

Morphological and molecular marker-based characterization of winter wheat genotypes for enhanced adaptation and yield

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Abstract

Winter wheat is a major staple crop in temperate regions and generally shows higher yield potential than spring wheat; however, productivity is often constrained by high temperature stress. This study evaluated morphological variability and genetic characteristics among 134 winter wheat genotypes. Morphological traits were analyzed using analysis of variance (ANOVA) in IBM SPSS Statistics 25. Significant variation ($p < 0.05$) was observed for germination, flag leaf dimensions, plant height, peduncle length, awn length, spike length, stem diameter, biomass, spike weight, tillers, and harvest index. Qualitative traits, including crown morphology, leaf architecture, flowering and anthesis behavior, lodging, and leaf rust susceptibility, also varied among genotypes. Pearson correlation analysis revealed strong positive associations ($p < 0.05$) among most traits, whereas harvest index showed weak correlations with germination and flag leaf width, indicating a trade-off between vegetative growth and reproductive allocation. Molecular screening identified VRN1 in three genotypes and NAM-B1 in two lines.

Introduction

Wheat (*Triticum aestivum* L.) is a staple food for a significant proportion of the world's population, supplying approximately 20% of global food calories and protein. Its demand and market size are expected to grow in the coming years (Erenstein et al. 2022). Wheat grain is a rich source of proteins, starch, and fiber, but it also contains lipids, minerals, vitamins, and phytochemicals in minor quantities that make it a nutritionally healthy diet (Iqbal et al. 2022). Climate change and a growing human population with an already stressed environment are serious threats to global food security (Kompas et al. 2024). This necessitates plant improvement through the creation of resilient plant varieties that have higher yields under unfavorable growth conditions (Karri and Nalluri 2024). This target might be attained by exploring and scrutinizing the genetic diversity in available wheat germplasm, crop management practices, and improving plant genetics (Chachar et al. 2024). Molecular or genetic markers show polymorphism and are used to differentiate organisms or species (Doveri et al. 2024). These markers generally exist near the gene of interest and act as a tag for that gene (Gupta and Singla 2024). The process of using genetic markers to select a phenotype or trait based on the presence or absence of a genetic marker or genotype is known as Marker Assisted Selection (MAS) (Hasan et al. 2021). MAS has the potential in plant breeding programs in order to improve major food crops like wheat (Song et al. 2023). Microsatellite or simple sequence repeats (SSR) markers show many advantages, such as being easily visualized, stable, highly polymorphic, co-dominant, and are used in plant breeding programs (Nair and Pandey 2024).

Wheat is generally categorized into spring wheat which is planted in spring and winter wheat which is sown in fall (Tosun et al. 2024). Winter wheat has received more attention worldwide because of some of its potential advantages like higher yield (Slafer et al. 2023). The principal adaptation of winter wheat is its ability to withstand freezing temperatures during vegetative growth and development. In other words, winter wheat needs exposure to low temperatures (as low as 5-10°C) or chilling treatment for the induction of flowering or reproductive growth, and this process is known as vernalization (Milec et al. 2023). A plant hormone called florigen helps in the induction of flowering and is activated as a consequence of the vernalization period (Izawa 2021). VRN1 and VRN2, both responsible genes for the control of vernalization, while it is also well known that VRN1 loci allelic differences are involved in variation in the growth habit in wheat. As far as VRN2 is concerned, it works in an antagonistic manner to the VRN1 gene, and low-temperature treatment causes the downregulation of VRN2 (Izawa 2021, Afshari-Behbahanzadeh et al. 2024). In wheat, vernalization is controlled and regulated by alleles at three orthologous loci, including Vrn-A1, Vrn-B1, and Vrn-D1 present on chromosomes 5A, 5B, and 5D (long arm), respectively. This group of three genes is called the VRN1 loci (Afshari-Behbahanzadeh et al. 2024).

NAC domain-containing products of gene expression, like No Apical Meristem (NAM) in *Petunia*, ATAF1/2, and Cup Shaped Cotyledon 2 (CUC2) in *Arabidopsis thaliana* plays a critical role in plant growth. NAC is an abbreviation for these three homologous genes (Bokolia et al. 2023). These transcription factors contain a parallel N-terminal domain called NAC. These proteins bind to cis-acting promoter elements to regulate the expression of targeted genes (Bokolia et al. 2023, Li et al. 2025). The NAM gene was discovered as a quantitative trait locus on the chromosome arm 6BS in wheat. NAM-B1 ancestral allele accelerates the maturity of wheat and holds back nutrients from vegetative parts to seeds, ultimately increasing the protein, zinc, and iron contents of the seed (Kiseleva et al. 2023). On the other hand, the non-functional allele of NAM-B1 is involved in delaying maturity in wheat, thus resulting in high yield but compromising protein, iron, and zinc contents in the grains (Li et al. 2024). Hence, this research aimed to assess the morphological characteristics and analyze the winter wheat lines and varieties for the presence of VRN1 and NAM-B1

genes. The goal was to investigate the genetic variability within and among various winter wheat germplasm selections based on morphological traits, the *VRN1*, and the *NAM-B1* genes using specific SSR markers.

Materials and Methods

Plant Material and Field Trial

The germplasm used in this study was obtained from multiple sources. A total of 134 experimental winter wheat lines were acquired from the Molecular Lab in Zurich. Additionally, six winter wheat varieties were imported from Mitchell L. Montgomery at the University of Nebraska-Lincoln in the USA. Local genotypes were provided by the Agriculture University in Peshawar, Pakistan. The Chinese Spring variety was provided by Kansas State University. Chromosome substitution lines were developed by R. Moris in the USA (Supplementary File 2). The experiment was conducted at the Biotechnology Research Farm, COMSATS University Islamabad, Abbottabad Campus. To minimize external sources of variation such as light and soil, a Randomized Complete Block Design (RCBD) experimental design was chosen for the study.

Evaluation of Morphological Traits

Morphological traits were evaluated from one week after seed planting until harvest and included both pre-harvest and post-harvest traits. The pre-harvest quantitative morphological traits evaluated in this study included flag leaf width, flag leaf length, flag leaf sheath length, plant height, peduncle length, awn length, spike length, stem diameter, number of tillers, and dry biomass weight. The post-harvest quantitative morphological traits evaluated in the current study included the weight of spikes and harvest index. Qualitative morphological traits assessed included crown morphology, leaf shape, leaf orientation, flowering, anthesis, lodging, and resistance to leaf rust disease.

