Isolation and Characterization of Begomovirus(es) from *Cestrum nocturnum*.

BY

Javeria Khan

MS (Plant Biotechnology)

NUST201260345MASAB91012F

Supervisor

Dr. Muhammad Tahir

Assistant Professor/HoD Plant Biotechnology

Atta-ur-Rahman School of Applied Biosciences (ASAB),

National University of Sciences & Technology (NUST),

Islamabad, Pakistan. 2014

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"This thesis is dedicated to my parents, without their faith and selfless affection, none of this would have been possible, friends who have supported and encouraged me my entire life and my supervisor who has been a constant source of knowledge and inspiration.

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Abbreviations:

ACMBFV	*African cassava mosaic Burkino Faso virus*
ACMC	*African cassava mosaic virus*
AEV	*Ageratum Enation virus*
A-rich	Adenine-rich
AYVV	*Ageratum Yellow Vein virus*
AYVV-Gx	*Ageratum yellow vein virus-Guangxi*
AYVV-P	*Ageratum Yellow vein virus-Pakistan*
C^o	Centigrade
CCSV	*Cotton Chlorotic spot virus*
ChLCB	*Chili leaf curl beta satellite*
CLCuD	Cotton leaf curl disease
CLCuKV	*Cotton leaf curl Kokran virus*
CLCuMV	*Cotton leaf curl Multan virus*
CLCuShV	*Cotton leaf curl Shahadpur virus*
CP	Coat Protein
CTAB	Cetyl tri ammonium bromide
EACMV	*East African Cassava mosaic virus*
EDTA	Ethy lene diamine tetraacetic acid

EuLCuV	*Euphorbia leaf curl virus*
HYVMV	*Hollyhock Yellow vein mosaic virus*
IR	Intergenic region
LB	Lauria Broth
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
ToLCKeV	*Tomato leaf curl Kerala virus*
ORF	Open reading frame
PaLCuV	*Papaya leaf curl virus*
PCR	Polymerase Chain reaction
PedLCV	*Pedilanthus leaf curl virus*
PepLCLV	*Pepper leaf curl virus*
RaLCuV	*Raddish leaf curl virus*
RCA	Rolling circle amplifictaion
REn	*Replication enhancer protein*
Rep	*Replication associated protein*
RhYMYuV	*Rhynchosia yellow mosaic Yuctan virus*
Rpm	revolutions per minute
SCR	Satellite Conserved region

SLCCNV	*Squash leaf curl China virus*
SPLCV	*Sweet potato leaf curl virus*
ssDNA	Single stranded DNA
TbCSV	*Tobacco curly shoot virus*
ToLCBDV	*Tomato leaf curl Bangladesh virus*
ToLCJaB	*Tomato leaf curl Java betasatellite*
ToLCJoV	*Tomato leaf curl Jodhepur virus*
ToLCKeV	*Tomato leaf curl Kerala virus*
TYLCV	*Tomato yellow leaf curl virus*
w/v	Weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-(galactopyranosidase)
ZLCV	*Zinnia leaf curl virus*
α	alpha
β	Beta
µM	micromolar
(NH4)2SO4	Ammonium Sulphate
%	Percent
µg	micro gram

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Abstract

Night blooming jasmine (*Cestrum nocturnum*; family *Solanacea*) is an evergreen ornamental plant, grown widely across the tropical and subtropical regions of the world. Severe leaf curling and mild yellowing symptoms were observed on the leaves of night blooming jasmine. Leaves from symptomatic plants and non-symptomatic plants were collected from Shifa International hospital (Isolate CN-146) and National University of Sciences and Technology (NUST; Isolate CS-152), around Islamabad, Pakistan during 2013. Their total DNA was extracted using Cetyl Trimethyl Ammonium Bromide (CTAB) method. The presence of a begomovirus was confirmed with diagnostic PCR using diagnostic pair of primers: CPF/CPR (for the amplification of coat protein gene of begomovirus) and NSPF/NSPR (for the amplification of Nuclear Shuttle Protein gene). The products of expected sizes: (approx. 750bp) for CP and (approx… 900bp) for NSP was obtained from symptomatic samples of both isolates. There was no amplification from non-symptomatic samples. Full length amplification products were obtained using abutting primer pairs; SonAF/SonAR and KTBF/KTBR, for the amplification of DNA A and DNA B, respectively. The expected size products of (approx. 2800bp) were obtained from both the samples. The products were T/A cloned and potential clones were sequenced in both orientation. The complete nucleotide sequence for DNA A of isolate CN-146 and isolate CS-152 were determined to be 2755 bp and 2757bp, respectively in length. The isolate CS-152 and the isolate CN-146 showed 84.8 nucleotide sequence identity between them while highest nucleotide sequence identity (89% and 88.9, respectively) to *Pedilanthus leaf curl virus* (accession no. AM948961), indicating to be new species of begomovirus for which we proposed the names "Cestrum leaf curl virus (for isolate CN-146) and Cestrum leaf curl Islamabad virus (for isolates CS-152)". The complete nucleotide sequences of DNA B, from isolate CS152 and the isolate CN-146 were determined to be of 2694 and 2692bp, respectively and showed highest nucleotide sequence identity (99%) with *Tomato leaf curl New Delhi Virus* DNA B (accession no.AM 849547). Since night blooming jasmine is widely grown as an ornamental plant in Pakistan, the presence of two different begomoviruses in the same host plants within short distance is a serious threat to other plants and to agriculture economy. This indicates that night blooming jasmine is a good host for begomoviruses. This is the first report of Cestrum leaf curl virus and Cestrum leaf curl Islamabad virus affecting night blooming jasmine.

1 INTRODUCTION

1.1 Geminiviruses

Geminiviruses (family *Geminiviradae*) are economically important group of phytopathogenic viruses, encapsidaing a small, circular single stranded DNA genome of approx. 2.5-3.0 kb in length as shown in Fig.1.1 (Moffat, 1999). Geminiviruses have been seen to infect both monocots and dicot plants (Gutierrez, 2000). The name *Geminiviradae* has been derived from the zodiac sign, Gemini, meaning twins (Harrison *et al.*, 1977).

Geminiviruses cause devastating losses to many economically important plant species. Diseases like beet curly top virus, maize streak virus and cassava mosaic virus were identified as serious threats to crop production century ago, but now the situation has aggravated and Geminiviruses still continue to cause worldwide agriculture problems. Many epidemics due to newly emerging Geminiviruses have occurred and have affected cotton, tomato, grain legumes and many other important crop plants. Economic losses observed due to Geminivirus infection are estimated to be US 1300 to US 2300 million dollars for cassava in Africa (Thresh and Cooter, 2005), losses to cotton were estimated to be US 5 billion dollar observed in Pakistan (Briddon & Markham, 2000), while for legumes, losses observed were of US 300 million dollars (Varma and Malathi, 2003).

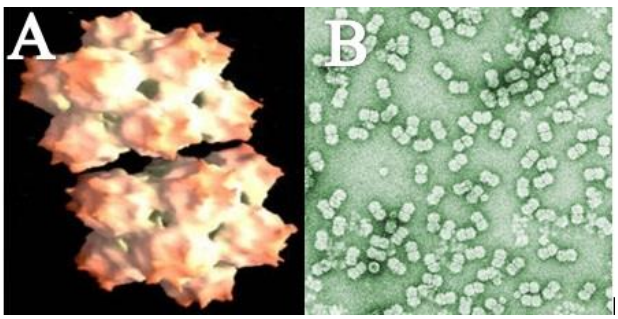

Figure 1.1 Structure of a typical Geminivirus: A. Computer generated model. B. Scanning Electron Micrograph.

1.2 Classification of family *Geminiviridae*

The family *Geminiviridae* is divided into seven genera based upon the genome organization, type of insect vector and host range; *Becurtoviruses, Eragroviruses, Turncurtoviruses, Mastreviruses, Curtoviruses, Topocuviruses and Begomoviruses* as shown in Table 1.1. (Adam *et al.*, 2013).

Table 1 .1 Seven genera of family *Geminiviridae,* based upon genome organization, type of insect vector and host range.

Genera	Type Specie	Genomic Organization	Geminivirus Host Range	Insect Vector
Becurtovirus	Beet curly top Iran virus	Monopartite	Monocots	Leaf hoppers and unknown vectors
Eragrovirus	Eragrostis curvula streak virus	Monopartite	Monocots	Leaf hoppers and unknown vectors
Turncurtovirus	Turnip curly top virus	Monopartite	Dicots	Leaf hoppers and unknown vectors
Topocuvirus	Tomato pseudo-curly top virus	Monopartite	Dicots	Tree hoppers
Curtovirus	Beet curly top virus	Monopartite	Dicots	Tree hoppers Leaf hoppers
Mastevirus	Maize streak virus	Monopartite	Monocots	Leaf hoppers
Begomovirus	Bean golden mosaic virus	Monopartite or Bipartite	Dicots	Whiteflies

1.3 Begomoviruses:

Begomovirus is the largest and the most important genus of the family *Geminiviridae.* Begomoviruses are ubiquitously transmitted by *Bamesi tabaci* as shown in Figure 1.2. Begomoviruses usually infect dicot plants in a circular non propagative manner (Fauquet *et al.*, 2003). During the last 30 years, begomoviruses have been seen to become a major threat to agriculturally important crops, vegetables and many ornamental plants (Brown *et al.*, 2012).

The symptoms caused by begomoviruses include leaf curl, yellow mosaic, vein yellowing, enation, leaf distortion, vein thickening, chlorosis, leaf crumple and stunting of plants. Some begomovirus symptoms are shown in Fig. 1.3.

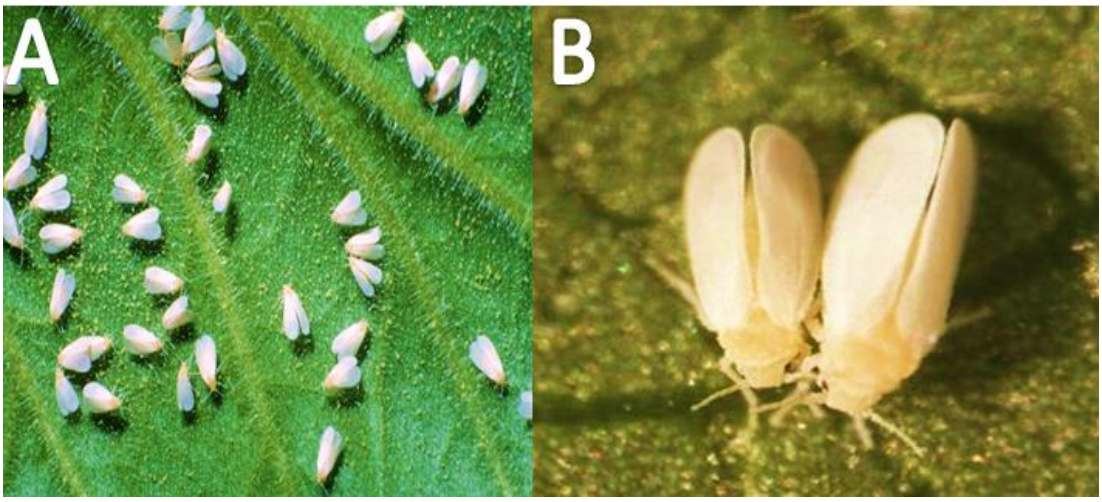

Fig.1.2 Begomovirus vector A. Whiteflies present on plant leaf. B. Whiteflies transmitting begomovirus.

1.4 Genome Organization of Begomoviruses

Begomoviruses can be divided into two subtypes; either containing a monopartite genome (having a single genomic component) or a bipartite genome (containing two genomic components as shown in Fig. 1.4. (Seal *et al.*, 2006).

1.4.1 Bipartite Begomoviruses:

Some begomoviruses contain two genomic components which are separately encapsidated; DNA A and DNA B (Brown *et al.*, 2012). Each component is a circular single stranded DNA molecule with an approximate size of 2700 nucleotides. DNA A and DNA B have been seen to share an approx. 200 nucleotide region within the intergenic region, known as the ‘common region’. The common region contains a conserved stem-loop structure and a highly conserved nonanucleotide sequence, TAATATT/AC.

