1 The 55K SNP-Based Exploration of QTL for Spikelet Number Per
2 Spike in a Tetraploid Wheat (*Triticum turgidum* L.) Population:
3 Chinese Landrace ‘Ailanmai’ × Wild Emmer

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25 **Abstract**

26 The spikelet number per spike (SNS) is a primary factor determining wheat yield.
 27 Common wheat breeding reduces the genetic diversity among elite germplasm
 28 resources, leading to a detrimental effect on future wheat production. It is, therefore,
 29 necessary to explore new genetic resources for SNS to increase wheat yield. A
 30 tetraploid landrace ‘Ailanmai’ × wild emmer wheat recombinant inbred line (RIL)
 31 population was used to construct a genetic map using the wheat 55K single
 32 nucleotide polymorphism (SNP) array. The linkage map containing 1150 bin markers
 33 with a total genetic distance of 2411.8 cM was obtained. Based on phenotypic data
 34 from eight environments and best linear unbiased prediction (BLUP) values, five
 35 quantitative trait loci (QTL) for SNS were identified, explaining 6.71-29.40% of the
 36 phenotypic variation. Two of them, *Q_{Sns.sau-AM-2B.3}* and *Q_{Sns.sau-AM-3B.2}*,
 37 were detected as the major and novel QTL. Their effects were further validated in
 38 two additional F₂ populations using tightly linked Kompetitive Allele-Specific PCR
 39 (KASP) markers. Potential candidate genes within the physical intervals of
 40 corresponding QTL were predicted to participate in inflorescence development and
 41 spikelet formation. Genetic associations between SNS and other agronomic traits
 42 were also detected and analyzed. This study demonstrates the feasibility of wheat
 43 55K SNP array developed for common wheat in genetic mapping of tetraploid
 44 population and shows the potential application of wheat related species in wheat
 45 improvement programs.

46 **Keywords:** Tetraploid wheat, high-quality genetic map, spikelet number per spike,
 47 QTL mapping, genetic correlations, potential application

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52 **Introduction**

53 Wheat (*Triticum* sp.) is among the most widely planted crops globally that provides
54 about 20% of the calories consumed by humans (<http://www.fao.org/faostat>).

55 Breeding high-yield wheat cultivars is a sustainable approach to meet the demands
56 of a growing human population. Wheat yield is determined by spike number per unit
57 area (SNPA), kernel number per spike (KNS), and kernel weight (KW). KNS is
58 jointly determined by the spikelet number per spike (SNS) and the kernel number
59 per spikelet (KNL). SNS depends on the number of lateral spikelets produced before
60 the spike meristem transitions to the terminal spikelet. It is a complex quantitative
61 trait greatly affected by genetic and environmental factors (Gao et al., 2019; Kuzay
62 et al., 2019). Therefore, dissecting the genetic mechanism of SNS at the QTL level
63 can provide more insights into the role of SNS in yield formation (Ma et al., 2019a;
64 Yao et al., 2019; Chen et al., 2020; Li et al., 2021).

65 A few genes related to spikelet number and morphology have been reported. For
66 example, the *WHEAT FRIZZY PANICLE* (WFZP) gene encodes a member of the
67 APETALA2/ethylene response transcription factor (AP2/ERF) family, which
68 influences the supernumerary spikelet trait in common wheat (Dobrovolskaya et al.,
69 2015). Regulatory expression of the two pivotal flowering genes, *Photoperiod-1*
70 (*Ppd-1*) and *FLOWERING LOCUS T* (*FT*), increases the number of fertile spikelets
71 (Boden et al., 2015). The inflorescence growth rate and the development of paired
72 spikelets are controlled by the *TEOSINTE BRANCHED1* (*TBI*) expression level
73 (Dixon et al., 2018). Similarly, the ortholog of the rice *APO1*, *WHEAT ORTHOLOG*
74 *OF APO1* (*WAPO1*) affects the spikelet number (Kuzay et al., 2019).
75 Overexpression of *TaTFL-2D* increases the number of spikelets and florets (Wang et
76 al., 2017).

77 More loci for SNS should thus be identified and utilized owing to its important role
 78 in yield formation. Nonetheless, common wheat breeding reduces the genetic
 79 diversity among elite germplasm resources, thus adversely affecting wheat yield
 80 (Cavanagh et al., 2013; Sthapit et al., 2020). Notably, the numerous genetic
 81 resources for agronomic traits and disease resistance from wheat related species may
 82 effectively solve future wheat production challenges (Zaïm et al., 2017; El Haddad et
 83 al., 2021). As the progenitor of modern cultivated tetraploid and hexaploid wheat,
 84 wild emmer wheat (*T. turgidum* subsp. *dicoccoides*) is the secondary gene pool of
 85 common wheat. It is resistant to various diseases and possesses important agronomic
 86 traits, thereby playing a potentially significant role in wheat breeding (Xie and Nevo,
 87 2008; Yu et al., 2020). For example, *Yr15* derived from wild emmer wheat confers
 88 resistance to yellow rust (Klymiuk et al., 2018). The reduced-function allele *GNI-A1*
 89 increases the number of fertile florets per spikelet (Sakuma et al., 2019). Cognizant
 90 of this, exploiting the genetic resources of wild emmer wheat is crucial in solving the
 91 existing bottleneck in wheat breeding. Wheat landraces are also primary materials
 92 for wheat improvement. They contain unique gene resources preserved under
 93 long-term natural selection and human intervention with strong adaptability to local
 94 environmental conditions and high production potential (Lopes et al., 2015; Crossa
 95 et al., 2016).

