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Parental Polymorphic Marker Survey and Genetic Diversity Studies among the Popular Maintainer Lines of Hybrid Rice (*Oryza sativa* L.) for Stigma Exsertion Trait

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Parental polymorphic survey using rice satellite (RM) simple sequence repeats (SSR'S) is a pre-requisite for genotypic screening to identify the loci associated with trait of interest among mapping population. In the present study, eight popularly used rice maintainer lines viz., APMS-6B (Improved for Bacterial leaf blight. [1], IR58025B, IR68897B, IR79156B, DRR-6B, DRR-9B, BF-16B and BF2096B were used to study stigma exsertion trait as a single, double and total stigma exsertion. A total of 630RM markers were used to study parental polymorphism among eight maintainer lines and also to map their association with stigma exsertion trait. Among 630, 253 RM markers showed polymorphism with 635 alleles among the eight maintainers which were distributed across twelve chromosomes of rice. The overall parental survey revealed 40.18 per cent of polymorphism among the maintainer lines with a maximum and minimum frequency of 5 and 2 alleles, respectively. The genetic similarity coefficient for the most number of pairs ranged between of 0.2-0.9 with the average value of 0.60 for all possible combinations, indicating moderate genetic diversity among the chosen genotypes. The genotypes grouped according to their place of origin

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and represents genetic closeness between them. The identified RM polymorphic markers could be used to construct the linkage map and subsequently, to identify the stigma exsertion related QTLs from mapping population developed from different combinations of the rice maintainer lines.

Keywords: Simple sequence repeats (SSR); parental polymorphism; diversity; rice maintainer lines; stigma exsertion; marker assisted selection.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population USDA (2016) and it occupies 23 percent of the total area in the world under cereal production. The development of hybrid rice breeding technology involves improvement and evaluation of parental lines, evaluation of the degree of heterosis for yield and techniques for seed production. Customarily to produce hybrids on a commercial scale, it is essential to change the function of male and female reproductive systems of rice plants.

The low yield of F_1 seed production, and the availability of F_1 seed at reasonable prices, has been cited as a major constraint to the wide adoption of hybrid rice in countries outside China [2,3,4]. The availability of affordable hybrid seeds to farmers is crucial to the success of hybrid rice commercialization since farmers have to use fresh hybrid seeds in each crop season.

In self pollinated crops like rice, hybrid breeding appeared to be difficult, as the floral traits are unfavorable for out crossing. Use of male sterility system has immensely helped in hybrid breeding [5]. It has been reported that out crossing is influenced by many floral traits like size of pistil and stamen, stigma exsertion, angle of glume opening. Among them, stigma exsertion is emphasized as a major component in increasing pollination and seed set [6]. Previous studies have demonstrated that the stigma exsertion rate of the male sterile line, the female parent in production of hybrid rice, is a key factor contributing to the efficient improvement of hybrid seed production, since exserted stigmas remain viable up to about 4 days and could continue to accept pollens [7,8]. A male sterile line with high stigma exsertion rate is expected to trap more pollen, thus improving the efficiency of hybrid seed production. With an increase in the efficiency of stigma exsertion in male sterile lines of hybrid rice, the seed- setting rate in hybrid seed production and the yield of hybrid seed also increased [9].

Previous studies have shown that stigma exsertion is controlled by quantitative trait loci (QTL) and affected by environmental conditions. Several QTLs have been identified for stigma exsertion trait in different rice materials. The wild rice (*Oryza rufipogon*) often has large exserted stigmas, two QTLs were identified for rate of exserted stigma (qRES-5 and qRES-10) between the indica line Pei-kuh and the common wild rice accession W1944 [10]. *O. longistaminata* is allogamous species, with a self-incompatibility system, and shows the extreme maximum values of stigma and anther length and number of pollen grains within the sativa species group [11]. This can be utilized as a genetic resource in breeding programme to introgress few of its allogamous floral traits in *O.sativa*. There were three QTLs identified on chromosomes 2, 6, and 8 for stigma exsertion rate (SER) in a cross between indica cultivar Guangluai-4 and the wild rice accession W1943 [12]. Two QTLs were identified for percentage of exserted stigma (qPEST-5 and qPEST-8) in a cross between Dongxiang wild rice and the indica cultivar Guichao 2 [13]. In general, indica rice has longer and more exserted stigmas than japonica rice. Nine QTLs for frequency of stigma exsertion were detected in recombinant inbred lines (RILs) derived from a cross between japonica cultivar Asominori and indica cultivar IR24 and further identified a major QTL for exserted stigmas, qES3, in the same genomic region as the GS3 (Grain Size 3) gene on chromosome 3 [14,15]. Recently, 11 QTLs identified for SER in a genome-wide association study (GWAS) of 217 indica CMS lines, and 23 genomic loci that significantly affected SER among diverse rice accessions [16,17]. These results shows that the stigma exsertion trait is complex and controlled by many genes and that different rice material may carry different QTLs for stigma exsertion rate.