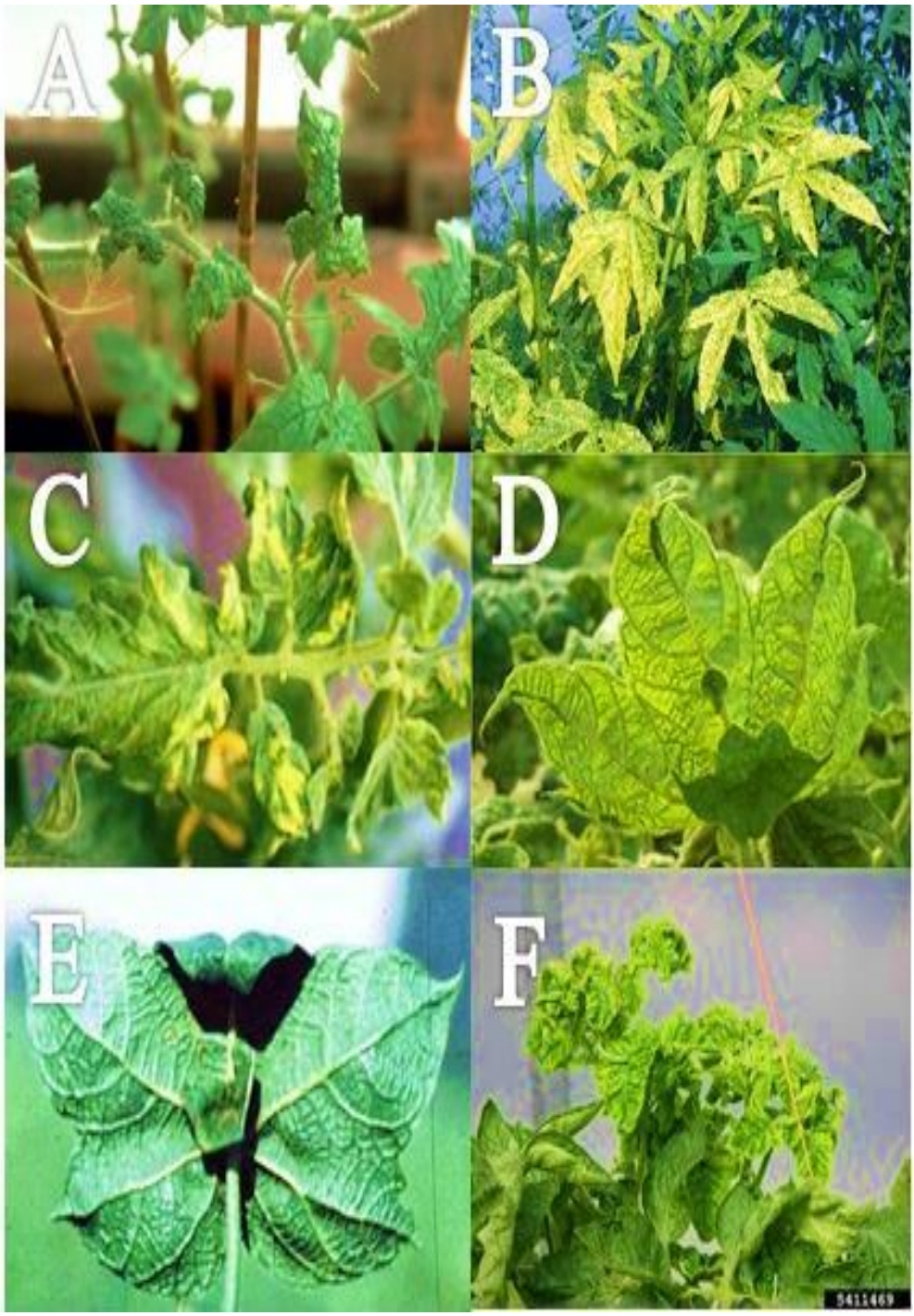

Fig.1.3. Typical symptoms caused by begomoviruses. A.Leaf curl B. Mosaic C. Leaf Yellowing D. Vein Thickening D. Enations F. Stunting

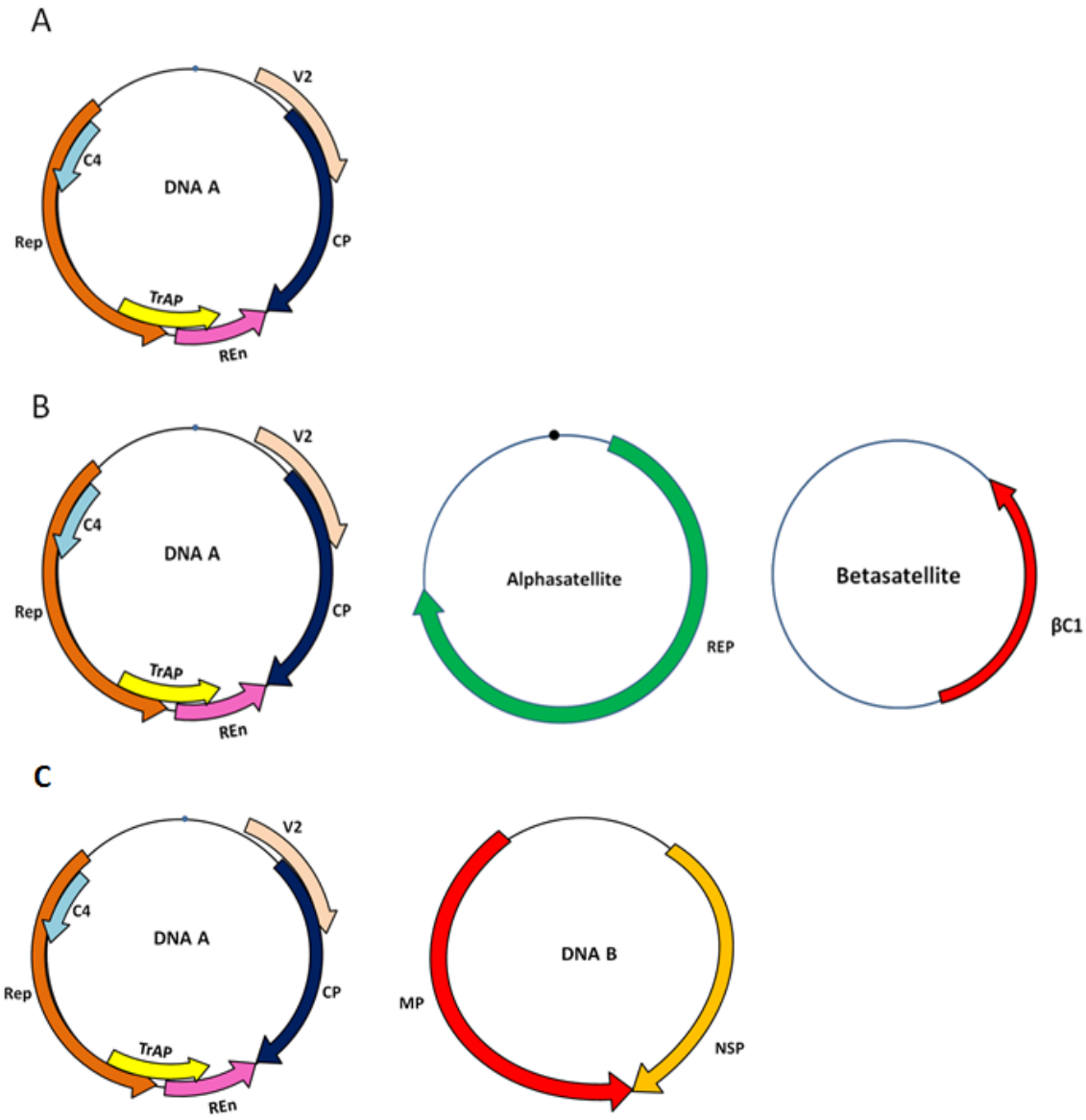

Fig.1.4.Genome organization of begomoviruseses. (A). Monopartite begomovirus DNAA. (B). Monopartite begomovirus alongwith betasatellite and alpha satellite molecule. C. Bipartite begomovirus; DNA A and DNA B.

The DNA A encodes for all proteins that are required for the viral DNA replication, control of gene expression, encapsidation and overcoming host defenses. While DNA B encodes for two proteins required for movement and symptom expression.

Table 1.2 shows the genes encoded by DNA A and DNA B along with the viral functions they perform.

1.4.2 Monopartite Begomoviruses:

There are majority of begomoviruses which are observed to be containing a single genomic component DNA A which performs all the viral functions. Monopartite begomoviruses may be associated with DNA satellite molecules; alphasatellites and betasatellites. For example: *Cotton leaf curl Multan virus* (CLCuMV) has been found associated with *Cotton leaf curl Multan betasatellite* (CLCuMB). While some monopartite begomoviruses, are not associated with satellite molecules. For example: *Tomato yellow leaf curl virus* (TYLCV) (Scholthof *et al.*, 2011).

1.4.3 Begomovirus Satellite molecules:

Monopartite begomoviruses may or may not be associated to satellite molecules known as alphasatellites and betasatellite. Recent studies have shown satellite molecules also associated with bipartite begomoviruses (Paprotka *et al.*, 2010).

1.4.3.1 Betasatellite molecules:

Betasatellite molecules are usually half the size of full length begomoviruses, approx. 1350 nucleotides in length. Betasatellites are found to be encoding a single gene known as ßC1 which is involved in symptom induction, suppression for post transcriptional gene silencing, movement and determination of host (Saunders *et al*., 2000; Briddon *et al*., 2001). ; Zhou *et al*., 2003). Betasatellite molecules contain an adenine rich region (A-rich) and a region conserved among all betasatellite molecules known as satellite conserved region (SCR). SCR is found near the hairpin loop structure, and is thought to interact with other begomoviruses for trans-replication, while the hairpin loop structure is thought to be involved

Table.1.2. Functions of begomoviral genes:

Protein	**Function**	**References**
Coat Protein (CP)	Ecapsidation, insect transmission and movement.	(Sharma & Ikegami, 2009; HanleyBowdoin *et al.*, 1999)
Pre-coat Protein (V2)	RNA Silencing suppressor, movement between the plant, Peri nuclear distribution of begomoviruses between Endoplasmic Reticulum and cytoplasmic strands.	(Rojas *et al.*, 2005; Sharma *et al.*, 2011; Sharma and Ikegami, 2010; Yadava *et al.*, 2010; Zrachya *et al.*, 2007)
Replication associated Protein (Rep)	Initiation of replication by producing a nick in the nonanucleotide sequence and it also recognizes iterons.	(Gopal *et al.*, 2007; Hussain *et al.*, 2007; Mubin *et al.*, 2010; Trinks *et al.*, 2005; Yang *et al.*, 2007)
Transcriptional activator protein (TrAP)	RNA silencing suppressor and is involved in overcoming virus induced hypersensitive cell death	(Gopal *et al.*, 2007; Hussain *et al.*, 2007; Mubin *et al.*, 2010; Trinks *et al.*, 2005; Yang *et al.*, 2007).
Replication enhancer protein (REn)	Facilitates virus replication.	(Pasumarthy *et al.*, 2011; Pedersen and Hanley-Bowdoin, 1994)
C4 protein (C4)	Pathogenecity determinant and suppressor for post transcriptional gene silencing (PTGS).	(Pandey*et al.*, 2009; Vanitharani *et al.*, 2004).
Nuclear Shuttle Protein (NSP)	Transfer of viral DNA from the nucleus into the cytoplasm.	(Malik *et al.*, 2005; Sanderfoot *et al.*, 1996).
Movement Protein (MP)	Movement of viral DNA across the plasmodesmatal boundaries, involved in viral pathogenic properties.	(Hussain *et al.*, 2007; Jeffrey *et al.*, 1996; Sanderfoot and Lazarowitz, 1996).

in the replication of virion strand and is the binding site for Rep protein (Replication associated protein) to produce a cut and initiate rolling circle amplification (RCA; Laufs *et al.*, 1995).

1.4.3.2 Alphasatellites:

Alpha satellites, previously known as DNA 1 are half the size (~1375 nts) of their helper begomovirus genome. They contain a single ORF which is found conserved in all alpha satellite molecules. This ORF codes for Replication initiator protein (Rep). Alpha satellites have always been found associated with monopartite begomoviruses (Idris *et al.*, 2011). But recently alpha satellite molecules have been found associated to bipartite begomoviruses (Paprotka *et al.*, 2010). Alpha satellite molecules are not involved in symptom induction like beta satellites. The function of alpha satellites is still unknown.

1.5 Begomoviruses and their importance in ornamental plants:

Ornamental plants are widely distributed across the world and have high levels of environment adaptability. Many reports suggest ornamental plants to be alternative hosts for the survival as well as for the spread of begomoviruses (Bedford *et al*., 1998). They are also considered as bases for new viruses and reservoirs for unidentified and economically important viruses which are often neglected during diversity studies. Ornamental plants have also been seen to act as reservoirs and alternative hosts for the survival of begomoviruses during off season.

Night blooming jasmine (*Cestrum nocturnum*; family Solanacea) is a well-known evergreen shrub, grown widely across the tropical and subtropical regions of the world. It is usually grown for its fragrance as an ornamental plant. *Pedilanthus leaf curl virus* is the only virus which has been isolated from night blooming jasmine uptill now. In the present study,

two distinct begomoviruses have been isolated from night blooming jasmine plant making night blooming jasmine an adaptive host for begomoviruses.

Other ornamental plants seen infected by begomoviruses include; *Lantana camara, Tecoma Stans, Justicia Adhatoda, J. sambac, M.hortensis, Vinca Rosea, Raphanus sativus, Calendula officinalis, Golden Duranta, chrysanthemum indicum* and *Croton*.

1.6 Aims of Study:

The main focus of research is the characterization of begomovirus(es) from ornamental plant night blooming jasmine (*C. Nocturnum).*

- Detection and cloning of begomovirus(es), if present.
- DNA sequencing and sequence analysis
- Phylogenetic analysis of characterized virus.

