Genome-Wide Association Study (GWAS) For Cold Tolerance at The Bud Burst Stage in Rice Using SNP Markers

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Abstract

Background: Rice is a crop that is very sensitive to low temperature, and its morphological development and production are greatly affected by low temperature. Therefore, understanding the genetic basis of cold tolerance in rice is of great significance for mining favorable genes and cultivating excellent rice varieties. However, there were limited studies focusing on cold tolerance at the bud burst stage, therefore, considerable attention should be paid to the genetic basis of cold tolerance at the bud burst stage (CTBB).

Results: In this study, a natural population consisting of 211 rice landraces collected from 15 provinces of China and other countries were firstly used to evaluate the cold tolerance at the bud burst stage. Population structure analysis showed that this population divided into three groups and was rich in genetic diversity. Our evaluation results confered that the *japonica* rice was more tolerance to cold at the bud burst stage than *indica* rice. Genome-wide association study (GWAS) were performed through the phenotypic data of 211 rice landraces and 36,727 SNPs dataset under a mixed linear model, and 12 QTLs ($P < 0.0001$) were identified according to the seedling survival rate (SSR) treated at 4 ℃, in which there are five QTLs (*qSSR2-2*, *qSSR3-1*, *qSSR3-2*, *qSSR3-3* and *qSSR9*) which were co-located with previous studies, and seven QTLs (*qSSR2-1*, *qSSR3-4*, *qSSR3-5*, *qSSR3-6*, *qSSR3-7*, *qSSR4* and *qSSR7*) which were reported for the first time. Among these QTLs, *qSSR9*, harboring the highest-peak SNP, explained biggest phenotypic variation. Through bioinformatics analysis, five genes (*LOC_Os09g12440*, *LOC_Os09g12470*, *LOC_Os09g12520*, *LOC_Os09g12580* and *LOC_Os09g12720*) were nominated as candidates for *qSSR9*.

Conclusion: This natural population consisting of 211 rice landraces with high density SNPs will serve as a better choice for identifying rice QTLs/genes in future, and the detected QTLs associated with cold tolerance in rice bud burst stage will be conducive to further mining favorable genes and breeding of rice varieties under cold stress.

Background

Originated in the tropical and subtropical, rice is one of the main staple foods in the world. Low temperature has a huge impact on rice. More than 15 million hectares of rice cultivation area in the world have been persecuted by low temperature. Severe cold damage exists in many countries, mainly in Japan, South Korea, the United States and China [1]. Rice is cultivated in a wide area in China, ranging from 53 ° 27 'to 18 ° 90' north latitude, especially in the provinces of the Yangtze river basin in China, from 1951 to 1980, *japonica* rice and *indica* rice in the Yangtze river suffered from cold injury severely, the disaster areas lose 5 to 10 billion kg of rice every year [2]. Early rice in the Yangtze river basin in China is often affected by cold injury at the bud burst stage, resulting in low germination rate and failure to emerge. Therefore, it is very necessary to study the cold tolerance in rice at the bud burst stage.

Cold tolerance is a complex quantitative trait that is often controlled by multiple genes and environment, and researchers often use bi-parental populations to look for QTLs associated with cold tolerance. The

researchers excavated more than 250 QTLs in the bi-parental population using traditional QTL mapping methods during various stages of rice growth [3]. Among more than 250 QTLs, many genes throughout the rice whole growth stages have been isolated. At the germination stage, *qLTG3-1* was the first gene to be linked to germination at low temperatures [4]. During the seedling stage of rice, many QTLs/genes related to cold tolerance of rice were isolated, including *qCTS12* [5], *qCTS4* [6], *qCtss11* [7], *qSCT1* and *qSCT11* [8], *qLOP2* and *qPSR2-1* [9], *COLD1* [10] and *qCTS-9* [11]. *qCTS12* was the first identified cold-tolerance gene at seedling stage. *COLD1* is another important gene related to cold tolerance in rice seedling stage, and it is also the first cold-tolerance gene involved in signal transduction. At the booting stage of rice, several genes have been isolated, including *Ctb1* [12], *qCT8* [13], *qCTB7* [14], *qCTB3* [15] and *qCT-3-2* [16], *Ctb1* is the first gene to be linked to cold tolerance at booting stage in rice. Although bi-parental mapping populations have played a great role in traditional gene mapping, the construction of bi-parental populations entails a major investment in time, and has therefore limited the number of genes excavate to date [17].

In order to excavate more new genes associated with target traits, the exploration of QTLs/genes through the natural population with weak genetic relationship using GWAS has become one of the most popular methods. This method eliminates the need to construct a mapping population, and can simultaneously analyze multiple alleles, using the recombination information from the long-term evolution of natural populations. In recent years, some studies have applied this method to study rice cold tolerance. 17 QTLs were detected related to the rice germination at low temperatures using 63 Japanese varieties [18]. 51 QTLs were mapped by GWAS with the population of 174 rice accessions from China [19]. 132 QTLs were identified from 527 rice varieties for both rice natural chilling and cold shock stresses [20]. 67 QTLs were mapped for cold stress at the seedling stage of rice, 56 QTLs were newly discovered [21]. 42 QTLs were found associated with cold tolerance in rice seedling stage, 20 of which have not been mentioned in previous reports [22]. 31 QTLs were detected related to low temperature germination of rice seeds at rice germination stage using 200 *japonica* rice varieties [23]. 47 QTLs were identified for cold tolerance at the bud burst stage using 249 *indica* rice accessions [24]. 26 QTLs were found related to cold tolerance in rice seedling stage by using a core collection of landraces of rice from 2,262 accessions of Ting's collection [25]. In addition, 31 distinct QTLs regions were identified in a panel of 257 rice accessions from all of the world for low temperature germination [26]. However, there are still few studies using GWAS to explore cold tolerance in rice at the bud burst stage. In order to understand the genetic mechanism of cold tolerance in rice at early stage, it is necessary to search for QTLs related to cold tolerance in rice at the bud burst stage.

