MOLECULAR ANALYSIS OF PHYLOGEOGRAPHY OF CASSAVA MOSAIC DISEASE

by

JAYAKRISHNAN J. T.

(2011-09-102)

THESIS

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DEPARTMENT OF PLANT BIOTECHNOLOGY

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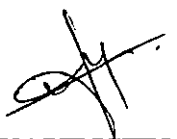
2016

DECLARATION

I hereby declare that the thesis entitled **"MOLECULAR ANALYSIS OF PHYLOGEOGRAPHY OF CASSAVA MOSAIC DISEASE"** is a bonafide record of research done by me and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.



Place: Vellayani
Date: 5/12/16

JAYAKRISHNAN J. T.
(2011-09-102)

भा कृ अनु प-केन्द्रीय कंद फसल अनुसंधान संस्थान
(भारतीय कृषि अनुसंधान परिषद, कृषि और किसान कल्याण मंत्रालय, भारत सरकार)
श्रीकार्यम, तिरुवनन्तपुरम - 695 017, केरल, भारत

ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE
(Indian Council of Agricultural Research, Ministry of Agriculture and Farmers Welfare, Govt. of India)
Sreekariyam, Thiruvananthapuram-695 017, Kerala, India

ISO 9001:2008

Dr.T.MAKESHKUMAR
Principal Scientist
makeshkumar.t@icar.gov.in ; makeshctcri@gmail.com ; (M) 9447158546

21th November 2016

CERTIFICATE

Certified that this thesis, entitled **"Molecular analysis of phylogeography of cassava mosaic disease"** is a record of research work done independently by **Mr. Jayakrishnan J. T.** (Admission No **2011-09-102**) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

21/11/16

Dr. T. Makeshkumar
(Major Advisor, Advisory Committee)
Principal Scientist
Division of Crop Protection
Central Tuber Crops Research Institute
Sreekariyam
Thiruvananthapuram – 695 017

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Jayakrishnan J. T. (2011-09-102)**, a candidate for the degree of **Master of Science (Integrated) in Biotechnology**, agree that the thesis entitled **"MOLECULAR ANALYSIS OF PHYLOGEOGRAPHY OF CASSAVA MOSAIC DISEASE"** may be submitted by Mr. Jayakrishnan. J. T, in partial fulfillment of the requirement for the degree.

Dr. T. Makeshkumar
(Major Advisor, Advisory committee)
Principal Scientist
Division of Crop Protection
ICAR-Central Tuber Crops Research Institute, Sreekariyam
Thiruvananthapuram- 695 017

Dr. B. R. Reghunath
Dean and Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram- 695 522

Dr. J. Sreekumar
Principal Scientist
Division of Social Sciences
ICAR-Central Tuber Crops Research Institute, Sreekariyam,
Thiruvananthapuram- 695 017

Dr. K. Umamaheswaran
Associate Director (RARS (SR)) and Professor
Department of Plant Pathology
College of Agriculture, Vellayani
Thiruvananthapuram-695 522

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JAYAKRISHNAN J. T.

DEDICATED TO MY PARENTS

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
3'	Three prime
5'	Five prime
A	Adenine
A_{405}	Absorbance at 405 nanometer
ACMBFV	African cassava mosaic Burkino Faso virus
ACMV	African cassava mosaic virus
ALP	Alkaline phosphatase
~	Approximate
AS	Average symptom severity
AYVV	Ageratum yellow vein virus
BCTV	Beet curly top virus
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CBB	Cassava bacterial blight
CGM	Cassava green mite
cm	Centimetre
cm^2	Centimeter square
CMG	Cassava mosaic geminivirus
CMMGV	Cassava mosaic Madagascar virus
CP	Coat protein
CR	Common Region
CTAB	Cetyl trimethyl ammonium bromide

CTCRI	Central Tuber Crops Research Institute
DIBA	Dot Immuno Binding Assay
DNA	Deoxyribo nucleic acid
DNA ß	DNA beta
dNTPs	Deoxy nucleotide tri phosphates
dsDNA	Double stranded DNA
DSMZ	Deutsche Sammlung Von Mikroorganismen und Zelkulturen GmbH
E. coli	*Escherichia coli*
EACMCV	East African cassava mosaic Cameroon virus
EACMKV	East African cassava mosaic Kenyan virus
EACMMV	East African cassava mosaic Malawi virus
EACMV	East African cassava mosaic virus
EACMZV	East African cassava mosaic Zanzibar virus
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
F	Forward primer
FAOSTAT	Food and Agriculture Organization Statistical Database
g	Gram
h	Hour
ha	Hectare
ICMV	Indian cassava mosaic virus
IgG	Immunoglobulin
kb	Kilobases
kDa	Kilo Dalton
kg	Kilogram
L	Litre

m	Meter
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
MP	Movement Protein
NASH	Nucleic Acid Spot Hybridisation
NCBI	National Centre for Biological Information
NCM	Nitrocellulose membrane
nm	Nanometer
NSP	Nuclear Shuttle Protein
nts	Nucleotides
°C	Degree Celsius
OD_{405}	Optical density at 405 nanometer
ORF	Open Reading Frames
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
p*M*	Picomolar
R	Reverse primer
RCA	Rolling circle amplification
RCR	Rolling Circle Replication
Rep	Replicase gene
RF	Replication Factor
RNAi	RNA interference
RNase	Ribonuclease
Rpm	Revolutions per minute

RTC	Root and Tuber crops
SACMV	South African cassava mosaic virus
SCR	Satellite Conserved Region
SDM	Spray dried milk
Sec	Second
SEGS	Symptom Enhancing Geminivirus Sequences
SLCMV	SriLankan cassava mosaic virus
SNP	Single nucleotide polymorphism
SqLCV	Squash leaf Curl Virus
ssDNA	Single stranded DNA
T	Tonnes
TBIA	Tissue Blot Immunoassay
TBS	Tris Buffered Saline
TLCV	Tomato Leaf Curl virus
Trap	Transcription activator protein
U	Unit
Ug-V	Ugandan Varient
V	Volt
Ml	Milliliter
Φ	Phi

INTRODUCTION

1. INTRODUCTION

Root and tuber crops comprising of cassava, sweet potato, yams and aroids serves as inevitable part of agriculture providing staple food for millions of people in different parts of the world. It has now become the source of most of the daily carbohydrate intake for a large number of populations in many parts of the tropics, besides being used as a raw material for industrial use and animal feed.

Among the different root and tuber crops, cassava (*Manihot esculenta* Crantz, Family *Euphorbiaceae*) is of much importance because of its consumption value and numerous agro-industrial uses. Because of the storage capacity in the ground for up to three years, this crop provides consistency to food security and represents a household food bank that can be drawn upon when adverse climatic conditions limit the production of other food crops (Lebot *et al.*, 2008). Fresh tubers of cassava are suitable for consumption by both humans and animals, and provide the most important dietary source of calories for more than a billion people in about 105 countries. Cassava starch is being exploited for its numerous industrial applications, including bio ethanol, processing for the paper industry, pellets for animal feed, and thickeners in the food industry (Blagbrough, 2010).

Like other vegetatively propagated crops, cassava is also more prone to systemic diseases carried through propagation material. Among the various diseases which affect the cassava crop, cassava mosaic disease (CMD) has emerged as one of the most serious limiting factors all over the world, resulting in annual yield losses of about 88 % in susceptible varieties and 50 % in field tolerant varieties (Malathi *et al.*, 1985; Obonyo, 2007). This disease is caused by cassava mosaic geminiviruses (CMGs) (genus *Begomovirus*; family *Geminiviridae*) and the main spread of this disease is due to the indiscriminate use of infected planting material (primary infection) and by the whitefly (*Bemisia tabaci*) vector. Worldwide, eleven species of CMGs were identified so far and only two among them are widespread in India

namely *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV) causing yield loss up to 80% in highly susceptible varieties and 20-30% in improved varieties (Malathi *et al.*, 1985; Hegde *et al.*, 2010).

CMD is one of the major problems receiving attention by researchers in India over the last three decades. As both ICMV and SLCMV was reported in India showing high variability (Patil *et al.*, 2005), the major concern is that of a high recombinogenic potency of SLCMV over ICMV. SLCMV has the capability to trans-replicate other viral DNA components (Saunders *et al.*, 2002) resulting in more virulent strains. So, control measures like development of transgenic cassava using CMG clones exploiting RNA interference needs to be updated frequently. Association with satellite molecules like beta increases the infectivity and virulence of these viruses (Saunders *et al.*, 2002) and this is also a major concern while developing control measures against CMD. Therefore several studies were carried out in different parts of India on different aspects like survey and surveillance, symptomatology, pathogen detection, yield loss estimation, transmission and epidemiology in order to develop proper measures to manage the infection. However, in recent years, the work has focused mainly on molecular diagnostics and the identification of the viruses/strains involved for understanding the biodiversity of these viruses (Hegde *et al.*, 2008).

The alarming proportions of CMD in the major cassava growing areas of India, especially Kerala and Tamil Nadu demands the elimination of CMGs from the field before they evolve new, more virulent recombination's after mixed infections (Thresh *et al.*, 2005; Anitha *et al.*, 2011). Identification of the range of species and strains of viruses present in a particular area provides important information for devising strategies to control CMD, allowing new varieties to be tested for resistance/tolerance specifically to the range of viruses identified in the target region. In this context, the present study is carried out to evaluate the biodiversity and

variation among different cassava mosaic viruses present in Kerala mainly focussing on the following objectives

- Identification of cassava mosaic viruses present in major cassava growing areas of Kerala
- Identify the occurrence of beta satellites or other defective DNA molecules in the cassava mosaic diseased samples

Findings from this study can provide a platform for monitoring novel virus species and strains that evolved or are introduced and this will aid in assessment of diversity of cassava mosaic virus across the country in future.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS (RTC) AND ITS IMPORTANCE

Tuber crops are underground crops. Plants that produce tubers use these tubers as storage organs, mainly for storing starch. Technically, a tuber is either a modified stem, as in white potato (*Solanum tuberosum*) and cocoyam (*Colocasia esculenta*) also known as a corm, or a modified root such as in sweetpotato (*Ipomoea batatas*) and cassava (*Manihot esculenta*).

Root and Tuber crops (RTCs), including cassava, sweet potato, yams, potato, cocoyams and other root crops are important to the agriculture and food security of more than 100 countries and overall are a component of the diet for 2.2 billion people as well as contributing to animal feeds and industry.

The annual world production of root and tuber crops is about 83.8153483 million tonnes (t) consisting of potatoes (385074114 t), cassava (270278871 t), sweet potatoes (104453966 t), yams (68114520 t), and taro and other aroids (10232012 t) (FAOSTAT 2014).

Tuber crops have a higher biological efficiency as producers of carbohydrates than the cereals. They can be used as excellent raw materials for the production of a wide variety of industrial products such as alcohol, starch, sago flour, liquid glucose, vitamin C etc., as well as animal feed (Edison, 2002). Being an important part of the diet, RTCs produce more edible energy per hectare per day than any other crop thus plays an important role in food security, nutrition and climate change adaptation. They also provide important sources of income through direct sale and value-addition via processing for food and non food uses (Scott *et al.*, 2000).

2.1.1 Cassava (*Manihot esculenta* Crantz)

Cassava (*Manihot esculenta* Crantz) is a vegetatively propagated perennial shrub with edible tubers, and leaves belonging to the family Euphorbiaceae. Of the most recent evidence based on molecular markers regarding its geographical origins, suggest that this plant was domesticated within the south western rim of the Amazon basin (in modern day Brazil) and is derived from its closest wild relative, *Manihot esculenta* ssp. *flabellifolia* (Pohl) (Olson and Schaal, 1999; Le´otard *et al.*, 2009).

Cassava plants typically reach 1to 4 meter in height at physiological maturity, and the tuberous roots produced may be harvested from 6 months to 4 years after planting. These roots, which typically have a dry matter content of 30 to 40 percent, provide an important source of starch. Being monoecious, cassava plants produce fertile seeds, but the standard cultivation system makes use of stem cuttings for propagation and establishing a new crop. However, despite of ensuring uniformity of a crop variety from season to season, sustenance of pathogen populations from one cropping cycle to the next is a negative consequence regarding this. Being a serious issue, this fact is particularly significant in the epidemiology of viruses infecting this plant.

Though this crop has been domesticated since 10,000 years in Latin America, it was introduced to Africa by the Portuguese traders during the 16th century (Carter *et al.*, 1997). It was introduced into Asia during the 18th and 19thcentury (Onwueme, 2002). By the start of the 21stcentury, cassava was being widely cultivated throughout the tropics and since ever, it became a globally important crop, providing essential source of carbohydrates to hundreds of millions of people and offering diverse commercial and industrial applications via transformation processes. Cassava was introduced in India during the 17th century as a food crop but the actual potential of this crop was recognized only during the 20th century i.e., when the Second World War has begun. During that time, this crop was truly the 'famine saver' which

protected south India, especially Kerala from the aftershock of famine (Edison, 2000). Presently it has got commercial status in Tamil Nadu and Andhra Pradesh for processing into starch and sago (Nair and Makeshkumar, 2000)

In India, cassava is grown in an area of 2.28 lakh ha with an annual production of 81.39 lakh tonnes (FAO, 2014) mostly in Kerala, Tamil Nadu and Andhra Pradesh and also in a few North Eastern states (Hegde *et al.*, 2008; Anitha *et al.*, 2011).

Being a tropical crop, cassava is important for food security and income generation for many poor farmers in several Asian and African countries. Fresh tubers of cassava are suitable for consumption by both humans and animals, and provide the most important dietary source of calories for more than a billion people in about 105 countries. Cassava starch is being exploited for its numerous industrial applications, including bio ethanol, processing for the paper industry, pellets for animal feed, and thickeners in the food industry. Tolerance towards unfavourable conditions and abiotic stress made cassava an excellent crop, in comparison with other cereals such as wheat, rice and maize, for small-scale farmers with limited resources (Blagbrough, 2010).

2.1.2 Threats in cassava production

Among the diverse set of constraints causing deleterious decline in the production of cassava worldwide, the most important areof pests and diseases. Most important among them are cassava mealybug (*Phenacoccus manihoti* Mat.-Ferr.] (CM), cassava green mite [*Mononychellus tanajoa* (Bondar)] (CGM), and cassava bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *manihotis*. Apart from these, cassava is affected by a number of viral, fungal, bacterial and mycoplasmal infections causing substantial losses in yields (Lozano and Booth, 1974).

There are only fewer virus groups affecting cassava in Africa and fewer still in Asia. Among them, cassava mosaic geminiviruses (the family Geminiviridae) and cassava brown streak potyvirus (Potyviridae) cause significant yield losses in cassava throughout the tropical and sub-tropical parts of the world (Thresh *et al.*, 1997; Patil and Fauquet, 2009; Castillo *et al.*, 2011).

2.2. GEMINIVIRUSES

Geminiviruses (the family Geminiviridae) are circular single-stranded DNA (ssDNA) plant pathogens, which cause damage to many crop plants in various continents and are transmitted by insect vectors. Based upon their host range, nature of the insect vector and genome arrangement, the geminiviruses have been classified into seven genera: *Begomovirus, Curtovirus, Mastrevirus Topocuvirus, Becurtovirus, Turncurtovirus* and *Eragrovirus* (Hull, 2002; Brown *et al.*, 2012; Adams *et al.*, 2013). Members of the genus *Begomoviruses* (the family Geminiviridae) are transmitted by whiteflies of the species *Bemisia tabaci* (Sanderfoot and Lazarowitz, 1996) and consist of one (monopartite) or two (bipartite) circular single stranded DNA components designated as DNA-A and DNA-B, both having about 2.7 kb in size (Kushawaha *et al*, 2015). DNA-A components encodes the proteins necessary for viral encapsidation and replication whereas DNA-B components encode proteins necessary for the movement of viral DNA within and between the host cell (Kheyr-Pour *et al.*, 1991; Fauquet *et al.*, 2008).A 200 base pair common region (CR) is present within the intergenic region of both DNA components, showing a high similarity with each other (Harrison and Robinson, 1999). The common region is highly conserved containing a hairpin structure with the loop sequence TAATATTAC (known as nonanucleotide sequence), which forms the part of virion strand DNA replication having some viral promoters and responsible for the initiation of viral replication (Stanley, 1995; Hong *et al.*, 1995; Dutt *et al.*, 2005).DNA-A encodes the genes responsible for Replication [AC1 (Rep) Replication associated protein, and AC3 (Ren) replication enhancer protein], regulation of gene expression

[AC2 (Trap)], particle encapsidation [AV1 (CP)], and symptom determination [AC4] while the DNA-B encodes two proteins, BC1 [(MP) Movement protein] and BV1 [(NSP) Nuclear shuttle protein], involved in cell-to-cell movement within the plant, host range and symptom modulation (Stanley *et al.*, 2004). AC1 and AC4 have also been reported to act as suppressors of RNA interference (RNAi), a defence response of plants against viruses, thus contributing towards the viral virulence functions (Vanitharani *et al.*, 2004). Majority of New world begomoviruses have bipartite genome containing both DNA-A and DNA-B whereas a large number of Old world begomoviruses are either strictly monopartite having only DNA-A component or monopartite DNA-A associated with subviral DNA components such as alpha and beta satellites. In the case of bipartite begomoviruses, both DNA-A and DNA-B are required for both encapsidation and movement respectively. Whereas in monopartite begomoviruses, DNA-A encodes both of these functions (Nawaz-ul-Rehman *et al.*, 2009a; 2009b; Patil *et al.*, 2010).

2.2.1 Cassava Mosiac Disease

Among the diseases and pests of cassava, cassava mosaic disease (CMD) is one of the main biotic constraint in cassava production, limiting the productivity of cassava and thereby becoming the most important threat to food security worldwide in all cassava growing areas especially in Africa and Asia (Kushawaha *et al.*, 2015; Chikoti *et al.*, 2015).

Cassava mosaic disease (CMD) is caused by several distinct whitefly transmitted viruses named cassava mosaic geminiviruses (CMGs) or cassava mosaic geminiviruses (CMGs) (genus *Begomovirus*; family Geminiviridae) which are bipartite in nature containing two sub genomic components namely DNA-A and DNA-B each of about 2.8 kb size (Fig. 1) (Stanley *et al.*, 2004).

Worldwide, eleven species of CMGs has been identified yet, namely *African cassava mosaic virus* (ACMV),*South African cassava mosaic virus* (SACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Zanzibar virus* (EACMZV), *East African cassava mosaic Malawi virus* (EACMMV), *and East African cassava mosaic Kenya virus* (EACMKV), *African cassava mosaic Burkina Faso virus* (ACMBFV),*Cassava mosaic Madagascar virus*(CMMGV), *Indian cassava mosaic virus* (ICMV) *and Sri Lankan cassava mosaic virus* (SLCMV) (Legg and Fauquet, 2004; Bull *et al.*, 2006; Patil and Fauquet, 2009; Alabi *et al.*, 2011; Harimalala *et al.*, 2012; Tiendre'be'ogo *et al.*, 2012).

Among these, the ICMV was the first CMG recorded from South Asia (Malathi *et al.*, 1985), followed by *Sri Lankan cassava mosaic virus* (SLCMV) (Saunders *et al.*, 2002, Dutt *et al.*, 2005).

In India, CMD was 1st reported by Abraham (1956). Later on, a similar disease in Sri Lanka caused by a different begomovirus, *Sri Lankan cassava mosaic virus* (SLCMV), was reported by Austin (1986).

The first clone of ICMV (ICMV-Ker) was obtained by Hong *et al.* (1993) and subsequently, Saunders *et al.* (2002) cloned SLCMV.

Although SLCMV was initially reported from Sri Lanka, it occurs subsequently in southern India, together with ICMV (Patil *et al.*, 2005; Anitha *et al.*, 2011). Anitha *et al.* (2011) recently reported the presence of ACMV along with ICMV and SLCMV, from southern India, Kerala.

In India, CMD causes yield loss of about 88 percent in susceptible varieties and 50 percent in field tolerant varieties (Edison, 2002; Obonyo, 2007). It was also reported that the starch content and protein content of cassava tubers was found to be reduced by about 20% and 0.15% respectively due to CMD infection (Rageshwari *et*

al., unpublished data, 2013; unreferenced).Overall incidence of CMD is highest in the two main cassava-growing states in India: Kerala (23%) and Tamil Nadu (30%) (Alvarez *et al.*, 2012). It also appears in Andhra Pradesh (Less than 1%) and Karnataka (5%), which are outside the main cassava growing areas (Calvert and Thresh, 2002).

2.2.2 Cassava mosaic virus replication

The cassava mosaic virus (geminivirus) replicates via a Rolling-circle replication (RCR) mechanism. RCR occurs in three stages. In the first stage, viral single stranded DNA (ssDNA) (plus strand) enters the host cell and is converted into a covalently closed double stranded DNA (dsDNA) replicative form (RF) in a process involving host-directed, RNA-primed synthesis of a complementary (minus) strand. The RF serves as template for viral transcription as well as atemplate for further replication. During the second step, additional RF DNA molecules will be generated by making a small nick in the plus strand at a specific sequence by the Rep protein. Following phosphodiester bond cleavage, Rep protein covalently binds to the *5* ' terminus via a phosphotyrosine linkage. The 3'-OH terminus is used as a primer for the synthesis of nascent plus strand, which displaces the parental plus strand from the intact minus-strand template. Synthesis again is carried out by host replication proteins. Completion of the nascent plus strand regenerates the origin of replication, which again is nicked by Rep, this time acting as a terminase to release the displaced unit-length plus strand, which is simultaneously ligated to circular form by the closing activity. In the process, Rep is transferred to the newly created *5'* terminus.
Early in the replication cycle, the circularized ssDNA is used as template for synthesis of minus-strand DNA, resulting in the amplification of RF. The third or the last stage of RCR which occurs late in the replication cycle, is responsible for the accumulation of viral genomes for encapsidation. This stage is similar to the second stage except that priming is prevented and ssDNA is the predominant product. Later

on, viral DNA will be encapsulated and new viruses are released. (Saunders *et al.*, 1991; Stenger *et al.*, 1991).

Though cassava mosaic viruses do not encode a gene product with polymerase activity, they rely on host machinery to replicate their chromosomes.

2.2.3 Transmission of Cassava Mosaic Disease

The cassava mosaic disease spreads primarily through the indiscriminate use of infected planting material. The secondary spread is through the whitefly (*Bemisia tabaci* (Gennadius)) (Fig. 2). However, it has been proved that CMD is not transmitted through seeds (Mathew, 1989; Makeshkumar *et al.*, 2005), but it can be easily transmitted through grafting (Edison *et al.*, 2002). It has been reported that cassava mosaic viruses are transmitted by mechanical inoculation from cassava to different species of *Nicotiana* sp., *Datura stramonium, Manihot glaziovii ,Nicandra phylosalodes* and *Petunia hybrida* (Malathi *et al.*, 1983; Mathew *et al.*, 1993; Anitha *et al.*, 2008).

Apart from cassava, CMGs are seen associated with many economically important crops. Rajinimala *et al.* (2007) reported ICMV causing yellow mosaic disease in bittergourd from Tamil Nadu, India. Gao *et al.* (2010) reported ICMV infection in Jatropha (*Jatropha curcas*) and in Caster bean (Invasive species compendium, 2016). During a survey conducted on chilli fields in Kerala, India, Khan *et al.* (2011) reported that CMGs also infects chilli causing mosaic disease in these crops. Recently, Shery (2016) reported that a new variant of CMG cause mulberry mosaic disease in India.

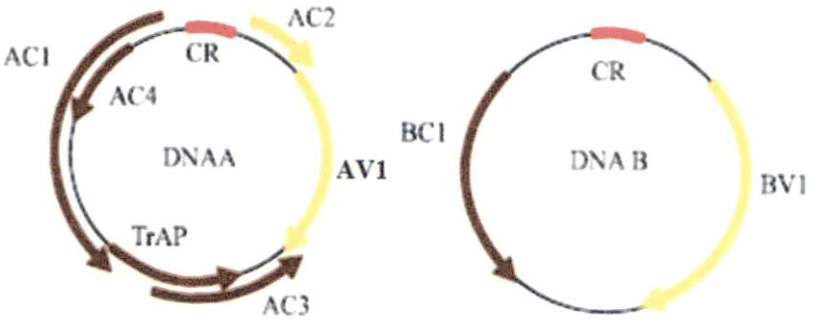


Fig. 1: Typical genomic organization of a cassava mosaic virus.AV1- coat protein (CP),AC1- Replication associated protein (Rep), AC2- transcriptional activator (TraP), AC3- replication enhancer (Ren), AC4- Silencing suppressor, CR- Common region, BV1- nuclear shuttle protein (NSP) and BC1- movement protein (MP).

Fig. 2: Whitefly *Bemisia tabaci.*

2.2.4 Recombinant Strains and Subviral Agents Associated with CMD

Geminiviruses have the capacity to evolve rapidly in response to changes in their environment (such as alterations in cropping systems and or population dynamics of insect vectors). Cassava mosaic begomoviruses are subjected to frequent recombinations which promote their adaptation towards changing environments and helps them to spread widely on new ecological niches (Pita *et al.*, 2001; Owor *et al.*, 2007).It has been reported that multiple begomovirus infections facilitate evolution of begomoviruses through pseudo-recombination and recombination (Sung *et al.*, 1995).

In 1990s, a major epidemic broke out due to the emergence of a recombinant strain of EACMV referred as EACMV-Uganda Variant (EACMV-Ug) in the cassava plantations of East Africa resulting in devastation of acres of cassava growing fields and thereby starvation in areaswhere cassava was formerly the staple food (Otim-Nape *et al.*, 1996). It has been reported that DNA-A of a geminivirus associated with this severe CMD in Uganda has arisen by interspecific recombination (Zhou *et al.*, 1997). Similarly, in India, Rothenstein et al. (2006) reported that majority of the newly identified CMG isolates in south India are variants which are originated by recombination between parental viruses resembling SLCMV and ICMV. It was also reported that sequences of ICMV and SLCMV shows a separate clad as that of ACMV during phylogenetic tree construction and this proves that ICMV and or SLCMV have been evolved through a special recombination event from its geminiviral ancestor. This relative isolation of cassava infecting geminiviruses may be due to the specific relationship of cassava with a specialized whitefly biotype (Rothenstein *et al.*, 2006).

In a PCR-RFLP analysis to identify the diversity of geminiviruses associated with cassava mosaic disease in India, it was found that both ICMV and SLCMV were found in mosaic affected cassava in which SLCMV was widespread and ICMV was

restricted to certain geographical regions. Presence of novel patterns identified from a higher proportion of samples indicates that polymorphism is present within the CMGs due to point mutation. Presence of these novel RFLP patterns attribute to high variability of ICMV and SLCMV in field grown cassava plants (Patil *et al.*, 2005).

Recently a new species of African cassava mosaic virus namely African cassava mosaic Burkina Faso virus (ACMBFV) has been reported in West Africa. By recombination analysis, it was revealed that this new virus arisen by interspecific recombination between bipartite and monopartite begomoviruses in Africa (Tiendrébéogo *et al.*, 2012).

Apart from recombination, another event named pseudorecombination, i.e., reassortment of DNA components, results in diversity among many begomoviruses. During a study in Uganda, it has been reported that stable pseudorecombinants exits in nature within different strains of a species as well as between different species (Pita *et al.*, 2001).

