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DETECTION OF POLYMORPHIC MARKERS AND HYBRIDITY CONFIRMATION FOR THE CROSSES DERIVED BETWEEN ELITE CULTIVARS AND WILD INTROGRESSION LINES IN RICE

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ABSTRACT

The research investigated was undertaken to assess the hybrid authenticity of experimental F_1 plants derived from crosses between selected wild introgression lines and high-yielding rice cultivars popular in southern India. Crossing of wild introgression lines with elite cultivars includes cross combinations, viz. ADT-51 × C10-21 (DBS-21), C10-21 × Telangana rice-1289 (DBS-23), DRR Dhan 74 × Swarna (DBS-24), and Swarna × NPK-56 (DBS-30). Polymorphism survey using ten microsatellite markers between the parents of all the four crosses revealed that marker RM8004 was found to be polymorphic in all the four cross combinations, followed by RM17377 and RM163 exhibiting polymorphism in three crosses and two crosses respectively and marker RM171 was polymorphic in single cross, while markers RM312, RM3183, RM1, RM178 were monomorphic. Successful hybrid authentication was achieved by employing the most effective polymorphic markers specific to each cross combination, where marker RM17377 validated the hybrid nature of F_1 plant from DBS21 and DBS30 crosses through detection of paternal bands and heterozygous patterns, while marker RM163 served to authenticate F_1 plants from DBS23 and DBS24 crosses, collectively confirming true hybridity in 19 out of 20 tested F_1 individuals by demonstrating the presence of alleles from both parental sources.

***Key words*:** Polymorphic Markers, Rice (*Oryza sativa* L.), Hybridity

Introduction

Rice (*Oryza sativa* L.), a key member of the Poaceae family, stands as one of the most essential cereal crops globally, providing a staple food source for billions, particularly across Asia where nearly 90% of its production and consumption occur. As the population in these regions continues to grow rapidly, there is an urgent need to develop high-yielding, climate-resilient rice varieties to meet escalating food demands. In countries like India, achieving a significant increase in rice production by 2025 is critical to ensuring national food security (FAO, 2009). However, the widespread practice of crossing among elite lines of *Oryza sativa* in current breeding programs has led to a narrowed genetic base, limiting genetic variation and hampering the development of superior recombinants (Tanksley and McCouch, 1997), reduced variability has contributed to a plateau in yield improvement, highlighting the necessity of broadening the genetic pool through pre-breeding and strategic hybridization efforts.

Wild relatives of cultivated rice, particularly from the genus *Oryza*, offer a rich reservoir of genetic diversity and harbor valuable traits for crop improvement. Historic breeding successes, such as the transfer of grassy stunt virus resistance from *Oryza nivara* to cultivated rice (Khush *et al.*, 1977), have demonstrated the potential of wild species to enhance biotic and abiotic stress tolerance, as well as yield-related traits. To leverage these advantages, wild introgression lines are now increasingly used in hybridization programs. In the context of breeding, assessing polymorphism is crucial for identifying true hybrids and confirming genetic diversity. It also aids in

Table 1: Parental polymorphism analysis using SSR Markers in Rice crosses.

CROSS	Female	Male	Polymorphic Markers
DBS-21	ADT-51	C10-21	RM17377, RM171, RM8004
DBS-23	C10-21	Telangana rice-1289	RM163, RM17377, RM8004
DBS-24	DRR Dhan 74	Swarna	RM163, RM8004
DBS-30	Swarna	NPK-56	RM17377, RM8004

understanding trait inheritance and selecting desirable traits for crop improvement. (Pranay *et al.*, 2024) The conventional method of identification of true F_1 s based on morphological traits was quite successful, but is time-consuming as involves a complete crop season for phenotypic comparison of progeny with the parents, also influenced by the environment and based on existence of observable morphological variations among the parents. Molecular markers, especially microsatellites or Simple Sequence Repeats (SSRs), have revolutionized this process by offering precise, environment-independent tools for genotypic characterization even at seedling stage. SSRs are particularly valuable due to their co-dominant nature, genome-wide distribution, cost-effectiveness, and reproducibility (Mason 2015). In this study, F_1 plants were developed by crossing high-yielding varieties with wild introgression lines, and their hybridity was evaluated using SSR markers. This molecular approach ensures accurate identification of true hybrids, facilitating efficient advancement to segregating generations and supporting the development of improved rice varieties.

