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Genome-Wide Association Study for Spike Traits and Distribution of Two QTLs for Grain Number in Chinese Wheat Cultivars

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Abstract: Spike traits play an important role in improving wheat yield. However, the application of reported spike-related loci remains unclear. Here, we assessed six spike-related traits across seven different environments using 406 wheat accessions. A negative correlation was observed between two components of wheat yield: thousand kernel weight (TKW) and grain number per spike (GN). Nonetheless, TKW and GN were significantly higher in modern cultivars compared to landraces. Two reliable quantitative trait loci (QTLs) related to GN, *QGN.nwafu-4A* and *QGN.nwafu-7A*, were identified through genome-wide associate analysis. *QGN.nwafu-4A* showed pleiotropy on GN, kernel number per spikelet and spike length. Both elite haplotypes of *QGN.nwafu-7A* were prominently present in Chinese modern cultivars, particularly those released after the year 2000. Elite haplotype A of *QGN.nwafu-7A* was significantly prevalent in Chinese Yellow and Huai wheat zone, while elite haplotype D of *QGN.nwafu-7A* was concentrated in other wheat-growing regions of China. This observation suggests distinct preferences in wheat cultivation across various agro-ecological regions. Conversely, elite haplotype A of *QGN.nwafu-4A* was more common in introduced cultivars from abroad. This divergence may be attributed to the reduced TKW of haplotype A. Overall, these findings provide valuable insights into the application of these two QTLs in high-yield wheat breeding.

Keywords: grain number; modern breeding; haplotype selection and application; wheat

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Citation: Wang, J.; Chen, Y.; Lai, X.; Liu, Z.; Liu, M.; Xu, S. Genome-Wide Association Study for Spike Traits and Distribution of Two QTLs for Grain Number in Chinese Wheat Cultivars. *Agronomy* **2023**, *13*, 2538. https://doi.org/10.3390/agronomy13102538

Academic Editor: Andras Cseh

Received: 19 August 2023
Revised: 28 September 2023
Accepted: 28 September 2023
Published: 30 September 2023

1. Introduction

Wheat (*Triticum aestivum* L.) is a major staple food crop worldwide, contributing approximately 20% of the global calories and protein in human diets [1]. Increasing wheat yield stands as a long-term goal for breeding and remains crucial for ensuring global food security. Wheat yield is a complicated trait primarily determined by three components: thousand kernel weight (TKW), grain number per spike (GN), and spike number per unit area (SN) [2]. GN plays a crucial role in determining the potential yield of wheat, and recent studies have indicated that variations in grain number per spike have a more significant impact on wheat grain yield than variations in grain size [3,4]. The architecture of the spike inflorescence significantly influences GN [5]. The wheat spike consists of a rachis, numerous spikelets, and each spikelet produces an indeterminate number of florets along the rachilla [6,7]. GN can be dissected into two subcomponents: spikelets per spike and grains per spikelet [8]. Consequently, the morphology and development of the spikelet or floret are key factors determining the number of grains per spike in wheat.

In recent decades, significant advancements in wheat genome sequencing and high-throughput genotyping systems have propelled association analysis into a pivotal tool for understanding the basis of yield potential and gene identification [9–12]. Numerous genetic loci associated with grain number per spike have been successfully identified [2,5,13], and several genes regulating floret fertility and spikelet number have been cloned. For example, *Grain Number Increase 1* (*GNI1*), which encodes an HD-Zip I transcription factor, has been found responsible for increasing both fertile floret and grain number [14]. *TEOSINTE BRANCHED1* (*TB1*) regulates wheat inflorescence architecture and increases wheat grain numbers [15]. *TaCol-B5* modifies spike architecture and enhances grains per spike in wheat [16]. Both *DUO-B1* and *FRIZZY PANICLE* (*FZP*) genes, encoding APETALA2/ethylene response transcription factors, regulate spike inflorescence architecture and grain numbers [7,17,18]. The *Q* gene, a significant component in the domestication process of wheat, plays a crucial role in controlling spike morphogenesis and contributing to higher grain yield [5,19]. Additionally, both the dwarfing genes *Rht-B1* and *Rht-D1*, as well as the photoperiod sensitivity gene *Ppd-D1*, are associated with the number of grains per spike [20,21]. Previous studies have focused on the function of these genes, but the selection and application of genes in molecular breeding programs remain largely unexplored.

Significant influence of the environment and intricate relationships among yield component traits are evident across the ten distinct ecological wheat zones in China, which collectively encompass the full spectrum of environmental conditions experienced by wheat globally [22]. Different wheat regions exhibit diverse ecological environments, thereby influencing the overall spike structure of plants. Extensive variations in allele frequency, driven by ecological selection, have played a pivotal role in shaping plant architecture and influencing the growing season during yield improvement [23]. Understanding the genetic basis of grain number variation and uncovering the mechanisms governing their selection and application across different regions of China assumes paramount significance. The integration of this knowledge into forthcoming breeding programs is the key to continual advancements in wheat yields.

In this study, a resampled genome-wide association study (GWAS) analysis was conducted to investigate six distinct spike traits across diverse environments and identify valuable spike-related QTLs. A total of 66 significant QTLs were identified, among which two reliable QTLs were directly associated with grain number (GN). Further investigation was performed to understand the distribution of different haplotypes in various cultivars and wheat-growing regions. Two haplotypes of *QGN.nwafu-4A* and four haplotypes of *QGN.nwafu-7A* were identified. Haplotype A of the *QGN.nwafu-7A* was significantly enriched among Chinese modern cultivars, particularly in those released after the year 2000 and in the Yellow and Huai wheat zone of China. These findings provide valuable guidance for the deliberate selection and application of these QTLs in modern wheat breeding, taking into consideration their distinctive concentrations within different wheat-growing environments.

2. Materials and Methods

2.1. Plant Materials and Field Trials

A total of 406 bread wheat accessions collected from China, the United States, and the International Center for Agricultural Research in the Dry Areas (ICARDA) were used in this study. The accessions comprised 60 breeding lines, 87 landraces (LA), and 259 modern cultivars (MC). Among the modern cultivars, there were 207 Chinese MC and 52 introduced accessions, originating from different regions, including Chinese Yellow and Huai wheat zone (Y&H), Yangtze River wheat zone (YTS), other wheat zones of China (OTW), and abroad zones (Table S1).

