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Marker-trait associations for improving cooking quality in glutinous rice

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Glutinous rice is an important cereal crop of Eastern India. Understanding the genetic structure of glutinous rice is a key point for its further utilization in breeding programmes. In the present study, the marker association with cooking and eating quality (amylose content, gelatinization temperature & gel consistency) was studied in 138 glutinous rice accessions grown in different places of Assam using 60 simple sequence repeat (SSR) markers. Majority of the genotypes were waxy type with amylose content <0.05%. The genotypes could be divided into 9 subpopulations based on STRUCTURE analysis. Allele frequency divergence among 9 subpopulations ranged from 0.215 to 0.454. A total of 356 alleles were detected with an average of 6.23 alleles per SSR locus. Nei's gene diversity revealed that glutinous rice accessions of Brahmaputra valley were more diverse. A clear-cut divergence of *japonica* accessions from other glutinous rice was observed. Genome-wide scanning detected a total of 20 significant marker-trait associations (P<0.01), with the R^2 values ranging from 5.0 to 43.0%. Those markers showing strongest effects could provide ideal candidates for further study in marker-assisted selection.

Keywords: Association mapping, cooking and eating quality, glutinous rice, marker assisted breeding, SSR polymorphism

Introduction

In North-East India rice plays a pivotal role in the socio-cultural life of the people. Among different classes of rice, waxy rice or glutinous rice, known as *bora* in vernacular, is an important class of rice. It is a type of short-grained rice that is especially sticky when cooked and is also grown in Japan, Korea, China, Philippines, Thailand, Indonesia and Vietnam. This class of rice was introduced into Assam from Thailand or Burma a considerable time ago[1]. Glutinous rice of greater Assam has significance in social and religious ceremonies and forms a popular daily breakfast diet in rural Assam. Milled rice is also used in the preparation of snacks, flat rice, puffed rice, bamboo rice, sweet rice beer and other dishes. The multiplicities of uses make the glutinous rice very popular among farmers, which are reflected by the cultivation of many landraces of glutinous rice in Assam, in spite of the advent of modern high yielding rice varieties. In general, diverse landraces traditionally cultivated by farmers around the centers of diversity and domestication of crops are considered as key natural resources[2], and their conservation is important for maintaining the future food security in light of the changing climate. Marker aided studies already revealed the existence of sufficient genetic diversity in glutinous rice of Assam[3,4]. However, most of these previous studies assessed either few marker loci or few genotypes without taking in to account of marker-trait association.

In rice, the three key components determining cooking and eating quality are amylose content, gelatinization temperature and gel consistency. Amylose content (AC) is regarded as the most important indicator in classifying rice varieties[5], because it influences texture and retro-gradation potential of cooked grains[6]. Most of these grain quality traits of rice are controlled by quantitative trait loci (QTLs) showing continuous variation in rice progeny[7]. Molecular marker technology has facilitated the understanding of the genetic basis of complex quantitative traits, such as, eating quality in rice[8]. So far, several studies reported the QTLs for rice grain quality by different populations[9]. Association mapping is an effective approach to detect marker-trait association, and enables researchers to use modern genetic technologies to exploit natural diversity and locate valuable genes in the genome[10]. Population structure is an important component in association with mapping analysis to reduce spurious

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association between genotypic and phenotypic variation because of the unequal allele frequency distribution between subgroups[11]. Using SSR markers, association mapping has been successfully done in rice[12].

Despite several reports on the population genetic structure of *Oryza sativa* germplasm at a global scale[13], region specific studies with a particular class of genotypes, as in the case of glutinous rice of Assam, are limited. Moreover, no information on markers associated with cooking quality traits are available in this class of rice, which evolved in a region of high genetic diversity. Because of the wide genetic diversity, we presume the presence of genetic associations between marker and cooking quality traits useful for breeding rice. This presumption was tested by comparing genetic diversity in glutinous rice landraces of Brahmaputra and Barak valley of Assam, and evidence of genetic associations between SSR markers and three main cooking quality traits was identified by taking account of population structure in this class of germplasms.

