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Genetic Diversity, Population Structure and Agronomic Performance of Modern Bulgarian Barley Based on 50K SNP Array

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Abstract

The systematic characterization of genetic architecture within elite barley (*Hordeum vulgare* L.) germplasm is essential for optimizing breeding strategies. In this study, an Illumina 50k iSelect SNP array was utilized to the assess genetic diversity, population structure, and selection signatures in 92 modern Bulgarian barley genotypes. Filtered datasets including 14,477 and 8674 high-quality SNPs were used for downstream analyses. The studied genotype set showed relatively low average genetic diversity (0.169) and polymorphic information content (0.144), though the genetic diversity value was higher in the six-row barley pool. The elevated observed heterozygosity (Ho = 0.0803) indicated a substantial reservoir of valuable allelic variation. Model-based population structure inference, PCA, and UPGMA analyses revealed two major sub-populations differentiated by ear-row type, with substructures and genotypes clustering according to breeding programs tailored to local agro-climatic conditions. Genome-wide scans identified directional selection signatures on chromosomes 2H and 4H, corresponding to loci for grain number, plant height, and ear-row type, with a prominent cluster on 4H. A biplot of phenotype data for ten agronomic traits across three seasons clearly discriminated between two-row and six-row genotypes in good congruence with the population structure inferred from the SNP data. This molecular and phenotypic framework establishes a robust foundation for transitioning toward predictive, genotypic data-driven crop improvement in Bulgarian barley breeding programs.

Keywords: Barley (*Hordeum vulgare* L.); SNP markers; genetic diversity; population structure; agronomic performance; selection signature

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1. Introduction

Barley (*Hordeum vulgare* L.) is among the earliest domesticated cereal crops, with archeological evidence tracing its cultivation back approximately 10,000 years to the Fertile Crescent [1]. Due to its exceptional ecological plasticity and broad adaptive capacity, barley remains one of the four most important cereal crops globally, with annual production ranging between 145 and 159 million tones [2,3].

Traditionally utilized as animal feed and as a key raw material in the malting and brewing industries, barley has recently gained increasing recognition as a functional food crop [4]. Compared with other major cereals such as wheat, rice, and maize, barley grain is particularly rich in dietary fiber, especially β-glucans (3.7–7.7%), and phenolic compounds (1.2–2.9 mg/g GAE), which contribute significantly to its health-promoting properties [4,5]. These bioactive components have been associated with reduced risks of metabolic syndrome, hypertension, and cardiovascular diseases, thereby enhancing barley's relevance in human nutrition [4]. Moreover, its favorable biochemical composition comprising approximately 80% carbohydrates, 11.5–14.2% proteins, and 4.7–6.8% lipids makes barley a valuable raw material for industrial applications [5].

At the global scale, the leading barley producers and exporters include the Russian Federation, Australia, France, Germany, and Canada. In Bulgaria, barley is predominantly cultivated in the southeastern and northeastern agro-ecological zones, where climatic and edaphic conditions ranging from temperate continental to Mediterranean-influenced regimes are particularly favorable. Barley ranks third among cereal crops in Bulgaria after wheat and maize in terms of both its production area and total output. The average annual production is approximately 1 million tones, reaching up to 1.1 million tons in 2024, reflecting recent recovery trends in national cereal production (https://www.mzh.government.bg/media/filer_public/2025/09/16/ra460_publicationcrops2024_en.pdf; accessed on 29 July 2026).

However, the adverse abiotic factors dictated by global climate change pose a critical challenge to the sustainable intensification of crop production on a global and national scale. Crop genetic improvement fundamentally depends on the availability and exploitation of genetic variation within germplasm collections [6]. A broad genetic diversity is essential for the development of modern cultivars with high yield potential, improved grain quality, and enhanced resilience to abiotic and biotic stresses. Such diversity reduces crop vulnerability to drought, salinity, heat stress, and pathogen pressures, thereby contributing to sustainable agricultural systems and global food security [7,8].

Since its domestication from wild barley (*Hordeum spontaneum* (C. Koch) Thell.), cultivated barley has undergone continuous selection and breeding, resulting in modern high-yielding cultivars. However, despite the significant genetic gains achieved through conventional breeding and modern biotechnological approaches [9], historical bottlenecks, genetic drift, and inbreeding have substantially reduced the genetic diversity of cultivated barley relative to its wild relatives [10,11]. Consequently, many adaptive alleles present in landraces and wild germplasm have been lost, reducing the adaptive potential of modern cultivars under changing environmental conditions [12].

To address these limitations, contemporary barley breeding programs increasingly focus on identifying and incorporating novel sources of genetic variation to broaden the genetic base of elite germplasm [13–16]. Enhancing genetic diversity is critical for improving yield stability, grain quality, and stress tolerance under increasing climatic variability [8,17,18].

Barley cultivation in Bulgaria is strongly influenced by regional agro-ecological conditions, particularly temperature regimes, precipitation patterns, and soil types. The crop is mainly concentrated in Southeastern and Northeastern Bulgaria, which represent the primary production zones due to their favorable environmental conditions [19–22]. Southeastern Bulgaria is characterized by a transitional continental to Mediterranean climate, with mild winters and frequent summer droughts. These conditions are particularly suitable for winter barley, which efficiently utilizes soil moisture accumulated during the autumn–winter period and avoids severe summer heat stress [23,24]. In contrast, Northeastern Bulgaria is characterized by a temperate continental climate and highly

fertile chernozem soils. This region represents the most stable and productive barley-growing area in the country, contributing substantially to national grain production and exhibiting a lower interannual yield variability [25]. Barley is also cultivated in smaller proportions in Central and Western Bulgaria, including the Plovdiv and Pleven regions, where it is integrated into crop rotation systems but often competes with wheat and maize for arable land [26].

The spatial distribution of barley cultivation reflects both its ecological plasticity and its sensitivity to environmental stressors, particularly drought and temperature extremes. Winter barley predominates due to its higher yield potential and efficient use of winter–spring moisture, whereas spring barley is cultivated on limited areas, mainly for malting purposes and under specific agronomic conditions [27–31].

Barley breeding in Bulgaria has been a long-established tradition and plays a key role in their national cereal improvement programs. Systematic breeding began in the early 20th century at institutions such as the Institute of Plant Genetic Resources (Sadovo) and the Institute of Agriculture (Karnobat), and were later complemented by the Dobrudzha Agricultural Institute (General Toshevo). Early breeding efforts focused on improving local populations through the individual selection of six-row and four-row barley types.

Systematic winter barley breeding at the Institute of Agriculture—Karnobat commenced in 1961, resulting in the release of 13 cultivars by 1990, including the widely adopted cultivar Obzor [25,32]. Subsequent breeding efforts have led to the development of both feed and malting cultivars such as Zherun, Veslets, Panagon, Aheloy 2, Korten, Aster, Perun, and Emon [32]. After 2000, breeding priorities have shifted towards the development of drought-tolerant and climate-resilient cultivars. Cultivars such as Orfey, Lardeya, Zagorets, Imeon, Asparuh, Kuber, and Sayra were developed, which are characterized by their high yield potential, ecological plasticity, and improved stress tolerance [32].

Modern Bulgarian barley breeding integrates classical and advanced approaches, including hybridization, selection, mutagenesis, and doubled haploid (DH) technology, enabling the development of cultivars with improved yield stability, lodging resistance, winter hardiness, and disease resistance [33–36].

Despite these advances, the molecular characterization of Bulgarian barley germplasm remains limited and has predominantly relied on a small number of genotypes analyzed using marker systems such as Restriction Fragment Length Polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and Simple Sequence repeats (SSRs) [37–39]. This highlights the need for more comprehensive genomic investigations.

The evaluation of barley genetic resources is essential for identifying novel sources of genetic variation for breeding applications. Various approaches have been employed, including morpho-physiological, biochemical, and molecular analyses [6], among which the high-throughput genotyping is the most prominent. The release of the draft barley (*H. vulgare*) genome (International Barley Genome Sequencing Consortium (IBSC) [40,41] and the sequential release of the high-quality cv. Morex v1, v2, and v3 assemblies [42–44] has allowed the development of widely accepted and accessible single-nucleotide polymorphism (SNP) arrays [45–47], that, together with the SNPs based on Genotyping-By-Sequencing (GBS) and RNA-sequencing analysis, have been widely used for the assessment of genetic diversity, population structure, evolutionary origin as well as for association studies, linkage map's construction, genomic selection (GS) and breeding applications in barley [48–58].

