

## Genome-wide association study on agronomic traits of temperate japonica rice (*Oryza sativa* L.)

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**Abstract:** *Temperate japonica rice plays a very important role in food security. In this study, a set of 191 temperate japonica accessions from 30 countries were planted in two sites in China, and 12 agronomic traits were measured. The results showed a wide range of variation for the traits measured. Most of the accessions are short; three accessions with large panicles and seven accessions with large grains were identified. Sixty-two quantitative trait loci (QTLs) were identified for 12 traits that were measured. Among them, twenty-one QTLs were identified in both experiments, and 41 QTLs were identified in only one site. Some known genes are located in the QTL regions identified in our study. SNP markers for grain size were identified and could be efficiently used for breeding selection. This study provided useful information for future gene validation and marker assisted selection for some important agronomic traits of temperate japonica rice.*

**Keywords:** *Genome sequencing, quantitative trait locus, single nucleotide polymorphism.*


### INTRODUCTION

Temperate japonica rice occupies 20% of the rice cultivation area worldwide, mainly distributed in high altitude and high latitude areas. It generally has better plant structure and higher yield than indica rice. With its high yield and high grain quality, temperate japonica rice plays a very important role in world food security and high-end consumption. Rice yield components include number of panicles per given area, the number of spikelets per panicle, the percent of filled grains per panicle, and the weight of each grain. Among these components, panicle size and grain size (weight) are very important for increasing rice yield. Many QTLs have been identified and some important genes related to yield components have also been cloned. However, since all the yield-related traits are controlled by multiple genes, it is important to identify more QTLs/genes and useful alleles of the known genes from the natural germplasm.

Recent development of high density markers, such as SNPs, enables identification of trait-marker association through association mapping; and genome-wide association studies (GWAS) for many important agronomic traits have been reported (Huang et al. 2010, Zhao et al. 2011, Huang et al. 2012). Recently, 3000 rice accessions were systematically sequenced, and around 18.9

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million SNPs were identified (Li et al. 2014). Phylogenetic analyses based on SNP data confirmed differentiation of the *O. sativa* gene pool into 5 varietal groups – *indica*, *aus/boro*, *basmati/sadri*, *tropical japonica*, and *temperate japonica* (Li et al. 2014). Among those groups, there are 203 *temperate japonica* accessions from more than 30 countries. We previously evaluated the salinity tolerance of these temperate *japonica* accessions, and identified 27 salinity tolerant accessions and 26 QTLs related to salinity tolerance traits (Batayeva et al. 2018). Here we report the evaluation and genetic analysis of some important agronomic traits of these temperate *japonica* accessions. The objectives of this study were to identify accessions with useful agronomic traits and QTLs associated with these traits for future breeding application.

## MATERIAL AND METHODS

### Plant materials

The 3K Rice Genomes Project dataset includes genome sequences derived from 3,000 accessions of rice with global representation (from 89 countries) of genetic diversity (Li et al. 2014). There were 203 accessions in the temperate *japonica* group; however, seed availability was limited to 191 accessions (<https://www.irri.org/international-rice-genebank>), which were used in this study (Table S1).

### Measurements of agronomic traits

The seeds of selected accessions were treated at 50 °C for 5 days in an oven to break seed dormancy, and then divided into two sets. The first set was sown in a wet seedling bed in the field in the city of Yuanjiang (lat 23° 36' 15" N, long 101° 58' 29" E and alt 433 m asl) of Yunnan province, China. The seedlings were transplanted in 100 x 200 cm plots (5 rows x 20 plants per row). All the plots were randomly arranged in a single field with completely randomized design. Crop management was the same as the local farms. At maturity, the number of panicles or effective tillers per

