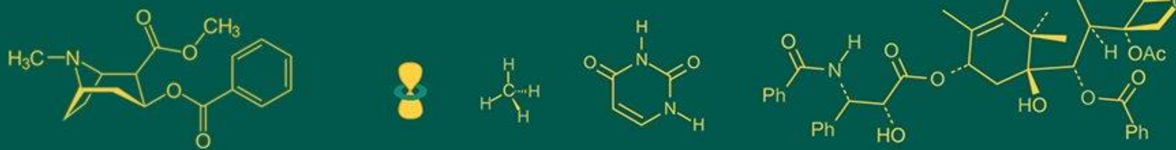

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Confirmation of F_1 hybridity in upland cotton (*Gossypium hirsutum*) by molecular assay

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Abstract
The F_1 population was developed by crossing two species of Upland Cotton *(Gossypium hirsutum)*. During the experiment, ICAR-CICR Rajat Bt had insect resistance, good adaptability, high yielding was choosen as a donor parent and a recently released non-bt variety Suvarna Shubra (AKH-09-5) that was resistant to bacterial leaf blight, grey mildew, Myrothecium leaf spot, and jassids was used as a female parent. The F_1 hybrids exhibiting insect resistance were confirmed through PCR amplification by utilization of a SSR molecular marker. After screening 60 F_1 plants, the *cry1Ac gene* was confirmed in 20 F_1 hybrids. These hybrids might be an important genetic resource to carry on the bollgard resistance gene to further advanced generations.

Keywords: Bt, F_1 population, hybrid, insect resistance, upland cotton

Introduction
Gossypium spp., a plant belonging to the Malvaceae family, is widely grown as an oilseed crop and the most widely utilized natural fiber in the world. It has long been for breeding, and genetic research because of the importance it brings to the agricultural and manufacturing industries. India is the world's highest producer of cotton, with 45 diploid varieties referred to as "old world" and five allotetraploid species known as "new world cotton" worldwide (Fryxell PA, 1992) [4]. Two substances in cotton that may benefit people and animals are gossypol, a polyphenolic with possible contraceptive effects, and trans-caryophyllene, a terpenoid with anti-inflammatory and cytotoxic qualities (Fernandes *et al*. 2007; Amiel *et al*. 2012) [3, 1]. There are only four farmed species of cotton at present, two of which are classified as tetraploids [*G. hirsutum* L. and *G. barbadense* L., ($2n = 4x = 52$)] and two diploid species [*G. herbaceum* L., ($2n = 2x = 26$) and *G. arboreum* L. *Gossypium hirsutum*, called upland cotton, is indigenous to the Caribbean, Mexico, and Central America. Ninety percent of the cotton produced worldwide is derived from *G. hirsutum*. Upland cotton fibers are incredibly short in comparison to other species. It is widely used in a variety of consumer goods, where manufacturers look for high-quality, pure natural fibers. In the current work, two *Gossypium* hirsutum cotton varieties were crossed to transfer the insect-resistant trait character to the F_1 plants during the Kharif season. This trait was then further transferred to the following generation using a marker-assisted backcross breeding technique.

Materials and Methods
During the *Kharif* season, the crossing block was raised in 2019-20, and the non-Bt female variety Suvarna Shubhra (AKH-09-5) was crossed with the donor parent ICAR-CICR Rajat Bt for obtaining F_1s. However, the donor parent ICAR-CICR Rajat Bt and the female parent Suvarna Shubra (AKH-09-5) were maintained in the demo field just before the research plot During the second *Kharif* season, 2020-21 60 F_0 seeds were planted on a field of Cotton Research Unit, Dr. PDKV., Akola. Out of the total of 60 F_0 seeds, 60 seeds were germinated and tested for confirmation of hybrids during *Kharif 2020* in the field conditions. These plants along with parents were used for recording data on molecular analysis. Young cotton leaf was used for the isolation of DNA from F_1 plants and their parents followed by PCR amplification using Cry1Ac SSR marker and agarose gel electrophoresis for confirmation of cotton hybrids.