In this context, the aim of the present study is to evaluate the genetic diversity and

population structure of 92 modern barley cultivars and advanced breeding lines developed at the Institute of Agriculture—Karnobat, Bulgaria over the past three decades. Utilizing a high-density SNP genotyping platform, this research seeks to identify valuable allelic variations for the development of future high-yielding, climate-resilient barley cultivars, while also assessing the suitability of this germplasm for subsequent quantitative trait analyses.

2. Materials and Methods

2.1. Plant Material

The study used a set of 92 elite winter barley genotypes from the collection in the Institute of Agriculture (IA)—Karnobat, Bulgaria (Table S1). The panel consists of 8 cultivars and two advanced breeding lines of foreign origin (Turkey, Serbia, Germany, Denmark/Sweden and France), a half of which were two-rowed and another half of which were half-rowed. The remaining 82 genotypes were of Bulgarian origin. The Bulgarian panel includes sixty-eight two-rowed and fourteen six-rowed genotypes, among which 26 were cultivars registered in the last 40 years. Twenty-five of Bulgarian cultivars were created at the Institute of Agriculture—Karnobat and 1 was selected at the DAI—General Toshevo (cv. Kaskadyor 3). Three of these cultivars (cvs. Obsor, Emon and Kaskadyor 3) are old and have been used as the national standards for the selection of two-rowed barley while cv. Veslets was used as a national standard for six-rowed barley. The group of advanced Bulgarian lines (56) consists of 50 two-rowed and 6 six-rowed genotypes developed by the IA-Karnobat in the last 20 years.

2.2. Phenotypic Evaluation

Field experiments were conducted in three crop seasons (2021–2023) in the fields of the Institute of Agriculture—Karnobat, Southeastern Bulgaria, a region typical for barley breeding and production. Sowing was carried out by the end of October each year. Field trials were organized in plots of 10 m^2 each, using a completely randomized design with four replications following the Methodology of Executive Agency of Variety Testing, Field Inspection and Seed Control (IASAS/EAVTFISC) in Bulgaria. Each genotype was sown with 450 germinated seeds per m^2. A single nitrogen (N) fertilization with a concentration of 100 kg/ha active substance nitrogen was applied in February each year and 92 kg/ha active substance P_2O_5 was applied before barley sowing.

Weather conditions varied considerably across the three growing seasons (Table S2). The mean air temperatures were higher than the long-term average (8.4 °C) in all three seasons, ranging from 9.2 °C in 2021/22 to 10.3 °C in 2022/23. The total precipitation was highest in 2020/21 (622.0 mm), exceeding the long-term average (424.6 mm) by 46.5%. In contrast, precipitation in 2021/22 (447.8 mm) and 2022/23 (423.3 mm) were close to the long-term average. Differences in the seasonal distribution of precipitation were also observed, particularly during the winter and spring months.

At full maturity, 10 plants from each replication were selected for biometric measurements. The following characteristics were evaluated and measured during each growing season according to EAVTFISC and CPVO Methodology: plant height (PH), (cm); number of productive tilers (NPT); productive spikes per m^2 (NSS); spike length (SL), (cm); number of kernels per spike (KS); number of sterile spikelets (SS); kernels weight per spike (WKS), (g); weight of kernels per plant (WKP), (g); thousand-grain weight (TKW), (g); and grain yield (GY).

Frequency distribution histograms were constructed to visualize the distribution of trait values among the 92 barley genotypes, and normality was assessed using the Shapiro–Wilk test [59]. The descriptive statistics and frequency distributions are pre-

sented in Tables S3 and S4. We used Principal Component Analysis (PCA) to examine how the genotypes from the populations defined by the Discriminant Analysis of Principal Components (DAPC) method are clustered according to their performance in the ten quantitative phenotypic traits described above. The PCA was computed from the mean values of ten quantitative phenotype traits (NPT, PH, NSS, SL, KS, SS, WKS, WKP, TKW and GY) across the three experimental seasons by using the package *factoextra* v2.2.0 [60] and visualized by the package *ggplot2* v4.0.4 [61] in R.4.5.2 [62].

2.3. SNP Genotyping, Data Quality Control (QC) and Filtering

A DNA extraction and SNP genotyping of the panel of 92 barley genotypes was performed by SGS Institut Fresenius GmbH TraitGenetics Section (Gatersleben, Germany). The genomic DNA was extracted from 100 mg of fresh leaves from a bulk of 10-day-old seedlings using DNeasy™ Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The genotyping was performed by the SGS Institut Fresenius, Trait Genetics GmbH (Gatersleben, Germany) using the barley Illumina 50k iSelect SNP array (Illumina, San Diego, CA 92122, USA) [47]. The array contained 49,267 SNP markers, of which 44,040 were scorable in GenomeStudio (Illumina, San Diego, CA 92122, USA). Among them, 6251 were from the 9K iSelect platform. The SNP positions were obtained from barley cv. Morex v3 physical map [47].

The initial filter for selecting only bi-allelic SNPs was by the removal of monomorphic SNPs and those with more than 50% missing calls. The merged SNP dataset was further filtered using PLINK v1.9 [63] to remove SNPs with more than 3% missing data and accessions with more than 4% missing calls (--geno 0.03, --mind 0.04), only keeping the bi-allelic-SNPs, thus retaining final set of 92 individuals and 14,477 SNPs for genetic diversity estimations. The merged dataset used for the inference of genetic structure and the DAPC analysis was first filtered for SNPs and accessions with more than 10% missing data, then filtered for pairs in high linkage disequilibrium (LD) ($r^2 > 0.1$) using PLINK v1.9 and imputed by beagle 5.5 (version: 27Feb25.75f) [64], yielding a curated LD pruned SNP dataset of 8674 SNPs. The resulting imputed vcf file was converted to a STRUCTURE format using TASSEL5 [65] and PLINK v1.9.

2.4. Genetic Diversity and Population Structure Analysis

All analyses and visualizations of gene diversity, DAPC, PCA and Analysis of Molecular Variance (AMOVA) were done using the official docker image of ShiNyP Platform v1.1.0 [66]. In this platform, the gene diversity indices [minor and major allele frequency, expected heterozygosity (He or Nei GD), observed heterozygosity (Ho), polymorphic information content (PIC), nucleotide diversity (Tajima's Pi) and the fixation indices (Fit, Fst, Fis)] are calculated by the *popgen* function of the R package SNPready [67]. The R package *pcadapt* v4.4.1 [68] was also utilized within the ShiNyP Platform to detect genome-wide SNPs under selection. The *pcadapt* analysis was performed using the LD pruned SNP dataset comprising 8674 SNPs. The first four PCs were retained for the analysis based on Cattell's rule and the Benjamini and Hochberg (FDR) ($\alpha = 0.10$) method was applied.

The population structure analysis for the whole genotypes panel was estimated with a curated LD pruned SNP dataset consisting of 8674 SNP markers using STRUCTURE V2.3 [69]. The Bayesian model-based clustering approach was used to define the number of sub-populations (k) given an admixture model with the correlated allele frequencies. Several STRUCTURE simulations were performed with the number of presumed clusters from $K = 1$ to $K = 10$. Ten independent runs were used for each subpopulation (k-value). For each run, simulations were run for 50,000 burnin and 500,000

Markov Chain Monte Carlo (MCMC) iterations. The Delta k statistic of [70] was estimated using STRUCTURE SELECTOR [71] to determine the best k value. The accessions with a membership probability lower than 0.70 were assigned to an 'admixed' group.

Standard QC filters were applied to the resultant dataset to remove low-quality SNPs and samples: SNPs with missing calls in 10% accessions, as well as accessions with missing calls in 10% of the SNPs, as well as SNPs in high LD ($r^2 > 0.1$) were removed from the curated dataset consisting of 8674 high-quality SNPs.

The genetic relationships among accessions were further evaluated by cluster analysis based on the Nei83 D_A genetic distance matrix [72] and computed across 92 individuals using the curated dataset containing 14,477 SNPs. An unweighted pair group method with an arithmetic mean (UPGMA) tree was constructed from the Nei83 D_A distance matrix with the Power Marker v3.25 software [73]. The visualization and annotation of the tree was done using the Evolview v2 webserver [74].

3. Results

3.1. SNP Markers Quality Distribution

Ninety-two barley genotypes were used to elucidate the genetic diversity within the gene pool of the Bulgarian barley developed and collected in the Institute of Agriculture —Karnobat. This panel includes elite germplasms and key cultivars which have had strong impact on the development of the recent barley cultivars in Southeastern Bulgaria. The panel was genotyped by using a 50K Illumina iSelect SNP array that contained 44,040 markers. After a quality control filtering of the dataset, 14,477 SNPs were used for analysis. For each SNP marker, chromosomal positions were obtained from the James Hutton Institute, Scotland (https://bioinf.hutton.ac.uk/iselect/app/index.pl). The high-quality SNPs were well distributed across the barley genome and presented in (Figure 1). Among the seven chromosomes, the highest number of markers was observed on chromosome 2H, 19.16% (2775 SNPs); followed by the chromosomes 5H, 18.29% (2649 SNPs); and 7H, 16.49% (2385 SNPs); while chromosome 6H had the lowest number of SNPs with a percentage of 8.01% (1161 SNPs).