The wheat accessions were planted and harvested at two different locations: Chongzhou (Sichuan Province, 30°63′ N, 103°67′ E) in 2018–2019 (E1) and Yangling (Shaanxi Province, 34°28′ N, 108°70′ E) in 2018–2021. This provided a total of seven different growth environments (Table S1). At Yangling, field trials were conducted with two different sowing dates: normal sowing (NS) at around 2 October and late sowing (LS) at around 15 January of the following year. The experiment employed a random block design with three replicates. Each individual line comprised ten plants, and the row span was set at 100 cm, with a 20 cm gap between rows. Field management followed local wheat production practices.

2.2. Phenotyping and Data Analysis

Upon reaching the maturation stage, a comprehensive phenotypic evaluation was conducted on the wheat population across seven environments. For phenotypic evaluation, five representative plants per genotype from each replication were selected. The traits that were evaluated included spike length (SL), grain number per spike (GN), spikelet number per spike (SNPS), fertile spikelets per spike (FS), thousand kernel weight (TKW), and kernel number per spikelet (KPS). SL was measured from the base to the tip of the spike, excluding awns. GN, SNPS, and FS were counted as the number of grains, total spikelets, and fertile spikelets per spike, respectively. The grain number data are missing in environment E5 due to adverse weather conditions during the anthesis and harvest stages. TKW was measured by randomly counting a thousand grains from each replication using an automatic seed test and a thousand kernels weighter (Wanshen-SC-G, Hangzhou, China). KPS was calculated as the ratio of GN to SNPS. Furthermore, the infertile spikelet ratio (ISR) was calculated as the ratio of (SNPS-FS) to SNPS.

The best linear unbiased prediction (BLUP) for target traits across different environments was calculated using the R package "lme4" [24]. Phenotypic variation, frequency distribution, and Pearson's correlations among different environments were obtained using the BLUP data in SPSS 25 (IBM SPSS, Armonk, NY, USA). Broad-sense heritability (h^2) across environments was estimated following the method described by Smith et al. [25]. Statistical significance was assessed using Student's t test ($p < 0.05$) and one-way ANOVA, followed by Tukey's test ($p < 0.05$) with GraphPad Prism (v8.0.1).

2.3. Genome-Wide Association Study and Linkage Disequilibrium Analysis

The genome-wide association study (GWAS) was conducted using FarmCPU [26] by the GAPIT version 3 in R (v3.6.1). This approach incorporates population structure and kinship results as covariates to reduce the occurrence of false positives [27,28]. We conducted association mapping using a resampling-based multiple SNP model, following a method previously applied successfully in maize [29,30]. The SNP data were generated using population RNA-seq, as described by Wang et al. [31]. In total, 157,050 SNPs were randomly distributed across all 21 wheat chromosomes and utilized for genotyping. Detailed information regarding these SNP data can be found in our earlier study [32].

The phenotypic data used in this study comprised the mean values from three replicates for each trait within a single environment, as well as the BLUP values amalgamated across all environments. Resample GWAS [29] was performed using R software with 100 iterations, where each iteration randomly selected 60% of the inbred lines. Given that the p value adjusted by a false discovery rate of 0.05 was overly restrictive, we considered SNPs detected at $p < 1.0 \times 10^{-4}$ and identified at least 5 times in 100 resamples (resample model inclusion probability (RMIP) threshold of 0.05) as significant associations. The SNP with the minimum p value for each locus was defined as the peak SNP (pSNP). Manhattan and quantile–quantile (Q–Q) plots were visualized using the "qqman" R package.

Linkage disequilibrium (LD) was estimated by calculating the squared allele frequency correlation using the "$-r^2$" command in PopLDdecay software (v3.4.2) [33]. The LD decay was calculated and plotted with the following parameters: "-bin1 1000 -break 5000 -bin2 5000". The LD decay distance for the whole genome was approximately 3 Mb, as shown in Figure S2. This corresponds to $r^2 > 0.1$ and was considered to represent the limits of a QTL/locus. The corresponding effect and phenotypic variance explained (PVE) were estimated and outputted using R software.

2.4. Haplotype Analysis

QTL information relevant to the candidate genes was meticulously extracted from the obtained QTL dataset, which exclusively included biallelic QTLs. Based on the results of LD block analysis, we performed haplotype classification using significant markers that were closely linked to the peak SNP within each QTL. Haplotype analysis was conducted in R (v3.6.1) to investigate whether these loci can induce phenotypic change through a random sampling substitution test involving 1000 iterations. The significance of the results was assessed using Student's *t*-test or one-way ANOVA, followed by Tukey's test. To effectively visualize and present the findings, the outcomes were elegantly depicted using GraphPad Prism (v8.0.1).

3. Results

3.1. Variation and Correlation Analysis of Spike-Related Traits

A comprehensive collection of 406 bread wheat accessions was amassed for this study (Table S1). To characterize these accessions, six spike-related traits were evaluated, including spike length (SL), grain number per spike (GN), spikelet number per spike (SNPS), thousand kernel weight (TKW), kernel number per spikelet (KPS), and infertile spikelet ratio (ISR). This evaluation was conducted across seven environments, and it was observed that late sowing significantly reduced the performance of five spike-related traits, except for kernel number per spikelet (Table S1).

The distribution patterns of all six traits exhibited a continuous variation (Figure 1a), implying that these spike-related traits are quantitative in nature, influenced by a multitude of genes or QTLs. Across various environments, SL showed the highest heritability, while ISR showed the lowest heritability. Notably, two key components of wheat yield, namely thousand kernel weight (TKW) and grain number per spike (GN), exhibited strong heritability exceeding 70% (Table 1).