Recombination events are a major driving force of geminivirus evolution. The diversity of viruses and frequent recombination events found in virus genomes influence the development of epidemics and the emergence of new viruses. Different variants of ICMV and SLCMV were identified from India which *viz.*, ICMV-[Ker], ICMV-[Ker2], ICMV-[Mah], ICMV-[Mah2], SLCMV-[KerC4], SLCMV-[Ker15], SLCMV-[Ker17], SLCMV-[ker20], SLCMV-[Tam2], SLCMV-[Tam6], SLCMV-[Tam7], and SLCMV-[Col] and the phylogeny of these viruses suggests that they are evolved from recombination between parental viruses resembling ICMV and SLCMV (Rothenstein *et al.*, 2006).

It has been reported that the widespread occurrence of SLCMV and its recombinogenic tendency pose grave threats to the cassava crop in south India, especially in the newly cultivated regions which highlights the urgent need to take effective antiviral measures for this crop (Borah *et al.*, 2012).

DNA satellites are short DNA strands found either in the cell nucleus or in the cytoplasm that interact with viral genomes resulting in modulation of symptom phenotypes. Episomal DNA satellites are found in the cytoplasm while integrated DNA satellites are integrated in the viral genome (Mwatuni *et al.,* 2015).

In majority of monopartite begomoviruses, only DNA A is required for the systematic infection (Kheyr-Pour *et al.*, 1991; Navot *et al.*, 1991; Dry *et al.*, 1993). Recently, it has been reported that, apart from DNA A, a type of satellite component named DNA satellite or satellite DNA, are seen associated with many of the monopartite begomoviruses. These are subviral particles composed of ssDNA approximately 1.3 kb in size, associated with the viral replication, encapsidation, insect transmission and movement in plants and also responsible for the induction of the characteristic viral symptoms in the host plant (Saunders *et al.,* 2000; Jose *et al.*, 2003; Briddon *et al.*, 2004).

To date, two types of DNA satellites are seen associated with begomoviruses: betasatellites (DNA ß) and alphasatellites (previously known as DNA-1) (Olive *et al.*, 2012). Beta satellites are satellite molecules associated with monopartite begomoviruses, having almost 1360 base pairs (bp) in size. Betasatellites encode a single gene, ßC1 (13 kDa protein), in the complementary strand of their genome, and contain an adenine-rich region of nearly 240 nucleotides (nts) as well as a satellite-conserved region (SCR) of nearly 220 nts, which is highly conserved among all betasatellites having a hairpin structure with the loop sequence TAA/GTATTAC, similar to that of the origin of replication of geminiviruses and nanoviruses (Briddon *et al.*, 2003). Betasatellites are completely dependent on the helper viral component (DNA-A) for their replication, encapsidation, movement in plants and transmission by whitefly vectors. Along with that, they augment the accumulation of their helper begomoviruses and enhance the symptoms induced in some host plants (Saunders *et al.*, 2000; Briddon *et al.*, 2001; Nawaz-ul-Rehman and Fauquet, 2009; Patil and

Fauquet, 2010), probably due to the silencing suppressor activityof the ßC1protein (Cui *et al.*, 2005; Saeed *et al.*, 2005).

Like betasatellites, alphasatellites are approximately half the size of begomovirus components (1375 bp) capable of self-replication in host plants but require a helper begomovirus for movement within the plant as well as for insect transmission. Like beta, alphasateliites also have a common organization consisting of a single ORFcoding for a Rep protein, an A-rich region and a hairpin structure with the sequence TAGTATTAC (Mansoor *et al.*, 1999; Saunders and Stanley, 1999; Saunders *et al.*, 2000; Briddon *et al.*, 2004).

With respect to cassava, it has been reported that through the interaction of the DNA satellites with begomoviruses, the symptom expression of CMD varies with increasing disease severity. It plays a direct role in symptom enhancement with characteristic stem curling and vein-swelling phenotypes thus impacting host-range determination and facilitating accumulation of both the begomovirus molecules and the encoded pathogenicity factors. The leaves of the infected plants having CMGs with DNA satellites assumed a sickle shape thus distinguishing them from other CMGs infected leaves (Saunders *et al.*, 2004; Stanley *et al.*, 2004; Mansoor *et al.*, 2006; Mwatuni *et al.*, 2015).

Recently, two novel DNA satellite sequences have been discovered from CMG infected cassava in Africa, designated as SEGS-1 and SEGS-2 (Sequences Enhancing Geminivirus Symptoms). It has been identified that when co-inoculated with ACMV, EACMV, or EACMV-Ug Variant, both these SEGS enhanced symptom severity leading to the development of filiform leaves and bleaching in cassava plants. Also, it is reported that when co-inoculated with EACMV-Ug, SEGS-1 overcomes the resistance of a cassava landrace carrying the CMD2 resistance locus (Ndunguru *et al.*, 2016).

2.2.5 Symptoms of CMD

A wide range of symptoms was expressed by the cassava plants infected by CMGs depending upon the virus species or strain, environmental conditions and the sensitivity of the cassava host. Typical symptoms include yellow or pale green chlorotic mosaic on leaves, commonly accomplished by distortion and crumbling. Young and emerging leafs of infected plants appear as chlorotic specs in leaf lamina. These specs enlarge as the leaf grows, intermix with the green tissue resulting in a typical mosaic pattern which may sparsely present or spread throughout the lamina. Affected leaves are reduced in size, misshapen, twisted and distorted to give shoestring appearance. In severe infection, all leaves become deformed, reduced in size resulting in stunting of the plant with bushy appearance. Infected plants usually express symptoms, unless the plant has only recently been infected or conditions are unfavourable like in drought conditions were most leaves are shed. Tolerant varieties may "recover" i.e., begin to produce symptomless leaves during the later stages of crop growth and particularly during hot, dry weather (Malathi *et al.*, 1985; Edison *et al.*, 2002; Anitha *et al.*, 2011).

Accurate and reliable visual assessment of symptoms is very important to assess the incidence, prevalence, intensity and systemicity of cassava mosaic disease. However, CMD symptoms can be confused with the symptoms of leaf damage due to cassava green mite (CGM) (*Mononychellus tanajoa*), cassava mealybug (*Phenacoccus manihoti*), cassava bacterial blight (*Xanthomonas campestris* pv. *manihotis*), drought and mineral deficiencies (Sseruwagi *et al.*, 2004). Most important among them is that of CGM and mineral deficiencies especially with that of Zinc (Fulton and Asher, 1997). However, CMD symptoms can be easily distinguished by the unequal mosaic distribution in both sides of the leaf lamina whereas both mineral deficiency and mite damage can be seen similar on each half of the lamina on the either side of the mid-rib (Sseruwagi *et al.*, 2004).

Fig. 3: Typical mosaic symptom of cassava mosaic disease.

Table. 2. The cassava mosaic symptom scale of 1-5 (Hahn *et al,* 1980).

Scale	Description
1	Unaffected shoots, no symptoms.
2	Mild chlorosis, mild distortions at bases of most leaves, while the remaining parts of the leaves and leaflets appear green and healthy.
3	Pronounced mosaic pattern on most leaves, narrowing and distortion of the lower one-third of the leaflets.
4	Severe mosaic distortion of two thirds of most leaves and general reduction of leaf size and stunting of shoots.
5	Very severe mosaic symptoms on all leaves, distortion, twisting, misshapen and severe leaf reductions of most leaves accompanied by severe stunting of plants.

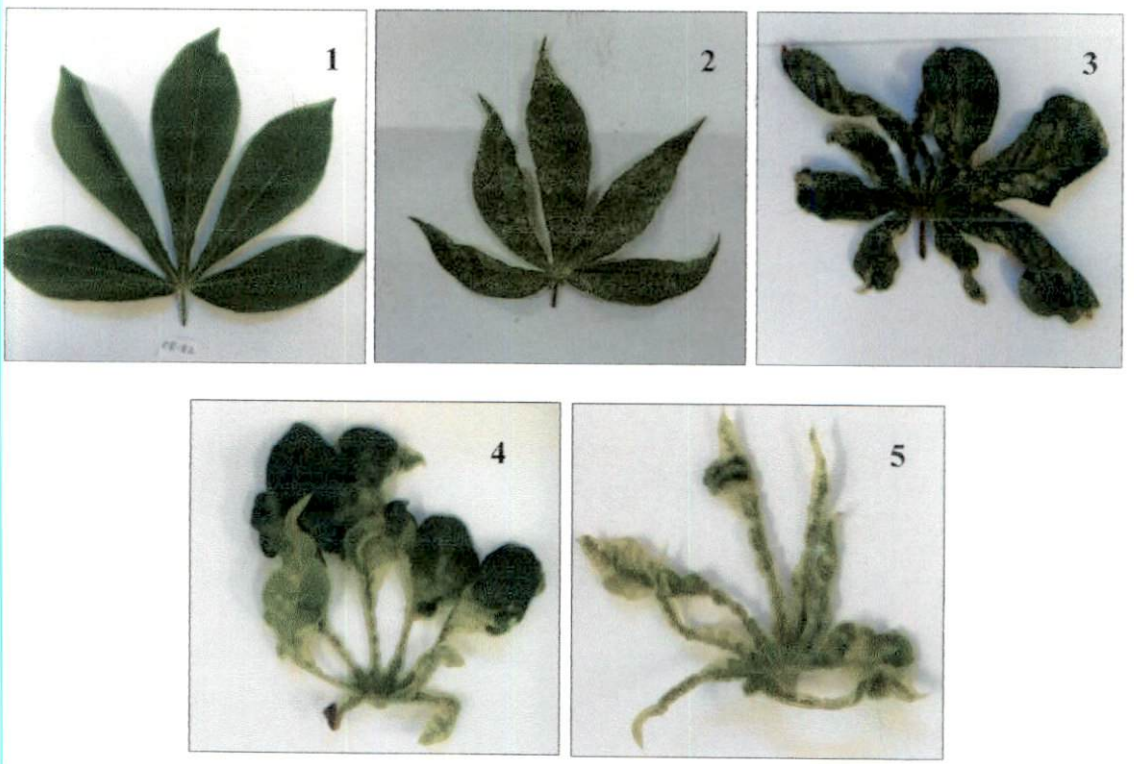


Fig. 4: Cassava leaves exhibiting different disease score.

The symptom severity of CMD is usually measured by visual assessment using an arbitrary scale. Scales of 0-5 (Cours, 1951; Fauquet and Fargette, 1990) and 1-5 (Hahn *et al.*, 1980) are commonly used for CMD, wherein 0 or 1 represent no symptoms and 4 or 5 the most severe symptoms, including distortion of leaves and stunting of plants, respectively.

2.2.6 Detection and Diagnosis of CMD

During recent years, cassava mosaic viruses have emerged as one of the most devastating plant virus declining the production and improvement of cassava in Africa and Asia (Legg *et al.*, 2015; Kushawaha *et al.*, 2015). Initially there was only one cassava mosaic geminivirus attributed to CMD in Africa and the Indian sub-continent (Bock and Harrison, 1985). Subsequently, three separate geminiviruses were distinguished, of which two occur in Africa: ACMV and EACMV (Swanson and Harrison, 1994) and one in India: ICMV (Hong *et al.*, 1993). Now, eleven species of cassava mosaic viruses have been identified yet worldwide, each of them emerged through recombination from their parental strains. The increasing number of CMGs identified demands the need of development of very efficient, ultrasensitive and more specific techniques for the detection of cassava mosaic disease.

Visual assessment of CMD symptoms provides a reliable indication of the incidence of virus infection in cassava growing fields (Sseruwagi *et al.*, 2004). However, using this technique complicates detection of CMD from recovered plants, because if they are partially resistant cultivars, symptoms may be localised and sometimes absent in young shoots.Apart from having these disadvantages, visual assessments indeed play a crucial role in surveying for CMD and the occurrence of CMGs.

Enzyme Linked Immunosorbent Assay (ELISA) is a very common method used for the detection of CMD in different parts of the world. The advantages of sensitivity, specificity, rapid detection, effectiveness using crude plant extracts and

especially, detection of plant pathogens as low as 10 to 100 ng/ml (Voller *et al.*, 1976), gave a universal acceptance to ELISA for using it as routine diagnostic technique for the detection of CMD worldwide (Malathi *et al.*, 1985; Makeshkumar & Nair, 2001; Hegde *et al.*, 2010).

Triple antibody sandwich ELISA (TAS-ELISA) has been widely used for the detection of cassava mosaic viruses in Africa and India using a panel of monoclonal antibodies against ACMV, EACMV and ICMV (Thomas *et al.*, 1986, Makeshkumar *et al.*, 2001). In India, CMD diagnosis is usually done with TAS-ELISA using both polyclonal and monoclonal antibodies of ICMV (Makeshkumar *et al.*, 2001). However, these monoclonal antibodies will not differentiate between ICMV and SLCMV as they bind with both the viruses. This limits the use of ELISA as it can't distinguish different CMGs from samples having mixed infection and this paved the way towards more precise DNA based methods of detection. Along with ELISA, Dot Immunobinding assay (DIBA) and Tissue immunoblot assay (TBIA) are also used for CMD detection in India (Hegde *et al.*, 2010).

DNA based techniques are more reliable for the detection of CMD as they provide precise information about the virus at molecular level. Nucleic Acid Spot hybridisation (NASH) and Polymerase Chain Reaction are the two methods in this regard. Makeshkumar *et al.* (2005) reported the use of NASH for the detection of cassava mosaic in India. Though this technique is unique, sensitive and specific than ELISA, it has certain limitations such as: during hybridisation, non specific binding can occur because of the presence of common conserved nucleotide sequences in many of the CMGs and the sensitivity is not good for very low level viral genome (10^{-15} g) present in the leaf tissue samples (Kushwaha *et al.*, 2010).

Polymerase Chain reaction (PCR) has been used for the specific detection of different CMGs occurring in several African countries (Chikoti *et al.*, 2015; Freddy *et al.*, 2015) and in India (Makeshkumar *et al* 2005; Rajinimala *et al.*, 2007; Khan *et al.*,

2011; Shery *et al.*, 2016). General primers designed from conserved regions of geminiviruses are used to detect any geminivirus infection in the sample (Wyatt and Brown, 1996) and specific primers designed from full length sequences of cassava mosaic viruses are widely used for the detection of ACMV, ICMV and SLCMV in CMD affected plants (Makeshkumar *et al.*, 2001; Dutt *et al.*, 2005; Patil *et al.*, 2005).Multiplex PCR is widely used for the detection and differentiation of ACMV and EACMV in Africa (Alabi *et al.*, 2008) as well as ICMV and SLCMV in India (Dutt *et al.*, 2005; Patil *et al.*, 2005). Universal primer designed from conserved regions of satellite DNA molecules can be used for the detection of these subviral betasatellite molecules from infected samples (Briddon *et al.*, 2002).

Rolling circle amplification (RCA) is one of the most recent techniques that revolutionized the detection of geminiviruses. It allows reliable diagnosis of all viruses with small single stranded circular DNA genomes utilising the bacteriophage Φ29 DNA polymerase to exponentially amplify single or double stranded circular DNA templates by rolling circle amplification using a very little amount of any circular DNA sample (Lizardi *et al.*, 1998; Dean *et al.*, 2001; Inoue-Nagata *et al.*, 2004). Using this method, it is able to produce microgram quantities of DNA from picogram quantities within a few hours ensuring high fidelity DNA replication by the proof reading activity of Φ29 DNA polymerase (Estaban *et al.*, 1993; Johne *et al.*, 2009). The RCA amplified products restricted with single cutting restriction enzymes can be used for direct sequencing (Inoue-Nagata *et al.*, 2004). Advantages of low cost, smooth handling, no need of expensive equipment's and detection of all infecting circular components in a single step without any sequencing knowledge, gave wide acceptance for this technique all over the world for the detection of CMGs (Kushwaha *et al.*, 2010). Using RFLP analysis, RCA amplified viral DNA can be directly sequenced upto 900 bases in a single turn without any cloning and plasmid purification and using this technique, it is possible to predict whether a geminivirus

has a monopartite or bipartite genome, without knowing anything about its sequence, just by calculating the sum of restriction fragment sizes (Haible *et al.*, 2006).
Haible *et al.* (2006) also reported that the cloning efficiency of the complete genome of a circular plant DNA virus increases while using RCA-RFLP technique rather than conventional PCR technique.

Wu *et al.* (2008) demonstrated a simple method employing RCA-RFLP technique to construct infectious clones of three geminivirus species. Two monopartite species [ageratum yellow vein virus (AYVV) and tomato leaf curl virus (TLCV)] and one bipartite species [squash leaf curl virus (SqLCV)] were used in the study, in which the RCA products digested with restriction enzymes were used to generate tandemly repeated conacatemers of whole genomes and these genome fragments were used to construct infectious clones. The integrity and infectivity of the begomovirus clones were assayed by agroinfection and confirmed using restriction analysis (Grimsley *et al.*, 1986; 1987; Elmer *et al.*, 1988; Hayes *et al.*, 1988).

While comparing to southern hybridisation, RCA-RFLP technique shows an excellent reliability to detect and distinguish viral DNAs in field-infected plant samples and thus proven to be an excellent tool for diagnostic practice (Haible *et al.*, 2006).

2.2.7 Management of CMD

Efficient management is inevitable to control this devastating disease from fields. Methods like phytosanitation (selection of disease free planting material), rouging (removal of diseased plants) (Fargette *et al.*, 1985; Fauquet and Fargette, 1990; Otim-Nape, 1993; Thresh and Otim-Nape, 1994; Thresh *et al.*, 1998), proper disposal of crop debris and use of virus resistant cultivars are some of the efficient methods to reduce the risk of CMD infection in cassava.

An extensive literature showcasing the losses due to CMD is available, as determined in field experiments conducted using diverse cultivars and improved specially bred cultivars in different countries (Anitha *et al.*, 2011; Samura *et al.*, 2014; Chikoti *et al.,* 2015) and these losses range from negligible to almost total depending upon several factors such as virulence of the virus(es) present, sensitivity of the host, stage of growth when infection occurs, soil fertility and growing conditions (Calvert and Thresh, 2002). Data obtained from these field surveys are vital in devising appropriate control measures to manage the disease in severely affected areas and to make adequate preparations in threatened areas. Therefore, regular diagnostic surveys on key cassava growing areas is to be undertaken to develop CMD resistant cassava, emphasizing on important aspects like the spread of CMD both in space and time and its epidemic characteristics, different CMGs present and their geographical distribution, distribution of whiteflies, their population and above all, the occurrence, frequency, amount and type (resistant or susceptible) of cassava cultivars being grown (Sseruwagi *et al.*, 2004; Samura *et al.*, 2014).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Molecular analysis of phylogeography of cassava mosaic disease" was carried out at the Division of Crop Protection, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016. Details pertaining to the experimental material and procedures used in the study are elaborated in this chapter

3.1 COLLECTION OF SAMPLES AND SYMPTOMATOLOGY STUDIES

During the course of study, a survey was conducted to record the incidence of cassava mosaic disease in cassava growing areas of all the 14 districts of Kerala. A survey sheet was prepared to record the details and a sample is listed in Table. 1. A total of 53 fields were surveyed and from each field, healthy and infected cassava leaf samples were collected and the symptom severity of each sample were recorded using the standard 1-5 scale described by Hahn *et al.*(1980) (Fig. 4) and the scale is represented in Table. 2. Farmers provided sufficient information on the varieties grown, but were unable to provide names of all. Altogether 115 samples were collected from different areas and were serially numbered according to the order of collection made. Plants showing no observable symptoms were also sampled to check the possibility of latent infection. The samples were photographed, put in sterile polythene bags and stored at -80°C for subsequent study.

3.2 INITIAL SCREENING OF SAMPLES FOR VARIOUS VIRUSES BY SEROLOGICAL ASSAYS

Enzyme Linked Immunosorbent Assay (ELISA) and Dot Immuno Blot Assay (DIBA) were employed for the detection of virus infection in cassava leaf samples collected during the survey. This procedure was carried out to screen the samples for CMD infection using ACMV polyclonal antibody obtained from DSMZ, Germany.

Table 1. The survey sheet to record the details of samples collected during the survey

SURVEY OF CASSAVA MOSAIC DISEASE IN KERALA

Name of the farmer:
Details of the cassava variety grown:
Name of the variety:

Sl. No.	Date of sample collection	District	Region	Field area (Acre)	Name of variety grown in the field	Cropping method (Intercrop/ Monocrop)	Crop duration (in months)	Total no. of plants in the field

No. of CMD infected plants	CMD incidence (%)	No. of samples taken		Symptom score (1-5)	CMD infection		Whitefly population (in top 5 leaves)
		Recovered samples	Non-recovered samples		Cutting infection	Whitefly infection	

3.2.1 Enzyme Linked Immunosorbent Assay (ELISA)

Triple Antibody Sandwich ELISA (TAS-ELISA) for CMD detection was carried out using collected cassava leaf samples showing different symptoms of virus infection. A healthy non host sample collected from the field was used as the negative control.

Wells of ELISA micro titre plates were coated with 200 µl of purified IgG (ACMV Polyclonal antibody) diluted to 1:1000 in coating buffer (Appendix I) and incubated at 37°C for 3 hrs. The plates were washed three times with PBS-Tween (Appendix I), soaked for three minutes during each wash and dried by tapping upside down on a tissue paper spread on the bench. When the plates became completely dried, 200 µl of blocking solution (Appendix I) was added to each well (blocking) and incubated at 37°C for 30 min. After the incubation period, the blocking solution was removed, tap dried the plates and again washed three times using PBS-Tween solution. After the plates become dried, 200 µl of test samples was loaded in duplicate wells and incubated overnight at 4°C. The test samples were prepared by grinding 100 mg test leaves in sample extraction buffer (Appendix I) and centrifuged at 8000 rpm for 10 minutes and the supernatant was taken. After the incubation period, plates were washed thrice using PBT-Tween solution and tap dried. Then the plates were coated with 200 µl of monoclonal antibody (ICMV/SLCMV SCR 58 or 60) diluted to 1:500 in conjugate buffer (Appendix I) and plates were incubated at 37°C for 3 hrs. After the incubation period, the plates were washed thrice using PBS-Tween solution and tap dried. Then the plates were coated with 200 µl of conjugate antibody [Alkaline Phosphatase (ALP) conjugated anti-mouse IgG] in appropriate conjugate buffer (Appendix I) incubated at 37°C for 2 hrs. Then 200 µl aliquots of freshly prepared substrate (Appendix I) dissolved in 10 ml of substrate buffer (Appendix I) was added to each well and incubated at room temperature in dark condition.

The A405 for sample in each well was measured in a BIO-RAD iMark Microplate Reader (USA). The readings were taken at 0 hr, 30 min, 1 hr, 2 hr and overnight.

3.2.2 Dot Immuno Blot Assay (DIBA)

Different infected leaf samples from each districts and a healthy non host were screened for DIBA using ACMV antibody. A desired size of nitrocellulose membrane (NCM) was cut and 1 cm^2 squares were drawn on it. The NCM was wetted by floating it in TBS (Appendix II) and was air dried. 5 µl of partially purified cassava leaf samples were spotted on respective squares. After air drying, the membrane was immersed in blocking solution (Appendix II) with gentle shaking for 1 h at room temperature. It was then rinsed once in TBS for 10 min. This was followed by incubating the NCM with primary antibody (ICMV/SLCMV-SCR 58 or 60) diluted to 1:1000 in TBS-SDM (Appendix II) for 1 hr at room temperature or overnight at 4°C. Then the membrane was washed thrice with TBS at 10 min interval and incubated with secondary antibody Ram-AP (ALP conjugated anti-mouse IgG) diluted 1:1000 in TBS-SDM for 1 hr at room temperature or overnight at 4°C. After rinsing thrice with TBS, the NCM was incubated in substrate solution (BCIP/NBT) (Appendix II) at room temperature in dark condition for 10 to 15 min. It was then observed for colour (pink) development. The membrane was rinsed with distilled water and then air dried. The colour formation and intensity were analyzed.

3.3 NUCLIEC ACID EXTRACTION

For carrying out polymerase chain reaction (PCR) based detection of the viruses infecting cassava, DNA isolation is a prerequisite. PCR was performed on DNA isolated from infected leaf samples using different virus specific primers.

3.3.1 DNA isolation

CTAB method of DNA extraction (Lodhi, *et al.*, 1994) was adopted for genomic DNA isolation. β-mercaptoethanol was added fresh to the CTAB extraction buffer (Appendix III) to give a final concentration of 2% (v/v). The buffer was pre-heated to 60°C in water bath (ROTEK, India). The samples (100 mg) were chilled and pulverized to a fine powder in liquid nitrogen using a sterile mortar and pestle and transferred in to a sterile 2 ml centrifuge tubes containing 1 ml of freshly prepared warm extraction buffer. The content was homogenized by gentle inversion and then incubated at 60°C in water bath for 30 min with intermittent shaking. Then it was centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was transferred to another sterile centrifuge tubes with a sterile pipette tip. To this 10 µl activated RNase (10 mg/ml) was added and incubated at 37°C for 1 hr. The homogenate was then extracted twice with an equal volume of 24: 1 (v/v) chloroform/isoamyl alcohol (Appendix III) and mixed well by inversion for 5-10 min and centrifuged (Hermle, Table top refrigerated centrifuge) at 15000 rpm for 10 min at 4°C. To the aqueous phase, 0.8 volume of chilled isopropanol was added and mixed by inversion. The mixture was then incubated at -20°C for at least 1 hr or overnight to precipitate the nucleic acid. After incubation, the precipitated DNA was pelletized by centrifugation at 15000 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was washed in 0.5 ml ethanol (70 %) and centrifuged at 15000 rpm for 10 min at room temperature. Supernatant was discarded and the pellet was air dried for 30-40 min and dissolved in 50 µl of TE buffer (Appendix III) or deionised water. The extracted DNA samples were then stored at -20°C (Vest frost Low Temperature Cabinet, India).

3.4 ANALYSIS OF THE EXTRACTED DNA

3.4.1 Agarose gel electrophoresis

The integrity and quality of the extracted DNA was checked in agarose gel electrophoresis. An agarose gel of 0.8 % was prepared in 1X TAE buffer and ethidium bromide (EtBr) was added, 0.5 µl/l. An aliquot of the DNA sample (2 µl) mixed with the loading dye was loaded in each of the wells of the gel. The gel was run at 5 V/cm for 30 min. The gel was then visualized under UV light and the image was documented using AlphaImager gel documentation system. The quantity of DNA was also measured using spectrophotometer (Denovix DS 11+ spectrophotometer).

3.5 MOLECULAR DETECTION OF VIRUSES USING POLYMERASE CHAIN REACTION

In order to detect the viruses associated with cassava and characterisation of identified viruses through PCR, different sets primers were used and their details were provided in the table. 3. These primers were synthesised from Eurofins (India). The synthesized primers (100 pM) were diluted to a final concentration of 10 pM with sterile water to obtain the working solution.

Table 3. Details of primers used for diagnosis of cassava mosaic viruses from collected samples.

