstrap replicates to infer the relationships among species and subspecies (Huson and Bryant 2006). Analysis of molecular AMOVA with 1000 permutations was performed in GenAlex 6.4 to determine genetic differentiation between the species under study (Peakall and Smouse 2006). A heatmap clustering (ClustVis) was employed to define the dependent variable parameters. Furthermore, to explicitly test for gene flow and reticulation, which are common in the genus *Hordeum*, reticulation analysis was performed using Diversity Analysis and Representation for Windows (DARwin) software ver. 6 (Perrier et al. 2006), based on the method described in Legendre and Makarenkov (2002). The software is freely available at https://darwin.cirad.fr/. An NJ phylogenetic tree was constructed to support the analysis. Reticulation branches were added iteratively to minimize the least squares criterion at each stage of the algorithm (Legendre and Makarenkov 2002, Makarenkov and Lapointe 2004). TCS networks (Clement et al. 2002) were constructed using Population Analysis with Reticulate Trees (PopART) (ver. 1.7; http://popart.otago.ac.nz) to resolve fine-scale phylogenetic relationships among populations and visualize potential haplotype connections. To assess genetic structure and infer potential population stratification (admixture), Bayesian clustering analysis was performed using STRUCTURE ver. 2.3.4 (Pritchard et al. 2000) on a dataset of 67 individuals. The admixture model with correlated allele frequencies was applied. For each K value ranging from 1 to 10, 10 independent simulations were conducted, each comprising a burn-in of 10^5 iterations followed by 10^6 MCMC repetitions. The most likely number of clusters was determined using the ΔK method of Evanno et al. (2005), as implemented in StructureSelector.

Importantly, we note that the molecular markers used are primarily informative within species or closely related species groups. Therefore, while some interspecific comparisons are included, results involving distantly related species are interpreted with caution, acknowledging the potential limitations of the markers for detecting deep evolutionary divergence.

Leaf micromorphological studies

Binary or multistate data (standardized data; mean = 0, variance = 1) were coded. For the morphological data, multivariate statistical analyses, including cluster analysis and ordination, were performed using PAST software (ver. 4.10) (Hammer et al. 2012). The unweighted pair group method with arithmetic mean (UPGMA) was applied to generate a hierarchical view of phenotypic similarities, while principal component analysis (PCA) was used to identify the key diagnostic traits contributing most to species divergence.

Results

Molecular investigation

ISSR analysis

A total of 57 ISSR scorable and reproducible bands were produced. The highest number of bands and genetic variation were observed in *H. murinum* subsp. *glaucum* (Steud.) Tzvelev and *H. vulgare* subsp. *spontaneum* (C. Koch) Thell, while *H. marinum* Huds. subsp. *gussoneanum* (Parl.) Arcang and *H. bulbosum* showed the lowest values, indicating substantial interspecific variation. Species-specific bands were observed in all taxa, with *H. murinum* subsp. *glaucum* and *H. vulgare* subsp. *spontaneum* possessing the greatest numbers. Each species possessed specific bands (those restricted to a single taxon), with species *H. murinum* subsp. *glaucum* having the highest number (4). *Hordeum murinum* subsp. *glaucum* had the highest degree of genetic polymorphism (50.88) and Shannon's information index (I = 0.276). *Hordeum vulgare* subsp. *spontaneum* had the highest values for effective alleles (Ne = 1.265). Moreover, the number of observed alleles (Na) ranged from 0.281 to 1.158 (Table 4). The lowest values for genetic variation parameters were observed in *H. bulbosum*. The average marker index (MI) representing the efficiency of a marker was 0.002, and UBC_{812} (0.003) had the highest values. All the primers showed high polymorphic information content (PIC) values (~ 0.358). The highest PIC was obtained with UBC_{817} (0.366) while the lowest PIC was found with UBC_{812} (0.324) (Table 5).

Among the studied species, the AMOVA test revealed significant genetic differences (p = 0.001). Furthermore, 52% of the total genetic variation occurred among species (i.e. between different species), while 48% occurred within species (i.e. among individuals within the same species). Gene flow (Nm) was low (0.40), reflecting limited exchange among species.

Based on the NeighborNet diagram, the *Hordeum* species were grouped according to genetic differences (Fig. 2). The two subspecies, *H. vulgare* subsp. *vulgare* and *H. vulgare* subsp. *spontaneum*, were clearly separated into distinct

Table 4. Genetic parameters in five *Hordeum* species for ISSR and SCoT markers. N, number of individuals; Na, mean number of alleles; Ne, number of effective alleles; I, Shannon's information index; He, expected heterozygosity; UHe, unbiased expected heterozygosity; P%, percentage of polymorphic loci.

Sp.	N	Na	Ne	I	He	UHe	%P
ISSR							
H. vulgare subsp. *spontaneum*	10	0.947	1.265	0.225	0.153	0.161	40.35
H. vulgare subsp. *vulgare*	10	0.544	1.189	0.133	0.095	0.100	19.30
H. bulbosum	10	0.439	1.013	0.016	0.010	0.010	5.26
H. brevisubulatum	10	1.053	1.243	0.221	0.146	0.153	43.86
H. murinum subsp. *glaucum*	10	1.158	1.315	0.276	0.186	0.195	50.88
H. murinum subsp. *leporinum*	10	0.947	1.214	0.197	0.130	0.136	38.60
H. marinum subsp. *gussoneanum*	7	0.281	1.018	0.016	0.011	0.011	3.51
SCoT							
H. vulgare subsp. *spontaneum*	10	1.414	1.342	0324	0.211	0.222	67.24
H. vulgare subsp. *vulgare*	10	1.017	1.256	0.227	0.149	0.157	48.28
H. bulbosum	10	1.121	1.252	0.240	0.154	0.163	53.45
H. brevisubulatum	10	1.000	1.222	0.227	0.144	0.152	50.00
H. murinum subsp. *glaucum*	10	1.276	1.333	0.309	0.202	0.213	63.79
H. murinum subsp. *leporinum*	10	0.948	1.171	0.183	0.113	0.119	46.55
H. marinum subsp. *gussoneanum*	7	0.517	1.130	0.109	0.074	0.080	18.97

clusters. A small number of *H. vulgare* subsp. *vulgare* individuals were located at the edge of their cluster, close to the *H. vulgare* subsp. spontaneum cluster in the network. The perennial species (*H. bulbosum* and *H. brevisubulatum*) appeared to be closely related. In addition, the two *H. murinum* subspecies (subsp. *glaucum* and subsp. *leporinum*) form distinct clusters but are located within the same major lineage of the network. STRUCTURE analysis supported the NJ tree and NeighborNet diagram, revealing evidence of genetic admixture among *H. brevisubulatum*, *H. murinum* subsp. *glaucum*, and *H. murinum* subsp. *leporinum* (Fig. 3).

SCoT analysis

The SCoT primers amplified a total of 58 loci. Among the species analyzed, *H. vulgare* subsp. *spontaneum* exhibited the highest number of bands (43), whereas *H. marinum* subsp. *gussoneanum* showed the lowest (19). Several species-specific bands were detected, with *H. vulgare* subsp. *spontaneum* having the greatest number (4). The average MI and PIC for SCoT primers were slightly lower than those for ISSR, consistent with ISSR's higher efficiency. Moreover, analysis revealed that 33% of the total genetic variation was attributable to differences among species, while 67% resulted from genetic differentiation within species. As detailed in Table 4, genetic diversity parameters demonstrated pronounced variation among species: *H. vulgare* subsp. *spontaneum* exhibited the highest genetic polymorphism (67.24%), followed by *H. marinum* subsp. *gussoneanum* (18.97%). *Hordeum vulgare* subsp. *spontaneum* also showed the greatest values for effective alleles (Ne = 1.342), Shannon's index (I = 0.324), and gene diversity (He = 0.211), whereas *H. marinum* subsp. *gussoneanum* displayed the lowest genetic variation across all measured parameters. The average marker index (MI) representing the efficiency of a marker was 0.001, with $SCoT_{22}$ (0.002) showing the highest value. It was calculated that the average PIC for SCoT primer combination was 0.294.