Materials and Methods

Materials and Phenotype Analysis

Rice landraces were collected randomly from different parts of Assam and, in total, 138 accessions were collected (†Suppl Table S1). Among them, 3 were high yielding varieties of Assam; 8 were *japonica* accessions collected from International Rice Research Institute (IRRI), Philippines. The rest were traditional rice germplasms collected from different parts as well as farmer's field of Brahmaputra valley (lying in between 25°44′ N-28° N latitude and 89°41′ E-96°02′ E longitude) and Barak valley of Assam (lying in between 24°08′ N-25°8′ N latitude and 92°15′ E-93°15′ E longitude), which were being maintained in two Regional Agricultural Research Stations of Assam Agricultural University, Jorhat. Seeds were purified by growing for one generation and data on cooking quality measure were taken thrice for each genotype. Amylose content was determined by the method as described by Juliano[14]. Gelatinization temperature (GT) was assessed indirectly as the alkali spreading value (ASV) of hulled kernels as per modified procedure of Little *et al*[15]. Gel consistency (GC) was determined by the method of Cagampang *et al*[6].

PCR Assay and Genetic Diversity Analysis

Young leaves were harvested during seedling stage for DNA extraction. The DNA extraction was performed according the Plaschke *et al*[16] with slight modification. SSR markers were screened at even distribution across the rice genome based on the published map information[17]. Those markers which showed clear-cut polymorphism with at least 10 bp differences among the amplicons in randomly chosen 20 genotypes were selected for the present work. Altogether, 60 polymorphic markers distributed across 12 chromosomes were selected from 150 markers initially used for the polymorphism survey. The PCR amplification was done with conditions optimized in our laboratory[18].

Data were entered in the form of single-individual genotypes. Genetic diversity parameters, *viz.*, number of alleles (*Na*), effective number of alleles (*Ne*) per locus, observed heterozygosity (*Ho*), Shannon's Index (*I*), and Nei's genetic diversity index (*He*) as given by Nei[19] were evaluated using POPGENE V 1.31[20].

Population Structure and Association Analysis

The presumption of association of SSR markers with AC, GC and GT in the presence of population structure was tested using a mixed linear model (MLM), as described by Yu *et al*[21], in the program TASSEL 2.0.1 (http://www.maizegenetics.net/) by taking into account multiple levels of both gross population structure (Q) and finer-scale relative kinship (K). The K matrix was generated based on 60 SSRs using kinship matrix function in TASSEL. Population structure consisted of a Q matrix that describes the percent subpopulation parentage for each line in the analysis. These percentages were calculated by STRUCTURE 2.3.3 software[22]. We set k (the number of subpopulations) from 2 to 20 and performed 3 runs for each k value. Length of burn-in period as well as numbers of iterations were set at 1,50,000 with the admixture model and correlated to allele frequency according to Falush *et al*[23]. A graphical representation of the genetic distances among the K Structure clusters was computed by applying the neighbor-joining algorithm to the matrix of allele-frequency divergence among clusters[24] using the program NEIGHBOR[25] and implementing Saitou and Nei's[26] "Neighbor Joining Method". The plot was produced using DRAWTREE[25].

Results and Discussion

A significant difference was observed among the accessions for AC, GC and GT (Table 1). The AC of the accessions varied from 0.05 to 29.6%. Likewise, GC varied from 18 to 100 mm and the GT score varied from 2 to 7. Of 138 accessions studied, 0-5%

Table 1—Variations among 138 accessions of glutinous rice under study

Parameters	Minimum	Maximum	Mean	Std. Error
Amylose content (AT) (%)	0.050	29.600	5.591	0.721
Gel consistency (GC)	18.000	100.000	91.442	1.255
Gelatinization temperature (GT)	2.000	7.000	6.080	0.126

AC was found in 95 accessions. Similarly, 5-10, 10-15, 15-20, 20-25 and 25-30% AC were found in 10, 5, 9, 14 and 5 accessions, respectively.

