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MOLECULAR SCREENING FOR *Rf3* AND *Rf4* GENES TO IDENTIFY FERTILITY RESTORER LINES IN ADVANCED SLENDER GRAIN RICE (*Oryza sativa* L.)

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ABSTRACT

Hybrid technology offers a proven pathway to enhance productivity in rice cultivation, but its success hinges on the availability of effective fertility restorer lines. Identification of genotypes carrying the major restoration genes *Rf3* and *Rf4* is therefore a critical step in hybrid seed production programs based on the Wild Abortive Cytoplasmic Male Sterility (WA-CMS) system. The present study was undertaken at Institute of Biotechnology (IBT), Professor Jayashankar Telangana Agricultural University (PJTAU), Rajendranagar, Hyderabad, Telangana, with the objective of screening advanced slender grain rice lines for fertility restorer genes using SSR markers linked to *Rf3* and *Rf4*. A total of 112 rice genotypes were evaluated, including 110 advanced breeding lines sourced from the Rice Research Unit (RRU), ARI, PJTAU, Hyderabad and two reference checks, KMR-3R and B-56, to validate positive and negative banding patterns for the two genes. Marker analysis revealed that 50 genotypes (45.4%) carried the *Rf3* restorer allele and 57 genotypes (51.8%) carried the *Rf4* restorer allele. Notably, 22 genotypes possessed both *Rf3* and *Rf4* restorer genes marking them as strong candidates for effective fertility restoration. These findings demonstrate that marker-assisted screening is an efficient and reliable approach for identifying superior fertility restorer genotypes, which can serve as valuable genetic resources for developing stable restorer lines and high-yielding WA-CMS-based hybrid rice.

Keywords **:** Hybrid technology, Fertility restorer genes, *Rf3* and *Rf4*, SSR markers, Marker-assisted selection, WA-CMS, Slender grain rice.

Introduction

Rice (*Oryza sativa* L.) (2n = 24) is one of the most important cereal crops belonging to the family Poaceae and serves as the staple food for more than half of the world's population. It is cultivated across diverse agro-ecological environments ranging from irrigated lowlands to rainfed uplands, reflecting its remarkable adaptability (Degenkolbe *et al*., 2009). Rice is a major source of dietary energy, containing approximately 80% carbohydrates, 7–8% protein, 3% fat, and 3% dietary fibre, while rice bran is rich in bioactive compounds such as tocopherols, tocotrienols, and oryzanols with significant antioxidant properties (Juliano, 1985; Lloyd *et al*., 2000). Globally, rice is cultivated in about 168 million hectares with an annual production of 713.10 million tonnes (USDA, 2024–25). In India, it occupies 477.30 lakh hectares with a production of 1364.37 lakh tonnes, highlighting its crucial role in national food security (India Stat, 2024–25).

The increasing global population and the consequent rise in food demand necessitate substantial

improvements in rice productivity. Hybrid rice technology has emerged as one of the most effective approaches for enhancing yield potential, offering a yield advantage of 15–30 per cent over conventional inbred varieties. The success of hybrid rice breeding is largely dependent on the efficient exploitation of cytoplasmic male sterility (CMS) systems and the availability of suitable fertility restorer lines. The three-line breeding system, comprising CMS lines, maintainer lines, and restorer lines, has been widely adopted for commercial hybrid rice development (Virmani *et al*., 1997; Krishnan *et al*., 2012).

Among the various CMS systems available in rice, the Wild Abortive Cytoplasmic Male Sterility (WA-CMS) system is the most extensively utilized in hybrid rice breeding programmes across Asia, including India (Lin and Yuan, 1980; Virmani and Wan, 1988). The effectiveness of this system depends on the identification of reliable restorer lines capable of restoring fertility in CMS-based hybrids. Fertility restoration is governed by nuclear-encoded restorer of fertility (*Rf*) genes, which suppress the sterility-inducing effects of the cytoplasm. Among the identified fertility restoration genes, *Rf3* located on chromosome 1 and *Rf4* located on chromosome 10 are the major genes responsible for restoring fertility in WA-CMS lines (Yao *et al*., 1997; Zhang *et al*., 1997).

