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ORIGINAL ARTICLE

Simultaneous improvement of grain yield and grain protein concentration in durum wheat by using association tests and weighted GBLUP

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Abstract

Key message **Simultaneous improvement for GY and GPC by using GWAS and GBLUP suggested a significant application in durum wheat breeding.**

Abstract Despite the importance of grain protein concentration (GPC) in determining wheat quality, its negative correlation with grain yield (GY) is still one of the major challenges for breeders. Here, a durum wheat panel of 200 genotypes was evaluated for GY, GPC, and their derived indices (GPD and GYD), under eight different agronomic conditions. The plant material was genotyped with the Illumina 25 k iSelect array, and a genome-wide association study was performed. Two statistical models revealed dozens of marker-trait associations (MTAs), each explaining up to 30%. phenotypic variance. Two markers on chromosomes 2A and 6B were consistently identified by both models and were found to be significantly associated with GY and GPC. MTAs identified for phenological traits co-mapped to well-known genes (i.e., Ppd-1, Vrn-1). The significance values (*p*-values) that measure the strength of the association of each single nucleotide polymorphism marker with the target traits were used to perform genomic prediction by using a weighted genomic best linear unbiased prediction model. The trained models were ultimately used to predict the agronomic performances of an independent durum wheat panel, confirming the utility of genomic prediction, although environmental conditions and genetic backgrounds may still be a challenge to overcome. The results generated through our study confirmed the utility of GPD and GYD to mitigate the inverse GY and GPC relationship in wheat, provided novel markers for marker-assisted selection and opened new ways to develop cultivars through genomic prediction approaches.

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Introduction

Durum wheat (*Triticum turgidum* ssp. *durum*) is a tetraploid species widely cultivated in the countries of the Mediterranean basin, in Canada, in the desert areas of the southeastern USA, in northern Mexico and in other minor areas (De Vita and Taranto 2019). The protein concentration, the starch composition, and the vitreousness of the kernels make this species particularly suitable to produce pasta (Troccoli et al. 2000; Kaplan Evlice 2022) whose consumption has grown steadily over the past few decades to reach over 16 million tons in 2021 (IPO 2021; https://internationalpasta.org/about-ipo/). This is mainly attributable to the beneficial effect of pasta on human health due to the low amount of fats and carbohydrates available (Augustin et al. 2017; Huang et al. 2017), and its reduced ecological footprint (Ruini et al. 2013). Grain protein concentration (GPC) and starch composition play a key role in defining the functional properties of

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semolina (Shewry et al. 2003; Lafiandra et al. 2014) as they are directly responsible for the firmness of the pasta during cooking and for the absence of surface stickiness of cooked pasta (Bonomi et al. 2012; Bresciani et al. 2022).

The possibility of increasing GPC through traditional plant breeding is hampered by the strong influence of the environment and by its negative relationship with grain yield (GY) (Blanco et al. 2006; 2012; Rapp et al. 2018; Giunta et al. 2022; Taranto et al. 2023). Indeed, the increase in GY achieved in the last decades was associated with a reduction in the protein amount in the kernels when genotypes are grown with similar nitrogen (N) availability (De Vita et al. 2007; Subira et al. 2014; Giunta et al. 2018); the latter depends directly on the absorption of N supplied as fertilizer (Colecchia et al. 2013; Blandino et al. 2015; Carucci et al. 2021). Extensive studies have suggested that GY and GPC are controlled by many quantitative trait loci (QTLs) with small effects and localized on all chromosomes of both tetraploid and hexaploid wheat (Gupta et al. 2017; Kumar et al. 2018; Colasuonno et al. 2021; Ding et al. 2022; Arriagada et al. 2022; Marcotuli et al. 2022).

The strategies mainly used to understand the genetic basis of these traits have been QTL mapping, using biparental populations, and genome-wide association studies (GWAS) (Taranto et al. 2018; Arriagada et al. 2020). For durum wheat, dozen of QTLs have been reported for both GY (Blanco et al. 2012; Patil et al. 2013; Graziani et al. 2014; Russo et al. 2014; Sukumaran et al. 2018b; Fatiukha et al. 2020; Mangini et al. 2018, 2021) and GPC (Blanco et al. 2006; 2012; Maccaferri et al. 2008; Laidò et al. 2014). In addition, QTL meta-analysis performed by combining different consensus maps and types of markers was performed for these two traits to aid marker-assisted selection (MAS) with the identification of candidate genes (Maccaferri et al. 2019; Soriano et al. 2021; Marcotuli et al. 2022; Arriagada et al. 2022). Unfortunately, only a few of these QTLs have been exploited in breeding programs through MAS, without breaking the negative association between GY and GPC. For example, the *Gpc-B1* locus, encoding a *NAC* transcription factor (*TtNAM-B1*), accelerated the senescence process and N translocation in the grain (i.e., GPC), but reduced grain weight by limiting GY (Brevis et al. 2010; Tabbita et al. 2017; Velu et al. 2017). Conversely, the identification of QTLs for grain weight and number of grains in the wild emmer wheat Zavitan, and the successful introgression into the background of the durum wheat Svevo (Avni et al. 2018; Golan et al. 2019) led to an increase in yield but no information emerged on the effect of the two QTLs on GPC. The grain protein deviation (GPD), a derived index based on the residuals of the regression of protein concentration on grain yield (as explaining factor), has been proposed as a concurrent selection criterion (Monaghan et al. 2001) so that genotypes with a high GPD show higher protein concentration at different yield level (Oury and Godin 2007). This adjusted phenotypic breeding value has already shown some potential to mitigate the aforementioned negative trade-off when used in a recurrent selection scheme (Mosleth et al. 2015; Rapp et al. 2018; Thorwarth et al. 2018). Nigro et al. (2019) identified four QTLs for GPD suggesting that selecting for GPC could not affect the final grain yield. Rapp et al. (2018) using GY and GPC-derived indices, such as GPD, yield protein and grain yield deviation (GYD), identified several MTAs, showing how a combination of indices allowed for a better balance between the two main traits. In all these latter reports, GWAS have revealed a complex genetic architecture for all traits with most QTLs having a very small effect not useful for a MAS program.

In this context, genomic prediction (GP) is a promising option to resolve the potential trade-off between the GY and GPC (Bentley et al. 2014; Guo et al. 2014; Bassi et al. 2016). Recently, GP has been widely used in durum wheat to study different traits such as yield and grain quality (Haile et al. 2018; Rapp et al. 2018), phenological indices (Montesinos-López et al. 2019), and disease resistance (Steiner et al. 2019), allowing the reduction in labor costs compared to conventional breeding (Meuwissen et al. 2001; Lorenzana and Bernardo 2009; Bhat et al. 2016).

To be attractive, the GP models should achieve a moderate level of prediction accuracy (PA), being this latter directly proportional to genetic gain (Heffner et al. 2010). PA is calculated from the correlation between the genomic estimated breeding values (GEBVs) and the true breeding values (TBVs) (Heffner et al. 2009) and depends on various parameters, such as population size (Crossa et al. 2013), the genetic architecture of the target trait(s) (Sallam et al. 2015), marker density (Poland and Rutkoski 2016), and the statistical model (Lozada and Carter 2019). Therefore, the successful implementation of genomic prediction strategies in breeding programs requires careful consideration of all these factors.

One of the most widely used methods for evaluating genomic prediction models is k-fold cross-validation, in which the original dataset is randomly split into subsets, typically five or ten. All but one of the subsets are used as the training population, while the remaining subset is used as the validation population. Several genomic prediction studies have applied this method for model validation (Crossa et al. 2010; Albrecht et al. 2011; Resende et al. 2012). However, this approach has a drawback since the same original population is part of both the training and test populations, the accuracy of the prediction from cross-validation could be biased, resulting in overly optimistic predictions, as also reported by Amer and Banos, (2010) and Michel et al. (2016). Conversely, validation using an independent study

could provide higher prediction accuracy by avoiding overfitting, as reported by Amer and Banos (2010) and Hofheinz et al. (2012).

Genomic best linear unbiased prediction (GBLUP) is one of the most applied models due to its ease of use and low computational power required (Meuwissen et al. 2001).

The GBLUP model calculates GEBVs using phenotypes and a genomic relationship matrix (G) assuming a normal distribution for single nucleotide polymorphism (SNP) effects (Contaldi et al. 2021; Cappetta et al. 2021). The G matrix is routinely estimated assuming the same effect for all SNPs (VanRaden 2008; Goddard and Hayes 2009). This represents one of the most relevant limitations of the GBLUP model, which is biologically flawed since most traits depend on different sets of genes and chromosomal regions (Zhang et al. 2010a, b, c). Breaking this assumption, Zhang et al. (2010a, b, c) performed a weighted GBLUP (WGBLUP) assuming an unequal contribution for all SNPs. More recently, WGBLUP has been successfully implemented in animal breeding providing improved accuracy prediction of carcass weight and backfat thickness in Hanwoo cattle (Lopez et al. 2020). In an empirical plant breeding study, WGBLUP slightly increased the prediction accuracy for yield in alfalfa (*Medicago sativa* L.) (Medina et al. 2021).

Therefore, in this study, GWAS and WGBLUP were performed to better elucidate the genetic basis of the mechanisms regulating individually or simultaneously important and complex agronomic traits. To this end, a large panel of durum wheat elite varieties were grown in different years and agronomic conditions to (i) investigate the genetic variance, heritability and correlations between GY, GPC, GPD, GYD and some other related traits; (ii) identify QTLs/genes that could be used in molecular-assisted breeding to improve GPC without reducing GY; (iii) evaluate the potential of genomic prediction using WGBLUP; (iv) validate the trained models using an independent panel of lines for their implementations in breeding programs.