Pre-harvesting Traits Evaluation

After 4-5 days of sowing, germination was observed, and morphological data were scored daily till harvesting. Plants' numbers were scored in each experimental unit. After plant height reached 30-40 cm, the crown morphology was recorded as totally flat (1), little cup-shaped (2), cup-shaped (3), not fully erect (4), and erect (5). As soon as the first genotype was observed having emerged spike, the flowering data was recorded till all the genotypes were observed with spikes. Anthesis data were recorded daily. The appearance of anthers on the spikes of the first genotype indicated that the fertilization process had occurred in the respective spike with seed formation. Before harvesting, tillering data was scored once, and the average number of tillers was estimated for each plant in the experimental unit. Lodging data was scored before harvesting based on the percentage. From each experimental unit, the number of lodged plants was counted as a percentage value using the percent formula. Flag leaf shape was noted by using the parameters 'C' for curly and "F" for flat by noticing the flag leaf in all the plants in an experimental unit. Leaf orientation was observed by observing the flag leaf in all the plants in an experimental unit and assigning 'E' for erect, 'S' for straight, and 'D' for drooping. A digital Vernier Caliper was used to measure the stem diameter from the two randomly selected plants in each experimental unit. A measuring scale was used to measure the flag leaf length and flag leaf sheath length from two randomly selected plants in each experimental unit in centimeters, and finally, the average value was estimated. Two flag leaves were analyzed randomly for each genotype. Flag leaf width was measured by determining the width of the flag leaf at three spots, *i.e.*, leaf base, mid of the leaf, and 2 centimeters proximal to the flag leaf tip, while values were finally averaged. A measuring scale was used to measure peduncle length (start of the flag leaf to the point where the spike emerges) and spike length in centimeters from two plants in each experimental unit to calculate the spike length average. Before harvesting, plant height was scored by randomly selecting two plants, and finally, it was averaged. The awn length (from the base to the tip of the awn) was estimated by randomly selecting two spikes in each experimental unit. Dry biomass (weight of stems and spikes) for each genotype was measured in grams using an analytical balance, having a precision of 0.0001g at the time of harvesting.

Post-Harvesting Traits Evaluation

Spike's weight for each entry was calculated in grams using an analytical balance. The harvest Index was estimated with the help of the following equation:

$$HA = \text{weight of spikes/weight of dry biomass}$$

Leaf rust disease data were scored by observing the whole plant using a magnifying glass for clear observation in the middle of May.

Molecular Marker-Based Analysis of Wheat Genotypes

Extraction of DNA

Genomic DNA was extracted from seeds using a modified CTAB method (Aboul-Maaty and Oraby 2019). Two seeds of each plant sample were squashed into a fine powder with the help of a sterile pestle and mortar. The sample was shifted to a 1.5 ml Eppendorf tube and added with 0.5 ml of preheated (at 65°C) CTAB extraction buffer (5M NaCl, 1M Tris HCl (Ph 8.0), 2% CTAB, 0.5M EDTA (pH 8.0), 1% BME dH₂O). The mixture was incubated for 1 hour (h) at 65°C during which each sample was vortexed at 15-minute intervals. 0.5 ml of chloroform: isoamyl alcohol (24:1) was mixed in the mixture, and centrifugation was carried out (13,000 rpm) (15 min) to separate the solid and liquid phases. While discarding the solid phase, the liquid phase was added with chilled isopropanol to precipitate the DNA. After gentle mixing, the sample was incubated at -20°C (30 min) and then centrifuged at 13000 rpm (10 min). The resultant pellet was retained, and the aqueous layer was discarded carefully. 70 % ethanol was used to wash the pellet and left for air drying (30 min). Finally, TE Buffer (100 µl) was used to resuspend the pellet for future analysis.

RNase Treatment and Purification

To remove any residual RNA, the DNA sample (80µl) was gently mixed with RNase-A (2µl), and this mixture was centrifuged at 3,000 rpm (30 sec, 4°C). After centrifugation, the mixture was incubated at 37°C (1 hr) and then centrifuged at 3,000 rpm (30 sec, 4°C). The sample was now ready to mix with 2 volumes of isopropanol (160 µl) and 1/10th volume of sodium acetate (8 µl) in the Eppendorf tube. Again, centrifugation was carried out at 3,000 rpm (5 min, 4°C), and the resultant supernatant was discarded. 95% ice-cold ethanol (100 µl) was added to the sample and allowed for centrifugation at 3,000 rpm (2 min, 4°C) to obtain a clear pellet. The pellet was finally dissolved in 100µl TE buffer, and the concentration of DNA was determined using a UV Spectrophotometer (Mapada UV 1200).

SSR Marker Analysis

Three SSR primers (Genelink, USA) were used to screen the *VRN1* and *NAM-B1* genes in experimental lines (Table 1). PCR amplifications were performed in 15µl reaction volume, including template DNA (5 µg), Taq polymerase (1U), Taq buffer (1 X), MgCl₂ (1.5 mM), dNTPs (0.8mM), and 1.9µM of each forward and reverse primer for the *VRN1* gene. PCR amplification and conditions were maintained as follows: initial denaturation at 94°C (10 min), 40 cycles of (denaturation: 94°C (45 sec), annealing: 50°C (45 sec), and elongation: 72°C (1 min), and final extension: 72°C (5 min). For *NAM-B1*, the PCR master mix (15µl) included template DNA (5µg), Taq polymerase (1 U), Taq buffer (1X), MgCl₂ (1.2 mM), each dNTP (0.2 mM), and 0.2µM of each forward and reverse primer for the *VRN1* gene. The PCR profile was initial denaturation at 94°C (5 min), 40 cycles of (denaturation at 94°C (30 sec), annealing at 61°C (30 sec), and elongation at 72°C (45sec)), and final extension at 72°C (5 min).

Gel-Electrophoresis

For visualization of DNA, 0.8 g agarose gel was dissolved in 100 ml 1X TBE (Tris Base, Boric Acid, EDTA, dH₂O). A clear solution of gel was obtained by heating the mix in a microwave oven for about 30 sec and then allowed to cool down a little bit to add ethidium bromide. The gel was poured into a casting tray and inserted with the comb to make wells for sample loading. After the solidification of the gel, it was transferred to the gel apparatus. The DNA sample was prepared by using 6X Bromo phenol blue dye in double distilled water, and a total of 12µl sample was loaded in the well. The electrophoretic process was initiated by applying a 100V (25 min), and finally, the gel was examined in the Gel-Doc system (BIOTOP 750).

