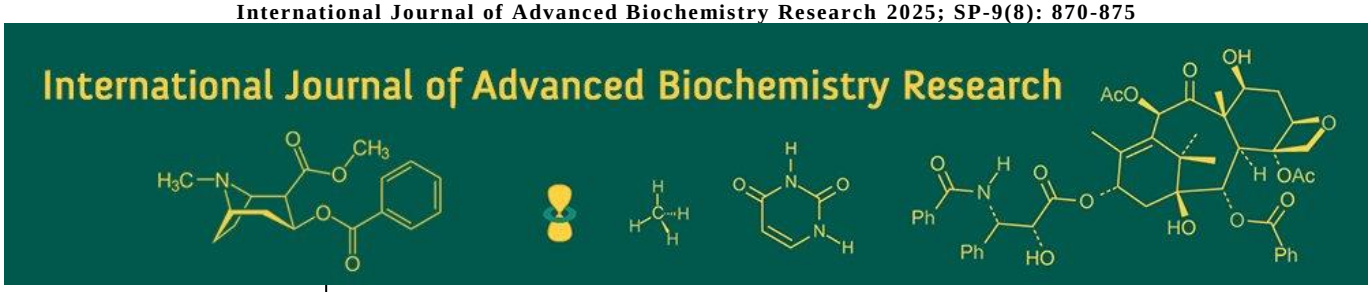

ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2025): 5.29
IJABR 2025; SP-9(8): 870-875
www.biochemjournal.com
Received: 25-05-2025
Accepted: 30-06-2025

V Keerthana
Master's Student, Department of Genetics and Plant Breeding, College of Agriculture, Rajendranagar, Hyderabad, Telangana, India

K Gopala Krishna Murthy
Professor, Department of Genetics and Plant Breeding, College of Agriculture, Rajendranagar, Hyderabad, Telangana, India

Y Chandramohan
Principal Scientist, Plant Breeding, Rice Research Unit, Rajendranagar, Hyderabad, Telangana, India

CH Anuradha
Professor, Molecular Biology and Biotechnology, Institute of Biotechnology, College of Agriculture, Rajendranagar, Hyderabad, Telangana, India

Corresponding Author:
V Keerthana
Master's Student, Department of Genetics and Plant Breeding, College of Agriculture, Rajendranagar, Hyderabad, Telangana, India

Molecular screening and identification of restorer lines for fertility restorer genes *Rf3* and *Rf4* in Rice (*Oryza sativa* L.)

V Keerthana, K Gopala Krishna Murthy, Y Chandramohan and CH Anuradha

DOI: https://www.doi.org/10.33545/26174693.2025.v9.i8Sm.5267

Abstract
As the primary food source for over 50% of the global population, rice (*Oryza sativa* L.) plays a crucial role in worldwide food security. The development of hybrid rice varieties, particularly through the Wild Abortive Cytoplasmic Male Sterility (WA-CMS) breeding approach, represents a promising and ecologically friendly strategy to overcome current productivity constraints. The restoration of fertility within this breeding system depends primarily on the action of two nuclear genetic factors: *Rf3* and *Rf4*. This investigation involved the evaluation of thirty-six candidate restorer genotypes through molecular marker technology, utilizing RM-SF21-5 (associated with *Rf3*) and RMS-PRR-9-1 (associated with *Rf4*) markers. Through polymerase chain reaction amplification and electrophoretic separation techniques, researchers identified the *Rf3* genetic variant in nineteen breeding lines, including genotypes SN 3171, SN 3194, and SN 3250. Additionally, five lines demonstrated the presence of the *Rf4* variant, notably SN 3184, SN 3190, and SN 3423. The identification of a characteristic 172 base pair amplification product served as confirmation for the presence of fertility restoration genes within these genetic materials. The identified breeding lines represent promising genetic resources for developing fertile hybrid combinations within WA-CMS breeding programs. The application of molecular marker-based selection strategies for *Rf* gene identification enhances the efficiency of restorer line development, ultimately contributing to the production of superior hybrid rice cultivars with improved yield potential and stress tolerance for addressing global food production challenges.

