MOLECULAR CLONING AND CHARACTERIZATION OF VIRUS CAUSING LEAF CURL DISEASE OF *Capsicum* spp.

by

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(2016-11-109)

THESIS

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2019

DECLARATION

I hereby declare that the thesis entitled **"Molecular cloning and characterization of virus causing leaf curl disease of *Capsicum* spp."**is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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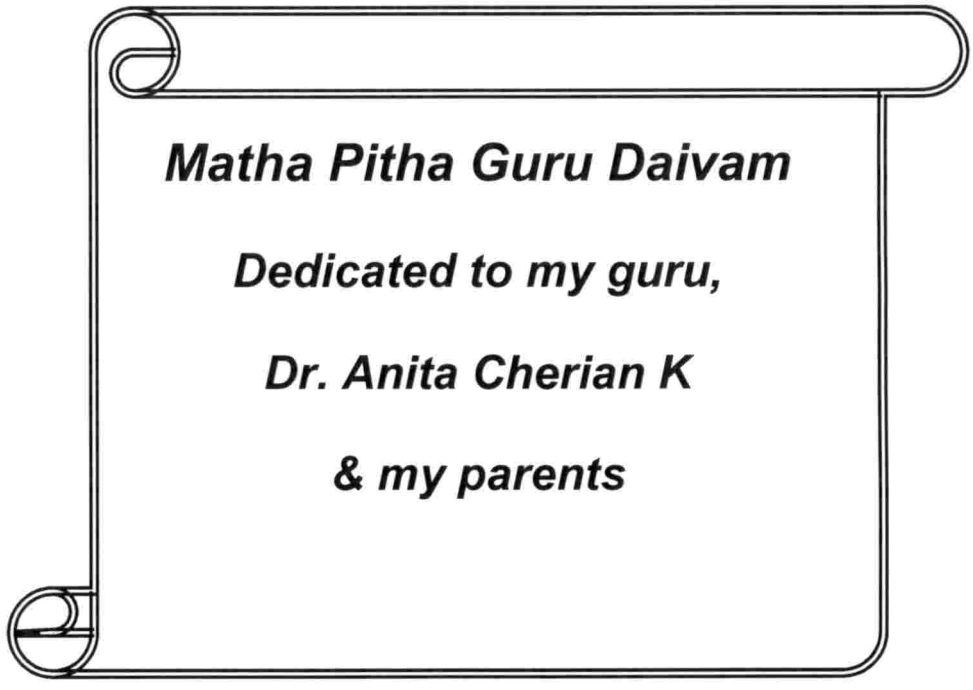
Matha Pitha Guru Daivam
Dedicated to my guru,
Dr. Anita Cherian K
& my parents

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ABBREVIATIONS

%	:	Percentage
@	:	At the rate
<	:	Less than
>	:	Greater than
A	:	Adenine
ai	:	Active ingredient
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
C	:	Cytosine
cm	:	Centimetre
CP	:	Coat Protein
CPBMB	:	Centre for Plant Biotechnology and Molecular Biology
CTAB	:	CetylTrimethyl Ammonium Bromide
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic Acid
DNase	:	Deoxyribonuclease
dNTPs	:	Deoxyribo Nucleoside Triphosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
FAO	:	Food and Agriculture Organization
g	:	Gram
G	:	Guanine
h	:	Hour
ha	:	Hectare
IPTG	:	Isopropyl thiogalactoside
KAU	:	Kerala Agricultural University
Kb	:	Kilo base pairs
kDa	:	Kilo Dalton
L	:	Litre
M	:	Molar
MT	:	Million Tonnes
mg	:	Milligram
$MgCl_2$	:	Magnesium Chloride
min.	:	Minutes

ml	:	Millilitre
mM	:	Milli molar
NCBI	:	National Centre for Biotechnology Information
ng	:	Nanogram
NHB	:	National Horticulture Board
nm	:	Nanometer
°C	:	Degree Celsius
OD	:	Optical Density
ORF	:	Open Reading Frame
PCR	:	Polymerase Chain Reaction
pH	:	Hydrogen ion concentration
PVP	:	Poly vinyl pyrolidone
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rpm	:	Revolutions per minute
s	:	Seconds
T	:	Thymine
Taq	:	*Thermusaquaticus*
TAE	:	Tris Acetate EDTA
TE	:	Tris EDTA
U	:	Unit
UTR	:	Untranslated Region
UV	:	Ultra violet
V	:	Volts
v/v	:	Volume per volume
w/v	:	Weight per volume
X-Gal	:	β - D- galactopyranoside
β	:	Beta
µg	:	Microgram
µl	:	Microlitre

INTRODUCTION

INTRODUCTION

Chilli (*Capsicum annuum* L.) is considered as one of main spice crops which is cultivated in all over the world. It belongs to the genus *Capsicum* and family Solanaceae. Worldwide, the green and dried fruits of chilli are used as condiments. As it requires prolonged, warm climate for growth and development, across the globe, large scale cultivation of chilli is limited to tropical regions.

An immense stake of world's spice trade is with India, the 'Land of spices' is the leading producer, consumer and exporter of spices in the world. In India, the crop covers an area of 0.316 million hectare with total production of 3.63 MT (NHB, 2017). Malaysia is the biggest importer of Indian chilli, followed by other conventional traders such as Bangladesh, Sri Lanka, USA and UAE (FAO, 2015). According to the statistics of National Horticulture Board (NHB, 2017), the crop covers an area of 1860 ha in Kerala with an average production of 12470 tonnes.

Despite its economic importance, the farmers are not in a position to produce good quality chilli with high productivity due to various biotic problems like incidence of pest and diseases, abiotic and crop related problems. Of the several diseases, those caused by viruses are very important and causes symptoms like mosaic, curling, yellowing, vein clearing, etc. leading to severe yield loss on chilli (Villalon, 1981). Among the different viral diseases, chilli leaf curl disease caused by *Chilli leaf curl virus* which is transmitted by whitefly (*Bemisia tabaci* G.) is one of the most important disease, limiting the production of *Capsicum* spp. causing an yield loss of up to 100 per cent (Saha *et al.*, 2005).

About 65 viruses have been reported to infect chilli worldwide, including *Begomovirus* causing chilli leaf curl disease (Nigam *et al.*, 2015). Chilli leaf curl disease was recorded as a viral disease in 1960s, but its connection with *Begomovirus* was identified only in 2007 (Senanayake *et al.*, 2007). Symptoms such as leaf curling and puckering are the characteristic features of *Chilli leaf curl virus* infection. The

infected leaves exhibit yellowing, vein banding and vein thickening. The infected crops are heavily stunted giving a bushy appearance and when the disease persist, flower buds would abscise and anthers would set without pollen grains, leading to improper fruit setting with fruit being distorted or underdeveloped (Bhatt *et al.*, 2016).

Chilli leaf curl virus belongs to the genus *Begomovirus*, family *Geminiviridae* and characteristically these have circular ssDNA genomes enclosed as twin particles. Geminiviruses have been grouped into nine genera *viz., Becurtovirus, Begomovirus, Topocuvirus, Turncurtovirus, Capulavirus, Curtovirus, Grablovirus, Mastrevirus* and *Eragrovirus*, relying on their vector, choice of host and genomic characteristics (Zerbini *et al.*, 2017). More than 80 per cent of the known geminiviruses are transmitted by whiteflies (*B. tabaci*) in a persistent manner. Most of the begomoviruses have bipartite genome consisting of DNA-A and DNA-B components. DNA-A is involved in genome replication, modulation of gene expression, inhibition of gene silencing and particle encapsulation, while DNA-B codes for proteins engaged in choice of host, virion distribution in the host, and symptom expression (Malathi *et al.*, 2017).

Recently in Kerala, more thrust has been given on the cultivation of vegetable crops *viz.* capsicum, cabbage, cauliflower etc. under protected conditions like polyhouses and rain shelters. But the microclimate prevailing in such enclosed structures is congenial for multiplication and spread of plant pathogens. High density cropping and monocropping of high yielding genotypes also make the plants under poly house predisposed to pathogens like fungi and viruses. Indistinct measures have been tested without much success, such as pesticide application to control vectors, exclusion of diseased plants and agronomic procedures. Vector control is also important for viral disease management as the B biotype of the insect vector (*B. tabaci*) has spread worldwide at a significant rate over the two last decades. Synthetic pesticides, physical obstacles and agronomic methods are generally used to manage the vector. Since, the immature forms of the pest are discovered below the leaves and lower portion of the

canopy commonly, standard chemical control for whiteflies are very challenging (Glick *et al.*, 2009). Also the symptoms of chilli leaf curl disease is often confused with that of mite infestation or nutritional disorders and hence, accurate and early diagnosis of the disease is very important to adopt timely management measures against the disease and to save the crop. Early detection and diagnosis is also important since symptomless hosts and weeds might carry the viral inoculum. The development of diagnostic probes like clones would help to detect viruses during latent period or in asymptomatic plants and could be used in chilli breeding programs.

Considering the importance of early diagnosis and timely management of the disease, the present study on 'Molecular cloning and characterization of virus causing leaf curl disease of *Capsicum* spp.' was undertaken in Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Plant Pathology, College of Horticulture, Vellanikkara. The objectives of the study were to investigate the incidence and symptomatology of leaf curl disease of *Capsicum* spp., to develop clones and also to characterize the coat protein gene of *Chilli leaf curl virus* isolates.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Chilli is one of the major spice crop raised all over India for vegetable, spice and industrial purposes. It is prone to various biotic and abiotic stresses that limit its production and productivity. Of the several biotic stresses, viruses are known to cause heavy loss on chilli crop (Villalon, 1981). Among the viral diseases, chilli leaf curl disease caused an yield loss up to 100 per cent especially when the infection starts at an early stage of the crop (Saha *et al.*, 2005). The disease is caused by *Chilli leaf curl virus* belonging to the genus *Begomovirus* and family *Geminiviridae*. Begomoviruses are vectored by whiteflies (*Bemisia tabaci* G.) in a persistent manner (Brown *et al.*, 1995).

2.1. OCCURRENCE AND GEOGRAPHICAL DISTRIBUTION

Hussain in 1932 was the first to describe the leaf curl disease in chilli and its association with whitefly from the Punjab of Integrated India which is now in Pakistan. Later in 1942, Pruthi and Samuel reported standard leaf curl symptoms on 15 - 20 per cent of chilli plants under field trial and reported *Tobacco leaf curl virus* (TLCV) as the causative agent of the disease. Mishra *et al.* (1963) experimentally proved the viral etiology of chilli leaf curl disease and it was caused by TLCV. Seth and Dhanraj (1972) and Raychaudhry (1977) showed that TLCV causes leaf curl disease in chilli and the virus was persistently transmitted by whitefly.

Amin (1979) reported that chilli leaf curl disease, which was locally known as 'Murda', is caused by infestation of insect pest complex of thrips and mites and not any infectious agents.

Besides India, chilli leaf curl disease was described from many other countries like Nigeria (Alegbejo, 1990), USA (Stenger *et al.*, 1990), Pakistan, Bangladesh and Indonesia (Fauquet and Stanley, 2003). Dharmasena (1998) reported that the prevalence of leaf curl disease in chilli cultivating areas in North central province of

Sri Lanka (1996 - 97) was due to infestation of whiteflies (*Bemisia tabaci* Genn.), mites (*Hemitarsonormus latus* Banks) and thrips (*Scirtothrips dorsalis* Hood) in chilli, causing yield loss upto 100 per cent.

Later, Venkatesh *et al.* (1998) reported that chilli leaf curl disease was caused by the combination of geminivirus and insect pests such as thrips (*Scirtothrips dorsalis*) and mites (*Polyphagotarsonemous latus*).

Although the viral nature of chilli leaf curl disease was confirmed in 1960s, its association with begomoviruses was only confirmed in 2006 by Khan *et al.* The first report of *Tomato leaf curl New Delhi virus* infecting chilli was given by Khan *et al.* (2006) from Lucknow, Uttar Pradesh and that of *Chilli leaf curl virus*-[Pakistan: Multan] by Senanayake *et al.* (2007) from Jodhpur, Rajasthan. Senanayake *et al.* (2012) reported that internationally, there are at least six different begomoviruses have been identified from chilli plants with leaf curl disease.

Worldwide, infection of more than 65 viruses have been described in chilli (Nigam *et al.*, 2015).

The geographical distribution of *Begomovirus* infecting chilli in India is given in Table 2.1.

Table 2.1: Geographical distribution of begomoviruses infecting chilli in India

Sl no	Year	Place	Virus isolate	Reference
1	2006	Lucknow	*Tomato leaf curl New Delhi virus* (AY883570)	Khan *et al.*, 2006
2	2007	Joydebpur	*Tomato leaf curl Joydebpur virus* (AJ875159)	Shih *et al.*, 2007
3	2007	Rajasthan	*Chilli leaf curl virus-[Pakistan: Multan]* (AF336806)	Senanayake *et al.*, 2007
4	2007	Varanasi	*Chilli leaf curl virus-[Pakistan: Multan]* (AF336806)	Chattopadhayay *et al.*, 2008
5	2007	Ghazipur	*Pepper leaf curl Bangladesh virus* (HM007097)	Chattopadhayay *et al.*, 2008
6	2008	Kanpur	*Chilli leaf curl Kanpur virus* (NC038441)	Chattopadhayay *et al.*, 2008
7	2010	Uttar Pradesh	*Tomato leaf curl Joydebpur virus* (AJ875159)	Rai *et al.*, 2010
8	2011	Palampur	*Chilli leaf curl Palampur virus*	Kumar *et al.*, 2011
9	2012	Kerala	*Chilli leaf curl Vellanad virus* (NC038442)	Kumar *et al.*, 2012
10	2012	Salem	*Chilli leaf curl Salem virus* (HM007119)	Kumar *et al.*, 2012
11	2013	Uttar Pradesh	*Pepper leaf curl Gonda virus* (KJ957157)	Khan and Khan, 2017

2.1.1. Disease incidence and economic impact

The incidence of leaf curl disease is a major challenge in the cultivation of this crop in chilli growing traits of India. The incidence of a sort of leaf curl symptoms which produced leaf enations on chilli has been reported in India as early as 1968 by Dhanraj *et al*. Considering the destructive nature of viral diseases in chilli, Gouda and Pandurange (1979), Bidari (1982) and Ilyas and Khan (1996) did studies and reported that the level of yield deficit due to leaf curl compound varied anywhere between 25 to 80 per cent.

Greenleaf (1986) reported that the chilli fields infected with begomoviruses rendered the chilli cultivation uneconomical, which led the farmers to abandon the crop prior to harvest. Singh *et al.* (1998) reported aphids (*Myzus persicae* S.), whiteflies (*Bemisia tabaci* G.) and thrips (*Scirtothrips dorsalis* H.) as the major sucking pests of chilli which could be the vectors of virus diseases like mosaic and leaf curl, these caused heavy reduction in yield. Saha and Nath (2005) reported that the prevalence of chilli leaf curl disease was more severe throughout summer season as oppposed to winter season causing yield loss up to 80 per cent when the crop gets infected early. Whereas Senanayake *et al.* (2007) reported chilli leaf curl disease caused 100 per cent yield loss when left untreated.

Varma and Malathi (2003) observed the epidemics of chilli leaf curl disease triggered by the surfacing of different strains of geminiviruses owing to recombination events in new areas that were previously free of these viruses. Several begomoviruses such as *Chilli leaf curl virus* (ChLCV), *Tomato leaf curl New Delhi Virus* (ToLCNDV) and *Tomato leaf curl Joydebpur virus* (Khan *et al.*, 2006; Senanayake *et al*., 2007) have been reported to infect chilli in India causing severe disease incidence (up to 100 %) and considerable yield loss.

Varma *et al.* (2011) confirmed that more than one *Begomovirus* could infect single crop and at least four species of begomoviruses have been isolated from infected

chilli. They also reported begomoviruses to be the key limitation in the cultivation of many other crops in the Indian subcontinent. Senanayake *et al.* (2013) studied the incidence and severity of *Chilli leaf curl virus* in Rajasthan and Madhya Pradesh and reported it as the most prevalent *Begomovirus* infecting chilli in India.

2.2. SYMPTOMATOLOGY

Peiris (1953) and Joshi and Dubey (1976) reported symptoms such as leaf curling, puckering, vein clearing and swelling as the characteristic symptoms of leaf curl disease in chilli. The size of leaves and branches of severely affected plants considerably diminished, giving a bushy appearance to the infected plants. Such plants bear very few flowers and fruits.

Symptoms such as leaf curling or yellowing in chilli were previously associated with begomoviruses such as *Cotton leaf curl Multan virus* (CLCuMV) (Hussain *et al.*, 2004) and *Pepper yellow leaf curl Indonesia virus* (PepYLCIV) (Tsai *et al.*, 2006). Hussain *et al.* (2004) reported that in chilli, yellowing and curling of the foliage followed by reduction of leaf area and stunting of the entire plants is induced by the infection of *Chilli leaf curl virus*. Saha and Nath (2005) reported that chilli plants with *Chilli leaf curl virus* infection exhibited symptoms such as inward rolling of leaves, wrinkling, yellow mosaic, stunted growth and chlorosis of leaf which resulted in notable reduction in yield.

Senanayake *et al.* (2007) reported that the initial symptoms of *Chilli leaf curl virus* infection include upward curling, puckering, vein clearing and reduction in size of the leaves. While the severely infected plants showed stunted growth and failed to produce flowers and fruits. Chattopadhyay *et al.* (2008) reported the incidence of chilli leaf curl disease in Varanasi. The symptoms included mild yellowing, severe leaf curling, leaf deformation, blistering of petioles, shortening of internodes and stunting of plants. Akther *et al.* (2009) reported that the chilli plants infected with *Tomato leaf*

curl New Delhi virus exhibited symptoms of severe leaf curl such as upward curling of leaves, cup-shaped leaves, yellowing of leaves and stunted plant growth.

Various kinds of symptoms of leaf curl disease have been recorded from all of chilli growing areas of the country. Shafiq *et al.* (2010) reported that the infected chilli plants showed severe leaf curl which later caused upward curling and cupping of leaves finally resulting in stunted growth. They also reported that, since chilli and tomato crops often cultivated simultaneously, the symptoms like yellowing, leaf curling, reduction in leaf size and stunting were comparable to *Tomato leaf curl virus* infection also. Kumar *et al.* (2011) observed that the healthy leaves of chilli plants inoculated with *Chilli leaf curl virus* exhibited leaf curling followed by puckering and yellowing of leaves. Symptoms were more severe in newly emerged leaves as compared to the older leaves. Compared to healthy plants, the infected plants showed stunted appearance. Sinha *et al.* (2011) reported that downward curling of the leaves accompanied by puckering, vein clearing and subsequently vein thickening, distortion, inflation of the veins and veinlets on dorsal side as the prominent symptoms of chilli leaf curl disease. Sivalingam *et al.* (2012) observed that the newly emerging leaves of the infected plants appeared more green and thicker than that of healthy leaves. The plants infected at an early stage of growth become severely stunted leading to bushy appearance and become unproductive in the later stages of the crop. Senanayake *et al.* (2013) reported that the leaf curl disease of chilli in India and Sri Lanka indicated similar etiology. It was characterized by severe upward curling and thickening of the leaves with vein clearing, stunted plant growth and severely affected plants hardly produced any fruits.

The typical begomoviral symptoms were curling of leaves, vein thickening and stunted plant growth in the initial stages of disease, while in the later stages of infection, the flower buds were abscised and anthers were underdeveloped without pollen grains which finally resulted in poor fruit production and fruit malformation (Kumar *et al.*, 2015). Zehra *et al.* (2017) reported, leaf curling, rolling, puckering, blistering of

interveinous regions, vein thickening and swelling, shortening of internodes and petioles, bunching of leaves, stunting, older leaves turning leathery and brittle were the characteristic symptoms of chilli leaf curl disease.

2.3. TRANSMISSION STUDIES

The first report on transmission of chilli leaf curl disease by whitefly was done by Hussain (1932). He reported that the infestation of insect pests *viz., Bemisia tabaci* and *B. gossypiperda* produced symptoms like leaf curl or leaf crinkle in chilli plants.

2.3.1. Vector transmission

Muniyappa and Veeresh (1984) reported that the chilli leaf curl disease was transmitted by whiteflies. They inoculated healthy chilli plants using viruliferous whiteflies and reported typical symptoms of leaf curl disease after 2 - 6 weeks. Premchand and Prasad (1990) conducted host range studies and reported that the disease was transmitted by the vector *B. tabaci* and they also noticed that the virus survived on a broad spectrum of wild or perennial host plants.

Senanayake *et al.* (2012) studied the virus - vector relationship and host range of some *Chilli leaf curl virus* isolates that caused the outbreak of chilli leaf curl disease in Jodhpur, Rajasthan. They reported that eight or more viruliferous whiteflies in a plant resulted in 100 per cent transmission of the virus and even single viruliferous whitefly could transmit the virus. The whiteflies remained viruliferous for up to five days after acquisition of virus and the minimum acquisition access period (AAP) and inoculation access period (IAP) required by the whiteflies were 180 min and 60 min respectively. The results of host range studies of *Chilli leaf curl virus* established that, apart from chill, the virus could infect only four species of host *viz., Carica papaya*, *Solanum lycopersicum*, *Nicotiana tabacum* and *N. benthamiana*.

Several crop species have been reported as hosts for different *Chilli leaf curl virus* isolates from India. Malathi *et al.* (2017) reported that crops such as chilli,

tomato, papaya, eggplant, amaranthus, *Solanum nigrum*, kenaf, petunia, mint and *Phaseolus aureus* were the potent hosts for *Chilli leaf curl virus* in India.

2.3.2. Graft transmission

Park and Fernando (1938) and Bos (1967) reported grafting of infected scion with healthy rootstock to be a successful method of plant virus transmission.

In India, Mishra *et al*, (1963) were the first to experimentally establish the viral nature of chilli leaf curl disease through graft transmission. The studies conducted by Dhanraj and Seth (1968) concluded that chilli leaf curl viruses were transmitted by whiteflies as well as through wedge grafting. Jamsari *et al.* (2015) reported that grafting methods such as top cleft grafting and side cleft grafting had 71.40 per cent success rate for the transmission of virus with an inoculation period ranging from 20-29 days.

2.3.3. Other methods of virus transmission

Other methods for artificial inoculation of geminiviruses includes mechanical infection *via.* agroinoculation (Leiser *et al.*, 1992, Mutterer *et al.*, 1999), wounding (Mayo *et al.*, 2000) and particle bombardment (Lapidot *et al.*, 2007).

Jamsari *et al.* (2015) reported injection method as an effective method of transmission of virus in chilli. They injected geminiviral particles extracted from infected plants into healthy chilli plants using suitable buffers and observed typical leaf curling symptom in 60 per cent of the plants one week after inoculation.

Chauhan *et al.* (2018) described three methods of transmission by sap inoculation for *Chilli leaf curl virus viz.,* inoculation by syringe, rubbing and immersion dip. Among the three methods used, syringe inoculation was the most efficient method of virus transmission which was followed by immersion dip method.

2.4. MOLECULAR CHARACTERIZATION

Different begomoviruses cause chilli leaf curl disease in India. Khan *et al.* (2006) and Senanayake *et al.* (2007) exploited partial DNA-A sequence data of a monopartite *Begomovirus* isolate from Pakistan to demonstrate the correlation between *Chilli leaf curl virus* and the chilli leaf curl disease in India. *Tomato leaf curl Joydebpur virus*, reported on tomato from Joydebpur, were also found to be associated with chilli leaf curl disease in Punjab (Shih *et al.*, 2007).

2.4.1. Geminiviruses

Viruses belonging to the family *Geminiviridae* have comparatively small genome size ranging between 2.5 and 3 Kb. The genomic DNAs, consisting of either one or two circular ssDNA molecules, containing different coding sequences interrupted by 5' intergenic regions. In plant cells, geminiviruses replicates *via.* a non - encapsidated, supercoiled replicative double stranded DNA which contributed only few factors for their replication and expression of viral genes and these geminiviruses relies almost completely on the host machinery (Palmer *et al.,* 1997; Hanley-Bowdoin *et al.,* 1999). Upon entry into host, the ssDNA molecules were converted to dsDNA virion particles known as replicative form DNA (RF-DNA) using the host cellular machinery. This RF-DNA act as template for genome replication by rolling circle amplification (RCA) and expression of viral genes.

Geminiviruses form the second largest family of plant viruses *i.e., Geminiviridae*. They have a unique twin particle morphology with either monopartite or bipartite ssDNA genomes. Based on genome organization, biological properties and host range, geminiviruses are divided into four genera *viz., Mastrevirus, Curtovirus, Topocuvirus* and *Begomovirus* (Fauquet *et al.*, 2008). Genus *Begomovirus* has either one genomic component (monopartite) which are translated to six proteins or two genomic elements (bipartite) with five (DNA-A) and two ORFs (DNA-B).

Begomoviruses infects dicotyledonous plants and are transmitted exclusively by whiteflies (*Bemisia tabaci* G.).

Based on latest data on genomic constitution, insect vector and host range, family *Geminiviridae* were ranked into nine genera *viz., Becurtovirus, Begomovirus, Topocuvirus, Turncurtovirus, Capulavirus, Curtovirus, Grablovirus, Mastrevirus* and *Eragrovirus* (Zerbini *et al.*, 2017).

2.4.2. Begomoviruses

Begomoviruses have become a major limitation in the production of crops like chilli, tomato, cucurbits, cassava, beans and cotton (Varma and Malathi, 2003). More than 80 per cent of the known geminiviruses belong to the genus, *Begomovirus* with *Bean golden mosaic virus* as the type species (Briddon *et al.,* 2008). Most of the begomoviruses have bipartite genomes designated as DNA A and DNA B. Vast majority of begomoviruses seen in the eastern hemisphere have a monopartite genome, whereas no such observable genome type has been identified for the begomoviruses native to the western hemisphere (Idris *et al.,* 2011).

The particle size of the *Begomovirus* virions are approximately 18 x 30 nm. The A component of bipartite begomoviruses contains five open reading frames (ORFs) capable of translating into proteins larger than 10 kDa. The two elements of the bipartite begomoviruses share a well conserved common region (CR) of about 200 nucleotides long. The CR consists of the origin of replication and regulatory regions for bidirectional transcription (Brown *et al.,* 2001).

The strains, species and taxonomic lineages of begomoviruses can be effectively predicted using the coat protein gene sequence, which is the substantially conserved gene in the family *Geminiviridae* (Rybicki, 1994). Padidam *et al.* (1996) suggested that the highly variable 200 nucleotides long begomoviral coat protein gene segment present at the 5' end can be used for assessing taxonomic links within the

genus *Begomovirus*. As per the guidelines of International Committee on the Taxonomy of Viruses (ICTV), provisional classification of begomoviruses can be performed using the complete CP gene sequences because the identification and classification by complete genome sequencing requires substantial time and expense (Brown *et al.*, 2001). Based on the guidelines of ICTV, a CP gene sequence sharing >90 per cent similarity with a virus would constitute a strain of an existing virus, whereas <90 per cent shared identity would suggest a separate species.

2.4.2.1. Genome organization of begomoviruses

The DNA-A of bipartite and the single component of monopartite and bipartite begomoviruses contain five (sometimes six) ORFs, one (AV1) or two (AV1 and AV2) in the viral sense strand and four (AC1, AC2, AC3 and AC4) in the complementary sense strand whereas DNA-B of bipartite begomoviruses contains two ORFs (BV1 and BC1) (Plate 2.1). Both the genomes *i.e.*, DNA-A and DNA-B are ~2.8 kb in size. A part of noncoding Intergenic region (IR; ~500 bp long) region is conserved between the two DNA components in bipartite begomoviruses and it contains the origin of replication, where the replication associated protein binds for initiating Rolling Circle Replication. The IR also harbors the promoter/ regulatory elements for expression of the viral genes in both V-sense and C-sense strand (Harrison *et al.*, 2002).

Dry *et al.* (1997) reported the presence of one or more small circular DNA molecules, about 1.4 kb in size, known as satellite DNAs (sat-DNA) associated with a *Geminivirus* infection. Satellites DNAs or sat-DNAs are defined as subviral nucleic acids that depend on co-infection with a helper virus for their replicative processes. The sat-DNA sequence showed least sequence homology to DNA A or DNA B associated with begomoviruses except for the universally conserved nonanucleotide sequence element (TAATATTAC) containing the initiation site of rolling circle replication. Begomoviruses are seen together with two classes of satellite molecule, known as alphasatellites (Mansoor *et al.*, 1999 and Briddon *et al.*, 2004) or

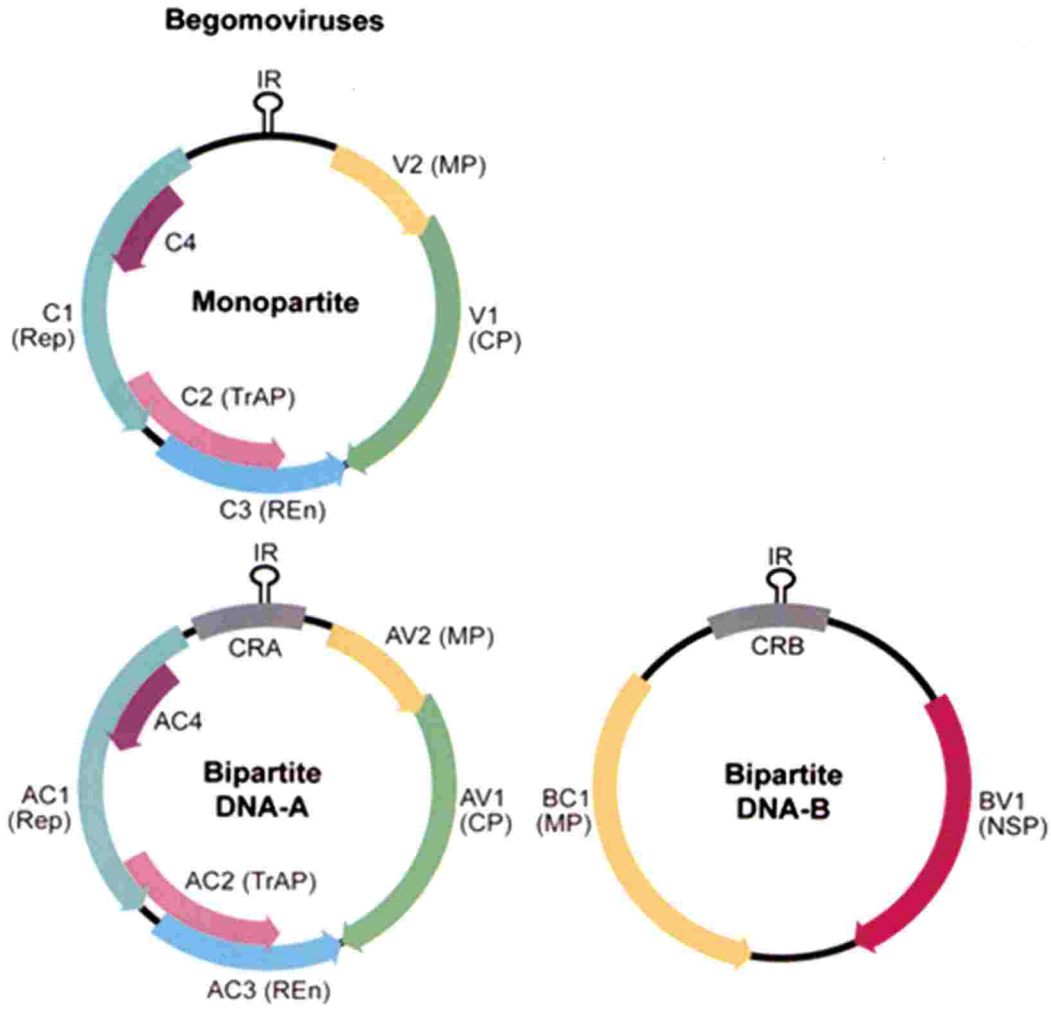


Plate 2.1: Genomic organization of begomovirus showing various ORFs

The ORFs are denoted as being encoded on the virion-sense (V) or complementary-sense (C) strand, preceded by component designation (A or B) if bipartite (bottom). Corresponding protein products are indicated. ORF AV2/V2 is not present in begomoviruses from the New World. The "common regions" CRA and CRB (bottom) that are shared between the two genomic components of bipartite viruses are shown as grey boxes within the intergenic region (IR). The position of the stem-loop containing the conserved TAATATTAC sequence located in the IR is shown. CP, coat protein; Rep, replication-associated protein; TrAP - transcriptional activator protein; REn, replication enhancer protein; MP, movement protein; NSP, nuclear shuttle protein.

betasatellites (Briddon *et al.*, 2008). The alphasatellites (previously known as DNA-1) are capable of autonomous replication in host plants cells by encoding a nanovirus-like replication-associated protein. Whereas the betasatellites (previously known as DNA-β), carry a single ORF (βC1), encoding a multifunctional protein, with RNA silencing activity and depend upon the helper virus for replication (Cui *et al.*, 2004). A notable difference between the two satellite DNAs is that, the alphasatellites are believed to be nonessential and do not influence to disease development (Briddon and Stanley, 2006).

