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Identification and validation of SSR$_S$ linked to fiber quality traits in cotton using RILs derived from *Gossypium hirsutum* var. MCU5 and TCH 1218

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Abstract
In order to increase the fiber productivity in cotton, it is imperative to genetically improve the fiber component traits and such effort can be quickened by use of molecular markers. Totally, seventy-four simple sequence repeat (SSR) markers that were previously shown to be linked to fibre strength, boll weight, and fibre elongation were used to differentiate *Gossypium hirsutum* var. MCU5 and TCH1218 and among them two were found to be polymorphic markers. These two polymorphic markers *viz.*, TMB 0409 and CGR 6889 were used to genotype 220 F$_{13}$ recombinant inbred lines (RILs) derived from MCU5 and TCH1218 and single marker analysis was used to confirm their linkage with target traits using the phenotypic data collected on cotton fiber yield component traits such as plant height, days to 50 percent flowering data, boll weight, number of monopodial and sympodial branches and bolls. These identified and validated markers across genetic background can be employed to genetically improve cotton through marker assisted selection.

Keywords: Fibre quality, RIL population, *Gossypium hirsutum,* Intra specific cross

Introduction
Cotton fiber, the largest contributor of natural fiber used by mankind, is the backbone of textile industry. Cotton is "king of fibres" and popularly known as "White Gold". India is the largest producer of cotton in the world (~ 35.2 million bales) accounting for about 22% of world cotton production (https://cotcorp.org.in/national_cotton.aspx) [13]. India's climate, specifically, the north and south part of country, is very favourable for cotton production.

The genus *Gossypium* contains four cultivated species and among them, the tetraploids (2n=4x=52), *G. hirsutum* and *G. barbadense* are known as new world cotton. The rest of two cultivated species *viz.*, *G. arboreum* L. and *G. herbaceum* L. are diploids (2n=2x=26) and known as old world cotton. Among these four cultivated species, *Gossypium hirsutum* is predominantly cultivated in more than 95 per cent of global cotton area as it has global demand for its natural textile fibre and vegetable oil (Zhang *et al.*, 2015) [15].

Genetic improvement of fiber quality and quantity is the main objective of cotton breeding program. Both yield and quality are controlled by multiple genes interacting with growing environment (Zhang *et al.*, 2011) [16]. For improvement of fiber quality, there is need to improve both fiber strength and length (Yang *et al.*, 2016) [14]. Hinderance of negative correlation of yield and fiber quality limits the process of evolving elite cotton cultivar and thus, conventional method of breeding found to be challenging as it involves complex inheritance of fiber yield and quality traits. Recent advances in molecular markers and QTL mapping paved way for alternative route to speed-up the cotton breeding program and several QTLs have been identified (Kushanov *et al.*, 2021) [6]. However, it is important to validate the association of marker with the fiber yield or quality traits in the parents that are used in the regional breeding program. As validated marker for genetic improvement of fiber quality traits in cotton are not available, this study was designed to identify and validate stable QTL linked to fiber traits in cotton.

In the present study, intraspecific F$_{13}$ RIL population derived from *G. hirsutum* var. MCU 5 and TCH 1218 was evaluated using published SSRs linked to fiber traits and two markers linked to fiber strength were identified and validated in the above RILs.

Materials and Methods

Plant Materials: An intraspecific F_{13} population with 220 recombinant inbred lines was developed in which *G. hirsutum* var. MCU 5 and TCH 1218 were used as parents. The F_1 was developed at Tamil Nadu Agricultural University (TNAU), Coimbatore (Boopathi *et al.*, 2014) [4] and advanced to F_{13} generation. The female parent MCU 5 is a medium staple fiber and during flowering stage it cannot withstand water stress whereas TCH 1218 male parent is drought tolerant.

Phenotypic data

A two-row randomised block design with 26 plants was laid out during August, 2021. The spacing adopted between plant to plant was 45cm and between rows was 90cm. Data on days to 50% flowering was collected 45 days after sowing (DAS) and plant height (cm), number of monopodial branches, number of sympodial branches, number of bolls per plant, boll weigh (g), number of locules per boll were collected from the RILs at the time of harvest. Throughout the growing season, essential intercultural operations including pesticide application were carried out consistently in all plots at regular intervals.

