“Biological and Molecular Characterization of Chilli Leaf Curl Virus (ChiLCV) in South Gujarat”

A

THESIS
SUBMITTED TO THE
NAVSARI AGRICULTURAL UNIVERSITY
NAVSARI

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR

THE AWARD OF THE DEGREE

OF

MASTER OF SCIENCE
(AGRICULTURE)

IN
PLANT PATHOLOGY

BY

DORE R
B.Sc. (Agri.)

DEPARTMENT OF PLANT PATHOLOGY
N.M. COLLEGE OF AGRICULTURE
NAVSARI AGRICULTURAL UNIVERSITY
NAVSARI - 396 450
GUJARAT STATE

JULY- 2017

REGISTRATION NO.: 2010115038

ABSTRACT

"Biological and Molecular Characterization of Chilli Leaf Curl Virus (ChiLCV) in South Gujarat"

Name of student	Major Advisor
Mr. DORE R	Dr. Lalit Mahatma

DEPARTMENT OF PLANT PATHOLOGY
N. M. COLLEGE OF AGRICULTURE
NAVSARI AGRICULTURAL UNIVERSITY
NAVSARI

A B S T R A C T

Study was undertaken to study the Chilli leaf curl virus (ChiLCV) in South Gujarat. High incidence of the disease in the different fields of NAU as well as the adjoining area could be seen during the survey in 2016. ChiLCD manifesting typical leaf curl symptoms were seen in all the areas surveyed ranging from 10-35 per cent. The variation in disease incidence was mainly due to the different stages of the crop at different place and the different management practices followed. In every infected field surveyed, whiteflies were found invariably. However, jassids and thrips were the other insect pests also noticed. The typical symptoms of the disease observed during the investigation were typical leaf curl symptoms with the upward/downward curling of leaves, puckering and reduced size of leaves along with the thickening and swelling of veins. Affected plants grew slowly and became stunted or dwarfed. Fruit, if produced at all, were small, dry and unmarketable. Quality viral DNA was isolated and in PCR a part of DNA-A molecule of~1200 bp was amplified by the *Begomovirus* specific primers confirming it to be *Begomovirus*. A 932 bp nucleotide sequence was obtained on sequencing. Analysis and comparison of the sequence with the other standard DNA-A

molecule of the *Begomovirus* indicated that the amplified fragment have two genes viz., virus coat protein (*V1*) gene and pre coat protein (*V2*) gene. The sequenced virus showed highest identity 99% with Chilli leaf curl India virus isolate India:Sonipat:TC290:2010 segment DNA-A, [KJ649706.1]. According to the recent criteria for the nomenclature of the virus was considered and named as Chilli leaf curl India virus – India: Sonipat: Navsari: 2016 abbreviated as ChiLCIV-[IN:Son:Nvs:16].

Alignment of the recently sequenced virus during present investigation [ChiLCIV-[IN:Son:Nvs:16] with other 45 different viruses showed the evolution pattern and similarity of the virus with the other Begomoviruses in the world. Different isolates of Chilli Leaf Curl Virus (ChiLCV) align distinctly in separate clad in the phylogenetic tree prepared.

Dr. Lalit Mahatma
Associate Professor
Dept. of Plant Pathology
N. M. College of Agriculture
N.A.U., Navsari – 396 450

C E R T I F I C A T E

This is to certify that the thesis entitled "**Biological and Molecular Characterization of Chilli Leaf Curl Virus (ChiLCV) in South Gujarat"** submitted by **Mr. DORE R** in partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE (AGRICULTURE) in PLANT PATHOLOGY of the NAVSARI AGRICULTURAL UNIVERSITY** is a record of bonafied research work carried out by him under my guidance and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Place: Navsari
Date: / /2017

(Lalit Mahatma)
Major Advisor

DECLARATION

This is to declare that the whole of the research work reported in the thesis in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **PLANT PATHOLOGY** by the undersigned is the result of investigations done by me under direct guidance and supervision of **Dr. Lalit Mahatma,** Associate Professor, Dept. of Plant Pathology, N. M. College of Agriculture, Navsari Agricultural University, Navsari and no part of the work has been submitted for any other degree so far.

Place: Navsari **(DORE R)**

Date: / /2017

Countersigned by

(Lalit Mahatma)
Associate Professor
Dept. of Plant Pathology
N. M. College of Agriculture
N.A.U., Navsari – 396 450

Acknowledgement

For with God nothing shall me impossible. I would consider this work nothing more than incomplete without attending to the task of acknowledging the overwhelming help I received during this endeavour of mine.

I am thankful to my Major guide ***Dr. Lalit Mahatma*** *and I also indebted to thank my Advisory committee members*

I sincerely express my special thanks and regard to Hon. Vice chancellor, Principal, Director of Research & Dean PGS and the authorities of Navsari Agricultural University, Navsari for providing me this golden opportunity to pursue my master degree in the discipline of Plant Pathology in this esteemed university.

My heartfill thanks to Lab technicians and gratefully acknowledge the unconditional help and support that I received from my dear fellowmates, Vivek T.S, Bhagyashree, Sanket V, Kishan K, Bipinchandra V, Arjunsinh S, Virendra J, Nirmal Y, Hasmuk K, Krupa R, Sonal J, Mahesh M, Dinesh H, Dasharath A, and Shashikanth

I appreciate the help and support of my seniors and juniors namely Vanthana M, Ghevariya Tushar V, Ankith Harishbai, Mital I. Patel, Jehani Muljibhai D, Sreenivas G, Sree Ganesh, Chaithanya, Rangaswamy, and Jagadeesh,

Personally I would like to express my deep sense of gratitude to the person who loved me more than my mother my beloved aunt puttamma, to my dear parents who believed in me my father Rajaiah P. K, mother Revamma and my lovable brother Santhosh kumar R and sister Kavya shree and brother-in-law Krishna and sister-in-law Asha and all my family members.

Acknowledgement

My candy close friends Chethan kumar, Yogesh, Lohith, Madukumar, Prakash, Chaitra, Chethan kumari, Noushad, Raghuram Pawar, Manasa, Nandini, for their everlasting love, constant encouragement, inspiration, prayer, support and guidance throughout my life.

Place: *Navsari*

Date: (*DORE R*)

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INTRODUCTION

I. INTRODUCTON

Chilli (*Capsicum annuum* L.) is an important vegetable and spice crop, belongs to the genus *Capsicum*, family Solanaceae. It has originated in Mexico (Villalon, 1981). Commercial cultivation of chilli is mostly confined to the tropical regions of the world. Chilli is known from prehistoric remains in Peru and was widely cultivated in Central and South America in early times. It was first introduced in India by Portuguese towards the end of 15th century (Basu and Krishna, 2003). Now a day's chilli has become an important crop all over India. It occupies a prime place as a spice and vegetable crop (Kumar and Rai, 2005).

Capsicum contains approximately 20-27 species, 5 of which *viz*., *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*, are domesticated and cultivated in different parts of the world. Among those *C. annuum* is one of the most commonlly cultivated crop throught the world followed by *C. frutescens* (Tong and Bosland, 1999; Bosland and Votava, 2003).

Chilli has great genetic diversity, appears in different sizes, shapes and colour. The fruit size of some varieties is more than hundred times that of others. The shape may be elongated or round, and distal end pointed, blunt or sucked in, corrugated, leathery or smooth may be the touch on outer skin, and small orange fruit. Colour varies from green, orange, red, and yellow.

In India chilli is an important commercial crop cultivated for vegetable, spice and industrial (oleoresin and capsaicin extraction) purposes. Chilli is used all over the world and is preferred by both vegetarian and non vegetarian masses to increase the palatability and taste of cooked food and vegetables.

Chilli has many culinary advantages because of the mainly capsaicinoids. Chilli comprises numerous chemicals including steam-volatile oils, fatty oils, capsaicinoids, carotenoids, vitamins, proteins, fibres and mineral elements. Chillies are low in sodium and rich in vitamin A, vitamin C, vitamin E, and are good source of potassium and folic acid. Fresh green chilli contain more vitamin C, than citrus fruits, and fresh red chilli has more vitamin A, than carrot (Osuna-Garcia *et al*., 1998; Marin *et al*., 2004). Capsaicinoids are alkaloids that make hot chilli pungent. A large number of carotenoids provide high nutritional value and the colour to chilli (Britton and Hornero-Mendez 1997; Hornero-Mendez *et al*., 2002; Perez- Galvez *et al*., 2003).

Both fresh and dried fruit contain phenolic compound, capsaicin in the placenta, which is responsible for pungency in chilli. Capsaicin, the active principle of chilli is an effective counter irritant and hence chilli extracts are used in pharmaceutical drugs as a powerful stimulant carminative, the bright colour at the ripening stage is due to the pigment of capsanthin.

Chilli has very high nutritional value according to Joshi and Singh (1975), 100 g edible part of chilli contains 92.4 percent water, 1.2 g protein, 0.8 g fat, 10 g carbohydrates, 2.6 g fiber, 11 mg calcium, 61 mg phosphorous, 2 Cal energy and rich source of vitamins like carotene 870 I.U., ascorbic acid 175 mg, riboflavin 0.03 mg, and niacin 0.58 mg.

India is the largest producer, consumer and exporter of chilli in the world and it exporting to USA, Canada, UK, Saudi Arabia, Singapore, Malaysia, Germany and many countries across the world. Other prominent exporting countries besides India include Spain, Japan and Thailand. Indian hot pepper is mainly exported in the form of curry powder and curry paste.

In India chilli is mainly cultivated in Andhra Pradesh, Karnataka, Maharashtra, Orissa, Rajastan, Tamil Nadu, Gujarat, West Bengal and Punjab states. Chilli occupies an area of 7.61 lakh hectares with an annual production of 16.05 lakh MT and 2.1 MT/hectare of productivity (dry chillies), (Anon 2015).

Gujarat contributed 2.1 per cent production of the total country's production with 33.3 thousand MT production from 9 thousand hectares area with the productivity of 1.7MT/ha (Anon 2015).

Chilli leaf curl virus disease (ChiLCVD) complex is often seen and considered the most severe limiting factors for the successful cultivation of the crop. Leaf curling due to the sucking insect pest and viral diseases is often confusing in most of the cases it has been assumed that the disease complex is caused by a whitefly transmitted *Begomovirus*. However, the ChiLCVD complex is caused by the feeding of thrips, mites and whiteflies as well as by the infection of whitefly transmitted *Begomovirus* (Dharamasena, 1998; Mahatma *et al.,* 2014). Thrips, *Thrips tabaci* (Lindeman) has piercing-sucking mouthparts and instead of feeding exclusively in the phloem sieve tube, also feed on the mesophyll and epidermal cells of leaf tissue using a single stylet in the mouth and then inserting a set of paired stylet, which lacerate and damage cell tissues and function to imbibe cellular fluids. As a result, thrips feed on a multi-tube of food types within plants. Infested plant showed leaf scarring, distorted growth, sunken tissues on leaf undersides and deformation. The plant showed bright appearance due to the influx of air after the removal of plant fluids in severe infestation. Damage to plant leaves also occurred when females, using their sharp ovipositor, insert egg into plant tissue. These symptoms were termed as "pseudo leaf curl symptoms" for better

differentiation of the leaf curl caused by the virus infection and leaf curl caused by the sucking insect pest (Mahatma *et al.*, 2014).

Viruses are most destructive pathogens reducing yield of various crops qualitatively and quantitatively. Cent per cent losses of marketable chilli fruit have been reported due to the viral diseases (Marte and Wetter, 1986). Chilli leaf curl virus (ChiLCV) is considered as most destructive disease which causes substantial losses in yield by infecting the crops at all the stages (Muniyappa and Veeresh, 1984; Kumar *et al*., 2006). Apart from this, natural occurrence of more than 45 viruses, including, Chilli veinal mottle virus and Chilli vein banding virus have been reported worldwide with varied level of significance at different places and seasons (Prakash and Singh, 2006).

ChiLCV belongs to genus *Begomovirus*, family *Geminiviridae* is characteristically have circular single stranded DNA genomes encapsidated in twin particles. Most Begomoviruses have a bipartite genome consisting of a DNA-A and DNA-B components. DNA-A encodes for genes responsible for viral replication, regulation of gene expression, suppression of gene silencing and particle encapsidation, while DNA-B encodes for proteins involved in viral movement, host range determination and symptom development (Lazarowitz, 1992).

First report of the leaf curl and leaf crinkle type of symptoms from the chilli and its association with whitefly was reported in 1932 from the Punjab of Integrated India now in Pakistan (Hussain, 1932). Further, Uppal (1940) observed that symptom of the leaf curl in chilli consists of abaxial and adaxial curling of leaves accompanied by puckering and blistering of interveinal areas and thickening and swelling of the veins. In advance stages, axillary buds are stimulated to produce clusters of leaves, which are reduced in size. The whole plant assumes busy appearance with stunted growth.

Flowers and fruits are few being curled at the end. The disease has been further reported in India by Vasudeva, (1954) and Mishra *et al*., (1963). Subsequently the disease has been observed from the different parts of the country by the different researchers.