2. MATERIALS AND METHODS

In this study, eight hybrid rice maintainer lines APMS-6B (Improved for Bacterial leaf blight), IR58025B, IR68897B, IR79156B, DRR-6B, DRR-9B, BF-16B and BF2096B were used as parents. APMS6B: A maintainer line of APMS6A, which is a female parent of popular medium slender Indian rice hybrid DRRH3, medium slender grain

type and medium (106 days) duration. This line has been bred from APRRI, Maruteru and improved for bacterial leaf blight and blast at ICAR-IIRR, Hyderabad [1]. IR68897B: A maintainer line of IR68897A, which is a female parent of early maturing rice hybrid DRRH2 suitable to develop early duration (90-95 days) long slender grain type hybrids. IR58025B: A maintainer line of IR58025B, which is a female parent for a number of popular hybrids KRH2 and DRRH1, Sahyadri, CORH2, with long slender grain type and IR79156B: A maintainer line of IR79156A, with long slender grain type and medium duration (101-104 days). DRR-9B: A medium slender grain and early maturity with moderate stigma exsertion maintainer line of DRR-9A. DRR-6B: A medium slender grain and early maturity with moderate stigma exsertion, maintainer line of DRR-6A. BF-16B: Improved maintainer line with good stigma exsertion with medium bold grain type. BF-2096B: Improved maintainer line with good stigma exsertion with medium bold grain type (Fig. 1).

2.1 Genomic DNA Extraction

The genomic DNA from the fresh leaves of the eight genotypes was extracted by cetyl-trimethyl ammonium bromide method (CTAB) as described [18]. The quality and quantity of extracted DNA was estimated through agarose gel electrophoresis (Alpha Imager UV gel documentation system, M/s Alpha Innotech Corporation, USA) and NanoDrop (ND100 spectrophotometer, NanoDrop Technologies Inc., USA), respectively. DNA samples with 260/280 ratio between 1.8 -1.9 were used for PCR to study parental polymorphism.

2.2 Primers Used in the Study

For studying the parental polymorphism among eight maintainer lines viz., APMS-6B (Improved for Bacterial leaf blight), IR58025B, IR68897B, IR79156B, DRR-6B, DRR-9B, BF-16B and BF2096B, total 630 SSR markers were used. The information regarding chromosomal location and sequences of primers were obtained from www.gramene.org.

2.3 PCR Analysis

The polymerase chain reaction (PCR) was carried out in thermal cycler (Applied Bio systems, USA) using 630 SSR markers. The PCR reaction mix includes the following: 20-50ng of genomic DNA, 1x Buffer (containing 1.5 mM $MgCl_2$), 125 µM of dNTPs, 0.2 µM of each (forward and reverse) primer and 0.5 unit of Taq DNA polymerase (Bangalore Genei, India). The PCR profile was included with initial denaturation at 94˚C for 5 min followed by 35 cycles (denaturation at 94˚C for 30 s + annealing at 55˚C for 30 s + extension at 72˚C for 1 min) and the final extension at 72˚C for 5 min. The PCR amplicons were resolved in a 3% agarose gel prestained with ethidium bromide in 1X TAE (40mM Tris-acetate and 2mM EDTA pH ~8.0) buffer. The electrophoresed products were visualized under UV light and documented using Alpha lmager Documentation System (M/s Alpha Innotech, USA).