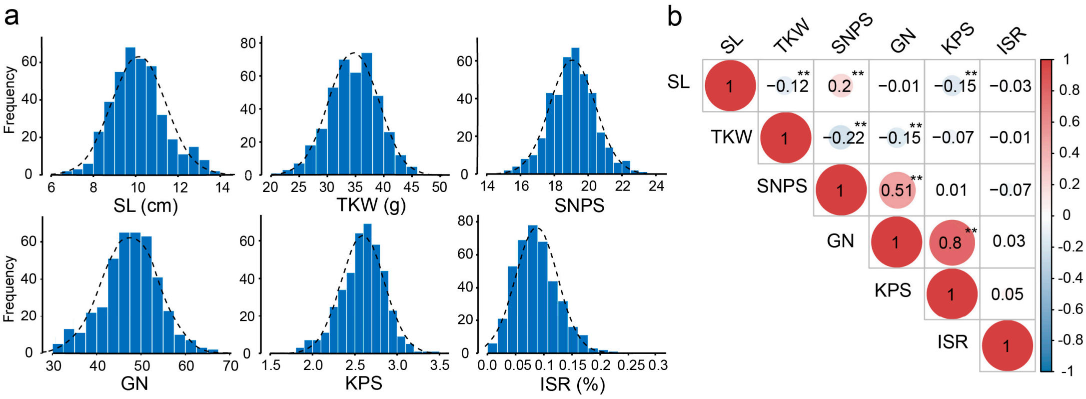

Figure 1. Variation and correlations for spike-related traits. (**a**) Frequency distribution of spike-related traits in the 406 wheat accessions based on BLUP values. SL: spike length; GN: grain number per spike; SNPS: spikelet number per spike; TKW: thousand kernel weights; KPS: kernel number per spikelet; ISR: infertile spikelet ratio. (**b**) Pearson's correlation coefficients of spike-related traits in the 406 wheat accessions. Correlation coefficients among the six traits were calculated based on the BLUP values across seven environments. "**": $p < 0.01$.

Table 1. Statistics of six spike-related traits in seven environments based on BLUP values.

Trait	Mean	Min	Max	CV (%)	h^2
SL (cm)	10.06	6.00	16.56	16.95	0.92
TKW (g)	35.09	12.33	71.71	21.37	0.82
SNPS	19.39	12.33	25.80	12.03	0.81
GN	48.72	21.00	78.60	20.33	0.78
KPS	2.53	1.14	3.81	18.74	0.72
ISR	0.12	0.00	0.59	43.95	0.46

Note: SL: spike length; GN: grain number per spike; SNPS: spikelet number per spike; TKW: thousand kernel weights; KPS: kernel number per spikelet; ISR: infertile spikelet ratio. CV: coefficient of variation. h^2: broad-sense heritability.

Correlation analysis revealed that TKW was negatively correlated with GN, SNPS, and SL. Conversely, GN showed positive correlations with KPS and SNPS (Figure 1b). These intricate interconnections indicate the complexity of wheat yield traits.

3.2. Variation of Spike-Related Traits during Modern Breeding

Improving spike-related traits stands as a crucial objective in the pursuit of high-yield wheat breeding [34]. Considering this, an in-depth investigation was conducted, focusing on the distinctions between landraces (LA) and modern cultivars (MC). In comparison to LA, MC exhibited significantly reduced SL but higher levels of TKW, GN, and KPS (Figure 2). Notably, TKW and GN showed remarkable improvements of 12.33% and 8.93%, respectively (Figure 2). Furthermore, in a converse trend, MC exhibited a lower ISR as compared to LA (Figure 2), highlighting a distinct preference for high-yield characteristics in modern breeding processes.

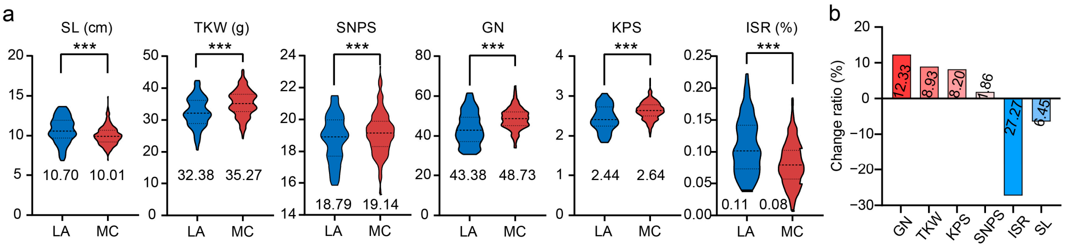

Figure 2. Variations of spike-related traits during modern breeding. (**a**) Variations of spike-related traits between landraces (LA) and modern cultivars (MC). "***" indicate significant differences by Student's test ($p < 0.001$). (**b**) Change ratio of spike-related traits between LA and MC.

3.3. GWAS of Spike-Related Traits in Seven Environments

GWAS were performed for six spike-related traits using the resample model inclusion probability [35]. QTLs that were identified across environments (based on BLUP value, E0) and detected in at least two environments were considered stable QTLs. Additionally, QTLs that have been previously identified as major QTLs were also regarded as stable. As a result, a total of 66 stable QTLs ($p < 1.0 \times 10^{-4}$, RMIP ≥ 5) were identified in at least two environments or reported by previous studies (Table S2, Figures 3 and S1).

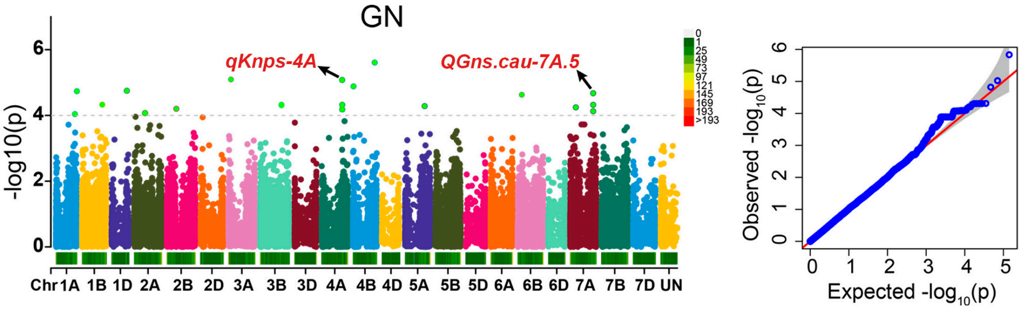

Figure 3. Manhattan and Q–Q plots for grain number per spike (GN). The physical location of the point indicated by the arrow coincides with the reported QTLs *qKnps-4A* [36] and *QGns.cau-7A.5* [37], respectively.