In this study, we selected 211 rice landraces from different regions to form a natural population and performed high-throughput sequencing using microarray. The 211 rice landraces were mainly composed of *indica* and *japonica* rice, which provided abundant genetic diversity for studying cold tolerance of rice. We treated the natural population with low temperature at 4 ℃, and then recovered it at room temperature. A total of 12 QTLs and 5 candidate genes for *qSSR9* related to cold tolerance were identified by genome-wide association study of seedling survival rate (SSR), which provided valuable gene

resources for cold tolerance research in rice and laid a solid foundation for breeding cold tolerant rice varieties.

Results

Cold tolerance of the 211 rice varieties

In our study, SSR were used for evaluate the CTBB **(Table S2)**. Due to the abundant landrace germplasm resources, the phenotypes of rice varieties within the natural population varied greatly after 4℃ treatment **(Table 1;Fig. 1)**. We classify cold tolerance into five levels of SSR: Extremely sensitive ($0 \leq X \leq 20$), Sensitive ($20 < X \leq 40$) , Light sensitive ($40 < X \leq 60$) , Tolerance ($60 < X \leq 80$) , Extremely tolerance ($80 < X \leq 100$). Of the 101 rice varieties that were extremely sensitive to low temperature, 99 were *indica* and 2 were *japonica*. Correspondingly, Of the 69 rice varieties that were extremely tolerance to low temperature, 5 were *indica* and 64 were *japonica*. According to SPSS software 26.0 analysis results, the SSR was significantly correlated with *indica* and *japonica*, and the correlation coefficient was 0.851 **(Table 2)**, suggesting that *japonica* was more cold tolerance than *indica* at the bud burst stage. On the other hand, we found that the cold tolerance of rice was significantly related to its geographical distribution. and the correlation coefficient was 0.714 **(Table 2)**. The higher the latitude, the stronger the cold tolerance of rice, which may be due to the significant correlation between *indica* or *japonica* types with latitude **(Table 2)**.

Population structure and relative kinship

Based on the 36,727 SNPs, the STRUCTURE, Neighbor-joining (NJ) tree method, Principal Component Analysis (PCA) and Kinship were used to analyze the population structure of the natural population **(Fig. 2)**. According to the STRUCTURE analysis, the log likelihood increased gradually from K = 1 to K = 10. The maximum *ad hoc* measure 1K was observed for K = 3, which indicated that the entire population could be divided into three subgroups **(Fig. 2A)**. The 211 rice varieties could be divided into 3 subgroups by NJ tree **(Fig. 2B)** and three principal components from this panel **(Fig. 2C)**. In addition, in the relationship analysis, we found that there were two major groups of subgroups and a middle subgroup in the 211 rice varieties **(Fig. 2D)**, suggesting that the landraces population germplasm resources were abundant, which was beneficial for performing GWAS.

GWAS analysis for CTBB

Based on genotype and phenotype data, association analysis was performed under a mixed linear model using PCA and KINSHIP as co-variables. A total of 12 QTLs (*qSSR2-1*, *qSSR2-2*, *qSSR3-1*, *qSSR3-2*, *qSSR3-3*, *qSSR3-4*, *qSSR3-5*, *qSSR3-6*, *qSSR3-7*, *qSSR4*, *qSSR7* and *qSSR9*) were identified in SSR with well-fitted quantile–quantile (Q–Q) plots ($p<0.0001$) **(Fig. 3 and Fig. 4; Table 3)**. A QTL, *qSSR2-2,* on chromosome 2 overlapped with *OsWRKY71* [28]. Another QTL on chromosome 2, *qSSR2-1*, has not been previously reported. On chromosome 3, there were three QTLs overlapping with previous studies [29-31], namely *qSSR3-1*, *qSSR3-2* and *qSSR3-3*, the other four QTLs on chromosome 3 are newly discovered and have not

been reported by previous studies. The other two QTLs on chromosome 4 (*qSSR4*) and 7 (*qSSR7*) were also newly discovered in this study. In addition, we also found a QTL on chromosome 9, *qSSR9*, which harbored the highest-peak SNP, which explained biggest phenotypic variation. It is located around 7.1 Mb on chromosome 9, and this QTL co-located with *clr9* [32] .

Candidate gene analysis

Among these QTLs, we conducted further candidate genetic analysis of the *qSSR9*. According to the LD decay analysis, a total 244-kb region was identified as the candidate region **(Fig. 5).** There are 39 genes in this region, including 3 hypothetical proteins, 4 transposon proteins, 7 retrotransposon proteins and 16 functionally annotated genes **(Table S3)**. In order to find possible candidate genes, we analyzed the homology between these 39 genes and 20 characterized cold-tolerance genes, *LOC_Os09g12440*, *LOC_Os09g12470*, *LOC_Os09g12520*, *LOC_Os09g12580* and *LOC_Os09g12720* were found having a high degree of homology with *COLD1*, *Ctb1*, *LTG1*, *OsWRKY71* and *OsbZIP73***(Fig. 6Table 4)**.