Fig. 1. Figure showing the panicle and grain type of various maintainer lines used in the study

3. RESULTS AND DISCUSSION

The ratio of UV absorbance at OD260/OD280 ranged between 1.8-1.9, and hence DNA samples were rated as good and standard. The quantity of DNA in the isolated samples ranged from 1100 to 1500ng/µl. The parental polymorphism survey indicated that a clear polymorphism was observed among the parents where 630 SSR markers mapped on all the 12 chromosomes (Fig. 3) including 8 reported markers (Table 2 & Fig. 5) for stigma exsertion trait and highly variable rice microsatellite markers (HRM) were used among the parents. The 8 reported markers and their chromosome number and physical position on chromosome given in Table 2. Out of 630 markers 253 SSR primer pairs were exhibited polymorphism among the eight parents and remaining 377 primers were monomorphic. Percentage of polymorphism highest (Table 3) on chromosome 1 (57.14) and least on chromosome 7 (17). Out of 8 reported markers [19], 4 markers were showing minor polymorphism among eight maintainers, they are RM3642 on chromosome 1 for DSE, RM5 on chromosome 1 for SSE & DSE, RM105 on chromosome 9 for SSE & TSE, RM25669 on chromosome 10 for SSE & TSE. The reported markers the earlier study [19] did not work well with the present set of genotypes. This might be because of novel regions contributing for the stigma exsertion trait. The average per cent of polymorphism on all the chromosomes was 43.39. The lack of detectable polymorphism among the eight parents would be due to the fact that all the parents are indica lines. Lack of molecular marker polymorphism among the indica genotypes has been earlier noticed in studies by [20,21].

3.1 Parental Line Phenotyping for Stigma Exsertion Traits

The 253 rice microsatellite (SSR) markers identified as polymorphic among the eight parents will be useful as a pointer to the existence of different alleles at each of the loci. As the parents differ from each other with respect to stigma exsertion traits (e.g. single stigma exsertion (SSE), double stigma exsertion (DSE), Total stigma exsertion (TSE) and no stigma exsertion (NSE)). Based on the mean values of different genotypes, the highest mean value of 80.25% of total stigma exsertion was performed by BF16B, followed by BF2096B with 78.03%, DRR6B with 61.46% for TSE. The minimum or lowest mean value was recorded for APMS6B with 14.97%, followed by IR68897B (36.78%) and DRR9B (42.46%) (Table 1 and Fig. 2).

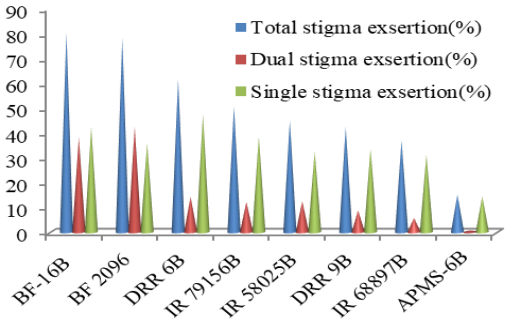

Fig. 2. The chart depicting the total, dual and single stigma exsertion percentages for various maintainer lines

Comparison of these mean values using the LSD value (7%) indicated that BF16B and BF2096B did not differ significantly whereas the difference between BF16B and APMS6B was significant, with the highest and lowest mean values performance. APMS6B recorded the lowest mean value for DSE (0.67%) and for SSE (14.3%). BF2096B had shown maximum mean value for DSE (42.34%), for SSE (35.69%). BF16B was shown second highest mean value with 38.04% for DSE, where the highest mean value for SSE with 42.21%. DRR6B, IR79156B and IR58025B were performed the moderate mean values for TSE with 61.46%, 50.54%, and 44.86%, respectively. The genotypes BF16B, BF2096B, DRR6B, and IR79156B had recorded more than 50% for TSE, where as all the other genotypes of mean values were below 50% for DSE and SSE. The genotypes DRR6B, IR79156B, IR58025B, DRR9B, IR68897B and APMS6B have exhibited less than 25% DSE and where as BF2096B and BF16B have exhibited more than 50%. Further, the association of identified polymorphic markers to stigma exsertion trait can be studied through QTL mapping.

