Detection & Cloning of Begomovirus from Ornamental Plants

Ayesha Ashraf
Registration # 00000172464

Master of Science in Plant Biotechnology

DEPARTMENT OF PLANT BIOTECHNOLOGY
ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES
NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY
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Dr. Muhammad Tahir
Assistant Professor
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Thesis Supervisor:

Dr. Muhammad Tahir

Assistant Professor

Thesis Supervisor's Signature: _____

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Examination Committee Members

1. **Name:** Dr. Muhammad Qasim Hayat. Signature:_____

2. **Name:** Dr. Najam – us – Sahar Sadaf Zaidi. Signature:_____

Supervisor: Dr. Muhammad Tahir. Signature:_____

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Supervisor:_____

Dr. Muhammad Tahir

Assistant Professor

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences & Technology.

Head of Department:_____

Dr. Muhammad Tahir

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences & Technology.

Principal:_____

Dr. Peter John

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences & Technology.

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Ayesha Ashraf
Master of Science in Plant Biotechnology
Registration # 00000172464

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Dedicated to

Allah Almighty

The Lord of the worlds

Prophet Muhammad (P.B.U.H)

A mercy to mankind

And

My respected parents, teachers and beloved brother & sisters, who have been my constant source of inspiration, they have given me the drive and discipline to tackle any task with enthusiasm and determination.

Abstract

Begomoviruses are the most damaging plant pathogens that effect the economical important crops including cotton, cassava, and tomato etc., in the world. Ornamental plants are considered as a reservoir for economically important begomoviruses. Leaf samples of ornamental plants, *Hibiscus rosa sinensis*, *Hibiscus shizuki*, *Iresine* and *Ageratum*, showing leaf curl and vein thickening symptoms were collected from areas around Lahore and Islamabad during 2017-2018. Their total DNA was extracted by Cetyle Trimethyl Ammonium Bromide (CTAB) method and subjected to diagnostic PCR using universal set of primers. All samples except for *Ageratum* gave expected size band between 1.1 to 2.8kb. Amplification PCR products of 1.5kb and 1.4kb were obtained using primer pair WTGF/WTGR and Beta01/Beta02 primer, respectively from two samples; *Hibiscus rosa-sinensis* (isolate AY1) and *Hibiscus shizuki* (AY2). The products were T/A cloned and sequenced. The nucleotide sequence of isolate AY1 shared 96% (over a stretch of 719/749 nucleotide) identity with *cotton leaf curl Kokhran virus – Burewala* while isolate AY2 showed 93% (over a stretch of 920/990 nucleotide) nucleotide sequence identity with cotton leaf curl Multan betasatellite. The present study concluded that *Hibiscus rosa-sinensis* and *Hibiscus shizuki* are reservoir host of *Cotton leaf curl Kokhran virus* which are major cause of cotton leaf curl disease in Pakistan, India and China. It is recommended to avoid cultivation of *Hibiscus* species in cotton growing regions to avoid virus pressure in cotton plants.

Abbreviations

%	Percent
$(NH_4)_2SO_4$	Ammonium Sulphate
°C	Degree Celsius
Approx.	Approximately
BGYVV	*Bitter gourd yellow vein virus*
BYVMV	*Bhendi yellow vein mosaic virus*
ChiLCSLB	*Chilli leaf curl Sri Lanka Betasatellite*
ChiLCSLV	*Chilli leaf curl Sri Lanka virus*
ChiLCV	*Chilli leaf curl virus*
ChLCB	Chilli leaf curl betasatellite
CLCuBuV	*Cotton leaf curl Burewala virus*
CLCuD	Cotton Leaf Curl Disease
CLCuGV	*Cotton leaf curl Gezira virus*
CLCuKoV	*Cotton leaf curl Kokhran virus*
CLCuMB	*Cotton leaf curl Multan betasatellite*
CLCuMuV	Cotton leaf curl Multan virus

CLCuShV	*Cotton leaf curl Shahdadpur virus*
CoMoV	*Coccinia mosaic virus*
CP	Coat protein
CTAB	Cetyltrimethylammonium bromide
DiAYVB	*Digera arvensis yellow vein betasatellite*
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside triphosphate or deoxynucleotides
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
et al.	et alia
g	gram
H_2O	Water
i.e.	id est means “that is”
ICTV	International Committee on Taxonomy of Viruses
IPTG	Isopropyl-β-D-thiogalactopyranoside
ISEM	Immunosorbent electron microscopy
kb	kilobases
LYMV	*Loofa yellow mosaic virus*

M	Molar
$MgCl_2$	Magnesium Chloride
min	Minute
ml	milliliter
mM	millimolar
MYMV	*Mungbean yellow mosaic virus*
MYVYNV	*Malvastrum yellow vein Yunnan virus*
NaCl	Sodium Chloride
NF H_2O	Nuclease free water
ng	nanogram
nm	nanometer
PaLCV	*Papaya leaf curl virus*
PCR	Polymerase Chain Reaction
PeLCV	*Pedilanthus leaf curl virus*
PepLCLV	*Pepper leaf curl Lahore virus*
PYMPV	*Potato yellow mosaic Panama virus*
RaLCuV	*Radish leaf curl virus*
Ren	Replication Enhancer protein

Rep	Replication associated protein
RoLCuV	*Rose leaf curl virus*
rpm	Round per minute
RT	Room Temperature
SCR	Satellite Conserved Region
SDS	Sodiumdodecylsulfate
ssDNA	single stranded DNA
TAE Buffer	Tris-Acetate-EDTA Buffer
TbLCB	*Tobacco leaf curl betasatellite*
TE Buffer	Tris-EDTA Buffer
ToLCB	*Tomato leaf curl betasatellite*
ToLCBDV	*Tomato leaf curl Bangladesh virus*
ToLCKV	*Tomato leaf curl Karnataka virus*
ToLCNDV	*Tomato leaf curl New Delhi virus*
ToLCSiV	*Tomato leaf curl Sinaloa virus*
ToLCuD	Tomato leaf curl disease
TrAP	Transcriptional activator protein
Tris	Trisma

Tris-HCl	(hydroxymethyl)aminomethane- Hydrochloride
TYLCV	*Tomato yellow leaf curl virus*
TYMoV	*Tomato yellow mottle virus*
UV	Ultra-violet
V	volt
X-gal	5-Bromo-4-chloro-3-indolyl-β-galactopyranosidase
α	alpha
β	beta
µg	micro gram
µl	microliter

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CHAPTER 1: INTRODUCTION

Virus as biological entity

Virus is an ultramicroscopic obligate parasite entity that explained as "organism whose genome is only comprises of nucleic acid and it's require cellular synthetic machinery of other living cells for survival, replication and movement from cell-to-cell" (Luria *et al.*, 1978). Viruses have ability to pass through ultra-filters of 0.22 µm and they are disease causing agent (Moreira & López-García, 2009). All organisms from *archaea*, *prokaryotes* and *eukaryotes* life domains are infected with viruses (Pearson, 2008). Virus is a Latin term named as poison (Raoult & Forterre, 2008). Viruses are functionally inactive outside the host body which termed as obligate intracellular parasites. They always require host body for propagation and are self-replicating (Wimmer *et al.*, 2009). They trap biological mechanism of their host cell for their essential activities as replication, translation and transcription. It's still topic of debates either they are non-living or living entities because they show both non-living and living features. Presence of a genome in viruses is only feature of living organisms excluding this they are non-living infectious particles and should not be included in microorganisms (Van Regenmortel & Mahy, 2004). It's difficult to define viruses because these are neither living nor non-living organisms and according to classification of five kingdom, viruses can't classify (Pace, 2006). Viruses are parasites that infect life of every type entities including plants, animals, archaea and bacteria (Koonin *et al.*, 2006). Now a days viruses may perhaps considered as pristine genes functional analogues (Mau, 2014).

Virus classification based on their various features as their morphology, genome type (either consist of RNA or DNA, double stranded or single stranded), transmission vector, host range and viral proteins (van Regenmortel *et al.*, 2000). Viruses' classification system proposed by Baltimore in 1971 according to their nucleic acid genomes. Based on classification of Baltimore, viruses were divided in 7 groups (single-stranded DNA (ssDNA), dsDNA (double-stranded DNA), dsRNA (double-stranded RNA), negative

sense ssRNA (single- stranded RNA), positive ssRNA and reverse transcribing viruses having genome either dsDNA or ssRNA) (Baltimore, 1971; Borden *et al.*, 1971). Currently the ICTV (International Committee on Taxonomy of Viruses) handled viruses' taxonomy and nomenclature. According to ICTV 9th meeting, viruses have divided into 6 orders, 87 families, 19 subfamilies, 349 genus and around 2284 species (Brown *et al.*, 2012; King *et al.*, 2011). While according to 10th report of ICTV, viruses have been grouped in 8 orders that contain 122 families and 35 subfamilies, 735 genera and approximately 4404 species (Lefkowitz *et al.*, 2018).

Plant viruses

Plant viruses are causal agent for plants to cause infection (Pallas & García, 2011). With different concepts viruses were not characterized even at a single point. In 1981 a plant virologist Heinz stated:

"Just as the five blind men may describe an elephant differently, so plant pathologists, virologists, tobacco growers, and biochemists surely see TMV differently" (Taylor, 2014).

The plant virology field were initiated with the discovery of TMV (*Tobacco mosaic virus*) by Martinus Beijerinck and in 1898 it's abbreviated as TMV (Kathiria *et al.*, 2010; Mayer *et al.*, 1942). He observed that the tobacco mosaic disease infected plants sap even after pass through a filter of porcelain remains infectious that suggests these infectious agent were smaller as compare to bacteria because porcelain filter cannot pass bacteria (Kathiria *et al.*, 2010; Kratochvílová, 2016). After electron microscopy invention, crystalline form of these infectious agents were observed and reported as these infectious agents were typically made up from RNA and protein particles (Stanley, 1935).

In 1950 era, work on RNA purification revealed that plant viruses mostly have RNA genomes while a few viruses have DNA genome to control host machinery (Covey & Al-Kaff, 2000; Dreher & Miller, 2006). Plant viruses spread through a vector from one to another host. Mostly plant viruses vectors are insects like whitefly, leafhopper and aphid. In plants symptoms of Viral infections include leaf curling, vein yellowing, leaf

distortion, color changes, stunting of growth and reduction in production by both low quality and quantity. Plant viruses of family *Geminiviridae* are responsible for crops economic losses throughout the World.

There are two categories of plant viruses: viruses (badnaviruses and caulimoviruses) which use enzyme reverse transcriptase during replication to form RNA intermediate, from double stranded circular DNA (dsDNA) while other class of viruses (*Geminiviridae* and *Nanoviridae*) which use mechanism of rolling circular amplification to form intermediates of dsDNA from single stranded cicular DNA (ssDNA) in replication process (Molnár *et al.*, 2005). With the passage of time, viral diversity, interaction with different hosts and characterization of viruses on large scale are the major focus (Waterhouse *et al.*, 2001). Now a days, researchers major focus on novel modes for the manipulation and reconstruction of viruses and plants to achieve resistance and to prevent our large crops from malicious viral diseases(Goldbach *et al.*, 2003).

Family *Geminiviridae*

Geminiviruses belongs to family *Geminiviridae* are in charge for a numeral viral plant diseases all over the world. *Geminiviridae* are the well characterized family of plant viruses because of their small genome size, vectors availability and manipulate easily through molecular approaches because of their DNA genome. These viruses infect monocotyledonous along with dicotyledonous plants (Brown, 2012; Rojas *et al.*, 2005). These viruses transferred through arthropod vectors in non-propagative but circulative manner (Hogenhout *et al.*, 2008; Power, 2000). In 752 A.D, geminivruses disease early or first promising description was given in a poem by Empress Koken. The poet describes the *Eupatorium* plants yellowish color (Inouye & Osaki, 1980). Recently it has been revealed that geminivirus was the cause of Eupatorium's yellowish color disease (Saunders *et al.*, 2003).

Geminiviruses have circular ssDNA genome encapsidate in geminate particles ("geminus" meaning twin) having quasi-icosahedral coat protein structure (Davies, 1987; Goodman, 1977; Harrison *et al.*, 1977; Stanley & Townsend, 1985). They have name

because of unique geminate morphology. Geminiviruses inadequate two quasi-icosahedral capsid are approximately 38 nm long and 2nm in diameter. Geminiviruses genome size ranges from 2.5 kb to 3.1 kb (Stanley, 2005).

In World, geminiviruses are major limitation for the crop plants production throughout tropical and subtropical regions. Human trade and environmental changes are direct causal agent for the spreading of viruses throughout World (Navas-Castillo *et al.*, 2011). Geminiviruses are infected wide varieties of cereal crops like cassava, cotton, tomato, beans and maize and vegetables and they cause disease in them (Legg & Fauquet, 2004; Malathi *et al.*, 2005; Morales & Anderson, 2001). The geminiviruses have specific host and vectors. Different geminiviruses cause infection in specific plants corresponding TYLCV (*Tomato yellow leaf curl virus*) damaged plants of tomato (Navot *et al.*, 1991; Salati *et al.*, 2002), BGMV (*Bean golden mosaic virus*) destroyed bean plants in America (Morales & Anderson, 2001), ACMV (*African cassava mosaic virus*) destroyed cassava plants (Legg & Fauquet, 2004), BCTV (*Beet curly top virus*) destroyed crops of sugar beet (Soto & Gilbertson, 2003) and *Maize streak virus* (MSV) damaged maize crop in Africa (Palmer & Rybicki, 1998). In Pakistan, the major reported constraint for the cotton production is CLCuMuV (*Cotton leaf curl Multan virus*) (Briddon *et al.*, 2000).