Conventionally, fertility restorers are identified through test-crossing with CMS lines followed by evaluation of pollen and spikelet fertility in the resulting hybrids. Although effective, this approach is laborious, time-consuming, and influenced by environmental factors (Govinda Raj and Virmani, 1988; Pranathi *et al*., 2016). The advent of molecular marker technology has enabled rapid and precise identification of fertility restoration genes, thereby accelerating breeding programmes. Molecular markers linked to *Rf* genes facilitate early-generation screening and improve selection efficiency by eliminating environmental influences. Previous studies have demonstrated the close association between fertility restoration alleles and CMS systems, emphasizing the importance of molecular characterization of *Rf* genes in developing superior restorer lines and high-yielding hybrids (Nematzadeh *et al*., 2003; Venkanna *et al*., 2022).

Therefore, the present investigation was undertaken to identify fertility restoration genes *Rf3* and *Rf4* among advanced slender grain rice genotypes using SSR markers linked to these loci. The identification of effective restorer lines will contribute to the development of superior hybrid rice combinations and strengthen hybrid rice breeding programmes aimed at enhancing productivity and grain quality.

Materials and Methods

Experimental Material

The present investigation was conducted during 2025 Institute of Biotechnology (IBT), Professor Jayashankar Telangana Agricultural University (PJTAU), Rajendranagar, Hyderabad, Telangana. The experimental material comprised 110 rice (*Oryza sativa* L.) genotypes along with two check varieties, KMR-3R and B-56. KMR-3R, (Table1) carrying the fertility restoration genes *Rf3* and *Rf4*, was used as the positive control, whereas B-56 served as the negative control.

Genomic DNA Isolation and Quantification

leaf samples from young plants were collected from all genotypes at approximately 25-30 days after sowing and stored at −80 °C until further use. Genomic DNA was isolated using the CTAB method described by Dellaporta *et al*. (1983) with minor modifications. Approximately 100 mg of leaf tissue was homogenized in 2% CTAB extraction buffer and incubated at 65°C for 45 min. The lysate was extracted using chloroform: isoamyl alcohol (24:1), and DNA was precipitated with ice-cold isopropanol. The resulting DNA pellet was washed with 70% ethanol, air-dried, and dissolved in 50 µL of TE buffer.

DNA concentration and purity were determined using a Nano Drop ND-1000 spectrophotometer. Samples exhibiting A260/A280 ratios between 1.8 and 2.0 were considered suitable for molecular analysis and were diluted to a working concentration of approximately 50ng/µl

SSR Marker Analysis and PCR Amplification

Molecular screening for fertility restoration genes was carried out using two SSR markers, RMS-SF21-5 (linked to the *Rf3* gene on chromosome 1) and RMS-PPR9-1 (linked to the *Rf4* gene on chromosome 10), following Pranathi *et al*. (2016) (Table 2). As shown in (Table 3), RMS-PRR9-1 amplified a 114 bp product in the restorer line and 159 bp in the non-restorer line, while hybrids showed both bands (114 and 159 bp). Similarly, RMS-SF21-5 amplified 172 bp in the restorer line and 127 bp in the non-restorer line, with hybrids showing both fragments (172 and 127 bp). This dual-band pattern confirmed both markers as reliable co-dominant tools for distinguishing restorer, non-restorer, and hybrid genotypes.

Polymerase chain reaction (PCR) was carried out following the procedure of Chen *et al*. (1997) in a

reaction volume of 10 µL containing 50 ng of 2 µl template DNA, 4 µl of 2× Takara Emerald PCR Master Mix, 1 µl each of forward and reverse primers (2.5pm/ µl), and 2 µl nuclease-free water (Table 4).

Amplification was performed with an initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 1 min, and extension at 72 ℃ for 1 min. A final extension was carried out at 72 ℃ for 7 min, after which the reactions were held at 4 ℃. (Table 5)

Agarose Gel Electrophoresis and Marker Scoring

The amplified PCR products were separated on 3% agarose gels prepared in 1× TAE buffer containing ethidium bromide. Electrophoresis was conducted at 120 V using a 100 bp DNA ladder as a molecular size standard. Following electrophoresis, gels were visualized under ultraviolet light and documented using a BIO-RAD Gel Documentation System.

The amplification profiles generated by SSR markers were analyzed for the presence of fertility restoration-specific alleles. The marker data was subsequently used to identify putative fertility restorers carrying *Rf3* and/or *Rf4* genes.