DNA isolation and genotyping

Genomic DNA was extracted from the young and fresh leaves of parents and 221 RILs using cetyltrimethylammonium bromide (CTAB) technique (Aboul-Maaty and Oraby, 2019) [3]. Using a Nano Drop spectrophotometer, the purity of DNA was determined and DNA was electrophoresed through 0.8% (w/v) agarose gel.

PCR amplification

PCR reaction (Proflex PCR system) for the DNA extracted samples was carried out using 74 SSR markers belonging to TMB, NAU, HAU, BNL, JESPER, CIR, DPL, CGR, CM, DC and GH series (Table 1) linked to fibre quality and boll weight. These markers were used to analyse parental polymorphism and were further screened among the recombinant inbred lines derived from these two parents. The PCR product was resolved using 3% (w/v) agarose gel and the gel was documented using documentation unit (UVITEC, Cambridge).

Table 1: Details of SSR markers used in this study

S. NO	SSR		Sequence 5' TO 3'	Trait	Chr.	Temp. (^{O}C)	PVE	Reference
1	CM076	F	TTAATTTTCAAAGGGCTCTTAGAAAG	Fiber elongation	15	61.02	9.15	Sun *et al*., 2012
2		R	GTATAATGGTAGGAGAGAAGGGTTAGGG					
3	BNL3649	F	GCAAAAACGAGTTGACCCAT	Fiber length	1	56.28	4.71	Sun *et al*., 2012
4		R	CCTGGTTTTCAAGCCTGTTC					
5	NAU2600	F	CTTCCTGACAAGGCAAAGAT	Fiber length	1	54.23	4.71	Sun *et al*., 2012
6		R	AGCCGATAAACCAAAAACAG					
7	HAU2489	F	GGCACGAGGAGAAAATGAAAGA	Fiber elongation	15	60.92	47.1	Zhang *et al*., 2012
8		R	GATCGGATTCTGGGTCCCGC					
9	HAU1044	F	TGGTCTGTATCCGTTCATTG	Fiber length	1	54.23	48.5	Zhang *et al*., 2012
10		R	TTTTCGTATTTGTGGTGGTG					
11	NAU3145	F	AAAACAGAGGCCTAATATCTCA	Fiber length	1	54.96	55.8	Zhang *et al*., 2012
12		R	TGTTGGTTTACCTGTTGTGC					
13	CIR213	F	TCAAGTGCATCAAGAAAC	Fiber strength	1	49.13	9.2	Zhang *et al*., 2012
14		R	CACTCCTAACAATGGAAA					
15	DC40175	F	TTGCTCAGGTTTTGATGTC	Fiber strength	1	53.02	7.92	Liang *et al*., 2013
16		R	AGGTGATGACCATCGGTA					
17	HAU1417	F	CAAAACTTGTTGCTCTTCCA	Fiber strength	1	53.2	12.46	Liang *et al*., 2013
18		R	TAACTGAAACCCCAAAAGGA					
19	NAU3254	F	GCTTTGCTTTGGAATGAGAT	Fiber strength	1	53.2	6.85	Zhang *et al*., 2016 [14]
20		R	TTGGTGCAGATAGCAAGAAA					
21	DPL0490	F	AGTATCGTCACTTGTCAAAGTCCA	Fiber length	1	59.09	7	Shao *et al*., 2014
22		R	CTCATGCATGCTTATCACACATC					
23	NAU2165	F	TAAATTTTGAGATGGCAGCA	Fiber length	15	53.2	18.1	Shao *et al*., 2014
24		R	CAAGGTGAAGGCAAAGAGAT					
25	HAU1619	F	AAAAACAATGGAAACGGTGT	Micronaire	1 or 15	51.15	16	Shao *et al*., 2014
26		R	CTTGGTTTGCCAATATGAAA					
27	NAU3291	F	GGTAGGGCTAAGGACAACAA	Fiber yellowness	12	56.28	0.0479	Wang *et al*., 2007
28		R	AATATGTTGCAGGTGGAGGT					
29	NAU3346	F	ACCTGAACCTGAATGTCCAC	Fibre length	1	55.25	7.7	liu *et al*., 2017
30		R	CCGTTCCATGTTTTTGTGTA					
31	BNL3994	F	TTGAGGGCATCCAAATCCAT	Boll weight	12	57.3	0.0614	Wang *et al*., 2007
32		R	CCTCCACCATACACGTGCTA					
33	BNL2495	F	ACCGCCATTACTGGACAAAG	Boll weight	12	55.25	0.0479	Wang *et al*., 2007
34		R	AATGGAATTTGAACCCATGC					
35	JESPR234	F	GCATAGTTATGAATGACTCTC	Boll weight	12	56.28	0.0614	Wang *et al*., 2007
36		R	CTAACTCGAATCCGTCAC					
37	NAU2355	F	ACAAACAAAACGCCTTCTTC	Boll weight	1	53.83	0.13	Sassel *et al*., 2011
38		R	AACACAAAAACGGTTCCAGT					
39	NAU2820	F	GCCACCAATAAAGCAACTCT	Boll weight	1	53.2	0.22	Wang *et al*., 2007
40		R	TGCATCCTGAAGAAGAGACA					
41	NAU5380	F	CTTTGCCTCCTCATTACCAT	Boll weight	1	55.25		Wei *et al*., 2011

