

Article

Assessment of Genetic Diversity of Bread Wheat Genotypes for Drought Tolerance Using Canopy Reflectance-Based Phenotyping and SSR Marker-Based Genotyping

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Abstract: This study investigated the genetic diversity of bread-wheat genotypes using canopy reflectance-based vegetation indices (VIs) and simple sequence repeat (SSR) marker-based genotyping for drought tolerance. A total of 56 wheat genotypes were assessed using phenotypic traits (combination of VIs and yield traits) and 30 SSR markers. The data of the phenotypic traits were averaged over two growing seasons under irrigated and drought-stressed conditions. The hierarchical clustering of the wheat genotypes unveiled three drought-tolerant groups. Cluster 1 genotypes showed minimal phenotypic alterations, conferring superior drought tolerance and yield stability than clusters 2 and 3. The polymorphism information content values for the SSR markers ranged from 0.434 to 0.932, averaging 0.83. A total of 458 alleles (18.32 alleles per locus) were detected, with the most polymorphic markers, *wmc177* and *wms292*, having the most alleles (24). A comparative study of SSR diversity among phenotypic clusters indicated that genotypes under cluster 1 had higher genetic diversity (0.879) and unique alleles (47%), suggesting their potential in future breeding programs. The unweighted neighbor-joining tree grouped the wheat genotypes into five major clusters. Wheat genotypes from all phenotypic clusters were distributed throughout all SSR-based clusters, indicating that genetically heterogeneous genotypes were allocated to different drought-tolerant groups. However, SSR-based clusters and model-based populations showed significant co-linearity (86.7%). The findings of the present study suggest that combining reflectance-based indirect phenotyping with SSR-based genotyping might be an effective technique for assessing genetic diversity to improve the drought tolerance of bread-wheat genotypes.

Keywords: bread-wheat; drought tolerance; vegetation indices; SSR markers; genetic diversity; unique alleles; cluster analysis; population structure



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1. Introduction

Drought stress, a major abiotic factor restricting wheat farming around the world, decreases wheat productivity by altering the crop plant's physiological and biochemical processes [1]. Drought is expected to become more frequent, longer, and more severe in many parts of the world in the twenty-first century as a result of global warming and other climate-change-driving factors [2,3], reducing wheat production in the future. As a result, actions to increase wheat production must be implemented as soon as possible, because the primary crop yield could be decreased by more than half, owing to a lack of irrigation water [4]. The introduction of new wheat genotypes with greater yield potential and enhanced drought tolerance is the most acceptable strategy for improving wheat

production [5]. Drought-tolerant genotypes are often chosen for economic features based on the presence of genetic variation in the genetic pool [6]. Genetic diversity facilitates the identification of tolerant genotypes as parental lines in the breeding programs to develop superior progenies for selection [7], without excluding species of the genus *Aegilops*, the wild ancestor of cultivated wheat [8].

It is commonly recognized that phenotypic and molecular methodologies have been used to investigate the variety and differentiation of wheat genetic resources, either separately or in combination [7–11]. There are several phenotypic tools for measuring genetic diversity; among them, crop ideotypes, phenology, and yield attributes are used extensively because of their reliability in measuring genetic variation within a gene pool [4,7,12]. Most phenotyping approaches, on the other hand, are damaging, costly, time-consuming, and resource-intensive, particularly when a high number of genotypes are being assessed [4]. Canopy-reflectance-sensing has proven to be a valuable high-throughput phenotyping alternative for precisely and indirectly monitoring drought tolerance traits across a large number of genotypes in a quick and non-destructive manner [4,12]. The crop canopy reflects a certain wavelength of the light spectrum because of its structural features and many biochemical and physiological processes. These wavelengths are commonly employed to assess canopy pigment content, senescence, photosynthetic activity, biomass build-up, leaf-area index, grain production, and plant hydration status [12]. Using the visible ($\lambda = 400\text{--}700\text{ nm}$) and near-infrared ($\lambda > 700\text{ nm}$) wavelength ranges of the canopy-reflected spectrum, several vegetation indices (VIs) are computed to forecast various agronomic and physiological aspects. Though VIs provides a rapid and non-destructive evaluation of phenotypic and yield features, the application of both techniques in a target environment is critical, since VIs and phenotypic traits are strongly related under drought conditions compared to irrigated conditions [12,13].

Drought phenotyping with morpho-physiological and yield parameters has been shown to be useful in genetic diversity evaluations and variety production, despite the restrictions imposed by environmental variables and polygenic nature [9,14,15]. Molecular markers, on the other hand, complemented phenotype-based genetic diversity and mitigated many of the disadvantages of this strategy [9,15,16]. In addition to indirect diversity evaluations based on phenotypic traits, the majority of molecular markers provide a direct estimate of genetic variation. The genetic diversity of genotypes has been assessed utilizing DNA-based markers, avoiding environmental effects, including Random Amplified Polymorphic DNA, Amplified Fragment Length Polymorphism, SSR (Simple Sequence Repeat), and Single Nucleotide Polymorphism. SSR or microsatellite markers are the best candidates for studying wheat genetic variation because of their widespread prevalence in wheat genomes, chromosomal specificity, multi-allelic composition, co-dominant nature, ease of assaying, and high polymorphism rates [17–19]. A higher rate of polymorphism, along with the multi-allelic nature of SSR markers, aids in establishing relationships among genotypes, even with fewer markers [20]. Some recent studies using SSR markers that successfully analyzed wheat genetic diversity showed that genotypes were significantly diverged and should be considered in drought-tolerance breeding programs [7,11,19,21,22].

Introgressing the drought-tolerance potential in new wheat varieties and ensuring their genetic potential is a primary goal for breeders. In order to achieve this goal, researchers used a combination of traditional phenotypic data and SSR markers to analyze the genetic diversity of candidate genotypes [7,23–25]. Nevertheless, the use of high-throughput-origin indirect phenotypic data along with the SSR markers to explore wheat genetic diversity is scant [26,27]. Keeping this lacuna in mind, we used canopy-reflectance-based vegetation indices and yield parameters, as well as 30 SSR markers, to assess the genetic diversity of 56 bread-wheat genotypes under irrigated control and irrigation-simulated dry situations.

The specific objectives of this study were (i) to compute and assess the level of genetic diversity in wheat genotypes using phenotypic traits (VIs and yield attributes) as well as genotypic data from SSR markers, and (ii) to extrapolate the neighbor-joining trees and population structure of wheat genotypes using SSR markers. The findings of this study

will open up the possibility of integrating canopy-reflectance-based indirect phenotypic data and SSR-marker-based genotypic data in the genetic diversity assessment of wheat genotypes for drought tolerance.

2. Materials and Methods

2.1. Phenotyping

2.1.1. Experimental Conditions and Measurement of VIs and Yield Traits

The experiment used 56 different wheat genotypes, composed of 17 varieties, 1 advanced line, 2 mutant lines, and 36 accessions (Table S1) that were selected from a previous trial at the seedling stage [28]. Seeds were sown on November 18 for two years (2017–2018 and 2018–2019) at the research field of the Bangabandhu Sheikh Mujibur Rahman Agricultural University (24.038° N latitude, 90.397° E longitude), Gazipur, Bangladesh. The experimental soil had a silt loam texture (sand 26%, silt 50%, and clay 24%) and attained full field capacity at 30.6% volumetric soil water content. The experiment was designed as a randomized complete block split-plot with three replications. The main plots were divided into two growing conditions: “control” (regular irrigated) and “drought” (discontinued regular irrigation after 45 days of seed sowing), while wheat genotypes were assigned at random to the sub-plots. Figure 1 depicts the mean soil water content (%) and mean air temperature of the control and drought-stressed plots throughout the reproductive phases. Importantly, canopy reflectance, canopy temperature, and soil and plant analysis development (SPAD) values were recorded at heading, anthesis, and 7, 14, and 21 days after anthesis.