**Table 1.** Correlations among the traits measured

Trait	#Site	AL	GD	GL	GLWR	GPP	GT	GW	PH	PL	SF	TGW
GD	FM	-0.145										
	YJ	-0.115										
GL	FM	-0.081	-0.113									
	YJ	-0.032	0.05									
GLWR	FM	-0.108	-0.073	0.846***								
	YJ	-0.091	-0.001	0.851***								
GPP	FM	-0.093	0.866***	-0.172*	-0.125							
	YJ	-0.074	0.903***	0.03	0.008							
GT	FM	0.016	0.037	-0.26***	-0.58***	0.039						
	YJ	0.109	-0.21**	-0.206**	-0.443***	-0.18*						
GW	FM	0.08	0.001	-0.19*	-0.674***	0.026	0.747***					
	YJ	0.109	0.007	-0.095	-0.572***	-0.037	0.61***					
PH	FM	0.137	0.061	-0.075	-0.134	0.391***	0.099	0.145				
	YJ	0.265***	0.141	0.087	0.063	0.408***	-0.027	-0.001				
PL	FM	0.071	-0.129	-0.08	-0.086	0.362***	0.011	0.058	0.707***			
	YJ	0.075	0.124	0.009	0.038	0.518***	-0.087	-0.089	0.701***			
SF	FM	-0.067	-0.243***	-0.151*	-0.107	-0.285***	0.097	0.001	-0.172*	-0.111		
	YJ	0.015	-0.117	0.089	0.046	-0.096	0.024	0.103	0.019	0.02		
TGW	FM	0.032	-0.165*	0.541***	0.208**	-0.28***	0.265***	0.356***	-0.149*	-0.189*	0.095	
	YJ	0.066	-0.111	0.712***	0.351***	-0.13	0.286***	0.446***	0.025	-0.058	0.135	
TN	FM	-0.097	-0.239***	0.005	0.039	-0.249***	-0.128	-0.105	0.033	-0.025	0.03	-0.12
	YJ	-0.103	-0.326***	-0.239**	-0.172*	-0.359***	0.058	-0.023	-0.225**	-0.198*	-0.17*	-0.2*

# Site: FM = Fumin, YJ = Yuanjiang. Significance: p < 0.05\*, p < 0.01\*\*, p < 0.0005\*\*\*;

Abbreviations: awn length (AL), Grain density (GD), grain length (GL), Grain length and width ratio (GLWR), number of grains per panicle (GPP), grain thickness (GT), grain width (GW), plant height (PH), panicle length (PL), Spikelet fertility (SF), thousand-grain weight (TGW), effective tillers per plant (TN).

plant (TN), plant height (PH) and panicle length (PL) of five plants were measured, and five panicles were harvested for the following measurements: awn length (AL), number of empty and filled grains per panicle (GPP), grain length (GL), grain width (GW), grain thickness (GT), and thousand-grain weight (TGW). All the traits were measured with five replications. The number of grains per panicle (GPP) was calculated as empty grains + filled grains. Spikelet fertility (SF) was calculated as number of filled grains/number of grains per panicle x 100%. Grain length and width ratio (GL/GW) was calculated as grain length/grain width. Grain density (GD) was calculated as number of grains per panicle/panicle length.

The second set was sown in a wet seedling bed in the field in the city of Fumin (lat 25° 12' 31" N, long 102° 30' 54" E and alt 1695 m asl) of Yunnan province, China. The experimental design and management were the same as the first set. At maturity, the same dataset was collected for analysis.

## Data analysis

Since some accessions did not head because of photoperiod sensitivity, phenotypic data was only collected from 160 accessions in Yuanjiang and 181 accessions in Fumin. The mean values of five replications were calculated and used for the analysis. Basic statistical information of the traits and correlations among different traits were calculated by using MINITAB V14.0 (Minitab Inc.).

The core SNP V2.1 of the 191 temperate *japonica* rice accessions selected were downloaded from the SNP-seek system (<http://snp-seek.irri.org>) (Alexandrov et al. 2015) and used for data analysis. The dataset with 365,710 SNPs were analyzed by using TASSEL program V5.2.18 (Bradbury et al. 2007). For the dataset for Yuanjiang, the SNP sites were filtered at a maximum count of 144 of the 160 accessions and a minimum frequency of 0.05 for the minor allele. There were 54,124 SNPs that met the above criteria. For the dataset for Fumin, the SNP sites were filtered at a maximum count of 163 of the 181 accessions and a minimum frequency of 0.05 for the minor allele. Ultimately, 65,460 SNPs were used for analysis. The genotyping data were first used for generating a kinship matrix with centered IBS. A united data file with the genotype and phenotype of the lines was created by using union join. The united file, along with the kinship matrix, was used to analyze marker-trait associations using a mixed linear model (MLM). The compression level was set to optimum level, and estimation of variance components was set to P3D. The criteria for claiming a QTL was  $p < 1 \times 10^{-4}$  ( $-\log_{10}$  p-value > 4.0) and marker  $R^2 > 0.1$ . The identified QTLs were named using the CGSNL nomenclature (McCouch 2008).