Statistical Analysis

The morphological data were formatted and organized according to the requirements of IBM SPSS to perform the ANOVA test effectively. The analysis provided the degree of freedom (df), the sum of squares, mean squares, F-value, and P-value for the blocks and genotypes against each trait. Additionally, Pearson correlation coefficients were calculated to determine the relationships among the morphological traits and between the morphological traits and molecular data.

Results

Morphological Traits of Wheat

Significant variations were observed in traits such as germination, flag leaf width, flag leaf length, flag leaf sheath length, plant height, peduncle length, awn length, spike length, stem diameter, number of tillers, dry biomass weight, spike weight, and harvest index, as revealed by ANOVA analysis (Supplementary File 1). To provide an overall perspective of trait variability across the evaluated winter wheat genotypes, descriptive statistical parameters—including range, mean, standard deviation (SD), and coefficient of variation (CV%)—were calculated and are presented in Table 2. These parameters reflect substantial diversity among the 127 genotypes for both pre- and post-harvest morphological traits. Germination percentage ranged widely from 6.67% to 100%, with a mean of 81.21% and a CV of 18.92%, indicating moderate variability. Among the traits, tillering exhibited the highest coefficient of variation (33.51%), suggesting strong genotypic influence and environmental interaction. Similarly, high variability was observed for spike weight (CV = 42.54%) and dry biomass (CV = 31.16%), highlighting the potential for selection based on these traits in breeding programs. Conversely, traits such as flag leaf width (CV = 11.26%) and flag leaf sheath length (CV = 13.51%) showed relatively lower variation, pointing toward greater uniformity. These summary statistics corroborate the findings from the ANOVA and further support the genetic diversity present in the studied germplasm.

Quantitative Morphological Traits Evaluation

Pre-harvesting Traits Evaluation

Germination

A high F-value (4.382) with a significant p-value (Sig. = .000) indicates that winter wheat varieties and lines significantly influence germination percentage, as reported in Supplementary File 1. There is a clear difference in germination performance among the 134 genotypes. However, replication effects in the experiment do not have a significant effect on germination percentage, suggesting minimal block-to-block variation. Notably, the germination rate was 100% for certain genotypes, particularly WWLINES 4, 34, 41, 47, 53, 67, 96, WI (CNN3A), and WI (CNN6A). Conversely, WWLINES 74, 108, OK-00514, OK-00421, *T. aestivum*, and W-47 displayed a 0% germination rate. Chinese Spring, on the other hand, did not exhibit any growth following germination, as observed in Supplementary File 2.

Flag Leaf Width and Length

Genotype (winter wheat varieties and lines) is a significant factor for both flag leaf width and flag leaf length, demonstrating genetic diversity in these traits. As shown in Supplementary File 1, the analysis of variance (ANOVA) revealed a significant difference in p-value (Sig. = .000) for flag leaf width. It is apparent from Supplementary File 2 that the maximum flag leaf width of 1.2 cm was observed by WWLINES 35 and WWLINES 124, followed by AUP-4008 at a minimum flag leaf width of 0.3 cm (Supplementary File 2).

The F-value (23.999) and significant p-value (Sig. = .000) reveal significant variation in flag leaf length among the 134 genotypes, as reported in Supplementary File 1. WWLINES 15 (37.4 cm) and WWLINES 13 (37.1 cm) exhibited the maximum flag leaf lengths, whereas OK-00421 (16.9 cm) and Khyber 87 (16.9 cm) displayed the minimum flag leaf lengths. WWLINES 15 (37.4 cm) and WWLINES 13 (37.1 cm) showed the maximum flag length, while OK-00421 (16.9 cm) and Khyber 87 (16.9 cm) showed the minimum flag length. Interestingly, both WWLINES 31 and WWLINES 32 had a flag leaf length of 39.4 cm, as shown in Supplementary File 2.

Flag Leaf Sheath Length

The analysis of variance (ANOVA) for flag leaf sheath length showed that the effect of genotypes was highly significant ($F = 22.025$, $p < 0.05$), indicating considerable variation among winter wheat varieties and lines, as reported in Supplementary File 1. It

implies that genetic differences between varieties and lines have a meaningful effect on this trait. WI (CNN6A) showed a maximum 24.6 cm flag leaf sheath length, followed by WWLINE 25 and WWLINE 87, both showing 24.3 cm. Minimum flag leaf sheath length was observed in AUP-4008 (5.5 cm), followed by WWLINE93 (10.4 cm), and Ok-00421 (10.9 cm) (Supplementary File 2).

Plant Height and Peduncle Length

ANOVA revealed a significant difference ($F = 19.489$, $p < 0.05$) for plant height across the 134 genotypes (Supplementary File 1). This highlights that genetic variability has a major influence on this trait. WWLINE 19 (160.2 cm), WWLINE 60 (160 cm), and WWLINE 25 (158.9 cm) showed the maximum plant height as compared to AUP-4008, which reached the minimum plant height (34.3 cm) (Supplementary File 2).

The F-value (26.664) and significant p-value (Sig. = .000) indicate that peduncle length differs significantly among the 134 genotypes as described in Supplementary File 1. This suggests strong genetic control over this trait. Among the genotypes, WWLINE 98 (60.3 cm) and WWLINE 25 (59.8 cm) showed the maximum peduncle lengths as compared to WWLINE 128 which showed a minimum peduncle length of 27.8 cm. Additionally, WWLINE 76 scored 58.3 cm, WWLINE 94 scored 56.8 cm, and WWLINE 97 exhibited a peduncle length of 56 cm, as shown in Supplementary File 2.

Awn Length and Spike Length

The F-value (20.926) and significant p-value (Sig. = .000) suggest significant variation in awn length among the 134 genotypes, highlighting strong genetic influence on this trait (Supplementary File 1). WWLINE 121 showed the maximum (12 cm) spike length in contrast to WWLINE 93, which showed the minimum (4.7 cm). WWLINE 122 scored 11.6 cm, WWLINE 44 scored 11.1 cm, and WWLINE 32 scored 10.5 cm spike lengths (Supplementary File 2).

Similarly, ANOVA revealed the F-value (18.016) and significant p-value (Sig. = .000), which indicates that spike length varies significantly among the 134 genotypes, confirming genetic variability in this trait, as mentioned in Supplementary File 1. WWLINE 1 displayed the maximum awn length of 11.6 cm, while AUP-4008 had the minimum awn length of 4.8 cm, as reported in Table 3. CNN (WI6A) exhibited an awn length of 11.4 cm, and both WWLINE 53 and WWLINE 104 showed an awn length of 11.3 cm (Supplementary File 2).

