MOLECULAR CHARACTERIZATION OF BLACKEYE COWPEA MOSAIC VIRUS CAUSING MOSAIC DISEASE IN COWPEA (*Vigna unguiculata* (L.)*Walp*) IN KERALA.

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By

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(2016-09-027)

THESIS

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DECLARATION

I hereby declare that the thesis entitled "Molecular characterization of *blackeye cowpea mosaic virus* causing mosaic disease in cowpea (*Vigna unguiculata* (*L.*) walp) in kerala" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellayani **TANIA MATHEW PAMPACKAL**

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CERTIFICATE

Certified that this thesis entitled "Molecular characterization of blackeye cowpea mosaic virus causing mosaic disease in cowpea (*Vigna unguiculata* (L.) *walp*) in Kerala" is a record of research work done independently by **Ms. TANIA MATHEW PAMPACKAL** (2016-09- 27) under my guidance and supervision and this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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LIST OF ABBREVATIONS

%	Percentage
^{o}C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
A	Adenine
Amp	Ampicillin
A260	Absorbance at 260 nm wavelength
A280	Absorbance at 280 nm wavelength
BCMV	Bean Common Mosaic Virus
BlCMV	Blackeye Cowpea Mosaic Virus
BLAST	Basic local alignment search tool
bp	Base pair
C	Cytosine
CABMV	Cowpea Aphid-Borne Mosaic Virus
$CaCl_2$	Calcium Chloride
cDNA	Complementary deoxyribonucleic acid

cm	Centimeter
CMV	Cucumber Mosaic Virus
CP	Coat Protein
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide tri phosphates
G	Guanine
g	gram
hrs	Hours
ha	Hectare
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kb	Kilo bases
kg	Kilogram
L	Litre
LB	Luria Broth
m	Meter
M	Molar
mg	milligram
mins	Minutes
ml	Millilitre

mm	Millimeter
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NCBI	National center for biotechnology information
Ng	Nanogram
Nm	Nanometer
OD	Optical Density
ORF	Open Reading Frame
pM	Pico Molar
ppm	Parts per million
Rpm	Revolutions per minute
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
UTR	Untranslated Region
T	Thymine
Tm	Melting Temperature
X-GAL	5-Bromo-4-chloro-3-Indolyl β-D Galactopyranoside

INTRODUCTION

1. INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp), which is commonly referred to as Black eye pea or Southern pea is an annual herbaceous legume, belonging to the family of Fabaceae (Papilionaceae) (Oyewale and Bamaiyi, 2013). The name 'cowpea', has originated from the fact that the plant had been a prime source of hay for the cows in southeastern United States and in other parts of the world. It is one among the most favored staple and fodder crops in arid and semi-arid regions that include parts of Asia, Africa, the Caribbean, Central, and South America, Southern Europe, Southern United States, and Australia (Quiles *et al.,* 2013). In India, cowpea cultivation is spread across 0.5 million ha with an average yield of 600 to 750 Kg ha^{-1} (Singh *et al.*, 2012) and while considering the case of Kerala, it is being extensively cultivated in all the ecological zones. Annual cowpea production has hiked to 4.5 million metric tons in 2015 from about 6.3 million tons in 2008 (Animasaun *et al.,* 2015).

Cowpea serves as a rich palette of proteins and carbohydrates, with its leaves and seeds holding 43 and 33 percent proteins respectively (Hall and Ehlers, 1997). It can also be used as green manure and animal fodder. The seed is reported to accommodate 24 per cent crude protein, 53 per cent carbohydrate, and 2 per cent fat (FAOSTAT, 2012).

Cowpea is recognized as a host for an immense number of pests, bacterial, fungal and viral diseases, with majority of them, being transmitted through seeds (Hampton *et al.,* 1992). Among them, the viral diseases are most disastrous, as they upset the cowpea production totally. Shoyinka *et al.,* (1997) has reported that cowpea is naturally infected by 140 varieties of viruses, out of which 20 are worldwide.

The *Potyviridae* family with its six genera poses a great danger to vegetables, fodders, fruits and oilseeds (Mohamed *et al.,* 2016). *Blackeye cowpea mosaic virus* (BlCMV), *Cowpea aphid borne mosaic virus* (CABMV) and *Papaya ring spot virus* (PRSV) are the major ones belonging to this family. Out of these, BlCMV is one of the most catastrophic, powerful and cosmopolitan seed borne viruses. They cause immense

yield loss at rates up to 87 percent and contributes to an overall stunted growth, vein banding, chlorosis, leaf mottling, a mosaic, serious cutback in leaf size, distortion, and poor pod formation (Thottappilly and Rossel, 1993). The variations in viral symptoms may bank on the plant genotype and the virus strain. It was primarily isolated and described by Anderson (1955) as causing mosaic disease of cowpea in Florida. They are transmitted by aphids in a non-persistent manner. The field infection may strengthen up to cent percent within two months, depending on the abundance and activity of the vector (Hao, *et al.,* 2003).

The coat protein gene of the virus is responsible for its biological properties such as host range, replication, vector transmission, pathogenicity and cross-protection (Shukla and Ward, 1988). These biological properties of the virus differ among isolates worldwide (Bashir *et al.,* 2002). Therefore coat protein gene is often used for studying the genetic diversity in plant viruses.

The molecular characterization of virus is important from academic point of view. It helps in determining the evolutionary missing links that should be addressed to give an accurate picture of the nature of the plant virosphere and to increase our understanding of virus evolution and ecology. The studies (Gonçalves *et al.,* 2016) describe that plant virus with single-stranded RNA genomes form a very diverse group with a mammoth of variation in genome structure and gene expression strategy. They could be classified into subgroups based on phylogenetic relationships determined by sequence homologies among the conserved virus genes, including those for the coat protein (CP), and movement protein (MP). Coat protein primer is a potent tool in understanding the inter-relationship between different *potyviruses*. Based on the level of identity from pair-wise comparisons of the conserved core region of the coat protein sequences, Ward and Shukla (1994) further proposed differentiation between distinct virus species and strains of a single virus.

It is therefore essential to have an understanding of sequence variability so that universally applicable diagnostic tests and transgenic resistance can be developed or, if not, then the limitations of these can be understood. Therefore this study on symptomatology and

molecular characterization of the *Blackeye cowpea mosaic virus* can contribute to the development of the methods for early detection and development of the disease.

Upon analyzing the significance of *potyviruses* infecting cowpea (*Vigna ungiculata)* it was of prime interest to look into the molecular characterization of the coat protein gene of *Blackeye Cowpea mosaic virus* and accordingly the work has been planned with the following objectives.

- To isolate the RNA of the BlCMV from infected cowpea samples.
- To amplify coat protein gene using specifically designed primers.
- To compare the sequences obtained from five different agro-ecological zones.
- To analyze the variability at molecular level through sequence analysis.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Vigna ungiculata (L.) Walp., (2n=2x=22) is one of the cardinal legume that was originated in Africa. On grounds of its high protein content, it is often referred as "poor man's meat" (Reis and Frederico, 2001). It is a highly profitable commodity of income for farmers and traders (Langyintuo *et al*., 2003). Though beans are the preliminary focus of the cowpea plant, their flowers and leaves are also consumed for their nutritional value. They generate edible commodities that provide subsistence peasants with food supply throughout the budding to maturing period (Butkute *et al.,* 2018).

Due to its high beneficiary properties they are presently cultivated across varied regions of the world. On an average, the dry weight of cowpea seed accumulates to about 23-32 per cent of protein (Jayathilake *et al.,* 2018), 50-60 percent of carbohydrate (Khalid and Elharadallou, 2013), and 1 percent of fat (Kirse and Karklina, 2015). Cowpea has an overall protein content two to four fold greater than any other cereal or tuber crops especially for amino acids like lysine and tryptophan (Gonclave *et al.,* 2016). It can be cultivated whole throughout the year, across all the ecological zones of Kerala, irrespective of the seasons (Pradeep kumar *et. al.,* 2016). However, places with average rainfall are the most preferred.

Cowpea is exceedingly prone to the attack of various viral, bacterial and fungal diseases due to which the yield become primarily limited. Numerous viruses that are capable of depleting the yield by 13 to 62 per cent of cowpea have been reported. Among them, the Bean common mosaic virus (BCMV) plays an indispensible role in limiting the yield.

Sequence variability studies of virus are of chief priority since variations have been observed between field strains of the virus. This helps in developing universally applicable diagnostic tests and transgenic resistance.

2.1. COLLECTION OF BLACKEYE COWPEA MOSAIC VIRUS INFECTED SAMPLES

In the present study, purposive sampling was done from different agro-ecological zones of Kerala in order to study the variations that occur in the coat protein gene of the virus due to geographical locations. The concept of agro-ecological sketches of Kerala was undertaken in 2012 by ICAR- NBSS&LP (National Bureau of soil survey and Land Use Planning), Bengaluru under the project organized by Kerala State Planning Board (KSPB). The idea was put forward by Food and Agricultural Organization (FAO) with the aim to delineate agriculturally important areas suitable for each crop or combinations of crops based on agro-climatic conditions. The technical support was given by the UN and the financial support was provided by the World Bank (Rajwi, T., 2020). The division is based on land use, rainfall, altitude, soil variability and geographical morphology (Envis centre: KSCSTE, 2021).

The agro-ecological zones have been divided into five (KAU, 2016). They are as follows:

(i) Coastal plain (ii) Midland plain (iii) Foothill (iv) High hills (v) Palakkad plain

- **Coastal plain:** It includes regions along the coast at an elevation below 30 meters, lying between sea and midlands. This zone covers 4, 60,074 ha (11.84 percent) that comprises low-lying areas such as broad valleys, swamps, estuaries, backwaters, submerged lands, marshes, and Kayal lands. It also includes sandy plains, beaches, and coastal laterites. It mainly includes coastal areas of Thiruvananthapuram, Kollam, Kasargode, Kozhikode, Malappuram, Thrissur, Ernakulam, Alappuzha (Babu, K., 2019).

- **Midland plains:** This zone covers 10, 56,385 ha (27.18 percent) of land that extends from the southern end to the northern end of the state. The elevation ranges are between 30 to 300m and include foothills and hills on the east and lands interspersed with narrow valleys between the coastal plain on the west.

- **Foothills:** This zone covers 4, 60,074 ha (11.84 percent) that includes low hills between the midland plain on the west and high hills on the eastern side.

- **High hills:** This is the largest zone and covers (39.97 percent) 15, 53, 225 ha. It Includes Western Ghats and highland plateaus that are 600m above mean sea level. The Western Ghats comprises Central and south Sahyadri and the Nilgiris. The mountains are plateau leftovers of two or three altitudinal zone.

- **Palakkad Plain:** It is an inland plain with low elevation covering 1, 60,006 ha (4.16 percent). It is along the Valley of Bharathapuzha River. It includes gently sloping lands flanked by Kuthiran on the east, Nelliyampathy hills and Attappady hills on the south and north.

Blackeye cowpea mosaic virus (BlCMV) was first reported by Anderson (1955) from Florida, USA and later on detailed by Zettler and Evans (1972). Occurrence of the virus has also been outlined from California (Pio-Riberio *et al.,* 1978), India (Mali and Kulthe, 1980), Africa, Japan (Taiwo *et al.,* 1982), Thailand (Tsuchizaki *et al.,* 1987) and Carolina (Murphy *et al.,* 1987). These literature pieces of evidence points out the wide spread geographical distribution of virus.

Kalikar and Mali (1983) documented an incidence of 18 to 54 per cent from Marathwada region due to the attack of cowpea mosaic virus. Also a few months later, after a survey for three consecutive seasons in Chotanagpur of Bihar reported an increment in disease incidence of Blackeye cowpea mosaic virus infection from18 to 57 per cent.

Saric (1991) isolated a filamentous virus particle from the wild cowpea revealing yellow and dark green mosaic patterns from the island of Yugoslavia. The virus was identified as Blackeye cowpea mosaic virus. During a three year survey for disease incidence and distribution of the virus in Nigeria, Shoyinka e*t al.,* (1997) detailed that the

virus was detected in 390 samples out of 649 samples collected from various agro-ecological zones.

Later on Putturaju *et al.,* (2000) carefully studied the incidence of BlCMV in the fields of Karnataka, India. He reported that the virus was recorded in 18 fields out of 21 surveyed fields at a rate of 1-70 per cent, based on variations in the climatic conditions and the susceptibility of the variety.

Prakash and Shetty (2003) examined the natural occurrence of BlCMV infection in the fields and it was found to vary from 1.00 to 70.00 per cent. Shilpasree (2006) carried out a survey in Belagum district of North Karnataka and reported that during the Kharif season of 2005, disease incidence ranged from 10-36 per cent and during summer season it extended up to 18- 48 per cent.

Udayashankar *et al.,* (2009) collected 136 samples of cowpea from different legume growing regions of India such as Tamil Nadu, Andhra Pradesh, Karnataka, Maharashtra, New Delhi, Haryana and Gujarat. The incidence was in range of 0.6 per cent to 13.49 per cent. He compared the 3'-UTR samples of virus from each location and also showed that strains of BlCMV shows variations in their genes due to the differences in the geographical conditions. Aliyu *et al.,* (2012) conducted a comprehensive field survey in 2011 by including thirty locations of Nigeria to indicate that the symptoms observed on the cowpea plant were specific to those associated with virus infection.

2.2. SYMPTOMATOLOGY

Symptomatology plays an outstanding part in the delineation of *potyviruses* and their strain in the gone by days, and even today they continue to exist as the first criteria for identification of strains. As the majority of the *potyviruses* have confined host range, they can be often differentiated on the basis of the peculiar symptoms they produce in certain hosts. However, dependency on these criteria has created a lot of uncertainty in the recognition of *potyviruses* infecting members of some plant families such as *Leguminosae* (Dijkstra *et al.,* 1987).

Different *potyviruses* appears to bring out similar symptoms in some hosts; while different climatic conditions or different genetic lines of the same plant species can have extreme effects on the susceptibility to infections (Hollings *et al.,* 1981). For instance, discrete *potyviruses* causing mosaic disease of soybean in United States produced almost identical symptoms and couldn't be differentiated from each other.

Additionally, Bos and co-workers (Bos L., 1982) remarked that discrete *potyviruses* transmitting infections to legumes had hosts in common and differed only to some degree in their range of effects on host plant. Several of these troubles can be disentangled by standardization of hosts, varieties, and climatic conditions. Host range and symptomatology can play a significant role in recognition and classification of viruses if their biochemical properties are established primarily (Shukla *et al.,* 1989).

It has been reported that Cowpea Aphid-Borne Mosaic Virus (CABMV) and *Blackeye Cowpea Mosaic Virus* strain of *Bean Common Mosaic Virus* (BCMV-BlCMV) are two cosmopolitan viruses that possess potential catastrophes to cowpea production in many regions especially India (Puttaraju and Santhosan, 2000), South and North America, Europe, Africa (Albersio *et al.,* 1979), Pakistan (Bashir and Ahmed, 2002) and Thailand.