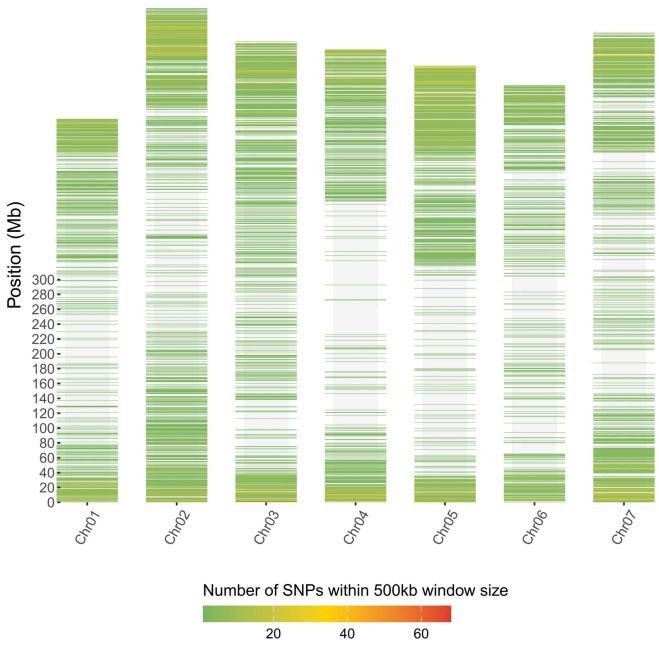


Figure 1. Distribution of 14,477 SNP markers (produced after quality filtering) based on the physical map of the barley genome. The marker density (number of SNPs within 500kb window size) is

shown in the color legend on the bottom.

3.2. Genetic Diversity

The genome-wide genetic diversity was assessed using the filtered dataset comprising 14,477 high-quality SNP markers. The selected set of SNP markers was able to differentiate all genotypes with the exception of two pairs of closely related sister lines, the six-rowed lines of German origin (SUEI and SUE II) and the Bulgarian six-rowed lines Kt2207 and Kt2208. The GD values ranged from 0.1295 (Chr. 5H) to 0.2004 (Chr. 3H) with an average of 0.1690 (Table 1). The average PIC in the barley genome was 0.1443 and ranged from 0.1130 (Chr. 5H) to 0.1693 (Chr. 3H) (Table 1). The minor allele frequency (MAF) varied between 0.0808 on Chr. 5H to 0.1330 on Chr. 3H with an average of 0.1100. A high genome-wide observed heterozygosity in SNP markers was observed for the 92 genotypes (Ho = 0.0803), ranging from 0.0634 in 5H to 0.0981 in 6H. The nucleotide diversity (Pi) was the highest in Chr. 3H (0.2026) followed by Chr. 4H (0.1904) and 6H (0.1900) but lowest in Chr. 5H (0.1309).

Table 1. Number of SNP counted, minor (MAF) and major (MAF) allele frequency, expected heterozygosity (He or Nei GD), observed heterozygosity (Ho), polymorphic information content (PIC), nucleotide diversity (Tajima's Pi) estimation and the fixation indices (Fit, Fst, Fis) in a panel of 92 barley genotypes.

Chr.	No of SNPs	Minor Allele	Major Allele	GD (He)	Ho	PIC	Pi	Fis	Fst	Fit
1H	1520	0.1184	0.8816	0.1816	0.0857	0.1546	0.1836	0.524	0.0078	0.5275
2H	2775	0.095	0.9050	0.1479	0.0696	0.1274	0.1495	0.4923	0.01	0.498
3H	2278	0.133	0.867	0.2004	0.0979	0.1693	0.2026	0.4887	0.0055	0.4913
4H	1709	0.1249	0.8751	0.1883	0.0731	0.1586	0.1904	0.5965	0.0094	0.6002
5H	2649	0.0808	0.9192	0.1295	0.0634	0.113	0.1309	0.5094	0.0101	0.5146
6H	1161	0.1282	0.8718	0.1879	0.0981	0.1579	0.1900	0.4485	0.0084	0.453
7H	2385	0.1131	0.8869	0.1767	0.0877	0.1515	0.1786	0.4936	0.0078	0.4975
Mean	**14,477**	**0.1100**	**0.8900**	**0.1690**	**0.0803**	**0.1443**	**0.1709**	**0.5072**	**0.0085**	**0.5115**

The comparative analysis of genetic diversity parameters between the two row-types of barley revealed distinct variations between them (Table 2). The GD and PIC values were lower in the group of two-row barley (0.1423 and 0.1218, respectively) than in the six-row barley group (0.1955 and 0.1616, respectively). Pi was also lower in the group of two-row barley. Conversely, the Ho was higher in the group of two-row barley (0.0836) compared to six-row genotypes (0.0678) despite a larger number of fixed alleles in the group of six-row barley. Furthermore, the Fis was substantially higher in the six-row barley group (0.6503) than in the two-row group (0.4083).

Table 2. Expected heterozygosity (He or Nei GD), observed heterozygosity (Ho), polymorphic information content (PIC), nucleotide diversity (Tajima's Pi) estimation and the fixation indices (Fis), number of the exclusive and fixed alleles in the groups of two-row and six-row barley genotypes.

Group	Sample Size	He (Nei GD)	Ho	PIC	Pi	Fis	Exclusive Allele	Fixed Allele
Two-row	73	0.1423	0.0836	0.1218	0.1443	0.4083	3587	1957

Group	Sample Size	He (Nei GD)	Ho	PIC	Pi	Fis	Exclusive Allele	Fixed Allele
Six-row	19	0.1955	0.0678	0.1616	0.2065	0.6503	1957	3587
Mean	**92**	**0.1690**	**0.0804**	**0.1443**	**0.1709**	**0.5221**	**NA**	**NA**

3.3. Population Structure Analysis

To analyze the genetic structure in the studied collection, we used a model-based approach implemented in the STRUCTURE software by using the LD pruned dataset comprising 8674 SNPs as input. The analysis with the Evano ΔK method identified a peak at $K = 2$ (Figure 2A). However, the log-likelihood values (LnP(K)) continued to increase until $K = 10$ (Figure S1), suggesting that additional substructures might be present in the studied set of barley genotypes. The resulting two sub-populations at $K = 2$ divided the genotypes in a pronounced ear type manner with minor exceptions (Figure 2 B). The first sub-population (Cluster 1) included sixty-eight two-row barley cultivars and advanced breeding lines with origins from Bulgaria, Turkey and Serbia. The second sub-population (Cluster 2) included 18 cultivars and lines from Bulgaria, France, Germany and Sweden, out of which sixteen were six-rowed and two were two-rowed barley. The STRUCTURE bar plot (Figure 2B) also provides information on the level of admixture in the collection. Six of the genotypes were not assigned to any of the described populations and were considered admixed. The admixed genotypes included three six-row and three two-row types of barley with origins from Bulgaria and France.

