Journal of Plant Protection Research eISSN 1899-007X

ORIGINAL ARTICLE

Molecular characterization of cotton leaf curl Multan beta-satellite occurance on chili plants in Multan, Pakistan

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DOI: 10.24425/jppr.2024.150248

Received: September 12, 2023
Accepted: February 14, 2024
Online publication: May 13, 2024

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Responsible Editor:
Huan Zhang

Abstract

The prevalence of cotton leaf curl disease (CLCuD) has significantly hampered chili (*Capsicum* spp.) production, presenting a formidable challenge in Pakistan. During a chili field survey in 2018, distinct symptoms, including stunted growth, yellowing, and severe leaf curling, were observed on several plants. Subsequently, a comprehensive sampling effort was undertaken, collecting a total of 39 symptomatic samples from diverse locations across Multan, Punjab. The DNA extraction from these samples was conducted at the plant virology laboratory at Bahauddin Zakariya University, Multan, marking a crucial step in the investigation of this debilitating disease and its impact on chili production in the region. Molecular analysis with PCR using Av/Ac Core, Beta 01/02, and CLCuMuBF11/R33 primers confirmed begomovirus infection in chili plants. Positive amplification demonstrated a 71.79% infection rate, with 579 bp, 1.4 kb, and 481 bp amplicons for Av/Ac Core, Beta 01/02, and CLCuMuBF11/R33, respectively. Sequencing identified cotton leaf curl Multan beta-satellite (MT668934) infecting the chili plant. Effectively managing these begomoviruses is crucial to curbing their multiplication and protecting vital crops like chili. Addressing the distributions of beta-satellites in agricultural fields, particularly chili, is imperative to prevent further viral spread.

Keywords: begomovirus, cotton leaf curl virus, Geminiviridae, phylogenetic analysis, stunted growth

Introduction

Pakistan, located in South Asia, is famous for its abundant cultural heritage and stunning landscapes. However, it has earned even greater renown due to its valuable agricultural resources and fertile lands (Hyder *et al.* 2018; Ali *et al.* 2020). Chili is an indispensable spice utilized in diverse culinary traditions and possesses medicinal properties that enhance its value as a useful commodity on the global market. Chili is an essential spice used in various cuisines and has medicinal properties that make it a valuable commodity on the global market. In recent years, Pakistan has assumed a notable role as a substantial chili producer and exporter, significantly contributing to the nation's economic prosperity (Arin 2019). According to the latest study by Abdullah *et al.* (2021), Pakistan produced 716.6 thousand metric tons of chili in the years 2020–2021, making it the fourth-largest producer of chili in the world. Chili production in Pakistan

is primarily centered in the provinces of Sindh and Punjab, where the climate is particularly conducive to its cultivation. As global demand for chili continues to rise, Pakistan is capitalizing on this trend by exporting its chili to a variety of countries, including the United States, the United Kingdom, and China. This export activity is contributing to Pakistan's economic growth and expanding its role on the international chili market (Rais *et al.* 2021). In recent years, the export of chili from Pakistan has experienced a notable increase of 35%, contributing substantially to the country's revenue.

The chili plant is renowned for its spiciness and serves as a bountiful source of essential vitamins, minerals, and antioxidants (Azlan *et al.* 2022; Sahid *et al.* 2022). Despite their high economic importance, chili plants are vulnerable to many diseases caused by various viruses, bacteria, and fungi. Numerous viruses and other pathogens pose a significant threat to chili crops, causing substantial economic setbacks for the agricultural industry. Globally, there is a documented presence of at least 65 distinct viruses known to infect chili plants, compounding the challenges faced by chili growers and highlighting the urgency of effective disease management and crop protection measures (Nigam *et al.* 2014; Nigam 2021). These viruses include begomoviruses that cause chili leaf curl virus disease (ChiLCVD). The begomoviruses are a group of plant viruses that cause devastating diseases in several important agricultural crops around the globe. The genome sequences of begomoviruses infecting chili, viz, chili leaf curl Vellanad virus, chili leaf curl virus (ChiLCV), tomato leaf curl New Delhi virus (ToLCNDV), chili leaf curl India virus, chili leaf curl Palampur virus (Khan and Khan 2017), and tomato leaf curl Joydebpur virus (Kumar *et al.* 2011), have been characterized. In severe cases, reports indicate that chili growers (have experienced yield losses of up to 100% in both pre-harvest and post-harvest fruits (Senanayake *et al.* 2012; Rai *et al.* 2014). Typical symptoms consist of crowding of leaves, rolling, leaf curling, shortening of internodes and petioles, puckering, swelling and thickening of the veins, blistering of intravenous areas, and stunting of the whole plant. The transmission of these chili viruses by mites (*Polyphagotarsonemus latus*), thrips (*Scirtothrips dorsalis*), and whiteflies (*Bemisia tabaci*) has been documented in prior studies (Chakraborty and Ghosh 2022*).*