Materials and methods

Plant material and genotyping

The plant material consisted of 200 durum wheat (*Triticum turgidum* ssp. *durum*, $2n = 4\times = 28$; AABB genome) genotypes adapted to the southern European climate, including modern varieties and advanced breeding lines. This group of genotypes will be referred hereafter to as the Cross-Validation Panel (CVP). Plants were grown at CREA Research Centre for Cereal and Industrial Crops (CREA-CI), Foggia, Southern Italy (41° 27′ 36″ N, 15° 30′ 05″ E) and at Giovanni Santacroce Spa, Deliceto, Foggia, Southern Italy (41° 26′ 59″ N, 15° 46′ 73″ E) for two consecutive seasons (2017–2018 and 2019–2020). At both sites, the genotypes were sown on the recommended dates (late fall) and randomly arranged in a split-plot design with two agronomic treatments as main plots (irrigation water supply and high nitrogen input vs. rainfed conditions and low nitrogen input) and two replicates. The plots included eight rows of 7.5 m in length with a row spacing of 0.17 m. In the high input treatment, 240 kg/ha of N fertilizer was divided into three applications (120, 70, and 50 N kg/ha at tillering, stem elongation, and flowering time, respectively), and the plots were irrigated using a drip irrigation system maintaining soil moisture not less than 20% of field capacity. Soil moisture probes evenly distributed across the fields provided data to help ensure target field capacity was maintained. The low input treatment was carried out under rainfed conditions and with a single application of 60 kg/ha of nitrogen in the tillering phase.

The sowing density has always been 350 seeds m^2. During pre-sowing, 45 kg/ha N and 115 kg/ha P_2O_5 were supplied; an additional dose of 85 kg/ha N was applied each year in the case of high input. Weeds, pests, and fungal diseases were chemically controlled. Genotypes were evaluated under 8 conditions resulting from site × treatment × growing season combination. Genotyping was performed using the Illumina wheat 25 K iSelect Array developed by TraitGenetics GmbH, Gatersleben, Germany (www.traitgenetics.com). The entire dataset can be downloaded at https://data.mendeley.com/datasets/rt2gmzbvmz/1. Raw genotyping data were processed with PLINK (Purcell et al. 2007) using a call rate value of less than 95% and a minimum allele frequency (MAF) of less than 5%. After filtering, a total of 6795 SNPs distributed across all 14 chromosomes were used for downstream analysis.

An Independent Validation Panel (IVP), consisting of 40 F7–F8 advanced breeding lines derived from the ongoing breeding program at CREA-CI, was used to evaluate the GP models. The advanced breeding lines were grown at CREA-CI during two consecutive seasons (2020–2021 and 2021–2022) in rainfed conditions and arranged in a randomized complete block design with plots of 10 square meters and two replicates. The durum wheat lines were grown under standard agronomic conditions (i.e., rainfed and 120 kg/ha of N fertilizer) and evaluated for the same traits (except for flag leaf appearance (FLA) and Nitrogen uptake in the grain (N_upt) resulting from site x growing-season combination. IVP was genotyped using the Illumina wheat 15 K iSelect Array developed by TraitGenetics, and only markers shared with the 25 K were used for analysis. The raw dataset describing the 40 advanced breeding lines under study can be downloaded at the following link: https://data.mendeley.com/datasets/rt2gmzbvmz/1.

Agronomic traits and statistical analysis

The plots of the fields were harvested with a combine harvester. Grain yield was determined in t ha^{-1} and adjusted for moisture concentration at 13%. Grain protein concentration was determined by near-infrared spectroscopy (ICC standard method 159, ICC, Vienna, Austria). FLA (growth stage 41; Zadoks et al. 1974) was expressed in days since April 1st. Heading date (HD) was recorded when about half of the culms showed emerging spikes and was expressed in days since April 1st (growth stage 55; Zadoks et al. 1974). The measurements of test weight (TW), expressed as kg hl^{-1}, were obtained using a Schopper Chondrometer equipped with a 1 L container. N_upt, expressed in Kg N ha^{-1}, was calculated by multiplying the N percentage of the total biomass at the harvest date by the grain yield. The data underwent a general linear mixed model statistical analysis during the present study, the latter being able to model non-normal distributed data (Suardi et al. 2020). In addition to the evaluated GY and GPC, two yield and protein indices were derived: grain protein deviation (GPD) and grain yield deviation (GYD). The IVP was phenotyped for the same traits as CVP except for FLA and N_upt as they were not collected.

BLUEs were calculated using the following linear mixed model:

$$y_{ijk} = \mu + g_i + t_j + r_{k(j)} + gt_{ij} + e_{ijk} \quad (1)$$

where y_{ijk} are the observed values of the ith genotype within the kth replicate at the jth environment, μ is the overall mean, g_i corresponds to the effect of the ith genotype assuming it was a fixed effect, t_j is the jth environment (all combination treatment-year-location) effect modeled as the random effect with $t_j \sim N\left(0, \boldsymbol{I}\sigma_t^2\right)$, $r_{k(j)}$ is the effect of the kth replicates within the trial considered as random factor with $r_{k(j)} \sim N\left(0, \boldsymbol{I}\sigma_r^2\right)$, gt_{ij} represents the genotype by environment interaction modeled as random effect with $gt_{ij} \sim N\left(0, \boldsymbol{I}\sigma_{gt}^2\right)$, and e_{ijk} is the residuals effect considered as random factor and assuming to have a normal distribution $e_{ijk} \sim N\left(0, \boldsymbol{I}\sigma_e^2\right)$. This model was constructed within the R environment using the 'lmer' function from the 'lme4' package (Bates et al. 2015). To calculate the broad sense heritability (H^2) per trait across all environments, the variance components were estimated using the same model used for the BLUEs, with the only exception that the ith genotype effect (g_i) was considered as a random factor with $g_i \sim N\left(0, \boldsymbol{I}\sigma_g^2\right)$. Therefore, the following formula was used to calculate the broad sense heritability:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{ge}^2}{\#Env} + \frac{\sigma_e^2}{\#Env*\#Rep}} \quad (2)$$

where σ_g^2 is the genotypic variance; σ_{ge}^2 is the variance resulting from the genotype x environment interaction; and σ_e^2 is the error variance component. Next, we used grain yield and grain protein concentration BLUEs to calculate the derived indices GYD and GPD. GYD was expressed as the deviation from the regression between GPC and GY. This was done by estimating the residuals from the regression of grain yield on protein concentration based on BLUEs previously calculated (Rapp et al. 2018) using the following formula:

$$GYD = GY - \alpha - \beta GPC \quad (3)$$

where GY and GPC are the measurements of grain yield and grain protein concentration, respectively; α is the intercept, and β is the regression coefficient. By inverting the role of GY and GPC in the last equation, we were able to calculate GPD (Oury and Godin 2007) as follows:

$$GPD = GPC - \alpha - \beta GY \quad (4)$$

A graphical assessment of the normality was carried out using the residuals obtained from the mixed linear model expressed in Eq. (1) for each trait and drawn using the R environment (R Core Team 2023), as reported by Kozak and Phiepo (2018).

Population structure, linkage disequilibrium, and marker-trait association analysis

The CVP structure was assessed by a Principal Component Analysis (PCA) matrix and a kinship matrix (K), which were used in a mixed linear model (MLM) as a variance–covariance matrix across individuals. Both were calculated and tested in GAPIT3 (Wang and Zhang 2021). The linkage disequilibrium (LD) decay value was calculated using the LD Adjacent Pairs Analysis function as part of the SNP and Variation Suite (SVS) software package (version 8.4.0, Golden Helix Inc.) and then, used to define the confidence interval for the identification of candidate genes. As for GWAS, BLUEs and five models were used to identify the MTAs for each environment (Esposito et al. 2022). The five models were: (i) Mixed linear Model (MLM; Yu et al. 2006); (ii) compressed mixed linear model (CMLM; Zhang et al. 2010a, b, c); (iii) Fixed and random model Circulating Probability Unification (Farm-CPU; Liu et al. 2016); (iv) Linkage-disequilibrium Iteratively Nested Keyway (BLINK; Liu et al. 2016); (v) Settlement of MLM Under Progressively Exclusive Relationship (SUPER; Wang et al. 2014). Manhattan plots and quantile–quantile (Q–Q) plots were

automatically generated by GAPIT3. The MLM model is the most popular approach to control for spurious associations using population structure and a kinship matrix as covariates, even though these might be confounding factors. The CMLM method was developed to solve the MLM confounding problem and to improve statistical power by grouping individuals into groups and adjusting their genetic values (not the genetic effects of individuals) as random effects. In the SUPER model, the number of genetic markers used to define individual relationships is strongly reduced to small bins, each of which is represented by the most significant markers.

A maximum likelihood method is then used to optimize the number of bins selected by excluding markers that are in LD with respect to the test marker, regardless of local distance. The last two models (FarmCPU and BLINK) are instead considered as multi-locus models, and both allow the evaluation of large datasets while reducing false positives and negatives (Huang et al. 2019). FarmCPU was developed to control for false positives and confounding effects between test markers and cofactors in an iterative way. The associated markers are fitted as cofactors to control for false positives to test the remaining markers in a fixed-effect model, and then, a random effect model is used to select the associated markers. BLINK was developed to increase statistical power and efficiency (Huang et al. 2019). It differs from FarmCPU assumption that causal genes are evenly distributed in the genome, which improves throughput, as optimization of bin size and the number is no longer required (Huang et al. 2019).