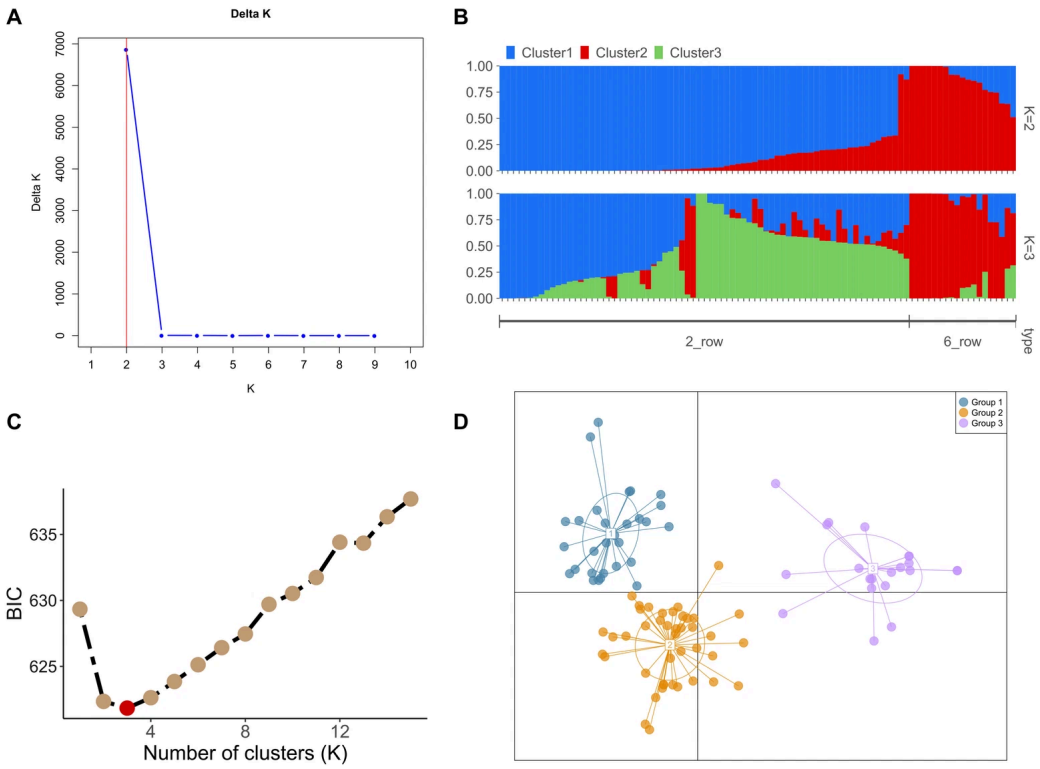


Figure 2. Genetic structure of the studied set of 92 elite barley genotypes. Most probable number of clusters, defined with the Evanno method. (**A**) Bar plot defining cluster membership of genotypes according to STRUCTURE analysis. (**B**) The most probable number of groups for DAPC was defined using BIC plotted against the number of clusters. (**C**) Grouping of the genotypes in DAPC (**D**).

To examine the hypothesis of additional substructures beyond the primary division, we also produced a $K = 3$ STRUCTURE bar plot. At $K = 3$, the first sub-population (Cluster 1) was additionally sub-divided into two sub-populations (Cluster 1 and Cluster 3), respectively. The second sub-population (Cluster 2) remained unchanged. Part of the genotypes from Cluster 1 fell into the group of the mixed genotypes, as their number increased from six to 36 and finally it included three six-rowed Bulgarian and French cultivars and 33 two-rowed Bulgarian, Serbian and Turkish genotypes.

The Bayesian information criterion (BIC) plot defined the most probable number of groups for the DAPC analysis as three (Figure 2C). The groups resulting from the DAPC analysis were in agreement with those formed at $K = 3$ in the model-based population structure analysis. The above-described sub-populations were completely preserved (Figure 2D). DAPC group 1 comprises 29 genotypes originating from Cluster 1, with local germplasm represented by twelve two-rowed Bulgarian cultivars and seventeen two-rowed advanced lines. DAPC group 2 includes ten two-rowed genotypes belonging to the Cluster 3 (2 Bulgarian cultivars and 8 advanced lines) and 32 admixture genotypes (9 cultivars from Bulgaria, Serbia, Turkey and 23 advanced Bulgarian lines) not assigned to any of the three sub-populations at $K = 3$ while DAPC group 3 comprises 21 genotypes belonging to the Cluster 2 at $K = 3$ (8 cultivars and 10 advanced breeding lines, mostly six-rowed barley originating from Bulgaria, France, Germany and Sweden).

With the STRUCTURE runs at $K = 10$, the two row-type groups were further divided into 10 sub-populations, distinguished based on their pedigree and breeding purposes (Figure S1), thus confirming the presence of substructures in the studied set of barley genotypes.

To validate these findings, a PCA was performed. The PCA separated the genotypes into two distinct groups consistent with the barley ear-row type as is shown on Figure 3.

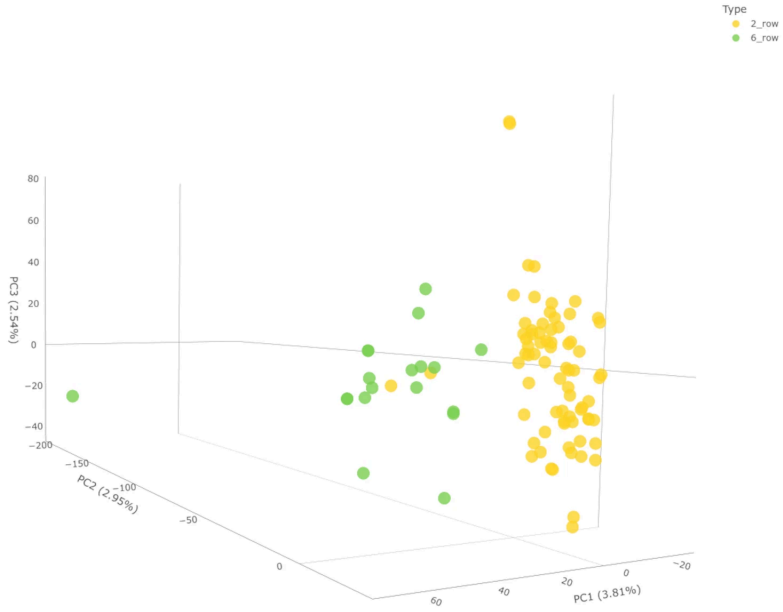


Figure 3. A Principal Component Analysis 3D scatter plot showing the clustering of 92 barley genotypes using 8674 SNP markers. The genotypes are grouped into two main groups. The colored dots are genotypes with different ear types, according to the legend.

AMOVA was conducted on the three groups defined by the DAPC analysis to partition the observed variation in accessions into sources of genetic differentiation (Figure 4). The results revealed that the level of variation among the groups of barley is lower

(17.03%) than the variation among the individuals within the groups (44.65%) and the variation within the individuals (38.31%) (Figure 4A). The significance test (Figure 4B) showed that all three variance components are highly statistically significant ($p < 0.001$), thus proving that the genetic differentiation observed among groups, among individuals, and within individuals is not due to random chance.

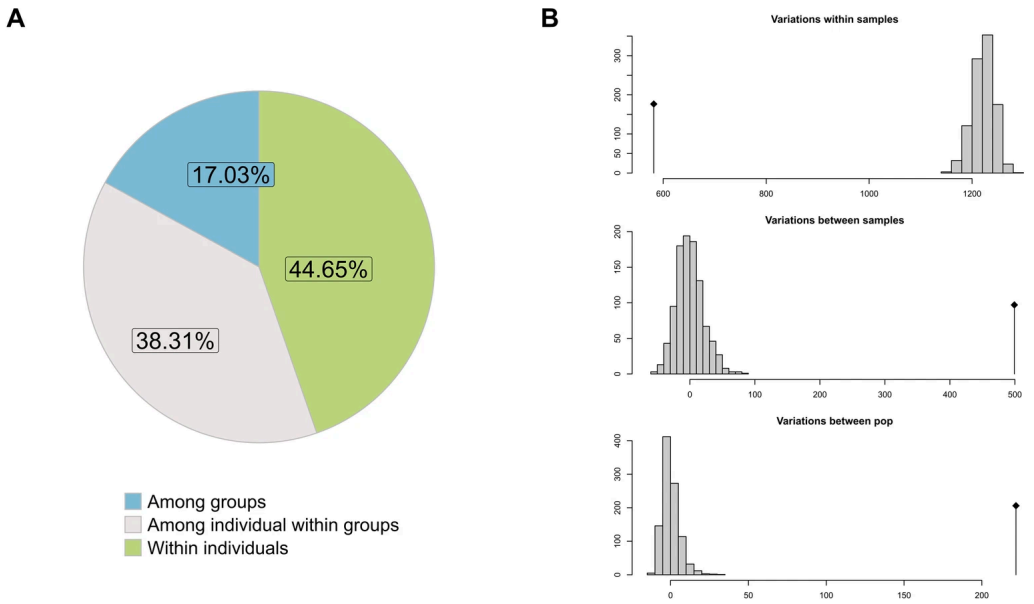


Figure 4. AMOVA analysis of the studied barley population. Pie chart summarizing AMOVA results for the three DAPC defined barley groups. (**A**) Significance test of population strata among defined DAPC groups. (**B**) The three histograms show the null distributions generated by randomly shuffling the data (1000 permutations), with the black line representing the genetic variance components.

3.4. Phylogenetic Analysis

The phylogenetic tree (Figure 5) delineated the 92 varieties into two main clades in agreement with the observed two main clusters inferred by the model-based approach at $K = 2$ in the genetic structure analysis. The first main clade (Clade 1) was composed entirely of the foreign and advanced breeding lines of six-rowed type, together with cv. Verty (six-rowed) as an outgroup. In this clade the lines SUE I and SUE II, with origins from Germany, and the Bulgarian advanced lines Kt2207 and Kt2208 were identical at the studied SNP loci. The second clade encompassed all cultivars and lines with origins from Bulgaria and Balkan peninsula (Serbia, Turkey) together with six-rowed line KT3041 as an outlier. The second clade was subdivided into three sub-clades (2a, 2b and 2c), the first of which (2a) is composed of two two-rowed genotypes (Kt1258, Kt3039) and ten six-rowed genotypes (Kt1706, Kt2213, Veslets, Yuli Byan, Siela, Kt3040, Ahinora, Aleksan, Bozhin and IzBori). The sub-clade 2b and 2c includes two-row genotypes except the positioning of one six-rowed Bulgarian cultivar Zemela in the sub-clade 2b.

