Received: 01 Aug 2025 | Revised: 31 Aug 2025 | Accepted: 01 Sep 2025

Electronic Journal of Plant Breeding

Research Note

Molecular identification of restorers for *Rf3* and *Rf4* fertility genes in rice (*Oryza sativa* L.) using traditional and high yielding varieties of Kerala

Roshni Vijayan[1]*, Jiji Joseph[1], Deepu Mathew[1], P. Sindhumole[1] and Rose Mary Francies[2]

[1]College of Agriculture, Vellanikkara, Kerala Agricultural University, Thrissur
[2]Agricultural Research Station, Mannuthy, Kerala Agricultural University, Thrissur
***E-Mail:** roshni.v@kau.in

Abstract

Hybrid rice technology, offering significant yield advantages, is a promising solution for addressing the global food security challenges. Fertility restorer genes *Rf3* and *Rf4 are* pivotal for advancing hybrid rice breeding initiatives. Owing to the enormous variability observed in the varieties and landraces in Kerala, India, possibility of finding new alleles of important genes from these varieties is hypothesised. Finding new alleles of *Rf3 and Rf4* genes will be extensively useful in hybrid rice technology. This work examined 152 rice varieties of Kerala, encompassing both High Yielding Varieties (HYVs) and Traditional Rice Varieties (TRVs), to mine the novel alleles of *Rf3 and Rf4.* The varieties were screened using the molecular markers functionally linked to these genes. A distinct distribution of allelic combinations, with *Rf4* displaying higher prevalence (52%) compared to *Rf3* (32%) was observed. Twenty-two varieties were identified as putative restorers, while forty-eight served as maintainers, offering a precise direction for selecting the parental lines with superior restoration potential. The findings highlight the pivotal role of *Rf4* in governing fertility restoration, emphasizing its critical significance for optimizing hybrid seed production.

Keywords: Allele mining; CMS; Hybrid Rice; Male Sterility;

Global food production must increase by over 70% to meet the nutritional demands of the growing population (FAO, 2009), and as of 2025, this benchmark remains central to food security. However, the focus has shifted from mere yield expansions to sustainable intensification and climate resilience (OCED/FAO 2022). In this context, hybrid rice breeding emerges as a strategic solution, particularly through the development of robust cytoplasmic male sterile lines and genetically diverse restorer systems. Hybrid rice technology is a transformative approach, demonstrating a 15–20% gain in yield performance over high-yielding varieties under comparable conditions (Shrivastava *et al*., 2019). Hybrid rice has seen widespread adoption in China, accounting for 57% of the nation's total rice cultivation area (Qian, *et al*., 2021). In contrast, India falls significantly behind, with hybrid rice covering only 6.8% of its rice-growing regions (Rout*, et al*., 2021). It is predominantly developed using the three-line system, involving a cytoplasmic male sterile (CMS) line (A line), a maintainer line (B line), and a restorer line (R line) containing fertility restorer (*Rf*) genes. The CGMS system utilises restorers which possess nuclear genes (Rf) that restore the fertility of CMS lines. The WA-CMS (Wild Abortive Cytoplasmic Male Sterility) system, derived from *Oryza rufipogon*, is indispensable for hybrid rice development in Kerala, as its success relies on the restoration of fertility through specific nuclear genes. In rice, 17 alleles have been identified for fertility restoration, all except *rf17* are dominant. Among these fertility restorations in WA-CMS is mediated by *Rf3* gene positioned on chromosomes 1 and *Rf4* gene located on chromosomes 10. The role of *Rf3* and *Rf4* locus in fertility restoration and its location has been a core area of study by many researcher groups (Lu *et al*., 1997; Tan *et al*., 2008; Jing *et al*., 2001; Zhang *et al*., 2002; Mishra *et al.,* 2003; Ahmadikhah and Karlov 2006; Ahmadikhah *et al.,* 2007; Sheeba *et al*., 2009; Ngangkham *et al*., 2010; Suresh *et al.,* 2012; Kazama and Toriyama, 2014). New restorer genes and improved marker-assisted selection methods are critical for developing superior restorer lines, improving partial restorers, and expanding hybrid rice cultivation in diverse agroecologies.

In the present study, 152 rice genotypes evaluated in this study, 108 are high yielding varieties officially released by Kerala Agricultural University, while the remaining represent traditional rice cultivars native to Kerala. These genotypes are systematically assessed to identify potential restorers and maintainers suitable for hybrid rice development in the region. Identification of a promising restorer is a challenge in hybrid rice technology in Kerala; it can significantly boost hybrid rice adoption and production. The main aim of this work is to identify good restorers which have high restoration capacity by confirming the presence of both *Rf3* and *Rf4* genes through molecular profiling.

A comprehensive evaluation was conducted on 152 rice varieties from Kerala, India encompassing both High Yielding Varieties (HYVs) and Traditional Rice Varieties (TRVs) (**Table 1**). These varieties raised in the experimental fields of Regional Agricultural Research Station, Kerala Agricultural University, Pattambi, during the *Kharif* season of 2023-24, were used to extract total genomic DNA from emerging leaves of seedlings aged 10–15 days, following the protocol devised by Zhang *et al*. (1997). Isolated DNA was quantified and the concentration was adjusted to 50 ng/μL. For genotyping, microsatellite markers functionally linked with the fertility restoration genes RMS-PPR9-1 for *Rf4* gene, and RMS-SF21-5 for *Rf3* gene were employed (**Table 2**). PCR amplification was carried out using 10μL reaction with ingredients as follows: 1μL of 1xPCR buffer [10mM Tris, pH 8.4, 50mM KCl, 1.8 Mm $MgCl_2$], 1μL of 2.5mM dNTP mix, 0.5μL of 10μM each of Forward and Reverse Primer, 0.6UTaqDNA Polymerase and Milli-Q water. The PCR thermal cycles set for reaction composed of initial denaturation at 94 ^{0}C for 5 minutes followed by 35 cycles of 1 min denaturation at 94^{0}C, 1 minute of annealing at 55^{0}C and 2 minutes of primer extension at 72 ^{0}C. Concluding with a final elongation step allowed for 7

