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Full Length Article

Identification and Molecular Characterization of Cotton Leaf Curl Begomovirus Complex Infecting Cotton in Baluchistan, Pakistan

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Abstract

Cotton leaf curl disease (CLCuD) is a serious threat to cotton productivity throughout the world, caused by whitefly transmitted single stranded DNA viruses belonging to the genus *Begomovirus*. Typical begomovirus disease symptoms were observed on cotton crop in 2017, in the Barkhan district, Baluchistan province, Pakistan. Symptomatic leaves were sampled and subjected to PCR amplification using both universal and specific primers for *begomovirus* and their satellites. The amplicons were cloned and sequenced; analysis of the resulting full-length viral sequences identified the begomovirus as a strain of cotton leaf curl Multan virus (CLCuMuV), which was associated with the cognate cotton leaf curl Multan betasatellite (CLCuMuB), and two alpha-satellite molecules cotton leaf curl Multan alphasatellite (CLCuMA) and okra leaf curl alphasatellite (OLCA). To the best of our knowledge, this is the first report of the begomovirus-satellite complex causing CLCuD in the Baluchistan province of Pakistan.

Keywords: Begomoviruses; Beta-satellites; Alpha-satellites; Cotton leaf curl Multan virus (CLCuMuV); Cotton leaf curl Multan beta-satellite (CLCuMuB); Cotton leaf curl Multan alphasatellite (CLCuMA)

Introduction

Cotton (*Gossypium hirsutum*) crop is a very important agricultural commodity and the export of cotton fibre and cotton goods play a critical role in the agriculture-based economies of many cotton-growing countries, including India and Pakistan. Cotton leaf curl disease (CLCuD) has a significant impact on cotton production in Pakistan and northern India (Sattar *et al.* 2013; Uniyal *et al.* 2019). This devastating disease was noted for the first time in late 1960s, close to the city of Multan, Pakistan (Hussain and Mahmood 1988; Ali *et al.* 2019) and quickly spread to practically all cotton-growing districts in the Punjab province and the north-western areas of India near the Pakistan border. Although CLCuD attracted minor attention initially, during the 1990s it emerged as an epidemic and caused major yield loss of cotton. CLCuD not only reduced the yield of cotton but also negatively impacted its quality-determining characteristics, such as fitness of fibre, length of staple, bundle strength of fibre, *etc*. This was due to compositional changes in fibre components including cellulose, protein, pectin, and wax (Farooq *et al.* 2011; Monga and Sain 2021). Cotton breeders established certain CLCuD-tolerant types in the late 1990s, through traditional breeding and selection, and cotton output in Pakistan was returned to pre-epidemic levels. Unfortunately, CLCuD symptoms reappeared in these tolerant types during the 2001–2002 growing season, originally near the town of Burewala in the Punjab province, indicating that the cotton resistance had been compromised (Mansoor *et al.* 2003). This marked the start of a second CLCuD epidemic, which quickly expanded over Pakistan and India's northern territories.

While Punjab sustained heavy losses of cotton crops during the first epidemic, other provinces of Pakistan including Sindh, Khyber Pakhtunkhwa and Baluchistan remained unaffected. Begomoviruses were reported in Sindh, infecting crops, non-crop weeds, and ornamental plants (Sanz *et al.* 2000), but the Sindh begomoviruses and their transmitting whitefly vectors were different from those in Punjab (Simón *et al.* 2003). CLCuD was initially reported in Sindh during 1997–1998, but losses were minimal as compared to those observed in Punjab (Mansoor *et al.* 1998). Since 2003–2004, however, CLCuD has been inflicting considerable losses in central and lower Sindh, as a result of introducing cotton types extremely sensitive to CLCuD and not approved for cultivation by the Pakistani government.

