Distribution and Molecular Characterization of Cassava Mosaic Geminiviruses and associated DNA Satellites in Kenya

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DECLARATION

This is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this work to my dear father. He cherished education and wanted to go to Makerere University while in Seta College, Uganda but didn't make it for school fees problems.

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ABSTRACT

CMD was widely distributed in the country with an average incidence of 57.3 Cassava (*Manihot esculenta* Crantz) is an important food crop in Kenya. It is popular with communities in Western, Nyanza, part of Eastern and Coast provinces. It is an important source of dietary carbohydrates and cushions these communities against hunger due to its drought tolerance characteristics. However, the crop has come under severe attack by various diseases of which viral diseases have had a very devastating effect on the overall yields. An epidemic of Cassava mosaic disease (CMD) caused by Cassava mosaic geminiviruses (CMG's) severely hindered cassava production across eastern and central Africa which led to farmers giving up on the crop especially in western Kenya.

Although quite some work has been done on the identification of plant viral diseases, not much has been done on the molecular characterization, genetic diversity and distribution in all cassava growing areas in the country. This study builds on the previous studies by determining the cassava mosaic geminiviruses infecting cassava and the associated DNA satellites in all cassava growing regions of the country.

A countrywide survey was carried out in four distinct regions which are also administrative regions namely provinces. The provinces under survey were Eastern, Nyanza, Western and Coast provinces. A total of 94 cassava fields were surveyed with 350 leaf samples of suspected CMG's and associated DNA satellites were collected and

assayed by PCR molecular techniques for detection and identification of viruses and the associated DNA satellites. Sequence diversity for CMG's and the associated DNA satellites were determined. Whitefly counts were also done on all visited farm fields to determine the method of virus transmission % with Coast province recording the highest (73.8%) incidence. The prevalence of CMD countrywide was 84.6% with Nyanza province recording the highest (96.2%) prevalence. Sequence identity and phylogenetic analysis revealed less variability within the viral species African cassava mosaic virus (ACMV). However variability amongst isolates in the species East African cassava mosaic virus (EACMV) was more distinct. The Kenyan ACMV isolates under study were closely related to ACMV isolates from Kenya, Uganda and Namibia with sequence identities of 97%-98%. Sequence identity and phylogenetic analysis for EACMV indeed showed that the Kenyan EACMV isolates are closely related to the Ugandan isolates. Sequence analysis of the DNA satellites associated with Cassava mosaic begomoviruses showed greater variability amongst themselves and the DNA satellites from the gene bank. Most of the DNA satellite sequences determined in this study had a low similarity to the NCBI genebank sequences of begomoviruses associated DNA III satellites from Tanzania and the Mentha leaf satellite DNAII from India at only 30% sequence similarity for both.

These results have shown emergence of ACMV in coast and eastern provinces with 20% cases of dual infection between ACMV and EACMV species of CMGs. Breeding programmes for resistance or tolerance to these CMG's need to take into considerations

the possibility of inter and intra species recombination. There is need also to evaluate yield losses due to the symptom severity of cases where CMG's and their associated DNA satellites have been identified.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Cassava is a major factor in food security across sub-Saharan Africa. In Kenya, it was introduced by Portuguese sailors in the eighteenth Century and has become an important food crop for many communities in Western, Eastern and Coastal areas of the country (Were *et al.*, 2004a). Despite the crop's rejection during the introduction phase by indigenous people, the crop has now spread and gained prominence as a major staple food for many communities in sub-Saharan Africa. On a worldwide scale, cassava is ranked among the top 10 most significant food crops produced in developing countries and as a major source of carbohydrates: The total world production in the year 2011 was 250.2 million tonnes, with 113 million tonnes being produced in Africa alone (FAO, 2011). In addition, it is a cash crop, feed crop, and a source of raw materials for industrial uses such as starch and alcohol production. Thus, cassava constitutes an important source of income in rural and often marginal areas (FAO, 2011).

1.2 Origin and botany of cassava (*Manihot esculanta* Crantz)

Manihot esculenta is not known in the wild state. Some 98 species of the genus *Manihot* have been found in the Western hemisphere, and it appears that *M. esculenta* must have arisen by mutation or hybridization, the probable centre being southern Mexico/Guatemala or north-eastern Brazil, or both (Fauquet and Fargette, 1986). It was

early domesticated and was cultivated in Peru 4 000 and in Mexico 2 000 years ago. It was subsequently spread throughout Central and tropical South America, and was taken by the Portuguese to Africa in the 16th Century. Its spread in Africa was slow until the end of the 19th and first half of the 20th Century (Martin, 1970). However, Africa now produces almost 45.3 per cent of the world's total production (FAO, 2011). The crop has become important throughout the tropics, under a wide range of conditions of climate and soil, with West Africa, Brazil, Indonesia and Thailand being major producers.

Cassava is a perennial shrub, with latex in all its parts, which produces enlarged tuberous roots. The height ranges from about 1 to 3 m or more. The stems are usually slender and glabrous, with leaves borne near the apex; the lower parts of the stems have nodes made conspicuous by prominent leaf scars. Branching is variable; some cultivars branch near the base and are spreading in form, others are erect and branch nearer the apex. Stems vary in colour, being grey or silvery, green, greenish-yellow, reddish-brown, or streaked with purple. The leaves, which are spirally arranged with phyllotaxis 2/5, have petioles 5-30 cm long, usually longer than the blades; the blades are deeply palmately divided with 5-7 (occasionally 3-9) lobes, each 4-20 cm long and 1-6 cm wide, obovatelanceolate, pointed and with entire margins (Martin, 1971). They vary in colour from green to reddish; the petiole and midrib may be deep red. Older leaves are shed leaving the prominent leaf scars mentioned above. The flowers are borne in axillary racemes near the ends of branches, and are monoecious, pale yellow or red, 1-1.5 cm in diameter. The fruit is a six-winged capsule with 3 ellipsoidal seeds each about

12 mm long. Root tubers develop by a process of secondary thickening as swellings on adventitious roots a short distance from the stem (Martin, 1970).

Great variation is shown in the number, shape and size of the roots and the angle at which they penetrate the ground. There are usually 5-10 roots per plant, cylindrical or tapering, 3-15 cm in diameter and 15-100 cm long, occasionally longer. Hydrocyanic glycoside is present in varying quantity (Nartey, 1981). Cassava clones are often classified by taste as 'sweet' or 'bitter', but contrary to the commonly stated notion; this does not always reflect a direct relationship with the cyanogenic glycoside content of the root. The two types have sometimes been regarded as different species, the former being called *M. esculenta* and the latter *M. palmata* or *M. dulcis*. Further, the toxicity of a cultivar varies according to environmental growth conditions (Bokanga *et al*., 1994).

1.3 Cassava production

According to FAO estimates, 250.2 million tonnes of cassava was produced worldwide in 2011. Africa accounted for 45.3%, Asia for 35%, and Latin America and the Caribbean for 19.7% of the total world production. Around 60 percent of global production is concentrated in five countries namely Nigeria, Brazil, Thailand, Indonesia and the Democratic Republic of Congo. World cassava production since the 1970s has risen on average by 2.2 percent per annum. This growth has relied on an expansion of plantings rather than on rising productivity (FAO, 2008).

In 2010, Nigeria produced 38 million tonnes making it the world's and Africa's largest producer (FAO, 2011). In terms of area harvested, a total of 18.2 million hectares was planted with cassava throughout the world in 2011; about 64% of this was in sub-Saharan Africa (FAO, 2011). The average yield in 2005 was 10.9 tonnes per hectare, but this varied from 1.8 tonnes per hectare in Sudan to 27.3 tonnes per hectare in Barbados. In Nigeria, the average yield was 10.6 tonnes per hectare (FAO, 2011). In Africa, a total of 113.3 million tons was produced during the year of 2011. Nigeria being the largest producer as earlier mentioned followed by the Democratic Republic of Congo (DRC) which produced 14 million tones the same year. Other large producers include Ghana, Angola, Tanzania Mozambique just to mention but a few in that order. In East and Central African region, a total of 22 million tons were produced during the period still according to the FAO estimates for the year 2011 (FAO, 2011). Tanzania leads in both production and yields in this region.

In Kenya, cassava is grown on over 90,000 hectares with an annual production of about 540 000 tons (Munga, 2009). Cultivation is mainly in western Kenya comprising of Nyanza and Western provinces (60%), Eastern (10%), and Coast provinces (30%). The crop is grown by small holder poor households for subsistence and is important for food security. The yields recorded range between 5-10t/ha while the potential is 32t/ha (Munga, 2000).The yields are low due to both biotic and abiotic constraints earlier mentioned. The crop has a lot of potential in increased acreage and yields once the two

constraints are addressed. Already the potential has been witnessed in western Kenya by introducing varieties tolerant to CMD (Obiero *et al.,* 2007).

1.4 Cassava utilization

Cassava is the staple food of the poorer section of the population of many tropical countries, and has been estimated to provide 37, 12 and 7 per cent of the energy in the diet of the tropical areas of Africa, America and Asia, respectively (Holzman and John, 1986). The fresh peeled roots are eaten as a vegetable after boiling. The roots are also eaten after roasting. They are often boiled and pounded into a paste and added to soups and stews. It is also a practice in Brazil to make a soup by boiling cassava roots with other vegetables (Balagopalan *et al.,* 1988). Cassava is used as a raw material in the manufacture of processed food, animal feeds and industrial products like ethanol, starch and biofuels. Wider utilization of cassava products can be a catalyst of rural industrial development and raise the income of producers, processors and traders. It can also contribute to food security status of its producing and consuming households (Plucknett *et al*., 1998).

In Kenya, cassava utilization can conveniently be grouped into two categories, utilization at farmer level and utilization at processor level. At farmer level, whole tuber is consumed as boiled, fried, roasted or raw. About 51% of farmers use it as flour from pound or ground dried cassava chips. This mode of utilization accounts for 80% of farmers where the dried chips are mixed with other cereals like sorghum and millet. The

flour is used in preparing some form of ‘Ugali’ mostly when mixed with finger millet flour. The mixed flour also makes delicious porridge after fermentation. Approximately 15% of farmers utilize the young leaves as vegetables. Cassava is utilized as chips, crisps or flour after processing. Cassava is also fed to livestock such as cattle, goats and chicken by about 55% of farmers growing it (Kiura *et al*., 2007).

1.5 Statement of the problem

The biggest constraints to cassava production are pest attack (49.6%) followed by drought (22.9%), weeds (14.7%), and shortage of clean planting materials (12.8 %) (Ntawuruhunga *et al*., 2007). The major arthropod pests of cassava in Africa are the cassava green mite, the cassava mealybug, and the variegated grasshopper. The main diseases affecting cassava are cassava mosaic disease, cassava brown streak disease, cassava bacterial blight, cassava anthracnose disease, and root rot. Pests and diseases, together with poor cultural practices, combine to cause yield losses that may be as high as 60.5% in Africa (Ntawuruhunga *et al*., 2007).

Crops that are vegetatively propagated are prone to virus infection due to handling of the planting materials and cassava is no exception (Gibson and Otim-Nape, 1997; Chellappan *et al*., 2004). At least seventeen different viruses of cassava have been described worldwide, of which eight are known to occur in Africa (Thresh *et al*., 1994). The main area of attention in Africa has been on the viruses causing cassava mosaic and cassava brown streak diseases which are the subject of this study. Relatively little

attention has been given to the other viruses of cassava (the diseases they cause) and their DNA satellites. There is limited information on their distribution and on their effects on growth or yield (Bull *et al*., 2006). These are issues which are addressed in this study especially in the distribution of the various species of CMD, the complexity of the DNA satellites of cassava mosaic virus and the severity of the two diseases.

Cassava mosaic disease (CMD) has been a hindrance to cassava production in Africa throughout the twentieth century. In Kenya, CMD is caused by geminiviruses African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), Uganda variant (EACMV-UG), East African Cassava Mosaic Zanzibar Virus (EACMZV) and recently the East African Cassava Mosaic Virus-KE2 (EACMV-KE2) of the genus Begomovirus (Maruthi *et al*., 2004;Bull *et al*., 2006). Studies show an increase in diversity and geographical distribution of these pathogens in East Africa region (Bull *et al*., 2006).

1.6 Rationale of the study

Cassava is a strategic food crop which can mitigate against hunger in many communities in Kenya and sub Saharan Africa. However the crop is devastated by viral diseases which greatly reduce the yields. This study focused on determining the distribution and the genetic diversity of these viruses.

The two major species of Cassava mosaic begomoviruses cause the disease but their distribution and incidences of dual infections and genetic diversity is scarcely known. It is thus important for the various species, strains and isolates of CMGs through molecular characterization to be mapped out as their occurrence and distribution will lead to researchers to design breeding programmes for the CMGs in relation to the geographical areas and the strains, species endemic in those areas. This is also important for the extension services for the knowledge on the distribution in terms of disease incidence, prevalence and severity will facilitate coining of appropriate extension packages to address the farmers need. The symptom expression in relation to the varieties and regions where the crop is grown was also assessed to understand the disease aetiology and epidemiology.

Little knowledge exists on the DNA satellites that are associated with these geminiviruses. The DNA satellites of Cassava mosaic viruses have been shown to cause more severe symptoms of the disease (Briddon *et al.*, 2008). The study therefore focused on determining the type of the DNA satellites associated with CMD in Kenya, their distribution and genetic diversity in the country. As such molecular characterization of the DNA satellites associated with CMGs will help in understanding their diversity and their effect on the disease severity and hence designing of appropriate mitigation measures. A few studies illustrate the increasing diversity and geographical distribution of CMGs and associated DNA satellites in this region. Such information will be useful in devising measures aimed at reducing the impact of CMD

and ensuring that breeding programmes produce resistant cassava varieties that target relevant virus species and strains. The findings of the study also aimed at providing a platform for monitoring novel virus species and strains that evolve. This supports the suggestion of Ndunguru *et al.* (2005) that eastern Africa serves as the 'melting pot' for CMD begomovirus diversity and evolution from which the viruses spread throughout the cassava growing areas of the continent.

1.7 OBJECTIVES

1.7.1 Overall objective

To determine the distribution and genetic diversity of cassava mosaic geminiviruses and their associated DNA satellites in Kenya

1.7.2 Specific objectives

i. To determine the distribution of cassava mosaic geminiviruses and their associated DNA satellites in Kenya

ii. To determine the genetic diversity of cassava mosaic geminiviruses

iii. To determine the genetic of DNA Satellites associated with cassava mosaic begomoviruses

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Cassava mosaic disease (CMD)

2.1.1 Introduction

Cassava mosaic disease was first described in 1894 in Tanganyika which is part of present day Tanzania. The disease was later reported in many other countries of East, West and Central Africa (Legg, 1999). It is now known to occur in all the cassava-growing countries of Africa and the adjacent islands and also, in India and Sri Lanka. A report of the disease in Indonesia in 1931 has not been confirmed and the mosaic disease of cassava in South America is caused by a different virus (Legg 1999).