Table 1. List of Kerala rice HYVs & TRVs screened for *Rf3* & *Rf4* genes

S.No	Name	Abb.	S.No	Name	Abb.
1	Aryan	PTB 1	2	Ponnaryan	PTB 2
3	Eravapandy	PTB 3	4	Vellari	PTB 4
5	Velutharikayama	PTB 5	6	Athikkiraya	PTB 6
7	Parambuvattan	PTB 7	8	Thavalakkanan	PTB 8
9	Thavalakkanan	PTB 9	10	Thekkanchera	PTB 10
11	Thekkanchitteni	PTB 12	12	Kayama	PTB 13
13	Maskathi	PTB 14	14	Kavunginpoothala	PTB 15
15	Kavunginpoothala	PTB 16	16	Jeddu halliga	PTB 17
17	Eravappandi	PTB 18	18	Athikkiraya	PTB 19
19	Vadakkanchitteni	PTB 20	20	Thekkan	PTB 21
21	Veluthavattan	PTB 22	22	Cheriya Aryan	PTB 23
23	Chuvanna vattan	PTB 24	24	Thonnoran	PTB 25
25	Chenkayama	PTB 26	26	Kodiyan	PTB 27
27	Kattamodan	PTB 28	28	Karuthamodan	PTB 29
29	Chuvannamodan	PTB 30	30	Elappapoochampan	PTB 31
31	Aruvakkari	PTB 32	32	Arikkirai	PTB 33
33	Valiyachampan	PTB 34	34	Annapoorna	PTB 35
35	Rohini	PTB 36	36	Aswathy	PTB 37
37	Triveni	PTB 38	38	Jyothi	PTB 39
39	Sabari	PTB 40	40	Bharathy	PTB 41
41	Swarnaprabha	PTB 43	42	Rasmi	PTB 44
43	Matta Triveni	PTB 45	44	Jayathi	PTB 46
45	Neeraja	PTB 47	46	Nila	PTB 48
47	Kairali	PTB 49	48	Kanchana	PTB 50
49	Aathira	PTB 51	50	Aiswarya	PTB 52
51	Mangala Mashuri	PTB 53	52	Karuna	PTB 54
53	Harsha	PTB 55	54	Varsha	PTB 56

S.No	Name	Abb.	S.No	Name	Abb.
55	Swetha	PTB 57	56	Anashwara	PTB 58
57	Samyuktha	PTB 59	58	Vaishakh	PTB 60
59	Supriya	PTB 61	60	Akshaya	PTB 62
61	Vyttila 1	VTL 1	62	Vyttila 2	VTL 2
63	Vyttila 3	VTL 3	64	Vyttila 4	VTL 4
65	Vyttila 5	VTL 5	66	Vyttila 6	VTL 6
67	Vyttila 7	VTL 7	68	Vyttila 8	VTL 8
69	Vyttila 9	VTL 9	70	Vyttila 10	VTL 10
71	Vyttila 11	VTL 11	72	Harshwa	M1
73	Manurathna	M2	74	Manuvarna	M3
75	Ezhome 1	E1	76	Ezhome 2	E2
77	Ezhome 3	E3	78	Ezhome 4	E4
79	Jaiva	E5	80	Mithila	E6
81	Bhagya	K1	82	Onam	K2
83	Dhanya	K3	84	Sagara	K4
85	Dhanu	K5	86	Amrutha	K6
87	Deepthi	W1	88	Bhadra	Mo4
89	Asha	Mo5	90	Pavizham	Mo6
91	Karthika	Mo7	92	Aruna	Mo8
93	Makom	Mo9	94	Remya	Mo10
95	Kanakom	Mo11	96	Ranjini	Mo12
97	Pavithra	Mo13	98	Panchami	Mo14
99	Remanika	Mo15	100	Uma	Mo16
101	Revathy	Mo17	102	Karishma	Mo18
103	Krishnanjana	Mo19	104	Gouri	Mo20
105	Prathyasha	Mo21	106	Sreyas	Mo22
107	Pournami	Mo23	108	Mullukuruva	W2
109	Kalladiaryan	W3	110	Punnadanthondi	W4
111	Veliyan	W5	112	Mannu veliyan	W6
113	Peruvaya	W7	114	Thonnuran	W8
115	Kodak veliyan	W9	116	Velumbala	W10
117	Thondi 1	W11	118	Puttavetta	W12
119	Velluthanjavara	TRV 303	120	Chembavu	TRV 296
121	Vellapuranam	TRV 318	122	Cheriyakunjan	TRV 337
123	Ponnadan Thondi	TRV 362	124	Mullanpuncha	TRV 363
125	Kunjukunju	TRV 368	126	Mattachamban	TRV 370
127	Undachamban	TRV 395	128	Arimodan	TRV 94
129	Nadankuruva	TRV 2	130	Kalladiaryan	TRV 93
131	Aryankayama	TRV 13	132	Onuttan	TRV 20
133	Rajameni	TRV 24	134	Kanali	TRV 57
135	Kumbali	TRV 58	136	Adukkan	TRV 59
137	Erunnazhi	TRV 61	138	Thottachera	TRV 88
139	Karanjavara	TRV 90	140	Chettuvirupu	TRV 91
141	Kaladiaryan	TRV 93	142	Vellathondi	TRV 233
143	Thovan	TRV 407	144	Karithaalikannan	TRV 410
145	Cherupunja	TRV 413	146	Arampottan	TRV 414
147	Kochuthonnuran	TRV 417	148	Malippurampokkali	TRV 420
149	Englishamanakkari	TRV 424	150	Mullankuruva	TRV 412
151	Ahalya	TRV 216	152	Kargi	TRV 219