Among the six spike-related traits, SL and TKW exhibited the highest number of identified QTLs, and SNPS, GN, and KPS fell in the middle range, while the number of QTLs associated with ISR was the lowest (Table S3). This observation is consistent with the heritability levels of these respective traits (Table 1).

A total of 17 QTLs harboring reported genes or loci were identified, with five of them previously reported and documented previously reported and documented (Table 2, Figure 4). *QSL.nwafu-2D.1*, *QSL.nwafu-2D.2*, and *QSL.nwafu-5A.5* have been consistently observed in various studies focusing on SL. Both *QGN.nwafu-4A* and *QGN.nwafu-7A* showed the same or close physical locations with previous studies. Notably, *QGN.nwafu-4A* was previously regarded solely as a locus influencing GN. However, our study revealed its role as a pleiotropic locus, impacting not only GN but also KPS and SL (Table 2). This discovery indicates a genetic association among these traits. Consequently, these five loci, which have been reported multiple times (Figure 4), possess significant credibility as reliable QTLs. Specifically, the two QTLs associated with GN were singled out for further analysis to elucidate their potential application in high-yield breeding initiatives.

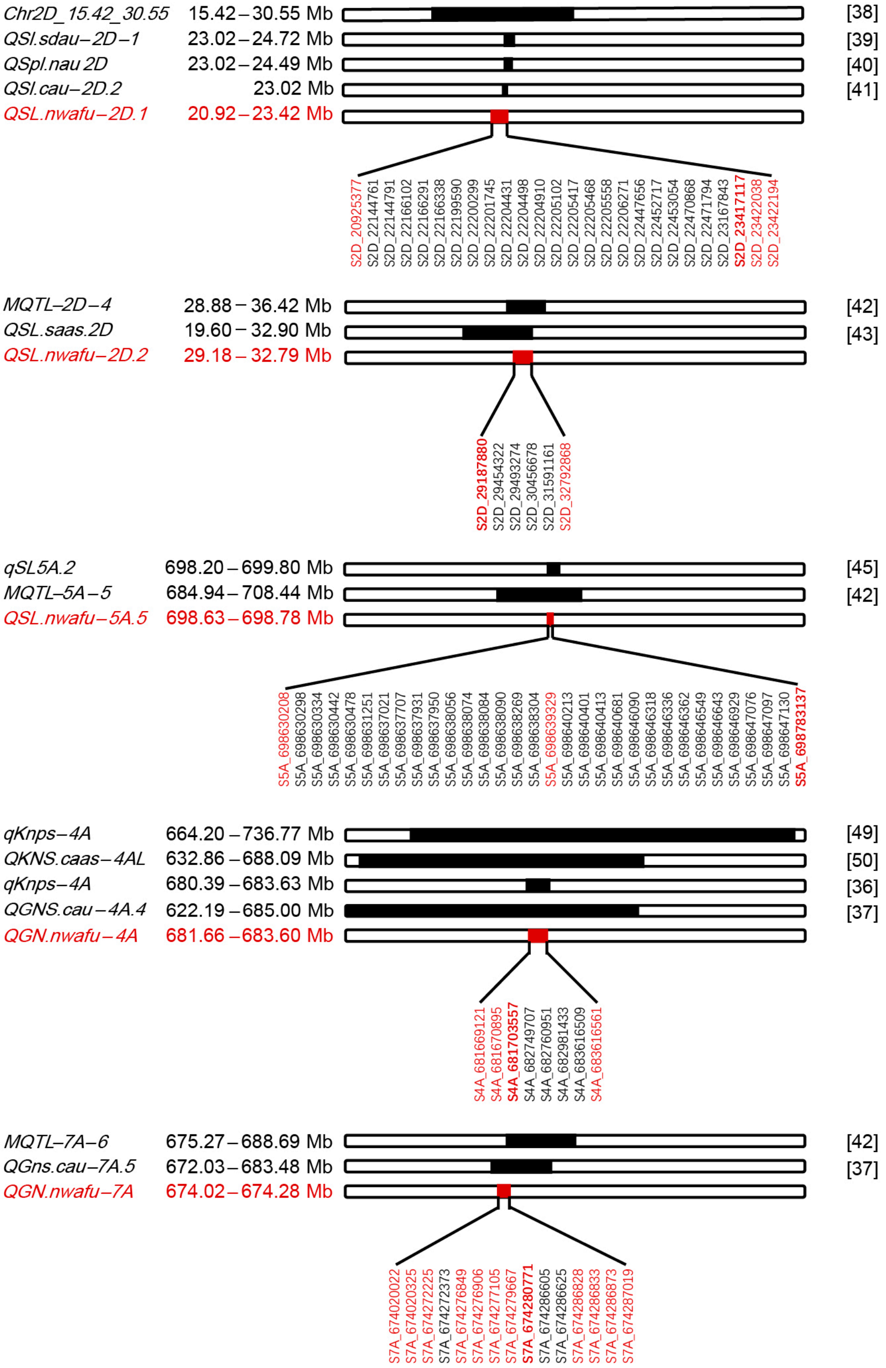

Figure 4. The physical position of five reliable QTLs and previous loci. The black marks the reported QTL and the SNPs of reported QTL, the red marks the QTLs and the significant SNPs identified by our GWAS. The bold red marks the peak SNP.

Table 2. Some of the stable QTLs for spike-related traits identified by resampling GWAS.

