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Research Article

Morphological and molecular analysis of genetic diversity in multiple cross derivatives of cotton (*Gossypium hirsutum* L.)

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Abstract
Cotton farmers in India were compelled to adopt high density planting system (HDPS) in the recent past owing to its cultivation in un favourable ecologies. In order to develop varieties suitable for HDPS, hundreds of multiple cross derivates were evaluated and 52 stable genotypes with compact plant characteristics were used in the present study, conducted at Agricultural College, Aswaraopet during *Kharif*' 2013 to compare the pattern of clustering through metroglyph analysis, D^2 statistics and SSR markers. The material was sown in RBD replicated thrice and the data was recorded as mean values of five competitive plants per replication on nine morphological characters. All the 52 genotypes were grouped into 12, 8 and 7 clusters through metroglyph, D^2 statistics and SSR markers respectively. Results of the present study clearly indicated that, there are striking differences among different groups formed through three analyses with respect to number of groups and group constellation. Keeping in view the objectives of the study, the multiple cross derivatives that possessed compact characters *viz*., MC 4-3, MC 5-1, MC 9-1, MC 16-3, MC 17-6, MC 23-2, NH 630, MC 3-2, MC 17-1, MC 19-2, MC 22-2, MC 11-1, MC 17-2 were selected for further crossing to identify hybrids possessing short compact plant characteristics.

Key words
Gossypium hirsutum, metroglyph analysis, D^2 statistics, SSR markers.

Introduction

Cotton (*Gossypium* spp.) popularly called as "White Gold" and "King of Fibre Crops" is the most important renewable natural fibre crop of global importance enjoying a premier position among all the commercial crops. It occupies the predominant position in the Indian textile industry, despite stiff competition from the man-made synthetic fibres. In India it is grown in area of 10.50 Mha with a production of 35.10 million bales and productivity of 568 Kg ha^{-1} (www.cotcorp.gov.in).

Cotton genotypes that are presently cultivated in India have an inherent defect associated with the large bushy plant type as compared to compact genotypes. High Density Planting System (HDPS) introduced in recent years has to be popularized with straight varieties rather than Bt hybrids, which the farmers cannot afford. Development of varieties and hybrids suitable for HDPS is one of the options to mitigate the hardship of the cotton farmers to some extent.

It is in this context, a systematic programme was initiated in 1997 at ARS, Adilabad to identify cotton genotypes with short and compact plant stature having short sympodia and zero or lowest number of monopodia through multiple crossing (Pradeep and Sumalini, 2005). As a result a number of genotypes having such characters were selected in F_2 generation and were further evaluated in segregating generations to assess their performance with respect to plant type. Finally 52 genotypes possessing short and compact plant characters with consistent performance were identified.

In the present study, an attempt was made to study the magnitude of genetic diversity among the newly developed multiple cross derivatives of cotton and to compare the extent of agreement between most commonly used methods for studies on genetic diversity *viz*., metroglyph analysis, D^2 statistics and SSR markers. Accordingly, the data were subjected to D^2 statistics (Mahalonobis, 1936), metroglyph analysis (Anderson, 1957) and SSR marker analysis.

Materials and methods

The present investigation was carried out at Agricultural College, Aswaraopet during *Kharif*, 2013. Fifty two multiple cross derivatives (Table. 1) developed by crossing eight strains of cotton *viz.*, Renuka, Narasimha, LRA 5166, L 604, MCU 5, DHY 286, ADB 39 and NDL 1588 were sown in RBD replicated thrice at a spacing of 60 cm x 60 cm. The plot size was 36 m^2. Recommended package of practices were followed to raise the crop. The data were recorded on nine morphological characters *viz.*, days to 50% flowering, plant height (cm), number of monopodia, number of sympodia, length of sympodia (cm), number of bolls per plant, boll

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weight (g), 100- seed weight (g) and yield per plant (g). The data collected were subjected to statistical analysis by using Fishers' method (1958) of variance technique. Metroglyph and D^2 analyses were performed to group the multiple cross derivatives based on index scores and Tochers' method as suggested by Rao (1952) respectively. Molecular diversity analysis was carried out at Institute of Biotechnology, College of Agriculture, Rajendranagar, PJTSAU, Hyderabad by using the selfed progeny of 52 multiple cross derivatives and 57 genome-wide SSR markers (*Genei, Bangalore*) which were selected from cotton data base (*http://www.cottonmarkersdatabase.org.in*) to understand the genetic relationship among them.