Classification of family Geminiviridae

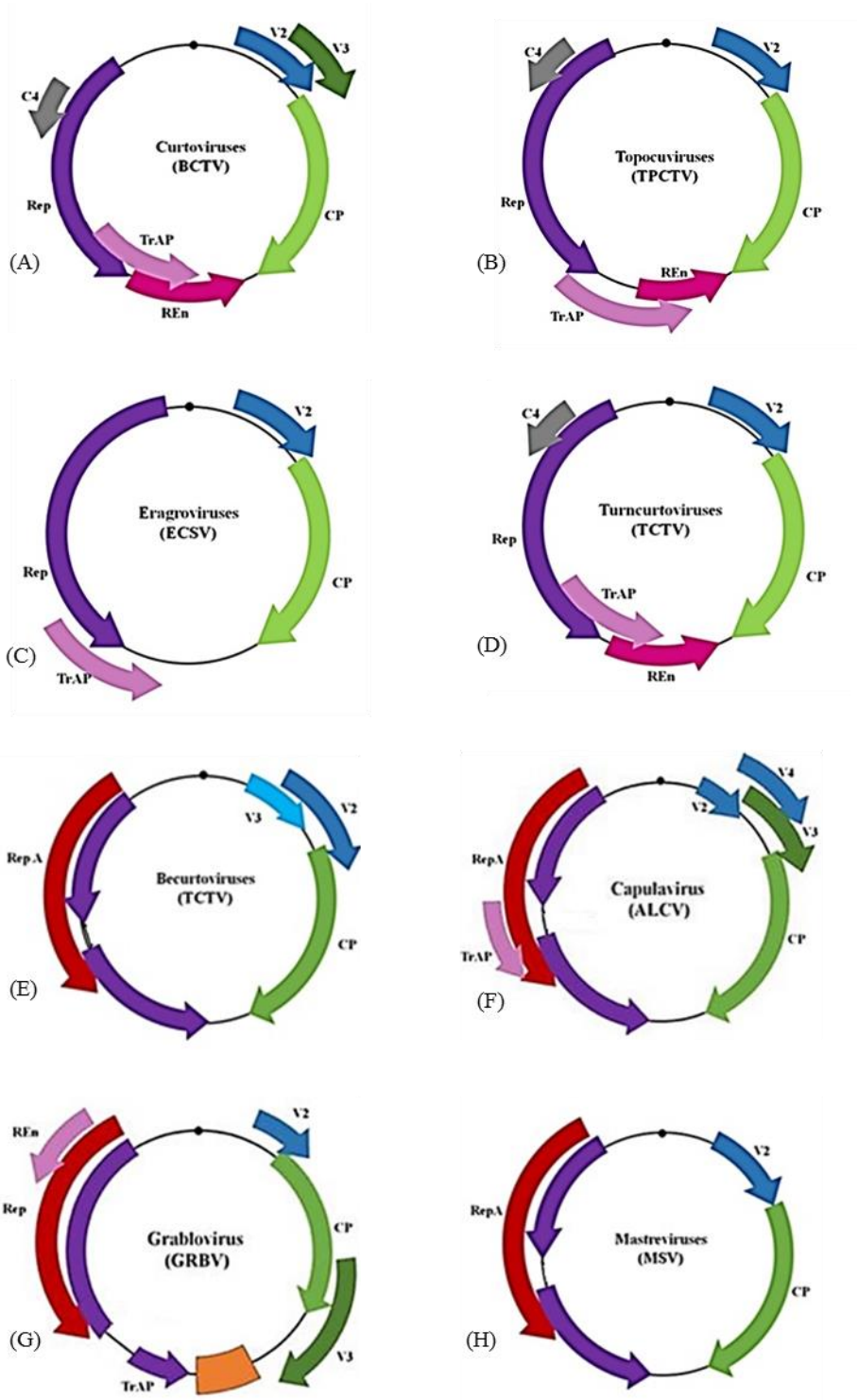
Geminiviruses classification was based on host range of the virus, insect vectors and organization of genome (Briddon *et al.*, 1996; Rybicki, 1994; Stanley, 2005). According to ICTV 9th report (Brown *et al.*, 2012), Geminiviruses grouped into 4 genera. Further studies reported and added 4 new genera to the taxonomy of *Geminiviridae* family (Adams *et al.*, 2015; Adams *et al.*, 2013; Varsani *et al.*, 2014). ICTV 10th report proposed a new *Grablovirus* genus in taxonomy of family *Geminiviridae* (Zerbini *et al.*, 2017). To date, geminiviruses have beyond 300 official species belongs to 9 genera (*Begomovirus, Mastrevirus, Eragrovirus, Turncurtovirus, Topocuvirus, Curtovirus, Becurtovirus, Capulavirus and Grablovirus*) and reported (Adams *et al.*, 2013; Brown *et al.*, 2012; Roumagnac *et al.*, 2015; Varsani *et al.*, 2014; Varsani *et al.*, 2017; Zerbini *et al.*, 2017). 9

genera of family *Geminiviridae* with their genome type, associated vectors and host ranges are given below.

Table 1. Recent classification of Geminiviridae family with their genome, associated vectors and host ranges (Zerbini *et al.*, 2017).

Genus	**Genome**	**Vector**	**Host Range**
Begomovirus	Monopartite/bipartite	Whiteflies	Dicotyledonous
Mastrevirus	Monopartite	Leafhoppers	Monocotyledonous and few Dicotyledonous
Topocuvirus	Monopartite	Treehoppers	Dicotyledonous
Curtovirus	Monopartite	Leafhoppers	Dicotyledonous
Turncurtovirus	Monopartite	Leafhoppers	Dicotyledonous
Becurtovirus	Monopartite	Leafhoppers	Dicotyledonous
Eragrovirus	Monopartite	Aphid	Monocotyledonous
Capulavirus	Monopartite	Aphid	Monocotyledonous/Dicotyledonous
Glabrovirus	Monopartite	Treehoppers	Dicotyledonous

Genome organization



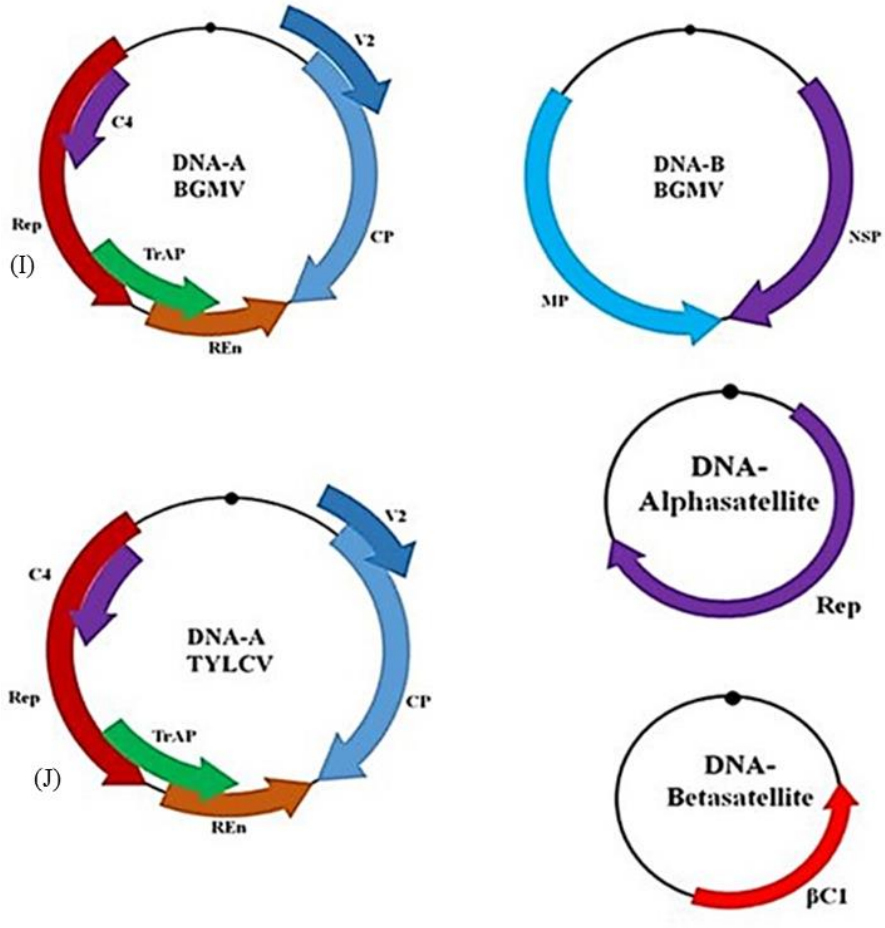


Figure 1. Genome organization of family *Geminiviridae* genera.

Each genera has type species and it represent perspective genera. (A) BCTV (*Beet curly top virus*) of *Curtovirus*, (B) TPCTV (*Tomato pseudo curly top virus*) of *Topocuvirus*, (C) ECSV (*Eragrostis curvula streak virus*) of *Eragrovirus*, (D) TCTV (*Turnip curly top virus*) type species of *Turncurtovirus*, (E) BCTIV (*Beet curl top Iranian virus*) of *Becurtovirus*, (F) ALCV (*Alfalfa leaf curl virus*) of *Capulavirus*, (G) GRBV (*Grapevine red blotch virus*) type species of *Grablovirus* and (H) MSV (*Maize streak virus*) of *Mastrevirus*. (J) Monopartite begomovirus is TYLCV (*Tomato yellow leaf curl virus*) with DNA satellites while (I) BGMV (*Bean golden mosaic virus*) is bipartite begomovirus with two genomic component DNA A component and DNA B component. Black circle represent stem-loop structure that have nona-nucleotide (TAATATTAC) sequence, CP (coat protein), NSP (nuclear shuttle protein), REn (replication enhancer protein), MP (movement protein), V2 (pre-coat protein), Rep (replication associated protein) and TrAP (transactivator protein).

Old World originating viruses are mostly monopartite like viruses of genus *Curtovirus, Turncurtovirus, Becurtovirus, Topocuvirus, Mastrevirus, Eragrovirus, Capulavirus,*

Grablovirus and some *Begomovirus* while few *Begomovirus* are bipartite. All new World originating begomoviruses are bipartite and they lack V2 ORF on DNA A virion-sense strand.

Genus *Begomovirus*

Genus *Begomovirus* is the major and dangerous genus of *Geminiviridae* family comprising almost 300 species and most central plant infecting viruses (Brown *et al.*, 2012; Brown *et al.*, 2015). Begomoviruses insect vector is *Bemisia tabaci* Whitefly (Brown, 1994). *Begomovirus* genus infect only dicot plants and it appear both in new world (America) and Old world (Asia, Africa, Australia, Europe). These viruses spoil several economically essential crops like squash, beans, tomato, cassava and cotton. Remarkable cases of agricultural losses due to begomoviruses include *Cassava mosaic virus* (Thottappilly, 1992) and *Tomato yellow leaf curl virus* (TYLCV) in Africa and Mediterranean region which extended to Florida respectively (Czosnek & Laterrot, 1997; McGlashan *et al.*, 1994).

On the base of organizations of their genome, they are divided in two groups. One is Bipartite begomoviruses and other is monopartite begomoviruses. Bipartite begomoviruses comprise of two single stranded circular genomic DNA components denoted to as DNA-A component and DNA-B component (Bisaro *et al.*, 1982). DNA-A component and DNA-B component size ranges ~2.6-2.8 kb and they generally occur in NW but some members are originating in OW too. There is no identity between these 2 components except a short ~200 nucleotide sequence known as common region (CR). CR lies within IR and is extremely conserved (more than 90% resemblance) between 2 DNA (DNA-A component and DNA-B component) (Briddon *et al.*, 2010; Fernandes *et al.*, 2008; Hamilton *et al.*, 1984).

Bipartite Begomoviruses

Bipartite Begomovirus have DNA-A genomic component and DNA-B genomic component with estimated 2.6 kb size. Both genomic particles has intergenic region (IR). C-sense strand of DNA-A encodes four ORFs linked to genes, replicating protein Rep (A

replication associated protein), TrAP (trans-activator protein gene), REn (Replication enhancing protein) which interact with Rep to up-regulate the replication, C4 linked with pathogenesis and viral movement. V-sense strand has 2 ORFs: AV1 involve in CP, V2 is pre-coat protein. DNA-B encodes 2 proteins. Virus genomic C-sense strand encodes MP and V-sense strand encodes NSP. Both these were primarily related with viral moment and appearance of disease symptoms (Yazdi *et al.,* 2008).

Monopartite Begomoviruses

Monopartite begomoviruses having single DNA genomic component which may or may not have alphasatellites and betasatellites. Single genome of monopartite begomovirus able to bring out all the imperative virus function and it is just a homolog of DNA-A of bipartite begomovirus. C-sense strand possess AC1 to AC4 genes while AV1 to AV2 genes are present on V-sense strand. Bulk amount associated to monopartite begomovirus has ssDNA satellite molecule with rare genes that stimulate the pathogenesis (Prasanna *et al.*, 2010).

Begomovirus with its satellite-complexes

Satellites have whole set of guidelines for mythical overview of disease on their genome either RNA or DNA however they depend upon virus for replication and transmitting (Hanley-Bowdoin *et al.,* 2013). Begomovirus has betasatellite or one nanovirus like DNA satellite recognized as alphasatellite. They are generally associated with the OW virus identified as betasatellite (previously called as DNA β but studies showed that some NW bipartite begomovirus are related with alphasatellite (Paprotka *et al.*, 2010; Romay *et al.*, 2010).

Betasatellite

Betasatellite are linked with monopartite begomovirus has ss-circular DNA by adding new viral particles in host plants by its helper virus, brings symptoms of disease on plants (Nawaz-ul-Rehman *et al.,* 2009). Betasatellite stem loop has the conserved nona-

nucleotide sequence like its helper virus. They enclose a A-rich (adenine) and satellite conserve region, 150-200 nts (SCR) of betasatellite (Xie *et al.*, 2010). Complementary-sense strand have merely one ORF identified as βC1 that is adept for symptoms initiation by doing various biochemical procedures in infected host plant (Mubin *et al.*, 2009b). Magically this single gene of betasatellite captures the host defense system in numbers of ways and intermixes with various signaling pathways of plants. Just like alphasatellite of adenine gilded region, betasatellite also have Adenine nucleotide crowded region (Fiallo-Olivé et al., 2012). Betasatellite size is 1350 nucleotides which are just half of the helper virus (Mubin *et al.*, 2009b).

Alphasatellite

Ancient name of alphasatellite is DNA-1 with the size of 1400 nucleotides (Nawaz-ul-Rehman *et al.*, 2010). Alpha particles have Rep proteins but for all additional functions they depend upon their helper virus. This Rep protein is like with nanovirus particles and believes that during co-infection certain evolutionary events may happen (Idris *et al.*, 2011). This Rep protein contain in PTGS and suppress infected plant silencing mechanism but still the function of alphasatellite is still ambiguous (Mandadi & Scholthof, 2013).

Importance of Begomoviruses/Ornamental plants in Pakistan

As a geographical capabilities of human actions, unrestricted movement of host plant and vectors globally, the commercial and modern way of farming methods increased together with climate variation, viruses are rapidly evolving. The geminiviruses especially begomoviruses, the prevalent group of plant viruses and are very diverse in natural surroundings.

Geographically, in genetic variability and severity terms, Pakistan lies on geminiviruses / begomoviruses hot plate. In last three decades, this region had an account for begomoviruses epidemic like African region. Many economical important crops were

infected with begomoviruses and significant losses were observed. It was quite substantial losses for agricultural regions.

Although, in the emergence of new begomoviruses, many factors are involved including the climate change, more harvesting, vectors environmental conditions and increased recombination of geminiviruses especially begomoviruses. The recombination phenomenon occures between every taxonomic level of viruses are very frequent and its a substantial contributor in emergence of new geminiviruses (Ferro *et al.*, 2017; Fondong *et al.*, 2000).

Begomoviruses are also reported in ornamental plants. Emergence of new and recombinant begomoviruses are major threat for ornamental plants, food and fibers including cotton, tomato, tobacco, cassava, bean, squash, cucurbits, muskmelon and many more from three decades worldwide. Recent reports show begomoviruses presence in *hibiscus* plants in Philippines (Briddon *et al.*, 2000; Brown, 1994; Fargette *et al.*, 2006; Mansoor *et al.*, 2006; Morales & Anderson, 2001; Moriones & Navas-Castillo, 2000; Polston & Anderson, 1997; Ribeiro *et al.*, 2003; Rybicki & Pietersen, 1999; Varma & Malathi, 2003).

