Plant Archives Vol. 26, No. 1, 2026 pp. 1364-1370 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2026.v26.no.1.185

VALIDATION OF SMD RESISTANCE SSR MARKERS FOR HYBRIDITY CONFIRMATION IN PIGEONPEA (*CAJANUS CAJAN*)

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(Date of Receiving : 05-01-2026; Date of Revision : 26-02-2026; Date of Acceptance : 11-03-2026)

ABSTRACT

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is a vital grain legume contributing to food and nutritional security in the semi-arid tropics, but its productivity is severely constrained by sterility mosaic disease (SMD). Development of resistant hybrids requires reliable confirmation of true hybridity, which is often difficult using morphological traits alone. The present investigation aimed to develop pigeonpea hybrids using SMD-resistant and susceptible parents and to validate F_1 hybridity through simple sequence repeat (SSR) markers. Two crosses, Rajeshwari × BSMR-243 and ICP-8863 × BSMR-571, were generated during *Kharif* 2023 and evaluated in *Kharif* 2024. A total of 15 SSR primers associated with SMD resistance were screened, of which 12 were amplified and four (AHSSR-20, AHSSR-34, CCttc018 and PKS-31) were polymorphic, revealing 33.33% polymorphism with allele sizes ranging from 120 to 248 bp. These polymorphic markers were employed for molecular screening of F_1 populations using PCR and agarose gel electrophoresis. In Cross I, out of 39 F_1 plants, 10 were confirmed as true hybrids, while in Cross II, 10 out of 34 F_1 plants were validated based on the presence of heterozygous and male parent-specific alleles. SSR markers effectively distinguished parental genotypes and enabled precise identification of hybridity at the seedling stage. The study demonstrates that SSR marker-based screening is a robust, efficient and reliable approach for confirming hybridity and facilitating early selection of SMD-resistant genotypes in pigeonpea breeding programmes, thereby ensuring genetic purity and accelerating varietal development.

***Keywords*:** Pigeonpea, *Cajanus cajan,* Sterility mosaic disease, SSR markers, Hybridity confirmation, Molecular screening, PCR.

Introduction

Pigeonpea *[Cajanus cajan (L.) Millsp.]* is an important grain legume crop of the semi-arid tropics and plays a crucial role in food and nutritional security in India and other tropical regions. It belongs to the sub-tribe Cajaninae, having a diploid genome with eleven pairs of chromosomes ($2n = 2x = 22$) and genome size around 833.07 Mbp (Greilhuber and Obermayer, 1998, Varshney *et al*., 2011). It serves as a major source of dietary protein for the predominantly vegetarian population and contributes to soil fertility through biological nitrogen fixation, thereby supporting sustainable agricultural systems (Saxena *et al*., 2010). With the growing demand for pulses and constraints on area expansion, genetic improvement of pigeonpea for higher yield and disease resistance remains a major breeding objective.

Among the various biotic stresses affecting pigeonpea, sterility mosaic disease (SMD) caused by pigeonpea sterility mosaic virus and transmitted by the eriophyid mite *Aceria cajani* is considered the most destructive, often causing yield losses up to 90–95 per cent under severe infection (Reddy and Nene, 1981; Gnanesh *et al*., 2011). The disease leads to profuse vegetative growth, mosaic mottling and complete or partial failure of flowering, thereby severely affecting productivity. Deployment of resistant cultivars is the most economical and eco-friendly approach for managing SMD.

Hybridization through conventional breeding is widely used in pigeonpea to combine desirable traits such as high yield potential and disease resistance. Crossing resistant and susceptible parents helps in understanding inheritance and developing superior hybrids with enhanced tolerance to SMD. However, confirmation of true hybridity and identification of resistance-associated genotypes using only morphological traits is difficult, time-consuming and often influenced by environmental factors.

