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Exploring the genetic diversity of upland cotton (*Gossypium hirsutum* L.) genotypes using EST-SSR markers

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Cotton is a paramount multi-purpose crop cultivated intensively for the leading source of naturally occurring fiber, animal feeds, and oilseeds. Understanding the genetic diversity of cotton species is necessary for the development of new varieties or cultivars with enhanced yield, fiber quality and disease resistance. EST-SSR markers are widely used for assessing genetic variation owing to their high polymorphism and reproducibility. This investigation used 28 EST-SSR markers to unveil the genetic diversity of 30 upland cotton genotypes collected from Central Cotton Research Institute, Multan, Pakistan. Seven EST-SSR primers out of 28 were found polymorphic at different SSR loci. A total of 37 alleles were ascertained using the 28 EST-SSR primers. The ordinary number of alleles per locus was 1.5, with a range from 1 to 3. The polymorphic information content (PIC) extended from 0.065 to 0.82 with an average of 0.403. Cluster analysis was executed using an un-weighted pair group method with arithmetic. The average algorithm grouped cotton genotypes into four major clusters except DNH-105, Cyto-178 and FH-326, acting as an out-group. Genotypes such as IR-3701, Sitar-008, CIM-598, and CIM-625 exhibited maximum similarity coefficient. Nau-1231 indicated the lowest confusion probability (Cj) value (0.531) along with the greatest PIC (0.82) and discriminating power (Dj) (0.82) values. This study provides valuable information on the genetic diversity of upland cotton, which could be useful for cotton breeders as they evolve strategies for the conservation and implementation of cotton germplasm resources.

Keywords: *Gossypium hirsutum*, Genetic variation, Molecular marker, Similarity coefficient, Cluster analysis

Cotton is a major fiber crop all over the world[1]. Cotton belongs to the Malvaceae family and genus *Gossypium*. It has 52 species, of which diploid and tetraploid species are 45 and 7, respectively[2]. *Gossypium* is cultivated in semi-arid and arid tropical areas. Presently, there are four cultivars of cotton, two of them are diploid and two are allotetraploid. Estimating the amount and magnitude of genetic variation at a molecular level is important for cotton breeding. Allotetraploid cotton species, especially *G. hirsutum*, occasionally known as upland cotton, are the most widely cultivated species[3,4]. Nearly 96–97% of the worldwide cotton farming areas are accounted for by *G. hirsutum*, while *G. barbadense* accounts for only 2–3%, and only 1% of the world's cotton plantations grow *G. arboreum* and *G. herbaceum*[5,6]. Almost 100 countries cultivate *Gossypium* species throughout the world.

Pakistan's textile industry relies mostly on cotton as a source of raw materials because it is a substantial cash crop. It contributes 4.1% to the overall value added in the agricultural sector and 0.8% to GDP. After China, India, USA and Brazil, Pakistan is the world's fifth largest cotton production country[7]. During 2019 to 2020, Pakistan's contribution to global cotton production was 6%. Cotton is a significantly profitable crop used in a variety of industries[8]. Its seeds are processed to produce oil, which is used in the manufacturing of numerous products, including