96 In the current research, a high-quality genetic map was constructed based on a RIL
 97 population developed from the cross between a Chinese landrace ‘Ailanmai’ and
 98 wild emmer using the wheat 55K SNP array to identify QTL for SNS, and major and
 99 novel QTL were validated in the different genetic backgrounds by KASP markers.

100 **Materials and Methods**

101 **Plant Materials**

102 A tetraploid wheat population (AM population) containing 121 F₈ recombinant
 103 inbred lines (RILs, including two parental lines) derived from a Chinese landrace

104 ‘Ailanmai’ (AL, *T. turgidum* L. cv. Ailanmai, 2n=28, AABB) and a wild emmer
105 accession (LM001, *T. turgidum* subsp. *dicoccoides*) were used in this study. AL is a
106 durum wheat landrace native to Sichuan Province, China. It has the advantages of
107 dwarf, multiple floret and strong environmental adaptability (Liu et al., 1999). AM
108 RIL population was developed aiming at identifying favorable alleles from AL and
109 LM001 to speed up their breeding utilization. The spike morphology of the parents
110 and representative lines are shown in Figure 1. Major and novel SNS QTL identified
111 in the AM population were validated in two populations including LM001 ×
112 PI193877 (*T. turgidum* ssp. *dicoccon*) (139 F₂ lines) and AL × AS2268 (*T. carthlicum*
113 Nevski) (100 F₂ lines). The above plant materials were preserved by Triticeae
114 Research Institute, Sichuan Agricultural University.

115 **Trait Measurement and Data Analysis**

116 Trait measurements of AM population were performed in eight environments:
117 Chongzhou (103° 38’ E, 30° 32’ N) in 2017, 2018, 2019, 2020, and 2021 (E₁, E₂, E₃,
118 E₄, and E₇), Wenjiang (103° 51’ E, 30° 43’ N) in 2020 and 2021 (E₅ and E₈), and
119 Ya’an (103° 0’ E, 29° 58’ N) in 2020 (E₆). Field experiments adopted a randomized
120 complete block design, and were managed following the conventional practices of
121 wheat production. The plot for a single line was space-planted (0.1-m) in a single
122 1.5-m row with a 0.3-m of row spacing. The agronomic traits were measured using
123 the methods described by Liu et al. (2020a). Spikelet number per spike (SNS) was
124 measured by counting the spikelet number of the main-spike per plant; plant height
125 (PH) was determined by the distance from the soil surface to the tip of the
126 main-spike (excluding awns) per plant; spike length (SL) was measured by the
127 length from the base to the tip of the main-spike (excluding awns) per plant; kernel
128 number per spike (KNS) was counted as the kernel number of the main-spike per
129 plant; spike density (SD) was calculated by dividing the SNS by SL; thousand kernel
130 weight (TKW) was calculated as 10 times the average weight of 100 seeds with three
131 repetitions in each line. Anthesis date (AD) was the number of days between sowing

132 and half of the plants flowering in each line (Ma et al., 2019a). Spike extension
133 length (SEL) was measured as the distance between the base of the main-spike and
134 the petiole of the flag leaf per plant (Li et al., 2020). At least four disease-free plants
135 with consistent growth status were selected for measurements. The environmental
136 information of surveyed agronomic traits of AM population is listed in
137 Supplementary Table 1. Two F₂ validation populations were grown in Chongzhou in
138 2021 and disease-free plants were selected to measure SNS.

139 The IBM SPSS Statistics 25 (Armonk, NY, USA) was used to analyze the
140 phenotypic data, including Pearson's correlation, frequency distribution, standard
141 error, and the Student's *t*-test ($P < 0.05$). The broad-sense heritability (H^2) and best
142 linear unbiased prediction (BLUP) of agronomic traits from multiple environments
143 were calculated using SAS version 8.0 (Cary, North Carolina).

144 **Construction of the Genetic Map**

145 High-quality genomic DNA was isolated from fresh leaves using the Plant Genomic
146 DNA Kit (Tiangen Biotech, Beijing, China). The DNA of 119 RILs and two parental
147 lines was subsequently genotyped by CapitalBio Technology (Beijing, China) using
148 the wheat 55K SNP array, and RILs were used for linkage map construction.

149 The genetic map was constructed following the methods described by Liu et al.
150 (2018) and Lin et al. (2021). Firstly, the Poly High-Resolution markers from A and
151 B genomes showing a minor allele frequency (≥ 0.3) among the mapping population
152 were retained for subsequent analysis. Secondly, the BIN function of QTL
153 IciMapping 4.1 (Meng et al., 2015) was used to analyze the remaining markers based
154 on their segregation patterns in the mapping population, with the parameters
155 'Distortion value' and 'Missing rate' being set as 0.01 and 20%, respectively. A
156 single marker with the lowest 'Missing rate' in each bin (bin marker) was selected to
157 construct genetic maps. Finally, the bin markers were grouped and sorted using the
158 Kosambi mapping function in IciMapping 4.1 with the logarithm of odds (LOD) ≥ 6

159 after preliminary analysis of SNP markers with LOD values ranging from 2 to 10.
160 The genetic maps were drawn using MapChart 2.3.2 (Voorrips, 2002). The syntenic
161 relationships between the genetic and physical maps of the bin markers were further
162 presented using the Strawberry Perl 5.24.0.1 (Rothwell, 2019).