2 LITERATURE REVIEW

During the last 30 years, begomoviruses have turned into viral plant pathogens, evolving rapidly, and posing a major threat to crop yields for food, fiber and ornamental plants in tropical and subtropical regions of the world. A lot of work has been carried out on the isolation, characterization, diversity analysis and developing resistance strategies against begomoviruses. Some of the previously published work is stated as under:

George *et al.* (2014) collected symptomatic leaves showing curling, leaf crinkling and leaf distortion symptoms in Amaranthus, from areas in the vicinity of Rajasthan (India). Three full length clones were obtained and characterized; mono partite begomovirus, an associated beta satellite and an associated alpha satellite. Complete nucleotide sequence of begomovirus from Amaranthus showed highest levels of nucleotide sequence identity with Chili leaf curl virus, depicting it to be an isolate of chili leaf curl virus. Highest levels of nucleotide sequence identity with Tomato yellow leaf curl virus were seen for beta satellite sequence and for alpha satellite highest levels of nucleotide sequence identity were seen with Chili leaf curl alpha satellite. This was said to be the first report of a new host being infected by begomovirus and their associated beta and alpha satellites.

Hameed *et al.*, (2013) assessed the genetic diversity as well as the biotype status of Bamesia tabaci by using Random amplified polymorphic DNA polymerase chain reaction. The study showed non biotype B prevalent in the provinces of Sindh and Punjab, while B biotype was seen to be restricted to Sindh only.

Srivastava *et al.* (2013) investigated crape jasmine (Tabernaemontana coronaria) and night blooming jasmine (Cestrum nocturnum) with yellow mottling and leaf curl symptoms. DNA was amplified by using Rolling circle amplification. The two plants had 95 percent

sequence identity to each other while 93 percent nucleotide sequence identity with Pedilanthus leaf curl virus (PeLCV). It was reported as a new strain of PeLCV.

Hussain *et al.* (2013) isolated nucleotide sequences of alpha satellites being associated with monopartite begomoviruses from the plant Xanthim strumarium L. Samples were collected from Pakistan. Highest levels of nucleotide sequence identity (96.7 percent) for alpha satellite was seen with a Gossypium darwinii isolate. The second highest nucleotide sequence identity (85.8 percent) was seen with Papaya leaf curl alpha satellite isolate. Therefore, the name given was Gossypium darwanii symptomless alphasatellite isolate Xanthium. It was said to be a new isolate of Gosyypium darwani symptomless alpha satellite species.

Hina *et al.* (2012) screened different kinds of cotton plants to check for begomoviral infection. Viruses were amplified through PCR using specific primers, then cloned and sequenced. The study revealed Cotton Burewala Virus alone to be responsible for destroying the most important economic crop. Pairwise sequence comparison of isolates from this study showed 98.9 percent to 99.55 percent homogenity with cotton leaf curl Burewala virus. Among all, one of the virus isolates had lacked Rep region, which is always found in begomoviruses.

Tahir *et al.*, (2011) identified cotton leaf curl Gezira virus (CLCuGV) associated with cotton leaf curl disease in Africa, from infected cotton samples collected from Southern Punjab. All attempts failed in identifying Cotton leaf curl Gezira betasatellite. Two new beta satellites were seen to be associated with CLCuGV; Cotton leaf curl Multan beta satellite (CLCuMB) and Chilli leaf curl beta satellite (ChLCB). There was successful agro-inoculation of CLCuGV with CLCuMB and ChLCB in N. benthimiana. But typical enation symptoms were seen when there was a presence of CLCuMB.

Zulfiqar *et al.* (2011) collected leaf samples of Morning glory exhibiting yellow mosaic and vein yellowing symptoms from different provinces of China. The genomic component, DNA A was amplified from both the samples, one to be 2827 nucleotides while other to be 2801 nucleotides. They were named as JS-1 and Y338. JS-1 showed highest levels of nucleotide sequence identity, 97 percent with sweet potato leaf curl virus (SPLCV) while DNA A of Y338 showed highest levels of nucleotide sequence identity, 97.8 percent with SPLCV isolate from China. After phylogenetic analysis, JS-1 was crowded with SPLCV-US and y338 being crowded with SPLCV-CN, confirming them to be new strains of sweet potato leaf curl virus.

Tahir *et al.* (2010) collected leaf samples with yellow mosaic symptoms from Cucurbita pepo plants from Lahore. Both genomic components; DNA A and DNA B were cloned and then sequenced. DNA A showed 98.4 percent nucleotide identity, while DNA B showed 89.6 percent nucleotide identity to an Indian Squash Leaf Curl China Virus (SLCCNV) strain.

Tahir *et al.* (2010) were able to clone full length begomovirus with its associated beta satellite from capsicum species. Begomovirus isolated from capsicum was a new specie, thus given the name Pepper leaf curl Lahore virus (PepLCLV). It was thought to be a recombinant of Papaya Leaf curl virus and chili leaf curl virus. Associated beta satellite was confirmed to be Chili leaf curl beta satellite (ChLCB). It was said to be a cognate of chili leaf curl beta satellite, which was first seen to be infecting potato. PepLCLV is among the increasing monopartite begomoviruses, which are seen to be accompanying a betasattelite, and among the numerous species infecting capsicum. In consideration of only been recognized in Pakistan, PepLCLC and ChLCB, both signify a geographically new begomovirus betasatellite complex.

Tahir *et al.* (2010) in 2004 collected M. charantia leaf samples containing yellow vein symptoms, possibly begomoviral infection from areas in the vicinity of Lahore. Full length clones of begomoviruses were obtained from infected leaves and sequenced. DNA A nucleotide sequence, showed 86.9 percent nucleotide sequence identity to one of the isolates from Tomato Leaf curl New Delhi Virus (ToLCNDV), a distinct specie, thus given the name Bittergourd Yellow Vein Virus (BYVV). It was shown to have resulted from interspecific recombination between ToLCNDV and Tomato leaf curl Bangladesh virus (ToLCBDV). Nucleotide sequence identity of DNA B was 97.2 percent to an Indian strain of Squash leaf curl China Virus.

Amrao *et al.* (2010) cloned begomoviruses and their associated components from six samples in the areas of Sindh. Out of the six samples, one was seen as Cotton leaf curl Kokhran virus isolate. Sequence analysis of the other five clones showed nucleotide sequence identity to be 90 percent with begomoviruses seen to be involved in CLCuD. The names given to these isolates were Cotton leaf curl Shahadpur virus (CLCuShV). After reviewing CLCuShV in detail, it was seen to have arisen due to recombination events. Two samples showed the association of an alpha satellite, making them a newly discovered specie. All six samples were seen to be associated to beta satellites. Sequence analysis of two beta satellites showed them to be CLCuMB, both of these were recombinants. Result from this study suggests that begomovirus causing cotton leaf curl disease in Sindh is entirely different from the begomovirus responsible for causing cotton leaf curl disease in Punjab.

Herna´ndez-Zepeda *et al.* (2010) collected leaf samples from Rhynchosia minima exhibiting bright golden mosaic symptoms typical of begomoviruses from Yuctan. Both genomic components, DNA A and DNA B were cloned and sequenced. Nucleotide comparison of the two components showed highest levels of sequence identity, (87 percent for DNA A and 71 percent for DNA B) with cabbage leaf curl virus, thus distinct specie and

named as Rhynchosia yellow mosaic Yuctan virus (RhYMYuV). Phylogenetic analysis showed Squash leaf curl virus to be the closest relative of RhYMYuV. After taking a look at recombination analysis, it was seen that DNA A had arisen due to inter molecular recombination.

Mubin *et al.* (2009) studied Digera arvensis for possible begomovirus infection. Five samples infected of Begomoviruses and their associated beta satellites were sequenced and cloned. 98 percent sequence identity was seen with Cotton leaf curl Rajasthan virus. Two associated beta satellites were isolated, one confirmed to be the isolates of Ageratum yellow leaf curl virus while the other was found to be a recombinant consisting major part of Tobacco leaf curl beta satellite and minor part from Cotton leaf curl Multan betasatellite. They also isolated an alpha satellite. The associated alpha satellite was similar to the one which was previously isolated from potato. D. arvensis is a weed, and begomovirus isolated from weed shows weeds to be an excellent reservoir for begomoviruses and providing begomoviruses a platform for recombination events.

Haider *et al.* (2007) collected leaf samples from three different plants (Zinnia elegans, Solanum nugrum and Ageratum conyzoides) showing possible begomoviral infection. The samples were subjected to PCR using degenerate primers specific for Coat protein. The coat protein gene was amplified, cloned and sequenced. Multiple sequence alignment and phylogenetic tree were constructed. The isolates of coat protein were named as Zinnia leaf curl virus (ZLCV) and Ageratum yellow vein virus- Pakistan (AYVV-P). These viruses were seen to be close relatives of Tomato leaf curl virus-India, Indian cassava mosaic virus and cotton leaf curl virus. One isolate named as Solanum yellow leaf curl virus (SYLCV) was seen as a mechanically infectious strain of tomato leaf curl virus-India, while Zinnia leaf curl virus was seen to be a mechanically infectious strain of Ageratum yellow vein virus-Pakistan.

Tahir *et al.* (2006) collected symptomatic leaf samples from an ornamental plant (Duranta Repens; Common name Pigeon berry). After DNA extraction, samples were subjected to PCR amplification which later confirmed the presence of begomovirus infection. Both the genomic components were cloned and sequenced. DNA A showed highest levels of nucleotide sequence identity (91 percent) to Croton yellow mosaic virus segment A. DNA B showed highest levels of nucleotide sequence identity to Tomato leaf curl New Delhi virus segment B. This was the first report stating leaf curl infection due to bipartite begomovirus in Duranta repens.

Ogbe *et al.* (2006) conducted a survey, in which they visited 418 farms. 48 percent of the cassava from these farms was severely and semi severely infected, while 52 percent cassava showed mild symptoms. Of the total leaf samples, 1397 that were collected, symptoms were seen in 1106 samples. When the infected samples were subjected to PCR, 74.1 percent samples were infected with African cassava mosaic virus (ACMV), infection to EACMV was seen in 0.3 percent samples while 24.4 percent samples were seen to be containing mixed infection. ACMV was found majorly in single and mixed infection samples. Two new variants of ACMV were identified from fields. They also discovered ACMV and EACMV in Senna occidentalis (L.) and in a weed (Combertum confertum Lams). These were said to be two new natural hosts of these viruses.

Tahir and Haider (2005) collected infectious leaf samples showing yellow blotch symptoms in Bittergourd from the areas of Lahore. The presence of begomovirus was confirmed through PCR amplification by using primers specific for coat protein sequence. The amplified product was cloned and then sequenced. Nucleotide sequence identity of 95 percent was seen with Tomato leaf curl New Delhi virus (ToLCNDV) revealing it to be a new strain ToLCNDV found for the first time in bittergourd.

Hameed *et al.*, (2004) tested monoclonal antibodies prepared against African Cassava mosaic virus and Okra leaf curl virus against begomovirus infected plants in Pakistan. Epitope profiles and phylogentic analysis showed that the mungbean samples obtained from Pakistan containing begomoviral infection were the isolates of Mungbean mosaic India virus. This represents a distinct lineage of begomoviruses.

Briddon *et al.* (2004) studied DNA 1 components which were thought to be similar to satellite like molecules, single stranded and are found associated to begomoviruses as a helper component to induce symptoms in the host. These satellite like molecules were seen to be causing different diseases in association to begomoviruses such as cotton leaf curl disease, okra leaf curl disease from Pakistan and Ageratum yellow vein disease from Singapore. They cloned and sequenced 17 different DNA 1 molecules which were associated with majority of begomovirus- DNA complexes. The sequence showed a conserved and a common organization of a single ORF in virion sense, an adenine rich sequence and a predicted hairpin structure. Phylogenetic analysis showed the grouping of DNA 1 molecules according to geographic origin other than host plant origin. The function and origin of these DNA-1 molecules were discussed.

Briddon *et al.* (2003) DNA-Beta is a symptom inducing, single stranded molecule that is found to be associated with monopartite begomoviruses. These satellite molecules have been seen to be inducing symptoms in infected Ageratum yellow vein virus from Singapore and cotton leaf curl Multan virus from Pakistan. 26 samples of beta satellites were isolated, cloned and sequenced. Sequence analysis showed high levels of conservation for a single open reading frame, for an adenine rich region and for a satellite conserved region (SCR). This was the region containing the hairpin loop sequence. Phylogenetic analysis revealed two groups of satellite molecules, one that had originated from Malvaceae, and the other group of plants that had originated from Solanaceae and Compositae. DNA molecules

showed close connections between both the host and geographic origin. This study showed the adaptation of begomoviruses with their associated satellite molecules.

Mansoor *et al*. (2003) studied cotton leaf curl Multan virus and its associated novel DNA satellite molecule designated as CLCuD DNA b. The satellite molecule is replicated in trans by cotton leaf curl Multan virus and is responsible for inducing symptoms in the susceptible plants. DNA b does not have an iteron sequence. Keeping diversity of begomoviruses in mind, it was seen that this sole satellite molecule was found associated with different begomoviruses.