Genetic differentiation both among and within species was consistent with the NeighborNet and NJ analyses. The reticulation dendrogram (Fig. 4) revealed patterns of gene flow between taxa. Outcrossing species, such as *H. bulbosum* and

Table 5. Marker parameters of genetic variation calculated for ISSR and SCoT primers. 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).

ISSR primers	H	PIC	EMR	H.av	MI	D	Rp
UBC_{807}	0.434	0.353	4.776	0.0004	0.002	0.899	4.746
UBC_{812}	0.497	0.324	6.044	0.0005	0.003	0.784	6.298
UBC_{817}	0.403	0.366	4.194	0.0004	0.001	0.922	5.343
$(AGC)_5GA$	0.424	0.358	4.269	0.0004	0.001	0.922	3.522
Mean	0.439	0.350	4.820	0.0004	0.002	0.878	4.978
SCot primers							
$SCoT_2$	0.370	0.293	3.672	0.0003	0.001	0.940	5.642
$SCoT_{22}$	0.420	0.273	3.000	0.0006	0.002	0.910	5.940
$SCoT_{36}$	0.337	0.305	4.298	0.0002	0.001	0.954	8.507
$SCoT_{44}$	0.336	0.305	2.776	0.0003	0.001	0.954	5.015
Mean	0.366	0.294	3.436	0.0004	0.001	0.940	6.276

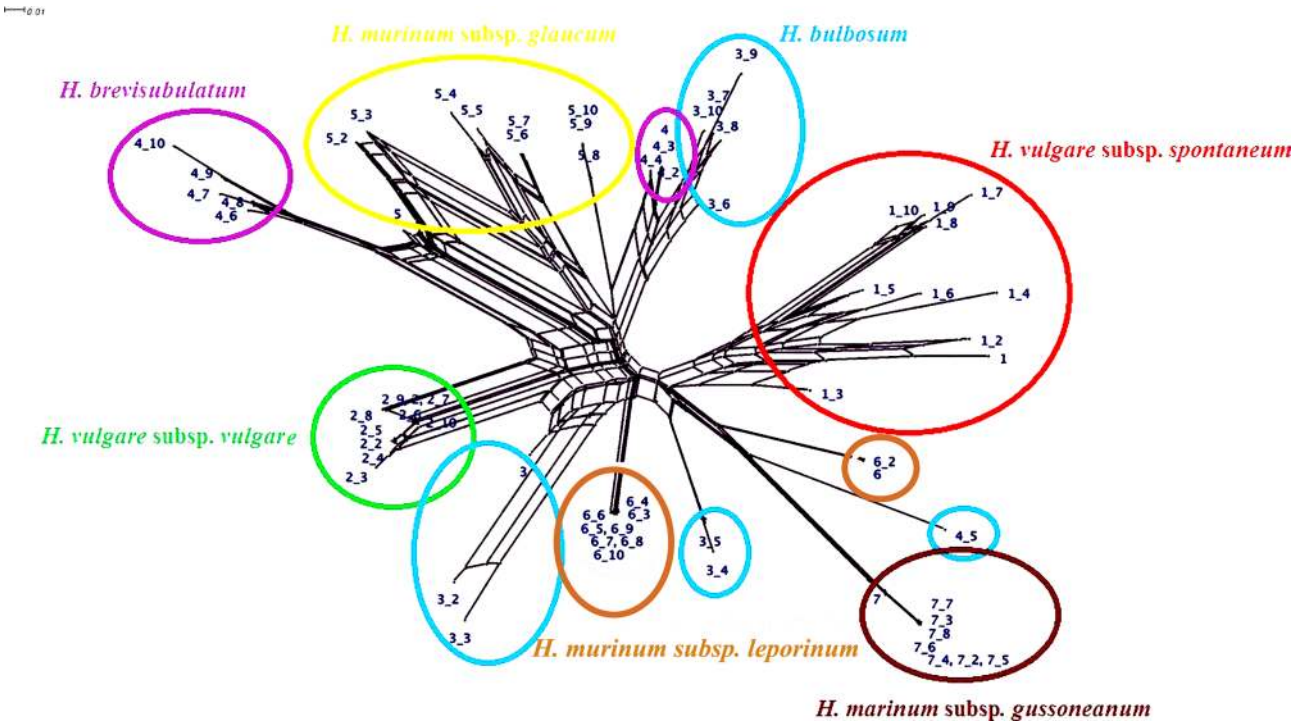


Figure 2. NeighborNet diagram illustrating the genetic relationships among the studied *Hordeum* species based on ISSR marker data. The network reveals patterns of genetic similarity and divergence among species, reflecting shared evolutionary history rather than direct gene flow.

H. brevisubulatum, exhibited strong genetic cohesion, forming distinct yet interconnected clusters within the network. In contrast, *H. vulgare* subsp. *spontaneum* appeared close to *H. murinum* subsp. *glaucum* in the TCS network (Fig. 5), suggesting a higher degree of allelic sharing or a common evolutionary origin between these lineages. These findings indicate that *Hordeum* species display substantial interspecific variability, likely shaped by successive mutational events, as evidenced by the numerous mutational steps between major haplotypes, with shared alleles contributing to the observed genetic affinities.

Combined data analysis

Combined data from ISSR and SCoT markers were utilized to conduct a more robust cluster analysis, improving the resolution and reliability of genetic relationships among the studied taxa. The NeighborNet diagram illustrated that *Hordeum* species clustered according to genetic differentiation, with each species forming a distinct group, confirming the patterns observed in individual marker analyses (Fig. 6). *Hordeum murinum* subsp. *glaucum* showed closer genetic affinity to *H. brevisubulatum*, whereas *H. murinum* subsp. *leporinum* grouped with *H. marinum* subsp. *gussoneanum*, consistent with the patterns revealed by the NeighborNet network. *Hordeum brevisubulatum* showed closer genetic affinity to *H. murinum* subsp. *glaucum*, whereas *H. bulbosum* grouped primarily with *H. vulgare*, consistent with the patterns observed in the NeighborNet network.

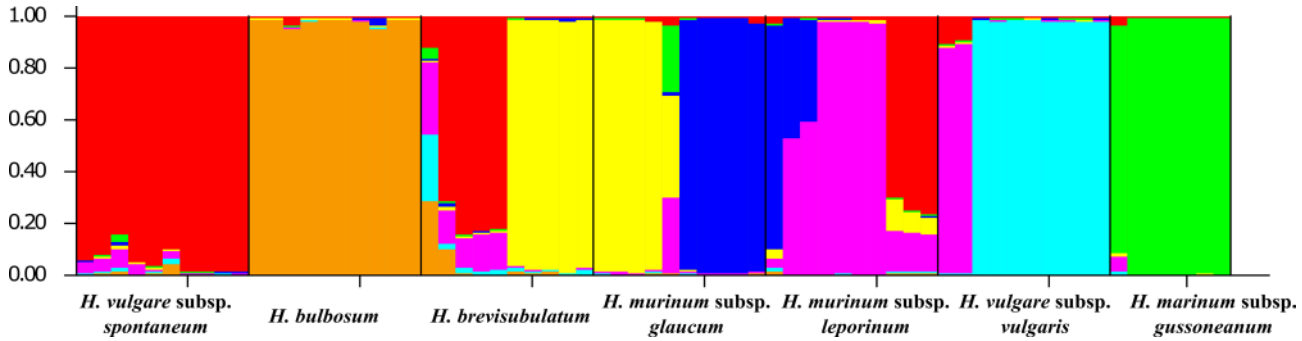


Figure 3. Structure analysis of the studied *Hordeum* species based on ISSR data, which revealed the best number of K = 7. Different colors represent distinct genetic groups, illustrating the population structure and admixture among taxa.