Table 2. List of molecular markers used for genotyping of fertility restorer genes in Kerala Rice varieties

Fertility Restorer gene	Molecular Marker	Annealing Temp (^{0}C)	Primer Sequence	Amplification Product size in restorer (bp)	Amplification Product size in non-restorer (bp)	Reference
Rf3	RMS-SF21-5	56	F: GAGTTGGGGGTCGAGAAATC R: CGTACGTGCGGCTAGGATCAA	172	127	Prasanthi *et al.* (2016)
Rf4	RM-PPR9-1	55	F: GAGTTTTGAATGATTTACGTGTGA R: AGTGTCCAGATTCGTAGTAATGC	114	159	

minutes at 72^{0}C. The PCR amplified DNA fragments were electroporated on a 4% agarose gel prepared in TAE buffer [242 g Tris Base, 51.7ml glacial acetic acid and 100 ml 0.5 ml EDTA (pH 8.0) dissolved in distilled water and finally made up to 1 litre]. Ethidium bromide was added at the rate of 5µL/100ml of agarose solution. The gels were loaded with 5µL of a 100-bp DNA ladder serving as a molecular size reference. Electrophoresis was carried out at 75 V for about 3 hours and gels were visualised under a UV light source in a gel documentation unit (Eppend of, USA) and documented.

The genotypes were categorized based on their amplification pattern gels, which were scored for presence and absence of bands as restorers and non-restorers. The sequence of primers and their amplification product sizes are presented in **Table 2**. This classification determined whether the *Rf3* and *Rf4* alleles were present or absent, enabling the TRVs and HYVs to be categorized according to their allelic configurations of restorer of fertility genes (**Table 3**).

The study utilized molecular markers to investigate 152 rice varieties from Kerala, with the objective to identify the restorer of fertility genes, *Rf3* and *Rf4*. The presence of the fertility restoration specific alleles was screened using the functional SSR markers RM-PPR9-1 and RMS-SF21-5. The molecular genotyping revealed that 15% of the varieties carried dominant alleles for both genes (*Rf3Rf4*), whereas 17% exhibited a dominant *Rf3* allele paired with a recessive *rf4* allele (*Rf3rf4*). A significant proportion, 56% of the varieties, possessed a dominant *Rf4* allele with a recessive *rf3* allele (*rf3Rf4*), while 31% showed recessive alleles for both genes *rf3rf4*) (**Fig.1**). The allelic distribution observed among the rice genotypes reveals a distinct skew towards the dominant *Rf4* allele, either singly or in combination with *Rf3*, indicating preferential selection for *Rf4* in hybrid breeding program utilizing WA-CMS systems. The earlier studies by Katara *et al*. (2017) and Cai *et al*. (2013), highlights the superior restoration efficiency of *Rf4* over *Rf3*. The chromosomal independence of *Rf3* (Chromosome 1) and *Rf4* (Chromosome 2), coupled with potential epistatic interactions and modifier loci, may further influence allelic segregation and retention. Marker assisted genotyping using tightly linked SSRs and functional markers ensured reliable allele identification, however minor allelic variants or undetected QTLs may also contribute to the observed diversity. These findings underscore the importance of integrating molecular diagnostics with pedigree analysis to delineate restorer gene dynamics and optimize parental selection in hybrid rice breeding. It further reinforces the identification and characterization of effective restorers and maintainers guided by allelic profiling of *Rf3* and *Rf4* constitute a final step in establishing robust parental lines for three-line hybrid rice breeding.

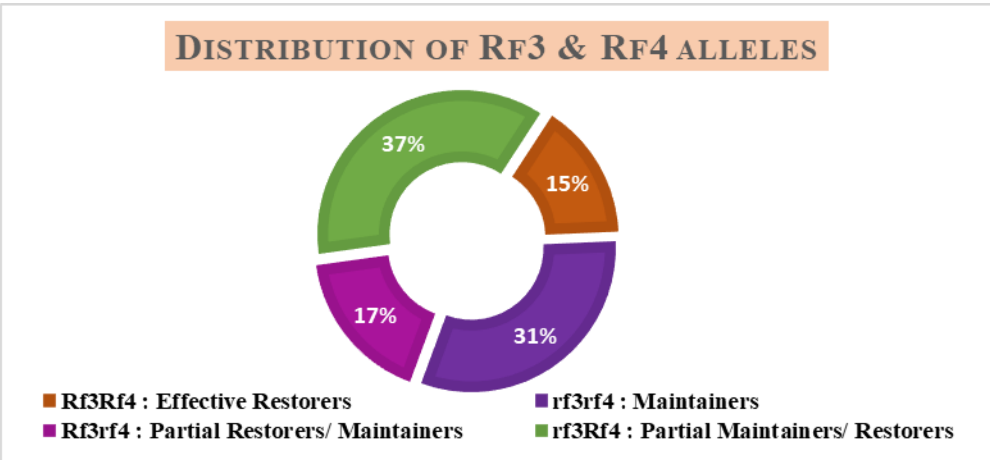

Fig.1. The frequency distribution of *Rf3* & *Rf4* genes based on genotyping of 152 Kerala Rice varieties

