Microsatellite Marker Based DNA Fingerprinting of Cotton (*Gossypium Spp.*) Hybrids and Their Parents

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Abstract

Cotton production in India by vast majority comes from cotton hybrids whose genetic purity is of great significance in seed production chain and trade. Therefore, there is need to develop a rapid, reliable and reproducible technique to assess the genetic purity of cotton hybrids as traditional, morphological traits-based 'Grow-Out Test' is resource intensive, time consuming, tedious and not an infallible procedure. In this regard, a study was planned to understand the genetic diversity among the hybrids and their parents, and also to identify SSR markers for confirmation of genetic purity or hybridity. One intra-*arboreum* hybrid, CICR2 (DS 5 GMS × LD 327 Sel.), four intra-*hirsutum* hybrids viz., CSHH198 (CSH 19 × CSH 8), CSHH238 (SH 2379 9Y × PIL 8 Sel.), CSHH243 (CSH 2013 × CSH 43), CSHH1862 (GMS 16A × CB 33) and one *hirsutum* × *barbadense* hybrid, Phule 388 (RHC-006 × RHCb-001) along with their respective parental lines were selected for molecular characterization. Of the total 215 SSR markers surveyed, 60 markers conveyed polymorphism. The information conveyed by the polymorphic SSR markers was utilized to assess the molecular divergence among the study material. Maximum genetic dissimilarity of 0.66 was noted between Phule 388 and LD 327 (Sel.), and between RHC-006 and DS 5 (GMS). Minimum genetic dissimilarity of 0.07 was observed between CSHH1862 with CB 33, followed by 0.11 between CICR2 with DS 5 (GMS). SSR markers were highly efficient in capturing both intra-species and inter-species level diversity. The clustering and factorial analysis was in congruence with the species of *Gossypium*. The diploid species genotypes were clustered separately and distinctly from the rest of the genotypes. All the *hirsutum* hybrids and their respective parents were found closely clustered. The inter-specific hybrid, Phule 388 along with its parents was found grouped closely. The genetic purity of the hybrids was confirmed using identified SSR markers [GH486, BNL1421, BNL3594, JESPR151 for *G. hirsutum* hybrid CSHH198; GH486, BNL2449, JESPR151, TMB0436 for *G. hirsutum* hybrid CSHH238; BNL2449, JESPR151, JESPR152 for *G. hirsutum* hybrid CSHH243; and, GH527, BNL3812, TMB1484, TMB1645, NAU1190, BNL3816 for inter-specific *G. hirsutum* × *G. barbadense* hybrid Phule 388]. The SSR markers were efficient in analysis of hybrid seed purity. The information generated in the present study about genetic diversity and genetic purity testing will greatly facilitate quality seed production of these cotton hybrids and thus, better cotton production.

Introduction

Cotton is the world's most important source of natural textile fibre and a significant oilseed crop. India leads the world in area and production of cotton with an estimated area of ≈ 13 million hectares with a production estimate of ≈ 37 million bales (<https://cotcorp.org.in/statistics.aspx>). Cotton production in India by vast majority comes from cotton hybrids. Hybrids have an advantage of heterosis by the virtue of genomic heterozygosity and are produced through hybridization between two genetically diverse purelines (near homozygous and homogenous) having better trait complementation. These hybrid seeds then need to be authenticated for genetic purity, parentage and quality potential before they are released for cultivation by the farmers. Mechanical handling, outcrossing and ecological adaptation and at times, mutations may deteriorate the identity and purity of the hybrid seeds. The success of hybrid cotton production depends upon timely production and adequate supply of genetically pure hybrid seeds to the farmers. It is estimated that for every 1% impurity in the hybrid seed, there will be yield reduction of 100 kg per hectare (Mao et al. 1996). Thus, the genetic purity of hybrid is of great significance in seed production chain and trade.