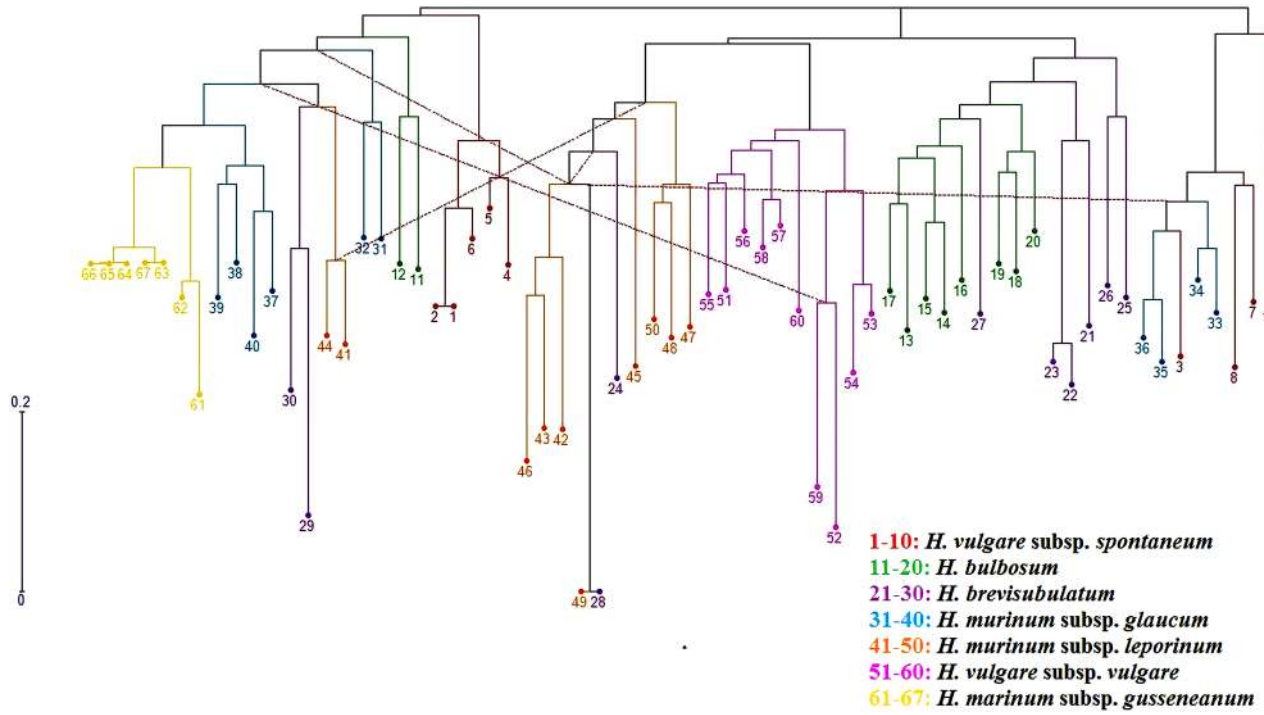


Figure 4. Reticulation dendrogram of the studied taxa based on SCoT markers, where dotted lines show gene flow among them.

The heatmap generated from combined ISSR and SCoT marker data (Fig. 7) visually represents pairwise genetic similarity among the studied *Hordeum* taxa. In this plot, rows and columns correspond to individual accessions, and the color gradient reflects genetic affinity, with warmer colors indicating closer genetic similarity and cooler colors indicating greater genetic divergence. The hierarchical clustering dendrograms flanking the heatmap provide a robust visualization of the genetic structure, grouping accessions according to their allelic profiles. The heatmap clearly illustrated

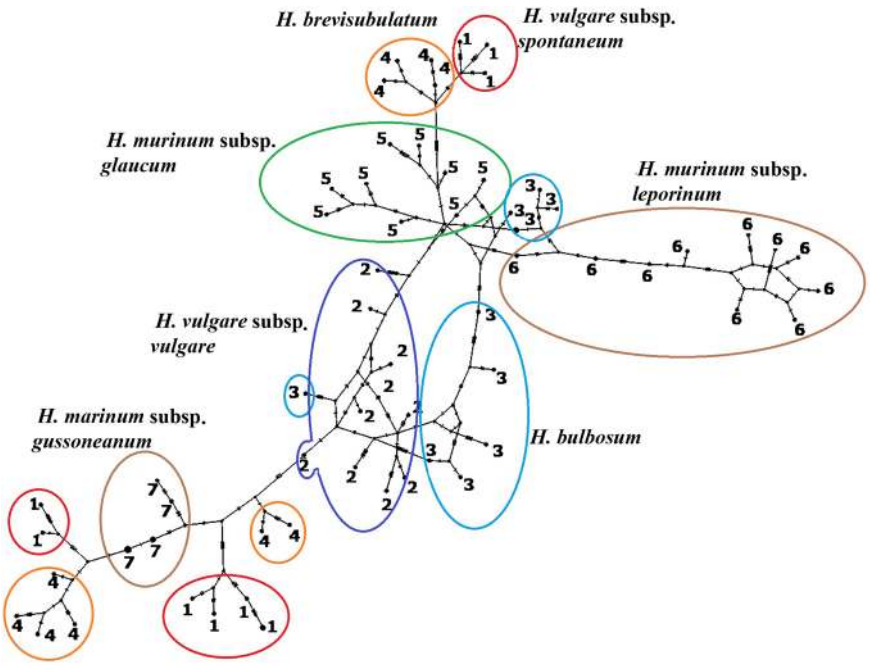


Figure 5. TCS network of the studied *Hordeum* taxa based on SCoT data (the number of mutational differences for each taxon is indicated within the network).