The molecular characterization of cotton leaf curl Multan beta-satellite (CLCuMuB) and its association with leaf curl diseases in chili plants in Multan, Pakistan, is a significant research topic due to its implications for agriculture and food security in the region. Emergence of Cotton leaf curl Multan beta-satellite (CLCuMuB) is a component of the cotton leaf curl Multan virus (CLCuMuV) complex, which primarily affects cotton crops. However, in recent years, this begomovirus-beta-satellite complex has expanded its host range to include a variety of solanaceous plants, including chili. This expansion has raised concerns in agricultural communities in Multan, Pakistan, where chili is a vital crop. Chili is a crucial cash crop in Multan, and the presence of CLCuMuB in chili plants has been associated with leaf curl diseases, which result in reduced yields and damaged fruit quality. This is a concerning issue that demands investigation. Conducting a phylogenetic analysis of beta-satellites with CLCuMuV's hosts and vectors is essential to trace the evolutionary relationships between these components. This can provide insights into the origin of beta-satellite strains that are infecting chili in Multan, Pakistan. This research aimed to contribute to the sustainable cultivation of chili and address the emerging threat posed by the expansion of this begomovirus-beta-satellite complex to new hosts. The proteins required for replication initiation and for recruitment of the host replication machinery are formed from the transcription of dsDNA intermediates. The virus encodes two proteins which are imperative for effective virus replication, i.e. C1 (Rep.) and C3. C1 protein serves as the launching factor and occurs midway between origin detection and DNA cleavage/ligation to begin and end the rolling circle replication process. The C3 protein alters the C1 activity and helps in the enrolment of host replication enzymes which help in accretion of high levels of viral DNA.

CLCuMuV, a whitefly-vectored begomovirus, was initially identified as the principal causative agent of Cotton leaf curl disease (CLCuD) in Multan, Pakistan (Briddon and Markham 2000; Mansoor *et al.* 2003). A single whitefly was able to transmit the virus, and eight or more whiteflies per plant resulted in 100% transmission. The minimum acquisition access period (AAP) and inoculation access period (IAP) were 180 and 60 min, respectively. CLCuMuV is also associated with a beta-satellite known as cotton leaf curl Multan beta-satellite (CLCuMuB). Since then, the virus has swiftly disseminated to a considerable number of cotton-growing nations, primarily in South Asia. A recent study by Farooq *et al.* (2021) reveals the geographical distribution of highly recombinant and fast-evolving CLCuMuV populations across key cotton-producing countries. The study also emphasizes the significant genetic diversity within these populations. However, the exact source and route of virus transmission from Pakistan to China or other countries have not been comprehensively documented (Afzal *et al.* 2023a), despite the fact that local transmission of CLCuMuV is linked to the whitefly cryptic species complex (Chen *et al.* 2019).