Discussion

Population structure and phenotypic assessment of a natural population

For this study, GWAS was used as a method to reveal complicated genetic variations of cold tolerance. However, population structure is an important factor affecting the results of GWAS and increases the false positive rate. In this study, a natural population consisting of 211 rice landraces (130 *indica* rice and 81 *japonica* rice) was used to assess cold tolerance in rice at the bud burst stage. Most of the rice varieties come from 15 provinces in China, with three from Japan and one from Philippines. The geographical regions spans from the north latitude 15°to 48° including temperate zone, tropics and subtropics. This natural population is newly constructed and has rich genetic diversity. Population structure analysis divided the natural population into three groups. Subsequently, the results of PCA and NJ trees support this result that low relatedness were showed from the relative kinship analysis **(Fig. 2)**, which makes it suitable for GWAS.

We used the SSR as the indicator to evaluate the cold tolerance of natural populations. The results show that the SSR ranges from 0% to 100% **(Table S2)**, *indica* rice is extremely sensitive to temperature, and its SSR was low after cold treatment. Some *indica* rice varieties even die in the recovery time, while *japonica* rice is with the characteristic of cold tolerance, and the SSR of most *japonica* rice is above 90%. This result showed that *japonica* rice was more tolerance to cold than *indica* rice at the bud burst stage.

Identification of QTLs/candidate genes controlling CTBB

In this study, we found 12 QTLs using SSR as indicator. Among these QTLs, seven of them (*qSSR2-1*, *qSSR3-4*, *qSSR3-5*, *qSSR3-6*, *qSSR3-7*, *qSSR4* and *qSSR7*) were reported for the first time, and the other five QTLs (*qSSR2-2*, *qSSR3-1*, *qSSR3-2*, *qSSR3-3* and *qSSR9*) were co-located with previous studies. The physical distance of the peak SNP for *qSSR2-2* was located at 4.4Mb on chromosome 2. It overlapped

with *OsWRKY71* that is a transcriptional suppressor that encodes GA signaling in aleurone cells and cold-tolerant [28]**)**. *qSSR3-1*, *qSSR3-2* and *qSSR3-3* were co-located with *qLVG3* [29], a QTL for low-temperature vigor of germination. In *qLVG3* region,, there were two characterized cold-tolerance genes (*OsMYB2* and *OsCIPK03*). *OsMYB2* is a MYB transcription factor and plays a regulatory role in tolerance of rice to salt, cold injury and dehydration stress [30], while *OsCIPK03* is a calcineurin B-like protein-interacting protein kinases, the overexpression of *OsCIPK03* transgenic plants significantly improved the tolerance to cold stress [31]. The QTL *qSSR9* explained the largest phenotype variation in our study overlapping with *clr9* that is a QTL associated with culm length growth rate under cold stress [32], however, the knowlwdge of candidate genes underlying *qSSR9* is still gaping.

The abundant SNPs dataset of our nature population through chip strategy makes it feasible to locate *qSSR9* on a small genomic region. The analysis of candidate genes shows that there are 39 candidate genes undelying *qSSR9*, among these candidate genes, five genes (*LOC_Os09g12440*, *LOC_Os09g12470*, *LOC_Os09g12520*, *LOC_Os09g12580* and *LOC_Os09g12720*) might be the target genes, because these five candidated genes share the same branch of the characterized cold tolerance genes *COLD1*, *Ctb1*, *LTG1*, *OsWRKY71* and *OsbZIP73,* respectively **(Fig. 6)**. For theses characterized cold tolerance genes, *COLD1* encodes a G protein signal regulator and it can interact with RGA1, the α subunit of G protein, to sense low temperature, activate Ca^{2+} channel, and enhance the activity of G protein GTP-enzyme to enhance cold tolerance of rice [10]; *Ctb1* encodes F-box protein, which interacts with an E3 ubiquitin ligase subunit SKP1 and is involved in cold tolerance at booting stage [12]; *LTG1* encodes casein kinase and regulates cold response in rice and affects auxin transport, synthesis and signal transduction, and positively regulates low temperature tolerance of rice during vegetative growth period [33]; $OsbZIP73^{Jap}$ is up-regulated by low temperature and the plant hormone abscisic acid (ABA), suggesting that *OsbZIP73* is involved in ABA-dependent low temperature signaling pathways [34]. However, that other genes underlying *qSSR9* cannot be ruled out, such as *LOC_Os09g12360, LOC_Os09g12390, LOC_Os09g12450, LOC_Os09g12615, LOC_Os09g12640* and *LOC_Os09g12650*. Although these genes do not share the same branches with the characterized cold tolerance genes, they are very homologous with some the characterized cold tolerance genes **(Fig. 6)**. Further studies, such as, gene complementation analysis, are necessary to elucidate which allele is more favourable.

Conclusions

In this study, a natural population consisting of 211 rice landraces were used to assess CTBB by using GWAS under a mixed linear model, 12 QTLs were detected on chromosomes 2, 3, 4, 7, and 9, five genes (*LOC_Os09g12440, LOC_Os09g12470, LOC_Os09g12520, LOC_Os09g12580 and LOC_Os09g12720*) might be the target genes for *qSSR9* after candidate gene analysis, these QTLs/genes will be conducive to further mining favorable gene resources and breeding of rice varieties.

