Research Paper

Genome-wide association mapping focusing on a rice population derived from rice breeding programs in a region

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Plant breeding programs in local regions may generate genetic variations that are desirable to local populations and shape adaptability during the establishment of local populations. To elucidate genetic bases for this process, we proposed a new approach for identifying the genetic bases for the traits improved during rice breeding programs; association mapping focusing on a local population. In the present study, we performed association mapping focusing on a local rice population, consisting of 63 varieties, in Hokkaido, the northernmost region of Japan and one of the northern limits of rice cultivation worldwide. Six and seventeen QTLs were identified for heading date and low temperature germinability, respectively. Of these, 13 were novel QTLs in this population and 10 corresponded to the QTLs previously reported based on QTL mapping. The identification of QTLs for traits in local populations including elite varieties may lead to a better understanding of the genetic bases of elite traits. This is of direct relevance for plant breeding programs in local regions.

Key Words: genome-wide association mapping, local population, *Oryza sativa* L., plant breeding programs, rice.

Introduction

The causal relationship between DNA polymorphisms and phenotypes among populations is the fundamental genetic force to improve traits in plant breeding programs. Genetic approaches have become important for identifying the genes for quantitative traits in natural variation. QTL mapping is a powerful method to identify chromosomal regions co-segregating with target traits in populations. Many important genes for domestication and agronomic traits have been identified by QTL analyses (Alonso-Blanco *et al.* 2009, Yamamoto *et al.* 2009). However, QTL mapping has some limitations in understanding the genetic architecture of traits involved in local populations. QTL mapping can identify the related genes to biparental variations. Mapping resolution depends on the number of recombinations occurred in the process of the development of mapping populations. Furthermore, suitable mapping populations for study is time consuming.

Genome wide association study (GWAS) has recently become popular for identifying QTLs. GWAS has the potential to overcome the limitations of QTL mapping. It has the ability to detect the genes involved in the population being analyzed. A genetically diverse population involves numerous recombinations. GWAS is advantageous for the identification of causal loci at a higher resolution than that of QTL mapping. Many aspects related to plant growth and development have been approached successfully using GWAS (Ogura and Busch 2015). Some factors must be considered to perform GWAS precisely; sample size, population composition, and statistical methods (Hamblin *et al.* 2011, Korte and Farlow 2013, Ogura and Busch 2015). The difficulties associated with GWAS have been attributed to genetic confounding and the complexity of genetic bases for traits among the population.

To overcome these genetic cues and identify the genes for traits, we herein proposed a new approach, genome-wide association mapping focusing on a local population derived from breeding programs in local regions. Most varieties in a population are involved in a single pedigree relationship, indicating that they may be genetically identical to the progenies derived from multiple cross combinations with the same genetic relationships. Association mapping focusing on such populations is beneficial for identifying QTLs. Such one advantage is a reduction in the genetic complexity and the genetic architecture of traits among the population, which makes it easy to perform statistical analyses of associations between genotypes and phenotypes. Another

advantage is the precise evaluation of phenotypes. The expression of a phenotype is a result of a genotype under environmental conditions. These varieties have been bred in plant breeding programs in local regions and cultivated in the area, and, thus, have better adaptability to local environmental conditions.

In the present study, we performed association mapping focusing on a local rice population from Hokkaido, the northernmost region of Japan and one of the northern limits of rice cultivation worldwide. We previously reported the process underlying the establishment of this local population (Fujino *et al.* 2015, Shinada *et al.* 2014), which was divided into six genetic groups over the history of rice breeding programs in Hokkaido (Shinada *et al.* 2014). In the last two decades, the genetic base of the local population has markedly shifted to the current variety type (Fujino *et al.* 2015). The trace of insertions and deletions (indels) found in the variety Kitaake compared with the reference Nipponbare revealed that pre-existing mutations in wild rice with the A-genome were continuously introduced into the local population via ancestral populations. The rapid accumulation of pre-existing mutations may play major roles in establishing and shaping adaptability to local regions in current rice breeding programs.

We previously performed association analysis using candidate genes among a local population from Hokkaido. *Hd5* for heading date and *qLTG3-1* for low temperature germinability played important roles in variations in these traits among the population (Fujino *et al.* 2013, Fujino and Iwata 2011, Fujino and Sekiguchi 2011). To confirm whether the approach proposed in the present study is suitable for identifying genes, heading date and low temperature germinability were targeted. The results showed that this approach had great potential to identify the QTLs involved in the genetic bases of traits in plant breeding programs in local regions.

