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Heterosis studies and molecular characterization of three-line rice hybrids

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ABSTRACT

The present investigation was undertaken at the Hybrid Rice plot of TCA, Dholi, and Muzaffarpur, and molecular analysis was conducted in the Molecular Laboratory of Postgraduate Dept. of Genetics & Plant Breeding, RPCAU, and Bihar to generate heterosis studies for 18 traits and molecular characterization using SSR markers. The experimental material comprised 31 three-line rice hybrids and 3 commercial checks evaluated in the RBD design. Among the tested varieties, Rajendra Sweta performed best in terms of grain yield per plant. Two rice hybrid genotypes, namely, IR68897A × KMR-3R and Rajendra-3A × RRR–4, exhibited superior standard heterosis over all three tests for trait grain yield per plant. By utilizing 12 primer pairs, a total of 33 shared alleles and 13 unique alleles were produced as amplified products. Among the 12 primers, seven primers were found to be comparatively informative for all nineteen hybrids and eleven parents. Only five primers, namely, MRG2894, RM515, RM520, RM538, and RM555, were able to confirm the hybridity (F_1) with the respective parental lines.

Introduction

Rice (*Oryza sativa* L. 2n = 2x = 24) is one of the world's most significant food crops, serving more than 50% of the people worldwide (Brar and Khush 2006; Kumar *et al*., 2020). It is a major grain crop grown in many regions of the globe (Muthayya *et al.,* 2014). In India, the average productivity of rice is 2659 kilograms per hectare (USDA 2020-2021), which is even less than the global productivity, which is nearly 4620 kilograms per hectare (USDA 2020-2021). The Bihar hybrid paddy is currently grown on approximately 6 lakh hectares, and the demand for hybrid paddy seeds among farmers is increasing daily. At the RPCAU, Pusa, Bihar sincere efforts are made to breed hybrid paddy varieties, and they are in an advanced stage with screening of two promising cytoplasmic male sterile lines along with five restorer lines. With the help of three cms lines and three restorer lines obtained from other institutes, five cms lines and eight restorers were crossed during Kharif-2020. Of the forty crosses made, sufficient quantities of seeds were obtained from thirty-two crosses, which were used for the current research purpose. The most vital and challenging variable researched in plant breeding is grain yield in rice. Grain yield enhancement is the principal objective in the majority of breeding initiatives (Yan *et al.,* 2002; Liu *et al*.,2008). The existence of genetic variation is a prime requirement for obtaining high-yielding varieties. Heterosis is based on the scientific concept that F1s or progenies obtained by crossing two diverse parents have a yield advantage in comparison to either the best

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yielder parent (heterobeltiosis) or commercial varieties with the same maturity duration, *i.e.*, standard heterosis (Saravanan *et al*., 2018; Zhang *et al*., 2021; Ramakrishna *et al.,* 2023). The use of molecular markers is an effective tool for assessing genetic connections within and across species, revealing variations in accessions at the DNA level (Akter *et al*., 2022; Vabna *et al.*, 2021).

Material and Methods

Experimental Design and Location

The experiment was conducted using thirty-one rice hybrids, including three strains, namely, Rajendra Sweta, Arize Gold 6444 and Rajendra Bhagwati. The design used for the experiment was the RBD, where each genotype was planted randomly in three replications at the Dholi, Muzaffarpur, and Bihar farms during *Kharif* 2021 from July to December, and molecular work was carried out from June-July 2022 at the Molecular Laboratory, Postgraduate Dept. of Genetics & Plant Breeding, RPCAU, Pusa, Bihar, India. From each plot, 5 competitive individuals were picked at random to record observations and to evaluate all the quantitative characteristics under study except days to 50% flowering and days to maturity, which were monitored on a line basis.

Standard heterosis

Standard Heterosis (SH) is expressed as a percentage of the mean F1 performance relative to the standard check with respect to the intended direction. It was estimated as previously suggested by Meredith and Bridge, 1972.

$$\mathbf{H\ (\%) = \frac{F_1 - S_P}{S_P}\ x\ 100}$$

Molecular characterization:

The cetyltrimethylammonium bromide (CTAB) method, as described by Murray and Thompson in 1980 with slight modifications, was used to standardize the protocol for genomic DNA isolation. PCR was performed using a thermal cycler followed by gel electrophoresis. Only clear and unambiguous bands of markers were scored using a gel documentation system. The details of the primers used in the present study are given in Table 1.

