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# Association mapping of morpho-physiological traits in bread wheat under drought-stressed and non-stressed conditions

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**ABSTRACT**

Drought is one of the main abiotic stresses limiting wheat growth and productivity worldwide. The main objective of this work was to determine population structure and marker-trait association (MTA) of 13 morpho-physiological traits of bread wheat for drought-tolerance breeding. To this end, twenty-five diverse wheat cultivars and promising lines were genotyped using AFLP. The phenotype evaluation steps of studding wheat genotypes were performed under normal and drought-stress conditions during 3 years. Low heritability estimates were obtained for spike length, heading date (DTH), and shoot biomass (24.87-28.8%) while, a high heritability was observed for the number of kernels per spike (KPS) (89.21-90.55%). The results exhibited high polymorphic level ranged from 84.62 to 100%, proving that AFLP method can be an effective tool in assessing genetic variability in any wheat breeding programs. Population structure analysis showed five subpopulations with at least 65% membership ancestry to their allocated sub-clusters, which was highly consistent with the results of cluster analysis. Mixed linear method association analysis identified 66 significant MTAs with *p*-values $10^{-06}$ to $10^{-04}$, justifying 7.8 to 38.7% of the phenotypic variation, observed under both environmental conditions. There were two pleiotropic markers for grain yield (GY) and KPS under normal and one pleiotropic marker for GY, thousand kernel weight (TKW) and KPS under stress conditions. The common MTAs were detected for DTH, plant height, peduncle length, and TKW under both environmental conditions. The identified linked markers with GY and its components in this study could be desirable candidate genes for future studies and marker assisted selection to develop drought-tolerant genotypes in wheat breeding programs.

***Key words:*** Bread wheat, Drought tolerance, Marker-trait association, Structure analysis.

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## INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is the most important crop in terms of production worldwide cultivated in 220.7 million hectares (FAO, 2021), mainly grown in arid and semi-arid regions of the world where water deficit is causing significant performance declines (Bijalwan *et al.,* 2022). Drought stress is one of the most challenging environmental stresses that has limited the survival and growth of wheat in arid and semi-arid regions (Sallam *et al.,* 2019). Thus, it is important to improve drought tolerance and detection of genomic loci and basic candidate genes associated with drought tolerance in wheat (Bhatta *et al.,* 2018).

Drought tolerance is a complex quantitative trait, controlled by multiple genes and plant traits, with minor effects (Serba and Yadav, 2016). Due to the low heritability of drought tolerance, selection for this feature should be carried out in more than one year and/or location in the target environments (Sallam *et al.,* 2019). To obviate the low heritability of drought tolerance, plant breeders used DNA molecular markers in their programs with good effect on improving drought tolerance in cereals (Thabet *et al.,* 2018). The AFLP is a PCR-based fingerprinting technique, developed by Vos *et al.* (1995). The advantages of this method include considerable repeatability (Jones *et al.,* 1997; Paun and Schonswetter, 2012; Reshma and Das, 2021), simultaneous study of multiple loci, needless to initial information for primer design, total genome investigation capacity to represent polymorphism and produce a large number of repeatable bands over short periods of time (Vos *et al.,* 1995; Zhu *et al.,* 2013). Producing a large number of polymorphic loci in AFLP, could balance the lack of information caused by its dominant nature (Paun and Schonswetter, 2012). Kumar *et al.* (2015) stated that AFLP is an indicator of genetic classification, developing of linkage maps, mapping of essential traits and assigning parentage.

Despite the fact that valuable genetic information exists from factors involved in drought tolerance in plants, as a result of the identification of QTLs after the development of molecular markers, but in many studies known QTLs have a high genetic distance with their flanking markers. This results from many factors such as the lack of saturation of the linkage map and the low segregation of individuals in the studied synthetic populations. These factors are limiting the use of marker assisted selection (MAS) in the breeding programs and the gene cloning based on the map. Recently, in order to overcome respective limitations, association analysis method has been introduced that not only allows for the accurate locating of genes and QTLs, but also identifies other chromosomal locations that are not possible in linkage-base studies. In this method, development of a segregating population that requires a great deal of time is not necessary, but it is better to use multi-year phenotypic data (Wang *et al.,* 2017). The association analysis that is also common to LD analysis has significant advantages over linkage analysis. Firstly, due to using natural population in such studies, a greater genetic diversity is observed compared to bi-parental population. Secondly, depending on the population, LD analysis has a much higher accuracy, because in this method all meiosis events that are accumulated during the evolutionary history of the plant are considered (Zhang *et al.,* 2016). It is important to conclude associations between markers and traits to develop utilization of prevalent breeding methods. Association analysis between different traits and markers under contrasting moisture regimes has been performed by plant breeders in bread wheat (Ayalew *et al.,* 2018; Sukumaran *et al.,* 2018a; Bhatta *et al.,* 2018; Khalid *et al.,* 2019; Lin *et al.,* 2019; Mathew *et al.,* 2019; Maulana *et al.,* 2020; Merida-Garcia *et al.,* 2020; Liu and Qin, 2022). A number of functional markers have been identified for important genes in wheat such as, genes associated with grain yield and related traits (Mwadzingeni *et al.,* 2017; Qaseem *et al.,* 2018; Liu *et al.,* 2019; Marzougui *et al.,* 2019; Ballesta *et al.,* 2020; Zhu *et al.,* 2020; Hu *et al.,* 2021; Govta *et al.,* 2022; Rabieyan *et al.,* 2022; Said *et al.,* 2022; Firouzian *et al.,* 2023). However, the rarity of genetic markers and limited investigations on MTAs for physiological traits such as RWC (Abou-Elwafa, 2016; Khalid *et al.,* 2019; Lin *et al.,* 2019; Ahmed *et al.,* 2022) and flag leaf area (Bhatta *et al.,* 2018; Ahmed *et al.,* 2021) despite their significant consequences for drought tolerance as well as, the inherent complexities of assessing drought stress and its related responses (Verslues *et al.,* 2014; Kang *et al.,* 2015; Bac-Molenaar *et al.,* 2016), prevent the use of MAS in expanding breeding populations for drought tolerance in bread wheat. Several recent studies used AFLP for identification of markers associated with important traits in multiple plants including tobacco, sugarcane, safflower, chickpea, pea, cumin, maize, durum wheat, proso millet and *Psammochloa villosa* (Trin.) (Dadras *et al.,* 2014; Gouy *et al.,* 2015; Kumar *et al.,* 2015; Ebrahimi *et al.,* 2017; Saeed and Darvishzadeh, 2017; El-Esawi *et al.,* 2018; Archangi *et al.,* 2019; Giordani *et al.,* 2019; Roncallo *et al.,* 2019; Yazdizadeh *et al.,* 2020; Lv *et al.,* 2021).

Although the association analysis has been used

to identify suitable alleles for different traits in bread wheat but there are few reports using this method in wheat, especially for multi-environment data including water-stress conditions in Iranian wheat genotypes. Accordingly, the objectives of the present study were to characterize the population structure within wheat genotypes and to identify AFLP markers associated with yield-related characteristics as well as RWC and RWL using association analysis for future marker-assisted breeding to improve drought tolerance in wheat.

## MATERIALS AND METHODS

### Plant materials and phenotyping

Field trials were conducted in the Miandoab Agricultural Research Station, West Azerbaijan Province, Iran, located at 36°58′ N and 46°06′ E. The soil texture of this site was loamy silt with pH 7.9 and the soil field capacity (FC) at a depth of 30 cm was 28.7. Climatic parameters are shown in supplementary Figure 1 (Figure S1). A total of twenty five diverse wheat genotypes containing cultivars and promising lines (Table 1), were included in the drought tolerance study. These lines were developed by several breeders at various research stations/institutes of Iran and the International Maize and Wheat Improvement Center (CIMMYT).