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vegetable oil and soaps. Cotton plays a significant role in the textile industry serving as a primary source of raw materials for a wide range of products, including textiles, towels, and papers[9]. Additionally, cotton is utilized in biofuel production. With the anticipated global population reaching 11 billion by 2050[10], a growing demand for food, feed, fiber, and fuel, placing considerable strain on agricultural crop production. Factors contributing to this strain include climate change, the genetic diversity of cotton germplasms, and limited access to the latest generations of genetically engineered (GE) cotton seeds. To meet future needs, it is necessary to implement genetic modifications aimed at increasing yield and quality and enhancing resistance to biotic and abiotic stresses[11]. It is crucial to examine the degree of proximity of various genomes and their genetic strength for increased resistance to various stresses to meet current and future problems. This will help cotton species become more productive. Utilizing genetic diversity and linkages can help identify accessions that can endure shifting environmental conditions. It can be accomplished by enhancing it with useful gene-based molecular markers that are unaffected by changes in environmental conditions[12]. Molecular markers like AFLP, ISSR, RAPD and SSR have been employed to assess cotton diversity. SSRs have the advantages of vivid polymorphism, strong repeatability, codominance, and ease of use among various marker types[13]. SSR markers are considered a promising tool for assessing genetic diversity and relationships among cotton resources at the genomic level, primarily due to their high reproducibility and accuracy[14,15]. The information gained from these markers is beneficial for enhancing breeding techniques. This study was directed to assess the genetic diversity of 30 upland cotton genotypes using EST-SSR markers which help cotton breeders to improve productivity and quality in cotton breeding programs.

Materials and Methods

Plant materials

The research was executed in the Department of Plant Breeding and Genetics at Bahauddin Zakariya University, Multan. A set of 30 diverse genotypes of cotton (*G. hirsutum*) were collected from Central Cotton Research Institute (CCRI), Multan, Pakistan, and Cotton Research Station, Multan, Pakistan (Table 1). The cotton genotypes were cultivated within a greenhouse environment (temperature 25±2°C, relative humidity 65–70%). The accessions were planted in polyethylene bags (25×5 cm) and filled with about 1.15 kg of soil composed of peat, sand, and soil in a 1:1:1 ratio. Prior to planting, all bags were thoroughly flooded to reach field capacity. The overnight soaked seeds were sown at 2 to 3 cm depth with four seeds per bag for each genotype. As the seeds started germinating, only one plant per bag was retained, and the rest were removed.

Genomic DNA extraction

For DNA extraction, fresh, young and intact leaves were collected from each cotton genotype at a specific time and immediately placed on ice to prevent desiccation. These samples were then stored at -20°C

Table 1 — List of *G. hirsutum* genotypes used to identify genetic diversity

Name of accessions	Origin
FH-Lalazar	AARI, Faisalabad, Pakistan
AA-802	Ali Akbar Seeds, Pakistan
CIM-616	CCRI, Multan, Pakistan
Sitar-008	Aziz Group, Pakistan
IR-3701	NIBGE, Pakistan
CIM-599	CCRI, Multan, Pakistan
SLH-1	CRS, Sahiwal, Pakistan
MNH-786	CRS, Multan, Pakistan
CIM-625	CCRI, Multan, Pakistan
CIM-632	CCRI, Multan, Pakistan
FH-118	CRI, Faisalabad, Pakistan
CEMB-33	CEMB, Lahore, Pakistan
SLH-13	CRS, Sahiwal, Pakistan
FH-952	CRI, Faisalabad, Pakistan
CIM-622	CCRI, Multan, Pakistan
BH-160	CRS, Bahawalpur, Pakistan
FH-142	CRI, Faisalabad, Pakistan
FH-326	CRI, Faisalabad, Pakistan
SLH-19	CRS, Sahiwal, Pakistan
CIM-598	CCRI, Multan, Pakistan
DNH-105	ARI, Pakistan
IUB-222	IUB, Bahawalpur, Pakistan
Cyto-178	CCRI, Multan, Pakistan
NIBGE-2	NIBGE, Pakistan
FH-114	CRI, Faisalabad, Pakistan
SLH-6	CRS, Sahiwal, Pakistan
CIM-612	CCRI, Multan, Pakistan
NIAB-78	NIAB, Faisalabad, Pakistan
MNH-886	CRS, Multan, Pakistan
SLH-12	CRS, Sahiwal, Pakistan