There are great differences between regions in the overall prevalence of CMD and in the severity of the losses caused. The available information from surveys and yield loss assessments is summarized by Thresh *et al*. (1997), which estimates the losses in Africa to be 15–24%. When CMD was first described, the causal agent was assumed to be a virus, This view was consistent with the results of early studies showing that the disease was transmitted by whiteflies, now known to be *Bemisia tabaci* (Hunter *et al*., 1998) However, proof of the viral aetiology was not obtained until the 1970s and 1980s, when sap inoculations to herbaceous hosts were successful and virus isolates obtained in this way were purified and characterized (Monger *et al*., 2001a). After initial uncertainty, the isolates were shown to cause CMD, Koch's postulates were fulfilled and the various

isolates from Africa and India were regarded as strains of a single virus of the geminiviruses group and designated African cassava mosaic virus (ACMV). Subsequent studies have led to the recognition of several distinct but similar viruses: African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV) Indian cassava mosaic virus (ICMV) and South African cassava mosaic virus (SACMV) (Thresh *et al*., 1994)

What appears to be a hybrid recombinant between ACMV and EACMV has been reported in Uganda, Kenya, Tanzania, Sudan and Democratic Republic of Congo and designated EACMV-UgV (Deng *et al*., 1994). The different viruses have very similar properties and they are all members of the newly created family: Geminiviridae; Genus: Begomovirus (type member, Bean golden mosaic virus). Each of the cassava mosaic geminiviruses (CMGs) can cause CMD and there is evidence that virus combinations are more damaging than single infections (Pita *et al.*, 2001).

2.1.2 Distribution of cassava mosaic geminiviruses in Kenya

In Kenya, Cassava Mosaic Disease (CMD) is caused by begomoviruses in the family Geminiviridae. These include African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), and Uganda variant (EACMV-UG) of the genus Begomovirus. The viruses are spread by the whitefly (*Bemisia tabaci* Gennadius) and

by planting infected cuttings. Affected plants are stunted and have greatly diminished tuberous root yield (Munga, 2000).

Members of six species of begomoviruses have been identified in association with CMD in Africa: African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), East African cassava mosaic Cameroon virus (EACMCV), East African cassava mosaic Malawi virus (EACMMV), East African cassava mosaic Zanzibar virus (EACMZV) and South African cassava mosaic virus (SACMV) (reviewed by Legg and Fauquet, 2004). These viruses are believed to have evolved from indigenous African viruses adapted to cassava upon its introduction during the 18th century. Initially they existed in distinct geographical regions (Hong & Harrison, 1995), but more recently their distribution has become more complex as a result of two possible factors. Firstly, cassava is propagated vegetatively, a process that not only perpetuates the virus, but also leads to dissemination of the virus in infected planting material (Gibson and Otim-Nape, 1997; Chellappan *et al*., 2004). Second, both trade and human migration due to drought and conflict can result in the spread of infected planting material over great distances.

Representatives of only three begomovirus species have previously been reported from Kenya, namely ACMV, EACMV and EACMZV (Stanley and Gay, 1983; Bull *et al*., 2003; Were *et al.*, 2004a;b), The recombinant strains of EACMV, EACMV-UG have been identified in the east and west of the country. EACMZV is a recently identified

begomovirus species that is believed to have originated on the island of Zanzibar, but is now also found in coastal areas of Kenya (Bull *et al*., 2003; Maruthi *et al.*, 2004). All major cassava-growing areas of Kenya were surveyed to assess the current diversity and geographical distribution of begomovirus associated with CMD.

2.1.3 Symptoms of cassava mosaic disease

The symptoms of CMD are characteristic leaf mosaic patterns that affect discrete areas determined at an early stage of leaf development. The chlorotic areas fail to expand fully so that stresses set up by unequal expansion of the lamina cause malformation and distortion. Severely affected leaves are reduced in size, misshapen and twisted, with yellow areas separated by areas of normal green colour. The plants are stunted and the young leaf abscises (Storey and Nichols, 1938; 1951).

The leaf chlorosis may be pale yellow or nearly white, or just discernibly paler than normal. The chlorotic areas are usually clearly demarcated and vary in size from the whole leaflet to small flecks or spots. Leaflets may show a uniform mosaic pattern or the pattern is localized to a few areas which are often at the bases of the leaflets. Distortion, reduction in leaflet size and general growth retardation, appear to be secondary effects associated with symptom severity. The photographs (Plate 1 and 2) below show the typical foliar symptoms as compared to the healthy leaves of the crop. The sickle shaped leaves in Plate 1 shows a combination of CMG's and DNA satellites

Plate 1:Cassava plants infected with CMD and DNA satellites

Plate 2:Healthy cassava plant leaves without CMD symptoms

2.1.4 Transmission of cassava mosaic begomoviruses

A number of studies have been done by Chant (1958) and Dubern (1994, 1979) confirming that the whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) transmits CMGs. Transmission was shown to be persistent, transtadial but not transovarial (Dubern, 1994), with minimum times for acquisition, latent period, and inoculation of 3.5 hours and 5–10 minutes, respectively. Reports on transmission efficiency have varied from very low (0.15–1.7% of individuals infective) for field-collected insects (Fargette *et al*., 1985), to moderate (4–13%) for laboratory-reared insects (Dubern 1994; Maruthi *et al.*, 2002). Recent evidence also suggests that there is only limited co-adaptation between CMGs and their vector within Africa, as the

frequencies of transmission of different CMGs by *B. tabaci* populations from geographically distant locations in Africa were not significantly different (Maruthi *et al*., 2002).

High incidences of begomovirus are associated with high populations of whiteflies and serious losses in several crops in the Americas, Africa and the Caribbean Basin (Brown and Bird, 1992; Polston and Anderson, 1997; Morales and Anderson, 2001).

Unlike the mosaic symptoms of CMD, the foliar symptoms of CBSD are less conspicuous and farmers are often unaware of the problem until the roots are harvested and the corky, yellow-brown necrotic rot becomes evident. In both cases, the diseases are also spread by way of infected planting materials such as the stem cuttings. The use of infected equipment in preparation of the cuttings for vegetative propagation leads to contamination of otherwise clean stem cuttings from healthy plants. Cross boundary movement has also contributed a lot to the spread of the diseases. Naturally, it is a tradition in most parts of Africa for people to carry vegetative planting materials from sources that are perceived to be superior in terms of yield and quality. This has led to movement of these plant materials through vast distances.

Several government and non-governmental agencies are involved in the sourcing and distribution of clean planting materials for cassava for the farmers. However, it is an uphill task to physically determine whether the plant material is infected by the virus or not. This has posed a serious challenge since even materials which are presumably regarded to be clean having been found to harbour the virus thus causing the disease and spread to the farmers' fields.

2.1.5 Genome organization of cassava mosaic begomoviruses

Begomoviruses are small (18-30 nm long) plant viruses with single-stranded circular DNA genomes that are encapsulated in twinned quasi-icosahedral particles. They belong to the Geminiviridae family. begomoviruses are transmitted by whiteflies, and infect dicotyledonous plants; their genomes can be mono or bipartite (Lazarowitz, 1992). They cause significant and often total yield losses of important food and industrial crops in tropical and subtropical regions of the Western and Eastern Hemispheres (Morales and Anderson, 2001; Navas- Castillo *et al*. 1998; Polston and Anderson, 1997). begomoviruses are mostly bipartite, but some are monopartite. Bipartite begomoviruses have two components, designated A and B. Each component has 2,600 nt. The genes on the A component are involved in encapsulation and replication, whereas the genes on the B component are involved in the movement of virus through the plant, host range, and symptom expression (Lazarowitz, 1992; Gafni and Epel, 2002). One of the five genes in the A component, the coat protein (*CP*) gene, is transcribed in the viral sense or clockwise direction. The other four genes replication-associated protein (*Rep*), transcriptional activator protein (*TrAP*), replication enhancer (*REn*), and *AC4* are transcribed in complementary sense or counter clockwise direction (Lazarowitz, 1992). The two sets of genes overlap and are separated by an intergenic region (IR), which begins with the start codon of the *Rep* and ends with the start codon of the *CP*. This region does not encode any protein and its sequence varies widely among begomoviruses, except that there is a conserved GC-rich inverted repeat

sequence, which has the potential to form a stem-loop structure (30 nt) with the invariant nanomeric TAATATT (↓) AC sequence or loop of the stem-loop structure. The nanomeric sequence contains the initiation site (↓) of rolling circle DNA replication (Gutierrez, 2000; Laufs *et al*. 1995a), the TATA box, and the forward and inverted repeats. In bipartite begomoviruses, the IR also contains an identical sequence of 200 nt in the A and the B components called the common region (CR) (Lazarowitz, 1992). The CR sequence is different among different begomoviruses and is used to identify the A and B components of the same virus (Gafni and Epel, 2002).

The *CP* is required for encapsulation of progeny virions, vector transmission, virion structure, and host specificity. For bipartite begomoviruses, the *CP* is not required for either local or systemic viral spread. In contrast, in all monopartite begomoviruses, the *CP* is essential for viral spread (Gafni and Epel, 2002). The *Rep* is the only gene essential for replication, being required for transcription of both A and B components (Argüello-Astorga *et al*., 1994). Begomoviruses replicate in the nucleus of infected cells through a double-stranded DNA intermediate via a rolling circle mechanism. The Rep protein has two functional targets or DNA elements in the begomovirus origin of replication. The nanomeric sequence, where the Rep introduce a site-specific nick to initiate virus replication for the rolling circle mechanism and secondly tandem repeated motif located at variable distances from the conserved hairpin sequence, which is bound specifically by its cognate Rep protein. This motif functions as a major recognition element of the replication origin in begomoviruses. These *cis*-acting elements belong to

a series of iterate DNA motifs called introns (Argüello-Astorga and Ruiz-Medrano, 2001). A transcription factor binds to a cis-regulatory element, associated with the 5′ border of the stem-loop sequence, and creates a nucleosome-free region in its neighbourhood. The transcriptional factor interacts with the TATA binding protein, by means of its activation domain, looping the intervening DNA. This event would place the stem-loop structure in an accessible position so that the Rep complex can nick the viral (+) strand in the loop of the hairpin structure. The stem-loop structure may acquire a cruciform structure as a consequence of the interactions with the transcription factor or/and Rep (Argüello-Astorga *et al*. 1994).

TrAP is a trans activator of the expression of the *CP* and the *nuclear shuttle protein (NSP)* genes (Sunter *et al*. 1990; Sunter and Bisaro, 1991). The *TrAP* along with the two proteins encoded by the B component is indirectly involved in the systemic movement of the virus through the plant (Gafni and Epel, 2002). The *REn* is not essential for viral replication. However, viral DNA replicates at higher levels when *REn* is present (Sunter *et al*., 1990). The *AC4* gene is involved in symptom expression of monopartite begomoviruses, but to date does not appear to have a function in bipartite begomoviruses.

The B component has two genes: the *NSP* gene, which is transcribed from the viral-sense strand, and a *movement protein* (*MP*), which is transcribed from the complementary-sense strand. The *NSP* is implicated in nuclear shuttling of the viral

genome, and *MP* is involved in cell-to-cell movement of the virus via plasmodesmata (Gafni and Epel, 2002). The *MP* appears to be a symptom-inducing element or a determinant of pathogenicity of bipartite begomoviruses. Mutation studies suggest that the 3' region of the *MP* gene is associated with symptom development (Gafni and Epel, 2002). Bipartite begomoviruses often spontaneously produce approximately half-sized defective DNA B components that function as defective interfering (DI) DNA. The (DI) DNA may have a biological role during infection to reduce the severity of the disease by competing with the genomic components for cellular resources (Stanley *et al*., 1990). Monopartite begomoviruses have small circular single-stranded DNA satellites, named DNA β. These depend on begomoviruses for their proliferation and, in turn, they affect the accumulation and symptom expression of begomoviruses (Mansoor *et al*., 2003).

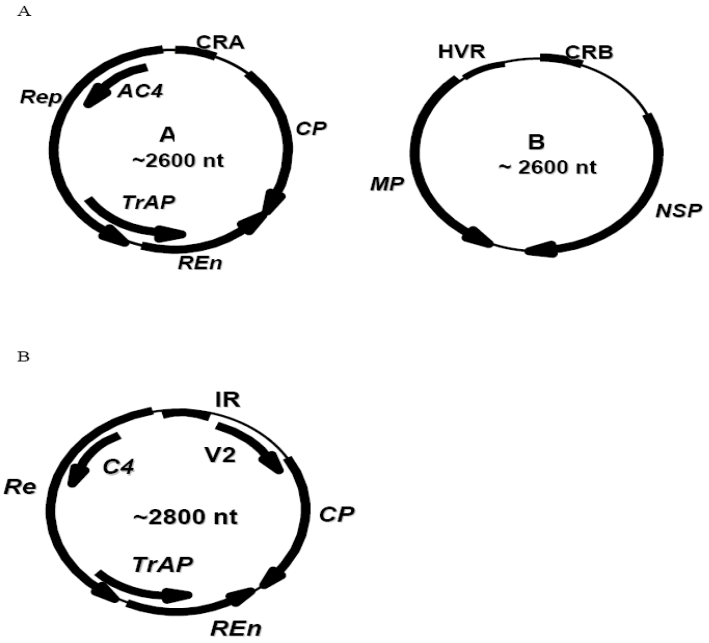

Figure 1:Genomic structure of begomviruses. A) Bipartite begomoviruses and B) Monopartite begomoviruses

2.2 DNA Satellites associated with begomoviruses

Geminiviruses are often associated with sub-viral agents called DNA satellites that require proteins encoded by the helper virus for their replication, movement and encapsulation. Hitherto, most of the single-stranded DNA satellites reported to be associated with members of the family Geminiviridae have been associated with monopartite begomoviruses (Basavaprabhu and Fauquet, 2010). Cassava mosaic disease is known to be caused by viruses belonging to nine different begomovirus species in the African continent and the Indian subcontinent. In addition to these species, several strains have been recognized that exhibit contrasting phenotypes and infection dynamics. It is established that Sri Lankan cassava mosaic virus can trans-replicate beta satellites and can cross host barriers (Kumar *et al.*, 2007). Cassava-infecting geminiviruses have showed a contrasting and differential interaction with the DNA satellites, not only in the capacity to interact with these molecules but also in the modulation of symptom phenotypes by the satellites mosaic disease complex (Briddon *et al.*, 2004). Detection techniques for these circular DNA molecules have been pegged on the nucleotide sequences. Primers have been designed to amplify some specific regions of the DNA molecule (Dry *et al*., 1997).

2.3 Detection of begomoviruses

begomoviruses have been detected in plants or insects by different techniques, such as visualization of nuclear inclusion bodies by light microscopy, ultrastructural localization of virions in plant cell by transmission electron microscopy, serological assays using polyclonal or monoclonal antibodies (Hunter *et al*. 1998; Konate *et al*.

1995; Pico *et al*. 1999; Polston *et al*, 1989), DNA hybridization assays (Lotrakul *et al*. 1998), Polymerase chain reaction (PCR) (Deng *et al.*, 1994; Ghanim *et al*. 1998; Lotrakul *et al*. 1998; Mehta-Prem *et al*. 1994; Pico *et al*. 1999; Rosell *et al*. 1999), immunocapture PCR (Rampersad and Umaharan, 2003), and print-PCR (Navas-Castillo *et al*. 1998) among others. Molecular cloning and DNA sequencing of viral genomes have become the tools of choice, allowing virus identification and evaluation of relationships with other virus isolates (Brown *et al*. 2001; Padidam *et al*. 1995; Paximadis *et al*. 1999; Rybicki, 1994).