The functions of each ORFs present in DNA-A and DNA-B as reported by Snehi *et al.* (2017) is detailed in Table 2.2.

Table 2.2 Gene order of *Begomovirus* DNA-A and DNA-B components with their putative protein products and predicted function (Snehi *et al.*, 2017)

Genome	Gene	Putative protein	Predicted function
DNA - A	AV1	Coat protein (CP)	Encapsidation
	AV2	Pre - coat protein	Cell to cell movement protein
	AC1	Replication initiation protein (Rep)	Replication initiation
	AC2	Transcriptional activator protein (TrAP)	Transcription activators
	AC3	Replication enhancer protein (Ren)	Replication enhancement
	AC4	_	Suppressor of RNA silencing
DNA - B	BV1	Nuclear shuttle protein (NSP)	Nuclear trafficking
	BC1	Movement protein (MP)	Cell to cell movement and pathogenicity determinant

The components of DNA- β were reported form a variety of hosts and the comparison of DNA sequence demonstrates elevated variability among strains. Saunders *et al.*, (2000) reported the co-infection of DNA-A (2.7 Kb) of *Ageratum yellow vein mosaic virus* (AYVV) and DNA-β that is approximately half its size (1.3 Kb) in *Ageratum conyzoides* L., exhibiting yellow vein symptoms. They also reported that the DNA- β satellites depend upon their helper viruses (DNA-A) for replication, movement in plants and transmission between plants. Briddon *et al.* (2008) stated that the majority of begomoviruses without the presence of DNA- β component, could not naturally infect plants.

2.4.3. Isolation of viral DNA

Usually, geminivirus genomes are electrophoretically isolated from the total nucleic acid and high quality DNA is necessary for this approach (Hamilton *et al.*, 1982). However, due to the presence of polysaccharides and polyphenols in several of the host plants, the isolation of best quality total genomic DNA is a challenging task. The polysaccharides in the host cells are viscid and have a gluey consistency rendering the DNA obstinate and unfit for PCR as they hinder *Taq* polymerase activity (Fang *et al.*, 1992). However, the viral DNA must be extracted from the hosts to characterize all the viral genomic elements, together with the satellite DNAs responsible for infection (Saunders *et al.*, 2000).

Hamilton *et al.* (1982) reported that the plant sap should be stirred overnight in the presence of Triton X-100 to the release geminiviral particles from inclusion bodies, prior to differential centrifugation and purification in sucrose density gradients. Czosnek *et al.* (1988) used sucrose gradient fractions and phenol - chloroform methods for isolation and purification of geminiviral particles from infected plant samples.

Muniyappa *et al.* (1991) reported a method for isolation and identification of *Indian tomato leaf curl geminivirus* (ITmLCV) from infected tomato samples. The method described purification of viral particles using chloroform-clarified extracts in

citrate buffer by precipitation with polyethylene glycol, ultracentrifugation and sucrose density gradient centrifugation.

Srivastava *et al.* (1995) developed a protocol for concentrating the double stranded replicative form of viral DNA from tomato plants infected *Indian tomato leaf curl virus*. The protocol used alkaline denaturation similar to that used for isolating *Escherichia coli* plasmid DNA for concentrating the viral RF - dsDNA in the total genomic DNA extracted from the infected crops.

Palmer *et al.* (1997) standardized a simple method for geminiviral DNA isolation using a low pH extraction buffer followed by an acidification step to denature many contaminating plant proteins.

Jose and Usha, (2000) compared three methods of DNA isolation *viz.*, method developed by Dellaporta *et al.* (1983), CTAB method (Doyle and Doyle, 1990) and the citrate buffer extraction with alkali lysis (Jose and Usha, 2000), from *Bhendi yellow vein mosaic virus* (BYVMV) infected leaf samples for concentrating geminiviral DNA in total nucleic acid obtained. They reported that, using both CTAB and citrate methods single stranded viral DNA could be obtained. The citrate method with alkali lysis was found to be superior among the three extraction methods evaluated, as the full length viral genome extracted using the citrate method which remained intact in southern analysis.

2.5.2. Polymerase Chain Reaction

The conception of a precise and modest methodology for rapid and accurate *Begomovirus* identification is crucial due to the recent reports of uncharacterized begomoviruses isolated from various hosts and locations around the world. Brown *et al.* (2001) stated that serology is not suitable for the characterization of begomoviruses because of the difficulty in preparing high titre antisera and insufficient specificity. Therefore, nucleic acid based diagnostic advances, comprising of amplification using

polymerase chain reaction (PCR) and DNA sequencing replaced protein based methodologies for taxonomic categorization of begomoviruses. The PCR amplification and sequencing of DNA-A and coat protein gene were used for identification and classification of begomoviruses.

2.5.2.1. Amplification of DNA-A

Stanley *et al.* (1992) reported that geminiviruses could be detected and identified by PCR due to the formation of dsDNA replicative form (RF-DNA) in the infected plant cells, which could serve as the template for PCR amplification. Rojas *et al.* (1993) developed degenerate primers (PAL1v1978/PAR1c496) for the amplification of whitefly transmitted geminiviruses using the sequences of twenty two virus isolates obtained from the America, Carribean Basin and Africa. These primers were designed to amplify 1.1 Kb fragment of the highly conserved common region (CR) of the bipartite genome. Briddon and Markham (1994) designed a set of degenerate primers from the aligned sequences of *African cassava mosaic virus* isolates for the PCR amplification of more or less complete genome of geminiviruses that infect dicotyledonous plants. These primers produced an amplicon of size 2.7 Kb which could be used for genome sequence determination and for cloning due to the presence of single-cutting restriction endonuclease sites. Chattopadyay *et al.* (2008) used the viral RF - DNA obtained from leaf curl infected chilli plants as a template for PCR using the degenerate primers, PAL1v1978/PAR1c496, which yielded an amplicon of size 1.1Kb.

2.5.2.2. Amplification of coat protein gene

Coat protein (CP) genes have traditionally been used to identify and classify plant viruses (Idris and Brown, 1998). For predicting taxonomic lineages within the genus *Begomovirus*, Mayo and Pringle (1998) suggested using the exceptionally diverse 200 nucleotide long 5' end of the begomoviral CP gene. Brown *et al.* (2001)

reported that the primers fabricated from the flanking region of begomoviral CP gene could not replicate the complete CP gene because of the 200 nucleotide variable region at the 5' end of the CP gene. However, the AV1 gene of genus *Begomovirus* comprises of a core coat protein region (core CP) which include variable and conserved regions that could be amplified using universal degenerate primers. The core CP region includes seven of the eight highly sustained amino acid motifs in the coat protein sequence throughout all members of the *Begomovirus* genus and is distinguished by segments of more or less the same sequences interspersed with variable bases. The inclusive arrangement of the entire CP sequence can be effectively represented through the core CP sequence as it contained adequately conserved and variable nucleotide segments (Sinha *et al.*, 2013).

Deng *et al.* (1994) developed a pair of universal degenerate primers, Deng 540 / 541, to amplify the *Begomovirus* AV1 gene (CP gene). The primers amplified the core coat protein region of *Begomovirus* isolates from Africa, America, Europe and India and yielded an amplicon of size 500 to 600bp.

Wyatt and Brown, (1996) used *Begomovirus* coat protein gene sequences of both bipartite and monopartite viruses from GenBank database to align and design a new set of degenerate primers. The primers named AV 494 and AC 1048, were fabricated from the conserved motifs bordering the core region located within the 770 bp long coat protein gene. These primers specifically amplified the middle or core region of the AV1 gene of whitefly transmitted geminiviruses (WTG) which yielded an amplicon of size 550 bp.

Senanayake *et al.* (2013) characterized different isolates of *Chilli leaf curl virus* infecting chilli in India and observed that the variation in sequence between the virus isolates was randomly distributed along the genome. Hence, it could be difficult to design species specific primers to duplicate the complete AV1 (CP) gene of the begomoviruses affecting chilli.

Sinha *et al.* (2013) devised specific primers for virus causing leaf curl disease in chilli in Varanasi which produced an amplicon of size 750 bp. The sequence data confirmed that the amplicons were viral ORF of AV1 gene of begomoviruses.

Rienzie *et al.* (2016) used two sets of degenerate (universal) primers *viz.*, Deng 540 / 541 and AV 494 / AC 1048 to detect begomoviruses causing bean yellowing disease (BYD) epidemic in Sri Lanka. They obtained the anticipated core coat protein amplicons of size 550 bp consistently from the DNA extracted from leaf samples with bean yellowing disease, thus confirming the infection by begomoviruses.

2.5.3 Molecular cloning

Srivastava *et al.* (1995) described a new method of cloning *Indian Tomato leaf curl virus* (ITLCV) genome using the supercoiled viral RF-DNA. Using this method they obtained about 95 per cent of transformants with plasmids with an insert of either DNA-A (2.75 kb) or DNA-B (2.55 kb). For validation, they used the cloned DNA-A and DNA-B fragments of virus as the probes and detected the presence of ITLCV in total genomic DNA obtained from the infected tissue.

Senanayake *et al.* (2007) was the first to clone and characterize *Chilli leaf curl virus* infecting chilli in India. They cloned and sequenced the 995 bp PCR product which yielded from the amplification of viral DNA obtained from the isolates collected from farmers' fields in Rajasthan.

Kumar *et al.* (2012) cloned an amplicon of ~2.8 kb into Kpn I-linearized pBluescript II KS(+) and the sequence analysis revealed sequence identity between 64 and 76 per cent with other isolates of begomoviruses infecting chilli in the Indian subcontinent. The reported virus isolate was distinctly placed in a distinct clade in the phylogenetic tree and designated as *Chilli leaf curl Vellanad virus* (ChiLCVeV). According to the ICTV guidelines, it has been considered a new *Begomovirus* species.

Sinha *et al.* (2013) cloned and characterized the 750 bp PCR amplicon of CP gene of *Chilli leaf curl virus* from chilli samples collected from Varanasi using specific primers. Phylogenetic analysis of CP sequence of the reported virus isolate with other *Begomovirus* sequences from GenBank revealed close relationship of these with other Indian and other Asiatic isolates.

2.5.4 *In silico* analysis

Shukla and Ward, (1988) compared sequences of nucleic acid and proteins of viral origin, along with structural and biological criteria to classify plant viruses. Padidam *et al.* (1995) reported that it could be possible to classify geminiviruses based on different sequence data and a short region of the genome would be sufficient to classify an isolate.

Chattopadhyay *et al.* (2008) performed sequence similarity studies on the cloned elements of a *Begomovirus* isolates from India with other begomoviral sequences in the GenBank database. They reported that the sequences of Indian isolates showed more resemblance with begomoviruses from Asia with maximum sequence similarity with ChiLCV-PK [PK:Mul:98] (*Chilli leaf curl virus* Multan, Pakistan isolate). On the basis of sequence identity data of individual viral genes of different isolates, they suggested that discrete segments of the viral genome have distinct ancestries.

Sinha *et al.* (2013) studied phylogenetic relationship between coat protein genes of *Chilli leaf curl virus* isolates. The data obtained after different phylogenetic analyses suggested distinct lineages for Old World and New world begomoviruses with a small group of isolates from the Indian subcontinent coming in one of the six well supported Old World *Begomovirus* lineages.

MATERIALS AND METHODS

MATERIALS AND METHODS

The present study on "Molecular cloning and characterization of virus causing leaf curl disease of *Capsicum* spp." was carried out in Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Plant Pathology, College of Horticulture, Vellanikkara during 2016 - 2018. The details of the materials and methods used in the experiments are described below.

3.1. SURVEY FOR CHILLI LEAF CURL DISEASE INCIDENCE AND COLLECTION OF SAMPLES

A purposive sampling survey was conducted in selected eleven locations of Thrissur district, *viz.,* Vellanikkara, Kattilapoovam, Kanimangalam, Valakkavu, Kodakara, Vellangallur, Kottanellur, Karumathra, Kannara, Parapukkara and Kodaly during 2017 - 2018 (Plate 3.1). These surveys were conducted with an objective to document the incidence and severity of chilli leaf curl disease in chilli growing areas under open field and protected conditions. The observations on growth stage of the crop, variety, total number of plants, number of infected plants and symptoms expressed on different parts of the plant were recorded.

Twenty plants were randomly selected from each field to assess the per cent disease incidence and severity of leaf curl disease in each field. The per cent disease incidence at each location was estimated using the given formula:

$$\text{Per cent disease incidence (PDI)} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Per cent disease severity was calculated using the standard score chart for leaf curl disease developed by Banarjee and Kaloo (1987) using 0 - 5 scale as given in Table 3.1.

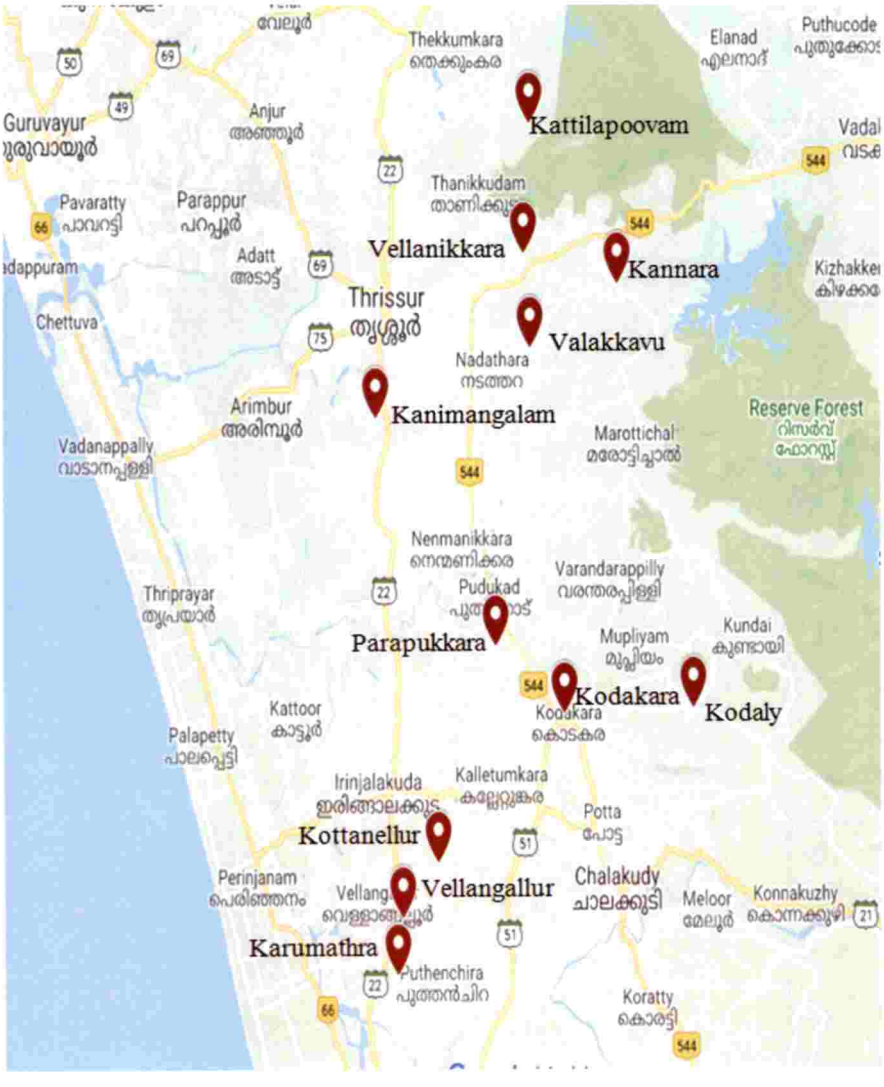


Plate 3.1: Locations of purposive sampling survey

Table 3.1: Standard score chart used for leaf curl disease scoring

Score	Symptoms
0	No symptom
1	0-5% curling and clearing of upper leaves
2	6-25% curling, clearing of leaves and swelling of veins
3	26-50% curling, puckering and yellowing of leaves and swelling of veins
4	51-75% leaf curling and stunted plant growth and blistering of internodes
5	More than 75% curling and deformed small leaves, stunted plant growth with small flowers and no or small fruit set

Using the scoring chart, per cent disease severity (PDS) was assessed using the given formula:

$$PDS = \frac{\text{No. of plants X each degree}}{\text{No. of plants evaluated X highest degree}} \text{ X } 100$$

The samples showing typical symptoms of leaf curl disease were collected and carried to the laboratory for further studies on identification and characterization of the virus associated with the disease.

3.2. SYMPTOMATOLOGY OF CHILLI LEAF CURL DISEASE

The symptoms expressed on different parts of plants under natural conditions *viz.*, open field and protected conditions as well as artificial conditions were documented.

3.2.1. Symptoms under natural field conditions

During the survey symptom expression on different parts of the infected plants *viz*., leaves, internodes and fruits were documented in detail. The leaves showing distinctive symptoms were collected from fields in polybags and brought to the laboratory for further characterization of the pathogen. The infected plants with clear symptoms were also collected from the field and maintained in the Plant virology glass house of Department of Plant Pathology under insect proof conditions.

3.2.2. Symptoms under artificial conditions

The development of symptoms under artificial conditions were studied by artificial inoculation of the virus isolates from the infected plant samples collected from the field during surveys into the healthy chilli seedlings. The artificial inoculation was done by two methods *viz.,* vector transmission and graft transmission.

3.2.2.1. Vector transmission

a) Collection and rearing of whiteflies: Non-viruliferous whiteflies were collected from brinjal plants during early morning and late evening hours using aspirator made up of glass and plastic tubes. The collected whiteflies were then released into insect proof cages containing healthy brinjal seedlings for rearing of whiteflies.

b) Acquisition of virus: The non-viruliferous whiteflies reared on brinjal seedlings were then released on to chilli plants infected with *Chilli leaf curl virus*, which were kept inside insect proof cage for acquiring the virus. Thirty whiteflies were released to feed on infected plant for 24 h. for virus acquisition, thereby making the whiteflies viruliferous.

c) Virus transmission: After acquisition access period, *i.e.,* the time the virus-free vector (here, whiteflies) was permitted to access the source of the virus (infected chilli plant), the viruliferous whiteflies were collected using an aspirator and released on healthy chilli seedlings. The inoculated chilli seedlings were further kept inside insect proof cages for expression of the symptoms.

The steps followed for vector transmission are given in Plate 3.2.

3.2.2.2. Graft transmission

Healthy chilli seedlings were used as root stock for apical wedge grafting whereas infected chilli seedlings were used as scion.

a. Preparation of root stock: The apical segment of the root stock was excised. A vertical cut of about 2.5 - 5 cm long was made on the stem to make a cleft to insert scion stem.

b. Preparation of scion: The scion was cut out from infected chilli seedling. The basal end was cut into V - shaped wedge, matching the cleft on the rootstock.

c. Wedge grafting: The basal wedge of the scion was inserted into the cleft at the top of the rootstock. The graft was carefully wrapped using a plastic strip. The grafted plants were then provided with high humidity by covering with polythene covers. These were regularly monitored for symptom expression on newly emerged leaves.

The steps followed for grafting are given in Plate 3.3.

3.3. MAINTENANCE OF VIRUS CULTURE

The infected plants collected from different locations during the survey were brought to the Plant virology glass house of Department of Plant Pathology and the virus culture was maintained under insect proof conditions by repeated insect transmission using whiteflies (Plate 3.4).

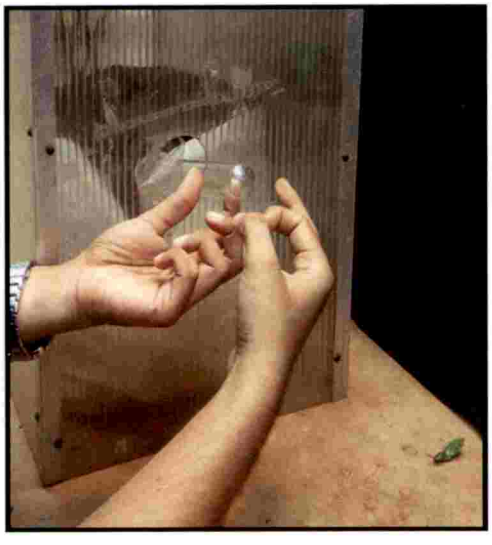
3.4. MOLECULAR CHARACTERIZATION

3.4.1. Molecular detection

Molecular detection and characterization of the virus isolates were carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Plant Pathology, College of Horticulture, Vellanikkara. Collection of leaf samples showing typical symptoms were done during early morning and the samples were

a) Collection of non-viruliferous whiteflies

b) Rearing of whiteflies

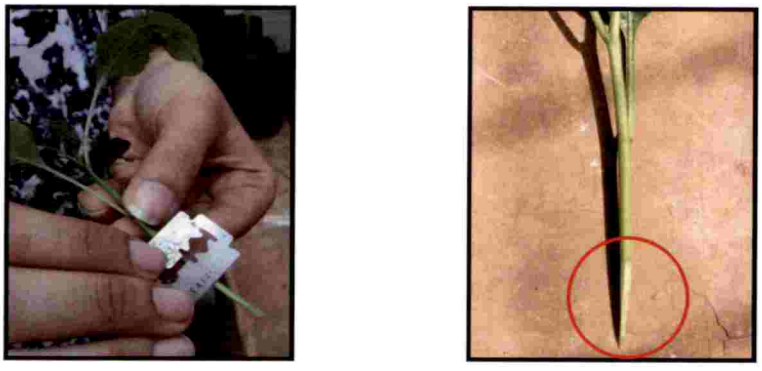


c) Acquisition of virus

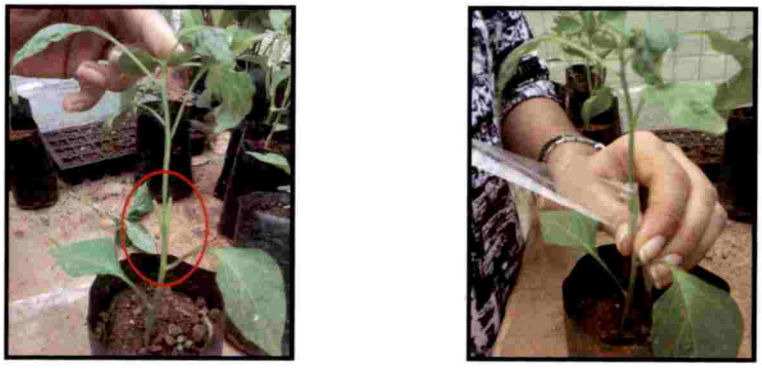
d) Virus transmission

Plate 3.2: Vector transmission

a) Preparation of root stock



b) Preparation of scion



c) Wedge grafting

d) Grafted plants

Plate 3.3 : Graft transmission

Plate 3.4: Maintenance of virus culture

brought to the laboratory in an ice box. The molecular detection of virus associated with chilli leaf curl disease was carried out by isolating total genomic DNA from the infected leaf samples followed by PCR amplification of the isolated DNA using two *Begomovirus* specific degenerate primers, AV494 / AC1048 (Wyatt and Brown, 1996) and Deng 540 / 541 (Deng *et al.*, 1994) and two designed primers, CLCVF / CLCVR and ChVelF / ChVelR. The molecular characterization was done further by sequencing the amplicons.

3.4.1.1. Isolation of total genomic DNA

The leaf samples collected from fields during survey were cleaned thoroughly with distilled water and total genomic DNA was isolated by standardizing three isolation methods, CTAB method, modified CTAB method and using commercial DNA isolation kit (Qiagen DNeasy® plant mini kit). The materials used for isolation of DNA *viz.*, mortar and pestle, spatula, centrifuge tubes and micropipette tips were autoclaved at 121°C at 15 lbs pressure for 20 min. These were then air dried at 50°C in an oven for 6 h before isolation. The composition of various stock solutions and extraction buffers used for the isolation of total genomic DNA is given in Appendix I.

3.4.1.1.1. Isolation of total genomic DNA – Method 1

The isolation of total genomic DNA from the infected leaf sample was done by CTAB method (Doyle and Doyle, 1990). Approximately, 0.5g - 0.8g of infected leaf sample was weighed and ground into fine powder using liquid nitrogen along with a pinch of polyvinylpyrrolidone (PVP) in a chilled mortar and pestle. One ml of 5 per cent CTAB buffer pre-warmed at 65°C was added to the powdered sample and again ground properly to a fine homogenate. The homogenate was transferred into 2 ml centrifuge tube and 30 µl β-mercaptoethanol (BME) was added. The tube was invert mixed thoroughly and incubated at 65°C for 30 min with random gentle mixing. Equal volume of chloroform-isoamyl alcohol (24:1) mixture was blended with the homogenate by inverting the tube and centrifuged at 12000 rpm for 15 min at 4°C. The

content got separated into three discrete phases *viz.*, aqueous top layer with DNA and small quantity of RNA, middle layer with protein and fine particles and last layer with chloroform, pigments and cell debris. The top aqueous layer was carefully moved to another 2 ml centrifuge tube. Equal volume of chloroform: isoamyl alcohol (24:1) and one tenth volume of 10 per cent CTAB was added to the tube and gently inverted until the contents were properly mixed. The mixture was then centrifuged at 12,000 rpm for 15 min at 4°C. The top layer or the aqueous phase was transferred into a 2 ml centrifuge tube. 2 µl of RNase was added and incubated at 37°C for 15 min. To this mixture equal volume of chloroform-isoamyl alcohol (24:1) mixture was added and after gentle mixing, it was centrifuged at 12000 rpm for 15 min at 4°C. The aqueous phase was relocated to a 1.5 ml centrifuge tube and 0.6 volume of chilled isopropanol was blended by temperate inversions till the DNA precipitated. These tubes were held at -20°C for 2 h for complete precipitation of DNA. Later, the tubes were centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was gently decanted without disturbing the DNA pellet formed at the base of the tube. The DNA was washed twice with 200 µl of 70 per cent ethanol by centrifuging at 10,000 rpm for 5 min. The supernatant was carefully poured out and the pellet was air dried for 10 - 20 min. The DNA pellet was suspended in 70 µl sterile distilled water and stored at -80°C until further use.

3.4.1.1.2. Isolation of DNA – Method 2

The total genomic DNA of the infected leaf sample was also isolated by modified CTAB method. The concentration of CTAB in the extraction buffer was reduced to two per cent in method-2 and the volume of β-mercaptoethanol added was also reduced to 5 µl. But the incubation time of the homogenate at 65°C was extended to 60 min. To prevent degradation of DNA, centrifugation with chloroform-isoamyl alcohol mixture (24:1 ratio) was done only once and the treatment with RNase was avoided. Chilled 100 per cent ethanol was used to precipitate DNA instead of isopropanol.

Approximately, 0.1g - 0.5g of infected leaf sample was weighed out and transferred to a chilled mortar and pestle. The samples were ground into fine powder using liquid nitrogen along with a pinch of polyvinylpyrrolidone (PVP). The powdered samples were then transferred to a 2 ml centrifuge tube and homogenized with 1.5 ml of pre-warmed (65°C) 2 per cent CTAB buffer with β-mercaptoethanol (5 µl). The homogenate was mixed thoroughly and incubated at 65°C for 60 min. The homogenate was mixed by inverting 2 - 3 times during incubation. After incubation for 60 min, the homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then transferred to another centrifuge tube and mixed with equivalent amount of chloroform - isoamyl alcohol mixture (24:1 ratio). The tubes were then inverted carefully for proper mixing of the contents followed by centrifugation at 7000 rpm for 7 min at 4°C, which resulted in the formation of three separate phases. The top aqueous layer containing DNA was carefully transferred to a new centrifuge tube which was followed by the addition of one ml chilled 100 per cent ethanol. The contents in the tube was blended thoroughly and incubated at -20°C for 60 min. The homogenate was then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was disposed dispensed without disturbing the pellet at the base. The pellet was then washed twice with 70 per cent ethanol and the supernatant was again cleared out. The DNA pellet formed was then air dried for about 10 min and dissolved in 50 µl sterile distilled water and stored at -80°C for further use. The total genomic DNA isolated from healthy leaves following the same protocol served as the control.

3.4.1.1.3. Isolation of DNA – Method 3

The isolation of DNA was also tried using Qiagen DNeasy® plant mini kit following the manufactures' protocol. 0.1 g of infected leaf sample was ground into fine powder using liquid nitrogen in a chilled mortar and pestle. The powdered leaf sample was shifted to 2 ml centrifuge tube. 400 µl of Buffer AP1 and 4 µl of RNaseA was stirred together with the homogenate by vortexing. The homogenate was then incubated at 65°C for 10 min. and the homogenate was inverted 2 - 3 times throughout

the incubation period. 130 µl of Buffer P3 was properly admixed with the contents in the tube by pipetting. The tube was chilled on ice for 5 min and centrifuged at 14,000 rpm for 5 min. The resultant supernatant was carried to QIAshredder spin column and centrifuged at 14,000 rpm for 2 min. The liquid homogenate collected in the column was displaced to a new tube without disturbing the pellet. The contents in the tube was properly mixed with 1.5 volume Buffer AW1 by pipetting. 650 µl of this admixture was transferred into DNeasy Mini spin column and centrifuged at 8,000 rpm for 1 min. The liquid homogenate collected in the column was removed and this step was repeated with the left over admixture. The DNeasy Mini spin column was positioned onto a new 2 ml collection tube. 500 µl Buffer AW2 was put in to the spin column and centrifuged at 8,000 rpm for one min. Flow through was discarded and another 500 µl Buffer AW2 was added. It was again centrifuged at 14,000 rpm for 2 min. For DNA elution, the spin column was transferred to 1.5 ml collection tube and 100 µl Buffer AE was added onto it. The column was then incubated at room temperature (25°C ± 1) for 5 min and centrifuged at 8,000 rpm for one minute. This step was repeated once more with Buffer AE (100 µl). The eluted DNA was then transferred to 1.5 ml eppendorf tube and stored at -80 °C for further studies.