Table 3. Allele status of restorers and non-restorers with gene specific markers

S.No	Rice Accession		Rf specific marker amplification pattern		Classified as R/ PR or PM/M
			Rf3 RMS-SF21-5	*Rf4* RM-PPR 9-1	
1	M2	Manurathna	+	+	R
2	E6	Mithila	+	+	R
3	E3	Ezhome 3	+	+	R
4	E4	Ezhome 4	+	+	R
5	W9	Kodak veliyan	+	+	R
6	W3	Kalladiaryan	+	+	R
7	PTB 28	Kattamodan	+	+	R
8	MO5	Asha	+	+	R
9	VTL 3	Vyttila 3	+	+	R
10	VTL 8	Vyttila 8	+	+	R
11	VTL 5	Vyttila 5	+	+	R
12	VTL 2	Vyttila 2	+	+	R
13	VTL 1	Vyttila 1	+	+	R
14	PTB 10	Thekkancheera	+	+	R
15	PTB 53	Mangalamahswari	+	+	R
16	PTB 38	Triveni	+	+	R
17	TRV 20	Onuttan	+	+	R
18	TRV 61	Erunnazhi	+	+	R
19	TRV 296	Chembavu	+	+	R
20	TRV 363	Mattachamban	+	+	R
21	PTB 35	Annapoorna	+	+	R
22	PTB 36	Rohini	+	+	R
23	E1	Ezhome 1	+	-	PR/PM
24	W11	Thondi 1	+	-	PR/PM
25	MO17	Revathy	+	-	PR/PM
26	MO22	Sreyas	+	-	PR/PM
27	W1	Deepthi	+	-	PR/PM
28	VTL 11	Vyttila 11	+	-	PR/PM
29	VTL 9	Vyttila 9	+	-	PR/PM
30	K2	Onam	+	-	PR/PM
31	PTB 25	Thonnoran	+	-	PR/PM
32	PTB 43	Swarnaprabha	+	-	PR/PM
33	PTB 60	Vaishakh	+	-	PR/PM
34	PTB 57	Swetha	+	-	PR/PM
35	PTB 59	Samyuktha	+	-	PR/PM
36	PTB 32	Aruvakkari	+	-	PR/PM
37	PTB 22	Veluthavattan	+	-	PR/PM
38	PTB 23	Cheriya Aryan	+	-	PR/PM
39	PTB 39	Jyothi	+	-	PR/PM
40	E2	Ezhome 2	+	-	PR/PM
41	PTB 13	Kayama	+	-	PR/PM
42	TRV 88	Tottachera	+	-	PR/PM
43	TRV 303	Veluthanjavara	+	-	PR/PM
44	TRV 420	Malippurampokkali	+	-	PR/PM
45	TRV 417	Kochuthonnuran	+	-	PR/PM
46	TRV 414	Arampottan	+	-	PR/PM
47	-	Arupathamkuruvai	+	-	PR/PM
48	TRV 93	Kalladiaryan	+	-	PR/PM
49	E5	Jaiva	-	+	PR/PM
50	MO4	Bhadra	-	+	PR/PM
51	MO10	Remya	-	+	PR/PM

https://doi.org/10.37992/2025.1603.041

S.No	Rice Accession		Rf specific marker amplification pattern		Classified as R/ PR or PM/M
			Rf3 RMS-SF21-5	*Rf4* RM-PPR 9-1	
52	MO9	Makom	-	+	PR/PM
53	MO8	Aruna	-	+	PR/PM
54	VTL 10	Vyttila 10	-	+	PR/PM
55	K3	Dhanya	-	+	PR/PM
56	VTL 6	Vyttila 6	-	+	PR/PM
57	VTL 7	Vyttila 7	-	+	PR/PM
58	K6	Amrutha	-	+	PR/PM
59	K4	Sagara	-	+	PR/PM
60	PTB 1	Aryan	-	+	PR/PM
61	PTB 21	Thekkan	-	+	PR/PM
62	PTB 7	Parambuvattan	-	+	PR/PM
63	PTB 48	Nila	-	+	PR/PM
64	PTB 47	Neeraja	-	+	PR/PM
65	PTB 45	Matta Triveni	-	+	PR/PM
66	PTB 44	Rasmi	-	+	PR/PM
67	PTB 58	Anashwara	-	+	PR/PM
68	PTB 29	Karuthamodan	-	+	PR/PM
69	PTB 27	Kodiyan	-	+	PR/PM
70	PTB 26	Chenkayama	-	+	PR/PM
71	PTB 61	Supriya	-	+	PR/PM
72	PTB 62	Akshaya	-	+	PR/PM
73	PTB 12	Thekkanchitteni	-	+	PR/PM
74	PTB 18	Eravapandi	-	+	PR/PM
75	PTB 20	Vadakkanchitteni	-	+	PR/PM
76	PTB 2	Ponnaryan	-	+	PR/PM
77	PTB 3	Eravapandy	-	+	PR/PM
78	PTB 4	Vellari	-	+	PR/PM
79	PTB 5	Velutharikayam	-	+	PR/PM
80	PTB 6	Athikkiraya	-	+	PR/PM
81	PTB 12	Thekkanchitteni	-	+	PR/PM
82	PTB 15	Kauginpoothala	-	+	PR/PM
83	PTB 18	Eravapandi	-	+	PR/PM
84	PTB 19	Athikkiraya	-	+	PR/PM
85	PTB 20	Vadakkanchitteni	-	+	PR/PM
86	PTB 33	Arikkirai	-	+	PR/PM
87	PTB 34	Valiyachampan	-	+	PR/PM
88	PTB 37	Aswathy	-	+	PR/PM
89	W6	Mannuveliyan	-	+	PR/PM
90	W8	Thonnuran	-	+	PR/PM
91	W10	Velumbala	-	+	PR/PM
92	MO11	Kanakom	-	+	PR/PM
93	TRV 57	Kanali	-	+	PR/PM
94	TRV 13	Aryankayama	-	+	PR/PM
95	TRV 94	Arimodan	-	+	PR/PM
96	TRV 90	Karanjavara	-	+	PR/PM
97	TRV 368	Kunjukunju	-	+	PR/PM
98	TRV 337	Cheriyakunjan	-	+	PR/PM
99	TRV 93	Kalladiayan	-	+	PR/PM
100	TRV 363	Mullanpuncha	-	+	PR/PM
101	TRV 410	Karithalikannan	-	+	PR/PM
102	TRV 91	Chettuviripu	-	+	PR/PM
103	TRV 407	Thovan	-	+	PR/PM
104	TRV 219	Kargi	-	+	PR/PM

