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Original scientific paper

LINKAGE DISEQUILIBRIUM ANALYSIS OF RICE SHEATH BLIGHT RESISTANCE MARKERS OF RICE GROWN IN THE COLD REGION OF NORTHEAST CHINA

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A total of 192 native rice germplasm lines from the cold region of Northeast China were analyzed for rice sheath blight resistance at the late tillering stage using the toothpick embedding method. Disease rating, absolute disease lesion height, relative lesion length and lesion length served as indicators of sheath blight resistance. Germplasm resources were then subjected to population structure analysis and linkage disequilibrium analysis in order to identify disease resistance markers for use in marker-assisted breeding for sheath blight resistance. The germplasm resources were segregated into three main groups .Notably, a total of nine markers were significantly associated with rice sheath blight resistance; eight of these markers(OSR23, RM270, RM339, RM470, RM536, RM569,RM1022, RM1163) were associated with disease rating. The additional marker RM283 was associated with both relative lesion length and lesion length and made a high contribution to the rate of phenotypic variation. Ultimately, the sheath blight resistance

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markers described in this study could be useful for rice marker-assisted selection of sheath blight resistant cultivars breeding.

Keywords: Japonica rice (*Oryza sativa* L.), cold region, sheath blight, linkage disequilibrium analysis, marker-assisted selection (MAS).

INTRODUCTION

Rhizoctonia solani, a common fungus present in soil, is the main cause of rice sheath blight, across all rice crops cultivated area (PAN and RUSH,1997). Symptoms of the disease are observed mainly in rice leaf and leaf sheath and also affect the decreased seed setting rate, reduced yield across large cultivation areas and decreased rice grain quality. Rice sheath blight reduced rice yield by 10%~30%, and even in serious disease areas, rice decreased by 50%, and it has become one of the most serious diseases affecting rice growing areas in South China (CHEN *et al*., 2016; JIANG *et al*., 2016; LIU *et al*., 2016). Subsequently, in recent years higher incidence and greater damage by rice sheath blight have been observed even in the cold northeastern region of China, due to several factors which have facilitated the spread of the fungus. Some of these factors include large area cultivation of improved dwarf rice lines and hybrids, increases in use of fertilizer (especially fertilizers that boost nitrogen) and increased planting density (SONG *et al*., 2002; XIN *et al*., 2000; WANG, 2002). Consequently, sheath blight has become one of the major emerging diseases which threaten rice production in this cold region.

At present, the application of chemical fungicides is the main method used to control rice sheath blight in cold regions (HONG *et al*., 2016; ZHANG, 2016). However, perennial use of pesticides has increased *Rhizoctonia* fungicide resistance, thus weakening fungicidal effectiveness while concurrently causing environmental pollution. Therefore, the breeding of sheath blight-resistant rice varieties is being investigated as a fundamental and effective way to control this disease. Marker-assisted breeding is a promising approach that uses DNA markers closely linked to sheath blight resistance traits to accelerate development of resistant rice varieties. This MASB method is dependent upon development of blight resistance identification systems, selection of resistant germplasms, localization of resistance markers and genetic breeding of sheath blight-resistant varieties. While much progress has been made in this area for southern rice varieties grown in warmer climates (XU *et al.*, 2015; ZUO *et al.*, 2014; WANG *et al.*, 2009; YIN *et al.*, 2008, 2009), Whereas little progress has been worked for cold climate rice varieties. Instead, research for cold climate rice varieties has mainly focused on chemical control or monitoring of disease incidence (SUN and LIN, 2009; ZHAO, 2013; LI, 2012). *Japonica* rice planted in Northeast China is known its high quality that has made it popular with consumers. Thus, the establishment of a resistance identification system, selection of resistant germplasm resources, genetic localization and molecular-assisted breeding of cold region rice sheath blight-resistant varieties are urgently needed.

A large number of QTLs for rice sheath blight resistance were mapped all over the word (PAN *et al*., 1998; XIE *et al*., 2008; FU, 2011). Unfortunately, only a few genes have been fine-mapped (CHANNAMALLIKARJUNA *et al*., 2009; ZUO *et al*., 2013), while most blight resistance genes have only been broadly localized (WANG *et al*., 2010; KUNIHIRO YASUFUMIL *et al*., 2002; XIE, 2009). Consequently, the results of QTL mapping have only rarely been useful for molecular assisted breeding of rice strains resistant to sheath blight, due to the large QTL mapping interval.