The DNA was extracted from the leaves of selfed progeny of 52 multiple cross derivatives by using Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method (Doyle and Doyle, 1987) with few modifications. PCR amplification reactions were done in 10 µl reaction mixtures containing 2.5 µl of pure DNA, 1.0 µl 10X PCR buffer, 0.65 µl dNTPs,, 1.0 µl of $MgCl_2$, 0.25µl of each primer with 0.1 µl of 5U/ µl Taq DNA polymerase (*Jonaki, Hyderabad*) and added 4.25 µl of double distilled water. A DNA thermal cycler (Eppendorf vapo.protect) was used along with the following PCR profile: an initial denaturation step for 5 minutes at 94^{o}C (hot start and strand separation) followed by 35 cycles of denaturation (94^{o}C), annealing (56^{o}C) and primer elongation (72^{o}C) for 45s each and then a final extension at 72^{o}C for 10 minutes and hold at 4^{o}C. Amplified products were stored at -20^{o}C until further use. Prior to electrophoresis, each PCR product was mixed with gel loading dye (6X) and electrophoresis was carried out on 3% metaphore-agarose gel in 1X TAE buffer. The samples were loaded in each well along with standard 50bp DNA ladder (*Genei, Bangalore*) and run at 80 V for 90 minutes. The gel after electrophoresis was scanned using an UV transilluminator and gel documentation system (*Gene View*) linked to a computer.

SSR data analysis

The size of the most intensely amplified fragment was determined by comparing the migration distance of amplified fragments relative to the molecular weight of known size marker *viz.,* 50 bp DNA ladder. The allelic data was recorded and the data was analysed with *Darwin software 6.0.013* (Perrier and Jacquemoud-Collect, 2016) to construct a UPGMA (Unweighted Pair Group Method with Arithmatic Average) dendrogram showing the distance based interrelationship among the genotypes.

Polymorphic Information Content (PIC) values for all the markers were calculated by using allele size as produced by SSR primers over 52 multiple cross derivatives by using *Power marker* V 3.25 (Liu and Muse, 2005).

Results and discussion

Analysis of variance (Table. 2) for nine morphological characters indicated that the mean sum of squares due to genotypes showed significant differences at 1% level of significance, ssuggesting that the genotypes were genetically divergent and hence, there is an ample scope for selection of promising genotypes from the multiple cross derivatives possessing compact plant type characteristics . The presence of large amount of variability might be due to multiple crossing between selected strains as well as environmental influence on the phenotypes.

The scattered diagram of metroglyph analysis, dendrogram of D^2 statistics and SSR markers are shown in Fig. 1, 2 and 3 respectively. The scatter diagram of metroglyph analysis, dendrogram of D^2 statistics and UPGMA dendrogram of SSR markers had revealed twelve, eight and six clusters respectively (Table 3) whereas the clusters I and III were sub grouped into I a, I b and III a, III b respectively. The clusters *viz.,* I, II, IV, V, VII & VIII (metroglyph, Fig. 1), I, II, III, IV & VII (D^2 statistics, Fig. 2) and I, II & III clusters formed from SSR marker analysis (Fig. 3) had included multiple cross derivatives with compact plant characteristics.

The results of SSR marker analysis further revealed that, out of 57 markers studied, seven markers were monophorphic where as remaining 50 markers have shown PIC values ranging from 0.04 to 0.54 with the average PIC value of 0.32. Total numbers of alleles were 111 with an average of 2.22 alleles per locus. The gene diversity was ranged from 0 to 0.6. Out of 50 polymorphic markers, the markers *viz*., JESPR 152, JESPR 101, CIR 61, CM 13 were found to be more polymorphic with the PIC values more than 0.50.