Genomic prediction

Genomic Best Linear Unbiased Prediction (GBLUP) and Weighted Genomic Best Linear Unbiased Prediction (WGBLUP) were applied using the following equation:

$$y = 1\mu + Zg + e \tag{5}$$

where y is the vector of the BLUEs; 1 is the vector of ones, μ is the grand mean; Z is the design matrix of random effects;g is the vector of genomic breeding values; and e is the vector of random residuals assuming $e \sim N\left(0, I\sigma_r^2\right)$ where σ_e^2 is the variance of the residuals and I is the scaled matrix of markers (VanRaden 2008). In the GBLUP formula, it is assumed that $g \sim N(0, G\sigma_g^2)$, where G is the genomic relationship matrix and σ_g^2 is the additive genetic variance. Here, the genomic relationship matrix was constructed by assuming equal weight of the markers (GBLUP) and assigning a specific weight to each marker (WGBLUP). In conventional GBLUP, according to VanRaden (2008), the G matrix was generated as follows:

$$G = \frac{MM'}{\sum_{i=1}^{m} 2p_i\left(1 - p_i\right)} \tag{6}$$

where M is the matrix of centered genotypes; m corresponds to the marker number and p_i represents the minor allele frequency of the ith SNP. In this formula, the equal contribution of each marker is assumed. By contrast, the weighted G matrix (G^*) in the WGBLUP was generated as follows:

$$G^* = \frac{MDM'}{\sum_{i=1}^{m} 2p_i\left(1 - p_i\right)} \tag{7}$$

where elements such as M, m, and p_i are the same as in Eq. 6, and D represents the diagonal matrix where each value corresponds to the weight of the SNP.

In this study, 10 rounds of fivefold cross-validation were performed. In the WGBLUP scenario, the weight corresponded to $-\log_{10}$ (p-values) obtained by performing GWAS on the training population at each round of the fivefold was used. Fifty random training populations, each including 160 genotypes, were constructed and used as input to perform GWAS. Then, p-values from the BLINK and FarmCPU models at each GWAS were used to weight fifty G matrices for each trait and obtain predictions. Prediction accuracies were calculated using the *sommer* R package (Covarrubias-Pazaran 2016), whereas all genomic relationship matrices were computed using the R package *AGHmatrix* (Amadeu 2016). PA in the IVP was calculated using the GBLUP and WGBLUP models implemented considering the whole CVP.

Comparison of MTAs with known QTLs

The MTAs identified in the present study were also compared with known QTLs/MTAs. The physical positions of all SNPs on Svevo chromosomes (Maccaferri et al. 2019) were obtained by aligning sequences harboring each SNP to the reference genome by BLAST, retrieving only hits with a full-length alignment. The physical position of all significant MTAs was also visualized in the Grain Genes Genome Browser (https://wheat.pw.usda.gov). The coordinates of the boundaries of the QTL intervals identified in the present study were compared with those detected by Saini et al. (2021) (for GY, HD, PH, and grain morphometric traits), Gudi et al. (2022) (for GPC), Nigro et al. (2019) and Thorwarth et al. (2018) (for GY-GPC indices).

Results

Descriptive statistics of phenotypic traits

BLUEs values showed a good degree of variability for the target traits, thus demonstrating that the population under study is suitable for GWAS (Supplementary Table 1). The variance explained by each component of Eq. (1) was reported in Supplementary Table 2, whereas normality was assessed using residuals obtained from the mixed linear model expressed in Eq. (1) for each trait (Fig. 1). Except for FLA, which exhibited a multimodal distribution, and GY, which was normally distributed, most of the traits under study displayed heavy-tailed distributions (Fig. 1).

The coefficient of variation ranged from 0.02 (TW) to 0.75 (GPD), whereas broad-sense heritability ranged from 0.41 (N_upt) to 0.93 (HD). In detail, higher values of heritability ($H^2 > 0.60$) were found for all traits except for N_upt, which had a lower H^2 (0.41).

Pearson's and Spearman's correlations were also employed to further understand pairwise relationships between traits (Supplementary Fig. 1, Supplementary Table 3). The highest positive correlation was observed between GYD and N_upt (+0.94; *p*-value = 2.2e−16), and between GY and GPC with their respective indices, namely GYD and GPD (+0.87 and +0.86, respectively; *p*-values < 0.001) (Supplementary Fig. 1). A negative correlation between GY and GPC (−0.46; *p*-value = 6.0e−12) and between TW and GPC (−0.28; *p*-value = 6.7e−5), whereas no relationship was found between GY and GPD. Low correlation values were also found between phenology-related traits (HD and FLA) and all other target traits (Supplementary Fig. 1).

Genotyping, principal component analysis and linkage disequilibrium decay

A total of 6795 high-quality SNPs, 43.8% mapped on genome A and 56.2% on genome B, were used (Fig. 2). As durum wheat chromosomes differ in length, SNP markers were not evenly distributed among chromosomes. The lowest number of SNPs was on chromosome 4, with fewer SNPs on chromosome 4A; on the other hand, chromosome 1B is the one with the greatest number of SNPs (Fig. 2).

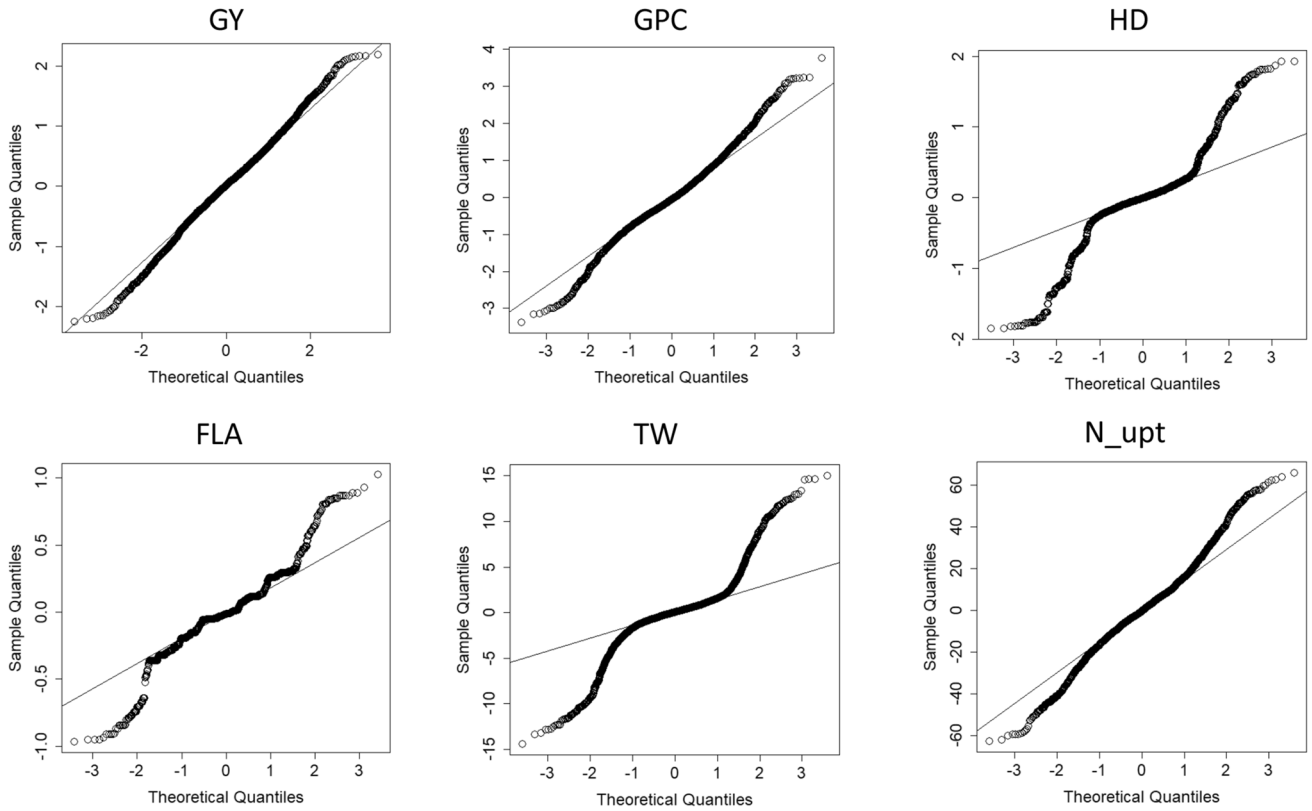


Fig. 1 QQ-plot of residuals obtained from Eq. (1) for each trait, with the exception of the derivate indexes (GYD, and GPD), for which values were not available. GY grain yield (t ha^{-1}), GPC grain protein concentration (%), HD heading date (days), FLA flag leaf appearance (days), TW test weight (g), N_upt nitrogen uptake (kg N ha^{-1})

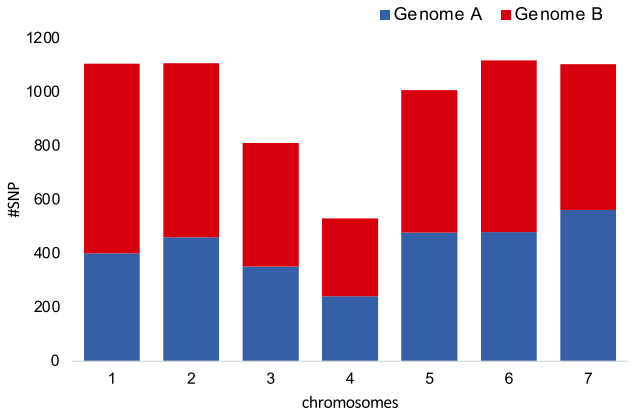


Fig. 2 Bar chart showing the distribution of SNPs along genomes A and B

PCA showed the individuals to be quite scattered in the plot, with the top three principal components accounting for only 30% of the phenotypic variation (Fig. 3A). This result suggested that the population under study was poorly stratified as also confirmed by the Kinship matrix used in GWAS (Fig. 3B). Genome-wide LD decay was observed at ~1.8 Mb (Supplementary Fig. 2).