Materials and Methods

Plant material

The Hokkaido Rice Core Panel (HRCP) was used for the association analysis (**Supplemental Table 1**). HRCP included 63 landraces and breeding lines that represented genetic diversity among the local population in Hokkaido (Shinada *et al.* 2014). HRCP clearly differentiated into six genetic groups over the history of rice breeding programs. Varieties among HRCP were evenly distributed into these groups. Two F_2 populations derived from crosses between varieties with different alleles at QTLs for the heading date were developed to confirm the results of the association analysis. One F_2 population, HK, was derived from the cross between Hayayuki and Kyouwa. The other F_2 population, NH, was derived from the cross between Nanatsuboshi and Honoka224. Seeds were provided by the Local Independent Administrative Agency Hokkaido Research Organization and Hokkaido Agricultural Research Center.

All rice varieties and F_2 populations were cultivated in an experimental paddy field at Hokkaido Agricultural Research Center (Sapporo, Hokkaido, Japan, 43°00′N latitude) in 2012 and 2013, respectively. Sowing and transplanting were performed in late April and late May, respectively. Leaf samples of each plant were collected for DNA extraction.

Phenotype evaluation

Heading date was individually recorded and days to heading (DTH) of the earliest heading panicle among individuals was calculated for each plant as the number of days required from sowing to heading. In addition to DTH at Sapporo in 2012, DTH at Pippu in 2012 (43°51′N latitude) in our previous study (Shinada *et al.* 2014) was used for the association analysis. To evaluate low temperature germinability, seeds were incubated at 15°C in the dark as described previously (Fujino *et al.* 2004, 2008). The arc-sine transformation of the average of triplicates in germination rate on sixth day after the start of the incubation was used in the data analysis.

DNA analysis

Total DNA was isolated from young leaves using the CTAB method (Murray and Thompson 1980). Genotypes at 115 SSR marker loci including 63 markers in our previous study (Shinada *et al.* 2014) were analyzed. Furthermore, two markers linked to *Hd5* and *qLTG3-1* were used; 19DEL for the *Hd5* gene controlling heading date (Fujino *et al.* 2013) and S103 for the *qLTG3-1* gene controlling low temperature germinability (Fujino *et al.* 2008, Fujino and Sekiguchi 2011). PCR, electrophoresis, and sequencing were performed as described previously (Fujino *et al.* 2004, 2005).

Data analysis

An association analysis between genotype and phenotype was performed using the program FATESer (http://fateser.ist.hokudai.ac.jp/). The populations used in this study, HRCP, were closely related to each other. FATESer was originally developed to specifically elucidate associations among such populations. The genotypes from 117 markers and phenotypes of three traits were used. Statistical analyses were performed using the *t*-test, *U*-test, or Tukey's test depending on normality/homogeneity of variance of the trait values and number of alleles at the SSR marker loci with the threshold $P < 0.001$. Minor alleles found in less than 5% were eliminated from the calculation to avoid statistical errors.

Results

Variations in heading date and low temperature germinability among HRCP

A wide variation in heading date was observed from 82.0 days of Norin No. 11, Norin No. 15, and Norin No. 19 to 101.5 of Minakuchiine (**Fig. 1**A). A single peak around 94–96 days was observed in HRCP involving the medium

A

B
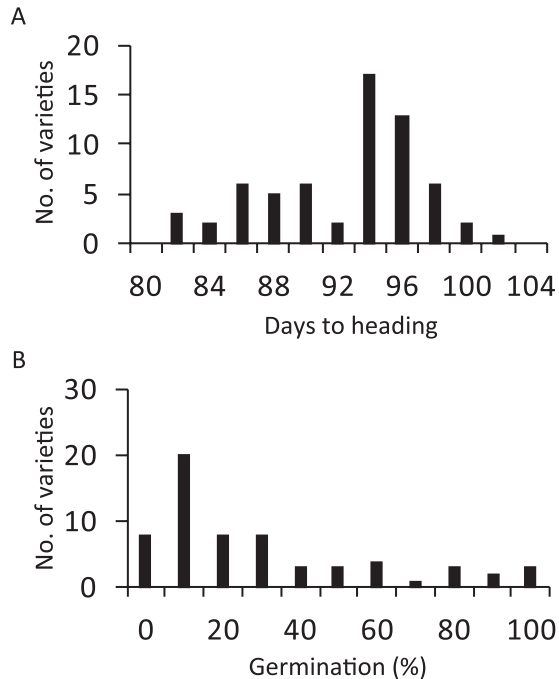
Fig. 1. Frequency distributions of heading date (A) and low temperature germinability (B) among HRCP. The heading date is expressed by days from sowing to heading. Low temperature germinability is expressed by the germination rate at 15°C on sixth day after the start of the incubation.