Virus target	Primer name	Sequence (5'-3')	Amplified region	Amplicon size	Reference
ICMV/SLCMV					
Multiplex	ICMV_A_F	GCTGATTCTGGCA TTTGTAN	Common region and part of AC5	600 bp (SLCMV) 900 bp (ICMV)	(Patil *et al.*, 2005; Makeshkumar *et al.*, 2005)
	SLCMV_A_F	TGTAATTCTCAAA AGTTACAGTCN			
	I/SLCMV _A_R	ATATGGACCACATC GTGTCN			
Coat Protein gene	CP-F	GGA TCC ATG TCG AAG CGACCA	Coat protein gene	770 bp	(Makeshkumar *et al.*, 2005)
	CP-R	AAG CTT TTA ATT GCT GAC CGA			
Replicase initiation protein coding gene	Rep-F	TACAGTGGTGGATT CGCGA	Replicase initiation protein coding gene	900 bp	(Makeshkumar *et al.*, 2005)
	Rep-R	GACCTTGATTGGGA CCTGAGT			
Movement protein gene	MP-F	ATG GAG AAT AAT AGT AGC AA	Movement protein gene	900 bp	(Makeshkumar *et al.*, 2005)
	MP-R	TTA TAC ATT TTT			

		GGA TAC AT			
Cassava mosaic viruses in Africa					
ACMV	ACMV AL1/F	GCG GAA TCC CTA ACA TTA TC	Coat protein gene	1000 bp	(Zhou *et al.*, 1997)
	ACMV ARO/R3	GCT CGT ATG TAT CCT CTA AGG CCT G			
EACMV	EACMV LCP	TCT TTA TTA ATT TGT CAC TGC AT	Coat protein gene	1500 bp	(Zhou *et al.*, 1997)
	EACMVT 588U20	CAC TGG TAT GGT CCG ATG TG			
Ug-V	ACMV CP/R3	TGC CTC CTG ATG ATT ATA TGTC	Coat protein gene	1500 bp	(Zhou *et al.*, 1997)
	UVAL1/F1	TGT CTT CTG GGA CTT GTC TG			
Beta satellite DNA					
β-DNA	BETA- 01	GGT ACC ACT ACG CTA CGC AGC AGC C	Conserved region nearby hairpin loop	1000bp (minor band) 500bp (major band)	(Briddon *et al.*, 2002)
	BETA- 02	GGT ACC TAC CCT CCC AGG GGT ACA C			

3.5.1 Detection and differentiation of ICMV and SLCMV

Multiplex PCR was carried out with the total DNA isolated from the samples for the detection and differentiation of ICMV and SLCMV. For this three primers were used: SLCMV-A-F, ICMV-A-F, ICMV/SLCMV-A-R (Patil *et al.*, 2005; Makeshkumar *et al.*, 2007).

3.5.1.1 Multiplex PCR Analysis with ICMV/SLCMV specific primers

The components of the mixture were optimized as listed below:

10x buffer for		
Dynazyme polymerase II (Thermoscientific)	:	2.5 µl
dNTP (10 mM)	:	1 µl
ICMV-A-F	:	0.5 µl
SLCMV-A-F	:	0.5 µl
ICMV/SLCMV-A-R	:	0.5 µl
Taq polymerase	:	0.3 µl
Water	:	12.7 µl
Template DNA	:	2 µl
Total volume	:	20 µl

PCR was carried out in BioRad C1000 Touch Thermocycler (Germany). PCR programme was set with initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. Final extension was done at 72°C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1%). The gel was viewed under gel documentation system.

3.5.2 Detection of other cassava mosaic virus (African viruses) in infected samples

Samples which had mosaic symptoms, but not shown positive for ICMV / SLCMV were used for detection of other cassava mosaic viruses. PCR was carried out with the total DNA isolated from such samples (Zhou *et al.* 1997).

3.5.2.1 PCR Analysis with specific primers

The components of the mixture were optimized as listed below:

10x buffer for		
Dynazyme polymerase II (Thermoscientific)	:	2.5 µl
dNTP (10 mM)	:	0.5 µl
Forward primer	:	0.5 µl
Reverse primer	:	0.5 µl
Taq polymerase	:	0.25 µl
Water	:	18.25 µl
Template DNA	:	2.5 µl
Total volume	:	25 µl

PCR was carried out in BioRad C1000 Touch Thermocycler (Germany). PCR programme was set with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 45 sec and extension at 72°C for 1.5 min. Final extension was done at 72°C for 10 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1%). The gel was viewed under gel documentation system.

3.6 RESTRICTION ANALYSIS FOR IDENTIFYING THE VARIATION AMONG THE VIRUSES IDENTIFIED DURING THE SURVEY

3.6.1 Amplification of restriction enzyme rich region of DNA-A using specific primers

To analyze the variability among different CMGs (ICMV and SLCMV) identified from infected samples and confirmed using various serological assays and PCR, a novel approach exploiting RFLP technique was used in the present study. From NCBI, different isolates of ICMV and SLCMV reported from Kerala, DNA-A components were analyzed and identified the restriction enzyme- rich region. Four primer combinations were used for amplifying this target region as listed below (Primer details mentioned in Table 3).

1. Multi forward primer (ICMV-A-F or SLCMV-A-F for ICMV, SLCMV samples respectively) + CP reverse primer

2. I/SLCMV-A-R+ Rep reverse primer

3. CP forward primer + Rep forward primer

4. Rep reverse primer + Multi reverse primer

For all these combinations, the PCR was carried out in BioRad C1000 Touch Thermocycler (Germany) using the PCR programme set with initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. Final extension was done at 72°C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1%). The gel was viewed under gel documentation system.

Of the 4 combinations used, multi reverse + rep reverse primer pair gave amplification and further analysis was done using this combination of primers.

From each district, one sample each having ICMV, SLCMV and mixed infection (if present) separately were selected and amplified using the above primer combination.

Different restriction enzyme sites present within the amplified region were identified from the available ICMV and SLCMV isolates in the NCBI nucleotide database. From the data obtained, six enzymes EcoRV, XhoI, HindIII, ClaI, BamHI and KpnI were selected for restriction digestion for identifying the variation in the samplesas particular restriction patterns.

3.6.1.1 Restriction analysis of Multi- reverse and Rep-reverse products

Amplified products (1.7 kb) from each districts (having ICMV, SLCMV and Mixed infection separately) were subjected to restriction digestion with the six enzymes separately for obtaining particular restricted fragments.

The components of the restriction reaction mixture were optimized as listed below:

Cutsmart buffer (10X)	:	1.0 µl
Restriction enzyme	:	0.5 µl
Water	:	6.5 µl
Template DNA	:	2.0 µl
Total volume	:	10 µl

Restriction digestion was carried out at 37°C for 25 min followed by incubation at 65°C for 10 min for enzyme inactivation. The restricted products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1%). The gel was viewed under gel documentation system for identifying the variation.

Samples showing variation in restriction pattern were selected, multiplex PCR was done and the amplified products were purified from the gel (QIAEX-II gel extraction kit). The purified fragments were given to Eurofins Pvt. Ltd (Banglore) for sequencing. The rest of the samples were used for whole genome amplification using RCA.

3.7 WHOLE GENOME AMPLIFICATION USING ROLLING CIRCLE AMPLIFICATION (RCA)

Rolling Circle Amplification (RCA) was carried out (Malathi *et al.*, 2011) with the total DNA isolated from the selected samples for whole genome amplification. This amplification needs only little amount of DNA (10-20 ng) as template. The reaction mixture composed of 2µl of Φ 29 DNA polymerase buffer (10X), 2µl of exo-resistant random hexamer primers (500µM) and 2µl of dNTPs (10mM). Template DNA was added to this mixture, mixed well and denatured for 3 min at 94°C and cooled down to room temperature. After cooling, 4 µl of Pyrophosphatase (0.1 U/ µl) and 0.7 µl of Φ 29 DNA polymerase (10 U/ µl) were added and incubated for 18-20 hrs at 30°C followed by heat inactivation at 65°C for 10 min. The products of RCA were analysed in 0.8% gel.

3.7.1 Restriction analysis of RCA products

RCA products were subjected to restriction digestion with PstI and KpnI for obtaining 2.7 kb fragments which represents full length genomic DNA-A and DNA-B respectively. Similarly, pUC18 (Appendix IX) vector was also restricted with PstI and KpnI separately for ligating the restricted RCA products.

The components of the restriction mixture were optimized as listed below:

Cutsmart buffer (10X)	:	1.0 µl
PstI/ KpnI	:	0.5 µl
Water	:	6.5 µl

Template DNA / pUC18 vector DNA	:	2.0 µl
Total volume	:	10 µl

Restriction digestion was carried out at 37°C for 25 min followed by incubation at 65°C for 10 min for enzyme inactivation. The restricted products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1%). The gel was viewed under gel documentation system.

3.7.2 Gel elution of whole genome amplified RCA fragments and vector fragment

Extraction of the RCA restricted DNA-A or B, products and linearised vector was carried out with QIAEX-II gel extraction kit (QIAGEN, Germany). The PCR products were resolved on agarose gel (1%) and the amplicon was excised from the gel using a clean sharp scalpel. The gel slice was placed into a pre-weighed 2 ml tube and its weight was recorded. Then, thrice the volume gel solubilising buffer was added to the gel slice and it was incubated at 50°C for 10 min occasionally inverting it every 3 min until the gel slice dissolved completely. Then the sample was centrifuged at 13,000 rpm for 30 sec and the supernatant was discarded. The pellet was washed with 500 µl QX1 buffer, vortexed and centrifuged for 30 sec at 13,000 rpm and removed the supernatant completely with a pipette for removing residual agarose contaminants. The pellet was washed twice with 500 µl PE buffer, vortexed and centrifuged for 30 sec at 13,000 rpm and removed the supernatant completely with a pipette for removing residual salt contaminants. The pellet is air-dried for 10-15 min or until it becomes white. Sterile distilled water (20 µl) was added to the pellet, resuspended by vortexing and incubated for 5 min at room temperature. The sample was centrifuged for 30 sec and the supernatant containing purified DNA was transferred to a fresh tube and stored at -20°C.

3.8 CLONING AND TRANSFORMATION

3.8.1 Cloning of RCA restricted products

The eluted DNA A or B fragment was cloned into pUC18 (Appendix IX) and transformed into *E. coli* DH5α using manual method as described by Sambrook et al. (2000). The recombinant clones obtained were analyzed by colony PCR method.

The cloning and transformation protocol was performed in three consecutive days.

Day 1:

E. coli DH5α cells revived in Luria agar medium (Appendix V) were used for the transformation procedure. The ligation mix was prepared with the components as listed below.

Vector pUC18 (restricted with PstI/ KpnI)	:	2.0 µl
10x ligation buffer	:	1.5 µl
dNTP (10 mM)	:	1.5 µl
RCA product (restricted with PstI/ KpnI)	:	8.0 µl
T4 DNA ligase	:	2.0 µl
Total volume	:	15.0 µl

After vortexing, the ligation mix was centrifuged for a few sec and incubated overnight at 4°C.

Day 2:

Preparation of competent cells

To fresh 50 ml LB broth (Appendix VI), 500µl overnight grown *E. coli* DH5α cells were inoculated and incubated at 37 °C with shaking at 200 rpm for 90 min. The cells were pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. Supernatant

was discarded and the pellet was washed with 10 ml 100mM $MgCl_2$, mixed well and centrifuged at 10,000 rpm for 10 min at 4°C. The cell was then dispersed with 10 ml 100 mM $CaCl_2$ and kept on ice for 1hr. After 1hr, centrifuged at 10,000 rpm for 10 min. To the pellet, 1 ml of 100 mM $CaCl_2$ was added, mixed well and again kept in ice for 1 hr. The cells will now become competent for transformation.

Transformation

A volume of 50 µl of competent cells were added to each tube containing ligation mix and kept on ice for 1 hr. The mixture was heat shocked at 42°C for 2 min and immediately the tubes were quenched into ice. Then, 1ml of the LB medium was added to each tube and incubated at 37°C with shaking at 200 rpm for 1 hr. The cells were then concentrated by centrifuging at 7000 rpm for 1 min. Finally the cell suspensions were spread on LA ampicillin /X gal/ IPTG agar plates (Appendix VII). The plates were incubated overnight at 37°C and observed for the growth of colonies next day.

Day 3:

Analysis of recombinant colonies

Analysis of the positive colonies containing the insert was confirmed through colony PCR [PCR using coat protein specific (CP) primers for DNA-A and movement protein specific (MP) primers for DNA-B]. The primer details are listed in Table.3.

3.8.2 Colony PCR for recombinant clone analysis

The recombinant clones were analyzed for the presence and orientation of the sequence insert by colony PCR using their respective primers (Eurofins, India). The PCR reaction mix was formulated as:

10x buffer for		
Dynazyme polymerase II (Thermoscientific)	:	2.5 µl
dNTP (10 mM)	:	0.5 µl
Forward primer	:	0.5 µl
Reverse primer	:	0.5 µl
Taq polymerase	:	0.25 µl
Water	:	20.75 µl
Total volume	:	25 µl

A single white colony was selected and resuspended in the PCR reaction mix. A short strike was made over the culture plate (containing selective medium) in order to save the clone for re propagation. The PCR was carried out in BioRad C1000 Touch Thermocycler (Germany). PCR programme was set with initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 5°C for 2 min and extension at 72°C for 3 min. Final extension was done at 72°C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1%). The gel was viewed under gel documentation system.

3.8.3 Plasmid isolation of transformed white colonies

Colonies which showed positive in colony PCR were selected for plasmid isolation for carrying out the downstream applications like further confirmation for the presence of inserts through restriction and sequencing.

Plasmid isolation was done using the manual method formulated as follows.

A single colony (Transformed white colony) was inoculated on 2 ml LB broth with appropriate antibiotic [Here, ampicillin (50µg/ml)] and incubated overnight with shaking of 150 rpm at 37°C. Next day, bacterial cells were pelletized by centrifuging

at 10,000 rpm for 10 min at room temperature. After discarding the supernatant, the pellet was resuspended in 100 µl Buffer P1 (Appendix VIII) and vortexed. To this, 10 µl activated RNase (10 mg/ml) was added and incubated for 5 min at 37°C. To this, 200 µl buffer P2 (Appendix VIII) was added and gently mixed by inverting 6 to 7 times and incubated on ice for 5 min. To this, 150 µl of buffer P3 (Appendix VIII) was added, mix gently by inverting 6 to 7 times and incubated on ice for 5 min. The mixture was centrifuged at 15,000 rpm for 30 min. The supernatant was collected and again centrifuged at 15,000 rpm for 20 min. To the supernatant was collected, 0.8 volume of isopropanol was added and incubated on -20°C for 1 hr. After incubation, the mixture was centrifuged at 15,000 rpm for 15 min and the supernatant was discarded. The pellet was washed with 70% ethanol by centrifuging for 15,000 rpm for 15 min. After discarding the supernatant, the pellet was air-dried, resuspended in sterile distilled water and stored at -20 °C.

3.8.4 Confirmation of recombinant clones using restriction analysis

Restriction digestion using particular enzymes was performed for the confirmation of insert in the particular white colony.

The components of the restriction mixture were optimized as listed below:

Cutsmart buffer (10X)	:	1.0 µl
PstI/ KpnI	:	0.5 µl
Water	:	6.5 µl
Plasmid DNA	:	2.0 µl
Total volume	:	10 µl

Restriction digestion was carried out at 37°C for 25 min followed by incubation at 65°C for 10 min for enzyme inactivation. The restricted products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1 percent). The gel was viewed under gel documentation system.

3.9 CHARACTERISATION OF VIRUSES

3.9.1 DNA sequencing

Gel elutes of multiplex PCR products and the plasmid containing ICMV and SLCMV DNA-A clones were sequenced at Eurofins Pvt. Ltd (Bangalore).

3.9.2 Sequence analysis

Multi PCR product and full genome sequences obtained were analyzed and blasted using the basic alignment search tools (BLASTN) in the NCBI website. The sequences having more than 80% identity were selected from NCBI and multiple sequence alignment was done using the CLUSTALW program with default parameters settings using BIOEDIT version 7.2.5. The aligned sequences were used to generate a phylogenetic tree using the neighbor-joining and bootstrap options in MEGA 6.1 (Kumar *et al.*, 2004; Tamura *et al.*, 2011) with a 1000 replicate bootstrap search. Beet curly top virus (BCTV) was used as out-group species in the tree.

3.10 IDENTIFICATION OF BETA SATELLITES OR OTHER DEFECTIVE DNA MOLECULES IN CASSAVA MOSAIC INFECTED SAMPLES

3.10.1 Beta PCR for detection of beta satellite molecules

All the multiplex PCR positive samples and those which didn't show amplification with multiplex PCR and other viruses PCR, and having very severe mosaic, were selected and processed for PCR amplification with universal beta primers to detect the presence of beta satellite molecules.

Beta PCR was carried out for the detection of beta satellite molecules using the total DNA isolated from the infected samples. For this two primers were used: BETA- 01 and BETA- 02 (Briddon *et al.*, 2002)(Table. 3).

3.10.1.1 PCR Analysis with beta specific primers

The components of the mixture were optimized as listed below:

10x buffer for		
Dynazyme polymerase II (Thermoscientific)	:	2.5 µl
$MgCl_2$ (25 mM)	:	1.5 µl
dNTP (10 mM)	:	2 µl
BETA-01	:	1.3 µl
BETA-02	:	1.3 µl
Taq polymerase	:	0.2 µl
Water	:	12.9 µl
Template DNA	:	3.3 µl
Total volume	:	25 µl

PCR was carried out in BioRad C1000 Touch Thermocycler (Germany). PCR programme was set with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min. Final extension was done at 72°C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The positive control used was papaya leaf curl viral DNA containing beta satellites (obtained from TNAU). The amplified products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1%). The gel was viewed under gel documentation system.

RESULTS

4. RESULTS

The results of the study entitled "Molecular analysis of phylogeography of cassava mosaic disease" conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016 are presented in this chapter.

4.1 COLLECTION OF SAMPLES AND SYMPTOMATOLOGY STUDIES

During the survey conducted to record the incidence of cassava mosaic disease, 53 fields were visited from all the 14 districts of Kerala and a total of 115 samples were collected. These samples were serially labelled and symptomatology was recorded by visual observation as per the cassava mosaic symptom scale of Hahn *et al.* (1980). Of the 115 samples collected, 11 samples shown scale 1, 36 shown scale 2, 52 shown scale 3, 13 shown scale 4 and 3 samples shown scale 5 of very severe mosaic having distorted and misshapen leaves with stunting of plants. The details of samples collected during the survey are shown in Table. 4A and 4B and representative samples from all districts are shown in Fig. 5.

4.2 INITIAL SCREENING OF SAMPLES FOR VARIOUS VIRUSES BY SEROLOGICAL ASSAYS

To confirm the presence of cassava mosaic virus in the infected samples collected during the survey, serological tests like Enzyme Linked Immunosorbent Assay (ELISA) and Dot Immuno Blot Assay (DIBA) were employed.

Table 4A: Details of samples collected during the survey and the CMD symptom severity in different districts

Districts	**Number of regions surveyed**	**Varieties grown**	**CMD symptom severity (1-5 scale)**
Thiruvananthapuram	11	Kochangamuttan, Gandharippadappan, Karutha cheeni, Aarumasakkappa, M4, Ettumasakkappa , Kariyilapothiyan, Ramanthala, Karutha gandharippadappan, Vella gandharippadappan, Manja noorumuttan, Karukennan	2.8
Kollam	6	Aarumasacheeni, Block cheeni, Vella cheeni, Chuvanna kappa, Quintal kappa, Thumban vella, Odiyan kappa	2.45
Pathanamthitta	2	Vella block cheeni, Chuvappu block cheeni, Chuvanna kappa, karutha kappa	2.20
Alappuzha	3	Vellakkappa, Chuvanna kappa, Mulamoodan, Number kappa, Velankanni	3.28
Ernakulam	4	Karutha M4, M4, Karutha micher, Silon kappa, Aarumasakappa	3.00
Thrissur	3	Vellayani hraswa, M4, Sree Vijaya, Sree Athulya,	2.42
Kottayam	3	Velutha micher, Chuvanna micher Karutha micher, Kottayam chulli	2.00
Idukki	4	Nadan kappa, Aambakkadan cross, Ramapuram kappa, Aambakkadan, Silon kappa, Block thandu	2.28
Palakkad	5	Micher thari, Moothachi, Deevan, Kolaraman, Ramanthari, Ariyanthari, Aarammasathari	2.50
Malapuram	2	Varma, Pathatti, Moothachi, 1-4, Diwan, Aambakkadan	3.40

Table 4A continued

Districts	**Number of regions surveyed**	**Varieties grown**	**CMD symptom severity (1-5 scale)**
Kozhikode	4	CWRDM, Chuvanna kappa, Vella N4, Diwan, Aambakkadan, Thaalees, Quintal kappa, Ethakkappa	2.67
Wayanad	3	Diwan, Aambakkadan	4.00
Kannnur	2	Micher kappa	2.20
Kasargod	1	Kurishumoodan (Silon Kappa), Chullikkatan, Vellakkattan	2.67

Table 4B: Details of samples collected during the survey

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
1	CWRDM	17-02-2016	Vellannoor, kunnamangalam	Kozhikode	Chlorosis, stunting, leaf curling	Severe mosaic	3
2	Chuvantha Kappa	17-02-2016	Thottilpalam	Kozhikode	Chlorosis	Mild mosaic	2
3	Vella M4	17-02-2016	Thottilpalam	Kozhikode	Chlorosis	Mild mosaic	2
4	Diwan kappa	17-02-2016	Vellannoor, kunnamangalam	Kozhikode	Chlorotic veins, leaves completely curled, stunted growth	Severe mosaic	5
5	Aambakkadan	17-02-2016	Thottilpalam	Kozhikode	Chlorosis	Mild mosaic	2
5A	Aambakkadan	17-02-2016	Vellannoor, Kunnamangalam	Kozhikode	Chlorosis, stunting, leaf curling	Severe mosaic	3
6	Thaalees	17-02-2016	Thottilpalam	Kozhikode	Chlorosis	Mild mosaic	2
7	Quintal Kappa	17-02-2016	Kaayanna, Perambra	Kozhikode	Chlorosis	Mild mosaic	2
8	Unknown	17-02-2016	Vaaloor, Perambra	Kozhikode	Chlorosis, stunting, leaf curling	Severe mosaic	3
9	Unknown	17-02-2016	Vaaloor, Perambra	Kozhikode	Chlorosis, stunting, leaf curling	Severe mosaic	3
10	Ethakkappa	17-02-2016	Thottilpalam	Kozhikode	Chlorosis, stunting, leaf curling	Severe mosaic	3
11	Ethakkappa	17-02-2016	Thottilpalam	Kozhikode	Chlorosis	Mild mosaic	2
12	Micher thari	23-02-2016	Valliyod, Vadakkkancheri	Palakkad	Chlorosis	Mild mosaic	2
13	Moothachi	23-02-2016	Manishery, Ottappalam	Palakkad	Chlorosis, stunting, leaf curling	Severe mosaic	3

Table 4B continued

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
14	Deevan	23-02-2016	Manishery, Ottappalam	Palakkad	Chlorosis, leaf curling anddistortion	Mosaic	4
15	Kolaraman	23-02-2016	Valliyod, Vadakkancheri	Palakkad	Chlorosis	Mild mosaic	2
16	Ramanthari	23-02-2016	Kizhakkumcheri	Palakkad	Chlorosis	Mild mosaic	2
17	Ariyanthari	22-02-2016	Vadakkancheri	Palakkad	Chlorosis	Mild mosaic	2
18	Unknown	23-02-2016	Vadakkancheri, Alathur	Palakkad	Chlorosis, stunting, leaf curling	Severe mosaic	3
19	Aaraamasathari	23-02-2016	Kizhakkumcheri	Palakkad	Chlorosis	Mild mosaic	2
20	Diwan kappa	19-02-2016	4-aam mile, Mananthavadi	Wayanad	Chlorotic veins, leaves completely curled, stunted growth	Severe mosaic	5
21	Unknown	19-02-2016	Karimpummal, Panamaram, Kalpetta	Wayanad	Chlorosis, leaf curling and distortion	Mosaic	4
22 H	Aambakkadan	19-02-2016	4-aam mile, Mananthavadi	Wayanad	Healthy	Symptomless	0
23	Unknown	19-02-2016	Puthuppadi, Kurishupalli, Thamarasery	Kozhikode/Wayanad border	Chlorosis, stunting, leaf curling	Severe mosaic	3
24	Kurishumoodan (Ceylon Kappa)	21-02-2016	Vellarikkundu	Kasargod	Chlorosis, stunting, leaf curling	Severe mosaic	3
25	Chullikkattan	21-02-2016	Vellarikkundu	Kasargod	Chlorosis, stunting, leaf curling	Severe mosaic	3
26	Vellakkattan	21-02-2016	Vellarikkundu	Kasargod	Chlorosis	Mild mosaic	2

Table 4B continued

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
27	Varma	16-02-2016	Koottilangadi	Malappuram	Chlorosis, leaf curling and distortion	Mosaic	4
28	Pathatti	16-02-2016	Koottilangadi	Malappuram	Chlorosis	Mild mosaic	2
29	M4	16-02-2016	Koottilangadi	Malappuram	Chlorosis, stunting, leaf curling	Severe mosaic	3
30	Moothachi	16-02-2016	Koottilangadi	Malappuram	Chlorotic veins, leaves completely curled, stunted growth	Severe mosaic	4
31	Deevan	16-02-2016	Vandoor, Nilambur	Malappuram	Chlorosis, leaf curling and distortion	Mosaic	4
32	Micher Kappa	18-02-2016	Vilamana, Iritty	Kannur	Chlorosis	Mild mosaic	2
33	Unknown	18-02-2016	Vilamana, Iritty	Kannur	Mild mosaic and leaf spot	Healthy	1
33A	Unknown	26-10-2015	Thalassery	Kannur	Chlorosis, stunting, leaf curling	Severe mosaic	3
33B	Unknown	26-10-2015	Thalassery	Kannur	Chlorosis, stunting, leaf curling	Severe mosaic	3
34	Unknown	07-03-2016	Kampanippadi, Kothamangalam	Ernakulam	Chlorosis, stunting, leaf curling	Mosaic	3
35	Unknown 1	07-03-2016	Kampanippadi, Kothamangalam	Ernakulam	Chlorosis, stunting, leaf curling	Mosaic	3
36	Unknown 2	07-03-2016	Kampanippadi, Kothamangalam	Ernakulam	Chlorosis	Mild mosaic	2

Table 4B continued

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
37 A	Karutha M4	07-03-2016	Kampanippadi, Kothamangalam	Ernakulam	Chlorosis, stunting, leaf curling	Mosaic	3
37 B	Karutha M4	07-03-2016	Kampanippadi, Kothamangalam	Ernakulam	Chlorosis, stunting, leaf curling	Mosaic	3
38	M4	07-03-2016	Nellikuzhi, Kothamangalam	Ernakulam	Chlorosis, leaf curling and distortion	Mosaic	4
39 A	Karutha micher	07-03-2016	Nellikuzhi, Kothamangalam	Ernakulam	Chlorosis, stunting, leaf curling	Mosaic	3
39 B	Karutha micher	07-03-2016	Nellikuzhi, Kothamangalam	Ernakulam	Chlorotic veins, leaves completely curled, stunted growth	Severe mosaic	4
40	Silon Kappa	07-03-2016	Maramballi, Aluva	Ernakulam	Chlorosis	Mild mosaic	2
41	Arumasakkappa	07-03-2016	Maramballi, Aluva	Ernakulam	Chlorosis, stunting, leaf curling	Mosaic	3
42 A	Vellayani hraswa	08-03-2016	Vellanikkara	Thrissur	Mild chlorosis	Very mild mosaic	2
43	M4	08-03-2016	Vellanikkara	Thrissur	Chlorosis	Mild mosaic	2
44	Sree vijaya	08-03-2016	Vellanikkara	Thrissur	Chlorosis, stunting, leaf curling	Mosaic	3
45	Athulya	08-03-2016	Vellanikkara	Thrissur	Chlorosis, stunting, leaf curling	Mosaic	3
45 A	Unknown	16-10-2015	Mudathur	Thrissur	Mild chlorosis	Very mild mosaic	2