Test materials were evaluated phenotypically comprising two trials under non-stressed (well-watered) and water-deficit stressed (rain-fed) conditions. Each field experiments were arranged based on a randomized complete blocks design (RCBD) with three replications and conducted over three cropping seasons (2014/2015, 2015/2016 and 2016/2017). The total rainfall during the cropping seasons of 2014/2015, 2016/2015 and 2016/2017 were 298.6, 306.4 and 185 mm, respectively, with an average of 263.3 mm, which compared to the long-term average, there was a decrease of 9.21% (Figure S1). Under non-stress conditions, the genotypes were irrigated when mean soil water content fell to 80% of FC. Each plot consisted of six rows, 4-m-long and 20 cm row spacing. Farm management advice for each environment was followed in every yield experiment. In each trial, evaluations were carried out for the following traits according to assigned protocols (Pask *et al.,* 2012): Number of days to 50% heading (DTH,

**Table 1.** Codes and the pedigree of diverse wheat cultivars and Promising lines used in this study.

| Code | Pedigree/Name | Type | Origin |
|---|---|---|---|
| G1 | Varan | Cultivar | IRAN |
| G2 | Rasad | Cultivar | IRAN |
| G3 | Azar 2 | Cultivar | IRAN |
| G4 | Sardari | Cultivar | IRAN |
| G5 | Unknown 11 | Promising line | IRAN |
| G6 | Saein | Cultivar | IRAN |
| G7 | Seafalah/3/Sbn//Trm/K253 | Promising line | IRAN |
| G8 | F10S-1//ATAY/GALVEZ87 | Promising line | IWWIP |
| G9 | Sardari-101 | Promising line | IRAN |
| G10 | Azar2/87Zhong291-149 | Promising line | IRAN |
| G11 | Homa | Cultivar | IRAN |
| G12 | Ohadi | Cultivar | IRAN |
| G13 | Sabalan/4/Vrz/3/Or F1.148/Tdl//Blo | Promising line | IRAN |
| G14 | Sabalan//Cno79/Prl"S"/3/Pf82200/4/Ebvd99-1 | Promising line | IRAN |
| G15 | SARDARI-HD84//UNKN/HATUSHA | Promising line | IRAN |
| G16 | F130-L-1-12/LAGOS | Promising line | IWWIP |
| G17 | Sara-PBWYT-85-86-22-5 | Promising line | IWWIP |
| G18 | PYN/BAU//BONITO | Promising line | IWWIP |
| G19 | Sabalan/84.40023//Seafallah | Promising line | IRAN |
| G20 | SUBEN-7 | Promising line | IWWIP |
| G21 | Azar2/78Zhong291-99 | Promising line | IRAN |
| G22 | Sardari//Ska/Aurifen | Promising line | IRAN |
| G23 | TIRCHMIR1/LCO//SABALAN | Promising line | IWWIP |
| G24 | TAST/TORIM/3/MLC/4/CWW339.5/SPN/5 | Promising line | IWWIP |
| G25 | BJN C 79/4/KVZ/CUT75/3/YMH//61.15 | Promising line | IWWIP |

IWWIP: International Winter Wheat Improvement Program.

**Table 2.** Description of primer combinations used for AFLP analysis in wheat genotypes.

| Restriction Enzyme | *Eco*RI | *Mse*I |
|---|---|---|
| Adapters | 5'-CTCGTAGACTGCGTACC-3'<br>3'-CTGACGCATGGTTAA-5' | 5'-GACGATGAGTCCTGAG-3'<br>3'-TACTCAGGACTCAT-5' |
| Pre-selective amplification | 5'-GACTGCGTACCAATTCA-3' | 5'-GATGAGTCCTGAGTAAC-3' |
| Selective amplification | E-AGG:5'-GACTGCGTACCAATTCAGG-3' | M-CTT:5'-GATGAGTCCTGAGTAACTT-3' |
| | E-AGC:5'-GACTGCGTACCAATTCAGC-3' | M-CTT:5'-GATGAGTCCTGAGTAACTT-3' |
| | E-ACT:5'-GACTGCGTACCAATTCACT-3' | M-CTC:5'-GATGAGTCCTGAGTAACTC-3' |
| | E-AGG:5'-GACTGCGTACCAATTCAGG-3' | M-CTC:5'-GATGAGTCCTGAGTAACTC-3' |
| | E-ACG:5'-GACTGCGTACCAATTCACG-3' | M-CTG:5'-GATGAGTCCTGAGTAACTG-3' |
| | E-AGG:5'-GACTGCGTACCAATTCAGG-3' | M-CTG:5'-GATGAGTCCTGAGTAACTG-3' |
| | E-ACT:5'-GACTGCGTACCAATTCACT-3' | M-CTT:5'-GATGAGTCCTGAGTAACTT-3' |
| | E-ACG:5'-GACTGCGTACCAATTCACG-3' | M-CAA:5'-GATGAGTCCTGAGTAACAA-3' |

day), plant height (PH, cm), flag leaf area (FLA, $cm^2$), spike length (SL, cm), dry weight per spike (DWPS, g), number of kernels per spike (KPS), thousand kernel weight (TKW, g), peduncle length (PL, cm), peduncle weight (PW, g), shoot biomass (SB, kg $ha^{-1}$) and grain yield (GY, kg $ha^{-1}$). For the evaluation of physiological traits, after anthesis stage, fresh leaves were taken from each genotype and weighed instantly to record fresh weight (FW). Then leaves were soaked in distilled water for 4 h at 25 °C and reweighed to record turgid weight (TW), and oven-dried for 48 h at 72 °C to obtain the dry weight (DW). The relative water content (RWC, %) and relative water loss (RWL, gr/gr.hr) were calculated, as explained by Ritchie *et al.* (1990) and Yang *et al.* (1991), respectively:

$$(1) \qquad \mathrm{RWC} = \{(\mathrm{FW} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW})\} \times 100$$

$$(2) \qquad \mathrm{RWL} = (\mathrm{FW} - \mathrm{ADW})/(\mathrm{t} \times \mathrm{DW})$$

Where ADW is wilt weight after 2 h at 30 °C, and t is the time in hour at the wilt weight.

**DNA extraction and AFLP analysis**

Young leaf tissues of four-week-old seedlings were used for genomic DNA extraction following the CTAB method described by Saghai Maroof *et al.* (1984). The DNA concentration in each sample was adjusted to a working solution of 50 ng $\mu L^{-1}$. The DNA samples were then exposed to AFLP analysis using the protocol explained by Vos *et al.* (1995) with some modifications. Based on the polymorphism information content and the effective multiplex ratio, eight AFLP primer pairs displayed a higher variability from twenty primer sets and were employed to assess the genotypes (Table 2). Genomic DNA (500 ng) were digested with 5U of each *Eco*RI (Tag Copenhagen A/S, Frederiksberg, Denmark; 12 h at 37 °C) and *Mse*I (Tag Copenhagen A/S, Frederiksberg, Denmark; 12 h at 65 °C) restriction enzymes. The restricted DNA fragments were ligated via $T_4$ DNA ligase (1U/μL) to adapters with the known sequences *Eco*RI F (5'-CTCGTAGACTGCGTACC-3'), *Eco*RI R (3'-CTGACGCATGGTTAA-5'), *Mse*I F (5'-GACGATGAGTCCTGAG-3') and *Mse*I R (3'-TACTCAGGACTCAT-5') at 22 °C for 1 h. The adaptor-ligated DNA was diluted to 1:5 by water and was used for pre-selective amplification with *Eco*RI and *Mse*I primers containing one selective base at the 3′ end (*Eco*RI-A and *Mse*I-C). Selective amplification was performed using diluted DNA from the pre-amplification reaction and eight *Eco*RI/*Mse*I primer sets (Table 2). The amplification PCR was carried out under the following conditions: After an initial denaturation step at 94 °C for 2 min, 13 cycles of 94 °C for 30 s, 65 °C for 30 s as touchdown with 0.7 °C lowering for each cycle, and 72 °C for 60 s. The PCR was followed by a subsequent 23 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s, and one final cycle of extension at 72 °C for 5 min.

The amplified fragments were transferred directly from the thermocycler into the QIAxcel System (QIAGEN, Hilden, Germany) and analyzed using the QIAxcel DNA High Resolution Kit on the system with the 0M700 method. The QIAxcel system is able to separate fragments of 12 DNA samples at 3 min in high resolution without the need for agarose. The AFLP bands were scored for presence as (1) and absence (0) via BioCalculator software (v. 3.2; QIAGEN) and only bands showing clear polymorphism were used to make a binary data matrix.