Table 1: Advanced slender grain type of rice lines evaluated for fertility restoration gene-linked markers (*Rf3* and *Rf4*) in the study

S.NO	ENTRY
1	Trupti
2	Shree101(T/L)
3	Sonam(T/L)
4	Sadhana(T/L)
5	Padma
6	Poornima
7	Sonali
8	Paddy Gold-66 laxmi gold
9	Paddy Gold-88 shri ram
10	Paddy Gold-99 super mohini
11	Paddy Gold-505 shighra
12	Indus-101
13	indus-555
14	Indus-606
15	Indus-gold
16	Jyothi
17	Kedhar
18	Pragathi
19	Pushpa
20	Soniya
21	Srivalli
22	JGL-27356
23	JGL-28545
24	KNM-33124
25	KNM-733
26	KNM-7715
27	KNM-12368
28	Sampurna
29	KNM-12510
30	KPS-6251
31	KPS-10329
32	KPS-10631
33	Divya sree(P-2/4)
34	Gayatri(P-2/5)
35	Paddy Bunt(P-204)
36	Paddy Gangotri(P-203)
37	Paddy Sona(N8)
38	Paddy godavari
39	Paddy Silk
40	KNM-12472
41	RNR-28355
42	RNR-29088
43	RNR-31479
44	RNR-31668
45	RNR-31672
46	KNM -7715
47	RNR-35192
48	KNM*12510
49	RNR-35989
50	RNR-38948
51	RNR-44277
52	RNR-44286
53	RNR-44292
54	RNR-44299
54.	RNR-44301
56.	RNR-52221
57.	RNR-44311
58.	RNR-44314
59.	RNR-44318
60.	RNR-44320
61.	RNR-44326
62.	RNR-44370
63.	RNR-44380
64.	RNR-44382
65.	RNR-44392
66.	RNR-44411
67.	RNR-44415
68.	RNR-44421
69.	RNR-46065
70.	RNR-46072
71.	RNR-46086
72.	RNR-46090
73.	RNR-46098
74.	RNR-49499
75.	RNR-49500
76.	RNR-52046
77.	RNR-52059
78.	RNR-52060
79.	RNR-52063
80.	RNR-52066
81.	RNR-52070
82.	RNR-52073
83.	RNR-52074

84.	RNR-52075	
85.	RNR-52082	
86.	RNR-52094	
87.	RNR-52097	
88.	RNR-52102	
89.	RNR-52107	
90.	RNR-52110	
91.	RNR-52113	
92.	RNR-52117	
93.	RNR-52120	
94.	RNR-52126	
95.	RNR-52130	
96.	RNR-52134	
97.	RNR-52177	
98.	RNR-52182	
99.	RNR-52205	
100.	RNR-52219	
101.	SN-2071	
102.	SN-2183	
103.	SN-2756	
104.	SN-2696	
105.	SN-2744	
106.	SN-2769	
107.	SN-2754	
108.	RNR-15048	
109.	BPT-1010	
110.	MTU-5204	
1	**CHECK**	**KMR-3R (*Rf3 & Rf4)***
2	**CHECK**	**B-56**

Table 2: List of SSR primers for fertility restoration

S. NO	Primers	Chromosome number	Primer sequence (5'-3')	Annealing Temperature °C
1	RM SF 21-5 (*Rf3*)	10	F- GAGTTGGGGGT CGAGAAATC R- CGTACGTGCGGC TAGGATCAA	55°C
2	RMS PRR 9-1 *(Rf4)*	1	F- GAGTTTTGAATAGA TTTACGTGTGGA R- AGTGTCCAGATTC GTAGTAATGC	55°C

Table 3: Amplification sizes of the markers used in the study

S. No.	Primer name	PCR product size (bp)		
		Restorer line	Non-restorer line	Hybrid lines
1	RMS-PRR9-1	114	159	114,159
2	RMS-SF21-5	172	127	172,127

Table 4: PCR Reaction mixture

Reagent	Stock concentration	Volume (µl)
DNA template	50ng	2
PCR Master mix (TAKARA)	2X Premix	4
Primer (forward)	5pm/µl	1
Primer (reverse)	5pm/µl	1
Sterile and nano pure H_2O		2
Total		**10**

Table 5: PCR conditions used for DNA amplification

Activity	Temperature (ºC)	Duration (min)	No. of cycles
Initial denaturation	94 ºC	5 minutes	1
Denaturation	94 ºC	30 seconds	35
Annealing	55 ºC	1minute	
Extension	72 ºC	1minute	
Final extension	72 ºC	7 minutes	1
Storage	4 ºC		

Results and Discussions

Identification of *Rf3* Fertility Restorer Gene Using the Functional Marker RM-SF21-5