DNA A and DNA B are integral components of the bipartite genome of begomoviruses (Akram *et al.* 2022). DNA A consists of a total of six open-reading frames. Two of these frames are in the virion sense (AV1 and AV2), and the other four are in the complementary sense (AC1, AC2, AC3, and AC4). On the other hand, DNA B possesses just two open reading frames, one that reads in the virion sense (BV1) and one that reads in the complementary sense (BC1). Begomoviruses are categorized into two groups: Old World begomoviruses (OWBego) and New World begomoviruses (NWBego). OWBego and NWBego are abbreviations commonly used in the context of begomoviruses, particularly in relation to their DNA components. OWBego stands for "Old World Begomovirus," referring to begomoviruses that are predominantly found in the Old World, which includes regions like Asia and Africa. NWBego stands for "New World Begomovirus," indicating begomoviruses that are primarily present in the New World, encompassing regions like the Americas. These terms are often used to classify begomoviruses based on their geographic distribution. It's important to note that begomoviruses often interact with satellite DNA molecules known as beta-satellites. Beta-satellites are small, circular, single-stranded DNA molecules that depend on helper begomoviruses for replication and encapsidation. The interaction between begomoviruses and beta-satellites is integral to the symptom induction and disease development observed in infected plants. The specific relationship between OWBego, NWBego, and beta-satellites can vary based on the individual characteristics of the viruses and their associated satellites. In the case of DNA A, it is recognized as the OWBego component, as evidenced by the fact that the virion sense encodes both the coat protein and the V2/AV2 protein. The replication-associated protein, also known as the C1/AC1 protein, the transcriptional protein, also known as the C2/AC2 protein, the replication enhancer protein, also known as the C3/AC3 protein, and the C4/AC4 protein, which has an unclear function, are all encoded by the complementary sense. According to Fondong (2013), the ORF region of AV2 was only discovered in the bipartite begomoviruses of the OWBego and not in any viruses from the NWBego. The monopartite begomovirus (single nucleus) is made up of open reading frames, each of which encodes one of the six genes that make up DNA A. A plethora of monopartite begomoviruses is associated with a novel class of single-stranded DNA satellites known as beta-satellites. These satellites rely on a helper virus for replication, displaying limited nucleotide sequence homology to the helper virus and are not crucial for its multiplication. Beta--satellites, approximately 1350 bp in size (half that of helper begomoviruses) play a pivotal role in symptom induction, determining host range, and overcoming host defenses (Briddon and Stanley 2006). Symptom development in plants infected with helper viruses is significantly influenced by the presence of satellite RNAs, ranging from symptom alleviation to increased severity. Research has shown that beta-satellites enhance viral symptoms without affecting viral DNA levels in hostplants (Khan *et al.* 2013). Additionally, betasatellites can impact the levels of helper viral DNA A and DNA B; their presence results in a 16-fold increase in DNA B, while the accumulation of beta-satellites is reduced by 60% in the presence of DNA B (Jyothsna *et al.* 2013). The interaction of bipartite begomoviruses and beta-satellites also leads to rapid changes in begomovirus complexes (Akhter *et al.* 2014). For instance, the pathogenicity of ChiLCBV (chili leaf curl Bijnour virus) in chilies is determined by bC1 of the chili leaf curl beta-satellite, influencing disease symptoms (Tahir and Mansoor 2011). Similarly, the ChiLCGV (chili leaf curl Gonda virus) and tomato leaf curl Bangladesh beta-satellite (ToLCBDB) complexes induce severe leaf curl symptoms in *Nicotiana benthamiana* (Khan and Khan 2017). This intricate interplay between begomoviruses and beta-satellites showcases the dynamic nature of plant viral infections.

Many monopartite begomoviruses are linked to satellite DNAs, including alpha-satellites, beta-satellites, and delta-satellites. These satellite DNAs either play a crucial role in the development of typical disease symptoms (beta-satellites) or have no discernible impact on or regulation of disease symptoms (alpha-satellites). Delta-satellites, on the other hand, do not contribute to the development of typical disease symptoms. However, it is important to note that only monopartite begomoviruses infecting tomatoes exhibit typical disease symptoms in their natural host when beta-satellites are absent (Melgarejo *et al.* 2013). Many monopartite begomoviruses are capable of infecting and causing symptoms in *Nicotiana benthamiana* even when there is no beta-satellite present. Replication of delta-satellites, encapsidation of delta-satellites, cell-to-cell transmission of delta-satellites, and transmission of delta-satellites by whiteflies are all dependent on the begomovirus genome (Fiallo-Olivé *et al.* 2016). Therefore, there is a dire need to stop the further spread of this destructive beta-satellite to improve chili production.

