

Identifying Genetic Diversity of Cotton Leaf Curl Virus in Commercial Cotton (*Gossypium hirsutum* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Plant viruses have become a major issue to the crop production around the world. These viruses have become an unavoidable limiting factor by causing rigorous crop deficit for those major crops which are economically important. Among these viruses, begomoviruses belonging to family *Geminiviridae*, have brought the major devastation to both monocots and dicots in tropical and subtropical regions. These begomoviruses are transmitted through white fly and cause diseases like mosaic, yellow mosaic, yellow leaf curling, and yellow vein etc. The objective of this study is to evaluate the association between the genetic diversity of begomoviruses and phenotypic data to identify the ideal cotton genotype for breeding. In this study, 30 leaf samples with CLCuD symptoms were analyzed using rolling circular amplification and PCR. Obtained results expressed the presence of begomovirus with its associated satellites (i.e. alpha and betasatellites) in six varieties, association of begomovirus and betasatellites in four varieties and combination of begomovirus with alphasatellite in six varieties. Severe disease symptoms were exhibited with these combinations in

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selected varieties but betasatellite-begomovirus complex demonstrated a deadly impact. Few varieties expressed the resistance against the begomoviruses, so these varieties can further be manipulated for breeding.

Keywords: *Begomovirus; bemisia tabaci; diversity; alphasatellite; betasatellite; CLCuD.*

1. INTRODUCTION

Begomovirus is the largest genus of family *Geminiviridae* with almost 388 species [1] and it is being transmitted via *Bemisia tabaci* [2]. Begomoviruses have the large host range infecting dicots, ornamental and non-cultivated plants. Typical disease symptoms expressed in infected plants are stunting, leaf curling, leaf distortion and enation etc. Begomovirus causes severe yield loss in plants at seedling stage [3]. This virus has become a serious threat by infecting a variety of crops and vegetables [4] and its pathogens are exhibiting continuous emergence in temperate, tropical and subtropical regions [5]. The most destructive strains of CLCuD are Cotton leaf curl Multan virus (CLCuMuV), Cotton leaf curl Kokhran virus (CLCuKoV), Cotton leaf curl Bangalore virus (CLCuBaV), CLCuKoV-Bu (Burewala strain), Cotton leaf curl Alabad Virus (CLCuAIV) and Cotton leaf curl Rajasthan virus (CLCuRaV). Burewala strain which is basically a recombinant of CLCuMuV and CLCuKoV exhibited a disastrous effect on the cotton varieties. As this strain has the capability to infect the crops which were previously resistant to CLCuD, so in 2001, burewala strain causes infection in varieties resistant to Multan strain previously and also exhibited the disease symptoms. So, this *Burewala* strain is very hazardous and an enduring threat to cotton crop in Pakistan. Plants infected with *Burewala* strain shows disease

symptoms of dark and swelled leaves, enations, stunted and bushy appearance and downward cupping in younger leaves. In highly infected varieties, significant adverse effect exhibits on the yield components, lint yield and on the fiber quality of the cotton and then eventually it affects the parameters of yarn quality.

Begomoviruses are divided in two groups New World (NW) and Old World (OW) depending upon their geographical distribution [6]. They have circular and single-stranded genome with almost 2.5 to 3 kb genome size [7]. Their DNA is either bipartite or monopartite [8]. There are two DNA components i.e. DNA-A & DNA-B in bipartite genome [7] and one DNA component i.e. DNA-A in monopartite genome of begomoviruses [9]. Proteins required for replication and encapsidation of viral genome are encoded by DNA-A while DNA-B encodes proteins for symptoms induction and plant to plant movement [10]. The nucleotide sequences of these two DNA components are quite dissimilar from each other except common region (CR) (Mahatma et al., 2016) which is a short segment of almost 180-200 nucleotides [11]. DNA-A encodes five to six ORFs viz. AC1/C1, AC2/C2, AC3/C3, AC4/C4 in antisense strand and AV1/V1 & AV2/V2 in sense strand. DNA-B possesses two ORFs i.e. BV1 in sense orientation and BC1 in antisense orientation [9]. Begomoviruses replicate their genomes via rolling circular replication (RCR) [12].

