Vol. 14(25), pp. 1058-1072, 20 June, 2019
DOI: 10.5897/AJAR2018.13826
Article Number: A2E705361241
ISSN: 1991-637X

http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

Association mapping for flag leaf thickness in an *indica* rice population from South China

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Received 19 December, 2018; Accepted 4 March, 2019

Flag leaf is the most important source of photosynthate for developing rice grains, and flag leaf thickness is an important morphological trait in rice plant-type breeding programs. In the present study, we carried out association mapping for flag leaf thickness in a local rice population which consisted of 86 cultivars derived from breeding programs and planted in large areas in South China. Phenotyping was conducted in the field using nondestructive leaf thickness measurements. Two hundred and thirty-six SSR markers covering 12 chromosomes were employed to genotype the accessions. The association analysis was carried out using a unified mixed-model approach. The Q+K model was selected for investigating marker-trait associations. A total of eleven marker-trait pairs with significant marker-trait associations were identified which were distributed on eight chromosomes. Four of these loci had already been identified as related to flag leaf thickness in previous studies, while the other seven were novel QTLs. The locus PSM163 had the highest r^2-marker value of the seven novel loci, explaining 21.54 and 18.49% of the phenotypic variation in 2008 and 2009, respectively. Three of four QTLs, which were detected in a F_2 mapping population in the validation study, could correspond to a significant locus in AM, respectively. The six alleles which had the highest phenotypic values at their respective loci should be considered as favored alleles in breeding programs. Pyramiding the favored alleles for flag leaf thickness identified in the study might be a valuable approach to construct an ideal plant architecture in rice breeding.

Key words: Rice, flag leaf thickness, breeding programs, association mapping, mixed linear model.

INTRODUCTION

Rice is a staple food for about 50% of the global population. With the ongoing reduction in arable landcaused by urbanization and industrialization, breeding rice varieties with greater yield potential will be a very important component of meeting the increased food demand of a growing global population. Genetic improvement of plant morphology is the backbone of increasing rice yielding potential (Khush, 1995; Yuan, 1997). Flag leaf is the most important source of photosynthate for developing rice grains. Over 50% of the carbohydrate accumulated in rice grains is produced by flag leaves (Gladun and Karpov, 1993). Flag leaf

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morphology is a major determinant of plant architecture, canopy photosynthesis and grain yield potential in rice (Jin et al., 2018). Rice leaf thickness is significantly negatively correlated with Ls (stomatal limitation under high light) and Ls_{low} (stomatal limitation under low light) but significantly positively correlated with Fv/Fm (maximum PSII efficiency), showing that thick leaves are beneficial in increasing the photosynthetic rate and carbohydrate assimilation (Qu et al., 2017). Thick leaves are favorable for improving P_n and canopy photosynthesis in rice, so leaf thickness has been regarded as one of the major indices in rice cultivation and plant-type breeding programs (Peng, 2000; Zhu et al., 2016). In recent decades, several plant-type models proposed by cultivators and breeders have employed thick flag leaves as a main selection index in rice cultivation and breeding programs. Matsushima (1976) suggested that the flag leaf should be “short, thick, erect” for the rice ideotype. Similarly, thick flag leaves were proposed in other ideotype models in rice breeding (Yang et al., 1984; Kush, 1995; Yuan, 1997).

The relationship between leaf thickness and grain yield and yield traits in *indica* rice had been analyzed by Chen et al. (2011) and Liu et al. (2014). The studies revealed a tight correlation between leaf thickness and panicle traits. In the studies, the thickness of the top three leaves had a significant negative correlation with leaf angle and the number of panicles per plant and a significant positive correlation with leaf length, panicle length, number of primary branches, number of secondary branches, filled grains per panicle, grain density, grain weight per panicle, and number of spikelets per panicle. There was little correlation with seed setting rate, 1000-grain weight, and harvest index. Thicker leaves were not just favourable to larger panicles and higher grains weight per panicle, but also to the construction of an ideal plant architecture in rice (Liu et al., 2014). The results also showed that flag leaf thickness is closely related to the thickness of other leaves on the same stem, suggesting that rice leaf thickness traits share a single genetic system controlled by multiple genes or quantitative trait loci (QTL) (Liu et al., 2014).

In recent years, many genes or QTLs related to the flag leaf morphological traits in rice, such as flag leaf length (Jiang et al., 2010; Shen et al., 2012), flag leaf width (Fujino et al., 2008; Qi et al., 2008), flag leaf area (Wang et al., 2011), flag leaf angle (Sakamoto et al., 2006), and rolling leaf (Zhou et al., 2010), have been cloned or fine-mapped. However, so far no QTLs for flag leaf thickness have been identified based on phenotypic data directly measured in the field, although a few QTLs for SLW or SLA of flag leaf had been identified in rice (Laza et al., 2006; Kanbe et al., 2008; Zhao et al., 2008; Takai et al., 2010).

Association mapping (AM) is a high-resolution method for the identification of QTLs for complex genetic traits in plants (Mackay and Powell, 2007). It has at least three benefits compared with traditional linkage analysis: consuming far less time, a higher mapping resolution, and a greater allele number (Breseghello and Sorrells, 2006a; Zhu et al., 2008). Association mapping has recently been successfully used to identify marker-trait associations in various plant species, such as maize (Pace et al., 2015), *Arabidopsis thaliana* (Davila Olivas et al., 2017), barley (Kraakman et al., 2006; Wang et al., 2017), wheat (Sabiel et al., 2017), and soybean (Che et al., 2017). In rice, Agrama et al. (2007) used a mixed linear model method to detect marker-trait associations for yield and its components in 103 accessions genotyped using 123 SSR markers. Twenty-five associations were identified. Zhao et al. (2013) genotyped 130 rice accessions using 170 SSR markers to identify marker–trait associations for physicochemical traits affecting eating quality. In total, 101 marker–trait associations (p<0.05) were identified with 52 different SSR markers covering 12 chromosomes. Fujino et al. (2015) used 115 SSRs for genotyping in an association analysis of 63 cultivars derived from rice breeding programs in Hokkaido, Japan. Six QTLs were identified for heading date and seventeen for low temperature germinability. Dong et al. (2018) newly found four loci associated with flag leaf inclination in rice by association mapping. Today, association analysis has become a powerful method of gene digging for complex traits in rice (Zhai et al., 2018; Huang and Han, 2018).