163 **QTL Analysis**

164 QTL analysis was performed using the inclusive composite interval mapping (ICIM)
165 in the Biparental Populations (BIP) module of QTL IciMapping 4.1. The LOD
166 threshold was set as 3.4 based on a test of 1000 permutations in IciMapping which
167 was used to determine the threshold that corresponded to a genome-wide false
168 discovery rate of 5% ($P < 0.05$), and loci detected in only single environment were
169 removed. The QTL-by-Environment interaction of SNS trait was analyzed using the
170 function of multi-environment trials (MET-ADD) in IciMapping (Step = 1 cM, PIN
171 = 0.001, and $\text{LOD} \geq 3.4$). Epistatic interaction of SNS QTL was also identified using
172 the multi-environmental trials (MET-EPI) pattern of IciMapping. Based on the initial
173 QTL mapping results, we developed new KASP markers to densify the genetic map
174 and narrow the mapping interval. The genomic DNA of two parental lines was
175 collected and genotyped by CapitalBio Technology (Beijing, China) using the wheat
176 660K SNP array. Polymorphic SNPs detected in initial mapping region of two
177 parental lines were converted to KASP markers to genotype the mapping population
178 and integrated into the genetic map.

179 QTL identified in at least five environments (including BLUP) and explained more
180 than 10% of the phenotypic variation were regarded as major ones, while those
181 having common flanking markers were treated as a single one. QTL was then named
182 following the Genetic Nomenclature provision
183 (<http://wheat.pw.usda.gov/ggpages/wgc/98/Intro.htm>). In the naming system, ‘Sau’
184 represented ‘Sichuan Agricultural University’. The RILs’ name ‘AM’ was also added
185 to the QTL’s name to distinguish them from others reported previously.

Sequences of the markers were blasted against the durum wheat (Svevo) (Maccaferri et al., 2019), wild emmer (Zavitan; v2.0) (Zhu et al., 2019), and common wheat genotype ‘Chinese Spring’ (CS; v2.1) (Zhu et al., 2021) genomes to identify their physical positions. The Svevo and Zavitan genomes were used when considering the physical positions of QTL detected from AL and LM001, respectively. Predicted genes mapped between the flanking markers and their function annotations and expression patterns were obtained from the Triticeae Multi-omics Center (<http://202.194.139.32/>).

QTL Validation

The tightly linked SNP markers of major and novel SNS QTL were subjected to develop KASP markers. KASP primers were designed and applied following the method of Tan et al. (2017). Each validation population was divided into two haplotype groups (with homozygous alleles from any parent) according to their alleles present in parents and progenies. Phenotypic differences between the two groups were analyzed using Student’s *t*-test ($P < 0.05$).

Results

SNP Markers and Genetic Map

As suggested by the Affymetrix Company, the high probability probes from the Poly High-Resolution group only were reserved. Thus, 8724 SNPs with minor allele frequency (≥ 0.3) among the mapping population were retained for following analysis. Subsequently, 823 markers from the D genome were rejected. Finally, 7901 SNP markers were classified into 1192 bins, and a single marker with the lowest ‘Missing rate’ in each bin (bin marker) was screened to construct the genetic map. The linkage analysis results revealed that 1150 bin markers were mapped on 15 linkage groups. One linkage group was constructed for each chromosome except chromosome 3B, which had two (Table 1 and Supplementary Table 2). Based on the bin information, 6537 mapped markers (including 1150 bin markers and 5387

co-segregated markers) were integrated into the genetic map with a total length of 2411.8 cM (Table 1 and Supplementary Table 3). The length of genetic map in each linkage group ranged between 49.7 cM (3B1) and 225.1 cM (5A). The average distance of bin markers ranged between 1.09 cM (4B) and 3.64 cM (3B2), with an average density of one bin marker per 2.10 cM. The number of mapped markers ranged between 37 on chromosome 3B1 and 944 on chromosome 2A (Table 1 and Supplementary Table 3). Notably, 56% and 44% of the mapped markers were distributed on A and B subgenomes, respectively.

Comparison of Genetic and Physical Maps

Considering that the parent accessions AL and LM001 were durum wheat and wild emmer wheat, respectively, the sequences of the 6537 mapped markers were blasted against durum wheat and wild emmer genomes to identify their physical positions (Supplementary Table 3). The genetic chromosomal locations of the 1150 bin markers were compared with their physical chromosomal locations in the durum wheat genome. Among them, 1087 (94.52 %) markers showed good consistencies (Figure 2a), and 63 (5.48%) markers had inconsistent chromosomal locations. In the wild emmer genome, the genetic chromosomal locations of 71 (6.17%) markers were inconsistent with physical chromosomal locations, while the remaining markers (1079, 93.83%) were well matched (Figure 2b). Notably, 1026 (89.22%) bin markers had consistent chromosomal locations in genetic map, durum wheat genome and wild emmer genome. Similarly, the genetic maps of the 6537 mapped markers were compared with their physical maps (Supplementary Table 3). 6242 (95.49%) and 6249 (95.59%) markers showed consistent chromosomal locations in the durum wheat and wild emmer genomes, respectively, while 6031 (92.26%) markers had consistent chromosomal locations in genetic map and two physical maps.

