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The Molecular Characterization and Evolutionary History Analysis of *Cotton leaf curl Multan virus* (CLCUMUV) Infecting (*Hibiscus sabidarrifa*, *Hibiscus rosa-sinensis*) in Punjab Province of Pakistan

Muhammad ARIF[1*]

[1] Department of Plant Protection, Faculty of Agriculture, Sakarya University of Applied Sciences, muhammadarif@subu.edu.tr

ABSTRACT

The plants (*Hibiscus sabidarrifa* and *Hibiscus rosa-sinensis*) are cultivated in many tropical and subtropical climates for their versatile usage. Plant viruses have a significant capacity to cause the maximum losses in agricultural production. It is assumed that these viruses are responsible for the emergence of about half of plant diseases which are a major threat to food safety or ecological integrity worldwide. Geminiviruses are family of plant viruses with characteristic twinned quasi-icosahedral virions and a small, single-stranded circular DNA genome. Some plants (*H. sabidarrifa* and *H. rosa-sinensis*) showing the characteristic symptoms of viruses were observed. The presence of whitefly vector presumed that these plants might have begomovirus. A 550 bp of amplicon size as a plasmid was obtained from the infected plants after PCR reaction. The obtained amplicon was amplified with sequence specific primers to get the full length genome. The sequence analysis has proved the existence of *Cotton leaf curl Multan virus* (CLCUMUV) from symptomatic plants. The full genome sequence analysis revealed a 2.4-2.5 kb full genome of CLCUMUV infecting Roselle species. A phylogenetic and evolutionary history analysis has confirmed the association of this virus with other aligned viruses. Overall, we have identified one new species as host of begomovirus in this area.

Keywords: AC2 gene, Begomovirus, nucleotide comparison, phylogenetic analysis, Roselle

[*] Corresponding Author's email: muhammadarif@subu.edu.tr

1. Introduction

Hibiscus genus a member of malvaceous family mostly belongs to the tropical and subtropical regions and has approximately 300 species [1]. Its cultivation was started from Sudan in 60000 years ago and become famous as Sudanese tea. The *H. sabdariffa* commonly known as Roselle is one of most famous specie of

Hibiscus which is cultivated in tropical regions for leaves, stem, seeds and most importantly its dry calyces used as a tea, syrup and jams worldwide. The cultivation of Roselle in Asian territories is for the production of bast fiber which is obtained from its stems by the process of retting [2].

The *H. rosa-sinensis* is also a member of the genus *Hibiscus*. This plant is mostly used as herbal medicine in China and India. The World health organization (WHO) has declared this plant as emerging folk medicine for many health problems. As this plant contains a many antioxidants and bioactive compounds which are used as anti-ovulatory, infertility, antifungal, antiviral, anti-inflammatory, hypertension, liver disorder, pyrexia and antibacterial [3, 4].

Begomoviruses are a group of plant viruses possess a small circular single-stranded DNA of 2.6–2.8 kb genome and geminate capsid morphology. The most of begomoviruses have two modules like DNA A and DNA B, which are important for viral propagation [5]. This group of plant viruses have ssDNA that leads to the major losses in economic crops throughout the world. The non-cultivated plants act as harbor and the second host of many begomoviruses, and as mixing vessels at recombination site. These viruses are extremely recombination-prone and show nucleotide substitution rates and are equivalent to other RNA viruses [6, 7].

The CLCuMuV and its associated betasatellites have versatile host range ranging from cultivated crops like cotton, tomato, tobacco, sunflower okra, hemp and non-cultivated plants including Ageratum, Hibiscus, Kudzu and many other weeds. It is estimated that this devastating virus has almost more than 400 host species. Weeds are serving as a major inoculum reservoir of this virus. The host range of this virus is increasing quickly as by detection of this virus from alternate hosts [8, 9].

These begomoviruses are divided into two groups like old world and new world on the basis of genomic studies. The ssDNA molecule of begomovirus is further categorized into two components like DNA-A and DNA-B as having their genome structure. Both DNA-A and DNA-B have broad functions and varied form of open reading frames (ORFs). Proteins encoded by DNA-A of begomovirus are associated with viral DNA replication, vector transmission and encapsidation, while those encoded by DNA-B are required in the intercellular and intracellular movement of viral particles. These proteins have multiple functions including host gene regulation, virus replication, vector transmission, viral assembly and silencing suppression [10-12].