Baluchistan is the largest province of Pakistan, but agriculture is limited due to the plethora of mountainous and

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arid regions. Most of the land consists of dry, barren hilly regions and is not arable; however, some valleys are very fertile and suitable for agriculture. In Baluchistan, cultivation of cotton started in the early 1970s and has recently expanded manifolds. For instance, only 300 ha were used of cotton cultivation in 1981 which increased up to 41,000 ha in 2015 (Batool and Saeed 2017). Baluchistan has a high potential for cotton cultivation. cotton cultivation is increasing in Baluchistan and numerous districts, such as Lasbella, Jafferabad, Nasirabad, Kachhi, Sibi, Kohlu, Dera Bugti, Barkhan, Khuzdar, Turbat (Kech), Kharan, Naushki, and Loralai, have cotton fields where a sufficient quantity of water is available (GOB 2014; Kakar *et al.* 2018). In Pakistan, CLCuD was reported initially in Punjab and subsequently in Sindh; however, no investigations have been performed in Baluchistan to date. In the current study, we focus on the identification and molecular characterization of a begomovirus complex associated with CLCuD affecting cotton crops in Baluchistan. To our knowledge, this is the report of a cotton leaf curl begomovirus complex infecting cotton crop in the province Baluchistan, Pakistan.

Materials and Methods

Sample collection, extraction of plant genomic DNA and PCR amplification

During the 2017 growing season, cotton leaves exhibiting CLCuD symptoms were collected from different fields of the Barkhan district in the Baluchistan province, Pakistan. Total genomic DNA was extracted using the modified CTAB method as described in Akram *et al*. (2017) and subjected to rolling circle amplification (RCA). The RCA product was used as a template for PCR amplification with universal and sequence-specific primers, including universal back-to-back primer pairs Begomo F/Begomo R (Shahid *et al.* 2007) and Beta01/Beta02 (Briddon *et al.* 2002; Bull *et al.* 2003; Hussain *et al.* 2003), together with the sequence-specific primer pairs CLCV1/CLCV2 and DNA101/DNA102.

Cloning and sequencing of amplified product

The amplicons were cloned in the pGEM®-T Easy Vector and Sanger sequencing of both strands was performed by Macrogen Inc. Korea using primer walking. The complete genome sequence of the virus, including beta-satellite and alpha-satellite molecules, was assembled using Lasergene (DNA-Star Inc., Madison, WI, USA) and submitted to the GenBank database (accession numbers MW594402, MW603840, MW645089 and MW645090).

Sequence analysis

Similar sequences were retrieved from public databases, such as GenBank, using the Basic Local Alignment Search Tool (BLAST; Zhang *et al.* 2000) and multiple sequence alignments were performed using Clustal W, as implemented by Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 (Kumar *et al.* 2016). Pairwise sequence comparisons were done using MegAlign, as implemented by Lasergene, and Sequence Demarcation Tool (SDT; Muhire *et al.* 2014). Phylogenetic analysis was conducted by constructing a phylogenetic tree using the neighbor-joining algorithm, as implemented by MEGA 7.0 with 1,000 bootstrap replicates. Virus species names were retrieved from ICTV (http://www.ictvonline.org/virustaxonomy.asp) and abbreviations of begomovirus names and their satellites are shown as previously (Varsani *et al.* 2017). The online National Centre for Biotechnology Information (NCBI) tool ORF-finder was used to identify open reading frames (ORFs) in the sequence of the virus and the related satellites (https://www.ncbi.nlm.nih.gov/orffinder/).

Results

Sanger sequencing of cloned genomic components revealed that the begomovirus (CT4-begomo clone, MW594402) was 2738 bp, the two CT13 alpha-satellites (CT13-alpha clone, MW645089 and CT45-alpha, MW645090) were respectively 1370 bp and 1371 bp and the beta-satellite (CT4-beta clone, MW603840) was 1358 bp in length. For these and similar sequences retrieved from public databases, pairwise distance matrices were constructed using MegAlign and SDT and phylogenetic analyses were performed using MEGA.

Pairwise distance matrix (Fig. 1a) and phylogenetic analysis (Fig. 1b) of the CT4-begomo complete nucleotide sequence confirmed its identity as a CLCuMuV strain since it grouped together with other CLCuMuV strains from Pakistan and India and showed 98.7% sequence similarity with CLCuMuV strains (MG373551 and MG373556) isolated from hollyhock plants (*Alcea rosea*) in New Delhi, India (Kumar *et al.* 2020).

