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Research Article

Studies on genetic divergence and screening of parental lines for *Rf3* and *Rf4* genes through molecular markers in hybrid rice (*Oryza sativa* L.)

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Abstract
Sixty four parental lines of hybrid rice were evaluated for genetic divergence by taking into account of nine traits during two seasons of year 2018. By resolving the parental lines into ten groups, Ward's clustering method demonstrated the parental lines having significant genetic diversity. Cluster X had the most parental lines (13) out of all the clusters, followed by cluster II (12 genotypes). The parental line clusters IX, VIII, VII, V, and IV, which correspond to SD-5, SD-2, HSRV-16, GR-37, and SD-39, respectively, showed the significant degree of variability. Screening for fertility restoration was done in 64 parental lines with the help of SSR molecular markers. Among them, 44 potential restorers were identified (*Rf4* and *Rf3* present) with a hundred percentage efficiency supported molecular screening. The traits number of productive tillers per plant, length of the panicle and number of grains per panicle were significantly correlated with the trait grain yield per plant. Path analysis showed that the traits number of grains per panicle, the number of productive tillers per plant, test weight and panicle length had positive direct effects on grain yield and indicating that selection for these yield contributing traits could lead to an improvement in single plant yield as a whole.

Keywords: Genetic Diversity, Character Association, Fertility Restoration.

INTRODUCTION

With the development of F_1 rice hybrids using cytoplasmic male sterility and fertility restoration method, heterosis breeding has been effectively used to increase rice yield in China. The hybrids out yielded the simplest pure line varieties by 20-30% (Lin and Yuan, 1980). Hybrid rice technology is one of the most promising, dependable, and well-established methods for increasing rice output, with a yield gain of 15-20% over inbred types. More than half of China's rice field is now cultivated with hybrid rice and other nations that grow rice are heading the same way (Virmani *et al*., 2003). However, still it's at infancy due to the non-availability of stable male sterile lines, maintainers and a low degree of fertility restoration by restorer lines. In order to commercially exploit hybrid rice in India, it is required to identify maintainers and restorers. A promising alternative method for overcoming these latest cultivars output ceiling is hybrid rice. Nuclear genes' restoration of male fertility permits commercial use of the CMS system to create hybrid seeds (Newton 1988, Kazama *et al*., 2008). The use of molecular markers connected to *Rf* genes

that restore fertility can improve the effectiveness of choice, reduce time, and prevent problems associated with phenotype-based screening. For WA-type CMS, chromosomes 1 and 10 are the locations of the fertility restorer genes *Rf*3 and *Rf*4, respectively (Yao *et al*., 1997). The production of improved recombinants depends on genetic variety, which is a requirement for any crop improvement programme (Arunachalam, 1981). Finally, grouping genotypes offers a clear image of how genotypes interact and aids in the selection of suitable divergent parents to be used in the next hybridization programmes. This study was conducted to identify maintainers and restorers for the generation of heterotic rice hybrids as well as to assess the genetic divergence of 64 parental lines of hybrid rice.

MATERIALS AND METHODS

The Indian Institute of Rice Research (IIRR), Hyderabad provided the 64 parental lines of hybrid rice, which were used in this investigation (**Table 1**). This study examined the genetic divergence and fertility restoration of 64 hybrid rice parental lines over the course of two seasons (*Kharif* and *Rabi*) in 2018 at the Indian Institute of Rice Research (IIRR) farm, Hyderabad. Two replications of the Randomised block design were used to lay out the experimental materials. Data was collected from five randomly chosen plants from each replication and observations including days to 50% flowering, plant height, panicle length, pollen fertility, the number of productive tillers per plant, the number of filled grains per panicle, spikelet fertility percentage, 1000 grain weight and grain yield per plant were included in the study to estimate genetic divergence, correlation and path analysis. Analysis was carried out by WINDOSTAT software. Following the Wards Clustering approach, genetic divergence was investigated (Wards, 1963). By using the CTAB procedure, total genomic DNA was recovered from young leaves (Sambrook, 1989). SSR markers were resolved using DNA quantification, PCR amplification, and agarose gel electrophoresis. 50 ng/l of template DNA, 2.5 mM of each dNTP, 0.5 l of each forward and reverse primer, 0.2 l of Taq DNA polymerase, and 10X PCR reaction buffer were used in PCR reactions that were conducted in a thermal cycler (Eppendorf, Nexus Gradient, USA). The amplified PCR products and molecular markers were separated on 3.0% Seakem® LE agarose gel and documented using Syngene Ingenius gel documentation system. In rice, the restoration of fertility in cases of wild-abortive cytoplasmic male sterility (WA-CMS) is known to be mediated by two important nuclear genes, *Rf*3 and *Rf*4. In the current investigation, 64 parental lines were tested for fertility restoration to identify maintainers and restorers using four published SSR markers associated to the *Rf*4 and *Rf*3 genes (RMS-PPR 9-1, RMS-PPR 762, RMS-SF-21-5, and DRRM-RF3-10). The primer sequences for the rice *Rf*4 and *Rf*3 markers are listed in (**Table 1**).