Materials And Methods

Plant material

A natural population of 211 rice varieties was used to evaluate the cold tolerance of rice at the bud burst stage **(Table S1)**. These varieties were mainly collected from 15 different provinces in China as well as from Philippines and Japan. The geographical position spans from the north latitude 15° to 48°. These regions including temperate, tropical and subtropical regions. Of the 211 rice varieties, 81 were classified as *japonica* rice , and 130 were classified as *indica* rice. The rice materials were collected in accordance with local laws without any dispute of interest. The population was developed in the experimental field at Jiangxi Agricultural University in Nanchang, Jiangxi Province, and Linwang, Hainan Province for more than two generations.

Cold tolerance evaluation at the bud burst stage

The tested seeds were placed in an oven at 45℃ for 48 hours to break seed dormancy. The seeds were disinfected with sodium hypochlorite solution and rinsed with sterile water for three times. Next, the seeds were soaked in water for about 48 hours and allowed to germinate for 24 hours. 30 seeds, 5mm in coleoptile length, were selected and placed in a petri dish with two sheets of filter paper. The petri dish were placed in a growth incubator at 4℃ , and treated in darkness for 10 days. After cold treatment, the petri dishes containing the seeds were placed in an incubator at 25℃ (14-h light/10-h dark) and recovered for 5 days. the seedlings survival rate (SSR) was calculated after 5 days of recovery of growth and used as the indicator of CTBB. seedlings survival rate (%) = surviving seed/30×100, All experiments were repeated for three times.

GWAS Mapping of Rice CTBB QTLs/genes

Tassel 5.0 software was used for GWAS of rice (CTBB). Principal component analysis (PCA) and kinship analysis were performed using 36,727 SNP genotype dataset. After filtering, standardized phenotype data, SNP data and principal component data were merged, and GWAS was performed with kinship data as co-variables under a mixed linear model. Plotting with R package (R_MVP). QTL regions were identified when 3 or more significant SNPs occurred within 400kb interval. Candidate gene analyses of *qSSR9* were conducted by the RAP-DB (https://rapdb.dna.affrc.go.jp/) and NCBI (https://www.ncbi.nlm.nih.gov/). The DNA sequences of the putative genes and cold tolerance genes were downloaded from NCBI and used in MEGA-X software (https://www.megasoftware.net/).

Statistical and genetic analyses

A correlation analysis of the SSR, latitude and *Indica* or *japonica* type was carried out by SPSS Statistics 26 software.

Genomic DNA extraction, sequencing and genotyping

The CTAB method was used to extract DNA from about 100 mg of fresh young leaves, and the quantity and quality of DNA were measured using a Denovix DS-11 spectrophotometer. In addition, purity was determined by the result of running the DNA for 1 h at 60 V in 1% agarose gel electrophoresis. The 50 K

rice gene SNP microarray 'OsSNPNKs' was used for genotyping. The microarrays were evenly distributed throughout the genome, with an average distance of < 1 Kb between each other. Genotyping based on Affymetrix AXIOM ®2.0. The Target Prep Protocol QRC (P/N 702990) kit manual was used for DNA amplification, DNA fragmentation, microarray hybridization, DNA binding single base extension, and signal amplification. Staining and scanning were performed using a GeneTitan® multi-channel instrument [27].

Abbreviations

Seedling survival rate (SSR), quantitative trait loci (QTL), genome-wide association study (GWAS), Single nucleotide polymorphism (SNP), cold tolerance at the bud burst stage (CTBB), Principal component analysis (PCA), Neighbor-Joining (NJ).

Declarations

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Authors' contributions

C, L is the first author of this article, designed and performed experiments, analyzed data and wrote the manuscript. J. L, J. B participated in analysis data. T. J, B. Z, J. T, X. Z, P. W, G. W, Q. C, Y. W, Q. Z, S. H, M. Y, T. H, S.L, C.L participated in performing experiments. H.H and J.B. conceived and supervised the experiments. All authors reviewed the manuscript.

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Availability of data and materials

The data supporting the conclusions of this article are included within the article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1
SSR (seedling survival rate) of cold tolerance at the bud burst stage (CTBB)

Seed survival rate (%)	The total number	Number of *indica*	Number of *japonica*
$0 \leq X \leq 20$	101	99	2
$20 < X \leq 40$	19	17	2
$40 < X \leq 60$	11	8	3
$60 < X \leq 80$	11	1	10
$80 < X \leq 100$	69	5	64

Table 2
Correlations between SSR, latitude and *indica* or *japonica* type

Traits	SSR	Latitude	*Indica* or *japonica* type
SSR	1	0.714**	0.851**
Latitude		1	0.910**
Indica or *japonica* type			1
**Indicates significance at the 1 % levels			

Table 3
Summary of the significant SNPs detected by GWAS and the overlapped QTLs/genes reported previously

QTL ID	Trait	Chr.	Peak SNPs	P value	Previous QTL/genes	References
qSSR2-1	seedling survival rate	2	3379509	8.99E-06		
qSSR2-2	seedling survival rate	2	4435145	9.70E-07	*OsWRKY71*	Kim et al. (2016)
qSSR3-1	seedling survival rate	3	10230171	3.16E-06	*qLVG3*	Han et al. (2006)
qSSR3-2	seedling survival rate	3	10552889	1.48E-06	*qLVG3*	Han et al. (2006)
qSSR3-3	seedling survival rate	3	11316517	1.64E-06	*qLVG3*	Han et al. (2006)
					OsMYB2	Yang et al. (2012)
					OsCIPK03	Xiang et al. (2007)
qSSR3-4	seedling survival rate	3	13860165	1.72E-08		
qSSR3-5	seedling survival rate	3	15042189	2.19E-08		
qSSR3-6	seedling survival rate	3	29271308	4.89E-05		
qSSR3-7	seedling survival rate	3	33444741	7.77E-06		
qSSR4	seedling survival rate	4	11487556	2.46E-05		
qSSR7	seedling survival rate	7	29111646	1.77E-05		
qSSR9	seedling survival rate	9	7106185	4.07E-09	*clr9*	Oh et al.(2004)

