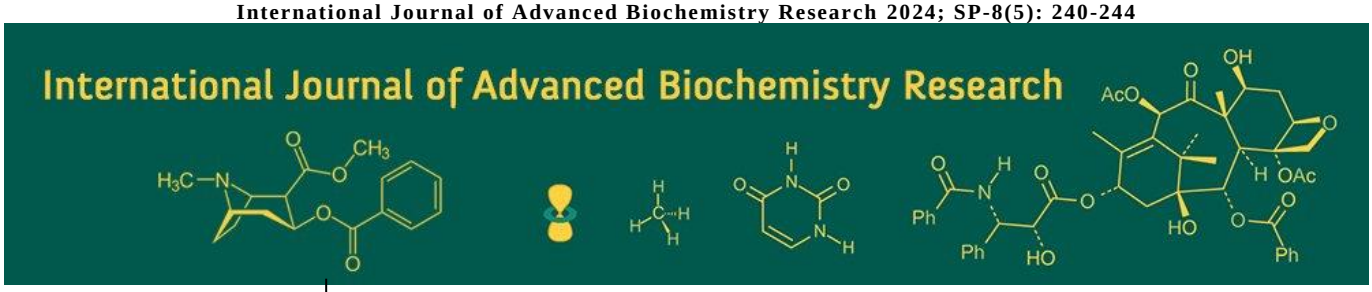


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# Advancing cotton breeding through SSR marker-assisted genetic purity evaluation: Insights from inter-specific cotton hybrid Phule Ekta

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**Abstract**
This study examines the utility of SSR markers in hybridity testing, focusing on the "Phule Ekta" cotton hybrid. Through SSR analysis, significant genetic polymorphisms were identified between the female and male parents. SSR markers are essential for accurately assessing hybrid purity and developing resilient cotton varieties. SSR marker 6019 displayed polymorphisms between the female and male parents of the Phule Ekta hybrid, with allele sizes differing, the female allele being approximately 30 bp larger. This polymorphism signifies genetic disparities between parental lines, enhancing the hybrid's genetic diversity and heterozygosity, indicating genetic variation at the SSR locus. The study highlights the importance of molecular tools, especially in heterosis breeding, aiming for hybrids with high yields, superior fibre properties, and resistance to pests and diseases. Overall, this research provides valuable insights for cotton breeders to select superior hybrid cultivars, addressing the evolving demands of agriculture.

**Keywords:** Hybridity testing, cotton breeding, SSR markers, Phule Ekta, heterosis breeding

## Introduction
India is a pioneer in commercial cotton hybrid cultivation, covering over 50% of cotton-growing areas [1]. These hybrids offer a 50% productivity increase compared to traditional varieties, along with broader adaptability, resistance to stresses, and superior fibre quality [2]. They can be developed more quickly than straight varieties, maintaining high productivity and uniform fibre quality [3]. Hybridity testing serves as a crucial step in ensuring the genetic purity and authenticity of hybrid seeds before they reach farmers, guaranteeing the availability of high-quality seeds with consistent and desirable traits [4]. The rise of new cotton hybrids necessitates rigorous hybridity testing.

While traditional morphological assessments are widely employed, biochemical assays such as isozymes offer superior precision in verifying genetic purity [5]. Nevertheless, the widespread adoption of DNA methods is hindered by cost limitations [6]. Emerging technologies hold promise in achieving greater throughput and potentially surpassing biochemical methods, ultimately elevating genetic purity standards and enabling more effective utilization of diverse genetic resources [7]. Achieving success in a hybrid program involves considering multiple factors beyond just yield and fibre quality, including aspects like earliness, disease and storm resistance, plant height, and structure [8]. The main drive behind producing hybrid cotton is to capitalize on the substantial heterosis already present in the crop [9]. The commercial acceptance of interspecific hybrids relies on finding methods to maintain heterosis for yield within manageable plant varieties [10]. Hybrid cotton seeds are produced by crossing two genetically distinct parental lines, typically referred to as the female (seed or ovule donor) and male (pollen donor) parents. The resulting $F_1$ hybrid seeds possess desirable traits from both parents, leading to hybrid Vigor [11]. Cotton hybrid seed production can be carried out using traditional methods involving manual emasculation and pollination, or through innovative approaches based on male sterility [12].