S.No		Rice Accession	Rf specific marker amplification pattern		Classified as R/ PR or PM/M
			Rf3 RMS-SF21-5	*Rf4* RM-PPR 9-1	
105	M3	Manuvarna	-	-	M
106	MO20	Gouri	-	-	M
107	MO12	Ranjini	-	-	M
108	MO21	Prathyasha	-	-	M
109	MO14	Panchami	-	-	M
110	MO18	Karishma	-	-	M
111	MO15	Remanika	-	-	M
112	MO19	Krishnanjana	-	-	M
113	MO13	Pavithra	-	-	M
114	MO7	Karthika	-	-	M
115	MO6	Pavizham	-	-	M
116	MO16	Uma	-	-	M
117	MO23	Pournami	-	-	M
118	K5	Dhanu (K)	-	-	M
119	K1	Bhagya(K)	-	-	M
120	PTB 8	Thavalakkannan	-	-	M
121	PTB 50	Kanchana	-	-	M
122	PTB 54	Karuna	-	-	M
123	PTB 41	Bharathy	-	-	M
124	PTB 55	Harsha	-	-	M
125	PTB 49	Kairali	-	-	M
126	PTB 56	Varsha	-	-	M
127	PTB 52	Aiswarya	-	-	M
128	PTB 51	Aathira	-	-	M
129	PTB 31	Elappapoochampan	-	-	M
130	PTB 30	Chuvannamodan	-	-	M
131	PTB 40	Sabari	-	-	M
132	PTB 9	Thavalkannan	-	-	M
133	PTB 21	Thekkan	-	-	M
134	PTB 22	Veluthavattan	-	-	M
135	PTB 24	Chuvannavattan	-	-	M
136	W5	Veliyan (W)	-	-	M
137	W4	Ponnadan Thondi (W)	-	-	M
138	W2	Mullukuruva(W)	-	-	M
139	W7	Peruvaya (W)	-	-	M
140	W12	Puttuvetta(W)	-	-	M
141	TRV 2	Nadankuruva	-	-	M
142	TRV 59	Adukkan	-	-	M
143	TRV 24	Rajameni	-	-	M
144	TRV 318	Vellapuram	-	-	M
145	TRV 395	Undachemban	-	-	M
146	TRV 233	Vellathondi	-	-	M
147	TRV 424	Englishamanakkari	-	-	M
148	TRV 362	Ponnadan Thondi	-	-	M
149	TRV 216	Ahalya	-	-	M
150	TRV 58	Kumbali	-	-	M
151	TRV 413	Cherupunja	-	-	M
152	TRV 412	Mullukuruva	-	-	M

(+) Restorer allele (172 bp and 114 bp for RM-PPR9-1and RMS-SF21-5, respectively); (-) Non restorer allele (127 bp and 159 bp for RM-PPR9-1 and RMS-SF21-5, respectively); R-Restorers; PR/PM-Partial Restorers or Partial Maintainers; M- Maintainers

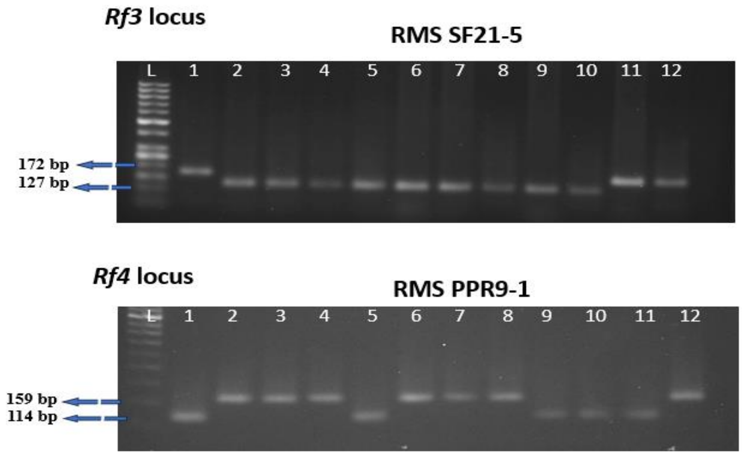

Fig. 2. Molecular profiling of *Rf3* and *Rf4* genes in selected rice varieties/TRVs functional markers RMSSF21-5 for *Rf3* and RMS PPR9-1 for *Rf4*