Due to begomoviruses, heavy losses were faced in national economy of Pakistan. In 1992-97, around 5 billion US $ losses in cotton due to CLCuD only (Briddon *et al.*, 2000). The Cucumis melo commonly known as muskmelon had yield loss of 100% in some regions of Punjab because of begomoviral disease (Malik *et al.*, 2006). Sindh and Punjab province had 30-40% loss in tomato production because of tomato leaf curl disease. In Pakistan, with the time passage the infection of begomoviruses has increased to inordinate extent throughout previous decade to ornamental plants like *Sesbania bispinosa* (Zaidi et al., 2016), *Hibiscus rosa-sinensis* (Riaz *et al.*, 2015), *Hibiscus syriacus* (Akhtar *et al.*, 2014), *Rosa chinensis* (Khatri *et al.*, 2014), *Duranta repens* (Mustujab *et al.*, 2015), *A. alpinus* (Zia-ur-Rahman *et al.*, 2013), *Luffa cylindrical* (Srivastava *et al.*, 2013), *Ricinus communis* (Fareed *et al.*, 2012), *Vinca minor* (Haider, Tahir, Saeed, *et al.*, 2007), *Amaranthus cruentus L.* (Srivastava *et al.*, 2015), *Malvastrum coromandelianum* (Jiang & Zhou, 2005), *Duranta erecta* (Iram *et al.*, 2005), *Solanum*

nigrum, Zinnia elegans and *Ageratum conyzoides (Haider, Tahir, Evans, et al., 2007), Codiaeum variegatum Sonchus arvensis* (Anwar *et al.*, 2012), (Mubin *et al.*, 2010). Other plants legumes *Rhynchosia minima* (Ilyas *et al.*, 2010)(Ilyas *et al.*, 2009), *Vigna aconitifolia Momordica charantia* (Qazi *et al.*, 2006)(Tahir *et al.*, 2010b), Potato (Mubin *et al.*, 2009a), *Capsicum spp.* (Shafiq *et al.*, 2010; Tahir *et al.*, 2010a), Bell pepper, tomato, pepper and chilli (Tahir *et al.*, 2010a)(Hussain *et al.*, 2004; Shih *et al.*, 2003) also have begomoviral infection.

Objectives

- Molecular detection and cloning of Begomovirus from Ornamental Plants species.
- DNA sequencing and sequence analysis of Begomovirus isolates.
- Phylogenetic analysis of Begomovirus isolates.

CHAPTER 2: LITERATURE REVIEW

Islam *et al.* (2018) describes begomoviruses are plant pathogens and transmitted by whiteflies. These plant pathogens are major threat to different economically important cash crops. Samples of whiteflies were collected from different regions of Pakistan. Begomoviruses were amplified by using PCR with degenerative primers from whiteflies DNA. 177 sequences were analyzed and 14 different species of begomoviruses were revealed, containing five new species which were not reported yet in Pakistan. ChiLCV (*Chilli leaf curl virus*) and CoYVV (*Corchorus yellow vein virus*) were identified and novel strains showing <90 % identity with available taxa. The majority of begomoviruses and five different type of begomoviruses were identified from Sindh area. Furthermore, CLCuMuVRa (*Cotton leaf curl Multan virus–Rajasthan*) were dominant in cotton growing areas. The reported data may helpful in control measure development against begomoviruses.

Sahu *et al.* (2018) observed spinach plant leaves with symptoms of vein yellowing, growth stunting, in Rajasthan. This spinach yellow vein disease associated with novel begomovirus, as a result of recombination of viruses. Recombination of viruses occur in the absence of main host. Like *Papaya leaf curl virus* extent to further crops in the absence of their main host. Sample analysis showed that spinach plants with vein yellowing were infected with novel begomovirus that show <91 % sequence similarity with other viruses and its associated betasatellite. Begomoviruses show inter species recombination and betasatellite recombinants have high variability in nucleotides. By recombination process, the formation of gene pool occur in the begomovirus associated betasatellite infecting crops. Evolution of new or novel begomoviruses were due to recombination and mutation, these were also driving forces for the emergence of new begomovirus species and an increase in begomovirus host range.

Kushawaha *et al.* (2018) reported in south Asia and the African continent cassava mosaic disease prevalent and caused by single stranded DNA viruses, ICMV (*Indian cassava mosaic virus*) and SLCMV (*Sri Lankan cassava mosaic virus*). Previously, SLCMV and ICMV diversity were studied according to a sequence-dependent method

using PCR in India. To amplify novel associated viruses with cassava mosaic disease sequence independent method of amplification used. According to rolling circle amplification (RCA) majority of samples were infected with SLCMV and few samples were infected with both SLCMV and ICMV. Variants showed 97–99% nucleotide sequence identity with previously reported SLCMV that suggest begomoviruses infecting cassava had low level of genetic variability.

Zerbini *et al.* (2017) described summary of ICTV report on family *Geminiviridae* taxonomy. Family *Geminiviridae* are plant pathogens that contain non-enveloped small, single stranded circular DNA genome of ~2500 to 5200 bases. Transmission of geminiviruses are through a number of insects. Whiteflies are the transmissible factor of genus *begomovirus* while *Curtovirus, Turncurtovirus, Grablovirus, Becurtovirus* and *Mastrevirus* genera transmission occurred by specific leaf hoppers. Transmission of one member of the *Capulavirus* genus by an aphid and *Topocuvirus* genus transmission by treehopper. In tropical, subtropical areas of World, the geminiviruses are important plant pathogens that cause economical important diseases and losses.

Versani *et al.* (2017) reported two new genus in *Geminiviridae* family consisting of seven genera (*Begomovirus, Eragrovirus, Becurtovirus, Curtovirus, Mastrevirus, Turncurtovirus* and *Topocuvirus*). With new molecular approaches and tools, discovering rate of new emerging viruses significantly increased. Two new genera establishment has been reported, *Grablovirus* with one new species and *Capulavirus* with new four species. ALCV (species of *Capulavirus*) vector are *Aphis craccivora*, a species of aphids while *Grablovirus* likely vector *Spissistilus festinus*, species of treehopper. Two new species are highly divergent, one infecting citrus plants and assigned to *Citrus chlorotic dwarf associated virus*, one other infecting to mulberry plants and assigned to *Mulberry mosaic dwarf associated virus*. ICTV not assigned these species to a genus because Insect vectors and particle morphology are unknown.

Lima *et al.* (2017) describes begomoviruses are responsible for major economic losses in agricultural field. They show highest degree of genetic variation with in host and quickly develop new variations. Begomoviruses genome have ability of combination. Continuous recombination and mutation leads to genetic variation of begomoviruses. Genetic variability of begomoviruses datasets estimated worldwide

and their record is present in online databases. Begomoviruses analysis clear that recombination variation occurred in length of coat protein and rep genes. They observed that mutation is main reason of genetic variation in all begomoviruses and relative contribution of recombination's and mutations are not compulsory a function of their relative rates. Results shows that fraction of genetic variation assigned to recombination is always lower than due to mutation, its indicate begomoviruses diversification is primarily possible through mutational dynamics.

Brown *et al.* (2017) describes viruses have potential to spread in other vegetable and cotton growing areas worldwide through global transportation of plants that were alternative hosts of begomoviruses like ornamental plants. Recently five new species of begomovirus have been emerged that reduced the yield and fiber quality of cotton. They describes the strategy for primer designing and validation for begomoviruses that cause severe damage in cotton production. Begomoviral-satellite complex genomic diversification was apparently based on two factors, alignments of nucleotide sequences and single nucleotide polymorphism analysis that cause challenges for primer designing. Due to high similarity with in viruses, additional steps required for the validation of primer designing. Molecular diagnostic plants screening prior to import and export, dramatically helps to control viruses, affect productivity of cotton, other vegetable and ornamental crops.

Ho *et al.* (2017) describes Geminiviruses are plant pathogen that cause severe diseases in agriculture worldwide and destructs the farmer's livelihood, especially in developing countries. Due to small genome, they change genome rapidly and have viable and diverse genome in nature. Researchers used genomic approach to explain virus infectious mechanism. A popular repository, NCBI Viral Genome website that provide a centralized source of genomic information. NCBI kept bipartite begomoviruses separately as individual genomes. Their goal to build an extensive Geminivirus genomic database that include virus genome characteristics and biological related annotations. 508 genomes and 161 associated satellites acquired from four major genera of *Geminiviridae* and NCBI databases. Data on website available for public that helps researchers to understand the diverse nature and complex relationships among the viruses of family *Geminiviridae*.

Barboza *et al.* (2017) studied begomoviruses as important plant pathogen that spread worldwide in tropical and subtropical areas. In 1970, begomoviruses were reported in Costa Rica but still poorly identified there. The viruses are diverse based on host, production system and geographical location. A linkage between begomovirus species and geographical regions were observed. In Costa Rica, agricultural system and climate change factors were involved in distribution of begomoviruses with in the country. These results might help to manage begomoviruses in Costa Rica.

Zaidi *et al.* (2016) observed *Sesbania bispinosa* growing in cotton fields infected by cotton leaf curl disease with typical severe symptoms of begomovirus, vein thickening, leaf curling and yellowing in Pakistan. Amplification and sequencing of begomovirus with its betasatellite were performed from these affected samples. *Pedilanthus leaf curl virus* (PeLCV), monopartite begomovirus were linked to disease showed through complete nucleotide sequence and 98.73% sequence identity shared with a previously reported clone from soybean. Characteristics of betasatellites were showed 96-97% highest sequence similarity with previously isolated *Tobacco leaf curl betasatellite* (TbLCB) from soybean. It's the first report of *Sesbania bispinosa* affected with PeLCV and TbLCB.

Saleem *et al.* (2016) examined recombination phenomena for those begomoviruses that were being tangled in progression of new begomoviruses through recombination. *Cotton leaf curl Burewala virus* was the best example of recombinant between CLCuKoV and CLCuMuV that cause cotton epidemic in Pakistan and India. In addition, Rep was usually donated by CLCuMuV whereas CLCuKoV donated CP in majority cases. Moreover, In Asian countries like Pakistan, India, China, CLCuMuV propagation was at an alarming rate and a near future threat for cash crops like cotton.

Snehi *et al.* (2016) reported begomoviruses in *Jatropha* ornamental species throughout the World. Samples were collected from a garden with mosaic symptoms at Lucknow and processed to identification of a begomovirus in diagnostic PCR by using specific primers. Phylogenetic relationships shows close relationship with *papaya leaf curl virus, Jatropha mosaic India virus* and *tomato leaf curl Patna virus.* Multiple type of begomovirus infecting *Jatropha* ornamental species were a new report in India. Current research highlighted new emerging diseases of begomovirus

on ornamental *Jatropha* species that helps researchers to improve *Jatropha* cultivation and enhance oil productivity.

Srivastava *et al.* (2016) observed severe begomovirus symptoms like enation, vein thickening and leaf curling on most of the ornamental *Hibiscus* (*Hibiscus rosa sinensis*) plants in garden at lucknow during 2011. DNA from healthy and infected plants were extracted and coat protein identify by using universal primers in a diagnostic PCR. By using RCA (rolling circle amplification), full length virus were identified from two samples while universal primers used for the identification of beta satellite. Infected samples of *Hibiscus* ornamental plants shows 98% nucleotide identity with CLCuMB and CLCuMuV. Hibiscus infected with CLCuMuV-CLCuMB complex was the first report in India.

Leke *et al.* (2016) describes vegetable and crops production have vital role in human health, diet, nutrition and livelihood in Cameroon. Fluted pumpkin is widely cultivated homegrown vegetable in South Africa but the major limitation for fluted pumpkin are plant viral diseases that caused by a plant pathogen. Samples with mosaic symptoms were collected and processed. Complete sequence of genome showed unreported bipartite begomovirus having same viral coding region arrangement on genomic components as the OW bipartite begomoviruses have. DNA-A component have 78% identity with ChaYMV (*chayote yellow mosaic virus*) while DNA-B component have 64% identity with SbCBV (*soybean chlorotic blotch virus*). The new Old World bipartite begomovirus named as *telfairia mosaic virus* (TelMV) and it's the first explosion of plant pathogen begomovirus affecting members of *telfairia* genus.

Tahir *et al.* (2015) collected samples from Pakistan and Nepal that exhibit virus like symptoms from different species like two *Ageratum conyzoids*, one *turnip* and one *Sonchus oleraceus*. Full length clone sequences shows >89.1% nucleotide identity with *Ageratum enation virus* (AEV) and thus isolates of AEV. Betasatellite clone sequences shows >90% identity with AYLCB (*Ageratum yellow leaf curl betasatellite*) sequences. AEV proposed two different strains, one name as Nepal and other one India. Infectivity of AEV clones isolates with their AYLCB in *A. conyzoides, N. tabacum, Nicotiana benthamiana,* and *Solanum lycopersicon* with

agrobacterium mediated transformation showed leaf curl symptoms only when diseased with AEV and its AYLCB but displayed no symptoms when effected by AEV or AYLCB. Finding exhibited AEV has capacity to infect crops and AYLCB linked with varying degree of symptoms and diseases, it's a virus of weeds.

Gupta *et al.* (2015) collected infected samples of chili, a member of *Capsicum* genus from India (Tirupati). Infected samples observed with distinctive indications of begomovirus likevein yellowing and leaf curling. Total DNA were extracted from symptomatic and normal leave samples and then amplified through PCR by means of using specific primers. A product of 1300 nucleotide DNA fragment was amplified and by analysis, they found begomovirus was the cause of *chili leaf curl disease* in infected chili samples.

Sahu *et al.* (2015) observed leaves with symptoms like reduce size and vein yellowing of spinach in India. Total genomic DNA was extracted and processed. DNA A and DNA betasatellite was cloned from these infected spinach samples. By sequence analysis, they found 88% nucleotide identity to PaLCV (*Papaya leaf curl virus*) showed by DNA A name as *Spinach yellow vein Sikar virus* and it's the discovery of new begomovirus species.

Rishishwar *et al.* (2015) collected samples of infected okra with BYVMD (Bhendi yellow vein mosaic disease) from different 10 locations of Bhubaneswar, India. Molecular characterization through PCR was done using diagnostic primers of coat protein. Analysis showed that seven samples of them have identity with BYVMV (*Bhendi yellow vein mosaic virus*) and three samples have identity with MeYVMV (*Mesta yellow vein mosaic virus*). Recombination analysis showed that complete viruses have recombinant resulting from MYVYNV (*Malvastrum yellow vein Yunnan virus*) and MeYVMV (*Mesta yellow vein mosaic virus*) as parents. Its first discovery of *Malvastrum yellow vein Yunnan virus* (MYVYNV) effecting okra in areas of India. For the okra production, Bhendi yellow vein mosaic disease was a major limitation and causes severe economic crop losses in India.

Shuja *et al.* (2014) reported a distinctive strain of CLCuBuV (*Cotton leaf curl Burewala virus*) genome have no gene for transcriptional activator protein (TrAP). In 2001 outbreak of cotton, only this virus was prevalent after breaking resistance

disease. It's a recombinant evolved with recombination of CLCuMV and CLCuKoV Sequence analysis showed that this virus was less than 93% identical with CLCuBuV that showed this virus was a new strain whereas associated betasatellite of CLCuBuV 97.6% identical to CLCuMB (*cotton leaf curl Multan betasatellite*).

Khatri *et al.* (2014) studied infected rose (*Rosa chinensis*) an ornamental plant. Samples were collected of infected rose leaves with typical symptoms like stunted growth and leaf curling. For virus amplification, total genomic DNA was extracted and amplify through PCR and RCA. Sequence analysis of amplified cloned product of DNA A *Rose leaf curl virus* (RoLCuV) showed 83% nucleotide identity to *Tomato leaf curl Pakistan virus*. While cloned product of betasatellite DNA showed 96% nucleotide identity to DiAYVB (*Digera arvensis yellow vein betasatellite*).