The genetic purity of the hybrid is assessed traditionally by Grow-Out Test (GOT) which is based on morphological traits (Tatineti et al. 1996). This procedure is resource intensive, time consuming, tedious and not an infallible procedure. These morphological traits are sensitive to environmental variations and prone to subjective assessment. Owing to this, it can be difficult to distinguish the morphological differences between true hybrids and off types, especially when the parents share closer pedigree (Selvakumar et al. 2010). The resource demanding and time consuming GOT may lead to delay in planting and resultantly can affect seed viability (Ali et al. 2008). Therefore, it is necessary to develop a rapid, reliable and reproducible technique to assess the genetic purity of cotton hybrids. Earlier studies have explored DNA marker systems such as RFLP (Pendse et al., 2001), RAPD (Mehetre et al. 2007), AFLP (Rana and Bhat, 2004), SSR (Saravanan et al. 2007; Selvakumar et al. 2010; Menka et al. 2016) and ISSR (Rana et al. 2006) to rapidly screen the genetic purity of hybrid seed. These molecular markers precisely assess the genotype, and not the phenotype (Sundaram et al. 2008). Among these markers, SSR markers are widely preferred for genetic purity testing (Saravanan et al. 2007; Selvakumar et al. 2010; Menka et al. 2016; Bora et al. 2016; Ben Romdhane et al. 2018), DNA fingerprinting (Santhy et al. 2019; Santosh et al. 2020), genetic diversity analysis (Abd El-Moghny et al. 2017) apart from other plant breeding applications owing to their reproducibility, co-dominant inheritance, genome-wide presence, robustness, higher polymorphism and analytical simplicity (Rakshit et al. 2011). Therefore, this study was planned to understand the genetic diversity among the hybrids and their parents and to identify SSR markers for confirmation of genetic purity or hybridity

Material And Methods

The study material included one Intra-*arboreum* hybrid, CICR2 (DS 5 GMS × LD 327 Sel.), four intra-*hirsutum* hybrids viz., CSHH198 (CSH 19 × CSH 8), CSHH238 (SH 2379 9Y × PIL 8 Sel.), CSHH243 (CSH 2013 × CSH 43), CSHH1862 (GMS 16A × CB 33) and one *hirsutum* × *barbadense* hybrid, Phule 388 (RHC-006 × RHCb-001) and their respective parental lines. The detailed information about the study material is provided as Table 1.

Table 1
List of hybrids along with their parentage and salient features

S. No.	Name	Species*	Year of release	Institution	Pedigree	Crop maturity (days)	Average yield (q/ha)	Ginning percent (%)	Fibre length (mm)	Bundle Strength (g/tex)	Mic value (µg/inch)
1	CICR2 (CISSA2)	A×A	2005	CICR, Sirsa	DS 5 (GMS) × LD 327 (Sel.)	160–170	21	38.4	19.2	16.3	7.0
2	CSHH198 (Shresth)	H×H	2005	CICR, Sirsa	CSH 19 × CSH 8	160–165	22	32.8	26.7	25.7	4.5
3	CSHH238 (Hybrid Kalyan)	H×H	2007	CICR, Sirsa	SH 2379 9Y × PIL 8 (Sel.)	150–160	21	33.5	27.6	22.5	4.5
4	CSHH243	H×H	2008	CICR, Sirsa	CSH 2013 × CSH 43	165–170	34	33.7	27.5	23.5	4.6
5	CSHH1862	H×H	2011	CICR, Sirsa	GMS 16A × CB 33	160–170	21	34.5	27.8	21.9	4.2
6	Phule 388 (RHB – 388)	H×B	2002	MPKV, Rahuri	RHC-006 × RHCb-001	170–180	17–20	34	35	23.8	3.4
* H – <i>Gossypium hirsutum</i> ; B – <i>Gossypium barbadense</i> ; A – <i>Gossypium arboreum</i>											

