

Research Article

Cite this article: Amounane F, Backhaus A, Amri A, Belqadi L, Alsamman AM and Kehel Z (2026) Genetic diversity, population structure and enhancing selection efficiency in wheat pre-breeding using the CGIAR mid-density DArTag panel. *Plant Genetic Resources: Characterization and Utilization* **24**, 356–368. <https://doi.org/10.1017/S1479262126100525>

Received: 25 June 2025

Revised: 28 January 2026

Accepted: 3 February 2026

First published online: 24 April 2026

Keywords:

bread wheat; DArTag mid-density wheat panel; genetic diversity; population structure; pre-breeding

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Genetic diversity, population structure and enhancing selection efficiency in wheat pre-breeding using the CGIAR mid-density DArTag panel

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Abstract

Bread wheat (*Triticum aestivum* L.) is a vital crop with production demand increasing due to population growth. However, bread wheat is facing challenges, including the emergence of pests and diseases, reduced genetic diversity and climate change. To meet food security, pre-breeding can help to develop varieties by incorporating desirable traits from wild relatives and landraces. Although pre-breeding is crucial, it does not often take full advantage of recent genotyping technologies. The DArTag mid-density wheat panel, developed by CIMMYT as part of the CGIAR initiative, offers a low-cost genotyping solution, but its applicability in pre-breeding programs has not been widely evaluated. This study aims to assess population structure, genetic diversity and genomic selection in a set of 484 pre-breeding bread wheat lines derived from crosses with wild relatives, using the DArTag mid-density wheat panel to investigate the ability of this genotyping approach to validate pedigrees, identify specific trait subsets and detect potential introgressions for targeted selection. Our results demonstrate that the panel captured genetic diversity in the pre-breeding population, identifying seven subpopulations with different genetic compositions; analysis confirmed the existence of private alleles associated with important agronomic traits, highlighting the role of wild relatives, especially *Aegilops bicornis* and *Aegilops ovata*, in genetic variation. In addition, the genotyping also facilitates pedigree validation and generates trait-specific sub-populations for phenotyping. The sub-populations and markers found in this work are useful resources that can be effectively used to accelerate wheat improvement, offering new insights into the contribution of wild relatives and landraces to wheat diversity.

Introduction

Annually, more than 200 million hectares of wheat are cultivated worldwide, of which 95% represents bread wheat (Li *et al.* 2019; Robbana *et al.* 2019; Erenstein *et al.* 2022). Global production of this vital crop reached 767 million tons in 2020 and 771 million tons in 2021 (FAOSTAT, 2024). Wheat is an important source of nutrients that are essential for health, including dietary fibre, minerals, vitamins and phytochemicals (Javid Iqbal *et al.* 2022). Given current population growth and food demands, the annual production of bread wheat must rise by 2% per year (Hatfield and Beres 2019; Singh *et al.* 2019). Historically, crop wild relatives and farmer varieties, also termed landraces, have served as valuable sources of novel diversity that can be easily accessed via global gene banks (Thattantavide and Kumar 2023).

With 21 pairs of chromosomes (AABBDD genome), bread wheat is a hexaploid cereal species, whose origins can be traced back to hybridization between three different grass species within the fertile crescent region of Syria and Turkey (Feldman 2001). The first hybridization event took place between 500,000 and 150,000 years ago, between *Triticum urartu* (AA genome) and an unidentified species closely related to *Aegilops speltoides* (BB genome), creating a new tetraploid species with 14 pairs of chromosomes, namely *Triticum turgidum* L. ssp. *dicoccoides* (AABB genome). The derived domesticated species is termed *T. turgidum* ssp. *dicoccum*, which is also the ancestor of modern durum wheat. A second hybridization event occurred around 8,500–9,000 years ago, between the domesticated tetraploid *T. dicoccum* and the wild diploid species *Aegilops tauschii* (DD genome) to give rise to the hexaploid species *Triticum aestivum* L. (AABBDD genome) (Charmet 2011; Mastrangelo and Cattivelli 2021;



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Levy and Feldman 2022). *Ae. speltooides*, *T. timopheevii*, *T. dicoccoides* and *Ae. tauschii* are species that are closely related to bread wheat and have been successfully used to give resistance to common diseases and pests, including leaf rust, yellow rust, stem rust, powdery mildew, Hessian fly and Russian wheat aphid to cultivated wheat (Helguera *et al.* 2003; Vikal *et al.* 2004; Liu *et al.* 2005; Marais *et al.* 2005; Hiebert *et al.* 2007; Miranda *et al.* 2010; Klindworth *et al.* 2017; Niranjana *et al.* 2017; Sadeghabad *et al.* 2017; Klymiuk *et al.* 2020). Several studies examined the contribution of wheat relatives in improving resistance to significant abiotic stresses, including heat, salinity and drought (Rasheed *et al.* 2018). In particular, *Ae. tauschii* is contributing significantly to bread wheat improvement by developing synthetic hexaploid wheat through interspecific crosses between this wild species and durum wheat (Kishii 2019; Aberkane *et al.* 2021; Gaurav *et al.* 2022; Gudi *et al.* 2024).

Molecular markers can be a useful tool to examine the genetic diversity and evolutionary links between plant accessions (Khan *et al.* 2014). Single nucleotide polymorphisms (SNPs) are considered as one of the most widely used type of markers in gene banks to examine the genetic diversity, population structure and relationships between accessions, which help in the effective use of genetic resources for pre-breeding as well as their management and protection (Arif *et al.* 2010; Robbana *et al.* 2019). In plant breeding, markers like SNP are useful to create kinship matrices for genomic selection (GS), identifying quantitative trait loci (QTL) for gene discovery and to facilitating marker-assisted selection (MAS) for desirable traits (Kamaluddin *et al.* 2017).

Recently, CGIAR has developed mid-density DArTag marker panels for several crops to make genotyping more accessible and cost-effective, particularly for researchers in low- and middle-income countries. These panels aim to optimize genotyping approaches, support breeding programs and accelerate the introduction of new improved varieties to the farmers (Sansaloni *et al.* 2011; Singh *et al.* 2019; CGIAR 2024). However, the use of the mid-density panel for pre-breeding has been limited. Pre-breeding has become an important link between gene banks and breeding initiatives but often lacks sufficient funding. Also, the use of modern breeding technologies, including genotyping with low-cost panels, can help in this context of pre-breeding efforts.

In this study, we start from the hypothesis that the CGIAR mid-density DArTag panel can provide useful information on the genetic diversity and structure of our 484 bread wheat pre-breeding lines derived mainly from crosses with wild parent accessions, making it a valuable tool for pre-breeding. Specifically, our objectives are to (I) assess the panel's ability to capture genetic variation across a set of pre-breeding germplasm, (II) investigate population structure with a focus on the contribution of exotic and elite parents and (III) identify allelic variation of potential selective value, including private alleles (PAs) that may be associated with adaptive traits linked to heat stress performance, which can be strategically exploited in pre-breeding to improve adaptation to drought and climate change. By testing this hypothesis, we aim to demonstrate the applicability of the mid-density DArTag panel and provide new insights into how wild relatives and landraces contribute to the genetic diversity available for wheat breeding.