Table 4B continued

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
45 B	Unknown	16-10-2015	Mudathur	Thrissur	Mild chlorosis	Very mild mosaic	2
46	Velutha micher	09-03-2016	Uzhavoor	Kottayam	Mild chlorosis	Very mild mosaic	2
47	Karutha micher	09-03-2016	Uzhavoor	Kottayam	Chlorosis, stunting, leaf curling	Mosaic	3
48	Karutha micher	09-03-2016	Areekkara	Kottayam	Mild mosaic and leaf spot	Healthy	1
49	Kottayam chulli	09-03-2016	Ammancheri, near Kottayam medical college	Kottayam	Chlorosis	Mild mosaic	2
50	Vella micher	09-03-2016	Ammancheri, near Kottayam medical college	Kottayam	Mild chlorosis	Very mild mosaic	2
51	Chuvanna micher	09-03-2016	Ammancheri, near Kottayam medical college	Kottayam	Mild chlorosis	Very mild mosaic	2
52	Karutha micher	09-03-2016	Ammancheri, near Kottayam medical college	Kottayam	Chlorosis	Mild mosaic	2
53	Nadan Kappa	10-03-2016	Thodupuzha	Idukki	Chlorosis, stunting, leaf curling	Mosaic	3
54	Aambakkadan cross	10-03-2016	Thodupuzha	Idukki	Chlorosis, stunting, leaf curling	Mosaic	3
55	Aambakkadan cross	10-03-2016	Thodupuzha	Idukki	Mild mosaic and leaf spot	Healthy	1

Table 4B continued

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
56	Ramapuram Kappa	10-03-2016	Alakkode	Idukki	Chlorosis, stunting, leaf curling	Mosaic	3
57	Aambakkadan	10-03-2016	Alakkode	Idukki	Chlorosis, stunting, leaf curling	Mosaic	3
58	Ceylon Kappa	10-03-2016	Alakkode	Idukki	Mild mosaic and leaf spot	Healthy	1
59	Block thandu	10-03-2016	Alakkode	Idukki	Chlorosis	Mild mosaic	2
60	Vella Kappa	20-03-2016	Mavelikkara	Alappuzha	Chlorosis, stunting, leaf curling	Mosaic	3
61	Chuvanna Kappa	20-03-2016	Mavelikkara	Alappuzha	Chlorosis, stunting, leaf curling	Mosaic	3
62	Mulamoodan	20-03-2016	Mavelikkara	Alappuzha	Chlorosis, stunting, leaf curling	Mosaic	3
63	Number Kappa	20-03-2016	Mavelikkara	Alappuzha	Mild chlorosis	Very mild mosaic	2
64	Vella Kappa	20-03-2016	Thamarakkulam	Alappuzha	Chlorosis, stunting, leaf curling	Mosaic	3
64 A	Velankanni	23-06-2016	Karuvatta	Alappuzha	Chlorotic veins, leaves completely curled, stunted growth	Severe mosaic	5
64 B	Unknown	23-06-2016	Karuvatta	Alappuzha	Chlorosis, leaf curling and distortion	Mosaic	4
65	Block cheeni (Chuvappu)	25-03-2016	Thatta, Panthalam	Pathanamthitta	Chlorosis	Mild mosaic	2

Table 4B continued

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
66	Block cheeni (Vella)	25-03-2016	Thatta, Panthalam	Pathanamthitta	Chlorosis	Mild mosaic	2
67	Chuvanna Kappa	25-03-2016	Thatta, Panthalam	Pathanamthitta	Chlorosis, stunting, leaf curling	Mosaic	3
68	Karutha Kappa	25-03-2016	Thatta, Panthalam	Pathanamthitta	Chlorosis, stunting, leaf curling	Mosaic	3
69	Block cheeni (Chuvappu)	25-03-2016	Thatta, Panthalam	Pathanamthitta	Mild mosaic and leaf spot	Healthy	1
70 A	Aarumasacheeni	27-03-2016	Mailam, Kottarakkara	Kollam	Chlorosis, stunting, leaf curling	Mosaic	3
71	Aarumasacheeni	27-03-2016	Pattazhi, Kottarakkara	Kollam	Chlorosis	Mild mosaic	2
72	Block cheeni	27-03-2016	Pattazhi, Kottarakkara	Kollam	Chlorosis	Mild mosaic	2
73	Vella cheeni	27-03-2016	Pattazhi, Kottarakkara	Kollam	Chlorosis	Mild mosaic	2
74	Quintal Kappa	27-03-2016	Pattazhi, Kottarakkara	Kollam	Chlorosis, stunting, leaf curling	Mosaic	3
75	Thumban vella	27-03-2016	Pattazhi, Kottarakkara	Kollam	Chlorosis, stunting, leaf curling	Mosaic	3
76	Odiyan Kappa	27-03-2016	Thazhamel, Anchal	Kollam	Chlorosis, stunting, leaf curling	Mosaic	3
77	Vella Kappa	27-03-2016	Thazhamel, Anchal	Kollam	Chlorosis, stunting, leaf curling	Mosaic	3
78	Chuvanna Kappa	27-03-2016	Thazhamel, Anchal	Kollam	Mild mosaic and leaf spot	Healthy	1

Table 4B continued

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
78 A	Unknown	09-01-2016	Chithara	Kollam	Chlorosis, leaf curling and distortion	Severe mosaic	4
79	Kochangamuttan	03-04-2016	Uroottukala, Neyyattinkara	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
79 A	Kochangamuttan	03-04-2016	Athiyannoor, Neyyattinkara	Thiruvananthapuram	Chlorosis, leaf curling and distortion	Mosaic	4
80	Gandharipadappan	03-04-2016	Uroottukala, Neyyattinkara	Thiruvananthapuram	Chlorosis	Mild mosaic	2
81 A	Karutha cheeni	03-04-2016	Athiyannoor, Neyyattinkara	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
82	Aarumasakappa (Green petiole)	09-04-2016	Mukkalaykkal, Nedumangad	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
83	Aarumasakappa	09-04-2016	Mukkalaykkal, Nedumangad	Thiruvananthapuram	Mild mosaic and leaf spot	Healthy	1
84	Aarumasakappa	09-04-2016	Nedumangad	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
85	M4	09-04-2016	Nedumangad	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
86	Ettammasakappa	09-04-2016	Nedumangad	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
87	Kariyilappothiyan	09-04-2016	Nedumangad	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
88	M4	09-04-2016	Nedumangad	Thiruvananthapuram	chlorosis, leaf curling and distortion	Mosaic	4

Table 4B continued

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
89	Raamanthala	09-04-2016	Pangappara	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
90	Karutha gandharipadappan	11-04-2016	Thozhukkal, Neyyattinkara	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
91	Manja noorumuttan	11-04-2016	Thozhukkal, Neyyattinkara	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
92	Kochangamuttan	11-04-2016	Thozhukkal, Neyyattinkara	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
93	Vella gandharipadappan	11-04-2016	Thozhukkal, Neyyattinkara	Thiruvananthapuram	Mild mosaic and leaf spot	Healthy	1
94	Unknown	30-01-2016	Thozhukkal, Neyyattinkara	Thiruvananthapuram	Mild mosaic and leaf spot	Healthy	1
95	Unknown	30-01-2016	Thozhukkal, Neyyattinkara	Thiruvananthapuram	Chlorosis, leaf curling and distortion	Mosaic	4
96	Karukennan	16-01-2016	Balaramapuram	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
97	Unknown	02-02-2016	Balaramapuram	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
98	Unknown	02-02-2016	Balaramapuram	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
99	Karukennan	02-02-2016	Balaramapuram	Thiruvananthapuram	Chlorosis, leaf curling and distortion	Mosaic	4
100	Unknown	07-01-2016	Attingal	Thiruvananthapuram	Mild chlorosis and leaf spot	Very mild mosaic	2

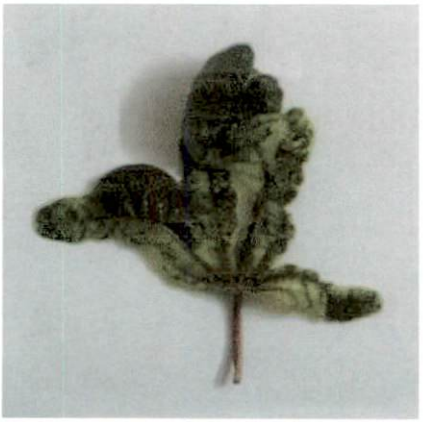
Table 4B continued

Sample no.	Variety name	Date of sampling	Place of sampling	District	Symptom features	Symptom severity	Score
101	Unknown	26-10-2016	Pallipuram	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
102	Unknown	26-10-2016	Pallipuram	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
103	Unknown	26-10-2016	Pallipuram	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
104	Unknown	26-10-2016	Pallipuram	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
105	Unknown	26-10-2016	Pallipuram	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3
106	Unknown	26-10-2016	Pallipuram	Thiruvananthapuram	Chlorosis, stunting, leaf curling	Mosaic	3

MALAPPURAM

Variety name: Varma
Symptom severity scale: 4
DOS: 16/2/16, 4 MAP

WAYANAD



Variety name: Deevan
Symptom severity scale: 5
DOS: 19/2/16, 4 MAP

KANNUR

Variety name: Micher Kappa
Symptom severity scale: 2
DOS: 18/2/16, 12 MAP

KOZHIKODE

Variety name: Deevan
Symptom severity scale: 5
DOS: 17/2/16, 6 MAP

KASARGOD

Variety name: Chullikkattan
Symptom severity scale: 3
DOS: 21/2/16, 7 MAP

PALAKKAD

Variety name: Deevan
Symptom severity scale: 4
DOS: 23/2/16, 5 MAP

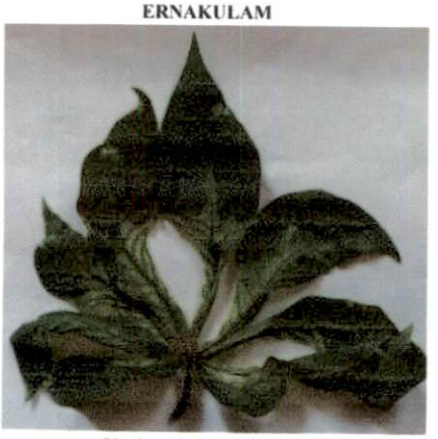
KOTTAYAM

Variety name: Vella micher
Symptom severity scale: 2
DOS: 9/3/16, 9 MAP

IDUKKI

Variety name: Aambakkadan
Symptom severity scale: 3
DOS: 10/3/16, 1 1/2 MAP

ERNAKULAM



Variety name: H4
Symptom severity scale: 4
DOS: 7/3/16, 2 MAP

THRISSUR

Variety name: Vellayani Hraswa
Symptom severity scale: 2
DOS: 8/3/16, 5 MAP

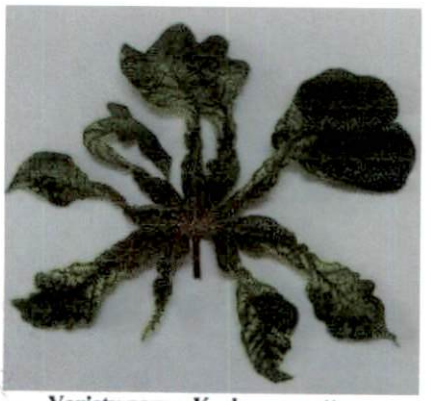
PATHANAMTHITTA

Variety name: Chuvanna kappa
Symptom severity scale: 3
DOS: 25/3/16, 2 MAP

ALAPPUZHA

Variety name: Unknown
Symptom severity scale: 4
DOS: 23/6/16, 8 MAP

TRIVANDRUM



Variety name: Kochangamuttan
Symptom severity scale: 4
DOS: 3/4/16, 3 MAP

KOLLAM

Variety name: Odiyan Kappa
Symptom severity scale: 3
DOS: 27/3/16, 3 1/2 MAP

Fig. 5: Representative samples showing different kinds of cassava mosaic disease symptoms.

4.2.1 Enzyme Linked Immunosorbent Assay (ELISA)

Triple Antibody Sandwich ELISA (TAS-ELISA) results shown that, out of the 115 samples collected, only 81 samples were positive for CMD. The TAS-ELISA readings (at 405 nm) of samples collected during the survey are shown in Table. 5.

Table 5: TAS-ELISA results of the collected samples

Sample no.	Rating of ELISA read	Sample no.	Rating of ELISA read	Sample no.	Rating of ELISA read	Sample no.	Rating of ELISA read	Sample no.	Rating of ELISA read
1	-	26	++	45A	-	66	+	88	+
2	++	27	+++	45B	+	67	++	89	++
3	-	28	+++	46	-	68	++	90	+
4	+++	29	+	47	+	69	-	91	+
5A	-	30	+++	48	-	70A	++	92	+
6	-	31	+++	49	-	71	++	93	+
7	-	32	+	50	-	72	-	94	+
8	++	33	-	51	++	73	-	95	++
9	-	33A	-	52	+	74	+	96	+
10	-	33B	++	53	+	75	+	97	+
11	-	34	+	54	+	76	-	98	+

12	+	35	++	55	-	77	-	99	+
13	+++	36	++	56	+	78	-	100	-
14	++	37A	++	57	++	78A	+++	101	++
15	++	37B	+	58	+	79	++	102	+
16	++	38	++	59	+	79A	+++	103	-
17	++	39A	+++	60	+	80	++	104	++
18	-	39B	++	61	+++	81A	+	105	+
19	+	40	-	62	++	82	+	106	-
20	+++	41	+	63	+	83	+		
21	++	42B	+	64	+	84	-		
23	-	43	-	64A	+++	85	++		
24	-	44	-	64B	+	86	+		
25	-	45	+	65	+	87	-		

+++: OD_{405} range of 0.700-1.000 ++: OD_{405} range of 0.400-0.699

+: OD_{405} range of 0.250-0.399

Negative control (Healthy plant): OD_{405} range of 0.140-0.150

Buffer control value: OD_{405} range of 0.050-0.100

4.2.2 Dot Immuno Blot Assay (DIBA)

Dot Immuno Blot Assay (DIBA) results were analysed for the samples collected, by visual assessment of intensity of purple spot in NCM. It was found that,

out of the 115 samples tested, only 78 samples were DIBA positive. A representative sample showing the dot blot assay of DIBA is shown in Fig. 6.

The DIBA results of all 115 samples collected during the survey were tabulated and shown in Table. 6.

Table 6: DIBA results of the collected samples

Sample no.	DIBA rating	Sample no.	DIBA rating	Sample no.	DIBA rating	Sample no.	DIBA rating	Sample no.	DIBA rating
1	-	26	-	45A	-	66	+	88	+
2	+	27	+	45B	+	67	+	89	+
3	-	28	+	46	-	68	+	90	+
4	++	29	+	47	+	69	-	91	+
5A	-	30	-	48	-	70A	++	92	+
6	-	31	+	49	+	71	++	93	+
7	-	32	-	50	+	72	+	94	+
8	+	33	+	51	+	73	-	95	+
9	-	33A	-	52	+	74	+	96	+
10	-	33B	-	53	+	75	+	97	+
11	-	34	+	54	-	76	-	98	+
12	+	35	-	55	-	77	-	99	+
13	++	36	+	56	-	78	-	100	-

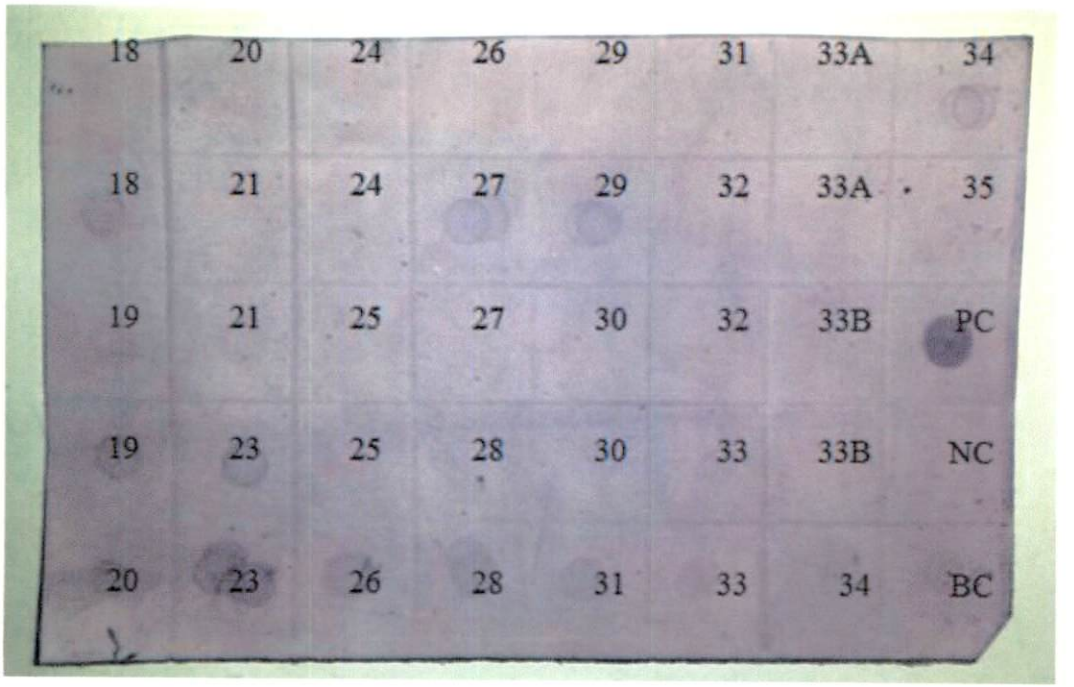


Fig. 6: DIBA results showing the cassava mosaic virus infection in different districts (sample no. 18- 35) as purple dots on NCM; PC- positive control ; NC- negative control; BC- buffer control.

14	+	37A	+	57	+	78A	++	101	+
15	+	37B	+	58	+	79	+	102	+
16	+	38	+	59	+	79A	++	103	-
17	+	39A	++	60	+	80	+	104	+
18	+	39B	+	61	++	81A	+	105	+
19	++	40	-	62	+	82	+	106	-
20	+	41	+	63	+	83	+		
21	-	42B	-	64	+	84	-		
23	++	43	-	64A	++	85	+		
24	-	44	-	64B	+	86	+		
25	-	45	+	65	+	87	-		

++ : High intensity spots (positive);

+ : Low intensity spots (positive)

- : no spot (negative)

4.3 NUCLEIC ACID EXTRACTION

4.3.1 DNA isolation

DNA was extracted from infected samples collected during the survey and the quality and quantity were analysed using agarose gel electrophoresis and spectrophotometer readings respectively. The gel image showing good quality DNA obtained as bright bands are shown in Fig. 7.

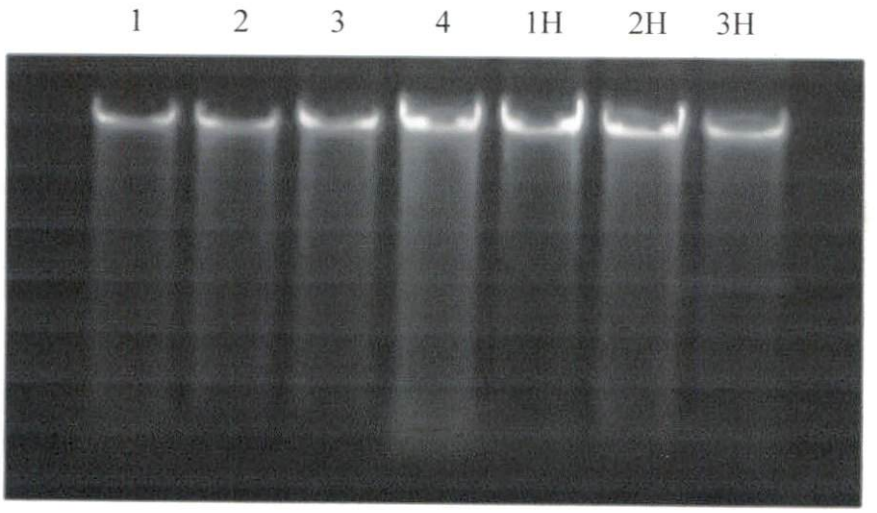


Fig. 7: Good quality DNA isolated from cassava mosaic infected leaf samples from Kozhikode (1-4) and tissue culture derived in-vitro cassava plant leaf samples (1H, 2H and 3H for H226, H165A and SreeAthulya respectively for using as negative control).

4.4 MOLECULAR DETECTION OF VIRUSES USING POLYMERASE CHAIN REACTION

4.4.1 Multiplex PCR for detection and differentiation of ICMV and SLCMV

Multiplex PCR was performed for the detection and differentiation of ICMV and SLCMV in the infected samples.

Of the 115 samples collected, 9 samples had only ICMV infection, 68 samples with only SLCMV infection, 18 samples had both ICMV and SLCMV (mixed infection) and 20 samples had neither ICMV nor SLCMV infection (Table. 7). The gel images showing multiplex PCR results of the samples collected during the survey were shown in Fig. 8.

4.4.2 PCR Analysis for the detection of cassava mosaic viruses of Africa in infected samples

Of the 115 samples collected, samples showing unusual symptoms and those were negative for both ICMV and SLCMV were selected (20 samples) and PCR were employed using specific primers of ACMV, EACMV, Ug-V to detect any such viruses presence.

Of the 20 such samples used, only 3 samples gave approximately 700 bp product wherein the ACMV positive sample gave an amplificon size of 1000bp. They were also SLCMV positive (65, 67 and 69) (samples from Pathanamthitta district). The gel image showing the ACMV PCR products of these samples is shown in Fig. 9. No amplification was observed with PCR reaction using EACMV and Ug-V primers, which showed that these viruses were absent in the samples tested.

PCR results of samples collected during the survey were shown in Table. 7. The overall distribution of cassava mosaic viruses identified from different districts of Kerala was shown in Fig. 10 and Table. 8.

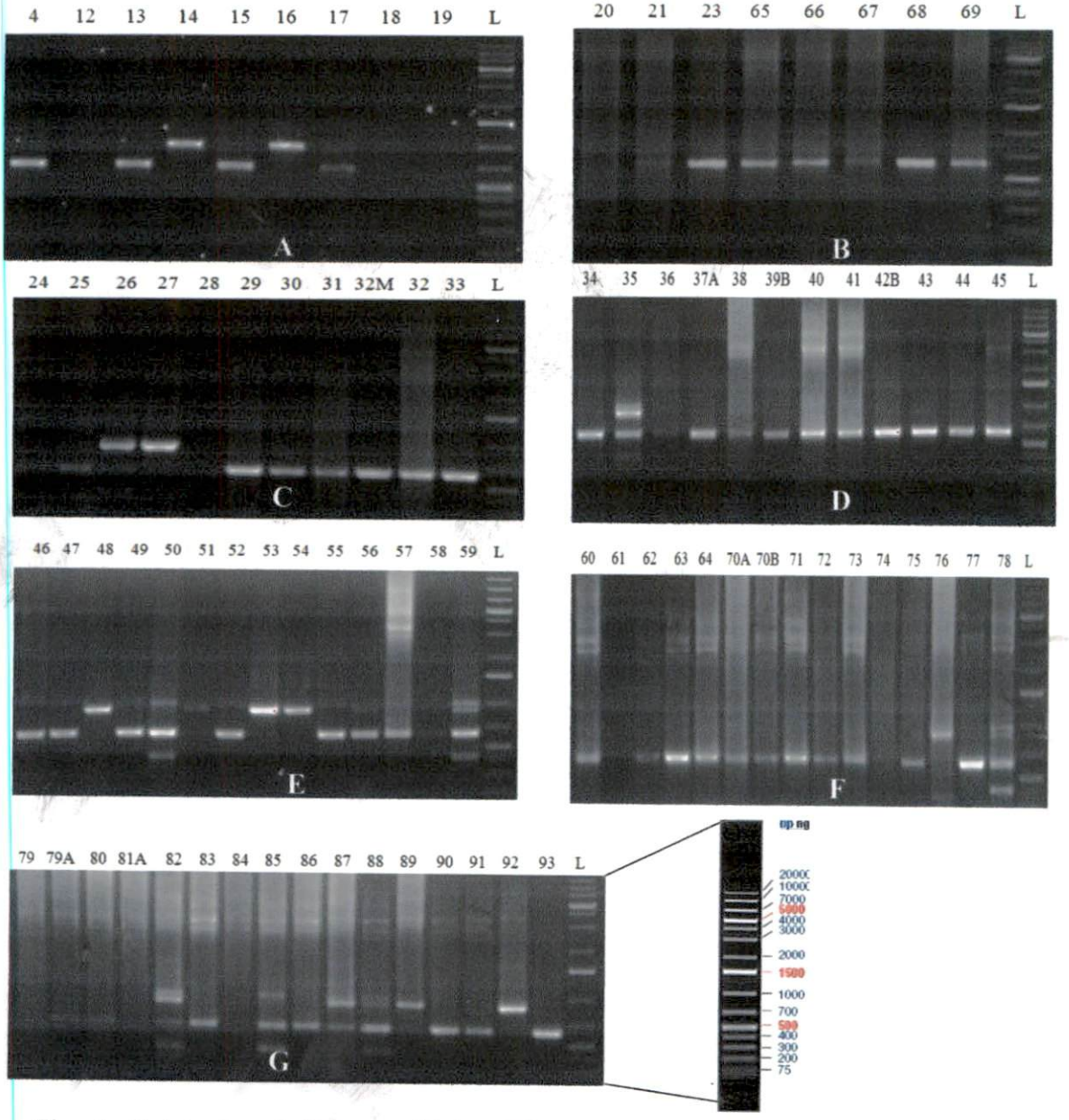


Fig. 8: Gel image showing multiplex PCR products of samples collected from different districts of Kerala. A: Kozhikode (1) and Palakkad (12,19); B: Wayanad (20,23) and Pathanamthitta (65, 69); C: Kasargod (24, 26), Malappuram (27,32M) and Kannur (32, 33); D: Ernakulam (34- 41) and Thrissur (42B- 45); E: Kottayam (46-52) and Idukki (53-59); F: Alappuzha (60-64) and Kollam (70A -78); G: Thiruvananthapuram (79-93); L- 1 kb plus DNA ladder (Thermo Scientific).

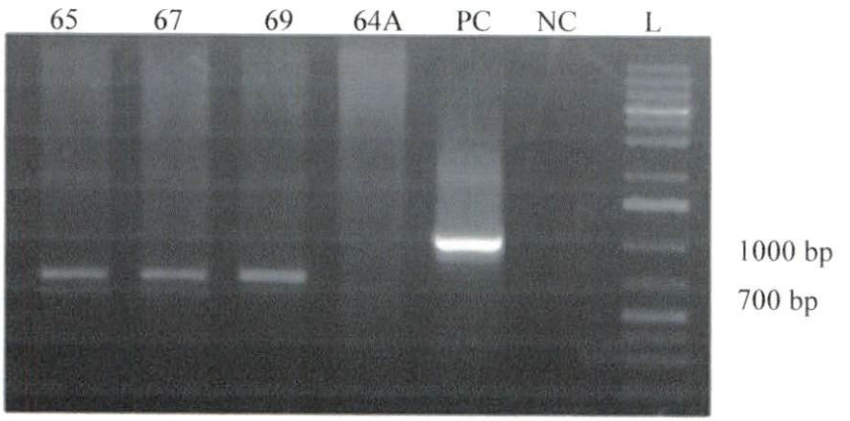


Fig. 9: ACMV PCR product analysis of samples from different districts (65-64A) on 1% gel. PC- ACMV positive control; NC- negative control; L- 1 kb plus DNA ladder (Thermo Scientific).

