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Full Length Article

Introgression of Root Trait Genes for Drought Tolerance to a Malaysian Rice Variety by Marker-Assisted Backcross Breeding

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Abstract

Drought gives a huge impact in declining rice yield. To overcome this problem, rice variety with ability to withstand limited water availability need to be developed. The study was conducted to introgress root trait genes from aerobic rice variety, AERON1, to a high yielding rice variety of Malaysia, MRQ74, through marker-assisted backcross breeding (MABC). Two foreground markers, RM242 and RM263, and 57 background markers were used along the breeding program. The Chi-square (χ^2) analysis showed a good fit to the expected segregation ratio (1:1) for a single dominant gene model (d.f. =1, P>0.05) for both foreground markers. The Chi-square values in BC_1F_1 and BC_2F_1, for RM242 and RM263 were χ^2=3.1 and χ^2=0.03, and χ^2=1.01 and χ^2=0.22, respectively. The recurrent parent genome (RPG) percentage for BC_1F_1 and BC_2F_1 were 71.0% and 89.6%, respectively. Eventually, six best rice lines in BC_2F_1 population were identified to carry root trait genes for drought tolerance. The lines were closely related to the recurrent parent by having the highest recovery percentage and approximately similar phenotypic appearance. Hence, the development of drought tolerance rice lines through marker-assisted backcross breeding was believed to help the researchers in understanding the importance of molecular markers in enhancing the ability of rice crop towards biotic and abiotic constraints and accelerating the development of new varieties.

Keywords: Drought tolerance; Marker–assisted backcross breeding; Foreground marker; Background marker

Introduction

Drought impinges on approximately 19 million ha of upland rice and more than 14 million ha of rain fed lowland rice (Lafitte *et al.*, 2006). In fact, traditional varieties are more tolerance to drought stress and low yielders but improved varieties are susceptible to drought stress and have high grain yields (Shashidhar *et al.*, 2013). Rice merely can extract water as deep as 60 cm under the soil surface, making it to be considered as having a shallow root system as compared to other cereal crops (Sandhu *et al.*, 2012). The scientists believed that a deep and thick root system could aid rice plant in facing water-limited condition, yet it is not fully understood. Root traits measurement methods are destructive for plants and indirectly make root traits as complicated and time-consuming to evaluate besides hard to integrate in the conventional breeding programs (Shen *et al.*, 1999; Farooq *et al.*, 2009a, b). Scientists are now hardly working on producing aerobic rice varieties by introgressing the drought-resistant traits of upland varieties into high-yielding characteristics of lowland varieties (Sandhu *et al.*, 2012). Hence, marker-assisted backcross breeding serves as a promising tool in developing stress tolerant varieties that can manage vast environmental changes (Linh *et al.*, 2012). It is widely used in improving traits such as disease resistance to pathogens and quality of some crop products besides enhancing complex traits such as yield and abiotic tolerance. Molecular markers are used to reveal the polymorphisms in the nucleotide sequence to create a difference in molecular marker alleles (Francia *et al.*, 2005).

Marker-assisted backcross breeding includes foreground and background selection. In foreground selection, plants were selected based on the desired traits determined by plant breeders. The target locus is preserve until the final backcross step before the selected plants were self-pollinated. In background selection, all genomic regions that contain recurrent parents marker alleles is chosen except the target locus, where target locus is preferred regarding to the plants phenotype (Hasan *et al.*, 2015a). By using molecular markers, numbers of backcrosses required to recover the recurrent parent genome is reduce besides precisely detecting the presence of demanded characteristics

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from donor parent (Hasan *et al.*, 2015b). However, breeders are facing difficulties in integrating high-yielding rice cultivars with drought tolerance traits because it is hard to find genetic segments that can reiterate yield effects under drought stress (Lafitte *et al.*, 2006). Factually, many integrated traits of drought tolerance ability had been discovered, making it is as one of the complex task ever (Francia *et al.*, 2005). For example, more than 30 root morphological parameters were accounted to be controlled by roots traits-related QTLs (Vasant, 2012). Merely, four mapping populations that was derived from crosses between Indica and Japonica were published for drought QTL experiments, which were CO39/Moroberekean, CT9993/IR62266, Azucena/Bala and IR64/Azucena.