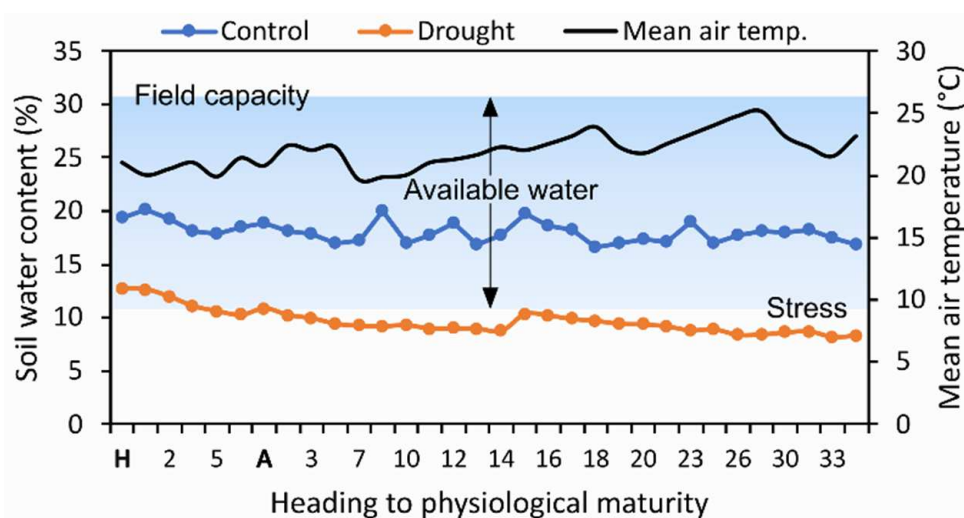


Figure 1. Soil water status (% moisture content) of control and drought-stressed plots and mean air temperature at the reproductive stages of wheat genotypes. Soil water content (%) was measured every day from the randomly selected plots ($n = 15$) of control and drought-treated blocks. H—heading and A—anthesis. Data are averaged over two growing seasons.

Using a microprocessor-equipped digital soil moisture meter (PMS-714, Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan), the soil water content (%) at a depth of 15 cm was measured from 15 randomly selected plots in each of the control and drought-treated blocks. Between 11.30 a.m. and 12.30 p.m., a hand-held infrared thermometer, coupled with a sensor to record the instant air temperature (Model: IR-818, URCERI, Highland, CA, USA; the distance-spot ratio of 13:1), was used to measure the temperature of the canopy and ambient air. Measurements were obtained at an angle of roughly 30° to the horizontal line, one meter away from the spotted canopy. Ten readings were collected from various locations of each plot. The canopy temperature depression (CTD) was estimated as the ambient temperature minus the canopy temperature using Fischer’s approach [29]. The

chlorophyll content was measured using a portable Chlorophyll Meter (Model: SPAD-502, Konica Minolta Inc., Osaka, Japan) on 10 preselected main shoot flag leaves from each plot.

Between 11 a.m. and 2 p.m., the plant-canopy reflectance was measured using a handheld multispectral radiometer (Model: MS-720, EKO Instruments Co. Ltd., Tokyo, Japan) maintaining a field of view of 25° and from 40 cm above the canopy, avoiding shadows, clouds, and strong winds (Figure S1). The reflectance by the radiometer was measured at 1 nm intervals from 350 to 1050 nm of the light spectrum. The reflectance data were calibrated using a white reflectance panel and then used to calculate the vegetation indices (VIs) outlined by Gizaw et al. [12] and is shown in Table 1.

Table 1. Vegetation indices (VIs) evaluated for the drought tolerance of bread-wheat genotypes.

| Vegetation Index | Formula ^a |
|---|---|
| Simple ratio (SR) | R_{800}/R_{680} |
| Normalized difference vegetation index (NDVI) | $(R_{800} - R_{680})/(R_{800} + R_{680})$ |
| Green-NDVI (GNDVI) | $(R_{780} - R_{550})/(R_{780} + R_{550})$ |
| Enhanced vegetation index (EVI) | $2.5 \times (R_{NIR} - R_{RED})/(R_{NIR} + 6 \times R_{RED} - 7.5 \times R_{BLUE} + 1)$ |
| Normalized water band index (NWI) | $(R_{900} - R_{970})/(R_{900} + R_{970})$ |
| Normalized chlorophyll pigment ratio index (NCPI) | $(R_{680} - R_{430})/(R_{680} + R_{430})$ |
| Photochemical reflectance index (PRI) | $(R_{530} - R_{570})/(R_{530} + R_{570})$ |
| Anthocyanin reflectance index (ARI) | $R_{800} \times (1/R_{550} - 1/R_{700})$ |
| Xanthophyll pigment epoxidation state (XES) | R_{531} |

^a The letter “R” followed by three-digit numbers stands for the wavelength of respective reflectance. R_{NIR} , R_{RED} , and R_{BLUE} indicate the mean reflectance of the near-infrared (770–895 nm), red (630–690 nm), and blue (450–510 nm) region of the spectra, respectively.

At physiological maturity, four-linear-meter plants from the middle of the plots were harvested at the ground level, and their yield traits and yields were measured. The biological yield (BY) [straw + grain + chaff] and grain yield (GY) were reported as the sun-dried weights with a moisture content of 12% and were represented as t ha⁻¹. The number of kernels per spike (NKS), the weight of kernels per spike (WKS), and the hundred kernel weight (HKW) were acquired by sun-drying, threshing, weighing, and counting grain numbers of 10 main-shoot spikes collected separately from each plot.

2.1.2. Statistical Analysis

Statistical analyses were carried out by R version 4.1.0 (<http://CRAN.R-project.org/>) (accessed on 15 November 2021). Growth-stage-specific VIs, SPAD, and CTD values were averaged and, together with the phenotypic traits, subjected to a combined analysis of variance (ANOVA) in the general linear model using the library lme4 [30]. Tukey’s HSD post hoc test was employed to assess the mean differences using the library agricolae [31], with differences at $p < 0.05$ considered significant. Boxplots and correlograms were prepared using the library ggplot2 along with ggpubr and reshape2 [32] and a web-based application MVApp [33], respectively. The package dendextend [34] was used to construct trees of hierarchical clusters (distance = Euclidean and method = wardD2) based on trait relative values.

2.2. Genotyping

2.2.1. SSR Markers and Extraction of Genomic DNA

To characterize the genetic diversity of 56 wheat genotypes, 30 SSR markers comprising most of the chromosomes of the hexaploid wheat genomes were employed. The SSR markers were chosen because they were highly polymorphic in the previous investigations of drought tolerance and genetic diversity and offered substantial genome coverage. Information about the markers was collected from the Agricultural Research Service–GrainGenes 2.0 (<http://wheat.pw.usda.gov>) (accessed on 23 July 2019) and is presented in Table S2. Genomic DNA of leaf samples was extracted from the 15-day-old pot-grown seedlings using the modified cetyltrimethylammonium bromide (CTAB) method [35].

2.2.2. Polymerase Chain Reaction (PCR) Amplification and Gel Electrophoresis

PCRs were performed according to Ciucă and Petcu [36], with 50 ng μL^{-1} DNA. Briefly, the PCR was performed in 10 μL reaction mixture containing 3 μL DNA, 1 μL 10 \times reaction buffer, 2 μL 25 mM MgCl_2 , 0.8 μL of 25 mM dNTP, 0.5 μL each of 10 μM forward and reverse primers, and 0.2 μL of Taq DNA polymerase. A single-channel pipette was used for transferring DNA from the dilution plate to the PCR plate. After the initial denaturation of the mixture for 5 min at 95 $^\circ\text{C}$, a total of 36 cycles were run where denaturation was performed for 45 s at 95 $^\circ\text{C}$, annealing was performed for 45 s at 55 $^\circ\text{C}$, and extension was performed for 1 min at 72 $^\circ\text{C}$ at each cycle with a final extension for 5 min at 72 $^\circ\text{C}$. The DNA amplicons obtained from the PCR were separated on 3.5% metaphor agarose gel [37] and then visualized and documented using a gel-documentation system (Figure S2).