The comparison of clustering pattern obtained through using three different methods for analysing morphological and genetic diversity among the multiple cross derivatives, it was found that there are striking differences among the three different methods with regard to number of clusters formed, cluster constellation etc., Nearly 80% of the multiple cross derivatives of cluster I of metroglyph scatter diagram were represented in Cluster II of D^2 statistics whereas 75% of the genotypes in cluster II of metroglyph were included in Cluster II of D^2 statistics. Only 3 genotypes each from cluster I and II were included in cluster I obtained through SSR markers. The genotypes present in cluster VIII of metroglyph were included in cluster III of both D^2 statistics and SSR marker analysis. The genotype *viz.,* MC 17-4 had formed a separate group both in metroglyph

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and D^2 statistics whereas it was included in cluster III derived through SSR marker dendrogram. The multiple cross derivatives MC 4-3 and MC 23-2 have formed separate groups whereas both of them were included in the same cluster i.e., I and II of metroglyph and D^2 statistics respectively, indicating the significant differences in the cluster formation through the methods using morphological characters (metroglyph and D^2 statistics) and DNA. In order to compare the extent of agreement between dendrograms derived from morphological characters and SSR markers, a distance matrix was constructed for each assay and compared using the Mantel (1967) matrix correspondence test. Accordingly, the correlation (r) between morphological and SSR dissimilarity matrices of all accessions was positive (0.03) but it is very low and near to zero indicating the combined use of both morphological and molecular diversity for selection of the parents for creation of variability through hybridization.

Conclusion

In the present study, it was observed that there are clear cut differences in respect of no. of clusters and no. of multiple cross derivatives included in each cluster when clustering pattern through metroglyph analysis, Mahalanobis D^2 statistics and SSR markers was compared. Further it was also revealed that, there is low correlation between morphological and molecular distance matrices. The experimental results were in agreement with the findings of several workers in different crops (Sundar *et al*. 2014 in cotton, Salem *et al*. 2008 in wheat, Ammar *et al*. 2015 in faba bean, Beyne *et al*. 2005 in maize, Singh *et al*. 2014 in wheat, Amabile *et al*. 2013, Koebner *et al*. 2003 in barley, Yadav *et al*. 2015 in barley). Further, it was also observed that, there is lack of correlation between morphological dissimilarity matrices and molecular dissimilarity matrices, similar results were also reported by Cholostova and Knotova, 2012 in alfalfa, Fikiru *et al*. 2010 in lentil, Zhang *et al*. 2010 in white clover. The reasons for differences among different methods with regard to cluster formation and constellation besides environmental influence may be due to the criteria used for clustering. In metroglyph analysis, the pattern of clustering is based on two highly variable characters which are used as ordinates on X and Y axis whereas in Mahalanobis D^2 statistics, clustering is based on pooled mean of all the characters (Kumar *et al.*, 2012). The reasons for low correlation between SSR markers and methods based on morphological traits is due to coverage of coding and non-coding regions and less subject to artificial selection by the DNA based markers (Salem *et al.* 2008) and the correspondence between different methods might be improved by analyzing more morphological characters and DNA markers (Marti'nez *et al*. 2005). A low correlation between phenotypic distance and distance measured using SSR markers was also reported by other workers (Bagavathiannan *et al.*, 2010, Crochemore *et al.*, 1998, Zhang *et al.*, 2010 and Martinez *et al.*, 2005).

Based on the present study, the multiple cross derivatives with early duration, short plant stature with lower number of monopodia, maximum number of shorter sympodia, average to high number of bolls per plant, boll weight, test weight and yield per plant that were scattered in different groups can be selected. Accordingly, the multiple cross derivates having above characteristics were selected from the clusters *viz.*, I, II, IV, V, VII & VIII (metroglyph), I, II, III, IV & VII (D^2 statistics) and I, II & III clusters formed from SSR marker data. Keeping in view the objectives of the present study, the multiple cross derivatives *viz.*, MC 4-3, MC 5-1, MC 9-1, MC 16-3, MC 17-6, MC 23-2, NH 630, MC 3-2, MC 17-1, MC 19-2, MC 22-2, MC 11-1, MC 17-2 were selected based on both morphological and molecular genetic variation for further crossing to identify hybrids suitable for HDPS system.

