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EST-SSR BASED ANALYSIS REVEALED NARROW GENETIC BASE OF IN-USE COTTON VARIETIES OF PAKISTAN

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Abstract

Genetic improvement of cotton relies on extent of variation in germplasm. The in-use cultivars of Pakistan have been selected to assess genetic dissimilarities. A total of 44 cotton varieties/cultivars were selected for molecular analysis. These varieties were screened by 100 EST-SSR primers, selected on the basis of wider genome coverage. Analysis showed that the range of bands per primer varied from one to six. The average number of bands was 2.23 per primer. The range of PIC value varied from 0.28 to 0.94. The maximum PIC value of 0.95 was recorded for NAU-2503, considered as highly informative marker. A large number of markers, 73.07%, showed PIC value higher than 0.60 and considered highly informative. The results of dendogram showed that the in-use cotton varieties of Pakistan had very narrow genetic base. High similarity index existed among BT varieties as well. We observed very low genetic diversity in the selected varieties which are commonly grown in the country. There is need to include wide range of variation for genetic improvement in cotton. There is need to Introduce exotic varieties, Inter-specific hybridization, mutation breeding etc. may be used to avoid genetic bottle neck.

Key words: Cotton**,** Diversity, EST-SSR, Genetic base, Polymorphism.

Introduction

Cotton was domesticated in east around 6000 BC (Moulherat *et al*., 2002). A total of fifty species of cotton have been divided in two groups on the basis of ploidy level. One group contains five allotetraploid whereas other contains 45 diploid species (Wendel & Cronn, 2003). *Gossypium hirsutum* L., the upland cotton is the widely cultivated species all around the world (Cantrell, 2004). The upland cotton being grown worldwide is reported to be originated from four varietal types namely Stoneville, Acala, Deltapine, and Coker. Among these, three varieties Stonville, Coker, and Deltapine had common origin from a variety "Bohemian" in 1860 (Niles, 1980). It could be a cause of low diversity in the available germplasm of cotton. The low genetic diversity of tetraploid cotton is considered as major cause affecting the yield as well as quality of the crop (Esbroeck & Bowman, 1998; Paterson & Smith, 1999). The problem can be overcome by inputting modern tools of exploiting genetic diversity of cotton to reveal genetic control of important traits at molecular level. For this purpose, genetic markers especially EST-SSR have been proved to be highly informative and polymorphic (Jia *et al*., 2014; Kencharaddi *et al*., 2018) and are successfully applied in cotton genomic studies (Frelichowski *et al.,* 2006; He *et al.,* 2007). The marker could also be used for identification of hybrid (Selvakumar, 2010; Kencharaddi *et al*., 2018).

In breeding programs for cotton improvement, presence of genetic similarity among its varied genotypes is a major hurdle (Becelaere *et al.*, 2005). Studies have been conducted to observe genetic diversity which exposed low polymorphism in cotton cultivars (Rungis *et al*., 2005). Modern molecular application is beneficial approach to assess the genetic diversity (Zhang, 2011). For genetic diversity analysis and precise evaluation, molecular marker could play pivotal role (Mohammadi & Prasanna, 2003).

The diversity in cotton at molecular level has been assessed using various molecular techniques such as AFLP (Li *et al.*, 2008), RAPD (Chaudhary *et al*., 2010; Tyagi *et al*., 2015), ISSR (Reddy *et al*., 2002) and SSRs (Rakshit *et al*., 2010; Tyagi *et al*., 2015). SSR markers, among these, proved to be reliable for analyzing molecular variations in a crop species (Gutierrez *et al.*, 2002; Kencharaddi *et al*., 2018). Most common reasons are that these markers are co-dominant in nature (Akkaya *et al*., 1995), distributed genome wide (Hakki *et al*., 2001), highly specific for loci and high degree polymorphism (Maroof *et al*., 1994). The EST-SSRs derived from SSRs, targets only expressed part of genome, are more effective among genera and species (Park *et al*., 2005) as well as considered very informative in diversity studies in cotton (Zhu *et al*., 2009). SSR and EST-SSR are used for diversity analysis in many other important crop plants (Lu *et al.*, 2020; Yunli *et al.*, 2020; Qamar *et al.*, 2020).

The improvement in cotton yield and quality is stagnant mainly because of narrow genetic base of in-use cotton cultivars. A number of cotton varieties released in the field could not show distinguished impact. Genetic evaluation of varieties at molecular level would provide useful information about similarity among in-use cotton varieties.