Materials and methods

Plant material

This study utilized 484 pre-breeding accessions of bread wheat (*T. aestivum* L.), developed through interspecific crosses performed by

ICARDA-Genetic Resources team and selected at the experimental stations Marchouch (33°36' N 6°42' W) and Annoceur (33°41' N 4°51' W) in Morocco. The crosses were made using different landraces and wild species. The selection method employed was the Bulk and pedigree method, which allowed for an efficient identification and propagation of lines exhibiting desirable traits. Pedigrees, species type used in cross and selection histories of all accessions are presented in the supplementary Table S1.

Genotyping and marker quality control

All 484 accessions were genotyped using the mid-density DArTag panel of 3,900 markers. This panel, designed for bread wheat genetic diversity research and GS, includes random and gene/QTL-specific markers (*Wheat 3.9K Mid-Density Genotyping Services*, 2021). Selection criteria of markers in this panel include haplotypes, minor allele frequency and genome and chromosome allocation. To increase its coverage and application, the genotyping panel was modified in 2021 to obtain 3,900 SNP markers distributed over 21 chromosomes (Velu *et al.*, 2016; Sehgal *et al.*, 2020). As a result, 3,900 SNP markers were distributed among the 21 bread wheat chromosomes. DNA extraction and genotyping were performed by Diversity Array Technology (Australia). Markers were filtered by two criteria: missing data $\leq 20\%$ and minor alleles $> 5\%$ and heterozygosity ≤ 0.95 , using the *ASRgenomics* (Gezan *et al.*, 2022) package in R version 4.4.3.

Population structure analysis

The population structure of our pre-breeding accessions was studied using sparse non-negative matrix factorization (sNMF) first introduced in 2014 by Frichot *et al.* and implemented in the LEA package of R version 4.4.3 (Frichot & François, 2015). We evaluated a range of K values from $K = 2$ to $K = 10$. For each K value, five repetitions were applied, the alpha parameter was set to 10, the tolerance to 10^{-5} and the number of iterations to 200. The principal component analysis (PCA) was also conducted on the genomic data for bread wheat using R package *ASRgenomics* (Gezan *et al.*, 2022) to present the population structure and genetically distinct groups within our population, using the first two principal components.

Analysis of genetic variation and population structure using AMOVA, genetic distances and diversity indices

To validate the population structure found in the previous analysis and to quantify genetic variation within and among resulting subpopulations, an analysis of molecular variance (AMOVA) implemented in the R package *poppr* (Kamvar *et al.*, 2014) was performed. Population differentiation was measured using pairwise F_{ST} values calculated with the *hierfstat* R package (Goudet, 2005). The genetic diversity indices including Nei's unbiased gene diversity, PAs (i.e., the alleles unique to the subpopulation) and allelic richness were calculated using the R *poppr* package (Kamvar *et al.*, 2014).

Results

Characterization of the CGIAR wheat DArTag panel in pre-breeding

We evaluated the quality and the relevance of the CGIAR mid-density DArTag marker panel for wheat pre-breeding population,

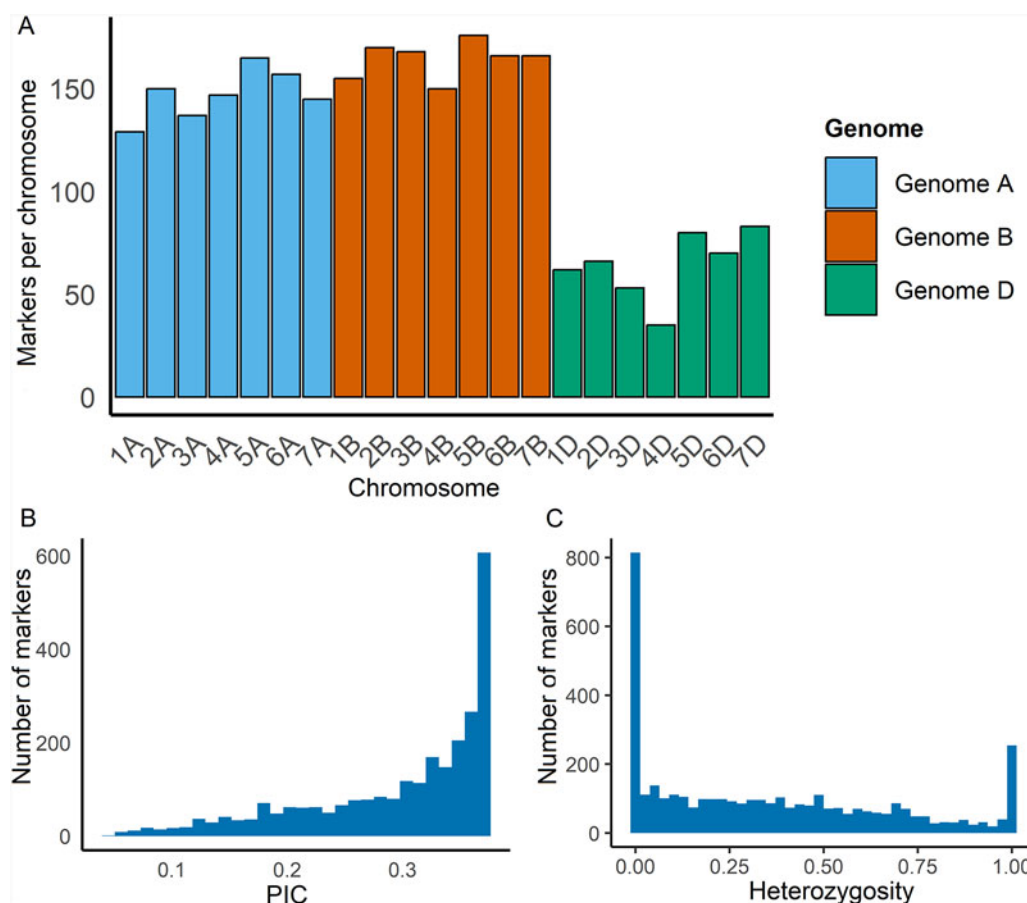


Figure 1. Distribution of 2,633 DArTag markers based on marker characteristics and chromosomal distribution. (A) Number of DArTag markers across the 21 chromosomes of bread wheat for 484 pre-breeding wheat accessions. (B) Polymorphism information content (PIC) distribution. (C) Distribution of the percentage of heterozygosity.

consisting of 484 genotypes derived from crosses between elite germplasm, landraces and crop wild relatives, by analysing marker frequency and distribution across the polyploid genome. Out of the 3,900 markers in the panel, 2,633 were retained after filtering. Of the 2,633 markers, 1,153 (43.81%) were found on the B genome, 1,030 (39.13%) on the A genome and only 449 (17.06%) on the D genome (Supplementary Table S2, Fig. 1A). This distribution of markers reflects the unbalanced distribution of markers in the mid-density panel, which has only 21% of the markers designed for the D genome. The telomeric sections of the 21 bread wheat chromosomes had more markers than the pericentromeric regions, showing a similar distribution to the one reported for the same marker panel used with elite CIMMYT material (CGIAR Excellence in Breeding 2024) (Supplementary Figure S1).

The heterozygosity across all markers ranged from 0.00 to 0.946. About 2% of the markers exhibited heterozygosity between 0.80 and 0.946. For a total of 1,509 markers, heterozygosity was below 0.05 (Fig. 1C, Supplementary Table S3). A total of 2,002 markers had a polymorphism information content (PIC) value greater than 0.25, with a range of 0.05–0.38. Chromosomes 4A, 5A, 6A, 3B and 1D had the highest PIC value (0.38; Supplementary Table S2, Fig. 1B).