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Table 1. List of fifty two multiple cross derivatives of cotton and their parentage

SI.No.	Name of the Multiple Cross Derivati	Parentage
1.	MC 2-1	SRT – 1 × Renuka
2.	MC 2-3	SRT – 1 × Renuka
3.	MC 3-2	ADB 11 × L 604
4.	MC 4-2	AC 738 × ADB 320
5.	MC 4-3	AC 738 × ADB 320
6.	MC 3-3	ADB 11 × L 604
7.	MC 5-1	ADB 11 × L 604
8.	MC 5-2	ADB 11 × L 604
9.	MC 5-3	ADB 11 × L 604
10.	MC 6-1	ADB 11 × L 604
11.	MC 6-2	ADB 11 × L 604
12.	MC 6-3	ADB 11 × L 604
13.	MC 8-1	(ADB 11 × NDL 1588) × (MCU 5 × LRA 5166)× (ADB 39)
14.	MC 8-2	ADB 11 × NDL 1588 × (MCU 5 × LRA 5166 × ADB 39)
15.	MC 12-1	ADB 11 × Renuka
16.	MC 12-2	ADB 11 × Renuka
17.	MC 15-3	ADB 11 × NDL 1588
18.	MC 15-4	ADB 11 × NDL 1588
19.	MC 16-1	ADB 11 × NDL 1588
20.	MC 16-3	ADB 11 × NDL 1588
21.	MC 13-1	NHH 44 × NDL 188 × (MCU 5 × LRA 5166 × ADB 39)
22.	MC 15-1	ADB 11 × NDL 1588
23.	MC 17-1	ADB 11 × NDL 1588
24.	MC 17-2	ADB 11 × NDL 1588
25.	MC 17-3	ADB 11 × NDL 1588
26.	MC 15-2	ADB 11 × NDL 1588
27.	MC 17-4	ADB 11 × NDL 1588
28.	MC 17-6	ADB 11 × NDL 1588
29.	MC 18-1	ADB 11 × NDL 1588
30.	MC 19-1	AC 738 × NDL 1588
31.	MC 19-2	AC 738 × NDL 1588
32.	MC 19-3	AC 738 × NDL 1588

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33.	MC 20-2	AC 738 × NDL 1588
34.	MC 23-2	ADB 11 × L 604 × (MCU 5 × LRA 5166 × ADB 39)
35.	MC 24-1	ADB 11 × NDL 1588 × (DHY 286 × Narasimha × Renuka)
36.	MC 2-4	SRT – 1 × Renuka
37.	MC 4-1	AC 738 × ADB 320
38.	MC 22-2	ADB 11 × DHY 286
39.	CNH 1105	CICR, Nagpur
40.	NH 630	CRI, Nanded
41.	MC 2-2	SRT – 1 × Renuka
42.	CN 28-I	--
43.	MC 11-1	ADB 11 × Renuka
44.	MC 22-3	ADB 11 × DHY 286
45.	ARBC 64	ARS, Arabhavi
46.	MC 3-1	ADB 11 × L 604
47.	MC 14-1	NHH 44 × NDL 1588 × (MCU 5 × LRA 5166 × ADB 39)
48.	MC 9-1	ADB 11 ×NDL 1588 × (MCU 5 × LRA 5166 × ADB 39)
49.	MC 23-1	ADB 11 × L 604 × MCU 5 × LRA 5166 × ADB 39
50.	ADB 39	ARS, Adilabad
51.	MC 17-5	ADB 11 × NDL 1588
	MC 11-2	ADB 11 × Renuka

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Table 2. Analysis of Variance for different morphological traits in multiple cross derivatives of cotton

S.No.	Character	Mean Sum of Squares		
		Replications (df=2)	Genotypes (df=51)	Error (df=102)
1	Days to 50% flowering	0.39	10.12**	1.94
2	Plant height (cm)	9.68	614.17**	5.59
3	No. of monopodia	0.09	0.63**	0.16
4	No. of sympodia	7.19	14.06**	5.25
5	Length of sympodia (cm)	2.15	25.64**	1.64
6	No. of bolls per plant	3.42	319.87**	3.50
7	Boll weight (g)	0.014	0.93**	0.012
8	100-seed weight (g)	0.06	5.43**	0.03
9	Yield per plant (g)	2.93	5448.89**	18.37

** Significant at 1% level

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Table 3. Group constellation through metroglyph analysis, D^2 statistics and SSR markers