Table 1. Changes of average in days to heading (DTH) and low temperature germinability (LTG) during rice breeding programs in Hokkaido

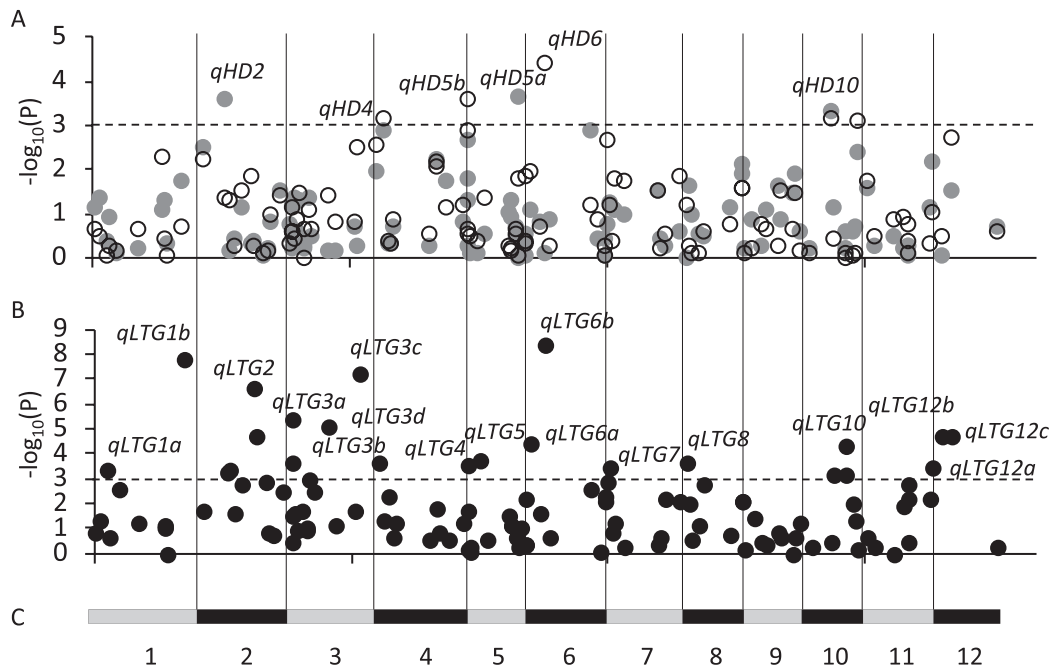| Group	n	DTH	LTG
I	11	89.4 ± 6.0 a	43.8 ± 23.9 a
II	10	93.5 ± 5.0 a	42.3 ± 17.9 a
IIIa	12	91.9 ± 4.4 a	36.8 ± 18.7 ab
IIIb	6	91.2 ± 5.3 a	17.9 ± 8.9 bc
IV	10	90.7 ± 5.0 a	7.0 ± 6.2 c
V	13	92.6 ± 3.4 a	7.8 ± 8.5 c

Different letters indicate a significant difference at $P < 0.05$ by the Tukey test.

Kitaibuki as an extremely early maturing type. The average of DTH in each genetic group were similar, 89.4–93.5 days, without significant difference (**Table 1**).

Low temperature germinability varied between 0% in eight varieties and 96.7% in Kuroge (**Fig. 1**B). Thirty-six varieties among HRCP exhibited weak low temperature germinability (less than 30%), while eight varieties exhibited vigorous low temperature germinability (more than 80%). The eight vigorous varieties belonged to groups I and II (**Supplemental Table 1**). The average of low temperature germinability significantly decreased during the rice breeding programs (**Table 1**).