Materials and Methods

One hundred and eleven cotton genotypes, on the basis of high cultivation record in Punjab, were obtained from different cotton research stations of Pakistan such as Central Cotton Research Institute Multan, Cotton Research Station Multan, Cotton Research Station Vehari, Cotton Research Station Sahiwal, Cotton Research Station Faisalabad, and Cotton Research Institute Sakrand. Among these one hundred and eleven genotypes, we selected a total of 44 varieties for this

research work (Table 1). The selected varieties were grown in controlled conditions. The genotypes were assigned in completely randomized design with three replications. All required agronomic inputs were followed for normal growth of plants. DNA was extracted from selected plants for PCR work.

Molecular work: DNA was extracted using standard CTAB method (Doyle & Doyle, 1987). One hundred EST-SSR markers (Annexure-I) were used for PCR analysis. PCR products were checked through Agarose Gel Documentation. The amplified PCR products were coded on the basis of presence or absence of bands according to software instructions. The unclear or ambiguous bands were not scored.

Data analysis: A standard statistical method was used on the basis of presence and absence of bands to calculate the allele frequencies, heterozygosity and effective number of alleles (Liu *et al*., 2006). Genetic divergence and cluster analysis was done using NTSYS-pc software version 2.02 (Exeter Software, NY, USA (Rohalf, 2000).

PIC value calculation: Polymorphism Information Content (PIC) value of all the primers was calculated by using the following equation (Botstein *et al*., 1980):

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2 = 1 - \sum_{i=1}^{n} p_i^2 - \left(\sum_{i=1}^{n} p_i^2\right)^2 + \sum_{i=1}^{n} p_i^4$$

Results

Molecular genetic diversity in selected varieties was assessed by screening of 100 EST-SSR markers of Nanjing Agriculture University (NAU) series. Mostly the observed bands were as according to the reported band size of the marker. Different primers had different band size. Band size in this analysis was ranged from 150-bp to 250-bp. PCR profiles of a few markers amplified for selected genotypes is shown in Fig. 1.

Diversity analysis: The dendogram generated exposed close genetic similarity among all the genotypes. Varieties emerged divided in three clusters, showing no genetic distinction within and among clusters. In first cluster, the genotype CIM-496 and CRIS-121 showed closeness even though one is from Punjab and other is from Sindh. Next genotypes, CIM-554, SLH-317 and CIM-595 assembled in common group. As these genotypes shared a common cluster which depicted further resemblance with each other on molecular basis. The genotypes AC-134, MNH-147, S-12, 124-F, BH-36 and CIM-448 were grouped in a same cluster which showed clear evidence of genetic resemblance among them. These genotypes were further divided in couple of small sub-clusters each of which containing three genotypes. Next cluster containing a total of eight varieties was further separated into two sub-clusters with four genotypes each. The first sub-cluster possessed genotypes 199-F, CRIS-134, SLH-8 and NIAB-999 while the second sub-cluster contained genotypes CRIS-134, CIM-506, Cyto-124 and CIM-240. The genotypes CIM-608, CRIS-129, CIM-600, Cyto-177, CIM-620, CRIS-508 and 149-F grouped in neighboring cluster. An adjoining line with this cluster shows a single variety CRIS-508, exhibiting resemblances with these varieties but at the same time seems to be distinguishable and different from all of them. The genotypes CIM-496 and S-14 were present at maximum distance from earlier discussed varieties but at the same time showed some molecular resemblance with all of them. The genotype S-14, among all genotypes used in the experiment, proved to be the most different at molecular level (Fig. 2).

The level or intensity of similarity has been assessed by calculating dissimilarity index; the analysis shows that how much selected varieties are genetically similar or different. The dissimilarity index applied for selected 44 cotton varieties using EST-SSR markers data shows that the in-use cotton possessed very narrow molecular genetic background. The cotton varieties/genotypes used in Pakistan for research as well as production purpose are genetically very similar to each other. The 44 cotton varieties used in this experiment showed maximum dissimilarity index (4.0) indicating that these genotypes possessed 96% similar molecular background. In other words, we observeed only 4% molecular diversity among them. Whereas, the mean molecular dissimilarity among the selected genotypes/varieties was observed 2.74. The lowest molecular dissimilarity index zero was found among CIM-496, CIM-573 and CIM-598. The varieties CIM-496 and CIM-573 from same cotton research institutes also proved to be originated from a common genetic source in this experiment. Overall very low molecular genetic diversity has been detected among the selected genotypes/varieties (Fig. 3).