Materials and Methods

Plant Material and Cross Development

The experimental material comprised six genetically diverse parental lines sourced from various rice research institutions across India. High-yielding regional cultivars included Swarna (Agricultural Research Station, Maruteru, Andhra Pradesh), ADT-51 (Tamil Nadu Rice Research Institute, Aduthurai), and Telangana rice-1289 (Regional Agricultural Research Station, Warangal), while wild introgression lines C10-21, NPK-56, and DRR Dhan 74 were obtained from the Indian Institute of Rice Research (IIRR), Hyderabad.

Controlled hybridization was performed during the *Kharif* season of 2024 at the experimental fields of ICAR-Indian Institute of Rice Research, Rajendranagar. Four distinct cross combinations were established through emasculation and hand pollination techniques: DBS-21 (ADT-51 × C10-21), DBS-23 (C10-21 × Telangana rice-1289), DBS-24 (DRR Dhan 74 × Swarna), and DBS-30 (Swarna × NPK-56) and presented in Table 1. These crosses yielded a total of 20 F_1 plants distributed as follows: one plant from DBS-21, ten from DBS-23, six from DBS-24, and two from DBS-30. The resulting F_1s along with their respective parents were subsequently cultivated during the *Rabi* season of 2024-25.

DNA Extraction and Molecular Analysis

Genomic DNA was extracted from fresh leaf tissues of four-week-old F_1 plants and parental lines using the standard Cetyl Trimethyl Ammonium Bromide method as described by Doyle and Doyle *et al.*, 1987. Approximately 0.1 g of young, tender leaf material was ground using a mortar and pestle with CTAB extraction buffer containing 4% CTAB, 100 mM Tris-HCl, 20 mM EDTA, and 1.4 M NaCl. The extracted DNA was quantified and diluted to a working concentration of 50 ng/ìL in TE buffer for subsequent PCR analysis.

PCR Amplification and Electrophoresis

Molecular characterization was performed using ten microsatellite markers covering six rice chromosomes, with primer information retrieved from the Gramene database (www.gramene.org). PCR reactions were conducted in a 25 µL volume containing 1X Taq buffer, 2 mM $MgCl_2$, 0.1 mM dNTP mix, 10 pM each of forward and reverse primers, 0.25 U Taq polymerase, and 50 ng/

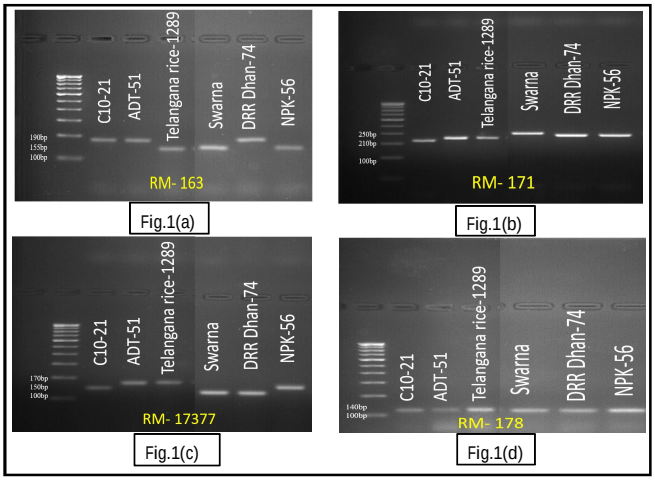

Fig. 1: Polymorphic survey among parental lines using SSR Markers. **(a)** RM163 showed polymorphism between C10-21 and Telangana rice -1289 (DBS23), Swarna showed polymorphism with DRR Dhan-74 (DBS24); **(b)** RM171 showed polymorphism between C10-21 and ADT-51(DBS-21) & C10-21 and Telanganarice-1289 (DBS-23) respectively, Swarna showed polymorphism with DRR Dhan-74 in cross DBS-24; **(c)** RM17377 showed polymorphism of C10-21 with ADT-51 (DBS21) and Telanganrice-1289(DBS23), Swarna showed polymorphism with NPK-56 (DBS30); **(d)** RM178 showed monomorphic bands in all the parental lines.

Table 2: Details of the microsatellite markers used in the present investigation.