Genome-wide association studies

Five statistical models were run in GAPIT3 (CMLMM, MLM, SUPER, FarmCPU, and BLINK) using 6,795 high-quality SNPs to identify genetic loci associated with target traits (Fig. 4). We tested five models to determine the optimal one for each trait based on quantile–quantile (QQ) plots. We observed a non-uniform distribution of *p*-values in the SUPER model for almost all phenotypical traits, suggesting that this model may be inappropriate for identifying associated markers. MLM and CMLM were instead strongly conservative since they identified few associated markers and increased the number of false negatives. By contrast, FarmCPU and BLINK appear to better control false positive and false negative associations (Table 1). As an example, Fig. 4b shows the QQ plot for each of the five tested models for GPC; the remaining QQ plots are reported in Supplementary Fig. 3.

FarmCPU and/or BLINK identified 32 markers significantly associated with seven traits except N_upt; seven markers were scored by both methods and therefore, were marked as more robust associations (Table 2; Fig. 5, Supplementary Fig. 4). Three additional markers were found to be significantly associated with GY, GPD and TW by a single model, while for the remaining one they are just below the Bonferroni threshold. Associated markers were located on all chromosomes with the sole exception of chromosome 4B (Table 1; Fig. 5). AX-94576974 on

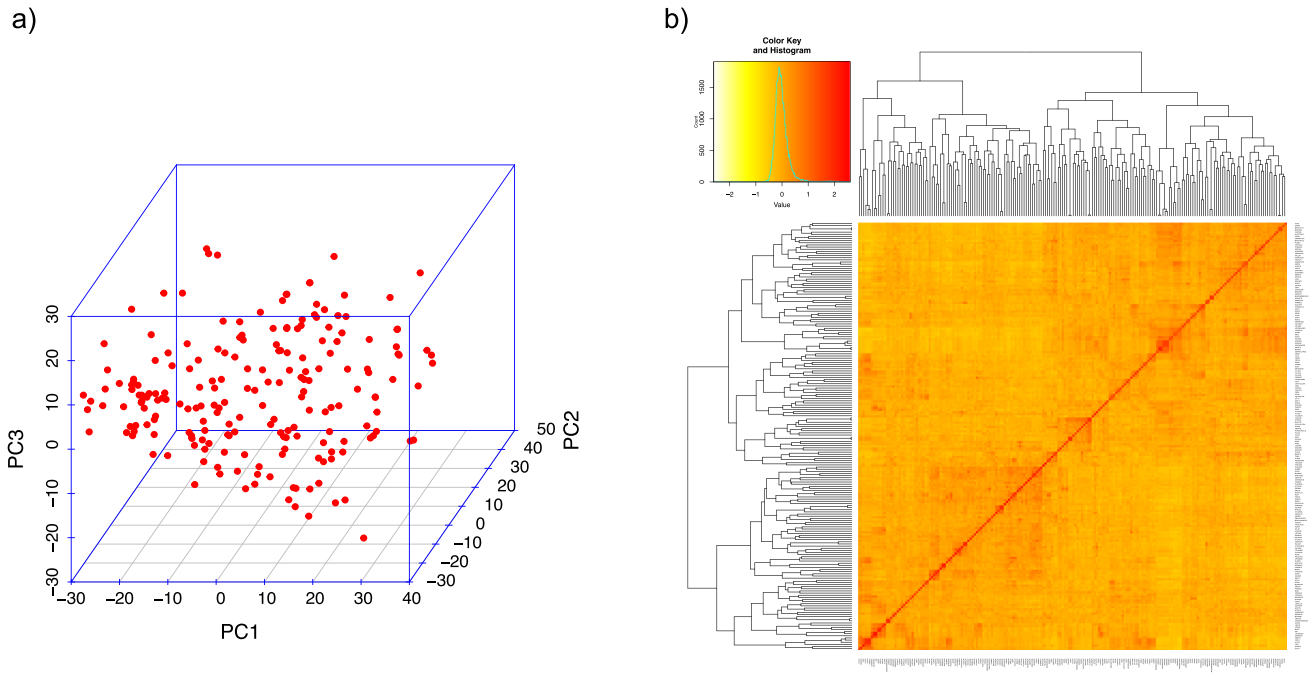


Fig. 3 **a** Three-dimensional plots showing the top three principal components of the SNPs ($N=6795$) × individuals ($N=200$) data matrix. **b** Heat map of the Kinship matrix calculated by the identity-by-descent based on genomic relationship matrices (GRM)

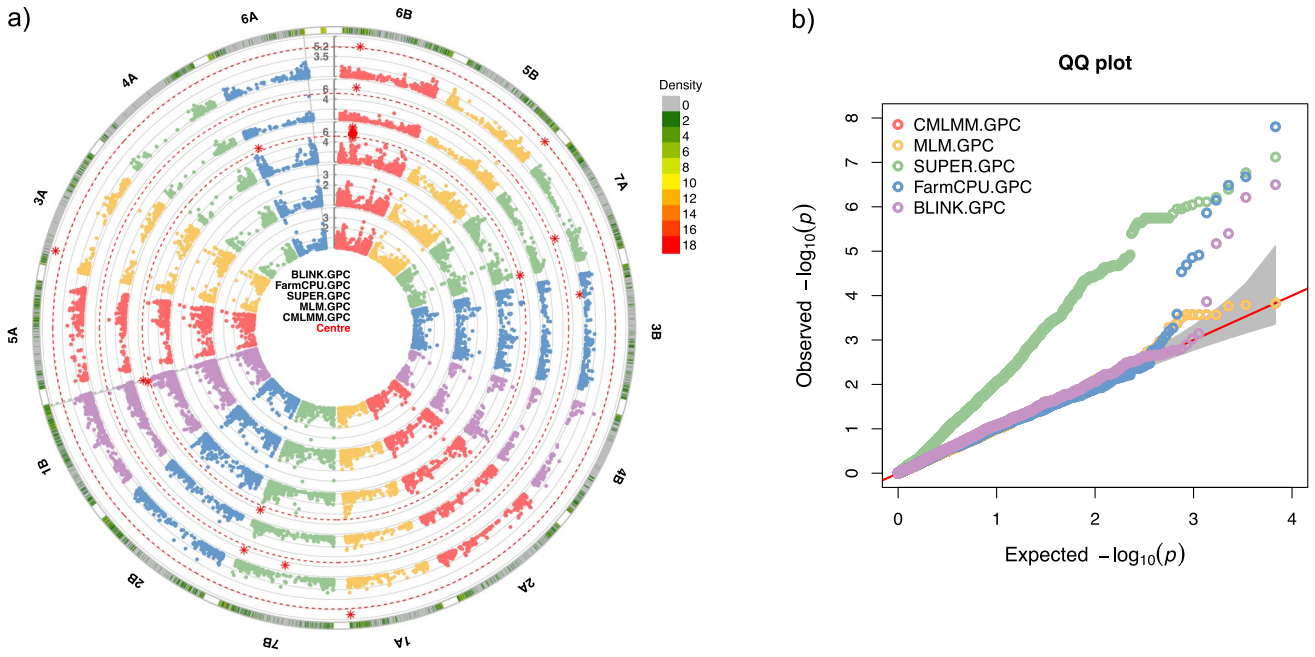


Fig. 4 **a** Circular Manhattan plot showing results for each of the statistical method used (BLINK, FarmCPU, SUPER, MLM and CMLMM). Significant associations are marked with red asterisks. **b** Quantile–quantile (QQ) plot of the five statistical models tested for the "grain protein concentration (GPC)" trait. The red line is the expected distribution under the null hypothesis (color figure online)

Table 1 Summary statistics and heritability for eight phenotypic target traits in a panel of 200 durum wheat genotypes

Trait	Mean	Min	Max	SD	CV	H^2
GY	6.77	5.54	7.77	0.46	0.07	0.67
GYD	0.00	−0.95	1.12	0.39	0.73	–
GPC	14.50	13.11	17.10	0.72	0.05	0.86
GPD	0.00	−1.63	1.81	0.61	0.75	–
HD	29.33	22.33	36.83	3.19	0.11	0.93
FLA	3.42	1.00	5.00	1.11	0.32	0.91
TW	81.43	76.48	84.38	1.47	0.02	0.92
N_upt	166.84	142.51	198.96	9.94	0.06	0.41

SD = Standard Deviation, CV = Coefficient of Variation. GY grain yield (t ha^{-1}), GYD grain yield deviation (index), GPC grain protein concentration (%), GPD grain protein deviation (index), HD heading date (days), FLA flag leaf appearance (days), TW test weight (g), N_upt nitrogen uptake (kg N ha^{-1}), SD standard deviation, CV coefficient of variation, H^2 heritability

chromosome 2A was found by BLINK to be associated with GY and GYD. The same marker was scored with a $-\log_{10}$ (p-value) = 4.8 by FarmCPU, just below the Bonferroni threshold. AX-109345149 on chromosome 6B was found to be associated with GPC by both statistical models. The marker BS00078413_51 on chromosome 1B was found to be associated with GPC and GPD. Association tests revealed the utility of GY and GPC indices. Indeed, several markers on chromosomes 2B, 5B, 6A, and 7A were associated with GPD but not with GPC, and most of them were independent of GY and GYD (Fig. 5).

Two markers, one on chromosome 2A and one on chromosome 5B, were found to be associated with HD by both methods. The marker AX-110933998 on chromosome 5B exceeds the threshold set only according to FarmCPU.