At least six Begomoviruses have been shown to be associated with chilli leaf curl disease in different countries. Among these, Tomato leaf curl New Delhi virus (ToLCNDV) has been reported from India (Khan *et al.,* 2006) and Pakistan (Hussain *et al*., 2004), Chilli leaf curl virus (ChiLCV), Cotton leaf curl Multan virus (CLCuMV) and Tomato leaf curl Joydebpur virus (ToLCJV) from Pakistan (Hussain *et al*., 2003; Shih *et al*., 2003), Pepper yellow leaf curl Indonesia virus from Indonesia (Tsai *et al*., 2006); and Cabbage leaf curl virus from Cuba (Zubiaur *et al*., 2006).

Leaf curling due to sucking insect pest is reversible and can be managed through insect control; however, leaf curl due to Chilli leaf curl virus (ChiLCV) is irreversible and cannot be controlled. Therefore, a robust technique to identify the pathogen in the field and further its characterization is one of the critical stages in the management of the disease. However, the satisfactory information to clearly differentiate the symptoms produced by the sucking insect pests and ChiLCV as well as molecular characterization of the virus causing leaf curl in south Gujarat are not available. Therefore, the present investigation was carried out to generate information about the same under the title "Biological and Molecular Characterization of Chilli Leaf Curl Virus (ChiLCV) in South Gujarat" with the following objectives.

1. Study of the symptoms produced by the Chilli Leaf Curl Virus on chilli.
2. Amplification and sequencing of a part of DNA-A molecule of ChiLCV infecting Chilli.

3. Diversity analysis of the sequence and comparison with other Begomoviruses.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Chilli leaf curl disease incited by the different species of the *Begomoviruses* is the most significant biological factor in limiting production and productivity of chilli across the countries**.** No systematic studies have been carried out on the different aspect of the disease in the South Gujarat, therefore the present investigation was carried out under the title "Biological and Molecular Characterization of Chilli Leaf Curl Virus (ChiLCV) in South Gujarat". Information available in the literature on the similar aspects *viz*., incidence, symptomatology, genome organization, nomenclature, taxonomy and diversity analysis of Begomoviruses have been reviewed and presented in different sub headings hereunder.

2.1 Incidence and symptoms produced by the Chilli Leaf Curl Virus (ChiLCV)

2.1.1 Disease incidence, symptoms and the diversity in the virus

Chilli leaf curl virus disease (ChiLCVD) complex is often seen and considered the most severe limiting factors for the successful cultivation of the crop. Leaf curling due to the sucking insect pest and viral diseases is often confusing. Most of the cases it has been assumed that the disease complex is caused by a whitefly transmitted *Begomovirus*. However, the ChiLCVD complex is caused by the feeding of thrips, mites and whiteflies as well as by the infection of whitefly transmitted *Begomovirus* (Dharamasena, 1998; Mahatma *et al.,* 2014). Thrips, *Thrips tabaci* (Lindeman) has piercing-sucking mouthparts and instead of feeding exclusively in the phloem sieve tube, also feed on the mesophyll and epidermal cells of leaf tissue using a single stylet in the mouth and then inserting a set of paired stylet, which lacerate and damage cell tissues and function to imbibe cellular fluids. As a result, thrips feed on a

multi-tube of food types within plants. Infested plant showed leaf scarring, distorted growth, sunken tissues on leaf undersides and deformation. The plant showed bright appearance due to the influx of air after the removal of plant fluids in severe infestation. Damage to plant leaves also occurred when females, using their sharp ovipositor, insert egg into plant tissue (Mahatma *et al*., 2014).

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Abaxial curling of the leaves accompanied by puckering, thickening and swelling of the veins were also observed by Muniyappa and Veeresh, (1984).

Sastry and Singh (1973) reported that the plants infected by ChiLCV within 20 days of transplanting remain stunted in growth and produce fewer leaves and fruits than those infected 35 to 50 days following transplanting. Early infection results in 97.3% yield reduction as compared with 74.1 to 28.9% yield reduction in plant infected 35 to 50 days after transplanting.

Singh *et al*., (1979) observed that the disease caused heavy loss in yield and quality of fruits. If the plant get infected within 20-25 days after transplanting the loss in yield goes up to 80-90%, but in case of later infection loss is comparatively less.

Saha and Nath (2005) reported that the disease is more sever during summer season in comparison to winter season in the plains of West Bengal and loss of yield goes upto 80% when the plants gets infected early.

The typical symptom of ChiLCV includes curling and puckering of leaves and stunted growth of the plants. Epidemics of ChiLCV can result in 100% yield loss resulting in severe economic consequences (Senanayake *et al*., 2006).

Tomato leaf curl Joydebpur virus symptoms on chilli were as mild yellowing, severe leaf curling, leaf distortion, stunting and blistering observed in the field of Ludhiana, Punjab by Shih *et al*., (2006)

Senanayake *et al*., (2007) reported that Leaf curl disease of Chilli has emerged as a serious problem in the Jodhpur district, the major chilli growing area of Rajasthan state. During December 2004, very high disease

incidence (up to 100% of plants) was observed in farmer's fields in Narwa and Tinwari villages.

Symptoms showing upward and downward curling, leathery and brittle leaves, reduced leaf size and stunted plant growths characterize the incidence of Leaf curl virus in pepper (Osaki and Inouye, 1981; Sinha *et al*., 2011).

Itoo *et al*., (2013) recorded the important symptoms were upward as well as downward curling, leathery and brittle leaves, reduction in leaf size, thickening of veins along with vein clearing, puckering, crinkling and stunting of the plants. This symptom variability could be attributed to the cultivation of different hybrids/variety, virus, stage of the plant and climatic factors.

The monopartite Begomoviruses and betasatellites associated with ChiLCD have spread to major chilli-growing regions of the Indian subcontinent. In India, chilli leaf curl virus (ChiLCV), chilli leaf curl India virus (ChiLCINV), chilli leaf curl Vellanad virus (ChiLCVV), tomato leaf curl Joydebpur virus and tomato leaf curl New Delhi virus (ToLCNDV) are known to be associated with ChiLCD; Senanayake *et al*., 2007; Shih *et al*., 2007; Kumar *et al*., 2011).

2.1.2 Transmission of the disease (Vector)

All the Begomoviruses including the virus causing chilli leaf curl disease are transmitted by whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in a persistent manner (Hussain, 1932; Pal and Thandon, 1937; Park and Fernando, 1938; Mishra *et al*., 1963; Dhanraj and Seth, 1968). The first firm evidence of transmission of Begomovirus by a whitefly (*Bemisia* sp) was obtained from Democratic Republic of Congo (Kufferath and Ghesquiere, 1932) in cassava crop. After acquisition, whiteflies can transmit the virus for 5

to 20 days, or even for longer period and sometimes entire life span (Muniyappa *et al*., 2003; Rojas, 2004).

There was no report of seed transmission of any of the *Begomovirus* in any of the crop till 2008. During 2008 it was observed that some of the plants of the mungbean (*Vigna radiata* L.), Gujarat, India, were found to infect the first trifoliate and have typical yellow-colored symptoms on the pod and seed coat. PCR with the *Begomovirus* specific primers revealed presence of *Begomovirus* in the different part of the seeds, viz., pod, seed coat, and cotyledon; however, it was absent in embryo. Callus developed by using the seed cotyledon as explants confirmed that the virus remains viable in the cotyledon of the seed (Pawar, 2010). Accordingly the route of movement of *Begomovirus* indirectly through the seed from one crop season to another was traced (Pawar *et al*., 2015; Mahatma and Pawar, (2015) and Mahatma *et al*., 2016). Germination of mungbean is epigeal and the seedling emerges within 4–6 days, rupturing the crust of the soil. Critical examination suggests that cotyledon softens enough and becomes suitable for the feeding of whiteflies by imbibing water. Few whiteflies that feed on the emerging cotyledon acquire virus which is subsequently transmitted to the developing seedling on feeding. Division of cotyledonary cells and the virus does not take place at the stage; therefore, it does not get transmitted from cotyledon to emerging seedling; however, seed indirectly serves as a source of primary inoculum for the initiation of the disease in the population, which rapidly spreads in the entire field if the vector is available (Pawar *et al*., 2015; Mahatma and Pawar, (2015) and Mahatma *et al*., 2016).

A single whitefly was capable of transmitting the virus and eight or more whiteflies per plant resulted in 100% transmission. The minimum acquisition access period (AAP) and inoculation access period (IAP) were 180

and 60 min, respectively. The virus persisted in whiteflies for up to 5 days post acquisition.

Hussain (1932) reported that the leaf curl or leaf crinkle occurring on chillies was caused by *Bemisia tabaci.*

Kapoor and Varma, (1950) found that Chilli leaf curl virus is only transmitted by whitefly (*Bemisia tabaci*) in circulative non propagative manner. Several attempts to transmit ChiLCV by other methods were made but none of them were successful except whitefly.

When the chilli leaf curl is artificially transmitted by means of whitefly (*Bemisia tabaci*). Inoculated chilli plants showed typical leaf curl symptoms after 2-6 weeks Mishra *et al*., (1963); Muniyappa and Veeresh (1984).

B. tabaci can also transmit chilli leaf curl virus disease in a persistant manner (Thresh, 1974 and Brown, 1994). *B. tabaci* can rapidly disseminate viruses in the field even when populations are not appreciable, and cause severe crop damage in susceptible plantings.

Alao *et al*., (1992) investigated that typical symptoms exhibited 13-30 days after cross transmission of viruses by whiteflies (*Bemisia tabaci*) in capsicum. He further observed that an acquisition access feeding period of at least 24 hours was required by vector and transmission was achieved after a minute inoculation period of 30 minutes. A longer inoculation feeding gave a higher transmission, up to maximum at 120 minutes. The medium latent period in the vector was 4-72 h with a retention period of 2-6 days and all that white flies died within 10 days.

Efficiency of transmission of virus was found to increase with increase in number of adults per plant (Mehta *et al*., 1994), while female

whiteflies were more efficient than the male (Muniyappa *et al*., 2000).

Meena *et al*., (2006) recorded low whitefly population on symptomatic *Datura stramonium* and *Verbicini*a and symptom less *Calotropis gigantean*, *Chenopodium album* and *Amaranthu*s, suggesting that these perennial hosts might be playing an important role in virus perpetuation and transmission.

2.2 Begomoviruses Genome organization

A vital character of the virus is its genome organization. Members of the family *Geminiviridae* characteristically have circular single-stranded DNA genomes packaged within twinned (so-called geminate) particles. The bipartite genome comprises two single-stranded DNA (ssDNA) components of similar size (2.5-2.8 kb), arbitrary referred to as DNA-A and DNA-B. The nucleotide sequences of DNA-A and DNA-B are quite different, except for a short common region of ~ 200 nucleotides found to be very similar in the two DNAs. The common region includes a stem-loop structure, with the loop containing the nonanucleotide TAATATTAC, conserved across the genomes of all seven *Geminivirus* genera. It also includes the origin for rolling circle replication (Eagle *et al*., 1994; Laufs *et al*., 1995; Padidam *et al*., 1996; Orozco *et al*., 1998; Harrison and Robinson, 1999; Harrison and Robinson, 2002; Zhou *et al*., 2003).

Begomovirus indigenous to the western hemisphere has bipartite genome with components referred to as DNA-A and DNA-B. DNA-A encodes genes responsible for viral replication, regulation of gene expression, suppression of gene silencing and particle encapsidation. DNA-B encodes proteins involved in virus movement, host range determination and symptom development (Lazarowitz, 1992). But in the case of chilli, the (PepLCV) has

monopertite genome that contains DNA-A and segment.

Begomovirus genomes have a number of characteristics that distinguish Old World and New World viruses. All New World Begomoviruses are bipartite, whereas in the Old World, most of the viruses are monopartite and the majority of these associate with recently identified satellite molecules. In addition, all Old World Begomoviruses, with an exception of two unusual viruses, have an extra ORF (*AV2*) on the genome of DNA-A components that is not present in New World Begomoviruses (Rybicki, 1994; Stanley *et al*., 2005). Both DNA components contain protein-coding regions in the viral as well as complementary strand. Six such genes are universally present, DNA- A contains two genes (*V1* and *V2*) in the viral strand and three genes (*C1*, *C2*, *C3* & *C4*) in the complementary strand. DNA-B contains one gene (*V1*) in the viral strand and one gene (*C1*) in the complementary strand (Fig 2.1). Some of the known functions of those proteins are summarized in Table 2.1. Most monopartite Begomoviruses are associated with the satellites known as betasatellites and in many cases, these *Begomovirus*–betasatellite complexes are additionally associated with the satellite-like alphasatellites. The betasatellites have a single gene (*C1*) in the complementary sense and a region of sequence highly conserved between all betasatellites, known as the satellite conserved region (SCR), and a region of sequence rich in adenine (A-rich). The alphasatellites have a single large protein (Rep) and contain an A-rich sequence. For each component the conserved hairpin structure, containing the nonanucleotide sequence TAATATTAC (TAGTATTAC for most alphasatellites) within the loop structure, is shown at position zero (Sattar *et al*., 2013).