Molecular marker technology provides a precise and reliable approach for genetic analysis in crop improvement. Simple sequence repeat (SSR) markers are co-dominant, highly polymorphic and reproducible, making them suitable for distinguishing closely related genotypes (Varshney *et al*., 2005). SSR markers enable detection of allelic variation between resistant and susceptible parents and allow confirmation of hybridity through the presence of parental alleles in F_1 progenies. Marker-based screening also facilitates early selection of resistant genotypes without exposure to the pathogen.

In pigeonpea, the use of SSR markers for disease resistance studies and genetic purity testing has gained importance (Odeny *et al*., 2007; Saxena *et al*., 2009). Identification of markers differentiating resistant and susceptible genotypes for SMD can accelerate breeding programmes by integrating molecular information with conventional selection.

Therefore, the present study was undertaken to develop pigeonpea hybrids through conventional breeding using SMD-resistant and susceptible parents and to employ SSR markers for molecular screening to distinguish resistant and susceptible genotypes as well as to confirm hybridity. This approach aims to enhance selection efficiency and support the development of improved pigeonpea genotypes with better resistance to sterility mosaic disease.

Materials and Methods

Plant materials

For the present investigation four parents *viz*., Rajeshwari (Sterility Mosaic Disease Susceptible), BSMR-243 (Sterility Mosaic Disease Resistant), ICP-8863 (Maruti) (National Susceptible check for Sterility Mosaic Disease) and BSMR-571 (Sterility Mosaic Disease Resistant) were selected. All the F_1 seeds of both cross (Rajeshwari × BSMR-243 and ICP-8863 × BSMR-571) along with their parents were sown at PGI farm, MPKV, Rahuri during *Kharif* 2024-25. Leaf samples of parents and F_1s were used for DNA isolation.

Extraction and purification of genomic DNA

DNA was extracted from individual plants of parents and F_1s of both the crosses. Succulent leaves were collected from the field, sterilized with 70 percent alcohol, and frozen in liquid nitrogen. The leaf samples were stored at – 80^0C until further use. The genomic DNA was extracted following the standard CTAB method with minor modifications.

PCR Amplification

In the present investigation, a set of fifteen SSR primer pairs were used to confirm F_1 hybrids for SMD resistance. The primers were selected based on previous studies on SMD resistance in Pigeonpea (Patil *et al*., 2016, Saxena, *et al*., 2009). The sequence of primers was compiled from the literature and synthesized by Custom Oligo Synthesis Division, Merck Specialties Pvt. Ltd., Bangalore. The details of the SSR primers used in the present investigation are given in Table 1.

The Polymerase chain reaction (PCR) reaction was carried in a 0.2 ml sterile thin-wall PCR tube, and the following components were mixed as each tube (20 µl) consisting 1.5 µl 50 ng/µl DNA template, 2 pmol of primer, 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM (2.5mM each) dNTPs mix, 0.5 µl of Genei Taq DNA polymerase 5U/µl and 13.5 µl sterilized distilled water. The PCR reaction was performed in a Korbett Research master cycler in a 96 well plate. Temperature cycling was done by the "Touchdown" method (Mellersh and Sampson, 1993). In touch-down PCR, the amplification of the non-specific sequences can be avoided by adapting high annealing temperature during the earliest steps of a touchdown polymerase chain reaction cycle. Therefore, a touchdown PCR profile with 4 min initial denaturation cycle followed by first five cycles of 94^0C for 30 secs, 60^0C for 30 secs, 72^0C for 30 secs with 1^0C decrease in annealing temperature depends upon Tm of primer for 30 sec and 72^0C for 30 sec followed by final extension for 20 min at 72^0C were adopted.

Table 1 : List of Genic SSR primers used for confirmation of F_1 hybrid.