Putturaju et. al. (2000) has reported that the blackeye cowpea mosaic virus can cause a yield reduction from 2500kg/ha to 50kg/ha. The BlCMV inoculated cowpea shows 92-100 per cent infection at the trifoliate stage. The virus reduced the yield of adzuki beans by 33 percentage (Lizuka, 1990). At least, 36 species in 7 dicot families are vulnerable to this virus, with cowpea being the paramount natural host. Depending upon the environmental conditions, viral strain, and susceptibility of the crop the yield loss can be from 1 to 98 per cent (Bashir *et al.,* 2002).

BlCMV infected plants show field symptoms that include discoloration of the leaves, inter-veinal chlorosis, drastic mosaic patterns and dark green vein banding (Collins *et al*., 1985). Towards the later stages, the leaves show mottling, vein yellowing, and yellow spots

(Anderson, 1955). Affected plants may also show stunting (growth reduction) and reduction in pod formation.

Mali and Kulthe (1980) studied the seed-borne nature of *potyvirus* and the manifestations produced by these viruses in cowpea and reported that symptoms include mild and non-uniform mosaic patterns followed by yellow mottling, slight distortion, puckering, and arching of trifoliate leaves. There will be severe green and yellow mosaic, yellow spots, vein banding, leaf roll, mottles and leaf deformation for the whole plant.

Provvidenti (1986) has reported the presence of the virus in cotyledons and embryo axes. The symptomatology and host range of BlCMV was studied by mechanical inoculation into different plants. The signs produced on inoculated plants were chlorosis, yellow mosaic, and vein banding in leaves of cowpea (Saric, 1991). The virus produced infection in *Phaseolus vulgaris, Pisium sativum, Nicotiana clevelandii* and *Glycine max.* Plants like *Vica faba, Trifolium pretense, Ocimum basilicum* and *N. tabacum* were free of infection. Localized manifestations were observed in *Gomphrena glohosa, Phaseolus lunatus, Chenopodium amaranticolor* and Chenopodium quinoa. Radhika (1999) mentioned that yellow chlorotic local lessions were observed in the virus-inoculated *Chenopodium amaranticolor* leaf.

Givord (1981) remarked that BlCMV inoculated cowpea plants produced symptoms at seven days after inoculation (DAI). It included leaf chlorosis and vein clearing. At thirteen days after inoculation (DAI), growth reduction, chlorosis and dark green blotches in leaves were observed; and after 16 DAI, vein banding, mosaic, leaf distortion, puckering of leaves, and stunting of plants were seen.

According to Ouattara and Chambliss (1991), mechanically inoculated cowpea plants produced manifestations within 14 days of inoculation. The signs include interveinal chlorosis, stunting, light vein clearing, dark and light green patches, mosaic mottling, vein banding and malformation in leaves. The affected plants produced malformed flowers and pods (Hao *et al*., 2003). According to Alex (2017), BlCMV virus that affects cowpea plants

produced vein clearing on leaves of cowpea followed by mosaic mottling, vein banding, leaf distortion, flower malformation, and the infected plants become stunted.

In addition, Huguenot *et al.,* (1993) have reported that CABMV and BlCMV cause similar mosaic diseases in cowpea. Mixed infection of both CABMV and BlCMV along with cucumber mosaic virus (CMV) results in serious stunting and nearly total yield loss depending on the virus strain.

2.3. PRIMER DESIGNING

PCR is an absolute revolutionary technique used universally for the invitro amplification of DNA. The indispensible essentials need for running a PCR reaction encompasses template DNA, primers, Taq polymerase, dNTPs, PCR buffer, and the instrument, thermocycler.

For a successful PCR reaction, high quality primers are necessary. During primer designing it is vitally important to precisely adhere to the criteria of primer designing. The following are some prime considerations detailed by Etebu (2013) directed to obtain specific amplifications with high yields.

- The length of the primer is crucial and it should lie between 18-30 bp.
- Primer sequences must not contain more than 4 GC residues.
- The melting temperature (Tm) of both reverse and forward primers must be roughly the same. Suggs and co-workers (1981) described an easy equation, 2˚C (A+T) + 4˚C (G+C), to compute the Tm of primers. As per the works of Dieffenbach et al., (1993) Tm should be higher than 54˚C.
- Primer dimers should be rejected.
- One GC clamp at the 3’ end is sufficient to ensure specificity.
- Primers must not be complementary at 3’ end and the possibility of forming secondary structure (hairpin structure) must be avoided.

Primer designing can be performed with various user friendly computational tools. Each of these tools poses their own guidelines for designing. Primers can be either synthesized from known sequences which are already deposited in the nucleotide sequence database or from unknown sequences.

In case of unknown sequences, primers are designed based on the conserved sequences (Sibhatu, 2003). Various softwares such as MEGAX, CLUSTAL etc. can be considered for identifying the conserved sequences using multiple sequence alignment programs. Based on the conserved sequences primers are produced using softwares like Primer3plus, IDT, NCBI primer BLAST etc.

2.4. AMPLIFICATION OF COAT PROTEIN GENE

Blackeye cowpea mosaic virus (BlCMV) is a single-stranded positive sense RNA virus that belongs to the *Potyviridae* family and genus *Potyvirus* (Harrison and Gudauskas, 1968). They are filamentous particles of 600-900 nm length and 11-15 nm width (Uyemoto *et. al.,* 1973). They have approximately 2000-3000 copies of capsid protein that encapsulates an RNA genome of 10Kb length and contains one long open reading frame (ORF) expressed as a polyprotein precursor (Riechmann et al., 1992). BlCMV is transmitted by aphids in a non-persistent manner (Bashir and Hampton, 1994). The N-terminal region of the ORF contains a motif which is involved in binding the coat protein to the aphid styles (thereby aids in transmission of virus).

Coat protein structure of the virus provides unique way of identifying the virus for the successive reasons. Primarily, the coat protein is a distinctive gene product with amino acid composition that is specific for the group (Fauquet *et al.,* 1986). Secondly, the coat protein accounts for 95 per cent of the *potyvirus* particle and is the only gene product in the virion (Domier *et al.,* 1988). The sequence comparison of the coat protein genes is a reliable tool for the differentiation of viruses or strains; likewise they have promoted the classification of the *potyviridae* family members into genera, distinct and related species, and strains (Shukla *et al.,* 1992). Even other regions of the genome such as 3’ UTR is also

used to classify *potyviruses* (Habera *et al.,* 1994). However, amongst all the genes, CP gene of *potyvirus* is most customarily used for genetic diversity studies.

2.4.1. cDNA Synthesis

RNA cannot directly serve as a template for PCR reactions. The process in which the DNA is synthesized from RNA template using reverse transcriptase enzyme is called complementary DNA (cDNA) synthesis. Reverse transcriptase makes use of an RNA template and a short primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA. Usually total RNA or Poly A+ RNA is used as template and primed with oligo dT primers or hexamers (Pathak and Rastogi, 2009). Primer selection is mainly based on our intended purpose. This formed strand can be then used as a template for the polymerase chain reaction (PCR).

2.4.2. Amplification of coat protein

The cDNA formed can then be specifically amplified with coat protein gene primers via normal PCR reactions to carry out the phylogenetic studies. The primers are usually synthesized from the conserved region of coat protein gene. This method, of using RT-PCR is very sensitive and is capable of detecting even one infected leaf from ninety nine healthy leaves. The two most commonly used RT enzymes include Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) and Moloney murine leukemia virus reverse Transcriptase (MMLV-RT). MMLV-RT is preferred for longer transcripts as it displays reduced RNAse H activity with add on proofreading 3'-5' exonuclease activity,(DeStefano et al., 1991) whereas AMV-RT become a choice when our transcript is having complex secondary structure (Merck, 2020).

Salem *et al.,* (2010) has reported the identification of CAMBV, and Cucumber mosaic virus (CMV) using RT-PCR with specific primers. It was also shown that this technique can identify the homology between CAMBV and its closely related viruses (Abreu and Medeiros, 2012).

It was reported by Gillaspie *et al.,* (2011) that RT-PCR showed more viruses in seeds infected with CABMV than that in infected seedlings. The works of Sharma *et al.,* (2013), shows that the DNA fragments from CABMV infected plants have been amplified by RT-PCR using specific pair of coat protein primers and 3'UTR.

2.5. CLONING AND SEQUENCING

Cloning is the process in which numerous copies of a gene are produced in increased number by inserting the gene fragment within a bacterial cell. There are different methods in cloning, such as TA cloning, Gibson cloning etc. out of which TA cloning shows better efficiency. It is a process in which the terminal transferase activity of the polymerase enzyme is made use of. The enzyme adds single 3' A overhangs to the ends of the PCR product, thereby allowing it to get cloned directly into a linearized vector possessing 3'-T overhangs. These gene fragments are then sequenced for phylogenetic studies.

The coat protein sequence is the major deciding factor of phylogenetic analysis due to its involvement in the morphology of the filamentous particle, serological relationships, and to the vector relations. Moreover, the coat protein sequence of the *potyvirus* is easy to discover among the plant viruses as it can be easily purified in relatively high yields for direct sequencing and also because the coat protein and the 3' NCR are the sequence immediately proximal to the 3' poly (A) tail, that is used for oligo-dT priming for reverse transcription from viral RNA for successive cDNA cloning (Shukla *et al.,* 1992).

2.6. PHYLOGENETIC ANALYSIS

Sequence comparison describes the way of arrangement of DNA sequences, in order to identify the regions of similarity. It helps to infer the structure, function, and evolutionary relationship between the sequences.

Closely related sequences which are roughly of same size are usually compared through global alignment method. Here the alignment is executed from beginning till end of the sequence to observe the best possible results. However, sequences which are thought to

have likeliness or even dissimilar sequences can be compared using local alignment tools such as BLAST. Hence local alignment tools are usually preferred for phylogenetic studies (Palanga *et. al.,* 2016).

Konate *et al*., in 2017 conducted molecular characterization of virus isolates from *potyvirus* genus that infected *Vigna subterranea*. He extracted the RNA of the virus from symptomatic leaves, collected from three agro-climatic zones of Burkina Faso and identification of the *potyvirus* (CABMV and BCMV-BlCMV) in the sample was done using degenerate primers. Further sequencing was done using coat protein gene primers such as P105 and P106. The sequenced genes were then compared with other sequences available in Basic Local Alignment Search Tool (BLAST) and a phylogenetic tree was constructed. Finally it was reported that the isolates had 97.3-100 per cent nucleotide identity.

Molecular and biological characterization of Bean Common Mosaic virus (BCMV) strain was done in *Phaseolus lunatus* (Lima bean) in 2017 by isolating the whole genome followed by amplification using BCMV-specific primers RT-PCR. In pair-wise comparison, BCMV strains shared 86 per cent sequence identity.

Udayashankar *et al.,* (2012) found that BCMV infecting French bean and BlCMV infecting urdbean clusters in BCMV subgroup. For this he amplified the coat protein gene of four isolates that infects common bean and urd bean. The PCR products of 700bp were cloned to pGEM vector using TA cloning kit and then transformed into E. *coli* JM 109 cells by heat shock method. They were further sequenced and compared with other isolates in Gen Bank database. Following this, phylogenetic tree was established.

The genetic diversity of cowpea mild mottle virus on soybean in several region of Indonesia was demonstrated by Sutawati *et. al.,* (2018) by amplifying the coat protein gene of the virus using forward primer 5’- ATTAAGGATCCGAGTTGATTTAAAT AAGT-3’ and reverse primer 5’- ATTAAGAATTCCTTGTGATTGAAATTGCG-3’. The amplified PCR products were cloned into pTZ57R/T easy vector system. The nucleotide sequences were aligned with those sequences present in Gen Bank database using ClustalX.

The gene sequences of watermelon leaf mottle virus was compared with 17 other *potyviruses* by Hiebert and Purcifull (1979) and they reported that 55.6-63.5 per cent of the coat protein sequences were identical. Likewise, 3' UTR (untranslated region) showed 37.2-48.3 per cent similarity.

The sequences of the viral components are very important to identify the disease causing entity and to design management strategies. Knowledge about the sequence relationship used in plant transformation, very specifically coat protein sequences helps to develop a more rational design of resistance as the coat protein accounts for 95 per cent of the virus particle.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Molecular characterization of *Blackeye cowpea mosaic virus* causing mosaic disease in cowpea (*Vigna unguiculata* (*L.*)Walp) in Kerala" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram. Details regarding the experimental materials used and the methodology followed for various experiments are presented in this chapter.

3.1. COLLECTION OF BLACKEYE COWPEA MOSAIC VIRUS FROM AGRO-ECOLOGICAL ZONES OF KERALA

The study was conducted in districts categorized under five agro-ecological zones of Kerala such as Coastal plain, Midland plain, Foothills, High hills, Palakkad plain and accordingly, Trivandrum was placed under coastal plain, Thrissur was under Midland Plain, Kottayam was considered under Foothill zone, Palakkad under Palakkad plain and Wayanad under High-hill zone.

3.1.1. Disease Incidence

The data of cowpea cultivators were identified in consultation with the extension workers of Vegetable and Fruit Promotion Council Kerala (VFPCK) of the respective district. The GPS co-ordinates of the location selected from each zone were measured and projected on corresponding district maps to get the exact location of the survey area (table 1).

The survey was undertaken during the major cultivated seasons in each district and during that period about 85 per cent of the commercial fields were at the flowering stage. From each district, a farm field consisting of a large plot under cowpea cultivation was randomly chosen for study and the percentage of disease incidence in each of this area was calculated as:

$$\text{Disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants}} \times 100$$

Incidence of virus infection was assessed by counting the total number of plants and number of plants showing distinct symptoms of blackeye cowpea mosaic virus in each field.

Table 1: Location under study

Sl. No.	Location	GPS coordinates
	Thiruvananthapuram	
1.	Pappanchani	N 8°44’13.40” , E 76°98’08.22”
	Palakkad	
2.	Puthucode/thekkepotta	N 10°38’18.17” , E 76°26’13.82”
Thrissur		
3.	Vellanikkara College	N 10°54’84.24”, E 76°28’44.26”
Kottayam		
4.	Edayazham	N 9°68’73.09”, E 76°42’03.65”
Wayanad		
5.	Meenaghadi	N 11°63’95.06”, E 76°15’90.90”

3.2. SYMPTOMATOLOGY

Symptomatology was studied by observing the naturally infected plants. The expression of symptoms as a consequence of virus infection was analyzed by noticing the burgeoning of symptoms in the infected cowpea plants. Symptoms expressed in the plants collected from different geographical areas were studied. From the total plants observed for taking the observations, symptomatic plants were selected.

3.3. PRIMER SYNTHESIS

A primer specific to the coat protein gene of Blackeye cowpea mosaic virus was designed using Primer3 plus tool. The steps involved are as follows:

1. Partially characterized sequence of Blackeye cowpea mosaic virus was retrieved from NCBI (www. Ncbi.nlm.nih.gov/) under nucleotide database and the FASTA sequence of the following accession number: >AY575773.1 was downloaded.
2. The sequence of coat protein gene was provided as query sequence in nucleotide BLAST, a local alignment search tool, in order to find out the various other sequences of the same virus with maximum similarity.
3. Thus the coat protein gene of the virus from different regions such >AY575773.1 (strain Coimbatore), >FR775796.1 (strain Southern Thailand), >Y17823.1 (strain Florida), >DQ925423.1 (strain Vietnam), >AF395678.1 (strain China), and >AH004380.2 (strain Vellayani), was downloaded in FASTA format.
4. Multiple sequence alignment of these biological sequences was performed using Clustal Omega.
5. Based on the output from clustal omega, the closely related sequences were analyzed for similarities and the conserved regions were detected. The conserved residues will be denoted using asterisks‘*’ symbol.
6. The conserved residues were then pasted in a primer designing software Primer3 Plus to pick suitable primers.
7. In Primer 3plus, from the ‘Task’ drop down menu select ‘cloning’. Enter the desired included regions in the text box and then click on ‘Pick Primers’.