RAC875_c1643_1548_1 on chromosome 2A had the highest significance ($-\log_{10}$ (p-value) > 12 for both models) and overlapped the genomic region harboring *Ppd-A1*, whereas AX-110933998 on chromosome 5B was close to the *Vrn-B1* locus. FarmCPU identified two additional markers associated with HD (on chromosomes 2A and 7B, respectively). BLINK association tests returned four markers (Ku_c269_2643 on chromosome 2A, Tdurum_contig54925_225 on chromosome 2B, wsnp_RFL_Contig4307_5006558 on chromosome 5A, and AX-158559793 on chromosome 7A)

Table 2 Characterization of MTAs identified by FarmCPU and BLINK models for eight traits

Trait	QTN	MTA	Chromosome	Position	*p*− value	Model	PVE (%)
GY	*Q.GY-2A*	AX-94576974	2A	699002865	1.05E−08	BLINK*	19.62
	Q.GY-4A	Tdurum_contig7992_605	4A	698878120	2.77E−08	FarmCPU	5.12
GYD	*Q.GYD-2A*	AX-94576974	2A	699002865	2.57E−09	BLINK	13.68
GPC	*Q.GPC-1A*	BS00024107_51	1A	576972389	6.17E−07	BLINK	7.68
	Q.GPC-3A	Tdurum_contig10426_280	3A	102225240	3.17E−07	BLINK	5.02
	Q.GPC-3B	Tdurum_contig43263_243	3B	148004098	1.58E−08	FarmCPU	27.81
	Q.GPC-5B	Kukri_c637_517	5B	699936252	6.74E−06	BLINK	11.30
	Q.GPC-6B	AX-109345149	6B	119530509	3.27E−07; 4.02E−06	FarmCPU; BLINK	1.18;6.18
	Q.GPC-7A	BS00091168_51	7A	517554461	1.37E−06	FarmCPU	2.07
	Q.GPC-7B	Tdurum_contig11521_102	7B	368924124	2.11E−07	FarmCPU	13.76
	Q.GPC-7B.2	Excalibur_c23777_74	7B	701398438	7.11E−07	FarmCPU	2.13
GPD	*Q.GPD-1B*	BS00078413_51	1B	654103642	8.08E−08	FarmCPU*	2.47
	Q.GPD-2B.1	AX-95256064	2B	2550907	1.43E−06	FarmCPU	3.10
	Q.GPD-2B.2	wsnp_Ex_c29445_38480890	2B	523912577	3.45E−06	FarmCPU	17.16
	Q.GPD-5B	AX-94449281	5B	411135705	2.66E−07	BLINK	0
	Q.GPD-6A	AX-94531527	6A	28543900	6.73E−08	FarmCPU	1.11
	Q.GPD-7A	BobWhite_c5396_296	7A	437594549	1.11E−06	FarmCPU	3.14
	Q.GPD-7B	Excalibur_c41736_124	7B	671888191	6,57E−06	FarmCPU	5.22
HD	*Q.HD-2A.1*	RAC875_c1643_1548_1	2A	36290496	7.07E−14; 1.78E−14	FarmCPU, BLINK	3.30;15.23
	Q.HD-2A.2	BS00063368_51	2A	750951682	2.79E−06	FarmCPU	30.16
	Q.HD-5B.1	AX-110933998	5B	535310058	5.74E−07	FarmCPU	5.89
	Q.HD-5B.2	AX-158525875	5B	670640309	2.26E−08; 1.97E−09	FarmCPU; BLINK	42.81;30.16
	Q.HD-7B	tplb0060b03_432	7B	717786400	3.94E−09	FarmCPU	8.62
FLA	*Q.FLA-2A*	Ku_c269_2643	2A	36293520	5.31E−07	BLINK	1.19
	Q.FLA-2B	Tdurum_contig54925_225	2B	626536953	6.56E−06	BLINK	6.71
	Q.FLA-5A	wsnp_RFL_Contig4307_5006558	5A	356083833	1.08E−09	BLINK	5.29
	Q.FLA-7A	AX-158559793	7A	35081074	3.75E−10	BLINK	6.71
TW	*Q.TW-2A*	AX-94476292	2A	101228899	1.90E−06; 1.57E−08	FarmCPU; BLINK	4.65;3.24
	Q.TW-3A.1	AX-94461370	3A	5229999	3.57E−09; 5.34E−08	FarmCPU; BLINK	4.59;3.68
	Q.TW-3A.2	BS00061179_51	3A	602570712	1.69E−07; 1.35E−07	FarmCPU; BLINK	8.13;3.51
	Q.TW-6B	Excalibur_c91980_139	6B	153041419	2.38E−06	BLINK*	5.81
	Q.TW-7A	AX-94750198	7A	687170561	2.34E−06; 5.5E−06	FarmCPU; BLINK	9.92;9.17

The asterisks indicate the MTA with a significance level just below the fixed Bonferroni threshold in the alternative model. GY grain yield, GYD grain yield deviation, GPC grain protein concentration, GPD grain protein deviation, HD heading date, FLA flag leaf appearance, TW test weight, QTN Quantitative trait nucleotide, MTA Marker-trait association. PVE percentage of total phenotypic variance explained

associated with FLA, of which those on chromosomes 2A and 5A overlap with *Ppd-A1* and *Vrn-A1*.

Finally, four markers (on chromosomes 2A, 3A, and 7A) were scored by both methods to be associated with TW. An additional significant association (Excalibur_c91980_139) was found on chromosome 6B only by BLINK. The 32 markers associated with the seven traits were subjected to the Wilcox test at $p \leq 0.05$. In detail, the population was divided into two groups based on allele profiles and we tested whether the BLUE values varied significantly between the two groups (Fig. 6). Almost all MTAs had a significant effect on the corresponding trait, with few exceptions. For example, *Q.GPD-7B* for GPD and GPC, *Q.HD-2A.1* and *Q.HD-7B* for HD and *Q.FLA-2A* for FLA showed a non-significant *p*-value (> 0.05).

Genomic prediction using a weighted GBLUP

The significance values (*p*-values) that measure the strength of the association of each SNP marker with the target traits were used to perform genomic prediction with WGBLUP. The results returned by this approach along with those obtained by the GBLUP model are shown in Fig. 7. WGBLUP presented prediction accuracies higher than GBLUP up to 10.1% (FLA) when $-\log_{10}$ (*p*-value) computed by BLINK were used as weights. The lowest rate of improvement in

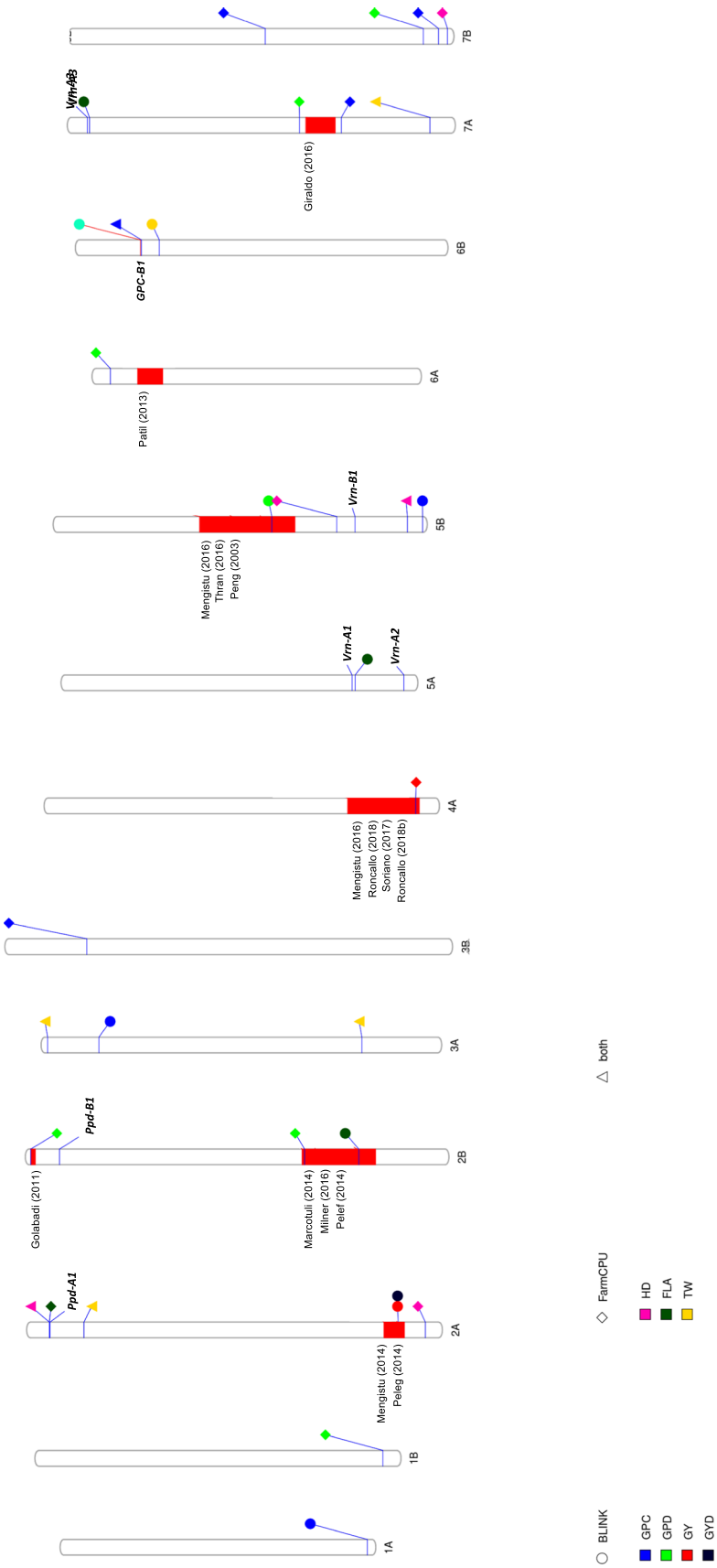


Fig. 5 PhenoGram used to plot MTAs along 12 durum wheat chromosomes (chr. 4B was omitted as no significant MTAs were found). Lines on chromosomes correspond to the physical location of each MTA. Colored circles represent the corresponding phenotype associated with the marker. Symbols indicate the GWA model (FarmCPU, BLINK, or both) used to identify MTAs. QTLs known in the literature and related to grain yield were reported on the left of each chromosome. Red boxes indicate the physical length of known QTLs. Know genes (i.e., *Vrn-A1*, *Vrn-A2*, and *Ppd-A1)* were also reported. GY = Grain Yield, GYD = Grain Yield Deviation, GPC = Grain Protein Concentration, GPD = Grain Protein Deviation, HD = Heading Date, FLA = Flag Leaf Appearance, TW = Test Weight (color figure online)

prediction accuracy was observed for N_upt (2.2%) and GPC (2.6%). Overall, prediction accuracy was slightly higher when BLINK weights were used, except for GYD and N_upt (Fig. 7). In detail, a significant improvement in prediction accuracy of 6.7% was observed by applying BLINK-WGBLUP for GY, whereas PA increased by 3.9% using FarmCPU-WGBLUP. An improvement in prediction accuracy (up to 5.4% and 6.2%) was observed for HD and TW, respectively, using WGBLUP-BLINK, whereas reaching 4.7% and 4.1% with FarmCPU-WGBLUP.