Table 4
Candidate genes in the *qSSR9* region

QTL	Putative genes	Putative protein functions	Reference genes
qSSR9	LOC_Os09g12440	retrotransposon protein, putative, unclassified, expressed	*COLD1*
	LOC_Os09g12470	hypothetical protein	*CTB1*
	LOC_Os09g12520	hypothetical protein	*LTG1*
	LOC_Os09g12580	expressed protein	*OsWRKY71*
	LOC_Os09g12720	zinc finger, C3HC4 type domain containing protein, expressed	*OsbZIP73*

Figures

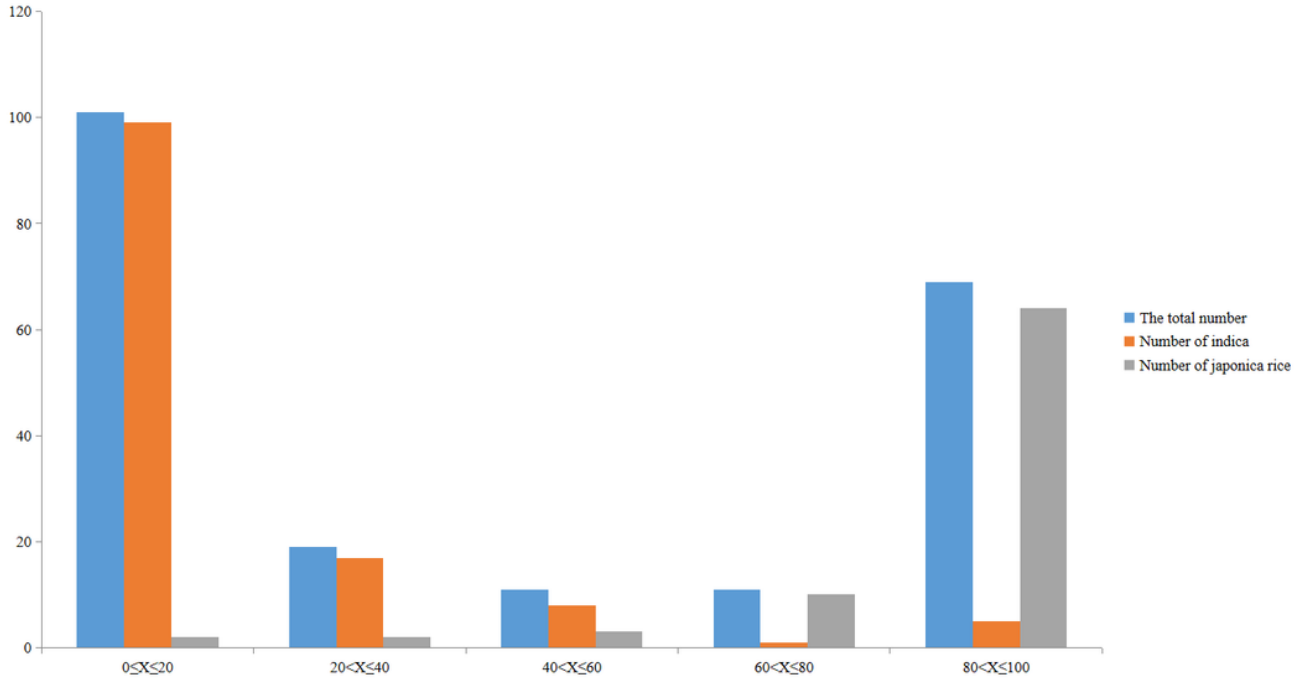

Figure 1

SSR of histogram. The blue area represents the total number of rice, the orange area represents indica rice, and the gray area represents japonica rice, and the horizontal axis represents the cold tolerance level.