8. From the result obtained primers of 18-24 base pairs were selected for further analysis. Sequences possessing 1-2 GC pairs at both the ends were chosen.
9. Different parameters such GC content, melting temperature, primer dimer formation (Primer pairs should not have complementary regions) were analyzed for these chosen sequences using Primer Express software.
    i. GC content should be between 40-60 per cent.
    ii. Melting temperature (T_m) must be in the range of 50-60 ^{o} C Primer pairs must have a melting temperature within 5^o C.
    iii. Primer sequences should have a maximum of 4 GC residues.
    iv. The sequences should not be complimentary at 3'ends and the chances of forming secondary structures such as hairpins must be avoided.
    v. One GC clamp at 3'end is enough to ensure specificity.

10. To find out the specificity of primers, NCBI primer BLAST was used.
11. Finally two sequences with finest parameters were chosen as forward and reverse primers.

3.4. AMPLIFICATION OF COAT PROTEIN GENE

3.4.1 Trizol method of RNA isolation

The RNA extraction was done using modified RDP Trio reagent (Himedia Catalogue No.MB566) as per manufactures instruction. The protocol performed is as given below.

3.4.1.1 Sample Preparation

A 100 mg of cowpea leaf tissue showing distinct symptoms of the virus was collected in either RNase later solution or quick-freezed in liquid nitrogen (immediately after collection).The sample was then taken in a sterile DEPC treated mortar and pestle and

liquid nitrogen was added to freeze the leaf tissues. It was then lysed and homogenized in 1mL TRIzol reagent and kept for five minutes. The homogenate was transferred to a 2mL pre chilled microfuge tube. Then 0.2mL of chloroform per 1mL of TRIzol reagent was added; and shaken vigorously for 15s. It was then incubated at room temperature for five minutes followed by incubation on ice for ten minutes. The samples were then centrifuged at 12,000g for 15 minutes at 4^{o}C. The mixture got separated to lower red phenol-chloroform, and interphase and a colorless upper aqueous phase. The upper aqueous phase containing RNA was transferred to a new 2mL centrifuge tube.

3.4.1.2. RNA Isolation

Added 0.5mL of chilled isopropanol (100 per cent) per mL of TRIzol used for lysis, to the tube and incubate for ten minutes in room temperature Centrifuge at 12,000g for ten minutes at 4 degree celsius. The total RNA precipitates and forms a white gel like pellet at the bottom of the microfuge tube.

3.4.1.3. RNA wash

The supernatant is discarded and the pellet is re-suspended in1mL of 75 ethanol ethanol per 1mL of TRIzol. The sample is briefly vortexed and then centrifuged at 7500g for 5 minutes at 4° C. Again the supernatant is discarded and the pellet is air dried for 5-10 minutes.

3.4.1.4. RNA resuspension

The pellet was finally suspended in 30 µl of RNAase free water and incubated at 55-60 for 10 minutes. It was then stored at -80°C.

3.4.2. Quality and Quantity assessment of RNA samples

The quality of RNA was analyzed by running the samples in 2 per cent agarose gel. It was prepared by melting 0.8g of agarose in 40ml1X TBE buffer (prepared in RNAase free water). Once the gel was cooled to 60-65 C, 3µl of ethidium bromide was added to the

melted gel and casted in a horizontal gel electrophoresis unit (Hoefer Power pack, Germany).

The solidified gel was placed in electrophoresis tank containing 1X TBE buffer prepared in RNAase free water (Appendix II). Then 5µl of RNA sample was mixed with 2µl of 6X loading dye (Appendix I) was dispensed into the wells of the gel. The voltage was maintained at $5Vcm^{-1}$. When the dye has run three-fourth of the entire distance of the gel, it was taken out and visualized in a UV trans-illuminator system (Bio-Rad) and documented in Gel Doc system (Gel DOC ^{TM} XR+).

The quantification of the RNA sample was done by measuring the absorbance in UV- visible spectrophotometer at a wavelength of 260nm and 280nm. The absorbance of 1.0 at 260nm indicated that 40ng μl^{-1} of RNA was present in samples. The concentration of RNA was ascertained by the following formula:

Concentration of RNA in the sample ($ng\mu l^{-1}$) = A_{260} x 40 x Dilution factor

Where, A_{260} = absorbance at 260 nm

The quality of the RNA samples was determined from the ratio of OD values at 260nm and 280nm. Those samples which have A_{260} /A_{280} value between 1.8 and 2.0 will have the best quality.

3.4.3. cDNA Synthesis

The RNA isolated was subjected to cDNA synthesis as per the manufacturer's instructions (Thermo scientific Verso cDNA Synthesis kit).

The synthesis of cDNA was carried out in a 20 µl reaction volume which contained: 5x cDNA synthesis buffer 4.0 µl, 5mM dNTPs (dATP, dGTP, dCTP and dTTP) 2.0 µl, RNA sample 4.0µl, Random Hexamer primer (400ng/µl) 0.5µl, RT enhancer 1µl, Verso reverse Transcriptase enzyme 1 µl, and 7µl nuclease free water. The reaction mixture was

run in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems) under the following conditions mentioned in table 2.

Table 2: Conditions for synthesis of cDNA:

Conditions	Temperature	Time	No. of cycles
cDNA synthesis	42°C	30 min	1 cycle
Inactivation	92°C	2 min	1 cycle

3.4.4. Confirmation of cDNA synthesis

The synthesized cDNA was assessed with specific house-keeping genes such as actin primers to corroborate the conversion of RNA to cDNA. A standard PCR mix was prepared for 20µl of total reaction (table 3). The primers used were in reference with the work of Amorim *et al.,* (2018):

Forward: GGAACATCCCGTTCTCTTGA

Reverse: CTCTCAGGAGGAGCAACCAC

Table 3.Components for PCR using actin primers

SI. No.	Components	Volume
1.	10X reaction buffer (1X)	2 µl
2.	dNTP mix (100 µM each)	1 µl
3.	Forward Primer	1 µl
4.	Reverse Primer	1 µl

5.	Taq DNA Polymerase	0.3 µl
6.	Template DNA	3 µl
7.	Nuclease free water	11.7 µl
	Total Volume	**20 µl**

cDNA samples of all five zones were amplified by an initial denaturation of 95 for 3 mins, followed by 30 cycles of denaturation for 15s at 95 , 15s of annealing at 60 and extension for 45 s at 72 . The final extension was carried on at 72 for 5 mins.

Amplified products were then examined in 1.5 per cent agarose gel prepared in 1X TBE buffer containing 0.5 µg/ml ethidium bromide. 2µl of 100 bp ladder was added in one of the wells as an indicator. Electrophoresis was executed at 60 V until the loading dye reached three fourth of the gel. The gel was finally documented using Gel-Doc (Bio-rad) unit.

3.4.5. Polymerase Chain Reaction (PCR)

PCR is a technique universally used to amplify small segments of DNA into billions of copies. The PCR detection is more sensitive and accurate. The synthesized cDNA was laid to PCR amplification with primers specific to the coat protein gene of *Blackeye cowpea mosaic virus* designed during the study.

The PCR amplification reactions were carried out in a 20 µl reaction volume which contained: 10x PCR buffer (contains 1.5 mM $MgCl_2$) 2.0 µl, 10*mM* dNTPs (dATP, dGTP, dCTP and dTTP) 1.0 µl, 3.0 µl cDNA, pand 0.3 µl (2.5 Units) Taq polymerase enzyme, forward and reverse primers (5*pM*) 0.5 µl each and 12.7 µl sterile distilled water. The reaction mixture was run in a thermocycler (BIORAD T100^{TM} Thermal cycler) under the following conditions (table 4).

Table 4. PCR Conditions for amplification of coat protein gene of BlCMV

Conditions	Temperature	Time
Initial denaturation	94°C	5 min
No. of cycles-35		
Denaturation Annealing Extension	94° C 54° C 72°C	30 sec. 30 sec. 20 sec.
Final Extension	72°C	5 min.

3.4.6. Agarose Gel electrophoresis of PCR products

The PCR products were analyzed in 1.2 per cent agarose gels prepared in 1X TBE buffer containing 0.5 µg/ml ethidium bromide. 1µl of 6X loading dye was added with 5 µl of PCR products and loaded into the wells. To one of the wells 2 µl of 100bp DNA ladder was added as a measure. Then electrophoresis was performed at 70V power supply with 1X TBE as electrophoresis buffer for about 1-2 h. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.4.7. Gel Elution

3.4.7.1. Gel Extraction

The gel extraction was done using Hiper Gel Extraction teaching kit Himedia (Catalogue No. HTBM010), as per manufacturer's instructions. The DNA band to be eluted was excised out from the agarose gel with a clean scalpel blade and kept in a sterilized, pre-weighed micro-centrifuge tube. The weight of the gel band was calculated and the Gel Bind buffer was added in the ratio of 10µlof buffer for each 10mg of gel slice. The sample was then incubated at 55°C and mixed vigorously by vortexing until the agarose gets completely dissolved (approximately 15 minutes).

3.4.7.2 Load sample on to the column

Samples were then loaded on to Hi Elute Mini Prep Spin Column and centrifuged at 13000rpm for 1min. The supernatant was transferred to a DNA binding column placed inside a collection tube. The flow-through was discarded from the collection tube and 500 µl of gel bind buffer was added on to the column. This was then centrifuged at 12000rpm for 1min at room temperature.

3.4.7.3 Wash

The column was then placed in a fresh collection tube and 750 µl of gel wash buffer was added on to the glass fiber matrix. It was then vortexed for an additional 1min at 13000rpm and the spin column was transferred to a new collection tube.

3.4.7.4. Elution

50 µl of elution buffer was added to the spin column and incubated for 1min at room temperature and finally it was centrifuged at 13000 rpm for 1 min. This step causes the elution of DNA sample.

3.5. CLONING AND SEQUENCING

Cloning is the process in which identical copies of the DNA segment is being produced. PCR product is used as the template which is ligated to the cloning vector and is maintained within bacteria. PCR detection followed by sequencing of PCR products after cloning in plasmids is mostly done to confirm the virus.

3.5.1. Standardization of procedure for competent cell preparation

- From the pure stock of *Escherichia coli* strain DH5α (glycerol stock) a loop full of cells are taken and is added to 2mL LB broth directly and is incubated overnight(16-20 hrs) at 37^{o}C.
- The overnight culture will have a silky appearance. It was then streaked onto an LB agar plate prepared by adding 1.5 per cent of agar into 100mL LB Broth. The plates are then incubated at 37^{o}C overnight.
- Using a sterile inoculating loop the single colonies were picked out and inoculated in a 25 mL LB broth. It was then incubated overnight at 37^{o}C with shaking.
- Inoculated 500µL of the above culture into 25 mL LB broth in 250 mL flask and it was allowed to grow for 2 - 3 hrs till the OD - 600 reached 0.3 to 0.4 (silky appearance). Then harvest the cells by spinning the bacterial culture taken in microfuge tubes at 4000 rpm for 5mins. The supernatant was discarded and the pelleting was repeated using fresh culture until sufficient amount of bacterial pellet was obtained. Finally the pellet was resuspended in 1 mL chilled 0.1M$CaCl_2$ and centrifuged at 4000 rpm for 5 mins at4^{o}C. The obtained pellet was resuspended in 1 mL chilled 0.1M $CaCl_2$ and kept on ice for 30mins.

3.5.2. Competency check

Transformation was done by heat shock method using the competent cells. The vial with competent cells was thawed on ice and pUC18 vector (50ng) was added to the vial. The contents were then mixed gently and kept on ice for 40minutes. After that the tube was taken from ice and heat shock was given at 42 ^{o}C for 2 mins by immersing the tube in

heated water bath. Then heat shock tubes are immediately placed back on ice for 5min. 250 µl of LB broth was then added to the vial under sterile conditions and the tubes were inverted twice to mix the contents. It was then incubated at 37 ºC for 1 hour with shaking to allow expression of ampicillin resistance gene in transformed cells. 150 µl of this transformed culture is poured on LB/Amp/IPTG/X-Gal plates (Appendix III) and spread plate using sterile glass rod. The plates are then incubated overnight at 37ºC.

3.5.3. TA Cloning

Cloning was performed using Mighty TA-Cloning kit (Catalogue No. 6028) as per the manufacturer's instructions.

3.5.3.1. Competent cell preparation

The single colonies were picked up using sterile loop from a petri plate and inoculated into a 25ml LB broth. It was then incubated overnight at 37ºC with shaking. A 500µL of the above culture was inoculated into a 25 mL LB broth and it was allowed to grow for 2 - 3 hrs till the OD - 600 reached 0.3 to 0.4 (silky appearance). The cells were aseptically transferred to a centrifuge tube and chilled on ice for 10mins. Then the cells were harvested by spinning the bacterial culture at 4000 rpm for 5mins. The supernatant was discarded and the pelleting was repeated using fresh culture until sufficient amount of bacterial pellet was obtained. Finally the pellet was resuspended in 1 mL chilled 0.1M$CaCl_2$ and centrifuged at 4000 rpm for 5 mins at4ºC. The obtained pellet was then dissolved in 1 mL chilled 0.1M $CaCl_2$ and kept on ice for 30mins.

3.5.3.2. Ligation reaction

To separate micro tubes, 1µl each of the eluted and purified PCR product was added. To another tube, 1µl positive control insert DNA was added (control reaction). Then to all these tubes 1µl of pMD20-T vector and 3µl of sterile distilled water was added and mixed well. This was followed by the addition of 5µl of Ligation Mighty mix. The mixture was then shaken gently and the tubes were incubated at 16ºC for 30 mins. Following this, transformation was carried out by heat shock method using the above obtained mixture and 100µl of competent *E. Coli* DH5α cells.

3.5.4. Confirmation of insert DNA

The colonies obtained on LB agar were screened for recombinants via colony PCR. Viral coat protein specific primer itself was used in this process. White colored colonies were selected randomly and a small fraction of the colony was picked using sterile tooth pick and dispensed into 5 µl sterile water taken in PCR tubes. Along with that 5 µl of master mix containing, 2 µl 2X reaction buffer, 0.2 µl each of M13 primer RV and M4, 0.2 µl dNTP and 0.2 µl of taq polymerase enzyme were added to each tube. This mixture was mixed thoroughly and amplified in a thermal cycler.

PCR conditions:

Initial denaturation	- 95	- 10 minutes
Denaturation	- 95℃	- 30 seconds
Annealing	- 56	- 30 seconds
Elongation	- 72° C	- 1 minute
Final elongation	- 72	- 10 minute
Hold	- 4	- Infinity

30 Cycles

Electrophoresis was performed on 1 per cent agarose gel containing ethidium bromide to identify the recombinant colonies. The positive colonies were selected and inoculated in 5 ml of LB broth containing 100 µg/ml of ampicillin. This culture is then intended for isolating plasmid DNA for sequencing.