CT13-alpha and CT45-alpha were respectively revealed as strains of okra leaf curl alphasatellite (OLCuA) and cotton leaf curl Multan alphasatellite (CLCuMuA). Pairwise distance matrix analysis (Fig. 2a) showed that CT13-alpha had 78–99.5% similarity to other alpha-satellite molecules found in Pakistan: 99.5% with OLCuA (LN811059), 99.4% with Ageratum enation alphasatellite (AEA; MH510295, MH510296) and 96% with Ageratum conyzoides symptomless alphasatellite (ACSLA; KX656850) sequences. Similarly, CT45-alpha had 82.7–98.7% similarity with different isolates of CLCuMuA (MK357286, MT966813 and MT966814), found in different districts of Punjab, Pakistan. Phylogenetic analysis (Fig. 2b) also illustrated that CT13-alpha grouped with different isolates of OLCuA, AEA and ACSLA, while CT45-alpha grouped with different isolates of CLCuMuA.

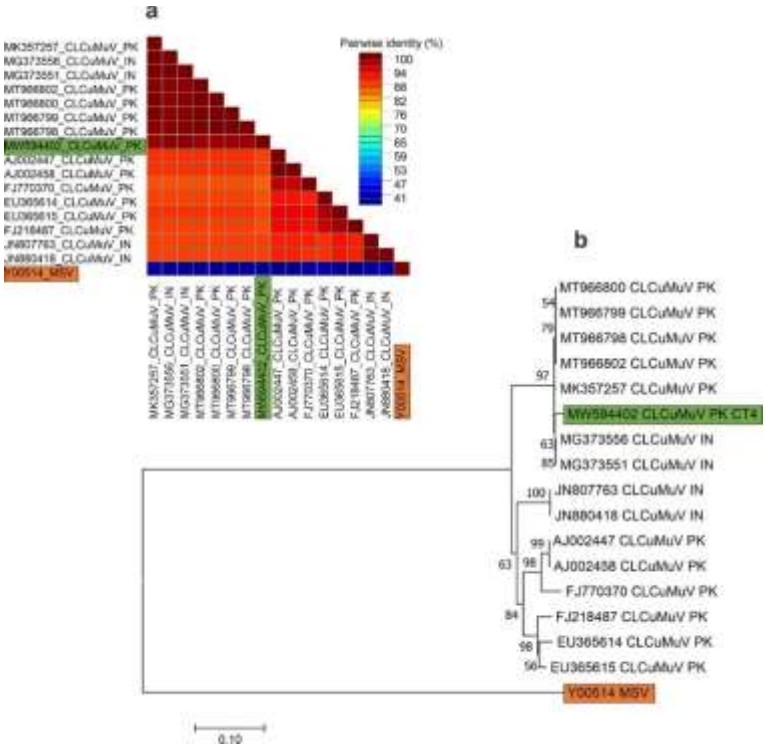

Fig. 1: (**a**) Pairwise distance matrix of CT4-begomo clone (MW594402_CLCuMuV_PK) sequence aligned by CLUSTAL W using sequence demarcation tool (SDT). The % identity of CT4-begomo clone with similar sequences from public databases are shown. (**b**) neighbor-joining phylogenetic tree constructed based on the alignment of CT4-begomo with closely related begomovirus sequences. CT4-begomo clone which was obtained in the current study is highlighted in green. The sequence of maize streak virus (MSV) serves as the outgroup and is highlighted in orange