## RESULTS

### Phenotypic variation among the accessions

Most of the traits are normally distributed with skewness and kurtosis less than 2.0, except for awn length and spikelet fertility (Table S2). There are only 27 accessions with awn, possibly because of breeding selection. The maximum and minimum temperatures during the crop season at Yuanjiang were higher than at Fumin (Figure S1). At Yuanjiang, there were 32 days of high temperature above 35 °C from April 4 to May 13, except from April 17-24. Thus, there was mild high temperature stress during the reproductive stage at Yuanjiang. At Fumin, the minimum temperature was always below 20 °C. There was mild low temperature stress during the crop season. Thus, some heat and cold susceptible accessions showed low spikelet fertility. At Yuanjiang, 25 accessions had spikelet fertility below 80%, and the lowest spikelet fertility was 50.5%. At Fumin, 51 accessions had spikelet fertility below 80%, and the lowest spikelet fertility of the accessions was 38.9%.

There were 60 accessions with high values for one or more of the traits measured at Yuanjiang or Fumin (Table S3). This set of temperate *japonica* accessions had relatively short plant height; only 10 accessions had plant height over 110 cm at both Yuanjiang and Fumin, and Rubi from Portugal was the tallest (135.6 cm at Yuanjiang and 145.5 cm at Fumin). The panicle sizes of these accessions were relatively small; only two accessions had panicle length over 25 cm at both Yuanjiang and Fumin (Hong Pi Nuo from China and IBO400 from Portugal), and three accessions had more than 240 spikelets per panicle (C722323 from Taiwan, Ota and Rubi from Portugal). Seven accessions with large grains (thousand grain weight over 35 g) were identified.

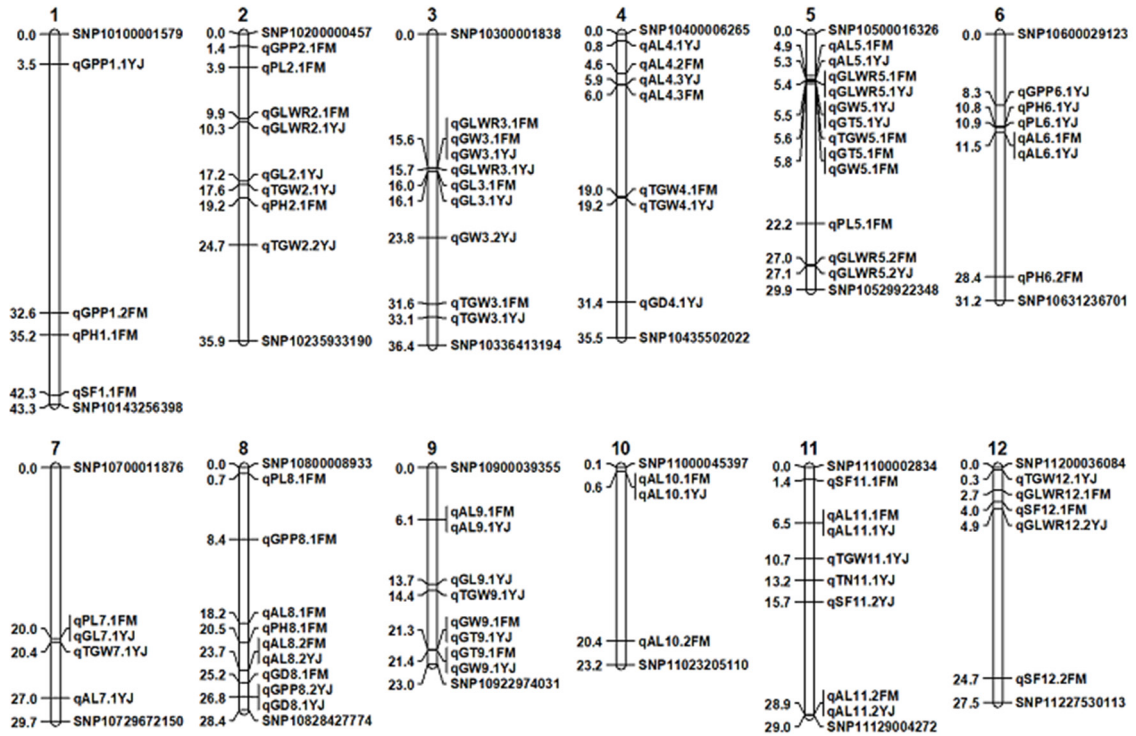


Figure 1. QTLs for traits measured. The numbers on the left side were physical distances in Mb. FM = Fumin, YJ = Yuanjiang.