Table 1. List of varieties with year of release.

No.	Variety	Year	No.	Variety	Year	No.	Variety	Year	No.	Variety	Year
1.	CIM-496	2005	12.	NIAB-111	2004	23.	CIM-608	2013	34.	CRIS-134	2004
2.	CIM-534	2006	13.	AC-134	1959	24.	CRIS-129	2014	35.	199-F	1946
3.	CRIS-121	2006	14.	MNH-147	1992	25.	Bt CIM-600	2017	36.	S-14	1995
4.	CIM-554	2009	15.	S-12	1988	26.	Bt Cyto-177	2017	37.	CIM-506	2004
5.	CIRS-342	2010	16.	124-F	1945	27.	Cyto-124	2015	38.	FH-Lalazar	2013
6.	CIM-573	2012	17.	BH-36	1992	28.	CIM-620	2016	39.	CRIS-9	1993
7.	Bt CIM-598	2012	18.	CIM-448	1996	29.	SLH-8	2016	40.	CIM-473	2002
8.	BH-167	2012	19.	NIAB-999	2003	30.	CRIS-533	2017	41.	CIM-240	1992
9.	SLH-317	2012	20.	CIM-707	2004	31.	CRIS-510	2017	42.	Bt CIM-602	2016
10.	CIM-595	2013	21.	BH-160	2004	32.	CRIS-508	2017	43.	M-4	1942
11.	CIM-599	2013	22.	149-F	1971	33.	CIM-598	2017	44.	CYTO-179	2017

Source: Pakistan Central Cotton Committee (PCCC)

Annexure-I. EST-SSR Markers and their respective chromosome number.

No	Oligo name	Chromosome	No	Oligo name	Chromosome	No	Oligo name	Chromosome	No	Oligo name	Chromosome
1	NAU862	03	26	NAU1023	13	51	NAU2758	21	76	NAU3769	08, 24
2	NAU1014	11	27	NAU1063	11	52	NAU2881	08, 16	77	NAU2773	08, 24
3	NAU1045	09	28	NAU1070	03, 14	53	NAU3009	04	78	NAU3777	04, 22
4	NAU1048	07	29	NAU1162	11	54	NAU3093	04	79	NAU3812	12
5	NAU1151	04, 05, 06, 12	30	NAU1215	13, 18	55	NAU3120	14	80	NAU3813	20, 10
6	NAU1190	03	31	NAU1048	07	56	NAU3158	24,21	81	NAU3380	07
7	NAU1231	26	32	NAU1070	03, 14	57	NAU4086	11	82	NAU3897	12
8	NAU2715	12, 26	33	NAU1207		58	NAU3201	08, 24	83	NAU 3913	14
9	NAU2741	01, 19	34	NAU2024	11	59	NAU3234	11	84	NAU3920	26
10	NAU2758	21	35	NAU2220		60	NAU3239	14	85	NAU3942	22
11	NAU2836	03, 17	36	NAU1231	26	61	NAU3306	25	86	NAU4014	20, 21
12	NAU2838	25	37	NAU1232	11	62	NAU3368	20	87	NAU4039	21
13	NAU2868	12, 26	38	NAU2274	05, 19	63	NAU3367	11	88	NAU4047	12
14	NAU2869	10, 20	39	NAU1350	24	64	NAU3374	21	89	NAU4065	14
15	NAU2954	25, 23	40	NAU1301	12	65	NAU3427	06	90	NAU4090	26
16	NAU2967	06	41	NAU1362	07	66	NAU3394		91	NAU4105	18
17	NAU3203	13, 18	42	NAU2252	05	67	NAU3554		92	NAU4871	18
18	NAU3279	16	43	NAU2002	07, 05	68	NAU3558	08	93	NAU4912	26
19	NAU3427	06	44	NAU2016	11, 21	69	NAU3590	08	94	NAU4922	
20	NAU4014	20, 21	45	NAU1366	21	70	NAU3598	14	95	NAU5005	19
21	NAU4105	18	46	NAU2272	14	71	NAU3626	02	96	NAU5024	16
22	NAU5046	22, 05	47	NAU2773	06, 25	72	NAU3633	22	97	NAU5027	14
23	NAU5269	06	48	NAU2675		73	NAU3731	11, 21	98	NAU5046	22, 05
24	NAU915	12, 26	49	NAU2575	09, 23	74	NAU3735	07	99	NAU5061	16
25	NAU967	11	50	NAU2651	11	75	NAU3754	21	100	NAU5109	18
									101	NAU5129	08