SSR Polymorphism and Genetic Diversity

Sixty markers distributed evenly on all the 12 chromosomes were chosen to assess the genetic relationships among the 138 genotypes. Altogether 356 alleles were amplified by these SSRs with an average *Na* of 6.23 per locus, with the *Na* ranging from 4 to 10 (†Suppl Table S2). The overall size of amplified products ranged from 85 bp (in locus RM276) to 400 bp (in locus RM592). The average *Na* detected by these SSR primers was least in *japonica* accession (3.3), whereas approx 5 alleles per locus were detected in the population of Brahmputra and Barak valley. Yu *et al*[27] studied 193 rice accessions drawn from 26 countries using 101 SSR primer pairs and detected an average Na of 6.3 per locus. Lapitan *et al*[28] also reported an average of 5.89 alleles per microsatellite locus. However, Ghneim *et al*[29] reported lower average of 4.23 alleles per locus in Venezuelan rice cultivars. Furthermore, higher average *Na* per locus was also observed in other previous studies. For example, Kuroda *et al*[30] reported an average of 9.28 alleles per locus over 7 SSR loci, while Luce *et al*[31] reported an average of 9.1 alleles per locus during the analysis of 419 rice accessions from the gene banks in five European countries using 16 SSR loci (different from the ones we selected). The higher average value compared to the present study could probably be as a result of larger number of accessions used in those studies.

The SSR markers RM51 and RM475 produced the highest *Ne* in all the populations. However, the maximum *Ne* (2) was produced by RM19 and RM25 in *japonica* population, RM153 and RM592 (1.8) in Barak valley population, and RM541 and RM423 (1.9) in Brahmaputra valley population. These values are comparable (2.19) with Kibria *et al*[32], but were lower than the value of obtained by Tabkhkar *et al*[33] (3.74). Nei's[19] gene diversity or *He* is another common diversity index in population genetics and is equivalent to polymorphism information content (PIC). *He* for microsatellite loci ranged from 0.06 (in RM307) to 0.46 (in RM541) with an average of 0.16 (†Suppl Table S2). The average *He* value of the present study is lower compared to the earlier studies[29,33]. However, smaller value (0.119) was recorded by Kibria *et al*[32] in case of aromatic rice genotypes using three SSR markers. In the present study, the low *He* values might be due to inclusion of majority of genotypes from *indica* group, which is in corroboration with the findings of Choudhary *et al*[34]. Among the *japonica* group, *He* values ranged from 0.04 (in RM182) to 0.5 (in RM25), with an average of 0.21. Similarly, among the Karimganj population, *He* values ranged from 0.004 in RM55 to 0.58 in RM72 with an average of 0.05. On the other hand, among the Brahmaputra valley population, *He* values ranged from 0.08 (in RM424) to 0.46 (in RM153), with an average of 0.15. The average genetic diversity as measured by *I* was 1.448 in all the genotypes. This value was within comparable estimates of average genetic diversity in Barak and Brahmaputa valley populations (*I*=1.3). These results also indicate that *japonica* population is more diverse than *indica* population. *Ho* per locus on average was 0.04, with a range of 0 to 0.19 (†Suppl Table S2), which is obvious for the self-pollinated nature of the rice crop.