The AC2 genes encode as main pathogenicity factor in many Begomoviruses and family *Geminiviridae*. This gene serves as a resistance breakage in many cultivated and non-cultivated plants including cotton. This gene has multifunctional protein which is involved in transcriptional control, silencing of gene and regulation of basal biosynthesis [13]. The main symptoms of begomoviruses are vein thickening, yellowing, curling and vein enations underside the leaves. The DNA β sequence of *cotton leaf curl Gezira virus*

interestingly show similar symptoms but in different ecological locations. One DNA molecule can relate with four other main CLCuD which have also the same type of typical symptoms [14].

Begomoviruses have a variety of host species including cultivated and uncultivated plants. Studying the begomoviruses of cultivated and wild plants and their dynamics of existing and dispersal are key factors for understanding virus evolution and epidemiology [15]. It is understood fact that development of new epidemics of geminiviruses in a certain area is because of introducing the new crops [16]. Wild plants produce new viruses and their strains by playing the role of "melting pots" for range and recombination with begomoviruses [17]. The interaction consequences between host plants and begomoviruses depend upon the capability of virus to respond plant defense mechanism [18, 19].

Mostly sequence of betasatellite has three important functions in many begomoviruses including an adenine rich sequence region, satellite conserved region for stem-loop structure and replication and single βC1 gene [20, 21]. The main role of βC1 is intermediated by typical encoded protein. The βC1 gene has many functions which include pathogenicity determination, post-transcriptional gene silencing suppressor and also have the interaction of virus movement [22].

Only *Cotton leaf curl Burewala virus* (CLCuBuV) shows the absence of compliment genes despite that begomoviruses has extensive sequencing. Most of CLCuBuV isolates originates from *G. hirustum* which has many stop codons in full TrAP gene. This TrAP product can be cut into 35 amino acids (aa) but other wild-type TrAP of other begomoviruses contain 150-aa-protein. It has been assumed that CLCuBuV can reduce host resistance from other amino acid sequence of TrAP [23].

In Pakistan this virus is infecting multiple crops and most importantly cotton. Many up and down have been occurred Punjab and Sindh Province of Pakistan because of this virus. The farming community and cotton industry is facing huge economic losses caused by this virus in recent years. The host range of this virus is increasing in cotton zone of Pakistan. The presence of vector and alternate hosts of this virus is a significant factor for the establishment of this virus on new hosts [24-26]. The purpose of this study was to check the presence of begomovirus in these plant species. Keeping in view the importance of this virus and its associated satellites, we have identified a new host of CLCuMuV from Punjab, Pakistan. We have reported the sequence-based characterization of this begomovirus which was found to be associated on Roselle species in areas of Tehsil Alipur, Punjab, Pakistan.

2. Research Methodology

2.1. Sample Collection

The twenty leaf samples of Roselle species (*H. sabidarrifa* and *H. rosa-sinensis*) containing virus symptoms were collected from the garden area of Seetpur-Alipur having coordinates including latitude and altitude 29.239510299220743, 70.83905928465599 and Sultanpur-Alipur with coordinates 29.329072, 70.813566 in 2022. The typical symptoms including vein clearing, vein thickening, leaf curling, leaf rolling and yellowing were prominent on the infected plants. The visible observation suggested that these plants might have infection of any plant virus. The presence of white fly population on infected plants has boosted the hypothesis of confirmation of any begomovirus. The 20 symptomatic and healthy leaf samples were collected from both locations. All the symptomatic and healthy leaf samples were well-preserved in airtight

polythene bags. All the obtained samples were brought into the plant virology laboratory for further analysis. All these samples were preserved in -80 °C refrigerator for avoiding the disintegration of plant virus.