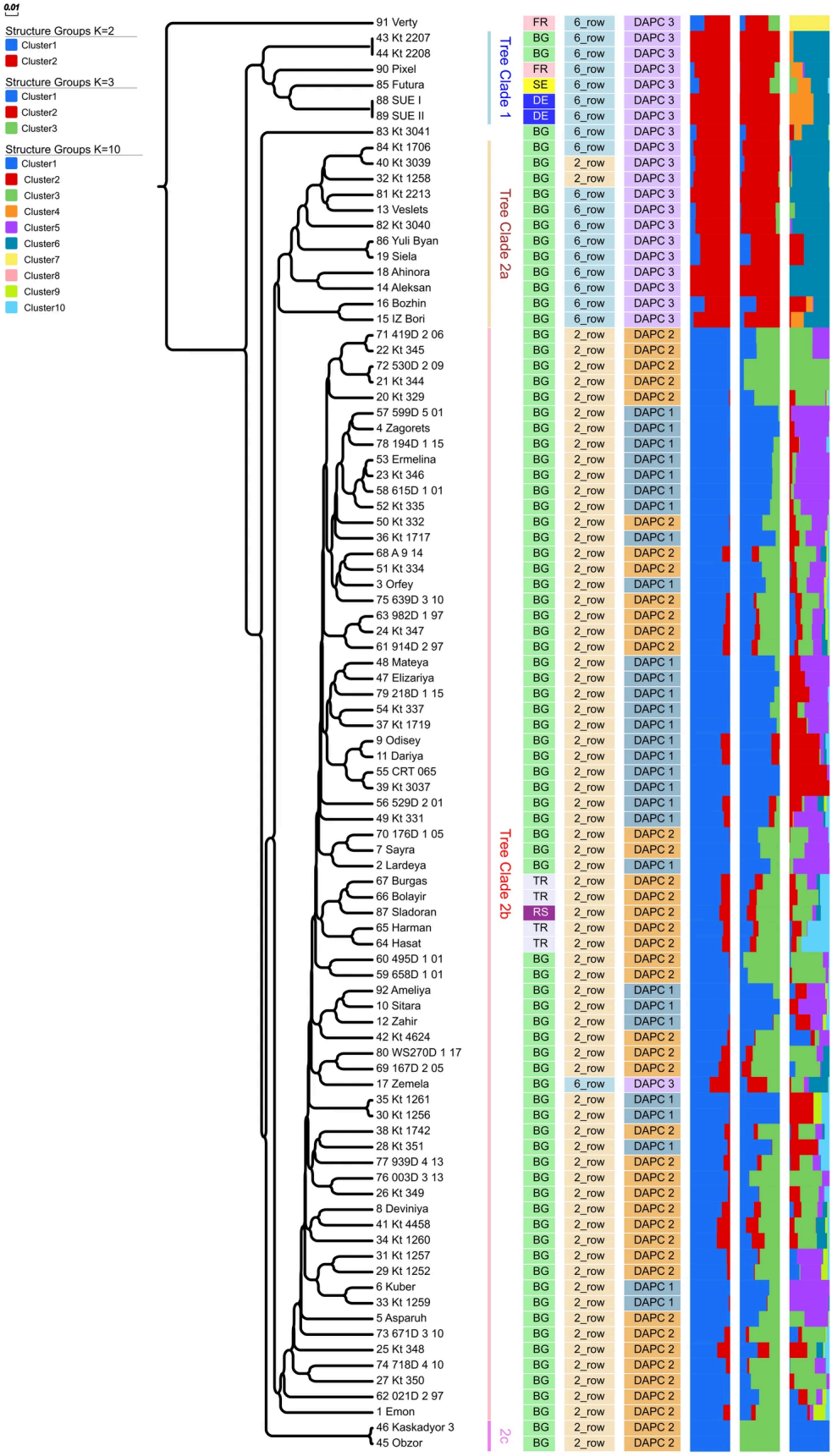


Figure 5. A phylogenetic tree of the collection of 92 modern Bulgarian and foreign barley cultivars and advanced breeding lines. The tree was built using an UPGMA algorithm based on the Nei83

D_A distance computed from the allele frequencies at SNP loci. The geographic origin of each genotype is represented by abbreviations highlighted with different colors. The bar plots to the right represent the membership to sub-populations inferred by the model-based approach at $K = 2$, $K = 3$ and $K = 10$. The two main clades of the phylogenetic tree are marked by vertical bars.

3.5. Genome-Wide Scan for Selection Signatures

To identify the loci subjected to selection, a genome-wide scan was performed using the *pcadapt* R package within the ShiNyP v1.1.0 framework, which controls for the background population structure identified in the PCA. The genomic distribution of the selection signatures is illustrated in the Manhattan plot (Figure 6). To control the proportion of false positives arising from multiple testing, a false discovery rate (FDR) threshold of ($\alpha = 0.10$) was applied. A Q-Q plot and a histogram of the distribution of *p*-values are shown in Supplemental Figure S2. Several SNPs exceeded this significance threshold, representing robust candidate loci under directional or diversifying selection within the studied barley population. Only four out of the nine significant SNPs (1 on Chr. 2 and 3 on Chr. 4) are annotated as related to GN, PH and row type. Notably, one of them, located on Chr. 4, was annotated to be related to row type (Table S5).

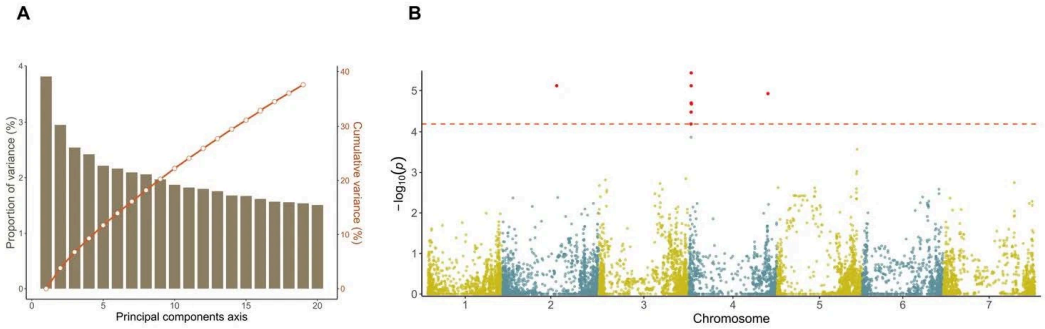


Figure 6. Detection of the SNPs under selection in the studied set of barley genotypes. (**A**) Genetic variance explained by PCA eigenvalues, with the *x*-axis representing the number of PCs, the primary *y*-axis showing the variance proportion (%), and the secondary *y*-axis displaying cumulative variance (%). The first four PCs were retained for further analysis based on Cattell's rule. (**B**) Manhattan plot of natural selection signatures identified through *pcadapt* analysis, with false discovery rate control ($\alpha = 0.10$) applied for multiple testing.

3.6. Comparison of Genetic and Phenotypic Structures

A considerable phenotypic variation was observed among the 92 barley genotypes in the field experiments across three growing seasons (Table S3). The coefficients of variation differed substantially among traits, with the highest values recorded for SS (102.18–136.21%), followed by KS (38.80–42.69%), WKS (30.82–32.32%), and WKP (26.98–35.57%). Grain yield exhibited a moderate variation (CV = 11.06–15.91%). The Shapiro–Wilk test indicated no significant deviation from normality for grain yield in any of the three years ($p > 0.05$), whereas several other traits showed significant deviations from normality (Table S4).