Stem Diameter and Tillering

ANOVA showed a significant difference ($F = 19.992$, $p < 0.05$) in terms of stem diameter among the studied genotypes of winter wheat, emphasizing genetic diversity for this trait (Supplementary File 1). Among the genotypes, WWLINE 125 exhibited a maximum stem diameter of 4.4 mm, while AUP-4008 had a minimum stem diameter of 1.2 mm. Furthermore, as indicated in Supplementary File 2, the stem diameter of both WWLINE 120 and WWLINE 122 is 4.2 mm, while that of WWLINE 58 is 4.1 mm. Similarly, ANOVA also showed a significant difference ($F = 40.087$, $p < 0.001$) for tillers, pointing to substantial genetic influence, as cited in Supplementary File 1. WI(CNN6A) demonstrated the highest number of tillers, with an average of 37.3 per plant, whereas WWLINE 119, OK-98g508W, and Khyber 87 exhibited 7 tillers per plant. Furthermore, WWLINE 119 and WWLINE 31 displayed 31.2 and 29.8 tillers per plant, respectively, as shown in Supplementary File 2.

Weight of Dry Biomass

A significant difference ($F = 3.756$, $p < 0.05$) was revealed by ANOVA for dry biomass weight, though the effect size (mean square) is smaller compared to other traits (Supplementary File 1). The maximum dry biomass was measured for WWLINE 129 (409 gm) as compared to the minimum for OK-00421 (21.3 gm) (Supplementary File 2). However, genotypes including WWLINE 105, WWLINE 112, WWLINE 120, and WWLINE 127 also scored significantly higher dry biomass weight as compared to other genotypes (Supplementary File 2).

Post-harvesting Traits Evaluation

Weight of Spikes per Entry

The F-value (3.820) and significant p-value (Sig. = .000) reveal significant differences in the weight of spikes among the 134 genotypes, highlighting the role of genetic variability (Supplementary File 1). WWLINE 129 showed the maximum (204.3 gm), while AUP-4008 scored the minimum (12 gm) spike weight (Supplementary File 2). However, several other genotypes, including WWLINE 125 (148.7 gm), WWLINE 127 (136.3 gm), and WWLINE 126 (116.7 gm), also showed significantly increased spike weight as compared to other lines and varieties (Supplementary File 2).

Harvest Index

ANOVA revealed a significant difference ($F = 2.437$, $p < 0.05$) for the harvest index, suggesting genetic variability in resource allocation efficiency, as reported in Supplementary File 1. WWLINE 47 displayed the highest harvest index of 2.1, while W-52 and AUP-4008 had the lowest harvest index of 0.38, as shown in Supplementary File 2. Additionally, other winter wheat genotypes that exhibited better harvest indexes included Ok-00421 (1.9), WWLINE 125 (1.7), WWLINE 125 (1.6), and WWLINE 10 (1.5), as reported in Supplementary File 2.

Qualitative Morphological Traits Evaluation

Crown Morphology

After the plants grew to a height of 30-40 cm, their crown morphology was assessed and classified into different categories. The recorded crown morphologies included totally flat (1), little cup-shaped (2), cup-shaped (3), not fully erect (4), and erect (5). The majority of the genotypes examined displayed either erect or not fully erect crown morphologies. However, a few genotypes also exhibited a cup-shaped crown morphology. Interestingly, none of the genotypes observed showed a crown morphology that was classified as little cup-shaped, as shown in Supplementary File 3.

Leaf Shape and Orientation

Most of the genotypes exhibited flat leaf shapes, but WWLINE 120, 124, and 129 displayed curly leaf shapes, as indicated in Table 4. While the majority of the leaves had a straight orientation, WWLINE 124 exhibited a drooping leaf orientation, as observed in Supplementary File 3.

Flowering, Anthesis, and Lodging

The genotypes WWLINE 102, 107, 119, 121, 122, 123, 124, 125, 126, 127, 128, 129, WI (CNN3A), and WI (CNN6A) indicated early flowering. Specifically, early anthesis was observed in WWLINE 119, 124, 125, 126, 127, and 129. The highest levels of lodging were observed in the genotypes WWLINE 30, 33, 49, 50, 51, 52, 53, 54, 55, 56, and 57. On the contrary, as shown in Supplementary File 3, no lodging was observed in WWLINE 4, 7, 10, 14, 15, 18, 19, 20, 24, 26, 31, 32, 35, 48, 60, 74, 93, 119, WI (CNN3A), and Ok-00421.

Leaf Rust Disease

The presence of isolated uredinia on the upper leaf surface was observed, indicating an infection of leaf rust caused by the fungus *Puccinia triticina*. Several genotypes, including WWLINE 16, 17, 22, 25, 40, 44, 47, 51, 52, 53, 56, 59, 60, 61, 62, 64, 65, 68, 69, 71, 73, 75, 76, 78, 79, 85, 91, 92, 94, 96, 98, 100, 101, 102, 103, 104, 105, 107, 109, 110, 112, 119, 120, 124, 125, 126, 128, CNN (WI3A), WI (CNN6A), OK98G508W, and Khyber 87, showed symptoms of infection (Supplementary File 3). These genotypes demonstrated susceptibility to *Puccinia triticina* infection. However, further research is necessary to identify the specific genes responsible for conferring resistance against this fungal infection in the resistant genotypes.

Molecular Marker-Based Analysis of Wheat Genotypes

SSR Marker Analysis for the *VRN1* Gene

The amplification of DNA samples was confirmed using a Gel-documentation system. Once the optimal concentrations of reagents and PCR profile were determined, the same conditions were applied to 27 experimental genotypes. In the control experimental winter wheat lines (OK-40021, OK00514, AUP-4008), an amplified DNA band at approximately 710bp was observed

using the specific marker *Intr/B/F/Intr1/B/R3* (Figure 1). This confirmed the presence of the *VRN* gene in these lines. However, none of the genotypes displayed any amplification with the specific marker *VRN1AF/VRN1-INT1R*, as outlined in Table 3.

SSR Marker Analysis for *NAM-B1* Gene

The presence of the *NAM-B1* gene in experimental wheat lines was analyzed using an SSR primer. Through successful amplification of a 126bp DNA band, it was confirmed that two control genotypes possessed the *NAM-B1* gene, as depicted in Figure 2. This amplification served as evidence for the presence of the *NAM-B1* gene. The summarized results for all genotypes can be found in Table 3.