3.5.5. Plasmid isolation

Plasmid isolation was based on the modified method of Birnboim and Doly (Birnboim, 1979). The *E.Coli* strain containing the plasmid of interest was grown overnight at 37 with shaking at 160 rpm in LB broth with adequate quantities of antibiotics.

The overnight grown culture is then aliquoted to micro-centifuge and pelleted by centrifugation at 12000 rpm for 5 minutes. The medium was removed by aspiration and the pellet was resuspended in 200 µl of solution I (Appendix IV) by vortexing. To this 300 µl of freshly prepared solution II (Appendix V) was added and the contents were mixed by inversion.

The tubes were then incubated on ice for 5 minutes. Subsequently, 300 µl of solution III (appendix VI) was added to neutralize this solution. The samples were mixed thoroughly by inverting the tubes and were again incubated on ice for another 5 minutes. The cell debris was then removed by centrifugation at 12000 rpm for 10 minutes at room temperature. The supernatant was transferred to a fresh tube to which ribonuclease A was added to a final concentration of 20µg/ml. The sample was incubated at 37˚C for half an hour. After RNase A treatment, the supernatant was extracted twice with 400 µl of chloroform to remove the proteins present. This was followed by centrifugation at 12000 rpm for 1minute to separate the phases. The upper aqueous phase containing the plasmid DNA was transferred to a fresh tube. An equal volume of 70 per cent isopropanol was added to those tubes to precipitate out the DNA. The contents were again mixed well and centrifuged at 12000 rpm for 10 minutes at room temperature. Then isopropanol was removed completely by aspiration.

The DNA pellet was washed with 500 µl of 70 per cent ethyl alcohol to remove any adhering salts. After aspirating out the alcohol, the pellet was air-dried and the plasmid DNA was dissolved in TE buffer. The quality of the prepared plasmid was checked by 0.8 per cent agarose gel containing ethidium bromide.

3.5.6. Sequencing using Sanger's dideoxy chain termination method

The isolated plasmid DNAs from recombinant colonies were sent to Athmic Biotech, Trivandrum for sequencing and the sequencing was done based on Sanger's dideoxy chain termination method. The sequencing PCR was done using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 2.2. Sequencing was

done using ABI 310 Automated DNA sequencer (Applied Biosystems, Life Technologies, California, and USA).

The ready reaction mix consists of the following components:

- A-Dye Terminator labeled with dichloro (R6G)
- C-Dye Terminator labeled with dichloro (ROX)
- G-Dye Terminator labeled with dichloro (R110)
- T- Dye Terminator labeled with dichloro (TAMRA)
- Deoxy nucleotide triphosphate (dATP, dCTP, dGTP, dUTP)
- AmpliTaq DNA Polymerase, FS
- $MgCl_2$
- Tris-HCL buffer, pH 9.0

The 20 µl sequencing reaction mixture consisted of 25-50 ng of template (plasmid DNA), 10 picomoles of the primer, 1.4 mM $MgCl_2$, 2 µl of the reaction buffer and 1 µl of the reaction premix. The ramp time of the thermal cycler was set at 1^o C. The thermo-cycling conditions are given below (table 5).

Table 5.The thermo-cycling conditions for Sanger sequencing

Step I	Initial denaturation	96°C	3 minutes
Step II 25 cycles	Denaturation	96°C	10 seconds
	Annealing	50°C	5 seconds
	Extension	60°C	4 minutes
Step III	Hold	4°C	

The PCR product obtained after sequencing was purified and subjected to ABI sequence reading.

3.6. PHYLOGENETIC TREE CONSTRUCTION

The sequences availed from Athmic Biotech's DNA sequencing facility, were compared, to detect any differences among sequences.

The construction of phylogenetic tree was done using Mega 6.0., molecular evolutionary genetic analysis software that validates the given input by mapping names in the topology to the names in the alignment data. Initially, pairwise alignment of the sequences was performed and its data was used to create the phylogenetic tree by neighbor joining method.

Also, Clustal omega, an online multiple sequence alignment tool was used to check the sequence alignment of the sequences under study. Access Clustal omega website (https://www.ebi.ac.uk/Tools/msa/clustalo/) and feed the obtained sequences of BlCMV in the input box. Submit the sequences by selecting "Submit" button. This enables sequence wise alignment to take place. After the alignment process the result will displayed in the "Alignments" tab. "Phylogenetic tree" tab allows the generation of the tree which will be displayed in a new window in the form of cladogram as well as Phylogram

RESULTS

4. RESULTS

The present research work entitled 'Molecular characterization of *Blackeye cowpea mosaic virus* causing mosaic disease in cowpea (*Vigna unguiculata* (*L.*)Walp) was carried out in College of Agriculture, Vellayani, during the year 2020-2021, with the objectives to characterize and determine the variations in Blackeye cowpea mosaic virus collected from the infected cowpea leaves belonging to the five ecological zones of Kerala. The results of the study are detailed here.

4.1. COLLECTION OF BLACKEYE COWPEA MOSAIC VIRUS FROM AGRO-ECOLOGICAL ZONES OF KERALA

4.1.1 Disease Incidence

(table 6). The Collection sites (Plate 1) chosen from coastal zone was Pappanchani, Trivandrum and that from Palakkad plain was Puthucode, Meenaghadi, Wayanad represented the high hills zone. The infected cowpea field in foot hill zone was from Edayazham, Kottayam and that from midland zone was Vellanikara, Thrissur.

Table 6: Percentage of disease incidence

SI No.	Agro-ecological Zone	Location	Total No. of plants surveyed	Total No. of plants infected	Disease Incidence (%)
1.	Coastal Plain	Pappanchani, Trivandrum (T1)	100	55	55.0
2.	Midland Plain		120	43	35.8
3.	Foot hills	Edayazham, Kottayam (T3)	100	34	34

4.	High hills	Meenaghadi, Wayanad (T4)	65	24	36.9
5.	Palakkad Plain	Puthucode, Palakkad (T5)	200	40	40

4.2. SYMPTOMATOLOGY

Under natural field conditions, the signs and the symptoms of infected cowpea plant during the survey is as follows.

The major symptoms observed in all six fields were severe mosaic patterns and variable amounts of dark green vein banding in the leaves. Diseased trifoliate leaves of cowpea showed leaflet distortion and interveinal chlorosis. The leaves also showed puckering, chlorotic leaf spot, reduced flowering. The Pappanchani field which had 55 per cent of disease incidence showed reduced pod formation and stunted growth. Infected plants also showed characteristic yellowing of leaves towards the later stages.

4.3 PRIMER SYNTHESIS

The FASTA sequences of the following accession numbers such as >AY575773.1 (strain Coimbatore), >FR775796.1 (strain Southern Thailand), >Y17823.1 (strain Florida), >DQ925423.1 (strain Vietnam), >AF395678.1 (strain China), >AH004380.2 (strain Vellayani), were given in the multiple sequence alignment tool, Clustal omega to obtain the conserved regions. The results of Clustal omega is given with (fig. 2) '*'symbol indicating the region of conserved sites.

These conserved sequences were then given in the Primer3 Plus tool to pick the primers. The output of Primer3Plus gave a list of primers along with their characteristics as shown in figure 3. Then by checking the parameters such as GC content, Primer dimer

Plate 1. Field view of collection sites (a) Pappanchani, Trivandrum (b) Puthucode, Palakkad (c) Meenaghadi, Wayanad (d) Edayazham, Kottayam

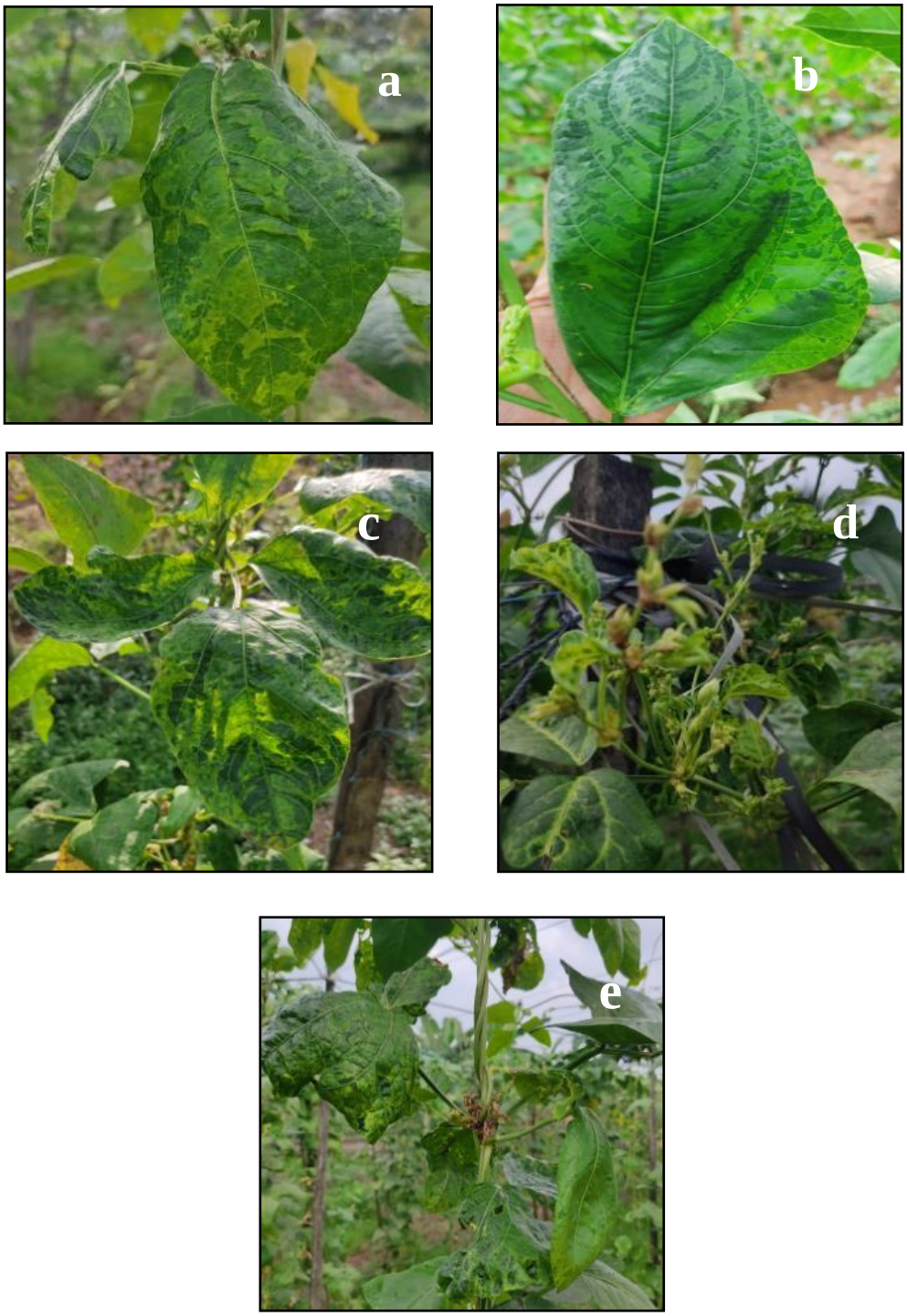

Plate 2. Symptoms of BlCMV infection in cowpea (a) chlorosis (b) Dark green vein banding (c) Mosaic pattern (d) Reduced flower formation (e) Puckered leaves