To analyze how the genetic structure inferred by the DAPC method is represented in the phenotypic performance of the studied genotypes, a PCA bi-plot analysis was performed with the mean values of ten agronomically important phenotypic traits (NPT, PH, NSS, SL, KS, SS, WKS, WKP, TKW and GY) across the three experimental seasons (Figure 7). The first two composite axes of the PCA explained 62.06% of the phenotypic variation—45.39% and 16.66, respectively, for PC1 and PC2. The kernel weight per spike (WKS), kernel weight per plant (WKP), number of sterile spikelets (SS) and number of

kernels per spike (KS) had the strongest correlations with the first axis in the positive direction, and the productive spikes per m^2 (NSS), thousand-grain weight (TKW) and spike length (SL) had the strongest contributions in the negative direction of the same axis, while grain yield (GY) had the strongest contribution to the second axis. Along the first axis the accessions were clearly separated according to their row type. The six-row genotypes separated in the right part of quadrant 1 and 4, and the majority of the two-row genotypes clustered near the origin of the coordinate system in quadrants 2 and 3. That distribution reflected a higher kernel weight per spike (WKS) and kernel weight per plant (WKP) overall for the six-row genotypes, and higher NSS, TKW and SL for those situated in the mid part of quadrant 3. Along the second axis, on the other hand, the accessions were separated mainly to their GY and PH.

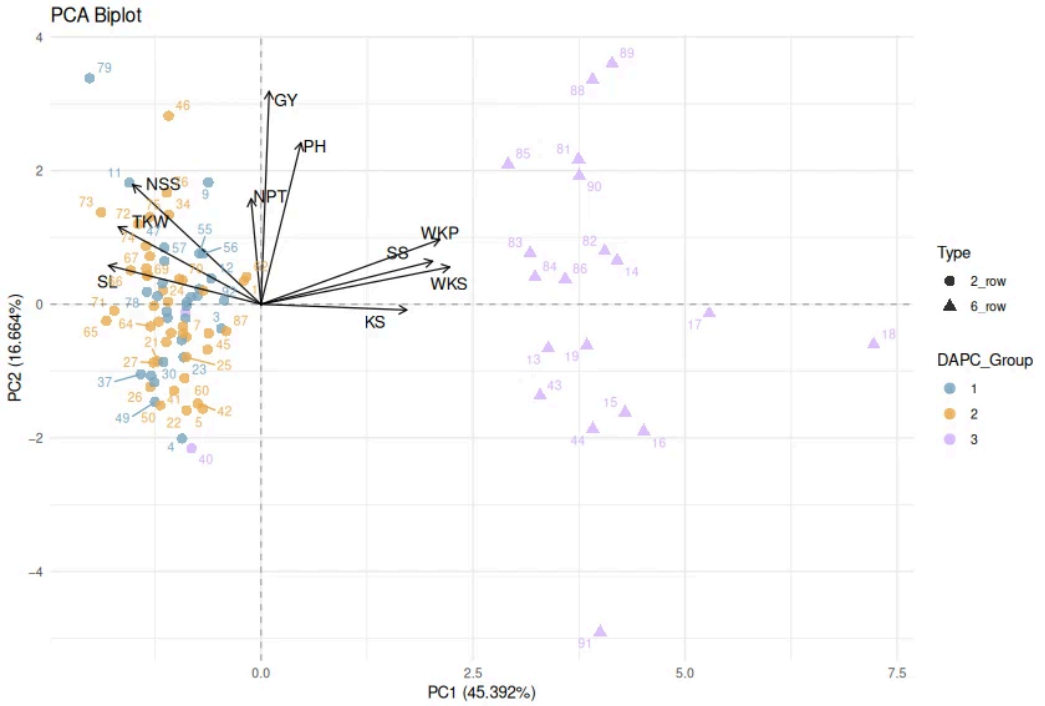


Figure 7. A PCA bi-plot of ten quantitative traits and the distribution of the genotypes in the three groups inferred by the DAPC analysis. The group memberships are coded in different colors and the spike type in different shapes. Numbers next to the symbols represent the studied barley genotypes. The studied traits, plant height (PH); number of productive tilers (NPT); productive spikes per m^2 (NSS); spike length (SL); number of kernels per spike (KS); number of sterile spikelets (SS); kernels weight per spike (WKS); weight of kernels per plant (WKP) (g); thousand kernel weight (TKW), and grain yield (GY) are drawn as arrows.

4. Discussion

The genome-wide SNP diversity analysis of elite barley genotypes serves as an important tool to optimize germplasm conservation and accelerate breeding programs. This approach could assist the development of climate-resilient cultivars by leveraging polymorphic loci to identify optimal parental lines for targeted trait introgression while preserving the important economic traits of barley, detection of historical selective sweeps and establishment of representative core collections [18,75–77].

4.1. Genetic Diversity Patterns Within the Bulgarian Barley Panel

The current study is the first attempt to assess the genetic diversity of barley in Bulgaria using the Illumina iSelect SNP array, starting with the collection in the

Agricultural institute-Karnobat, Southeastern Bulgaria consisting of 92 genotypes. A filtered dataset of 14,477 high-quality SNP markers distributed across the barley genome with the highest number of markers on the chromosome 2H (2775 SNPs) and the lowest on the 5H chromosome (1161 SNPs) was employed for genetic diversity studies. Our data demonstrates that chromosome 5H shows the lowest genetic diversity, PIC, Ho and Pi values. Since most of the barley genotypes studied in the present study are of the facultative winter type, the low diversity in chromosome 5H could be partially attributed to fixation of Fr-H1/VRN-H1 super-locus in winter barley [78]. Another possible cause for the low diversity in chromosome 5H in Bulgarian barley panel could be a reduced recombination in pericentromeric regions, leading to the creation of large haplotype blocks that have little or no recombination. In a European spring barley collection, it was recently discovered that a large 250 kb haplotype block in the pericentromeric region has not recombined in the last 70 years of breeding [79]. This finding outwardly contrasts with three decades of marker-based studies (e.g., RFLP, SSR, and SNP arrays) that consistently identified chromosome 1H as the primary low-polymorphism locus in barley [52,53,80–83]. Overcoming the limitations of the observed recombination "coldspots" in some barley chromosomes reported here and in other studies remains a major obstacle for targeted crop improvement.

The mean genome-wide gene diversity in the present study was low (GD = 0.169). This value is lower than that observed in barley genotypes sampled from various agro-ecological regions of Ethiopia (0.253) but comparable to the GD reported for ICARDA barley collection (0.151) when the sample of genotypes were divided according to their breeding origin [56]. The mean GD value of Bulgarian barley collection is also lower in comparison to those observed in the wild and worldwide barley (GD = 0.388) [84], in the ICARDA spring barley collections (GD = 0.366) [85], in Nordic spring barley collections (GD = 0.359) [83], Western Canadian barley (GD = 0.341) [53] and Egyptian barley collections (GD = 0.550) [50]. These contradictions in GD levels across studies stem largely from the differences in germplasm composition subjected to investigations and project purposes. Specifically, higher diversity is observed in studies that pool elite lines across temporal scales, combine materials selected for divergent breeding objectives (e.g., yield optimization vs. abiotic stress tolerance), or evaluate expansive collections that span wild barley, landraces, and elite cultivars. The use of diverse germplasm collections can capture vast allelic diversity lost in elite lines during intensive modern breeding. The studies highlight that the assessment of modern barley genotypes is of utmost importance for reprogramming current objectives, most of which are prioritizing the improvement of traits like yield stability and disease resistance, like in the case of the breeding objectives in IA-Karnobat, Bulgaria. However, these priorities narrow the genetic base of the germplasm, thereby compounding the risk of genetic erosion and increasing their vulnerability to emerging biotic pressures and unpredictable abiotic fluctuations under future climatic challenges.

The polymorphic information content (PIC) values observed in this study (average = 0.1443, ranging between 0.1130 and 0.1693) supports a low-to-moderate level of genetic diversity within the investigated barley genotypes. These values are lower, or comparable to previous reports on barley germplasm. For instance, the PIC values presented here fall slightly below those reported for Ethiopian barley (mean = 0.216; range = 0.151 to 0.274) [53] and the broader collection evaluated by Cappo-chichi et al. [53] (mean = 0.2345; range = 0.094 to 0.375). The average Ho (0.083) in Bulgarian barley is higher than the Ho reported for the Ethiopian barley (Ho = 0.045) [56], Canadian barley (0.055) [53], and Shanghai region landraces (0.0696) [55].

Our finding of a higher genetic diversity (GD) in six-rowed Bulgarian barley

(0.1955) relative to two-rowed genotypes (0.1423) contrasts slightly with the findings by Bouhlal et al. [86]. They reported equivalent values for Nei's unbiased gene diversity (He = 0.33) between six- and two-row types in a Global Barley Panel (GBP) of 500 genotypes representing a wide range of row types, end-uses, growth habits, geographical origins, and environments. However, both studies demonstrate that significant genetic variation is maintained across different six-row-type populations. In contradiction, most studies on barley reported higher GD in two-row in comparison to six-row barley [83,87], underscoring that regional germplasm collections can deviate significantly from global benchmarks due to specific agro-climatic adaptations and historical local breeding preferences.