New technics like the Next Generation Sequencing (NGS) can be used for identifying pathogens especially viruses if you are not sure of the specific strain or species (Adams *et al*., 2012).

2.3.1 Serological techniques

Begomoviruses can be detected in plant tissues using serological assays. In this procedure, polyclonal antiserum is prepared to the different begomoviruses coat proteins. These are usually expressed in *E- coli* serving as useful serological probes for begomoviruses in ELISA, Western blots and leaf imprint blots. ELISA is the most commonly used technique with the prepared plant extracts reacting with the polychlonal antisera for the begomoviruses prepared (Cancina *et al.*, 1995). Absorption readings are done and figures evaluated.

2.3.2. Nucleic acid- based techniques

The polymerase chain reaction (PCR) is a powerful technique that has widespread application in molecular biology. This technique is used to amplify a specific nucleic acid fragment that lies between two regions of known nucleotide sequence, and often from an extremely small amount of target nucleic acid in biologically complex samples. The polymerase chain reaction (PCR) was first proposed by Gobind Khorana *et al*. in 1971, but several elements required for PCR, particularly thermostable DNA polymerase, had not yet been discovered at that time. They did not further develop their idea. In 1983, Kary Mullis independently developed PCR.

Begomoviruses are either monopartite or bipartite. The coat protein (CP) gene is highly conserved among begomoviruses originating from the same geographical region (Maruthi *et al.*, 2002). Smaller fragments comprising the core coat protein gene (core CP), a partial 575-579 base pair (bp) sequence of the Coat Protein gene (Brown *et al.*, 2001), or the complete CP sequence have also been used to establish provisional species identification owing to the highly conserved nature of the viral CP sequence. As such primers for the coat protein gene are always designed to amplify either the partial or complete protein gene through the PCR process. This detection method is now very popular due to its accuracy if other procedures especially in DNA extraction, amplification and gel electrophoresis are done as required.

2.4 Importance of cassava mosaic geminiviruses

Proliferation and rapid dissemination of begomoviruses that infect important food and industrial crops in Latin America and Africa have been the consequence of drastic changes in traditional cropping systems (Morales and Anderson, 2001), along with the introduction of the B biotype of *Bemisia tabacii* in the mid 1980s (Polston and Anderson, 1997). The B biotype of *B. tabacii* has displaced many indigenous biotypes, because of its broader host range, higher fecundity, dispersal capacity, virus-transmission efficiency, and resistance to insecticides traditionally used against whiteflies (Brown *et al*. 1995). Begomoviruses have been reported as limiting factors in the production of several crops in the Americas such as cotton, common bean, tomato, pepper and cucurbits, among others (Morales and Anderson, 2001; Polston and Anderson, 1997).

In the 1990s, Cassava mosaic begomoviruses caused a major regional pandemic of Cassava mosaic disease (CMD) (affecting parts of at least five countries in Africa, Kenya inclusive) that led to massive economic losses and destabilization of food security (Legg and Thresh, 2000). A key factor in the genesis and spread of the pandemic was the recombination of two distinct cassava mosaic begomoviruses to produce a novel and more virulent hybrid (Pita *et al*., 2001). Resistance was developed

originally in Tanzania, providing effective CMD control in current pandemic-affected areas of East Africa. (Legg and Thresh, 2000).

CHAPTER THREE

DISTRIBUTION OF CASSAVA MOSAIC GEMINIVIRUSES AND ASSOCIATED DNA SATELLITES IN KENYA

Abstract

A countrywide survey was conducted to determine the incidence, prevalence and severity of cassava mosaic disease (CMD) and the associated DNA satellites in Kenya. The survey focused on the areas in which cassava is grown as a food crop. Disease incidence, prevalence and severity were assessed in all the selected fields visited. Whitefly counts were done on plants randomly selected in the fields visited. Method of disease transmission either by whitefly or infected cuttings was also determined. PCR detection method was used in the detection of these viruses and the associated DNA satellites using the DNA extracted from the samples collected from the field. CMD was widely distributed in the country with an average incidence of 57.3% countrywide whereas Coast province recorded the highest incidence (73.8%). The prevalence of CMD countrywide was 84.6% with Nyanza province recording the highest (96.2%) prevalence, whereas Eastern province had the least (66.7%) prevalence. The spread of CMD through use of infected cuttings accounted for 80.6% of the infected plants compared to the whitefly-borne infections which only accounted for 19.4%. East African Cassava Mosaic Virus (EACMV) and African Cassava Mosaic Virus (ACMV) accounted for 51% and 20% of samples, respectively. Co-infection of cassava plants with the two viruses was detected in only 9% of the samples. EACMV was detected in samples collected from all the provinces surveyed with nearly all the districts visited

recording the presence of EACMV. ACMV on the other hand was mostly prevalent in the districts in Western and Nyanza provinces although for the first time, ACMV was detected in samples collected from Eastern and Coast provinces for the first time. Nyanza province had the highest whitefly count with Western province registering the least whitefly counts per plant. The method of transmission of CMD was mainly through the distribution or use of infected cassava cuttings with 100% transmission by whiteflies in Coast province. DNA satellites associated with these Begomoviruses were distributed across the areas under survey with 41.4% of the samples collected testing positive for the DNA satellites. There was a marked increase in symptom severity in plants infected by CMGs and the associated DNA satellites compared to those infected with CMGs only. There is need for the identification of varieties resistant to these viruses and pooling regional efforts in the characterization of the viruses to further understand reasons behind the high disease severities in some areas. The begomovirus symptom modulation by the DNA satellites need to be further investigated to determine any effect on the disease severity and yield of cassava.

3.1 Introduction

Cassava (*Manihot esculenta* Crantz) is a major staple food for many communities in sub- Saharan Africa. In Kenya, cassava is grown on over 90,000 ha with an annual production of about 540 000 tons (USAID, 2009). Cultivation is concentrated in Nyanza and Western provinces (60%), Eastern (10%), and Coast provinces (30%). The crop is grown by resource poor households for subsistence where it is an important food

security crop. The available information from surveys and yield loss assessments due to CMD is summarized by Thresh *et al*., (1997), which estimates the losses in Africa to be 15–24%. In Kenya, yields recorded range between 5 and 10t/ha against a potential of 32t/ha (Munga, 2000).

CMD is transmitted by a whitefly vector known as *Bemisia tabaci* but proof of viral aetiology was not obtained until the 1970s and 1980s, when sap inoculations to herbaceous hosts were successful and virus isolates obtained in this way were purified and characterized (Monger *et al*., 2001a). After initial uncertainty, the isolates were shown to cause CMD, Koch's postulates were fulfilled and the various isolates from Africa and India were regarded as strains of a single virus of the geminiviruses group and designated African cassava mosaic virus (ACMV). Subsequent studies have led to the recognition of several distinct but similar viruses namely African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), Indian cassava mosaic virus (ICMV) and South African cassava mosaic virus (SACMV) (Briddon *et al*., 2004).

In Kenya, cassava mosaic disease (CMD) is caused by begomoviruses in the family Geminiviridae. These include African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), and Uganda variant (EACMV-UG) of the genus begomovirus. Previous studies have shown ACMV, EACMV, EACMV-UG and EACMZV to be present in Kenya (Were *et al*., 2004: Bull *et al*., 2006). Earlier reports

indicate that EACMV, EACMV-UG and EACMZV have distinct geographical distributions (Bull *et al.,* 2006).

The whitefly vector, *Bemisia tabacii* (Gennadius) (Aleyrodidae, Hemiptera) transmits Cassava mosaic begomoviruses (CMBs) from plant-to-plant. Long-distance spread of CMD occurs by the distribution of infected stem cuttings (Olufemi *et al.,* 2008). Whitefly presence on plants does not necessarily suggest that the disease is spread by the insects. Affected plants are stunted and have greatly diminished tuberous root yield. Cassava is also affected by the DNA satellites associated with Cassava mosaic geminiviruses (Nduguru *et al.,* 2007).

This survey focused on determining the status and distribution of the CMG's and the DNA satellites particularly their incidence, prevalence and severity in all major regions where cassava is grown in the country.

3.2 Material and methods

3.2.1 Sampling sites

The survey was carried out in four distinct regions which are also administrative regions namely provinces. The provinces surveyed were Eastern, Nyanza, Western and Coast provinces. These are the major regions where cassava is grown as one of the major food crops. The districts within these regions where sampling was done were selected according to the importance of cassava as a food crop and where the disease under study has caused serious problems. Fields having a cassava crop as a pure stand or intercropped with other crops were selected and randomly surveyed along selected routes at 5-10 km intervals. A total of 94 cassava fields were surveyed. In each field, the coordinates and altitude were recorded using a global positioning system (GPS; Magellan GPS 315, San Dimas, CA).

In Nyanza province, the survey and sampling was done in the following districts; Kisii central, Gucha, Kuria west, Migori, Rongo, Homa Bay, Rachuonyo, Gem, Bondo and Siaya. In Eastern province, sampling was done in Imenti south, Tharaka south, Maara, Meru south, Embu, Mbeere north, Mbeere, Kitui, Kitui central, Mwala, Makueni, Kangundo and Kathiani districts. In Western province, survey and sampling was done in the following districts; Kakamega south, Butere, Mumias, Busia, Bumula, Teso North, Teso South, and Bungoma west. Finally sampling was done in Coast province in the following districts; Kilifi, Malindi, Kwale, Msambweni and Taita.

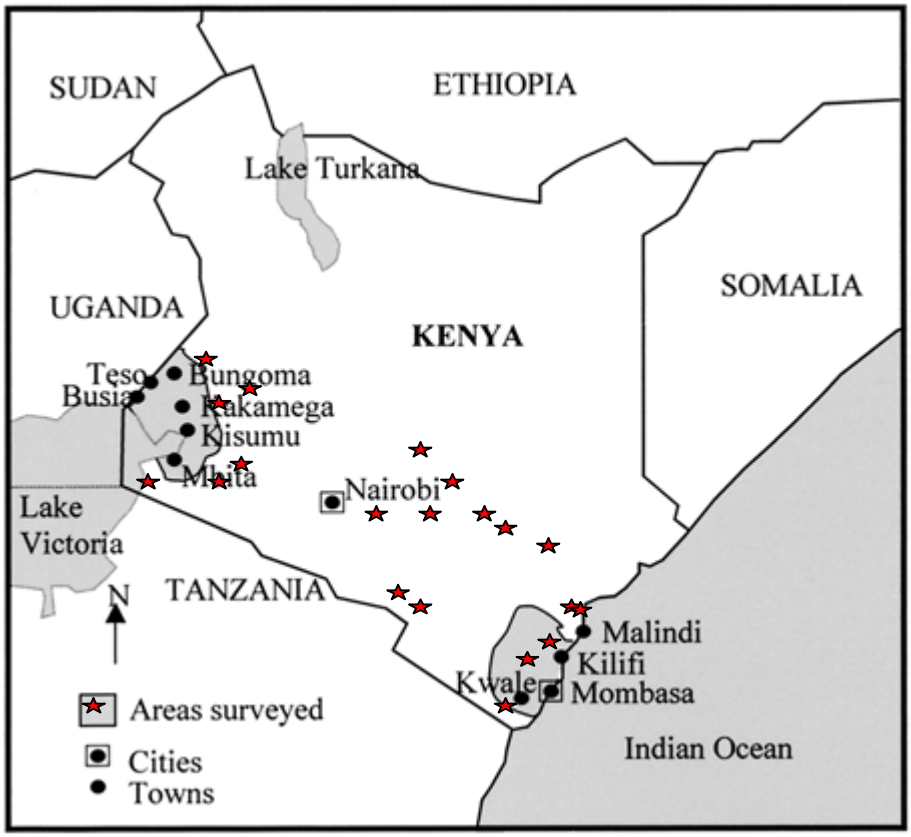

Figure 2: Areas surveyed for CMGs and associated DNA Satellites

3.2.2 Sampling procedure

Sampling was done using the stratified random sampling method. The geographical regions hereby defined as provinces formed the four strata. During sampling, representative plants were selected along X-shaped transects in each field for the

determination of disease incidence and severity (Otim-Nape, 1993; Otim-Nape *et al.*, 1998a; Sseruwagi *et al*., 2003).

An imaginary line (transect) was drawn diagonally in the field from both directions thus ending up with two transects within one field. A total of 15 plants were examined for the symptoms of both Cassava Mosaic Virus disease (CMD) and the associated DNA satellites on each transect. The symptoms of DNA satellites are characterized by sickle shaped deformed leaves infected with CMD. In total, 30 plants in every field were examined. The prevalence of the viral diseases was evaluated in every region by calculating the number of fields in which at least one cassava plant presented symptoms of viral diseases divided by the total number of fields observed in that region. The disease severity symptoms for both diseases were established with disease severity scale (1-5) (James, 1974) which is internationally accepted and adopted. For CMD, the plants were observed for the foliar symptoms and their satellites symptoms.

Farms or fields having cassava crop as a pure stand or intercropped with other crops were selected and randomly visited along the selected routes within the region. In each region, a particular representative route that captures the area of interest was discussed and agreed upon by the survey team and adopted. Amongst issues considered include the sample area and availability of suitable cassava fields. Farmers' fields were selected after every 5 km in densely populated areas due to close proximity of the small scale farms growing cassava such as in Western province and some parts of Coast, Nyanza

and Eastern province. In marginal and sparsely populated areas like Ukambani districts in Eastern province, a distance interval of 10 km was adopted. In all, 94 fields were visited during the survey. In each field, the coordinates were recorded using a global positioning system (GPS; Magellan GPS 315, San Dimas, CA).

Cassava plants in farmers' fields were observed for virus disease symptoms. Foliar samples from plants infected by CMD and the DNA satellites were picked and preserved in bottles containing silica gel granules. The tender young leaves are the ones that were picked avoiding the old leaves and woody parts. In each field, 3 – 4 samples were taken with a total of 350 samples. The leaf samples were put in vials containing silica gel for desiccation and were taken to the laboratory for analysis.

3.2.3 Whitefly counts and mode of transmission

This study determined whitefly counts and also investigated the method of transmission of the cassava mosaic geminiviruses. The population of adult whiteflies was determined on the five top-most apical leaves of the tallest shoot of each sampled plant. This was done in early morning hours since the flies become active as the day warms up. This makes it difficult to count the whiteflies in the cassava fields after 10 in the morning.
Plants exhibiting symptoms on upper leaves indicated inoculation by whiteflies while those showing symptoms in all parts of the plant indicate transmission of CMD through cuttings. As such, scoring for whitefly infected fields was denoted by letter W while those infected by cuttings by the letter C.