The study of cotton leaf curl Multan virus (CLCuMuV) and its associated beta satellite, cotton leaf curl Multan beta-satellite (CLCuMuB), in chili crops is of significant importance for several reasons. Here are some key points highlighting the importance of studying CLCuMuV and CLCuMuB in chili crops. Firstly, there is an emerging threat to chili production. CLCuMuV and CLCuMuB have been primarily associated with cotton crops, but there is evidence that they are now infecting a wide range of solanaceous plants,

including chili. Understanding the extent and impact of this expansion is crucial to assessing the potential threat to chili production. Secondly, for disease management, knowledge about the presence of CLCuMuV and CLCuMuB in chili crops is essential for implementing effective disease management strategies. This can include strategies like breeding for resistance, vector control, and crop rotation to mitigate the impact of these pathogens. The economic importance of chili cultivation makes it imperative to consider the potential economic consequences of the spread of CLCuMuV and CLCuMuB (Sattar *et al.* 2013; Saleem *et al.* 2020). This spread can result in diminished chili yields and a decline in the overall quality of chili produce, thereby posing a significant economic threat. This is a concern for both farmers and the chili industry. The objective of the present study was to detect begomoviruses and beta-satellites in new chili fields in Multan, Pakistan, at the molecular level through PCR. The molecular detection of begomoviruses and beta-satellites in newly cultivated chili fields serves as a proactive measure for disease control, crop protection, and contributes to broader agricultural research efforts.

Materials and Methods

Field survey for sample collection

During the present study, the survey was conducted to collect virus-infected chili leaf samples from five locations (10 samples from Qasba Marla, seven samples from Lar, six samples from 5-Faiz, six samples from Makhdoom Ali and, 10 samples from Rattan Hatti) of the district Multan, Punjab, Pakistan. A total of 39 leaf samples displaying typical viral symptoms, including leaf curling, yellowing, vein thickening, and puckering, was collected. Samples were carefully placed in polythene bags and stored at –20°C for subsequent processing in the laboratory.

DNA extraction

The DNA from the collected virus-infected fresh chili leaf samples was extracted using the CTAB method (Doyle and Doyle 1990). The fresh CTAB extraction buffer was prepared by adding (polyvinyl pyrrolidone (PVP) 1%, cetyl trimethylammonium bromide (CTAB) 2%, tris-HCl 100 mM, ethylene diamine tetra-acetic acid (EDTA) 20 mM, sodium chloride (NaCl) 1.4 M, and β-mercaptoethanol (0.2%). A 100 mg leaf sample was ground into a fine powder using liquid nitrogen with an autoclaved pestle and mortar. Subsequently, the fine powder was transferred into a 1.5 ml centrifuged tube (Eppendorf). To each Eppendorf tube, 700 µl of extraction buffer was added, and the tubes were then placed in a water bath for 1 hour at 65°C. During this incubation, the tubes were gently inverted every 5–6 minutes. An equal volume of chloroform isoamyl alcohol (24 : 1) was added and centrifuged at 10,000 rpm for 10 minutes. After centrifugation, the supernatant was taken and 0.6 volume cold 2-propanol for DNA precipitation was added. It was mixed well and placed in the tube at –20°C for 30 minutes and then centrifuged for 10 minutes at 12,000 rpm. Finally, the DNA pellet was washed with 70% ethanol. The pelleted DNA was air-dried before being dissolved in 100 µl of TE buffer. To assess the quality and quantity of DNA per microliter, nano-drop quantification was employed.

PCR amplification

PCR amplification for the detection of begomovirus from chili samples was done using the following primers (Table 1). A total of 25 µl of PCR reactions comprised of 12 µl 2 × FastTaq PCR master mix (ZOMANBIO), 10 µl water, 1 µl of forward primer, 1 µl of reverse primer, and 1 µl genomic DNA. The primers used in the study are mentioned in Table 1, along with their PCR profile. The PCR product was run on 1.5% of agarose gel with ethidium bromide for the confirmation and presence of virus in PCR samples and a Gel Doc with UL was used to visualize the PCR amplification. The Av/Ac Core primer was employed for the detection of begomovirus and coat protein was the targeted region. The targeted gene of Beta 01/02 and CLCuMuBF11/R33 primers were BC1 and A-rich region utilized for beta-satellite detection.

Sequencing and phylogenetic analysis

The cleaned amplified PCR product was cloned and sent to the Macrogen company for Sanger sequencing. The obtained sequences were cleaned and BLAST on NCBI was carried out to compare with other related sequences submitted to Genbank from other countries. Sequence alignment was aggregated of begomovirus sequences with the Clustal W method using MEGA X7 software (Kumar *et al.* 2018). The phylogenetic tree was created with 'Mega X7 software having 1000 bootstrap replication.

Results

Observed symptoms during the study

The collected chili leaves and field plants clearly showed begomovirus symptoms like leaf curling, yellowing, vein thickening, and puckering (Fig. 1).