3.4.1.2. Assessment of quality of DNA

The superiority of the extracted DNA was analyzed by agarose gel electrophoresis in 0.8 per cent agarose gel. For this, 0.8 g agarose was added to 100ml 1X TAE buffer, boiled until the agarose absolutely suspended and then it was allowed to cool till lukewarm temperature. Ethidium bromide (EtBr) was added to a final concentration of 0.5 µg/ml as the DNA intercalating dye, for visual tracking of DNA migration during electrophoresis. It was then dispensed into the casting tray, the comb was positioned appropriately and the gel was then left undisturbed for proper solidification. After the gel was properly set, the comb was removed and transferred to an electrophoresis tank holding 1X TAE buffer with the wells towards the cathode end and fully immersed in the electrophoresis buffer. An aliquot of DNA sample (5 µl) was

blended with 1 µl of 6X gel loading dye and loaded to each of the wells in the agarose gel. In one lane 1 Kb molecular marker was also loaded. The samples were resolved by performing electrophoresis at a constant voltage of 75 V for 30 - 35 min. The DNA samples was then portrayed under UV light and image was documented using BIORAD Molecular Imager (GelDoc^{TM} XR^{+}). The details of different reagents used for agarose gel electrophoresis is given Appendix II.

3.4.1.3. Assessment of quantity of DNA

The quantity of isolated DNA samples was determined using NanoDrop® ND 1000 spectrophotometer. The instrument was initialized with 1µl of distilled water and then calibrated to zero using sterile distilled water as blank. The concentration of nucleic acids and their absorbance at 260 nm and 280 nm wavelength were recorded. The purity of the sample was indicated by the absorbance ratio (OD or $A_{260/280}$) at 260 nm and 280 nm wavelength.

3.4.1.4. Polymerase Chain Reaction (PCR)

PCR amplification of the isolated DNA was carried out in Applied Biosystems Thermocycler using two *Begomovirus* specific degenerate (universal) primers, namely, AV494 / AC1048 (Wyatt and Brown, 1996) and Deng 540 / 541 (Deng, *et al.*, 1994) and designed primers, namely, CLCVF & CLCVR and ChVelF & ChVelR. The details of the sequences of the reported primers used are given in Table 3.2.

Table 3.2: Nucleotide sequence of the reported primers used for PCR reaction

Primer	Sequence (5' - 3')	Length (bp)
AV 494 (forward)	GCCYATRTAYAGRAAGCCMAG	21
AC 1048 (reverse)	GGRTTDGARGCATGHGTACATG	22
Deng 540 (forward)	TAATATTACCKGWKGVCCSC	20
Deng 541 (reverse)	TGGACYTTRCAWGGCCTTCACA	23

Ambiguity characters according to IUPAC nomenclature are represented as B = (C, G, T); D = (A, G, T); H = (A, C, T); K = (G, T); M = (A, C); R = (A, G); S = (C, G); V = (A, C, G); W = (A, T); Y = (C, T)

3.4.1.4.1. Primer designing

Apart from the reported primers, attempts were also made to design primers to specifically amplify the 770 bp long complete coat protein gene of begomoviruses. The designing of primers *viz.,* CLCVF & CLCVR and ChVelF & ChVelR were done manually. Complete AV1 gene or coat protein gene sequences of *Chilli leaf curl virus* isolates were downloaded from NCBI Genbank database (https://www.ncbi.nlm.nih.gov). Multiple sequence alignment was executed using ClustalW (https://www.genome.jp/tools-bin/clustalw). Two pairs of forward and reverse primers were selected based on the ideal primer parameters given below.

a) GC content of primer sequences should be between 50 to 60 per cent
b) Melting temperature (T_m = 4GC + 2AT) ranged between 60°C and 70°C
c) Annealing temperature (T_a = T_m - 5°C) ranged between 50°C and 60°C
d) Difference between the annealing temperatures of the primer pairs should be between 3°C - 5°C

e) Preferably a G or C base at the 3' end of the sequence
f) Avoidance of single base repeats of more than 2 bases within the primer sequence
g) Nucleotide length of each primer between 18 to 24 bases
h) Primer pairs should differ in length by less than 3 base pairs
i) Avoid any complementarity between forward and reverse primers

From the alignment data created using ClustalW software, degenerate primer pairs of nucleotide length 20 bp were selected and used to design the primers: CLCVF (forward) & CLCVR (reverse). The complete sequence of *Chilli leaf curl Vellanad virus* AV1 gene / coat protein gene was downloaded from NCBI GenBank (Accession no. NC038442.1). From the nucleotide sequence, a pair of primers were fabricated to duplicate the complete coat protein gene with an amplicon size of 770 bp and named as ChVelF (forward) & ChVelR (reverse).

Primers designed as per the guidelines mentioned above were synthesized from Sigma Aldrich Chemical Pvt. Ltd. and validated using OligoAnalyzer Tool, IDT (https://eu.idtdna.com/pages/tools/oligoanalyzer).

3.4.1.4.2. Standardization of annealing temperature

The annealing temperature of PCR reaction was standardized by gradient PCR in Applied Biosystems Thermocycler. The range of temperature used for standardization of annealing temperature for the reported primers AV494 / AC1048 and Deng 540 / 541 and designed primers CLCVF / CLCVR and ChVelF / ChVelR are given in Table 3.3. The temperature at which good amplified products obtained was selected as the optimum annealing temperature for each primer pair.

Table 3.3: Range of annealing temperature tested

Sl no.	Temperature (°C)	
	AV494 / AC1048 and Deng 540 / 541	CLCVF / CLCVR and ChVelF / ChVelR
1	55	53
2	56	54
3	57	55
4	58	56
5	59	57
6	60	58

3.4.1.4.3. Standardization of annealing time

Different combinations of annealing temperature and time were considered to retrieve the ideal permutation of conditions. Variations in annealing time from 30s to 120s was tested for reported primers for good product concentration (Table 3.4). The results were inferred based on the gel documentation images after agarose gel electrophoresis of PCR products.

Table 3.4: Range of annealing time tested

Primers	Time (s)				
AV494 / AC1048	30	45	60	90	120
Deng 540 / 541					

3.4.1.4.4. PCR thermal profile

PCR was carried out under standardized conditions using the reported primers as given in Table 3.2. The reaction mixture (Table 3.5) was allotted into 0.2 ml PCR tubes. The PCR thermal profile used for the reactions is given in the Table 3.6.

Table 3.5: Composition of reaction mixture

Sl no.	**Components**	**Concentration of the stock**	**Volume added**	**Final concentration**
1	Template DNA (20 ng /µl)	20 ng/ µl	2 µl	20 ng
2	PCR buffer	10X	2.5 µl	1X
3	dNTP mix	10 mM	2.0 µl	0.8 mM
4	*Taq* DNA polymerase	3 U/µl	0.5 µl	3 U
5	$MgCl_2$	25 mM	1.5 µl	1.5 mM
6	Forward primer	10 µM/ µl	1 µl	0.4 µM
7	Reverse primer	10 µM/ µl	1 µl	0.4 µM
8	Sterile distilled water		14.5 µl	
	Total volume		**25 µl**	

Table 3.6: Programme of PCR

Steps	AV494 / AC1048		Deng 540 / 541	
	Temperature	Time	Temperature	Time
Initial denaturation	94°C	5 min	94°C	5 min
Denaturation	94°C	30 s	94°C	30 s
Annealing	*	*	*	*
Extension	72°C	45 s	72°C	45 s
Final extension	72°C	10 min	72°C	10 min

*Annealing temperature and time standardized under sections 3.4.1.4.2 and 3.4.1.4.3 using gradient PCR

3.4.1.4.5. Analysis of PCR amplicons

The quality of the PCR amplicons were analyzed by agarose gel electrophoresis in 1.2 per cent agarose gel. 1.2 g agarose was added to 100 ml of 1X TAE buffer, heated till the agarose was absolutely dissolved and then cooled to lukewarm temperature. Ethidium bromide (EtBr) was added to a final concentration of 0.5 µg/ml as an intercalating dye of DNA. An aliquot of PCR product (5 µl) was blended with 1 µl of 6X gel loading dye and loaded to each of the wells in the agarose gel next to either 100 bp DNA ladder or Low range DNA ruler plus (GeNei, Banglore) as molecular marker to detect the size of amplicons. The samples were resolved by performing electrophoresis at a constant voltage of 80 V for one hour or until the molecular marker was fully resolved. The amplified products were then pictured under UV light and image was documented using BIORAD Molecular Imager (GelDoc^{TM} XR^{+}).

3.4.1.4.6. Elution of PCR amplicons

The PCR products were loaded on 1 per cent agarose gel and desired band was eluted using QIAquick Gel Extraction Kit following the manufactures' protocol. The PCR product was carefully cut out from the agarose gel using a clean scalpel and transferred to a 2 ml centrifuge tube and weighed. Three volumes of Buffer QG was blended with one volume of gel (100 mg ~ 100 µl) and incubated at 50°C for 10 min or until the gel slice was entirely suspended. The gel was softened by vortexing the tube every 2 - 3 min throughout the incubation period. After the gel slice was fully suspended, one gel volume of isopropanol was added and mixed by pipetting. The mixture was transferred to QIAquick spin column with 2 ml collection tube which was then centrifuged for one min. The flow-through was discarded. 0.5 ml of Buffer QG was added to QIAquick spin column and centrifuged for one min followed by addition of 750 µl of Buffer PE to QIAquick column. This was centrifuged for one min and the flow through was discarded and an additional centrifugation at 13,000 rpm for one min was carried out to remove the presence of any extra buffer. The QIAquick column was then positioned into a 1.5 ml microcentrifuge tube. To elute DNA from the column, 30 µl - 50 µl of pre-warmed Buffer EB was pipetted to the centre of the QIAquick membrane and incubated at room temperature for one minute and subsequently centrifuged at 15,000 rpm for one minute. Finally, the eluted product was stored at -20°C and also sent for sequencing for molecular characterization.

3.4.2. Molecular cloning

Transformation of competent bacterial cells for cloning of plasmid vector pTZ57R/T was carried out using Thermo Scientific InsTAclone PCR cloning kit. The TA cloning system was based on the terminal transferase activity of *Taq* DNA polymerase and other non - proofreading DNA polymerases which attach single 3' - A overhang to the extremities of the PCR products. These PCR products favored direct

cloning into linearized cloning vector pTZ57R/T with 3'- ddT overhangs. Such overhangs also prevented the re-circularization of the vector.

3.4.2.1. A - Tailing of PCR product

A - Tailing was carried out by adding multiple adenine (A) bases or A- tail to the end of the amplified product obtained at section 3.4.1.4.6. A-tailing was performed at 72°C for 20 min. The A-tailing reaction mixture was prepared using 10X PCR buffer, *Taq* DNA polymerase, dNTPs and gel elution product (template DNA). Composition of the reaction mixture is given in Table 3.7.

Table 3.7: Composition of reaction mixture for A - Tailing

Sl no.	Components	Volume added
1	10X PCR buffer	3 µl
2	*Taq* DNA polymerase	0.5 µl
3	dNTP	0.5 µl
4	Template DNA	20 µl
	Total volume	**24 µl**

Since, A- tails obtained were not stable, the A-tailed products were immediately used for ligation with plasmid vector pTZ57R/T which was further used for cloning.

3.4.2.2. Ligation of PCR product with plasmid vector

The vector pTZ57R/T, supplied by Thermo Scientific, USA was used for ligation and cloning (Plate 3.5). It is a linearized and ddT tailed plasmid for precise assistance in TA cloning of PCR products, generated by polymerases which insert a spare adenine to the edge of the PCR products. The cloning site of the vector pTZ57R/T is at 650 - 651 bp position which is 3'- ddT tailed for ligation with insert. It has a LacZα

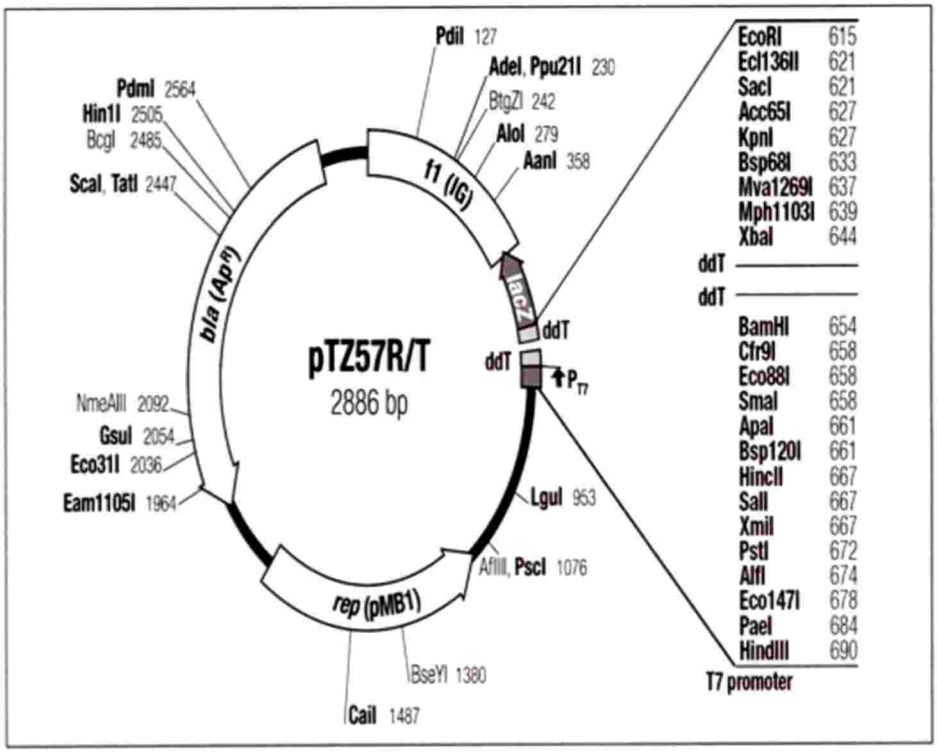


Fig 3.5: Map of the pTZ57R/T cloning vector with unique restriction sites

- peptide element at 449 - 739 bp position for blue/white screening and excision of the cloned insert.

The reaction mixture for ligation was prepared with vector (pTZ57R/T), 5X ligation buffer, T4 DNA ligase (Thermo Scientific, USA) and PCR product from section 3.4.2.1. The required amount of PCR product was calculated according to the spectrophotometer reading (OD value) of the eluted product and the data given by the manufacturers (Table 3.8). Composition of reaction mixture for ligation is given in the Table 3.9. The reaction mixture was prepared with optimal insert/vector ratio of 3:1 and following the manufactures' protocol. It was then incubated at room temperature for one hour followed by incubation at 4°C overnight. 2.5 µl of the ligated product was directly used for bacterial transformation.

Table 3.8: Recommended amount of PCR product for the ligation reaction

Length of PCR product (bp)	Optimal PCR product quantity for ligation reaction (ng)
100	17
300	51
500	86
1000	172
2000	343
3000	515
4000	686
5000	858

44

Table 3.9: Composition of reaction mixture used for ligation

Sl no.	Reagents	Volume added (µl)
1	Vector pTZ57R/T (0.17 pmol)	3
2	5X Ligation buffer	6
3	PCR product (0.52 pmol)	*
4	Water (nuclease free)	19
5	T4 DNA Ligase	1
	Total volume	**30 µl**

*Volume of PCR product was determined by comparing the data from Table 3.8 and concentration of the product obtained from section 3.4.2.1.

3.4.2.3. Preparation of bacterial cells for transformation

DH5α strain of *Escherichia coli* was employed for transformation and cloning. Single colony of 18 h old *E. coli* DH5α was streaked (quadrant streaking method) on to a Luria Bertani (LB) agar plate. It was incubated overnight at 37°C. From the culture, single colony was selected for chemical competent cell preparation and transformation.

3.4.2.4. Preparation of reagents for transformation

Prior to transformation ampicillin stock solution (50 mg/ml), X-Gal stock solution (20 mg/ml) and IPTG stock solution (100 mM) were prepared. Luria Bertani (LB) agar plates were prepared with 50 µg/ml ampicillin and 40 µl each of X-Gal (20 mg/ml) and IPTG (100 mM). These plates were incubated at 37°C (pre-warming) for at least 20 min.

a) Ampicillin stock solution (50 mg/ml): 2.5 g of ampicillin sodium salt was taken and suspended in 50 ml of sterile distilled water. X-Gal stock solution (20

mg/ml): 200 mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was weighed and suspended in 10 ml DMSO.

b) IPTG stock solution (100 mM): 1.2 g IPTG (isopropyl- β -D-thiogalactopyranoside) was weighed and suspended in 50 ml sterile distilled water

c) LB-ampicillin X-Gal/IPTG plates: LB agar media was prepared and cooled to 55°C. One ml of ampicillin stock solution (50 mg/ml) was added to the media to get a final concentration of 50 µg/ml ampicillin LB plates. To the solidified LB agar plates, 40 µl of X-Gal stock solution (20 mg/ml) and 40 µl of IPTG 100 mM was added and spread evenly with a sterile L-rod.

3.4.2.5. Transformation of bacterial cells

E. coli DH5α cells were made competent and transformed with the ligated product using Thermo Scientific InsTAclone PCR cloning kit. The *E. coli* DH5α cells were streaked on LB agar media from a master plate and was incubated overnight at 37°C. Two ml of C-media was inoculated with a single bacterial colony from the overnight culture prepared and kept for overnight at 37°C in shaker- incubator. The C - media (1.5 ml) and the LB agar plates (augmented with ampicillin, X-Gal and IPTG) were pre-warmed at 37°C for 20 min in an incubator. T - solution was formulated by mixing 250 µl of T - solution (A) and T - solution (B) (provided along with kit) in a 1.5 ml microcentrifuge tube and maintained on ice. 150 µl overnight bacterial culture was added to 1.5 ml of pre-warmed C - media and incubated at 37°C for 20 min in shaker - incubator. These bacterial cells were allowed to settle down by centrifuging for one minute and the remaining solution was cast-off. The cells were again suspended in 300 µl T - solution and held on ice for five minutes the mixture was centrifuged and for one minute and the remaining solution was disposed. The cells was again resuspended in 120 µl T - solution and kept on ice for 5 min. One µl of supercoiled control DNA I and control DNA II were taken in new microcentrifuge tubes and refrigerated on ice for 2 min. Each tube containing DNA was supplemented with 50 µl

of prepared competent cells. The components were mixed and incubated on ice for 5 min. These were then straightaway plated onto the pre warmed LB-ampicillin X-Gal/IPTG plates and the plates were incubated overnight at 37°C.

3.4.2.6. Selection of transformed cells

Transformed *E. coli* DH5α cells were plated onto the pre warmed LB-ampicillin X-Gal/IPTG plates. The plates were maintained at 37°C overnight. The transformed colonies were selected based on blue/white screening and further confirmation of presence of insert was done by colony PCR.

3.4.2.7. Cloning of transformed cells

The white colonies were subcultured in LB-ampicillin plates and incubated at 37°C overnight. To identify the true recombinant colonies, colony PCR was performed using AV494 / AC1048 primers. All the true recombinant colonies were selected by colony PCR which was further subcultured and stored at 4°C.

3.4.2.8. Confirmation of insert: Colony PCR

For the selection of true recombinants with the desired insert, colony PCR was performed. The transformed colonies were selected and resuspended in 20 µl sterile distilled water. The bacterial cells were denatured at 98°C for 5 min followed by centrifugation at 10,000 rpm for one minute. The supernatant was taken as the template to perform PCR reaction using the AV494 / AC1048 primers. The composition of PCR reaction mixture is given in the Table 3.10.

PCR reaction was performed with the program described in section 3.4.5.3. The amplicons were resolved on 1.2 per cent agarose gel and pictured under UV light and image was documented using BIORAD Molecular Imager (GelDoc^{TM} XR^{+}). The colonies with amplicons of expected size were selected as true recombinants.

Table 3.10: Composition of reaction mixture used for colony PCR

Sl no.	Reagents	Concentration of the stock	Volume added	Final concentration
1	Template DNA		10 µl	
2	PCR buffer	10X	2.5 µl	1X
3	dNTP mix	10 mM	2.0 µl	0.8 mM
4	*Taq* polymerase	3 U/ µl	0.1 µl	0.3 U
5	$MgCl_2$	25 mM	1.2 µl	1.2 mM
6	Forward primer	10 µM/ µl	0.5 µl	0.2 µM
7	Reverse primer	10 µM/ µl	0.5 µl	0.2 µM
8	Sterile distilled water		3.2 µl	
	Total volume		**20 µl**	

3.4.2.9. Maintenance of clones

Pure culture of the recombinant clones were prepared. Single white colonies were used to streak LB agar plates containing ampicillin (50 mg/ml) to an absolute concentration of 50 µg/ml in quadrant streaking method. The plates were incubated overnight at 37° C and further stored at 4°C.

3.4.3. Sequencing of PCR product

The amplified products were outsourced to Agri Genome, Kochi for sequencing.

3.4.4. *In silico* analysis of sequences

The sequence information obtained was further examined using various bioinformatics tools for characterization of the virus isolates.

3.4.4.1. Homology search

The sequence data obtained was processed in TrimEST software (http://bioinfo.nhri.org.tw/cgi-bin/emboss/trimest) to remove the poly A-tail and Cap3 software (http://doua.prabi.fr/cgi-bin/run_cap3) to assemble the sequences into contigs. The processed sequences were contrasted with the sequences of *Chilli leaf curl virus* available in National Centre for Biotechnology Information (NCBI) database using BLAST (Basic Local Search Alignment Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide BLAST analysis using BLASTn software was implemented on the viral sequences to find out the best aligned sequences available in NCBI. From the BLASTn results, sequences with minimum E - value, query coverage of more than 95 per cent and identity above 90 per cent were selected and used for further analyses. BLASTx (translated nucleotide - protein BLAST) was carried out for the viral sequence data to find the best aligned protein sequence.

3.4.4.2. Detection of Open Reading Frames (ORF)

Open Reading Frames (ORF) in the viral sequences were identified using ExPASy Translate tool (https://web.expasy.org/translate/). Using the default settings, the sequence was translated into six possible ORF. From the results displayed, the longest ORF was selected for further analysis and interpretation.

3.4.4.3. Amino acid analysis

Amino acid sequence of the viral genome was predicted using ExPASy Translate tool (https://web.expasy.org/translate/). The deduced amino acid sequence was used for further analysis and interpretation.

3.4.4.4. Phylogenetic analysis

Multiple Sequence Alignment (MSA) was employed using the processed sequences of virus isolates from the study and the sequences obtained from NCBI GenBank database using CLUSTALW software. The MSA file was processed using MEGA-X software and a phylogenetic tree with bootstrap value of 1000 was generated through Maximum - Likelihood method applying Tamura - Nei model (Tamura and Nei, 1993).

RESULTS

RESULTS

The results of the research project on "Molecular cloning and characterization of virus causing leaf curl disease of *Capsicum* spp." carried out during 2016 - 2018 at Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Plant Pathology, College of Horticulture, Vellanikkara are presented in this chapter.

4.1. SURVEY TO ASSESS DISEASE INCIDENCE AND COLLECTION OF SAMPLES

During 2016 - 2018, purposive sampling surveys were conducted in different locations of Thrissur district to assess the disease incidence and severity of chilli leaf curl disease (Plate 4.1). A total of 16 fields in twelve locations were surveyed. Among these,five were protected cultivation units and eleven open fields. During the survey, various parameters such as the growth stage and variety of the crop, number of infected plants of chilli leaf curl disease were recorded. The per cent disease incidence (PDI) and per cent disease severity (PDS) in different fields were also assessed.The results are presented in Table 4.1. The infected leaf samples collected during the survey were brought to the laboratory for further studies.

4.1.1. Assessment of disease incidence and severity

During the survey it was observed that,PDI under open field conditions,variedbetween 43.30 and 85.00 and PDS variedfrom 43.60 to 81.54. Under protected conditions, PDI ranged from 45.75 to 79.40 and PDS ranged from 49.40 to 87.50.

Among the various fields surveyed, the highest disease incidence of 85.00 per cent was recorded in Karumathra on local variety of chilli in Vellangallurpanchayath followed by Kottanellur (84.62%). Whereas the lowest per cent disease incidence of 43.30 per cent was recorded on chilli var. Ujwala in Vellanikkara.

Plate 4.1: Survey at different locations

Table 4.1: Details of surveys conducted at Thrissur district

Sl no.	Location	Crop	Stage of crop	Variety	PDI (%)	PDS(%)
1	Vellanikkara (protected cultivation plot 1)	Capsicum	10 MAP	Indra	64 .00	52.00
2	Vellanikkara (protected cultivation plot 2)	Capsicum	10 MAP	Indra	62.50	49.40
3	Vellanikkara	Chilli	7 MAP	Ujwala	43.30	58.50
4	Kattilapoovam	Chilli	6 MAP	Kanthari	60.00	29.50
5	Vellanikkara (protected cultivation plot 3)	Capsicum	5 MAP	Indra	45.70	87.50
6	Kanimangalam	Chilli	3 MAP	Local	62.00	38.70
7	Valakkavu	Chilli	4 MAP	Local	70.00	59.00
8	Vellanikkara (protected cultivation plot 4)	Chilli	5 MAP	Ujwala	79.40	84.60
9	Kodakara	Chilli	10 MAP	Local	67.80	43.60
10	Vellangallur	Chilli	3 MAP	Local	80.00	72.80
11	Kottanellur	Chilli	4 MAP	Local	84.62	81.54
12	Karumathra	Chilli	8 MAP	Local	85.00	78.30
13	Kannara	Chilli	11 MAP	Kanthari	62.50	68.00
14	Parapukkara	Chilli	9 MAP	Kodali	76.30	52.30
15	Kodaly	Chilli	9 MAP	Kodali	78.62	68.90
16	Vellayani (protected cultivation)	Chilli	8 MAP	Ujwala	64.50	75.00

The PDS was expressed by calculating the vulnerability index based on the 0 - 5 scale developed by Banerjee and Kalloo in 1987 as mentioned in section 3.1. The highest PDS was recorded under protected cultivation unit (plot 3) of Vellanikkara with PDS of 87.50 on capsicum variety Indra, which was followed by 84.60 on chilli var. Ujwala under protected cultivation unit (plot 4) of Vellanikkara. While the lowest PDS of 29.50 was recorded in Kattilapoovam of Madakkathara panchayath on chilli (*Capsicum frutescens*) var. Kanthari.

However, in fourteen fields, the PDI was more than 60 per cent except at Vellanikkara plot 2 and 3 where capsicum was raised under protected conditions. But in the case of disease severity more than 50 per cent was recorded in thirteen fields. The results revealed that chilli leaf curl disease is a critical glitch in chilli grown under open as well as protected conditions.

4.2. MAINTENANCE OF VIRUS CULTURE

During the survey, the infected plants exhibiting typical symptoms of chilli leaf curl disease were uprooted and planted in pots and brought to the laboratory. This was used as the source of primary inoculum of the virus and was inoculated to healthy chilli plants maintained in the net house of Department of Plant Pathology, Vellanikkara under insect proof conditions by insect transmission using whiteflies and graft transmission as described in section 3.2.2. These were maintained by repeated transfers of virus to healthy chilli plants and was used as the source of inoculum for further studies.

a) Under open field conditions

b) Under protected conditions

c) Under open field conditions

Plate 4.2: Upward curling of leaves

a) Under protected condition

b) Under open field condition

Plate 4.3: Inward rolling of leaf margins

a) Under protected condition

b) Under open field condition

Plate 4.4: Puckering of leaves

Under open field conditions

Plate 4.5: Crinkling of leaves

Under open field condition

Plate 4.6: General yellowing of leaves

4.3. SYMPTOMATOLOGY

The symptoms of chilli leaf curl disease expressed on different parts of the plant *viz*., leaves, internodes and fruits under natural conditions were documented during the survey. The symptom expression on healthy chilli seedlings following artificial inoculation were also documented.

4.3.1. Symptoms observed under natural conditions

The symptoms of chilli leaf curl disease observed on different parts of the infected plant *viz*., leaves, internodes, fruits and the whole plant under natural field conditions were documented.

4.3.1.1. Symptom expression on leaves

Upward curling of leaves was the characteristic symptom exhibited by leaves of the infected chilli plants (Plate 4.2). The leaf margins of infected plants tends to bend inward resulting in upward curling of the leaf blade. Curling was equally prominent in both young as well as old leaves of the infected plants. Under severe conditions, the upward curling of leaves resulted in leaf cupping. Excessive upward curling of leaf lamina was noticed in plants exhibiting more than 75 per cent infection. Apart from curling, some leaves of the infected plants exhibited inward rolling of leaf margins (Plate 4.3).

All the *Chilli leaf curl virus* infected plants showed puckering of leaves (Plate 4.4). The interveinal area of the leaves were contorted resulted in puckered appearance of leaves. The lamina had raised or elevated regions between the veins. The newly emerging leaves also exhibited puckering. The infected leaves having puckering symptom were thick and leathery in texture when compared to the healthy leaves.

Along with puckering, crinkling of leaves were also observed in plants grown under both protected and open field conditions (Plate 4.5). Such leaves exhibited extensive wrinkling and curling of leaf lamina.

Apart from curling, puckering and crinkling symptoms, interveinal chlorosis of infected leaves was also noticed (Plate 4.6). The infected leaves initially showed yellow patches in interveinal area which later coalesced between the margins. In later stages of infection the green veins became prominent on yellow lamina leading to vein banding symptom (Plate 4.7). Interveinal chlorosis was mostly seen on older leaves grown under natural field conditions.

Along with vein banding symptoms, thickening of veins was also observed in capsicum grown under protected conditions (Plate 4.8). Vein thickening was prominent on older leaves when compared to young leaves. This symptom was predominantly observed on infected capsicum plants grown under protected conditions.

Reduction in leaf area of younger leaves was observed in infected plants as compared to healthy plants (Plate 4.9). There was a considerable reduction in size of older leaves towards the apex, while the younger leaves failed to attain the full size giving a bushy appearance to the infected plant. In the case of severe infection, partial suppression of the lamina was observed especially near the petiolar end resulting in the formation of narrow strap-shaped leaves. The malformed leaves were brittle and undersized compared to the healthy leaves. This symptom along with elongation of petioles resulted in shoestring appearance of infected leaves (Plate 4.10). This type of symptom was noticed only under open field conditions

In advanced stages of the disease, the apical meristem was aborted and consequently the development of axillary buds was also affected. The axillary buds were stimulated to produce cluster of minute, thickened and brittle leaves giving a rosette-like appearance.

4.3.1.2. Symptom expression on other parts

The internodal length of diseased plants decreased towards the apex. The size reduction of younger leaves along with decrease in internodal length together

contributed to the tapered appearance of the infected plants. In the later stages of infection, the plants exhibited bunching of leaves. If the infection started early, there was significant stunting of plant growth giving a bushy appearance (Plate 4.11).

The severely infected with plants did not produce any flowers. Fruits produced were malformed with truncated appearance or curling at the stylar end (Plate 4.12). In case of bell pepper (*Capsicum annuum* L. var. grossum Sendt), the fruits formed were underdeveloped and deformed, while in the case of chilli (*Capsicum annuum* L.) the fruits developed were curled and small compared to healthy fruits (Plate 4.13).

4.3.2. Symptoms observed under artificial conditions

Artificial inoculation of healthy chilli seedlings with virus isolates collected during surveys for studying expression of symptoms under artificial conditions was carried out following two methods *viz.*, insect vector transmission of virus using viruliferous whiteflies and graft transmission of virus from virus infected scion to healthy rootstock.