The aims of this research were to identify the most suitable markers responsible for root traits in drought conditions and to improve Malaysian rice variety by enhancing the ability to compete with water deficit environments through molecular breeding.

Materials and Methods

Plant Materials and Breeding Scheme

An aerobic rice variety (AERON1) and a high yielding rice variety (MRQ74) were crossed and backcrossed to generate F_1, BC_1F_1 and BC_2F_1 populations, respectively. MRQ74 is a Malaysian aromatic rice variety but susceptible to drought condition. AERON1 was chosen as donor parent to transfer the root trait genes to MRQ74. MRQ74 acted as the female parent/recurrent parent, while AERON1 acted as the male/donor parent. The plants were cultivated in Glasshouse Field 10, the crossing process was done in a crossing house, Field 10, Rice Research Center, Universiti Putra Malaysia, Malaysia (3° 02' N, 101° 42' E), and the marker analysis was done in Breeding Laboratory, Faculty of Agriculture, Universiti Putra Malaysia, Malaysia. The experiments were conducted from September 2013 to June 2015. The temperature in the glasshouse range from 25°C to 35°C and the light intensity range from 250 to 375 µmol/m/s. All plants were cultivated under aerobic soil condition and the soil water pressure was maintained by using tensiometer. The plants were managed by recommended fertilizers and pesticides such as urea (50 kg N/ha), muriate of potash (MOP) (100 kg P/ha), triple super phosphate (TSP) (100 kg K/ha), NPK blue (15:15:15), NPK green (17:15:15:2), Malathion, Diazinon etc. During the early stage, a cross between MRQ74 and AERON1 was done to produce F_1 population. Next, backcrosses were carried out between BC_1F_1 lines (F_1 lines/MRQ74) and BC_2F_1 lines (BC_1F_1 lines/MRQ74) with recurrent parent plant (MRQ74). Germinated seeds of BC_1F_1 lines were planted in pots. Among two hundred seeds, hundred and 29 seedlings survived. Then, seeds collected from previous crossing (BC_1F_1/MRQ74) were planted as BC_2F_1 lines. Among two hundred seeds, only hundred and 44 seedlings survived. Plants with high recurrent parent genome recovery were chosen as female plants to cross with the recurrent parent plant. The selection for best rice line was carried out in BC_1F_1 and BC_2F_1 populations after considering the foreground selection.

Molecular Marker Analysis

Closely linked SSR markers to root traits viz. RM201, RM212, RM221, RM242, RM250, RM263, RM270 and RM302 (Nguyen *et al.*, 2004; Thanh *et al.*, 2006) were screened for foreground selection between parental lines. For background selection, 229 SSR markers referred from Gramene (2002), across all twelve chromosomes in rice were screened for polymorphism between parental lines.

DNA Extraction

The 29 day-old leaves were extracted by using Doyle (1990) method with some modifications. The DNA stock obtained was stored in -20ºC for further use. The stock was diluted with 1×TE buffer to get a concentration of 60–70 ng/µL for PCR analysis.

PCR Amplification and Gel Electrophoresis

The total of a mix solution for PCR was 15.0 µL which include 6.5 µL master mix, 5.5 µL deionized water, 1.0 µL reverse primer, 1.0 µL forward primer and 1.0 µL template of DNA. PCR amplification for both foreground and background markers was carried out in a Thermocycler (T100TM, BioRad) using methods from Miah *et al.* (2015). 3% metaphor-agarose gel solution was prepared for gel electrophoresis. For DNA loading: 2 µL of 50 bp ladder and 5 µL of PCR product were loaded into the gel wells, respectively. Then, the gel was placed into the gel electrophoresis machine [BIORAD (Power Pac Basic Sub-Cell Model 96)] and was run at 80 Volt for 80 min. The bands appearance was viewed using gel documentation machine (GelDoc TM XR+, Bio-Rad].