Based on these genotyping, the varieties were grouped into categories, with 22 identified as putative restorers and 48 as putative maintainers. The putative restorers included HYVs like Manurathna, Mithila, Ezhome 3, Ezhome 4, Kattamodan (PTB28), Asha, Vyttila1, Vyttila2, Vyttila3, Vyttila5, Vyttila8, Thekkancheera (PTB10), Mangalamahsuri (PTB53), Triveni (PTB38), Annapoorna (PTB35), Rohini (PTB36); and TRVs like Kodakveliyan, Kalladiaryan, Onuttan, Erunnazhi, Chembavu, Mattachamban) showed the presence of both the genes (**Table 3**). The remaining varieties were classified as either partial restorers or partial maintainers. Notably, the dominant allele of *Rf4* was significantly more prevalent, observed in 63% of the varieties, in contrast to the 19% frequency of the dominant *Rf3* allele. This disparity highlights the pivotal role of *Rf4* in facilitating fertility restoration and its strategic emphasis within contemporary breeding initiatives. Further categorization was achieved through the use of markers, such as RMS-SF21-5 for *Rf3* and RMS-PPR9-1 for *Rf4*. These markers enabled a comprehensive elucidation of allele combinations, thus driving the precise identification of elite restorer and maintainer lines, which are indispensable for the refinement of hybrid rice breeding strategies. The findings underscore the importance of *Rf4* in improving fertility restoration, thereby enhancing the efficiency of hybrid seed production systems.

*Understanding of fertility restorer genes Rf3 and Rf4 on hybrid breeding in Rice***:**The advancement of hybrid rice breeding relies on the efficient restoration of fertility within CMS-based systems, driven by enhanced pollen viability and optimal spikelet fertility. Achieving this requires a thorough understanding of the genetics of fertility restoration, particularly the interaction of *Rf* genes with CMS cytoplasm. The fertility restorer gene *Rf3* mapped to chromosomes 1 and *Rf4* in chromosome 10, plays pivotal role in restoring fertility in WA-CMS systems (Jing *et al*., 2001; Zhang *et al*., 1997). The molecular functions of *Rf4* and *Rf3* are distinct, each playing an equally vital role. *Rf4* mediates post-transcriptional degradation of transcripts originating from the sterility-inducing WA352 mitochondrial gene, whereas *Rf3* operates at the post-translational level, effectively blocking their translation. (Lu *et al*., 2013).

The molecular dissection of *Rf3* and *Rf4* genes provides a mechanistic framework that directly supports the interpretation of marker based classification outcomes in hybrid rice breeding. The distinct functional roles – *Rf4* mediating transcript degradation and *Rf3* inhibiting translation, highlights the necessity for both genes for complete fertility restoration in WA-CMS systems (Lu *et al*., 2013; Jing *et al*., 2001; Zhang *et al*., 1997). This complementarity is reflected in the genotypic distribution observed among the 152 rice varieties evaluated in the present study, where only 15% carried dominant alleles for both genes (*Rf3Rf4*), representing the most effective restorers. In contrast, 17% exhibited *Rf3rf4* genotype and 56% carried *rf3Rf4* indicating partial restoration potential, while 31% possessed recessive alleles for both the genes (*rf3rf4*), likely functioning as maintainers. These molecular profiles are consistent with the underlying genetic mechanisms; however their functional validity must be substantiated through test crosses using the profiled varieties as restorers, followed by assessments of spikelet and pollen fertility to confirm restoration capacity (Sundaram *et al*., 2010; Virmani *et al*., 2003). Such phenotypic evaluations are essential to validate the predictive reliability of marker assisted classification and ensure the molecular diagnostics accurately reflect restoration potential under field conditions. Thus, the integration of functional genomics with empirical validation reinforces the foundational role of *Rf* gene profiling in accelerating the identification of effective parental lines for three-line hybrid rice breeding.

Traditional methods of identifying restorer and maintainer lines through test crosses with CMS lines are labour-intensive and time-consuming (Ikehashi & Araki, 1984;

Virmani, 1996). Molecular screening using tightly linked markers, RMS-SF21-5, for *Rf3* and RMS-PPR9-1 for *Rf4*, provides an efficient alternative. This approach allows breeders to rapidly identify potential parental lines with desirable allele combinations, facilitating the development of hybrid rice varieties tailored to specific ecologies. The study found a skewed distribution of *Rf3* and *Rf4* alleles, likely due to historical breeding practices and pedigree relationships. The broader adoption of parental lines with dominant *Rf4* alleles reflects its superior functionality in restoring fertility. This is further supported by its molecular role as a post-transcriptional regulator, compared to the post-translational action of *Rf3*. Studies in radish (Murayama *et al*., 2004) and other crops similarly show the dominance of effective restorer alleles in breeding populations, favouring hybrids with high fertility restoration rates.

CMS and Restorer Genes: A Complex Interaction: Cytoplasmic male sterility, documented in more than 150 flowering plant species, arises from mitochondrial genome rearrangements that impair pollen formation (Hanson & Bentolila, 2004; Toriyama, 2021; Igarashi, 2016). In populations with CMS, nuclear genes such as *Rf3* and *Rf4* evolve to counteract these sterility-causing mitochondrial genes, maintaining fertility in hybrids (Bentolila *et al*., 2002; Kazama *et al*., 2008; Zhao *et al*., 2023). The theory predicts that CMS genes and their corresponding restorer alleles undergo swift fixation, facilitating the stable coexistence of sterility-inducing cytoplasms and their nuclear suppressors within plant populations (Nguyen & Pannell, 2025; Palanisamy, *et al*., 2019).

The dominance of *Rf4* in the tested rice varieties aligns with this evolutionary framework. Katara *et al*. (2017) found that genotypes with *Rf4Rf4* alleles restored fertility more effectively than those with *Rf3Rf3* alleles or a combination of recessive alleles. Double dominants (*Rf3Rf3*/*Rf4Rf4*) were more effective, but *Rf4Rf4* alone outperformed *Rf3Rf3*. The higher efficacy of *Rf4* may also be linked to its molecular role in degrading WA352 transcripts, compared to the translational suppression mechanism of *Rf3*. Molecular confirmations show the same effects in the present evaluations done for the 152 rice varieties, but it needs to be confirmed with the test cross using these varieties as restorers followed by the spikelet and pollen fertility studies to justify the molecular profiling.