[CCRI: Central Cotton Research Institute; CRS: Cotton Research Station; CRI: Cotton Research Institute; ARI: Agriculture Research Institute]

in the genomic laboratory of (DPB&G) Bahauddin Zakariya University, Multan, to prevent oxidation until DNA isolation. The CTAB protocol suggested by Doyle & Jeff[16] was employed for DNA extraction, with minor modifications by Baloch *et al.*[17]. Double-distilled water was used to dilute the isolated DNA and kept at -20°C to avoid denaturation. To assess the concentration and quality of the genomic DNA, we used an Implen Nanophotometer (Germany), and further confirmation of DNA quality was executed by running at 0.8% agarose gel. The final concentration of genomic DNA for the 30 samples, intended for polymerase chain reactions (PCR) was finalized to 30 ng/µL, and kept at -20°C until the commencement of PCR amplifications.

PCR amplification and electrophoresis detection

A total of 28 EST-SSR markers were employed to assess the genetic variability among studied cotton genotypes (Supplementary table 1). For the PCR reaction, the reagents were combined as follows: 1µL of DNA (30 ng μL^{-1}) was used as the template, 2 µL of (10×) PCR buffer containing 50 mM Tris at pH 8.3 and 500 mM KCl was added, 1.5 µL of dNTPs (10 mM), 2 µL of $MgCl_2$ (25 mM) and 1 µL of each forward and reverse primers (30 ng µL-1) were included. At the end, 0.2 µL (equivalent to 1U) of Taq DNA polymerase, obtained from Fermentas (USA), was added. To reach a final volume of 20 µL, 11.3 µL of ddH_2O was added. Amplification reactions were conducted using a Bio-Rad MyCycler thermal cycler (USA), with the following conditions: a first step of denaturation at 94°C for 10 min, followed by 30 cycles consisting of 30 s at 94°C, 1 min at 55-60°C, and 1 min at 72°C. Subsequently, a final extension was performed at 72°C for 10 min, and the tubes were subsequently stored at 4°C. Amplified products were visualized in an ultraviolet transilluminator, and gel images were captured using the "Gel Documentation System" (Photonyx, USA). The amplified fragment size was determined using a 100 bp DNA ladder (Fermentas, USA).

Data analysis

Only robust, clear and distinct bands were scored. Gel scoring was done using a binary method, with 1; denoting the presence of bands and 0; denoting the absence of bands. The band mobility and the molecular mass of the ladder were used to calculate the size of the amplicon. The data was used to determine genetic similarity based on the number of amplification products. The unweighted pair group arithmetic means approach (UPGMA) was utilized to create a dendrogram based on these similarity coefficients. The 30 cotton genotypes' genetic linkages were ascertained by using software (NTSYS-pc 2.0) to assess the similarity matrix and cluster the data using the UPGMA algorithm. The formula used to calculate the PIC value, as described by Anderson *et al.*[18], is $PICi = 1 - \sum P(i)\ 2$, where, pi; represents the frequency of allele. These PIC values offer an assessment ability of each locus to distinguish between alleles based on the number of alleles per locus and their relative frequencies in the population. Furthermore, according to Anderson *et al.*[18] the confusion probability (Cj) and discriminating power (Dj) for each primer pair was determined.

Results

In this study, we analyzed 30 cotton genotypes using EST-SSR primers. Out of the 28 primer pairs, seven primers showed a high polymorphism, while the remaining 21 were more similar (monomorphic). The primers produced different-sized amplicons ranging from 60 to 550 base pairs. The details of these primers are given in Table S1. Among the primers, Nau-4871, Nau-1231, and Nau-4014 produced the highest number of bands (3), while Nau-3158, Nau-1023, and others produced the lowest number of band (1) (Table 2).

Marker discriminating indices for SSRs

In present investigation, SSRs primers produced 37 alleles. The number of alleles per locus ranged from 1 to 3, with an average of 1.5 bands per primer (Table 2; Supplementary fig. 1). In this study, the PIC value ranged from 0.065 to 0.82, with an average of 0.407. Among the markers, Nau-1231 exhibited the highest PIC value (0.82) and Dj value (0.82), while having the lowest Cj value (0.531). On the other hand, primer Nau-4871 revealed the highest Cj value (1.55) and the lowest Dj (0.47) and PIC (0.48) values (Table 2).