**Correlation among the traits measured**

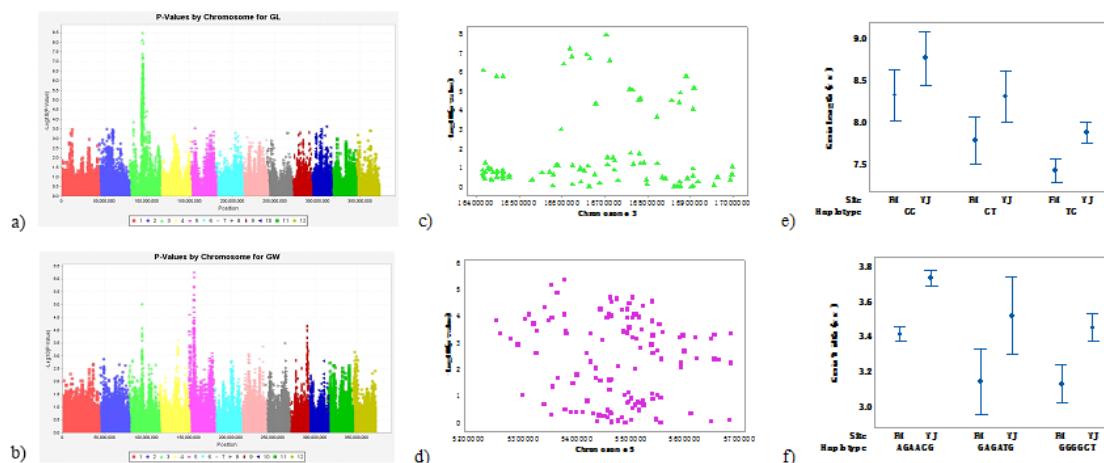
In the subset of temperate japonica that was used, plant height was correlated with panicle length and number of spikelets per panicle; tall plants had longer panicles and more spikelets on the panicle (Table 1). The number of tillers also correlated with grains per panicle and grain density. Awn length was not correlated with other traits, except for a weak correlation with plant height at Yuanjiang. In terms of grain size, grain length and width were not correlated, but both grain length and width were correlated with grain thickness. Grain length, grain width, and grain thickness were all correlated with grain length/width ratio and thousand-grain weight.

**Trait-marker association of the traits measured**

Based on the Manhattan plots from genome-wide association analysis (Figure S2), sixty-two QTLs were identified for 12 traits measured (Table S4). Among them, twenty-one QTLs were identified at both Yuanjiang and Fumin, and another 41 QTLs were identified at only Yuanjiang or Fumin. QTL clusters with QTLs for different traits were observed on chromosomes 3, 5, 6, 7, and 9 (Figure 1).

In the QTL regions of qAL4.3, qAL11.2, qGL3.1, qGLWR3.1, qGLWR5.1, qGT5.1, qGT9.1, and qGW5.1, many SNPs with low p values were lined up. These QTLs were very promising for the traits, and some of the known genes are located in related QTLs; for example, GS3 is located in QTL qGL3.1, and GW5 is located in QTL qGW5.1 and qGT5.1.

In the QTL region of qGL3.1, gene Os03g0407400 (chr03:16,729,501-16,735,109) in the RAP database corresponds to GS3, but there is no gene corresponding to GS3 in the MSU database. Gene Os03g0407400 is located between LOC\_Os03g29370 and LOC\_Os03g29380, and only two SNPs (10316723913 and 10316731513) were identified in this region, including one SNP in GS3 (SNP10316731513 with alleles C/T). There are three haplotypes for these two SNPs, and allele CC has the longest grains at both Yuanjiang and Fumin (Figure 2).



**Figure 2.** a, b) Manhattan plots of QTL for grain length and grain width; c, d) detailed position of the QTLs *qGL3.1* and *qGW5.1*; e, f) grain length and width of different haplotypes of SNPs around the known genes *GS3* and *GW5*, for grain size. The bars show a 95% confidence interval of the SE of mean. FM = Fumin, YJ = Yuanjiang.

In the QTL region of *qGW5.1*, gene *LOC\_Os05g09520* (chr05:5,365,122-5,366,701) corresponds to *GW5*, which is located between *LOC\_Os05g09510* and *LOC\_Os05g09530*, and six SNPs were identified in this region, but no SNP is located in gene *GW5*. Haplotype AGAACG had the widest grain at both Yuanjiang and Fumin (Figure 2).