Briddon *et al*. (2001) infectious clones of monopartite begomoviruses which were responsible for disease in cotton failed to produce any disease symptoms in host plants. Later on a single stranded DNA molecule was discovered which was of 1350 nucleotides. When monopartite begomovirus along with this single stranded DNA molecule were co inoculated in the host plant, possible disease symptoms were observed. The symptoms seen were leaf curling, vein yellowing, and vein darkening. This helper begomovirus molecule was termed as DNA b, which has been seen to be important for begomovirus replication and encapsidation. The same complex was later on discovered in Ageratum conyzoides which depicted this complex to be a new discovery of disease causing agent.

Briddon and Markham (2000) Cotton is Pakistan's cash crop and 60 percent of the foreign exchange earnings depend on cotton. Cotton leaf curl disease was initially reported in Punjab in 1967, later in 1985. By 1990, cotton leaf curl disease became a major epidemic in Pakistan. It has now spread to the surrounding regions such as West and South of Pakistan. Reports of cotton leaf curl disease were also found from some regions of India. Symptoms observed of cotton leaf curl disease were vein darkening, swelling, a leaf like out-growth at the underside of the leaf. It was seen that cotton leaf curl disease was spread due to a single

species of white fly known as Bamesia tabaci. The virus was ascribed to be begomovirus from the family geminivirus. But later in 1999, after studying the etiology of the disease it was seen to be much more complex than presumed.

3 MATERIALS AND METHODS

3.1 Collection of Samples:

Night blooming jasmine (*Cestrum Nocturnum*) leaf samples exhibiting severe leaf curling and yellowing (typical symptoms of begomoviruses) were collected from two different areas of Islamabad, Pakistan in 2013. The samples were washed, cleaned, labeled as CS-152 and CN-146 and stored at -20° C for further processing.

3.2 DNA Extraction:

The (Doyle, 1990) protocol was used to extract DNA from both the infected plant samples.1 gram of infected leaf tissue sample was taken in a mortar and was finely grounded with the help of a pestle using liquid nitrogen. This fine ground powder was later on added in warm CTAB isolation buffer (2% Cetyl trimethyl ammonium bromide, 100mM Tris HCl, 1.4 M NaCl, 20 mM EDTA, 0.2% mercaptoethanol with a pH8) which was kept at 60°C in a water bath. The finely ground powder was added in a 50ml blue capped falcon tube and a total of 25ml volume was achieved by adding CTAB isolation buffer.

The mixture was then incubated in a shaker for half an hour at 60°C. The mixture was then permitted to cool at optimum temperature for 3 minutes under a fume hood. The ratio 24:1 of Chloroform: Isoamyl alcohol was added in the mixture and a total of 50ml volume was achieved. The resultant slurry was then mixed and divided into two 50 ml falcon tubes. Both the falcon tubes were then subjected to centrifugation (Eppendorf centrifuge 5804 R) for fifteen minutes at 12000 rcf, 4^{o}C in order to separate phases. The upper aqueous phase was separated carefully and was transferred to another 50ml tube. Equal amounts of ice cold isopropanol (~20ml) were added to the supernatant. The tube was incubated for 16 hours,

overnight at optimum temperature and centrifuged the next day at 12000 rcf for 15 minutes. The supernatant was then removed and the pellet was washed with 1ml cold washing buffer (76% ethanol, 10mM ammonium acetate).

The pellet after washing was shifted to a 1.5 ml eppendorf tube and kept at room temperature for 20 minutes. It was later subjected to centrifugation for 5 minutes at 5976xg. The supernatant was decant and the pellet was allowed to dry for 30 to 60 minutes in a 37°C incubator. After being dried, resuspension of the pellet in 1ml TE buffer was performed (0.1 M Tris HCl, 0.001M EDTA) (Doyle JJ & Doyle JL, 1990). The total DNA solution was then poured into a 1.5ml sterile eppendorf tube and stored in -20°C freezer for further use.

3.3 Analysis of DNA:

Analysis of DNA was performed using Agarose gel electrophoresis. The total extracted DNA was analyzed on 1% agarose gel containing 40ml of 1X TAE (0.001M EDTA, 0.025M Tris and 0.005M Glacial Acetic acid with a pH of 8) was used to dissolve 0.4 grams of agarose, which was later heated in a microwave oven for 1-2 minutes. Finally a transparent solution was attained. It was permitted to cool and 0.01% of ethidium bromide was further added. The solution was then poured in a gel casting tray. A gel comb was selected and placed in the tray. After solidification, the comb was taken out and the gel was set into an electrophoresis tank which contained TAE buffer. The DNA samples for visualization were mixed in a 10X loading buffer containing 0.4% xylene cyanol FF, 25% Ficol and 0.4% bromophenol blue. The DNA samples were then loaded on the gel along with a DNA ladder (Fermentas 1 kb). The electrophoresis tank was connected to current and the gel was run at 90 volts for 30 minutes. The DNA bands were observed in the gel through Dolphin-Doc Plus Image System.

3.4 Amplification through Polymerase Chain Reaction:

The total PCR reaction volume 50µl consisted of 1X Taq Buffer $(NH_4)\ 2SO_4$, 0.2M dNTP's, 0.0015M $MgCl_2$, 100ng of total DNA, 2pM primers (SONA F/R; Tahir *et al.*, 2010), KTB F/R) and 0.5 units of Taq Polymerase. Dual block PCR machine (ESCO, Swift. Max.Pro) was used to place the PCR reaction so that amplification of a specific region could take place. Fig 3.1 and Fig. 3.2 shows the PCR conditions that were used for the amplification of begomovirus. Total number of cycles used were 30.

Table.3.1. Sequence of primers used for begomovirus amplification:

Primers	Sequences
SONA F	GGGCCCCCATGAACTCTTTAAAGTG
SONA R	GGGCCCAAAGGGACTGGCAATC
KTB F	CTGCAGAGGTCACCTTGTCATTTCCTTC
KTB R	CTGCAGCATCATTTGTGAGCGCATATTC

PCR Conditions for DNA A:

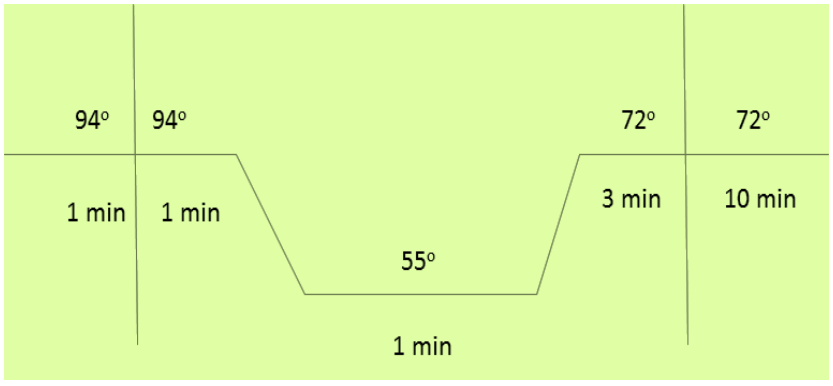

Fig.3.1 PCR Conditions for the amplification of DNA A.

PCR Conditions DNA B:

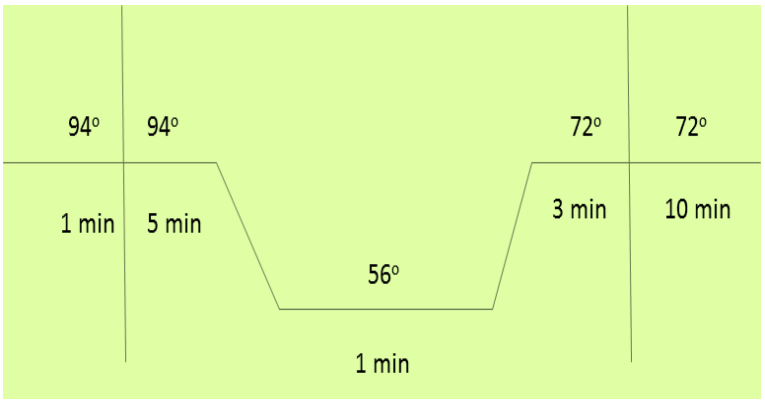

Fig.3.2. PCR Conditions for the amplification of DNA B.

3.5 Gel Elution of PCR Product:

The DNA was removed from gel by Silica beads DNA Gel Extraction Kit. PCR product that was amplified was cut out of the gel by using a clean blade and was then transferred into a 1.5ml eppendorf tube. 1ml binding buffer added to Eppendorf tube and was mixed thoroughly. It was then incubated for five minutes at 55 °C. After incubation, the mixture was centrifuged on 16162g for 30 seconds. After discarding supernatant, cold washing silica buffer approx. 500 µl was added and pellet was resuspended. Once again, it was subjected to centrifugation for 1 minute at 16162g and this step was again reiterated. 20 µl of 1X TE buffer was then added to the pellet with five minutes incubation at 55 °C. After 5 minutes, it was centrifuged for a minute at 16162g, after this supernatant was removed very carefully and shifted into a fresh tube for ligation to a vector.

3.6 T/A Cloning into pTZ57R/T Vector:

DNA purified by Silica beads gel extraction method was ligated to a pTZ57R/T cloning vector rendering to the protocol delivered by the company (InsTAclone^{TM} PCR Cloning Kit, Fermentas).

Table.3.2. Reaction mixture for ligation into pTZ57R/T vector:

Components	Volume
pTZ57R/T Vector	3µl
5X Ligation Buffer	6µl
PCR Product	20µl
T4 DNA Ligase	1µ
Total Volume	30µl

The ligation mixture was kept overnight and incubated at 4^{o}C for transformation into DH5α cells the next day.

3.7 Preparation of Competent Cells:

10ml of Lauria Bertani Broth (LB Broth) (sodium and yeast extract 0.5% weight by volume, Tryptone; 1% weight by volume, autoclaved for 15 minutes at 121^{o}C), was inoculated with a single colony of *Escherichia Coli* (E.Coli) DH5α strain incubated at 37^{o}C in a shaker incubator overnight. 50ml of LB media was taken the next day and culture was refreshed. The refreshed culture was again incubated at 37^{o}C. The culture was kept at 37^{o}C until the absorbance was seen to have reached a level of 600nm.

The culture was ice cooled for 10 minutes and was transferred to 50ml falcon tube. After being cooled, centrifugation for 15minutes at 4000 rounds per minute at 4^{o}C was done. The supernatant was then removed and ice cold 10ml 0.05M $CaCl_2$ solution was used to resuspend cells. The cells for fifteen minutes were kept on ice and were then subjected to centrifugation for 15 minutes at 4000 rpm. Once again the supernatant was removed and resuspension of cells was done in cold 2ml 50mMl $CaCl_2$ solution. Aliquots were made, containing 50µl of competent cells and they were stored in freezer at -80°C.

3.8 DH5α Cells Transformation:

Transformation was performed using heat shock method. DH5α competent cells were taken from -80^{o}C and they were added to the overnight ligated mixture. The reaction mixture (ligated material and DH5α cells) was kept in ice, for almost half an hour, incubated at 42^{o}C for two minutes. It was again incubated on ice, but for 2 minutes this time. After incubation, 800µl of Lauria Broth media was added in mixture and incubation at 37 ^{o} C for 3-4 hours was done. LB media plates were taken, containing nutrient agar, ampicillin, 20 µl X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranosidase), 20 µl of IPTG (isopropyl-beta-D-thiogalactopyranoside) and the culture was spread on these plates. The plates were incubated in a 37^{o}C incubator overnight.

3.9 Screening and Selection of Clones:

3.9.1 Isolation of Plasmid DNA:

White colonies four each, designated as C1a, C2a, C3a and C4a, respectively inoculated from the culture plate were added in 4 different 50ml falcon tubes. 10ml of LB media was added along with 100µg of ampicillin. These four colonies were incubated at 37^{o}C in a shaker for 16 hours. After 16 hours, plasmid was isolated from these colonies by using Alkaline Lysis Method.

5ml of overnight kept culture was taken for plasmid preparation, followed by centrifugation at maximum speed for a minute. The supernatant was then removed and resuspension of pellet was done in 100 µl solution 1 (25mM Tris-HCl, 10mM EDTA with a pH8). Then solution II (0.2 N sodium hydroxide (NaOH) was added and tubes were inverted 5-10 times. 150 µl amount of solution III (28.5 ml H_2O, 6ml of 3M potassium acetate, 11.5 ml acetic acid) was further added. It was vortex so that all solutions get mixed and later

centrifugation at maximum speed for ten minutes. Carefully removal of supernatant was done and it was transferred into a new eppendorf. An addition of 1ml ice cold 100% ethanol was done to the supernatant and left for 1 hour at room temperature. It was then centrifuged at maximum speed for ten minutes. Supernatant was then discarded and washing of pellet was done by using 70% ethanol and centrifugation was done again for five minutes at maximum speed. Removal of supernatant was again done and pellet was dried at room temperature. RNase A water of about 40 µl was added and the DNA was resuspended. The presence of plasmid DNA was confirmed on 1% agarose gel.