Phenotypic Variation

The SNS of AL and LM001 in multiple environments ranged between 25.00 and

240 27.00, and 23.33 and 26.00, respectively (Table 2). That of AM RILs ranged between
241 15.33 and 34.00. There were no significant differences in SNS between the two
242 parental lines. However, continuous variation and bidirectional transgressive
243 segregation was observed in the RIL population. These results suggested that it was
244 feasible to analyze the SNS loci in the current population.

245 The broad-sense heritability (H^2) of SNS ranged between 0.93 and 0.97 in each
246 environment (Table 2), indicating that the SNS trait was mainly controlled by genetic
247 factors. Strong interaction was detected between SNS and environments ($P < 0.01$),
248 with significant correlation coefficients ranging between 0.50 and 0.90 (Figure 3).
249 The frequency distribution of SNS in multiple environments indicated that it was
250 polygenically inherited (Figure 3).

251 **Genetic Correlations between SNS and other Agronomic Traits**

252 Pearson's correlations were analyzed between SNS and seven other agronomic traits
253 based on the BLUP values (Table 3). SNS was significantly and positively correlated
254 with PH, AD, SL, SEL, KNS, and SD. However, SNS was not significantly
255 correlated with TKW.

256 **Analysis of QTL Associated with SNS**

257 Five QTL for SNS were identified and distributed on chromosomes 5A (1 QTL), 2B
258 (2), and 3B (2), with LOD scores ranging between 3.55 and 21.49 (Table 4). They
259 explained 6.71-29.40% of the phenotypic variation. *Q_{Sns.sau-AM-5A}*,
260 *Q_{Sns.sau-AM-2B.3}*, and *Q_{Sns.sau-AM-3B.2}* were detected in at least five
261 environments and explained more than 10% of the phenotypic variation, which were
262 treated as major QTL. And the other two QTL were identified as minor QTL.

263 *Q_{Sns.sau-AM-5A}* was detected in five environments and located in a 19.1 cM region
264 between AX-108790581 and AX-111596791. It accounted for 8.27-12.09% of the
265 phenotypic variation (Table 4). AM population were divided into two haplotype

groups based on the genotype of QTL's flanking markers, and the effect of *Q_{Sns.sau}-AY-5A.2* increased SNS significantly by 4.54-10.48% (Figure 4a).

Q_{Sns.sau}-AM-2B.2 was stably identified in all environments, explaining 9.09-29.40% of the phenotypic variation (Table 4). It was firstly mapped between AX-111626925 and AX-111588559 (Figure 4b). AX-95072014 and AX-109422178 were polymorphic SNPs detected in initial mapping interval of two parental lines using the wheat 660K SNP array. They were converted to KASP markers (KASP-AX-95072014 and KASP-AX-109422178) to genotype the mapping population and integrated into the genetic map. Remapping results showed that *Q_{Sns.sau}-AM-2B.2* was located in a 2.2 cM region between KASP-AX-95072014 and AX-111588559. The additive effect of *Q_{Sns.sau}-AM-2B.2* was negative, indicating that the increasing SNS allele was contributed by LM001. The effects of *Q_{Sns.sau}-AM-2B.2* on increasing SNS in single environment ranged from 7.00% to 12.18% (Figure 4b). *Q_{Sns.sau}-AM-3B.2* was detected in five environments (Table 4). It explained up to 15.46% of the phenotypic variation. The positive allele of *Q_{Sns.sau}-AM-3B.2* was from AL, and RILs containing the positive allele of *Q_{Sns.sau}-AM-3B.2* had a higher SNS than those containing the negative one. The effect of *Q_{Sns.sau}-AM-3B.2* significantly increased SNS by 5.02-10.14% in single environment (Figure 4c).

The QTL-by-Environment interaction analysis identified 47 QTL (Supplementary Table 4). Five of them were the same as detected in individual environment analysis, indicating that they were stably expressed for SNS. In addition, epistatic interaction were also analyzed, and the results showed that there were no epistasis effects between five SNS QTL detected in the present study (Supplementary Table 5).

Effects of Major QTL on Increasing SNS

The effects of three QTL (*Q_{Sns.sau}-AM-5A*, *Q_{Sns.sau}-AM-2B.2*, and *Q_{Sns.sau}-AM-3B.2*) on increasing the SNS were further revealed in the mapping

293 population based on the genotypes of the flanking markers (Figure 5). Compared to
 294 the haplotype group that carried the negative alleles of three QTL, haplotype groups
 295 with the favorable alleles from at least one QTL significantly increased SNS. The
 296 haplotype group containing the favorable alleles from three QTL significantly
 297 increased SNS compared with those with two or single one, respectively. No
 298 significant differences were detected among three haplotype groups with any two
 299 positive alleles. *QSn.sau-AM-2B.2* had the largest individual positive effect on SNS.
 300 However, there was no significant difference between the individual effect of three
 301 QTL on increasing SNS.