3.2 SSR Polymorphism among Maintainer Lines of Hybrid Rice

All the 8 maintainer lines of hybrid rice were genotyped were selected for their ability to produce amplified product at optimum concentration, polymorphism level among the maintainers and consistency of the pattern. The banding pattern of different polymorphic markers among 8 genotypes of maintainer lines is shown in Figs. 4 & 5. The respective values for overall

genetic variability for polymorphism information content, resolving power (RP), major allele frequency, percentage of polymorphism, number of alleles across all the 8 genotypes are given in Supplementary Table 1. Highest PIC value (1) was observed for the primer HRM25754, RM258 and lowest PIC value (0.219) was recorded for the primer RM10209 (Supplementary Table 1) with an average of 0.503. The percentage of polymorphism values ranged from 100 to 62.50 with an average of 98.17. The resolving power (RP) is a feature of marker that indicates the discriminatory potential of the primer. RP ranged from 1 to 0.250 with an average of 0.600 for polymorphic marker. In case of polymorphic markers the major allele frequency ranged from 0.156 to 0.781 with an average of 0.501 (Supplementary Table 1). The allele number per locus varied from 2 to 5 with an average of 3 alleles per locus (Supplementary Table 1).

Table 1. Table showing the different type of stigma exsertion percentages of eight maintainer lines used in the study

S. No.	Genotype	Total stigma exsertion(%)	Dual stigma exsertion(%)	Single stigma exsertion(%)
1	BF-16B	80.25	38.04	42.21
2	BF 2096	78.03	42.34	35.69
3	DRR 6B	61.46	14.2	47.27
4	IR 79156B	50.54	12.15	38.4
5	IR 58025B	44.86	12.44	32.42
6	DRR 9B	42.26	8.78	33.48
7	IR 68897B	36.78	5.72	31.06
8	APMS-6B	14.97	0.67	14.3

Table 2. List of eight reported markers [19] for different type of stigma exsertion traits employed for polymorphism study

Sl. No.	Marker	Chromosome number	Position (cM)	Traits
1	RM5	1	98.5	SSE & DSE
2	RM3642	1	102.3	DSE
3	RM178	5	104.4	DSE & TSE
4	RM133	6	0.5	SSE
5	RM455	7	78.9	DSE & TSE
6	RM44	8	46.9	DSE
7	RM105	9	7.8	SSE & TSE
8	RM25669	10	55.2	SSE & TSE

Table 3. Chromosome wise total markers used, polymorphic markers, monomorphic markers and percentage of primers showing polymorphism among the parental lines

Sl. no.	Chromosome	Total markers used on each chromosome	Polymorphic markers on each chromosome	Number of monomorphic primers on each chromosome	% of primers showing polymorphic on each chromosome
1	Chromosome-1	49	28	21	**57.14**
2	Chromosome-2	37	19	18	51.35
3	Chromosome-3	44	22	22	50.00
4	Chromosome-4	45	20	25	44.44
5	Chromosome-5	35	18	17	51.42
6	Chromosome-6	45	18	27	40.00
7	Chromosome-7	100	17	83	**17.00**
8	Chromosome-8	57	30	27	52.63
9	Chromosome-9	39	16	23	41.02
10	Chromosome-10	47	22	25	46.80
11	Chromosome-11	65	26	39	40.00
12	Chromosome-12	67	17	50	25.37
	Total	630	253	377	43.09