DNA fragment analysis was performed using the NTSYS-PC (Numerical Taxonomy System, version 2.11 W) software (Rohlf, 2000). The SIMQUAL program was used to calculate the Dice coefficient, and a common estimator of genetic identity and similarity matrices based on the Dice coefficient were calculated.

Table 1: Details of primers used in the study

S No	Primer	Forward	Reverse	Annealing Temperature	Repeat Motifs
1	MRG 2894	TATGCTCTCTCCTTCAGGCC	CTTACCAACTCCGCACTTGC	58^{0}C	(GT)15
2	RM 252	TTCGCTGACGTGATAGGTTG	ATGACTTGATCCCGAGAACG	55^{0}C	(CT)19
3	RM 319	ATCAAGGTACCTAGACCACCAC	TCCTGGTGCAGCTATGTCTG	55^{0}C	(GT)10
4	RM 321	CCAACACTGCCACTCTGTTC	GAGGATGGACACCTTGATCG	58^{0}C	(CAT)5
5	RM 416	GGGAGTTAGGGTTTGGAGC	TCCAGTTTCACACTGCTTCG	55^{0}C	(GA)9
6	RM 431	TCCTGCGAACTGAAGAGTTG	AGAGCAAAACCCTGGTTCAC	55^{0}C	(AG)16
7	RM 515	TAGGACGACCAAAGGGTGG	TGGCCTGCTCTCTCTCTCTC	56^{0}C	(GA)11
8	RM 520	AGGAGCAAGAAAAGTTCCCC	GCCAATGTGTGACGCAATAG	55^{0}C	(AG)10
9	RM 538	GGTCGTTGAAGCTTACCAGC	ACAAGCTCTCAAAACTCGCC	55^{0}C	(GA)14
10	RM 555	TTGGATCAGCCAAAGGAGAC	CAGCATTGTGGCATGGATAC	55^{0}C	(AG)11
11	RM 276	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA	55^{0}C	(AG) 8A3 (GA)33
12	RM 558	GAACTCCTCGAACTCGATGC	AGGCATTCAACCTGTTCGAC	55^{0}C	(ATTG)5

A dendrogram of all 11 parental rice genotypes was constructed using unweighted pair group method with arithmetic average (UPGMA) cluster analysis. The polymorphism information content (PIC) of the SSR markers was obtained by calculating the value according to the formula (Anderson *et al*., 1993). The polymorphism information content (PIC) of the SSR primer pairs was calculated as follows:

$$PIC_i = 1 - \Sigma^{k}_{j=1} P^2_{ij}$$

where
k is the total number of alleles detected for a locus of a marker, P is the frequency of the j^{th} allele for the i^{th} marker, and the summation extends over k alleles.

Results and Discussion

Heterosis

The current study estimated heterosis over standard checks (standard heterosis) in 31 hybrids for yield traits to determine the ideal parent combination and characterize the parents for their potential future use in breeding programmes. Estimates of standard heterosis over three checks for grain yield per plant are given in Table 2.

Standard heterosis across Rajendra Bhagwati

Numerically significant heterosis for grain yield per plant in Rajendra Bhagwati ranged between -17.13% in the cross IR68897A×RRR-1 and 9.90% in the cross Rajendra-3A×RRR-4. Sixteen hybrids were found to have significant negative heterosis. The seven hybrids that exhibited significant positive heterosis were IR68897A × KMR - 3R, Rajendra - 1A × RRR – 4, Rajendra - 3A × RRR – 4, Rajendra - 3A × DR714 - 1- 2, Rajendra - 3A × MSN- 36 R, IR - 58025A × MSN - 36R and CMR 32A × RRR – 2. The abovementioned results were similar to the findings of Sreelakshmi *et al*. (2019) and Ramakrishna *et al.,* 2023.