Genomic DNA isolation
Implementing the cetyltrimethylammonium bromide (CTAB) extraction method (Doyle, 1990) with minor modifications, high-quality genomic DNA was isolated from the young cotton leaves by using three different stocks of 0.5 M EDTA Na_2, 5 M NaCl, and 1 M Tris HCl along with other reagents were used. During the extraction technique, 2% CTAB, 1.4M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA (pH 8.0), and 100 mM Tris HCl were used to prepare the extraction buffer. Using a mortar and pestle, three punches of fresh cotton leaf samples were macerated in liquid nitrogen during the process to produce a fine powder. Following a component of 850µl of CTAB buffer was added to 2 ml micro centrifuge tubes containing the powdered materials. Subsequently 1 hour of incubation at 65ºC in a water bath with intermittent shaking every 10 minutes, the samples were centrifuged for 20 minutes at room temperature. The supernatant was transferred to a new centrifuge tube, and to emulsify the two components, an equal volume of chloroform: isoamyl alcohol (24:1) was added and gently stirred. The mixture was centrifuged for 20 minutes at room temperature at 9,000 rpm. After extraction, the aqueous phase was transferred to a different micro centrifuge tube. An equivalent volume of chilled isopropanol was added. To extract the most genomic DNA possible, samples were subsequently incubated at -20 ºC for an entire night. Samples containing genomic DNA were centrifuged for 15 minutes at 4ºC and 10,000 rpm. The bottom of the micro centrifuge tubes contained a pellet developed after centrifugation. Following a 120 µl 100% ethanol wash, the pellet was centrifuged for 10 minutes at 10,000 rpm and 4 ºC. After discarding the supernatant, the pellet was allowed to air dry for 15 to 20 minutes at room temperature. Tapping dissolved the pellet in 30 µl of TE buffer (8.0 pH). When kept at 4 ºC without stirring, the pellets could dissolve entirely during a single night. Using a Nano photometer (Implen), DNA sample amount and quality were evaluated. The absorbance at 260 and 280 nm was measured. The ratio A260/280 value between 1.7 and 1.9 was chosen for PCR amplification.

PCR amplification
The following PCR components were used to amp up the DNA samples using a conventional 35-cycle PCR reaction: 2.0µl of 10 X PCR buffer with 17.5 mM MgCl2, 0.4µl dNTPs (10 mM), 0.5µl Taq polymerase (5U/µl), 0.7µl Cry1Ac SSR forward and reverse primer (10 pmol), 1.3µl of DNA (~100 ng/µl), and 19.2µl of Nuclease Free water. Three minutes of initial denaturation at 94 °C, thirty seconds of denaturation at 94 °C, 45 seconds of annealing at 59.7 °C, one minute of elongation at 72 °C, and ten minutes of final elongation at 72 °C comprised the PCR temperature cycle. The details are given in Table 1.

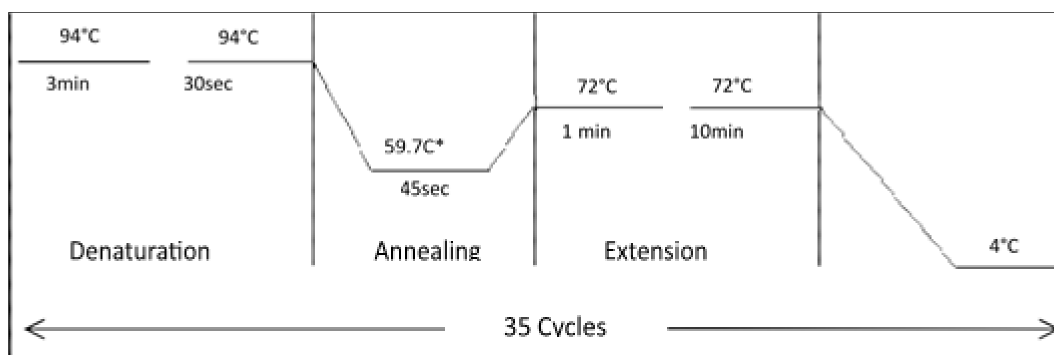


Fig 1: Reaction conditions for the amplification of Cry1Ac SSR marker

Table 1: Marker used for confirmation of hybrids

S.N.	Marker name	(5'-3')	(3'-5')	Product size (BP)
1	Cry1Ac	GGGAGGAGATGCGTATTCA	CTATACCCTGGGCAGAACCA	486bp