Genotypic data of the pre-breeding population reveals distinct sub-populations are due to the gene bank and elite parent

We conducted PCA using the filtered set of 2,633 DArTag markers to evaluate the genetic diversity of the 484 pre-breeding accessions.

The first two principal component (PC1: 10.6% and PCA2: 8.6%) explained 19.2% of the total genomic variance and grouped the lines into four clusters (Fig. 2A). Cluster 1 consisted mainly of accessions derived from crosses with *Ae. ovata* and *Ae. bicornis*. While clusters 2 and 4 contained a mixture of lines, including lines from wild and elite varieties.

Cluster 3 was composed mainly by accessions from crosses between *Ae. bicornis* and winter bread wheat breeding accession (GRIS29/5/VRZ/3/ORF1.148/TDL//BLO/4/PONY/OPATA/) as the elite parent. Additionally, this cluster includes six accessions that, according to the pedigree were derived from a cross between the mentioned winter wheat *Ae. bicornis* accession, back-crossed to the spring elite breeding accession 6920/6/F9.70/MAYA//4105W/3/PLK70/LIRA/4/88 ZHONG 257//CNO79/PRL/5/SB-360-1. Grouped with the winter material, suggesting the backcross was unsuccessful and showcasing the role of genotyping for pedigree validation in pre-breeding. Overall, the PCA indicates that both wild and elite parents influence the genetic diversity of the pre-breeding material (Fig. 2A).

The population structure of the pre-breeding population was further analysed using the sNMF algorithm, indicating the existence of 6–8 subpopulations (Supplementary Figures S2, S3 and S4).

Based on the conducted analysis, $K = 7$ was identified as the most suitable choice of number of subpopulations (Fig. 2B). At $K = 7$ as compared to $K = 6$, a distinct subpopulation comprising 48 lines emerged exclusively from crosses with *Ae. ovata*,

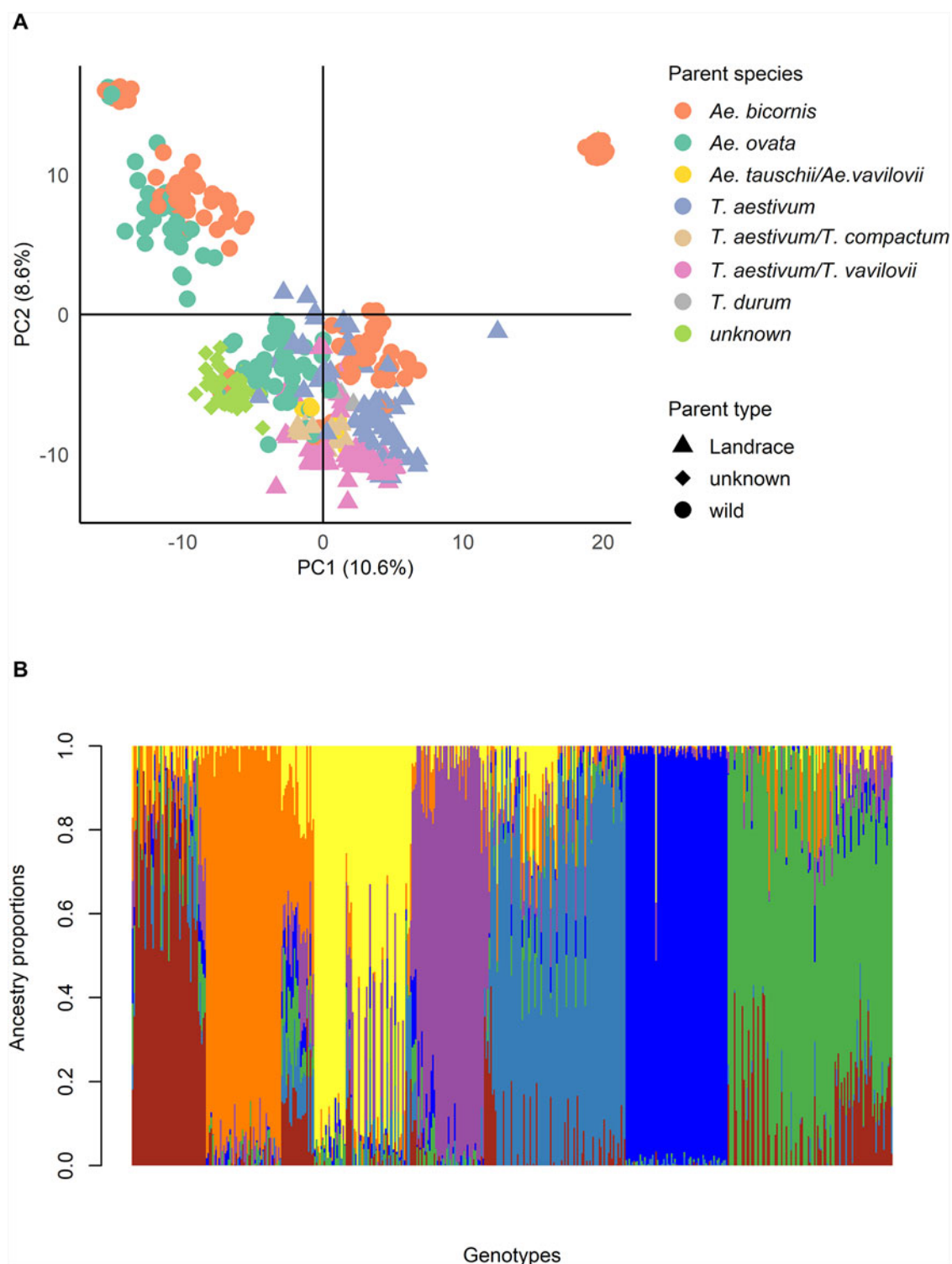


Figure 2. Population structure of 484 bread wheat pre-breeding lines. (A) Principal component analysis of 484 bread wheat pre-breeding lines. (B) Ancestry matrix of 484 bread wheat pre-breeding lines at $K = 7$. Each vertical bar represents one genotype, and the colours indicate the proportion of ancestry from each of the seven subpopulations.

highlighting a clear separation of genetic material and providing a coherent grouping with pedigree information. Furthermore, the subpopulations at $K = 7$ are distinct and interpretable on the basis of parental type and species, whereas at $K = 8$, the emergence of a small subpopulation with no biological relevance may be a sign of overfitting (Supplementary Figures S3 and S4).