42		R	TTGAGTTGGGGGCTTAGTAG					
43	GH542	F	TTCAATTCTGATTCTAACGCCATCAG	Boll weight	1	60.54		
44		R	TACCCAGAATCGATGAGACACATG					
45	BNL3033	F	TTTTTTGTTTCCACCCAAGC	Boll weight	1	56.86	0.48	
46		R	GTCGCCCCATCCGATGTC					
47	BNL2882	F	CAACCTTTGGTAATCTTCTTTCG	Boll weight	1	56.17	0.48	Luan *et al.* 2009
48		R	CGCTAACGCATTTGACATCT					
49	BNL3099	F	GCCCATGTTCAAATCAATCA	Boll weight	1	56.28	0.51	
50		R	CCCCGACCTGAATCTAACCT					
51	BNL2986	F	TAGAGCCAAGTGGTGATCCC	Boll weight	1	57.3	0.0966	Wang *et al.*, 2007
52		R	AAAGGGGGGAATGATTATGC					
53	CIR062	F	CCTCCACCAAACAGACATC	Boll weight	15	57.75		Wei *et al.*, 2011
54		R	GTCTGGGAGAGGTTGAGTG					
55	BNL2920	F	TTCTTGCATTGAATAATACTGGC	Boll weight	15	55.34	0.07	Wu *et al.*, 2009
56		R	CTTAATTCTAAAAATCAATAAATTTAGCC					
57	NAU1156	F	ACACTCTCTCAGCTGGAACC	Boll weight	15	59.34		Wei *et al.*, 2011
58		R	GGTCTCCCTCTAGCTTGTTG					
59	NAU5107	F	CGATGAAGACGATGCTATTG	Micronaire	1	56.28	6.82	Wang *et al.*, 2015 [16]
60		R	GTAGCCTTTGGTCTTCGTGT					
61	HAU1693	F	TGGATGATGTGGAAAAACAG	Micronaire	1	53.2	9.6	
62		R	AAATCCAAAAACACCACCAC					
63	HAU1038	F	TCAACAGGTTCATCATCAGC	Micronaire	1	54.23	9.6	Wang *et al.*, 2016 [14]
64		R	ACAAAGTTACCCATGGCATT					
65	HAU4220	F	TTCGGGGTTTCACATCCCCT	Fiber strength	15	60.38	8.4	
66		R	CCCTCTTGGCTGTTTCCACC					
67	BNL830	F	TTCCGGGTTTTCAATAAACG		15	54.53		
68		R	GTTAATACTTTTTTTCTTTTGTGTGTG					
69	CGR5826	F	GGTGACAATGGCCTGAACTT	fiber uniformity	15	57.3	26.3	Zhang *et al.*, 2012
70		R	CGTCTGGCCCATAAAGGTTA					
71	DPL0790	F	ACAATGGCGGATTGGATTC	Fiber strength	1	57.17	5	Ma *et al.*, 2017
72		R	TTCCAAGTGTCACCCTCTCAC					
73	DPL0090	F	CACCTACTGGTCCTACCACCTAAG	Fiber elongation	1	61.87	12.51	Ma *et al.*, 2017
74		R	GTTGTTGTCGTCTTGCAGATTATG					
75	BNL3902	F	GAGTTTGGGGGCTGTGTATG	Micronaire	15	59.35	0.116	
76		R	GGGGTGCTTATGTCAGACGT					