302 **Validation of Major and Novel SNS QTL**

303 The SNP markers closely linked to *QSn.sau-AM-2B.2* (AX-111588559) and
 304 *QSn.sau-AM-3B.2* (AX-110548993) were converted to KASP markers, to verify the
 305 effects of them in two other populations (Supplementary Table 6 and Figure 6).
 306 Since the favorable alleles of *QSn.sau-AM-2B.2* and *QSn.sau-AM-3B.2* were from
 307 LM001 and AL, respectively, haplotype group with homozygous alleles of LM001
 308 showed a significant increase in SNS by 8.33% (Figure 6a) and those from AL
 309 increased SNS significantly by 6.00% (Figure 6b).

310 **Discussion**

311 **Exploration of the SNS Loci from Tetraploid Wheat Species Using the Wheat** 312 **55K SNP Array**

313 Previous studies have reported on SNS in tetraploid wheat at the QTL level. Peng et
 314 al. (2001) identified four SNS QTL, explaining a total of 49.3% of the phenotypic
 315 variation in a wild emmer × durum wheat F₂ population genotyped using restriction
 316 fragment length polymorphisms (RFLP) markers. Genotyping of the durum ×
 317 cultivated emmer wheat RIL population with the 9 K iSelect SNP chip identified
 318 four SNS QTL on chromosomes 1B, 3B, 7A, and 7B (Faris et al., 2014a). In the
 319 same time, a linkage map of chromosome 2A generated in the Langdon ×

320 Langdon-wild emmer wheat substitution lines population consisting of 23 simple
321 sequence repeat (SSR) markers was used to identify an SNS QTL, *QSpn.fcu-2A*,
322 explaining 19.4% of the phenotypic variation (Faris et al., 2014b). SNS QTL,
323 *QSpn.fcu-1B*, and *QSpn.fcu-5A*, have been identified in a durum $\times$ cultivated emmer
324 wheat population genotyped using the 90K iSelect array (Sharma et al., 2019). These
325 reports enhance our understanding of the genetic mechanism of SNS in tetraploid
326 wheat.

327 The wheat 55K SNP array was developed based on the selection and optimization of
328 the 660K SNP array which was designed based on hexaploid, tetraploid and diploid
329 wheat, and has the advantages of being efficient and genome-specific (Sun et al.,
330 2020). It has been widely used for genotyping and genetic loci identification of
331 agronomic traits and disease resistance in bread wheat (Ren et al., 2018; Ma et al.,
332 2019b; Liu et al., 2020b; Ma et al., 2020; Tu et al., 2021). To the author's knowledge,
333 this is the first study to adopt the wheat 55K SNP array to construct the genetic
334 linkage map of a tetraploid wheat RIL population. Our results confirm the feasibility
335 of using a 55K SNP array developed for common wheat for genetic mapping of
336 tetraploid wheat, thus proving its broad utilization.

337 **Comparison of the Major SNS QTL Identified with those of Previous Studies**

338 There was no significant difference for SNS detected between the parental lines in
339 multiple environments (Table 2). The phenotypic data of SNS in the six
340 environments showed approximately normal distribution (Figure 4). Three major and
341 stably expressed QTL for SNS were identified. This phenomenon is attributed to the
342 transgressive inheritance in progenies that result in the bidirectional segregation of
343 SNS. Several genes controlling SNS may inhibit each other in a given parental
344 genotype resulting in the absence of the corresponding phenotype in the parents.
345 However, a single locus produced by genetic recombination between parental
346 genotypes can display a conspicuous phenotype in the progenies without interference
347 from other loci that control the same trait. The major SNS loci in the AM RIL

population can thus be identified. This is not uncommon in the past studies of QTL identification based on RIL populations (Liu et al., 2020a; Shang et al., 2020).

Physical locations of known SNS QTL were detected to verify whether major QTL were novel (Supplementary Table 7). In wild emmer genome, *QSns.sau-AM-5A* was physically located on chromosome arm 5AL at 557.72-571.41 Mb, overlapping with *QSpS.ccsu-5A.2* (568.96-662.44 Mb) (Kumar et al., 2007) and *QTL1755_5A* (569.50-579.91 Mb) (Peng et al., 2003), indicating that they are likely alleles. *QSns.sau-AM-2B.2* was located at 731.25-731.60 Mb on chromosome arm 2BL in wild emmer genome. Previous studies have detected SNS QTL on chromosome arm 2BS. However, none has been reported on 2BL. For example, *Qspn-2007* was located on chromosome arm 2BS with the closest marker Xgwm429 (Manickavelu et al., 2011). *QFsn.sdau-2B-1* was located on chromosome arm 2BS, explaining up to 28.43% of the phenotypic variation (Deng et al., 2017). Some genes regulating SNS, such as *Ppd-B1* (Mohler et al., 2004) and *WFZP-2B* (Dobrovolskaya et al., 2015), have also been detected on chromosome arm 2BS. In the durum wheat genome, *QSns.sau-AM-3B.2* was located on chromosome arm 3BS at 65.87-72.59 Mb. Similarly, Cui et al. (2012) identified two QTL, *QSpn.WJ.3B.1* and *QSpn.WY.3B.1*, all closely linked to the locus Xgwm566. Azadi et al. (2015) detected three QTL for SNS on chromosome arm 3BL. Comparing the physical positions of three major QTL with those previously reported indicates that *QSns.sau-AY-2B.2* and *QSns.sau-AY-3B.2* are probably novel loci controlling SNS in tetraploid wheat.