Table 1. Primer sequence of *Rf4* and *Rf3* markers in rice.

Name of the Primer	Primer Sequence (5' - 3')	Gene tagged	Chromosomal location	Reference
RMS-PRR 9-1	GAGTTTTGAATAGATTTACGTGTGGA AGTGTCCAGATTCGTAGTAATGC	*Rf4*	10	(Pranathi *et al.,* 2016)
RMS-PPR 762	TTGCCAGCATGTTCTCAGTT GCAAAGCCCATGAAGGATTA	*Rf4*	10	(Pranathi *et al.,* 2016)
RM SF 21-5	ACTTACACAAGGCCGGGAAAGG TGGTAGTGGTAACTCTACCGATGG	*Rf3*	1	(Pranathi *et al.,* 2016)
DRRM-Rf3-10	GATGGCAAGCTTCAGAACA CTAATTCTGGGCGAGCAAAG	*Rf3*	1	(Balaji *et al*., 2012)

Table 2. Pooled analysis of variance for yield and its component traits in hybrid rice.

Source of variation	Df	DFF	PH	PT	PL	PF	SF	GPP	TW	SPY
Replication	1	4.0	32.81	31.39	15.65	4.17	107.52	346.12	12.80	9.10
Treatments	127	97.32**	739.69**	16.26**	10.12**	81.18**	71.63**	3748.83**	17.67**	129.39**
Error	127	7.78	13.75	2.62	3.12	4.83	10.94	123.63	0.17	0.16
Total	255	52.36	375.37	9.53	6.66	42.85	41.55	1930.0	8.94	64.56
CV (%)		2.90	3.60	13.51	7.83	2.51	3.82	8.83	2.02	1.76
CD		5.52	7.33	3.20	3.50	4.34	6.54	22.00	0.81	0.79
SE		1.97	2.62	1.14	1.25	1.55	2.33	7.86	0.29	0.28

RESULTS AND DISCUSSION

Analysis of variance indicated substantial differences for all the variables examined, showing that the 64 parental lines have enough genetic diversity (**Table 2**). In the present investigation, the genetic divergence among 64 parental lines was studied by Wards clustering analysis at 6 coefficients, and it distributed 64 genotypes into 10 clusters (**Table 3, Fig. 1**). Among different clusters, cluster 10 had maximum parental lines (*i.e*., 13) followed by cluster II (*i.e*., 12) parental lines. A high degree of genetic heterogeneity was indicated by the clusters IX, VIII, VII, V, and IV, which correspond to SD-5, SD-2, HSRV-16, GR-37, and SD-39, respectively. With this perspective, it is determined that genotypes from clusters X, VIII, VII, V, and IV could be employed as parents in the hybridization programme to provide breeding material with high genetic diversity.

In the present study, four reported SSR markers, RMS-PPR 9-1, RMS-PPR 762, RMS-SF-21-5 (Pranathi *et al.,* 2016) and DRRM- RF3-10 (Balaji *et al*., 2012) were used. Among them, RMS-PPR 9-1, RMS-PPR 762 were linked to *Rf4* and RMS-SF-21-5, DRRM- RF3-10 were linked to *Rf3* are used efficiently for identifying restorer lines in crop improvement programmes. Of these, 60 lines were found positive with RMS-PPR 9-1 (*Rf*4), 62 lines positive with RMS-PPR 762 (*Rf*4), 54 lines positive with DRRM-RF3-10 (*Rf*3) and 49 lines positive with RMS-SF-21-5 (*Rf*3). It is presented in **Table 4** and **plate no. 1, 2, 3 and 4**. Among the four reported markers RMS-PPR-9-1 marker is best compared with other markers for identifying restorers. Shidenur *et al*. (2019) and Pranathi *et al*. (2016) found similar findings for the identification of restorers. Out of 64 genotypes, 44 parental lines were found positive with all

Table 3. Clustering pattern of 64 hybrid rice parental lines.

Cluster Number	Number of Genotypes	Name of the Genotypes
I	5	SD-63, SD-77, PSV-397, DP-204, BCW-56
II	12	SD-4, SD-57, DP-207, SD-66, SD-49, SD-62, PSV-250, SD-67, SD-73, DP-230, SD-12, PSD-30
III	1	SD-46
IV	8	SD-39, SD-38, SD-7, SD-8, SD-32, SD-44, SD-13, SD-43
V	7	TCP-964, GR-22, GR-37, HSRV-16, GR-29, HSRV-19, GR-6
VI	2	HSRV-1, ATR-394
VII	3	GR-7, RPHR-517, IBL-57
VIII	4	SD-2, SD-9, SD-55, SD-61
IX	9	GR-9, GR-40, SD-5, GR-15, RPHR-619-20, GR-11, GR-14, TCP-1483, SD-64
X	13	HSRV-4, HSRV-5, HSRV-6, KMR-3R, P-63, TCP-718, TCP-1369, RPHR-1005, GR-2, TCP-2069, TCP-950, TCP-583, RPHR-2