Association mapping of heading date and low temperature germinability

Six QTLs were identified for the heading date (**Fig. 2**A and **Table 2**). *qHD2* and *qHD5a* were identified in the heading date at Sapporo, and the allelic difference at both loci was approximately 10 days. *qHD4*, *qHD5b*, and *qHD6* were

maturing variety Hoshinoyume (Fujino 2003), while that of the early maturing variety Kitaibuki was 89.3 days. Sixteen varieties exhibited an earlier heading date than that of

Fig. 2. Genome-wide P values from FATESer. (A) Heading date. White and gray circles show data for the heading date in Pippu and Sapporo, respectively. (B) Low temperature germinability. (C) Chromosomal locations of the markers examined. Horizontal dotted bar indicates the threshold of probability (0.001%).

Table 2. QTLs for heading date and low-temperature germinablity by an association analysis

Trait	QTL	Marker	Chromosome	Position	P-value	A No	A Mean±SD	B No	B Mean±SD	C No	C Mean±SD	D No	D Mean±SD	E No	E Mean±SD	F No	F Mean±SD	G No	G Mean±SD	H No	H Mean±SD	I No	I Mean±SD	QTLs reported
DTH Sapporo	qHD2	RM6911	2	9,009,029	0.0002484	20	92.3±4.1 ab	10	90.9±5.3 ab	9	91.6±4.5 ab	6	84.6±1.7 a	17	93.5±3.7 b									
	qHD5a	RM3160	5	20,043,639	0.0002320	6	95.8±2.9 a	4	93.6±3.3 ab			8	88.8±4.9 ab					12	92.6±4.5 ab	15	94.1±2.3 a	5	85.1±2.7 b	
	qHD10	RM3283	10	12,383,411	0.0004819	45	93.0±4.2	18	88.5±4.9 *															
DTH Pippu	qHD4	RM5414	4	2,034,676	0.0007049	34	90.7±4 ab	13	91.7±4 ab	8	86.1±3.6 a	7	94.7±4.8 b											
	qHD5b	RM1248	5	93,969	0.0002526	13	89.1±2.6 ab	25	88.9±3.7 a	24	93.6±4.7 b													
	qHD6	RM1169	6	7,661,599	0.0000368	41	92.2±4.5	21	88.0±2.9 *															
	qHD10	RM3283	10	12,383,411	0.0007063	44	92±4.2	18	87.8±3.8 *															Hd1[1]
LTG	qLTG1a	RM6451	1	4,797,375	0.0003922	19	40.6±21.0	44	20.0±20.0 *															qSD-1[2], Sdr6[3], qSD1[4], qDGE1[5]
	qLTG1b	RM5501	1	34,548,947	0.0000000	22	43.5±22.0 a	10	31.9±20.0 ab			30	11.6±10.0 b											qSD-1[6]
	qLTG2	RM6165	2	19,374,071	0.0000002	50	31.0±22.0	13	7.8±8.5 *															
	qLTG3a	SI03	3	219,977	0.0000043	10	46.0±25.0 a	20	38.0±18.0 a	33	13.1±13.0 b													qLTG3-1[7]
	qLTG3b	RM6676	3	14,494,362	0.0000071	50	30.7±22.0	13	9.2±10.0 *															
	qLTG3c	RM3601	3	25,959,692	0.0000001	54	30.9±22.0	12	6.3±8.1 *															
	qLTG3d	RM1038	3	33,660,363	0.0001982	34	14.8±16.0 a	6	34.4±14.0 ab	14	39.5±22.0 b	6	31.7±15.0 ab											Sd3.2[8], qSl3-2[9]
	qLTG4	RM5879	4	35,112,390	0.0002563	30	30.3±22.0 ab	14	38.9±21.0 a	19	10.4±12.0 b													
	qLTG5	RM3777	5	4,113,575	0.0001960	8	51.4±19.0	53	22.1±19.0 *															qLTG-5[10], qPHS-5[11], Sdr2[12], qSD-5[2]
	qLTG6a	RM1369	6	1,563,617	0.0000359	30	36.1±21.0 a	5	5.0±4.9 ab	20	11.3±10.0 b			4	24.5±15.0 ab									grm6.1[13]
	qLTG6b	RM1169	6	7,661,599	0.0000000	41	35.5±21.0	22	9.0±8.9 *															
	qLTG7	RM5344	7	1,905,597	0.0003187	41	20.1±20.0 a	12	46.8±16.0 b	5	42.6±22.0 ab	5	10.8±12.0 ab											Sdr10[3], qSD6[14]
	qLTG8	RM5647	8	2,892,870	0.0002175	37	35.1±23.0 a	18	11.5±12.0 b	8	18.1±16.0 ab													
	qLTG10	RM1125	10	17,842,706	0.0006376	12	12.4±15.0 a	21	39.2±21.0 b			27	22.1±19.0 ab											qGR-10[15]
	qLTG12a	RM1880	12	747,745	0.0003380	6	51.9±18.0 a	17	14.5±18.0 b	7	30.4±23.0 ab	4	46.3±20.0 a	17	15.9±10.0 b	6	39.5±16.0 ab							
	qLTG12b	RM7619	12	4,829,808	0.0000191	44	32.7±21.0 a			4	16.6±12.0 ab	14	5.3±6.8 b											
	qLTG12c	RM1036	12	8,797,117	0.0000192	13	41.4±20.0 a	15	14.7±13.0 b	15	40.7±20.0 a	12	8.8±9.3 b											

