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MOLECULAR DIVERSITY AMONG DROUGHT-RESPONSIVE UPLAND COTTON (GOSSYPIUM HIRSUTUM L.) GENOTYPES ASSESSED WITH SSR MARKERS AND ITS USE IN PARENT SELECTION FOR RAINFED

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ABSTRACT
Cotton (Gossypium spp.) is one of the most important commercial fiber crops cultivated throughout the world. Despite its economic significance, cotton productivity is influenced by several environmental factors and stresses. Among the various abiotic stresses drought limits the yield of rainfed cotton and breeders choosing parents for drought tolerance need to know how much genetic variation exist among them. We used 15 simple sequence repeat (SSR) markers to characterise 60 upland cotton (Gossypium hirsutum L.) genotypes that had earlier been evaluated under irrigated and rainfed conditions. Eleven primers amplified reproducibly; eight were polymorphic and together produced 39 alleles (3–7 per primer, mean 4.75), with fragment sizes of 100–600 bp. Polymorphism information content (PIC) ranged from 0.326 (NAU3203) to 0.771 (JESPR153), with a mean of 0.594 and gene diversity ranged from 0.358 to 0.800 (mean 0.642). JESPR153, DPL0045 and NAU2954 were the most informative markers, although DPL0045 amplified in only about half of the genotypes. Jaccard dissimilarity between genotype pairs ranged from 0.32 to 0.89 and UPGMA clustering divided the panel into five groups of 21, 14, 13, 9 and 3 genotypes. The four genotypes classed as drought tolerant on yield-based indices (CBMH-24, CBMH-41, CBMH-38 and CBMH-60) fell in four different clusters and highly susceptible genotypes shared clusters with tolerant ones, so molecular grouping did not follow drought response. Because the marker set was small, the clusters give only a coarse picture of relatedness. Crosses among the tolerant genotypes, which are molecularly distinct from one another, are candidate combinations for broadening the genetic base of drought tolerance in rainfed upland cotton.

KEYWORDS: Gossypium hirsutum; SSR markers; genetic diversity; drought tolerance; polymorphism information content; UPGMA

INTRODUCTION
Cotton belongs to the genus Gossypium under the tribe Gossypieae of the family Malvaceae is one of the most important commercial fiber crops cultivated throughout the world and is popularly known as “white gold” because of its immense economic value (Shahrajabian et al., 2020). Upland cotton (Gossypium hirsutum L.) supplies most of the world’s natural textile fibre and is also a source of edible oil and protein meal. Cotton is grown in more than 100 countries under a wide range of agro-ecological conditions. Major cotton producing countries include India, China, United States, Brazil and Pakistan (Abdullah and Khan., 2022). Among these countries, India occupies the first position in terms of area under cotton cultivation. In India a large part of the cotton area is rainfed, so yield depends heavily on how much moisture the crop receives. Water deficit disturbs plant growth, flowering and boll set, physiological efficiency and fibre quality and the final loss in seed cotton yield depends on the timing, intensity and duration of the stress as well as on the genotype (Shani et al. 2025). Breeding cultivars that hold their yield under moisture stress has therefore been a standing objective of cotton improvement programmes.

Parents for such programmes are only useful if their genetic differences are known. Morphological and agronomic data help, but stress-related traits are strongly affected by the environment and phenotypic similarity can hide genetic differences or exaggerate them. DNA markers avoid this problem. Genetic diversity analysis using molecular markers have been superior over conventional breeding (Melchinger et al., 1991). Simple sequence repeats (SSRs) are reproducible, spread across the genome and inexpensive to run and are in principle co-dominant; they have been widely used in cotton to measure diversity, group germplasm and select parents (Abdalla et al., 2025; Azimov and Shavkiev, 2026). Cultivated upland cotton is also known to have a narrow genetic base, which makes it more important to know how much variation a chosen set of parents actually carries.