Standard heterosis over check Arize Gold 6444:

Numerically significant heterosis for grain yield/plant over Check ARIZE GOLD 6444 ranged between -12.68% in the cross IR68897A × RRR – 1 to 15.80% in the cross Rajendra-3A × RRR-4. Out of 31 crosses, seven showed negative heterosis, whereas the other crosses, *i.e.,* IR68897A × RRR– 5, IR68897A × DR714 - 1- 2, IR68897A × KMR- 3R, Rajendra-1A × RRR– 2, Rajendra-1A × RRR– 3, Rajendra- 1A × RRR– 4, Rajendra-1A × RRR – 5, Rajendra-1A × DR714 - 1- 2, Rajendra-1A × KMR - 3R, Rajendra-1A × MSN- 36 R, Rajendra-3A × RRR – 1, RAJENDRA - 3A × RRR – 3, RAJENDRA - 3A × RRR – 4, RAJENDRA - 3A × RRR – 5, Rajendra - 3A × DR714 - 1- 2, Rajendra-3A × MSN- 36 R, IR - 58025A × MSN - 36R, CMR 32A × RRR – 1 and CMR 32A × RRR – 2, exhibited positive significant heterosis. Other research studies carried out by Ranjith *et al*., 2020; Azad *et al*., 2022 also presented similar results.Standard heterosis over the Rajendra Sweta variety:Numerically significant heterosis for grain yield per plant in Rajendra Sweta ranged between -19.47% in the IR68897A × RRR–1 cross and 6.80% in the Rajendra-3A × RRR–4 cross. Most of the crosses were found to have negative standard heterosis with this check, but two crosses, namely, IR68897A × KMR-3R and Rajendra-3A × RRR–4, exhibited significant positive results. These findings are similar to the results of Shukla *et al*. (2020) and Aananthi and Jebaraj (2023).Out of 31 three-line rice hybrid genotypes, 19 hybrids were superior in terms of yield and other related traits. Thus, significant standard heterosis for these characteristics indicated a high level of genetic variability among the hybrids, and the unidirectional distribution of allelic constitution contributed to desirable heterosis in the present study. Numerous crosses expressing negative heterosis for traits such as days to 50% flowering plant height and days to maturity indicated that the hybrids outperformed the parents in these areas and that the heterotic effects were in the right direction. To identify better transgressive segregants in subsequent generations, more research can be done on the crosses that showed strong heterotic expression in F_1.

Molecular experimental findings

The number of alleles and polymorphism information content (PIC, Table 3) values for the 12 primers included in the present study were used to assess the degree of polymorphism among the eleven parental rice genotypes.

SSR primer-based amplification of genotypes

Twelve SSR primers were used for amplifying the genomic DNA isolated from 11 parental genotypes and 19 three-line rice hybrid genotypes. Amplification was carried out among the parents, and the results revealed polymorphisms. Therefore, further studies were performed on each genotype.

Table 2: Estimates of standard heterosis for grain yield per plant

Sl. No.	Genotypes	Rajendra bhagwati	Arize gold 6444	Rajendra sweta
1	IR68897A × RRR - 1	-17.13**	-12.68**	-19.47**
2	IR68897A × RRR - 2	-12.90**	-8.22**	-15.32**
3	IR68897A × RRR - 3	-8.12**	-3.19**	-10.72**
4	IR68897A × RRR - 4	-16.24**	-11.74**	-18.60**
5	IR68897A × RRR - 5	0.21	5.59**	-2.62*
6	IR68897A × DR714 - 1- 2	-3.04**	2.16*	-5.78**
7	IR68897A × KMR - 3R	7.60**	13.38**	4.57**
8	IR68897A × MSN- 36 R	-16.23**	-11.73**	-18.59**
9	RAJENDRA - 1A × RRR - 1	-8.73**	-3.83**	-11.31**
10	RAJENDRA - 1A × RRR - 2	-1.80	3.47**	-4.57**
11	RAJENDRA - 1A × RRR - 3	-2.81**	2.41*	-5.56**
12	RAJENDRA - 1A × RRR - 4	4.93**	10.57**	1.97
13	RAJENDRA - 1A × RRR - 5	-2.66**	2.57**	-5.41**
14	RAJENDRA - 1A × DR714 - 1- 2	1.48	6.93**	-1.39
15	RAJENDRA - 1A × KMR - 3R	0.63	6.04**	-2.21*
16	RAJENDRA - 1A × MSN- 36 R	-0.62	4.72**	-3.42**
17	RAJENDRA - 3A × RRR - 1	-2.00*	3.26**	-4.77**
18	RAJENDRA - 3A × RRR - 2	-7.18**	-2.20*	-9.80**
19	RAJENDRA - 3A × RRR - 3	-2.21*	3.04**	-4.98**
20	RAJENDRA - 3A × RRR - 4	9.90**	15.80**	6.80**
21	RAJENDRA - 3A × RRR - 5	1.27	6.71**	-1.59
22	RAJENDRA - 3A × DR714 - 1- 2	2.01*	7.49**	-0.87
23	RAJENDRA - 3A × MSN- 36 R	4.65**	10.27**	1.70
24	IR - 58025A × MSN - 36R	3.30**	8.85**	0.39
25	CMR 32A × RRR - 1	-0.83	4.49**	-3.63**
26	CMR 32A × RRR - 2	3.80**	9.38**	0.87
27	CMR 32A × RRR - 3	0.25	1.63	-2.58*
28	CMR 32A × RRR - 4	-3.25**	1.94	-5.98**
29	CMR 32A × RRR - 5	-3.09**	1.92	-5.83**
30	CMR 32A × KMR - 3R	-6.11**	-1.07	-8.77**
31	CMR 32A × MSN - 36 R	-3.49**	1.69	-6.21**