2.2.3. SSR Marker Data Analysis

Five of the 30 SSR markers did not produce any bands in any of the 56 wheat genotypes and were thus excluded from the following diversity analysis. Genetic diversity estimates for individual markers, such as the frequency of allele and number of alleles, the gene diversity, and the polymorphic information content (PIC) were quantified using Power Marker 3.23 software [38]. Phenotypic cluster- and population-based genetic diversity, analysis of molecular variance (AMOVA), principal coordinate analysis (PCoA), and Wright's *F* statistics were calculated using GenAlEx 6.5 [39]. The Bayesian clustering approach in the STRUCTURE 2.3.4 program was used to analyze the population structure [40], with 10 runs for each *K* (assumed number of subpopulations [1–10]) and Monte Carlo chain replicates of 100,000 iterations, and the burn-in period for every run was 100,000 steps. A Structure Harvester (<http://taylor0.biology.ucla.edu/structureHarvester/>) (accessed on 20 November 2021) was used for the determination of delta *K* and the final populations [41,42]. Harvested data for *K* = 3 and 7 were used to create admixture barplots in the shiny app StructuRly [43]. Dissimilarity coefficient analysis was executed using the SSR data [44] using Power Marker 3.23 [38] and the dissimilarity matrix was visualized using the library pheatmap in R v.4.1.0 [45]. The dissimilarity coefficients were used to construct the unweighted neighbor-joining (NJ) tree by MEGA X [46]. The co-linearity between the SSR-marker-based clusters and model-based populations was measured using the online interactive tool Venny 2.1 [47].

3. Results

3.1. VIs and Yield Traits

The evaluation of the VIs and yield traits (together termed as 'phenotypic traits' hereafter) in the control and drought environments revealed significant variation in wheat genotypes (Table S3). For all phenotypic traits, the genotype by treatment interaction was highly significant, suggesting that the response of the diverse genotypes differed with changes in water availability. The drought treatment resulted in substantial variations in phenotypic traits (Figure 2). Among the VIs, Simple ratio (SR), Normalized difference vegetation index (NDVI), Green-NDVI (GNDVI), Enhanced vegetation index (EVI), Normalized water band index (NWI), and Photochemical reflectance index (PRI) were the highest under the control condition and significantly dropped as a result of drought. The normalized chlorophyll pigment ratio index (NCPI), the Anthocyanin reflectance index (ARI), and the Xanthophyll pigment epoxidation state (XES), on the other hand, were the lowest in the control and increased substantially during the drought. Under drought conditions, the SPAD value, canopy temperature depression (CTD), days to heading (DTH), plant height (PH), number of kernels per spike (NKS), weight of kernels per spike (WKS), hundred kernel weight (HKW), BY, and GY reduced considerably.

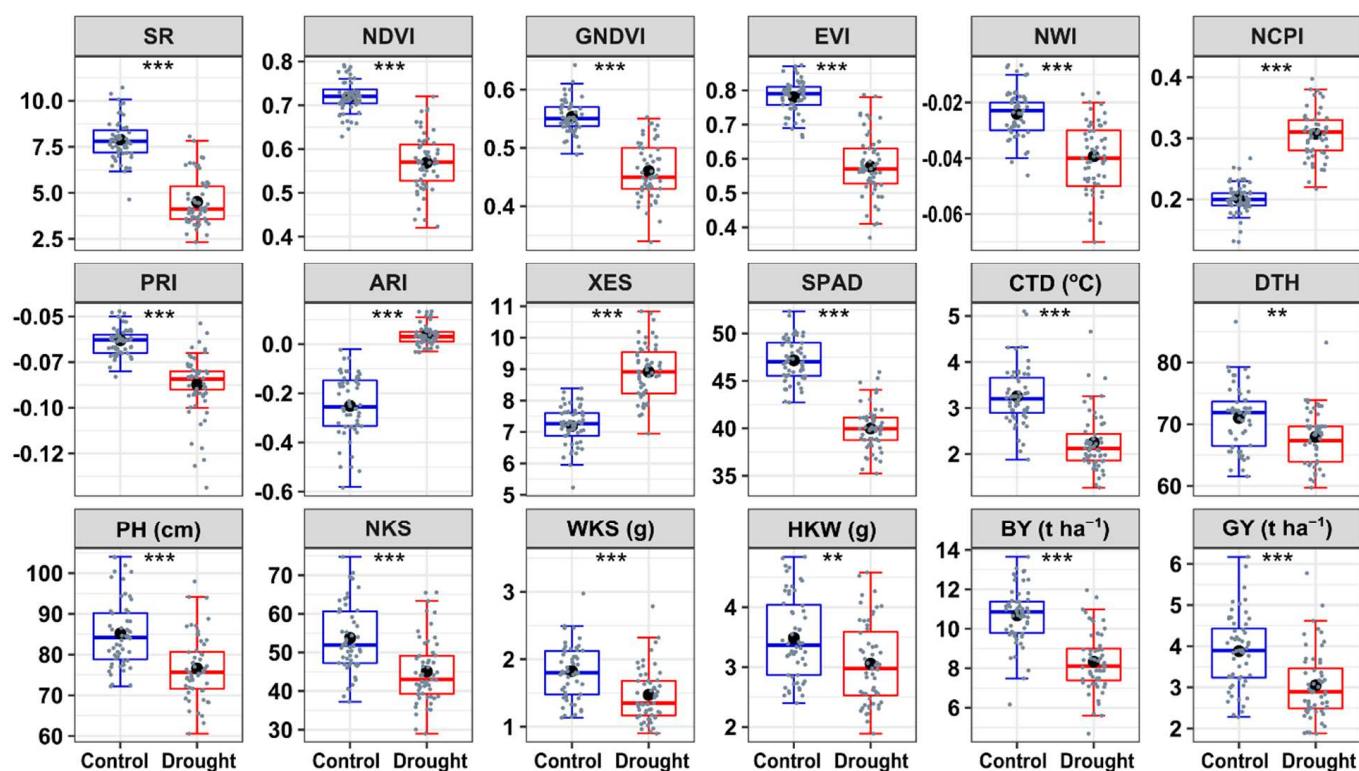


Figure 2. Descriptive summary of the studied phenotypic traits of 56 bread-wheat genotypes under control and drought treated plots in two growing years. ** and *** denote statistically significant at $p \leq 0.01$ and 0.001 , respectively. The median and mean are represented by horizontal thickened line and black circle within the box, respectively. Q1 (first quartile/25th percentile), Q3 (third quartile/75th percentile), $(Q1 - 1.5IQR)$, and $(Q3 + 1.5IQR)$ are represented by the lower and upper limit of the box, lower and upper whisker, respectively. IQR—interquartile range. The distribution of 56 wheat genotypes is indicated by slate color dots on the boxes. SPAD—chlorophyll index, CTD—canopy temperature depression, DTH—days to heading, PH—plant height (cm), NKS—number of kernels per spike, WKS—weight of kernels per spike (g), HKW—hundred-kernel weight (g), BY—biological yield ($t\ ha^{-1}$), and GY—grain yield ($t\ ha^{-1}$). Additional details are shown in Table 1.

3.2. Association among Phenotypic Traits

After combining data from the two wheat-growing seasons, the relationship between the phenotypic traits was determined under control and drought conditions (Figure 3). Under control conditions, both BY and GY exhibited insignificant and inconsistent associations with VIs; however, under drought stress, these relationships were significant and stronger. With the exception of PH, GY and BY were positively associated with NKS, WKS, and HKW under drought (Figure 3). In the water-limiting situations, GY was substantially and positively correlated with the SR, NDVI, GNDVI, EVI, NWI, PRI, SPAD, and CTD, whereas NCPI, ARI, and XES were negatively correlated. All the VIs, SPAD, and CTD exhibited stronger correlations among them under drought compared to control.