3.9.2 Confirmation through Restriction Enzyme Digestion:

The presence of insert of suspected virus from night blooming jasmine (*Cestrum Nocturnum*) was checked by digestion with *EcoR*1, *Hind*III, *Pst*I, *Apa*I (Fermentas). A total of 30 µl volume was achieved in which there was 5U of enzyme, enzyme specific buffer and plasmid. The mixture was incubated for 3 to 5 hours at 37^{o}C and the digested product was visualised on 1% agarose gel.

3.10 DNA Sequencing:

Samples found positive, containing full length viral genome were selected. Their plasmids were extracted and sent for sequencing. The sequences of the primers required for sequencing were also sent along to Macrogen, Korea. The sequences of the primers required for sequencing are shown in Table 3.3.

3.10.1 Sequence Analysis:

Sequences were assembled using BLAST (Basic Local Alignment Search Tool) and analyzed. Phylogenetic trees were constructed using Clustal X and trees were printed using tree view.

Table.3.3. Sequencing primers for Full length begomovirus DNA A and DNA B.

M13F	GTAAAACGACGGCCAGT
M13R	GCGGATAACAATTTCACACAGG
1JK1F	TTGCATACCATTTGCGCAG
1JK1R	ATTTAGCTCCCTGAATG
2JK1F	TCTATTTTGATCCGTTCAGATC
3JK1F	TTCAAATTCCCTATAGCGGC
4JK1F	TTTGGCATATATTTGAAAACGC
1JK2F	TAACATACACCTAAAACCGTGAACG

4 RESULTS

4.1 Collection of Samples:

Leaf samples of an ornamental plant, *Cestrum Nocturnum*, commonly known as-night blooming jasmine, exhibiting leaf curl symptoms, typical of begomovirus, were collected from two different localities of Islamabad: National University of Sciences & Technology (NUST) - isolate CS-152; and near Shifa International hospital - isolate CN-146 during July, 2013. Apparently symptomless leaf samples were also collected for negative controls. Fig.4.1 shows symptomatic leaves of isolate CS-152, isolate CN-146 and apparently healthy leaves of *C. nocturnum.* Samples were cleaned with a piece of cloth and kept at -80^{o}C until further processing.

Fig.4.1 Night blooming jasmine (*C. Nocturnum*) leaves showing leaf curl symptoms.A)Isolate CS-152 B) Isolate CN-146 C) Apparently symptomless plant.

4.2 Isolation of DNA:

Total DNA was extracted from both type of samples, symptomless and infected leaves (see section 3.2). Extracted total DNA was run on 1% Agarose gel electrophoresis (see section 3.3.1) visualized and examined on UV Trans illuminator. Good quality of DNA was obtained when observed on UV trans illuminator as shown in Fig.4.2.

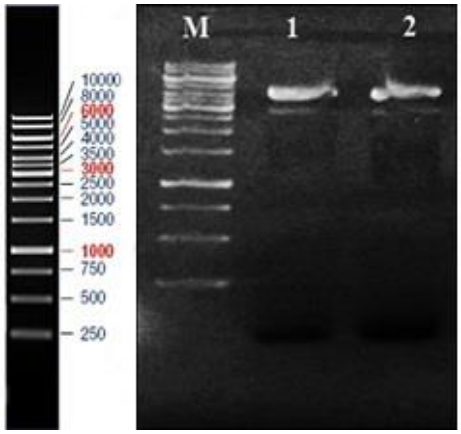

Fig.4.2. Agarose gel electrophoresis of total DNA isolated from night blooming jasmine (*C.Nocturnum*). Lane 1. Isolate CS-152, Lane 2. Isolate CN-146, Lane M standard 1kb ladder.

4.3 Detection of Begomovirus through PCR:

A product of approx. 2750 nucleotides, equaling to full length begomovirus DNA A, was observed in PCR by using abutting primer pair SONAF/SONAR (see section 3.4) from both samples (isolate CS-152 and isolate CN-146) as shown in Fig.4.3. No amplification was observed from symptomless leaf samples.

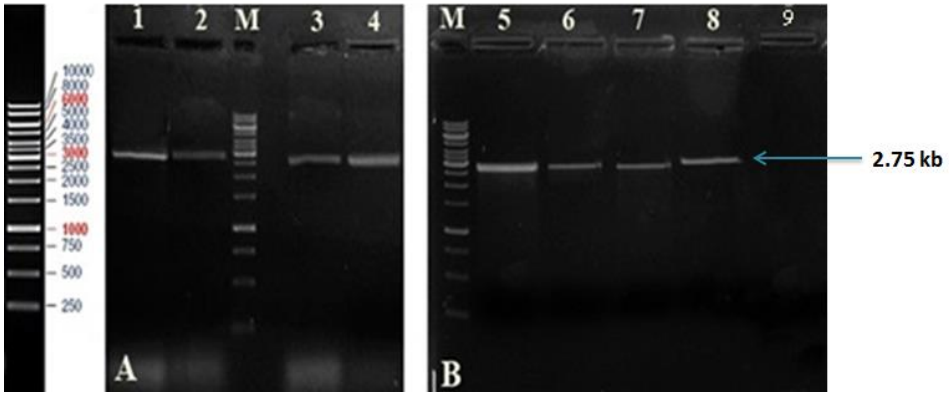

Fig.4.3. Agarose gel electrophoresis of amplified full length begomovirus from night blooming jasmine (*C. Nocturnum*) plant. (A) Isolate CS-152; expected size band, at approx. 2.75 kb of DNA A (Lane 1,2) and DNA B (Lane 3,4), (B) isolate CN-146; expected size band , at approx. 2.75 kb of DNA A (Lane 5,6) and DNA B(Lane 7,8). Lane 9 shows negative control and Lane M showed 1kb standard ladder.

Amplification product of approx. 2750 nucleotides were obtained, using abutting primer pair KTBF/KTBR, from both infected samples (CS-152, CN-146) corresponding to DNA B of begomoviruses as shown in Fig.4.3.

4.4 Gel Elution of PCR Product:

Amplified PCR products were removed from agarose gel were purified (Section 3.5). The purified products were run on agarose gel and expected size bands were observed (~2750bp) from both the samples on UV trans-illuminator as shown in Fig.4.4.

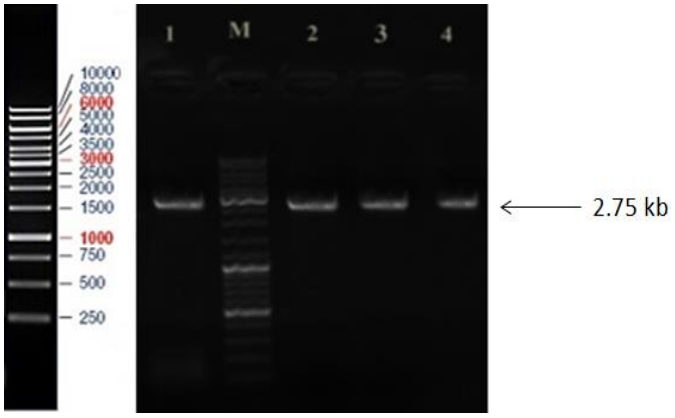

Fig.4.4 Agarose gel electrophoresis of gene eluted products. Lane 1 and 2 showed DNA A and DNA B, respectively of isolate CS-152 while Lane 3 and 4 showed DNA A and DNA B, respectively of isolate CN-146. Lane M showed standard 1 kb marker.

4.5 T/A Cloning

The purified PCR product was ligated to a T/A cloning vector, pTZ57R/T vector (see section 3.6). The ligated product was used to transform DH5α cells of *E. coli* strain. The transformed mixture was spread on LB plates, and incubated at 37^{o}C overnight (see section 3.8). Blue and white colonies were observed after 14 hours of incubation.

4.6 Plasmid Purification:

A single white colony of each clone, in triplicate, was inoculated in to LB broth and incubated overnight (see section 3.9.1). Plasmid DNA was purified from the overnight

culture and run on agarose gel. A good quality plasmid DNA was obtained as shown in Fig.4.5.

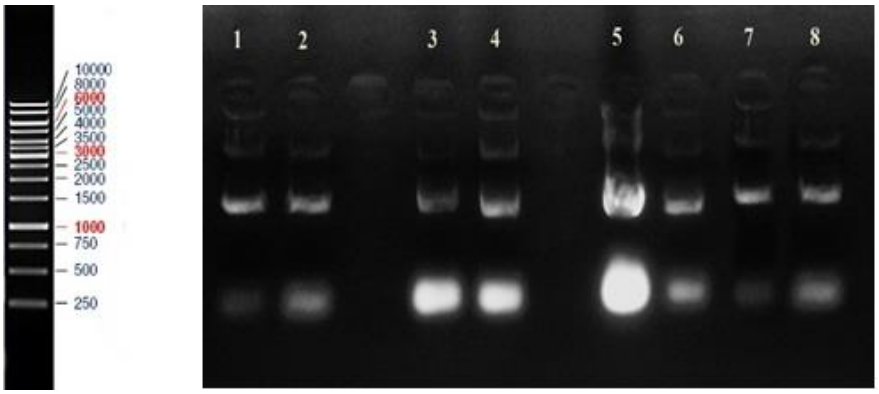

Fig.4.5. Agarose gel electrophoresis of isolated plasmid: Lane 1,2,3,4,5,6,7 and 8 shows isolated plasmids.

4.7 Confirmation of Clones by Restriction Digestion:

4.7.1 Restriction analysis for Begomovirus DNA A:

DNA A was amplified using abutting primer pair, SONAF/SONAR, containing a unique restriction site *Apa*I, the plasmid DNA was digested either with *Apa*I or *EcoR*I. A single band at approx. 2.8 kb was obtained with restriction enzyme *Apa*I, corresponding to full length DNA-A component and pTZ57R/T vector (approx. 2.8 kb) while a band at 5.6 kb was obtained with *EcoR*1, representing DNA A component (2.8 kb) and vector (2.8 kb). Restriction enzyme digestion of begomovirus DNA A is shown in Fig.4.5.

4.7.2 Restriction analysis for Begomovirus DNA B:

DNA B products were digested either with *Pst*I or *Hind*III restriction enzymes. Expected bands at approx. 2.8 kb were observed while digested with *Pst*1 enzyme (since abutting primers contain unique *Pst*I restriction site), and a single band at 5.6 kb was observed after digestion with *Hind*III, correspond to vector and DNA B component (2.8+2.8kb=5.6 Kb) as shown in Fig.4.6.

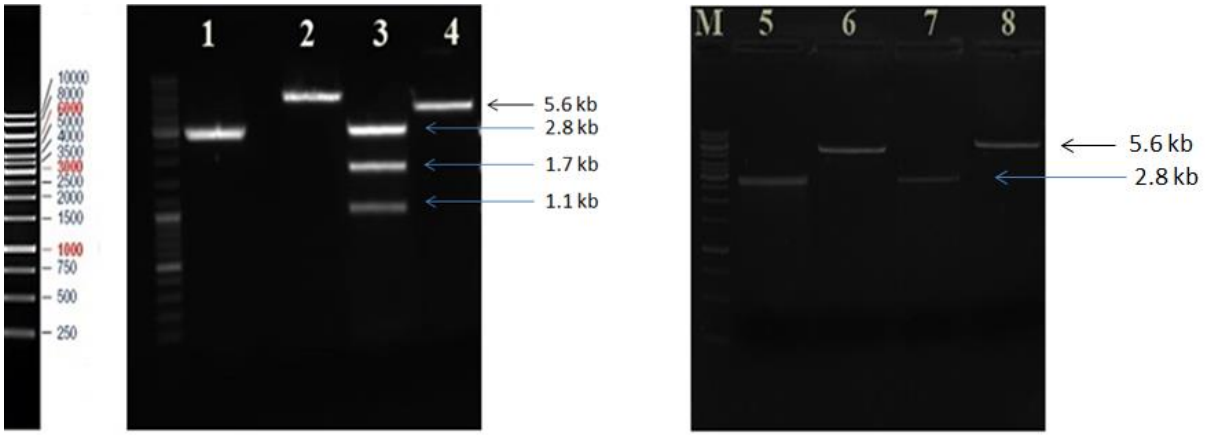

Fig-4.6. Agarose gel electrophoresis of restriction digestion products. Lane 1-4 showed restriction digestion of DNA A while lane 5-8 showed restriction digestion of DNA B products. Lane 1, 2 showed single digestion of isolate CS-152 while Lane 5, 6 showed single digestion of isolate CN-146, using *Apa*I and *EcoR*I restriction enzymes. Single digestion of isolate CS-152 (Lane 3,4) and CN-146 (Lane 7, 8) using *Pst*I and *Hind*III. Lane M showed standard 1kb marker.

4.8 DNA Sequencing and Analysis:

The potential clones were sent to Macrogen Korea for DNA sequencing. Each sample was sequenced in both the orientations. The sequences showed that both isolates CS-152 and CN146 are bipartite begomoviruses having two genomic components, DNA A and DNA B.