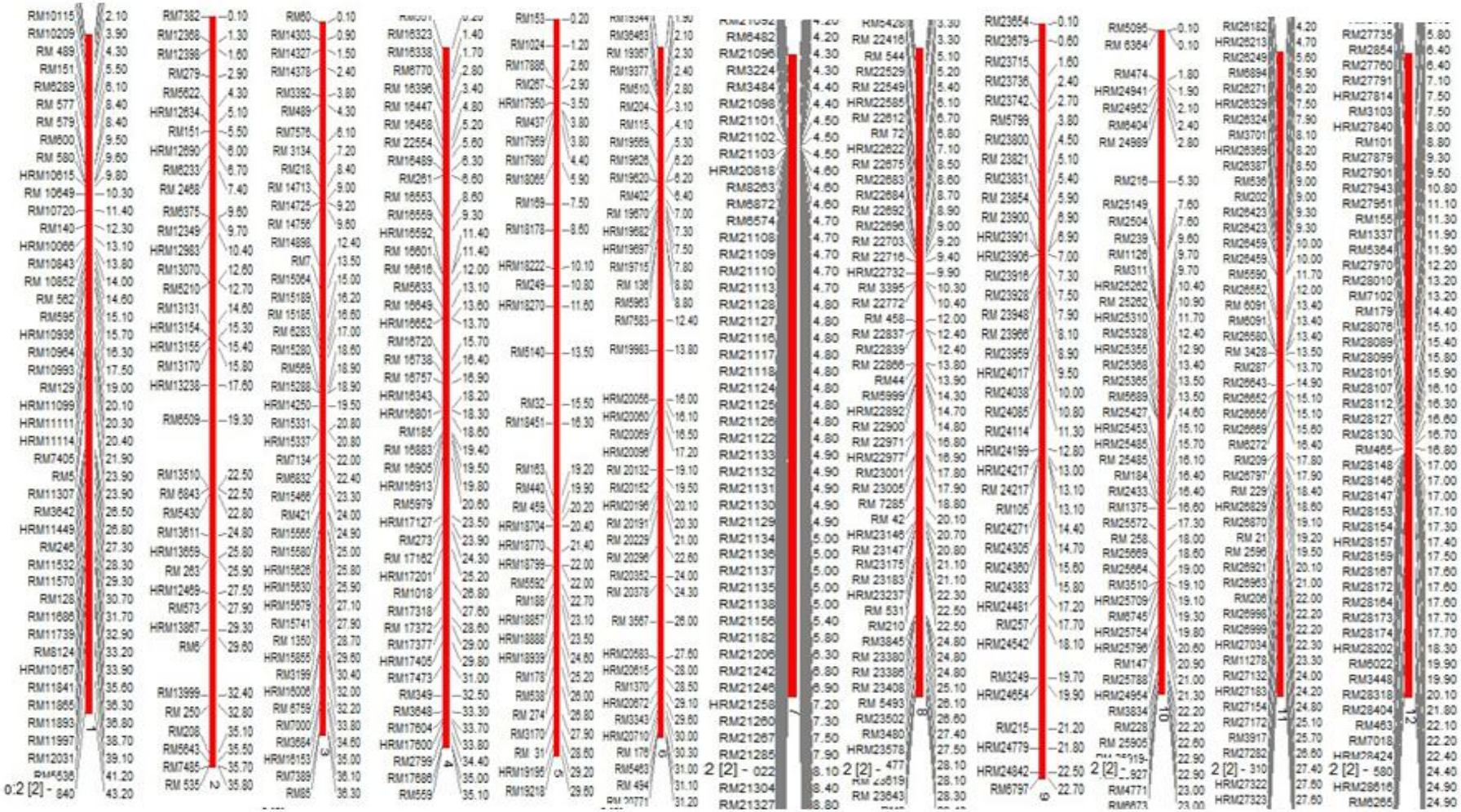

Fig. 3. Physical map of the 12 rice chromosomes showing location of 630 HRM and RM markers using Graphical genotyping (GGT)