The pure seeds of these hybrids and their male and female parent were received from their respective breeders/institutions. The freshly opened young leaves of each of the hybrid and their parents were taken for extraction of genomic DNA and maintained in ice cold condition. The genomic DNA was extracted using the quick Cetyl Trimethyl Ammonium Bromide method (Paterson et al. 1993). Extracted DNA was quantified on 0.8% agarose gel and quality was assessed using spectrophotometer. The genomic DNA of these 18 genotypes (6 hybrids and their respective parents) was profiled to identify DNA polymorphisms using 215 genomic SSR markers and polymorphic markers were identified. The information conveyed by the polymorphic SSR markers was utilized to assess the molecular divergence among the study material. PCR amplification was carried out in 15µl reaction using the touchdown PCR protocol in Veriti® 96 well Fast Thermal Cycler (Applied Biosystems). The PCR amplification programme (Rakshit et al. 2010) consisted of an initial denaturation step at 94°C for 7 min (step-1), followed by 9 cycles (step-2) of 94°C for 15s, 65°C for 30s and 72°C for 60s with touch down by 1°C in each cycle from 65°C to 56°C followed by 40 cycles (step-3) of 94°C for 15s, 55°C for 30s and 72°C for 60s. Final extension was carried out at 72°C for 7 min (step-4). The PCR amplicons were electrophoresed on 4% agarose gel stained with ethidium bromide and visualized under UV transillumination. The molecular profiles were visually scored based on the product size in comparison with the standard 50bp DNA ladder (Thermo Scientific). The allelic data was converted into 1 (presence) – 0 (absence) binary matrix to estimate the genetic dissimilarity indices based on Jaccard's similarity coefficient. The genetic dissimilarities among the genotypes was utilized for clustering of the genotypes using Neighbour Joining method and factorial analysis employing DARwin 6.0 software (Perrier et al. 2003). In order to confirm the hybridity or genetic purity, the genomic DNA of hybrid vis-à-vis its male and female parent was assayed with polymorphic SSR markers. The SSR markers clearly distinguishing the male and female parent of each of the hybrid was identified among the 60 polymorphic SSR markers. The markers producing multiple bands with heterozygosity were excluded for genetic purity analysis. SSR markers which revealed different, homozygous alleles in parents with distinct difference in allele size were identified for each of the hybrid. The genetic purity was confirmed in each of the hybrid using identified markers distinctly polymorphic between respective parents.

Results And Discussion

SSR polymorphism

Of the total 215 SSR markers employed for polymorphism survey among the hybrids and their parents, 60 markers conveyed a polymorphism percent of 27.9%. The list of polymorphic SSR markers and their details is provided as supplementary table 1. The higher polymorphism percent observed in the present study might be due to inclusion of different species of cotton in the study. In our earlier diversity studies, we observed SSR polymorphism of 16% and 27% in *G. arboreum* (Santosh et al. 2020) and tetraploid cotton (Santhy et al. 2019), respectively. Earlier, Selvakumar et al. (2010) observed 30% polymorphism during genetic purity analysis of three cotton hybrids using SSR markers. Menka et al. (2016) noted 20% polymorphism while studying hybrid purity in two cotton hybrids. Marker polymorphism depends on many factors such as breeding behaviour of the species, genetic diversity in the study material, sample size, sensitivity of genotyping method and location of primers in the genome used for study. The information conveyed by the polymorphic SSR markers was utilized to assess the molecular divergence among the study material.

Genetic dissimilarity

The genetic dissimilarity among the genotypes under study is presented as Table 2. Maximum genetic dissimilarity of 0.66 was noted between inter-specific H×B hybrid, Phule 388 with *G. arboreum* line LD 327 (Sel.), and, between *G. hirsutum* line RHC-006 with *G. arboreum* line DS 5 (GMS). The dissimilarity of 0.65 was observed between inter-specific H×B hybrid, Phule 388 with intra-*arboreum* hybrid, CICR2 and its male parent DS 5 (GMS), between *G. hirsutum* line CSH 43 with *G. arboreum* line LD 327 (Sel.), between *G. hirsutum* line RHC-006 and *G. arboreum* line LD 327 (Sel.). The lesser genetic diversity was observed between the hybrids and its parents. The minimum genetic dissimilarity of 0.07 was observed between intra-*hirsutum* hybrid, CSHH1862 with its female parent CB 33, followed by 0.11 between intra-*arboreum* hybrid, CICR2 with its male parent DS 5 (GMS). The genetic similarity of 0.85 was noted between intra-*hirsutum* hybrid, CSHH243 with its male parent CSH 2013, and between intra-*hirsutum* hybrid, CSHH1862 with its female parent GMS 16A, and between GMS

16A with CB 33. The SSR markers were highly efficient in capturing both intra-species and inter-species diversity (Abd El-Moghny et al. 2017; Santhy et al. 2019; Santosh et al. 2020) as they revealed higher genetic diversity between different species and lesser diversity within species or between hybrids and their parents.