Table 7: PCR results of samples collected during the survey

Sample no.	PCR results			Sample no.	PCR results		
	ICMV	SLCMV	ACMV		ICMV	SLCMV	ACMV
1	-	-	-	53	+	+	-
2	-	-	-	54	+	-	-
3	-	-	-	55	-	+	-
4	-	+	-	56	+	+	-
5	-	-	-	57	-	+	-
5A	-	-	-	58	-	+	-
6	-	-	-	59	+	+	-
7	-	-	-	60	-	+	-
8	-	-	-	61	-	-	-
9	-	-	-	62	-	+	-
10	-	-	-	63	-	+	-
11	-	-	-	64	-	+	-
12	-	-	-	64 A	+	+	-
13	-	+	-	64 B	-	+	-
14	+	-	-	65	-	+	+
15	-	+	-	66	-	+	-
16	+	-	-	67	-	+	+
17	+	+	-	68	-	+	-
18	-	-	-	69	-	+	+
19	-	-	-	70 A	-	+	-
20	-	-	-	71	-	+	-
21	-	+	-	72	-	+	-
22 H	-	-	-	73	-	+	-
23	-	+	-	74	-	-	-
24	-	+	-	75	-	+	-
25	-	+	-	76	+	-	-
26	+	+	-	77	-	+	-
27	+	+	-	78	+	+	-
28	-	+	-	78 A	-	+	-
29	-	+	-	79	-	-	-
30	-	+	-	79 A	-	+	-
31	-	+	-	80	-	+	-
32	-	+	-	81 A	-	-	-
33	-	+	-	82	+	+	-

Table 7 continued

Sample no.	PCR results			Sample no.	PCR results		
	ICMV	SLCMV	ACMV		ICMV	SLCMV	ACMV
33A	-	+	-	83	-	+	-
33B	-	+	-	84	-	-	-
34	-	+	-	85	+	+	-
35	+	+	-	86	-	+	-
36	-	+	-	87	+	+	-
37 A	-	+	-	88	+	+	-
37 B	-	-	-	89	+	-	-
38	-	+	-	90	-	+	-
39 A	-	-	-	91	-	+	-
39 B	-	+	-	92	+	-	-
40	-	+	-	93	-	+	-
41	-	+	-	94	+	-	-
42 A	-	-	-	95	-	+	-
43	-	+	-	96	-	-	-
44	-	+	-	97	-	-	-
45	-	+	-	98	-	+	-
45 A	-	-	-	99	-	+	-
45 B	-	+	-	100	-	+	-
46	-	+	-	101	-	+	-
47	-	+	-	102	+	+	-
48	+	-	-	103	-	-	-
49	-	+	-	104	+	-	-
50	+	+	-	105	+	+	-
51	+	+	-	106	+	+	-
52	-	+	-				

Table 8: Distribution of cassava mosaic virus in different districts of Kerala

District	No. of samples collected	No. of samples having only ICMV infection	No. of samples having only SLCMV infection	No. of samples having mixed infection	No. of samples having both ACMV and SLCMV infection	No. of samples showing symptoms and no viruses detected	ICMV infection (%)	SLCMV Infection (%)	Mixed infection (%)
Thiruvananthapuram	29	4	12	7	0	6	13.8	41.3	24.1
Kollam	11	1	8	1	0	1	9	72.7	9
Pathanamthitta	5	0	2	0	3	0	0	100	0
Alappuzha	7	0	5	1	0	1	0	71.4	14.2
Ernakulam	8	0	7	1	0	0	0	87.5	12.5
Thrissur	6	0	5	0	0	1	0	83.4	0
Kottayam	7	1	4	2	0	0	14.3	57.1	28.5
Idukki	7	1	3	3	0	0	14.3	42.8	42.8
Palakkad	8	2	2	1	0	3	25	25	12.5
Malappuram	6	0	5	1	0	0	0	83.4	16.7
Kozhikode	11	0	1	0	0	10	0	9	0
Wayanad	3	0	2	0	0	1	0	66.7	0
Kannur	4	0	4	0	0	0	0	100	0
Kasargod	3	0	2	1	0	0	0	66.7	33.3

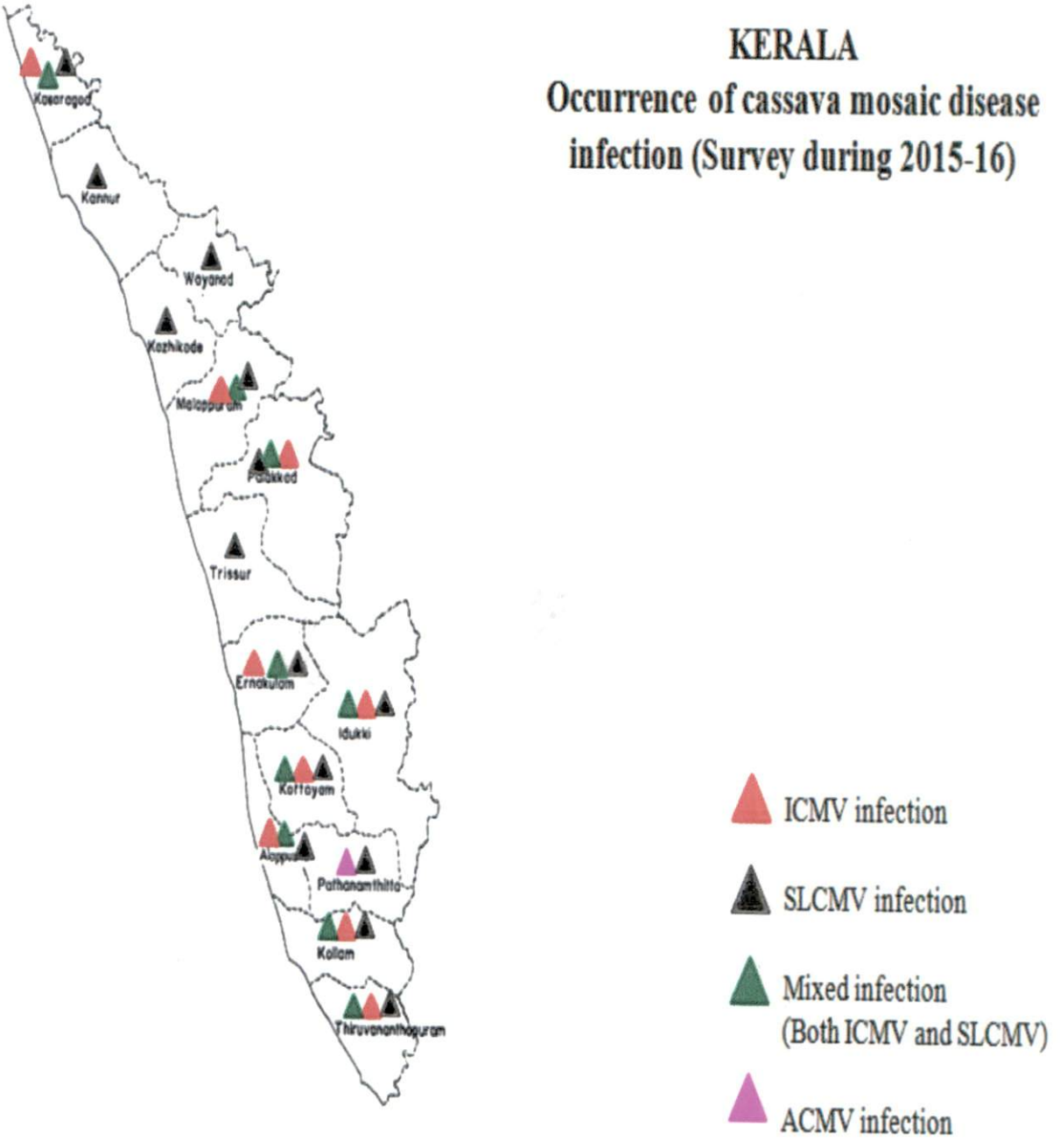


Fig. 10: The overall occurrence of cassava mosaic disease in Kerala obtained during the survey.

4.5 RESTRICTION ANALYSIS FOR IDENTIFYING THE VARIATION AMONG THE VIRUSES IDENTIFIED DURING THE SURVEY

4.5.1 Amplification of restriction enzyme rich region of DNA-A using specific primers

From different isolates of ICMV and SLCMV reported from Kerala and Tamil Nadu (Table 9), DNA-A components were analyzed and identified the restriction enzyme- rich region and found that position no. 1543 to 2757 and 1 to 534 were rich in enzymes mainly EcoRV, HindIII, XhoI, ClaI, BamHI and KpnI at positions 316, 2514, 2500, 2269, 1714 and 1543 respectively (Fig. 11). For amplifying this region, four combinations of primer pairs were used (Multi forward primer+ CP reverse primer, I/SLCMV reverse primer + Rep reverse primer, CP forward primer + Rep forward primer and Rep reverse primer + CP reverse primer). Of these 4 combinations, only I/SLCMV reverse primer + Rep reverse primer pair gave positive amplification with a band size of 1.7 kb encompassing the Replicase, CR and partl of CP region in the DNA-A component (position no. 1543 to 2757: 1 to 534) was obtained using this pair of primers (Fig. 12).

From the total samples collected, one sample each having ICMV infection, SLCMV infection and mixed infection, from each district were selected and amplified using this primer pair and the amplified products were used for restriction analysis for identifying the variation. The amplified 1.7 kb fragment was restricted with these respective enzymes EcoRV, HindIII, XhoI, ClaI, BamHI and KpnI separately. A sum total of 7 ICMV positive samples, 14 SLCMV positive samples and 3 mixed infected samples were used for restriction analysis using these enzymes.

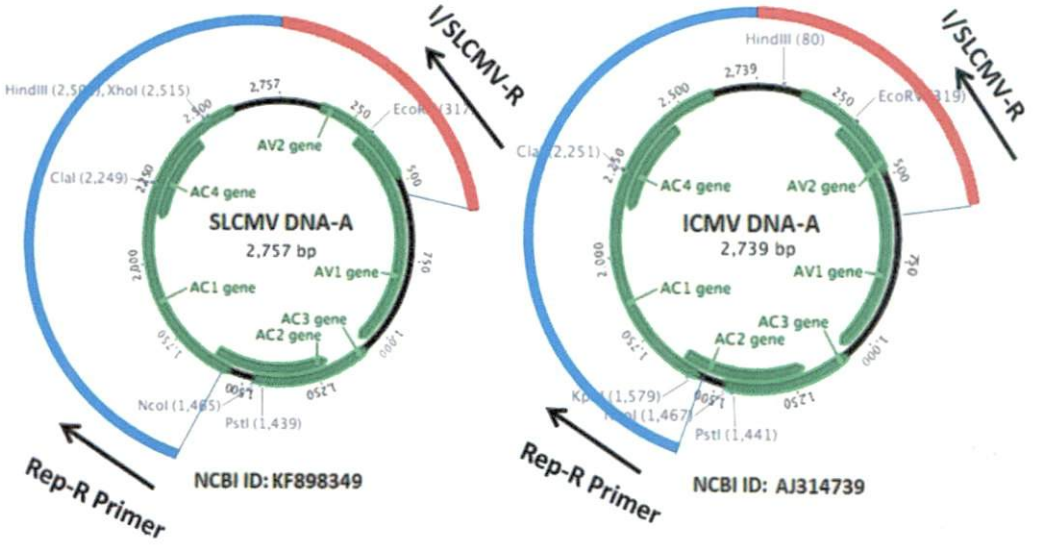


Fig. 11: ICMV and SLCMV DNA-A circular genome map showing positions of different restriction enzymes (Source: NCBI) and amplification strategy.

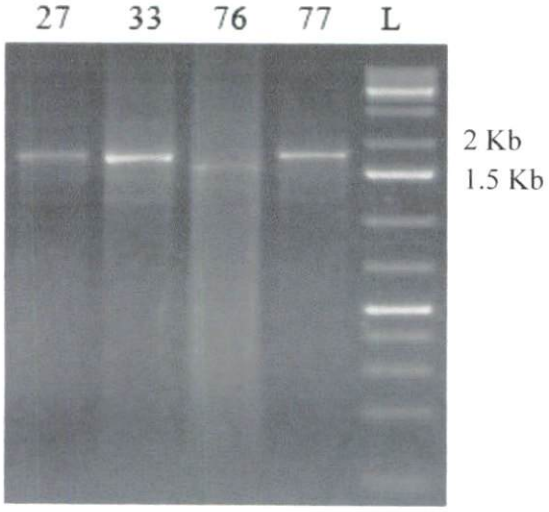


Fig. 12: Gel analysis of 1.7 kb PCR products of samples of different districts (23-77) run on 1% gel. L- 1 Kb plus DNA ladder (Thermo Scientific).

Table 9: List of ICMV and SLCMV isolates available in NCBI which are reported from Kerala and Tamil Nadu and the restriction map of their DNA-A components

NCBI ID	Sequence description	EcoRV	HindIII	XhoI	ClaI	BamHI	KpnI
KU308385.1	ICMV isolate TVM4	+	-	-	+	-	-
AJ575819.1	ICMV isolate Adivaram 2	+	-	-	-	-	-
KP455486.1	SLCMV isolate TVM3	+	+	-	++	-	-
KR611579.1	SLCMV isolate TVM1	+	+	-	+	-	-
KR611577.1	SLCMV isolate Malappuram	+	+	+	++	-	-
AJ579307.1	SLCMV isolate Adivaram	+	+	+	+	-	-
AJ607394.1	SLCMV isolate Salem	+	+	+	+	-	-
AJ890224.1	SLCMV isolate Kerala 15	+	+	+	+	-	-
AJ890225.1	SLCMV isolate Kerala 17	+	+	+	+	-	-
AJ890226.1	SLCMV isolate Kerala C4	+	-	+	+	-	-
AJ890227.1	SLCMV isolate Tamil Nadu 2	+	+	+	+	-	-
AJ890228.1	SLCMV isolate Tamil Nadu 6	+	+	+	+	-	-
AJ890229.1	SLCMV isolate Tamil Nadu 7	+	+	+	+	-	-
KC424490.1	SLCMV India[Attur:2009]	+	+	+	++	-	-
KF898349.1	SLCMV isolate Erode	+	+	+	+	-	-
KP455484.1	SLCMV isolate Attur 2	+	+	-	++	+	+
KP455486.1	SLCMV isolate TVM3	+	+	-	++	-	-
KR611577.1	SLCMV isolate Malappuram	+	+	+	++	-	-

The expected band sizes of restricted fragments were: 200 bp and 1.5 kb (EcoRV); 950 bp and 800 bp (HindIII); 970 bp and 770 bp (XhoI); 700 bp and 1000 bp (ClaI); 170 bp and 1.6 kb (BamHI) and; 270 bp and 1.4 kb (KpnI). The gel image showing restriction of Multi reverse + Rep reverse PCR amplified products of all districts are shown from Fig.13.

In contrast with the selected NCBI sequences (Table. 9), it was found that XhoI and HindIII sites were additionally present in two ICMV positive samples (27, 76) along with EcoRV and ClaI sites (Table. 10) indicating variation from the available sequences.

Table 10: Restriction analysis result of ICMV infected samples

Samples having ICMV infection from different districts	**EcoRV**	**XhoI**	**HindIII**	**ClaI**	**KpnI**	**BamHI**
NCBI sequences	+	-	-	+	-	-
Kasargod (sample no. 26)	+	-	-	+	-	-
Malappuram (sample no. 27)	+	+	+	+	-	-
Palakkad (sample no. 14)	+	-	-	+	-	-
Kottayam (sample no. 48)	+	-	-	+	-	-
Idukki (sample no. 54)	+	-	-	+	-	-
Kollam (sample no. 76)	+	+	+	+	-	-
Thiruvananthapuram (sample no. 92)	+	-	-	+	-	-

+: one site; -: restriction site absent

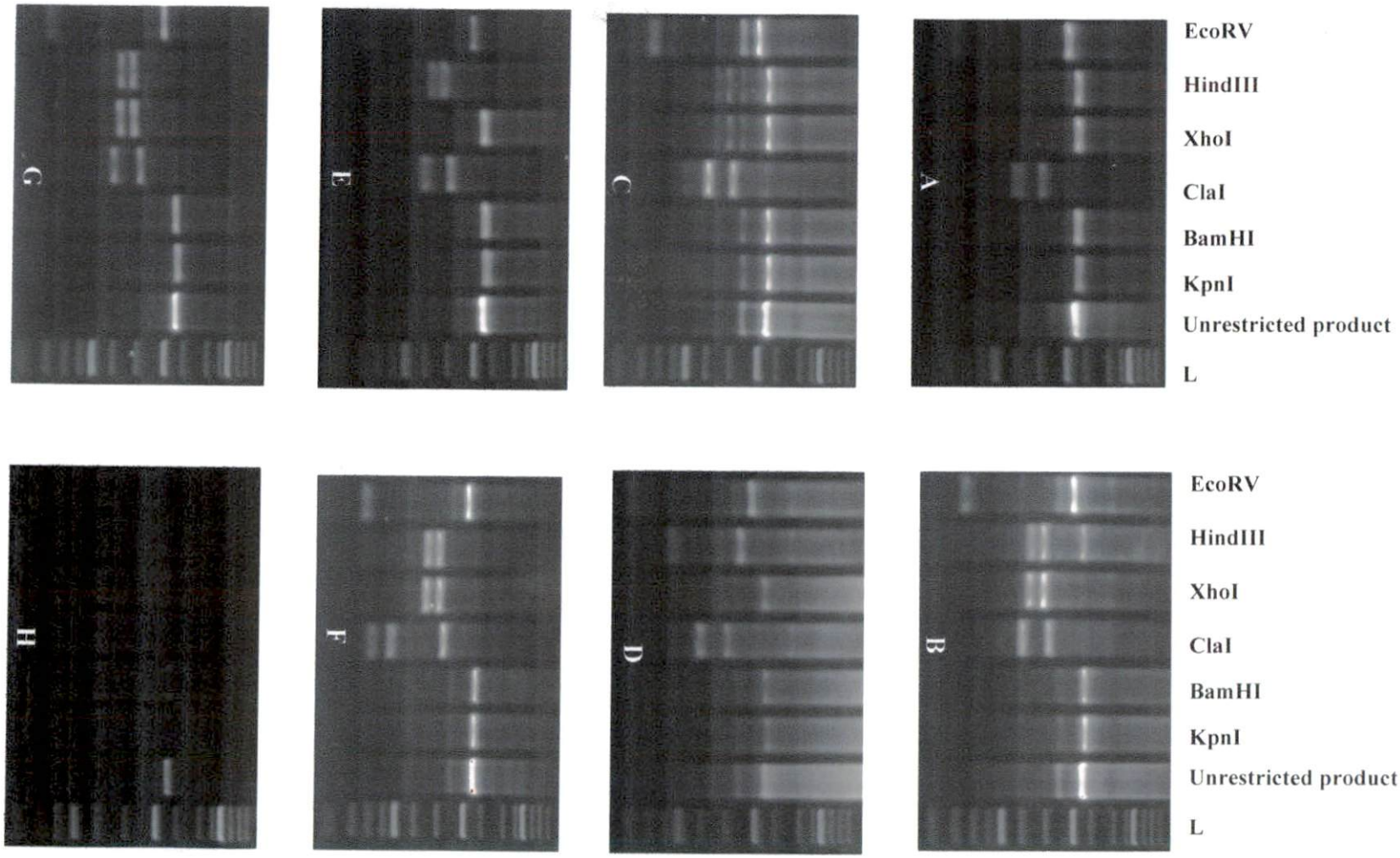
Restriction analysis of SLCMV positive samples from different districts shown that major variation existed within restriction sites of EcoRV, XhoI, HindIII and ClaI. All these four restriction sites were present in the selected NCBI sequences (Table. 9). In contrast, EcoRV site was absent in two samples (49, 55) and XhoI site

was absent in five samples (26, 33, 49, 55, 77). Similarly HindIII site was absent in five samples (26, 33, 49, 55, 68). ClaI sites are present in two positions in almost all NCBI sequences selected for the study, but were absent in three samples (33, 49, 55) (Table. 11).

Table 11: Restriction analysis result of SLCMV infected samples

Samples having SLCMV infection from different districts	**EcoRV**	**XhoI**	**HindIII**	**ClaI**	**KpnI**	**BamHI**
NCBI sequences	+	+	+	++	-	-
Thiruvananthapuram (sample no. 83)	+	+	+	+	-	-
Kollam (sample no. 77)	+	-	+	+	-	-
Pathanamthitta (sample no. 68)	+	+	-	+	-	-
Alappuzha (sample no. 63)	+	+	+	+	-	-
Ernakulam (sample no. 34)	+	+	+	+	-	-
Thrissur (sample no. 42B)	+	+	+	+	-	-
Kottayam (sample no. 49)	-	-	-	-	-	-
Idukki (sample no. 55)	-	-	-	-	-	-
Palakkad (sample no. 13)	+	+	+	+	-	-
Malappuram (sample no. 29)	+	+	+	+	-	-
Kozhikode (sample no. 4)	+	+	+	+	-	-
Wayanad (sample no. 23)	+	+	+	+	-	-
Kannur (sample no. 33)	+	-	-	-	-	-
Kasargod (sample no. 26)	+	-	-	+	-	+

+: one site; ++: two sites; -: restriction site absent.


G
E
C
A
EcoRV
HindIII
XhoI
ClaI
BamHI
KpnI
Unrestricted product
L
H
F
D
B
EcoRV
HindIII
XhoI
ClaI
BamHI
KpnI
Unrestricted product
L

EcoRV
HindIII
XhoI
ClaI
BamHI
KpnI
Unrestricted product
L
I
J
K
L
M
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P

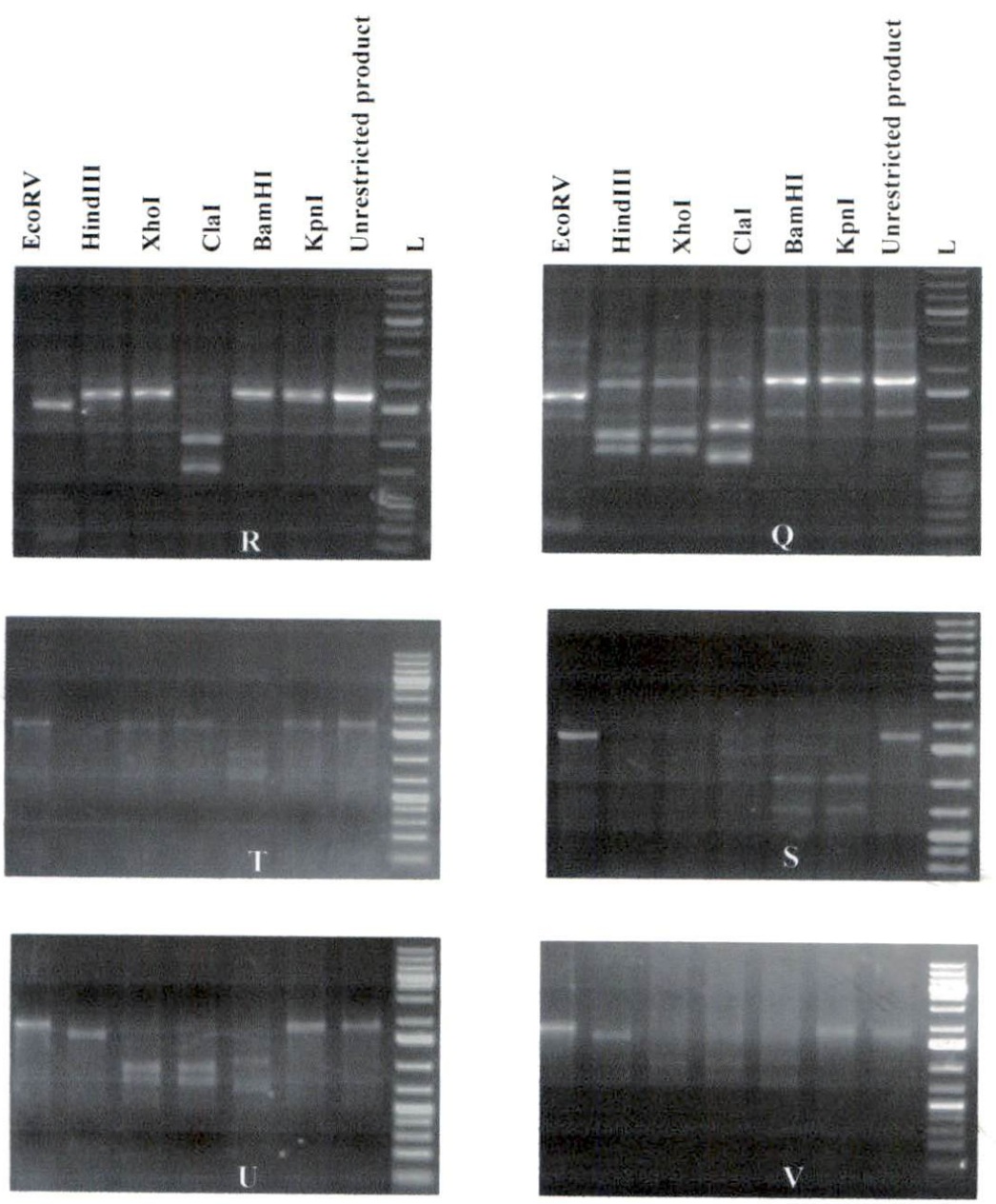


Fig. 13: Restriction analysis of PCR amplified 1.7 kb product (Multi-R + Rep-R primer pair) of samples of different districts. A: Thiruvananthapuram ICMV, B: Thiruvananthapuram SLCMV, C: Thiruvananthapuram mixed, D: Kollam ICMV, E: Kollam SLCMV, F: Alappuzha SLCMV, G: Wayanad SLCMV, H: Idukki ICMV, I: Idukki SLCMV, J: Thrissur SLCMV, K: Ernakulam SLCMV, L: Ernakulam mixed, M: Pathanamthitta SLCMV, N: Kannur SLCMV, O: Malappuram ICMV, P: Malappuram SLCMV, Q: Malappuram mixed, R: Kasargod ICMV, S: Kasargod SLCMV, T: Palakkad ICMV, U: Palakkad SLCMV, V: Kozhikode SLCMV.

4.6 SEQUENCE ANALYSIS

4.6.1 Sequence analysis of multiplex PCR products

From the restriction analysis results, it was found that totally 8 samples shown high variation which comprises, two ICMV samples (76, 27) and six SLCMV samples (26, 33, 49, 55, 68, 77). Among these, 5 samples [3 ICMV (26, 76, 92) and 2 SLCMV (23, 39A)] were selected for amplifying with multiplex PCR and these PCR products were sequenced at Eurofins Pvt. Ltd. (Bangalore).

The sequences obtained were analysed and consensus sequence for each sample was obtained using GENEIOUS version 9.1. These sequences were used for further analysis and are listed below.