Sr. No.	SSR marker	Primer	Sequence(5'-3')	Ta (^{0}C)
	Earlier reported by Patil *et al.* (2016)			
1	AHSSR 20	F	AATGTTCTATTGTTTTACGAGTG	55
		R	AATTTCTCGTGTGATTGTGAT	
2	AHSSR 34	F	TCTTGAGTAAGTGAACATTCAAA	54
		R	GGTGAAACTCAACTCAACACT	
3	AHSSR 50	F	AGTTTTTGTGTTTTCAACCTG	55
		R	GAGCAAATAATCATTCAAACAC	
	Earlier reported by Saxena, *et al.* (2009)			
4	CCac012	F	ACCTTGCTTGTTTCGCTTTT	59
		R	AAGGGAGGTGGACTACAAGGA	
5	CCac013	F	GTGAGTGAGAGTGAGTGTATTTGTG	56
		R	GCTCTGATGCCAAATGTTGA	
6	CCat011	F	TGCTCTAATGGCTAGTTCATCC	59
		R	AAACACTCATGGGTTAGATTCTCC	
7	CCB1	F	AAGGGTTGTATCTCCGCGTG	56
		R	GCAAAGCAGCAATCATTTCG	

8	CCB4	F	GGAGCTATGTTGGAGGATGA	56
		R	CCTTTTTGCATGGGTTGTAT	
9	CCtc004	F	GGAAAACCCCGAGACAAAAG	48
		R	GGGCAACCCATAAACCCTAA	
10	CCtc012	F	GAGGATTGCACCAAGCAACT	48
		R	GCACTGCTGGCCTTACCATA	
11	CCttc003	F	ACACCACCATGCTAAAGAACAAG	56
		R	CCAAGCAAGACACGAGTAATCATA	
12	CCttc006	F	GTAGAGGAGGTTCCAAATGACATA	56
		R	ATCTGTCTGGTGTTTTAGTGTGCT	
13	CCttc007	F	CTCTTGCTTACGCGTGGACT	48
		R	CTTTTGCTTTTGCGTGCTT	
14	CCttc018	F	ATGGGCATGGTAGAGGAGGT	48
		R	CGCTCATCATCGTCATCAAA	
15	PKS31	F	CCAATCCTGGGCAGTTTCT	56
		R	GCGGGCTTCATGACAACTT	

Agarose gel electrophoresis

The amplified PCR products were checked on 1.2% agarose gel. The amplified product was fractionated using capillary electrophoresis. Allele sizing of electrophoretic data was obtained from the UV trans-illuminator/ Gel documentation unit (KODAK Molecular Imaging Software with a TWAIN Device).

Results and Discussion

Reliable discrimination of hybrids and parental lines can be achieved using DNA-based markers through a process known as DNA fingerprinting, which offers greater precision than conventional approaches. Morphological markers in pigeonpea are often influenced by environmental conditions and require extensive time and labour for evaluation. Biochemical markers such as isozymes and protein profiles, though relatively stable, generally exhibit low polymorphism and fail to distinguish closely related genotypes. DNA markers overcome these limitations and allow clear identification of hybrids, parental lines and off-types at the genomic level.

Simple sequence repeat (SSR) markers are particularly suitable for genetic analysis in pigeonpea because they are co-dominant, highly polymorphic and reproducible. SSRs enable detection of allelic variation between sterility mosaic disease (SMD) resistant and susceptible parents and facilitate confirmation of hybridity through the presence of both parental alleles in F_1 plants. Their high polymorphic information content makes SSRs effective tools for molecular fingerprinting and early screening in pigeonpea breeding programmes.

Molecular analysis

Agarose gel electrophoresis showed 33.33 percent polymorphism in the DNA banding pattern. Out of 15 SSR primers, only 12 were amplified, and four primers showed polymorphism while the remaining 8 were monomorphic. Total number of polymorphic alleles were 5 and size of the amplified product ranged from 120 - 248 bp (Table 2).

Table 2 : Details of the SSR primers used for amplification of genomic DNA of pigeonpea.