Statistical Analysis

In both foreground and background selections, the banding patterns of each primers displayed by each genotype were scored as 'A' for bands pattern as similar as AERON1, 'B' for bands pattern as similar as MRQ74 and 'H' for bands having similar pattern with both parental lines. In foreground selection, Chi-square (χ^2) analysis was used to calculate the goodness of fit to 1:1 segregation ratio of each backcross generations by using this formula, $\chi^2 = (O\text{-}E)\ 2/E$, where O is the observed value and E is the expected value. In background selection, marker analysis was done by using Graphical GenoTyper (GGT 2.0) software. In GGT 2.0 software, the percentages of homozygous alleles for donor

Table 1: Foreground markers information

Marker	Sequence (5'-3')	Chromosome number	Repeat motif
RM242	F:GGCCAACGTGTGTATGTCTC R:TATATGCCAAGACGGATGGG	9	(CT)26
RM263	F:CCCAGGCTAGCTCATGAACC R: GCTACGTTTGAGCTACCACG	2	(CT)34

Table 2: Polymorphic background markers according to the chromosome number

Chromosome number	Polymorphic markers
1	RM5, RM9, RM23, RM104, RM165
2	RM208, RM262, RM1234, RM3355, RM452
3	RM130, RM168, RM232, RM251, RM5639
4	RM252, RM261, RM280, RM303, RM348
5	RM13, RM247, RM274, RM289, RM1237
6	RM3, RM190, RM204, RM3827, RM7193
7	RM11, RM192, RM542, RM1132, RM248
8	RM72, RM284, RM308, RM556, RM3572
9	RM 215, RM205, RM257, RM23865
10	RM216, RM222, RM271, RM1375, RM3123
11	RM21, RM144, RM229, RM552
12	RM12, RM19, RM235, RM277, RM3331

Table 3: Chi-square test (χ^2) for foreground marker in BC_1F_1 and BC_2F_1 population derived from cross of MRQ74 and AERON1

Marker	Population	Marker analyzed		χ^2	Probability
		Aa	aa	(1:1)	
RM242	BC_1F_1	54	75	3.10	0.078
	BC_2F_1	67	77	1.01	0.316
RM263	BC_1F_1	65	64	0.03	0.860
	BC_2F_1	75	70	0.22	0.633

d.f. = 1; $\chi 2$ (0.05, 1) =3.84

Aa indicated heterozygous plants, while aa indicated homozygous recipient plants

and recipient parent, and heterozygous alleles were calculated.

Result

Eight potential markers related to root traits *viz.* RM201, RM212, RM250, RM270, RM263, RM242, RM221 and RM302 recognized by Nguyen *et al.* (2004) and Thanh *et al.* (2006), were screened for their polymorphism. However, only two markers, which were RM263 and RM242, showed clear polymorphism between parental plants. Therefore, they were chosen as foreground markers to recognize the desired progenies. The information of polymorphic foreground markers was shown in Table 1. During foreground selection, fifty F_1 plants from a cross of MRQ74/AERON1 were tested for their hybridity using two polymorphic foreground markers. All plants exhibited polymorphic bands pattern and signified that they were carrying the linked markers of root traits. The best four plants of F_1 population with root trait genes were backcrossed with MRQ74 to generate 129 BC_1F_1 plants, while the best three plants with root trait genes in BC_1F_1 were backcrossed with MRQ74 to produce 144 BC_2F_1 plants. All plants in BC_1F_1 and BC_2F_1 populations were evaluated with foreground markers. Fig. 1 showed several plants in BC_1F_1 and BC_2F_1 populations that were tested by both foreground markers, respectively. Results showed that the calculated Chi-square (χ^2) were smaller than the critical value of 3.84 (for d.f. =1) in both BC_1F_1 and BC_2F_1 populations. Therefore, the Chi-square (χ^2) analysis for two foreground markers (RM242 and RM263) showed a good fit to the expected segregation ratio (1:1) for a single gene model (d.f.=1, P>0.05). In BC_1F_1 and BC_2F_1 populations, the Chi-square (χ^2) values for RM242 and RM263 were χ^2=3.10 and χ^2=0.03, and χ^2=1.01 and χ^2=0.22, respectively. This result explained that RM242 and RM263 have an association with root traits in rice. Table 3 showed marker segregation analysis of BC_1F_1 and BC_2F_1 population using RM263 and RM242, respectively.