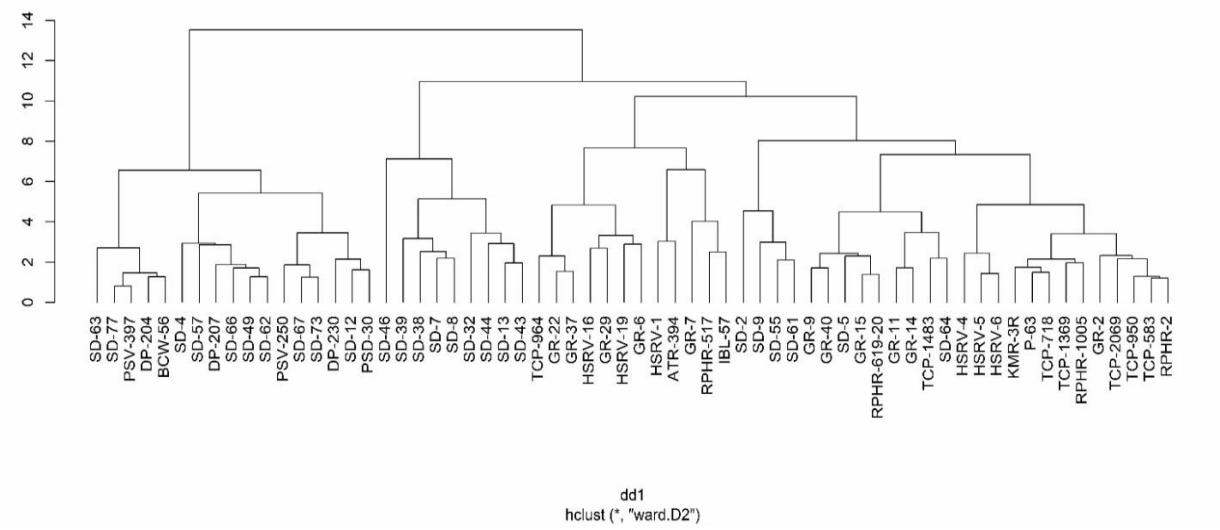


Fig. 1. Clustering by Ward's method in 64 parental lines of hybrid rice.

Table 4. Screening of parental lines for fertility restoration through *Rf4* and *Rf3* markers in hybrid rice.

S. No	ENTRIES	*Rf4* (RMS-PPR-9-1)	*Rf4* (RMS-PPR-762)	*Rf3* (DRRM-RF3-10)	*Rf3* (RMS-SF-21-5)
1	HSRV-1	*Rf4*	*Rf4*	*Rf3*	*Rf3*
2	HSRV-4	*Rf4*	*Rf4*	*Rf3*	*Rf3*
3	HSRV-5	*Rf4*	*Rf4*	*Rf3*	*Rf3*
4	HSRV-6	*Rf4*	*Rf4*	*Rf3*	*Rf3*
5	HSRV-16	*Rf4*	*Rf4*	*Rf3*	*Rf3*
6	HSRV-19	*Rf4*	*Rf4*	*Rf3*	-
7	GR-2	*Rf4*	*Rf4*	*Rf3*	*Rf3*
8	GR-6	*Rf4*	*Rf4*	*Rf3*	*Rf3*
9	GR-7	-	-	*Rf3*	*Rf3*
10	GR-9	*Rf4*	*Rf4*	*Rf3*	*Rf3*
11	GR-11	*Rf4*	*Rf4*	*Rf3*	*Rf3*
12	GR-14	*Rf4*	*Rf4*	*Rf3*	*Rf3*
13	GR-15	*Rf4*	*Rf4*	*Rf3*	*Rf3*
14	GR-22	*Rf4*	*Rf4*	*Rf3*	*Rf3*
15	GR-29	*Rf4*	*Rf4*	*Rf3*	*Rf3*
16	GR-37	*Rf4*	*Rf4*	*Rf3*	*Rf3*
17	GR-40	*Rf4*	*Rf4*	*Rf3*	*Rf3*
18	P-63	*Rf4*	*Rf4*	*Rf3*	*Rf3*
19	TCP-583	*Rf4*	*Rf4*	*Rf3*	*Rf3*
20	TCP-718	*Rf4*	*Rf4*	*Rf3*	*Rf3*
21	TCP-950	*Rf4*	*Rf4*	*Rf3*	*Rf3*
22	TCP-964	*Rf4*	*Rf4*	*Rf3*	*Rf3*
23	TCP-1369	*Rf4*	*Rf4*	*Rf3*	*Rf3*
24	TCP-1483	*Rf4*	*Rf4*	*Rf3*	*Rf3*
25	TCP-2069	*Rf4*	*Rf4*	*Rf3*	*Rf3*
26	ATR-394	*Rf4*	*Rf4*	*Rf3*	*Rf3*
27	KMR-3R	*Rf4*	*Rf4*	*Rf3*	*Rf3*
28	RPHR-2	*Rf4*	*Rf4*	*Rf3*	*Rf3*
29	RPHR-517	*Rf4*	*Rf4*	*Rf3*	-
30	RPHR-619-20	*Rf4*	*Rf4*	*Rf3*	*Rf3*
31	RPHR-1005	*Rf4*	*Rf4*	*Rf3*	*Rf3*
32	IBL-57	*Rf4*	*Rf4*	*Rf3*	*Rf3*
33	DP-204	*Rf4*	*Rf4*	*Rf3*	-
34	DP-207	*Rf4*	*Rf4*	*Rf3*	*Rf3*
35	DP-230	*Rf4*	*Rf4*	*Rf3*	*Rf3*
36	SD-4	*Rf4*	*Rf4*	*Rf3*	*Rf3*
37	SD-57	*Rf4*	*Rf4*	*Rf3*	*Rf3*
38	SD-64	*Rf4*	*Rf4*	*Rf3*	*Rf3*
39	SD-66	*Rf4*	*Rf4*	*Rf3*	*Rf3*
40	SD-67	*Rf4*	*Rf4*	*Rf3*	*Rf3*
41	SD-12	*Rf4*	*Rf4*	-	-
42	SD-13	*Rf4*	*Rf4*	-	-
43	SD-32	*Rf4*	*Rf4*	-	-
44	SD-38	*Rf4*	*Rf4*	*Rf3*	-
45	SD-39	*Rf4*	*Rf4*	*Rf3*	-