Similarity matrix and diversity analysis for SSRs

The genetic similarity or diversity of 30 cotton genotypes was determined by analysing amplified DNA amplicon profiles. The similarity matrix based on the SSR markers revealed a higher level of genetic similarity of 0.93 among the selected cotton genotypes. Furthermore, pairwise comparisons using the EST-SSR markers showed that the similarity

Table 2 — SSR markers discriminating indices

Primer name	No. of loci	Allele size (bp)	PIC	Cj	Dj
NAU915	1	210	-	-	-
NAU1014	2	180-200	0.19	0.8069	0.19
NAU1023	1	200	0.065	0.9333	0.065
NAU1063	2	200-240	-	-	-
NAU1070	1	160	-	-	-
NAU1207	1	200	-	-	-
NAU1231	3	240-260	0.82	0.531	0.82
NAU1301	1	210	-	-	-
NAU2002	1	370	-	-	-
NAU2220	1	200	-	-	-
NAU3009	1	310	-	-	-
NAU3093	1	275	-	-	-
NAU3158	1	240	-	-	-
NAU3234	1	180	-	-	-
NAU3239	1	200	-	-	-
NAU3279	1	340	-	-	-
NAU3306	1	350	0.128	0.811	0.128
NAU3427	1	200	-	-	-
NAU3735	1	540	-	-	-
NAU3558	1	220	-	-	-
NAU3773	1	330	-	-	-
NAU3897	2	180-190	0.594	0.726	0.594
NAU4014	3	200-290	0.573	1.2689	0.573
NAU4047	1	340	-	-	-
NAU4065	1	550	-	-	-
NAU4871	3	60-160	0.479	1.554	0.479
NAU4912	1	150	-	-	-
NAU5046	1	220	-	-	-

[Cj: Confusion probability; PIC: Polymorphic information contents; Dj: Discriminating power; bp: Base pair]

ranged from 0.78 to 1.00 among the designated cotton germplasm (Supplementary table. 2). A total of 28 EST-SSR produced 37 alleles and dendrograms were constructed using the UPGMA. The dendrogram partitioned thirty genotypes into four primary clusters by truncating at a genetic similarity level of 0.93. However, three genotypes, namely DNH-105, Cyto-178, and FH-326, were identified as independent (Fig. 1).

Cluster 1 consisted of two genotypes, AA-802 and BH-160 FH-Lalazar, while CIM-616 belonged to Cluster 2. Cluster 3 comprised 21 genotypes, which were further subdivided into six sub-clusters denoted as 3a, 3b, 3c, 3d, 3e, and 3f. Sub-cluster 3a consists of 4 genotypes (IR-3701, Sitar-008, CIM-625, CIM-598) and CIM-622 and SLH-19 were independent, and 3b consisted of 6 genotypes, *i.e.*, MNH-786, SLH-1, SLH-12, FH-142, SLH-6, CEMB-33, while IUB-222 was independent. Subcluster 3c consisted of 2 genotypes (CIM-599 and FH-118). Subcluster 3d also contained 2 genotypes (CIM-632 and FH-952). Subcluster 3e comprised CIM-612, NIAB-78 while Cluster 3f consisted of two genotypes, *i.e.*, NIBG-2 and MNH-886. Cluster 4 also consisted of 2 genotypes (FH-114 and SLH-113).