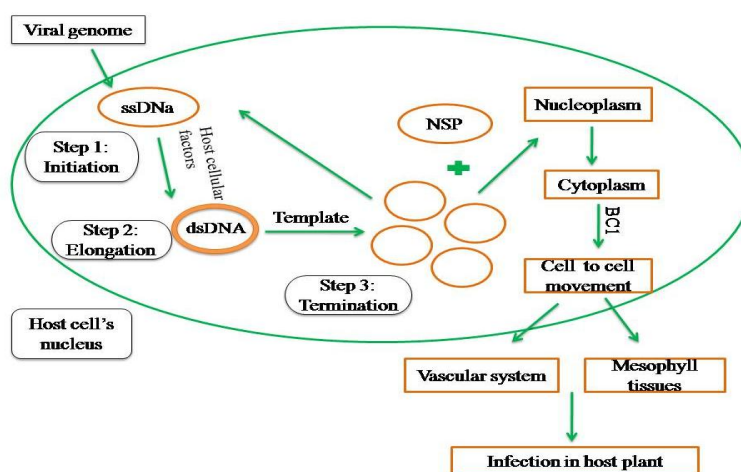


Fig. 1. Simplified mechanism of replication of begomovirus DNA

Usually, begomoviruses are found to be associated with DNA satellite molecules named as betasatellites and alphasatellites. Recently, a third type of DNA satellite molecule viz. deltasatellite has been identified. These satellite molecules carry circular and single-stranded DNA genome with almost 1.4 kb genome size [13,14] while the genome size of deltasatellite is almost 700 bp [15]. Betasatellites have expressed their role in symptoms modulation [16] and alphasatellite has been reported to interrupt the replication process of betasatellites but it exerts no effect on replication of begomovirus while there is clear evidence of alphasatellites' role in pathogenicity [17]. Although, the precise role of deltasatellite is not clear yet [15] but it has been reported that deltasatellite reduces the accumulation of begomovirus DNA and also contributes in suppressing symptoms of infection caused by helper viruses [14]. So, deltasatellite is not required by begomoviruses for disease occurrence [18].

The purpose of this study is to assess the genetic diversity among different cotton varieties for the resistance against cotton leaf curl disease (CLCuD) and phenotypic characters to isolate the ideal genotypes for breeding purpose.

2. MATERIALS AND METHODS

2.1 Sample Collection, Viral DNA Amplification and Detection

Leaf samples of cotton plants were gathered from Cotton Research Station (CRS) and Central Cotton Research Institute (CCRI) Multan Pakistan in October and November. Almost five

to six leaves were plucked from each of the 30 randomly selected plants (Table 1). [19], these tolerant and susceptible lines were used for the genotypic study by using different molecular markers. Phenotypic data were also noted down for the selected traits. Selected plants were classified into susceptible and tolerant lines based on the severity of disease. The varieties with high disease severity were placed into susceptible line and those varieties which depicted less severity of infection were put into the tolerant lines.

Data for phenotypic characters was calculated from three fully matured and uninjured plants from each line at 30, 60 and 90 days. Data calculated included plant height (cm), monopodial branches, sympodial branches, leaf length (cm), leaf width (cm), petiole length (cm), flowers per plant, bolls per plant, locks per boll, seeds per plant, boll weight (g) and CLCuD.

Genomic DNA was extracted via CTAB method (Doyle and Doyle 1987) with a modification, i.e. addition of Polyvinyl pyrrolidone (PVP). The extracted circular DNA was then amplified through rolling circular amplification (RCA) using phi 29 DNA polymerase [13]. Then, polymerase chain reaction (PCR) was performed on all 30 samples to identify the full-length begomoviruses by using BegomoRe-F/BegomoRe-R primer pairs [20], for alphasatellite detection primers DNA 101/ 102 were used [21] and the presence of betasatellites were checked by using Beta 01/02 primers [22]. All the amplified PCR products were then examined via gel electrophoresis in 0.8% agarose by using 1 kb DNA ladder [23].

Table 1. Selected Cotton Varieties

Sr. #	Variety name	Origin	Sr. #	Variety name	Origin
1	CIM 473	CRS	16	CYTO 535	CCRI
2	CIM 506	CRS	17	CYTO 533	CCRI
3	CIM 554	CRS	18	MAC 07	CCRI
4	CIM 573	CRS	19	DNWC	CCRI
5	NIAB KIRAN	CRS	20	CIM 446	CRS
6	AGC 999	CRS	21	CIM 482	CRS
7	ALSEMI 151	CRS	22	CIM 496	CRS
8	BH 160	CRS	23	CIM 598	CCRI
9	BH 184	CRS	24	CIM 610	CRS
10	Bt 121	CRS	25	CIM 632	CCRI
11	CIM 678	CCRI	26	CIM 707	CRS
12	CIM 632	CCRI	27	CYTO 608	CRS
13	CIM 789	CCRI	28	FH 114	CRS
14	CIM 775	CCRI	29	FH 118	CRS
15	CIM 735	CCRI	30	FH Lalazar	CRS

2.2 Statistical Analysis

Analysis of mean data for all phenotypic characters was performed by correlation analyses through a statistical software Minitab v 16 [24]. Statistical software, SPSS v 19 was used to perform principal component analysis and cluster analyses [25].