Table 3. Analysis of primer pairs utilized for amplification of genomic DNA extracted from eleven parental rice genotypes

Sl. No	Primer	Size range (bp)	Amplicon difference range (bp)	No. of Alleles	No. of Unique Alleles	No. of Shared Alleles	PIC
1	MRG2894	256-72	184	7	3	4	0.84
2	RM 252	288-228	60	3	2	1	0.31
3	RM 319	124-83	41	3	0	3	0.63
4	RM 321	121-95	26	3	1	2	0.43
5	RM 416	132-109	23	1	0	1	0.00
6	RM 431	246-226	20	1	0	1	0.00
7	RM 515	259-125	134	6	1	5	0.83
8	RM 520	258-100	158	6	2	4	0.81
9	RM 538	263-75	188	6	1	5	0.83
10	RM 555	282-76	206	5	1	4	0.76
11	RM 276	174-157	17	4	2	2	0.70
12	RM 558	148-128	20	1	0	1	0.00

All 12 SSR primers used for amplification produced amplified products. Generally, SSR markers are codominant and polymorphic, and the primers used for the present study produced 1 to2 bands. The primers MRG 2894, RM 515, RM 520, RM 538, and RM 555 were able to differentiate the F_1 generations, which confirms that these hybrids were developed by crossing these 11 parents, as represented in figures 1-5. According to the PIC values, there was a variety of allele frequencies and diversity among the rice genotypes, ranging from 0.00 in RM 416, RM 431 and RM 558 to 0.84 in MRG 2894 (Singh *et al*., 2011; Toppo *et al*., 2018).

Clustering and Similarity coefficient

Examining the dendrogram (Table 4) revealed that the entries were essentially split into five clusters. Cluster A included 1 genotype, viz. IR58025A. Cluster B had four genotypes, *i.e.,* RRR – 1, MSN-36 R, RAJENDRA - 3A, and IR 68897A. Cluster C consisted of DR714-1-2 and RAJENDRA-1A. Cluster D consisted of RRR – 2 and RRR – 4. Cluster E had RRR -3 and RRR – 5. More diversity was found in Cluster A, which included 1 genotype, viz. IR58025A. (Singh *et al.,* 2016). A diagram of the dendrogram is shown in Figure 6. The extent of the dice similarity coefficient between RAJ-3A and IR 68897A (0.917) was found to be the greatest, followed by RRR-2 and RRR-4 (0.833), MSN-36R and RRR-1 (0.818), RAJ-1A and DR-714-1-2 (0.750) and MSN-36R, RAJ-1A and RAJ-3A (0.636) and the minimum for IR 58025A and RRR-1, RRR-5, DR-714-1-2 (0.240). A total of 33 shared alleles and 13 unique allelic variants were produced as amplified products. All primer pairs generated both unique and shared alleles, except RM-319, RM-416, RM-431 and RM-558, which generated only shared alleles without any unique alleles. The PIC ranged from 0 to 0.84. The PIC value thus showed that each of these primers was extremely informative and able to differentiate between genotypes. Through cluster analysis, it is clear that the genotypes Raj 3A and IR68897-A are more similar and less diverse, whereas the genotype IR58025A is more diverse than all other parents. With an average Jaccard coefficient similarity index of 0.57, the genotypes in the study exhibited a high degree of diversity. The similarity coefficients ranged from 0.24 to 0.91. A lower similarity coefficient indicates that the genotypes are more diverse and can be used as parents in breeding programs. A higher value indicates less diversity among the genotypes. In this study, the wider range of genotype similarity values