South China is one of the major rice-producing and consuming regions in China, which expands the area between the latitude of 18°43ǫ and 26°24ǫ N and the longitude of 104°26ǫ and 117°19ǫ E including Guangdong, Guangxi, and Hainan provinces (Figure 1). In this region, the tropical and subtropical monsoon climate is typical, and the temperature and precipitation resources are rich (more than 300 d with the daily mean temperature of 10°C and annual rainfall of 1400–2000 mm). Indica rice is traditionally planted in a double-cropping system in South China, and the rice area accounted for 15.1% of the total national rice acreage (Liu and Zhang, 2010).

In the present study, an association analysis was carried out between flag leaf thickness and SSR markers employing a set of elite rice cultivars derived from breeding programs in South China during the past 60 years. The objective of this study was to identify major QTL(s) associated with flag leaf thickness which could help us to detect the genetic mechanism of flag leaf thickness in rice and be used in rice molecular breeding to construct an ideal plant type.

MATERIALS AND METHODS

Plant material and phenotyping

Eighty-six semidwarf *indica* rice cultivars were used as the association panel in this study, which included 80 cultivars

Figure 1. Map of the area of South China in which double-cropping rice are cultivated. *The marked black area in the map is the area of South China.

developed in South China from 1949 to 2006, four cultivars introduced from IRRI, and two landraces which were used as core parents in rice breeding programs in South China (Table 1). Seeds of the cultivars were preserved in the rice germplasm repository of the Rice Research Institute, Guangdong Academy of Agricultural Sciences.

Field experiments were carried out at the test station of the Rice Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, in the late-cropping seasons in 2008-2009. The field trials design and field management were conducted according to the methods described by Liu et al. (2014). Five to seven days after full heading, 10 plants with uniform growth were sampled from each plot to determine the thickness of the flag leaf blade on the main stem. The measurement the protocol as described by Liu et al. (2014) was used for measuring the thickness of the flag leaf. To avoid interference from leaf water status, the field was kept flooded while measuring of flag leaf thickness.

SSR genotyping

Two hundred and thirty-six polymorphic SSR markers were employed to genotype the cultivars. The average distance between loci was about 6.4 cM. The number of markers on chromosomes 1 to 12 was 27, 24, 25, 21, 16, 19, 18, 21, 16, 14, 18 and 17, respectively (Figure 2). One hundred and fifty-two SSR markers were obtained from the Gramene database (http://www.gramene.org), while the remaining SSR markers (labeled ‘‘PSM’’) were developed in the State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources based on the sequence of the delimited region from the International Rice Genome Sequencing Project (IRGSP) database (http://rgp.dna.affrc.go.jp/IRGSP/index.html). Mini-scale DNA was extracted using the modified SDS protocol reported in Zheng et al. (1995). PCRs were conducted according to the method described by Panaud et al. (1996) in a 20 µl reaction mix containing 50 ng

Table 1. Rice cultivars included in this study and their origin.

S/N	Cultivar name	Released or introduced year	Subpopulation*
1	CYZ-18	1974	4
2	QIU-EA	1970	2
3	GYA-C17	1976	5
4	GQA	1963	2
5	GNA-1	1969	2
6	QBZ-3	1970	2
7	YXYZ	2005	2
8	FAZ	1999	1
9	TSA-2	1992	3
10	YQL	1982	2
11	WHA-1	1983	2
12	YXZ	1998	1
13	ZGA-1	1990	3
14	CG-314	1980	2
15	ZERZ	2001	2
16	ST-1	1999	1
17	ZYQ-8	1973	6
18	LQZ-1	1988	1
19	CYZ-18-X	1974	4
20	AZZ-4		4
21	QDZ	1992	1
22	PG-2	1971	4
23	RPA	1964	2
24	QFA	1971	7
25	GLA-4	1969	4
26	XZ-69	1973	6
27	FBZ	2001	1
28	GC-2	1976	2
29	MBYZ	2001	3
30	FAZ-5	1998	5
31	TXZ-25	1998	6
32	GE-104	1976	3
33	GEA-5	1963	7
34	GYA-121	1976	2
35	AXZ	2003	1
36	SZZ	1966	6
37	JEA	1967	7
38	MXZ	1968	7
39	HMZ	1967	7
40	MLSM	2005	1
41	FMZ	2005	7
42	GJ-9	1964	4
43	GC-13	1977	2
44	ZZA-11	1962	4
45	JX-89	1991	6

Table 1. Contd.

46	YFZ	2001	3
47	XXZ	1995	5
48	HHZ	2005	3
49	GNZ	2005	7
50	QLSM	2004	7
51	IR24	1971	6
52	QLA	1990	5
53	IR22	1971	6
54	IR8	1971	6
55	SG-36	1986	5
56	FQA	1992	7
57	SG-1	1982	5
58	XX-299	1992	7
59	QGZ-25	1985	5
60	QJZ	1986	5
61	YXZ-8	2005	6
62	QIN-EA	1975	7
63	FHZ	2002	7
64	SC-169	1983	7
65	TXZ-13	1996	6
66	MLXZ	2001	5
67	QXZ-3	1992	6
68	SEA	1983	7
69	YEZ	2005	3
70	JDL		4
71	AJNT	1957	4
72	GSA	1984	7
73	TQ-2	1984	7
74	YG-146	1988	5
75	QSZ	1991	7
76	FAZ-1	1997	7
77	QXJZ	1960	4
78	SYA	1992	7
79	GCA-3784	1959	4
80	JLXSZ	1982	6
81	GES	1979	5
82	SY-2	1994	7
83	GCA-4182	1959	4
84	LHZ	1996	7
85	IR20	1971	6
86	AQZ	1995	7

template DNA, 200 µM dNTP, 1×PCR buffer, 1U *Taq* DNA polymerase, and 0.15 µM forward and reverse primers. DNA amplification was performed using a PTC-100™ 96 Plus thermal cycler. The reaction program was as follows: 94°C for 5 mins followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min with a final extension of 5 minutes at 72°C. PCR products were separated by size with 6% polyacrylamide gel electrophoresis and detected by silver staining.