Table 4. Continued..

S. No	ENTRIES	*Rf4* (RMS-PPR-9-1)	*Rf4* (RMS-PPR-762)	*Rf3* (DRRM-RF3-10)	*Rf3* (RMS-SF-21-5)
46	SD-55	*Rf4*	*Rf4*	-	*Rf3*
47	SD-61	*Rf4*	*Rf4*	-	*Rf3*
48	SD-5	*Rf4*	*Rf4*	*Rf3*	*Rf3*
49	SD-2	*Rf4*	*Rf4*	*Rf3*	*Rf3*
50	SD-7	*Rf4*	*Rf4*	*Rf3*	*Rf3*
51	SD-8	-	-	*Rf3*	*Rf3*
52	SD-9	*Rf4*	*Rf4*	*Rf3*	*Rf3*
53	SD-43	*Rf4*	*Rf4*	*Rf3*	-
54	SD-44	-	*Rf4*	*Rf3*	-
55	SD-46	*Rf4*	*Rf4*	-	*Rf3*
56	SD-49	*Rf4*	*Rf4*	*Rf3*	*Rf3*
57	SD-62	*Rf4*	*Rf4*	*Rf3*	*Rf3*
58	SD-63	-	*Rf4*	-	-
59	SD-73	*Rf4*	*Rf4*	*Rf3*	*Rf3*
60	SD-77	*Rf4*	*Rf4*	*Rf3*	*Rf3*
61	GP-15	*Rf4*	*Rf4*	*Rf3*	-
62	GP-59	*Rf4*	*Rf4*	*Rf3*	*Rf3*
63	PSV-250	*Rf4*	*Rf4*	-	-
64	PSV-397	*Rf4*	*Rf4*	-	-
	Number of lines confirmed	**60**	**62**	**54**	**49**