Source: Cottonmarkerdatabase.org

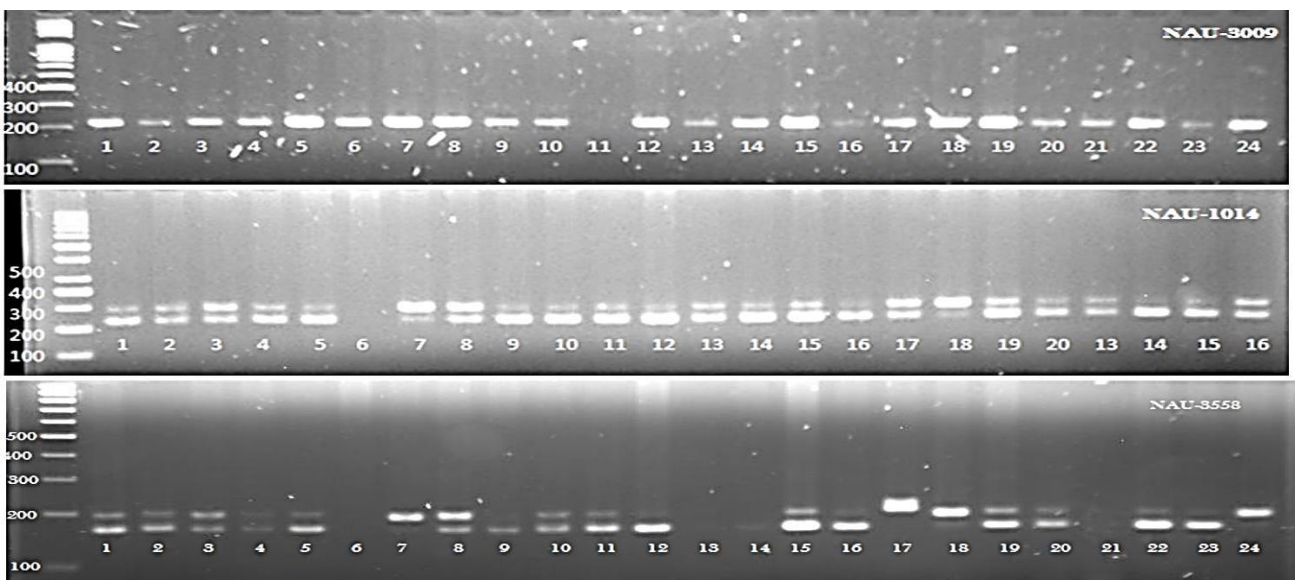

Fig. 1. Amplification product of primers NAU-3009, NAU-1014, and NAU-3558 using varieties 1= CIM-496, 2 = CIM-534, 3 = CRIS-121, 4 = CIM-554, 5 = CIRS-342, 6 = CIM-573, 7 = Bt CIM-598, 8 = BH-167, 9 = SLH-317, 10 = CIM-595, 11 = CIM-599, 12= NIAB-111, 13 = AC-13, 14 = MNH-147, 15 = S-12, 16 = 124-F, 17 = BH-36, 18 = CIM-448, 19 = NIAB-999, 20 = CIM-707, 21 = BH-160, 22 = 149-F, 23 = CIM-608, and 24 = CRIS-129.

Polymorphism information content (PIC): The information generated by primer is calculated through Polymorphism Information Content (PIC) value, which shows primer's ability to evaluate number of heterozygous alleles. The PIC values varied for different primers used on samples. Higher the PIC value, higher would be the potential to show molecular diversity. The range of PIC value was from 0.29-0.95. Mean PIC value for all markers was 0.70. The range of bands/primer varied from one to six and with an average of 2.23 numbers of bands/primers. The maximum PIC value (0.95) was observed for NAU-2503 showing high value of the marker. Whereas, lowest PIC value (0.29) was observed for NAU-1215. Among 100 markers used there was only one marker low polymorphism (lowest PIC value) whereas six markers (PIC: 0.30 to 0.59) were moderately informative. A large set of markers (PIC: >0.60) were proved to be highly informative as they targeted maximum number of locus in cotton. These markers could be selected for molecular genetic diversity analysis of cotton (Annexure-I).