* indicates the significant difference at P < 0.001 by t-test. Different alphabets indicate the significant difference at P < 0.001 by Tukey test.

QTLs are defined by the markers showing the lowest P value within the 1 Mb region.

Alleles with more than 3 varieties were used for the calculation.

1) Yano et al. 2000, 2) Miura et al. 2002, 3) Marzougui et al. 2012, 4) Gu et al. 2006, 5) Li et al. 2011, 6) Wan et al. 2005, 7) Fujino et al. 2008, 8) Lee et al. 2005, 9) Zhang et al. 2005, 10) Miura et al. 2001, 11) Dong et al. 2003, 12) Lin et al. 1998, 13) Thomson et al. 2003, 14) Gu et al. 2004, 15) Ji et al. 2009.

Table 3. Genotype and DTH of parental varieties for the linkage analysis

| | | DTH | | QTL | qHD2 | qHD4 | qHD5a | qHD5b | qHD6 | Hd5 | qHD10 |
| | | | | Chr | 2 | 4 | 5 | 5 | 6 | 9 | 10 |
| | | | | Marker | RM6911 | RM5414 | RM3160 | RM1248 | RM1169 | 19DEL | RM3283 |
Population	Parental varieties	Sapporo	Pippu								
HK	Hayayuki	85.2	89		C	D	G	B *	A	A	B
	Kyouwa	94.6	97		B	A	E	C	A	A	B
NH	Nanatsuboshi	95.4	92		A	A	H	B *	B *	A	A
	Honoka224	97.1	97		E	B	H	C	A	B	A

* indicates alleles with a significant difference by the association analysis.

identified at Pippu, and allelic differences were 8.6, 4.7, and 4.2 days, respectively. *qHD10* was identified at both places, and the allelic difference was approximately 5 days. *Hd5* was previously shown to contribute to variations in the heading date among the varieties in Hokkaido (Fujino *et al.* 2013). However, no significant difference was detected at the marker 19DEL for *Hd5* at either place. In this study, 16 varieties with the deletion allele at *Hd5* as a nonfunction allele were used. The 16 varieties showed the wide range of DTH, 82.5–97.1 days. Among them, eight were common in the previous study. Because heading date is controlled by complex genetics, the genotype of heading date among the common varieties may be different from that of the re-mained varieties.

Seventeen QTLs were identified for low temperature germinability (**Fig. 2**B and **Table 2**). The largest allelic difference was detected at *qLTG12a*, 37.4 between 51.9 of allele A and 14.5 of allele B in an arc-sine transformation value of the germination rate. At other QTLs, allelic differences varied from 20.6 at *qLTG1a* to 32.6 at *qLTG12c*. We previously reported that *qLTG3-1* contributed to variations in low temperature germinability among the varieties in Hokkaido (Fujino and Iwata 2011). A significant association was detected at the marker S103 for *qLTG3-1*, *qLTG3a*.