Implications for hybrid Rice breeding: Pinpointing robust restorers and maintainers plays a pivotal role in driving progress in hybrid rice breeding initiatives. This study by molecular screening approach, coupled with detailed allele frequency analysis, provides a roadmap for selecting superior parental lines. The integration of molecular markers into breeding programs offers a faster, more precise method for developing hybrids with high fertility restoration and adaptability to diverse agroecological conditions. Further exploration of minor or modifier genes influencing fertility restoration is warranted, particularly in cases where genotypes with recessive alleles (*rf3rf3rf4rf4*) demonstrated unexpected fertility restoration. At the core of the CMS system lie fertility restorer (*Rf*) genes, which play an indispensable role in enabling the formation of functional male gametophytes in plants harbouring mitochondrial CMS genes. These *Rf* genes, residing within the nuclear genome, counteract the expression of the CMS-inducing gene, thereby restoring male fertility (Toriyama, 2021). This highlights the complexity of nuclear-cytoplasmic interactions and the potential for uncovering additional genetic factors contributing to fertility restoration in CMS systems (Chen *et al*., 2017).

This study underscores the importance of *Rf4* and *Rf3* in hybrid rice breeding, with *Rf4* playing a dominant role in fertility restoration. From the identified 22 restorers having the both *Rf* genes can be further utilized for the development of hybrid rice for Kerala, with the suitable A lines. The integration of molecular screening into breeding workflows enhances the efficiency of identifying restorer and maintainer lines, addressing the challenges of labour-intensive traditional methods. The findings contribute to the development of hybrids with improved fertility, adaptability, and yield potential, supporting efforts to ensure global food security through hybrid rice technology.

REFERENCES

Ahmadikhah. A. and Karlov, G.I. 2006. Molecular mapping of fertility restoration gene *Rf4* for WA-cytoplasmic male sterility in rice. *Plant Breed,* **125**: 363-367. [Cross Ref]

Ahmadikhah, A., Karlov, G.I., Nematzadeh, G.H. and Ghasemi Bezdi, K. 2007. Inheritance of the fertility restoration and genotyping of rice lines at the restoring fertility (*Rf*) loci using molecular markers. *Int J Plant Prod,* **1**(1):13–21.

Bentolila, S., Alfonso, A. A. and Hanson, M. R. 2002. A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. *Proc. Natl Acad. Sci., USA* **99:** 10887—10892. [Cross Ref]

Cai, J., Liao, Q.P., Dai, Z.J., Zhu, H.T., Zeng, R.Z., Zhang, Z.M. and Zhang, G.Q. 2013. Allelic differentiations and effects of the *Rf3* and *Rf4* genes on fertility restoration in rice with wild abortive cytoplasmic male sterility. *Biologia Plantarum*, **57**(2): 274–280. [Cross Ref]

Chen, L.K., Yan, X.C., Dai, J.H., Chen, S. P., Lui, Y.Z., Wang, H., Chen, Z.Q. and Guo, T. 2017. Significant association of the novel Rf4-targeted SNP marker with the restorer for WA-CMS in different rice backgrounds and its utilization in molecular screening. *J. Integrative Agri.*, **16**(10): 2128–2135. [Cross Ref]

FAO. 2009. How to feed the world in 2050. Report of the FAO High level expert forum meeting, Rome, 1-4.

Hanson, M. R. and Bentolila, S. 2004. Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *Plant Cell,* **16:** S154—S169. [Cross Ref]

Igarashi, K., Kazama, T. and Toriyama, K. 2016. A gene encoding pentatricopeptide repeat protein partially restores fertility in RT98-type cytoplasmic male-sterile rice, *Plant Cell Physio*., **57**(10): 2187–2193. [Cross Ref]

Ikehashi, H. and Araki, H. 1984. Varietal screening of compatibility types revealed in F1 fertility of distant crosses in rice. *Japan J. Breed*., **34:** 304—313. [Cross Ref]

Jing, R., Li, X., Yi, P. and Zhu, Y. 2001. Mapping fertility-restoring genes of rice WA cytoplasmic male sterility using SSLP markers. *Bot. Bull. Acad. Sin*., **42:** 167—171.

Katara, J.L., Verma, R.L., Nayak, D., Ngangkham, U., Ray, S., Subudhi, H., Behera, L., Samantaray, S., Rao, R.N., Singh, O.N. and Mohapatra, T. 2017. Frequency and fertility restoration efficiency of Rf3 and Rf4 genes in Indian rice. *Plant Breed*, **136**(1): 74–82. [Cross Ref]

Kazama, T., Nakamura, T., Watanabe, M., Sugita, M., Toriyama, K., Kazama, T., Nakamura, T., Watanabe, M., Sugita, M. and Toriyama, K. 2008. Suppression mechanism of mitochondrial ORF79 accumulation by *Rf1* protein in BT-type cytoplasmic male sterile rice. *Plant J*., **55**: 619—628. [Cross Ref]

Kazama, T. and Toriyama, K. 2014. A fertility restorer gene, *Rf4*, widely used for hybrid rice breeding encodes a pentatricopeptide repeat protein. *Rice,* **7:** 28 . [Cross Ref]