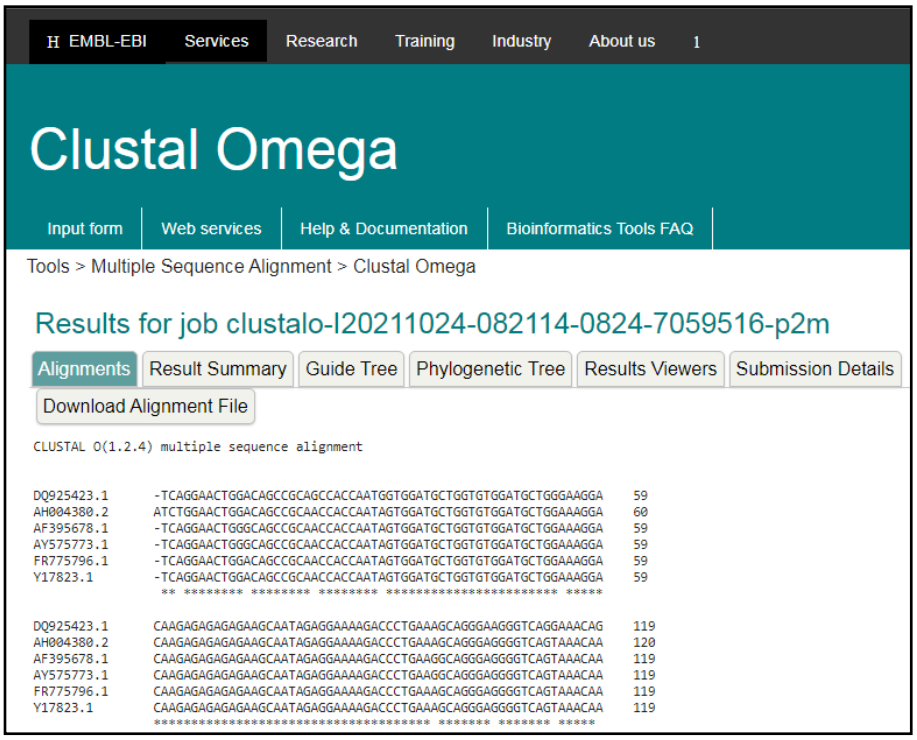

Fig. 1. Output of multiple sequence alignment using Clustal omega

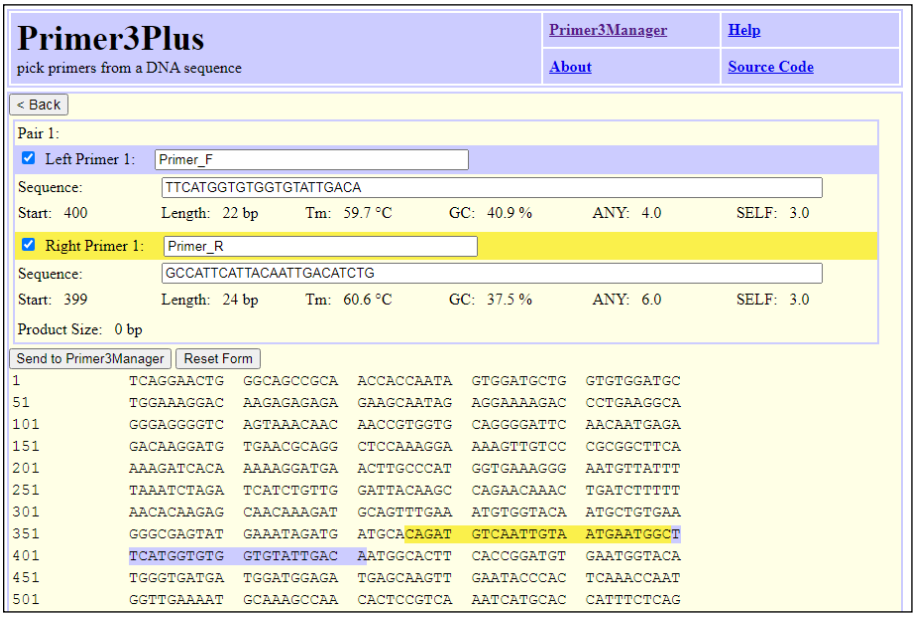

Fig. 2. Output of Primer3Plus

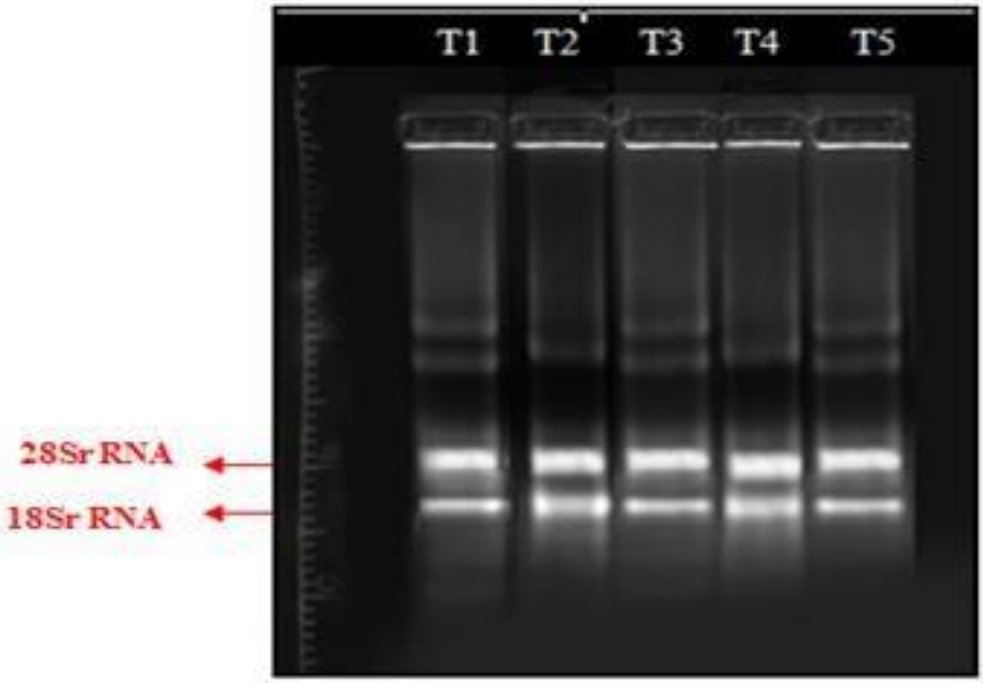

Plate 3. Gel image of isolated RNA samples

Lane 1. Pappanchani Lane 2. Vellanikara Lane 3.Edayazham Lane 4. Meenaghadi Lane 5. Puthucode

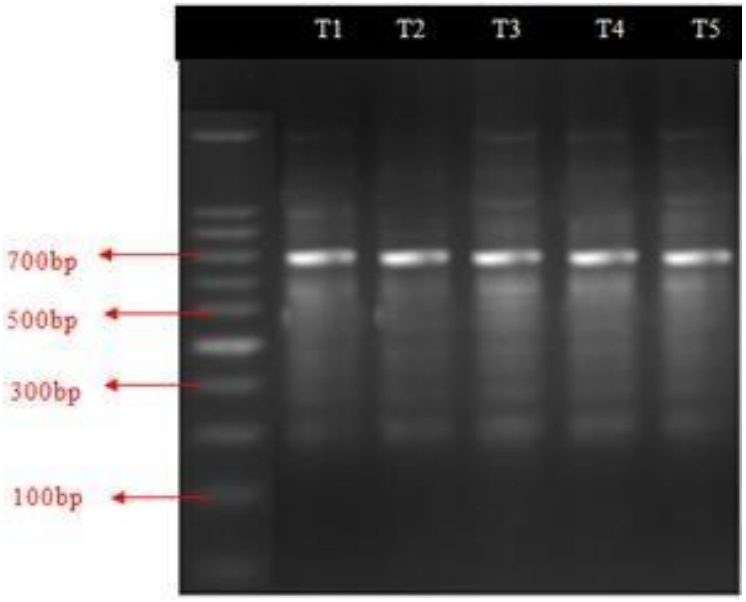

Plate 4. Gel image of PCR with β- actin primers

Lane 1. 100 bp ladder Lane 2.Pappanchani Lane 3. Vellanikara Lane 4. Edayazham Lane 5. Meenaghadi Lane 5. Puthucode

formation, melting temperature and secondary structure formation, GC clamp, a primer pair was selected. The coherence of the selected primers was checked using *in silico* PCR tool, NCBI Primer–BLAST. Finally the selected primer pair is:

Forward Primer: TCAGGAACTGGGCAGCCGC
Reverse Primer: CTCGATCCGATGTTTTGGATG

The forward primer has a length of 19bp, 58.42 per cent GC content and 63.14 melting temperature whereas the reverse primer has a length of 20 bp, 47.62 per cent of GC content and 57.8 melting temperature.

4.4. AMPLIFICATION OF COAT PROTEIN GENE

4.4.1. Quality and quantity assessment of the RNA sample

The overall quality of the RNA sample collected from each agro-ecological zone was confirmed by spectrophotometric readings. The ratio of OD values at 260nm and 280nm were taken (table 7) and all the samples had A_{260} /A_{280} value between 1.8 and 2.0. Therefore, all the isolated samples had good quality RNA. The quantity of isolated RNA was between 2900-3400 ng/µl.

Further the quality of the samples was assessed through agarose gel electrophoresis. All the RNA isolated, appeared on gel as two distinct bands, 18SrRNA and 26SrRNA, ensuring good quality.

Table 7. Spectrophotometric readings of RNA sample

SI.No.	Sample	A_{280}	A_{260}	A_{260}/A_{280} value	DNA Concentration (ng/µl)
1.	Pappanchani (T1)	0.0574	0.1129	1.96	3387
3.	Vellanikara (T2)	0.0629	0.1209	1.92	3627
4.	Edayazham (T3)	0.0549	0.00968	1.76	2904
5.	Meenaghadi (T4)	0.0538	0.0982	1.82	2946
6.	Puthucode (T5)	0.0518	0.1036	2.0	3108

4.4.2. cDNA Synthesis

The synthesis of cDNA was performed using Verso cDNA synthesis kit and it was authenticated by carrying out PCR amplification with house-keeping gene specific primers *i.e.,* β-actin. The PCR product acquired showed an amplicon in expected size range of 708bp (Plate 4).

4.4.3. Amplification of coat protein gene

PCR with primers particularly designed for the coat protein of BlCMV were made use of to amplify the coat protein sequence of the virus. It was followed by 1.5 per cent agarose gel electrophoresis for the specific detection of bands of the virus that

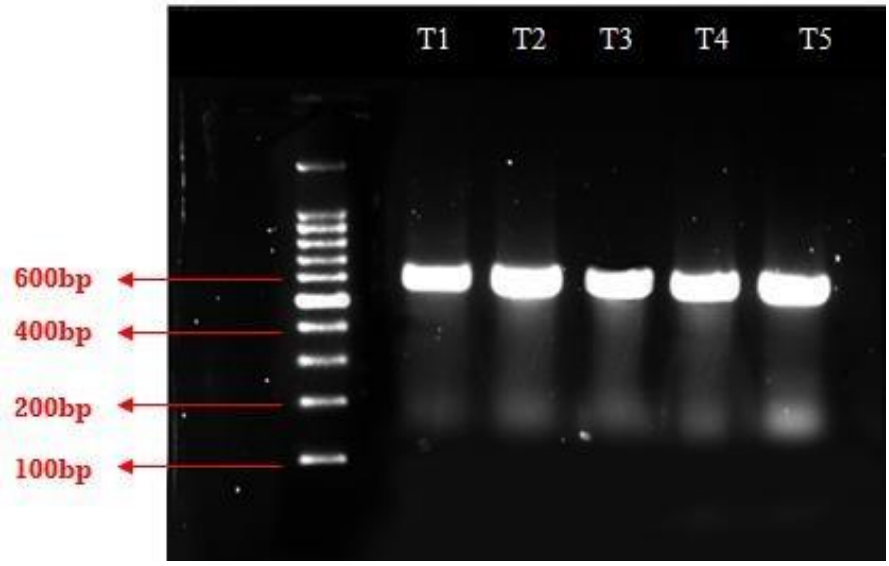

Plate 5. Gel image of cDNA amplified with BlCMV coat protein primers
Lane 1. 100 bp ladder Lane 2.Pappanchani Lane 3. Vellanikara
Lane 4.Edayazham Lane 5. Meenaghadi Lane 5. Puthucode

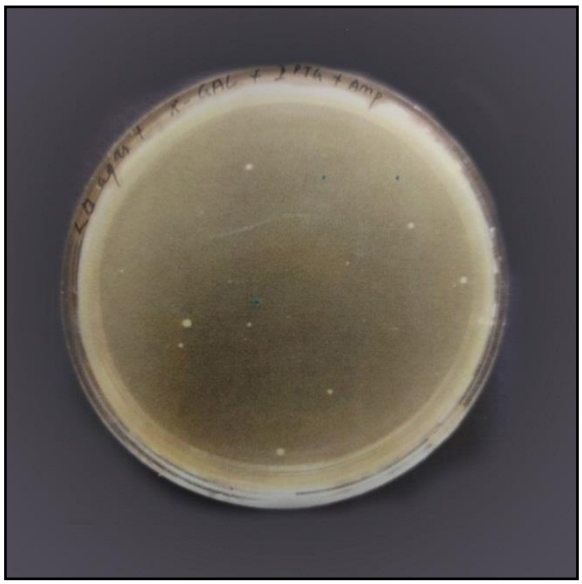

Plate 6. Transformed colonies on LB ampicillin agar plates

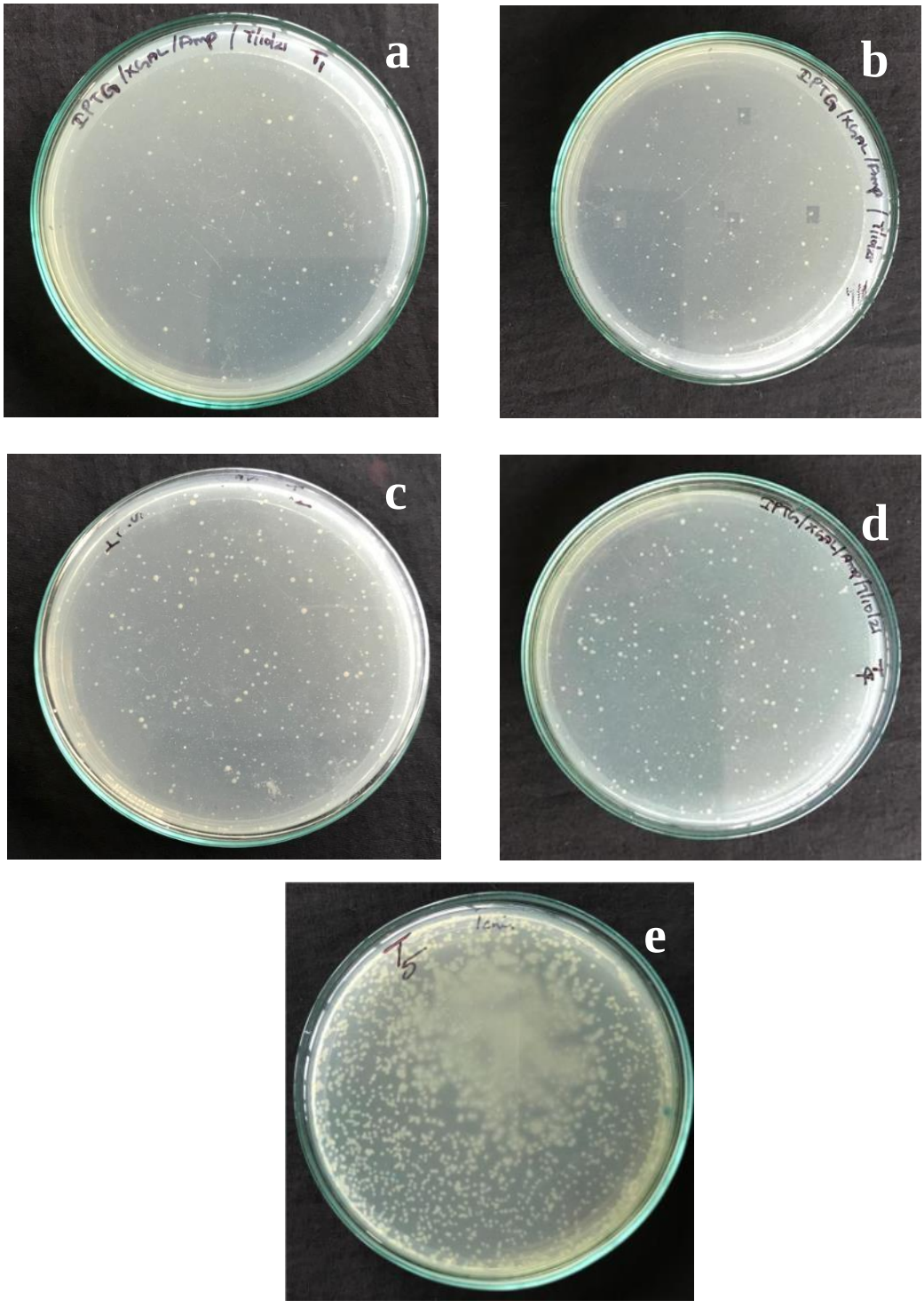

Plate 7. Blue white screening of transformed colonies (a) T1-Trivandrum (b) T2-Thrissur (c) T3-Kottayam (d) T4-Wayanad (e) T5-Palakkad

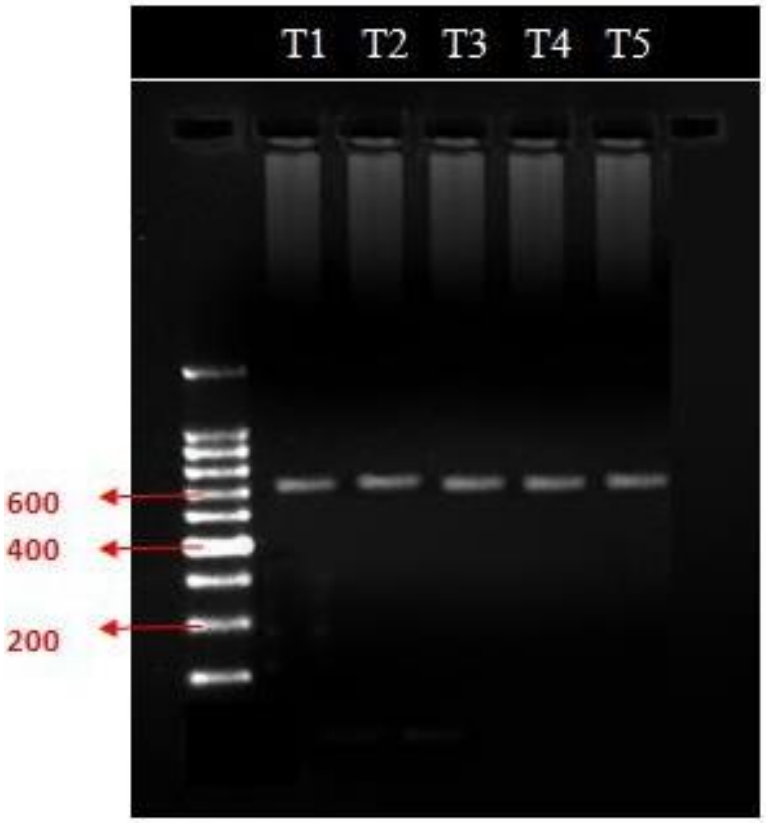

Plate 8. Gel image of the results of colony PCR
Lane 1. 100 bp ladder Lane 2.Pappanchani Lane 3. Vellanikara
Lane 4. Edayazham Lane 5. Meenaghadi Lane 5. Puthucode

4.5.3. Confirmation of recombinant clones using colony PCR

Distinct white colonies from the plates were picked for performing colony PCR. The products obtained after PCR were examined in 1.5 per cent agarose gel electrophoresis and it showed bands in the expected size range (plate 8). In order to confirm that no contaminant colonies appeared, a control plate was also kept.