George *et al.* (2014) observed infected plant sample of *Amaranths*. Infected leaves have symptoms of leaf crinkling, leaf curling, leaf distortion and yellow leaf margins. From infected plant samples of *Amaranthus*, molecular characterization was done of a complete monopartite begomovirus. From sequence analysis found that DNA alphasatellite showed identity to *Chilli leaf curl alphasatellite*. DNA betasatellite was identical with *Tomato yellow leaf curl Thailand betasatellite*.

Ullah *et al.* (2014) observed Eggplants having severe symptoms of mottling and leaf mosaic. Virus amplification was done through RCA and its complement with betasatellite specific primers. Product were cloned and sequenced. Sequence analysis indicated 99% nucleotide sequence identity to CLCuBuV and its complement showed 97% nucleotide sequence identity to (CLCuMB). According to species demarcation threshold, both virus and its complement were the variant of CLCuMB and CLCuBuV. It's the first explosion of eggplant in Pakistan as the alternate host for cotton linked begomoviruses.

Srivastava *et al.* (2014) described the PedLCV (*Pedilanthus leaf curl virus*) had association with *Tabernaemontana coronaria* and *Cestrum nocturnum* in Lucknow, India. Infected plants of night blooming jasmine (*C. nocturnum*, family *Solanaceae*) having symptoms of leaf curling whereas crape jasmine (family *Apocynaceae, T. coronaria*) having symptoms of leaf yellowing, dwarfing and mottling. In order to ascertain begomoviral infection, molecular characterization and Rolling circle

amplification were performed. On sequencing, virus showed the nucleotide identity to PedLCV (*Pedilanthus leaf curl virus*).

Ilyas *et al.* (2013) cloned two isolates of begomovirus from *Catharanthus roseus* (Madagascar periwinkle). Madagascar periwinkle a famous medicinal ornamental plant in Pakistan. One isolated clone showed 86.5 % nucleotide identity to unpublished ChiLCIV then had 84.4 % nucleotide identity to PaLCV (*papaya leaf curl virus*). That suggests it's a new species and "*Catharanthus yellow mosaic virus*" name were proposed. Other isolated clone sequence have 95% to 99 % nucleotide identity to PaLCrV (*papaya leaf crumple virus*). Sequence analysis identified these isolates were recombinant of CrYVMV (*croton yellow vein mosaic virus*) and PedLCV (*Pedilanthus leaf curl virus*).

Marwal *et al.* (2013) collected infected samples of Spanish Flag (*Lantana camara*) that is an ornamental plat having severe symptoms of vein yellowing disease. Molecular characterization for begomoviral infection, total genomic DNA was extracted and amplification for begomoviruses were done through PCR using specific degenerate primers of Coat protein (CP). Southern blot hybridization were done for further confirmation. Phylogenetic analysis showed *Lantana camara* infecting begomovirus had cluster with *Tomato leaf curl Bathinda virus* isolate. It's the first report of begomovirus infecting *Lantana camara*.

Anwar *et al.* (2012) collected symptomatic samples of *croton* diseased plants that evergreen shrub grown in gardens of Lahore in 2012. Samples had symptoms of leaf yellowing and mild leaf curling. Total genomic DNA were extracted and amplification of full length virus were done. On sequencing, sequence analysis showed 99.1% nucleotide sequence identity with ClYMV that suggests it's a new variant of ClYMV. Hence amplification attempts for DNA b or associated betasatellite component were failrd. It's the first report of croton having begomovirus infection and serve as host plant for begomoviruses.

Fareed *et al.* (2012) observed *caster bean* plants with typical begomovirus symptoms vein thickening and leaf curling that were growing as an ornamental plant for oil production in these region. Total DNA extraction was done from leaf samples and amplified for begomovirus through PCR. Initially used degenerate primers for

begomovirus detection and abutting primers were used for amplify full length virus. Associated betasatellite were amplified by using universal primers. On sequencing, clone showed 98% sequence identity with CLCuKoV-Bu and associated betasatellite showed 96% nucleotide sequence identity with CLCuMB. According to species demarcation threshold, that suggests both cloned were variant of the CLCuKoV-Bu and the CLCuMB. It's the first report of caster bean infecting with complex of CLCuBuV, CLCuMB and serve as an alternate host for begomoviruses.

Zulfiqar *et al.* (2012) observed yellow vein symptoms on leaves of *Vernonia cinerea* plant. Molecular characterization were done for begomoviral amplification. On sequencing, cloned showed 78.9% highest identity to VeYVV (*Vernonia yellow vein virus*) recently reported in India. Linked alphasatellite and betasatellite also cloned. Alphasatellite showed 70.7% identity to *Gossypium mustelinium symptomless alphasatellite* (GMusSLA). While betasatellite had 51.2% maximum nucleotide identity to *Vernonia yellow vein virus betasatellite* (VeYVVB). According to species demarcation criteria, cloned virus with associated alphasatellite and betasatellite were the new begomoviruses species named as *Vernonia yellow vein Fujian virus* (VeYVFJV).

Gaur *et al.* (2011) observed *Mimosa pudica L.* plant having symptoms of small yellow leaf and stunted growth. Sample were collected from infected plants. *Bemesia tabaci* were present on infected leaves that may indicate the presence of begomovirus infection. Begomovirus amplification was done through PCR by using coat protein specific primers. Amplified product were cloned. On sequencing, clone showed 97% highest sequence identity with AYVV (*Ageratum yellow vein virus*) and with AYVV-Gx (*Ageratum yellow vein virus-Guangxi*). It's the first explosion of *Mimosa pudica* infected with begomovirus and serve as alternate host for begomoviruses.

Cuong *et al.* (2011) collected tomato and papaya leaf samples exhibiting leaf curling symptoms from northern areas of Vietnam. Samples molecular characterization were done and cloned. Sequences isolates and named as tomato-100, tomato-89 and papaya-31. After sequencing, sequence of isolates were submit to GenBank. Later amplification of these samples, it's confirmed, these isolate viruses have identity with *Tomato leaf curl Hainan virus*. It's the first detection report of the isolate viruses in

Vietnam. Papaya was the new host for begomoviruses while tomato isolated virus were the distinct species and identified as *Tomato leaf curl Hanoi virus*. It's a recombinant resulting from *Ageratum leaf curl Virus* and *Papaya leaf curl China virus*.

Zulfiqar *et al.* (2011) observed Morning glory plants exhibiting symptoms of vein yellowing and yellow mosaic pattern. Samples were collected from different regions of China. From both samples, the genomic DNA-A component were amplified of length 2801 and 2827 nucleotides and named as Y338 and JS-1 respectively. JS-1 showed 97% highest nucleotide identity to SPLCV (*Sweet potato leaf curl virus*) while DNA-A component of Y338 have 97.8% identity to isolate of SPLCV from China. Phylogenetic analysis showed these isolates were SPLCV new strains.

Tahir *et al.* (2011) collected leaf samples of *Ageratum conyzoides* (ACL) and *Sonchusoleraceous* (SOL) exhibiting leaf yellowing and leaf curling symptoms. Isolates were amplified for full length virus. On sequencing isolates showed 92.5%-98.7% identity to each other. DNA A and associated betasatellite of SOL isolate had and ACL isolates had 92.2%-97.8% sequence identity to Nepalese isolate (CAN) AEN [Nepal: 01] while isolates of ACL had 93.5%-96.0% nucleotide identity to AYLCuB (*Ageratum yellow leaf curl betasatellite*). AEV reported first time in Pakistan. These ACL and SOL isolates were used to partial repeat construct production.

Tahir *et al.* (2010) reported a new genus *begomovirus* species named as *Pepper leaf curl Lahore virus* (PepLCLV) from capsicum plants. Full length virus were cloned with associated DNA betasatellite and thaught that these cloned were recombinant resulting from PaLCuV and *Chili leaf curl virus*. Its associated betasatellite have identity to *Chili leaf curl betasatellite* (ChLCB). Its first time reported to infecting potato plants and assumed as chili leaf curl betasatellite cognate. PepLCLV were among the monopartite begomoviruses with associated betasatellite, and among the numerous begomoviruses infecting *capsicum*.

Tahir *et al.* (2010) observed *Cucurbita pepo* leaves with symptoms of yellow mosaic pattern and collected infected leaf samples from Lahore. Full length virus were cloned and sequenced. DNA-A component showed highest 98.4% nucleotide identity, while

DNA-B component have 89.6% sequence identity with a strain of SLCCNV (*Indian Squash Leaf Curl China Virus*).

Tahir *et al.* (2010) collected *M. charantia* plant leaf samples exhibiting symptoms of yellow vein probably begomovirus infection symptoms from Lahore adjacent areas in 2004. From infected leaf samples full-length genomic components of begomoviruses were cloned and sequenced. The DNA A component sequence had 86.9 % nucleotide identity with one of ToLCNDV (*Tomato Leaf curl New Delhi Virus*) isolate, which suggests it's a distinct species and named as BYVV (*Bitter gourd Yellow Vein Virus*). It's a recombinant resulting from ToLCBDV (*Tomato leaf curl Bangladesh virus*) and ToLCNDV through interspecific recombination. Whereas DNA B component had 97.2% identity with *Squash leaf curl China Virus*, an Indian strain.

He *et al.* (2009) reported begomovirus isolates from infected *allamanda* plants in Guangdong, China. The virus were amplified and sequenced, comprising 2755 nucleotides with basic begomovirus arrangement in six overlapping ORFs. Sequence analysis were performed withprevious available sequences reported in database. Cloned had highest 81.2% nucleotide identity with isolate *Tomato leaf curl Guangdong virus* (ToLCGuV). According to species demarcation threshold, isolated virus were distinct species from *allamanda* plant and proposed name was AlLCV (*Allamanda leaf curl virus*).

Mubin *et al.* (2009) reported begomoviral components from *Digera arvensis* (a common weed) related to vein yellowing disease. Isolated cloned of full-length virus had 2752 nucleotides that showed 98% nucleotide sequence identity to *Cotton leaf curl Rajasthan virus* while Another isolated clone had 1386 nucleotides sequence that had typical arrangement of alphasatellites and related with *Potato leaf curl alphasatellite* (PotLCA). Whereas, two betasatellite components were also isolated that showed identity with AYVB and recombinant of CLCuMB and *Tobacco leaf curl betasatellite* from the *Digera arvensis* samples. They suggests *Digera arvensis* serve as an alternative host for begomoviruses that were highlighted the importance of weeds.

Haider *et al.* (2008) observed the presence of begomoviral infection in plants growing in surrounding areas of Punjab University in 2008 from Lahore, Pakistan.

Vinca minor growing ornamental plant exhibiting severe begomovirus symptoms. Symptomatic and non-symptomatic plant leafs were collected, total genomic DNA were isolated for further amplification. Amplification for begomoviral infection were done through diagnostic PCR by using primers for coat protein. 0.7 kb product were amplified and cloned. On sequencing, isolated clone have 93% highest nucleotide sequence identity to *Pedilanthus leaf curl virus*. It's the first detection report of ornamental plants infected with PedLCV, a begomovirus infection in Pakistan.

Saeed *et al.* (2007) studied the association of a *cotton leaf curl disease* (CLCuD) associated DNA betasatellite of monopartite viruses with ToLCNDV (*Tomato leaf curl New Delhi Virus*) which a bipartite virus. DNA betasatellite had the same function as DNA-B. Betasatellite encoded bC1 like DNA B had movement function. DNA betasatellite and DNA A co-infection further exhibit systematic infection in plants while individual DNA A Inoculation exhibit no symptoms. Similarly, inoculation of mutant bC1 exhibit no symptoms. Hence new infection emergence resulting from betasatellite association with other mono/bipartite begomoviruses.

Tahir *et al.* (2006) observed *Duranta repens* (pigeon berry) plants exhibiting mild leaf symptoms grown in Pakistan as an ornamental plant. Samples was collected from symptomatic and healthy plant leaves and total genomic DNA were extracted. Amplification was done through PCR by using 1.5 kb size primers pair. Sequence analysis showed isolate had 91% highest identity with the *Croton yellow vein mosaic virus*. While DNA-B component were amplified of 2.8kb size and its sequence analysis performed and showed highest 94% nucleotide sequence identity with segment b of *Tomato leaf curl New Delhi virus*. This study identified begomovirus presence in *D. repens* exhibiting leaf curl disease.

Khan *et al.* (2005) observed several ornamental *Calendula* plants exhibiting vein yellowing net disease in India. Whiteflies naturally transform disease from infected to healthy plants or seedlings. Genomic DNA were extracted from infected and healthy leaf samples. Virus amplification was done through PCR using Coat protein primers set and amplified product was cloned. Cross hybridization was done for further confirmation of PCR authenticity by using ToLCNDV as a DNA-A probe. On sequencing, the isolated clone virus had 93% nucleotide identity to *Tomato leaf curl*

Bangladesh virus, 94 % nucleotide identity to *Ageratum enation virus* while 95% highest identity to *Tobacco curly shoot virus*. It's the first begomovirus detection report of infecting *Calendula officinal*.

Tahir and Haider (2005) collected infected samples of bitter gourd leaves exhibiting symptoms of yellow blotch from different areas of Lahore. To confirm begomovirus presence, DNA was extracted from samples and amplified through PCR by using specific coat protein primers and cloned. On sequencing, isolate had highest 95% identity to ToLCNDV (*Tomato leaf curl New Delhi virus*) that suggests it's new strain and first time reported in bitter gourd.

Hussain *et al.* (2004) studied CLCuD to find its alternative hosts. Collected symptomatic samples of chili leaves from Khanewal and Vehari. Genomic DNA were extracted from infected chili leaves. Amplification for begomoviral infection was done through PCR and Southern blot hybridization was done to further confirm PCR result authenticity. In southern blots DNA beta probe of CLCuD were used. In result Strong signal were produced that showed the CLCuD presence in Chili. That suggests chili as an alternative host for CLCuD.

Wang *et al.* (2004) studied comparative analysis of six papaya leaves sample that exhibit symptoms of downward leaf curl. By Comparative analysis two different begomovirus species were identified, one infecting isolates of group II named as PaLCuGDV (*Papaya leaf curl Guangdong virus*) while other one infected isolates of group I and named as PaLCuCNV (*Papaya leaf curl China virus*). On sequencing, amino acid sequence of coat protein had 97% and 94% identity to PepLCV (*Pepper leaf curl virus*) respectively. Comparison analysis of amino acid sequences suggests that coat protein of these isolate viruses had common ancestors.

Jose & Usha. (2003) studied okra plant to confirm the interaction in BYVMV (*Bhendi yellow vein mosaic virus*) and its associated DNA betasatellites and okra plants were threatened to begomoviral infection due to that interaction in India. Partial repeat construct were designed and insert in binary vector to observe symptoms in plants. Agro-inoculation were done through binary vector. BYVMV inoculation produced symptoms of leaf curl while interaction of both BYVMV and its associated DNA betasatellite were produced vein yellowing and mosaic pattern symptoms.

Briddon *et al.* (2002) reported a region located to upstream hairpin loop that extremely conserved. From conserved sequence, they designed abutting primers pair to amplify full-length virus DNA component betasatellite of 1350 nucleotides from unhealthy plants. Betasatellite that is single stranded DNA component depends on helper begomovirus to transmit and replicate in other host plant.