Table 1. Primers used for the detection of begomovirus

Primers	Primers sequence	Amplicon size	PCR profile	References
Av/Ac Core	GCCHATRTAYAGRAAGCCMAGRAT GGRTTDGARGCATGHGTACAN GCC	579 bp	95°C, 5 min; (30 sec at 95°C; 30 sec at 50°C; and 45 sec at 72°C with 35 cycles)	Abdel-Salam *et al.* (2016)
Beta 01/02	GGTACCACTACGCTACGCAGCAGCC GGTACCTACCCTCCCAGG GGTACA C	1.4 kb	95°C, 5 min; (30 sec at 95°C; 30 sec at 56°C; and 45 sec at 72°C with 35 cycles)	Briddon *et al.* (2002)
CLCuMuBF11/R33	GGTCCCACTGCTTGTCTTGA GGTTCATAGTCGACGTTCGC	481 kb	95°C, 5 min; (30 sec at 95°C; 30 sec at 56° C; and 45 sec at 72°C with 35 cycles)	Brown *et al.* (2017)

Fig. 1. A – leaf curling, mild puckering and stunted growth; B – yellowing, puckering and less curling; C – severe leaf curling and puckering

PCR amplification for the detection of begomovirus

Molecular level detection for the confirmation of begomovirus from all 37 collected chili samples was done through the PCR by using three well known begomovirus detection primers (Av/Ac core, CLCuMuBF11/R33, and Beta 01/02). The PCR amplification confirmed that 28 out of 39 showed positive amplicon with 579 bp, 481 bp and 1.4 kb, respectively (Fig. 2). Table 2 showed the incidence and presence of begomovirus location-wise with all tested primers.

Samples from the 5-Faiz location exhibited a 50% disease incidence based on PCR detection (three out of six), representing the lowest recorded value compared to other locations. In Qasba Marla, six out of 10 samples indicated a 60% incidence with Av/Ac core primer. Additionally, four out of six samples showed positive results, representing a 67% incidence with samples collected from Makhdoom Ali. For samples collected in Lar (a total of seven), five displayed positive results with the begomovirus and beta-satellite primer, resulting in a 71% detection percentage. In Rattan Hatti, where 10 samples were collected, eight exhibited positive results with Av/Ac core, CLCuMuBF11/R33, and beta 01/02 primers. The beta 01/02 primer which showed 1400 bp product size, confirmed the association of beta-satellite with chili samples of Multan. An amplicon of 481 bp obtained with CLCuMuBF11/R33, universal beta-satellite primer. The product size of 579 bp was achieved by using the Av/Ac core primer.

Results of phylogenetic analysis

Phylogenetic analysis of the obtained sequences during the present study was conducted by using MEGA X7 software, incorporating data from already reported sequences from other countries of the world. The closest sequence accession was retrieved (Table 3) from the NCBI after BLASTing our obtained sequence (MT668934) (Fig. 3). The phylogenetic tree was bifurcated into two distinct groups, labeled as A and B. Notably, our obtained sequence from the present study was grouped within category A, alongside other closely related accessions. Our sequencing result revealed that Cotton leaf curl Multan beta-satellite was infecting chili plants in Multan, Pakistan. However, Cotton leaf curl Multan Beta-satellite was also found on cotton plants as well as whiteflies that act as their vector. Phylogenetic trees show that spinach, tomato, papaya, hollyhock, and hibiscus are also infected with cotton leaf curl Multan beta-satellite (Fig. 3).

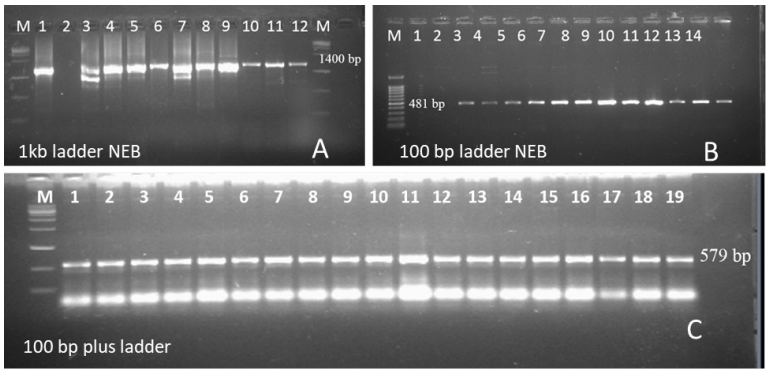

Fig. 2. A – a positive sample of chili with Beta 01/02 primers, with amplicon of 1400 bp; B – a positive sample of chili with CLCuMuBF11/R33 primers showed the band of 481 bp; C – a positive sample of chili with Av/Ac core primers showing the band of 579 bp on 1.5% of agarose gel