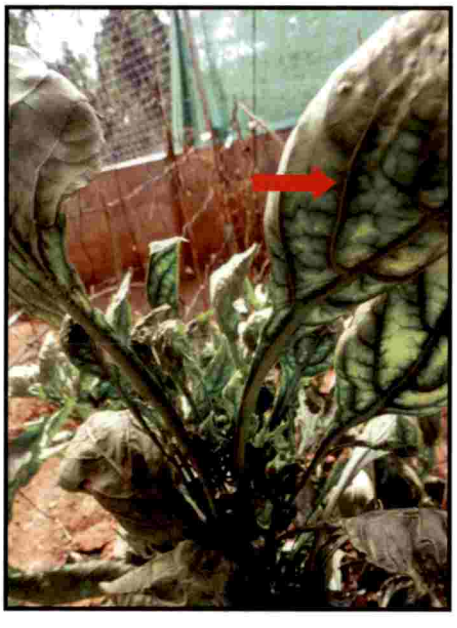
4.3.2.1. Vector transmission

The disease symptoms on the inoculated chilli seedlings were recorded seven days after artificial inoculation. Initially, the newly emerged leaves showed puckering symptoms followed by curling of the leaves (Plate 4.14). As the leaves matured, puckering symptom became very prominent. Interveinal chlorosis was not observed on artificially inoculated chilli seedlings.

The artificially inoculated seedlings exhibited stunted growth along with a reduction in internodal length as compared to healthy seedlings. The observations of vector transmission studies are given in Table 4.2.

a) Under protected condition b) Under open field condition

Plate 4.7: Vein banding



Under protected condition

Plate 4.8: Vein thickening

a) Under protected condition

b) Under open field condition

c) Under open field condition

Plate 4.9: Malformation of leaves

Under open field conditions

Plate 4.10: Shoestring appearance of leaves

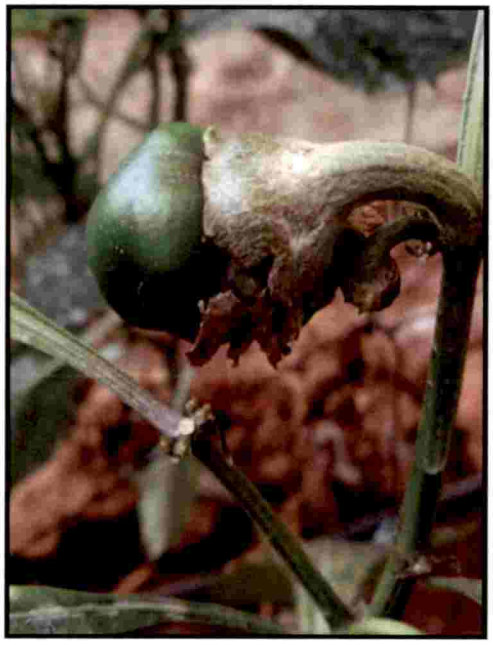
Under open field conditions

Plate 4.11: Stunted growth

a) Under protected condition

b) Under open field condition

Plate 4.12: Deformation of fruits



a) Under protected condition

b) Under open field condition

Plate 4.13: Reduction in fruit size

a) Puckering of leaf

b) Upward curling of leaf

c) Crinkling of leaves

Plate 4.14 : Symptoms under artificial conditions
Vector transmission

Table 4.2: Vector transmission

Sl no.	Particulars	Number
1	Inoculated seedlings	10
2	Infected seedlings showing symptoms	6
3	Per cent transmission	60%
4	Average days taken for symptom expression	7

4.3.2.2. Graft transmission

Top wedge grafting method was adopted for artificial inoculation of virus from infected scion to healthy rootstock as mentioned in section 3.2.2.2. The healthy chilli rootstock grafted with infected scion started expressing symptoms 14 days of post inoculation. Initially, the newly emerged leaves from the rootstock showed typical leaf curling symptoms (Plate 4.15). Whereas puckering symptoms developed on both newly emerged as well as the existing leaves on rootstock. The observations of graft transmission studies are given in Table 4.3.

Table 4.3: Graft transmission

Sl no.	Particulars	Number
1	No. of grafts	15
2	No. of successful grafts	12
3	Grafts which produced symptoms	12
4	Per cent transmission	100
5	Average days taken for symptom expression	14

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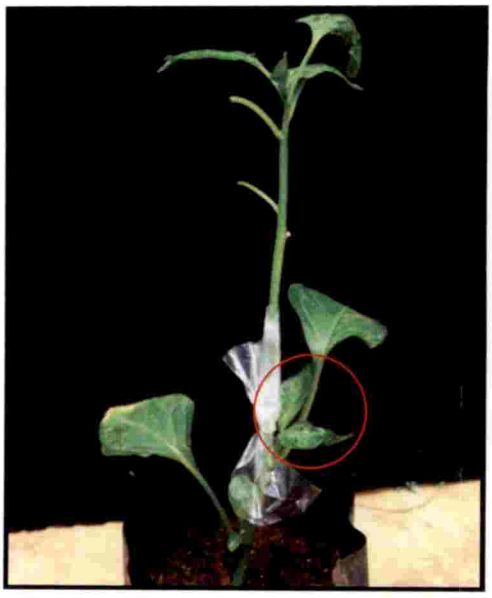
a) Puckering of leaf



b) Upward curling of leaf

c) Crinkling of leaves

Plate 4.15 : Symptoms under artificial conditions
Graft transmission

4.4. MOLECULAR CHARACTERIZATION

Molecular characterization of coat protein gene of six isolates *viz.,* four isolates collected from Thrissur district and one isolate each from Palakkad and Vellayani were undertaken. It is inferred that *Chilli leaf curl virus* belongs to genus *Begomovirus*, family *Geminiviridae*.

Total DNA was isolated from the infected leaf samples and were subjected to polymerase chain reaction. Two *Begomovirus* specific degenerate (universal) primers were used to test the presence of virus in the samples. Both primers amplified the core coat protein region (500 bp) of *Begomovirus*. The amplicons were cloned into the pTZ57R/T cloning vector and the clones were outsourced to Agri Genome, Kochi for sequencing. The sequence data were subjected to phylogenetic analysis.

4.4.1. Isolation of DNA

For molecular detection of *Chilli leaf curl virus* from the infected leaf samples, the total DNA was isolated using three methods *viz.,* CTAB method, modified CTAB method and using Qiagen® DNeasy plant minikit. The DNA isolated from the healthy chilli leaf sample served as the negative control. The quantity and quality of isolated DNA samples were determined using NanoDrop® ND 1000 spectrophotometer. The concentration and OD values of the DNA obtained using modified CTAB method and commercial kit are given in the Table 4.4. The total DNA was then visualized in agarose gel under UV light and image was documented using BIORAD Molecular Imager (GelDoc™ XR+). The samples with OD value ($A_{260/280}$) between 1.8 and 2.0 and with clear band on agarose gel were selected for polymerase chain reaction.

4.4.1.1. DNA Isolation using CTAB Method

In CTAB method, 5 per cent CTAB buffer was used for the extraction of total genomic DNA. The quality of DNA was not satisfactory with presence of high amount of polyphenolic compounds and polysaccharides. Also, DNA obtained using this method did not produce any amplicons from PCR reactions when used for the detection of virus in the sample.

4.4.1.2. DNA Isolation using Modified CTAB Method

Total DNA from leaf samples expressing leaf curl symptoms were isolated using modified CTAB method to get good quality and quantity DNA. The standardized CTAB method yielded better quantity of DNA from diseased chilli leaf samples but the quality of the isolated DNA was poor compared to DNA obtained using commercial DNA isolation kit (Plate 4.16). Also, the PCR amplifications of the isolated DNA did not yield good quality amplicons.

4.4.1.3. DNA isolation using commercial DNA isolation kit

The genomic DNA from diseased chilli leaf samples was isolated using the commercial DNA isolation kit (Qiagen DNeasy plant kit) (Plate 4.17). The quantity of the DNA isolated by modified CTAB method and commercial DNA isolation kit are given in Table 4.4.

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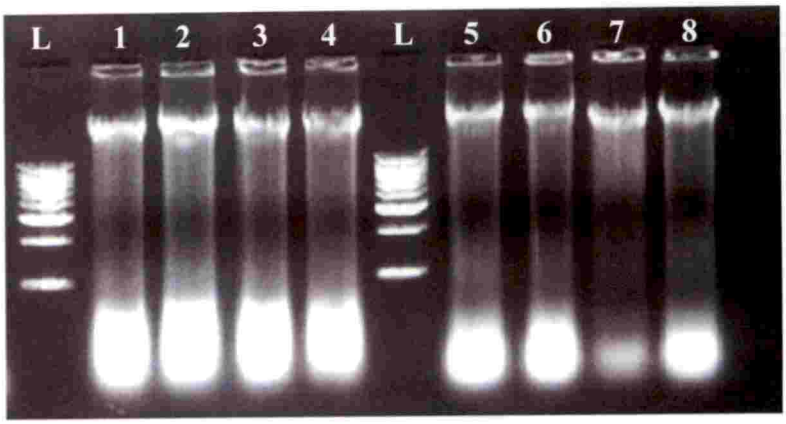


Plate 4.16: Genomic DNA isolated using modified CTAB method
L: Ladder (1kb), Lane 1-4, 5-8: DNA samples

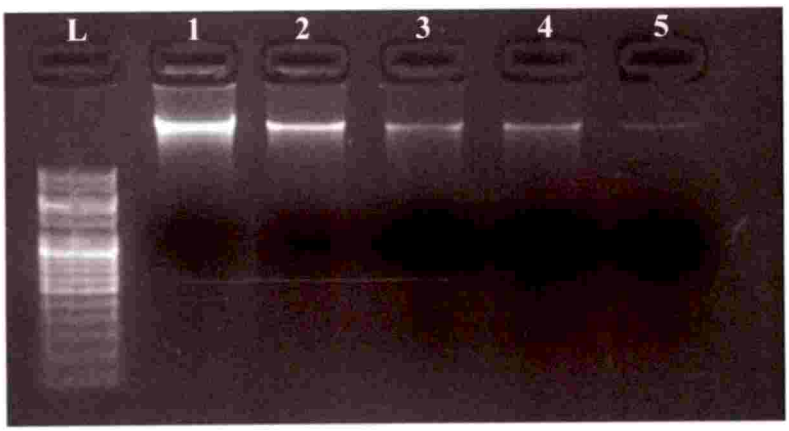


Plate 4.17: Genomic DNA isolated using commercial DNA isolation kit
L: Ladder (100bp to 3kb), Lane 1-5: DNA samples

Table 4.4: Quality and quantity of isolated DNA obtained using modified CTAB method and commercial DNA isolation kit

Sl no.	Modified CTAB method		Qiagen® DNeasy plant kit	
	DNA yield (ng/µl)	OD value (A 260/280)	DNA yield (ng/µl)	OD value (A 260/280)
R1	306.60	1.54	85.64	1.84
R2	289.60	1.68	23.20	1.80
R3	327.30	1.75	30.65	1.87
R4	178.60	1.73	21.50	1.82
R5	147.20	2.21	19.15	1.79
Average	**249.86**	**1.79**	**36.03**	**1.83**

4.4.1.4. Quality of genomic DNA

The agarose gel electrophoresis of the isolated DNA produced good DNA with intact bands for all the six isolates.

4.4.1.4. Quantity of DNA

The OD value (A 260/280) ranged from 1.8 to 1.96 which implied the integrity of the isolated DNA. The quantity of DNA (ng/µl) present per microliter of samples and the OD value are given in Table 4.5 (Plate 4.20).

Table 4.5: Quantity of DNA and OD value of the isolates obtained

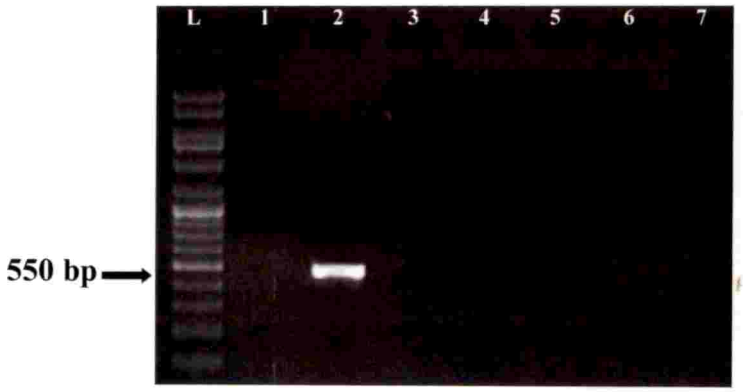
Location	Isolate	DNA yield (ng/µl)	OD value (A 260/280)
Vellanikkara	VKA1	21.30	1.80
	VKA2	20.50	1.92
Kannara	KAR1	40.50	1.82
Kodali	KOD4	52.80	1.96
Vithinassery	PKD1	25.60	1.95
Vellayani	VLNY1	21.03	1.82

4.4.3. Molecular detection using Polymerase Chain Reaction (PCR)

The presence of *Chilli leaf curl virus* in the total genomic DNA obtained from the infected samples were confirmed by PCR amplification using two sets of primer *viz.*, AV494 / AC1048 and Deng 540 / 541 mentioned in section 3.4.1.4.4. The optimum annealing temperatures for the primers and amplification profiles were standardized for both the primers. The presence of expected band confirmed the presence of the virus.

4.4.3.1. Primer Designing

Two pairs of forward and reverse primers were fabricated for the amplification of coat protein gene (AV1) of the virus isolates by multiple sequence alignment of coat protein nucleotide sequences retrieved from NCBI GenBank (Table 4.6). The primers were devised from the promising permutation of the conserved regions in the multiple sequence alignment. The primers designed were named as CLCVF & CLCVR and ChVelF & ChVelR which were expected to produce an amplicon of size 770 bp. The details of the designed primers are given in Table 4.7 and Table 4.8.



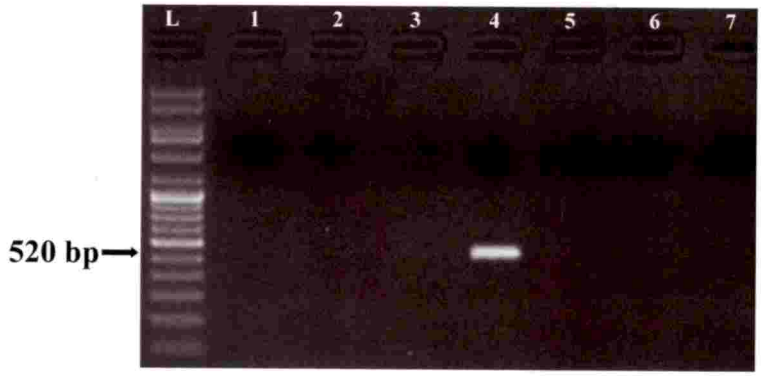
L - Ladder (100bp to 3kb)

Lane 1 – 55°C ; Lane 2 - 56°C ; Lane 3 - 57°C

Lane 4 - 58°C ; Lane 5 - 59°C ; Lane 6 - 60°C

Lane 7 - Negative control

Plate 4.18 : Standardization of annealing temperature for AV494/AC1048 primers using gradient PCR



L - Ladder (100bp to 3kb)

Lane 1 – 55°C ; Lane 2 - 56°C ; Lane 3 - 57°C

Lane 4 - 58°C ; Lane 5 - 59°C ; Lane 6 - 60°C

Lane 7 - Negative control

Plate 4.19 : Standardization of annealing temperature for Deng 540/541 primers using gradient PCR

4.4.3.2. Standardization of Annealing temperature

The annealing temperature for PCR amplification of the isolated DNA using the reported and designed primers were standardized using gradient PCR. The reported primer AV494 / AC1048 produced faint band at 56°C (Plate 4.18) while the reported primer Deng 540 / 541 produced faint band at 58°C (Plate 4.19). The designed primers CLCVF/ CLCVR and ChVelF/ ChVelR failed to produce any amplicons at all the tested temperatures. The results of standardization of PCR for annealing temperature of the primers are shown in Table 4.9.

Table 4.6: Details of the selected coat protein gene sequences of *Chilli leaf curl virus* isolates used for primer designing

Virus isolate	GenBank Accession no.
Chilli leaf curl virus clone A1 coat protein (AV1) gene, partial cds	KY769275.1
Chilli leaf curl virus coat protein (AV1) gene, partial cds	FJ403045.1
Chilli leaf curl virus isolate India:UP:Matera:Potato:2014 coat protein (AV1) gene, complete cds	KJ590964.1
Chilli leaf curl virus isolate Lucknow coat protein (AV1) gene, complete cds	JF682241.1
Chilli leaf curl virus isolate Narwan AC1 protein (AC1), AV2 protein (AV2), and AV1 protein (AV1) genes, partial cds	DQ445255.1
Chilli leaf curl virus coat protein (CP) gene, partial cds	FJ558515.1
Chilli leaf curl virus [India:UP:Lucknow:Capsicum sp.11b:2011] coat protein (AV1) gene, complete cds	JN896946.1
Chilli leaf curl Multan virus AV2 gene for pre-coat protein and partial AV1 gene for coat protein	FN252382.1
Chilli Leaf curl Vellanad virus India/Vellanad/2008 clone pChVelK52 AV1 gene	NC038442.1
Chilli leaf curl virus-[Soyla:India:2009] coat protein-like (AV1) gene, complete sequence	HM004433.1
Chilli leaf curl virus-India isolate Ratlam coat protein (AV1) gene, complete cds	KT868894.1
Chilli leaf curl virus isolate Salvia1 clone Salvia1 AV1 coat protein gene, complete cds	KY216072.1
Chilli leaf curl virus isolate Nasik coat protein gene, partial cds	KY863455.1

Table 4.7: Degenerate primers for CP gene of *Chilli leaf curl virus*

Name of the primer	Sequence	Length (bp)	Expected amplicon size
CLCVF	5'TTAATTYSAWACKGAATCRTA 3'	21	770 bp
CLCVR	5'ATGTCSAAGCGDCCAGCA 3'	18	

Table 4.8: Primers for CP gene of *Chilli leaf curl Vellanad virus*

Name of the primer	Sequence	Length (bp)	Expected amplicon size
ChVelF	5' ATGTGGGACCCCCTAGTCAAT 3'	21	770 bp
ChVelR	5'GCGGCCTTAATTTGATACTGAATCG3'	24	

Table 4.9: Standardization of annealing temperature of reported primers

Temperature	Primer	
	AV494 / AC1048	**Deng 540 / 541**
55°C	No band	No band
56°C	Good band	No band
57°C	No band	No band
58°C	No band	Good band
59°C	No band	No band
60°C	No band	No band

L - Ladder (100bp to 3kb)

1 – VKA1 ; 2 – VKA2 ; 3 – KAR1

4 – KOD4 ; 5 – PKD1 ; 6 – VLNY1

Plate 4.20: Total genomic DNA of virus isolates

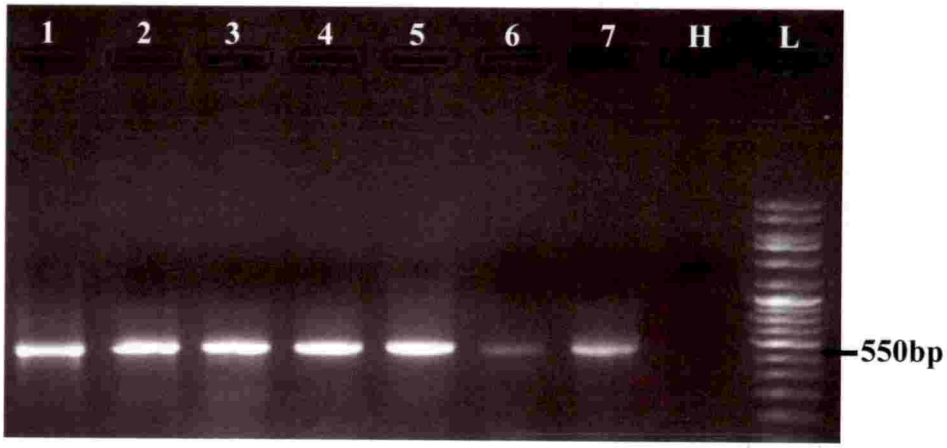


Plate 4.21: PCR gel profile of virus isolates

1 - Positive control

2 - VKA1 ; 3 – VKA2 ; 4 – KAR1

5 – KOD4 ; 6 – PKD1 ; 7 – VLNY1

H - Negative control ; L - Ladder (100bp to 3kb)

4.4.3.3. Standardization of Annealing Time

The standardization of annealing time for the standardized annealing temperature (section 4.4.3.1) was carried out in Applied Biosystems Thermocycler. The primer AV494 / AC1048 produced good band at 56°C for annealing time of 120s and the primer Deng 540 / 541 produced good band at 58°C for annealing time of 30s. The designed primers CLCVF/ CLCVR and ChVelF/ ChVelR failed to produce any amplicons. The outcomes are shown in Table 4.10.

Table 4.10: Standardization of PCR for annealing time

Time	Primer			
	AV494 / AC1048	**Deng 540 / 541**	**CLCVF/ CLCVR**	**ChVelF/ ChVelR**
30s	No band	Good band	No band	No band
45s	No band	Faint band	No band	No band
60s	Faint band	No band	No band	No band
90s	Faint band	No band	No band	No band
120s	Good band	No band	No band	No band

4.4.3.3. PCR amplification

The PCR reaction was performed using the standardized annealing temperature and time. The PCR amplification of coat protein gene of the obtained virus isolates was carried out using the degenerate primers *viz.*, AV494 / AC1048 and Deng 540 / 541 (Plate 4.21). The details of the PCR thermal profile is given in Table 4.11.

4.4.3.3.1. Analysis of PCR products

The isolated DNA amplified with primers AV494 / AC1048 and Deng 540 / 541 and generated amplicons of size 550 bp and 520 bp, respectively, when visualized on 1.2 per cent agarose gel. Whereas in control no bands were produced.

4.4.4. Elution of PCR product

The PCR products were analyzed in 1.2 per cent agarose gel. The 550 bp bands were eluted from agarose gel following the method given in section 3.4.1.4.6. The eluted products were used for transformation and cloning.

Table 4.11: PCR thermal profile

Steps	AV494 / AC1048		Deng 540 / 541	
	Temperature	Time	Temperature	Time
Initial denaturation	94° C	5min	94° C	5min
Denaturation	94° C	30s	94° C	30s
Annealing	56°C	120s	58°C	30s
Extension	72° C	45s	72° C	45s
Final extension	72° C	10min	72° C	10min

4.4.5. A - Tailing and ligation of PCR product

The desired amplicons (550 bp) eluted from agarose gel was then A - tailed and the products were analyzed. The products were then used for ligation with the cloning vector pTZ57R/T as mentioned in section 3.4.2.2.

Plate 4.22: Transformed colonies (White) in LB-ampicillin X-Gal/IPTG plates

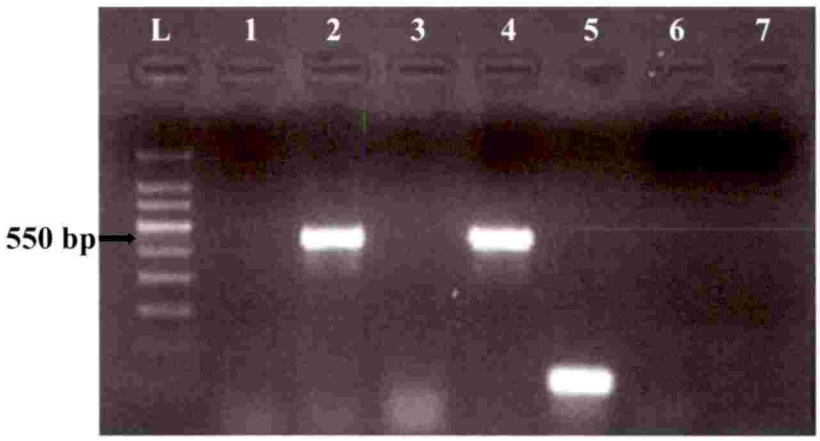


Plate 4.23: Colony PCR
L: Ladder (100bp), Lane 1-7: Clones 1-7

4.4.6. Transformation and Cloning

The PCR product, ligated to the vector pTZ57R/T was used for transforming *Escherichia coli* DH5α cells. Both blue and white colonies were formed after 16h incubation at 37°C. White colonies were considered as the transformants and analyzed using colony PCR for presence of the desired insert/coat protein gene (Plate 4.22). Number of blue and white colonies seen in the LB-ampicillin X-Gal/IPTG plates is given in Table 4.12.

Table: 4.12 Colony count from LB-ampicillin X-Gal/IPTG plates

Plate	No. of blue colonies	No. of white colonies
1	2	9
2	20	16

4.4.6.1. Colony PCR

The colony PCR amplification profile generated an amplicon of size 550 bp confirming the insert (Plate 4.23). The true recombinant colonies were subcultured and further stored at 4°C.

4.4.6. Sequencing of coat protein gene

The amplified products were outsourced to Agri Genome, Kochi for sequencing. The nucleotide sequence data of the six *Chilli leaf curl virus* isolates obtained after sequencing is given in Fig. 4.1a and 4.1b.

> **VKA1**

GAAGCCCAGGATGTACAGGATGTACAGAAGCGCTGATGTGCCTAAAGG
ATGTGAAGGCCCATGTAAGGTCCAGTCGTTTGAGTCTAGGCATGACGTA
ACCCATGTAGGTAAGGTAATGTGTATTAGTGATGTTACTCGGGGTAGTG
GGCTTACCCATAGAGTAGGTAAACGTTTTTGTGTGAAGTCCGTTTATGT
GTTGGGTAAAATATGGATGGATGAAAATATTAAAACGAAGAATCACAC
CAACAGTGTGATGTTCTTTCTAGTCCGTGATAGAAGACCCGTGGATAGA
CCCCAGGATTTCGGAGAAGTCTTCAACATGTTCGATAATGAACCCAGTA
CTGCAACCGTGAAGAATGTACATCGTGACAGATATCAAGTTCTGAGGA
AATGGCATGCAACTGTGACCGGTGGTCAGTACGCATCGAAGGAGCAGG
CATTAGTTAGGAAGTTTGTTAGGGTTAACAATTATGTAATTTATAACCA
GCAAGAGGCTGGCAAG

> **VKA2**

TAATGCATTCTCCTGGTGATTCTCATACTTGCCAGCCTCTTGCTGGTTAT
AAATTACATAATTGTTAACCCTAACAAACTTCCTAACTAATGCCTGCTC
CTTCGATGCGTACTGACCACCGGTCACAGTTGCATGCCATTTCCTCAGA
ACTTGATATCTGTCACGATGTACATTCTTCACGGTTGCAGTACTGGGTTC
ATTATCGAACATGTTGAAGACTTCTCCGAAATCCTGGGGTCTATCCACG
GGTCTTCTATCACGGACTAGAAAGAACATCACACTGTTGGTGTGATTCT
TCGTTTTAATATTTTCATCCATCCATATTTTACCCAACACATAAACGGAC
TTCACACAAAAACGTTTACCTACTCTATGGGTAAGCCCACTACCCCGAG
TAACATCACTAATACACATTACCTTACCTACATGGGTTACGTCATGCCT
AGACTCAAACGACTGGACCTTACATGGGCCTTCACATCCTTTAGGCACA
TCAGCGCTTCTGTACATCCTGTACATCCTGGG

> **KAR1**

GGCATGAGTACATGCCATATACAACATTAATGCATTCTCCTGGTGATTC
TCATACTTACCAGCCTCTTGCTGGTTGTAAATTACATAATTGTTAACCCT
AACAAACTTCCTAACTAATGCCTGCTCCTTCGATGCGTACTGACCACCC
GTCACAGTTGCATGCCATTTCCTCAGAACTTGATATCTGTCACGATGTAC
ATTCTTCACGGTTGCCGTACTGGGTTCATTATCGAACATGTTGAAGACTT
CTCCGAAATCCTGGGGTCTATCCACGGGTCTTCTATCACGTACTAGAAA
GAACATCACACTGTTAGTGTGATTCTTCGTTTTAATATTTTCATCCATCC
ATATTTTACCCAACACATAAACGGACTTCACACAAAAACGTTTACCTAC
TCTATGGGTCAGCCCACTACCCCGAGTAACATCACTAATACACATTACC
TTACCTACATGGGGTACGTCATGCCTAGACTCAAACGACTGGACCTTAC
ATGGGCC

Figure 1a: Nucleotide sequences of CP gene of virus isolates

> **KOD4**

GCCATATACAACATTAATGCATTCTCCTGGTGATTCTCATATTTACCAG
CCTCTTGCTGGTTATAAATTACATAATTGTTAACCCTAACAAACTTCCT
AACTAAAGCCTGCTCCTTCGATGCCTACTGACCACCCGTCACAGTTGCA
TGCCATTTCCTCAGAACTTGATATCTGTCACGATGTACATTCTTCACGG
TTGCAGTACTGGGTTCATTATCCAACATGTTGAAAACTTCTCCCAAATC
CTGGGGTCTATCCACGGGTCTTCTATCACGGACTAAAAAGAACATCAC
ACTGTTAGTGTGATTCTTCGTTTTAATATTTTCATCCATCCATATTTTAC
CCAACACATAAACGGACTTCACACAAAACCGTTTACCTACTCTATGGG
TCAGCCCACTACCCCGAGTAACATCACTAATACACATTACCTTACCTAC
ATGGGGTATGTCATGCCTAGACTCAAACGACTGGACCTTACATGGGCC
TTCACAT

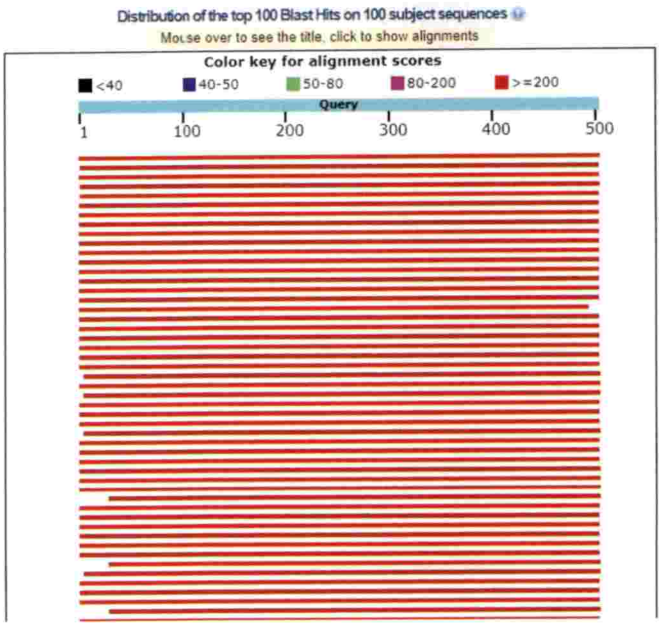
> **PKD1**

GCCCAGGATGTACAGGATGTACAGAAGCGCTGATGTGCCTAAAGGATG
TGAAGGCCCATGTAAGGTCCAGTCGTTTGAGTCTAGGCATGACGTAAC
CCATGTAGGTAAGGTAATGTGTATTAGTGATGTTACTCGGGGTAGTGG
GCTGACCCATAGAGTAGGTAAACGTTTTTGTGTGAAGTCCGTGTATGTG
TTGGGTAAAATATGGATGGATGAAAATATTAAAACGAAGAATCACACT
AACAGTGTGATGTTCTTTCTAGTCCGTGATAGAAGACCCGTGGATAGA
CCCCAGGATTTCGGAGAAGTCTTCAACATGTTCGATAATGAACCCAGT
ACTGCAACCGTGAAGAATGTACATCGTGACAGATATCAAGTTCTGAGG
AAATGGCATGCAACTGTGACTGGTGGTCAGTACGCATCGAAGGAGCAG
GCATTAGTTAGGAAGTTTGTTAGGGTTAACAATTATGTAATCTATAACC
AGCAAGAGGCTGGTAAGTATGAGA