2.2. DNA Extraction, Amplification and Sequencing

The DNA was isolated from leaves containing virus symptoms (15 from *H. sabidarrifa* and 5 from *H. rosa-sinensis*) as well as from healthy samples with the help of Cetyl trimethyl ammonium bromide (CTAB) method [27, 28]. Symptomatic leaves of each sample were crushed in liquid nitrogen to transform into a fine powder by using pre-chilled pestle and mortar. The resulting tissue powder was shifted instantly into 50 ml autoclaved polypropylene centrifuge tube comprising of 20ml pre-warmed (65 centigrade) CTAB extraction buffer. The mixture of extraction buffer containing CTAB 1.5%, Tris HCL (pH 8.0) 100mM, Nacl 1.4M, EDTA (pH 8.0) 20mM, β-Mercaptoethanol 2% and Polyvinylpyrolidone (PVP) 2% was used. The whole tissue powder was adjourned carefully in extraction buffer by rotating and inverting the mixture tubes. The incubation of homogenate mixture was allowed at 65 centigrade for a period of 1h in the water bath. This mixture was regularly assorted during the incubation period. A 15 ml quantity of chloroform and isoamyl alcohol with (24:1) ratio was added to each tube after incubation. All the mixture tubes spun until it transforms into the dark green emulsion. Then, mixture tubes were shaken 30 minutes into a rotary shaker. After shaking, all tubes were allowed to shake at 4000 RPM (g) for 20 minutes in the centrifugal shaking machine. This supernatant mixture was shifted into fresh, sterilized 50 ml tube. 2µl RNase (10mg/ml of RNase) was mixed into each tube and then placed for incubation in the water bath for approximately one hour at 37 ^{0}C. This step was optionally applied and also can be completed right after purification steps. Chloroform, isoamyl alcohol, and centrifugation procedures were repeated after RNAse treatment.

The concentration of obtained DNA/per gram of leaf tissue from each purified sample was measured by Smartspec Plus spectrophotometer (BioRad) at 260-nanometer wavelengths. The concentration of each sample was obtained as 50 µg per mL of isolated DNA. The total nucleic acid was filtered with the help of extraction buffer and then re-suspended in cold TE (10 mM Tris, 1 mM EDTA, pH 8.0). The complete nucleotide sequence of amplified product was obtained with the help of degenerate primers. The CLCuMuV AC2 was obtained via PCR amplification by using the specific primers of begomoviral DNA AV494/Dep as described in table 1. Virus and betasatellites were perceived from the DNA extracts with the help of prescribed primers pairs in a PCR [29].

The amplification reaction was carried out in a final volume of 25 µl volume. PCR products were analyzed in 1% agarose gel electrophoresis having tris-acetate-EDTA (TAE) and ethidium bromide. The amplified DNA was eluted from agarose gel with the help of purification kit (QuikGene, Qiagen Inc., USA). The purified PCR product was allowed to ligate into the Pgem-t-vector and later in *Escherichia coli* competent cells [30]. The PCR products of both species were digested with the help of sequence specific forward and reverse primer pairs as described in table 1. The cloned gene of both species were confirmed by the restriction release of fragments and selected clones were sequenced from the commercial Company (Takara).

Table 1: *Primer pairs used to detect the virus.*

Host	Location	Virus genome	Primers sequence 5′-3′	Total no. of sample[Ω]
H. sabidarrifa	Seetpur-Alipur, Punjab, Pakistan	CLCuMuV DNA A	AV494 /Dep3[α] Begomo-F-(5′ GGATCCTTTGTTGAACGCCTTTCC-3′)[*] Begomo-R-(5′ GGATCCCACATGTTTAAAGTAAAGC-3′)[*]	15
H. rosa-sinensis	Sulltanpur-Alipur, Punjab, Pakistan	CLCuMuV DNA-A	CLCMuV-GGATCCATGACGAGGAGCAAAACAA and CLCMuV-CTGCAGAACGGTGAACTTCTTATTGA[*]	5

[α]Degenerate primer used for detection of virus from infected plant samples.
[*]Sequence specific primer pairs
[∝]Degenerate primer used for detection of betasatellite.
[Ω]No. of samples from which PCR amplicons of begomovirus were obtained.