The nucleotide diversity (Pi) was higher in the six-rowed barley, and this group also exhibited a higher number of fixed alleles and lower observed heterozygosity (Ho). Conversely, the larger two-rowed group mostly consisting of Bulgarian barley, shows lower Pi and number of fixed alleles along with elevated Ho values. Since the studied set of barley genotypes contains a substantial number of advanced breeding lines, the elevated Ho values in two-rowed pool might be a result of recent parental admixture, i.e., crosses between genetically distinct elite lines for new varieties' development, continued segregation or selective sweeps that reduce local Pi around high-value agronomic genes while flanking regions retain transient heterozygosity. This distinction is further supported by the elevated inbreeding coefficient (Fis) in the six-rowed Bulgarian genotypes (0.6503) compared to the two-rowed ones (0.4083). Taken together, these data indicate that the two-rowed population may have undergone intense selection or genetic drift, compromising their overall genetic diversity. On the other hand, given its geographically diverse composition (including germplasm from Bulgaria, France, Germany, and Sweden) the six-rowed pool may harbor unique adaptive traits shaped by local environmental pressures, which could be a valuable reservoir for further crop improvement.

4.2. Genetic Differentiation and Row-Type Variation in Bulgarian Barley

To infer the population structure, we used the Bayesian-model-based structure analysis which is widely used for both human and plant species [88]. The model-based analysis of the studied barley population based on the LD pruned dataset retaining 8674 high-quality SNPs revealed a clear genetic structure within the investigated barley germplasm. At the optimal $K = 2$, the accession panel was partitioned into two distinct genetic clusters, primarily reflecting row type (two-row vs. six-row) (Figure 2B) which was also supported by UPGMA phylogenetic and PCA analyses. The results of the cluster analysis at $K = 3$ (Figure 2B) and further at $K = 10$ (Figure S1) demonstrate that the genetic structure of the studied barley panel is jointly determined by the row type (two-row/six-row), pedigree and the goals of the practical breeding towards tolerance to abiotic stress (cold and drought), productivity, and to some extent, the malting/forage production. In contrast to STRUCTURE, the BIC plot (Figure 2C) in the DAPC analysis suggested that the most probable number of groups is three. The results of the DAPC analysis (Figure 2D) are in good congruence of the STRUCTURE clustering at $K = 3$. The DAPC Groups 1 and 3 almost completely correspond to STRUCTURE Cluster 1 and Cluster 2 whereas Group 2 of DAPC includes Cluster 3 and a large part of the admixed genotypes. As the number of clusters increases (from $K = 3$ to $K = 10$), a finer differentiation was observed, manifested in the subdivision of the main clusters into distinct subgroups (Figure 2D, Figure 5), especially evident for Cluster 1 which splits into five distinct sub-clusters at $K = 10$ that generally agree with the sub-clades in the UPGMA tree.

The AMOVA analysis of the three groups defined by DAPC partitioned the variance components into 44.65% among individuals, 38.31% within individuals and 17.03% be-

tween groups indicating a substantial level of variability among individuals and a relatively low-to-moderate genetic differentiation between the predefined DAPC groups. The observed high level of variance among individuals is evidence for the presence of genetically unique individual barley genotypes in the collection, which is important for discovering rare and valuable alleles. Other studies also reported higher genetic variation among individuals within a population than among populations [89–91].

4.3. Breeding-Driven Selective Sweeps and Phylogeny

The UPGMA cluster analysis demonstrated a genetic stratification based primarily on ear-row type, which separated the investigated barley germplasm into two major clades. This clear differentiation aligns with previous global, European, American, and Nordic evaluations confirming that growth habit, together with ear type, represent the primary determinants of population structure in barley [83,92–95]. Conversely, the SNP panel utilized in this study did not effectively resolve macro-geographic differentiation or seasonal growth habits, a limitation mirroring previous observations in the European and Serbian collections [87,96,97].

Clade 1 grouped few introduced six-row genotypes from Central and Western Europe alongside three advanced Bulgarian six-rowed lines with foreign germplasm in their pedigrees. Notably, identical SNP profiles were shared between the German (SUE I and SUE II) and the Bulgarian sister lines (Kt2207 and Kt2208), validating the utility of this SNP marker set for identifying genetic duplicates. The clustering of elevated diversity observed in Kt2207 and Kt2208 in Clade 1 is probably driven by their complex ancestry, which includes the Western European parental line PG 4440 (derived from cv. Vada, Netherlands) and the Eastern European selection K-6823, both contributing to the dual resistance to *Ustilago nuda* and *Drechslera graminea*. The rest of Bulgarian six-row genotypes are grouped in a separate Clade 2a. This divergence in the six-row pool supports the established genetic distinction between Eastern and Western European barley [50].

A cluster analysis delineated a distinct structural hierarchy within Clade 2a, entirely composed of Bulgarian barley germplasm consisting of ten six-rowed and two two-rowed genotypes and successfully separating them into discrete functional groups. For example, the six-rowed cultivars Siela and Yuli Byan clustered together, which is consistent with their shared pedigree and parallel agronomic performance, particularly regarding their high productivity and robust tolerance to abiotic stress, including low temperatures and drought. Furthermore, the separation of the six- and two-rowed lines (Kt1706, Kt3039, and Kt1258) into sub-clusters further validates the functional relevance of the observed genomic diversity. This specific sub-cluster was unified predominantly by their advanced physiological adaptation to drought stress. The clustering of the two two-row advanced lines (Kt3039, and Kt1258) together in Clade 2a, also confirmed by the structure and DAPC analyses (Figure 5), is surprising. It might be related to an incomplete genetic differentiation between barley row types as have been reported by Bustos-Korts et al. [98] and Bouhlal et al. [86]. These findings indicate that spike row type is an important, but not exclusive factor in shaping the genetic structure of barley germplasm. Shared ancestry and breeding history may also contribute to the observed genetic relationships.

Clade 2b clustered the majority of Bulgarian two-rowed genotypes along with Turkish and Serbian accessions, reflecting the germplasm exchange and directional selection under similar Balkan Peninsula agro-climatic conditions. An exception was the six-rowed Bulgarian cv. Zemela, which was nested within this two-rowed cluster (Clade 2b). This line was obtained by the gamma irradiation treatment of an F2 cross (Table S1)

and it falls into an admixed group in structure analysis. Therefore, this erroneous clustering could be explained by induced mutations or heterozygosity at the genetic loci governing the row type. However, further studies are needed to prove the above hypotheses. The Clade 2b displayed a compact grouping with moderate-to-low internal diversification, indicative of a shared ancestral gene pool within national breeding programs. The distinct genetic clustering observed among the evaluated two-rowed barley genotypes highlights a classic paradigm of pedigree-driven stratification. Furthermore, the internal topology of this clade directly aligns with the well documented genetic architecture characteristic of long-term European barley breeding programs [87]. Beyond broad regional pedigree effects, our results reveal a highly specialized clustering model driven by local selection pressures, which have generated discrete functional groups. The micro-evolutionary dynamics within Clade 2b elucidate this adaptive stratification, as evidenced by the emergence of several distinct sub-clusters reflecting sustained efforts to enhance environmental resilience, and malting and brewing characteristics. For example, one of the prominent sub-clusters encompasses a specific group (cv. Orfey, A9 14, Kt334, and 639D 3 10) distinguished by a very good drought tolerance. The close phylogenetic grouping of these materials is natively tied to their pedigree, owing to the introgression of drought-resilient Ethiopian landraces. Concurrently, a highly compact sister sub-cluster within Clade 2b delineates an elite pool of germplasm consisting of four cultivars (Darya, Odisey, Elizariya, and Mateya) and five advanced breeding lines (Kt3037, CRT065, 218D-1/15, Kt337, and Kt1719). The tight clustering of this group highlights a successful breeding synergy, effectively combining robust winter hardiness (cold tolerance) with high yield potential and brewing characteristics. The phylogenetic adjacency of cvs. Sayra and Lardeya, alongside line 176D-105, provides further validation of a strong, conserved genetic signal for low-temperature tolerance within this specific evolutionary branch. The strategic introgression of elite Western European germplasm, specifically from France, Germany, and Denmark (e.g., cvs. Lambic, Sonja, and Merian, and line HVW1.2.803), to augment the quality and yield parameters of domestic Bulgarian barley is clearly reflected in the topology. This foreign genetic influx resulted in the bifurcation of high-yielding, malting genotypes into two independent groups: the first comprising 914D 2 97, Kt347, and 982D 1 97, and the second encompassing lines 419D 2 06, Kt345, 530D 2 09, and Kt344. Taken together, these findings demonstrate that a targeted selection for dual-purpose agronomic traits (resilience and quality) drives predictable genomic partitioning in the winter type two-row barley germplasm.