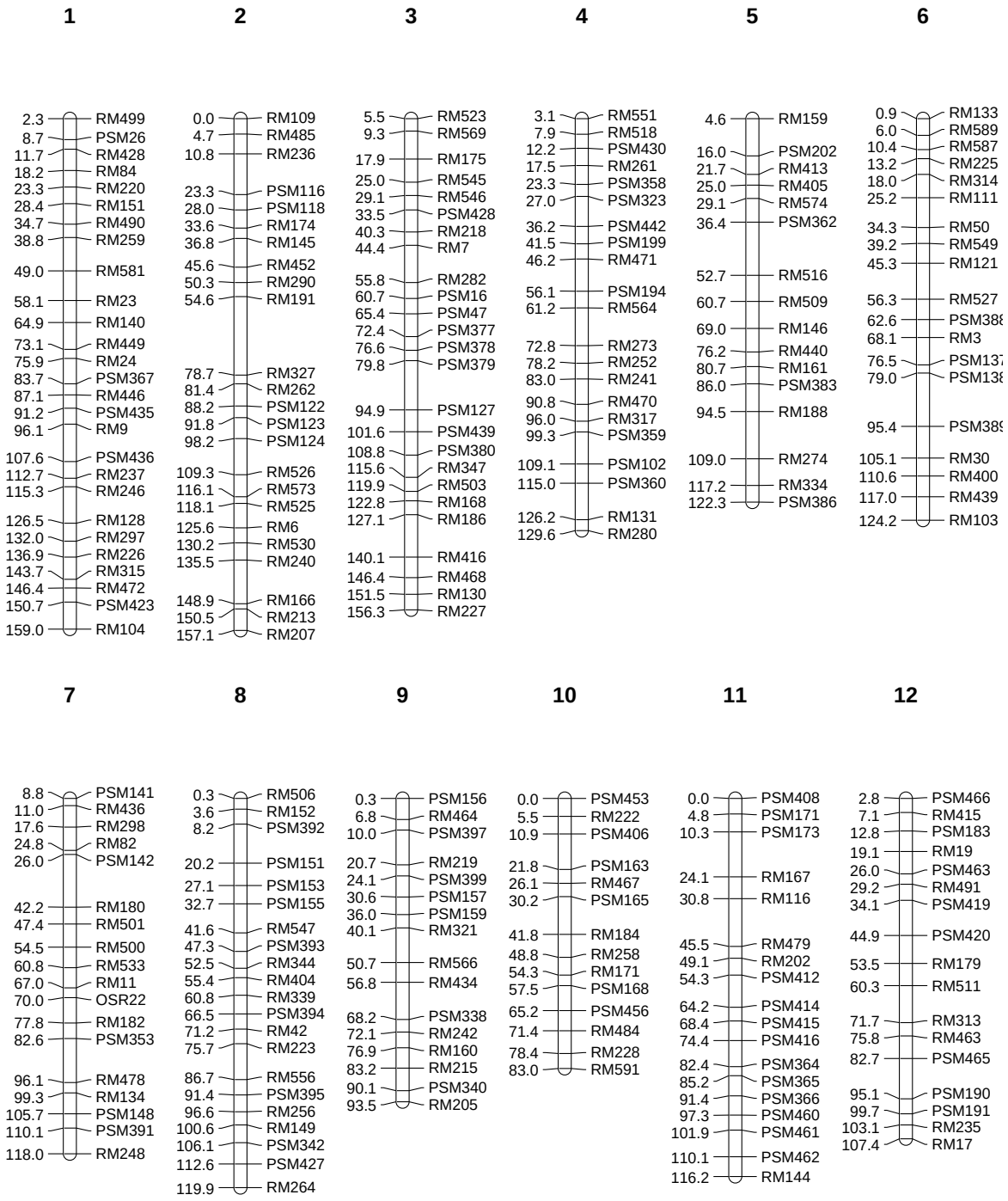

Figure 2. Distribution of 236 tested SSR markers on 12 rice chromosomes.

Genetic diversity and association analysis

Phenotypic data analysis

The data collection and basic processing was done in Excel 2007 on Windows XP. The distribution and histogram of flag leaf thickness were determined in SPSS 16.0 (SPSS Inc., Chicago, IL, USA) using the FREQUENCIES option in DESCRIPTIVE STATISTICS. Analysis of variance was carried out in SPSS 16.0 using the general linear model (GLM) and assuming a random effects model on multiple environments. Broad-sense heritability was calculated as Zhang et al. (2014).

Allelic diversity and demographic analysis

All cultivars were treated as pure lines. A few heterozygous loci were treated as missing data which were detected in this study. The

number of alleles and polymorphism information content (PIC) per locus were calculated with the Power Marker 3.25 program (Liu and Muse, 2005). A phylogenetic tree was constructed using the neighbor-joining method in MEGA 5.1 (Tamura et al., 2011). The population structure of the association mapping population was determined with the model-based STRUCTURE 2.3 program (Pritchard et al., 2000) using a burn-in of 10,000, a run length of 100,000, and a model allowing for admixture and correlated allele frequencies. Five runs were performed, and the number of sub-populations (K) was set from two to ten. The most likely number of sub-groups was estimated by LnP(D) in the STRUCTURE output and an ad hoc statistical ΔK following Evanno et al. (2005). Rare alleles, those with a frequency of less than 5% in the panel were treated as missing data for the structure analysis. Analysis of molecular variance (AMOVA) was performed with the program ARLEQIUN 3.11 (Excoffier et al., 2005). The F_{ST} value, which measures the degree of differentiation of each subpopulation, was calculated through AMOVA.

Association mapping

Association tests were performed with the mixed linear model (MLM) method in TASSEL 2.1 (http://www.maizegenetics.net). To reduce the type I error rate, four models, namely the Simple model, Q, K, and Q+K models, were used to evaluate the marker-trait associations. The population structure matrix (Q) was determined by running Structure 2.3 with the most likely number of sub-groups, K. The relative kinship matrix (K-matrix) was obtained using the software SPAGeDi (Hardy and Vekemans, 2002). Output from SPAGeDi was formatted to a text file readable by TASSEL 2.1. The best-fit model for the marker-trait association was determined using the Bayesian information criterion (BIC) to evaluate the four models: Simple model, Q, K, and Q+K. For controlling the type I error rate, *p*-values were compared to a Bonferroni threshold to identify significant loci (Nakagawa, 2004). The Bonferroni threshold was 1/236=0.0042, where 236 was the number of association tests for the trait in this study. The allelic effects at a marker locus were estimated using the Probability of Difference (PDIFF) procedure in SAS 9.1 (SAS institute Inc, Cary, NC, USA) on the least square means (LSMEANS) of the phenotype data.