Genetic evaluation of 110 advanced slender grain type rice lines using the codominant functional marker RM-SF21-5 effectively detected genotypes harbouring the *Rf3* fertility restoration gene. Molecular analysis showed that 50 accessions (45.4% of the tested material) displayed the characteristic 172 bp amplification product, confirming the presence of the active *Rf3* allele. This positive group included valuable breeding material such as Trupti, Sadhana (T/L), Padma, Paddy Gold-99 Super Mohini, Indus-101, Jyothi, Soniya, Srivalli, JGL-27356, JGL-28545, KNM-733, KNM-7715, KNM-12368, Sampurna, KNM-12510, KPS-6251, KNM-12472, RNR-31672, RNR-35192, RNR-44286, RNR-44292, RNR-44299, RNR-44320, RNR-44370, RNR-44411, RNR-46086, RNR-46090, RNR-46098, RNR-49499, RNR-49500, RNR-52046, RNR-52059, RNR-52060, RNR-52063, RNR-52066, RNR-52070, RNR-52073, RNR-52075, RNR-52094, RNR-52097, RNR-52107, RNR-52110, RNR-52219, SN-2744, SN-2769, SN-2754, RNR-15048, and BPT-5204 (Table 6 and Fig 1).

In contrast, 55 genotypes (50.0% of the collection) produced exclusively the 127 bp amplification fragment, indicating the absence of functional *Rf3* allele. These included Shree101 (T/L), Sonam (T/L), Poornima, Sonali, Paddy Gold-66 Laxmi Gold, Paddy Gold-88 Shri Ram, Paddy Gold-505 Shighra, Indus-555, Indus-606, Indus-gold, KNM-33124, KPS-10329, KPS-10631, Divya Sree (P-2/4), Gayatri (P-2/5), Paddy Bunt (P-204), Paddy Gangotri (P-203), Paddy Sona (N8), Paddy Godavari, Paddy Silk, RNR-28355, RNR-29088, RNR-31479, RNR-31668, RNR-35989, RNR-38948, RNR-44277, RNR-44301, RNR-44311, RNR-44314, RNR-44318, RNR-44326, RNR-44392, RNR-44415, RNR-44421, RNR-46065, RNR-46072, RNR-52074, RNR-52082, RNR-52102, RNR-52113, RNR-52117, RNR-52120, RNR-

52126, RNR-52130, RNR-52134, RNR-52177, RNR-52182, RNR-52205, RNR-52221, SN-2071, SN-2183, SN-2756, SN-2696, and MTU-1010.

Five advanced slender grain rice lines - Kedhar, Pragathi, Pushpa, RNR-44380, and RNR-44382 - exhibited both the 172 bp and 127 bp amplicons simultaneously, indicating heterozygosity (*Rf3*/*rf3*) at this locus, i.e., the presence of one restoring and one non-restoring allele. These findings demonstrate the efficacy of marker-assisted screening for distinguishing restoration-competent from restoration-deficient material, with RM-SF21-5 showing clear and reproducible polymorphism (Figure 1). The substantial frequency (45.4%) of *Rf3*-carrying lines within this genetic resource collection indicates considerable utility for three-line hybrid rice development programmes employing the WA-CMS approach.

Identification of *Rf4* Fertility Restorer Gene Using Functional Marker RMS-PRR9-1

Genetic characterization of the 110 rice breeding lines using the codominant functional marker RMS-PRR9-1 effectively detected genotypes harbouring the *Rf4* fertility restoration gene. Fifty-seven accessions (51.8% of the tested material) - namely Shree101 (T/L), Sonam (T/L), Poornima, Sonali, Indus-101, Indus-555, Kedhar, Soniya, JGL-28545, KNM-733, Sampurna, KNM-12510, Paddy Bunt (P-204), Paddy Godavari, KNM-12472, RNR-29088, RNR-31479, RNR-31668, RNR-31672, RNR-35192, RNR-35989, RNR-38948, RNR-44286, RNR-44292, RNR-44299, RNR-44301, RNR-44311, RNR-44314, RNR-44318, RNR-44320, RNR-44326, RNR-44370, RNR-44380, RNR-44382, RNR-44392, RNR-44415, RNR-44421, RNR-46065, RNR-46072, RNR-46086, RNR-46090, RNR-46098, RNR-52074, RNR-52082, RNR-52113, RNR-52120, RNR-52205, RNR-52219, RNR-52221, SN-2071, SN-2183, SN-2756, SN-2696, SN-2744, and MTU-1010-showed the characteristic 114 bp amplification product, confirming the presence of the active *Rf4* allele.(Table 6 and Fig 2)