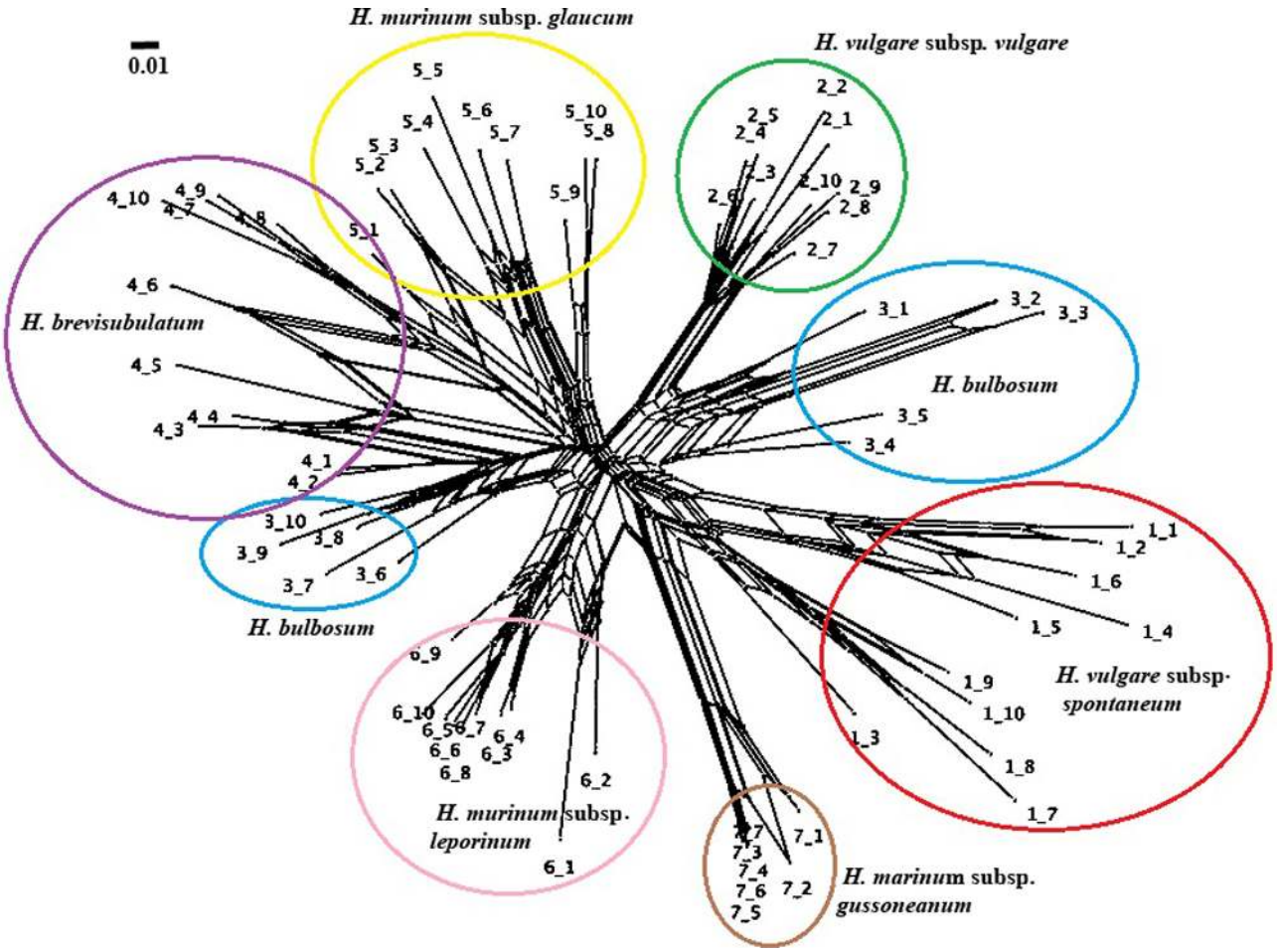


Figure 6. NeighborNet diagram illustrating the genetic relationships among the studied *Hordeum* taxa based on combined ISSR+SCoT markers data. The network reveals clustering patterns reflecting genetic similarities and divergence among species and subspecies.

the genetic separation of *H. vulgare* subsp. *vulgare* and *H. marinum* subsp. *gussoneanum* from the other species, reinforcing the distinct genetic backgrounds within the group. Specifically, the intense color blocks within certain clusters denote high levels of intra-specific homogeneity. These clustering patterns, together with NeighborNet results, emphasize consistent genetic differentiation among species while highlighting affinities between specific taxa, such as *H. murinum* subsp. *glaucum* and *H. brevisubulatum*, which are positioned in adjacent clusters due to their shared molecular fragments.

Micromorphological examination

SEM observations and leaf epidermal traits

Epidermal characteristics of leaves can provide useful information for distinguishing Poaceae taxa. SEM observations revealed notable variation in the abaxial leaf surfaces across the studied species (Fig. 8), including differences in stomatal distribution, presence of prickles, silica bodies, and macrohairs. Considerable variation was observed among taxa in stomatal length-to-breadth ratios, prickle lengths, long cell shapes, and epicuticular wax morphology, with distinctive traits particularly evident in *H. murinum* subsp. *leporinum* and *H. brevisubulatum* (Fig. 9). Macrohairs were present on the abaxial leaf surfaces of *H. murinum* subsp. *leporinum* and *H. bulbosum*. The long cells displayed either fusiform or rectangular shapes, while the wax structures primarily consisted of membranous platelets and granules. Notably, unique wax structures were observed in *H. murinum* subsp. *leporinum* (Fig. 9, 10; Table 6).

UPGMA and PCA analyses

The UPGMA dendrogram (Fig. 11) grouped the studied taxa into two main clusters. The first cluster included *H. vulgare* subsp. *spontaneum*, *H. bulbosum*, and *H. brevisubulatum*, which exhibited strong morphological affinities, while *H. vulgare* subsp. *vulgare* formed a relatively distinct branch within this cluster. The second cluster consisted of *H. murinum* subsp. *leporinum* and *H. marinum* subsp. *gussoneanum*. Notably, *H. murinum* subsp. *glaucum* was identified as the most distinct taxon, forming a separate lineage at the base of the phenogram.

To further clarify the relationships among these groups and the specific traits listed in Table 6, a PCA biplot was constructed (Fig. 12). The first two principal components explained 55.27% of the total variation. The biplot revealed

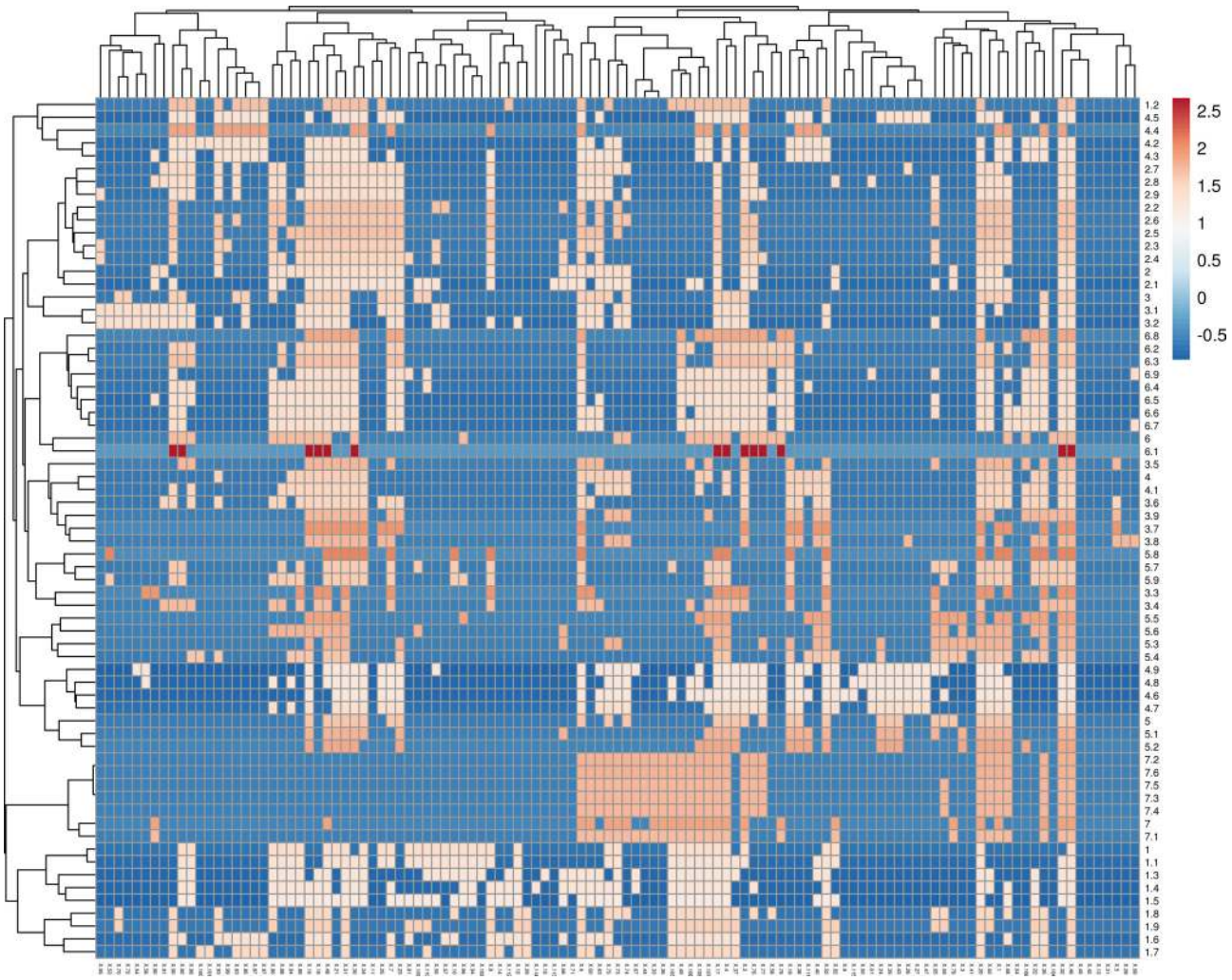


Figure 7. Heatmap clustering of the studied taxa using 115 ISSR+SCoT fragments (number indicate taxa names: 1- *Hordeum vulgare* subsp. *spontaneum,* 2- *H. vulgare* subsp. *vulgare,* 3- *H. bulbosum,* 4- *H. brevisubulatum,* 5- *H. murinum* subsp. *glaucum,* 6- *H. murinum* subsp. *leporinum,* 7- *H. marinum* subsp. *gussoneanum*). The heatmap illustrates the genetic similarity and clustering patterns among taxa based on combined molecular marker data.

that the separation of the *H. vulgare* and *H. bulbosum* group (Cluster I) was mainly driven by stomatal cell wall shape (Trait 13), cell wall shape between long cells (Trait 16), and prickle length (Trait 3), all of which showed high positive loadings on the first component. In contrast, the distinct position of *H. murinum* subsp. *glaucum* was strongly associated with macrohair density/presence (Trait 4) and the stomatal length-to-breadth ratio (Trait 7). These statistical associations confirm that the hierarchical clustering observed in the UPGMA dendrogram is grounded in specific micromorphological differences in epidermal cell patterns and trichome types.