Based on the pedigree information, subpop7.1 included 50 genotypes, derived from crosses between the wild parent *Ae. bicornis* (IG47572, IG46854 and IG47620) and elite winter bread wheat lines, corresponding to cluster 4 identified in the PCA. Subpop7.2 consisted of 65 accessions, of which 83.07% resulted from crosses with *Ae. bicornis* (IG46854) and the elite winter bread wheat

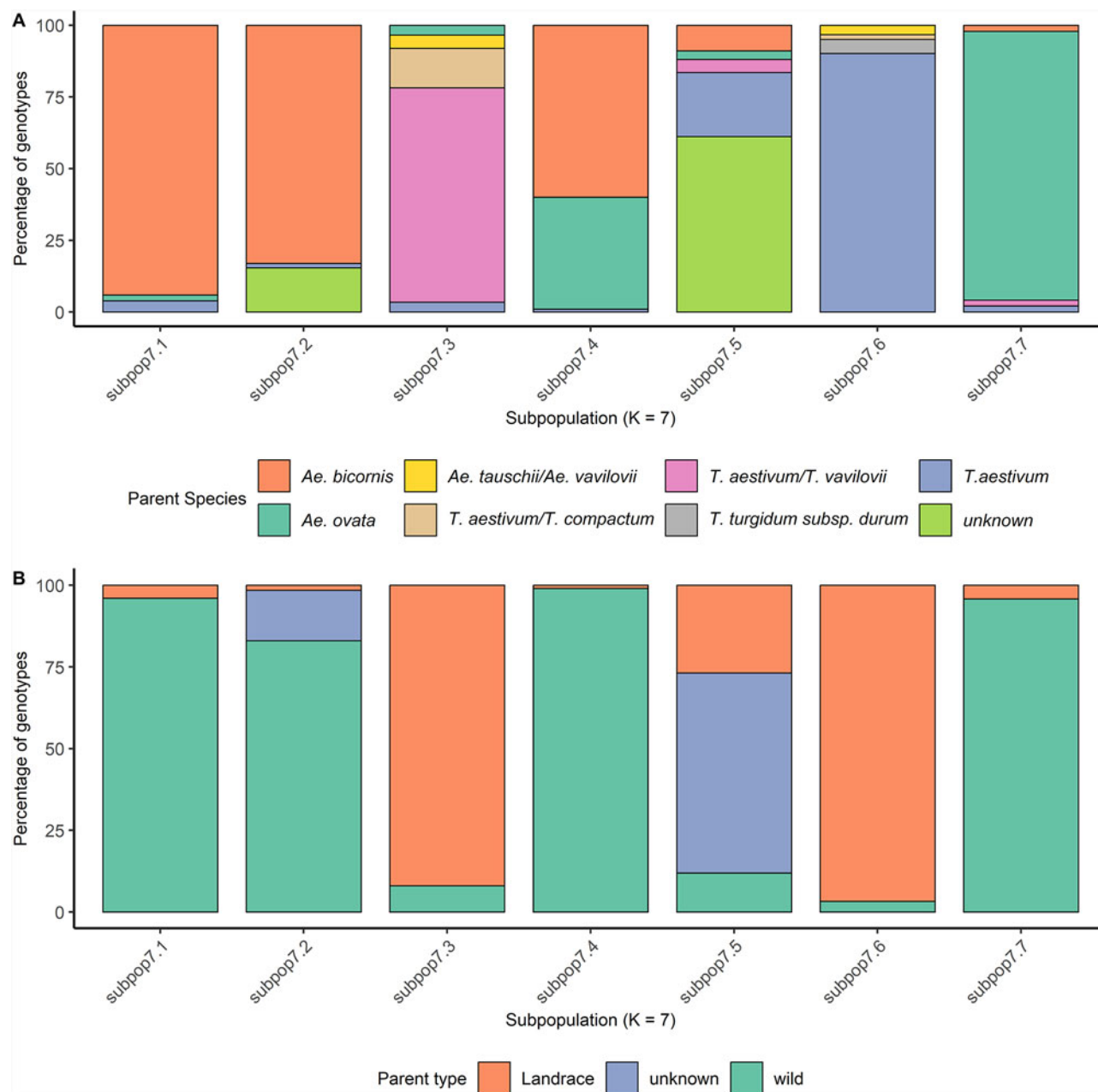


Figure 3. Percentage of parent types and parent species of the 484 bread wheat pre-breeding lines in subpopulations at $K = 7$. (A) Percentage of parent types in subpopulations at $K = 7$. (B) Percentage of parent species in subpopulations at $K = 7$.

GRISSET-9/5/VRZ/3/ORF1.148/TDL//BLO/4/PONY/OPATA, and the six lines with the same wild and elite parent but back-crossed to a spring variety, corresponding thus to PCA cluster 3 (Figs. 2A and 3). Subpop7.4 contained 105 lines that were also mostly derived from crosses to *Ae. bicornis* (IG47572 and IG47403; 60% of the sub-population), but the elite parent included accessions derived from durum varieties (Icaracha and Merzak) crossed to bread wheat lines (SERI.1B//KAUZ/HEVO, Najia and Demo 104; Fig. 3, Supplementary Table S1). In the same sub-population, there were also accessions derived from *Ae. ovata* (IGAECO96-21, IGAECO96-62 and IGAECO96-23) crossed with bread wheat Aguila, Najia, Tilila and SERI-1B//KAUZ. The pedigree information thus suggests that sub-populations are defined by the wild parent as all three populations (subpop7.1, subpop7.2 and

subpop7.4) contain accessions derived from *Aegilops*. However, the sub-populations are also heavily influenced by the elite parent, especially subpop7.4 demonstrates that accessions derived from two different wild species cluster together due to the similar elite parents in the pedigree. Subpop7.3 was predominantly (96.55%) composed of lines resulting from crosses with parents belonging to the *Triticum* genus (*T. aestivum*, *T. aestivum* subsp. *compactum* and *Triticum vavilovii*) to the Tunisian bread wheat varieties Florence, Aurore, Ariana 66, Haidra 99, Tahent and Utique. Subpop7.5 was composed of 61% accessions with unresolved pedigrees and 27% accessions from landraces crossed to the bread wheat varieties Nesma and Cham8, suggesting that the accessions with unresolved pedigrees were from landrace by elite crosses (Fig. 3, Supplementary Table S1). Subpop7.6 predominantly (96.72%)

Table 1. AMOVA results for the genetic variation among and within populations for $K = 7$

Component	Df	Sum Sq	Mean Sq	Sigma	%	Phi
Between population	6	178,803.91	29,800.61	281.31	25.828	0.258
Between samples within population	9	19,934.06	2,214.896	175.627	16.125	0.217
Within samples	468	295,883.37	632.229	632.229	58.047	0.42
Total	483	494,621.34	1,024.061	1,089.166	100	

Note: Df: degrees of freedom; Sum Sq: sum of squares; Mean Sq: mean square.

consisted also of accessions resulting from crosses with landraces; however, the elite parent used is a winter wheat accession. Subpop7.7, comprising 48 genotypes that were exclusively derived from crosses with *Ae. ovata* (IGAECO96-21 and IGAECO96-62), crossed to Faiza, Kharouba and Kanz (Fig. 3, Supplementary Table S1).

Furthermore, genetic variation between and within subpopulations was investigated using AMOVA. The results of the AMOVA for $K = 7$ are summarized in Table 1. AMOVA confirmed significant structure at $K = 7$, with 25.8% of variation explained between subpopulations, 16.1% between samples within populations and 58% within samples (Table 1; Supplementary Figure S5).

Identification of potential introgressions from mid-density genotyping uncovers genetic differences within sub-populations

In addition to the results of our population structure analysis, we identify potential introgressions (PIs) by screening for blocks of consecutive missing markers, which may indicate genomic regions from crop wild relatives not captured by DArTag markers as the markers were designed to amplify in the wheat genome but not in wild relatives.