Admixture Model Based Population Clusters

Population structure is a consequence of departures from random mating in the sampling population that result in some individuals being more closely related than others. Population structure represent genetic relatedness between samples at different scales, and is a confounding factors in genome-wide association studies (GWAS) that can decrease power and increase the false positive rate (Type I error) of tests of association[35]. Such effects of population structure have been amply demonstrated in autogamous species, such as, barley and rice[36]. Association mapping requires population structure to be taken into account to avoid false positive associations. In the present study, model-based Bayesian clustering methodology was used to analyze 60 linked SSR markers in all 138 accessions. The number of clusters did not peak in the range of two to ten subpopulations,

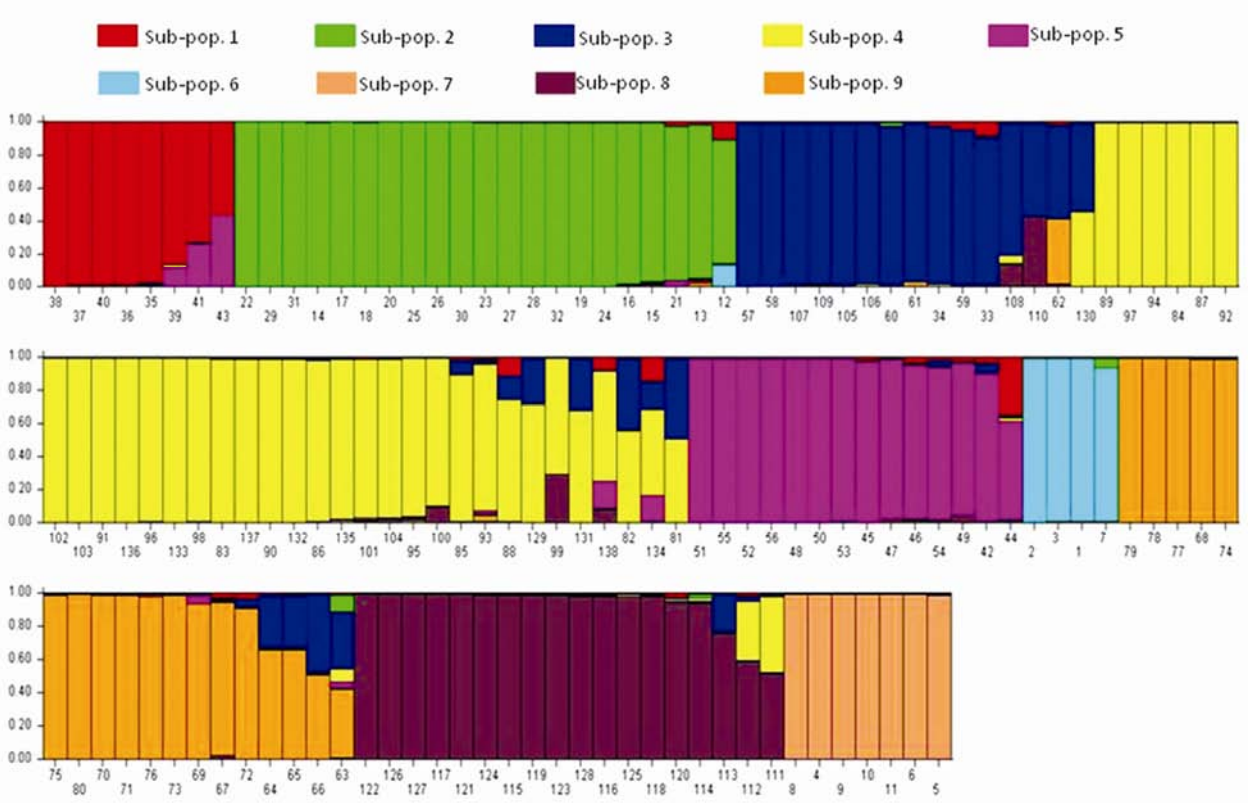

Fig. 1—Bar plot showing genetic diversity for 138 glutinous rice genotypes using the program STRUCTURE. [Each accession is divided into a number of hypothetical subpopulations based on the proportional membership (a vertical bar expressed as %) from K=2 to K=20, with the most divergent subpopulations were obtained at K=9. Each group is represented by a different colour as listed: green, pink, yellow, cyan, brown, blue.]