We evaluated the 60 genotypes examined here under irrigated and rainfed conditions for morphological, physiological, biochemical and seedling traits. Yield-based tolerance indices identified four drought-tolerant genotypes (CBMH-38, CBMH-24, CBMH-41 and CBMH-60) and four highly susceptible ones (CBMH-53, CBMH-71, CBMH-64 and CBMH-85). The present paper reports the molecular characterisation of the same panel. Our objectives were (i) to measure the polymorphism and informativeness of a set of SSR markers in these germplasm, (ii) to estimate genetic dissimilarity among the 60 genotypes and group them by UPGMA clustering and (iii) to examine whether molecular grouping relates to drought response and to identify divergent genotypes that could serve as parents for improving drought tolerance under rainfed conditions.

2. MATERIALS AND METHODS

2.1 PLANT MATERIAL AND FIELD EVALUATION

The material comprised 60 genotypes of upland cotton from the cotton breeding material of research farm.The genotypes were evaluated under irrigated (E1 and E3) and rainfed (E2 and E4, with pre-sowing irrigation only) conditions for two years (kharif 2023 and kharif 2025) at the Research Farm of the Cotton Section, Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar, in a randomised block design with two replications. Drought tolerance was classified with the yield stability index (YSI; Bouslama and Schapaugh, 1984) and the drought susceptibility index (DSI; Fischer and Maurer, 1978), both calculated from seed cotton yield under the two water regimes.

2.2 GENOMIC DNA ISOLATION

Genomic DNA was isolated from young leaves of all 60 genotypes by the cetyl trimethyl ammonium bromide (CTAB) method of Saghai-Maroof et al. (1984), with minor modifications to the extraction buffer (200 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 2% PVP and 1% β-mercaptoethanol). Leaf tissue (250–300 mg) was ground in liquid nitrogen and incubated in pre-heated extraction buffer at 65 °C. The lysate was extracted with chloroform:isoamyl alcohol (24:1) and the DNA was precipitated with chilled isopropanol, washed twice in 70% ethanol, air-dried and dissolved in TE buffer. RNA was removed with RNase (10 mg/mL) at 37 °C for 1 h. DNA concentration and purity were checked by spectrophotometry (A260/A280 of 1.8–2.0) and integrity by electrophoresis on 1.0% agarose gels against lambda DNA standards.

2.3 SSR AMPLIFICATION AND GEL ELECTROPHORESIS

Fifteen SSR markers from the NAU, JESPR and DPL series, chosen to give wide coverage of the genome, were used for amplification. Reactions of 10 µL were run in a Bio-Rad T100 thermal cycler. Each contained 1.0 µL of 5X Green GoTaq Flexi buffer, 1.0 µL of 25 mM $MgCl_2$, 0.2 µL of 10 mM dNTPs, 0.4 µL each of 10 µM forward and reverse primers, 1.0 µL of genomic DNA (50 ng/µL), 0.1 µL of Taq DNA polymerase (5 U/µL) and sterile water to volume. Cycling consisted of initial denaturation at 94 °C for 4 min; 34 cycles of 94 °C for 1 min, annealing at the primer-specific temperature (46–55 °C) for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 7 min. Amplified products were separated on 3% agarose gels in 1X TBE at 90–110 V for 2–3 h, stained with ethidium bromide, viewed under UV light and sized against a 100 bp DNA ladder.

2.4 DATA SCORING AND ANALYSIS

Bands were scored as present (1) or absent (0) for each marker in each of the 60 genotypes and fragment sizes were estimated against the 100 bp ladder. Distinct band sizes amplified by a primer were treated as alleles. For every polymorphic primer we calculated the number of alleles, major allele frequency, gene diversity ($H = 1 - \Sigma pi2$; Nei, 1973) and PIC (Botstein et al., 1980), where $PIC = 1 - \Sigma pi2 - \Sigma\Sigma 2pi2pj2$ and pi is the frequency of the ith allele. Allele availability was taken as the proportion of the 60 genotypes that gave a scorable product with a primer. Pairwise genetic dissimilarity was calculated from the band data with the Jaccard coefficient (Jaccard, 1908) in DARwin 6.0 (Perrier and Jacquemoud-Collet, 2006) and the dissimilarity matrix was clustered by the unweighted pair group method with arithmetic mean (UPGMA) to obtain a dendrogram.