A more useful approach for mapping resistance genes relies on linkage disequilibrium analysis (LDA). LDA is a method for identifying the relationship between target traits and candidate genes in a population by analyzing the linkage disequilibrium between allelic variants of different loci. This method uses natural populations, which are rich in genetic diversity, and does not require construction of a special population. Moreover, LDA can analyze multiple alleles simultaneously, as well as closely spaced variations at the level of a single gene (MACKAY and POWELL, 2007). Due to these advantages, linkage disequilibrium analysis has been continuously used in genetic mapping of many traits (LAI *et al*., 2013; LIU *et al*., 2013; ZHAO *et al*., 2015; GUO *et al*., 2015). In this study, rice germplasm lines adapted to growth in cold regions were used to map rice sheath blight resistance genes using LDA, in order to provide important technical support and a theoretical foundation for rice resistance variants breeding in northeastern China.

MATERIALS AND METHODS

Materials

Rice germplasms

In this study, 192 cultivars and breeding lines (Table S1) of *japonica* rice with various distinct growth periods from the cold region of Northeast China were selected as experimental materials. The experimental materials were provided and preserved by the Jiamusi Rice Research Institute of Heilongjiang Academy of Agricultural Sciences. All the experimental materials were planted in the disease resistance identification nursery in 2016. This study was randomized block design and repeated 3 times. Each rice gemplasm was planted in 2 rows by using a dry-nursery planting approach, the length of a row was 1 m and the plot dimensions for seedling transplantation were 30 cm × 10 cm

Pathogen of rice sheath blight in cold regions

Diseased plants were collected in the field of the Jiamusi Rice Research Institute of Heilongjiang Academy of Agricultural Sciences in 2014. The isolation and cultivation of the pathogen was conducted according to the method developed by Shen Yongan (SHEN *et al*., 1995).

Simple sequence repeats (SSR) markers of rice

809 pairs of SSR markers distributed among all 12 chromosomes of the rice genome were used to identify polymorphisms. Primers were download from http://archive.gramene.org/markers/ and synthesized by Heilongjiang Bokuai Laboratory Equipment Co.

Methods

Identification of rice strains resistant to rice sheath blight in cold regions

Experimental rice plants were inoculated at the late tillering stage using the toothpick embedding method developed by Pan Xuebiao (PAN *et al*., 1997). Toothpicks were each cut to a length of 0.8-1.0 cm, sterilized, then laid on the bottom of a sterile culture dish. Sterile PDA medium (autoclaved at 121°C for 15 min) was poured into the culture dish so that the medium depth covering the toothpicks was 0.5-1.0 cm. A rice sheath blight fungal strain was used to inoculate culture dishes containing the toothpicks and the dishes were cultured at 25°C for 3-5 d. Toothpicks were used to inoculate rice plants when fungal growth was observed in the culture dish.

Three plants were inoculated for each germplasm tested and three stems of each rice plexus were inoculated. Inoculation involved insertion of fungus-infested toothpicks into the third sheath using tweezers. Disease rating, relative lesion length, lesion length, lesion height, absolute lesion height and plant height were determined 36 days after heading. The methods of inoculation and identification were based on the method of PAN XUEBIAO (2016) with modifications according to the characteristics of rice sheath blight in cold regions, as described in the report of LI XIUPING (2017).

The specific method of investigation was as follows: disease rating was determined using a nine-level rating system as reported by RUS (ZUO *et al.*, 2006) ; lesion length is the distance between the upper and lower lesion boundaries and defines the length of a single lesion; the lesion length of each plant was the sum of all lesion lengths for an entire plant (cm); lesion height was the distance from the upper boundary of a lesion to the soil surface (cm); plant height was the distance from spike top to soil surface (cm); relative lesion length was calculated as (lesion length / plant height) × 100%; absolute lesion height was (lesion height / plant height) × 100%.

SSR analysis of rice sheath blight

Rice genomic DNA extraction

Genomic DNA was prepared from tissue samples of rice germplasm lines in cold regions by extraction with SDS. Final DNA concentration was detected using 1% agarose gel electrophoresis (LI *et al.*, 2008).