77	TMJ24	F	GGCTCCAAAATTGAAACGTG	Micronaire	15	55.25	0.116	
78		R	GTGGACATTGGCATTCATTG					Shen *et al.*, 2005
79	NAU913	F	CCCATGATCAAAAGACAACA	Fiber elongation	1	54.23	7.1	
80		R	GCTTAAAGATCGAGGACGAA					
81	NAU974	F	GCTTAAAGATCGAGGACGAA	Fiber elongation	1	54.23	29.5	
82		R	CCCATGATCAAAAGACAACA					
83	NAU2343	F	GCTTTGCTTTGGAATGAGAT	Fiber elongation	15	55.25	5.02	San *et al.*, 2012
84		R	ATACTGCAACCCCTCACACT					
85	DPL0322	F	AAACCTCGTAGTCATAGGCTCAAA	Fiber elongation	15	59.3	8.67	
86		R	AACTATGCACACAGATTTGGTACG					Sun *et al.*, 2012
87	CIR334	F	ACCCTTGACAGTTACCAC	Fiber elongation	15	50.82	9.15	
88		R	TGCCCATTTAGGTATGA					
89	NAU3881	F	AATAGTGATGCTCCCTTTGG	Fiber length	12	56.28		Dong *et al.*, 2018
90		R	TGCCCACTAAAGAGTTAGCC					
91	NAU3084	F	GATCCTCCTCTTCCTCTTCC	Boll weight	12	57.3		Zhang *et al.*, 2013
92		R	GATGAAAGCGGTGGTTAAGT					
93	BNL3867	F	TAATTGAGTTGTTTTCTTACTTGCC	Boll weight	12	54.81	2.18	Dai *et al.*, 2019
94		R	TGCCAATTTAGCAATCACCA					
95	BNL2921	F	CGAGAGATTTTAAAGGGAAACA	Boll weight	1	55.98		Zeng *et al.*, 2009
96		R	GGGAGTGGTCTGATGGAAAA					
97	CIR307	F	GACTTGAAAAGATTACACAC	Fiber elongation	15	48.13	0.07	Shang *et al.*, 2015
98		R	GAATTTGCTGGCTCT					
99	BNL1693	F	CCCTTGGGAATAGCAGGTG	Fiber elongation	15	60.47	12.47	Zhang *et al.*, 2016 [14]
100		R	CATGTGTCTCCGTGTGTGTGTG					
101	CGR6889	F	AGACACCAGCATCCACATCA	Fiberstrength	15	57.3	5.64	Shang *et al.*, 2015
102		R	CCGCTTCCCATTTAGGTATG					
103	DPL0182	F	TTTGAGTGGAGACTGAGAGCG	Fiber strength	15	58.18	7.92	Liang *et al.*, 2013
104		R	TGGCTTAGAGCTTTGAATTTGG					
105	NAU2437	F	CTTGGAAAAAGGAAGAGCAG	Fiber uniformity	1	54.23	6.5	Tan *et al.*, 2015
106		R	TTAAAAGACCAAAGGCAAGG					
107	DPL0526	F	GTTCTTGGTCATGCTGGTAAGAAA	Fiber elongation	1	59.3	7.1	Tan *et al.*, 2015
108		R	TAGCCATATCCACCTTAGCAGATT					