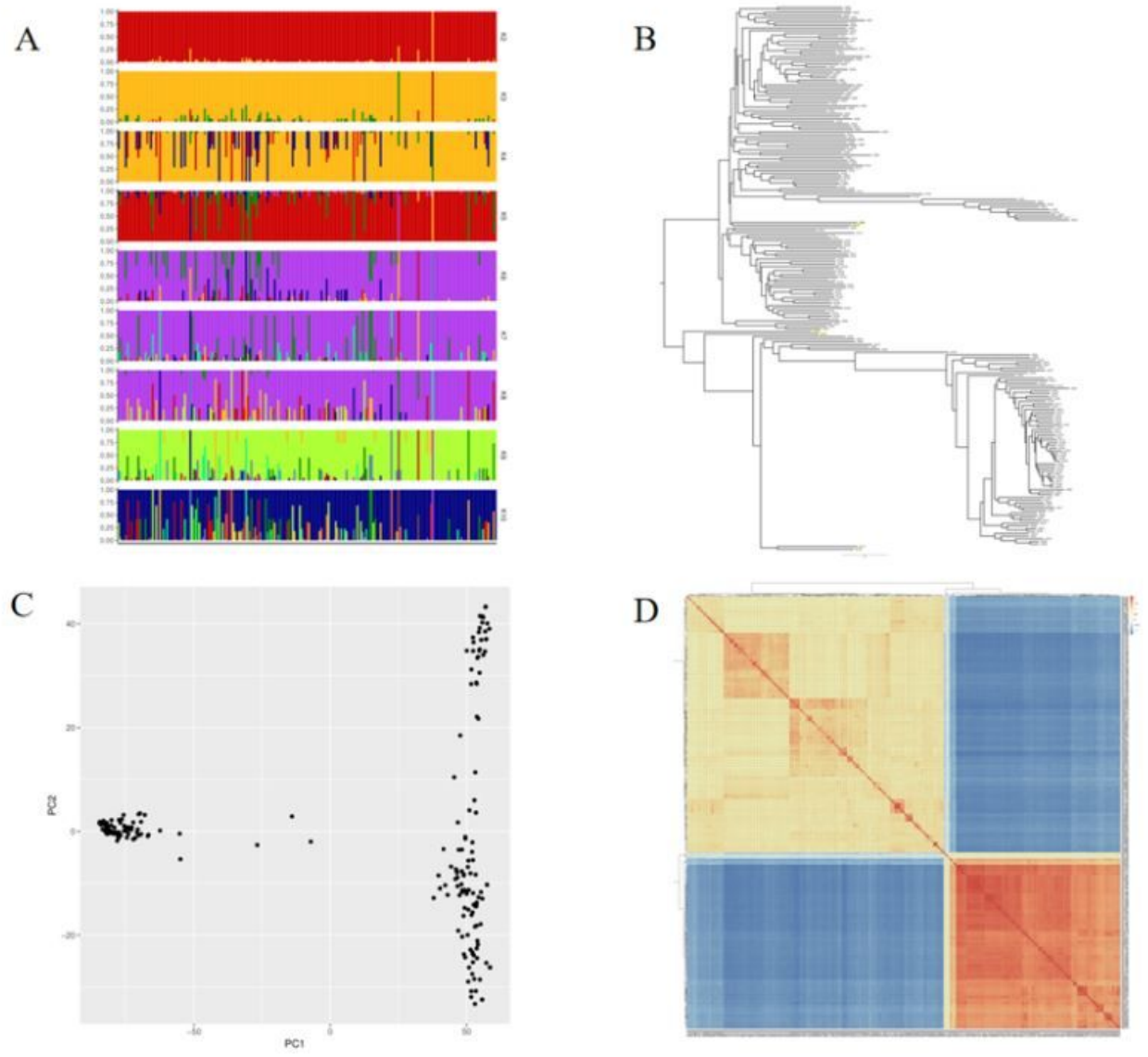

Figure 2

Population structure of 211 landraces A. Subgroups (K=3) inferred using structure software, yellow, red, and green represent subgroup I, II, and III, respectively; B. NJ tree based on Nei's genetic distances; C. PCA of 211 rice varieties; D. Pairwise relative kinship analysis of rice panel.

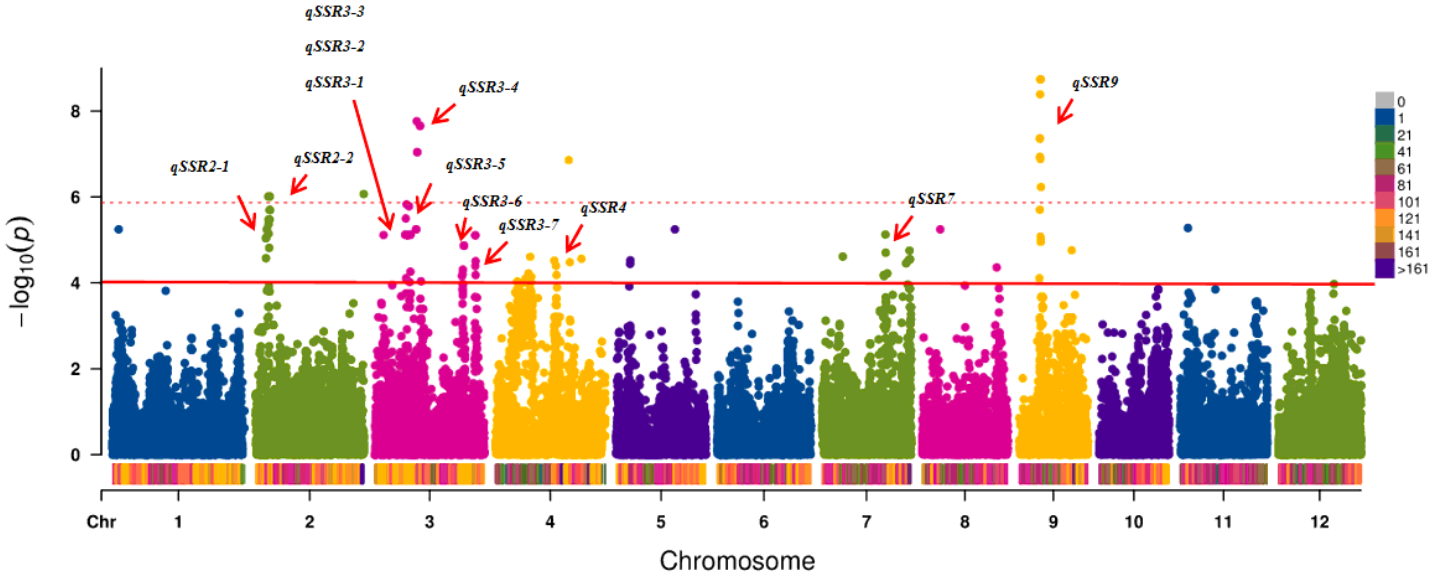

Figure 3

Manhattan plots of GWAS for SSR. The dotted line represents the Bonferoni correction and the solid line represents P =0.0001