PCR amplification and PAGE gel electrophoresis detection

The PCR amplification reaction system and cycles were as follows:

PCR reaction（20μL）		PCR cycles	
Template DNA（50 ng/μL)	3μL	Pre-denaturation at 95 ℃ for 5 min	
10×PCR buffer	2μL	Denaturation at 94 ℃ for 30 s	
dNTP (I0mM)	0.3μL	Annealing at 55 ℃ for 30 s,	
Tag DNA Polymerase（5 U/μL)	0.2μL	Enlongation at 72 ℃ for 30 s	35 cycles
$MgCl_2$	1.5μL	Enlongation at 72 ℃ for 5min	
SSR Primers	3μL	Hold at 4℃	
ddH_2O	9.9μL		

PCR products were analyzed using polyacrylamide gel electrophoresis (PAGE) according to the report of LI XIUPING (2008).

Linkage disequilibrium analysis of rice sheath blight

Molecular marker data preprocessing

The preprocessing of polymorphic marker data was conducted using the quality control function of Plink version 1.07 as follows: allele loci were eliminated if their minor allele frequency was less than 1%, if they contained deletions greater than 5% of total experimental

materials, if their allelic frequencies deviated greatly from Hardy-Weinberg equilibrium ($p<1\times10^{-6}$), if they exhibited greater than 10% genotype deletion, if they exhibited heterozygous genotype or if they exhibited unknown genotype (WEN *et al.*, 2008).

LD measurement

D' (standard imbalance coefficient) was used to measure the LD between the alleles.

$$D' = \sum_{i=1}^{u} \sum_{j=1}^{v} p_i q_j \left| D'_{ij} \right| ,$$

In the formula, u and v represent the number of allelic variations at two loci, p_i represents the allelic variation i of the A locus and Pj represents the frequency of allelic variation j of the B locus (WEN *et al.*, 2008).

Structure analysis of the mapping population of rice sheath blight

Structure V2.3.1 was used for classification of the mapping population of rice sheath blight using a mathematical model and calculation of the corresponding Q value of each material (the probability of genomic variation in material i derived from group k). The specific method used for structure analysis was derived from methods according to the report of WEN (2008). The number of groups was set to 2-10 and the loci were assumed to be independent. A suitable K value was selected based on the principle of maximum likelihood.

Linkage disequilibrium analysis of rice sheath blight

The Mixed Linear Model program (MLM) of TASSEL was used for group corrections and for regression analysis of phenotypic data and marker variability. The specific method is outlined in the report of Wen (WEN *et al*., 2008). Population structure and Kinship coefficient matrix data were used as covariates to analyze the correlation between molecular markers and target traits. Evaluation of the strength and significance of their associations was performed using P-maker.

RESULTS

Germplasm population structure analysis of rice grown in a cold region in China

According to the results of SSR classification and pretreatment, 164 pairs of polymorphic markers were selected from 890 pairs of SSR markers from *japonica* rice germplasm resources for cold regions. Within these 164 markers, 625 alleles were detected, the variation range was 2-10 and the polymorphism information content (PIC) value of the average polymorphism was 0.59. The variation range of the minimum allele frequency was 1.04%~46.9% and the average variation was 9.82%. The distribution of molecular markers among rice chromosomes is shown in Figure 1. Most markers were distributed on chromosomes 1, 2, 3, 5, 6, 7, 8, 11 and 12, while few polymorphic markers mapped to chromosomes 4, 9 and 10.

Using 164 SSR markers distributed among the 12 chromosomes of rice, the population structures of 192 rice germplasm were analyzed using Structure V2.3.1 (Figure 2). The 192 germplasm lines segregated into 3 groups. The first group was further divided into 2 subgroups. The first subgroup included 64 rice germplasm, most of which were grown in the third temperature zone of Heilongjiang province and included varieties bred by Jiamusi Rice Institute, Heilongjiang Academy of Agricultural Sciences, as well as Kongyu 131, Kongyu 133 and other varieties sharing

consanguinity. The second subgroup included 86 rice germplasm, most of which were grown in the second temperature zone of Heilongjiang province. The varieties bred by Heilongjiang Academy of Agricultural Sciences, varieties bred by the Suihua Institute and varieties introduced from Japan were assigned to this subgroup. The second group included 26 germplasm, most of which were grown in the first temperature zone of Heilongjiang province. The varieties bred by researchers from Northeast Agricultural University and the Wuchang Rice Institute, including 'Tetep' and susceptible control variety 'Lemont', were also in this group. The third group included 16 other germplasm.