Correlation Analysis

Most of the morphological traits displayed a positive correlation among themselves; however, Harvest Index (HI) shows relatively weak correlations with other traits, particularly with Germination (Ger.), Flag Leaf Width (FLW), and others, suggesting that harvest index may not be directly related to early growth traits or leaf characteristics in this dataset. The correlation values involving HI are generally below 0.1, and none of them reach the level of statistical significance ($p > 0.01$). Traits like Plant Height (PH) and Peduncle Length (PL) show very high correlations ($r = 0.882$ and 0.911 , respectively), indicating that taller plants tend to have longer peduncles. Awn Length (AL) and Spike Length (SL) are also highly correlated ($r = 0.859$), suggesting that these two traits increase together. However, none of the scored morphological traits showed a significant correlation with the molecular analysis. The summarized results for all genotypes can be found in Table 4.

Discussion

The current investigation was accomplished to explore the potential of the winter wheat genotypes concerning morphological traits and molecular parameters. The winter wheat proved successful in yield as compared to the spring wheat (Slafer et al. 2023). However, due to high temperatures throughout the year, the capacity of winter wheat is not fully explored in temperate and semi-temperate regions. This study assessed and analyzed 134 winter wheat genotypes for morphological and molecular parameters using the Statistical Analysis System (SAS). All the morphological traits evaluated showed a significant difference at $p < 0.05$. These findings align with a related study by Rajper et al. (2018) that reported similar results concerning the inheritance and relationship of morphological traits in wheat. Correlation studies showed a significant positive correlation ($p < 0.01$) between most of the morphological parameters evaluated, as depicted in Table 4. A positive correlation between the morphological parameters might indicate the presence of linked genes, epistasis, or environmental factors. It is commonly reported that traits such as plant height, crown morphology, leaf shape, stem diameter, and tillering tend to exhibit positive correlations with each other. These traits are often influenced by similar genetic factors and physiological processes, leading to their co-regulation and positive associations (Hassani et al. 2022). The positive correlations among these morphological traits suggest a coordinated growth and development pattern within the studied genotypes.

However, the negative correlation observed between most morphological traits and the harvest index is an interesting finding. This indicates that genotypes with higher values for traits such as plant height, crown morphology, leaf shape, stem diameter, and tillering tend to have lower harvest index values. This inverse relationship could be attributed to trade-offs between vegetative growth and reproductive allocation. It is well-known that high levels of vegetative growth, such as increased plant height and tillering, may lead to a greater allocation of resources towards vegetative biomass production rather than grain production (Feng et al. 2021). As a result, genotypes with more extensive vegetative growth may allocate fewer resources towards grain filling and thus exhibit lower harvest index values.

Although multiple factors can lead to a reduction in yield, they can be grouped into environmental, technological, and biological categories (Liliane and Charles 2020). On the other hand, the pressure to achieve greater yield resulted in soil degradation due to practices like irrigation, the use of large quantities of inorganic fertilizers and synthetic chemicals for weeds, and pest control (Hossain et al. 2022). All these activities harm the environment and are a big obstruction to sustainable agriculture development. It has been reported recently that climate change had a serious impact on the winter wheat varieties and it has been difficult for winter wheat varieties to adapt to climate change (Kaya 2021). There is a need for introducing new plant crop varieties that perform well in terms of yield and also impose minimal adverse impact on the environment.

None of the morphological traits revealed a significant correlation with the molecular analysis. The lack of significant correlations between the assessed morphological parameters and the molecular analysis indicates that the studied morphological characteristics may not be directly related to the molecular markers used in the analysis. These results are consistent with the previous findings, as several genes have been reported to be responsible for the regulation of plant morphological characteristics. On the contrary, molecular markers target specific genes or genetic regions (Liu et al. 2020). Thus, the lack of significant correlations between these two sets of traits indicates that the studied morphological traits may be influenced by a combination of genetic factors that are not directly captured by the molecular markers utilized.

Winter wheat needs a spell of low-temperature treatment to initiate flowering (Milec et al. 2023). Winter wheat possesses vernalization genes that are expressed under low-temperature treatment (Skalicky et al. 2020, Izawa 2021). Genetic differences in growth habits are regulated and managed by *VRN1*, *VRN2*, and *VRN3* genes, and these genes have two or more alleles (Skalicky et al. 2020, Afshari-Behbahanizadeh et al. 2024). Dominant alleles of these genes inhibit the need for low-temperature treatment thus manifesting the spring growth habit while recessive alleles favor the winter growth habit (Afshari-Behbahanizadeh et al. 2024). This requirement of winter wheat makes it vulnerable to increased average global temperature through inadequate vernalization (Yousefi et al. 2025). On the other hand, the genes responsible for less or more vernalization requirements in winter wheat cultivars are still largely unknown (Milec et al. 2023). In the current study, SSR markers were employed to analyze the *VRN1* gene in winter wheat genotypes. Three of the winter wheat genotypes indicated the presence of the *VRN1* gene by amplification of the 709bp DNA band, whereas other genotypes didn't show any amplification, indicating either they might not contain the *VRN1* gene or it may be masked by the *VRN2* repressor gene (Benaouda 2022). In the germplasm investigated in the current analysis, we discovered a lack of *VRN1* loci; however, the genotypes that showed the presence of *VRN1* didn't show any correlation with the morphological traits studied. Wheat CAP (Wheat Coordinated Agriculture Projects), funded by USDA-CSREE, utilized eight primers for the detection of vernalization genes in the winter wheat genome. The primers utilized in the present study were furnished by the Wheat CAP Program. Genotypes that are likely to contain the *VRN1* gene can be released for trials in temperate and semi-temperate regions for further studies.