4.5.4. Plasmid isolation

The plasmid DNA was isolated from the chosen positive colonies after culturing them for overnight at 37 on LB broth. The plasmid DNAs obtained through isolation were then given to Athmic Biotech, Trivandrum for sequencing.

4.5.3 Sequencing

Dideoxy chain termination method of sequencing was performed. The sequences had lengths ranging from 669-674 bp. The identified sequences are given below:

The T1 (Pappanchani, Trivandrum) sample had a length of 674 bp.

>T1_BlCMV_F

TCTGGGTGCTGGCCGGAATACCGAATGCTGGGGAAAGACAAGAGAGAGAGAA
GCAATAGAGGAAAAGACCCTGAAAAGCAGGGTTAAGGGGTCAGGAAACAACA
ATTCCGTGGTGCAGGAGATTCAACAATGAGAGACAAGGATGTGAACGCAGGCT
CCAAAGGAAAGTTGTCCCACGGCTTCAAAAGATCACAATAAAGGATGAACTTG
CCCATGGTGAAGGGGAATGTTATTAATCTAGACCATCTGTTGGATTACAAGCC
AGAACAAACTGATCTCTTTAACACAAGAGCAACAAAGATGCAGTTTGAAATGT
GGTACAATGCTGTGAAGGGCATGAGATAGATGACGCACAGATGTCAATTGTAA
TGAATGGTTTCATGGTGTGGTGTATCGACAATGGCACTTCACCGGATGTGAATG
GTACGTGGGTGATGATGGATGGAGATGAGCAAGTTGAATACACTCAAACCAAT
GGTTGAGAATGCAAAACCAACACTCCGTCAAATCATGCACCATTTCTCAGATG
CAGCTGAAGCATACATTGAGATGAGAAATTCCGAGAAACCGTACATGCCTAGG

TACGGATTACTTCGGTTTGAGGGATAAAAATCTAGCTCGCTACGCTTTTGATTT
CTATGAGGTGACATCCAAAACATCCGGGACCAGAGAAG

The T2 (Vellanikara, Thrissur) sample had a length of 674 bp

>T2_BlCMV_F

TCTGGGTGCTGGCCGGGAATGCTGGGGAAAGACAAGAGAGAGAGAAGCAATA
GAGGAAAAGACCCTGAAAAGCAGGGAGGGGTCAGGAAACAACAACCGTGGTG
CAGGAGATTCAACAATGAGAGACAAGGATGTGAACGCAGGCTCCAAAGGGAA
AGTTGTCCCACGGCTTCAAAAGATCACAAAAAGGATGAACTTGCCCATGGTGA
AGGGGAATGTTATTTTGAATCTAGACCATCTGTTGGATTACAAGCCAGAACAA
ACTGATCTCTTTAACACAAGAGCAACAAAGATGCAGTTTGAAATGTGGTACAA
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CGTGGGTGATGATGGATGGAGATGAGCAAGTTGAATACCCACTCAAACCAATG
GTTGAGAATGCAAAACCAACACTCCGTCAAATCATGCACCATTTCTCAGATGC
AGCTGAAGCATACATTGAGATGAGAAATTCCGAGAAACCGTACATGCCTAGGT
ACGGATTACTTCGGAATTTGAGGGATAAAAATCTAGCTCGCTACGCTTTTGATT
TCTATGAGGTGACATCCAAAACATCCGGGACCAGAGAAG

The T3 (Edayazham, Kottayam) sample had a length of 673 bp.

>T3_BlCMV_F

CCAGGAGCGGGCCGGGAATGCTGGGAAAGAACAAGAAGAGAGAGAAGCAAT
AGAGGAAAAGACCCTGAAAGCAGGGAGGGGTCAGGAAACAACAACCGTGGTG
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AAAGTTGTCCCACGGCTTCAAAAGATCACAAAAAGGATGAACTTGCCCATGGT
GAAGGGGAATGTTATTTTGAATCTAGACCATCTGTTGGATTACAAGCCAGAAC
AAACTGATCTCTTTAACACAAGAGCAACAAAGATGCAGTTTGAAATGTGGTAC
AATGCTGTGAAGGGCGAGTATGAGATAGATGACGCACAGATGTCAATTGTAAT
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ATTTCTATGAGGTGACATCCAAAACATCCGGAACGCGAAGAA

5.5. PHYLOGENETIC ANALYSIS

The sequenced samples from Trivandrum, Thrissur, Kottayam, Wayanad and Palakkad upon BLAST analysis showed their percentage similarity with the reported sequences of Bean common mosaic virus.

T1 (Trivandrum) sample (accession number: OL343665), showed a maximum of 95 per cent similarity with Bean common mosaic virus (BCMV) polyprotein gene. The graphical output of BLAST after comparison with the top hundred BLAST hits was generated. Likewise, T2 (Thrissur) sample (accession number: OL343666) showed up to 96 per cent similarity with the sequence of Bean common mosaic virus.

The graphical output of BLAST after comparison with top hundred reported sequences was produced. T3 (Kottayam) sample (accession number: OL343667) had likeness up to 96 per cent with BCMV when compared with top the 200 BLAST hits. The graphical output of BLAST was generated after comparison with the top hundred reported sequences. T4 (Meenaghadi) sample (accession number: OL343668) showed maximum of 98 per cent resemblance with BCMV as shown in the graphical output of BLAST after comparison with top 197 reported sequences. T5 (Palakkad) sample (accession number: OL343669) showed up to 98 per cent parallelism with the sequence of Bean common

caused mosaic disease in cowpea. It produced an amplicon of 670bp from the cDNA reverse transcribed from isolated viral RNA (Plate 5).

4.5. CLONING AND SEQUENCING

Molecular cloning was performed using pMD 20-T vector, TA cloning method.

4.5.1. Standardization of procedure for competent cell preparation

Initially the *Escherichia coli* strain DH5α (glycerol stock) was revived and made competent using Calcium chloride method. These competent cells were then transformed with pUC 18 vector through heat shock method and the transformed culture was incubated on LB/Amp/IPTG/X-Gal plates. After an overnight incubation 9 transformed white colonies and 3 blue colored colonies appeared on the petri plate (plate 6).

4.5.2. TA Cloning

Cloning was performed using Mighty TA-Cloning kit.

4.5.2.1 Ligation reaction

All the five PCR amplicons obtained on the gel were extracted and eluted using Hiper Gel Extraction Kit Himedia. Each eluted sample was then ligated to the pMD20-T vector in a thermo-cycler at16˚C by incubating the reaction mix including ligation mighty mix, vector and insert for 30 minutes.

4.5.2.2 Transformation

Competent cell colonies transformed with different vector ligated products were obtained on each LB ampicillin plates. Roughly, 100 distinct colonies appeared on all five Amp+ LB agar plates. The transformed *E.coli* cells exhibited white coloured colonies and the non-transformed ones exhibited blue coloured colonies (Plate 7).

AATGGTTTCATGGTGTGGTGTATCGACAATGACACTTCACCGGATGTGAATGGT
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GTTGAGAATGCAAAACCAACACTCCGTCAAATCATGCACCATTTCTCAGATGC
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ACGGATTACTTCGGAATTTGAGGGATAAAAATCTAGCTCGCTACGCTTTTGATT
TCTATGAGGTGACATCCAAAACATCCGGAACGCGAAGAA

The T4 (Meenaghadi, Wayanad) sample had a length of 669 bp.

>T4_BlCMV_F

TTTGTGCTGGCGGTTGGAATGCTGGGAAAGAACAAGAGAGAGAGAAGCAATA
GAGGAAAAGACCCTGAAAGCAGGGAGGGGTCAGGAAACAACAACCGTGGTGC
AGGAGATTCAACAATGAGAGACAAGGATGTGAACGCAGGCTCCAAAGGGAAA
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GCTGTGAAGGGCGAGTATGAGATAGATGACGCACAGATGTCAATTGTAATGAA
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GCTGAAGCATACATTGAGATGAGAAATTCCGAGAAACCGTACATGCCTAGGTA
CGGATTACTTCGGAATTTGAGGGATAAAAATCTAGCTCGCTACGCTTTTGATTT
CTATGAGGTGACATCCAAAACATCGGAATCAAGA

The T5 (Puthucode, Palakkad) sample had a length of 674 bp.

>T5_BlCMV_F

CCAAGGGAGCGGGCCGGGAATGCTGGGAAAGAACAAGAAGAGAGAGAAGCA
ATAGAGGAAAAGACCCTGAAAGCAGGGAGGGGTCAGGAAACAACAACCGTGG
TGCAGGAGATTCAACAATGAGAGACAAGGATGTGAACGCAGGCTCCAAAGGG

mosaic virus. The graphical output was produced after comparison with top hundred reported sequences.

The sequences under study from Trivandrum, Thrissur, Kottayam, Wayanad and Palakkad were compared with other reported sequences from India (West Bengal, Calicut, New Delhi, Coimbatore and Kanpur) and the Neighbour-Joining phylogenetic tree constructed in Mega 6.0 conveyed that the tree could be divided into two clusters. Cluster I included sequences from South India and cluster II included sequences from North India (Fig. 4).

Multiple sequence alignment of the sequences under study was done in clustal omega. It compared all the sequences pairwise and gave the conserved, semi-conserved and non-conserved sequences (fig. 5, fig. 6). '*' indicated the conserved site, '.' designated the non-conserved sites and ':' indicated the semi-conserved region. Finally, neighbour - joining (bootsrap consensus tree) gave a phylogenetic tree with four clusters and they had bootstrap value of 99 per cent (fig. 7). Table 8 shows the sequences that are placed under each cluster.

Cluster I includes isolates from Trivandrum (T1, accession number: OL343665) and Thrissur (T2, accession number: OL343666), Wayanad (T4, accession number: OL343668) and Palakkad (T5, accession number: OL343669). Cluster II included sequences from Kottayam (accession number: OL343667).

Table 8. Coat protein sequences of samples subjected for study grouped based on Dendrogram

Clusters	**Coat protein sequences**
I	T1(Trivandrum), T2 (Thrissur), T4 (Wayanad), T5 (Palakkad),
II	T3 (Kottayam)

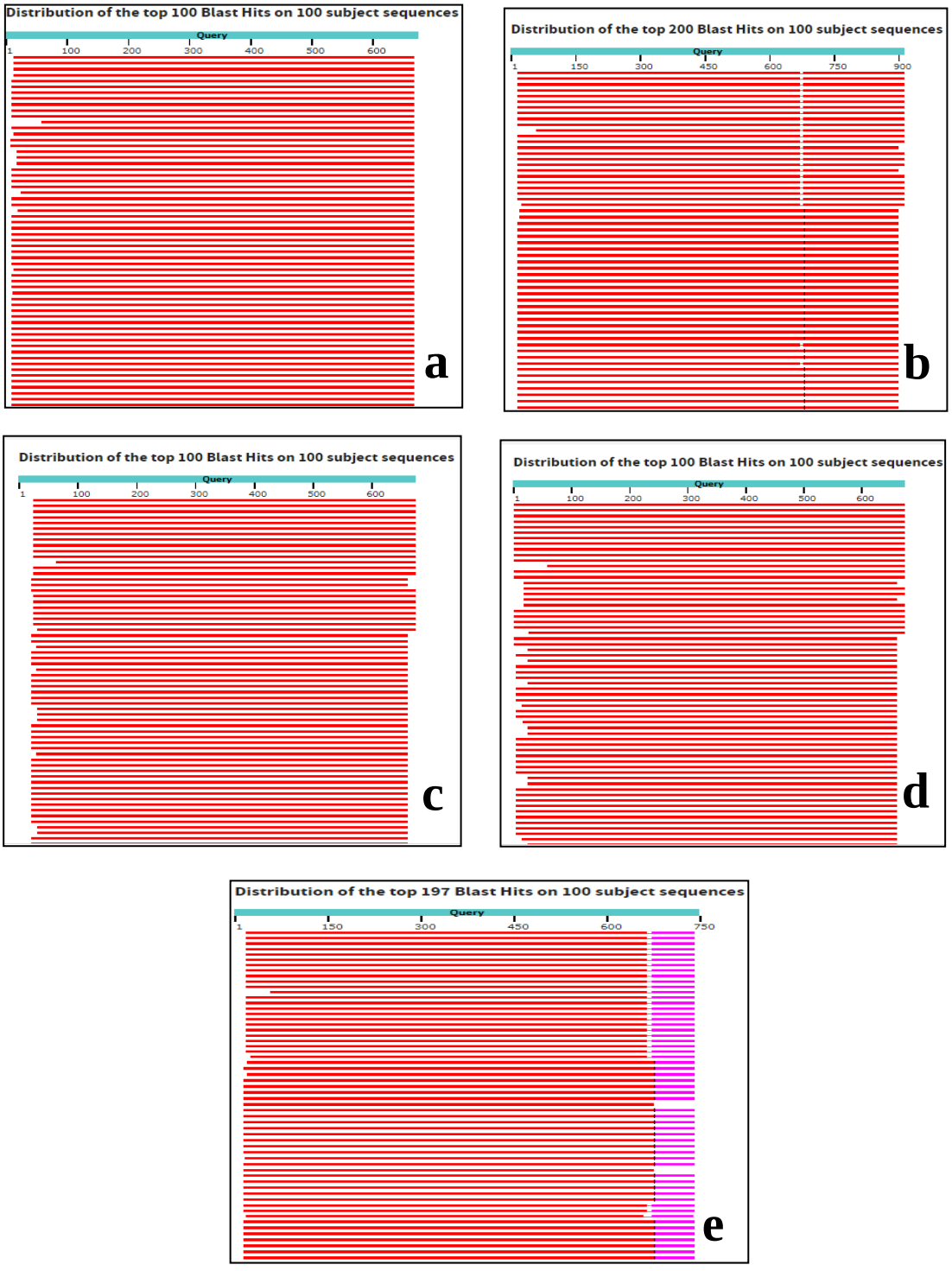

Fig. 3. Graphical output of BLAST analysis (a) T1-Trivandrum (b) T2-Thrissur (c)T3- Kottayam (d) T4-Wayanad (e) T5- Palakkad