and beyond nine, the increase was not significant and hence K=9 was selected as the optimal cluster number. Therefore, the structure results of K=9 were considered the best possible partition as they showed a high consistency clustering based on genetic distance (Fig. 1). Thus, 22, 15, 13, 12.4, 12, 10, 6, 5 and 3% of the accessions were assigned to yellow, green, coffee, blue, light brown, pink, red, brown and cyan subgroups, respectively. Fig. 1 also shows the number of lines in each cluster, and the percent mixing of each line within each cluster, a useful visualization of admixture. Altogether 37 genotypes were found be admixtured in varying proportion. Graphical representation of genetic distance among the k=9 subpopulations applying the neighbor-joining algorithm to the matrix of allele-frequency divergence among subpopulation is given in Fig. 2. Allele frequency divergence among groups measured as net nucleotide distance[35] in Table 2 shows that allele frequency distance ranged from 0.454 (between subpopulation 1 and subpopulation 9) to 0.215 (between subpopulation 1 and subpopulation 5).

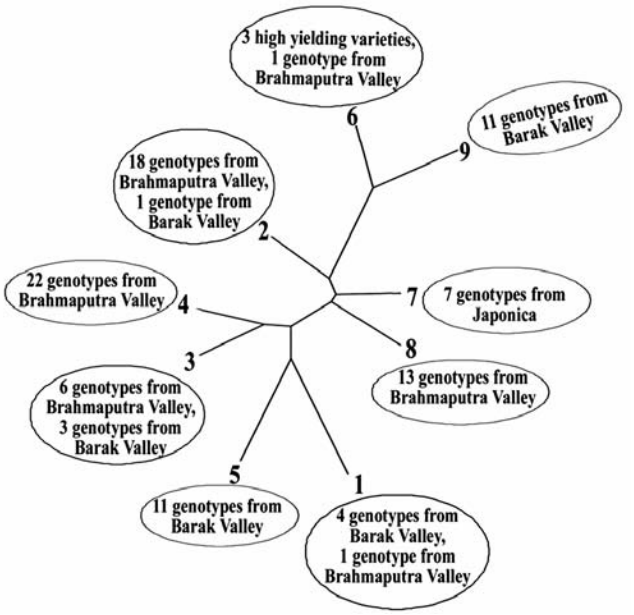

Fig. 2—Graphical representation of the genetic distances among the K Structure clusters. The trees were computed by applying the neighbor-joining algorithm.

Table 2—Allele frequency divergence among groups measured as net nucleotide distance computed by point estimate (k=9) in STRUCTURE 2.2

Subpopulation	1	2	3	4	5	6	7	8	9
1	0.370	0.379	0.250	0.277	0.215	0.388	0.348	0.342	0.454
2		0.423	0.405	0.358	0.325	0.335	0.284	0.301	0.424
3			0.383	0.247	0.313	0.363	0.328	0.343	0.441
4				0.440	0.273	0.377	0.324	0.287	0.409
5					0.457	0.393	0.264	0.309	0.443
6						0.439	0.350	0.381	0.296
7							0.473	0.292	0.449
8								0.393	0.445
9									0.395

(Diagonal values indicate average distances between individuals in same subpopulation)

Table 3—Genetic diversity parameters in 9 subpopulations of glutinous rice used in the study