Keywords: Rice, WA-CMS, Rf3, Rf4, molecular markers, hybrid breeding

1. Introduction
Rice (*Oryza sativa* L.) serves as a fundamental cereal grain supporting global populations and maintaining profound socioeconomic importance throughout multiple nations. Within this botanical genus, 24 species exist—comprising 22 indigenous varieties and 2 cultivated forms—demonstrating remarkable genetic variability. The chromosomal architecture varies between nine tetraploid species (containing 48 chromosomes) and the remaining diploid species (possessing 24 chromosomes). Within India's agricultural landscape, rice cultivation represents the predominant farming activity, encompassing the most extensive cropping area while substantially contributing to the nation's nutritional security. Contemporary agricultural statistics (Indiastat, 2023-24) [3] reveal production across 47.82 million hectares, generating approximately 138.72 million metric tons with mean productivity reaching 2, 882 kg/ha. The state of Telangana exemplifies exceptional production efficiency, achieving 16.87 million tons from 4.68 million hectares, resulting in superior yield rates of 3, 602 kg/ha. Meeting escalating worldwide food requirements necessitates productivity enhancement through sophisticated breeding methodologies (Veeresha *et al.*, 2015) [9]. Hybrid rice development, integrating conventional breeding approaches with contemporary molecular tools, has demonstrated remarkable success in creating superior F_1 hybrid varieties (Huang *et al.*, 2014; Khush, 2013; Vaz Mondo *et al.*, 2016) [2, 4, 8]. China maintains its position as the foremost adopter of hybrid rice technology globally, while India has progressively incorporated these methodologies to enhance agricultural output. A revolutionary breakthrough emerged during the 1970s with the identification of Wild Abortive Cytoplasmic Male Sterility (WA-CMS) within a naturally occurring mutant of *Oryza sativa*

f. *spontanea*. The inaugural commercial CMS line, derived from a sterile specimen discovered on Hainan Island in 1973, established the groundwork for the three-line hybrid methodology, currently representing the most extensively employed technique (Shih-Cheng *et al.*, 1980; Yuan, 1998; Virmani *et al.*, 1983) [7, 12, 10]. This breeding system, alternatively termed cytoplasmic-genic male sterility (CGMS), functions through three essential components: a male-sterile (CMS) line, a maintainer line, and a restorer line containing fertility-restoring (*Rf*) genetic elements. Indian hybrid rice production predominantly utilizes the WA-CMS-based three-line approach, although securing dependable restorer lines continues to present significant challenges. Among the 17 documented fertility restoration genes in rice—predominantly exhibiting dominant inheritance patterns excluding *rf17*—the *Rf3* gene (located on chromosome 1) and *Rf4* gene (positioned on chromosome 10) demonstrate essential functions in WA-CMS hybrid development (Zhang *et al.*, 1997; Yao *et al.*, 1997) [13, 11]. Molecular marker technology, including systems established by Alavi *et al.* (2009) [1], facilitates effective marker-assisted selection (MAS) protocols for restorer line identification, reducing dependence on time-intensive test crossing procedures. Research findings (Revathi *et al.*, 2013) [6] demonstrate 85-92% precision in forecasting functional restorer performance through marker implementation Revathi *et al.*, (2013) [6]. Verified restorer lines subsequently undergo hybridization with superior A-lines to produce high-performance hybrids, followed by comprehensive assessment of agronomic characteristics, grain attributes, and combining capacity. The objective of this investigation was to validate molecular markers linked with *Rf3* and *Rf4* genes to enhance the efficiency of identifying effective restorers within WA-CMS hybrid breeding initiatives.

2. Materials and Methods

This investigation was executed throughout the 2024 *Kharif* cropping period at the Rice Research Unit (RRU) within the Agricultural Research Institute of Professor Jayashankar Telangana Agricultural University, situated at Rajendranagar, Hyderabad, in Telangana state, India. The experimental material comprised 36 rice genetic resources sourced from dual origins: the local RRU facility at ARI, Rajendranagar, and the International Rice Research Institute (IRRI) based in the Philippines. Following the initial season, molecular characterization of these 36 breeding lines was performed during the succeeding *Kharif* period (2025) to identify the presence of fertility restoration loci *Rf3* and *Rf4* using marker-based detection methods. The genetic screening protocol incorporated targeted molecular markers specifically linked to these restoration genes: RM-SF21-5 and RMS-PRR9-1. A comprehensive listing of all genotypes subjected to this molecular characterization process is presented in Table 1.