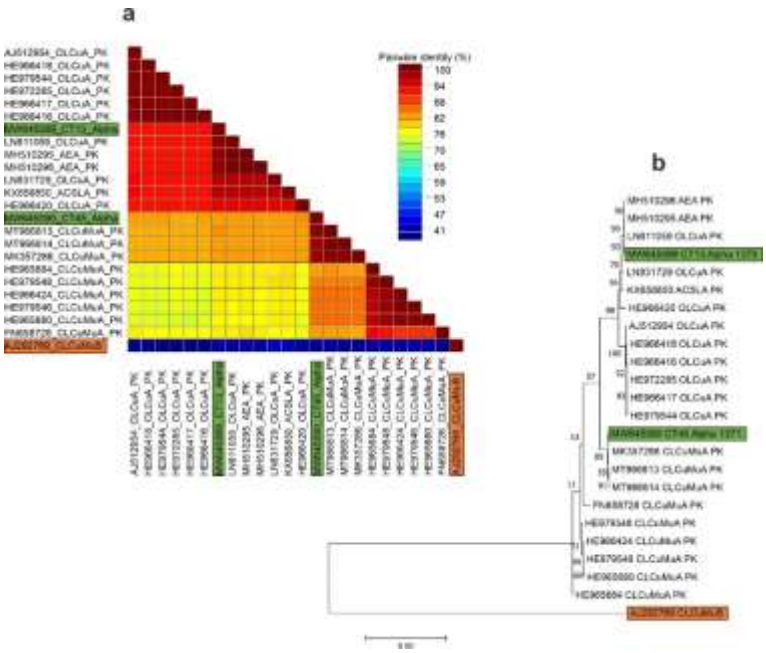

Fig. 2: (**a**) SDT pairwise distance matrix of isolated 2 alpha satellites (MW645089_CT13_alpha and MW645090_ CT45_alpha) sequences which were aligned by CLUSTAL W tool in SDT. The % identity of isolated alpha-satellites molecules with similar sequences from public databases are shown. (**b**) neighbor-joining phylogenetic tree constructed based on the alignment of CT13-alpha and CT45-alpha with closely related alpha-satellite sequences. CT13-alpha and CT45-alpha are highlighted in green. The sequence of CLCuMuB serves as the outgroup and is highlighted in orange

Table 1: Positions and coding capacity of predicted genes and ORFs percentage sequence identity for the clone of CT4 full genome virus, CT4 beta-satellite, CT13, CT45 alpha-satellite molecules isolated from cotton Baluchistan

Name	Strand	Frame	Start----Stop	Length nt/aa	Highest Percentage similarity (Virus name and Accession number)
CT4 clone full genome virus					
Rep/C1	-ve	3	2583-1495	1089/362	97.15% (CLCuMuV (KX656795)
TrAP/ C2	-ve	1	1598-1146	453/150	99.56% (CLCuMuV (MG373556)
REn/C3	-ve	2	1453-1049	405/134	99.75% (CLCuMuV (MG373556)
C4	-ve	1	2429-2127	303/100	98.68% (PeLCV (MN910265)
C5	-ve	1	791-60	732/243	100% (CLCuV (KY120361)
Coat protein/V1	+ve	3	276-1046	771/256	100% (CLCuV (KY120361)
V2	+ve	2	116-472	357/118	100% (CLCuMuV (MG373551)
CT4 beta-satellite genome ORF					
BetaC1	-ve	2	550-194	357/118	99.44% (CLCuMuB (MN910267)
CT13 alpha-satellite genome ORF					
Replication associated protein (Rep)	+ve	1	82-1029	948/315	99.68% (OLCuA (LN811059)
CT45 alpha-satellite genome ORF					
Replication associated protein (Rep)	+ve	2	77-1024	948/315	99.22% (CLCuMuA (MK357290)