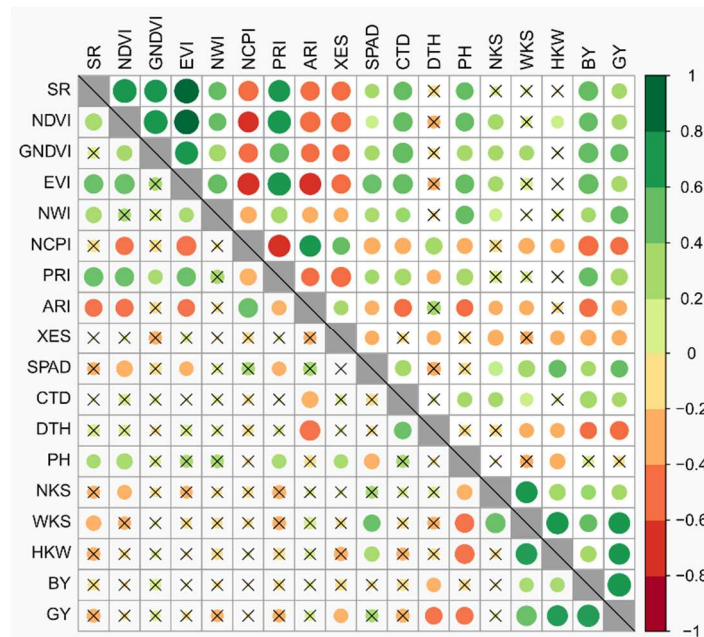


Figure 3. The pair-wise associations among phenotypic traits of 56 bread-wheat genotypes were shown by correlation coefficients as assessed under control (lower diagonal) and drought-stress conditions (upper diagonal). Data are averaged over two growing seasons. The higher the size and color intensity of circles, the higher is the correlation coefficient. Crossmark (×) indicates a nonsignificant correlation at $p < 0.05$. Table 1 and Figure 2 provide further details.

3.3. Hierarchical Cluster Analysis of Wheat Genotypes

Phenotypic trait values of two growing seasons were averaged, and trait relative values were calculated. Using the relative values of phenotypic traits, wheat genotypes were classified into distinct clusters of identical genotypes using hierarchical cluster analysis (distance = Euclidean and method = wardD2). Three clusters were generated based on the dissimilarity of the investigated phenotypic traits and presented as a cluster dendrogram (Figure 4). Cluster 2 has the most wheat genotypes (31), followed by clusters 1 (13) and 3 (12) (Figure 4).

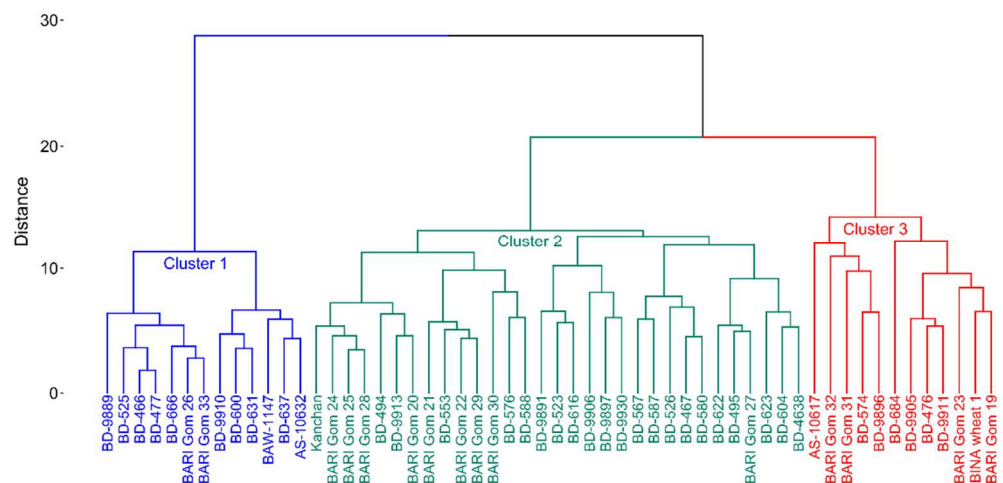


Figure 4. Hierarchical clustering trees (distance = Euclidean and method = wardD2) of the wheat genotypes based on traits relative values. Among the clusters, genotypes of cluster 1 showed a greater degree of tolerance to drought followed by the genotypes of clusters 2 and 3.

3.4. Cluster-Based Changes in Phenotypic Traits under Drought

Drought had a substantial impact on the SR, NDVI, GNDVI, EVI, NWI, PRI, and yield traits, with the magnitude of the decrease being smaller in cluster 1 genotypes than in cluster 2 and 3 genotypes (Figure 5). On the contrary, NCPI, ARI, and XES increased significantly during drought, with a lesser increase in cluster 1 genotypes than in the other two clusters (Figure 5). Under drought, the SPAD value declined by 12, 16, and 17 percent in the genotypes of clusters 1, 2, and 3, respectively (Figure 5). The genotypes of cluster 1 had the lowest decrease (15%) in CTD, whereas the genotypes of clusters 2 and 3 had significantly greater decreases (35 and 38%) (Figure 5). Cluster 1 genotypes had a smaller decline in the DTH, PH, NKS, WKS, HKW, and BY under drought conditions than cluster 2 and 3 genotypes (Figure 5). Due to drought, the GY decreased by 7, 21, and 35% in clusters 1, 2, and 3, respectively (Figure 5).

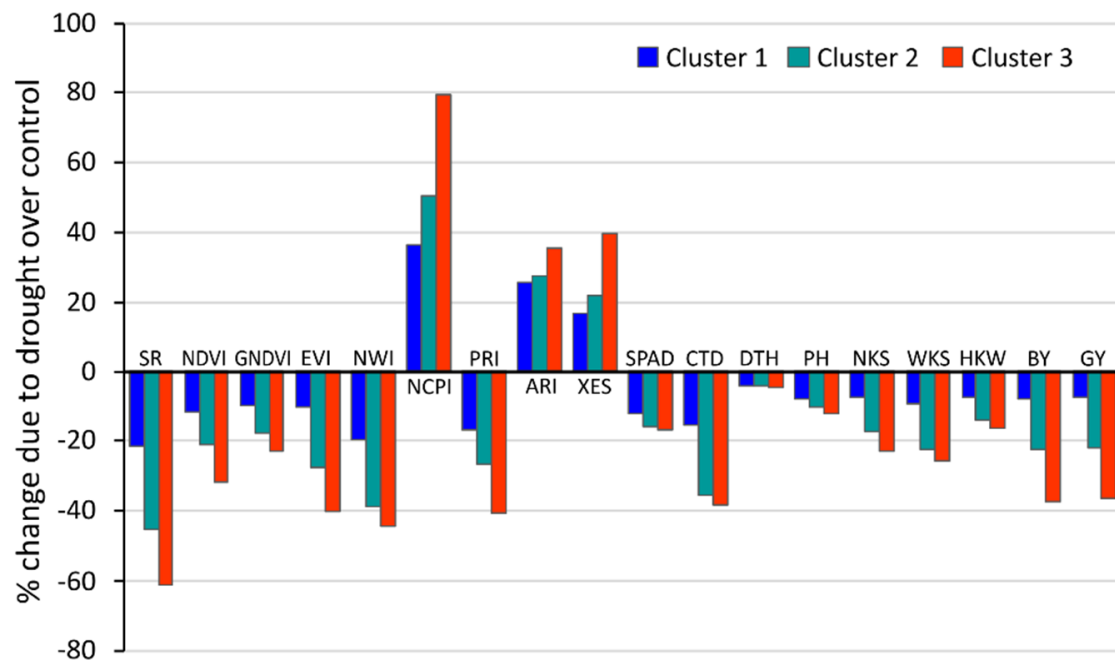


Figure 5. Variations in VIs and yield traits of wheat genotypes (% change due to drought over control). Data are the means of two years and five growth stages for each parameter (heading, anthesis, 7, 14, and 21 days after anthesis). Table 1 and Figure 2 provide further details.

3.5. Genetic Diversity Estimates of SSR Markers

A total of 30 SSR markers were used to detect polymorphism among the 56 wheat genotypes, five of which produced no bands in the genotypes and were thus excluded from further processing (Table 2). A total of 56 bread-wheat genotypes showed a considerable amount of genetic diversity when evaluated using the specified SSR markers. The number of alleles amplified by the markers varied between loci, ranging from 8 to 24 alleles per locus. The markers *wmc177* and *wms292* were the most polymorphic microsatellite markers, each with 24 alleles, followed by *wmc179*, which had 23, and *wms136*, *wms337*, *wms304*, and *wms149*, which all had 22 alleles (Table 2). A total of 458 polymorphic alleles were detected from screening 56 bread-wheat genotypes using the 25 SSR markers, with an average of 18.32 alleles per locus.