Sub-population	Genotypes	Na	Ne	I	He	No. of polymorphic loci	% polymorphic loci
1	Kmj Bora1, Kmj Bora2, Kmj Bora5, Kmj Bora9, Kola Ampakhi (5 genotypes)	1.569	1.446	0.320	0.209	23	38.33
2	Tilesh Biroin, Pakhori Bora, Til Bora, Boka Bora, Bora, Doha Bora, Kati Bora, Muga Bora, Xuagmoni Bora, Boka Chakuwa, Chakuwa Black, Da Chakuwa, Moina Bora, Moumoha Bora, Temai Bora, Ronga Pekhi, Munin Kichi, Sigan (19 genotypes)	2.716	2.057	0.697	0.399	43	71.67
3	Kmj Bora47, Kmj Bora48, Kmj Bora52, Ronga Bora1, Ronga Bora2, Ronga Bora3, Ronga Bora6, Kola Bora4, Kola Bora5 (9 genotypes)	2.035	1.754	0.535	0.336	39	65
4	Mon Bora1, Mon Bora2, Malbog Bora1, Malbhog Bora2, Mikir Bora, Manipuri Bora, Mouguti Bora, Misiri Bora, Narul Bora, Nashinggeti Bora, Neoli Bora, Nolchutia Bora, Nol Bora, Poita Bora1, Poita Bora2, Pakhiloga Bora, Pakhori Bora, Tita Phulia Bora2, Tita Pholia Bora3, Til Kochu Bora, Tuloxee Bora, Kuki Bora. (22 genotypes)	2.424	1.965	0.674	0.411	49	81.67
5	Kmj Bora19, Kmj Bora21, Kmj Bora25, Kmj Bora26, Kmj Bora27, Kmj Bora31, Kmj Bora33, Kmj Bora34, Kmj Bora35, Kmj Bora40, Kmj Bora44 (11 genotypes)	2.254	1.898	0.599	0.362	38	63.33
6	Ranjit, Mahsuri, IR36, Bokul Bora (4 genotypes)	2.033	1.857	0.559	0.351	40	66.67
7	Rikuto Norin Mochi20, Malagkit Songsong, Miyagi Aikuu, Pyedo, Hao Nai Huan, C101, Guad Nang (7 genotypes)	2.000	1.687	0.526	0.338	42	70
8	Sikora Bora, Saru Bora, Sorai Bhanu Bora, Silatika Bora1, Sontuki, Saudang Bora2, Sukoni Bora1, Sukoni Bora2, Saukha Bora, Singphow Bora, Tangun Bora1, Tangun Bora2, Tangun Bora3 (13 genotypes)	1.982	1.614	0.477	0.301	40	66.67
9	Kmj Bora63, Kmj Bora65, Kmj Bora69, Kmj Bora74, Kmj Bora79, Kmj Bora76, Kmj Bora80, Kmj Bora86, Kmj Bora89, Kmj Bora90, Kmj Bora91 (11 genotypes)	2.118	1.759	0.536	0.326	35	58.33

Similarly, an average distance between individuals in same subpopulation ranged from 0.473 in subpopulation 7 to 0.370 in subpopulation 1. These results provide evidence for the existence of population substructure among the 138 accessions.

The population composition and genetic diversity analyzed in the subpopulations are given in Table 3. In subpopulation 4, largest numbers of genotypes were included and all of them were from Brahmputra valley; 81% of loci were polymorphic in this

subpopulation. The Nei's[19] gene diversity (*He*) was also the highest in this subpopulation. But the highest *Na* and *Ne* was recorded in subpopulation 2. Average distances between individuals in same subpopulation ranged from 0.369 (in subpopulation 3) to 0.473 (n subpopulation 7). A clear-cut divergence of *japonica* accessions from other glutinous rice was observed. These suggest sufficient genetic diversity among the subpopulations and within the subpopulation.

Association Mapping

Marker-trait associations are present in glutinous rice for cooking quality as we presumed. Twenty significant markers were associated with cooking quality traits (P<0.01) as given in Table 4.

AC

Of seven significant associations for AC, two markers each on chromosomes 6 and 8, and one marker each on chromosomes 2, 4 and 11 were linked. AC is a major determinant of rice eating quality and is mainly studied through the waxy gene (*wx*) of rice along with other modifier genes[8,37]. Lanceras *et al*[38] have also found four QTLs for AC on chromosomes 2, 3, 4 and 7. Beside the *wx* locus, some other loci regulating AC were identified and mapped on chromosomes 2, 4, 5, 6, 7 and 9[39]. The identified marker-traits associations in the present study confirm earlier findings. Regarding the association of a marker on chromosome 8 and 11 with AC, available literature did not support such effect on these chromosomes, which further suggests the presence of new QTL for AC and it requires further confirmation.