Table 2. Location-wise virus infections incidence detection through PCR using all three tested primers

Sr. no	Location	Number of samples	Av/Ac core [%]	CLCuMuBF11/R33 [%]	Beta 01/02 [%]
1	Qasba Marla	10	6/10 (60)	6/10 (60)	6/10 (60)
2	Lar	7	5/7 (71)	5/7 (71)	5/7 (71)
3	5-Faiz	6	3/6 (50)	3/6 (50)	3/6 (50)
4	Makhdoom Ali	6	4/6 (67)	4/6 (67)	4/6(67)
5	Rattan Hatti	10	8/10 (80)	8/10(80)	8/10 (80)
	Total	39	26/38 (68)	26/38 (68)	26/38 (68)

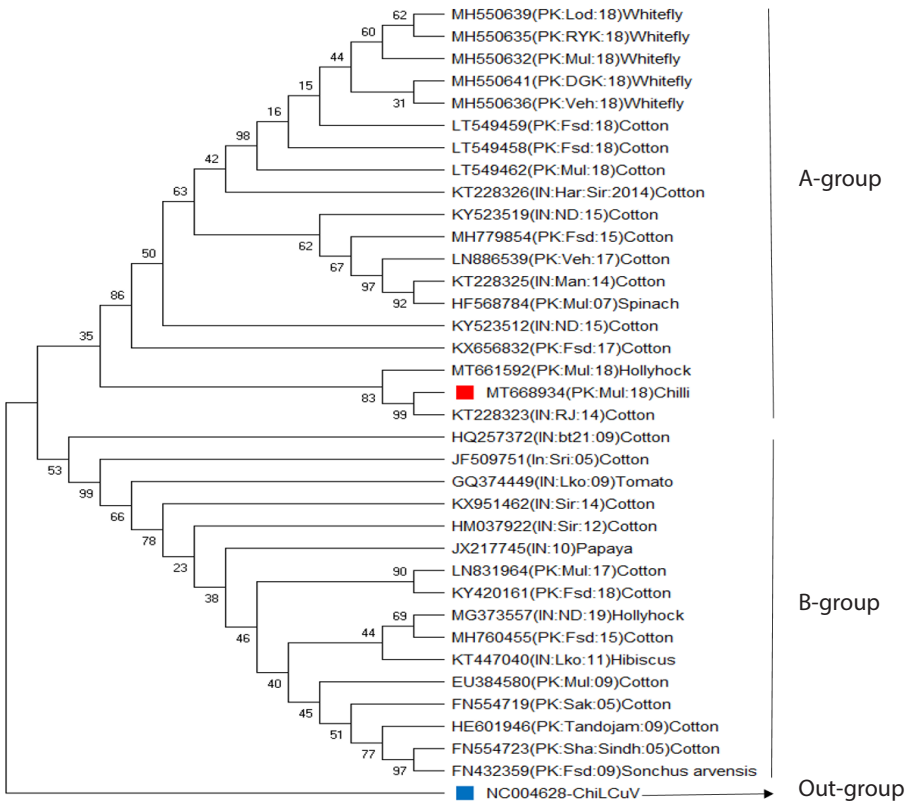

Fig. 3. Phylogenetic tree of cotton leaf curl Multan beta-satellite (CLCuMuB) with the closest sequence of other host species and an outgroup NC004628 (chili leaf curl virus)

Table 3. The closest sequence related to our obtained sequence used to analyze the phylogenetic tree and their description