3. RESULTS

3.1 SSR AMPLIFICATION AND POLYMORPHISM

Molecular characterization of 60 upland cotton genotypes was carried out using simple sequence repeat (SSR) markers to assess the extent of genetic diversity among the experimental material. A total of 15 SSR primers were employed for amplification of genomic DNA through polymerase chain reaction (PCR). Out of these, 11 primers showed successful amplification, whereas four primers failed to amplify. Among the amplified primers, eight primers were polymorphic while three primers were monomorphic. The amplified PCR products were separated through agarose gel electrophoresis and clear polymorphic banding patterns were observed among the genotypes. The amplified fragment size ranged from 100 to 300 bp.

3.2 Allelic variation and marker informativeness

The eight polymorphic primers produced 38 alleles, with 3 to 7 alleles per primer (mean 4.75; Table 2). NAU3203 gave the fewest alleles (3) and NAU2954 the most (7). JESPR153 amplified six alleles, JESPR135, NAU4105 and DPL0045 five each and NAU1151 and NAU2836 four each. Major allele frequency ranged from 0.294 (JESPR153) to 0.785

(NAU3203). JESPR153 had the lowest value, meaning its alleles were spread more evenly across the genotypes than those of any other marker.

PIC ranged from 0.326 to 0.771 with a mean of 0.594. JESPR153 had the highest PIC (0.771), followed by DPL0045 (0.733) and NAU2954 (0.717); NAU3203 had the lowest (0.326). Six of the eight primers had a PIC above 0.5, the level Botstein et al. (1980) used to define highly informative markers and the remaining two (NAU1151, 0.388; NAU3203, 0.326) fell in the reasonably informative range of 0.25–0.5. Gene diversity ranged from 0.358 (NAU3203) to 0.800 (JESPR153), with a mean of 0.642. Allele availability was 90.7–99.3% for seven primers but only 52.7% for DPL0045, which therefore produced a scorable product in roughly half of the genotypes.

3.3 GENETIC DISSIMILARITY AMONG GENOTYPES

Jaccard dissimilarity coefficients among the 60 genotypes ranged from 0.32 to 0.89, which points to considerable molecular variation in the panel. Low values indicate closely related genotypes and high values indicate wide divergence.

3.4 CLUSTER ANALYSIS

All the genotypes were scored for presence and absence of bands for the specific alleles of each SSR markers. UPGMA clustering of the Jaccard dissimilarity matrix divided the 60 genotypes into five clusters (Fig. 2; Table 3). Cluster I was the largest with 21 genotypes, followed by Cluster IV with 14, Cluster II with 13 and Cluster III with 9. Cluster V was the smallest and contained three genotypes (CBMH-7, CBMH-9 and CBMH-10). Genotypes within a cluster were more similar to each other than to genotypes in other clusters. The membership of each cluster is given in Table 3.

3.5 Molecular grouping and drought response

The four drought-tolerant genotypes were spread over four of the five clusters: CBMH-24 (DSI 0.481; YSI 0.93) in Cluster I, CBMH-41 (DSI 0.493; YSI 0.93) in Cluster II, CBMH-38 (DSI 0.099; YSI 0.99) in Cluster III and CBMH-60 (DSI 0.505; YSI 0.93) in Cluster IV. The highly susceptible genotypes were scattered in the same way. CBMH-53 (DSI 2.754) and CBMH-64 were in Cluster IV, alongside the tolerant CBMH-60 and CBMH-85 (DSI 2.138) and CBMH-71 were in Cluster I, alongside the tolerant CBMH-24. Tolerant genotypes therefore did not group together and Clusters I and IV each held both tolerant and susceptible entries.

