



# Association mapping of yield and its components in rice cultivars

H. A. Agrama  C. Eizenga  W. Yan

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**Abstract** To make advances in rice breeding it is important to understand the relatedness and ancestry of introduced rice accessions, and identify SSR markers associated with agronomically important phenotypic traits, for example yield. Ninety-two rice germplasm accessions recently introduced from seven geographic regions of Africa, Asia, and Latin America, and eleven US cultivars, included as checks, were evaluated for yield and kernel characteristics, and genotyped with 123 SSR markers. The SSR markers were highly polymorphic across all accessions. Population structure analysis identified eight main clusters for the accessions which corresponded to the major geographic regions, indicating agreement between genetic and predefined populations. Linkage disequilibrium (LD) patterns and distributions are of fundamental importance for genome-wide mapping association. LD between linked markers decreased with distance and with a substantial drop in LD decay values between

20 and 30 cM, suggesting it should be possible to achieve resolution down to the 25 cM level. For the 103 cultivars, the complex traits yield, kernel width, kernel length, kernel width/length ratio, and 1000-kernel weight, were estimated by analysis of variety trial data. The mixed linear model method was used to disclose marker-trait associations. Many of the associated markers were located in regions where QTL had previously been identified. In conclusion, association mapping in rice is a viable alternative to QTL mapping based on crosses between different lines.

**Keywords** Linkage disequilibrium · Unified mixed-model method · Population structure · Kinship coefficient · Relatedness

## Abbreviations

SSR Simple sequence repeat  
QTL Quantitative trait loci  
cM CentiMorgan

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H. A. Agrama (✉)  
University of Arkansas Rice Research and Extension  
Center, Stuttgart, AR 72160, USA  
e-mail: hagrama@uark.edu

G. C. Eizenga · W. Yan  
United States Department of Agriculture ·  
Agricultural Research Service, Dale Bumpers  
National Rice Research Center, Stuttgart, AR 72160-  
1090, USA

## Introduction

Rice (*Oryza sativa* L.) genetic mapping often involves the development, genotyping, and phenotyping of doubled haploid (Li et al. 2003

Hittalmani et al. 2002), recombinant inbred (Ge et al. 2005; Guo et al. 2005), or advanced backcross (Li et al. 2004; Jing et al. 2005) lines derived from an  $F_1$  cross between different cultivars. For these mapping populations there is extreme disequilibrium between linked loci (Mather et al. 2004) and there should be no population structure, because of the random sampling, or disequilibrium between non-linked loci favoring the detection and approximate mapping of quantitative trait loci (QTL). In contrast, genetic mapping in humans relies on analysis of genotypic and phenotypic data sampled non-randomly from existing populations of complex but often unknown structure. In these populations, linkage disequilibrium (LD) may decline over relatively short or long distances in the genome, making fine mapping possible. In sugar beet (*Beta vulgaris* L.), LD extended to 3 cM (Kraft et al. 2000) and LD in some *Arabidopsis* populations exceeded 50 cM (Nordborg et al. 2002). LD as a function of genetic distance is very common for distances <10 cM (Kraakman et al. 2004) in barley (*Hordeum vulgare* L.), in contrast with maize (*Zea mays* L.), for which the LD diminished after 2000 bp (Remington et al. 2001). LD decay between all pairs of SNP (single nucleotide polymorphism) loci in the region around the rice *xa5* locus approached 0.1 after only 100 kb (Garris et al. 2003) and in *Arabidopsis* the pattern of polymorphism LD decayed rapidly, within 50 kb (Nordborg et al. 2005), in contrast with one human population for which the LD extended over 3 cM (Eaves et al. 1998). Most studies of species variation begin by sampling from populations defined on the basis of culture or geography and might not reflect underlying genetic relationships (Rosenberg et al. 2002; Foster and Sharp 2002). If a whole-genome scan is to be undertaken, trait mapping by allele association requires high marker density (Lander and Schork 1994; Jorde 1995; Jorde 2000; Risch 2000). SSRs are particularly useful for studying the population structure and demographic history of domesticated species such as rice (Garris et al. 2005), and are being extensively used to genotype rice germplasm collections (Yang et al. 1994; McCouch et al. 1997; Ishii and McCouch 2000; Ishii et al. 2001; Ni

et al. 2002; Lu et al. 2005). Use of SSR markers to interpret population structure results in much greater resolution than use of other types of marker, because of the high level of polymorphism at SSR loci (Akkaya et al. 1992; Cho et al. 2000). In rice the highly polymorphic nature of SSR motifs is coupled with a low level of homoplasmy observed in *O. sativa* cultivars (Chen et al. 2002), providing an appropriate tool for population genetic studies. Although polymorphic SSRs are excellent molecular markers, because of their multi-allelism and the resulting high informativeness (Weber and May 1989), they may not be frequent enough for association studies (Ching et al. 2002). Size homoplasmy of SSR alleles and allele reversion could also be a problem in some populations (Estoup et al. 1995; Viard et al. 1998). Rice has the smallest genome size (estimated as approx. 430 Mb) among cereal crops, which makes it most manageable at the whole-genome level.

The potential of LD and regression methods to identify and characterize loci/genes associated with different complex traits in true breeding lines has been demonstrated in barley and maize (Kraakman et al. 2004; Wilson et al. 2004). In contrast with calculating associations between pairs of loci or genes by the traditional LD technique, or using a single marker-trait regression scheme, a multivariate approach, known as discriminant analysis, has been used for whole-genome scans of microsatellite markers associated with economic traits in unrelated inbred lines of rice (Zhang et al. 2005). Of particular interest to rice breeders is the possibility of using existing germplasm resources for gene and allele discovery on the basis of association mapping strategies (Kruglyak 1999; Jorde 2000; Farnir et al. 2000). Understanding population structure is important to avoid identifying spurious associations between phenotype and genotype in association mapping (Pritchard and Rosenberg 1999; Pritchard et al. 2000; Pritchard and Donnelly 2001).