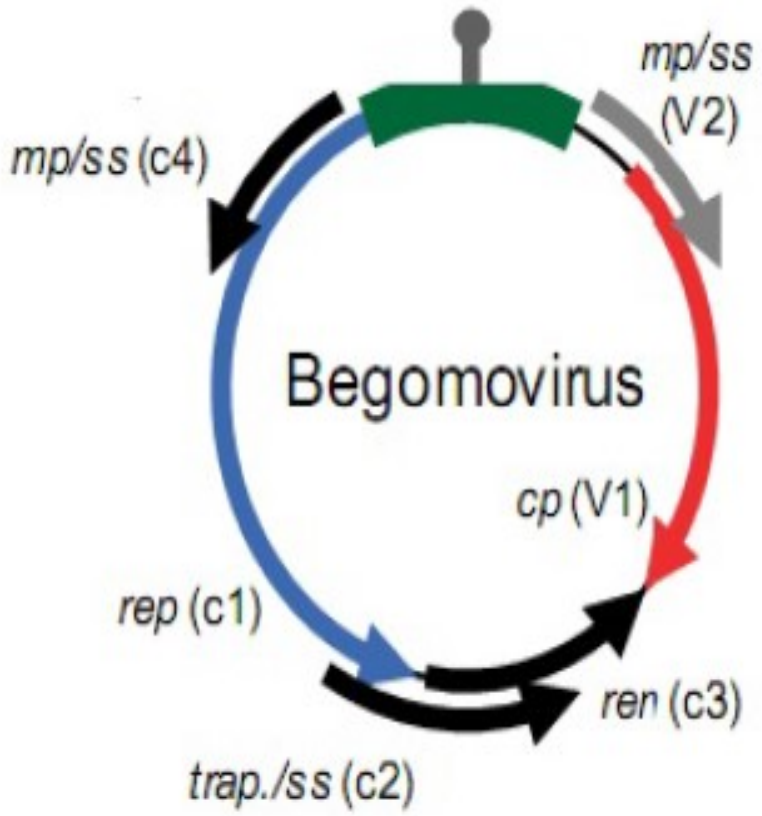


Fig 2.1 Schematic representation of different components of *Begomovirus* and their ORF

Table 2.1 Some known functions of *Begomovirus* genes

Gene	Protein	Functions
V1	CP	Whitefly-mediated transmission, virion capsid, Entry in nucleus
V2	AV2	Symptom development and viral DNA accumulation
C1	Rep	Replication associated protein. Viral DNA replication
C2	TrAP	Transcriptional activator for the virus-sense genes, suppresses RNA silencing and other host defense responses. Activate expression of the capsid protein gene
C3	REn	Enable viral replication
C4	NSP	Shuttling of viral DNA in nucleus
V1	NSP	Transport of viral DNA between the nucleus and cytoplasm and host range, properties of the virus
C1	MPB	Mediates the cell-to-cell movement and viral pathogenic properties

2.2.1 Nomenclature and Classification of Begomoviruses

Geminiviruses were earlier divided into four genera on the basis of their genome organizations and biological properties. (1) ***Begomovirus*:** The most important member of the group formed of viruses transmitted by the whitefly, *B. tabaci* (Gennadius) (Hemiptera: *Aleyrodidae*) to dicotyledonous plants, with Bean golden yellow mosaic virus (now Bean golden mosaic virus –Puerto Rico) as the type species. (2) ***Mastrevirus***: Virus with monopartite genome and transmitted by leafhopper vectors, primarily in monocotyledonous plants, represented by Maize streak virus as type species. (3) ***Curtovirus***: Viruses that had monopartite genomes distinct from those of the *Mastreviruses* and are transmitted by leafhopper vectors to dicotyledonous plants, having

Beet curly top virus as the type species. *(4)* ***Topocuvirus****:* Recognized late, having monopartite genome and transmitted by a tree hopper to dicotyledonous plants with only a single species, Tomato pseudo-curly top virus (also classified the type species), (Van *et al.,*1997; Fauquet *et al*., 2003; Fauquet *et al*., 2008). Further, International Committee on the Taxonomy of Viruses (ICTV) created a *Geminiviridae*. Study Group for assessing the taxonomic proposal of *Geminiviruses* under the chairmanship of Prof J. K. Brown, who proposed three more genera viz., ***Becurtovirus, Eragrovirus*** and ***Turncurtovirus*** with their type species Beet curly top Iran virus, Eragrostis curvula streak virus and Turnip curly top virus respectively. Among the different genera *Begomovirus* is the largest genera (288 species) followed by *Mastrevirus* (29 species), *Curtovirus* (3 species*), Becurtovirus (2 species).* Remaining three genera viz*., Topocuvirus, Eragrovirus* and *Turncurtovirus* have only one species each (Anon 2016).

The following guidelines are proposed for classification into species and strains:

1. A BLASTn analysis of the ‘‘non-redundant nucleotide’’ database should be performed to identify the species whose members have sequences most similar to the new sequence. The nucleotide sequence database at the NCBI website (http://www.ncbi.nlm.nih.gov/nuccore/) can also be searched using the search term‘‘txid10814 [Organism: exp] AND 2500:3500[SLEN]’’, which will return all *Begomovirus* nucleotide sequences that are between 2500and 3500 nucleotides long.
2. The new sequence should be added to a dataset of full length genomes or DNA-A components created based on the BLAST results, and saved in FASTA format. All sequences must start at

the same genomic coordinate (the first nucleotide after the nicking site within the conserved nonanucleotide at the origin of replication is the recommended standard).

3. The MUSCLE option in SDT v1.2 (freely available at http://www.cbio.uct.ac.za/SDT) or any other program that uses the MUSCLE alignment algorithm with pairwise deletion of gaps should be used to calculate identities between every pair of sequences in the dataset. If using SDT, these pairwise identities may be saved in either a column or matrix csv format that can be viewed in a spreadsheet program such as Microsoft Excel. Percent identities must be rounded to the nearest full percentile.
4. If the new sequence shares <91% genome-wide pairwise identity to any other known *Begomovirus* sequence, appropriate species and virus names should then be proposed (see below for guidelines on doing so).
5. If the sequence shares <94 % genome-wide pairwise identity to all isolates described for that species, a strain name should then be proposed.

2.2.2 Guidelines for naming new species that include newly discovered Begomoviruses

This is the ICTV-accepted name of a group of Begomoviruses sharing ≥ 91 % pairwise sequence identity for the full-length genome or DNA-A component. If the sequence has <91% sequence identity to all Begomoviruses previously classified as members of distinct species, the virus should be considered a member of a new species, and a unique name that is not currently in use for any ICTV recognized species should be assigned. This name should follow the template ‘‘Host symptom virus’’ (e.g., Bean golden

mosaic virus). Although it was common practice for Begomoviruses, the *Geminiviridae* Study Group recommends that country, city, town, village or province names not be used in naming new viruses and new viral species (e.g., Tomato yellow leaf curl Thailand virus), as this may cause misunderstandings when a virus named after a country or city is subsequently found in other locations within that country or in other countries. (Previously accepted names using this practice will not be changed to avoid conflicts in the literature.)

2.2.3 ChiLCV Classification and Taxonomic Position

Identification and characterization of the viruses is very difficult. Most of the viruses are identified by using different molecular techniques including PCR, however, only PCR is not sufficient to identify the virus, therefore, sequencing is often done for comprehensive identification, characterization and nomenclature of the viruses. ChiLCV belongs to the genus *Begomovirus* family *Geminiviridae*. The genus *Begomovirus* contains viruses that are transmitted by the whitefly, *B. tabaci* (Gennadius) (Hemiptera: *Aleyrodidae*) to dicotyledonous plants, with Bean golden yellow mosaic virus (originally Bean golden mosaic virus – Puerto Rico) as the type species. In the Old World (OW; Africa, Asia, Australasia and Europe), most Begomoviruses are monopartite, with a few having a bipartite genome. *Begomoviruses* native to the New World (NW; the Americas) are almost exclusively bipartite, with only a single monopartite virus having been identified.

2.3 Amplification and Sequencing of a part of DNA-A Molecule

2.3.1 Amplification and Sequencing

Hussain *et al*., (2004) reported that the presence of ToLCNDV in chilli (pepper), plants from several locations in the Punjab province of Pakistan and collected samples to confirm infection of ToLCNDV, a PCR procedure

was carried out using degenerate Begomovirus. DNA-A primers, and products were obtained from these samples. A nearly full-length clone of this DNA-A product was partially sequenced and was found to share 95% sequence identity with DNA A of ToLCNDV. These results confirmed ToLCNDV infection in chilli.

Senanayake *et al*., (2007) observed that 50–100% of chilli test plants, which produced vein clearing, curling and stunting symptoms. Electron microscopic examination of field samples revealed few, typical geminate particles. The presence of a *Begomovirus* was confirmed by PCR using the degenerate primers AVF28 5′ -GCCCACATYGTCTTYCCNGT-3′and AV29R 5′-GGCTTYCTRTACATRGG-3′, which gave a ca. 1·0 kb product. Cloning and sequencing of the PCR product yielded a 995 bp sequence (Acc. No. DQ445255). A BLAST search of GenBank revealed close similarity of the sequence with the intergenic region and part of the replication initiator protein, AV1 and AV2 genes of Chilli leaf curl virus-[Pakistan: Multan] (ChiLCuV-[Pk:Mul]; AF336806).

Kumar *et al*., (2011) found that chilli plants exhibiting leaf curl symptoms, collected from Palampur, Himachal Pradesh region of India, were associated with a *Begomovirus* and a betasatellite-like molecule. Viral DNA was amplified, cloned and sequenced. The begomoviral genome and the betasatellite consisted of 2775 and 1376 nucleotides respectively. The virus appeared to be monopartite. The genome sequence had <87% identity with all other *Begomovirus* sequences, below the threshold for species demarcation, suggesting that the isolate represents a distinct species for which the name Chilli leaf curl Palampur virus (ChiLCPaV) is proposed.

Two more *Begomovirus* species, *Cotton leaf curl Multan virus* (CLCuMV) (Hussain *et al*., 2003) and *Pepper yellow leaf curl Indonesia virus*

(PepYLCIDV) (Tsai *et al*., 2006), have been reported to be associated with chilli leaf curl in Pakistan and Indonesia respectively. Sequence analysis showed that the virus isolated from Jodhpur was distantly related (59.1- 67.9 % identity) to CLCuMV, PepYLCIDV and ToLCNDV. However, it shared 96.5% identity with ChiLCuV[Pak:Mul]. Given the close sequence identity with ChiLCuV[Pk:Mul], the virus isolated from Jodhpur is considered to be ChiLCuV

Saeed *et al*., (2014) reported that leaf yellowing, mosaic crinkling, and cupping symptoms were observed in spearmint (*M. spicata*), in the experimental fields and farmer fields of Uttar Pradesh province, India. Eighteen symptomatic samples were collected and screened for the presence of a *Begomovirus*. DNA from these samples was used as PCR template to amplify a 771bp fragment using *Begomovirus* coat protein gene specific primers. Eleven of the 18 samples (61.1%) were positive. PCR products were cloned into the pGEMT Easy and sequenced using the universal M13F/M13R primers. The sequence showed similarity with that of Chilli leaf curl India virus. To amplify the full length DNA A/ B and a possible β satellite, detection was conducted using the rolling circle amplification method. Sequence analysis showed maximum nucleotide identity (99%) with Chilli leaf curl India virus (FM877858). This is thought to be the first report of Chilli leaf curl India virus infecting *Mentha spicata* in India.

Khan and Khan (2016) extracted Full-length genomes of a *Begomovirus* and an associated betasatellite were amplified, cloned and sequenced. The size of the *Begomovirus* genome and the betasatellite were 2760 bp and 1374 bp, respectively. The nucleotide sequence of the *Begomovirus* genome shared maximum identity (89%) with pepper leaf curl Bangladesh virus-India isolate Chhapra (PepLCBV, JN663853) Sequence

analysis showed that the *Begomovirus* is a potential recombinant between viruses related to PepLCBV and chilli leaf curl virus (ChiLCV). The name chilli leaf curl Gonda virus (ChiLCGV) is being proposed.

Jaidi *et al*., (2017) isolated the total DNA from the leaves of 17 diseased and one asymptomatic *M. jalapa* plants and subjected to PCR using *Begomovirus* degenerate primers. An amplicon of the expected size (c. 1.2 kb) was produced from all 17 diseased samples, confirming *Begomovirus* infection. The full length virus genome (c. 2.7 kb) was amplified using the rolling-circle amplification method, cloned and sequenced (GenBank Accession No. KX951415). The sequence showed (92-99%) nucleotide sequence identity and a close phylogenetic relationship with isolates of Chilli leaf curl India virus (ChiLCINV) identified in *Capsicum sp*. (FM877858), and *Mentha arvensis* (KT779820) and *spicata* (KF312364) from India. This shows the virus detected in *M. jalapa* to be an isolate of ChiLCINV.