Searching for these PIs systematically, we gathered all regions with at least three consecutive markers missing and where the same block of missing data occurs in at least two accessions. We detected a total of 49 PIs (Fig. 4, Supplementary Table S4), which were predominantly identified in accessions derived from crosses with *Aegilops* species (98.97%). We detected PIs in accessions derived from crosses with *Ae. ovata* (29) in subpop7.4 and subpop7.7, *Ae. bicornis* (7) in subpop7.1, subpop7.4 and subpop7.5 and *T. vavilovii* (10) in subpop7.3, subpop7.5 and subpop7.6. In subpop7.4, accessions with the pedigree MERZAK/*Ae. bicornis* IG47403//Demo 104 share a potentially introgressed region from marker TaDAR TAG006220 to TaDAR TAG002597 on chromosome 3B with accessions of the pedigree MERZAK/*Ae. bicornis* IG47403//SERI.1B/KAUZ. As these accessions are all derived from the same original cross (Merzak by *Ae. bicornis*), this suggests that genetic material from *Ae. bicornis* was introgressed into the Merzak genome during cross-over in the meiotic division, which was retained in both top-crosses (with Demo and SERI.1B/Kauz). Of the 40 accessions derived from the original crosses, only 18 carried the PI while the other accessions had DArTag data for the three consecutive markers, supporting the idea that the missing data are not due to quality issues.

Additionally, 8 (out of 14) accessions with the pedigree MIK13/*Ae. ovata* IGAECO96-62//Kharouba and (6 out of 13) accessions MIK13/*Ae. ovata* IGAECO96-62//Kanz carried a PI in chromosome 3D (TaDAR TAG002861-TaDAR TAG002868), suggesting a similar inheritance of a PI as described above. Among

eight accessions resulting from crosses with *Ae. ovata* IGAECO96-62 (pedigree: MIK13/*Ae. ovata* IGAECO96-62//Kharouba), all carried PI in chromosome 1D, 3D and 4D. However, three of these accessions (GWP2101873, GWP2100307 and GWP2100349) carried only the PI in chromosome 4D (Fig. 4, Supplementary Table S4).

Accessions with and without the PI clustered closely together as the genome wide differences between them are only marginal and could not be detected with these methods. However, introgressions from the wild can have substantial phenotypic effects and are highly interesting to pre-breeding (Boehm & Cai, 2024; Heuberger et al., 2024). Our analysis suggests that clustering based only on genome-wide differences to form core collections is not advisable when working with pre-breeding material derived from crop wild relatives. The information of PI allows us furthermore to link specific phenotypic variations to introgression once the accessions are evaluated phenotypically and thus measure the benefit of each introgression in comparison to the sister line. The information on PIs can thus help to reduce population size during population advancement, focusing on retaining as many introgressions in the smallest possible population size.

A considerable proportion of markers in these introgressed regions are linked to key traits, including rust resistance, grain yield, heat tolerance and grain quality. This indicates that introgressions could be valuable for breeding programs aimed at improving the adaptability and productivity of wheat under various environmental conditions. Furthermore, the stability of these introgressions across accessions implies that they could be reliable targets for MAS, which could accelerate the development of improved wheat varieties with the desired introgression.

Genetic diversity between sub-populations identifies *Ae. ovata*-derived material as most diverse

Genetic diversity ranged from 0.27 to 0.34 for the seven subpopulations (Table 2). The highest count of PAs was found in subpop7.4 and subpop7.3 with 207 and 129 PAs, respectively. These two subpopulations were the ones with the largest number of genotypes.

Subpop7.4, which is composed of diverse crosses involving both, *Ae. ovata* (IGAECO96-62, IGAECO96-21 and IGAECO96-23) and *Ae. bicornis* (10.18730/7NS17 and 10.18730/7NRP~), displayed a wide variation in PAs, ranging from 1 PA per accession to as many as 10 in the accession GWP2100412 with the pedigree Aguila/*Ae. ovata* IGAECO96-21//Aguila. The diversity of parental lines in this subpopulation, combining genetic material from wild relatives with traditional farmer varieties (Aguila, Najia, Icaracha, Merzak and MIK13), likely contributed to the high number of PAs. This suggests that the integration of wild species with locally adapted varieties can introduce unique genetic variations that may be beneficial if large population sizes for each cross are maintained (Supplementary Table S5). While subpop7.3