2.1 Molecular analysis: DNA isolation and marker detection

Total genomic DNA isolation from 36 candidate restorer genotypes was accomplished through a conventional CTAB-based extraction methodology. Fresh leaf material (roughly 100 mg) was harvested into sterile, pre-coded 2 mL microcentrifuge vessels containing a sterilized metallic bead and promptly preserved at-80 °C prior to analysis. During DNA isolation procedures, cryopreserved plant tissue underwent mechanical disruption in 500 µL CTAB extraction buffer utilizing a tissue homogenizer (300 rpm, 2 min duration), subsequently supplemented with an additional 500 µL CTAB buffer and maintained at 65 °C for 45 minutes. Following high-speed centrifugation (12, 000 × g, 15 min, 4 °C), the clarified supernatant underwent purification using chloroform: isoamyl alcohol (24:1 ratio). DNA precipitation was achieved through isopropanol treatment at 4 °C for overnight duration, followed by 70% ethanol washing, and final reconstitution in 50 µL TE buffer solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Spectrophotometric analysis determined DNA quantity and quality parameters.

Table 1: Rice germplasm accessions evaluated for fertility restoration gene-linked markers (*Rf3* and *Rf4*) in the study

S. No.	Genotype
1	SN 2888
2	SN 3063
3	SN 3171
4	SN 3173
5	SN 3184
6	SN 3190
7	SN 3194
8	SN 3205
9	SN 3217
10	SN 3239
11	SN 3250
12	SN 3274
13	SN 3275
14	SN 3287
15	SN 3289
16	SN 3300
17	SN 3310
18	SN 3315
19	SN 3323
20	SN 3342
21	SN 3353
22	SN 3375
23	SN 3378
24	SN 3379
25	SN 3389
26	SN 3398
27	SN 3409
28	SN 3422
29	SN 3423
30	SN 3457
31	SN 3465
32	SN 3476
33	SN 3484
34	SN 3492
35	SN 3495
36	SN 3520

Polymerase chain reaction amplification utilized 10 µL reaction volumes comprising 20 ng template DNA, 1× PCR master mix, 0.25 µL forward and reverse primers each, and 3 µL molecular biology-grade water. Thermal cycling conditions included initial template denaturation at 94 °C for 5 minutes, succeeded by 35 amplification cycles consisting of denaturation (94 °C, 30 seconds), primer annealing (55 °C, 30 seconds), and DNA synthesis (72 °C, 1 minute), culminating with final extension at 72 °C for 7 minutes. Amplified products underwent electrophoretic separation on 3% agarose gel matrices prepared in 1× TAE buffer

containing ethidium bromide, with concurrent 100 bp molecular weight marker analysis. Gel electrophoresis proceeded at 120V until tracking dye migrated approximately two-thirds through the gel matrix. DNA band visualization occurred under ultraviolet illumination and image capture utilized a digital gel documentation system.

Molecular genotyping employed two sequence-tagged site (STS) markers: RM-SF21-5 targeting Rf3 and RMS-PRR-9-1 specific for Rf4 loci. Amplification products of 172 bp confirmed the presence of restoration alleles, whereas 127 bp fragments or absent amplification indicated non-restorer variants. Complete genotyping results were systematically compiled and evaluated for marker authentication across all 36 candidate lines. Comprehensive primer sequence details and marker specifications are documented in Table 2.