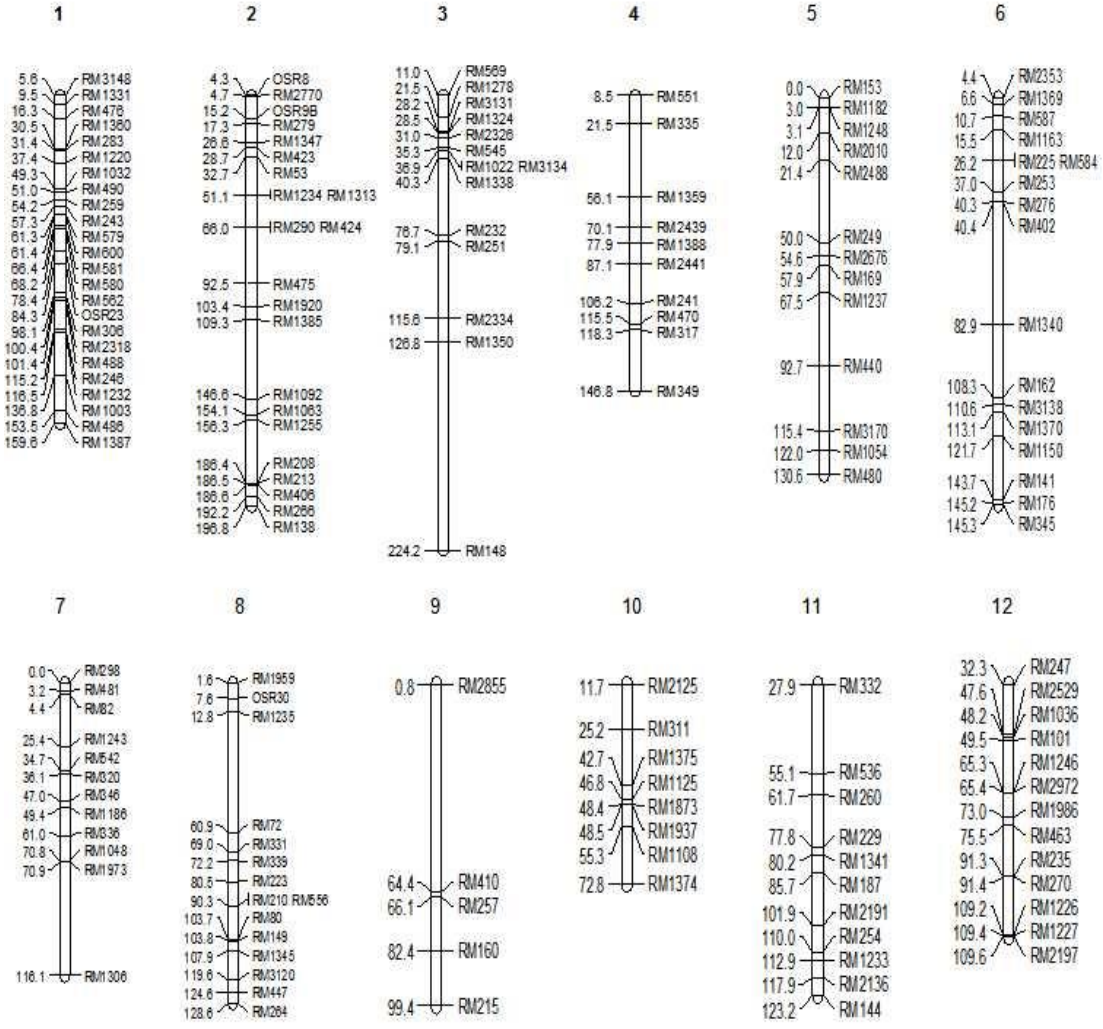

Fig. 1 The genetic map established by 164 pairs of polymorphic SSR marker

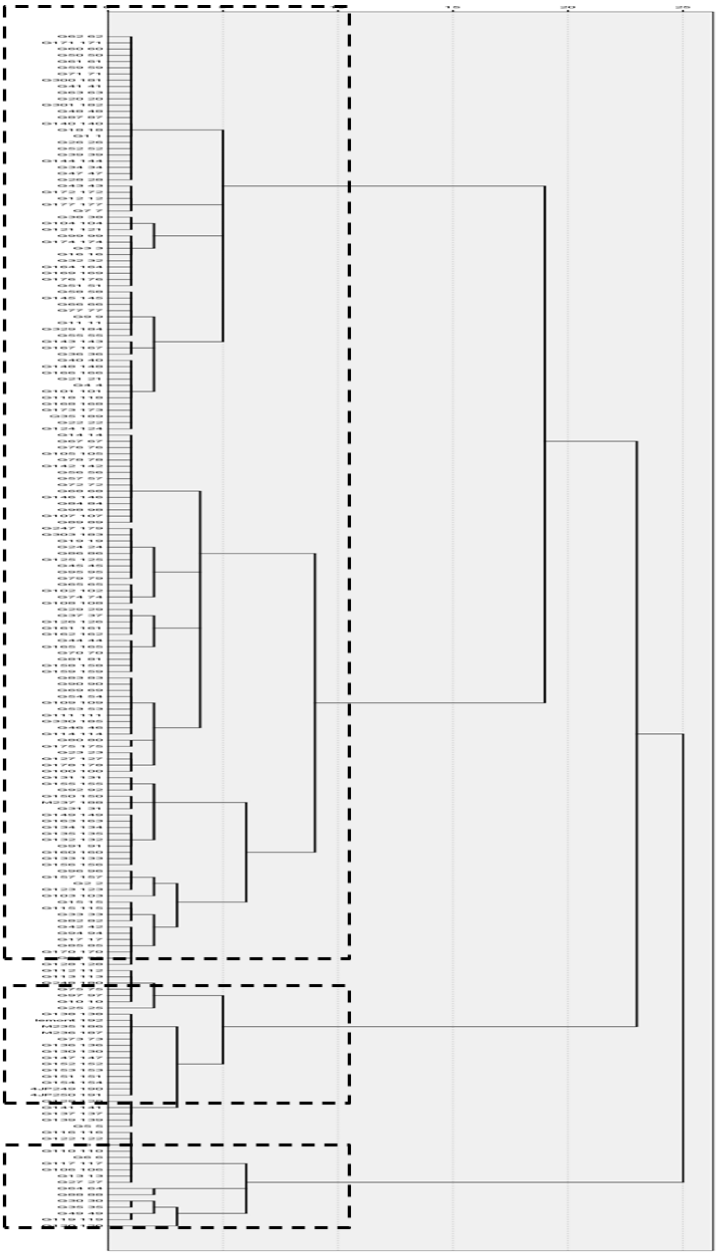

Fig.2 Cluster analysis of rice germplasm of sheath blight in cold region

Linkage disequilibrium analysis and association of SSRs with blight resistance of rice grown in a cold region

LD (linkage disequilibrium) is the basis of linkage disequilibrium association analysis. Studies of the linkage disequilibrium between SSR loci in the rice genome have been straight forward and progress has been made to delineate the linkage disequilibrium of the rice genome. Linkage disequilibrium between SSR loci in the rice genome is shown for 164 SSR markers located on the 12 rice chromosomes (Figure 3). The results demonstrate a certain degree of linkage disequilibrium for both collinearly arranged marker combinations (on the same chromosome, linked) and non-collinearly arranged marker combinations (different chromosomes, non-linked), which are represented by colored small squares above the black diagonal. Unbalanced alignments are represented by colored pixels below the diagonal line (the statistical probability of D', $p<0.001$).