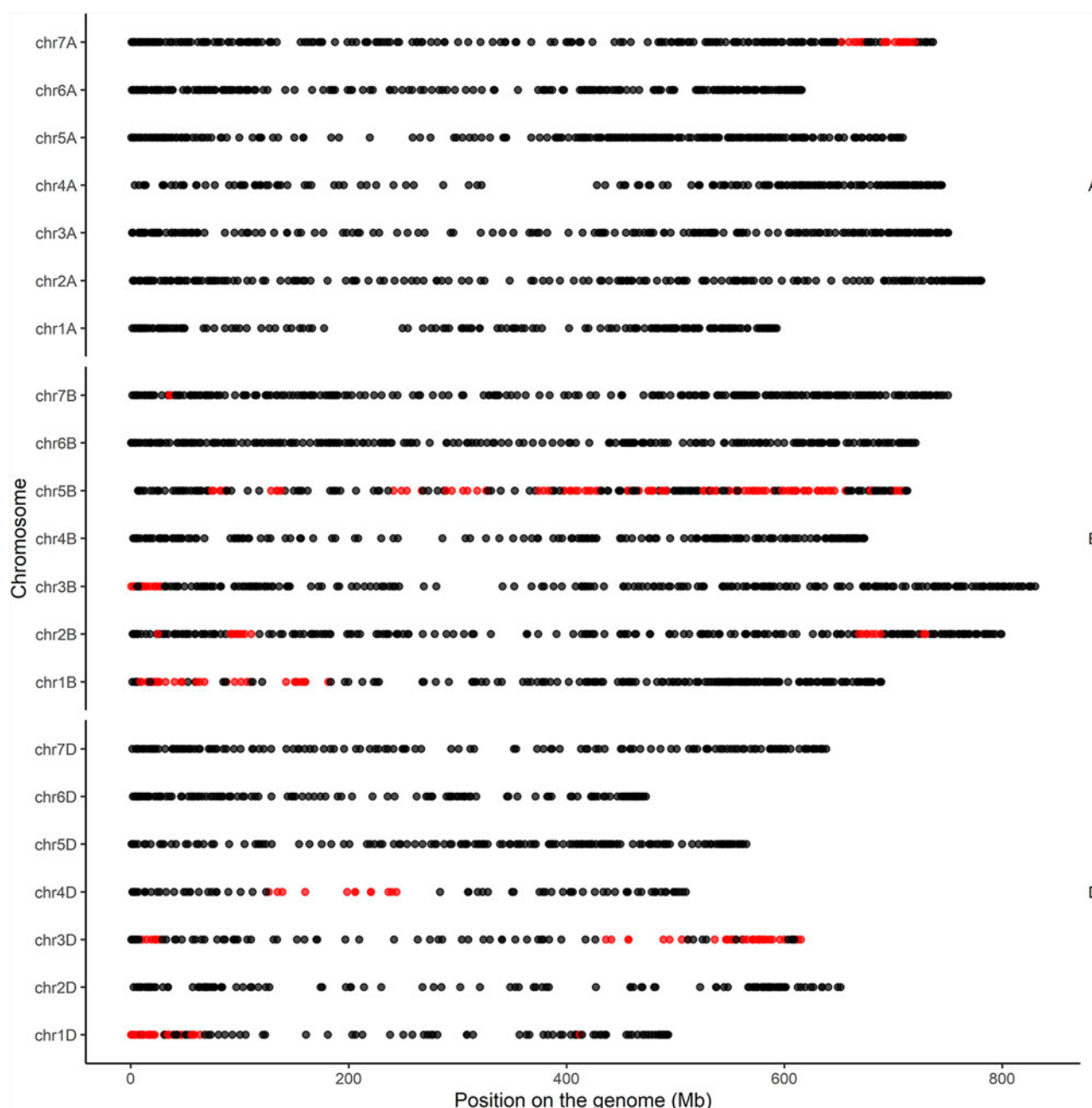


Figure 4. Distribution of DArTag markers across wheat chromosomes highlighting potential introgressions. Markers are presented along chromosomes grouped by genome (A, B and D). Red points indicate the position of markers within introgressed regions; black points represent non-introgressed markers. Genomic positions are in megabases (Mb).

was primarily composed of genotypes resulting from crosses involving *T. vavilovii*, this subpopulation included 64 genotypes with PAs, ranging from 1 allele found in 5 genotypes derived from crosses like *T. aestivum* subsp. *aestivum* (DOI:10.18730/9J03Z)/*T. vavilovii* (DOI:10.18730/9HE6D)//*T. aestivum* subsp. *aestivum* (DOI:10.18730/9J58B) to 4 PAs identified in 3 lines from crosses involving *T. aestivum* subsp. *aestivum* (DOI: 10.18730/9J3M~)/*T. vavilovii* (DOI: 10.18730/9HE6D)//*T. aestivum* subsp. *aestivum* (DOI: 10.18730/9HWX3; Supplementary Table S5). The presence of these PAs, particularly in genotypes derived from multiple crosses with *T. vavilovii*, indicates that such combinations may be effective in introducing unique genetic variations without use of

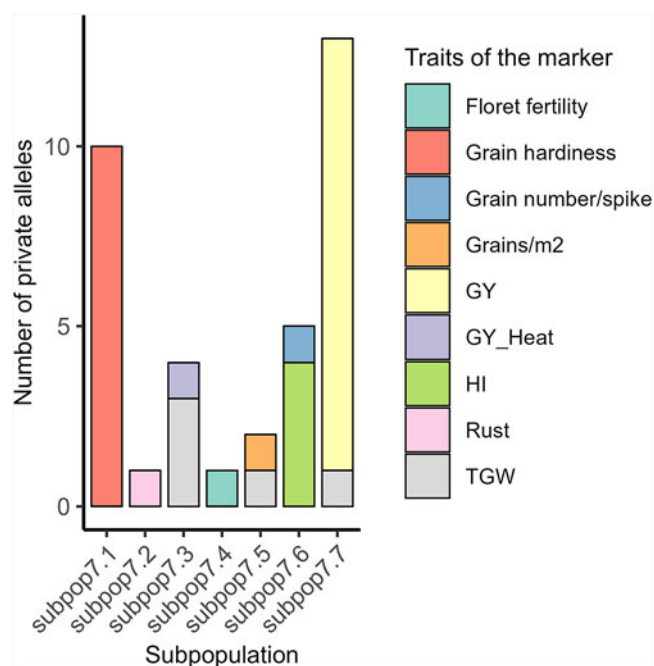
crop wild relatives. This genetic diversity could be biologically significant for breeding programs focused on enhancing diversity per se (Supplementary Table S5).

Despite having the lowest number of genotypes (48), subpop7.7 exhibited the highest unbiased Nei genetic diversity ($H_e = 0.38$) and allelic richness (1.38), with 66 PAs identified, 6 of which are associated with grain yield (Fig. 5). This higher diversity in subpop7.7 is likely due to its exclusive composition of genotypes derived from crosses with *Ae. ovata* (IGAECO96-21 and IGAECO96-62) and different farmer varieties such as Aguila, Kanz, MIK13, Najia, Arrehane, Merzak, Faiza, Kharouba, Najia and Rajae (Fig. 5). Whereas 65 PAs were found in subpop7.1 with 10

Table 2. Population genetics parameters among subpopulations at $K = 7$

Pop	N	No. of PA	Lines with PA	Ar	He
Subpop7.1	51	65	38	1.33	0.33
Subpop7.2	65	6	5	1.27	0.27
Subpop7.3	87	129	64	1.34	0.34
Subpop7.4	105	207	58	1.32	0.32
Subpop7.5	67	37	30	1.31	0.31
Subpop7.6	61	57	40	1.34	0.34
Subpop7.7	48	66	24	1.38	0.38

Note: N: number of genotypes; PA: private allele; Ar: allelic richness; He: Nei's unbiased gene diversity.

**Figure 5.** Total private alleles by subpopulation and trait at $K = 7$. GY: grain yield; GY_Heat: grain yield under heat stress; HI: harvest index; TGW: thousand grain weight.

related to grain hardness (Fig. 5), subpop7.2, which consisted of 65 genotypes with 83.07% resulting from crosses with *Ae. bicornis* (DOI: 10.18730/7NQM), showed the lowest values in terms of PAs (6) with one related to rust, allelic richness (1.27) and genetic diversity ($He = 0.27$).

Overall, these results indicate comparatively better diversity in subpop7.7 that was predominantly composed of crosses with the wild parents *Ae. ovata* (IGAECO96-21 and IGAECO96-62) crossed to Faiza, Kharouba and Kanz.

The population differentiation matrix gives insights into the genetic structure of the seven subpopulations of pre-breeding bread wheat lines (Table 3). The Nei genetic distance values, shown below the diagonal, indicate that subpop7.2 is the most genetically distinct, with values ranging from 0.297 (with subpop7.1) to 0.393 (with subpop7.4). On the other hand, subpop7.5 and subpop7.7 have smaller genetic distances of 0.068, suggesting a closer genetic relationship. The F_{ST} values, shown above the diagonal, underline this differentiation even more. Subpop7.2 has higher F_{ST} values, of 0.191 (with subpop7.5) and 0.166 (with subpop7.3), indicating significant genetic differentiation. In contrast, subpop7.7 has

lower F_{ST} values with several subpopulations, such as 0.066 (with subpop7.3) and 0.083 (with subpop7.6), reflecting less genetic differentiation.

DARtag genotyping can be used to assemble smaller trait-specific subsets that capture full population diversity of target traits

One benefit of the DARtag mid-density marker set is its inclusion of several markers linked to known genes and QTLs for a range of phenotypes. By using this information, we examined the 6 known markers associated with septoria and 11 with rust resistance (CGIAR Excellence in Breeding 2024). We systematically analysed markers for resistance to rust and septoria in order to identify genetic variation within each cross. For each pedigree, we defined the allele combinations of markers linked to each disease (septoria and rust) in order to represent genetic variation in our material. Based on these combinations and the number of lines having each combination, we selected the minimum number of lines needed to capture at least one representative of each unique haplotype (allele combination) in a new subset. Figure 6 illustrates the distribution of selected lines among groups and pedigrees (see Supplementary Table S6). Notably, subpop7.4 and subpop7.7 exhibited higher percentages (more than 46%) of selected lines for either septoria and rust markers, suggesting that the population has particularly high genetic variation for the two traits of interest. In contrast, subpop7.2 displayed a lower proportion of lines selected for septoria and rust. Overall, our methodology enabled us to select 189 (39% of the population) lines with unique haplotypes associated with septoria resistance and 270 (56% of the population) lines with unique haplotypes linked to rust resistance, allowing us to capture the complexity of the allelic compositions present in the whole panel within much smaller sub-panels. This selection approach could lead to more cost-effective and more traits targeted evaluation of pre-breeding populations. For instance, for septoria, the evaluation of only 40% of the initial population would be sufficient to assess all possible unique septoria haplotypes.