> **VLNY1**

GTGAAGGCCCATGTAAGGTCCAGTCTTTTGAGTCCAGGCATGACGTGA
CCCACGTAGGGAAGGTTATGTGTATTAGTGATGTTACACGAGGTAGTG
GGCTTACCCATAGAGTAGGCAAACGTTTTTGTGTCAAGTCAGTGTATGT
GTTAGGTAAAATATGGATGGATGAAAATATCAAAACGAAAAACCACA
CTAACAGTGTGATGTTCTTCCTTGTCCGTGATAGAAGGCCTGTGGATAG
ACCCCAGGATTTTGGAGAAGTGTTCAACATGTTTGACAATGAACCCAG
TACCGCAACCGTGAAGAACGTACATCGTGACAGGTATCAAGTTTTGAG
GAAATGGCATGCAACTGTCACCGGAGGTCAATACGCATCGAAGGAGC
AGGCATTAGTTAGGAAGTTTGTTAGGGTTAACAATTATGTAATTTATAA
CCAGCAGGAAGCTGGTAAGT

Figure 1b: Nucleotide sequences of CP gene of virus isolates



a. Graphical output of BLASTn of isolate VKA1

Sequences producing significant alignments:

Select: All None Selected 0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chilli Leaf curl Vellanad virus [India/Vellanad/2008] clone [illegible] segment DNA A, complete sequence	723	723	100%	0.0	93%	NC_[illegible]
Croton yellow vein mosaic virus, complete sequence, clone HYDNA A	531	531	99%	1e-146	86%	FN[illegible]
Ageratum enation virus [India UP Bahraich Croton bonplandianum[illegible] 2011] coat protein (AV1) gene, complete cds	527	527	99%	2e-145	86%	JN[illegible]
Croton yellow vein virus, complete genome, clone 1	527	527	99%	2e-145	86%	LN[illegible]
Papaya leaf curl virus isolate DP1, complete genome	525	525	99%	6e-145	86%	KX[illegible]
Chilli leaf curl virus coat protein (CP) gene, partial cds	521	521	99%	7e-144	85%	FJ[illegible]
Papaya leaf curl virus clone Rad07, complete genome	518	518	99%	9e-143	85%	KY[illegible]
Papaya leaf curl virus clone Rad38, complete genome	518	518	99%	9e-143	85%	KY[illegible]
Ageratum enation virus [India UP Bahraich Croton bonplandianum7 2011] coat protein-like (AV1) gene, complete sequence	510	510	99%	2e-140	85%	JN[illegible]
Chili leaf curl Pakistan virus isolate YN1045, complete sequence	510	510	99%	2e-140	[illegible]	HM[illegible]
Tobacco curly shoot virus isolate [illegible] segment DNA A, complete genome	505	505	99%	7e-139	85%	KM[illegible]
Tobacco curly shoot virus isolate TbCSV-CN[illegible] Ru 01 24 Tom 19, complete genome	505	505	99%	7e-139	85%	KM[illegible]
Croton yellow vein mosaic virus isolate India UP Bahraich Croton bonplandianus 2012 coat protein (AV1) gene, complete cds	505	505	99%	7e-139	85%	KC[illegible]
Radish leaf curl virus isolate Varanasi, complete genome	505	505	99%	7e-139	85%	[illegible]
Radish leaf curl virus segment A, complete sequence	505	505	99%	7e-139	85%	EF175733.1
Papaya leaf curl virus AV1, AV2, AC1, AC2, AC3, AC4, AC5 genes and IR region	505	505	99%	7e-139	85%	Y15934.1
Chili leaf curl virus coat protein (AV1) gene, partial cds	501	501	97%	1e-137	86%	FJ[illegible]
Ageratum enation virus isolate TC228 segment DNA A, complete sequence	499	499	99%	3e-137	85%	KP[illegible]

b. Text output of isolate VKA1

Figure 4.2: BLASTn analysis of CP region of isolate VKA1

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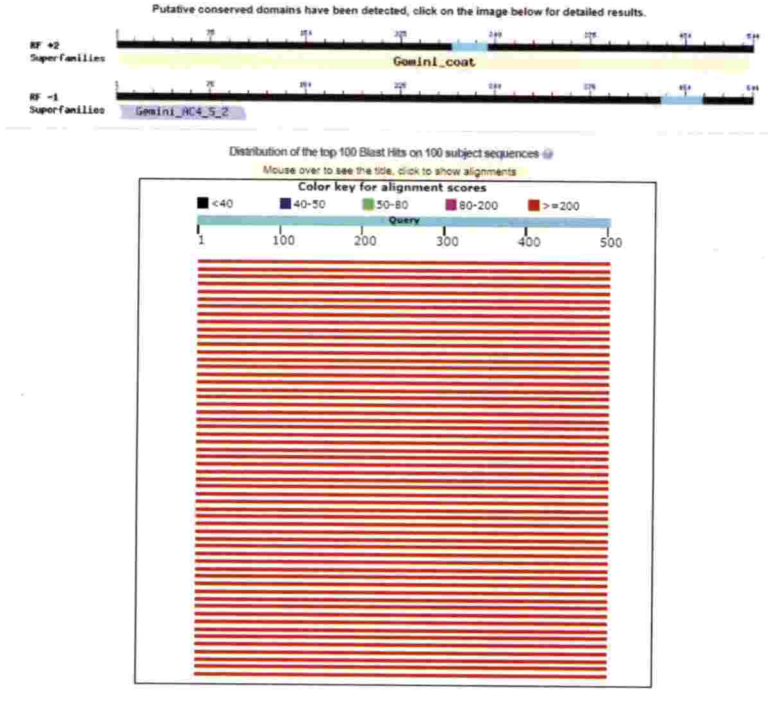


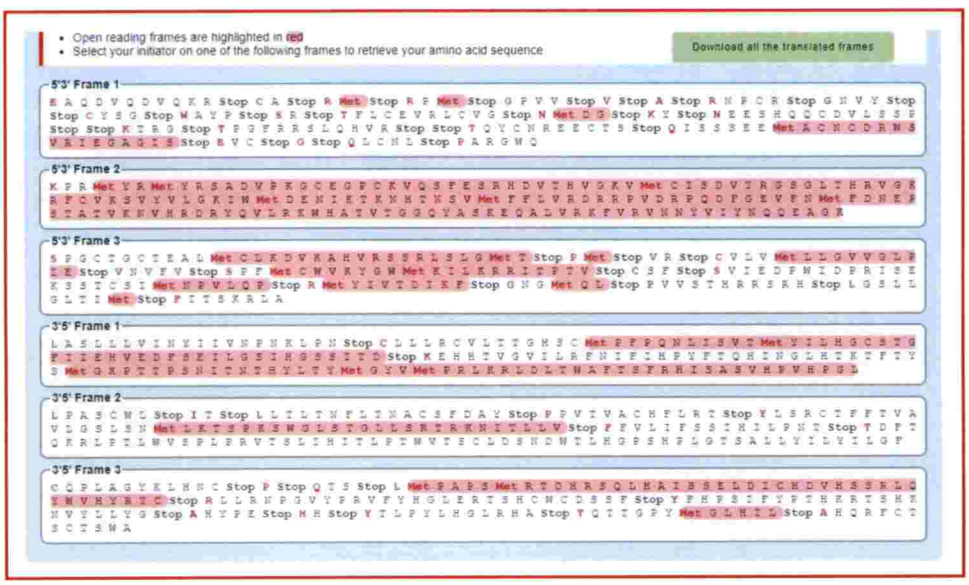
a. Graphical output of isolate VKA1

b. Text output of isolate VKA1

Figure 4.3: BLASTx analysis of CP region of isolate VKA1

MYRMYRSADVPKGCEGPCKVQSFESRHDVTHVGKVMCISDVTR
GSGLTHRVGKRFCVKSVYVLGKIWMDENIKTKNHTNSVMFFLV
RDRRPVDRPQDFGEVFNMFDNEPSTATVKNVHRDRYQVLRKWH
ATVTGGQYASKEQALVRKFVRVNNYVIYNQQEAGK

a. Amino acid sequence of CP region of isolate VKA1



b. Open reading frames in translated sequence of isolate VKA1

Figure 4.4: Translation of nucleotide sequence of isolate VKA1 generated on ExPASy Translate

4.4.7. *In silico* analysis

Annotation of nucleotide sequences of different virus isolates using nucleotide BLAST analysis (BLASTn) established significant homology with *Chilli leaf curl vellanad virus* (accession NC038442.1) from Vellanad, Thiruvanathapuram district of Kerala. Also, BLASTx analysis of the viral sequences revealed the sequence similarity with *Chilli leaf curl Vellanad virus* coat protein (accession no. YP_009506391.1).

4.4.7.1. Homology search of isolate VKA1

Nucleotide BLAST (BLASTn) analysis of sequence data of isolate VKA1 revealed 92.63 per cent sequence similarity with the virus isolate *Chilli leaf curl Vellanad virus* (NC038442.1) from Vellanad, Thiruvanathapuram district of Kerala (Fig. 4.2) . The isolate also had more than 80 per cent similarity to *Begomovirus* isolates of *Papaya leaf curl virus*, *Radish leaf curl virus*, *Cotton leaf curl virus, Tobacco curly shoot virus, Croton yellow vein mosaic virus* and *Ageratum enation virus.* Isolate VKA1 also exhibited similarity to *Chilli leaf curl virus* isolates from Varanasi (KP868762, MH346125, FJ558515 and FJ403045), Ludhiana (KR074211), Sangrur (KM098113), Chhapra (JN663852), Pakistan (DQ116877, DQ116879 and DQ114477) and Yunnan (HM587709).

BLASTx (translated nucleotide - protein BLAST) analysis performed on the sequence data of isolate VKA1 revealed 99.40 per cent sequence identity with *Chilli leaf curl Vellanad virus* coat protein (accession no. YP_009506391.1) (Fig. 4.3). The analysis revealed similarity of the isolate with putative conserved domain of *Geminivirus* coat protein/nuclear export factor (BR1 family).

The sequence data obtained was also used to deduce corresponding amino acid sequence by ExPASy Translate tool. The sequence was translated into six possible Open Reading Frame (ORF). Among which the longest was frame 2 (5'-3') commencing from base 11 to 502, having a stretch of 492 nucleotides with 163 amino acids (Fig. 4.4).

The sequence data was deposited in GenBank, NCBI database using BankIt tool (https://www.ncbi.nlm.nih.gov/WebSub/) and the accession number MN231252 was assigned to the isolate VKA1 from Vellanikkara.

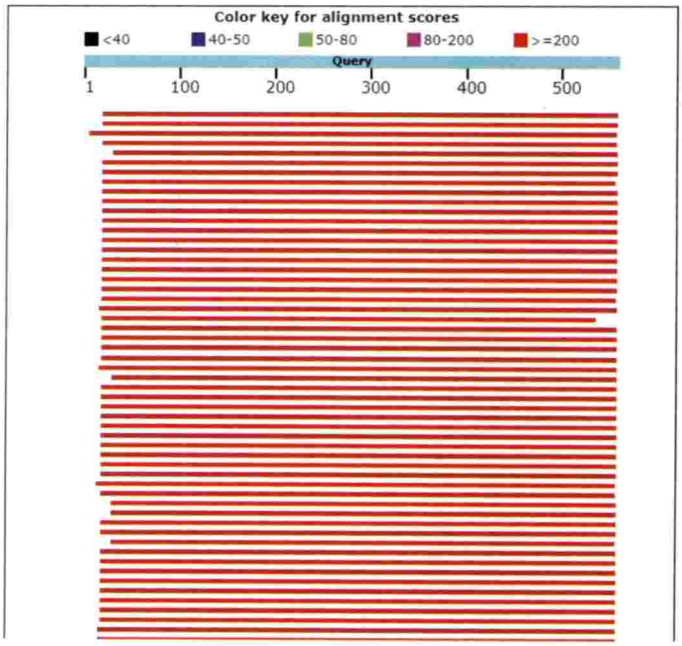
4.4.7.2. Homology search of isolate VKA2

Nucleotide BLAST (BLASTn) analysis isolate VKA2 showed 92.57 per cent sequence similarity with the virus isolate *Chilli leaf curl Vellanad virus* (NC038442.1) (Fig. 4.5). The isolate exhibited more than 80 per cent similarity to *Chilli leaf curl virus* isolates from Guntur (HM007100 and KT835649), Varanasi (FJ558515 and FJ403045), Ludhiana (KR074211), Nagpur (JN663865), Chhapra (JN663852) and Pakistan (DQ116877 and DQ116879). Apart from leaf curl virus from chilli, isolate VKA2 showed similarity with different isolates of viruses such as *Tomato leaf curl virus, Papaya leaf curl virus*, *Radish leaf curl virus*, *Cotton leaf curl virus, Tobacco curly shoot virus, Croton yellow vein mosaic virus* and *Ageratum enation virus*.

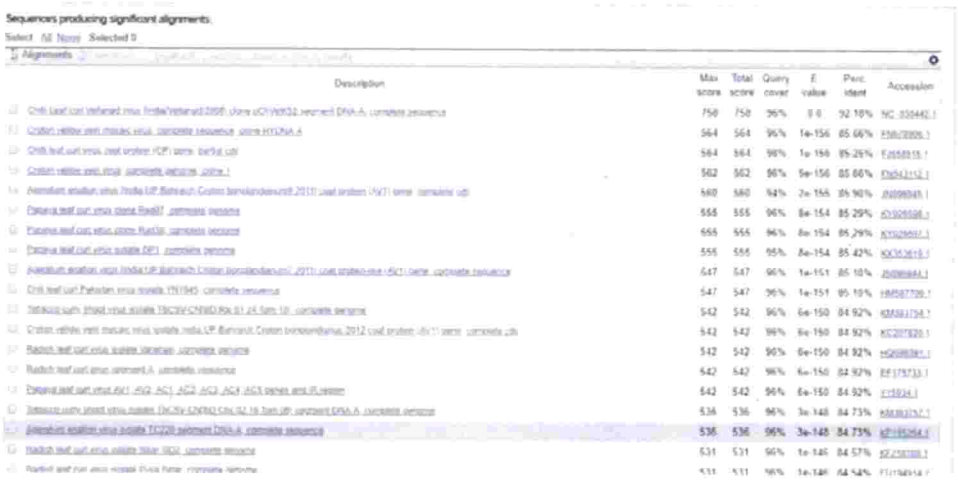
BLASTx (translated nucleotide - protein BLAST) analysis performed on the sequence data of isolate VKA2 revealed 99.43 per cent sequence identity with *Chilli leaf curl Vellanad virus* coat protein sequence (accession no. YP_009506391.1) (Fig. 4.6). The analysis revealed similarity of the isolate with putative conserved domain of *Geminivirus* coat protein/nuclear export factor (BR1 family).

The sequence data obtained was also used to deduce corresponding amino acid sequence by ExPASy Translate tool. The sequence was translated into six possible Open Reading Frame (ORF). Among which the longest was frame 1 (3'-5') commencing from base 1 to 519, having a stretch of 519 nucleotides with 172 amino acids (Fig. 4.7).

The sequence data was deposited in GenBank, NCBI database using BankIt tool (https://www.ncbi.nlm.nih.gov/WebSub/) and the accession number MN231249 was assigned to the isolate VKA2 from Vellanikkara.

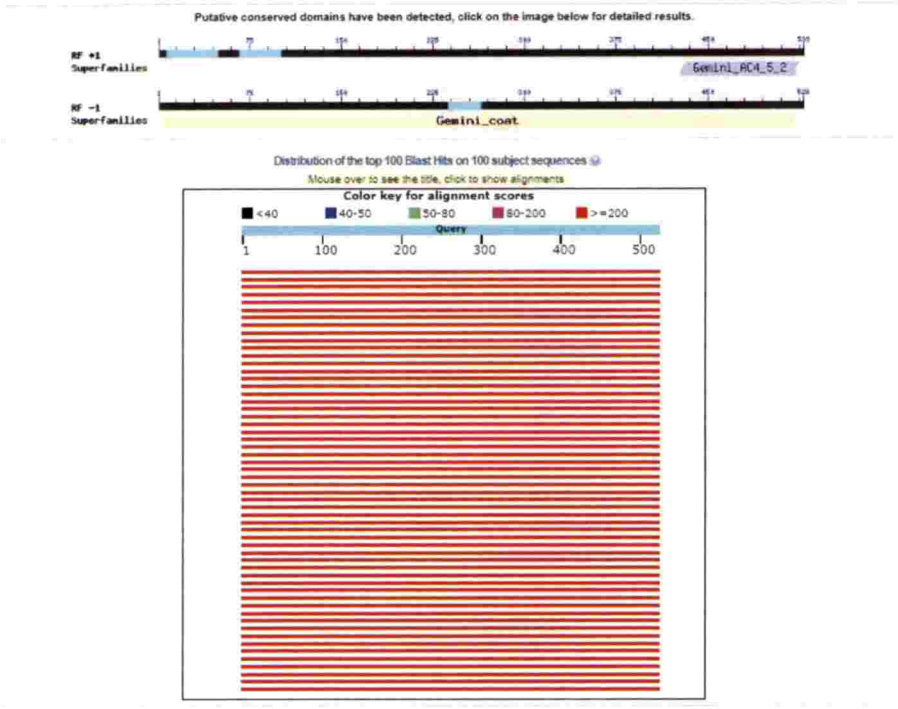


a. Graphical output of BLASTn of isolate VKA2



b. Text output of isolate VKA2

Figure 4.5: BLASTn analysis of CP region of isolate VKA2



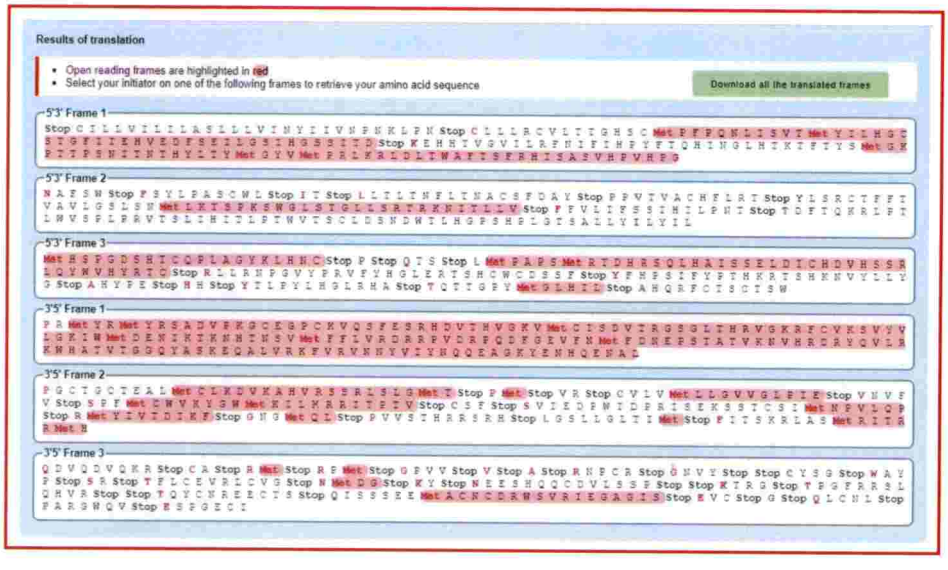
a. Graphical output of isolate VKA2

b. Text output of isolate VKA2

Figure 4.6: BLASTx analysis of CP region of isolate VKA2

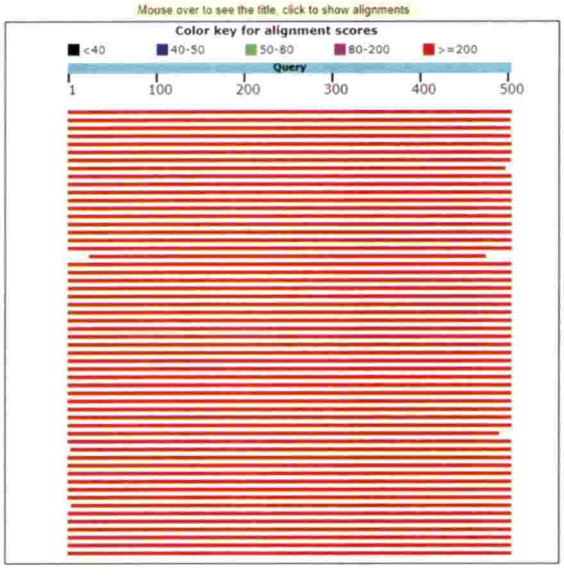
MYRMYRSADVPKGCEGPCKVQSFESRHDVTHVGKVMCISDVTRGS
GLTHRVGKRFCVKSVYVLGKIWMDENIKTKNHTNSVMFFLVRDRR
PVDRPQDFGEVFNMFDNEPSTATVKNVHRDRYQVLRKWHATVTG
GQYASKEQALVRKFVRVNNYVIYNQQEAGKYENHQENAL

a. Amino acid sequence of CP region of isolate VKA2



b. Open reading frames in translated sequence of isolate VKA2

Figure 4.7: Translation of nucleotide sequence of isolate VKA2 generated on ExPASy Translate



a. Graphical output of BLASTn of isolate KAR1

Sequences producing significant alignments:

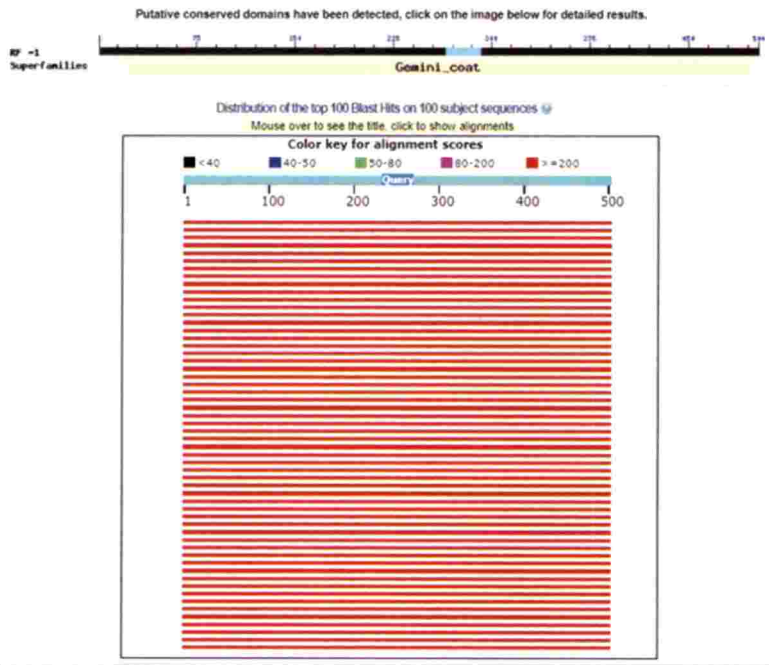
Select All None Selected 0

Alignments

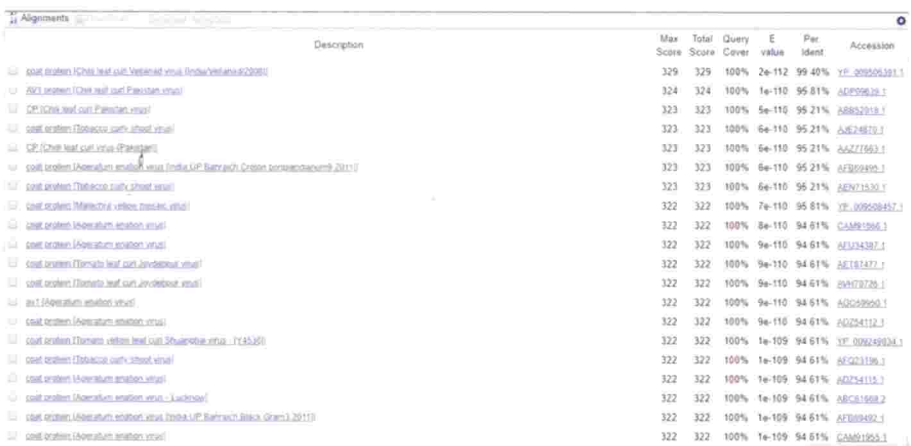
Description	Max score	Total score	Query cover	E value	Ident	Accession
Chilli Leaf curl Vellanad virus 'India/Vellanad/2009' clone pCrVelK52 segment DNA-A, complete sequence	699	699	100%	0.0	91.82%	NC_038442.1
Papaya leaf curl virus isolate DP1, complete genome	531	531	99%	1e-146	85.80%	KX053619.1
Croton yellow vein mosaic virus, complete sequence, clone HYDNA-A	531	531	99%	1e-146	85.80%	FN678906.1
Ageratum enation virus 'India UP Bahraich Croton bonglandlanum9 2011' coat protein (AV1) gene, complete cds	525	525	99%	6e-145	85.60%	JN996845.1
Papaya leaf curl virus clone Rad07, complete genome	523	523	100%	2e-144	85.43%	KY026596.1
Papaya leaf curl virus clone Rad08, complete genome	523	523	100%	2e-144	85.43%	KY026597.1
Croton yellow vein virus, complete genome, clone 1	520	520	99%	3e-143	85.46%	FN543112.1
Chilli leaf curl virus coat protein (AV1) gene, partial cds	520	520	98%	3e-143	85.63%	FJ403045.1
Tobacco curly shoot virus isolate TbCSV-CNRBD Cju 02 16 Tom 06 segment DNA-A, complete genome	516	516	100%	3e-142	85.23%	KM383757.1
Tobacco curly shoot virus isolate TbCSV-CNRBD Rja 01 24 Tom 10, complete genome	516	516	100%	3e-142	85.23%	KM383754.1
Tomato leaf curl Guanxi virus isolate TC49 segment DNA-A, complete sequence	516	516	100%	3e-142	85.23%	KP178726.1
Ageratum enation virus isolate TC228 segment DNA-A, complete sequence	516	516	100%	3e-142	85.23%	KP196264.1
Ageratum enation virus 'India UP Bahraich Croton bonglandlanum7 2011' coat protein-like (Av1) gene, complete sequence	516	516	100%	3e-142	85.23%	JN896844.1
Papaya leaf curl virus AV1, AV2, AC1, AC2, AC3, AC4, AC5 genes and IR region	516	516	100%	3e-142	85.23%	Y15934.1
Radish leaf curl virus isolate Pusa Bihar, complete genome	505	505	100%	7e-139	84.83%	EU194914.2
Tobacco curly shoot virus isolate TbCSV-CNRBD Chi 02 14 Tom 06, complete genome	499	499	100%	3e-137	84.63%	KM383753.1

b. Text output of isolate KAR1

Figure 4.8: BLASTn analysis of CP sequence of isolate KAR1



a. Graphical output of isolate KAR1

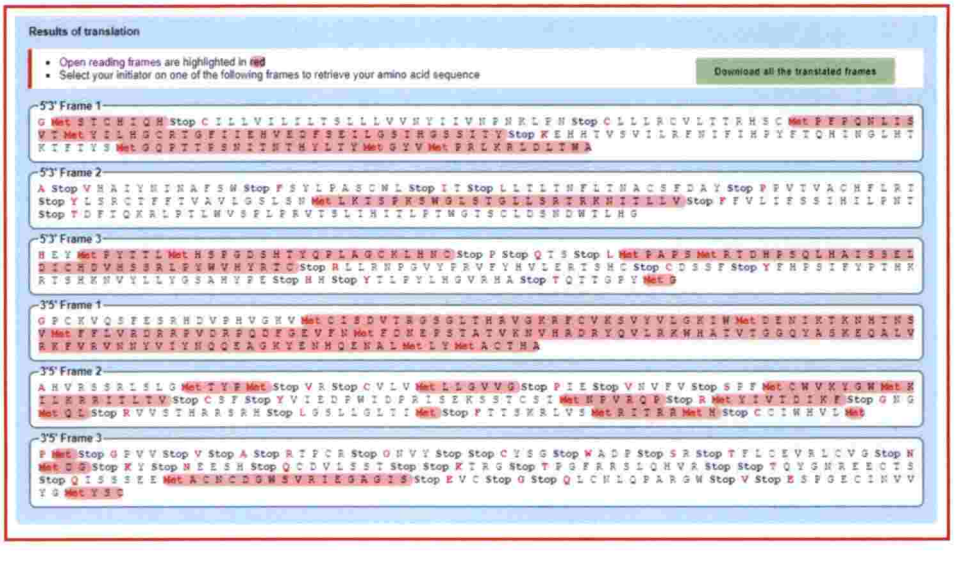


b. Text output of isolate KAR1

Figure 4.9: BLASTx analysis of CP region of isolate KAR1

MCISDVTRGSGLTHRVGKRFCVKSVYVLGKIWMDENIKTKNHTNS
VMFFLVRDRRPVDRPQDFGEVFNMFDNEPSTATVKNVHRDRYQVL
RKWHATVTGGQYASKEQALVRKFVRVNNYVIYNQQEAGKYENHQ
ENALMLYMACTHA

a. Amino acid sequence of CP region of isolate KAR1



b. Open reading frames in translated sequence of isolate KAR1

Figure 4.10: Translation of nucleotide sequence of isolate KAR1 generated on ExPASy Translate

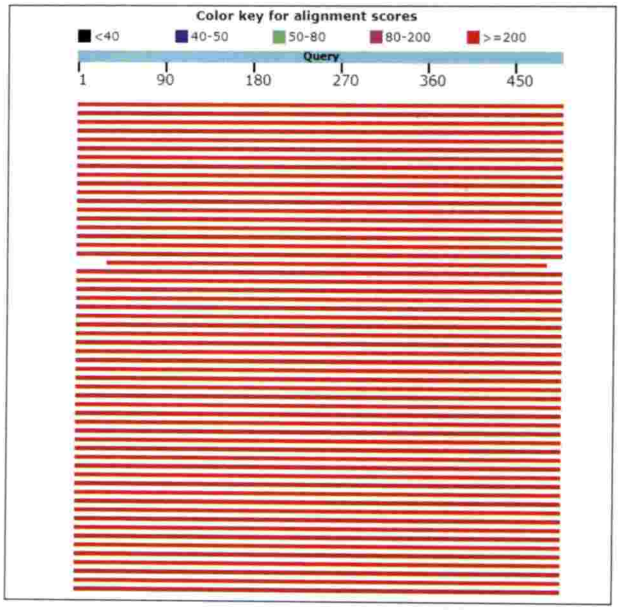
4.4.7.3. Homology search of isolate KAR1

Nucleotide BLAST (BLASTn) analysis isolate KAR1 showed 92.02 per cent sequence similarity with the virus isolate *Chilli leaf curl Vellanad virus* (NC038442.1) with 100 per cent query coverage and minimum E-value (Fig. 4.8). In the 100 BLAST hits for isolate KAR1, there were only three *Chilli leaf curl virus* accessions; NC038442.1 from Vellanad and FJ403045 & FJ558515 from Varanasi. Accession no. FJ403045 and FJ558515 (*Chilli leaf curl virus* coat protein (AV1) gene, partial cds.) showed 85.63 per cent and 84.63 per cent sequence identity, respectively, with 98 per cent query coverage and 3e-143 E-value. The BLASTn analysis of the isolate KAR1 sequence, it revealed more than 85 per cent similarity with different isolates of *Tomato leaf curl virus* (KP178726.1), *Papaya leaf curl virus* (KX353619.1, KY026598.1 and Y15934.1), *Tobacco curly shoot virus* (KM383757.1 and KM383754.1), *Croton yellow vein mosaic virus* (FN678906.1 and FN543112.1) and *Ageratum enation virus* (JN896945.1, KP195264.1 and JN896944.1).