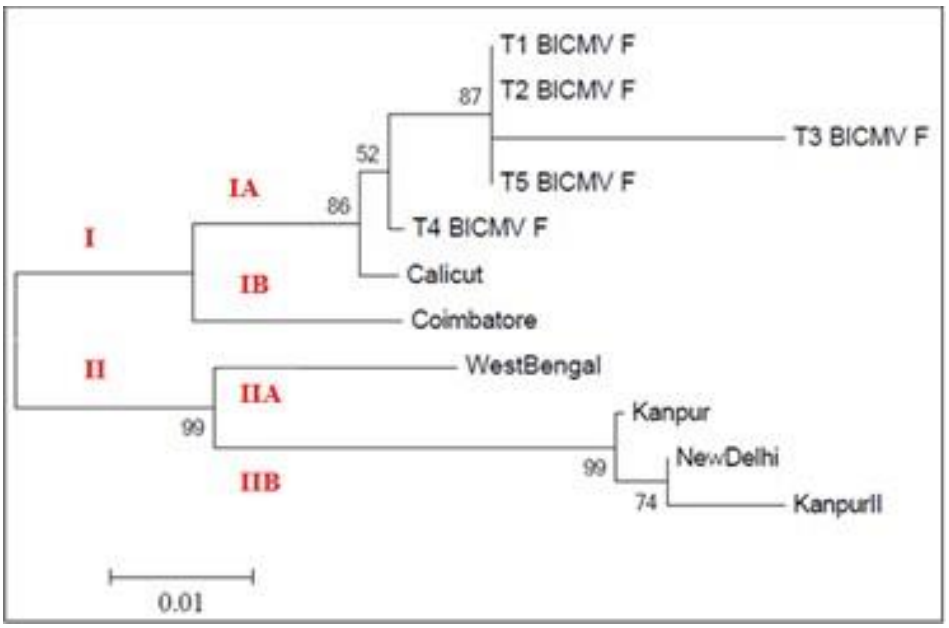

Fig. 4. Phylogenetic tree generated in Mega 6.0 using sequences under study and reported sequences of Bean common mosaic virus in India

Results for job clustalo-I20211027-201721-0539-43892943-p1m

Alignments | Result Summary | Guide Tree | Phylogenetic Tree | Results Viewers | Submission Details

Download Alignment File | Show Colors

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CLUSTAL O(1.2.4) multiple sequence alignment


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1021_490_007_PCR_T5_BLACKEYCOAT_F_C05.ab1      CCAAGGGGCGGGCCGGG------AATGCTGGGAAAGAACAAGAAGAGAGAAGCAATAG     54
1021_490_001_PCR_T1_BLACKEYCOAT_F_A05.ab1      TCTGGGTGCTGGCCGGAATACCGAATGCTGGGGAAAGACAAG-AGAGAGAGAAGCAATAG     59
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1021_490_001_PCR_T3_BLACKEYCOAT_F_A05.ab1      -CCAGGAGCGGGCCGGG------AATGCTGGGAAAGAACAAGAAGAGAGAGAAGCAATAG     53
                                                        *  ***      *********.**..***** ****************


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                                               ************** * ******   *****************  :************


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1021_490_003_PCR_T2_BLACKEYCOAT_F_B05.ab1      GCTTCA-AAAGATCACAAAAAGGATGAACTTGCCCATGGTGAAGGGGAATGTTATTTTGA     227
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                                               **** . *: .:  ..*:************. ********************    *
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Fig. 5. Output of Clustal Omega generated by Multiple Sequence Analysis (MSA) of obtained sequences

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1021_490_001_PCR_T1_BLACKEYCOAT_F_A05.ab1      ATCTAGACCATCTGTTGGATTACAAGCCAGAACAAACTGATCTCTTTAACACAAGAGCAA    295
1021_490_003_PCR_T2_BLACKEYCOAT_F_B05.ab1      ATCTAGACCATCTGTTGGATTACAAGCCAGAACAAACTGATCTCTTTAACACAAGAGCAA    287
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                                               ************************************************************

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1021_490_003_PCR_T2_BLACKEYCOAT_F_B05.ab1      CAAAGATGCAGTTTGAAATGTGGTACAATGCTGTGAAGGGCGAGTATGAGATAGATGACG    347
1021_490_001_PCR_T3_BLACKEYCOAT_F_A05.ab1      CAAAGATGCAGTTTGAAATGTGGTACAATGCTGTGAAGGGCGAGTATGAGATAGATGACG    347
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1021_490_001_PCR_T1_BLACKEYCOAT_F_A05.ab1      CACAGATGTCAATTGTAATGAATGGTTTCATGGTGTGGTGTATCGACAATGGCACTTCAC    411
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Fig. 6. Output of Clustal Omega generated by Multiple Sequence Analysis (MSA) of obtained sequences

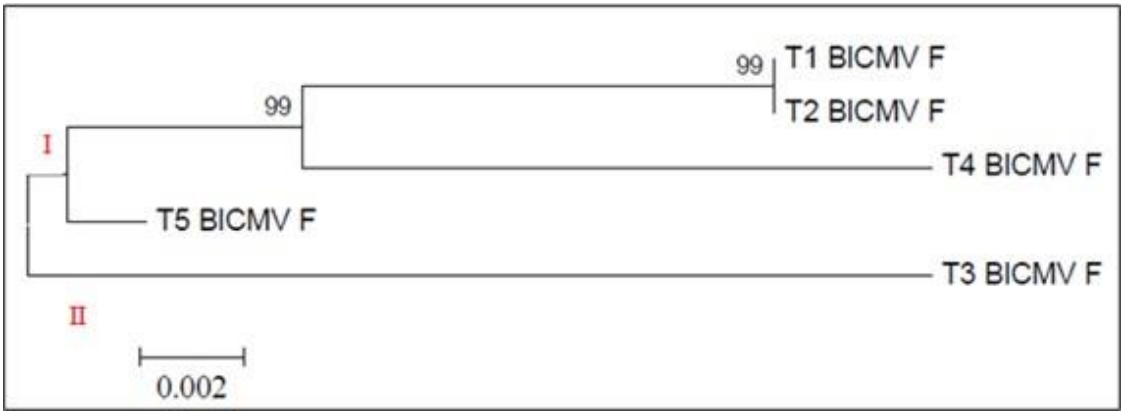

Fig.7. Dendrogram showing the relationship of samples subjected for study constructed using Mega 6.0

DISCUSSION

5. DISCUSSION

The results of the study, Molecular characterisation of Blackeye cowpea mosaic virus collected from the five agro-ecological zones of Kerala, along with their symptomatology and disease incidence from each zone is discussed in this chapter.

Cowpea *(Vigna ungiculata (L.)Walp)* which belongs to the Leguminosae family is a salient dual purpose legume, widely grown in the tropical and subtropical regions. They are prone to the attack of various viruses especially,the *Potyvirus*genus, one of the most devastating genera of plant viruses. They are single stranded, positive sense RNA as their genetic material. The *Potyvirus* taken here for the study is *Blackeye cowpea mosaic virus* (BlCMV) which causes mosaic diseases in cowpea, owing to their widespread occurrence among the cultivars in Kerala.

Taking into consideration the losses caused by the *Blackeye cowpea mosaic virus,* molecular characterisation was performed to clone and sequence the coat protein gene of Blackeye cowpea mosaic virus causing mosaic disease in cowpea (Vigna ungiculata (L.) Walp.) in Kerala and to analyse its sequence.

5.1 DISEASE INCIDENCE

The collection of BlCMV infected cowpea samples was conducted during the months of January-March, 2020 in various districts under each agro-ecological zones of Kerala *viz.*, Trivandrum (Coastal Plain), Kottayam (Foot hills), Thrissur (Midland plain), Wayanad (High hills) and Palakkad (Palakkad plain). *Potyvirus* infection is recognised as a major disease in all regions.

Disease incidence varied widely in the range of 30 per cent–55 per cent, from location to location with a higher percentage shown in Trivandrum isolate and lowest in Kottayam. This percentage of incidence was in accordance with the reports of Pavithra (2013), who suggested a percentage ranging from 1.6 per cent- 60 per cent. Mangeni *et al.*,

(2019) had adopted similar method of disease incidence wherein incidence of disease from different locations of Africa was scored based on the presence and absence of symptoms. He covered areas that are as low as 1,164m above the sea level (low lands) to areas as high as 1600m above the mean sea level (high land).

The highest percentage of incidence recorded was 47.6 per cent which is similar to the observation made. The percentage of disease incidence from each zone was depicted in graphical format taking percentage of incidence along the X-axis (fig. 8).Udayshankar *et al.,* (2009) has stated that the transmission of BCMV-BlCMV in cowpea is as high as 39.9 per cent.

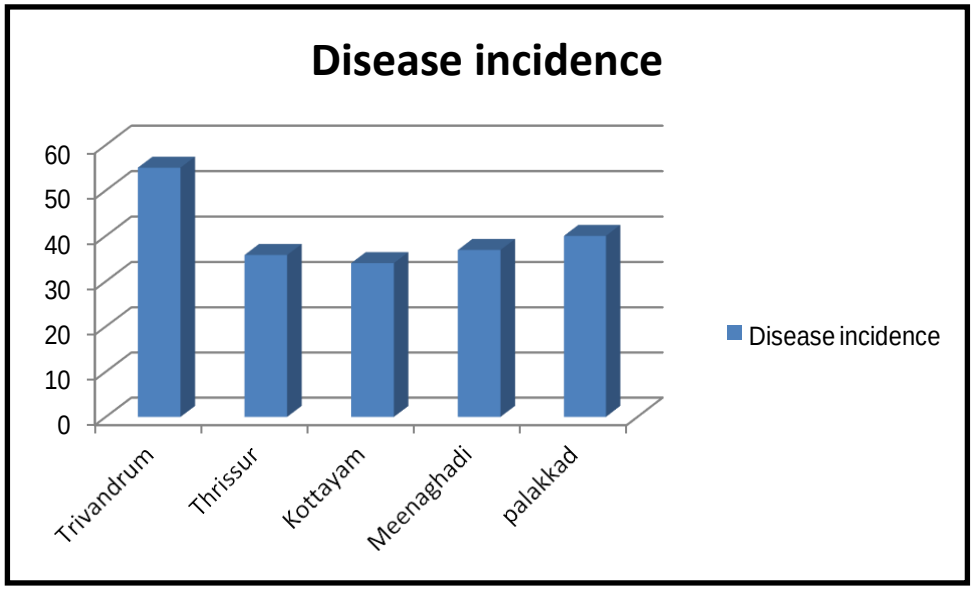

Fig. 8. Graph showing the percentage of disease incidence

5.2. SYMPTOMATOLOGY

Symptomatology of the BlCMV was studied in the surveyed locations. The plants produced various symptoms viz., dark green vein banding, chlorosis, severe and distinct islands of mosaic patterns, reduced flowering and pod formation, leaf deformation, and puckering of leaves.Similar symptoms like mosaic pattern, blistering, distortion of leaves and stunting of the plants were reported by Radhika (1999) as characteristic symptoms of BlCMV infection.

Fajinmi, A., (2019) reported that BlCMV causes vein clearing, mosaic and cholorosis during the early stages of infection and as the infection prolongs it causes severe mosaic patterns, change in texture of the leaf (dullness), leaf stunning and halted stem extension.

Ogunsola *et al.,* (2020) reported that BCMV-BlCMV infection produces systemic foliar symptoms of mosaic, and mottling on susceptible lines.Reduced leaf size with deformation, mottling, and leaf stunning were also observed.

The symptoms recorded in this study were in-line with reports of Alex (2017), who also detailed the symptoms like malformed leaves and reduced flowering in infected plants.

In 2003, Hao *et al.* has also described the various symptoms of BlCMV infection in cowpea including vein banding, stunting of plants, vein clearing, chlorosis, malformation of leaves and downward curling.

5.3. PRIMER SYNTHESIS

Primers are significant compounds that ascertain the success of a PCR reaction (Dieffenbach *et al.,* 1993). Primer was constructed from the conserved sequences of the coat protein gene.

The conserved sequences were identified using multiple sequence alignment programmes such as clustal omega as detailed in the works of Sibhatu, 2003. Based on this data, forward and reverse primers of the coat protein gene were designed using the

softwarePrimer3Plus. It's a web interface available at http://www.bioinformatics.nl/primer 3plus. To know about the specificity of primers, an *in silico* PCR tool, NCBI primer BLAST, was made use. It's a global alignment algorithm to figure out primers against the PCR template (in order to avoid non- specific amplifications through primer dimer formation).

Etebu (2013) had recommended BLAST analysis to ensure the specificity and quality of primer. The forward and reverse primers were 19bp and 20bp long respectively and were estimated to produce an amplicon off 670bp long.

5.4 AMPLIFICATION OF COAT PROTEIN GENE

In this study, RNA was isolated from the infected cowpea leaves collected from different agro ecological zones. The isolation was performed using TriZol reagent (Konate *et al.,* 2017) and the quality was analyzed in 2 per cent agarose gel.

Desbeiz *et al*., (2004) isolated RNA from the Watermelon mosaic virus, a strain that belongs to *Potyviridae* family with TriZol reagent and she has reported TriZol procedure as an efficient method to obtain high quality RNA from the virus. Trizol reagent is a monophasic solution of phenol and guanidinium isothiocyanate that has the capacity to simultaneously denature the protein as well as solubilize the biological material while maintaining the RNA integrity. The chloroform addition helps in separating the solution into an aqueous phase and an organic phase. RNA will be present in the aqueous phase and it can be precipitated with isopropanol. Isolated RNA appeared as two distinct bands (28S and 18S rRNA) as described in the works of Boxtel *et al.,* (1999).The bands observed showed a rough intensity of 2:1 ratio.

The clarity and concentration of samples were analyzed through spectrophotometric readings and it showed that the A_{260} /A_{280} values were between 1.8 and 2.0 and the concentration was in between 2900-3400 ng/µl. This implied that the isolated samples were suitable for cDNA synthesis.

cDNA synthesis was performed using Verso cDNA synthesis kit. A similar preparation method of cDNA was performed by Karanja *et al.,* (2017). It contained RNA dependent DNA polymerase enzyme called Reverse transcriptase which utilizes the total RNA as a template to produce the cDNA.

The synthesized cDNA, was authenticated using reference gene called β-actin as described in the works of Amorim *et al.,* (2018). All the samples produced a distinct band at 700 bp region. This indicates that all the RNA samples have been efficiently synthesized to cDNA.

The resulting cDNAs were amplified using specifically designed coat protein primers in a total PCR volume of 20 µL. The results of agarose gel electrophoresis showed that all the amplicons nearly had the same size range of 670 bp. Similar data was obtained by Ouattara, (1991) who also reported that, PCR is extremely sensitive in the detection of BCMV in infected tissues.

Amplified PCR product was extracted from the gel and used as a template for the nucleotide sequence determination.

5.5. CLONING AND SEQUENCING

In the present work, cloning was done using pMD 20 T-vector, TA cloning method. The PCR-product obtained using Taq DNA polymerase has a single base deoxyribo adenosine (dA), added at the 3’ end due to the non-template dependant activity of the enzyme.