Detection of marker-trait associations in breeding germplasm has potential advantages over classical linkage analysis and QTL mapping (Jannink and Walsh 2002). For example, broader genetic variation in a more representative genetic background can be included in the analysis, LD mapping may achieve higher resolution, and

multi-trait phenotypic data stored in databases can be linked to marker characterizations of the cultivars involved. The last advantage is especially important when evaluation of the trait is time-consuming and expensive, as it is for yield, adaptability, and stability (Kraakman et al. 2004). This approach may, however, be limited by the structure of the population, eventually leading to spurious, inappropriate levels of LD for QTL mapping, and insufficient phenotypic or genetic diversity available within the gene pool (Condon and Smith 2005). The number of markers needed for genome-wide LD scanning depends on the level of LD, however; to be effective, LD-mapping requires a marker density compatible with the distances across which LD extends in the population of interest. Association mapping could complement and enhance previous QTL information for marker-assisted selection in wheat (Breseghello and Sorrells, 2006), barley (Kraakman et al. 2006), and maize (Yu and Buckler 2006). Within different sets of barley germplasm there was frequent disequilibrium among non-linked SSR markers, suggesting that association mapping without consideration of population structure would result in a high rate of false positive Type I errors (Mather et al. 2004). Use of a unified mixed-model approach to account for multiple levels of relatedness simultaneously, as detected by use of genetic markers, has improved control of both type I and type II error rates (Yu et al. 2006).

Multi-allelic markers, for example SSRs, have been used to characterize population structure in maize (Flint-Garcia et al. 2005; Remington et al. 2001) and rice (Garris et al. 2003); and LD-based associations in wheat, (Kruger et al. 2004), barley (Maccaferri et al. 2005), and *Lolium* (Skjelt et al. 2005). The objective of this research was to use a large collection of blast (*Magnaporthe oryzae* B. Couch) resistant rice accessions to determine the utility of population structure analysis, linkage disequilibrium (LD), and association mapping of yield traits in evaluating rice germplasm accessions.

#### Materials and methods

Included in this study were 92 blast-resistant rice germplasm accessions from seven different

regions of Africa, Asia, and Latin America (Table 1). The 91 accessions were recently added to the USDA-ARS National Plant Germplasm System. The eleven US cultivars were Bengal (PI561535), Cocodrie (PI606331), Drew (PI596758), Katy (PI527707), Kaybonnet (PI583278), LaGrue (PI568891), Lemont (PI475833), Newbonnet (PI474580), Wells (Moldenhauer et al. 2000), Saber (PI633624), and Zenith (Clor7787). TeQing (PI536047) is an older cultivar from Guangdong, China. Further information on the accessions and cultivars is available from the USDA-ARS National Plant Germplasm System (<http://www.ars-grin.gov/npgs>).

The test was seeded in April 2000 and April 2001 in a split-block design (group as main block) with four replications at the University of Arkansas Rice Research and Extension Center in Stuttgart, AR, USA. The plots were 4.5 m long and six rows wide with 20 cm spacing between rows. At maturity, 3.6 m from the center, two rows in each plot were harvested for grain yield (GY). Other aspects of field management and measures of grain quality were as described by Dilday et al. (2001) and Yan et al. (2003). Seed (30 g) of each accession was dehulled to determine the kernel length (KL), kernel width (KW) and kernel length/width ratio (LWR) with the GrainCheck 312 video image system (FOSS Food Technology). Thousand kernel weight (TKW) was determined by weighing 1000 rice kernels.

Genomic DNA was extracted from leaf tissue by the methods described in Eizenga et al. (2006). One-hundred and twenty-three microsatellite markers located on the twelve chromosomes were selected from the core set developed and mapped by McCouch et al. (2002). The forward primers were labeled with FAM, TET, NED, or HEX fluorescent dyes and the reverse primers were unlabeled. DNA amplification was performed using an MJ Research PTC-100 96 Plus thermal cycler. PCRs were conducted in a 10 $\mu$ L reaction mix containing 37.5 ng template DNA, 1 $\times$  PCR buffer, 0.025 units Taq DNA polymerase (Qiagen, Valencia, CA, USA), 0.2 mmol dNTPs, and 0.8 pmol forward and reverse primers. Information on primer sequences and PCR amplification conditions for each set of primers are available at

Table 1 Rice germplasm accessions included in this study and their country of origin<sup>a</sup>

No.	Accession name	Country of origin <sup>b</sup>
1	Bhujon Kolpo	Bangladesh
2	Bogra	Bangladesh
3	Khoia	Bangladesh
4	Iac 47	Brazil
5	IRGA409	Brazil
6	Guang-6ai-4	China
7	02428	China-CD
8	Chunzhi No. 11	China-CD
9	Fu No83	China-CD
10	Kechengnuo No. 4	China-CD
11	Sheng 10	China-CD
12	Shufeng 117	China-CD
13	Shufeng 122	China-CD
14	Tie 90-1	China-CD
15	Tiejing No. 4	China-CD
16	Zhang 32	China-CD
17	Xiangzaoxian No. 1	China-HN
18	71198	China-HZ
19	Aijiaonante	China-HZ
20	Zanuo No. 1	China-HZ
21	Zhongyu No. 1	China-HZ
22	Zhongyu No. 6	China-HZ
23	Zhongzao No. 1	China-HZ
24	460	China-JT
25	2410	China-JT
26	4484	China-JT
27	4593	China-JT
28	4594	China-JT
29	4596	China-JT
30	4597	China-JT
31	4607	China-JT
32	4611	China-JT
33	4612	China-JT
34	4632	China-JT
35	4633	China-JT
36	4642	China-JT
37	4641(1)	China-JT
38	GP-2	China-JT
39	Gui 99	China-JT
40	R 147	China-JT
41	R 312	China-JT
42	Dian No. 1	China-KM
43	Egyptian Jasmine	Egypt
44	GZ-1368-5-4	Egypt
45	GZ-5578-2-1-2	Egypt
46	GZ-5594-23-1-2	Egypt
47	GZ-5830-48-2-2	Egypt
48	Ad 9246	Ivory Coast
49	Fkr 19 (Tox728-8)	Ivory Coast
50	Fkr 48	Ivory Coast
51	32 Xan Sc	Ivory Coast
52	Ita 406	Ivory Coast
53	Ita 416	Ivory Coast