3. RESULTS

Varieties selected for this study were divided into 3 groups named as resistant (0-1), moderate (2-3) and susceptible (4-6) depending upon their CLCuD infection scale. In susceptible group, combination of betasatellite, alphasatellite and begomovirus were detected in many varieties such as FH 142 & Sitara 008 (disease index is 5.3 and 5.0, respectively) displaying that these varieties have high susceptibility towards CLCuD. In some varieties e.g. MNH 886 (5.0 disease index), only betasatellite has been found with begomovirus while the association of alphasatellite with begomovirus has been identified in just one highly susceptible variety i.e. IUB 222 (4.7 disease index). Combination of begomovirus with betasatellite badly affected the genotype of plants and the clear symptoms of a highly susceptible group such as severe leaf twisting, vein inflammation, development of enations and malformed internodes were observed which affected plant's morphology showing that betasatellites are more detrimental than the alphasatellites. Some varieties of moderate group like Tarzan 5 (1.67 infection rate) expressed both satellite molecules with begomovirus but the infection rate is very less. Presence of both virus and satellites with this reduced infection rate describes that this variety is effective against the virus and satellite combination because no remarkable harm is observed either in plant genetics or in its phenotype. Symptoms were not obvious but presence of very few leaf enations and slight vein thickening were manifested. Few varieties of the same group such as CIM 473 (2.33 infection level) depicted the presence of betasatellite and begomovirus only which means that betasatellite raise the disease scale and put some severe impact on the phenotypic characters of this cotton variety whereas alphasatellite also expressed its presence with begomovirus in varieties like CRSM 38 (infection level is 1.33) but this association is not very noxious to the plant because it is clearly demonstrated from the low infection level that alphasatellite didn't cause much harm to the phenotype of this variety.

Negligible vein thickening, slightly distorted internodes and insignificant decrease in leaf size were displayed by these varieties.

Genotypes in resistant group exhibited latent infection because no illustrated disease symptoms were present in these varieties, but the PCR analysis supplied the evidence of the presence of virus in this group. The combination of alphasatellite and begomovirus has been identified in Bahar 2017 variety with 1.00 infection rate; but no drastic effect has been seen from this virus-satellite complex. However, virus couldn't be detected in other resistant varieties because of its low titer.

To begin any breeding program, it is essential to obtain information about relationship amongst many characters because this can help in selecting a genotype with desirable traits [26]. Different statistical software were used in this study to describe the variation between the genotypes and the association between specific traits. In many breeding plans, similar approaches have been used effectively for multiple crops [27].

3.1 Correlation Analysis

Correlation analysis as indicated in Table 2 shows the relationship among 14 different phenotypic traits. First monopodial nodes per plant (FMN) expressed highest positive association (0.489) with monopodial branches per plant (MPP) and plant height (PH) (0.283). There is a significant positive correlation between sympodial branches per plant (SPP) and FMN i.e. 0.319, whereas, SPP showed negative correlation MPP i.e. -0.048. A highest positive association was found between First sympodial nodes per plant (FSP) and FMN i.e. 0.682. There was a remarkable positive correlation of Leaf width (LW) with leaf length (LL) with 0.951 value while, LW had minimum positive association with FMN i.e. 0.12. Negative correlation between LW and SPP have been shown i.e. -0.258. Plant length (PL) indicated the highest positive interaction with LW i.e. 0.596 and indicated least positive interaction with SPP i.e. 0.011, while PL indicated negative correlation with FSP and PH i.e. -0.063 and -0.16, respectively. Flower per plant (FP) had expressed significantly positive interaction with MPP i.e. 0.489 and also with other traits except PH with -0.106 value.