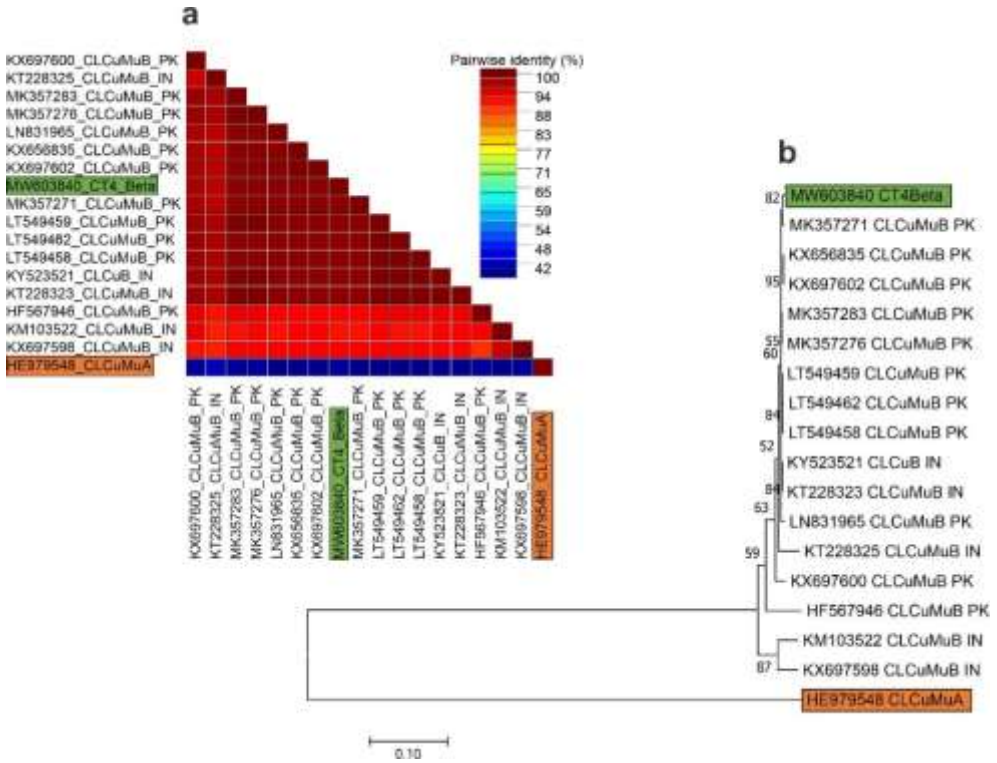

Fig. 3: (**a**) Pairwise distance matrix of isolated beta-satellite molecule (MW603840_CT4_Beta) sequence aligned by CLUSTAL W using sequence demarcation tool (SDT). The % identity of CT4-beta molecule with similar sequences from public databases are shown. (**b**) neighbor-joining phylogenetic tree constructed based on the alignment of CT4-beta with closely related beta-satellite sequences. CT4-beta is highlighted in green. The sequence of CLCuMuA serves as the outgroup and is highlighted in orange

CT4-beta was revealed as a strain of cotton leaf curl Multan betasetallite (CLCuMuB). Pairwise distance matrix (Fig. 3a) and phylogenetic analysis (Fig. 3b) showed up to 99.34% similarity with several other strains of CLCuMuB (LT549459, KT228323, KY52352, LT549459, MK357271, MK357276 and MK357283) found in Pakistan and India. CT4-beta was revealed as a strain of cotton leaf curl Multan betasetallite (CLCuMuB). Pairwise distance matrix (Fig. 3a) and phylogenetic analysis (Fig. 3b) showed up to 99.34% similarity with several other strains of CLCuMuB (LT549459, KT228323, KY52352, LT549459, MK357271, MK357276 and MK357283) found in Pakistan and India.

Potential gene sequences and their hypothetical protein sequences were analyzed using the NCBI ORF finder tool. In the case of CT4-begomo, two ORFs, V1 (coat protein) and V2 (pre-coat protein) were identified on the virion strand, whereas five ORFs, C1, C2, C3, C4 and C5, were identified on the complementary strand. CT13-alpha and CT45-alpha had a single ORF encoding a replication-associated protein on the complementary strand. CT4-beta also had a single

ORF known as beta C1 (*β*C1) on the complementary strand. The ORFs length, their coordinates, the number of encoded amino acids and their % homology with the most closely related virus and satellite genes available in public databases are described in Table 1.

Discussion

Cotton (*Gossypium* spp.) has a major economic impact in cotton-producing countries worldwide. After China, the United States, India, and Brazil, Pakistan is the fifth-largest producer of cotton (Aslam *et al.* 2022), with the average cotton yield being about 570.99 $kg.hm^{-2}$. Currently, cotton production is declining due to climate change and various biotic stresses (Razzaq *et al.* 2021). Various factors influence cotton yield, the most important being CLCuD caused by begomoviruses. CLCuD was initially reported in 1912 in Nigeria, while in Pakistan it was reported for the first time in 1967 in the Punjab province (Farooq *et al.* 2014; Ali *et al.* 2019) and the Sindh province remained unaffected until 1997 (Mansoor *et al.* 1998, 2006). Pakistan is the most productive country in the world in terms of research related to CLCuD and more than 217 articles have been published on this topic (Khan *et al.* 2020). However, no investigations for CLCuD have been conducted and no CLCuD has been reported in the Baluchistan province of Pakistan. Here we report for the first the identification and characterization of CLCuMuV and its associated satellites as a complex causing CLCuD in cotton plants collected from Baluchistan, Pakistan.