2.3. BLAST Analysis

The obtained sequences were allowed to run on BLAST website. The obtained full genome sequence was designated for homolog check. A Blastn search of the NCBI non-redundant nucleotide database was applied for comparing all the obtained sequences with already identified sequences in GenBank (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Evolutionary History and Phylogenetic Analysis

To understand the ancestry of begomoviral species with their satellites, the associated sequences were recovered from the non-redundant database of NCBI. A phylogenetic tree was fabricated for this virus with the help of aligned viral sequences. This phylogenetic tree was concluded by employing the neighbor joining method. The bootstrap consensus tree was deduced from the one thousand replicates which were obtained to denote the transformative history of analyzed taxa [31]. This transformative analysis was help in the software of MEGA7 [32]. A nucleotide comparison of this sequence with its aligned sequences was carried out by following the [32] in MEGA11 software. All the obtained sequences were compared on the basis of their frequencies in a percent form. The related viruses' domains were assembled from NCBI to check the frequency difference of nucleotides with their codons.

3. Result and Discussion

The begomoviruses and their associated satellites have become a major concern in modern agriculture for their quick development into a new host. These begomoviruses have approximately more than 400 new host species including cultivated and non-cultivated plants. But these non-cultivated plants which are mostly naturally growing including weeds, hedges, shrubs mostly act as reservoir and vessels for the emergence of new plant virus and its evolution. In most cases, insect vector can complete their life cycle in the absence of its original host and virus can move from the body of insect vector into that non-cultivated plant.

The total 20 samples of both species were analyzed to check the presence of begomovirus. It is presumed from many previous studies that first amplicon obtained by using degenerate or universal primers varied in the form of its size. Its size mostly depends on the concentration of DNA, accuracy of PCR and suitable primer pairs. Here in this study, 550 bp amplicon size was obtained from the infected samples. This amplicon was further amplified to get the full length genome of this virus. Most of DNA viruses have plasmid size of approximately 550 bp. Typically, the CLCuMuV has almost 2.2 to 2.6 kb full length genome size so it was predicted that these infected plant samples might have begomovirus infection. A same study was carried out to check the presence of CLCuMuV in *Hibiscus sabidarrifa* in Fujian province of China. They have successfully identified this virus from the infected Roselle species in 2008, 2010 and later in 2017 [33, 34]. The CLCuMuV infecting *H. rosa-sinensis* was also identified in Philippines in 2012 [35]. The *Cotton leaf curl Burewala virus* infecting *H. rosa-sinensis* was also identified in 2014 in Pakistan [36] and 2016 in India [37].

A routine survey was carried out to check the presence of begomoviruses and its associated satellites in areas of south Punjab. Some Roselle plants showing typical begomoviruses symptoms were identified in Seetpur and Sulltanpur. The presence of insect vector has boosted our hypothesis to analyze these samples as these infected plants might have begomovirus infection. The complete genome sequence of CLCuMuV was obtained from the symptomatic samples of Roselle plants. The symptomatic plants were showing the typical symptoms of cotton leaf curl disease including leaf curling, leaf rolling, enation, vein clearing and growth stunting. The samples were analyzed by using the degenerate primers of begomovirus. The transmission of geminiviruses in field condition entirely depend on its insect vectors whitefly, a principal vector to transmit these viruses. It is depicted from many studies that geminiviruses cannot be transmitted via seed, but in some cases can be mechanically and graft-transmissible under experiment conditions [38, 39].

Figure 1: The observed symptoms on both plant species. A: *H. sabidarrifa* and B: *H. rosa-sinensis*

To get the full genome sequence of both isolates, both amplicons were allowed to run on rolling circular amplification (RCA). It was confirmed from RCA that full length has sequence of approximately 2.3-2.4 kb size as shown in figure 2. Both the products were sent to commercial company to get the full-length genome sequences. The isolate of *H. sabidarrifa* obtained from the area of Seetpur-Alipur showed the 2738 while *H. rosa-sinensis* obtained from Sultanpur-Alipur has the 2744 which were 5-10 less or more with already submitted databases. Initially, it was confirmed from the sequencing that amplicon clone (plasmid) of both locations has size of 550 bp which was quite similar to already submitted sequences of CLCuMuV in database of NCBI by running the BLASTn search.