Discussion

For future breeding and conservation, it is crucial to understand how *Hordeum* species differ and how their genetic resources are organized. These genetic resources are not only fundamental for understanding species differences but also serve as the basis for developing improved cultivars with desirable traits such as stress tolerance and disease resistance, and for prioritizing conservation strategies that preserve rare alleles and diverse gene pools (Salgotra and Chauhan 2023). By examining genetic structure, divergence, and gene pools across populations and species (Tabaripour and Keshavarzi 2021), we can gain valuable insights that help manage germplasm collections and guide effective crop improvement strategies. To enhance the genetic base of modern cultivars, plant breeders utilize the genetic diversity present in wild progenitors of cultivated cereals, particularly those found near centers of diversity, to introgress beneficial traits into crops (Khodayari et al. 2014). Iran, located in southwestern Asia, boasts a wide variety of ecological conditions. Therefore, a comprehensive assessment of genetic diversity in Iranian *Hordeum* germplasm is of critical importance, as it provides essential insights into the structure and extent of

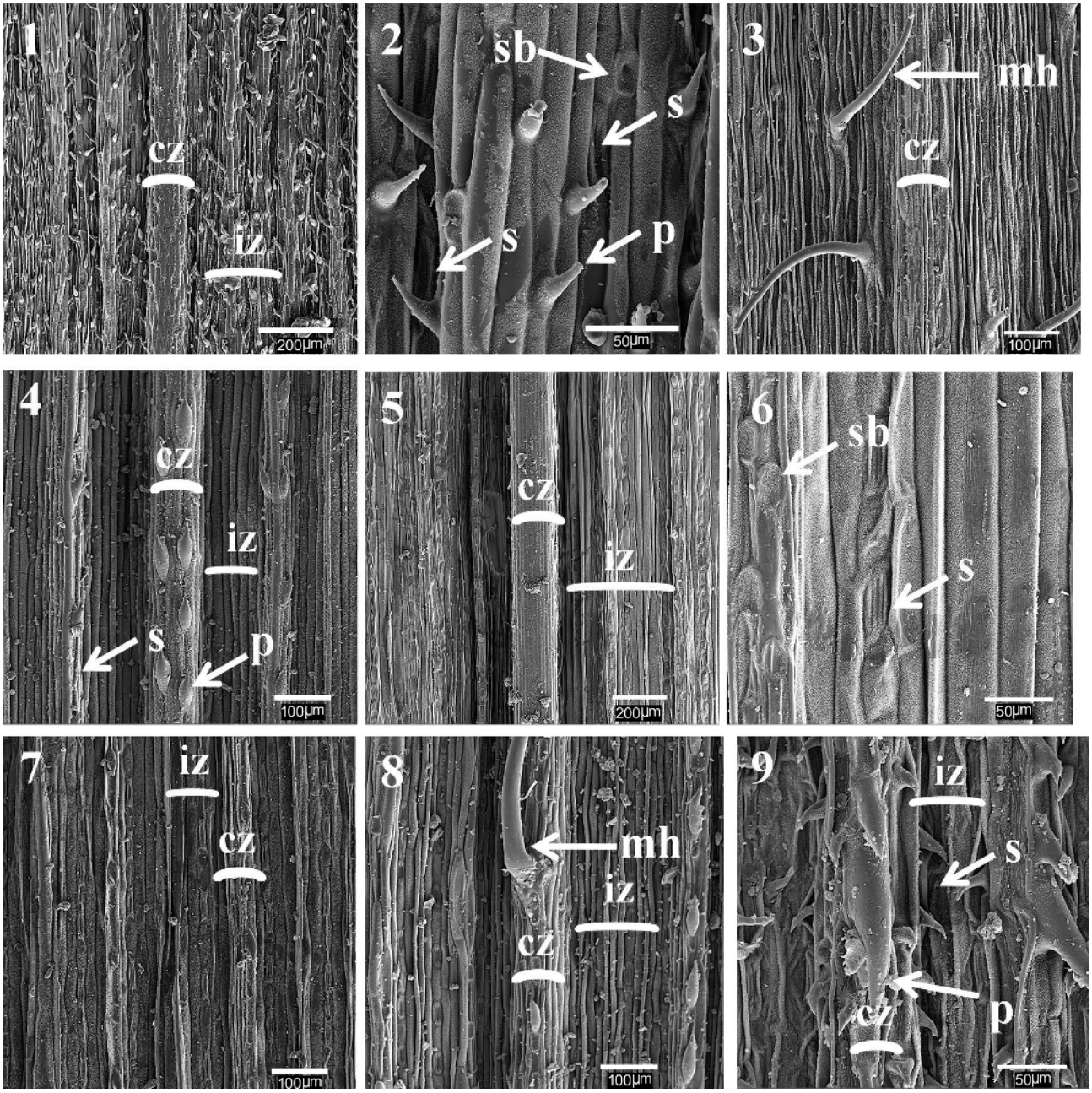


Figure 8. SEM observations of the abaxial leaf surfaces. 1 and 2 - *Hordeum marinum* subsp. *gussoneanum;* 3 - *H. murinum* subsp. *leporinum;* 4 - *H. murinum* subsp. *glaucum;* 5 and 6 - *H. vulgare* subsp. *vulgare;* 7 - *H. vulgare* subsp. *spontaneum;* 8 - *H. bulbosum;* 9 - *H. brevisubulatum*. cz, costal zone; iz, intercostal zone; s, stoma; p, prickle; sb, silica body; mh, macro hair.

genetic variation, which can directly inform both conservation efforts and targeted breeding programs.

We used both ISSR and SCoT markers in this study to identify genetic diversity and species boundaries in *Hordeum*. While SCoT markers have shown greater discriminatory power in certain cases, ISSR markers provide valuable complementary information by targeting different regions of the genome. ISSR markers target inter-simple sequence repeat regions, which are highly polymorphic and thus particularly informative for detecting genetic variation. By combining ISSR and SCoT markers, we obtained a comprehensive overview of genetic relationships, reducing redundancy in results and highlighting key interspecific patterns. For example, in a study by Safari et al. (2019), 90 accessions from 18 *Bromus* species were analyzed using both marker types. The results showed that SCoT markers offered superior discriminatory power, and their combined use significantly enhanced the resolution of taxonomic relationships by clearly distinguishing interspecific genetic differences.