Idris *et al.* (2002) observed hollyhock and okra plants exhibiting symptoms of leaf crumpling and leaf curl respectively. Full-length genomic component were amplified and cloned. On sequencing hollyhock isolate had 84% identity while okra isolate have 95% identity with OW begomoviruses. On Alignment, hollyhock and okra isolates individually showed 98% and 99% sequence identity with AREV and OKEV respectively. Satellites DNA ranging from 741 to 1350 nucleotides were also amplified that showed identity with enation symptom viruses from both the samples.

Bigarre *et al.* (2001) isolate viruses having symptoms of leaf curling and enation on *Althea rosea* from Egypt characterized at cytopathological and molecular levels. By microscopic study it's observed that enations was differentiation and reorganization of vascular tissues. This isolate sequenced and exhibit similar characteristics as Old World begomovirus. Although show significant sequence dissimilarity <69% with any other geminivirus, thus it's represent a new viral species AREV. When it's inject in *N. benthamiana* it show symptoms of vein distortion and leaf curling but not cause any infection. To find if AREV associated with similar disease in Okra Egypt, Partial sequence of coat protein gene was determined and it show 97% nucleotide similarity with isolate (hollyhock) that show genetic heterogeneity within population of begomovirus that related with enation disease.

Xie *et al.* (2001) reported a distinct begomovirus species which named as *Tobacco curly shoot virus*. Collected symptomatic samples of tobacco plants exhibiting symptoms of curly shoot from Yunnan. Full length virus were amplified from infected samples and cloned. On sequencing, sequence analysis showed isolated DNA A clone had highest 85% nucleotide identical to *Tomato leaf curl virus* that was from India while sequence of Coat protein had highest 98% identity to CLCV from Pakistan.

Briddon *et al.* (2000) cloned CLCV that were similar to bipartite begomovirus DNA A component. *Nicotiana benthamiana* and cotton were infected with isolated CLCV

clone, in result leaf yellowing and leaf curling symptoms were observed. That suggests CLCV may contribute in disease transmission.

Lotrakul *et al.* (2000) reported a distinct species of bipartite begomoviruses from infected *Dicliptera sexangularis* plant, which grown in Lee County as an ornamental plant from Florida. Molecular characterization for begomoviral infection were done through PCR and southern blot hybridization. Full length genomic component of viruses DNA-A, DNA-B were amplified and cloned successfully. On sequencing, sequence analysis showed it's a distinct species based on species demarcation threshold thus named as *Dicliptera yellow mottle virus* (DiYMV). Phylogenetic analysis suggests that distinct species may be a recombinant or diverged away due to sudden shift from *Potato yellow mosaic virus* (PYMV).

CHAPTER 3: MATERIALS AND METHODS

Sample Collection

Samples of begomovirus infected ornamental plants were collected. These samples have severe symptoms of begomovirus infection like vein thickening, vein yellowing, leaf curling and mosaic pattern etc. These infected samples were stored at -80◦C until DNA extraction.

Total DNA Extraction

Genomic DNA extraction of infected plant samples were done through Cetyl Tri methyl Ammonium Bromide (CTAB) method. In 1990, Doyle first introduced CTAB method. Approximately 1g of leaf samples were taken and grounded into powder form by using liquid nitrogen in pestle and mortar. 25ml CTAB buffer (2% Cetyl Trimethyl Ammonium Bromide, 1.4 M NaCl, 20 mM EDTA, 100 mMTris HCl, and 0.2% mercaptoethanol) were taken in a 50ml labelled falcon tube and preheated at 60◦c for 20-30 min in a water bath. Preheated CTAB buffer was added in powdered form of samples and mixed well. Incubated falcon tubes at 60◦c for 30 min with continuous shaking in a shaking water bath. After incubation, mixture was allowed to cool at room temperature in a fume hood for 3 min and then mixture was divided equally in two 50ml falcon tubes and then added equal volume of chloroform: isoamylalcohol (24:1) in mixture and mixed. Then centrifugation (Eppendorf Centrifuge 5804R) of both tubes was done at 5,000 rpm for 15 min at room temperature. After centrifugation, the upper clear aqueous phase were transferred into new 50ml falcon tube. Approximately 20ml were transferred into new 50ml labelled falcon tube. Then equal volume of ice cold isopropanol was added to falcon tubes and mixed gently by inverting tube. Tube were placed at room temperature for overnight incubation. Next day, centrifugation was done at 5,000 rpm for 10 min at room temperature. Carefully discarded supernatant and pellet was transferred in 1.5ml eppendorf. Pellet was washed with cold 1ml DNA washing buffer (76% ethanol, 10 mM ammonium acetate) and centrifuged (Eppendorf centrifuge, 5415R) at 10,000 rpm for 2 min at room temperature. Supernatant was discarded and pellet was allowed

to air dry at 37◦c in incubator for 20-30 min. pellet was dissolved in 1ml TE buffer (10mM Tris-HCl, 1mM EDTA) and stored at -20°C.

DNA Quantification Analysis

DNA concentrations were analyzed through visualizing DNA on 1% agarose gel prepared in TAE buffer. Quantified through nanodrop. DNA quality were analyzed by using Agarose Gel Electrophoresis. 5µl of total genomic DNA were encumbered on agarose gel beside with a DNA marker and quantified visually through UV-light and compared with marker. DNA quantity were analyzed by using Nano drop. 1µl of DNA sample were loaded on Nano drop lens and quantify DNA. The TE buffer was used as blank.

Dilution of Total DNA

Total DNA was diluted in sterile water or TE buffer according to 1:10 ratio in 1.5 ml micro-centrifuge tube. Formula used to calculate ratio was

$$m1v1=m2v2$$

Polymerase Chain Reaction

Amplification of begomovirus in sample was amplified by performing Polymerase chain reaction. Total reaction mixture for PCR was 50µl and contained: 10X Taq buffer $(NH4)SO4$ (5µl), 1.5 mM $MgCl2$ (3µl), 2mM dNTPs (2µl), 2 µl of each 50µM primers (both Forward and Reverse), 100 ng of total DNA, 0.5 units *Taq Polymerase* (1µl). ProFlex™ PCR System (Life Technologies) was used to perform PCR reaction and particular temperature was set for the amplification of desired product. Particular conditions for amplification of desired product used in Polymerase chain reaction are given in Figure and primers name and sequences are given in table.

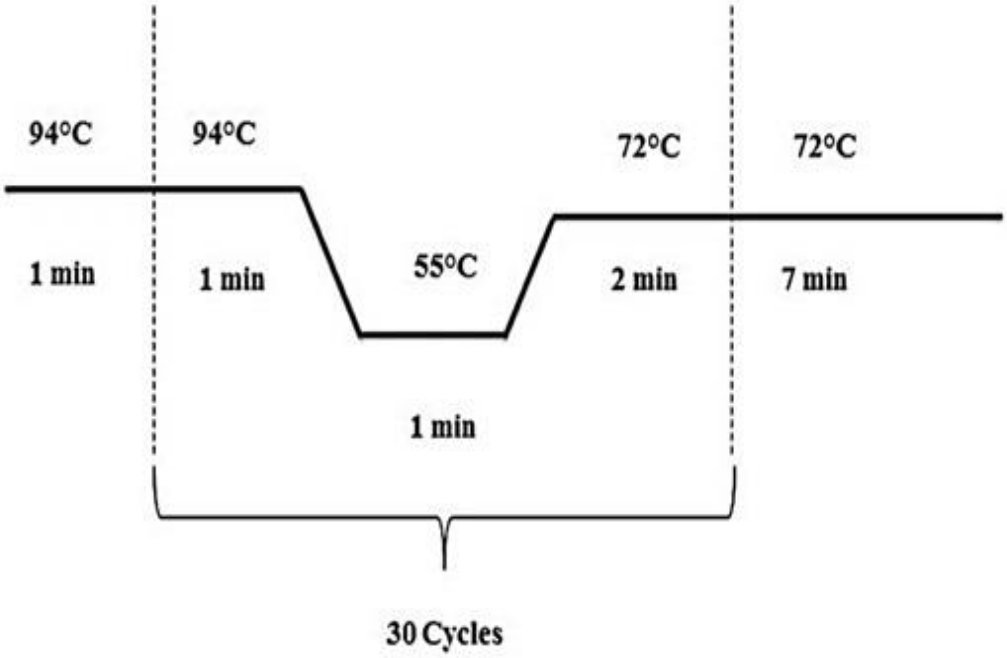


Figure 2. Conditions for PCR amplification of DNA A of begomovirus.

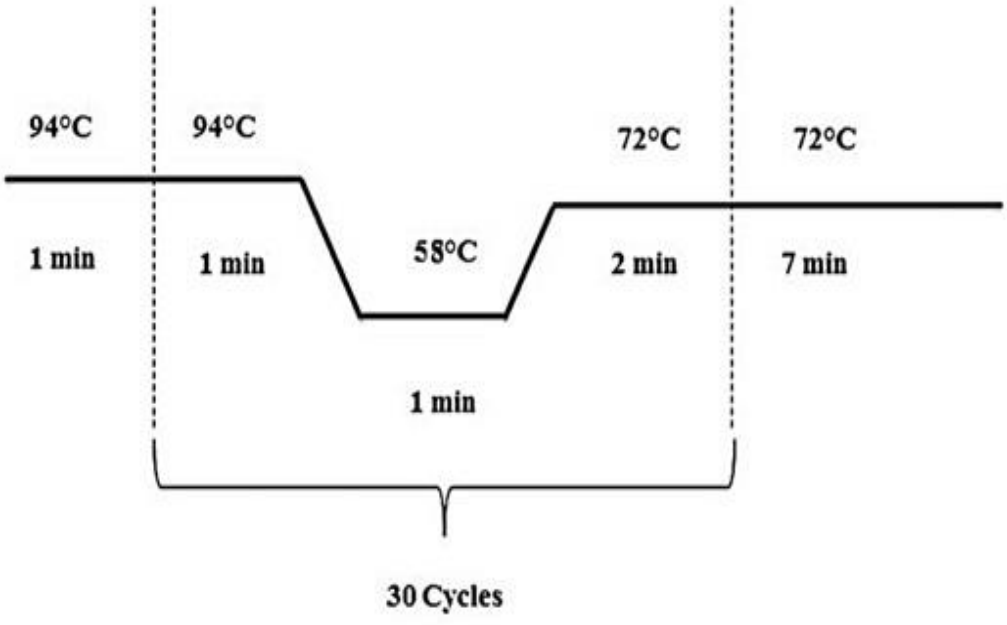


Figure 3. PCR condition for amplification of betasatellite of begomovirus.

Table 2. Primer Name, sequence and used for amplification.

Primer Name	**Sequence (5'-------3')**	**Used to Amplify**
WTG-F WTG-R	5'-GATTGTACGCGTCTAATTTGAAYBGG-3' 5'-TANACGCGTGGCTTACATGGGCCTDT-3'	DNA A
CLCV1 CLCV2	5'-CCGTGCTGCTGCCCCCATTGTCCGCGTCAC-3' 5'-CTGCCACAACCATGGATTCACGCACAGGG-3'	DNA A
Beta-01 Beta-02	5'-GGTACCACTACGCTACGCAGCAGCC-3' 5'-GGTACCTACCCTCCCAGGGGTACAC-3'	Betasatellite
CP-F CP-R	5'-ATGHSVAAGCGWMCMGSMGATAT-3' 5'-TTAATTBVHDAYHSHRTCATARAARTA-3'	Coat Protein of DNA-A
BurX-F BurX-R	5'-CTCGAGAGTGTCCCCGTCCTTGTCG-3' 5'-CTCGAGTGGGGAGAGTTTCAGATCG-3'	DNA-A
BurN-F BurN-R	5'-CCATGGTTGTGGCAGTTGATTGACAGATAC-3' 5'-CCATGGATTCACGCACAGGGGAACCC-3'	DNA-A
KTB-F KTB-R	5'-CTGCAGAGGTCACCTTGTCATTTCCTTC-3' 5'-CTGCAGCATCATTTGTGAGCGCATATTC-3'	DNA-B

Agarose Gel Electrophoresis

To prepare 1% (w/v) agarose gel, 0.5g agarose were added in 1x 50 ml TAE buffer (25 mM Tris and 1mM EDTA, 5Mm NaOH) in 250 ml flask. Then flask covered with aluminum foil and kept in microwave oven for 2 min or until solution became clear. Cooled it for about 10 minutes. Then 5µl of 0.1% (w/v) ethidium bromide was added in melted agarose. Gel was poured in gel tray with appropriate comb. DNA loading buffer was mixed in PCR product and loaded in well along with DNA ladder. The gel was run at 100 volts for 30 min. To visualize DNA bands, UV-trans-illuminator with short wave ultraviolet light was used.

DNA Isolation from Agarose Gel

Amplified virus DNA product were extracted by using the Silica Bead DNA Gel Extraction Kit (Thermo Fisher Scientific) as manufacturer's instructions. Amplified DNA Specific band fragment was cut from agarose gel and cutting band sited in eppendorf tube. Weighed size of cutting band on electrical weighing balance and then added 1:3 DNA Binding Buffer in it (in 100 mg gel 300 µl DNA Binding buffer). Place on electrical heat block at 55 ◦c for 5 min or until gel dissolved in DNA Binding buffer. Vortex Silica Beads and 5 µl added in it and mix thoroughly, again place at 55 ◦c for 5 min. Centrifuged (Eppendorf centrifuge, 5415R) for 30 sec at 10,000 rpm and room temperature. Discarded supernatant and wash pellet with 1 ml washing buffer and centrifuged for 30 sec at 10,000 rpm and this step were repeated for 3 times. Discarded supernatant and air dried pellet. Dissolved pellet in 23 µl TE buffer and centrifuged for 30 sec. Supernatant were carefully transferred into new eppendorf and stored at -20 ◦c.

Ligation of amplified product

The amplified DNA fragments of required size were ligated in cloning vector (pTZ57R/T) using T/A cloning kit (Thermo Fisher Scientific) following the directions of manufacturer. 17 µL of ligation mixture was prepared consisting of 100-540 ng or 10 µl of PCR product (depending upon the length of DNA fragment), 3 µL of ligation buffer, 13µL (50-100 ng) of pTZ57R/T vector, 1 µl of T4 DNA ligase. The mixture

was kept in water at 4 ˚C refrigerator for overnight incubation. Next day, it was transformed into *E. coli* cells.

Preparation of *DH-5α* Electro-Competent Cells

A single colony of *DH5α* strain of *E. coli* was inoculated into 10ml LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated overnight at 37 ◦c in shaking incubator with continuous shaking. 0.2 ml of the overnight culture was inoculated into 50 ml of LB broth medium in a 250 ml flask. The flask was incubated at 37 °C with continuous shaking in a shaking incubator for 2-3 hours. The culture was chilled and centrifuged (Eppendorf Centrifuge 5804R) at 5000 rpm for 10 min at 4 °C for pellet down. The supernatant was remove and pellet were re-suspended by vortexing in 10 ml ice cold, sterile 10% glycerol. Then made the volume up to 40 ml with 10% chilled glycerol. Tubes was centrifuge on 5000 rpm for 10 min at 4 °C. The washing step was repeated twofold with 10% chilled glycerol making the volume 20ml and 10ml respectively. Finally, the pellet was dissolve in 1 ml 10 % ice chilled glycerol and 50 µl aliquoted in sterile 1.5 ml eppendorf and stored these aliquots at -80 °C.