Table 1 Continued

No.	Accession name	Country of origin <sup>b</sup>
54	Let 3137	Ivory Coast
55	S 992-F4-2-5-1-B	Ivory Coast
56	Tnau 7893	Ivory Coast
57	Tox 3093-35-2-3-3-1	Ivory Coast
58	Tox 3211-49-1-1-3-2	Ivory Coast
59	Tox 3241-21-2-2-3-2	Ivory Coast
60	Tox 3241-22-3-3-3	Ivory Coast
61	Tox 3241-31-2-1-3-1	Ivory Coast
62	Tox 3441-123-2-3-2-2-2	Ivory Coast
63	Tox 3553-34-3-2-3-2-2	Ivory Coast
64	Tox 3706-60-3-3-3	Ivory Coast
65	Tox 3706-6-3-3-2	Ivory Coast
66	Tox 3716-4-3-2-2-2-2	Ivory Coast
67	Tox 3717-25-3-1-3	Ivory Coast
68	Tox 3717-25-3-3-1	Ivory Coast
69	Tox 3717-25-3-3-2	Ivory Coast
70	Tox 3717-76-2-2-3	Ivory Coast
71	Tox 3717-81-1-1-3	Ivory Coast
72	Tox 3770-17-2-2-1	Ivory Coast
73	Tox 3771-144-2-1-1	Ivory Coast
74	Tox 3772-38-2-2-3	Ivory Coast
75	Tox 3772-40-3-2-2	Ivory Coast
76	Tox 3772-94-1-1-1	Ivory Coast
77	Tox 3779-51-2-2-2	Ivory Coast
78	Tox 3867-19-1-1-3-1-1-1	Ivory Coast
79	Tox 3869-34-1-3-1-1-3-3	Ivory Coast
80	Tox 3872-61-3-3-3-2-1	Ivory Coast
81	Tox 3894-41-2-3-1	Ivory Coast
82	Tox 4136-38-2	Ivory Coast
83	Tox 4251-313-3	Ivory Coast
84	Tox 3749-71-1-1-3-2-2	Ivory Coast
85	Wab450-24-3-2-P18-hb	Ivory Coast
86	Wab450-l-B-P-62-hb	Ivory Coast
87	Pyongyang 23	Korea, N.
88	IR56450-28-2-2 1	Philippines
89	Nj70507 17578	Philippines
90	RP2199-16-2-2-1	Philippines
91	S972B-22-1-3-1-1	Philippines
92	Bengal	USA
93	Cocodrie	USA
94	Drew	USA
95	Katy	USA
96	Kaybonnet	USA
97	LaGrue	USA
98	Lemont	USA
99	Newbonnet	USA
100	Saber	USA
101	Te Qing	China/USA
102	Wells	USA
103	Zenith	USA

<sup>a</sup> Adapted from Eizenga et al. (2006)

<sup>b</sup> Source of Chinese germplasm: CD - Chengdu, HN - Hunan, HZ - Hangzhou, KM - Kunming, JT - Joshua Tao

<http://www.gramene.org/> (verified 3 July 2006). PCR products were separated by size using an ABI 3700 DNA analyzer (Applied Biosystems (ABI), Foster City, CA, USA). SSR fragment sizing was performed with Gene Scan software (ABI) using the local Southern method and default analysis settings after which alleles were identified with Genotyper software (ABI) and binned manually. For most markers fractional numbers were rounded to the nearest integer and alleles differing by 1 bp were declared different. SSR data, obtained from genotyping US cultivars with the same ABI 3700 DNA analyzer (Lu et al. 2005), was included for comparison. The map position of most of the SSR loci was inferred from McCouch et al. (2002).

The model-based software Structure (Pritchard et al. 2000) was used to infer population structure using a burn-in of 10,000, run length of 100,000, and a model allowing for admixture and correlated allele frequencies. Ten independent runs yielded consistent results. A model-based clustering algorithm was applied that identified subgroups with distinctive allele frequencies. This procedure, implemented in computer structure, places individuals into  $K$  clusters, where  $K$  is chosen in advance but can be varied for independent runs of the algorithm. The degree of admixture,  $\alpha$ , was inferred from the data. When  $\alpha$  is close to zero, most individuals are essentially from one population or another whereas  $\alpha > 1$  means that most individuals are admixed (Falush et al. 2003). The range of possible tested  $K$ s was from 2 to 10.

Distance-based analysis of the accessions using Euclidean inferred ancestry for each accession and the key for identifying the accessions was shown in the neighbor-joining tree using the unweighted pair group method using arithmetic averages (UPGMA). This is a hierarchical algorithm for clustering accessions into similar groups. The output of this clustering procedure is a dendrogram or tree with distance along the horizontal (top) axis and the accessions listed vertically down the side. Levels of genetic variation within and among populations identified by the cluster analysis were estimated from allelic frequencies using analysis of molecular variance, AMOVA (Weir and Cockreham 1984; Weir

1996). The software Arlequin 3.0 (Excoffier et al. 2005) performs the AMOVA procedure using SSR and standard multi-locus frequency data. Wright's  $F$ -statistics are a hierarchical series of fixation indices where  $F_{IS}$  represents the deviation from Hardy-Weinberg expectation within populations (approximately equal to the mean  $F$  across populations),  $F_{ST}$  measures the fixation of different alleles in different populations, and  $F_{IT}$  measures deviations from Hardy-Weinberg expectation across the population system as a whole.

Decay of LD with distance in cM between SSR loci within the genome was evaluated using PowerMarker 3.23 (Liu and Muse 2004). The LD decay was calculated using the statistical coefficient of determination ( $R^2$ ) which is a measurement of correlation between a pair of variables (Hill and Robertson 1968). All association tests were run with the mixed linear model (MLM) method as described by Yu et al. (2006) in TASSEL 1.9.4 (<http://www.maizegenetics.net>), a recently developed unified mixed-model method simultaneously taking into account multiple levels of both gross level population structure ( $Q$ ) and finer scale relative kinship ( $K$ ). The population structure matrix ( $Q$ ) was identified by running Structure at  $K = 7$ . The relative kinship matrix ( $K$  matrix) was obtained by running SPAGeDi (Hardy and Vekemans 2002). Output from SPAGeDi was formatted to a text file readable by TASSEL. The  $P$ -value determines whether a QTL is associated with the marker and the  $R^2$ -marker evaluates the magnitude of the QTL effects (personal communication, Zhiwu Zhang, Cornell University).