				Trait	Chr.	Temp	PVE	
109	BNL1454	F	AGGAAGGAGCGAAGGAAGAG	Fiber elongation	15	58.33	8.5	Tan *et al*., 2015
110		R	CTTTCCCCTCCCTTTTCAAG					
111	NAU3384	F	TCATAACGGAAGCATTTTAC	Fiber uniformity	15	53.2	5.3	Tang *et al*., 2015
112		R	GTTGGCTTCTCTTTGATCGT					
113	CIR234	F	AGCACTCATCCATCACA	Micronaire	15	50.89	5.5	
114		R	GCACCCTTTAGAAACAAG					
115	CGR5372	F	GGGCTCACCTCTTCAGAGAA	Micronaire	1	58.33	9.84	Wang *et al*., 2016 [14]
116		R	ATATGGGAGGTGTGGGAACA					
117	NAU0902	F	GGAGAGTGAAAATGGAATGTG	Fiber length	15	54.56	50.8	Zhang *et al*., 2012
118		R	ACGAGAAGTTATTCGACAGA					
119	TMB0409	F	CAGAGGACGAAGGTAGCAG	fiber elongation	1 or 15	57.04	6.15	Wang *et al*., 2015 [15]
120		R	TGGTGGGTTTCACTTTCACA					
121	BNL1053	F	AGGGTCTGTCATGGTTGGAG	Fiber strength	1 or 15	58.33	4.87	
122		R	CATGCATGCGTACGTGTGTA					
123	BNL3345	F	CGAAGCGCGATTAAGAGAAC	Fiber strength	1 or 15	57.3	4.87	
124		R	AAAGCGAAGCCAACAGTCTC					
125	HAU3074	F	CAGAGCCAGTTGCCGAGGAG	Micronaire	1 or 15	62.43	6.29	
126		R	CGGCTTCCTCTTTGGGTGCT					
127	BNL2599	F	ATTGCCACAACCACAATCAA	Micronaire	1 or 15	52.18	6.29	
128		R	TATTTTTTTGGGCTTGCTGA					
129	GH216	F	TCCACATTCCCATGCACTACTC	Fiber elongation	1 or 15	59.76	6.98	
130		R	CTAAAACCTTATACATACAAAATGCAGC					
131	DPL0052	F	GCTTACGTGTATGATTAAATCGCC	Fibre elongation	1	60.16	7.74	
132		R	CAGAGGACTTGTAAACAACACTGC					
133	CGR6586	F	CTCGCCTCTTCAGAGAAAGAA	fiber elongation	1 or 15	57.59	7.74	
134		R	ATATGGGAGGTGTGGGAACA					
135	HAU3923	F	TGGCCAGTAACACCGAGACA	Fiber elongation	1 or 15	59.35	11.63	
136		R	GGCCTTCGCCTTTTCTTCCT					
137	CGR5001	F	TCTCCATGTATCCACCCACA	Fiber elongation	1 or 15	56.28	11.63	
138		R	ATAGCGAATGCAGATCGTGA					
139	HAU1001	F	ACAGGATGTGCATGTTATGG	Fiber elongation	1 or 15	54.23	6.54	
140		R	ATCTCTTGATTTGGGGTCAA					
141	HAU3319	F	ATCTCTTGATTTGGGGTCAA	Fiber elongation	1 or 15	57.3	6.54	
142		R	GATGAGGGTCAAAGGCGGCA					
143	HAU035	F	CAGAAAGAAGAAGGGAAGACC	fiber elongation	1 or 15	55.54	12.81	
144		R	TTTTGGAGAAAATGGTCAGC					
145	HAU4228	F	CGGCAGGTTCGACAACGTAA	Fiber elongation	1 or 15	60.38	12.81	Wang *et al*., 2015 [15]
146		R	CTCTTGCAGCTCCGTCTTCC					
147	NAU5138	F	TCGCCATCTTCACTATTCTTC	Fiber elongation	15	54.56	8.14	Yu *et al*., 2013
148		R	CATGGCGAATTTCCTTACTT					