Traits	QTLs	pSNP	Chr	Pos (Mb)	Env	PVE (%)	RMIP	QTLs Detected in Previous Studies	
								Traits	Bibliography
SL	***QSL.nwafu-2D.1***	**S2D_23417117**	**2D**	**20.92–23.42**	**0/1/2/3/4/5/6/7**	**7.59–11.32**	**5–44**	**SL**	[38–41]
	QSL.nwafu-2D.2	**S2D_29187880**	**2D**	**29.18–32.79**	**/0/4/5 6/7**	**9.66**	**17–38**	**SL**	[42,43]
	QSL.nwafu-4A.1	S4A_681703557	4A	681.70–683.61	0/3/5	4.69–5.99	5–46	SL	[44]
	QSL.nwafu-5A.2	S5A_586308393	5A	586.3	0/1/3 /5	4.48–6.08	6–12	SL	[42]
	QSL.nwafu-5A.5 *	**S5A_698783137**	**5A**	**698.63–698.78**	**6/7**	**4.21–5.08**	**5–39**	**SL** **GN, TKW**	[45] [42]
TKW	*QTKW.nwafu-3A*	S3A_33423910	3A	33.42–35.39	0/4/5	5.07–6.41	5–13	TKW	[46]
	QTKW.nwafu-4B.1	S4B_40752468	4B	36.15–40.75	0/4/7	4.15–6.64	5–8	GN	[45]
	QTKW.nwafu-4B.2	S4B_44185162	4B	44.18	0/3/7	4.28–5.36	6–18	GN	[45]
	QTKW.nwafu-7D	S7D_55412560	7D	55.41	0/4/5	3.11–4.57	5–12	TKW	[2]
SNPS	*QSNPS.nwafu-2D*	S2D_628937584	2D	628.93	0/4/6	4.34–5.32	5–30	FS	[47]
	QSNPS.nwafu-4A	S4A_597024251	4A	597.02	0/6	5.83–7.55	6	SNPS	[48]
GN	*QGN.nwafu-2A*	S2A_715307274	2A	715.30–715.33	0/2/4	4.56–5.38	5–20	TKW	[37]
	QGN.nwafu-4A *	**S4A_681703557**	**4A**	**681.7**	**0/6**	**4.69–7.88**	**7–10**	**GN**	[36,37,49,50]
	QGN.nwafu-7A	**S7A_674280771**	**7A**	**674.02–674.28**	**0/2/4**	**5.28–8.68**	**5–31**	**GN**	[37,42]
KPS	*QKPS.nwafu-4A* *	S4A_681703557	4A	681.66–681.70	0/6	7.56–7.88	5–9	GN	[36,49,50]
	QKPS.nwafu-4B.1	S4B_27522453	4B	27.52–30.86	0/2/6 7	8.07–11.49	5–9	GN, KPS	[21]
	QKPS.nwafu-4B.2 *	S4B_41013810	4B	36.15–41.01	0/2	5.58–7.39	5–19	GN	[45]

SL: spike length; GN: grain number per spike; SNPS: spikelet number per spike; TKW: thousand kernel weights; KPS: kernel number per spikelet; ISR: infertile spikelet ratio. pSNP: peak SNP; Chr: chromosome; Pos: physical position of the QTL in the reference genome; Env: environments; PVE: phenotypic variation explained; RMIP: resample model inclusion probability. Bold indicates the QTL was reported repeatedly and identified by the same trait as previous studies. Underline indicates the QTL showed pleiotropy. * Marks the QTL that has been reported, even if they were only identified in two environments.

3.4. Haplotype Analysis of Reported QTLs of GN in Modern Wheat Breeding

Haplotypes were defined based on the combination of significant SNPs identified for the two QTLs of GN. Two and four haplotypes were identified for *QGN.nwafu-4A* and *QGN.nwafu-7A*, respectively (Figure 5). Among them, the major haplotype for the pleiotropic *QGN.nwafu-4A*, referred to as Hap-A, showed a significantly higher GN, KPS, and SL. However, it exhibited a diminishing effect on TKW, and no statistical difference was observed between SNPS and ISR, which is consistent with the negative correlation between GN and TKW. Regarding *QGN.nwafu-7A*, although Hap-A was the major haplotype, both Hap-A and Hap-D displayed significantly higher GN and KPS, as well as lower SL and ISR (Figure 5). This observation aligns with modern breeding selection trends that aim for increased grain number (Figure 2). Notably, only Hap-D of *QGN.nwafu-7A* exhibited a simultaneous increase in GN and TKW, highlighting a potential application of this allele in future high-yield breeding.

3.5. Selection and Application for QGN.nwafu-4A and QGN.nwafu-7A during Modern Breeding

The distribution frequency of haplotypes within the germplasm was analyzed, revealing a significant concentration of only Hap-A from *QGN.nwafu-7A* in MC when compared to LA (Figure 6a,b). While both Hap-A and Hap-D of *QGN.nwafu-7A* exhibited notably higher grain numbers than other haplotypes, Hap-D did not demonstrate a pronounced enrichment in MC, whereas Hap-A showed a distinct enrichment, especially in cultivars released after the year 2000 (Figure 6c,d). For the two haplotypes of pleiotropic *QGN.nwafu-4A*, no significant variance in frequency distribution was observed between MC and LA. However, the frequency of the superior Hap-A from *QGN.nwafu-4A* exhibited a declining trend as the breeding process advanced (Figure 6c). This observation could be attributed to the lower TKW associated with Hap-A (Figure 5b), highlighting the complexity of wheat high-yield breeding and the need to strike a balance among yield components.