## Results

The 103 accessions had a mean grain yield (GY) of 7,301 kg ha<sup>-1</sup>, a mean kernel length (KL) of 6.7 mm, a mean kernel width (KW) of 2.3 mm, a mean kernel length/width ratio (LWR) of 2.9, and a mean 1000-kernel weight (TKW) of 21.2 g (Table 2). Correlations of TKW with GY and with KW, and of KL with LWR were very significant ( $P < 0.0001$ ). Correlation of KL with TKW was significant ( $P < 0.001$ ) as was the

Table 2 Descriptive statistics for yield (GY), dehulled kernel length (KL), kernel width (KW), kernel length/width ratio (LWR), and 1000-kernel weight (TKW)

	GY (kg ha <sup>-1</sup> )	KL (mm)	KW (mm)	LWR	TKW (g)
Average	7301	6.7	2.3	2.9	21.2
Range	8822	2.4	1.1	3.0	11.2
Minimum	2897	5.4	1.8	1.0	16.2
Maximum	11719	7.8	2.9	4.0	27.4
Std. Dev.	1950	0.6	0.2	0.5	2.1
Correlations					
GY					
KL	0.019				
KW	0.186*	0.481**			
LWR	0.162*	0.846***	0.748***		
TKW	0.348***	0.395**	0.496***	0.064	

\*P < 0.01, \*\*P < 0.001, \*\*\*P < 0.0001

correlation of GY with KW ( $P < 0.01$ ). There was a positive correlation between GY and KL but it was not significant. Significant negative correlations of LWR with KW ( $P < 0.0001$ ) and GY ( $P < 0.01$ ) were found. This indicates that increasing KW and KL results in increase kernel weight and, to a lesser extent, improves GY. The correlations also suggest that increased KW has more effect on GY than increased KL. The negative correlation between KW and KL ( $P < 0.001$ ) indicates that as KW increases KL decreases.

All 123 SSR markers were polymorphic and produced a total of 1009 alleles among the 103 accessions assayed. The average number of alleles per locus was 8.2, ranging from 2 (RM338 on chromosome 3) to 21 (RM206 on chromosome 11). The average genetic diversity over all SSR loci was 0.635, ranging from 0.115 (RM512 on chromosome 12) to 0.881 (RM304 on chromosome 10).

Analysis of genetic distance and population structure provided evidence of significant population structure in these rice accessions and identified the highest likelihood value at  $K = 7$  for all ten replicates (Fig. 1). Analysis of these data identified the major substructure groups when the number of populations was set at three, however, which was consistent with clustering based on genetic distance. In this worldwide sample, accessions from the same predefined population nearly always shared

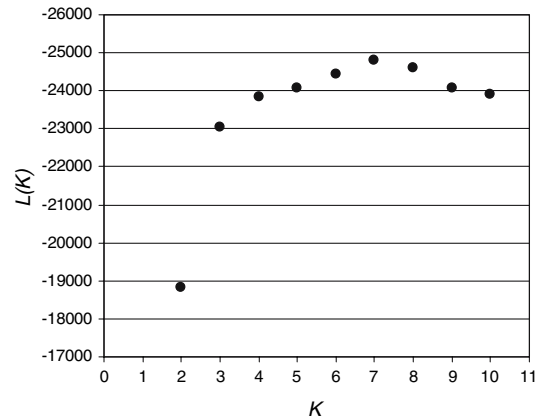
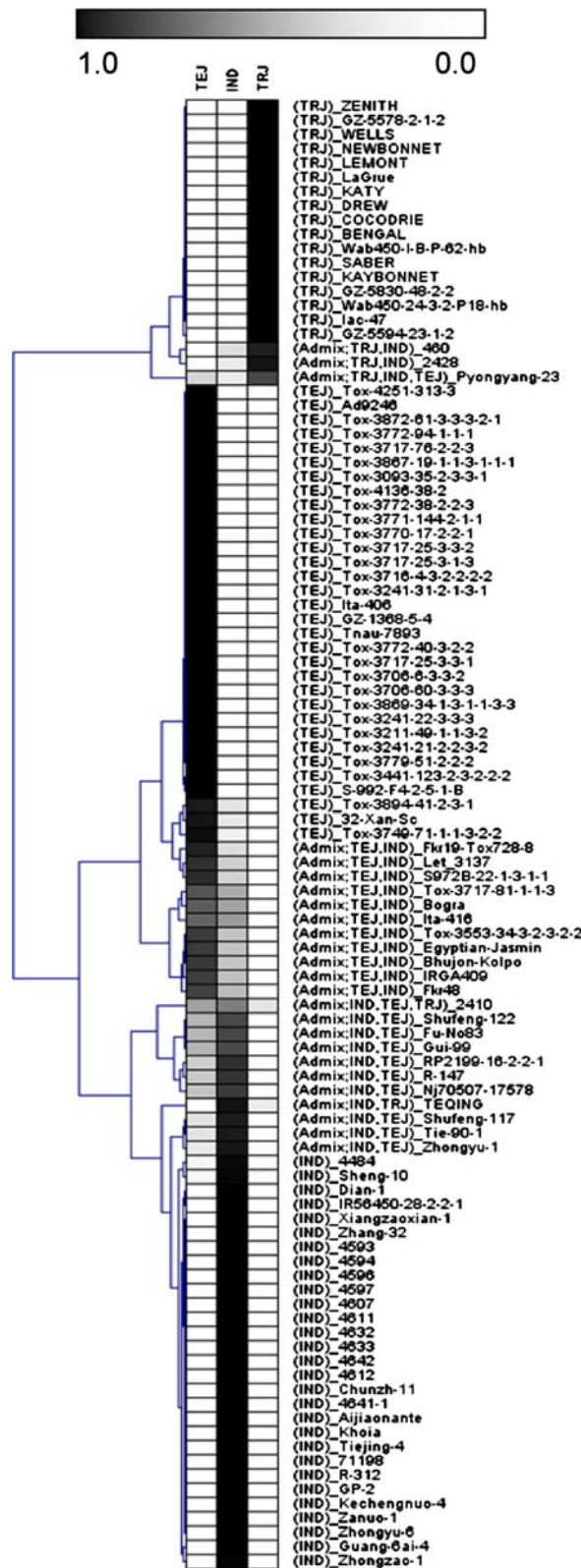


Fig. 1 Log probability of data,  $L(K)$ , averaged over the replicates

similar membership coefficients in inferred clusters (Fig. 2). At  $K = 3$ , most accessions were classified into one of the three groups, which corresponded to the traditional rice sub-species indica (29 accessions), temperate japonica (32), and tropical japonica (17) separated by relatively large genetic distance. In addition to these 78 accessions that were clearly assigned to a single population, where more than 85% of their inferred ancestry is derived from one of the model-based populations, 25 accessions in the sample were categorized as having a mixed ancestry, defined as an "admixed" (Fig. 2). Although most of these were identified as admixture between indica and temperate japonica groups, other admixture combinations were also present.