Potential Candidate Genes in the Physical Intervals of *QSns.sau-AM-2B.2*

QSns.sau-AM-2B.2 was physical located in a 0.35 Mb region of wild emmer genome, and 4 genes and 3 functional orthologs were annotated (Supplementary Table 8a). *QSns.sau-AM-3B.2* was located in a 6.72 Mb interval of durum wheat genome with 62 genes and 38 functional orthologs (Supplementary Table 8b). Since the physical interval of *QSns.sau-AM-2B.2* contains only 3 orthologs, it is valuable to analyze function annotations and expression patterns to predict candidate genes.

376 *TRIDC2BG075290* encodes a protein kinase which involves in cell signal
 377 transduction and plant growth. Previous research reported wheat protein kinase gene
 378 *TaSnRK2.9*□5A was significantly correlated with high thousand kernel weight and
 379 high kernel per spike (Ur Rehman et al., 2019), suggesting that protein kinase can
 380 have a positive impact on agronomic traits. In addition, *Stomatal opening factor 1*
 381 (*OST1*, *SnRK2.6*) is a homologous gene of *TRIDC2BG075290* in *Arabidopsis*. *OST1*
 382 can promote the expression of *FLOWERING LOCUS C (FLC)* to participate in the
 383 regulation of flowering by ABA (Wang et al., 2013), which may affect inflorescence
 384 development and the formation of spikelets. *TRIDC2BG075310* and
 385 *TRIDC2BG075320* all encode SAUR-like auxin-responsive protein. It has been
 386 reported that plant SAUR-like auxin-responsive protein family is involved in the
 387 growth of roots, hypocotyls, leaves and stamens (Chae et al., 2012; Hou et al., 2013).
 388 Expression patterns analyses showed that *TRIDC2BG075290* has higher expression
 389 levels in spike and flag leaf (Supplementary Figure 1). *TRIDC2BG075310* has a high
 390 expression level throughout the spike development stage, while *TRIDC2BG075320*
 391 is only expressed in spike. Therefore, we speculate that these 3 functional orthologs
 392 may affect the wheat inflorescence development, which can be further determined by
 393 fine mapping and gene cloning in future.

394 **Phenotypic Correlations of Agronomic Traits**

395 SNS was significantly and positively correlated with PH in the present study (Table
 396 3). This finding was consistent with that of Ajmal et al. (2009) and Wu et al. (2012).
 397 We speculate that SL is part of PH, and the taller plants have a long spike with more
 398 spikelets (Yao et al., 2011). There was a significant and positive relationship between
 399 SNS and AD (Table 3), consistent with the results of Ma et al. (2019a). It is supposed
 400 that wheat plants that take longer to flower tend to produce longer spikes with more
 401 spikelets (Shaw et al., 2013; Kamran et al., 2014; Boden et al., 2015). These results
 402 indicate that AD plays an important role in spikelet formation.

403 Similarly, there was a strong significant and positive correlation between SNS and

404 SL (Table 3). The increase in SL requires a longer spike development stage
 405 combined with optimal temperature and light to promote spikelet germination (Shaw
 406 et al., 2013). On the whole, floret fertility determines the KNS in wheat. More
 407 spikelets produce more fertile florets, thereby increasing the KNS (Gao et al., 2019;
 408 Sakuma et al., 2019). This reason potentially caused the SNS to be significantly and
 409 positively correlated with KNS (Table 3). Moreover, SNS was significantly and
 410 positively correlated with SD because SD is the ratio of SNS to SL (Table 3).

411 It is postulated that there is a negative correlation between SNS and TKW (Li et al.,
 412 2007; Ma et al., 2019a). The negative correlation is attributed to source limitations
 413 during kernel filling or more kernels with lower KW in the spike's distal positions
 414 (Quintero et al., 2018). Previous studies also suggest that SNS is negatively
 415 correlated with SEL because of competition in product assimilation between SNS
 416 and SEL (Bancal, 2008; Li et al., 2020). However, these conclusions are inconsistent
 417 with the findings of this study. Herein, SNS was positively correlated with TKW and
 418 SEL (Table 3). It probably indicates that the loci controlling SNS play an individual
 419 role without interacting with SEL. Previous studies postulate that SEL has a special
 420 function in photosynthesis and nutrients and water storage (Bridgemohan and
 421 Bridgemohan, 2014; Ávila-Lovera et al., 2017). Therefore, a longer SEL is more
 422 conducive for ventilation and transportation of more nutrients and water to kernels
 423 thereby increasing the KW.

424 These conclusions are vital in comprehensively understanding the complex
 425 relationships in agronomic traits, and provide new insights into wheat yield
 426 improvement. KNS is one of the primary determinants of grain yield. Therefore,
 427 wheat breeding programs should focus on increasing SNS under moderate SD in the
 428 present study.