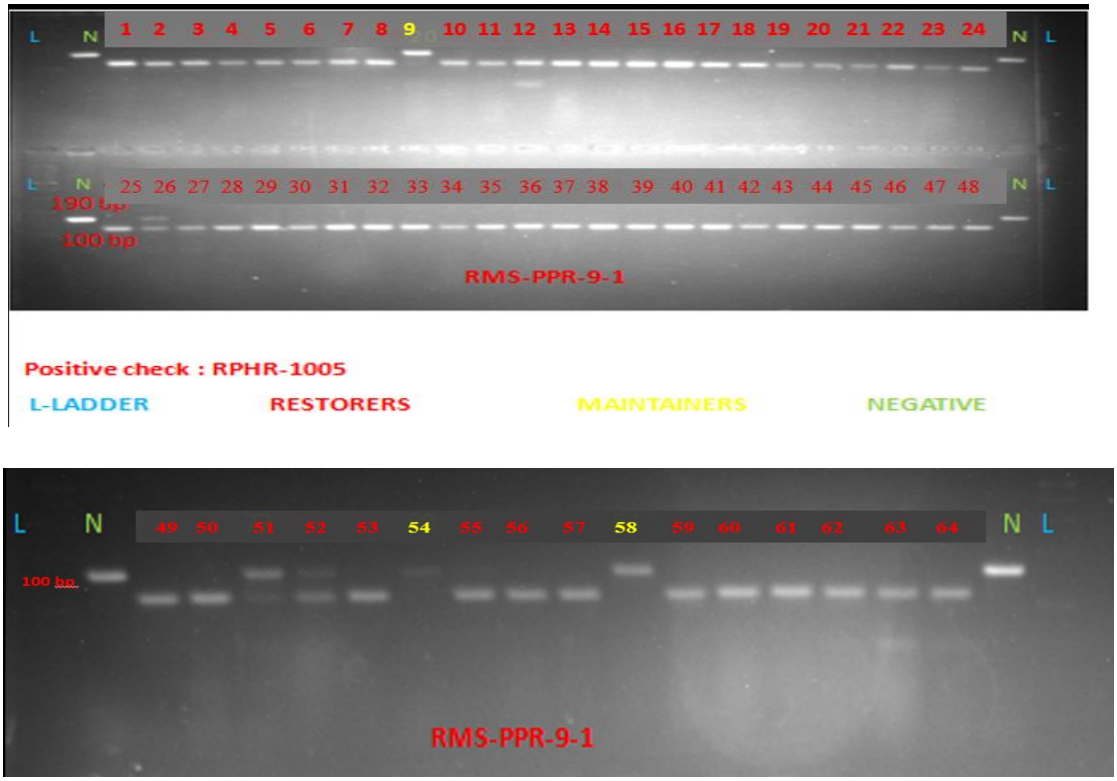


Plate No. 1. Representative gel picture for screening of parental lines for fertility restoration with RMS-PPR-9-1 marker in hybrid Rice.

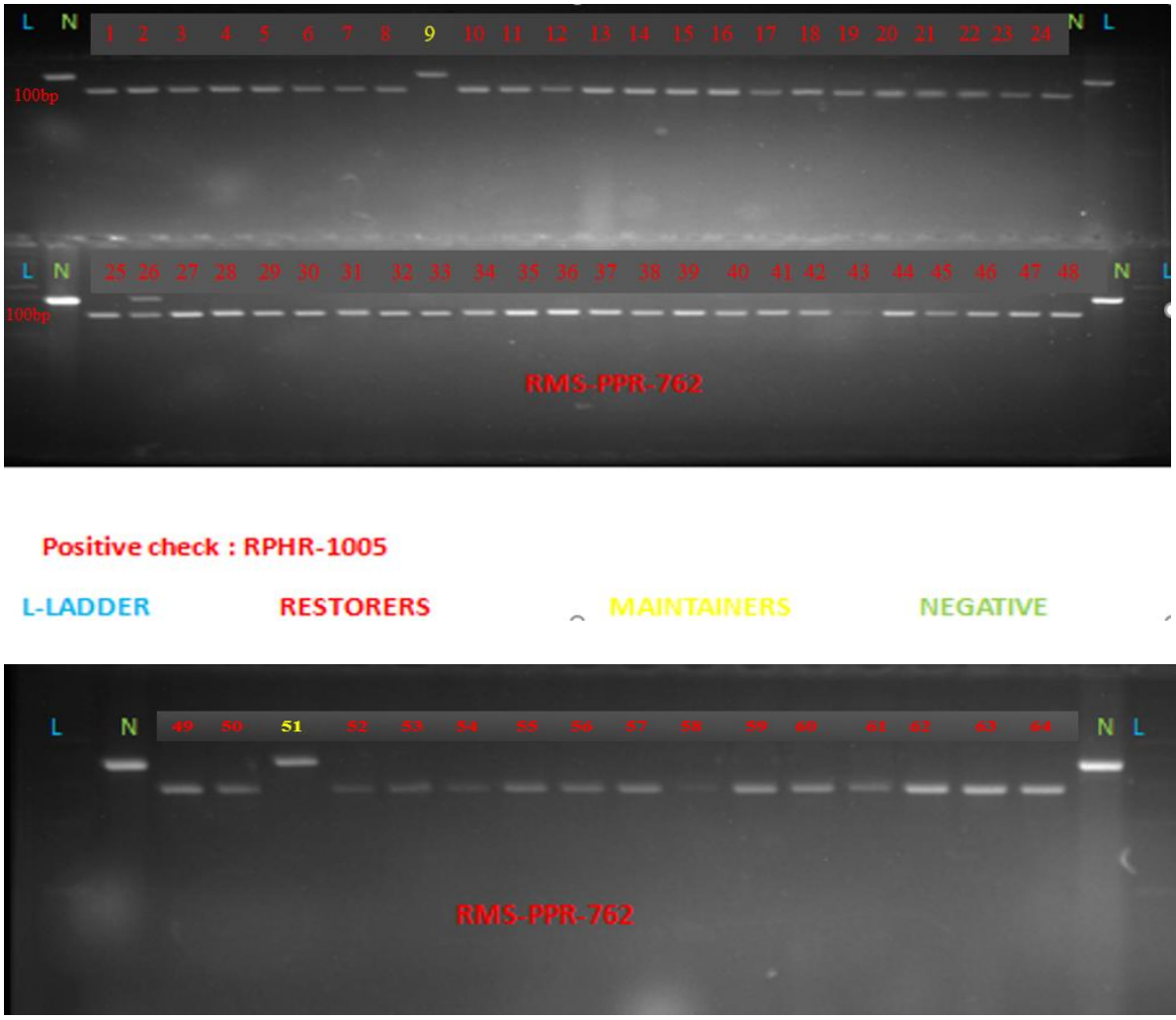
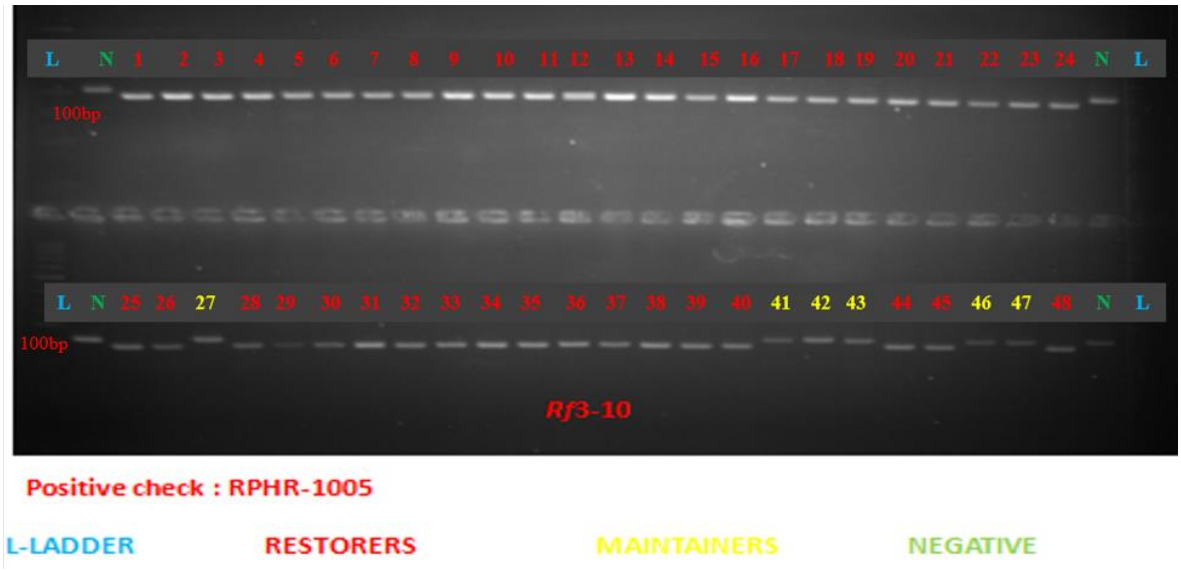