3.2.4 Detection of cassava mosaic geminiviruses in collected samples

3.2.4.1 Nucleic acid extraction and detection of cassava mosaic geminivirus

All begomoviruses code for coat protein which act as the protective coat of the virus particle and determine vector transmitability of the viruses by whitefly vector *B. tabacii.* Thus, the CP gene is highly conserved among begomoviruses originating from the same geographical region and adapted to transmission by local vector populations (Maruthi *et al*., 2002). Smaller fragments comprising the core coat protein gene (core CP), a partial 575-579 base pair (bp) sequence of the coat protein gene (Brown *et al.,* 2001), or the complete CP sequence have also been used to establish provisional species identification owing to the highly conserved nature of the viral CP sequence.

Total nucleic acid (TNA) was extracted from the dry leaf samples using the CTAB based method (Lodhi *et al.,* 1994). About 0.03g of the dried leaf samples was ground in 1.5ml of CTAB extraction buffer. About 750µl of the sample was poured into a 1.5ml eppendorf tube and incubated at 65^{o}C for 30min. The samples were then mixed with an equal volume 750µl of chloroform: Isoamyl alcohol (24:1). They were mixed by gentle shaking before being centrifuged at 1200 rpm for 10min. The top aqueous phase was transferred into a new eppendorf tube and an equal volume (750µl) of chloroform:Isoamyl alcohol(24:1) was added, mixed and centrifuged again as in the previous step. 300µl of the top aqueous phase was transferred into a new eppendorf tube and DNA was precipitated by adding two volumes (600µl) of ice cold isopropanol. The samples were then centrifuged at 8000 rpm for 10min and the resulting supernatant

discarded. The pellet was then washed in 0.5ml of 70% ethanol by vortexing and then centrifuged at 8000 rpm for 5min. Ethanol was removed gently and the pellet air dried for 30min. The pellet was suspended in sterilized water and stored at -20^{o}C.

The PCR mix consisted of GoTaq green (Promega), 10µl of each primer (Forward primer EAB555F and reverse primers EAB555R) of the template DNA. Go Taq green contains Taq polymerase enzyme and dNTPs. The final reaction volume was 20µl. Universal primers were used to detect African cassava mosaic virus (ACMV) with an expected amplicon of 774bp (Ndunguru *et al.,* 2005). The universal primers used for detection of ACMV were JSP001 (5'-ATGTCGAAGCGACCAGGAGAT-3') ACMV the forward primer and (AV1/CP) JSP002 (5'-TGTTTATTAATTGCCAATACT-3') ACMV (AV1/CP) the reverse primer. The PCR detection of EACMV was done using EAB555 F/R primers whose sequences were EAB555/F (5'-TACATCGGCCTTTGAGTCGCATGG-3') EACMV DNA-B and EAB555/R (5'-CTTATTAACGCCTATATAAACACC-3') EACMV DNA-B.

These primers are designed to amplify a 556 bp fragment of EACMV DNA B component (Ndunguru *et al*., 2005). The cycling regimes was as follows; the first step (initial denaturation) was at 94^{o}C for 3 minutes, second step was at 94^{o}C for 1min, the third cycle at 72^{o}C and the final cycle at 48^{o}C (annealing) for 1min. The reaction was set for 31 cycles. After the 31st cycle, the PCR reaction tubes were removed from the thermocycler and stored temporarily at 4^{o}C awaiting gel electrophoresis. The PCR

cycling regimes were the same as those of EACMV detection. The annealing temperature of 48°C worked perfectly with generation of well amplified DNA bands after agarose gel electrophoresis.

3.2.4.2 Nucleic acid extraction for detection of DNA satellites

The CMD viral DNA was also analyzed for the detection of DNA satellites associated with Cassava mosaic geminiviruses. Specific primers designed for the amplification of the integrated and episomal satellites were used in the PCR based detection technique. Nucleic acid extraction was carried out in a similar method as for the testing for CMD.

The primers used for the detection of DNA Integrated satellites which amplify the DNA-B with and expected 306bp PCR product were ;

SAT III F-5'-AGGCCTCGTTACTAAAAGTGC-3'

SAT III R-5'-ACCTGACGGCAGAAGGAAT-3'

The mastermix was prepared with one of the set ups for 17 samples. PCR cycling regimes or program was as follows; Initial denaturation 94^{o}C for 3min, denaturation 94^{o}C for 1min, annealing 55^{o}C for 1.5min and extension of 72^{o}C for 1min. The final step in PCR extension was for 4mins at 72^{o}C.

3.2.5 Statistical analysis

Data on disease prevalence, incidence and severity were subjected to one way Analysis of variance (ANOVA) using Genstat discovery edition software (2005). Mean

comparison of the incidence, severity were done using student t –test at 95% confidence level. ANOVA test was used to determine any significant differences between the means of the three independent variables of CMD incidence, prevalence and severity. The t test was used to separate the means.

3.3 Results

3.3.1 CMGs incidence, prevalence and severity based on symptomatology

A total of 94 fields, 23 in Eastern province, 26 in Nyanza province, 25 in western province and 20 in Coast province were visited during the survey. A total of 350 samples with symptoms of CMGs and the DNA satellites associated with the CMGs were collected from the fields. Table 1 shows the disease incidence, prevalence, symptom severity and types of infection within the districts surveyed in the four provinces surveyed.

Table 1:CMD incidence, prevalence, symptom severity and type of infection in sampled Kenyandistricts in 2009.

Province	District	No.of fields	Disease incidence (%)	Prevalence (%)	Severity(1-5 scale)	Type of infection
Western	Kakamega	3	73±1.15	50±0.33	3.1±0.11	C
	Butere	3	66±1.15	100±0.00	2.8±0.11	C
	Mumias	2	75±0.57	100±0.00	2.3±0.05	C and W
	Busia	3	22±1.15	75±1.15	2.1±0.05	C
	Teso South	4	31±0.57	80±0.57	2.4±0.11	C and W
	Teso North	4	25±1.15	66±1.15	2.8±0.11	C
	Bumula	3	26±1.73	85±2.3	2.1±0.05	C and W
	Bungoma W.	3	63±1.73	100±0.00	3.9±0.05	C and W
	Mean		**47.6**	**82**	**2.7**	
Nyanza	Siaya	4	58±1.73	100±0.00	3.7±0.17	C
	Bondo	3	62±1.15	100±0.00	3.1±0.05	C
	Rachuonyo	3	36±1.15	100±0.00	3.2±0.11	C
	Homa Bay	3	55±1.15	60±0.57	3.3±0.11	C
	Rongo	3	46±0.57	100±0.00	2.8±0.11	C
	Migori	3	6±0.57	100±0.00	3.0±0.11	C
	Kuria West	3	54±1.73	100±0.00	3.8±0.17	C
	Gucha	1	13±1.15	100±0.33	2.0±0.12	W
	Kisii Central	2	70±1.73	100±0.00	3.5±0.11	C
	Mean		**44.4**	**95.5**	**3.2**	
Eastern	Kathiani	2	53±0.33	50±2.98	3.4±0.11	C and W
	Kangundo	1	30±1.73	100±0.00	2.3±0.11	C and W
	Makueni	2	68±1.15	90±2.3	3.3±0,11	C and W
	Mwala	2	16±0,57	50±0.57	2.5±0.12	W
	Kitui Central	3	100±0.00	100±0.00	4.3±0.08	C
	Mbeere South	2	0	0	-	-

Province	District	No.of fields	Disease incidence (%)	Prevalence (%)	Severity(1-5 scale)	Type of infection
	Mbeere North	2	0	0	-	-
	Embu	1	33±0.11	33±0.57	2.3±0.05	C
	Meru South	2	6.6±1.15	45±0.57	2.1±0,12	C
	Maara	2	81.6±0,3	100±0.33	4.1±0.55	C
	Tharaka South	2	96±1.15	100±0.00	3.8±0.11	C
	Imenti South	2	76±0.57	100±0.00	2.1±0.05	C and W
	Mean		**46.7**	**64**	**2.8**	
Coast	Kilifi	4	80±1.73	100±0.00	3.5±0,11	C
	Malindi	4	98±0.57	100±0.00	4±0.55	C
	Msambweni	4	68±1.15	100±0.00	3.3±0.12	C
	Kwale	5	52±2,3	100±0.00	2.8±0.11	C
	Taita	3	71±1.15	66±0.57	3.2±0.11	C
	Mean		**73.8**	**93.2**	**3.4**	
	Total means		**57.3**	**81.6**	**3.1**	
	LSD0.05		**6.13**	**34.05**	**0.52**	

C-cuttings borne infections W- whiteflies borne infection

Incidence and prevalence is expressed in percentages while severity in the scale 1 – 5.

Table 2 depicts the analysed data for disease incidence, prevalence, severity and whitefly counts at the provinces level. There was a significant difference in CMD prevalence between all the provinces where the survey was done. The same trend was evident with the disease incidences in the four provinces under study. However, there

was no significant difference of CMD severity in all the provinces surveyed apart from Western province.

Cassava mosaic disease was observed in major areas where cassava is grown in Kenya. The disease is widely distributed countrywide with an average incidence of 57.3% (Table1). Coast province had the highest average CMD incidence (74.0%) followed by Eastern province recording a mean incidence of 57.0%. Western and Nyanza province had the lowest CMD incidence of 47.0% and 51.0%, respectively. Overall CMD prevalence was 81.6% with Nyanza province recording the highest (96.0 %) prevalence followed by coast province with a disease prevalence of 93.0%. Eastern province had the least disease prevalence of 78.0% (Table 2).

Coast province had the highest CMD symptom severity (3.4) but this was not statistically significant from other provinces except Western (2.7). A mean severity of 3.1 countrywide indicates the severe symptoms prevalent in the survey areas.

Table 2: Incidence, Prevalence, Severity of cassava mosaic disease and the whitefly counts in the four major cassava growing provinces in Kenya(2009)

Province	No.of fields	CMD incidence	CMD prevalence (%)	Whitefly counts per leaf	CMD severity
Eastern	23	57.4±0. 3[b]	78.0±2.0[d]	1.86±0.16[b]	3.1±0.3[a]
Nyanza	26	51.0±0.4[d]	96.0±2.0[a]	3.18±0.17[a]	3.2±0.2[a]
Western	25	47.0±0.3[c]	82.0±3.0[c]	1.16±0.07[c]	2.7±0.2[b]
Coast	20	74.0±2.0[a]	93.0±2.0[b]	2.99±0.21[a]	3.4±0.1[a]
Means		**57.3**	**81.6**	**2.3**	**3.1**

Means with the same subscripts down the columns denotes no significant differences between the means at p=0.05

Table 3 summarizes the molecular detection work for Cassava mosaic virus amongst the samples collected. ACMV was for the first time detected in Eastern and Coast province. Dual infection of EACMV and ACMV were common in Nyanza, Western and Coast province.

Table 3: Detection for EACMV and ACMV in the four provinces under survey

Province	No. of Samples tested	Positive for EACMV	Positive for ACMV	Dual infections
Nyanza	97	21	4	4
Western	110	11	3	2
Coast	62	11	3	3
Eastern	78	11	1	0
Total samples	**350**	**51**	**11**	**9**

From the PCR-based detection, EACMV was more widespread than ACMV in the country. EACMV occurred in all the provinces surveyed (Table 3). Nearly all the districts under survey showed the presence of EACMV. However, ACMV was mostly prevalent in Western, Nyanza and for the first time in Coast and Eastern province (Table 3). The distribution was not so much intense as EACMV. About 18 out of 61 samples had ACMV constituting 29.5% in Western province. ACMV was recorded only in one sample from Kathiani district in Eastern province. The presence of ACMV was detected in leaf samples collected from several fields in Kilifi, Msambweni and Kwale districts of Coast province, an area previously presumed to be ACMV-free. Co-infection of 8% EACMV and ACMV was recorded in field samples collected. Co-infection was more prevalent in Nyanza and Western province and to some extent in Coast province. Teso North, Teso South and Bungoma West districts in Western Kenya had the highest co infection rates of the two viruses.

3.3.2 Survey of CMG's and associated DNA satellites

3.3.2.1 Detection of cassava mosaic geminiviruses

The PCR product of 556bp was evident as expected for the amplification of the DNA – B with EAB555F/R primers for the detection of EACMV (Plate 3). For the detection of ACMV, the expected PCR product of 774bp was realized after amplification of the ACMV coat protein gene by the primer set JSP001/002.

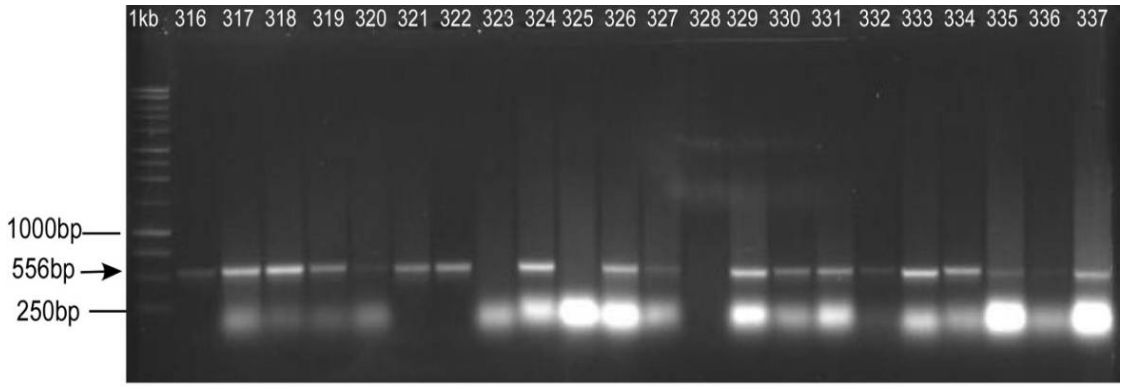

Plate 3:PCR products (556bp) of East African cassava mosaic virus (EACMV) from infected cassava leaf samples total nucleic acid.
Lane 1 is the 1kb DNA marker. The numbers in the gel picture are sample numbers.

3.3.2.2 PCR detection of the CMD associated DNA Satellites III

The PCR products after the amplification and gel electrophoresis were of the expected size of 306 base pairs (Plate 4). The 1kb molecular marker was used thus perfectly giving the expected PCR product as shown in plate 4. Some samples were negative for the DNA integrated satellites but the majority of the samples collected from the field with typical symptoms of the satellites associating with the CMGs tested positive. The integrated satellites were common amongst the samples collected during the survey. Out the 350 samples collected from the field during the survey, 145 tested positive for the

integrated CMD - DNA satellites accounting for 41.1%. The episomal DNA satellites for CMD on the other hand were very rare with just a few samples testing positive for the

primers used SAT III F/R.