Table 4: Estimates of 12 primer pair-based similarity coefficients among 11 parental rice genotypes

Genotypes	GP-1	GP-2	GP-3	GP-4	GP-5	GP-6	GP-7	GP-8	GP-9	GP-10
GP-2	0.434									
GP-3	0.400	0.636								
GP-4	0.400	0.636	0.583							
GP-5	0.320	0.546	0.500	0.917						
GP-6	0.480	0.818	0.500	0.500	0.583					
GP-7	0.320	0.455	0.417	0.417	0.417	0.417				
GP-8	0.320	0.546	0.417	0.417	0.417	0.500	0.500			
GP-9	0.240	0.364	0.333	0.333	0.333	0.333	0.833	0.417		
GP-10	0.240	0.455	0.250	0.250	0.250	0.417	0.333	0.583	0.500	
GP-11	0.240	0.364	0.750	0.333	0.333	0.333	0.417	0.417	0.500	0.417

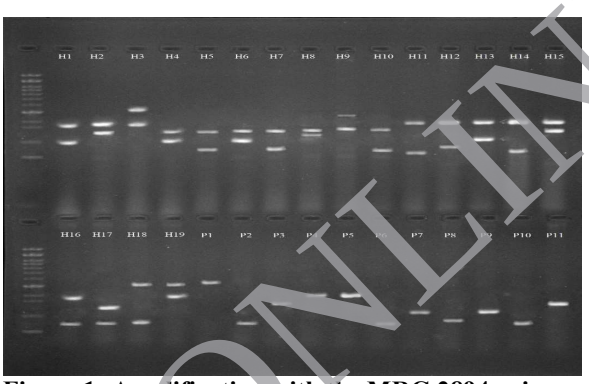

Figure 1: Amplification with the MRG 2894 primer

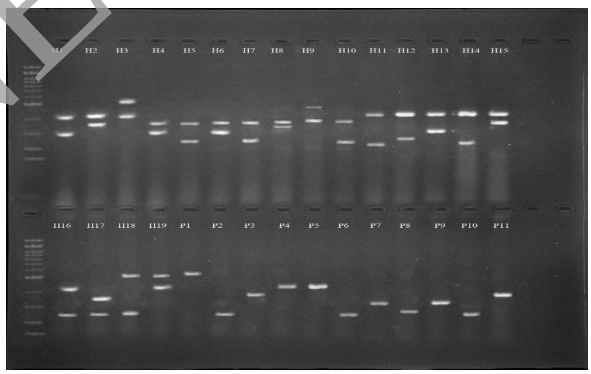

Figure 2: Amplification with the primer RM 515

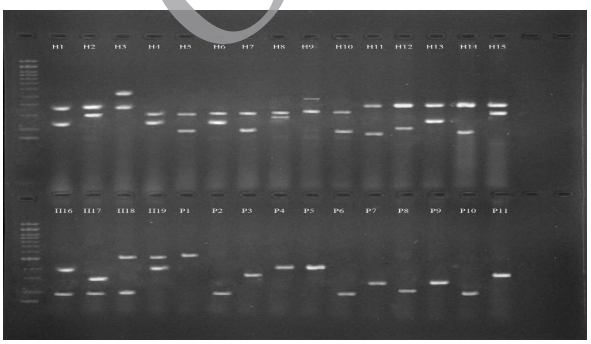

Figure 3: Amplification with the primer RM 520

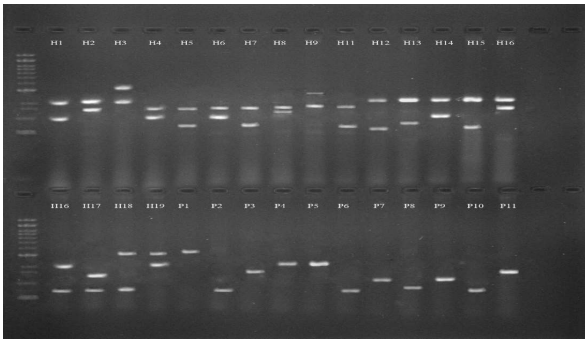

Figure 4: Amplification with the primer RM 538

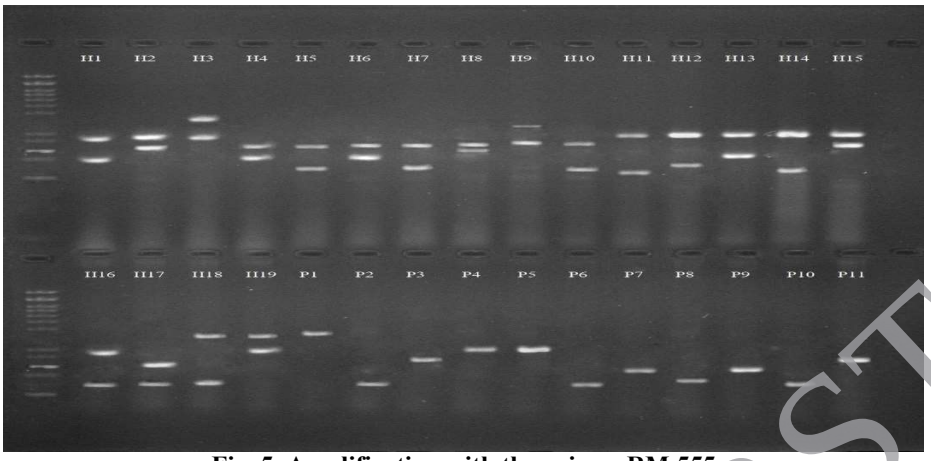

Fig. 5. Amplification with the primer RM 555

PARENTS	HYBRIDS	
P1-IR 58025A	H1-RAJENDRA - 1A × RRR - 1	H12-RAJENDRA - 3A × RRR - 4
P2-MSN-36R	H2-RAJENDRA - 1A × RRR - 2	H13-RAJENDRA - 3A × RRR - 5
P3-RAJ-1A	H3-RAJENDRA - 1A × RRR - 3	H14-RAJENDRA - 3A × DR714 - 1- 2
P4-RAJ-3A	H4-RAJENDRA - 1A × RRR - 4	H15-RAJENDRA - 3A × MSN- 36 R
P5-IR 68897A	H5-RAJENDRA - 1A × RRR - 5	H16-IR - 58025A × RRR-1
P6-RRR-1	H6-RAJENDRA - 1A × DR714 - 1- 2	H17-IR - 58025A × RRR-2