2.4 Diversity analysis of the sequence and comparison with other Begomoviruses

Senanayake *et al.,* (2006) reported that the genome of ChiLCV-[Nar] (EU939533) is 2756 nt long and contains six open reading frames (AC1, AC2, AC3, AC4, AV1 and AV2). The genome organization of the virus isolate was similar to ChiLCV reported previously. The sequence comparison of ChiLCV-(Nar) isolate with nine other ChiLCV isolates occur-ring in the Indian subcontinent revealed up to (12.3%) sequence diversity. ChiLCV-[Nar] was closes to the ChiLCV isolates reported from Potato in Lahore, Pakistan (FM179613), and chilli in Varanasi, India (EF190217), sharing (96.1%) sequence identity. The present isolate showed significant sequence diversity from one each chilli isolate reported from India (DQ673859) and Pakistan (DQ116 b878).

Chattopadhya *et al*., (2008) performed Phylogenetic analyses based on a multiple alignment of the present isolate and 48 other well characterized Begomoviruses. The present virus isolate clusters together with Begomoviruses infecting chillies in Pakistan and India and is most closely related to ChiLCV-PK[PK:Mul:98] and ChiLCV-Kha[PK:Kha1:05].However, ChiLCV-PK[IN:Var:06] is distantly related to chilli-infecting *Begomoviruses* from Bangladesh, such as PepLCBDV-BD[BD:Bog:99] and PepLCBDVPK [PK:Kha:04].

Rai *et al*., (2010) reported that understanding the diversity and distribution of ChiLCV variants/strains is crucial for effective screening of Capsicum germplasm against virus. The emergence and existence of different variants of ChiLCV, infecting chilli in North India. Phylogenetic analysis the CP DNA sequences of four isolates and 25 representative sequences from GenBank were used to generate the phylogenetic tree for the aligned sequences formed three major clusters. The first cluster consisted of 20 isolates, which were further separated into four sub-clusters. The second and third clusters consisted of 5 and 4 isolates, respectively. The Varanasi isolate (ChiLCV-VNS) grouped with ChLCV[AMT] (GU136803.1) in first cluster. Isolates from Gorakhpur (ChiLCV-GKP), Mahrajganj (ChiLCV-MAH) and Mirzapur (ChiLCV-MZP) showed (99%) nucleotide identity and clustered with ToLCJV (DQ673859.1 and EF194765.1) in second cluster. Percentage sequence identity within each cluster was in agreement with the criterion of <90% CP sequence identity representative for different geminivirus species.

Sinha *et al*., (2011) identified CP-PepLCV consists of 792 nucleotide, which encodes 194 amino acid residues with a mass of 28 kD. The nucleotide sequence is accessible as accession number FJ968525at Gen Bank data base (www.ncbi.nlm.nih.gov) . The analysis of coat protein coding region

sequence data and its alignment with other chilli leaf curl virus isolates revealed its 98% identity with pepper leaf curl virus previously reported from Narwan, Amritsar and Varanasi regions of India. It also showed (97%) identity to papaya leaf curl virus from New Delhi, 96% identity to chilli leaf curl virus from Multan (Pakistan) and 95% identity to pepper leaf curl Bangladesh virus. This indicates that, the virus is an isolate of PepLCV.

Kumar *et al*., (2012) reported that the Vellanad virus have highest levels of nucleotide sequence identity (77%) with a 'Pakistan" strain of Pepper leaf curl Bangladesh virus (AF314531). With other chilli-infecting Begomoviruses in the Indian sub-continent, there was between 64 and 76% sequence identity. The present virus is distinctly placed in a separate clade in the phylogenetic tree. It has to be considered a new *Begomovirus* species for which the name Chilli leaf curl Vellanad virus (ChiLCVeV) is proposed. The associated betasatellites (GenBank Accession Nos.JN663876,1355 nt and JN663877,1372 nt) each contain a single ORF C1. They share maximum identity of 95.6% and 96.9% with Radish leaf curl betasatellite (EF175734) and Tomato leaf curl Bangladesh betasatellite (AY438558), respectively.

Khan *et al.,* (2013) conducted survey during 2010-2011 to collect samples and assess the diversity of Begomoviruses associated with leaf curl disease of Pepper. A virus previously only identified on the Indian subcontinent, chili leaf curl virus (ChiLCV), was found associated with Tomato and Pepper diseases in all vegetable grown areas of Oman. Some of the infected plant samples were also found to contain a Betasatellite. A total of 19 potentially full-length *Begomovirus* and eight Betasatellite clones were sequenced. The *Begomovirus* clones showed >96% nucleotide sequence identity, showing them to represent a single species. Comparisons to sequences available in the databases showed the highest levels of nucleotide sequence

identity (88.0-91.1%) to isolates of the "Pakistan" strain of ChiLCV (ChiLCV-PK), indicating the virus from Oman to be a distinct strain, for which the name Oman strain (ChiLCV-OM) is proposed.

Sing *et al*., (2013) performed a phylogenetic analysis based upon alignment of CP gene of detected *Begomovirus* with other selected Begomoviruses sequences showed that the CP gene forms a cluster with other ToLCNDV isolated from northern India. Nucleotide sequence identity matrix build on alignment of CP gene sequence of detected *Begomovirus* and other selected Begomoviruses revealed that the CP gene sequence was 97.2% identical to Tomato Leaf Curl Virus coat protein gene (AY691902) while the lowest nucleotide homology of 94.4% was found to be with Squash Leaf Curl China Virus isolate (GQ225732). In phylogenetic analysis the reported betasatellite clustered with ToLCB (HM143901) and ChLCB (HM143904) Nucleotide sequence identity matrix build on alignment of betasatellite sequence of detected *Begomovirus* and other selected Begomoviruses betasatellites showed that detedcted betasatellite was (95%) identical to Chilli leaf Curl Betasatellite reported from Panipat (HM143904).

George *et al.,* (2014) reported that complete nucleotide sequence of the *Begomovirus* isolated from Amaranthus was found to be of 2,755 nt (KF471061) and sequence analysis showed the genome organization of a typical of OW Begomoviruses with six open reading frames (ORFs). Full-length sequence and all the encoded ORFs of the identified *Begomovirus* share maximum per cent identity of 95% with chilli leaf curl virus. It also segregated with chilli leaf curl virus in the phylogenetic dendrogram and it is considered to be an isolate of chilli leaf curl virus. The alphasatellite of 1,392 nt (KF471047) has the capacity of encoding 315 aa protein (coordinates 76–1,023 nt) similar to Rep protein of nanoviruses, A-rich region (coordinates 1,010–

1,157 nt) and a predicted hairpin structure with the loop sequence of TAGTATTAC. Alphasatellite identified here shares a maximum nucleotide identity of 96 % with unreported chilli leaf curl alphasatellite.

Mugisa *et al*., (2014) prepared two phylogenetic trees from the aligned sequences using the neighbour-joining method and bootstrap option of tree conversion (1000 bootstrap replicates). In the first phylogenetic tree total of 45 different viruses were aligned together. Whereas in the second phylogenetic tree all the legume viruses were aligned together. Results of the first phylogenetic tree revealed that all the Begomoviruses separated in to two distinct clads. One clad comprised all the Begomoviruses infecting different crops *viz*., cotton, cassava, alternanthera, ageratum, clerodendron, okra, tomato, papaya and chilli and another clad consisted variants of different infecting legumes. This indicated that the Begomoviruses infecting legumes are entirely distinct from the Begomoviruses infecting other crops.

Nehra and Gaur (2015) studied all the fifteen positive clones were sequenced and analyzed for the identity. All the sequences showed (100%) identity from each other, therefore, only two sequences of each DNA-A (KJ700653 and KJ700656) and betasatellites (KJ700654 and KJ700655).The full length sequences of both the clones of DNA-A and betasatellites show maximum identity of 99% with the chilli leaf curl virus and hence are considered as isolates of ChLCuV which infect new host Petunia hybrid.

Comparison of results with other Begomoviruses phylogenetic analysis of both the clones of DNA-A demonstrated most proximity with each other. Both showed the highest similarity with ChLCuV: HM007104 isolate as indicated in the dendogram. Both clones of identified *Begomovirus* from petunia isolates shared 99% sequence identity with ChLCuV: HM007104. The analysis of both betasatellite clones with other Indian Begomoviruses

illustrated close relationship with ChLCuV: JF706231. They also showed 99 % identity with ChLCuV as indicated in the cluster of phylogenetic tree. Phylogenetic relationship with other Indian Begomoviruses revealed clear evidence of phylogenetic conflicts. The presences of this clusters clearly indicates that the epidemiology of these viruses may be more threatening during the coming years.

Kumar *et al*., (2015) diversity and phylogenetic analysis of ChiLCD associated Begomoviruses analysis showed that 35 out of the 41 molecules were 2.8 kb in size with a DNA-A/DNA-A-like *Begomovirus* genome arrangement, whereas six molecules had a DNA B-like genomic organization. Betasatellites were present in all the samples irrespective of whether the *Begomovirus* genome detected was either monopartite or bipartite. Based on 91% nucleotide sequence identity as the species demarcation threshold for DNA-A and by pairwise sequence comparison, these DNA-A/DNA-A like isolates were categorized into seven distinct species including one newly identified species (HM007119). When compared with the genomic sequences available in GenBank, the majority of these sequences had high similarity with previously reported Begomoviruses associated with chilli.

Khan and Khan (2016) found that ChiLCGV (Gonda *Begomovirus* isolate) shares the highest nucleotide sequence identity (89%) with (pepper leaf curl Bangladesh virus) PepLCBV. This is less than the threshold value (91%) for species demarcation in Begomoviruses according to ICTV guidelines. ChiLCGV and ToLCBDV have been described and characterized for the first time from the Gonda region of India where, in combination, they represent a new *Begomovirus*-betasatellite complex, infecting capsicum.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

The experimental materials utilized, methods employed and techniques adopted during the course of present investigation entitled "Biological and Molecular Characterization of Chilli Leaf Curl Virus (ChiLCV) in South Gujarat" are described hereunder.

3.1 Glassware and chemicals

All the glasswares used in the experimental work were of Borosil grade (Corning glass). Chemicals were procured from Biogene, USA, Biorad, USA, Applied Bioscience, USA, Merck India, Bangalore, Hi-Media Laboratories Pvt. Ltd. Mumbai, India, etc. Primers got constructed from Ocimum biosolutions Ltd. Hyderabad, India. All the glasswares were cleaned up with chromic acid and thereafter autoclaved at 15 lbs pressure for 20 minutes. The instruments and devices used during experimentation have been marked with notation wherever necessary.

3.2 Symptomatology of diseases

The symptoms of Chilli Leaf Curl Virus (ChiLCV) were studied from the naturally infected chilli plants first in the field. Specific plant was collected and brought to the greenhouse. Symptoms were also observed from the different field in and around Navsari Agricultural University farm, Navsari. Healthy uninfected plant of the same age was maintained for the comparison of the symptoms and its gradual changes.

3.3 Amplification and Sequencing of a part of DNA-A Molecule of chilli leaf curl virus infecting chilli

3.3.1 Isolation of viral DNA

Viral DNA was isolated by the CTAB method given by (Doyle and Doyle, 1987) with slight modification. Different buffers and steps followed for the isolation of viral DNA from the different tissues of the plants were as under.

Table 3.1: DNA extraction buffer

Sr. No.	Stocks	Final concentration	Quantity for buffer (10 ml)
1.	CTAB (Cetyl Trimethyl Ammonium Bromide)	2 per cent	200 mg
2.	Tris buffer stock, 1M (pH 8.0)	100 mM	1 ml
3.	EDTA stock 0.5M (pH 8.0)	20 mM	400 µl
4.	PVP (Polyvinyl Pyrrolidone)	2 per cent	200 mg
5.	2-Mercapto ethanol	1 per cent	100 µl
6.	Sterile Millipore Water		Volume made up to 10 ml

3.3.2 Materials / Equipments used

1. Water bath
2. Conical flask
3. Mortar and pestle
4. Tissue paper
5. Centrifuge machine
6. Micropipettes and Micropipette tips

3.3.3 Viral DNA was isolation (CTAB method and its modification) as follows

1. Collected tissue (500mg)
2. Crushed the tissue in liquid nitrogen (N_2) with in a sterile pastel and mortar
3. Collected the crushed material in eppendorf tube and added 700µl CTAB extraction buffer
4. Kept the eppendorf tube at 60-65^0 C for one hour in water bath
5. Added 700 µl Chloroform: Isoamyl Alcohol (24:1) after one hour and vortex it
6. Spun at 5000 rpm for 15 min in centrifuged machine (Eltek Equipment (Mumbai) Pvt. Ltd.)
7. Took supernatant (approx 450 µl)
8. Added 700 µl Chloroform: Isoamyl Alcohol (24:1) in the supernatant
9. Mixed it thoroughly
10. Spun at 5000 rpm for 15 min.
11. Took supernatant and added 700 µl Isopropyl Alcohol (Shake it vigorously)
12. Incubated the tubes at -20^0 C for 2 hours
13. Spun at 5000 rpm for 20 min.
14. Discarded supernatant and added 70 per cent ethyl alcohol.
15. Spun at 10000 rpm for 10 min.
16. Discarded supernatant and added 70 per cent ethyl alcohol
17. Spun at 10000 rpm for 10 min.
18. Collected the pallet

19. Dried it at room temperature
20. Added 50 µl TE buffer
21. Incubated over night at 4^0 C.
22. Measured the quantity of DNA with the help of spectrophotometer (Nanodrop)
23. Tested DNA quality by running on 0.8 per cent agarose gel
24. Documented the gel by GelDoc (SYNGENE, UK).