S.No	Marker	Sequence	Chr. No.
1.	RM163	F:CGCCTTTATGAGGAGGAGATGG; R:AAACTCTTCGACACGCCTTGC	5
2.	RM1	F:GCGAAAACACAATGCAAAAA; R:GCGTTGGTTGGACCTGAC	1
3.	RM9	F:GGCCCTCATCACCTTCGTAGC; R:CGTCCTCCCTCTCCCTATCTCC	1
4.	RM171	F:CGATCCATTCCTGCTGCTCGCG ; R:CGCCCCCATGCATGAGAAGACG	10
5.	RM8004	F:TTGACCAAAGGTGATTGTAAT; R:CTTGATGAGTTTCATGAGCA	1
6.	RM312	F:GTATGCATATTTGATAAGAG; R:AAGTCACCGAGTTTACCTTC	1
7.	RM17377	F:ATATTACTTCGACGCTGGATCAGG; R:GTCAGTTCGTCAGGCACAACG	4
8.	RM3183	F:GCTCCACAGAAAAGCAAAGC; R:TGCAACAGTAGCTGTAGCCG	6
9.	RM552	F:CGCAGTTGTGGATTTCAGTG; R:TGCTCAACGTTTGACTGTCC	11
10.	RM178	F: TCGCGTGAAAGATAAGCGGCGC; R: GATCACCGTTCCCTCCGCCTGC	5

ìL template DNA. Amplification was performed using a Biorad thermal cycler with the following program: initial denaturation at 94°C for 7 minutes, followed by 35 cycles (94°C for 30 seconds), annealing (55°C, 30 seconds), and extension (72°C, 60 seconds), with a final extension step at 72°C for 10 minutes. The amplified products were separated on 2.5% agarose gels prepared in 1X TAE buffer and electrophoresed at 150 V for one hour. Visualization was achieved through ethidium bromide staining, and gel images were documented using a gel documentation system (Vilber- BIO-PRINT cx4 Edge-Fixed pad Container, France).

Results and Discussion

The crossing among the selected parents was accomplished with a future objective of developing high-yielding rice varieties with enhanced tolerance to abiotic and biotic stresses and improved climate resilience. A comprehensive molecular analysis was conducted using ten SSR markers distributed across different chromosomes. Four of the ten SSR markers (RM312, RM1, RM9 and RM8004) are from chromosome 1, and the two markers RM163, RM178 from chromosome 5 and remaining five markers RM17377, RM3183, RM171, RM552 are from chromosomes 4, 6, 10 and 11 respectively. The markers demonstrating male and female parent-specific bands and which are showing clear polymorphism between them were utilized to assess the hybridity of the derived experimental F_1 hybrids. Parental polymorphism among parental lines using SSR Markers are illustrated in Fig. 1. Among the microsatellite markers used for parental polymorphism, RM8004, RM17377, RM163 and RM171 were found polymorphism in most of the crosses, while remaining six markers were found to be monomorphic. Of the polymorphic markers, RM8004 proved most informative, showing polymorphism in all four cross combinations, followed by RM17377 and RM163 exhibiting polymorphism in three crosses and two crosses respectively and marker RM171 was polymorphic in single cross. In contrast, the other markers RM312, RM3183, RM1, RM178 were monomorphic. The microsatellite profiles of the parental combinations and their experimental F_1s are illustrated in Fig. 2. In the F_1s of cross DBS-21, marker RM17377 revealed a male-specific allele at 150 bp, indicating successful inheritance