Table 2. Genetic diversity estimates in 56 genotypes by using 25 SSR markers.

| Marker (Location) | Major Allele Freq. | Allele Number | Allele Size (bp) | Shannon Index | Gene Diversity | PIC |
|---------------------|--------------------|---------------|------------------|---------------|----------------|--------------|
| <i>wms136</i> (1A) | 0.286 | 22 | 100–300 | 1.634 | 0.885 | 0.879 |
| <i>wmc177</i> (2A) | 0.125 | 24 | 115–295 | 1.598 | 0.936 | 0.932 |
| <i>wms304</i> (2A) | 0.161 | 22 | 135–350 | 1.468 | 0.920 | 0.915 |
| <i>wms369</i> (3A) | 0.286 | 20 | 180–350 | 1.354 | 0.872 | 0.863 |
| <i>wms165</i> (4A) | 0.393 | 21 | 100–290 | 1.370 | 0.823 | 0.815 |
| <i>wms186</i> (5A) | 0.732 | 8 | 85–260 | 0.468 | 0.450 | 0.434 |
| <i>wms169</i> (6A) | 0.196 | 16 | 140–265 | 1.323 | 0.890 | 0.881 |
| <i>psp3071</i> (6A) | 0.304 | 12 | 150–270 | 1.001 | 0.844 | 0.829 |
| <i>barc108</i> (7A) | 0.464 | 21 | 100–260 | 1.237 | 0.768 | 0.760 |
| <i>wmc9</i> (7A) | 0.357 | 21 | 150–300 | 1.363 | 0.846 | 0.839 |
| <i>wms260</i> (7A) | 0.304 | 18 | 100–225 | 1.420 | 0.869 | 0.861 |
| Genome A | 0.328 | 18.6 | — | 1.294 | 0.828 | 0.819 |
| <i>wms11</i> (1B) | 0.214 | 18 | 135–295 | 1.444 | 0.889 | 0.880 |
| <i>wms257</i> (2B) | 0.125 | 19 | 155–285 | 1.674 | 0.920 | 0.914 |
| <i>wms389</i> (3B) | 0.214 | 16 | 145–245 | 1.336 | 0.879 | 0.868 |
| <i>wms149</i> (4B) | 0.268 | 22 | 100–300 | 1.431 | 0.893 | 0.887 |
| <i>wms375</i> (4B) | 0.125 | 15 | 200–300 | 1.542 | 0.916 | 0.910 |
| <i>wms118</i> (5B) | 0.679 | 14 | 100–240 | 0.832 | 0.530 | 0.521 |
| Genome B | 0.271 | 17.3 | — | 1.377 | 0.838 | 0.830 |
| <i>wmc179</i> (1D) | 0.179 | 23 | 110–300 | 1.499 | 0.928 | 0.924 |
| <i>wms337</i> (1D) | 0.250 | 22 | 120–300 | 1.434 | 0.895 | 0.888 |
| <i>wms30</i> (2D) | 0.214 | 15 | 170–240 | 1.411 | 0.885 | 0.875 |
| <i>wms484</i> (2D) | 0.161 | 15 | 110–250 | 1.534 | 0.911 | 0.905 |
| <i>wms161</i> (3D) | 0.179 | 21 | 160–300 | 1.498 | 0.914 | 0.908 |
| <i>wms292</i> (5D) | 0.268 | 24 | 115–285 | 1.521 | 0.895 | 0.889 |
| <i>psp3200</i> (6D) | 0.696 | 15 | 110–460 | 0.627 | 0.508 | 0.500 |
| <i>wms295</i> (7D) | 0.214 | 14 | 185–290 | 1.155 | 0.879 | 0.867 |
| Genome D | 0.270 | 18.6 | — | 1.335 | 0.852 | 0.845 |
| Mean | 0.296 | 18.3 | — | 1.327 | 0.838 | 0.830 |

25 SSR markers did not produce any band in 56 genotypes. PIC— polymorphic information content.

The major (most numerate) allele frequencies of 25 SSR loci varied from 0.125 (*wmc177*, *wms257*, and *wms375*) to 0.732 (*wms186*), with an average of 0.296 (Table 2). The higher mean Shannon index of 1.327 emphasizes the genetic richness of the test population. The highest gene diversity value was recorded for *wmc177*, with a value of 0.936, followed by 0.928 for *wmc179*, and the lowest for *wms186*, with a value of 0.450 (Table 2). The PIC values varied from 0.434 (*wms184*) to 0.932 (*wmc177*), with a mean PIC of 0.830. A PIC value greater than 0.80 was found in 21 microsatellites with more than 10 alleles (Table 2). Although the microsatellite markers *wms118*, *psp3200*, and *barc108* contained more than 10 alleles, their PIC values ranged from 0.500 to 0.768, since many alleles might be rare.

When comparing various wheat genomes, the SSR markers from the A and D genomes produced more alleles per locus (18.6) than the SSR markers from the B genome (17.3) (Table 2). The SSR markers from genome D had the highest mean gene diversity and PIC (0.852 and 0.845, respectively), followed by genomes B (0.838 and 0.830) and A (0.828 and 0.819). SSR markers from genome B, on the other hand, produced the highest mean Shannon information index (1.377) than those from genomes D (1.335) and A (1.294).

The dissimilarity matrix revealed that the majority of the 56 wheat genotypes have significant genetic dissimilarities with others (Figure 6). Among the genotypes, BARI Gom 20, BARI Gom 26, BARI Gom 33, Kanchan, BINA wheat 1, BAW-1147, and AS-10632 were genetically the most distant from the other genotypes.

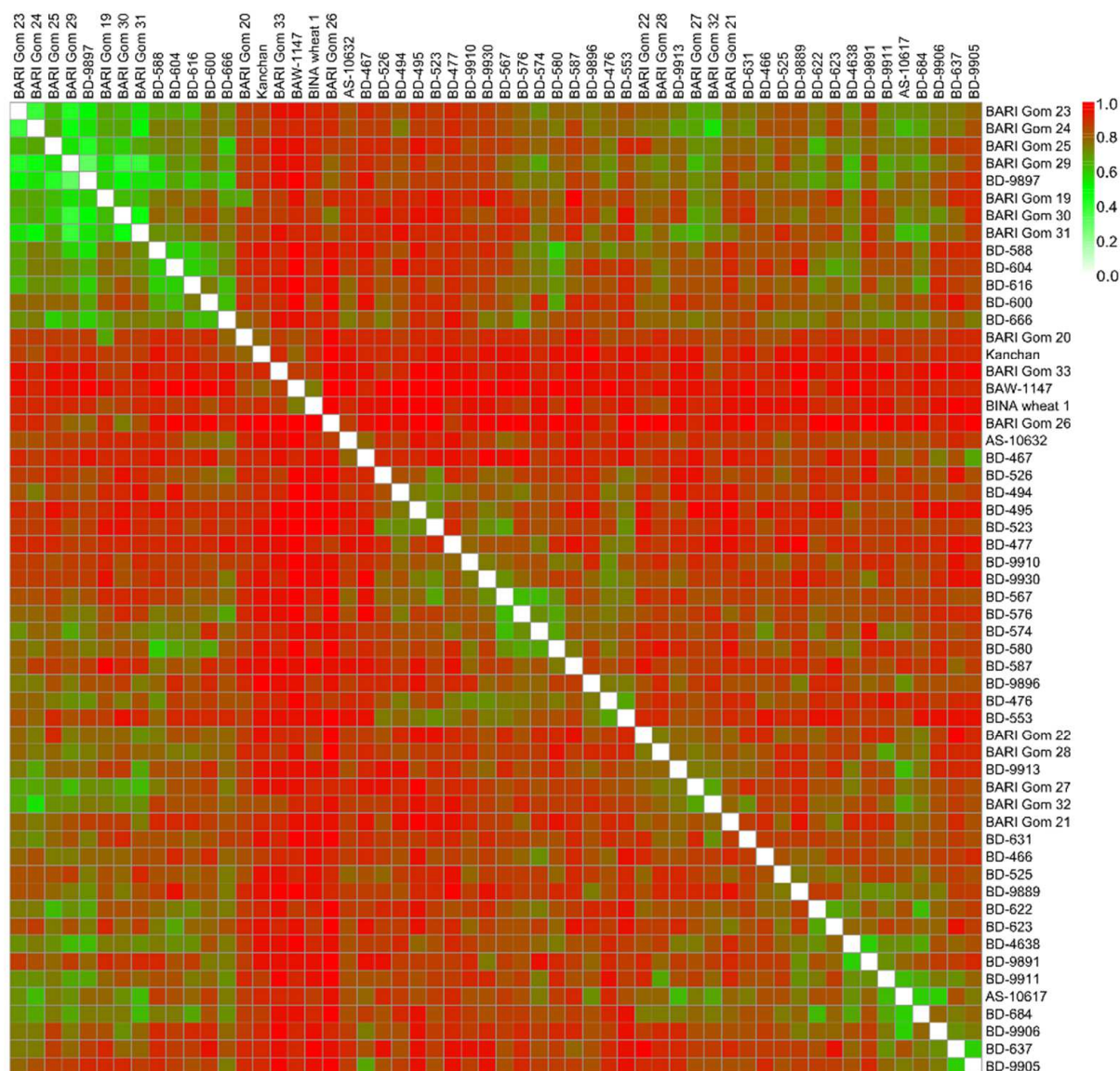


Figure 6. Dissimilarity matrix based on 25 SSR markers showing the genetic distances among the genotypes. The highest dissimilarity and the lowest genetic distance are represented by red and green colors, respectively.