Discussion

The genetic diversity of 30 cotton genotypes was investigated in this study. The investigation of the genetic differentiation within plant breeding population is a crucial aspect, as it serves as the foundation for any cultivar breeding and enhancement initiative[19]. Because of this, novel alien genes can be introduced, resulting in cultivars/hybrids better equipped to withstand the effects of a changing climate[20]. The existence of genetic variability is a necessary condition for successful development of novel cultivars that can fulfil our current and future needs. The present investigation involved the screening of 28 SSR markers, and a subset of 7 markers were chosen based on their high level of polymorphism, reproducibility and appropriateness for the multiplicity paneling of 30 cotton genotypes. Although a relatively lower level of polymorphism was displayed by the EST-SSR markers, the genetic diversity shown by the EST-SSR was found to be promising as these are functional markers[14]. SSR markers are widely favoured due to their codominant and inheritance nature, high level of informativeness, and transferability, rendering them a preferred marker for plant germplasm enhancement programs[21].

The existence of genetic variability is crucial for cotton and other field crop breeders when it comes to interspecific and intraspecific hybridization. The evaluation of genetic variability and the utilization of DNAbased fingerprinting are crucial in identifying genotypes and grouping them into distinct heterotic categories for the purpose of crop improvement programs that rely on hybridization techniques[22]. Various types of molecular markers, including Restriction Fragment Length Polymorphisms (RFLPs); Random Amplified Polymorphic DNA (RAPD); Amplified Fragment Length Polymorphisms (AFLPs); “Simple Sequence Repeats (SSRs); ;Single Nucleotide Polymorphisms (SNPs), are utilized in studies of genetic diversity, DNA fingerprinting, and variability[23]. The utilization of Simple Sequence Repeats (SSRs) has proven to be effective in cotton DNA fingerprinting, genetic diversity analysis and QTL mapping[23]. This improved the conservation of