The TA cloning method utilises a T-vector which has single 3’-T overhangs on both ends. This allows direct cloning of PCR products, by means of complementarity. The 3’A overhang of the PCR product and 3’ T-overhangs of the vector combines directly (Wang *et. al.,* 2009). This method enables high-efficiency ligations.

The pMD 20-T, used in this study is a linearized vector having 2736 bp. They possess LacZ gene, Ampicillin resistance gene, and an ori region. The multiple cloning

sites in the LacZ gene have been removed from this vector, but its beta-galactosidase activity is uninterrupted. Therefore, transformant colonies containing recombinant plasmids can be recognized through blue-white screening technique. They have single 3'T overhanging's at each ends that enables quick and easy ligation. Another advantage in using this vector is that they can take up small DNA fragments that allow uncomplicated ligation and introduction into host cells (Zhang *et al.,* 2012).

The recombinant vectors after the ligation reaction were transformed using the most commonly used host strain, *E.Coli* DH5α after making them competent using calcium chloride via heat shock method (Sambrook *et al.,* 1989). Out of the handful of microbes used as host strains, *E. Coli* seem to be an efficient system to support genes from various different phyla (Hernandez-Leon, *et al*., 2010). They are highly manageable and have high transformation efficiency.

Blue-white screening technique was used for the selection of the transformants by inoculating the transformed cells onto LB agar plates containing significant concentrations of ampicillin, IPTG, and X-GAL (Khan *et al*., 1993).

The white coloured colonies serve as a harbinger of transformed cells with insert whereas blue coloured colonies indicates transformed cells without the insert. Most of the colonies on the plate appeared white, compared to very few blue coloured colonies. This indicated that the transformation efficiency was higher. White colonies were picked up and grown in LB broth to isolate the plasmid.

The PCR product of 669 bp - 675 bp was sequenced through Sanger's dideoxy chain termination method that gave results in the form of chromatogram wherein red colour indicated thymine (T), Adenine (A) by green, Cytosine (C) by blue and Guanine (G) by yellow colour. A similar method of sequencing was carried out by Boxtel *et al.,* (2000) in his work on the phylogenetic analysis of two *potyvirus* strains that affected cowpea.

5.6.PHYLOGENETIC ANALYSIS

The sequenced fragment was analysed using Basic Local Alignment Search Tool (BLAST) software, a computer algorithm available at National centre for Biotechnology Information (NCBI) to rapidly align and compare the query sequence with the database sequences. Sequences from the five virus isolates were compared with reported strains of BCMV and also with each other which happened to show 95-99 per cent.

Sharma *et al.*, (2015) in their work in Bean common mosaic virus attacking *Phaseolus Vulgaris* showed that the coat protein sequence of 819bp shared 83-99 per cent sequence identities with reported strains.

Multiple sequence alignment of the coat protein nucleotide, of reference BCMV and BCMV- BlCMV isolates obtained was performed in Mega 6.0. software and the phylogenetic tree construction was carried out based on Neighbour-joining method, a distance based procedure. A similar kind of comparison was made by Moradi and Mehvar (2019). It makes use of clustering approach to construct the tree.

The branching of the corresponding eleven sequences from India gave two major clusters with each of them being separated into two different sub-clusters. Cluster I, II included sequences from South India and North India respectively.

The sub-cluster IA contained sequences from Kerala only and sub-cluster IB appended Coimbatore sequence. Sub-cluster IIA encompassed sequence from West Bengal and sub-cluster IIB incorporated sequences from Kanpur, and New Delhi.

The robustness of the internal branches from the resulting tree was statistically tested by bootstrap analysis from 1000 bootstrap replications. The observations were in accordance with the statements of Udayashankar *et al.*, (2012).

In-line with the present findings, Krishnapriya *et al.,* (2015) isolated a 110 bp of coat protein region isolated from the viral RNA of the infected leaf sample showed 97 per cent homology with the Bean common mosaic virus strain Blackeye from Karnataka on comparative nucleotide sequence alignment with NCBI.

Phylogenetic tree between the identified sequences of coat protein, showed 99 per cent similarity with each other. This shows considerable amount of homology. A similar phylogenetic tree of partially characterized polyprotein gene of BCMV-BlCMV was constructed by Pavithra (2013) using clustal X which resulted in 95-97 per cent similarity.

It was found that the partially characterized coat protein gene of BlCMV isolated from different ecological zones of Kerala was strongly related with each other.

The partially characterized nucleotide sequences of BlCMV from Thiruvananthapuram, Thrissur, Kottayam, Wayanad and Palakkad have been submitted to EMBL/GenBank nucleotide sequence database and have been assigned the following accession numbers OL343665, OL343666, OL343667, OL343668 and OL343669 respectively.

On the basis of results obtained, the present study concludes that the coat protein sequence of the *Blackeye cowpea mosaic virus*, isolated from five agro-ecological zones of Kerala namely Thrissur (midland plain), Thiruvananthapuram (coastal plain), Kottayam (foothill zone), Wayand (high hill zone) and Palakkad (Palakkad plain) had 94 to 98 per cent homology to the reported sequences of BCMV.

This also indicates that the coat protein gene of the *Blackeye cowpea mosaic virus* is a conserved site. This could form the basis of future lines of studies in Lateral flow Immuno Assay (LFIA) for the rapid and early detection of the virus at field levels.

SUMMARY

6. SUMMARY

The *Potyvirus*, *Blackeye cowpea mosaic virus* causing mosaic disease in cowpea was selected for the study on account of their extensive occurrence among the cultivars in Kerala. The present research work entitled 'Molecular characterization of *Blackeye cowpea mosaic virus* causing mosaic disease in cowpea (*Vigna unguiculata* (*L.*)Walp) in Kerala' was carried out in Department of Plant Biotechnology and Department of Plant Pathology, College of Agriculture, Vellayani, during the year 2020-2021, with the objectives to clone and sequence the coat protein gene of *Blackeye Cowpea mosaic virus* causing mosaic in cowpea (*Vigna unguiculata* (*L.*)Walp.) in Kerala and to analyse its sequence.

Samples were collected from the five agro-ecological zones of the state namely Pappanchani, Thiruvananthapuram (Coastal zone), Puthucode (Palakkad plain), Meenaghadi, Wayanad (High hill zone), Vellanikkara, Thrissur (Midland plain) and Edayazham, Kottayam(Foothill zone) after consultation with the extension workers of Vegetable and Fruit Promotion Council Keralam (VFPCK) of the respective district..

From each district, a farm field consisting of a large plot under cowpea cultivation was randomly chosen for study. Plots were surveyed and disease incidence was evaluated. Highest percentage of disease incidence was recorded from Pappanchani (Thiruvananthapuram) (55 per cent).

The burgeoning field symptoms associated with the disease included severe mosaic patterns, dark green vein banding, puckering of leaves and interveinal chlorosis. Leaf distortion and reduction in leaf size were also observed. In Thiruvananthapuram, infected plants exhibited reduced flower formation and stunted growth.

Primers of coat protein gene were designed based on the conserved sequences reported from India, retrieved from NCBI. Primer designing was accomplished using Primer3plus tool and its coherence was analysed using Oligo and Primer Express software.

Later, primer specificity was probed using NCBI Primer BLAST. After passing all the quality yardsticks, pair primers were chosen. The forward primer (TCAGGAACTGGGCAGCCGC) had a length of 19 bp, 58.42 per cent guanine-cytosine (GC) content and 53.14 melting temperature whereas the reverse primer (CTCGATCCGATGTTTTGGATG) had a length of 20 bp, 47.62 per cent of GC content and 57.8 melting temperature.

RNA was isolated from the infectious leaves of cowpea via Trizol reagent method. Quality and quantity of the RNA samples were analysed through spectrophotometric readings which gave an absorbance value of 1.8 to 2.0 for all the samples. The concentration of the samples was between 2900-3400 $ng\mu l^{-1}$. Agarose gel electrophoresis resulted in two clear distinct bands that correspond to 28SrRNA and 18SrRNA.

The cDNA synthesised, was authenticated by house-keeping gene, β-actin, which produced a band of 708bp in 1.5 per cent agarose gel electrophoresis.

cDNA was amplified with designed coat protein specific primers that produced an amplicon of 670 bp. Amplicon was eluted from the gel, ligated with pMD20-T vector and cloned to competent *E.coli DH5α* using Mighty TA cloning kit. Transformants were screened through blue white screening technique on LB ampicillin/X-GAL/IPTG plates wherein recombinant transformed cells appeared as distinct white colonies and non-recombinant cells showed themselves in blue colour. Colony PCR of the transformed colonies that gave band in the expected size range were chosen for plasmid isolation and sequencing. The resultant sequences from Thiruvananthapuram, Thrissur, Kottayam, Wayanad, and Palakkad samples had a length of 674bp, 674bp, 673bp, 669bp and 674bp respectively.

Comparative nucleotide sequence alignment of the virus with available databases from National Centre for Biotechnological Information (NCBI) revealed Thiruvananthapuram isolate to have 95 per cent homology, Thrissur isolate to have 97 per cent, Kottayam isolate to have 96 per cent, Wayanad isolate to have 98 per cent and

Palakkad isolate to have 98 per cent homology with *Bean common mosaic virus* (BCMV) strain.

The sequences deposited in GenBank obtained accession numbers such as OL343665, OL343666, OL343667, OL343668, OL343669 for virus isolate from Thiruvananthapuram, Thrissur, Kottayam, Wayanad and Palakkad sample respectively.

Phylogenetic tree constructed with sequences under study, four reported sequences from North India and two reported sequences from South India got categorised into two clusters viz., North India and South India. The obtained sequences were classified under the cluster of South India and subcluster of Kerala at 99 per cent similarity.

Comparative sequence analysis and phylogenetic tree constructed with sequences under study alone formed into two clusters and had 99 per cent homolgy. The Trivandrum isolate and Thrissur isolate were more similar to each other when compared with sequences from other zones.

The present study revealed that the partially characterized coat protein sequence of the *Blackeye cowpea mosaic virus*, isolated from five agro-ecological zones of Kerala namely Thrissur, Thiruvananthapuram, Kottayam, Wayand and Palakkad had 99 per cent homology to the reported sequences of BCMV. This also indicates that the coat protein gene of the *Blackeye cowpea mosaic virus* is a conserved site. In addition to that, the present work could form the basis of future lines of studies in Lateral flow Immuno Assay (LFIA) for the rapid and early detection of the virus at field levels.

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APPENDIX

APPENDIX I

Gel loading dye

- Bromophenol blue 25 mg
- Xylene cyanol FF 25mg
- Glycerol 3.3ml
- 0.1% DEPC Treated Distilled water 6.7ml

APPENDIX II

1X TBE (Tris Borate EDTA) :1000 ml

- Tris base 27g
- Boric acid 13.75g
- 0.5M EDTA (pH 8.0) 10ml
- Final volume made up to 500ml with autoclaved distilledwater. From 5X TBE, 1X was prepared before use.
- **TBE Buffer (1X)**
- TBE Buffer (5X) 200ml
- Distilled water 800ml

APPENDIX III

LB/Amp/IPTG/X-GAL plate : 100 ml

- LB Broth 2.5 g
- Agar 1.5 g
- IPTG (20 mg/ml) 40 µl
- X-GAL (60 mg/ml) 50 µl
- Ampicillin (60 mg/ml) 100 µl

APPENDIX IV

Solution I – Resuspension Buffer

- Tris – HCl 25 mM
- Glucose 50 mM
- EDTA 10 mM

APPENDIX V

Solution II- Denaturing solution

- NaOH 0.2 N
- SDS 1%

APPENDIX VI

Solution III – Renaturing solution

- Pottasium acetate (5M) 120 ml
- Glacial acetic acid 23 ml
- Distilled water 57 ml

ABSTRACT

MOLECULAR CHARACTERIZATION OF BLACKEYE COWPEA MOSAIC VIRUS CAUSING MOSAIC DISEASE IN COWPEA (*Vigna ungiculata* (L.) Walp) IN KERALA

By

TANIA MATHEW PAMPACKAL

(2016-09-027)

Abstract of Thesis

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2021

9. ABSTRACT

The present research work entitled ‘Molecular characterization of *Blackeye cowpea mosaic virus* causing mosaic disease in cowpea (*Vigna unguiculata* (*L.*)Walp) in Kerala’ was carried out in Department of Plant Biotechnology and Department of Plant Pathology, College of Agriculture, Vellayani, during the year 2020-2021, with the objectives to clone and sequence the coat protein gene of *Blackeye Cowpea mosaic virus* causing mosaic in cowpea (*Vigna unguiculata* (*L.*)Walp.) in Kerala and to analyse its sequence.

Samples collected from five agro-ecological zones of the state namely Pappanchani, Thiruvananthapuram (Coastal zone), Puthucode (Palakkad plain), Meenaghadi, Wayanad (High hill zone), Vellanikkara, Thrissur (Midland plain) and Edayazham, Kottayam(Foothill zone) showed severe mosaic patterns, dark green vein banding, puckering of leaves and interveinal chlorosis. Plots were surveyed and infected leaves of cowpea were collected. Highest disease incidence was recorded from Pappanchani (Thiruvananthapuram) (55 per cent).

Primers of coat protein gene were designed based on the conserved sequences reported from India using Primer3plus tool and the coherence was analysed using Oligo and Primer Express software. The forward primer (TCAGGAACTGGGCAGCCGC) had a length of 19bp whereas the reverse primer (CTCGATCCGATGTTTTGGATG) had a length of 20 bp.

Quality and quantity of the RNA samples isolated from infected leaves were analysed, which gave an absorbance value of 1.8 to 2.0. The concentrations of the samples were between 2900-3400 $ng\mu l^{-1}$. cDNA amplified with designed coat protein specific primers produced an amplicon of 670bp. Amplicon was eluted from the gel, ligated with pMD20-T vector and cloned to competent *E.coli DH5α.* Transformants were screened through blue white screening technique on LB ampicillin/X-GAL/IPTG plates wherein recombinant transformed cells appeared as distinct white colonies. Colony PCR of the transformed colonies that gave band in the expected size range were chosen for plasmid

isolation and sequencing. The resultant sequences from Thiruvananthapuram, Thrissur, Kottayam, Wayanad, and Palakkad sample had a length of 674bp, 674bp, 673bp, 669bp and 674bp respectively.

BLAST analysis of resultant sequences showed that Thiruvananthapuram (95 per cent), Thrissur (97percent), Kottayam (96per cent), Wayanad (98per cent) and Palakkad virus isolate (98per cent) had similarity with other reported *Bean common mosaic virus* (BCMV) sequences. The sequences deposited in GenBank obtained accession numbers such as OL343665, OL343666, OL343667, OL343668, OL343669 for virus isolate from Thiruvananthapuram, Thrissur, Kottayam, Wayanad and Palakkad sample.

Phylogenetic tree constructed using resultant sequences, four reported sequences from North India and two reported sequences from South India got categorised into two clusters viz., North India and South India. The obtained sequences were classified under the cluster of South India and subcluster of Kerala at 99 per cent similarity. Phylogenetic tree constructed with only isolated sequences formed into two clusters at a similarity of 99 per cent.

To conclude, the result of the study indicates that the coat protein sequence of the Blackeye cowpea mosaic virus, isolated from five agro-ecological zones of Kerala, had 99 per cent similarity to the reported sequences of BCMV.