Divergence among the accessions was found using all measures of population structure. The Structure model-based analysis for several theoretical population sizes (defined in this study as ancestral backgrounds) with the highest posterior probability was for a model with seven different backgrounds (Fig. 3, center). For these ancestral backgrounds, a burn of 10,000 runs followed by data collection on 100,000 runs seemed to be sufficient, giving reasonably consistent values of  $\ln \text{prop data} (X|K)$  across replicates. At  $K = 7$ , the backgrounds largely corresponded to major geographic regions with one background being US accessions. Several accessions, for example "Tox3717-25-3-3-2" and "Shufeng-122", had par-



◀Fig. 2 Estimated population structure ( $K = 3$ ) for the 103 accessions as rice subgroup tropical japonica (TRJ), temperate japonica (TEJ), and indica (IND). Each accession is represented by row, which is partitioned into  $K$  colored segments according to the individual's estimated membership fractions in each of the  $K$  clusters

tial membership in multiple backgrounds. At  $K = 7$ , the cluster that included tropical japonica populations split into two clusters, of which one includes all US genotypes (Fig. 3).

Distance-based analysis of the 103 accessions with Euclidean distance detected eight major clusters (Fig. 3, left and right sides). The accessions were classified into eight clusters by the UPGMA and algorithm neighbor joining tree based on the genetic similarity matrix. These clusters usually agreed with the origin of the accessions and the clustering previously defined by Eizenga et al. (2006). All US accessions were grouped in cluster 1; cluster 3 contained Chinese lines from J. Tao, cluster 4 contained the breeding lines obtained from the Africa Rice Center (WARDA), Bouake, Ivory Coast, cluster 7 mainly included Chinese lines from Chengdu, and cluster 8 contained the Chinese lines from Hangzhou (Fig. 3). Clusters 2, 5, and 6 had more than one ancestral background, defined as an admixture, and these clusters included accessions from Bangladesh, Brazil, Egypt, North Korea, the Philippines, and China.

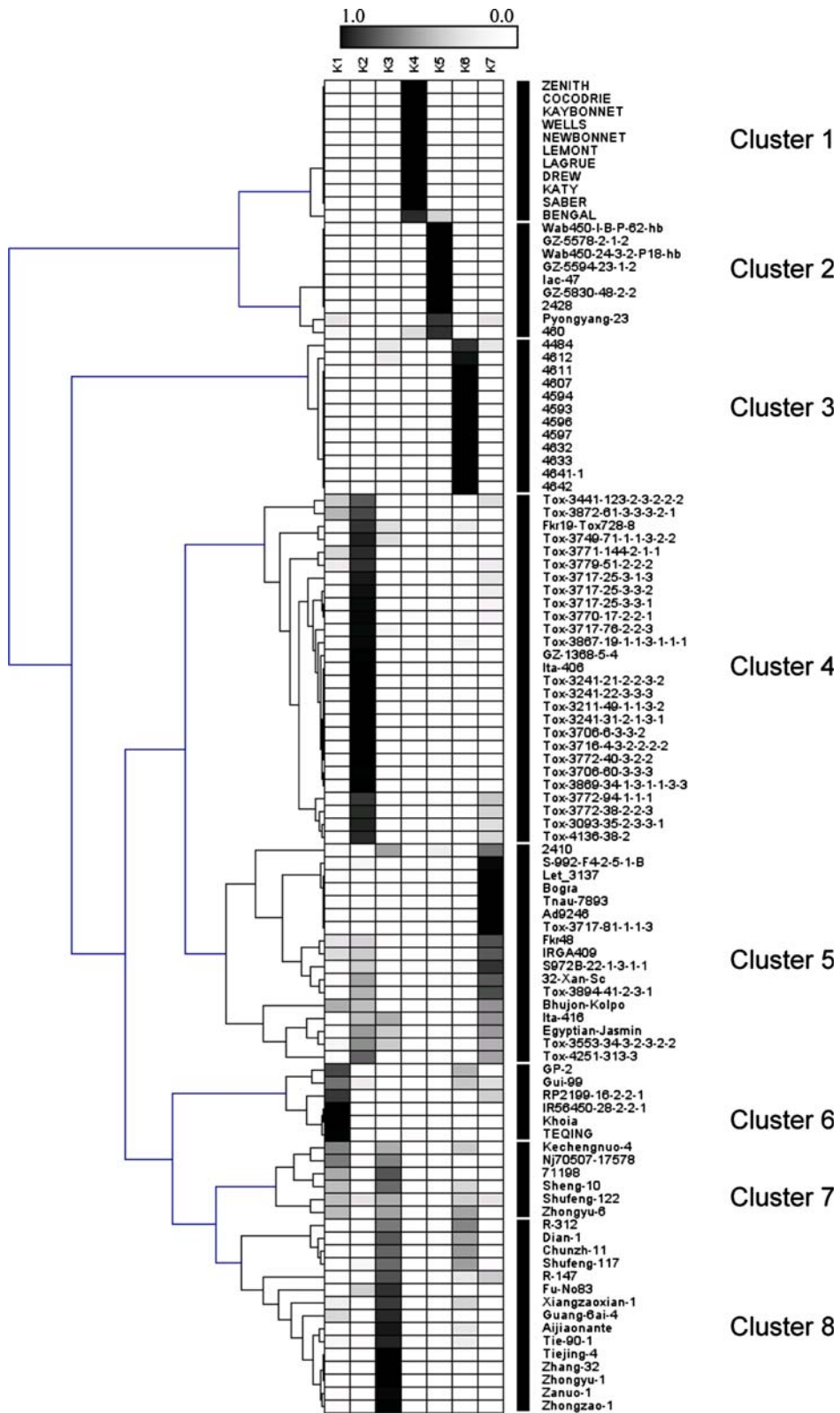
The distribution of molecular genetic variation among and within the eight clusters of accessions was estimated by AMOVA, which revealed 41% of total variation was among the clusters whereas 51% of the variation was within the clusters (Table 3). Calculation of Wright's  $F$  statistics for all SSR loci revealed  $F_{IS}$  was 0.66, suggesting there was deviation from Hardy-Weinberg expectation for molecular variation within the clusters,  $F_{IT}$  was 0.71, signifying non-equilibrium conditions across clusters and a deficiency of heterozygous SSR loci, and  $F_{ST}$  was 0.312 indicating 31.2% of the total genetic variation was among the clusters.