Table4. Detection of DNA satellites associated with Cassava Mosaic geminiviruses

Province	No. of samples tested	No. Of Positive Satellite samples	No. of Negative samples
Western	89	36	53
Nyanza	90	37	53
Eastern	77	33	44
Coast	94	39	55
Total	**350**	**145(41.4%)**	**205(58.5%)**

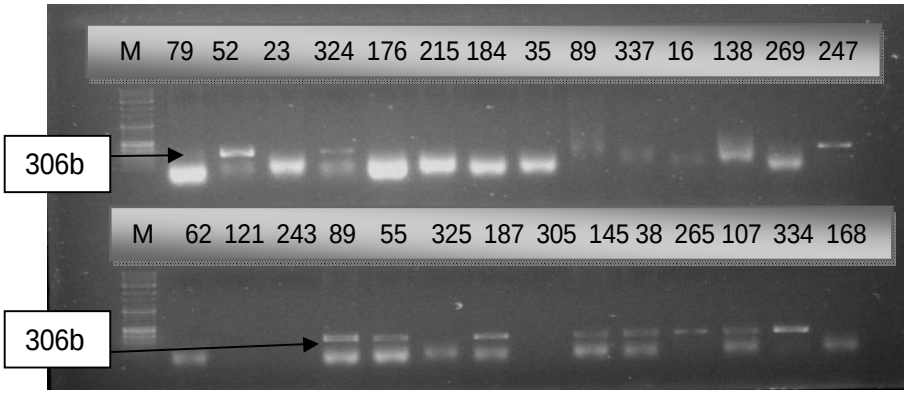

Plate 4:Agarose gel electrophoresis of the integrated DNA satellites specific PCR products of 30bp. The numbers denotes sample numbers from the field.

3.3.3 CMD severity compared with whitefly count and methods of CMD infection

Nyanza region had the highest (3.2) adult whiteflies per plant which was not significantly (P=0.05) higher than the population recorded in Coast region (2.9). The lowest whitely population was recorded in Western province (Table 5). There was no significant difference in whitefly infestation in coast and Nyanza provinces. Likewise, there was no significant difference in cuttings and whitefly method of transmission in eastern and western provinces.

Table 4: CMD severity, whitefly count, cuttings infection and whitefly count per plant in the sampled areas

Province	CMD Severity(1-5)	Whitefly Infection (%)	Cuttings infection (%)	Whitefly counts
Eastern	3.1^{b}	33.3^{c}	66.6^{a}	1.9 ± 0.16^{b}
Nyanza	3.2^{d}	11.1^{b}	88.8^{b}	3.2 ± 0.17^{a}
Western	2.7^{c}	33.3^{c}	66.6^{a}	1.2 ± 0.07^{c}
Coast	3.4^{a}	0^{a}	100^{c}	2.9 ± 0.21^{a}
Mean	**3.1**	**19.6**	**80.5**	**2.3**

The infection due to cuttings is correlated to the high severity symptoms. There is significant difference in white fly infection across the provinces. However, there is no

significant difference in cuttings borne infections in Eastern and western provinces. Cutting-borne infection of CMD accounted for 80.6% compared to the whitefly infection of 19.5%.

3.4 Discussion

This survey of viruses infecting cassava in Kenya was the most comprehensive covering the entire country including Eastern province and the Mt. Kenya region which has not been studied. The plants showing symptoms of cassava mosaic disease were easily identified due to the symptoms they exhibited. Typical symptoms of CMD observed were leaf chlorosis which ranged from pale yellow to white and others were paler that the normal leaf colour. Defined mosaic patterns, leaf malformation and distortion were associated with more severe symptoms of the disease. Symptoms of CMD with the associated DNA satellites showed the same symptoms as described but with more leaf distortion assuming a sickle shape.

Cassava mosaic disease was reported in all the major areas where cassava is grown in Kenya. CMD incidence was observed to be highest in Coast province compared to other provinces. Western and Eastern provinces had the least CMD incidence. On the other hand, Nyanza province had the highest CMD prevalence followed by Coast province with Eastern province registering the lowest disease prevalence. A mean severity of 3.1 countrywide indicates the severity of CMD in the surveyed areas is high. However, Coast province had the highest CMD severity (3.4). Farmers in this province indeed

expressed the fear that the symptoms are nowadays more severe compared to the recent years. Nyanza province recorded the second most CMD severe symptoms of 3.2 with Western province posting the least severity symptoms of 2.7. CMD was very severe in the late 1980's to early 1990's but the disease severity was greatly reduced due to the introduction of resistant and tolerant varieties by KARI and the Ministry of Agriculture (Obiero *et al*., 2007). The same measures were not taken in Coast and Nyanza districts at that time.

Nyanza province had the highest whitefly count in the country followed by Coast province. This was followed by Eastern province with Western province registering the least whitefly counts per plant. It is vividly clear that infection by cuttings is more rampant than that caused by whiteflies. Though whiteflies carry the CMD viruses, the method of transmission through distribution or use of infected cuttings is widespread. This phenomenon has also been observed in Togo (Adjata *et al*., 2012) It is quite contrasting for Coast where the average whitefly count per plant is 2.99 but has 0% infection due to whiteflies. All the plant sampled in coast showed that the method of CMD infection is purely (100%) due use of infected cuttings for planting. The same replicates for Nyanza province where the whitefly infection accounts for 11.1% and through infected cuttings accounting for 88.8%. Eastern province had the highest whitefly method of infection at 33.3% but still infection by cuttings is more prevalent there at 66.6%.

The PCR detection from the samples collected in the nationwide survey showed that EACMV is more widespread than ACMV in the country. EACMV occurs in all the provinces and was distributed across the country. Nearly all the districts under survey showed the presence of EACMV. ACMV was recorded only in Kathiani district in Eastern province signalling the first recorded occurrence of ACMV species in this region. In Coast province, an area presumed to be free of ACMV reported the presence of ACMV also for the first time. ACMV was detected in several farmers' fields in Kilifi, Msambweni and Kwale districts which had been presumably been thought to be free from this species of CMD. Previous studies show indeed that EAMCV is the most common species of CMV in Kenya than ACMV (Were *et al*., 2004). However, in this study, ACMV was only detected in western and Nyanza provinces but none in Eastern and Coast provinces.

The DNA satellites associated with CMGs in this study were common across the country amongst the samples collected during the survey. A total of 145 from the 350 samples collected during the survey tested positive for the integrated Begomoviruses DNA satellites accounting for 41.1%. The episomal DNA satellites for CMD on the other hand were very rare with just a few samples showing positive for the satellites after DNA amplification. The interaction of the DNA satellites with Begomoviruses leads to different symptoms expression of Cassava mosaic Begomoviruses with a likelihood of increasing the disease severity (Nduguru *et al.,* 2007). The leaves

exhibiting these symptoms were definitely also having typical symptoms of the cassava mosaic Begomoviruses.

It is likely that ACMV and EACMV are synergistically interacting leading to severe symptoms as reported by farmers. The study shows that the method of infection is predominantly due to use of infected cuttings with farmers almost not utilizing any management practices (Njenga *et al*., 2005). The same trend was noted with CMD symptom severity where again Coast province recorded the highest symptom severity of 3.36. This observation was amplified by the respondents' interviewed during the survey. The farmers whose fields were sampled expressed that they have known the disease symptoms of the disease and still were able to get some yields. They have also noted that the disease symptoms are now quite severe and that the yields have greatly reduced.

The detection of ACMV in Kathiani district and several districts in Coast province present challenges in the management of CMD in these regions. Dual infections of EACMV and ACMV in these regions point to a possibility of more severe forms of CMD due to synergism and genetic recombination between EACMV and ACMV (Pita *et al.,* 2001).

The interaction of the DNA satellites with Begomoviruses leads to enhanced symptom severity of Cassava mosaic Begomoviruses (Nduguru *et al.,* 2007). In this study, the symptom phenotypes modulation by the DNA satellites on the CMGs symptoms was

quite evident. DNA satellites species are often associated with geminivirus infection (Stenger et al., 1992).These DNA molecules can either enhance symptoms severity or even ameliorate the symptoms in some cases (Ian *et al*., 1997). The leaves of the plants infected assumed a sickle shape thus distinguishing them from other CMGs infected leaves. It was also established that varieties infected with CMGs and DNA satellites exhibited more severe symptoms compared to the same varieties infected only with CMGs. The effect of the DNA satellites on the quality and yield of cassava is not known. Studies in Sri Lanka show that each of the cassava-infecting geminiviruses showed a contrasting and differential interaction with the DNA satellites, not only in the capacity to interact with these molecules but also in the modulation of symptom phenotypes by the satellites (Kumar *et al*., 2006).

CHAPTER FOUR

MOLECULAR CHARACTERIZATION OF KENYAN CASSAVA MOSAIC GEMINIVIRUS ISOLATES

Abstract

Cassava mosaic disease is caused by cassava mosaic geminiviruses which belong to the genus Begomovirus and family Geminiviridae. The two dominant species are East African cassava mosaic virus (EACMV) and African cassava mosaic virus (ACMV) in Kenya. Cassava mosaic begomoviruses caused a major regional pandemic of Cassava mosaic disease (CMD). Kenya was also affected and the pandemic led to massive economic losses. This therefore posed a great threat to food security in Eastern Africa. This study aimed at determining the diversity of the two species of cassava mosaic virus namely EACMV and ACMV. Primers were designed for amplification of the DNA-B and partial coat protein (CP) of EACMV and ACMV, respectively. Resultant PCR products were purified and sequenced at BecA – ILRI using the Sanger sequencing ABI platform. Sequence analysis was done by multiple sequence analysis for sequence identity, pairwise nucleotide sequence identity and phylogenetic analysis of the isolate sequences and those from the NCBI gene bank. Both sequence identity analysis and phylogenetic analysis revealed low variability (92% - 98%) within the coat protein (CP) gene of ACMV. Most of the isolates of ACMV showed high level homology with sequences from the gene bank thus showing they are strains of ACMV species that is dominant in the East African region. However, variability amongst isolates in the species EACMV was more common thus confirming assertions by most studies that

recombination within this species of CMD is more rampant than in the species ACMV. This study reveals the complexity of Cassava mosaic geminiviruses and therefore underscores the need for further study to facilitate comprehensive breeding programmes for developing resistant or tolerant varieties of cassava to CMGs in this region.

4.1 Introduction

Begomoviruses are a major constraint to the successful cultivation of horticultural and agricultural crops throughout the world. The severity and incidences of these viruses in Kenya have increased in recent years, especially in vegetatively propagated crops such as cassava (Gibson and Otim-Nape, 1997; Chellappan *et al.,* 2004). begomoviruses typically have bipartite ssDNA genomes with essential genes encoded on the DNA-A and DNA-B. Many monopartite begomoviruses that have single DNA molecules are also reported from tomato and cotton (Briddon and Markham, 2000) with additional satellite molecules called DNA-β which regulates symptom expression (Briddon *et al.,* 2001).

All Begomoviruses code for coat protein which act as the protective coat of the virus particle and determine vector transmitability of the viruses by the whitefly vector *B. tabacii*. Thus, the CP gene is highly conserved among begomoviruses originating from the same geographical region and adapted to transmission by local vector populations (Maruthi *et al.*, 2002). Smaller fragments comprising the core coat protein gene (core CP), a partial 575-579 base pair (bp) sequence of the coat protein gene (Brown *et al.*,

2001), or the complete CP sequence have also been used to establish provisional species identification owing to the highly conserved nature of the viral CP sequence. The core CP primers have been illustrated to amplify a fragment for most, if not all, begomoviruses irrespective of old or new world origin, making possible the rapid detection followed by prediction of provisional species affiliation by comparison with reference to begomovirus core CP sequences (Wyatt and Brown, 1996; Brown *et al.*, 2001; Harrison *et al.*, 2002).

In Kenya, cassava mosaic disease (CMD) is caused by begomoviruses in the family Geminiviridae. These include African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV) and Uganda variant (EACMV-UG) of the genus begomovirus. EACMV and the recombinant strain EACMV-UG have been identified in the east and west of the country, respectively (Bull *et al.*, 2003; Were *et al.*, 2004a, b). The viruses are spread by the whitefly (*Bemisia tabaci* Gennadius) and by planting infected cuttings. Affected plants are stunted and have greatly diminished tuberous root yield. The occurrence of CMD epidemics, the most recent of which initiated in or around northern Uganda during the 1990s, swept through Uganda and surrounding countries including Kenya and continues to affect cassava throughout central Africa (Legg and Fauquet, 2004). The severe CMD phenotype that is associated with this epidemic is caused by the synergistic interaction between ACMV and a distinct recombinant strain of EACMV (EACMV-UG), commonly known as the 'Uganda variant' (Zhou *et al.*, 1997; Pita *et al.*, 2001). This study further characterized the CMGs

to ascertain the strains or any novel species since most studies suggest a high level of recombination in the genus begomovirus.

4.2 Materials and Methods

4.2.1 DNA extraction

Total nucleic acid (TNA) was extracted from the dry leaf samples using the CTAB based method (Lodhi *et al.*,1994). The DNA pellets obtained were washed in 70% ethanol and suspended in sterilized water and stored at -20^{o}C for subsequent PCR procedures for viral detection.

4.2.2 Polymerase chain reaction (PCR) and gel eelectrophoresis

The PCR mix reaction consisted of GoTaq green (Promega), forward primer EAB555F and reverse primers EAB555R and the template DNA for the detection of EACMV. This primer set was designed to amplify a 556 bp fragment of DNA B component (Ndunguru *et al.*, 2005). Universal primers (JSP001/JSP002) were used to detect African Cassava Mosaic Virus (ACMV) with an expected amplicon of 774bp (Ndunguru *et al.*, 2005). The PCR cycling regimes were the same for both ACMV and EACMV detection with annealing temperature of 48°C. The PCR products were subjected to 1.5% agarose gel electrophoresis and pictures of the gel taken after 45 minutes of electrophoresis. The gels were illuminated with ultraviolet light and photographed with a digital Canon camera model EOS Rebel T4i.

4.2.3 Purification of PCR products for sequencing

PCR was done on all DNA samples of ACMV and EACMV which had been selected for sequencing. The appropriate primer sets were used for detection of ACMV and EACMV as was done in the initial detection work for the two CMV species. The samples were selected on the basis of location (Table 6) to represent all the areas under the study. The amplified DNA fragments were excised from the gels and purified using the QIA quick PCR products purification kit from Qiagen. The purified PCR products (DNA fragments) were then sent to Segoli, BecA – ILRI for sequencing. Sequencing was done using ABI Sanger sequencing platform. The sequences received from BecA were converted into a FASTA files' format for the sequence alignment procedures**.**

Table 5: Isolates of ACMV and EACMV selected for molecular characterization

Isolate	Sample no.	Seq. length (bps)	Expected seq length (bp)	Location collected	Symptoms severity scale 1-5	Varieties
EACMV	307	522F 521R	556bp	Maara	2	Mianga Mikwazi
	14	515F 517R	"	Coast	3	Kibanda meno, Karembo
	294	517F 519R	"	Teso south	3	Matiuja Serere Uganda
	324	520F 517R	"	Teso south	4	Magana, Matuja, Serere
	36	283F 259R	"	Homa bay	3	Mariwa
	314	427F 506R	"	Malindi	3	Kibandameno
	101	491F 512R	"	Mumias	3	Serere, Nigeria
ACMV	157	763F 762R	774bp	kilifi	3	Kibandameno karembo
	214	764R 762F	"	Kitui	2	Musherisheri Kibandameno
	129	766F 766R	"	Maara	3	Mianga, Mikwazi
	300	774F 762R	"	Teso north	4	Matuja, Serere, Uganda
	172	761F	"	Tharaka south	2	Mikwazi
	5	550F 644R	"	Coast	3	Kibandameno
	337	395F	"	Butere	3	Nigeria, Serere Nigeria

EACMV- East African Cassava Mosaic Virus. **ACMV** – African Cassava Mosaic Virus

4.2.4 Sequence analysis

Sequences were manually edited for quality by the Chromas-lite 2.33 software programme (www.softpedia.com/get/Science-CAD/**Chromas-Lite**.shtml). The sequences were sorted into separate files for ACMV and EACMV, respectively. The Basic Local Alignment Search Tool (BLAST) was performed against the NCBI database. The begomovirus sequences from the database that had the highest similarity to each BLAST query sequence were selected for later sequence similarity and phylogenetic analysis. Multiple alignments of the generated sequences and the begomovirus sequences (selected by the similarity values) were performed using DNAMAN software.