4.9 Analysis of DNA A component from the isolates CS-152 and CN-146:

The nucleotide sequences of DNA A from isolate CS-152 and isolate CN-146 were determined to be of 2757 and 2755 nucleotides, respectively in length. Nucleotide sequences were submitted in NCBI databases under the accession number: LM645010 for isolate CN-146 while the sequence for isolate CS-152 is under the process of submission. Nucleotide sequences obtained from the isolate CS-152 and the isolate CN-146 contained six predicted ORF's with an arrangement typical of begomoviruses DNA A. DNA B contains two ORF's; one on virion sense strand while second on the complementary strand. Characteristic features of DNA A and DNA B of isolate CS-152 and isolate CN-146 are shown in Table.4.1.

Table.4.1. Characteristic features of DNA A and DNA B of isolate CS-152 and isolate CN-146:

Isolates	ORFs	Start Codon	Stop Codon	Predicted size (no. of amino acids)	Molecular Weight (kDa)
CS-152 (DNA A)	V2	147	503	118	13.097
	CP	307	1077	256	28.416
	Rep	2614	1556	352	39.072
	Ren	1478	1074	134	14.874
	C4	2457	2155	100	11.1
	TrAP	1626	1219	135	14.985
CS-152 (DNA B)	NSP	439	1245	268	29.748
	MP	2148	1303	281	31.302
CN-146 (DNA A)	V2	146	511	121	13.431
	CP	306	1076	256	28.638
	Rep	1525	2610	361	13.357
	Ren	1477	1073	134	14.874
	C4	2453	2151	100	11.1
	TrAP	1622	1218	134	14.985
CN-146 (DNA B)	NSP	439	1245	268	29.748
	MP	2148	1303	281	31.302

The predicted amino acid sequence identities of all gene products of isolate CS-152 and isolate CN-146 are shown in Table 4.2.

The sequences of the intergenic region were aligned using Clustal X alignment. The intergenic region was seen to contain a series of *cis*-acting elements required for the transcription of *Rep* gene and for the replication of DNA (Arguello-Astroga *et al*., 2001). All these components are present in the isolate CS-152 and isolate CN-146. These components include; direct and inverted repeats upstream (binding site for Rep protein) known as iterons, TATA box and a highly conserved nonanucleotide sequence forming the hairpin loop (the

structure where *Rep* protein produces a nick in order to initiate viral DNA replication). The *Rep* iteron related domain (IRD) 'MAPPKR[FKVQ]' for the isolate CS-152 and 'MAPPKR[FQIY]' for the isolate CN-146, recognizes and binds to the iteron sequence AATTGGAG in case of isolate CS-152 and ATCGGTACT in case of isolate CN-146, respectively within the intergenic region, thereby producing a nick in the nonanucleotide sequence and initiating rolling circle replication, showing the sequences of stem-loop and nonanucleotide to be highly conserved. The iteron sequences for isolate CS-152 and isolate CN-146 are shown in Table.4.2. Percentage nucleotide sequence identity matrix was also produced from the common region of DNA A for the isolate CS-152 and isolate CN-146, compared with the common region of closely related begomovirus sequences from the databases. The percentage nucleotide sequence identity matrix is shown in Table.4.3.

Table.4.2. Iteron sequences within the intergenic region for isolate CS-152 and isolate CN-146.

Isolates	Iteron Sequences	
	DNA A	DNA B
CS-152	ATT[GGAG]	CTT[GGAG]
CN-146	ATC[GGTACT]	TCC[GGTACT]

4.10 Comparison of DNA A sequence from CS-152 and CN-146 isolates to other begomovirus sequences:

The nucleotide sequences of isolate CS-152 and CN-146 showed 84.4% nucleotide sequence identity between them. While the complete nucleotide sequences of the isolate CS-152 and isolate CN-146 showed high nucleotide sequence identity 89% and 88.9%, respectively with *Pedilanthus leaf curl virus (*PedLCV). Based on the species demarcation criteria [set at 89% nucleotide sequence identity for the whole genome (or DNA A genomic component for bipartite begomoviruses); Fauquet *et al.,* 2003], isolate CS-152 and isolate CN-146 are two distinct species begomoviruses for which we proposed the name Cestrum leaf curl Islamabad virus (for isolate CS-152) and Cestrum leaf curl virus (for isolate CN-146).

Table.4.3: Maximum amino acid nucleotide sequence similarities of DNA A and DNA B gene products of virus isolate CS-152 and isolate CN-146 with sequences available in the databases

	V2	CP	Rep	REn	C4	TrAP	MP	NSP
Isolate CS-152	97% CaYMV	99% ToLCBDV	92% PedLCV	95% PaLCV	89% CoLCuV	87% PaLCV	99% ToLCNDV	99% ToLCNDV
	95% PedLCV	99% CLCuKoV	91% ToLCHnV	93% PedLCV	89% ToLCPaV	87% PedLCV	96% BYVMV	95% ToLCNDV
	94% PaLCV	99% PedLCV	91% EuLCV	93% RoLCuV	84% PedLCV	85% CYVMV	95% TLCNDV	93% ToLCNDV
Isolate CN-146	92% ToLVJoV	80% PedLCV	96% PedLCV	97% PaLCV	91% RaLCV	94% PaLCV	99% ToLCNDV	98% ToLCNDV
	89% PaLCrV	79% PaLCrV	93% EuLCuV	96% PedLCV	87% CoLCuV	92% PedLCV	95% BYVMV	94% ToLCNDV
	89% RoLCuV	79% CaYVMV	94% PedLCV	93% PaLCV	87% ToLCPaV	91% ToLCKV	94% ToLCNDV	92% ToLCNDV

Table.4.4. Percentage nucleotide sequence identity matrix of DNA A common regions of the isolate CS-152 and the isolate CN-146, with common region of other begomovirus sequences selected from NCBI databases. Each isolate of the most closely related species was selected.

	TbCSV (1)	**ToLCKeV (2)**	**ToLCuGNV (1)**	**AEV (2)**	**EuLCV (2)**	**SuLCV (1)**	**PedLCV (6)**	**CaYMV (2)**	**PaLCuV (1)**	**ToLCKV (1)**	**CLCuKoV (1)**	**ToLCuBDV (1)**	**CLCuMV (2)**	**CN-146 (1)**	**CS-152 (1)**
CS-152	53.6	51.9	63.7	50.7	51.7	52.2	57.9	54.2	72.7	51.2	37.2	52.2	55.6	39.4	***
CN-146	55.5	47.9	65.4	53.5	51	51.4	55.5	67.5	57.8	48.3	40.7	45.9	55.1	***	
CLCuMV	40-44.6	45.7-70.5	36.8-44.9	39.6-53	39.3-44.9	43.9-56.5	43.9-61.6	36.1-37.1	40.4-41	41.2-54.2	39.6	48.3			
ToLCuBDV	61.6	50	63.7	55.2	50.3	52.7	50	52.4	55	52.4	37.2				
CLCuKoV	40.4-42.3	67-70.6	44.9-46.9	49.5-51.4	36.8-41.6	51.9-54.6	57.3-65.2	35.1	37.1	40.4-42.3					
ToLCKV	61.6	69	67.8	67.1	50	86.7	65.2	53.8	52.2						
PaLCuV	56.4	53.3	57.4	51.4	50.3	51.6	59.8	79.7							
CaYMV	52.1	53.8	58.7	50	50	55.9	59.1								
PedLCV	50.6-61.6	54.6-76.8	54.3-77.5	49-64	49-58	51-76.2									
SuLCV	65.8	68.9	66.8	73.1	52.1										
EuLCV	52.4-59.8	49-47.2	50-57.3	53.1-57.7											
AEV	68.5-72.2	59-59.4	58.4-74.3												
ToLCuGNV	62.6	62.6													
ToLCKeV	54.8														

A table 4.4 has been constructed showing percentages nucleotide sequence identity of the isolate CS-152 and isolate CN-146 with the isolates of closely related species.

Two kinds of phylogenetic trees; Neighbor joining and UPGA(Unweighted pair group method with Arithmetic mean) were constructed based upon similar sequences and sequences of closely related species shown in Fig.4.7 and Fig.4.8. It was observed that the isolate CS-152 and isolate CN-146 occupy distinct positions in the tree, thus representing to be new species compared to all the previously identified begomovirus species. The sequences were supported with maximum (100) bootstrap value.

Table.4.5. Percentage nucleotide sequence identity of DNA A of the isolate CS-152 and the isolate CN-146, with other begomovirus sequences selected from NCBI databases. Each isolate of the most closely related species was selected.

	HYVMV (2)	**CLCuMV (2)**	**PedLCV (4)**	**ToLCKV (13)**	**ToLCKeV (3)**	**ToLCJoV (21)**	**ToLCuGNV (1)**	**ToLCuBDV (4)**	**TbCSV (16)**	**SHLDV (3)**	**RaLCuV (6)**	**PaLCuV (12)**	**EuLCuV (4)**	**CLCuKV (7)**	**AEV (28)**	**CN-146 (1)**	**CS-152 (1)**
CS-152	67.7-70.5	74.6-72.5	86.1-88.9	76.1-80.2	80.4-81.7	78.4-80.3	83.3	77.8-81.1	78.1-81.5	79.2-79.3	81.5-86.2	75.6-84.1	78.7-82.4	79.2-81.7	74.6-82.2	84.4	***
CN-146	67.6-72.6	69.9-73.3	86.5-89	76.2-82.2	80.9-82.9	78.6-81.4	84.3	79-81.1	77.6-81.7	81.3-81.4	80-86	73.4-80.9	79.4-83.8	77.1-80.6	75.8-83.7	***	
AEV (28)	65.2-72.3	68.4-73.4	74.5-86.5	71.4-84.2	73.5-82.4	72.3-81.9	77.4-84.9	72.9-85.5	74.1-87.4	72.2-82.1	70.9-87.3	66.1-84.9	73-81	70.7-81.9	80-100		
CLCuKV (7)	68.8-73.9	71.7-84.4	80.2-85.1	74-82.1	79.3-83	73.1-77	78.1-80.5	72.8-80.1	73.6-80.5	77.5-80.6	74.3-82	68.6-80.3	72.3-79.1	89.4-100			
EuLCuV (4)	63.7-71.3	68.6-74.1	77.6-84.3	73.2-80.4	76.1-80.6	76-80	77.1-81.7	76.2-79.7	76.2-79.7	76.1-80.2	71-83.9	72.3-79.4	89.7-100				
PaLCuV (12)	58.4-70.3	62.1-69.9	74-88.7	70.6-82.8	71.2-80.2	70.1-79.9	73-81.4	70.7-81.3	70.6-82.1	67.2-77.8	68.3-84.3	79.9-100					
RaLCuV (6)	66.5-70.4	71.4-75.6	80.6-88	70.7-81.9	75-83.2	72.3-81.9	79.6-85.5	72.8-84.4	62.5-86.9	67.4-87.6	78.5-100						
SHLDV (3)	64.5-73.2	66.3-74.6	83.4-83.8	73.4-78.7	81.4-82.4	74.3-77.3	78.9-79	74.9-81	78.1-81.5	100							
TbCSV (16)	63.2-66.6	67.6-72.8	77.9-84.2	76.5-84.9	77.4-80.4	77.8-87.5	77.7-81	77.1-89.5	84.2-100								
ToLCuBDV (4)	62.4-69.9	67.5-72.4	78.8-84.3	76.9-86.9	77.8-80.8	80-87.7	79.2-81.5	80.5-100									
ToLCuGNV (1)	63-69.7	67.5-72.2	83.9-87.1	80.1-87.5	85.8-87.8	78.9-81.3	100										
ToLCJoV (21)	68.5-73	70.6-72.8	77.8-83	77.2-83.4	76.9-79.3	84.2-100											
ToLCKeV (3)	63.2-69.4	67.2-71.8	82.5-87.1	80.1-85.7	95-100												
ToLCKV (13)	67-71.2	70.2-76.9	77.5-83.7	84.1-100													
PedLCV (4)	63.9-73.2	67.5-72.7	91-100														
CLCuMV (2)	74.4-77.4	88.1-100															
HYVMV (2)	89.2-100																