Plate No. 2. Representative gel picture for Screening of parental lines for fertility restoration with RMS-PPR-762 marker in hybrid Rice.



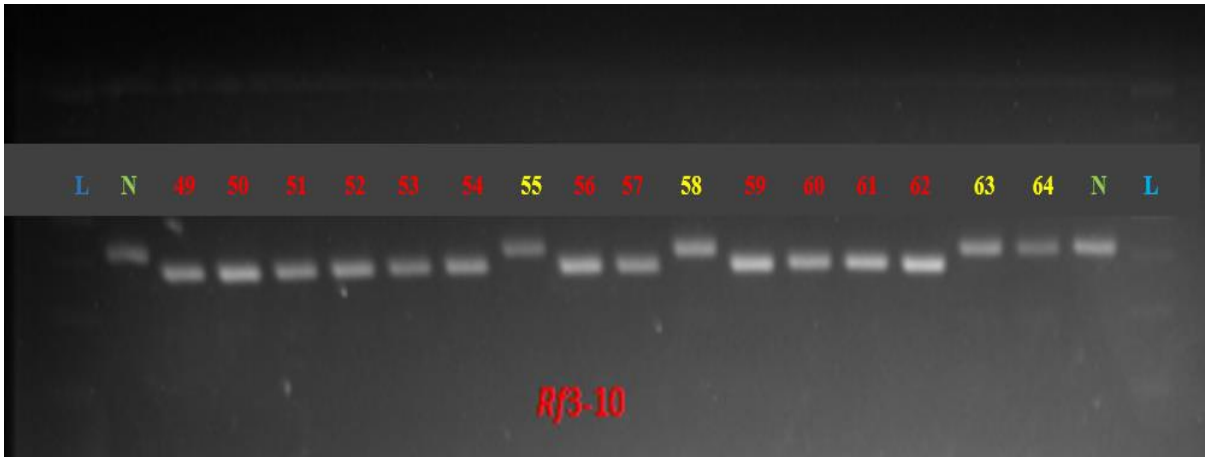
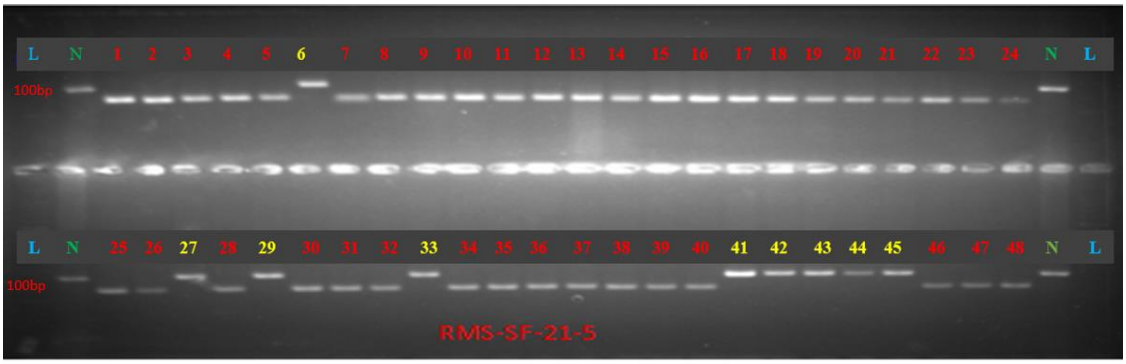


Plate No. 3. Representative gel picture for Screening of parental lines for fertility restoration with DRRM- Rf3-10 marker in hybrid rice.