4. DISCUSSION

Eight of the 11 primers that amplified (72.7%) were polymorphic across the 60 genotypes. They produced 38 alleles and a mean PIC of 0.594. This is above the mean PIC of 0.38–0.53 reported for several upland cotton panels (Altaf et al., 2024; Celik, 2022; Ali et al., 2025; Azimov and Shavkiev, 2026), but the comparison needs care. Our mean was calculated only for polymorphic primers, which raises it relative to studies that average over every primer tested and the fragments were resolved on agarose, where bands of similar size may not separate. JESPR153, DPL0045 and NAU2954 were the most informative markers and are good candidates for fingerprinting drought-responsive breeding material. DPL0045 should be used with caution, however, because it gave data for only about half of the genotypes and its PIC may change once the missing data are filled.

Jaccard dissimilarity of 0.32–0.89 confirms that the CBMH series carries broad molecular variation, in keeping with the wide phenotypic variation the same panel showed for morphological, physiological, biochemical and seedling traits. The range is wider than those reported for Brazilian cultivars (dissimilarity 0.13–0.73; Moiana et al., 2015) and Chinese cultivars (similarity 0.53–0.99, equivalent to dissimilarity 0.01–0.47; Zhang et al., 2011) and wider than the narrow base found in Pakistani drought-tolerance panels (Javaid et al., 2017). Differences in marker number and in the coefficient used limit such comparisons and with only eight polymorphic loci each pairwise value is coarse (Shukla et al., 2021; Kushanov et al., 2021). We therefore take the range as evidence of exploitable variation in the germplasm, not as a precise measure of it.

Cluster membership agreed with pedigree only partly. CBMH-9 (H 1226 × RS 875) was in Cluster V, whereas CBMH-32 and CBMH-39, which come from the same cross, were together in Cluster I. CBMH-7 (H 1226 × PIL 8) joined CBMH-9 in Cluster V, but CBMH-4 (H 1117 × H 1226) was in Cluster II. Lines with H 1117 in their pedigree (CBMH-4, CBMH-16 and CBMH-38) were spread across Clusters II and III and the two H 1470 × H 1316 lines, CBMH-50 and CBMH-52, were in Clusters I and IV. Related lines sometimes grouped together but often did not. This is what would be expected after recombination and selection have reshuffled the parental genomes, although sampling error from a small marker set may also contribute. Earlier work on upland cotton likewise found that molecular clusters matched pedigree only in broad terms (Ali et al., 2025). The dendrogram was not tested by bootstrapping and Cluster V contains only three genotypes, so the deeper branching should be treated as provisional.

The main finding is that molecular grouping and drought response were unrelated. Each of the four tolerant genotypes sat in a different cluster and susceptible genotypes shared Clusters I and IV with tolerant ones. This is what one would expect if the SSRs, which sample the genome more or less at random, reflect general relatedness and are not linked to the loci that control drought response. It is also consistent with tolerance in this panel being controlled by many loci, although this study cannot show that directly. For breeding, the scatter of tolerant genotypes across clusters is helpful. Crosses among them, such as CBMH-24 × CBMH-38 or CBMH-41 × CBMH-60, bring together tolerant parents that are molecularly distinct, which should improve the chance of transgressive segregants for yield stability under rainfed conditions (Moiana et al., 2015; Fatima et al., 2025). Which of these crosses to make should be decided from the pairwise dissimilarity values and from agronomic data on complementary traits, not from cluster membership alone.