Escherichia coli Transformation

10 µl of ligation mixture were added in electro-competent cells (50 µl) and assorted gently. The assorted mixture were shifted to ice-cold electroporation cuvette place on ice. Then electroporation cuvette was placed in MicroPulser (Bio-Red) chamber base between contacts and the MicroPulser charge button was pushed to charge cuvette and placed in chamber until a sound like beep was produced. Cuvette were removed and 0.8 ml LB broth medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) were mixed and the mixture were shifted to sterile eppendorf tube. Mixture was incubated in shaking incubator for 60 min at 37 °C with shaking. Then mixture was spread on LB solid medium containing the ampicillin antibiotic. Plates were placed in an incubator for incubation at 37 °C overnight.

Preparation of Glycerol Stocks

For bacterial cultures preservation, glycerol stocks were prepared. For glycerol stock preparation 200 µl of 100% glycerol was added in eppendorf tubes and autoclaved.

Then in glycerol autoclaved tubes 800 µl of bacterial culture was added and stored at -80 ◦c.

Plasmid Isolation (miniprep)

Method of alkaline lysis with modifications made by Doly and Birnboim (1979) and Burke and Ish-Horowicz (1981) was used for plasmid isolation. Select one transformed colony and added in 10 ml LB broth medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 10 µl Ampicillin antibiotic in it and placed at 37 ◦c for overnight with continuous shaking in a shaking incubator. Next day, 4.5 ml culture were pelleted by centrifugation (Eppendorf centrifuge, 5415R) at 10,000 rpm for 2 min at room temperature for miniprep. Ependorf tubes were placed on ice and added 100 µl of ice cold Solution-I (50 mM glucose, 25 mM Tris-Cl [pH 8.0] and 10 mM EDTA [pH 8.0]) in it and dissolved pellet with pipetting, then added 200 µl freshly prepared solution II (0.2 N NaOH and 1% SDS) in it and mix by inverting tubes 5-7 times. Then added 150 µl solution III (3M potassium acetate [pH 8.0]) and mix gently by inverting tubes. Centrifuged at 10,000 rpm for 7-10 min and transferred supernatant or upper clear phase into new eppendorf and added 1 ml 100% ethanol in it and place at room temperature for 30 min. Centrifuged for 2-5 min at 10,000 rpm. Discarded supernatant and added 1 ml 70% ethanol in it. Centrifuged for 2 min at 10,000 rpm. Discarded supernatant and air dried pellet at 37 ◦c. Dissolved pellet in 30-40 µl TE buffer and stored at -20 ◦c.

Restriction Digestion

To confirm plasmid, digestion was performed using restriction endonucleases following manufacturer's instructions. A total reaction of 30µl containing 8 µl plasmid DNA, 3µl Tango buffer, 1µl *Hind*III, 1µl *Eco*RI and 15µl sterile distilled water was used and kept for 2 hours at 37 ◦c in an incubator. Band was seen on 1% agarose gel to confirm the digestion products.

DNA Sequencing

For sequencing purpose, isolated plasmid AY1 and AY2 was referred towards Macrogen, Korea. Universal primers M13R and M13F for sequencing were used.

Sequence Analysis

For sequence analysis, NCBI tool BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used. Assembled nucleotide sequences of AY1 and AY2 was used for analysis. Searches for sequence similarity were performed to compare AY1 and AY2 sequences with other online available begomoviruses/betasatellites in the database.

For genome features, find open reading frame locations in sequences, an online ORF (open reading frame) finder (https://www.ncbi.nlm.nih.gov/orffinder/) tool of NCBI used.

Phylogenetic Analysis

For phylogenetic analysis, identified similar sequences through BLASTn (Basic Local Alignment Tool) were collected and organized. MEGA7 software used to studied phylogenetic relationship. Multiple sequence alignment were performed through MUSCLE in MEGA7 and then these aligned sequences were used to construct phylogenetic phylogram by using Maximum Likelihood (ML) algorithm in MEGA7.

CHAPTER 4: RESULTS

Sample Collection

Symptomatic and asymptomatic leaf samples of ornamental plants were collected from surrounding areas of Islamabad and Lahore during the year 2017-2018. Ornamental plants samples showing begomoviral disease like symptoms; vein thickening, vein yellowing, mosaic pattern etc. Samples were categorized properly in plastic zipper bags and placed at -80 ◦C for future processing.

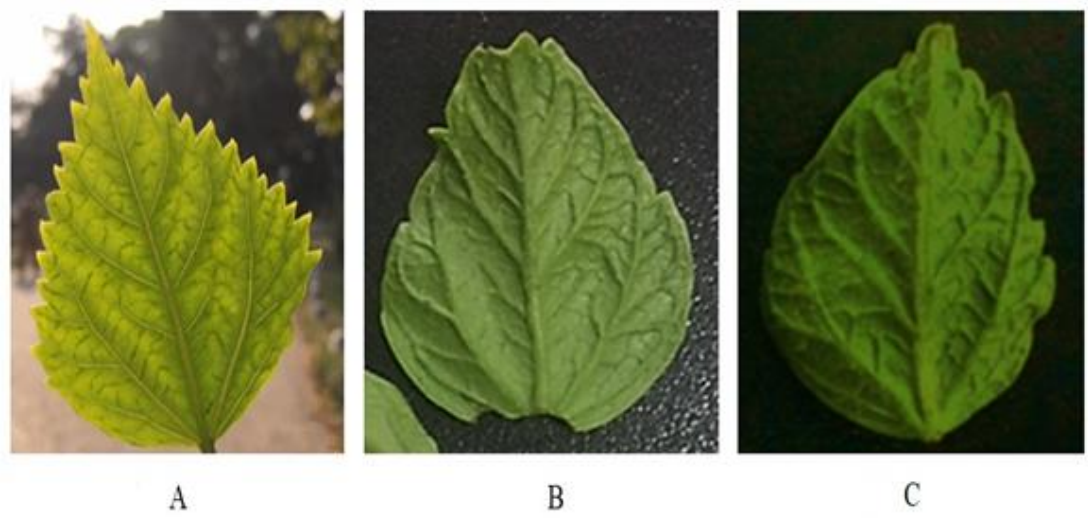


Figure 4. Symptomatic leaf samples.

Symptomatic leaf samples were collected from areas of Islamabad and Lahore for this study. Infected leaf showing vein thickening and leaf curling. A show vein thickening while B and C show vein thickening and mild leaf curl.

Table 3. Sample name, symptoms and sample location described.

Sample name	**Sample code**	**Symptoms**	**Location**
Hibiscus species	HSI-1	Vein thickening	Islamabad
Hibiscus species	HSL-2	Vein thickening and mild leaf curling	Lahore
Hibiscus rosa sinensis	HRI-1	Vein thickening	Islamabad
Hibiscus rosa sinensis	HRI-2	Vein thickening	Islamabad
Hibiscus rosa sinensis	HRL-3	Vein thickening	Lahore
Iresine species	IRI-1	Mild Leaf curling	Islamabad
Iresine species	IRL-2	Leaf curling	Lahore
Ageratum	Ag-1	Vein thickening	Islamabad

Extraction of Genomic DNA

Total genomic DNA was extracted by using CTAB method and stored at -20 ◦c. DNA quantification was done by using agarose gel electrophoresis and nano drop. Total genomic DNA run on 1% agarose gel along with DNA marker and observe under UV trans-illuminator. 5 µl DNA was loaded on agarose gel and analyzed.

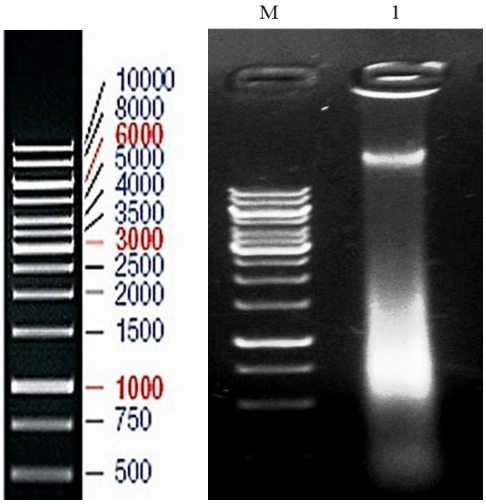


Figure 5. Agarose (1%) gel Electrophoresis of DNA extraction of symptomatic sample.

From left to right first marker M (standard 1 kb gene ruler) and bands on lane-1 *Hibiscus rosa sinensis* DNA.

Detection of Begomoviruses and Associated Satellites

Symptomatic samples were amplified through polymerase chain reaction by using diagnostic primers with total genomic DNA to confirm the presence of a begomovirus. First CLCV1/CLCV2 primer set was used to amplify begomoviruses specific to cotton infection and amplify approximately 1100 bp product which consist of CP in virion sense strand and TrAP. Then another primer set WTGF/WTGR (Mansoor *et al.*, 2000) was used to amplify all whitefly transmitted viruses and it amplify 1500 bp product approximately which covers IR and most of Rep gene region. Begomoviruses related to cotton infection are mostly monopartite and have an associated satellite component. To amplify betasatellite, Beta01/Beta02 a universal primer set (Briddon *et al.*, 2002) was used and it's amplified approximately 1400 bp product which covers full length betasatellite.

Hibiscus rosa sinensis and *Hibiscus species* samples were positive for WTGF/WTGR, CLCV1/CLCV2 and Beta01/Beta02 primers set while *Iresine species* samples were positive for CLCV1/CLCV2 and Beta01/Beta02 primers set. *Ageratum* samples had no virus amplification.

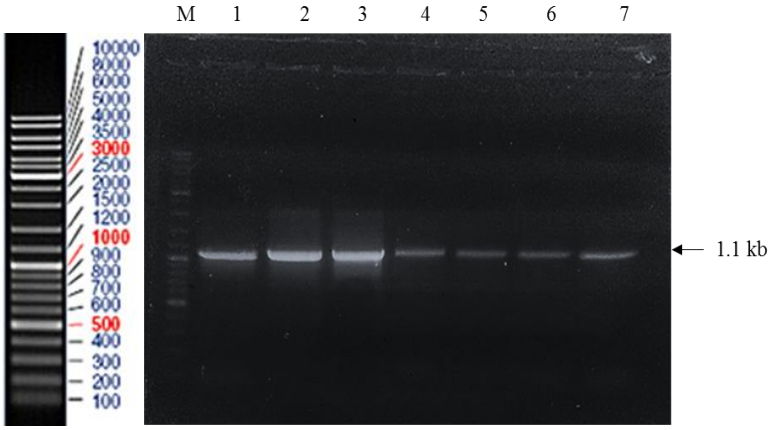


Figure 6. Agarose (1%) gel Electrophoresis of amplified product by CLCV1/2 primers.

From left to right first marker M (standard 1 kb gene ruler). Bands on lane (1-3) are amplified product from *Hibiscus rosa sinensis* samples and bands on lane-4 and lane-5 are amplified product from *Hibiscus species* samples while bands on lane-6 and lane-7 are amplified product from *Iresine species* samples of approximately 1.1 kb with reference to M (standard 1 kb gene ruler).

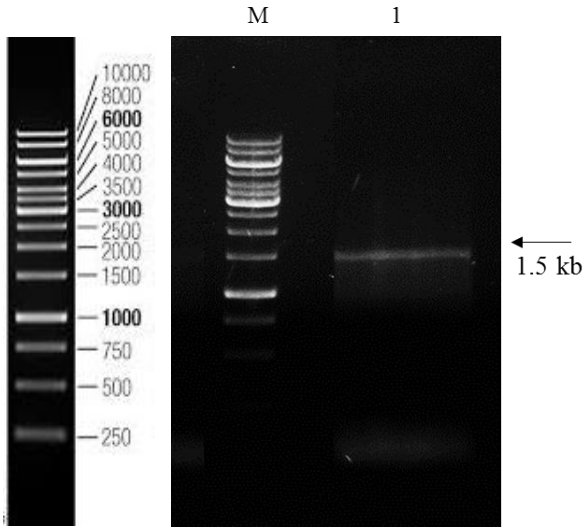


Figure 7. Agarose (1%) gel Electrophoresis of amplified product by WTG primers. From left to right first marker M (standard 1 kb gene ruler). Bands on lane-1 are amplified product from *Hibiscus* samples of 1.5 kb size as reference with M (standard 1 kb gene ruler).

To amplify full length DNA A, DNA B and other regions by using specific primers like KTBF/KTBR, BurXF/BurXR, BurNF/BurNR and CPF/CPR, many attempt were made resulting no amplification.

Betasatellite amplification was done with Beta01/Beta02 primer set. It amplified full length betasatellite of approximately 1.4 kb size. *Hibiscus species, Hibiscus rosa sinensis* and *Iresine species* samples were positive for betasatellite primers. Results are shown in figure.

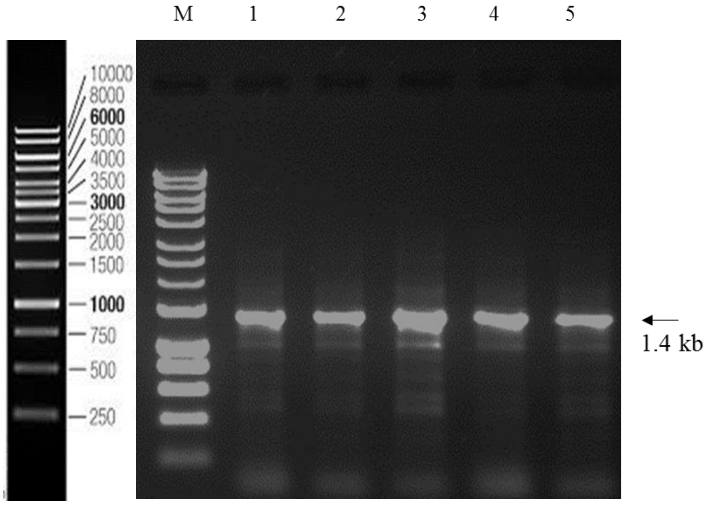


Figure 8. Agarose (1%) gel Electrophoresis of amplified product by Beta01/02 primers.

From left to right first ladder L (standard 1 kb gene ruler). Bands on lane-1-5 are amplified product from *Hibiscus* samples of 1.4 kb size as reference with L (standard 1 kb gene ruler).

DNA Gel Elution of PCR Product

All the desired size bands of DNA A and associated betasatellites from the agarose gel were excised and elute through Silica Bead DNA Gel Extraction Kit. The eluted products i.e., gene clean were then analyzed and compared with standard 1kb marker on 1% agarose gel. Bands were exactly the same size as were observed in PCR amplification on 1% agarose gel.

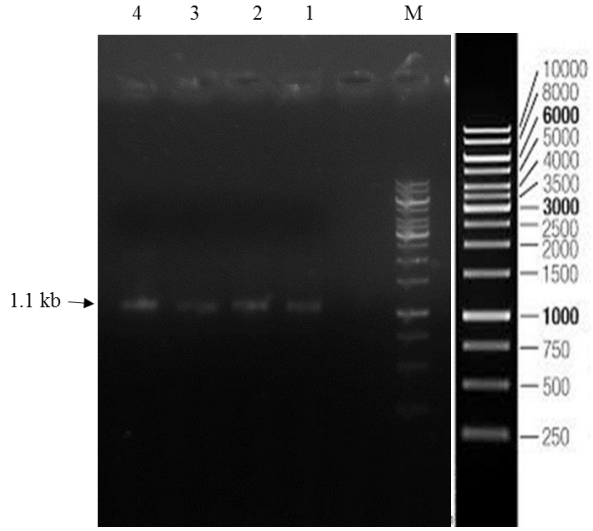


Figure 9. Agarose (1%) gel Electrophoresis of gene clean of amplified product from Hibiscus samples.