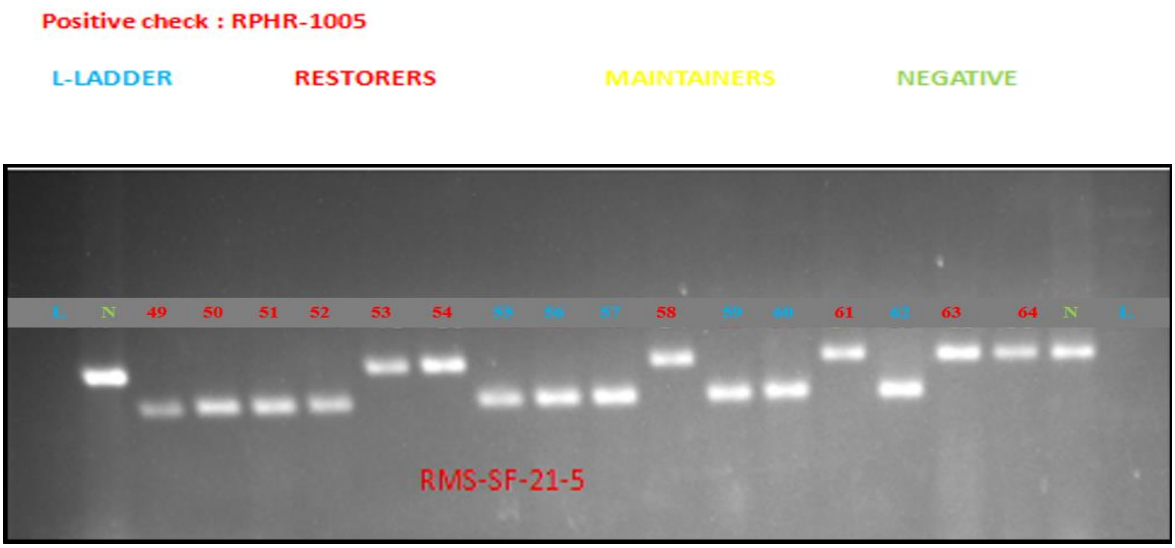


Plate No. 4. Representative gel picture for Screening of parental lines for fertility restoration with RMS-SF-21-5 marker in hybrid rice.

four markers. Forty four genotypes are HSRV-1, HSRV-4, HSRV-5, HSRV-6, HSRV-16, GR-2, GR-6, GR-9, GR-11, GR-14, GR-15, GR-22, GR-29, GR-37, GR-40, P-63, TCP-583, TCP-718, TCP-950, TCP-964, TCP-1369, TCP-1483, TCP-2069, ATR-394, RPHR-2, RPHR-619-20, RPHR-1005, IBL-57, DP-207, DP-230, SD-4, SD-57, SD-64, SD-66, SD-67, SD-5, SD-2, SD-7, SD-9, SD-49, SD-62, SD-73, SD-77, TCP-1483. The combination of the candidate gene-specific markers *Rf*4 and *Rf*3 can aid in the precise selection of lines that restore fertility. These 44 parental lines served as restorers in a programme to increase yield in the future.

The strength and direction of the relationship between a character's yield and its component characters as well as among those characters themselves determines how well the genotypes are selected based on corresponding traits. According to character association studies, the number of productive tillers per plant, the length of the panicle, and the number of grains per panicle were all significantly positively correlated with grain yield per plant. A positive non-significant association of grain yield per plant was observed for days to 50% flowering, plant height and test weight; while a negative significant correlation was noticed for pollen fertility and a negative non-significant for spikelet fertility (**Table 5**). The results are in accordance with Nanda *et al*. (2019) for productive tillers per plant, the number of filled grains per panicle, Kiranmayee *et al.* (2018) for days to 50% flowering & spikelet fertility, Prasad *et al*. (2017) for plant height, Hasan *et al*. (2010) for plant height, Mahendra *et al*. (2015) for test weight and Shobhana *et al*. (2018) for days to 50% flowering & plant height. Days to 50% flowering had a positive significant correlation with spikelet fertility. Plant height had a positive significant correlation with panicle length & spikelet fertility. Panicle length had a positive significant correlation with the number of filled grains per panicle and pollen fertility had a positive significant correlation with spikelet fertility. Correlation gives only the relation between two variables whereas path coefficient analysis allows the separation of the direct effect and their indirect effects through other attributes by partitioning the correlations (Wright, 1921). Data related to results of the path analysis is presented in **Table 6**, which showed that the number of grains per panicle had the greatest direct effect on grain yield, followed by the number of productive tillers per plant, test weight, panicle length, and plant height, and that selection for these characters is likely to result in a general improvement in single plant yield. Whereas days to 50% flowering, pollen fertility and spikelet fertility exhibited a negative effect on grain yield. The results are in accordance with Prasad *et al*. (2017) for plant height, Kiranmayee *et al.* (2018) for days to 50% flowering, spikelet fertility and Shobhana *et al*. (2018) for days to 50% flowering and plant height