Finally, to validate the trained models, we predicted the agronomic performances of 40 durum wheat advanced breeding lines (Table 3; Supplementary Table 4). A similar prediction accuracy was achieved for GY (0.45) by testing the trained model developed within the 200 genotypes (0.42) (Table 3). Similarly, no evident changes in prediction accuracy were observed using CVP and IVP also for the traits GYD and TW (from 0.30 to 0.33 and 0.63 to 0.59, respectively). By contrast, a decrease in prediction accuracy was observed for GPC, GPD, and HD (from 0.56 to 0.43, from 0.47 to 0.30, and from 0.63 to 0.59, respectively) (Table 3). Using WGBLUP, no clear differences in prediction accuracy were observed for all traits, with the single exception of GY, which passed from 0.42 (GBLUP) to 0.46 by (BLINK-WGBLUP). However, it is interesting to note that approximately half of the associated SNPs detected in CVP are missing in the 15 K array used to genotype the IVP, perhaps explaining why WGBLUP was less effective.

Discussion

The improvement of GY and GPC at one time is considered one of the top priorities in durum wheat breeding programs. These traits are the ultimate expression of the multiple and complex plant physiological processes, which are influenced by climate and the environment during crop growth. Unfortunately, the inverse relationship between them represents one of the biggest constraints for wheat improvement. The main hypotheses that could explain the negative correlation are the competition between carbon and N for energy (Munier-Jolain and Salon 2005). In addition, the polyploid nature and functional gene redundancy of durum wheat makes a genetic approach for the selection of a desired phenotype time-consuming and, in some cases, impossible because of gene linkage, gene drag (Gaut et al. 2018; Borrill et al. 2019) or the small effect of individual QTLs on the target traits.

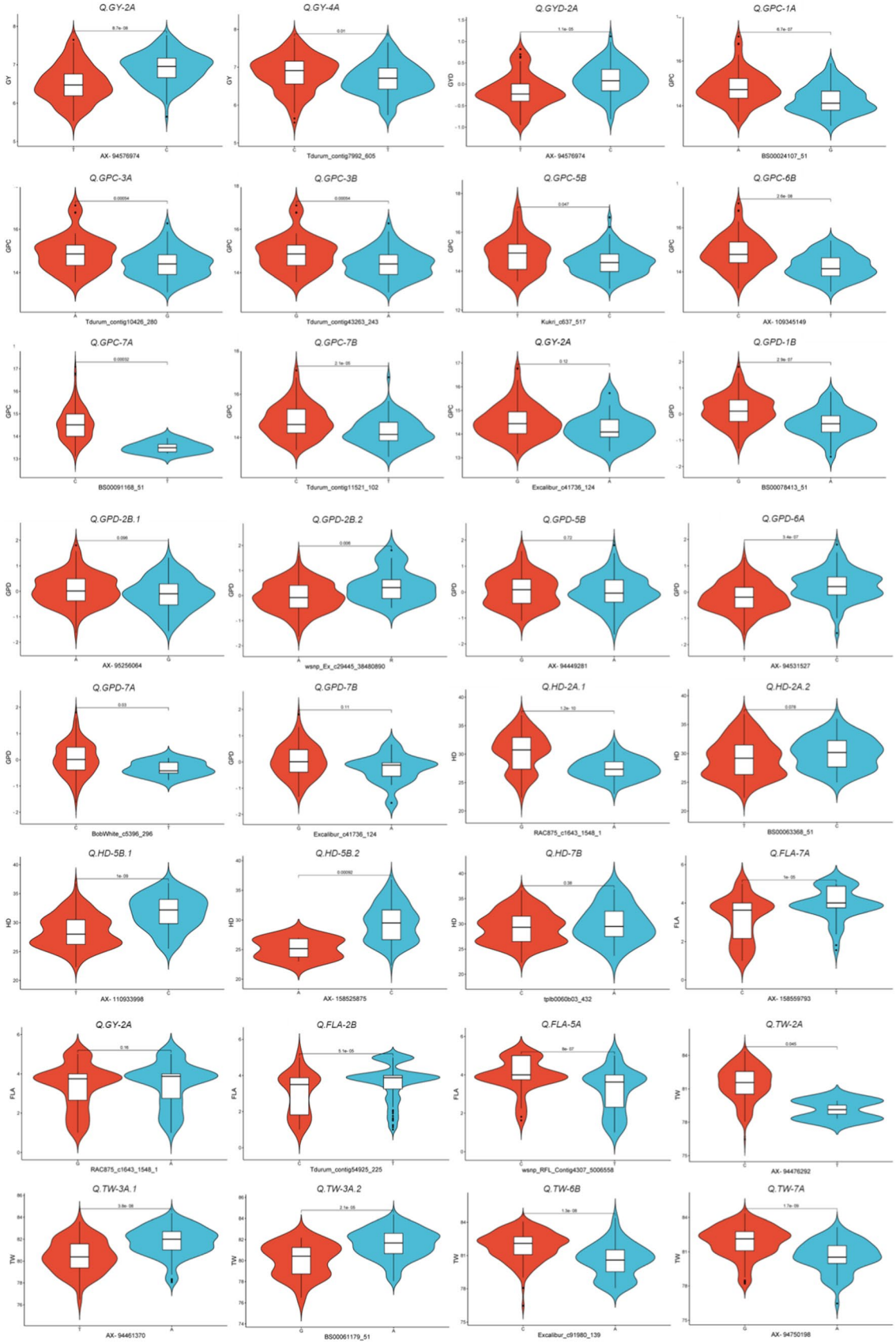
Independent QTLs for GY and GPC

In this study, we detected putative QTLs associated with GY and GPC with small effects each explaining a small proportion of the phenotypic variance, confirming the typical characteristics of a quantitative trait, which is usually influenced by many loci with small effects and by environmental factors (Maccaferri et al. 2008; Blanco et al. 2012; Soriano et al. 2017). In particular, two MTAs were identified for GY on chromosomes 2A (*Q.GY-2A*) and 4A (*Q.GY-4A*). *Q.GY-2A* (AX-94576974) mapped to the region previously identified by Mengistu et al. (2016), Peng et al. (2003) and Peleg et al. (2009) as associated with different yield-related traits including kernels per plant, spike dry matter and spikes per plant. Peleg et al. (2003) studied the genetic architecture of domesticated-related traits in *Triticum dicoccoides,* the ancestor of tetraploid and hexaploid cultivated wheat, reporting on chromosomes 1B, 2A, and 5A the most significant QTLs for GY ($LOD > 5$, $P < 0.001$). Subsequently, Peleg et al. (2009) also remarked the importance of chromosome 2A in the control of yield-related traits even under stressful conditions.

Recently, Adhikari et al. (2021) confirmed the role of the same chromosome as a hotspot for QTLs associated with different agronomical and physiological traits under drought conditions, suggesting that chromosome 2A carries several QTLs associated with variability for physiological indices and agronomic traits under normal and stressful conditions, as also described by Liu et al. (2019a, b, c). *Q.GY-2A* is also 50 Mb away from the well-known *Ppd-A1* gene, thus confirming the relationship between phenology and yield or yield-related traits (Wang et al. 2011; Kamran et al. 2014; Maphosa et al. 2014).

The marker "Tdurum_contig7992_605" on chromosome 4A was also found to be associated with two yield-related traits (i.e., the number of grains per spikelet and the number of grains) by Dubcovsky and colleagues in bread wheat (unpublished data, https://wheat.pw.usda.gov/GG3/). In durum wheat, the region was found to be associated with grain filling duration (Soriano et al. 2017), grain yield per plant (Roncallo et al. 2017), and plant biomass (Mengistu et al. 2016; Roncallo et al. 2017). The same region was also identified by the recent meta-QTL analysis conducted by Arriagada et al. (2022). Instead, chromosome 4A was not reported as a QTL hotspot in the consensus map of durum wheat developed by Marcotuli et al. (2022). Soriano et al. (2021) found QTLs on chromosome 4A, but none overlapped the one identified here. By contrast, chromosome 4A was considered a hotspot for GY in bread wheat. Liu et al. (2019a, b, c) reported the largest number of QTLs for GY on this chromosome, of which one was considered stable and major. Recently, Isham et al. (2021) identified four genomic regions in bread wheat controlling yield-related traits, including one on chromosome 4A.