4.2.5 Phylogenetic analysis and genetic distance evaluation

Phylogenetic analysis was carried out using DNAMAN software. The sequences of the isolates which were found to align well and with similar or close sequence size in terms of base pairs selected previously and saved in a FASTA files' format were well arranged to ensure that the process is continuous as per the entries of the sequences. Some sequences from genebank were used for comparison with the isolate sequences generated in the study (Tables 7 and 8). A pairwise nucleotide sequence analysis was done under this program to determine the percentage nucleotide sequence identity and the evolutionary distance of all the selected isolates from gene bank and those in the study.

The DNAMAN program was used to construct the phylogenetic and homology distance trees for the aligned sequences.

Table 6: Sequences of ACMV used for phylogenetic analysis derived from gene bank

Isolate	Country	Accession No.	Reference
ACMV-Nam	Namibia	AF423177.1	Maruthi *et al.*, 2001
ACMV- Clv W.Keny844	Kenya	JO2057	Robinson,D. 1996
ACMV-Uganda Mild1	Kenya	AY562429.1	Sseruwagi *et al.*, 2001
ACMV-Ybi Cam	Cameroon	FN436276.1	Winter *et al.*, 2007
ACMV-Uganda	Uganda	Z83252.1	Robinson,D. 1996
ACMV-Uganda Mild	Uganda	AF126800.1	Pita *et al.*, 2001
ACMV-Uganda severe	Uganda	AF126802.1	Pita *et al.*, 2001
ACMV-KE1	Kenya	X68318.1	Briddon *et al.*, 1993
ACMV-Nigeria-Ogo	Nigeria	AJ427910.1	Briddon 2003
ACMV-Cameroon	Cameroon	AF112962.1	Maruthi *et al.*, 2001
ACMV-Burkina Faso	Burkina Faso	FM877473.1	Tiendrebeogo, F. 2009

Table 7: Sequences of EACMV used for phylogenetic analysis derived from gene bank

Isolate	Country	Accession No.	Reference
EACMV-TZT	Tanzania	AY800262.1	Ndunguru *et al*., 2005
EACMV- KE2[K197]	Kenya	AJ704973.1	Bull *et al.*, 2006
EACMV- UG[K66]	Kenya/Uganda	AJ704954.1	Ndunguru *et al*., 2005
EACMV-UG[K72]	Uganda	AJ704974.1	Ndunguru *et al*., 2005
EACMV-KE2[K25]	Kenya	AJ704950.1	Ndunguru *et al*., 2005
EACMV-TZ2	Tanzania	AY800253.1	Ndunguru *et al*., 2005
EACMKV[K238]	Kenya	AJ704971.1	Bull *et al.*, 2006
EACMV-Cam(Ivory cst)	Ivory Coast	FJ826890.1	Fondong 2009
EACMV/Ug3/Svr	Uganda	AF126807.1	Pita *et al.*, 1999
EACMV UG K81	Kenya	AJ170959.1	Bull *et al.*, 2006
EACMV/Ug3/Mld	Uganda	AF126805.1	Pita *et al.*, 1999
EACMV-TZ6	Tanzania	AY800257.1	Ndunguru *et al*., 2005
EACMV-UG(Cam055)	Cameroon	FN 668380.1	Winter *et al.*, 2010
EACMV-Ug1	Uganda	AF 230375.1	Pita *et al.*, 2001

4.3 Results

4.3.1 Sequence comparisons and phylogenetic analysis of ACMV

The isolates of ACMV showed a high level of similarity within themselves as per the sequence alignment (BLAST) results, pairwise nucleotide identities and through phylogenetic analysis. These isolate sequences showed identities of 92% to 98%. Upon comparison with the sequences in the NCBI genebank by multiple alignment of the coat protein gene, the Kenyan isolates were closely related to Ugandan and Namibian ACMV isolates with sequence identities of 98% and 97%, respectively. Similarly, the Kenyan isolates showed substantial homology with sequences of ACMV Cameroon (FN436276.1), ACMV-Nigeria (AJ427910.1) and ACMV-Namibia (AF423177.1) with sequence identities of 96%, 97% and 98% respectively inspite of the geographical distances between the isolates through homology sequence analysis. However, there was some reduced identity to the isolates of ACMV-Ivory coast at 94% and ACMV-Burkina Faso at 92% sequence identity. The same is also depicted in the phylogenetic tree of ACMV with the Kenyan isolates in the same branch and the others in the other branch on the phylogenetic tree generated (Fig 3).

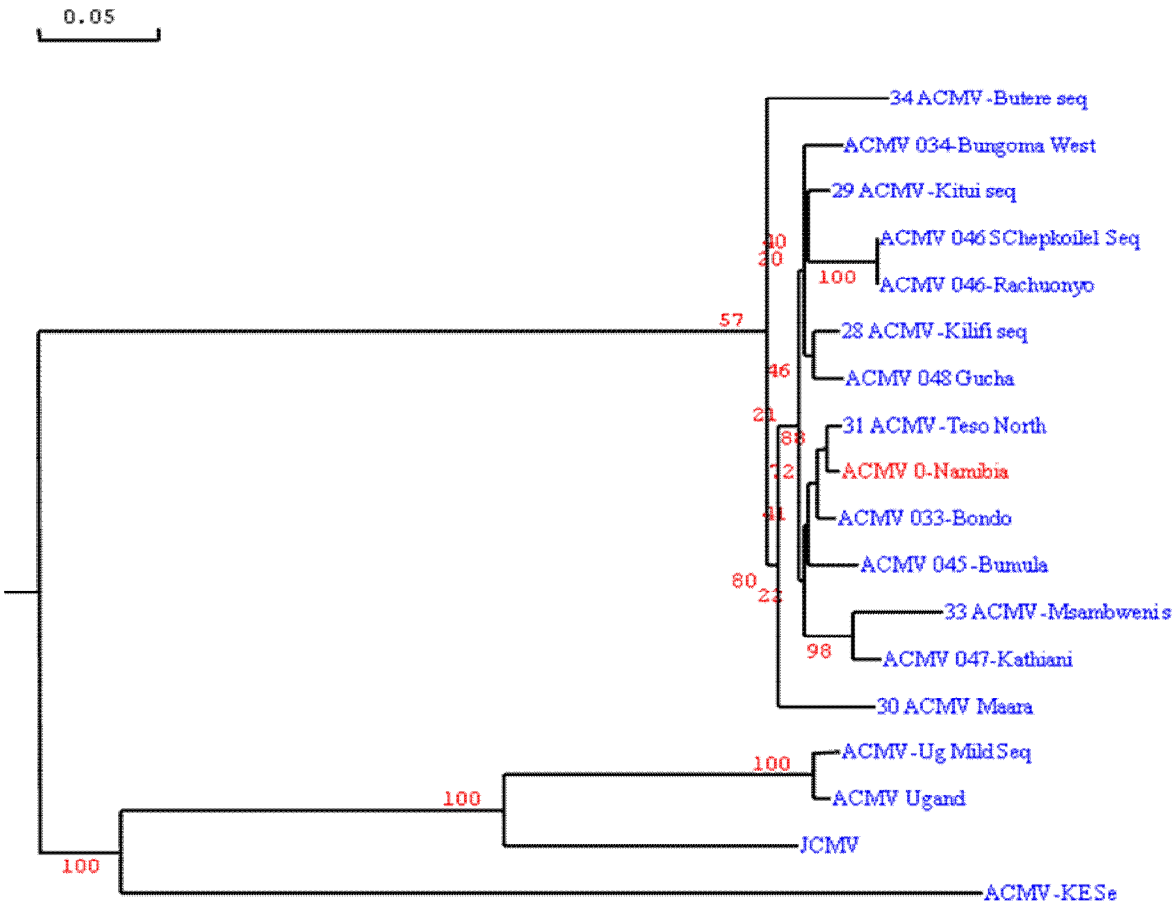

Figure 3: Phylogenetic tree of Kenyan ACMV isolates based on the coat protein gene

ACMV isolates from Kenya also showed less variability between them through the genetic distance evaluation. This is evaluated on the basis of the nucleotide substitution between the nucleotide sequences under comparison. It is evident that the sequences from genebank also showed close relationship between them Fig.5.

4.3.2 Sequence comparisons and phylogenetic analysis of EACMV DNA-B gene.

The sequences of the Kenyan isolates of EACMV subjected to various tools of analysis amongst themselves and those sequences in the gene bank gave contrasting results to ACMV. Multiple sequence alignment and pairwise nucleotide sequence identity showed that the Kenyan isolates have greater variability within the EACMV species (Fig. 5). However, the Kenyan isolates showed similarity to the Ugandan isolates (EACMV-Ug), Tanzania (EACMV-TZT) and Zanzibar (EACMZV) with sequence identities of 92%, 87% and 86% respectively (Table 10). The Malindi and Rachuonyo isolates surprisingly showed high sequence identity (97%) though geographically far apart. Isolates from Maara, Matuga and Teso south also showed similarity of 93%-95%. These two groups however differ from each other considerably (Fig. 4 and Table 9).

Table 8: Pairwise DNA-B nucleotide sequence homology matrix of 18 isolates sequences using the DNAMAN software

	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	20_EACMV Maara	100																	
2	003_EACMVMalindi	85.8	100																
3	004_EACMV-Rachu	87.3	97.2	100															
4	EACMV-KE2[K197]	26.5	25.0	25.6	100														
5	21_EACMV-Matuga	94.6	87.5	89.4	25.8	100													
6	22 EACMV-Teso S.	92.4	84.2	85.9	26.5	93.6	100												
7	ACMV-Nigeria_Ogo	35.0	31.1	30.8	33.4	33.9	34.8	100											
8	EACMV-[K27]_seq	26.1	24.8	25.4	97.0	25.8	26.1	33.1	100										
9	EACMV-Ug_seq	35.3	31.3	31.5	32.1	34.2	34.7	97.2	31.8	100									
10	EACMVKV-[K298]	27.1	26.0	26.6	86.3	26.2	26.9	34.7	86.5	33.0	100								
11	EACMV-TZ2_seq	0.1	0.1	0.1	72.1	0.1	0.1	32.3	71.4	0.1	64.6	100							
12	EACMV-TZ6_seq	0.1	0.1	0.1	82.2	0.1	0.1	32.2	81.3	0.1	94.1	65.9	100						
13	EACMV-Ug3-Mld	26.3	25.4	25.8	86.7	25.4	26.1	34.5	86.4	33.5	96.2	66.4	97.5	100					
14	EACMV-Ug3-Svr	27.1	26.0	26.4	86.4	26.2	26.9	34.9	86.0	33.8	96.1	65.4	97.5	98.3	100				
15	ICMV	30.7	29.1	28.6	30.0	29.8	30.2	70.9	30.3	75.2	30.3	29.9	29.2	30.9	31.2	100			
16	EACMV-UG[K66]	26.3	25.0	25.6	97.1	25.6	26.3	33.2	98.3	32,0	86.6	71.8	82.0	86.5	86.2	30.0	100		
17	EACMV-UG[K72]	26.3	25.0	25.6	96.9	25.6	26.3	33.1	98.2	32.0	86.5	71.9	81.8	86.4	86.2	30.1	99.8	100	
18	EACMZV-[K270]	27.5	26.4	26.8	90.9	26.8	27.3	33.8	91.9	32.3	85.6	71.0	81.8	85.0	85.0	30.9	91.3	91.2	100

The isolates were distantly related to the EACMV – Cam (Ivory coast) (FJ 826890.1) isolates from Ivory Coast and EACMV-UG (Cam 055) (FN 668380.1 from Cameroon with sequence identities of 85%.

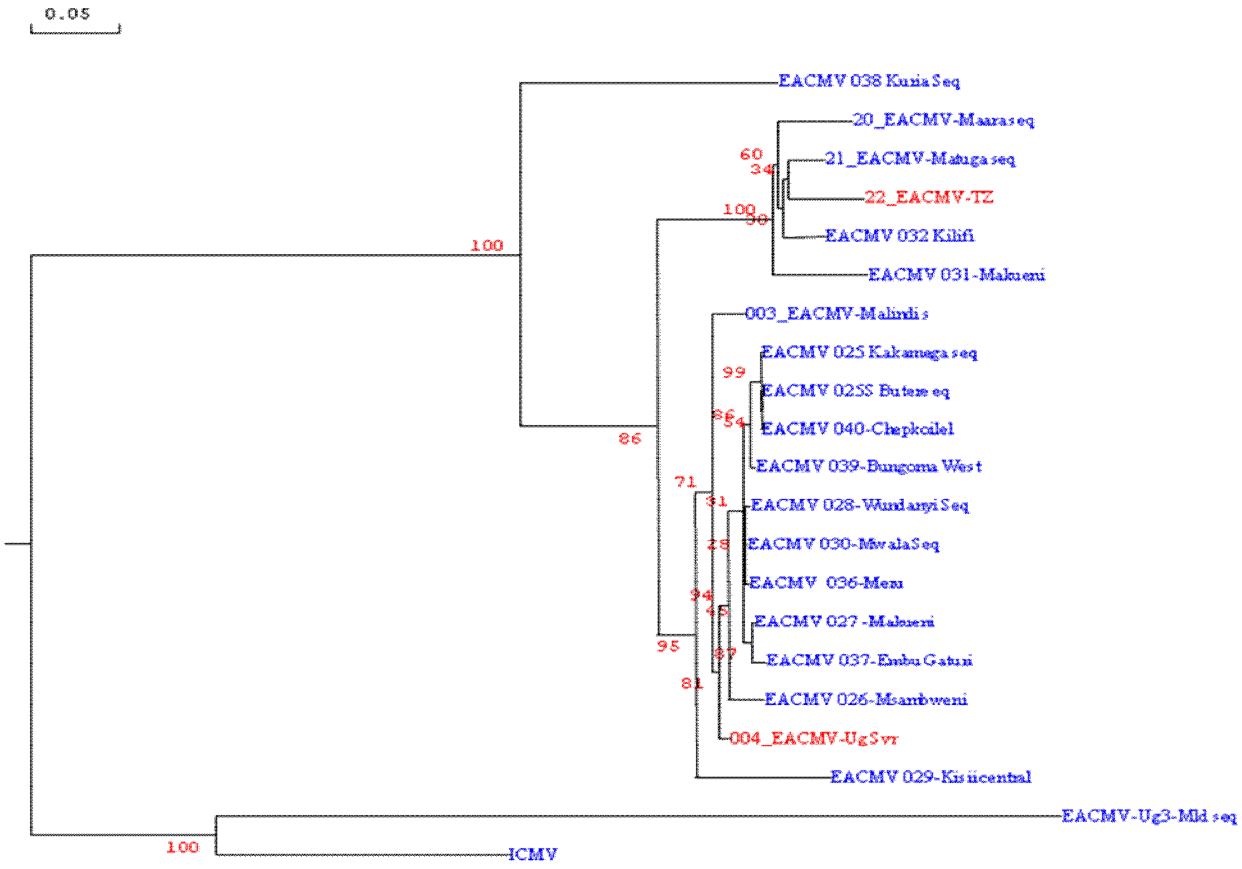

Figure 4: Phylogenetic tree for Kenyan and NCBI genebank EACMV isolates

The Kenyan isolates of EACMV from Maara and Teso districts according to the phylogenetic analysis showed that they are closely related to EACMV-Ug/Mld but those from Nyanza province are closely related to the virulent Kenyan EACMV (EACMV-UGK66) and the severe Ugandan EACMV strain (EACMV-Ug/Svr) (Fig.4).