Sr. No.	Particulars	Observation
1.	Total number of primers used	15
2.	Number of primers amplified DNA	12
3.	Total number of polymorphic markers	4
4.	Percentage of polymorphic markers	33.33%
5.	Total number of alleles	13
6.	Total number of polymorphic alleles	5
7.	Total number of monomorphic alleles	8
8.	Average number of alleles	1.08
9.	Size of amplified product range	120 - 248 bp

Confirmation of F_1s through Molecular Markers for Hybridity Test

Cross I: Rajeshwari × BSMR-243

For Cross I, BSMR-243 (male parent) was crossed with Rajeshwari (female parent) during *Kharif* 2023 and 39 F_1 seeds were obtained. These 39 F_1s were sown during *Kharif* 2024, and all germinated successfully under field conditions. Out of 15 SSR primers tested, 12 showed amplification, of which four primers namely AHSSR-20, AHSSR-34, CCttc018 and PKS-31 exhibited polymorphism, while the remaining primers were monomorphic.

The F_1 plants were tested for hybridity using these four polymorphic markers. These markers were polymorphic between the parental genotypes Rajeshwari and BSMR-243 and were earlier reported to be associated with sterility mosaic disease by Patil *et al*. (2016) and Saxena *et al*. (2009). Different numbers of plants showed heterozygosity with these markers (Table 3). Based on the resolution of gel images, 10 plants were confirmed as heterozygous across the four markers and were considered true F_1s. Only these confirmed F_1 plants were used for selfing, and the selfed seeds were utilized for the development of F_2 populations.

Molecular confirmation of F_1 plants derived from the cross Rajeshwari × BSMR-243 during Kharif *2024* was carried out using SSR markers AHSSR-20, AHSSR-34, CCttc018 and PKS-31 (Table 3). The female parent Rajeshwari showed an allele size of 168 bp for AHSSR-20 and absence of amplification for the remaining markers, whereas the male parent BSMR-243 exhibited distinct alleles of 172 bp, 188 bp, 248 bp and 191 bp, respectively (Plate 1,2,3and 4).

The F_1 plants were screened for heterozygosity by comparing banding patterns with both parents. The presence of heterozygous (±) or male parent-specific (P) alleles confirmed hybridity, whereas plants showing only recurrent parent alleles or absence of donor alleles were considered doubtful. Most of the analyzed F_1s exhibited the expected heterozygous or donor-specific bands across the markers, confirming successful hybridization between Rajeshwari and BSMR-243. Similar results were also reported by Kandarkar *et al.* (2023).

Table 3: List of number of confirmed F_1 plants identified from the cross Rajeshwari x BSMR- 243 in *Kharif* 2024.

Parents/F_1	**Markers used for F_1 confirmation**			
	AHSSR 20	**AHSSR 34**	**CCttc 018**	**PKS 31**
Rajeshwari (♀)	**168 bp (+)**	**Absent (A)**	**Absent (A)**	**Absent (A)**
BSMR- 243 (♂)	**172 bp (-)**	**188bp (P)**	**248bp (P)**	**191bp (P)**
1	+	P	P	P
2	±	P	P	P
3	±	P	P	P
4	+	P	P	P
5	±	P	P	P
6	+	P	P	P
7	±	P	P	P
8	+	P	P	P
9	+	A	P	P
10	±	A	P	P
11	+	A	P	P
12	+	P	P	P
13	±	A	P	P
14	+	P	P	P
15	+	P	P	P
16	±	A	P	P
17	+	P	P	P
18	±	P	P	P
19	±	A	P	P
20	+	P	P	P
21	±	P	P	P
22	±	P	P	P
23	+	P	P	P
24	+	P	P	P
25	+	P	P	P
26	±	P	A	P
27	±	P	P	P
28	±	P	P	P
29	+	P	P	P
30	+	P	P	P
31	±	P	P	P
32	+	P	P	P
33	+	P	P	P
34	+	P	P	P
35	+	P	P	P
36	+	P	P	P
37	+	P	P	P
38	+	P	P	P
39	+	A	P	P

± Heterozygous, + same as that of recurrent parent, - same as that of donor parent
A→ Absent, P → Present

Cross II: ICP-8863 × BSMR-571

Similarly, for Cross II, BSMR-571 (male parent) was crossed with ICP-8863 (female parent) during *Kharif* 2023 and 34 F_1 seeds were generated. These 34 F_1s were sown during *Kharif* 2024, and all germinated successfully in the field. The F_1 plants were tested for hybridity using the four SSR markers AHSSR-20, AHSSR-34, CCttc018 and PKS-31, which were polymorphic between the parental genotypes ICP-8863 and BSMR-571. These markers were earlier reported to be associated with sterility mosaic disease by Patil *et al*. (2016) and Saxena *et al.* (2009).