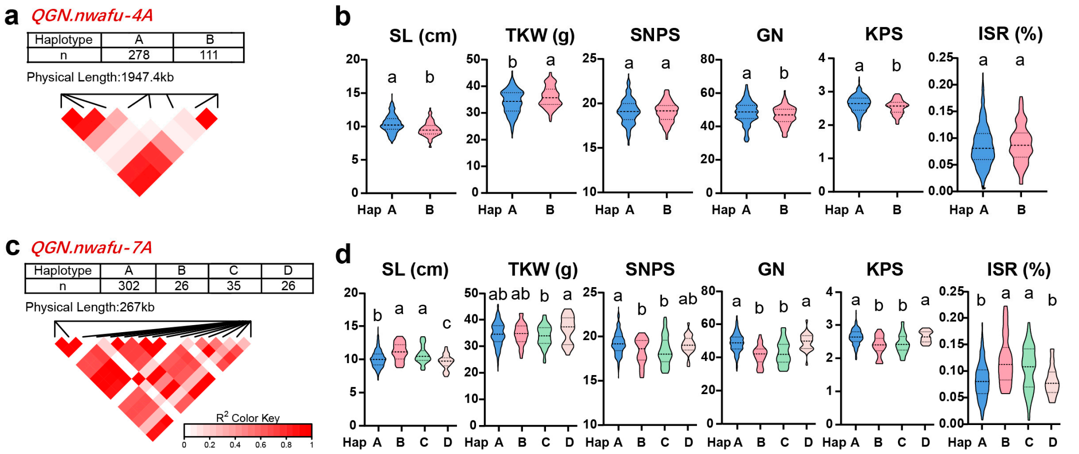

Figure 5. Variations in haplotypes of five QTLs controlling spike-related traits. Linkage disequilibrium (LD) heatmap of SNPs within the LD block of *QGN.nwafu-4A* (**a**) and *QGN.nwafu-7A* (**c**). The haplotypic effects of *QGN.nwafu-4A* (**b**) and *QGN.nwafu-7A* (**d**). Hap: haplotype. Different letters indicate significant differences calculated by one-way ANOVA, followed by Tukey's test ($p < 0.05$).

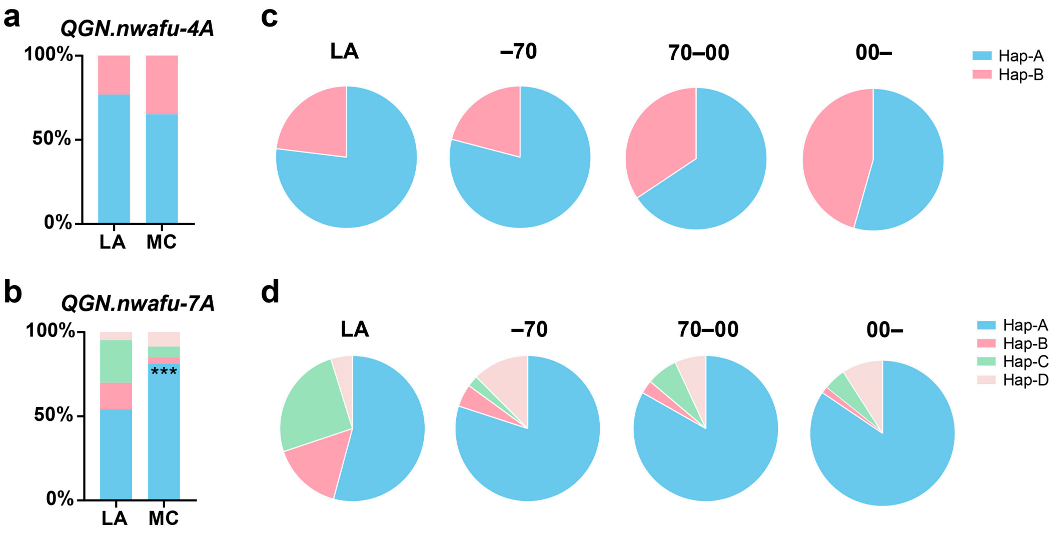

Figure 6. Frequency variation of different haplotypes during modern breeding. (**a**,**b**) The distribution frequency and changes of each haplotype for *QGN.nwafu-4A* (**a**) and *QGN.nwafu-7A* (**b**) in LA and MC. (**c**,**d**) The distribution frequency of each haplotype for *QGN.nwafu-4A* (**c**) and *QGN.nwafu-7A* (**d**) in different years. The significance was tested by chi-square test, "***": $p < 0.001$. LA: landraces. MC: Modern cultivars. −70: cultivars developed before 1970 year; 70−00: cultivars developed from 1970 to 2000 year; 00−: cultivars developed after 2000 year.

3.6. Selection and Application for QGN.nwafu-4A and QGN.nwafu-7A in Different Chinese Wheat Regions

The grain number exhibited a significant increase in both cultivars from Chinese modern cultivars (CMC) or introduced abroad cultivars (IC), with a particularly significant increase observed in CMC from the Yellow and Huai wheat zone (Y&H), as compared to LA (Figure 7a,b). Although no significant selection preferences were found for *QGN.nwafu-4A* between LA and MC, the frequency of Hap-B of *QGN.nwafu-4A* increased in CMC in Y&H, while the frequency of Hap-A of *QGN.nwafu-4A* increased in IC compared to LA (Figure 7c). These results suggest that the elite haplotype of *QGN.nwafu-4A* is not fully selected in Chinese modern wheat breeding, indicating the potential utilization of excellent germplasm from abroad for future wheat variety improvement. Conversely, the frequency of elite haplotypes of *QGN.nwafu-7A* increased in CMC, with haplotype A and haplotype D concentrated in Y&H and other wheat zones of China (OTW), respectively (Figure 7d). Interestingly, no significant selection preferences were found for each haplotype of *QGN.nwafu-4A* and *QGN.nwafu-7A* in the middle and lower reaches of the Yangtze River winter wheat zone (YTR), which is consistent with the lower GN observed in this Chinese wheat region (Figure 7b). This implies a potential application of this locus for future wheat breeding in this region.