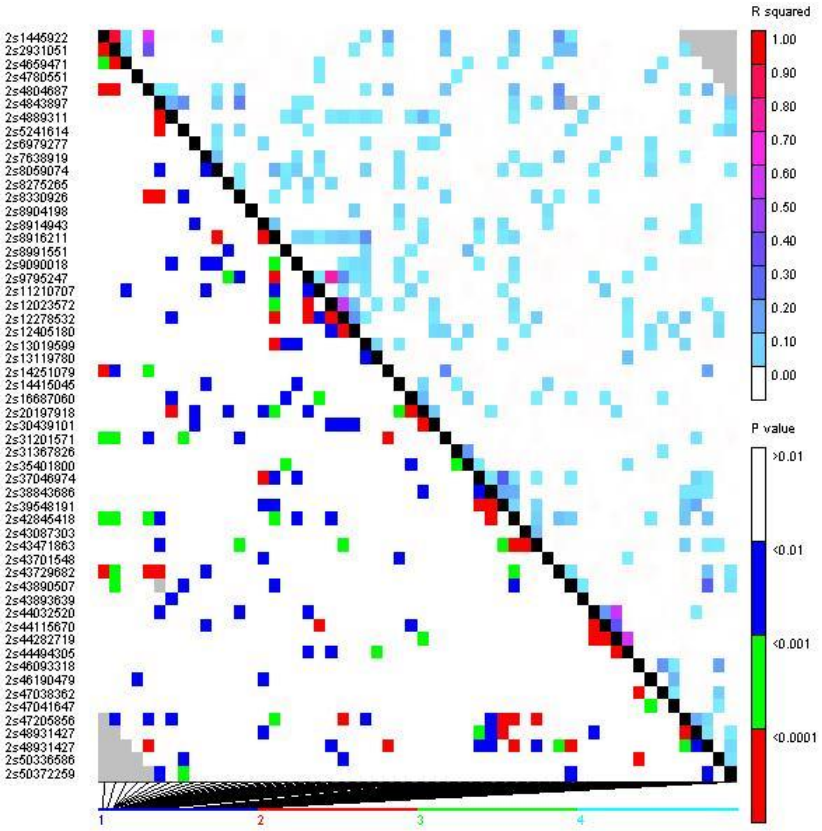

Fig.3 Linkage disequilibrium distribution of 164 SSR markers. The colored pixels above the black diagonal represent the D' values of paired points. The colored pixels below the diagonal represent the P values of the LD of paired points.

The Q-Q diagram (Figure 4) showing results of the genome-wide association analysis of four phenotypic traits (disease rating, absolute lesion height, relative lesion length and lesion length) was

obtained by TASSEE 5.0 and shows that the structure of the associated population was controlled well. Based on population structure analysis, regression analysis of the rice sheath blight resistance index for each SSR marker locus was conducted using the Q value of each individual as covariant (Table 1, Figure 5).

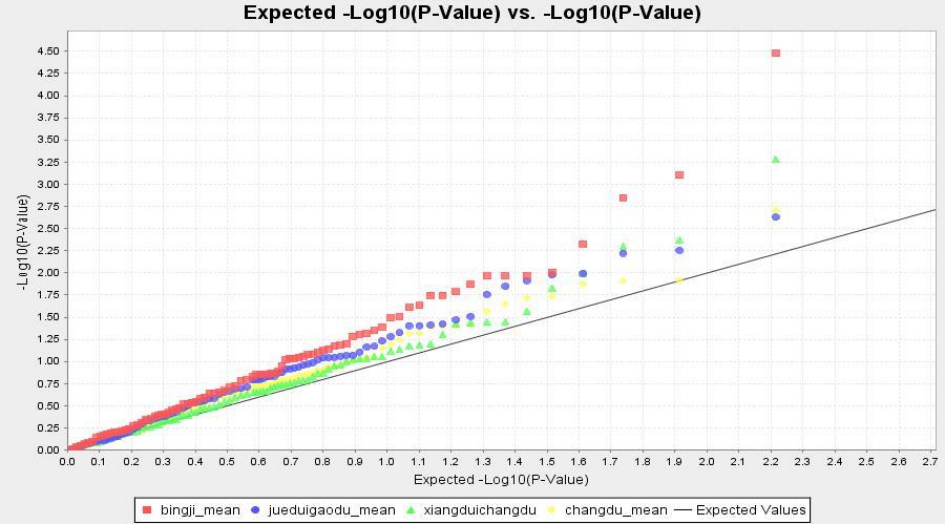

Fig.4 Quantile-Quantile plot for 4 different traits

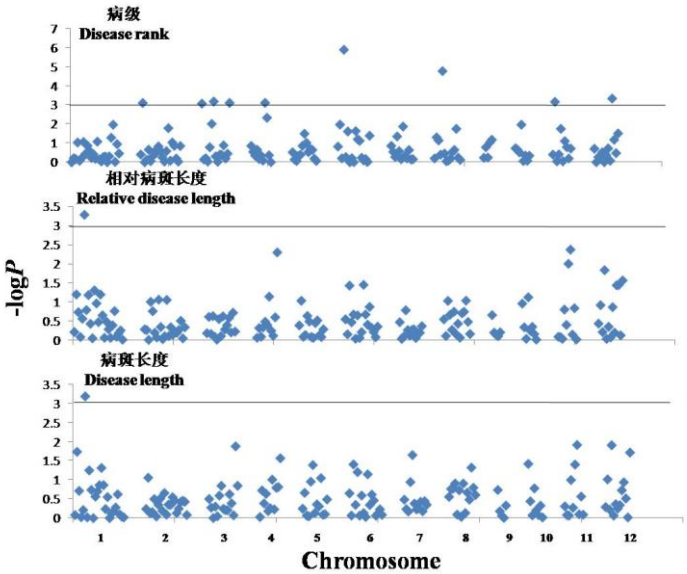

Fig.5 Genome-wide association study of rice sheath blight in cold region with mixed linear model

Table1 Genome-wide association study of rice sheath blight in cold region. R^2 represented the rate of each SSR local explained the variance, that was contribution rate.