From right to left first ladder L (standard 1 kb gene ruler). Bands on lane-1, lane-2, lane-3 and lane 4 are gene clean of amplified product from *Hibiscus* samples as reference with L (standard 1 kb gene ruler).

Escherichia coli Transformation

The desired products after gene clean were ligated into PTZ57R/T vector. These ligated DNA was transformed into electro-competent cells of *E. coli DH5α* strain by electroporation method. These transformants were spread on LB agar medium and place at 37 ◦c for overnight growth. Blue and white colonies were observed on the agar plates shown in figure (12). White colonies were picked and inoculated. After about 16 hour's incubation, the plasmid DNA were isolated and to confirm clone, subjected to the endonuclease enzyme restriction digestion.

Confirmation of Transformants

To confirm transformants AY1 and AY2, first restrict plasmid with endonuclease restriction enzymes and analyze. If plasmid were restricted with endonuclease restriction enzymes then sequencing of the clone were performed and analyze.

Restriction Enzyme Digestion

Isolated plasmid were double digested with endonuclease restriction enzymes *Hin*dIII and *Eco*R1 and analyzed and compared with DNA marker on 1% agarose gel. For double digestion, two clear band were observed, one with desired product size and

other same as vector size shown in figure (14&15). Presence of these desired bands confirm the clone.

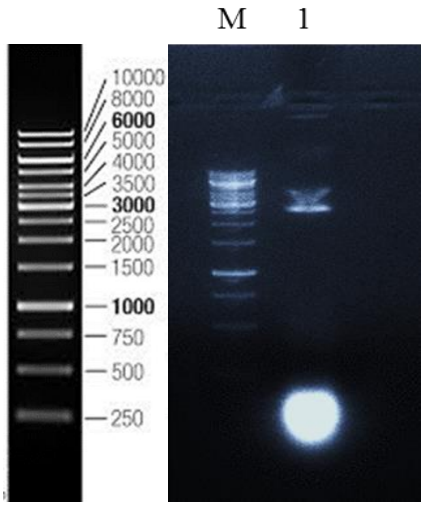


Figure 10. Agarose gel Electrophoresis of plasmid isolation of clone (AY1) from *Hibiscus* samples.

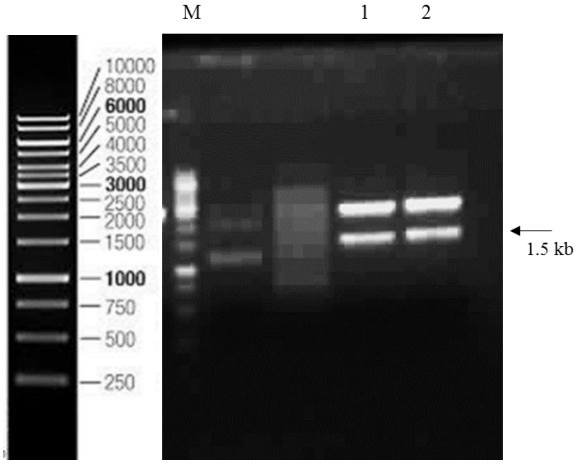


Figure 11. Agarose (1%) gel Electrophoresis of restricted DNA A (AY1) clone from *Hibiscus* samples.

From left to right first ladder M (standard 1 kb gene ruler). Bands on lane-1 and lane-2 are as a result of restricted DNA A (AY1) clone with *Eco*R1 and *Hind*III of 2.8 kb and 1.5 kb size as reference with M (standard 1 kb gene ruler).

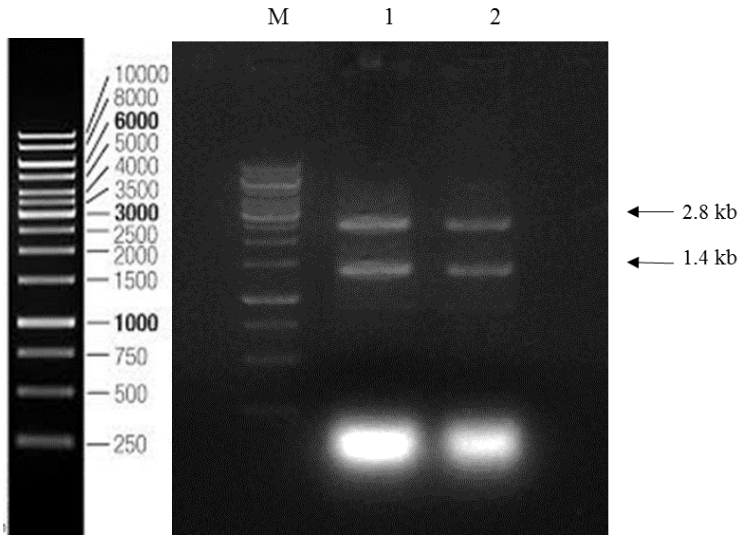


Figure 12. Agarose (1%) gel Electrophoresis of restricted Beta clone from *Hibiscus* samples.

From left to right first ladder M (standard 1 kb gene ruler). Bands on lane-1 and lane-2 are as a result of restricted desired beta clone with *Eco*R1 and *Hind*III of 2.8 kb and 1.4 kb size as reference with M (standard 1 kb gene ruler).

DNA Sequencing

To confirm transformants AY1 and AY2, sequencing were performed by dideoxy chain termination method (Sanger *et al.*, 1977) and get data in the contigs form. The nucleotide sequences were determine by assembling sequences data that present in contigs form. Sequences of both partial clone trimmed to remove vector sequences.

Sequence Analysis

Sequence analysis were performed by using NCBI BLASTn tool BLASTn used to find sequence identity and find sequences of viruses/satellites that resembled our clone sequences and complete sequence identity were detected. Retrieved various sequences that resembled to our isolate sequences from database NCBI. Sequence analysis showed partial sequence of DNA A had ≤ 96% identity with *Cotton leaf curl kokhran Virus-Burewala* and betasatellite had 93% identity with *Cotton leaf curl Multan Betasatellite*. Components AY1 and AY2 are given below in figure.

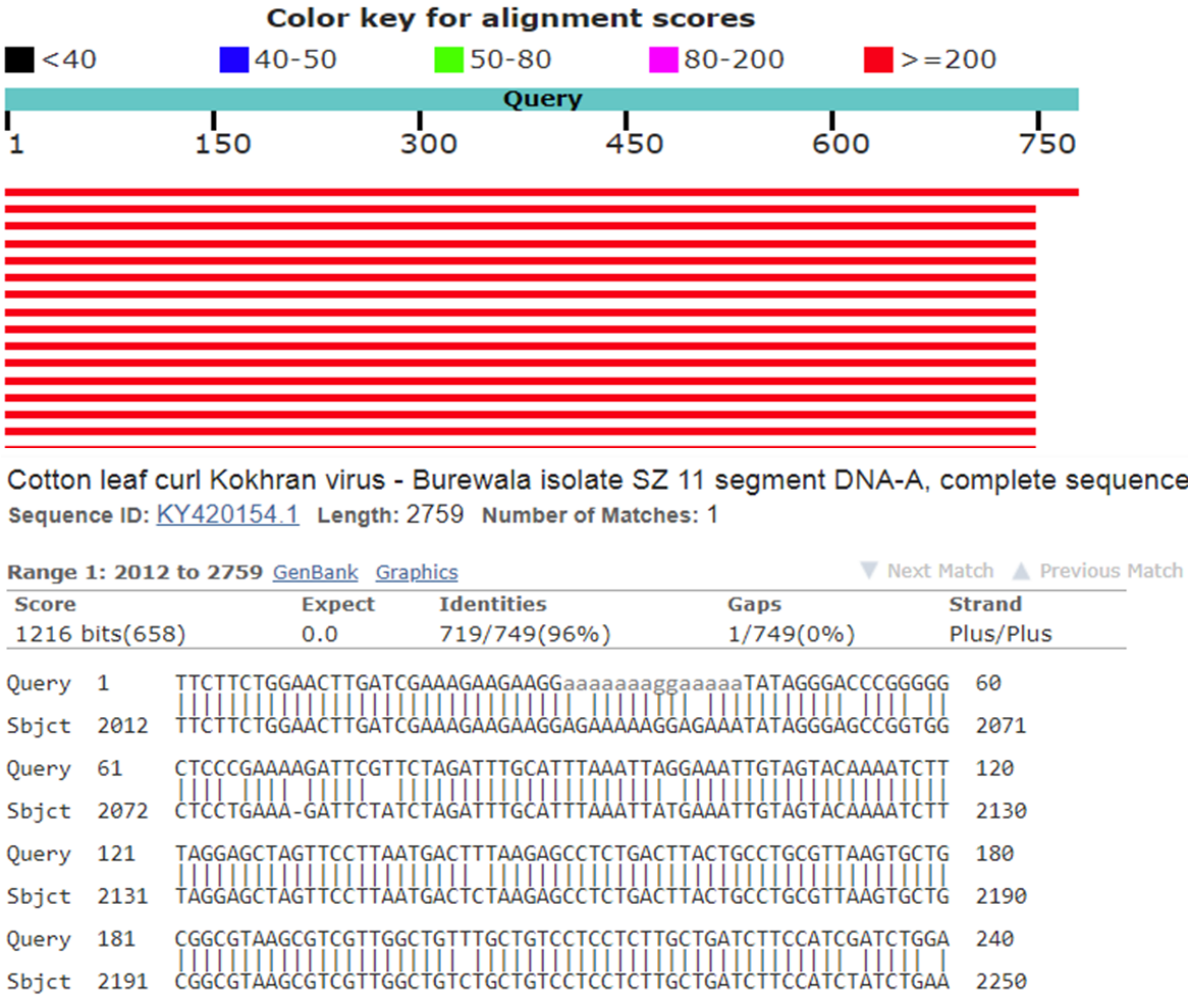


Figure 13. BLAST sequence of *Hibiscus rosa sinensis* AY1 isolate. 96% identity was found with *Cotton leaf curl Kokhran virus-Burewala.*

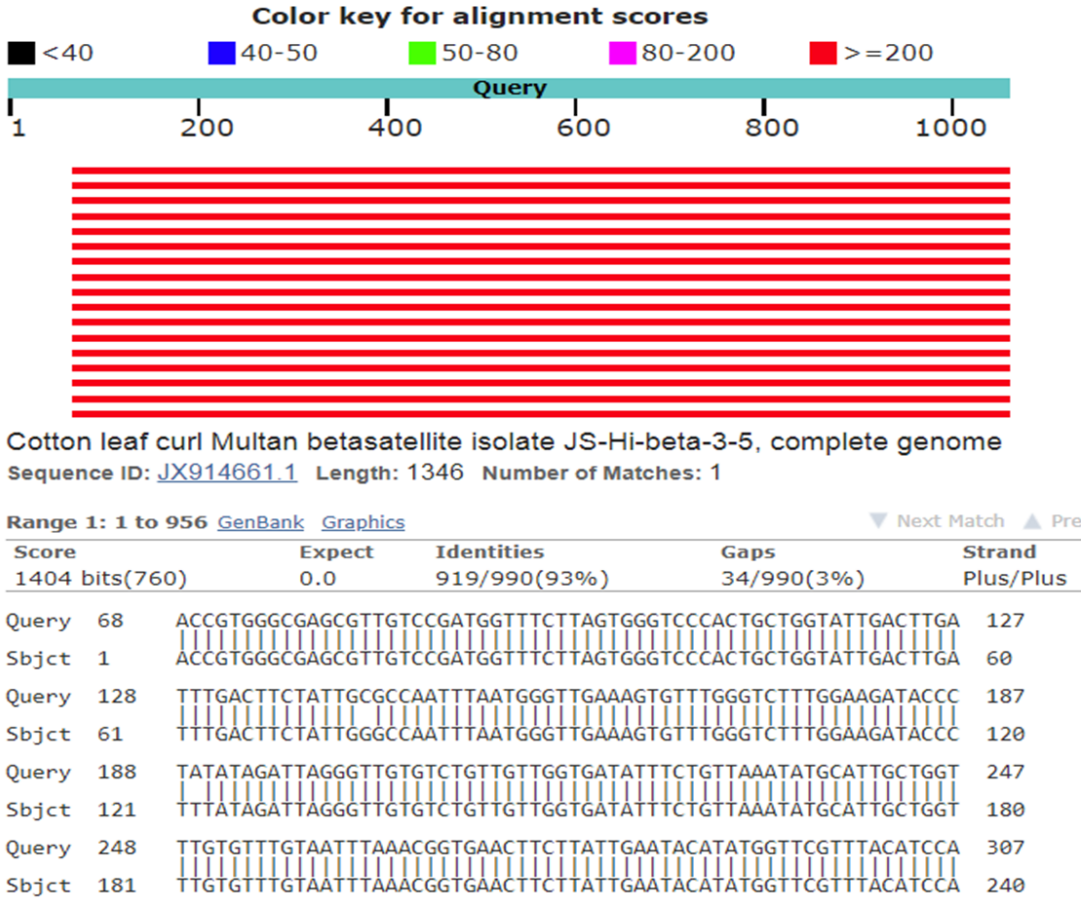


Figure 14. BLAST Sequence *Hibiscus species* AY2 isolate. 93% identity was found with *Cotton leaf curl Multan betasatellite*.

Genome Features

For genome features, open reading frames find by using ORF finder. Isolate of DNA A have C4 complete gene, V2, V1 with 3' incomplete and Rep with 5' incomplete region and have conserved region as CLCuKoV-Bu have while isolate of betasatellite have BC1 complete gene, A-rich region and SCR.

Table 4. Genome features of AY1 and AY2 isolates.

Genome Feature	IR/ SCR	Iterons	IRD	Nona-nucleotides	ORF	Start codon	Stop codon	Nucleotides	Seq identity	Amino acids	Seq identity	Complete/ Incomplete
DNA-A (AY1)	193	GGAGA	FKVQ	TAATATTAC	**C4**	2684	2138	546	98%	181	92%	Complete
					Rep	2595	2011	585	97%	194	96%	5’ Incomplete
					V2	126	453	327	97%	108	94%	Complete
					V1	275	467	192	97%	63	98%	3’ Incomplete
DNA-Beta (AY2)	83	-	-	TAATATTAC	**BC1**	550	194	357	100%	118	99%	Complete

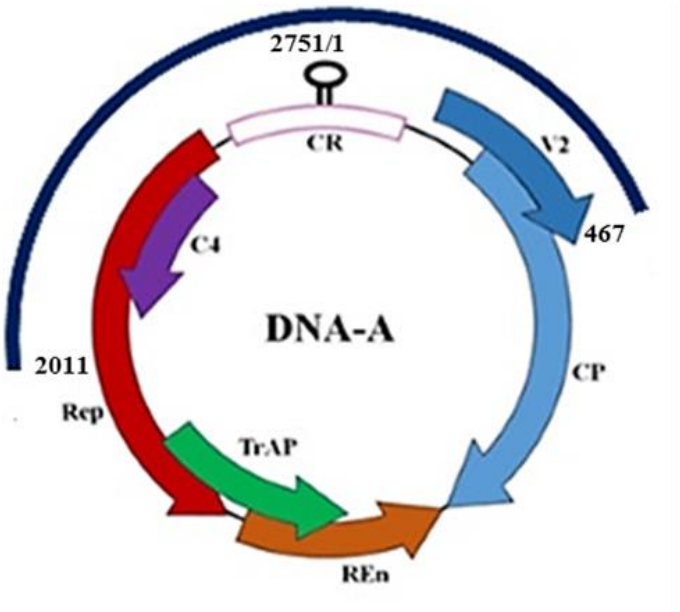


Figure 15. Genome map of DNA A isolate (AY1).