**Analysis of phenotypic data**

Phenotypic data were exposed to the Kolmogorov–

Smirnov test for normality in SPSS v. 19 software. Analysis of variance (ANOVA) was performed using Genstat 14th edition to assign the main effects and interactions using F test ($P$<0.05). Descriptive statistics along with phenotypic coefficient of variation ($CV_p$) and genotypic coefficient of variation ($CV_g$) were calculated in Genstat 14th. The $H^2$ values for each trait under both environments were computed according to Nyquist (1991) as follows:

$$(3) \qquad H^2 = \delta_g^2 / \left(\delta_g^2 + \delta_{gy}^2/y + \delta_e^2/ry\right)$$

Where $\sigma^2_g$, $\sigma^2_{gy}$ and $\sigma^2_e$ are the variance components for genotype, genotype×year, and residual, respectively, while y and r are the number of years and replications per year, respectively.

**Analysis of molecular data**

For each primer pair, total amplified bands, number of polymorphic bands and the percentage of polymorphic bands (PPB) were calculated. AFLP markers were used to evaluate genetic diversity indices under the assumption that populations were in Hardy-Weinberg equilibrium (HWE), such as polymorphic information content (PIC) (Roldan-Ruiz *et al.,* 2000), the effective number of alleles per locus (Ne) (Hartl and Clark, 1989), marker index (MI) (Varshney *et al.,* 2007), Nei's gene diversity or heterozigosity (He) (Nei, 1973) and Shannon's Information Index (I) (Lewontin, 1972) using GenAlEx v. 6.503 software (Peakall and Smouse, 2012). To evaluate the variance within and among subpopulations derived from structure analysis, an analysis of molecular variance (AMOVA) was carried out using GenAlEx with 999 permutations. The PhiPT statistics were computed to determine the genetic differences among subpopulations as follows:

$$(4) \qquad PhiPT = AP/(WP + AP)$$

Where AP and WP are the estimates of genetic diversity among and within subpopulations, respectively.

**Genetic relatedness among genotypes and population structure**

Analysis of population structure based on data from AFLP markers was investigated using the model-Bayesian STRUCTURE v. 2.3.4 software (Pritchard *et al.,* 2000) considering admixture model and correlated allele frequencies. Parameters were set at burn-in period of 10,000 and 100,000 Markov Chain Monte Carlo (MCMC) repetitions after burn-in. The membership of any genotype was run for the range of genetically distinctive clusters (K) from 1 to 10 with five iterations for each K. To decrease the risk of spurious positive associations, the best K-value with the highest likelihood was assigned for evaluating an appropriate population size for the dataset (Gupta *et al.,* 2014). Thus, the true number of K using the ΔK approach described by Evanno *et al.* (2005) was determined by STRUCTURE HARVESTER (Earl and VonHoldt, 2012). Finally, based on the optimal K determined by Evanno method, the Q matrix (population structure matrix) was extracted from the population structure results.

Alternately, a cluster analysis was performed to assess the genetic relatedness between genotypes using a distance-based method. For this purpose, the simple matching (SM) similarity matrix was subjected to classify the studied wheat genotypes using NTSYS-pc, v. 2.02 based on unweighted pair group method with arithmetic means (UPGMA), complete linkage, and neighbor-joining with 1,000 bootstrap replicates algorithms. Finally, the complete linkage clustering was used since it kept a justly stable topology over the different distance matrices that were tested.

**Association analysis**

The association analysis among AFLP marker alleles and phenotypic traits (P-matrix) measured under non-stressed and drought-stressed conditions was performed using TASSEL v. 4.2.1 software (Bradbury *et al.,* 2007) via both mixed linear method (MLM) and general linear method (GLM) (Yu *et al.,* 2006). The Q-matrix resulted from structure analysis (at maximum ΔK) was used like a covariate to correct population structure in both methods. Moreover, the kinship matrix (K matrix) based on the results of marker data obtained from the SPAGeDi program (Hardy and Vekemans, 2002) was used in the MLM (Q+K). The phenotypic variation explained per each marker ($R^2$) was calculated to assign the fraction of the total variation justified by the marker. The markers with minor allele frequency (MAF) <0.05 were not considered for the analysis as previously explained by Mwadzingeni *et al.* (2017). The significant threshold for association among loci and traits was set at $P$<0.001 and false discovery rate of 5%, which was supposed to be very strict to decrease the risk of spurious marker trait associations (Sukumaran *et al.,* 2018a).

## RESULTS

**Phenotypic variation between genotypes and contrasting moisture regimes**

The results of combined ANOVA for morpho-

**Table 3.** Mean squares after combined analysis of variance for morpho-physiological traits of wheat genotypes investigated over three years under two different moisture regimes.

| Source of variation | df | Mean of square | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DTH | PH | FLA | SL | KPS | DWPS | PL | PW | TKW | SB | GY | RWC | RWL |
| Year (Y) | 2 | 570.61** | 765.51 | 58.49 | 8.53* | 29.24 | 0.03 | 100.97 | 0.02 | 249.73** | 5792296.89 | 869319.63** | 314.45 | 0.012* |
| Moisture regime (M) | 1 | 610.28** | 5404.85 | 3.15 | 89.11* | 4378.28* | 5.81* | 1266.10 | 1.04** | 5539.64* | 777035496.34* | 4459185 6.83** | 3888.12* | 0.054* |
| Y×M | 2 | 2.36* | 1415.94* | 9.47 | 5.48 | 57.81 | 0.02 | 108.78 | 0.02 | 180.48* | 8967671.9 | 72475.61 | 229.35 | 0.001 |
| R (Y×M) | 12 | 0.46 | 216.42 | 76.46 | 1.37 | 48.09 | 0.03 | 30.30 | 0.02 | 27.87 | 13267081.32 | 1199714.28 | 98.39 | 0.002 |
| Genotype (G) | 24 | 12.74 | 2635.75* | 168.66 | 2.87** | 652.87** | 0.13** | 343.43** | 0.17** | 335.57* | 15756404.74* | 6295561.24** | 690.79* | 0.021** |
| G×Y | 48 | 20.94** | 1010.77** | 83.52 | 3.72** | 42.33** | 0.02 | 57.65** | 0.01 | 110.96** | 9304975.16* | 1986267.49** | 222.36** | 0.005** |
| G×M | 24 | 2.30* | 167.80 | 92.21 | 1.5* | 88.50** | 0.07** | 22.28* | 0.02** | 12.28 | 6908562.76** | 1451751.18** | 56.80* | 0.017** |
| G×Y×M | 48 | 0.97 | 86.03 | 15.61 | 0.43 | 7.48 | 0.004 | 9.22 | 0.002 | 7.03 | 878668.05 | 265680.94 | 25.00 | 0.004 |
| Residual | 288 | 0.61 | 98.93 | 16.79 | 0.5 | 11.40 | 0.03 | 12.61 | 0.01 | 7.10 | 3491337.19 | 317612.71 | 34.93 | 0.002 |
| Coefficient of variation (%) | | 11.2 | 8.34 | 26.62 | 7.16 | 10.43 | 24.74 | 8.76 | 19.16 | 6.41 | 16.28 | 11.25 | 7.28 | 16.54 |

df: Degrees of freedom, DTH: Number of days to heading, PH: Plant height, FLA: Flag leaf area, SL: Spike length, KPS: Number of kernels per spike, DWPS: Dry weight per spike, PL: Peduncle length, PW: Peduncle weight, TKW: Thousand kernel weight, SB: Shoot biomass, GY: Grain yield, RWC: Relative water content, RWL: Relative water loss.

*, **: Significant at 5% and 1% level of probability, respectively.

physiological traits showed the year, genotype and moisture regimes effects significantly influenced SL, TKW, GY and RWL (Table 3). Except for PH and TKW, the significant genotype×moisture regime interaction on all traits was observed indicating genotypes responded differently to non-stressed and drought-stressed conditions. The genotype×year interaction was significant on all traits except DWPS and PW. The experimental CV ranged from 6.41 to 26.62 that except for DWPS and FLA, the most values were less than 20%.