The study has several limitations. Only eight polymorphic loci were scored, which is enough to show broad relatedness but not fine structure and we did not attempt model-based inference of subpopulations. Fragments were resolved on agarose gels and distinct band sizes were counted as alleles even though G. hirsutum is an allotetraploid in which a primer may amplify homoeologous loci. Four primers failed to amplify and DPL0045 gave data for only about half of the panel. The cluster assignments should be validated with a denser marker set, preferably with fluorescent or capillary genotyping and the relationship between molecular and phenotypic distance should be tested formally, for example with a Mantel test on the matrices from this panel, before the clusters are used to direct crosses. Marker–trait association analysis would be the logical next step for drought-responsive traits.

Table 1. The 60 upland cotton (Gossypium hirsutum L.) genotypes evaluated. Serial numbers correspond to lanes 1–60 in Fig. 1 and to labels G1–G60 in Fig. 2.

Sr. no	Genotype	Pedigree
1	CBMH-1	H 974 x J 34 P2-3-1-1-1-1
2	CBMH-4	H1117 x H 1226 P1-1-1-1-1
3	CBMH-7	H1226 x PIL 8 P2-2-2-1-3-2-1
4	CBMH-9	H 1226 x RS 875 P5-5-4-1-1-1-1
5	CBMH-10	JK 1050 BG II P2-1-1-2-1-1
6	CBMH-11	Pancham P2-1-1-1-1-1
7	CBMH-12	Khakhi Kapas P1-1-3-1-1
8	CBMH-16	F 2228 x H1117 P1-2-1-1-1-1
9	CBMH-18	H 1508 P1
10	CBMH-19	Bio 6488-2 BG II P2-1-1-2-1
11	CBMH-24	GM 19-1-2-1-1
12	CBMH-27	GM 27-2-1-1-1
13	CBMH-28	GM 34-2-2-2-1
14	CBMH-29	GM 33-1-1-1-1
15	CBMH-32	H 1226 X RS 875 P2-3-1-1-1-1
16	CBMH-35	Coker P2-1-1-1-1
17	CBMH-36	H974 x RS 875 P1-2-2-1-1
18	CBMH-38	H 1117 x PIL 8 P4-1-2-1-1-1
19	CBMH-39	H 1226 x RS 875 P3-8-3-1-1-1
20	CBMH-41	H-1464
21	CBMH-42	H 1490 P1-2
22	CBMH-46	H 1098I X H 1442 P1-1-1-2-1
23	CBMH-47	H 1477 P2-2
24	CBMH-49	LH 2108 X H 1098 P5-1-1-2-1
25	CBMH-50	H 1470 X H 1316 P3-1-1-1-1
26	CBMH-52	H 1470 X H 1316 P5-2-1-1-1
27	CBMH-53	H 1481 X H 1353 P1-2-1-1
28	CBMH-54	H 1489 P1-1
29	CBMH-55	H 1481 X H 1465 P2-1-1-1
30	CBMH-56	H 1480 X H 1353 P1-2-1-1
31	CBMH-58	GTHH-217 P1-1-1-1
32	CBMH-59	FHH 261 P2-1-1-1-1
33	CBMH-60	H 1487 P1-2-1

Sr. no	Genotype	Pedigree
34	CBMH-61	RCH 791 P3-1-2-1-1-1
35	CBMH-62	Ankur 3224 P1-1-1-2-1
36	CBMH-63	H1475 x H 1300 P1-1-1-1-1
37	CBMH-64	PCH 402 Bt P1-1-2-1-1
38	CBMH-65	PCH 402 Bt P1-2-3-1-1
39	CBMH-66	PCH 402 Bt P2-1-1-1-1
40	CBMH-68	NCS 9030 BGII P2-4-2-1-1
41	CBMH-69	NCS 1916 Bt P1-4-1-1-1
42	CBMH-70	Deltapine 66
43	CBMH-71	Fergusan
44	CBMH-73	PIL 43
45	CBMH-74	Unknown
46	CBMH-75	P22
47	CBMH-76	P25
48	CBMH-77	PRT 35
49	CBMH-78	J 2 P 7
50	CBMH-79	GP 321
51	CBMH-80	Mex 685-3
52	CBMH-81	HS 90-8
53	CBMH-83	Bio-100-P3-1-1-2-1
54	CBMH-84	Bio-100-P3-5-1-1-1
55	CBMH-85	Bio-100-P3-10-1-1-1
56	CBMH-87	Bio-100-P12-2-1-1-1
57	CBMH-89	Bio-100-P12-5-1-1-1
58	CBMH-92	GCH-3-5-1
59	CBMH-93	H-1530
60	CBMH-94	H 1480