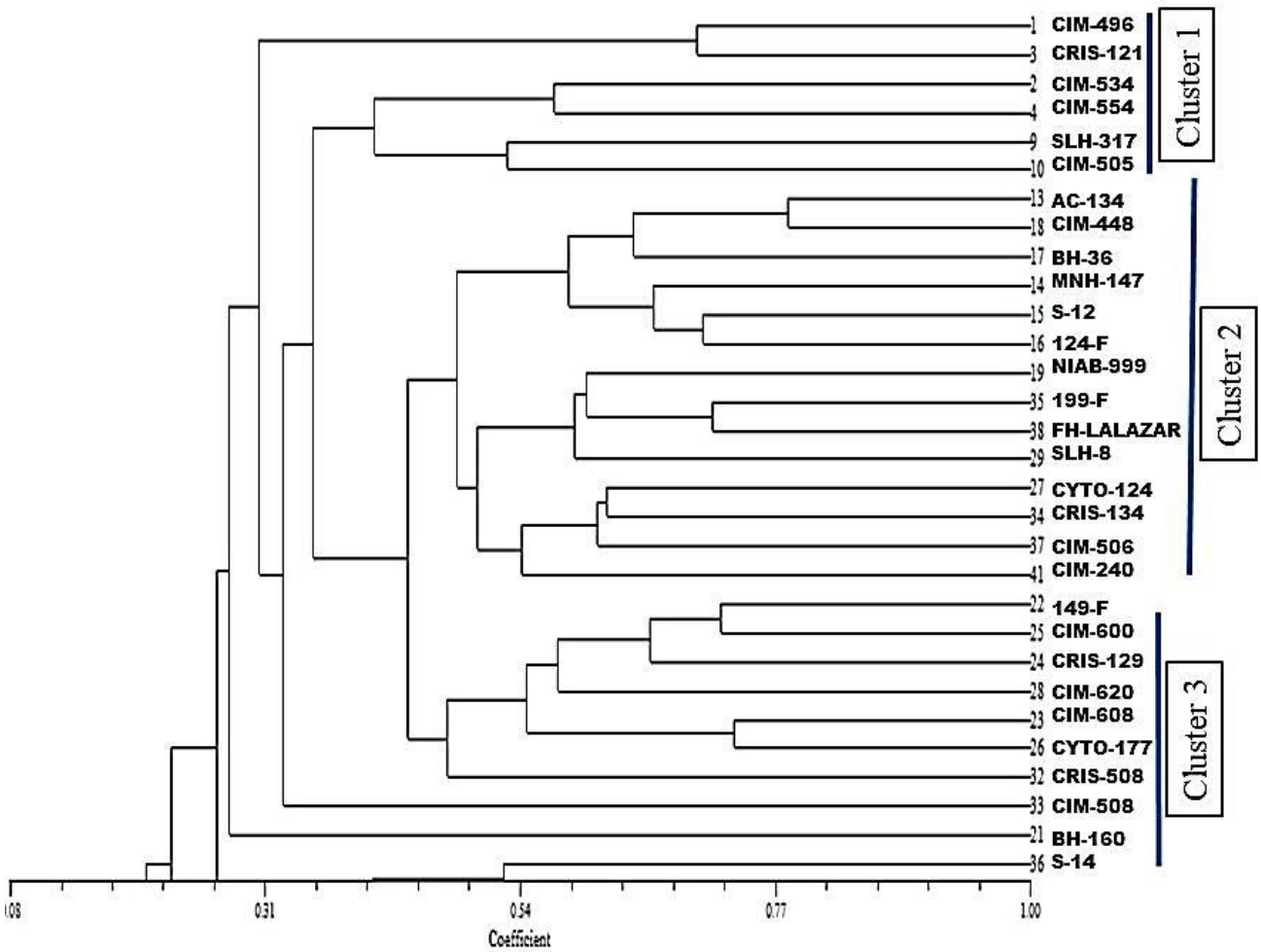

Fig. 2. Dendogram illustrating diversity among selected varieties.