3.6. Drought Tolerance Pattern and Diversity of SSR Markers

The total number of alleles, mean alleles per locus, number of cluster-specific alleles (unique alleles), and gene diversity were calculated to test the drought-tolerance pattern and genetic diversity of the three clusters (Table 3). Cluster 2 had the highest number of alleles (529) and mean alleles per locus (21.2), whereas cluster 1 (13 genotypes) had higher mean alleles per genotype (20.3) than clusters 2 (31 genotypes) and 3 (12 genotypes). Cluster 3 genotypes showed fewer total alleles (218) and mean alleles per locus (8.7). The number of unique alleles varied from 92 in cluster 3 to 215 in cluster 2 and 123 in cluster 1, accounting for 42, 41, and 47% of the total alleles detected, respectively. Cluster 1 has the most gene diversity (0.879), followed by Cluster 2 (0.849) and Cluster 3 (0.807).

Table 3. Genetic diversity across 25 loci and 56 wheat genotypes divided into three drought-tolerance clusters using VIs and yield traits.

| Category | Cluster 1 | Cluster 2 | Cluster 3 |
|---------------------------|-----------|-----------|-----------|
| Number of genotypes | 13 | 31 | 12 |
| Number of alleles | 264 | 529 | 218 |
| Mean alleles per loci | 10.6 | 21.2 | 8.7 |
| Mean alleles per genotype | 20.3 | 16.5 | 19.8 |
| Number of unique alleles | 123 | 215 | 92 |
| % unique alleles | 47 | 41 | 42 |
| Gene diversity | 0.879 | 0.849 | 0.807 |

Wright's F statistic (F_{ST}) was 0.053 (max. 0.174), but F_{IS} , F_{IT} , and N_m were 1.00, 1.00, and 4.01, respectively, despite the fact that all statistics were significant (Table 4). The pairwise F_{ST} values between the clusters were calculated and used to examine the genetic differentiation among the clusters (Table S4). The results showed that the pairwise F_{ST} values were statistically significant ($p < 0.001$) between the clusters. A higher pairwise F_{ST} value was recorded between clusters 1 and 3 (0.082), followed by clusters 1 and 2 (0.050), and clusters 2 and 3 (0.044). Despite the AMOVA being highly significant ($p < 0.001$), the among-variance component for all clusters accounted for just 5% of the overall variation, while the great majority of variation was allocated to the within-cluster component (95%) (Table 4). Based on genotyping data from 25 SSR loci, the relationships of individual genotypes were analyzed using PCoA (Figure S4A). The first two principal coordinates explained a decent amount of variance (6.2 and 4.7%, respectively). The PCoA revealed that genotypes did not generally group based on their membership in the clusters created by VIs and yield attributes, while the majority of cluster-1 genotypes were separated clearly.

Table 4. Analysis of molecular variance (AMOVA) and Wright's F statistics: effect of phenotypic clusters. * significant at $p < 0.001$.

| Source of Variation | df | Est. Variance * | % Variation |
|---------------------|-------|------------------|-------------|
| Among clusters | 2 | 0.580 | 5 |
| Within clusters | 53 | 10.306 | 95 |
| Total | 55 | 10.886 | 100 |
| F-statistics | Value | P (rand >= data) | |
| F_{ST} | 0.053 | 0.001 | |
| F_{IS} | 1.000 | 0.001 | |
| F_{IT} | 1.000 | 0.001 | |
| N_m | 4.010 | | |

3.7. Population (Pop) Structure and Diversity of SSR Markers

The harvested data from the population structure analysis clearly indicated 2 peaks ($K = 3$ and 7) near to each other. The subsequent analysis for $K = 3$ was presented, as its Pops showed relatively higher co-linearity (86.7%) with neighbor-joining clusters, and the analysis for $K = 7$ was annexed to the Supplementary Materials (Figures S3 and S6). The analysis of the population structures classified the 56 wheat genotypes into three subpopulations based on Evanno's $K = 3$ [35] (Figure 7A,B). Pop 1 had 20 genotypes, 16 of which were accessions, a mutant line and three varieties. Pop 2 consisted of 14 varieties, a mutant line, two accessions, and an advanced line, while 17 accessions were placed in Pop 3. Among the genotypes, 53 were categorized as pure, while only 3 were regarded as admixtures.

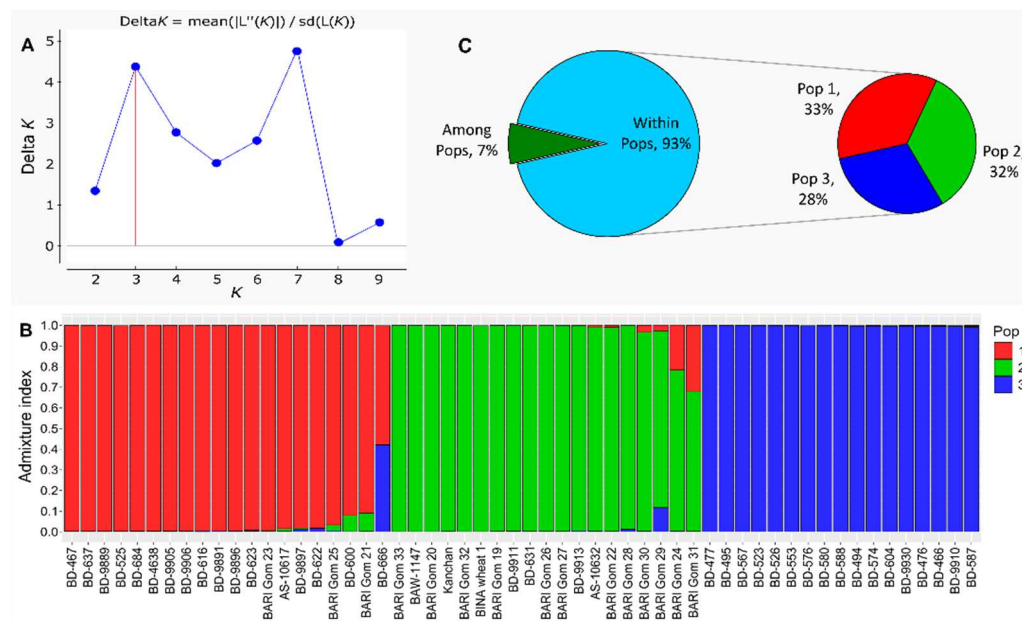


Figure 7. (A). The first peak at $K = 3$ determines the number of populations in our study, (B) Estimated population structure of 56 bread-wheat genotypes with 25 SSR markers for $K = 3$, and (C) The population obtained by the STRUCTURE-based method was used to analyze the molecular variance (AMOVA) of 56 wheat genotypes. Population structure for $K = 7$ is presented in the Supplementary materials (Figure S3).