Meanwhile, 57 polymorphic SSR markers were found for background markers during parental plants survey, concerning all twelve rice chromosomes. List of all polymorphic background markers was shown in Table 2. The ratio of polymorphic markers during parental survey is approximately 25.33%. Most chromosomes were carrying five markers except chromosomes 1, 7 and 11 that only bear four markers. As shown in Table 4, in BC_1F_1 population, the best rice lines were 45-6, 50-39 and 45-27. Rice line 50-39, found to have the highest recurrent parent genome percentage (RPG%) that was 71.4%, while the average of the RPG% for the selected lines was 71.0%. Meanwhile, in BC_2F_1 the best rice lines were 45-27-3, 50-39-8, 45-27-10, 45-27-8, 50-39-7 and 45-6-24. Rice line 45-6-24 discovered to have the highest RPG% that was 90.8%, while the average of RPG% for selected lines was 89.6%. Fig. 2 and Fig. 3 displayed the coverage of background markers with respect to all twelve chromosomes in rice line 50-39 and 45-6-24.

Discussion

Researchers are now emphasizing on the use of root-related QTL to improve drought tolerance ability via marker-assisted-selection (MAS) rather than markers associated with leaf water potential, spikelet sterility and flowering delay (Bernier *et al.*, 2008). Utilization of microsatellite markers or SSR markers has become promising in improving cultivars where development of new plant lines previously are facilitated through phenotype evaluation (Miah *et al.*, 2013). Screening of polymorphic markers as foreground and background markers in targeted population is a fundamental step to differentiate the specialty of each parental plant (Hasan *et al.*, 2015b). In this study, the ratio of polymorphic markers on parental survey is approximately 25.33%.

Table 4: Background and introgressed segment analysis in selected lines of (a) BC_1F_1 and (b) BC_2F_1 populations

(a) Rice line	A (%)	B (%)	H (%)	Total cM	H-segments
45-6	6.1	70.2	23.7	1187.2	8
50-39	6.1	71.4	22.5	1187.2	10
45-27	6.1	71.3	22.6	1187.2	6
Average	6.1	71.0	22.3	1187.2	8
(b) Rice line	A (%)	B (%)	H (%)	Total cM	H-segments
45-27-3	6.1	90.2	3.7	1187.2	2
50-39-8	6.1	89.2	4.8	1187.2	2
45-27-10	6.1	90.5	3.4	1187.2	2
45-27-8	6.1	88.6	5.3	1187.2	2
50-39-7	6.1	88.4	5.5	1187.2	3
45-6-24	6.1	90.8	3.1	1187.2	3
Average	6.1	89.6	4.3	1187.2	2.3

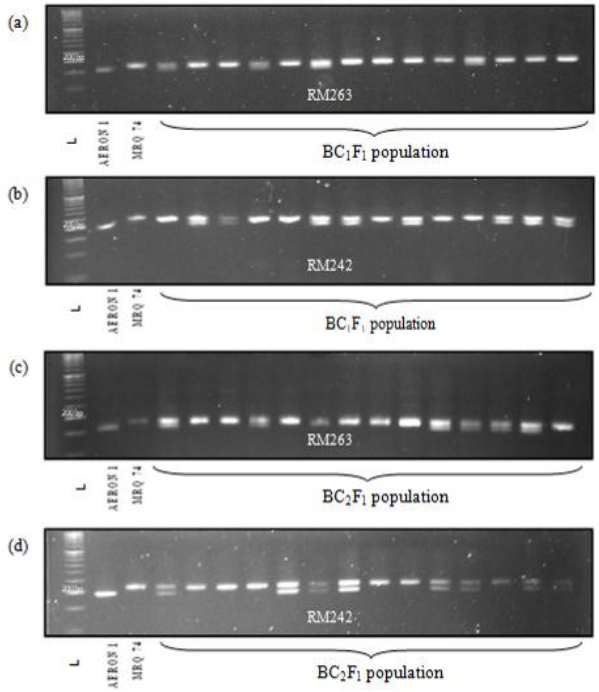

Fig. 1: Screening of BC_1F_1 population and BC_2F_1 population using (a)(c) RM263 and (b)(d) RM242 marker (L= 50bp DNA ladder, bp =base pair). Double banding pattern showed rice lines with both donor and recurrent parent allele