3.3.4 Polymerase Chain Reaction (PCR)

3.3.5 Materials/Equipments used

1. Micropipettes
2. Micropipette tips
3. Thermo cycler
4. Micro centrifuge
5. Eppendorf tube

3.3.6 Primer

PCR amplification using primer pair LVF+LVR (LVF=TCTCAACTTCGACAGCCCATC+LVR=ATAGGTCCAGTGGCGTT TGA) for the amplification of the DNA-A molecule of *Begomovirus* will be used for the amplification of the DNA-A molecule of the virus. PCR amplification will be carried out in 25 µl reaction volume containing 50 ng genomic DNA, 2.5 µl PCR buffer (MBI Fermentas, Hanover, USA), 200 µM dNTPs (Merk, Bangaluru, India), 1.5 U Taq DNA polymerase (MBI Fermentas) and 0.4 µM primer using a thermal cycler (Eppendorf, Germany).

Table 3.2 PCR mixture

Sr. No.	Stocks	Quantity
1.	*Taq* Buffer (10x)	2.5 µl
2.	*Taq* DNA polymerase (3 U/µl)	0.6 µl
3.	dNTPs	0.6 µl
4.	$MgCl_2$	2.0 µl
5.	DNA template	2.0 µl
6.	Forward primer	2.0 µl of (10 pmoles/ml)
7.	Reverse primer	2.0 µl of (10 pmoles/ml)
8.	Sterilized Millipore water.	13.3 µl

3.3.7 Protocol of PCR

1. Extracted DNA of the samples by CTAB method with slight modification as present in the Extraction of DNA.
2. PCR reaction mixture was prepared by mixing different chemicals, buffers, primer, etc. as presented in the Table 3.2
3. Filled the PCR tubes with 23 µl PCR mixture.
4. Added 2 µl DNA from the test sample in above tube.
5. Added 2 µl sterilized Millipore water in water control. Similarly added 2 µl DNA extracted from the healthy leaf for the negative control and 2 µl DNA extracted from the characterized infected plants leaves for the positive control.
6. Programmed the Thermocycler as given in the Fig 3.1 and Table 3.3
7. Run PCR products on 2.0 per cent (w/v) agarose gel in 0.5XTBE buffer at 100 mV for 1 hr. Gels with amplified

fragments can visualized and photographed under UV light using SYNGENE Bio imaging system.

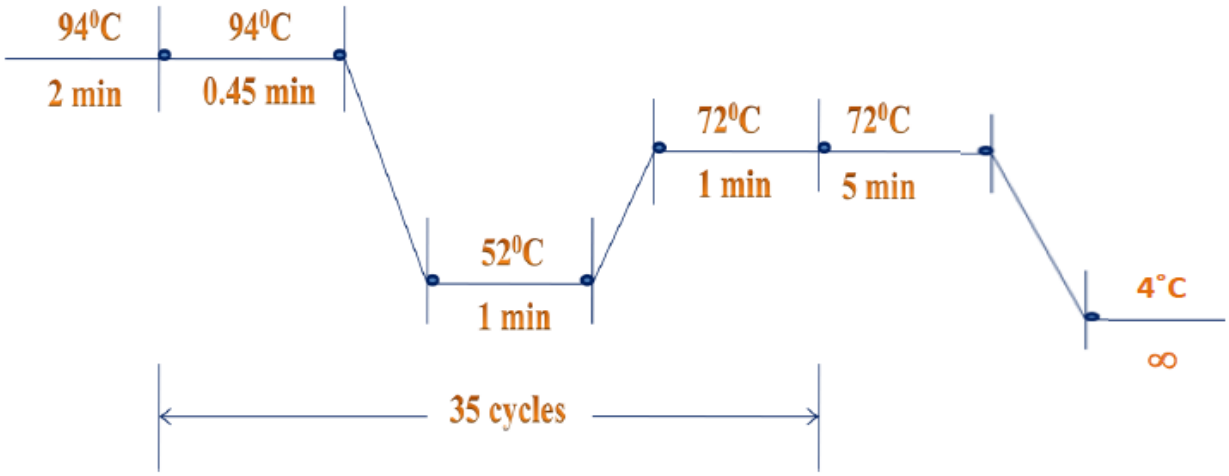


Fig: 3.1 Schematic representation of programme of the Thermocycler for the amplification of viral DNA

Table 3.3: Programme of the Thermocycler for the amplification of viral DNA

Stage	Time	Temperature	Cycles
Denaturation	2 min	94^{0}C	
Denaturation	45 sec	94^{0}C	
Annealing	1 min	52^{0}C	35
Extension	1 min	72^{0}C	
Final Extension	5 min	72^{0}C	
Storage	∞	4^{0}C	

3.4 Running of DNA on Agarose gel (Electrophoresis)

3.4.1 Materials/Equipments used

1. Electrophoresis apparatuses, electrodes and power supplies
2. Micropipette
3. Micropipette tips

3.4.2 TBE buffer (1 lit 5x stock solution)

Tris base : 53.0 g
Boric acid : 27.5 g
EDTA : 20 ml

3.4.3 Gel electrophoresis process

1. Placed the comb at the negative electrode.
2. Took 8g of agarose and added to 100 ml sterilized Millipore water to make 0.8 gel. This was stored at the room temperature and used as and when required.
3. The above agarose mixture was melted in microwave oven at the time of use.
4. Approx 7 µl Ethidium Bromide was added to the luke warm agarose gel and poured in the gel casting tray to make approximately 5 mm thick gel.
5. Gel tray was kept undisturbed to achieve uniform solidification of the gel.
6. Tray was kept in the electrophoresis tank filled with TB running buffer.
7. Gently removed the comb and filled the wells with the marker and DNA/PCR product.
8. Connected electrophoresis unit with the power pace and run the unit at 80 mV (max = 100 mV) for 10-15 minutes or the

tracking dye reaches to the end.

3.5 Purification and Sequencing amplified products

1. Prepared reaction mixture by adding 3.0 µl of 3 M sodium acetate (NaOAc), pH 4.6, 62.5 µl of non-denatured 95% ethanol (EtOH), 14.5 µl of deionized water to make final volume 80 µl for each sample.
2. Added 80 µl of this ethanol/sodium acetate solution was to20 µl of reaction mixture from the thermal cycler.
3. Sealed the tubes with strip caps.
4. Inverted tubes few times to mix well.
5. Incubated the tubes at room temperature for 15 minutes to precipitate the extension products.
6. Centrifuged the content at 12000g for 20min at RT
7. Discarded supernatant by inverting the tube onto a paper towel.
8. Added 150 µl of 70% ethanol to each pellet.
9. Caped and inverted tubes few times to mix well.
10. Spun tubes for 10 minutes at 12000g
11. Dried pellet and added 15µl of Hi-Di formamide for denaturation of DNA.
12. The samples were snap chilled and then transferred 10µl of sample to 96- well sequencing plate.
13. Performed the reaction.

3.5.1 Sequencing of Amplified product

1. Prepared reaction mixture by adding Terminator Ready Reaction Mix (Big Dye sequencing kit 3.1v provided by Applied Bio systems) 4.0 µl, Primer 3.2 pmol, 20 ng PCR product, Deionized water to make a total volume of 20 µl.
2. Mixed reaction mixture well and spun briefly.
3. Placed tubes in a thermal cycler, the volume set to 20 µl and sequencing done with the following programme. (Table 3.4)
4. Data were retrieved from the sequencer and further analyzed.

Table 3.4: Programme of the Thermocycler for the sequencing PCR

Stage	Time	Temperature	Cycles
Denaturation	2 min	96^0C	
Denaturation	10 sec	96^0C	
Annealing	10 sec	50^0C	35
Extension	4 min	60^0C	
Final Extension	5 min	60^0C	
Storage	∞	4^0C	

3.6 Diversity analysis of the sequence and comparison with other Begomoviruses

3.6.1 Multiple alignment and Similarity Index

Database searches for *Begomoviruse* sequences, Basic local Alignments and similarity index analysis were carried out using NCBI-BLASTN program (http://blast.ncbi.nlm.nih.gov). Multiple nucleotide (nt) sequence alignments were performed for part of DNA-A of the virus isolate

with other Begomoviruses reported from India and worldwide by using CLUSTALW (2.1) programme (http://www.ebi.ac.uk/clustalw).

3.6.2 Finding of Open Reading Frame (ORF)

ORFs of our Sequence were determined Using Open reading Frame finder of the NCBI software (www.ncbi.nlm.nih.gov/projects/gorf/).

3.6.3 Construction of Phylogenetic Tree

Representative species, strains and variants of different *Begomovirus* species from the different crops and geographical location were selected for the phylogenetic analysis. Full length sequences of these viruses were downloaded from NCBI website. Downloaded sequences were chopped corresponding to the sequence of our amplified gene. Phylogenetic tree was constructed by using online software using Neighbor-joining method with 1000 bootstrap replication in the MEGA version 4.0 (Tamura *et al*., 2007).

RESULTS AND DISCUSSION

IV. RESULTS AND DISCUSSION

Chilli leaf curl disease incited by the different species of the *Begomovirus* is the most significant biotic disease in limiting production and productivity of chilli across the countries. Leaf curling due to the sucking insect pest and viral diseases is often confuse. Most of the cases it has been assumed that the disease complex is caused by a whitefly transmitted *Begomovirus*. However, the ChiLCVD complex is caused by the feeding of thrips, mites and whiteflies as well as by the infection of whitefly transmitted *Begomovirus* (Dharamasena, (1998); Mahatma *et al.,* 2014). Leaf curling due to sucking insect pest is reversible and can be managed through insect control; however, leaf curl due to chilli leaf curl virus (ChiLCV) is irreversible and cannot be controlled. Therefore, a robust technique to identify the pathogen in the field and further its characterization is one of the critical stages in the management of the disease. However, the satisfactory information to clearly differentiate the symptoms produced by the sucking insect pests and ChiLCV as well as molecular characterization of the virus causing leaf curl in south Gujarat is are not available. Therefore, the present investigation was carried out to generate information about the same under the title "Biological and Molecular Characterization of Chilli Leaf Curl Virus (ChiLCV) in South Gujarat". Results obtained during the course of present investigation have been presented hereunder the different heads and have been discussed with the support of available literature.

4.1 Incidence of the disease in the different field

High incidence of the disease in the different field of NAU as well as the adjoining area could be seen during the survey in 2016. Total 7 different fields of chilli in the villages around the Navsari Agricultural University, viz., Supa, Munsad, Ugat, Gandevi, Bardoli and Gandeva were

surveyed. The crop grown in the Vegetable breeding Farm and Horticulture farm of N.A.U., Navsari fields also surveyed. Typical leaf curl symptoms were seen in all the area surveyed ranging from 10-35 per cent (Table 4.1). Maximum 35 per cent incidence was observed in farmer's field of Gandeva having local selection. The farmer was poorly managed and almost all the plants were either having leaf curling due to *Begomovirus* or due to sucking insect pests. Minimum 10 per cent infection was observed in the farm of village Supa and Ugat, in both the field farmer sown local variety. Variation in disease incidence was mainly due to the different stages of the crop at different place and the management practices followed. Heavy incidence of the disease is very common in the different parts of the country and have been documented by Hussain, 1932; Uppal, 1940; Mishra *et al*., 1963; Singh *et al*., 1979; Rishi and Dhawan, 1988; Sanger *et al*., 1988; Singh *et al*., 1990; Mallick and Chowdhury, 1996. The crop was at various stages of growth and typical leaf curl symptoms with the upward/downward curling along with the vein clearing of leaves, stunting, vein thickening, vein swelling, shrinking of leaves were observed during the present investigation. (Plate-I&II). In every infected field surveyed, whiteflies were found invariably. Mites and thrips were the other insect pests noticed.

4.2 Study of the symptoms produced by the ChiLCV infection

Early infected plant becomes stunted. Fruit bearing reduced drastically on the infected plant. Early infected plant generally did not have flower, even if few flower were developed they dropped or produce very few small sized fruits. Infected Chilli plants showed typical leaf curl symptoms with the upward/downward curling of leaves, puckering and reduction in size of leaves along with the thickening and swelling of veins. Affected plants grew slowly and became stunted or dwarfed. Severity of the symptoms varied from place to place because of the environmental condition, host resistance and virus strain and isolates. Symptoms were in

accordance to the previous observations of Uppal 1940; Mishra *et al*., 1963; Muniyappa and Veeresh, 1984.