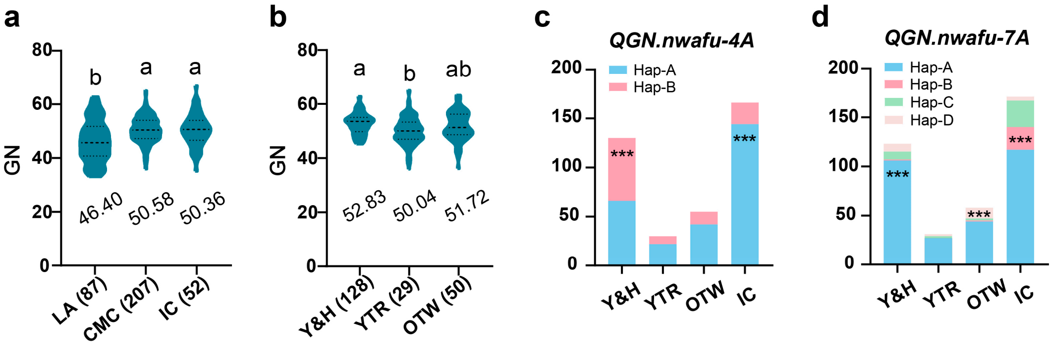

Figure 7. The grain numbers and haplotype distribution of two GN QTLs in different wheat regions. (**a**) The grain number per spike among LA, CMC, and IC. Different letters indicate significant differences calculated by one-way ANOVA, followed by Tukey's test ($p < 0.05$). (**b**) The grain number per spike in Chinese different wheat regions. Different letters indicate significant differences calculated by one-way ANOVA, followed by Tukey's test ($p < 0.05$). (**c**,**d**) The distribution frequency of each haplotype for *QGN.nwafu-4A* (**c**) and *QGN.nwafu-7A* (**d**) in different wheat regions. Y&H: Yellow and Huai wheat zone. YTR: the middle and lower reaches of the Yangtze River winter wheat zone. OTW, other wheat zones of China; IC, introduced abroad cultivars. The significance was tested by chi-square test, "***": $p < 0.001$.

4. Discussion

4.1. The Contribution of Grain Number to Wheat Yield

Wheat (*Triticum aestivum* L.) is one of the most widely cultivated food crops. Enhancing grain yield is a crucial task to ensure global food and nutritional security. The complexity of wheat yield is attributed to the interaction of three vital components: thousand kernel weight (TKW), grain number per spike (GNS), and spike number per unit area (SN) [2]. Among these components, GNS, which is affected by spike development and structure, exerts a substantial influence on grain productivity and overall yield [51]. Therefore, achieving an optimal spike structure and grain number is a pivotal objective in wheat breeding efforts.

However, improving grain yield through the selection process encounters significant challenges primarily due to three factors: (1) the pronounced interaction between genotypes and environmental conditions, (2) relatively low heritability, and (3) the complex genetic architecture of this trait, which depends on several related traits [52]. Thus, selecting for grain yield indirectly via highly heritable and related traits would be a practicable way. In our study, both GN and TKW showed higher broad-sense heritability (h^2) (Table 1), indicating the significance of these two traits in determining wheat yield. Most studies have attributed the increased wheat yields to increases in GN, TKW, or both [2,53,54]. However, recent investigations from Ireland [3] and the winter wheat regions of north China [4] have suggested that wheat grain yield is more profoundly impacted by fluctuations in grain number per spike than alterations in grain size. In the north China region, breeding and selection have been focused on increasing both grain number per spike and grain weight, depending on the specific positions of the grains within the spike. It has been observed that grain number may have a more significant impact on yield than grain weight [4]. Interestingly, our findings indicate that GN is significantly higher in the Yellow and Huai wheat region (located in north China), in contrast to the Yangtze River Winter wheat zone (located in middle China). These results support the conclusion that GN could be a crucial and potentially more important trait determining high wheat yield in this wheat region. Moreover, GN exhibited a negative correlation with TKW. Conversely, GN showed significant positive correlations with KPS and SNPS (Figure 1b). These results highlight the complexity and correlations between GN and other yield-related traits.

4.2. Enrichment of Spike-Related QTLs Based on GWAS Assay

In contrast to bi-parental mapping populations, which have limitations in identifying a restricted set of alleles, genome-wide association studies (GWAS) based on natural populations provide a broader genetic landscape and have proven to be an effective strategy for detecting numerous genetic loci associated with complex traits [49,55–57]. GWAS for yield-related traits is an effective method for detecting multi-allelic variation [53,58]. In this study, a total of 406 wheat accessions were genotyped in GWAS using 157,050 high- quality SNPs. We identified 66 stable and reported QTLs, among which 17 QTLs overlapped with previously mapped QTLs. Additionally, five QTLs were repeatedly reported, showing close physical location and association with the same spike trait as previous studies (Tables S2 and 2). These findings underscore the reliability of our GWAS results and provide additional insights into the reported wheat QTLs.

In addition, we observed a relatively high number of QTLs associated with TKW and GN, while the number of QTLs identified for ISR was comparatively lower (Table S3), consistent with the heritability of these traits (Table 1). Our analysis identified five reliable QTLs, among which *QGN.nwafu-4A* and *QGN.nwafu-7A* were found to be reliable QTLs for GN (Figure 4), in agreement with [36,49,50]. Specifically, *QGN-4A* showed a pleiotropy for GN, KPS, and SL in our study (Table 2, Figures 3 and 4).

4.3. Repetitive and Novel QTLs of Spike-Related Traits Compared with Previous Research

Although spike-related traits are complex quantitative traits and greatly influenced by environments, previous studies have reported the identification of QTLs associated with these traits distributed across all wheat chromosomes [2,36,37,42,50,59–63]. In this study, we detected 66 QTLs governing six spike-related traits, primarily located on chromosomes 1B, 2A, 2B, 2D, 3B, 4A, 4B, 5A, 5B, 6B, and 7A (Table 2 and Table S2). Among these QTLs, we identified 16, 21, 11, 8, 7, and 3 QTL for SL, TKW, SNPS, GN, KPS, and ISR, respectively.

In previous studies, a QTL for GN has been repeatedly detected in the genomic interval of 622.19–731.77 Mb on chromosome 4A [36,37,49,50]. In this study, we detected *QGN.nwafu-4A* within the genomic interval of 681.66–683.60 Mb on 4A chromosome (Table 2), which aligns with the previously reported region. Intriguingly, *QGN.nwafu-4A* partially overlaps with *QSL.nwafu-4A.1* and *QKPS.nwafu-4A* (Table S2), indicating a correlation among these spike-related traits. In addition, we identified *QGN.nwafu-7A* as a stable QTL, with Hap-A contributing to the improvement of grain number during wheat breeding (Figure 6b,d). *QGN.nwafu-7A* maps to the same region as a previously detected QTL [37,42] and colocalizes with the recently described *WAPO-A1* gene (at 674.07 Mb), which affects the total number of spikelets per spike [64]. This suggests that *WAPO-A1* could be the candidate gene for *QGN.nwafu-7A*. Additionally, *QGN.nwafu-2A.2* showed a similar genomic region as previously reported (Table S2), but it is distant from the *GNI-A1* gene (on chromosome 2A, 626.0–631.4 Mb), which is a major gene determining floret fertility and grain number [14]. Thus, we speculated that *QGN.nwafu-2A.2* is not allelic to *GNI-A1*.