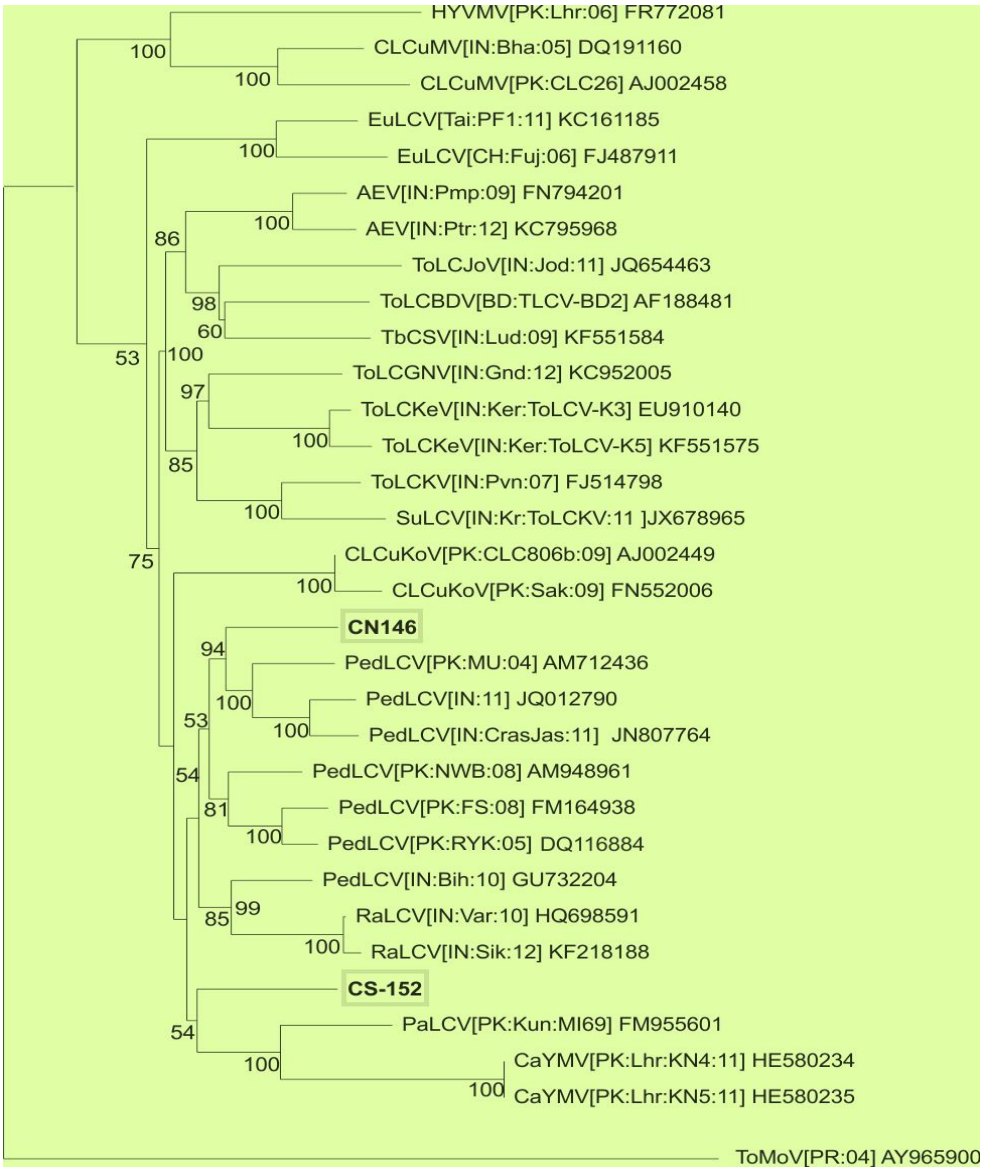

Fig.4.7. Neighbor-Joining phylogenetic tree showing both isolates CS-152 and CN-146 on separate clades along with sequences from closely related species. DNA -A of begomoviruses reported in this study are highlighted. Viruses used are *Cotton leaf curl Multan virus* (CLCuMV), *Hollyhock yellow vein mosaic virus* (HYVMV), *Cotton leaf curl Kokran virus* (CLCuKV), *Euphorbia leaf curl virus* (EuLCuV), *Papaya leaf curl virus* (PaLCuV), *Radish leaf curl virus* (RaLCuV), *Pedilanthus leaf curl virus* (PedLCV), *Ageratum enation virus* (AEV), *Tomato leaf curl Jodhepur virus* (ToLCJoV), *Tomato leaf curl Bangladesh virus* (ToLCBDV), *Tobacco curly shoot virus* (TbCSV), *Tomato leaf curl Karnataka virus* (ToLCKV), *Tomato leaf curl virus* (ToLCuV), *Sunflower leaf curl virus* (SuLCV), *Tomato leaf curl Gandhinagar virus* (ToLCGNV), *Catharanthus yellow mosaic virus*(CaYMV) and *Tomato leaf curl Kerala virus* (ToLCKeV). The tree has been rooted with *Tomato Mottle virus*(ToMoV).

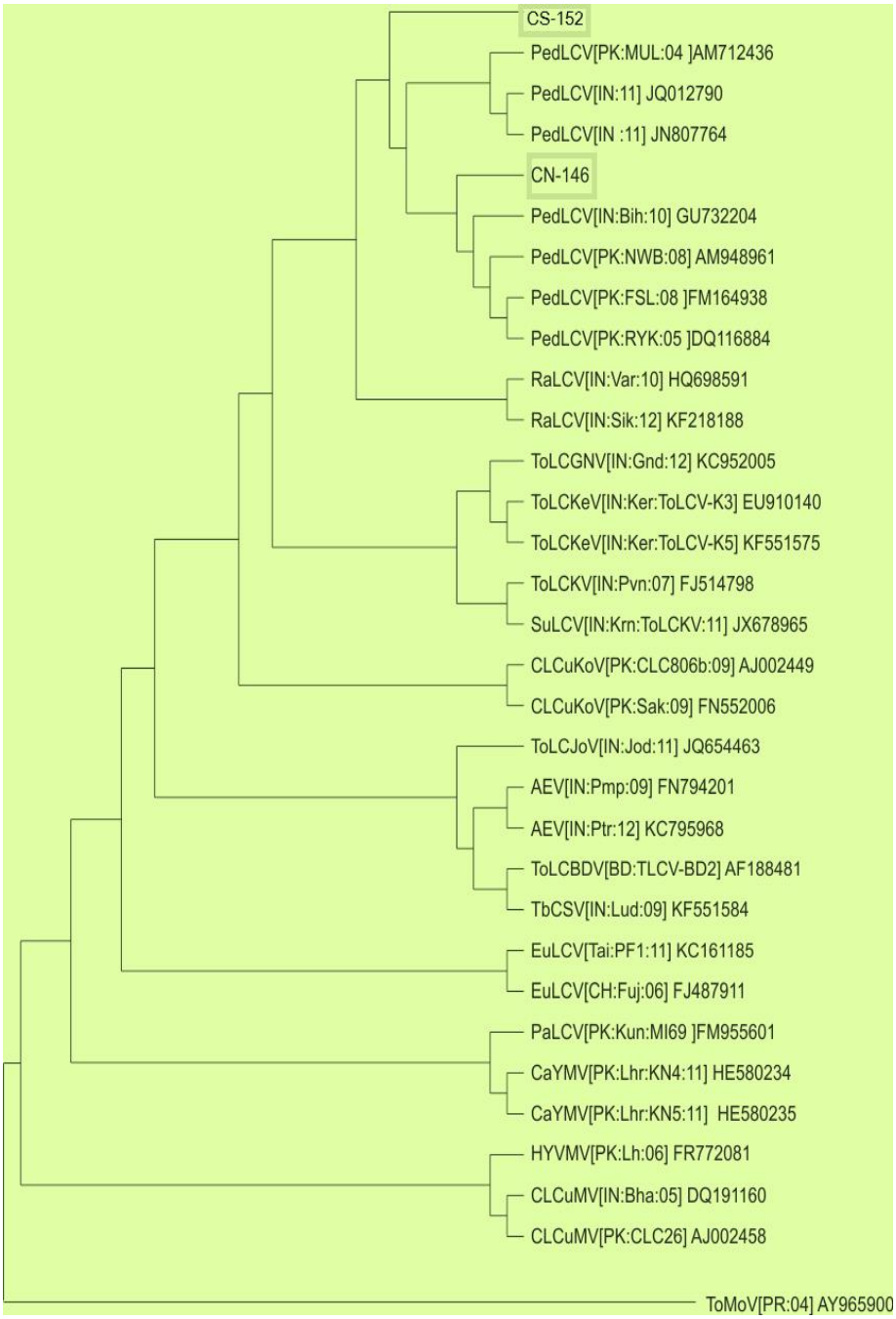

Fig.4.8. UPGMA(phylogenetic tree (Unweighted Pair Group Method with Arithmetic Mean) showing isolate CS-152 and isolate CN-146 on separate clades along with sequences from closely related species. DNA -A of begomoviruses reported in this study are highlighted. Viruses used are *Cotton leaf curl Multan virus* (CLCuMV), *Hollyhock yellow vein mosaic virus* (HYVMV), *Cotton leaf curl Kokran virus* (CLCuKV), *Euphorbia leaf curl virus* (EuLCuV), *Papaya leaf curl virus* (PaLCuV), *Radish leaf curl virus* (RaLCuV), *Pedilanthus leaf curl virus* (PedLCV), *Ageratum enation virus* (AEV), *Tomato leaf curl Jodhepur virus* (ToLCJoV), *Tomato leaf curl Bangladesh virus* (ToLCBDV), *Tobacco curly shoot virus* (TbCSV), *Tomato leaf curl Karnataka virus* (ToLCKV), *Tomato leaf curl virus* (ToLCuV), *Sunflower leaf curl virus* (SuLCV), *Tomato leaf curl Gandhinagar virus* (ToLCGNV), *Catharanthus yellow mosaic virus*(CaYMV) and *Tomato leaf curl Kerala virus* (ToLCKeV). The tree has been rooted with *Tomato Mottle virus* (ToMoV).

4.11 Analysis of DNA B component of isolate CS-152 and isolate CN-146:

The nucleotide sequences of DNA B from both the isolate CS-152 and isolate CN-146 were determined to be of 2692 and 2694 nucleotides, respectively. Sequences were submitted to NCBI databases under the accession number:LM645011and LM645012. Sequences from the isolate CS-152 and isolate CN-146 showed to be containing two ORF's with an arrangement typical of DNA B of begomoviruses. Characteristic features of DNA B from both the isolates are shown in Table. 4.1.

The complete nucleotide sequences of the isolate CS-152 and isolateCN-146 showed highest nucleotide sequence identity (99%) with *Tomato leaf curl New Delhi virus*, DNA B (accession no.AM849547) , followed by 88% nucleotide sequence identity with Bhindi yellow vein mosaic virus (HQ586007).

Two different types of phylogenetic trees; Neighbor joining and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) were constructed based upon complete nucleotide sequences of DNA B from NCBI databases and the sequences of the present study. The most similar sequences from the databases were included and were found on separate clades, indicating species affiliation and *Tomato Mottle virus* (acc. no. AY965900) was used as an outgroup. It is apparent from the trees as shown in Fig.4.9. and Fig.4.10, that the isolate CS-152 and isolate CN-146 cluster with ToLCNDV. The sequences were supported with maximum (100) bootstrap value.

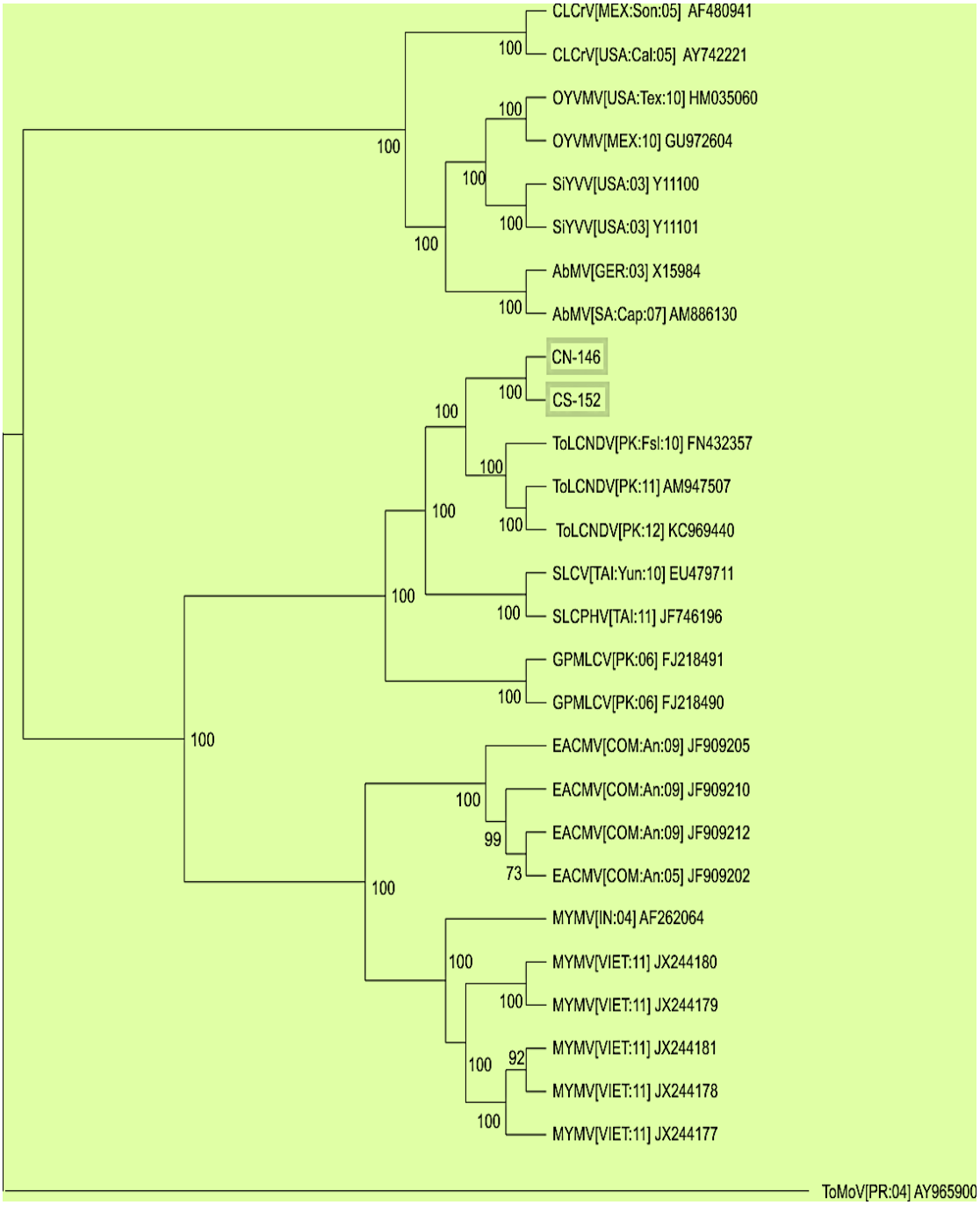

Fig.4.9 Neighbor joining phylogenetic tree based on allignment of DNA B Full length nucleotide sequences with the sequences selected from NCBI databases. Each clade is labelled with a bootstrap value (maximum 100). DNA B sequences reported in this study are highlighted. Sequences of the viruses used are *Tomato leaf curl New Delhi virus*(ToLCNDV),*Squash leaf curl Phillipines virus*(SLCPHV), *Gossypium Punctatum mild leaf curl virus*(GPMLCV), *Mungbean Yellow Mosaic virus*(MYMV), *East African Cassava mosaic virus*(EACMV), *Abutilon mosaic virus*(AbMV), *Sida Yellow vein virus*(SiYVV), *Okra Yellow vein mosaic virus*(OYVMV) and *Cotton leaf crumple virus*(CLCrV). The tree has been rooted with *Tomato Mottle virus* (ToMoV).