Sr. No	Isolate	Host	Abbreviation	Country	Accession No.	Reference
1	HA27	hollyhock	CLCuMB	Pakistan	MT661592	(Azeem *et al*. 2022)
2	DG.Khan-5	whitefly	CLCuMB	Pakistan	MH550641	(Afzal *et al*. 2023b)
3	SJ102	cotton	CLCuMB	Pakistan	LT549458	(Ijaz *et al*. 2020)
4	ARS 14-3	cotton	CLCuB	India	KT228323	(Balram *et al*. 2017)
5	Clone 34	cotton	CLCuMB	Pakistan	KX656832	(Zubair *et al*. 2017)
6	Sir-14	cotton	CLCuB	India	KX951462	(Gupta *et al*. 2020)
7	Lodhran-1	cotton	CLCuMB	Pakistan	MH550639	(Afzal *et al*. 2023a)
8	SriGanganagar:2005	cotton	CLCuMB	India	JF509751	(Rajagopalan *et al*. 2012)
9	NIBGE:mw23	cotton	CLCuMB	Pakistan	MH779854	(Naqvi *et al*. 2019)
10	1(flower)	hibiscus	CLCuMB	India	KT447040	(Kumar *et al*. 2011)
11	CLCuMB	papaya	CLCuMB	India	JX217745	(Sinha *et al*. 2016)
12	Clone Sak	cotton	CLCuB	Pakistan	FN554719	(Amrao *et al*. 2010)
13	VIRO 933	hollyhock	CLCuB	India	MG373557	(Sharma *et al*. 2019)
14	Multan-1	whitefly	CLCuB	Pakistan	MH550632	(Afzal *et al*. 2023a)
15	Rahimyar.Khan-4	whitefly	CLCuB	Pakistan	MH550635	(Afzal *et al*. 2023a)
16	Hmg15-6	cotton	CLCuB	India	KY523519	(Balram *et al*. 2017)
17	Ma-14-3	cotton	CLCuRaB	India	KT228325	(Sohrab *et al*. 2016)
18	NIBGE:mw23	cotton	CLCuMB	Pakistan	MH760455	(Shakir *et al*. 2019)
19	Si-14-1	cotton	CLCuBuB	India	KT228326	(Sohrab *et al*. 2016)
20	AR-25	cotton	CLCuMB	Pakistan	LN831964	(Sattar *et al*. 2017)
21	SJ102	cotton	CLCuMB	Pakistan	LT549458	(Ijaz *et al*. 2020)
22	Vehari-4	whitefly	CLCuMB	Pakistan	MH550636	(Afzal *et al*. 2023b)
23	H247	cotton	CLCuMB	Pakistan	LN886539	(Hassan *et al*. 2017)
24	Mohanpura-Rajasthan	cotton	CLCuB	India	HM146307	(Chakrabarty *et al*. 2020)
25	Bihar	tomato	CLCuB	India	GQ374449	(Shih *et al*. 2009)
26	ARSB15-1	Cotton	CLCuB	India	KY523512	(Balram *et al*. 2017)
27	Sirsa-Haryana-En(P)	cotton	CLCuB	India	HM037922	(Chakrabarty *et al*. 2020)
28	SZ 192	cotton	CLCuMB	Pakistan	KY420161	(Zubair *et al*. 2017)
29	Pakistan:Multan:2007	spinach	CLCuMB	Pakistan	HF568784	(Sattar *et al*. 2013)
30	bt 21	cotton	CLCuMB	India	HQ257372	(Kumar *et al*. 2011)
31	Pun-beta-18	cotton	CLCuMB	Pakistan	EU384580	(Rahman *et al*. 2017)
32	sha	cotton	CLCuMB	Pakistan	FN554723	(Amrao *et al*. 2010)
33	SJ9	cotton	CLCuMB	Pakistan	HE601946	(Akhtar *et al*. 2014)
34	Fsd:09	perennial sow thistle	CLCuMB	Pakistan	FN432359	(Mubin *et al*. 2010)

Discussion

Cotton leaf curl Multan beta-satellite (CLCuMuB), identified in the current study, is the main cause of leaf curl diseases in chili plants in Multan, Pakistan. The virus belongs to the Geminiviridae family and is transmitted by the whitefly *Bemisia tabaci* (Zubair *et al*. 2017). The symptoms of CLCuMuB infection in chili plants include stunted growth, curling of leaves, and yellowing of foliage. The molecular characterization of CLCuMuB is essential to understanding the mechanisms of viral infection and developing effective strategies to control its spread. Recent studies have provided insight into the genome organization and gene expression of CLCuMuB (Sharma *et al*. 2019; Baig *et al*. 2021; Tabein *et al*. 2022). The virus has a circular single-stranded DNA genome of approximately 1.3 kb in length. The genome encodes two open reading frames (ORFs), designated as V1 and V2. The V1 ORF encodes the coat protein (CP) of the virus, which is responsible for the formation of virus particles. The V2 ORF encodes a multifunctional protein that is involved in several aspects of viral replication, including DNA replication, transcription, and suppression of host defense responses (Ali *et al*. 2019; Gnanasekaran