Out of the 16 QTLs detected for SL, five showed partial or complete overlap with previously reported QTLs. Notably, *QSL.nwafu-2D.1* and *QSL.nwafu-2D.2* overlap with several previous reported QTLs, and *QSL.nwafu-5A.5* has been detected in equivalent regions and reported in multiple studies. Moreover, the identification of *QSL.nwafu-2D.2* and *QSL.nwafu-5A.5* narrowed the physical region to 3. 61 Mb and 0.15 Mb, respectively (Table 2), which further advances the search for genes related to spike length.

Regarding TGW, four QTLs, namely *QTKW.nwafu-3A*, *QTKW.nwafu-4B.1*, *QTKW.nwafu-4B.2*, and *QTKW.nwafu-7D*, were found to show the same or very close physical location as previous studies [2,45,46] (Table S2). For KPS, *QKPS.nwafu-4A*, *QKPS.nwafu-4B.1*, *and QKPS.nwafu-4B.1*, were detected on chromosomes 4A and 4B, consistent with earlier reports. Notably, *QKPS.nwafu-4B.1* colocalizes with the *Rht-B1* gene, which has been reported as a regulator of grain weight [65]. Thus, we speculate that *Rht-B1* might be a potential candidate gene influencing KPS, given its association with grain yield [21]. Additionally, two significant QTLs for SNPS, *QSNPS.nwafu-2D* and *QSNPS.nwafu-4A*, were detected on chromosomes 2D and 4A, aligning with findings from previous studies [47,48] (Table S2).

Furthermore, we identified 11 novel stable QTLs for SL located on chromosomes 1B, 3B, 4A, 5A, 5B, and 6B. Additionally, we discovered 17 novel stable QTLs for TKW located on chromosome 4A. Moreover, nine novel stable QTLs for SNPS were detected on chromosomes 1B, 1D, 2A, 4B, 5B, 6B, and 7A. For GN, 5 novel stable QTLs for GN were found on chromosomes 1A, 2B, 5A, and 6B. In addition, four novel stable QTLs for KPS were detected on chromosomes 1B, 2A, 2B, and 5A. Finally, three novel stable QTLs for ISR were detected on chromosomes 2B and 6B (Table S2). These newly identified QTLs provide valuable resources for the exploration of genes related to spike traits.

4.4. Selection and Application of GN-Related QTLs in Wheat Breeding

Although numerous QTLs underlying grain number per spike have been identified in wheat [2,66–68], the practical selection and integration of these QTLs in the breeding process have not been extensively explored. Haplotype analysis, a powerful technique for allele assessment, has been effectively employed to investigate the functionality, selection, and evolution of genes related to yield, as demonstrated in studies such as that of *TaCWI* [69]. In this study, we conducted haplotype analyses on two previously identified QTLs related to GN using a combination of discerned significant SNPs. Hap-A of the pleiotropic QTL *QGN.nwafu-4A* showed a superior effect, and both Hap-A and Hap-D of *QGN.nwafu-7A* exhibited a superior effect on GN, KPS, and ISR (Figure 5).

Compared to LA, the traits of TKW, SNPS, GN, and KPS showed significant improvement in MC, corresponding to the goal of achieving high grain yield in wheat breeding [2]. Furtherly, the Hap-A of *QGN.nwafu-7A* displayed a higher frequency in the MC than in LA, especially in cultivars released after the year 2000. However, no distinct haplotype pattern of the pleiotropic *QGN.nwafu-4A* was observed between MC and LA (Figure 6). Moreover, the haplotypes of *QGN.nwafu-7A.2* were concentrated across different wheat regions in China. The superior Hap-A was concentrated in Yellow and Huai wheat zone, while the superior Hap- D was enriched in other wheat zones of China (OTW) (Figure 7). This highlights a wide applicability of the elite *QGN.nwafu-7A* haplotype in Chinese modern wheat breeding, indicating distinct selection preferences among different wheat regions in China. In contrast, the superior haplotype of *QGN.nwafu-4A* was significantly enriched in the varieties from abroad, with no significant enrichment in the main wheat zones of China. This variation in distribution frequency across different wheat regions may be attributed to the ecological selection effect driven by differing climate conditions in these regions.

5. Conclusions

In summary, a total of 66 stable and previously reported QTLs associated with wheat spike traits were detected through GWAS. Among them, two reliable QTLs related to GN were identified. Haplotype analysis showed that the superior haplotypes of these two GN-related QTLs have been selected during modern breeding process, particularly in cultivars released after the year 2000. The frequency of haplotype distribution showed that the superior haplotypes of *QGN.nwafu-7A*, but not *QGN.nwafu-4A*, were concentrated in Chinese wheat zones, providing a clue for further high-yield breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13102538/s1, Figure S1: Manhattan and Q–Q plots for spike-related traits; Table S1: The information and phenotypes of 406 wheat accessions in seven environments; Table S2: Summary of stable and reported QTLs identified by GWAS of six spike-related traits; Table S3: Statistics of QTL numbers for six spike-related traits in multiple environments.

Author Contributions: J.W. and Y.C. conducted the experiments and wrote the paper; J.W., Y.C. and X.L. constructed the graphics; Y.C. and Z.L. collated and statistically analyzed the data; S.X. designed the experiment; S.X. and M.L. reviewed the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Hainan Yazhou Bay Seed Laboratory (B21E18101), "Integration of Two Chains" Key Research and Development Projects of Shaanxi Province "Wheat Seed Industry Innovation Project", and Key R&D of Yangling Seed Industry Innovation Center (Ylzy-xm-01).

Data Availability Statement: The datasets supporting the results of this article are included within the main text and the Supplementary Materials. The phenotypes and SNP data are available at https://iwheat.net/resource/ (accessed on 10 April 2021) for free download.

Conflicts of Interest: The authors declare no conflict of interest.

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