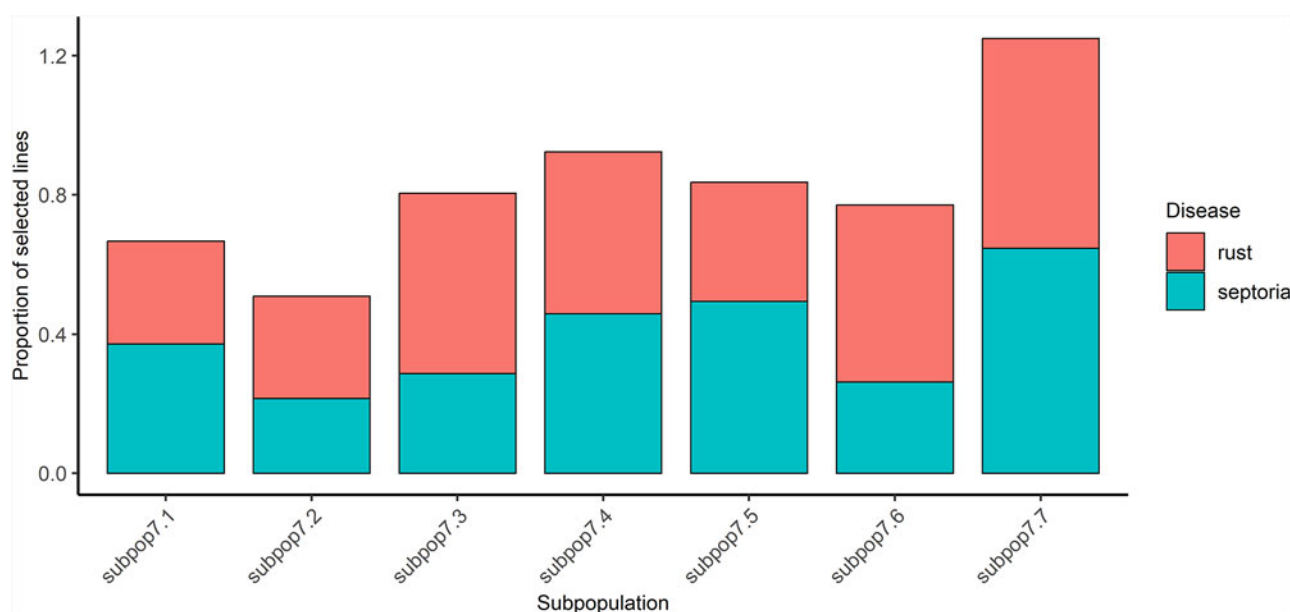
Interestingly, in some cases, specific gene-based resistance gene markers segregated within a sister-line population between a known allele and an unknown one. For example, 15 accessions all with the same pedigree had either the allele '1' for marker TaDARtag000180 or the marker did not amplify, suggesting that there could be a new allele present in these accessions. Therefore, it would be highly valuable to prioritize the phenotypic assessment of these accessions to explore if the unknown allele harbours a novel resistance gene.

Discussion

This study demonstrated the relevance of DARtag technology as a reliable and cost-effective tool for assessing the diversity within and between accessions from crosses between gene bank material including wild parents and elite varieties. This technique yielded a large number of polymorphic and informative markers spread in the A (39.1%), B (43.8%) and D (17.1%) genomes allowing high coverage of the genomes of the bread wheat pre-breeding lines compared to other molecular techniques used previously for wheat cultivar, landraces and advanced lines (Winfield *et al.* 2016; Ul Islam *et al.* 2023; Uddin *et al.* 2024). The large coverage of the genomes can serve to undertake association mapping in the studied germplasm, including finding new allelic variations for major pre-breeder and breeders sought traits such as QTLs corresponding to

Table 3. Population differentiation matrix of bread wheat pre-breeding lines based on Nei's genetic distance (below the diagonal) and pairwise F_{ST} values (above the diagonal)

	Subpop7.6	Subpop7.1	Subpop7.5	Subpop7.2	Subpop7.7	Subpop7.3	Subpop7.4
Subpop7.6		0.085	0.099	0.163	0.083	0.086	0.13
Subpop7.1	0.197		0.1	0.159	0.088	0.098	0.125
Subpop7.5	0.235	0.225		0.191	0.068	0.098	0.107
Subpop7.2	0.325	0.297	0.392		0.163	0.166	0.19
Subpop7.7	0.232	0.236	0.175	0.358		0.066	0.083
Subpop7.3	0.217	0.243	0.243	0.354	0.186		0.119
Subpop7.4	0.319	0.288	0.238	0.393	0.201	0.292	

**Figure 6.** Proportion of selected lines in subpopulations at $K = 7$ for rust and septoria markers.

yield component, protein content, salinity tolerance and disease resistance (L. Chou *et al.* 2022; Tyrka *et al.* 2023; Chen *et al.* 2025). Mid-density DArTag technology along with other high throughput and genotyping by sequencing molecular techniques are increasingly used to study the genetic diversity of different crops as they allow to study the genetic diversity of large number entries and complex genomes with low-cost (T. Chen *et al.* 2018; Nadeem *et al.* 2018; Ongom *et al.* 2024).

The PIC is widely used in population genetics studies because it provides information about the variability and frequency of different alleles within a population (Botstein *et al.* 1980). In our study, 76% of markers had values above 0.25, with an average of 0.28, which is consistent with previous studies using the DArTag and various marker panels for triticale and wheat accessions (Badea *et al.* 2011; Novoselović *et al.* 2016).

While the mid-density DArTag panel for bread wheat offers a practical and cost-effective method for quality control, diversity analysis and GS, as well as for integrating markers linked to QTLs, genes and quality control in order to balance genome coverage with trait relevance, its lower marker density compared to high-density panels limits its resolution for accurate mapping or the discovery of new variants (CGIAR Excellence in Breeding 2024). On the other hand, the 35K Axiom platform and other high-resolution

arrays offer better interpretation of studies due to reduced missing data and greater uniformity for population structure analysis and GWAS analysis, as well as broader genomic coverage (Kumar *et al.* 2020; Wang *et al.* 2022). The ideal panel could be further optimized to specifically mark SNPs across all *Triticum* and *Aegilops* lines, although this requires a lot of work and is not necessary for most breeding programmes. As a practical alternative, the use of mid-density panels, such as DArTag in parallel with parental genotyping, may offer a balance between cost, resolution and forward analysis performance.