Seven different regions were instead associated with GPC, of which *Q.GPC-6B* was the only one detected by both


Q.GY-2A
Q.GY-4A
Q.GYD-2A
Q.GPC-1A
Q.GPC-3A
Q.GPC-3B
Q.GPC-5B
Q.GPC-6B
Q.GPC-7A
Q.GPC-7B
Q.GY-2A
Q.GPD-1B
Q.GPD-2B.1
Q.GPD-2B.2
Q.GPD-5B
Q.GPD-6A
Q.GPD-7A
Q.GPD-7B
Q.HD-2A.1
Q.HD-2A.2
Q.HD-5B.1
Q.HD-5B.2
Q.HD-7B
Q.FLA-7A
Q.GY-2A
Q.FLA-2B
Q.FLA-5A
Q.TW-2A
Q.TW-3A.1
Q.TW-3A.2
Q.TW-6B
Q.TW-7A

◂**Fig. 6** Violin plot for MTA with significant effects (p-value < 0.05) on corresponding traits. For each of them, the durum wheat genotypes were divided into two groups based on allele profiles. The x-axis represents the two alleles for each associated MTA, while the y-axis corresponds to BLUE values of the corresponding trait (color figure online)

FarmCPU and BLINK and independent of GY, and therefore, of particular interest for the simultaneous improvement of GY and GPC. The 119 Mb region on the Svevo genome is close to the position of the previously cloned *Gpc-B1* gene, identified in wild emmer (Uauy et al. 2006). The presence of the *Gpc-B1* allele accelerates senescence in flag leaves by producing pleiotropic effects on nitrogen remobilization, total GPC, and grain size (Uauy et al. 2006a). To verify its role, in 2014 Pearce and colleagues (Pearce et al. 2014) generated loss-of-function mutants in tetraploid wheat and found that the mutants delayed senescence and reduced the protein, zinc, and iron content of the grain. In this framework, this study confirmed the importance of this region for identifying new alleles for GPC improvement.

Markers associated with GY and GPC-derived indices

The absence of a negative correlation between GY and GPD recorded in this study highlighted the utility of these derived indices (Bogard et al. 2010; Taulemesse et al. 2016; Nehe et al. 2020). GPD is known to be under genetic control (Bogard et al. 2010; Latshaw et al. 2016; Oury and Godin 2007), with several QTLs identified by GWAS (Nigro et al. 2019). Two recent studies in durum wheat highlighted the usefulness of exploiting the genetic variability of GPD to develop improved cultivars. Rapp et al. (2018) using two different panels (159 and 189 genotypes) grown in multiple locations revealed a complex genetic architecture of GPD, as it is controlled by many panel-specific QTLs having small effects and being detected in only one panel. Later, Nigro et al. (2019) by using a set of ~ 250 genotypes grown in seven different field trials identified four stable QTLs associated with positive GPD. Of all MTAs, *Q.GPD-5B* was within 1.5 Mb of the QTL *QGpd.mgb-5B.1* identified by Nigro et al. (2019) at 54.4 cM on chromosome 5B. The same MTA was also detected by Groos et al. (2003), Habash et al. (2007), Wang et al. (2011), and Rapp et al. (2018).

However, compared with those previous studies, we found no association for GPD on chromosome 4A, possibly due to the different approach or the statistical models used for detecting MTAs. For example, Groos et al. (2003), Habash et al. (2007) and Wang et al. (2011) used mapping populations (both early and late generations) obtained from crossing different parental lines (i.e., Renan/Récital; Line 3228/Jing 4839), and identified QTLs using inclusive composite interval mapping (ICIM) or composite interval mapping (CIM) approaches. Rapp et al. (2018) instead identified MTAs in a population of durum wheat called the "Central European panel" using a mixed linear model with a kinship matrix to correct for population structure. Given the difficulty in unambiguously identifying QTL from different studies due to the different marker systems (SNP, DARTseq, and SSR), linkage maps, and statistical approaches (ICIM, CIM, MLM) used, we believe that putative QTLs detected in early studies might be panel-specific, as also pointed out by Rapp et al. (2018).

With respect to the other MTAs, *Q.GPD-2B.1* co-localizes with a QTL previously described by Golabadi et al. (2011) as associated with harvest index (HI), while *Q.GPD-2B.2* co-mapped in the same region detected for spikelet per spike (Distelfeld et al. unpublished), kernel weight (Faris et al. 2014) and plant height (Milner et al. 2016).

Similarly, *Q.GPD-6A* overlapped with a QTL associated with grain yield per spike as described by Patil et al. (2013), although Blanco et al. (2002) confirmed the results reported here by describing the same marker associated with GPC. Furthermore, *Q.GPD-7A* identified in this study on the long arm of chromosome 7A, falls in the same region previously identified to be associated with GPC (Suprayogi et al. 2009) but also with spikelet per spike (Giraldo et al. 2016), and kernels per fertile spikelet (Roncallo et al. 2017). Recently, Mulugeta et al. (2023) performed GWAS using 10,045 SNP scored in a panel of Ethiopian durum wheat genotypes revealing MTAs for grain yield and GYD on chromosomes 1B, 5A and 7A. Notably, the MTA on chromosome 7A overlapped with that identified by FarmCPU in this study for the same trait, although the marker showed a level of significance below the fixed threshold.

MTAs associated with other traits

As the investigation of grain yield-related traits has great importance to increase yield potential and protein concentration, we also identified here MTAs for HD, FLA, TW, and N_upt. Heading date and flowering time are closely related to grain yield in wheat, due to their key role in maintaining the right balance between making full use of resources and avoiding environmental stresses. Two MTAs found on chromosomes 2A and 5B for HD (*Q.HD-2A.1* and *Q.HD-5B.1*) co-mapped with well-known adaptive genes (i.e., *Ppd-1* and *Vrn-1*), confirming the robustness of the dataset and the panel of genotypes used for the association tests. Four MTAs were identified for FLA of which *Q.FLA-2A* co-mapped with *Q.HD-2A.1*. Indeed, the short physical distance between the associated markers suggested that it may be the same, while *Q.FLA-2B* overlapped a genomic region previously described by Peleg et al. (2009) and Marcotuli et al. (2022) as associated with GPC suggesting the possible role of crop phenology on the expression of this trait.

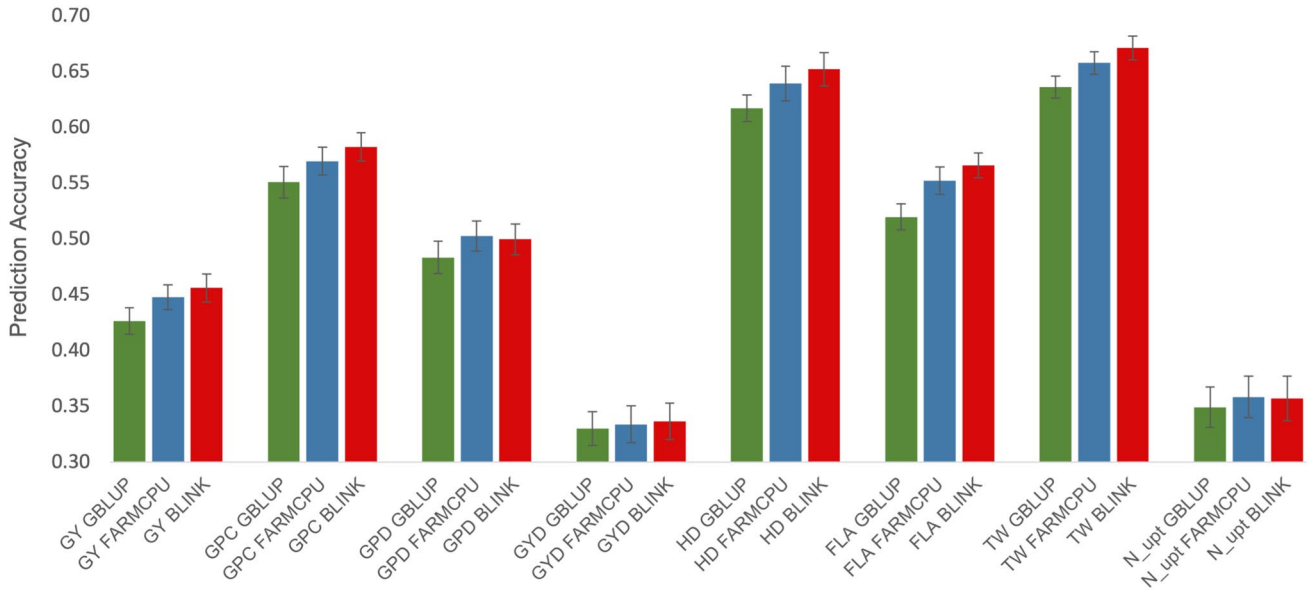


Fig. 7 Prediction accuracy obtained for all target traits. The genomic best linear unbiased prediction (GPLUP) model (orange bars) was compared with the weighted GBLUP (WGBLUP) model that used the −log10 (*p*-values) from BLINK (green) and FarmCPU (blue) as "weights". GY grain yield, GYD grain yield deviation, GPC grain protein concentration, GPD grain protein deviation, HD heading date, FLA flag leaf appearance, TW test weight, N_upt nitrogen uptake. Bars indicate standard errors (color figure online)

Table 3 Comparison between fivefold Cross-Validation (CV) and Independent Validations (IV) for all target traits

Trait	Validation method	Prediction Accuracy (PA)		
		GBLUP	WGBLUP (FarmCPU)	WGBLUP (BLINK)
GY	CV	0.42	0.45	0.46
	IV	0.45	0.45	0.49
GYD	CV	0.30	0.33	0.34
	IV	0.33	0.34	0.29
GPC	CV	0.56	0.57	0.59
	IV	0.43	0.41	0.41
GPD	CV	0.47	0.50	0.50
	IV	0.30	0.31	0.24
HD	CV	0.60	0.64	0.65
	IV	0.51	0.47	0.48
FLA	CV	0.51	0.55	0.57
	IV	–	–	–
TW	CV	0.63	0.66	0.67
	IV	0.59	0.62	0.62
N_upt	CV	0.35	0.36	0.36
	IV	–	–	–

Phenotypic data points for FLA and Nupt under the independent validation scheme were not available (–).GY grain yield, GYD grain yield deviation, GPC grain protein concentration, GPD grain protein deviation, HD heading date, FLA flag leaf appearance, TW test weight, Nupt nitrogen uptake

Interestingly, *Q.FLA-7A* was located close to *Vrn-3*, i.e., the wheat ortholog of the *Arabidopsis FLOWERING LOCUS T* (Yan et al. 2006) gene located on the group of homeologues chromosomes 7. Several studies have demonstrated the importance of this gene during the flowering period, and the possible pleiotropic effect on yield and its components (Sanna et al. 2014; Giunta et al. 2018).