The *NAM-B1* gene was first identified in the population of a cross between wild emmer and durum wheat as a quantitative trait loci (QTL) for protein contents of grain on chromosome 6BS (Shoormij et al. 2023). After that, this QTL was recognized as a single Mendelian locus, i.e., *Gpc-B1* (Leonova et al. 2022). Due to its similarity to *Arabidopsis* protein No Apical Meristem, it was retitled as *NAM-B1* (Shoormij et al. 2023). The ancestral functional allele of the *NAM-B1* gene showed involvement in the early senescence and increased grain protein, zinc, and iron contents. At the same time, this early senescence is responsible for the low yield of crops. Among the identified three alleles of the *NAM-B1*, only the wild-type allele is functional, and two other alleles are null (Andleeb et al. 2023). Three alleles of the *NAM-B1* gene have been reported; however, the wild-type allele is the only functional allele while the remaining two alleles are null (Shoormij et al. 2023). The mutated form of *NAM-B1* is concerned with delayed maturity, hence causing low protein, zinc, and iron contents in the grains. It was also reported that during the domestication of the wheat ancestral allele of *NAM-B1* was deleted from hexaploid wheat (Kumar et al. 2021, Shoormij et al. 2023). In the current analysis, SSR markers were employed to analyze the presence or absence of *NAM-B1*. The results indicated the presence of the *NAM-B1* gene (126bp DNA band) in the two winter wheat genotypes. While most of the genomes analyzed in the current analysis didn't show any presence of the *NAM-B1* gene. To increase the grain mineral and protein content the positive genotypes for *NAM-B1* can be manipulated using molecular and breeding techniques with an emphasis on increasing grain mineral and protein content without compromising the yield.

Recent advancements in plant biotechnology, particularly CRISPR/Cas genome editing, offer promising solutions for enhancing abiotic stress tolerance in wheat and other crops. This powerful tool allows precise editing of stress-responsive genes such as *DREB2A*, *NHX1*, *OsPYL9*, and *GmNAC12*, thereby improving drought and salinity resilience without compromising growth or yield. Unlike traditional breeding methods, CRISPR/Cas enables multiplex editing, base editing, and even transgene-free modifications, accelerating the development of climate-resilient cultivars (Farooq et al. 2026). In the context of winter wheat, future efforts can leverage CRISPR-based approaches to fine-tune vernalization genes (*VRN1*, *VRN2*) and nutrient remobilization genes like *NAM-B1*, which were explored in this study. Targeted manipulation of such loci may enhance adaptation to warming climates and improve grain protein and micronutrient contents. Integration of CRISPR with marker-assisted breeding and omics tools could thus transform wheat improvement pipelines, enabling the creation of elite genotypes with improved agronomic traits and stress tolerance (Farooq et al. 2026).

Conclusions

Overall, the results presented in this study contribute to our understanding of the complex relationships between morphological traits, harvest index, and molecular markers in wheat. These findings highlight the importance of considering multiple traits and their interactions when breeding for improved wheat varieties with desirable agronomic characteristics and higher grain yield potential. Considering crop yield is the most critical trait for food security in today's world, it can be concluded that the genotypes that showed better spike weight and harvest index, including WWLINE 129, WWLINE 125, WWLINE 127, WWLINE 126, WWLINE 47, and Ok-00421, need further field trials to perceive better insights regarding their morphological parameters. The current study will contribute to the release of some of the genotypes for field trials that showed better agronomic traits for temperate and semi-temperate regions of the world.

Future research is necessary to recognize the molecular mechanisms and specific genes underlying the observed correlations between the harvest index and morphological traits. Quantitative trait locus (QTL) mapping and genome-wide association studies (GWAS) approaches can be employed to pinpoint candidate genes and genetic regions linked to the harvest index and morphological traits. Understanding the genetic basis of these correlations will provide valuable insights into the basic physiological and molecular mechanisms that determine the trade-offs between wheat grain production and vegetative growth.

Declarations

Ethics and Consent to Participate

All plant materials used in this study complied with relevant institutional, national, and international guidelines. The winter wheat (*Triticum aestivum* L.) germplasm utilized in this research was either obtained from recognized research institutions or developed under controlled breeding programs, and no wild or endangered species were involved.

The plant materials acquired from international sources, including the University of Nebraska–Lincoln (USA) and Kansas State University (USA), were obtained through formal academic collaboration and material sharing for research purposes, with appropriate permissions from the respective providers. Similarly, the germplasm obtained from the Molecular Lab in Zurich and the Agriculture University Peshawar, Pakistan, was shared under institutional research agreements.

All experimental work was conducted at the Biotechnology Research Farm, COMSATS University Islamabad, Abbottabad Campus, in accordance with institutional guidelines for field experimentation. No specific permits or licenses were required for the use of cultivated wheat genotypes, as they are not protected species and are routinely used in agricultural research.

The authors confirm that all necessary permissions for the acquisition and use of plant materials were obtained, and the study complied with all applicable regulations.

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Competing Interests

The authors declare that they have no competing interests.

Data Availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

All authors contributed to the study conception and design. M.F. performed the investigation and conducted the experiments. A.K. assisted in experimental design and data analysis. A.H. contributed to writing, reviewing, and editing the manuscript and supported the research facilities. M.M.S. contributed to writing, reviewing, and editing the manuscript, provided research facilities, and supervised the study.

Clinical Trial Registration

Not applicable.

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Tables

Table 1: List of molecular marker primers used in the study with their sequences.

S/No	Primer	Target gene	Sequence
1	<i>Intr/B/F/Intr1/B/R3</i>	<i>VRN1</i>	Forward 5'- CAA GTG GAA CGG TTA GGA CA -3' Reverse 5'- CTC ATG CCA AAA ATT GAA GAT GA -3'
2	<i>VRN1AF /VRN1-INT1R</i>	<i>VRN1</i>	Forward 5'- GAA AGG AAA AAT TCT GCT CG -3' Reverse 5'- GCA GGA AAT CGA AAT CGA AG -3'
3	<i>NAM-B1</i>	<i>NAM-B1</i>	Forward 5'-TTGTCCACTGCGCCAGC-3' Reverse 5'-GGCTCCGACCAAACAGTTTC-3'

Table 2: Statistical parameters of evaluation of morphological traits for 127 winter wheat varieties and lines.