The richness of alleles, total number of alleles, gene diversity, and the presence of unique alleles were the selected parameters to estimate the level of genetic diversity within and between populations (Table 5). The highest allelic richness (6.88) was found in population 1 (Pop 1), followed by Pop 3 (6.54) and Pop 2 (6.43). There was a substantial variation in the unique (population-specific) alleles, with 73 in Pop 2 and 106 in Pop 3, with the latter population accounting for the highest 39% of the unique alleles. The AMOVA, which was obtained using the SSR data, revealed significant genetic variations among the wheat genotypes (Figure 7C). The AMOVA showed that the among-population component accounted for 7% of the total genetic diversity, while 93% of the genetic differences were attributed to the within-population component. The within-population variance was split into 33, 32, and 28% of 3 wheat populations. There were two significant differences ($p < 0.01$) in pairwise F_{ST} values across the model-based populations (Table S5). A higher and significant pairwise F_{ST} value was recorded between Pop 1 and 2 (0.057), followed by Pop 1 and 3 (0.055), while the value between Pop 2 and 3 (0.017) was non-significant. Nonetheless, the PCoA revealed that the 56 genotypes did not actually group according to their population membership (Figure S4B).

Table 5. Genetic diversity across 25 loci and 56 wheat genotypes divided into 3 model-based populations.

| Category | Population | | |
|--------------------------|------------|-------|-------|
| | 1 | 2 | 3 |
| Number of Genotypes | 20 | 19 | 17 |
| Number of alleles | 388 | 351 | 272 |
| Mean allelic richness | 6.88 | 6.43 | 6.54 |
| Number of unique alleles | 98 | 73 | 106 |
| % unique alleles | 25.3 | 20.8 | 39.0 |
| Gene diversity | 0.827 | 0.798 | 0.834 |

3.8. Cluster Analysis of Marker-Based Allelic Data

The unweighted neighbor-joining cluster dendrogram among the genotypes was derived from the SSR-marker information (presented in Figure 8). The genotypes were divided into five primary groups using cluster analysis based on SSR data. The number of genotypes in the clusters was 8, 14, 13, 9, and 12, respectively (Figure 8). The distribution of the genotypes in the clusters might be explained by genetic descent and similar ancestry. The genetic distance between wheat genotypes ranged from 0.32 to 1.00, with an average of 0.853, showing that the genotypes have a high level of genetic diversity. The majority of the varieties were assigned to clusters I and V, whereas most of the accessions were allocated to clusters II and IV. The topologies of the phenotypic and SSR-based clusters were not identical, and genotypes from various drought tolerance groups were dispersed throughout all SSR-based clusters (Figure 8). Cluster I had the most drought-tolerant genotypes (four), followed by Cluster II (three), III (three), IV (two), and V (one).

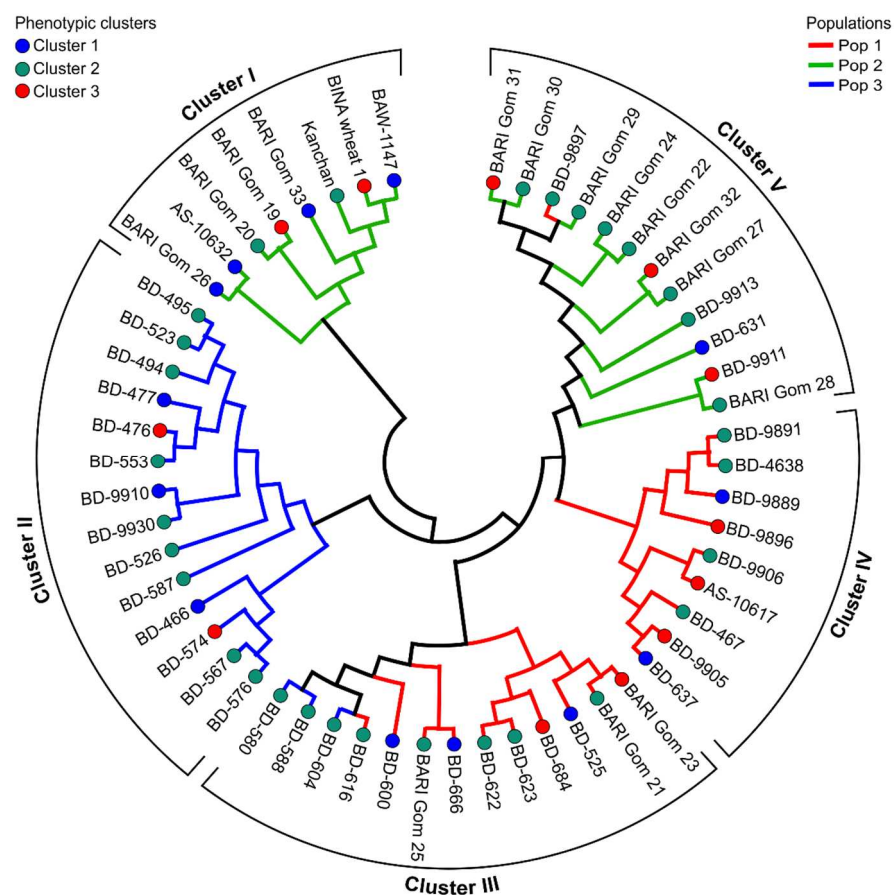


Figure 8. Unweighted neighbor-joining clustering trees of 56 wheat genotypes based on 25 SSR markers and their co-linearity with phenotypic clusters and model-based populations. Different colored circles indicate phenotypic clusters, while branch colors denote structure-based populations. Nei's genetic distance was used for neighbor-joining clustering.

3.9. Co-Linearity between SSR-Based Clusters and Model-Based Populations

A Venn diagram was used to compare the co-linearity of the model-based analysis to the unweighted neighbor-joining clusters (Figure S5). Clusters I and V together shared 95% of their genotypes with Pop 2, while cluster II shared 82.4% of its genotypes with Pop 3. Similarly, clusters III and IV shared 82.6% of their genotypes with Pop 1. Overall, the co-linearity between SSR-based neighbor-joining clusters and model-based populations was 86.7%.

4. Discussion

VIs derived from canopy spectral reflectance often indicate a quick and non-destructive technique to assess phenotypic features such as the biomass accumulation, radiation usage efficiency, and grain yield of various crops under abiotic stressors [48]. Under water-limiting conditions, the plant water status was monitored using VIs calculated from the visible, near-, and far-infrared spectra in numerous crop plants, including wheat [12,49,50]. The use of VIs as markers for phenotypic traits, biochemical properties, and grain yield is important in the collective use of canopy-reflectance data in target-environment breeding projects [12]. The current study found that most of the plant VIs declined under drought, with the exception of NCPI, ARI, and XES, which showed a significant rise across the years. A decrease in the DTH, PH, NKS, WKS, HKW, BY, and GY under drought stress in this study (Figure 2) supported the earlier reports [51–53], where a substantial drop in grain yield and yield-contributing features in rainfed bread-wheat was reported.

In the present study, SPAD values and CTD had a strong positive association with most of the VIs, suggesting that cooler canopies and higher stomatal conductance were related to improved plant health. Combining VIs, low canopy temperature, and stay-green traits such as the SPAD index may result in compounding responses that enhance stress tolerance in wheat [54], which is corroborated by the findings of the current study. The VIs evaluate the canopy's health and water status, biomass capacity, photosynthetic activity, pigment abundance, and the composition of other protectant molecules under a variety of stressful environments [12,55]. In the present investigation, the stronger associations between the VIs and the biological and grain yield imply that these indices might be used as indirect phenotypic criteria for selecting drought-tolerant genotypes under water-stressed environments. Nonetheless, in a target environment, the combination of proximally sensed VIs and morpho-physiological traits is crucial, and it has been found that VIs are substantially associated with grain yield, biomass accumulation, and leaf-area index in response to drought [12,56].

We used a hierarchical cluster analysis to select drought-tolerant genotypes rather than looking at each phenotypic trait independently. By analyzing the contribution of the phenotypic traits, the cluster analysis produced three distinct clusters from 56 wheat genotypes (Figure 4). Genotypes of cluster 1 are the best at coping with drought stress, as evidenced by the smaller changes in phenotypic traits and higher yield stability under drought, followed by clusters 2 and 3 (Figures 5 and 6). Recently, Mohi-Ud-Din et al. [28], Grzesiak et al. [57] and Islam et al. [58] employed cluster analysis to determine genotypic dissimilarity and categorization for drought tolerance in wheat.