Table 4.1: Survey of Chilli Leaf Curl Virus Disease incidence in Navsari during late winter to onset of summer of the year 2016

Sr. No.	Village	Variety	Disease incidence (%)
1.	Supa	Local	10.00
2.	Vesma	Local	15.00
3.	Munsad	Tejas – 4	20.00
4.	Ugat	Local	10.00
5.	Gandevi	Indam - 42	20.00
6.	Bardoli	Local	18.00
7.	Gandeva	Local	35.00
8.	N.A.U. Vegetable breeding farm	Breeding materials	12.00
9.	N.A.U. Horticulture farm	Varietal trial	15.00

Plate-I : Field view of Chilli plants showing natural severe symptoms of ChiLCV infection

A- Healthy plant

B- Infected plant showing typical upward leaf curling

Plate-II: Symptoms observed on chilli plant due to ChiLCV infection

A- Early infected plant showing stunting

B- Mild vein clearing on the leaves

4.3 Amplification and sequencing of a part of DNA-A Molecule of Chilli Leaf Curl Virus infecting Chilli

4.3.1 Isolation of viral DNA

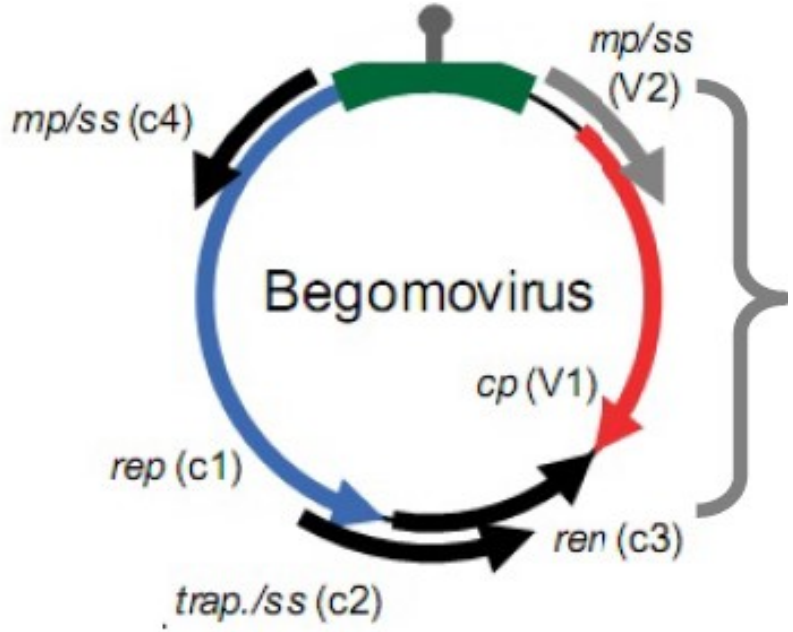
Viral DNA was isolated from younger symptomatic leaves of Chilli plants by CTAB method given by Doyle and Doyle, (1987) with slight modification as described in the chapter three. After DNA extraction, the DNA concentration was determined using UV spectrophotometry to ascertain its quality and quantity. Accordingly different quantities of the DNA were used for the PCR reaction to have uniform result. Isolated DNA was resolved by running on 0.8 % Agarose gel and visualised under Gel Doc. The method found very versatile for the isolation of DNA from the leaf tissue.

4.3.2 Genome amplification

PCR amplification using primer pair LVF + LVR (LVF=TCTCAACTTCGACAGCCCATC+LVR=ATAGGTCCAGTGGCG TTTGA) was done to amplify a part DNA-A molecule and it showed ~1200 bp band of DNA-A molecule (Plate-III). Amplification of the DNA-A fragment with the specific primers suggested that the virus in question is a *Begomovirus*. This primer pair has earlier been used by different scientists (Chakraborty *et al*., 2003 and Hussain *et al*., 2004) for the amplification of viral DNA-A molecule.

4.3.3 Sequencing of the part of the DNA-A molecule of ChiLCV

Amplified fragment was purified, and sequenced in an automated DNA sequencer by the Cycle sequencing method (Big Dye sequencing kit 3.1v). A 932 bp nucleotide sequence was obtained (Appendix-I). ORF obtained by the ORF finder of the NCBI software indicated that the sequence is having two ORF. Comparison of the sequence with the other standard universal ORF of the DNA-A molecule indicated that



Part of the DNA-A molecule sequenced

Plate-III: Genome organization of the whole DNA-A particle verses part of the DNA-A sequenced

the amplified fragment have two genes viz., virus coat protein (*V1*) gene and precoat protein (*V2*) gene. This genome organization is in agreement with the typical genome organization of Begomoviruses (Van *et al*., 2000; Navot *et al*., 1991). Amplification of the DNA-A fragment with the specific primers suggested that the virus in question is a *Begomovirus*. Further matching of ORF with other Begomoviruses confirmed the virus to be a *Begomovirus*.

4.4 Diversity analysis of the sequence and comparison with other Begomoviruses

4.4.1 Multiple alignment and Similarity Index

Database searches for *Begomovirus* sequences (Table 4.3), Basic local alignment and similarity index analysis were carried out by NCBIBLASTN program (http://blast.ncbi.nlm.nih.gov/). Multiple nucleotide (nt) sequence alignments were performed for part of DNA-A of the virus isolate with other Begomoviruses reported from India and worldwide by using CLUSTALW (2.1) programme (http://www.ebi.ac.uk/clustalw). The sequenced virus showed highest identity 99% with Chilli leaf curl India virus isolate India:Sonipat: TC 290:2010 segment DNA-A, [KJ649706.1] followed by 98% sequence identity with Chilli leaf curl virus-India isolate Ahmedabad segment DNA-A, [JN663846.1] and Chilli leaf curl virus isolate Narwan segment DNA –A [EU939533.1], and other viruses which were close related were ChiLCV[IN:ND:03], ChiLCV-[Mul]-[Pak:Mul:03], ChiLCV-[Pak]-VIRO-[IN:ND:15], having 97% sequence identity and some Begomoviruses of chilli are ChiLCV[IN:Amr:Pap:09], PepLCV-Pa-[IN:09], PepLCV-Jorehat-[IN:09], PepLCV-Nagpur-[IN:09], ChiLCMVI-[IN:Gun:09], ChiLCV-Taq2-[Oma:11], ChiLCV-Sh1-[Omn:11], (Table 4.2).

Table 4.2: Percent identities (nucleotide) between part of DNA- A ChiLCIV-[IN:Son:Nvs:16] with the selected Begomoviruses reported worldwide

Description	Acronym	Accession number	% Nucleotide sequence
Chilli leaf curl India virus isolate India:Sonipat: TC 290:2010 segment DNA-A, complete sequence	ChiLCIV-[IN:Son:TC290:10]	KJ649706.1	99%
Chilli leaf curl virus-India isolate Ahmedabad segment DNA-A, complete sequence	ChiLCVI-[IN:Ahm:09]	JN663846.1	98%
Chilli leaf curl virus isolate Narwan segment DNA A, complete sequence	ChiLCV-Nar-[IN:04]	EU939533.1	98%
Chilli leaf curl virus isolate New Delhi segment DNA-A, complete sequence	ChiLCV-[IN:ND:03]	JN663866.1	97%
Chilli leaf curl virus-[Multan] segment DNA-A, complete sequence	ChiLCV-[MUL]-[Pak:Mul:03]	AF336806.1	97%
Chilli leaf curl virus-[Pakistan] isolate VIRO 764 segment DNA-A, complete sequence	ChiLCV-[Pak]-VIRO-[IN:ND:15]	KR957353.1	97%
Chilli leaf curl virus isolate India:Amritsar:Papaya:2009 segment DNA A, complete sequence	ChiLCV[IN:Amr:Pap:09]	GU136803.1	96%
Pepper leaf curl virus isolate Palampur segment DNA-A, complete sequence	PepLCV-Pa-[IN:09]	JN663870.1	96%
Pepper leaf curl Varanasi virus isolate Jorehat segment DNA-A, complete sequence	PepLCV-Jorehat-[IN:09]	JN663861.1	94%
Pepper leaf curl Lahore virus isolate Nagpur segment DNA-A, complete sequence	PepLCV-Nagpur-[IN:09]	JN663864.1	93%
Chilli leaf curl Multan virus-India [India/Guntur/2009] segment DNA-A, complete sequence	ChiLCMVI-[IN:Gun:09]	HM007100.1	93%
Chilli leaf curl virus isolate Taq2, complete genome	ChiLCV-Taq2-[Oma:11]	JN604493.1	92%
Chilli leaf curl virus isolate Sh1, complete genome	ChiLCV-Sh1-[Omn:11]	JN604491.1	91%

Table 4.3: Begomoviruses with their accession numbers from GenBank database used for sequence analysis and phylogenetic comparison

Viruses	Virus-Acronym	Accession numbers
African cassava mosaic virus-[Nigeria:Ricinus communis:2003]	ACMV-[NG:Rc:03]	EU685324.1
African cassava mosaic virus-[Nigeria:Glycine max:2007]	ACMV-[NG:Sb:07]	EU685325.1
African cassava mosaic virus-[Benin: Con1017:2014]	ACMV-[BN:Con1017:14]	KR476371.1
South African cassava mosaic virus	SACMV	NC_003803.1
Mungbean yellow mosaic India virus	MYMIV	NC_004608.1
Mungbean yellow mosaic virus-[India:Varanasi:2011]	MYMV-[IN:Var:11]	KC019303.1
Mungbean yellow mosaic virus [India:Namakkal:2006]	MYMV-[IN:Nam:06]	DQ865201.1
Mungbean yellow mosaic virus-[Cambodia:PhnomPenh:2003]	MYMV-[Cam:Pho:03]	AY271892.1
Mungbean yellow mosaic virus -[Thailand: Mungbean 1]	MYMV-[Tha:Mg1]	AB017341.1
Mungbean yellow mosaic virus -[Viet Nam 1:2011]	MYMV-[VN1:11]	JX244172.1
Tomato leaf curl Patna virus -[India:Patna:2008]	ToLCPV-[IN:Pat:08]	EU862323.1
Papaya leaf curl China virus-Tobacco[China: Guangxi:2015]	PaLCCV-[CH:Gua:Tob:15]	KX273342.1
Tomato yellow leaf curl virus, isolate Tom96	ToYLCV- Tom96	LN680631.1
Tomato leaf curl virus -[Nepal:Panchkhal:2000]	ToLCV-[NP:Pan:00]	AY234383.1
Croton yellow vein mosaic virus-[India:Bhubaneswar:2009]	CrYVMV-[IN:Bhu:09]	JN663850.1
Tomato leaf curl Joydebpur virus-[India:Amadalavalasa1:Kenaf:2007]	ToLCJV-[IN:Am:ken:07]	EU431116.1
Papaya leaf curl virus – Amaranthus [India: Lucknow:Amaranthus:2011]	PaLCuV-Ama[PK:Luc:Am:11]	JN135233.1
Bhendi yellow vein Delhi virus-[India:New Delhi:Palem:2004]	BYVDV-[IN:ND:Pa:04]	NC_011919.1
Tomato leaf curl New Delhi virus-[India:Aurangabad:OY164A:2006]	ToLCNDV-[IN:Aur:OY164A:06]	GU112088.1
Tomato leaf curl New Delhi virus-Chilli [India:2007]	ToLCNDV-[IN:07]	EU309045.1
Cotton leaf curl Multan virus-[Pakistan:1998]	CLCMV-[Pak:98]	NC_004607.1