429 **Conclusion**

430 Herein, the wheat 55K SNP array was successfully used to construct a high-quality

genetic map that identified five QTL for SNS in a tetraploid wheat RIL population. The additive effects of the major SNS QTL in increasing SNS were revealed in the mapping population. *Qsns.sau-AM-2B.2* and *Qsns.sau-AM-3B.2* were major and novel QTL, and their effects were successfully validated in other genetic backgrounds using closely linked KASP markers. The genetic correlations between SNS and other agronomic traits were also evaluated. The novel genetic loci of SNS and tightly linked KASP markers will be valuable in further fine mapping and gene cloning.

Data Availability Statement

All data generated or analyzed during this study are included in this published article and its supplementary information files, further inquiries can be directed to the corresponding author.

Author Contributions

ZQM and JZ conducted the study and drafted this manuscript. JTW and JGZ participated in phenotype measurement and data analysis. QX and HPT helped with field work and data analysis. YM, MD, QTJ, and YXL participated in data collection and analysis. GYC, JRW, PFQ, and WL did QTL analysis and manuscript revision. YMW and YLZ discussed the results and revised the manuscript. XJL guided the study and revised the manuscript. JM designed the experiments, guided the entire study, participated in data analysis, wrote and extensively revised this manuscript. All authors participated in the research and approved the final manuscript.

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464 **Conflict of Interest**

465 The authors declare that they have no competing interests.

466

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468 **References**

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688

Table 1 Information of SNP markers in the constructed genetic map

Chromosome	Group	Number of bin markers	Number of mapped markers	Length (cM)	Density (cM/bin)
1A	1	101	586	153.3	1.52
1B	1	69	488	149.3	2.16
2A	1	66	944	161.8	2.45
2B	1	92	424	183.7	2.00
3A	1	99	517	161.0	1.63
3B	1	17	37	49.7	2.92
	2	27	174	98.4	3.64
4A	1	80	282	199.9	2.50
4B	1	77	422	83.7	1.09
5A	1	92	298	225.1	2.45
5B	1	121	674	207.5	1.72
6A	1	60	636	167.0	2.78
6B	1	58	160	162.5	2.80
7A	1	92	395	214.2	2.33
7B	1	99	500	194.8	1.97
A genome	7	590	3658	1282.2	2.17
B genome	8	560	2879	1129.6	2.02
Total	15	1150	6537	2411.8	2.10

Table 2 Phenotypic data and heritability (H^2) of spikelet number per spike (SNS) for the AM population in multiple environments

Environment	Parents		AL $\times$ LM001 (AM)			
	AL	LM001	Min-Max	Mean	SD	H^2
E ₁	25.00	24.33	20.00-31.67	25.96	2.56	0.95
E ₂	26.00	23.33	15.33-33.00	24.41	3.22	0.97
E ₃	26.33	26.00	17.00-31.25	24.81	2.80	0.96
E ₄	26.00	25.40	18.00-32.00	26.21	2.87	0.96
E ₅	27.00	24.60	18.50-31.00	24.99	2.42	0.95
E ₆	25.80	25.40	20.00-34.00	26.76	2.69	0.96
E ₇	23.40	24.25	15.75-31.00	23.68	2.62	0.95
E ₈	25.44	24.67	18.50-30.67	24.93	2.55	0.95
BLUP	25.60	24.78	18.41-30.10	25.22	2.12	0.93

BLUP phenotype values based on the best linear unbiased prediction, *SD* standard deviation, H^2 the broad-sense heritability

Table 3 Correlations between spikelet number per spike (SNS) and other agronomic traits in the AM population

Trait	Spikelet number per spike (SNS)
Plant height (PH)	0.30**
Anthesis date (AD)	0.34**
Spike length (SL)	0.70**
Spike extension length (SEL)	0.23*
Kernel number per spike (KNS)	0.36**
Spike density (SD)	0.32**
Thousand kernel weight (TKW)	0.14

*Significance level at $P < 0.05$, **Significance level at $P < 0.01$

Table 4 Quantitative trait loci (QTL) for spikelet number per spike (SNS) identified in multiple environments in the AM population