The SB varied from 10058 to 14291 kg $ha^{-1}$ under non-stressed, whereas it ranged between 5014.9 and 9949.4 kg $ha^{-1}$ under drought-stressed conditions (Table 4). A 39.4% decline in mean SB due to drought stress was obtained. The highest RWC obtained was 91.44% under non-stressed conditions, while the lowest obtained was 57.72% under stressed conditions. On mean, GY reduced by 34% under drought conditions. The estimated $H^2$ indicated that the highest and lowest $H^2$ were found for KPS (89.21% under non-stressed and 90.55% under stressed conditions) and SB (28.8% under non-stressed and 24.87% under stressed conditions), respectively. The broad-sense heritability obtained for grain yield was 68.46% under non-stress and 37.50% under rain-fed conditions (Table 4).

**AFLP Genotyping**

The eight AFLP primer sets in the wheat genotypes produced 127 clear and reliable bands with a mean of 15.88 bands per primer, of which 119 bands were polymorphic (Table 5). The polymorphism percentage ranged from 84.62% (E-AGC/M-CTT and E-ACT/M-CTC) to 100% (E-AGG/M-CTC, E-AGG/M-CTG and E-ACG/M-CAA) with an average of 93.14%. To recognize the most instructive AFLP primer pair, the PIC values were computed for each primer combination that varied from 0.267 for E-AGC/M-CTT to 0.351 for E-ACT/M-CTT with a mean of 0.298 (Table 5). Another criterion for assessing the efficiency of markers for determining polymorphism is the Shannon's Index, which the highest and lowest values were assigned to E-ACT/M-CTT (0.543) and E-AGC/M-CTT (0.365) primer sets, respectively. In the present study, the Shannon index was positively correlated with PIC (r=0.867, $P<0.01$). MI ranged between 2.08 (E-AGG/M-CTC) and 6.59 (E-AGG/M-CTT) with a mean value of 4.07 per combination. The primer combination E-ACT/M-CTT had the maximum He (0.368), while E-ACG/M-CAA had the minimum one.

**Table 4.** Summary statistics and heritability estimates of morpho-physiological traits calculated in wheat genotypes across three cropping seasons under drought-stress and non-stress conditions.

| Trait | Conditions | Mean | Min | Max | SEM | CVg (%) | CVp (%) | $H^2$ (%) |
|---|---|---|---|---|---|---|---|---|
| DTH | NS | 193.09 | 191.02 | 195.18 | 0.35 | 1 | 1.06 | 88.53 |
| | DS | 180.88 | 179.85 | 182.3 | 0.21 | 0.61 | 0.75 | 64.71 |
| PH | NS | 117.54 | 100.46 | 135.06 | 3.29 | 6.56 | 9.64 | 46.31 |
| | DS | 101.38 | 80.27 | 128.43 | 4.63 | 13.02 | 16.21 | 64.47 |
| FLA | NS | 15.53 | 5.70 | 29.40 | 0.61 | 21.78 | 26.96 | 50.14 |
| | DS | 15.24 | 3.27 | 45.63 | 0.91 | 33.68 | 41.10 | 67.86 |
| SL | NS | 10.41 | 5.99 | 14.70 | 0.20 | 8.10 | 9.98 | 65.81 |
| | DS | 9.24 | 3.80 | 14.97 | 0.21 | 6.44 | 7.75 | 67.53 |
| KPS | NS | 35.61 | 22.84 | 44.08 | 2.34 | 21.93 | 23.22 | 89.21 |
| | DS | 24.08 | 13.33 | 36.72 | 1.74 | 29.31 | 30.81 | 90.55 |
| DWPS | NS | 1.38 | 0.67 | 2.39 | 0.04 | 16.52 | 20.54 | 64.71 |
| | DS | 0.85 | 0.40 | 1.64 | 0.03 | 18.21 | 21.76 | 70 |
| PL | NS | 40.78 | 32.36 | 47.96 | 1.5 | 11.21 | 13.08 | 73.53 |
| | DS | 33.68 | 27.91 | 40.81 | 1.42 | 12.98 | 16.41 | 62.60 |
| PW | NS | 0.58 | 0.42 | 0.86 | 0.04 | 23.71 | 25.88 | 83.89 |
| | DS | 0.4 | 0.26 | 0.61 | 0.03 | 20.61 | 30.56 | 45.45 |
| TKW | NS | 44.75 | 35.38 | 50.48 | 1.55 | 10.05 | 11.16 | 82.54 |
| | DS | 31.65 | 25.39 | 37.52 | 1.29 | 12.67 | 15.34 | 68.24 |
| SB | NS | 11797 | 10058 | 14291 | 408.36 | 10.09 | 18.8 | 28.80 |
| | DS | 7151 | 5014.9 | 9949.4 | 384.73 | 15.01 | 30.09 | 24.87 |
| GY | NS | 3418.87 | 2454.94 | 4742.87 | 218.55 | 25.28 | 29.32 | 68.46 |
| | DS | 2254.88 | 1700.39 | 3057.83 | 134.29 | 10.31 | 24.18 | 37.50 |
| RWC | NS | 80.66 | 71.54 | 91.44 | 2.09 | 8.63 | 10.13 | 72.53 |
| | DS | 68.92 | 57.72 | 76.91 | 1.86 | 7.40 | 11.73 | 39.78 |
| RWL | NS | 0.228 | 0.105 | 0.379 | 0.022 | 7.93 | 10.68 | 66.17 |
| | DS | 0.368 | 0.173 | 0.652 | 0.038 | 6.88 | 9.08 | 52.55 |

DTH: Number of days to heading, PH: Plant height, FLA: Flag leaf area, SL: Spike length, KPS: Number of kernels per spike, DWPS: Dry weight per spike, PL: Peduncle length, PW: Peduncle weight, TKW: Thousand kernel weight, SB: Shoot biomass, GY: Grain yield, RWC: Relative water content, RWL: Relative water loss, NS: Non-stressed conditions, DS: Drought-stressed conditions, Min: Minimum value, Max: Maximum value, SEM: Standard error of mean, CVg: Genotypic coefficient of variation, CVp: Phenotypic coefficient of variation, $H^2$: Broad sense heritability.

**Table 5.** Genetic variation statistics generated by AFLP primer combinations in wheat genotypes.

| Primer combination | TB | PB | PPB | Ne | PIC | Rp | MI | I | EMR | He |
|---|---|---|---|---|---|---|---|---|---|---|
| E-AGG/M-CTT | 28 | 26 | 92.86 | 1.42 | 0.273 | 9.658 | 6.59 | 0.396 | 24.14 | 0.252 |
| E-AGC/M-CTT | 13 | 11 | 84.62 | 1.37 | 0.266 | 4.162 | 2.48 | 0.365 | 9.31 | 0.228 |
| E-ACT/M-CTC | 13 | 11 | 84.62 | 1.50 | 0.269 | 4.166 | 2.504 | 0.45 | 9.31 | 0.294 |
| E-AGG/M-CTC | 6 | 6 | 100 | 1.60 | 0.347 | 3.334 | 2.083 | 0.508 | 6 | 0.342 |
| E-ACG/M-CTG | 17 | 16 | 94.12 | 1.40 | 0.3 | 6.998 | 4.523 | 0.404 | 15.06 | 0.255 |
| E-AGG/M-CTG | 20 | 20 | 100 | 1.35 | 0.277 | 7.5 | 5.542 | 0.372 | 20 | 0.229 |
| E-ACT/M-CTT | 9 | 8 | 88.89 | 1.64 | 0.351 | 4.334 | 2.493 | 0.543 | 7.11 | 0.368 |
| E-ACG/M-CAA | 21 | 21 | 100 | 1.45 | 0.3 | 8.666 | 6.306 | 0.416 | 21 | 0.268 |
| Total | 127 | 119 | | 11.73 | | | | | | |
| Mean | 15.88 | 14.88 | 93.14 | 1.47 | 0.298 | 6.102 | 4.065 | 0.432 | 13.99 | 0.28 |

TB: Total bands; PB: Polymorphic bands; PPB: Percentage of polymorphic bands; Ne: Effective number of alleles; PIC: Polymorphic information content; Rp: Resolving Power; MI: Marker index; I: Shannon's information index; EMR: Effective multiplex ratio; He: Nei's gene diversity or heterozygosity.