From genetic diversity among the ten clusters, genotypes from clusters X, VIII, VII, V, IV might be utilised as parents in the hybridization programme to create breeding material with high genetic diversity. Forty four genotypes were found positive for fertility restoration (*Rf3* and *Rf4*) genes with the whole four gene based markers, which indicates that the efficiency of molecular markers in identifying restorers and its deployment in hybrid rice breeding programme. These restorers could also be directly used as parental lines in the development of heterotic hybrids for irrigated and unfavourable ecosystems. The correlation coefficient studies revealed that grain yield per plant showed a positive significant association with the number of productive tillers per plant, panicle length and the number of grains per panicle. Thus one should select these characters for direct selection. Path analysis showed that the number of grains per panicle, the number of productive tillers per plant, test weight and panicle length all had the best direct effects on grain yield, indicating that selection for these characters is likely to directly lead to an improvement in single plant yield as a whole.

Table 5. Estimates of correlation coefficient between SPY (Single plant yield) and its component traits in hybrid rice.

Character	DFF	PH	PT	PL	PF	SF	GPP	TW	SPY
Days to 50% flowering (DFF)	1.000	-0.019	-0.185*	-0.097	-0.004	0.199*	0.163	-0.086	0.004
Plant height (PH)		1.000	0.106	0.197*	0.019	0.313**	-0.005	-0.115	0.103
Productive tillers (PT)			1.000	0.152	0.034	0.124	0.026	-0.055	0.301**
Panicle length (PL)				1.000	-0.053	-0.165	0.220*	0.145	0.322**
Pollen fertility (PF)					1.000	0.228**	-0.049	-0.022	-0.173*
Spikelet fertility (SF)						1.000	-0.168	-0065	-0.097
No. of grains per panicle (SGP)							1.000	-0.255**	0.563**
Test weight (TW)								1.000	1.000

*5% level of significance; **1% level of significance

Table 6. Path coefficients for grain yield per plant and it's contributing traits in hybrid rice

Days to 50% flowering vs Grain yield per plant	r = 0.004	**Pollen fertility vs Grain yield per plant**	r = -0.173
Direct effect	-0.0072	Direct effect	-0.1313
Indirect effect via PH	0.0001	Indirect effect via DFF	0.0003
Indirect effect via PT	0.0012	Indirect effect via PH	-0.0023
Indirect effect via PL	0.0006	Indirect effect via PT	-0.0034
Indirect effect via PF	0.0000	Indirect effect via PL	0.0081
Indirect effect via SF	-0.0012	Indirect effect via SF	-0.0274
Indirect effect via GPP	-0.0010	Indirect effect via GPP	0.0068
Indirect effect via TW	0.0006	Indirect effect via TW	0.0034
Plant Height vs Grain yield per plant	r = 0.103	**Spikelet fertility vs Grain yield per plant**	r = -0.097
Direct effect	0.1036	Direct effect	-0.0069
Indirect effect via DFF	-0.0015	Indirect effect via DFF	-0.0011
Indirect effect via PT	0.0108	Indirect effect via PH	-0.002
Indirect effect via PL	0.0185	Indirect effect via PT	-0.007
Indirect effect via PF	0.0018	Indirect effect via PL	0.0011
Indirect effect via SF	0.0299	Indirect effect via PF	-0.0014
Indirect effect via GPP	-0.0002	Indirect effect via GPP	0.0010
Indirect effect via TW	-0.0117	Indirect effect via TW	0.0004
Productive tillers per plant vs Grain yield per plant	r =0.301	**Filled grains per panicle vs Grain yield per plant**	r = 0.563
Direct effect	0.2589	Direct effect	0.6245
Indirect effect via DFF	-0.0437	Indirect effect via DFF	0.1103
Indirect effect via PH	0.0269	Indirect effect via PH	-0.0056
Indirect effect via PL	0.0266	Indirect effect via PT	0.0123
Indirect effect via PF	0.0066	Indirect effect via PL	0.1476
Indirect effect via SF	0.0262	Indirect effect via PF	-0.0290
Indirect effect via GPP	0.0084	Indirect effect via SF	-0.1255
Indirect effect via TW	-0.0116	Indirect effect via TW	-0.1625
Panicle length vs Grain yield per plant	r =0.322	**Test weight vs Grain yield per plant**	r = 1.00
Direct effect	0.0600	Direct effect	0.3102
Indirect effect via DFF	-0.0050	Indirect effect via DFF	-0.0265
Indirect effect via PH	0.0107	Indirect effect via PH	-0.0364
Indirect effect via PT	0.0061	Indirect effect via PT	-0.0209
Indirect effect via PF	-0.0037	Indirect effect via PL	0.0526
Indirect effect via SF	-0.0092	Indirect effect via PF	-0.0058
Indirect effect via GPP	0.0128	Indirect effect via SF	-0.0233
Indirect effect via TW	0.0078	Indirect effect via GPP	-0.0807

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