Table 2. Major allele frequency, number of alleles, allele availability, gene diversity and PIC of the eight polymorphic SSR markers. Total number of alleles = 39.

Sr. no	Primer	Major allele freq.	Alleles	Allele availability	Gene diversity	PIC	Band size (bp)
1	NAU1151	0.693	4	0.913	0.453	0.388	120-280
2	NAU2836	0.497	4	0.993	0.666	0.618	100-400
3	NAU3203	0.785	3	0.960	0.358	0.326	100-250
4	JESPR153	0.294	6	0.907	0.800	0.771	140-600
5	NAU2954	0.348	7	0.940	0.755	0.717	100-420
6	JESPR135	0.366	5	0.947	0.718	0.671	100-400
7	NAU4105	0.634	5	0.967	0.556	0.522	150-450
8	DPL0045	0.316	5	0.527	0.770	0.733	150-600
	Mean	**0.492**	**4.88**	**0.893**	**0.635**	**0.593**	

Table 3. Distribution of the 60 upland cotton genotypes into five UPGMA clusters (from Fig. 2).

Cluster	No. of genotypes	Genotypes
I	21	CBMH-1, CBMH-11, CBMH-12, CBMH-18, CBMH-19, CBMH-24, CBMH-28, CBMH-29, CBMH-32, CBMH-36, CBMH-47, CBMH-49, CBMH-50, CBMH-54, CBMH-61, CBMH-62, CBMH-63, CBMH-71, CBMH-85, CBMH-89, CBMH-93
II	13	CBMH-4, CBMH-41, CBMH-42, CBMH-46, CBMH-56, CBMH-59, CBMH-68, CBMH-69, CBMH-70, CBMH-73, CBMH-76, CBMH-77, CBMH-78
III	9	CBMH-16, CBMH-27, CBMH-38, CBMH-79, CBMH-80, CBMH-83, CBMH-84, CBMH-87, CBMH-94
IV	14	CBMH-35, CBMH-39, CBMH-52, CBMH-53, CBMH-55, CBMH-58, CBMH-60, CBMH-64, CBMH-65, CBMH-66, CBMH-74, CBMH-75, CBMH-81, CBMH-92
V	3	CBMH-7, CBMH-9, CBMH-10

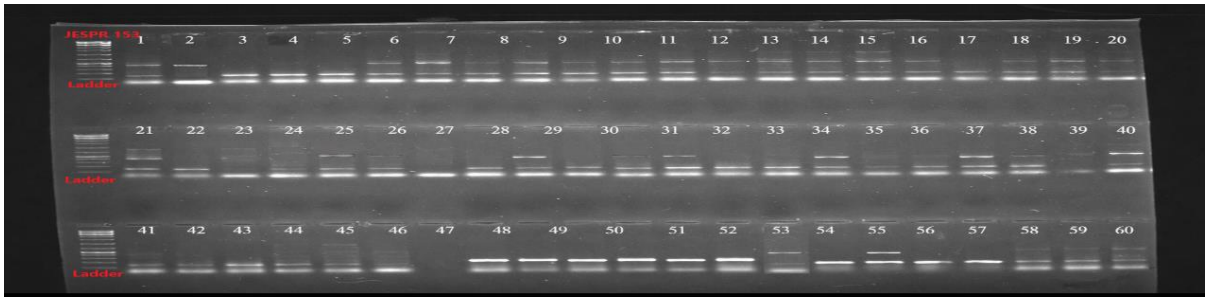

Fig. 1. Amplification profile of primer JESPR153 across the 60 upland cotton genotypes on a 3% agarose gel (lanes 1–60, in the order of Table 1; ladder, 100 bp).