Trait = Trait to which marker is linked, Chr. – Chromosome number; Temp (ºC) = annealing temperature of the given primer which was used to set PCR profile, PVE = Phenotypic variance explained (%).

Results and Discussion
In this study, 220 F_{13} RILs derived from *G hirusutum* var MCU5 and TCH 1218 were evaluated phenotypically for quantitative traits and significant variation for plant height (20 – 160 cm), boll number (1 - 19), boll weight (1.2 - 5.6g), sympodial branches (2 - 29), days to 50 percent flowering (70 – 85 DAS) and monopodial branches (0 - 3) were noticed (Table 2).

Table 2: Variation in phenotypic data of 220 F_{13} RILs derived from MCU 5 and TCH 1218

Traits	Mean	Minimum value	Maximum value
Plant height (cm)	78.95	20	160
Number of bolls	4.6	1	19
Boll weight	3.8	1.2	5.6
Days 50% flowering data	72.6	70	85
Number of Sympodial branches	14.9	2	29
Number of Monopodial branches	1.22	0	3

Genotyping with seventy-four SSR markers exhibited that two markers *viz*., TMB 0409 and CGR 6889 (which had amplicon size of 221 bp and 131 bp, respectively) that were already shown to be linked with fiber strength (Table 1) were also found to be polymorphic between the investigated parents (Figure 1).
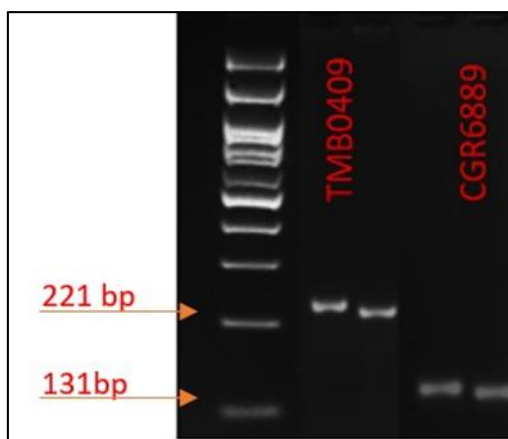
Fig 1: Agarose gel electrophoresis of PCR products amplified by TMB 0409 and CGR 6889. Lane 1- 100bp ladder; Lane 2 and 3- PCR product amplified by TMB 0409 in MCU 5 and TCH 1218, respectively; Lane 4 and 5- PCR product amplified by CGR 6889 in MCU 5 and TCH 1218, respectively

On further screening among the recombinant inbred lines (RIL) derived from these two parents with identified two polymorphic markers, a total of 183 RIL and 198 RIL progenies were found to possess the allele for fiber strength, respectively. While 137 RILs found to have allele for both the polymorphic markers.

Genotypic mean performance of progenies was found to be more when compared with parents for quantitative traits like plant height (cm), boll weight (g), number of bolls, days to 50 percent flowering data, monopodial branches.