As TW influences grain weight, this trait is closely related to yield and GPC. Therefore, it is important to identify TW-associated QTLs that do not coincide with GY and GPC, but unfortunately in many studies this information was either missing or referred to bread wheat.

In this study, five MTAs were found to be associated with TW and were not expected to co-localize with known QTLs. The *Q.TW-2A* identified on chromosome 2A may be different from the *QTw.macs-2A* described by Patil et al. (2013) as it was more than 20 Mb away from the closely linked marker (Xgwm71.2). Two other MTAs identified on the short arm of chromosomes 6B (*Q.TW-6B*) and 7A (*Q.TW-7A*) may coincide with those reported by Elouafi et al. (2004). However, in this case it was not possible to compare their physical positions on the Svevo genome, due to the absence of common markers.

Weighted genomic prediction

Given the severe negative relationship between GY and GPC, and the presence of many minor QTLs distributed across the whole genome of durum wheat, our findings suggested that genomic prediction could help counteract

the inverse relationship, facilitating the selection of high-yielding varieties with high protein concentration (Michel et al. 2019). GBLUP is the most widely used statistical model for genomic prediction as its computational demand is low. However, it is less sensitive because it assumes that each marker has the same variance. Here, we used *p*-values obtained from two GWAS models (BLINK and FarmCPU) to perform a weighted GBLUP for each target trait. Consistent with the results obtained by Annicchiarico et al. (2022) and Medina et al. (2021), we observed an increase in prediction accuracy with WGBLUP. This is of particular interest as clues to WGBLUP in durum wheat are still scarce. We hypothesize that the weighted matrix applied in the WGBLUP model, which assigns weights to each of the SNPs involved in the process breaking the conventional GBLUP assumption, contributed to the observed outcomes. However, several criticisms need to be considered when assigning weights to each SNP. For example, *p*-values are usually calculated solely based on the phenotypic information of the training population, thus requiring a population size for GWAS large enough to ensure accurate *p*-value estimates and avoid potential negative impacts on their reliability. Furthermore, the contribution as a weight for some key genes, such as the *Reduced Height* (*Rht*) or *Vernalization* (*Vrn*) genes, which play well-established roles in specific phenotypic traits, should not be based exclusively on *p*-values obtained from GWAS but explicitly considered in the matrix weighted according to their established significance. Compared with the literature, the results herein reported are consistent with Sukumaran et al. (2018a), which used nine GBLUP-based models to achieve a prediction accuracy of 0.20 to 0.40 for grain yield in a panel of 208 durum wheat genotypes under different growing conditions. However, we obtained a higher accuracy up to 5.9% for the same trait by weighting the markers with *p*-values from both BLINK and FarmCPU. A similar increase in prediction accuracy was also observed by Zaïm et al. (2020), who incorporated significant QTLs for grain yield into the genomic prediction model (ridge regression BLUP). Sehgal et al. (2020) incorporated robustly associated loci identified in GWAS as fixed effects in genomic prediction models to predict grain yield in spring bread wheat. The authors found that the model that accounted for the haplotype-based GWAS loci as fixed effects led to an increase in prediction accuracy of up to 9–10%, which is in line with the improvements we have achieved. Instead, comparing the GYD accuracy of this study with that obtained by Rapp et al. (2018) a lower prediction accuracy was shown (PA ~ 0.5%), although the same authors reported a lower accuracy (PA = 0.41) when the model was assessed on a different panel.

A moderate accuracy of 0.56 for GPC using the GBLUP model was found in this study, compared with ~ 0.60 obtained by Haile et al. (2018) in a double haploid population of durum wheat. However, the results shown here improved by 8% with weighted GWAS models. As for GPD, Michel et al. (2019) running two genomic prediction models (genomics- and assisted-based) in a population of 1,114 F4:6 individuals and doubled haploid winter wheat breeding lines, reported prediction accuracy values of up to 0.58, which was in line with what we achieved using WGBLUP (BLINK; 0.57). Such improvements in prediction accuracy were also observed for the other two traits evaluated in the present study (i.e., HD and TW). We obtained a higher prediction accuracy for HD using WGBLUP and comparable results using GBLUP to what was highlighted by Crossa et al. (2016). Similarly for TW, when GBLUP were used, the results reported here were in agreement with those by Fiedler et al. (2017), but as expected, using WGBLUP prediction accuracy slightly increased.

As a final goal, we tested whether trained GP models could be employed in a plant breeding program to select the most promising lines with enhanced traits. Indeed, the assessment of the GP model in new breeding programs is still a big challenge. Although the high and robust prediction accuracy has promised reasonably good identification of the highest performing lines, this is still a difficult task for breeders.

Given the fact that the most widely used method for evaluating genomic prediction models (k-fold cross-validation) has a drawback since the same original population is part of both the training and test populations (Crossa et al. 2010; Hofheinz et al. 2012; Michel et al. 2016), the accuracy of the prediction from cross-validation could be biased, resulting in overly optimistic predictions. Here, we have opted for an independent validation using an unconnected trial to support the results of the cross-validation method.

Compared with the cross-validation method, slightly lower accuracy was obtained for all traits under investigation using GBLUP except for GY, GYD and TW which remained approximately constant. Our results are in line with those by Schillaci et al. (2021), who predicted bulk density for both topsoil and subsoil achieving higher accuracy using cross-validation compared with the independent panel.

Similarly, prediction accuracy decreased when switching from cross-validation to independent validation (IV) for all traits using WGBLUP.

The trend is consistent with the assumption that independent validation is a one-off evaluation and cannot be used to adjust model parameters explaining the lower accuracy compared to the iterative cross-validation method. The lower accuracy achieved by WGBLUP in the independent validation scheme could be related to the lack of significantly associated markers in the 15 K array used to perform the genotyping in the independent panel, thus opening new opportunities to exploit GWAS information in predictive models to help and guide breeders in their decisions. In fact,

while some studies have reported no significant differences in predictive ability using independent validation, we believe this strategy will become the benchmark for evaluating any model.

Finally, the results presented here are consistent with those by Annicchiarico et al. (2019) who applied the same validation procedure. In this regard, the authors found higher prediction accuracy in intra-population than inter-population for pea phenotypic traits such as grain yield, the onset of flowering, seed weight, and lodging susceptibility. Similar to the results reported here, low prediction accuracy has been recorded for independent predictions of grain yield, heading date, and test weight using different sets of wheat double haploid and recombinant inbred populations (Charmet et al. 2014). Wang et al. (2020) also estimated higher prediction accuracy (0.60) using cross-validation across all the scenarios than applying an independent validation scheme using one- as and two-year datasets as training population (from 0.23 to 0.32 and from 0.31 to 0.42, respectively).

Plant breeding programs often involve multiple trials performed in different environments over several years, testing different genotypes. An independent validation strategy can provide a reliable degree of prediction accuracy by training the model on one population and subsequently predicting untested genotypes in untested trials (Jiang et al. 2017). Enhancing prediction accuracy through this validation strategy is crucial for the application of genomic prediction approaches to plant breeding programs. While the exploitation of information from GWAS was not effective in improving prediction accuracy, integrated approaches such as incorporating climate and soil parameters, crop management and companion organism information, high-throughput phenotyping data points, or even microbial profiles into predictive models might enhance prediction accuracy.

Conclusions

This study provides new insights into the simultaneous improvement of grain yield and grain protein concentration in durum wheat using GWAS and GP approaches. Two significant and independent MTAs on chromosomes 2A and 6B were identified for GY and GPC. These might be usable for assisted breeding programs and for further studies aimed at identifying candidate alleles/genes underlying the target phenotype. Other independent QTLs were found for GPD, revealing its utility for identifying high-yielding genotypes with a reduced penalty in protein concentration. The WGBLUP model produced slightly higher prediction accuracy, revealing an improvement up to 9% for FLA. Independent validation conducted on different genetic materials confirmed the robustness and effectiveness of the proposed approach even though further improvement in prediction accuracy could be achieved if the training and breeding populations will share the same number of molecular markers.

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Author contribution statement P.D.V. conceived the work, coordinated and supervised the activities; I.P. and V.N. increased seed stocks and performed the phenotypic analyses; S.E. and P.D.V. wrote the manuscript with the help of P.V.; S.S., N.D.A, F.T. and M.R. S.S and P.V organized the dataset and performed the preliminary analyses; S.E., performed association study, bioinformatic analyses and data visualization; P.V. performed all genomic predictions; S.E. and F.T identified candidate genes; All authors critically revised and approved the final manuscript.

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Data availability The genotyping data related to the whole dataset can be downloaded using the link https://doi.org/10.6084/m9.figshare.22132796.v1.

Declarations

Conflict of interest The authors declare no conflict of interest.

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