Genetic distances of the EACMV isolates from Kenya also confirmed the close similarity between them but not as closely related as was the case of ACMV isolates (Fig. 5). The gene bank sequences also showed divergent relationship between the EACMV isolates.

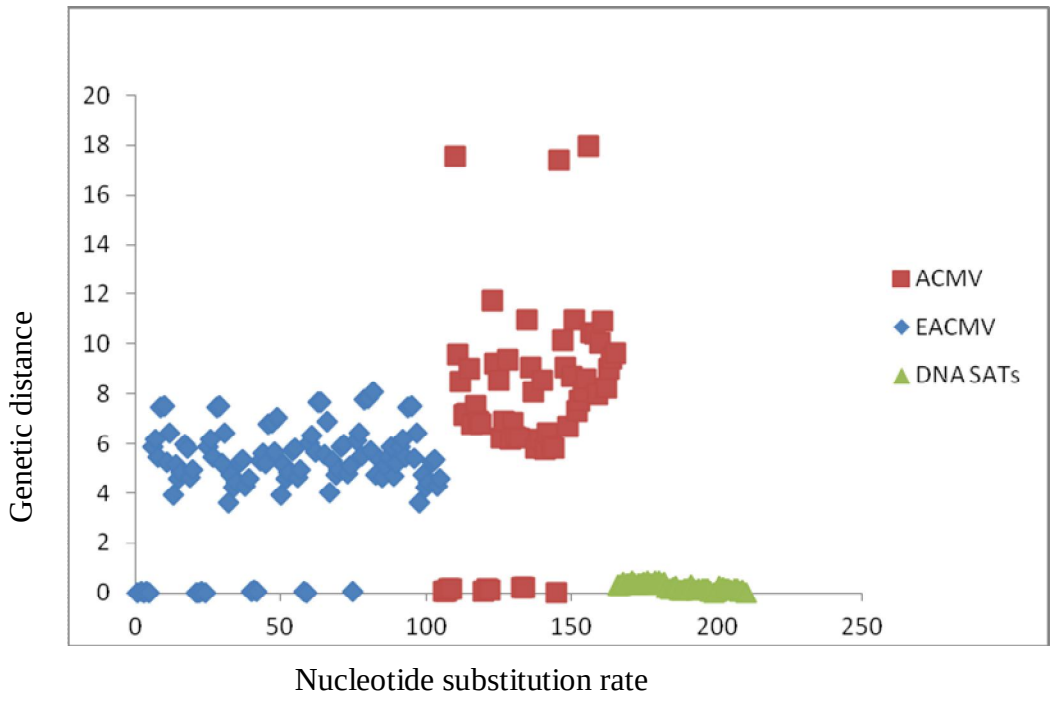

Figure 5 : Genetic distance within the CMG species under study and their associated DNA satellites

There is higher variability amongst isolates of EACMV as depicted in Fig.5. The genetic distances in the five isolate sequences had up to 75 nucleotides substitution. This is also evident with the isolates from the gene bank with distances spreading to 100 nucleotides. Genetic variability within ACMV species is of low genetic distance of 40 for the most distant isolates. There is more variability for the DNA satellite associated with these begomoviruses where the genetic variability is about 65 nucleotides for isolates under study but higher divergence with those in the genebank.

This scenario is also witnessed in the phylogenetic analysis results of ACMV and EACMV isolates.

4.4 Discussion

This study determined the molecular diversity of the Kenyan ACMV and EACMV species. The Kenyan ACMV isolates were closely related to ACMV isolates from Uganda and Namibia with sequence identities of 97%-98%. The Kenyan isolates also showed substantial similarity with sequences of ACMV from Cameroon (FN436276.1), Nigeria (AJ427910.1) and Namibia (AF423177.1) with sequence identities of 96%, 97% and 98%, respectively. According to the International Committee of Taxonomy of Viruses, geminiviruses of 90% to 99% are strains of the same virus. This indicates that the isolates under study are strains of ACMV since all gave sequence identities of greater than 90%. There was some low similarity to some isolates of ACMV-IC at 94% and ACMV-Burkina Faso at 92% but still indicating that they are strains of ACMV.

This is in line with most literature which ascertains that there is less genetic variability amongst viruses of this species (Were *et al*., 2004; Bull *et al.*, 2006).

Genetic distance evaluation of the Kenyan isolates and those from the gene bank also gave the same trend with sequence samples showing less variability amongst the isolates. The same trend was also shown in the phylogenetic tree constructed from these sequences where the Kenyan ACMV isolates were in the same branch while the others in the different branch on the rooted tree generated. This genetic distance evaluation as stated before is on the basis of the nucleotide substitution between the nucleotide sequences under comparison. The high sequence identity of 96% to the ACMV – Uganda severe isolate points at the possibility of these Kenyan isolates to cause a severe form of the disease as witnessed in the field during the survey. Disease severity scores of 4 were recorded in many areas in Coast and Nyanza provinces. The fact that ACMV has now been officially detected in the coast region, more studies need to be undertaken to determine the etiology and epidemiology of these begomoviruses.

There was however a marked contrast in the diversity of the Kenyan EACMV isolates. The isolates exhibited similarity to the isolates of Uganda (EACMV-Ug), Tanzania (EACMV-TZT) and Zanzibar (EACMZV) with sequence identities of 92%, 87% and 86%, respectively. This supports previous reports of reduced sequence homology

between isolates in Eastern Africa (Nduguru *et. al*., 2005). South African Cassava Mosaic Virus (SACMV, AF 155807.2) had a sequence identity of 88% with the EACMV isolates in this study. The isolates were distantly related to the EACMV – Cam (Ivory Coast) (FJ 826890.1) isolates from Ivory Coast and EACMV-UG (Cam 055) (FN 668380.1 from Cameroon with sequence similarity ranging from 65% to 98%. However in the phylogenetic analysis, the Kenyan isolates were closely related to the EACMV-Ug isolates.

Previous studies (Njenga, 2005) showed the presence of the EACMV-Ug isolates in coast province. This was in contrast to earlier reports that the EACMV-UG isolates are only common in Western and Nyanza provinces which are close to Uganda, the origin of this virulent form of EACMV-Ug (Deng *et al.*, 1997). The phylogenetic tree for EACMV indeed showed that the Kenyan EACMV isolates are closely related to the Ugandan isolates sandwiched between various strains of EACMV –Ug. This may partly explain the high severity score of the disease in coast, Nyanza and western provinces. EACMV isolates still exhibited low similarity to the Cameroon, Ivory Coast and Tanzanian isolates. This study therefore shows that the CMGs infecting cassava in Kenya are the two species, ACMV and EACMV of begomoviruses. The discovery of ACMV in eastern and coast region in this study showed a high possibility of interspecies recombination with EACMV, EACMV Ug and ACMV which may serve as an explanation for the severe symptoms of the disease recorded in these regions. Dual

infection of these two species may present cases of synergy between them with distinct viral epidemiology thus need to further study this phenomenon.

CHAPTER FIVE

MOLECULAR CHARACTERIZATION OF DNA SATELLITES ASSOCIATED WITH CASSAVA MOSAIC GEMINIVIRUSES

5.0 Abstract

DNA satellites are subviral agents that require proteins encoded by the helper virus for their replication, movement and encapsulation. The interaction of the DNA satellites with begomoviruses leads to different symptom expression of CMD with a likelihood of increasing disease severity. The symptoms manifest themselves with leaves infected by begomoviruses assuming a sickle shape. This study aimed at characterizing the DNA satellites associated with the CMGs in Kenya and further describe symptom modulation of the disease. Plants with severe symptoms associated with begomoviruses were sampled during the survey in 34 districts in Eastern, Western, Nyanza and Coast provinces. The samples were then tested by PCR using Sat III F/R primers in the laboratory. Selected positive samples were sequenced and characterized by sequence identity analysis, phylogenetic analysis and genetic distance evaluation. The integrated satellites in this study were distantly related to begomoviruses associated DNA III satellites (AY 836367.1) found in other parts of Eastern Africa with a sequence similarity of 30%. They were also distantly related to the EACMV- Di (defective interference) DNA molecules (AY 676464) and the Tobacco leaf curl virus defective DNA satellite (AF 368275) from Zimbambwe at sequence similarity of 35%. The sequences of the satellites from the study exhibited high levels of variability ranging

from 40% to 95% with some showing low similarity (29%) with genebank sequences of the Mentha leaf DNA II satellites from India. Evolutionary distance analysis showed high variability with over 60 nucleotide substitution. Cassava varieties co-infected with CMGs and the associated DNA satellites were characterized by enhanced symptom severity compared to the same varieties infected with CMGs only. More studies in the CMD symptom modulation in the presence of DNA satellites and the economic importance on the crop need to be initiated.

5.1 Introduction

Geminiviruses are often associated with subviral agents called DNA satellites that require proteins encoded by the helper virus for their replication, movement and encapsulation. Most of the single-stranded DNA satellites reported to be associated with members of the family Geminiviridae have been associated with monopartite begomoviruses (Briddon *et al.*, 2008). Cassava mosaic disease is known to be caused by viruses belonging to nine different begomovirus species in the African continent and the Indian subcontinent (Kumar *et al.,* 2006). In addition to these species, several strains have been recognized that exhibit contrasting phenotypes and infection dynamics. It is established that Sri Lankan cassava mosaic virus can trans-replicate beta satellites and can cross host barriers (Kumar *et al*., 2006).

Cassava mosaic virus DNA interacts with some DNA molecules either in the cytoplasm or in the nucleus. These DNA satellites give the disease some unique symptoms in that

leaves infected by CMD assume a sickle shape curling inwards (Nduguru *et al.,* 2007). The integrated satellites have the defective sequence of the DNA satellites integrated in the viral genome in the nucleus. The episomal DNA satellites on the other hand interact with the viral genome during the replication process in the cytoplasm conferring some unique expression of the viral genes for the disease aetiology. The aim of this experiment was to determine the type of the CMD associated DNA satellites in Kenya and their genetic diversity.

5.2 Materials and methods

5.2.1 DNA extraction, PCR procedure and gel electrophoresis

The leaf samples with DNA satellite symptoms were sampled form the fields visited during the survey. The leaf samples were preserved in vials with silica gel to desiccate them.Total nucleic acids (TNA) were extracted from the dry leaf samples collected from the fields during the survey using the CTAB based method (Lodhi *et al,*. 1994). The primer set SAT III F/R was used with an expected PCR amplicon of 306 bp. The primers amplify DNA B of the CMGs where the integrated nucleotides of the DNA molecules are embedded in the viral genome. PCR cycling conditions were done as explained by Ndunguru *et al*., (2004) for amplification of the integrated DNA satellites. The resultant PCR reaction mixtures were subjected to 1.5% gel electrophoresis to determine the positive samples in comparison with a molecular marker of 1kb. The positive samples of the DNA III integrated satellites were selected from various parts of the country for diversity analysis as shown in Table 10.

5.2.2 Purification and sequencing PCR products

The PCR products were purified using the Qiagen purification kit according to the manufacturer's instructions before taking them for sequencing at BecA, ILRI. Sequencing was done under the ABI Sanger sequencing platform.

5.2.3 Sequence analysis, Phylogenetic analysis and genetic distance evaluation

The DNA satellites sequences obtained from ILRI were edited in the same way as for the ACMV and EACMV sequences. Multiple sequence alignment was done with the sequences of the DNA satellites from the study compared with those from the gene bank. DNA satellite sequences from the gene bank that showed similarities to the DNA satellite sequences in the study were the begomovirus associated DNAIII satellite (AY 836367.1) and the Mentha leaf deformity associated Satellite DNA II (EU 862815.1) for diversity analysis. Phylogenetic analysis and pairwise nucleotide distance matrix comparison was done using the DNAMAN software as earlier described in chapter four.

5.3 Results

The symptom severity of the CMGs alone was demonstrated to be less severe than the symptom severity for the same varieties infected by CMGs and the associated DNA satellites (Table 10). This observation was found to occur in all the areas surveyed across the country.

The DNA satellite sequences from this study showed a high variability within themselves with sequence homology ranging from as low as 22% to 95%. Kitui and Makueni DNA satellites sequences showed 95% similarity. These two sequences showed a sequence similarity of 92% to the Homa Bay and Malindi sequences.

Table 9: Effect DNA satellites on CMD symptomatology o various cassava cultivars

Where collected	CMGs symptoms only	Symptom severity(CMGs alone)	Symptoms observed with DNA satellites	Symptom severity with DNA satellites	Variety
Busia	Interveinal leaf chlorosis	2.0	Leaf chlorosis and curly leaves	3.2	Nigeria
Mumias	Interveinal leaf chlorosis	2.3	Leaf chlorosis and curly leaves	2.8	Serere
Bondo	Interveinal leaf chlorosis	3.0	Leaf chlorosis and curly leaves	3.2	Nyakanegi , Muogo
Rachuonyo	Interveinal leaf chlorosis	2.5	Leaf chlorosis and curly leaves	2.8	Mariwa
Homa Bay	Interveinal leaf chlorosis	2.0	Leaf chlorosis and curly leaves	3.5	Mariwa
Kuria	Interveinal leaf chlorosis	2.5	Leaf chlorosis and curly leaves	3.5	Embetoto
Gucha	Interveinal leaf chlorosis	3.0	Leaf chlorosis and curly leaves	2.6	Muogo
Malindi	Interveinal leaf chlorosis	2.4	Leaf chlorosis and curly leaves	4.0	Kibandameno
Kitui	Interveinal leaf	2.8	Leaf chlorosis and curly	3.1	Musherisheri,

	chlorosis		leaves		Kibandameno
Makueni	Interveinal leaf chlorosis	2.5	Leaf chlorosis and curly leaves	2.9	Manga

However, there was less homology between Mumias, Busia sequences and Bondo, Rachuonyo sequences at 30% sequence similarity (Table 11).