The majority of hybrids developed so far are of the conventional type, which entails identifying and cultivating male and female parent plants, emasculating the female parent, and then pollinating it with the selected male parent [13]. Cotton, being mainly cross-pollinated, in diploid cotton varieties, the traditional method is not cost-effective due to low boll setting caused by small flower size and brittle pedicels. Alternatively, male-sterility-based hybrids offer a solution by eliminating the need for emasculation, as the female parent plants have sterile anthers lacking pollen [14] This approach reduces production costs, although manual pollination remains necessary Cotton employs two main types of male sterility genetic male sterility and cytoplasmic genetic male sterility for seed production. Following hybrid seed production, field trials are undertaken to assess the performance of the hybrids across various environmental conditions and agronomic practices [15]. Field trials assess traits like yield, fibre quality, disease resistance, and adaptability, vital in breeding programs. Stability across environments is pivotal in genotype selection. Hybridity testing involves collecting samples like leaves for DNA extraction using [16]. Molecular markers, such as Simple Sequence Repeats (SSRs), Single Nucleotide Polymorphisms (SNPs), or Amplified Fragment Length Polymorphisms (AFLPs), are used to analyse the genetic composition of the collected samples [17]. SSR markers, in particular, are valuable due to their high level of polymorphism, reproducibility, and co-dominant inheritance [18]. Marker-Assisted Selection (MAS): Molecular marker data are analysed to identify specific alleles or genetic markers unique to the hybrid genotype [19]. These markers serve as molecular signatures that distinguish hybrids from their parental lines [20]. MAS facilitates rapid and accurate identification of hybrids, aiding breeders in discarding impure seeds and maintaining genetic integrity. Hybridity testing prevents inadvertent seed mixing or contamination, ensuring genetic purity and uniformity crucial for hybrid cotton varieties' performance and market value. By employing molecular markers, hybridity testing verifies seed genetic purity, guaranteeing high-performance varieties with desirable traits for farmers and the cotton industry [21]. Hybridity testing is essential in cotton breeding, ensuring the purity and authenticity of hybrid seeds before distribution to farmers [22]. This process is crucial because hybrid seeds offer improved traits such as higher yield, better fibre quality, and increased resistance to pests and diseases compared to their parent lines [23]. By verifying the genetic purity of hybrid seeds, cotton breeders ensure that farmers receive high-quality seeds with consistent and desirable characteristics, leading to enhanced crop performance and profitability [24]. Marker-assisted approaches further streamline hybridity testing by using molecular markers like Simple Sequence Repeats (SSRs) or Single Nucleotide Polymorphisms (SNPs) to analyze the genetic makeup of plant samples [25]. These techniques accurately identify superior hybrids by distinguishing them from parental lines and pinpointing specific genetic traits like disease resistance or fibre quality. Combining traditional breeding with molecular methods, cotton breeders supply high-performing hybrids meeting modern agriculture's needs. SSR markers, or microsatellites, are brief DNA sequences with repeated nucleotide patterns [26]. SSR markers are extensively used in genetic analysis for detecting genetic variation within populations or closely related species. Crucial for purposes like assessing genetic diversity, mapping genes, understanding population structure, and conducting forensic analyses, SSR markers play a vital role in genetic research [27]. Their versatility stems from their high polymorphism, reproducibility, and co dominant inheritance, making them valuable tools for researchers studying evolutionary processes, conservation efforts, and breeding programs. Overall, SSR markers play a crucial role in advancing our understanding of genetic diversity, population dynamics, and evolutionary relationships across different organisms.

In the dynamic landscape of cotton cultivation, the emergence of hybrid varieties has redefined the standards of productivity and quality [28]. Phule Ekata (RHB-1008) emerges as an innovative interspecific cotton hybrid, showcasing remarkable prowess with a seed cotton yield of 1853 kg/ha and superior fibre properties. Its staple length of 35.6mm, micronaire value of 3.2, and strength of 36.5 g/tex exemplify excellence in fibre quality. Resilient against alternaria leaf blight, bacterial leaf blight, and grey mildew, it also displays tolerance to sucking pests and bollworms, thriving in India's central zone. Developed through meticulous hybridization and rigorous testing, Phule Ekata's systematic development ensured high performance and resilience. With a maturity period of 170-180 days and moderate susceptibility to diseases, it promises enhanced productivity, making it suitable for timely sown conditions. Its pedigree, traced to parent lines RHC-0577/3-3 and NDGB-17, provides a solid genetic foundation. Phule Ekata recorded the highest seed cotton yield of 1702 kg/ha, surpassing checks by significant margins, with superior fibre quality meeting long staple cotton standards. While moderately susceptible to diseases, its tolerance to pests makes it a recommended choice for cultivation in India's south zone.