**Genetic structure analysis**

The admixture model-based Bayesian cluster analysis using STRUCTURE program was employed with 119 AFLP polymorphic bands to investigate the nature of genetic relationships among genotypes. The bilateral charts for determining K value (the number of appropriate clusters) was shown in Figure S2. As presented in Figure S2, ΔK parameter resulted with the method offered by Evanno *et al.* (2005) was highest at K=5, revealing five main clusters in the population. Membership of each individual to a special subpopulation was based on at least 65% ancestry (Mathew *et al.,* 2019) otherwise, it was defined as 'admixed' genotype.

Figure 1A displays the population structure for K=5 where each color shows a distinct genetic cluster. The membership probability (Q matrix) of each genotype to each sub-cluster for the K=5 is presented in Table 6. Of all genotypes, 88% were allocated into the relevant subgroups, and the rest of them were classified into the 'admixed' genotypes based on their Q-values. Sub-cluster 1 had the highest membership with 28% of the population, whereas the lowest was sub-cluster 5 only with 8%. Average distances (expected heterozygosity) between individuals in the same cluster ranged from 0.12 (cluster 1) to 0.28 (cluster 2). Clusters 5 and 2 represented the highest (0.56) and the lowest (0.06) level of the mean fixation index ($F_{st}$) among clusters, respectively.

Population structure in the wheat genotypes was also studied by means of cluster analysis based on complete linkage method using SM similarity coefficient that classified the studied genotypes into five clusters (Figure 1B). The calculated cophenetic coefficient was 0.803 revealing a high correlation among similarity matrix and dendrogram and displays the complete

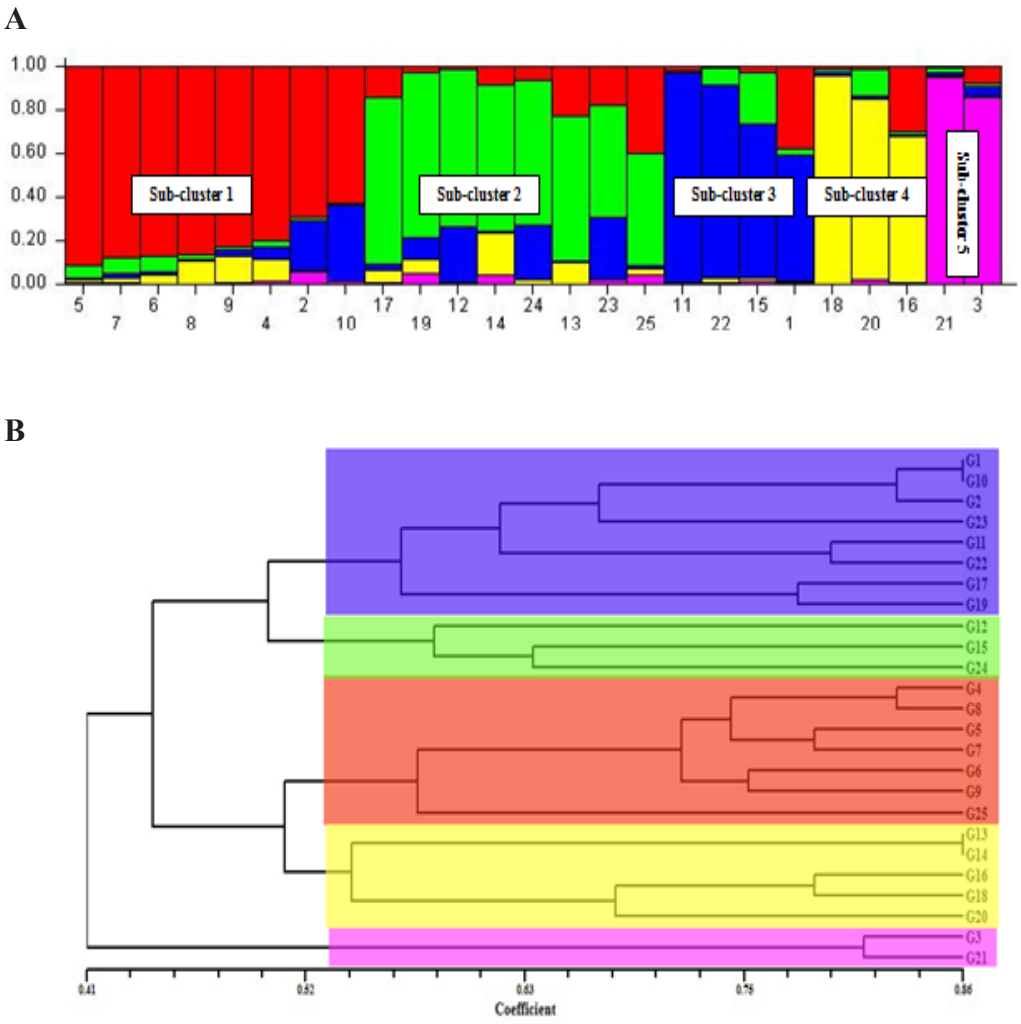


**Figure 1.** Comparison of population structure resulted from **A:** Bayesian model (STRUCTURE) and **B:** complete linkage cluster analysis based on SM distances using AFLP markers in the bread wheat genotypes.
See Table 1 for genotypes characteristics.

**Table 6.** Five sub-clusters with their member genotypes, proportion of membership, expected heterozygosity, and the mean fixation values obtained from structure analysis.

| Sub-cluster | Genotypes* | Membership (%) | Expected heterozygosity | Mean fixation index (Fst) |
|---|---|---|---|---|
| 1 | G2, G4, G5, G6, G7, G8, G9 | 28 | 0.12 | 0.38 |
| 2 | G12, G13, G14, G17, G19, G24 | 24 | 0.28 | 0.06 |
| 3 | G11, G15, G22, G1 | 16 | 0.16 | 0.4 |
| 4 | G16, G18, G20 | 12 | 0.15 | 0.47 |
| 5 | G3, G21 | 8 | 0.14 | 0.56 |

* See table 1 for genotypes characteristics.

linkage as a suitable method for this cluster analysis. According to SM genetic distance matrix, the genetic distance between the wheat genotypes varied from 0.41 to 0.86, with an average distance of 0.61. In the present study, two genotypes G13 and G14 along with G1 and G10 had the highest genetic distance from other genotypes (Data not shown).

The percentage of variance justified between and within the various genetic subpopulations resulted from STRUCTURE software was determined through AMOVA based on PhiPT parameter (Table S1). According to AMOVA, the percentage of total variance was 81.64% within and 18.36% among subpopulations. The PhiPT index was significant ($P$<0.01), indicating the presence of genetic structure.

**Marker-trait associations under different environments**

Due to the more effective and stable results of the MLM model (G+P+Q+K) (Yu *et al.,* 2006; Zhang *et al.,* 2016), in this study, only the results of the MLM are reported. Table 7 represents the number of AFLP markers (MAF>0.05) identified for each trait evaluated under non-stressed and drought stress conditions.

***Non-stress***

Under non-stressed conditions, 36 MTAs were detected in $P$<0.001. The $R^2$ ranged from 0.078 to 0.387 (Table 7). The highest number of MTAs was observed for PH (5), followed by GY, SL, and RWC (4). Four markers were associated with supposed QTLs for GY, explaining 0.136 to 0.323 of the total phenotypic variation and three markers were associated with KPS, explaining 0.078 to 0.337 of the total phenotypic variation; two of these shared both traits. A total of three MTAs were identified for each of traits SB, PL, PW, KPS and FLA. The markers E-ACT/M-CTC-11 ($R^2$=0.382), followed by E-AGG/M-CTT-24 ($R^2$=0.204) displayed the strongest associations with PL and RWC, respectively. These two markers were the most consistent regions associated with multiple traits like KPS, PL, and RWC. Several pleiotropic loci were detected that included marker E-ACT/M-CTC-11, which was associated with DWPS, KPS, PL, PW, RWC, GY, and FLA. PL, RWC, and KPS were associated with marker E-AGG/M-CTT-24, and SB, RWC, and SL were associated with marker E-ACT/M-CTT-7. The locus E-AGG/M-CTG-17 was associated with GY and KPS, E-ACT/M-CTT-8 was associated with PH and TKW and finally E-AGG/M-CTT-14 was associated with SL and FLA.