BLASTx (translated nucleotide - protein BLAST) analysis performed on the sequence data of isolate KAR1 revealed 99.40 per cent sequence identity with *Chilli leaf curl Vellanad virus* coat protein sequence (accession no. YP_009506391.1) (Fig. 4.9). The analysis revealed similarity of the isolate with putative conserved domain of *Geminivirus* coat protein/nuclear export factor (BR1 family).

The sequence data obtained was also used to deduce corresponding amino acid sequence by ExPASy Translate tool. The sequence was translated into six possible Open Reading Frame (ORF). Among which the longest was frame 1 (3'-5') commencing from base 1 to 441, having a stretch of 441 nucleotides with 146 amino acids (Fig. 4.10).

The sequence data was deposited in GenBank, NCBI database using BankIt tool (https://www.ncbi.nlm.nih.gov/WebSub/) and the accession number MN231250 was assigned to the isolate KAR1 from Kannara.



a. Graphical output of BLASTn of isolate KOD4

Sequences producing significant alignments:

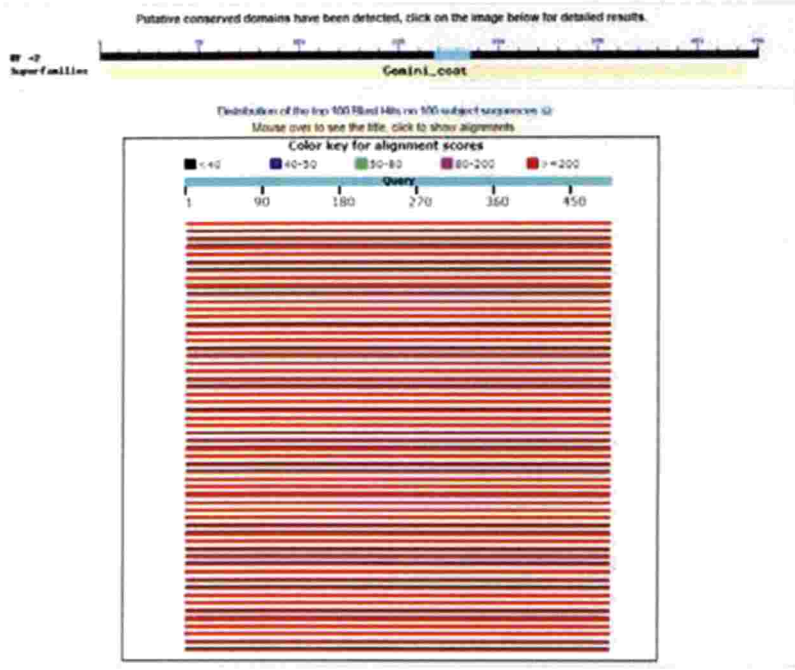
Select: All None Selected 0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chilli leaf curl Vellanad virus India/Vellanad/2008 clone pChiVelK02 segment DNA-A, complete sequence	671	671	100%	0.0	91.11%	[illegible]
Croton yellow vein mosaic virus, complete sequence, clone [illegible] DNA-A	544	544	100%	2e-150	86.46%	[illegible]
Croton yellow vein virus, complete genome, clone [illegible]	532	532	100%	3e-147	86.06%	[illegible]
Chilli leaf curl virus coat protein (AV1) gene, partial cds	532	532	100%	3e-147	86.06%	[illegible]
Papaya leaf curl virus clone [illegible], complete genome	523	523	100%	2e-144	85.66%	[illegible]
Papaya leaf curl virus clone [illegible], complete genome	523	523	100%	2e-144	85.66%	[illegible]
Papaya leaf curl virus isolate DF1, complete genome	521	521	100%	7e-144	85.66%	[illegible]
Tobacco curly shoot virus isolate TbCSV CMBD [illegible], complete genome	516	516	100%	3e-142	85.46%	[illegible]
Ageratum enation virus isolate TC276 segment DNA-A, complete sequence	516	516	100%	3e-142	85.45%	[illegible]
Ageratum enation virus India UP Bahraich Croton bonplandianum 2011 coat protein (AV1) gene, complete cds	516	516	100%	3e-142	85.45%	[illegible]
Ageratum enation virus India UP Bahraich Croton bonplandianum2 2011 coat protein-like (AV1) gene, complete sequence	516	516	100%	3e-142	85.45%	[illegible]
Chilli leaf curl virus coat protein (CP) gene, partial cds	507	507	99%	2e-139	85.25%	[illegible]
Tobacco curly shoot virus isolate TbCSV CMBD [illegible], complete genome	505	505	100%	7e-139	85.06%	[illegible]
Croton yellow vein mosaic virus isolate India UP Bahraich Croton bonplandianum 2011 coat protein (AV1) gene, complete cds	505	505	100%	7e-139	85.06%	[illegible]
Radish leaf curl virus isolate Pusa Bihar, complete genome	505	505	100%	7e-139	85.05%	[illegible]
Papaya leaf curl virus AV1, AV2, AC1, AC2, AC3, AC4, AC5 genes and IR region	505	505	100%	7e-135	85.05%	[illegible]
Papaya leaf Curl virus	499	499	100%	3e-137	84.85%	[illegible]
Tobacco curly shoot virus isolate TbCSV CMBD [illegible], complete genome	499	499	100%	3e-137	84.86%	[illegible]

b. Text output of isolate KOD4

Figure 4.11: BLASTn analysis of CP region of isolate KOD4



a. Graphical output of isolate KOD4

Alignments

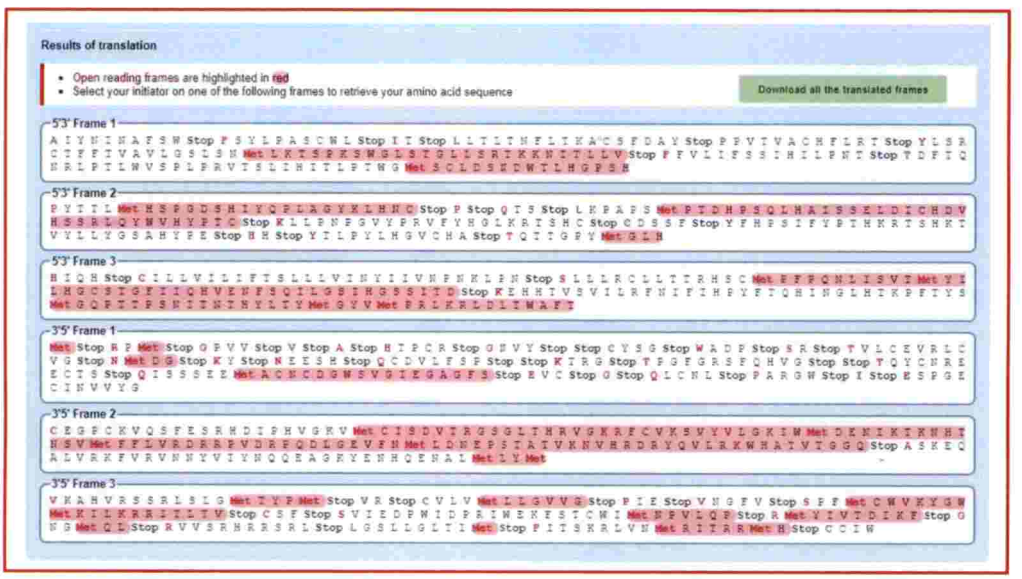
Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
coat protein [Chilli leaf curl Vellanad virus (India:Vellanad:2009)]	314	314	99%	1e-106	96.95%	YP_009506391.1
AV1 protein [Chili leaf curl Pakistan virus]	310	310	99%	4e-105	94.51%	ADP09639.1
CP [Chili leaf curl Pakistan virus]	309	309	99%	1e-104	93.90%	ABB52019.1
coat protein [Ageratum enation virus (India:UP:Bahraich:Croton bonplandianus:8:2011)]	309	309	99%	1e-104	93.90%	AFB69495.1
coat protein [Tobacco curly shoot virus]	309	309	99%	1e-104	93.90%	AJE24870.1
CP [Chilli leaf curl virus (Pakistan)]	309	309	99%	1e-104	93.90%	AAZ77863.1
coat protein [Tobacco curly shoot virus]	309	309	99%	1e-104	93.90%	AEN71530.1
coat protein [Ageratum enation virus]	309	309	99%	2e-104	93.29%	AFU34387.1
coat protein [Chilli leaf curl virus]	306	306	99%	2e-104	93.90%	AC023318.1
coat protein [Pepper leaf curl virus isolate Mahurangan]	307	307	99%	2e-104	93.29%	AEQ23042.1
coat protein [Ageratum enation virus]	309	309	99%	2e-104	93.29%	ADZ54112.1
coat protein [Ageratum enation virus]	309	309	99%	2e-104	93.29%	CAM91666.1
av1 [Ageratum enation virus]	309	309	99%	2e-104	93.29%	AGO59950.1
coat protein [Tomato leaf curl Bangladesh virus]	308	308	99%	2e-104	93.29%	NP_803246.1
AV1 [Tobacco curly shoot virus]	308	308	99%	2e-104	93.29%	ADR75362.1
coat protein [Ageratum enation virus]	308	308	99%	2e-104	93.29%	ADZ54114.2
coat protein [Tobacco curly shoot virus]	308	308	99%	2e-104	93.29%	AFQ23196.1
coat protein [Ageratum enation virus (India:UP:Bahraich:Ageratum conyzoides6:2011)]	308	308	99%	2e-104	93.29%	AFB69494.1
coat protein [Tomato yellow leaf curl Shuangbai virus - [Y45:35]]	308	308	99%	2e-104	93.29%	YP_009249834.1
coat protein [Ageratum enation virus]	308	308	99%	2e-104	93.29%	AJE24738.1

b. Text output of isolate KOD4

Figure 4.12: BLASTx analysis of CP region of isolate KOD4

MCISDVTRGSGLTHRVGKRFCVKSVYVLGKIWMDENIKTKNHTNS
VMFFLVRDRRPVDRPQDLGEVFNMLDNEPSTATVKNVHRDRYQVL
RKWHATVTGGQ

a. Amino acid sequence of CP region of isolate KOD4



b. Open reading frames in translated sequence of isolate KOD4

Figure 4.13: Translation of nucleotide sequence of isolate KOD4 generated on ExPASy Translate

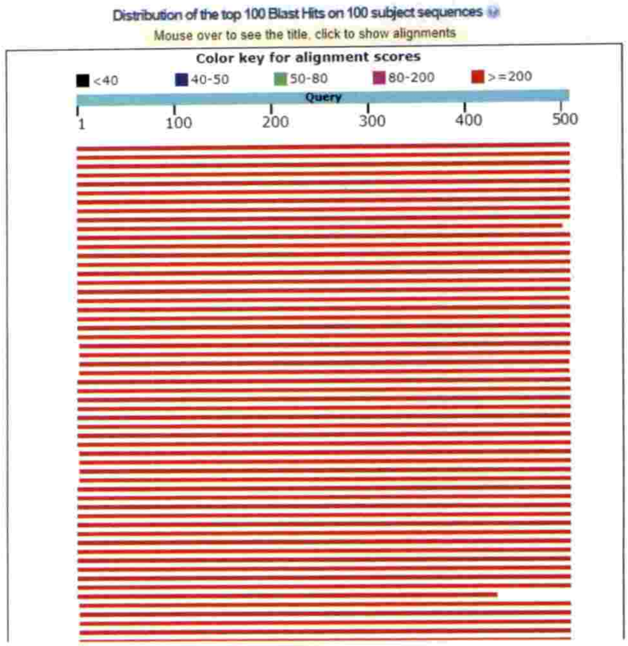
4.4.7.4. Homology search of isolate KOD4

Nucleotide BLAST (BLASTn) analysis isolate KOD1 exhibited maximum similarity of 91.52 per cent with the virus isolate *Chilli leaf curl Vellanad virus* (NC038442.1) with minimum E-value (Fig. 4.11). Similar to isolate KAR1, the isolate KOD1 also had similarity with only three *Chilli leaf curl virus* accessions; NC038442.1 from Vellanad and FJ403045 & FJ558515 from Varanasi. Among the 100 BLASTn hits, there were nine accessions with more than 85 per cent sequence similarity to the isolate KOD1 *viz.*, *Chilli leaf curl virus* (FJ403045 and FJ558515), *Papaya leaf curl virus* (KY026598.1 and KY026597.1), *Tobacco curly shoot virus* (KM383752.1, KM383753.1 and KM383754.1) and *Ageratum enation virus* (JN896945.1, KP195264.1 and JN896944.1).

BLASTx (translated nucleotide - protein BLAST) analysis performed on the sequence data of isolate KOD4 revealed 96.95 per cent sequence identity with *Chilli leaf curl Vellanad virus* coat protein sequence (accession no. YP_009506391.1) (Fig. 4.12). The analysis revealed similarity of the isolate with putative conserved domain of *Geminivirus* coat protein/nuclear export factor (BR1 family).

The sequence data obtained was also used to deduce corresponding amino acid sequence by ExPASy Translate tool. The sequence was translated into six possible Open Reading Frame (ORF). Among which the longest was frame 2 (3'-5') commencing from base 428 to 123, having a stretch of 306 nucleotides with 101 amino acids (Fig. 4.13).

The sequence data was deposited in GenBank, NCBI database using BankIt tool (https://www.ncbi.nlm.nih.gov/WebSub/) and the accession number MN231248 was assigned to the isolate KOD4 from Kodali.



a. Graphical output of BLASTn of isolate PKD1

Sequences producing significant alignments:

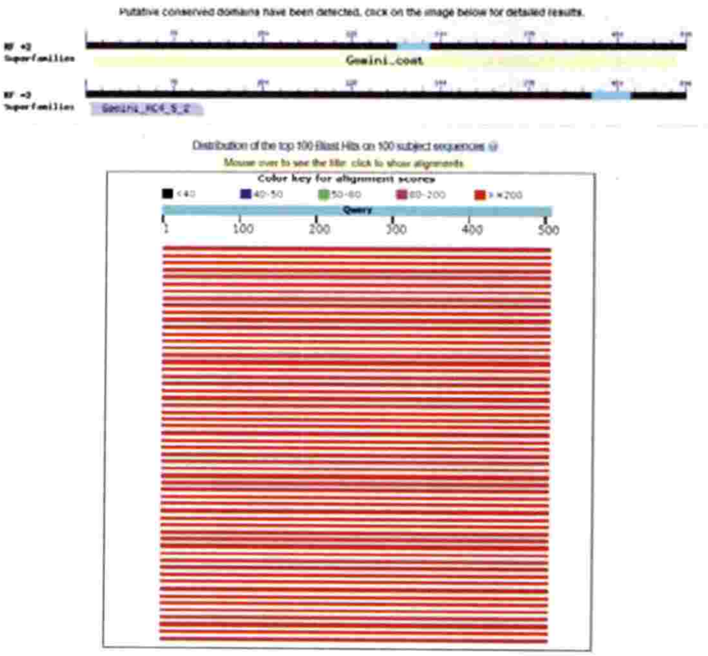
Select: All None Selected 0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chilli Leaf curl Vellanad virus India/Vellanad/2008, clone pChVelK52 segment DNA-A, complete sequence	730	730	100%	0.0	93%	NC_038442.1
Ageratum enation virus isolate UP Bahraich Croton bonplandianum8 2011 coat protein (AV1) gene, complete cds	542	542	100%	6e-150	86%	JN896945.1
Chilli leaf curl virus coat protein (CP) gene, partial cds	536	536	100%	3e-148	86%	FJ558515.1
Croton yellow vein mosaic virus, complete sequence, clone HYDNA-A	531	531	100%	1e-146	86%	FN678906.1
Papaya leaf curl virus isolate DP1, complete genome	525	525	100%	6e-145	85%	KC353619.1
Ageratum enation virus isolate UP Bahraich Croton bonplandianum7 2011 coat protein-like (AV1) gene, complete sequence	525	525	100%	6e-145	85%	JN896944.1
Croton yellow vein virus, complete genome, clone 1	525	525	100%	6e-145	85%	FN543112.1
Papaya leaf curl virus clone Rad07, complete genome	521	521	100%	7e-144	85%	KY026598.1
Papaya leaf curl virus clone Rad36, complete genome	521	521	100%	7e-144	85%	KY026597.1
Chilli leaf curl virus coat protein (AV1) gene, partial cds	520	520	98%	3e-143	86%	FJ493345.1
Tobacco curly shoot virus isolate TbCSV-CN[BD:Raj:01:24:Tom:1], complete genome	514	514	100%	1e-141	85%	KM383754.1
Croton yellow vein mosaic virus isolate India UP Bahraich Croton bonplandianus 2012 coat protein (AV1) gene, complete cds	514	514	100%	1e-141	85%	KC207820.1
Ageratum enation virus isolate TC228 segment DNA-A, complete sequence	508	508	100%	6e-140	85%	KP195264.1
Chilli leaf curl virus clone Pep-Ser coat protein-like gene, partial sequence	508	508	100%	6e-140	85%	KM098113.1
Papaya leaf curl virus AV1, AV2, AC1, AC2, AC3, AC4, AC5 genes and IR region	508	508	100%	6e-140	85%	Y15934.1
Chilli leaf curl virus isolate Luchiana clone pae1 coat protein gene, partial cds	503	503	100%	3e-138	85%	KR074211.1
Radish leaf curl virus isolate Pusa Bihar, complete genome	503	503	100%	3e-138	85%	EU194914.2
Papaya leaf Curl virus	496	496	99%	4e-136	84%	LN878129.1

b. Text output of isolate PKD1

Figure 4.14: BLASTn analysis of CP region of isolate PKD1



a. Graphical output of isolate PKD1

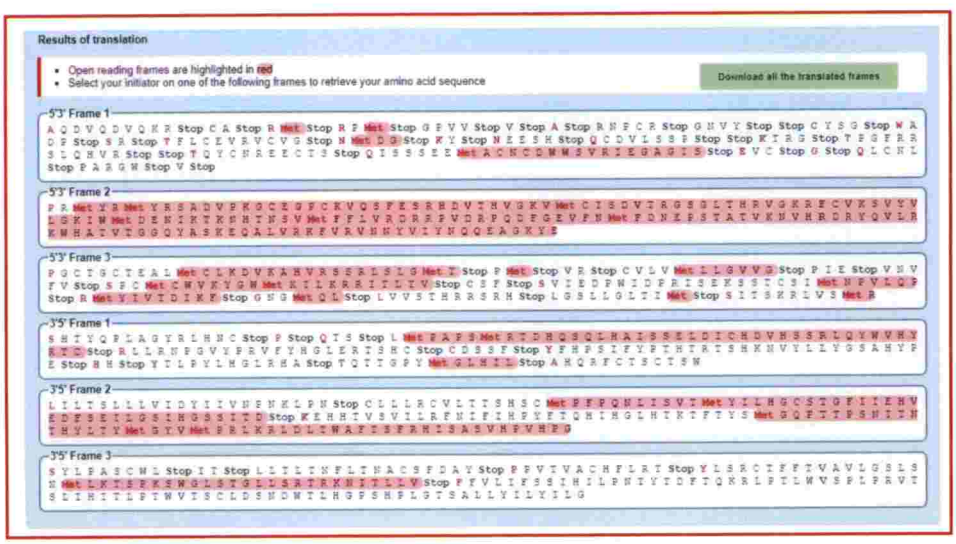
Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
coat protein [Chili leaf curl Vellanad virus [India/Vellanad/2008]]	331	331	99%	3e-113	99.40%	YP_009506391.1
AV1 protein [Chili leaf curl Pakistan virus]	325	325	99%	6e-111	95.24%	ADP09639.1
coat protein [Tobacco curly shoot virus]	325	325	99%	1e-110	94.64%	BBA21851.1
coat protein [Malachra yellow mosaic virus]	325	325	99%	1e-110	95.24%	YP_009508457.1
coat protein [Whitefly-transmitted Indian begomovirus]	324	324	99%	1e-110	94.64%	ABC70517.1
coat protein [Ageratum enation virus [India UP Bahraich Croton bonplandianum9 2011]]	324	324	99%	1e-110	94.64%	AFB89495.1
coat protein [Tomato leaf curl Joydebpur virus]	324	324	99%	2e-110	94.05%	AJE24835.1
coat protein [Croton yellow vein virus]	324	324	99%	2e-110	95.24%	YP_003864095.1
coat protein [Pepper leaf curl virus isolate Maharajganj]	322	322	99%	2e-110	94.05%	AEO23042.1
coat protein [Tomato leaf curl virus]	323	323	99%	2e-110	94.64%	AKA60698.1
coat protein [Sunflower leaf curl virus]	324	324	99%	2e-110	94.05%	AFX72833.1
coat protein [Pepper leaf curl virus isolate Varanasi]	322	322	99%	2e-110	94.64%	AEO23044.1
coat protein [Tomato leaf curl Karnataka virus]	324	324	99%	2e-110	94.64%	ACJ65693.1
coat protein [Begomovirus sp.]	324	324	99%	2e-110	94.64%	AOY34812.1
coat protein [Chili leaf curl Multan virus]	324	324	99%	2e-110	94.05%	ALN99432.1
coat protein [Tobacco curly shoot virus]	324	324	99%	2e-110	94.64%	AJE24870.1
coat protein [Tomato leaf curl Karnataka virus]	323	323	99%	2e-110	94.64%	CUR44406.1
coat protein [Tomato leaf curl Karnataka virus]	324	324	99%	2e-110	94.64%	AFX95949.1
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

b. Text output of isolate PKD1

Figure 4.15: BLASTx analysis of CP region of isolate PKD1

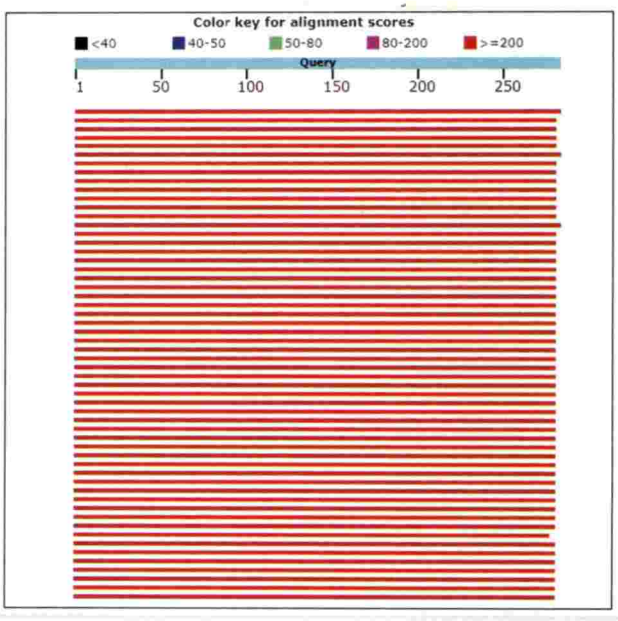
MYRMYRSADVPKGCEGPCKVQSFESRHDVTHVGKVMCISDVTRGS
GLTHRVGKRFCVKSVYVLGKIWMDENIKTKNHTNSVMFFLVRDRR
PVDRPQDFGEVFNMFDNEPSTATVKNVHRDRYQVLRKWHATVTG
GQYASKEQALVRKFVRVNNYVIYNQQEAGKYE

a. Amino acid sequence of CP region of isolate PKD1



b. Open reading frames in translated sequence of isolate PKD1

Figure 4.16: Translation of nucleotide sequence of isolate PKD1 generated on ExPASy Translate



a. Graphical output of BLASTn of isolate VLNY1

Sequences producing significant alignments:

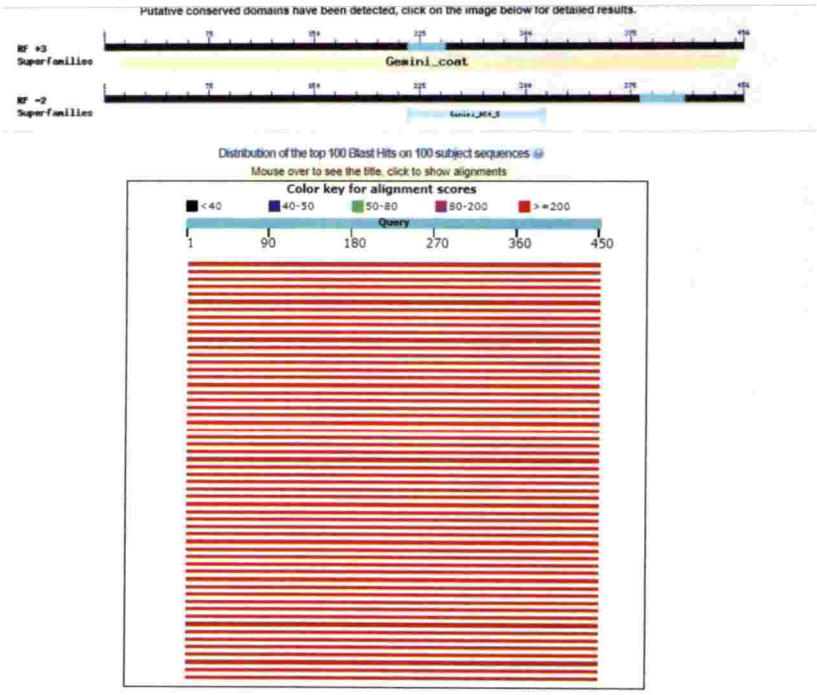
Select All None Selected 0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chilli Leaf curl Vellanad virus (India/Vellanad/2008) clone pCNV6Kb2 segment DNA A, complete sequence	484	484	100%	6e-133	98%	NC_038442.1
Chilli leaf curl virus clone Pep-Sgl coat protein-like gene, partial sequence	307	307	98%	1e-79	86%	KM098113.1
Chilli leaf curl virus isolate Jhugsi coat protein gene, partial cds	302	302	98%	6e-78	86%	MF400985.1
Chilli leaf curl virus isolate India:Amritsar Papaya:2009 segment DNA A, complete sequence	302	302	98%	6e-78	86%	GU136493.1
Chilli leaf curl Multan virus AV2 gene for pre-coat protein and partial AV1 gene for coat protein	302	302	98%	6e-78	86%	FN252382.1
Chilli leaf curl virus partial pc gene for coat protein, clone CHL4	302	302	100%	6e-78	86%	HE21044/8.1
Chilli leaf curl virus-[Multan] segment DNA-A, complete sequence	302	302	98%	6e-78	86%	AF338608.1
Pepper leaf curl Bangladesh virus isolate sl 11 segment DNA-A, complete sequence	296	296	98%	3e-76	86%	KY470149.1
Chilli leaf curl virus isolate New Delhi, complete genome	296	296	98%	3e-76	86%	KX533910.1
Chilli leaf curl virus complete genome, clone RM279	296	296	98%	3e-76	86%	LN885552.1
Chilli leaf curl virus clone A1 coat protein (AV1) gene, partial cds	296	296	98%	3e-76	86%	KY982/29.1
Papaya leaf curl virus isolate India/New Delhi/Papaya/2016 segment DNA-A, complete sequence	296	296	98%	3e-76	86%	KY300906.1
Chilli leaf curl virus isolate New Delhi, complete genome	296	296	98%	3e-76	86%	KX499526.1
Chilli leaf curl virus cotton Ludhiana clone pep2 coat protein gene, partial cds	296	296	100%	3e-76	86%	KR074212.1
Chilli leaf curl virus isolate Ludhiana clone pep1 coat protein gene, partial cds	296	296	98%	3e-76	86%	KR074211.1
Chilli leaf curl virus isolate LC coat protein gene, partial cds	296	296	98%	3e-76	86%	KM923895.1
Chilli leaf curl Multan virus av2 gene for pre coat protein, segment DNA-A, isolate LN	296	296	98%	3e-76	86%	HG932561.1
Chilli leaf curl virus clone Rat-P4U coat protein gene, partial cds	296	296	98%	3e-76	86%	KM068112.1

b. Text output of isolate VLNY1

Figure 4.17: BLASTn analysis of CP region of isolate VLNY1



a. Graphical output of isolate VLNY1

Alignments

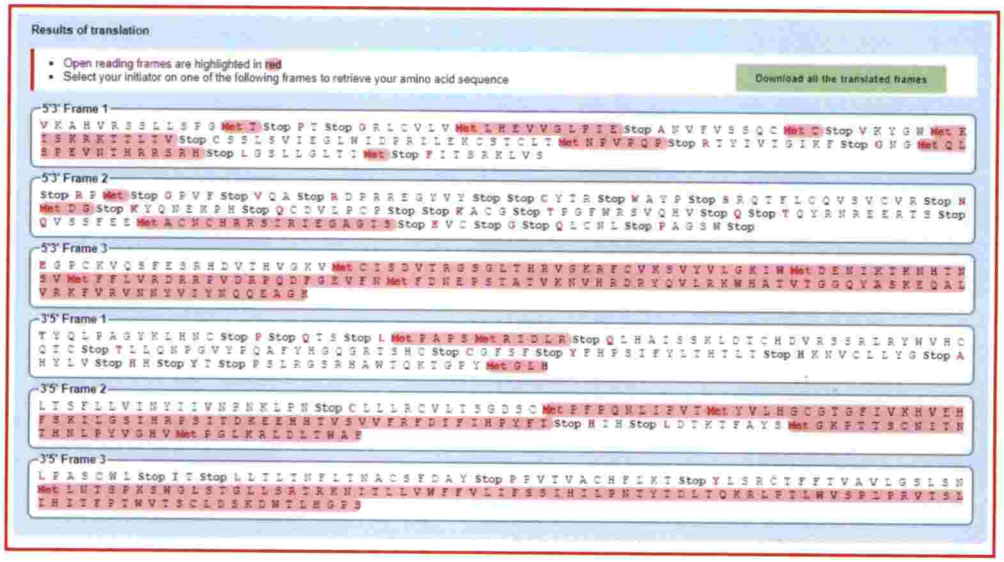
Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
coat protein [Chilli leaf curl Vellanad virus (India/Vellanad/2008)]	294	294	99%	6e-99	100.00%	YP_009506391.1
AV1 protein [Chili leaf curl Pakistan virus]	289	289	99%	7e-97	96.00%	ADP09639.1
coat protein [Malachra yellow mosaic virus]	288	288	99%	1e-96	96.00%	YP_009598457.1
coat protein [Whitefly-transmitted Indian begomovirus]	288	288	99%	2e-96	95.33%	ABC79517.1
coat protein [Chilli leaf curl virus-DU [India:New Delhi:Papaya:2009]]	288	288	99%	2e-96	95.33%	ADQ95322.1
coat protein [Tomato leaf curl Karnataka virus]	288	288	99%	2e-96	94.67%	SAM29905.1
coat protein [Tomato leaf curl virus]	287	287	99%	2e-96	95.33%	AKA60698.1
coat protein [Pepper leaf curl virus isolate Varanasi]	286	286	99%	2e-96	95.33%	AEO23044.1
coat protein [Pepper leaf curl virus isolate Maharajganj]	286	286	99%	2e-96	94.67%	AEO23042.1
coat protein [Ageratum enation virus [India:UP:Bahraich:Croton bonplandianum9:2011]]	288	288	99%	2e-96	95.33%	AFB69495.1
coat protein [Tobacco curly shoot virus]	288	288	99%	2e-96	95.33%	AJE24870.1
coat protein [Tobacco curly shoot virus]	288	288	99%	2e-96	95.33%	AEN71530.1
CP [Chili leaf curl Pakistan virus]	287	287	99%	3e-96	95.33%	ABB52018.1
coat protein [Chilli leaf curl Multan virus]	287	287	99%	3e-96	94.67%	ALN96432.1
coat protein [Tomato leaf curl virus]	287	287	99%	3e-96	94.67%	ANS81419.1
coat protein [Tomato leaf curl Karnataka virus]	287	287	99%	3e-96	95.33%	ACJ65693.1
coat protein [Ageratum enation virus]	287	287	99%	3e-96	94.67%	AFU34387.1
coat protein [Tomato leaf curl Karnataka virus]	286	286	99%	3e-96	95.33%	CUR44406.1

b. Text output of isolate VLNY1

Figure 4.18: BLASTx analysis of CP region of isolate VLNY1

MCISDVTRGSGLTHRVGKRFCVKSVYVLGKIWMDENIKTKNHTNS
VMFFLVRDRRPVDRPQDFGEVFNMFDNEPSTATVKNVHRDRYQVL
RKWHATVTGGQYASKEQALVRKFVRVNNYVIYNQQEAGK

a. Amino acid sequence of CP region of isolate VLNY1



b. Open reading frames in translated sequence of isolate VLNY1

Figure 4.19: Translation of nucleotide sequence of isolate VLNY1 generated on ExPASy translate

4.4.7.5. Homology search of isolate PKD1

Nucleotide BLAST (BLASTn) analysis isolate PKD1 showed 92.69 per cent sequence similarity with the virus isolate *Chilli leaf curl Vellanad virus* (NC038442.1) (Fig. 4.14). The isolate exhibited more than 83 per cent similarity to *Chilli leaf curl virus* isolates from Varanasi (KP868762, MH346125, FJ558515 and FJ403045), Sangrur (KM098113), Ludhiana (KR074211), New Delhi (KR957353 and HM140370), Chhapra (JN663852), UP (KJ590964), Noida (HM007114), Lucknow (JF682241), Palampur (FM210477) and Taiwan (AF336806).