In contrast, 48 genotypes (43.6% of the collection) - Trupti, Sadhana (T/L), Padma, Paddy Gold-66 Laxmi Gold, Paddy Gold-88 Shri Ram, Paddy Gold-99 Super Mohini, Paddy Gold-505 Shighra, Indus-606, Indus-gold, Jyothi, Pushpa, Srivalli, JGL-27356, KNM-33124, KNM-7715, KNM-12368, KPS-10329, KPS-10631, Divya Sree (P-2/4), Gayatri (P-2/5), Paddy Gangotri (P-203), Paddy Sona (N8), Paddy Silk, RNR-44277, RNR-44411, RNR-49499, RNR-49500, RNR-52046, RNR-52059, RNR-52060, RNR-52063, RNR-52066, RNR-52070, RNR-52073, RNR-52075, RNR-52094, RNR-52097, RNR-52102, RNR-52107, RNR-52110, RNR-52126, RNR-52130, RNR-52177, RNR-52182, SN-2769, SN-2754, RNR-15048, and BPT-5204 - amplified the 159 bp fragment, indicating the absence of the restorer allele at this locus.

Five slender grain rice lines - Pragathi, KPS-6251, RNR-28355, RNR-52117, and RNR-52134 - displayed both amplicon sizes concurrently, establishing their heterozygous (*Rf4*/*rf4*) constitution at the *Rf4* locus. Notably, the genotype Pragathi was heterozygous at both the *Rf3* and *Rf4* loci simultaneously, making it a particularly valuable breeding resource for further fixation through successive selfing. The distinct polymorphic patterns obtained between restoration-capable and restoration-deficient genotypes (Figure 2) confirm the reliability of the RMS-PRR9-1 marker system for *Rf4* detection.

Identification of Genotypes Carrying Both *Rf3* and *Rf4* Restorer Genes

Comparison of the marker profiles across both loci revealed that 22 genotypes carried both the Rf3 and Rf4 restorer genes (Table 7). These double restorer genotypes viz., Indus-101, Soniya, JGL-28545, KNM-733, Sampurna, KNM-12510, KNM-12472, RNR-31672, KNM-7715(b), RNR-35192, KNM-12510(b), RNR-44286, RNR-44292, RNR-44299, RNR-44320, RNR-44370, RNR-46086, RNR-46090, RNR-46098, RNR-52066, RNR-52219, and SN-2744 represent exceptionally valuable genetic resources for hybrid rice improvement. Combining both major restoration loci within a single line increases the likelihood of complete and stable fertility restoration in WA-CMS-based hybrids, making these genotypes promising candidates for use as restorer parents in future hybrid breeding programs.

The marked difference in allele frequency between *Rf3* (45.4%) and *Rf4* (51.8%) among the tested accessions suggests differing selection pressures or independent genetic maintenance of these two restoration loci during the development of this breeding material. Overall, genotypes carrying dominant alleles at either or both loci represent promising candidates as fertility restorers, while the heterozygous lines identified constitute valuable intermediate genetic resources that can be advanced to homozygosity through successive selfing cycles. The restorer genotypes identified in this study constitute significant genetic material for the development of high-yielding, WA-CMS-based hybrid rice varieties, thereby contributing to future food security goals.

Similar observations of heterozygous genotypes exhibiting both positive and negative marker alleles for

fertility restorer genes were reported by Kumar *et al*. (2017), who identified heterozygous individuals for *Rf3* and *Rf4* using gene-linked molecular markers in rice restorer lines. Such heterozygous lines are valuable genetic resources in hybrid rice breeding programmes, as they carry the desired fertility restoration allele that can be fixed through selfing and marker-assisted selection. Subsequent generations can be screened to identify homozygous dominant (*Rf3* and *Rf3*) plants, which can be utilized as stable restorer lines. Furthermore, these heterozygous lines can serve as donor parents in breeding programmes aimed at introgressing and pyramiding fertility restorer genes into elite genetic backgrounds, thereby enhancing the efficiency of developing effective restorers for WA-CMS-based hybrid rice breeding systems (Kumar *et al*., 2017; Suresh *et al*., 2012).