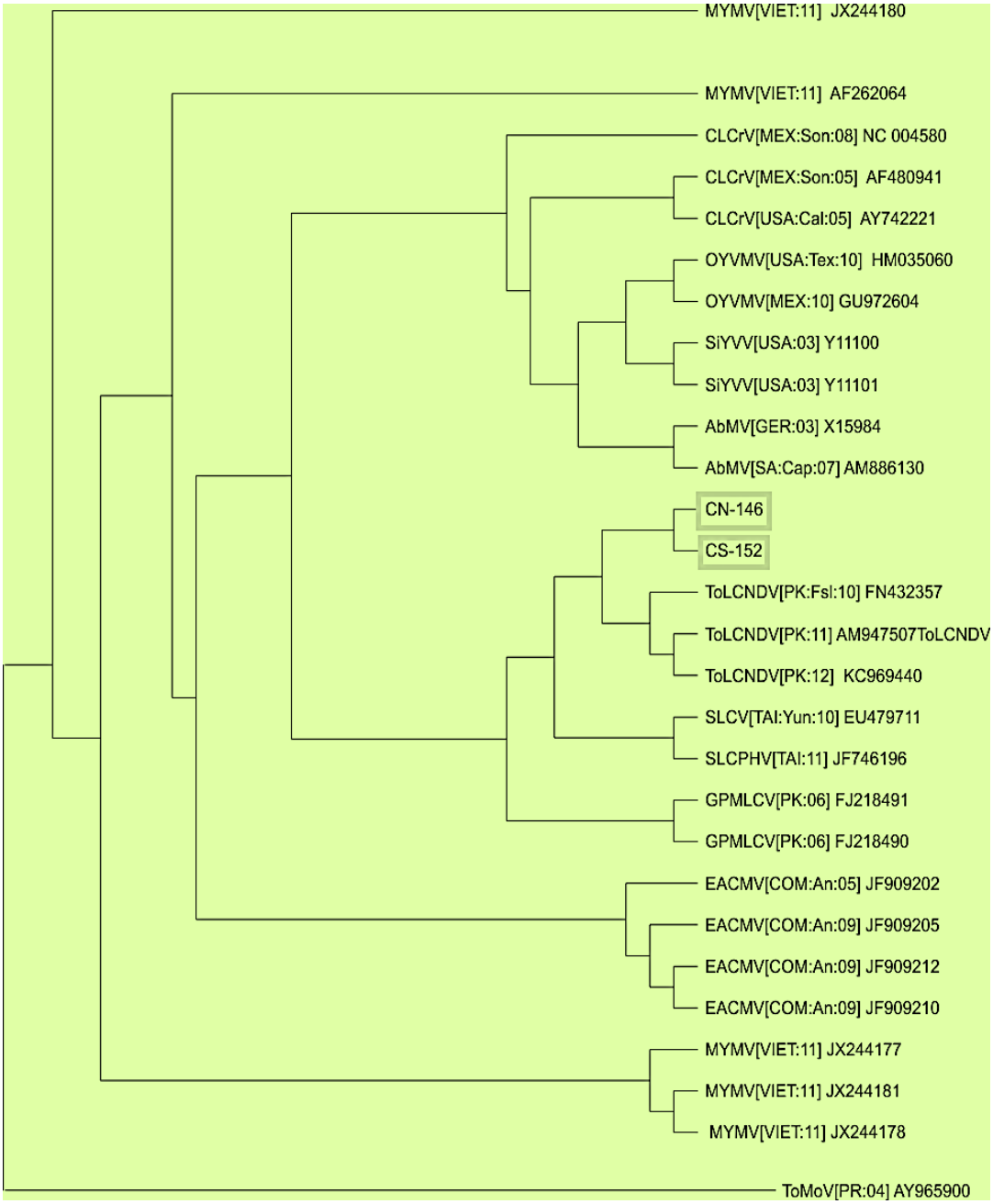

Fig.4.10. UPGMA tree (Unweighted Pair Group Method with Arithmetic Mean) based on allignment of DNA B Full length nucleotide sequences with the sequences selected from NCBI databases. Each clade is labelled with a bootstrap value (maximum 100). DNA B sequences reported in this study are highlighted. Sequences of the viruses used are *Tomato leaf curl New Delhi virus*(ToLCNDV),*Squash leaf curl Phillipines virus*(SLCPHV), *Gossypium Punctatum mild leaf curl virus*(GPMLCV), *Mungbean Yellow Mosaic virus*(MYMV), *East African Cassava mosaic virus*(EACMV), *Abutilon mosaic virus*(AbMV), *Sida Yellow vein virus*(SiYVV), *Okra Yellow vein mosaic virus*(OYVMV) and *Cotton leaf crumple virus*(CLCrV). The tree has been rooted with *Tomato Mottle virus* (ToMoV)

5 Discussion:

Geminiviruses are plant pathogenic viruses consisting of small circular, single stranded genome encapsidated in an approx. 20x30 nm geminate particle. Geminiviruses came into form in 1979 (Matthews, 1979) and the group was later upgraded to *Geminiviradae* in 1995 (Murphy *et al.*, 1995). At present, the family is divided into seven genera; *Becurtovirus, Eragrovirus, Turncurtovirus, Topocuvirus, Curtovirus, Mastrevirus* and *Begomovirus*. The division is based upon genome organization, host range and insect vector. There are almost 199 officially recognized species of Geminiviruses, out of which 181 belong to *Begomoviruses* (Fauquet *et al*., 2008). This explains the diversification and economic importance of *Begomoviruses*. *Begomoviruses* are the most damaging viruses and cause devastating economic losses. They can only be transmitted by a single species of white fly known as *Bamesia tabaci* and affects dicotyledonous plants. During the last two decades, begomoviruses have been the sole reason of worldwide economic losses. In Pakistan, losses due to cotton leaf curl disease were up to US 5 billion (Briddon and Markham, 2001). 1.3 to 2.3 billion US dollar losses were observed for cassava in Africa (Thresh & Cooter, 2005), in Florida massive losses estimated to be 140 million US dollars were observed due to tomato leaf curl virus (Moffat, 1999). While in India the losses observed were to be of US 300 million for legumes (Varma and Malathi., 2003).

In present study, two bipartite begomoviruses were isolated and characterized from night blooming jasmine (*C. nocturnum*). The plant samples were collected from two different areas of Islamabad; near Shifa International Hospital (isolate CS-152) and from the lawns of ASAB (Atta-ur-Rahman School of Applied Biosciences), NUST (isolate CN-146). The study showed the presence of two different begomoviruses, *Cestrum leaf curl Islamabad virus* and

Cestrum leaf curl virus, from the isolate CS-152 and the isolate CN-146, respectively along with DNA B of *Tomato leaf curl New Delhi Virus.*

Cestrum leaf curl virus and *Cestrum leaf curl Islamabad virus* from the isolate CN-146 and isolate CS-152, respectively showed 84.4% nucleotide sequence identity among each other, while, 88.9% and 89% nucleotide sequence identity, respectively to *Pedilanthus leaf curl virus.*

Pedilanthus leaf curl virus is a monopartite begomovirus which was reported from Redbird flower (*Pedilanthus tithymaloides*; Tahir *et al*., 2009). It was found to be associated with *Tobacco leaf curl betasatellite* (Tahir et al., 2009). *Pedilanthus leaf curl virus* has also been reported from Vinca minor L. (common name Periwinkle) an ornamental plant, from Lahore without the association of a beta satellite. About 30-40% of the periwinkle plants surveyed showed begomoviral symptoms (yellowing, leaf curl and distorted leaves) making it an alternative host of the virus (Haider *et al.,* 2008). *Pedilanthus leaf curl virus* is also seen to infect some legumes species (Ilyas *et al.,* 2010). In 2014, *Pedilanthus leaf curl virus* was reported from night blooming jasmine plant again as a monopartite begomovirus (Srivastava *et al.,* 2014). It is apparent from all the early reports that *Pedilanthus leaf curl virus*, which shows maximum nucleotide sequence identity(88% and 88.9% respectively) to the isolate CS-152 and the isolate CN-146, respectively is a monopartite begomovirus which may or may not be found associated with a beta satellite.

Up till now, no begomovirus other than *Pedilanthus leaf curl virus* has been reported infecting night blooming jasmine (Srivastava *et al.,* 2014). This is the first occurrence of *Cestrum leaf curl virus* and *Cestrum leaf curl Islamabad virus*, infecting night blooming jasmine plant making it an adaptive host of begomoviruses.

The DNA B from the isolate CS-152 and isolate CN-146 showed 99% nucleotide sequence identity to DNA B of *Tomato leaf curl New Delhi virus.* Night blooming jasmine is seen infected by a monopartite begomovirus, the presence of a bipartite begomovirus in night blooming jasmine is a serious threat to agriculturally important crops.

Tomato leaf curl New Delhi virus, DNA B has previously been reported from Ash gourd (Roy *et al.*, 2011), Pumpkin (Maruthi *et al.*, 2007), Luffa acutangula (Jyothsna *et al*., 2010), Chillies (Vinothkumar *et al.*, 2013) and bottle gourd (Ito *et al.*, 2009). A report shows the presence of ToLCNDV, DNA B along with the presence of a beta satellite molecule (Roy *et al.*, 2013). In the current study, ToLCNDV, DNA B has been seen associated with Cestrum leaf curl Islamabad virus and Cestrum lead curl virus.

A phylogenetic tree was also constructed for the analysis of DNA A and DNA B of the isolate CS-152 and the isolate CN-146. The phylogenetic tree showed the isolate CS-152 and isolate CN-146 to be present on separate clades, a further confirmation of isolate CS-152 and the isolate CN-146 to be new species. Phylogenetic tree for DNA B showed isolate CS-152 and isolate CN-146 to cluster with *Tomato leaf curl New Delhi virus*, making them close relatives of the virus.

Night blooming jasmine is an ornamental plant grown across the tropical and subtropical regions of the world for its fragrance. In some countries like Aucland and NewZealand, it is categorized as a weed and is difficult to eradicate (Keyserver.lucidcentral.org,2014) In Pakistan, it is grown widely as an ornamental plant.

In the present study, it has been seen that night blooming jasmine works as a reservoir to harbor new begomoviruses and has become a very good host for the survival of begomoviruses. This is the first report of the occurrence of Cestrum leaf curl virus (LM and Cestrum leaf curl Islamabad virus in night blooming jasmine.

APPENDICES

```
DE  Cestrum yellow leaf curling virus, complete genome
XX
KW  complete genome.
XX
OS  Cestrum yellow leaf curling virus
OC  Viruses; Retro-transcribing viruses; Caulimoviridae; Soymovirus.
XX
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RP  1-2755
RA  Tahir M.;
RT  ;
RL  Submitted (27-JUN-2014) to the INSDC.
RL  Tahir M., Plant Biotech, ASAB, National University of Sciences &
RL  Technology, ASAB, National University of Sciences &, Technology, Islamabad
RL  44000, PAKISTAN.
XX
RN  [2]
RA  Khan J., Tahir M.;
RT  "First report of cestrum leaf curl virus affecting cestrum nocturnum";
RL  Unpublished.
XX
RN  [3]
RA  Khan J.;
RT  "Isolation and characterization of begomovirus(es) from Cestrum nocturnum";
RL  Unpublished.
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