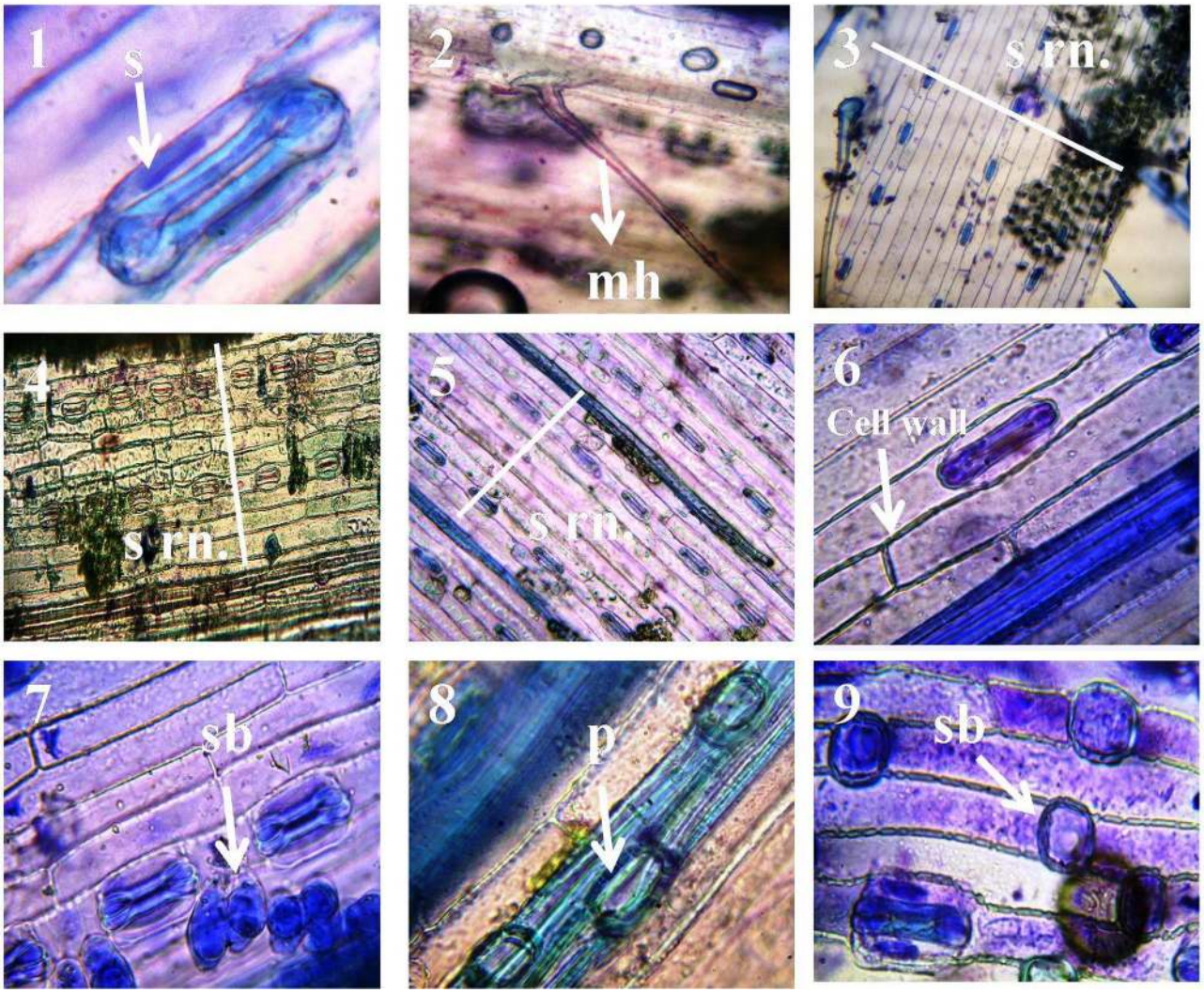


Figure 9. Abaxial leaf surfaces of *Hordeum* taxa. 1 - *H. marinum* subsp. *gussoneanum;* 2 - *H. murinum* subsp. *leporinum;* 3 - *H. murinum* subsp. *glaucum;* 4 - *H. vulgare* subsp. *vulgare;* 5 and 6 - *H. vulgare* subsp. *spontaneum;* 7 and 8 - *H. bulbosum;* 9 - *H. brevisubulatum*. s: stoma, p: prickle, sb: silica body, mh: macro hair, s rn.: stomata row number.

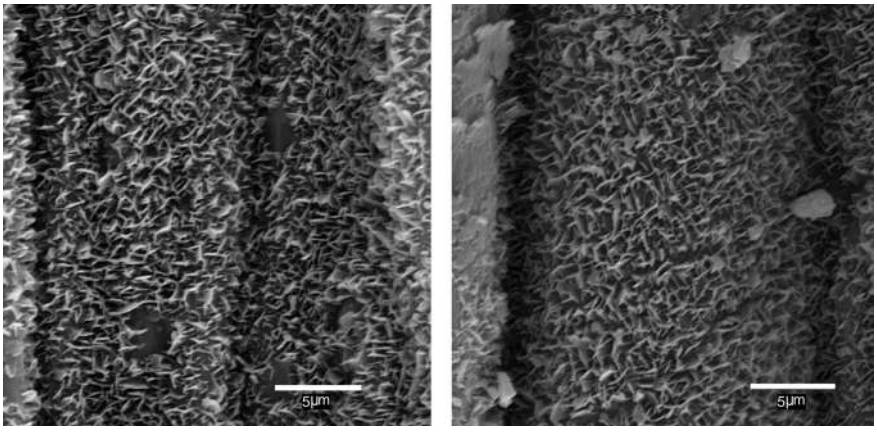


Figure 10. Micrographs of different epicuticular wax types in *Hordeum* species studied. (a) Membranous platelets in *H. murinum* subsp. *leporinum;* (b) membranous platelets and granules in *H. bulbosum*.

Table 6. The abaxial foliar epidermal features of the *Hordeum* taxa studied.

Abaxial epidermal characters	*H. marinum* subsp. *gussoneanum*	*H. murinum* subsp. *leporinum*	*H. murinum* subsp. *glaucum*	*H. vulgare* subsp. *vulgare*	*H. vulgare* subsp. *spontaneum*	*H. bulbosum*	*H. brevisubulatum*
Qualitative characters							
Prickle in the costal zone	absent	present	present	absent	present	present	present
Prickle in the intercostal zone	present	absent	absent	absent	absent	absent	present
Macro hair	absent	present	absent	absent	absent	present	absent
Silica bodies in the costal zone	present	absent	absent	absent	present	present	absent
Silica bodies in the intercostal zone	absent	present	absent	present	present	absent	absent
Wax shape	membranous platelets and granules	membranous platelets	membranous platelets and granules	membranous platelets and granules	membranous platelets and granules	membranous platelets and granules	membranous platelets and granules
Short cell in intercostal zone	present	present	present	absent	absent	absent	absent
Stomata cell wall shape	parallel	parallel	parallel	low domed	low domed	low domed	quadrate
Stomata row number	2	2	4	2	2	2	2
Anticlinal wall pattern of long cells	diagonal	diagonal	straight-diagonal	straight-diagonal	straight	straight	straight
Long cell shape	fusiform	rectangular	fusiform	fusiform	rectangular	rectangular	rectangular
Quantitative characters							
Prickle length (μm)	42.61 ± 6.82	62.94 ± 22.32	74.72 ± 11.00	-	51.09 ± 12.38	74.11 ± 19.54	83.74 ± 33.84
Stomata length (μm)	51.24 ± 7.86	53.67 ± 8.13	52.07 ± 4.59	46.86 ± 6.24	45.25 ± 3.12	42.66 ± 3.87	54.53 ± 9.21
Stomata breadth (μm)	9.62 ± 1.93	12.99 ± 2.60	13.49 ± 3.08	9.85 ± 2.14	12.34 ± 2.37	16.76 ± 3.21	13.16 ± 3.21
Length/breadth ratio	5.33	4.13	3.86	4.76	3.67	2.54	4.14
Interstomatal length (μm)	54.46 ± 34.24	67.31 ± 15.10	127.07 ± 40.96	57.52 ± 10.89	67.32 ± 15.22	69.70 ± 18.21	65.46 ± 19.04