QTL	Env.	Interval (cM)	Left Marker	Right Marker	LOD	PVE (%)	Add	Physical position (Mb)
<i>QSns.sau-AM-5A</i>	E ₁	116.1-131.9	AX-108790581	AX-111499991	5.62	12.07	-0.95	557.72-571.41 ^a
	E ₃	131.9-135.2	AX-111499991	AX-111596791	4.01	8.27	-0.83	
	E ₄	116.1-131.9	AX-108790581	AX-111499991	3.55	10.34	-0.95	
	E ₇	116.1-131.9	AX-108790581	AX-111499991	8.17	9.1812.0	-0.85	
	BLUP	116.1-131.9	AX-108790581	AX-111499991	6.61	9	-0.75	
<i>QSns.sau-AM-2B.1</i>	E ₄	113.7-114.6	AX-109421413	AX-109506426	3.90	15.66	-0.75	710.11-717.87 ^a
	E ₈	113.7-114.6	AX-109421413	AX-109506426	3.82	7.54	-0.63	
<i>QSns.sau-AM-2B.2</i>	E ₁	131.3-132.2	KASP-AX-109422178	AX-111588559	9.02	17.99	-1.16	731.25-731.60 ^a
	E ₂	131.3-132.2	KASP-AX-109422178	AX-111588559	5.10	9.09	-1.06	
	E ₃	131.3-132.2	KASP-AX-109422178	AX-111588559	11.14	26.57	-1.48	
	E ₄	131.3-132.2	KASP-AX-109422178	AX-111588559	7.35	15.79	-1.17	
	E ₅	130.0-131.3	KASP-AX-95072014	KASP-AX-109422178	4.97	20.39	-0.85	
	E ₇	130.0-131.3	KASP-AX-95072014	KASP-AX-109422178	21.49	25.60	-1.41	
	E ₈	131.3-132.2	KASP-AX-109422178	AX-111588559	8.87	19.32	-1.00	
	BLUP	131.3-132.2	KASP-AX-109422178	AX-111588559	15.97	29.40	-1.16	
<i>QSns.sau-AM-3B.1</i>	E ₄	22.7-42.5	AX-110399975	AX-109050064	6.61	17.54	1.26	65.87-72.59 ^b
	E ₆	22.7-42.5	AX-110399975	AX-109050064	3.97	11.85	1.02	
	E ₈	22.7-42.5	AX-110399975	AX-109050064	7.63	16.78	0.95	
<i>QSns.sau-AM-3B.2</i>	E ₁	48.3-49.7	AX-109393346	AX-110548993	4.82	9.04	0.85	
	E ₂	47.0-48.3	AX-109876826	AX-109393346	3.86	6.71	0.94	
	E ₃	48.3-49.7	AX-109393346	AX-110548993	5.84	12.67	1.05	
	E ₇	47.0-48.3	AX-109876826	AX-109393346	14.77	15.46	1.12	
	BLUP	48.3-49.7	AX-109393346	AX-110548993	8.64	14.27	0.83	

Env. Environment, *BLUP* best linear unbiased prediction; *LOD* logarithm of odds; *PVE* phenotype variance explained; *Add* additive effect, positive values: positive alleles from AL, negative values: positive alleles from LM001. *Physical position*^a the flanking marker sequences of QTL which positive alleles detected from LM001 were blasted against wild emmer genome to get physical positions; *Physical position*^b those detected from AL were blasted against durum wheat genome to get physical positions

Figure Legends

Figure 1 The spike morphology of the parents and representative lines from the AM population. (scale bar = 2 cm).

Figure 2 The syntenic relationships between the genetic and physical maps of bin markers. Gen-1A to Gen-7B represented the 15 chromosomal genetic maps used in the current study; Phy-1A to Phy-7B represented the 14 chromosomal physical maps of (a) durum wheat and (b) wild emmer genomes.

Figure 3 The phenotype, frequency distribution, and correlation coefficients of spikelet number per spike (SNS) in multiple environments. **Significance level at $P < 0.01$.

Figure 4 AM RILs were divided into two haplotype groups based on the genotype of flanking markers, and the SNS differences caused by the corresponding QTL were represented. Genetic maps of (a) *QSns.sau-AM-5A*, (b) *QSns.sau-AM-2B.2*, (c) *QSns.sau-AM-3B.2* and their effects. *Significance level at $P < 0.05$, **Significance level at $P < 0.01$.

Figure 5 The additive effects of *QSns.sau-AM-5A*, *QSns.sau-AM-2B.2*, and *QSns.sau-AM-3B.2* on increasing SNS. ‘+’ and ‘-’ represented haplotype group with and without the positive allele of the corresponding QTL based on the genotype of flanking markers, respectively. Letters a, b, c, and d represent significant difference among haplotype group.

Figure 6 Validation of (a) *QSns.sau-AM-2B.2* (KASP-AX-111588559) and (b) *QSns.sau-AM-3B.2* (KASP-AX-110548993) in LM001 × PI193877 and AL × AS2268 populations by Student’s *t*-test, respectively. **Significance level at $P < 0.01$.

Supplementary Materials

Supplementary Figure 1 Expression patterns at the different development stage of 3 functional orthologs in the physical interval of *Q_{Sns.sau-AM-2B.2}*. (a) *TRIDC2BG075290*; (b) *TRIDC2BG075310*; (c) *TRIDC2BG075320*.

Supplementary Table 1 Environmental information of surveyed agronomic traits in AM population.

Supplementary Table 2 The 1150 bin markers were used for QTL mapping.

Supplementary Table 3 Comparison of the genetic and physical positions of 6537 mapped markers (including bin markers and co-segregated markers).

Supplementary Table 4 Quantitative trait loci (QTL) detected in the QTL-by-Environment interaction module.

Supplementary Table 5 Epistasis interaction analysis of QTL for spikelet number per spike (SNS).

Supplementary Table 6 Details of KASP markers.

Supplementary Table 7 Comparison of the major quantitative trait loci (QTL) with loci previously reported for spikelet number per spike (SNS).

Supplementary Table 8 Predicted genes in the physical interval of *Q_{Sns.sau-AM-2B.2}* and *Q_{Sns.sau-AM-3B.2}*.

AL

LM001

#71

#99

#118

#26

#132

(a)

(b)