An accelerating climate volatility in Southeastern Europe (shifting precipitation, reduced winter snow cover, and erratic spring frosts) necessitates alternative phenological adaptations. Consequently, to mitigate these hazards and secure yield stability, Bulgarian breeders developed intermediate winter–spring (facultative) lines 167D 2 05 and WS270D 1 17, which cluster tightly due to their shared pedigree (cv. Ludine × cv. Scarlet). Furthermore, the close phylogenetic grouping of Turkish (Hasat, Harman, Burgaz, Bolayir) and Serbian (Sladoran) cultivars within Clade 2b indicates a highly specialized, shared allelic architecture which support the fact that Clade 2b represents a critical genetic reservoir for breeding climate-resilient European barley. Similar groupings for Bulgarian and Turkish barley cultivars has been observed in the study by Güngör et al. [99], who analyzed the genetic diversity of 17 modern Bulgarian and 34 Turkish barley cultivars using iPBS-retrotransposon and SCoT markers.

The low genetic differentiation observed among the local two-rowed barley genotypes within Clade 2b strongly align with the macro-level findings in [87]. Their comprehensive analysis of 207 European two-row spring barley varieties demonstrated a highly homogenous, European spring barley cluster driven by intensive commercial breeding

which optimizes agronomic performance but often compromises their resilience to climatic changes. So, to mitigate the risks of genetic bottleneck, it is necessary to broaden the local gene pool by introducing wild barley relatives and globally sourced genebank accessions to provide the raw allelic variance necessary to combat novel climatic pressures and ensure long-term food security.

In contradiction to the modern two-row barley pool, the old cvs. Obzor and Kaskadyor 3 (var. *erectum*) emerged as the most genetically diverse Bulgarian two-rowed barleys, and they split into a separate Clade 2c. Developed in the early 1980s via hybridization with the German cultivars Triumph and Kaskada, these genotypes successfully introduced critical agronomic traits, specifically the semi-dwarf stature and enhanced lodging resistance into the domestic gene pool. Together with cv. Emon (Clade 2b), which exhibits a unique combination of high yield, excellent malting quality, and good environmental plasticity, these three cultivars constitute the current Bulgarian national standards. Crucially, the elevated genetic diversity observed in these older cultivars underscores their value as an indispensable allelic reservoir for future breeding programs.

4.4. SNPs Under Selection in Bulgarian Barley Panel

The detection of SNPs under selection relies on identifying the distinctive signatures of selective sweeps, extreme allele frequency differentiation, or statistically significant marker-trait associations. We used the *pcadapt* R package which utilizes PCA to identify selective outliers relative to population structure. Since the PCA analysis defined two distinct groups based on ear type (Figure 3), the detected SNPs under selection should be related to selection of two-row vs. six-row type. In the present study, several SNPs located on chromosomes 2H and 4H exceeded the established significance thresholds. These loci represent robust candidate regions that have likely undergone directional selection within the investigated set of barley genotypes. Notably, the highest density of these selected loci was observed on chromosome 4H, which harbored a prominent cluster of significant SNPs (Table S5).

A functional annotation of some of these SNP loci revealed strong associations with critical agronomic traits, specifically grain number (GN), plant height (PH), and ear-row type. These findings align with the previous literature establishing these genomic regions as key determinants of barley morphology [100–102]. Furthermore, chromosomes 2H and 4H are well documented repositories for essential agronomic loci, including those modulating flowering time, photoperiod response, and environmental stress tolerance. The identification of selection signatures within these regions underscores their pivotal role in both the adaptive evolution and targeted breeding of the studied barley germplasm. In addition, the identification of these robust and selection-responsive loci holds significant potential for Marker-Assisted Selection (MAS) programs.

4.5. Phenotypic Differentiation of Row-Type Genotypes

The PCA, based on 10 phenotype traits averaged across three growing seasons, clearly separated the two-rowed and six-rowed genotypes along the PC1 according to the row-type. The primary contributors to this axis were kernel weight per spike (WKS) and kernel weight per plant (WKP), both of which were significantly higher in the six-rowed genotypes. Productive spikes per m^2 (NSS), thousand kernel weight (TKW) and spike length (SL) correlated negatively with the first axis emphasizing their higher values in the two-rowed genotypes. Grain yield (GY) and plant height (PH) contributed to the second axis, both having higher values in the six-rowed barley. Although six-rowed genotypes exhibited numerically higher mean grain yields across the three growing sea-

sons, the differences between the row types were not statistically significant (Table S6). The distributions of grain yield showed considerable overlap between the two spike types in each year (Figure S3).

Our findings differ from previous studies, which reported higher grain yield (GY) and lower thousand-kernel weight (TKW) in six-rowed barley genotypes [91,103] and this could be attributed to low number of six-row genotyped used in our study. Plant height (PH) exhibited the strongest positive correlation with GY, aligning with the established literature [104–106]. Furthermore, phenotypic PCA clusters strongly coincided with SNP-based PCA and DAPC groupings, validating the utility of SNP markers for parental selection.

5. Conclusions

The study is the first genome-wide characterization of the *H. vulgare* L. collection in the Agricultural Institute, Karnobat, Bulgaria, utilizing a high-density SNP genotyping platform. By systematically dissecting the genetic diversity, population structure, and selection signatures of 92 modern barley genotypes, this research establishes a critical molecular foundation for regional crop improvement.

Integrating high-density single-nucleotide polymorphism (SNP) data with multi-season agronomic traits confirmed that despite a low genetic diversity driven by intensive directional selection and shared ancestry within a closed breeding program, the germplasm showed substantial genetic flexibility and environmental resilience. On the other hand, the present study also identified bottlenecks that reduce genetic diversity, especially in two-row barley breeding pools, and highlights an urgent need to incorporate local landraces and foreign varieties into breeding programs to expand the genetic reservoir, ensuring long-term climate resilience and sustainable yield security.

The clear partitioning of population structure based on spike architecture provides a definitive directive for a targeted parental selection in future breeding programs. Furthermore, the dense clusters of significant SNPs identified under directional selection on chromosome 4H offer high-priority candidates for the subsequent fine mapping and functional validation of genes regulating grain number, plant height, and row type.

Ultimately, these insights effectively bridge the gap between empirical breeding and modern crop genomics in Bulgaria. This work provides the empirical framework necessary to initiate large-scale genome-wide association studies (GWAS) and genomic selection (GS) models using a large panel of Bulgarian barley genotypes, optimizing barley breeding pipelines for challenging Mediterranean and continental environments.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/doi/s1. Table S1: Origin, type and growth habit of the studied barley from the collection of Institute of Agriculture—Karnobat, Bulgaria. Table S2: Average air temperature and monthly precipitation during three growing seasons in Karnobat, Southeastern Bulgaria, compared with long-term averages. Table S3: Phenotypic variation among 92 barley genotypes during 2021–2023. Table S4: Shapiro–Wilk test for normality of phenotypic traits distribution among 92 barley genotypes in three growing seasons (2021–2023). Table S5: pcadapt_Significant_SNPs_8674snps_4_PCs_FDR. Table S6: Year-specific comparisons of grain yield between two-rowed and six-rowed barley genotypes. Figure S1: Genetic structure of the studied set of 92 elite barley genotypes. The most probable number of clusters, defined with the LnPK method. Figure S2: QQ plot (A) and Histogram of *p*-values (B) in the *pcadapt* approach genome scan for selection signatures. Figure S3: Distribution of grain yield (t ha^{-1}) among two-rowed ($n = 73$) and six-rowed ($n = 19$) barley genotypes across three growing seasons (2021–2023).

Author Contributions: Conceptualization, E.G.T.; methodology, E.G.T.; formal analysis, N.K.C.; investigation, N.K.C., D.V. (Darina Valcheva), B.D. and D.V. (Dragomir Valchev); resources, D.V. (Darina Valcheva), B.D., D.V. (Dragomir Valchev), M.G., D.D. and T.P.; data curation, N.K.C.; writing—original draft preparation, E.G.T., N.K.C., D.V. (Darina Valcheva) and S.T.; writing—review and editing, E.G.T., N.K.C., S.T., D.V. (Darina Valcheva) and B.D.; visualization, N.K.C.; supervision, E.G.T.; project administration, E.G.T.; funding acquisition, E.G.T. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

RFLP	Restriction Fragment Length Polymorphism
RAPD	Random Amplified Polymorphic DNA
SSR	Simple Sequence repeat
SNP	Single nucleotide polymorphism
GD	Genetic diversity
PIC	Polymorphic information content
Pi	Nucleotide diversity
Ho	Observed heterozigosity
PCA	Principal Component Analysis
DAPC	Discriminant Analysis of Principal Components
AMOVA	Analysis of Molecular Variance
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

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