BLASTx (translated nucleotide - protein BLAST) analysis performed on the sequence data of isolate PKD1 revealed 99.40 per cent sequence identity with *Chilli leaf curl Vellanad virus* coat protein sequence (accession no. YP_009506391.1) (Fig. 4.15). The analysis revealed similarity of the isolate with putative conserved domain of *Geminivirus* coat protein/nuclear export factor (BR1 family).

The sequence data obtained was also used to deduce corresponding amino acid sequence by ExPASy Translate tool. The sequence was translated into six possible Open Reading Frame (ORF). Among which the longest was frame 2 (5'-3') commencing from base 5 to 505, having a stretch of 498 nucleotides with 165 amino acids (Fig. 4.16).

The sequence data was deposited in GenBank, NCBI database using BankIt tool (https://www.ncbi.nlm.nih.gov/WebSub/) and the accession number MN231251 was assigned to the isolate PKD1 from Palakkad.

4.4.7.6. Homology search of isolate VLNY1

Nucleotide BLAST (BLASTn) analysis isolate VLNY1 exhibited maximum similarity of 96.69 per cent with the virus isolate *Chilli leaf curl Vellanad virus* (NC038442.1) with minimum E-value (Fig. 4.17). The virus isolates showing

similarity with the isolate VLNY1 included 63 of *Chilli leaf curl virus* isolates from India and other countries such as Pakistan and Oman.

BLASTx (translated nucleotide - protein BLAST) analysis performed on the sequence data of isolate VLNY1 revealed 100 per cent sequence identity with *Chilli leaf curl Vellanad virus* coat protein sequence (accession no. YP_009506391.1) (Fig. 4.18). The analysis revealed similarity of the isolate with putative conserved domain of *Geminivirus* coat protein/nuclear export factor (BR1 family).

The sequence data obtained was also used to deduce corresponding amino acid sequence by ExPASy Translate tool. The sequence was translated into six possible Open Reading Frame (ORF). Among which the longest was frame 3 (5'-3') commencing from base 66 to 458, having a stretch of 387 nucleotides with 128 amino acids (Fig. 4.19).

The sequence data was deposited in GenBank, NCBI database using BankIt tool (https://www.ncbi.nlm.nih.gov/WebSub/) and the accession number MN231253 was assigned to the isolate VLNY1 from Vellayani.

4.4.7.6. Phylogenetic analysis

Sequence data of different *Chilli leaf curl virus* isolates from India accessible from NCBI GenBank database was downloaded in Fasta format (Table 4.13). The sequences were edited using the software BioEdit 7.0 to obtain core coat protein gene sequence (550 bp). The edited gene sequences were used for Multiple Sequence Alignment (MSA) in ClustalW program (https://www.genome.jp/tools-bin/clustalw) using its default clustering pattern, Neighbor - Joining method.

Based on the alignment of different isolates with the sequences obtained from the study, different accessions were selected for generating phylogenetic tree in MEGA-X software. To analyze the diversity of the isolates *viz.*, PKD1, VKA1, VKA2, KAR1, KOD4 and VLNY1, three different phylogenetic trees were created with 1000

replications in Maximum - Likelihood method. The dendrograms generated were used to assess: a) the diversity among the obtained isolates (Plate 4.26), b) the diversity between the isolates and leaf curl virus isolates from only southern part of India (Plate 4.27) and c) the diversity between the isolates and leaf curl virus isolates from all over India (Plate 4.28).

Phylogenetic tree generated using the isolates obtained from the study revealed similarity between the six isolates (Plate 4.26). The isolates clustered into two groups with three isolates in each group. The first group consists of the isolates VKA2, KAR1 and KOD4. The second group in the phylogenetic tree consisted of isolates PKD1, VKA1 and VLNY1. The phylogenetic tree revealed the presence of a common ancestor for all the six isolates collected from different locations. The isolates VKA2, KAR1 and KOD4 appeared to be very closely related with a common ancestor. The results from the dissimilarity index indicates that, the isolates KAR1 and KOD4 evolved from a single ancestor.

Second phylogenetic tree was generated to assess the diversity of *Chilli leaf curl virus* isolates from South India using the annotated data of the sequences, *Chilli leaf curl Vellanad virus* (NC038442.1), *Chilli Leaf curl Salem virus* (HM007119.1), *Chilli leaf curl Multan virus* India, Guntur isolates (KT835649.1, HM007100.1), *Chilli leaf curl Multan virus* India, Bangalore isolate (KP195266.1), *Chilli leaf curl virus* Bhavanisagar isolate (HM992939.1) and the six isolates obtained from the study. All the above mentioned isolates clustered into four groups with the isolates VKA2, KAR1 and KOD4 as a outgroup (Group 4 Plate 4.27). Isolates PKD1, VKA1 and VLNY1 clustered into a group (Group 3 Plate 4.27) along with the isolate from Vellanad (NC038442.1). The analysis revealed that the isolate VLNY1 is directly related to Vellanad isolate (NC038442.1). The cluster Group 1 consisted of Guntur isolates; KT835649.1 and HM007100.1 along with the Bangalore isolate (KP195266.1) of *Chilli leaf curl Multan virus* India and Group 2 consisted of *Chilli Leaf curl Salem virus* (HM007119.1) and *Chilli leaf curl virus* Bhavanisagar isolate (HM992939.1).

Based on the Multiple Sequence Alignment (MSA) data using ClustalW program, ten *Chilli leaf curl virus* isolates described from India were selected for phylogenetic analysis along with the virus isolates from the study. The phylogenetic tree generated suggested a common ancestor for the isolates PKD1, VKA1 and VLNY1 from the study and other Indian isolates from New Delhi (MH346125.1 and HM140370.1), Varanasi (KP868762.1), Ahmedabad (KM880103.1), Vellanad (NC038442.1), Guntur (HM007100.1 and KT835649.1) and Bangalore (KP195266.1). Again the isolates VKA2, KAR1 and KOD4 still remained in a different cluster (Group 5 Plate 4.28). The isolates PKD1, VKA1 and VLNY1 clustered with Vellanad isolate (NC038442.1) into a single group.

The MSA data revealed significant similarity only between the isolates VKA2, KAR1 and KOD4 (Plate 24, 25). These three isolates did not show much similarity towards other isolates from the present study and towards other *Chilli leaf curl virus* isolates described from India. Also, the alignment of the isolates VKA2, KAR1 and KOD4 with other *Chilli leaf curl virus* isolates was not agreeable due to the presence of multiple gaps in the alignment. These three isolates have a six nucleotides (CCTGGG) long insertion which was absent in all other virus isolates used for alignment. These results indicate that, the three isolates *viz.,* VKA2, KAR1 and KOD4 could be new strains of *Chilli leaf curl virus* infecting chilli in Thrissur district. It is inferred that the virus belongs to the genus *Begomovirus*, family *Geminiviridae*.

Table 4.13: Accessions of *Chilli leaf curl virus* isolates used for phylogenetic analysis

Virus isolate	Accession no.
Chilli Leaf curl Vellanad virus [India/Vellanad/2008] clone pChVelK52 segment DNA-A, complete sequence	NC_038442.1
Chilli leaf curl virus-[Bhavanisagar:India:2010] segment DNA A, complete sequence.	HM992939.1
Chilli Leaf curl Salem virus-India [India/Salem/2008] clone pChSalH36 segment DNA-A, complete sequence.	HM007119.1
Chilli leaf curl Multan virus coat protein (AV1) gene, complete cds.	KT835649.1
Chilli leaf curl Multan virus-India [India/Guntur/2009] clone pChGuB16 segment DNA-A, complete sequence.	HM007100.1
Chilli leaf curl Multan virus isolate TC96 segment DNA-A, complete sequence.	KP195266.1
Chilli leaf curl virus isolate AKS1-VNS segment DNA-A, complete sequence.	MH346125.1
Chilli leaf curl virus isolate TC-Vns segment DNA A, complete sequence.	KP868762.1
Chilli leaf curl virus-Najafgarh2 [India:New Delhi:Papaya: 2009], complete genome.	HM140370.1
Chilli leaf curl Ahmedabad virus-India [India/Ahmedabad/2014], complete genome.	KM880103.1

DISCUSSION

Chilli (*Capsicum annuum*L.) which originated from the Latin American tropical regionsbelongs to the genus *Capsicum* and family Solanaceae. Itis animportant spice crop cultivated all over the world for its nutritional, medicinal and economic benefits.In the international scenario, India is the largest producer of chillicontributing 25 per cent of the total production, of which only 4 per cent is exported because of the high demand for domestic consumption.Despite its economic importance, growers are not in a position to produce good quality chilli with high productivity due to the incidence of various biotic problems like pest and diseases, abiotic and other crop related problems.

Till date 65 viruses have been reported to infect chilli worldwide, including *Begomovirus* prompting chilli leaf curl disease (Nigam *et al.*, 2015). Begomoviruseshave become a significant threat to crops such as chilli, tomato, cucurbits,cassava, beans and cottonrestricting its production and productivity (Varma and Malathi, 2003).Of the different begomovirusesthat naturally infect chilli, 11 viruses have been reported fromIndia, *viz.*, *Cucumber mosaic virus*, *Tobacco leaf curl virus*, *Indian chilli mosaic virus*, *Potato virus Y*, *Potato virus X*, *Tobacco ring spot virus*, *Pepper veinal mottle virus*,*Pepper vein bending virus*, *Chilli leaf curl virus*, *Tomato leaf curl New Delhi virus* and *Capsicum chlorosisvirus*.Among these viral diseases, chilli leaf curl disease, transmitted by whitefly (*Bemisiatabaci*Gennadius) significantly limits the production of chilli and capsicum causing almost 100per centshortfallin revenue (Saha*et al.*, 2005). It is due to *Chilli leaf curl virus* belonging to the genus *Begomovirus*, family *Geminiviridae*.

Hussain in 1932 was the first to report leaf curl disease in chilli and its association with whitefly in India from the Punjab of Integrated India now in Pakistan. Years later it was reported in India by Vasudeva (1954). The disease was

initially thought to be triggered by feeding of insect pest complex of thrips and mites (Amin, 1979). Mishra *et al.* (1963) reported the *Tobacco leaf curl virus* as the causative agent of leaf curl disease of chilli in India and experimentally proved it by grafting.

Currently, 322 species of *Begomovirus* have been officially described throughout the world, about 82 of which are reported in India. (Malathi*et al.*, 2017).Symptoms such as vein yellowing, yellow mosaic and leaf curl in plants are the easily recognizable symptoms of *Begomovirus*infection. The association ofbegomoviruseswith leaf curl disease of chilli in India was confirmedby Khan*et al.*(2006) who reported *Tomato leaf curl New Delhi virus*as the virus causing chilli leaf curl disease in Lucknow, Uttar Pradesh.Restraining chilli leaf curl disease by chemicals or cultural management practices has not been effectual. So far,only the use of resistant cultivars extendsabeneficial way to confine these viruses. Recently, in Kerala there has been an outbreak of leaf curl disease in *Capsicum* spp. cultivated under protected conditions and open field conditions. The severe occurence of leaf curl virus could be due to the change in climatic conditions and the spread of virus through insect vectors.

Recently, in Kerala more thrust has been given on the cultivation of vegetable crops viz. capsicum, cabbage, cauliflower etc. under protected conditions like polyhouses and rain shelters. But the microclimate prevailing in such enclosed structures is congenial for multiplication and spread of plant pathogens. High density cropping and monocropping of high yielding genotypes also makes the plants under poly house predisposed to pathogens like fungi and viruses. Considering the importance of the disease, this project was undertaken to clone and characterize the *Chilli leaf curl virus* isolates from Thrissur district and to study the diversity of the isolates. The virus isolates obtained from the study were analyzed to determine the diversity among the isolates and its similarity with other leaf curl viruses in India.

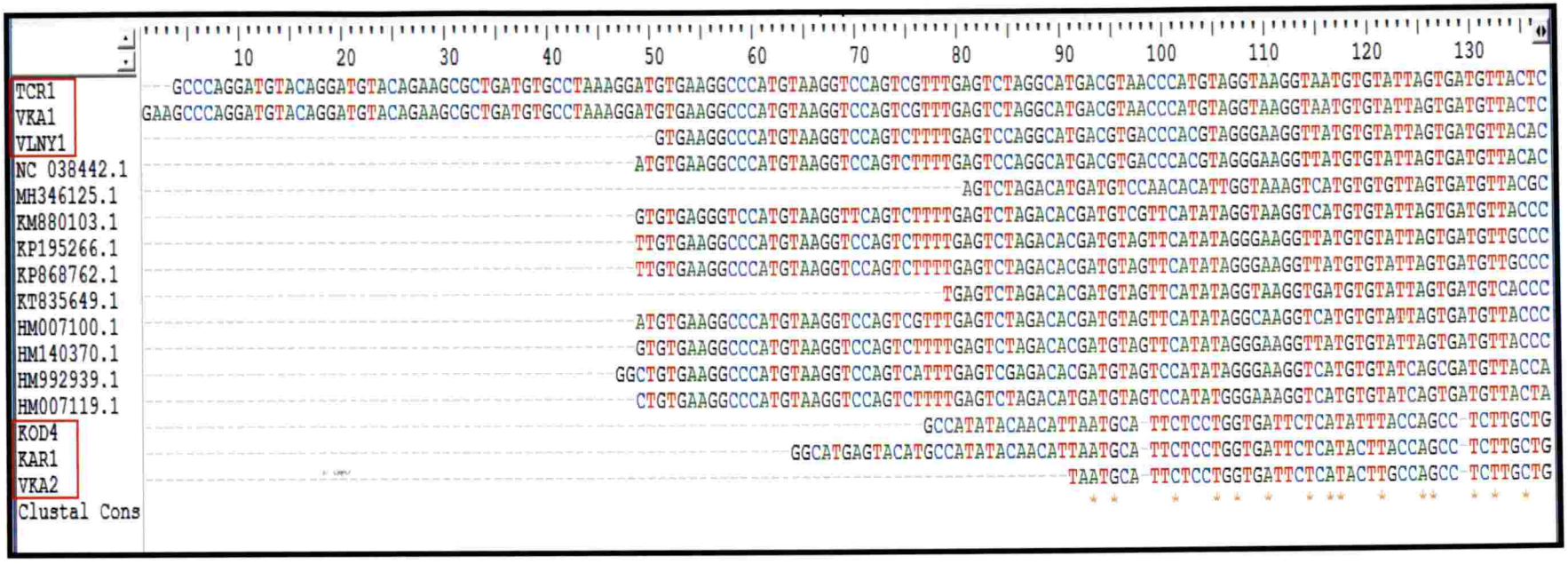


Plate 4.24: Multiple Sequence Alignment – CLUSTALW

Isolates from the study are indicated by red box.

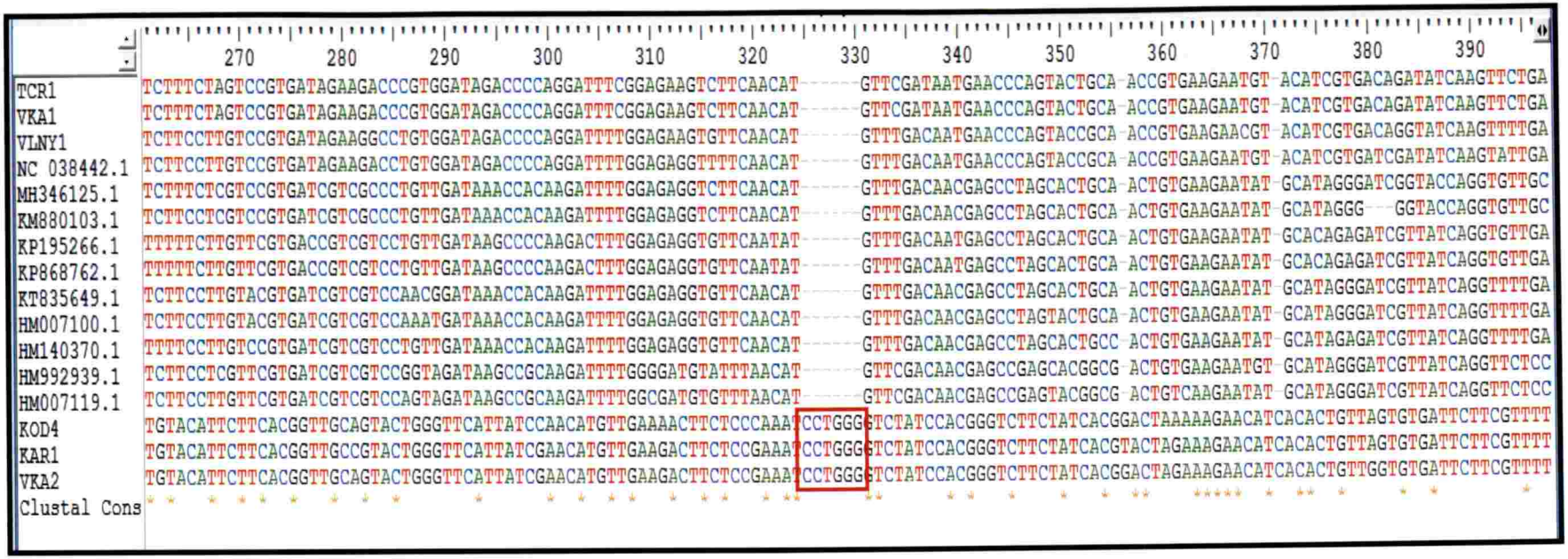


Plate 4.25: Multiple Sequence Alignment – CLUSTALW

The three isolates *viz.*, VKA2, KAR1 and KOD4 have a six nucleotides (CCTGGG) long insertion which was absent in all other virus isolates used for alignment.

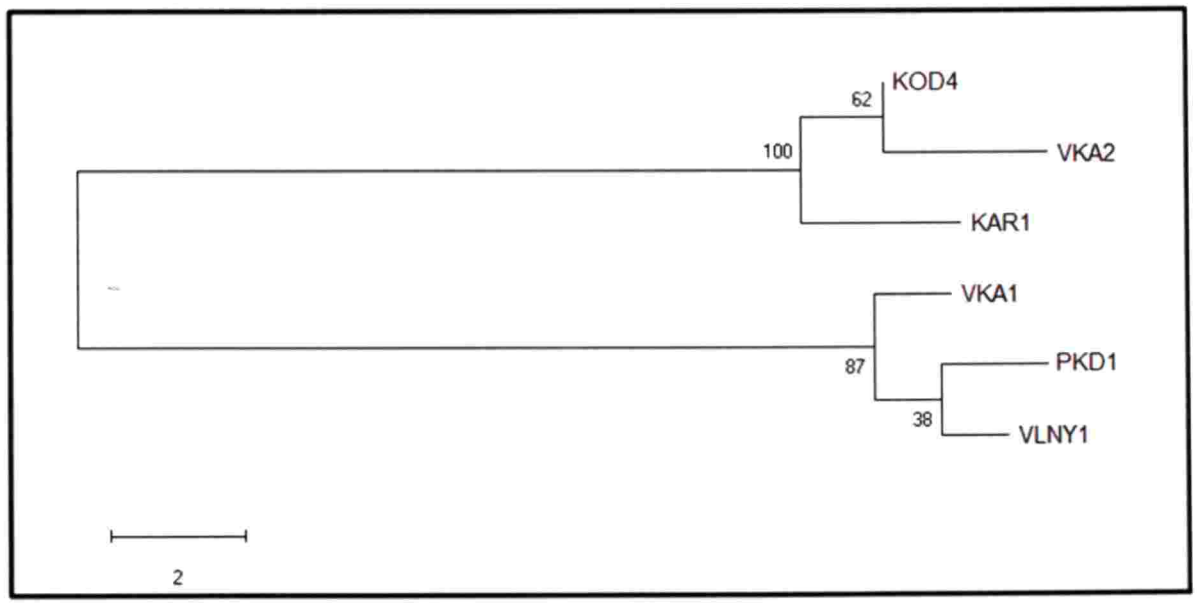


Plate 4.26: Phylogenetic analysis of coat protein gene of isolates obtained from the study
Tree was constructed by Maximum – Likelihood method using MEGA X
Numbers are the percentage support of branching based on bootstrap analysis (1000 replications)
Scale bar indicates 2.0 substitution per site

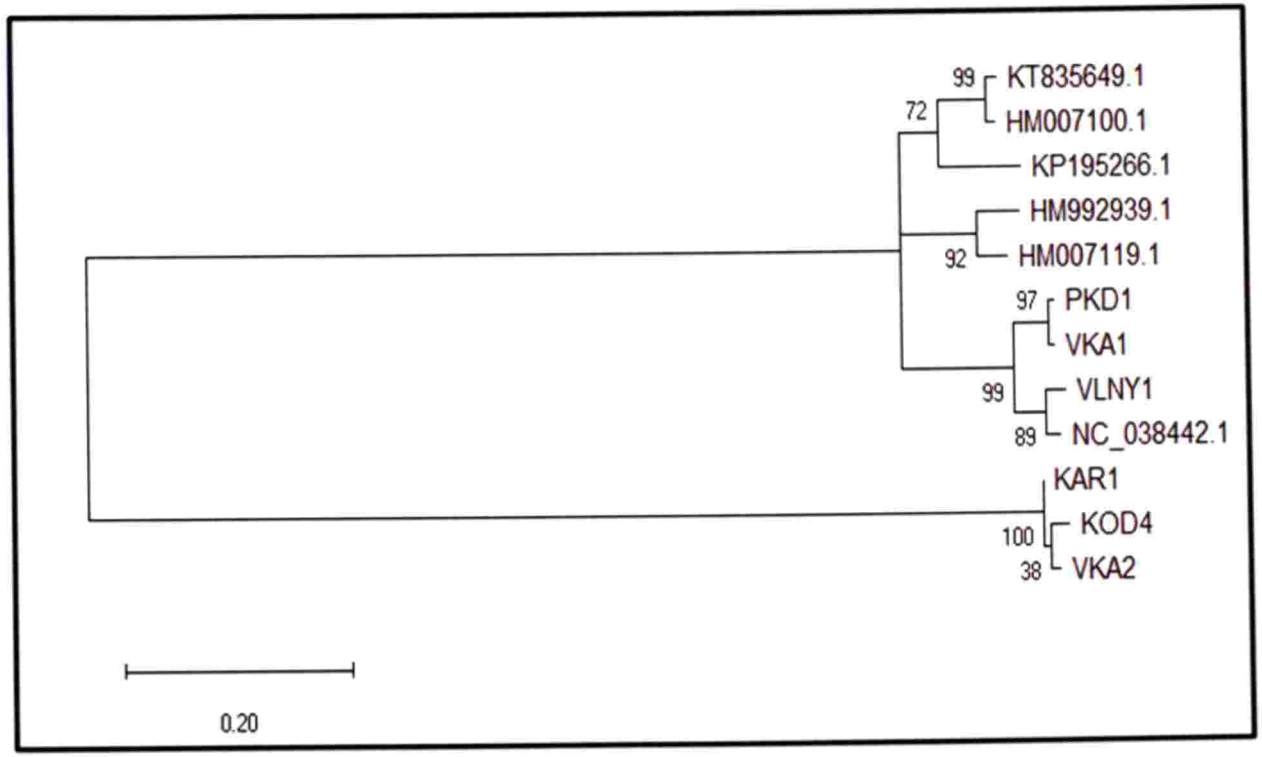


Plate 4.23: Phylogenetic analysis of coat protein gene of South Indian isolates of *Chilli leaf curl virus*
Tree was constructed by Maximum – Likelihood method using MEGA X
Numbers are the percentage support of branching based on bootstrap analysis (1000 replications)
Scale bar indicates 0.02 substitution per site

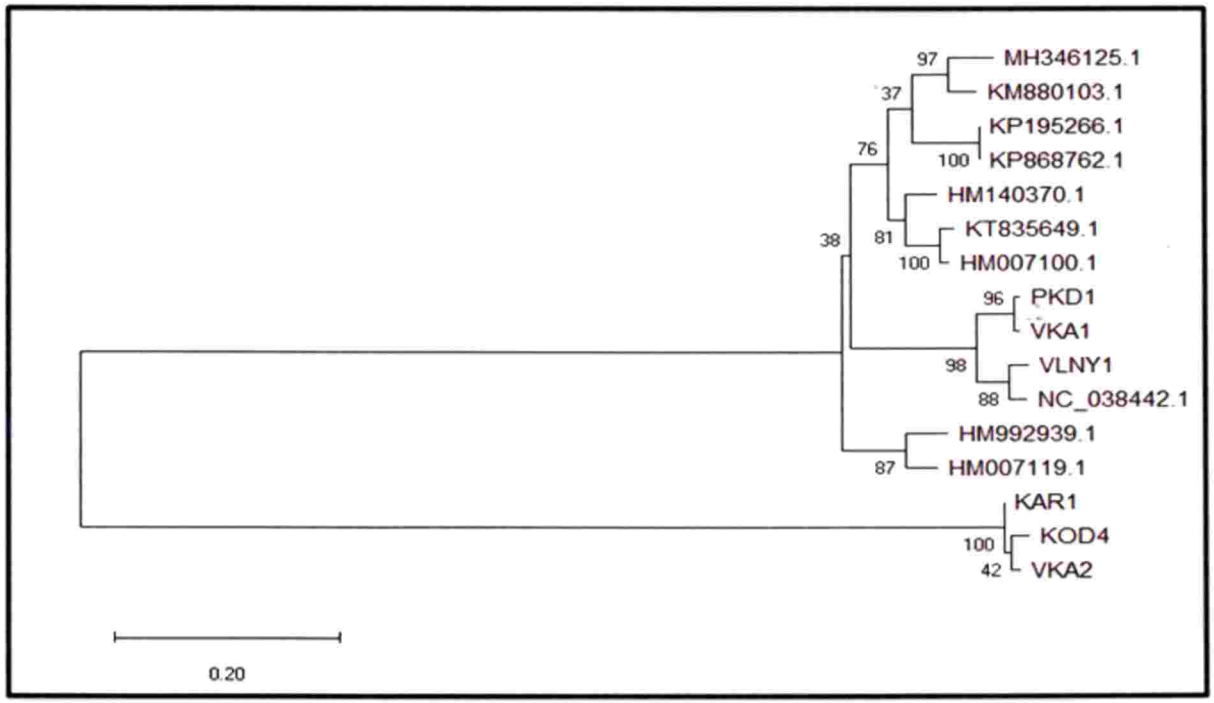


Plate 4.28: Phylogenetic analysis of coat protein gene of Indian isolates of *Chilli leaf curl virus*
Tree was constructed by Maximum – Likelihood method using MEGA X
Numbers are the percentage support of branching based on bootstrap analysis (1000 replications)
Scale bar indicates 0.02 substitution per site

DISCUSSION

DISCUSSION

Chilli (*Capsicum annuum* L.) which originated from the Latin American tropical regions belongs to the genus *Capsicum* and family Solanaceae. It is an important spice crop cultivated all over the world for its nutritional, medicinal and economic benefits. In the international scenario, India is the largest producer of chilli contributing 25 per cent of the total production, of which only 4 per cent is exported because of the high demand for domestic consumption. Despite its economic importance, growers are not in a position to produce good quality chilli with high productivity due to the incidence of various biotic problems like pest and diseases, abiotic and other crop related problems.

Till date 65 viruses have been reported to infect chilli worldwide, including *Begomovirus* prompting chilli leaf curl disease (Nigam *et al.*, 2015). Begomoviruses have become a significant threat to crops such as chilli, tomato, cucurbits, cassava, beans and cotton restricting its production and productivity (Varma and Malathi, 2003). Of the different begomoviruses that naturally infect chilli, 11 viruses have been reported from India, *viz.*, *Cucumber mosaic virus*, *Tobacco leaf curl virus*, *Indian chilli mosaic virus*, *Potato virus Y*, *Potato virus X*, *Tobacco ring spot virus*, *Pepper veinal mottle virus*, *Pepper vein bending virus*, *Chilli leaf curl virus*, *Tomato leaf curl New Delhi virus* and *Capsicum chlorosis virus*. Among these viral diseases, chilli leaf curl disease, transmitted by whitefly (*Bemisia tabaci* Gennadius) significantly limits the production of chilli and capsicum causing almost 100 per cent shortfall in revenue (Saha *et al.*, 2005). It is due to *Chilli leaf curl virus* belonging to the genus *Begomovirus*, family *Geminiviridae*.

Hussain in 1932 was the first to report leaf curl disease in chilli and its association with whitefly in India from the Punjab of Integrated India now in Pakistan. Years later it was reported in India by Vasudeva (1954). The disease was initially

initially thought to be triggered by feeding of insect pest complex of thrips and mites (Amin, 1979). Mishra *et al.* (1963) reported the *Tobacco leaf curl virus* as the causative agent of leaf curl disease of chilli in India and experimentally proved it by grafting.

Currently, 322 species of *Begomovirus* have been officially described throughout the world, about 82 of which are reported in India. (Malathi*et al.*, 2017).Symptoms such as vein yellowing, yellow mosaic and leaf curl in plants are the easily recognizable symptoms of *Begomovirus*infection. The association ofbegomoviruseswith leaf curl disease of chilli in India was confirmedby Khan*et al.*(2006) who reported *Tomato leaf curl New Delhi virus*as the virus causing chilli leaf curl disease in Lucknow, Uttar Pradesh.Restraining chilli leaf curl disease by chemicals or cultural management practices has not been effectual. So far,only the use of resistant cultivars extendsabeneficial way to confine these viruses. Recently, in Kerala there has been an outbreak of leaf curl disease in *Capsicum* spp. cultivated under protected conditions and open field conditions. The severe occurence of leaf curl virus could be due to the change in climatic conditions and the spread of virus through insect vectors.