***Drought stress***

Under drought stress, 30 significant MTAs were observed for various traits (Table 7). Of these traits, RWL had the highest number of MTAs (4). Marker E-ACT/M-CTT-8 explained the highest ratio of the phenotypic variation (0.393) in TKW while marker E-AGC/M-CTT-2 explained the minimum ratio (0.082) of the phenotypic variation obtained for RWL. Three MTAs were identified for GY ($P$<0.001), explaining 0.15 to 0.309 of the total phenotypic variation; one of these was also associated with TKW. Markers E-ACG/M-CTG-2 ($R^2$=0.336), followed by E-ACT/M-CTT-8 (with the highest $R^2$) represented the highest tight associations with RWL and TKW, respectively. In this study, a total of three MTAs were observed for each of the traits DTH, SL, DWPS, KPS and FLA. Marker E-AGG/M-CTT-24, which was more closely linked to PL, was also associated with SB, KPS, and FLA. PH and TKW were associated with marker E-ACT/M-CTT-8, and DWPS and RWL were associated with marker E-ACG/M-CTG-2. The locus E-AGG/M-CTT-16 was associated with DTH and FLA, and finally E-ACG/M-CAA-13 was associated with SL and DWPS.

***Comparison of MTAs for GY, KSP, RWC, and TKW under various environments***

A comparison of the MTAs for GY, KSP, TKW, and RWC detected a marker as the most common locus for GY, KPS, and RWC under non-stressed conditions. Another pleiotropic locus belonged to KPS, GY, KPS as well as RWC (Table 7). No common loci were identified for GY, KPS, and TKW and some loci for GY were independent of KPS, TKW and RWC. Under

**Table 7.** AFLP markers with high association with morpho-physiological traits under non-stress and drought-stress conditions using mixed linear model (MLM) in wheat genotypes.

| Trait | Non-stressed | | | Drought-stressed | | |
|---|---|---|---|---|---|---|
| | Marker | *P*.value | $R^2$ | Marker | *P*.value | $R^2$ |
| DTH | E-AGG/M-CTT-16 | 4.3E-4 | 0.346 | E-ACG/M-CAA-21 | 2.7E-5 | 0.225 |
| | | | | E-AGG/M-CTT-3 | 2.8E-5 | 0.233 |
| | | | | E-AGG/M-CTT-16 | 1.2E-4 | 0.128 |
| PH | E-AGG/M-CTG-3 | 5.9E-4 | 0.079 | E-ACT/M-CTT-8 | 1.5E-4 | 0.184 |
| | E-ACT/M-CTT-8 | 1.1E-4 | 0.171 | E-ACT/M-CTC-2 | 5.2E-4 | 0.262 |
| | E-ACT/M-CTC-2 | 4.8E-5 | 0.276 | | | |
| | E-AGG/M-CTT-3 | 1.1E-4 | 0.159 | | | |
| | E-ACT/M-CTT-9 | 2.2E-4 | 0.081 | | | |
| SL | E-AGG/M-CTT-14 | 7.2E-4 | 0.179 | E-AGG/M-CTC-3 | 1.4E-5 | 0.273 |
| | E-ACT/M-CTT-7 | 2.6E-4 | 0.239 | E-ACG/M-CAA-14 | 4.8E-5 | 0.153 |
| | E-AGG/M-CTT-17 | 4E-4 | 0.157 | E-ACG/M-CAA-13 | 5.6E-5 | 0.155 |
| | E-AGG/M-CTC-4 | 9.8E-4 | 0.086 | | | |
| DWPS | E-AGC/M-CTT-7 | 6.9E-4 | 0.097 | E-ACG/M-CTG-2 | 1.2E-4 | 0.108 |
| | E-ACT/M-CTC-11 | 1.9E-4 | 0.357 | E-AGG/M-CTT-25 | 1.3E-4 | 0.310 |
| KPS | E-ACT/M-CTC-11 | 4.3E-4 | 0.337 | E-AGG/M-CTT-24 | 2.6E-5 | 0.351 |
| | E-AGG/M-CTT-24 | 5.8E-4 | 0.267 | E-ACG/M-CAA-6 | 1.1E-4 | 0.126 |
| | E-AGG/M-CTG-17 | 9.7E-4 | 0.078 | E-ACG/M-CAA-20 | 3.3E-4 | 0.147 |
| PL | E-ACT/M-CTC-11 | 2.3E-5 | 0.382 | E-ACT/M-CTC-11 | 1E-5 | 0.279 |
| | E-AGG/M-CTT-24 | 1.1E-4 | 0.132 | E-AGG/M-CTT-24 | 3.5E-6 | 0.3 |
| | E-AGG/M-CTG-8 | 5.6E-5 | 0.118 | | | |
| PW | E-ACG/M-CAA-9 | 5.4E-5 | 0.097 | - | | |
| | E-ACG/M-CAA-5 | 8.6E-5 | 0.072 | | | |
| | E-ACT/M-CTC-11 | 3E-5 | 0.357 | | | |
| FLA | E-ACT/M-CTC-11 | 6.6E-4 | 0.387 | E-AGG/M-CTT-16 | 2.3E-4 | 0.359 |
| | E-ACG/M-CAA-4 | 6.8E-4 | 0.174 | E-AGG/M-CTT-24 | 8.6E-4 | 0.165 |
| | E-AGG/M-CTT-14 | 8.7E-4 | 0.146 | E-AGG/M-CTG-10 | 9.5E-4 | 0.125 |
| RWC | E-ACT/M-CTT-4 | 5.5E-5 | 0.219 | E-ACG/M-CAA-2 | 9.8E-4 | 0.366 |
| | E-AGG/M-CTT-24 | 3.8E-5 | 0.204 | | | |
| | E-ACT/M-CTC-11 | 2.5E-4 | 0.141 | | | |
| | E-ACT/M-CTT-7 | 5.2E-4 | 0.103 | | | |
| RWL | - | | | E-ACG/M-CTG-2 | 1.3E-6 | 0.336 |
| | | | | E-AGC/M-CTT-7 | 5.3E-6 | 0.144 |
| | | | | E-ACT/M-CTT-9 | 4E-6 | 0.116 |
| | | | | E-AGC/M-CTT-2 | 5.8E-6 | 0.082 |
| TKW | E-ACT/M-CTT-8 | 1.6E-4 | 0.323 | E-ACT/M-CTT-8 | 2.1E-6 | 0.393 |
| | | | | E-ACG/M-CAA-20 | 5.6E-6 | 0.142 |
| SB | E-ACG/M-CAA-13 | 3.1E-4 | 0.214 | E-AGG/M-CTT-24 | 1.2E-4 | 0.281 |
| | E-ACG/M-CAA-11 | 3.8E-4 | 0.108 | | | |
| | E-ACT/M-CTT-7 | 4.2E-4 | 0.119 | | | |
| GY | E-ACT/M-CTC-4 | 9.8E-4 | 0.136 | E-ACG/M-CAA-20 | 5.2E-5 | 0.309 |
| | E-ACT/M-CTC-11 | 5.1E-4 | 0.223 | E-AGG/M-CTT-6 | 4.8E-5 | 0.15 |
| | E-AGG/M-CTG-17 | 6.7E-4 | 0.145 | E-AGG/M-CTG-16 | 4.9E-4 | 0.17 |
| | E-AGC/M-CTT-6 | 6.6E-4 | 0.135 | | | |

DTH: Number of days to heading, PH: Plant height, SL: Spike length, DWPS: Dry weight per spike, KPS: Number of kernels per spike, PL: Peduncle length, PW: Peduncle weight, FLA: Flag leaf area, RWC: Relative water content, RWL: Relative water loss, TKW: Thousand kernel weight, SB: Shoot biomass, GY: Grain yield, $R^2$: Phenotypic variation explained by marker.

drought stress, one common locus was detected for GY, TKW, and KPS. Marker E-ACT/M-CTC-11 was associated with GY, RWC, PW, KPS, and DWPS under non-stressed condition as well as PL under both drought-stressand non-stress conditions. Moreover, KPS and PL under both non-stress and drought conditions as well as RWC under non-stressconditions and SB and FLA under stress conditions were associated with the marker E-AGG/M-CTT-24. In the resent study, Marker AGG/M-CTT-16 for DTH and Marker ACT/M-CTT-8 for PH and TKW, were the common marker under either of non-stressed and drought conditions. For all two environments, the most common marker for different traits was E-AGG/M-CTT-24. All evaluated traits were displayed via at least one significant trait-specific MTA under both of the two moisture?? Irrigation? regimes.