Da *et al.* (1999) emphasized on importance of selecting demanded genetic markers for QTL mapping and other DNA related uses. Huyen *et al.* (2012) found 12.6% of polymorphism between parental lines during screening analysis of 500 markers between parental lines AS996 and FL478 of rice. Meanwhile, Linh *et al.* (2012) found 18.7% polymorphism of markers among 477 SSR markers tested between parental lines BT7 and FL478 of rice. In this study, two polymorphic foreground markers are used in each backcross generation to monitor the introgression of root trait genes for drought tolerance from AERON1 to MRQ74. RM263 and RM242 are chosen as the foreground markers, because they are the linked markers to rice root trait genes (Nguyen *et al.*, 2004; Kumar *et al.*, 2005; Thanh *et al.*, 2006; Siangliw *et al.*, 2007; Reddy *et al.*, 2013). Furthermore, they have proven to be useful in marker-assisted backcross breeding since they exhibited polymorphic banding pattern between parental plants. Foreground selection in backcross method is prominently used to preserve the desired heterozygous allele at the targeted locus of donor parent. Foreground selection reduces labor input and time compared to phenotype measurements. For instance, traits at reproductive stage can be identified during seedling stage and further selection of suitable plant for backcrossing can be made.

Ye *et al.* (2009) emphasized that the necessity for foreground and background selection to be pooled to introgress the desired genes to reduce linkage drag and genome of the donor parent. Employment of tightly linked and unlinked markers of background selection is necessary to evaluate the genome recovery percentage of recurrent parent (Akhtar *et al*., 2010). In this study, 57 polymorphic SSR markers demonstrated suitability to be used in background selection. Utilization of marker information in background selection have been demonstrated to facilitate recovery rate of recipient's genetic background (Jun-yan *et al*., 2006). In conventional breeding, it is difficult to identify backcross progeny visually since a precise attention is required to distinguish the recurrent parent and individual backcross plants in each backcross generation (Ananna, 2014). Background selection is a regular and prominent tool in plant breeding as proven in a maize inbred line that has been introgressed with toxin gene coding of *Bacillus thuringienesis* (Scheible *et al*., 2005).

The recurrent parent genome recovery by molecular genotyping and selection enhance marker-assisted backcrossing competence and reduce time taken compared to conventional breeding (Linh *et al.*, 2012). Scheible *et al.* (2005) found that after six generations of backcross without marker-assisted background selection, the recurrent parent genome rate is 96.8%. The same recovery rate is also achieved if 80 markers are used in a population size of 100 individuals in each generation of BC_1 to BC_3. The increase in recurrent parent genome of marker-assisted backcross breeding is encouraged by selection intensity and the backcross process itself, whereas donor parent genome is declining by half in each backcrossing generation, regardless of the number of genome in the non-recurrent parent. During background selection, majority of the chromosomes carried five markers except chromosome 1, 7 and 11 that carried only four markers. BC_1F_1 population showed a lower recurrent parent genome percentage (71.0%), while in BC_2F_1 population, higher recurrent parent genome percentage was observed (89.3%). Huyen *et al.* (2012) found higher recurrent parent genome percentage in BC_1F_1 (86.3 to 89.06%) and BC_2F_1 (93.18 to 94.03%) during introgression of *Saltol* QTLs in a Vietnamese elite rice variety.