Different numbers of plants showed heterozygosity with these markers (Table 4). Based on gel image resolution, 10 plants were identified as heterozygous across the four markers and confirmed as true F_1s. Only these confirmed F_1 plants were used for selfing, and the selfed seeds were used for the development of F_2 populations.

In this cross, the female parent ICP-8863 did not show amplification for any of the primers (AHSSR-20, AHSSR-34, CCttc018 and PKS-31). However, the male parent BSMR-571 showed bands of different sizes for the polymorphic primers, namely 172bp in AHSSR-20, 188bp in AHSSR-34, 220bp in CCttc018 and 191bp in PKS-31, as depicted in Plates 5, 6, 7 and 8 (Table 4). The presence of these male parent-specific alleles in F_1 plants confirmed their hybridity.

Similarly, For the cross II, BSMR-571 (male parent) was crossed with ICP-8863 (female parent) in *Kharif* 2023 and 34 F_1 seeds were generated. 34 F_1s were sown in *Kharif* 2024 out of which all 34 germinated in field. The F_1 plants were tested for true hybrid using four markers i.e., AHSSR 20, AHSSR 34, Ccttc018 and PKS 31. These markers were found polymorphic between parental genotypes ICP-8863 and BSMR-571. These four markers were earlier reported to be associated with sterility mosaic disease by Patil *et al.* (2016) and Saxena *et al.* (2009). Different numbers of plants were found heterozygous using these four markers (Table 4). Based on the resolution of the gel images 10 plants were found to be heterozygous for these four markers. Only the heterozygous plants were used for selfing. The selfed seed of true F$_1$s from the cross were used to develop F$_2$ plants.

Table 4: List of number of confirmed F$_1$ plants identified from the cross ICP-8863 x BSMR- 571 in *Kharif* 2024.

Parents / F$_1$	Markers used for F$_1$ confirmation			
	AHSSR 20	AHSSR 34	CCttc018	PKS 31
ICP-8863 (♀)	Absent (A)	Absent (A)	Absent(A)	Absent(A)
BSMR- 571 (♂)	172 bp (P)	188bp (P)	220bp (P)	191bp (P)
1	P	A	P	P
2	A	P	P	P
3	P	P	P	P
4	P	P	P	P
5	P	P	P	P
6	P	P	P	P
7	A	A	P	P
8	P	A	P	P
9	P	A	P	P
10	P	P	P	P
11	P	P	P	P
12	P	P	P	P
13	P	P	P	P
14	A	A	P	P
15	P	P	P	P
16	A	A	P	A
17	P	A	P	A
18	A	P	P	A
19	A	A	P	A
20	A	P	P	P
21	A	P	P	P
22	P	A	P	A
23	A	P	P	P
24	P	P	P	A
25	A	P	P	P
26	P	P	P	A
27	A	P	P	A
28	P	A	A	A
29	P	P	P	P
30	A	P	P	P
31	A	P	P	P
32	A	P	P	P
33	A	P	P	P
34	A	P	P	P