**Materials and Methods the DNA extraction**

The leaf tissue samples from the male parent, NDGB-17, and the female parent, RHC-577/3-3, and hybrid Phule Ekata (RHB-1008), were collected from field-grown plants. Careful attention was paid during sample collection, with leaf tissue samples obtained from multiple plants within each hybrid.

**Details of development of hybrid Phule Ekata (RHB-1008).**

| **Female Parent** | **Male Parent** |
|---|---|
| **RHC-577/3-3** | **NDGB-17** |
| *(G. hirsutum L.)* | *(G. barbadense L.)* |

**x**

↓

**RHB-1008**

**(Development of hybrid)**

A standard CTAB protocol facilitated DNA isolation, involving grinding of plant tissue in liquid nitrogen (Ref). Post-extraction, the quality and quantity of isolated DNA

were evaluated through agarose gel electrophoresis. A 0.8% agarose gel was prepared, and DNA samples, alongside known uncut λ DNA ladder amounts, were loaded. Electrophoresis separated DNA fragments by size, enabling visualization to confirm intact genomic DNA presence and quantity estimation. This crucial step ensured DNA samples were of adequate quality and quantity for downstream molecular analyses, like SSR marker analysis, facilitating accurate assessment of genetic purity and diversity in cotton hybrids by SSR Marker Analysis [29].

**A. PCR Reaction Mixture**. (Table no. 1)

**B. B.PCR Amplification**: Genomic DNA samples extracted from the cotton leaf tissues were subjected to PCR amplification using 15 SSR primer pairs (Table. 2).Execute the optimized cycling program for each SSR marker as follows: The PCR begins with a single denaturation step at 94°C for 5 minutes, separating DNA double strands into single strands. Subsequently, annealing initiates at 94°C for 30 seconds for 10 cycles, followed by a drop to 65°C for 30 seconds to promote specific primer binding. Extension then proceeds at 72°C for 45 seconds for 25 cycles, allowing DNA polymerase to elongate primers. Finally, a 7-minute extension at 72°C ensures complete strand extension, followed by a hold at 4°C to terminate the PCR process and preserve amplified DNA for further analysis. (Table no. 3)

**C. Preparation of Agarose Gel:** Prepare a 4% agarose gel by dissolving agarose powder in 1X TAE buffer. Heat the mixture until the agarose is completely dissolved, then allow it to cool to around 50°C. Add ethidium bromide (EtBr) dye to the agarose solution to a final concentration of 0.4 µg/ml. Mix well by swirling gently. Pour the agarose solution into a gel tray and insert a well comb to create wells for sample loading. Allow the gel to solidify at room temperature for about 20-30 minutes [30].

**Loading of PCR Products:** Once the gel is solidified, carefully remove the well comb. Mix each PCR product with loading dye (3 µl/sample) in separate microcentrifuge tubes. Load the PCR products along with a DNA size marker into the wells of the agarose gel using a micropipette, ensuring that each well is properly filled.

**Gel Electrophoresis:** Submerge the gel tray with loaded samples into an electrophoresis tank containing 1X TAE buffer, connecting electrodes to a power supply and running the gel at 120 volts for 60 minutes. Ensure the positive electrode (anode) is connected to the end with loaded wells, monitoring DNA fragment migration towards the positive electrode during electrophoresis [31].

**Visualization and Analysis:** After electrophoresis, carefully remove the gel from the tank and place it on a UV transilluminator. Turn on the UV light and visualize the gel under UV illumination. Ethidium bromide-stained DNA bands will fluoresce under UV light. Capture an image of the gel using a gel documentation system or a camera-equipped UV transilluminator. Analyse the gel image to determine the sizes of the SSR marker bands. Compare the band sizes with the DNA size marker to identify the allele sizes and genotypes of the samples.