There is a significantly positive relation of locks per boll (LB) with LL (0.4), LW (0.374) and other

characters except SPP (-0.291), PH (-0.322) & FMN (-0.034). A negative correlation was represented by bolls per plant (BP) and FMN i.e. -0.166 and also by BP and FSP i.e. -0.212. But there happen to be a positive correlation between BP and rest of the traits. Seeds per boll (SB) had negative interaction with all the selected traits except SPP i.e. 0.018. Highest positive correlation was found between boll weight (BW), LW (0.312) and LL (0.397), whereas, BW depicted lowest positive correlation with PH i.e. 0.009. BW had negatively interacted towards other traits like FMN (-0.223), SPP (-0.266) and FP (-0.011). A maximum positive association was described by CLCuD with BP, PH, BW and SB i.e. 0.383, 0.166, 0.10 and 0.068, respectively while it had negative correlation with other characters.

3.2 Cluster Analysis

In cluster analysis, 32 cotton genotypes were grouped into five clusters to overcome the concern of categorizing the genotypes in the same species. Similar genotypes were clustered on the base of minimal distance. Cluster 4 contains maximum number of genotypes i.e., 11, whereas the cluster 1 comprises minimum number of genotypes i.e. 1. Cluster 2, 3 and 5 possesses 7, 6 and 7 genotypes, respectively. All the genotypes exhibited the highest proportion of plant height in all clusters, whereas boll per plant also indicated high amount of content in cluster 2. Cluster 3 and 4 exhibited maximum value of seeds per boll (Table 3).

3.3 Principal Component Analysis (PCA)

Out of 14 principal component, 5 components were extracted with Eigen value of >1. PC1-PC4 contributed 62.53 % of the total variability, while PC5 explained only 8.51 % of the variation. Thus, PC1-PC5 explained total variance of 71.12 %. Rest of the PCs contributed 28.88 % of total variance. The maximum share of the variance by PC1 is 23.9 %, PC2 16.7 %, PC3 12.4 %, PC4 9.5 % and PC5 8.5 %.

Traits like MPP, FMN, FSP had expressed positive factor loading whereas, PH and SPP exhibited minimum negative factor loading on PC1 (Table 4). The PC2 was explained by

variance due to PH, MPP, FMN, SPP, FSP and FP having positive factor loading, whereas, the other traits in that PC obtained negative loading. The PC3 was described only by PH, MPP and SPP with positive factor loadings, however, FMN and FSP loaded negatively on the same PC. The traits including PH, FMN, FSP displayed positive factor loading on PC4, meanwhile, MPP and SPP have significant negative loadings.

4. DISCUSSION

To successfully control the plant viral infections, it is essential to identify the source of initial viral inoculum [28]. Cotton crop is referred as silver fiber in Pakistan [29]. Very first attack of this virus was reported in Nigeria [19] in 1912. The initial observation of this infection in Indian sub-continent [30] was recorded in 1960 and in Multan; it emerged as local infection for next two decades [31]. In the current study, begomovirus have been identified in association with alphasatellite and betasatellite molecules causing infection to this crop.

Similar studies have been conducted in which the infected plant samples expressed the presence of begomovirus and betasatellite [32,33,34] while in another study presence of alphasatellite and begomoviruses have been detected [4]. Positive PCR amplification for alphasatellite, betasatellite and begomovirus complex has been demonstrated in various infected plants [35,36]. A similar kind of study has been reported which displayed the presence of bipartite begomovirus and betasatellite via RCA and PCR in symptomatic plants of kidney bean. The full-length sequences were also analyzed which displayed the virus to be *Mungbean yellow mosaic Indian virus* (MYMIV) and betasatellite Tomato leaf curl betasatellite (ToLCB). Thus, sequencing is a valuable technique to find out the available strains and to identify the new strains. Another study identified the presence of cotton leaf curl virus through quantitative real-time PCR (qPCR) assay. This technique detected virus in symptomatic plants with very low virus & satellite titer and in asymptomatic plants with high virus and satellite titer. So, qPCR assay can also be helpful in the detection of cotton leaf curl virus even at low titer.