The integrated satellites associated with begomoviruses in this study were distantly related to the ones found in other parts of Eastern Africa, Sothern Africa and India. They showed a sequence similarity of 30% to the begomoviruses associated DNA III satellites (AY 836367.1) (Ndunguru *et al*., 2004). The DNA III satellites associated with begomoviruses from this study also exhibited high variability with genebank sequences of the Mentha leaf DNA II satellites (EU 862815.1) (Kumar *et al*., 2006) from India with the same sequence identity of 30%. The DNA satellite sequences in this study also showed low similarity to the EACMV associated defective DNA molecules and the Tobacco leaf curl virus at 35% sequence similarity. These two begomoviruses associated DNA satellites are found in the Southern Africa region.

Evolutionary distance analysis revealed a great variability amongst the sample DNA sequences and those from the genebank with nucleotide substitution of about 60 (Fig.5). Phylogenetic analysis gave rather comprehensive results showing the Rachuonyo

satellites being closely related to the Malindi satellites inspite of the geographical satellites distance between the two locations as illustrated in Fig. 6.

Table 10: Pairwise percent DNA-A nucleotide sequence similarity among 14 begomovirus associated DNA satellites

	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	TLCV - DI-Zimb	100													
2	Begomovirus assc	35.2	100												
3	Bondo	38,5	30.4	100											
4	Busia	40.0	30.9	67.8	100										
5	EACMV -DI seg	60.8	29.9	31.6	30.0	100									
6	Gucha	36.6	24.7	77.0	71.0	29.3	100								
7	Homa Bay	38.2	31.4	84.4	74.1	31.2	86.7	100							
8	Kitui	37.4	29.4	86.0	72.7	30.9	88.4	95.1	100						
9	Kuria	40.2	30.9	77.7	75.1	33.2	81.7	85.8	86.2	100					
10	Makueni	37.4	28.8	84.2	73.1	29.4	90.1	93.3	95.4	85.8	100				
11	Malindi	39.5	27.6	84.2	73.0	29.8	88.7	91.5	94.0	84.2	94.0	100			
12	Mentha l DNA Sat	28.6	38.9	24.3	27.8	23.7	24.5	23.8	22.0	25.5	22.6	23.3	100		
13	Mumias	39.9	32/1	69.3	80.5	30.4	72.2	76.6	74.2	75.0	75.3	74.1	28.1	100	
14	Rachuonyo	38.6	25.9	76.6	67.2	30.1	79.0	81.5	82.7	74.9	82.7	86.1	20.2	67.9	100

The Bondo satellite sequence was distantly related to all the other sequences across the country with sequence similarity ranging from 67% to 86% as illustrated in Table 11 and in Fig. 6. The Southern Africa DNA satellites were on the same branch hence showing some similarity between them.

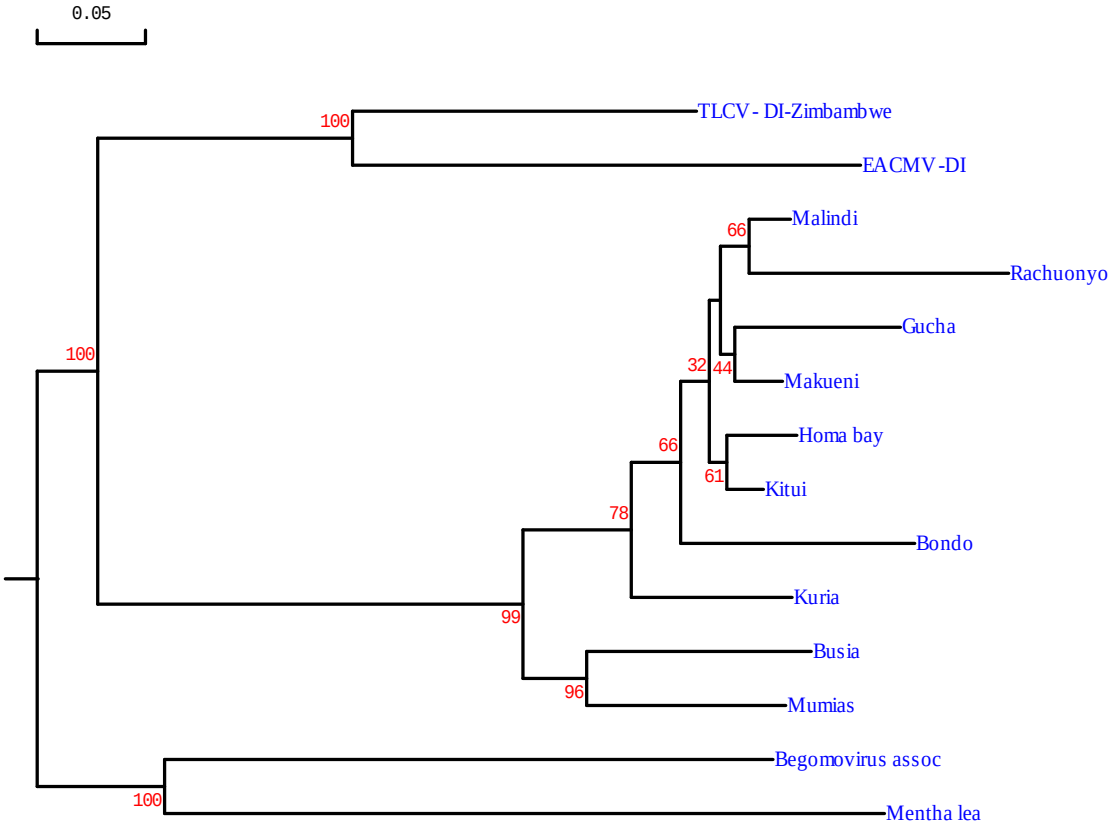

Figure 6: Phylogenetic tree for CMGs associated DNA satellites

5.4 Discussion

Molecular characterization of DNA satellites associated with begomoviruses that cause Cassava Mosaic Disease (CMD) is described in this study. Indeed, the occurrence of these DNA satellite molecules in nearly all the cassava growing areas is an indication of how this occurrence is widespread. The satellites exhibit contrasting phenotypes and infection dynamics which in this case are characterized by sickle shaped leaf shapes.

The DNA satellite sequences determined in this study had a low similarity to the NCBI genebank sequences of begomoviruses associated DNA III satellites (AY836367.1) from Tanzania and the Mentha leaf satellite DNAII from India at only 30% sequence similarity for both. They were also distantly related to the EACMV- DI DNA molecules (AY 676464) and the Tobacco leaf curl virus defective DNA satellite (AF 368275) from Zimbambwe at sequence similarity of 35%.There is increased variability amongst DNA satellites from East and South African region (Marie *et al*., 2012). Both evolutionary distance evaluation and the phylogenetic analysis show a large variability amongst the DNA satellites under this study. The evolutionary distances indicates nucleotide substitution rates of about 60 nucleotides hence high variability amongst the sample sequences (Fig.5). Most of the satellite sequences in this study had a sequence similarity of 85% and below. This concurs with the observation that the DNA satellites associated with begomoviruses are not closely related though they occur in the East African region with similar begomovirus symptom modulation (Nduguru *et al.,* 2009). This study has therefore clearly demonstrated that there are four distinct groups of begomovirus associated DNA satellites with two groups being predominant in Kenya, one in Eastern Africa and the other one in Southern Africa.

The DNA satellite sequences from Busia, Mumias, Rachuonyo, Bondo, and Kuria suggests a new group of novel DNA III satellites associated with CMGs in Kenya as is the case of another group of isolates from Kitui, Makueni and Malindi. This phenomenon needs to be investigated further especially on the mode of interaction with

the identified CMGs common in those areas. It was also clearly demonstrated that the symptom severity is enhanced on varieties infected with both DNA satellites and the CMGs as compared to varieties infected only with CMGs. This view is supported by some studies which have demonstrated that defective DNA molecules have been shown to ameliorate, delay and attenuate symptoms in plants infected with the helper virus (Stanely *et al*., 2004). Symptom severity did not appear to depend entirely on continuously decreasing pattern in levels of DNA-A or DNA-B over time in *N. benthamiana* and cassava (Marie *et al*., 2012). The effect of the DNA satellites on the quality and yield of cassava is not known. Studies in Sri Lanka showed that each of the cassava-infecting geminiviruses a contrasting and differential interaction with the DNA satellites, not only in the capacity to interact with these molecules but also in the modulation of symptom phenotypes by the satellites (Kumar *et al*., 2006).

CHAPTER SIX

6.0 GENERAL DISCUSSION

Cassava is an important food crop in Kenya and in sub-Saharan Africa due to its richness in dietary carbohydrates and tolerance to drought. It can also thrive relatively well in marginal soils. However, the crop is heavily attacked by various diseases of which viral diseases are more common reducing yields to as much as 50% - 80% (Thresh *et al.*, 1977). The study identified and characterized the Cassava mosaic geminiviruses and the DNA satellites associated with these geminiviruses. The incidence, prevalence and severity of these viruses were also determined.

The survey and virus detection work using molecular based detection methods gave findings of the occurrence of Cassava mosaic geminiviruses in all areas where cassava is grown in Kenya. The sampling was based on the plants which exhibited the typical symptoms of CMD and the associated DNA satellite. Defined mosaic patterns, leaf malformation and distortion were associated with more severe symptoms of the disease. Symptoms of CMD with the associated DNA satellites showed the same symptoms as described but with more leaf distortion assuming a sickle shape.

CMD incidence was observed to be highest in Coast province (3.4) compared to other provinces. The farmers in Coast province indeed expressed concern that the disease is more severe compared to the previous years (Njenga *et al*., 2005). Western and Eastern provinces had the least CMD incidence. On the other hand, Nyanza province had the

highest CMD prevalence followed by Coast province with Eastern province registering the lowest disease prevalence. A mean severity of 3.1 countrywide indicates the severity of CMD in the surveyed areas is high. However, Coast province had the highest CMD severity (3.4). Farmers in this province indeed expressed the fear that the symptoms are nowadays more severe compared to the recent years. Nyanza province recorded the second most CMD severe symptoms of 3.2 with Western province posting the least severity symptoms of CMD.

Nearly all the districts under survey showed the presence of EACMV and were distributed across the country. However, ACMV was not widely distributed in the country as was the case with EACMV. ACMV was detected in some samples in Eastern and Coast thus being the first ever recorded presence of this species of CMV in this part of the country. Dual infection by the two species of CMGs was recorded in 33 samples and was related to the severe symptoms of CMGs. The occurrence of EACMV and ACMV increase the chances of inter species genetic recombination which is typical with geminiviruses (Pita *et al*., 2009). There is also a possibility of intra species recombination between DNA-A and DNA-B leading to novel viruses with different aetiology and epidemiology (Deng et al., 1994). The interaction of the two species leads to synergy between them giving a more severe form of the disease (Pita *et al.*, 2009).

This study also determined that CMGs are widely spread by use of infected cuttings than by the whitefly (*Bemisia tabaci*). In some areas in the cassava growing regions,

infection of CMD was 100% by cuttings. This observation was also made by Njenga *et al.,* (2006). This phenomenon confirms that farmers are almost not utilizing any management practice at the moment to reduce the transmission rates of these CMGs through infected cuttings.

In this study, the coat protein (CP) gene sequence analyses showed that the Kenyan ACMV isolates were closely related to ACMV isolates from Uganda and Namibia with sequence identities ranging from 97% to 98%. Generally, ACMV sequences showed low variability amongst the Kenyan isolates and those in the genebank. On the other hand, EACMV isolates of the Cassava mosaic begomoviruses showed more variability within the DNA-B sequence under this study. The phylogenetic tree for EACMV indeed showed that the Kenyan EACMV isolates are closely related to the Ugandan isolates sandwiched between various strains of EACMV –Ug in the same branch. The DNA satellites obtained from this study are different in sequence identity with the begomoviruses associated DNA III satellites from Tanzania and the Mentha leaf satellite DNAII from India. However, there was much higher variability amongst the sequences with the Kenyan DNA sequences showing low similarity to the DNA satellites from India.

6.1 CONCLUSIONS

EACMV is more prevalent than ACMV and the two viral species of the cassava Mosaic virus disease are now well mapped in the country. The study has revealed cases of dual infection accounting for 21% of all the samples analyzed for the presence of the virus. The increased symptom severity is attributed to the dual infections of the two CMV species and the combined infection of CMD and the associated DNA satellites. It is vividly clear that infection by cuttings is more rampant than that caused by whiteflies. Though whiteflies carry the CMD viruses, the mode of transmission distribution or use of infected cuttings is widespread. Even in the provinces where the whitefly infestation is high like in Coast, the dominant mode of transmission of the virus is by infected cuttings. There exist DNA satellite molecules which associate with the viral DNA of Cassava mosaic virus. The symptoms severity score correlated well with the molecular detection of the DNA satellite molecules. The DNA integrated satellites were far more prevalent and are distributed across the county than the episomal satellites as determined from this study.

This study has revealed that Cassava mosaic geminiviruses in Kenya are caused by the two species of CMD namely EACMV and ACMV. Kenyan EACMV strains have a high homology to the EACMV – Ug strains. The high sequence identity of 96% to the ACMV –Uganda severe isolate points at the possibility of these Kenyan isolates to cause a severe form of the disease as witnessed in the field during the survey.

The DNA satellites obtained from this study exhibited low sequence identity with the begomoviruses associated DNA III satellites East African region and India. There is a large genetic variability amongst the DNA III satellites characterized in this study. This study has therefore clearly demonstrated that there are four distinct groups of begomovirus associated DNA satellites with two groups being predominant in Kenya, one in Eastern Africa and the other one in Southern Africa The DNA satellites identified in this study are distantly related to those from other parts of east Africa, South Africa and India.

6.2 RECOMMENDATIONS

The detections of ACMV in Kathiani district of Eastern province and several districts in Coast province in this study present challenges in the management of CMD in these regions and the county at large. Dual infections of EACMV and ACMV in these regions point to a possibility of more severe forms of CMD due to synergism and genetic recombination between EACMV and ACMV (Pita *et al.*, 2001). As such there is need to continue evaluating varieties resistant or tolerant to these viruses and pooling regional efforts in the characterization of the viruses. The existing varieties that are resistant or tolerant to CMD can now be deployed in areas where the disease severity, prevalence and incidence have been determined to be high. This will lead to reduced severity levels hence increased yields.

Breeders can now target resistance to the two main species of CMD i.e. ACMV and EACMV since the two species are now characterized, Genetic modification techniques or conventional breeding techniques can now be tailored to coming up with resistant and tolerant varieties to mitigate this situation. Further characterization studies are therefore required to ascertain the isolates from Coast and Nyanza where exceptionally high severity symptoms were recorded in the study.

The DNA satellites associated with the CMD virus had a strong correlation between the symptoms expression and the molecular detection especially the DNA integrated satellites for the plant viruses under study. There is need to evaluate the Integrated DNA satellites associated with CMGs to determine their modulation of symptom expression of the CMGs and the possibility of causing more severe symptoms of the disease. The effect on the yield of cassava also needs to be evaluated.

These field observations of the symptom severity could be extrapolated to field situations in order to hypothesize about the possibility of acquisition of such DNA satellites currently associated with other begomoviruses. These results call for more detailed analyses of these sub viral components and an investigation of their possible interaction with the cassava mosaic disease complex. There is need to investigate the above mentioned phenomenon with special interest on interaction of the DNA satellites with plants having dual infection of the two species of CMD, ACMV and EACMV.

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