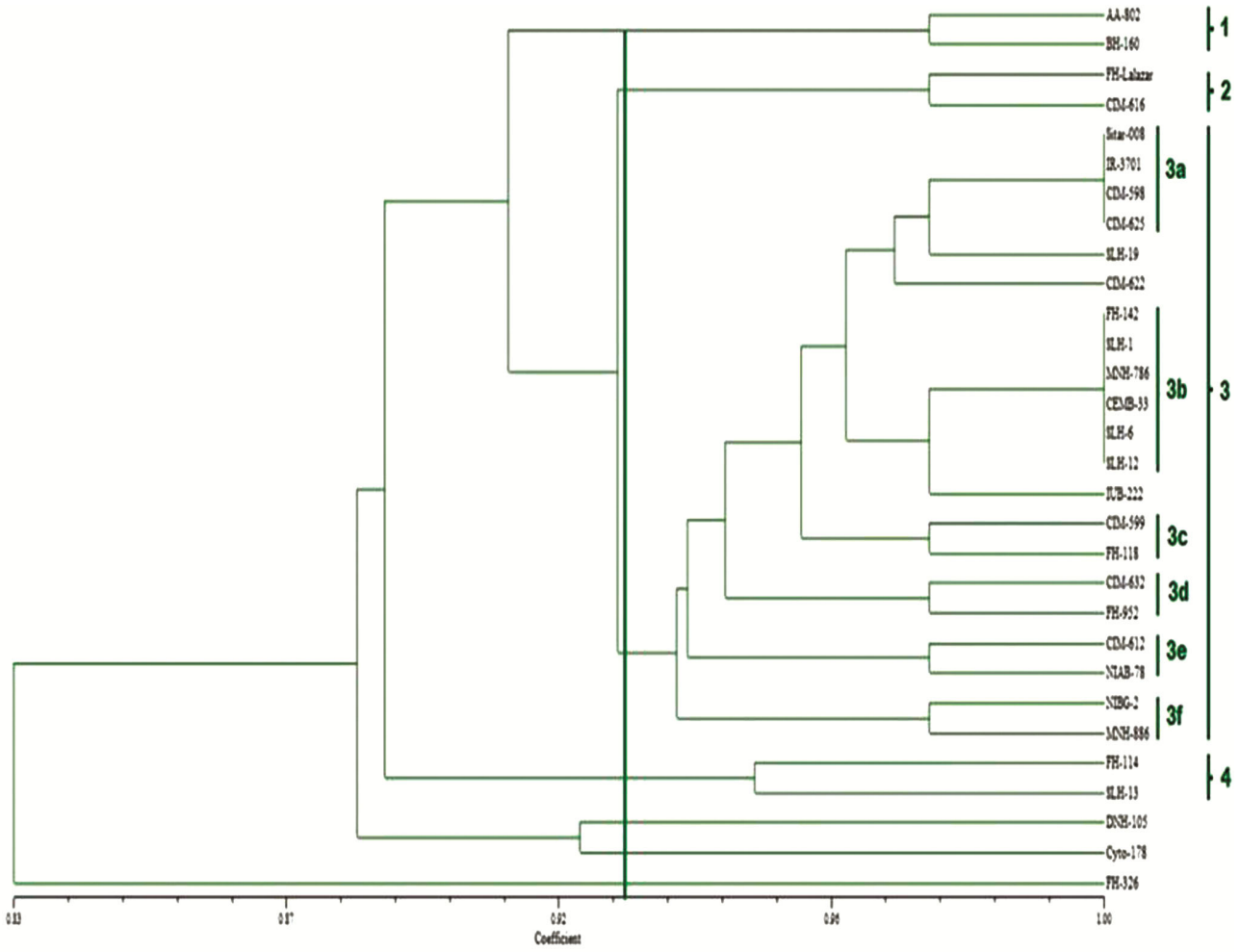

Fig. 1 — UPGMA dendrogram of thirty cotton genotypes produced using EST-SSR data that shows the genetic distances. The identified major clusters and sub-clusters are shown on the right.

cotton germplasm possessions and promoted the genetic enhancement of cotton varieties.

The molecular data obtained from the aforementioned markers was subsequently analysed to ascertain the genetic similarities among the accessions. The current research exhibits a substantial level of genetic resemblance among the examined germplasm. Ullah *et al.*[24] demonstrated that 19 genotypes of Bt cotton exhibit a considerable degree of genetic similarity utilizing SSR markers. Likewise, the study carried out by Yasmin *et al.*[25] investigated the presence of a significant degree of genetic resemblance among various cotton genotypes. In our investigation, the dendrograms generated using EST-SSR markers revealed variations in the entire number of clusters and the arrangement of genotypes within these clusters. The observed variation could potentially be attributed to the distinct genomic regions identified by various markers[26]. The dendrogram clustering analysis utilizing EST-SSR markers revealed that the majority of clusters comprised of genotypes from public and private sectors. The observed similarity could potentially be attributed to the recurrent utilization of exceptional gene pools, resulting in a limited genetic base for existing germplasms[15].

In addition, typically breeders incorporate the same elite parental lines in their breeding initiatives, resulting in a high degree of relatedness among cotton breeds. In their study, Khan *et al.*[27] employed SSR markers to group a total of forty genotypes into three distinct clusters, with an average similarity ranging from 36 to 89%. Anderica *et al.*[18] analysed the genetic distance between different cotton genotypes using 62 SSR markers which resulted in the classification of the 48 genotypes into three main groups. Kuang

et al.[21] employed SSRs to investigate the genetic diversity of cotton accessions through cluster analysis, resulting in the categorization of all accessions into five groups at a similarity coefficient of 0.57.

The amplification products generated per primer for cotton genotypes exhibited a range of 1 to 3, with a mean of 1.32 bands per primer. The findings of the present investigation are consistent with those of Khan *et al.*[27] earlier research, which examined the genetic variability of cotton germplasms through the use of SSR markers. The current study reports a slightly lower mean number of alleles compared to previous investigations on genetic diversity in cotton. Javaid *et al.*[28] studied genetic diversity of 22 cotton accessions, and observed an average of 3.72 alleles per locus utilizing 30 SSR markers. Likewise, Gurmessa *et al.*[29] documented 3.8 alleles per locus in cotton genotypes. Conversely, McCarty et al. [30] found a relatively elevated alleles i.e., 7.9 per locus. The PIC values for various primers vary among different genotypes. Markers with higher PIC values possess the capacity to capture greater genetic diversity and allelic differentiation. The PIC value in our study varied between 0.065 and 0.82. Cai *et al.*[31] conducted an analysis of 99 *G. hirsutum* and two *G. barbadense* genotypes and reported PIC value of 0.4 for 20 SSRs, which is consistent with the findings of our study.