**Results and Discussion**:

SSR Marker Analysis

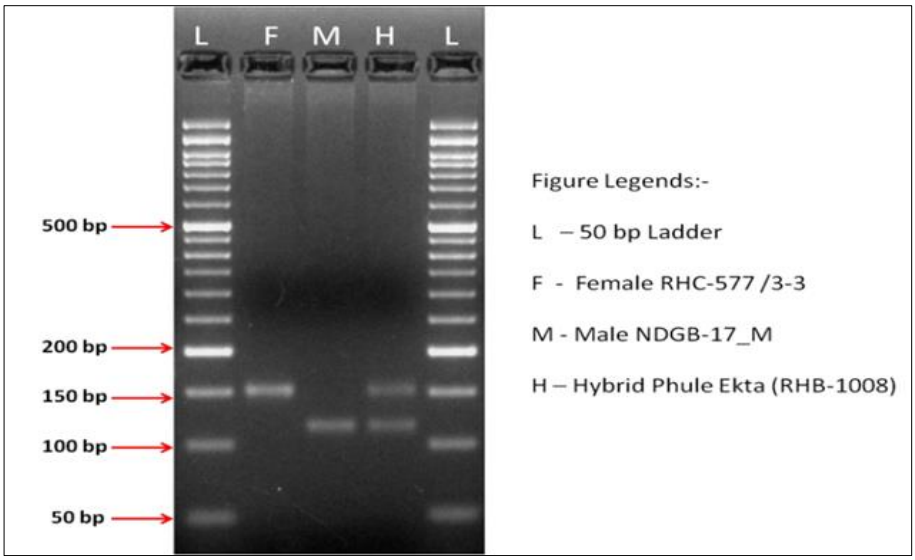


**Fig 1:** Agarose gel image showing DNA Fingerprinting of Cotton Hybrid "Phule Ekta" with SSR marker 6019.

**SSR Marker Identification: Results of SSR Marker Analysis for Cotton Hybrid "**Phule Ekta**"**

**SSR Marker 6019, Primer Sequences:** Forward: CTGGACTAAAAACCTTAACTGG, Reverse: CTCGATTCTAACTCAATCACG,

**Allele Sizes:** Phule Ekta Female: ~150 bp, Phule Ekta Male: ~120 bp,

**Polymorphism Levels and Allele Diversity:** SSR marker 6019 displayed polymorphisms between the female and male parents of the Phule Ekta hybrid, with allele sizes differing, the female allele being approximately 30 bp larger. This polymorphism signifies genetic disparities between parental lines, enhancing the hybrid's genetic diversity and heterozygosity, indicating genetic variation at the SSR locus. The analysis of SSR marker 6019 identified genetic polymorphisms between the female and male parents of the Phule Ekta cotton hybrid, offering insights into its genetic diversity and composition, aiding in characterization and breeding endeavours.

### Hybridity Testing Using SSR Markers

Hybridity Testing Using SSR Markers: The identified SSR markers were utilized in hybridity testing, where PCR amplification of DNA samples from cotton plants facilitated the detection of SSR loci and their alleles. Comparisons of SSR profiles between putative hybrid plants and parental lines determined hybridity by allele inheritance patterns. Effectiveness of SSR Markers in Distinguishing Hybrid Plants: SSR markers effectively differentiated hybrid from non-hybrid cotton plants by detecting specific SSR alleles unique to parental lines, enabling identification of hybrid plants through hybrid-specific SSR profiles containing alleles from both parents. Conversely, non-hybrid plants lacked parental SSR profiles, indicating their purebred status. SSR markers offered reliable verification of hybridity, ensuring genetic purity and authenticity of hybrid cotton varieties. Analysis of SSR marker 6019 in the cotton hybrid " Phule Ekta" revealed significant genetic polymorphisms between its female and male parents, indicating diverse genetic contributions and enriching the hybrid's diversity, potentially improving agronomic traits. The observed allele size disparity highlights genetic variation at the SSR locus, shaping the hybrid's unique composition and aiding its adaptability across environments. Such insights are crucial for cotton breeding, deepening understanding of the hybrid's genetics and guiding selection of superior cultivars. SSR markers play a pivotal role in hybridity testing, ensuring genetically pure hybrids and maintaining seed quality for farmers. The distinct allele sizes, facilitated by specific primer sequences, accentuate genetic diversity within the hybrid, enhancing heterozygosity and trait potential. These findings underscore SSR markers' importance in assessing genetic variation and selecting superior hybrids in cotton breeding. Identifying genetic polymorphisms through SSR analysis enriches hybrid genetic diversity and aids in parent line selection, improving agronomic performance. Effective SSR marker use ensures accurate hybrid purity assessment, preserving seed integrity for farmers. Our study highlights molecular tools' significance in cotton breeding, advancing high-yield, resilient varieties for modern agriculture. Findings align with genetic diversity demonstrations in " Phule Ekta," but future research could explore additional markers and their agronomic impacts, addressing program limitations to enhance cultivar development.