## DISCUSSION

In modern rice breeding, new plant type with short stem, fewer tillers, large panicle, and large grain is an important way to improve yield (Chen et al. 2001). Therefore, it is important to identify genetic resources with these favorable traits and use them in breeding programs. In the present study, we identified three accessions with more than 240 spikelets per panicle, and seven accessions with large grains (TGW>35g). These accessions are very important resources for improving rice yield.

In rice, the long-awn trait appears to have been lost during domestication and breeding selection (Toriba and Hirano 2014). In the present study, awn length was not correlated with other traits, except for a weak correlation with plant height. This may be because the accessions with awn are usually traditional tall varieties, and plants with awn were eliminated during selection for short high-yielding varieties. The awn trait in rice is a typical quantitative trait controlled by multiple genes. More than 20 QTLs related to the awn trait have been identified (Kubo et al. 2001, Cai and Morishima 2002, Sato et al. 2004, Wang et al. 2013), and some genes such as *An-1* (*LOC\_Os04g28280*), *An-2* (or *LABA1*, *LOC\_Os04g43840*), and *An-3* (*LOC\_Os08g37890*) have been cloned (Luo et al. 2013, Gu et al. 2015, Hua et al. 2015, Yano et al. 2016, Liu et al. 2017b). In our study, we identified 13 QTLs related to awn length on chromosomes 4, 5, 6, 8, 9, 10, and 11. Eight of them were identified at both Yuanjiang and Fumin, and six of them had the same peak SNP in both experiments. The cloned awn gene *An-3* is located in QTL *qAL8.2*. QTL *qAL11.2* explained high phenotypic variation (about 18% at both Yuanjiang and Fumin), which may be a novel QTL for further genetic validation.

There have been many studies on panicle length and number of grains per panicle. At least 253 QTLs for panicle length, 353 QTLs for number of grains per panicle, and 29 QTLs for grain (spikelet) density have been detected (<http://www.gramene.org>) (Youens-Clark et al. 2011). However, rice panicle length is sensitive to the growth environment (Hittalmani et al. 2003). In the present study, we identified five QTLs for panicle length, six QTLs for number of grains per panicle, and two QTLs for grain density. Only one QTL for grain density (*qGD8.1*) was identified at both Yuanjiang and Fumin; other QTLs were identified in only one location. Some related genes have been cloned, for example, genes for short panicle (*SP1*, *LOC\_Os11g12740*) (Li et al. 2009), long panicle (*LP1*, *LOC\_Os09g28300*; *qPL6*, *LOC\_Os06g45460*)

(Zhang et al. 2015, Liu et al. 2016), dense and erect panicle (*DEP1*, *LOC\_Os09g26999*; *DEP2*, *LOC\_Os07g42410*; *DEP3*, *LOC\_Os06g46350*) (Huang et al. 2009, Li et al. 2010, Qiao et al. 2011), and number of grains (*Gn1a*, *LOC\_Os01g10110*; *gnp4*, *LOC\_Os04g32510*; *OsEBS*, *LOC\_Os05g51360*) (Ashikari et al. 2005, Zhang et al. 2011, Dong et al. 2013). But none of the above known genes were located in the QTLs for number of grains per panicle identified in our study. QTLs *qGPP8.2* and *qGD8.1* were overlapped on the long arm of chromosome 8 at about 26.7-26.8 Mb. The effect of this locus needs to be further validated.