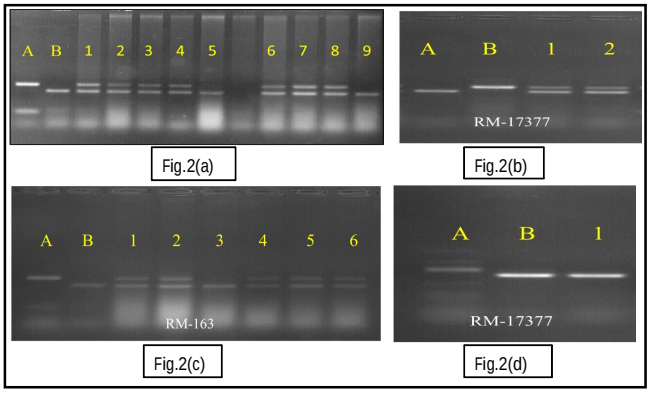

Fig. 2: Gel images of F_1s Confirmation. **(a)** DBS23 Cross A=C10-21; B=Telangana rice-1289; 1, 2, 3, 4, 5, 6, 7, 8, 9 are true F_1s; **(b)** DBS30 Cross A= Swarna; B= NPK-56; 1, 2 are true F_1s; **(c)** DBS24 Cross A= DRR Dhan 74; B = Swarna; 1, 2, 3, 4, 5, 6 are true F_1s; **(d)** DBS21 Cross A = ADT-51; B = C10-21; 1 is true F_1

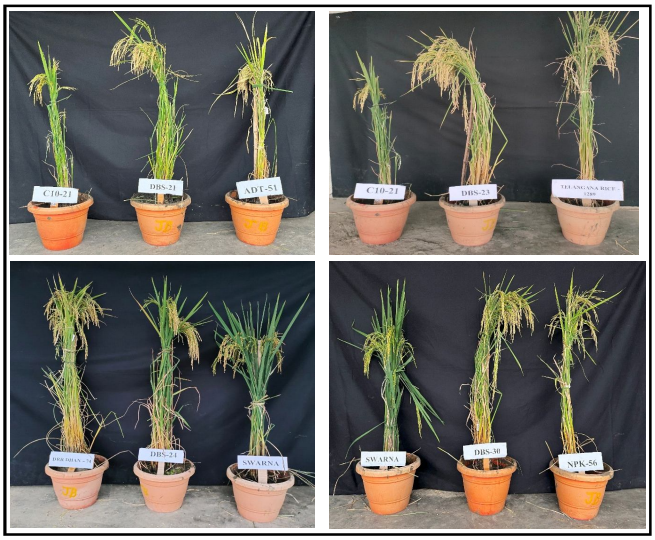

Fig. 3: Morphological Comparison of F_1 Hybrids with Parental Lines Across All Cross Combinations.

of the male allele. In cross DBS-23, seven of the F_1 individuals displayed heterozygosity for marker RM163 (alleles at 155 bp and 190 bp), while two F_1s exhibited only the male-specific band. Similarly, F_1s from cross DBS-24 showed heterozygosity at RM163 (alleles at 150 bp and 170 bp) in five individuals, whereas one F_1 plant displayed only the male allele. In Cross DBS-30, heterozygosity for marker RM17377 (alleles at 155 bp and 190 bp) was observed in two F_1s.

The presence of alleles from both parental lines in the F_1 microsatellite profiles confirmed heterozygosity at specific loci, thereby validating the hybridity of the F_1 plants. Similar findings have been reported in earlier studies on rice by (Manohara *et al*., 2020, Ranjitha *et al*., 2016, Gimhani *et al*., 2014, Tamilkumar *et al*., 2009, and Sundaram *et al*., 2007). Furthermore, SSR markers have proven effective for hybridity testing in other crops such as pigeon pea (Bohra *et al*., 2015), maize (Sudharani *et al*., 2014), sunflower (Pallavi *et al*., 2011), and lentils (Solanki *et al*., 2010). After confirming the hybrid plants using molecular techniques, the F_1 plants were also checked morphologically to verify they were true hybrids. The visual observations showed that the F_1 plants displayed similarities with both parent plants, exhibiting intermediate traits that indicated successful cross-breeding as showed in Fig. 3. The morphological features of the F_1 plants, particularly their plant height, tillers and panicles provided additional evidence supporting their hybrid status. This combination of molecular and morphological confirmation methods ensured that the F_1 plants were true hybrids rather than self-pollinated offspring from either parent variety.

In conclusion, this study underscores the effectiveness of microsatellite markers for rapid and precise detection of hybridity in experimental F_1 rice lines. This approach helps eliminate selection of selfed plants for advancement, thereby reducing the risk of segregation distortion in the succeeding generations. Notably, markers such as RM8004 and RM17377, which demonstrated high polymorphism across multiple crosses, show strong potential for future use in hybridity assessments, thereby minimizing the need for screening new random markers. The true F_1s were further selfed and were further advanced as F_2 population and raised during *Kharif* 2025 for the development mapping populations and climate resilient rice varieties.

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