It was confirmed from the sequence analysis that samples collected from both locations have CLCuMuV infection. As, this virus was not identified on this host earlier in this area, so we have detected a new host of CLCuMuV from this area. Moreover, this virus was detected previously from vector (whitefly) also from cotton plants from this area. The increasing number of hosts of this virus is an alarming situation in this area as this area is a hub of cotton production. It is believed that geminiviruses responsible to cause the infection in cotton, corn, cassava, tomato can lead to ample economic losses or even starvation in many underdeveloped countries. The topographical enlargement of agriculture is considered as a rambling factor for spread of many plant viruses. The family *Geminiviridae* is increasing day by day due to increase in number of plant viruses and host enlargement and currently comprise of nine genera including *Becurtovirus*, *Begomovirus*, *Capulavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Mastrevirus*, *Topocuvirus* and *Turncurtovirus* [40-43].

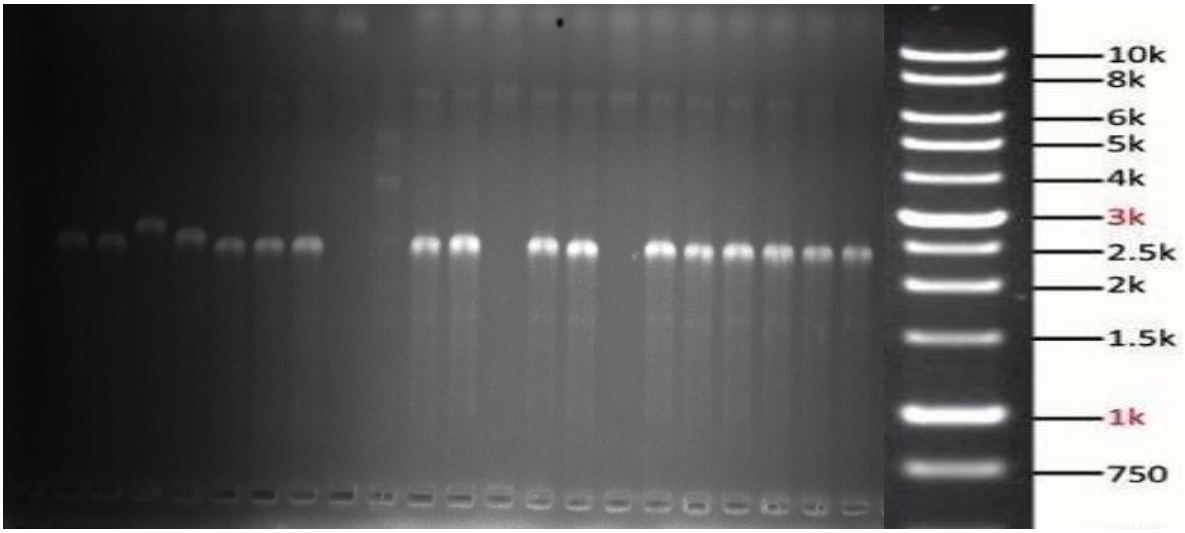

Figure 2: The expression of amplified genome on gel-electrophoresis. A 10 kb DNA ladder was used to check the exact size of amplicon.

The obtained sequence showed maximum similarity index with already submitted sequences of cotton leaf curl disease in NCBI database. All the related sequences were retrieved from NCBI database to develop a phylogenetic analysis. All the sequences were converted into FASTA format. The obtained sequence with its related sequences were aligned in MEGA11 software. A phylogenetic analysis was carried out to check the ancestry of this plant virus. ICTV has developed a solid criterion for mapping and organizing a fresh virus isolate with its genus and group. According to this criteria, if new sequence has similarity with more than 89% then this will fall within that family or genus but if the sequence similarity is less than 89% then

it can be added into a new virus. Our obtained sequence has more than 89% similarity in NCBI with its related sequences, so it can be added as new host in the begomovirus group. While it was revealed from the phylogenetic analysis that CLCuMuV infecting *H. rosa-sinensis* has almost more than 89% similarity with other submitted databases, so it can be considered as same virus with already identified begomovirus group with same name and genus.

There are definite directions for the classifications of plant viruses into species, strains and variants [44]. The amount of categorized geminiviruses is increasing quickly in this decade. A unique nomenclature structure is applied for addition of new viruses into the family. The species name: strain description: symptoms: host: place: and/or a letter A: B:C etc. Strain/variant description include Country, Place, Host, Year [45]. Our study has fulfilled the criteria of ICTV for mapping and organizing the new begomoviruses and its associated satellites. In the 1980s a new group of plant viruses was classified into a new family *Geminiviridae* [46]. This name was originated from "Gemini" the Zodiac symbol characterized by twins [43, 47]. The viruses of *Geminiviridae* family is structurally categorized by joined (geminate), quasi-icosahedral capsids and consist of monopartite or bipartite genome components [48].