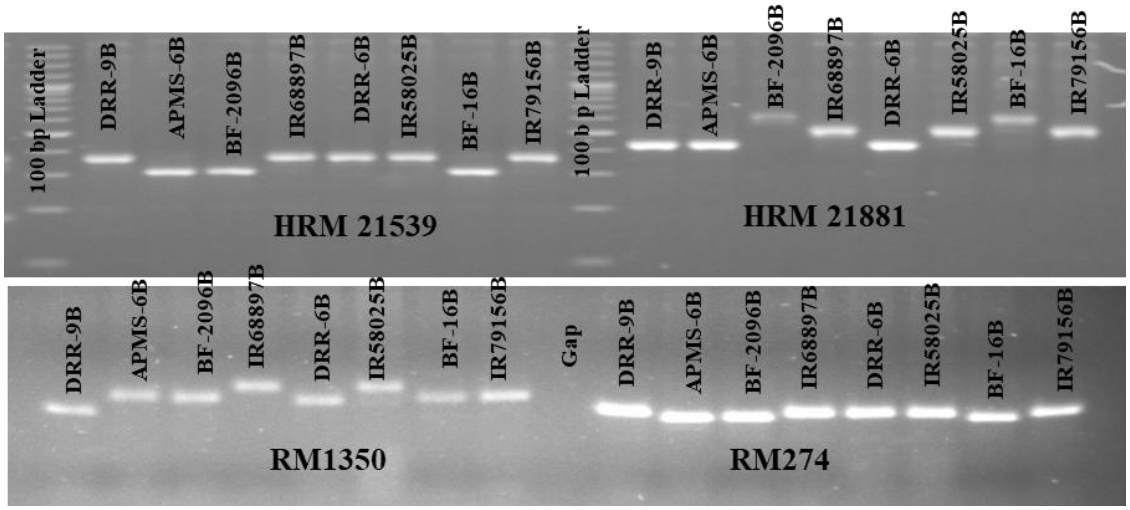

Fig. 4. Gel picture showing the polymorphic banding pattern of highly variable SSRs (HRM) and SSRs (RM) among eight maintainer lines of hybrid rice

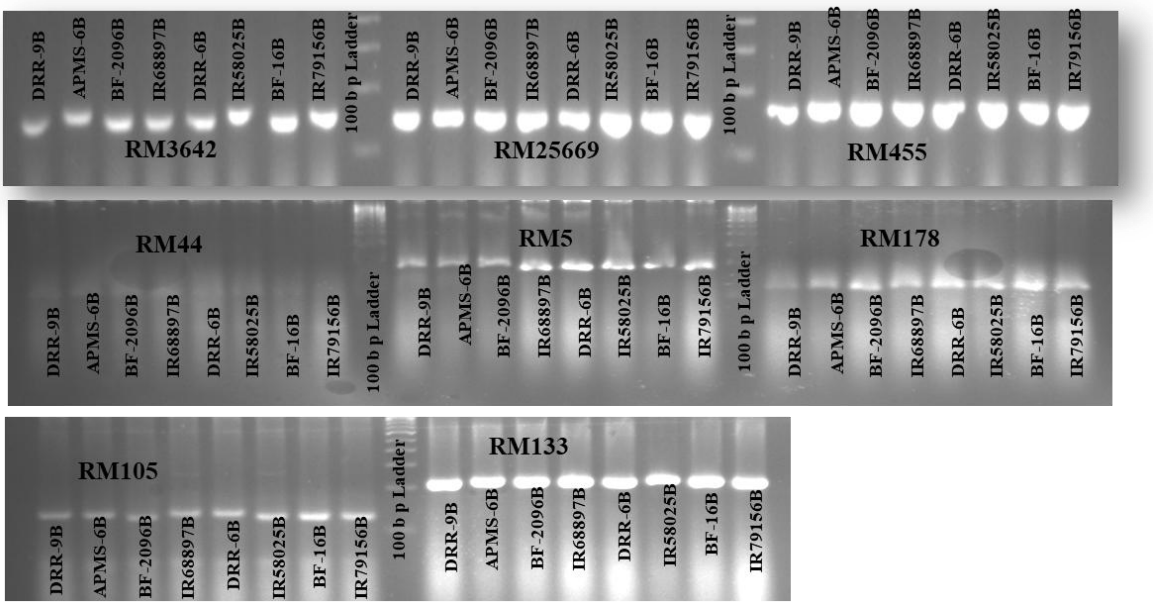

Fig. 5. Gel picture showing the banding pattern of 8 reported markers [19] among 8 maintainer lines

3.3 Genetic Relationship

To find out the genetic relationship between different maintainer rice genotypes, SSR data were used for analysis using NTSYSpc version 2.02e. The genetic similarity coefficients found in the genotype comparison matrix were relatively moderate. A dendrogram was constructed to understand the diversity among eight popularly used maintainer lines using genotypic data of 253 polymorphic markers. The cluster analysis was performed using UPGMA method on the basis of Jaccard's coefficients with one possible tie found between the closest pairs. The neighbour-joining tree based on all SSR fragments grouped eight germplasm accessions into three major clusters (Fig. 6).