Table 2: Characteristics of molecular markers targeting fertility restoration genes

S. No	Primer Sequence	Chromosome	Annealing temperature °C	Reference
1. RM SF 21-5 (*Rf3*)	F GAGTTGGGGGTCGAGAAATC R CGTACGTGCGGCTAGGATCAA	1	55 °C	Pranathi *et al.*, 2016 [5]
2. RMS PRR 9-1 (*Rf4*)	F GAGTTTTGAATAGATTTACGTGTGGA R AGTGTCCAGATTCGTAGTAATGC	10	55 °C	Pranathi *et al.*, 2016 [5]

3. Findings and Interpretation

Genetic characterization of 36 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 system for *Rf3* detection yielded the characteristic 172 bp restoration allele in 19 genotypes (representing 52.8% of tested materials), notably including lines SN 3171, SN 3194, and SN 3250, whereas remaining accessions demonstrated either the 127 bp non-restoration variant or exhibited amplification failure. Molecular analysis targeting *Rf4* through RMS-PRR9-1 marker deployment revealed considerably lower frequencies, with merely 5 breeding lines (constituting 13.9% of the collection) displaying the diagnostic 172 bp amplification product. Among these positive genotypes were SN 3184, SN 3190, and SN 3423. The pronounced disparity in allelic distribution frequencies between *Rf3* (52.8%) and *Rf4* (13.9%) indicates this restoration loci potentially exhibit divergent evolutionary trajectories or perform specialized functions within fertility restoration mechanisms.

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.

3.1 Validation of Rf3 Fertility Restorer Gene Using Functional Marker RM-SF21-5

Genetic evaluation of 36 rice breeding lines through application of the codominant functional marker RM-SF21-5 effectively detected genotypes harbouring the *Rf3* fertility restoration gene. Molecular analysis demonstrated that 19 accessions (representing 52.8% of tested materials) displayed the characteristic 172 bp amplification product, validating the presence of the active *Rf3* genetic variant. This positive cohort encompassed valuable breeding materials including SN 3171, SN 3194, and SN 3250, alongside 16 additional genotypes: SN 3173, SN 3205, SN 3217, SN 3239, SN 3275, SN 3342, SN 3353, SN 3378, SN 3379, SN 3398, SN 3409, SN 3457, SN 3465, SN 3476, and SN 3484.

Conversely, the remaining 17 breeding lines (constituting 47.2% of the collection) produced exclusively the 127 bp amplification fragment, signifying the absence of functional *Rf3* restoration capacity. These findings underscore the efficacy of marker-assisted breeding strategies for detecting candidate restorer genotypes, with the RM-SF21-5 marker system exhibiting distinct polymorphic differentiation between restoration-competent and restoration-deficient materials. The substantial frequency (52.8%) of *Rf3*-carrying lines within this genetic resource collection indicates these accessions possess considerable utility for three-line hybrid rice development programs employing the WA-CMS breeding approach. The distinct electrophoretic band patterns documented (Figure 1) further confirm the dependability of this molecular marker system for extensive screening applications across breeding populations.

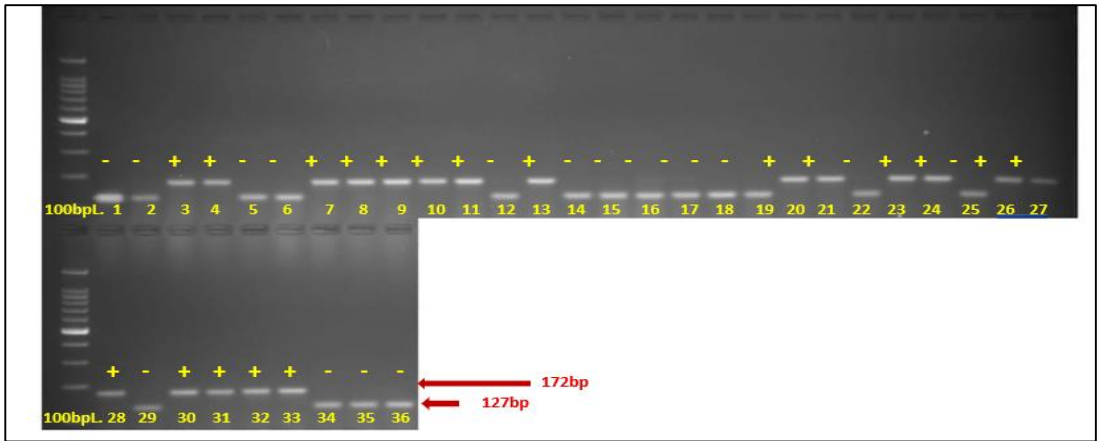

Fig 1: Genotypic profiling of *Rf3* fertility restorer gene using RM-SF21-5 functional marker