Determination of  $F_{ST}$  for the polymorphic SSR loci across all accessions revealed  $F_{ST}$  ranged from 0.06 for RM120 on chromosome 11 to 0.94 for RM124 on chromosome 4 with an average of 0.381, indicating 38.1% of the total variation in

allele frequency of the 103 accessions was because of genetic differences among clusters. Determination of the pair-wise  $F_{ST}$  values between the eight clusters (Table 4) indicated that genetic differentiation among clusters was highest for the combination of clusters 1 and 3 ( $F_{ST} = 0.719$ ). The  $F_{ST}$  values for cluster 1 paired with clusters 4, 5, 6, 7, and 8 ranged from 0.649 to 0.533 and  $F_{ST} = 0.381$  for clusters 1 and 2. These values confirm that the US accessions were quite different from most other accessions except those in cluster 2. The combination of clusters 7 and 8 had the lowest  $F_{ST}$  value (0.110), because of the shared ancestry of these clusters. The average  $F_{ST}$  value for all eight cluster combinations was 0.391.

The distribution of data points in the plot of LD ( $R^2$ ) decay against distance (cM) within the twelve chromosomes (Fig. 4) showed that LD was not a simple monotonic function of the distance between markers. LD was very common for distances <30 cM and occasionally, LD occurred between SSR loci that were further apart. The  $R^2$  between unlinked markers on different chromosomes was mainly <0.4. Some unlinked SSR markers associated with blast resistance were identified using these accessions by Eizenga et al. (2006) and had higher  $R^2$ . For example,  $R^2$  values were 1.0 (RM206 on chromosome 11, OSM89 on chromosome 12), 0.96 (RM208 on chromosome 2, RM5963 on chromosome 6), and 0.91 (RM3431-ch 6, RM144-ch 11). In contrast with a-priori expectation, some marker pairs that were close together on the Gramene map were not correlated across the genotypes and so were in linkage equilibrium.

Association analysis (Table 5) identified marker-trait associations ( $P < 0.05$ ) for all the traits evaluated, these included GY, KW, and LWR, associated with markers each, KL, with six markers, and TKW, with four markers. A total of 25 marker-trait associations were identified with 21 different SSR markers. Four markers were associated with two traits: RM85 on chromosome 3 was associated with KW and TKW, RM122 on chromosome 5 was associated with KL and TKW, RM459 also on chromosome 5 was associated with KL and TKW, and RM228 on chromosome 10 was associated with GY and LWR. Seventeen of the 25 associations were in regions where QTL associated with the given trait had previously



◀Fig. 3 Population structure and distance-based analysis of the 103 rice accessions using Euclidean-inferred ancestry for each accession. Initially, the accessions were divided into clusters based on UPGMA and neighbor joining (left and right sides). Next, the accessions were divided into seven ancestral backgrounds defined at (center) based on analysis in Structure. Accessions were assigned to a single background or to two or more backgrounds if the genotype indicated the accession was admixed with membership in two or more different backgrounds and estimated on a scale from 1.0 (accession is from one background only) to 0.0 (accession is not from this background)

been identified (<http://www.gramene.org>). Of the 25 marker-trait associations, seven were identified as explaining 20% or more of the total variation ( $R^2$ , Table 5) for GY (RM261, RM228), KL (RM284), LWR (RM7, RM228), and TKW (RM440, RM122). Only RM284 associated with KL was not in the region of a previously identified QTL for the associated trait.

## Discussion

The genetic structure of rice has previously been documented (Glaszmann 1987, Parsons et al. 1999, Ni et al. 2002, Garris et al. 2005) but this analysis uses accessions recently introduced into the USA that have blast resistance (Eizenga et al. 2006). By use of Structure software with  $K = 3$ , the *O. sativa* rice accessions were significantly differentiated into three subgroups, temperate japonica, tropical japonica, and indica. The analysis revealed that several accessions had partial ancestry in more than one background. These accessions probably had a complex breeding history involving intercrossing and introgression between germplasm from diverse backgrounds, overlaid with strong selection pressure for agronomic and quality characteristics (Mather et al. 2004). Population structure analysis identified

Table 4 Pair-wise  $F_{ST}$  values between eight clusters as identified using Euclidean distance in Fig. 3

Population	1	2	3	4	5	6	7	8
1	0							
2	0.381	0						
3	0.719	0.601	0					
4	0.609	0.485	0.496	0				
5	0.561	0.418	0.392	0.169	0			
6	0.649	0.467	0.479	0.362	0.198	0		
7	0.589	0.412	0.360	0.291	0.168	0.142	0	
8	0.533	0.400	0.301	0.291	0.157	0.208	0.110	0

eight main clusters for the accessions that corresponded to major geographic regions. General agreement between the genetic and predefined clusters suggests that knowledge of the ancestral background can facilitate choices of parental lines in rice-breeding programs (Rosenberg et al. 2002). Although both rice-breeding efforts and domestication have had large effects on structuring the diversity of rice, the independent population histories of the groups have also shaped the gene pools (Garris et al. 2005).

Values of  $F_{ST}$  were high when the eight clusters were considered, thus identifying large differences between the accessions. It has been demonstrated that markers with higher  $F_{ST}$  values have greater resolving power and produce more consistent genetic distance estimates (Watkins et al. 2003). The significant  $F_{ST}$  among the clusters suggests a real difference between these clusters, and heterosis might be observed for crosses between the accessions made to improve yield (NõGoran et al. 2000).