Table 6 : Restorer gene status of the 110 advanced slender grain rice lines

S. No	Genotype	Restorer gene for *Rf3*	Restorer gene for *Rf4*
1.	Trupti	1	0
2.	Shree101(T/L)	0	1
3.	Sonam(T/L)	0	1
4.	Sadhana(T/L)	1	0
5.	Padma	1	0
6.	Poornima	0	1
7.	Sonali	0	1
8.	Paddy Gold-66 laxmi gold	0	0
9.	Paddy Gold-88 shriram	0	0
10.	Paddy Gold-99 super mohini	1	0
11.	Paddy Gold-505 shighra	0	0
12.	Indus-101	1	1
13.	indus-555	0	1
14.	Indus-606	0	0
15.	Indus-gold	0	0
16.	Jyothi	1	0
17.	Kedhar	1, 0	1
18.	Pragathi	1, 0	1, 0
19.	Pushpa	1, 0	0
20.	Soniya	1	1
21.	Srivalli	1	0
22.	JGL-27356	1	0
23.	JGL-28545	1	1
24.	KNM-33124	0	0
25.	KNM-733	1	1
26.	KNM-7715	1	0
27.	KNM-12368	1	0
28.	Sampurna	1	1
29.	KNM-12510	1	1
30.	KPS-6251	1	1, 0
31.	KPS-10329	0	0
32.	KPS-10631	0	0
33.	Divya sree(P-2/4)	0	0
34.	Gayatri(P-2/5)	0	0
35.	Paddy Bunt(P-204)	0	1
36.	Paddy Gangotri(P-203)	0	0
37.	Paddy Sona(N8)	0	0
38.	Paddy Godavari	0	1
39.	Paddy Silk	0	0
40.	KNM-12472	1	1
41.	RNR-28355	0	1, 0
42.	RNR-29088	0	1
43.	RNR-31479	0	1
44.	RNR-31668	0	1
45.	RNR-31672	1	1
46.	KNM -7715	1	1
47.	RNR-35192	1	1
48.	KNM*12510	1	1
49.	RNR-35989	0	1
50.	RNR-38948	0	1
51.	RNR-44277	0	0
52.	RNR-44286	1	1
53.	RNR-44292	1	1
54.	RNR-44299	1	1
55.	RNR-44301	0	1
56.	RNR-52221	0	1
57.	RNR-44311	0	1
58.	RNR-44314	0	1
59.	RNR-44318	0	1
60.	RNR-44320	1	1
61.	RNR-44326	0	1
62.	RNR-44370	1	1
63.	RNR-44380	1, 0	1
64.	RNR-44382	1, 0	1
65.	RNR-44392	0	1
66.	RNR-44411	1	0
67.	RNR-44415	0	1
68.	RNR-44421	0	1
69.	RNR-46065	0	1
70.	RNR-46072	0	1
71.	RNR-46086	1	1
72.	RNR-46090	1	1
73.	RNR-46098	1	1
74.	RNR-49499	1	0
75.	RNR-49500	1	0
76.	RNR-52046	1	0
77.	RNR-52059	1	0
78.	RNR-52060	1	0
79.	RNR-52063	1	0
80.	RNR-52066	1	0
81.	RNR-52070	1	0
82.	RNR-52073	1	0
83.	RNR-52074	0	1
84.	RNR-52075	1	0
85.	RNR-52082	0	1
86.	RNR-52094	1	0
87.	RNR-52097	1	0
88.	RNR-52102	0	0
89.	RNR-52107	1	0
90.	RNR-52110	1	0

91.	RNR-52113	0	1
92.	RNR-52117	0	1, 0
93.	RNR-52120	0	1
94.	RNR-52126	0	0
95.	RNR-52130	0	0
96.	RNR-52134	0	1, 0
97.	RNR-52177	0	0
98.	RNR-52182	0	0
99.	RNR-52205	0	1
100.	RNR-52219	1	1
101.	SN-2071	0	1
102.	SN-2183	0	1
103.	SN-2756	0	1
104.	SN-2696	0	1
105.	SN-2744	1	1
106.	SN-2769	1	0
107.	SN-2754	1	0
108.	RNR-15048	1	0
109.	BPT-5204	1	0
110.	MTU-1010	0	1

Note: '1'= *Rf* genes present; '0'= *Rf* genes absent and '1,0'= heterozygote

Table 7 : Rice Genotypes with both the *Rf3* and *Rf4* fertility restoration genes