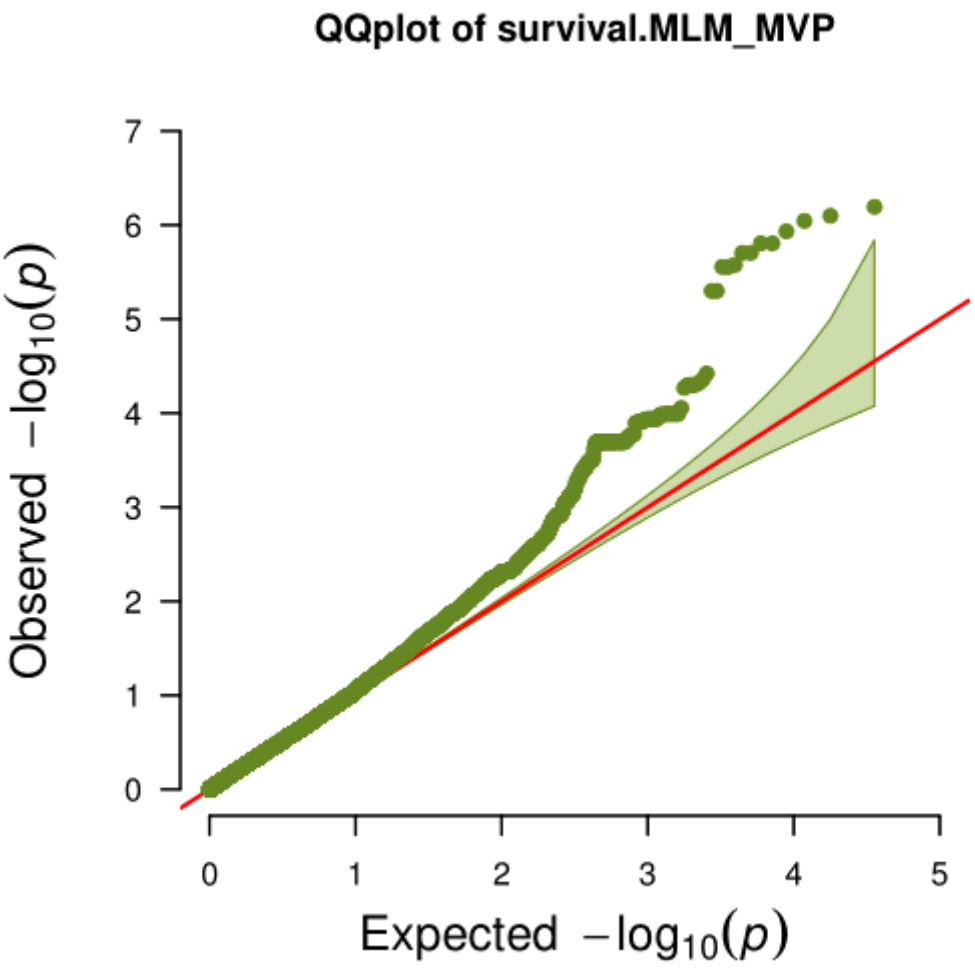

Figure 4

Quantile–quantile (Q–Q) plot of GWAS for SSR

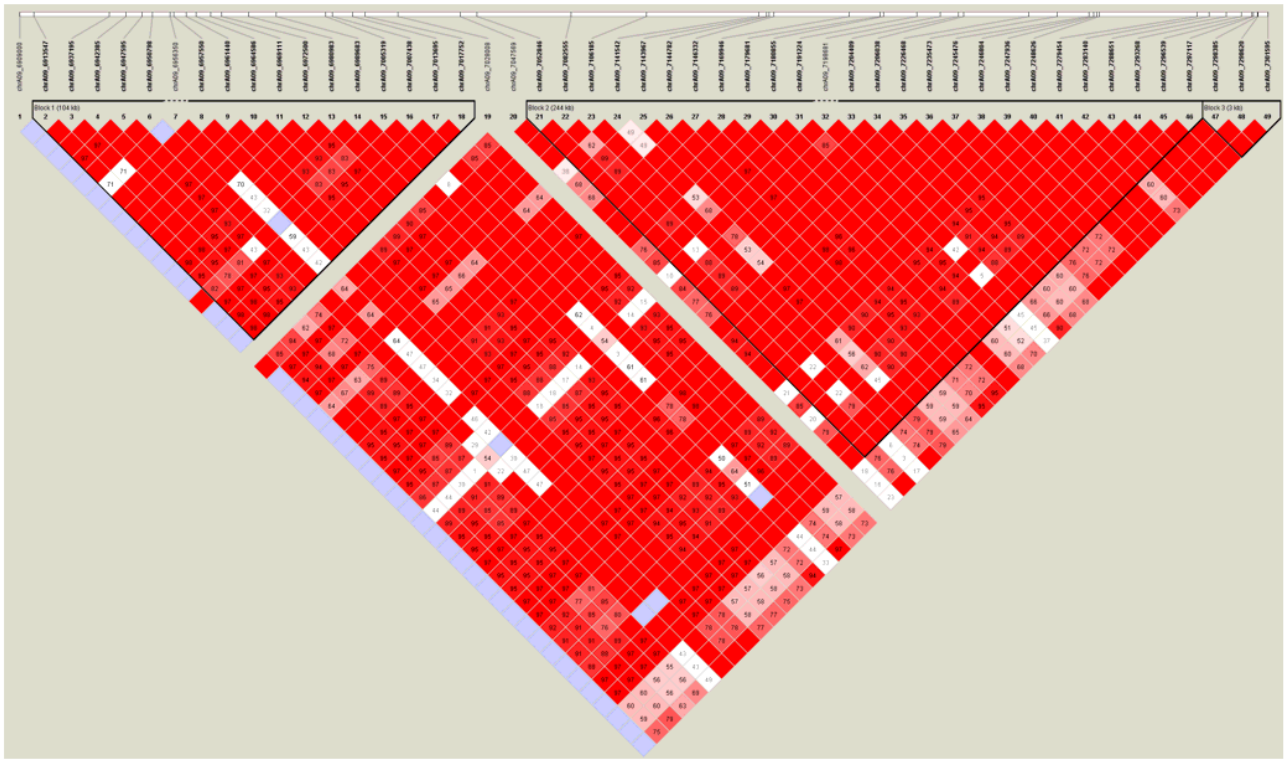

Figure 5

LD heatmap around peak on chromosome 9