3.2 Validation of Rf4 Fertility Restorer Gene Using Functional Marker RMS-PRR9-1

Genetic characterization of the 36 rice breeding lines utilizing the codominant functional marker RMS-PRR9-1 effectively detected genotypes harbouring the *Rf4* fertility restoration gene. Five accessions (representing 13.9% of tested materials)-specifically SN 3184, SN 3190, SN 3422, SN 3423, and SN 3492-demonstrated the characteristic 172 bp amplification product, validating the presence of the active *Rf4* genetic variant. The balance of 31 breeding lines (constituting 86.1% of the collection) produced either the 127 bp non-restoration fragment or, in three instances, failed to generate amplification products, indicating probable null allelic variants.

The distinct polymorphic patterns documented between restoration-capable and restoration-deficient genotypes (Fig. 2) confirm the utility of the RMS-PRR9-1 marker system for *Rf4* gene detection. The comparatively modest frequency (13.9%) of *Rf4*-carrying lines relative to *Rf3* (52.8%) within this genetic resource assembly indicates varying selective pressures operating on these dual restorer loci throughout breeding program development. These molecular findings contribute significant genetic assets for hybrid rice cultivation, especially for establishing WA-CMS breeding lines possessing reliable fertility restoration mechanisms. The five *Rf4*-positive accessions identified constitute essential parental components for three-line hybrid development frameworks.

Genetic characterization outcomes demonstrated distinctive distribution profiles of fertility restoration loci across the 36 rice accessions examined. Nineteen breeding lines (representing 52.8% of tested materials) were validated to harbour the Rf3 restoration gene, encompassing genotypes SN 3171, SN 3173, SN 3194, SN 3205, SN 3217, SN 3239, SN 3250, SN 3275, SN 3342, SN 3353, SN 3378, SN 3379, SN 3398, SN 3409, SN 3457, SN 3465, SN 3476, and SN 3484, as demonstrated by detection of the 172 bp amplification product utilizing the RM-SF21-5 marker system.

Conversely, merely five accessions (constituting 13.9%)-specifically SN 3184, SN 3190, SN 3422, SN 3423, and SN 3492-exhibited the Rf4 gene, as confirmed through generation of the distinctive 172 bp DNA fragment employing the RMS-PRR9-1 marker approach. The pronounced disparity in occurrence frequencies between Rf3 (52.8%) and Rf4 (13.9%) carriers indicates probable differences in selective forces or genetic maintenance between these dual restoration mechanisms throughout rice improvement initiatives.

Remarkably, one accessions SN3422 were determined to possess both *Rf3* and *Rf4* restoration genes simultaneously, constituting exceptionally valuable genetic assets for hybrid rice advancement. These molecularly-authenticated restorer accessions, as documented in Table 3, furnish critical parental components for marker-assisted breeding approaches directed toward producing superior WA-CMS hybrid cultivars exhibiting dependable fertility restoration mechanisms.