Sample no. 76 (889 bp)

TGAGAAGTGCAGATGAGGCTCCCCATTCTCATGTAGCTCTCTGCATATTTT
GATGAATTTAGGGTTTGTAGGTGTTTGAAAGCTCCGAATTTGAGAGAGAG
CCTCTTCTTTAGTTAAGGAGCATCGAGGGTAAGTGAGGAAATAGTTTTTA
GCGTTTATTTGAAAGCGCTTAGGTGGTGACATTCTCTCTCCTCAATCGGTA
CTCAGATAGTTTAGCCCCCATATTAGGTACTCAATATATACATGAGTACC
AAATGGCATAGAAGTAAATAATGGGAATATAATTTGAAATCAAAAGCGG
CCATCCGTATAATATTACCGGATGGCCGCGCCCCCCGCTTTGTGGTGGCCC
CCTCCCCCACGTGGAGATGTCCCCCACTCAGAACGCTCGCTCAAAGCTTG
TATATGTGTGGTCCCCCTTTAAGTACTTGGGCAACAAGTTGTTGCCTGTAC
AATGTGGGACCCTTTACTAAACGAGTTCCCTGAGTCTGTCCACGGTTTCCG
GTGTATGCTCGCCGTGAAATACCTTCAGCTGGTTGAATGTACCTATTCTCC
GGATACACTCGGTTACGATTTAATCAGAGATTTGATCTCTGTTATTAGGGC
GAAGAATTATGTCGAAGCGACCAGCAGATATCATCATTTCAACTCCCGCC
TCGAAGGTGCGTCGCCGTCTGAACTTCGACAGCCCATACAGCAGCCGTGC
TGCTGTCCCCACTGTCCGCGTCACAAAAAGACAAGCCTGGACAAACAGGC

CCATGAATCGGAAGCCCAGGTGGTACAGGATGTACAGAAGCCCAGATGT
TCCTAAGGGCTGTGAAGGCCCATGTAAGGTCCAGTCATCGAGTCGAGACA
CGATGTGGTCCAATTATACAAATAAGAGACTGGT

Sample no. 92 (884 bp)

TTAGAGAGTGCAGATGAGGCTCCCCATTCTCATGTAGCTCCCTGCATATTT
TGATGAATTTAGTGTTTGTAGGTGTTTGAAAGTTCCTAATTTGAGAGAGA
GCCTCTTCTTTAGTTAAGGAGCATCGAGGGTAAGTGAGGAAATAGTTTTT
AGCGTTTATTTGAAAGCGCTTAGGTGGTGACATTCTCTCTCCTCAATCGGT
ACTCAGATAGTTTAGCCCCCATATTAGGTACTCAATATATACATGAGTAC
CAAATGGCATAGATGTAAATAATGGAAATATAATTTGAATTCAAAAGCGG
CCATCCTTATAATATTACCGGATGGCCGCGCCCCCCGCTTTGTGGTGGACC
CCCCCACGTGGAGATGTCCCCCACTCAGAACGCTCCCTCAAAGCCTGTAT
AGTTGTGGTCCCTCTTTAAGTACTTGCTCAGCAAGTTGTAATCTGCACAAT
GTGGGACCCTTTGGTAAACGAGTTCCCGGATTCAGTTCACGGTTTCCGGT
GTATGCTTGCCGTGAAATATCTTCAGCTAGTTGAAGGTACTTATTCCCCCG
ATACACTCGGTTACGATTTAATCAGAGATTTGATCTCTGTCATCAGGGCCA
AAAATTATGTCGAAGCGACCAGCAGATATCATCATTTCAACTCCCGCCTC
GAAGGTTCGTCGCCGTCTGAACTTCGACAGCCCATACAGCAGTCGTGCTG
CTGTCCCCACTGTCCGCGTCACAAAAAGACAAGCCTGGACAAACAGGCCC
ATGAATCGGAAGCCCAGGTGGTACAGGATGTTCAAAAGCCCAGATGTTCC
TAGGGGATGTGAAGGCCCATGTAAGGTTCAATCGTTTGAGTCCAGACACG
ATGTGGGTCAATATAAACAAAAAGGTAT

Sample no. 26 (843 bp)

GGCAGCAGGAGTGCAGATGAGGCTCCCCATTCTCATGTAGCTCCCTGCAT
ATTTTGATGAATTTAGGGTTTGTAGGTGTTTGAAAGTTCCTAATTTGAGAG
AGAGCCTCTTCTTTAGTTAAGGAGCATCGAGGGTAAGTGAGGAAATAGTT

TTTAGCGTTTATTTGAAAGCGCTTAGGTGGTGACATTCTCTCTCCTCAATT
GGTACTCAGATAGTTTAGCCCCCATATTAGGTACTCAATATATACATGAG
TACCAAATGGCATAGATGTAAATAATGGGAATATAATTTGAATTCAAAAG
CGGCCATCCGTATAATATTACCGGATGGCCGCGCCCCCCGCTTTGTGGTG
GACCCCCCCCCACGTGGAGATGTCCCCCACTCAGAACGCTCCCTCAAAGTC
TGTATAGTTGTGGTCAAATCTTTAAGTACTTGCTCAGCAAGTTGTAATCTG
CACAATGTGGGACCCTTTGGTAAACGAGTTCCCTGAATCAGTTCACGGTT
TCCGGTGTATGCTTGCCGTGAAATATCTTCAGCTAGTTTGAAGGTACTTAT
TTCCCCCGATACACTCGGTTATGATTTAATCAGAGATTTGATCTCTTGTCA
TCAGGGCCAAAAATTATGTCGAAGCGACCAGCAGATATCATCATTTCAAC
TCCCGCCTCGAAGGTTCGTCGCCGTCTGAACTTCGACAGCCCATACAGCA
GTCGTGCTGCTGTCCCCACTGTCCGCGTCACAAAAAGACAAGCCTGGACA
AACAGGCCCATGAATCGGAAGCCCAGGTGGTATCGGATGTACAGAAGCC
CAGATGTCCTAAGGGCTGTGAAGGCCCAGAAGTTCAGAG

Sample no. 39A (604 bp)

GTCGTGATGAGATCAAAGCGGCCATCCGTATAATATTACCGGATGGCCGC
GCCCCCCGCTTTGTGGTGGACCCCCCCCCCACGTGGAGATGTCCCCCACTC
AGAACGCTCCCTCAAAGCCTGTATAGTTGTGGTAAACTCTTTAAGTACTTG
CTCAGCAAGTTGTAATCTGCACAATGTGGGACCCTTTGGTAAACGAGTTC
CCGGAGTCAGTTCACGGTTTCCGGTGTATGCTTGCCGTGAAATATCTTCAG
CTAGTTGAAGGTACTTATTCCCCCGATACACTCGGTTACGATTTAATCAGA
GATTTGATCTCTGTCATCAGGGCCAAAAATTATGTCGAAGCGACCAGCAG
ATATCATCATTTCAACTCCCGCCTCGAAGGTTCGTCGCCGTCTGAACTTCG
ACAGCCCATACAGCAGTCGTGCTGCTGTCCCCACTGTCCGCGTCACAAAA
AGACAAGCCTGGACAAACAGGCCCATGAATCGGAAGCCCAGGTGGTACA
GGATGTTCAAAAGCCCAGATGTTCCTAGGGGATGTGAAGGCCCATGTAAG
GTTCAATCGTTTGAGTCCAGACACGATGTGGTTCCATATTAAAAAAATTT

Sample no. 23 (641 bp)

AAAGTACCGTCCAAATTTAAAATTGAAATTGAGGTTCCAAAGCGGCCCTC
CTTATAATATTACCGGATGGCCGGCGCCCCCCGCTTTTGTGGTGGGCCCCC
CCCCCACGTGGAGATGTCCCCCCCTTCAGAACGCTCCCTCAAAGCCTGTTT
AGTTGTGGTCCCTCTTTAATTACTTGCTCAGCAATTTGTAATTCTGCACAA
TGTGGGAACCTTTTGGTAAACGATTTCCCGGATTCATTTCACGGGTTTCCG
GTGTATGGCTTGCCGTGAAATATCTTCAGCTAGTCCGAAGGTATTTATTCC
CCCGATACACTTCGGTTACGATTTAATCCAGAGATTTGATCTCTGTCTTCG
GGGCCAAAAATTTTGGTCGAAGCGACCGGCAGATTTCATCCTTTTCAATT
CCCGCCTCGAAGGTTCGTCGCCGTCTGAAATTTCGACAGCCCATTACAGC
ATTTCGTGCTGGTGGTCCCCACTGGTCCGGGTCACAAAAAGACAAGCCTG
GACAAACAGGCCCATGGAATCGGAAGCCCAGGTGGTACGGGATGTTCAA
AGGCCCGAGATGTTCTTAGGGGATGTGAAAGGCCCATGGTAATGGGTTCA
TCCTTTGGATTCAGAACCCAAGGTGGCCATAAAATA

By blast analysis of the multiplex PCR consensus sequences, it was found that all the 5 samples showed more than 80% similarity with the available ICMV and SLCMV sequences in the NCBI. Sample no. 76 showed maximum similarity with isolate ICMV-Mah (AJ314739.1) having 95 % query cover and 92 % identity. Sample no. 92 showed maximum similarity with 2 sequences namely isolate ICMV-Mah (AJ314739.1) and isolate ICMV-Mah2 (AYJ30035.2) both having 41% query cover and 99% identity. Sample no. 26 showed maximum similarity with 3 sequences namely isolate SLCMV-Tamil Nadu 7 (AJ890229.1), isolate Adivaram (AJ579307.1) and isolate Attur (KC424490.1), all having 94 % query cover and 93% identity. Sample no. 39A showed maximum similarity with 2 sequences namely isolate SLCMV-TVM3 (KP455486.1) and isolate SLCMV-TVM1 (KR611579.1) both having 95 % query cover and 89 % identity. Sample no. 23 showed maximum

similarity with isolate SLCMV-TVM3 (KP455486.1) having 50 % query cover and 96 % identity.

Based on these sequence analysis it was confirmed that the samples 26,76,92 belongs to ICMV and 23 & 39A belongs to SLCMV.

To identify the molecular level variation in the above samples sequenced, multiple sequence alignment was done using GENEIOUS 9.1 with alignment type "Global alignment with free gaps" and cost matrix "93% similarity (5.0/9.026168)". All the 5 samples were aligned with available I/SLCMV DNA-A sequences (Table. 12) and the analysis results are shown below (Fig. 14).

It was observed that all the 5 samples (ICMV - sample No. 26,76,92 & SLCMV - sample no. 23, 39A) aligned with the reference sequences within the 553 bp conserved region (position 1-553 with respect to reference sequences). Sample no. 23, 76 and 92 shown maximum identity within them having only 5 SNPs between them.

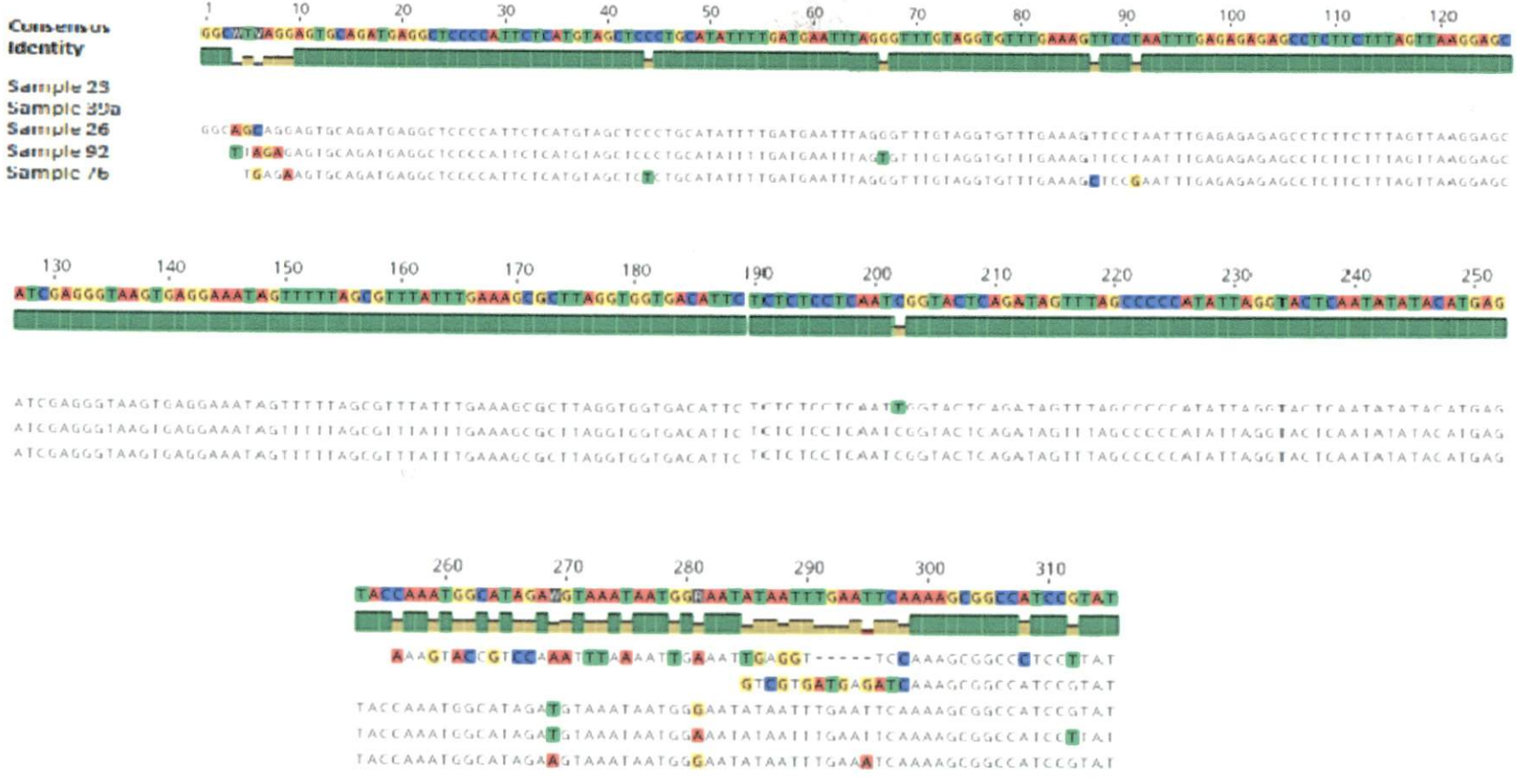
Between the 5 samples aligned with the reference sequences, sample no. 23 had maximum variability with respect to other samples having 23 nucleotide additions (position no. 5, 12, 23, 61, 128, 147, 177, 192, 217, 260, 295, 298, 321, 373, 382, 392, 401, 442, 480, 502, 509, 527), 2 deletions (position no. 59, 531) and 34 single nucleotide polymorphisms (SNPs) (position no. 33, 58, 84, 87, 88, 105, 120, 143, 158, 171, 217, 218, 226, 279, 282, 309, 316, 322, 329, 360, 380, 389, 405, 463, 475, 488, 513, 522, 528, 531, 534, 536, 538, 540) followed by sample no. 76 with 4 additions (position no. 82, 95, 128, 551), 6 deletions (position no. 79, 125, 126, 523, 524, 525) and 14 SNPs (131, 149, 150, 170, 173, 222, 227, 236, 278, 281, 287, 290, 345, 385) and sample no. 26 with 4 additions (position no. 96, 97, 231, 276), 4 deletions (position no. 486, 508, 510, 513) and 6 SNPs (position no. 79, 167, 251, 462, 463, 553). Sample no. 39A had 6 additions (95, 96, 97, 217, 545, 551) and 2

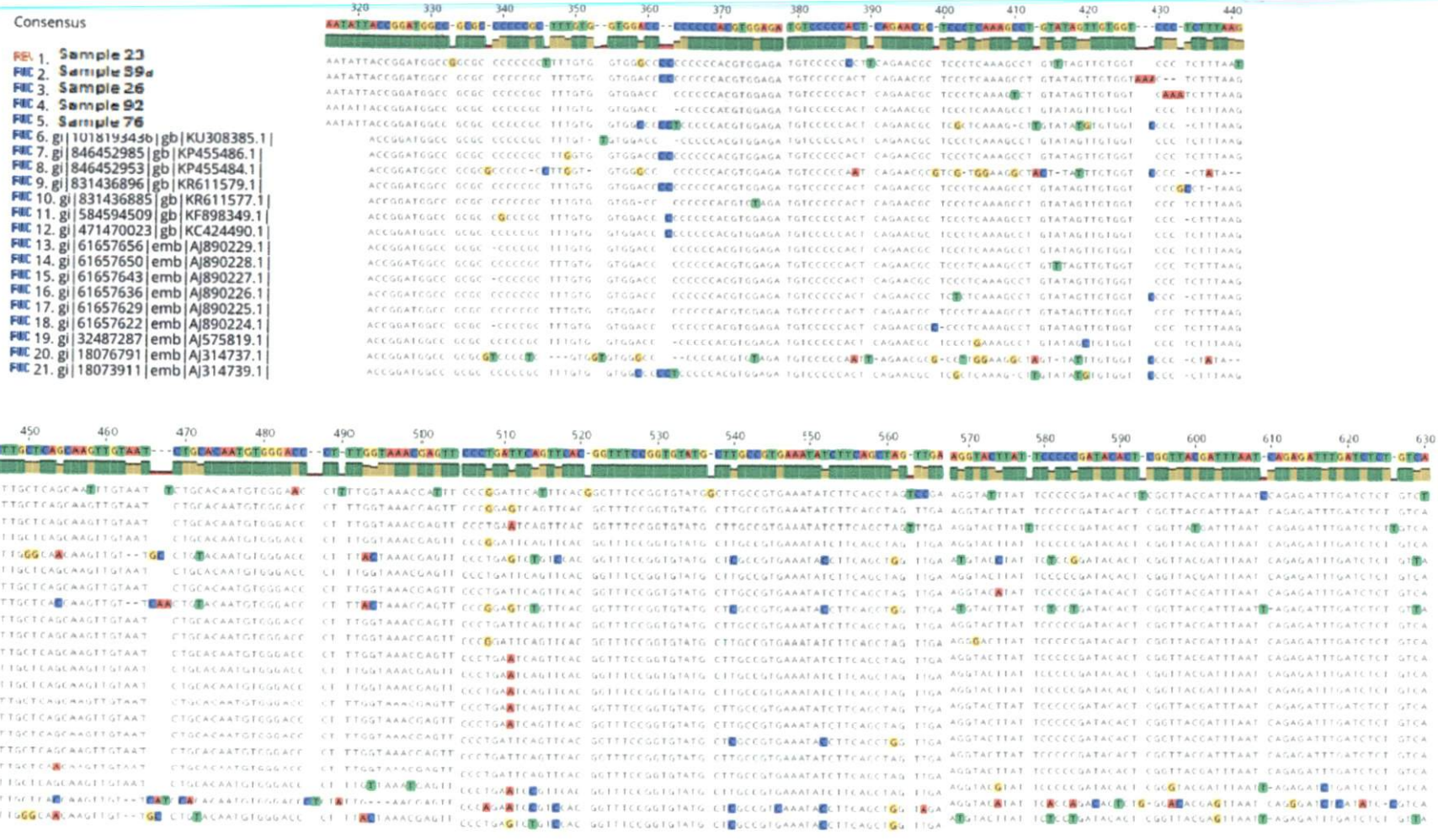
deletions (position no. 96, 97) but no SNPs. The least variability was observed in sample no. 92 with 2 nucleotide additions only (position no. 545, 547).

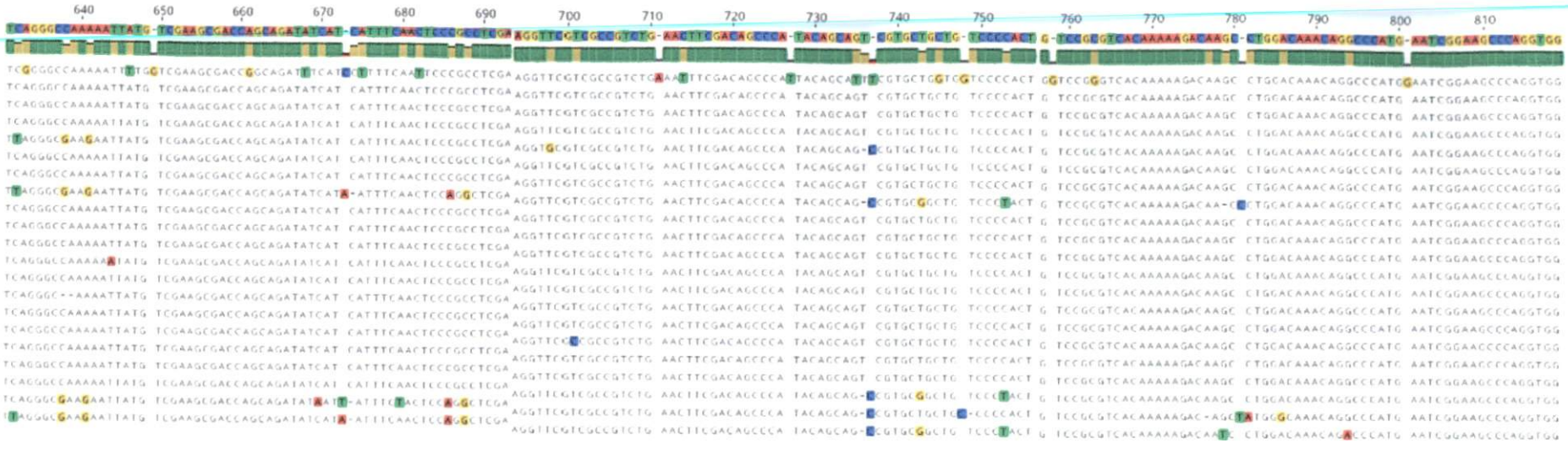
Table 12: Reference sequences of cassava mosaic viruses DNA-A of ICMV & SLCMV used for multiple sequence alignment

NCBI ID	**Sequence description**
AJ314737.1	SLCMV isolate SLCMV-Col
AJ314739.1	ICMV isolate ICMV-Mah
AJ575819.1	ICMV isolate Adivaram 2
AJ890224.1	SLCMV isolate Kerala 15
AJ890225.1	SLCMV isolate Kerala 17
AJ890226.1	SLCMV isolate Kerala C4
AJ890227.1	SLCMV isolate Tamil Nadu 2
AJ890228.1	SLCMV isolate Tamil Nadu 6
AJ890229.1	SLCMV isolate Tamil Nadu 7
KC424490.1	SLCMV [Attur:2009]
KF898349.1	SLCMV Erode
KP455484.1	SLCMV isolate Attur 2
KP455486.1	SLCMV TVM3
KR611577.1	SLCMV isolate Malappuram
KR611579.1	SLCMV isolate TVM1
KU308385.1	ICMV isolate TVM4
NC003861.1	SLCMV isolate [Colombo]

Fig. 14: Sequences alignment results of multiplex PCR samples aligned with reported sequences (NCBI) obtained using GENEIOUS 9.1




Consensus
REV 1. Sample 23
REV 2. Sample 39d
REV 3. Sample 26
REV 4. Sample 92
REV 5. Sample 76
REV 6. gi|1018195456|gb|KU308385.1|
REV 7. gi|846452985|gb|KP455486.1|
REV 8. gi|846452953|gb|KP455484.1|
REV 9. gi|831436896|gb|KR611579.1|
REV 10. gi|831436885|gb|KR611577.1|
REV 11. gi|584594509|gb|KF898349.1|
REV 12. gi|471470023|gb|KC424490.1|
REV 13. gi|61657656|emb|AJ890229.1|
REV 14. gi|61657650|emb|AJ890228.1|
REV 15. gi|61657643|emb|AJ890227.1|
REV 16. gi|61657636|emb|AJ890226.1|
REV 17. gi|61657629|emb|AJ890225.1|
REV 18. gi|61657622|emb|AJ890224.1|
REV 19. gi|32487287|emb|AJ575819.1|
REV 20. gi|18076791|emb|AJ314737.1|
REV 21. gi|18073911|emb|AJ314739.1|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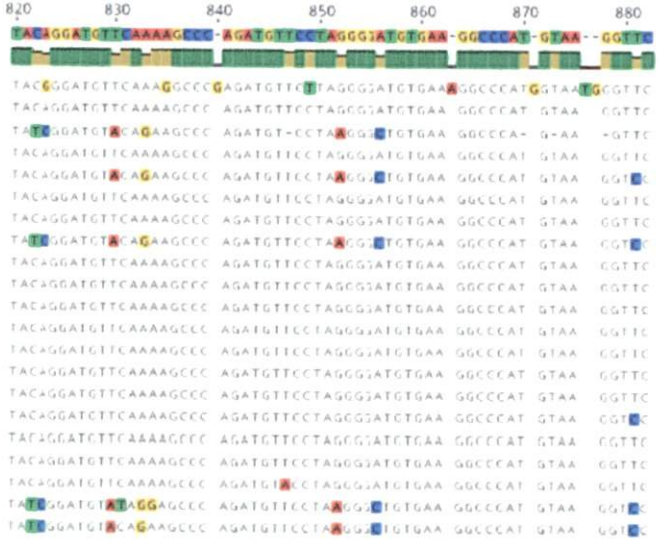
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4.6.2 Construction of phylogenetic tree

Using 27 reference sequences (Table. 13) and 5 samples from this study, multiple alignments was done in CLUSTALW program with default parameters settings in BIOEDIT version 7.2.5. and generated a phylogenetic tree using the neighbor-joining and bootstrap options in MEGA 6.0 with a 1000 replicate bootstrap search (Fig. 15). Beet curly top virus (BCTV) sequence was used as the out-group species.

Table 13: Reference sequences used for phylogenetic tree construction

NCBI ID	**Sequence description**
AJ314737.1	SLCMV isolate SLCMV-Col
AJ314739.1	ICMV isolate ICMV-Mah
AJ575819.1	ICMV isolate Adivaram 2
AJ579307.1	SLCMV isolate Adivaram
AJ607394.1	SLCMV isolate Salem
AJ890224.1	SLCMV isolate Kerala 15
AJ890225.1	SLCMV isolate Kerala 17
AJ890226.1	SLCMV isolate Kerala C4
AJ890227.1	SLCMV isolate Tamil Nadu 2
AJ890228.1	SLCMV isolate Tamil Nadu 6
AJ890229.1	SLCMV isolate Tamil Nadu 7
AM231025.1	ICMV recombinant defective DNA (SLCMV-[Col]A and SLCMV-[Ker4]B)
AY188956.2	ICMV-[Lucknow] AV2 protein (AV2) gene, complete cds; and coat protein (AV1) gene, partial cds
AY730035.2	ICMV isolate Mah-2
DQ302764.1	SLCMV segment A, partial sequence

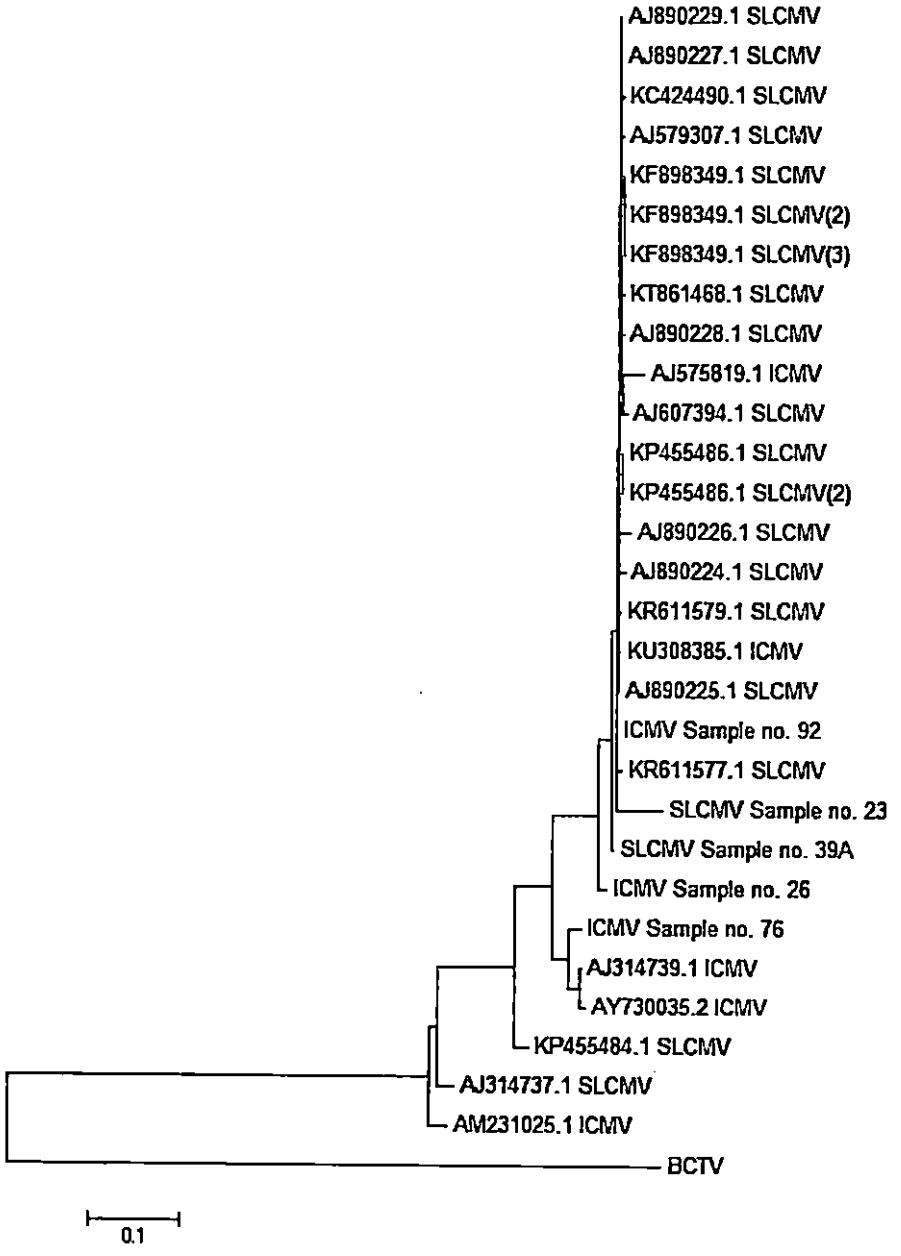

Fig. 15: Phylogenetic tree of the multiplex sequenced samples obtained using MEGA 6.0.