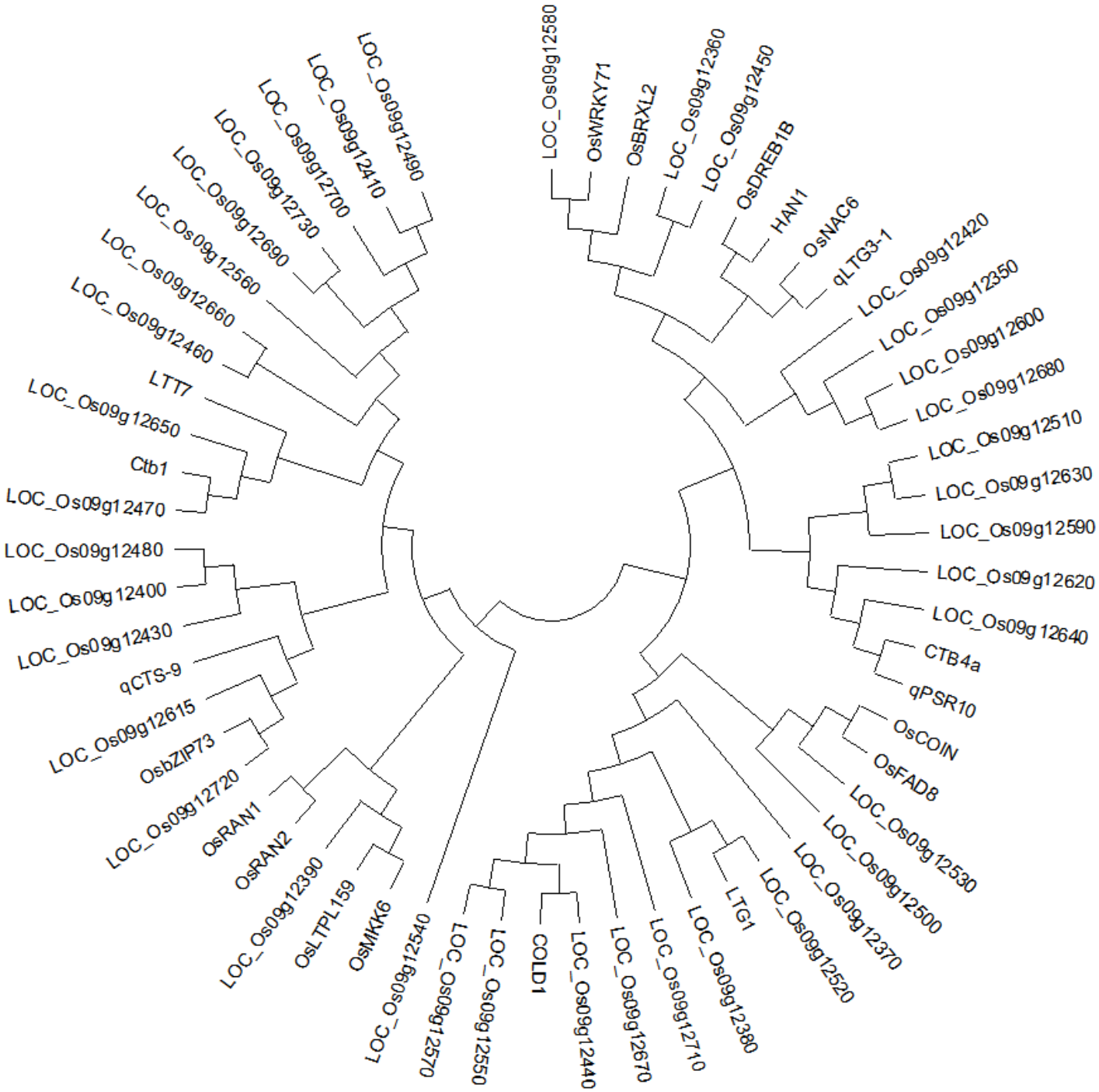

Figure 6

Homology analysis among 59 genes(39 putative genes and 20 reference genes)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xls
- TableS2.xls
- TableS3.xls