GC

GC was found to be associated with five markers on chromosomes 1, 2, 5, 9 and 12. The marker RM592 on chromosome 5 accounted for 34% of variation. GC is regarded as good index of cooked rice texture. Genetic analysis for GC indicated the control of *Wx* or another gene tightly linked to the *Wx* locus for variation in GC[8,38]. Mapping efforts identified different QTLs for GC on chromosomes 2, 6 and 7[38,40]. Govindaraj *et al*[41] detected 8 QTLs for GC on chromosomes 4, 5, 6, 8 and 12. The present study could not detect association of markers on chromosome 6 linked to AC but the comparative analysis showed that the markers RM592 on chromosome 5 and RM12 on chromosome 12 associated with GC mapped close to the marker identified by Govindaraj *et al*[41]. This shows the similarity of genetic effect for the gene that controls GC on the chromosomes indicated in previous reports.

Table 4—Association (R^2) of SSR markers with cooking quality traits of glutinous rice studied using MLM approach in TASSEL

Trait*	Locus	p_Marker	R^2	Chromosome no.	Map position
AC	RM541	0.0014	22.49	6	75.5
AC	RM3	0.0034	12.21	6	74.3-75.5
AC	RM307	0.022	9.76	4	0
AC	RM21	0.023	5.05	11	85.7
AC	RM154	0.0357	6.11	2	4.8
AC	RM152	0.0366	6.97	8	9.4
AC	RM25	0.0486	3.55	8	52.2
GC	RM592	0.000	33.92	5	31.4
GC	RM9	0.004	21.98	1	92.4
GC	RM154	0.013	11.28	2	4.8
GC	RM278	0.024	11.56	9	77.5
GC	RM12	0.006	9.9	12	109.1
GT	RM252	0.000	43.26	4	99
GT	RM423	0.001	11.74	2	28.7
GT	RM234	0.001	15.76	7	88.2
GT	RM276	0.001	11.84	6	40.3
GT	RM424	0.002	13.21	2	66-68.9
GT	RM206	0.010	13.4	11	102.9
GT	RM149	0.021	13.66	8	103.7
GT	RM249	0.031	9.52	5	50.2

*AC = Amylose contents; GC = Gel consistency; GT = Gelatinization temperature

GT

GT determines the time taken to cook the rice. For the trait GT, we identified 8 markers on chromosomes 2, 4, 5, 6, 7, 8 and 11 with a maximum effect of RM252 on chromosome number 4, explaining 44% of variation. The inheritance of GT was reported to be complex, showing quantitative variation along with some minor modifier genes[42,41]. However, several QTL mapping studies reported a major QTL on chromosome 6, explaining high phenotypic variation[43]. Significant influencs of another locus on chromosome 6, encompassing alkali degeneration (*Alk*) gene[44], encoding for soluble starch synthase IIa[45] has also been reported to control GT. We confirm a major marker effect for GT on chromosome 6 through the marker RM276. These markers can be used for screening of other rice germplasms globally for quality improvement in terms of GT.

Conclusion

By carrying out genome-wide scanning, we detected a total of 20 significant marker-trait associations (P<0.01). In particular, the marker RM541 linked to AC, RM592 linked to GC and

RM252 linked to GT showed strongest effects. These markers are promising candidates for marker-assisted selection and, after adequate validation, can be used for improving cooking quality of rice. Therefore, we believe that after adequate validation these markers can be used practically in breeding programmes for improving cooking quality of rice. The present results suggest substantial phenotypic and genotypic diversity exist in this germplasm set, allowing for genome-wide association mapping and candidate gene mapping for eating quality.

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