A→ Absent, P → Present

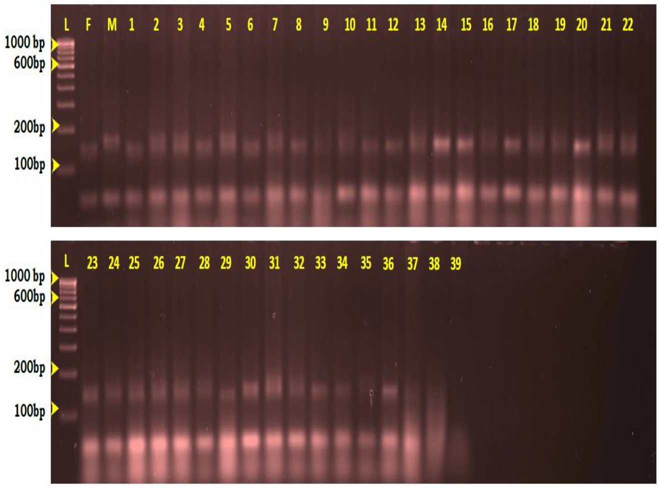
Conclusion

The present investigation demonstrated the effectiveness of DNA-based SSR markers for reliable confirmation of hybridity in pigeonpea. Unlike morphological and biochemical markers, which are often influenced by environmental conditions and exhibit limited polymorphism, SSR markers provided precise, reproducible and co-dominant detection of allelic variation at the genomic level. This enabled accurate discrimination between parental lines and true hybrids at early growth stages, thereby saving time and labour in breeding programmes.

Molecular analysis revealed 33.33 per cent polymorphism among the SSR primers tested. Out of 15 primers, 12 were successfully amplified and four markers (AHSSR-20, AHSSR-34, CCttc018 and PKS-31) were polymorphic with amplified product sizes ranging from 120 to 248 bp. These polymorphic SSRs proved highly informative for detecting parental alleles and confirming heterozygosity in F$_1$ populations. The association of these markers with sterility mosaic disease resistance further enhances their utility in pigeonpea improvement programmes.

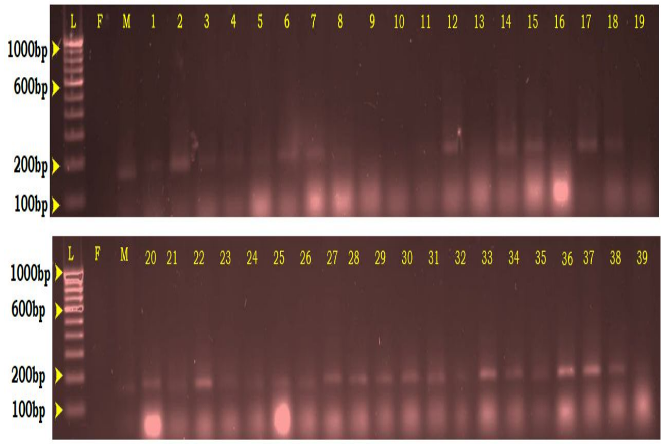
In Cross I (Rajeshwari × BSMR-243), molecular screening of 39 F$_1$ plants identified 10 true hybrids based on the presence of heterozygous and male parent-specific alleles. Similarly, in Cross II (ICP-8863 × BSMR-571), 10 out of 34 F$_1$ plants were confirmed as true hybrids using the same set of SSR markers. The presence of donor-specific bands in the F$_1$ plants clearly validated successful hybridization in both crosses. Only confirmed F$_1$s were advanced for selfing and development of F$_2$ populations, ensuring genetic purity in subsequent generations.

Overall, the study confirms that SSR marker-based hybridity testing is a robust, efficient and reliable approach for pigeonpea breeding. The use of polymorphic SSR markers facilitates early and accurate identification of true hybrids, improves selection efficiency and strengthens breeding strategies aimed at developing sterility mosaic disease-resistant and high-yielding pigeonpea varieties. This molecular approach can be effectively integrated into routine pigeonpea improvement programmes for maintaining genetic purity and accelerating varietal development.