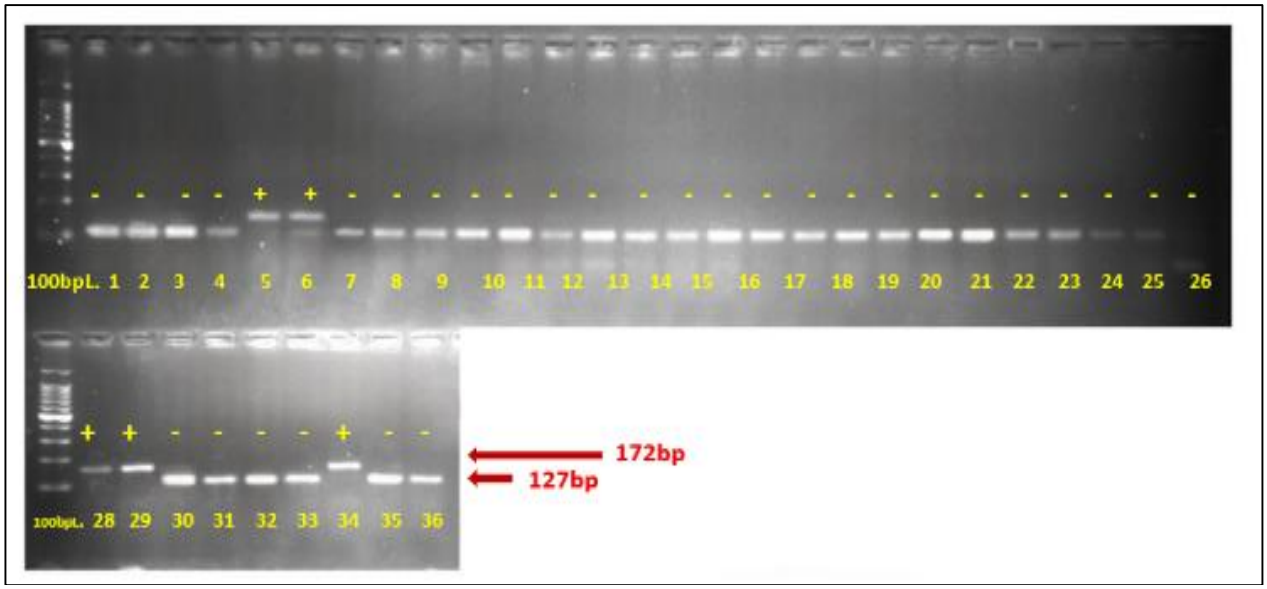

Fig 1: Genotypic profiling of Rf4 fertility restorer gene using RMS PRR9-1 functional marker

Table 3: These molecularly-authenticated restorer accessions, as documented

S. No	Genotype	Restorer gene	
		Rf3	*Rf4*
1	SN 2888	0	0
2	SN 3063	0	0
3	SN 3171	1	0
4	SN 3173	1	0
5	SN 3184	0	1
6	SN 3190	0	1
7	SN 3194	1	0
8	SN 3205	1	0
9	SN 3217	1	0
10	SN 3239	1	0
11	SN 3250	1	0
12	SN 3274	0	0
13	SN 3275	1	0
14	SN 3287	0	0
15	SN 3289	0	0
16	SN 3300	0	0
17	SN 3310	0	0
18	SN 3315	0	0
19	SN 3323	0	0
20	SN 3342	1	0
21	SN 3353	1	0
22	SN 3375	0	0
23	SN 3378	1	0
24	SN 3379	1	0
25	SN 3389	0	0
26	SN 3398	1	0
27	SN 3409	1	0
28	SN 3422	1	1
29	SN 3423	0	1
30	SN 3457	1	0
31	SN 3465	1	0
32	SN 3476	1	0
33	SN 3484	1	0
34	SN 3492	0	1
35	SN 3495	0	0
36	SN 3520	0	0

Restorer = 1, Non restorer = 0

4. Conclusion

This investigation effectively characterized 19 rice breeding genotypes harboring the *Rf3* genetic variant and five accessions possessing the *Rf4* element through molecular characterization utilizing RM-SF21-5 and RMS-PRR9-1 marker systems, respectively. Significantly, genotype SN3422 was determined to contain both *Rf3* and *Rf4* restoration elements simultaneously, establishing it as an exceptionally important genetic asset for hybrid rice development initiatives.

The deployment of these codominant marker technologies delivers a streamlined and dependable methodology for marker-assisted breeding, presenting numerous benefits compared to traditional cultivation approaches. Particularly, this molecular strategy facilitates preliminary screening of restorer accessions during early developmental phases, substantially minimizing temporal and resource investments relative to conventional test-crossing procedures. The marker systems exhibited exceptional precision, with the 172 bp amplification product consistently confirming active restorer genetic variants.These outcomes establish a robust molecular framework for expediting the advancement of superior hybrid rice cultivars through accurate detection of fertility restoration genes within breeding collections. The characterized restorer accessions, especially those harbouring dual Rf elements, constitute important genetic materials for improving hybrid rice performance and consistency.