## DISCUSSION

The wide genetic diversity of wheat genotypes assessed in the present study for morpho-physiological traits showed that selection for drought adapted genotypes was possible in the studied germplasm. All evaluated traits were significantly reduced under drought stress as compared with non-stress conditions approving that traits have phenotypic flexibility. This flexibility could be used to improve wheat drought tolerance to reduce water deficiency (Dalal *et al.,* 2017). In the present study, we obtained the highest ratio of $CV_g/CV_e$ for KPS, followed by TKW under both environmental conditions, revealing the existence of a wide genetic diversity and good genetic gain by selection. $H^2$ estimates varied from low to high heritability, indicating the genetic instability of these traits between environmental conditions. Similar $H^2$s have been reported for most of the respective traits in previous research studies (Sehgal *et al.,* 2017; Bhatta *et al.,* 2018; Mohammadi *et al.,* 2018; Shamuyarira *et al.,* 2019; Sun *et al.,* 2019; Bhatta *et al.,* 2020; Gao *et al.,* 2021). A significant reduction in grain yield heritability was also reported under drought conditions in line with Dodig *et al.* (2012), Mathew (2018), Sukumaran *et al.* (2018a) and Said *et al.* (2022) findings. The grain yield has been shown to be a complex trait and its heritability is severely reduced under stress (Eid, 2009). High broad sense heritability (>50%) estimates were obtained between the studied traits approving the validity of the data in the present marker-trait association mapping. This is supported by Alqudah *et al.* (2020) and Bhatta *et al.* (2020) who explained the relation of traits that had high heritability for QTL analyses.

Although the average polymorphic value in present study was high (93.14%), however, the mean number of the polymorphic bands per primer sets (14.88) was moderate as compared with prior research studies such as Roncallo *et al.* (2019) that identified 30 polymorphic bands per combination in their AFLP analysis of durum wheat collection. These differences can be attributed to factors such as the variety and size of the accessions, the method of detection and the evaluation of the length of the amplified fragments. The number of amplified alleles from each loci is directly influenced by the degree of heterozygosity, genotypic frequency, and the polymorphism index content (Mohammadi Maibody and Golkar, 2019). Nevertheless, Ejaz *et al.* (2015) detected 113 polymorphic band with a mean of 8.7 per primer in their study on wheat genotypes. Actually, the association between genotypes, their origins, genetic similarity, and other factors could be effective in analyzing genetic polymorphism. According to Balta *et al.* (2014), in wheat AFLP analysis, one of the *Eco*RI/*Mse*I primer pairs often revealed the highest polymorphism. Diversity index estimates for AFLP primer sets exhibited that E-ACT/M-CTT and E-AGG/M-CTC combinations had better marker performance based on higher PIC, Shannon index, and He values. Since, the maximum PIC values for dominant markers such as AFLP are reported 0.5 (Roldan-Ruiz, 2000), in this research, four primer pairs revealed PIC estimate ≥0.3. In the present research, the Shannon index was positively correlated with PIC, showing that the highest values of respective parameters can be used as a criterion for selection of the best primer set. Considering the results of allele diversity, AFLP has a high potential for distinction of wheat genotypes because accessibility of high numbers of polymorphic bands enables the effective assessment of genetic variation. These results are consistent with other reports on AFLP markers as an appropriate marker for detecting the differentiation of various plants (Kumar *et al.,* 2015; Saeed and Darvishzadeh, 2016; Ebrahimi *et al.,* 2017; Jamali *et al.,* 2017; Giordani *et al.,* 2019; Archangi *et al.,* 2019; Yazdizadeh *et al.,* 2020). According to Roncallo *et al.* (2019), the AFLP markers have a better capability than the SNP markers to distinguish sister lines and have a greater degree of resolution.

Genetic associations in the studied wheat genotypes were evaluated through various statistical ways to recognize genetic variation level and population structure. According to the method of Evanno *et al.* (2005), the wheat genotypes divided based on K values into five separate main clusters. Wright's F-statistics ($F_{st}$) related with the five subpopulations

varied from 0.06 to 0.56, supporting a potentially distinction between the clusters and existence of genetic structure. The obtained population structure and genetic distances among pairs of subpopulations also verified the presence of 'admixed' and kinship. The admixed and kinship patterns observed were imputed to participation of common ancestry between some of the genotypes (Mathew *et al.,* 2019). For example, genotypes G13, G14, G19 and G23 in sub-cluster 2 shared a common parent Sabalan. Parent Sardari was common for genotypes G11, G15 and G22 in sub-cluster 3 and genotype G3 along with genotype G21 (with common parent Azar2) were assigned to sub-cluster 5. In this study, the results derived from the clustering distance-based method and those obtained with structure analysis had relatively high conformity together and were able to form five main clusters. Investigation of population structure in wheat collection for purposes such as controlling false positive associations between marker loci and phenotypic traits, understanding the genetic variation between genotypes and assessing heterotic groups of wheat germplasm has been highlighted by many researchers (Qaseem *et al.,* 2018; Bhatta *et al.,* 2019; Rufo *et al.,* 2019; Soumya *et al.,* 2021). Result from the AMOVA analyses displayed that genetic variance among subpopulations was significant and accounted for 18.36% of the total variance of AFLP data. Using a various panel of genotypes can prepare more worthy conclusion compared with bi-parental populations (Vos-Fels *et al.,* 2017) profiting high allelic variation (Ayalew *et al.,* 2018).

For complex traits like drought tolerance, the knowledge of MTAs can be utilized for MAS breeding to enhance the efficiency of selection in segregating populations (Bennani *et al.,* 2022). A total of 66 significant MTAs ($P$<0.001) were detected using MLM models. Drought tolerance is highly affected by genotype×environment interaction which is explicated by the higher number of significant MTAs detected under non-stressed than drought conditions (Mwadzingeni *et al.,* 2017). Higher MTAs were detected for evaluated traits under non-stressed in comparison with stress conditions, which indicated that traits were likely controlled by a greater number of different genes under non-stress conditions than drought conditions. In the current study, comparison of association analysis by GLM and MLM procedures under non-stress and drought-stress conditions showed that the number of significant markers was reduced in the MLM. According to Guo *et al.* (2015), Giordani *et al.* (2019) and Kumar *et al.* (2022), the MLM has more power than the GLM model due to the lower false positive MTAs. Thus, the AFLP markers identified based on the MLM can be considered the most interesting candidate markers for future studies. Due to differences in structure of the population, environmental conditions and the methods of QTLs detection, it might be difficult to compare QTLs identified in this study with those previously reported (Lakew *et al.,* 2013). Most of the detected MTAs were different under non-stress and drought conditions. It shows the influence of environment on the traits, that shows why; different QTLs were detected under different environments (Abou-Elwafa and Shehzad 2020; Negisho *et al.,* 2022). Marker E-ACG/M-CAA-20 was closely linked to GY also presented highly significant relationships with TKW and KPS under drought conditions. Identification of common markers is very important in plant breeding, because simultaneous selection of numerous traits is possible (Guo *et al.,* 2018). Furthermore, the markers that exhibit powerful effects on the traits represent ideal candidates for future research studies using MAS. Genotypes with high GY, KPS, and TKW are targeted by wheat breeding; thus, if the effectiveness of these loci in the genetic control of respective traits is approved, they could be useful tools for wheat molecular breeding programs for enhancing drought tolerance. The common MTAs observed for GY, RWC, PL, KPS, and TKW, and unique MTAs detected for any trait, suggesting the traits RWC, PL, KPS, and TKW can be manipulated freely of GY, as individual MTAs were found for them under different conditions. There are few studies on the identification of MTAs in physiological traits such as, FLA, RWC, and RWL (Gupta *et al.,* 2012; Bhatta *et al.,* 2018; Lin *et al.,* 2019; Ahmed *et al.,* 2022). Khalid *et al.* (2019) in a study on advanced lines derived from synthetic hexaploid wheats detected five KASP assays for *Ppd1* homeologous genes were significantly related with DTH, GY, RWC, SL, and TKW. In the present study, Marker E-ACT/M-CTC-11 was common for GY, KPS, RWC, FLA, PL, and PW under non-stress conditions. Most functional genes in the genome might contribute directly or indirectly to the yield, and most released fine-mapped QTLs and the genes detected as affecting yield present pleiotropic effects on at least one trait (Mangini *et al.,* 2021). Markers E-ACT/M-CTC-11, E-AGG/M-CTTT-24, and E-ACT/M-CTT-8 showed significant associations with several traits including GY, RWC, KPS, PL, and SB. Association between a single marker and various phenotypes could be due to pleiotropic effects or closely linked genes influencing diverse traits synchronously. In fact, most of complex traits display linkage and selection of pleiotropic genes