The mean number of alleles per locus in this study was much higher in comparison with the 3.10 to 10.06 alleles reported by Arora et al. [10], Ateş Sönmezoğlu and Terzi [19], and Belete et al. [7] who evaluated the genetic diversity of bread-wheat genotypes with SSR markers. The allelic diversity in a population is influenced by the genetic composition, which in turn determines the number of alleles identified per locus [7]. The mean number of effective alleles per locus in the current study was 18.32, indicating that the SSR marker set are robust, and the diversity of the test genotypes is notable. The degree of gene diversity identified in this study was greater, correlating with the findings of Henkrar et al. [59], Ateş Sönmezoğlu and Terzi [19], and Belete et al. [7] regarding wheat genotypes. The PIC value is a reliable indicator to evaluate the genetic diversity in a plant. A PIC value higher than 0.5 denotes a higher diversity, whereas a PIC value less than 0.25 denotes a lower diversity [60,61].

A higher mean PIC value (0.830) and higher-than-0.800 PIC in 21 SSR markers (out of 25) implies the primers used in this study were highly informative and effective. In this study, the higher mean values for Shannon index, gene diversity, and PIC suggested that the genotypes demonstrated significant variability in drought tolerance. The results obtained using these SSRs are prospective markers that may be utilized to aid in selection for drought tolerance through molecular plant breeding. Furthermore, the findings are consistent with the effectiveness of SSR markers related to diversity in drought tolerance

in wheat reported by Honore et al. [62], Ateş Sönmezoğlu and Terzi [19], Slim et al. [23], Belete et al. [7], and El-Rawy and Hassan [11]. In the present study, the A and D genomes contains the highest mean numbers of alleles per locus compared to the B genome. The D genome has the highest mean gene diversity and PIC, followed by the B and A genomes, whereas the B genome has the highest Shannon index. Overall, the B and D genomes have more diversity in drought tolerance than the A genome. This finding agrees in part with recent findings by Jaiswal et al. [63], El-Rawy [64], and El-Rawy and Hassan [11], who reported substantial genetic diversity among wheat genotypes in the B genome.

The present research employed the high level of genetic information generated using SSR markers to differentiate genotypes, as presented by neighbor-joining clustering, AMOVA, PCoA, and the Bayesian-model-based population structure. The modest and considerable F_{ST} value, along with a high F_{IS} and N_m values, suggests moderate differentiation and a considerable degree of inbreeding across the clusters [65]; nonetheless, there were actual variations in genetic diversity and genotypic dissimilarity among the clusters, which might have been caused by genetic drift and/or gene flow [66]. The drought-tolerance pattern of the phenotypic clusters revealed that the pairwise F_{ST} values among the clusters were statistically significant ($p < 0.001$). In the case of the model-based populations, there were two significant differences ($p < 0.01$) in the pairwise F_{ST} values among the subpopulations. The results suggest that there was a substantial difference in genetic diversity between the phenotypic clusters as well as among most of the model-based subpopulations. This is in line with the findings of Dodig et al. [9], who reported similar results in wheat. Variance among-phenotypic clusters and among-subpopulations accounted for just 5% and 7% of overall variation, respectively. As a result, selection techniques that make use of between-population variance would only produce a limited response to selection [7]. This research, on the other hand, found greater and highly significant variability within clusters and within subpopulations. Similar findings were also reported in the genetic diversity studies of wheat by Dodig et al. [9], Arora et al. [10], Henkrar et al. [59], and Belete et al. [7]. In general, higher percentages of unique alleles were found in all phenotypic clusters (41 to 47%) and model-based populations (21 to 39%), with the drought-tolerant cluster 1 and subpopulation 3 having the largest percentage of unique alleles. It would be crucial to incorporate genotype groups with a larger percentage of unique alleles in future breeding programs in drought-prone areas in order to maximize genetic diversity [9].

The SSR-based cluster analysis indicated that genotypes from all phenotypic clusters were dispersed throughout all SSR-based clusters, implying that genetically diverse genotypes were assigned to various drought-tolerant groups. As a result, there were no marked correlations between phenotypic and SSR-based clustering, which was consistent with the findings of Dodig et al. [9] and Belete et al. [7] in wheat and Verma et al. [18] in rice. This sort of relationship is obvious, since the SSR markers employed in the current study were random SSR markers, and the phenotypic trait diversity is controlled by genotype and environmental factors [67]. Nonetheless, SSR-based clusters and model-based population groupings showed significant co-linearity (86.7%) in the present study. Singh et al. [68] and Verma et al. [18] reported similar findings in rice. The modest differences in genotype categorization in both techniques might be ascribed to differences in methodology. The findings of this study clearly demonstrate that the use of microsatellite markers is an effective approach for the differentiation of diverse wheat genotypes.

The genetic diversity of a gene pool of bread-wheat had previously been assessed by utilizing both traditional morphological and SSR-marker datasets extensively [7,9,11,23–25]. However, the application of high-throughput origin-indirect phenotypic data along with SSR markers for the genetic diversity analysis of wheat is limited. In this study, we successfully integrated canopy-reflectance-based phenotypic (VIs and yield) data with SSR-marker-based genotypic data for the evaluation of the genetic diversity of 56 wheat genotypes. The findings of the study corroborate the outcomes of prior studies that used traditional phenotypic data with SSR-marker-based genotypic data to estimate the genetic diversity of bread-wheat genotypes. Based on the findings of this study, it is plausible

to infer that the genetic bases of these genotypes revealed by canopy-reflectance-based indirect phenotypic and SSR-marker-based molecular data differ significantly, allowing wheat breeders to integrate these genetic variabilities into their breeding programs to increase the drought tolerance of wheat.

5. Conclusions

A high level of genetic diversity has been accounted for in a genotypic collection of bread-wheat by using both phenotypic and molecular data where morphological dataset alone cannot explain total variation. However, combining canopy-reflectance-based indirect phenotypic and SSR-marker-based molecular data was a strong technique for identifying divergent parents. The genetic diversity discovered among the investigated genotypes using phenotypic and genotypic data can widen the genetic basis of drought tolerance and can be used in breeding efforts to develop drought-tolerant bread-wheat varieties. Despite the phenotypic and molecular-marker data showing smaller associations, their use in breeding programs is still credible, because the information generated by the markers was complementary rather than an alternative to phenotyping. Further research with a higher number of markers is warranted to precisely infer the genetic bases and drought tolerances of the genotypes using canopy-reflectance-based phenotyping blended with marker-based genotyping.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su14169818/s1>, Figure S1: Measurement of canopy reflectance using a multispectral radiometer; Figure S2: The SSR marker profiles of bread-wheat genotypes using *wmc177* (A), *wms292* (B), *wms260* (C) and *wms186* (D) SSR primers. M indicates molecular weight. A list of 56 wheat genotypes is present in the supplementary Table S1; Figure S3: A. The peak of $K = 7$ determines the number of populations in our study, B. Estimated population structure of 56 bread-wheat genotypes with 25 SSR markers for $K = 7$, and C. The population obtained by the STRUCTURE-based method was used to analyse the molecular variance (AMOVA) of 56 wheat genotypes. Figure S4: Principal coordinate analysis (PCoA) of SSR marker allelic data for 56 wheat genotypes with respect to phenotypic clusters (A), and model-based subpopulations (B). Individuals, clusters, and populations differences are visualized in the figure. Individuals from different clusters and populations are intermixed across the coordinates; Figure S5: Venn diagram showing co-linearity between SSR-based NJ clusters and model-based populations. Overall, 86.7% co-linearity exists between NJ clusters and extracted wheat populations when $\Delta K = 3$; Figure S6: Venn diagram showing co-linearity between SSR-based NJ clusters and model-based populations. Overall, 80.6% co-linearity exists between NJ clusters and extracted wheat populations when $\Delta K = 7$; Table S1: List of wheat genotypes used in the exploratory study; Table S2: Details of SSR primers used in this study [9,69–73]; Table S3: Combined analysis of variance (mean square values) across years for mean VIs, and yield traits of bread-wheat genotypes; Table S4: Pairwise F_{ST} values among phenotypic clusters. F_{ST} values in the below diagonal and p values in the upper diagonal; Table S5: Pairwise F_{ST} values among subpopulations. F_{ST} values in the below diagonal and p values in the upper diagonal.

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