Group No.	Metroglyph Analysis		D^2 Statistics		SSR Markers	
	No. of multiple cross derivatives	Name of the multiple cross derivatives	No. of multiple cross derivatives	Name of the multiple cross derivatives	No. of multiple cross derivatives	Name of the multiple cross derivatives
I	**11**	MC 4-3, MC 5-1, MC 8-2, MC 11-2, MC 16-1, MC 17-6, MC 23-1, MC 23-2, MC 24-1, CN 28-I, NH 630	**15**	MC 2-1, MC 3-1, MC 3-3, MC 4-1, MC 4-2, MC 5-2, MC 5-3, MC 9-1, MC 11-2, MC 12-1, MC 16-1, MC 15-3, MC 19-3, CNH 1105, MC 22-3	**I a - 05**	MC 2-4, MC 22-2, MC 4-1, CNH 1105, MC 24-1
					I b - 09	MC 2-2, MC 3-1, MC 9-1, MC 11-1, MC 14-1, MC 22-3, NH 630, ARBC 64, CN 28-I
II	**08**	MC 2-1, MC 2-2, MC 2-4, MC 3-2, MC 8-1, MC 9-1, MC 18-1, ADB 39	**20**	MC 2-2, MC 2-3, MC 2-4, MC 3-2, MC 4-3, MC 5-1, MC 8-1, MC 8-2, MC 12-2, MC 16-3, MC 17-6, MC 18-1, MC 20-2, MC 23-1, MC 23-2, MC 24-1, ARBC 64, NH 630, CN 28-I, ADB 39	**04**	MC 11-2, MC 17-5, MC 23-1, ADB 39
III	**02**	MC 12-2, MC 17-3	**12**	MC 6-1, MC 6-2, MC 6-3, MC 11-1, MC 13-1, MC 14-1, MC 15-1, MC 15-2, MC 15-4, MC 19-1, MC 19-2, MC 22-2	**III a - 10**	MC 13-1, MC 15-1, MC 15-2, MC 16-1, MC 16-3, MC 17-1, MC 17-2, MC 17-3, MC 17-4, MC 17-6
					III b - 05	MC 19-2, MC 19-3, MC 19-1, MC 20-2, MC 18-1
IV	**02**	MC 12-1, MC 16-3	**01**	MC 17-1	**IV - 13**	MC 3-3, MC 5-1, MC 5-2, MC 5-3, MC 6-1, MC 6-2, MC 6-3, MC 8-1, MC 8-2, MC 12-1, MC 12-2, MC 15-3, MC 15-4
V	**08**	MC 3-1, MC 4-1, MC 4-2, MC 5-2, MC 17-2, MC 19-3, MC 20-2, CNH 1105	**01**	MC 17-5	**V - 05**	MC 2-1, MC 2-3, MC 3-2, MC 4-2 MC 4-3
VI	**08**	MC 2-3, MC 3-3, MC 5-3, MC 13-1, MC 14-1, MC 19-1, ARBC 64, MC 15-3	**01**	MC 17-3	**VI - 01**	MC 23-2
VII	**02**	MC 17-5, MC 22-2	**01**	MC 17-2	--	--
VIII	**02**	MC 15-2, MC 19-2	**01**	MC 17-4	--	--
IX	**01**	MC 19-3	--	--	--	--
X	**05**	MC 6-1, MC 6-2, MC 6-3, MC 11-1, MC 17-1	--	--	--	--
XI	**02**	MC 15-1, MC 15-4	--	--	--	--
XII	**01**	MC 17-4	--	--	--	--

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Table 4. Cluster mean values for nine morphological characters (Tochers' method)

Cluster	DFF	PH	NM	NS	LS	NB	BW	HSW	YPP
I	63.27	91.19	0.95	21.47	17.99	57.22	2.52	6.38	148.40
II	64.22	85.07	0.85	20.29	16.42	49.53	2.35	8.87	121.17
III	63.83	104.15	0.76	21.83	21.34	61.25	3.13	7.99	194.46
IV	63.00	102.27	1.33	20.00	17.40	79.93	2.90	6.17	237.03
V	61.67	68.73	1.27	21.87	18.47	78.67	2.30	7.54	188.46
VI	67.33	118.33	0.27	17.27	18.38	45.40	2.23	7.17	106.72
VII	65.00	94.80	0.20	19.47	16.58	39.27	4.13	6.03	167.57
VIII	63.33	60.33	0.27	17.87	19.16	75.60	3.70	7.54	286.89

DFF – Days to 50% flowering, PH – Plant height, NM – Numberof monopodia, NS – Number of sympodia, LS – Length of sympodia, NB – Number of bolls per plant, BW – Boll weight, HSW – Hundred seed weight, YPP – Yield per plant