Table 2
Genetic dissimilarity among the genotypes under study

Dissimilarity co-efficient	LD 327 (Sel.)	DS 5 (GMS)	CICR2	CSH 8	CSH 19	CSHH198	PIL 8 (Sel.)	SH 2379 9Y	CSHH238	CSH 43	CSH 2013	CSHH243	CB 33	GMS 16A	CSHH1
DS 5 (GMS)	0.25														
CICR2	0.17	0.11													
CSH 8	0.61	0.59	0.58												
CSH 19	0.64	0.63	0.61	0.40											
CSHH198	0.61	0.59	0.57	0.24	0.18										
PIL 8 (Sel.)	0.59	0.57	0.56	0.28	0.44	0.32									
SH 2379 9Y	0.64	0.62	0.62	0.45	0.20	0.27	0.42								
CSHH238	0.60	0.58	0.56	0.31	0.29	0.18	0.26	0.22							
CSH 43	0.65	0.64	0.62	0.35	0.45	0.31	0.20	0.45	0.27						
CSH 2013	0.62	0.61	0.59	0.29	0.29	0.26	0.33	0.29	0.25	0.33					
CSHH243	0.61	0.58	0.57	0.26	0.36	0.21	0.24	0.34	0.18	0.18	0.15				
CB 33	0.58	0.57	0.55	0.30	0.32	0.19	0.27	0.36	0.24	0.29	0.30	0.22			
GMS 16A	0.57	0.58	0.54	0.30	0.37	0.28	0.32	0.40	0.32	0.35	0.36	0.31	0.15		
CSHH1862	0.55	0.53	0.51	0.31	0.34	0.23	0.28	0.38	0.22	0.31	0.32	0.26	0.07	0.15	
RHCb-001	0.63	0.63	0.63	0.54	0.55	0.52	0.55	0.57	0.55	0.59	0.55	0.54	0.55	0.55	0.55
RHC-006	0.65	0.66	0.63	0.37	0.40	0.34	0.39	0.45	0.37	0.45	0.31	0.36	0.39	0.46	0.40
Phule 388	0.66	0.65	0.65	0.46	0.53	0.43	0.50	0.55	0.48	0.53	0.50	0.47	0.50	0.53	0.51

Clustering and factorial analysis

The information on genetic dissimilarity among the genotypes was utilized for clustering and factorial analysis. Both clustering based on unweighted Neighbour Joining (Fig. 1) and factorial analysis (Fig. 2) depicted pattern of genetic diversity and the grouping of genotypes was in congruence with the ploidy of the species. The diploid species (*G. arboreum*) hybrid, CICR2 along with their parents [DS 5 (GMS) and LD 327 (Sel.)] were clustered separately and distinctly from the rest of the genotypes. All the *hirsutum* genotypes (Hybrids CSHH198, CSHH238, CSHH243, CSHH1862 and their respective parents were also found closely clustered. Similarly, the inter-specific hybrid, Phule 388 along with its *G. hirsutum* (RHC-006) and *G. barbadense* (RHCb-001) parent formed a distinct group. Factorial coordinate analysis provides overall representation of diversity while, clustering tends to faithfully represent the individual relations (Santosh et al. 2017). Clustering based on molecular makers revealed that particular hybrid and its parents were grouped together as cluster and hybrid was positioned in a near midway between its two parents (Rana et al. 2006; Chauhan et al. 2016). A similar pattern of distinct grouping was observed for the hybrids Phule 388, CSHH1862 and CICR2.