S.NO.	Genotype	*Rf3* status	*Rf4* status
1	Indus-101	Present (1)	Present (1)
2	Soniya	Present (1)	Present (1)
3	JGL-28545	Present (1)	Present (1)
4	KNM-733	Present (1)	Present (1)
5	Sampurna	Present (1)	Present (1)
6	KNM-12510	Present (1)	Present (1)
7	KNM-12472	Present (1)	Present (1)
8	RNR-31672	Present (1)	Present (1)
9	KNM-7715 (b)	Present (1)	Present (1)
10	RNR-35192	Present (1)	Present (1)
11	KNM-12510 (b)	Present (1)	Present (1)
12	RNR-44286	Present (1)	Present (1)
13	RNR-44292	Present (1)	Present (1)
14	RNR-44299	Present (1)	Present (1)
15	RNR-44320	Present (1)	Present (1)
16	RNR-44370	Present (1)	Present (1)
17	RNR-46086	Present (1)	Present (1)
18	RNR-46090	Present (1)	Present (1)
19	RNR-46098	Present (1)	Present (1)
20	RNR-52066	Present (1)	Present (1)
21	RNR-52219	Present (1)	Present (1)
22	SN-2744	Present (1)	Present (1)

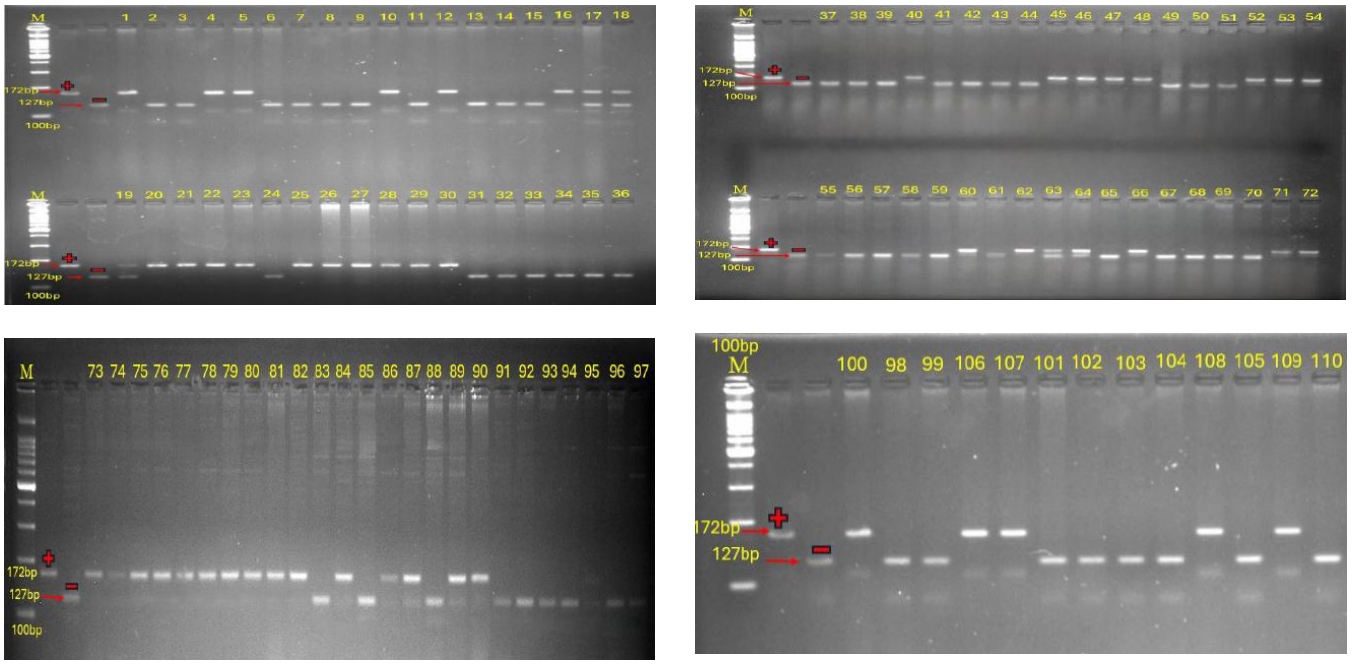

Fig. 1 : Amplification pattern of RM SF21-5 linked to fertility restorer (*Rf3*) gene M= 100bp ladder, 172bp=positive allele, 127bp= negative allele, no. of samples studied 1-110