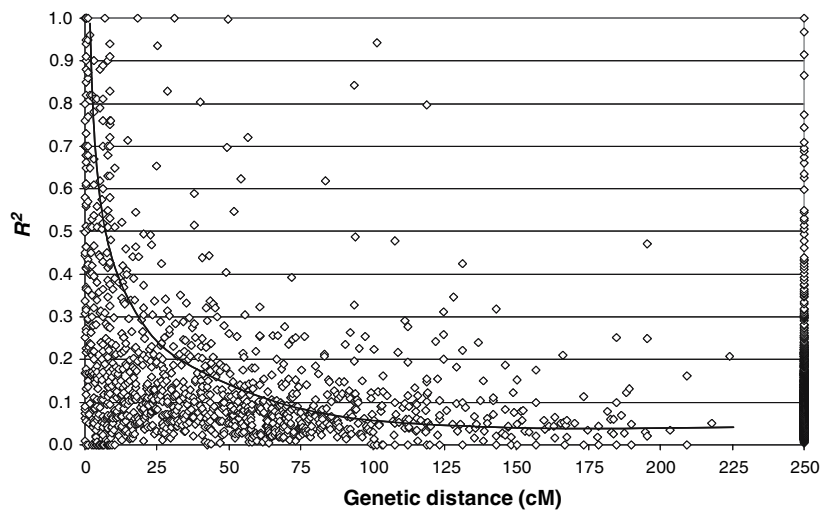
Linkage disequilibrium (LD) studies have now been conducted for more than a dozen plant systems, both at the individual gene level and at the level of whole genome. In individual species, these studies included:

Table 3 Analysis of molecular variance (AMOVA) for the eight clusters of rice accessions identified in Fig. 3

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation	P-value
Among clusters	7	1193.29	6.14	41.37	<0.001
Among accessions within clusters	95	1707.25	8.32	50.71	<0.001
Within accessions	103	136.50	1.32	7.92	<0.001
Total	205	3037.04	15.79		

Fixation indices:  
 $F_{IS} = 0.663$ ,  $F_{ST} = 0.312$ ,  
 $F_{IT} = 0.710$

Fig. 4 Scatterplot of the LD ( $R^2$ ) of marker pairs as a function of the intermarker distance in cM. A genetic distance of 250 cM was chosen to represent unlinked loci on different chromosomes. LD analysis was performed on the entire population (103 accessions)



- (i) estimation of the extent of LD in different plant genomes or in different parts of the genome of an individual species,
- (ii) measure of nucleotide diversity/haplotype structure,
- (iii) assessment of the effect of selection/domestication,
- (iv) identification of marker-trait associations, Gupta et al. (2005).

Table 5 Association ( $R^2$ ) of SSR markers with yield (GY), dehulled kernel length (KL) and width (KW), length/width ratio (LWR), and 1000 dehulled kernels weight (TKW), as described in Table 2

Trait	SSR Marker <sup>a</sup>	Chromosome no.	Position (cM)	P	$R^2$ <sup>b</sup>
GY	RM416	3	191.6 <sup>c</sup>	0.0515	0.1244
GY	RM261	4	35.4 <sup>c</sup>	0.0016	0.2032
GY	RM447	8	124.6	0.0370	0.1950
GY	RM271	10	59.4 <sup>c</sup>	0.0111	0.1909
GY	RM228	10	96.3 <sup>c</sup>	0.0118	0.2542
KL	RM259	1	54.2 <sup>c</sup>	0.0237	0.1016
KL	RM16	3	131.5 <sup>c</sup>	0.0317	0.1155
KL	RM122	5	0.0 <sup>c</sup>	0.0167	0.1226
KL	RM284	8	83.7	0.0197	0.3258
KL	RM202	11	54.0	0.0105	0.1012
KL	RM287	11	68.6	0.0491	0.1994
KW	RM468	3	202.3	0.0060	0.1351
KW	RM85	3	231.0	0.0103	0.1451
KW	RM459	5	93.6 <sup>c</sup>	0.0041	0.1305
KW	RM248	7	116.6	0.0106	0.1867
KW	RM152	8	9.4 <sup>c</sup>	0.0022	0.1318
LWR	RM109	2	0.0	0.0009	0.1287
LWR	RM7	3	64.0 <sup>c</sup>	0.0171	0.2359
LWR	RM245	9	112.3	0.0006	0.1748
LWR	RM228	10	96.3 <sup>c</sup>	0.0203	0.3213
LWR	RM147	10	99.8 <sup>c</sup>	0.0139	0.1330
TKW	RM449	1	78.4 <sup>c</sup>	0.0118	0.2199
TKW	RM85	3	231.0 <sup>c</sup>	0.0429	0.1914
TKW	RM122	5	0.0 <sup>c</sup>	0.0039	0.2661
TKW	RM459	5	93.6 <sup>c</sup>	0.0509	0.1750

<sup>a</sup> Only SSR markers with a significant marker-trait association are reported ( $P < 0.05$ )

<sup>b</sup>  $R^2$  indicates the percentage of the total variation explained

<sup>c</sup> QTL for the given trait previously reported in the same region (<http://www.gramene.org>)

The LD between unlinked loci, for example RM208 and RM224 on chromosomes 2 and 11, and RM3431 and RM144 on chromosomes 6 and 11, was occasionally significant. These markers are linked with three blast-resistance (*Pi*-) genes located on these chromosomes (Fjellstrom et al. 2004). LD was high among unlinked SSR markers around *Pi* that were associated with blast resistance in these accessions. Our results suggest that mapping strategies exploiting LD around the region of *Pi*-loci may be particularly effective in rice, as it was in exploiting the haplotype diversity and LD surrounding the *xa5* locus (Garris et al. 2003). Further research will be conducted to describe the diversity and decay of linkage disequilibrium in this region of the rice genome, to characterize the extent of LD in resistant accessions, to determine if it is possible to reduce the number of candidate genes, and to analyze haplotype diversity in the context of population structure to determine the distribution of the resistance allele among ecotypes and to make predictions about the allelic diversity underlying the *Pi* phenotype. In contrast with the situation in the human, genome-wide LD mapping of which may require a marker density two times of magnitude higher than that required for conventional linkage mapping, the available 150 microsatellites could be sufficient for first-pass LD screening in rice genotypes (Kruglyak 1999). The corollary of this observation, however, is that the mapping resolution to be gained from LD is likely to be limited in these populations also. In this work we still observed a substantial drop in LD values between 20 and 30 cM, suggesting it should, nevertheless, be possible to achieve resolution down to the 25 cM level. The same observation on LD at larger distances was found in *Arabidopsis* (Nordborg et al. 2002) and barley (Kraakman et al. 2004). In sugar beet lines, however, LD was <3 cM (Kraft et al. 2000), in *Lolium perenne*, LD was <3.4 cM (Skjelt et al. 2005), and in maize LD diminished over a distance of 2000 bp (Remington et al. 2001). Significant LD between pairs of unlinked markers was observed (McRae et al. 2002; Skjelt et al. 2005), emphasizing the advantage of both linkage and LD testing. Many factors affect LD (Ardlie et al. 2002), but the most probable cause of the