et al. 2019). The V2 protein has been shown to interact with several host proteins, including transcription factors and RNA silencing components, which play a crucial role in regulating gene expression in plants (Ali *et al.* 2021). The role of beta-satellites in adapting to new hosts is a fascinating area of research. Beta-satellites are small, circular, single-stranded DNA molecules that are associated with certain begomoviruses. They play a crucial role in the interaction between the virus and its host plant. When it comes to adapting to new hosts, beta-satellites are known to influence a host range by helping begomoviruses overcome host barriers and facilitate their replication and movement within the plant. This process can involve mutations or recombination events that allow the virus-beta--satellite complex to infect new plant species. The present study described the detection of cotton leaf curl Multan beta-satellite CLCuMuB) at the molecular level with PCR using degenerated primers. This is the easiest way to confirm infection in chili plants which show symptoms of yellowing, leaf curling, puckering, and stunted plant growth observed in this study. The varying severity, infection level, and variations in symptom developments among chili-growing regions suggested that many begomoviruses and mites were involved. The extensive application of insecticides over the last 5–10 years to control the vector of chili leaf curl disease has become a commercial method to manage the vector, but the vector has developed resistance against these insecticides, and, this strategy may not be useful in the future. The severity and occurrence of the chili leaf curl disease on chili in Punjab province deliver a warning signal about the province's potential for economic production. In addition, the PCR methodology that utilized degenerate primer pairs such as Av/Ac Core enabled quick, sensitive, and accurate detection of begomoviruses in chili with minimal sample preparation; hence, it has the potential to be a very useful method for displaying the current state of begomovirus in a region.

This phylogenetic study revealed that our obtained sequence (MT668934) was placed in group A with other closely related accessions of whitefly, cotton, hollyhock, and spinach. Our sequencing result revealed that cotton leaf curl Multan beta-satellite is infecting chili plants in Multan, Pakistan. However, cotton leaf curl Multan beta-satellite was also found on cotton plants, as well as whiteflies that act as their vector. Phylogenetic trees show that spinach, tomato, papaya, hollyhock, and hibiscus are also infected with cotton leaf curl Multan betasatellite (Fig. 3). The phylogenetic tree in Figure 3 illustrates that, in addition to chili plants, other crops and plant species such as spinach, tomato, papaya, hollyhock, and hibiscus are also susceptible to infection by cotton leaf curl Multan beta-satellite. The obtained sequence (MT668934) shows the highest identity with the contributions of India and Pakistan that were previously submitted on the NCBI site. This similarity proves that the CLCuMuB was most prevalent in India and Pakistan, and that regulatory control is necessary to prevent the spread of this virus in both countries. It is a crucial prerequisite for ensuring future food security and achieving higher chili yield production. This broader host range highlights the adaptability and potential impact of the beta-satellite on various plant species, emphasizing the need for comprehensive research and effective disease management strategies across multiple crops.

Conclusions

In conclusion, the findings of this study shed light on the prevalence and impact of cotton leaf curl Multan beta-satellite (CLCuMuB) in chili plants in Multan, Pakistan. The identification of CLCuMuB as a key contributor to leaf curl diseases in chili plants underscores its significance in agricultural contexts. This research has advanced our understanding of the molecular characteristics of CLCuMuB, contributing valuable insights into the mechanisms of viral infection in chili crops. The implications of this research are not limited to Multan, Pakistan, but extend to regions where chili cultivation is economically vital. The knowledge gained in this study can enhance the development of targeted strategies for disease management and crop protection, ultimately benefiting chili farmers and the chili industry. Further research in this area, including continuous surveillance and genetic characterization of CLCuMuB strains, will be essential for staying ahead of the evolving challenges posed by begomoviruses and their associated beta-satellites. This knowledge will aid in the development of resilient chili varieties and sustainable agricultural practices. In essence, this study represents a crucial step in the ongoing efforts to combat the spread of CLCuMuB and its devastating effects on chili crops. It underscores the importance of proactive research and vigilant disease management to secure the future of chili production in the region and beyond.

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