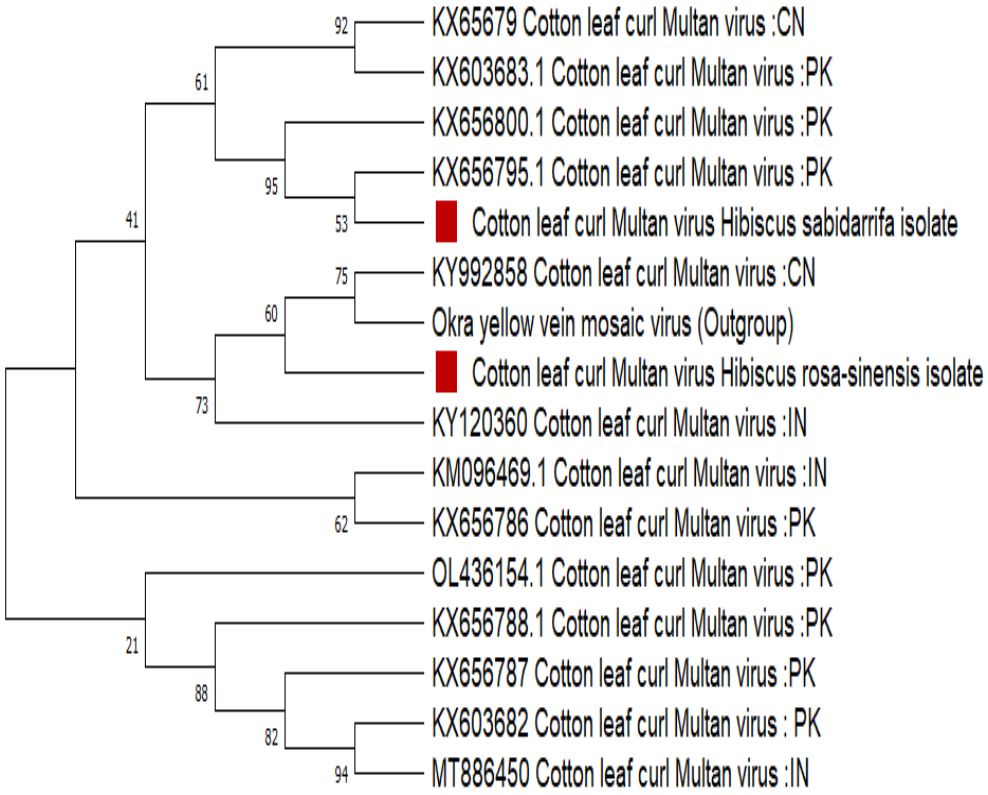

Figure 3: A phylogenetic analysis of obtained sequences with already submitted sequences in NCBI. The Phylogenetic tree was constructed by using maximum likelihood method in MEGA11 Software. The bootstrap tree was inferred from 100 replicates. The red sign indicates the new isolates of this virus. The acronym used in this tree are: PK-Pakistan, IN-India, CN-China. The okra yellow vein mosaic virus was also an India isolate.

Table 2: *Nucleotide frequencies of cotton leaf curl isolates with their codons and total nucleotides*