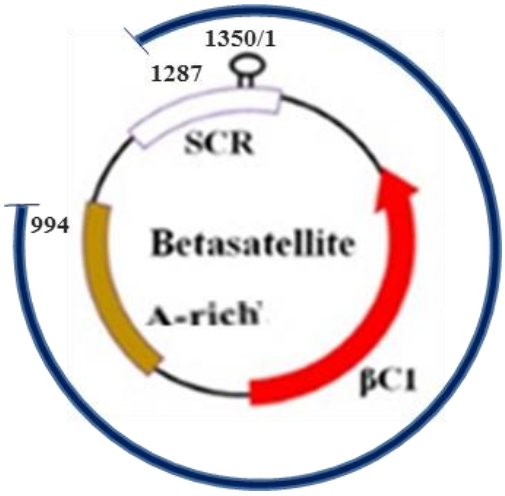


Figure 16. Genome map of betasattelite Isolate (AY2).

Phylogenetic Relationships of Isolates

MEGA 7 software used to construct phylograms of isolates to show the relationships with other similar sequences available in database NCBI. Multiple sequence alignment was performed by using MUSCLE alignment method.

Genetic diversity of DNA A clone of Begomovirus (AY1)

Only one clone of Begomovirus DNA A (AY1) was isolated from *Hibiscus rosa sinensis* samples. Phylogenetic relationship of AY1 clone with similar sequences of begomoviruses retrieved from databases shown in figure.

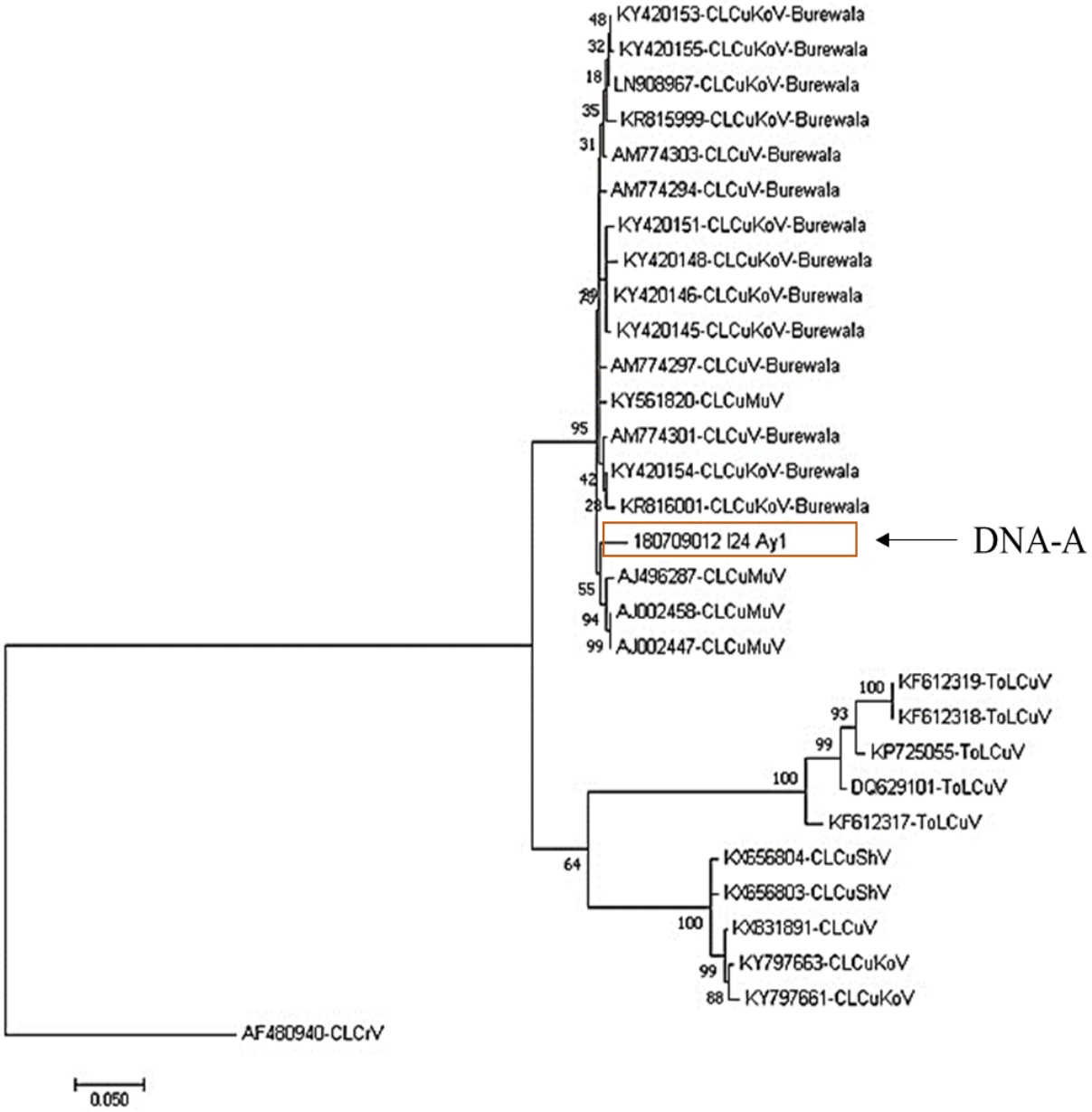


Figure 17. Phylogram of partial DNA A clone of begomovirus (AY1).

Phylogram of DNA A partial clone showed identity with CLCuKoV-Bu and CLCuMuV and found with CLCuMuV in clade because it's a partial clone of

CLCuKoV-Bu which is recombinant of CLCuMuV and CLCuKoV shown in figure 18.

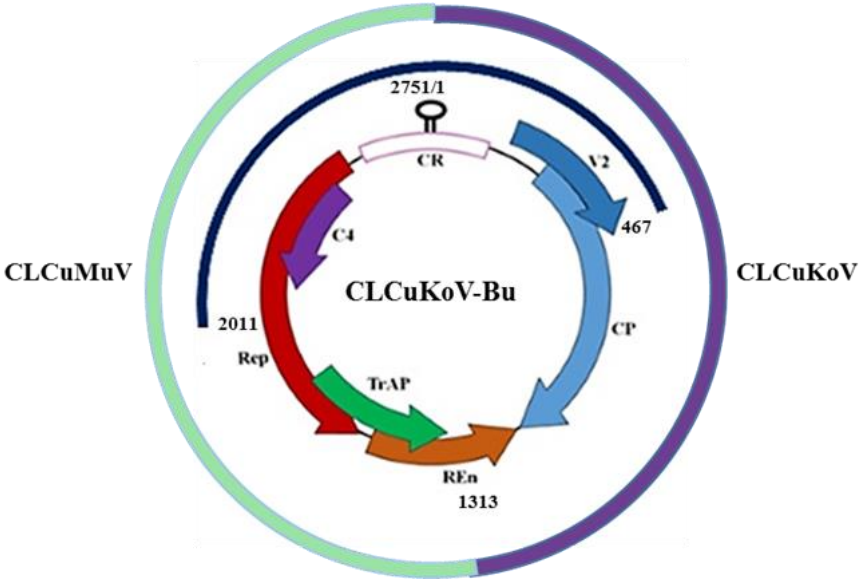


Figure 18. Genome structure of recombinant CLCuKoV-Bu.

Genetic diversity of betasatellite clone (AY2)

One clone of betasatellite was isolated from *Hibiscus shizuki* samples. Phylogenetic relationship of betasatellite clone AY2 with similar sequences of betasatellite retrieved from databases shown in figure.

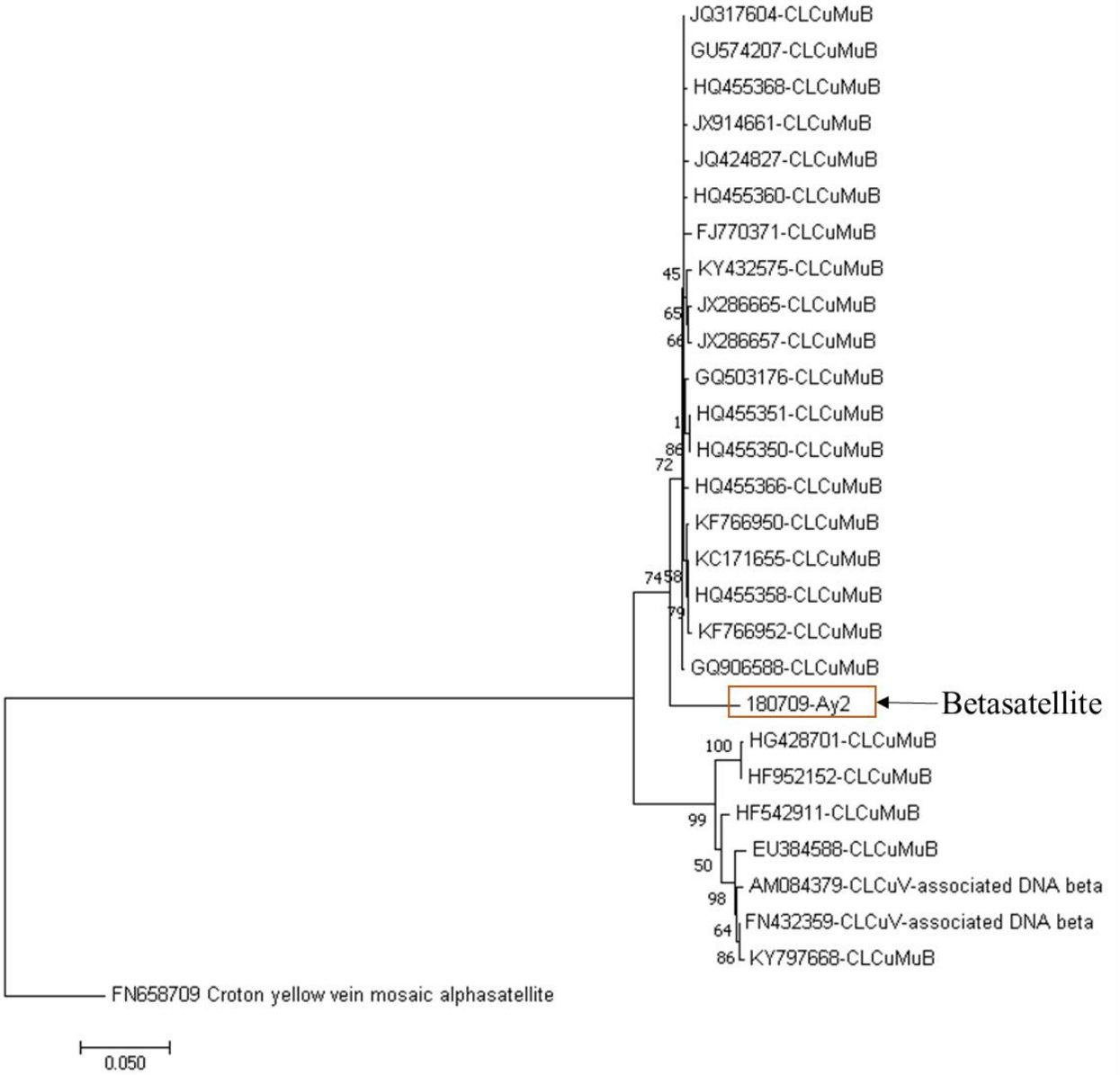


Figure 19. Phylogram of betasatellite clone (AY2).Phylogram of betasatellite partial sequence showed identity with CLCuMuB and form a clade with CLCuMuB.

CHAPTER 5: DISCUSSION

Phytopathogenic viruses' have extensive history and responsible for huge losses in agricultural productivity in regions throughout tropical and subtropical especially in developing countries that face more hardship and losses. There are a lot of factors involved that elucidation for losses and they contain the fact which describe the prevalence and plant viruses diversity for the importance of agriculture are maximum. Moreover environmental circumstances are more promising for virus transmitting vectors. Expenditures of chemical agents to control vectors cannot afford by farmers because of low or lack of budget along with lack of standard education and efficiently use training.

Geographical position of Pakistan along with other southern Asia countries serve as center of hub for all plant infecting taxonomic group of viruses that infecting all type of plants and take part in agricultural productivity reduction in country that hampers the economic prosperity (Ali *et al.*, 2004; Crawford *et al.*, 2006; Iram *et al.*, 2005; Mali & Rajegore, 1979; Mandal *et al.*, 2004; Naidu *et al.*, 1989; Raikhy *et al.*, 2003; Tahir *et al.*, 2010a; Verma *et al.*, 2004). Begomovirus is the prime virus among phytopathogen viruses of family *Geminiviridae* transmitted by whiteflies, *Bemisia tabaci* (Brown *et al.*, 2015; Monga *et al.*, 2011).

Agricultural country Pakistan depends on agriculture for its economy and crops have major role in Pakistan economy that reduces productivity due to plant viruses of family *Geminiviridae*. The main factor for cotton yield reduction is disease of cotton leaf curl complex in Pakistan that reduces production of cotton fiber leading to reduce in economy of Pakistan. Genus *Begomovirus*, are responsible main agents for CLCuD.

Begomoviruses are monopartite and bipartite. Monopartite begomoviruses have satellite molecules i.e. betasatellite and alphasatellite of size approximately 1.4kb. Begomoviruses genetic makeup have ability to mutate rapidly and undergo recombination (Monci *et al.*, 2002; Sanz *et al.*, 2000). These events of recombination and mutations lead to the new virulent strains emergence having the tendency for novel disease cause, wide host range to infect and overcome the barriers of resistance

(García-Andrés *et al.*, 2007; Zhang *et al.*, 2010). In Pakistan, it's a big challenge to protect cotton and begomovirus complex to control after 2001 resistance breakdown is a major concern(Mansoor *et al.*, 1999). With the emergence of *Cotton Leaf Curl Kokhran virus-Burewala* strain (CLCuKoV-Bur), a new virus recombinant, the varieties of conventional resistant become susceptible to viral disease especially CLCuD.

In order to discover and handle any novel and new mutations and recombination's, it's highly needs to be featured begomoviruses periodically. The betasatellites diversity studies also be considered on a parallel basis with the same importance to viruses.

Present study focus on identification and cloning of begomoviruses and its satellites from ornamental plants. Samples of infected plants leaf were collected with begomovirus symptoms from Lahore and Islamabad. Total DNA extracted from samples, amplified begomovirus and associated satellite was cloned and then sequenced. After that sequence and phylogenetic analysis were performed.

One betasatellite were identified from *Hibiscus shizuki* samples belongs to CLCuMB and one DNA A were isolated from *Hibiscus rosa sinensis* samples had identity with CLCuKoV-Burewala.

Present research finding reveals that ornamental plants are alternate host of begomoviruses and they harbor different viruses. Ornamental plants play role in the evolution and emergence of new viruses by different species recombination. To control CLCuD, suggests viral host ornamental plants especially *Hibiscus* plants should be avoided to grow in cotton areas.

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