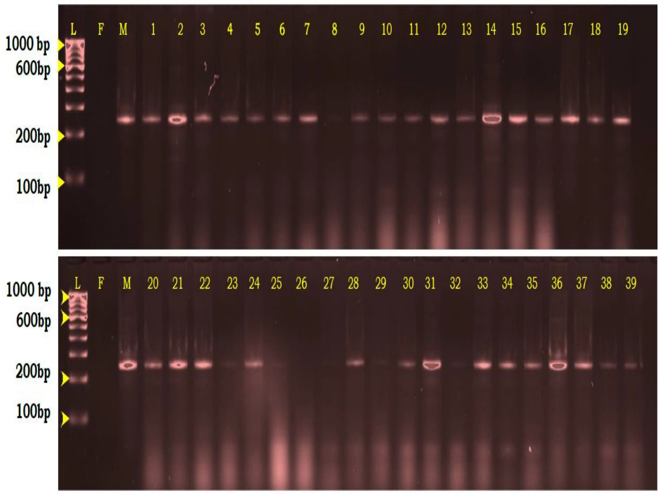
L- 100bp Ladder F- Rajeshwari M- BSMR-243 F1 Plants- 1-39

Plate 1: Screening of F1 in Cross Rajeshwari x BSMR-243 for confirmation of true hybrids by using SSR marker AHSSR 20 during *Kharif* 2024



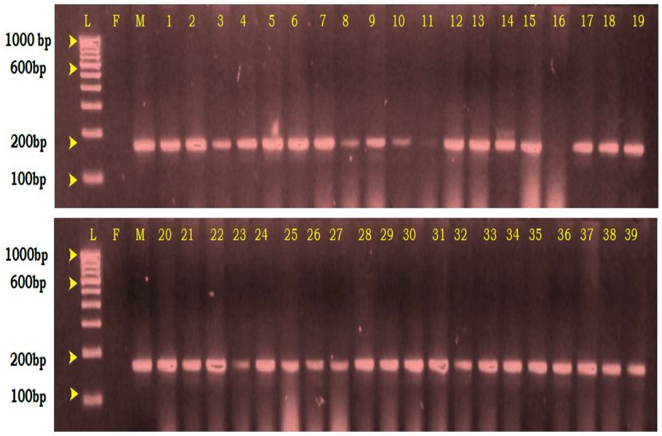
L- 100bp Ladder F- Rajeshwari M- BSMR-243 F1 Plants- 1- 39

Plate 2: Screening of F1 in Cross Rajeshwari x BSMR-243 for confirmation of true hybrids by using SSR marker AHSSR 34 during *Kharif* 2024



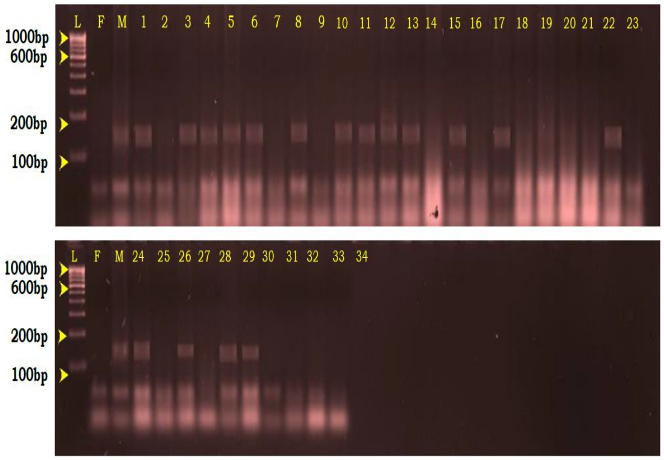
L- 100bp Ladder F- Rajeshwari M- BSMR-243 F1 Plants- 1- 39

Plate 3: Screening of F1 in Cross Rajeshwari x BSMR-243 for confirmation of true hybrids by using SSR marker Ccttcc018 during *Kharif* 2024