Domain (Data) Virus name with isolate	T (U)	C	A	G	Total (nucleotides) base pairs*
KX65679 Cotton leaf curl Multan virus :CN isolate	30.2	20.2	26.2	23.4	2739
KX656800.1 Cotton leaf curl Multan virus :PK isolate	30.2	20.2	26.2	23.4	2737
KM096469.1 Cotton leaf curl Multan virus :IN isolate	30.2	20.2	26.2	23.4	2739
OL436154.1 Cotton leaf curl Multan virus :PK isolate	30.3	20.1	26.2	23.4	2739
KX603683.1 Cotton leaf curl Multan virus :PK isolate	30.3	20.1	26.1	23.5	2739
KX656795.1 Cotton leaf curl Multan virus :PK isolate	30.2	20.2	26.1	23.5	2737
KX656788.1 Cotton leaf curl Multan virus :PK isolate	30.2	20.1	26.2	23.5	2733
KY992858 Cotton leaf curl Multan virus :PK isolate	30.4	20.0	26.4	23.3	2738
KX656787 Cotton leaf curl Multan virus :CN isolate	30.2	20.1	26.4	23.2	2738
KX656786 Cotton leaf curl Multan virus :PK isolate	30.3	20.1	26.3	23.4	2738
KX603682 Cotton leaf curl Multan virus :PK isolate	30.4	20.1	26.4	23.1	2738
MT886450 Cotton leaf curl Multan virus :PK isolate	30.3	20.1	26.4	23.2	2738
***Cotton leaf curl Multan virus H. sabidarrifa* isolate**	30.2	20.2	26.2	23.4	2738
***Cotton leaf curl Multan virus H rosa-sinensis* isolate**	30.8	19.9	25.7	23.6	2744
KY120360 Cotton leaf curl Multan virus :IN isolate	31.0	19.9	25.6	23.5	2750
GU181356.1 *Okra yellow vein mosaic virus (Outgroup):IN isolate*	27.1	25.7	21.4	25.7	2743
Average	30.3	20.1	26.1	23.4	2739

An evolutionary history was carried out in MEGA11 software to check the difference of start codons and stop codons. The obtained sequences were compared with already submitted domains of Cotton leaf curl Multan virus with their start codons and stop codons. The data of already submitted sequences was retrieved from the NCBI. It was revealed from this evolutionary history analysis that our sequences have quite similarity with already submitted sequences. The 14 other sequences were selected to check the nucleotide difference with our obtained sequences. The average nucleotide difference was 30.3 in start codons while stop codons showed the difference of 23.4. The complete nucleotide difference can be found in table. 2. The values of start codons (T&U) while stop codons values are in (C&A&G) form. Okra yellow vein mosaic virus was selected as an outgroup for this analysis which is also a member of this devastating begomovirus genus. The number of start and stop codons for this outgroup were 27.1, 25.7, 2.14 and 25.7 with total number of sequence was 2743 base pair.

The genomic studies with evolutionary history and phylogenetic analysis has proved that begomoviruses are categorized into two groups, including new world (NW) and old world (OW) viruses [38]. This division might be happened either by the relationship of monopartite viruses with betasatellite or by dislodgment of DNA-B from an OW bipartite begomovirus as revealed in the case of *Srilankan cassava mosaic virus* (SLCMV) [49]. Our phylogenetic analysis has confirmd that this virus has quite similar with already submitted database in NCBI. Our virus has showed approximately 89% similarity with already identified begomiviruses with their host, genome organization and physical appreance.

Major influential factors responsible for appearance and dispersal of new geminiviruses are progression of new variants, presence of B biotype insect vector *Bemisia tabaci* and rise of its population. The genomic recombination in geminiviruses, not only between variants of the same virus, but also between species and

even genera, caused the speedy virus modification. Most of the infectious geminiviruses originated from recombination of viral genomes including those connected with tomato leaf curl, cassava mosaic and cotton leaf curl diseases. Heterologous recombinants comprising parts of the host genome and sequences from satellite-like components provided infinite evolutionary prospects. Human activities are also playing major role in disperse of geminiviruses globally by modifying the cropping systems, transferring the infected planting materials, introducing the new crops and introducing the susceptible germplasm [50].

4. Conclusion

In this study, plants of Roselle genus showing begomovirus infection were characterized. These plants have versatile usage and a major source of bast fiber in many countries. Here in this study, we have successfully detected the CLCuMuV infecting the Roselle species in Seetpur and Sultanpur areas of Punjab, Pakistan. Previously, these two species were serving as alternate host of this devastating plant virus. This study has confirmed the establishment of begomovirus on 8th malvaceous and 6 seed-propagated species of plants. Many factors are responsible for increasing the host species of this virus like B or Q type insect vector and speedy viral mutation. To manage this virus, it is necessary to control the spread of insect vector.

5. Declaration

Author declare that no conflict of interest exists.

5.1. Funding source

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

6. Human and Animal Related Study

This work does not involve in the study of humans and animals.

Data availability

All data available within the manuscript.

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