Trait	Range	Mean $\pm$ SD	CV%
Germination (%)	6.67-100.00	81.21 $\pm$ 15.37	18.92
Flag Leaf Width (cm)	0.32-1.21	0.96 $\pm$ 0.11	11.26
Flag Leaf Length (cm)	10.00-37.43	25.39 $\pm$ 4.91	19.32
Flag Leaf Sheath Length (cm)	5.50-25.70	19.43 $\pm$ 2.62	13.51
Plant Height (cm)	34.33-160.23	131.14 $\pm$ 19.18	14.62
Peduncle Length (cm)	24.50-60.25	43.28 $\pm$ 7.51	17.36
Tillering	6.33-37.33	17.95 $\pm$ 6.02	33.51
Awn Length (cm)	4.75-11.55	9.01 $\pm$ 1.28	14.16
Spike Length (cm)	4.73-12.00	7.93 $\pm$ 1.26	15.94
Stem Diameter (mm)	1.16-4.38	3.19 $\pm$ 0.50	15.60
Dry Biomass (gm)	21.33-409.00	178.55 $\pm$ 55.63	31.16
Spike Weight (gm)	12.00-204.33	60.85 $\pm$ 25.89	42.54
Harvest Index	0.38-2.10	1.04 $\pm$ 0.31	29.44

Table 3. PCR results for the amplification of *VRN1* (using *Intr/B/F/Intr1/B/R3* and *VRN1AF/VRN1INT1R* SSR markers) and *NAM-B1* (using the *NAM-B1* SSR marker) in different winter wheat varieties and lines.

S. No.	Variety/Line	SSR Marker	Category	Presence or Absence	
				VRN1	NAM-B1
1	OK-00514	<i>Intr/B/F/Intr1/B/R3</i>	Positive Control	+	Nil
2	OK-00421	<i>Intr/B/F/Intr1/B/R3</i>	Positive Control	+	Nil
3	AUP-4008	<i>Intr/B/F/Intr1/B/R3</i>	Positive Control	+	Nil
4	<i>T. aestivum</i> ssp. <i>Compactum</i>	NAM-B1	Positive Control for ancestral allele	Nil	+
5	Khyber-87	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Positive Control	-	+
6	Chinese Spring	<i>Intr/B/F/Intr1/B/R3</i>	Negative Control	-	Nil
7	OK98G508W	VRN1AF/VRN1INT1R	Positive Control	-	Nil
8	W-47 (Pakistan)	NAM-B1	Negative Control	Nil	-
9	WWLINE 16	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Experimental	-	-
10	WWLINE 26	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Experimental	-	-
11	WWLINE 31	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-
12	WWLINE 32	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-
13	WWLINE 33	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-
14	WWLINE 34	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Experimental	-	-
15	WWLINE 35	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Experimental	-	-
16	WWLINE 36	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-
17	WWLINE 37	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-
18	WWLINE 38	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Experimental	-	-
19	WWLINE 39	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-
20	WWLINE 40	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Experimental	-	-
21	WWLINE 41	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Experimental	-	-
22	WWLINE 44	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Experimental	-	-
23	WWLINE 45	▲ NAM-B1 ▲ <i>Intr/B/F/Intr1/B/R3</i>	Experimental	-	-
24	WWLINE 47	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-

S. No.	Variety/Line	SSR Marker	Category	Presence or Absence	
				VRN1	NAM-B1
25	WWLINE 49	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-
26	CNN (WI3A)	▲ NAM-B1 ▲ Intr/B/F/Intr1/B/R3	Experimental	-	-
27	CNN (WI6A)	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-
28	WI (CNN3A)	▲ NAM-B1 ▲ VRN1AF/VRN1INT1R	Experimental	-	-
29	WI (CNN6A)	▲ NAM-B1 ▲ Intr/B/F/Intr1/B/R3	Experimental	-	-

+, - for presence and absence of amplified band, respectively. Nil: Marker was not analyzed for this variety/line. The SSR Marker column consolidates information for both VRN1 and NAM-B1.

Table 4. Pearson correlation analysis of germination (Ger.), flag leaf width (FLW), flag leaf length (FLL), flag leaf sheath length (FLSL), plant height (PH), peduncle length (PL), awn length (AL), spike length (SL), stem diameter (SD), number of tillers (Tiller), dry biomass weight (DBW), spikes weigh (SP), and harvest index (HI).

Traits	Ger.	FLW	FLL	FLSL	PH	PL	AL	SL	SD	Tiller	DBW	SW	HI
Ger.	1	.803**	.710**	.817**	.821**	.719**	.817**	.784**	.690**	.525**	.736**	.714**	.062
FLW	.803**	1	.820**	.892**	.846**	.703**	.892**	.827**	.888**	.565**	.875**	.727**	.079
FLL	.710**	.820**	1	.830**	.794**	.655**	.830**	.788**	.731**	.393**	.741**	.558**	.072
FLSL	.817**	.892**	.830**	1	.911**	.787**	1.000**	.859**	.836**	.482**	.858**	.665**	.114
PH	.821**	.846**	.794**	.911**	1	.882**	.911**	.892**	.731**	.382**	.800**	.630**	.086
PL	.719**	.703**	.655**	.787**	.882**	1	.787**	.820**	.594**	.350**	.688**	.496**	.136
AL	.817**	.892**	.830**	1.000**	.911**	.787**	1	.859**	.836**	.482**	.858**	.665**	.114
SL	.784**	.827**	.788**	.859**	.892**	.820**	.859**	1	.708**	.357**	.754**	.556**	.112
SD	.690**	.888**	.731**	.836**	.731**	.594**	.836**	.708**	1	.562**	.882**	.683**	.124
Tiller	.525**	.565**	.393**	.482**	.382**	.350**	.482**	.357**	.562**	1	.586**	.758**	.022
DBW	.736**	.875**	.741**	.858**	.800**	.688**	.858**	.754**	.882**	.586**	1	.689**	.141
SW	.714**	.727**	.558**	.665**	.630**	.496**	.665**	.556**	.683**	.758**	.689**	1	.065
HI	.062	.079	.072	.114	.086	.136	.114	.112	.124	.022	.141	.065	1

** The correlation is significant at the 0.01 level of probability. This table presents correlations for a set of traits measured in a Randomized Complete Block Design (RCBD) experimental design.