Confirmation of genetic purity

Out of the 215 SSR markets surveyed, 60 were observed as polymorphic among the material included in the present study. Polymorphic markers which clearly differentiated the male and female parent of each of the hybrid were identified from the 60 polymorphic markers. The genetic purity was confirmed in each of the hybrid using identified markers that differentiated male and female parents of each hybrid by clear, scorable and unambiguous amplified fragments. The markers producing multiple bands with heterozygosity were excluded for genetic purity analysis. Microsatellite markers in cotton are known to reveal multiple banding patterns per locus (Rudmann-Maurer et al. 2007; Rana et al. 2006; Selvakumar et al. 2010; Chauhan et al. 2016), which may be the result of polyploidy or amplification of repetitive sequences or due to pollen contamination.

The markers *GH486*, *BNL1421*, *BNL3594* and *JESPR151* differentiated the parents (CSH19 and CSH8) of *G. hirsutum* hybrid, CSHH198 and confirmed the genetic purity of the hybrid by producing alleles from both the parents (Fig. 3). The SSR markers *viz.*, *GH486*, *BNL2449*, *JESPR151* and *TMB0436* produced parent-specific alleles in the SH2379-9Y and PIL8 Sel. and hybridity was confirmed in *G. hirsutum* hybrid, CSHH238 by producing both the parental alleles (Fig. 4). The parents (CSH2013 and CSH43) of *G. hirsutum* hybrid, CSHH243 produced genotype specific alleles for the markers *BNL2449*, *JESPR151* and *JESPR152* (Fig. 5). These markers produced heterozygous bands specific to male and female parents of the hybrid, thus confirming the hybrid purity. The parents of inter-specific hybrid, Phule 388 were found to be homozygous for different alleles of *GH527*, *BNL3812*, *TMB1484*, *TMB1645*, *NAU1190* and *BNL3816* (Fig. 6). The hybrid produced both *G. hirsutum* and *G. barbadense* parent specific alleles for each of these markers, thus confirming genetic purity of the hybrid. Markers distinctly differentiating the parents of intra-*arboreum* hybrid, CICR2 and intra-*hirsutum* hybrid CSHH1862 and also unambiguously

confirming the genetic purity of these hybrids were not observed in the study. SSR markers are known for their efficiency in genetic purity analysis and were utilized for genetic purity testing of different cotton hybrids (Rana et al. 2006; Selvakumar et al. 2010; Rao et al. 2015; Chauhan et al. 2016; Menka et al. 2016).

Phenotyping based on morphological traits is very important as they represent the expressed part of the genome. Since, most of these morphological traits are quantitative in inheritance and environmentally influenced, more often, there exists a risk of categorising genetically different cultivars as similar or vice-versa owing to subjective assessment (Santhy and Meshram, 2015). The SSR markers can be used in efficient analysis of hybrid seed purity since this technique is simple to use, more accurate and not affected by environment when compared with GOT. Moreover, SSR based clustering is known to have better correlation with the pedigree than the dendrogram from morphological data (Giancola et al. 2002). Pattanaik et al. (2018) carried out the comparison of traditional grow-out test and DNA-based PCR assay to estimate F₁ hybrid purity in cauliflower and proposed that molecular marker-based hybrid purity assessment may serve as an effective substitute to traditional GOT. A combination of SSR markers and morphological descriptors are proposed for comprehensive and unambiguous cultivar identification and differentiation (Santhy et al. 2019; Santosh et al. 2020). The present study has identified polymorphic SSR markers which can be used in hybrid purity testing. The information generated in the study about genetic diversity and genetic purity testing will greatly facilitate seed production of these cotton hybrids. The polymorphic SSR markers identified in the study will facilitate their robust identification and thus, their licensing and commercialization.

Declarations

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Figures

Figure 1

Clustering of genotypes as revealed by polymorphic SSR markers

Factorial analysis: Axes 1 / 2

Figure 2

Factorial analysis

Figure 3

DNA fingerprinting of *G. hirsutum* hybrid CSHH198

Figure 4

DNA fingerprinting of *G. hirsutum* hybrid CSHH238

Figure 5

DNA fingerprinting of *G. hirsutum* hybrid CSHH243

Figure 6

DNA fingerprinting of *G. hirsutum* × *G. barbadense* interspecific hybrid Phule 388

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