The findings (PIC= 0.40) of Bertini *et al.*[32], who utilized markers (SSR) to examine the genetic relationships among diverse Brazilian cultivars of cotton (*G. hirsutum*), were consistent with the average PIC results found in our study. This PIC value was slightly lower than the 0.46 reported by Tu *et al.*[33], who evaluated the genetic relationships among various genotypes of *G. hirsutum*. Our results also fell short of the PIC value of 0.62 presented by Guang & Xiong-Ming [34], who used SSR markers to assess the genetic diversity across various upland cotton cultivars. Furthermore, our results (PIC value) were lower than Zhang *et al.*[35], who used EST-SSR markers to study genetic diversity across different cultivars of cotton (*G. hirsutum*) from China's key cotton-cultivating regions. The fluctuation observed in the values of PIC can be attributed to the genetic composition of the primer material. The concept of polymorphism has exhibited notable variations across various species, as evidenced by the divergent mean polymorphic information content (PIC) values observed in different cotton genotypes[13]. Ge *et al.*[4] stated that loci exhibiting a PIC value between 0.25 and 0.5 are indicative of moderate polymorphism. Thus, it can be inferred that the utilization of SSR markers is a viable approach for investigating the genetic relatedness of cotton, as indicated by the intermediate PIC value obtained. Menezes *et al.*[36] reported that markers exhibiting higher PIC values are more effective in identifying polymorphism within a given population. In their study, Yu *et al.*[37] determined the mean PIC value of 0.713 for a set of 25 SSRs across 12 cotton genotypes that were representative of six distinct *Gossypium* species. Tyagi *et al.*[38] obtained average PIC value of 0.17 from the investigation of 378 accessions utilizing 120 SSR markers. Moiana *et al.*[39] reported a PIC value of 0.361 based on the analysis of 20 accessions using 27 SSR markers. Qin *et al.*[40] obtained PIC value of 0.3 on average from the analysis of 241 accessions employing 333 SSR markers. Kuang *et al.*[21] determined the SSR markers PIC across 79 cotton genotypes. The PIC values ranged from 0.1841 to 0.9043, with 0.6494 being the average. is in contrast to prior findings. The observed level of polymorphism in this investigation is attributed to the utilization of distinct materials derived from two distinct cultivated species of cotton. Furthermore, the diverse genotypes belonging to a particular species were chosen from distinct agro-climatic regions. The efficiency of primers is a crucial parameter, particularly when they are utilized for studies on genetic diversity. The Nau-1231 exhibited the highest PIC value (0.82) among the EST-SSR, indicating its excellent capability to detect allelic variation. Moreover, these primers demonstrated a higher propensity to differentiate between two genotypes due to their high Dj and low Cj values[41].

Conclusion

In conclusion, the investigation revealed a relatively low genetic diversity, with 37 alleles detected across the SSR markers. Importantly, the high polymorphic information content (PIC) value, averaging 0.403, underscores the utility and reproducibility of EST-SSR markers for germplasm characterization. These findings emphasize the significance of understanding genetic variability in cotton breeding programs, providing valuable insights to enhance yield, fiber quality, and disease resistance. Moving forward, further research employing diverse marker systems, particularly EST-SSR markers, holds promise for exploring cotton's interaction with

climatic changes and stress tolerance, thereby contributing to the development of improved cotton varieties.

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Conflict of interest

The authors declare no conflict of interest.

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