P7-RRR-2	H7-RAJENDRA - 1A × KMR - 3R	H18-IR - 58025A × RRR-3
P8-RRR-3	H8-RAJENDRA - 1A × MSN- 36 R	H19-IR - 58025A × MSN - 36R
P9-RRR-4	H9- RAJENDRA - 3A × RRR - 1	
P10-RRR-5	H10-RAJENDRA - 3A × RRR - 2	
P11-DR-714-1-2	H11-RAJENDRA - 3A × RRR - 3	

provided by microsatellite markers increases the certainty of genetic diversity and relationship assessments, which can be applied in subsequent breeding initiatives (Thete *et al*., 2023, Deepika *et al.,* 2022).

Conclusion

Through heterotic studies, two crosses, namely, IR68897A×KMR-3R and Rajendra-3A×RRR-4, were found to be the best cross combinations for yield traits among all the other checks considered. Thus, these cross combinations can be further utilized for commercial use and for obtaining transgressive segregants in the F_2 generation. On the other hand, molecular analysis revealed that the PIC varied from 0.00 for RM 416, RM 431 and RM 558 to 0.84 for MRG 2894, indicating that out of 12 primers, only 9 showed polymorphisms. The parents IR 58025A and RRR-1, RRR-5, and DR-714-1-2 had minimum similarity coefficients, which revealed the diversity among these parents. The above genotypes that showed the highest polymorphism and greatest diversity may be tested for hybridization by breeders in an effort to improve genetic variability in rice and aid in the breeding of promising rice genotypes.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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