Figures

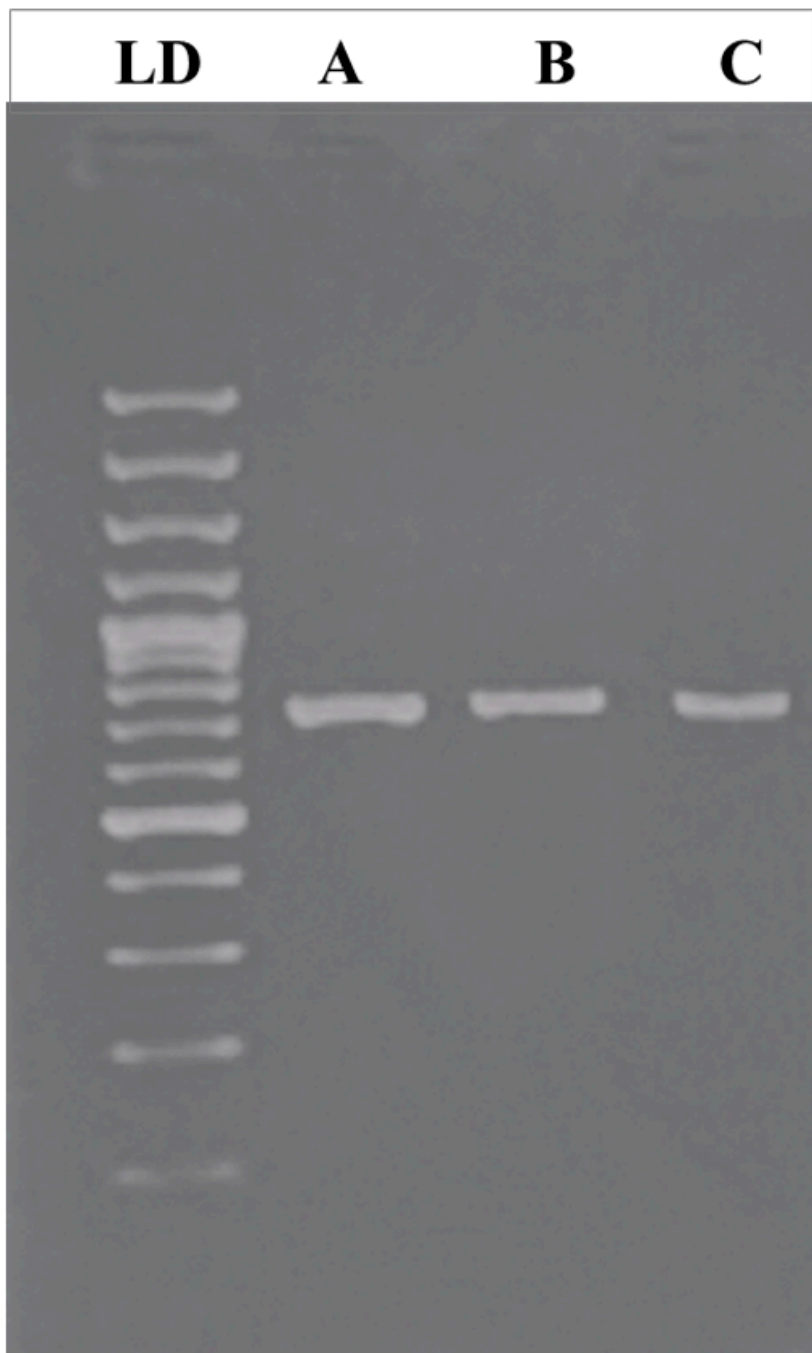


Figure 1

Gel electrophoresis image showing the amplified DNA fragments of various wheat genotypes using SSR *VRN1*-specific molecular markers. Lane LD contains the 100bp molecular ladder. Lanes B, C, and D represent the genotypes OK-40021, OK00514, and AUP-4008, respectively, which show successful amplification.

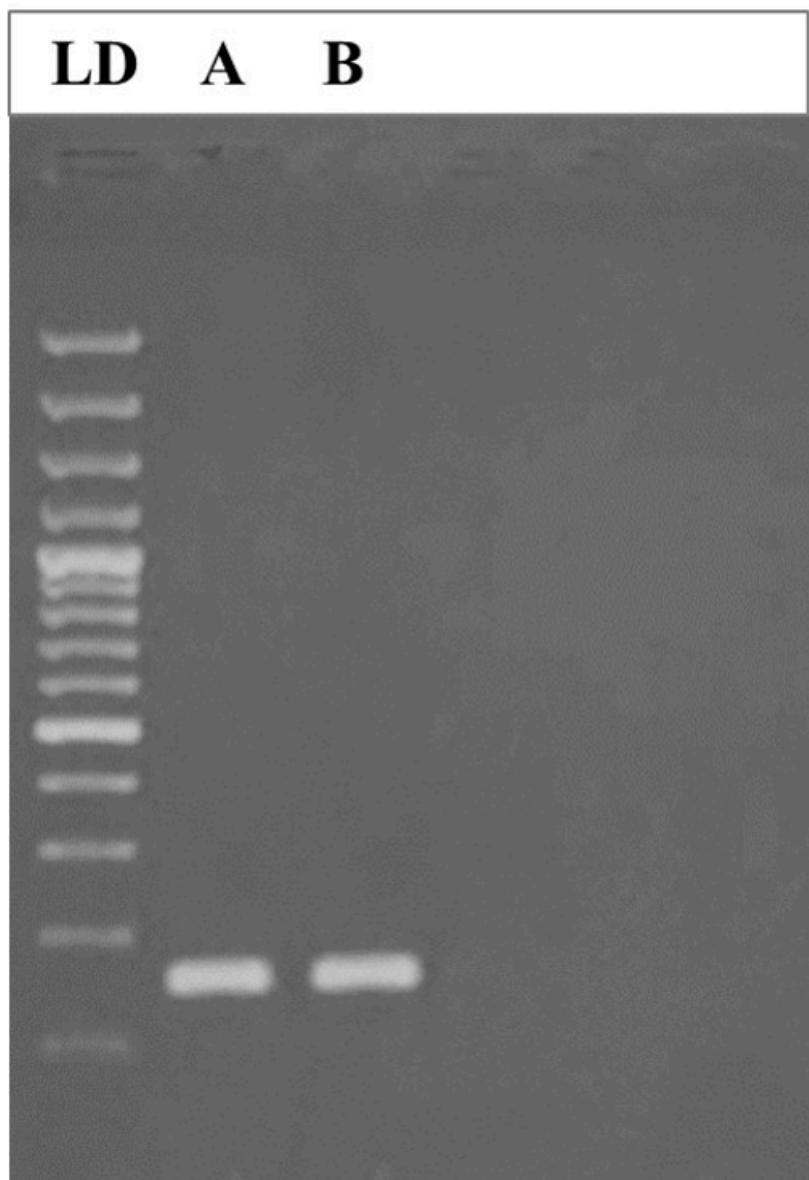


Figure 2

Gel electrophoresis image showing the amplified DNA fragments of various wheat genotypes using SSR *NAM-B1*-specific molecular markers. Lane **LD** contains the 100bp molecular ladder. Lanes **B** and **C** represent the genotypes *T. aestivum* ssp. *Compactum* and Khyber-b7, respectively, which show successful amplification.

Supplementary Files

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