Recently, in Kerala more thrust has been given on the cultivation of vegetable crops viz. capsicum, cabbage, cauliflower etc. under protected conditions like polyhouses and rain shelters. But the microclimate prevailing in such enclosed structures is congenial for multiplication and spread of plant pathogens. High density cropping and monocropping of high yielding genotypes also makes the plants under poly house predisposed to pathogens like fungi and viruses. Considering the importance of the disease, this project was undertaken to clone and characterize the *Chilli leaf curl virus* isolates from Thrissur district and to study the diversity of the isolates. The virus isolates obtained from the study were analyzed to determine the diversity among the isolates and its similarity with other leaf curl viruses in India.

In this study purposive sampling surveys were carried out under both open field and protected conditions to document the incidence and severity of chilli leaf curl disease in the selected locations of Thrissur district. Under open field conditions the per cent disease incidencevaried between43.30 and 85.00 and per cent disease severity varied from 43.60 to 81.54. Under protected conditions, the per cent disease incidence ranged from 45.75 to 79.40 and per cent disease severity ranged from 49.40 to 87.50.

Among the surveyed locations, disease incidence was highest in an open field at Karumathra followed by Kottanellur of Vellangallurpanchayath with per cent disease incidence of 85.00 and 84.62, respectively in the local variety of chilli. While the least per cent disease incidence of 43.30 was recorded at Vellanikkara on chilli var. Ujwala. The low disease incidence at Vellanikkara might be due to the well maintained fields with recommended spacing and nutritional applications with regular insecticidal sprays when compared with other plots surveyed. The per cent disease incidence recorded was above 60 per cent in all the surveyed locations except at Vellanikkara. This general increase in per cent disease incidence of chilli leaf curl disease might be due to the climate change, change in agricultural practices and the establishment of various biotype of whitefly vector into additional zones. Similar findings were reported by Kumar *et al.* (2008).

The highest per cent disease severity recorded in open field conditions was at Kottanellur (81.54), which was followed byKarumathra (78.30). This might be because, both the plots were poorly maintained along with decreased plant spacing and without any insectide application which favored high vector population. The least per cent disease severity of 29.50 was recorded in Kattilapoovam of Madakkatharapanchayath on chilli var. Kanthari. In the plot surveyed at Kattilapoovam the chilli was grown as an intercrop along with gliricidia (*Gliricidiasepium*) and other vegetables such as snake gourd, cowpea which are non-

host and less preferred hosts of the vector. This might have hindered the vector multiplication anddisease spread.

Under protected conditions, highest per cent disease incidence of 79.40 was recorded in chilli var. Ujwala while the lowest PDI of 49.40 was recorded in capsicum var. Indra. The maximum per cent disease severity of 87.50 was at Vellanikkara on capsicum var. Indrafollowed 84.60 on chilli var. Ujwala. This was in accordance with observations made by Kumar *et al.* (2008) andVattakunnel and Sajitharani, (2016) who reported that the controlled conditions inside protected cultivation units leads to better growth of chilli plants which directly contributed to multiplication of vectors and pathogens which gained entry into the polyhouses leads to increased disease severity on crops grown in protected cultivation units.

Due to the recent climatic changes and rise in temperature, spread of begomoviruseswould become rapid through its insect vectors, whiteflies (*Bemisiatabaci*G.). Reports on leaf curl disease on solanaeceous crops like chilli, tomato, potato etc. and non - solanaeceous crops are likeamaranthus, bitter gourd, papaya etc. from India caused yield loss from 90 to 100 per cent (Saha*et al.,* 2005; Senanayake*et al.,* 2012; Saeed *et al.*, 2014; George *et al.*, 2014; Malathi*et al.,* 2017).

The expression of symptoms on different parts of the plant was studied in detail under natural field conditions as well as artificial conditions. The symptoms of chilli leaf curl disease were observed in different parts of an infected plant *viz.,* leaves, internodes, fruits and whole plant. The symptoms witnessed on the leaves of infected chilli plants under natural conditions include upward curling, inward rolling of leaf margins, crinkling, puckering, vein banding, vein thickening, interveinal chlorosis, shoestring appearance and leaf malformation. Upward curling and puckering of leaves were the prominent symptoms noticedin infected plants where high per cent disease severity was recorded. The lamina of the infected leaves with puckering symptoms had raised or elevated regions between the veins. This might be

due to the disproportionate cell growth in lamina brought about by virus infection. Thetissue configuration between the epidermis *i.e.*, palisade layer and the spongy parenchyma might havedentedand therefore width of blades was notably reduced in infected plants(Kumar *et al.,* 2018).

The fruits produced by the infected plants showed significant size reduction and deformation. When the disease persisted in later stages, infected mature plants hardly flowered and if the flower buds were to develop, it got abscised and anthers set without pollen grains. This resulted in underdevelopedfruits and the fruits with truncations or curling at the stylar end. Similar observations were recorded by Bhatt *et al.* (2016).

When the virus infection occurs at later stages of the plant, the newly emerged leaves had significant reduction in leaf area and the internodal length towards the apex reduced. This has led to the tapered appearance for the infected chilli plants. However if the infection starts in the early stage of plant growth, the infected plants would become severely stunted with bushy appearance at maturity. Similar observations were reported on chilli plants infected with *Chilli leaf curl virus* by many workers (Khan *et al.,* 2006; Chattopadhyay*et al.*, 2008; Senanayake*et al.*, 2007; Shafiq*et al.,* 2010;Kumar *et al.,* 2011; Sinha *et al.*, 2011; Sivalingam*et al.,* 2012; Senanayake*et al.*, 2013; Kumar *et al.,* 2015; Zehra*et al.,* 2017).

The artificially inoculated chilli seedlings with *Chilli leaf curl virus* developed symptoms 10 - 14 days post inoculation using whiteflies. The newly emerged leaves after artificial inoculation showed curling and crinkling symptoms along with the stunting of plant growth. Similar findings were reported after transmission and host range studies carried out by Premchand and Prasad (1990).

The transmission studies using wedge grafting were also carried out. The *Chilli leaf curl virus* infected seedling (scion) was grafted to healthy one month old

chilli seedlings (rootstock) and symptom expressions were monitored. Initially, curling of the newly emerged leaves from the rootstock after grafting was noted after 10 days of grafting. Later these leaves developed puckering symptoms. Artificial inoculation studies using whiteflies and grafting revealed 60 per cent and 100 per cent virus transmission, respectively. Comparable conclusions were stated by Park and Fernando, (1938), Mishra *et al.* (1963) andDhanraj and Seth, (1968) on transmission of virus causing chilli leaf curl disease by grafting.

An attempt was made to standardize molecular detection techniques to identify and characterize the virus associated with leaf curl disease of *Capsicum* spp. Molecular approaches have gained much importance in the detection of plant viruses since serology is not suitable for the characterization of begomoviruses due to difficulty in preparing high titre antisera and lack of sufficientspecificity (Brown *et al.,* 2001). As a result, diagnostic methods based on DNA, including amplification using Polymerase Chain Reaction (PCR) and DNA sequencing, have substituted serology for *Begomovirus* detection, identification and classification.In the present study molecular characterization of the five virus isolates collected from various locations of Thrissur district *viz.,*VKA1 VKA2, KAR1 and KOD4 and two isolates collected from Vithinasseri, Palakkad *viz.,*PKD1 and Vellayani, Thiruvanathapuram district *viz.,* VLNY1 were undertaken.

Total DNA was isolated from infected leaf samples following three methods *viz.*, CTAB method, modified CTAB method and commercial plant DNA isolation kit method. Since the concentration of geminiviral particles in the infected tissue is low compared to other plant viruses, its isolation could be tedious and the success of any isolation protocol is highly dependent on the type of virus and host plant (Palmer *et al.*,1997).In the present study, DNA isolated using commercial plant DNA isolation kit was found to be superior with better quality DNA having OD value ranging from 1.8 to 2.0 and gave positive results on PCR amplification. Although higher quantity DNA was

obtained using CTAB method as well as modified CTAB method, the quality of DNA was poor with spectrophotometer readings ($A_{260/280}$) ranging from 1.4 to 2.0. The OD value ($A_{260/280}$) of DNA obtained using theprotocols with CTAB buffer indicated contaminationof proteins and other phenolic compounds which gave negative results on PCR amplification of viral DNA. This might be due to poor quality and less stability of geminiviral DNA in Trisbuffer. These outcomes were in accordance with the observations reported by Muniyappa*et al.* (1991) and Jose and Usha, (2000).

DNA-based diagnostic methods, involving amplification by polymerase chain reaction (PCR) and DNA sequencing, substituted protein - baseddiagnosis for ascertaining the taxonomic lineage of *Begomovirus*(Brown *et al.*,2001). In this study, Polymerase Chain Reaction (PCR) assays using degenerate primers were performedanticipating theamplificationof the coat protein gene of the *Begomovirus*causing chilli leaf curl disease. The coat protein gene is the fundamentally conserved gene in thefamily *Geminiviridae*and this gene sequencecould be essentially used to envisagethe discretestrains, species, and taxonomic lineages ofbegomoviruses(Wyatt and Brown, 1996). The core coat proteinregion (core CP)/ AV1 gene of *Begomovirus*were amplified using the primers AV 494 / AC 1048 and Deng 540 / 541 which yielded amplicons of size 550 bp and 520 bp, respectively. The result of the present study is analogous to the outcome reported byRajeshwari*et al.* (2005), Raj *et al.*(2008), Govindappa*et al.*(2011)Bandaranayake*et al.*(2014) andRienzie*et al.* (2016) who stated that the primersAV 494 / AC 1048 and Deng 540 / 541 were efficient tools to detect begomoviruses from infected plant samples.

Apart from these, primers were also designed by aligning the complete AV1 gene or coat protein gene sequences of *Chilli leaf curl virus*isolates available in NCBI GenBank database. Attempts were also made to amplify the complete coat protein (AV1) gene of the virus isolates using two designed primers (CLCVF /

CLCVR and ChVelF / ChVelR). However, it did not produce any amplification. This might be due to thepresence of a highly variable 200 nucleotides long gene segment present at the 5' end of the begomoviralcoat protein gene as suggested byPadidam*et al.* (1995). Also, Brown *et al.* (2001) reported that designing universalprimers that are bordering the begomoviralCPgene are not functional because of the 5' variable region flanking the coat protein geneof genus*Begomovirus*. This prevents the universal amplification of the 5' - 200 nucleotidevariable CP region, or the complete CPgene using a particular set of primers. Similarly, Senanayake*et al.*(2007, 2013) reported significant sequence difference between the virus isolates ofleaf curl viruses affecting chilli in India. Hence, they stated that, it would be difficult to design species specific primers to duplicate the complete coat protein gene of the begomoviruses infecting chilli in India.

In order to detect and characterize a virus at the molecular and biological levels, it is obligatory to generate an infectious clone.For most of the geminiviruses, cloning is reasonablysimple due to the presence of the virusesin the form of double-stranded (replicative) DNA particles in the infected plants (Boulton, 2008). Attempts were made to clone the amplified viral coat protein gene segment of the six viral isolates *viz.,* PKD1, VKA1 VKA2, KAR1, KOD4 and VLNY1 using Thermo Scientific InsTAclone PCR cloning kit. The PCR products of size 550 bp obtained through PCR amplification were eluted from 1.2% agarose gel using QIAquick Gel ExtractionKit following the manufactures' protocol. The method of cloning used in the present study was TA cloning. The eluted PCR products were subjected to A-tailing using *Taq* DNA polymerase. The A-tailed products were ligated with plasmid vector pTZ57R/T which has a 3'- ddT tailed cloning site at 650 - 651 bp position and LacZα - peptide element at 449 - 739 bp position for blue/white screening. The ligation mixture containing the plasmid vector ligated to the viral coat protein gene was used to transform the competent cells of DH5α strain of *Escherichia coli*. The transformed bacterial colonies were recognized based on blue/white screening and

the white colonies were subcultured in LB-ampicillin plates.Further confirmation of presence of desired insert was done by colony PCRusing AV494/ AC1048 primers which yielded an amplicon of similar size (550 bp). This product was then outsourced to AgriGenome for sequencing. The results obtained was in accordance with the reports of Senanayake*et al.*(2007, 2013), Kumar *et al.* (2011),Sinha *et al.*(2013) and Rienzie*et al.*(2016).

In silico analysis of the coat protein gene of the six virus isolates revealed that more than 90 per cent sequence homology with *Chilli leaf curlVellanad virus*(accession no. NC038442.1) available in the NCBI GenBank database. Therefore the present study indicated theinvolvement of *Chilli leaf curl virus*with the leaf curl disease of chilli in Thrissur district. These conclusions were in compatible with reports of Kumar *et al.* (2012) who reported that*Chilli leaf curlVellanad virus*causal agent of chilli leaf curl disease in Kerala. For further validation, BLASTx analysis was performed and the results revealed more than 96 per cent sequence similarity of the virus isolates with *Chilli leaf curl Vellanad virus* coat protein sequence (accession no. YP_009506391.1).Additionally, the nucleotide sequence data obtained was also used to deduce corresponding amino acid sequence by ExPASy Translate tool.

Attempts to analyze the genetic diversity among the six virus isolates revealed that the isolates from the present study could be grouped into two separate clades. The isolates PKD1, VKA1 and VLNY1 were grouped into a single clade and had maximum similarity with *Chilli leaf curlVellanad virus*(accession no. NC038442.1). Whereas the isolates VKA2, KAR1 and KOD4 clustered together to form an outgroup. This indicated that there is a significant diversity among the *Chilli leaf curl virus* isolates in Thrissur district. Phylogenetic analysis of the virus isolates from Thrissur with the South Indian isolates of *Chilli Leaf curl virus*showed a common ancestry for all the South Indian isolates including the isolates from the study. Isolate

VLNY1 showed maximum similarity with the accession no. NC038442.1 and along with the isolates VKA1 and PKD1, it formed a subgroup. Isolate VKA1 from Vellanikkara was found to be most closely related to the isolate PKD1 from Vithinasseri. But the two isolates from Vellanikkara *viz.,* VKA1 and VKA2, did not show any similarities and the isolate VKA2 clustered with the isolates KAR1 and KOD4. Similar findings were reported by Senanayake*et al.* (2013) from Sri Lanka, when two leaf curl virus isolates obtained from infected plants separated only by a few kilometers from their origin showed significant sequence divergence on phylogenetic analysis. Also, Fauquet*et al.* (2003)stated that sequence analysis of begomoviruseswithinterchangeable biological properties frequentlydisclosesdistinctive viruses,lots ofwhich could bedivergent at the species level.

Phylogenetic analysis of the virus isolates from Thrissur with other *Chilli leaf curl virus*isolates from India available in NCBI GenBank database was performed. The resulting phylogenetic tree had five groups with isolates PKD1, VKA1, VLNY1 and NC038442.1 grouped together into a single clade.These clustering pattern could be because of the AV1 region that we used for diversity analysisbeing one of the recombination hotspots in begomoviruses as reported by George *et al.*, (2014).

On analyzing the MSA data and resultant phylogenetic tree, three isolates *i.e,* VKA2 from Vellanikkara, KAR1 from Kannara and KOD4 from Kodalithree isolates didnot show similarity towards other isolates from the present study and towards other *Chilli leaf curl virus* isolates reported from India and clutsered into a separate clade.Also, the alignment of the isolates VKA2, KAR1 and KOD4 with other *Chilli leaf curl virus*isolates was notagreeable due to the presence of multiple gaps in the alignment. In addition to that these isolates have six nucleotides (CCTGGG) long insertion which was absent in all other virus isolates used for alignment.Hence these

isolates could be proposed as new strains of *Chilli leaf curl virus* infecting chilli in Thrissur district.In recombination analysis of begomoviral components from India, Kumar *et al.* (2015) detected significant evidence ofrecombination in the vast majority of isolates, where the breakpoints were located within the common region (CR) of AV1 gene and there is evidence for predominant intra - species recombination among different chilli-infecting begomoviruses. The clustering pattern obtained through the study could also be due to the fact that geminiviruses have a higher tendency for recombination*via* the substitution of minor sections of few nucleotides as well assignificantunits of about 2000 nucleotides or more from their entire genomeas stated by Padidam*et al.* (1996). Similar findings were observed when Chattopadhyay*et al.* (2008) studied sequence similarity between *Begomovirus*isolates of viruses causing leaf curl disease in India and regions of Asia using the sequence data in NCBI GenBank database. On the basis of sequence homology data of viral coat protein gene (AV1) among different viral isolates, Chattopadhyay*et al.* (2008) suggestedthat the viral genome of Indian isolates have different origin.

Although, the cultivation of chilli is affected by many pests and diseases, during the last decade, the threat posed by the emerging *Begomovirus* infecting solanaceous crops is a major concern for chilli cultivation in Kerala. Three, possibly new strains of *Chilli leaf curl virus* infecting chilli have been identified and hence the study highlights the need for monitoring the emergence of new strains of plant viruses especially begomoviruses infecting solanaceous crops of Kerala. The detailed information on the symptomatology of chilli leaf curl disease and PCR mediated molecular diagnosis can be applied for timely diagnosis and effective management of the disease.

Future line of work

The molecular clones developed in the study could be used as a tool to develop diagnostic tools such as protein hybridization techniques or nucleic acid hybridization techniques. Identification and characterization of betasatellite component of the identified virus isolates and its infectivity status can be explored. Further investigations on virus - vector relationships, host range, detection and sequence comparison of virus isolates from other parts of Kerala could be carried out.

SUMMARY

SUMMARY

Chilli (*Capsicum annuum* L.) is an economically influential spice crop grown all over the world for its pungent fruits.Although, the cultivation of chilli is affected by many pests and diseases, during the last decade, the threat posed by the emerging *Begomovirus* infecting solanaceous crops is a major limitation for chilli production in India. Leaf curl disease of chilli is caused by *Chilli leaf curl virus*,transmitted by whitefly (*Bemisia tabaci* G.) belongs to genus *Begomovirus*, family *Geminiviridae*.

The study entitled "Molecular cloning and characterization of virus causing leaf curl disease of *Capsicum* spp." was carried out in the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Plant Pathology, College of Horticulture, Vellanikkara during 2016 - 2018. The study was commenced with an objective to assess the incidence, severity and symptomatologyof leaf curl disease of chilli. The project also intended to characterize the coat protein gene of *Chilli leaf curl virus* causing chilli leaf curl disease and to clone the coat protein gene.

The project initiated with purposive sampling surveys conducted in eleven different locations of Thrissur district, Kerala to document in detail the symptomatology of leaf curl disease on chilli plants. The infected plant samples showing typical leaf curl disease symptoms were collected and maintained in insect proof net house of Department of Plant Pathology for further characterization of the virus associated with the disease.

During surveys, the incidence and severity of the disease on the crop was also assessed based on the standard score chart for leaf curl disease developed by Banarjee and Kaloo (1987) using 0 - 5 scale. Under open field conditions, the disease incidencevaried between 43.30 and 85.00 per cent and disease severity varied from 43.60 to 81.54 per cent. Under protected conditions, the disease incidence ranged

from 45.75 to 79.40 per cent and disease severity ranged from 49.40 to 87.50 per cent.

Highest per cent disease incidence was noted under open field condition while per cent disease severity was recorded maximum in protected cultivation unit. Maximum disease incidence of 85.00 per cent was recorded in Karumathra of Vellangallur panchayath followed by Kottanellur (84.62%) on local chilli variety. The highest percent disease severity of 87.50 per cent was recorded in Vellanikkara on capsicum variety Indra (87.50%) followed by chilli variety Ujwala (84.60%) both under protected conditions.

The symptomatology of chilli leaf curl disease on different parts of the plant such as leaves, internodes, fruits and the whole plant under natural conditions were documented during the survey. The symptoms observed on the leaves of infected chilli plants under natural conditions include upward curling, crinkling, puckering, vein banding, interveinal chlorosis, size reduction of lamina and leaf malformation. Fruits produced by the infected plants showed significant size reduction and deformation. Infected chilli plants were stunted and bushy in appearance.

The key diagnostic symptoms of the disease were expressed on the leaves of infected chilli plants. Severe upward curling of leaf lamina was noticed followed by cupping symptom. All the infected plants exhibited puckering of leaves. Upward curling and puckering of leaves were the prominent symptoms noticedin infected plants where high per cent disease severity was recorded. The texture of the infected leaves with puckering symptoms were thick and leathery when compared to the healthy leaves. Interveinal chlorosis was mostly seen in older leaves and such plants rarely flowered. Newly emerged leaves from infected plants started showing puckering symptoms very early. In the case of severely affected plants, size reduction of lamina was observed, especially near the petiolar end resulting in the formation of narrow strap-shaped leaves. However, if the infection starts in the early stage of plant

growth, the infected plants became severely stunted with bushy appearance. Infected plants showed reduction in internodal length towards the apex and the younger leaves failed to attain full size. If the disease persisted in later stages of plant growth, the infected plants hardly flowered and fruits produced were underdevelopedand deformed with truncations or curling at the stylar end. In bell pepper (*Capsicum annuum* L. var. grossum Sendt), the fruits formed were underdeveloped and deformed with asymmetrical surface while in the case of chilli (*Capsicum annuum* L.), the fruits developed were curled and small compared to healthy fruits.

The symptom expression on artificially inoculated plants through vector transmission and grafting on chilli seedlings were studied. The newly emerged leaves following artificial inoculation expressed symptoms such as curling and crinkling along with considerable stunting of plant growth. Inoculated chilli seedlings developed leaf curl symptoms seven days after inoculation using whitefly transmission while grafted seedlings took an average of fourteen days for symptom expression.

Molecular characterization of four virus isolates collected from Thrissur district and one isolate each collected from Palakkad and Vellayani were carried out. Total genomic DNA was extracted from virus infected chilli leaf samples followed by amplification of viral DNA *via.* Polymerase Chain Reaction (PCR) were standardized. Three methods were adopted for isolation of DNA *viz.*, CTAB method, modified CTAB method and using Qiagen® DNeasy plant minikit. Protocols using CTAB buffer were found to yield higher quantity of DNA but when subjected to PCR, it failed to produce proper amplification from viral DNA. Protocol using Qiagen® DNeasy plant kit yielded better quality DNA with OD values between 1.8 and 2.0. DNA obtained using Qiagen® DNeasy plant kit following the manufactures' protocol yielded better amplicons of viral DNA after PCR amplification. Thus DNA isolation using commercial plant DNA isolation kit for amplification of viral DNA using PCR was found superior over the other methods.

Total DNA isolated from the infected leaf samples and were subjected to PCR amplification using two reported primers, *viz.,* AV494 / AC1048 (Wyatt and Brown,1996) and Deng 540 / 541 (Deng*et al.*, 1994) and designed primers, *viz.,* CLCVF & CLCVR and ChVelF & ChVelR. The reported primers used in the study were *Begomovirus* specific degenerate (universal) primers which amplifies the core coat protein region of *Begomovirus* species and produces an amplicon of size 550 base pairs. Optimum annealing temperature and time for PCR amplification using the reported primers were standardized. Good bands of size 550 bp obtained using the reported primers were eluted and cloned into the pTZ57R/T cloning vector using Thermo Scientific InsTAclone PCR cloning kit following the manufactures' protocol. The transformed clones were selected based on the blue/white selection and were subjected to colony PCR for the selection of true recombinants with the desired insert. The clones were maintained at 4°C.

The sequence data obtained in the study was subjected to *in silico* analyses for studying the diversity of the isolates. The nucleotide BLAST (BLASTn) and translated nucleotide - protein BLAST (BLASTx) analyses of all the six virus isolates revealed more than 90 per cent sequence identity with *Chilli leaf curl Vellanad virus* (accession no. NC038442.1) and more than 96 per cent sequence similarity with *Chilli leaf curl Vellanad virus* coat protein sequence (accession no. YP_009506391.1). The coat protein sequences of all the six isolates were translated into corresponding amino acid sequence by ExPASy Translate tool. The sequences were translated into six possible Open Reading Frame (ORF) and the longest ORF was selected for further analysis and interpretation.

Diversity analysis was carried out using CLUSTALW and MEGA X software. The Multiple Sequence Alignment (MSA) data obtained using CLUSTALW and the different phylogenetic trees (Maximum - Likelihood method; Tamura-Nei model)generated by MEGAX software revealed significant sequence

variations in the coat protein sequences of the virus isolates from the study. The phylogenetic analysis of the sequences revealed that, the isolates VKA2, KAR1 and KOD4 could be different strains of *Chilli leaf curl virus* since these formed a different cluster which is distinct from the other isolates. While, the isolates VKA1, PKD1 and VLNY1 clustered into single group, are most closely related to Vellanad isolate of *Chilli leaf curl virus*.

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APPENDICES

ANNEXURE –I

Composition of various stock solutions and extraction buffers used for total genomic DNA isolation

1. Stock solutions used for DNA isolation

a. **1 M Tris buffer (100ml)**

Dissolve 12.114g Tris buffer in 100ml distilled water. Adjust pH to 8.0.

b. **0.5 M EDTA (100ml)**

Dissolve 18.612g EDTA-Na salt in 100ml distilled water. Adjust pH to 8.0 using sodium hydroxide pellets.

c. **4 M Sodium chloride (100ml)**

Dissolve 23.376g NaCl in 100ml distilled water.

d. **2 per cent CTAB (100ml)**

Dissolve 2g CTAB in 100ml distilled water by intermittent heating.

2. Composition of CTAB buffer (100ml)

a. 1M Tris buffer – 10ml
b. 0.5 M EDTA – 4ml
c. 4 M NaCl – 35ml
d. 2 per cent CTAB – 40ml
e. Distilled water – 11ml

3. Chloroform - Isoamyl alcohol mixture (24:1)

Mix 24 ml of chloroform with 1 ml of isoamyl alcohol to prepare 25 ml of chloroform - isoamyl alcohol mixture. Always use freshly prepared mixture for isolation.

4. Ethanol (70 per cent and 100 per cent)

Mix 70 ml of 100 per cent ethanol with 30 ml distilled water to prepare 100ml of 70 per cent ethanol. Both 70 per cent and 100 per cent concentrations should be stored in 4°C and used in chilled condition for isolation.

ANNEXURE– II

Composition of various buffers and dyes used for Agarose Gel Electrophoresis

a. Agarose(SRL)

0.8 per cent (w/v)

b. 50X TAE buffer (pH 8.0)

i. Tris base -242.0 g

ii. Glacial acetic acid - 57.1 ml

iii. 0.5 mM EDTA - 100 ml

c. Tracking/loading dye (6X)

i. Bromophenol blue - 0.25%

ii. Xylene cyanol - 0.25%

iii. Glycerol - 30%

The dye was prepared and stored at 4°C.

d. Ethidium bromide(Intercalating dye)

The dye was prepared as a stock solution of 10 mg/ml and stored in amber colored bottles at 4°C. Later a working concentration of 0.5 µg/mlwas prepared and used for staining.

MOLECULAR CLONING AND CHARACTERIZATION OF VIRUS CAUSING LEAF CURL DISEASE OF *Capsicum* spp.

by

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ABSTRACT

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ABSTRACT

Chilli is one of the most important crops cultivated across the globe, as vegetable, spice and for industrial purposes. According to the statistics of National Horticulture Board (2017), the crop covers an area of 1860 ha in the state of Kerala with an average production of 12470 tonnes. During the last decade, the threats posed by the emerging begomoviruses infecting solanaceous crops have affected the economic cultivation of chilli. Chilli leaf curl disease caused by *Chilli leaf curl virus* belonging to the genus *Begomovirus* and family Geminiviridae is a serious constraint to chilli production in India which causes upto 100 per cent yield loss especially when infected at an early stage of the crop. Considering the importance of the disease, the present study was undertaken with the objective to study the incidence and symptomatology of chilli leaf curl disease and to characterize and clone the coat protein gene of the *Chilli leaf curl virus* isolates.

The project initiated with purposive sampling surveys conducted in eleven different locations of Thrissur district, Kerala to document the incidence and symptomatology of leaf curl disease on chilli plants. The disease incidence recorded during the survey ranged from 43.30 to 85.00 per cent under open field conditions and from 45.75 to 79.40 per cent under protected conditions while the disease severity ranged from 43.60 to 81.54 per cent and from 49.40 to 87.50 per cent, respectively under open field conditions and protected conditions.

The symptomatology of chilli leaf curl disease on different parts of the plant such as leaves, internodes, fruits and the whole plant under natural conditions was documented during the survey. The symptoms observed on the leaves of infected chilli plants under natural conditions include upward curling, crinkling, puckering, vein banding, interveinal chlorosis, size reduction of leaf lamina and leaf malformation. The fruits produced by the infected plants showed size reduction and deformation. The infected plants were stunted and bushy in appearance. The transmission of the virus by insect vector, *Bemisia tabaci* and grafting was studied and the symptoms were documented. The newly emerged leaves after artificial inoculation expressed symptoms such as curling, puckering and crinkling along with stunting of plant growth.

Molecular characterization of the four virus isolates collected from various locations of Thrissur district *viz.*, VKA1 VKA2, KAR1 and KOD4 and two isolates *viz.*, VLNY1 and PKD1 collected from Vellayani, Thiruvanathapuram district and from Vithinasseri, Palakkad district, respectively were undertaken. The total genomic DNA from virus infected chilli leaf samples was isolated and subjected to PCR amplification of viral coat protein gene to confirm the presence of virus infection. PCR amplification of the isolated DNA was carried out using two *Begomovirus* specific degenerate (universal) primers, namely, AV494 / AC1048 (Wyatt and Brown, 1996) and Deng 540 / 541 (Deng *et al.*, 1994). The amplicons of size 550 bp were obtained and were sequenced. The partial coat protein gene of size 550bp were also cloned into the vector pTZ57R/T and transformed into DH5α strain of *Escherichia coli* and the true recombinants with desirable insert were confirmed by colony PCR.

The sequence data obtained in the study were subjected to *in silico* analysis to assess the diversity of the isolates. The nucleotide BLAST (BLASTn) analysis revealed more than 90 per cent sequence identity with *Chilli leaf curl Vellanad virus* isolate (Accession No. NC038442.1) from Vellanad region of Thiruvanathapuram district, Kerala. The translated nucleotide - protein BLAST (BLASTx) analysis of the viral sequences revealed more than 96 per cent sequence identity with *Chilli leaf curl Vellanad virus* coat protein sequence (accession no. YP_009506391.1). The coat protein sequences of all the six isolates were translated into corresponding amino acid sequence by ExPASy Translate tool and were used for further analysis and interpretation. The phylogenetic analysis revealed that, the isolates VKA2, KAR1 and KOD4 had very distinct sequence alignment when compared to other *Chilli leaf curl virus* isolates from India. The results indicated that, the three isolates *viz.*, VKA2, KAR1 and KOD4 could be new strains of *Chilli leaf curl virus* infecting chilli.

Three, possibly new strains of *Chilli leaf curl virus* infecting chilli have been identified and hence the study highlights the need for monitoring the emergence of new strains of plant viruses especially begomoviruses infecting solanaceous crops grown in Kerala. As this disease is one of the most important challenges to chilli cultivation, the information generated from the study could also be applied for the timely detection and effective disease management.