Trait	SSR markers	Chr.	Genetic distance（cM）	*p* value	R^2（%）	Allele	Phenotypic effect value
Disease rating	OSR23	1	84.3	7.87E-04	6.1099	120	1.2800
						123	0.0000
Disease rating	RM270	12	93.1	4.69E-04	11.1019	184	-0.1357
						178	-0.0743
						173	-1.4452
Disease rating	RM339	8	72.2	1.72E-05	12.2214	168	-0.0490
						512	-1.0681
						212	-0.6134
Disease rating	RM470	4	115.5	7.78E-04	10.4545	151	1.2886
						163	1.1808
						142	1.1666
Disease rating	RM536	11	55.1	6.92E-04	9.3401	323	0.0689
						294	-0.0811
						278	-0.6476
Disease rating	RM569	3	11	8.57E-04	10.3311	174	-0.1711
						171	-0.0923
						177	-0.1957
Disease rating	RM1022	3	36.9	6.68E-04	10.6489	123	-0.1621
						110	0.1400
						154	-0.1258
Disease rating	RM1163	6	15.5	1.32E-06	18.7350	200	-0.0440
						199	-0.1955
						175	-2.1818
Relative disease length	RM283	1	31.4	8.12E-04	7.6979	123	-0.0523
						151	-0.1373
Disease length	RM283	1	31.4	6.59E-04	7.6044	123	-5.0204
						151	-10.9268

The results show that nine SSR markers were associated with resistance to rice sheath blight, in which eight SSR markers are associated with disease rating (Table 1). These eight SSR markers, OSR23, RM270, RM339, RM470, RM536, RM569, RM1022 and RM1163, are located on chromosome 1, 12, 8, 4, 11, 3, 3 and 6, respectively. The contribution rates of these eight SSR markers to disease rating variations were 6.1099%, 11.1019%, 12.2214%, 10.4545%, 9.3401%, 10.3311%, 10.6489% and 18.7350%, respectively. These eight SSR markers accounted for 88.9428% of the variation in disease rating, with the largest contribution rate observed for RM1163. RM283 was associated with both relative lesion length and lesion length and was located on chromosome 1. The contribution rates of RM283 to relative lesion length and lesion length were 7.6979% and 7.6044%, respectively.

The allelic effects of different loci within the same marker varied and could be divided into additive effects and subtractive effects. Allelic variants of loci within three such markers were identified as OSR23 (120 bp), RM470 (151 bp, 163 bp, 142 bp) and RM536 (323 bp), which were all shown to be associated with disease rating, with allelic effects of 1.2800, 1.2886, 1.1808, 1.1666 and 0.0689, respectively. The allelic effect values of RM1163 (175 bp), RM270 (173 bp) and RM339 (512 bp) were -2.1818, -1.4452 and -1.068, respectively, indicating that these three loci were more useful than other loci. Meanwhile, RM283 exhibited blight resistance allelic effects for both relative lesion length and lesion length. The allelic effects of two loci within this marker on these two traits were -0.0523 (123 bp), -0.1373 (151 bp) and -5.0204 (123 bp), -10.9268 (151 bp), respectively. The variations of these SSR loci were also more useful than other SSR loci.

DISCUSSION

With recent rapid increases in rice cultivation in cold regions in Northeast of China, the occurrence of rice sheath blight has become increasingly more frequent, with increasingly serious rice yield losses now approaching 40%-60% (LI and MU, 2015). Because the rice sheath blight host range is broad and disease-resistant germplasm resources for *japonica* rice are rare, progress in breeding of resistant japonica rice has progressed slowly. To counter the emergence of this sheath blight in *japonica* rice grown in cold regions, the resistance identification method, which is suitable for cold regions in China for *japonica* rice with good resistant germplasm, is very important. The markers which were closely linked with rice sheath blight could accelerate the molecular marker-assisted breeding of resistant varieties. Ultimately, breeding of resistant varieties of *japonica* rice that grow in cold regions of China would be an economical and effective method to control the spread of rice sheath blight before it gains a foothold in cold regions.