Validation of QTLs identified through AM

To validate the significant loci for flag leaf thickness through association mapping, we constructed an F_2 population with the cross QSZ/P205. QSZ was a modern *indica* rice cultivar with flag leaf thickness of 255.3 cm selected from the association panel. P205 was a japonica variety with flag leaf thickness 382.5 cm we screened from the germplasms preserved in the rice germplasm repository of the Rice Research Institute, Guangdong Academy of Agricultural Sciences. 297 individuals were investigated for phenotypic and genotypic assay. Identification of flag leaf thickness and SSR genotyping performed using the method described above. QTL analysis was conducted using the approach of composite interval mapping in the computer package Windows QTL Cartographer version 2.5 (Wang et al., 2007).

RESULTS

Natural variation in flag leaf thickness

Flag leaf thickness of the 86 cultivars was measured in the field on the fifth to seventh day after full heading in the late-cropping seasons of 2008-2009. The flag leaf thickness values were normally distributed (Figure 3). The minimum, maximum, mean, and standard deviation (*SD*) were 251.9, 371.3, 309.4, and 24.4 μm in 2008 (Figure 3A) and 255.6, 372.5, 312.3, and 24.2 μm in 2009 (Figure 3B), respectively. The broad sense heritability (h^2) of flag leaf thickness was 88.6%.

Genetic diversity

We identified 781 polymorphic loci across the 86 accessions. The number of alleles per locus varied from two to twelve, with the average being 3.309. We detected 113 heterozygous loci, accounting for 0.557% of the total of 20,296 loci. The average Nei's genetic diversity index was 0.442, ranging from 0.042 to 0.839. The average PIC value was 0.415, ranging from 0.041 to 0.792.

Population structure

Analysis of the population structure was performed using the model-based program STRUCTURE 2.3. The model of ΔK value calculation was used to determine the most probable K. A sharp peak of ΔK at K=7 was observed (Figure 4A), indicating that the population could be optimally grouped with K=7. We therefore divided the population into seven subpopulations, S1 to S7. A graphical bar plot was then generated showing the posterior membership coefficients for each accession (Figure 4B). A neighbor-joining tree based on the genetic distance matrix was constructed using MEGA 5.1 (Figure 4C). It revealed genetic relationships that were relatively consistent with the STRUCTURE-based membership assignments of the cultivars. Most of the cultivars in the same subpopulation were classified in the same cluster. However, for a few cultivars, such as QFA, GQA and GEA-5, the classified cluster did not coincide with the corresponding subpopulation.

Estimation of relative kinship

The relative kinship matrix (K-matrix) was obtained using the software SPAGeDi. 42.05% of the pairwise relative kinship estimates were equal to zero, 32.04% were less than 0.05, 10.8% were between 0.05 and 0.1, 5.39% were between 0.1 and 0.15, 9.73% were between 0.15 and 0.5, and the remaining 0.014% of the estimates were >0.5 (Figure 5).

Population differences

The distribution of molecular genetic variation among and within the seven subpopulations was estimated by

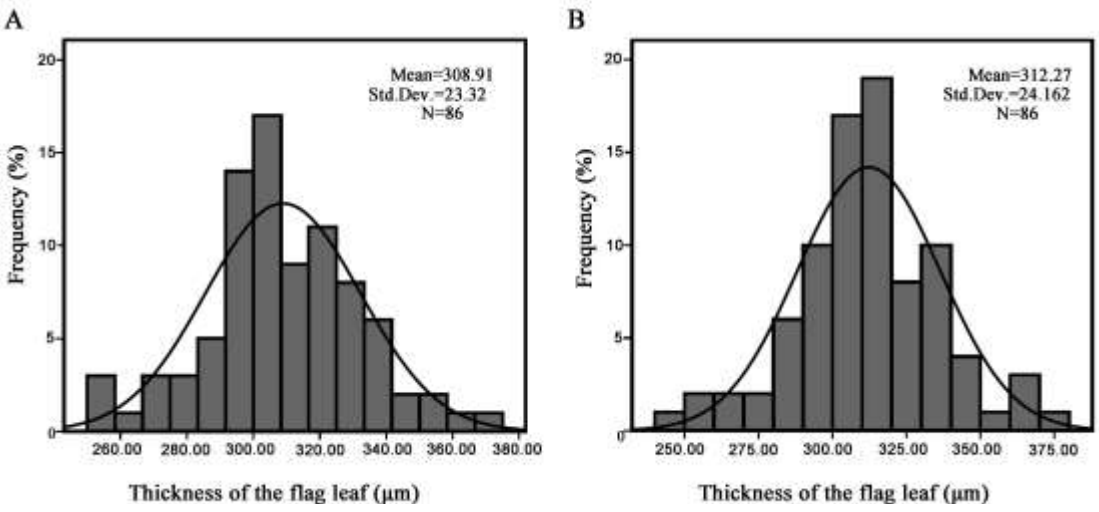

Figure 3. Histograms showing the frequency distribution of flag leaf thickness. (A) 2008; (B) 2009.