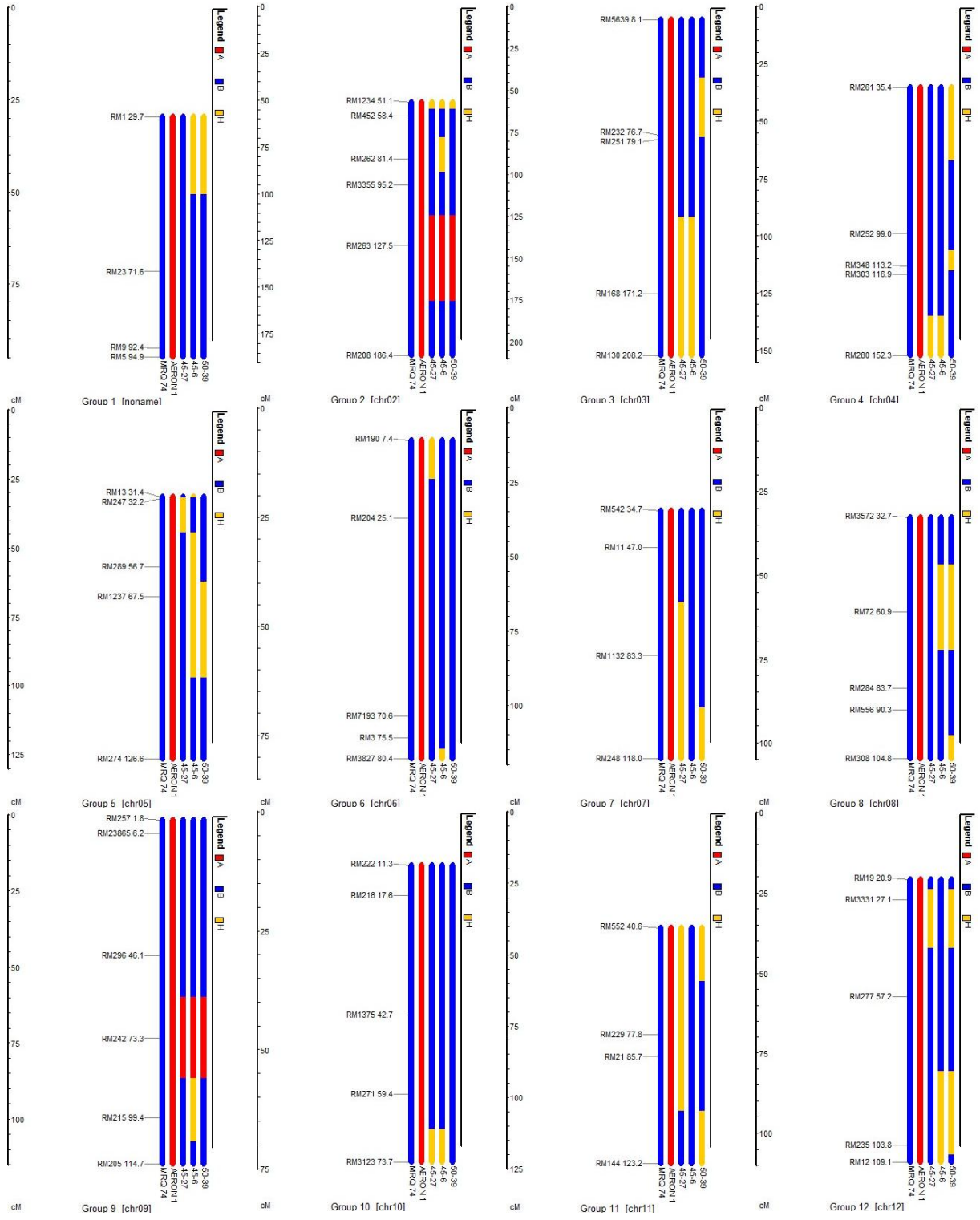

Fig. 2: Recurrent parent genome recovery of selected BC_1F_1 plants in regards to all twelve rice chromosomes. Blue color indicates homozygous regions for MRQ74 alleles, red color indicates homozygous regions for AERON1 alleles, and yellow color indicates heterozygous regions