**Table 1:** PCR Reaction Components and Volumes

| Sr. No. | Components | Working on centration | Volume(µl) |
|---|---|---|---|
| 1 | Emerald Amp GT PCR Master mix (2x Premix) | 1X | 4 |
| 2 | Forward Primer (10 pM/µl) | - | 0.5 |
| 3 | Reverse Primer (10 pM/µl) | - | 0.5 |
| 4 | PCR grade Water | - | 4 |
| 5 | DNA Template | 50 ng /µl | 1.5 |
| | Total Volume | - | 10.5 |

**Table 2:** List of markers screened for Fingerprinting of Phule Ekta

| Sr. No. Marker | Name | Primer Seq Forward | Primer Seq Reverse |
|---|---|---|---|
| 1. | 1332 | 5' AAAAATCAGCCAAATTGGGA 3 | 5' CGTCAACAATTGTCCCAAGA 3' |
| 2. | 726 | 5' TGAAGATTTGGAGGCAATTG 3' | 5' GAAATCAAGCCTCAATTCGG 3' |
| 3. | 1071 | 5'ACCATCCCAAAGAATCATCCTC3' | 5' ACTAAAACCAAGGCAATAAAGTG 3' |
| 4. | 1092 | 5' TACCACATTGGATGTTTGCAAACCC 3' | 5' ATAGCAAACTGGAATCACTCCAAGC 3' |
| 5. | 1047 | 5' AGCCAGAACCTGATTGAATCA 3' | 5' ACCGCTATATTTCTTTAAGCACC 3' |
| 6. | 1055 | 5' CTCCTGCCTATGCTGCTATG 3' | 5' AGGAACTAGCACTTGTCTACATTGA 3' |
| 7. | 1091 | 5' GATTGATAAAGATAGGGTTGTGTCAC3' | 5' CATTATGTGCCTTCAAATTCCTGGC 3' |
| 8. | 894 | 5' ATCTGAACCATCATCCTC 3' | 5' TTCTGATTGGCACTTTC 3' |
| 9. | 5798 | 5' TTAGGGTTTAGTTGAATGG 3' | 5' ATGAACACACGCACG 3' |
| 10. | 6019 | 5' CTGGACTAAAAACCTTAACTGG 3' | 5' CTCGATTCTAACTCAATCACG 3' |
| 11. | 755 | 5' CAATATCTCACTTGGACCT 3' | 5' TGCTACACATCATAGTTGG 3' |
| 12. | 3387 | 5' ATCCAAACCAACCATGCAAT 3' | 5' GAAGGGGTTTTGCATTTCAA 3' |
| 13. | 746 | 5' CTTTCCACGTGTAATTTGTTGATA 3' | 5' GATCTTAACTCTTGCTCTCTCTCTCTC 3' |
| 14. | 6020 | 5' CGCTCGGTTATTTAGTTT 3' | 5' AAATTCCAGCTCATGGT 3' |
| 15. | 6021 | 5' CAATACCTGGAACATAGACAAATG 3' | 5' CTTGAGGCTTGCAAAAAATG 3' |

**Table 3:** PCR conditions/ PCR profile

| Temp (˚C) | Time | Remarks |
|---|---|---|
| 94 | 5 min | 1 Cycles (Denaturation) |
| 94 | 30 sec | 10 Cycles (Annealing) |
| 65* | 30 sec | |
| 72 | 45 sec | |
| 94 | 30 sec | 25 Cycles (Extension) |
| 55 | 30 sec | |
| 72 | 45 sec | |
| 72 | 7 min | 1 Cycle (Final Extension) |
| 4 | ∞ | Stop (Hold) |

### Conclusion

In conclusion, our study demonstrates the utility of SSR markers in hybridity testing for cotton breeding. Through SSR marker analysis of the cotton hybrid "Phule Ekta," we identified significant genetic polymorphisms between its female and male parents, indicating unique genetic contributions and enriching the hybrid's genetic diversity. This highlights the importance of SSR markers in accurately assessing hybrid purity and ensuring the integrity of cotton seeds marketed to farmers. Our findings underscore the implications for cotton breeders and geneticists, emphasizing the value of molecular tools in advancing breeding efforts and facilitating the development of high-

yielding and resilient cotton varieties tailored to meet the evolving demands of agriculture.

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