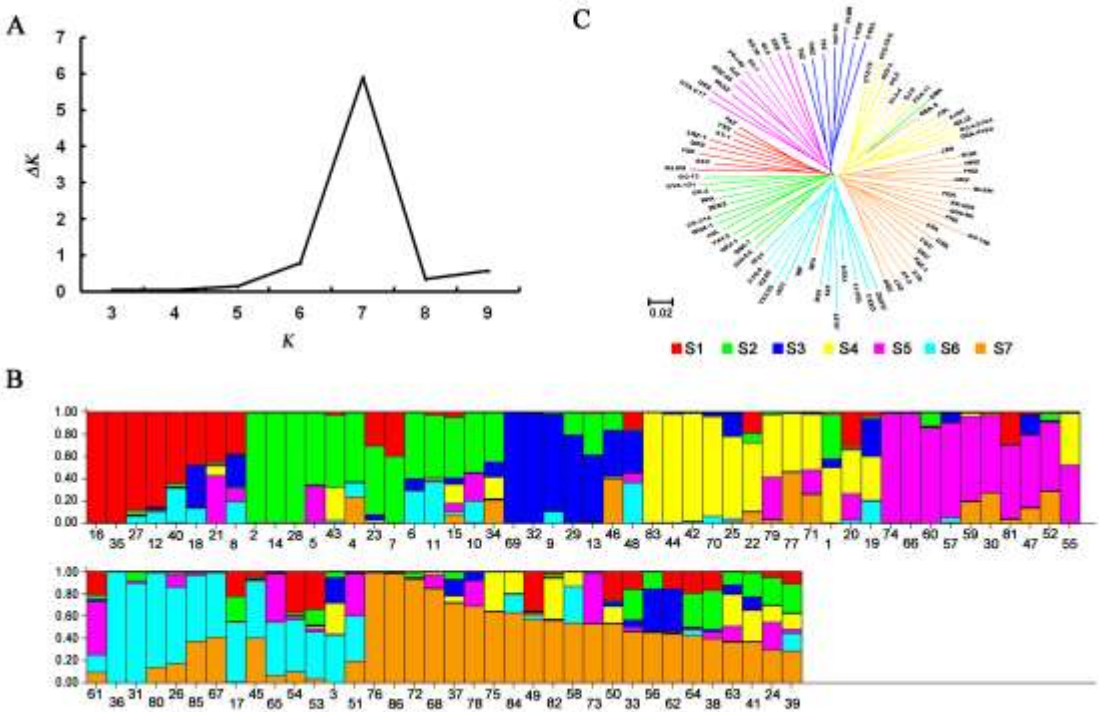

Figure 4. Population structure of 86 cultivars based on 236 SSR markers. (A) Changes of ΔK with the number of subpopulations; (B) Population structure analysis of 86 cultivars showing seven subpopulations (S1-S7), with the estimated membership probability listed on the y-axis and each cultivar represented by a thin vertical line in a different color; (C) Neighbor-joining tree analysis of the 86 rice accessions. The colors (S1-S7) correspond to the model-based populations.

AMOVA (Table 2). The variation among the subpopulations accounted for 7.18% of the total variation, whereas 92.82% of the variation was within the subpopulations. The pair-wise F_{ST} values between the seven subpopulations indicated that the levels of genetic divergence among subpopulations were medium to low (Table 3), on the whole. The highest was subpopulation S1 with S5 (0.105), and the lowest was S6 with S7 (0.025). The overall F_{ST} value was 0.072. It can be concluded that there is moderate genetic differentiation in

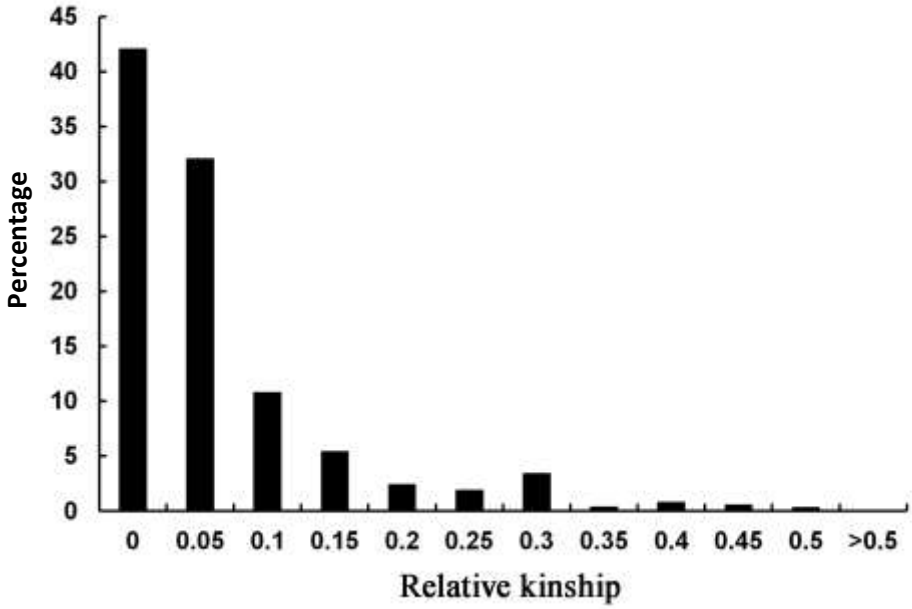

Figure 5. Distribution of pairwise relative kinship estimates among the 86 rice cultivars.

Table 2. AMOVA analysis of the seven rice accession clusters identified by STRUCTURE 2.3.

Source of variation	d.f.	Sum of squares	Mean squares	Estimated variance	Percentage variation (%)	*p*-value
Among clusters	6	114.73	19.12	0.71	7.18	<0.001
Among accessions within clusters	79	725.60	9.18	9.18	92.82	<0.001
Total	85	840.33	28.30	9.89		

Table 3. Pair-wise F_{st} values between the seven subpopulations as identified using Euclidean distance by the program STRUCTURE 3.1.

Cluster	S1	S2	S3	S4	S5	S6	Overall
S2	0.084	-					
S3	0.095	0.073	-				
S4	0.098	0.081	0.061	-			
S5	0.105	0.082	0.068	0.046	-		
S6	0.094	0.075	0.077	0.063	0.038	-	
S7	0.102	0.079	0.063	0.066	0.043	0.025	
Overall							0.072

the population.

Association mapping

To control for false positives (type I errors), four association mapping models (Simple model, Q, K, and Q + K models) were compared based on the Bayesian Information Criterion (BIC) and plots of observed-versus-expected *p*-values. Of the four models, the Q+K model had the smallest BIC values, the K model had the closest values to the Q+K model, and the Simple model had the largest BIC values (Table 4). We plotted the observed - versus-expected –log10(*p*) of the four models and found that the Q+K model had the smallest deviation between the observed and expected *p* values (Figure 6). The K model performed similarly to the Q+K model. This analysis indicates that the Q+K model is the best fit model for the correction of false positive in this association analysis, followed by the K model. The Q+K

Table 4. Fitness analysis of the mapping models for flag leaf thickness using Bayesian information criterion (BIC) among 86 cultivars genotyped with 236 molecular markers and phenotyped in Guangzhou, China in 2008-2009.