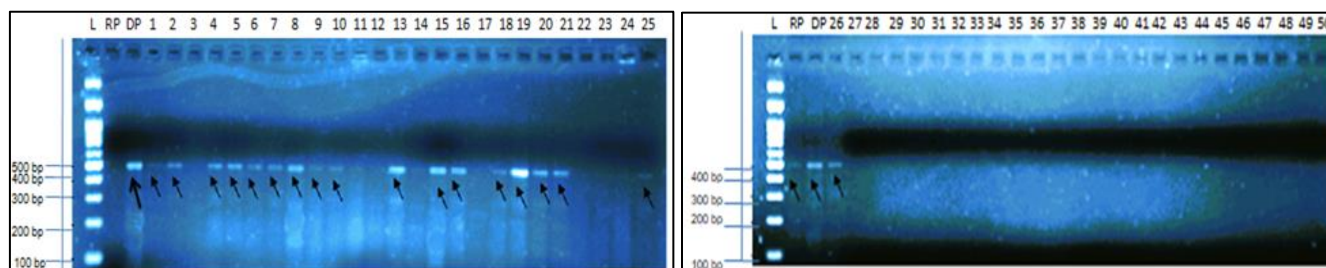
Agarose gel electrophoresis
To assess the quality of PCR products, samples were run on 2.5 percent agarose gel. Agarose gel of 2.5% was prepared in 1X TBE buffer with ethidium bromide 10µl/100ml of gel volume in a horizontal gel electrophoresis system. The samples from each genotype with 6X loading dye were loaded in each well. The gel was run at 80-100 V for 1-1.5 hours. After completion of the run, the gel was visualized under UV light on a UV trans illuminator, and photographs were taken using a gel documentation system (Alpha Innotech) and the samples were confirmed.

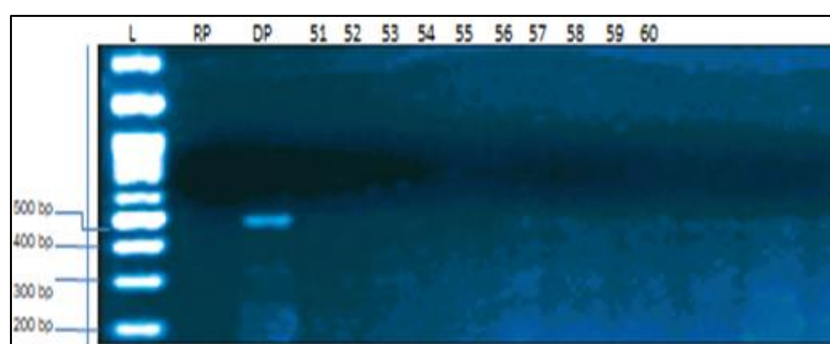
Results and Discussions
60 F_1 plants obtained from the crossing program were tested for hybridity assessment during the *Kharif* season 2020-21 by using SSR molecular marker. This marker had confirmed the hybridity of the *cry1Ac* gene in 20 F_1 plants as true crosses (Table 2) with 33.33% positive plants. The cry1Ac SSR marker was amplified on 486bp among the parents and their tested F_1 individuals. The representative gel image of hybridity confirmation is given in figure 2. a similar SSR molecular during their experiment to detect two genetically modified cotton events. Shashi Bhushan Chauhan *et al.* (2016) used cotton-specific SSR markers to evaluate the genetic purity in two cotton hybrids. Based on amplifying "140bp & 100bp alleles" of the lines, results showed that just one SSR marker, CM43, could detect the hybrid's heterozygosity (CSHH198). Similarly, Harpreet Kaur *et al.* (2016) verified the interspecific hybrid by molecular research, utilizing *Gossypium hirsutum* cv. F 1861 as the female parent and *G. armourianum* cv. PAU 1 as the male parent. The findings demonstrated that, out of the 33 polymorphic SSR markers between the parents, 8 markers supported the interspecific hybrid status. By using molecular techniques, Muthuraj *et al.* (2019) [6] confirmed the F_1 hybridity and concluded that, of the 11 SSR markers that

were polymorphic between parents, five demonstrated the interspecific hybrid status. Similarly, while generating two sets of F_1 population and using two male parents-DHMAS and RML 22 and K 343 as the common female parent. Confirmed F_1 hybrids. Of the 12 F_1 plants resulting from the cross K 343×DHMAS, 6 were real hybrids, and 8 of the 10 F_1 plants from a different cross, K 343×RML22, were hybrids for both parents.

Table 2: Hybridity assessment in F_1 population

S.N.	Marker name	(ICAR-CICR Rajat Bt) Donor parent amplicon size (bp)	(Suvarna Shubhra) Female parent amplicon size (bp)	Expected amplicon size (bp) in F_1 hybrid individual	Plant screened	Confirmed Plants
1	Cry1Ac	486	-	486 bp	60	20





L: Ladder, RP: Recurrent Parent, DP: Donor Parent, 1-60: F1 plants

Fig 2: Hybrid confirmation in F_1 population

Conclusion

Twenty plants exhibited the ICAR-CICR Rajat Bt allele (Donor parent) and were deemed to be genuine hybrids after the hybridity confirmation in the F_1 generation was conducted using agarose gel electrophoresis with the SSR marker. In the meantime, chosen plants were employed to produce the generations to come.

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