Table 4.3: Continued

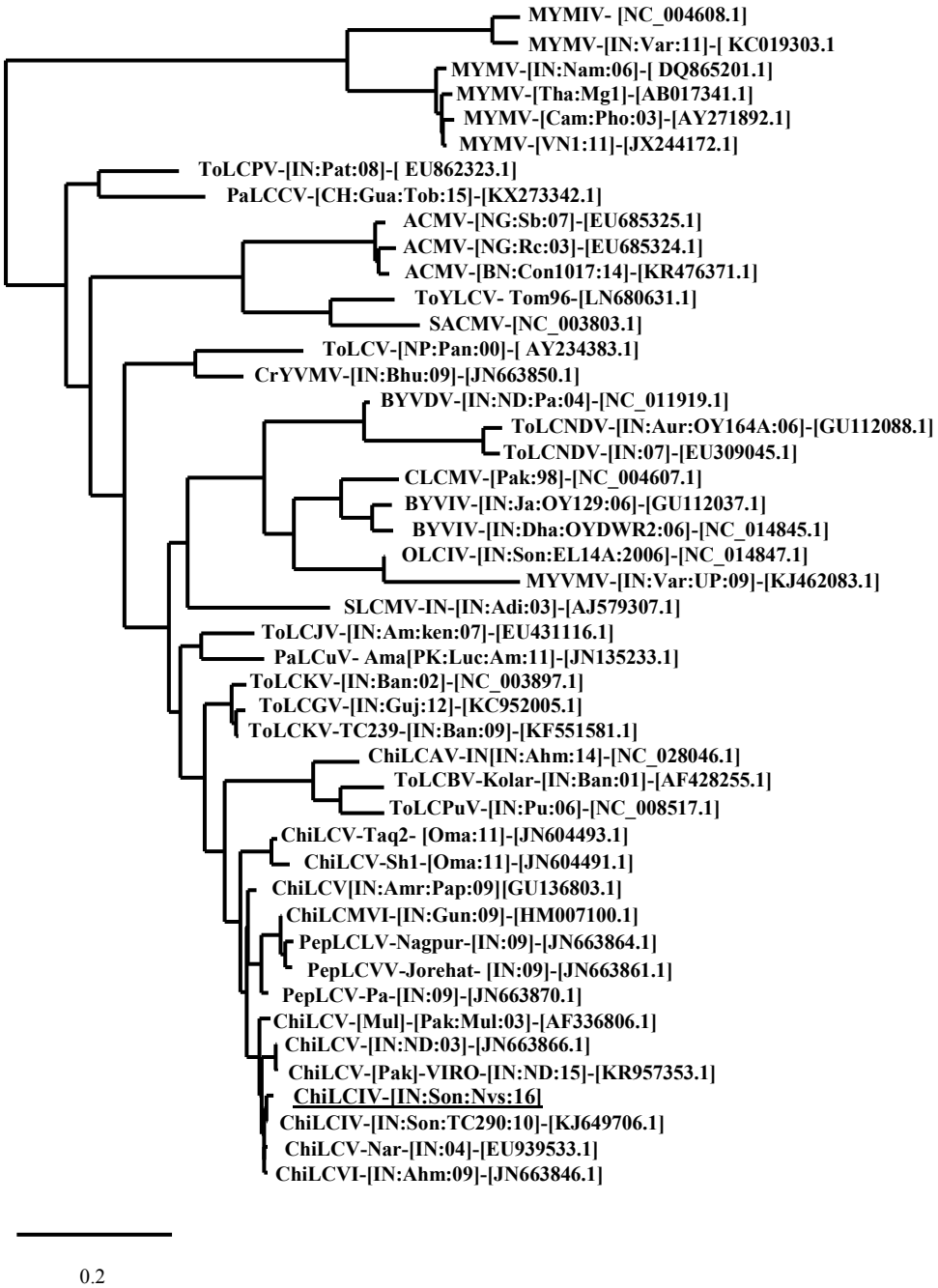
Viruses	Virus-Acronym	Accession numbers
Bhendi yellow vein India virus-[India:Jalgov:OY129:2006]	BYVIV-[IN:Ja:OY129:06]	GU112037.1
Bhendi yellow vein India virus-[India:Dharwad OYDWR2:2006]	BYVIV-[IN:Dha:OYDWR2:06]	NC_014845.1
Okra leaf curl India virus-[India:Sonipat:EL14A:2006]	OLCIV-[IN:Son:EL14A:2006]	NC_014847.1
Mesta yellow vein mosaic virus [India: Varanasi:UttarPradesh:2009]	MYVMV-[IN:Var:UP:09]	KJ462083.1
Tomato leaf curl Karnataka virus-[India:Bangalore:2002]	ToLCKV-[IN:Ban:02]	NC_003897.1
Tomato leaf curl Gandhinagar virus-[India:Gujarat:2012]	ToLCGV-[IN:Guj:12]	KC952005.1
Tomato leaf curl Karnataka virus-IsolateTC239-[India:Bangalore:2009]	ToLCKV-TC239-[IN:Ban:09]	KF551581.1
Chilli leaf curl Ahmedabad virus-India [India:Ahmedabad:2014]	ChiLCAV-IN[IN:Ahm:14]	NC_028046.1
Tomato leaf curl Bangalore virus-[Kolar]-[India:Bangalore:2001]	ToLCBV-Kolar-[IN:Ban:01]	AF428255.1
Tomato leaf curl Pune virus-[India:Pune:2006]	ToLCPuV-[IN:Pu:06]	NC_008517.1
Chilli leaf curl virus-isolate Taq2-[Oman:2011]	ChiLCV-Taq2- [Oma:11]	JN604493.1
Chilli leaf curl virus- Sh1-[Oman:2011]	ChiLCV-Sh1-[Oma:11]	JN604491.1
Chilli leaf curl virus-[India: Amritsar: Papaya:2009]	ChiLCV-[IN:Amr:Pap:09]	GU136803.1
Chilli leaf curl Multan virus-India [India:Guntur:2009]	ChiLCMVI-[IN:Gun:09]	HM007100.1
Pepper leaf curl Lahore virus-Nagpur-[India::2009]	PepLCLV-Nagpur-[IN:09]	JN663864.1
Pepper leaf curl Varanasi virus-isolate Jorehat- [India:New Delhi:2009]	PepLCVV-Jorehat- [IN:09]	JN663861.1
Pepper leaf curl virus- isolate Palampur -[Indian:2009]	PepLCV-Pa-[IN:09]	JN663870.1
Chilli leaf curl virus-[Multan]-[Pakistan: Multan:2003]	ChiLCV-[Mul]-[Pak:Mul:03]	AF336806.1
Chilli leaf curl virus-[India:New Delhi:2003]	ChiLCV-[IN:ND:03]	JN663866.1
Chilli leaf curl virus-[Pakistan]- VIRO 764-[India:NewDelhi:2015]	ChiLCV-[Pak]-VIRO-[IN:ND:15]	KR957353.1
Chilli leaf curl India virus-India:Sonipat:TC290:2010	ChiLCIV-[IN:Son:TC290:10]	KJ649706.1
Chilli leaf curl virus isolate Narwan-[India:2004]	ChiLCV-Nar-[IN:04]	EU939533.1
Chilli leaf curl virus-India [India:Ahmedabad: 2009]	ChiLCVI-[IN:Ahm:09]	JN663846.1

Chilli leaf curl India virus – India:Sonipat:Navsari:2016	ChiLCIV-[IN:Son:Nvs:16]	GU00000.0
Sri Lankan cassava mosaic virus -India [India:Adivaram:2003]	SLCMV-IN[IN:Adi:03]	AJ579307.1

For the nomenclature and demarcation of species, strains and variants of the species of the virus the recent criteria proposed by Fauquet *et al*., (2008) and subsequent guidelines given by ICTV (Anon 2016) were used. Accordingly the sequenced isolate has been considered as tentative strain of chilli leaf curl India virus –India:Sonipat:TC290:2016 and named as Chilli leaf curl India virus – India:Sonipat:Navsari:2016 and is abbreviated as ChiLCIV-[IN:Son:Nvs:16].

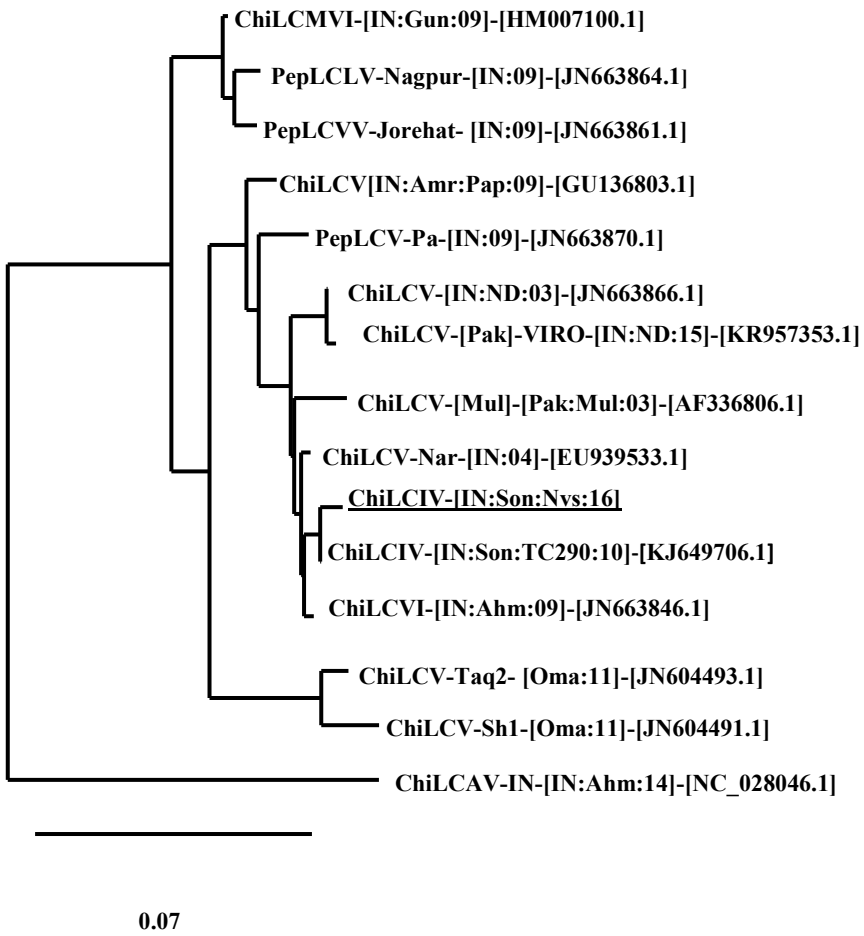
4.4.2 Diversity analysis of the Isolated Virus ChiLCIV-[IN:Son:Nvs:16]

Representative species, strains and variants of different *Begomovirus* species from the different crops and geographical location were selected for the phylogenetic analysis. Full length sequences of these viruses were downloaded from NCBI website. Downloaded sequences were chopped corresponding to the sequence of amplified gene. Two Dendrograms were constructed from the aligned sequences using the neighbor-joining method and bootstrap option of Tree conversion (1000 bootstrap replicates). In the first phylogenetic tree (Fig 4.1) a total of 46 different viruses including recently sequenced virus during present investigation were aligned together. Whereas in the second phylogenetic tree (Fig 4.2), 15 chilli viruses including the virus sequence obtained during the present investigation were aligned together.



The tree was constructed by the full optimal alignment in the CLUSTALW2.0 and the neighbor joining method with 1000 boot strap replications available in the MEGA4.0.

Fig 4.1: Phylogenetic tree of sequences of ChiLCIV-[IN:Son:Nvs:16] and previous reported Begomovirus [Table 4.3].



The tree was constructed by the full optimal alignment in the CLUSTALW2.0 and the neighbor joining method with 1000 bootstrap replications available in the MEGA4.0.

Fig 4.2: Phylogenetic tree of part of DNA-A sequence of ChiLCIV- [IN:Son:Nvs:16] with other viruses infecting Chilli plant.

Study of the Phylogenetic tree (Fig 4.1) of the viruses from the different crops and geographical area revealed that all the *Begomovirus* claded in two distinct broad clads. One clad comprises all the Begomoviruses infecting different crops viz., cotton, cassava, okra, tomato, papaya and chilli. Another clad consists of species, strains and variants of different *Begomovirus* infecting legumes. This indicated that the *Begomovirus* infecting legumes are entirely distinct from the Begomoviruses infecting other crops. The results were in accordance to the observation of Mugisa *et al*., (2014). The phylogenetic study was also in agreement with the findings of Rishi (2004) regarding MYMV being serologically related to other whitefly transmitted viruses including Bean Golden Mosaic Virus (BGMV).

Cassava mosaic disease is being caused by three distinct virus species viz., South African Cassava Mosaic Virus (SACMV), African Cassava Mosaic Virus (ACMV) and Sri Lankan Cassava Mosaic Virus (SLCMV). In the present investigation SACMV (SACMV-D-[NC_003803.1] and ACMV (ACMV-[NG:Sb:07]-[EU685325.1],ACMV-[NG:Rc:03]-[EU685324.1], ACMV- [BN:Con1017:14]- [KR476371.1]) were align closely however were in two distinct clad. The third clad comprised of SLCMV (SLCMV-IN [IN:Adi:03]-[AJ579307.1]) predominantly found in Sri Lanka and India found entirely different. The result indicated that the virus has been originated distinctly therefore, align distinctly. Geographically also the African continent and Indian subcontinent including India and Sri Lanka are extremely far having different agro climatic situation which have differentially affected the evolution of the virus.

Among the 46 total isolates taken for the generation of the phylogenic tree fifteen viral isolates were of chilli. These viruses claded into four distinct clusters. Among these Chilli leaf curl Ahmedabad virus-ChiLCAV-IN-[IN:Ahm:14]-[NC_028046.1] aligned separately and was

found close with Tomato leaf curl Bangalore virus (ToLCBV-Kolar-[IN:Ban:01]-[AF428255.1] and tomato leaf curl pune virus ToLCPuV-[IN:Pu:06]-[NC_008517.1]) These viruses produce typical leaf curl type of symptoms.

Similarly, Chilli leaf curl virus isolate Amritsar (ChiLCV [IN:Amr:Pap:09][GU136803.1] was also aligned distinctly and was close to the Chilli leaf curl Multan virus-India ChiLCMVI- [IN:Gun:09]-[HM007100.1] This virus too produces typical leaf curl type of symptoms.