Lu, Y., Virmani, S.S., Zhang, G., Bharaj, T.S. and Huang, N. 1997. Mapping of the *Rf-3* nuclear fertility-restoring gene for WA cytoplasmic male sterility in rice using RAPD and RFLP markers. *Theor Appl Genet,* **94**:27–33. [Cross Ref]

Lu, D. P., Xu, H., Liu, Z. L., Guo, J. X., Li, H. Y., Chen, L. T., Fang, C., Zhang, Q. Y., Bai, M., Yao, N., Wu, H., Wu, H., Ji, C., Zheng, H., Chen, Y., Ye, S., Li, X., Zhao, X., Li, R. and Liu, Y. G. 2013. A detrimental mitochondrial-nuclear interaction causes cytoplasmic male sterility in rice. *Nat. Genet*, **45**: 573—577. [Cross Ref]

Mishra, G.P., Singh. R.K., Mohapatra, T., Singh, A.K., Prabhu, K.V., Zaman, F.U. and Sharma, R.K. 2003. Molecular mapping of a gene for fertility restoration of wild abortive (WA) cytoplasmic male sterility using a basmati rice restorer line. *J Plant Biochem Biotechnol* **12**:37–42. [Cross Ref]

Murayama, K., Yahara, T. and Terachi, T. 2004. Variation of female frequency and cytoplasmic male-sterility gene frequency among natural gynodioecious populations of wild radish (*Raphanus sativus* L.). *Mol. Ecol*. **13**, 2459—2464. [Cross Ref]

Ngangkham, U., De Sandip, Singh, S.K., Parida, S.K., RajKumar, K.A., Singh, A.K. and Mohapatra, T. 2010. Genic markers for wild abortive (WA) cytoplasm based male sterility and its fertility restoration in rice. *Mol Breed,* **26**(2):275–292. [Cross Ref]

Nguyen, M.T. and Pannell, J.R. 2025. The evolution and maintenance of trioecy with cytoplasmic male sterility. *Heredity*. **134:**1–9. [Cross Ref]

OECD/FAO (2025), OECD-FAO Agricultural Outlook 2025-2034, OECD Publishing, Paris/FAO, Rome, https://doi.org/10.1787/601276cd-en. [Cross Ref]

Palanisamy, D., Marappan, S., Ponnuswamy, R.D. Mahalingam, P.S., Bohar, R. and Vaidyanathan, S. 2019. Accelerating hybrid rice breeding through the adoption of doubled haploid technology for R-line development. *Biologia,* **74**: 1259–1269. [Cross Ref]

Qian, Q., Zhang, F. and Xin, Y. 2021. Yuan longping and hybrid rice research. *Rice,* **14:** 101. [Cross Ref]

Rout, D., Jena, D., Singh, V., Kumar, M., *et al*. 2021. Hybrid Rice Research: Current Status and Prospects. *IntechOpen*. doi:10.5772/intechopen.93668. [Cross Ref]

Sheeba, N.K., Viraktamath, B.C., Sivaramakrishnan, S., Gangashetti, M.G., Pawan, K.and Sundaram, R.M. 2009. Validation of molecular markers linked to fertility restorer gene(s) for WA-CMS lines of rice. *Euphytica,* **167** :217–227. [Cross Ref]

Shrivastava, A., Singh, S., Koutu, G.K. and Tomar, A.K. 2019. Hybrid rice seed production technology in India. Proceedings of international conference on agriculture and allied sciences: The Productivity, Food Security and Ecology, 137-145. ISBN-978-93-85822-71-1

Sundaram, R.M., Sakthivel, K., Rani, N.S., Balachandran, S.M. and Neeraja, C.N. 2010. Genetic and molecular basis of fragrance in rice. *Biotechnology Advances*, **28**(4): 451–461. [Cross Ref]

Suresh, P. B., Srikanth, B., Hemanth, K. V., Subhakara, R. I., Vemireddy, L. R., Dharika, N., Sundaram, R. M., Ramesha, M. S., Sambasiva, R. K. R. S., Viraktamath, B. C. and Neeraja, C. N. 2012. Fine mapping of *Rf3* and *Rf4* fertility restorer loci of WA-CMS of rice (*Oryza sativa* L.) and validation of the developed marker system for identification of restorer lines. *Euphytica,* **187**: 421—435. [Cross Ref]

Tan, X.L., Vanavichit, A., Amornsilpa, S. and Trangoonrung, S. 1998. Genetic analysis of rice CMS-WA fertility restoration based on QTL mapping. *Theor Appl Genet,* **96**: 994–999. [Cross Ref]

Toriyama, K. 2021. Molecular basis of cytoplasmic male sterility and fertility restoration in rice. *Plant Biotech.* **38**(3):285-295. [Cross Ref]

Virmani, S. S. 1996. Hybrid rice. *Adv. Agron*., **57:** 328—462. [Cross Ref]

Zhang, G., Bharaj, T. S., Virmani, S. S. and Huang, N. 1997. Mapping of the Rf-3 nuclear fertility restoring gene for WA cytoplasmic male sterility in rice using RAPD and RILP markers. *Theor. Appl. Genet*., **94:** 27—33. [Cross Ref]

Zhang, Q. Y., Liu, Y. G. and Mei, M.T. 2002. Molecular mapping of the fertility restorer gene *Rf4* for WA cytoplasmic male sterility. *Acta Genet Sin,* **29**:1001–1004.

Zhao, Z., Ding, Z., Huang, J., Meng, H., Zhang, Z., Gou, X., Tang, H., Xie, X., Ping, J., Xiao, F., Liu, Y., Xie, Y. and Chen, L. 2023. Copy number variation of the restorer Rf4 underlies human selection of three-line hybrid rice breeding. *Nat Commun*. **14:** 7333. [Cross Ref]