Validation of association mapping results

A linkage analysis was carried out using two F_2 populations to validate the results of association mapping for the heading date. Two F_2 populations were developed based on the genotypes of the six QTLs for the heading date identified in this study (**Table 3**). In HK F_2 population, most plants showed similar heading dates to the parental varieties and a small number of late transgressive segregants were noted (**Fig. 3**A). Among the six QTLs for the heading date,
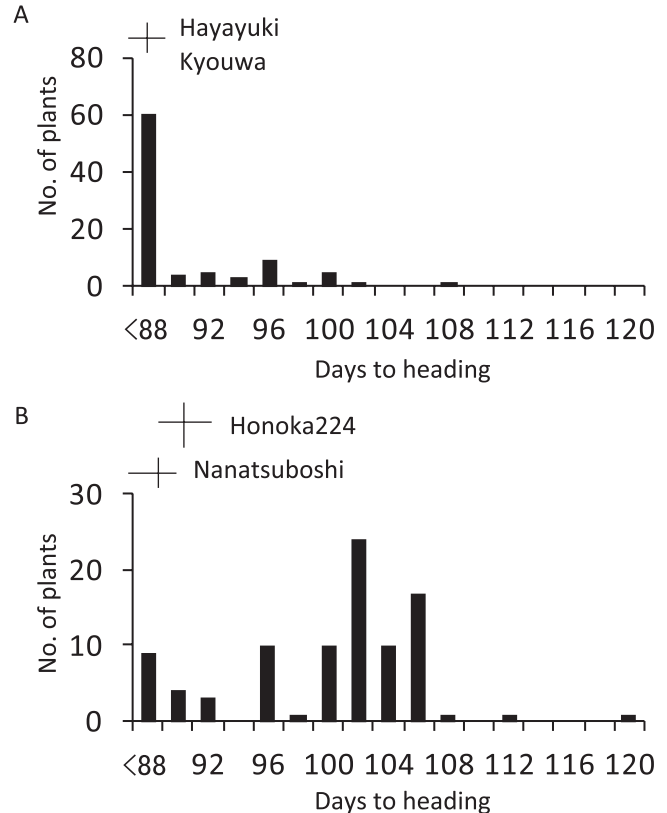
Fig. 3. Frequency distributions of the heading date in F_2 populations derived from crosses between Hayayuki and Kyouwa (A) and between Nanatsuboshi and Honoka224 (B). The vertical and horizontal bars indicate the mean and range of varieties, respectively.

allelic differences between the parental varieties were observed at a single locus, *qHD5b*, (**Table 3**). A significant difference was detected at *qHD5b* (**Table 4**). The Hayayuki

Table 4. QTLs of the heading date in F_2 populations as determined by the *t*-test

| Combination (Parent 1/ Parent 2) | Marker | QTL | Mean of DTH | | | | | | Probability |
| | | | Parent 1 | | Parent 2 | | Heterozygous | | |
			n	Mean	n	Mean	n	Mean	
Hayayuki/Kyouwa	RM1248	*qHD5b*	20	88.9	18	93.6	39	89.5	0.0005
Nanatsuboshi/Honoka224	RM1248	*qHD5b*	23	101.0	23	101.8	41	98.4	0.6510
	RM1169	*qHD6*	26	100.5	19	99.5	45	100.3	0.5890
	19DEL	*Hd5*	23	102.5	30	96.1	33	102.5	0.0000

Probability shows the difference between the mean of Parent 1 and 2 types.

allele (88.9 days) showed an earlier heading date than that of the Kyouwa allele (93.6 days).

Although the parental varieties showed a similar heading date, a wide variation was observed from 88 to 120 days, including many late transgressive segregants in NH F_2 population (**Fig. 3**B). Among the six QTLs for the heading date, allelic differences between the parental varieties were observed at two loci, *qHD5b* and *qHD6* (**Table 3**). No significant differences were detected at the two loci (**Table 4**). *Hd5* was examined in addition to these two QTLs because Nanatsuboshi and Honoka224 carry wild type and loss-of-function type alleles at *Hd5*, respectively. The Honoka224 allele at *Hd5* locus expressed a significantly earlier heading date (96.1 days) compared with the Nanatsuboshi allele (102.5 days) (**Table 4**).

Discussion

Genomes in local populations are structured by artificial selection of the genotype × environmental conditions in recurrent cycles of hybridizations among local populations or with an exotic germplasm during plant breeding programs (Shinada *et al.* 2014). These processes may generate genetic variations that are desirable to local populations and shape adaptability during the establishment of local populations (Fujino *et al.* 2015). One of the most important objectives in plant breeding programs is the control of adaptability to local environmental conditions. In the present study, we proposed a new approach, association mapping focusing on a local population, to identify the genetic bases for traits improved during rice breeding programs.

Plant breeding programs generate intensive selection pressures that focus on shaping adaptability to local environmental conditions, cultivation methods, and market demands. The establishment of local populations reflects the combination of pre-existing mutations widely distributed throughout wild rice into the local population via cultivated rice over the world (Fujino *et al.* 2015). These selections have restricted genetic diversity among local populations, establishing an ideotype for the objectives of current breeding programs (Dilday 1990, Fu *et al.* 2003, Le Clerc *et al.* 2005, Roussel *et al.* 2005, Yamamoto *et al.* 2010). This genetic feature may enhance association analyses to eliminate the complexity of genetic bases, population structure, and genetic architecture of traits among the population.