high level of LD in rice is that it is a self-pollinated species. Selection can also increase LD, for instance, by a hitchhiking effect in which the alleles at flanking loci of a locus under selection can be rapidly swept to high frequency or fixation (Kraakman et al. 2004). Further analyses will be required to evaluate the benefit of LD mapping at the sub-cM level in these populations.

For LD between two multi-allelic loci,  $r^2$  (statistical coefficient of determination) and  $D'$  (absolute ratio of deviation of haplotype frequencies from disequilibrium compared with its maximum value) are the most widely used measure of LD for each pair of alleles, or even for overall LD between all the alleles at two loci (Gupta et al. 2005). Whereas  $D'$  measures only recombination differences,  $r^2$  summarizes recombination and mutation history. Also,  $r^2$  is indicative of how markers might be correlated with the QTL of interest, so  $r^2$  is often preferred for association studies (Abdallah et al. 2003). In the sets of cultivated rice examined here, linkage disequilibrium as  $r^2$  is present on a scale that could be useful for association mapping. Genome-scale association mapping should be possible, if adequate methods are implemented to control the effects of population structure. Model-based analyses of population structure, for example those conducted here, may be helpful for providing information that could be incorporated into association mapping analyses.

Fear of false-positive outcomes arising from population stratification has virtually dictated progress in human association study design and analysis methodology (Cardon and Bell 2001; Yu et al. 2006). Genome-scale association mapping should be possible if adequate methods are implemented to control for the effects of population structure. Association mapping without consideration of population structure would have a high rate of Type I error (false positive) because of spurious associations between non-linked loci. Model-based analysis of population structure similar to that conducted here has provided information that has been incorporated into association mapping analysis.

In the eight clusters identified in this study,  $F_{IS}$  was high, suggesting that most of the populations

deviated from the Hardy–Weinberg expectation within populations. The  $F_{IT}$  value, indicating nonequilibrium conditions across populations and deficiency of heterozygotes, was also high. Testing for association between the SSR multilocus genotype data associated with quantitative variation in the presence of population structure (Thornsberry et al. 2001; Remington et al. 2001) was applied using the MLM procedure in TASSEL (Yu et al. 2006). The positive results from these studies should encourage the further testing of these methods in different genetic systems, in the same way as we applied these procedures to rice. Association between markers and grain yield, dehulled kernel length, and width, length/width ratio, and 1000-kernel weight was examined in two ways—significance of marker-trait association ( $P$ -values) using TASSEL software and marker-trait associations found in other QTL studies reported in <http://www.gramene.org/>. This indicates that QTL detected in mapping populations from biparental mapping populations were widely presented in this set of accessions, and that they could be detected with LD mapping. Association between traits and marker regions that had not been implicated before to affect trait suggest new QTL. The trait markers association was indicative of a rapid decrease in correlation, suggesting LD across a short distance. Within different sets of barley germplasm there was frequent disequilibrium among non-linked SSR loci, suggesting that association mapping without consideration of population structure would have a high rate of false positive Type I error (Mather et al. 2004). A unified mixed-model approach to account for multiple levels of relatedness simultaneously, as detected by genetic markers, has resulted in improved control of both type-I and type-II error rates (Yu et al. 2006).

Plant genetics has an important and challenging goal of identifying the genetic variants that underlie complex traits. Two main approaches are available for mapping the relevant genes and identifying the variants that associate with the traits: linkage mapping in families and population-based genetic association studies. Linkage mapping has been very successful in finding genes for rare, Mendelian, monogenic disease resistance. For complex traits that involve variants at

several loci, each of which contribute small amounts to the overall genetic contribution, linkage studies mainly identify only those loci with the strongest influence, however. The most significant finding of this paper is that the LD in this set of germplasm did not decay until 20–30 cM. These results could have important implications for association testing in rice. There are two previous studies of LD in rice. The first is that of Garris et al. (2003) who found LD to decay at 100 kb across one region on chromosome 5. In a second study Olsen et al. (2006) analyzed a 500-kb region on chromosome 6 and found a 250 kb selective sweep at the waxy locus that led to elevated LD in that region. This would indicate that LD decay at 250 kb was unusually high, because of selection on the waxy locus. Although the amount of LD will vary across the genome (because of recombination rates, selective pressures, etc.), these studies seem indicative of LD decaying in rice at 1 cM or less (assuming an average of 250 kb/cM across the genome). This is in contrast with the LD decaying at 20–30 cM in the current study. LD in some Arabidopsis populations exceeds 50 cM (Nordborg et al. 2002), however, and LD as a function of genetic distance is very common for distances <10 cM (Kraakman et al. 2004) in barley.

In theory, genetic association mapping has greater power than linkage studies to identify variants with weak effects that might contribute risk for common complex traits (Risch and Merikangas 1996). Whole-genome association studies have the advantage of enabling the entire genome to be assessed for trait-associated variants, rather than analyzing specific candidate genes. The disadvantage of such studies, however, is that a large amount of genotyping is required. This can be reduced by using a subset of markers to report on neighboring linked markers within the genome (Smith and O'Brien 2005). Application of association mapping to plant breeding seems to be a promising means of overcoming the limitations of conventional linkage mapping (Stich et al. 2005). Our results have shown that LD studies are an efficient means of indicating novel genes for important agronomic characters that subsequently can be validated in specific biparental crossing populations, and for con-

ing QTL that have been detected in mapping populations.

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