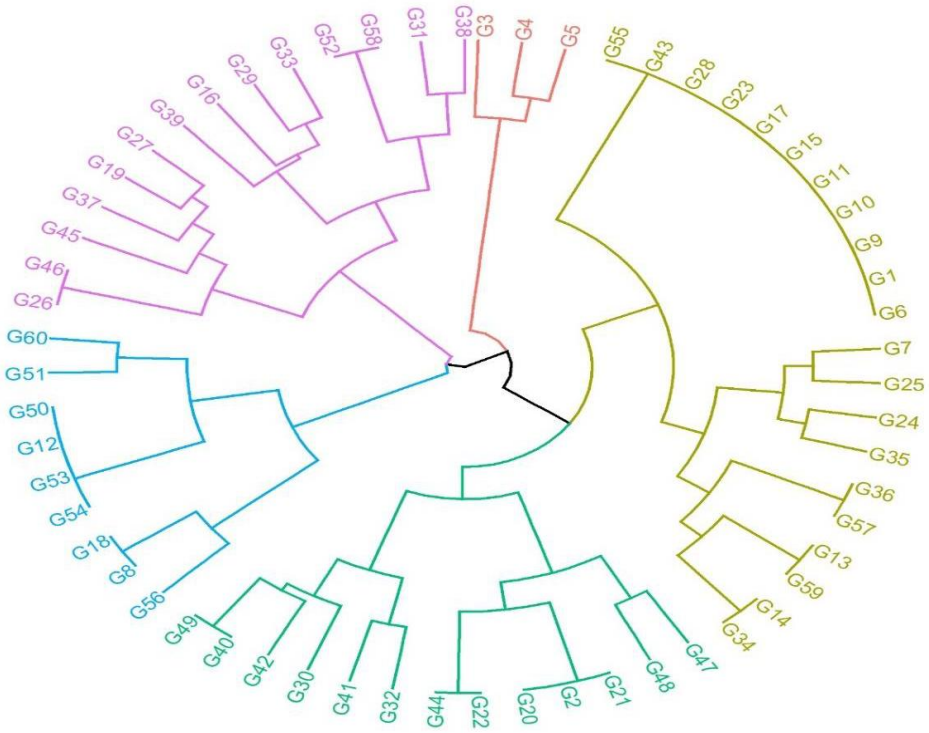

Fig. 2. UPGMA dendrogram based on Jaccard dissimilarity coefficients for the 60 upland cotton genotypes. Labels G1–G60 are the serial numbers in Table 1. Colours mark clusters: olive, I; green, II; blue, III; pink, IV; red, V.

5. CONCLUSION

Eight polymorphic SSR markers with a mean PIC of 0.593 revealed considerable molecular variation among 60 drought-responsive upland cotton genotypes, which UPGMA clustering separated into five groups at Jaccard dissimilarities of 0.32–0.89. JESPR153, DPL0045 and NAU2954 were the most informative markers, although DPL0045 needs to be re-tested because it amplified in only about half of the panel. The drought-tolerant genotypes CBMH-24, CBMH-41, CBMH-38 and CBMH-60 each fell in a different cluster and susceptible genotypes occupied the same clusters as tolerant ones, so molecular similarity did not predict drought response in this material. Crosses among the tolerant genotypes and between them and other divergent genotypes from different clusters, are candidates for generating segregating populations aimed at yield stability under rainfed conditions. Their value should be confirmed in field trials and the cluster assignments with a denser marker set, before they are used to direct a breeding programme.

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