cause major synchronous changes in the traits (Touzy *et al.,* 2022).

The common genetic markers for DTH, PH, TKW, PL, and KPS were observed under stress and non-stress conditions. This demonstrates that the genetic basis of respective traits was assigned by a similar mechanism in both environmental conditions. An earlier research on durum wheat using drought-stress and yield potential environments, the common genetic markers for TKW and grain number $m^{-2}$ were identified on chromosomes 2A and 2B under DT and YP conditions (Sukumaran *et al.,* 2018a). Ideally, the effects of these genomic regions may not be affected by the environmental variation. Such loci could be effective in MAS or gene introgression when breeding for wide compatibility (Sukumaran *et al.,* 2018b). Plant height is reported to be a serious morphological trait in wheat for improving dwarf varieties with high harvest index, and its relation with the yield component traits could be important for indirect selection through plant height (Thomas, 2017). Therefore, markers associated with plant height can also be desirable candidates for efficient wheat breeding efforts. The AFLP markers could be changed into sequence characterized amplified region (SCAR) markers that have advantages such as, detection as separate bands in agarose gels, easy scoring, less sensitivity to reaction conditions, and high repeatability (Wei *et al.,* 2009).

In conclusion, the results of the present study represented that AFLP markers have a considerable potential for association analysis especially for multi-environment experiments including contrasting water regimes. This study detected a total of 66 highly significant MTAs under non-stressand drought-stress conditions. Under non-stress conditions Marker E-ACT/M-CTC-11 was associated with GY, PL, PW, RWC, KPS and FLA. Marker E-ACG/M-CAA-20 was associated with GY, TKW and KPS under stress conditions. The significant MTAs identified would be beneficial for MAS and trait introgression in wheat breeding programs to develop drought-tolerant genotypes for arid and semi-arid areas, and for fine mapping and cloning of the fundamental genes and QTL. However, the markers detected should be accredited by testing their effectiveness in the identification of the target phenotypes in larger populations and different genetic backgrounds, supporting by the multiple loci mixed model (MLMM) as suggested by Segura *et al.* (2012).

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## SUPPLEMENTAL DATA

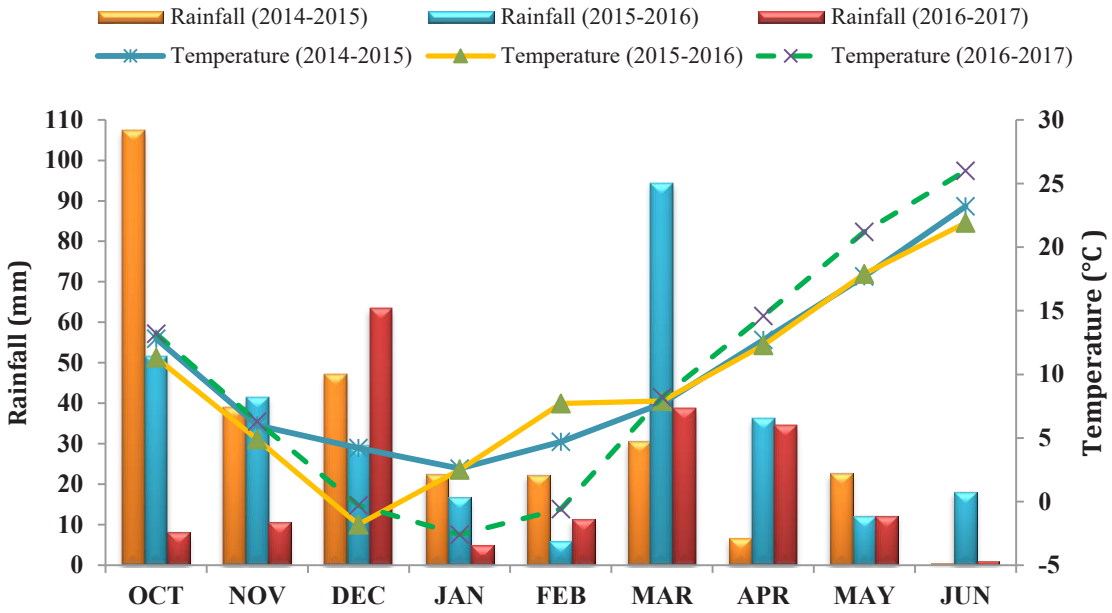


**Figure S1.** Monthly patterns of temperature of air and rainfall recorded during the course of the experiment.

**Table S1.** Analysis of molecular variance for five subpopulations derived from structure analysis in wheat genotypes using AFLP markers.

| Source of variation | df | Mean of square | | | |
|---|---|---|---|---|---|
| | | MS | Est. variance | Variance (%) | *PhiPT* |
| Among subpopulations | 4 | 40.603 | 4.449 | 18.36 | 0.184** |
| Within subpopulations | 20 | 19.783 | 19.783 | 81.64 | |
| Total | 24 | | 24.232 | 100 | |

df: Degree of freedom, MS: Mean of squares, ** Significant at 1% level of probability.

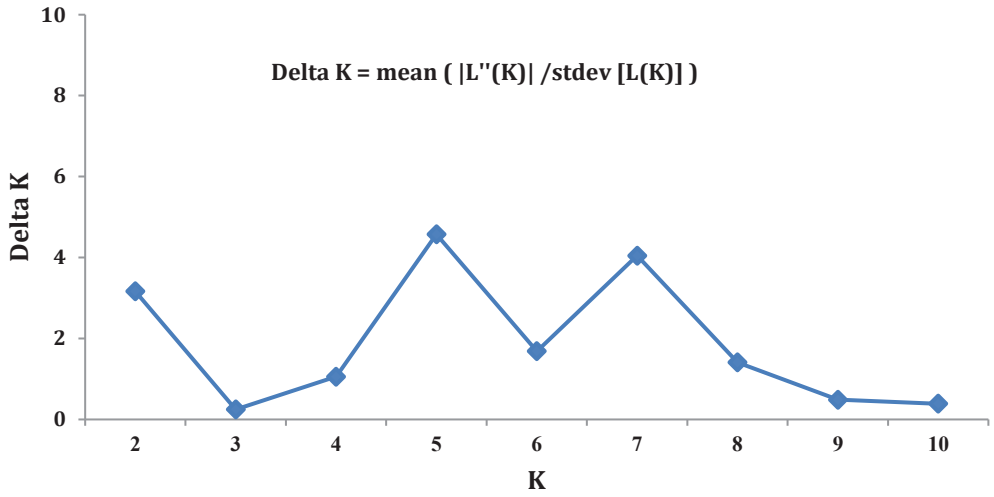


**Figure S2.** The Delta K calculated by the Evanno method displaying the classification of the population into five main clusters.