Table 2. Phenotypic correlations among 14 traits of 32 accessed genotypes

	PH	MPP	FMN	SPP	FSP	LL	LW	PL	FP	LB	BP	SB	BW
PH	1												
MPP	-0.11*												
	0.527**												
FMN	0.283**	0.188*											
	0.116*	0.3											
SPP	0.064	-0.04	0.319**										
	0.724**	0.792**	0.074										
FSP	0.097	0.26**	0.682**	0.022									
	0.595**	0.149*	0.000016	0.904**									
LL	-0.27**	0.183*	0.08	-0.28**	0.216**								
	0.127*	0.313**	0.662**	0.118*	0.234**								
LW	-0.28**	0.204**	0.12*	-0.25**	0.156*	0.951**							
	0.119*	0.26**	0.51**	0.153*	0.392**	5.742**							
PL	-0.16*	0.148*	0.13*	0.011	-0.06	0.52**	0.596**						
	0.376**	0.415**	0.475**	0.948**	0.73**	0.002	0.0003						
FP	-0.10*	0.489**	0.311**	0.294**	0.13*	0.105*	0.097	0.199*					
	0.562**	0.004	0.082	0.102*	0.477**	0.566**	0.596**	0.274**					
LB	-0.32**	0.262**	-0.034	-0.29**	0.182*	0.4**	0.374**	0.248**	0.065				
	0.071	0.146*	0.85**	0.105*	0.317**	0.023	0.034	0.17*	0.72**				
BP	0.049	0.34**	-0.166*	0.067	-0.21**	0.111*	0.073	0.103*	0.335**	0.119*			
	0.786**	0.056	0.363**	0.711**	0.243**	0.542**	0.69**	0.571**	0.06	0.515**			
SB	-0.01	-0.17*	-0.32**	0.018	-0.27**	-0.09	-0.058	-0.13*	-0.19*	-0.16*	-0.07		
	0.943**	0.329**	0.073	0.918**	0.134*	0.595**	0.75**	0.46**	0.28**	0.379**	0.698**		
BW	0.009	0.142*	-0.22**	-0.26**	0.067	0.397**	0.312**	0.108*	-0.01	0.221**	0.245**	0.066	
	0.957**	0.436**	0.218**	0.139*	0.712**	0.024	0.081	0.552**	0.951**	0.224**	0.175*	0.715**	
CLCuD	0.166*	-0.26**	-0.19*	-0.10*	-0.20**	-0.19*	-0.23**	-0.06	-0.06	-0.20**	0.383**	0.068	0.102*
	0.363**	0.144*	0.278	0.575**	0.266**	0.296**	0.189*	0.736**	0.724**	0.26**	0.03	0.708**	0.574**

** Significant correlation at the 0.01 level (2-tailed)

* Significant correlation at the 0.05 level (2-tailed)

Table 3. Cluster Analysis of 14 Phenotypic Traits

Traits	Initial Cluster Centers			
	Cluster 1	Cluster 2	Cluster 3	Cluster 4
PH	274.3	122.1	97.0	122.9
MPP	2.22	2.66	2.55	2.11
FMN	8.77	7.11	3.44	7.44
SPP	15.6	16.7	19.3	17.1
FSP	9.66	7.66	6.88	8.77
LL	7.28	8.3	8.28	6.72
LW	7.94	8.72	9.1	7.75
PL	7.44	7.85	7.11	6.85
FP	2	3.55	3	2.66
LB	3.55	3.77	4.11	3.44
BP	28.1	42.3	37	18.1
SB	27.2	24	28.8	29.8
BW	2.94	3.2	2.82	2.77
CLCuD	5.02	5.1	4.08	4.27

Table 4. Principal Component Analysis of 14 Phenotypic Traits of Cotton Genotypes

Trait	Components			
	PC1	PC2	PC3	PC4
PH	-0.35	0.28	0.09	0.62
MPP	0.50	0.29	0.40	-0.06
FMN	0.22	0.85	-0.12	0.20
SPP	-0.24	0.52	0.25	-0.40
FSP	0.34	0.62	-0.28	0.42
LL	0.86	-0.21	-0.14	0.08
LW	0.86	-0.19	-0.17	-0.007
PL	0.61	-0.07	0.07	-0.20
FP	0.34	0.44	0.59	-0.25
LB	0.61	-0.16	-0.07	-0.01
BP	0.18	-0.17	0.85	0.12
SB	-0.27	-0.40	-0.07	-0.17
BW	0.38	-0.40	0.16	0.46
CLCuD	-0.31	-0.31	0.46	0.41
Eigen value	3.34	2.33	1.74	1.33
% of variance	23.9	16.7	12.4	9.5
Cumulative %	23.9	40.5	53.0	62.5

5. CONCLUSION

It has been investigated from this study that complex of betasatellite and begomovirus put a terrible effect on crops while no specific role is being played from alphasatellites in disease occurrence. Although presence of begomovirus and the associated satellite molecule has been observed in Tarzan 5 & Bahar 2017 but these varieties expressed resistance against infectious particles and virus couldn't cause much harm to them so these two varieties can further be used for breeding purpose. This study is able to confirm the presence of begomovirus with different disease symptoms in cotton plants and

this information is very useful for disease management. Thus, a careful monitoring is needed to assess the threat of this begomovirus in crop fields.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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