Analysis of the complete genome sequence of the begomovirus we discovered showed maximum nucleotide sequence identity with CLCuMuV isolates from New Delhi, India (Kumar *et al.* 2020). According to Zerbini *et al.* (2017), CLCuMuB is required for the development of CLCuD symptoms by the majority of viruses that cause CLCuD. In this study, we have also identified a beta-satellite that exhibited maximum similarity with different isolates of CLCuMuB. In addition to the beta-satellite, we isolated two alpha-satellites from the same cotton samples. The first alpha-satellite showed maximum similarity with different isolates of CLCuMuA, first identified in cotton plants from central Punjab (HE965684, HE979548, HE965680, HE966424, HE979546; Mansoor *et al.* 1999; Siddiqui *et al.* 2016) and also from Burewala (misnamed as CLCuBuA in GenBank; FN658728), Punjab, Pakistan (Hameed *et al.* 2014). The second alpha-satellite showed the highest similarity with OLCuA, AEA and ACSLA, three alpha-satellite molecules isolated from Pakistan. OLCuA was first isolated from okra plants in Pakistan (AJ512954; Briddon *et al.* 2004) and subsequently found in cotton samples from Punjab, Pakistan (*e.g.*, HE966420, HE966418, HE979544, HE966417, HE966416, HE972285; Siddiqui *et al.* 2016). Interestingly, begomoviruses possess the strategy of recombination and pseudo recombination, allowing them to overcome the plant defense mechanisms for *bona fide* infection. This is a serious problem as the virus can modify itself by recombination and can evolve into a more virulent complex by capturing diverse satellite components. According to literature, the Old-World alpha-satellites have been demonstrated to play a role in the suppression of gene silencing (Nawaz-ul-Rehman *et al.* 2010; Abbas *et al.* 2019), and they also reduce disease symptoms (Luo *et al.* 2019; Kumar *et al.* 2021). However, research on New World alpha satellites (Nogueira *et al.* 2021) elucidated that their presence can enhance the severity of symptoms. Their ability to suppress gene silencing may be one reason why these alpha satellites are maintained by helper viruses. Furthermore, the alpha satellites interfere with the transmission of the virus by the whitefly vector. Therefore, it can be presumed that alpha satellites suppress the plant's natural immunity and provide the necessary platform for viral infection during the initial stages. However, they might compete with the helper virus and lower its replication during the later infection stages.

Interestingly, a recent study highlighted that the presence of a beta-satellite impairs the maintenance of the alpha-satellite and mutations in the CP of helper virus further reduce its titer (Iqbal *et al.* 2022). In our study, two different alpha-satellites together with a beta-satellite are found to be associated with a helper virus. The molecular and cellular mechanisms underlying their pattern of interactions between these alpha and beta-satellites could be an interesting research topic for the future and help in understanding the maintenance of alpha-satellites by the helper virus.

Conclusion

In the current study, we conclude that CLCuMuV is associated with different alpha-satellites and a dominating cotton leaf curl Multan beta-satellite to infect cotton. The limitation of the study is that the samples were collected exclusively from Barkhan, an eastern district connected to the Punjab province. More extensive studies with comprehensive sampling from all cotton-growing districts of Baluchistan are needed to establish the complete CLCuD-causing complex of begomoviruses in Baluchistan.

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Author Contributions

KH designed the project and studies. KR, MA and KH collected the samples and performed the experiments. KR, KH, MT and SS analyzed data. KR, KH and IKL wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

Authors declare no conflict of interests and all authors read and approved the manuscript and agreed to submit it in IJAB for publication.

Data Availability

The full genome sequences of CT4-begomo clone is under accession number MW594402, alpha-satellites CT13 and CT45 alpha-satellites under accession numbers MW645089 and MW645090 respectively and CT4-beta betasatellite under accession number MW603840 in Genbank database.

Ethics Approval

The present research does not involve any animal as experimental organism therefore approval from ethical committee was not needed.

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