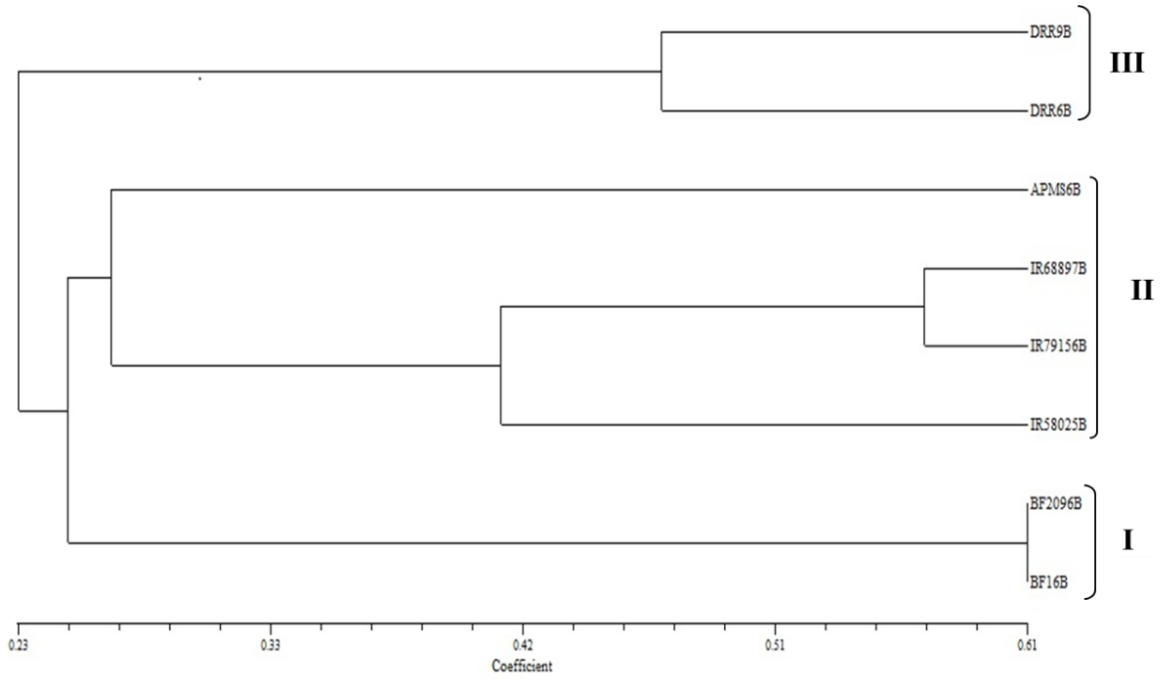

Fig. 6. Dendrogram depicting the diversity among eight maintainer lines of hybrid rice

The first cluster (P1) is formed between two accessions BF16B and BF2096B evincing 0.61% similarity with each other where these two B lines developed by Barwale foundation with higher stigma exsertion percentages. The other phenon (P2) is constructed between IR79156B and IR68897B showing mere 0.57% similarity with P1 and joined with IR58025B with a much higher distance. These three B lines developed at IRRI, Philippines and grouped under one cluster. The three B lines connected with other B line APMS6B where this line bred at APRRI, Maruteru with medium slender grain type. The third cluster (P3) is of two accessions of DRR6B and DRR9B at 0.47% similarity. These lines were developed at ICAR-IIRR, Hyderabad. The genotypes grouped according to their place of origin and represents genetic closeness between them.

In the present study, total 635 alleles were detected among 8 rice genotypes with an average number of 3 alleles per locus and average polymorphism information content (PIC) of 0.503, an average percentage of polymorphism 98.17, average resolution power (RP) of 0.600 and an average major allelic frequency of 0.501. The genetic diversity observed in the present study is similar to earlier studies [22], they detected 4.8 alleles per locus and an average PIC value of 0.50. Three alleles per locus with an average PIC value of 0.41 among 88 Indian rice varieties collected from different agro-climatic regions of India were also reported [23]. Similarly, the average PIC values of 0.405, an average RP values of 1.01, the average values of major allelic frequencies of 0.74, an average number of 3 alleles per locus detected among the 141 basmati rice accessions were also reported [24]. Similarly, the average PIC value of 0.44 was observed among 43 Thai and 57 IRRI germplasm of rice [25]. In another study, an average PIC value of 0.45 was observed among the 183 Indonesian rice landraces on the Islands of Borneo [26]. A slightly lower genetic diversity was reported with an average of 2.75 alleles per locus and average PIC value of 0.38 among 40 rice accessions of Pakistan [27]. Similarly, a lower SSR diversity was also observed in a study with 36 polymorphic HvSSRs in which they detected 2.22 alleles per locus and an average PIC value of 0.25 in 375 Indian rice varieties collected from different regions of India [28].