Correlation analysis based on linkage disequilibrium analysis results was used here to identify the association of target traits with genetic markers or candidate genes. This method has been used in past research to study rice quality, resistance to cold, rice yield and seed characteristics (ZHANG *et al*., 2014; XU, 2013; YANG, 2013; CUI, 2011; LU, 2014; HE, 2014; JIN, 2009). As an example, for analysis of resistance to rice sheath blight, Sun *et al.* identified 13 SSR loci associated with resistance after performing correlation analysis of 456 rice germplasm with 144 SSR markers distributed across the rice genome. The phenotypic variation rates of these 13 SSR loci were in the range of 1.84%-8.42% (SUN *et al.*, 2014).

In this study of a natural population comprised of 192 rice germplasm, nine SSR markers were identified that were associated with resistance to rice sheath blight after correlation analysis of 164 SSR markers distributed among all 12 rice chromosomes. The allelic effects of different loci of a given SSR marker varied and eight SSR markers were associated with disease rating. The allelic effects of RM1163 (175 bp), RM270 (173 bp) and RM339 (512 bp) were -2.1818, -1.4452 and -1.0681, respectively. The contribution rates of these three loci to observed disease rating were 18.7350%, 11.1019% and 12.2214%, respectively. Moreover, these three SSR markers were more useful for rice marker-assisted selection of sheath blight resistant cultivars breeding. In addition, RM283 was identified as associated with both relative lesion length and lesion length, both of which also be useful for MAS. Therefore, these SSR markers, which were closely linked with sheath blight resistance, hold promise for use in molecular-assisted breeding to create varieties of resistant rice in cold regions in China and provide a technical foundation for subsequent genetic research studies of *japonica* rice.

By combining the results of identification of resistant germplasm resources with variation characteristics of four SSR markers for japonica rice linked with sheath blight resistance, RM1163 (175 bp), RM270 (173 bp), RM339 (512 bp) and RM283 (123 bp, 151 bp), resistant germplasm could ultimately be selected. The selected germplasm could then serve as excellent parental breeding stock to create sheath blight-resistant rice. Moreover, these four markers should be useful for marker-assisted selection of progeny populations and thus help to accelerate progress in creating sheath blight-resistant japonica rice varieties adapted to cold regions in Northeast China.

CONCLUSIONS

The natural population of 192 rice germplasm lines isolated from a cold region of China was used as experimental material for this study. After inoculation of rice plants with fungus at the late tillering stage using the toothpick embedding method, disease rating and resistance to sheath blight were evaluated using measurements of absolute lesion height, relative lesion length and lesion length. Association analysis of 164 pairs of SSR primers distributed throughout the rice genome with sheath blight resistance of rice grown in cold regions was conducted using linkage disequilibrium analysis. Using population structure analysis, the original 192 experimental lines were segregated into 3 groups. Subsequently, nine SSR markers were identified that were associated with rice sheath blight resistance by linkage disequilibrium analysis; eight of these markers were associated with disease rating. RM283 was associated with both relative lesion length and lesion length. The contribution rates of allelic variation of RM1163 (175 bp), RM270 (173 bp), RM339 (512 bp) and RM283 (123 bp, 151 bp) to disease resistance were high, which indicated that these markers could be useful for molecular-assisted breeding of sheath blight-resistant rice in cold regions of China.

Additional files

Addtional file 1: Table S1. Rice germplasms

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LINKAGE DISEQUILIBRIUM ANALIZA MARKERA ZA OTPORNOST NA OBEZBOJAVANJE LISNOG RUKAVCA KOD PIRINČA GAJENOG U HLADNOM REGIONU SEVEROISTOČNE KINE

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Izvod

Ukupno 192 autohtone linije pirinča iz hladnog regiona severoistočne Kine analizirane su za otpornost na obezbojavanje lisnog rukavca u završnoj fazi klasanja korišćenjem metode *toothpick embedding*. Kao indikatori za ocenu bolesti korišćeni su apsolutna dužina lezije, relativna dužina lezije i dužina lezije. Linije su potom bile podvrgnute analizi strukture populacije i *linkage disequilibrium*-a; linije su podeljene u tri glavne grupe kako bi se identifikovali markeri otpornosti na bolest za upotrebu u oplemenjivanju pomoću markera. Utvrđeno je da je ukupno devet markera značajno povezano sa otpornošću kod pirinča; osam markera OSR23, RM270, RM339, RM470, RM536, RM569, RM1022, RM1163) je bilo povezano sa ocenom bolesti. Dodatno, marker RM283, bio je povezan i sa relativnom dužinom i sa dužinom lezije, i značajno je doprineo oceni fenotipske varijabilnosti. Markeri za otpornost opisani u ovom radu, mogu biti korisni za selekciju pomoću markera na otpornost kod pirinča.

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