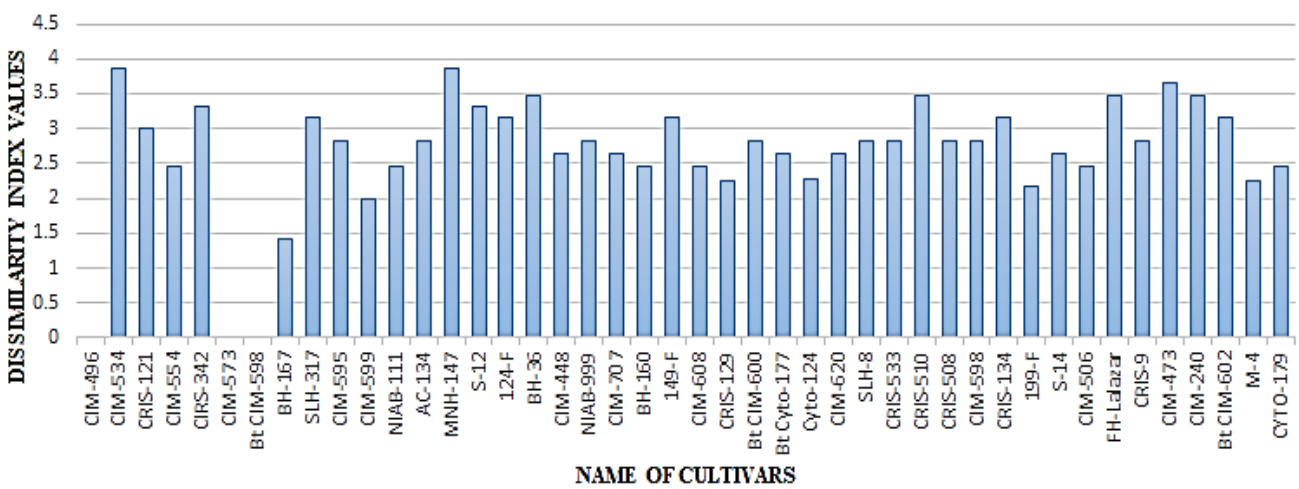

Fig. 3. The analysis of dissimilarity index among selected genotypes.

Discussion

Pakistan generated income through cotton. The crop produces fibre to be utilized in textile industry. Breeding efforts to produce improved cultivars has been hindered by genetic bottleneck or narrow molecular base of cotton varieties mostly used for cultivation. This study also showed that at molecular level, most in-use cotton varieties have same genetic background. The results verified previous studies that the artificial evolution in cotton has increased homogeneity (Iqbal e*t al*., 2001). The diversity analysis grouped all the verities into three main clusters but there was no prominent distinction among the clusters. The molecular results showed that CIM-496 and CRIS-121 had same genetic background but had been developed by different institute *i.e.* CCRI, Multan, and CRI-Sakrand. Similarly, CIM-534, CIM-554, SLH-317 and CIM-598 are similar molecularly to each other. The molecular results also

confirmed higher level of similarities among Pakistan BT cotton varieties as well. Six genotypes including AC-134, MNH-147, S-12, 124-F, BH-36, and CIM-448 grouped together having maximum genetic similarity, despite the fact that these do not have same research center of origin. The varieties 199-F, CRIS-134, SLH-8, and NIAB-999 are grouped together showing same genetic background. These varieties have different origins but the results shows that these may not be breed from different sources. Furthermore, CRIS-134, CIM-506, Cyto-124, and CIM-240 screened in one group and the succeeding group contained 10 varieties, CIM-608, CRIS-129, CIM-600, S-14, Cyto-177, CIM-620, BH-160, CRIS-508, 149-F, and CIM-598. Similar results have been reported by researchers who found minimum genetic diversity in cotton germplasm of Pakistan (Rahman *et al*., 2008). The main reason for this genetic bottle neck is continuous breeding among varieties of same origin resulted in very low level of variation left to breed new resistant cultivars against biotic and abiotic stresses, as it is considered as the most important factor to improve crop (Pereira *et al.*, 2015). The speedy way out is "Introduction" of new variation to breed new cotton varieties with maximum genetic diversity and good yield potential.

Conclusion

Molecular analysis showed low genetic diversity among popular cotton varieties of Pakistan hinders genetic improvement. Strategy like introduction, inter-specific hybridization, mutation breeding may be included for new spectrums of variation.

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