NCBI ID	Sequence description
DQ303479.1	ICMV isolate ICMV-TN
EU439256.1	ICMV-[Lucknow] precoat protein (AV2) and coat protein (AV1) genes, complete cds
KC424490.1	SLCMV [Attur:2009]
KF898349.1	SLCMV Erode segment DNA-A
KP455484.1	SLCMV isolate Attur 2
KP455486.1	SLCMV isolate TVM3
KR611577.1	SLCMV isolate Malappuram
KR611579.1	SLCMV isolate TVM1
KT276920.1	Beet curly top virus isolate CTS11-88
KT861468.1	SLCMV isolate SLCMA_A segment DNA-A
KU308385.1	ICMV isolate TVM4
Z24758.1	ICMV encoding AR0 complete CDS

4.6.3 Sequence analysis of full genome:

Six samples showing variation in the restriction analysis [ICMV (27) and SLCMV (26, 33, 49, 55, 68)] were selected for their whole genome amplification using RCA. The amplified products were obtained as a shear of concatemers while running on 1% gel (Fig. 16).

While analysing DNA-A genome of the available ICMV and SLCMV isolates from NCBI (Table. 12), it was found that PstI restriction site is present in DNA-A, but absent in DNA-B. Similarly, KpnI restriction site is present in DNA-B, but absent in DNA-A. So, these two enzymes were used separately to digest RCA products and the whole genome fragments (2.7 kb) of DNA-A (Fig. 17) and DNA-B (Fig. 18) were obtained. The fragments were further confirmed as DNA-A or DNA-B by PCR using coat protein gene and movement protein gene specific primers.

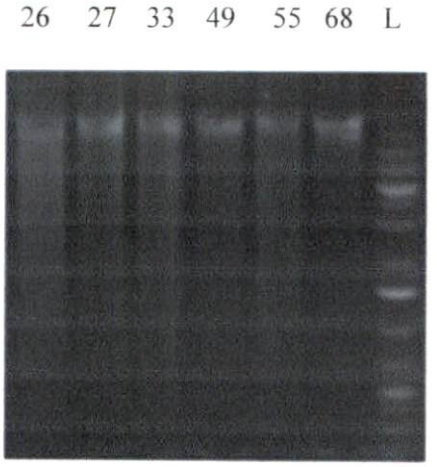

Fig. 16: Gel image showing RCA products of samples from different districts (26-68) as a smear of concatemers; L- 1 kb plus DNA ladder (Thermo Scientific).

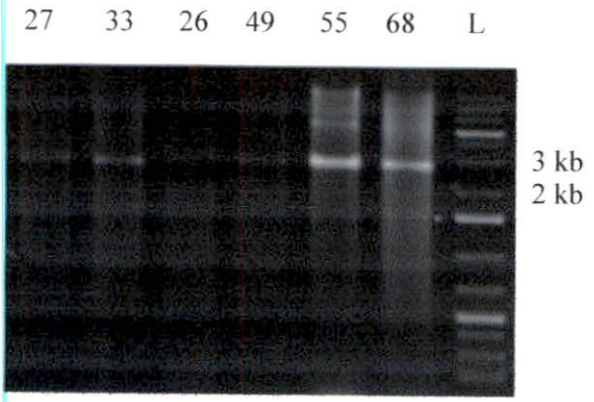

Fig. 17

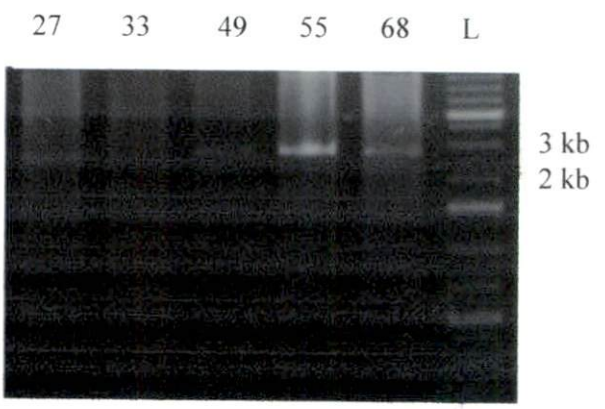

Fig. 18

Fig. 17: RCA amplified products of different districts (26-68) restricted with PstI.

Fig. 18: RCA amplified products of different districts (27-68) restricted with KpnI.

L- 1 Kb plus DNA ladder (Thermo Scientific).

The RCA restricted products were gel purified using QIAEX-II gel extraction kit (QIAGEN, Germany) and elute check was done in 1 % gel. The eluted fragment were cloned into pUC18 (Appendix IX) and transformed into *E. coli* DH5α using manual method. The recombinant clones obtained for DNA-A and DNA-B were analyzed by colony PCR using coat protein gene and movement protein gene specific primers respectively. Positive clones were obtained for DNA- A (27, 33, 55) and DNA-B (55, 68) components. Plasmids were isolated from these positive clones and checked on 1% gel and then they were sequenced at Eurofins Pvt Ltd. (Bangalore).

While sequencing (partial genome), only two samples (27, 33) of DNA-A gave quality sequence reads. These sequences were aligned and consensus sequence for each sample was obtained using GENEIOUS version 9.1. The sequences obtained are listed below and the same was used for further analysis.

Sample no. 27 (1428 bp)

GACCCAGCTCGGGGGAAACGCTGGAGTCGATCTCTCAGGGAGGAGTTCC
ACGGCGGGTGAATGCATGGTTTAAAAAAAGGGGGATGTTTTGATAAGTG
AAGCGAACTCAAGAGAAGGCGTGGGTAACATGGGGCGGTGCGGAACAAG
TGGGATGTATCACCTTAAATATAACCTCAGTAGATCCGCGCAAAAAGAGC
GCCCCTGATCTTGTTGTTCGGCGCCAGCGCAAAGAGGGCACTGCTGGTTA
CAGGGGCTTGGATGATTGGGAACGTGAATTCGACACTGTAATCCCGTCGC
TTGGTATCTTACTTCCTGACGTCTCATTACCTTGTCCTTGTCTCAGGCTCGC
ATGGAAGCGAATGGCCAGACAGAGGAACAAGGAGCCGCCCTGAAATAAA
GAAAGTCAATTAGCTAATTAGCAATGAATAAAAGTAAAAAAGAATTGAA
AAAGATTAGGAATGACCCATAACCCGTTGGCGCGCTCAGCGATGATGGCG
TAAATATCTGGGACGTTCCAAATCCCCTCTATTTCAGGATCATCCAGCATT
TAACGACAGCCGGCACGTAGAAAATCAACAGGCCGTGGGACATAATAAC
CATTCAGATACGCGTTCAACCACAACCTGAAGGACGAAGCGCAATTTGGG
ACTACATCAATGCTGAATGGATTCCTCAAGGTCTGGACGACCTTACAGCC

TCAGACCTTTTCGGCGTTTCTTGAGGGTATTTAAGACCCAAGTATTAAAAT
ACCTCGATAGATTAGGGATTCTATGTATTAATACGATTGTAAACGCAGTA
GAACATGTATTATATAATGTAATCGCTGGGACTGACAGTATTGAGCAGTC
TAATTTAATAAAAAATTTAATGTTTATTAATTGCTGAACGAATCATAGAA
GTAGATTCTAATCTTCAGCGTAGCGTATACAGGCGTTAGCGAGGCATGAG
TACACGCCATGTACAGCATCAATGCATTCTCGGTATGATTTTCATACTTGC
CAGCCTCTTGCTGGTTATACACAACATAATTATTAACTCTAAAAAAACGC
CTAACTAAAGCCTGTTCCTTGCTCGCATACTGACCACCAGTGACAGTGGC
ACTCCCACTTCCTGAGGACTTTGATAACGATCACGATGCATGTTCTTCACC
GTAGCTGTACTAAGTTCATTATCGAACATATAAACACTTTAGCAAAATCC
TGGGGGCTTATCAACGGGGCCTACGATCCCTTAGCAGGAAAAAACATCAC
AGCAAATCTTAAGAGTCCTTGGTCCTTATCATTTTTCGTCCGTCCAGAACG
TTACACGGGACATAAAGGGAATTTACGAGAGACCTATTTAACCAAGGAG
AGAAGTAACCCAAATGCAACAAGTGCGCATAGACAAACATATTAGATGA
GCCTGTTTAGACGACGCCGGGTGTGGAGG

Sample no. 33 (1623 bp)

CGCGTGATGAGTTCCCTGTGCGTGAATCCATGGTTGTGGCAGTTGATGTG
CACGTAGTATGAGCAGCCGCAATTGAGGTCTACCCTCCGTCGCCGAATGG
CCTTACGCTTAGCTGCCCTGTGTTGGACCTTGATTGGAACCTGAGTAGAGC
GGCTCGCTGAGGGAGATGAAGGTCGCATTCTTCAGAGCCCAAGCCTTCAA
TGCGCTATTCTTCGCCTCGTCAAGGAATTCTTTATAGCTGGAATTGGGCCC
AGGATTGCAGAGGAAGATAGTGGGAATTCCCCCTTTAATTTGAACTGGCT
TCCCGTACTTGGTGTTTGACTGGCAGTCTCTTTGGGCCCCCATGAATTCCT
TAAAGTGTTTTAGATAGTGCGGATCTACGTCATCAATTACGTTGTACCATG
CATCATTGCTGTAAACCCTAGGACTCAAGTCCAGATGTCCACACAGATAA
TTGTGTGGACCTAATGACCTAGCCCACATTGTTTTGCCTGTACGACTATCG
GCCTCGATGACTATACTATTAGGTCTCAAAGGCCTCGCAGAGGCACCCAT

GACGTTCTCTGACACCCACTCCTCAAGTTCATCTGGAACTTGGTCAAATGA
ATAGGCTGAGAAGGGAGACACATAAACCTCGGGAGGAGGTGTAAAAATC
CTATCTAAATTAGCATTTAGATTATGAAATTGTAAAACATAATCCTTGGGT
GCTAACTCCTTAATGGGACTCTAAGAGCCTCTGGCTTACTGCCTGCAGGTC
AAGTGCTGCATGGCGTATACGTCGTTTGCTGTCTGCTGTCCCCTCTATTTG
CAGATCTTCCAGCCGACGATCTGAAGTTCACCCCAGTCCGTGGGACATAG
TAACCATCCAGATACGCTTCATCTACAACCTGACGTCTGCGTTGGGACTA
CATCACTGCTGGATGGATTTCAAGGTCTGGACGACCTTACAGCCTCATAC
CTGGCGTTTCTTGAGGGTATTTAAGACCCAAGTAGTAAAATACCTTGATA
GATTAGGGATTATATGTATTAATACGGTTGTAAACGCAGTAAAACATGTA
TTATATAATGTAATAGCTGGGACTGACAGTATTGAGCAGTCTAATTTAAT
AAAATTTAATGTTTATTAATTGCTGACCGAATCCTAGAAGTAGATTCTAAT
CTTCAGCGTATCGTATACAGGGTTAGAGGCATGAGTACACGCCATGTACA
GCATCAATGCATTCTCGGTATGATTTTCATATTTGCCAGCCTCTTGCTGGT
TATACACAACATAATTATTAACTCTAAAAAAAAACGCCTAACTAAAGCCTG
TTCCTTGCTCGCATACTGACCACCAATGACAGTGGCACTCCACTTCCTGAG
GACTTGATAACAATCACCATGCATGTTCATCACCGTAGCTGGACTAGGTT
CATCATCAACATATTAAAGACTTCACCAAAATTCCTGGGGCTTATCAACA
GGCCTTCATCCCTTACAGGAAAGAACATCACATTATTCGTATGATTCTTGG
TCTTACATTTTCATCCATTCGTATCCTTACTCAGGATATTAAACGAATTTA
TGCAAAACCTCTTATCCACTCCGATGAGTTAGCCCCACTTCCCCCGAGTGA
CCATCAGAT

By sequence analysis, it was found that obtained sequence of sample no. 33 having 1623 bp lies within the AC3 and Rep region showing high variability. By blast analysis of these sequences, it was found that sample no. 27 showed maximum similarity with 5 SLCMV sequences namely isolate Erode:2011 (KU550961.1), isolate Kerala17 (AJ890225.1), isolate Kerala15 (AJ890224.1), isolate TVM3 (KP455486.1) and isolate TVM1 (KR611579.1) and all of them shown 99% query

cover and 97% identity. Sample no. 33 showed maximum similarity with 4 SLCMV sequences namely isolate TVM1 (KR611579.1), isolate Adivaram (AJ579307.1), isolate Erode:2011(KU550961.1) and isolate TVM3 (KP455486.1) all having 51% query cover and 91% identity.

To identify the molecular level variation of the above samples sequenced, multiple sequence alignment was done using GENEIOUS 9.1 with alignment type "Global alignment with free gaps" and cost matrix "93% similarity (5.0/9.026168)". SLCMV positive RCA samples were aligned with 14 SLCMV DNA-A sequences and the sequence results are shown below.

Sequence analysis results showed that sample no. 33 (1623 bp) shows high variability among all the reference sequences. Within the conserved region of 1323 bp (position no. 1433-2756 with respect to reference sequences), it was found that 20 nucleotide additions (position no. 1513, 2043, 2057, 2065, 2082, 2156, 2157, 2187, 2198, 2234, 2247, 2257, 2258, 2272, 2369, 2375, 2384, 2414, 2494), 23 deletions (position no. 1434, 1437, 1449, 2044, 2045, 2066, 2081, 2271, 2293, 2294, 2299, 2303, 2304, 2372, 2392, 2419, 2458, 2502, 2550, 2588, 2590, 2594) and 29 SNPs (position no. 2204, 2232, 2275, 2278, 2279, 2286, 2297, 2306, 2322, 2378, 2424, 2426, 2428, 2429, 2442, 2446, 2454, 2482, 2497, 2526, 2528, 2536, 2604, 2607, 2609, 2612, 2697, 2748, 2753) were present.

4.6.3.1 Construction of phylogenetic tree

Using 26 reference sequences (Table. No. 13), multiple alignments of these 2 samples was done using CLUSTALW program with default parameters settings in BIOEDIT version 7.2.5. and they were used to generate a phylogenetic tree using the neighbor-joining and bootstrap options in MEGA 6.0 with a 1000 replicate bootstrap search (Fig. 19). Beet curly top virus (BCTV) sequence was used as the out-group species.

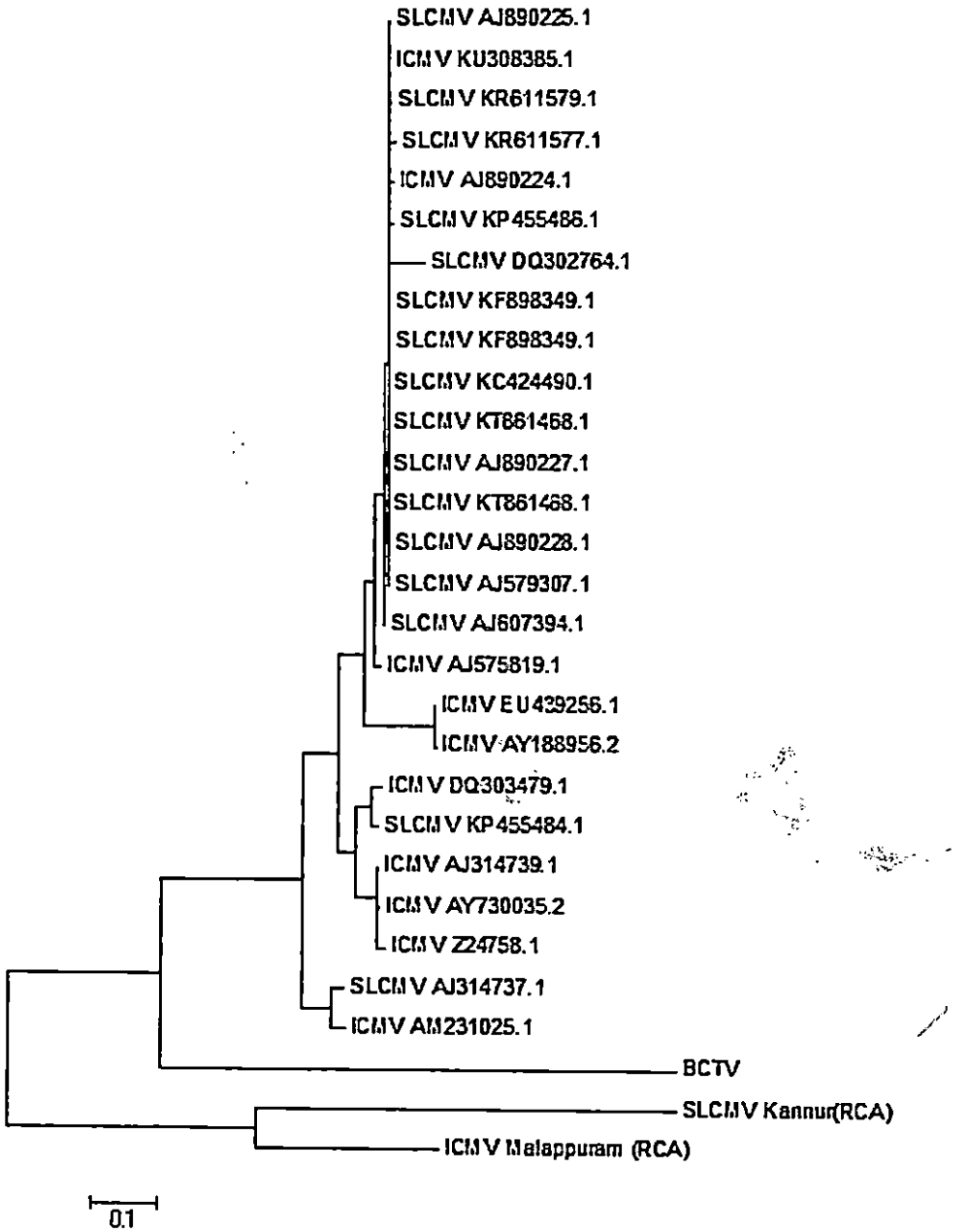

Fig. 19: Phylogenetic tree of the RCA DNA-A sequenced samples obtained using MEGA 6.0.

4.6.4 Construction of phylogenetic tree using both multiplex and RCA samples

For identifying the variation among the RCA DNA-A sequences and multiplex PCR sequences, multiple sequence alignment was done using all these samples with NCBI sequences (Table. 14) using the CLUSTALW program with default parameters settings in BIOEDIT 7.2.5. The aligned sequences were used to generate a phylogenetic tree (Fig. 20).

While analysing the phylogenetic tree, it was found that the multiplex samples sequenced shown molecular level variations in the form of SNPs eventhough they lays within the group. Also, two among the 26 reference sequences shown very much similarity with the sample sequences, namely isolate SLCMV [Colombo] (AJ314737.1) and ICMV defective DNA SLCMV-[Col]A and SLCMV-[Ker4]B) (AM231025.1).

Also, it was found that RCA DNA A sequences forms an out-group explicating its high variability among other sequences.

4.7 IDENTIFICATION OF BETA SATELLITES OR OTHER DEFECTIVE DNA MOLECULES IN CASSAVA MOSAIC INFECTED SAMPLES

4.7.1 Beta PCR for detection of beta satellite molecules

Of the samples used, only one sample showed beta positive with beta PCR [Trivandrum mixed infection (sample no. 82) sample] with bands at 1000 bp and 500 bp (Fig. 21).

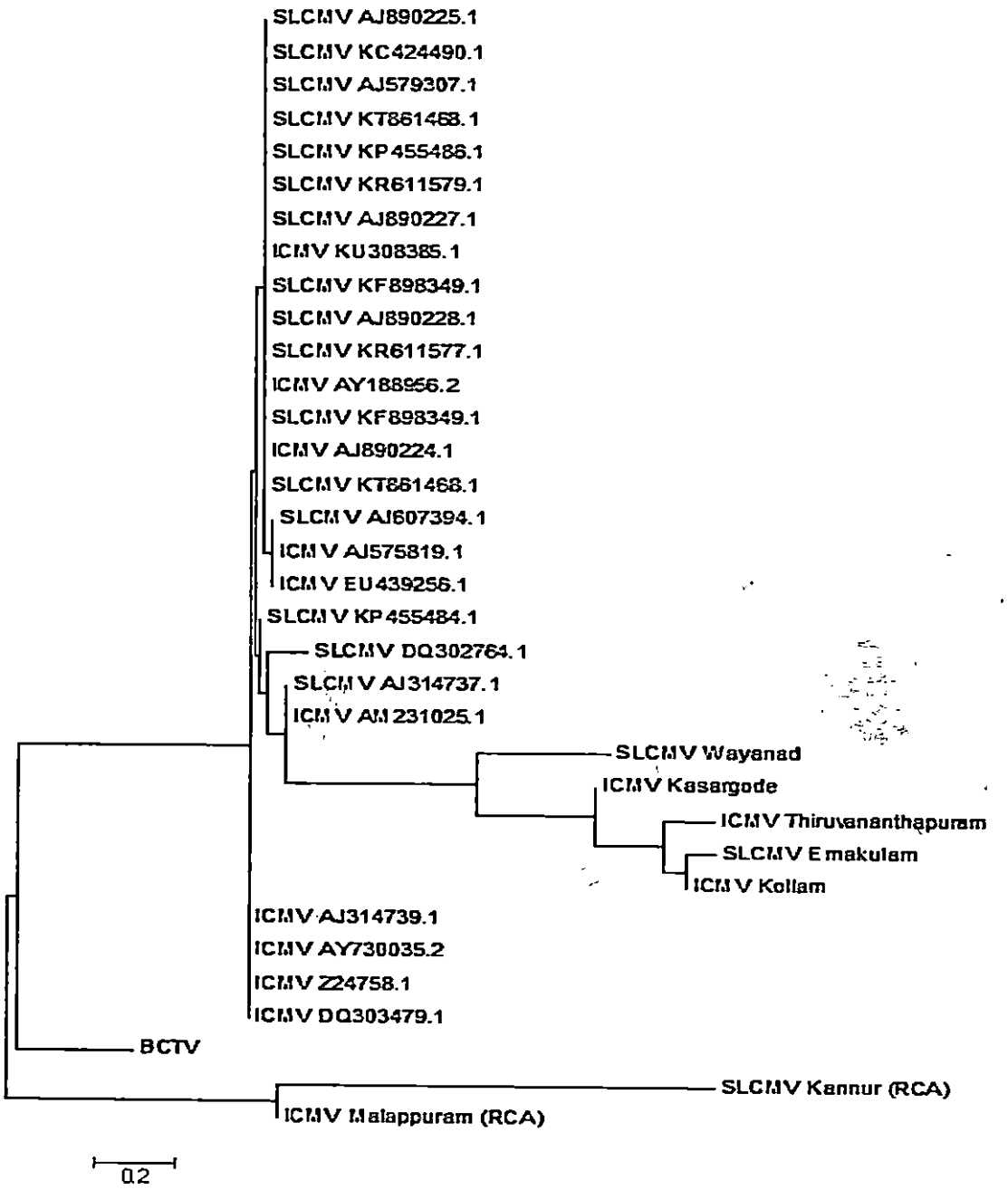

Fig. 20: Phylogenetic tree of the sequenced samples (multiplex and RCA sequences) obtained using MEGA 6. 0.

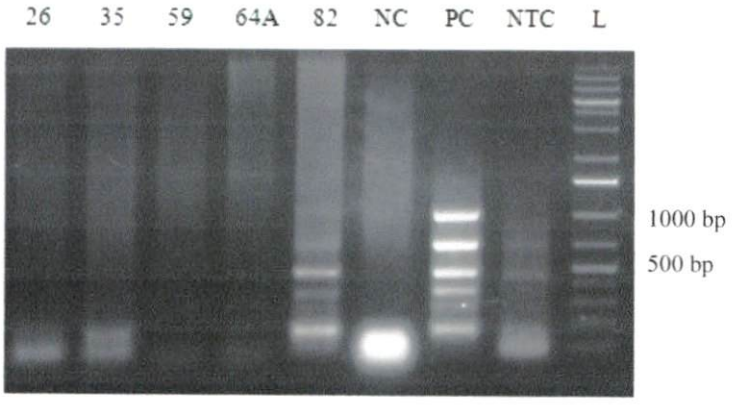

Fig. 21: Beta PCR product run on 1% gel. Lane 26- Kasargod sample; 35- Ernakulam sample; 59- Idukki sample;64A- Alappuzha sample; 82- Kollam sample; PC- positive control; NC- negative control; NTC- non-template control, L -1 kb plus DNA ladder (fermentas).

DISCUSSION

5. DISCUSSION

Tropical root and tuber crops (cassava, sweet potato, yams and aroids) are the third most important food crops in the world after cereals and legumes making significant contributions to income generation, sustainable development and household food security in developing countries (Hegde *et al.,* 2008).

Among these crops grown in India, cassava (*Manihot esculenta* Crantz) is first with respect to area, production and productivity. The high yield potential and easiness to be grown under a wide range of upland conditions even on poor soils and in areas of low or unpredictable rainfall made cassava one of the most important food crops in India and the "food of last resort" (Howeler *et al.*, 2014).

Among the various biotic and abiotic factors affecting the cassava, cassava mosaic disease (CMD) is one of the serious limiting factor affecting the yield and productivity worldwide. In all cassava growing areas especially in Africa and Asia, yield loss of about 20-80 % were reported (Malathi *et al.*, 1985; Anitha *et al.*, 2011 Kushawaha *et al.*, 2015; Chikoti *et al.*, 2015). This disease is caused by cassava mosaic geminivirus (CMG) (genus *Begomovirus*, family Geminiviridae) and the spread of the disease is through indiscriminate use of infected planting material (primary spread) and the whitefly vector [*Bemisia tabaci*] (secondary spread).