Model	2008	2009
Simple model	1675.56	1630.18
Q	1660.78	1635.40
K	1591.54	1572.70
Q+K	1566.76	1567.92

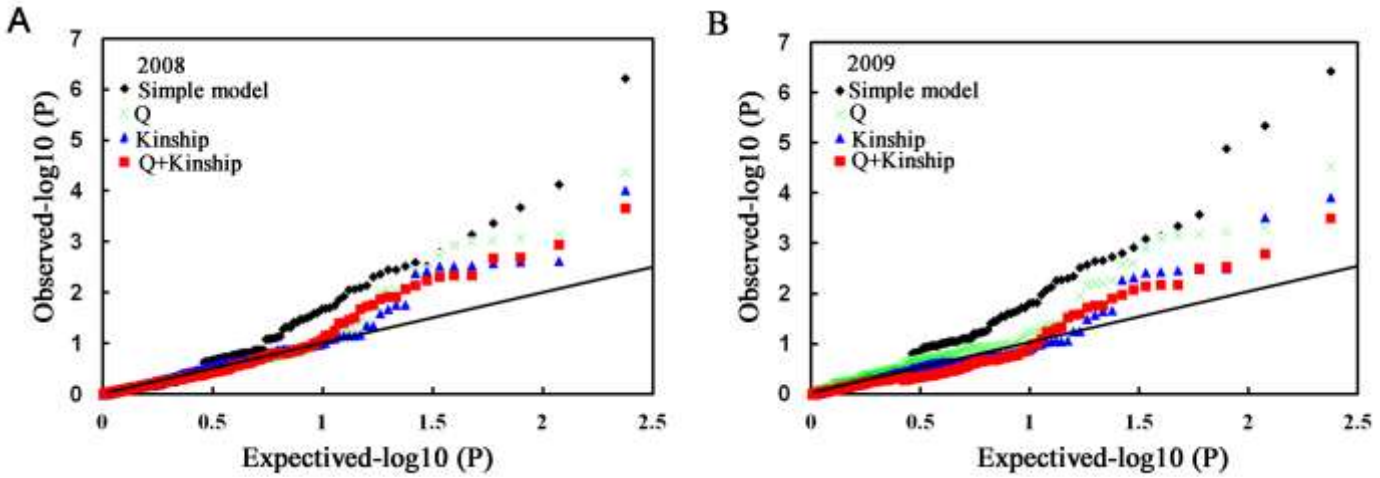

Figure 6. Quantile-quantile plots of estimated –log10(p) from association analysis using four models in flag leaf thickness. The black line is the expected line under the null distribution.

Table 5. Association of SSR markers with flag leaf thickness in *indica* rice.

Locus	Chr.	Position (cM)	2008		2009		Reference
			p-marker	r^2-marker	*p*-marker	r^2-marker	
RM297	1	132	-	-	0.0031	0.1214	
RM315	1	143.7	0.0019	0.1022	0.0009	0.1358	
PSM124	2	98.2	0.0031	0.1273	-	-	
RM227	3	156.3	0.0034	0.1136	0.0023	0.1406	Zhao et al. (2008); Khowaja and Price, 2008
RM471	4	46.2	-	-	0.0037	0.0736	
PSM362	5	36.4	-	-	0.0039	0.0928	Laza et al. (2006); Khowaja and Price, 2008
PSM353	7	82.6	0.0009	0.2507	0.0003	0.2261	Kanbe et al. (2008)
RM478	7	96.1	0.0019	0.0925	0.0008	0.1135	Zhao et al. (2008)
PSM163	10	21.8	0.001	0.2154	0.0006	0.1849	
PSM414	11	64.2	-	-	0.0035	0.0657	
PSM364	11	82.4	0.0024	0.1728	0.0037	0.1552	

model was therefore selected for the association mapping.

Association analysis was performed based on the Q+K model using the MLM procedure in TASSEL 2.1. A total of eleven marker-trait pairs were identified to have significant (p<0.0042) marker-trait associations (Table 5). These markers were distributed on eight chromosomes. On chromosomes 1, 7 and 11, two loci were identified. Only one locus was identified on chromosomes 2, 3, 4, 5 and 10. Four of these loci such as RM227 (Zhao et al., 2008; Khowaja and Price, 2008), PSM362 (Laza et al., 2006; Khowaja and Price, 2008), PSM353 (Kanbe et al., 2008) and RM478 (Zhao et al., 2008) had been already identified as related to flag leaf thickness. However the

Table 6. Comparison of the allelic effects of the six marker loci associated with flag leaf thickness.

Locus	Allele size (bp)[a]	Number of varieties	FLT (μm)[b]	
			2008	2009
RM315	134	33	297.3±22.6^{Cb}	303.6±26.5^{Bb}
	136	3	282.9±16.8^{Cb}	280.9±20.3^{Cc}
	139	32	338.6±26.7^{Aa}	341.6±29.2^{Aa}
	143	18	316.1±22.8^{Bb}	319.6±21.9^{Bb}
RM227	100	25	340.8±26.5^{Aa}	342.9±27.2^{Aa}
	106	41	305.6±31.6^{Bb}	308.6±32.3^{Bb}
	108	20	279.2±19.9^{Cc}	281.5±20.3^{Cc}
PSM353	293	49	288.5±23.5^{Bb}	291.6±22.7^{Bb}
	297	37	337.1±25.7^{Aa}	339.5±26.3^{Aa}
RM478	197	30	303.2±33.2^{Bb}	306.5±32.6^{Bb}
	199	26	338.2±25.7^{Aa}	339.8±26.7^{Aa}
	205	13	311.1±22.2^{Bb}	309.6±20.9^{Bb}
	208	17	275.7±19.1^{Cc}	282.1±16.4^{Bc}
PSM163	202	23	350.4±29.7^{Aa}	352.7±27.8^{Aa}
	206	37	310.2±21.1^{Bb}	312.3±22.5^{Bb}
	212	26	271.5±27.3^{Cc}	276.3±24.1^{Cc}
PSM364[c]	174	4	310.5±24.5^{Bb}	313.6±23.6^{Bb}
	178	29	294.7±18.2^{BbCc}	293.9±21.6^{BbCc}
	184	19	348.3±29.6^{Aa}	352.2±31.4^{Aa}
	186	25	332.9±22.7^{Aa}	337.6±34.8^{Aa}
	192	6	281.2±15.6^{Cc}	285.7±18.8^{Cc}

Note: [a] Allele Size (bp) is PCR product amplified by SSR markers; [b] Within a column, mean value±*SD* followed by capital and small letters represent significant difference at α=0.01 and 0.05, respectively; [c] Three heterozygotes identified with this marker were excluded from the data, 83 cultivars were used for the statistical analysis.

other seven loci were novel (Table 4). Six of the eleven loci were detected in both years, including RM315, RM227, PSM353, RM478, PSM163 and PSM364. The locus PSM353 (on chromosome 7) had the highest r^2-marker value in both years and explained 25.07 and 22.61% of the phenotypic variation respectively in 2008 and 2009. The second strongest association was with PSM163 (on chromosome 10), which explained 21.54 and 18.49% of the phenotypic variation in 2008 and 2009, respectively.