Association mapping focusing on a local population in this study identified QTLs for heading date and low temperature germinability (**Table 2**). Of the 17 QTLs for low temperature germinability, nine were novel QTLs in this population and eight corresponded to QTLs previously reported based on QTL analysis. *qLTG3a* in the present study was co-localized with the gene *qLTG3-1* for low temperature germinability (Fujino *et al.* 2008). This gene is known to play an important role in variations in low temperature germinability among the varieties in Hokkaido (Fujino and Iwata 2011, Fujino and Sekiguchi 2011). *qLTG5* in the pres-

ent study was co-localized with QTLs for low temperature germinability and seed dormancy (Dong *et al.* 2003, Lin *et al.* 1998, Miura *et al.* 2001, 2002). The remaining six loci were co-localized with the QTLs for seed dormancy (**Table 2**). The allelic difference in *qLTG3-1* between Nipponbare and Koshihikari may have been associated with differences in both seed dormancy and low temperature germinability (Hori *et al.* 2010). These results also demonstrated the potentially close relationship between low temperature germinability and seed dormancy.

Of the six QTLs for the heading date, four were novel QTLs and two corresponded to QTLs previously reported (**Table 2**). *qHD6* in the present study was co-localized with the gene *Hd1* for the heading date, rice orthologue *CONSTANS* (Yano *et al.* 2000). QTLs for the heading date have been identified at the same region in the population derived from crosses between varieties in Hokkaido (Fujino and Sekiguchi 2008). Furthermore, the existence of *qHD5b* for the heading date was confirmed by genetic analyses (**Table 4**). In this chromosomal region, major QTLs for the heading date were identified among Asian rice accessions (Hori *et al.* 2015). These results strongly indicated that the approach proposed in the present study, association mapping focusing on a local population, is suitable to detect the genes involved in local populations.

This approach proposed herein may identify not only genes for traits improved by rice breeding programs, but also alleles for the traits in each variety among the population. In the present study, two populations were developed based on the genotype of the heading date (**Table 3**). *qHD6* was not confirmed by a genetic analysis and *Hd5* was not detected in an association analysis (**Tables 2**, **3**). Based on the segregation pattern, gene interactions such as epistasis were involved in the NH population (**Fig. 3**). The genotypes of the genes for the resulting traits indicated that association mapping supports screening suitable experimental materials for developing mapping populations.

In rice breeding programs, the selection for desirable traits is completed based on the evaluation of phenotypes. This process may generate a genetic force to change the genetic compositions of local populations. Allelic frequencies at the loci for the heading date clearly correlated with the history of rice breeding programs in Hokkaido. The alleles B at both loci, RM1248 and RM1169 linked to *qHD5b* and *qHD6*, respectively, was predominant in group V (**Table 5**). Allele B at RM1248 was predominant in groups I and II, but all varieties in groups I and II carried allele A at RM1169. Although no significant difference in the average heading date was detected between groups (**Table 1**), the combinations of favorable alleles in different loci in each group may lead to a desirable phenotype during rice breeding programs.

Many genes/QTLs for agronomic traits have been identified using GWAS in rice (Begum *et al.* 2015, Huang *et al.* 2010, 2012, Yang *et al.* 2014, Zhao *et al.* 2011). A set of varieties with abundant genetic diversity were used in these

Table 5. Distribution of alleles at marker loci RM1248 and RM1169 linked to *qHD5b* and *qHD6*, respectively, for the heading date among HRCP

Group	Allele				
	RM1248			RM1169	
	A	B	C	A	B
I	5	6	0	11	0
II	0	4	4	8	0
IIIa	1	1	11	12	1
IIIb	2	0	4	6	0
IV	5	2	4	4	7
V	0	12	1	0	13

studies, including *japonica* and *indica*. In contrast, varieties that were not so rich in genetic diversity were used in the proposed approach, which may identify local population-specific genes or alleles related to elite traits. The identification of QTLs for the traits in local populations including elite varieties may provide a clearer understanding of the genetic bases of elite traits. This is of direct relevance for plant breeding programs in local regions. Association mapping focusing on a local population proposed in the present study may identify genes for elite traits at a high resolution using high-density genetic markers such as SNPs.

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