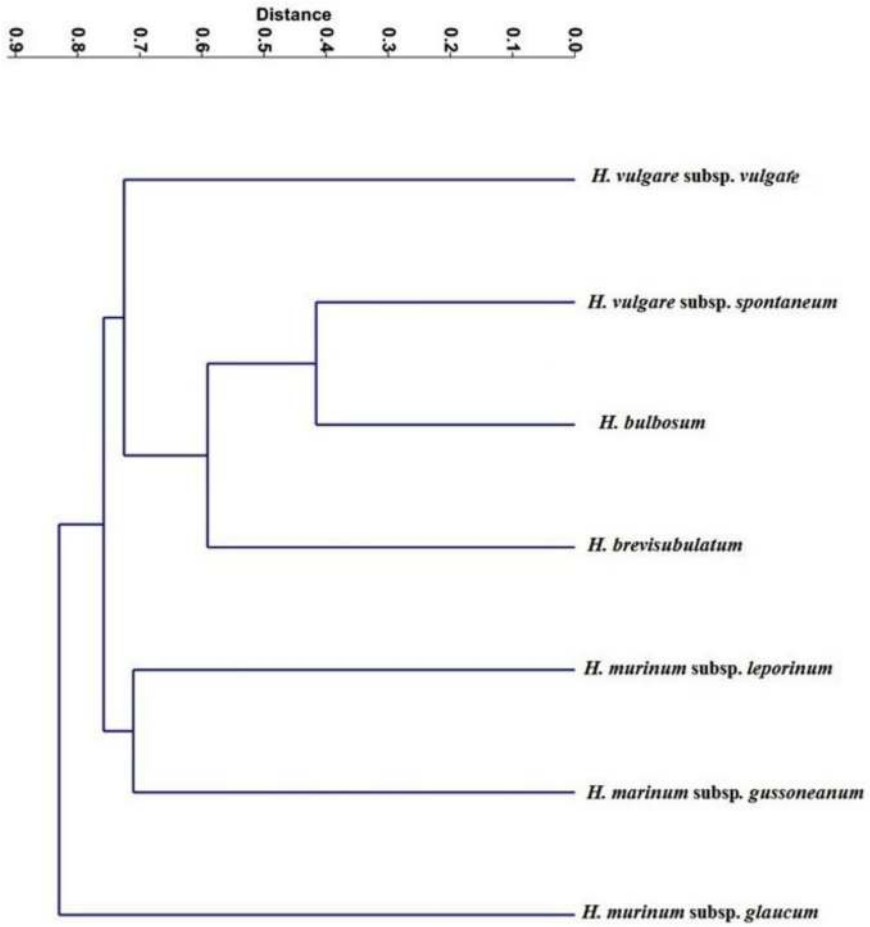


Figure 11. UPGMA dendrogram based on leaf micromorphology data showing the relationships within the genus *Hordeum*.

Although dominant markers such as ISSR and SCoT have provided valuable insights into the genetic diversity and structure of the studied *Hordeum* populations, our findings complement broader phylogenetic knowledge. We observed clear differentiation patterns among populations, highlighting important genetic variation within and between taxa. Previous studies using multilocus nuclear markers and next-generation sequencing (NGS) have achieved high resolution in resolving species relationships and polyploid origins within *Hordeum* (Brassac and Blattner 2015). While dominant markers provide less phylogenetic depth, they are particularly useful for practical applications in germplasm management and breeding.

Dominant markers are effective for revealing major genetic structures within species; however, observed interspecific relationships in our study differ from established phylogenies (Brassac and Blattner 2015), highlighting the need for caution when interpreting between-species patterns. Furthermore, our results provide insights into the genetic relationships among Iranian *Hordeum* accessions. The observed patterns reflect not only the variation within our dataset but also the broader subgenus classifications: both *H. vulgare* subspecies and *H. spontaneum* belong to subgenus *Hordeum*, whereas *H. marinum* subsp. *gussoneanum* and *H. brevisubulatum* are classified under subgenus *Hordeastrum*.

Hordeum brevisubulatum and *H. murinum* subsp. *glaucum* showed close genetic relationships, while cultivated and wild barley remained distinctly separated, consistent with previous studies (Petersen and Seberg 2003, Khodayari et al. 2014). Similar assessments in *Aegilops*, *Triticum*, and *Lolium* using ISSR and SCoT markers support these findings (Pour-Aboughadareh et al. 2018, Khodaee et al. 2021, Tabaripour and Keshavarzi 2021). Our STRUCTURE analysis also revealed genetic admixture among *H. brevisubulatum*, *H. murinum* subsp. *glaucum*, and *H. murinum* subsp. *leporinum*, reflecting historical gene flow and shared ancestral polymorphisms (Aghajani et al. 2025).

Our study highlights several *Hordeum* taxa that should be considered priority genetic resources for both breeding and conservation. In particular, wild populations of *H. vulgare* subsp. *spontaneum* and *H. brevisubulatum*, which show high genetic diversity and unique adaptive traits, represent valuable sources for introducing stress tolerance and disease resistance into cultivated barley. Moreover, taxa with restricted distributions or distinctive micromorphological features,

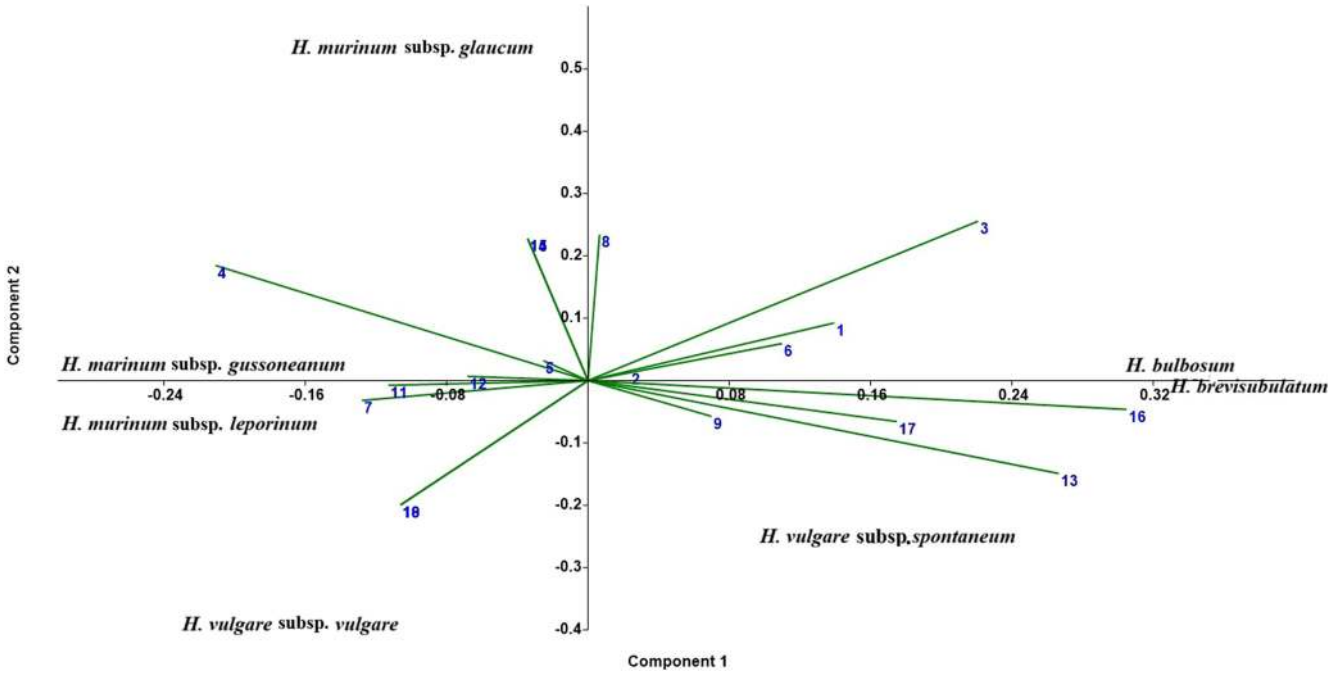


Figure 12. PCA biplot of the studied *Hordeum* taxa based on leaf micromorphological traits. The vectors (numbered 1–18) represent the characters listed in Table 6, and the points represent the studied *Hordeum* taxa.

such as the *H. murinum* subspecies, serve as important reservoirs of rare alleles and functional traits that could be crucial for future crop improvement initiatives.

The NeighborNet diagram based on ISSR and SCoT markers, together with the UPGMA dendrogram constructed from leaf micromorphological traits, consistently grouped all accessions of *H. murinum*, *H. bulbosum*, and *H. marinum* into closely related clusters. This agreement between molecular and micromorphological datasets emphasizes the main genetic patterns while avoiding redundant details about *H. vulgare* subsp. *spontaneum* and *H. bulbosum*. Previous molecular studies – including analyses of repetitive DNA sequences, RFLP, and SSR markers – support a shared ancestry between *H. bulbosum* and *H. murinum*, whereas the clustering of *H. marinum* likely reflects ancestral variation or homoplastic signals rather than direct gene flow (Sonina et al. 1989, Molnar et al. 1992, Khodayari et al. 2012, Brassac and Blattner 2015).