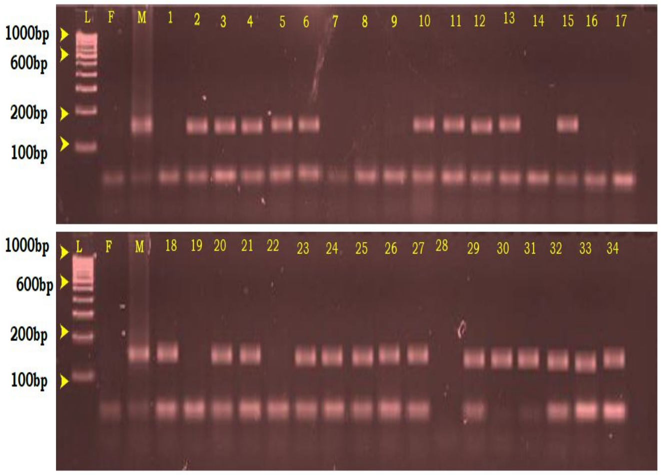
L- 100bp Ladder F- Rajeshwari M- BSMR-243 F1 Plants- 1- 39

Plate 4: Screening of F1 in Cross Rajeshwari x BSMR-243 for confirmation of true hybrids by using SSR marker PKS 31 during *Kharif* 2024



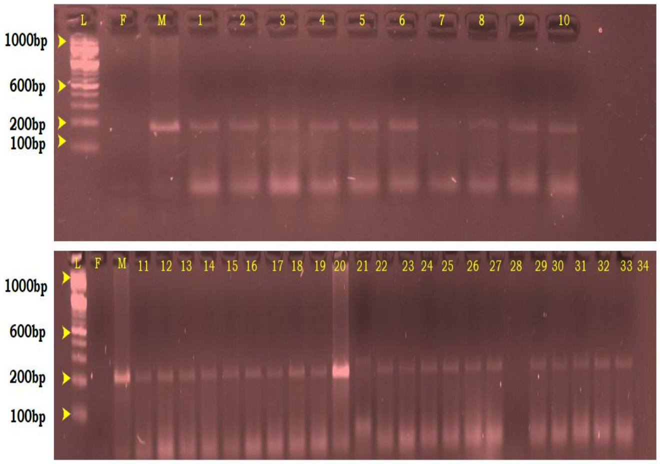
L- 100bp Ladder F- ICP 8863 (Maruti) M- BSMR-571 F1 Plants- 1-34

Plate 5: Screening of F1 in Cross ICP-8863 x BSMR-571 for confirmation of true hybrids by using SSR marker AHSSR 20 during *Kharif* 2024



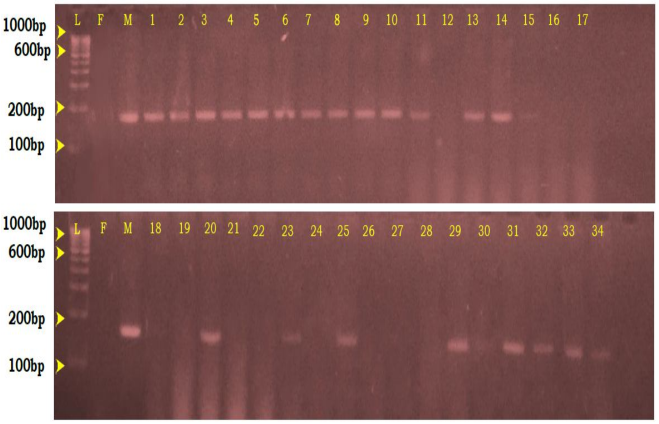
L - 100bp Ladder F- ICP 8863 (Maruti) M- BSMR-571 F1 Plants- 1-34

Plate 6: Screening of F1 in Cross ICP-8863 x BSMR-571 for confirmation of true hybrids by using SSR marker AHSSR 34 during *Kharif* 2024.



L - 100bp Ladder F- ICP 8863 (Maruti) M- BSMR-571 F1 Plants- 1-34

Plate7: Screening of F1 in Cross ICP-8863 x BSMR-571 for confirmation of true hybrids by using SSR marker Ccttcc018 during *Kharif* 2024



L - 100bp Ladder F- ICP 8863 (Maruti) M- BSMR-571 F1 Plants- 1-34

Plate 8: Screening of F1 in Cross ICP-8863 x BSMR-571 for confirmation of true hybrids by using SSR marker PKS 31 during *Kharif* 2024

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