One of the clad was exclusively made by the two isolates of chilli leaf curl virus from oman consisting of Chilli leaf curl virus isolate Taq2(ChiLCV-Taq2-[Oma:11]-[JN604493.1]) and Chilli leaf curl virus isolate Sh1 (ChiLCV-Sh1-[Oma:11]-[JN604491.1] were found to close with each.

Similarly, one cluster was formed separately which had chilli leaf curl multan virus India-(ChiLCMVI-[IN:Gun:09]-[HM007100.1], Pepper leaf curl Lahore virus isolate Nagpur PepLCLV-Nagpur [IN::09]-[JN663864.1],Pepper leaf curl Varanasi virus isolate Jorehat PepLCVV-Jorehat- [IN:09]-[JN663861.1]and Pepper leaf curl virus isoate Palampur PepLCV-Pa-[IN:09]-[JN663870.1]. These virus produce typical curling symptoms.

A distinct cluster was formed which contained 7 different virus isolate selected viz., Chilli leaf curl virus isolate New Delhi (ChiLCV-[IN:ND:03]-[JN663866.1]), Chilli leaf curl virus Pakistan isolate VIRO 764 (ChiLCV-[Pak]-VIRO-[IN:ND:15]-[KR957353.1]), Chilli leaf curl virus Multan (ChiLCV-[Mul]-[Pak:Mul:03]-[AF336806.1], Chilli leaf curl virus isolate Narwan (ChiLCV-Nar-[IN:04]-[EU939533.1]), Chilli leaf curl virus isolate India Sonipat (ChiLCIV-[IN:Son:TC290:10]-[KJ649706.1]), and Chilli leaf curl virus India isolate Ahmedabad (ChiLCVI-[IN:Ahm:09]-

]-[JN663846.1]) found on different crops and location. Our own isolated and sequenced virus ChiLCIV-[IN:Son:Nvs:16] sequence was also aligned in the same cluster only, near to the ChiLCIV-[IN:Son:TC290:10]-[KJ649706.1] and Chilli leaf curl virus isolate Narwan (ChiLCV-Nar-[IN:04]-[EU939533.1]). Our own sequenced virus was also aligned in this cluster and result indicated that this has high similarity with ChiLCIV-[IN:Son:TC290:10]-[KJ649706.1] Virus. Almost similar results have been observed by the different scientists from the different parts of the world Senanayake *et al.,* 2006; Sinha *et al*., 2011; Kumar *et al*., 2012; George *et al.,* 2014). Mugisa *et al*., (2014) observed similar results in the mungbean and other virus sequenced aligned. These viruses produce typical leaf curl type of symptoms.

From the above investigation it can be concluded that the Chilli Leaf Curl Virus studied and sequenced during the present investigation should produce typical leaf curl symptoms with the upward/downward curling of leaves, puckering and reduced size of leaves along with the thickening and swelling of veins. Affected plants grew slowly and became stunted or dwarfed. Fruit, if produced at all, were small dry and unmarketable. Interestingly similar symptoms were observed in the study of symptoms during the present investigation. The DNA-A sequence obtained during the present investigation and morphological features of the virus as expressed through symptoms were as per the expectation.

SUMMARY AND CONCLUSION

V. SUMMARY AND CONCLUSIONS

Chilli leaf curl virus disease (ChiLCVD) complex is often seen and considered the most severe limiting factors for the successful cultivation of the crop. Leaf curling due to the sucking insect pest and viral diseases is often confusing. In most of the cases it has been assumed that the disease complex is caused by a whitefly transmitted *Begomovirus*. However, the ChiLCVD complex is caused by the feeding of thrips, mites and whiteflies as well as by the infection of whitefly transmitted *Begomovirus.* Leaf curling due to sucking insect pest is reversible and can be managed through insect control; however, leaf curl due to Chilli leaf curl virus (ChiLCV) is irreversible and cannot be controlled. Therefore, a robust technique to identify the pathogen in the field and further its characterization is one of the critical stages in the management of the disease. Therefore, the present investigation was carried out to generate information about the same under the title "Biological and Molecular Characterization of Chilli Leaf Curl Virus (ChiLCV) in South Gujarat".

High incidence of the disease in the different fields of NAU as well as the adjoining area could be seen during the survey in 2016. ChiLCD manifesting typical leaf curl symptoms were seen in all the areas surveyed ranging from 10-35 per cent. The variation in disease incidence was mainly due to the different stages of the crop at different place and the different management practices followed. In every infected field surveyed, whiteflies were found invariably. However, jassids and thrips were the other insect pests also noticed. The typical symptoms of the disease observed during the investigation were typical leaf curl symptoms with the upward/downward curling of leaves, puckering and reduced size of leaves along with the thickening and swelling of veins. Affected plants grew slowly and became stunted or dwarfed. Fruit, if produced at all, were small, dry and unmarketable.

Study confirmed that the disease was of biotic nature particularly caused by the virus. Quality viral DNA was successfully isolated from symptomatic younger leaves of Chilli plants by CTAB method. In PCR a part of DNA-A molecule of~1200 bp was amplified by the *Begomovirus* specific primers confirming it to be *Begomovirus*. Amplified fragment was purified and sequenced in an automated DNA sequencer by the Cycle sequencing method and a 932 bp nucleotide sequence was obtained. Analysis and comparison of the sequence with the other standard DNA-A molecule of the *Begomovirus* indicated that the amplified fragment have two genes viz., virus coat protein (*V1*) gene and pre coat protein (*V2*) gene (*V2*). The sequenced virus showed highest identity 99% with Chilli leaf curl India virus isolate India:Sonipat: TC 290:2010 segment DNA-A, [KJ649706.1]. According to the recent criteria for the nomenclature of the virus was considered and named as Chilli leaf curl India virus - India:Sonipat:Navsari:2016 abbreviated as ChiLCIV-[IN:Son:Nvs:16].

Alignment of the recently sequenced virus during present investigation [ChiLCIV-[IN:Son:Nvs:16] with other 45 different viruses showed the evolution pattern and similarity of the virus with the other Begomoviruses in the world. All the *Begomovirus* claded in two distinct clads. One clad comprises all the Begomoviruses infecting different crops viz., cotton, cassava, okra, tomato, papaya, croton and chilli. Another clad consists of species, strains and variants of different *Begomovirus* infecting legumes. This indicated that the *Begomovirus* infecting legume are entirely distinct from the Begomoviruses infecting other crops. Different isolates of Chilli Leaf Curl Virus (ChiLCV) align distinctly in separate clad in the phylogenetic tree prepared.

Cassava mosaic disease is being caused by three distinct virus species viz., South African Cassava Mosaic Virus (SACMV), African Cassava

Mosaic Virus (ACMV) and Sri Lankan Cassava Mosaic Virus (SLCMV). In the present investigation SACMV (SACMV-D-[NC_003803.1] and ACMV (ACMV-[NG:Sb:07]-[EU685325.1],ACMV-[NG:Rc:03]-[EU685324.1], ACMV- [BN:Con1017:14]- [KR476371.1]) were align closely however were in two distinct clad. The third clad comprised of SLCMV (SLCMV-IN [IN:Adi:03]-[AJ579307.1]) predominantly found in Sri Lanka and India found entirely different. The result indicated that the virus has been originated distinctly therefore, align distinctly. Geographically also the African continent and Indian subcontinent including India and Sri Lanka are extremely far having different agro climatic situation which have differentially affected the evolution of the virus.

Among the 46 total isolates taken for the generation of the phylogenic tree fifteen viral isolates were of chilli. These viruses claded into four distinct clusters. Among these Chilli leaf curl Ahmedabad virus-ChiLCAV-IN[IN:Ahm:14]-[NC_028046.1] aligned separately and was found close with Tomato leaf curl Bangalore virus (ToLCBV-Kolar-[IN:Ban:01]-[AF428255.1] and tomato leaf curl pune virus ToLCPuV-[IN:Pu:06]-[NC_008517.1]) These viruses produce typical leaf curl type of symptoms.

Similarly, Chilli leaf curl virus isolate Amritsar (ChiLCV [IN:Amr:Pap:09][GU136803.1] was also aligned distinctly and was close to the Chilli leaf curl Multan virus-India ChiLCMVI-[IN:Gun:09]-[HM007100.1] This virus too produces leaf curl type of symptoms.

One of the clad was exclusively made by the two isolates of chilli leaf curl virus from oman consisting of Chilli leaf curl virus isolate Taq2(ChiLCV-Taq2-[Oma:11]-[JN604493.1]) and Chilli leaf curl virus isolate Sh1 (ChiLCV-Sh1-[Oma:11]-[JN604491.1] were found to close with each.

Similarly, one cluster was formed separately which had chilli leaf curl multan virus India-(ChiLCMVI-[IN:Gun:09]-[HM007100.1], Pepper leaf curl Lahore virus isolate Nagpur PepLCLV-Nagpur [IN:09]-[JN663864.1], Pepper leaf curl Varanasi virus isolate Jorehat PepLCVV-Jorehat- [IN:09]-[JN663861.1] and Pepper leaf curl virus isoate Palampur PepLCV-Pa-[IN:09]-[JN663870.1], these virus produce typical curling symptoms.

A distinct cluster was formed which contained 7 different virus isolate selected viz., Chilli leaf curl virus isolate New Delhi (ChiLCV-[IN:ND:03]-[JN663866.1]), Chilli leaf curl virus Pakistan isolate VIRO 764 (ChiLCV-[Pak]-VIRO-[IN:ND:15]-[KR957353.1]), Chilli leaf curl virus Multan (ChiLCV-[Mul]-[Pak:Mul:03]-[AF336806.1], Chilli leaf curl virus isolate Narwan (ChiLCV-Nar-[IN:04]-[EU939533.1]), Chilli leaf curl virus isolate India Sonipat (ChiLCIV-[IN:Son:TC290:10]-[KJ649706.1]), and Chilli leaf curl virus India isolate Ahmedabad (ChiLCVI-[IN:Ahm:09]-[JN663846.1]) found on different crops and location. Our own isolated and sequenced virus ChiLCIV-[IN:Son:Nvs:16] was also aligned in the same cluster only, near to the ChiLCIV-[IN:Son:TC290:10]-[KJ649706.1] and Chilli leaf curl virus isolate Narwan (ChiLCV-Nar-[IN:04]-[EU939533.1]) and result indicated that this has high similarity with ChiLCIV-[IN:Son:TC290:10]-[KJ649706.1] Virus.

However, the study concluded that the ChiLCD is predominantly caused by the Chilli leaf curl India virus – India:Sonipat:Navsari:2016. All the morphological, biological and molecular results were found supporting, the viral etiology of the disease. The disease produced typical leaf curl symptoms with the upward/downward curling along with the thickening and swelling of veins. Affected plants grew slowly and became stunted or dwarfed. Fruit, if produced at all, were small, dry and unmarketable.

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APPENDIX

Appendix –I Part of DNA-A ChiLCV sequence

TGTGGGATCCATTAGTAAACGAGTTTCCTGAAACCGTTCACGGTT
TTAGGTGTATGTTAGCAGTTAAATATCTGCAGCTACTAGAAAATA
CATATTCTCCAGACACTCTGGGGTATGATTTAATCAGGGATTTGA
TCTCCGTTATTAGGGCTAAGAATTATGTCCAAGCGACCGGCAGAT
ATCATCATTTCCACTCCCGCCTCGAAGGTACGCCGCCGTCTCAAC
TTCGACAGCCCTTATGCCAGCCGTGCTGCTGCCCCCACTGTCCGC
GTCACAAAGGCCAGAGCATGGGTGAACAGGCCCATGAACAGGA
AGCCCAGGATGTACAGGATGTACAGAAGCCCACATGTTCCTAGG
GGTTGTGAAGGCCCATGTAAGGTCCAGTCTTTTGAGTCTAGACAC
GATGTAGTTCATATAGGGCAGGTTATGTGTATTAGTGATGTTACC
CGTGGTACTGGGTTAACCCATAGAGTAGGTAAGCGATTCTGTGTT
AAGTCTGTGTACGTATTAGGGAAGATATGGATGGATGAGAATAT
CAAGACTAAGAATCACACGAATAGTGTGATGTTTTTCCTTGTCCG
TGATCGTCGTCCTGTTGATAAACCACAAGATTTTGGAGAGGTGTT
CAACATGTTTGACAACGAGCCTAGCACTGCTACTGTGAAGAATA
TGCATAGAGATCGTTATCAGGTTTTGAGGAAATGGCACGTAACG
GTTACTGGTGGACAGTACGCGTCGAAGGAACAGGCATTAGTTAA
GAAGTTCGTTAGGGTTAATAATTATGTTGTGTATAACCAGCAGGA
AGCTGGTAAATATGAGAATCATACGGAGAATGCATTGATGTTGT
ACATGGCGTGTACCCACGCTTCTAATCCTGTGTATGCTACCTTAA
AGATACGGATCTATTTCTTTGATTCAGTATCGAATTAATA

Appendix –II

C E R T I F I C A T E

This is to certify that I have no objection for supplying to any scientist only one copy of any part of this thesis at a time through reprographic process, if necessary for rendering reference services in a library or documentation center.

Place: Navsari.

Date: / /2017 (DORE R)