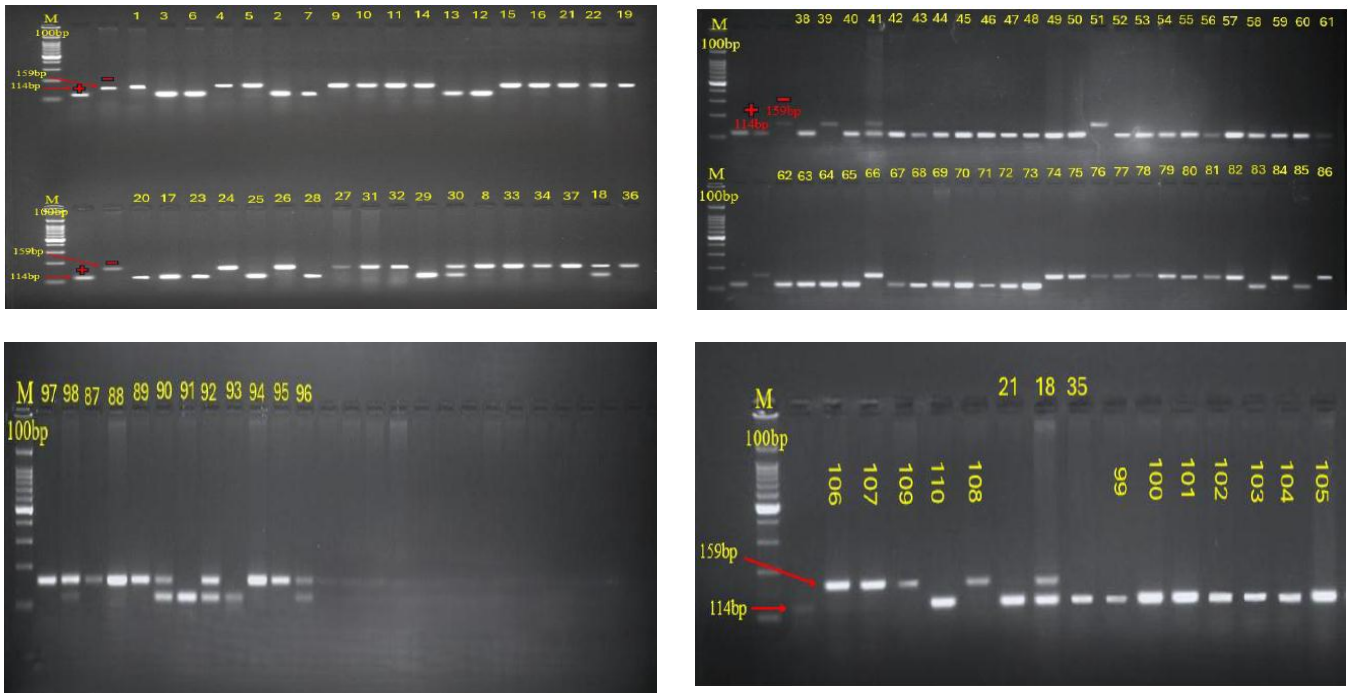

Fig. 2 : Genotypic profiling of *Rf4* fertility restorer gene using RMS PRR9-1 functional marker. M= 100bp ladder, 114bp=positive band size, 159bp= negative band size, no. of samples evaluated =110

Conclusion

Genetic characterization of 110 rice breeding lines through molecular marker analysis effectively revealed genotypes harbouring *Rf3* and *Rf4* fertility restoration loci utilizing locus-specific DNA markers. Application of the RM-SF21-5 marker for *Rf3* detection yielded the characteristic 172 bp restoration allele in 50 genotypes (representing 45.5% of tested materials), while 55 accessions demonstrated 127 bp which is non-restoration variant (representing 55% of tested materials) and 5 lines exhibited heterozygous nature (4.5% of tested material). Molecular analysis targeting *Rf4* through RMS-PRR9-1 marker deployment revealed considerably lower frequencies, with merely 57 lines (51.8% of tested material) displaying 114 bp amplicon, 48 lines exhibiting 159bp (constituting the 43.6%of tested material) and 5 lines exhibited heterozygotes (4.5% of tested material). The pronounced disparity in allelic distribution frequencies between *Rf3* (45.5%) and *Rf4* (51.8%) indicates that restoration loci potentially exhibit divergent evolutionary trajectories or perform specialized functions within fertility restoration mechanisms.

Twenty-two genotypes revealed the presence of both the *Rf3* and *Rf4* restorer genes, and one genotype, Pragathi, was heterozygous at both loci. These molecular findings constitute important genetic assets for WA-CMS-dependent hybrid rice development initiatives, particularly valuable for identifying parental combinations possessing complementary restoration gene profiles to guarantee consistent fertility expression in hybrid progeny. The validated marker-gene relationships established through this investigation underscore the utility of marker-assisted breeding approaches for streamlining restorer line identification processes, potentially minimizing temporal and resource investments typically associated with traditional test-crossing assessment protocols.

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