In our study, population structure analysis using PCA revealed the genetic relationships between accessions, identifying four groups within the 484 bread wheat pre-breeding lines. A distinct cluster (3) was created by all accessions derived from winter wheat by *Ae. bicornis* crosses. However, within this cluster were also six accessions, which according to their pedigree were derived from crossing the winter *Ae. bicornis* to spring bread wheat varieties. The PCA results suggest that in these six cases the cross failed and the pedigree should be corrected. Another nine accessions derived from the same spring $\times$ (winter $\times$ *Ae. bicornis*) crosses were assigned to cluster 4 and 2, which otherwise contained spring wheat $\times$ *Ae. bicornis* crosses, suggesting that these were successful top-crosses. Furthermore, in 51 accessions, the information on

what gene bank species had been used was lost in the pedigree. All clustered with other accessions derived from crosses with landraces, suggesting that the 51 unresolved accessions were derived from landrace crosses as well. Population structure analysis using the sNMF algorithm enabled us to identify seven distinct subpopulations, four of them composed mainly of crosses with the wild parents *Ae. bicornis* and *Ae. ovata*. In particular, the analysis revealed that the subpop7.1 and subpop7.2 groups are associated with crosses with winter wheat, indicating that these constitute a unique subpopulation with characteristics of interest for breeding.

Subpop7.5, subpop7.6 and subpop7.3 were composed mainly of crosses with landraces and primitive wheat from the primary gene pool, which includes *T. aestivum* subsp. *compactum* and *T. vavilovii*. All subpopulations identified by the PCA and sNMF analyses were aligned, indicating that the selected elite parent and the wild parent from the gene banks have important effects on subpopulation structure. Also, a high degree of within subpopulation diversity was associated with the diversity of elite parents chosen. This underlines the importance of careful designing crosses in pre-breeding, considering both parents. Pre-breeding involves crossing diverse gene bank accessions to a limited number of elite accessions (Mujeeb-Kazi and Hettel 1995; Moore 2015; Dempewolf *et al.* 2017), such as Chinese Spring wheat, which is more successful in wide crosses with wild relatives. Similarly, the discovery of *Ph1* mutations has limited the diversity of elite parents in pre-breeding (Türkösi *et al.* 2022). Looking forward, especially in landrace crosses with low recombination barriers, pre-breeders must focus more on selecting the elite crossing partner, based on the trait desired from the cross, the adaptation to the local environment, and on reducing the loss of genetic gain by using younger elite accessions.

The contribution of wild relatives to genetic diversity was demonstrated by the analysis of PAs, which showed distinct differences between subpopulations. The greatest number of PAs was found in subpop7.7, which is derived from *Ae. ovata* crosses. This suggests that this species offers an important number of novel variations. On the other hand, subpop7.2, composed mainly of crosses with *Ae. bicornis*, had the fewest number of PAs. This aligns with previous studies demonstrating wild relatives' contribution to bread wheat gene pools (Khanna-Chopra and Viswanathan 1999; Farooq 2004; Pradhan *et al.* 2012; Tirnaz *et al.* 2022). In addition, several studies have shown that *Ae. ovata* provides salt tolerance-related alleles for bread wheat breeding (Farooq *et al.* 1989; Nevo *et al.* 1993; Mguis *et al.* 2008).

The population structure and genetic diversity identified in our material, particularly the PAs associated with traits related to heat and drought tolerance, provide useful information for improving pre-breeding efforts aimed at enhancing drought resilience and climate adaptability in wheat production systems in arid areas.

PIs of the CWR genomic sequence improve wheat's genetic diversity, disease resistance and grain quality. These introgressions increase wheat's adaptability and resilience to environmental changes and pests. Studies show that introgressions from *Triticum* and *Aegilops* species significantly increase wheat's genetic diversity (Farooq 2004; Kishii 2019; Johansson *et al.* 2020; Aberkane *et al.* 2021; Gudi *et al.* 2024). In our study, we identified 49 PIs in accessions derived from crosses with *Aegilops* species, with 98.97% of these introgressions occurring in accessions that include *Ae. ovata* and *Ae. bicornis*, specifically involving the B and D genomes. This high frequency of PIs, particularly in *Ae. ovata* accessions, suggests that these wild species contribute considerably different and novel genetic variation to the wheat genome. Although the presence of these PIs, based on missing marker data, provides a

first indication of their importance, further validation is essential to confirm their added value. Techniques such as targeted molecular markers, fluorescent in situ hybridization or genomic in situ hybridization are complementary tools for validating these introgressions and assessing their true contribution to wheat breeding. Previous studies have highlighted the role of these cytogenetic methods to detect introgressions and confirm their functional role in crop improvement (Chaumeil *et al.* 2003; Asmamaw Amogne and Awoke 2023).

Characterizing genetic diversity in pre-breeding material is essential for identifying beneficial alleles. In this study, we explored PAs and exploited the design of the DARtag panel, which includes 466 markers linked to known genes and QTLs. By focusing on disease resistance traits, particularly septoria and rust, we reduced the population from 484 to 189 accessions for septoria and to 270 for rust, while keeping all the genetic diversity. Accessions from CWR crosses for which data were missing were also retained, as they may carry new alleles not detected by current markers. These results show how the panel can be used to create trait-focused subsets from pre-breeding populations for specific traits of interest, making the process more targeted, decreasing downstream phenotyping costs and facilitating the discovery of new resistance genes.

Conclusion

In the present study, we used the DARtag mid-density panel to assess genetic diversity in bread wheat pre-breeding material. Our population showed significant genetic variation and several subgroups based on parent type and species, indicating its potential for future breeding programs aimed at developing new wheat varieties with desirable traits such as high yield and resistance to major biotic and abiotic stresses. In addition, the identification of PAs linked to important agronomic traits highlights the importance of incorporating diverse genetic resources into pre-breeding efforts. While DARtag technology is still efficient and low-cost, future panels should contain markers coming from related wild species and increase marker density in order to better understand changes in relations to wild species and enhance the success of introgression. This work highlights how crucial it is to combine pre-breeding with advanced molecular methods in order to maximize the utilization of genetic resources and manage challenges related to global food security.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S1479262126100525>.

Acknowledgements. This work is partially funded by the CGIAR Genebank initiative on gene banks. Work by AEB was supported by the UK-CGIAR Centre for Collaboration in Science and Innovation, project L24ROM137, funded by FCDO. We also acknowledge the contributions of all collaborators involved in the development and evaluation of the wheat pre-breeding material.

Author contributions. F.A.: Conceptualization (lead); methodology (lead); investigation (lead); data curation (lead); formal analysis (lead); visualization (lead); writing – original draft (lead); and writing – review and editing (equal). A.B.: Conceptualization (supporting); methodology (supporting); formal analysis (supporting); supervision (supporting); writing – original draft (supporting); and funding acquisition (supporting). A.A.: Resources (supporting); writing – review and editing (equal); supervision (lead); and validation (supporting). L.B.: Writing – review and editing (equal); supervision (lead); and validation (supporting). A.I.A.: Writing – review & editing (equal). Z.K.: Conceptualization (supporting); methodology (supporting); resources (lead); formal analysis (supporting); supervision (lead); writing – original draft (supporting); funding acquisition (lead); and validation (lead).

Competing interests. The authors declare no competing interests.

Data availability. The data generated and analysed during this study will be made available through ICARDA's institutional database at the following link: https://gigwa.icarda.org:8443/gigwa/?module=Wheat_Taestivum5.

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