Allelic effects and their interactions

The allelic effects of the six loci were estimated which were detected in both years (Table 6). For RM315, individuals carrying the 139 bp allele (RM315^{139bp}) had thicker flag leaves than those carrying alleles RM315^{134bp} and RM315^{136bp}. Allele RM315^{139bp} thus had the highest positive impact on flag leaf thickness at this locus. For the other loci, the alleles RM227^{100bp}, PSM353^{297bp}, RM478^{199bp}, PSM163^{202bp} and PSM364^{184bp} had the highest phenotypic values and thus the greatest positive impact on flag leaf thickness. We therefore suggest considering RM315^{139bp}, RM227^{100bp}, PSM353^{297bp}, RM478^{199bp}, PSM163^{202bp} and PSM364^{184bp} favored alleles in breeding programs. The effect of the number of favor alleles on phenotypic values was also analyzed. As the number of favored alleles in an individual increased, so did the phenotypic value (Figure 7). The correlation between the number of favored alleles and flag leaf thickness was significantly positive, with a correlation coefficient of 0.726 (p<0.001). Cultivars with more favored alleles thus had greater flag leaf thickness.

Validation of significant loci through AM

To examine whether the QTLs identified through AM could be detected by bi-parental QTL mapping, we

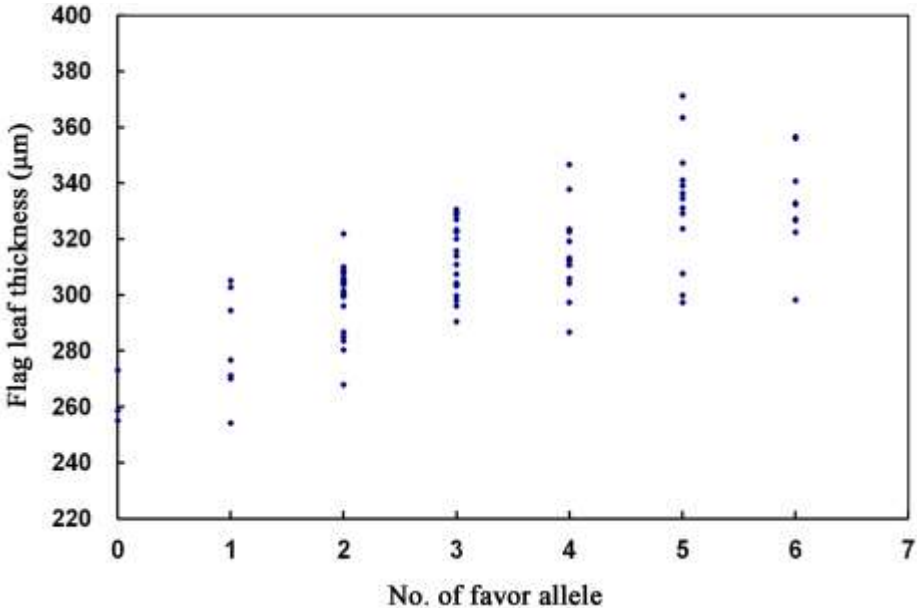

Figure 7. Relationship between the number of favored allele and flag leaf thickness in a cultivar.

Table 7. Summary of major QTLs for flag leaf thickness identified in the F_2 population of the cross QSZ/P205.

QTL	Chr.	Interval	LOD	Var (%)	Corresponding locus in AM
qFLT1.1	1	RM226-RM472	8.3	9.6	RM315
qFLT2.1	2	RM283-RM151	9.2	11.8	
qFLT7.1	7	RM505-RM18	12.1	15.7	PSM353
qFLT10.1	10	RM216-PSM165	9.6	10.5	PSM163

constructed one F_2 mapping population derived from the cross QSZ/P205. Four QTLs were detected in the study, which were located on chromosome 1, 2, 7 and 10, respectively (Table 7). In these loci, except qFLT2.1, all the other three QTLs could correspond to a significant locus in AM. qFLT1.1 explained 9.6% of the phenotypic variation, which was located in the interval between RM226 and RM472 on chromosome 1. The area of qFLT1.1 on chromosome overlaped the significant locus RM315 identified in AM. The QTL qFLT4.1 explained 15.7% of the phenotypic variation, which was located in the interval between PSM432 and RM18 on chromosome 4. The QTL qFLT4.1 was very near to the significant locus PSM353 detected in AM. The QTL qFLT10.1 explained 10.5% of the phenotypic variation, which was located in the interval between RM216 and PSM165 on chromosome 10 and overlaped the significant locus PSM163 in AM.

DISCUSSION

Flag leaf thickness is so important for high yielding in rice that breeders employed it as one of the major selecting targets in rice breeding programs (Yang et al., 1984; Krush, 1995; Yuan, 1997). In recent years, a few QTLs for flag leaf SLW and SLA, the alternative indicators of leaf thickness, have been identified in rice (Laza et al., 2006; Kanbe et al., 2008; Zhao et al., 2008; Takai et al., 2010). However, SLW and SLA are not direct phenotypic measurements, but the indexes converted from measurements of several traits. It is therefore difficult to ensure accuracy when these converted data are used as phenotypic data to identify genes (QTLs) for leaf thickness in rice. This may be the main reason why although several QTLs for SLW and SLA have been identified, no major QTL has been cloned or fine-mapped. In present study, we first carried out an association analysis between SSR markers and flag leaf thickness using the phenotypic data obtained with a nondestructive rice leaf thickness instrument and identified several QTLs for flag leaf thickness in *indica* rice.

Elite breeding materials in plant breeding programs could be used as a population for association analysis (Breseghello and Sorrells, 2006b; Zhu et al., 2008).