Expected heterozygosity (He) and Shannon's information index (I) were higher in *H. vulgare* subsp. *spontaneum* and *H. vulgare* subsp. *vulgare*, consistent with previous reports (Naghavi et al. 2011, Amiryousefi et al. 2018). Rather than repeating all PIC and marker index values, the key patterns can be summarized as follows: wild barley exhibits the highest genetic diversity, *H. bulbosum* shows lower diversity, and overall variation reflects both germplasm richness and the species' evolutionary history.

UPGMA analysis and PCA biplot confirmed that the observed clusters were largely influenced by specific leaf micromorphological traits, including stomatal cell wall shape, long cell wall shape, prickle length, and macrohair density. These similarities among taxa that are not closely related phylogenetically are likely the result of convergent adaptations, highlighting the importance of functional traits rather than strict phylogenetic relationships.

The high similarity observed between *H. murinum* subspecies further confirms their close taxonomic relationship, characterized by shared micro-structural traits such as silica body patterns, which consistently positioned them together in both the UPGMA dendrogram and PCA analysis. These results highlight that leaf micromorphological traits provide valuable taxonomic information for specific groups, such as the *murinum* subspecies, while additional molecular evidence may be necessary to resolve relationships in other groups. Notably, the distinctiveness of *H. murinum* subsp. *glaucum* was primarily driven by its unique macrohair density (Trait 4), a trait that clearly distinguishes it from all other studied taxa.

Support for these relationships is reinforced by the reproductive systems of the taxa: *H. bulbosum*, the sister species of *H. vulgare*, is self-incompatible, contrasting with the predominantly self-compatible *H. vulgare*, which likely influenced the evolution of reproductive traits in the genus. Moreover, recent molecular clock analyses estimate that these two species diverged from a common ancestor around 4.5 million years ago (Brassac and Blattner 2015), providing a temporal framework for understanding their evolutionary divergence.

As demonstrated by Mavi et al. (2011), the anatomical patterns of perennial species such as *H. bulbosum* and *H. vulgare* (including both cultivated and wild subspecies) are closely related, supporting their strong affinities. While Mavi et al. (2011) focused primarily on general anatomical traits like mesophyll structure, vascular bundle arrangement, and stomatal types, our study places a strong emphasis on leaf

micromorphology at the epidermal level, specifically identifying anticlinal wall patterns and trichome types as critical diagnostic features for species delimitation.

The taxonomic value of leaf micromorphological traits is clearly evident in our findings. For example, the lemma and palea surfaces exhibit diverse epicuticular wax structures with varying densities, reflecting adaptive responses to different environmental conditions (Mosaferi and Keshavarzi 2021, Tabaripour et al. 2022). By integrating these fine-scale epidermal traits, our study provides a more refined and practical approach to distinguish closely related *Hordeum* species, demonstrating the significant contribution of micromorphology to taxonomic research.

Our study reveals a clear genetic distinction between the cultivated barley (*H. vulgare* subsp. *vulgare*) and its wild progenitor (*H. vulgare* subsp. *spontaneum*), in agreement with previous molecular analyses of domesticated grasses. This differentiation underscores the impact of domestication on the genetic makeup of barley and highlights the importance of preserving wild germplasm for breeding and conservation efforts. In many cases, multilocus markers have confirmed that wild and cultivated gene pools are clearly separated (Lin et al. 2011, Tiruneh et al. 2024). Further evidence is provided by Brassac and Blattner (2015), who demonstrated that diploid and polyploid *Hordeum* taxa can be resolved in a single analysis and, importantly, that the wild progenitor *H. spontaneum* and cultivated *H. vulgare* form clearly distinct gene pools, while relationships among more distantly related species require careful interpretation. Furthermore, the micromorphological analysis of leaf traits revealed significant differences between the two subspecies, indicating that domestication has influenced both structural and molecular characteristics. This is consistent with findings by Jakob and Blattner (2006), who analyzed chloroplast haplotypes in *Hordeum* and discussed long-term persisting haplotypes, incomplete lineage sorting (ILS), and regional extinction, highlighting the challenges for phylogenetic inference between wild and cultivated barley subspecies.

Comparisons with ecologically and floristically similar regions, including Turkey, Iraq, Armenia, Azerbaijan, and Central Asia, show that the genetic diversity patterns of Iranian *Hordeum* species largely align with those reported from neighboring parts of the Fertile Crescent. Studies from Turkey and the Caucasus have revealed high levels of genetic diversity and strong population structuring in wild barley (*H. vulgare* subsp. *spontaneum*), reflecting historical isolation, local adaptation, and limited gene flow across heterogeneous landscapes (Jakob et al. 2004, Özkan et al. 2011). Iran's central location within the Fertile Crescent underscores its role as both a historical corridor for gene flow and a potential center of diversity for *Hordeum*. Such regional comparisons are crucial for understanding species distribution limits and historical isolation patterns, and for designing coordinated breeding and conservation strategies at a broader geographic scale.

Our study using ISSR and SCoT markers provides valuable insights into the genetic diversity and structure of Iranian *Hordeum*. Overall, combining molecular and micromorphological data offers valuable and practical insights for both targeted breeding and the conservation of *Hordeum* species, ensuring that their genetic resources are efficiently utilized and preserved for future generations (Salgotra and Chauhan 2023). Our study addresses critical gaps in the knowledge of Iranian *Hordeum* species by combining ISSR and SCoT molecular markers with detailed leaf micromorphology. This integrated approach provides novel insights into population structure, interspecific relationships, and diagnostic leaf traits that have not been reported previously. Together, these findings enhance our understanding of species variation and offer a solid foundation for future research, breeding programs, and conservation strategies.

Research on *Hordeum* taxa has largely focused on their biological characteristics, salt tolerance mechanisms, and disease resistance. However, constraints such as small grain size in wild species (except *H. vulgare* and *H. bulbosum*) and limited genetic data hinder effective breeding. Addressing these gaps through further research is critical to fully harness *Hordeum* germplasm and accelerate breeding programs.

Conclusion

In the genus *Hordeum*, polyploidization plays a crucial role in speciation. In this study, ISSR and SCoT markers, together with detailed leaf micromorphology, were used to explore genetic diversity, species boundaries, and variation among Iranian *Hordeum* species. While molecular markers provided useful insights into genetic structure, the most valuable contribution of this study lies in the micromorphological analysis of leaf traits, which effectively distinguished taxa and revealed patterns of variation across populations. Given that most *Hordeum* species are available in diverse genebanks worldwide, these analyses could be readily extended to all species, enabling standardized growth and comparative studies. The knowledge gained from this study may aid in understanding and managing the collection and conservation of *Hordeum* germplasm. Our results indicate the levels of genetic diversity within the studied wild barley species, highlighting their potential importance for understanding barley evolution and for future crop improvement efforts. This research fills key gaps in the assessment of genetic diversity and species delimitation in Iranian *Hordeum*, offering new perspectives that can directly support breeding, conservation, and future studies on *Hordeum* germplasm. Overall, integrating molecular and micromorphological data provides actionable insights for both targeted breeding and the conservation of *Hordeum* species, ensuring that genetic resources are effectively utilized and preserved for future generations.

Acknowledgements – The authors gratefully acknowledge the Vice-Chancellor of Alzahra University, Tehran, Iran, for financial support and for providing the necessary facilities to carry out this research.
Funding – The authors received no specific funding for this work.
Conflict of interest – The authors declare no conflict of interest.

Author contributions

Maryam Keshavarzi: Conceptualization (supporting); Investigation (equal); Project administration (supporting); Validation (equal); Writing – review and editing (equal). **Raheleh Tabaripour**: Conceptualization (equal); Formal analysis (lead); Investigation (supporting); Methodology (equal); Software (lead); Writing – original draft (lead); Writing – review and editing (equal). **Nasrin Farasat**: Data curation (equal); Resources (equal).

Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.866t1g25v (Keshavarzi et al. 2026).

Supporting information

The Supporting information associated with this article is available with the online version.

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