Worldwide, 11 species of cassava mosaic viruses were identified so far and among them, *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV) are seen in Asia (Mainly in India and Sri Lanka) (Malathi *et al.*, 1985; Saunders *et al.*, 2002; Dutt *et al.*, 2005; Patil *et al., 2005*).

In India, CMD was first reported by Abraham *et al.* (1956) and it was later identified as ICMV. The first clone of ICMV (ICMV-Ker) was obtained by Hong *et al.* (1993). Subsequently, Saunders *et al.* (2002) cloned SLCMV and the results obtained showed that SLCMV was more closely related to ICMV (DNA A, 84%;

DNA B, 94% nucleotide identity) than African cassava mosaic virus (ACMV) (DNA A, 74%; DNA B, 47% nucleotide identity). The presence of SLCMV on mainland India was first reported by Dutt *et al.* (2005). Later on, Patil and Dasgupta (2005) reported the presence of SLCMV in southern India through differential polymerase chain reaction (PCR) studies, and a distribution map of ICMV and SLCMV was developed. Subsequently, several ICMV and SLCMV isolates from southern India were cloned and their biodiversity and infectivity were studied (Dutt *et al.*, 2005; Patil *et al.*, 2005; Rothenstien *et al.*, 2006).

In India, CMD is widespread in south India, mainly in Kerala and Tamil Nadu. Recently a survey was conducted for identifying the incidence and severity of CMD in Kerala by Anitha *et al.* (2011) and found that CMD was present throughout Kerala at low to high incidences (44.5-96.75%) in the 35 regions surveyed all across Kerala. The disease incidence was higher in Thiruvananthapuram and Kollam districts and lower in Wayanad district. It was also found that SLCMV infection was wide spread in all districts of Kerala whereas ICMV infection was restricted to certain geographical locations. Presence of ACMV infection was also reported during this survey from Pathanamthitta district which demand the need of control measures as early as possible.

A similar survey was conducted in major cassava growing districts of Tamil Nadu (Salem, Trichy, Coimbatore, Erode, Dharamapuri, Namakkal, Tirunelveli and Kanyakumari) by Rajinimala *et al.* (2011) to identify the incidence and severity of this disease. The results shown that the incidence of CMD was more than 90% having more no. of cutting borne infection than whitefly infection in the areas surveyed showing indiscriminate use of infected planting material throughout the cassava growing field in Tamil Nadu.

From these field surveys, it was observed that CMD is emerging as a serious threat to cassava production in South India mainly in Kerala and Tamil Nadu

attributing to significant yield losses ranging from negligible to almost total. Information's from the above specified field surveys indicates that these viruses are becoming more virulent and aggressive leading to severe yield losses, frequently spreading towards larger areas and also to other economically important crops like bitterguard, chilli, mulberry, Jatropha etc (Rajinimala *et al.*, 2007; Khan *et al.*, 2011; Shery *et al.*, 2016). Recombinations may be the reason for this virulence and increased symptom severity. Up-to-date molecular information on these viruses are necessary in devising appropriate control measures to manage the disease in severely affected areas and to make adequate preparations in threatened areas. Regular diagnostic surveys on key cassava growing areas are to be undertaken to develop CMD resistant cassava, emphasizing on important aspects like the spread of CMD both in space and time and its epidemic characteristics, different CMGs present and their geographical distribution, distribution of whiteflies, their population and above all, the occurrence, frequency, amount and type (resistant or susceptible) of cassava cultivars being grown. The present study is relevant in this concern and was undertaken at the ICAR- Central Tuber Crop Research Institute, Thiruvananthapuram to identify the current status of cassava mosaic disease in Kerala and to detect whether any beta satellites or other sub viral agents present in cassava mosaic diseased samples.

As part of the study, a survey was conducted to record the incidence of cassava mosaic disease in cassava growing regions of all districts of Kerala. Totally 53 fields were visited from all the 14 districts of Kerala and altogether 115 samples were collected. These samples were serially labelled and symptomatology was recorded by visual observation as per the cassava mosaic symptom scale of Hahn, *et al.* (1980). All infected plants exhibited chlorotic areas on the leaves giving a mosaic pattern, severe leaf distortion and reduction in the size of leaf lamina, stunting of plant growth and twisted leaf. These symptoms were similar to those reported earlier (Malathi *et al.*, 1985; Anitha *et al.*, 2011). However, in the present study, inter-veinal

chlorosis and chlorotic leaves with shrunken base were also observed in infected samples collected from Ernakulam, Idukki and Thiruvananthapuram districts. During the survey unusual symptoms, similar to candle-stick symptoms of EACMV affected plants in Africa were observed from CMD infected fields in Wayanad.

During the survey, it was found that the incidence of CMD was higher in Wayanad, Kozhikode, Malappuram, Palakkad, Ernakulam, Pathanamthitta, Kollam and Thiruvananthapuram as compared with other. Disease prevalence was almost 100 percent in all districts of Kerala whereas intensity of CMD was highest in Wayanad district [Average Symptom severity (AS) of 4.00] followed by Malappuram, Alappuzha and Ernakulam districts having AS of 3.40, 3.28 and 3.00 respectively (Table. 7B.). This is contrary to the report of Anitha *et al* (2011) where less disease was recorded in Wayanad district. This change observed in the present study may be due to change in climatic condition.

During the survey, it was found that "Ethakkappa", "Micher kappa", "Vella M_4" and "Aambakkadan" varieties are resistant to CMD whereas "Diwan kappa", "Thaalees" and "Ceylon Kappa" varieties are highly susceptible to CMD causing significant yield loss.

It was observed that in almost all the cassava fields surveyed, cutting borne infection was more prevalent as compared to whitefly infection which is similar to previous studies (Anitha *et al.*, 2008). Knowingly or unknowingly, farmers are indiscriminately using infected planting material and this may be the reason for this widespread occurrence of cutting borne infection. From the information gathered during the survey, it was observed that most of the planting materials available with the farmers are infected by CMGs and disease free planting materials of cassava are almost extinct in farmer's holdings.

In the present study, whitefly mediated infection with more no. of whitefly population was observed from high altitude fields (Mananthavadi in Wayanad district

and Thottilpalam in Kozhikode district). This shows some contradictions to previous reports where less incidence of CMD with less no. of whitefly population was observed in high range fields owing to the cool climate present (Anitha *et al.*, 2011). Therefore, the interpretation from current survey is that whiteflies in high altitudes became more adapted to the cool climate and transmitted the virus which leads to mosaic disease with increased severity on these regions.

During the end of the survey, it was found that CMD was prevalent in all cassava growing areas of Kerala with increased aggressiveness and symptom severity, similar to the earlier reports (Anitha *et al.,* 2008), thus demanding a proper solution for elimination of CMD from the fields.

Using serological techniques like ELISA and DIBA, the presence of cassava mosaic virus in infected samples collected during the survey were identified as done by earlier workers (Makeshkumar *et al.*, 2001; Anitha *et al.*, 2008). Eventhough with this method, ICMV and SLCMV could not be distinguished, it can be used as efficient and cost effective method for primary diagnosis of CMD.

PCR-based diagnosis is the best method to confirm the presence of CMGs in the infected samples. Among the different primers designed to detect CMG, CP primer was found to be best (Makeshkumar *et al.,* 2005), but it cannot differentiate ICMV and SLCMV. So, in the present study, multiplex PCR (Dutt *et al.,* 2005; Makeshkumar *et al.*, 2007; Anitha *et al.*, 2011) was employed to differentially detect the presence of ICMV and SLCMV from infected samples and the results shown that SLCMV was widespread in all districts of Kerala with increased infectivity and symptom severity. ICMV infection was identified in only 9 districts namely Trivandrum, Kollam, Pathanamthitta, Alappuzha, Kottayam, Idukki, Ernakulam, Palakkad, Malappuram and Kasargod solely as well as mixed infection with SLCMV. Widespread distribution of SLCMV might be due to its more aggressive nature, the genotypes of cassava grown in those regions, to the vector whitefly populations and

possibly due to germplasm movement. Previous studies from Kerala showed that ICMV occurrence were very less as compared to SLCMV during that period (Patil *et al.*, 2005; Anitha *et al.*, 2011). But in the present study, ICMV occurrence was observed in more samples and this shows that the re-emergence of ICMV is taking place.

Anitha *et al.* (2011) reported the presence of ACMV in field infected cassava leaf samples from Kerala (Pathanamthitta). In the present study, PCR amplification with ACMV specific primers gave an amplification of ~700bp (Fig. 9) in three samples when compared to the positive control (1000bp). Further sequencing of the amplification product and analysis is essential to confirm the presence of ACMV.

Synergism or recombination between these viruses may result in the rise of a more virulent species and cause serious epidemic like that occurred in Africa (Thresh *et al.*, 1997; Zhou *et al.*, 1997; Thresh *et al.*, 1998; Pita *et al.*, 2001). The reported properties of SLCMV such as increased aggressiveness and capability of trans-replicating DNA components of other viruses (Saunders *et al.*, 2002) might have important contribution in this regard making the problem more complex leading to severe threat in future to cassava crop in Kerala. To tackle the situation, the primary measure to be undertaken is to prevent the spread of ACMV towards other districts. Along with that efficient control measures should be developed to eradicate this deadly disease from infected fields.

To identify the variation among different ICMV and SLCMV positive samples obtained from all districts, restriction analysis was done for Multi + Rep primer PCR products using six different enzymes *viz.*,EcoRV, HindIII, XhoI, ClaI, BamHI and KpnI.

While analyzing the results, it was observed that main variation occurs within the EcoRV, XhoI, HindII and ClaI sites. These four sites were present in available SLCMV sequences (NCBI), but XhoI and HindIII sites were absent in available

ICMV sequences (NCBI). ICMV positive samples of Malappuram and Kollam districts shows the presence of XhoI and HindIII sites attributing that recombination between DNA-A of ICMV and SLCMV may be reason for gaining of these sites. In the case of SLCMV positive samples, EcoRV site was absent in samples of 2 districts (Kottayam, Idukki) and XhoI site was absent in samples of 5 districts (Kollam, Kottayam, Idukki, Kannur, Kasargod). HindIII site was absent in samples of 5 districts (Pathanamthitta, Kottayam, Idukki, Kannur, Kasargod). ClaI sites are present in two positions in almost all NCBI sequences selected for the study, but were absent in samples of three districts (Kottayam, Idukki, Kannur).

XhoI and HindIII sites are the common variation points as observed by restriction analysis. As these sites are present nearby the conserved region of DNA-A genome of both ICMV and SLCMV, the presence or absence of these sites may contribute change in conserved domain leading to adverse effects. Therefore, it may be concluded that during the period of time, point mutations may happened resulted in the change (gain or loss) of these specific restriction sites which contributes in the variation of these samples. In the earlier reports of assessing the biodiversity of cassava mosaic virus in India, they have used the PCR-RFLP strategy with enzymes like EcoRI, HpI, RsaI, Sau3AI and TaqI to obtain specific polymorphic patterns (Patil *et al.,* 2005; Rothenstein *et al.*, 2006).

Between the DNA-A of ICMV and SLCMV, the most diverse region is the intergenic region (IR) and the most conserved region is the CP as reported by Saunders *et al.* (2002) and Patil *et al.* (2005). Also, Borah *et al.* (2012) reported the existence of a recombination hotspot between the IR and AC1 regions of both ICMV and SLCMV. It is easy to identify the variation if the IR is sequenced. As the multiplex PCR primers were designed to specifically amplify a 900 bp fragment from ICMV and a 600 bp fragment from SLCMV encompassing the 5' portion of AC1 and the IR, its PCR products can be used for identifying the variation and this strategy was followed in the present study.

While analysing the multiplex PCR sequences of different districts, it was found that so many single nucleotide polymorphisms (SNPs) exists within this region though it shows similarity with the available sequences in NCBI. Even SNPs occur within the intergenic region.

In sequence analysis, Kollam ICMV sample showed maximum similarity with isolate ICMV-Mah (AJ314739.1), still so much of polymorphisms and recombinations occurred within this sample. A total of 23 additions, 2 deletions, 34 SNPs were observed within 553 bp conserved region identified, followed by Kasargod SLCMV. Least variability was observed in Trivandrum ICMV as compared to other samples. Similar studies were reported earlier by Patil *et al.* (2005) in which a high proportion of samples had point mutations attributing to novel restriction patterns localized within specific regions (genes encoding coat protein, rep protein and movement protein). The increased incidence and severity of CMD reported from Kerala during the recent years demands the necessity to develop clones of whole genome sequences of ICMV and SLCMV DNA-A and DNA-B to develop CMD resistance cassava. In this context, the ICMV and SLCMV positive samples collected from different districts were used for whole genome amplification using RCA. Owing to the efficiency of this technique in characterizing viral DNA components from several geminiviral DNA components from natural and experimental host plant sources (Inoue-Nagata *et al.*, 2004; Haible *et al.*, 2006) the present study exploited this technique to obtain whole genome DNA-A component from the CMG positive samples.

Sequence analysis of RCA amplified DNA-A components showed that they showed high variability with the available sequences in NCBI.

Malappuram ICMV sample showed more similarity with the available SLCMV DNA-A sequences in contrast with ICMV DNA-A sequences. A total of 61

SNPs were observed within 200 bp conserved region identified when aligned with 4 ICMV DNA-A sequences showing high variability over other sequences.

Kannur SLCMV showed more than 90% identity with the available sequences. But within the 750 bp conserved region identified when aligned with 14 SLCMV DNA-A sequences, 21 SNPs were observed.

The phylogenetic analysis of these samples revealed that variability exists within all the 7 samples sequenced. Eventhough multi PCR product sequences lies within the group, RCA products shown a separate clad indicating hyper variabilty. Further investigation is necessary to study the extent of variation and to identify the presence of recombination events.

Beta satellite molecules are small single stranded DNA molecules (1.6 kb) usually seen associated with monopartite begomoviruses enhancing host viral replication and increasing the symptom severity (Saunders *et al.*, 2000; Briddon *et al.*, 2001; Nawaz-ul-Rehman and Fauquet, 2009; Patil and Fauquet, 2010). Beta satellites were never reported to be associated with cassava mosaic viruses till now and in the present study it was detectd and this is the first time ß DNA was reported from cassava mosaic infected sample of Thiruvananthapuram district. Therefore, this matter is of a serious concern because presence of these subviral agents can enhance the symptom severity in currently available susceptible cultivars. And also there is a chance to move these particles to other CMD resistant varieties through whiteflies. Further studies are needed to identify the sources of these defective DNA molecules in the infected samples, their virulence potential and effective methods should be formulated to control these risk factors from infecting healthy cassava plants.

SUMMARY

6. SUMMARY

A study on "Molecular analysis of phylogeography of cassava mosaic disease" was conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram, during 2015- 2016. The important findings of the above studies are summarised in this chapter.

The survey conducted across all districts in Kerala revealed that cassava mosaic disease (CMD) is widespread in Kerala having high symptom severity and increased aggressiveness as compared to earlier years. Maximum intensity of CMD was observed in Wayanad (4.00) followed by Malappuram (3.40), Alappuzha (3.28) and Ernakulam (3.00).

Serological tests like ELISA and DIBA were utilized to screen and categorize the samples having infection of ICMV / SLCMV. Further confirmation of these samples was done using multiplex PCR to identify the single or mixed infection of ICMV / SLCMV which was not revealed from the serological assays.

Studies using multiplex PCR with all the samples collected during survey showed that SLCMV is widespread in all districts of Kerala while ICMV infection is observed seen solely as well as combined with SLCMV (mixed infection) in 9 districts namely Thiruvananthapuram, Kollam, Alappuzha, Kottayam, Idukki, Ernakulam, Palakkad, Malappuram and Kasargod. Of the 115 samples collected, 9 samples had only ICMV infection, 68 samples only SLCMV infection, 18 samples had both ICMV and SLCMV (mixed infection) and 20 samples had neither ICMV nor SLCMV infection.

Apart from ICMV and SLCMV, possible occurrence of other cassava mosaic viruses were checked using cassava mosaic geminiviruses reported in Africa (ACMV, EACMV, Ug-V). Among all, only ACMV (suspected) was detected in samples from Pathanamthitta district.

Suspected beta satellite molecules were identified from the Thiruvananthapuram sample having mixed infection, and this may give rise to a situation to increase in disease severity. This is the first report of identification of beta satellite molecules associated with cassava mosaic disease in India.

By restriction analysis and sequence analysis, high variability was detected within and between the cassava mosaic viruses in Kerala. High sequence variability is found within and between the ICMV and SLCMV samples collected during this survey and this attributes to a large number of single nuclear polymorphisms (SNPs) found within the conserved regions.

From the phylogenetic tree constructed, it was observed that the sample sequences have varialbility eventhough lies within the group. Further investigation is necessary to study the extent of variation and to identify the presence of recombination events in these samples.

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APPENDICES

APPENDIX I

Reagents required for ELISA

1. Coating Buffer

Na_2CO_3	:	1.59 g
$NaHCO_3$	:	2.93 g
NaN_3	:	0.20 g

Adjust pH to 9.6 with 1 N NaOH and make up to 1L. Autoclave and store at 4°C

2. Phosphate Buffered Saline (PBS) (pH 7.4)

NaCl	:	8.0 g
Na_2HPO_4. $12H_2O$ (or 1.16 g Na_2HPO_4)	:	2.9 g
KCl	:	0.2 g
KH_2PO_4	:	0.2 g

Dissolve in 1L distilled water and check pH of the final solution

3. PBS-Tween (PBST)

Add 0.5 ml of Tween-20 to1 L PBS.

4. Sample Extraction Buffer

PBS-T + 2% PVP

5. Conjugate Buffer

PBS-T + 2% PVP + 0.2% egg albumin

6. Substrate Buffer

Diethanolamine	:	9.7 ml
Distilled Water	:	80 ml
NaN_3	:	0.2 g

Adjust the pH to 9.8 with 1 M HCl and make up to 100ml.

Autoclave and store at room temperature

7. Substrate for Alkaline phosphatase (freshly prepared)

Dissolve 0.5 mg/ml PNPP in substrate buffer

8. Blocking Buffer

PBS-T+ 2% spray dried milk

APPENDIX II

Reagents required for DIBA

1. Tris Buffered Saline (TBS)

Tris	:	0.02M
NaCl	:	0.5 M
Distilled Water	:	800 ml

Adjust the pH to 7.5 with 1 M HCl and make up to 1L

2. Blocking solution (TBS-SDM)

TBS + 5% spray dried milk

3. Substrate buffer

Tris	:	0.1 M
NaCl	:	0.1 M
$MgCl_2$	:	5 mM
Distilled Water	:	800 ml

Adjust the pH to 9.5 with 1 M HCl and make up to 1L

4. **Substrate solution**

Solution A

Dissolve 75 mg of NBT in 1ml N, N Dimethylformamide

Solution B

Dissolve 50 mg of BCIP in 1 ml N, N Dimethylformamide

Add NBT and BCIP to the substrate buffer just before use to give a final concentration of 0.33mg/ml and 0.175 mg/ml respectively

APPENDIX III

Reagents required for plant total DNA isolation

1. **CTAB DNA Extraction Buffer**

Tris HCl (pH 8.0)	:	100 mM
EDTA	:	20 mM
NaCl	:	1.4 M
CTAB	:	2 %
PVP	:	2 % (w/v)
β-mercaptoethanol	:	0.2 % (v/v)

(Freshly added prior to DNA extraction)

Autoclave and store at room temperature

2. **Chloroform-Isoamyl alcohol (24: 1)**

Chloroform	:	24 ml
Isoamyl alcohol	:	1 ml

3. **TE Buffer**

Tris HCl (pH 8.0)	:	10 mM
EDTA	:	1 mM

APPENDIX IV

Reagents required for Agarose gel electrophoresis

TAE Buffer (50X)

Tris base 242g

Glacial acetic acid 57.1 ml

0.5 M EDTA (pH 8.0) 100 ml

APPENDIX V

Luria Agar Medium

35 g of LA (HiMedia) in 1000 ml distilled water. Autoclave and store at room temperature

APPENDIX VI

Luria Broth Medium

20 g of LB (HiMedia) in 1000 ml distilled water. Autoclave and store at room temperature

APPENDIX VII

Preparation of LA Ampicillin /X gal /IPTG Plates

1. **Ampicillin stock (50 mg/ml)**
   Dissolve 2.5 g Ampicillin in 50 ml deionized water. Filter sterilize and store at -20°C in the dark

2. **X gal stock (20 mg/ml)**
   Dissolve 0.2 g X gal in 10 ml N, N Dimethylformamide. Store at -20°C in the dark

3. IPTG stock (100 mM)

Dissolve 1.7 g IPTG in 50 ml deionized water. Filter sterilize and store at 4°C in the dark.

Before pouring the plates, allow the LA medium (1L) to cool to 55°C, then add 1ml ampicillin stock (making the final concentration to 50µg/ml). Mix gently and pour the plates. Dry the plates opened at room temperature under UV light for 30 min. Add 40µl X gal stock solution to the plate and spread evenly using a sterile spatula. Allow it to diffuse completely into the medium for about 10 min. Then add 40µl IPTG stock solution, spread evenly using a sterile spatula.

Pre-warm the LA ampicillin /X gal /IPTG Plates at 37°C for 20 min before use.

APPENDIX VIII

Reagents required for plasmid isolation

1. Buffer P1

Tris (pH 8.0)	:	50 mM
EDTA(pH 8.0)	:	10 mM

Autoclave and store at 4°C

2. Buffer P2 (freshly prepared)

NaOH	:	200mM
SDS	:	1 %

Autoclave and store at room temperature

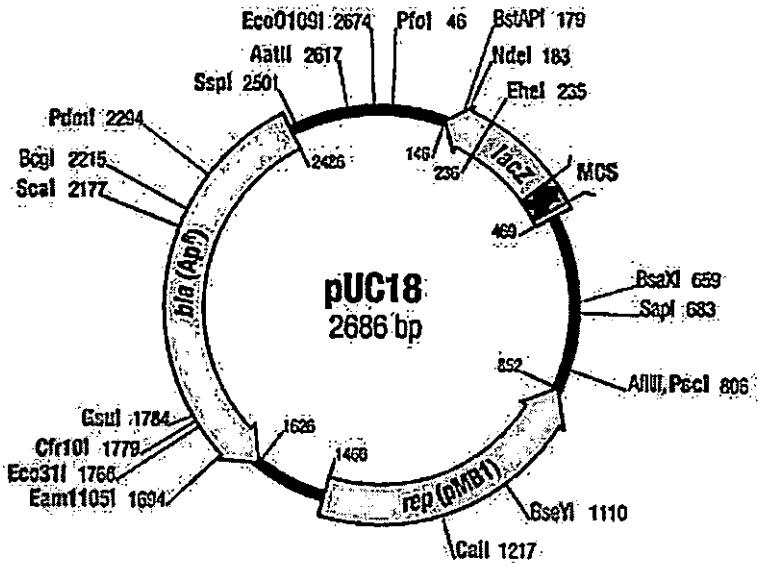
3. Buffer P3

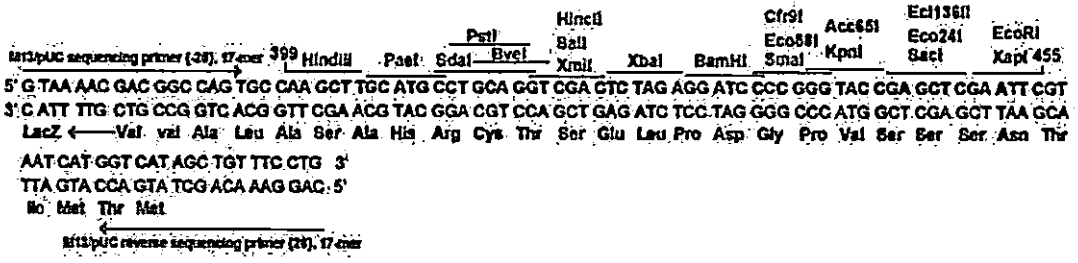
Potassium acetate	:	3 M

Adjust the pH to 5.5 with glacial acetic acid. Do not autoclave. Use autoclaved distilled water. Store at 4°C

APPENDIX IX

VECTOR MAP OF pUC18





ABSTRACT

MOLECULAR ANALYSIS OF PHYLOGEOGRAPHY OF CASSAVA MOSAIC DISEASE

JAYAKRISHNAN. J. T.

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Abstract of the thesis

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VELLAYANI, THIRUVANANTHAPURAM-695 522

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ABSTRACT

A study on "Molecular analysis of phylogeography of cassava mosaic disease" conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016.

During the study, a survey was conducted in all districts of Kerala to identify the different cassava mosaic viruses present in major cassava growing areas. The survey conducted revealed that cassava mosaic disease (CMD) is widespread in Kerala having high symptom severity and increased aggressiveness as compared to earlier years. Maximum intensity of CMD was observed in Wayanad followed by Malppuram, Alappuzha and Ernakulam districts.

From the 53 fields visited across all districts, 115 samples were collected and their symptomatology was recorded. Serological tests like ELISA and DIBA were done to detect the presence of cassava mosaic viruses in them and all the samples were further diagnosed using multiplex PCR to differentially detect ICMV and SLCMV. From the 115 samples, 9 samples were ICMV positive, 68 samples SLCMV positive, 18 samples had both ICMV and SLCMV (mixed infection) and 20 samples had neither ICMV nor SLCMV infection. These 20 samples were selected and PCR were employed using specific primers of ACMV, EACMV, UgV to detect any such virus presence and the results showed that 3 samples from Pathanamthitta had suspected ACMV infection which were also positive for SLCMV.

From PCR analysis, it was revealed that SLCMV is widespread in all districts of Kerala while ICMV infection was restricted to 9 districts only *viz.*, Thiruvananthapuram, Kollam, Alappuzha, Kottayam, Idukki, Ernakulam, Palakkad, Malappuram and Kasargod.

To identify the variation of cassava mosaic viruses within different districts, each ICMV and SLCMV representative samples from all districts were used for PCR-RFLP analysis using 6 different restriction enzymes *viz.*, EcoRV, HindIII, XhoI, ClaI, BamHI and KpnI. The results revealed that SLCMV samples from districts *viz.*, Kollam, Pathanamthitta, Kottayam, Idukki, Kannur and Kasargod shown variation within the EcoRV, HindIII, XhoI, ClaI sites wheras ICMV samples from Kollam and Malappuram showed variation within HindIII and XhoI sites.

To identify the molecular level variations within the conserved region of both ICMV and SLCMV, multiplex PCR amplified products of 5 representative samples (3 ICMV and 2 SLCMV) were sequenced and the result indicates that the SLCMV samples had more variability than the ICMV samples and also more number of SNPs were found within the conserved region of all these samples.

Whole genome amplification of selected samples was done using RCA to identify the variation within the DNA-A genome and the sequence result disclosed that high level of variability is present in the form of SNPs in the conserved Rep region within DNA-A of selected samples.

To identify the phylogenetic relationship of the sequenced samples with that of available accessions, dendrograms were made using MEGA 9.0 software and the tree showed that sequences has variability eventhough lies within the group.

To identify the presence of beta satellite molecules in the samples collected during the survey, multiplex positive samples and samples showing unusual symptoms and increased aggressiveness were selected and PCR was employed using beta specific primer. Only one sample (Thiruvananthapuram mixed infection) shown positive results for suspected beta molecule and this is the first report of identification of beta satellite molecules associated with cassava mosaic disease in India making the situation more crucial.