The dendrogram showed that all eight maintainer lines were grouped into three major clusters (Fig. 6). The genotypes were well clustered based on their place of collection and geographical region. The genotypes from Barwale foundation BF-16B and BF-2096B were grouped in cluster I. Similarly, the genotypes from IRRI, IR79156B, IR68897B, IR58025B and APMS-6B from APRRI Maruteru were clustered in cluster II and

genotypes from IIRR, DRR-6B and DRR-9B were grouped in cluster III. Thus, most of the IRRI maintainer lines were clustered in cluster II suggesting moderately less genetic diversity among these genotypes. It is because of similar breeding material were used for the development of these genotypes or in other words they have same ancestry. APMS-6B was distant in dendrogram, because of different types of material have been used for the breeding of this genotype.

Recently [29] Rice microsatellite (RM) markers were used to study the parental polymorphism between the selected two parents APMS-6B a popularly used maintainer line with low stigma exsertion (14.95%) and BF-16B, another maintainer line with high stigma exsertion (80.25%). The two parents were screened for parental polymorphism using 454 SSR markers, of which 118 markers exhibited polymorphism. The overall polymorphism level for the surveyed SSR markers was 25.99% across the 12 chromosomes. [15] Identified the major QTLs for stigma exsertion rate in F2 mapping population using 269 polymorphic SSR markers by crossing Koshihikari / 98SQ1496 of japonica rice genetic background and the population size of 150 segregating plants. Similarly, [30] mapping of minor QTLs for stigma exsertion rate in 225 NILs population using 171 SSR polymorphic markers derived from a cross between ZX and Cx29B. Similarly, [31] Identified a major QTL and its candidate gene for stigma exsertion trait on chromosome 3 in F_3 mapping population using 307 SNPs and 27 Indels by crossing ZS616 [*Oryza sativa* subsp. Xian (indica)], a male sterile line with a stigma exsertion rate (SER) as high as 94.5%, was crossed to DS552, a japonica line with almost no exserted stigmas.

The 253 rice microsatellite (SSR) markers identified as polymorphic among the eight parents in this study will be useful as a pointer to the existence of different alleles at each of the 253 marker loci.

The screening of markers for parental polymorphism among the rice cultivars forms the basis for tagging of the desired gene, fine mapping of the gene in the rice chromosome and in the subsequent Marker assisted selection (MAS) programmes. The polymorphic rice markers can be used in the fine mapping of the stigma exsertion trait and to study the mapping populations of crosses obtained from these parents.

4. CONCLUSION

This study majorly addressing high seed cost of hybrids which is one of the major constraints for large scale adoption of rice hybrids in India. Stigma exsertion is the crucial outcrossing floral trait that increases pollination and seed setting rate of maternal parents there by it improves hybrid seed production efficiency. Significant difference was observed among stigma exsertion donors (Improved maintainers for stigma exsertion trait) and recipient parents (popularly used maintainers in Indian hybrid breeding programme). These parents can be used for developing mapping populations for identifying major QTLs and desirable segregating materials to improve stigma exsertion trait in maintainer pool. Further these improved maintainers can be converted into CMS lines for good out crossing ability. The identified polymorphic markers in the present study can be utilized for QTL mapping studies of various stigma exsertion related traits along with mapping population developed from the crosses among the eight genotypes. These polymorphic markers can be used for background selection of these combinations during marker assisted breeding programmes. Moreover, these identified polymorphic markers can be used for diversity analysis and linkage analysis for various traits in rice.

SUPPLEMENTARY MATERIALS

Supplementary material is available in the following link:

https://www.journalijpss.com/index.php/IJPSS/libraryFiles/downloadPublic/20

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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