The mean phenotypic performance of quantitative traits for TMB 0409 marker and CGR 6889, as indicated in Figure 2, demonstrated that there was a significance difference for plant height, 50% flowering data, number of sympodial branches, boll weight and boll number.
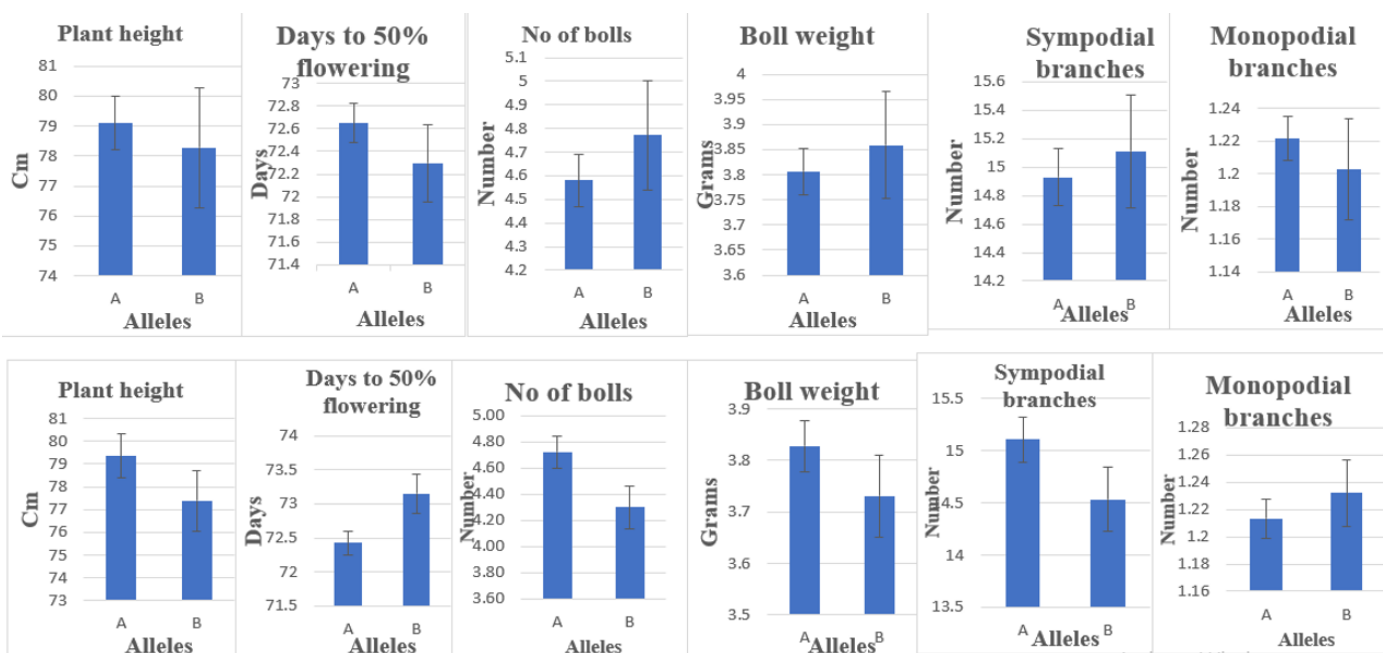
Fig 2: Histogram showing variation in quantitative traits with respect to marker alleles that were measured from 220 RILs derived from *Gossypium hirsutum* var MCU5 and TCH1218 for two SSR markers *viz*., TMB 0409 (1a to 1f) and CGR 6889 (1g to 1l).

A significance difference in the expression of quantitative trait between the two alleles indicates that the given marker is linked to particular QTL (Meena *et al.*, 2017) [9]. Studies on single marker analysis conducted by Semizer-cuming *et al.*, 2015 [10] identified 43 QTLs for fiber colour and fiber quality traits. Similarly, 11 markers linked to node of first fruit branch (NFFB) and first node of highest fruiting bodies (FNHB) were identified (Li *et al.*, 2012) [7]. In present study, single marker analysis of two polymorphic markers observed significance difference in the expression of various quantitative traits and this reveals that these markers were linked to fiber strength.

In earlier studies QTLs linked to wilt resistance were identified and they were precisely introgressed into *G hirsutum* from *G barbadense* through marker assisted backcross breeding program (Li *et al.*, 2013) [8]. By identifying markers linked to fiber quality traits and introgressing them from diverse Uzbek cotton germplasm (Abdurakhmonov *et al.*, 2008; Abdurakhmonov *et al.*, 2009) [1, 2], new varieties with fiber qualities traits such as "Ravnaq-1" and "Ravnaq-2" were developed (Darmanov *et al.*, 2015) [5]. Therefore, the major and consistent breeder friendly

markers *viz*., CGR 6889 and TMB 0409 (Shang *et al.*, 2015; Wang *et al.*, 2015) [11, 12] that were reported elsewhere have also been validated in this study using another independent mapping population.

Conclusion
As these markers that were linked to fiber strength in upland cotton are consistent across genetic backgrounds, they may be useful in efficient marker assisted selection for fiber quality trait improvement in cotton.

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