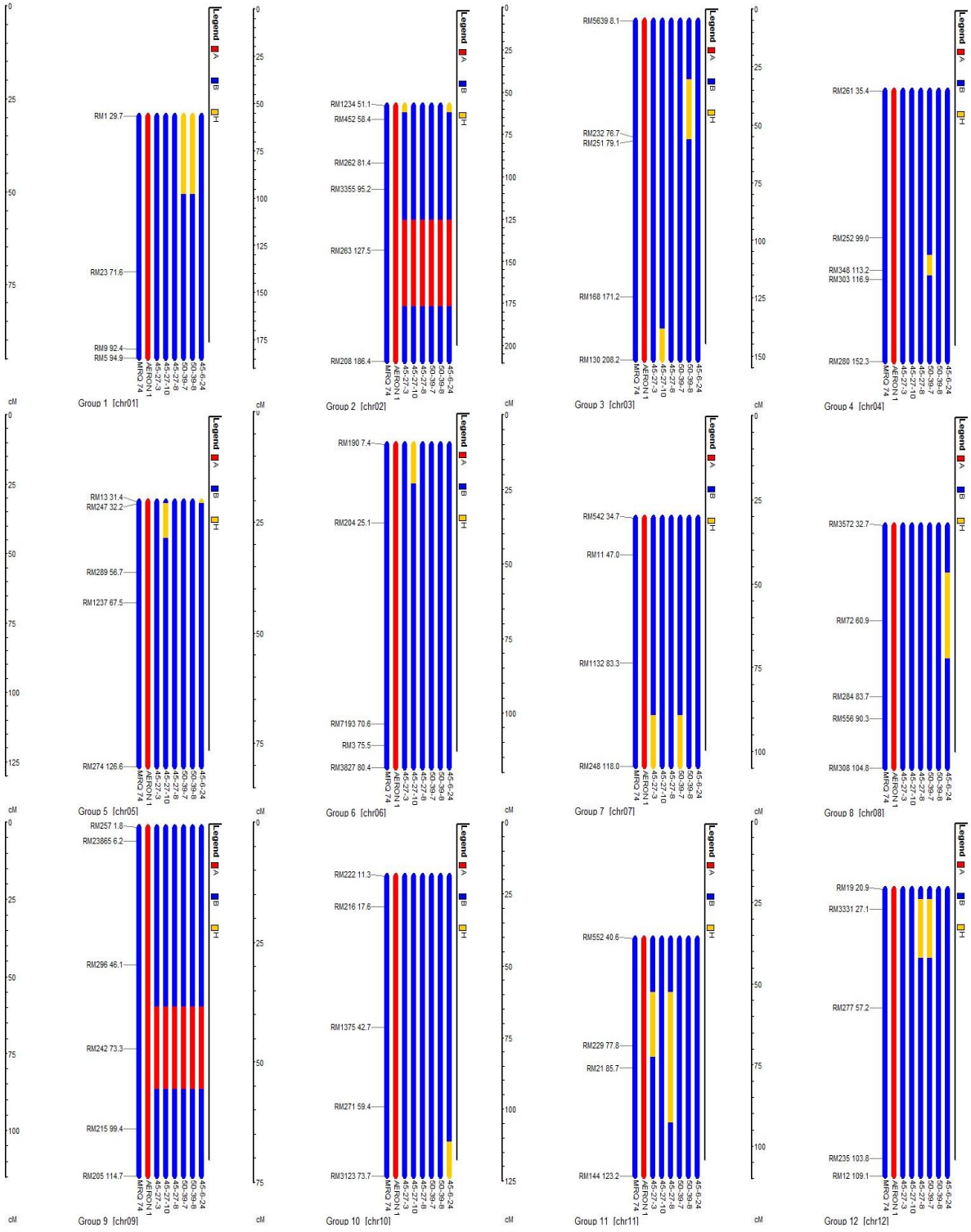

Fig. 3: Recurrent parent genome recovery of selected BC_2F_1 plants in regards to all twelve rice chromosomes. Blue color indicates homozygous regions for MRQ74 alleles, red color indicates homozygous regions for AERON1 alleles, and yellow color indicates heterozygous regions

However, Somers (2004) found individuals with 68 to 70% of RPG% in BC_1F_1, while 88 to 91% in BC_2F_1 during his bread wheat experiment. According to Collard *et al.* (2008), even though the whole BC_1 generation recurrent parent genome recovery averaged at 75%, several individuals in the generation might possess more or less recovery percentage than others, showing that there is a distribution occurrence of recurrent parent genome recovery in the BC_1 generation with means of 75%. Langridge and Chalmers (2004) stated that since recurrent parent background selection could save at least two generations of backcrossing, a large number of polymorphic markers alleles are needed to recover all the plant genome.

Conclusion

The discovery of the new rice lines showed that the introgression of root trait genes from donor parent (AERON1) into recurrent parent (MRQ74) using marker-assisted backcross breeding was successful in this study. Molecular markers are specially developed to help in detecting DNA level allelic variations in the individuals of a generation progeny and are not dependable on environmental condition; therefore, it is reliable during complex traits selections in contrast to conventional breeding.

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