Future Scope

The genetic characterization revealed 19 breeding accessions harbouring Rf3 and 5 genotypes possessing Rf4, supplying essential parental components for hybrid rice cultivation programs. These materials can be systematically hybridized with WA-CMS genotypes to generate superior hybrid combinations, subsequently undergoing thorough assessment of fertility restoration mechanisms (achieving 90%+ pollen viability and reproductive success), agricultural performance metrics (production capacity, environmental resilience), and kernel characteristics (processing efficiency, starch composition).The dual Rf3/Rf4 combination genotype SN3422 presents exceptional potential for establishing resilient restoration frameworks. Subsequent breeding initiatives should prioritize incorporating supplementary Rf elements and fertility-associated quantitative trait loci to construct polygenic restoration networks, thereby improving environmental stability and minimizing restoration failure risks.

This marker-guided methodology substantially expedites breeding timelines relative to traditional approaches, compressing assessment periods from 4-5 years to single seasonal cycles while preserving accuracy in restorer genotype development. The synthesis of molecular characterization with established breeding protocols will enable advancement of innovative hybrid rice cultivars featuring enhanced production consistency and expanded environmental adaptability.

Acknowledgment

The authors acknowledge Professor Jayashankar Telangana Agricultural University (PJTAU) for their generous financial assistance in support of the research work presented in this study.

Competing Interests

The authors hereby declare that no known competing financial interests or personal relationships exist that could have influenced the work reported in this study. This research was conducted objectively, and all results presented reflect an unbiased interpretation of the experimental data.

References

1. Alavi M, Ahamadikhan A, Kamkar B, Kalateh M. Mapping *Rf3* locus in rice by SSR and CAPS markers. International Journal of Genetics and Molecular Biology. 2009;7:121-126.
2. Huang JZ, E ZG, Zhang HL, Shu QY. Workable male sterility systems for hybrid rice: Genetics, biochemistry, molecular biology, and utilization. Rice. 2014;7:1-14.
3. IndiaStat. http://www.indiastat.com
4. Khush GS. Strategies for increasing the yield potential of cereals: case of rice as an example. Plant Breeding. 2013;132(5):433-436.
5. Pranathi K, Viraktamath BC, Neeraja CN, Balachandran SM, Rao PK, Revathi P, *et al.* Development and validation of candidate gene-specific

markers for the major fertility restorer genes, *Rf4* and *Rf3* in rice. Molecular Breeding. 2016;36(10):1-14.
6. Revathi P, Medoju P, Singh AK, Sundaram RM, Raju S, Senguttuve P, *et al.* Efficiency of molecular markers in identifying fertility restoration trait of WA-CMS system in rice. Indian Journal of Genetics and Plant Breeding. 2013;73(1):89-93.
7. Shih-Cheng L, Yuan LP. Hybrid rice breeding in China. In: Innovative approaches to rice breeding: Selected papers from the 1979 International Rice Research Conference. Los Baños (Philippines): International Rice Research Institute; 1980. p. 35-51.
8. Vaz Mondo VH, Nascente AS, Neves PDCF, Taillebois JE, Neto MOAC. Flowering synchronization in hybrid rice parental lines. Australian Journal of Crop Science. 2016;10(8):1213-1221.
9. Veeresha BA, Hanamaratti N, Salimath PM. Heterosis and combining ability studies for yield and productivity traits in rice. International Journal of Current Agricultural Research. 2015;4(5):120-126.
10. Virmani SS, Edwards IB. Current status and future prospects for breeding hybrid rice and wheat. Advances in Agronomy. 1983;36:145-214.
11. Yao FY, Yu SB, Gao YJ, Zhang Q. Mapping and genetic analysis of two fertility restorer loci in the wild abortive cytoplasmic male sterility system of rice (*Oryza sativa* L.). Euphytica. 1997;98:183-187.
12. Yuan LP. Hybrid rice breeding in China. In: Advances in Hybrid Rice Technology. Los Baños (Philippines): International Rice Research Institute; 1988. p. 27-33.
13. Zhang G, Bharaj TS, Lu Y, Virmani SS, Huang N. Mapping of the *Rf3* nuclear fertility-restoring gene for WA cytoplasmic male sterility in rice using RAPD and RFLP markers. Theoretical and Applied Genetics. 1997;94:27-33.