Genetic improvement programs of plant cultivars could be considered recurrent selection breeding programs in the breeding history of a local region. In each round of selection, genes introgressed from the primary groups or exotic germplasm caused phenotypic changes. Genomes in local populations have been structured by artificial selection of genotype × environment interactions during plant breeding programs (Shinada et al*.,* 2014). This offers two advantages for association mapping focused on elite lines derived from breeding programs in a local region: first, precise evaluation of phenotypes can be accomplished because the elite lines are genetically stable and well adapted to the local environmental conditions; second, elite lines are often used as parents for crossing in the next round of breeding, and significant markers associated with target traits could thus be used for marker-assisted selection in the progenies (Breseghello and Sorrells, 2006b; Zhu et al., 2008). Several association studies employing a local population derived from crop breeding programs have been conducted. Breseghello and Sorrells (2006a) performed association mapping for kernel size and milling quality using an association panel consisting of 95 elite soft winter wheat cultivars which were genotyped using 36 SSRs. The selected cultivars represented the variability of the current elite soft winter wheat cultivars in the eastern United States. The analysis identified 62 significant marker-trait association loci. Fujino et al*.* (2015) used 115 SSRs for genotyping in an association analysis of 63 cultivars derived from rice breeding programs in Hokkaido, Japan. Six QTLs were identified for heading date and seventeen for low temperature germinability. An association panel consisting of 109 German winter barley cultivars which were released in Germany between 1959 and 2003 was genotyped using 72 SSRs to detect loci related to grain yield traits, and 91 significant marker-trait loci were identified (Rode et al*.,* 2012). Three hundred and sixty-three elite breeding lines were selected for genotyping from an IRRI irrigated rice breeding program and genotyped for 71,710 SNPs using genotyping-by-sequencing (GBS), and 52 QTL for 11 agronomic traits were identified through association analysis (Begum et al., 2015). In the present study, an association panel consisting of 86 cultivars which had once been planted in large areas in South China was selected as a panel for association mapping of flag leaf thickness in rice. These cultivars, which were selected from 334 modern cultivars and some landraces in South China, included two landraces, four foreign germplasms from IRRI and 80 improved cultivars released in 1957-2005. This population was representative of the variability of the elite *indica* rice germplasm released in the South China since 1949. Two hundred and thirty-six SSR markers covering 12 chromosomes were employed for genotyping the accessions. The population size was similar to that in studies by Breseghello and Sorrells (2006a), Fujino et al*.* (2015), and Rode et al *.* (2012) (95, 63 and 109 varieties, respectively). However, the SSR density was significantly higher than that of the aforementioned three studies (which used 36, 115 and 72 SSRs, respectively). We identified 781 polymorphic alleles, and the mean number of alleles per locus was 3.309, with a range from 2 to 12. This was similar to the 3.88 alleles reported in a rice core collection (Zhang et al*.,* 2011) and the 3.9 alleles in an association mapping population identified by Jin et al*.* (2010). The average PIC value was 0.415, ranging from 0.041 to 0.792. This is similar to that reported by Jin et al*.* (2010) in a rice panel (0.4214), but slightly higher than the result determined by Cui et al*.* (2013) in a diverse rice panel (0.3137) and Xu et al. (2016) in rice collected from China (0.2465). The average Nei's genetic diversity index was 0.442, ranging from 0.042 to 0.839. This is similar to the 0.3413 reported by Cui et al*.* (2013) and the results reported by Yu et al. (2013).

Spurious associations between candidate markers and phenotypes can be caused by population structure, relatedness between individuals, selection, or genetic drift (Yu and Buckler, 2006). A unified mixed-model approach which demonstrated improved control over other methods for both type I and type II error rates was introduced by Yu et al*.* (2006). It was successfully used in association mapping (Breseghello and Sorrells, 2006a; Agrama et al., 2007; Yang et al*.,* 2010; Neumann et al*.,* 2011). In the present study, we compared four models, the Simple, Q, K, and Q+K models. The Q+K model had the smallest BIC value and best approximated the expected cumulative distribution of *p* values. This indicated that the Q+K model was the optimal model for the identification of marker-trait loci in a local population derived from breeding programs.

We identified 11 marker-trait association loci (Table 5). Seven of these were novel QTLs identified in this study, while four had been previously reported. The locus RM227 on chromosome 3 was detected in both the 2008 and 2009 data, and it has been reported as associated with both SLW (Zhao et al*.,* 2008) and SLA (Khowaja and Price, 2008). The locus PSM362 on chromosome 5 was detected in the 2009 data and has been identified as linked to SLA by Laza et al*.* (2006) and Khowaja and Price (2008). The locus PSM353 on chromosome 7 had the highest r^2-marker value in both years (0.2507 and 0.2261); it is located in the region of a reported QTL associated with SLW (Kanbe et al*.,* 2008). The locus RM478 was detected in both the 2008 and 2009 data and has been reported to affect SLW by Zhao et al. (2008). Of the seven novel loci, the locus PSM163 had the highest r^2-marker value, explaining 21.54% and 18.49% of the phenotypic variation in 2008 and 2009, respectively.

In the validation study, three of four QTLs detected by one F_2 mapping population could correspond to a significant locus in AM. It demonstrated that almost all of the significant loci identified in AM could be detected in bi-parental F_2 groups. The results also indicated that AM

is more efficient and has a greater allele number than bi-parental QTL mapping. Six alleles, RM315^{139bp}, RM227^{100bp}, PSM353^{297bp}, RM478^{199bp}, PSM163^{202bp} and PSM364^{184bp}, had the highest effect at their respective loci (Table 6). They should therefore be considered as favored alleles in breeding programs. The significantly positive correlation between the number of favored alleles and flag leaf thickness shows that pyramiding several of the favored alleles is a viable approach to improve flag leaf thickness and construct an ideal plant type in rice breeding programs.

Conclusion

Association mapping is an important approach for identifying QTLs based on linkage disequilibrium. In the present study, a total of eleven marker-trait pairs with significant marker-trait associations were identified. The putative QTLs were distributed on eight chromosomes. Seven loci were novel QTLs. Almost all of the significant loci identified in AM could be detected in bi-parental F_2 groups and indicated that AM is more efficiency and has a greater allele number than bi-parental QTL mapping. Pyramiding the favored alleles for flag leaf thickness identified in this study will be a valuable approach to construct ideal plant architecture in rice ideal plant type breeding programs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This study was supported by the Natural Science Foundation of Guangdong Province, China (2017A030313185), Guangdong Scientific and Technological Plan, China (2015B020231002), Scientific Research Institutions Reform and Innovation Program of Guangdong Province, China (2014B070706013, 2016B070701011) and Guangdong Key Agricultural Program, China (2017B020201003). We thank Assistant Prof. Chen Jianyou, Guangdong Academy of Agricultural Sciences, who kindly provided some of the test materials.

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