Grain length was not correlated with grain width. Thus, it is possible to develop varieties with different grain shape (length/width ratio) for different markets. There are 75 QTLs for grain length, 66 QTLs for grain width, and 315 QTLs for grain weight (<http://www.gramene.org>). Many genes controlling rice grain size have been cloned, including genes for grain length (*PGL1*, *LOC\_Os03g07510*; *PGL2*, *LOC\_Os2g51320*; *GL3.1*, *LOC\_Os03g44500*; *GL7/GW7*, *LOC\_Os07g41200*) (Heang and Sassa 2012a, b, Qi et al. 2012, Wang et al. 2015a, Wang et al. 2015b), grain width (*GW2*, *LOC\_Os02g14720*; *GW8*, *LOC\_Os08g41940*) (Song et al. 2007, Wang et al. 2012), grain size (*GS2*, *LOC\_Os02g47280*; *GS3*, *Os03g0407400* between *LOC\_Os03g29370* and *LOC\_Os03g29389*; *GS5*, *LOC\_Os05g06660*; *GS6*, *LOC\_Os06g03710*) (Fan et al. 2006, Weng et al. 2008, Li et al. 2011, Sun et al. 2013, Hu et al. 2015, McCouch et al. 2016, Liu et al. 2017a), and grain weight (*GW5*, *LOC\_Os05g09520*; *GW6a*, *LOC\_Os06g44100*; *TGW6*, *LOC\_Os06g41850*; *HGW*, *LOC\_Os06g06530*) (Li et al. 2012, Ishimaru et al. 2013, Song et al. 2015). In the present study, we identified four QTLs for grain length, four QTLs for grain width, two QTLs for grain thickness, six QTLs for grain length and width ratio, and nine QTLs for grain weight. Among these QTLs, the known gene *GS3* is located in QTL *qGL3.1*, *GL3.1* is located in *qGW3.2*, *GW5* is located in *qGW5.1*, and *qGT5.1*, *GL7/GW7* is located in *qTGW2.2*. In the QTL region of *qGL3.1*, there are 2 SNPs around gene *GS3*. Haplotype CC had the longest grains at both Yuanjiang and Fumin. In the QTL region of *qGW5.1*, there are 6 SNPs around gene *GW5*. Haplotype AGAACG had the widest grain at both Yuanjiang and Fumin. These SNP markers could be efficiently used for selection of grain size in future breeding programs.

Plant height was correlated with panicle length and number of spikelets per panicle. Thus, it is important to select relatively tall plants with strong stems and big panicles in rice breeding programs targeting a new plant type. There are 1011 QTLs for plant height in the Gramene database, and almost 10 genes for dwarf and semi-dwarf have been cloned, including the genes *sd1* (*LOC\_Os01g66100*) (Sasaki et al. 2002), *sd-g* (*LOC\_Os05g33730*) (Sui et al. 2012), *sdt* (*LOC\_Os06g44034*) (Zhao et al. 2015), *sd37* (*LOC\_Os03g04680*) (Zhang et al. 2014), *ssd1* (*LOC\_Os03g19080*) (Asano et al. 2010), *brd1* (*LOC\_Os03g40540*) (Hong et al. 2002), and *OsSIN* (*LOC\_Os03g22510*) (Han et al. 2005). The semi-dwarf gene *sd1* is the most widely used source for short plant (Monna et al. 2002). There is no SNP in *sd1* alleles of the temperate *japonica* accessions used in our study, but a QTL (*qPH1.1*) close to *sd1* was identified. QTLs for plant height were also identified on chromosome 2, 6, and 8. The known gene *sdt* is close to QTL *qPH6.2*. The QTLs on chromosomes 2 and 8 are novel loci for further validation.

The number of effective tillers is negatively correlated with the number of grains per panicle and grain density on the panicle; plants with more tillers usually have fewer grains on the panicle. Thus, we need to balance tiller number and panicle size in breeding programs. There are 213 QTLs for tiller number in the Gramene database, and some genes controlling tiller number were cloned, such as the monocolm genes *MOC1* (*LOC\_Os06g40780*), *MOC2* (*LOC\_Os01g64660*), *MOC3*, (*LOC\_Os04g56780*), and *OsTB1* (*LOC\_Os03g49880*) (Li et al. 2003, Yao et al. 2007, Koumoto et al. 2013, Lu et al. 2015). We identified only one QTL for number of effective tillers in this study. The QTL *qTN11.1* identified was located at a position similar to *qTN-11-2* (Yang et al. 2006).

At Yuanjiang, high temperature occurred during the reproductive stage of some accessions, which caused spikelet sterility. A QTL was identified for spikelet fertility (*qSF11.2*), but this QTL is not linked to any reported QTL for rice heat tolerance (Ye et al. 2012, Ye et al. 2015, Ishimaru et al. 2016). On the contrary, the minimum temperature at Fumin was always below 20 °C during the crop season, which may cause cold stress to the plants. Four QTLs for spikelet fertility were identified. QTLs *qSF1.1* and *qSF11.1* are located at positions similar to the QTLs *qCT-1* and *qCT-11* for booting stage cold tolerance (Takeuchi et al. 2001). These QTLs may be related to the cold tolerance of temperate *japonica* rice accessions; however, because of temperature variation in the field, it is still necessary to validate these QTLs under temperature-controlled conditions.

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